Pharmaceutical Dosage Forms:

Parenteral Medications

Third Edition

Volume 1: Formulation and Packaging

Edited by
Sandeep Nema
John D. Ludwig

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We dedicate this work to those who have inspired us.
To my parents Walter and Ruth Ludwig and my wife Sue Ludwig
To my parents Hari and Pratibha Nema and my wife Tina Busch-Nema

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Foreword

I was a faculty member at the University of Tennessee and a colleague of Dr. Kenneth Avis when he conceived, organized, and edited (along with H.A. Lieberman and L. Lachman) the first edition of this book series that was published in 1984. It was so well received by the pharmaceutical science community that an expanded three-volume second edition was published in 1992. Dr. Avis did not survive long enough to oversee a third edition, and it was questionable whether a third edition would ever be published until two of his graduate students, Drs. Nema and Ludwig, took it upon themselves to carry on Dr. Avis' tradition.

Their oversight of this third edition is work that their mentor would be highly pleased and proud of. From 29 chapters in the second edition to 43 chapters in this new edition, this three-volume series comprehensively covers both the traditional subjects in parenteral science and technology as well as new and expanded subjects. For example, separate chapter topics in this edition not found in previous editions include solubility and solubilization, depot delivery systems, biophysical and biochemical characterization of peptides and proteins, container-closure integrity testing, water systems, endotoxin testing, focused chapters on different sterilization methods, risk assessment in aseptic processing, visual inspection, advances in injection devices, RNAi delivery, regulatory considerations for excipients, techniques to evaluate pain on injection, product specifications, extractables and leachables, process analytical technology, and quality by design.

The editors have done an outstanding job of convincing so many top experts in their fields to author these 43 chapters. The excellent reputations of the authors and editors of this book will guarantee superb content of each chapter. There is no other book in the world that covers the breadth and depth of parenteral science and technology better than this one. In my opinion, the editors have achieved their primary objectives—publishing a book that contains current and emerging sterile product development and manufacturing information, and maintaining the high standard of quality that readers would expect.

Michael J. Akers
Baxter BioPharma Solutions
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Preface

Pharmaceutical Dosage Forms: Parenteral Medications was originally published in 1984 and immediately accepted as a definitive reference in academic institutions and the pharmaceutical industry. The second edition was published in 1993. The ensuing years have produced incredible technological advancement. Classic small-molecule drugs are now complemented by complex molecules such as monoclonal antibodies, antibody fragments, aptamers, antisense, RNAi therapeutics, and DNA vaccines. There have been significant innovations in delivery devices, analytical techniques, in-silico modeling, and manufacturing and control technologies. In addition, the global regulatory environment has shifted toward greater emphasis on science-based risk assessment as evidenced by the evolving cGMPs, quality by design (QbD), process analytical technology (PAT), continuous processing, real time release, and other initiatives. The rapidly changing landscape in the parenteral field was the primary reason we undertook the challenging task of updating the three volumes. Our objectives were to (*i*) revise the text with current and emerging sterile product development and manufacturing science and (*ii*) maintain the high standard of quality the readers expect.

The third edition not only reflects enhanced content in all the chapters, but also more than half of the chapters are new underscoring the rapidly advancing technology. We have divided the volumes into logical subunits—volume 1 addresses formulation and packaging aspects; volume 2, facility design, sterilization and processing; and volume 3, regulations, validation and future directions. The authors invited to contribute chapters are established leaders with proven track records in their specialty areas. Hence, the textbook is authoritative and contains much of the collective experience gained in the (bio)pharmaceutical industry over the last two decades. *We are deeply grateful to all the authors who made this work possible.*

Volume 1 begins with a historical perspective of injectable drug therapy and common routes of administration. Formulation of small molecules and large molecules is presented in depth, including ophthalmic dosage forms. Parenteral packaging options are discussed relative to glass and plastic containers, as well as elastomeric closures. A definitive chapter is provided on container closure integrity.

Volume 2 presents chapters on facility design, cleanroom operations, and control of the environment. A chapter discussing pharmaceutical water systems is included. Key quality attributes of sterile dosage forms are discussed, including particulate matter, endotoxin, and sterility testing. The most widely used sterilization techniques as well as processing technologies are presented. Volume 2 concludes with an in-depth chapter on lyophilization.

Volume 3 focuses on regulatory requirements, risk-based process design, specifications, QbD, and extractables/leachables. In addition, we have included chapters on parenteral administration devices, siRNA delivery systems, injection site pain assessment, and control, PAT, and rapid microbiology test methods. Volume 3 concludes with a forward-looking chapter discussing the future of parenteral product manufacturing.

These three volumes differ from other textbooks in that they provide a learned review on developing parenteral dosage forms for *both* small molecules and biologics. Practical guidance is provided, in addition to theoretical aspects, for how to bring a drug candidate forward from discovery, through preclinical and clinical development, manufacturing, validation, and eventual registration.

The editors wish to thank Judy Clarkston and Lynn O'Toole-Bird (Pfizer, Inc.) for their invaluable assistance and organizational support during this project, and Sherri Niziolek and Bianca Turnbull (Informa Healthcare) for patiently leading us through the publishing process.

We also acknowledge the assistance of Pfizer, Inc. colleagues Lin Chen and Min Huang for reviewing several of the chapters.

We would like to express special gratitude to the late Kenneth E. Avis (University of Tennessee College of Pharmacy) for his dedication to teaching and sharing practical knowledge in the area of parenteral medications to so many students over the years, including us. Finally, we acknowledge the contributions of Dr Avis, Leon Lachman, and Herbert A. Lieberman who edited the earlier editions of this book series.

Sandeep Nema
John D. Ludwig

Contents

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1 Parenteral dosage forms: introduction and historical perspective

John D. Ludwig

INTRODUCTION

Parenteral dosage forms are those administered directly into body tissues rather than via the alimentary canal. "Parenteral" is derived from the Greek words *para* (beside) and *enteron* (the intestine) and most often refers to subcutaneous (SC), intramuscular (IM), or intravenous (IV) administration of drugs. Parenteral drug delivery can pose significant risk to the patient since the natural barriers of the body (gut, skin, and mucous membranes) are bypassed. The highest standards for quality and purity must be maintained throughout dosage form manufacture to protect the patient from physical, chemical, and microbial contaminants. A single contaminated vial out of a batch of thousands can seriously injure a patient (or worse). Further, if improper or poor aseptic technique is used while administering an injection the patient could be similarly harmed. The minimum quality standards for pharmaceutical manufacturers are expressed in the current good manufacturing practices (cGMPs), which are constantly evolving as technology advances. An equal burden of responsibility is placed on physicians, pharmacists, nurses, and other health professionals to follow strict good aseptic practices (GAPs) as they administer parenteral dosage forms to patients. Nosocomial infections associated with parenteral drug therapy remain a significant issue (1–4).

ADVANTAGES AND DISADVANTAGES OF PARENTERAL DRUG DELIVERY

Parenteral drug delivery provides a number of advantages for the patient. The parenteral route provides an effective way to dose patients who are unconscious or those who cannot or would not take oral medications. A drug administered parenterally generally produces an immediate therapeutic effect and is therefore desirable in emergency situations. Parenteral administration also provides a mechanism for dosing drugs that are not bioavailable via noninjectable routes such as many protein and peptide therapeutics. Total parenteral nutrition can be provided for seriously ill patients where tube feeding is not an alternative. In addition, large amounts of fluid and electrolytes can be given relatively quickly via the IV route to patients with serious fluid loss from dehydration or gastrointestinal infections.

A significant disadvantage of injectable drug administration is that once a drug has been dosed it is difficult to reverse its effect. For example, in the event of a dosing error (overdose) with an oral tablet, gastric lavage, induced emesis, or activated charcoal can be employed. The options for reversing an IV overdose are usually very limited. Secondly, the risk of infection is always present with parenteral dosing both in the hospital/clinic setting as well as home administration. Finally, the cost per dose of parenteral drugs is typically higher than for oral medications.

PARENTERAL DRUG DELIVERY ROUTES

Routes of parenteral drug delivery are summarized in Table 1. SC, IM, and IV are the most common modes of administration. The fastest onset of action is achieved via the IV route since the injection is directly into a vein. Relatively large amounts of fluid can be delivered quickly and efficiently using the IV route. Slower and more variable onset of action typically occurs following SC and IM administration since the drug must be absorbed into the bloodstream from the site of injection. The absorption step can be exploited for drugs requiring chronic administration. Formulations can be designed to provide sustained-release profiles therefore reducing the number of injections required and the associated risk. Examples of "depot" formulations include DEPO-PROVERA® Contraceptive Injection, which is administered deep IM every 13 weeks and depo-subQ provera 104™ which is administered SC in the anterior thigh or abdomen every 12 to 14 weeks. Intravitreal dosing has increased significantly in recent

Table 1 Parenteral Drug Delivery Routes

Route	Administration volume
Subcutaneous (SC)	Low, generally <2 mL
Intramuscular (IM)	Medium, 2 mL–5 mL
Intravenous (IV)	High
Intravitreal	Low, generally <0.1 mL
Intradermal (ID)	Low, 0.1 mL
Intra-articular	Medium
Intrathecal	Low
Intraepidural	Low
Intracisternal	Medium
Intra-arterial	High
Intracardiac	Medium
Intrapleural	Medium
Intraperitoneal	High
Intraosseous	Medium

years because of new treatments for neovascular wet age-related macular degeneration (AMD) such as Lucentis® (ranibizumb injection) and Macugen® (pegaptanid sodium injection). The intradermal (ID) route is commonly used for very small volume injections (0.1 mL) such as the tuberculosis skin test [or tuberculin purified protein derivative (PPD) test]. Intra-articular injections directly into joint synovial fluid are routinely used to administer corticosteroids or hyaluronic acid derivatives to relieve the symptoms of osteoarthritis. Intrathecal (intraspinal) and intraepidural injections are used to deliver anesthesia, analgesics, anti-infectives, and some cancer therapies. Intracisternal administration is used to deliver critical therapeutics directly to the caudal region of the brain. Less common parenteral routes include intra-arterial, intracardiac (e.g., epinephrine for cardiac resuscitation), intrapleural, intraperitoneal, and intraosseous (bone) (5,6).

QUALITY ATTRIBUTES OF PARENTERAL DOSAGE FORMS

Quality attributes specific to parenteral dosage forms are shown in Table 2. Injectable products must be manufactured using the highest quality active drug substance and excipients. The regulatory review process requires that each ingredient in the formulation must be justified as

Table 2 Quality Aspects of Parenteral Dosage Forms

Attribute	Comment
Highest level of purity for the active drug substance and excipients	Highly purified "parenteral grade" excipients are available.
Formulation containing the fewest number and the simplest excipients possible	The presence and amount of each excipient must be justified in regulatory filings.
Physical and chemical stability	Minimal degradation during shelf-life.
Container-closure system with low extractable/ leachable profile	Minimize the impact of the container on product purity and stability.
Sterile	Sterility assurance is critical for patient safety.
Pyrogen free	Pyrogens cause febrile response. The most potent pyrogens are bacterial endotoxins.
Free from visible particulate matter	Subvisible particulate matter must be excluded as much as possible as defined by compendial requirements.
Container-closure integrity	Product container maintains microbiological integrity during shelf-life.
Injection site tolerability	Formulation does not cause significant injection site irritation or tissue damage. Products are frequently formulated as isotonic solutions.
Detailed dosing and administration instructions including evaluation of compatibility with coadministered drugs	In clinical practice, multiple drugs are frequently administered through the same IV line to avoid the risk of an additional venipuncture.

to why it was included and the relative amount. As a general rule, formulations with the fewest excipients and simplest composition are highly desired. The quality and robustness of the container-closure system must also be described and justified relative to extractables/leachables, container integrity (microbiological, oxygen transmission, moisture transmission), and intended clinical use. Parenteral products must be sterile, pyrogen-free, and free from visible particulate matter and remain so throughout shelf-life. Adverse injection site events are widely reported and can cause significant tissue damage. Often, the formulation can be modified to increase injection site tolerability, for example, by changing buffers and/or decreasing buffer concentration as well as rendering the dosing solution isotonic. The compatibility of the formulation should be assessed with the most likely drugs that will be coadministered with the new product. Compatibility results are generally included in the approved dosing instructions to assist pharmacists, nurses, and other health care providers.

MILESTONES IN PARENTERAL DRUG THERAPY

Various scholars have summarized the development of parenteral drug therapy (7–13). A compiled historical timeline is presented in Table 3. The reader should be aware there is disagreement in the literature about exact dates as well as who was "first," particularly for

Table 3 Historical Milestones in Parenteral Drug Delivery

Year	Milestone
1616	William Harvey described the circulation of blood. His findings were published in 1628.
1656	Christopher Wren infused dogs with opiates and alcoholic beverages using a sharpened quill and animal bladder.
1665	Johannes Escholtz described techniques for IV infusion of drugs into humans.
1796	Edward Jenner vaccinated children against smallpox using intradermal administration with cowpox virus.
1818	James Blundell performed a successful blood transfusion following postpartum hemorrhage.
1831	William O'Shaughnessy studied the blood of cholera patients and developed the concepts for IV water and electrolyte replacement therapy.
1832	Thomas Latta established the first clinical practice of IV infusions of water and salts to treat cholera patients, based on O'Shaughnessy's work.
1855	Alexander Wood developed the first modern hypodermic syringe with a steel barrel and hollow steel needle.
1867	Joseph Lister developed the concepts of antisepsis using carbolic acid (phenol) solutions to sanitize hands, instruments, and wounds to reduce postsurgery infections.
1860s–1880s	Louis Pasteur confirmed the germ theory of disease, discovered techniques for pasteurization of milk, and developed vaccinations against chicken cholera, bovine anthrax, and rabies.
1879	Charles Chamberland invented the autoclave.
1884	Charles Chamberland invented the "Chamberland filter" (porcelain) that removed bacteria from solutions prior to dosing.
1891	R.M. Matas demonstrated the effective use of IV saline solutions to treat shock.
1912	Using a rabbit model, E.C. Hort and W.J. Penfold determined the pyrogenic response following many IV injections was caused by a substance produced by gram-negative bacterial contamination of the solution (14–16).
1918	Richard Zsigmondy and W. Bachman developed technology to manufacture microporous membrane filters from cellulose esters (nitrocellulose, acetyl cellulose, cellulose acetate).
1923	Florence Siebert and L.B. Mendel developed a definitive rabbit pyrogen test model and showed that endotoxin from gram-negative bacteria was the substance responsible for the pyrogenic response following injection with sterile solutions (17–19,20).
1923	Frederick Banting and J.J.R. Macleod share the Nobel Prize in Physiology or Medicine for the extraction of insulin and demonstration of clinical efficacy.
1923	Purified insulin product marketed (Iletin®).
1924	R.M. Matas demonstrates continuous IV "drip" (21).
1933	L. Rademaker reported that after installation of a distilled water system for pharmaceutical production, pyrogenic reactions by surgery patients to parenteral injections dropped from 30% to 4% (22).
1938	Lloyd A. Hall and Carroll L. Griffith patented the use of ethylene oxide to sterilize and preserve spices. This technology was applied to sterile pharmaceutical product manufacturing during the 1940s.

(*Continued*)

Table 3 Historical Milestones in Parenteral Drug Delivery (*Continued*)

Year	Milestone
1942	Rabbit pyrogen test (Seibert and Mendel) published in the U.S. Pharmacopeia.
1940s	High Efficiency Particulate Air (HEPA) filters designed and installed for clean air supply in rudimentary cleanrooms at Manhattan project sites and biological weapons research laboratories at Fort Detrick, Maryland (10,23,24).
1946	Parenteral Drug Association founded.
1950s	Cleanrooms with HEPA filtered air supply widely used for pharmaceutical fill/finish (10,23,24).
1961	Willis J. Whitfield pioneered the concept of laminar air flow and constructed the first modern cleanroom at Sandia Corporation in Albuquerque, New Mexico (10,23,24).
1961	Arvid Wretlind and O. Schuberth formulated the first lipid emulsion, Intralipid®, suitable for IV infusion (7,25).
1964	Arvid Wretlind developed a total parenteral nutrition (TPN) program providing half of the calories from lipid and half from glucose. Recognized as the father of TPN (7,25).
1967	Stanley J. Dudrick reported comprehensive technique to provide long-term total parenteral nutrition (TPN) (7,25).
1969	DW Wilmore and Stanley J Dudrick used an in-line filter to reduce the risk of IV infusions (7, 25).
1971	James F. Cooper, Jack Levin, and H.N. Wagner Jr. pioneered use of the limulus amebocyte lysate test for screening parenteral drug products for endotoxin contamination (26).
1973	Infusion Nurses Society founded.
1976	Food and Drug Administration publishes *Current Good Manufacturing Practice in the Manufacture, Processing, Packing, or Holding of Large Volume Parenterals* (never formally adopted).
1978–1979	Human insulin cloned. Human growth hormone cloned.
1980s	First steps toward barrier isolator technology for aseptic fill/finish operations—gray side maintenance (24).
1980s	Sterilizable isolators introduced for compendial sterility testing (27).
1982	Humulin® (human insulin recombinant) marketed.
1985	Protropin® (somatrem for injection) and Somatonorm® (somatrem) marketed. (methionyl human somatropin).
1986	Orthoclone® OTK3 marketed to treat the rejection of transplanted organs.
1987	FDA publishes *Industry Guideline on Sterile Drug Products Produced by Aseptic Processing and Guideline on General Principles of Process Validation*.
1987	Humatrope® (somatropin recombinant) and Genotropin® [somatropin (rDNA) for injection] marketed.
1987	First dual chamber pen injector launched (KabiPen®).
1990s	Barrier isolator technology for fill/finish operations—Restricted Access Barrier Systems (RABS) and Isolators (24).
1992	The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) is established.
1994	FDA publishes *Guidance for Industry for the Submission Documentation for Sterilization Process Validation in Applications for Human and Veterinary Drug Products*.
1996	*Note for Guidance on Manufacture of the Finished Dosage Form* issued by the Committee For Proprietary Medicinal Products (CPMP), CPMP/QWP/486/95.
1997	First monoclonal antibody to treat cancer approved Rituxan® (rituximab).
1999	*Decision Trees for the Selection of Sterilization Methods* finalized by the CPMP, CPMP/QWP/054/98.
2003	Pharmaceutical Compounding—Sterile Preparations <797> became official in the U.S. Pharmacopeia.
2003	European Commission: Ad Hoc GMP Inspections Services Group, EC Guide to Good Manufacturing Practice Revision to Annex 1, Title: *Manufacture of Sterile Medicinal Products*.
2004	FDA publishes *Guidance for Industry Sterile Drug Products Produced by Aseptic Processing—Current Good Manufacturing Practice* (replaces 1987 version).
2006	Infusion Nurses Society publishes updated *Infusion Nursing Standards of Practice* (28).
2008	Heparin recalls due to intentional contamination during production of active pharmaceutical ingredient.
2009	European Commission: EudraLex—The Rules Governing Medicinal Products in the European Union, Volume 4, EU Guidelines to Good Manufacturing Practice, Medicinal Products for Human and Veterinary Use, Annex 1, *Manufacture of Sterile Medicinal Products* (replaces 2003 version).

Abbreviation: IV, intravenous.

discoveries prior to the 20th century. Therefore, the author attempted to arrive at reasonable dates after consulting multiple sources. It is clear early scientific findings were not disseminated quickly because of lack of modern communication tools, and scientists were often working without knowledge of similar research occurring in other laboratories. In addition, advancements were occasionally "forgotten" only to be rediscovered independently a century later, all adding to the fascinating history of medicines and health care. Specific references have been included in Table 3 for recent advances and milestones.

CONCLUSION

The advent of safe, effective parenteral therapy has resulted in tremendous improvement in the quality of medical care around the world. Those of us fortunate enough to work in this exciting area whether in research, dosage form development, manufacturing, or clinical practice share a common goal of providing the highest standard of care. To do so requires diligence at each step in the process, be it synthesis of the active ingredient and excipients, production of the container and closure, compounding of the formulation, or aseptic fill/finish of the final product. The minimum quality standards are provided in the cGMPs, but regulatory and ethical expectations go well beyond the written requirements. Providing the highest standard of care also requires strict adherence to GAPs as the health care professional or family member is preparing and administering the dose to the patient. The risk of introducing infection and causing harm is ever present. Maxine B. Perdue of the Infusion Nurses Society summarized these sentiments as follows (29):

> "My word for competency is *excellence*. Excellence is not perfection; it is stellar performance. It is keeping current and complying with evidence-based practice standards. It is not accepting the status quo, rather, being visionary and innovative and a catalyst for research. It is sharing information with others by writing articles. . .and speaking at meetings. Each day is an opportunity to step outside the box and look at how we practice infusion therapy and to focus on each aspect of what we do as a chance to improve infusion care."

The constant pursuit of *excellence* is what drives us to the highest standard of care. Our patients deserve nothing less.

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The author wishes to dedicate this chapter to Kenneth E. Avis, major professor and friend, who passed away in January 1999, and to Michael J. Akers for many years of mentoring and collaboration.

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2 Parenteral drug administration: routes of administration and devices

Himanshu Bhattacharjee and Laura A. Thoma

INTRODUCTION

The word Parenteral is coined from the greek words "*para enteron*" meaning "to avoid the intestines." Drugs administered via any route other than oral or rectal routes, are considered to be parenteral. However, common usage more closely associates the term as being synonymous with "injectable." These include drugs that are topically administered to the eye, ear, and skin or even inhaled may be considered broadly as parenterals. It is estimated that 40% of all drugs administered in hospitals are in the form of an injection. In some institutional settings, the percentage of injectables is greater than 40%. However, medical and pharmacy practioners of today generally limit the classification of parenterals to include only those drugs administered directly into tissues, tissue spaces, or compartments by injection or infusion.

Injectable products are sterile products and may require special handling and administration. Estimates indicate that over one billion disposable plastic syringes are used annually in American hospitals. With increasing complexity of the drugs being administered by the parenteral route, significant development with respect to techniques for parenteral administration have evolved in recent years and continue to do so. Moreover, development of site specific, efficacious, safe, and reproducible administration techniques have led to the development of highly advanced stand alone drug delivery devices. Some of these developments have addressed significant safety and efficacy concerns but the area of drug delivery device research is an active field of study. This chapter is an attempt to review and to update the current usage of parenteral drugs and their routes of administration. Additionally, this chapter will address currently available parenteral drug delivery devices and the trends of existing technology in the field.

PARENTERAL ADMINISTRATIONS CONCEPTS

Although oral administration is more prevalent in the current market place, parenteral administration of drugs has a number of distinct advantages over the former. Increasing complexity of new drug entities (e.g., biomolecules) and treatment regimens to treat life threatening diseases have led many formulation groups utilize parenteral routes. In some instances, parenteral administration is essential for the drug to be absorbed in active form. For example, almost all protein drugs are administered by injection, rather than administration by the oral route, because protein drugs are broken down by stomach acid and digestive enzymes. Absorption through the parenteral route is usually more rapid and predictable than when a drug is administered orally. Because of its predictable rate of absorption and bio-availability, parenteral drugs are routinely used in emergency therapy. If a patient is unconscious, uncooperative, or unable to retain anything administered orally, parenteral therapy may become a necessity.

Parenteral dosage forms ensure delivery of therapeutic concentrations of drug/s to its desired site/s of action (diseased tissues or target areas of the body). This factor becomes more significant especially when inadequate or marginal transport of drug/s into the tissues or target areas occurs or is anticipated. One such example is a direct intra-articular injection of drugs, (e.g. anti-inflammatory drugs such as the steroids) which exhibit poor transport characteristics into the synovial spaces between joints, may be used to reduce inflammation. Additionally, injectable drugs allow researcher to exert direct control over pharmacological parameters, such as the time of drug onset, serum peak and trough levels, tissue distribution, clearance and rates of elimination of the drug from the body; for example, sustained or prolonged action of intramuscular (IM) insulin administration. Parenteral administration of drugs, in some cases may aid in decreased side effects of the drug by avoiding the traditional

oral route. Methotrexate, an antimetabolite, used for blood malignancies, exhibits varied physiological side effects when administered via the intravenous (IV) route and shows poor blood-brain barrier (BBB) penetration. However in patients suffering from acute lymphoblastic leukemina (ALL) (1), methotraxate can be administered intrathecally to avoid systemic side effects. In a clinical setting, parenterally administered drugs are commonly employed for immediate correction of electrolyte or fluid imbalance, for example, dehydration or excessive blood loss due to trauma. Patients who require hyper alimentation can also be administered total parenteral nutrition consisting of minerals, amino acids, vitamins, and carbohydrates via the IV route.

Although parenterally administered drugs have a number of advantages they do suffer from certain shortcomings. One of the major disadvantages is the possiblity for infections resulting from inadequate aseptic technique during product administration. Asepsis must be maintained to avoid infection, particularly for an intravascular or intraventricular injection. Apart from infections, other life threatening conditions like AIDS (2) and hepatitis C (3) can be attributed to improper use of parenteral devices. Disinfection of the patient's skin with an antibacterial solution or rubbing alcohol before injection and using a new syringes and needles for each administration is considered a best practice. Since injecting a needle into vascular compartments or body cavities can be considered as invasive processes, pain may be an additional factor. This is especially a significant factor for patients who perform self-administration (e.g., insulin, human growth hormone). Many of the products in the current market are highly specialized drug products and expense is still a major consideration.

Although in many instances precaution are unique to the route to be utilized, several factors need to be emphasized. Needless to say good aseptic technique and sterile practices is an absolute necessity. The health practitioner should always examine the product carefully before administration to identify potential or real contamination by microorganisms or particulate matter unless the product is supplied as a suspension or emulsion. Adequate attention should be given to details with respect to dosage, mixing, potential drug interaction, and storage. Informed actions and precautions should be taken during handling of accessory or delivery devices necessary to accomplish the task of injection or infusion or to monitor the patient's conditions. Selection of correct equipment for administration of the drug product, careful assessment of the patient history, evaluation of risk factors (e.g., bleeding diathesis, previous drug interactions, predisposition to infection, etc.) and a careful observation of the patient during and after parenteral administration are recommended.

The need for good practices in storage and handling of parenteral drugs or infusions is also an important factor and should be appropriately emphasized. From the moment a parenteral drug product is manufactured, its purity and sterility are constantly threatened by handling or storage errors. Such problems are not unique to manufacturers but extend throughout the life of the product in all areas of delivery, receiving, and distribution. Difficulties encountered may range from inadequate temperature control of storage temperatures, to outdated shelf lives, to defective containers and closures (4). On the other hand errors encountered during handling or compounding usually occur at the hospital pharmacy or at bedside. Past attempts by hospital pharmacies emphasizing a "central additive programs" as a method of reducing such errors have led to reduced admixing errors (4). In such a setting sterile parenteral product received from the manufacturer is mixed in a central location (usually in the pharmacy) with specific agents or fluid formulas that physicians may have prescribed. The central location is isolated and compounding is performed aspectically under a laminar flow hood. Complex formulas are often generated in these specialized units to satisfy the therapeutic needs of an extremely difficult medical or surgical problem (e.g. hyperalimentation). Upon compounding, the product/s is shipped to the hospital ward for administration to the patient. Newer infusion devices like the "smart pumps" or "intelligent pumps" are now available that have shown to significantly reduce compounding errors related to dose accuracy (5). Central additive programs reduce the high risk of compounding and contaminating errors which may occur because of personnel variability.

In addition to these problems, difficulties exist in securing properly trained, highly intelligent, motivated health care personnel to employ correctly and responsibly the complicated methods often utilized in the modern hospital or clinic setting. Such personnel,

in addition to being expensive and scarce, must be constantly educated on new techniques and problems (continual education). Similarly, some of the devices employed in administration are not only expensive but also highly advanced, and in some instances possess inherent or generated problems too difficult to identify with 100% assurance with even the best quality control techniques. The actively engaged personnel or administrator must able to identify real and potential dangers associated with such delivery systems.

General hazards or complications are at risk of occurring regardless of the agent or class of drugs being administered, whereas specific hazards or complications are unique or peculiar to certain agents and methods of administration. An important fact to remember about all parenteral injections is that if a reaction or adverse side effect of any sort occurs, it is usually impossible to retrieve or locally neutralize the offending agent, whereas with oral agents, recovery or expulsion of the medication is possible.

ROUTES OF ADMINISTRATION

The major routes of parenteral administration are IV, subcutaneous (SC), and IM. These three routes satisfy to a large extent the four principal reasons for administering parenterals: (1) for therapy (definitive or palliative), (2) for prevention, (3) for diagnosis, and (4) for temporarily altering tissue function(s) to facilitate other forms of therapy. Besides these three primary routes, additional ones are utilized under special circumstances: for example, intrathecal, subconjunctival, intraocular, intrathecal, intra-articular, and so on. A comprehensive description of the most commonly used routes of administration is discussed in the following section.

Intravenous Route

Injections or infusions directly into a vein are termed as IV administration (Fig. 1). Such administrations of true solution drug products is considered to be 100%. Drug absorption and factors concerning absorption are circumvented by IV injection of drugs in aqueous solution. At the desired concentration of a drug in the blood an accurate and immediate action is obtained that is not always possible by other procedures. It is of the most common parenteral routes employed in hospitals for drugs, fluids, and/or electrolytes. It offers a convenient route for rapidly infusing large volumes of fluid. If the dose is administered over a few minutes, it is

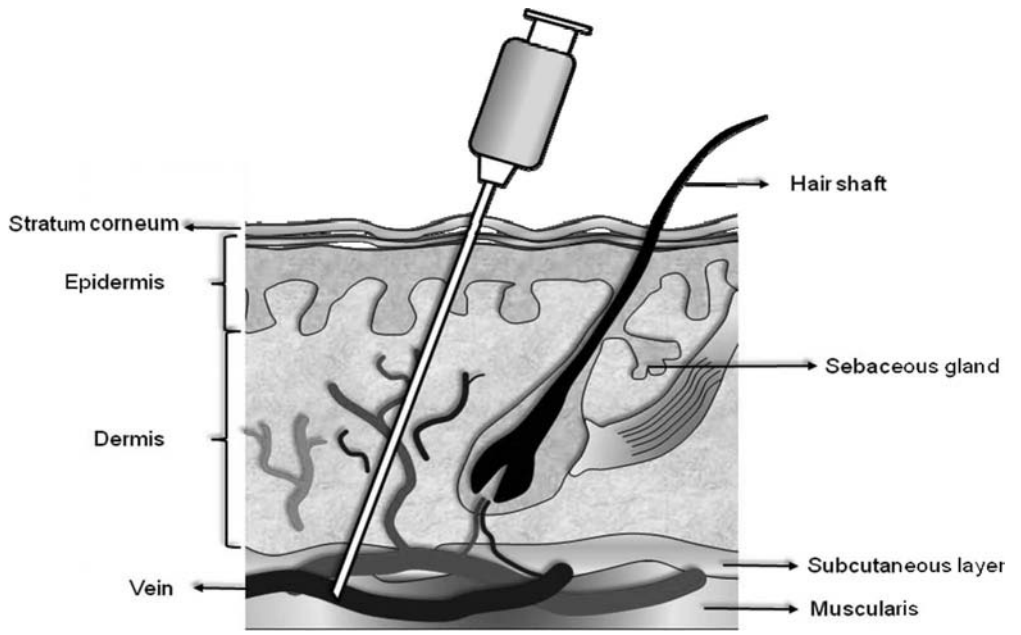

Figure 1 Schematic representation of an intravenous administration.

called a bolus dose and is primarily administered by a syringe directly into the vein. If the drug product is administered over hours from an infusion bag, it is termed an IV drip or infusion. Unfavorable reactions are prone to occur, since high concentrations of drug may be attained rapidly in both plasma and tissues. Repeated IV injections are dependent on the ability to maintain a patent vein. For prolonged IV use, flexible plastic catheters are better than sharp metal needles that may puncture through the other side of the vein.

Examples of drugs that are commonly administered by the IV route are analgesics, general anesthetics, antiviral agents, antibiotics, immunosuppressive agents, antifungal agents, antibacterial agents, antihypertensive agents, vasodilators, antiarrhythmic drugs, and chemotherapeutic agents. The preferred route for strong analgesics is a continuous IV infusion, because it produces less fluctuation in serum concentrations of the drug than do intermittent IM injections. Today, many IV drips are made in the pharmacy or by a special team rather than bedside preparations to insure accuracy of the drug product being administered.

The most common indication for use of this route are: (1) to guarantee delivery and distribution when hypotension or shock exists; (2) to restore rapidly electrolyte and fluid balance; (3) to achieve an immediate pharmacological effect, especially in emergencies, such as the treatment of certain arrhythmias or of seizures; (4) to treat serious, life threatening infections or conditions; (5) to provide continuous nutrition (hyperalimentation) when patients are unable to be fed by mouth; and (6) to avoid complications which might result if other administration routes are employed (e.g., hematomas at the site of IM injections in a patient with a bleeding diathesis). In addition, the IV route may be used for a variety of other purposes, such as plasmapheresis, blood transfusion, and hemodynamic monitoring, among others. Patient-controlled analgesia (PCA) is another unique mode of IV administration and is designed to deliver IV bolus doses in addition to a slow, continuous IV by this route for narcotic analgesics such as fentanyl, methadone, and morphine (6). Programmable infusion pumps with limited patient controls are often used for this type of administration and only allow the patient to receive an additional dose within limited time periods (7).

The IV route is not without adverse effects. Generally IV injections are administered directly into the venous circulation, and hence highly vascular and perfused organs, such as the heart, lungs, liver, and kidney, rapidly acquire the drug. However, a sudden increase in serum drug concentration may lead to toxicity and adversely affect the vital organs. This can be prevented by giving a slow IV bolus injection or controlling an IV drip. Some drugs with poor aqueous solubility may precipitate from solution and produce an embolism, for example, phenytoin IV injection. Hence, in such instances, it is important that proper selection of the diluent and slow IV administration be carried out; the latter allows for proper mixing of the drug into the circulation. Some vehicles may cause adverse effects in pediatric patients. For example, phenobarbital sodium when dissolved in propylene glycol may cause hyperosmolality in infants. In addition, because the alcohol and aldehyde dehydrogenase pathway that metabolizes propylene glycol is not well developed in infants and children younger than four years, repeated use of IV injections containing propylene glycol can lead to toxicity (8). Some lipid-soluble drugs, like diazepam, can cross the BBB and are effective when given by the IV route. Thus, lipid-soluble drugs, especially central nervous system (CNS) active drug, for example, sedatives, depressants, etc., often need to be administered by specialized routes of delivery that bypass the BBB. Other complications that may occur using the IV route are as follows: (1) thrombosis with or without complicating infection at the site of injection or infusion; (2) injection of microorganisms, toxins, particulate matter, or air; (3) the occurrence of physical or chemical incompatibilities between agents prior to or at the time of injection; (4) uncontrolled or excessive administration of drugs or fluids; and (5) extravasation of injections or infusions at the site of administration. When indwelling catheters are utilized, rarely the catheter tip may break off and lodge in a major vessel, in the heart, or in the lung and can cause fatalities.

To administer drugs through the IV route the upper extremities are chosen whenever possible for the site of injection or infusion. The most peripheral veins (e.g., over the hand) are selected for initial use. When arm sites are no longer available, the leg veins (femoral and saphenous) or dorsal foot veins may be utilized; and in small children the scalp veins. A recent improved in locating veins in pediatric and geriatric population is being used in clinical trials and is based on noninvasive infrared technology (Fig. 2). This unique device captures a near

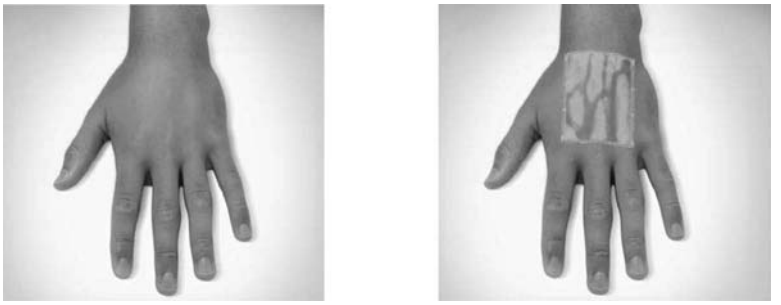

Figure 2 Visualization of veins using the proprietary VeinViewer® instrument form LuminetX, LLC.

infrared vein image, processes it, and projects it onto the skin using green light thus aiding phlebotomy (9). Selection of a vein depends on the size of the needle intended for use, type of fluids to be infused, flow rate anticipated, volume to be received, concomitant medications to be given, degree of patient mobility desired, and of course the skill of the person performing the venipuncture or catheterization. The veins in the antecubital fossa are among the most commonly chosen, because they are large and readily punctured. Other veins utilized commonly are basilic, cephalic, radial at the wrist, and the metacarpal and dorsal venous plexuses. Needles are generally preferred to indwelling IV catheters, as the risk of infection is believed to be less. Even after apparent exhaustion of all available venous sites, surgical cut downs of deep veins with insertion of catheters may be performed. When long-term, repeated usage is expected or when prolonged infusion is anticipated, the subclavian or internal jugular in the upper chest may be utilized. For peripheral veins and single or short-term usage, a 1 to 2 inch long, beveled, 18- to 22-gauge, stainless steel needle is commonly used.

For long-term and/or repeated IV administration, a sterile plastic catheter may be inserted into the vein percutaneously through or over the needle that was used for the initial puncture. The needle is then removed and the catheter is left in place. The indwelling needle or catheter, whichever is utilized, is anchored to the extremity or body by means of appropriate, sterile occlusive or nonocclusive dressings, often impregnated with an antibiotic ointment. Indwelling catheters may contain a heparin lock to ensure against clotting and loss of patency through venous thrombosis.

Intramuscular Route

An IM injection is defined as an injection directly into the body of a relaxed muscle (Fig. 3). The IM route is one of the most popular and convenient routes available, both for the administrator and for the patient, and a route of choice especially for pediatric subjects. Therefore, whenever it is possible and practicable, the IM route is used. The IM route provides a means for prolonged release of drugs formulated as aqueous or oily solutions or suspensions.

The IM route is preferred over the SC route when a rapid rate of absorption is desired for certain life threatening conditions. For example, administration of epinephrine via the IM route causes a higher peak plasma concentration compared with the SC route (10). However the rate of absorption is slow when compared with the IV route. One reason for using the IM route is because of the inability to administer the drug directly into the vascular compartment. Drugs commonly injected by IM administration include lidocaine, cephalosporins, aminoglycosides, diazepam, phenytoin, insoluble salts of penicillin G (procaine penicillin G), corticosteroids, narcotics, narcotic antagonists, and contraceptive steroids.

Although IM injections are much easier to administer than other injections, the main precaution is to avoid entering a blood vessel (especially an artery), which might lead to infusion of a toxic agent or a toxic vehicle directly to an organ or tissue. This can be prevented usually by pulling back on the plunger of the syringe; if blood does not appear, the needle is probably not in a vessel. Also, the accidental striking of or injection into a peripheral nerve may result in a peripheral nerve palsy with or without sensory damage. Occasionally, when a large bolus of drug is injected into the muscle, local damage or muscle infarction may result,

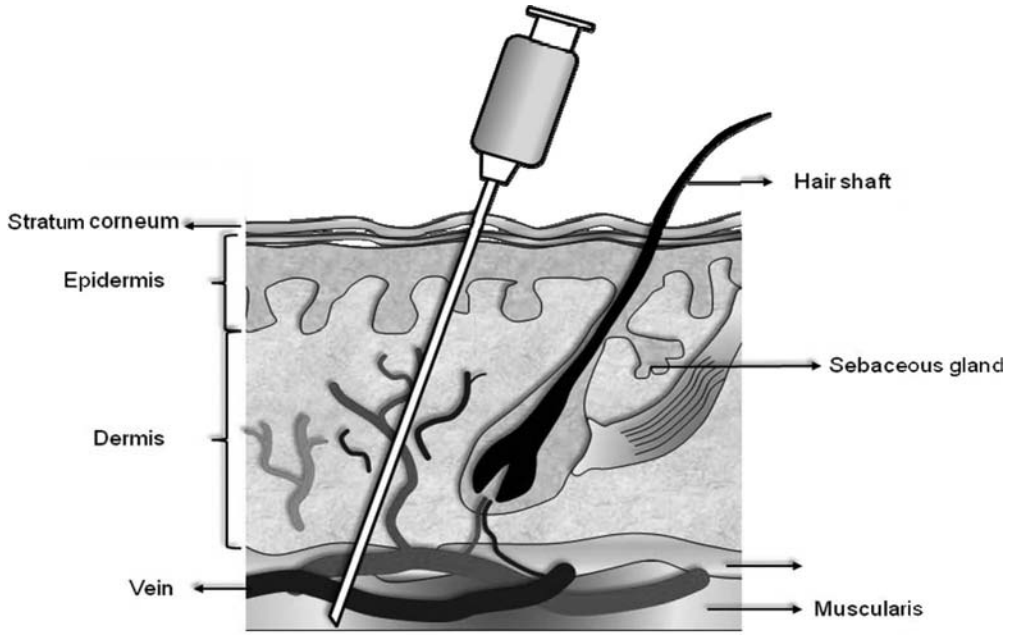

Figure 3 Schematic representation of an intramuscular administration.

leading to a sterile abscess or elevation of serum levels of muscle enzymes. The latter complication may present confusing diagnostic problems, especially in patients under suspicion of having a myocardial infarction or hepatitis.

If materials contaminated with microorganisms are injected, a septic abscess may result. Therefore, appropriate precautions must be taken to ensure sterility prior to injection. In patients with poor hygiene or skin care, microorganisms from the skin flora may be punched in by the needle at the time of injection, resulting in staphylococcal or streptococcal abscesses and rarely gas gangrene (11) or tetanus (12). An important note of caution: the IM route should never be employed in patients with significant heart failure or shock, where uptake into the vascular compartment may be expectantly poor. This caution should be followed especially if immediately high serum or plasma concentrations of the drug are desired or if rapid distribution to a distal organ is mandatory.

Various muscle sites are available for delivery, including the gluteal, deltoid, triceps, pectoral, and vastus lateralis muscles. In adults the site of choice often is the gluteal muscle, because large volumes of drug may be injected and tolerated. However, the vastus lateralis of the thigh may also be used because it not only tolerates large volumes of medication, but it is also away from any major vessels or nerves. For rapid absorption and small volumes ($<$2 mL), the deltoid muscle is preferred, as some studies suggest that blood flow in the deltoid muscle is 7% greater than that of the vastus lateralis and 17% greater than that of the gluteus maximus (4). In infants and small children, the vastus lateralis of the thigh is often preferred because it is better developed than other muscle groups.

With IM injections a beveled, 19- to 22-gauge, 1 to 2 inch long, stainless steel needle is used and no more than 5 mL of fluid is injected, depending on the site selected. The skin is first cleaned with alcohol or a suitable disinfectant, and the plunger on the syringe is always retracted prior to injection to be sure that the needle is not in a vessel. For deep IM injections, as might be used for irritating medications such as iron preparations, a "z-track" injection method is employed (4).

Subcutaneous Route

A SC injection (abbreviated as SC, SQ, sub-cu, sub-Q or subcut) is administered as a bolus into the subcutis, the layer of skin directly below the dermis and epidermis, collectively referred to

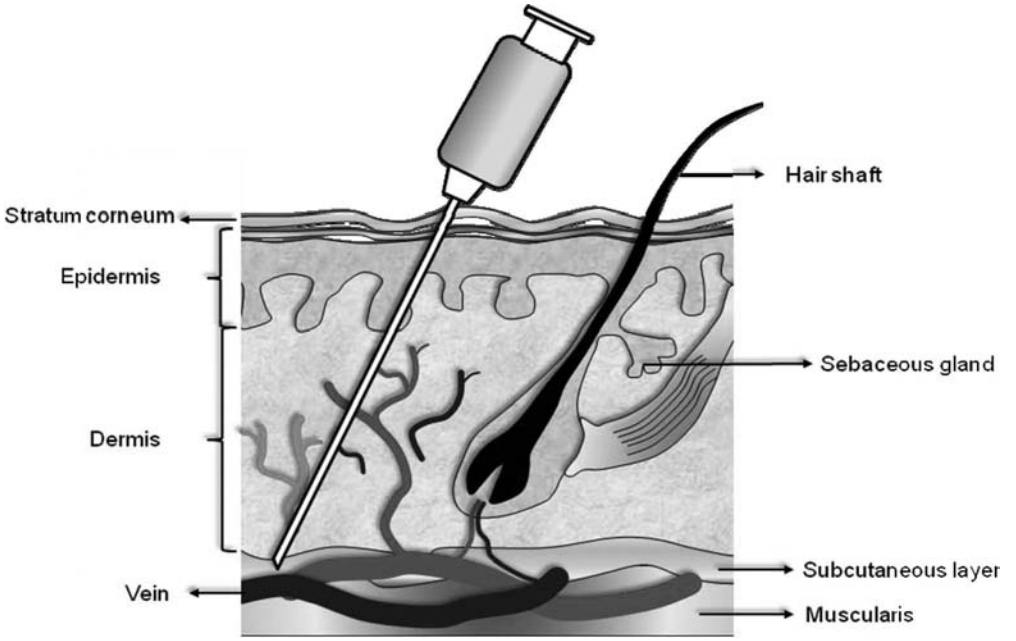

Figure 4 Schematic representation of a subcutaneous administration.

as the cutis (Fig. 4). SC injections are highly effective in administering vaccines and such medications as insulin, morphine, diacetylmorphine or goserelin. This route may be utilized if drugs cannot be administered orally because of lack of absorption from or inactivation by the contents of the gastrointestinal tract, if the patient is unable to ingest medications by mouth or if self-medication of parenterals (e.g., insulin) is desired. Drugs are more rapidly and more predictably absorbed by this route than by the oral route. However absorption of drugs via this route is slower and less predictable compared to the IM route and this effect can be attributed to the difference in vascularity of the muscle and dermis. Medications commonly administered subcutaneously include insulin, vaccines, narcotics, epinephrine, and vitamin B12. As with the IM route, if heart failure, shock, or vascular collapse exists, this route should not be depended on. Hypodermoclysis is a special form of SC administration, namely, the infusion of large amounts of fluid into the SC tissues when IV sites are not available. This form of administration is rarely (if ever) used today but in the recent past was a common mode of replenishment of fluid and electrolytes in infants and elderly patients.

Medications that are highly acidic, alkaline, or irritating, causing the production of pain, inflammation, and/or necrosis of tissues, should not be administered by this route. Infection, as with all parenteral injections, may occur, particularly in a patient with poor skin hygiene and particularly in situations where self-administration is practiced. Generally, a beveled, 24- to 25-gauge, 0.25 to 0.625 inch long, stainless steel needle is utilized. The volume injected generally does not exceed 0.5 to 1. 5 mL. Injection sites include the abdomen at the level of the umbilicus, the upper back, the upper arms, and the upper hip. The skin over the site of administration should be disinfected prior to injection with a sterile alcohol sponge. Prior to injection, aspiration should be attempted to be certain that the needle has not inadvertently entered a vessel. If blood does not appear in the syringe when the plunger is retracted, then the product is not injected.

It is advisable that the area of injection must be rotated for long-term therapies like administration of insulin or human growth hormone. Changing the injection site keeps lumps or small dents called lipodystrophies from forming in the skin. However, patients should try to use the same body area for injections that are given at the same time each day. Using the same body area for these routine injections lessens the possibility of changes in the timing and action of drugs like insulin.

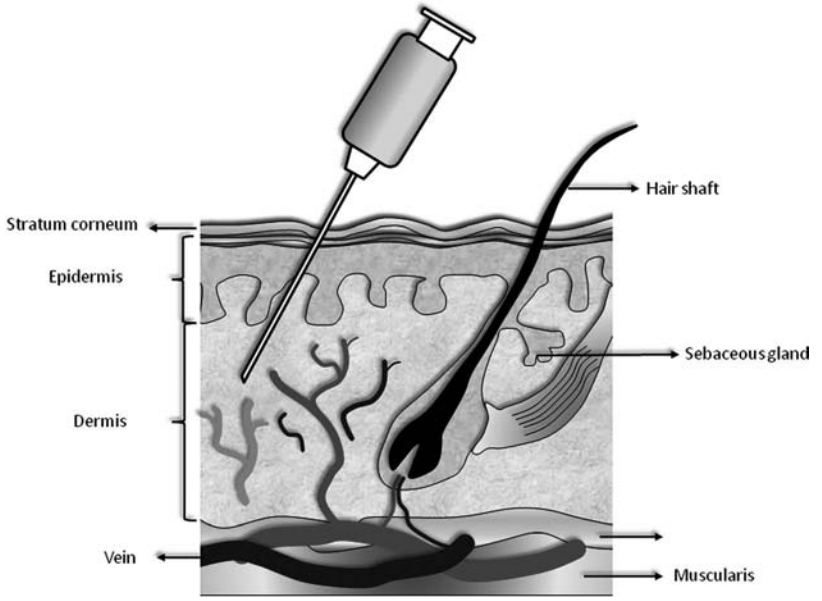

Figure 5 Schematic representation of an intradermal administration.

Intradermal Route

Injection into the dermis, located just beneath and adjacent to the epidermis is called an intradermal injection (Fig. 5). A number of diagnostic agents, antigens (e.g., tuberculin) and vaccines (e.g., smallpox) are administered by this route. The volume of fluid injected generally does not exceed 0.1 mL. Absorption by the intradermal route is very fast compared with the SC or IM route.

Generally a beveled, 26- or 30-gauge, 0.375 inch long, stainless steel needle is utilized. The skin at the site of administration should be cleaned prior to injection with 70% alcohol. Certainty of intradermal injection is evident by the appearance of a localized swelling of the skin, giving the appearance of an orange peel. The most common mistakes in intradermal injections are injecting beneath the skin rather than into it or permitting materials to leak out of the needle tip if it is not inserted completely into the skin.

Intra-arterial Route

The intra-arterial route is infrequently used route. Injection of a drug into an artery terminates in a target area, which may be an organ. Almost every artery is approachable by arterial catheterization and none are inaccessible to the skilled surgeon or radiologist.

The nature of the drug and the physiology of the circulatory system require IV injection to be diluted in the blood rather than going directly to an organ or tissue where the effects will be localized. The intra-arterial route is employed generally for diagnostic purposes, such as injecting radiopaque substances for roentgenographic studies of the vascular supply of various organs or tissues (e.g., coronary, cerebral, pulmonary, renal, enteric, or peripheral arteries). The usual reason for using the intra-arterial route is to introduce radiopaque materials for diagnostic purposes, such as for arteriograms. This route can be extremely hazardous, because products administered intra-arterially are not adequately diluted nor are they filtered by the lungs, liver, or kidneys before contact with peripheral tissue/s or vital organs nourished by the artery. Products contaminated with microorganisms, endotoxin, and/or particulate matter may result in serious complications or reactions, such as infection (either intra-arterial or extra-arterial) or arterial thromboembolism or vasospasm. This may result in ischemia, infarction, or gangrene of the tissues or organs supplied. In addition, if the technique of entry is faulty, damage to the arterial intima and vessel wall may occur resulting in serious hemorrhagic

extravagation or a dissecting aneurysm. If air is infused accidentally, air embolism with consequent ischemia and/or infarction of the tissue may occur; an event which usually does not occur when small amounts of air are infused into the venous system.

Usage of the intra-arterial route for treatment purposes is infrequent and limited generally to organ-specific chemotherapy, such as treating certain localized cancers (e.g., malignant melanomas of the lower extremities), where regional perfusion with high concentrations of toxic drugs (which when given intravenously may be associated with serious systemic reactions) can be achieved. Arterial spasm and subsequent gangrene present problems that make the intra-arterial route hazardous.

Either a suitably sized, smooth-bore, stainless steel needle or a short, flexible, plastic catheter is surgically inserted into the desired artery or a lengthy catheter is guided over a stylet or needle through a percutaneous entry site (sometimes under fluoroscopy) until the desired artery, organ, or tissue is reached; or the skin over the artery may be punctured directly, and the needle then inserted into the artery. Also, an open operative incision through the skin may be made (a "cut-down"), by which the artery is surgically exposed and under direct visualization is entered; a catheter is then inserted into the artery and sewn in place. Regardless of the method used, strict aseptic technique is practiced and appropriate occlusive or nonocclusive dressings are employed.

Intracisternal Route

Administration of drug products directly into the cisternal space surrounding the base of the brain is called as intracisternal injection. This route is employed mainly for diagnostic purposes. Additionally this route is used to decrease elevated intracranial pressures and reduce the risk of herniation of the brain if fluid is removed from the lumbar sac. Diseases involving the cisterns generally extend to nearby, contiguous structures are treated by utilizing the intraventricular route. Rarely, in order to locate and define a particular disease process; especially a spinal tumor or abscess, various contrast materials are injected into the cisterns. Intrathecal or intracisteral injections do not result in distribution of the drug into the ventricular space; thus disease within the ventricles would not be treated by these routes.

Many of the precautions concerning the use of the intraventricular route are applicable to the use of the intracisternal route, particularly as regards to aseptic practices and the threat of physicochemical irritation of the substances injected. One very serious drawback to the use of this route is the danger of producing permanent, serious, neurological injury or death due to possible damage to the midbrain. The space entered is relatively small, and insertion of a needle into it should be attempted only when other routes may not be used and only by the most experienced personnel. For intracisternal puncture the patient is placed in a head-down position and the entry approach is posterior between the occiput and the first cervical vertebrae. The cisterna magnum is punctured and extreme care is exercised to continue aspirating with a syringe while inserting the needle.

Intraventricular Route

Here the drug product is injected or infused directly into the lateral ventricles of the brain. This route is employed mainly in the treatment of infections (such as bacterial or fungal meningitis and/or ventriculitis) or of malignancies (such as leukemic infiltrates of the meninges or carcinomatoses) involving the membranes and cerebrospinal fluid surrounding the CNS. It is used especially in situations where the drugs involved are known to diffuse or pass poorly from the vascular compartment into the ventricles and subarachnoid space and/or where reduction of systemic side effects from a particular agent are desired. One such example is the treatment of fungal meningitis with amphotericin B (13) or in the therapy of leukemic infiltrates with methotrexate (14). Often, therapy via this route is complemented by the IV administration of the same agent which has been injected into the ventricles.

In the treatment of diseases of these areas, the intraventricular route often is preferred over the intracisternal or intrathecal. This is because the flow of cerebrospinal fluid is unidirectional and originates principally in the choroid plexus of the lateral ventricles and pursues a path through the third and fourth ventricles out the foramina of Luschka and

Magendie into the posterior fossa at the level of the pons, down over the spinal cord, and then finally reversing itself to flow up over the cerebral hemispheres. In addition, the ventricle provides a large fluid space in which to inject drugs, thereby diluting such drugs in a large volume of cerebrospinal fluid, thus minimizing potential, localized physicochemical irritation to the cells lining the ventricle and subsequent damage from a host reaction. In addition, if intracranial pressures are excessive, the risk of brain stem herniation may be avoided, a known risk factor for intracisternal route. Radiopaque tracers, radiolabeled, or dyes may be injected into the intraventricular space for studies of either the anatomy or patency of the system or for studies of the flow of cerebrospinal fluid.

Since cerebrospinal fluid bathes such critical organs as the brain and spinal cord and since one of its functions is believed to be a protective or cushioning fluid for these organs, any disturbance of this fluid or the membranes containing it may be deleterious and possibly lethal. Any foreign material, chemical or biological, when injected into the system may precipitate an inflammatory response anywhere or everywhere within the system. Strict aseptic techniques should be adhered to when entering the ventricles to prevent iatrogenic infections, and care should be exercised to be certain that the substances injected or infused are not irritating to the cells lining the ventricular or subarachnoid spaces. If irritating drugs are injected, ventriculitis or myelitis may result (sometimes progressive), producing obstruction of the system (hydrocephalus) or permanent neurological injury.

The vehicles employed for intraventricular injection should have physical characteristics as close to the cerebrospinal fluid as possible. If the ventricles are small or almost closed because of intracerebral edema, these spaces may be difficult to locate, and undesirable intracerebral injection of the drug with subsequent neurological injury may result. In addition, hemorrhages in the subdural, epidural, intraventricular, or intracerebral regions may occur. If the ventricular needle is inserted too far, passing through the ventricles, damage to the basal ganglia, thalamus, or other vital structures may occur. The procedure should be carried out only by experienced personnel.

To administer drug products via this route a 3.5 inch long, smooth-bore, 18-gauge, stainless steel, blunt-ended ventricular needle is used. The patient's skin is prepared as in any surgical procedure, taking extreme care to maintain strict aseptic technique. A twist drill puncture of the cranium is first performed, generally over the coronal suture about 2 cm from the midline and in line with the ipsilateral pupil. The needle, which is a special blunt, open-ended needle, is passed through the frontal lobe into the lateral ventricle. When repeated injections or infusions are required, use of an Ommaya (15) or Rickam (16) reservoir or similar silicone, elastomer, SC reservoir is recommended. Surgical placement of the reservoir may be accomplished in a variety of ways. Often with these devices no local anesthetic is required for reinjection, and the system may be sampled and injected repeatedly with minimum disturbance to the patient and with reduced risk of infection.

Intrathecal Route

Intrathecal (Latin *intra* "inside," Greek *theka* "capsule," "hull") is an adjective that refers to events that happen inside the spinal canal. An intrathecal injection (often simply called "intrathecal") is an injection into the spinal canal (intrathecal space surrounding the spinal cord), as in a spinal anesthesia or in chemotherapy or pain management applications (Fig. 6). This route is also used for some infections, particularly postneurosurgical. Drugs given intrathecally often have to be made up specially by a pharmacist or technician because they cannot contain any preservative or other potentially harmful inactive ingredients that are sometimes found in standard injectable drug preparations.

This route is a very popular for a single 24-hour dose of analgesia (opioid with local anesthetic). However extreme control had to be employed during dosing as most narcotic pain medications can cause a late onset respiratory depression when administered through this route. Often reserved for spastic cerebral palsy, intrathecally-administered baclofen is done through a intrathecal pump implanted just below the skin of the stomach with a tube connected directly to the base of the spine, where it bathes the appropriate nerves using low dose baclofen (17). Intrathecal baclofen also carries none of the side effects, such as sedation, that typically occur with oral baclofen. It is the preferred route for long-term management of

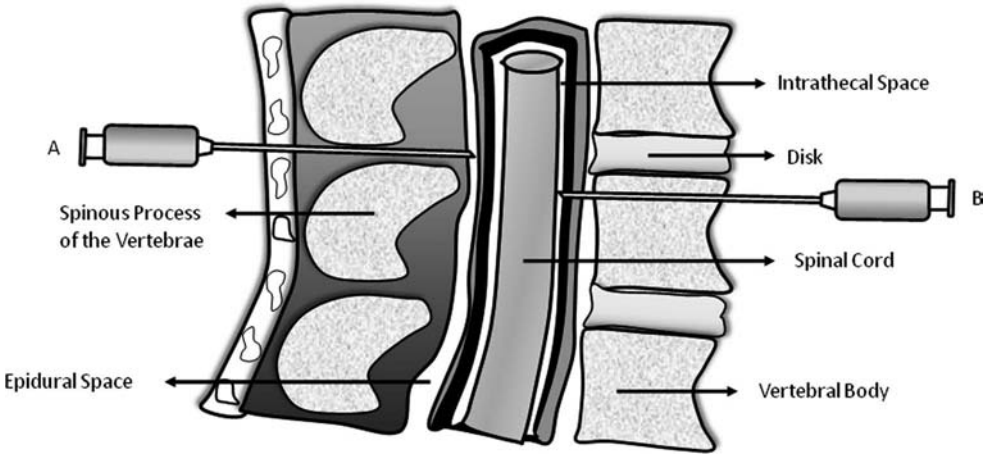

Figure 6 Schematic representation of an intrathecal administration (A) epidural route; (B) intrathecal route.

spasticity in people with cerebral palsy for whom other procedures, such as rhizotomy or orthopedic surgery, are inappropriate.

The same precautions required for intraventricular administration apply to use of the intrathecal route. In addition, a real threat of tonsilar or brain stem herniation (and possibly death) exists if this procedure is performed while intracranial pressure is elevated. Great care must be exercised to avoid this complication, which usually occurs one to two hours or sooner after removal of fluid. To administer via this route a 3.5 inch long, smooth-bore, beveled, 20- to 22-gauge stainless steel spinal needle is used for adults. The patient's skin is prepared as in any surgical procedure, taking the greatest caution to use aseptic technique. The needle is inserted posteriorly at the midline into any space below the third lumbar spinal process. The patient is in the lateral decubitus position with head, back, and thighs flexed. If intracranial pressure is diffusely elevated, the special precautions outlined above should be taken, but if intracranial masses are suspected, this procedure should not be done.

Epidural Route

The epidural space (or extradural space or peridural space) is a part of the human spine. It is the space inside the bony spinal canal but outside the membrane called the dura mater (Fig. 5). In contact with the inner surface of the dura is another membrane called the arachnoid matter. The arachnoid encompasses the cerebrospinal fluid that surrounds the spinal cord. The term epidural is often synonymous with epidural anesthesia, is a form of regional anesthesia involving injection of drugs through a catheter placed into the epidural space. The injection can cause both a loss of sensation and analgesia, by blocking the transmission of signals through nerves in or near the spinal cord.

Injecting medication into the epidural space is primarily performed for analgesia (18). This may be performed using a number of different techniques and for a variety of reasons. A patient receiving an epidural for pain relief typically receives a combination of local anesthetics and opioids (19). This combination works better than either type of drug used alone. Common local anesthetics include lidocaine, bupivacaine, ropivacaine, and chloroprocaine. Common opioids include morphine, fentanyl, sufentanil, and meperidine in the United States. These are injected in relatively small doses. Occasionally, other agents may be used, such as clonidine or ketamine.

When a catheter is placed into the epidural space, a continuous infusion can be maintained for several days, if needed. Epidural analgesia may be used for the following: (*i*) Analgesia alone especially where surgery is not contemplated. An epidural for pain relief (e.g., in childbirth) is unlikely to cause loss of muscle power, but is not usually sufficient for

surgery. (*ii*) An adjunct to general anesthesia. The anesthetist may use epidural analgesia in addition to general anesthesia. This may reduce the patient's requirement for opioid analgesics. This is suitable for a wide variety of surgery, for example, gynecological surgery (e.g., hysterectomy), orthopedic surgery (e.g., hip replacement), general surgery (e.g., laparotomy) and vascular surgery (e.g., open aortic aneurysm repair). (*iii*) As a sole technique for surgical anesthesia. Some operations, most frequently cesarean section, may be performed using an epidural anesthetic as the sole technique. Typically the patient would remain awake during the operation. The dose required for anesthesia is much higher than that required for analgesia. (*iv*) For postoperative analgesia, in either of the two situations above. Analgesics are given into the epidural space for a few days after surgery, provided a catheter has been inserted. Through the use of a patient-controlled epidural analgesia (PCEA) infusion pump (20), a patient may be given the ability to control postsurgical pain medications administered through the epidural. (*v*) For the treatment of back pain. Injection of analgesics and steroids into the epidural space may improve some forms of back pain. (*vi*) For the treatment of chronic pain or palliation of symptoms in terminal care, usually in the short or medium term. The epidural space is more difficult and risky to access as one ascends the spine, so epidural techniques are most suitable for analgesia for the chest, abdomen, pelvis or legs. They are much less suitable for analgesia for the neck, or arms and are not possible for the head.

There are certain instances where the risks of an epidural are higher than normal. Anatomical abnormalities, such as spina bifida, meningomyelocele or scoliosis could be a major limiting factor for using this route. If the patient has previous history of spinal surgery, which can lead to scar tissue, can potentially cause disruption in the distribution of the medication. Use of this route is not recommended for patient suffering from certain CNS disorders like multiple sclerosis. Certain heart-valve problems such as aortic stenosis, where the vasodilation induced by the anesthetic may impair blood supply to the thickened heart muscle, may be fatal.

A particular type of needle known as a *Tuohy* needle is used. This needle is specially designed for locating the epidural space safely, and has several specific features. The needle is inserted to the ligamentum flavum and a loss of resistance to injection technique is used to identify the epidural space. This technique works because the ligamentum flavum is extremely dense, and injection into it is almost impossible. The anesthesiologist attaches a syringe to the Tuohy needle and advances it slowly. The syringe may contain air or saline. The principles are the same, but the specifics of the technique are different because of the greater compressibility of air with respect to saline. When the tip of the needle enters a space of negative or neutral pressure (such as the epidural space), there occurs a "loss of resistance" and is possible to inject through the syringe (21).

Traditionally anesthesiologists have used either air or saline for identifying the epidural space, depending on their personal preference. However, evidence is accumulating that saline may result in more rapid and satisfactory quality of analgesia (22,23). In addition to the loss of resistance technique, real-time observation of the advancing needle is becoming more common. This may be done using a portable ultrasound scanner, fluoroscopy or real-time X-ray (1).

Intra-articular Route

Injection or infusion into the synovial sacs of accessible joints is termed as an intra-articular injection (Fig. 7). Antibiotics, lidocaine, and antiinlammatory drugs, like corticosteroid, may be administered into joints for the treatment of infections, pain, inflammation, or other problems resulting from inflammatory diseases (e.g., rheumatoid arthritis or trauma). Some agents are administered in single injections and some (e.g., antibiotics) via continuous infusion and "bathing" of the joint.

Intra-articular injections are easily accomplished in the knee, ankle, wrist, elbow, shoulder, phalangeal, sternoclavicular, and acromioclavicular joints. Joints deformed by any disease process (e.g., rheumatoid arthritis or trauma) may be more difficult to enter and inject. Usually, the intra-articular approach is utilized when no more than one or two joints are involved. Often it supplements systemic therapy since; when the synovium is inflamed it is

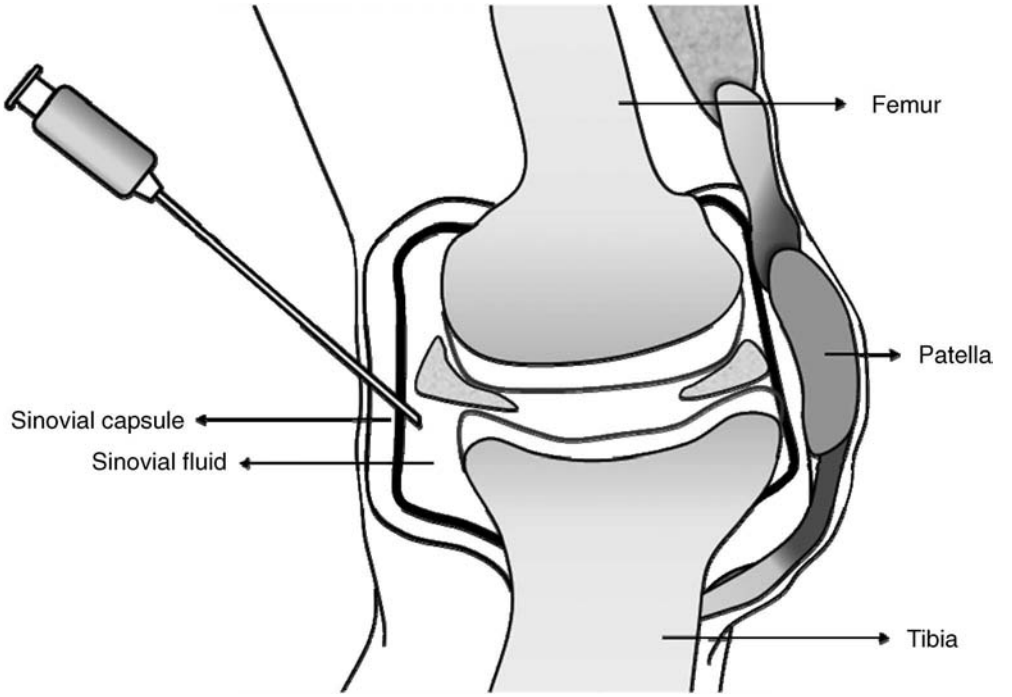

Figure 7 Schematic representation of an intra-articular administration.

often highly vascularized, permitting a multitude of agents to enter with ease from the intravascular compartment.

Iatrogenic infection is always a threat following intra-articular injection. The consequences of such infection may result in destruction of the joint. Administration of corticosteroids is particularly troublesome because if serious infection does occur, recognition may be delayed because of suppression of the local inflammatory response; thus destruction of the joint and the cartilage may occur before the identification of a complicating infection. Severe, recurrent, intra-articular hemorrhage may be produced if a bleeding diathesis, such as hemophilia or severe hypoprothrombinemia, is present. Ordinarily, such blood is resorbed, but with recurrent hemorrhage eventual destruction of weight-bearing joints may occur. If the therapist is inexperienced, tendons may be ruptured if appropriate administration technique is not employed.

The anatomy of the joint to be treated should be studied by X-ray or imaging techniques prior to injection. Entry should be at the point where the synovial cavity is most superficial and free of large vessels and nerves. The site of skin entry is cleaned and prepared as with any surgical procedure; strict aseptic technique is mandatory. A sterile, 19- to 22-gauge, stainless steel needle attached to a syringe is inserted into the synovial cavity. The synovial fluid should be first aspirated to ensure that the needle is within the joint space. The syringe is changed, and one containing the drugs to be injected is attached and administered.

Intra-abdominal Route

This route is also known as the intraperitoneal route. An injection or infusion directly into the peritoneal cavity via a needle or indwelling catheter or directly into an abdominal organ, such as the liver, kidney, or bladder is defined as a intra-abdominal injection. The intra-abdominal route may be employed to treat local or widespread intra-abdominal disease due to microbial infection or tumor. The route is also employed to dialyze (peritoneal dialysis) various toxic substances from the abdomen when severe renal failure prohibits excretion. Another

application of this route is to determine the patency, as well as the structure, of various vascular or lymphatic systems employing radio opaque agents.

The intra-abdominal route of administration can cause serious abdominal infection (peritonitis) and hemorrhage. The source of infection may be extrinsic (e.g., from skin or contaminated drugs or infusates) or intrinsic (e.g., from puncture of the bowel). The risk of infection is enhanced if an indwelling catheter, rather than a single injection using a sterile needle, is utilized. Such infections are particularly difficult to treat, especially in the presence of ascites; thus every precaution should be taken to prevent them. In addition, an aseptic peritonitis may be induced if the agent or fluid injected is highly irritable or contains endotoxin. The chance of inducing hemorrhage is related generally to the size of the needle employed, the anatomical site selected for injection, the skill of the technician, and any tendencies of the patient to bleed (i. e., coagulation problems). If hemorrhage is induced, it may be difficult to control and may require surgical intervention and repair.

Drugs injected into the intraperitoneal space are usually absorbed into the vascular compartment, and under certain pathological conditions this can be unpredictable. This can result in an uncontrolled risk of toxicity or therapeutic failure. To administer a drug intraperitonially, suitable aseptic preparation of the skin should be carried out. A 16- or 18-gauge, stainless steel needle is then inserted through the anterior abdominal wall just lateral to the rectus muscles. If ascites is present, there is little risk of bowel puncture; however, if the peritoneal cavity is "dry," puncture of the bowel may occur (indicated by aspiration of fecal contents). Bowel puncture may be avoided by shallow punctures and withdrawing on the plunger while advancing the needle.

Intracardiac Route

An injection directly into chambers of the heart or the cardiac muscle is called as an intracardiac injection. The use of this route is not common for delivery of drugs. Nevertheless, under unusual circumstances and in certain emergency situations, such as cardiac arrest, in which drugs may have to reach the myocardium immediately, intracardiac injections may be employed.

One of the major risk factors is the damage inflicted on the heart muscle, coronary arteries, or the conducting system due to trauma of an injecting needle or by the drug injected. Occasionally, hemorrhage into the myocardium or pericardium may result, leading to infarction or pericardial tamponade. If extracardiac structures such as the lung are inadvertently punctured, a pneumothorax may result and breathing may be impaired.

Selection of the route may be influenced by the presence of left or right ventricular hypertrophy, the former being better suited for the anterolateral approach and the latter being better suited for the medial approach, or any anatomical derangements of the chest which may exist. Generally, a beveled, 18- to 21-gauge, 4 to 6 inch long, stainless steel needle is used.

Intraocular Route

Injection of drug products directly into the various chambers of the eye is collectively termed as intraocular injection (Fig. 8). Four types of intraocular injections are utilized. These include (*i*) anterior chamber: injection or irrigation directly into the anterior chamber of the eye; (*ii*) intravitreal: injection directly into the vitreous cavity of the eye; (*iii*) retrobulbar: injection around the posterior segment of the globe; and (*iv*) subconjunctival (4). Although included under this heading, subconjunctival (and retrobulbar) injections are not intraocular (Fig. 9). Instead, such injections are adminstered beneath the conjunctiva, so that medication diffuses through the limbus and sclera into the eye. This route is generally used in the treatment of infections and inflammatory diseases of the eye which are not treated effectively by topical or systemic drug administration for anesthesia of the globe (retrobulbar) and occasionally for pupillary dilation with cycloplegics and mydriatics. Absorption of drugs into the eye is challenging, as intraocular transport and diffusion are poor. Intraocular injections are complemented frequently by IV infusions of the therapeutic drugs employed. Selection of the type of intraocular injection depends on the disease present and the precise location of that disease within the eye.

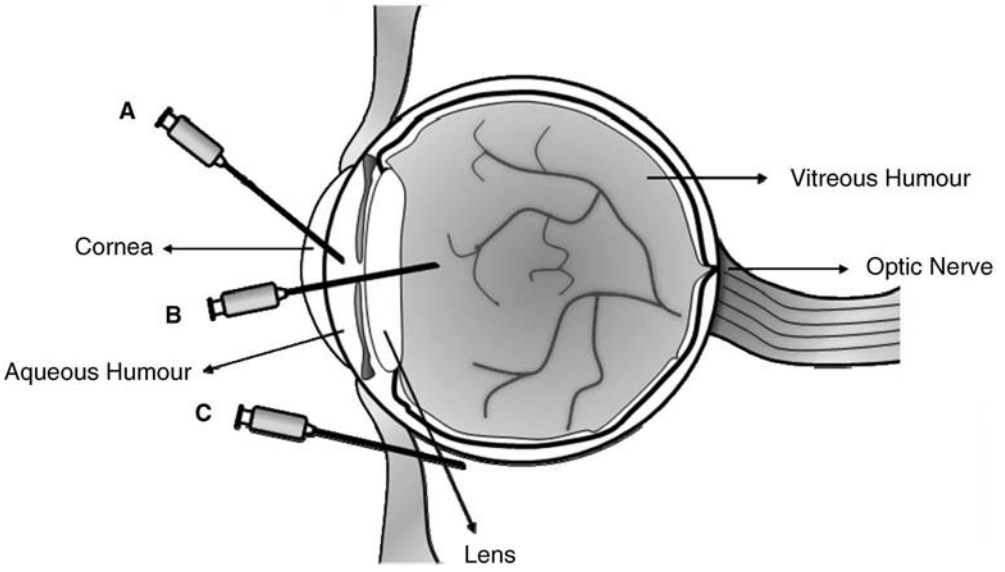

Figure 8 Schematic representation of an intraocular administration: (**A**) anterior chamber injection, (**B**) intravitreal injection, and (**C**) retrobulbar injection.

Extreme care and precise technique are required to minimize or prevent damage to the eye, especially to the corneal endothelium. Complications that can occur, depending on the route selected, are optic nerve damage, hemorrhage, retinal detachment, retinal necrosis, cataracts, and injection of the drug directly into the circulation with consequent systemic effects. Infection is always a threat and must be avoided as such infections may result in rapid destruction of the eye and/or blindness. The volume of solution that may be injected into the eye is severely restricted, generally to not more than 0.1 to 0.2 mL. Since an excellent knowledge of the anatomy and function of the eye is required, only an ophthalmologist should attempt these procedures.

The anterior chamber (containing the aqueous humor) is entered at a point located on the edge of the cornea (the limbus) with a 25-gauge or smaller, stainless steel needle, withdrawing a volume of fluid prior to injection equal to that to be instilled. For intraocular injections excluding the anterior chamber, a drop of 1:100,000 dilution epinephrine may be placed on the iris to dilate the pupil. Great care must be taken not to inject or damage the lens, as this may result in cataract formation.

Entry into the vitreous humor is accomplished by injection through the pars plana (junction of retina and ciliary body) with a 25-gauge stainless steel needle. The vitreous appears to be an inert fluid which is not replaced once removed. During injection, great care must be taken not to detach the retina. Again, a volume of fluid equal to that to be injected must be removed before instillation. Generally, not more than 0.1 mL may be injected. Injection of steroids into this chamber can be dangerous, resulting in destruction of the retina (retinal necrosis).

Entering the retrobulbar space involves insertion of the needle at the junction of the lateral and medial third of the orbital rim and then advancing the needle toward the apex of the orbit. Care must be taken not to inject the optic nerve directly. A 1 to 0.5 inch long, 25-gauge stainless steel needle is generally employed. Subconjunctival injections generally do not exceed volumes of 0.5 mL. This route is especially used in treating corneal abscesses. Injection of the sub-Tenon fascia is utilized for the treatment of uveitis (e.g., secondary to localized sarcoidosis) or chronic cyclitis. Again, care must be taken not to inject or nick the orbit.

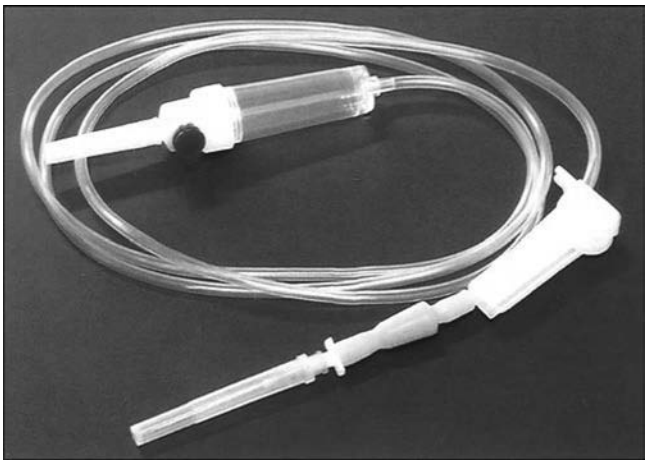

Figure 9 Picture of a standard infusion set indicating its components: a piercing spike; a vent; a drop chamber; a connection tubing; a roller clamp; a luer fitting; and a protective cap on the spike.

PARENTERAL DRUG ADMINISTRATION: METHODS AND DEVICES

This section describes the factors which determine the necessity of exact dosage as well as those which affect the flow of the infusion. Various infusion techniques such as gravity infusion, positive pressure infusion as well as other highly specialized types of infusion equipment will be discussed. Related information about their function and areas of application will be provided.

GENERAL CONCEPTS

Venous or arterial administration of a liquid into the circulatory system requires an accurate dosage and the infusion technique employed determines the accuracy of the dosage. The required dosage accuracy is generally dependent on the patient's status as well as on the type and amount of fluid to be infused, and the infusion equipment used. The flow of the infusion is affected by a range of factors including resistance in the channel of the piercing spike; resistance in the tubing and in the connector pieces; speed of drop formation; variability of the delivery pressure; and physicochemical characteristics of the solution.

GRAVITY INFUSION

The technique is the most frequently used one comprising of more than 80% of all infusions performed. The accuracy of the dosage and the infusion rate requirements are low for this type of infusion (±50%). The volume administered is based on the hydrostatic pressure differential between the patient and the infusion container. The rate of fluid administration can only be accelerated through compression of the container or by increasing the internal pressure of the container. Over the years, a standardised infusion set (Fig. 9) has been developed. Components used for this type of infusion are; a piercing spike; a vent; a drop chamber; a connection tubing; a roller clamp; a luer fitting; and a protective cap on the spike.

Depending on the type of container to be used with, the piercing spike is sharp for rubber stoppers or rounded and blunt for bag insertion sites. The infusion bag contains one channel for fluid and optionally a second channel for venting with a cap or stopper. Upon opening of a cap or stopper air flows into the container. The vent usually is equipped with a bacterial filter. A drop generator is located at the top of the drop chamber, which produces drops of a certain size. The chamber is partially filled with liquid to prevent air bubbles from entering the tubing. A particle filter is often located at the bottom outlet of the chamber. The connecting tube is usually 150 cm long and made of PVC. These are also available in other lengths and materials for special applications. The roller clamp supplied within the connecting tube is used to

regulate the flow rate of infusion by controlled compression of the tubing. The Luer fittings at the end of the line, guarantees a secure connection to all other products by means of the standardized Luer cone. In the lock version the lock connection is further secured against jerks and pressure by means of a screw thread. This prevents damage to the packaging and thus loss of sterility. The standardised infusion set is connected to a infusion container (bottle, bag) using the spike.

The rate of the infusion is a critical factor for gravity infusion and is mainly regulated by means of the roller clamp in most of the hospital settings. The roller clamp is positioned on the infusion tubing of the infusion set in such a way that the lumen of the infusion tubing is compressed from outside. With respect to gravity infusion the rate of infusion is calculated on the basis of number of drops/min. Most standard infusion sets are designed to deliver approximately 20 drops/min (equivalent to 1 mL/min). Specialized roller clamps are available that allow for drop rates of 60 drops/min. However, even with higher drop rates, the microdroppers (e.g., Dosifix® from B. Braun) still delivers only 1 mL/min; that is, 60 drops = 1 mL/min.

Another type of flow regulator is the tubing independent flow regulators that can replace the traditional roller clamp for improved control of dosage accuracy. The flow rate is controlled by varying the size of an accurately designed flow channel and flow rates can range from 3–200 mL/hr. These units are used for infusion solutions which are carrier solutions for drugs that need to be administered at a specific concentration for longer duration. It is important to note that an ideal flow regulator is the one that can maintain the desired flow rate irrespective of changes in the infusion height and patient activities.

PRESSURE INFUSION

In certain instances during IV administrations using infusion or transfusion bags, a pressure infusion may be performed. For this purpose a pressure cuff is used which is pumped up with an inflation bulb in a similar manner as with a blood pressure measurement instrument, thus exerting pressure on the container. A pressure of up to a maximum of 300 mmHg can be exerted on a regular infusion bag. Other types of positive pressure infusion equipments are available and employed for such infusions. They are especially used when the dosage accuracy is required or increased rate of infusion is needed or when a constant rate of delivery during long-term infusions is desired. The infusion equipment used should meet certain and the important criteria: (*i*) requirement-based infusion rate, (*ii*) exact dosage, (*iii*) robustness of equipment, (*iv*) quick functional readiness, (*v*) simple and safe operation, (*vi*) alarms for interruption of infusion or in the event of danger, (*vii*) mains-independent operation, and (*viii*) easy cleaning.

Depending of different applications and administrations to be performed, the required infusion rates extend over a wide range. Pressured infusion rates may vary from 1 mL/hr and $>$ 1000 mL/hr (e.g., shock therapy) for adult patients. Such a type of infusion is generally used in an intensive care medicine scenario. Cost of equipment for pressured infusion can also be a limiting factor for many settings. The degree of accuracy of dosage depends on the status of the patient, the solution to be infused and other factors. Also, the degree of accuracy a dosage can have is determined by the kind of infusion technique that is employed.

With regard to these techniques, distinctions are made between gravity infusion, pressure infusion and the use of infusion equipment. Additional infusion equipment is required when the dosage accuracy should be increased, the rate of infusion should be raised or when a constant rate of delivery during long-term infusions should be achieved. In equipment-supported infusion techniques, distinctions are made between infusion regulators, that is, electronic medical devices without a delivery drive, infusion pumps and syringe pumps. In contrast to the infusion regulators, infusion pumps have their own delivery drives. Depending on the type of drive, there is a distinction between roller pumps, peristaltic pumps and plunger or syringe pumps. The accuracy of the dosage mainly depends on how the pumps are regulated. Syringe pumps are pressure infusion devices which administer the content of one or more syringes simultaneously using a precision linear drive. This form of infusion is particularly suited for an exact administration of drugs.

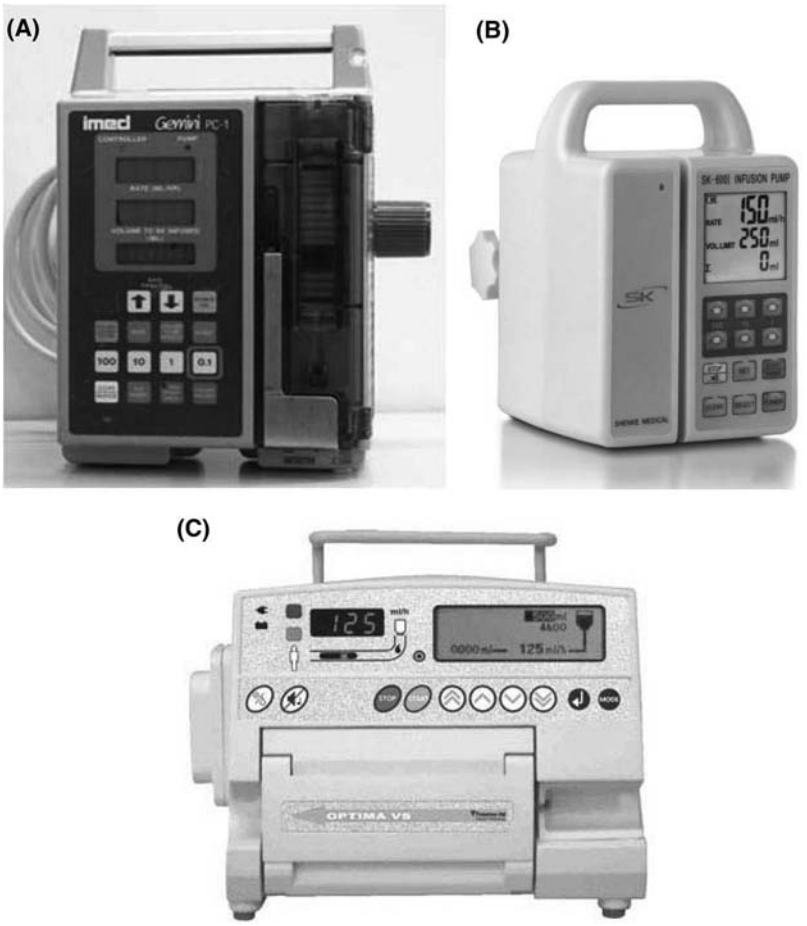

Figure 10 Examples of different types of infusion pumps: (**A**) a roller infusion pump, (**B**) a syringe-driven pump, and (**C**) a peristaltic infusion pump.

Types of Equipment

Over the recent years significant advances have occurred in the area of pressure infusion or positive pressure infusion. Most of the infusion systems available in today's market are highly sophisticated, precise, and electronically advanced requiring specialized training. They can be broadly classified into three distinct classes: (*i*) infusion regulators, (*ii*) infusion pumps, and (*iii*) syringe pumps. Other infusion devices like the disposable infusion pumps, smart pumps, and associated accessories are regularly employed in different medical settings (Figure 10).

a. *Infusion regulators*: Infusion regulators are electronic medical devices which do not have their own delivery drive. They regulate and monitor the supply of fluid in the flow process. Simply stated, they are mechanized roller clamps. The dosage accuracy is often sufficient for everyday clinical purposes and ranges between $\pm 10\%$ and 20%.
b. *Infusion pumps*: In contrast to the regulators, infusion pumps are equipped with their own delivery drive. Depending on the type of drive, it can be classified as roller pumps, peristaltic pumps and piston pumps (Fig. 10). The main purpose of an infusion pump is to deliver medication(s) at a regulated rate and thereby in a regulated dose. Control of infusion pumps can either be drop based or volume based. The basic design of infusion pumps comprise of a delivery drive, a control or regulating system, and an infusion set. The dosage mainly depends on how the

pump is regulated. Roller pumps and peristaltic pumps are examples of volume-based pumps. The delivery principle of a roller pump is based on the rollers bringing a set amount of fluid into the tubing which is then transported by help of rotation in the flow direction. On the other hand, delivery principle of a peristaltic pump depend on the successive compression of the tubing by the individual fingers, makes the fluid be advanced forward.

In the case of the drop regulated infusion pumps, the dosage accuracy of these pumps relates to the number of drops (per minute) and depends on the volume of the drops. The drop accuracy is subject to several important conditions such as the viscosity of a solution, the solution's surface tension and the flow behavior resulting from these factors. Dosage accuracy is ±1 0%.

Accurate fluid infusion and drug administration is crucial for the optimum management of a critically ill patient. Continuous and controlled IV delivery of common medications, such as inotropic agents, vasodilators, aminophylline, insulin, heparin, etc., via infusion pump is the preferred mode of therapy in acute care. This is especially true for drugs with short half lives, so as to maintain a desirable constant serum concentration and in situations when constant infusion of glucose is needed. Patients with compromised renal, cardiac or pulmonary function have limited fluid tolerance and hence it is essential to use infusion pumps so as to prevent inadvertent volume overload. For intensive care, more than one infusion pump is often used when drug dosage, concentration, interaction and fluid volume require separate infusion rates. The use of infusion pumps has been advocated over manual flow control system on the basis of assuring precise and accurate delivery of prescribed fluid volumes over a specified time and to help in better nursing management.

The performance of infusion pumps is generally acceptable for clinical use, but the volume that may be infused is limited by the syringe capacity and infusion must be stopped whenever it is necessary to replace or refill the syringe. The largest syringe accepted by these pumps accommodates 100 mL of drug product. The small weight and no interference of gravity and positioning makes these syringe pumps suitable for transport. These pumps can be mounted on an IV pole or on the operating table. In addition these are small and light weight and have an occlusion alarm pressure of 570 mmHg.

Recently introduced modern infusion pumps incorporate a soft key interface by which a range of body weight and drug concentrations can be entered. Bolus doses can be easily and rapidly administered at any time during the infusion. These systems are also modifiable to accept all syringe sizes from 10-100 mL and have two independent microprocessors to monitor and control infusion processes for consistent delivery.

c. *Syringe pumps*: The syringe pump has been defined as a power driven device for pushing the plunger of a syringe forward at an accurately controlled rate. These are pressure infusion devices which supply the content of one or more syringes simultaneously by means of a precision linear drive. The dosage accuracy with these pumps is ±2% since a precise syringe volume is delivered through these pumps and all the error sources involved in drop regulation do not apply. This form of infusion is particularly suited for an exact administration of drugs with a dosage rate of 0.1 to 200 mL/hr. Special syringes of 10, 20, and 50/60 mL are commercially available. Because infusion pumps work with a maximum pressure of 1 bar, all tubings connected with such pumps need to be pressure resistant for safety reasons.

Previous research has demonstrated that variation occurs when different types of syringes are used with electronic syringe drivers (Medical Device Amendment (MDA), 2003). For example, it has been reported that there is a difference in the amount of drug delivered and the occlusion to alarm time in two different types of syringe (24). Similar findings are associated with spring devices (25). Luer-lock syringes are commonly recommended to avoid separation of the syringe and infusion set. This is particularly important for subjects who may be restless or lack of

understanding about the importance of protecting the device. Clearly, the type of syringe should be standardized to avoid variation in infusion rate and ensuing symptom control. The MDA (2003) recommends using specific types of syringes as indicated by the manufacturer of the pump used.

Nonelectronic spring driven devices work on the principle that the syringe compresses the spring and the flow of liquid from the syringe is controlled by tubing with a restrictive narrow bore (Springfusor®). Such devices are reported to be advantageous in comparison with electronic devices in terms of cost and simplicity of use. A number of researchers have compared the two in terms of accuracy and reliability. One disadvantage of the Springfusor is that it is calibrated at 25°C and is affected by temperature variation. When the temperature rises, for example, if the device is close to the skin or under the bed clothes, the flow rate increases. Although this is not expected to cause clinical effects in adults, it may well have implications for children in terms of over-infusion (26).

SMART PUMPS

Studies indicate that although 38% of errors occur at the time of drug administration, only 2% are actually caught (27). Roughly 35% to 60% of all harmful IV medication errors can be directly associated with the use of an infusion pump device (28). Because many of these harmful errors occur with drugs that are classified as high-alert medications it is not a surprise that safety-minded organizations are choosing to convert their infusion pumps to the newest form of "smart infusion devices." The term "smart" or "intelligent" is used to describe this pump technology because these infusion devices contain error reduction software with the ability to store organization-specific dosing guidelines, and they produce real-time alerts for practitioners when attempts are made to program doses outside of the established safe range. Smart pumps are computerized infusion devices with dose-error reduction software designed to help avert IV programming errors, as well as other errors associated with infusions (29). Smart pumps differ from older pumps because they can be programmed to include facility customized drug libraries—lists of IV medications and their concentrations. Software provides point-of-care decision support for high or low infusion rates. The device prompts the user to choose a medication from the library, confirm the selection, input a volume to be infused, and input an infusion rate or dose. For all medications selected from the library, the keypad entry of an infusion rate in milliliters will automatically calculate the equivalent dose in units, milligrams or micrograms (5).

PATIENT-CONTROLLED ANALGESIA

One of the most common methods for providing postoperative analgesia is via patient-controlled analgesia (PCA). Although the typical approach is to administer opioids via a programmable infusion pump, other drugs and other modes of administration are available. There are several advantages of using a PCA (30). It reduces the time between when the patient feels pain and/or the need to receive analgesia and when it is administered (activation automatically pumps the dose into a preexisting IV line into the patient). It also reduces the workload of the nursing staff (an amount of the prescribed analgesic is preloaded into the PCA, enough for multiple doses) and the chances for medication errors. The PCA is programmed per the physician's order for amount and interval between doses and "locks out" the patient if he or she attempts excessive self-administeration. Patients can receive medicine when they need it, instead of having to wait for nurse practitioner or caretaker. Patients who use PCAs report better analgesia and lower pain scores than those patients who have to request analgesia from the nursing staff when they are in pain. Additionally careful examination of the syringes in a PCA provides a measurement of how much pain an individual patient is experiencing from one day to the next. It involves patients in their own care, giving them control and ultimately rendering better patient outcomes.

PCAs do suffer from certain disadvantages. Patients may be unwilling to use the PCA or be physically or mentally unable to. However, PCA pumps are rated among the world's most accessible pieces of equipment since all manufacturers must have alternative switch access

built into their PCA pumps. Most companies employ a TASH (The Association for Persons with Severe Handicaps) approved switch interface connection as TASH is one of the industry standards in accessibility switches (31). The pumps are often expensive and may malfunction.

DISPOSABLE INFUSION PUMPS

All nonelectric disposable pumps exploit the same physical principle: mechanical restriction within the flow path determines the speed of pressurized fluid. The pressure on the fluid is generated by a variety of mechanisms using nonelectric power, including a stretched elastomer or compressed spring, pressure generated during a chemical reaction (32), and pressure supplied from a cartridge of pressurized gas. The restriction of flow in all disposable pumps is caused by narrow-bore tubing. Tubing diameter has a determining influence on the device's flow rate. Therefore, flow restrictors are usually made of materials whose dimensions change little with temperature to maintain accuracy. Glass capillary-flow restrictors are typically used for devices infusing at a rate of 0.5–10 mL/hr; plastic is typically used for flow restrictors of pumps infusing at rates of 50–250 mL/hr. The flow restrictor is always integral to the administration set. The administration set can be integrated within or can be detachable from the pump reservoir.

Elastomeric infusion pumps are disposable devices, in which the pressure on the fluid is generated by the force of a stretched elastomer. Elastomeric disposable pumps consist of an elastomeric membrane, which contains the drug that is contained within an outer protective shell. The outer protective shell can either be a conformable elastomer (e.g., Homepump Eclipse®, BBraun) or a more rigid plastic (e.g., Infusor®). A soft elastomeric outer shell offers less protection against sharps puncture but requires less storage and disposal space. The membranes of elastomeric pumps are made of various elastomers, both natural and synthetic (e.g., isoprene rubber, latex, and silicon), and can be made of a single or multiple layers. The type of elastomer and the geometry of the elastomeric balloon determine the pressure generated on the fluid when the balloon is stretched (33). Multiple-layer elastomeric membranes can generate higher pressures than the single-layer membranes. Elastomeric pumps operate with a driving pressure of 260–520 mmHg and infuse at rates of 0.5–500 mL/hr.

Another type of disposable pump used is negative-pressure pumps. With negative-pressure pumps, a driving force is generated from the pressure difference across two sides of the pump's low-pressure chamber wall, with one side being at very low pressure (inside a vacuum chamber) and another side being at atmospheric pressure. The very low pressure in the vacuum chamber is created by the user while filling the device. Expansion of the drug reservoir, caused by the addition of fluid to the drug-containing reservoir, causes simultaneous expansion of the reduced pressure chamber, thus creating a significant vacuum. During infusion delivery, pressure on the movable wall plunger is generated by the large pressure difference between its two sides, causing it to move and compress the fluid in the drug-containing chamber.

SUMMARY

Although over the years the different routes of administration used for parenteral medications has remained the same, the science behind the design, development, and delivery of parenteral dosage forms have become complex. With continued and ever increasing need for superior dosage administration control, accuracy, and efficacy the development of newer dosage forms as well as parenteral drug delivery devices have become highly sophisticated. Additionally, new as well as older highly potent and difficult to formulate drug molecules are being rescrutinized and drugs once thought to be not viable because of poor oral bio-availability are seeing a comeback as parenteral dosage forms. These potent drug entities require accurate control of dose and higher safety margins. The advent of smarter and sleeker electronics and computers have helped to achieve this and also helped in the development of "error proof" infusion systems that have increased patient compliance and have lead to improved therapeutic outcomes. Some of these systems have considerably reduced the risks involved with parenteral administration of drugs and others show promise for safe and efficacious administration of drugs via this route.

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3 Biopharmaceutics of NCEs and NBEs

Balaji Agoram, Kazuko Sagawa, Ravi M. Shanker, and Satish K. Singh

INTRODUCTION

The term Biopharmaceutics is the study of the influence of formulation on the biological activity of a drug product, including its in vitro properties such as its physicochemical characteristics, formulation, and delivery technology (1). Pharmacokinetics (PK) is used to define the science of in vivo performance of a drug such as its bioavailability/absorption and systemic disposition, and is an important marker of the likely intensity and duration of the biological activity of the drug. Therefore, an understanding of the underlying processes governing drug absorption and disposition within the human body, methods of analyzing the characterizing the concentration-time profile, and the temporal relation between the measured concentration-time profile and the efficacy and safety time profiles are all critical elements in the design of appropriate dosage forms. This chapter has been designed to provide an overview of these topics.

The first part of this chapter focuses on the physicochemical properties of small-molecule drugs that influence their absorption by the parenteral route. An increasingly important category of injectable drugs now also includes biotherapeutics. Biotherapeutics (also called biologicals, biologics, or biopharmaceuticals) are compounds that are biologically produced as opposed to chemically synthesized. Some common examples of biotherapeutics are peptides, proteins and monoclonal antibodies. Most biotherapeutics are large hydrophilic molecules with complex tertiary structures. While the biopharmaceutical properties of small-molecule therapeutics have been extensively studied, the number of corresponding publications on injected biologics molecules is relatively rare (2). Therefore, biotherpeutics have also been considered in this chapter. However, many of the discussions on the basics of exposure (PK) and exposure-response (pharmacodynamics) analysis in this chapter are applicable to both biotherapeutics and small molecules. The impact of key physiological and physicochemical parameters on PK is also discussed in this chapter. A key biopharmaceutical aspect unique to biotherapeutics is their potential to cause immunological reactions, which can affect both PK and safety/efficacy profile. Immunogenicity, and the impact of formulation changes on immunogenicity is therefore covered in this chapter. Finally, the concept of comparability for biotherapeutics is discussed from the bioequivalence and PK perspective.

PHYSICOCHEMICAL PROPERTIES OF SMALL-MOLECULE DRUGS AFFECTING ABSORPTION BY THE PARENTERAL ROUTE

Takeru Higuchi, known as the "father of physical pharmacy" is credited with the introduction of many of the basic principles of physical chemistry that are known to influence the absorption, distribution, metabolism and excretion of drugs from the body. Although much of the literature on factors influencing absorption of drugs has focused on gaining detailed understanding after oral administration (3), the same physicochemical properties of molecules are important for absorption after administration via subcutaneous (SC), intramuscular (IM), intraperitoneal, and other extravascular routes delivered via injection. On the other hand, when a drug is injected directly into the vascular system, that is, via intravenous (IV) route then there are no physicochemical factors that affect absorption. Figure 1 provides a simplified, schematic overview of the relationships of administered dose of an injectable drug to the elicitation of the pharmacological effect, which includes therapeutic benefits as well as undesirable side effects. In the case of direct vascular injection via a bolus dose or as an infusion, the drug must be dissolved prior to administration to avoid the risks of causing

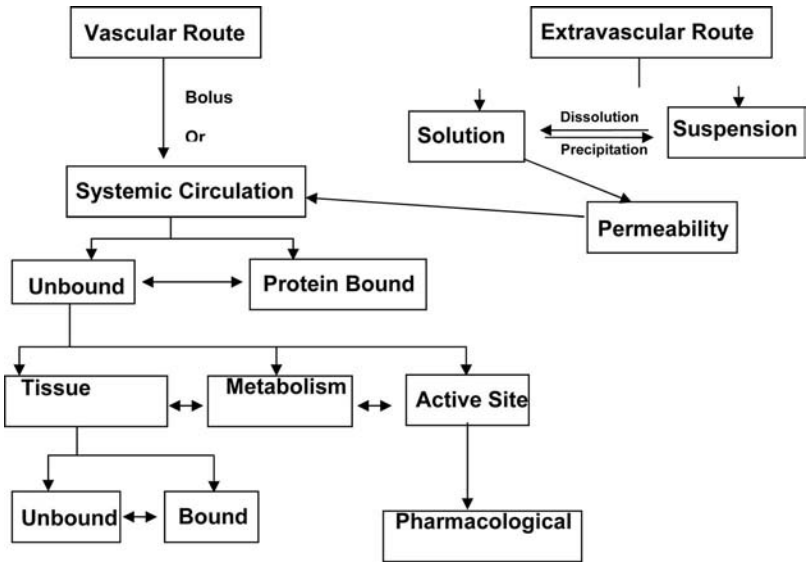

Figure 1 A schematic overview of fundamental relationships between routes of administration of injectable drugs to their ability to elicit pharmacological response.

blockage of capillaries that can affect the safety of the patient. However, injections through the extravascular route may be administered as either solutions or as suspensions of particles in aqueous or oil-based vehicles. Ultimately, for the drug to reach its intended target of the diseased tissue to elicit a pharmacological response, it is imperative that the drug must dissolve in the aqueous environment of the interstitial fluid and in the blood. Similarly, for the drug to reach the site of action from extravascular sites, it must have the ability to diffuse through cell membranes. These two essential properties of drug molecules: dissolution in aqueous and biological environment, and diffusing to reach the site of action, are governed by a multitude of physicochemical properties. The aim of this section is to provide the formulator of injectable drugs, a basic understanding of physicochemical properties of drugs that influence their PK as well as pharmacodynamics to assist with the design of drug products that can utilize these properties; to help identify formulation approaches to overcome limitations presented by any of these properties and also to assist in troubleshooting suboptimal performance of either novel or purportedly equivalent injectable drug products.

To consider the physicochemical properties of drugs that influence their absorption, distribution, metabolism and excretion it is essential to consider the anatomical and physiological characteristics of the vascular and extravascular injection sites. Detailed discussion of these factors and their impact on the design consideration of injectable dosage forms have been provided in preceding chapters. Similarly detailed discussions of preformulation and formulation approaches to quantitiatively understand the solubility and stability of a variety of injectable dosage forms are covered in various chapters. Factors such as the pH of blood, intracellular and extracellular fluid; the nature of ions and ionic strength of these physiological fluids; blood flow as well as number of capillaries at extravascular sites; the presence of lymphatic network; muscle movement; body temperature; nature of disease state; and, age of the patient are important considerations in understanding the PK and pharmacodynamics of drugs. The physicochemical properties of drugs may be broadly classified into two categories: (*i*) intrinsic properties and (*ii*) adjustable or changeable properties. Examples of intrinsic properties are molecular structure, functional groups, the ionization constant (pK_a—the negative logarithm of the ionization constant) of the functional groups, partition coefficient ($\log P$), melting point, and intrinsic aqueous solubility (of the unionized form of the drug). Examples of properties that can either adjusted or selected by the

formulator include salt forms of ionizable drugs, particle size, degree of crystallinity, amorphous form and solubilization via selection of excipients that can alter the solubility of the drug.

Ionization (pK_a)

A molecule or an atom group in a molecule may lose or gain a proton when the molecule is placed in an aqueous solution. The symbol K_a is used to describe the tendency of compounds to accept protons and is called the ionization constant. Expressed in mathematical terms, the negative logarithm ($-\log_{10}$) of the ionization constant (K_a) is defined as pK_a. Since pH is the negative logarithm of the hydrogen ion concentration ($-\log_{10}$ [H^+]), the relationship between pH and pK_a for an acidic drug can be expressed as follows:

$$\mathrm{pH} = \mathrm{p}K_a - \log \frac{[\text{Unionized}]}{[\text{Ionized}]}$$

This mathematical relationship provides an ability to calculate the fraction or percentage of ionized and unionized species of a drug in the pH of physiological interest by knowing the pK_a of the drug. This understanding of distribution of species is extremely important in predicting and quantifying the solubility, distribution coefficient (log$D$) and thus the drug's PK (ADME) and pharmcodynamics. The unionized form is the only species that diffuses through cell membranes; however, it is also the form that has the lowest aqueous solubility. Therefore, an injectable drug product when formulated at a pH to take advantage of its increased solubility in the ionized state stands the risk of precipitation of drug upon encountering physiological pH at the site of injection. This phenomenon of precipitation of drug can result in phlebitis as well as significant pain at the site of injection (4,5).

Partition Coefficient (log$P$ and log$D$)

Partition coefficient (P) of a drug is the ratio of its concentration in the two phases of a mixture of two immiscible solvents at equilibrium. Conventionally, one of the solvents chosen is water while the second is octanol (6). Logarithm of the partition coefficient is referred to as log$P$ as is defined as the ratio of the concentration of the unionized species in octanol divided by the concentration of unionized species in water.

$$P = \frac{[\text{Unionized Species}]_{\text{Octanol}}}{[\text{Unonized Species}]_{\text{Water}}}$$

Similarly, log$D$, refers to the logarithm of the distribution coefficient (D), which is defined as the ratio of the concentration of all the species, that is, unionized and ionized in octanol divided by the concentration of all species in water.

$$D = \frac{[\text{Unionized Species} + \text{Ionized Species}]_{\text{Octanol}}}{[\text{Unonized Species} + \text{Ionized Species}]_{\text{Water}}}$$

Since the fraction of unionized and ionized species in aqueous solution is governed by the pH of the solution and the pK_a of the molecule, therefore, the log$D$ or distribution of the drug is dependent on pH and pK_a. Since only the unionized molecule diffuses through biological membrane, therefore, the permeability of the drug is dependent on log$D$. The interrelationships between ionization, pH, and partitioning of the drug through biological membrane are often referred to as the "pH-partition hypothesis" (Fig. 2). These interrelations are summarized in Figure 3. The pH-partition hypothesis was first proposed to explain the influence of pH of the gastrointestinal tract on the oral absorption of drugs (7). The concept is extensively used for not only understanding oral absorption but also the toxicity of drug molecules as well as the accumulation of drugs in specific tissues. Therefore, the interrelationships between the degree of ionization, the pH of biological fluid and the distribution coefficient is important for understanding the biopharmaceutical aspects of drugs.

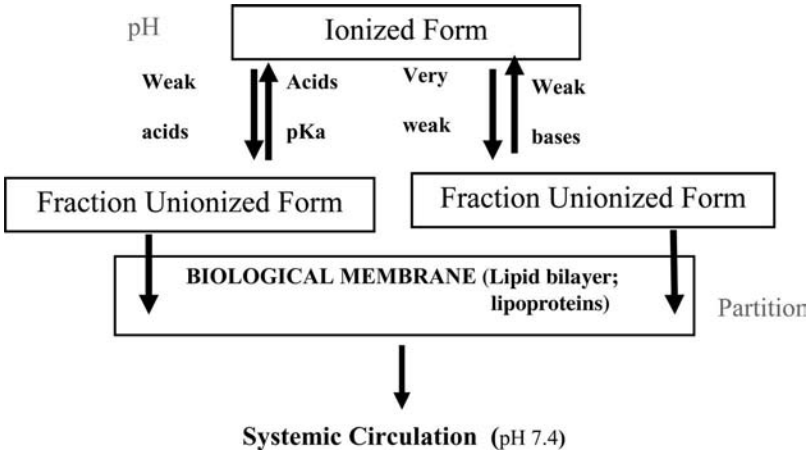

Figure 2 The pH-partition theory for the absorption of drugs across biological membrane from extravascular sites of administration of injections.

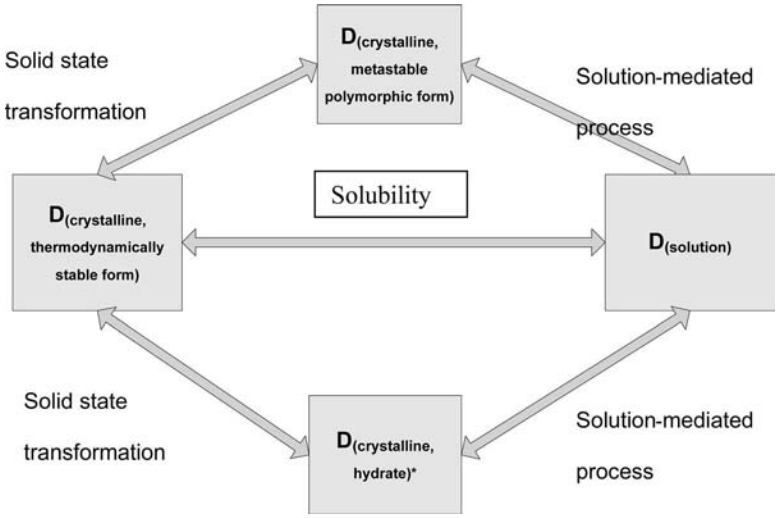

Figure 3 Potential pathways for transformation of solid form of drug (basic description for unionized drug since ionized form can undergo additional transformations to unionized form) during determination of equilibrium solubility or during transit through the body upon injection. Each arrow depicts a forward rate and a backward rate to maintain equilibrium.

Diffusion and Permeability (P_{app})

Molecular diffusion, often referred to as just diffusion is the physical phenomenon of transport of molecules via random molecular motion from a region of high concentration to one of low concentration. The phenomenon is typically described by Fick's laws of diffusion; the first law relates the diffusive flux to the concentration gradient and the second law predicts how the diffusion of molecules causes the concentration field to change with time. Mathematical expressions based on Fick's first law have been used to model transport processes in many systems including drugs across biological membranes. Fick's first law is expressed as follows:

$$J = -PA(C_2 - C_1)$$

where J is the diffusion flux in units of [(amount of substance) length^{-2} time^{-1}]; P is the permeability of the membrane (e.g., biological cell membrane) for a given molecule at a given

temperature; A is the surface area over which diffusion is taking place; and $C_2 - C_1$ is the difference in concentration or concentration gradient of the molecule across the membrane in the direction of flow of molecules ($C_1 - C_2$). Biological membranes consisting of lipid bilayer are semi-permeable in nature and are also known as selectively permeable membranes, that is, they allow certain molecules or ions to diffuse through. There are several factors that influence the permeability of organic molecules through biological, semipermeable membranes such as molecular size (molecular weight), charge on the molecule, lipohilicity (log$P$ or log$D$) of the molecule, polar surface area, number of rotatable bonds, etc. Although it is possible to utilize formulation factors to change the concentration gradient to influence the flux across the biological membrane, it is not possible to alter the intrinsic permeability of a compound using formulation approaches. Because of the challenges of experimentally determining the permeability of drugs across biological membranes, several in vitro approaches, based on cell cultures, have been utilized extensively to ascertain the apparent permeability (P_{app}). Understanding and predicting molecular descriptors that can influence permeability of drugs across biological membranes continues to be a matter of extensive fundamental and applied research (8,9).

Solubility, Dissolution, and Solubilization

The pharmaceutical literature in the past few decades has used multiple terms to describe solubility and dissolution of drugs which has often resulted in confusion and misunderstanding (10). Terms such as thermodynamic solubility, equilibrium solubility, intrinsic solubility, kinetic solubility, apparent solubility, intrinsic equilibrium solubility, dissolution rate, intrinsic dissolution rate, etc., have been used by researchers to describe different aspects of experimental observations. Aqueous solubility of solutes is a relatively straightforward thermodynamic concept, especially for crystalline drug molecules since solubility represents the concentration of drug in solution which is in an equilibrium two phase system consisting of the drug in the solid state and the solution state. This concept is often schematically illustrated by

$$\text{Solute}_{(\text{solid, crystalline, excess})} + \text{Solvent} \overset{K}{\Leftrightarrow} \text{Solute}_{(\text{solution})}$$

where K, the equilibrium constant, is the ratio of activity of solute in solution to that in the solid. Typically, when the solute concentrations are low then the solute activity coefficients are essentially unity. Since solubility is equilibrium constant, it is dependent on temperature and pressure. However, in the context of drug delivery and biopharmaceutics, pressure is not considered to be a variable. The above definition of solubility highlights the importance of characterizing the solid at equilibrium in addition to measuring the concentration of drug in solution. If the solid form undergoes a change in its solid state relative to the initial form that was used for experimentally assessing solubility then the equilibrium solubility is reflective of the new solid form (polymorph, hydrate, solvate, etc.) rather than the original form. Solid state transformations can either be mediated through solution or directly through the solid state. Figure 3 provides a schematic overview of potential transformations that can occur during experimental determination of solubility determination or during the time the dosage form is present in the body.

Since a change in drug's solid state (polymorph, hydrate, solvate, amorphous form, etc.) can result in significant change in its solubility as well as dissolution rate, such transformations as depicted in Figure 3 can have a direct impact on the biopharmaceutic performance of an injectable drug product. An additional aspect of understanding the equilibrium constant between the drug in solid state and drug in solution is the rates of the forward and backward processes.

If both the forward rate, that is, dissolution and backward rate, that is, crystallization as shown in Figure 3 were completely controlled by diffusion process, then these rates would be identical at equilibrium, which however is rarely the case. Crystallization is not merely based on diffusion but is known to be a stochastic (probability-driven) process that requires random collisions to form a critical size of nuclei before crystal growth can occur. Therefore, it is imperative to understand the impact of dilution and mixing of solubilizing excipients with biological fluids at the site of injection as the resulting decrease in solubility of the drug can

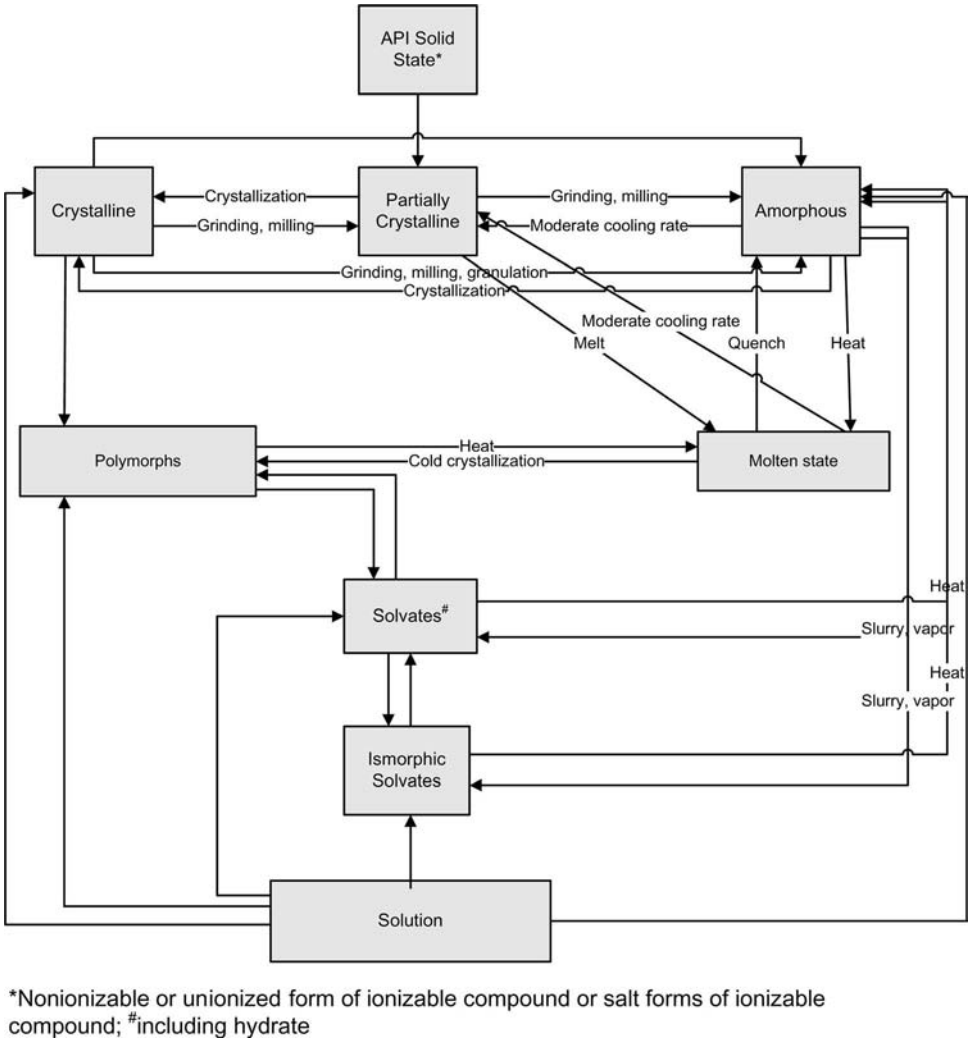

Figure 4 Processing options available for solid state transformation of drug to facilitate isolation of preferred API form for development of injectable formulations.

lead to precipitation of drug and consequently lead to decrease in available concentration of drug at the site, pain and phlebitis. Understanding of the thermodynamics and kinetics of interconversion of the solid state transformations can facilitate the development of processes that isolate the preferred stable form (thermodynamically or kinetically stable) for manufacture of dosage form. Figure 4 provides a schematic overview of the processing options available to the formulator.

In addition to equilibrium solubility, the time required to reach solubility, that is, dissolution is an extremely important phenomenon for the biopharmaceutics characteristics of an injectable suspension. There are several theories that model dissolution of solids to form solutions and the most frequently used relationship known as Nernst-Brunner equation, which was a modification of the original Noyes-Whitney equation (11). The Nernst-Brunner equation, shown below, is derived from Fick's law of diffusion and takes into account the presence of an aqueous diffusion boundary layer on the surface of the dissolving solid.

$$\text{Dissolution Rate} = -\frac{A_{\text{solid}(t)} D_{\text{Drug}}}{h_{(t)}} \left(S_{\text{Bulk}} - \frac{X_{\text{Solution}(t)}}{V_{\text{Bulk}}} \right)$$

where $A_{\text{solid}(t)}$ is the total surface area of the solid at time t; D_{Drug} is the diffusion coefficient of the drug; $h_{(t)}$ is the thickness of the diffusion layer at time t; S_{Bulk} is the solubility of the drug in the bulk liquid; $X_{\text{solution}(t)}$ is the amount of drug dissolved in bulk solution at time t; and V_{bulk} is the volume of the bulk solution. Although the Nernst-Brunner equation is useful, it is not always applicable for biopharmaceutical applications. Modeling of dissolution kinetics especially of powders is of significant practical importance especially for injectable drug products. To model dissolution of particles, knowledge of particle size distribution as well as an estimate of the thickness of the aqueous diffusion layer as a function of particle size are necessary. In terms of particle size, it is important to take into account the poydispersity of the particle size (12). Several mathematical relationships have been developed to model the dissolution of powders. However, it is important to note that the dissolution of particles from an extravascular site of injection does not follow these models adequately because of poor mixing and agitation at the site. Therefore, biopharmaceutical considerations of particle size-dependent dissolution for injectables requires the development of more complex mathematical models (13).

The influence of degree of ionization of an ionizable drug on its partitioning into biological membranes was discussed previously. Similarly, the degree of ionization greatly affects the solubility of the drug. The ionized form (either acid or base) has higher solubility than the unionized form. Theoretical pH-solubility profiles of ionizable drugs is given by the Henderson-Hasselbalch equation, which relates the solubility of the unionized form of the drug (S_0) to the dissociation constant (pK_a) to obtain the total solubility (S_T) of the drug.

The underlying assumption in these predictions is that the drug molecule does not self-associate in solution either in the unionized or ionized states. However, these relationships cannot predict the pH independent, limiting solubility of the salt forms of ionizable drugs. There are no theoretical methods available to predict the solubility product (K_{sp}) of a drug with a specific counterion. Therefore, it is essential to determine the K_{sp} experimentally. Although salt forms of ionizable drugs can provide wide ranges of solubility enhancement, it is not possible to a priori predict a preferred salt form for any drug on the basis of any basic principles. Furthermore, the rate of conversion of a salt form to its unionized state upon being subjected to a change in pH is also not predicted by any known theory or good empirical model. Knowledge of the expected solid form (as predicted by the phase rule) at equilibrium at any given pH is extremely useful in ascertaining whether the formulation as drug product or after administration at the injection site has reached equilibrium or is in the metastable state. Generally, according to the phase rule, the solid form at equilibrium is the unionized form of the drug at all pH values in the K_{sp} controlled region ($pH < pH_{max}$ for bases and $pH > pH_{max}$ for acids).

For monobasic compounds, the relationships are as follows:

$$S_T = S_0\left(1 + \frac{K_a}{[H^+]}\right) \quad \text{when } pH < pH_{max}$$

$$S_T = S_0\left(1 + \frac{[H^+]}{K_a}\right) \quad \text{when } pH > pH_{max}$$

For monoacidic compounds,

$$S_T = S_0\left(1 + \frac{K_a}{[H^+]}\right) \quad \text{when } pH < pH_{max}$$

$$S_T = S_0\left(1 + \frac{[H^+]}{K_a}\right) \quad \text{when } pH > pH_{max}$$

The importance of aqueous solubility of drug has been discussed specifically in the context of biopharmaceutical properties injectable drugs. It is equally important to discuss the fundamental factors that contribute to make drugs insoluble. Considering the general solubility equation (14) provides insights into the physicochemical reasons that make drugs insoluble.

$$\log S_0 = 0.5 - 0.01[T_m(^\circ C) - 25] - \log P$$

According to the above equation, aqueous solubility (S_0) of a drug would be reduced if the melting point (T_m) is high, that is, the solid in the crystalline state has high lattice energy. Alternatively, insolubility may also arise because of high log*P* or lipophilicity of the drug. If both factors, that is, lattice energy and lipophilicity are high then the drug would also be highly insoluble. There are many approaches available for the formulator to enhance the solubility of insoluble compounds. Knowledge of the key factors contributing to insolubility along with its molecular structure can assist in the selection of appropriate solubilization technologies. A few of the many solubility enhancing options available to the formulation scientist developing injectable drug products include pH adjustment, cosolvent solubilization, solubilization by lipids, micellar solubilization, complexation (e.g., with cyclodextrins), amorphous forms of drugs, emulsification, liposomes, etc. Similarly, enhancement of dissolution rates can be achieved by particle size reduction including generation of attrition milled nanoparticles. This discussion has briefly outlined why it is important to consider the various thermodynamic but also the kinetic aspects of equilibrium for all of the physicochemical properties in designing, optimizing and troubleshooting the biopharmaceutical properties of injectable drug products.

DRUG EXPOSURE/PHARMACOKINETICS

Pharmacokinetics (PK) refers to the in vivo time course of blood concentration of an exogenously administered drug. In most cases, this is described by observing the plasma or serum concentration of the drug over time. The various processes that govern the observed concentration-time profile are *a*bsorption, *d*istribution, *m*etabolism, and *e*xcretion. Together, these four processes are described by the acronym ADME. Any changes in the ADME processes, for example, due to disease in the target population, could have changes in the PK profiles and hence the observed safety/efficacy profile of the compound.

Figure 5 illustrates the events that occur after administration of a drug. If the drug is administered in a "depot" [e.g., orally, subcutaneously (SC), etc.], a delay may occur before the drug appears in the blood circulation, possibly because of the time taken for the transport from the site of administration to the circulation. The initial increase in the drug concentration corresponds to the accumulation of the drug in the blood because of absorption from the site of administration. When the absorption rate becomes slower the elimination rate of the drug, the observed plasma concentration profile enters the declining phase. The inflection point at the top of the curve represents the change in this balance of the absorption and elimination rates. A distribution phase occurs where the absorbed drug is deemed to have distributed from the central circulation to other tissues or components of the blood. A rapid decline in the observed concentrations in blood represents distribution from the blood to other tissues such as the liver, kidneys, fat, etc., rather than elimination from the body.

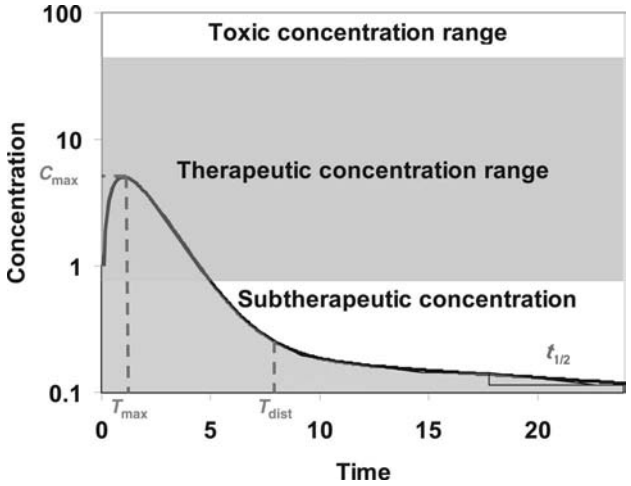

Figure 5 Absorption, distribution, metabolism, and excretion processes and pharmacokinetic summary parameters.

Elimination of the drug occurs as soon as a drug is absorbed and enters the systemic circulation. The last phase of the decline in blood concentrations typically corresponds to the elimination of the drug from the body because absorption is completed and distribution equilibrium is established. The elimination phase is also sometimes referred to as the terminal phase.

The concentration-time profile is often summarized using a set of PK parameters: the maximum concentration (C_{max}), the time to attain maximum concentration (T_{max}), the time taken for the concentration to decline by half (half-life; $t_{1/2}$), and the area under the plasma concentration versus time curve (AUC) (Fig. 5). C_{max} and T_{max}, can be read from the concentration time profiles. The half-life can be read as the slope of the concentration-time curve, where the concentrations are plotted on a log scale. The AUC is typically calculated from the observed concentration-time profile through standard area calculation algorithms.

Taken together, the PK parameters—C_{max}, T_{max}, and AUC—can be used to characterize the rate and extent of absorption of a drug. Thus, they can be used to compare the relative extents to which a particular compound is bioavailable—that is, reaches systemic circulation and is therefore available for therapeutic action—after administration through different routes or from different formulations. Typically, the bioavailability (denoted by the symbol *F*) after an intravenous (IV) administration is assumed to be 100%, and that after other routes of administrations are expressed as fractions of the IV bioavailability.

Most therapeutic compounds exert their pharmacological effect by reversibly interacting with their targets—for example, receptors, enzymes, ion channels, etc. When the systemic drug concentration declines, the extent of modulation of the target also reduces. Thus, the desired therapeutic effect for most compounds, which is the primary objective of the therapeutic dosing regimen, is obtained by maintaining a drug concentration above effective level (therapeutic concentration; Fig. 5). If the concentration is too low, loss of efficacy occurs because of lack of adequate modulation of the receptor. If the concentration is too high, toxicity might occur because of excessive modulation and potential exaggerated pharmacology or because of increasing expression of secondary pharmacological effects such as modulation of other subclasses of receptors. The difference (or the ratio) between the required therapeutic concentration and the toxic concentration is called the therapeutic index of a drug. Drugs with small differences between the therapeutic and toxic concentrations are referred to as narrow therapeutic index drugs and pose challenges in their clinical usage. A successful biopharmaceutical strategy would be effective in maintaining the concentration of the drug within the therapeutic concentration range.

Many biotherapeutics, especially macromolecules, because of their structure and physicochemical properties, possess distinct ADME properties from typical synthetic small molecules. As opposed to small molecules, a detailed understanding of these ADME mechanisms is not yet available for biotherapeutics. However, understanding the ADME processes for biotherapeutics is essential to appropriately design dosing regimens that maximize the therapeutic potential of these compounds.

Absorption

Before a drug can exert a pharmacological effect by modulating its target, it has to be absorbed from the site of administration into the bloodstream. For many synthetic small molecules, the oral route of administration is the preferred route of delivery because of the ease of administration and the related high level of patient compliance. However, biotherapeutics such as peptides, proteins and other macromolecules are, in general, not highly bioavailable after oral administration because of mainly two factors: (*i*) degradation in the gastrointestinal tract and (*ii*) lack of permeability across the GI mucosal barrier. Therefore, biotherapeutics such as monoclonal antibodies are typically administered through injections: IV, SC, and intramuscular (IM) routes being the preferred options. For example, of the 22 approved monoclonal antibodies (15), 4 are administered SC, 17 IV, and one each IM and intravitreally. Each of these sites of administration presents an absorption barrier with a unique set of properties.

For IV administration, there is no absorption barrier since the drug is directly delivered into the bloodstream. For extravascular routes of administration, the rate of absorption can vary widely depending on the site of administration.

SC doses are typically administered in the intradermal SC space in the shoulder, abdomen, thigh, or lower back. Similarly, IM doses are administered in the shoulder and gluteal muscles. After SC and IM injection, it is hypothesized that the drug is absorbed directly into systemic circulation via blood capillaries and through the lymphatic circulation. It has been shown through experiments in sheep that the lymphatic convective transport contributes substantially to the absorption of biotherapeutics after SC and IM administration and that the fraction of the drug absorbed through this process increases as the molecular weight increases (16). Consequently, it is hypothesized that for high molecular weight biotherapeutics such as monoclonal antibodies (approximate molecular weight of 150 kDa) are almost fully absorbed through the lymphatic system. Recent data in rats, where a low—<3%—contribution of the lymphatic route to the overall absorption was observed for erythropoietin, appear to contradict the findings in sheep. Suffice to say that a thorough quantitative understanding of the absorption processes after SC and IM administration of biotherapeutics is not yet available. Typically, absorption is slower for biotherapeutics than for small molecules with T_{max} values in the range of 24 to 72 hours post SC or IM dose.

The rate and extent of absorption from the extravascular site of administration depends on multiple factors and there is loss of drug prior to reaching systemic circulation (bioavailability is less than 100% compared with IV administration). A fraction of the drug administered after extravascular administered dose is subject to presystemic degradation, either at the site of administration, or during lymphatic transport—hence, these routes are clinically relevant only when a limited amount of drug is required to be administered for efficacy.

Other routes of administration such as intravitreal and inhaled routes have also been explored for biotherapeutics. The intravitreal route has been pursued for ranibizumab (Lucentis®), a vascular endothelial growth factor antibody fragment and pegaptinib sodium (Macugen®), a polyethylene glycol conjugated aptamer to promote a local effect. Administration of the drug directly into the site of action typically overcomes systemic PK limitations such as short half-life and minimizes side effects due to interaction with therapeutically inactive targets or targets at organs other than the site of action, thus improving the therapeutic index of the compound. Recently, the inhaled route is being widely explored as an option for biotherapeutics. Exubera® is an inhaled form of insulin for diabetic control. The large surface area of the lungs and the rapid transport of many molecules across the lung epithelial barrier provide attractive options for delivery, especially when the target is present in the airways (17,18). The rate and extent of systemic absorption for biotherapeutics administered at the site of action can vary widely depending on the physiology of the site of action—the density and porosity of the capillary bed, the lymphatic drainage of the site, any existing clearance mechanisms, and the effect of disease (see section "Absorption" under "Physiological Factors That Influence Pharmacokinetics of Injectable Drugs" for more details).

Distribution

Once the drug is absorbed from the site of administration into the blood circulation, it distributes to tissues, including the site of action, to exert its pharmacological effect. Unless the drug is designed to reach only a particular organ or tissue, this distribution of the drug occurs to various extents to all parts of the body. Within the PK field, the term distribution refers to the reversible partitioning of a drug to tissues within the body (19). The rate and extent of overall distribution of a drug from blood circulation to other tissues typically depends on many factors including the ability of the compound to cross tissue membranes, the perfusion rate of the tissues, partitioning into fat, and the tissue composition (20,21). Readers should note that the volume of distribution (V_d) commonly expressed as a PK parameter is a theoretical fluid volume that relates the administered dose and the observed blood concentrations and is not a strictly physiological quantity. For example, drugs that bind extensively to tissue targets have low blood concentrations after dosing, resulting in high estimated V_d, sometimes even higher than body volume (e.g., some basic drugs such as amphetamines)!

Except in the case of active transport, the distribution process for most small-molecule drugs is generally driven by concentration gradients. Therefore, at steady state, the free drug

concentrations in the blood and different tissues are at equilibrium. However, biotherapeutics are typically larger hydrophilic compounds with poor permeability across the tissue membranes. Entry into tissues is thought to be primarily through extracellular pathways (22,23), especially for tissues such as cerebrospinal fluid. Furthermore, return to blood from the tissue is in many cases through the lymphatic drainage (24), which is primarily a convective transport process not dependent on the concentration gradient and the biochemical properties of the compound such as permeability and tissue affinity. Therefore, the concentrations of the drug in blood and other tissues do not reach equilibrium, which is generally the case for small molecules. For example, the serum to cerebrospinal fluid concentration ratio of albumin is approximately 200:1 (22,23). Other investigations have shown that the blood: tissue ratio may also be dependent on the size of the biologic (24). Distribution of a drug to targets is another important factor to consider in the case of biotherapeutics. Many biotherapeutics, because of the very high affinity to their targets, are dosed at stoichiometrically equal molar concentrations to the target. Therefore, binding to the target constitutes a significant distribution pathway. Because the fraction of a drug bound to targets decreases with dose, target binding can lead to nonlinear distribution characteristics—that is, dose-dependant volume of distribution—for some biotherapeutics.

Metabolism

Most drugs begin to be metabolized after they enter the body. The majority of small-molecule drug metabolism is carried out in the liver by *redox* enzymes, termed cytochrome P (CYP)450 enzymes (ubiquitously expressed in the body). As metabolism occurs, a (parent) drug is chemically converted to metabolites. Metabolism eliminates the administered dose of a parent drug. When metabolites are pharmacologically inert, metabolism reduces pharmacological effects in the body as a parent drug is eliminated. Metabolites may also be pharmacologically active, sometimes more so than a parent drug (active metabolites).

The term catabolism is more relevant to describe the process by which biotherapeutics are broken down into smaller molecules such as amino acids. Proteolytic processes through enzymes such as proteases perform this function for biotherapeutics rather than CYP450 types of enzymes. The rate of proteolysis depends on many factors such as the size, carbohydrate content (glycosylation), potential for preproteolytic modification such as desialylation, the primary and tertiary structures (25). The sites of catabolism is also varied with liver, kidneys, and other extravascular sites such as sites of injection, for example, SC space have been implicated in protein catabolism. Many therapeutic proteins such as monoclonal antibodies are glycosylated proteins and are thought to interact with the asialoglycoprotein receptor (ASGPR) expressed on the sinusoidal surface of the parenchymal cells of the liver. ASGPR is believed to mediate the rapid removal and degradation of desialylated circulating proteins containing terminal galactose residues (26). It should be mentioned that characterizing the products of catabolism is substantially more difficult for biotherapeutics because of the wide range of catabolism products arising from an abundance of proteolysis sites and proteolytic enzymes.

An important site of catabolism of biotherapeutics is through the target. Binding of the biologic to the target has been shown to result in target-mediated endocytosis followed by lysosomal degradation for antibodies (27,28) and recombinant proteins (29).

Similar to V_d, clearance (CL) is a theoretical term that is the flow rate at a given concentration that is completely cleared of the drug in unit time and is calculated as the dose divided by the AUC under the assumption of constant clearance during drug elimination. Oxidative metabolism, catabolism, and other elimination processes all combine in achieving clearance of a xenobiotic.

Excretion/Elimination

Drugs and their metabolites are removed from the body via excretion, usually in the urine, in the feces or exhaled in the air. There are three major sites where drug excretion occurs. The kidneys, bile, and lungs. Many hydrophillic small molecules are cleared from the systemic circulation through the kidneys either intact or in the form of their metabolites (glomerular filtration) and excreted in the urine (renal elimination). Macromolecule biotherapeutics, because of their size are typically not cleared intact by filtration through the kidneys. However, small biotherapeutics such as some cytokines, insulin, granulocyte-colony stimulating factor,

interferon α, and erythropoietin have varying degrees of renal elimination, somewhat related to their size. In general, the renal elimination of intact biotherapeutics of molecular weight >30 kDa is expected to be negligible.

Larger molecular weight molecules are excreted into the bile and excrete in feces (biliary excretion). There are species differences in molecular weight cuttoffs for biliary excretion versus renal excretion. In human, the molecular weight cutoff required for biliary excretion is much greater than that for renal excretion. If the molecular weight is lower (e.g., <325–475 Da), the compound may be preferentially excreted in urine. Molecular weight from 325 to 850 Da may be eliminated via both renal and biliary routes. Excretion of molecules larger than 850 Da occurs mainly via biliary excretion. Physicochemical properties of the drug (polarity, lipophilicity, structure) are also critical to the extent of biliary excretion of a drug/metabolite. Biliary excretion has also been reported for biotherapeutics such as insulin (30) and epidermal growth factor.

PHYSIOLOGICAL FACTORS THAT INFLUENCE PHARMACOKINETICS OF INJECTABLE DRUGS

Physiological factors such as age, gender and disease states are known to alter PK of drugs. These factors can affect each component of PK—absorption, distribution, metabolism and elimination—described above.

Absorption

There is no absorption for IV administered drugs, as the drugs will directly circulate into the bloodstream. Therefore, the physiological factors, which influence absorption are minimal for IV dose. SC and IM administered drugs are taken up by the capillaries at the injection site and the permeability of the capillary wall membrane is affected by number of physiological factors.

Proteins larger than 16 to 20 kDa are generally taken up primarily by the lymphatic system and there is a linear correlation between molecular weight (MW 2,500–19,000) and the extent of recovery in the lymph (16). SC administered proteins generally exhibit a slower absorption and elimination compared with IV administration. Absolute bioavailability is generally low possibly because of protein degradation at the site of injection.

The factors affecting lymphatic transport of proteins after SC administration are summarized in the review by Porter and Charman (16). Lymph flow rate increases with exercise or mechanical injury (31). Massage is also known to increase lymph flow (32). Literatures show systemically administered insulin or gonadotropin increases capillary diameter and blood flow rate in rat cremaster muscle (33), although insulin-like growth factor-1 does not increase blood flow in human (34). The site of injection (injected to the abdomen vs. peripheral such as thigh or arm) influences the absorption (35) possibly because of differences in local blood flow and lymph flow.

SC blood flow increases in response to alterations in injection site, skin fold thickness, exercise, orthostatic changes, and ambient temperature (36).

Lymph flow is known to decrease with age (36,37). Membrane fluidity also decreases with age (38). Membrane permeability is also known to be altered with various disease states and with pharmacological agents (39).

Metabolism

Administered small-molecule drugs—either orally or injection—are mainly metabolized in the liver where the major metabolizing enzymes are located. Numerous literature reports suggest age and gender differences in CYP450 enzymes mediated metabolisms (40), however it is difficult to interpret those reports to general terms as those reports use probe drugs and majority of the studies is done in preclinical species.

The liver volume, liver blood flow and biliary function correlate well with body surface area (BSA). The liver size and blood flow decrease with aging, and therefore drug metabolism is reduced with advancing age (41). Renal clearance decreases with age and lower in women than in men at all ages.

Pelletier et al, demonstrated that the gut proteolytic activity is spread over a wide range of pH in younger animals than older ones with a shift from higher pH toward lower pH values with increasing age (42). A review article by Bota and Davies summarizes the regulation of

proteolytic enzymes in human diseases and ageing (43). Several disease states such as muscular dystrophy, cancer, Alzheimer's disease, neurological injury, ischemic injury, atherosclerosis, diabetes and cataract formation are known to alter the regulation of protease activities (44). Similarly, disease severity may also be related to increasing expression of the target and result in increased clearance of some biologics such as herceptin (cleared through the HER-2 receptor pathway) and omalizumab [cleared through immunoglobulin E (IgE)].

Distribution

Intravascular volumes, organ volumes and muscle volumes are generally smaller in elderly than younger people. The impact of reduced volumes is evident when the drug is distributed to those particular organs including muscles.

Drug distribution is also known to change with age because of relative changes in body fat. Lipophilic drugs such as midazolam and diazepam tend to get distributed to fatty tissue resulting in an increased volume of distribution(V_d) in elderly subjects (45,46). Divoll et al. studied PK of diazepam in young and elderly men and women (47). The authors found that the V_d was larger in women than in men but increased with age regardless of gender. Elimination half-life was longer in elderly than in young men partly because of the increased V_d as well as to a reduction in total metabolic clearance. It is noteworthy that the neither age nor gender influenced oral absorption and diazepam was nearly completely absorbed after IM administration (47). The level of α acid glycoprotein increase with age and as a consequence (48) the V_d can decrease for those drugs which bind to this particular protein.

As described above, biotherapeutics are distributed to tissues by blood or lymph, any disease states or aging which alter the blood flow and/or lymph flow can alter the tissue distribution of those large molecules. As mentioned earlier, the expression levels of target tissues (e.g., receptors) can be largely altered by the disease states as well. For example, the level of IgE correlates with the severity of asthma and the distribution of omalizumab, an anti-IgE monoclonal antibody, is related to the level of IgE present in the patient.

EXPOSURE-RESPONSE ANALYSIS

Pharmacokinetic Analysis

The primary aim of PK analysis is to summarize available plasma concentration versus time profiles (PK profiles) for interpretation, comparison, and predictions through the use of a set of parameters. These parameters can be obtained directly from an observation of the PK profile without the assumption of an underlying model quantitatively describing the different ADME processes. This is commonly referred to as a nonparametric, noncompartmental or model-independent analysis. These parameters include maximum concentration, time to reach C_{max}, area under the curve, the clearance [CL derived from the dose and AUC (CL = dose/AUC)]. While this analysis is simple and can represent simple PK characteristics of a compound, it has limited extrapolation ability beyond the studied regimen.

The PK profile can also be described by a set of PK parameters, assuming an underlying mathematical model—typically, a mammillary model with first-order kinetic processes describing the ADME process. This analysis is commonly referred to as compartmental modeling. A simple model is a one-compartment model, which represents central compartment (blood/plasma compartment) (Fig. 6). The rate of drug in (k_a, first-order absorption rate constant) and out (k_{el}, first-order elimination rate constant) of the central compartment is described by first-order kinetics.

Figure 6 Schematic of a simple compartmental pharmacokinetic model.

The versatility of the parametric analysis is founded in the ability of simple mathematical constructs to describe complex ADME phenomenon. By fitting the data to the right model, the model parameters can be estimated and these model parameters can be used to simulate time versus concentration curve with different dose or different routes of administration. Further complexity can be added to the simple one-compartment model to describe more complex PK; standard additions include second (and third) distribution compartments to describe distribution at different rates to different sets of tissues and multiple absorption routes and windows. It should be noted that in this approach, the parameters of the model Ka, CL, V_d, etc., do not have a direct physiological meaning even though they are related to physiological phenomenon.

Physiology-based pharmacokinetic modeling (PBPK) could be considered a special case of compartmental modeling, where the compartments and transfer rates correspond to physiological quantities such as tissues and organ volumes and blood flow rates. PBPK modeling is particularly useful when one wishes to predict the disposition in a particular organ.

Pharmacokinetic-Pharmacodynamic Analysis

The original concept of pharmacokinetic-pharmacodynamic (PK/PD) was described by Gerhard Levy in 1966 (49). PK is a study of a time and drug concentration relationship. Pharmacodynamics is a study of pharmacological responses. PK/PD analysis is a study of the relationship between PK and pharmacodynamics (PD). Understanding the PK/PD relationship is critical to determine the clinical dose and dosing regimen.

There are many different types of pharmacological responses. Mainly they can be categorized as either direct or indirect responses. A direct response is when the observed time course of response is temporally similar to the PK. A simple example of direct response is a receptor binding type response where the relationship between blood drug concentrations and the effect can be described with Hill function (50).

$$E = \frac{E_{\max} \times C^{\gamma}}{\mathrm{EC}_{50} + C^{\gamma}}$$

where $E_{\max}$ is the maximum efficacy (capacity), EC_{50} is the concentration to produce 50% of effect (sensitivity), and γ is Hill factor. Direct PK/PD responses are observed when the drug target is present in blood or when equilibrium is established rapidly between plasma concentration and biophase (Fig. 7). Examples of direct responses are neuromuscular blocking agents, etc., where the response is directly related to the drug concentration and pharmacological effect can be seen immediately.

Those target tissues are often not in the blood and therefore, it is necessary to establish the relationship between plasma concentrations (PK) and the concentrations at the target tissue to understand the PK/PD relationship. The concept of "biophase" (target tissue) was first introduced by Segre in 1968 (51). Indirect PK/PD response is used to describe the case where the time course of PD is time-shifted from that of the PK—that is, the maximum PD response does not occur at the maximum blood concentration (Fig. 8). Such responses occur when the pharmacological effects are results of a cascade of events such as induction, synthesis, secretion or cell trafficking. The very first work in this area was done with anticoagulants by Levy et al. (52,53). The diagram below shows the effect compartment model where the rate of onset and offset of effect is governed by the drug distribution and elimination from the biophase (effect compartment or target tissue).

Basic PK/PD Models

The relationship between PK and PD time courses is usually derived using a PK/PD model. Either observed or model-predicted blood concentrations are used as the forcing function for the PD response and the appropriate PD response parameters—for example, $E_{\max}$, EC_{50}, and γ in the Hill equation—are estimated. PK/PD modeling enables us to quantify pharmacological effects as a function of time in relation to drug concentrations. The direct effect and indirect

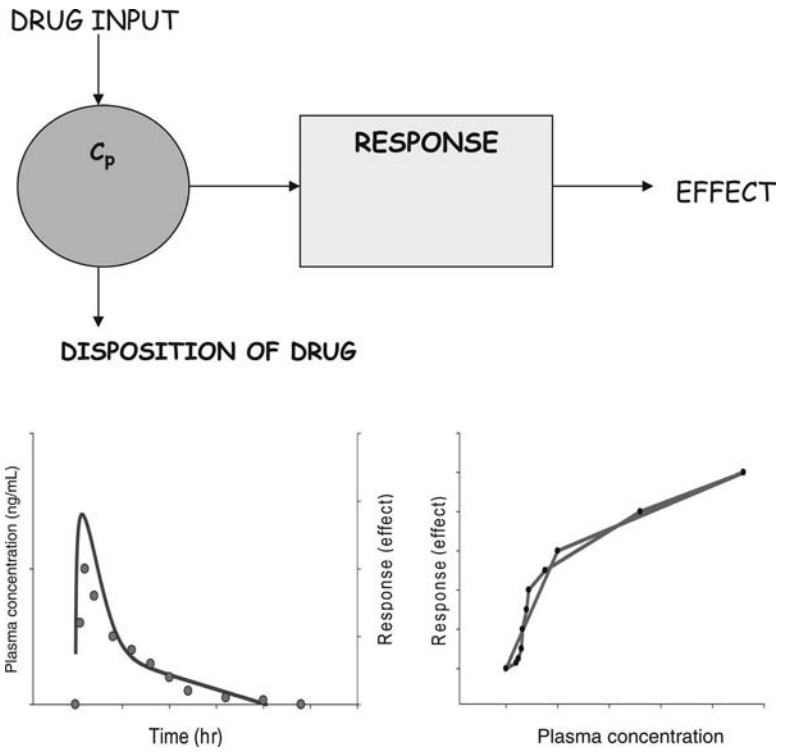

Figure 7 Illustration of a direct pharmacokinetic-pharmacodynamic model. The solid line represents PK profile and the dots represent the PD measures.

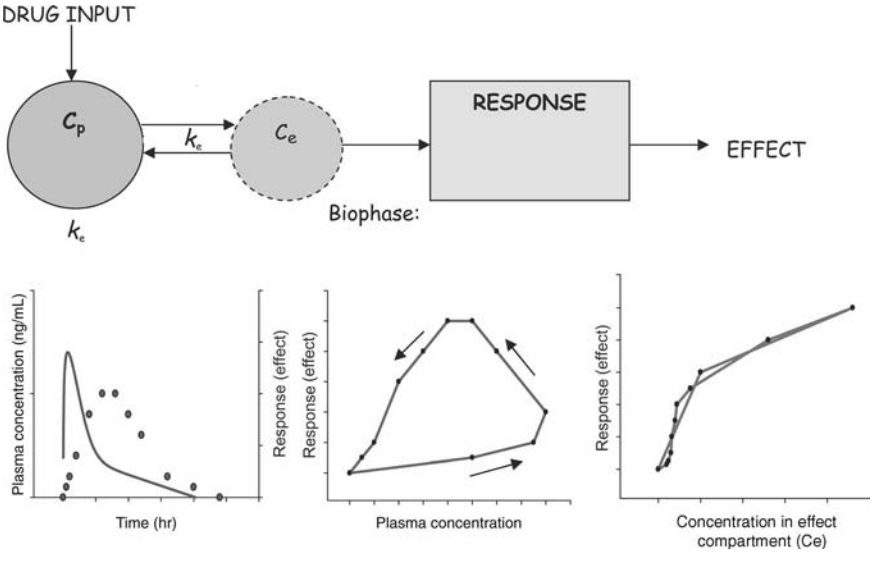

Figure 8 Illustration of an "indirect" or delayed pharmacokinetic-pharmacodynamic effect. In the left panel, the solid lines represent the PK profile and the dots represent PD measures.

effect compartment models shown above are two of the simplest models to describe PK/PD relationships. As stated above, there are many other types of pharmacological responses which cause delayed responses. Because of the diversity of in vivo pharmacological responses, the variety of PK/PD models is quite large and cannot be dealt with in detail here.

BIOTHERAPEUTICS FORMULATION AND IMMUNOGENICITY

All biotherapeutics are potentially immunogenic, and this immunological reaction has the potential to impact the biopharmaceutics of the product. Thus, understanding and mitigating the causes of immunogenicity are critical to the successful application of the biotherapeutic (54).

The causes of immunogencity of biotherapeutics vary widely, and is not necessarily related simply to the amino acid sequence being of foreign origin. General immunological or safety concerns with protein therapeutics include acute infusion or injection site reactions (anaphylactic or anaphylactoid), serum sickness, effects related to the generation of antibodies against the therapeutic, as well as antibodies to therapeutic that may cross-react with endogenous proteins. The latter type of immunological reaction carries the greatest risk because of its potential to impact both safety and efficacy (55,56).

Therapeutic proteins can lead to antibody induction via two pathways: a T cell–independent and a T cell–dependent pathway (57–60). Analysis of antibodies from clinical studies suggests that IgG antibodies make up the majority of the antidrug antibody (ADA) responses, implicating the T cell–dependent pathway as the primary mechanism.

The T cell–dependent pathway requires a cognate T cell–B cell interaction. To initiate the response, the protein must interact with antigen-presenting cells (APCs) such as dendritic cells (DCs), B cells, or macrophages. APCs internalize the antigen (i.e., therapeutic protein), digest it in the endosome, generate peptides that can be loaded into an appropriate MHC class II molecule and present them in a linear conformation on the surface as a complex. These peptides are called T-cell epitopes and may be recognized by T-cell receptors on naïve T (helper) cells in lymph nodes. In parallel naïve B cells also take up the antigen via their specific membrane-bound antigen (B-cell) receptors, process and subsequently present epitopes in MHC class II molecules on their surface. Helper T cells that have been already activated by recognizing the epitope on the APCs, must then proliferate, migrate and encounter B cells with the same epitope on the same MHC class II molecule at the lymphoid follicles. Binding of the T-cell receptor to the peptide:MHC class II complex on the B-cell surface then leads to the expression of costimulatory molecules and secretion of cytokines from T-cell surface that trigger the B cell to differentiate and mature into antibody-secreting cells. A mature but naïve B cell will initially produce an IgM response. Further helper T-cell interactions induce isotype switching to IgG (and other isotype) responses. This T cell–dependent immune response is usually long lasting and of high titer. Once the switch has occurred, some of the activated B cells become long-lived memory cells which react rapidly to rechallenge with the characteristic IgG production. This mechanism requires that B cells (via B-cell receptors) and T cells respond to the same antigen although not necessarily the same epitope. Another important requirement is a costimulatory signal to activate the T cells. These costimulatory molecules can be induced by infection or inflammation—a distress or danger signal in the form of cytokines such as tumor necrosis factor (TNF). In the absence of these distress signals, the peptide:MHC class II complex alone on the APC cannot activate the T cells, thus promoting anergy or tolerance in naïve T cells. On the other hand, the presence of additional molecules that are associated with the therapeutic protein that act like adjuvants (e.g., HCPs or endotoxins), can activate toll-like receptors on the APCs, and may result in reversing tolerance or abrogating T- and B-cell anergy, thus inducing the generation of an immune response.

B cells can also be activated without cognate T-cell help by the so-called T cell–independent pathway. For this purpose, the antigen has to be engulfed by specialized blood-borne peripheral DCs, and presented to B cells. B-cell stimulatory signals are generated when a number of B-cell receptors simultaneously bind to the antigen resulting in their crosslinking and subsequent cell proliferation. A costimulatory signal (e.g., a cytokine) is however required for the activation step. Antibodies produced in this situation are of the IgM type, transient, of low titer and poor specificity. Because of lack of affinity maturation, there is no class switching or generation of memory. This pathway is typically evoked by particulate antigens displaying repetitive epitopes termed pathogen-associated molecular patterns, usually found on bacteria. Again, delivery of a second signal by helper T cells or via pathways mediated by Toll-like receptors would allow for affinity maturation and class switching, creating a more efficient IgG response.

Table 1 Factors That May Impact Immunogenicity of Biotherapeutics

Product-related factors	Patient-related factors	Treatment-related factors
Protein structure (human/nonhuman, posttranslational or chemical modifications)	Disease state being treated	Dose
Product quality parameters (isoforms, chemical and physical degradants)	General immune status of patient	Route
Contaminants and impurities	Genetic background (MHC genotype, HLA phenotypes)	Frequency of dosing
	Concurrent illnesses and concomitant therapy	Length of treatment

Immune response to foreign (exogeneous) proteins also called the "classical" immune response, arises via the T cell–dependent pathway. On the other hand, the human immune system is usually tolerant or anergic to proteins of human origin. In the absence of a neoantigen, an immune response against a human protein though not impossible, is highly unlikely unless the protein is presented to the immune system in a fashion that can reverse tolerance or T- and B-cell anergy by the above T cell–dependent pathway. The likelihood of breakage of tolerance to proteins of human origin or recombinant autologous proteins, is considered a function of the abundance of the endogenous soluble protein. For proteins of low abundance, the immunological tolerance is not complete. T and B cells specific for low-abundance proteins (autoantigens) may not be completely eliminated during early development. Under sufficient provocation (e.g., presence of molecules with adjuvant-like characteristics), these might generate an immune response.

ADAs are broadly classified as binding (BAbs) or neutralizing (NAbs). For biologics of human origin, BAbs and NAbs are of concern because of the possibility of impacting efficacy and PK. BAbs bind to the protein but do not neutralize it. They may mediate infusion reactions or alter the PK/PD profile of the therapeutic. BAbs can enhance clearance or prolong systemic exposure. BAbs can be precursor or triggers for the generation of NAbs through epitope spreading. NAbs bind to the therapeutic molecule and disrupt its ability to bind to the target, that is, neutralize its function. When present at low titers, the impact on efficacy may be minimal but efficacy and biological activity may be impacted at high titers. The most serious type of NAbs response are those that cross-react and neutralize the function of the endogenous analog, especially one that serves a biologically unique function and has no redundancies (61).

There are many factors that can be involved in breaking of tolerance to a protein biotherapeutic and can be broadly classified into three categories as given in Table 1.

Although the factors are categorized above, in practice it is very difficult to deconvolute the impact of specific product attributes from the number of patient and dosing regimen related factors (62–64).

When considered from the perspective of a product development scientist, the causes of immunogencity can be divided into two broad categories.

1. Intrinsic to the molecule and treatment regimen
2. Extrinsic factors related to CMC aspects of the product

Immunogenicity as a Consequence of Molecule and Treatment Aspects of the Biotherapeutic

This category is concerned with the selection and design of the molecule itself and is often the result of a discovery effort intended to realize a certain therapeutic effect. A detailed consideration of this category is therefore outside the scope here but some relevant concepts are covered to provide a background for the subsequent discussion. A nonhuman protein (e.g., streptokinase, botulinum toxin) will induce antibodies by the classical immune response. A similar response can be generated in people who do not have tolerance to a certain protein. For

example, patients with severe hemophilia A involving large deletions or nonsense mutations of the factor VIII gene are more likely to have an antibody response to exogeneous factor VIII than patients with mild or moderate disease since patients with the severe form of the disease do not express functional factor VIII antigen and hence have no immune tolerance (65). In these instances, the generation of ADAs may be considered as a vaccine-like reaction to a foreign protein. As in vaccines, the response is related to a number of factors such as (number, frequency and amount) of dose administered, length of treatment, delivery route, and presence of "adjuvants" (66). More surprising is the observation that "self" proteins can induce an immunological response even in individuals who are not deficient in the protein, but simply produce an insufficient amount for the desired biological effect (67).

Foreign proteins can induce antibodies after a single injection while human proteins may require longer exposure of up to six months (68). Yet, as exemplified by insulin and growth hormone, chronic therapy need not compromise the therapeutic efficacy of the protein. The fact that both types of proteins can induce antibodies implies that the molecular characteristic evoking antibody response is at least more complex than simply being self or nonself to the human system. Nature of the therapeutic (immunostimulatory vs. immunosuppressive) proteins and host immune status also play a role in the observed effect. Cell surface binding therapeutic antibodies generally will have more potential to be immunogenic than those that interact with soluble targets.

The probability of an antibody immune response is considered highest after SC injection, followed by IM, intranasal, and intraveneous routes. SC administration localizes the protein to a small area with a short path to drain into the lymph nodes where B and T cells are present (69). Clinical experience with pulmonary administration of insulin suggests that this route also carries a high risk for generation of immunological reaction (70).

The type of disease plays a role, likely related to the immune status of the patient. Patients with weak or compromised immune systems or those on immune-suppression therapy are less likely to develop ADAs than those with intact immune systems. Acute therapy is less likely to be immunogenic than chronic therapy, although intermittent treatment is more likely to elicit a response than continuous therapy. Also, lower doses are generally more immunogenic than higher, probably related to the fact that the immune system is generally less tolerant of low-abundance proteins.

Porter (71) has prepared a comprehensive review of the literature on immune response to recombinant proteins used in therapy. Among the significant conclusions drawn are that the presence of antibodies has not necessarily been detrimental to the clinical efficacy and that no particular property of a protein has been identified as an obvious predictor of immunogenicity in humans.

Immunogenicity as a Consequence of Chemistry Manufacturing and Control (CMC) Aspects of the Biotherapeutic

The characteristics of a parenteral products are determined by three major factors: process, formulation and package. From the perspective of a product development scientist, the CMC aspects that can play a role in the immunogenicity profile of the product begin with the gene design and cell line selection. Gene sequences are mutated to avoid degradation and aggregation hotspots as well as antigenic epitopes, while maintaining potency (72–74). The choice of host cell line determines the presence (or absence) of glycosylation and the glycosylation pattern. The upstream (bioreactor/fermentation, harvest) process impacts the distribution of glycoforms and other product variants, for example, deamidated variants, disulfide scrambling, and also determines the type of host cell impurities that may ultimately remain in the product. The protein then further undergoes a complex series of processing steps for purification including viral removal. While the overall objective of the post harvest steps is to purify the protein by removing impurities (e.g., host cell proteins, DNA, endotoxins), and product related species (e.g., truncated, hydrolyzed, aggregated, deamidated, oxidized, and improperly glycosylated forms), it is nevertheless impossible to completely eliminate these. The current state of purification processes is such that impurities are routinely reduced to levels well below what is considered a risk in the particular case. Product variants are not as

easily eliminated and a certain minor fraction of some or all of these variants make their way into the final bulk solution. The bulk solution after the purification steps may be stored for a period of time either as a liquid or frozen, before it is finally filtered and pumped into vials or syringes. In some cases, it is subject to the final processing step of lyophilization, before being shipped over an appropriately designed (cold) transport chain to the clinic or pharmacy. Thus, an important objective of the process and formulation development is to stabilize the native state of the molecule and minimize physical and chemical degradation over the shelf-life of the product.

Impact of Process and Formulation

The process that a biotherapeutic undergoes in its product has a significant impact on the product characterisitc. The formulation is intended to stabilize the product during the process and during storage and use. Some aspects of the process and formulation that have the potential to impact immunogenicity are considered below.

Glycosylation. Glycosylation refers to the enzymatic addition of saccharides to the protein as a post-traslational modification. Glycosylation is present in approximately 50% of human proteins and an almost similar proportion of approved biopharmaceuticals. The presence and nature of the glycoform may impact primary functional activity, folding, stability, trafficking and immunogenicity. Although glycosylation is in a way intrinsic to the molecule, it can also be impacted by the production process. For this reason, the choice of the expression system is a critical activity in the development of a biotherapeutic. As mammalian expression systems produce mainly human glycans, these have become the dominant platform for production of therapeutic glycoproteins. However, these platforms require good process control since they display an inherent glycan heterogeneity that is sensitive to culture conditions. Glycosylation can have direct impact on immunogenicity through patterns that are not present in humans. CHO cells produce gycosylation patterns that are close to human, although these cells also express *N*-glycolylneuranimic acid (NGNA), a form of sialic acid not found in humans and reported as immunogenic. Mouse cell lines (e.g., NS0, SP2/0) also produce NGNA in addition to or instead of the *N*-acetylneuraminic acid (NANA) present in human IgGs (75,76). Galactose α(1-3) galactose linkages or terminal α(1-3) galactose can also be added by murine cells (e.g., C127, NS0, SP2/0). This residue has been shown to be recognized by up to 1% of circulating IgG in humans (77). Glycoslation can have an indirect effect on immunogenicty through its impact on folding solubility and (structural) stability. Glycosylation can affect local secondary structure and thereby direct the generation of tertiary structure. Altered or absent glycosylation can therefore alter or eliminate epitopes or expose/generate new ones. Glycosylation can increase solubility by shielding hydrophobic patches and reducing tendency to aggregate, and enhance stability by participating in intrachain stabilizing interactions (78–80).

Purity. Host cell proteins and DNA are contaminants that carry the risk of functioning as adjuvants and thus triggering an immunogenic reaction to the therapeutic given the appropriate antigenic determinants. Lundin et al. (81) summarized that the early pituitary preparations of hGH resulted in about 45% patients developing antibodies. Improvements in processing and purification led to a marked decrease in antibody formation to less than 10% (pituitary source), while it was $<2\%$ for the purest commercial pituitary preparation. Early recombinant preparations, on the other hand, also led to unexpectedly high antibody levels, but were related to *E coli* proteins remaining as impurities in the preparations (82). Bacterial DNA contains unmethylated CpG motifs that are known to activate Toll-Like receptors and are themselves being studied as adjuvants for vaccines. Process improvements resulting in greater purity by reduction of product-related and unrelated species have led to a clear reduction in ADA response. Current purification processes reduce host cell and process contaminants to very low levels.

Product-related impurities and degradation products. Product-related impurities and degradation products for biotherapeutics often overlap and are not readily distinguishable.

For example, charge variants encoded as a consequence of the cell line (e.g., sialylation) and/or generated in the upstream/downstream processes will often overlap with deamidation/isomerization products. Oxidation of susceptible residues can occur at any stage in the production process or subsequent storage and use, as can fragmentation/hydrolysis. Finally, size variants such as truncated, misfolded and aggregated species can also arise at all stages. However, among all the possible chemical and structural changes, the one that causes the most concern is aggregation involving association of multiple protein molecules in partially/wholly unfolded forms, and even in their native state.

Aggregates can form as a result of a variety of interactions between the protein molecules including hydrophobic interactions as well as because of covalent changes caused by chemical modifications such as oxidation. The protein molecules making up the aggregates can be in their native, partially or fully unfolded states. Aggregation is governed by the conformational, that is, thermodynamic stability self-association tendency, that is, colloidal stability of the molecule. A full discussion of the mechanisms of aggregation is outside the scope of this chapter but a number of good reviews are available [Chi et al. (83), Wang (84), Mahler et al. (85)]. Other factors that can impact the level of aggregation in a protein solution includes conditions such as pH, temperature, concentration, ions and ionic strength and stresses such as freeze/thaw, air/liquid and liquid/solid interfacial stress. Chemical modifications such as oxidation can also lead to loss of structural stability and aggregation. Since a protein can undergo aggregation by multiple pathways, all of these factors have to be addressed as part of the formulation development program for the biotherapeutic.

Aggregates are hypothesized to cause immunogenicity through their "repetitive" display of epitopes that are seen by the immune system as resembling the external surfaces of invasive species. As reviewed by Rosenberg (86), it is not the low MW aggregates such as dimers or trimers but the large multimers with molecular weights exceeding 100 kDa that are efficient inducers of immune responses. Native aggregates in which the protein retains a large part of its structure are of greater concern since antibodies could be generated against epitopes that are present on the native monomeric version. Antibodies generated against nonnative aggregates (generated by misfolded species or chemical modifications) could still result in increased clearance as well as raise potential safety concerns. Experiments on animal models have shown that aggregated proteins can lead to an immunological response, but the relevance to human experience is debated (61,87,88).

Aggregation is considered a strong risk factor for generation of immunological reaction and therefore has to be minimized by proper design of process and product. It is a fundamental attribute to assess the quality of a biotherapeutic and control of this parameter is an important aspect of biotherapeutic product development.

Container/Closure System

Container/closure are an integral part of a biological product, be it a vial/stopper, a prefilled syringe or a dual-chamber cartridge. Some component materials which come into contact with the product include the container (glass or plastic, vial or syringe), closure (stopper), administration and infusion components (syringes, bags, infusion lines). The concern for packaging component-dosage form interaction for biologics again arises because of the potential for alteration of the structure of the protein through aggregation or chemical degradation pathways such as oxidation. The impact on the protein can occur directly through the container interface, but also indirectly through any chemical compound that may leach out of the container. Some common leachables from the common container/closures used for biologics include metals, antioxidants, plasticizers, lubricants as well as degradation products of the various components. For example, tungsten residues left behind when preparing staked-needle syringes have been shown to cause oxidation of protein solutions. The FDA also considers the compatibility of container/closure with product as a key requirement in the development of parenteral products. The FDA guidance document on container closure considers inhalation aerosols and solutions, injections and injectable suspensions as products with the greatest level of concern when accounting for route of administration and risk for packaging component-dosage form interaction (89).

Silicone oil coating is commonly used on stoppers and on the inside of syringes or cartridges as a lubricant to enable movement of the plunger. Silicone oil contamination by the syringes used for injecting insulin has been well documented [Chantelau et al. (90), Bernstein (90,91)]. Current processes for siliconization of prefilled syringes or cartridges apply well controlled amounts and involves baking of the silicone emulsion. This tends to reduce the levels of silicone oil extracted into the formulation but the possibility exists. Fibrous aggregates have been shown to form in a a number of model proteins when incubated with silicone oil (92).

Selection of the container/closure system for any product is a critical task. The container/closure must provide adequate protection to the product from the environment and prevent contamination. It must also be compatible with the product and not leach any compunds that could harm the product or pose a safety risk. The experience with vials and stoppers is extensive, but use of devices such as inhalers and injectors increases the complexity of this task.

Safety/Tolerability of Excipients

As stated before, formulation development for a biologic is carried out to identify the optimal composition that will keep the biologic stable for an economically viable length of time. The product format can be a liquid or a lyophilized powder. Excipients are added to accomplish this. A review of the formulation composition for biologics shows that the vast majority comprise a buffer, a tonicity modifier, cryo- or lyoprotectant, and a surfactant. Other additives such as a chelator, antioxidant, and a preservative are occasionally found (93). Most common excipients are generally safe and have long precedence of use, although precautions may be in order in certain cases. Among the common ingredients is the surfactant polysorbate 20 or 80, comprising partial fatty acid esters of sorbital and its anhydrides copolymerized with ethylene oxide. These is known to cause anaphylactic reactions in dogs, and may have an allergenic potential in susceptible individuals. Intravenous immunoglobulin (IVIG) therapy has been connected with numerous episodes of acute renal toxicity and osmotic nephropathy because of a very high sucrose load. The sucrose is added to the product to reduce formation of aggregates as a consequence of the pathogen-removal steps in the process. Sucrose and sorbitol as well as maltitol and fructose can also be contraindicated in patients with hereditary fructose intolerance, the glucose-galactose malabsorption syndrome or sucrose-isomaltase deficiency.

Evaluation and Prediction of Immunogenicity

Animal models have traditionally been used to evaluate the safety of (bio)pharmaceuticals, but their utility in evaluation or prediction of clinical immunogenicity is controversial. Data generated from the animal models must be placed in context of the type of molecule. Bugelski and Treacy (94) group recombinant proteins into classes on the basis of preclinical immunogenicity. For some classes, for example, bacterial proteins, immunogenicity in animals is often predictive for humans. For others, such as fully human proteins, even data from nonhuman primates may have little predictive value. Nonhuman primates with a high level of sequence homology with humans are often seen as most relevant. However, the evidence for success is limited, and mainly governed by the degree of conservation across species. Limited homology means that the animal models are generally over-predictive of human immunogenicity. Transgenic mice that express the appropriate human transgene allow the protein to be tested without generating a xenogenic response. There are many caveats and limitations of this approach (62,94,95), the least of them being that the wild-type strain must be capable of making antibodies to the protein in questions. Limitation in the use of animal models is magnified when trying to decipher the relative impact on immunogenicity of a few percent of product degradants. To be able to detect such changes, the animal models must have a low baseline immune response or a slow development trajectory for immunogenicity, while the studies have to be carefully controlled. In summary, the utility of animal models would primarily lie in assessing the relative immunogenicity risk of CMC related factors.

Computational tools are also being developed for assessment of intrinsic immunogenicity of protein therapeutics including identification and modification or removal of T-cell epitopes (72). Further research is required to develop models with the ability to assess the impact of CMC factors in general and aggregation in particular on immunogenicity.

BIOTHERAPEUTICS BIOEQUIVALENCE/COMPARABILITY

Manufacturers of biotechnological/biological products frequently make changes to manufacturing processes of their products both during development and post-approval. These changes, however minor, could cause undetectable changes in the physicochemical composition of the primary active ingredient of the drug substance or in the profile coproduced compounds such as host cell proteins and other potential impurities. Also, as discussed in previous sections, the dose, frequency, and route of administration all have the potential to change the PK/PD and immunogenicity characteristics. Thus, even minor changes in the drug manufacturing and/or administration process have the potential to affect the overall safety/efficacy profile of the drug product. Demonstration of comparability of the pre- and postchange product is a sequential process, beginning with quality studies (limited or comprehensive) and supported, as necessary, by nonclinical, clinical and/or pharmacovigilance studies. For most changes to the manufacturing process, physicochemical and (quality-related) biological testing can demonstrate that there is no difference in quality of the product that could adversely impact the safety and efficacy of a product. Thus the comparability exercise may be limited to strict process validation of the change or be extended to various quality criteria such as in-process controls, thorough analytical and biological characterization of the product and stability data. However, sometimes an effect on efficacy and/or safety can be expected on the basis of observed difference(s) or cannot be ruled out in spite of the state of the art physicochemical and biological tests. In such cases, additional nonclinical and/or clinical studies will be necessary.

PK studies are a key component of the in vivo comparability testing and are typically performed when analytical characterization is not sufficient to detect differences, or the clinical implication of analytically detected differences is unknown. The study could be performed in animals, if a relevant animal model exists, or in humans. PK studies may not be appropriate for comparability testing when the PK variability of the reference product is in general very high or when the PK variability is of no clinical relevance, when PK is insensitive to clinically relevant changes to the active substance (e.g., in the case of misfolded proteins), drug is active at the site of administration and blood exposure is not a relevant biomarker for safety/efficacy. Despite these limitations, PK testing remains a valuable comparative tool.

Some of the key considerations of a PK for biologics study are: the study population—patients or subjects, dosing regimen—single or multiple doses, parallel or crossover study, the duration of sampling, route and method of administration, doses for evaluation in the study, PK parameters of interest, and the criteria for claiming equivalence. While many of these considerations are also relevant for a small-molecule drug, the PK characteristics of biologics pose a unique challenge in the design and conduct of these studies. The choice of study population depends on the PK and safety profile of the compound of interest and its mode of action. For compounds that are generally well-tolerated and where the PK in healthy subjects is known to be predictive of that in the target patient population, healthy subjects might be appropriate for comparative testing. In other cases, a patient PK study might be considered, especially where relevant PD information can also be gathered. Similarly, many biologics have a long half-life, from days to weeks. Therefore, standard crossover studies can pose limitations due to the duration of treatment and follow-up. Parallel studies could be considered if the duration of the study could become unfeasible. Furthermore, the potential for immunogenic reactions, typically observable after three to four weeks after a single dose, should also be considered in crossover designs. The route of administration should be in accordance with the intended clinical use. If the product is planned to be administered by more than one route (e.g., SC and IV), it may become necessary to test all routes. The selected dose should be in the steep portion of the dose-response curve to detect relevant differences, especially if PD markers are being monitored in the study. Apart from standard PK parameters describing absorption and

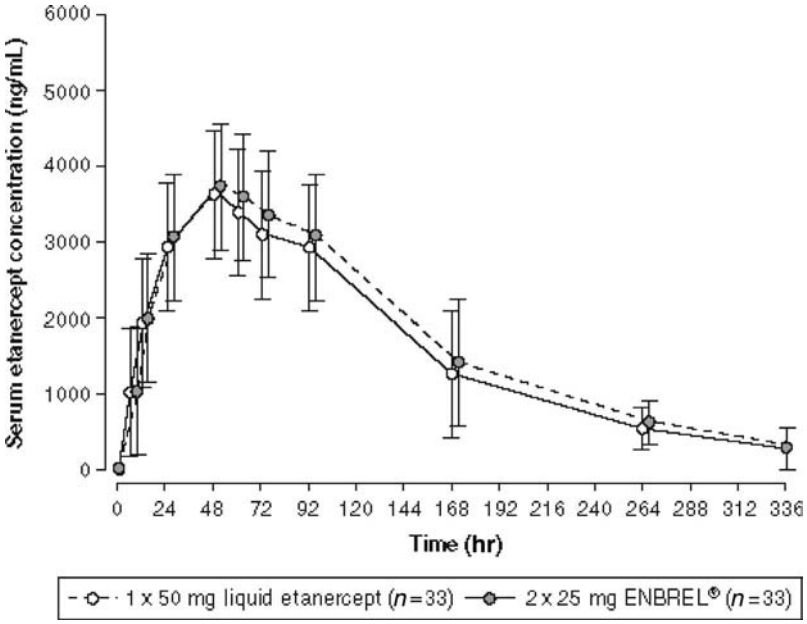

Figure 9 Illustration of pharmacokinetic equivalence for two formulations of etanercept. *Source*: From Ref. 97.

bioavailability (such as C_{max} and AUC), other PK parameters such as elimination half-life and clearance should also be considered for comparability, because of potential changes in the heterogeneity of active substance due to process changes.

The following example by Sullivan et al. (96) illustrate the concept of PK-based comparability assessment for a new formulation of Enbrel® (etanercept). Etanercept is a soluble, fully human, TNF receptor that competitively inhibits the interaction of TNF with cell surface receptors. Etanercept is currently approved for reducing signs and symptoms, inhibiting the progression of structural damage, and improving physical function in patients with rheumatoid arthritis. It is also approved for reducing the signs and symptoms and inhibiting the progression of structural damage in patients with psoriatic arthritis and for reducing the signs and symptoms of active ankylosing spondylitis, juvenile rheumatoid arthritis, and psoriasis. Etanercept was originally introduced commercially in vials containing 25 mg lyophilized powder requiring reconstitution, and to date most patients have received the reconstituted formulation. A 50-mg/mL liquid formulation supplied in a prefilled syringe was approved recently for commercial use. Sullivan et al. (96) present the results of a study in healthy volunteers comparing the PK of the liquid etanercept formulation with that of the reconstituted formulation (Fig. 9). The study was conducted in healthy male and female subjects, where each subject received both formulations (50 mg of etanercept per dose) in a crossover fashion with a minimum of 28 days washout period in between doses. The following PK parameters, obtained from the observed PK profile using noncompartmental analysis, were reported: AUC (to till the final sample collection timepoint and extrapolated to infinity), C_{max}, T_{max}, and terminal $t_{1/2}$. The point estimate of the ratio of geometric means of the PK parameters (AUC and C_{max}) were generated along with their 90% confidence intervals. Equivalence of the two formulations was concluded since the 90% confidence interval of the ratio of PK parameter means lay between 80% and 125%, which is the standard bioequivalence criterion.

Similarly, Paulson et al. (97) performed a PK comparability assessment for adalimumab (Humira) in healthy subjects between two administration routes—as an autoinjector pen and a prefilled syringe. Adalimumab is a murine monoclonal antibody prescribed for the treatment of rheumatoid arthritis, and has a half-life of two to three weeks (PI). Therefore, a parallel group study in 290 subjects was performed in this case to assess the PK equivalence in this

case. The duration of PK assessment was appropriately adjusted to account for the long half-life. The PK and statistical data analysis was similar to that described by Sullivan et al. Comparability was concluded in this case also.

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4 Preformulation

N. Murti Vemuri

INTRODUCTION

Parenteral medication refers to drugs administered by routes other than the oral, typically implying injectable medications. Injectable medications could be presented in various volumes (small volume and large volume), primary packaging (ampoules, vials, cartridges, bags) and specified routes (e.g., intravenous, intramuscular). Many of the preformulation and formulation principles applicable to injectable medications can often be extended to ophthalmic and nasal spray dosage forms as well.

Rational formulation development of parenteral medication should be based on the desired product profile, the physicochemical properties of the drug substance and its interaction with other formulation ingredients, primary packaging components under storage conditions defined by the product profile, as well as the pharmacokinetic properties of the drug substance. Preformulation research comprises pharmaceutical and analytical investigations in acquiring such knowledge base, and these investigations both precede and support formulation development.

On a drug development timescale, preformulation research enables data-driven decisions related to the drug substance and drug product such as salt form selection, polymorph selection, excipient selection, identification of suitable toxicology formulations, and, finally, selection of compositions for clinical and commercial formulations. Additionally, understanding the physical and chemical attributes of the drug substance can often help in troubleshooting formulation, stability, and processing issues that may arise.

Many good reviews and book chapters (1) have been written on the subject of preformulation and physicochemical characterization of drug substances. Although most articles focus on oral formulations, many of the principles carry over to development of parenteral medications. This chapter will attempt to focus more on aspects relevant to development of parenteral dosage forms. Much of the discussion will focus on small molecules and solutions dosage forms, but later sections will touch on specificities related to macromolecules and specialized dosage forms.

CHARACTERIZATION OF THE DRUG SUBSTANCE

Understanding the physicochemical properties of the drug substance is the first step (2) toward building quality into a product using rational formulation design. Drug substances are investigated at various levels of scrutiny to fully understand their behavior—at the molecular/material level, at the particulate level and also at a bulk property level. Table 1 shows a representation of this hierarchy of physicochemical properties. The intended dosage form often dictates where to place the greatest emphasis. For a solid dosage form, it is important to also fully understand the bulk properties, but for parenteral dosage forms, greater emphasis is on understanding the molecular and material properties of the active pharmaceutical ingredient (API).

Molecular Properties

Prior to initiation of preformulation studies, the molecular structure of the drug substance is identified and confirmed by appropriate spectroscopic (NMR, MS) evidence. The material is further identified by its characteristic IR and UV spectrum.

Physicochemical Constants

Two key physicochemical constants of importance are the partition coefficient and the ionization constant. The partition coefficient is an indication of the lipophilicity of a compound and is measured as a ratio of the equilibrium concentrations of the drug in an oily (e.g., octanol) and an aqueous (e.g., water) phase in contact with each other and held at a constant temperature. The

Table 1 Physicochemical Properties of Drug Compounds

Molecular properties *Properties defined by the molecular structure*	**Material properties** *Properties intrinsic to the material or particle*	**Bulk properties** *Properties related to bulk powders*
Molecular weight	Salt form	Powder flow
log *P*/log *D*, pK_a	Crystal form (XRPD)	Bulk density
Chemical stability	Crystal habit	Wettability
Melting point		Powder electrostatics
Solid-state stability		
Solubility		
Spectral characterization (UV, IR, NMR)	Particle size	
		Specific surface area
		Hygroscopicity

Abbreviation: XRPD, X-ray powder diffractometry.

logarithmic value of the ratio of these concentrations is often used and referred as log *P*, or partition coefficient. When an aqueous buffer solution (often pH 7.4) is used instead of water, the value is referred to as log *D*, or distribution coefficient. These coefficients, which are descriptions of the lipophilicity of a compound, are often correlated to the ability of a compound to cross biological membranes as well as their ability to dissolve in formulation vehicles.

The ionization constant (K_a), an intrinsic property of the molecule, describes the ionization behavior of a compound as a function of pH. The negative logarithm of K_a is often used and referred to as pK_a. The pK_a is equal to the pH value when the ratio of the ionized and unionized species is one. The pK_a is thus an important determinant in the pH dependence of ionization and hence solubility as well as salt formation ability of a molecule. These concepts will be further expanded elsewhere in this chapter. If a compound has multiple ionizable groups, each group has a corresponding pK_a value.

The molecular structure of the compound can be utilized for obtaining additional first estimate of properties such as dissociation constants and partition coefficients utilizing prediction software (e.g., from ACD/Labs, Simulations Plus, etc.). Such software packages can also provide a first estimate of the solubility and pH-solubility profiles. These data are especially useful during early development when compound supply is very short and there is a need to provide formulations for discovery pharmacology and early toxicology studies.

Solubility

Solubility is the concentration of drug in solution at equilibrium with excess solid. Typically, when the solid drug is brought in contact with a solvent, it dissolves into the solvent over a period of time and achieves equilibrium asymptotically. Aqueous solubility is of particular relevance to biological activity, bioavailability, and formulation strategy (3).

Solubility is experimentally measured by placing an excess solid in a test tube in contact with a particular solvent with mild agitation and determining the concentration of the drug in a supernatant solution over a period of time using appropriate analytical techniques such as UV spectrophotometry or high-performance liquid chromatography (HPLC). In determining equilibrium solubility, it is important to ascertain that (*i*) an asymptotic value has been achieved (constant over multiple time-points) and (*ii*) the identity of the solid in contact with the solvent is unchanged. The identity of the residual phase can be confirmed by analyzing the residue using techniques such as differential scanning calorimetry (DSC) or X-ray powder diffractometry (XRPD).

During preformulation studies, it is common to determine solubility of the drug compound in aqueous and nonaqueous vehicles used in pharmaceutical formulations. Aqueous systems include buffers, surfactant solutions, and complexant solutions. Nonaqueous

systems include cosolvents (e.g., ethanol, glycerol, polyethylene glycols) and oils (soyabean oil, glycofurol). A more detailed list of excipients is discussed later in this chapter.

pH-solubility profile. Many pharmaceutical compounds contain acidic or basic functional groups and hence show pH dependence in their aqueous solubility. Solubilities can vary significantly in accordance with the pK_a across acceptable pH range. Hence, adjusting pH to achieve requisite solubility can be an important tool in formulating injectable solutions.

The pH dependence of solubility of acids and bases is derived from the ionic equilibria occurring across the pK_a of a compound and is described by the Henderson-Hasselbalch equation (4).

$$\mathrm{pH} = \mathrm{p}K_a + \log\frac{[\mathrm{A}^-]}{[\mathrm{HA}]} \quad (\text{for an acid}) \tag{1}$$

$$\mathrm{pH} = \mathrm{p}K_a + \log\frac{[\mathrm{B}]}{[\mathrm{BH}^+]} \quad (\text{for a base}) \tag{2}$$

Taking the example of a free base, the total solubility of at any given pH is the sum of the solubility of the unionized species (S_0) and the ionized species.

$$S = S_0 + [\mathrm{BH}^+] \tag{3}$$

Figure 1 shows a hypothetical pH-solubility profile for a weak base. At a high pH (pH >> pK_a), the solubility is practically independent of pH and is essentially S_0. As the pH approaches the pK_a, the fraction of ionized species and hence the total solubility increase and are described by

$$S = S_0\left(1 + \frac{[\mathrm{H}^+]}{K_a}\right) \tag{4}$$

The ionized species can associate with a charged counterion to form a salt. This linear increase in solubility ends abruptly when the solubility of the salt form is reached, and at this point the solubility is governed by the solubility product (K_{sp}) of the salt form. For example,

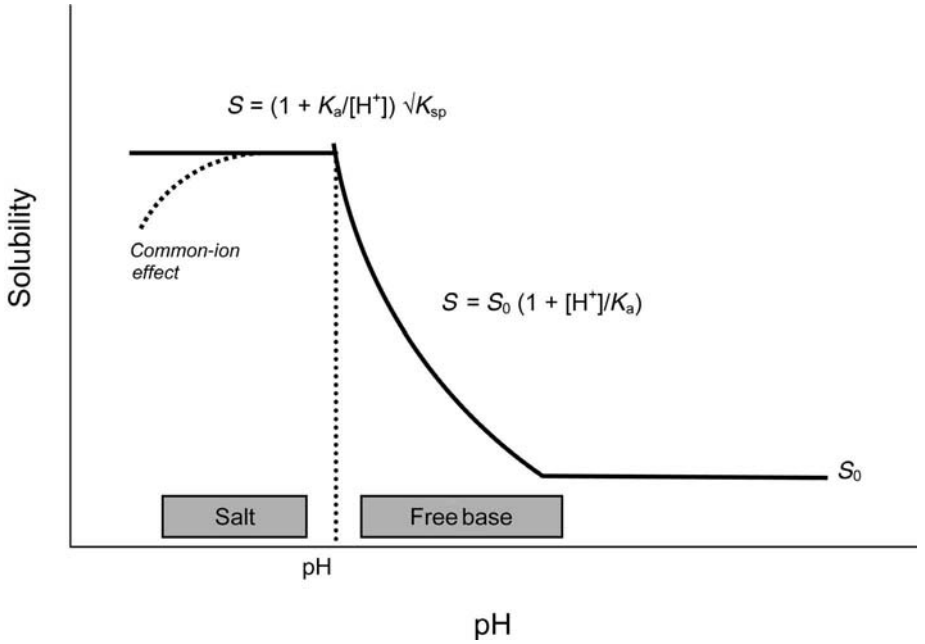


Figure 1 pH-solubility profile of a hypothetical weak base.

Table 2 Properties of Some Commonly Used Solvents

Solvent	Dielectric constant (ε)	log P	Surface tension (γ) (dynes/cm)
Water	81.0	−4.00	72.0
Glycerin	42.5	−2.60	64.9
Propylene glycol	36.7	−1.93	48.8
Ethanol	24.3	−0.31	22.2

assuming that the pH was being changed by titrating with hydrochloric acid, the solubility product is

$$K_{sp} = [BH^+][Cl^-] \tag{5}$$

and the total solubility at this pH_{max} would be

$$S = \left(1 + \frac{K_a}{[H^+]}\right)\sqrt{K_{sp}} \tag{6}$$

Rearranging the equation, the pH_{max} can be determined if the solubility product is known.

$$pH_{max} = pK_a + \log\frac{S_0}{\sqrt{K_{sp}}} \tag{7}$$

Common-ion effect or salting-out effect is also depicted in Figure 1, representing the pH-solubility profile of a weakly basic drug. From the pH of maximum solubility, as one moves toward lower pH values, there is an increase in the concentration of the counterion (e.g., $[Cl^-]$). Depending on the value of the solubility product (a function of the nature of the drug and the counterion), this increase may be compensated by a decrease in the concentration of the ionized drug molecule. This decrease occurs through a precipitation of the drug in its corresponding salt form. This phenomenon is known as "salting-out" or common-ion effect and can be an important consideration in selecting salt forms or buffer systems for formulations.

Solubility in cosolvent systems. Cosolvents such as ethanol, propylene glycol, polyethylene glycols, and glycerol are routinely used in formulating to a higher solubility when aqueous solubility alone is not sufficient to achieve required levels. In case of some drug compounds, the use of appropriate cosolvents can increase the solubility quite significantly. The mechanism behind the increased solubility is frequently related to modifying the polarity or dielectric constant of the solvent system. The principle of "like dissolves like" works—less polar molecules would be better dissolved in a less polar solvent system. Adding a cosolvent with a smaller dielectric constant to water will bring down the overall dielectric constant of the resultant solvent system and make it a better medium for dissolving a less polar or nonpolar molecule. Table 2 shows some physical parameters of common cosolvents (5).

Although cosolvents can be quite effective in achieving solubilization, it should be noted that as excipients these can have toxicological effects (e.g., hemolysis) and potential for local irritation depending on the concentrations used. Additionally, it is very important to consider the potential for the drug to precipitate upon dilution (6). This risk can be assessed both by calculating the degree of precipitation that could occur and by experimentally simulating the dilution that could occur and testing for precipitation potential (7).

Solubility in surfactant systems. Surfactants, a common class of excipients, are amphiphilic molecules (hydrophilic head group and hydrophobic tails), which strongly orient themselves at interfaces. In an aqueous system surfactant molecules would mainly be present at the water-air interface with a small but finite concentration in the bulk of the solution. Surfactants oriented at the water-air interface cause a reduction in the surface tension of water and thereby

improve wettability of drugs being exposed to such a system. With increasing concentration of surfactant in the system, the interface becomes crowded, and at a specific concentration, the surfactant molecules in the bulk orient themselves in micellar structures. Micelles consist of spherical structures with the hydrophobic (lipophilic) tails toward the core and hydrophilic heads forming the external surface. The concentration at which this occurs is called the critical micelle concentration (CMC). Above the CMC, aqueous surfactant systems would contain micellar structures in the bulk.

Lipophilic drugs can be incorporated into the core of micelles, thereby increasing the total solubility of a drug into aqueous systems. The lipophilic cores of micelles present a different environment to the drug molecule providing, in some instances, a stabilizing effect against chemical degradation. Surfactants will preferentially orient toward the surface of nuclei during a precipitation phenomenon and can prevent precipitation occurring due to dilution effects. Thus, surfactants can be a very useful tool in formulating aqueous injectable solutions and suspensions.

Examples of surfactants commonly used in injectable formulations include polyoxyethylene sorbitan monoesters (Tweens), polyoxyethylene-polyoxypropylene copolymers (Pluronics), sodium lauryl sulfate, and lecithins.

Solubility in complexant systems. A complex is an entity formed when two molecules, such as a drug and a solubilizing ligand, are held together by weak, noncovalent forces (dipole-dipole, hydrophobic, or hydrogen bond interactions). Cyclodextrins are a class of such solubilizing ligands that have found a significant application to pharmaceutical compounds. α-, β-, and γ- Cyclodextrins are cyclic oligomers of glucose containing six, seven, or eight glucose residues. Cyclodextrins have gained popularity from a pharmaceutical standpoint because of the ability of these materials to interact with poorly water-soluble drugs and drug candidates resulting in an increase in their apparent water solubility. The mechanism for this solubilization is rooted in the ability of cyclodextrin to form noncovalent dynamic inclusion complexes in solution. As a result of their structure, cyclodextrins present a hydrophilic exterior but a core that is more lipophilic and hence provides a microenvironment for lipophilic drug molecules to engage via hydrophobic interactions. In certain cases, the modified microenvironment of the cyclodextrin core results in improved chemical stability similar to micellar systems. The ability of the cyclodextrin to solubilize a drug compound depends on steric factors (size of the cavity) and thermodynamic factors (decrease in free energy of the system). Additionally, the solubility of the cyclodextrin in water is another key determinant. β-Cyclodextrin has relatively low water solubility (~18.5 mg/mL), but chemical modifications of the basic β-cyclodextrin have imparted improved solubility and lower toxicity. Two of the modified β-cyclodextrins that have gained greater acceptance are hydroxypropyl-β-cyclodextrin (HP-β-CD) and sulfobutylether-β-cyclodextrin (SBE-β-CD). These have water solubilities of about 600 mg/mL and 500 mg/mL, respectively. Both of these modified cyclodextrins have been used in developing injectable formulations that are now FDA-approved products.

During preformulation studies, it is common to assess the solubility of a poorly soluble drug candidate in such cyclodextrins. If solubilization via cyclodextrin complexation is identified as a potential formulation approach, then it is also important to fully characterize the interactions in terms of stoichiometry of the complex as well as the equilibrium constant for the complexation. A number of excellent reviews cover the theoretical and experimental considerations for such determinations in detail (8–10).

Stability and Drug Degradation

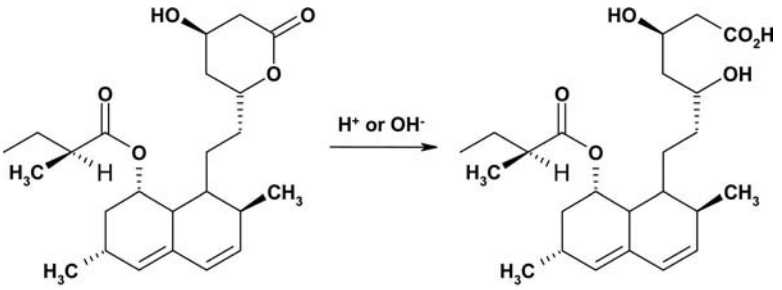
In addition to solubility, stability of the active drug compound is a key determinant in the viability of parenteral drug product. First, it is essential for a drug product to maintain potency relative to label claim over the shelf life to deliver an accurate dose. Second, degradation in the drug product can result in changes in appearance (color, precipitation) or bioavailability. Finally, degradation of the active compound can result in degradation products that may have toxicity that is more significant than that of the active drug substance. Depending on the daily

dose of the active and levels of such degradation products anticipated in a drug product, they may be subject to additional toxicological qualifications as described in ICH Guidance Q3B (R2) (11). When impurities or degradation products are identified as potentially genotoxic, they have to be controlled to very low levels if not completely avoided. This process is detailed in the EMEA Guidance on Limits for Genotoxic Impurities (12). Thus, it is essential to understand the stability and degradation of the active ingredient as a bulk drug substance and in formulation. Understanding the degradation pathways, kinetics, and mechanisms leads to development of a stable drug product (13,14).

During preformulation studies, the goal is to understand the modes of instability of a drug compound, kinetics of degradation, and factors (including formulation factors) influencing the kinetics of such degradation (15). One of the first steps is to develop a stability-indicating method that is capable of resolving and quantifying impurities and degradation products resulting from the drug compound. Typically, HPLC with UV detection is used in preformulation studies, but techniques such as LC-MS and NMR spectroscopy could often aid in the identification of degradation products. HPLC methods are developed to effectively resolve degradation products resulting from forced degradation studies (highly stressed condition of temperature, humidity, or pH).

Modes of degradation. Chemical degradation of small-molecule drugs can occur because of various chemical processes. However, a majority of these fall into three types of reactions.

Hydrolysis This is a very common pathway for drug degradation (16) and is essentially the cleavage of a molecule under the effect of water. Since water, either as a solvent or in the form of moisture in the air, is ubiquitous, the potential for this degradation pathway exists for most drugs. This is of particular relevance to parenteral products, which are mostly formulated in aqueous systems. Chemical bonds that commonly undergo hydrolytic degradation include lactam, ester, amide, and imide bonds. Aspirin is the most common example of a drug undergoing hydrolytic degradation. Lovastatin is a prodrug that undergoes activation through hydrolysis by carboxyesterases in vivo. In vitro it undergoes hydrolysis under acidic and basic conditions by cleavage of the lactone.



lovastatin
(hypolipidemic agent)

Hydrolytic reactions can be significantly influenced by the composition of the medium—pH, buffer concentration, ionic strength, etc. The relationship of the rate of reaction (expressed as rate constant k_{obs}) with pH is quite informative both for understanding the mechanisms involved as well as a guide for formulation. When the reaction is catalyzed by the hydronium (H^+) ion, it results in a slope of negative one on a log k_{obs} versus pH profile, and similarly, when the reaction is catalyzed by the hydroxide ion (OH^-), a slope of one is observed. If no other catalyses are involved, then these two lines meet, forming a V-shaped profile. The pH at which they meet represents pH of maximum stability and is important to know during selection of formulation pH. The shape of curves can be more complicated (U shaped, additional inflections, etc.) depending on the number of ionic species involved (15,17).

In addition to the pH of the medium, concentration of the buffer itself can play a catalytic role in hydrolysis. This can be studied by studying the reaction rate as a function of buffer type and concentration while holding the pH constant. Ester hydrolysis of an experimental compound GW280430A was shown to be catalyzed by citrate, malate, and tartrate buffers but not by a glycine buffer (18). This phenomenon is termed as general acid/base catalysis or buffer catalysis and can often be the cause of deviation from a slope of -1 or $+1$ described in specific acid/base catalysis in the previous paragraph. Additionally, reactions can also be affected by ionic strength, which can be studied by holding the pH and buffer concentration constant and studying the reaction rate as a function of concentration of added ions (e.g., NaCl). Typically, this is not a big effect in pharmaceutical systems.

In summary, hydrolysis is a key degradation pathway for many drug compounds. pH-stability profiles can vary from a simple V shape to more complex profiles depending on the number of ionization states and the different reactivities they present. While some of the pathways can be predicted on the basis of the structure, evaluation of the pH-stability profile and effect of buffer catalysis can be very important in designing the formulation strategy.

Oxidation Oxidation is another common mode of drug degradation. Oxidation can be broadly defined as a loss of electrons in a system; alternately, it could be considered as an increase in oxygen or a decrease in hydrogen atoms. The reaction occurs in concert with reduction of the other reactant, thus forming a redox reaction. If molecular oxygen is involved in the reaction, this is termed as "auto-oxidation." Trace metals and light can catalyze oxidation reactions by initiating free radical chain reactions. Once formed, the radical can be propagated until a termination reaction or a suitable chemical inhibitor intervenes. These reactions can happen in aqueous and nonaqueous media.

Excipients used in formulation can be a source of trace metals and also peroxides, which can have significant effect on oxidative drug degradation. Table 3 shows levels of hydroperoxides measured in some commonly used pharmaceutical excipients (19).

To control oxidation reactions, antioxidants are often included in a formulation. Antioxidants used in a formulation could affect different stages of an oxidation reaction. True antioxidants (e.g., butylated hydroxy toluene, α-tocopherol) react with free radicals, resulting in termination of the chain reaction. Reducing agents (e.g., ascorbic acid) get preferentially oxidized and hence reduce the level of oxygen or the oxidant in the formulation. Chelating agents such as EDTA sequester trace metals which can catalyze oxidation and thereby function as antioxidant synergistic agents. Depending on the reaction involved, a combination of such agents may help control the oxidative degradation (20). Also, during manufacturing and in the primary package, an inert atmosphere generated by nitrogen blanketing can help control oxidative degradation.

Photolysis Photolysis, also referred to as photodegradation, occurs as a result of absorption of light (or radiation energy) (21). When the absorbed energy dissipates through a chemical change in the molecule, photolysis occurs. The changes may result in a color change, precipitate formation or may not be visually detectable. However, there is always loss of potency that is accompanied. Toxicity of the decomposition products is also of concern,

Table 3 Levels of HPO in Some Commonly Used Excipients

Excipient	Number of batches tested	Average HPO (nmol/gm)	Range of HPO (nmol/gm)
Polyvinylpyrrolidone	5	7,300	3,600–11,000
Polyethylene glycol 400	4	2,200	1,000–3,300
Polysorbate 80	8	1,500	180–4,600
Poloxamer[a]	7	30	10–50
Mannitol	5	<10	<10
Sucrose	5	<10	<10–20

[a]Different grades (188, 338, and 407) and batches tested.
Abbreviation: HPO, hydroperoxides.

especially when such products can form by the action of sunlight on the skin or eyes after administration (phototoxicity) (22).

Photodegradation depends on wavelength of the incident light as well as intensity. Primary photochemical reactions usually occur at wavelengths where the drug absorbs light, that is, in regions where the UV/VIS absorption spectrum of the drug overlaps with the spectrum of incident radiation. In some instances it is possible that the energy absorbed by a nondrug molecule (photosensitizer) in the formulation is transferred to the drug molecule, which eventually degrades. Examples of some common drugs that undergo photolytic degradation include methotrexate, furosemide, and tetracyclines. For many drug substances, the kinetics of photodegradation varies significantly with the ionization state of the molecule. Examples would include ciprofloxacin, midazolam, mefloquine, and ameloride (23).

Once a photoinstability is identified, it can be addressed during formulation development through different means. A protective market pack is one of the simplest solutions. Control of pH, ionic strength, trace metals, or even use of complexants (24) can be formulation approaches to also address such instability.

In addition to these major modes of degradation, many other routes are involved in drug degradation such as decarboxylation, racemization/epimerization, acylation, etc. Understanding the causes of drug instability allows for a rational design of a formulation.

Preformulation stability studies. Typically, the drug substance is studied in solid as well as solution states. Stability studies might involve storing the samples under stressed conditions of temperature and humidity such as 40°C/75% RH and 50°C. If the drug is fairly stable, conditions such as 80°C/75% RH and 80°C may be employed to get a first view of drug instability in a reasonable amount of time. These studies are conducted over a short duration such as four to six weeks.

Additionally, the solid drug and an aqueous solution of the drug are exposed to a representative duration and intensity of light in appropriate photostability chambers [as per ICH Q1B (25)]. These studies may be able to indicate not only potential need for protecting the drug product from light but also the need for conducting other stability studies under light-protected conditions. Failure to know this early can produce confounding results.

pH-stability profiles are determined by preparing aqueous solutions of the drug at various pH values ranging from 2 to 12 and studying the kinetics of degradation (loss of active/growth of degradation products) at an appropriate elevated temperature. The solutions are sampled at regular intervals and analyzed using a stability-indicating method. The time course of degradation at a particular pH can typically be expressed as the first-order rate constant k_{obs} (k observed). A log k_{obs} versus pH plot is referred to as the pH-rate profile and can be quite revealing of the mechanisms involved in drug degradation. The pH of maximum stability would be targeted as the pH for the formulation as long as it agrees with the required solubility and local tolerability at that pH.

Form Selection

The solid form of the drug compound can have a significant effect on parenteral drug product processing. During late discovery or early development stages, the solid form of the drug compound needs to be defined and fixed to develop formulations and processes consistent with the expected physical and chemical properties of the API. The solid form is typically described by the salt form used and the crystal polymorph of the chosen salt.

Salt Form

Many drugs are either weak acids or weak bases and can consequently form a range of salts by reacting with various bases and acids, respectively. Salt formation may be employed to alter the physicochemical, biopharmaceutical, and processing properties of a drug substance without modifying the pharmacologically relevant moiety (26).

To form stable salts, the pK_a of the basic center should be greater ($\Delta pK_a \geq 2$) than the pK_a of the conjugate acid to be utilized. Thus, for a basic drug, pK_a of the basic center will determine what salts are feasible.

In the case of parenteral medications, increased solubility is often desired from chosen salts. In general, utilizing counterions with greater acidity, utilizing more hydrophilic counterions (hydroxy acids), and lowering the melting point of the resultant salt (decreased crystal lattice energy) can result in increased solubility. Agharkar et al. (27) demonstrated an increased solubility of an experimental antimalarial drug as a result of decreased crystal lattice energy due to salt formation.

In the case of solution formulations, it is not essential that salt formation is only employed for obtaining a suitable solid form. Salts can be formed in situ in solutions by using the appropriate acid or base to adjust pH of the formulation (28). Sometimes the high aqueous solubility achieved prevents a salt from being easily isolated but can still be utilized as an effective solubilization approach, as previously discussed in the context of pH-solubility profiles.

Polymorph Selection

Polymorphism is defined as the ability of a substance (of constant chemical composition) to exist in two or more crystalline phases that differ in crystal packing arrangement and/or conformation of the molecules in the crystal lattice. The different crystalline forms are then termed as polymorphs.

Crystals are made up of repeating blocks called unit cells. Different polymorphs have distinct unit cells. Polymorphs can differ in various physical, physicochemical, and physicomechanical properties. Differences such as melting point, enthalpy of melt, true density, and powder X-ray diffraction patterns help characterize and differentiate between polymorphs. One can screen for polymorphs by crystallizing a drug from different systems of solvents, evaporation and cooling profiles, and then examining crystals obtained. However, it is not easy to search exhaustively for all possible crystal forms, and often new forms are discovered during development. To reduce the risk, many automated crystallization systems have been developed, which help examine a larger experimental space.

Polymorphism is commonly of concern in the context of solid dosage form bioavailability and processing (29). However, polymorphs also differ in properties that impact a parenteral drug product formulation of which solubility, dissolution rate, and hygroscopicity are of most relevance. Polymorphs differ in their free energy as a result of their packing, and this manifests itself as differences in solubility. The most stable polymorphic form has the lowest solubility. If a metastable polymorph is used in a solution or suspension formulation, there will be a risk of growing crystals of the stable form over a period of time. Solvent maturation studies and temperature cycling of prototype formulations can help identify such problems early.

When a solvent molecule incorporates itself into a crystal lattice associated with a drug compound, it is said to form a solvate. When this solvent is water, it is termed as a hydrate. A hydrate form of the drug is more stable than an anhydrous form and will exhibit lower solubility in an aqueous system. Thus, it is also important to understand and characterize solvate and hydrate forms of the drug compound.

Characterization of Material Properties

Appearance and Microscopy

The solid form of a drug substance is characterized by its appearance in terms of color and subjective description. Additionally, examination under a microscope reveals further details such as crystal morphology and habit.

Crystallinity

Crystalline material can be identified by polarized light optical microscopy where the sample displays birefringence. Crystallinity is also commonly examined by XRPD. An X-ray diffraction pattern is generated because of constructive and destructive interference of X rays reflected off the crystal planes of a powder sample as the angle of incidence is varied. This is described by the Bragg equation.

$$n\lambda = 2d \sin\theta \tag{8}$$

where θ is the incident angle, λ is the wavelength of the X radiation, d is the distance between the crystal planes, and n is an integer representing the order of reflection.

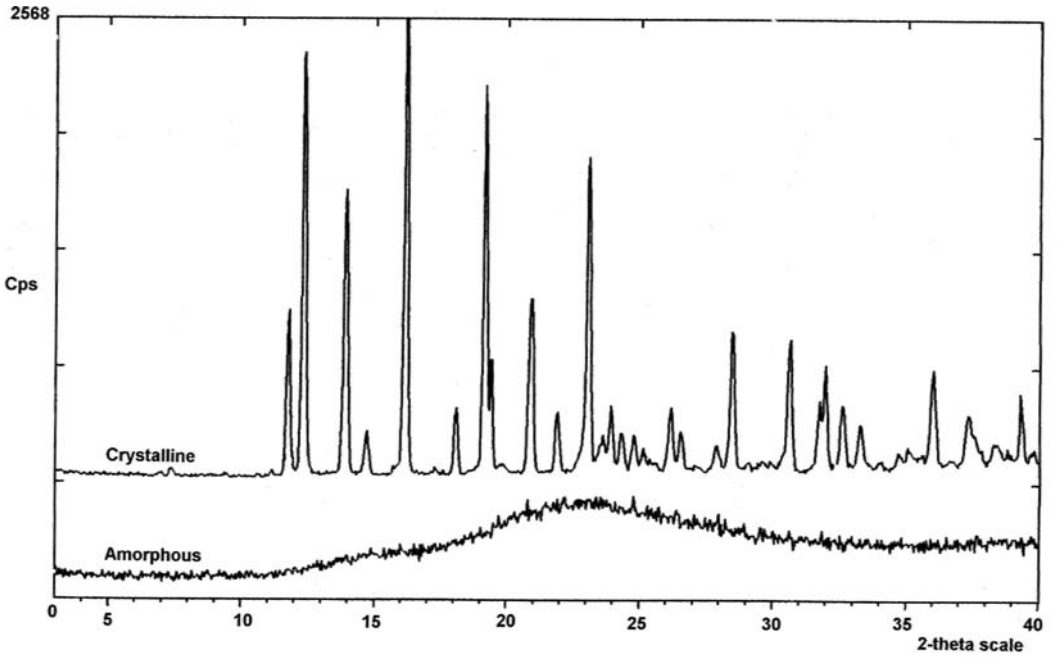


Figure 2 X-ray powder diffraction patterns showing amorphous and crystalline states of an experimental drug compound.

Crystalline forms are characterized by sharp characteristic peaks, while an amorphous material displays a broad halo (Fig. 2) (30). XRPD can be used to distinguish between different polymorphs, solvates, and hydrates. Further, this technique can also be used to quantify mixtures of polymorphs and degree of crystallinity of a crystal form.

Thermal Properties

DSC measures the difference in the amount of heat required to raise the temperature of a sample and a reference as a function of a change in temperature. A typical output shows heat flow into (endothermic event) or out of (exothermic event) the sample as a function of temperature. Melting of a crystalline material is observed as an endothermic event characterized by an onset temperature (melting point) and heat of fusion measured as the area under the endothermic curve. At the glass transition temperature, amorphous materials undergo a transition from a glassy rigid state to a rubbery state of greater mobility (a higher heat capacity), and this is observed on the DSC as a baseline shift characterized by temperature (T_g) and change in heat capacity (ΔC_p). The glass transition is sometimes followed by a small endotherm of enthalpic relaxation related to time-dependent relaxation of this phase. Figure 3 shows the DSC thermogram of an experimental drug compound displaying these transitions along with an overlay of corresponding changes to the X-ray diffraction patterns as observed by variable-temperature XRPD (31).

Modulated DSC (mDSC) is a related technique where an oscillation of temperature is introduced on top of a linear heating rate. This allows deconvolution of the output into reversing (thermodynamic) and nonreversing (kinetic) components, allowing a further understanding of the transitions measured. This can be of particular utility in studying amorphous materials (29).

Thermogravimetric analysis (TGA) measures the weight of the sample as a function of increasing temperature. Loss of water, solvents, or volatile decomposition products can be observed as a weight loss at characteristic temperatures. This analysis is a key technique in characterizing solvates and hydrates. The technique is sometimes further coupled with an IR spectrometer or a mass spectrometer to characterize the evolved volatile components that come off during heating of the sample.

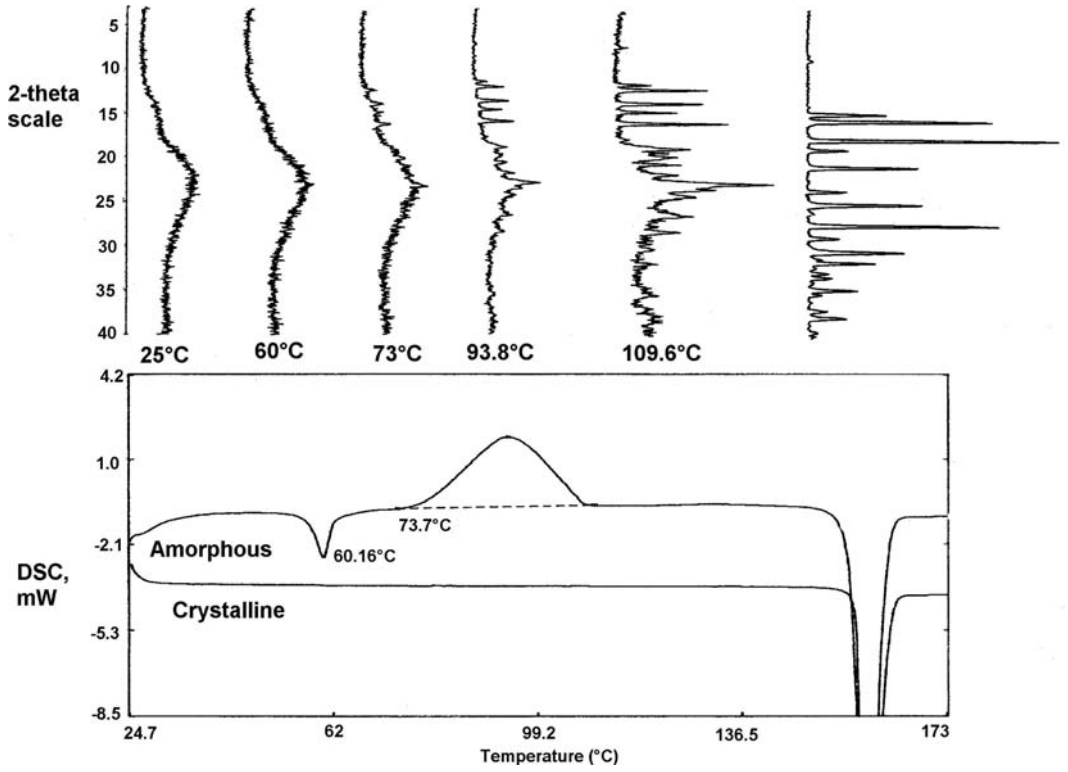


Figure 3 DSC curves of crystalline and amorphous phases of an experimental drug compound overlaid with XRD patterns of the amorphous phase obtained at temperatures corresponding to thermal events in the DSC curve. *Abbreviations*: DSC, differential scanning calorimetry; XRD, X-ray diffraction.

Vapor (moisture) Sorption Analysis

The weight of the sample is monitored as it is exposed to different relative humidities for a period of time approaching equilibrium. The output is a moisture sorption profile, which depicts the sample weight as a function of relative humidity. When a material picks up enough water that causes a change in its physical properties, the material is considered hygroscopic. Crystalline materials typically *adsorb* small amounts of water on the surface unless they pick up water molecules into the crystal lattice to form hydrates. Hydrates are characterized by picking up stoichiometric amounts of water and are physically stable over a range of %RH. Deliquescence occurs when the material adsorbs enough water to dissolve into it thereby turning liquid. This can sometimes happen with salts of hydrophilic molecules and is characterized by a sharp increase in moisture uptake at humidity values greater than a threshold %RH.

Amorphous materials *absorb* water and other solvents into the bulk. The absorbed solvent acts as a plasticizer and reduces the apparent glass transition temperature. When the apparent glass transition temperature drops below storage temperature, the material goes into a mobile rubbery state from which collapse of the structure (liquefaction) with possible recrystallization can occur. This relationship of glass transition temperature as a function of absorbed water is critical to understand when developing a lyophilization process.

INTERACTION BETWEEN THE DRUG SUBSTANCE AND FORMULATION COMPONENTS

Formulation Components

In formulating a parenteral drug product, a number of excipients are employed, and these often form the bulk of a drug product. These excipients are included to dissolve the drug substance, increase the chemical or physical stability of the drug product, give the product

Table 4 Excipients Used in Parenteral Formulations

Solvents and cosolvents
- Glycerin
- Propylene glycol
- Ethanol
- Polyethylene glycol (300, 400)
- *N,N*-dimethylacetamide
- Soyabean oil
- Corn oil
- Ethyl oleate
- Glycofurol

Surfactants (solubilizers, emulsifiers, and suspending agents)
- Polysorbate 80 (Tween 80)
- Polysorbate 20 (Tween 20)
- Polyoxyethylene-polyoxypropylene copolymers (poloxamers)
- Cremophor EL
- Lecithin

Complexants
- Hydroxypropyl-β-cyclodextrin
- Sulfobutylether-β-cyclodextrin (Captisol®)

Buffers
- Citrate
- Phosphate
- Tartrate
- Tromethamine (TRIS)

Chelating agents
- Disodium ethylenediaminetetraacetic acid

Antioxidants
- Ascorbic acid
- Butylated hydroxy anisole
- Butylated hydroxyl toluene
- Sodium bisulfite
- Propyl gallate
- α-Tocopherol

Preservatives
- Benzalkonium chloride
- Benzethonium chloride
- Benzyl alcohol
- Chlorbutanol
- Paraben (methyl, propyl)
- Thimerosal

Tonicity adjusters, bulking agents, lyoprotectants
- Sodium chloride
- Mannitol
- Glycine
- Sucrose
- Trehalose
- Dextran
- Povidone

microbiological protection, or control other product attributes. Since inclusion of new additives could require extensive pharmacological and toxicological evaluation, it is common for formulators to depend on materials already used in marketed parenteral products. Table 4 shows a representation of the classes of excipients that might be used in parenteral formulations and some examples of each of these categories. There is more discussion within this book on the functions and levels of these excipients. Additionally, the reader can refer to some excellent reviews that have been published on this topic (31,32). The FDA also maintains a listing of inactive ingredients used in approved products (33).

Designing Excipient Compatibility Studies

Excipients are often referred to as inactive or inert ingredients to distinguish them from the APIs. However, the lack of pharmacological activity does not necessarily result in a lack of chemical reactivity. Excipients can have significant expected and unexpected effects on the physical and chemical stabilities of a drug product. This is first assessed through well-designed excipient compatibility studies conducted at the preformulation stage (34).

Traditionally, thermal methods such as DSC have been employed as a first screen in determining incompatibilities (35). In these studies, the drug, excipient, and drug-excipient mixture are subjected to a temperature program. If the thermogram of the mixture is not representative (temperature and enthalpy) of the combination of the two single components, then an incompatibility could be suspected. Modifications such as a stepwise isothermal high-sensitivity DSC study have also been tried (36). However, DSC techniques have proved to be of limited predictability.

Isothermal heat conduction calorimetry is a technique that measures heat evolved or absorbed by a sample (relative to a suitable reference) with great sensitivity. Hence, even slow reactions occurring under isothermal (25°C, 45°C/75% RH) can be detected because of the

Table 5 A Plackett–Burman Design

	Variable											Response
Trial	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	Y
1	+	+	−	+	+	+	−	−	−	+	−	
2	+	−	+	+	+	−	−	−	+	−	+	
3	−	+	+	+	−	−	−	+	−	+	+	
4	+	+	+	−	−	−	+	−	+	+	−	
5	+	+	−	−	−	+	−	+	+	−	+	
6	+	−	−	−	+	−	+	+	−	+	+	
7	−	−	−	+	−	+	+	−	+	+	+	
8	−	−	+	−	+	+	−	+	+	+	−	
9	−	+	−	+	+	−	+	+	+	−	−	
10	+	−	+	+	−	+	+	+	−	−	−	
11	−	+	+	−	+	+	+	−	−	−	+	
12	−	−	−	−	−	−	−	−	−	−	−	

sensitivity of the technique (37). This technique has been used to compare the heat signal from a drug-excipient mixture with the sum of the curves generated by the individual components under the same conditions. The magnitude of this interaction curve (difference curve) is an indicator of the extent of the incompatibility (38). However, this technique generally suffers from the fact that it is nonspecific and it is important to carefully design appropriate control experiments to make sure that the recorded heat pertains to a specific chemical incompatibility.

Given some of the challenges described above, the conventional method of chemical analysis of mixtures stored under accelerated storage conditions is still the most commonly employed method. The prerequisite for this methodology is having a stability-indicating method, and most commonly, this is an HPLC method. Since in a parenteral product, the drug and excipients are in very close contact (at a molecular level in the case of solution products) with each other, the stabilizing or destabilizing effect of an excipient is best studied in the presence of all formulation components (prototype formulations) including the targeted primary packaging when possible. High and low levels of each excipient or formulation factor are identified for testing on the basis of conventional levels used in experience or levels approved for use by regulatory authorities.

Different experimental designs can be used for obtaining the required information from a limited number of experimental runs. In such studies excipients constitute factors (at two levels—high and low) in a factorial design of experiments. For such studies screening design is employed at first. A commonly used screening design is a fractional factorial design called a Plackett–Burman design. Table 5 represents a possible design for studying 11 factors by performing 12 trials. This design was employed for a parenteral preformulation study for Naproxen as described by Peswani and Lalla (39). In this study they looked at effects of five excipients, pH, buffer type, autoclaving, and nitrogen blanketing by conducting 12 trials. Although these designs are quite efficient in terms of number of trials, it should be noted that these designs are not capable of identifying interaction terms (e.g., if two factors interact to produce an effect). If such confounding is suspected and needs to be resolved, a full factorial design study could be conducted on a smaller number of identified factors. The reader can get details of the advantages and disadvantages of different experimental designs from other reviews of this specific topic (40).

INTERACTION OF THE DRUG WITH PACKAGING COMPONENTS AND MANUFACTURING SURFACES

Parenteral drug products are in close contact with the primary package of the drug product; so it is useful to carefully consider primary package in the same way other formulation ingredients are evaluated. These packaging materials would include glass vials (or ampoules),

rubber stoppers, infusion bags, etc. Glass vials are most commonly type I (borosilicate glass), but that too can undergo different surface treatments at the manufacturer. Rubber stoppers (commonly butyl or other synthetic rubber and rarely natural rubber because of its sensitizing potential) and bag materials can be quite complex in composition. The formulation scientist works closely with the rubber manufacturer as with the glass manufacturer to choose the appropriate rubber formulation having consistent specifications and characteristics to maintain product stability. It is important during preformulation studies to include an evaluation of likely primary packaging materials to assess potential issues such as adsorption and incompatibilities. Also important to consider are other likely surfaces to be encountered during manufacturing steps, for example, stainless steel, glass, tubing, and filters.

Adsorption

Adsorption occurs when a molecule is attached to another solid surface, most commonly because of Van der Waals forces, hydrogen bonding, or electrostatic interactions. This can often occur with low-solubility hydrophobic compounds as they may prefer another surface as opposed to being in water. When a covalent bond is involved, the adsorption is chemisorption, but this is not commonly observed in the systems being discussed here.

To evaluate adsorption, the formulation (at the most dilute concentration likely) is exposed to the surface and then assayed for loss of drug concentration. For filters and tubing, this might involve passing through the tubing and filters for a fixed duration of time that will exceed the likely duration of a manufacturing run. For stoppers, it might be done by adding a fixed number of stoppers to flasks containing the formulation and storing for a fixed period of time before assaying the concentration. During development of an injectable formulation of Abbott-72517, Gupta et al. observed a 6% of loss of drug (250 mL recirculated for four hours) using a Pall Nylon 66® filter but no loss with a Millipore Durapore disk membrane (41). If adsorption to potential surfaces is identified early on, then it can be used to select appropriate materials for packaging and manufacturing processes.

Compatibility

In addition to adsorption, the degradation of the drug molecule can also be effected by packaging material or manufacturing surfaces. Thus, when feasible, it is useful to conduct excipient compatibility studies using preferred container closure systems. An early readout on any potential incompatibility can lead to an early assessment of alternatives and prevent the loss of time during development. For instance, rubber stoppers can leach out trace quantities of zinc into the formulation and effect oxidation of the drug. If a drug is particularly prone to oxidation, a steel surface may aggravate the issue and a glass-lined tank may be an appropriate measure. Protein drugs could be especially sensitive to silicone that is used on rubber stoppers. A nonsiliconized rubber with a bonded coating may be the answer to the issue.

SPECIALIZED FORMULATIONS

Suspensions and Nanosuspensions

Sterile injectable suspensions comprise of the active compound dispersed in a liquid vehicle either as a ready-to-use formulation or as a dry powder for reconstitution. Such formulations may be engaged either when the drug has solubility that is too low for a solution formulation or for prolonging the release of the drug through depot formulations. Aristocort® is a suspension of triamcinolone diacetate and may be administered by the intramuscular, intra-articular, or intrasynovial routes depending on the situation (42). NPH insulin is a suspension of crystalline zinc insulin combined with the positively charged polypeptide protamine. When injected subcutaneously, it has an intermediate duration of action. Depo-Medrol® is an anti-inflammatory glucocorticoid for intramuscular, intra-articular, soft-tissue, or intralesional injection. One of the challenges of formulating such products involves an evaluation of suspension physical stability with regard to resuspendability and caking.

Another area of specific concern for suspensions is syringeability (drawing a uniform dose) and injectability (pressure applied to expel product through a needle of specified gauge) of the product. The flow properties of the suspension can be characterized using techniques such as rheometry. This technique characterizes the flow of a fluid in response to a range of

applied stresses, resultant strains, and temperatures. Many suspensions and emulsions do not show a linear relationship between applied stress and strain (non-Newtonian behavior) and hence cannot be characterized by a single value for viscosity. A full discussion of this topic is out of the scope of this chapter and is well captured in many reviews on this topic.

For suspension formulations, the solid-state properties are quite relevant. Particle size of the dispersed phase can have a significant impact on the physical stability and syringeability of a suspension. Particle size distributions in suspensions can change over time because of Ostwald ripening—a solution-mediated phenomenon during which larger particles grow at the expense of smaller particles dissolving. An appropriately selected medium and surfactant can minimize the impact of this phenomenon. During screening, subjecting prototype samples to temperature cycling can accelerate the event and help select systems that are the most stabilizing. Crystal growth can also occur because of a more stable polymorph precipitating or a salt being formed. A change in crystal habit can result in significant effects on syringeability and injectability. Hence, there is a greater emphasis to fully understand the solid properties of the drug being formulated as a suspension as opposed to a solution product.

Lately, there has been a growing interest in formulating poorly soluble drugs as nanoparticulate suspensions (43). For compounds that exhibit poor solubility in aqueous and oily vehicles, nanosuspensions could be a preferred formulation option resulting in improved bioavailability. Nanoparticles also form an interesting platform for attaching targeting moieties. Nanoparticles are produced by "top-down" (media milling) techniques (44) or by "bottoms-up" (controlled crystallization) approaches (45). More recently, there have been reports of generating engineered nanoparticles by printing techniques (46).

Well-formulated nanosuspensions are typically nonsettling and hence circumvent some of the concerns mentioned previously with conventional suspension formulations. In such formulations the natural tendency of these small particles to aggregate is overcome by a careful selection of stabilizers, which could include a mix of surfactants and polymers. Compatibility of the drug with a range of possible surfactants and polymers needs to be assessed in parallel to selecting the best options for stabilization. As in conventional suspensions, Ostwald ripening and crystal growth is a concern, and gaining a good understanding of the solid-state properties of the drug is very relevant. Prototype nanosuspensions can be stressed by temperature cycling and freeze-thaw studies to establish their physical stability. It is also useful to assess the physical and chemical stability of the formulated drug to autoclaving conditions to define the strategy for sterilization.

Emulsions

Injectable emulsions have been most commonly used for long-term parenteral nutrition (Intralipid®, Lipofundin®). However, emulsions can also be good carriers of drug substances with good lipid solubility (high log *P*) and poor aqueous solubility (47). Propofol (Diprivan®) and diazepam (Diazemul®) are examples of drugs formulated as emulsions (33), and there are reports on studies conducted with Taxol emulsions (48). With the increased interest in injectable lipid emulsions, there is also a greater awareness of safety issues surrounding such delivery (49).

Typical emulsion formulations consist of oils (long- and medium-chain triglycerides or high-quality food grade oils), emulsifiers (e.g., lecithins, poloxamers, Tweens, and Spans) and an aqueous phase containing appropriate additives to control pH, tonicity, etc. Antioxidants such as α-tocopherol could be included in the oil phase to prevent oxidation of the oils. The emulsions are typically prepared by dissolving the appropriate ingredients in the oil phase and water phase and then homogenizing (e.g., Microfluidizer®, Silverson® homogenizer) the two to obtain the emulsion.

Some attributes to be studied in the specific context of emulsion formulations include assessment of particle (droplet) size and surface charge. Droplet surface charge is measured in terms of the zeta potential. Essentially, zeta potential is the potential difference between the dispersion medium and the stationary layer of fluid attached to the dispersed particle. The zeta potential is determined using instruments that measure the elecrophoretic mobility of the particles. The surface change on droplets stabilizes emulsions because of electrostatic repulsion, which prevents coalescence of droplets. A zeta potential of ±30 mV or higher can

help stabilize a colloidal system. Measurement of zeta potential is equally useful while formulating suspensions and nanosuspensions.

SPECIFICITIES RELATED TO BIOLOGICS

Biotherapeutic molecules could range from small oligonucleotides or peptides synthesized using techniques such as solid-phase synthesis to proteins (including interferons, soluble receptors, antibodies, etc.) with tertiary and quaternary structures, which are often produced via genetic engineering technologies. Small oligonucleotides and peptides can often be formulated and analyzed by techniques similar to small molecules. More specialized analytical techniques and formulation considerations are needed for larger proteins. From a preformulation perspective, the goals are the same—to characterize the drug compound and understand the solubility and stability of the drug as well as the interactions with potential excipients that would be used to formulate the drug compound. Early results may determine the formulation strategy of either a ready-to-use solution or a lyophilized product for reconstitution. On the basis of this strategy, additional preformulation studies may be needed to support the formulation choice.

Characterization

In addition to the conventional characterization described earlier in the chapter, additional parameters relevant to protein drugs need to be assessed (50). These include determination of molecular weight, amino acid sequence, and disulfide bonds. Because of a large number of charged groups, proteins are generally soluble in water but can be physically unstable at high concentrations because of their complex interaction with surrounding water. Proteins are zwitterionic in nature as a consequence of the amino and carboxylic groups of individual amino acids. At low pH values, proteins would have a net positive charge, and at higher pH values, due to ionization of the carboxyl groups, they carry a net negative charge. The isoelectric point, pI, is the pH of an aqueous solution of a peptide (or protein) at which the molecules on average have no net charge. In other words, the positively charged groups are exactly balanced by the negatively charged groups. This is an important parameter, which is most commonly determined using an electrophoresis technique called isoelectric focusing.

From a solid-state point of view, protein drugs are frequently amorphous and quite hygroscopic. For large proteins made by genetic engineering technologies, it is also quite common not to routinely isolate the protein as a solid but to hold it in a solution or frozen buffered and stabilized solution.

Stability

The pharmacological activity of proteins and peptides is largely dependent on their intact primary, secondary, tertiary, and quaternary structures. Proteins and peptides are quite fragile and can undergo physical and chemical degradation under a variety of conditions.

Chemical Stability

Chemical degradation can be triggered by changes in temperature, pH, oxygen levels, and trace metals and under the influence of light. Methionine, cysteine, tryptophane, and histidine residues can undergo oxidation under the influence of trace metals and light and higher levels of oxygen. Hydrolysis of the side chains of asparagine and glutamine residues can result in deamidation reaction. Hydrolysis of the amide bond in the protein backbone is another degradation route, which is mainly influenced by the solution pH. β-elimination of cysteine, serine, threonine, and lysine residues is also affected by the solution pH, temperature, and ionic composition.

To characterize the degradation pathways, a multitude of analytical techniques are employed. These include different sequencing (*N*-terminal sequencing), spectroscopic (UV spectral analysis), separation (e.g., ion exchange, reverse phase, gel electrophoresis with protein staining, isoelectric focusing) of the intact proteins or enzymatically digested proteins (peptide map), and mass spectroscopic analysis of proteins to define the chemical modifications occurring. Circular dichroism is used to assess secondary and tertiary structures.

Physical Stability

Native protein structures are not very thermodynamically stable. Proteins easily unfold (denaturation) under the influence of increased temperature and concentration, pH change, buffer species, or chemical and physical stress. Completely or partially unfolded proteins can associate to form irreversible aggregates. Aggregation is not necessarily visible to the eye, but with increasing aggregation, aggregate size increases, and eventually, precipitation can occur, which is clearly visible.

Fluorescence measurements, light scattering techniques (sometimes in combination with reverse-phase or size exclusion chromatographic separation) and field flow fractionation can be used to assess aggregation. Conformational changes leading to aggregation can also be measured by DSC.

Protein unfolding, adsorption to surfaces, and aggregation can be modulated by pH, buffer species, choice of preservatives, and use of appropriate surfactants and stabilizers (sugars) in the formulation. The formulation factors have to be tailored to individual proteins through well-executed studies evaluating formulation, processing, and storage conditions. Other chapters in this book cover protein characterization and formulation aspects in detail.

SUMMARY

The aim of preformulation studies is to gain a thorough understanding of the drug molecule, its physical and chemical properties, as well as its interaction with other formulation ingredients and packaging materials to drive a rational formulation design. This chapter has provided an overview of preformulation studies related to development of parenteral medications.

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5 Formulation development of small and large volume injections

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INTRODUCTION

As described in the U.S. Pharmacopeia, USP-32/NF-27 (1), an injection is a preparation intended for parenteral administration and/or for constituting or diluting a parenteral article prior to administration. It is administered through the skin or other external boundary tissue, rather than through the alimentary canal, so that therapeutic substances, using gravity or force, can gain direct entry to a blood vessel, organ, tissue, or lesion. Parenteral products are required to meet pharmacopeial requirements for sterility, pyrogens, particulate matter, and other contaminants, and, where appropriate, contain inhibitors of the growth of microorganisms.

The USP (1) categorizes sterile preparations for parenteral use according to the physical state of the product as follows:

1. Liquid preparations that are drug substances or solutions thereof, for example, [drug] injection.
2. Dry solids that, upon the addition of suitable vehicles, yield solutions conforming in all respects to the requirements for injections, for example, [drug] for injection.
3. Liquid preparations of drug substances dissolved or dispersed in a suitable emulsion medium, for example, [drug] injectable emulsion.
4. Liquid preparations of solids suspended in a suitable liquid medium, for example, [drug] injectable suspension.
5. Dry solids that, upon the addition of suitable vehicles, yield preparations conforming in all respects to the requirements for injectable suspensions, for example, [drug] for injectable suspension.

Depending on the volume of injection in a package, the USP further designates injection, as either (*i*) small-volume injections or (*ii*) large-volume intravenous (IV) solutions. The term small-volume injection applies to an injection that is packaged in containers labeled as containing 100 mL or less. The large-volume IV solution applies to a single-dose injection that is intended for IV use and is packaged in containers labeled as containing more than 100 mL. Although the term sterile pharmaceutical is applicable to all injections (radiopharmaceuticals included), ophthalmic preparations, and irrigating solutions, this chapter emphasizes the formulation of injectable dosage forms.

FORMULATION OF SMALL-VOLUME INJECTIONS

In terms of number, the small-volume injections constitute the vast majority of all the injectable products in the market - small and large-volume injections combined. Whereas, large volume injections are administered exclusively as IV infusion, the small-volume injectables can be given by IV as well as other routes, although dictated by the volume of injection, as described later.

The goal of formulation development is to have a product that addresses all four requisites of an ideal product from a patient point of view: It should be safe, efficacious, stable, and acceptable/tolerable. From the point of marketing and commercial economics, the product should be easy to manufacture, relatively easy to use or present, and should have optimum shelf life at convenient storage conditions, such as room temperature. Although the preferred goal of the formulation scientist is to develop an injectable formulation that is ready to use (such as an aqueous solution), a number of codependent factors must be carefully evaluated in determining the most appropriate type of formulation. These factors are a) Biopharmaceutical considerations, b) Solubility, and c) Stability.

Biopharmaceutical considerations are aimed at achieving the required drug concentration for pharmacological response and include the intended mode of administration, desired onset of action, and the dose required. The formulation - the drug itself and the excipients used - must be compatible with body tissues, particularly taking care of properties such as hemolysis potential, pain on injection, precipitation of the drug upon administration, etc. Sterility, lack of pyrogenicity, and absence of particulate matter are other important considerations from general safety point of view.

Solubility issues become important when the drug does not have sufficient water solubility to achieve the target concentration in the formulation at physiologically acceptable pH range of 3–10. Various solubilization strategies must be employed to increase the solubility to achieve the required deliverable dose in a minimum possible volume. These techniques include use of buffers, salt formation, use of cosolvent, use of surfactants, etc.

Stability considerations are aimed at developing a formulation that provides sufficient shelf life, which is generally considered to be the time for 10% degradation. The product is optimized in such a way that its intrinsic degradation pathways, for example, the commonly encountered hydrolysis or oxidation, are minimized by appropriate modification of formulation composition, many times by using added substances, such as buffers, chelating agents, etc.

The successful formulation of an injectable small-volume preparation requires knowledge and expertise to effect rational decisions regarding the selection of

1. a suitable vehicle (aqueous, nonaqueous, or cosolvent),
2. added substances (buffers, antioxidants, antimicrobial agents, buffers, chelating agents, tonicity contributors, etc.), and
3. the appropriate container and closure components.

During the course of product development, formulation optimization is an iterative process and evolves as the product moves from the discovery to clinical to commercial stages. Inherent in the above decisions is the obligatory concern for product safety, effectiveness, stability, and reliability. As the injection formulation is finalized, a number of additional supportive studies must be undertaken to establish ruggedness of the formulation.

The majority of parenteral products are aqueous solutions, preferred because of their physiological compatibility and versatility with regard to route of administration. Survey of USP (1) shows that out of >300 pharmacopeial injection entries, nearly 70% are aqueous formulations (a similar trend is expected for nonpharmacopeial products as well). However, cosolvents or nonaqueous substances are often required to affect solution and/or stability of many compounds. Furthermore, for some other compounds, the desired properties must be attained through the use of an alternate dosage form such as suspension, emulsion, or even newer approaches such as liposomes and nanosuspensions.

Although each of these dosage forms have distinctive characteristics and formulation requirements, certain physical-chemical principles are common. Those common principles will be discussed in a general manner and the differences distinctive of each system will be emphasized. It is important to recognize that the pharmaceutical products derived from biotechnology are on the increase and the formulation of these products requires some unique skills and novel approaches. Formulation development aspects of these products are described elsewhere (see chap. 9).

Formulation Principles

Influence of the Route of Administration

Since parenteral preparations are introduced directly into the intra- or extracellular fluid compartments, the lymphatic system, or the blood, the nature of the product and the desired pharmacological action are factors determining the particular route of administration to be employed. The desired route of administration, in turn, places certain requirements and limitations on the formulations as well as the devices used for administering the dosage forms. Consequently, a variety of routes of administration (see chap. 2) are used.

One of the most important considerations in formulating a parenteral product is the appropriate volume into which the drug should be incorporated. The IV route is the only route

in which there are no strict limits of the volumes and as much as fifty milliliters can be administered by the IV route, via hypodermic injection, and several liters can be administered over the course of several hours through an IV administration system. Volumes up to 10 mL can be administered intraspinally, while the intramuscular route is normally limited to 3 mL, subcutaneous to 2 mL and intradermal to 0.2 mL.

The choice of the solvent system or vehicle is directly related to the intended route of administration of the product. IV and intraspinal injections are generally restricted to dilute aqueous solutions, whereas oily solutions, cosolvent solutions, suspensions, and emulsions can be injected intramuscularly and/or subcutaneously.

Isotonicity is another factor that must be taken into consideration. Although isotonic solutions are less irritating, cause less toxicity, and eliminate the possibility of hemolysis, it is not essential that all injections be isotonic. In fact, for subcutaneous and intramuscular injections hypertonic solutions are often used to facilitate absorption of drug because of local effusion of tissue fluids. With IV solutions, isotonicity becomes less important as long as administration is slow enough to permit dilution or adjustment in the blood. However, intraspinal injections must be isotonic because of slow circulation of the cerebrospinal fluid in which abrupt changes of osmotic pressure can give rise to severe side effects.

New routes of administration include intraarticular, directly into the synovial fluid for rheumatoidal diseases and even intradigital, between the fingers, in order to better target the lymphatics. The parenteral routes of administration will influence the design of novel dosage forms and drug delivery systems especially as more potent agents from biotechnology are developed.

This chapter focuses on the physicochemical aspects of formulating a stable product in a suitable container recognizing that safety must be established through evaluation of toxicity, tissue tolerance, pyrogenicity, sterility, and tonicity, and efficacy must be demonstrated through controlled clinical investigations.

Selection of Vehicle

Most parenteral products are aqueous solutions. Chemically, the high dielectric constant (DC) of water makes it possible to dissolve ionizable electrolytes and its hydrogen-bonding potential facilitates the solution of alcohols, aldehydes, ketones, and amines. Water for injection (WFI) is the solvent of choice for making parenterals. When it is not possible to use 100% aqueous solution for physical or chemical reasons, other means of solubilization including the addition of solubilizing agents or cosolvents may be necessary. For instance, nonpolar substances (i.e., alkaloidal bases) possess limited solubility in water and it is necessary to add a cosolvent such as glycerin, ethanol, propylene glycol, or polyethylene glycol. In other cases, to prevent chemical degradation (i.e., hydrolysis, oxidation, decarboxylation, or racemization) water may have to be eliminated partially or totally. Most proteins and peptides require an aqueous environment, and the addition of salt, buffer, or other additives for solubility purposes often leads to conformational changes. Consequently, parenteral product formulators should be aware of not only the nature of the solvent and solute in parenterals but also the solvent-solute interactions and the route of administration. Typically, aqueous solution formulations are prepared by simple solution of the drug and the excipients, by in situ salt formation of the drug in the solution (titrating against an acid or base), or by complexation of the drug with a complexing agent.

Solubility and solubilization. The solubility of a substance at a given temperature is defined quantitatively as the concentration of the dissolved solute in a saturated solution (i.e., the dissolved solute phase). Generally, drugs are present in solution at unsaturated or subsaturated concentrations; otherwise, crystallization of the drug may occur as a result of changes in pH, temperature, by seeding from other ingredients, or particulates in the solution.

The solubilization techniques for injectable formulations include pH adjustment, mixed aqueous/organic cosolvents, oily vehicles, surface-active agents, complexation, as well as formulating the drug in emulsion, suspension, liposomes, nanosuspensions, and combinations of techniques. An excellent review of the solubilizing excipients that could be used in the injectable formulations has been provided by Strickly (2).

Table 1 Expressions for Approximate Solubility

Term	Relative amount of solvent to dissolve
Very soluble	<1
Freely soluble	1–10
Soluble	10–30
Sparingly soluble	30–100
Slightly soluble	100–1,000
Very slightly soluble	1,000–10,000
Practically insoluble or insoluble	>10,000

Table 2 Typical Examples of Drugs Representing the Solubility Terms

Term	Drug	Solubility of drug
Very soluble	Chloral hydrate	>8 g/mL
Freely soluble	Isoniazid	0.330 g/mL
Soluble	Guaifensin	0.050 g/mL
Sparingly soluble	Pyrazinamide	0.015 g/mL
Slightly soluble	Salicylic acid	0.002 g/mL
Very slightly soluble	Griseofulvin	0.000,02 g/mL
Practically insoluble or insoluble	Diclofenec	0.000,002 g/mL

Source: Adapted from Ref. 3.

Solubility expressions. Solubility of a substance can be expressed in a number of ways. Generally, the concentration is expressed as percent (w/v), that is, grams per 100 mL of solution, but molarity and molality have been used. Molarity is defined as the number of moles per 1000 mL of solution. Molality is the number of moles of solute per 1000 g of solvent and, therefore, being a weight relationship, is not influenced by temperature. The USP lists solubility in terms of the number of milliliters of solvent required to dissolve 1 g of substance. If exact solubilities are not known, the USP provides general terms to describe a given range. These descriptive terms are listed in Table 1. Typical examples of drugs representing the solubility terms are listed in Table 2 (3).

Bonding forces. For a substance to dissolve, the forces of attraction that hold the molecules together must be overcome by the solvent. The solubility will be determined by the relative binding forces within the substance (solute-solute interactions) and between the substance and the vehicle (solute-solvent interactions). If an environment similar to that of the crystal structure can be provided by the solvent, then the greater the solubility (i.e., "like dissolves like"). Ionic compounds dissolve more readily in water by virtue of ion-dipole interactions, whereas hydrophobic substances dissolve more easily in organic solvents as a result of dipole or induced dipole interactions.

Often, the solubility of the drug substance is due in large part to the polarity of the solvent, generally expressed in terms of dipole moment, which is related to the DC. Solvents with high DCs dissolve ionic compounds and are water soluble, whereas solvents with low DCs are not water soluble and do not dissolve ionic compounds. The former are classified as polar solvents (e.g., water, glycerin, and ethanol), while the latter are nonpolar (e.g., chloroform, benzene, and the oils). Solvents with intermediate DCs (e.g., acetone and butanol) are classified as semipolar. The DCs of most pharmaceutical solvents are known (4) and values for a number of binary and tertiary blends have been reported (5) and, if not reported, can be readily estimated (6,7). Table 3 is a listing of the DCs of some solvents at 25°C.

The solubility profiles of a number of pharmaceuticals as a function of DC have been reported by Paruta and coworkers (8–10). By determining the solubility of a substance in a system at various DCs, a graph such as that shown in Figure 1 can be constructed to determine the DC that will provide the required solubility for a particular drug substance. As can be seen

Table 3 DCs of Some Solvents at 25°C

Solvent	DC
Water[a]	78.5
Glycerin[a]	40.1
N,*N*-dimethyl acetamide[a]	37.8
Propylene glycol[a]	32.0 (30°C)
Methanol	31.5
Cremophor EL (R) (polyoxyl castor oil 35)[a]	27.0
Ethanol[a]	24.3
N-propanol	20.1
Acetone	19.1
Benzyl alcohol[a]	13.1
Polyethylene glycol 400[a]	12.5
Cottonseed oil[a]	3.0
Benzene	2.3
Dioxane	2.2

[a]Solvents used in parenterals
Abbreviation: DC, dielectric constant.

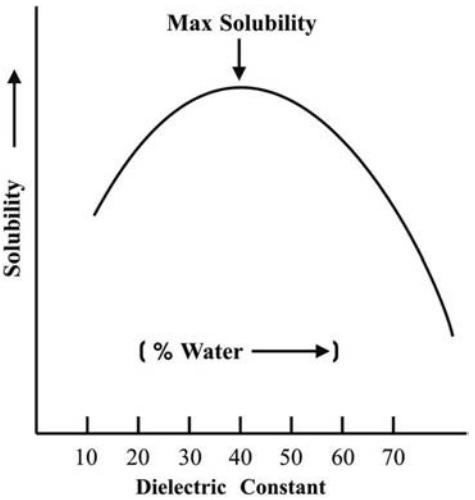


Figure 1 Hypothetical plot of solubility of a substance versus dielectric constant in various mixtures of dioxane and water.

from the plot, to obtain the maximum concentration, a DC of around 40 is required. Not all mixtures will show a maximum, but such a plot illustrates the required DC to obtain the desired concentration. For example, if a DC (DC) of 60 was selected, a mixture of water (DC = 78.5), polyethylene glycol (PEG) 400 (DC = 12.5) and ethanol (DC = 24.3) could be used. Selecting an amount of ethanol necessary to dissolve the drug (e.g., 10%), the percentages of PEG 400 and water can be calculated as follows:

$$(10)(24.3) + (X)(78.5) + (90 - X)(12.5) = (100)(60)$$

where X is the percentage of water required and is calculated to be 73.5%.

Therefore, the vehicle to provide a DC of 60 will have the following composition: Ethanol 10%, PEG 400 16.5%, and Water 73.5%

Since DC is a measure of the polarizability and dipole moment of a compound, several researchers have explored other parameters and polarity indices (11) which include molecular volume, solvent and solute interactions and specific interactions such as hydrogen bonding. In 1952, Hildebrand and Scott (12) introduced solubility parameters to predict solubility of regular solutions. Since pharmaceutical systems deviate from regular or ideal solutions, Martin and coworkers (13) modified the Hildebrand approach to include hydrogen-bonding and

dipolar interactions. The molecular surface area of the solute and interfacial tension between solute and solvent were further used by Amidon (14) and Yalkowsky (15) to predict solubility. Among the many theoretical models available to predict solubility in water, recent reports review the available models and discuss the potential and limitations of these computational approaches (16,17).

Hydrogen bonding, a type of dipole-dipole interaction, is an important determinant of solubility. Because of its small size, the hydrogen atom (proton donor) with its positive center, can approach the negative center (electron donor) of a neighboring dipole more closely than any other atom. As a result of this spatial maneuverability, both intramolecular bonding (i.e., between groups within a single molecule) and the intermolecular type (i.e., among molecules) can occur. The latter is responsible for association in most solvents and dissolution of most drugs. Alcohols dissolve in water by hydrogen bonding, up to an alkyl chain length of five carbon atoms. Phenols dissolve in water and alcohol and, as the number of hydroxyl groups increase, the water solubility is enhanced because of the increased opportunity for hydrogen bonding. Most aromatic carboxylic acids, steroids, and cardiac glycosides are not water soluble but dissolve in alcohol, glycerin, or glycols by hydrogen bonding.

Dipole-ion interaction is another important molecular property that is responsible for the dissolution of ionic crystalline substances in polar solvents (i.e., water or alcohol). Ions in aqueous solution are generally hydrated (surrounded by water molecules) by as many water molecules as can spatially fit around the ion. The attributes of a good solvent for electrolytes include: (*i*) a high-dipole moment; (*ii*) a small molecular size; and (*iii*) a high DC to reduce the force of attraction between the oppositely charged ions in the crystal. Water possesses all of these characteristics and is, therefore, a good solvent for electrolytes. The cation of the electrolyte is attracted to the negative oxygen atom, while the anion attracts the hydrogen atoms of the dipolar water molecules.

Symmetrical molecules, such as benzene and carbon tetrachloride, possess zero dipole moment and are nonpolar. Solubility of such molecules or their existence in a liquid state is due to van der Waals forces. Other intermolecular interactions, such as London forces or Debye interactions are also responsible for solubility of such nonpolar substances.

Effect of temperature. Substances generally dissolve faster if heat is applied to the system and the solubility of most solids is increased by an increase in temperature. This is true if the substance absorbs heat during the course of dissolution. The degree to which temperature can influence solubility is determined by the heat of solution, more specifically the differential heat of solution, ΔH, which represents the rate of change of the heat of solution per mole of solute in a solution of specified concentration. The higher the heat of solution, the greater is the influence of temperature on solubility.

The following equation shows the influence of temperature on solubility:

$$\frac{d\,Ln\,S}{dT} = \frac{\Delta H}{RT^2} \tag{1}$$

where S is the solubility or concentration of a saturated solution, often expressed in terms of molality, molarity, or mole fraction; R is the gas constant; and T is the absolute temperature. Equation (1) can be written as

$$\log S = \frac{\Delta H}{2.303R} \times \frac{1}{T} + \text{constant} \tag{2}$$

By plotting the logarithm of the solubility in moles per liter versus the reciprocal of the absolute temperature as shown in Figure 2, the differential heat of solution can be calculated from the slope of the line, which is equal to

$$-\frac{\Delta H}{(2.303)(1.987)}$$

A positive heat of solution indicates that the process is endothermic (i.e., the solute absorbs heat when dissolving). Therefore, an increase in temperature will increase solubility. A

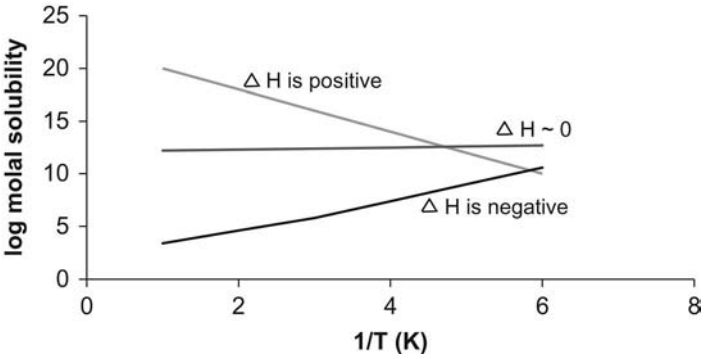


Figure 2 Effect of temperature on solubility of a substance. ΔH represents the differential heat of solution and is calculated from the slope of the line, $-\frac{\Delta H}{(2.303)(1.987)}$.

negative value indicates that the process is exothermic (i.e., the solute evolves heat when dissolving). In this case, increase in temperature results in a decrease in solubility. A differential heat of solution around zero indicates that the solubility is not significantly influenced by temperature.

Measuring solubility. Methods for determining the solubility of drug substances in aqueous solvents have been described (18,19). The standard way to determine the solubility of a compound is to use the "shake-flask" solubility method. This method is inherently low-throughput, labor intensive, and necessitates the addition of drug in a powder form. It involves adding an excess quantity of solid material to a volume of buffer at a fixed pH and the saturated solution is agitated (shake-flask) until equilibrium is reached, generally 12 hours to seven days. Following separation by filtration or centrifugation, the compound in solution is analyzed and quantified by a suitable analytical technique such as UV/Vis spectroscopy or high-performance liquid chromatography (HPLC). The other classical experimental methods used to determine solubility are turbidimetric ranking assays, HPLC-based assays, and potentiometric methods. The newer high-throughput methods which determine both kinetic and thermodynamic (equilibrium) solubilities are based on screening multiple solutes and solvents, in array of compositions, using 96-well format that allows for solubility analysis in a single plate with very low drug amount (19,20).

Solubilization techniques. A variety of approaches to increase the aqueous solubility of an otherwise less soluble or insoluble drug substance to a desired level for optimum injectable product have been reported and reviewed (2,21,22). These include: 1) pH adjustment, 2) salt formation, 3) use of cosolvents, 4) surfactants as solubilizers, 5) use of complexing agents, and others. Metabolizable oils as vehicles have has also been used for certain class of compounds. Beyond these solubilization approaches, it may become necessary in some cases to change the formulation from solution to dispersed system such as emulsion, suspension, and more recently liposomes and nanosuspensions.

pH adjustment Most organic drug substances are weak electrolytes and, therefore, exist in solution in dissociated and undissociated forms. The ratio of these forms is determined by the pH of the solution as per the Henderson-Hasselbach relationship. As a result, properties such as solubility, partition coefficient, and chemical stability, which are markedly different for the undissociated and dissociated forms are influenced by pH.

Many of the organic electrolytes used in parenteral systems contain a basic nitrogen atom in the molecules. These include antihistamines, alkaloids, local anesthetics, and so on, which are practically insoluble in water but dissolve readily in dilute solutions of acids because of salt formation. The addition of alkali to these solutions increases the pH and causes free base to precipitate. Examples are atropine sulfate, ephedrine sulfate, lidocaine hydrochloride, and pyribenzamine hydrochloride.

In compounds that contain an electron withdrawing group, such as oxygen, a positive center is created, which in turn attracts electrons from adjacent nitrogen, and if a hydrogen

atom is attached, the N-H bond is weakened. As a result, in alkaline solution a more soluble anion is formed. The examples are phenobarbital and sulfanilamide.

The addition of acid to the solutions of these compounds will cause the free acid form to precipitate. Even the addition of a salt of a strong acid such as morphine sulfate will result in precipitation.

Most marketed injection products are in the pH range of 4 to 8 for biocompatibility reason, however, some are outside of this range. The pH solubility and pH stability-rate profiles of a drug usually determines the pH at which a product is formulated (23). Additional formulation variables to be considered are the necessity of a buffer, buffer capacity, and drug concentration. These variables are described in details in a further section (see "Added Substances").

Salt formation Salts of acidic and basic drugs usually exhibit higher solubility than their corresponding acid or base forms. Therefore, salt formation is the most preferred and effective method of increasing solubility and dissolution rates of acidic and basic drugs (24,25).

Solubility-pH profiles of weakly acidic or basic organic drugs may be visualized on the basis of classical Henderson-Hasselbach relationship. In the case of monoprotic acid, a saturated solution can be defined by the following equations and corresponding constants (26).

$$\mathrm{HA} \Leftrightarrow \mathrm{H^+} + \mathrm{A^-} \mathrm{K_a} = \frac{[\mathrm{H^+}][\mathrm{A^-}]}{[\mathrm{HA}]} \tag{3}$$

$$\mathrm{HA(Solid)} \Leftrightarrow \mathrm{HA(Solution)} S_0 = [\mathrm{HA}] \tag{4}$$

where [HA] is the concentration of undissociated acid form, $[\mathrm{A^-}]$ is the concentration of corresponding salt form, $[\mathrm{H^+}]$ is the concentration of proton or dissociated hydrogen, and S_0 is the intrinsic solubility of the monoprotic acid. Solubility, S, at a particular pH is defined then as mass balance sum of the concentrations of all of the species dissolved in the aqueous phase.

$$S = [\mathrm{A}]^- + [\mathrm{HA}] \tag{5}$$

Rearranging equations (3), (4), and (5),

$$\begin{aligned} S &= K_a[\mathrm{HA}]/[\mathrm{H^+}] + [\mathrm{HA}] \\ &= S_0(K_a/[\mathrm{H^+}] + 1) \\ &= S_0\left(10^{-\mathrm{p}K_a+\mathrm{pH}} + 1\right), \text{ or} \\ \log S &= \log S_0 + \log\left(10^{-\mathrm{p}K_a+\mathrm{pH}} + 1\right) \end{aligned} \tag{6}$$

For a weakly acidic drug, depending on the pH of the solution, the term, $\log\left(10^{-\mathrm{p}K_a+\mathrm{pH}} + 1\right)$, changes solubility function according to the conditions below.

1. $\mathrm{pH} >> \mathrm{p}K_a$

   The exponent $(-\mathrm{p}K_a + \mathrm{pH})$ remains positive and very large number compared with 1, and hence, 1 is ignored, and

$$\begin{aligned} &\log\left(10^{-\mathrm{p}K_a+\mathrm{pH}} + 1\right) \text{ becomes } \log\left(10^{-\mathrm{p}K_a+\mathrm{pH}}\right) \text{ or} \\ &\left(-\mathrm{p}K_a + \mathrm{pH}\right)\log(10) \text{ or } \left(-\mathrm{p}K_a + \mathrm{pH}\right) \\ &\text{Therefore,} \\ &\log S = \log S_0 - \mathrm{p}K_a + \mathrm{pH} \\ &\text{Since } \mathrm{p}K_a \text{ is a constant.} \\ &\log S = (\log S_0 - \mathrm{p}K_a) + \mathrm{pH} \end{aligned} \tag{7}$$

   Equation (7) is of the form, $Y = c + mX$ or an equation of a straight line with associated intercept and slope, c and m, respectively.

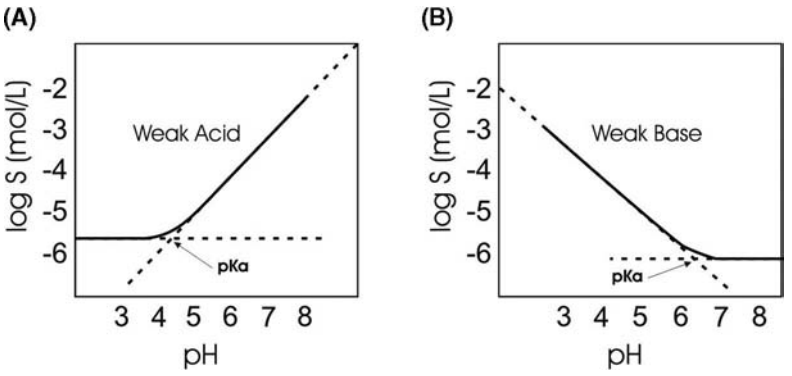


Figure 3 Solubility/pH profile for weak acid ($pK_a = 4.4$) and weak base ($pK_a = 6.1$). *Source*: Adapted from Ref. 26.

Therefore, a plot of log$S$ versus pH, will yield a straight line the slope of which will be equal to +1 and the intercept will be ($\log S_0 - pK_a$). A similar relationship can be made for a weakly basic drug, in which case, the slope will be equal to −1.

Figure 3 (26) shows the solubility-pH profile for a (*i*) weak acid (pK_a 4.4, logS_0 −5.6) and (*ii*) weak base (pK_a 6.1, log S_0 −5.9).

2. $pH = pK_a$, or at the inflection point in the curve.

   The exponent ($-pK_a + pH$) becomes zero and the term

   $$\log\left(10^{-pK_a+pH} + 1\right) \text{ becomes } \log\left(10^0 + 1\right) \text{ or } \log(1+1).$$

   Therefore,

   $$\log S = \log S_0 + 0.3$$

3. $pH << pK_a$, or at the flat line of the curve.

   The exponent ($-pK_a + pH$) remains negative.

   $$\log\left(10^{-pK_a+pH} + 1\right) \text{ becomes } \log\left(0.000\ldots + 1\right) \text{ or close to } 0.$$

   Therefore,

   $$\log S \cong \log S_0$$

Whether certain acidic or basic drugs would form salts and, if salts are formed, dissociation back to the free acid or base forms would depend on several factors, such as pH, pK_a, S_0 (intrinsic solubility), k_{sp} (solubility product) and pH_{max} (pH of maximum solubility). The aqueous solubility of an acidic or basic drug as a function of pH determines if the compound will form suitable salts within the physiologically acceptable pH range. Moreover, the common-ion effect of the salt-forming agents is also important in determining the final solubility. It has been reported that dissolution rates of a hydrochloride salt decrease as the pH of an aqueous medium is lowered when HCl is added or if NaCl is added to the medium. Similarly, the dissolution rate of a sodium salt decreases in the presence of added NaCl in the medium. There are numerous reports in the literature indicating such common-ion effects on salts having relatively low aqueous solubilities (27).

A review by Serajuddin about the principles of salt formation and its utility in formulation has recently been published (28). It surveyed about 120 salts approved by the FDA during the 12-year period from 1995 to 2006 and showed that the hydrochloride salt was the predominant salt form among the basic drugs and the sodium salt was the predominant form for acidic drugs. About 77% of the salts of basic drugs were prepared with relatively stronger counterions (hydrochloride, hydrobromide/bromide, sulfate/bisulfate and nitrate). Similarly, 14 out of 19 salts of acidic drugs were prepared with strong alkalies such as NaOH and KOH.

Use of cosolvents If the pH adjustment or salt formation approach still results in aqueous solubility of a drug well below its therapeutic dose, a mixture of solvents may be used to achieve sufficiently high solubility. A cosolvent is a water-miscible organic solvent that is used to increase the solubility of a poorly water-soluble compound. The addition of cosolvent results in reduction of polarity of water which in effect reduces the surface tension, DC, and solubility parameter of water. The increase in solubility by cosolvents is much more dramatic for nonpolar solutes (can be several orders of magnitude), than for solutes of intermediate polarity. Another advantage of using cosolvents is that a change in solvent property may help considerably in stability for drugs which may exhibit hydrolytic degradation by reducing the concentration of water in the formulation. Cosolvent may also enhance the stability of a drug by providing a less suitable environment for the transition state of the reactants, provided the transition state is more polar than the reactants. It is reported that cosolvents are employed in approximately 10% of the FDA approved injectable products (22).

Cosolvents and solubility J. H. Hildebrand, in a series of papers published begining in 1916, deescribed the basic principles of solutions and solubility and introduced the cosolvency approach (29) and experimental tests of a general equation for solubility (30). Since then, numerous theoretical cosolvency models have been proposed that correlate and/or predict the solubility of drugs in water cosolvent mixtures (31–34) and have been reviewed extensively by Jouban (35). The simplest experimental cosolvency model, that is, the log-linear model of Yalkowsky (36–38), provides an estimate of drug solubility in water-cosolvent mixtures using aqueous solubility of the drug. It is expressed as:

$$\log S_m = f \log S_c + (1 - f) \log S_w \tag{8}$$

Where S_m is the solute's solubility in water-cosolvent mixture, f is the volume fraction of cosolvent, S_c is the solubility of drug in pure cosolvent, and S_w is the solubility of drug in water. S_x values can be expressed in g/L, mole fraction, etc. Equation (8) can be further simplified as

$$\log S_m = \log S_w + f\sigma \tag{9}$$

where

$$\sigma = \log ac_w - \log ac_c \tag{10}$$

And ac_w and ac_c are the activity coefficients for the drug in water and cosolvent, respectively. In a given cosolvent system, σ will be constant. Therefore, if one plots log S_m versus f, the slope will be σ. Comparing slopes of different cosolvent-water systems can easily be done by using σ as a measure of the solubilization potential of the cosolvent. In practice, experimental methods of characterizing the solubility of cosolvent systems can be utilized with the aid of statistical experimental design. Advantage of the experimental approach is that one can use additional excipients, for example, surfactants, buffers, etc., in screening experimental designs.

Cosolvents and stability Cosolvents cannot only increase the solubility of drugs but may also increase the stability of some drugs (31). The addition of cosolvent reduces the collision probability between a water molecule and a drug molecule which is necessary for hydrolysis. As mentioned earlier, the degradation rate of a drug may change with the DC of the medium. Decreasing the polarity of the reaction medium by the addition of cosolvent unfavors the formation of the charged species. It stabilizes a solute against any reaction that produces charged products or proceeds through a charged transition state (39,40). As a general rule, for reactions leading to products that are less polar than the starting material, a less polar medium may accelerate the reaction. On the other hand, reactions leading to products that are more polar than the starting material may proceed rapidly in polar media.

Improvement of stability of a drug in the presence of cosolvent was reported by Ni, et al (41). The authors studied the stability of an anticancer compound, SarCNU (a nitrosourea derivative), in several pharmaceutically acceptable solvents such as water, EtOH, propylene

glycol (PG), propylene glycol monoester of medium chain fatty acids (Capmul PG), dimethylsulfoxide (DMSO), and in different combinations of these cosolvents at four different temperatures. The degradation of the drug was monitored by HPLC and was found to be catalyzed not only by general but also by specific acid and base and followed first-order kinetics. The t_{90} (time for 90% of the drug remaining intact) in pure cosolvent was 25–50 times higher than that in water or semi-aqueous vehicles. Figure 4 shows an Arrhenius plot of the observed rate constants of SarCNU in the solvent mixtures. There was no significant difference in the slopes for the different solvents, suggesting similar degradation mechanism of SarCNU in all solvent mixtures. Furthermore, the order of stabilization by these solvents was Capmul PG> /EtOH> /PE> /PG> /WPE> /water, which was in agreement with decreasing the polarities of the vehicles. The greatest SarCNU stability, as measured by the degradation rate constant derived $t_{90,}$ was observed with Capmul PG as shown in Table 4. Another example where the degradation was significantly reduced in the nonaqueous solvents is described for Eptifibatide, a peptide compound used as an inhibitor of platelet receptor glycoprotein (42). The use of cosolvent to help in solubilization may not, however, lead to favorable stability outcome at all the times. Trivedi, et al, (43) showed that as the fraction of organic solvents was increased, the degradation of zileuton also increased because of the solvolysis of the drug by the cosolvents used.

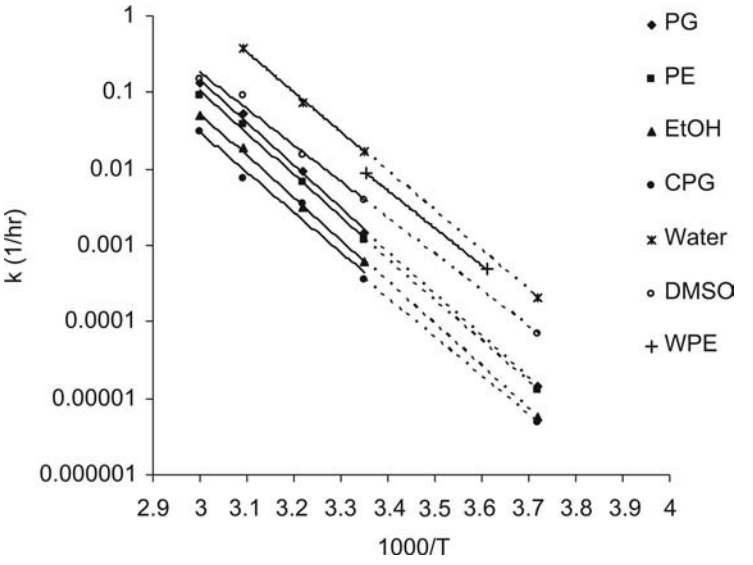


Figure 4 Stability behavior of SarCNU in the presence of various cosolvent.

Table 4 Degradation of SarCNU in the Presence of Various Cosolvent Mixtures

	t_{90} (days)	
Solvent	Room temperature (25°C)	Refrigeration (4°C)
Water	0.25	5.90
Water + propylene glycol + EtOH	0.50	8.96
DMSO	1.14	19.03
Propylene glycol	2.92	77.78
Propylene glycol + EtOH	3.64	89.50
EtOH	7.29	199.52
Capmul PG	12.50	242.57

Source: From Ref. 41.

Table 5 Examples of Marketed Injectable Products Containing Cosolvent Mixtures

Generic name	Trade name	Predominant cosolvent(s) in marketed vehicle
Carmustine	BiCNU	100% ethanol
Diazepam	Valium	Propylene glycol 40% Ethyl alcohol 10%
Digoxin	Lanoxin	Propylene glycol 40% Ethyl alcohol 10%
Melphalan	Alkeran	Propylene glycol 60% Ethyl alcohol 5%
Methocabamol	Robaxin	Polyethylene glycol 50%
Oxytetracycline	Terramycin	Propylene glycol 67–75%
Paricalcitol	Zemplar	Propylene glycol 30% Ethyl alcohol 20%
Phenobarbital Na	Nembutal	Propylene glycol 40% Ethyl alcohol 10%
Phenytoin Na	Dilantin	Propylene glycol 40% Ethyl alcohol 10%
Teniposide	Vumon	*N,N*-dimethylacetamide 6% Cremophor 50% Ethyl alcohol 40%
Docetaxel	Taxotere	Polysorbate 80 100%

Source: From Refs. 44 and 45.

Examples of drugs marketed in water-miscible systems include digoxin, phenytoin, diazepam and others as shown in Table 5 (44,45). These injections are formulated in a water-miscible system containing glycols and alcohol and adjusted to a suitable pH. Other cosolvents used in the past included glycerin in deslanoside, dimethylacetamide in reserpine and dimethylsulfoxide in chemotherapeutic agents undergoing clinical testing. Propylene glycol is used most frequently as a cosolvent, generally in concentrations of 40%. Although such systems are stable in individual vials, care must be exercised on administration. For example, phenytoin is dissolved as the sodium salt in a vehicle containing 40% propylene glycol and 10% ethanol and adjusted to a pH of 12 with sodium hydroxide. However, if this solution is added to a large-volume IV solution and the pH is lowered to a value close to the pK_a of the drug ($pK_a = 8.3$), precipitation of the drug can occur. This is due to the fact that in aqueous systems at pH below 11, the amount of undissociated phenytoin exceeds its solubility.

To be used as solubility/stability enhancer in injectable products, the cosolvent must have certain attributes such as it should be nontoxic, compatibile with blood, nonsensitizing, nonirritating and above all physically and chemically stable and inert. Many cosolvent formulations contain high concentrations of organic solvent and most are diluted prior to injection, however, some may be injected directly and in that case, care must be taken that the rate of injection remains slow.

Surfactants as solubilizers The ability of surfactants to enhance the solubility of otherwise poorly water-soluble compounds in aqueous solution is widely known and used in many injectable formulations. Surfactants are effective solubilizing agents because of their wetting properties and association tendencies as they are able to disperse water-insoluble substances. Surfactants are also used very widely in the biotechnology area for otherwise water-soluble monoclonal antibodies and other proteins and polypeptides, but the primary goal of using surfactant in these products is to minimize hydrophobic interaction related aggregation and not necessarily for the enhancement of solubility. This aspect will be discussed in detail in other chapters.

Surfactants can be either nonionic or ionic (i.e., the ability to lower surface tension rests with the anion or cation in the molecule). In nonionic surfactants, the head groups contain no charged moieties and their hydrophilic properties are due to the presence of hydroxyl groups. Nonionic surfactants are most frequently used in pharmaceutical systems because of their compatibility with other surfactants, stability, and relatively low toxicity. Some examples of

water-soluble nonionic surfactants include long-chain fatty acid analogs such as fatty alcohols, glyceryl esters, and fatty acid esters. Among the most widely used water-soluble nonionic surfactants in injectable products are polyethylene oxide (PEO) sorbitan fatty acid esters, or Polysorbates.

In anionic surfactants, the head groups are negatively charged. The most widely used anionic surfactants are those containing carboxylate groups, such as soaps, sulfonates, and sulfate ions. In cationic surfactants, the head groups are positively charged. Some examples include amine and quaternary ammonium salts. Cationic surfactants are not used in pharmaceutical systems because of their toxicity since they adsorb readily to cell membrane structures in a nonspecific manner, leading to cell lysis (46).

As shown in Figure 5, surfactants typically orient themselves at polar/nonpolar interfaces because of the presence of discrete hydrophobic and hydrophilic regions. As the bulk concentration of surfactant in solution is increased, the surfactant molecules begin to associate into small aggregates called micelles, whereby their hydrophobic regions are shielded from aqueous contact by their hydrophilic regions. All surfactant molecules in excess of that concentration associate into micelles, while the concentration of nonassociated surfactant molecules remains nearly constant. The concentration at which such association occurs is called critical micelle concentration (CMC). Using soap as a micelle forming substance, Lawrence proposed in 1937 that poorly soluble hydrophobic molecules locate in the hydrocarbon core of the micelle, while polar molecules would associate with the polar end (47). Molecules that contain polar and nonpolar groups align themselves between the chains of the micelle with the nonpolar part directed into the central region and the polar end extending out into the hydrophilic chains (Fig. 6).

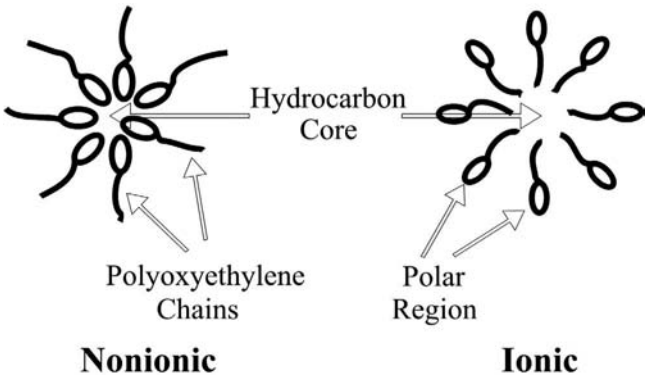


Figure 5 Illustration of spherical orientations of nonionic and ionic micelles.

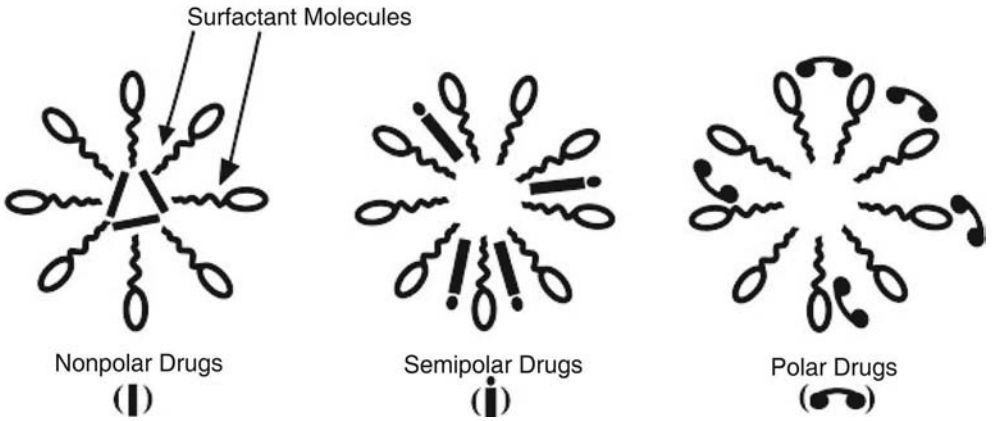


Figure 6 Schematic representation of mechanisms of miceller solubilization.

Table 6 Effect of Surfactants on the Solubility of Furosemide

	Distilled water		0.1 N hydrochloric acid	
Surfactant % (w/v)	Total solubility (μg/mL)	Miceller solubility (μg/mL)	Total solubility (μg/mL)	Miceller solubility (μg/mL)
0	41.2	–	15.0	–
Polysorbate 20 (C12)				
0.005	31.2	–	40.0	–
0.05	45.0	3.7	41.1	26.1
0.5	57.0	15.7	50.0	35.0
1.0	167.0	125.7	145.0	130.0
5.0	705.0	663.7	670.0	655.0
Polysorbate 40 (C16)				
0.005	32.5	–	25.0	–
0.05	45.0	3.7	22.5	7.5
0.5	112.5	71.2	72.5	57.5
1.0	143.7	102.4	137.5	122.5
5.0	792.5	751.2	887.0	872.0
Polysorbate 80 (C18)				
0.005	43.7	2.4	15.9	0.9
0.05	43.7	2.4	18.7	3.7
0.5	141.2	100.0	74.0	59.0
1.0	205.0	163.7	160.0	145.0
5.0	980.0	938.7	808.0	793.0

Source: From Ref. 49.

Generally, the solubilization capacity of a same amount of surfactant is high for those with lower CMC value. The solubilizing ability of nonionic surfactant toward water-insoluble drugs has been extensively studied (48). Akbuga and Gursoy (49) showed how the solubility of furosemide, a very insoluble compound commonly used as diuretic, was dramatically affected by the surfactant concentration and alkyl chain length (Table 6).

The CMC can be measured by a variety of techniques, for example, surface tension, light scattering, osmometry, all of which show a characteristic break point in the plot of the operative property as a function of concentration. Figure 7, a plot of surface tension against concentration of surfactant shows a break in the linearity of the curve, indicating the CMC (50). Many factors such as temperature, pH of the solution, electrolytes, and other ingredients affect micellization and hence solubilization (51,52). For nonionic surfactants, the CMC value decreases with increasing temperature whereas for ionic surfactants, it increases as the temperature increases (53). Since the pH can affect the equilibrium between ionized and nonionized solute species, it can have an effect on the capacity of micellar solubility as shown by Castro et al, for atenolol, nadolol, midazolam and nitrazepam (54). For ionic surfactant micelles, electrolyte addition causes a decrease in the CMC resulting in an increase in the micellar solubilization capacity (55), whereas in the case of nonionic surfactant, polysorbate 80, the solubility of furosemide increases in the presence of sodium chloride because of increased micellar packing and micelle volume (56). Other ingredients present in the formulation can also have a profound effect on the solubilizing capacity of surfactants. Surfactants may precipitate in the presence of some organic additives or micellization may be abolished if high enough concentrations of, for example, alcohols are present. Excipients such as phospholipids also affect the CMC. Many water-soluble drugs themselves are remarkably surface active: they lower the surface and interfacial tension of water, promote foaming, and associate into micelles, such as antibacterial (hydrochlorides of acridines, benzalkonium chloride, cetylpyridinium chloride) tranquilizers (hydrochlorides of reserpine and phenothiazine derivatives), local anesthetics (hydrochlorides of procaine, tetracaine, dibucaine, and lidocaine), nonnarcotic analgesic (propoxyphene hydrochloride) and narcotic analgesic (morphine sulfate and meperidine hydrochloride), antimuscarinic drugs (propantheline bromide, methantheline bromide, methixene hydrochloride), cholinergic agents (pilocarpine hydrochloride, and other

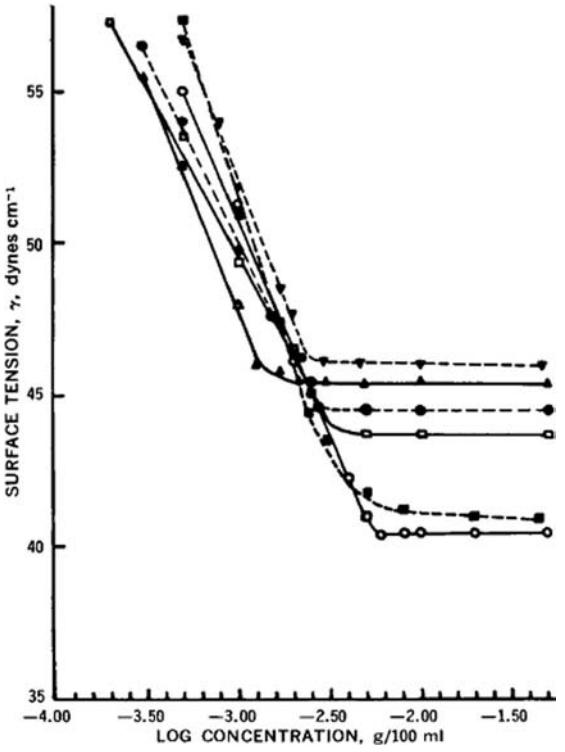


Figure 7 Surface tension versus concentration of surfactant. Break in the curve denotes CMC. *Source*: From Ref. 50.

alkaloidal salts), antihistamines (pyrilamine maleate, tripelennamine hydrochloride, chlorcyclizine hydrochloride, diphenhydramine hydrochloride), anthelmintics (lucanthone hydrochloride), and antibiotics (sodium fusidate, some penicillins, and cephalosporins) (46).

Selection of surfactant in the injectable products should be based on its safety and toxicology profile (LD50, tissue tolerance, hemolysis, etc.), solubility of the drug in the in surfactant, and drug-surfactant compatibility. Since surfactants act as nonspecific solubilizers, stabilizers, emulsifiers and wetting agents, they can also cause toxicity and disrupt normal membrane structure. As mentioned earlier, only nonionic surfactants are generally used in parenterals because of their relative less destruction to biological membranes. Table 7 lists some commonly used surfactants, their properties, and examples of marketed injection products that contain surfactants for the purpose of solubility enhancement. Polysorbate 80 is

Table 7 List of Some Surfactants in Injectable Products and Their Properties

Surfactant	Chemical name	HLB[a] value	CMC (% w/w)	Injection product (chemical/ brand/% surfactant)
Cremophor	Polyoxyethylated castor oil	12–14	0.02	Paclitaxel/taxol/52.7 Tenoposide/vumon/55 Cyclosporine/sandimmune/65
Solutol HS	Polyethylene glycol 660 hydroxystearate	14–16	0.03	Vitamin K /Aqua-mephyton/25
Pluronic-F68	Polaxomer	>24	0.1	Recombinant Growth hormone/accretropin/0.2
Polysorbates	Tween-80	15	0.0014	Amiodorone/cordarone/10 docetaxel/taxotere/100 Vitamin A palmitate/aquasol-A/12
–	Sodium desoxycholate	16	0.08	Amphotericin/fungizone/0.4
–	Sodium dodecyl sulfate	40	0.03	Aldesleukin/proleukin/0.018

[a]Hydrophilic Lipophilic Balance

the most commonly used surfactant and is used in the range from fraction of percent in many products to 100% in the case of taxotere injection.

Cyclodextrins as solubilizers Cyclodextrins are oligomers of glucose produced by enzymatic degradation of starch. The number of α-1,4-linked glucose units determine the classification into α, β, or γ cyclodextrins having six, seven, or eight glucose units, respectively (57–59). The cyclodextrins exert their solubilizing effect by forming soluble inclusion complexes in aqueous solutions. The cyclodextrins are amphipathic (i.e., the exterior is hydrophilic due to the hydroxy groups oriented on the exterior while the interior is hydrophobic) and can form soluble, reversible inclusion complexes with water-insoluble compounds. The unsubstituted cyclodextrins are too toxic for parental use but the chemically modified cyclodextrins appear to be well tolerated when administered parenterally and have been shown to effectively enhance the solubility of several drugs including steroids and proteins (60,61). The solubility of alfaxalone, an insoluble anesthetic, was increased by 5000 times to 19 mg/mL in 20% hydroxypropyl-β-cyclodextrin (62). Some other examples of injectables that are currently in the market which contain chemically modified cyclodextrin for the purpose of enhancement of solubility are: Aripiprazole (Abilify®) (63), ziprasidone (Geodon®) (64) and voriconazole (Vfend®) (65) containing sulfobutylether β cyclodextrin (SBECD), itraconazole (Sporanox®) (66) containing hydroxypropyl-β-cyclodextrin, and others.

Having reviewed the factors that govern solubility and solubilization during the formulation development of injectable products, the next considerations are the elements of formulations.

Types of vehicles

Aqueous The vast majority of injectable products are administered as aqueous solutions because of the physiological compatibility of water with body tissues. Additionally, the high DC of water makes it possible to dissolve ionizable electrolytes, and its hydrogen-bonding potential facilitates the solution of alcohols, aldehydes, ketones, and amines. The current USP (1) has monographs for purified water, sterile purified water, WFI, sterile WFI, bacteriostatic WFI, sterile water for inhalation, and sterile water for irrigation.

WFI is the solvent of choice for making parenterals. It must be prepared fresh and be pyrogen-free. It must meet all the chemical requirements for sterile purified water and in addition the requirements for bacterial endotoxins. The tests required for WFI are generally the same among the various pharmacopeias but differences do exist with regards to limits. WFI may be prepared by either distillation or reverse osmosis but the distillation method is by far the most common and accepted method. Because of the excellent solvent properties of water, it is both difficult to purify and maintain purity. Microorganisms, dissolved gases, organic and inorganic substances, and foreign particulate matter are the most common contaminants of water.

Prior to distillation, the water used as the source for WFI is usually subjected to chlorination, carbon treatment, deionization, and, sometimes, reverse osmosis treatment (forced passage through membrane materials). After distillation, it is filtered and then stored in a chemically resistant tank (stainless steel, glass, or blocked tin) at a cold temperature around 5°C or at an elevated temperature between 65°C and 85°C to inhibit microbial growth and prevent pyrogen formation. Generally, the hot water is continually circulated in the manufacturing areas during storage and usually filtered again prior to use. Sterile WFI and Bacteriostatic WFI are permitted to contain higher levels of solids than WFI because of the possible leaching of glass container constituents into the water during sterilization and storage. Bacteriostatic WFI, which generally contain 0.9% (9 mg/mL) of benzyl alcohol as a bacteriostatic preservative, should not be sold in containers larger than 30 mL to prevent injection of unacceptably large amounts of bacteriostatic agents (such as phenol and thimerosal).

Other water-miscible cosolvents These have been discussed earlier.

Nonaqueous vehicles Drugs that are insoluble in aqueous systems are often incorporated in metabolizable oils. Steroids, hormones, and vitamins are incorporated in vegetable oils such as peanut, sesame, corn, olive, and cottonseed. Oil injections are only administered intramuscularly. There are strict specifications for the vegetable oils used in manufacturing

Table 8 Official Injections Containing Oils as Vehicles

USP (1)	Oil commonly used
Desoxycorticosterone acetate	Sesame
Diethylstilbestrol	Sesame, cottonseed
Dimercaprol (suspension)	Peanut
Estradiol cypionate	Cottonseed
Estradiol valerate	Sesame
Estrone	Sesame
Ethiodized iodine	Poppyseed
Fluphenazine decanoate	Sesame
Fluphenazine enanthate	Sesame
Hydroxyprogesterone caproate	Sesame
Menadione	Sesame
Nandrolone decanoate	Sesame
Nandrolone phenpropionate	Sesame
Penicillin G procaine (combinations)	Vegetable
Propyliodone (suspension)	Peanut
Testosterone cypionate	Cottonseed
Testosterone enanthate	Sesame
Testosterone propionate	Sesame

intramuscular injections. Storage of these preparations is important if stability is to be maintained. For example, they should not be subjected to conditions above room temperature for extended periods of time. Although the oils used for injections are of vegetable origin, federal regulations require that the specific oil be listed on the label of a product, because some patients have exhibited allergic responses to certain vegetable oils.

Sesame oil is the preferred oil for most of the compendial injections formulated with oil. It is the most stable of the vegetable oils (except to light), because it contains natural antioxidants. Sesame oil has also been used to obtain slow release of fluphenazine esters given intramuscularly (67). Excessive unsaturation of oil can produce tissue irritation. In recent years, the use of injections in oil has diminished somewhat in preference to aqueous suspensions, which generally have less irritating and sensitizing properties. Benzyl benzoate may be used to enhance steroid solubility in oils if desired. Table 8 lists the oil injections official in the current USP (1).

Added Substances

Added substances such as buffers, antioxidants, antimicrobial preservatives, tonicity adjusting agents, bulking agents, chelating agents, solubilizing agents, and surfactants must frequently be incorporated into parenteral formulas in order to provide safe, efficacious, and elegant parenteral dosage forms. However, any such additive may also produce negative effects such as loss of drug solubility, activity, and/or stability. Any additive to a formulation must be justified by a clear purpose and function. No coloring agent may be added, solely for the purpose of coloring the finished preparation, intended for parenteral administration (1). The reader is encouraged to refer to a number of publications that provide comprehensive listing of formulation components used in all marketed injectable products (1,68–74). Hospital pharmacists who are involved in IV additive programs should be aware of the types of additives present in products that are being combined. Commonly used parenteral additives and their usual concentrations are listed in Table 9.

Pharmacopeias often specify the type and amount of additive substances that may be included in injectable products. These requirements often vary from compendia to compendia, so it is important to refer to the specific pharmacopeia that applies to the product in question. USP (1) specifies following maximum limits in preparations for injection that are administered in a volume exceeding 5 mL: for agents containing mercury and the cationic surface-active compounds, 0.01%; for chlorobutanol, cresol, phenol, and similar types of substances, 0.5%; and for sulfur dioxide, or an equivalent amount of the sulfite, bisulfite, or metabisulfite of

Table 9 Commonly Used Parenteral Additives and Their Usual Concentration

Added substance	Usual concentrations (%)
Antibacterial preservatives	
Benzalkonium chloride	0.01
Benzethonium chloride	0.01
Benzyl alcohol	1–2
Chlorobutanol	0.25–0.5
Chlorocresol	0.1–0.3
Metacresol	0.1–0.3
Phenol	0.5
Phenylmercuric nitrate and acetate	0.002
Methyl p-hydroxybenzoate	0.18
Propyl p-hydroxybenzoate	0.02
Butyl p-hydroxybenzoate	0.015
Thimerosal	0.01
Antioxidants	
Acetone sodium bisulfite	0.2
Ascorbic acid	0.01
Ascorbic acid esters	0.015
Butylhydroxyanisole (BHA)	0.02
Butylhydroxytoluene (BHT)	0.02
Cysteine	0.5
Nordihydroguaiaretic acid (NDGA)	0.01
Monothioglycerol	0.5
Sodium bisulfite	0.15
Sodium metabisulfite	0.2
Tocopherols	0.5
Glutathione	0.1
Chelating agent	
Ethylenediaminetetraacetic acid salts	0.01–0.075
DTPA	0.01–0.075
Buffers	
Acetic acid and a salt, pH 3.5–5.7	1–2
Citric acid and a salt, pH 2.5–6	1–5
Glutamic acid, pH 8.2–10.2	1–2
Phosphoric acid salts, pH 6–8.2	0.8–2
Tonicity adjustment	
Dextrose	4–5.5
Sodium chloride	0.5–0.9
Mannitol	4–5

potassium or sodium, 0.2%. Ethylenediaminetetraacetic acid derivatives and salts are sometimes used to complex and thereby inactivate trace metals that may catalyze oxidative degradation of drugs. The properties and function of these added substances will be reviewed next, except solubilizing agents and surfactant, which have been reviewed earlier.

Buffers. Maintenance of appropriate pH of the formulation is essential for proper solubility and stability. Changes in the pH of a formulation may occur during storage because of degradation reactions within the product, interaction with container components (i.e., glass or rubber), and absorption or evolution of gases and vapors. Buffers are added to many products to resist a change in pH. Excellent reviews on pH control within pharmaceutical systems by Flynn (75) and Nema et al (76) are recommended to the reader. A suitable buffer system should have an adequate buffer capacity to maintain the pH of the product at a stable value during storage, while permitting the body fluids to adjust the pH easily to that of the blood following administration. Therefore, the ideal pH to select would be 7.4, the pH of the blood. Extreme deviation from this pH can cause complications. Tissue necrosis often occurs above pH 9, while extreme pain and phlebitis are experienced below pH 3. The acceptable range for IV injections is 3 to 9 because blood itself is an excellent buffer and can very quickly neutralize the

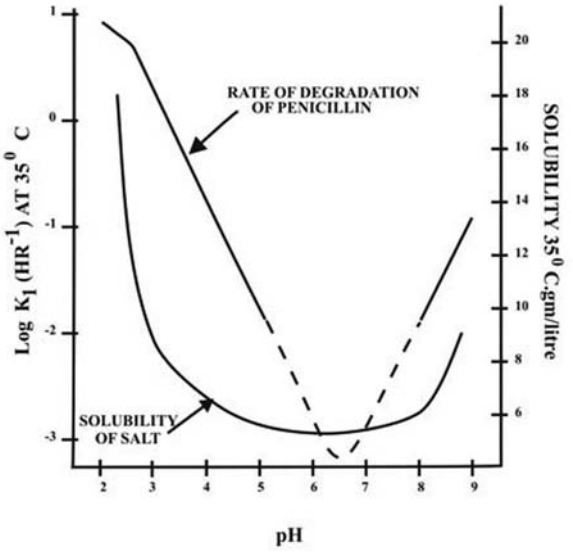


Figure 8 Solubility/stability pH profile of procaine penicillin. *Source*: From Ref. 77.

pH outside of 7.4. Parenterals administered by other routes are generally adjusted to a pH between 4 and 8.

A suitable buffer system can be selected from knowledge of a solubility/stability pH profile of the drug in solution. A typical pH profile of both solubility and stability is shown in Figure 8 for procaine penicillin G (77). By following the degradation over a given pH range and plotting the rate constants versus pH, the pH of maximum stability (pH 6.6) can be determined. In the case of procaine penicillin G, the solubility is lowest between the pH 6 and 7, which is desirable since the product is formulated as a suspension. Once the desired pH is determined, a buffer system that provides sufficient buffer capacity can be selected. The buffer capacity, β, is an indication of the resistance to change in pH upon the addition of either basic or acid substances and can be represented by the following expression:

$$\beta = \frac{dB}{d\text{pH}} = 2.303C\frac{K_a H^+}{(K_a + H^+)} \tag{11}$$

where

dB = change in concentration of base or acid,
dpH = change in pH,
C = molar concentration of buffer system, and
K_a = dissociation constant of the buffer.

A hypothetical plot of β versus pH-pK_a is illustrated in Figure 9 for a monobasic acid. A maximum value at zero indicates that the greatest buffer capacity occurs at a pH equal to the pK_a of the buffer system and further suggests that a buffer system with a pK_a within ±1.0 unit of the desired pH should be selected.

Buffer systems for parenterals generally consist of either a weak base and the salt of a weak base or a weak acid and the salt of a weak acid. Figure 10 shows the effective range of typical pharmaceutical buffers. The distance indicated by the arrows represents the effective buffer range for each system and the dashed lines represent the pK_a for the system. Commonly used buffers are phosphates, citrate, acetate, and glutamates.

The Henderson-Hasselbach relationship is used to calculate the quantities of buffer species required to provide a desired pH.

$$\text{pH} = \text{p}K_a + \log\frac{C_{salt}}{C_{acid}} \tag{12}$$

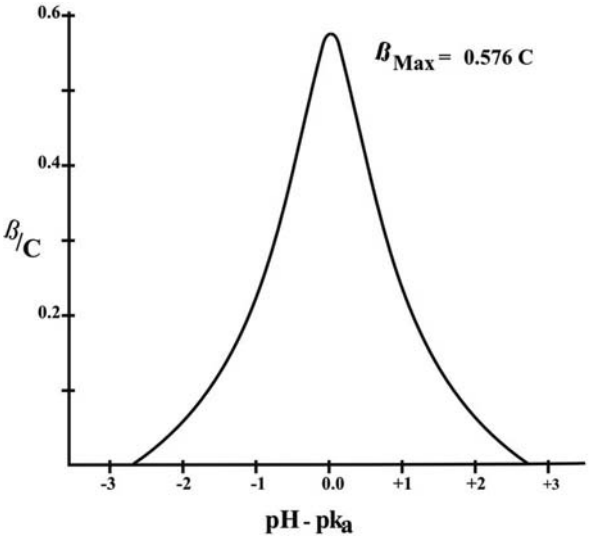


Figure 9 Theoretical buffer capacity curves of a monobasic acid.

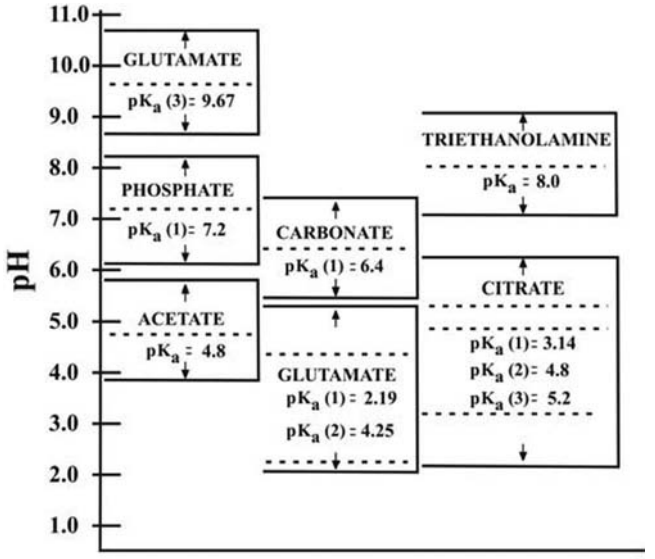


Figure 10 Effective range of pharmaceutical buffers, indicated by the arrows. The dashed line represents the pK_a value.

Where C_{salt} and C_{acid} are the molar concentrations of the salt form and the acid form, respectively. As shown from the following calculation, an acetate buffer system (pK_a = 4.8) consisting of 0.1 M acetic acid and 0.05 M sodium acetate would result in a pH of 4.5.

$$\text{pH} = 4.8 + \log\frac{0.05}{0.1} = 4.8 - 0.3 = 4.5$$

Although buffers assure the stability of pH of solution, the buffer system itself can affect other properties such as reaction kinetics and solubility aspects. Buffers can act as general acid or general base catalysts and cause degradation of some drug substances. Such a mechanism occurs with a number of amine and amine derivative drugs in systems containing polycarboxylic acids (e.g., citric, tartaric, and succinic). In one such case, as shown in Figure 11, the degradation of vitamin B_1 increases with increase in citrate buffer concentration (78).

The ionic strength contributions of the buffer system can also affect both isotonicity and stability. For example, if adjustment of pH is made with sodium hydroxide, say of a solution

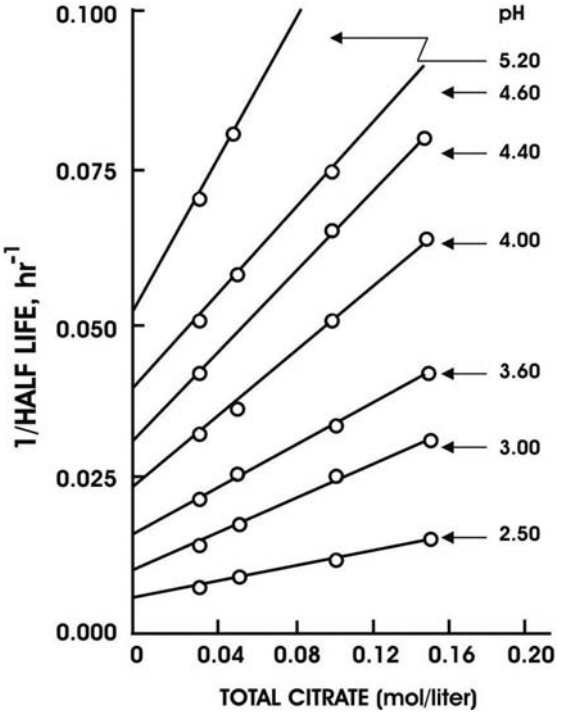


Figure 11 Effect of citrate buffer concentration on thiamine hydrolysis (vitamin B1) at 96.4°C at constant ionic strength and at different pH values. *Source*: From Ref. 78.

containing monosodium phosphate, the effect of the generation of disodium salt on isotonicity and the effect of HPO_4^{-2} must be taken into account (79,80).

Antioxidants. Many drugs in solution are subject to oxidative degradation. Such reactions are mediated either by free radicals or by molecular oxygen and often involve the addition of oxygen or the removal of hydrogen. For products in which oxygen is directly involved in the degradation, protection can be afforded by displacing oxygen (air) from the system. This is accomplished by bubbling nitrogen, argon, or carbon dioxide through the solution prior to filling and sealing in the final container. Oxidative decomposition is catalyzed by metal, hydrogen, and hydroxyl ions. Drugs possessing a favorable oxidation potential will be especially vulnerable to oxidation. For example, a great number of drugs are formulated in the reduced form (e.g., epinephrine, morphine, ascorbic acid, menadione, etc.) and are easily oxidized. Oxidation can be minimized by increasing the oxidation potential of the drug. As illustrated in Figure 12 (81), lowering the pH of the solution will increase the oxidation potential. This occurs because according to a simplified version of the Nernst equation:

$$E = E^0 + \frac{RT}{2} \log \frac{[H^+] \cdot [Ox]}{[Rd]} \tag{13}$$

an increase in hydrogen ion concentration causes an increase in the actual oxidation potential, E. In this equation E^0 is the standard oxidation potential, R the gas constant, T the absolute temperature, and constant 2 represents the number of electrons taking part in the oxidation-reduction reaction.

Agents that have a lower oxidation potential than the drug in question, and thus can be preferentially oxidized, are called antioxidants. Such agents are added to parenteral solutions either alone or in combination with a chelating agent or other antioxidant and function in at least two ways: (*i*) by being preferentially oxidized and thereby gradually consumed or (*ii*) by blocking an oxidative chain reaction in which they are not usually consumed.

Morphine in aqueous solution undergoes a pH-dependent oxidative degradation. The rate is slow and constant between pH 2 and 5, where morphine exists in the protonated form as

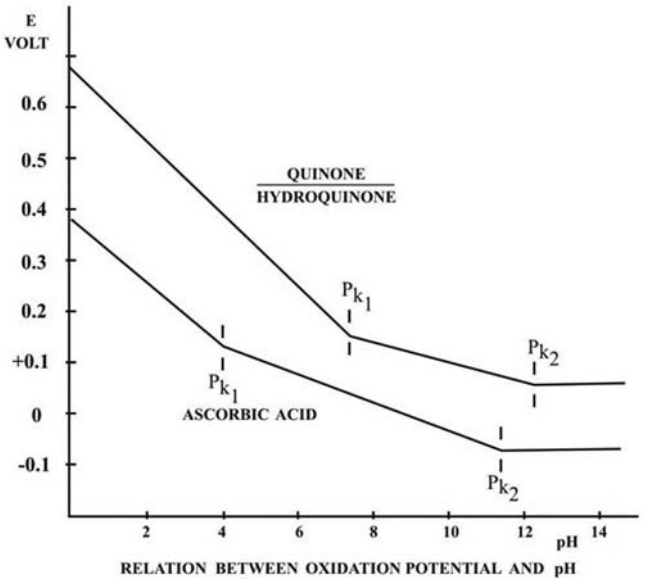


Figure 12 Relationship between oxidation potential and pH. *Source*: From Ref. 81.

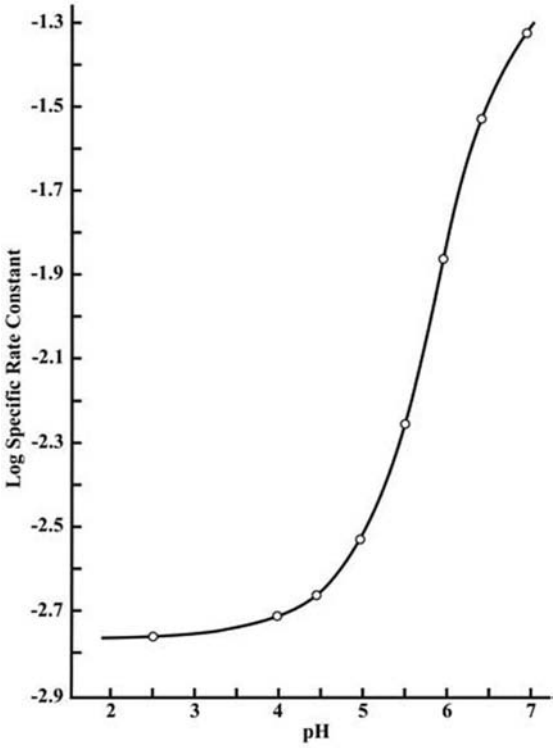


Figure 13 Reaction rate constant for the first-order oxidative degradation of morphine at 95°C as a function of pH. *Source*: From Ref. 82.

shown in Figure 13. However, above pH 5, the oxidation increases with increase in pH (82). Therefore, morphine can be stabilized by lowering the pH or by adding an antioxidant such as ascorbic acid which will be preferentially and reversibly oxidized between pH 5 and 7. Ascorbic acid, in turn, can act as an antioxidant for hydroquinone because it has a lower oxidation potential and will be preferentially oxidized. Table 10 lists some standard oxidation

Table 10 Some Commonly Used Antioxidants and Their Oxidation Potentials

Substance	E^a (V)	pH	Temperature (°C)
Riboflavin	+0.208	7.0	30
Dithiothreitol	+0.053	7.0	30
Sodium thiosulfate	+0.050	7.0	30
Thiourea	+0.029	7.0	30
Ascorbic acid[a]	+0.003	7.0	25
	−0.115	5.2	30
	−0.136	4.58	30
Methylene blue	−0.011	7.0	30
Sodium metabisulfite[a]	−0.114	7.0	25
Sodium bisulfite[a]	−0.117	7.0	25
Propyl gallate[a]	−0.199	7.0	25
Acetylcysteine[a]	−0.293	7.0	25
Vitamin K	−0.363	–	20
Epinephrine	−0.380	7.0	30
Hydroquinone	−0.673	–	–
Resorcinol	−1.043	–	–
Phenol[a]	−1.098	–	–

[a]Common in parenteral products

potentials. Salts of sulfur dioxide, including bisulfite, metabisulfite, and sulfite are the most common antioxidants in aqueous solutions. Irrespective of which salt is added to the solution, the antioxidant moiety depends on the final concentration of the compound and the final pH of the formulation (83). The metabisulfite is used at low pH values (84). Some drugs can be inactivated by bisulfites. For example, in the presence of bisulfite, epinephrine forms addition product as epinephrine sulfonate, which is inactive (85). Ortho or para-hydroxybenzyl alcohol derivatives such as parabens react in a similar manner.

While undergoing oxidation reactions, the sulfites are converted to sulfates. Since small amounts (picograms) of barium or calcium can be extracted even from type I glass, an insoluble sulfate can form in the solution (86). Therefore, additional care must be exercised to visibly inspect preparations containing sulfite antioxidants or sulfate drugs for the presence of fine particles which will appear, upon gently shaking, as a swirl originating from the bottom of the container. Sulfite levels are determined by the reactivity of the drug, the type of container (glass seal vs. rubber stopper), single or multiple-dose use, container headspace, and the expiration dating period to be employed.

Another antioxidant, Glutathione, an electron donor, stabilized the photooxidation of menadione, a synthetic analogue of Vitamin K by a charge transfer complex formation (87), thereby blocking the light-catalyzed oxidative chain reaction.

Often a single antioxidant may not be sufficient to completely protect the product. Certain compounds have been found to act as synergists, increasing the effectiveness of antioxidants, particularly those that block oxidative reactions, e.g., ascorbic acid and citric acid. Frequently, chelating agents such as ethylenediaminetetraacetic acid (EDTA) salts are used because these salts form complexes with trace amounts of heavy metals which otherwise would catalyze oxidative reactions. While incorporating such antioxidants, the formulator must be aware of their potential side effects. Although, very widely used, sulfites are associated with several effects upon parenteral administration, including flushing, pruritus, urticaria, dyspenia, and bronchospasm (88).

In practice, several approaches can be utilized by the formulator to protect the product from oxidative instability, such as purging the solution and headspace with inert gas to exclude oxygen, lowering the pH, and addition of an antioxidant. One must ensure use of high purity excipients since trace impurities, namely peroxides and metals, carried into a formulation through ingoing components, may also have a catalyzing effect on the auto-oxidation pathway. Well-protected, properly sealed packages that provide an acceptable headspace-to-product ratio can also provide some robustness to the product, thus making it

Table 11 List of Commonly Used Antibacterial Preservatives and Their MICs

Agent	MIC[a] range	Amount most often used (%)
Benzalkonium chloride	0.005–0.03	0.01
Benzethonium chloride	0.005–0.03	0.01
Benzyl alcohol	1.0–10.0	1.0
Chlorobutanol	0.2–0.8	0.5
Chlorocresol	0.1–0.3	0.1–0.25
Cresol	0.1–0.6	0.3
Parabens (methyl, ethyl,	0.05–0.25 methyl	0.18
propyl, butyl esters)	0.005–0.03 others	0.02
Phenol	0.1–0.8	0.5
Phenylmercuric nitrate	0.001–0.05	0.002
Thimerosol	0.005–0.03	0.01

[a]Affected by product pH, ionic strength, storage temperature, packaging materials, etc.
Abbreviation: MIC, minimum inhibitory concentration.

less sensitive to oxidation (89). Process control is required for assurance that every container is deareated adequately and uniformly.

Antimicrobial preservatives. Agents with antimicrobial activity must be added to preparations packaged in multiple-dose containers unless prohibited by compendial monograph or unless the drug itself is bacteriostatic, for example, methohexital sodium for injection and most of the cytotoxic anticancer products. A partial list of antimicrobial preservatives used in pharmaceutical systems along with their minimum inhibitory concentrations (MICs), is presented in Table 11.

An excellent review is published by Meyer et al (90) that provides a comprehensive summary of antimicrobial preservatives that are commonly used in licensed parenteral products to date. It was noted that the most commonly used eight antimicrobial preservatives in all parenteral products at the present are: phenol, benzyl alcohol, chlorobutanol, m-cresol, methylparaben, phenoxyethanol, propylparaben, and thimerosal with the three most commonly used preservatives in small molecule injection products are phenol, benzyl alcohol, and parabens.

Phenol is a bacteriostatic when present in 1% w/v solution and has activity against mycobacteria, fungi, and viruses (91). The solubility of phenol in water is 1 in 15 (w/w) at 20 °C. Aqueous solutions of phenol are stable, can be sterilized by dry heat or autoclaving, and should be maintained in containers that are protected from light. Phenol is incompatible with albumin and gelatin, which will result in precipitates possibly due to phenol-induced denaturation of these molecules. There is a low likelihood of adverse reactions from phenol in parenteral products due to the low concentrations used in these products.

Benzyl alcohol is an aromatic primary alcohol and is effective against most Gram-positive bacteria, yeast, and mold, but is less effective against gram-negative bacteria. Its solubility in water is 1 in 25 (w/w) at 25°C. The optimum antimicrobial activity occurs at pH less than 5 and is less active above pH 8.3. It may be stored in glass or metal containers or in polypropylene containers coated with Teflon or other inert fluorinated polymers (92).

Parabens are benzoic acid esters and have a broad spectrum of antimicrobial activity at a pH range of 4–8, but are more effective against yeasts and molds when compared with bacteria. Antimicrobial activity is normally enhanced when combinations of parabens are used with excipients such as propylene glycol, phenylethyl alcohol, and edetic acid (93). Aqueous solutions of parabens are stable at a pH range of 3 to 6, but degrade by hydrolysis at pH greater than 8. The solubility of methylparaben and propylparaben in water is 1 in 400 (w/w) at 25 °C, and 1 in 2500 at 20°C, respectively (92). Because of inherent low solubilities, sodium salts are frequently utilized in the final dosage forms.

Antimicrobial agents are specifically excluded in the large-volume injections that are used to provide fluids, nutrients, or electrolytes, such as dextrose and sodium chloride injection, dextrose injection, ringer's injection, lactated ringer's injection, and sodium chloride

injection. Bacteriostatic agents may be added to dextrose and sodium chloride injection when it is labeled for use as a sclerosing agent, because the amount of injection used for such purposes is small, and the quantity of antibacterial present would not be harmful to the patient.

The two main considerations while selecting an antimicrobial preservative in the injection products are their compatibility and effectiveness.

Many papers have been published describing the incompatibilities or binding of preservatives with surfactants, pharmaceuticals, and rubber closures (94–99).

Antimicrobial activity of preservative parabens, which was due to the concentration of the free form, was shown to be significantly reduced in the presence of polysorbate because of binding (96). Rubber closures and rubber extractives have also been found to influence significantly preservative loss from solution and antimicrobial activity, respectively. Lachman and coworkers (98,99) studied the interaction of preservatives with various types of rubber and found significant losses of a number of preservatives (i.e., chlorobutanol, chlorophenylethyl alcohol, methylparaben, and benzyl alcohol) with natural and neoprene rubber whereas the loss was minimal in the presence of butyl rubber.

The effectiveness of antimicrobial agents can be determined using a test described in compendia as "antimicrobial effectiveness testing." The test typically consists of inoculating 10^5–10^6 CFU/mL microorganisms (e.g., bacteria and fungi) per container at time zero, and evaluating the log reduction over time. The criterion used for passing this test is as follows:

> Bacteria: Not less than 1.0 log reduction from the initial calculated count at 7 days, not less than 3.0 log reduction from the initial calculated count at 14 days, and no increase from the 14 days' count at 28 days.
> Yeasts and molds: No increase from the initial calculated count at 7, 14, and 28 days.

It is recommended that this test should be performed with the formulation throughout and near the end of the expiration date to ensure that adequate levels of preservative are still available.

While the need for an antimicrobial is clearly obvious, there have been recent concerns and evidence of irritation from these agents. Therefore, it is essential to keep the concentration as low as possible, recognizing that these agents act by killing living cells and do not differentiate the good cells from the bad ones.

Tonicity. To minimize tissue damage and irritation, reduce hemolysis of blood cells, and prevent electrolyte imbalance upon administration of small-volume parenterals, the product should be isotonic, or nearly so. Isotonic solutions exert the same osmotic pressure as blood plasma. Solutions may also exert less (hypotonic) or more (hypertonic) osmotic pressure than plasma. Red blood cells (RBCs; erythrocytes) when introduced into hypotonic solution will swell and often burst (hemolysis) because of diffusion of water into the cell. If the cells are placed into hypertonic solutions, they may lose water and shrink (crenation). In isotonic solutions (e.g., 0.9% sodium chloride) the cells maintain their "tone" and the solution is isotonic with human erythrocytes. Isotonicity of formulation is not always feasible as a result of the high concentrations of drug utilized, the low volumes required for some injections, the wide variety of dose regimens and methods of administration, and product stability considerations. Historically, there has been concern over the osmolarity or tonicity of IV infusion fluids because of the large amounts of solution administered to hospitalized patients, but in the last few years there has also been interest in the osmolarity of other parental dosage forms.

Sodium or potassium chloride and dextrose are commonly added to adjust hypotonic solutions. There are several methods available to calculate tonicity (100). The sodium chloride equivalent method is the most convenient. The sodium chloride equivalent of a substance can be determined from its ability to lower the freezing point of water. A 1% sodium chloride solution has a freezing point of -0.58°C and is assigned a sodium chloride equivalent, E, of 1.00. The freezing point of blood (serum) is -0.58°C, the same as a 0.9% w/v solution of sodium chloride. If a 1% solution of a substance has a freezing point of -0.058°C, the E value will be 0.1. Therefore, 1.0 g of the substance will be equivalent to 0.1 g of NaCl.

Table 12 Sodium Chloride Equivalents and Freezing Point Depression for 1% Solutions

Agent	Sodium chloride equivalent	Freezing point depression (°C)
Atropine sulfate	0.13	0.075
Barbital sodium	0.30	0.171
Benzyl alcohol	0.17	0.09
Boric acid	0.50	0.288
Calcium chloride	0.51	0.298
Calcium disodium edetate	0.21	0.120
Calcium gluconate	0.16	0.191
Chlorobutanol	0.24	0.14
Citric acid	0.18	0.10
Codeine phosphate	0.14	0.080
Dextrose	0.16	0.091
Dimethyl sulfoxide	0.42	0.245
Edetate disodium	0.23	0.132
Ephedrine HCl	0.30	0.165
Isoproterenol sulfate	0.14	0.078
Mannitol	0.18	0.1
Penicillin G potassium	0.18	0.102
Phenol	0.35	0.20
Pilocarpine nitrate	0.23	0.132
Polyethylene glycol 300	0.12	0.069
Polyethylene glycol 400	0.08	0.047
Sodium bisulfite	0.61	0.35
Sodium cephalothin	0.17	0.095
Sodium chloride	1.00	0.576
Sodium citrate	0.31	0.178
Sodium phosphate, dibasic	0.42	0.24
Sodium sulfate, anhyd	0.58	0.34
Sucrose	0.08	0.047
Urea	0.59	0.34

To make 100 mL of a 1% solution of the substance isotonic, 0.8 g of sodium chloride must be added. A partial list of sodium chloride equivalents of variety of parenteral additives is shown in Table 12.

In the absence of a sodium chloride equivalent the L_{iso} method can be used as shown by Goyan, et al, in 1944 (101). The L_{iso} is the value at which a specific compound type will be isotonic with blood. It is related to sodium chloride equivalent in the following manner:

$$E = 17\frac{L_{iso}}{M} \tag{14}$$

where M is the molecular weight of the substance. Table 13 shows some L_{iso} values for various types of compounds. The calculation of tonicity is illustrated by the following example.

It is desired to make a 2 g/100 mL solution of sodium cephalothin isotonic using sodium chloride. Sodium cephalothin has a molecular weight of 238.

Table 13 L_{iso} Values for Various Types of Additives in Parenteral Formulations

Compound type	L_{iso}	Example
Nonelectrolyte	1.9	Sucrose
Weak electrolyte	2.0	Phenobarbital
Divalent electrolyte	2.0	Zinc sulfate
Univalent electrolyte	3.4	Sodium chloride
Unidivalent electrolyte	4.3	Sodium sulfate
Diunivalent electrolyte	4.8	Calcium chloride
Unitrivalent electrolyte	5.2	Sodium phosphate
Triunivalent electrolyte	6.0	Aluminum chloride

Table 14 Comparison of Measured Osmolality Values with Those Calculated from Sodium Chloride Equivalents

Solution (g/100 mL)	Measured osmolality mean mOsm ± SD	Sodium chloride equivalent method	
		Osmolality	Percent of measure
Dextrose			
5.0	262 ± 5.9	249	95.0
10.0	547 ± 6.2	499	91.2
20.0	1176 ± 14.9	998	84.9
Alanine glycine			
1.0	246 ± 0.5	256	104
2.0	480 ± 1.7	512	107
5.0	1245 ± 10.8	1281	103
0.2 NaCl in 5% dextrose	311 ± 5.85	312	100
0.45% NaCl in 5% dextrose	385 ± 5.48	390	98.7
Ringer's solution, USP	294 ± 4.98	281	95.6
Lactated ringer's, USP	264 ± 3.23	248	93.9
Travasol 5.5%	554 ± 11.4	596	107.6
67% travasol (5.5%) 33% dextrose (50%)	1330 ± 29.6	1323	91.9

As shown in Table 13 the L_{iso} for univalent electrolytes has a calculated value of 3.4. Therefore,

$$E = 17x\frac{3.4}{238} = \frac{57.8}{238} = 0.24g - \text{eq.}$$

Since 2 g of drug is used in the 100 mL of fluid, $2 \times 0.24 = 0.48$ g – eq. is contributed by sodium cephalothin toward the 0.90 g of sodium chloride needed for isotonicity.

Hence 0.90 g − 0.48 g = 0.42 g of sodium chloride must be added to 2 g of sodium cephalothin in 100 mL to achieve isotonicity of the resulting solution. The sodium chloride equivalent method was used for determining the osmolarity of a number of infusion solutions and compared with measured values. As shown in Table 14, there is good agreement between measured and calculated values until the concentrations become very high.

Isoosmosity, determined by physical methods, should be distinguished from isotonicity, determined by biological methods (i.e., the hematocrit method with human erythrocytes). This distinction is necessary because of the variable diffusibility of different medicinal substances across the cell membrane, which does not always behave as a truly semi-permeable membrane. Solutions that are theoretically isoosmotic with the cells may cause hemolysis because solutes diffuse through the cell membrane. For example, a 1.8% solution of urea has the same osmotic pressure as 0.9% sodium chloride, but the urea solution produces hemolysis, because urea permeates the cell membrane. If a solution is hypertonic, not much can be done with the formulation unless it can be diluted with water prior to administration. Administration of a hypertonic solution should be done slowly to permit dilution by the blood. In some cases, where injection of such solutions produces pain, as in an intramuscular injection, a local anesthetic may be added. The effect of isotonicity on reducing pain on injection is somewhat vague, although it may at least reduce tissue irritation.

Special Types of Parenterals

Suspensions. A parenteral suspension is a dispersed, multiphased, heterogeneous system of insoluble solid particles intended principally for intramuscular and subcutaneous injection.

Suspension formulation is desired when the drug is too insoluble or unstable to be formulated as a solution, as well as when there is a need to retard or control the release of drug from a suspension. The desirable parenteral suspension is sterile, stable, resuspendable, syringeable, injectable, and isotonic/nonirritating. Because a delicate balance of variables is required to formulate a suitable product, a suspension is one of the most difficult parenteral

forms to prepare. Such a product must not cake during shipping and storage, and should be easy to suspend and inject through 18- to 21-gauge needle throughout its shelf life.

To achieve these goals, it is necessary to control the crystallization, particle size reduction (micronization), and sterilization of the drug substance, as well as the processes involved in wetting of the drug with surfactants, aseptic dispersion and milling, and final filling into containers. Uniform distribution of the drug is required to ensure that an adequate dose is administered to the patient. Parenteral suspensions exhibit instability in ways not applicable to solutions and dry solids. This is due to the problem of crystal growth, caking, and product-package interactions.

Injectable suspensions may be made with either vegetable oils or aqueous vehicles. Many contain low concentrations of solids (5% or less) but a few, such as procaine penicillin G, may contain up to 58% w/v solids. Therefore, properties such as resuspendibility, zeta potential, rheology, and particle size distribution become important, and often need to be monitored as a part of a stability program for these products. When particles interact to form clumps or aggregates, the process is termed flocculation or agglomeration. The process of dispersing these aggregates into individual particles is called deflocculation. The size of individual particles may also change because of temperature fluctuation during storage and/or polymorphic changes. For example, if the solubility of a drug is very temperature dependent, individual crystals can dissolve or grow in size depending on the circumstances encountered. If the rate of absorption or injectability of the drug depends on the particle size distribution of the dispersed insoluble drug, the intended performance of the product may be altered.

The requirements for, limitations of, and difference between the design of injectable suspensions and other suspensions have been summarized by several authors (102,103). The requirements and limitations relate to (*i*) microbiological purity, (*ii*) ingredients allowed, and (*iii*) mechanical flow properties. The microbiological purity requirements, like all parenterals, involve sterility and freedom from pyrogens.

There are 38 official parenteral suspensions in the current USP (1). The wide variety of injectable suspensions can be illustrated with the following examples. Sterile Ampicillin for suspension, USP, represents a powder to which an aqueous diluent is added to make an injectable suspension. Sterile aurothioglucose suspension, USP, is an example of a ready-to-use suspension in vegetable oil. Aqueous ready-to-use suspensions include betamethasone acetate suspension, USP, and insulin zinc suspension, USP.

As shown in Table 15, a formula for an injectable suspension might consist of the active ingredient suspended in an aqueous vehicle containing an antimicrobial agent, a surfactant for wetting and preventing crystal growth (by reducing free surface energy), a dispersing or suspending agent, antioxidant, and perhaps a buffer or salt, etc. Table 16 lists materials commonly used to formulate parenteral suspensions.

Two basic methods are used to prepare parenteral suspensions: (*i*) sterile vehicle and powder are combined aseptically or (*ii*) sterile solutions are combined and the crystals are formed in situ. In the first method, an aqueous vehicle containing the water-soluble components are heat sterilized, when possible; or filtered through a 0.22 μm sterilizing membrane filter into a presterilized mixing/filling tank. The sterile drug powder is gradually added to the sterile solution, aseptically, while mixing. The sterile drug powder, in turn, is obtained by aseptically filtering a solution of the drug through a sterilizing membrane into a

Table 15 Examples of Injectable Suspension Formulations in the Market

Active/Brand/Conc.	Dexamethazone/ Decadron® (8 mg/mL)	Medroxyprogesterone Acetate/Depo-Provera® (100 and 400 mg/mL)	Triamcinolone Acetonide/Kenalog® (10 and 40 mg/mL)
Surfactant	Polysorbate 80	Polysorbate 80	Polysorbate 80
Suspending agent	Sodium CMC	PEG 3350	Sodium CMC
Antimicrobial agent	Benzyl alcohol	Parabens	Benzyl alcohol
Antioxidant	Sodium bisulfite	–	–
Others	Disodium edetate, sodium chloride, creatinine	Sodium chloride	Sodium chloride

Table 16 Partial List of Ingredients Used in Aqueous Parenteral Suspensions

Suspending agents
Aluminum monstearate
Gelatin (nonantigenic)
Mannitol
Povidone
Sodium carboxymethylcellulose
Sorbitol
Surfactants
Lecithin (soybean)
Polyoxyethylene-polyoxypropylene ethers
Polyoxyethylene sorbitan monolaurate
Polysorbate 80
Silicone antifoam
Sorbitan trioleate
Solubilizing agents
Polyethylene glycol 300
Propylene glycol
pH adjustment
Citric acid
Sodium citrate

sterile vessel into which a presterilized solution of antisolvent is introduced causing the drug to crystallize. The crystals or powder are separated aseptically by filtration or centrifugation, washed, dried, and sized through milling. After all tests have been completed on the bulk material, it is aseptically filled.

In the second method, the vehicle is prepared and sterilized by filtration. The drug is dissolved separately in a nonaqueous solvent and sterilized by filtration. The sterile drug solution is aseptically added to the sterile vehicle, causing the drug to crystallize. The resulting suspension is then diluted with sterile vehicle, mixed, the crystals are allowed to settle, and the supernatant solution siphoned off. The suspension is then brought to volume and filled in the normal manner. In few cases, the filled vials may be subjected to terminal sterilization if chemical properties and particle size characteristics remain unchanged post sterilization.

Rheologically, an injectable suspension can present some formidable challenges. While a suspension can usually be formulated so that it can be filled, shipped, and injected, it is frequently difficult to formulate a product in which these three qualities remain relatively unchanged throughout its shelf life. Rheological evaluation should be done with a recording viscometer that continuously measures the shear throughout the hysteresis loop.

The critical nature of the flow properties of parenteral suspensions becomes apparent when one remembers that those products are frequently administered through 1- to 1.5-in or longer needles, having internal diameters in the range of only 300 to 600 μm. In addition, microscopic examination shows a very rough interior needle surface, further hindering flow. The flow properties of parenteral suspensions are usually characterized on the basis of syringeability or injectability. Syringeability refers to the handling characteristics of a suspension while drawing it into and manipulating it in a syringe, clogging and foaming tendencies, and accuracy of dose measurement. The term injectability refers to the properties of the suspension during injection. It includes such factors as pressure or force required for injection, evenness of flow, aspiration qualities, and freedom from clogging. The syringeability and injectability characteristics of a suspension are closely related to viscosity and to particle characteristics.

Emulsions. An emulsion is a heterogeneous dispersion of one immiscible liquid in another.

This inherently unstable system is made possible through the use of an emulsifying agent, which prevents coalescence of the dispersed droplets (104). Parenteral emulsions are rare because it is necessary (and difficult) to achieve stable average droplets of less than 1 μm to prevent emboli in the blood vessels. In addition, they are also thermodynamically unstable

by nature, that is, on standing they will eventually separate into two phases. However, proper choice of emulsifier (generally 1–5%) and optimum preparation conditions can delay the separation of phases and thus lead to more desirable nominal shelf lives of >2 years. An emulsion can be characterized as oil-in-water (o/w), containing up to 40% oil or water in oil (w/o), depending on the identity of the dispersed and continuous phases (105).

Preparation of an emulsion requires mixing the two immiscible phases with the surfactant(s) and applying energy (generally mechanical) in order to create shear forces to deform the interface and form droplets, using sufficient force and/or time to achieve the required droplet size. This can be done in either batch or continuous modes of operation. Typically, the surfactant or mixture of surfactants is dispersed in the aqueous phase along with any water-soluble components by stirring and heating as necessary until a homogenous mixture is formed. The oil phase is then added with stirring or shaking to form a "premix" with large (>10 μm) droplets, which is then subjected to a high-energy mechanical homogenization. The final droplet size depends on the formulation composition as well as the operating conditions (e.g., temperature, homogenization pressure, and duration of homogenization) (106). The preferred method for sterilization of parenteral emulsion is terminal autoclaving. If the components of a particular drug-emulsion formulation preclude autoclaving because of stability problems, sterile filtration of the product may be a viable alternative, requiring that the emulsion droplets pass through a 0.22 μm sterilizing membrane filters. Apart from the requirements of sterility and absence of pyrogens, parenteral emulsion product must show acceptable physical stability properties such as particle (droplet) size distribution, viscosity, osmolarity, and zeta potential, as well as good chemical stability.

Parenteral emulsions are used for several purposes, including

1. water-in-oil emulsions of allergenic extracts (given subcutaneously),
2. oil-in-water sustained-release depot preparations (given intramuscularly), and
3. oil-in-water nutrient emulsions (given intravenously).

IV oil-in-water nutrient emulsions provide the source of calories and essential fatty acids for patients requiring parenteral nutrition for extended periods of time (usually for longer than five days). IV fat emulsions are prepared from either soybean (5–30%) or safflower oil (5–10%) and provide a mixture of neutral triglycerides, predominantly unsaturated fatty acids. The major component of fatty acids are linoleic, oleic, palmitic, stearic and linolenic acids. In addition, these products contain 1.2% egg yolk phospholipids as an emulsifier and glycerol to adjust tonicity. The emulsified fat particles are approximately 0.4 to 0.5 μm in diameter, similar to naturally occurring chylomicrons. The prime destabilizers of emulsions are excessive acidity (low pH) and inappropriate electrolyte content. Careful consideration must be given to additions of divalent cations (calcium and magnesium) which cause emulsion instability (107). Amino acid solutions, on the other hand, exert a buffering effect protecting the emulsion (108).

For IV oil-in-water nutrient emulsions, the current USP (1) specifies special requirement for the globule size: The volume-weighted, large-diameter fat globule limits of the dispersed phase, expressed as the percentage of fat residing in globules larger than 5 μm (PFAT5) for a given lipid injectable emulsion, must be less than 0.05%.

Liposomes. Liposomes are small, spherical vesicles which consist of amphiphilic lipids enclosing an aqueous core. The lipids are predominantly phospholipids which form bilayers similar to those found in biomembranes. Depending on the processing conditions and the chemical composition, liposomes are formed with one or several concentric bilayers.

Liposomes are often distinguished according to their number of lamellae and size. For example, small unilamellar vesicles (SUVs), large unilamellar vesicles (LUVs), and large multilamellar vesicles (MLVs) or multivesicular vesicles (MVVs). SUVs show a diameter of 20 to approximately 100 nm. LUVs, MLVs, and MVVs range in size from a few hundred nanometers to several microns. The thickness of the membrane (phospholipid bilayer) measures approximately 5 to 6 nm (109).

Liposomes are unique as drug carriers in that they can encapsulate drugs with widely varying polarities. Liposomal formulation can significantly increase the apparent aqueous solubility of a lipophilic drug, making possible delivery of a dose much higher than its water solubility, Therefore, a stable formulation with a water-insoluble drug is often achievable with no precipitation upon dilution. Drugs formulated in liposomes distribute differently in the body than conventional pharmaceuticals, since liposomes have distinct pharmacokinetic pathways of distribution and elimination (110). Encapsulation of drugs in liposomes thus results in an increase of drug levels at the targeted sites, such as inflammation, infection, or neoplasm, compared with the conventional formulations. This site-specific action reduces the toxicity of drugs without loss of their efficacies (111,112).

Phospholipids are the principal raw material of forming liposomes. These are susceptible to hydrolysis and oxidative degradation, latter due to unsaturated acyl chains. Large liposomes form spontaneously when phospholipids are dispersed in water above their phase transition temperature. The preparation of SUVs starts usually with MLVs, which then are transformed into small vesicles using an appropriate manufacturing technique.

Mechanical dispersion method is the most frequently used in the production of the large-scale liposomes. Usually it is two-step process: the film preparation and hydration step, and the particle size reduction step. The hand-shaken method and proliposome method are the two commonly used methods in the first step. For particle size reduction, sonication or microfluidization techniques are used. The liposomal preparations are then aseptically filtered through 0.22 μm membrane filter to render them sterile for IV use since both lipids and the structure of liposomes are unstable at high temperatures and hence conventional terminal steam sterilization is not suitable.

Currently, there are two liposomal formulations approved for the U.S. market by the FDA: AmBisome®, a liposomal formulation of amphotericin B, and DOXIL®, a liposomal formulation of doxorubicin.

Nanosuspensions. Nanosuspension can be defined as colloidal dispersion of nano-sized drug particles that are produced by a suitable method and stabilized by a suitable stabilizer. Nanosuspensions are used to formulate drugs that are poorly water soluble as well as poorly lipid or organic solvent soluble. A number of reports have been published on the nanosuspenion development in general (113–116), nanosuspension based injectable products (117–121), and their preclinical and clinical aspects (122). Major advantages of nano-suspensions for IV use are (*i*) avoidance of organic cosolvents, (*ii*) capability of packing higher mass-per-volume per dose, and (*iii*) potential stability improvement due to presence of unsolubilized solid-phase drug.

Nanosuspensions of drugs are typically produced either by controlled crystallization or by a high-energy particle size reduction process. Examples of the latter include wet milling and high-pressure homogenization (115,116). A third approach was reported recently, wherein crystallization and particle size reduction were combined to produce injectable nano-suspensions (117). Some of the important considerations in development of injectable nanosuspensions include: a) Nanoparticles should be stable and not susceptible to phenomena such as aggregation or Ostwald ripening, b) The nanosuspension should be free of contamination from any media used during processing, c) The nanoparticle manufacturing should be possible by aseptic processing, if terminal sterilization by heat or membrane filtration is not feasible, and d) Surfactants and excipients used should be acceptable for injectable applications.

Particle size distribution and its stability is an important element in the formulation in nanosuspension and requires careful optimization of surfactants to be used in the formulation. Adsorption kinetics and affinity of the surfactant to the newly formed crystal surface play a determining factor on the final particle size and stability of the nanosuspension. A number of surfactants have been explored for the stabilization of nano-crystals including polysorbates, phospholipids, phosphatidylcholine, etc.

Recently, a nanosuspension product containing Paclitaxel (a very water-insoluble anticancer agent), Abraxane®, has been approved by FDA for IV administration. Abraxane

contains lyophilized particles with 10% (w/w) paclitaxel and 90% (w/w) albumin. The particle size of the suspension is about 130 nm (123). Another example of IV nanosuspenion is sterile powder of busulfan, encapsulated in a mixture of phospholipids - dimyritoylphosphatidylcholine and dilauroylphosphatidylcholine - in a buffer containing mannitol (124).

Dried forms. Sterile solids are drugs or drug products packaged in a dry form which must be reconstituted or suspended in sterile vehicle prior to administration. Many drugs, particularly the cephalosporins and penicillins, are not sufficiently stable in aqueous solution to permit packaging them "ready to use." The dry solids which are intended to be reconstituted by the addition of suitable solvents to yield solutions, conforming in all respects to the requirements for injections (solutions for injection), are described by a title in the form "for injection or sterile." Examples are thiopental sodium for injection (USP), in which the preparation contains added substances in addition to the drug, and sterile nafcillin sodium (USP), in which there are no additional ingredients other than the drug. In any such labeling, the product is intended to be appropriately reconstituted as a solution. Some reconstituted products must be further diluted prior to use, an example being methohexital sodium for injection (1).

Dry products which are to be reconstituted as suspensions by the addition of a suitable vehicle to yield a product meeting all requirements for sterile suspensions are labeled as "sterile—for suspension." An example is sterile ampicillin trihydrate for suspension. Such preparations are manufactured and packaged as dry sterile solids by sterile filtration and freeze-drying or bulk sterilization and aseptic powder filling. The sterile bulk powder in the latter process can be achieved by either aseptic crystallization or spray-drying.

The powder filling procedure is briefly described below.

Powder filling. This method involves filling sterile powder into individual containers (vials) under aseptic conditions in which a measured quantity, either on a weight or volume basis, is delivered. If the material is free flowing, a machine method is used whereby the solid material is fed from a hopper to the container by means of an auger in the stem of the hopper or an adjustable cavity in the rim of a filling wheel.

Particle size and shape are important factors in powder filling since electrostatic charge, hygroscopicity, and flow are generally influenced by these properties. Additionally, the dissolution rate can be influenced by particle size. The humidity of the filling room should be carefully controlled. If the room is too dry, the powder will become electrostatically charged and will not flow. If the humidity is too high, compaction will occur because of moisture in the powder.

For parenteral products, the powder is generally prepared under aseptic conditions by crystallization or spray-drying, which provides greater assurance of sterility within the material. In the crystallization technique, the drug is dissolved in an appropriate solvent and sterilized by 0.2 μm membrane. Next, under controlled conditions, another sterile solvent in which the drug is not soluble is added to the solution to induce crystallization of the drug. The sterile crystals are removed, washed and dried, then usually tested for particle size distribution, dissolution rate, and correct crystalline form prior to filling.

In order to obtain a uniform product from lot to lot, strict adherence to the procedures developed for a particular crystallization must be followed, including control of pH, rates of addition, solvent concentrations, purity, temperature, and mixing rates. Each crystallization procedure has to be designed to ensure sterility and minimize particulate contamination. Subtle changes, such as using absolute ethyl alcohol instead of 95% ethanol during the washing step of crystallization procedure, can destroy the crystalline structure if the material being crystallized is a hydrate structure.

If the drug powder is to be prepared by spray-drying, as shown in Figure 14, a sterile solution of the drug is prepared in a similar manner as for aseptic crystallization but instead of crystallizing the drug by adding another solvent, the sterile solution or a resultant slurry is sprayed through an atomizer with a fine orifice into a drying chamber, generally conical in shape.

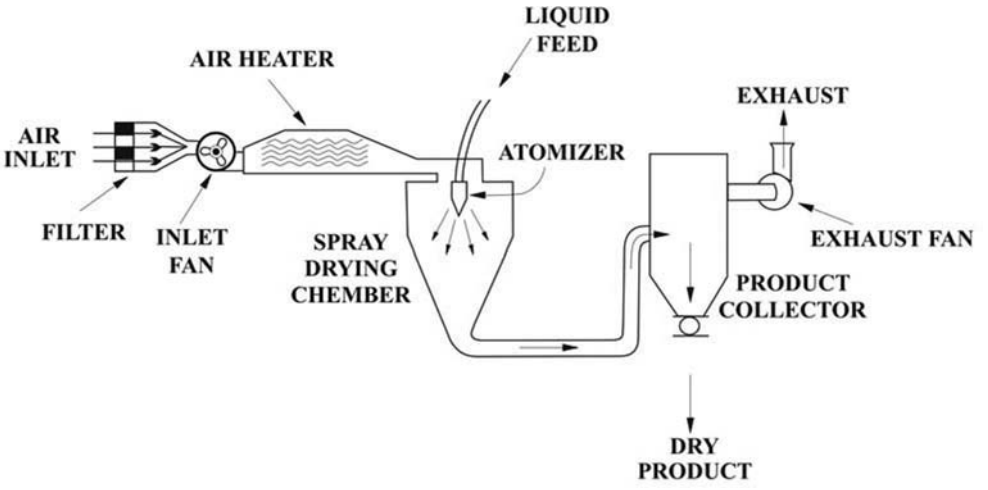


Figure 14 Schematic representation of spray-drying process.

Upon contact with a stream of hot sterile gas, the solvent rapidly evaporates and the resulting powder is collected in a sterile chamber. The type of atomizer and method of spraying, the concentration of the solution to be sprayed, the pressure at which it is atomized and the temperature and pressure of the gas in the chamber are factors that influence the particle size and porosity of the resultant powder. The drug powder, present as hollow spheres, is then filled into vials as a dry powder.

Freeze-drying or lyophilization. The freeze-drying or lyophilization of injectable products is described in sufficient detail elsewhere (chap. 17, volume 2), so only a brief discussion will be included here.

Freeze-drying, also known as lyophilization, is widely used for pharmaceuticals to improve the stability and long-term storage stability of labile drugs (125–127). Freeze-dried formulations not only have the advantage of better stability, but also provide easy handling (shipping and storage). There are currently more than 125 small molecule lyophilized injection products in the market and the number of lyophilized proteins and vaccines exceeds 50 (128). Most of these are formulated as lyophilized products because of their instability in aqueous solutions, however, as in the case of acyclovir sodium, lyophilization is necessary to minimize interaction of the alkaline formulation with glass material. Table 17 shows the examples of products of whose aqueous stability was only for few hours, but once they were converted into dry product by lyophilization, the resulting products had acceptable market shelf life.

Table 17 Comparison of Stability of Lyophilized and Solution Forms

Product	Bulking agent	Lyophilized product	Reconstituted product
Actreonam/Azactam	Arginine	3 yr	2 days (RT)
Amphotericin/Fungizone	–	2 yr	1 day (RT)
Cyclophosphamide/Cytoxan	Mannitol	3 yr	1 wk (13% loss)
Carboplatin/Paraplatin	Mannitol	2 yr	Particulates
Fosaprepitant/Emend	Lactose	>2 yr	24 hr at RT
Gemcitabine/Gemzar	Mannitol	>2 yr	24 hr at RT
Lansoprazole/Prevacid	Mannitol	>2 yr	1 hr at RT
Ixabepilone/Ixempra	None	>2 yr	1 hr at RT

Although there are those who would consider freeze-drying only as the last resort, there are others who view it as a panacea - a way to get into clinical trials quickly or a way to exclude contaminants and inert particles, especially in comparison with powder filling. Certainly, freeze-drying does offer the advantage over powder filling of accuracy of dosage, since the drug is filled into the final container as a solution. Microgram quantities can be filled precisely. The desired characteristics of a freeze-dried pharmaceutical dosage form include

1. an intact cake occupying the same shape and size as the original frozen mass,
2. sufficient strength to prevent cracking, powdering, or collapse,
3. uniform color and consistency,
4. sufficient dryness to maintain stability, and
5. sufficient porosity and surface area to permit rapid reconstitution.

Of course, as with any injectable dosage form, freedom from contamination (i.e., micro-organisms, pyrogens, and particulates) is an essential attribute. The desired characteristics can be achieved by proper formulation of the product and by employing optimum freeze-drying cycles.

A freeze-drying cycle essentially consists of three distinct phases: a) Freezing of the solution, b) primary drying or sublimation, and c) secondary drying. Loading of the filled vials in the chamber, maintenance of vacuum throughout the drying phases, supply of refrigeration during freezing and heat during the drying phases, and completion of the drying cycle by stoppering the dried vials and unloading them out of chamber are some other required actions. For a systematic approach to the development of a suitable freeze-dried product, knowledge of the various stages of the process is necessary. Comprehensive reviews of principles and practice of freeze-drying in pharmaceutical are widely reported in pharmaceutical literature (129–132).

The initial freezing process is of critical importance since it will influence the pattern of the sublimation phase. During freezing, pH change may arise from crystallization of buffer salts as well as large increase in ionic strength that may result into stability problems. The pH shift during freezing can be minimized by optimal choice of buffer salts or by reducing buffer concentrations. Upon freezing, the entire formulation must be in a completely frozen state otherwise collapse or meltback may happen during drying. The temperature above which the freeze-dried product loses macroscopic structure and collapses during freeze-drying is termed as collapse temperature or T_c and is usually about 2°C higher than T_g', which is often associated with the glass transition temperature in the frozen state (133). T_c equals the eutectic temperature (T_{eu}) if solutes are crystallized in the frozen solution. Well designed cooling cycle (ramp and hold times) must be used in order to obtain an appropriate structure of the frozen mass, which is a function of the rate of freezing and the final freezing temperature. The rate of freezing affects the size of ice crystals. Slower rate of freezing results in larger ice crystals and vice versa. If the frozen system exhibits metastable or amorphous-glassy structures, these structures may need to be ruptured by appropriate thermal treatment or annealing process (a succession of cooling and rewarming periods), thereby inducing crystallization of the amorphous material for efficient sublimation.

Most freeze-dried drug products are organic electrolytes which exhibit eutectic points or glass transition temperatures and super-cooling tendencies. Several methods have been used for determining eutectic temperatures: (*i*) thermal analysis, (*ii*) differential thermal analysis, and (*iii*) electric resistivity (131).

Knowledge of the eutectic temperature of the additive is essential since the addition of a salt such as sodium chloride to a drug with a eutectic significantly above that of sodium chloride would only succeed in lengthening the cycle because lower temperatures would have to be maintained. In addition, some additives, such as the phosphates, tend to form crusty-appearing cakes. This occurs during freezing and drying, probably because of the phenomenon of recrystallization. Volatile substances are generally considered to be of little value to the finished cake but can be used if they accelerate the drying cycle, for example, t-butanol (134,135). The next step in the freeze-drying process is primary or sublimation drying which is

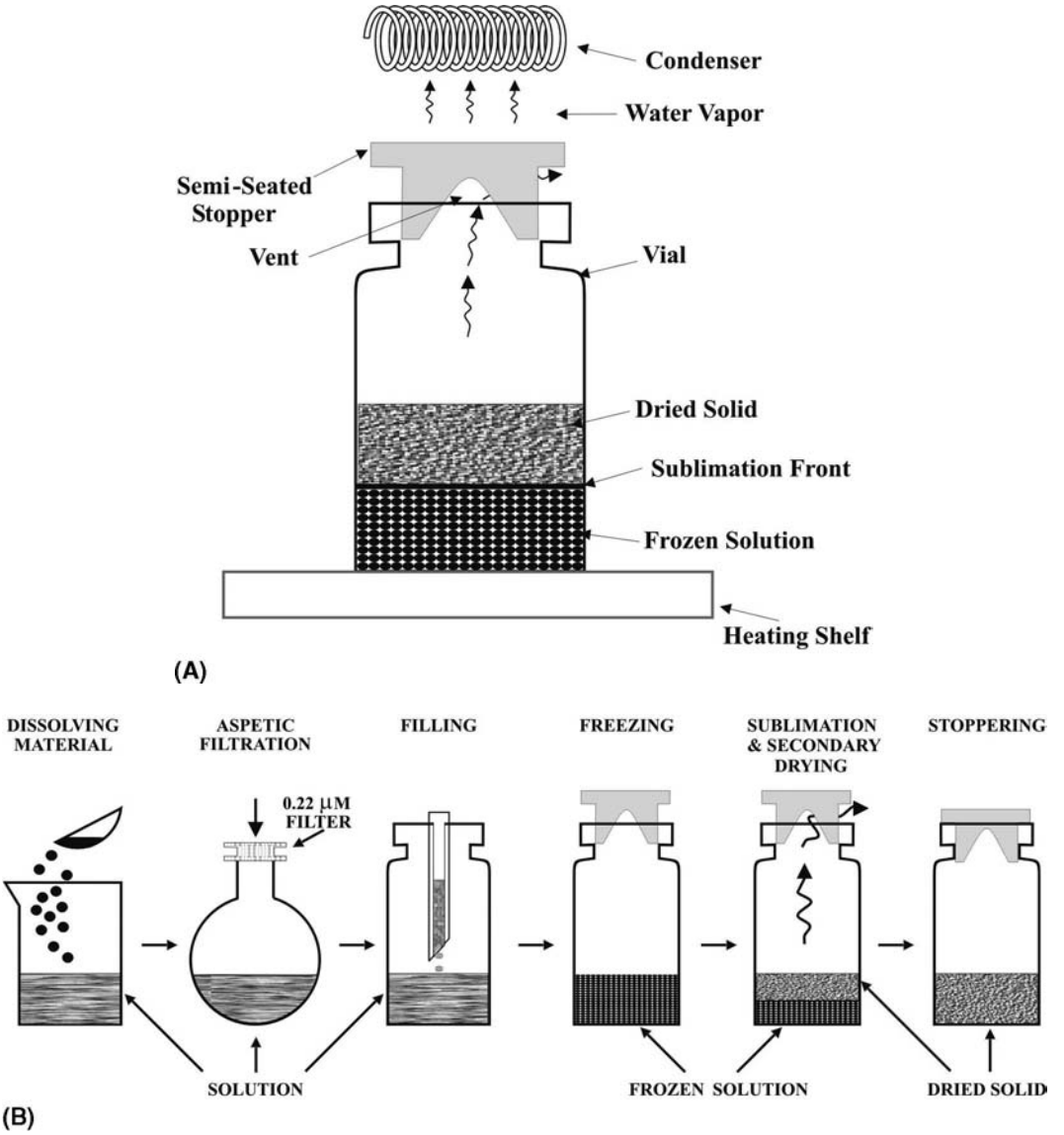


Figure 15 Schematic representation of freeze-drying process.

conducted under low chamber pressure conditions, for example, 200 mTorr or lower, under which sublimation of ice, as dictated by the ice/water-vapor equilibrium line of the phase diagram of water, takes place and the water vapor from the frozen matrix is transferred out of the vial, traveling into the headspace of the vial, through the vents of the closure, into the chamber, and eventually onto the cold condenser, where it is condensed again as ice (Figure 15A). Thus, frozen water from the vial is vaporized by sublimation and collected on the cold plates of condenser. The sublimation is a phase change, requiring energy, which must be supplied as heat from the carefully controlled heated shelf. The sublimation drying phase is a combined heat-mass transfer process in which both the transfer phenomena must be carefully balanced so that sustained drying rate (mass transfer) prevails without collapsing or melting of the frozen mass because of accumulation of heat from the heated shelf (heat transfer). During

the entire sublimation phase, the product temperature should always be several degrees below Tc in order to obtain a dry product with acceptable appearance. Factors influencing the rate of vaporization have been discussed extensively (136–139). The faster heat can he applied, the faster the drying proceeds, provided that, a) The temperature of the product remains below its liquefying point, and b) sufficiently low pressure is maintained in the system by efficient vacuum pumps. If a sufficiently low pressure is not maintained, the temperature of the product will rise resulting in the partial softening or puffing of the product.

In developing a formulation for freeze-drying, the optimal formula will permit the overall cycle to be carried out in the least amount of time, while providing a stable and efficacious product which contains a low moisture content, undergoes rapid reconstitution, and possesses the desired appearance. The potency of many lyophilized products is so high that relatively small amounts are required for the lyophilized injectable dosage form. Therefore, the need for suitable filler or bulking agent is often indicated. The percentage of solids in the frozen plug will vary depending on the dosage and nature of the active ingredient; generally, it should be above 5% and not exceed 30%, with a 10 to 15% content being optimum. Materials to choose from to add to the solution to improve the physical characteristics of the finished cake are limited but include mannitol, lactose, sucrose, dextran, amino acids, sorbitol, gelatin, mono- and dibasic sodium phosphate, albumin, sodium chloride, etc. It should be kept in mind when adding bulking agents that drying will be accelerated if the solute concentration is kept low. However, solutions with too low concentration ($\leq$ 1% w/w) may result in very brittle cake and there is a likelihood that some of the powder may fly off the cake into the chamber resulting in low drug content in the vial.

If degradation is a risk during freezing due to concentration effects or pH changes, stabilizers or buffers may have to be added. The problem of collapse has been discussed earlier (140) and if the substance is vulnerable to collapse, a rigidizer such as glycine or mannitol may need to be added. If damage during freezing is a problem, a cryo-protective agent such as sucrose or albumin may be added. If the ingredients that are added are found to adhere to the glass surface, such as albumin, then the containers with thin walls, such as ampuls and tubular vials, may need to be coated with silicone to minimize sticking. The depth of fill in a container is critical. While this depends on the volume of the container, a rule of thumb has been 1 to 2 cm in depth but never exceed one-half the capacity of the container otherwise breakage of vials may be seen.

Freeze-dried products are generally packaged in ampuls or vials. Ampuls would only be used for single-dose administration, and provide even drying because the tubing is thin and bottoms are reasonably flat. However, they must be sealed after removal from the chamber and reconstitution is sometimes cumbersome if shaking is required. Additionally, the generation of glass particles is a problem. Vials are used for both single- and multiple-dose application. If molded glass is used, there is greater incidence of variation of thickness and uneven bottoms. The containers must be sealed with a closure that can be accomplished inside the chamber, lessening the risk of contamination and providing an opportunity to seal under an inert gas or under vacuum.

The next stage in freeze-drying cycle is secondary drying. When sublimation drying phase is completed, the temperature of the product progressively rises (following the temperature of the shelves). The goal of desorption is to remove traces of moisture in the product (the majority of the water in the form of ice already been removed during the sublimation phase). The secondary drying process consists in removing the molecules by having the product under the highest possible shelf temperature (e.g., 20–35°C) compatible with its stability and the chamber pressure at its lowest value.

Typical process of freeze-drying is illustrated in Figure 15B. It involves: (1) dissolving the drug and excipients in a suitable solvent, generally water; (2) sterilizing the bulk solution by passing it through a bacteria-retentive filter; (3) filling into individual sterile containers with semi-stoppered closures; (4) freezing the solution by placing the open vials on cooled shelves in a freeze-drying chamber, (5) applying a vacuum to the chamber and heating the shelves in order to sublime the water from the frozen state, and (6) breaking the vacuum at

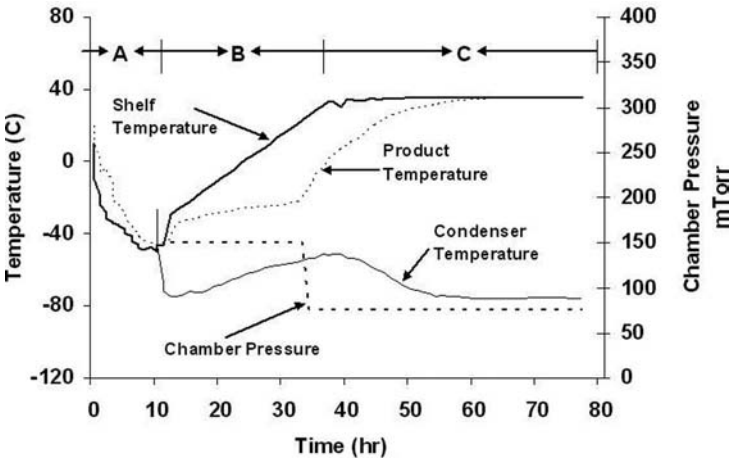


Figure 16 Typical product temperature/chamber pressure curve during freeze-drying.

the end of drying using sterile air or nitrogen, fully stoppering the containers, and unloading of the vials.

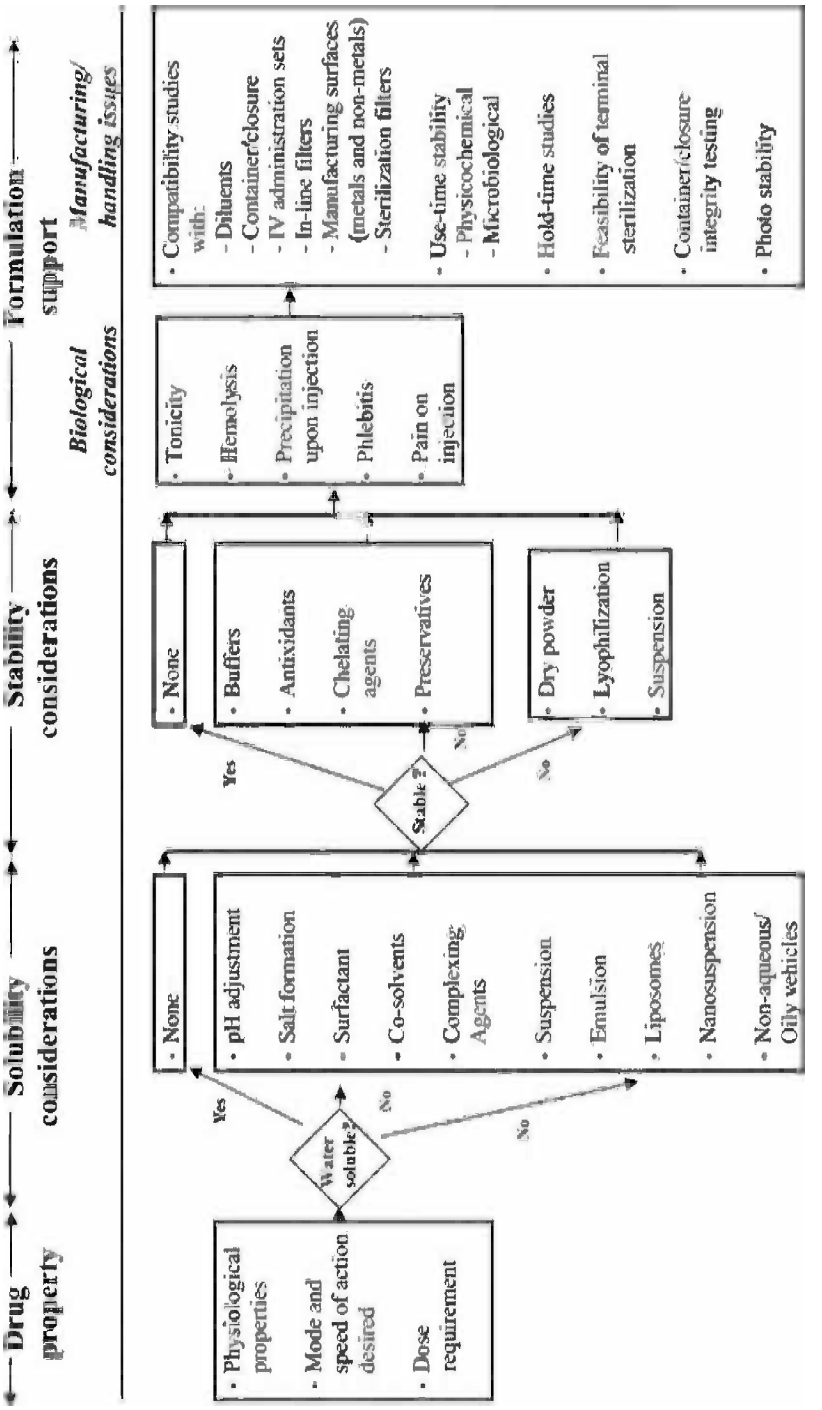
Temperature and pressure curves for a typical cycle are illustrated in Figure 16 for Mannitol solution (5% w/w) filled into 10 mL glass vial (5 mL/Vial). Freezing stage is denoted by "A," primary drying by "B," and secondary drying by "C." During freezing as the shelf temperature is lowered the product cools down and freezes and eventually reaches its target temperature of $<-40^{\circ}C$. At this time, the condensers are chilled to below very low temperature ($<-70^{\circ}C$) and the vacuum is initiated in the chamber. Once the vacuum has reached its target value, say 150 mTorr in this case, then the primary drying begins wherein the shelf is heated slowly to provide heat to sustain sublimation. Around 34 hours, at the end of phase "B, " the product temperature starts rising swiftly indicating that the ice is removed and the heat is consumed not just for the phase change in sublimation, but results in increase in the temperature of the product. The phase denoted by "C" is secondary drying where the continuously heated shelves provide heat to remove residual moisture by desorption process, aided by lower chamber pressure than before. At the end of secondary drying, the vials are fully stoppered, vacuum is broken to return the chamber to the atmosphere and the vials are unloaded.

Formulation Development Process

From preceding sections, it is clear that successful formulation of an injectable small-volume preparation requires a broad knowledge of physical, chemical, and biological principles as well as expertise in the application of these principles. Moreover, formulation is a highly specialized task requiring not only specific knowledge but also years of experience. During the course of development, formulation design and optimization is an iterative process and evolves as the product moves from the discovery to clinical to commercial stages. Although, most of the times, the development is an empirical approach based on principles mentioned earlier, there are number of strategies or decision trees that one can adopt to proceed with the product design. There are even published reports that the suggest use of "expert systems," comprising of databases and decision making processes, to aid parenteral development (141).

Table 18 summarizes one such approach that can be considered as a template for parenteral formulation development process which considers many of the essential factors necessary for the formulation design and lists various formulation-supporting studies that are needed from patient use, manufacturing, and marketability point of view. These

Table 18 Suggested Template for Small-Volume Injectable Formulation Development Process



studies are not mutually independent, though. Not only the formulator must arrive at an optimum formula from stability/solubility point of view alone, but he/she must ensure that the product is acceptable from patient's acceptability/tolerance point of view and it poses minimal difficulty or constraints from the manufacturing and/or marketing point of view.

Formulation-supporting studies. In finalizing the formulation, a number of supporting studies are needed to address the biological or patient-related issues, support the manufacturing process, and define the boundaries under which the product's qualities will be maintained throughout the shelf life of the product.

1. Biological considerations:
   a. Evaluation of impact of formulation toward hemolysis, precipitation, phlebitis, and pain on injection
   b. Tonicity
2. Manufacturing and handling support studies:
   a. Compatibility with commonly used diluents and IV administration sets, etc.
   b. Compatibility with manufacturing equipment
   c. Compatibility with membrane filters, if aseptic processing is used during the manufacture
   d. "In-use" stability studies
   e. Feasibility of terminal sterilization
   f. Photostability

Biological considerations.

Hemolysis, precipitation, phlebitis, and pain on injection Some injection products are prone to formulation-related problems such as hemolysis of the RBCs; precipitation of the drug and ensuing phlebitis; and pain at the site of injection.

Hemolysis results from disintegration of RBC membrane and release of the cellular contents into the plasma, particularly that of hemoglobin. Once outside of the RBC, hemoglobin molecule quickly dissociates into its component polypeptide chains which can pose many serious physiological problems, mainly the renal failure. Hemolysis usually results from hypotonicity or from the effect of drug or the formulation components on cell membranes (142,143).

Precipitation of the drug at the site of administration can happen once the solubilizing principles are diluted away or removed from the vicinity of the drug.

Phlebitis occurs because of inflammation of a vein with symptoms such as tenderness, edema, erythema, and a local temperature rise. In severe cases, it can lead to thrombus and even more severe complications. Although a number of factors have been implicated as causes of phlebitis; particulate matter, precipitation of drug, and local pH effects are the most likely causes (144–148).

Injectable formulations are often painful and irritating following injection as a result of cell damage such as phlebitis. Sometimes the pain/irritation response is associated with the active drug (s) present in the formulation, for example, macrolide antibiotic (149) and excipients (150). Pain on injection may occur during and immediately following the injection or it may be a delayed or prolonged type of pain which increases in severity after subsequent injections. The actual cause of the pain is often unknown and will vary significantly among patients according to the product. In some cases pain may be reduced by minor formulation changes such as adjusting tonicity and pH or adding an anesthetic agent such as benzyl alcohol or lidocaine hydrochloride. In other cases pain is more inherent to the drug and the problem is more difficult or impossible to resolve. Pain, soreness, and tissue inflammation are often encountered in parenteral suspensions, especially those containing a high amount of solids. A number of in vivo (animal studies) and in vitro studies to evaluate hemolysis, precipitation, phlebitis and pain upon injection have been published (151–154). It is important that the

formulator evaluate the potential of the formulation to causes of the above mentioned problems using these or other suitable techniques.

Tonicity Tonicity has been previously discussed under "Added Substances."

Manufacturing and handling support studies.

Compatibility with commonly used diluents and IV administration sets Many IV parenteral products are often administered via large-volume parenteral (LVP) solutions. In such cases, the solubilized portion of the product, either withdrawn directly from the ready-to-use solution or from the reconstituted dry product, is directly added to the diluent bag or added through the Y-site of the IV administration set. Obviously, the potential for drug stability and compatibility problem is great because of the long duration of contact time and exposure to ambient conditions of temperature and light (155). The potential physical and chemical incompatibilities associated with such dilutions are compiled in a treatise by Trissel (74) and is often the primary reference book on this subject in the practice of pharmacy.

Typically, compatibility of the drug product with the reconstitution diluents (precipitation and stability), at the recommended storage temperature and at the likely extreme concentrations of administration, is demonstrated with most commonly used diluents and IV fluids, such as normal saline, dextrose solutions, ringer's solution, etc., and combinations thereof (156). It is also important that compatibility information is generated for the drug in contact with potential delivery devices such as the IV administration sets, in-line filters, syringes, etc.

Compatibility studies with manufacturing equipment contact surfaces Various contact surfaces are encountered during the manufacture and storage of injection products. Compatibility studies of the drug product with such surfaces must be evaluated to ensure that there are no adverse interactions and the quality of the product is unaffected. Typical product contact surfaces during the manufacture are transfer tubing, manufacturing equipment, filtration surfaces and devices, filling machine parts (pumps, filling needles) surfaces, etc. These are comprised of variety of materials such as rubber, plastic, ceramics, and metals. Typically, the component under investigation is placed in contact with the drug product solution for 24 to 96 hours at room temperature, at which point the samples are analyzed for various physicochemical attributes such as pH, appearance, UV/FT-IR spectroscopy, and potency.

Compatibility with packaging components During the storage of the product in the final container, the product comes in contact with the rubber-based or polymeric stoppers, glass in the case of vials, or other plastic materials in the case of syringes and plastic bags. Compatibility studies of the drug product with such packaging components is performed similarly by contacting the packaging components with the drug product and analyzing for physicochemical attributes of both the solution and the components.

Compatibility with membrane filters Bulk solutions of many aseptically produced injection products are sterilized by membrane filtration using 0.22 μm filters. It is important that the compatibility of the drug product with that of material of the sterilization membrane filter (and prefilter, if used) as well as the filter assembly is evaluated to ensure that the product quality is not affected as well as no undesired components are added to the drug product. Some of the techniques used in practice for this purpose include the following:

1. Microbial membrane retention testing to demonstrate that the formulation of the product does not adversely affect the effectiveness of removal of any microbial contamination from the bulk solution. This is typically done by filtering a challenge solution containing large number of bacteria in the drug product solution (or its equivalent placebo) and testing for the filtrate for any microbial presence.
2. Membrane compatibility study to ensure that the prolonged exposure of the product does not affect the key membrane characteristics. This is typically done by soaking the membrane disks in the drug bulk solution for 24 to 48 hours and then evaluating

the filters for key parameters such as water permeability (flow rate), product bubble point, weight change, and appearance.
3. Filter extractability testing to assess the effect of formulation on the extractables from the filter. This is typically performed by subjecting the filter device to worst-case sterilization conditions (time, temperature, and repeated cycles) followed by extended exposure to organic solvents such as 100% denatured ethanol and then analyzing the extract for volatile and nonvolatile organic compounds.
4. Product specific bubble point measurement as a tool to monitor the integrity of the filter during routine manufacturing.

"In-use" stability Use-time stability studies are performed to establish the following:

1. How long the drug product solution is stable at ambient (use) conditions, if normally the drug product is supplied in dried form.
2. How long the drug product is stable at ambient (use) conditions, if normally kept at refrigerated storage.
3. In what diluent and how long the diluted drug solution is stable, from both physicochemical and microbiological perspectives.

The above information is then included in the package insert that is provided with the final drug product and forms the basis for the proper use of the drug and instructions for suitable use of diluents and delivery devices.

Feasibility of terminal sterilization Injection products are rendered microbiologically sterile by terminal sterilization by using steam or dry heat. Steam sterilization, which offers the greatest assurance of sterility, can be expected to cause some changes in the product, however subtle. Drugs are reactive substances and autoclave temperature (121°C) for 15 to 30 minutes could give rise to degradation processes and interactions with the container. Additionally, materials could leach form the rubber closure. In addition to loss of drug, antimicrobial agents and antioxidants can be absorbed or consumed during sterilization. Lately, it is becoming a well-accepted principle that sterile drugs should be manufactured by aseptic processing only when terminal sterilization is not feasible because of excessive thermal degradation of the product. There are many categories of the products that may qualify for not subjecting to terminal sterilization (157); however, regulatory agencies may require a written justification to address why a product is not being terminally sterilized. With such restrictions, the formulator of an injection product must assess the effect of terminal sterilization conditions on the stability of the product, the acceptable level of degradants, and offer alternate sterilization techniques such as aseptic processing or adjunct processing step(s) in addition to aseptic processing, for example, addition of heat exposure condition which may provide increased level of sterility confidence (158).

Photostability Exposure to irradiation such as light can influence the stability of the formulation, leading to changes in the physicochemical properties of some products. The most obvious result of drug photodecomposition is a loss of potency of the product. In few cases, trace amounts of photodecomposition products formed during storage and administration may lead to adverse effects (159). The excipients used may also often contribute to the photoreaction (160–163) and hence stability evaluation in the presence of excipients is important. The selection of a protective packaging must be based on knowledge about the wavelength causing the instability. A review by Tonnesen (164) has focused on practical problems related to formulation and stability testing of photolabile drugs. An ICH guideline, "Guidelines for the photostability testing of new drug substances and products," describes photostability methodology, including the decision flow-cart, choice of light source, sample preparations, and interpretation of results (165).

In the case of injection products, transparent glass or plastic vial offers little protection toward radiation (166). The stabilizing effect of amber glass as the only means of

photoprotection is not satisfactory for highly photolabile drugs like molsidomine (167). Even brown glass can offer inadequate protection as demonstrated for drugs like epinephrine, isoprenaline and levarterenol (168). In practice, a secondary container, such as a cardboard box or carton is often necessary to prevent photodegradation. Similarly, for extremely high light-sensitive drugs, the manufacturing operations (compounding, filling, and packaging) may also need to be carried out by minimizing light exposure or by using yellow lights in the process areas.

At the conclusion of the formulation development process, the formulator must be in a position to compile all the knowledge generated in the process for regulatory scrutiny. International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) has published guidance for industry, "Q8 Pharmaceutical Development," which provides necessary elements of the development process that includes the concepts of quality by design (QbD), use of quality risk management, and use of knowledge management (156). This guidance recommends summarizing the development of the formulation including identification of those attributes that are critical to the quality of the drug product. As per the guidance, the summary should highlight the evolution of the formulation design from initial concept up to the final design. This summary should also take into consideration the choice of drug product components (e.g., the properties of the drug substance, excipients, container closure system, any relevant dosing device), the manufacturing process, and, if appropriate, knowledge gained from the development of similar drug product(s). The guideline further describes the use of principles of quality by design (QbD) during the development of a drug product. The QbD identifies characteristics that are critical to quality from the perspective of the patients, translates them into attributes that the drug product should possess, and establishes how the critical process parameters can be varied to consistently produce a drug product with the desired characteristics. Reader is encouraged to study and practice the quality principles laid down by this guidance.

Container Effects on Formulation

Containers for parenteral products serve several purposes; facilitate manufacturing; maintain product protection including sterility and freedom from pyrogen; allow inspection of the contents; permit shipping and storage; and provide convenient clinical use. The container components must be considered as integral parts of the product because they can dramatically affect product stability, potency, toxicity, and safety, and therefore must be evaluated carefully with a variety of tests. For details on this topic, reader is directed to chapters 11 and 12 of this book.

Stability Evaluation

Throughout the world, there has been phenomenal increase in laws, regulations and guidelines for reporting and evaluating data on safety, quality and efficacy of new medicinal products. Although different regulatory systems have the same fundamental concepts to evaluate the quality, safety and efficacy, the process of evaluation has diverged over time to such an extent that the industry has found it necessary to duplicate many time-consuming and expensive test procedures, in order to market new products, internationally. To address this, initiation of ICH was pioneered by the European Community, in the 1980s, and later joined by the representatives of the regulatory agencies and industry associations of the United States and Japan. The key goals of the ICH have been the development of the "ICH process" for developing harmonized guidance on technical issues and under this process many guidance have been published. For details on the topic of stability studies regarding the stability procedures, sampling requirements, storage conditions, testing schedules, and evaluation of data, reader is directed to chapter 10, volume 3 of this book.

Process Effects

The processing of parenteral products has been covered elsewhere in this textbook, but some specific cautions associated with the effects on formulation will be highlighted. There comes a

point in the development process of a product to characterize the production process and assess its effect on the formulation. This requires scale-up procedures to identify the process and equipment variables and with knowledge of the formulation and package variables assess how product quality and manufacturing productivity will be affected. In the manufacture of a sterile product, the assurance that the finished product possesses the desired quality control characteristics depends on a number of independent but interrelated events commencing with the initial design of the dosage form and carrying forth through the process design and validation and culminating with the establishment of standard procedures for manufacturing.

To provide for the assurance that all quality attributes will be achieved on a repetitive basis, the following are essential: (1) the dosage form is designed with knowledge of the desired functional and quality control characteristics of the finished product; (2) the qualification procedures are adequate to ensure reliability of the equipment, effectiveness of the process, and the integrity of the processing environment; (3) personnel are trained in contamination control techniques; and (4) there is adequate documentation of all procedures and tests. Such a development sequence combined with validation requirements suggests a formalized program culminating in a product that can be reliably processed. The process characterization is a principal step in assuring that the process can be translated to manufacturing on a routine production basis. Although this chapter is not intended to cover processing in the broad sense, those responsible for developing formulations should have an understanding of the following:

1. Scale-up procedures
2. Preliminary technical documentation
3. Design of processing and validation protocols
4. Use of process analytical technologies (PAT) for monitoring and control purposes
5. Qualification/validation runs
6. Final technical documentation and authorizations

The overall approach must be organized, scientific, and thorough. Moreover, the issues in shipment of the product, especially if refrigeration or some other storage temperature restrictions apply must be addressed. Lastly, addressing the usual unplanned deviations in the manufacturing processes and the provision of rework or rescue procedures must also be considered.

FORMULATION OF LARGE-VOLUME INJECTIONS

Introduction

LVPs or injections are primarily used for IV nutritional therapy which is required when normal enteral feeding is not possible or is inadequate for nutritional requirements. Specific nutritional requirements and administration mode depends on the nutritional status of the patient and the duration of the parenteral therapy (45). To meet IV nutritional requirements, one or more of the following nutrients may be required:

- Protein substrates: These include various amino acids formulation used for general replacement purpose, for hepatic failure, for encephalopathy, and for metabolic stress conditions.
- Energy substrates: These include dextrose and IV fat emulsion.
- Electrolytes: Saline, ringer's solution, etc.
- Vitamins and trace metal supplements.

Besides providing the water, electrolytes, and simple carbohydrates needed by the body, LVPs also a) act as the vehicle for infusion of drugs that are compatible in the solution, b) provide solutions to correct acid-base balance in the body, c) act as plasma expanders, d) promote diuresis when the body is retaining fluids, d) act as dialyzing agents in patients

with impaired kidney function, and e) act as x-ray contrast agents to improve diagnostic abilities. It is now almost a standard practice to begin infusing a patient with a LVP, often dextrose and electrolytes, shortly after admission to the hospital. One of the reasons for this is to provide a readily accessible link to the central compartment if additional medications are required, while at the same time providing fluids and electrolytes to achieve an optimum balance for further treatment. IV administration, however, bypasses protective mechanisms of the body, and the onset of adverse reactions, including the cases of nosocomial bacteremias in hospitals (169), which may come about from many causes, can be as rapid as the beneficial effects. The National Intravenous Therapy Association (NITA) as well as many technical books have developed recommendations for procedures to be followed during IV therapy (170,171). The procedures are designed to minimize undesired reactions.

Formulation Principles

Physiological Parameters

The physiological parameters of a LVP formulation are limits on those characteristics of the solution that impart some effect on the biochemistry of the body.

Some constituents that are basic to the sustenance of life in the human organism can be influenced by IV therapy. These are water, electrolytes, carbohydrates, amino acids, lipids, and micronutrients such as vitamins, minerals, and trace elements.

The living cell, the body's basic unit, is bathed in tissue fluid kept constant in composition by the interaction of many processes, some of which are outside the scope of this chapter. Alteration in the amount or composition of tissue fluids can cause significant physiological derangements. Such imbalances may occur as a major or minor feature of illness, trauma, or surgical procedures. Under such circumstances it is necessary to anticipate and correct deficits and imbalances by administration of suitable fluids. The body fluids, named for the compartments in which they are found, are intravascular (within the blood vessels), intracellular (within the cells), and interstitial (within the space between cells). Extracellular fluid is the total of intravascular and interstitial fluids. The fluids consist of water containing a mix of electrolytes, neutral solutes in a wide range of high and low molecular weights, and undissolved substances. The composition of each fluid differs, yet a chemical balance is maintained in each fluid. Approximate figures for the electrolytic composition of body fluids are shown in Table 19.

Table 19 Electrolyte Composition of Body Fluid Compartments

	Intravascular	Interstitial	Intercellular
Electrolytes	(mEq/L)	(mEq/L)	(mEq/L)
Cations			
Sodium (Na^+)	142	145	10
Potassium (K^+)	4	4	160
Calcium (Ca^{2+})	5	5	2
Magnesium (Mg^{2+})	2	2	26
Total	154	156	198
Anions			
Chloride (Cl^-)	102	115	2
Bicarbonate (HCO_3^-)	27	30	8
Phosphate (HPO_4^{2-})	2	2	120
Sulfate (SO_4^{2-})	1	1	20
Organic acids	6	7	–
Protein	16	1	48
Total	154	156	198

Extracellular fluid is characterized by high concentrations of sodium and chloride ions. The intravascular fluid contains a much higher concentration of protein than is found in interstitial fluid because the large plasma protein molecules are not diffusible. The retention of protein anions on one side of, the semi-permeable membrane causes a redistribution of the anions that are permeable, in order to maintain chemical balance (172). As a result, the concentration of other anions is lower in intravascular fluid than in interstitial. Intracellular fluid is characterized by very high concentrations of potassium, phosphate, and protein.

An LVP formulation must be developed to ensure that desired levels of the solution are administered in a therapeutically active and available form. In order to obtain the desired response, the physiological intent of the formulation must be considered and the physiological, chemical, and physical properties of the formulation defined. The formulator must understand the biochemistry of the body and the chemistry of the in vivo parenteral because it is through their interaction that the result is achieved. These factors are discussed in the sections to follow.

Formulation Parameters

Physiological. Body fluids rapidly exchange both water and electrolytes between the cells and extracellular compartments, maintaining equilibrium within and between the compartments. The movement of solvent and solute through the semi-permeable membranes that separate the compartments is called osmosis. If the concentration of solutes in adjoining compartments differs, water moves very rapidly into the compartment with the higher concentration in the effort to establish equilibrium. Simultaneously, disassociated solutes diffuse at a slower rate to the compartment with the lower concentration. Because some components of the fluid cannot move through the semi-permeable membrane, the fluid in the compartment must make adjustments to maintain its own ionic equilibrium (mentioned previously with respect to the difference in the ions contained in extracellular and interstitial fluids).

The resistance to unrestricted movement between compartments is defined as osmotic pressure and is expressed as osmoles per kilogram (osm/kg) or, more conveniently, milliosmoles per kilogram (mOsm/kg). Osmolarity values of dilute solutions can be calculated and their levels expressed as milliosmoles per liter (mOsm/L) by using the formula:

$$\text{mOsmol/L} = \frac{\text{g/L of solute}}{\text{molecular weight of solute}} \times 1000 \times \text{number of ions}$$

Sodium chloride, for example, has a molecular weight of 58.5 and forms two ions, Na^+ and Cl^-, in solution. The osmolarity of 0.9% sodium chloride injection would be calculated as $\text{mOsm/L} = 9/58.5 \times 1000 \times 2 = 307.7$, rounded to 308.

An immediate concern of introducing large amounts of fluid into the body system is that of maintaining the "tone" of the living body cells, RBCs circulate in blood, which has an osmolarity of 306. Using osmolarity as a measure of tonicity, one would expect no physical change in the RBC if 0.9% sodium chloride injection, with an osmolarity of 308, were infused into the vein. This is the case, as can be demonstrated by putting red cells into the 0.9% Sodium Chloride Injection and microscopically examining the cells for physical change. No changes result, and the solution is termed isotonic. If RBC are placed in a hypertonic solution, for example, 20% dextrose (1010 mOsm/L), the water in the cell will diffuse out, causing the cell to shrivel. Conversely, RBC placed in a hypotonic solution, such as 0.45% sodium chloride (154 mOsm/L), will swell because of the flow of water into the cell and, if the effect is great enough, may rupture. For this reason, WFI, USP, which has no dissolved solids, despite its name is never injected alone. Table 20 shows the relationship between osmolarity and tonicity.

Tonicity, as defined by numerical calculation, is only one consideration that must be taken into account and it must be used with judgment. For example, a solution of 1.85% urea is isotonic but quite unsuitable for administration at the rate isotonic solutions are normally infused; it can cause hemolysis as well as upset the body's nitrogen balance. A solution of amino acids, which is hypertonic at about 850 mOsm/L, may be life sustaining and the

Table 20 Relationship Between Osmolarity and Tonicity

Osmolarity (mOsm/L)	Tonicity
>350	Hypertonic
329–350	Slightly hypertonic
270–328	Isotonic
250–269	Slightly hypotonic
0–249	Hypotonic

problems of tonicity can be overcome if it is introduced slowly into a large vein where there is ample blood volume to assure dilution. Hypertonic and hypotonic solutions can be used if administered slowly. The rates of shift of water into or out of the vascular system are determined by the rate of administration, rate of diffusion of the solute, and tonicity of the solution. Calculation of tonicity has been described in the earlier section.

Physicochemical

Solubility Compared with the solubility challenges in compounds used in small-volume parenteral as described earlier, most of the solutes used in LVP solutions are extremely soluble relative to their therapeutic concentrations. This means that solubility is rarely a consideration during formulation and, once in solution, the ingredients remain dissolved under normal storage and handling conditions. There are occasional reports of crystallization in highly concentrated solutions, such as 15% mannitol; this is caused by a reduction in solubility when the bottle is cold and the crystals go back into solution readily when the bottle is warmed. The solubility of mannitol is 13 g/100 mL water at 14°C; the package inserts for mannitol solutions caution the user that concentrations over 15% may show a tendency to crystallize.

In some cases, as with amino acid or high-concentration dextrose solutions, the temperature of the WFI is elevated during mixing. Although the ingredients are soluble at lower temperatures, minimizing the preparation time reduces the time the solution is exposed to ambient microorganisms. The order in which ingredients are added to the mix tank may have an effect on how rapidly the mix is completed or whether it can be completed. For example, when one is preparing amino acid solutions the pH changes after the addition of each amino acid and some amino acids are soluble only at specific, narrow pH ranges. Consequently, the order of adding the various amino acids can be critical unless preblended powdered amino acids are used. In general, solubility only becomes a consideration when the LVP is used as a carrier for other drugs.

pH The pH of a formulation must be considered from the following standpoints: the effect on the body when the solution is administered; the effect on stability of the product; the effect on the container closure system, and the possible degradation of drugs that are added. The pH of blood is normally between 7.35 and 7.45, and the immediate effect of intravenously introducing fluids outside this range depends on the buffer capacity of the solution, determined by the amount of weak acids or bases that are part of the formulation. The solution is rapidly diluted in the bloodstream, and the body's buffering system can maintain the proper pH level when high or low pH LVPs are administered, although it does so less easily if the solutions are highly buffered.

Because of its lower cost, type II glass, a flint glass with a surface treatment, is used for many LVPs that are packaged in glass. Solutions with pH values approaching or over 7.0 accelerate glass attack and must be packaged in the more expensive type I borosilicate glass. Since this glass is resistant to attack by alkaline solutions, it is used to prevent the pH from rising even higher. Other problems associated with degradation of the glass surface, such as the formation of glass flakes in the product, can be avoided by the use of type I glass. Chapter 11 provides a thorough discussion of glass containers that are used for packaging parenterals.

Vehicles WFI is the vehicle used for all LVPs. All ingredients are dissolved, and the resulting aqueous solution is clear and generally colorless. The IV fat emulsion, an LVP that may be administered alone or in combination with amino acid and dextrose solutions for total parenteral nutrition (TPN) therapy, is the exception. Triglycerides, egg phospholipids, glycerin, and WFI are homogenized to produce a stable emulsion with fat particles approximately 0.3 μm in size.

Physical Parameters

The sensitivity of a solution when exposed to light and extremes in temperature must be evaluated during the development of a formulation. Certain vitamin solutions require protection from light, for example, in the form of an amber bottle or an opaque unit carton. A light protective cover must be put over containers of solutions to which photodegradable drugs have been added. Solutions with high concentrations of dextrose or combinations with dextrose that have a tendency to develop slight discoloration with age will do so more rapidly if stored at high temperatures. The physical parameters that are defined for a solution are stated on the labeling and packaging inserts.

Packaging Parameters

The chapters on containers and closures in this textbook provide detailed information about the characteristics of materials available for packaging parenteral medications.

Stabilization of LVPs

Added substances. Buffering agents, chelating agents, antimicrobial preservatives, and antioxidants, commonly added to parenteral medications, are rarely used in LVPs. Buffering agents generally are not added as such, although acids and bases, which are used to adjust pH, can raise or lower the buffering capacity of the solution. By their nature and use, LVPs introduce large amounts of fluid and chemicals into the body. The active ingredients are present for a therapeutic effect, and although present in only very low percentages, added substances might, in total, have an effect on the patient who receives many bottles of solution during the course of treatment.

Very minute quantities of metals such as iron, copper, or calcium may be introduced into LVPs because of ingredients used and hence the quality of the incoming raw materials must be ensured. When drugs are administered orally, the gastrointestinal tract prevents aluminum from being absorbed into patient tissues; however, when the drugs are administered parenterally the aluminum can be deposited in tissues, potentially at toxic amounts. Therefore, according to the latest FDA guideline, the aluminum content of LVP drug products used in TPN therapy must not exceed 25 μg/L (173).

Antioxidants such as sodium bisulfite or sodium metabisulfite are part of some LVP formulations. They are added to protect the active ingredients from the action of oxygen in the solution or headspace of the container. The presence of oxygen, even very small amounts, can accelerate color formation or degradation of such products as 5% Dextrose in lactated ringer's or amino acid solutions. In lieu of the addition of an antioxidant, which might be added in concentrations of up to 0.1%, processing to displace the oxygen with an inert gas, usually nitrogen, may be done during mixing and filling operations. If both nitrogen and an antioxidant are used, the use of nitrogen will reduce the amount of bisulfite needed to protect the product during its shelf life.

Electrolytes, Carbohydrates, and Nutritionals

Typical examples of LVP formulations are shown in Tables 21 to 23. They are only a few of the many formula variations that represent the basic theme of each grouping.

Electrolyte solutions. The multiple electrolyte injection is an example of a solution that must be packaged in type I glass or plastic because its high pH, 7.3, can chemically attack type II

Table 21 Typical Examples of Electrolyte Solutions

Electrolyte	Plasma-Lyte R®	Isolyte S pH 7.4®	Normosol R®
Na^+	140	141	140
K^+	10	5	5
Ca^{++}	5	–	–
Mg^{++}	3	3	3
Cl^-	103	98	98
Lactate	8	–	–
Acetate	47	27	27
Gluconate	–	23	23
Phosphate	–	1	–
Osmolarity (mOsm/L)	312	295	294
pH	5.5	7.4	6.6

Source: From Ref. 45.

Table 22 Examples of Carbohydrate Solutions

Dextrose concentration		Caloric content	Osmolarity
%	g/L	(cal/L)	(mOsm/L)
2.5	25	85	126
5	50	170	253
10	100	340	505
20	200	680	1010
25	250	850	1330
30	300	1020	1515
40	400	1360	2020
50	500	1700	2525
60	600	2040	3030
70	700	2380	3535

Source: From Ref. 45.

Table 23 Examples of Nutritional Solutions

Solution	Amino acids with electrolytes	Intralipid® 10%
mOsm/L	357–1300	260
cal/L	–	1100
Total nitrogen (g/100 mL)	0.55–2.3	–
Formulation	May contain up to 8 essential	10% soybean oil
Electrolytes	and 11 nonessential amino acids and electrolytes (Na^+, K^+, Cl^- acetate, and phosphate)	1.2% egg yolk phospsolipids 2.25% glycerin –
Antioxidant	May be present	No
Buffering capacity	Moderate	Low
Light Protection	Yes	Yes
Container	Glass, plastic	Glass, plastic

Source: From Ref. 45.

glass surfaces. Each 100 mL of lactated ringer's injection contains 0.60 g sodium chloride, 0.03 g potassium chloride, 0.02 g calcium chloride, and 0.31 g sodium lactate (anhydrous) in WFI. The lactate ion in this solution is metabolized in the liver to glycogen, which becomes carbon dioxide and water, requiring the consumption of hydrogen ions; the result is an alkalinizing

effect. Again, the addition of dextrose, 5.0 g/100 mL, is for the caloric value and results in lower pH and higher osmolarity.

Electrolyte solutions make it possible to maintain or, in the case of specific clinical disorders, bring about the balanced levels of water and electrolytes required for proper body functioning.

Carbohydrate solutions. A standard solution that provides a source of water for hydration and carbohydrate calories contains Dextrose as a energy substance (Table 22). The dextrose is metabolized rapidly, and the water moves into other body compartments. If it is necessary to replace large losses of body water the injection can be administered, the patient's condition permitting, at a rate as high as 8–10 mL/min. Higher concentrations of the dextrose injection provide more calories without overloading the body with water.

Nutritional solutions. For proper nutrition an individual must have an intake of carbohydrates, amino acids, and fatty acids, along with trace minerals and vitamins. Carbohydrate and amino acid solutions have been available as injections for a number of years and can supply part of the patient's nutritional needs. Problems of toxicity, stability of the emulsion, particle size, and formation of free fatty acids had to be overcome before fat emulsions became viable products. Successful commercial production of fat emulsions that could be administered intravenously made it possible to provide the additional calories and essential fatty acids needed to implement TPN for the patient unable to take food enterally.

Fat emulsions typically contain a metabolizable vegetable oil, emulsifying agent, tonicity agent, and WFI. Table 23 shows a formula of fat emulsion in which each 100 mL contains 10 g soybean oil, 1.2 g egg yolk phospholipids as an emulsifying agent, 2.25 g glycerin as tonicity agent, and WFI. Sodium hydroxide is used to adjust the pH to approximately 8.0. In the soybean oil, the major fatty acids are linoleic (50%) and oleic (26%), with palmitic, linolenic, stearic, myristic, arachidic, and behenic acids making up the remainder. Size of the fat particles is controlled to about 0.3 μm. The emulsion is opaque, so the visible signs of incompatibility with additives might be concealed, although breaking of the emulsion results in visible free oil floating on the surface.

Complete amino acid solutions which contain L-amino acids provide the eight essential and as many as ten nonessential amino acids. Studies of blood serum levels of amino acids in normal individuals have established the ranges of each that are present and provide the basis for formulation. Each manufacturer of these solutions has particular combinations of amino acids that have been shown to be effective. There are over 70 amino acid injection formulations now being marketed including specialized amino acid injections (e.g., Aminess®, Aminosyn RF®, HepatAmine®, NephrAmine®, RenAmin®) for patients (e.g., those with renal or hepatic disease) who may have specialized requirements for amino acids or who may not tolerate amino acids contained in conventional solutions (45).

An essential amino acid cannot be converted to another amino acid and must be used by the body to fill a need for that particular one or be converted into uric acid. A nonessential acid may be used if needed, metabolized to another nonessential acid that is needed or converted to uric acid. When amino acids are administered parenterally, adequate calories must be provided concurrently to bring about synthesis of proteins; high-concentration dextrose injection or fat emulsion provides the source of calories. Concentrations of amino acid solutions vary from 3.5% to 15% depending on the indication for use. With some amino acids, however, there are limitations on the amount that will go into solution because the presence of other amino acids has an effect on solubility; the formulation of amino acid solutions is difficult because of this interaction and changing behavior.

Parenteral Nutrition

It has been estimated that approximately 40–55% of hospitalized patients are malnourished to some degree (174). Nutritional assessment and introduction of parenteral nutrition therapy based on the particular needs of the patient can reverse the nutritional status, minimize the harmful effects of poor nutrition, and accelerate the healing process.

Standard IV therapy usually provides dextrose, water, and electrolytes. Dextrose solutions are available in concentrations of 2.5% to 70%; a 5% solution supplies 170 cal/L and has an osmolality of 280 mOsm/L. These solutions are nutritionally incomplete, cannot supply enough calories without overhydrating the patient, and are suitable only for a few days as a source of nutrition. Electrolytes and vitamins may be added to correct imbalances and ensure normal body functions, including utilization of nutrients.

Amino acid therapy prevents nitrogen loss, is used for treatment of negative nitrogen balance, and provides the building blocks for the protein that is necessary for the return to proper health. These solutions may be given concurrently with oral feeding and, as with any IV solution, provide a route for other medications. They are, like the dextrose solutions, when used alone, nutritionally incomplete and should be given only in the short term to help preserve body protein in a stable patient.

TPN via the central venous route is used for patients with a need for calories and nutrients over a long period of time. High-concentration solutions of dextrose and amino acid solution, for example, 50% dextrose and 8.5% amino acid solution, are admixed in the hospital pharmacy. Trace elements, vitamins, or electrolytes are added to the mixture as needed. This solution will be quite hypertonic, with an osmolarity of around 2000 mOsm/L, and must be administered at a carefully controlled rate into a large vein with a high rate of blood flow to achieve proper dilution and minimize irritation of the vein. Infusion is accomplished by inserting a catheter with the tip extending into the superior vena cava and then, via an administration set, connecting the catheter to the bag that contains the admixture. The catheter may remain in place for as long as 30 days with proper care and precautions to avoid sepsis. The 10 or 20% fat emulsion may be administered intermittently through the central vein, through a peripheral vein, or be combined with the dextrose and amino acid solutions in the "mixing bag" prepared in the hospital pharmacy.

Stress Testing

Stress testing, testing after exposure to exaggerated conditions, is done throughout the developmental process and is designed to establish "safety factors." The data obtained from chemical, microbiological, biological, and physical tests, when compared with the results of tests on samples prepared under normal conditions, provide additional assurance that a safe and effective product will reach the market. Stress testing may take many forms.

Materials that will be in contact with the solution are subjected to extractions that far exceed the normal surface-volume ratios and the extracts are used for chemical, physical, biological, and toxicity testing. Tests for plastic and rubber are listed in various the pharmacopoeias. In addition, the LVP manufacturer may prepare concentrated extracts for tissue culture tests, a screening test for direct cell effects, and tests in rodents and other animals for indications of toxicity. The identity of the material extracted can be established chemically, quantified, and, with the results of the biological tests, related to its effect on humans.

During development of the sterilization cycles, temperature distribution and penetration studies are performed to ensure that the lethality is imparted to the entire sterilizer load. These studies are followed with evaluation lethality of biological indicators in the load. Often, the filled containers are subjected to two or three sterilization cycles and then checked for physical or chemical change.

Product filled containers are tested for drop tests, thermal shock tests, internal pressure tests, and impact resistance. The procedures for these tests are given in manuals that are available from the American Society for Testing Materials (ASTM). Alternating cycles of low and high temperatures provide information about how the solution and container react to adverse storage conditions. Such an evaluation may become part of the initial stability evaluation or the subject of a special stability study.

Stability Evaluation

Stability evaluation studies are aimed to support expiration dating of the product and also to provide labeling information about shipping and storage conditions, maximum and minimum

temperatures, or the necessity to prevent exposure to light. These studies encompass many aspects: physical (change of color or formation of a precipitate), chemical (change in pH or assay), microbiological (there are no antimicrobial agents in LVPs), or the packaging, which must be nonreactive and protect the solution during the shelf life. For details on the topic of stability studies regarding the stability procedures, sampling requirements, storage conditions, testing schedules, and evaluation of data, reader is directed to chapter 10, volume 3 of this book.

Processing Conditions Affecting Formulation of LVP

Some aspects of water quality, filtration, and sterilization are described below as they relate to LVP formulation (these have been described in detail in separate chapters elsewhere in this textbook).

WFI is the main ingredient of an LVP formula. Produced in large amounts by distillation or by reverse osmosis, the water must be tested frequently to assure that it is of the quality specified in the compendia. For particulate matter, pharmacopoeias require that each LVP unit must contain no more than 25 particles/mL that are equal to or larger than 10 μm and no more than 3 particles/mL that are equal to or larger than 25 μm in effective linear measurement. Particle generation from any source to which the solution will be exposed must be identified and controlled. Likely sources are air, processing liquids and gases, or components. Each source may contribute only a few particles but in combination can have a significant effect on the quality of the solution. Emphasis should be placed on reducing the generation of particles as well as effective filtration of liquids and gases at the point of use in the process.

LVPs are terminally sterilized, that is, sterilized after the product is filled and sealed in its final container. The sterilization methods generally used is steam under pressure. The type of container, size of container and solution has an effect on the cycle. Plastic containers, for example, are flexible and permeable. Air overpressure inside the sterilizer must be adjusted during the cycle to counteract the internal pressure in the container in order to avoid distortion. The air that prevents distortion also can enrich the oxygen content of the solution and airspace in the container; the result is that 5% dextrose in lactated ringer's develops more color in plastic than in glass. Amino acids are particularly susceptible to oxygen and all but a few are currently packaged in glass. Glass containers are rigid and impermeable but are subject to breakage because of thermal shock if the temperature differentials between the content of the bottle and sterilizer are excessive. The rate of heat up or cooling must be carefully controlled to avoid thermal shock. During sterilization of product in glass containers, the air overpressure in the sterilizer prevents lifting of the closure, which may be brought about by the internal pressure of the bottle. Cycle adjustments must be made for container size; smaller sizes have more surface area available per unit volume than larger sizes and may be used as worst-case samples for studying the effects of heat history.

Admixture Considerations

Of all LVPs infused, 60% to 80% are estimated to be admixed with one or more drugs (175,176). The number of new drugs and possible combinations is increasing steadily. Appropriate compatibility and stability studies must be performed to ensure that the drugs introduced into LVPs are compatible. The phenomenon of incompatibility occurs when the LVP and drugs produce, by physicochemical means, a product that is unsuitable for administration to the patient. Physical incompatibility may be detected by a change in the appearance of the solution, such as the formation of a precipitate, a haze, a change of color, or the breaking of an emulsion. Subtle incompatibilities, such as a change in pH or drug concentration, may not result in a visual change or may not become evident until a later time.

Instability occurs when an LVP product or admixture is modified because of sorption or such storage conditions as time, light, or temperature. The modified product may not be suitable for administration, and unless the combination has been studied in the laboratory, the only clue to a stability problem may, be failure to get the expected clinical result.

The parameters of tonicity, pH, solubility, and added substances, which were considerations in the design of the LVP formulation, also must be considered in a different context when drugs are added to the solution. The drug product may contain solvents, preservatives, stabilizers, buffers, antioxidants, and other ingredients that, when added to the LVP, can result in instability and incompatibility problems. Sodium benzoate, a preservative in some drugs, precipitates as benzoic acid when added to an LVP with an acidic pH. Copper, a trace metal needed by the body, can cause precipitation in amino acid solutions. Stability of the combination must be maintained after mixing and during infusion if the desired result is to be achieved. Stability problems may be caused by pH, solubility, sensitivity to light or temperature, absorption, or chemical incompatibility. Stability may also be related to time, and this is one reason that it is recommended that admixtures not be stored for prolonged periods.

One example of the role of pH would be that of ampicillin B in dextrose solutions. Unless the pH of the dextrose solution is greater than 5.0, the combination is incompatible. The monograph for Dextrose for Injection allows a pH range of 3.5 to 6.5. When the pH of 5% dextrose in lactated ringer's injection is below 5, some nerve-blocking agents, such as succinylcholine, will precipitate from solution.

Chemotherapeutic drugs and vitamin preparations generally should be protected from light. Sodium bisulfite, an ingredient added to some LVPs to reduce degradation caused by oxidation, may be present in only the quantity needed for protection of the solution during sterilization and shelf life. It may not be present in sufficient quantity to provide protection from the air that may be introduced to the container during admixing or storage in plastic containers.

The order of introduction of drugs to the LVP may either highlight or mask visible incompatibilities. If a drug is incompatible at a given pH and the pH of the LVP must be adjusted, the pH should be adjusted before the drug is added. A fat emulsion, white and opaque, masks reactions that might be visible in a clear solution, and the package insert cautions not to add electrolytes directly to the emulsion.

The potential physical and chemical incompatibilities associated with such dilutions are compiled by Trissel (74) and is often the primary reference book on this subject in the practice of pharmacy.

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6 Drug solubility and solubilization

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SOLUBILITY AND PARENTERAL PRODUCTS

This chapter provides a practical description of the physical phenomena leading to molecular level solubilization or dispersion of solutes (drugs) in a way that should enable the formulator to make informed decisions regarding formulation strategies for parenteral delivery. Solubility is discussed from the perspective of a thermodynamically defined equilibrium requiring several energetic steps in going from solute in a condensed phase to a solute in solution. Discussions will include the nonequilibrium state of supersaturation while focusing on the fit-for-purpose definition of solubility targeting parenteral drug delivery. The definition of solubility can relate to the solubility of any physical state of matter in another, or even in a similar state (miscibility), but this chapter will focus on solubility of a solid state in a liquid media, resulting in a solution mixture, which is of primary pharmaceutical importance for parenteral drug delivery (1).

Thermodynamic solubility can be described as the condition where the chemical potential of solute (μ_{solute}) in solution is in equilibrium with, and equal to, the chemical potential of the solute in its respective solid phase (μ_{solid}) under consideration (2). At a constant temperature and pressure, this equilibrium defines the saturated solution with respect to the designated solid phase and respective media. Any perturbation in the solute phase or solvent phase can result in a temporary metastable state of either supersaturation ($\mu_{solute} > \mu_{solid}$) or subsaturation ($\mu_{solute} < \mu_{solid}$), where the chemical potentials differ and the system will spontaneously attempt to reestablish equilibrium. Any effort to intentionally alter solubility will require a modification in the chemical potentials of either the solute solid state or the solute in solution.

To better understand strategies to modify solubility, three key energetic drivers for the solubilization process should be considered (2). The first step is the necessary energy input to overcome the intermolecular interactions of the solute in its respective condensed state (Fig. 1). The second step is the energy input necessary to overcome solvent-solvent interactions and create a cavity in the solvent which accommodates the solute. The unfavorable energy input to this point is then countered with the energy release occurring upon collapse of the solvent cavity around the solute and ensuing intermolecular interactions between solute and solvent.

Alterations in the solvent can influence both solvent-solvent interactions and subsequent solvent-solute interactions. This is the basis for many of the cosolvent strategies used for solubilization, wherein the μ_{solute} is decreased shifting the equilibrium toward increased amounts of drug in solution. Solubilization through changes in the solid form of a drug (amphorous, polymorphs, etc.) leads to increases in the μ_{solid}, which also shifts the equilibrium, but also runs the risk of conversion to a more thermodynamically stable and less soluble solid form with time. Solubilization obtained through alterations in the solute's molecular structure has the potential to significantly alter solubility by impacting specific solvent—solute interactions or solute—solute interactions. This is probably the preferred strategy for enhancing solubility, but such molecular modifications are difficult to introduce once the drug development process on an entity has been initiated. Hence, molecular design modifications are best instituted through interactions with medicinal chemists in the discovery organization prior to drug candidate selection.

One of the most commonly used strategies to provide apparent increases in solubility, or total drug in solution, is to create alternative equilibria for the drug or solute to reside in. While these equilibria enhance the total amount of drug in solution, the μ_{solute} remains equivalent to that of the solid phase, that is, the intrinsic solubility is not altered but instead the μ_{solute} residing in some additional equilibrium is reduced through specific interations or altered solvation. Creation of alternative equilibria to "sequester" drug provides the basis for solubilization strategies, such as micellar partitioning, chemical ionization, complexation, and partitioning into emulsions.

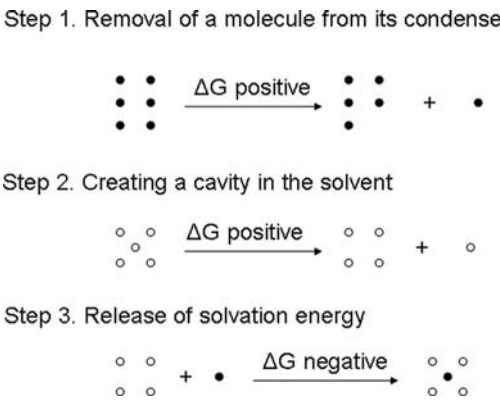

Figure 1 An illustration of the three steps needed for drug solubility.

In the simplest of terms, the solubility of a solute in a given solvent system, as defined by amount of drug dissolved, seems easily determined, but reliable, reproducible and meaningful numbers can be difficult to obtain. The more common methods are best described as "fit for use," wherein the solid phase of interest is incubated in solvent and the total amount of solute present in solution is measured. The method of solid-phase separation is critical and really defines the utility of the apparent solubility obtained. Typically, either filtration or centrifugation is used with subsequent assay of filtrate (filtration) or supernatant (centrifugation). Details of separation can be particularly important when colloid scale dispersions exist. Furthermore, as solubilities begin to drop below 1 μg/mL, issues of nonspecific adsorption to surfaces (filter, container), coupled with analytical detection limitations can result in highly variable values across labs.

Factors such as temperature, energy input and the nature of both the solid phase and the solvent can significantly impact how rapidly equilibrium is obtained. Approaching equilibrium from both a state of supersaturation and subsaturation taking measurements as a function of time is probably the best approach. At equilibrium both should approach similar values. When solubilities are >1 μg/mL, 24-hour incubation will generally approach 90% to 95% of equilibrium value, assuming particle sizes are small (3).

IMPLICATIONS OF SOLUBILITY FOR PARENTERALS

A common challenge in development of drugs intended for parenteral administration is the solubilization of a poorly soluble active ingredient (4). For intravenous (intravascular) injection, solubility of the active ingredient in the plasma needs to be below saturation upon dilution to prevent precipitation or formation of phlebitis. Injection of a drug into an extravascular site may establish a depot depending on the type of formulation administered. Drug absorption from a depot by passive diffusion and partitioning is dependent on drug solubility. Only the fraction of drug in solution is available for absorption. A critical difference between the pH of the administered drug solution and the physiological pH at the injection site (and/or solubility of the drug in a cosolvent vehicle and in physiological tissue fluid) can cause an unpredicted decrease in absorption due to precipitation of the drug at the injection site. Phenytoin is formulated as a sodium salt in a pH 12 solution of 40% propylene glycol, 10% alcohol and water for injection. When injected into muscle tissue, the large difference in pH and simultaneous dilution of propylene glycol with tissue fluids cause conversion of the sodium salt to less soluble free acid and precipitation at the injection site. Amphotericin B has a low aqueous solubility of 0.1 mg/mL at pH 2 or pH 11. However, Amphotericin B is highly soluble in liposomal intercalation and becomes an integral part of the lipid-bilayer membrane. These liposomal products permit administration by IV infusion. Another commonly studied low solubility drug is paclitaxel with an aqueous solubility of 0.1 μg/mL. Wheelar et al. manufactured an emulsion and liposome blend using corn oil, cholesterol and egg phosphotidylcholine containing 5 mg/mL of paclitaxel, a 50,000-fold increase in solubility (5).

PROPERTIES OF THE SOLVENT

A popular aphorism used for predicting solubility is "*like dissolves like*" (6). This statement indicates that a solute will dissolve best in a solvent that has a similar polarity to itself. This view is rather simplistic, since it ignores many solvent-solute interactions, but it is a useful rule of thumb. Strongly polar compounds like sugars or ionic compounds like inorganic salts dissolve only in very polar solvents like water, while strongly nonpolar compounds like oils or waxes dissolve only in very nonpolar organic solvents like hexane. The dielectric constant, solubility parameter and interfacial/surface tension are among the most common polarity indices used for solvent blending to improve solubility.

Generally, the dielectric constant of the solvent provides a rough measure of a solvent's polarity. It is the electric permittivity ratio of solvent to vacuum. It measures the solvent's ability to reduce the strength of the electric field surrounding a charged particle immersed in it. This reduction is then compared with the field strength of the charged particle in a vacuum. In general, polar solvents have higher dielectric constant values than nonpolar molecules. Solvents with a dielectric constant of less than 15 are generally considered nonpolar (7). The dielectric constants of some commonly used solvents and cosolvents in parenteral products are listed in (Table 1).

Gorman and Hall (10) studied the solubility of methyl salicylate in isopropanol-water mixtures, and obtained a linear relationship between log mole fraction of the methyl salicylate and the dielectric constant of the mixed solvent.

For a solution to occur, both solute and solvent molecules must overcome their own intermolecular attraction forces, so called van der Waals forces, and find their way between and around each other. This is accomplished best when the attractions between the molecules of both components are similar. The solubility parameters are defined to express the cohesion between like molecules. It is a numerical value that indicates the relative solvency behavior of a specific solvent and can be calculated from heats of vaporization, internal pressures, surface tensions, and other properties, as described by Hildebrand and Scott (11). The heat of vaporization in conjunction with the molar volume of the species, when available at the desired temperature, probably affords the best means for calculating the solubility parameter. It can be expressed as equation (1).

$$\delta = \left(\frac{\Delta H_v - RT}{V_l}\right)^{1/2} \tag{1}$$

where ΔH_v is the heat of vaporization and V_l is the molar volume of the liquid compound at the desired temperature, R is the gas constant, and T is the desired absolute temperature. Hildebrand and Scott include solubility parameters for a number of compounds in their book. A table of solubility parameters has also been compiled by Hansen and Beerbower (12), wherein the authors introduced partial solubility parameters δ_D, δ_p, and δ_H. The parameter δ_D accounts for nonpolar effects, δ_p for polar effects, and δ_H to express the hydrogen bonding

Table 1 Dielectric Constant, Solubility Parameter, and Surface Tension of Common Solvents and Cosolvents

Solvent	Dielectric constant	Solubility parameter $(cal/cm^3)^{1/2}$	Surface tension 20°C (dyne/cm)
Water	78.5	23.4	72.8
Ethanol	24.3	12.7	22.4
Propylene glycol	32	14.8	38.0
Glycerin	43	16.5	64.3
PEG 300 or 400	35	9.9	43.5 (PEG 200)
Benzyl alcohol	13	12.1	40.7
Dimethyl sulphoxide (DMSO)	47	12.0	43.5
N,N-dimethylacetamide (DMA)	38	10.8	36.7
N,N-dimethylformamide (DMF)	37	12.1	39.1
N-methyl-2-pyrrolidone (NMP)	32	23.0	40.8

Source: From Refs. 8 and 9.

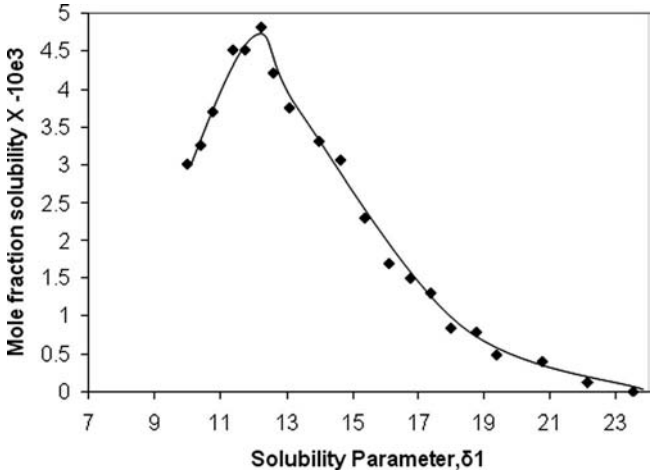

Figure 2 Solubility of trimethoprim in dioxane-water mixture of varying solubility parameter. *Source*: From Ref. 14.

nature of the solvent molecules. The sum of the squares of the partial parameters gives the total cohesive energy density $\delta^2_{(\text{total})}$ [eq. (2)]. Kesselring et al. have determined both total and partial solubility parameters using gas-liquid chromatography (13).

$$\delta^2_{(\text{total})} = \delta^2_{\text{D}} + \delta^2_{\text{P}} + \delta^2_{\text{H}} \tag{2}$$

The more alike are the δ values of two components, the greater is the mutual solubility, miscibility, of the pair. For example, the δ value of phenanthrene is 9.8; it would be expected to be more soluble in carbon disulfide with a δ value of 10 than in normal hexane with a δ value of 7.3. Conversely, δ of a drug can be estimated from measured solubility as a function of solvent solubility parameter (14) (Fig. 2).

Interfacial/surface tension is another solvent property caused by the attraction between the liquid's molecules by various intermolecular forces. It is a measure of the work required to create a cavity of unit area of surface from molecules in the bulk, hence relating to cavity formation for solutes. Polar solvent generally has higher surface tension than nonpolar solvent. Some surface tension and interfacial tension (against water) at 20°C are listed in Table 1 (15).

PROPERTIES OF THE SOLUTE

Drug molecules contain different structures and functional groups. The collective contributions from each functional group make the macroscopic physicochemical properties of the drug, which are a reflection of inter- or intramolecular interactions. For example, the stronger the attractions between molecules or ions, the more difficult it is to separate the molecules, therefore, the higher the melting point and poorer the solubility. The intra- or intermolecular forces are dictated by intrinsic molecular properties, such as polarizability, electronic factors, topology and steric factors, lipophilicity, hydrogen bonding, surface areas, volumes and connectivity, etc.

Molecular Properties

Polarizability and Electronic Factors

Polarizability is a characteristic property of the particular molecule. It is defined as the ease with which an ion or molecule can be polarized by any external forces. From electromagnetic theory, there is a relationship between polarizability α_{p} and dielectric constant ε of a molecule, where n is the number of molecules per unit volume [eq. (3)].

$$\frac{\varepsilon - 1}{\varepsilon + 2} = \frac{4}{3}\pi \cdot n \cdot \alpha_{\text{p}} \tag{3}$$

When a molecule cannot be represented by a single Lewis structure, that is, using an integral number of covalent bonds between two atoms, but rather has properties in some sense

intermediate to these, resonance structures are then employed to approximate the true electronic structure. Because of confusion with the physical meaning of the word resonance, as no elements actually appear to be resonating, it has been suggested that the term resonance be abandoned in favor of delocalization and delocalization energies (16).

An electric dipole is a separation of positive and negative charges. It can be characterized by dipole moment, μ, which is equal to the product of charge on the atoms and the distance between the two atoms bounded with each other. Many molecules have such dipole moments because of nonuniform distributions of positive and negative charges on the various atoms. Such is the case with polar compounds like hydroxide (OH^-), where electron density is shared unequally between atoms. Dipole moment is the polarity measurement of a polar covalent bond. The higher the polarity of a molecule the greater the dipole moment and the value can be calculated through the comparison of dielectric constant and the refractive index of the solutions.

Some drugs are known to form a charge-transfer complex with certain solvents. A charge-transfer complex (or CT complex, electron-donor-acceptor-complex) is a chemical association of two or more molecules, or of different parts of one very large molecule, in which the attraction between the molecules (or parts) is created by an electronic transition into an excited electronic state, such that a fraction of electronic charge is transferred between the molecules. The resulting electrostatic attraction provides a stabilizing force for the molecular complex. The association does not constitute a strong covalent bond and is subject to significant temperature, concentration, and host (e.g., solvent) dependencies and occurs in a chemical equilibrium with the independent donor (D) and acceptor (A) molecules.

The great majority of drugs contain ionizable groups; most are basic, some are acidic. The ionization constant (K_a) indicates a compound's propensity to ionize. It is a function of the acidity or basicity of group(s) in the molecule. Because of the many orders of magnitude spanned by K_a values, a logarithmic measure of the constant is more commonly used in practice, wherein the pK_a is equal to $-\log_{10} K_a$. The equilbria for acids [eqs. (4) and (5)] and for bases [eqs. (6) and (7)] are described as follows:

$$HA = H^+ + A^- \tag{4}$$

$$pK_a = -\log([H^+] \cdot [A^-]/[HA]) \tag{5}$$

$$HB^+ = H^+ + B \tag{6}$$

$$pK_a = -\log([H^+] \cdot [B]/[HB^+]) \tag{7}$$

Rearranging the pK_a equations give the well-known Henderson-Hasselbalch equations for both weak acid (HA) and weak base (B) and the ability to calculate the percentage of ionized species at any particular pH [eqs. (8) and (9)].

$$pH = pK_a + \log[A^-]/[HA] \tag{8}$$

$$pH = pK_a + \log([B]/[BH^+]) \tag{9}$$

When the pH is two units either side of the pK_a, then the drug will be almost completely ionized (BH^+, A^-) or unionized (B, HA). The solution pH and the pK_a are important because the charged form of a drug is more soluble than the neutral form. To have any realistic chance of significant pH-solubility manipulation for a parenteral, the pK_a for a base must be greater than 3 and for an acid less than 11.

Lipophilicity

Lipophilicty is the tendency of a compound to partition into a nonpolar lipid matrix versus an aqueous matrix. Lipophilicity is readily calculated, thanks to the work of Hansch and Leo (17).

It is a rapid and effective tool for initial compound property assessment. One traditional approach for assessing lipophilicity is to partition the compound between immiscible nonpolar and polar liquid phases. Traditionally, octanol is the nonpolar phase and aqueous buffer as the polar phase with the partition value, log*P* defined below [eq. (10)]. Log*P* is measured at a pH of the buffer where all of the compound molecules are in the neutral form.

$$\text{Log}\, P = \log \frac{[C_{\text{nonpolar}}]}{[C_{\text{polar}}]} \tag{10}$$

Hydrogen Bonding

The assumption that the solubility of a solute in a given solvent is related simply to the bulk properties of the pure components, that is, "like dissolves like," was originally intended strictly for systems involving only London dispersion forces. For quite polar solution components, the specific intermolecular interactions, such as hydrogen bonding, when they occur, are often the dominant factors in determining solubility (18).

A hydrogen bond is a special type of attractive interaction that exists between an electronegative atom and a hydrogen atom covalently bonded to another electronegative atom. Usually the electronegative atom is oxygen, nitrogen, or fluorine, which has a partial negative charge and is the hydrogen bond acceptor. The hydrogen then has the partial positive charge and is the hydrogen bond donor. The typical hydrogen bond is stronger than van der Waals forces, but weaker than covalent or ionic bonds and can occur intermolecularly, or intramolecularly. When hydrogen bonding between solute and solvent is possible, solubility is greater than expected for compounds of similar polarity that cannot form hydrogen bonds. Hansen and Beerbower (12) have introduced hydrogen bond partial solubility parameter, δ_H, to account for the nonideality effect from hydrogen bonding on total solubility (see above).

Topology and Steric Factors

It is believed that the variations in the magnitude of solubility of different solutes in water are caused by their dissimilar chemical structures and much attention has been paid to quantitative structure activity relationship (QSAR) studies of modeling the relationship between chemical structure and solubility of organic compounds. Molecular topology as one of the structure indices has been used widely to study the solubility of compound in different models (18,19).

Molecular topology is the mathematical description of molecular structure allowing a unique and easy characterization of molecules by means of invariants, called topological indices, which are the molecular descriptors to correlate with the experimental properties. Different from the conventional physicochemical descriptors, topological indices (TIs) allow the use of the QSAR relations to design new compounds from scratch. This is possible because, contrary to the physical parameters, the algebraic descriptors are not indirectly related to structure but they are a mathematical depiction of the structure itself.

Besides the chemical structure of the molecules, the spatial arrangement of their functional groups can play a significant role in compound solubility when it influences the degree of interaction between solute and solvent. For example, two isomers can exhibit very different solubilities in the same solvent (20). The influence of the location of the functional groups is referred to here as the steric effect. For strongly interactive solvents like water, the steric effect is particularly severe and sometimes dominating when it hinders or promotes hydrogen bonding interaction. On the other hand, structural alterations that are not in the vicinity of an interacting functional group and do not alter the functionality of the group, have little influence on solubility.

Surface Areas, Volumes, Connectivity

Theoretically, the dissolution process of a crystalline solid can be carried out in four hypothetical steps: (1) melting of the crystalline solute, (2) separation of a solute molecule from the molten bulk, (3) creation of a cavity in the solvent for accommodation of a solute, and (4) placement of the solute molecule into the cavity created. The energy required for these

processes can be characterized using the enthalpy of melting, the cohesive energy of the solute and solvents, and the adhesive energy at the interface, which are directly proportional to the interfacial area. Hence, solubility can be related to the molecular surface area of a solute.

The solubility in water of aliphatic compounds has been successively related to molecular surface area by Amidon and associates (21,22). They investigated the aqueous solubility of hydrocarbons, alcohols, esters, ketones, esters, and carboxylic acids. Excluding olefins, a linear relationship was found between log (solubility) and total surface area with 158 compounds that they investigated. Similarly, molar volume of the solute is another property impacting solubility. It is related to molecular weight and affects the size of the cavity that must be formed in the solvent to solubilize the molecule.

Molecular connectivity is a measure of extent of molecular branching and normally used as a connectivity index. The connectivity index, easily computed, based on the degree of connectedness at each vertex in the molecular skeleton, is shown to give highly significant correlations with water solubility of branched, cyclic, and straight-chain alcohols and hydrocarbons as well as boiling points of alcohols (23). These correlations are superior to those based on well-founded theory relating to solvent cavity surface area.

Macroscopic Properties

The melting point or freezing point of a pure crystalline solid is strictly defined as the temperature at which the pure liquid and solid exist in equilibrium. The heat absorbed when a gram of a solid melts, or the heat liberated when it freezes, is known as the latent heat of fusion. The heat added during the melting process does not bring about a change in temperature until the entire solid has disappeared, since this heat is converted into the potential energy of the molecules that have escaped from the solid into the liquid state.

The heat of fusion may be considered as the heat required to increase the interatomic or intermolecular distances in crystals, thus allowing melting to occur. Heat of fusion is dictated by crystal packing. A crystal that is packed by weak forces generally has a low heat of fusion and a low melting point, whereas one packed together with strong forces has a high heat of fusion and a high melting point.

Solubility, as discussed earlier, is strongly influenced by intermolecular forces, similar to melting point. This similarity was demonstrated by Guttman and Higuchi, who studied the melting points and solubilities of xanthines. When the side chain at 7 position changed from H (theophylline) to propyl (7-propyltheophylline), the melting point decreased from 270 to 100°C, while solubility in water at 30°C increased from 0.045 to 1.04 mol/L. An empirical equation was derived by Yalkowsky and Banerjee (24) to estimate solubility on the basis of the lipophilicity and melting point [eq. (11)].

$$\mathrm{Log}S = 0.8 - \log P_{\mathrm{ow}} - 0.01(\mathrm{MP} - 25) \tag{11}$$

Here S is solubility, $\log P_{\mathrm{ow}}$ is the octanol/water partition coefficient (a measure of lipophilicity), and MP is the melting point (a measure of crystal packing).

Polymorphs exist when two crystals have the same chemical composition but different unit cell dimensions and crystal packing. Compounds that crystallize as polymorphs generally have different physical and chemical properties, including different melting points, x-ray diffraction patterns, and solubilities. Generally, the most stable polymorph has the highest melting point and lowest solubility; other polymorphs are metastable and convert. A consideration of the data in the literature indicates that improvements in solubility of metastable crystal forms can be expected to be as high as twofold (25).

When the crystal lattice contains solvents that induce polymorphic changes, they are called solvates. If the solvent is water, these pseudo-polymorphs are called hydrates. These hydrates and solvates are easily confused with true polymorphism and lead to the term pseudo-polymorphism. The solvates may be discriminated by DSC/TGA, where an additional endotherm due to the solvent will be apparent in DSC provided the heating rate is slow, and weight loss at similar temperature is observed in TGA.

Hydrate formation generally leads to a lower solubility since the preexistence of water in the crystal lattice reduces the energy available for solvation. For example, glutethimide

anhydrate has melting point 83°C and solubility 0.42mg/mL, but its hydrate has melting point 68°C but solubility only 0.26mg/mL. However, solvates tend to have higher solubility than the neat form because of the weakening of the crystal lattice by the organic solvent. For example, succinylsulphathiazole neat has a solubility of 0.39mg/mL, and its pentanol solvate has solubility of 0.80mg/mL (26).

Amorphous solids may be considered as supercooled liquids in which the molecules are arranged in a random manner somewhat as in the liquid state and do not have melting points. Amorphous solids are in a high energy state relative to their respective crystalline solids, therefore, leading to differences in dissolution rate, chemical reaction rate and mechanical properties. Amorphous solids also have a higher solubility than their crystal form. The solubility advantage compared with the most stable crystalline counterpart was predicted to be from 10 to 1600 fold, as shown by Hancock and Parks (25). However, the experimental solubility advantage was usually considerably less than this, because determining solubility for amorphous materials under true equilibrium conditions is difficult because of the tendency for such materials to crystallize upon exposure to small quantities of solvents.

When particles are in the submicron range, a small increase in the saturation solubility is expected as described by the Freundlich–Ostwald equation [eq. (12)] (27,28).

$$\frac{RT}{V_m}\ln\frac{S}{S_0}=\frac{2\gamma}{r} \tag{12}$$

where S is the saturation solubility of nanosized particle, S_0 is saturation solubility of an infinitely large crystal, γ is the crystal-medium interfacial tension, r is the particle radius, V_m is the molar volume, R is a gas constant, and T is the temperature. Assuming a molecular weight of 500, density of 1 gm/mL, and a value of 60 to 70 mN/m for the crystal-water interfacial tension, the above equation would predict a 62% to 76% increase in solubility at a particle size of 100 nm.

IONIZATION AND THE SOLUBILITY PROFILE

The total solubility of a compound at a particular pH is the sum of the "intrinsic solubility" of the neutral species in solution plus the solubility of the charged species. For a weak base, when the aqueous medium at a given pH is saturated with free base, the total solubility at that pH may be expressed as described [eq. (13)]. The typical solubility profile of a weak base when $pH > pH_{max}$ is shown in (Fig. 3).

$$S_{base}(pH > pH_{max}) = [B]_s + [BH^+] = [B]_s\left(1+\frac{[H_3O^+]}{K_a}\right) \tag{13}$$

When there are counterions present in the solution, at low enough pH, the entire free base will be converted into salt form, and the salt is the solid form. In this case, the equilibrium solubility at a particular pH may be expressed by equation (14).

$$S_{base}(pH < pH_{max}) = [B] + [BH^+]_s = [BH^+]_s\left(1+\frac{K_a}{[H_3O^+]}\right) \tag{14}$$

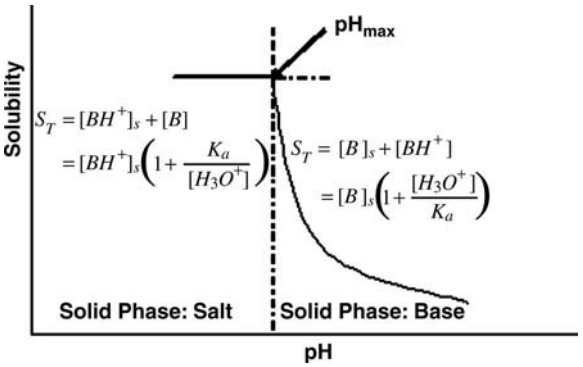

Figure 3 Schematic representation of the pH-solubility profile of a weakly basic compound.

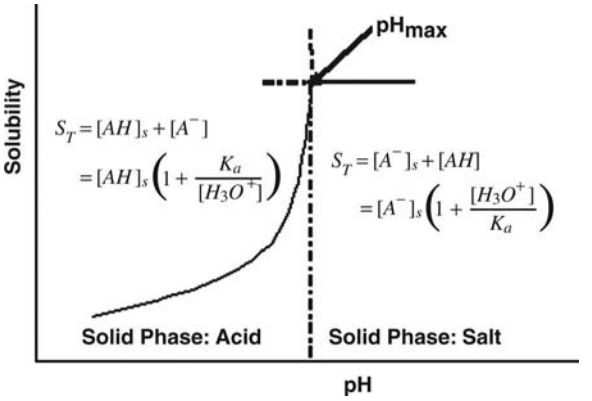

Figure 4 Schematic representation of the pH-solubility profile of a weakly acidic compound.

When these two independent curves in solubility pH profile intersect, the point is called pH_{max} as shown in the Figure 3. Similarly, the pH-solubility profile for a weak acid is also shown (Fig. 4).

Zwitterions refer to compounds with oppositely charged groups, but carry a total net charge of 0 and is thus electrically neutral. Solubility of zwitterions at certain pH is the combination of the contributions from all the charge groups. For compounds with two ionizable groups, solubility can be expressed by the following equation [eq. (15)].

$$S = S_0(1 + 10^{pK_{a1}-pH} + 10^{pH-pK_{a2}}) \tag{15}$$

It depends on its ionization constants, pH and intrinsic solubility, S_0, which is defined as the solubility of the neutral form of the compound. The solubility profile is U-shape characteristic for zwitterionic compounds.

For weak electrolyte drugs, salt formation is a common approach to improve solubility. Acids form salts with basic drugs and bases form salts with acidic drugs (29). For the salt of a basic drug, the dissolution equilibrium can be described as equation (16).

$$(BH^+X^-)_{solid} \leftrightarrow [BH^+]_s + [X^-] \tag{16}$$

Where $[BH^+]_s$ is the salt solubility and $[X^-]$ is the counterion concentration. The apparent solubility product K_{sp} can be derived as equation (17).

$$K_{sp} = [BH^+]_s[X^-] \tag{17}$$

In the absence of excess counterion, $[BH^+]_s = [X^-]$, solubility is the square root of K_{sp}. Under such conditions, drug solubility does not change with pH, as indicated in the figures above. On the other hand, if a significant amount of counterions exit in the formulation, decrease in solubility may be observed according to equation (18).

$$[BH^+]_s = K_{sp}/[X^-] \tag{18}$$

SOLUBILITY PREDICTION

A number of approaches to solubility prediction have been developed over the years and continue to be used (30). Recently many successful attempts were made for predicting aqueous solubility of compounds, but it is still a challenge to identify a single method that is best at predicting aqueous solubility (31). The first hurdle in the prediction of aqueous solubility is the estimation of melting point or enthalpy of sublimation (32). In addition, it is difficult to predict the solubility of a complex drug candidate on the basis of the presence or absence of certain functional groups. Conformational effects in solution may play a role in solubility and cannot be accounted for by a simple summation of contributing groups.

Because of the complexity involved in developing the prediction models, most models were completed using nonelectrolytes.

The prediction of aqueous solubility tends to use three approaches: methods correlating experimentally determined melting points and log$P$, correlations based on group contributions, and correlations with physicochemical and quantum chemical descriptors calculated from the molecular structure [quantitative structure property relationship (QSPR) approaches] (1).

Methods using melting point and log$P$ are best exemplified by the general solubility equation (GSE) model (33). The GSE model is based on the fact that the aqueous solubility of a nonelectrolyte solute depends on its crystallinity and its polarity, wherein the melting point and the octanol-water partition coefficient act as good surrogate measures, respectfully. For compounds with melting points $< 25^{\circ}$C, the melting point is taken to be 25°C. Ran, Yalkowsky and coworkers (34) revised equation 11 to equation (19).

$$\text{Log}S = 0.5 - \log P_{\text{ow}} - 0.01(\text{MP} - 25) \tag{19}$$

The theoretical treatment of this solubility prediction method is presented in more details elsewhere (1). With this prediction model, the absolute average error ranged from 0.5 to 1 log molar solubility unit for drug-like compounds (35).

The aqueous functional group activity coefficients (AQUAFAC) model is based on group contribution values, which are based on experimental aqueous solubilities (36). In this model, the molar aqueous solubility can be calculated using equation (20).

$$\text{Log}S = 1.74 - \log \gamma_{\text{w}} - \frac{\Delta S_{\text{m}}(T_{\text{m}} - T)}{2.303RT} \tag{20}$$

Where, γ_{w} is the aqueous activity coefficient of a compound, which is obtained from the AQUAFAC model. ΔS_{m} is the entropy of melting, T_{m} is the melting point, and T is the ambient temperature, both in Kelvin, R is the gas constant.

Using QSPR models, aqueous solubility is controlled predominantly by solute molecular size and shape, by its polar nature and hydrogen bonding capabilities. In addition, hydrophobicity, flexibility, electron distribution and charge have been found to play important roles in prediction (37). Many molecular property desciptors are now available computationally. Aqueous solubility has been modeled by correlating measured solubilities with one or more physicochemical and/or structure properties. Most methods use linear methods such as multiple linear regression (PLS) or nonlinear methods such as artificial neural networks (ANN). In general, nonlinear methods appear to provide better predictions (38). The root mean squared errors for models based on QSPR tends to range from approximately 0.7 log units to 1 log units. Recently, the effect of crystal packing on solubility has been added into the computational model (39).

Jain et al. applied two methods to compare aqueous solubility estimation of 1642 organic nonelctrolyte compounds ranging from 10^{-13} to 10^{0} in experimental molar solubility (33). The average absolute errors in the solubility prediction are 0.543 log units for AQUAFAC and 0.576 log units for the GSE. About 88.0% of the AQUAFAC solubilities and 83.0% of the GSE molar solubilities are predicted within one log unit of the observed values. The marginally better accuracy of AQUAFAC is assumed to be due to the fact that it utilizes fitted-parameters for many structural fragments and is based on experimental solubility data. The AQUAFAC also includes reasonable estimate of the role of crystallinity in determining solubility. The GSE on the other hand is a simpler, nonregression based equation, which uses two parameters (MP and logK_{ow}) for solubility prediction. The major assumption in the GSE is that octanol is an ideal solvent for all the solutes. This may not be true for strongly hydrogen bonding compounds, and consequently might result in larger error for such compounds.

With some computational packages it is now possible to make predictions on aqueous solubility that are as good as experimental measurements (± 0.5 log unit) for many compounds. However, all of the commercial programs were trained on selected organic chemicals and the predictive ability for drug-like compounds is still a challenge. When the commercial software programs do not yield good results for internal compounds, it may be necessary to evaluate various QSAR models and develop an in-house model (30).

SOLUBILIZATION AND "ENHANCED SOLUBILITY"

Modifications to the Solid State

Salt formation is probably the most common way to increase both the solubility and dissolution rate of ionizable drugs (29). The solid form, clearly distinct from the free acid or base solid form, provides significant enhancement in solubility through the provision of alternative equilibria, thus driving the total solubility (intrinsic + ionized) up significantly. This alternative equilibria results in a more readily solvated ionized form in hydrolytic solvents. As discussed earlier, the saturation solubility of the salt will be defined in conjunction with the Ksp, resulting pH and relative pK_a of the drug. As shown earlier (Figs. 3 and 4), changes in the pH or media composition can alter the solubility through common ion effects, or if the pH deviates well away from the pK_a, can actually result in precipitation of the free acid or base solid.

Selection of the counterion can actually be used to control the solubility by varying the Ksp. As pointed out by Anderson and Conradi (40), the impact of hydrogen bonding within the conjugate species can play a role in the Ksp and ends up also being translated into effects on the melting point of the salt. Common ion effects are manifested through the relationship defined by the Ksp. The solubility of the hydrochloride salt of the zwitterionic molecule lomefloxacin is a good example where excess chloride ion, as in admixtures with normal saline, can impact the solubility of the salt (41) (Fig. 5).

It is important to recognize that with any salt, the resulting pH of the media will be paramount in avoiding precipitation of the free base or acid. The strong acid conjugate salt of a weakly basic drug will end up driving the pH of the solution acidic, and conversely for strong base conjugate of weakly acidic drug. Care must be taken when such salts are dissolved into buffered systems where supersaturated solutions of the free base or acid may occur and have the propensity to precipitate with time. In such cases, a full understanding of the solubility versus pH curve is critical when using salts to provide improved solubility.

Cocrystals, similar to salts, provide a means to generate a crystalline form of the drug. While these solid phases can provide increased dissolution rates there has been minimal use of cocrystals to facilitate parenteral drug delivery. The properties and description of cocrystals has been discussed at length in a recent review (42).

The use of high energy amorphous solids can often result in temporary increases in solubility, but with a propensity to generate more stable crystalline forms. In parenteral

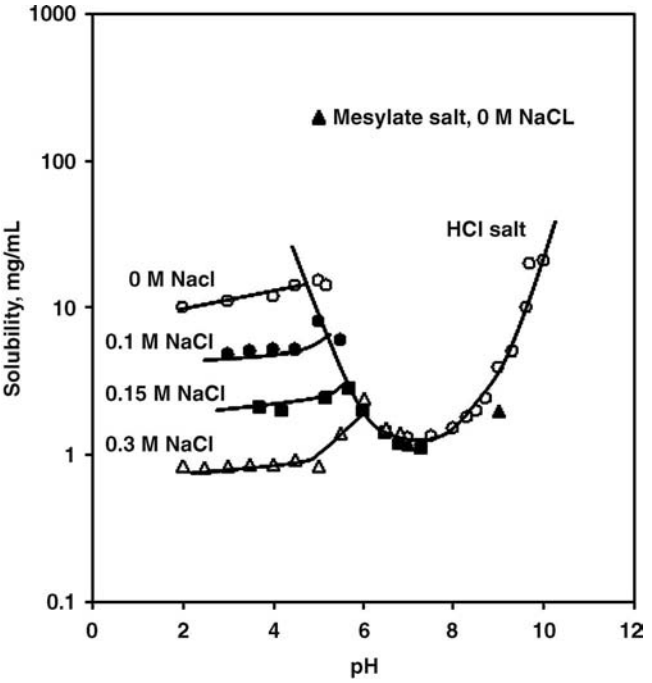

Figure 5 Effect of pH and NaCl concentration on the solubility of the zwitterionic quinolone lomefloxacin. *Source*: From Ref. 41.

products, the importance of metastable solids can many times play a role with lyophilized products upon reconstitution. The process of lyophilization often results in higher energy polymorphs or amorphous solids which allow for a very rapid dissolution and reconstitution back to the solution state. A thorough understanding of the dynamic nature of the lyophilized solid forms and the more stable crystalline forms which may exist is critical, whether they are hydrates, solvates, or polymorphs. The intentional use of such high energy states to increase solubility is limited because of its unpredictable behavior.

The best way to adjust solid form and impact solubility is via molecular modification, either as an analog or through formation of a prodrug. While these must be considered new chemical entities, they can provide a broad range of possible properties. Analog strategies are often focused on attempts to either decrease the lipophilicity and/or introduce hydrogen bonding groups which can enhance solvation in more hydrophilic media. In either case, especially with introduction of hydrogen bonding groups, increased interactions in the solid phase and its melt can actually increase as well, thus offsetting any gains afforded by increases in solvation. When possible, the introduction of ionizable groups can provide great solubility advantages (43).

In those cases where the perservation of the pharmacophore or desired biopharmaceutical properties does not permit molecular modifications leading to a more soluble molecule, a prodrug strategy can be invoked, overcoming immediate solubility limitations, yet when appropriately triggered, can release the active parent of interest (44).

Modifications to the Solution Phase

The use of cosolvents as was discussed earlier, has the ability to alter the dielectric constant of the solvent, influence the energy required to overcome hydrogen bonding forces in aqueous media and reduce the amount of energy necessary to create a cavity sufficient to accommodate the solute. Furthermore, these changes in solvent can greatly alter the degree of solvation of the solute once molecularly dispersed in the solvent. Soubility enhancement by addition of cosolvent is very typically log linear with respect to the cosolvent (Fig. 6). The degree of solubilization is dependent on both the lipophilicty, or log*P*, of the drug and type of cosolvent (45) (Fig. 6). Cosolvency and solubilization have been discussed by Rubino (46).

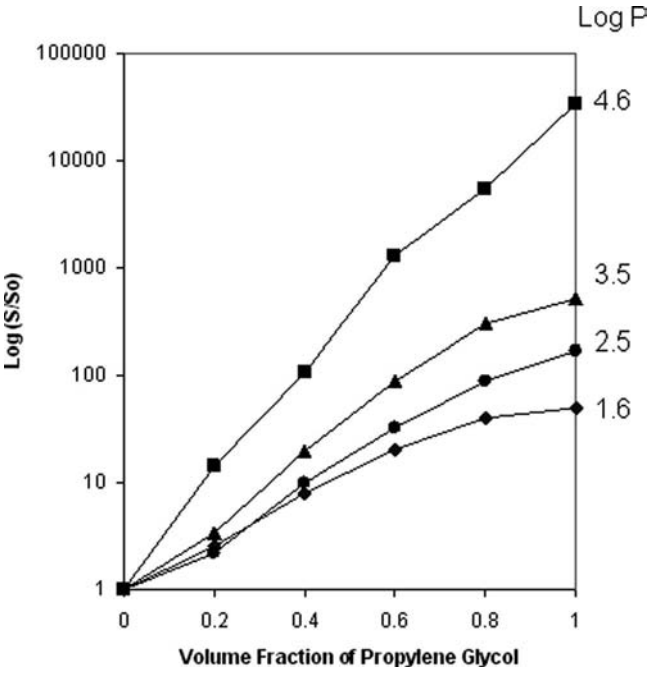

Figure 6 Propylene glycol solubilization of hydrocortisone esters. *Source*: From Ref. 45.

Modification due to Alternative Equilibria for Solute

An excellent overview of various methods to provide alternative equilibria for solubilization was presented by Yalkowsky (1). The rational selection of a solubilizing agent should be based on the structure of the drug to be solubilized and on the degree of solubilization needed to obtain the desired dose. The generation of alternative equilibria for the drug to exist in is one of the most commonly used methods to provide enhancements in the overall "apparent solubility" of the drug in solution. This strategy includes the use of ionization equilibria (discussed above in conjunction with salts), complexation equilibria, partitioning into surfactant micelles, partitioning into emulsion systems, and liposomal type systems.

Complexation and Association

Strategies of complexation include the use of chelating agents, organic molecular associations and inclusion complexes. The most common formulation strategies using complexation are centered around the use of cyclodextrins, with more emphasis generally placed on derivatized cyclodextrins because of their greater solubility and improved in vivo safety margin. Typically only those drugs with an aromatic ring or a nonpolar side chain are solubilized by cyclodextrin complexation (4). If complexation alone is insufficient, then a combination of complexation and pH modification or/and cosolvent may be used (47).

Complexation is an equilibrium process and the binding constant (or stability constant) for the formation of a 1:1 complex is given by equation (21).

$$\kappa_{1:1} = \frac{[\text{Drug}]_{\text{complex}}}{[\text{Drug}]_{\text{free}}[\text{Ligand}]_{\text{free}}} \tag{21}$$

$[\text{Drug}]_{\text{free}}$, $[\text{Ligand}]_{\text{free}}$ and $[\text{Drug}]_{\text{complex}}$ (m molecules of drug, n molecules of ligand) are the equilibrium molar concentrations of the free drug, the ligand and the drug in the complex form, respectively. Often, it is impossible to separate the individual binding constants and the apparent binding constant (κ_{app}) is used [eq. (22)].

$$\kappa_{\text{appm:n}} = \frac{[\text{Drug}_{\text{m}}\text{Ligand}_{\text{n}}]_{\text{complex}}}{[\text{Drug}]^{\text{m}}[\text{Ligand}]^{\text{n}}} \tag{22}$$

The total solubility of the drug in the presence of ligand is the sum of the intrinsic solubility of the drug in the absence of the ligand and the solubility of the drug in the ligand(s) [eqs. (23) and (24)].

$$[\text{Drug}]_{\text{total}} = [\text{Drug}]_{\text{intrinsic}} + \tau[\text{Ligand}]_{\text{total}} \tag{23}$$

$$\tau = \frac{\text{m}\kappa_{\text{appm:n}}[\text{Drug}]^{\text{m}}_{\text{intrinsic}}}{1 + \kappa_{\text{appm:n}}[\text{Drug}]^{\text{m}}_{\text{intrinsic}}} \tag{24}$$

A plot of $[\text{Drug}]_{\text{total}}$ versus $[\text{Ligand}]_{\text{total}}$ gives an intercept of $[\text{Drug}]_{\text{intrinsic}}$ and a slope τ. According to the above equation, the total solubility of a drug undergoing complexation is a linear function of the ligand concentration. The intercept of this line is equal to the solubility of the free drug and its slope is given by τ. Rearrangement of the equation allowed the calculation of the apparent binding constant, $\kappa_{\text{appm:n}}$ [eq. (25)].

$$\kappa_{\text{appm:n}} = \frac{\tau}{\text{m}[\text{Drug}]^{\text{m}}_{\text{intrinsic}} - \tau[\text{Drug}]^{\text{m}}_{\text{intrinsic}}} \tag{25}$$

The value of κ is a measure of the strength of the drug-ligand interactions and is dependent on the properties of the drug and the ligand molecules. For a particular ligand, the size, shape, aromaticity and the nonpolarity of the drug molecule play important roles in determining this strength. The properties of the solubilizing medium, such as temperature and polarity also influence the strength of these interactions (48–50).

Complexation of lomefloxacin with five metal ions (Al^{3+}, Ca^{2+}, Mg^{2+}, Bi^{3+}, and Fe^{3+}) was found to increase solubility of lomefloxacin (50). The stoichiometrics of the various complexes were different. In the presence of 0.25 M Ca^{2+} ion, solubility of lomefloxacin was raised by two to threefold at pH 5, while 0.25 M Al^{3+} increased the solubility by nearly 30 fold. The stability constants were determined from the solubility, which ranged from 11.2 for L:Ca^{2+} complexes to 2.34×10^{10} for L:Al^{3+} complexes. The authors concluded that the higher order of stability for lomefloxacin-Al ion complex was related to the higher charge density of the metal ion.

Hydrotropic agents (hydrotropes) have been used to increase the water solubility of poorly soluble drugs, and in many cases, the water solubility has increased by orders of magnitude (51). Several hydrotropic agents such as urea, caffeine and other xanthine derivatives, tryptophan, sodium benzoate, PABA-HCl, Procaine-HCl and nicotinamides have been identified. Solubilization diagram for riboflavine exhibits a positive deviation from linearity, which implies a greater solubilizing power at higher concentrations of PABA-HCl and is characteristics of hydrotropic solubilization (52). In the study to increase the solubility of paclitaxel, 5.95 M of *N*,*N*-doethylnicotinamide was found to raise the solubility by 1700 fold (from 0.30 μg/mL to 512 mg/mL or 0.6 M). The authors indicated that an effective hydrotropic agent should be highly water soluble while maintaining a hydrophobic segment (51). Almost all highly effective hydrotropic agents have a pyridine or a benzene ring in their structure.

Complexation of a drug molecule with a ligand molecule reduces the exposure of former's hydrophobic region to water resulting in an increase in its solubility. The practical and phenomenological implications of phase-solubility analysis were developed by Higuchi and Connors in their pioneering work published in 1965 (53). On the basis of the shape of the generated phase-solubility relationships, several types of behaviors can be identified (Fig. 7). The two major types are A and B. Only A-type of profile will be discussed in this Chapter.

In an A-type system, the apparent solubility of the substrate increases as a function of CD concentration. In A_L subtype, the solubility is increased linearly as a function of solubilizing concentration. A_P system indicates an isotherm wherein the curve deviates in a positive direction from linearity and the A_N system indicates a negative deviation from linearity. The equations related to complexation with cyclodextrin were presented in the previous section except that the ligand is replaced with cyclodextrin.

The use of CDs to enhance solubilization of a poorly soluble drug is often preferred to organic solvents (54). As a solution is administered, both the drug and CD concentration are reduced in a linear manner making precipitation is less likely. Drug release from parenteral administration of CD complexes is thought to be associated with complete and almost instantaneous dissociation via the dilution of the complex (49). For strongly bound drugs, or for those cases where dilution is minimal, contributions from competitive displacement by endogenous materials, drug binding to plasma and tissue components, uptake of the drug by tissue not available to the complex or CD, and CD elimination may also be important (55). In ophthalmic applications where the possibility for dilution is more limited, factors associated with partitioning and secondary equilibria may be the main mechanisms for drug release.

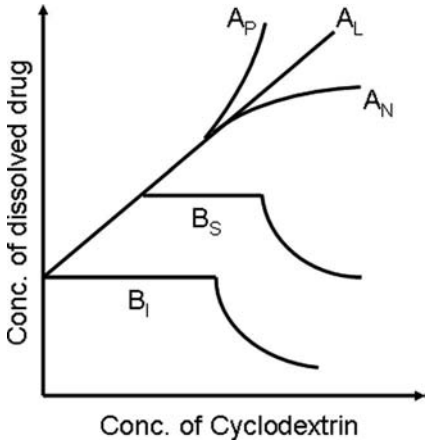

Figure 7 Graphical representation of A- and B-type phase-solubility profiles with applicable subtypes (A_P, A_L, A_N, and B_S, B_I). *Source*: From Ref. 53.

Inclusion complexation is restricted to drugs that have a hydrophobic region that can be inserted into a cavity that has the fixed dimensions. For α-, β-, and γ-cyclodextrins, the cross section of the solute protrusion must be less than 6, 8, and 10 Å, respectively. The αCD can preferentially accommodate aliphatic chains, and the βCD accommodates aromatic rings most efficiently. Fused ring or branched compounds can often best accommodate in the larger γCD cavity. Modified cyclodextrins are very water soluble and form moderately nonviscous solutions (1). Because of the large molecular weight and relatively high cost of cyclodextrins, their use is generally limited to solutes for which a low molar solubility is desired.

Cyclodextrins are cyclic oligosacchrides derived from starch containing six (αCD), seven (βCD), eight (γCD), nine (δCD), ten (εCD) or more (α-1,4)-linked α-D-glucopyranose units (54). In addition to increase the aqueous solubility of poorly water-soluble drugs and stability, CDs can be used to reduce or prevent irritation and prevent drug-drug interactions (56). The central cavity of the CD molecule carries lipophilic characteristic (57). In aqueous solution, the hydroxy groups form hydrogen bonds with the surrounding water molecules resulting in a hydration shell around the dissolved CD molecule (54). In general, the natural cyclodextrins exhibited less than 10-fold improvement in the solubility of compound.

The rates of formation and dissociation of drug:CD complexes are very close to diffusion rate-controlled with drug: CD complexes continuously being formed and broken apart (55). The equilibrium constants were reported to have a mean value of 130, 490 and 350 M^{-1} for αCD, βCD and γCD (58). A marketed parenteral solution, Caverject Dual® (alprostadil IV solution), contains αCD in which αCD is mainly excreted unchanged in the urine after IV injection and it has a higher solubility of 145 mg/mL at 25°C in water (59). βCD is limited in its parenteral application by its low aqueous solubility of 18.5 mg/mL at 25°C and adverse nephrotoxicity.

The natural CDs and their complexes are of limited aqueous solubility. Substitution of the hydrogen bond-forming hydroxyl groups results in improvement in their aqueous solubility. Modified CD include the hydroxypropyl derivatives of βCD (HPβCD) and γCD (HPγCD), the randomly methylated βCD (RMβCD) and sulfobutylether βCD (SBEβCD) (54). The modified cyclodextrin has been reported to increase solubility of progesterone by 3600 fold in with 300 mM of HPβCD (60). HPβCD and SBEβCD are considered nontoxic at low to moderate i.v. doses (54). HPβCD and SBEβCD are much more water soluble than natural βCD and have been used in several parenteral products, including Itraconazole (Sporanox) and Voriconazole (Vfend®, containing 16%w/v SBEβCD). After i.v. injection, HPβCD is almost exclusively eliminated through the kidneys. HPγCD has been incorporated in an eye drop solution and a parenteral diagnostic product.

Cyclodextrins can be used in combination with pH adjustment for synergistic drug solubility enhancement, according to the following equation [eq. (26)].

$$[\text{Drug}]_{\text{total}} = [\text{Drug}_u] + [\text{Drug}_i] + [\text{Drug}_u\text{CD}] + [\text{Drug}_i\text{CD}] \quad (26)$$

Where $[\text{Drug}_u\text{CD}]$ is unionized drug-cyclodextrin complex, and $[\text{Drug}_i\text{CD}]$ is ionized drug-cyclodextrin complex. The synergistic effect is generated because of the ionized drug-ligand complex $[\text{Drug}_i\text{CD}]$, which is absent in situations where pH adjustment or cyclodextrin is used alone (61). The interactions of charged and uncharged drugs with neutral (HPβCD) and anionically charged (SBEβCD) modified β-cyclodextrins have been studied (62). The authors found the binding constants for the neutral forms of the drugs to be greater with SBEβCD than with HPβCD. For the anionic drugs, the binding constants between SBEβCD and HPβCD were similar, while the binding constants for the cationic agents with SBEβCD were superior to those of HPβCD. Therefore, a clear charge effect on complexation, attraction in the case of cationic drugs and perhaps inhibition in the case of anionic drugs, was seen with the SBEβCD.

Micellar

If a drug is not solubilized by aqueous pH-modification, cosolvents, complexation, or combinations of these, surfactants are often used. The formulations are usually concentrated drug solutions in water-miscible organic solvent(s) that are diluted prior to intravenous administration (4). Water-miscible surfactant molecules contain both hydrophilic and

hydrophobic portions which self-associate to form micelles once the surfactant monomer concentration reaches the critical micelle concentration (CMC). Surfactants in parenterals can increase drug solubility through micellization, improve drug wetting, prevent drug precipitation upon injection, improve the stability of a drug in solution, modulate drug release or to prevent aggregation due to liquid/air or liquid/solid interfacial interactions (63).

A simple equation illustrates the principle of surfactant induced micellization and its impact on drug dissolution is as follows [eq. (27)].

$$[\text{Drug}]_{\text{total}} = [\text{Drug}]_{\text{aqueous}}(1 + \kappa[\text{Surfactant}]_{\text{m}}) \tag{27}$$

Where $[\text{Drug}]_{\text{total}}$ is the total solubility, $[\text{Drug}]_{\text{aqueous}}$ is the drug aqueous solubility, κ is a distribution coefficient, $[\text{Surfactant}]_{\text{m}}$ is the difference between the surfactant concentration and the CMC. The total drug in solution increases linearly with the linear increase in surfactant concentration once the surfactant concentration exceeds the CMC. While the linear response limits the degree of solubilization, it minimizes the potential for supersaturation or precipitation upon dilution.

The surfactants commonly used for intravenous infusion formulation include cremophor EL, cremophor RH60, and polysorbate 80. The solubilizing solvent is typically a mixture of surfactant and solvent(s) such as cremophor EL/ethanol/propylene glycol. The upper limit of surfactant administered in vivo is 10% for the cremophor EL and up to 25% polysorbate 80 for IV infusion. Cremophor EL is known to have significant side effects such as hypersensitivity reactions and liver damage (64).

Polysorbate 80 is a nonionic surfactant commonly used in parenteral formulations. Chlordiazepoxide (Librium™) comprises 4% of polysorbate 80 along with 20% propylene glycol and is injected undiluted intramuscularly. Quite often the surfactant containing formulation is diluted prior to intravenous administration to reduce toxicity. For example, amidarone hydrochloride has a water solubility of 0.7 mg/mL, is solubilized to 50 mg/mL in Cordarone™ by a combination of 10% polysorbate 80 and pH adjustment to 4.1. It is administered by intravenous infusion after a 25-fold dilution with dextrose 5%. Solutol HS-15 is a newer nonionic surfactant for parenteral formulation. Solutol HS-15 is used up to 50% to solubilize Propanidid, 7% to solubilize Vitamin K_1. Solutol HS-15 has also been used in preclinical formulations to prepare supersaturated injectable formulations of water-insoluble molecules (65).

Emulsions

Highly lipophilic, low melting point drugs can be quite soluble in oils and formulated for intravenous administration by employing an oil-in-water emulsion stabilized by surfactants in interfacial phases. A recent review by Strickley provides an excellent summary of excipients used in commercially available lipid-based formulations (4). Emulsions typically contain 10% to 20% oil and 2% glycerol for isotonicity, 1% phospholipid surfactant (e.g., lecithin), at pH 7 to 8 and an oil-soluble drug partitioned into the oil phase. The surfactant is applied to provide an energy barrier to agglomeration of the emulsion droplets. Lipid-based systems can exist in a wide variety of microstructures depending on the components used and their concentration, such as w/o or o/w emulsion and microemulsions, micelles, reverse micelles, bicontinuous phases, or mesomorphous pases (66). The solubilization capacity and drug release rate of the active molecules are related to the microstructure. Understanding solubility in lipid mixture is complicated by the fact that these systems are strongly affected by their interfacial nature, the nature of the oil, surfactant, cosurfactant, the size of the droplet and the preferred location of the drug within the system (67). The unique structural organization of the microemulsion results in additional domains which may increase their solubilization capacity as compared with nonstructured solutions containing the same fraction of components.

A marketed emulsion in the United States, Diprivan®, in which propofol, a water-insoluble compound is solubilized to 10 mg/mL in an emulsion composed of 10% soybean oil, is administered by IV bolus or IN infusion (4). There are other commercial emulsions in Europe and Japan, including diazepam, PGE1, dexamethasone palmitate and flurbiprofen.

Emulsions are being prepared with an energy input, such as ultrasonication, homogenization, or high-speed stirring and are thermodynamically unstable because of high

interfacial energy. Stabilization hinges on the ability to reduce interfacial tension, forming an interfacial film barrier to kinetically impede coalescence of droplets. There are four types of stabilizing agents: inorganic electrolytes, surfactants, macromolecules and solid particles. Detailed discussion is available elsewhere (68).

Microemulsions are a thermodynamically stable isotropically clear dispersion composed of a polar solvent, an oil, a surfactant, and a cosurfactant. The potential to form self-emulsifying drug delivery systems was evaluated by Pouton in 1985 (69). Recently, development of injectable microemlsuions has received considerable attention for IV delivery of drugs because of its potential to increase solubility (e.g., solubility of felodipine was increased by 10,000 fold in the microemulsion), reduce toxicity and hypersensitivity, reduce pain upon injection, as a long circulating formulation for drug targeting, and as a depot for IM delivery of drugs (70–72).

Microemulsions offer many advantages compared with macroemulsions: smaller particles (often <100 nm), require less energy to process and have higher physical stability (73). Microemulsions generally have very low interfacial tension at the water-oil interface, and form a highly fluid interfacial surfactant film. Because of the numerous small droplets, the surface area to volume ratio of microemulsions are very high and it forms easily because of the low surface tension, typically due to high levels of surface active species.

Most drugs that can be formulated in emulsions are generally liquids or low melting solids that have high octanol-water partition coefficients (74). In the Diprivan emulsion, Propofol has a high solubility in vegetable oil (>0 mg/mL), a low melting point of 18°C, and a large octanol-water partition coefficient (log*P* 3.83 in pH 6–8.5). Drugs with moderate to high melting point often cannot be formulated as emulsions because of the high lattice energy and low solubility in oil. High melting drugs possess some degree of polarity (i.e., presence of permanent dipoles and ability to form hydrogen bond), and these strong intermolecular forces cannot be readily overcome by the weak dispersion forces operating between solute and oil. Malcolmson studied the effect of oil on the solubility of testosterone propionate in nonionic o/w microemulsions and reported that larger molecular volume oils such as triglycerides miglyol 812 significantly increased the solubility of the compound over the corresponding micellar solution (75).

Predicting the solubility in lipid emulsions may be quite complicated because of the interfacial nature of the systems and the distribution of the drug in the continuous or dispersed phase and sometimes preferred location at the surfactant interface (67). If the drug preferentially resides at the interface in microemulsions, the creation of a larger interfacial area upon mixing the components may result in higher solubility. Testard studied the solubilization of a lipophilic molecule, lindane, in a microemulsion with a nonioinc surfactant. They found the solubility of lindane increased in the microemulsion region compared with the bulk oil; it was attributed to the incorporation of lindane in the surfactant interface (76). Addition of an amphiphilic block copolymer to medium chain surfactants has been shown to favorably alter the interfacial structure and significantly boost the solubilization capacity of microemulsions (77).

Surfactants are added to emulsion systems to reduce interfacial tension, reduce initial droplet size and size distribution, draw a liquid fill between droplets in areas where film thinning may have occurred, impart steric stabilization and in the case of charged surfactants give rise to charge distribution. The presence of surfactant and cosurfactant could make microemulsion supersolvents for drugs relatively insoluble in both aqueous and hydrophobic solvents (78). Using mixed oils and/or mixed surfactants in microemulsion may offer significant advantages over using pure single component materials (79). Prediction of absolute solubility in lipid vehicles is difficult since it requires similar knowledge as needed for aqueous solubility prediction, but also knowledge of the drug's specific interactions between the solute and formulation components, including an understanding of the lipid microstructure (67).

Liposome

Liposome formulations can be used as a means to solubilize some drugs for intravenous administration, to improve pharmacokinetics, enhance efficacy, and reduce toxicity (4). Liposomes are closed spherical vesicles composed of one or more bilayers of amphipathic lipid molecules enclosing one or more aqueous core compartments (80). Moderately hydrophobic

drugs can be solubilized by liposomes if the drug becomes encapsulated or intercalated within the liposome. Hydrophobic drugs can also be solubilized by liposomes as an integral part of the lipid bilayer. Water-soluble drugs reside within the aqueous inner core and are released as the liposome erodes in vivo or by leakage. A typical liposome formulation contains water with phopholipid at ~5 to 20 mg/mL, an isotonicifier, a pH 5 to 8 buffer, and potentially cholesterol.

Liposomes are injectd either by IV infusion or intrathecally. Upon IV administration, most conventional liposomes are easily taken up by the reticuloendothelial system (RES, in the body. There are several liposome formulations on the market. Amphotericin B, a compound with low aqueous solubility of ~0.1 mg/mL at pH 2 (anion) or pH 11 (cation), is solubilized to 5 mg/mL by liposomal intercaltion and becomes an integral part of the lipid bilayer (81). The amphotericin B liposomal products are being administered by IV infusion and have a longer in vivo half-life. Upon formulation in liposomes, paclitaxel, a low solubility drug (< 2 μg/mL), has been reported to achieve a solubility of 3.39 mg/mL in a liposomal formulation of polyethylene glycol 400, soybean phosphatidylcholine (PC) and cholesterol (82). Liposomes can be classified on the basis of liposome size or lamellarity as multilamellar large vesicles (MLVs), small unilamellar vesicles (SUVs), and large unilamellar vesicles (LUVs).

The lipids normally used are the unsaturated PC, phosphatidic acid (PA), phosphatidylglycerol (PG), and the saturated lipids L-a-dimyristoylphosphatidylcholine (DMPC), dipalmitoyl phosphatidylcholine (DPPC), dipalmitoyl phosphatidic acid (DPPA), and L-a-dimyristoylphosphatidylglycerol (DMPG). ABELCET® is an example of MLV consists of amphotericin B complexed with DMPC and DMPG in a 1/0.7/03 molar ratio. The complex assumes a flattened, ribbon-like mutilamellar structure with a particle size ranging from 1600 to 11,000 nm. Upon administration, ABELCET exhibits large volume of distribution, high clearance from blood and long terminal elimination half-life.

Large unilamellar liposomes (LUV) refer to vesicles > 100 nm in diameter bounded to single bilayer membrane. LUV provides higher encapsulation of water-soluble drugs, economy of lipids and reproducible drug release rates; however, these LUV liposomes are difficult to produce. Small unilamellar liposomes (SUV) are formed by dispersing multilamellar vesicles into water using sonication, extrusion through filters of various pore sizes, or homogenization to form optically clear suspensions. AmBisome® is an example of closed fluid-filled unilamellar bilayer liposomes made of a single phospholipid bilayer with amphotericin B intercalated within the membrane at drug:lipid molecular ratio 1:9, and particle size 45 to 80 nm. Upon injection, AmBisome exhibits smaller volume of distribution than the multilamellar ABELCET. Several excellent reviews on liposome technology and its application have been published (83,84).

Combined Solubilization Strategies

Various methods have been reported to enhance solubility of poorly soluble compounds by utilizing a combination of more than one of the solubilization techniques (54,85,86).

Combined use of pH with surfactants was reported to significantly increase drug solubility. The total solubility of a weak electrolyte undergoing ionization and micellization can be accounting for the free unionized drug D_u, free ionized drug D_i, micellized unionized drug D_uM, and micellized ionized drug D_iM as equation (28).

$$[\text{Drug}]_{\text{total}} = [\text{Drug}_u] + [\text{Drug}_i] + K_u[\text{Drug}_u][M] + K_i[\text{Drug}_i][M] \tag{28}$$

where K_u and K_i are the micellar equilibrium constants for the unionized and ionized drug, respectively. This equation is valid for surfactants that are either neutral or completely ionized in the pH range studied. Li discussed this approach using polysorbate 20 on flavopiridol, a weakly basic compound with an apparent pK_a of 5.69 and a low intrinsic solubility of 0.025 mg/mL for its zwitterionic form (87). The solubility of flavopiridol in 10% polysorbate 20 solution at pH 4.3 (27.3 mM) is much higher than that could be expected by increasing the total solubility through appropriate pH adjustment from pH 8.4 and solubilization of the unionized drug in the micelles (3.3 mM). The authors pointed out that high solubility of the ionized drug in the micelles is the source of synergism for solubility enhancement in the

pH-surfactant solutions. Furthermore, this formulation does not precipitate upon dilution with isotonic Sorensen's phosphate buffer.

Combination usage of pH control and cosolvent has been reported to increase solubility of flavopiridol (87). Since solubility of the unionized form is pH independent, the authors concluded the higher total solubility at low pH is attributed to the solubilization of the ionized species by the cosolvent. The pH related solubilization produced by cosolvent can be described by equation (29).

$$[\text{Drug}]_{\text{total}} = [\text{Drug}_u]10^{\sigma_u f} + [\text{Drug}_u]10^{(pK_a - pH)}10^{\sigma_i f} \quad (29)$$

Where f is the volume fraction of cosolvent, σ_u and σ_i are the solubilizing powers of the cosolvent for the unionized and the ionized species, respectively.

Redenti reported that hydroxylcarboxylic acids (such as citric acid, lactic acid, malic acid, tartaric acid), or bases (such as tromethamine, diethanolamine, triethanolamine) can be used in drug-cyclodextrin solutions to enhance drug solubility by several orders of magnitude through formation of a "multicomponent complex" while that of cyclodextrin can be enahanced more than10 fold (54). The synergistic effect was rationalized due to the specific interaction of the hydroxyl acid groups with the hydrogen bond system of the host and/or the modification of the hydrogen bond network of the surrounding water molecules. Astemizole, upon βCD multicomponent complexation with tartaric acid, achieved 27,600-fold enhancement of solubility. The resulting amorphous complex dissolved rapidly and generated supersaturation that remains stable for several days.

Loftsson reported that addition of small percentage of hydrophilic polymers in cyclodextrin-based formulation can further enhance drug solubility (88). With the addition of 0.25% polyvinylpyrrolidone, the solubility of a number of compounds was increased from 12% to 129% in a 10% (w/v) HPβCD vehicle. The authors suggested that the polymer increased the stability constants of the drug-cyclodextrin complexes because of increased negative enthalpy change together with an increased negative entropy change.

Pitha reported that gradual addition of ethanol decreased and eventually abolished the formation of inclusion complexes of testosterone with HPβCD in aqueous solutions (89) (Fig. 8). Initially, at ethanol concentration $<30\%$, the solvent acted as a competing for the cavity of HPβCD and reduced the solubility of testosterone; at higher ethanol concentrations

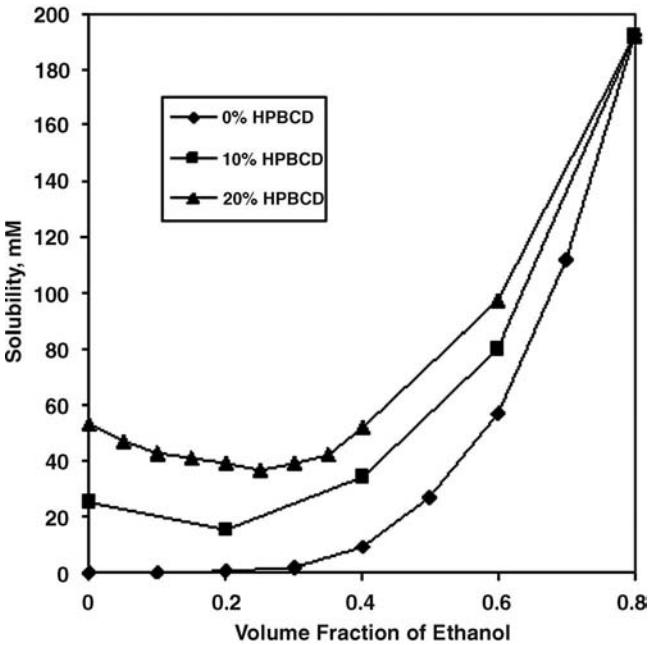

Figure 8 Effect of ethanol on solubilization of testosterone into aqueous solution containing hydroxyl-β-cyclodextrin. *Source*: From Ref. 89.

the solubility of testosterone started to rise, in which the dissolution primarily occurred through nonspecific solvent effects.

The effect of pH variation on complexation and solubilization of naproxen (a weak acid with pK_a 4.2) with natural βCD and various neutral, cationic and anionic βCD derivatives, and hydrophilic polymers has been investigated (86). The authors found the presence of 0.1% PVP increased the solubility of naproxen in the presence of 25 mM HPβCD complex by approximately 30%, at pH 1.1 and 6.5. This integrated strategy of pH control and polymer addition to the CD complexing medium allows a smaller quantity of CD be used to solubilize a given amount of drug.

Propylene glycol, PEG, ethanol, cremophor EL, cremophor RH60, and polysorbate 80 are water-miscible solvents and surfactants in commercially available injectable formulations. These solvents and surfactants are used in combination with each other, usually as a concentrate for dilution just prior to IV injection (4). In general, the cosolvent increases the CMC of the surfactant and increases solubility of the drug. Paclitaxel, a water-insoluble compound (aqueous solubility of 0.1 μg/mL), is solubilized in Taxol® to 6 mg/mL (i.e., 60,000-fold aqueous solubility) with 51% cremophor EL and 49% ethanol, and is diluted 5 to 20 fold with dextrose 5% or lactated Ringer's prior to administration. The final dosing formulation of Taxol is a micellar dispersion (90). The combination of cremophor EL and ethanol has also been used to solubilize teniposide, valrubicin, tacrolimus and cyclosporin.

Trace amount of polymer may decrease the precipitation rate (91), stabilize micelles and other type of aggregates in aqueous solutions and increase the solubility of the compounds by about twofold (92). Water-soluble polymers not only solubilize βCD and its complexes, but they are also able to enhance formation of complexes between drugs and βCD (54). Quarternary complexs of drug, cyclodextrin, polymer and tartaric acid have been reported to further enhance drug solubility (93). However, contrary results have been reported that formation of polymer/cyclodextrin complexes reduced the ability of the cyclodextrin to solubilize drug through complexation (54).

SUMMARY

The decisions regarding solubilization strategy often reside in the intrinsic solubility of the drug, solubilization capacity of the particular strategy, dose of drug to be delivered, infusion time, and potential safety concerns with the excipients, all coupled with the therapeutic area and unmet need. Technologies such as cosolvency and pH modification (indirectly salts) are often favored because of their very high capacity for solubilization. They typically result in exponential increases in solubility and can be very valuable for very low intrinsic solubility drugs (i.e., less than 10 mcg/mL), leading to apparent solubilities in excess of 50 mg/mL. However, given the exponential nature of solubilization and linear nature of subsequent dilution on administration, they are much more prone to precipitation upon dilution. Other approaches (micellar, complexation, emulsions, liposomes) often have lower capacity, but tend to solubilize in a more linear proportionality to concentration of solubilizer, thus being much less prone to precipitation upon dilution. These more linear alternative equilibrium type approaches are not likely to provide solubilization in excess of 20 mg/mL, often much less.

The risk in any sort of solubilization strategy is the propensity for precipitation upon administration and dilution into biological media. The presence of proteins and lipoproteins upon dilution can often facilitate supersaturation and allow for the time necessary to get further dilution and distribution in vivo. In essence, they often provide alternative equilibria for drug solubilization in vivo. The use of in vitro methods (94) and in vivo methods (95) to explore propensity for precipitation can often be very useful.

Solubility, coupled with dose and therapeutic indication, often define the ability to adequately deliver a drug parenterally. While the thermodynamic solubility ultimately dictates the actual chemical potential of the drug in solution under specified conditions, the total "solubilized drug" probably becomes the more relevant descriptor for drug delivery in parenteral systems. Efforts to solubilize drugs are highly dependent on altering either the conditions of the solvent system, creating alternative equilibria for the drug to reside in, changing the macroscopic solid form of the solute, or actually changing the solute at the molecular level (i.e., creating a new chemical entity). These alterations can increase the

escaping tendency from the solid state, facilitate the cavity formation in the solvent necessary for solute insertion, enhance the level of interactions between the solute and solvent, or simply provide an alternative state in which the molecule can reside. As will be discussed elsewhere in this book, the ultimate success of these strategies resides in the ability to deliver the molecule of interest to the in vivo milieu without deleterious results of precipitation upon administration.

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7 Formulation of depot delivery systems

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INTRODUCTION AND DRIVERS FOR DEPOT DELIVERY

Depot delivery systems, also known as sustained-release systems, are parenteral formulations containing multiple doses of drug that, when introduced into the body, are designed to release the drug over a specified, often prolonged, period of time. Depot formulations come in many forms, designed for several different administration routes, and have been in use for over half a century. In addition to the many depot pharmaceuticals approved for use today, the development of novel systems remains an active area of research because of the ability of depot systems to overcome several well-recognized challenges often associated with conventional delivery. These include variations in drug plasma levels between doses that can lead to adverse effects or compromised efficacy, poor patient compliance due to frequent dosing, and difficulty localizing exposures to the target organ or tissue.

Advantages of Depot Delivery Systems

As earlier chapters in this book have highlighted, parenteral drug delivery can overcome many of the challenges associated with oral delivery of bioactive molecules, including degradation in the gut, low permeation through intestinal mucosa, and high first pass metabolism. It is generally recognized that, for certain therapeutic molecules, such as proteins, parenteral dosing is indeed often the only viable way to deliver pharmacologically relevant doses. At the same time, relative to other routes of administration, injection is invasive and is generally less preferred as a means of administering therapeutics. Depot delivery systems provide one way to mitigate this issue by decreasing the frequency of administration. For example, BYDUREON™, pending FDA approval for the treatment of type II diabetes, promises to reduce the frequency of dosing from twice daily with the current product, BYETTA™, to once weekly with the depot formulation (1).

Certain classes of drugs have relatively narrow therapeutic windows, defined as the concentration in vivo above which a compound is therapeutically effective, but below that at which toxic effects are observed (2). For these drugs, it can be challenging to maintain plasma concentrations within the therapeutic window (Fig. 1). In some cases, such as when the molecule has a very short half life, and is not well absorbed along the length of the GI tract, oral dosing may simply not be feasible. The gold standard for maintaining precise control over plasma drug levels is continuous infusion, typically via the intravenous route (3). Clearly, despite the degree of control it offers, continuous IV infusion is often not practical because of heightened risk and the need for close medical supervision during treatment. Depot delivery systems can avoid the peaks and troughs in plasma concentrations common with conventional dosing, and maintain the plasma concentration within the therapeutic window, by providing an infusion-like profile without the drawbacks of IV delivery.

In some cases, such as cancer treatment, it may be desirable to limit drug exposure to the site of action, and minimize systemic exposure altogether. The GLIADEL® wafer, a depot formulation of carmustine, which is implanted at the surgical site after brain tumor resection, is one example of this approach (4). Intra-articular injection of corticosteroid depots is another example where local effects at the site of action can be maximized relative to systemic effects (5).

Poor compliance is increasingly recognized as a significant factor in the failure of therapy in certain patients and there is an inverse relationship between dose frequency and compliance (6). Schizophrenia is one such example, where compliance rates are estimated at about 50% (7). Depot formulations of antipsychotics were first introduced in the early 1960s, initially for patients with suicidal or violent tendencies, but later became well-accepted as maintenance therapies (7,8). Depot antipsychotics are also reported to reduce the frequency of side effects

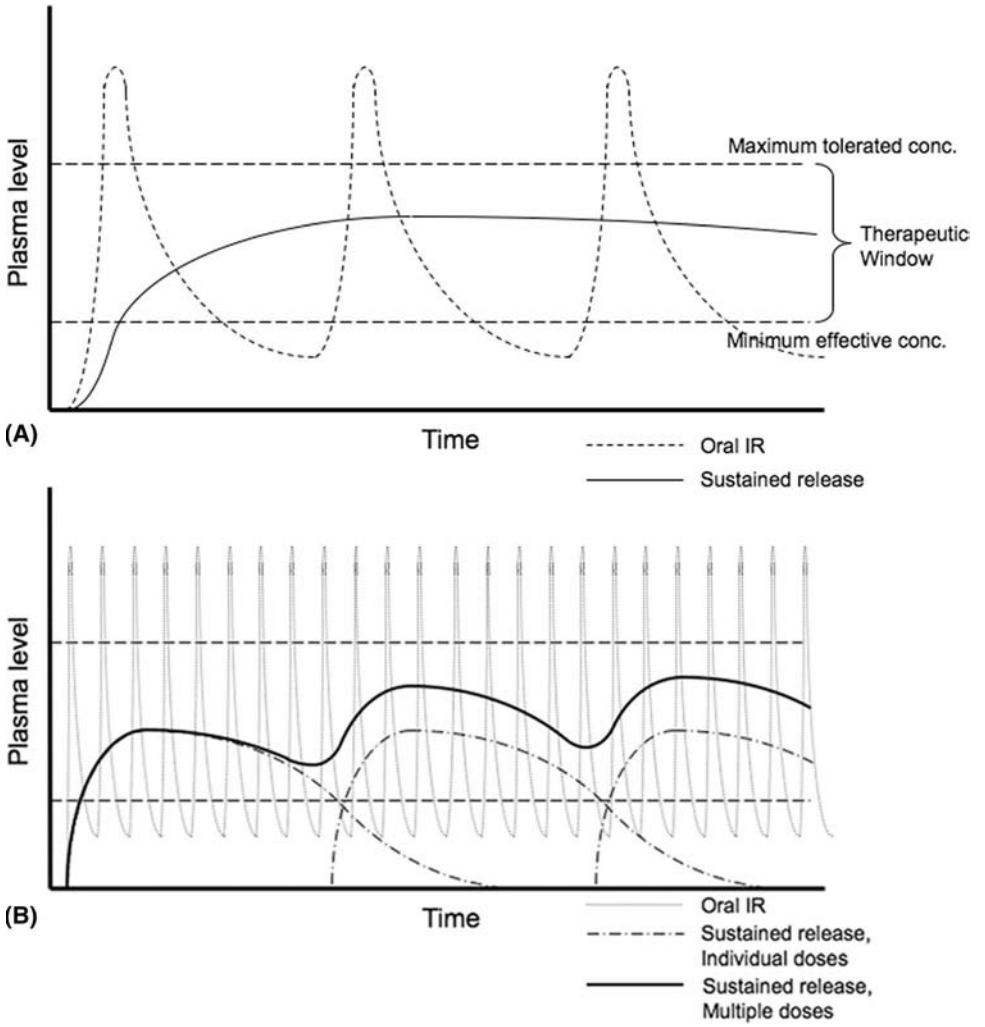

Figure 1 (**A**) An idealized representation of plasma concentration versus time obtained following oral dosing and administration of a sustained-release formulation. Note that oral dosing can result in large variations in plasma concentrations between doses, and that plasma concentrations may not be maintained within the therapeutic window. In contrast, sustained-release formulations are capable of maintaining relatively constant plasma profiles over time. (**B**) Plasma concentration versus time profiles for oral and multiple sustained-release doses over a longer period of time.

(7). RISPERDAL® CONSTA® was the first atypical antipsychotic approved as an injectable depot. As evidence of its impact, sales have grown to $870 million (IMS, 2006).

Despite their many advantages, there are some drawbacks to depot delivery, including difficulty in removing the dose once administered, lack of dosing flexibility, the need for injection or implantation, and potential local adverse tissue reactions (9). These limitations can, in many cases, be managed or overcome. In cases where it is necessary to maintain the ability to cease dosing, nondegradable implant systems can be utilized. Several products, such as Lupron Depot®, include formulations that release for varying periods of time to improve dosing flexibility. Most modern sustained-release formulations can be delivered through conventional needles (although admittedly large-bore by current standards), and the excipients used in the formulations are generally nonirritating. The properties of an ideal depot delivery system include extent and duration of release matched to the needs of the

indication, tolerability and lack of toxicity, and biodegradability (in most cases). Zero order release is often desired, although this can be difficult to achieve in practice, and many products have been commercialized without meeting this criterion. The ability to alter the release rate during administration, while not currently possible in commercialized systems, would also be a desirable option, and is an active area of research.

History and Types of Depot Formulations

Depot formulations have been in use for well over half a century; implantation of testosterone pellets was employed in the 1930s (10). The first widely marketed depot formulations, launched in the 1950s, were injectable intramuscular (IM) suspensions of drugs in aqueous and oily vehicles. A number of additional suspension and oily-vehicle depot formulations, based on poorly soluble alkane ester prodrugs, were developed and launched during the 1960s and 1970s. Use of the biodegradable polyester, poly(lactic-co-glycolic acid) (PLGA), for drug delivery began in the 1970s (11), culminating in the U.S. launch of the PLGA microsphere product, Lupron Depot®, in 1989. In the years following, a number of additional PLGA depot products were launched, including a microsphere formulation of a protein, and extruded PLGA rods. The 1990s saw the introduction of new polymers and lipid-based strategies for sustained-release delivery, as well as the development of implantable device-based depot systems. These strategies have enabled a number of product launches that have continued into recent years.

Requirements for Pharmaceutical Actives Suitable for Depot Delivery

Given the practical constraints and technical challenges associated with developing parenteral sustained-release formulations, pharmaceutical actives must meet certain requirements to be suitable for depot delivery. These requirements vary according to the specific depot strategy selected, but several criteria are general. Most importantly, actives should be potent to allow incorporation of the entire quantity of active needed for dosing over the lifetime of the depot, at a reasonable drug loading within the system. The required potency should be estimated by considering the desired duration of release, and injection volume and drug loading constraints. Note that, when oral PK/PD data exist, it is important to consider the impact that parenteral dosing may have on exposures; this often works to the advantage of the formulator in terms of reduced doses because of absorption limitations and first pass metabolism via oral dosing. Stability is the second criterion, as it is necessary to ensure that the active remains stable not only during the manufacturing process and over the shelf life of the product, but also after administration, within the environment of the body. Stability at body temperature, in an aqueous environment, and in the presence of proteins and enzymes, may become important considerations. Solubility, in aqueous media, solvents that may be used in the manufacturing process, and within the formulation itself, is the third important criterion. Specific solubility requirements will vary according to the formulation approach, and may indeed dictate the formulation strategy. Additional criteria include PK/PD profile (therapeutic window), lack of irritation of the active to local tissues, and the absorption profile of the active. In determining the suitability of an active for depot delivery, it is also important to consider the requirements of the therapeutic area. Therapeutic areas that require extended periods of dosing, high compliance rates, and localized delivery lend themselves to depot formulations. Specific examples of relevant therapeutic areas include hormone therapy (testosterone, estrogen, GnRH antagonists, etc.), corticosteroid treatment, basal insulin delivery, antipsychotics, and contraception.

SUSPENSION AND OILY-VEHICLE DEPOT SYSTEMS

Formulations based on suspensions of drug substance in aqueous or oily vehicles were amongst the first long-acting injectable delivery systems developed (Table 1). These systems rely in large part on the dissolution properties of the suspension particles to govern the release rate from the depot. When the solubility of the drug substance in an oily vehicle allows, an alternate approach is to formulate an oil solution of the drug; in this case the formulator relies

Table 1 A Partial List of Injectable Suspension and Oily-Vehicle Sustained-Release Products Approved in the United States

Trade name	Active	U.S. approval	Excipients (reference number)
Aristocort	Triamcinolone diacetate, 40 mg/mL	1961, Fujisawa	PEG 3350, 3% Tween 80, 0.2% Sodium chloride, 8.5 mg/mL Benzyl alcohol, 9 mg/mL pH ~ 6 (12)
Aristospan	Triamcinolone hexacetonide, 5, 20 mg/mL	1969, Fujisawa	Sorbitol, 50% Polysorbate 80, 0.2–0.4% Benzyl alcohol, 0.9% pH 4.5–6.5
Bicillin C-R	Penicillin G Benzathine, 300,000 U/mL, Penicillin G Procaine, 300,000 U/mL	1953, Wyeth-Ayerst	CMC, 0.55% Lecithin, 0.5% Povidone, 0.1% Methylparaben, 0.1% Propylparaben, 0.01% Sodium citrate pH 6–8.5 (12)
Bicillin L-A	Penicillin G Benzathine, 600,000, 300,000 U/mL	1958, Wyeth-Ayerst	Lecithin, 0.5% CMC, 0.6% Povidone, 0.6% Methylparaben, 0.1% Propylparaben, 0.01% Sodium citrate buffer
Celestone Soluspan	β-methasone sodium phosphate / acetate, 3 mg/mL	1965, Schering	Sodium phosphate dibasic, 7.1 mg/mL Sodium phosphate monobasic, 3.4 mg/mL EDTA, 0.1 mg/mL Benzalkonium chloride, 0.2 mg/mL pH 6.8–7.2 (12)
Cortone	Cortisone acetate, multiple strengths	1950	Sodium CMC, 5 mg/mL Tween 80, 4 mg/mL Sodium chloride, 9 mg/mL Benzyl alcohol, 9 mg/mL (12)
Decadron-LA	Dexamethasone acetate, 8 mg/mL	1973	Sodium CMC, 5 mg/mL Tween 80, 0.75 mg/mL Sodium chloride, 6.7 mg/mL Creatinine, 5 mg/mL EDTA, 0.5 mg/mL Benzyl alcohol, 9 mg/mL Sodium bisulfite, 1 mg/mL pH 5.0–7.5 (12)
Deca durabolin	Nandrolone decanoate, 25, 50 mg	1962, Organon	Arachis oil Benzyl alcohol,10%
Delalutin	Hydroxyprogesterone caproate, 125, 250 mg/mL		Castor oil Benzyl benzoate Benzyl alcohol (13)
Delatestryl	Testosterone enanthate, 200 mg/mL	1953, Squibb	Sesame oil Chlorobutanol, 5 mg/mL (12)
Delestrogen	Estradiol valerate, 10, 20, 40 mg/mL	1954, Squibb	Castor oil Benzyl benzoate Benzyl alcohol Ethanol (13)
Depinar	Cyanocobalamin-Zn-tannate	1980, Armour	Sesame oil Aluminum monostearate, 2% (14)
Depo-Estradiol	Estradiol cypionate, 5 mg/mL	1979, Upjohn	Cottonseed oil Chlorobutanol anhydrous, 5.4 mg/mL
Depo-Medrol	Methylprednisone acetate, 20, 40, 80 mg/mL	1959, Upjohn	PEG 3350, 3% Tween 80, 2 mg/mL Sodium phosphates, 2 mg/mL Benzyl alcohol 9 mg/mL Sodium chloride (isotonic) pH 3.5–7.0 (12)

(*continued*)

Table 1 A Partial List of Injectable Suspension and Oily-Vehicle Sustained-Release Products Approved in the United States (*continued*)

Trade name	Active	U.S. approval	Excipients (reference number)
Depo-Provera	Medroxyprednisolone (progesterone), acetate, 100, 400 mg/mL	1960, Upjohn	PEG 3350, 20–29 mg/mL Tween 80, 2.4 mg/mL Sodium chloride, 8.7 mg/mL Methylparaben, 1.4 mg/mL Propylparaben, 0.15 mg/mL (12)
Depo Sub Q Provera 104	Medroxyprogesterone acetate, 104 mg	2004, Pharmacia and Upjohn	PEG Polysorbate 80 Povidone Monobasic sodium phosphate Dibasic sodium phosphate Methionine Sodium chloride Parabens
Depo-testadiol	Estradiol cypionate, 2 mg/mL, testosterone cypionate, 50 mg/mL	1980, Upjohn	Cottonseed oil Chlorobutanol anhydrous, 5.4 mg/mL
Depo-testosterone	Testosterone cypionate, 200 mg	1979, Upjohn	Cottonseed oil (15)
Ditate-DS	Testosterone enanthate, 180 mg/mL, estradiol valerate, 8 mg/mL	1982, Savage	Ethyl oleate BP (15)
Haldol	Haloperidol decanoate, 50, 100 mg	1986	Sesame oil Benzyl alcohol, 1.2% (12)
HP Acthar	ACTH-Zn-tannate	1952, Armour	Gelatin,16% Phenol, 0.5% (16)
Hydeltra-TBA	Prednisolone Tebutate, 20 mg/mL	1956	Sorbitol Polysorbate 80 Sodium citrate Benzyl alcohol (17)
Hydro-cortone	Hydrocortisone acetate, 50 mg/mL	1951	Sodium CMC, 5 mg/mL Tween 80, 4 mg/mL Sodium chloride, 9 mg/mL Benzyl alcohol, 9 mg/mL (12)
Kenalog-10, 40	Triamcinolone acetonide, 10, 40 mg/mL	1960	Sodium CMC Polysorbate 80 Sodium chloride Benzyl alcohol (17)
Lantus	Insulin glargine, 100 U/mL	2000, Sanofi-Aventis	Glycerol 85% M-cresol Polysorbate 20 Zinc
Lunelle	Medroxyprogesterone acetate, 25 mg Estradiol cypionate, 5 mg	2000, Pharmacia and Upjohn	PEG, 28.56 mg/mL Polysorbate 80, 1.9 mg/mL Methylparaben, 1.8 mg/mL Propylparaben, 0.2 mg/mL Sodium chloride, 8.56 mg/mL
Percorten	Desoxycortisone pivalate, 25 mg/mL	Ciba	Methylcellulose Sodium CMC Polsorbate 80 Sodium chloride Thimerosal (17)
Plenaxis	Abarelix, 100 mg	2003, Praecis	CMC Reconstituted in sodium chloride
Prolixin Decanoate, 25 mg/mL	Fluphenazine decanoate	1972, Squibb	Sesame oil Benzyl alcohol, 1.2%
Prolixin Enanthate, 25 mg/mL	Fluphenazine enanthate	1967, Squibb	Sesame oil Benzyl alcohol

Table 1 (*continued*)

Trade name	Active	U.S. approval	Excipients (reference number)
Sus-phrine	Epinephrine HCl, 5 mg/mL	1951	Glycerin, 325 mg/mL Thioglycolic acid, 6.6 mg/mL Ascorbic acid, 10 mg/mL Phenol, 5 mg/mL (12)

Note: Note that some of these products have been discontinued. Approval dates were referenced from Drugs@FDA (http://www.accessdata.fda.gov/scripts/cder/drugsatfda/index.cfm?fuseaction=Search.Search_Drug_Name).
Abbreviation: CMC, carboxymethylcellulose.

chiefly on the oil/water partition coefficient and dispersion of the vehicle to govern release. We will discuss both approaches in more detail in this section. A third approach is adsorption of the active component to a solid adsorbent (3); this approach is commonly used in vaccine formulations, but will not be discussed in more detail here. Suspension and oily-vehicle formulations are generally suitable only for compounds with low aqueous solubility. If the aqueous solubility of the drug substance is too high to enable formulation by these approaches, solubility can be reduced by formation of a poorly soluble prodrug. One common approach is esterification with alkanes (e.g., to form enanthates, decanoates, or cypionates), an approach used extensively for hormones such as testosterone (3). Alternatively, poorly soluble complexes or salts can be formed, such as zinc-insulin and carboxymethylcellulose (CMC) complexes (3). Suspension and oily-vehicle depot formulations are most often administered by the IM route, although they can also be administered via the subcutaneous (SC), intra-articular, and intradermal routes.

Physical Stability of Suspensions

Injectable suspensions are dispersions of solid drug in an aqueous or oily liquid vehicle. The most common are coarse suspensions, which typically have a mean particle size of less than about 50 μm to ensure that they can be administered through a suitably sized hypodermic needle, and to slow the rate of settling (18). The rate of settling of particles in a dispersion is governed by Stokes' law.

$$\nu = \frac{\Delta\rho g d^2}{18\eta}$$

where ν is the velocity of sedimentation, $\triangle\rho$ is the density difference between the phases, g is the gravitational acceleration, d is the particle radius, and η is the viscosity of the continuous phase (18). Given the goal of slowing the sedimentation rate, Stokes' law instructs the formulator to decrease the particle size and density difference between the phases, and increase the viscosity of the continuous phase. We will later examine how excipients can be used to accomplish these goals.

Stokes' law assumes uniform and noninteracting particles. In reality, interactions between suspended particles are significant and include attractive van der Waals forces, and repulsive electrical double layer and solvation/hydration forces (19). One of the primary failure modes in the formulation of suspensions is caking, which results from the settling of particles and the formation of a densely-packed layer of solids (17). The distance between particles is sufficiently decreased within the cake so that attractive van der Waals forces dominate and cause irreversible aggregation of the particles, preventing their redispersion. One technique used to prevent caking is to formulate the suspension to flocculate. Flocculated particles interact to form a loosely aggregated structure, where interparticle distances are sufficiently large that the system is easily resuspended (e.g., by brief shaking). Formulation at the secondary minimum of the potential energy function can maximize the stability of the flocculated system (18).

A second failure mode, particularly in nanoparticulate systems, is particle growth over time through Ostwald ripening. This phenomenon is described by the Ostwald-Freundlich equation.

$$\ln \frac{C_1}{C_2} = \frac{2M\gamma}{\rho RT}\left(\frac{1}{r_1} - \frac{1}{r_2}\right)$$

where C_1 and C_2 are the saturation solubility at the surface of particles of radius r_1 and r_2, respectively. M is molecular weight, γ is the surface energy of the solid in contact with the solution, ρ is the density of the solid, R is the gas constant, and T is absolute temperature (17). The phenomenon is driven by the higher saturation solubility at the surface of small particles relative to larger ones, as a result of curvature effects. Drug therefore dissolves from the surface of small particles, diffuses to the vicinity of larger particles where saturation solubility is exceeded, and deposits on to the surface of the larger particles, causing a net upward shift in particle size of the system. It must always be appreciated that micro- and nanosuspension systems are thermodynamically unfavorable, and that one must rely on slowing the kinetics to ensure physical stability of these formulations.

Formulation of Parenteral Suspensions

The ideal parenteral suspension is easily resuspended with mild shaking and does not cake upon storage, does not settle rapidly and remains homogenous long enough to allow reproducible dosing, maintains stability and elegance over its shelf life, maintains sterility during storage and use, and is easily administered through a 20- to 25-gauge needle (17). Like all formulations, the formulation of injectable suspensions should begin with a thorough preformulation characterization including solubility in water over a range of pH and in the presence of stabilizing surfactants and polymers, chemical stability in both solid state and solution, and full characterization of drug forms including polymorphs, hydrates and solvates. Drug form can significantly influence the rate of absorption from the injection site (20). After IM administration, aqueous suspensions tend to form a loose agglomerate within the fibrous or membranous tissues between muscle fibers, while the vehicle is rapidly absorbed (21).

Particle Size

Drug particle size can have a significant impact on formulation physical stability, syringeability, and release rate, and therefore should be well characterized and controlled through approaches such as controlled crystallization or milling (17). It is important that the process used provide a narrow particle size distribution to minimize Ostwald ripening, and that the potential for form change, for example, to the amorphous form, be well-understood given the potential adverse impact on physical stability (22). Particle size reduction techniques include jet milling, spray drying, and supercritical fluid processing (18). Wet media milling can be used to generate nanocrystalline dispersions (23): the Elan NanoCrystal® technology is used in Janssen's product INVEGA SUSTENNA™. Compared with coarse suspensions, reduction of particle size to the submicron range enhances physical stability (reduced settling rate), homogeneity, syringeability (reduced viscosity), and options for sterilization (23).

Particle size has a significant effect on syringeability, and it is critical to evaluate suspension systems for syringeability and injectability. A typical recommendation to prevent particle "bridging" that could lead to clogging, is to limit the size of the largest particles to no larger than one-quarter to one-third the inner diameter of the needle (17). The viscosity of the formulation should be optimized to ensure a balance between physical stability of the suspension and syringeability. Thixotropy and shear-thinning behavior can be leveraged to accomplish both goals, as in the case of penicillin G procaine suspensions (24).

The impact of particle size on release rate and pharmacokinetics has been the subject of a number of published studies. Procaine penicillin G aqueous IM suspensions demonstrated faster release as particle size was reduced from 60 to 100 mesh to micronized size (25). The trend was similar for oil suspensions, unless the system was gelled by addition of aluminum monostearate, in which case the trend was reversed. In a separate study, in which aqueous

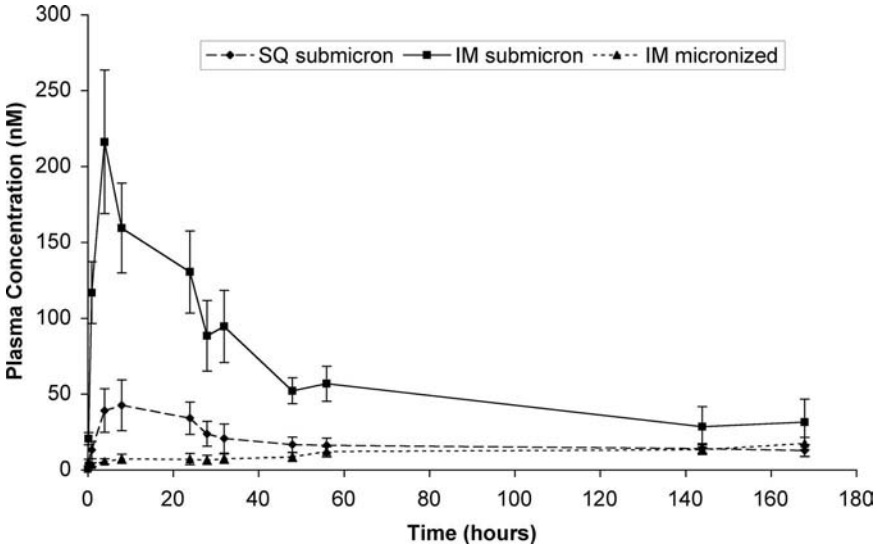

Figure 2 Mean plasma concentration versus time profiles for a poorly-soluble Merck compound after subcutaneous or intramuscular administration to rats of coarse or submicron drug suspension formulations. The composition of the suspension vehicle was the same for both suspensions, as was the dose (20 mg/kg). Data are mean ± SE, $n = 3$–4).

phenobarbital suspensions were administered intramuscularly to dogs, the area under the blood level curve was found to decrease as particle size increased from 6.63 to 29.96 μm (26). Studies of IM aqueous suspensions of model compounds in rats also demonstrated that the absorption rate constant increased with decreasing particle size (21). This effect was particularly apparent as particle size was decreased to 2 to 3 μm or smaller, possibly because of the ability of smaller particles to migrate more easily through the fibrous tissues at the injection site, enabling the depot to spread further following injection. In studies performed in our laboratories on aqueous suspension formulations of a poorly soluble drug, we similarly observed that reduction of particle size led to much faster absorption from a submicron suspension as compared with a coarse suspension (Fig. 2), by IM dosing. We also observed much faster absorption of the submicron suspension when administered by the IM route, compared with the SC route, highlighting the importance of administration route.

Theoretically, the release rate of drug from the depot under sink conditions is given by the following equation:

$$\left(\frac{Q}{t}\right)_{\mathrm{d}} = \frac{S_{\mathrm{a}} D_{\mathrm{s}} C_{\mathrm{s}}}{\delta_{\mathrm{s}}}$$

where Q is the amount of drug released in time t. S_{a} is the surface area of drug in contact with the surrounding fluid, D_{s} is the diffusion coefficient of drug molecules in the fluid, C_{s} is the saturation solubility of the drug, and δ_{s} is the thickness of the hydrodynamic diffusion layer surrounding the solid (3). The faster dissolution of smaller particles is explained by their higher surface area, but this relationship is only relevant if the particles remain at least partially dispersed after administration, as demonstrated by the results of the gelled oil system referenced previously.

Excipients

Earlier in this section we discussed the use of excipients to aid in the stabilization of suspensions. Nonionic surfactants, such as polysorbate 80, are commonly used to wet and sterically stabilize the drug particle surface (18). Povidone and lecithin have been used less

commonly for this purpose (17). Polymers such as CMC and high molecular weight polyethylene glycol (e.g., PEG 3350) are commonly used to increase the viscosity of the continuous phase. Additional excipients may include buffers, antimicrobial preservatives, and electrolytes such as sodium chloride. The latter may be used both to ensure tonicity, and to adjust ionic strength to impact flocculation (18). The total solids content in parenteral suspensions is often limited by the syringeability and injectability of the system, and may impose an upper limit on drug concentration.

Manufacture and Control of Parenteral Suspensions

Suspension formulations can be very challenging to develop and manufacture. They can be prepared either as ready to use suspensions, or as powders for reconstitution. It is typically not possible to sterilize suspension systems by sterile filtration, so they must either be manufactured under aseptic conditions, or terminally sterilized by heat or ionizing radiation. Two processes used to prepare parenteral suspensions are aseptic combination of sterile powder and vehicle, and in situ crystallization from sterile solutions (17). For the latter, sterile powder can be prepared by aseptic antisolvent crystallization, lyophilization, or spray drying (17). Particle size reduction is often required and can be accomplished by milling, and the vehicle is typically sterilized by either filtration or heat sterilization (17). Additional manufacturing considerations include entrapped air and foam, and particulate matter control (17).

Oily-Vehicle Solution Depot Systems

Compounds with low solubility, poor stability, or the potential for causing irritation in aqueous vehicles can be formulated as injectable solutions in oily vehicles. Advantages of this approach relative to suspension formulations include greater ease of manufacture, fewer physical stability concerns, and the ability to sterilize by filtration. Clearly, for this approach to be viable, the active must be sufficiently soluble and stable in the selected vehicle. As for suspensions, hydrophilic compounds can be converted to lipophilic prodrugs for formulation as a depot.

Ideally, oils for use in depot formulations should be chemically stable and inert to reactions with the drug, relatively low in viscosity, physically stable across a wide range of temperature, nonirritating, and free of antigenic properties (27). Oils acceptable for injection include fixed oils such as olive oil, corn oil, sesame oil, arachis oil, almond oil, peanut oil, poppyseed oil, soybean oil, cottonseed oil, and castor oil (28). Vegetable oils, as natural products, contain a variety of triglyceride components, including olein, linolein, stearin, palmitin, and myristin (29). Sesame oil is generally preferred because of its enhanced stability, imparted by natural antioxidants, however, it is light-sensitive (28). Isopropyl myristate, ethyl oleate, benzyl benzoate, polyoxyethylene oleic triglycerides (Labrafils), thin vegetable oil (fractionated coconut oil, Viscoleo) and PEGs are synthetic alternatives (28). Ethyl oleate is sometimes preferred because of lower viscosity. The fixed oils are generally well-tolerated, however, some patients may have allergic reactions to vegetable oils (28). Oily depots are typically administered intramuscularly, as SC injection has resulted in pain and irritation at the injection site (28).

Many oily vehicles are eliminated from the injection site slowly, by dissolution in body fluids or conversion to soluble species, or via the shedding and transport of oil microdroplets from the depot surface (29). Visual observation after IM administration has indicated that oil depots do not spread as extensively as aqueous systems and take on a flattened, pod-like shape (29). This is important because the surface area of the depot is expected to be a key determinant of release rate. The absorption of drugs from oil solutions has been shown to obey first-order kinetics in cases when the absorption of the vehicle is slow relative to the active. In this case, diffusion of the active through the aqueous phase surrounding the depot is rate limiting, and the rate constant is controlled by both the oil/water partition coefficient and the vehicle injection volume. By contrast, the absorption of drugs from oily suspensions can obey zero order kinetics, since the solubility of the drug in the vehicle is maintained at the saturation solubility until the suspension particles have completely dissolved (29).

DEGRADABLE POLYMERIC DEPOT DELIVERY SYSTEMS

Poly(Lactide-co-Glycolide) Systems

Polyesters of poly(lactic acid) (PLA) and copolymers of lactic and glycolic acids, referred to as PLGA, are the most commonly used polymers in biodegradable depot dosage forms. These biocompatible polymers undergo random, mostly nonenzymatic, ester linkage hydrolysis to form lactic acid and glycolic acid, which are normal metabolic compounds in the body. Resorbable sutures, clips and implants were the earliest applications of these polymers (30). The application of PLA and PLGA as biodegradable and biocompatible polymers for drug delivery was initiated in the 1970s (11,31,32). Southern Research Institute developed the first synthetic, resorbable suture (Dexon®) in 1970, and the first patent describing the use of PLGA polymers in a sustained-release dosage form appeared in 1973 (33).

PLGA is synthesized by means of a random ring-opening copolymerization of two different monomers, the cyclic dimers of glycolic acid and lactic acid. Thus, the polymers or copolymers may be produced by the polycondensation of the lactic acid and/or glycolic acid in the presence of an inorganic acid (34). Today, PLGA polymers are commercially available from multiple suppliers, including Boehringer-Ingelheim (Resomer®), PURAC (Purasorb®), Absorbable Polymers International (Lactel®), and Alkermes (Medisorb®). PLGA polymers are available commercially as end capped or acid terminated and with inherent viscosities ranging from 0.15 to 6.5 dL/g (35).

Polymer Selection and Degradation

Understanding the physicochemical and biological properties of a polymer is important prior to selection of a polymer for depot drug delivery. PLGA polymer can generally be characterized by molecular weight (inherent viscosity), polydispersity, lactide to glycolide ratio, and chemistry (end capped vs. acid terminated). The selection of the polymer for depot delivery would depend on the target release profile of the drug, with the drug release mainly governed by the degradation of the polymer. A vast amount of literature is available on the characterization of PLGA, its biodegradation, and drug release properties. The polymer PLA exists in an optically active (L-PLA; semicrystalline) and an optically inactive (DL-PLA; amorphous) form. The amorphous form is preferred, as it enables a more homogenous dispersion of the drug in the polymer matrix (36). The glass transition temperature of the DL-PLA and PLGA is about 30°C to 60°C and is represented by the following equation:

$$T_g = T^0{}_g - \frac{K}{M_n}$$

where $T^0{}_g$ (60.1°C for PLA) is a limiting T_g of a material of infinite MW, M_n is a number average MW, and K (37.1×10^4 °C for PLA) is a constant for the polymer (37,38).

Lactic acid is more hydrophobic than glycolic acid and hence, PLGA polymers rich in lactic acid are more hydrophobic, absorb less water, and degrade at a slower rate (31,39–41). Generally, a bulk erosion mechanism (a homogenous chain cleavage reaction throughout the matrix) has been considered as the main degradation pathway for PLA and PLGA (42,43). However, recent studies on the degradation of various PLGA copolymers have demonstrated a heterogeneous degradation mechanism. The degradation products generated in the interior autocatalytically accelerate the degradation process, because of an increased amount of carboxylic acid end groups and thus, a decrease in the microclimate pH (43–45). Enzyme catalyzed degradation has been hypothesized, but these studies are not convincing (46). The factors that can influence the hydrolytic degradation of lactide/glycolide homopolymer and copolymer include: water permeability and solubility (hydrophilicity/hydrophobicity), chemical composition, mechanism of hydrolysis (noncatalytic, autocatalytic, enzymatic), additives (acidic, basic, monomers, solvents, drugs), morphology (crystalline, amorphous), device dimensions (size, shape, surface to volume ratio), porosity, glass transition temperature (glassy, rubbery), molecular weight and molecular weight distribution, physicochemical factors (ion exchange, ionic strength, pH), sterilization, and site of implantation (47). The kinetics of biodegradation of PLGA microspheres were studied in rats using steroid microspheres prepared with radiolabeled PLGA of varying composition (48–50). The

degradation of PLGA ranged from 10 weeks (50:50 lactide to glycolide) to approximately 30 weeks (87:13 lactide to glycolide), and finally to 45 weeks with 100% lactide (49). PLGA has found application in multiple depot products in the market. Some of the marketed PLGA based depots are summarized in Table 2.

PLGA Microspheres

PLGA microspheres are by far the most commonly used polymer-based injectable depot drug delivery systems, and are advantageous for several reasons. PLGA microspheres are biocompatible, can be easily administered through a syringe, can provide sustained release for prolonged periods of time, and can encapsulate active molecules with wide-ranging physicochemical properties, including small molecules, peptides, proteins and nucleic acids (51).

A number of techniques have been developed for the microencapsulation of drugs, such as solvent evaporation and solvent extraction [oil-in-water (o/w) emulsion, water-in-oil-in-water (w/o/w) emulsion, and solid-in-oil-in-water (s/o/w) emulsion], phase separation or coacervation, spray drying, extrusion, and supercritical fluid based encapsulation. Although each process is associated with certain advantages and disadvantages, in general, the selection of the microencapsulation process is dependent on the nature of the polymer, the drug, the intended use, and duration of therapy (36,41,45–54). The microencapsulation method selected should (41,51,52,55)

- ensure stability or biological activity of the drug;
- yield microspheres in a desired size range (microparticles of size less than 250 μm, ideally less than 125 μm have been determined to be suitable for depot delivery);
- be reproducible with regards to the quality and drug release profile from the microspheres;
- be scalable to support clinical development and commercialization; and
- not exhibit microsphere aggregation or adherence.

A number of proprietary technologies, based on minor variations in the basic encapsulating techniques discussed above, have been developed for preparing microspheres. A brief listing of those technologies is provided in Table 3. We will be discussing the various encapsulation techniques briefly in the following section.

Solvent evaporation and solvent extraction.

Oil-in-water emulsion The o/w single emulsion/solvent evaporation technique is the most favorable technique to encapsulate hydrophobic drugs with poor aqueous solubility but good solubility in water-immiscible organic solvents, such as methylene chloride and ethyl acetate. In this process, the drug and the polymer are dissolved in the organic solvent, followed by emulsification of the organic (oil) phase in water to form the o/w emulsion (Fig. 3A). The water phase generally contains an emulsifier, such as polyvinyl alcohol (PVA) and polysorbate 80 (PS 80). It is desirable that the drug has low solubility in the planned aqueous phase to enhance encapsulation efficiency and yield. The volatile solvent is generally removed by either evaporation to a gas phase (56), which involves prior dissolution into the continuous phase (57), or is extracted into the continuous phase (58,59). The rate of solvent removal from both the evaporation and extraction processes is dependent on the temperature and solubility characteristics of the solvent, polymer and dispersion medium, and in the case of extraction process, on the ratio of the emulsion volume to the quench medium volume (60). Solvent removal by the extraction process is faster than that by the evaporation process, and hence the microspheres made by the extraction process are generally more porous than the ones made by the evaporation process under similar conditions (60).

The o/w method has been used for a large number of drug candidates, such as neuroleptics (thioridazine, chlorpromazine, bromperidol), local anesthetics, diazepam, L-methadone, anticancer compounds (aclarubicin, lomustine, and paclitaxel) and steroids (36,61). It should be noted that for high drug loading formulations, precipitation of the drug

Table 2 Marketed PLGA Depot Products

Product name	Active ingredient	Product owner	Partner	Drug release duration	Comments
Atridox®	Doxycycline hyclate	Tolmar, Inc.	–	1 wk	PLA-based in situ gelling formulation delivered to the gum for the treatment of chronic adult periodontitis. The system consists of 450 mg of the ATRIGEL® delivery system, which is a bioabsorbable, flowable polymeric formulation composed of 36.7% (PLA) dissolved in 63.3% *N*-methyl-2-pyrrolidone. Doxycycline hyclate equivalent to 42.5 mg doxycycline is mixed prior to administration.
Atrisorb FreeFlow-D	Doxycycline Hyclate	Tolmar, Inc.	–	–	PLA-based system for guided tissue regeneration
Decapeptyl SR	Triptorelin acetate	Debiopharm Group	Ipsen (ex–United States)	1, 3 mo	PLGA microspheres containing water-insoluble salts of triptorelin for prostate cancer. Other excipients include mannitol, carmellose sodium, and polysorbate 80.
Eligard	Leuprolide acetate	QLT, Inc.	Astellas Pharma, Inc. (Europe) Sanofi-Aventis (United States, Canada) Sosei (Japan) MediGene AG (Europe) Tecnofarma (Latin America), Luxembourg Pharmaceuticals (Israel), Han All (Korea), Tecnofarma International (Central and South America)	1, 3, 4, 6 mo	PLGA/PLA in situ forming gel administered SC for the treatment of prostate cancer.
Lectrum depot	Leuprolide acetate	Eriochem S.A. (Latin America and Asia)	–	1, 3 mo	Lyophilized PLGA/PLA-based microspheres for uterine leiomynomas, endometriosis, prostate cancer. or precocious puberty.
Leuprolide 14 day	Leuprolide acetate	Oakwood Laboratories, LLC	–	2 wk	PLGA microsphere of GnRH for the treatment of prostate cancer.
Leuprorelin acetate injection	Leuprolide acetate	Dongkook Pharmaceutical Co. (Korea)	–	1, 3 mo	PLGA microspheres for SC administration to treat breast and prostate cancer
Lupride 1 month	Leuprolide acetate	Sun Pharmaceutical Industries (India)	–	1 mo	PLGA microspheres for IM administration to treat prostate cancer and endometriosis.

(*continued*)

Table 2 Marketed PLGA Depot Products (*continued*)

Product name	Active ingredient	Product owner	Partner	Drug release duration	Comments
Lupron depot	Leuprolide acetate	Takeda Pharmaceutical Co. Ltd.	Abbott Laboratories, Inc. (United States, Canada); Orion Corp (Northern Europe)	1, 3, 4 mo	PLGA/PLA microspheres with diluent presented in prefilled syringe for IM administration to treat endometriosis and prostate cancer. The front chamber prefilled dual-chamber syringe contains leuprolide acetate, PLGA/PLA and D-mannitol. The second chamber of diluent contains CMC sodium, D-mannitol, polysorbate 80, water for injection, USP, and glacial acetic acid, USP to control pH.
Lupron depot—PED	Leuprolide acetate	Abbott Laboratories, Inc.	–	1 mo	PLGA microspheres for IM administration to treat central precocious puberty. Presentation similar to Lupron Depot.
Luprorelin/ luposhere 1 month	Leuprolide acetate	Peptron, Inc.	Daewoong Pharmaceutical Co. Ltd. (Korea)	1 mo	PLGA microspheres for SC administration for the treatment of breast cancer, prostate cancer, endometriosis, and precocious puberty.
Nutropin depot	Somatropin (growth hormone)	Genentech (Roche), Inc.	–	2 wk, 1 mo	PLGA microspheres for SC administration to treat growth failure. Supplied in vials containing somatotropin, zinc acetate, zinc carbonate, and PLGA. The diluent is composed of 3% CMC sodium salt, 0.1% polysorbate 20 in normal saline.
Risperdal Consta	Risperidone	Janssen Pharmaceutica Products LP	Janssen-Cilag N.V. (Europe, United Kingdom); Janssen-Cilag N.V. (Japan)	2 wk	PLGA microspheres with 38% drug loading administered IM for the treatment of schizophrenia, bipolar disorder. The microspheres are reconstituted in 2 mL diluent (polysorbate 20, sodium carboxymethyl cellulose, disodium hydrogen phosphate dihydrate, citric acid anhydrous, sodium chloride, sodium hydroxide, and water for injection) prior to administration.
Salvacy/Moapar 3 months	Triptorelin pamoate	Debiopharm Group	European Pharmaceutical Partner	3 mo	Lyophilized PLGA microspheres for SC administration for reversible reduction of testosterone to castrate levels. The diluent consists of 8.5% mannitol USP, 3% CMC sodium USP, 0.2% polysorbate 80 NF.

Sandostatin LAR	Octreotide acetate	Novartis AG	–	1 mo	PLGA microspheres administered IM after reconstitution for the treatment of acromegaly.
Sinofuan	Fluorouracil	Simcere Pharmaceutical Group (China)	–	–	Sustained-release implant for multiple cancer indications.
Somatuline LA	Lanreotide acetate	Ipsen (Europe)	Teijin Pharma Ltd. (Japan)	2 wk	PLGA microspheres for IM administration for the treatment of Acromegaly and for the relief of symptoms associated with neuroendocrine tumors.
Suprefact depot	Buserelin acetate	Sanofi-Aventis	–	2, 3 mo	PLGA-based rods for SC implantation for treatment of advanced prostate cancer.
Trelstar depot Trelstar LA	Triptorelin pamoate	Debiopharm Group	Watson Pharmaceuticals, Inc. (United States), Paladin Labs, Inc. (Canada), Ferring Pharmaceuticals (Asia, Europe), West Pharmaceutical services, Inc.	1 mo (depot) 3 mo (LA)	Lyophilized PLGA microspheres for IM administration for treating prostate cancer. The diluent consists of 8.5% mannitol USP, 3% CMC sodium USP, 0.2% polysorbate 80 NF.
Vivitrol injectable suspension	Naltrexone	Alkermes, Inc.	Janssen Pharmaceutica Products, LP (Russia)	1 mo	PLGA microspheres for reconstitution prior to IM administration with Naltrexone loading of ca. 33% for the treatment of alcohol abuse and opioid dependence. Delivery diluent contains CMC sodium salt, polysorbate 20, sodium chloride and water for injection.
Zoladex implant	Goserelin acetate	AstraZeneca PLC	–	1, 3 mo	Cylindrical 1mm–diameter PLGA-based implant administered SC using a 16G hypodermic needle for the treatment of advanced prostate cancer, breast cancer and endometriosis.

Abbreviations: PLGA, poly(lactide-co-glycolide); PLA, poly(DL-lactide); CMC, carboxymethylcellulose.

Table 3 Proprietary Encapsulation Technologies and Related Patents

Encapsulation technology	Company	Encapsulation process	Reference
PolyShell	Akina, Inc.	Solvent exchange—double emulsion	WO03053325 (A3), EP1404516 (A3)
Injectable depot technology by coacervation	Alkermes, Inc.	Coacervation	US2004228833
Medisorb	Alkermes, Inc.	Solvent evaporation/extraction—emulsion method	US2003113380, US6110921, US5650173
ProLease®	Alkermes, Inc.	Solvent evaporation/ extraction—emulsion method	US6051259
West Pharma injectable depot technology	Archimedes Pharma	Solvent extraction—emulsion method	US5869103
Microcoat	Biotek, Inc.	Solvent evaporation	US4623588
SRI/Brookwood injectable microspheres	Brookwood Pharmaceuticals (now SurModics)	Solvent extraction—emulsion method	US4897268, US5407609
Depocore	CeNeS Pharmaceuticals	Coacervation	US2003180368
Debio® PLGA-2	Debio	O/W emulsion method/phase separation	US5445832
Mimplant microgranules	Debio	Solventfree extrusion process	US6319512
Extruded injectable beads	Debio	Solventfree extrusion process	US5134122
SynBioSys	InnoCore	Solvent evaporation/extraction—emulsion method	WO2005068533, EP1555278
Oligosphere®	MacroMed, Inc.	Solvent evaporation/extraction—emulsion method	US5100669, US5665428
ChroniJect	Oakwood Laboratories	Solvent evaporation/extraction—emulsion method	US5945126
SmartDepot	Peptron	Spray drying	WO2004112752, WO2005023224
TheraPhase ProPhase CoPhase	PR Pharmaceuticals	Solvent evaporation/extraction—emulsion method	US6706289 and family
CriticalMix™	Critical Pharmaceuticals	Supercritical CO_2 without solvents or high temperature	US6414050, US6670407

out of the polymer phase is very likely and thus, understanding the phase behavior of the drug polymer system and kinetics of precipitation, including particle size and polymorphism of the drug, become critical factors influencing drug release from the matrix.

Solid-in-oil-in-water emulsion The s/o/w emulsion technique is applicable when a specific drug is not soluble in the carrier solvent or solvent mixture, or when extensive drug loss to the continuous phase cannot be avoided when employing a cosolvent system. A lot of early research on hydrophobic drug encapsulation (such as norethisterone) as a contraceptive utilized this technique (49). Recently, the s/o/w technique has been used for the encapsulation of hydrophobic drugs such as levonorgestrel (62), β-estradiol (63), haloperidol (64), and camptothecin and its derivatives (65). Since drug particles are encapsulated directly, it is important that the particle size of the drug is small and well controlled. Generally, particle sizes of less than 10 μm, preferably in the 1 to 2 μm range, are desirable to improve drug loading and the uniformity of drug distribution within and amongst microspheres. Besides small particle size of the drug, careful control of drug sedimentation (in the suspension medium) or floatation (due to adhesion of bubbles to hydrophobic surfaces) during the encapsulation process must be achieved. Drug particles adsorbed on the surface of prepared microspheres (especially if the drug particle size is large) could lead to a burst release (63). This

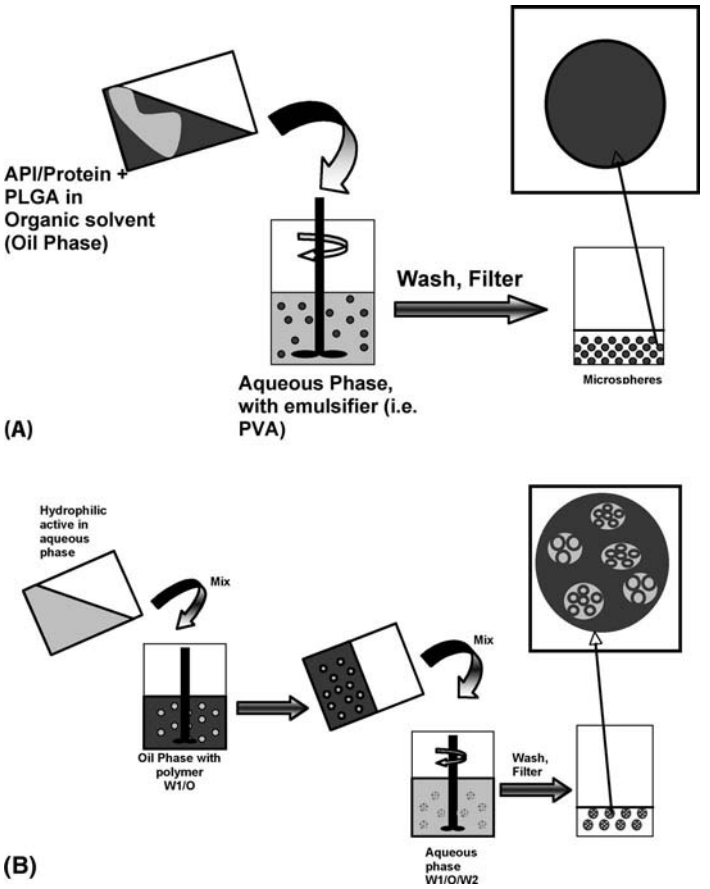

Figure 3 Schematic representations of the (**A**) single oil-in-water emulsion and (**B**) double water-in-oil-in-water emulsion processes for making microspheres.

issue could potentially be alleviated by addition of an extra polymer coating step for s/o/w microspheres, as has been suggested in the literature (66). The s/o/w microspheres tend to form large voids and channels as the drug particles dissolve, leading to better access of the dissolution medium into the microspheres, and resulting in a faster release profile as compared with monolithic microspheres prepared by the o/w emulsion technique.

Water-in-oil-in-water emulsion The $w_1/o/w_2$ encapsulation method is a commonly used method for hydrophilic compounds with high aqueous solubility, such as peptides, proteins, and vaccines (40,41,53). One of the first challenges with this technique was low encapsulation efficiency of hydrophilic molecules, as described by Okada et al. (U.S. patent 4652441), which was overcome by performing w_1 phase solidification. Briefly, the process comprises dissolving the active molecule in a suitable buffer, and then adding this to an organic phase (e.g., dichloromethane) containing dissolved PLGA, under controlled stirring to form the first w_1/o emulsion (Fig. 3B). This emulsion is then introduced with stirring into the second water phase, containing an emulsifier (e.g., PVA) to form the $w_1/o/w_2$ emulsion. The organic solvent is either removed by evaporation (reduced pressure or stirring) or extraction (dilution into a large quantity of water with or without surfactant). The microspheres are then washed, separated (e.g., by filtration, sieving, or centrifugation), and then dried or lyophilized to give the final product. During the development of this technique, various formulation and process variables were evaluated to optimize drug loading, encapsulation efficiency and release profiles (67–71).

Phase separation technique. Unlike the o/w emulsification technique, the phase separation, or coacervation, technique is suited for both water-soluble and water-insoluble drugs. However,

the coacervation process is mainly used for hydrophilic molecules, such as peptides and proteins. The process consists of precipitating (or phase separating) the polymer from the organic solution by the addition of a nonsolvent to yield drug-containing microspheres. In brief, the drug is either dissolved in water and then added to the polymer-containing organic phase (o/w emulsion), or directly added to form a solution in the organic phase. To this, an organic nonsolvent is added with stirring, which extracts the polymer solvent. This leads to phase separation of the polymer to form coacervate droplets, which entrap the drug. The microspheres thus formed are hardened by transferring to a larger quantity of organic nonsolvent, washed, filtered, sieved and dried (54,72). The various factors that influence the final product include addition rate of the first nonsolvent, concentration of the polymer, stirring rate, temperature, or addition of an additive (to alleviate stickiness of the coacervate droplets). Since the process does not utilize addition of an emulsion stabilizer, agglomeration might become a frequent problem.

Melting and spray-drying techniques. Melting and spray drying have been utilized to prepare microspheres in cases where conventional processing methods, such as o/w and w/o/w emulsion, do not provide the required throughput and product stability. Spray drying is rapid, convenient, easy to scale-up, utilizes mild conditions, and is less dependent on the solubility parameter of the drug and the polymer (41,73,74). Compared with the conventional emulsion methods, the spray drying method requires larger batch sizes (limitation if small amounts of bulk available), results in larger losses due to adhesion of microparticles to the apparatus, and is reported to cause agglomeration of the microparticles (74). Modifications to the spray drying technique have been incorporated, such as a double nozzle technique to reduce agglomeration. Spray dried formulations for a range of compounds, such as theophylline, progesterone, and piroxicam, have been reviewed in the literature (36).

Melting is a technique that avoids the use of organic solvents, but requires the dispersion or melting of the drug in a polymer melt. To generate microspheres from this hot melt, a water-soluble polymer that is not miscible with the drug/polymer melt can be employed. The resulting emulsion can be solidified by cooling, and the microspheres can be collected by dissolving the water-soluble polymer matrix in a large volume of water (European Patent EP 934 353). An alternative method is to grind/jet-mill the drug/polymer matrix after cooling (33,61,75). The improvements in this technology have focused on generating more uniform particles by introducing an extrusion step in the process, and getting spherical and smaller particles by emulsification in a hot solution containing an emulsifier (61). Microspheres produced by the melt technique generally lead to nonporous polymer matrices, which subsequently lead to slower release rates from the depot, especially for hydrophobic drugs.

PLGA Gel/Rod Systems

Although microspheres (Fig. 4A) have been predominant, other PLGA-based depot systems have also been developed, including in situ forming gels, and rods for implantation. In this section we will discuss some of the PLGA-based gel and rod systems briefly, and highlight the advantages and disadvantages of such systems.

The in situ gelling systems are presented as liquids or semi-solids with a wide range of viscosity, containing a biodegradable polymer and drug dispersed or dissolved in the liquid phase of the delivery system (solvent/cosolvent system). Upon SC or IM administration, a depot is formed at the site of injection (Fig. 4B). Such systems are usually manufactured through aseptic processing, however, γ-irradiation for terminal sterilization of the product has been evaluated as well. The in situ forming depots have been classified into different categories, depending on the depot-forming mechanism (76,77).

The in situ precipitating system consists of PLGA dissolved in a water-immiscible or partially miscible organic solvent, which also dissolves/disperses the drug to form a solution/suspension. Once administered, the organic solvent escapes, allowing water ingress and precipitation or phase separation of the drug/polymer system, leading to the formation of a depot. Depending on the solubility of the drug in the organic phase, these systems are generally associated with high initial burst. The initial burst is also dependent on the

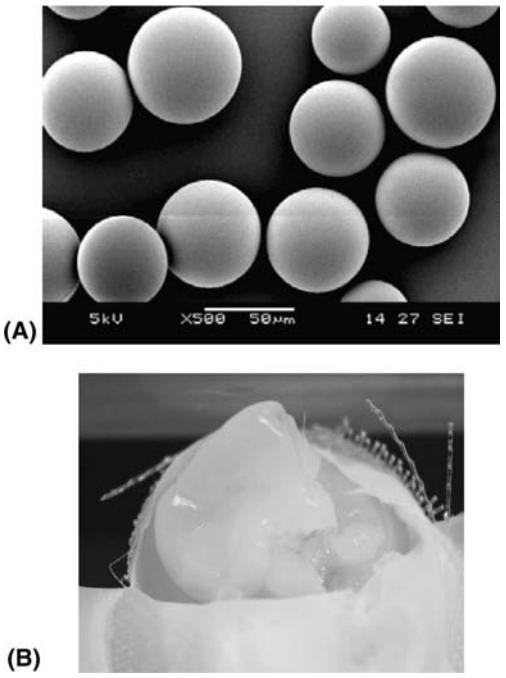

Figure 4 (**A**) Scanning electron micrograph of PLGA microspheres and (**B**) photograph of an in situ forming PLGA gel depot explanted from a rat. *Abbreviation*: PLGA, poly(lactide-co-glycolide).

hydrophobicity and concentration of PLGA, water miscibility of the organic solvent, and the aqueous solubility and loading of the drug. Subsequent drug release from the depot is dependent on the degradation/erosion of PLGA. Eligard®, which uses the Atrigel® technology from QLT, (78) has received regulatory approval. The Atrigel® technology involves the dissolution of polymer and drug in *N*-methyl-2-pyrrolidone, but has also utilized other organic solvents such as propylene glycol, dimethyl sulfoxide, tetrahydrofuran, triacetin and ethyl benzoate to control initial burst. The biocompatibility and systemic toxicity of these organic solvents, when administered intramuscularly or subcutaneously, have been of concern. Alzamer® technology, developed by Alza, also utilizes PLGA as a carrier for in situ depot formation, however, this technology utilizes more lipophilic solvents, such as benzyl alcohol, to reduce irritation and initial drug burst.

Thermally induced gelling systems are exemplified by the water-soluble ReGel® triblock copolymer, composed of the hydrophobic PLGA (A) and hydrophilic PEG (B) blocks in the ABA configuration, which is a solution under ambient conditions, but turns into a gel at body temperature. OncoGel® is a six-week sustained-release depot of paclitaxel that utilizes this system. The release from the ReGel polymer system is controlled by controlling the hydrophilicity/hydrophobicity, molecular weight, concentration and polydispersity of the copolymer (79).

Implantable PLGA-based biodegradable systems have also been explored. Zoladex® is a one- and three-month PLGA depot of goserelin acetate for the treatment of prostrate cancer. Durect is developing the PLGA-based Durin® implant, containing Leuprolide for Alzheimer's disease. Durin is a reservoir-type implant where the drug release is controlled by the drug loading, polymer molecular weight and composition, geometry of the device, and permeability of the membrane (WO03000156 from Southern Biosystems Inc).

Delivery of Proteins and Peptides

Peptides and proteins have become a vital class of therapeutics, however, many issues exist in the delivery of biologically active macromolecules to target tissues. Upon injection, peptides and proteins are rapidly cleared because of proteolytic degradation, efficient renal clearance, neutralization by antibodies, and rapid distribution to tissues outside the blood stream. The

rapid clearance results in the need to dose peptides and proteins on a very frequent basis, which is a painful and inconvenient dosing regimen and often results in poor patient compliance. Several strategies have evolved to overcome the challenge of short half-life, including increasing the molecular size of the protein via conjugation of high molecular weight biopolymers (80) and site-directed mutagenesis to remove proteolytic cleavage sites. Over the last 25 years, much pharmaceutical research has gone into developing improved delivery systems aimed at delivering real patient value by providing another means of overcoming these challenges. Formulation strategies include injection of crystalline or amorphous peptide or protein particles (81), implantable osmotic pump devices, and sustained-release polymeric depot systems.

The development of peptide and protein depot systems can involve significant challenges beyond those typically encountered with small molecules. Polypeptides are inherently unstable because of their physicochemical and biochemical properties, which stem in part from their large molecular size. Quite simply, more can go wrong with larger molecules. Proteins have secondary, tertiary and often quaternary structure that all contribute to the three dimensional orientation necessary for proper protein function. The processes outlined earlier for manufacturing depot systems, which can include high-shear mixing, pumping, organic solvent/aqueous interfaces, surfactants, contact with hydrophobic surfaces, sudden pressure differentials, heat, and drying, are all detrimental to the delicate structure of a protein. The more successful formulation strategies have sought to minimize protein unfolding and aggregation by reducing process stress and carefully considering the additives/solvents used. Additives and solvents can cause protein denaturation by perturbing their physicochemical stability, and the use of solvents is therefore an important consideration for polypeptide depot development (82). In addition to their inherent physicochemical instability, proteins are also sensitive to chemical degradation (83). In particular, asparagine deamidation and hydrolytic cleavage are accelerated as a result of the acidic environment created when PLGA breaks down via ester bond hydrolysis (84,85).

Despite the aforementioned challenges, several peptides are commercially available as sustained-release depots, including leuprolide, triptorelin, histrelin, goserelin and octreotide. Images include biodegradable microspheres and rods, as well as nonbiodegradable polymer rods and titanium-based implantable osmotic pump devices. Once-monthly Lupron Depot® (Leuprorelin acetate suspension for SC injection) was the first sustained-release peptide approved in the United States, in 1989 (38). Since this approval, longer-acting images have been produced and today three-, four-, and six-month and one-year delivery options are available.

The only protein depot to receive FDA approval was Nutropin Depot™. Nutropin Depot™, approved in 1999 as a treatment for growth hormone deficiency in pediatric patients, is a sustained-release form of Genentech's human growth hormone [somatropin (rDNA origin)] using Alkermes' PLGA-based ProLease® technology. The once or twice-monthly injection (based on the patient weight) offered an alternative to multiple weekly injections. Unfortunately, the product had a short lifetime and was pulled from the market in June of 2004, citing the high cost of production and commercialization. Although the drug was discontinued, the successful development and approval of this complex dosage form signified major success for those working on sustained-release dosage forms for biologics. There was a large leap in complexity in producing Nutropin Depot™ compared with the smaller octa-, nona- and decapeptides mentioned previously. These peptides do not possess the secondary structure of most proteins (alpha-helix or beta-sheet) and are quite stable, having properties more like small molecules. In contrast, human growth hormone contains 191 amino acids and both secondary and tertiary structure.

The Nutropin Depot™ approval took years of commitment and was the result of a well-designed manufacturing strategy, which focused specifically on stabilizing the protein structure (86–88). The manufacturing process, based on the work of Gombotz (89), was different from other, more conventional s/o/w microsphere manufacturing processes, as it utilized low temperature processing, excipient-based protein stabilization, and release-controlling agents. On the basis of this work, and the work of many others, many of the technical challenges inherent to developing PLGA-based sustained-release biologics have been defined, opening the way for rational design of molecules (especially peptides) for sustained-release delivery. Synthetic peptides can be designed and/or screened to be less sensitive to the low pH environment of a degrading microsphere. Reactive amino acids like lysine, with its

nucleophilic primary nitrogen, can be removed or capped to avoid amide formation that can result in covalent peptide-PLGA conjugates. If the desire is a PLGA-based protein delivery system, early forced degradation screening utilizing conditions which mimic PLGA degradation, as well as a screen of manufacturing stress conditions, should be conducted to select the protein with the highest stability. Having very early insight into the desired final product image will better allow for the rational design of the proper characteristics, which will, in turn, ensure manufacturability later in development.

Other Degradable Depot Delivery Systems

Natural and Synthetic Polymers

A number of natural and synthetic biodegradable polymers have been investigated for depot delivery, although only few of them have demonstrated biocompatibility. Natural biodegradable carriers like bovine serum albumin (BSA), human serum albumin (HSA), collagen, gelatin, and hemoglobin have been studied for drug delivery (41), but their use is limited by their high costs and questions over purity. Thus, in the last two decades, synthetic biodegradable polymers have been widely used. In this section we will summarize such biodegradable depot systems and highlight the various depot delivery technologies utilizing those polymers.

Polycaprolactones. Poly-ε-caprolactone (PCL) is a biodegradable polyester with a melting point around 60°C and a glass transition temperature of approximately −60°C (90). It is semicrystalline and is known to degrade slower than polylactide under physiological conditions and thus, is suitable for release extending to a period of greater than one year. A variety of drugs including antigens, antihypertensives, chemotherapeutic agents, and antibiotics have been evaluated with regards to encapsulation in PCL microspheres (91).

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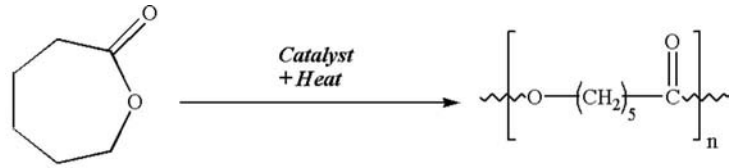

PCL can be an attractive polymer for encapsulating proteins since the degradation of PCL will not result in an acidic environment that is detrimental to protein stability (9). This has been exemplified with PCL microspheres of insulin (92). Block copolymers of caprolactone with PLA, PLGA, PEG, or PEO have also been evaluated for drug delivery (93–95). Capronor is a biodegradable polymer system for the sustained subdermal delivery of contraceptive steroids. Capronor utilizes PCL as the polymer and was evaluated in phase II clinical trials as a contraceptive however, the product did not make it to market.

Polyphosphoesters. Polyphosphoesters (PPE) are a group of structurally versatile biodegradable polymers (degrade via hydrolysis and possibly enzymatic digestion at the phosphoester linkages) that have found application in drug delivery because of their biocompatibility and similarity to bio-macromolecules such as nucleic acids (96).

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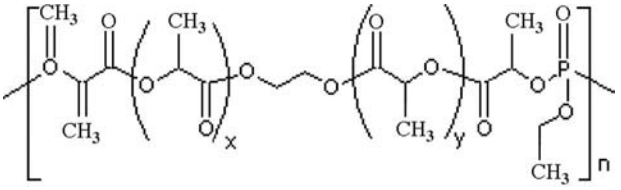

PPE has been used as a carrier for sustained delivery of low molecular weight drugs (97), proteins (98), and DNA (99). Guilford Pharmaceuticals (now MGI Pharma) had a product

candidate, Paclimer®, a poly (lactide-co-ethylphosphate) microsphere formulation of paclitaxel, designed to deliver paclitaxel over eight weeks for the treatment of ovarian cancer.

Polyanhydrides. Polyanhydrides (PA), as the name suggests, are biodegradable copolymers with a hydrophobic backbone of anhydride linkages formed by the condensation reaction of two fatty acids. Their applications in parenteral drug delivery have been reviewed for a variety of therapeutic agents such as growth hormone, anticancer agents, antibiotics, local anesthetics, anticoagulants, anti-inflammatory, and neuroactive drugs (100,101). Polyanhydride microspheres can be prepared by spray drying, hot-melt encapsulation or emulsion methods. Because of release mediated by surface erosion, they are believed to better protect unreleased drug from the release medium (9,101). Various types of homo- and hetero-PAs consisting of aliphatic, aromatic, heterocyclic and other monomers have been studied in detail and extensive work on PA carriers resulted in clinically used implants like Gliadel® (MGI Pharma, Inc.) and Septacin™ (Abbott Laboratories). Gliadel is a polyanhydride polymer matrix of poly[bis(p-carboxyphenoxy) propane] with sebacic acid P(CPP:SA) (20:80 molar ratio) containing BCNU for the treatment of brain tumor. BCNU is a nitrosourea with short half-life but is considered a "gold standard" for treating glioblastomas. Gliadel wafer is a sterile, off-white to pale yellow wafer with a diameter of 1.45 cm and 1 mm thickness. Each wafer contains 7.7 mg BCNU and 192.3 mg PA copolymer. Septacin™ is a PA implant consisting of P(FAD:SA) (1:1 weight ratio) polymer and gentamicin for the treatment of osteomyelitis. Each implant has five beads in a strand with each bead being 12 mm long and 4 mm in diameter weighing 150 mg (contains 20 mg gentamicin as gentamicin sulfate) (102).

Polyortho esters. Polyortho esters (POE) are generally synthesized by condensation of diols and a diketene acetal, and often involve copolymerization with a latent acid such as glycolic acid and lactic acid (a class of POE called Biochronomer®, which have been developed by AP Pharma) to allow control over the hydrolytic degradation of the ortho ester linkages (9,103). POEs are thermoplastic polymers that have been demonstrated to be stable to 24 kGy γ-irradiation and can be easily formulated as microspheres using extrusion followed by cryogenic milling (104,105). Various processes have been employed to prepare POE microspheres including spray congealing (106), emulsion-solvent evaporation (low encapsulation efficiency with water-soluble drugs) (107,108), and extrusion of block copolymers of PEG and POE to enhance encapsulation efficiency with water-soluble compounds (109).

Block copolymers of polybutylene terephthalate. Multiblock copolymers of hydrophilic PEG and hydrophobic polybutylene terephthalate (PBT), known as PolyActive™, have been developed by OctoPlus. The degradation of these biodegradable and biocompatible polyether ester copolymers occurs by hydrolysis of the ester bonds and oxidation of the ether linkages (110,111). OctoPlus is currently developing Locteron™, a microsphere formulation of interferon α, using this technology.

Cross-linked dextran. Cross-linked dextran is a biodegradable and biocompatible (112,113) hydrogel system for drug delivery, specifically protein delivery, which has been developed by OctoPlus. A modified dextran derivatized with hydroxyethyl methacrylate (dex-HEMA), referred to as OctoDEX®, has been reported to be able to tailor the release of proteins from microspheres from days to months (114–116).

Polyamino acid polymers. Polyamino acid polymers, as the name suggests, are composed of naturally occurring amino acids. The release duration can be tailored, in principle, by modifying the hydrophobicity of the participating amino acids in the block copolymer. Flamel Technologies has developed these polymer systems for protein delivery. An amphiphilic block copolymer, composed of L-leucine and L-glutamate, is referred to as Medusa I® (117). These are self-assembling systems, which are noncovalently associated with proteins. Insulin (Basulin®) is one of the proteins that is being investigated with this technology for type I diabetes, with a target release duration of two days. Flamel has also developed Medusa II®,

which is hydrophobically modified L-glutamate, for release over two weeks. Interferon α2b and Interleukin-2 are also being developed using this technology (118).

Cellulosic polymers. Water-soluble anionic polymers, such as CMC, have been utilized to form water-insoluble complexes with soluble cationic peptides. Such insoluble complexes, formed by ionic interactions, have been developed (Rel-Ease®) for sustained drug delivery by Praecis. Plenaxis® is an abarelix-CMC complex that utilized Rel-Ease® technology and was approved in 2003 for the treatment of advanced prostate cancer; however, it was withdrawn in 2005 because of financial considerations (119–121).

Cross-linked albumin. Use of cross-linked albumin for sustained-release applications is exemplified by the ProMaxx® drug delivery technology, which was developed by Epic Therapeutics, Inc, a wholly-owned subsidiary of Baxter Healthcare Corporation. ProMaxx is a protein matrix-based technology developed for protein, peptide, and small molecule delivery. The microspheres, in the particle size range of 0.5 to 40 um, are produced in an aqueous medium by mixing a carrier protein (e.g., HSA), a water-soluble polymer (e.g., hetastarch), a polyanionic polysaccharide (e.g., dextran sulfate, heparan sulfate, and polyglutamic or polyaspartic acid), and a divalent metal cation (e.g., Ca^{2+} and Mg^{2+}). The release from the microspheres can be controlled by varying the concentration of hetastarch, temperature, pH, albumin, or length of heat exposure of microspheres. Baxter is developing LeuProMaxx® (one- and three-month release of leuprolide acetate) using the ProMaxx technology, for the treatment of prostate cancer (9,122).

Other gel-forming polymer systems. The SABER® system, from Durect Corporation, consists of a hydrophobic polysaccharide, sucrose acetate isobutyrate (SAIB), as the drug release-controlling matrix. SAIB, along with the drug, is dissolved/dispersed in ethanol, benzyl alcohol, or other water-miscible solvents. Since this system has a relatively low viscosity, administration with a smaller gauge needle is easier compared with PLGA-based gel systems. Sustained-release formulations of bupivacaine (123) and rhGH (124) are being considered for feasibility assessment or development.

A cross-linked PEG-based copolymer (containing multiple thio (-SH) groups along the polymer backbone) which forms a hydrogel when mixed with α, omega-divinylsulfone-PEG (2 kDa) dissolved in a neutral phosphate buffer has been reported (125). The system has been proposed to achieve a release over two to four weeks, with application mostly suited toward large molecules. Mild adverse tissue reactions have been reported in biocompatibility studies in rabbits and rats.

GelSite® polymer, from DelSite biotechnologies, is a natural acidic polysaccharide extracted and purified from the aloe plant. The polymer forms a gel in the presence of calcium (in situ cross-linking) when injected subcutaneously or intramuscularly, and thus entraps a water-soluble drug (e.g., a protein) providing sustained release (U.S. patent 5929051). The polymer has also been shown to specifically bind to, and stabilize, heparin binding proteins, thus providing additional control over drug release without affecting the biological function (U.S. patent 6313103).

Chitosan is a pH-dependent cationic polymer (amino polysaccharide) that has been demonstrated to be biocompatible and biodegradable. Chitosan can form an in situ thermosensitive gelling system when combined with an anionic polyphosphate salt, glycerophosphate (GP) (126,127). A chitosan-GP gelling system has been evaluated for camptothecin delivery, providing zero-order release over four weeks (128).

Poloxamer® 407 is a triblock copolymer of polyoxyethylene and polyoxypropylene units in the ABA configuration. Mostly utilized as a nonionic surfactant, this water-soluble polymer demonstrates reverse gelling properties. A 20% or higher polymer solution is a liquid at low temperatures, but gels at body temperature (129). Although this approach potentially provides an exciting system for sustained release of large molecules, because of the lack of organic solvents, its application has been limited by a lack of biodegradability, cytotoxicity concerns, and reports of increased levels of plasma cholesterol in rats administered with poloxamer intraperitoneally (130).

Lipid-Based Systems

Conventional lipid-based depot systems, such as oil solutions or suspensions, have been discussed earlier in this chapter. Conventional lipid systems rely on the partition of drug from the oil phase into the aqueous phase at the injection site to control release. Advanced lipid-based dispersed systems, with particles in the submicron size range, have been developed for water-soluble and water-insoluble drugs for parenteral administration. Natural and synthetic phospholipids, with or without further chemical modifications, have not only been used in stabilizing triglyceride-based lipid formulations, but also are the major structural components of lipid vesicles. Though lipid-based systems including emulsions provide an opportunity for sustained release, the duration of release is seldom over one week. In this section we will briefly discuss a few such lipid-based systems.

Liposomes. Liposomes are vesicles composed of an inner aqueous core surrounded by a phospholipid bilayer. Liposomes are primarily categorized into three types—multilamellar vesicles (MLV), small unilamellar vesicles (SUV) and large unilamellar vesicles (LUV). Optimization of the bilayer composition, charge, and size of liposomes, as well as the internal aqueous composition, allows efficient incorporation of a wide variety of drugs (131). Liposomes, with or without surface pegylation, have been evaluated extensively for various compounds for intravenous administration (9). Doxil® (doxorubicin HCl) liposome injection from Alza Corporation was the first pegylated liposomal doxorubicin product approved for the treatment of refractory ovarian cancer and AIDS-related Kaposi's sarcoma. However, as a depot delivery system for SC and IM use, liposomes have not proven to be the best candidates despite being biocompatible and demonstrating positive results for efficacy (132). The primary reason for this lack of success is the relatively limited drug-loading capacity and short duration of release for the entrapped drugs. This is coupled with a complex manufacturing process, and physical stability considerations (9).

Multivesicular liposomes. SkyePharma developed the DepoFoam™ [now owned by Pacira Pharmaceuticals, Inc. (133)] technology, which consists of tiny, lipid-based particles, 10 to 30 μm in size, composed of hundreds to thousands of discrete water-filled chambers containing the encapsulated drug, with each chamber separated from adjacent chambers by a bilayer lipid membrane. The bilayer is composed of synthetic phospholipids (dioleoyl phosphatidylcholine and dipalmitoyl phosphatidylglycerol), cholesterol and triglyceride (134). Drug release from DepoFoam particles is achieved by diffusion through the walls, gradual erosion of the particles, and by processes involving the rearrangement of membranes. DepoCyt® is the first approved DepoFoam product containing cytarabine for the treatment of lymphomatous meningitis, administered intrathecally every two weeks. DepoDur® is a morphine sulfate formulation for postsurgical pain relief, given epidurally every two days. DepoBupivacaine®, a sustained-release formulation of bupivacaine, is in phase III development for local anaesthesia/pain. Proteins and peptides have also been evaluated with the DepoFoam technology with regards to in vitro and in vivo release (135).

Lipid microparticles. Lipid microparticles are solid lipid-based drug delivery systems composed of a dissolved or dispersed drug in a solid lipid matrix. The low mobility of the drug in the lipid matrix and hydrophobic nature of the lipids provide the required sustained-release properties (136). A maximum loading capacity of 25% has been reported for these systems (137). Various methods of encapsulation have been utilized to produce these microparticles, such as solvent-evaporation, melt-dispersion or spray-congealing methods (138). Lipid microparticles have been evaluated for the sustained release of small molecules such as local anesthetics and antibiotics, as well as proteins and peptides (139–142).

Cochleates. Cochleates are formed by the condensation of small, unilamellar, negatively charged liposomes composed of an anionic phospholipid, such as phosphatidylserine. The small liposomes fuse to form larger lipid bilayer sheets in the presence of a cation, such as calcium. These sheets roll up into cinnamon stick-like or cigar-like structures to minimize the interactions between water and the hydrophobic surface of the sheet. The cochleates are

characterized by a tightly-packed bilayer with little or no internal aqueous phase (143). Depending on the hydrophobicity and charge of the molecule, it could either be embedded in the bilayer, or encapsulated between the bilayers (144). The characteristics of cochleates lend themselves to application via the intravenous route to increase drug circulation time (e.g., amphotericin B cochleates), and ability to penetrate and accumulate in target tissue (145,146). Recently, delivery system for vaccines (147,148) and genes (149), have utilized cochleates as well.

IMPLANTABLE DEVICE-BASED AND NONDEGRADABLE DEPOT DELIVERY SYSTEMS

One of the key aspects of an implantable, nondegradable depot delivery system is the requirement for a minor surgery for implantation, and a similar procedure for explanation of the implant once the dose has been delivered. Hence, a longer duration of drug release is required to maintain patient acceptability. Although the administration involves an invasive procedure, in the case of adverse effects, removal is straightforward. Generally, implants would not be considered where the drug dose is dependent on body weight, since the dose and release from these systems is predetermined. However, in cases where a broad therapeutic window exists and sustained drug levels are required, implants present themselves as a viable option. In this section we will briefly discuss some of the nonbiodegradable implants including polymeric systems, osmotically driven systems and other device-based systems.

Polymeric Systems

The nondegradable polymers can be processed with drug to yield depot systems of various configurations, which can then be implanted subcutaneously. Two primary categories for nondegradable polymeric systems are the encapsulated reservoir system and the matrix-loaded system. One of the leading examples of the encapsulated reservoir system is the Norplant® implant from Wyeth. Norplant is a five-year levonorgesterol implant for contraception, approved by the FDA for use in women. The implant, which consists of six flexible closed capsules, is a reservoir system with each capsule consisting of 36 mg of active in silicone rubber tubing (silastic) of 2.4 mm diameter and 34 mm length (150). Wyeth has an analogous product in the form of Jadelle®, which was designed to require fewer capsules (two) for implantation, and thus improve insertion and removal. Jadelle has been approved in the United States, but is not marketed. On the basis of the publically available prescriber's information, Jadelle is a set of two flexible cylindrical implants, consisting of a dimethylsiloxane/methylvinylsiloxane copolymer core enclosed in thin-walled silicone tubing. Each implant contains 75 mg of the progestin levonorgestrel. The implants are sealed with polydimethylsiloxane adhesive and sterilized. Each implant is approximately 2.5 mm in diameter and 43 mm in length. The implants are inserted in a superficial plane beneath the skin of the upper arm. The calculated mean daily in vivo release rate of levonorgestrel provided by the implants is about 100 μg/day at month one, followed by a decline to about 40 μg/day at 12 months, and to about 30 μg/day at 24 months, with stabilization thereafter at about 30 μg/day. One of the major drawbacks with the reservoir system is the risk of "drug dumping" if there is a rupture of the releasing membrane.

Implanon®, from Organon (now part of Merck, Sharp & Dohme Corp), is a leading example of the matrix-loaded system. Implanon is an etonogestrel implant with each implant containing 67 mg of the active embedded in an EVA (ethyl vinyl acetate) matrix, which is then surrounded by a rate-controlling EVA membrane to form a rod measuring 40 mm in length and 2 mm in diameter (150). The implant is designed to release over three years and was approved by the FDA in 2004. One of the major drawbacks with the matrix-loaded systems is a more complex release mechanism (likely diffusion controlled) which presents a significant barrier toward achieving a zero order release profile. The Hydron® implant, from Valera Pharmaceuticals (acquired by Indevus Pharmaceuticals in 2006), is a hydrogel reservoir drug delivery system designed for delivery of drugs at a predetermined rate over a one-year period. The hydrogel nature of the implant is likely to cause less discomfort when compared with metal implants. The cylindrical implant is 26 mm long, 3.5 mm in diameter and 0.5 mm in wall thickness, and is composed of a cross-linked copolymer of hydroxypropyl methacrylate and 2-hydroxyl methacrylate. The core of the implant consists of the drug (e.g., 50 mg histrelin) and

stearic acid (as in Vantas®, which is a one-year histrelin implant) (151). The implant is packaged in a glass vial containing 1.8% sodium chloride solution, which allows hydration and priming of the implant prior to insertion.

Osmotically Driven Systems

As the name suggests, these systems utilize osmotic pressure for long-term delivery of potent therapeutic agents. The Duros® implant, from DURECT, is one such example, which consists of an outer titanium cylinder, an osmotic engine (containing sodium chloride), a piston and a drug chamber. One end of the outer cylinder is capped with a semi-permeable membrane (controls the rate), and the other end has an orifice, which releases the drug using a diffusion moderator. The implant holds a maximum of 200 μL of the drug solution, and can be up to 4 mm in diameter and 44 mm in length. A brief description of the steps involved in the functioning of the Duros systems will include (1) water influx into the osmotic engine, (2) expansion of osmotic engine, (3) displacement of the piston, and (4) contraction of drug formulation-containing chamber to release the drug through the exit port (152). Because of the volume constraints, the implant usage is limited to potent compounds with high formulation concentrations. Solution formulations with various aqueous and nonaqueous solvents, and suspensions with nonaqueous solvents, have been evaluated with the Duros implants. Viadur®, from Alza Corporation, is a one-year leuprolide acetate implant, which received FDA approval for prostate cancer (153). DURECT is also developing Chronogesic®, a three-month sufentanil implant, for which the clinical trials are currently suspended to improve the device to mitigate performance issues.

Other Device-Based Systems

Besides the polymeric implants and the osmotically driven systems, there are other devices, which have been utilized for the delivery of highly potent drugs. SynchroMed pump from Medtronic and Codman 3000 from Codman and Shurtleff are representative systems. The SynchroMed pump is an implantable, programmable, battery-powered device that stores and delivers medication according to instructions received from the programmer. The various models of the pump vary in size of the reservoir and the presence of a side catheter access port. The hold volume in the refillable pump can range from 10 to 40 mL. The CODMAN 3000 implantable drug delivery system features an inexhaustible power supply obviating the need for battery and provides continuous delivery with the refillable volume ranging from 16 to 50 mL. The Codman 3000 implantable pump is divided into inner and outer chambers by accordion-like bellows. The inner chamber contains the drug to be infused while the outer chamber contains propellant permanently sealed. The patient's own body temperature warms the propellant, which exerts a constant pressure on the bellows. This causes the drug to flow out of the inner chamber through a filter and flow restrictor then slowly out of the catheter.

Both these pumps require surgical insertion and removal, and the refill of these pumps will require expertise. One of the most common applications of device-based systems is for the administration of insulin. Such insulin pumps typically consist of the pump, the disposable drug reservoir, and an infusion set, which includes a canula for SC insertion. The pumps come in various models that include Ping (Animas), Cozmo® (Deltec), Spirit® (Disetronic), Paradigm 522/722 (MiniMed), OmniPod (Insulet) and Diabecare IIS (Sooil). The insulin pumps range in hold volume from a couple of milliliters, to as much as 90 mL, and are intended to deliver rapid- or short-acting insulin 24 hours a day through a catheter placed under the skin.

DEVELOPMENT CONSIDERATIONS FOR DEPOT DELIVERY SYSTEMS

Sustained-release parenteral formulations are generally complex dosage forms, and therefore often present significant challenges during development and scaleup. These challenges include sensitivity to changing API and excipient properties, maintaining critical formulation attributes during manufacturing process development and scaleup, ensuring sterility, evaluating drug release and establishing in vitro–in vivo correlations (IVIVC), setting specifications, ensuring product quality, and managing material and manufacturing costs. Regulations, standards, and science-based guidance are generally lacking for parenteral sustained-release dosage forms (154,155), and development timelines can be long. For these

reasons, it can be advantageous to initiate development of these dosage forms early in the product lifecycle.

In Vitro and In Vivo Release from Depot Delivery Systems

Selection of in vitro release methods remains a significant challenge in the development of depot formulations, and little in the way of science-based guidance for industry exists (156). Suitable in vitro release methods can reduce the dependence on in vivo testing and speed development timelines. Key uses for in vitro release methods include assessment of drug release (including burst release) during early formulation and process development and subsequent optimization, quality control to support batch release and stability evaluation, and definition of critical product attributes and critical process parameters (154–156). In vitro release methods should be biorelavent to enable a robust IVIVC for predicting in vivo release on the basis of in vitro evaluation; validated IVIVC could potentially support formulation bridging during development. For depot formulations that are designed to release over long periods of time (e.g., months), it is often not practical to rely on real-time in vitro release evaluations, and therefore accelerated methods are required (157).

In Vitro Method Development

In vitro release methods for parenteral depot formulations have been well-reviewed (157–159). Considerations for method development include the apparatus type and design, the release media, and rational selection of experimental variables such as temperature and agitation rate. Generally, three methods are used for evaluating in vitro release from parenteral depot formulations: separation methods, flow-through cells (open system), and dialysis techniques (156–160). Each has certain advantages and limitations. There are currently no regulatory standards for in vitro release testing of parenteral depot formulations, and available compendial apparati were not designed for this purpose and are generally not suitable, with the exception of USP IV (156). Of the available methods, the separation technique is the simplest and appears to be the most widely utilized. A quantity of the dosage form is placed in a vessel, along with a specified volume of release medium, and agitated at a controlled rate and temperature. At specified timepoints, the release medium is sampled and assayed for drug content, and fresh medium is returned to the vessel. Dispersed systems must first be separated by centrifugation or filtration prior to sampling, and aggregation of dispersed systems is a concern (156). The flow-through method allows for maintenance of sink conditions, but is more complex and potentially less robust (156). The dialysis technique may provide the best approximation of the confined environment at the injection site, coupled with sink conditions in the bulk release medium, but membrane stability and drug adsorption must be evaluated (156,160).

Selection of biorelavant release media is another important aspect of method development. Many reports specify phosphate buffered saline (PBS) at 37°C as the release medium to approximate in vivo conditions, although in some cases media with different pH, ionic strength, or protein content are more appropriate. Media volume is a critical variable, particularly for drugs with solubility limitations where sink conditions may not be achieved. Media should be selected on a case-by-case basis based on the properties of the active and the formulation. Other physiological variables to consider during selection of release media and development of the method include metabolism, tissue pH and buffer capacity, vascularity, level of exercise, and volume and osmolarity of the product (156).

Accelerated In Vitro Release Methods

Accelerated methods that can promote rapid release of the depot contents over a short (e.g., few days) time period are needed for quality control (e.g., product release) and formulation development applications, particularly for very long-acting formulations (157). These methods should be capable of discriminating formulation changes that can impact bioavailability, and detecting batch to batch variability and the impact of product instability over time (155). Acceleration of release is most commonly accomplished by raising temperature (e.g., to 50–60°C), altering pH, or adding surfactants (156,157). It is important to consider the impact of

factors such as polymer transition and degradation temperatures on the release mechanism (154). To accurately assess burst release, a real-time release evaluation may be conducted in parallel to the accelerated method (154).

In Vitro–In Vivo Correlation

The need to establish IVIVC for parenteral depot formulations is well recognized (154). This has historically been difficult to achieve, presumably because of the large number of parameters influencing release from depot formulations both in vivo and in vitro, including fluid volume, viscosity, tissue barriers (e.g., fibrous encapsulation), phagocytosis, and inflammation (161–163). There are, however, increasing numbers of successful reports in the literature establishing IVIVC for parenteral depot systems, and these have been recently reviewed (156,164,165). IVIVC becomes more likely as drug release from the depot is the rate-limiting step for absorption, and as release is governed primarily by diffusion, rather than polymer degradation, which can differ in vivo and in vitro (165). Steps to establish IVIVC are similar to those for oral dosage forms, and include in vitro method development, preparation of formulation variants that are expected to have different behavior in vivo, in vitro and in vivo testing, and modification/optimization of the in vitro release method to mimic in vivo results (156). Animal models, such as the rat, are suitable for formulation development and optimization, but would not be suitable for demonstration of human bioequivalence; larger species may be needed to evaluate relevant injection volumes (154).

Development of IVIVC can be particularly challenging for local delivery, such as ocular delivery, where it may be difficult or impossible to assess the local drug concentration in humans, and plasma levels may be extremely low and not indicative of local exposure. In animal models, local tissue exposure may be determined by sacrificing animals at specified timepoints and measuring tissue concentrations or drug content of explanted dosage forms, or by microdialysis methods (29,153,166).

Manufacturing Process and Scalability

Manufacturing processes for parenteral sustained-release formulations are often complex and many involve nonconventional unit operations. Considerations during process development include ensuring that stability and activity of the drug are not compromised, optimizing process yield and drug encapsulation (e.g., for microspheres), ensuring the release profile is reproducible within specified limits and, when relevant, that particle size is controlled to specifications (31). Scaling can be a challenge for many mixing operations; scalability can be improved by utilizing continuous processes, such as in-line mixing or extrusion (for implants). Solvent-based processes present additional environmental and safety challenges, such as the need for solvent-recovery handling, and ensuring residual solvent levels conform to specifications.

Terminal Sterilization

Ensuring sterility of parenteral sustained-release formulations is a significant challenge, given the dispersed nature of many of these systems, which often precludes sterile filtration. Sterile filtration may be feasible for systems formulated as solutions, such as oily-vehicle solutions and in-situ-forming depots dosed in organic solvents. Terminal heat sterilization may be possible for suspension formulations, and cloud point modifiers can be included to improve physical stability at high temperatures. This approach is not suitable for polymeric systems such as PLGA because of the relatively low T_g of the polymer (154). γ-Irradiation has been employed for terminal sterilization of PLGA microsphere and suspension systems, but the potential for polymer and drug degradation must be evaluated. As a result of these challenges, most parenteral sustained-release formulations are aseptically processed (154).

Regulatory Considerations

Depot delivery systems are subject to the same quality control requirements that govern other parenteral drug products, including sterility, pyrogen testing, drug content, impurities and degradates. The sterility test method used depends on the nature of the depot delivery system,

with the direct transfer method typically used for suspensions that cannot be solubilized in suitable solvents, and sterilized devices, and the membrane filtration method for other depot delivery systems (167). Although particulate matter testing is not required for suspensions, these systems can be dissolved in a suitable organic solvent to test for foreign particles (154). It is important to verify syringeability and injectability with appropriately-sized needles. While there is no regulatory guidance, viscosity measurements and evaluation of needle-clogging and plunger force can be used to assess these attributes. A fundamental understanding of release mechanisms and physicochemical changes within the delivery system is an important part of quality by design.

Development Strategy and Economics

The successful development and launch of parenteral sustained-release dosage forms can be a long and expensive process, owing to their high technical complexity, nonconventional unit operations, and long duration of action. It is critical to establish an early line of sight from the concept and compound properties to the market, to minimize additional cost and lost time. This involves establishing a target product profile early, including a thorough assessment of the market and target patient populations. It is important to ensure that the properties of the active are suitable for the desired sustained-release application, and that they are properly matched to the appropriate formulation approach. Ideally, if the need for sustained release is recognized early during discovery, compound potency and physicochemical properties, such as solubility and stability, should be designed to enable formulation using specified sustained-release technologies.

Sustained-release formulations are often developed as lifecycle management opportunities for compounds already in development or launched as conventional parenteral or oral formulations. As a result, there may be a significant body of existing safety and efficacy data in humans, and depot formulations typically do not enter clinical development until a thorough understanding of PK/PD and therapeutic window is available. In vitro release data and preclinical pharmacokinetic data should be used to design the dosage form to meet the target pharmacokinetic profile. As for any new formulation, preclinical safety studies must be run prior to initiation of clinical studies. Clinical dose ranging can be supported either by administering formulations with different release rates, or by administering different doses of a single formulation. It is desirable to initiate clinical studies with a formulation composition and manufacturing process that is representative of the intended commercial product to minimize the challenge of bridging formulation changes and process changes later in development. As this is rare in practice, and given a lack of regulatory bioequivalence guidelines for sustained-release dosage forms, formulation and process changes should ideally be supported by a validated IVIVC.

Development and product costs of sustained-release formulations are typically higher than conventional formulations because of their high technical complexity, long development timelines, nonconventional excipients and manufacturing unit operations, and higher doses of active per administration. This should be planned into the overall development strategy from the beginning.

FUTURE DIRECTIONS

The future of parenteral sustained delivery promises to be an exciting one, with the potential for significant advances that will meaningfully change the way medicines are administered. Technical advances will span from incremental improvements in existing technologies, to the introduction of new excipient materials, the development of systems that offer an improved level of control over drug release, and the emergence of new applications for depot delivery. This future will require pharmaceutical and formulation scientists to broaden their already multidisciplinary backgrounds even further into areas as diverse as microelectromechanical systems (MEMS), information sciences, and cell and tissue biology (168).

Incremental Enhancements of Existing Technologies

The pace of launching new parenteral sustained-release technologies over the last several decades has been relatively slow, due in part to the major challenges and costs inherent in

commercializing new delivery modalities. It is therefore reasonable to expect that incremental improvements in existing technologies will continue to dominate the near-term future of depot delivery. These improvements may include new manufacturing process techniques, new approaches to sterilization, novel packaging technologies, and novel combinations of existing technologies. Recent examples of these include the emerging use of supercritical fluid technologies to make polymeric microspheres (169), evaluation of electron-beam and ethylene oxide as methods of sterilization (170), increasing use of delivery devices, such as the Lupron Depot-PED® dual-chamber syringe, to enhance convenience during administration, and the integration of acid-neutralizing excipients in PLGA formulations to counteract acidification by hydrolysis products (44). Further value may be extracted from these technologies if leads are optimized during discovery specifically for sustained release, emphasizing potency and stability as key criteria.

Introduction of New Excipients

The acceptability of materials for parenteral use, from both the safety and regulatory points of view, continues to be a major constraint in the development of new depot delivery technologies. The hurdles to introduction of new excipients are significant, and few companies are willing to invest the significant time and money required to bring new or novel-use excipients through development to the market. PLGA enjoys the status of being a proven and well-accepted excipient, and continues to be the most common polymer used in parenteral sustained-release systems, further entrenching it in this application. Although PLGA is attractive in many respects, new polymeric materials are needed to provide a wider range of properties and potential release profiles, and to enhance the range of actives compatible with sustained-release approaches. In the short term, the most promising new candidates for approval are likely to be copolymers of currently-approved materials, such as copolymers of PLA and PEG, which can be expected to degrade to known materials. Longer-term, one approach to speed the introduction of new excipients could be the formation of jointly-funded industrial consortia, to advance the preclinical evaluation of novel materials.

Enhanced Control over Drug Release

Despite their many advances over the years, marketed depot delivery systems continue to offer a relatively limited ability to control release rate, relying on the intrinsic properties of the formulation (e.g., matrix degradation, API dissolution or partition, osmotic pressure, etc.) to govern drug release. The ability to rationally change drug release during dosing would represent a major step forward, and continues to comprise an active area of scientific inquiry. The ultimate goal is responsive systems, or smart delivery systems, which incorporate the ability to sense their surroundings and alter their function in response to specific signals generated in the body (171). Such systems will be particularly valuable in the treatment of diabetes and other metabolic disorders, and may also be useful in chronotherapy (172,173).

Several approaches have been evaluated in the pursuit of this goal, including environmentally responsive polymers and microprocessor-based devices. Novel polymers have been synthesized, which are capable of changing their properties in response to changes in their environment, including pH, temperature, ionic strength, solvent composition or electromagnetic radiation (174–178). These include the pH-sensitive methacrylates, which change in their degree of swelling as pH changes, and temperature-sensitive systems such as poly (*N*-isopropylacrylamide) (174). Microelectromechanical solutions include an electrothermally activated implantable silicon chip, under development by MicroCHIPS (179). The device is segmented into multiple wells, which can be sealed prior to implantation and then opened on demand. Depot delivery systems of the future will likely include integrated sensing of biomarkers, metabolites, or actives, feedback-control over drug release, and real-time output of information relating to the underlying pathology and treatment (168).

New Applications

A number of new applications for depot delivery are emerging, including targeted delivery, gene delivery, and tissue engineering. Fabrication of nanoparticles from PLGA offers a new platform for targeted delivery, amenable to IV administration (180). These systems are being

developed and studied for the targeted delivery of a range of therapeutics, from small molecules to nucleic acids. Nucleic acid delivery via sustained-release systems is an increasingly active field of research given the recent advent of RNAi technology and continued interest in local gene delivery (181,182). Tissue engineering and regenerative medicine strategies often require controlled delivery of bioactive molecules, with particular sensitivity to spatial and temporal control of release (183), to a particular cell type or in a particular region of the body (184). There are many potent growth factors including nerve growth factor, bone morphogenic protein and vascular endothelial growth factor, which are under investigation (185). Approaches for regenerating nerve tissues, repairing bone defects from fractures, infections and cancers, and the ability to accelerate blood vessel formation are all areas of active research. The field of parenteral sustained release promises to be an exciting and active area of research for many years to come, offering the potential to significantly increase the value of both existing and new therapeutics and address important unmet medical needs.

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8 Biophysical and biochemical characterization of peptide and protein drug product

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INTRODUCTION

Classes of Biotherapeutics

The biotherapeutics class of drugs that are commercially available encompass a range of compounds including recombinant or purified proteins, monoclonal antibodies (also proteins), peptides, conjugated or fused peptides, antibody conjugates, protein vaccines, oligonucleotides, protein-lipid complexes, enzymes, antibody fragments (Fabs), glycosylated proteins, and carbohydrates (Fig. 1). Additional molecule types are in preclinical and clinical development.

The biotherapeutics class contains a wide variety of recombinant proteins derived from microbial, mammalian, and yeast sources (Table 1). There are few products that are extracted from natural sources. The biotherapeutics class of drugs uses a variety of technologies for extending half-life such as conjugating to polyethylene glycol (PEG), fusion with antibody or Fab, and employing the antibody itself. This is especially true for peptides and other small entities that would be cleared via the kidneys without a half-life enhancing strategy such as conjugation or fusion. Table 1 illustrates the wide variety of biotherapeutics entities on the market.

Regulatory Guidance on Structural Characterization

Regulatory approval of a biotherapeutic entity requires meeting the guidelines for chemistry, manufacturing, and controls (CMC) put forth by the relevant regulatory agency. A complete CMC package includes a description of the characterization of the biotherapeutic entity, which includes the Elucidation of Structure and Impurities sections, which, for biological entities can be quite complex. It is expected that the applicant have a detailed understanding of the structure, heterogeneity, and stability of the biotherapeutic entity using a variety of analytical methods. Regulatory guidance on the characterization of biotherapeutic molecules can be found in several sources. The U.S. Food and Drug Administration (FDA), the European Medicines Agency (EMEA), and other regulatory agencies around the world often provide guidance documents on specific topics relating to the review and approval of drugs, and these can be excellent sources of information for applicants (www.fda.gov, www.emea.europa.eu). The International Committee on Harmonization (ICH) (www.ich.org) provides guidance documentation agreed on by the regulatory agencies of the United States, Europe, and Japan. The ICH guideline Q5 deals specifically with biotechnology products, and some information concerning characterization is available in this section, particularly Q5E on comparability. Q6B deals with specifications of biotechnology products, and provides further relevant information for biotherapeutic entities.

Proof of Structure

As part of the Elucidation of Structure section of a CMC package, a detailed analysis of the structure of the biotherapeutic is required. This evaluation is in addition to the normal batch release assays used for the product which ensure the safety and efficacy of each batch. The characterization assays included in this section are used for confirmation of the predicted primary structure, higher order structures, post-translational modifications, and degradation products that may form or increase on stability. The presence and levels of variant forms needs to be measured, and their impact on the safety and efficacy of the product needs to be assessed. The attributes investigated may be assessed using multiple analytical methods for each, as discussed in some detail below.

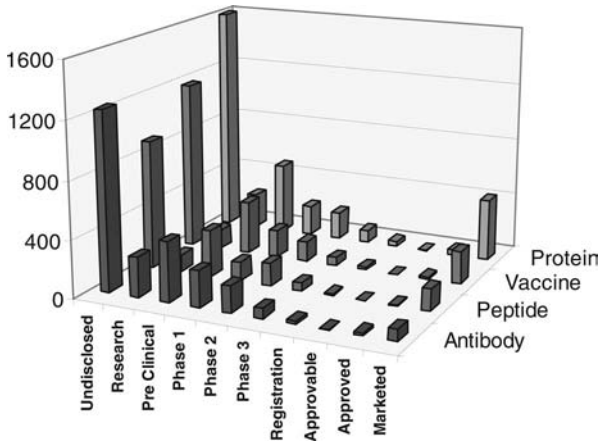

Figure 1 Portfolio of selected biotherapeutic class of drugs and drug candidates in various stages of development (data from PharmaCircle, March 2009). Numbers do not represent unique molecule types in any of the classes.

The confirmation of primary structure may include assays that demonstrate the product has the expected amino acid sequence, such as amino acid sequencing, mass spectrometry (MS), and electrophoresis. These methods ensure that there are no translation variants such as amino acid substitutions, terminal extensions, or unprocessed introns present in the product. Higher order structure may be assessed by biophysical and spectroscopic methods such as circular dichroism (CD) and fluorescence spectroscopy. This may include a determination of the disulfide bond connectivity, which can be critical for a protein to maintain its active conformation. Many post-translational modifications of proteins are possible, such as glycosylation. Other modifications may include related species formed as a consequence of degradation, such as oxidation and deamidation. For conjugated products, variants due to the conjugation process and degradation products of these need to be assessed and understood. In total, biotherapeutics may include a heterogeneous mixture due to all of the variant forms possible, and the applicant needs to demonstrate an understanding of the species present.

Potency Determination

For biologics, in most cases, a relevant potency assay for the biological entity is required for its approval. The assay needs to demonstrate "the specific ability or capacity of a product to achieve a defined biological effect." (ICH, Q6B, specifications: test procedures and acceptance criteria for biotechnological/biological products). One or more bioassays are typically included as part of batch release, and range from binding assays, cell-based assays, or in vivo animal assays. As part of characterization, it is expected that variant forms of the biological entity be assessed for potency. This involves isolation of the variant form and testing in the relevant bioassay(s) for the product. For species that form or increase on stability because of degradation, stress conditions can be used to generate sufficient material to perform potency assays.

Formulation Characterization

Most therapeutic biologics currently are administered via parenteral (intravenous or subcutaneous) route. The goal of biologics drug product formulation development is to minimize various degradation pathways to achieve a minimum shelf-life of 18 to 24 months at the intended storage condition. An emerging strategy in the biotherapeutics industry is to minimize investment in the early stages of preclinical and clinical development, and therefore, drug product formulation for early clinical trials may not be characterized in detail. Additionally, long-term stability data may be rarely available in early stage. However it is necessary to make an assessment of potential chemical and physical labilities that may impact long-term stability. A part of this assessment can be achieved by Preformulation work which is a combination of experimental and bioinformatics studies conducted in early stage prior to nominating a drug product formulation. "Formulation characterization" refers to

Table 1 Examples of Biotherapeutics Class of Molecules: Types, Sources, Technologies, and Molecules

Name of drug	Name of active substance	Class of molecule	Technology	Source	Indication	Company
Genotropin	Somatropin	Protein	Single polypeptide	*Escherichia coli* (rDNA)	Growth hormone deficiency, Turner syndrome and others	Pfizer
Somavert	Pegvisomant	Conjugated protein	Single polypeptide PEGylated at multiple sites	*E. coli* (rDNA)	Acromegaly	Pfizer
PEG-Intron Redipen	Peginterferon α-2b	Conjugated protein	Covalent conjugate of PEG to protein	*E. coli* (rDNA)	Infections, hepatitis C	Schering-Plough
Nplate	Romiplostim	Fusion protein	Fc-peptide fusion protein (peptibody)	*E. coli* (rDNA)	Thrombocytopenic purpura	Amgen
Survanta	Beractant	Lipid-protein mixture	Natural bovine lung extract containing lipids and surfactant-associated proteins, and added lipids	Bovine lung extract	Respiratory distress syndrome	Mitsubishi Tanabe; Ross (Abbott)
DigiFab	Digoxin immune Fab	Antibody fragment (Fab)	digoxin-specific Fab	Ovine serum	Digoxin toxicity or overdose	BTG International Ltd.; Nycomed
Lucentis	Ranibizumab	Antibody fragment (Fab)	Humanized IgG1 κ	*E. coli* (rDNA)	Age-related macular degeneration	Genentech and partners
Cimzia	Certolizumab pegol (CDP-870)	Antibody fragment (Fab) conjugate	Humanized antibody fragment Pegylated	*E. coli* (rDNA)	Crohn's disease	UCB and partners
Enbrel	Etanercept	Fusion protein	Dimeric fusion protein (extracellular portion of human tumor necrosis factor receptor linked to IgG1 Fc	Mammalian cell (CHO) (rDNA)	Rheumatoid arthritis; plaque psoriasis and others	Amgen, Wyeth; Takeda
Herceptin	Trastuzumab	Full length antibody	Humanized IgG1 κ	Mammalian cell (CHO) (rDNA)	Cancer (breast, stomach, pancreatic)	Genentech and partners

Vectibix	Panitumumab	Full length antibody	Humanized IgG1 κ	Mammalian cell (CHO) (rDNA)	Cancer (colorectal)	Amgen
Gardasil	Human papillomavirus quadrivalent vaccine	Protein vaccine (VLP)	Self-assembled VLP of capsid protein of HPV types 6, 11, 16, and 18—adsorbed into aluminium-containing adjuvant	*Saccharomyces cerevisiae* (yeast) (rDNA)	Prevention of several diseases caused by HPV	Merck
Prevnar	Pneumococcal 7-valent conjugate vaccine	Vaccine (glycoconjugate)	Saccharides of capsular antigens of *Streptococcus pneumoniae* serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F each conjugated to diphtheria CRM197 protein	Serotype from soy peptone broth; CRM197 from *Corynebacterium diphtheriae*	Immunisation against several diseases caused by *S. pneumoniae*	Wyeth (Pfizer)
Fragmin	Dalteparin sodium injection	Carbohydrate	Controlled depolymerization of sodium heparin	Porcine intestinal mucosa	Deep vein thrombosis and others	Pfizer
Pulmozyme	Dornase α	Enzyme (glycoprotein)	Recombinant human deoxyribonuclease I	Mammalian cell (CHO) (rDNA)	Cystic fibrosis	Genentech
Fabrazyme	Agalsidase β	Enzyme (glycoprotein)	Recombinant human α-galactosidase A	Mammalian cell (CHO) (rDNA)	Fabry disease	Genzyme

Abbreviations: IgG, immunoglobulin; Fab, antibody fragments; PEG, polyethylene glycol; VLP, virus-like particles; mAb, monoclonal antibody.

characterization of drug product formulation using biochemical and biophysical methods for adequate understanding of structural and functional correlations to stability in a stage appropriate manner. It should be noted that depending on the type of biologics candidate and its stability profile, it may be necessary to conduct additional formulation characterization studies especially when stability is poor and/or stability-bioactivity correlation is complex. In later stages of clinical development as well as for biologics license applications (BLA) it is expected that extensive formulation characterization studies are conducted.

Determination of Hot Spots

An important and first step in formulation characterization is to determine the potential liabilities in the amino acid sequence and other parts (for contents other than amino acid) of the biotherapeutic candidate. These liabilities are often referred to as "hot spots." There are some amino acids or groups of amino acids that exhibit common occurrences of chemical or physical degradation events such as oxidation and deamidation. For example, the amino acid methionine (Met) undergoes oxidation, especially in the presence of oxygen and when it is on the protein surface exposed to bulk solvent. Similarly, a surface-exposed pair of asparagine-glycine (Asn-Gly) when present in a loosely formed structural domain in the protein may be prone to deamidation under certain formulation conditions (1).

Linear sequence vs. folded structure. Determination of hot spots may not be trivial for all protein types. Prediction of lability of an amino acid based on primary structure [i.e., amino acid linkage (Table 2)] does not work well for folded proteins because surface exposure and flexibility in the three-dimensional structure are among the important criteria dictating propensity of degradation. For certain classes of biotherapeutics where adequate correlation between structural and chemical degradation is available, it might be possible to more accurately predict hot spots. For example, immunoglobulins (IgGs) of a given subtype may contain common hot spots in the conserved part of the sequence (Table 2). Similarly, degradation behavior of a nonconserved amino acid in a conserved structural motif in IgGs may be partially predicted on the basis of structural flexibility of the motif (unordered vs. helical or β sheet). While these approaches are quite useful in enlisting the common hot spots for chemical degradation, they may not predict physical degradation (aggregation) hot spots or unique chemical degradation events [e.g., tyrosine (Tyr)/tryptophan (Trp) oxidation].

The determination of hot spots needs information on folded structure but many biotherapeutic candidates will not have its crystal structure or other solution-based (e.g., NMR) structure available. In the absence of structure, homology modeling may be beneficial to derive qualitative structure using bioinformatics tools. In a recent study, Wang et al. (14) employed a novel use of bioinformatics tools to delineate common sequence segments across several antibodies and hypothesized that such segments may contribute to aggregation propensity on the basis of certain physicochemical properties of the contributing amino acids in these segments (rich in aliphatic/aromatic residues). Using full antibody atomistic molecular dynamics simulations, Chennamsetty et al. (15) identified the antibody regions prone to aggregation by using a technology called spatial aggregation propensity. Development of such bioinformatics tools is a good first step in understanding aggregation propensity, however it remains to be experimentally tested how accurately and widely such tools can be used for reliable prediction appropriate for drug development.

Physical and Chemical Degradations

Following determination of hot spots as described above, the next step in formulation characterization is to experimentally determine the major degradation pathway(s) and to understand the mechanism of degradation. Unlike small molecule drugs, protein-based biotherapeutics candidates have added complexity of several degrees of structure such as secondary, tertiary and quaternary structures that are critical to its stability and intended function. The degradations observed and/or predicted can be categorized into two types—chemical and physical degradations. Majority of the degradations cited in Table 2 are of

Table 2 Protein and Peptide Degradation Hot Spots

Labile groups	Type of degradation	Occurrence in IgG and other proteins
Asn-Gly	Deamidation, Isomerization	$NN^{386}G$ in CH3 (IgG2a) (2) $QN^{156}G$ in CL (IgG2a) (2) $LN^{316}G$ in CH2 (IgG1) (3) $SN^{385}G$ in CH3 (IgG1) (3)
Asn-Ser, Asn-Asn, Asn-Thr, Asn-Lys, Asn-His, Asn-Asp	Deamidation, Isomerization	$RN^{423}S$ in CH3 (IgG2a) (2) $PEN^{390}NY$ in CH3 (3) $VN^{30}T$ in CDR1 of LC (4) $SN^{329}K$ in CH2 (5)
Asp-Pro Asp-Gln	Clipping (peptide bond)	D^{274}-P^{275} (IgG1) (5) D-K in hinge (IgG1) (5) H-T in hinge (IgG1) (5)
Asp-Lys His-Thr		
Asp	Isomerization	$D^{102}G$ in CDR3 of HC (IgG1) (4)
Met	Oxidation	M^{34} in CDR1 of HC (IgG1) (6) M^{101} in CDR3 of HC (IgG1) (6)
Cys	Oxidation (to form disulfide)	C^{105} in CDR3 of HC (IgG2a) (2)
Trp	Oxidation	W^{54}, W^{55} in CDR2 of HC (IgG1) (6) W^{105} in CDR3 of HC (IgG1) (6)
Tyr	Oxidation	Oxidation of lens protein forms dihydroxyphenylalanine, o- and m-Tyr, and di-Tyr (7)
Pro	Proline isomerization	Trans-P^{32} isomer formation in β2-microglobulin (8)
Lys	Glycation	K^{49} in LC (IgG1) (9)
Fe-His/Asp/Tyr	Metal bond breakage	Iron loss by acidic pH, chelator in transferrin (10)
His-Fe (heme)	Metal bond breakage	Low-pH Fe-His breakage in hemoglobin (11)
Met-Fe (heme)	Metal bond breakage	Labile Fe-S (Met) bond in cytochrome c breaks under various conditions (12)
Amine and other reactive amino acids	Reaction with buffer/excipients	May form adducts such as carboxylate adduct with citrate/succinate (13)
Various hydrophobic segments	Aggregation	Potential hot spots for aggregation in IgG predicted using bioinformatics tools (14,15)

Abbreviations: IgG, immunoglobulin; LC, light chain of IgG; HC, heavy chain of IgG; Tyr, tyrosine; Met, methionine.

chemical nature, whereas physical degradation includes aggregation, particulate formation, and related structural degradation events associated with adsorption, misfolding, denaturation (by heat, chemicals, chaotropes, etc.), partial misfolding, nucleating species, and sometimes chemical degradation. Physical degradation is complex and may involve a wide variety of causative factors that may involve protein-protein interaction, native state conformational distortion, air-water interfacial tension, and conformational changes induced by solvents, additives, and processing. Therefore, a multitude of biophysical tools (in addition to biochemical characterization) is often necessary to achieve a comprehensive formulation characterization.

ASSESSMENT OF PRIMARY STRUCTURE

Simply put, the primary structure of a protein consists of its amino acid sequence. For recombinant proteins, the amino acid sequence can be predicted from the cDNA used in its production. This basic attribute of a protein determines the entirety of its biophysical and biochemical properties. The amino acid sequence of a protein determines its ability to fold properly, and thus determines its ability to maintain its function. Therefore, a small change in the primary structure, depending on its location, may have a range of effects on a protein's activity, from no effect to a very large impact. The amino acid sequence can also impact the chemical and physical stability of a protein, even when there is no measurable impact on activity. Thus, confirming the amino acid sequence of a protein is fundamental to understanding its overall structure and properties.

During production of recombinant proteins, several modifications to the primary structure are possible. These include errors in transcription or translation, generating such variant forms as amino acid substitutions, N- and C-terminal extensions, splice variants, and internal sequence extensions. Other changes to the primary structure may occur as a consequence of biochemical instability, such as deamidation or oxidation. All of these variant forms can have large impacts on the properties of the protein, and need to be detected and controlled during production and storage.

Amino Acid Composition Analysis

One of the most basic assessments of primary structure is the confirmation of the expected amino acid composition of the polypeptide. Recombinantly produced proteins have amino acid sequences predicted from the DNA sequence used in their production. The amino acid composition, therefore, is a predictable attribute, and can be confirmed using amino acid composition analysis. The technique can be broken down into three steps: complete hydrolysis of the polypeptide into its constituent amino acids, chemical labeling of the free amino acids with a chromophore or fluorophore, and separation of the amino acids by liquid chromatography (LC), with quantification of the individual amino acids by UV absorbance or fluorescence detection (16,17). Typically, overnight digestion with 6N HCl or other acids at high temperature or vapor phase hydrolysis with trifluoroacetic acid is used for complete hydrolysis. Derivatization can be achieved either prior to separation (precolumn) or after separation but prior to detection (post-column). Typical chemical labels include fluorescamine, o-phthalaldehyde (OPA), ninhydrin, and phenyl isothiocyanate (PITC). Separation can be accomplished for all twenty naturally occurring amino acids using reversed-phase or ion exchange chromatography (IEC), the former typically used with precolumn derivatization methods and the latter used in combination with post-column derivatization approaches.

The harsh conditions used for complete hydrolysis of the polypeptide can lead to destruction of particularly sensitive residues. Trp and cysteine residues are typically destroyed during acid hydrolysis, and cannot be confidently quantified using this approach. Also, amino acids with side chain amide groups, glutamine and asparagine, are modified to form their analogous amino acids with side chain acid groups. The levels of these amino acids are added to the levels for the glutamic acid and aspartic acid residues, and can be quantified as combinations of glutamine plus glutamic acid and as asparagine plus aspartic acid (Glx and Asx).

The relative amounts of the amino acids present in the protein are determined by comparison with quantitative standards. This is one of the most accurate methods for determining the protein quantity. The amino acid composition of a sample can be compared with the theoretical composition on a residue-by-residue basis. Each of the amino acid residues may have a different precision depending on the relative stability of the residue during hydrolysis and the chromatographic properties of the residue on a given system.

In combination with accurate absorbance measurements, amino acid composition analysis is commonly used for accurate determination of protein molar absorptivity, or extinction coefficient (18,19). Once an accurate extinction coefficient is determined for a given protein, the concentration of the protein in formulated solutions can be determined consistently using UV absorbance spectroscopy according to the Beer-Lambert law

$$A = \varepsilon l C \tag{1}$$

in which A is the measured absorbance at a given wavelength, ε is the molar absorption coefficient in $M^{-1}cm^{-1}$ at that wavelength, l is the pathlength used in measuring the absorbance in cm, and C is the protein concentration in M. So, after determining the molar absorptivity at, for example, 280 nm, the protein concentration can be reliably determined by measuring the absorbance at 280 nm.

N-Terminal Sequencing by Edman Degradation

Confirming the termini of polypeptides is fundamental to their characterization, and N-terminal sequencing using Edman degradation is a robust technology for achieving

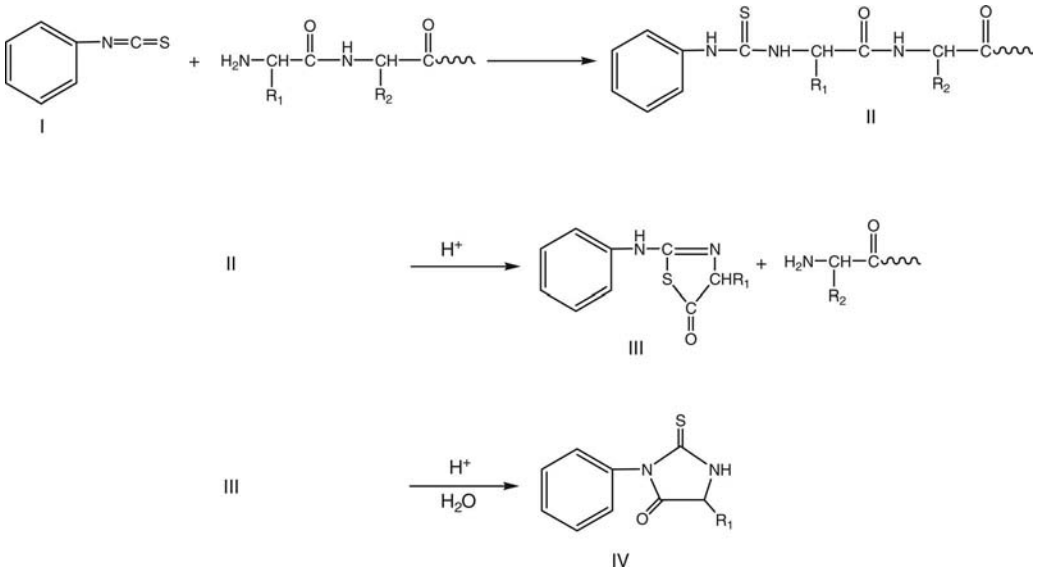

Figure 2 Edman degradation chemistry.

confirmation of the N-terminal residues (20). This technology uses amine-specific chemistry to remove the N-terminal residue, followed by chromatographic separation of the residue. By comparison of the retention time of the released residue with amino acid standards, the identity of the residue can be determined. After release of the N-terminal residue, a new N-terminal amine is generated, and the chemistry can be repeated in multiple cycles to deduce the N-terminal sequence of the protein. The reaction sequence is illustrated in the scheme below (Fig. 2). There are three steps to the Edman degradation reaction: (*i*) coupling of the Edman reagent, PITC, (*ii*) cleavage of the phenylthiocarbamyl polypeptide (*iii*) under acidic conditions to form an anilinothiazolinone (ATZ) derivative of the N-terminal amino acid, and a new N-terminus on the n-1 polypeptide, and (*iv*) conversion of the ATZ amino acid under acidic conditions to form a stable thiohydantoin (PTH) derivative of the N-terminal amino acid. The chemistry can be repeated after extraction of the PTH–amino acid to determine the next amino acid in the polypeptide, and so on. Sequencing instruments are available such that the entire process is automated.

The number of cycles that can be repeated for a protein is highly dependent on the sequence of the protein, the amount of protein in the sample, and the conditions of the reaction. Typically, up to twenty cycles is easily attainable for a recombinant protein.

In many instances, the free amine on the N-terminus of the protein may be blocked, typically by acetylation or cyclization, thus preventing the Edman degradation reaction from occurring (21). N-terminal acetylation is a common post-translational modification which can prevent Edman sequencing. There are strategies for unblocking or removing acetylated N-terminal residues using enzymatic or chemical methods, but these methods are not generally considered to be very efficient. If the N-terminal residue is glutamine, these residues undergo spontaneous cyclization, blocking the free amine to form pyroglutamic acid (22). Less common is cyclization of glutamic acid to form pyroglutamic acid (23). Pyroglutamic acid can be efficiently removed using a pyroglutaminase enzyme, generating a free N-terminus on the n +1 residue that can then be sequenced using Edman degradation (24).

Proteolytic Mapping

Proteolytic mapping of proteins is the most comprehensive method for the determination of primary structure. This method employs the use of residue-specific enzymes to cleave the protein into smaller peptides, which can then be separated using high-performance liquid chromatography (HPLC). The resulting chromatogram, or proteolytic map, can be extremely

reproducible and specific for the protein, and can be used as an identity method when compared with a reference standard of the protein. It is often used as a batch release test for this purpose (25). Proteolytic maps can be extremely efficient at detecting changes in the protein primary structure, since a single change of an amino acid at the peptide level can often generate a detectable shift in the retention time of the peptide. In combination with MS, it is often used as a characterization tool for detecting and quantifying impurities and degradants (26).

The proteolytic enzyme appropriate for use for a given protein depends on the amino acid sequence. An analysis can be performed utilizing theoretical digestion on the basis of the specificity of the enzyme to determine the most appropriate enzyme for a given protein. The goal is to generate a sufficient number of peptides that can be well separated chromatographically, typically using reversed-phase chromatography, to generate a highly specific proteolytic map.

The specificity of proteolytic enzymes suitable for mapping include trypsin (C-terminal to Arg and Lys), endoproteinase Lys-C (C-terminal to Lys), V8 protease (C-terminal to Glu and Asp), and endoproteinase Asp-N (N-terminal to Asp). There are many other less common or less specific proteases that can be used when appropriate. Trypsin is a very common enzyme used for proteolytic mapping because of its high fidelity for its substrate sites and its generation of highly specific proteolytic maps for many proteins. It has the added advantage of generating peptides with C-terminal Arg or Lys residues, which can be detected with high sensitivity when analyzed using MS because of the high ionization efficiency of basic peptides in the positive ion mode.

Mass Spectrometry

MS is a powerful method for confirmation of the primary structure of proteins and peptides (27). The use of MS for characterization of therapeutic proteins is typically performed as a part of structural elucidation for regulatory submissions, and not as a routine batch release test. Current MS instrumentation is capable of measuring the molecular mass of proteins to within 100 ppm for intact proteins, depending on the instrumentation used and the molecular mass being measured. This is sufficient mass accuracy to confirm the predicted molecular mass on the basis of the amino acid sequence and expected post-translational modifications. For example, a protein with a predicted molecular mass of 20 kDa can be measured to within 2 Da at 100 ppm mass accuracy. With this mass accuracy, many modifications of the primary structure can be detected and examined further if present. The exception to this is alterations in the sequence of amino acids, or modifications or substitutions that lead to mass changes of 2 Da or less, such as deamidation of asparagine residues (a 1 Da mass change). These types of changes require proteolytic mapping in combination with MS or other orthogonal methods for their detection.

There are many types of MS methods that can be used for analysis of biotherapeutic proteins and peptides. MS is categorized by the type of ionization method and the type of mass analyzer used. For proteins and peptides, either electrospray ionization (ESI) or matrix-assisted laser desorption/ionization (MALDI) are used almost exclusively as ionization methods. ESI is more commonly used, as it is directly compatible with LC/MS as long as volatile mobile phase components are used for the separation (28,29). For analysis of intact proteins, either ionization method can be used. There are many types of mass analyzers used for therapeutic proteins. MALDI is typically coupled with time-of-flight (TOF) mass analyzers, and is characterized by very good sensitivity and a fairly high tolerance of salts and other buffer excipients (30). Compared with ESI coupled with TOF analyzers, however, the resolution of MALDI-TOF is significantly lower, meaning that the mass accuracy is not as good and the ability to detect variant forms is diminished. ESI is very intolerant of salts and buffer components; adducts of alkali metal ions are common for impure samples, which can lower the effective sensitivity and lead to an inability to accurately determine the molecular mass. Therefore, significant sample preparation to desalt the sample is required. However, when coupled with LC/MS, the separation effectively ensures that a pure sample is introduced into the ionization source so that high-quality spectra can be acquired. An efficient approach to analyzing intact proteins is to use LC/MS in which a de-salting column is used prior to introduction of the sample into the

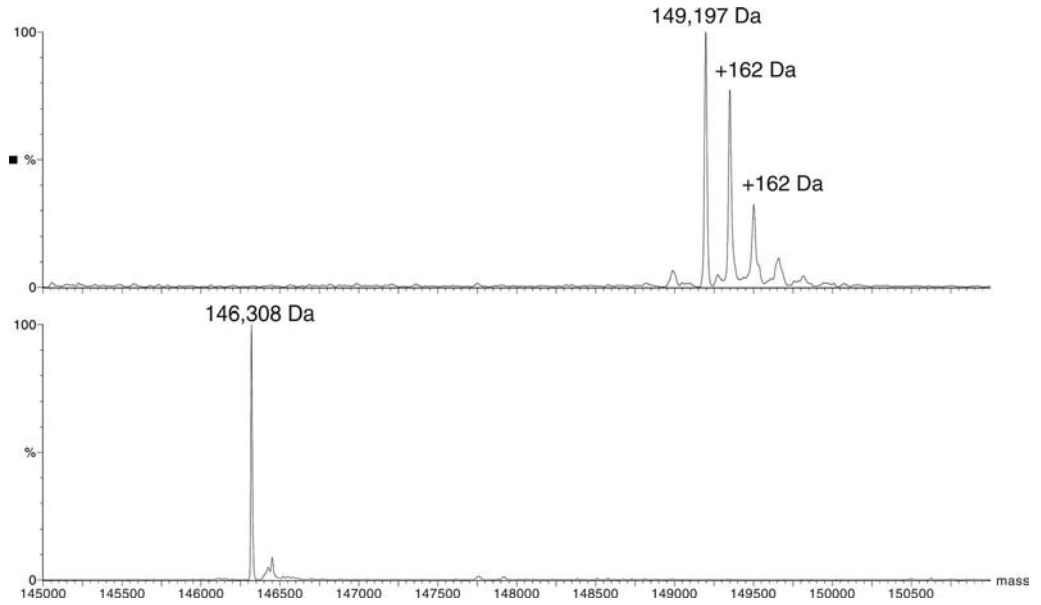

Figure 3 Mass spectra of an IgG (*top*) and the same IgG treated with PNGaseF to remove the N-linked glycans (*bottom*).

ionization source. A common approach for characterizing proteins and peptides using MS is to first analyze the molecule intact, and then perform analyses on samples which have been purposefully degraded in some way to generate smaller species which can be thoroughly characterized. For example, a multichain glycoprotein can be analyzed intact, reduced, deglycosylated, reduced and deglycosylated, etc., with proteolytic mapping as the final "degradation" approach. An example of this is shown below in Figure 3, which shows a therapeutic monoclonal antibody (mAb) before and after deglycosylation analyzed using LC/MS. In the top spectrum, the glycan heterogeneity is evident from the multiple signals observed in the spectrum, most of which differ by the mass of a single hexose moiety, 162 Da. The mass difference between the untreated and deglycosylated samples yields the molecular mass of the N-linked glycans removed from the mAb, in this case 2889 Da. This difference corresponds to the molecular weight of two G0 glycans, each with a monosaccharide composition of four *N*-acetylglucosamine residues, three mannose residues, and one fucose residue. This is a common type of glycan for IgG molecules. On the basis of this analysis, the molecular mass of the expected primary structure can be confirmed, and information concerning some of the modifications, in this case glycosylation, can be determined as well.

When used in combination with proteolytic mapping, MS is invaluable for detecting unpredicted modifications to the primary structure and post-translational modifications (26). By digesting the protein into smaller pieces, more detailed information can be obtained for any modifications to the primary structure. For a tryptic peptide of molecular mass 1000 Da, for example, a mass change of 1 Da from the predicted molecular mass can be easily determined. An example is shown in Figure 4, which shows an expanded version of the 214 nm UV absorbance chromatograms of a tryptic digest of an IgG (top) compared with the same molecule subjected to a pH of 8 for three days to induce deamidation (bottom). The control sample shows a peak, labeled Peak A, which has a molecular weight consistent with an expected tryptic peptide with a sequence of GFYPSDIAVEWESNGQPENNYK. Two new peaks show up in the stressed sample, labeled B and C. Figure 5 shows the mass spectra of these peaks. Peaks B and C show a 1 Da difference relative to Peak A. This is consistent with deamidation of an asparagine residue. The predicted peptide contains three asparagine residues, making this a reasonable interpretation of the data. Tandem MS, in which an ion

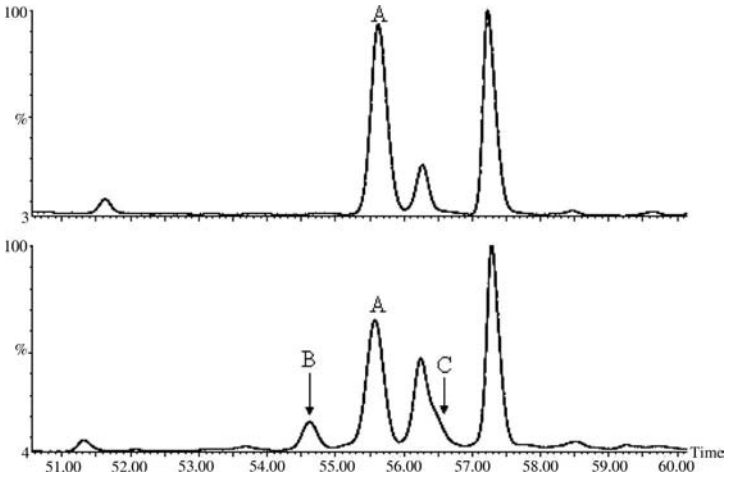

Figure 4 Expanded views of the tryptic maps (214 nm) of an IgG sample (*top*) and the sample treated with pH 8 for three days (*bottom*).

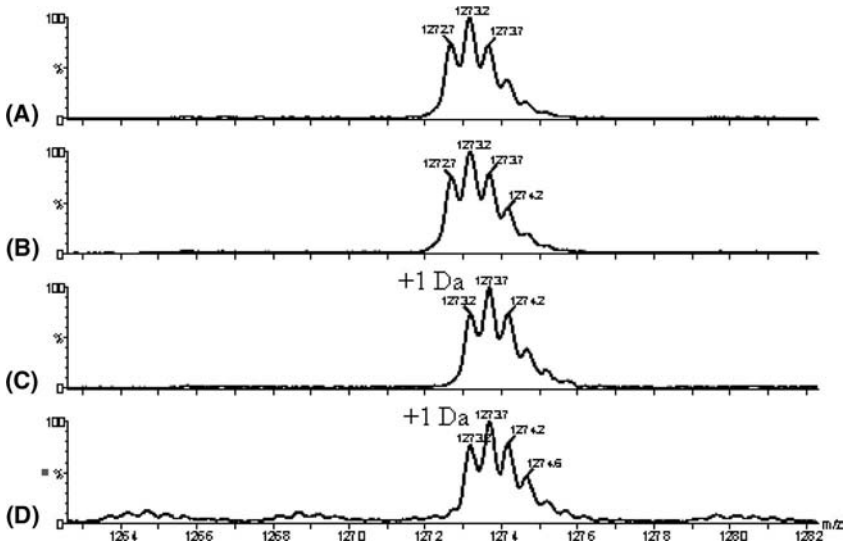

Figure 5 Mass spectra of the peaks shown in Figure 3. (**A**) IgG peak A. (**B**) Stressed IgG peak A. (**C**) Stressed IgG peak B. (**D**) Stressed IgG peak C.

formed in the ionization source is subjected to fragmentation and the resulting fragment ions are measured, is a powerful tool for determining the sites of modifications. In the example above, the precursor ion for the putatively deamidated peptide can be subjected to tandem MS to determine which asparagine in the peptide is the site of deamidation for each of the peaks.

Tandem mass spectrometry (MS/MS) can be accomplished using multiple modes of fragmentation. Most commonly used is collisionally activated dissociation (CAD), in which the precursor ion is accelerated in a collision cell in the mass spectrometer which is filled with a collision gas, such as argon, to impart internal energy into the ion, leading to fragmentation. For peptides, fragmentation tends to occur along the peptide backbone at the amide bonds. This leads to fragment ion spectra which differ in mass by the residue mass of the amino acids present in the peptide. In this way, the sequence of the peptide and the site of any modifications to the peptide can be determined. Fragmentation of the peptide can also be generated using other means, including electron transfer dissociation (ETD) in ion trap

instrument, or electron capture dissociation (ECD) or multiphoton dissociation (MPD) in ion cyclotron resonance (ICR) instruments.

ASSESSMENT OF SECONDARY STRUCTURE

Circular Dichroism

CD spectroscopy measures differences in the absorption of left-handed and right-handed circularly polarized light which arises from an optically active (chiral) molecule. The amide bonds in a protein absorb in the far ultraviolet (far UV), approximately 180 to 250 nm, where the peptide contributions dominate. The intrinsic CD of a protein in the far-UV region is influenced by the asymmetric environment as a consequence of the three-dimensional structure adopted by the molecule, and therefore is sensitive to the secondary structure (e.g., α-helical, β-sheet, β-turn) of the protein. This gives rise to characteristic CD profiles for each secondary structure type (31,32). Therefore, any changes in protein secondary structure due to unfolding or structural conversion can be conveniently monitored by CD spectroscopy.

In the wavelength range at greater than ~240 nm, typically ~240 to 300 nm (near UV), the amino acids Cys (at >240 nm and >320 nm), Phe (peaks at ~262 nm and ~268 nm), Tyr (maxima at ~275–282 nm) and Trp (~280–293 nm and ~265 nm) exhibit CD bands that are characteristic of the tertiary structure of the biomolecule. These bands can be used to monitor local conformational changes as well as large scale structural changes in the protein (31,32).

The biomolecules that contain non–amino acid groups in their active site (ligand- or substrate-binding site) such as porphyrin, heme, metal centers (Fe, Mo, Cu, etc.) coordinated to amino acids, Fe-S cluster, and many other groups may display characteristic CD bands depending on the local structure of the chromophore and its chiral properties. Such CD bands can be extremely useful probes for studying structure-function relationship in these proteins, especially the redox-induced events.

CD of protein-based biologics such as human growth hormone or monoclonal antibodies is measured in aqueous buffered solutions. A protein solution of approximately 0.1 to 1.0 mg/mL can be used, depending on protein molecular weight, its CD strength, and pathlength of measurement cell to measure far-UV CD spectra. Because the near-UV CD signal of proteins is far less intense than in the far UV, typically a ~10× higher concentration is needed for the near-UV range. Alternatively, with higher protein concentration, various pathlengths of sample cell (e.g., cuvette) can be used to accommodate CD measurements in wider wavelength ranges. One can push the measurable limit of high protein concentrations using conventional CD instrumentation by reducing the cell pathlength to much less than 1 mm. However, concerns of surface denaturation of protein due to interfacial tension, artifacts of solution drying, and inaccuracy of pathlength need to be considered to ensure the quality of CD data. To consistently acquire good quality CD spectra down to ~190 nm, the spectrometer along with the UV lamp and mirrors must be carefully maintained and purged with high quality nitrogen flow.

Far-UV and near-UV CD data are often used for assessment of secondary and tertiary structure, respectively, of a biologics candidate—for analytical reference material characterization as well as drug product formulation characterization. It is also used to establish comparability of drug substance between campaigns and/or batches. It should be noted that the near-UV CD spectral signature by itself generally does not point to any particular tertiary structural type of a protein, but instead can be used to compare changes between batches of recombinantly produced protein.

The CD spectrum of proteins in the far-UV range has distinct signatures for α-helical and β-sheet structures. For example, a majority α-helical content (e.g., human growth hormone) displays strong negative bands at ~208 nm and ~222 nm, and a positive band at ~192 nm, while a majority β-structure content (e.g., mAb) shows a negative peak at ~216 nm and a positive peak at ~200 nm depending on the mix of β-sheet (parallel or antiparallel β-sheet) and β-turn components, and any α-helical contributions. Because α-helical structure contributes much stronger to the CD spectrum in the far UV, the presence of even a small percentage of α-helical structure content can significantly change the CD peak positions of a majority β-sheet protein. Unordered (random coil, e.g., unfolded protein) structures can exhibit a strong negative band at ~195 to 200 nm (32). The CD spectrum in the far-UV range can be used to

make an empirical estimate of secondary structure using several algorithms including least squares fitting, singular value decomposition, and self-consistent method (SELCON) (31,33). SELCON is quite popular for secondary structural estimates, and it deconvolutes decent structural information for both α-helical and β-sheet/turn structural components. However, depending on the quality of CD spectra, the estimation of structure can vary significantly, and therefore such estimates should not be used for comparability purposes. A better protocol for drug substance comparability is to compare and overlap normalized CD spectra of protein samples of which accurate protein concentration data (of the identical samples that are used in CD measurements) are available. Normalized CD values can also be expressed in molar residue ellipticity (i.e., also normalized for number of amino acid residues) that is useful to compare CD value/spectra between different proteins belonging to the homologous structural class.

Although CD spectra in far and near UV are very useful in assessing as well as comparing the secondary and tertiary structures of a protein, it is very challenging to reproducibly detect small structural changes. Because of uncertainties introduced by the measurement protocol as well as interference from the drug product formulation matrix, it is difficult to determine an accurate limit of quantitation of the method.

Fourier Transform Infrared

Fourier transform infrared (FTIR) spectroscopy is another tool for probing secondary structure of protein- and peptide-based biologics candidates (34,35). The vibrational motions in a molecule when coupled with a change in dipole moment can be observed, in principle, in an FTIR spectrum. However, several factors including overlap with rotational motions result in significant band broadening under normal conditions relevant to biologics formulations. Additionally, the changes in dipole moment need to be sufficient for actually observing a vibrational frequency. For peptides and proteins, typically the amide region is tracked for secondary structure determinations (34,35). The amide region has multiple frequencies but practically three of them (Amide I, Amide II, and Amide III) are most useful. Modern FTIR spectrometers are capable of producing high quality spectra in the mid-IR range of approximately 1000 to 1800 cm^{-1} that is useful for protein secondary structures. The low frequency range (<1000 cm^{-1}), if desired for detecting out-of-plane bending modes in polypeptides, can be studied using accessories with appropriate IR grade materials (crystal). For example, an attenuated total reflectance (ATR) accessory with diamond crystal and compatible optics can go down to approximately 200 cm^{-1}. In addition to protein-related vibrational bands, one can choose to probe signature bands from excipients (e.g., sucrose) and other additives present in biologics formulations. Finally, the CO stretching vibration of carboxylic acid-containing side chains and other vibrational modes from polar and aromatic side chains of amino acids also can be seen in FTIR spectra of proteins, but these are typically much weaker than amide I and II bands (35).

Protein FTIR spectra show a strong amide I band in the 1600 to 1700 cm^{-1} range arising from primarily C = O stretching of the polypeptide backbone. Amide II ($\sim$1480–1580 cm^{-1}) and III ($\sim$1230–1300 cm^{-1}) bands are comprised of CN stretching and NH bending modes. The amide bands are sensitive to type of secondary structure (e.g., α-helical, β-sheet, β-turn) and therefore the band pattern (intensity and frequency) in the amide region of an FTIR spectrum can be used to distinguish protein structural types. The origin of this sensitivity (frequency and intensity pattern) is attributed to hydrogen bond strength of amide CO and NH groups, and associated dipole orientations (collectively) present in a particular secondary structure type.

Unfortunately, interference from water (water bending frequency at $\sim$1645 cm^{-1} overlaps with amide I) is a major issue for most biologics formulations, especially for aqueous solutions. Water being the major component ($\sim$55 M) in aqueous formulations gives rise to a strong band that requires careful subtraction by a reference spectrum. Obviously, the water issue is minimized when the biologics formulation is freeze-dried to make lyophilized powder with low water content. Water interference as well as other measurement errors can lead to erroneous assignment of secondary structure types. Several practical measures have been proposed to avoid some of the artifacts (36). These include ensuring (a) appropriate amide I/II ratio (1.2–1.7), (b) presence of amide III bands, (c) presence of C-H stretching modes,

Table 3 Fourier Transform Infrared Frequencies of Amide I Band in Polypeptides

Structure type	Amide I frequency, cm^{-1}
α-Helix	~1654 (range 1640–1660)
β-Sheet[a]	~1633 (range 1620–1641)
	~1684 (range 1670–1695)[a]
β-Turn[b]	~1672 (range 1650–1690)
3_{10}-Helix	~1660–1670
Unordered structure (random coil)	~1654 (range 1640–1660)
Denatured aggregate[c]	~1615
	~1695

[a]β-sheet amide I is often characterized by a shoulder at ~1670 to 1695 cm^{-1} in addition to the major band at ~1620 to 1640 cm^{-1}.
[b]Assignment of amide I for β-turn is highly variable and should therefore be used with caution.
[c]Aggregates formed by native state or nearly native state of proteins may not exhibit amide I frequencies similar to denatured aggregates.
Source: From Refs. 35–38.

(d) appropriate subtraction of vapor bands, (e) no artifact from protein adsorption on sample cell or ATR crystal, (f) appropriate baseline of spectrum, and (g) mismatch of pathlength between sample and reference spectra. An ATR accessory is particularly useful for versatile applications including lyophilized powder, suspensions, liquid, etc., that provide adequate surface contact on the crystal. Diamond crystal is scratch resistant and may help avoid excessive protein adsorption, therefore eliminating some of the artifacts noted above.

Determination of secondary structure is often achieved by examining the amide I frequency or group of frequencies (Table 3). This is possible when a protein or peptide has predominant helical or β structure. However, if structure content is mixed, it is difficult to readily assign a structural type. Additionally, as seen in Table 3, the range of amide I frequencies of multiple structure types overlap significantly (e.g., frequency overlap of α-helix and unordered structure). For an unknown structure, one can get a qualitative estimate by using various algorithms including curve fitting, and pattern recognition such as factor analysis. The derived numbers for content of structural component types are only qualitative, and they can be quite sensitive to the quality of an FTIR spectrum. Therefore structure content determinations may not be suitable for QC (quality control) environment.

Collecting FTIR spectra of low-concentration protein formulations (1 mg/mL or less) can be challenging for the detection of amide bands. However, many of the commercial and clinical biologics formulations employ relatively high active concentrations; therefore FTIR can be suitably used. In fact, for very high concentration protein formulations, FTIR is one of the very few techniques that do not require sample dilution. To prepare samples for FTIR measurements, KBr pelleting has been extensively used. This could be a problem for some sensitive proteins. The modern applications (using ATR and other state-of-the-art accessories), however, do not require sample manipulation, and therefore enables higher throughput as well as application to a wide variety of biologics samples.

ASSESSMENT OF TERTIARY STRUCTURE

Disulfide Bond Determination

The tertiary structure of a protein is often highly dependent on the formation of disulfide bonds. Disulfide bonds confer physical stability to the protein as well as ensuring that it maintains its active form. For recombinantly produced proteins, the confirmation of disulfide bonds is a fundamental part of the elucidation of structure, and any variants present because of incorrectly paired disulfides needs to be assessed. The number and arrangement of cysteine residues in a protein can lead to significant complexity for the determination of the disulfide connectivity.

A typical approach for the determination of disulfides in a protein involves proteolytic mapping under nonreducing conditions, followed by detection of the resulting disulfide-bound

peptides formed, often using mass spectrometric detection (39). For small proteins with few cysteines, this may be straightforward. For larger proteins with many cysteine residues, the complexity may require additional analyses to map all of the disulfides. A parallel analysis, in which all of the disulfides are reduced, with a comparison of which peaks have changed upon reduction, can aid in the detection of which peptides are involved in disulfide bonding. IgG molecules, which are a major class of biotherapeutics in the form of monoclonal antibodies (mAbs), have several disulfides predicted in the constant and variable regions of the molecule. These disulfides serve to connect the heavy and light chains together and to form the intrachain loops necessary for the IgG to maintain its functions. Most of the commercial therapeutic mAbs are IgG1 molecules, which is the major subclass of the IgG class of molecules. The disulfide bonding of IgG1 molecules has been well established. IgG2 molecules have been under development as biotherapeutic entities for some indications because of their low level of secondary activity, such as antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC). One IgG2 molecule, panitumumab, which is an antiepidermal growth factor receptor (EGFR) mAb, has been approved for use for the treatment of metastatic colorectal carcinoma (40). It was recently discovered that IgG2 molecules have an intrinsic heterogeneity in their disulfide connectivity, which leads to a mixture of at least three forms of disulfide isomers (41). These disulfide mediate isomers differ in the interchain disulfide bonds. As therapeutic entities, the levels of each form and their relative activities and properties are attributes that need to be determined.

Protein Intrinsic Fluorescence

Fluorescence spectroscopy is a powerful and widely used tool to monitor higher order structures in proteins (42). Most proteins have intrinsic fluorescence that originates primarily from Trp residues. Tyr and Phe residues also contribute to total protein fluorescence, although quantum yield of Tyr is much less than for Trp, and Phe is the weakest among the three. Fluorescence may also originate from other cofactors present in a protein such as flavin, porphyrin, etc. For most therapeutic proteins, Trp is widely used as a fluorescence probe because of its frequent presence in proteins as well as ease of use and wide applicability in formulation screening and characterization studies. The advanced uses of fluorescence include fluorescence lifetime measurement (time-correlated single photon counting method, phase modulation method in frequency domain), fluorescence resonance energy transfer (FRET), fluorescence correlation spectroscopy (FCS), single-molecule fluorescence, rotational correlation time by time-resolved anisotropy, decay-associated spectrum (DAS), and others. Discussion of these advanced uses is generally out-of-scope for this section.

When a fluorophore (e.g., Trp) is excited using a light source matching its absorption band (excitation wavelength) electrons are promoted from ground electronic state (S_0) to excited states (S_1, S_2, etc.). The cascade of events following excitation is often described by the Jablonski diagram (42). Fluorescence emission occurs from the lowest vibrational level of the excited state (S_1), and exhibits a red shift because of loss of energy in the process.

Trp(s) in proteins exhibit a relatively broad absorption band at approximately 280 nm. When excited at 280 nm, Trp emission occurs over a range of wavelengths up to approximately 450 nm and appears as a very broad band. Most folded proteins show Trp fluorescence emission maxima in the 320 to 350 nm wavelength range. Exceptions include azurin in which the Trp located in a highly hydrophobic environment exhibits an emission maximum at 308 nm—the most blue-shifted spectrum known of Trp in a protein. Typically, protein unfolding causes exposure of buried Trp to bulk solvents, and hence a red shift of the emission maxima to approximately 350 nm is observed.

A typical steady-state (i.e., not a fluorescence lifetime study) fluorescence measurement is quite straightforward. However, several precautions should be taken to avoid artifacts. An appropriate concentration of the protein or peptide in solution should be chosen to ensure that absorbance at 280 nm or the chosen excitation wavelength is not far greater than approximately 0.1 OD. High absorbance at or following the excitation wavelength causes nonlinearity and an artificial reduction of emission intensity called “inner filter effect” (loss of emitted photons due to absorption). Inner filter effect is caused by high absorbance of any

component in solution including protein, excipients and other additives if it overlaps with the emission wavelengths, and may lead to incorrect conclusions from fluorescence data, reported in literature [caused by sodium dithionite absorbance, (43,44)]. It is possible to collect partial emission spectra of higher concentration protein solutions (up to $\sim$0.5 OD at 280 nm) at $>$310 nm emission range and using a higher wavelength excitation (e.g., at 295 nm). However data interpretation must be conducted with great care keeping in mind that emission intensity may not be proportional to lower protein concentrations.

Appropriate baseline correction should be performed by subtraction of a reference spectrum of matching solvent. This is particularly important for low-concentration protein solutions and when quantum yield of Trp is very low (such as quenched by heme/metal, or Trp is oxidized). In these cases, the relative intensity of the water Raman band may appear as a prominent shoulder or peak in the emission spectrum. Location of the Raman band ($\sim$3450 cm^{-1}) in a fluorescence spectrum depends on the excitation wavelength (for 295 nm excitation, it appears at $\sim$329 nm).

Choice of excitation wavelength depends on what fluorophore is used as a probe. If a protein contains both Trp and Tyr residues, one can use either 280 nm or 295 nm to collect fluorescence contribution, respectively, from Trp plus Tyr or Trp only. If there are multiple Trps present in a protein, they all contribute to the emission spectrum. Therefore, if a change in fluorescence intensity and/or emission maximum is observed in a multi-Trp protein (such as a mAb), it is not easy to interpret the data because of the large number of possibilities as causative factors including local conformational change, global structural change, solvent effect (if relevant), quenching due to charge, quenching by oxygen/additives/side chain/bound groups/disulfide bond, change of quenching efficiency of quenchers present in native state, and many others.

Measurement of fluorescence lifetime is generally recognized as providing a more quantitative estimate of some of the fluorescence events. For example, if a fluorescence dye partitions itself between hydrophobic and solvent-exposed environments, simplistically it may yield two distinct lifetimes and one can determine the percentage population of each of the components. Measurement of Trp lifetime may not always help because each single Trp displays two prominent lifetime components arising from two rotamers (42,45,46). Therefore multi-Trp proteins are comprised of (theoretically) several lifetime components; however, there are practical difficulties of how many discrete lifetimes can be retrieved from fluorescence decay data. Analysis involving more than four lifetime components is unreliable, but one can employ lifetime distribution analysis aided by sophisticated mathematical algorithms such as Maximum Entropy Method (47). Trp lifetime data can sometimes help in understanding the impact of solvent relaxation and dynamic quenching.

The sensitivity of Trp fluorescence emission maximum in proteins is generally interpreted as excited Trp (indole ring) interacting with its microenvironment (45,46). For example, in azurin (also noted above), Trp side chain is surrounded by a nonpolar environment, whereas if the excited state interacts with a polar solvent or charged/polar side chains, it emits in the red. Emission maxima as well as quantum yield are also influenced by intramolecular quenching (for example, Fe-porphyrin in cytochrome c, Cu in hemocyanin).

For practical applications of Trp fluorescence in formulation characterization as well as for comparability purposes, steady-state fluorescence studies are quite sufficient to probe conformational changes or unfolding of a therapeutic biological candidate because of high sensitivity of fluorescence signal to local environment of Trp and high signal-to-noise ratio of fluorescence signal. The major goal in the application of Trp fluorescence spectroscopy in a comparability study is to interpret the fluorescence properties such as emission maxima and fluorescence intensity in terms of changes in protein structure. In other words, it is expected that comparison of fluorescence spectra will detect any significant changes in folding and structure of a biologics candidate arising from changes in manufacturing and process. Fluorescence quenching studies using acrylamide and sodium iodide provide valuable information on surface exposure of Trp. A conformational transition may change the exposure of Trp to solute quenchers (acrylamide, iodide, or CsCl), hence can be monitored by measuring Trp quenching (45). Steady-state fluorescence anisotropy is another fluorescence protocol that can be used to study rigidity (or lack of) of a protein segment and relative size of a protein.

Anisotropy value can change upon unfolding of a protein or complexation of a protein (e.g., aggregation, antigen binding).

Tyr fluorescence is less commonly studied because of its weaker fluorescence relative to Trp. Tyr absorption band appears at ~277 nm (tyrosinate at ~294 nm) and the corresponding fluorescence emission maximum is at ~303 nm (~340 nm for tyrosinate emission). Although the microenvironment of Tyr may have a strong effect on its emission intensity, the emission maximum of Tyr is relatively insensitive to local environment (48), in sharp contrast to the behavior of Trp.

Use of external fluorescence probes is very popular in all areas of biology, biological chemistry, and protein chemistry. There are literally thousands of fluorescent dyes for various purposes. For formulation characterization, a few of them are worth noting in this section. ANS (8-anilino-1-naphthalenesulfonic acid) and bis-ANS are used traditionally to probe hydrophobicity and change in surface exposure of hydrophobic groups in a protein. Thioflavin T and Congo red are generally used to look for the presence of amyloid-like structure (aggregate). Nile red is also known to be sensitive for aggregate detection.

POST-TRANSLATIONAL MODIFICATIONS

Most proteins are modified in some way after translation of the polypeptide chain. These modifications may impart specific function to the protein and can be integral to the protein activity or stability. For biotherapeutic proteins, common post-translational modifications include disulfide bond formation, N-terminal acetylation, or glycosylation. Degradation of amino acid residues can be considered as post-translational modifications, but are typically discussed separately as part of stability. However, the tools used for analysis of many types of post-translational modifications are the same. The types and propensity of these modifications are dependent on both the protein and the expression system used for its production. Some of the most common modifications and degradation products observed for biotherapeutic proteins are discussed below.

Glycosylation Analysis

Glycosylation of proteins is a common post-translational modification which can affect the physical properties and activity of the biotherapeutic protein. Glycosylation has been shown to affect the activity, *in vivo* clearance, immunogenicity, and stability of biotherapeutic proteins (49,50). For these reasons, the levels and types of glycosylation need to be determined and controlled for biotherapeutic proteins.

Glycoproteins can be either N-linked or O-linked, depending on the type of covalent modification of the glycan to the protein. The type of glycosylation is dependent on both the protein sequence and the expression system used to produce it. Glycosylation may commonly occur for proteins expressed in mammalian or yeast expression systems, but is not observed for proteins expressed in bacterial systems. N-linked glycosylation occurs only at asparagine residues in the consensus sequence of Asn-Xxx-Ser or Asn-Xxx-Thr, where Xxx is any amino acid except proline. While the presence of this sequon does not guarantee glycosylation, it makes N-linked glycosylation a predictable attribute. The amino acid sequence can be easily scanned for this sequon to determine if N-linked glycosylation is a possibility for a given biotherapeutic protein. Analysis of N-linked glycosylation, therefore, begins with an assessment of the site occupancy levels of any possible N-linked glycosylation sites in molecule, referred to as the macroheterogeneity. This can be accomplished using analytical methods which can distinguish size variants, such as electrophoretic or chromatographic separations, or MS. For glycoproteins with multiple glycosylation sites, macroheterogeneity can lead to complex mixtures. For example, the therapeutic glycoprotein interferon γ (IFN-γ) has two sequons for N-linked glycosylation. Therefore, there are four theoretical forms on the basis of occupancy alone: unoccupied, two different singly occupied forms, and one fully occupied form.

The identities of the glycans at a specific site can be extremely varied as well, contributing to additional complexity termed microheterogeneity. Microheterogeneity can be assessed by isolating the glycans associated with a given site and determining the glycan identity. There are a wide variety of analytical methods and approaches for assessing the levels

and identities of glycans present in a biotherapeutic protein. The methods used depend highly on the specific molecule being analyzed, the type of instrumentation and skill available in the laboratory performing the analyses, and the level of detail required for regulatory approval. For routine batch release of a glycoprotein, profiling for consistency may be appropriate, while more detailed structural characterization may be required to satisfy Elucidation of Structure expectations.

For N-linked glycans, there are enzymes such as PNGaseF, which are efficient at removing glycans, which can then be identified using orthogonal methods. Typically, chemical labeling of released glycans is necessary, since they lack a chromophore and thus a sensitive detection method. A common method for quantifying released N-linked glycans, termed glycan size profiling, employs enzymatic release of the glycans, removal of the protein by precipitation or filtration, labeling of the glycans with a fluorophore, and separation of the labeled glycans using normal-phase HPLC (NPLC) with fluorescence detection (51). This method is highly quantitative, since each glycan has one fluorescent label. For this reason, it can be used for routine batch release to ensure consistency in the types and levels of glycans.

Charge profiling is a common method for the determination of the relative amount of charged, or sialic acid containing, glycans. In this method, the glycans are prepared identically to size profiling: enzymatic release of the N-linked glycans followed by fluorescent labeling. The glycans are then separated by anion exchange chromatography, which separates neutral from singly charged from doubly charged glycans. This yields the relative levels of sialic acids in the glycan population.

There are several types of sialic acids possible, and these types depend on the production cell line. For example, murine cell lines such as NS0 produce mainly *N*-glycolylneuraminic acid, while CHO cell lines produce mainly *N*-acetylneuraminic acid. These sialic acid types can be distinguished using sialic acid typing, in which the sialic acid residues are removed from the glycans by acid hydrolysis, labeled with a fluorescent tag, and separated by reversed-phase HPLC. The identities of the sialic acids are determined by comparison of the retention times to a sialic acids reference panel of standards.

Glycan structure determination includes the assessment of monosaccharide composition, the sequence of the monosaccharides, the branching heterogeneity, and the linkage heterogeneity. While known structures can be confirmed using authentic standards, unknowns require a combination of methods, including MS and linkage-specific enzymes. The identities of the glycans can be determined using MS or by analysis of authentic standards. MALDI-TOF or electrospray MS of the released, labeled glycans yields accurate masses, which can be compared with the theoretical masses for confirmation of identity. For detailed structural information, tandem MS may be performed, which can be used for the determination of the sequence, linkage, and branching (52). In combination with chemical labeling methods such as permethylation or peracetylation, linkage information can be determined as well. The types of MS instruments utilized for tandem MS experiments include quadrupole time-of-flight (Q-TOF) hybrid instruments, which have an advantage of yielding accurate mass of product ions, or ion trap instruments, which are capable of multiple stages of fragmentation (MS^n) for potentially increased structural information. The degree of characterization performed is dependent on the nature and requirements of the molecule being developed.

Charge Heterogeneity

Biotherapeutic proteins may have intrinsic heterogeneity based on charge variants. These variants may be due to a variety of sources, including but not limited to, glycosylation with acidic or basic glycans, variably processed or modified N- or C-termini, degradation due to deamidation or cyclic imide formation, other modifications to basic or acidic residues, or peptide bond hydrolysis.

Deamidation may be a major degradation pathway for peptides and proteins containing asparagine residues. The mechanism for asparagine deamidation, shown in Figure 6, involves loss of NH_3 via a cyclic intermediate. The cyclic imide intermediate can be hydrolyzed to yield two potential products: an aspartic acid or an isoaspartic acid, which is a β amino acid. Both of these products are acidic variants of the original polypeptide and can be separated using charge-based separation methods, and both generate a change in mass of 1 Da relative to the

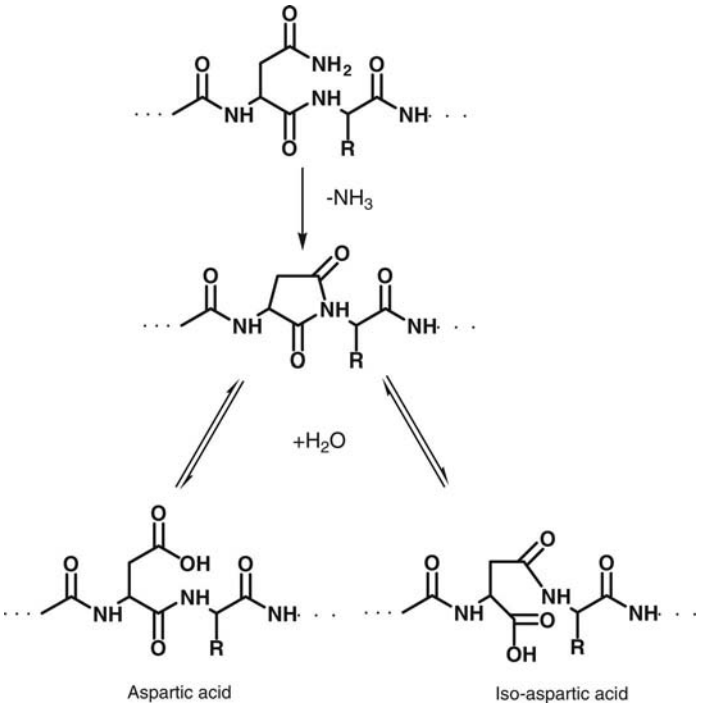

Figure 6 Mechanism for deamidation of an asparagine residue to form aspartic acid and isoaspartic acid via a cyclic imide intermediate.

original polypeptide. The degree of degradation is dependent on many factors, including neighboring residues, surface accessibility and conformation, and the pH of the formulation. The susceptibility and rate of deamidation of a given asparagine residue has been shown to be greatly influenced by the N+1 residue (1). Glycine in the N+1 position has been shown to give the highest rate of deamidation, followed by His, Ser, and Ala. A similar mechanism may occur for aspartic acid residues, in which cyclization followed by hydrolysis to yield either the starting material or its isomer, the isoaspartic acid residue may form. In this case, there is no difference in charge or mass relative to the original molecule. Exceptions to this are examples in which the cyclic imide intermediate is stable. For these species, the cyclic imide has a net basic shift in charge relative to the aspartic acid starting molecule, and is 18 Da lower in molecular mass.

Pyroglutamic acid formation is a common modification for proteins, and occurs spontaneously when the N-terminal residue is a glutamine, or less commonly, a glutamic acid. The formation of pyroglutamic acid from an N-terminal glutamine residue generates a net acidic shift and a loss of 17 Da. This is due to cyclization with the N-terminus with the loss of NH_3 from the side chain, which blocks the N-terminal amine. For monoclonal antibodies, N-terminal glutamine and glutamic acid residues are common for both heavy and light chains, and pyroglutamic acid formation is a very common post-translational modification for IgG molecules (22).

For monoclonal antibodies, variable levels of C-terminal lysine on the heavy chains lead to charge heterogeneity as well. The conserved heavy chain sequence of IgG molecules predicts a C-terminal lysine residue. This residue has been observed to be removed as a post-translational modification, and is thought to be due to proteolysis in the cell leading to a heterogeneous population (53). Typically, a mixture of species exists in which zero, one, or two heavy chains have the lysine removed.

Other modifications leading to charge heterogeneity include glycation of lysine (acidic shift), carbamylation of lysine (acidic shift), C-terminal amidation (basic shift), and N-terminal acetylation (acidic shift). These potential modifications of biotherapeutic proteins need to be detected, quantified, and controlled using analytical methods appropriate for their detection and quantification.

There are a variety of methods that are useful for detecting, characterizing, and quantifying charge variants in proteins. These include isoelectric focusing (IEF), capillary IEF, and ion exchange chromatography (IEC). The advantages of these methods are that they can separate and quantify overall charge heterogeneity. However, they give little or no information concerning the types or sites of charge heterogeneity present in the molecule. For monitoring stability, inherent charge variability may interfere with the ability to monitor degradation using these methods. An example would be the assessment of deamidation in a glycoprotein in the presence of significant heterogeneity in sialic acid levels. So, while these methods may be appropriate for routine batch release and monitoring of consistency, more detailed characterization is required to gain information on the presence of specific modifications leading to charge heterogeneity. Site-specific information can be assessed using approaches involving proteolysis and LC/MS. This approach can be used to characterize and quantify, for example, deamidation at a specific site in the presence of inherent heterogeneity elsewhere in the molecule.

IEF is a gel-based method which separates analytes in an immobilized pH gradient (54). Proteins will migrate in an electric field to their isoelectric point (pI), which is the pH at which the overall charge is net neutral. Charge variants can be well separated using this technique, with resolution as high as 0.01 pH units. The resolution can be determined by the gradient used in the separation. For high resolution separations a very narrow pH gradient may be used with long focusing times. IEF offers a reproducible method for establishing consistency of batches with regard to charge variants, as well as a powerful method for monitoring stability of protein therapeutics. This method is tried and true, but is not highly quantitative because of the need for general protein staining and densitometry, both of which may be highly variable.

Capillary IEF offers the possibility of high resolution with a more reproducible quantitation (55). In capillary IEF, the species are focused in a capillary to their pI, and then migrate to the detector using either electroosmotic flow or differential pressure. Detection can be performed by UV absorbance, offering reproducible and automated quantitation. For more sensitive detection, laser-induced fluorescence (LIF) detection can be used. Another mode of capillary IEF, termed imaged capillary electrophoresis (iCE), offers detection in the capillary without a mobilization step (56). This leads to increased resolution because no band broadening occurs as a consequence of the mobilization step. Also, detection is based on imaging of the entire capillary, so quantitation is generally more reproducible. This method offers high quality, quantitative data for charge variants. The acidic and basic species can be reproducibly quantified using this method.

IEC is a powerful method for separating charge species in an HPLC format (57). IEC separates charged species on the basis of electrostatic interactions of the analyte with a column resin. Anion exchange resins are positively charged, and bind negatively charged analytes, while cation exchange resins are negatively charged, and bind positively charged analytes. In an anion exchange separation, more acidic, or negatively charged analytes, will be retained more strongly and will elute later than less acidic species. Ion exchange resins can be considered strong or weak, depending on the type of resin used. A typical strong anion exchange resin contains a quaternary amine, which has a fixed positive charge or other strong basic species, and tightly binds negatively charges species. Weak anion exchange resins have basic species such as diethylamine functional groups, which bind negatively charged species, but not as tightly as strong anion exchange resins. Conversely, cation exchange resins are either strong acids, such as sulfate groups, or weak acids, such as carboxymethyl groups. Elution of analytes from ion exchange resins can be obtained using a salt gradient to compete with the charge on the resin, or by changing the charge on the analyte by changing the pH of the mobile phase over the course of the separation. As a consequence of IEC being an HPLC method, it has advantages over IEF methods in terms of throughput, ease of use, and quantitative precision. HPLC methods are extremely valuable in that individual fractions of separated species can be collected and characterized further. In an IEC separation, for example, an acidic variant can be separated and quantified, collected, assessed for potency relative to the parent molecule, and further characterized using orthogonal methods to determine the specific site(s) of modification in the molecule. This type of further characterization would be difficult or impossible using capillary electrophoretic methods.

Size Heterogeneity

Size heterogeneity of recombinant proteins may refer to truncated variants because of peptide bond hydrolysis or to the formation of aggregates.

Truncated Species

For truncated variants due to peptide bond cleavage, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a common method used for analysis. SDS-PAGE is based on the migration of a protein in an electric field. The proteins are first treated with SDS, which coats the proteins with a polyanion. In this way, the proteins are coated to similar size to charge ratios, and migration through a polyacrylamide gel is dependent on the size of the protein, with smaller proteins migrating faster through the gel, and larger proteins migrating a shorter distance. By comparison with standards, the molecular weight of the analytes can be estimated on the basis of the migration distance through the gel. Just about any protein analysis laboratory is set up to perform routine SDS-PAGE analysis as a first step in characterization. It gives a visual assessment of the quality of the material in terms of the purity and size heterogeneity. It can be extremely flexible in the type of detection used. General protein stains such as Coomassie blue are reliable and give a visual readout for the detection. For more sensitive detection, silver stain or some of the fluorescent stains such as Sypro Ruby offer the highest sensitivity. Finally, gels offer the possibility of immunoblotting or immunostaining of analytes once they are separated, which provides some additional functional information of the species being separated. Like other gel methods, SDS-PAGE is labor intensive and suffers from a difficulty in reliable quantitation. For routine characterization, SDS-PAGE is a reliable method for assessing the size heterogeneity of a formulated protein product, and is a powerful method for comparison of batches or stability in different formulations.

Capillary gel electrophoresis (CGE), also referred to SDS-CE, has the ability to resolve proteins from 10 to 200 kDa. It offers similar resolution to gel-based separations, but is more easily quantifiable because of the detection methods used (UV absorbance). Like SDS-PAGE, the proteins are coated with SDS and are separated on the basis of migration through an electric field, although in this case through a capillary. Since this method utilizes UV absorbance detection, the sensitivity may be limited for low levels of size variants that in gels could be detected with sensitive staining techniques such as silver stain. LIF detection can help overcome this limitation when applied to CGE. In combination with fluorescence labeling, LIF can lead to sensitive detection of separated species, including low level impurities and truncated variant species (58).

Aggregates and Particulates

Aggregation is a process in which one or more drug molecules combine physically and/or chemically to form nonnative oligomers which may remain soluble or become insoluble depending on their size and other physical properties. Protein aggregates and particulates form in a wide range of sizes (nanometer to centimeter, thereby spanning nearly million-folds in dimension) and shapes making it extremely challenging to comprehensively characterize particulates in a biologics formulation (59,60). Dimers and other smaller size aggregates are soluble in nature and typically range in size from few nanometers to tens of nanometers. The aggregate species that are in the size range of hundreds of nanometers may still remain soluble in the sense that they may not exhibit any change in appearance of the formulation. Some of the multimers can grow huge in size and may eventually appear as visible particulates. Although all multimeric species (referring to degradation products only and not a purposefully created multimeric therapeutic candidate) are generally termed as aggregates, particulates refer to the large size aggregates in the size range of tens of microns or larger species that are visibly detected. Subvisible particulates range in size from few microns to many tens of microns. An approximate size boundary for a particulate to be visibly detected is 100 μm (59).

Characterization of aggregates typically includes detection of soluble aggregates on the basis of size, and determination of physicochemical properties and nature of aggregated

species such as covalent, noncovalent, reversible, irreversible, etc. Covalent aggregates are generally irreversible. Example of a covalent aggregate is disulfide scrambled species, which often are dissociable by a reducing agent. Noncovalent aggregates can be held together either by strong association (i.e., not dissociated by simple dilution or mild treatments) or weak association (i.e., may be reverted to monomer by dilution). Aggregates in both of these structural categories can cover a wide range of sizes. Therefore, it may be more convenient to classify the aggregates in terms of their size in reference to the capability of various biophysical and particle analysis technologies. Insoluble aggregate is also referred to as particulate (subvisible and visible—as noted above), and precipitate (large size species that easily sediments). Insoluble aggregates require somewhat specialized protocols including enumeration (using light obscuration, light scattering, and light microscope) and characterization (imaging-based techniques, and spectroscopic methods such as FTIR or Raman), depending on the types of aggregates observed. Finally, finding the root cause of aggregation may involve all of the above and additional custom-designed protocols.

Formation of aggregates may occur under conditions such as storage, shipping, handling, manufacturing, processing, and freezing-thawing. One of the most challenging areas in aggregation, lately, is studying aggregate formation induced by freezing and thawing of biologics. It should be noted that the freeze-thaw induced aggregation phenomenon should not be confused with cold denaturation. Cold denaturation classically refers to denaturation induced by thermal factors per se without a change in the state of the bulk, and is linked to thermodynamically favored hydration of the hydrophobic core at low temperature (61). Freeze-thaw induced aggregation has been linked to secondary factors such as ice surface denaturation, freeze-induced change in solute concentration and pH, etc. (62), but theoretically may also include effects of cold denaturation. The study of freeze-thaw-induced protein denaturation and aggregation requires specialized equipments and protocols that can probe events in the frozen state.

Several aggregate separation methods are available depending on the type of information sought. Separation methods may either detect the presence of various species in a drug formulation [such as dynamic light scattering (DLS) and analytical ultracentrifugation (AUC)], or fractionate various species [such as size exclusion chromatography (SEC) and asymmetric flow field flow fractionation (aFFF)]. Fractionated species, if desired, may be collected for further analysis.

SEC is considered a "work horse" technique, especially for biologics, and major advantages include high throughput, automation, amenability to several detection systems, reproducibility, reliability, and operational compatibility in both the development and QC environments. Major applications include separation of drug monomer from higher molecular weight species that might accumulate during storage stability and processing. Disadvantages of SEC include concern for potential alteration/dissociation of aggregated species as a result of column/mobile phase interactions. Also, for a given biologics, the dynamic range for separation of various aggregated species is rather limited leaving large aggregates unfractionated or lost.

All four methods noted above (SEC, aFFF, AUC, DLS) are used in formulation characterization to monitor aggregate formation and to delineate the aggregation mechanism. Only SEC is used in a QC environment such as in GMP stability studies. Use of the other three techniques (AUC, aFFF, and DLS) in a QC environment is quite challenging because of the difficulty in adequately validating the methods and/or their low throughput. More detail of these techniques is covered in the last section of this chapter.

Although a relatively smaller number of techniques are available to study large particulates including protein precipitates, additional characterization can be accomplished by solubilizing the particulates using dispersing/denaturing solvents. Multiple biochemical assays can be utilized with solubilized particulates including SDS-PAGE or CGE. This characterization approach can be employed to estimate aggregate size after solubilization and determine if there are covalent linkages between protein molecules. However, the influence of hydrodynamic size may result in an inaccurate estimate of molecular mass for certain molecules, such as conjugated or pegylated proteins.

FORMULATION CHARACTERIZATION METHODS

Selected formulation characterization methods are described below. Use of these methods depends on the type of formulation (e.g., liquid, lyophilized powder, etc.), stage of clinical development, and type of information sought (e.g., to solve a process-related issue, to characterize a degradant, or delineate a stability issue).

Analytical Ultracentrifugation

AUC is an orthogonal method for size-based separation of high and low molecular weight species that employs centrifugal principles to determine size and shape (60). Two principal types of experimentations are conducted in AUC—sedimentation velocity and sedimentation equilibrium. Sedimentation rate (velocity) of the protein species (monomer, dimer, etc.) present in solution is measured as sedimentation coefficient which is governed by several factors including molecular mass, conformation and solvent properties.

AUC measurement does not involve any matrix (column, membrane) interactions, does not dilute the measured sample, and covers a wide size range, especially when multiple measurements are conducted using various centrifugal speeds. AUC typically uses absorbance as a probe. Additionally, interference (for higher concentration) and fluorescence (low-concentration) probes are also available. Therefore, AUC offers an independent confirmation of the presence of any aggregate species in a biologics formulation measured by SEC.

Equilibrium studies employ low centrifugal force to achieve a diffusion-controlled equilibrium, and are typically used to determine molecular mass as well as equilibrium binding constants (e.g., monomer-dimer reversible transition). One of the important applications of equilibrium studies in biotherapeutics is to detect any self-association (reversibly aggregated species).

AUC suffers from low throughput of measurement, lack of robustness, and artifacts from solvent and high concentration formulations. The majorities of the commercial as well as clinical biologics formulations cannot be studied "as is" with the absorbance probe, and require dilution. Although the concentration limit can be pushed higher by the use of an interference probe, several sources of "nonideality" (high concentration, sensitivity to excipients, protein shape factor) can cripple data interpretation. Because the sedimentation profiles by themselves do not provide an estimate of the protein species present, one needs good data analysis software to derive relative quantity of individual species. This is in contrast to SEC quantification, which relies on relative UV absorbance. Several instrument configuration parameters (rotor, cell, loading, probe alignment, wavelength, etc.) add sensitivity to analyzed data, and therefore it can be quite challenging to achieve consistent quantitative results.

Asymmetric Flow Field Flow Fractionation

aFFF uses cross flow onto a membrane in a channel with parallel flow where the smaller particles are transported more rapidly along the channel than the larger particles, hence achieving separation. Prior to migration of particles is initiated, the injected sample is focused onto a narrow area. aFFF is considered an orthogonal method of aggregate separation, using no column (a difference from SEC), and to achieve a wider dynamic range. However, potential interactions with membrane and concern of aggregate formation during its focusing step make aFFF rather unreliable (63). Like SEC, aFFF can also use one or more probes such as light scattering, UV, and refractive index to detect and characterize the fractionated species by hydrodynamic size, molecular weight, and conformational difference.

Light Scattering (Static and Dynamic)

Static Light Scattering

Static Light Scattering measures time-averaged value of scattered light intensity from a sample, typically over many seconds. SLS is used typically in conjunction with separation techniques such as SEC or aFFF. The intensity of the scattered light depends on protein concentration as well as scattering angle, and it is related to radius of gyration, hence molecular mass. SLS provides quite reliable determination of molecular mass of protein monomers and aggregates. For large size particles (such as protein aggregates larger than $\sim$60 nm, depending on wavelength of incident laser) angular dependence is significant, and measurement at several

angles can produce useful data on size. For most protein monomers ($< \sim 10$ nm) such angular dependence is diminished, and measurement at a 90° angle can be used to determine mass.

Dynamic Light Scattering

DLS relies on measuring fluctuations (microsecond and longer time scale) of the scattered light caused by Brownian motion of molecules in solution, and therefore relates to diffusion coefficient (63). With spherical approximation, hydrodynamic radius (Rh) can be extracted from diffusion coefficient values. DLS provides a relatively easy and fast measurement of size (Rh), and covers a large dynamic range (~ 1 nm to ~ 1 μm) in one single measurement. Additionally, the measurement can be done with liquid/suspension of formulated API or drug product without any alteration/dilution (unless strength is greater than approximately 0.3 mM). However, it can resolve species of various sizes only if their hydrodynamic sizes differ by more than 2-fold to 5-fold. This is a serious disadvantage because, for example, monomer and dimer cannot be separated by DLS, instead an average value of size will be measured.

DLS is also referred to as photon correlation spectroscopy (PCS) or, quasielastic light scattering (QELS). Some of the DLS equipments are also configured to measure zeta potential.

Imaging (Static and Dynamic)

Microscopy is an established technique for studying protein particulates. Typically, it requires the particulates to be filtered and examined in static mode. Microscope images can be used for enumeration (pharmacopeia method) as well as for directly visualizing size and shape. Advances in imaging technology enable analysis in dynamic mode where the particles remain suspended in fluid either in stationary or flow modes (59). Digital images of particulates are collected and analyzed to provide a digital archive of particle parameters such as Feret diameter, aspect ratio, circularity, and intensity. Also, if particulate formation in a protein formulation is relatively slow, the dynamic nature of size distribution can be tracked over time. Such data are valuable to characterize particulate formation during biologics formulation development as well as to find potential prevention strategies. Disadvantages include the inherent complexity in determining a true size distribution from imaging data for biologics particulates because of their often extreme irregularity in size and shape. Finally, the size distribution and particulate count from dynamic imaging cannot be directly compared with such information obtained from light obscuration or laser-diffraction analyses (63).

Raman Spectroscopy

Raman spectroscopy, discovered by C.V. Raman (64), is a powerful tool to record the vibrational frequency pattern of a molecule that can be used as a fingerprint for identification. Raman spectroscopy uses laser excitation in modern applications, and frequency shifts caused by the probed molecule relative to the excitation frequency are recorded to generate a Raman spectrum. It is a powerful tool for small molecule pharmaceutical applications that include API identification, determination of tablet depth, and study of polymorphs. Unlike FTIR, Raman spectra have minimal interference from water and therefore the technique is quite suitable for studying aqueous biologics formulations. But protein fluorescence is a significant problem, and Raman bands might completely disappear with elevated background from highly fluorescing proteins, especially in the near-UV region. For proteins, amide bands can be conveniently detected in the ~ 1200 to 1700 cm^{-1} spectral range (analogous to FTIR spectrum) for secondary structure determination. A more advanced application of Raman spectroscopy is Resonance Raman that uses a laser frequency excitation overlapping with a particular protein absorption band (chromophore). This enables detailed structural analysis (including local tertiary structure) of the desired chromophore (e.g., Tyr).

Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) is capable of measuring thermally induced transitions, and particularly the structural transitions of biological macromolecules, such as between the folded and the unfolded structure of a protein. DSC measures the excess heat capacity of a protein solution (Cp) as a function of temperature and the structural transition is recognized as

a sharp endothermic peak centered at the melting temperature (T_m). Generally, DSC is useful to study the energetics of protein thermal unfolding. The T_m of liquid protein formulations is often used as a probe for protein physical stability, that is, higher T_m value may indicate greater physical stability. However, it is recognized that comparisons of physical stability of different classes of proteins by T_m may not hold true. T_m values are also known to be sensitive to the solution matrix such as excipients, pH, buffer and surfactants. However, no clear correlation exists. While some of the stabilizing components may increase the T_m value, some of the stabilizers (e.g., surfactants) may actually result in lower T_m values. Because T_m cannot reliably predict physical stability, establishment of critical parameters in formulation screening and characterization should not be based solely on DSC data. Finally, for monoclonal antibodies, quite often multiple T_m values are observed which are typically assigned arbitrarily to structural domains on the basis of available T_m data of isolated domains in similar protein class. This makes structural interpretation of changes in T_m values as a function of pH or other additives very challenging.

Isothermal Titration Calorimetry

ITC measures heat change from binding interactions, such as antibody-antigen binding or receptor-ligand binding. It is quite versatile and can be applied to a wide variety of molecules in solution without any pretreatment (such as fixation of matrix). ITC can also detect weak interactions with dissociation constants in the sub-millimolar range. Appropriate control experiments must be conducted as several sources of heat change (e.g., heat of dilution) can introduce artifacts.

Near-Infrared Spectroscopy

A near-infrared (NIR) spectrum (12,000–14,000 cm^{-1}) represents combination and overtone bands that are harmonics of absorption frequencies in the mid-infrared region. Because each material has a unique NIR spectrum, NIR spectroscopy can be used as a positive identification of material. NIR is a versatile technique with reduced or eliminated sample preparation, decreased cost and analysis time, and the ability to record spectra through glass and packaging materials.

NIR measures vibrational spectra of a wide variety of materials including solids, liquids, powders, pastes and tablets. NIR has a variety of applications in the area of microbial and cell culture system monitoring and control. An important pharmaceutical application in injectables development is moisture analysis of freeze-dried samples without opening the vials. Determination of water content employs the strong water absorption bands in the NIR region, most prominently the first overtone of OH stretching at around 6800 to 7100 cm^{-1} and the combination band of OH stretching and bending at around 5100 to 5300 cm^{-1}. Karl Fischer method is the most commonly used method for measuring moisture content but it is a destructive method, may need method development, and requires careful handling of sample to not allow additional moisture when a vial is opened. On the other hand, NIR offers increased efficiency in measurement time (higher throughput than Karl Fischer) and reduced cost (especially for expensive biologics products) because the vials can be reused to conduct other assays following NIR measurement. It should be noted however that a standard curve must be generated and requires method development to establish a robust NIR protocol for moisture analysis. If formulation composition is changed, NIR spectrum may also change and additional method development may be necessary.

Powder X-Ray Diffraction

The primary use of powder X-ray diffraction (XRD) in characterizing biologics formulation is to probe the presence of amorphous and any crystalline states in the freeze-dried form. It also can detect the presence of polymorphs of certain excipients such as mannitol. Additionally, the X-ray diffraction can be used to study the phase behavior of the frozen state of protein solutions as well as placebos, using low-temperature accessories (65). Low-temperature XRD is a powerful tool to identify the phases that crystallize during cooling and annealing of frozen solutions.

Freeze-dried powder is often characterized for its crystalinity or amorphousness by the presence or absence of sharp diffraction peaks in the XRD profile. If formulations contain

mannitol, it is important to establish if any of the mannitol polymorphs are present. Mannitol is known to crystallize in multiple forms such as α, β, and δ forms. It also forms a metastable hydrate form that might negatively impact the stability of the freeze-dried formulation.

SUMMARY

Biotherapeutic entities include a variety of macromolecular compounds, each with distinct biochemical and biophysical properties. Extensive structural characterization must be performed for these molecules to be approved as drugs by worldwide regulatory agencies. Characterization should typically include assays to demonstrate that the molecule has the expected primary, secondary, tertiary structure, as well as the expected bioactivity. Any post-translational modifications or variant forms need to be adequately described in terms of the levels and any therapeutic effects such as potency or in vivo half-life. Similarly, the sponsor must demonstrate that the molecule has adequate chemical and physical stability for the proposed shelf-life in its formulation, and that the degradation pathways are well-understood. The analytical toolbox required for these investigations can be extensive, and a suite of assays specific for the biotherapeutic entity can be tailored to provide the required information.

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9 Formulation of protein- and peptide-based parenteral products

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INTRODUCTION

Since the early 1970s, scientific advances in molecular biology and genetic engineering have led to enormous success in protein- and peptide-based therapeutics for the treatment of many human diseases. They cover almost all therapeutic categories, including cardiovascular hemostasis, antineoplastic, diabetes and endocrinology, anti-infective, neuropharmacological, enzyme replacement, wound healing, respiratory, and bone cartilage. Protein-based therapeutics are emerging as a major class of new molecular entities in the pharmaceutical industry. Over 200 biotechnology and pharmaceutical companies are developing protein-based therapeutics. More than 150 biologics are currently marketed, and over 400 are in advanced stages of testing and clinical trials (1).

Unlike small molecules, which are typically synthesized through chemical processes, proteins are produced in living systems. The main technology used to produce proteins utilizes recombinant DNA techniques to produce protein molecules in a host cell. Several types of host cells have been employed, including *Escherichia coli*, yeast, mammalian cells [e.g., Chinese hamster ovary (CHO) cells and human fibroblasts], and plant-derived cells. Several other technologies are also used to produce therapeutic proteins. Small proteins and peptides, such as calcitonin, may be produced by chemical synthesis. Most human serum albumin is sourced from human blood, urokinase from urine, and streptokinase from fungi. Recombinant human antithrombin (ATryn®), a new product approved by the FDA in 2009, is produced by transgenic animals.

CHARACTERISTICS OF PROTEINS AND PEPTIDES

Compared with small-molecule drugs, protein-based pharmaceuticals are not only larger in molecular weight, but they also contain more complex compositions and higher order structures. Intrinsically, most proteins have poor stability and a very short half-life in vivo. Because of their poor oral bioavailability, most proteins require parenteral administration routes. In some cases, they require specific delivery systems targeting the specific site of action to achieve sufficient efficacy. Therefore, formulating these proteins as therapeutic agents with proper efficacy and safety profiles has been a challenging task. For successful product development, one needs to have a thorough understanding of the protein's physicochemical and biological characteristics, including stability, immunogenicity, and pharmacokinetic properties. The characterization of proteins is therefore an important step in formulation development.

Molecular Composition, Structure, and Heterogeneity

A protein, or polypeptide, is formed through the linkage of peptide bonds of amino acids. Generally, protein structures are described at four levels: primary, secondary, tertiary, and quaternary. Details about these can be found in the preceding chapter of this volume.

Because of their complex manufacturing process, from cell culture to downstream purification, protein products generally contain multiple species in terms of molecular weight or size, which could be due to various modifications to the polypeptide side chains or glycans, reversible or irreversible formation of oligomers by either noncovalent or covalent linkages, and formation of large soluble and/or insoluble aggregates. It is important to characterize and quantify all species, as they may directly affect product efficacy, safety, and immunogenicity.

Depending on its size and the nature of its associations, several analytical techniques can be used to characterize a protein's size. Routinely, electrophoretic and chromatographic (with multiangle light-scattering detector) techniques have been used to estimate protein size up to

oligomers. By combining a denaturing electrophoretic technique (sodium dodecyl sulfate polyacrylamide gel electrophoresis [SDS-PAGE]) with size-exclusion high-performance liquid chromatography (HPLC) or a native electrophoretic technique (Native PAGE), the size of proteins and the nature of their associations (covalent vs. noncovalent) in native and denatured states can also be estimated. To more accurately determine the size of proteins, mass spectrometry, such as matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) or liquid chromatography mass spectrometry (LC-MS), is often used. However, because of the matrix effect and the high energy applied, the molecular weight or size determined by this technique may not be the true size in solution.

To measure the size of a protein in solution up to 100 nm, several biophysical techniques may be feasible, including analytical ultracentrifugation (AUC), field flow fractionation (FFF), and dynamic light scattering. It should be noted that the size distribution of proteins in solution, especially for reversible association, may be highly dependent on the solution properties, including pH, salt concentration, and protein concentration. Therefore, the mobile phase used in these analyses is preferably the same as the formulation vehicle, and the impact of the dilution factor during analysis should be assessed.

Insoluble aggregates or particles larger than 100 μm can be observed by visual inspection with the unaided eye. Their size can be estimated by microscopy. Subvisible insoluble aggregates between 10 and 100 μm in size can be quantified and sized either by a light obscuration test or by a microscopic particle count test per USP method <788>. It is still technically challenging to accurately quantify and size particles between 0.1 and 10 μm. A technique using Micro-Flow™ imaging (MFI) has been used for particles as small as 0.75 μm (2).

Isoelectric Point

Proteins that contain both positively and negatively charged amino acids are amphoteric molecules. One property that characterizes a protein's charge profile is its isoelectric point, or pI. The pI of a protein is the pH at which it carries no net electrical charge. At a pH below its pI, a protein is positively charged; above its pI, it is negatively charged.

The pI may be approximately calculated from the amino acid composition data, that is, $pI = (pK_1 + pK_2 + pK_3 \ldots + pK_n)/n$ for n ionizable groups. However, because the dielectric constant in the immediate vicinity of an ionizable group depends on protein structure, and because hydrogen bonding may alter dissociation constants (K_a), the true pI can differ significantly from the calculated one. Several websites provide theoretical estimations of pI for proteins (e.g., http://www.scripps.edu/~cdputnam/protcalc.html, http://www.expasy.ch/tools/pi_tool.html, and http://www.nihilnovus.com/Palabra.html).

Some proteins have multiple species with different charge profiles, and each species has its own pI, so these proteins appear to have more than one pI. Some glycoproteins in particular exhibit complicated pI patterns because of the heterogeneity in their glycan composition. Also, some proteins comprise multiple deamidation species, which also results in complicated charge profiles that could be characterized by several techniques, including isoelectric focusing (IEF), ion exchange chromatography (IEC), and capillary electrophoresis (CE).

Proteins show a broad range of pIs, mostly in the range of 2 to 12. The pI of a protein may play an important role in solubility and stability. In general, protein solubility is at its minimum when the pH is near its pI. Also, because zero net charge at pI should presumably allow maximum interaction between salt bridges and exert the least interaction between protein molecules, it could be expected to be the most stable condition for conformation. However, studies have shown that the optimal pH for conformational stability can be quite different from the pI and in many cases is found at a pH corresponding to a large net charge of the protein (3).

Solubility

The varieties of functional groups (charged, hydrophobic, etc.) on the side chain of amino acids and glycans (for glycoproteins) make protein solubility dependent on the pH, salt concentration, and polarity of the solvent. The overall size of the protein does not necessarily influence solubility. For example, antibodies, which have molecular weights of approximately 150 kDa, can often achieve aqueous solubility greater than 100 mg/mL.

The aqueous solubility of a peptide or protein is not easy to determine because peptides and proteins at high concentrations may form gels, or may develop aggregates upon concentrating, thus making solubility assessment difficult. In addition, solubility varies significantly depending on the conformation. The solubility determined by most methods is apparent solubility, because the true solubility of a protein as a hydrocolloid is difficult to define. A common approach is to concentrate a protein solution using a semipermeable membrane with centrifugation until the highest protein concentration is reached. Another approach is to lyophilize a protein or peptide and then add water to the point where undissolved material is barely present. When a limited amount of protein is available, one approach is to determine solubility in polyethylene glycol (PEG) solution (typically 1–9%) and then extrapolate the solubility to 0% PEG to determine aqueous solubility (4).

The factors that determine a protein's solubility include its intrinsic properties and the composition of the solvent. The intrinsic properties are the composition of amino acids, the folded structure, and for glycoproteins, the composition and structure of glycans. Generally, a protein made of a large proportion of hydrophobic amino acids such as Phe, Tyr, and Trp will have low water solubility, and adding glycans increases water solubility. The solvent properties, including pH, salt concentration, and specific ligands, can also significantly affect the solubility. Protein solubility as a function of pH is typically in the shape of a U or V, where the minimum is at the pI. However, there are exceptions. The solubility of a protein at low ionic strength generally increases with the salt concentration, which is called the salting-in effect. As the salt concentration increases, the additional counter-ions shield the ionic charge and thereby increase the protein solubility. As salt concentration continues to increase, protein solubility decreases (the salting-out effect). At high salt concentration, the salts begin to compete with the ionic moieties of the protein for the solvation of the polar solvent, which results in decreasing solubility. A specific ligand or stabilizer that binds to the protein may also influence solubility. For example, increased solubility of fibroblast growth factor was observed in the presence of heparin or heparin-like substances (5). Also, alteplase solubility was increased by the addition of arginine (6). However, one needs to assess whether the ligand or excipient is acceptable for the intended clinical use before adding it into the final formulation.

Thermal Transition Midpoint

Because native proteins exhibit folded structure in solution, they can undergo transition from native form to unfolded or denatured form with increasing temperature. The thermal transition midpoint (T_m), defined as the temperature at which equal amounts of native and denatured forms exist in equilibrium, is an important characteristic of proteins, measuring their thermal stability. Generally, a higher T_m value indicates better thermal stability.

The most commonly used technique to determine T_m is differential scanning calorimetry (DSC), as this method not only provides an accurate measurement of T_m but also can assess reversibility of transition and estimate apparent enthalpy. Temperature-controlled spectrometry, including circular dichroism (CD), fluorescence, and ultraviolet (UV) absorbance spectroscopy, is also sometimes used to differentiate the transitions by tertiary structure from those by secondary structure.

Measurement of T_m has been widely used in preformulation and formulation development. The profile of T_m as a function of pH provides important information in selecting the optimal pH for formulation. This method has also been used in screening different stabilizers, as an excipient that elevates T_m is expected to be a potential stabilizer (7). However, it should be noted that in choosing the formulation, one also needs to consider other information, as T_m alone is only indicative of thermal stability.

Proteins in solid state also exhibit thermal transitions upon heating. These are typically determined by DSC. However, it is difficult to measure the true thermal transitions of solid protein, because in most cases other components present in the solid dosage form also contribute to the overall thermal transition. Recently, glass transition temperatures (T_g) for proteins have been estimated by extrapolating excipient concentration to zero using T_g values measured at a very fast scanning rate in binary mixtures of protein and another glass form excipient, such as sucrose, over a range of excipient concentrations (8).

Instability: Key Degradation Pathways

The structural complexity of proteins makes them susceptible to processing and handling conditions that can result in structural and functional modifications. A protein can undergo a variety of covalent and noncovalent reactions or modifications, which may be generally classified into two main categories: (*i*) physical or non–covalent bond degradation pathways and (*ii*) chemical or covalent bond degradation pathways. Common physical degradation pathways include denaturation or unfolding, adsorption, and aggregation due to noncovalent forces. Chemical degradation pathways include covalent-bonded aggregation, disulfide exchange, deamidation, isomerization, racemization, fragmentation, oxidation, β-elimination, Maillard reaction, diketopiperazine formation, and so on. Oftentimes, physical degradation facilitates chemical degradation, and vice versa. The fundamentals of these degradation pathways have been extensively described in several review articles and book chapters (9–14). A brief description of each degradation pathway, the factors responsible for degradation in some proteins, and remedies are presented below.

Denaturation

Denaturation is the process of altering protein structure (i.e., secondary, tertiary, or quaternary structures) from its native folded state. Denaturation may result in an unfolded state, which could further facilitate other physical and chemical degradations. Because a specific structure is required for proteins to exert physiological and pharmacological activities, denaturation causes loss of efficacy and incurs the risk of safety such as immunogenicity.

Many times, the denaturation process can be described as $N \leftrightarrow I \leftrightarrow D$. The folded native structure (N) unwinds and passes through a partially unfolded or intermediate state (I) to a denatured state (D). This process may be reversible or irreversible, depending on conditions. For reversible denaturation, the unfolded protein will regain its native state once the denaturing condition is removed.

Many factors can cause denaturation, including heat, freezing, extreme pHs, organic solvents, high salt concentration, lyophilization, surface adsorption, and mechanical stress. These denaturing conditions disrupt a protein's higher order structure, which is held together by intramolecular forces including hydrogen bonding, salt bridges or electrostatic forces, hydrophobic interactions, and van der Waals forces.

Hydrogen bonds are critical in determining overall protein conformation, since they are the major forces that stabilize the secondary α-helices and β-sheets, as well as the overall folded structure. Water, the nearly ubiquitous medium for proteins, contributes to this hydrogen bonding. Cosolvents such as ethanol and acetone and chaotropic agents such as urea and guanidine hydrochloride disrupt the hydrogen bonds and thus readily denature proteins.

The ionic side chains of aspartic acid, glutamic acid, lysine, arginine, and histidine, normally found on the surface of the protein, contribute to the stability of the native conformation by forming salt bridges. The pH of the solvent will determine the charge of the side chains on these amino acids and the extent of ionic bonding. Therefore, an extreme pH shift can disrupt these salt bridges and lead to denaturation. Furthermore, organic solvents will reduce dielectric constant and increase ionic forces or salt bridges, so inappropriate exposure to organic solvents can also result in denaturation.

Because hydrophobic side chains (i.e., phenyl, indole, and hydrocarbon chains) are usually tucked inside the protein's globular structure, significant stabilizing effects result from their hydrophobic interactions. These interactions, too, are sensitive to the effects of solvents. Disruption of hydrophobic interactions is also considered the mechanism of denaturation by surfactant, extreme temperature, and mechanical stress, all of which commonly occur during manufacturing processes.

Adsorption

Proteins are amphiphilic polyelectrolytes, so they tend to adsorb at liquid-solid, liquid-gas, and liquid-liquid interfaces. When adsorption of proteins occurs, the molecules exchange their interactions with the solvent and other solutes for interactions with the surface. Two mechanisms are primarily responsible for protein adsorption. One mechanism is charge-charge

or electrostatic interaction. For example, salmon calcitonin, as a positively charged protein, strongly binds to the negative potential of a glass surface through electrostatic interaction (15). The other mechanism is hydrophobic interaction. One example is bovine serum albumin, which near its isoelectric point has shown the highest affinity to the hydrophobic surface of polystyrene through hydrophobic interactions (16). Other interactions, including charge-dipole, dipole-dipole, and van der Waals forces, may also contribute to the adsorption.

These interactions may lead to altered structures, including secondary, tertiary, and quaternary structures, which could further facilitate other physical and chemical degradation, including aggregation and covalently bonded modification. Therefore, depending on the nature of the protein and of the contact surface, interfacial adsorption can significantly impact a protein drug's potency, stability, and safety, particularly in a low-concentration dosage form.

The key strategy to minimize or inhibit protein adsorption is either to adjust formulation parameters or to modify or avoid certain contact surfaces. The formulation parameters that potentially control adsorption include protein concentration, pH, ionic strength, and addition of specific excipients such as surfactants or albumin. For example, modification of the contact surface of siliconized vials has minimized interferon adsorption on the glass surface (17). When these approaches do not prevent significant adsorption, alternative contact surfaces should be considered during process or storage. In some cases, when the level of adsorption can be predicted, overage is required in the vials.

Aggregation by Noncovalent Linkage

Non–covalently linked aggregation often results from some degree of denaturation of proteins, since unfolding leads to the exposure of hydrophobic moieties previously buried in the protein interior, which is followed by the association of unfolded molecules via noncovalent interactions to form aggregates. Non–covalently linked aggregation can be a reversible or an irreversible process, depending on conditions.

Reversible aggregation is highly dependent on protein concentration, pH, salt concentration, and other formulation components. Generally, proteins tend to form high–molecular weight species (HMWS) at high protein concentration. Upon dilution, these HMWS or oligomers may dissociate into monomers or dimers. This self-association phenomenon may be characterized by AUC (18) or by static light scattering (19).

Irreversible aggregates can be soluble or insoluble, depending on the size and nature of the molecules. Generally, these aggregates can be induced by single or multiple stress conditions, including heat, extreme pH, mechanical pumping, high pressure, shaking or agitation, freezing, and freeze-drying. For example, acidic pH and a temperature of 37°C have resulted in irreversible aggregation of albumin (20).

To minimize aggregation, besides tight control of the process parameters, adjusting formulation parameters, such as adding sucrose, should be assessed. Sucrose and other polyols maintain protein molecules in a native compact form, so as to be resistant to external stress.

Aggregation by Covalent Linkage

The most commonly observed protein aggregation by covalent linkage occurs through intermolecular disulfide linkage, also called disulfide bond formation and scrambling. This intermolecular aggregation may occur to any protein containing cysteine or cystine.

Generally, proteins with a free thiol group tend to form aggregates more easily through disulfide bonds, especially when the free thiol group is solvent-exposed on the surface of the protein. Free thiol groups buried within the tertiary structure are less reactive. The formation of disulfide bonds in protein aggregates with free thiol groups can take place either through the two free thiol groups available on the surface of each of two protein molecules, or through thiol-disulfide exchange, whereby a reactive thiol group in one molecule attacks an existing disulfide bond in another molecule to form a new disulfide bond between the two molecules.

Proteins without free thiol groups may still form aggregates by disulfide bond scrambling through intermolecular disulfide exchange, especially in alkaline conditions. A cystine or disulfide bond in one molecule can be reduced into two free cysteines, which can react with cysteine or cystine in another molecule to form a new disulfide bond.

Aggregates formed through disulfide bonds may, through multiple disulfide scrambling reactions, result in high-molecular-weight aggregates, which could eventually precipitate from solution. In addition, the formation of a new disulfide bond may change the native conformation to a denatured form, which could further aggregate through noncovalent hydrophobic interactions due to exposed hydrophobic residues.

In neutral or alkaline pH conditions, disulfide-bonded aggregation generally becomes more severe as the thiol group becomes more reactive. However, extremely acidic pH may also cause disulfide-bonded aggregation (21).

To prevent or minimize disulfide-bonded aggregates, the main formulation parameter is pH. A slightly lower pH (e.g., pH 5) may significantly reduce aggregation. The addition of reducing agents such as cysteine, or of stabilizers that alter conformation such that free cysteine or reactive cystine becomes more buried into tertiary structure, may also minimize aggregation. For proteins with severe disulfide aggregation, lyophilized formulation should be considered, as reactivity in the solid state is reduced significantly.

Nonreducible aggregates through nondisulfide linkages have also been reported. The reactions involving these covalent linkages include (*i*) oxidation-induced reactions through Trp or Tyr linkage (22); (*ii*) reaction through transamidation, whereby an amino group of amino acids (e.g., lysine residue or N-terminal of a protein) in one molecule forms an isopeptide bond with the carbonyl group of either Asn or Gln in another molecule [examples are insulin (23) and lyophilized ribonuclease A (24); and (*iii*) reaction through a reactive dehydroalanine generated from β-elimination at alkaline pH, which forms nonreducible cross linkages with other amino acids such as Tyr, Lys, His, Arg, and Cys.

Intramolecular Disulfide Exchange

Disulfide exchange can also take place within a protein molecule when a cystine (disulfide) bond is reduced into two cysteines; one of the cysteine residues then reacts with another cysteine to form a new disulfide bond. Improper linkages of disulfide bonds were responsible for a reduction in biological activity of interleukin-2 (IL-2) (25). There are three cysteines in IL-2 at positions 58, 105, and 125. The native protein forms a disulfide linkage between the two cysteines at 58 and 105. The cleavage of this disulfide in IL-2 and the subsequent formation of two less active isomers with disulfide bonds at incorrect positions (Cys^{58}-Cys^{125} and Cys^{105}-Cys^{125}) are promoted by high pH and copper ions (25). Intramolecular disulfide exchange has also been reported for monoclonal antibodies.

To minimize this type of degradation, it is important to select a low formulation pH and minimize any impurities, such as peroxides or heavy metals, known to promote redox reactions.

Deamidation/Isomerization

Deamidation refers to the removal of ammonia from the amide ($RCONH_2$) moiety of an Asn or Gln side chain, resulting in a carboxylic acid. Deamidation is a major cause of instability of proteins and peptides in aqueous solution. In lyophilized solid state, the deamidation rate is slower than in solution.

Deamidation occurs through different pathways at different pH levels. In an acidic pH of 2 to 5, deamidation occurs by direct hydrolysis, which causes Asn or Gln residue to change into Asp or Glu residue, respectively. The type of neighboring amino acids does not affect the deamidation rate. Hydrolytic mechanisms in neutral or alkaline pHs are more complex, however. Under these conditions, the side chain carbonyl group on the Asn or Gln residue reacts with the nitrogen atom on the peptide backbone to form a cyclic imide (succinimide) intermediate (Asu). Depending on which bond in the cyclic imide breaks, the reaction product can be (*i*) the des-amido peptide (Asp), (*ii*) the isopeptide (IsoAsp), or (*iii*) D-isomers. The formation of isopeptides is called isomerization, or sometimes referred to as transpeptidation, because an extra methylene group is inserted to the peptide backbone. When deamidation occurs, the IsoAsp to Asp ratio is typically 3. Detailed descriptions of deamidation and isomerization can be found in a review by Wakankar and Borchardt (26).

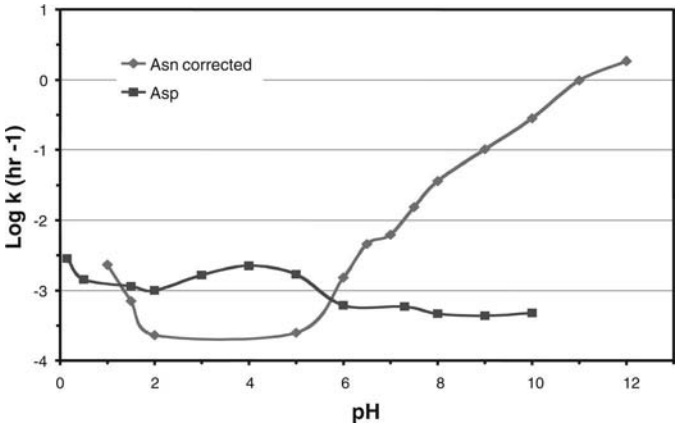

Figure 1 Comparison of Asn deamidation (*diamond*) and Asp dehydration (*square*) rates in a model peptide (Val-Tyr-Pro-Asx-Gly-Ala) at 37°C. Dehydration is the rate-limiting step for isomerization. *Source*: From Refs. 104 and 105.

At neutral to alkaline pHs, the rate of deamidation is significantly affected by the size of the amino acid on the C-terminal side of the Asn or Gln residue. In general, Asn is more labile than Gln and is most labile when adjacent to glycine, which is least obstructive to the formation of a cyclic imide. Since Asn-Gly is most susceptible to deamidation, protein engineers make significant efforts to avoid constructing a protein drug candidate with such a hot spot.

The deamidation rate profile as a function of pH is V-shaped, usually with a minimum rate at a pH of about 4 to 5 (Fig. 1). In a number of synthetic peptides, the half-life of deamidation reactions of Asn residues at 37°C in pH 7.2 phosphate buffer ranges from two days to nine years (27). Not all Asn residues are equally labile; those buried within the interior portion of a protein are inaccessible to water and thus less reactive. Secondary and tertiary structures play an important role in determining the site and the rate of deamidation. In insulin, for example, there are three asparagines. At acidic pH, the prevailing deamidated species was monodesamido-(A21)-insulin. At neutral pH, deamidation occurred to Asn at the B3 position (28). For growth hormone containing nine asparagines, deamidation occurred primarily at the Asn-149 position (29). These proteins and others—pramlintide (30), epidermal growth factor (31), IgG (32), IgG1 (33)—represent a small fraction of proteins that have shown deamidation.

Isomerization at Asp goes through the same cyclic imide intermediate, Asu. Because of the effect of pH on the leaving group (-OH), the rate-pH profile is significantly different from that of deamidation of Asn. Examples of protein drugs that undergo IsoAsp formation include insulin aspart (34), hirudin (35), and porcine somatropin (36). On the basis of the study of two monoclonal antibodies (37), the degradants from isomerization were detected by hydrophobic interaction chromatography.

The best way to avoid deamidation and isomerization is to mutate Asn-Gly or Asp-Gly sequence in the solvent-exposed region of the protein, if the mutation at these sites does not affect the biological activity. Otherwise, an appropriate pH (in the range of 5–6) should be selected to minimize the degradation rate. As the formation of cyclic imide intermediates does not depend on water, these reactions may occur even in anhydrous conditions such as 100% dimethyl sulfoxide (DMSO) (38)

Racemization

The racemization reaction is catalyzed by both acid and base. Racemization of peptides and proteins results in the formation of diastereomers. Racemization under basic conditions is hypothesized to proceed by abstraction of the α-proton from an amino acid in a peptide to yield a negatively charged planar carbanion. A proton can then be returned to this optically inactive intermediate, thus producing a mixture of D- and L-enantiomers for the individual amino acid. Since a peptide is composed of multiple chiral centers, the product formed is a diastereomer. Racemization is biologically significant because a peptide composed of D-amino acids is generally metabolized much more slowly than a naturally occurring peptide made

only of L-amino acids. For this reason, many new synthetic peptides, both agonists and antagonists, incorporate D-amino acids. A pH dependency for racemization was demonstrated in an aqueous degradation study of a decapeptide, RS-26306 (39), which found that at neutral and alkaline pHs, racemization contributed to more degradation than did deamidation.

Fragmentation

The peptide bond (RNH—CO—R) can undergo hydrolysis, resulting in peptide fragments. Generally, most peptide bonds are relatively stable. For example, oxytocin injection was reported to be stable at room temperature for five years (40). Protirelin, a tripeptide (PyrGlu-His-Pro), is stable for 20 hours at 80°C at both pH 3.3 and pH 6 (41).

The formulation factor that most influences the hydrolytic rate is solution pH. The rate of hydrolysis is in direct proportion to the activity of hydronium or hydroxide ions, when in acidic or alkaline pHs, respectively. Generally, the reaction becomes much faster in either extremely acidic or extremely alkaline conditions. Fragmentation of therapeutic peptides, including nafarelin (42), secretin (43), captopril, (44), and urokinase (45), has been reported in various pH conditions.

The type of neighboring amino acids also affects the susceptibility of the linkage to fragmentation. For example, the bond between aspartic acid and proline is sensitive to acid hydrolysis. A cleavage at Asp-Pro was found in basic fibroblast growth factor (bFGF) (46) and macrophage colony-stimulating factor (47). The resultant products are peptides with aspartic acid at the C-terminus. The C-terminal peptide bond adjacent to serine is also a reactive one, because of the neighboring-group effect of the alcohol on serine (48).

Another example of fragmentation is the cleavage at hinge regions of antibodies resulting in a Fab fragment. The hinge region in IgG1 heavy chain comprises about eight amino acids, including two cysteines responsible for the two disulfide linkages between the two heavy chains. Cleavage takes place at multiple sites, with the majority between Asp and Lys and between His and Thr (49). The extreme flexibility at the hinge region and the solvent exposure are the driving forces for such cleavage. The rate of hydrolysis at hinge region is minimal at pH 6 (50).

Besides chemically induced fragmentation, protein hydrolysis can also be mediated by some residual proteases remaining from production. The cleavage site in this case is dependent on the type of proteases present. As the proteolytic activity of proteases is typically pH dependent, the degradation rate is also dependent on solution pH. For example, a cathepsin D protease derived from CHO was identified as being responsible for degradation of an Fc-fusion recombinant protein. This protease belongs to an aspartic protease family and is preferentially active at acidic pH (51).

Oxidation

Several amino acid residues including Cys, Met, Trp, His, and Tyr are potential sites of oxidation. These residues can be oxidized by atmospheric oxygen or by peroxide impurities from a number of raw materials including formulation excipients such as polysorbates. Oxidation can also be induced by exposure to light or catalyzed by transition metal ions such as Cu^{2+} and Fe^{3+}. The most commonly observed oxidations in therapeutic peptides and proteins are described below.

Oxidation of cysteine. Under neutral or basic conditions, the free thiol (-SH) group of a cysteine is the most reactive moiety of all amino acid components. The disulfide (-S—S-) bond formed from the oxidation of two thiol groups results in significant changes in conformation both intramolecularly and intermolecularly.

Oxidation of the thiol group is promoted at both neutral and basic pH. The rate-pH profile for captopril, a quasi-dipeptide, shows an increase in oxidation rate starting at pH 5 (44). This reaction can also be catalyzed by heavy metals. For example, this reaction was effectively retarded by the addition of a metal chelating agent such as EDTA in FGF formulation (5).

Oxidation of methionine. The thiolether ($-CH_2-S-CH_3$) moiety on methionine is susceptible to oxidation to form sulfoxide ($-CH_2-SO-CH3$) derivatives. The susceptibility of methionine to

oxidation is highly dependent on its site in a protein. For example, of the three methionines in human growth hormone, Met-125 is most reactive, Met-14 is less, and Met-170 is not reactive at all (52). The reactive methionine is likely to be the one exposed on a protein surface, and the unreactive one buried within the core. Air in the headspace of formulated and freeze-dried growth hormone can cause 40% of the growth hormone molecules to be oxidized during a six-month storage period (53). Examples of other pharmaceutical proteins exhibiting Met oxidation are listed below.

- Interleukin-2 (54)
- Murine antibody (Orthoclone OKT3) (55)
- Herceptin® (trastuzumab) (56)
- Recombinant interferon γ (Actimmune) and recombinant tissue plasminogen activator (alteplase, Activase®) (57)
- Recombinant human granulocyte colony-stimulating factor (58,59)
- Parathyroid hormone (60)

As methionine can be easily oxidized by atmospheric oxygen, replacing oxygen with nitrogen or argon during manufacturing or in the headspace of the final product container is a common practice to minimize oxidation. In addition, adding free methionine as an antioxidant is also an effective approach to reducing oxidation (61).

Oxidation of tryptophan. Oxidation of tryptophan can generate multiple oxidized species. Stability studies of Trp amino acid alone in aqueous solution (62) and Trp residues in small peptides and lysozymes (63) and in bovine α-crystallin (64) clearly identified the main degradants as 5-hydroxy-Trp, oxy-indole alanine, kynurenine, and *N*-formylkynurenine. There are very few articles reporting the oxidation of Trp in therapeutic proteins. Davies et al. reported oxidized bovine serum albumin with oxygen radicals generated from cobalt radiation (65); Trp oxidation in monoclonal antibodies was recently reported by Yang et al. (66) and Wei et al. (67), and ozone and UV irradiation were used as stress conditions in these studies. That Trp oxidation has not been studied in depth is perhaps due to the fact that no model oxidizing condition has been adopted, and a system that promotes Trp oxidation is not easy to reproduce. In the case of Met oxidation, tert-butyl hydroperoxide (tBHP) and H_2O_2 are commonly used reagents to generate oxidized Met species. Most recently, a free radical generator, AAPH (2,2′-azobis(2-amidinopropane) dihydrochloride) was found to effectively oxidize tryptophan in peptides or large proteins (68). By using AAPH as an oxidation stressing agent, one can predict the vulnerability of a protein or the specific Trp residue in a protein.

As Trp oxidation appears to be mediated through oxygen radicals because of light exposure or peroxide residues from excipients, it is important for mitigation of Trp oxidation to limit direct light exposure and to use high-quality excipients.

β-Elimination

The disulfide bond between two peptide chains can be cleaved disproportionally, catalyzed by hydroxide ions to produce dehydroalanine and thiocysteine through the mechanism of β-elimination. The thiolate ion (HS-) is very reactive and will continue to react with other disulfide bonds, causing chain reactions. This is one of the operative mechanisms for the covalent aggregates in solid phase for bovine serum albumin (69) and ANP, a 25–amino acid peptide (70). β-elimination has also been attributed to the fragmentation of hinge regions in antibodies (71).

Maillard Reaction

The first substantial investigation of the reaction of reducing sugars with amino acids was carried out about 75 years ago by Maillard. The chemical reactions involved are, first, the reversible formation of a Schiff base between the aldehydic function group of reducing sugars (e.g., glucose) and the amino group of lysine residues in proteins, followed by a relatively

slow, but essentially irreversible, Amadori rearrangement, with the formation of ketoamines, which forms the hemiketal structure. As the Schiff base may also be involved in cross-linking, this reaction commonly leads to nonenzymatic browning, also called a browning reaction. This reaction occurs most readily in neutral to weakly alkaline conditions.

In protein formulation, reducing sugars such as glucose, maltose, fructose, and lactose should be avoided to prevent potential Maillard reactions. In addition, one needs to be concerned with acidic pH, which may cause hydrolysis of sucrose into glucose and fructose, leading to glycation of the protein. One example is freeze-dried bFGF. When the cake collapsed at elevated temperature, the acidity from its buffer, citric acid, caused hydrolysis of sucrose, which resulted in glycation of the protein with glucose (46).

Diketopiperazine Formation

Rearrangement of the N-terminal dipeptide results in the splitting off of a cyclic diketopiperazine at high pH. Proline and glycine in the N-terminal promote the reaction. Aspartame degrades through a similar mechanism (72), with the cyclization of aspartame minimal at a pH of 6 to 7, moderate at 7 to 8.5, and rapid above 8.5. Diketopiperazine formation has also been reported in the case of vascular endothelial growth factor (73).

PHARMACEUTICAL DEVELOPMENT PRINCIPLES

Because of their poor permeability to epithelium cell membranes and instability in gastrointestinal tract, proteins and peptides have very poor oral bioavailability and are therefore primarily formulated as injectable or parenteral products for intravenous (IV) infusion or subcutaneous (SC), intramuscular (IM), or intrathecal (IT) administration. Some proteins are also delivered through noninvasive administration routes, such as nasal and pulmonary formulations, to improve patient compliance. For local therapeutic effect, proteins such as growth factors are formulated for topical application and bone matrices.

Most protein products are in aqueous solution or in solid, freeze-dried form. Some are also formulated as suspensions in a crystalline form or in other lipid- or polymer-based delivery systems such as microspheres, liposomes, and nanoparticles. Rarely, some proteins, such as bovine growth hormone, are formulated in oleaginous vehicle.

The formulation development process for products that involve lipids or polymers as carriers is more complicated than the process for simple conventional dosage forms of liquid and lyophilized formulations. The principles described below focus mainly on the development process for the conventional dosage forms, some of which have also been discussed in several book chapters and review articles (74–79).

General Formulation Development Process in Industry

Generally, the manufacturing process for most protein products consists of two steps. One is the manufacture of drug substance, *aka* the active pharmaceutical ingredient (API), which is typically stored in bulk containers, such as plastic or stainless steel containers, in frozen condition for long-term storage purposes. The other step is the manufacture of the final drug product from drug substance. This is the final presentation to patients and healthcare professionals, typically stored in glass vials, prefilled syringes, or injection devices, at refrigerated condition for convenient use and distribution.

Formulations for drug substance and drug product may not be the same. Typically, the drug substance formulation is developed earlier, as it is the first step of the manufacturing process. However, drug substance should ideally be in a formulation vehicle that can be readily further formulated into drug product. Therefore, it is important to develop the drug product formulation as early as possible, so that the manufacturing process from drug substance to drug product will be harmonized and straightforward.

Prior to formulation development, it is important to conduct preformulation development activities, which serve to identify the key degradation pathways of the molecule and to develop stability-indicating assays to support formulation development studies. In general, preformulation activities should evaluate the biophysical and biochemical properties of the molecule under pharmaceutically relevant stress conditions, which include chemical-related

factors (such as extremes of pH, salts, buffers, oxygen, and peroxides) and process-related factors (such as high temperature, freezing, thawing, light, agitation, pressure, and shear stress). In these studies, biophysical characterization assays such as UV-visible spectroscopy, DSC, CD, Fourier transform infrared spectroscopy (FT-IR), AUC, and fluorescence spectroscopy are often used to determine which conditions result in the highest thermal stability and the least conformational changes. Biochemical methods such as size-exclusion HPLC, reversed-phase HPLC, hydrophobic interaction chromatography, capillary electrophoresis, peptide map, gel electrophoresis, ion-exchange HPLC, and potency assays are used to identify the key chemical degradation pathways, primarily through covalent bond changes, under these stress conditions.

Results from preformulation studies should identify the potential sources of the instability of the molecules and the key stability-indicating assays to monitor the degradation products, as these are needed for formulation screening and optimization studies. Once the leading formulation(s) are identified from the screening studies, appropriate container closure for final product presentation should be evaluated. In addition, the manufacturing process should be developed.

The choice of formulation, dosage form, and final product presentation is dependent on several factors. These choices not only need to offer the best stability and safety profiles, but they must also be easy to scale up for manufacturing and convenient to use during administration. The decisions are also dependent on the development stage of the product. At preclinical and early clinical stages, the goal of the project is to evaluate the proof of concept in product efficacy and safety as early as possible, so the shelf-life of early clinical products may only need to be 12 months or even less, and the storage or shipping conditions may not require the refrigerated or ambient temperature that is typically used for commercial products. Therefore, frozen or lyophilized formulations are typically chosen for preclinical and early clinical studies. At late clinical and commercial stages, there are more stringent requirements. At these stages, not only stability but also cost and marketing competitiveness are very important in making the final choice of the formulation. A very critical factor in a field of many competing, similar biotech products is patient compliance and acceptance, so prefilled syringes and auto-injection devices are in many cases essential for the success of the product's introduction to the market. As a result, evaluation of these dosage forms and devices must be initiated during phase III or pivotal clinical trials.

Overall, formulation development is a critical and evolving process step in product development. It is important to initiate the development effort as early as possible. It is also important to have input from multiple functional areas before finalizing the formulation choice.

Evaluation of Critical Formulation Parameters

To prepare a stable formulation suitable for patient use, the critical formulation parameters should be evaluated, including protein concentration, pH, buffers, stabilizers, tonicity modifiers, bulk agents (for lyophilized products only), and preservatives (for multidose products only). A discussion of evaluating each formulation parameter follows.

Protein Concentration

Protein concentration not only serves as a critical parameter in finalizing product presentation, but is also a key parameter in product stability. The following considerations are important in the selection of a viable protein concentration in a formulation:

- It should be below the protein's solubility in the selected formulation vehicle. Since protein solubility varies in different formulation vehicles and at different temperatures, the selected protein concentration should be below the "true" or thermodynamic solubility in the vehicle at the intended long-term storage temperature. However, measurement of true protein solubility is challenging, so this is an empirical exercise where real-time data needs to be evaluated on potential precipitation of the protein at the selected concentration and stored for the desired duration. This

assessment should be made for both drug substance and drug product before a protein concentration is finalized.

- It should maintain the protein's stability during long-term storage. Generally, proteins at high concentrations may lead to aggregation and precipitation. On the other hand, low protein concentrations may lead to significant loss of protein content due to adsorption onto various product-contacting surfaces (e.g., container closures during storage, filter membranes and silicone tubing during processing). Also, a higher relative ratio of any reactive impurities (such as traces of heavy metals, peroxides from surfactants, and sterilants used in the aseptic filling process) to the protein could cause degradation.
- It should be operable under manufacturing conditions. High protein concentrations achieved at lab-scale may not be operable at manufacturing scale. For example, a formulation with a high protein concentration may have high viscosity, requiring an undesirably long processing time for filtration or the ultrafiltration and diafiltration (UFDF) step. On the other hand, at low concentrations there may be significant protein loss from the filtration membrane due to the larger surface area at manufacturing scale. Therefore, it is critical to assess the scalability of the manufacturing process before choosing a protein concentration.
- It should minimize product waste during the manufacturing process, testing, and clinical use. Material losses during manufacturing (e.g., line loss), sample testing, and dose preparation for clinical use (e.g., residual in containers) are all volume based, so a high protein concentration will result in a large amount of protein waste. Therefore, the lowest protein concentration that delivers the required dose and maximizes production yield should be identified.
- It should meet the requirements for product presentation. Product presentation is selected on the basis of factors such as dose (size and frequency), administration route, convenience of dose preparation, patient weight distribution (for weight-based dosing schemes), number of manufacturing lots desired or manageable per year, and commercial considerations of cost and product differentiation. From these analyses, the amount of protein per vial is specified, and subsequently, the optimal volume and corresponding protein concentration is determined on the basis of factors such as solubility, stability, minimal protein waste, and manufacturability.
- It should take into account manufacturing process cycle time and cost. For lyophilized products, the major contributor to manufacturing cost is the lyophilization time. Reducing the fill volume by increasing protein concentration can significantly reduce the lyophilization time.

In summary, protein concentration, as a critical formulation parameter, should be chosen on the basis of multiple factors, including solubility, stability, manufacturability, cost, product presentation, and commercial considerations.

pH

As proteins containing both positively and negatively charged amino acids are amphoteric molecules, solution pH has a direct impact on the overall stability of proteins. When solution pH is far from the pI of the protein, electrostatic repulsions between like charges in the protein increase, causing a tendency to unfold. In addition to electrostatic interactions, pH also affects other interactions within proteins. Therefore, changing pH directly affects the conformational stability of proteins and their solubility in aqueous solution. In addition, since certain chemical reactions are highly pH dependent, solution pH also affects the chemical stability of proteins. The following areas should be assessed when selecting an optimal pH for a protein formulation:

- Profiles of conformational stability, chemical stability, and solubility as functions of pH in solution may not overlap each other for a given protein. The pH at maximum solubility may not be the same as the pH at maximum conformational stability or

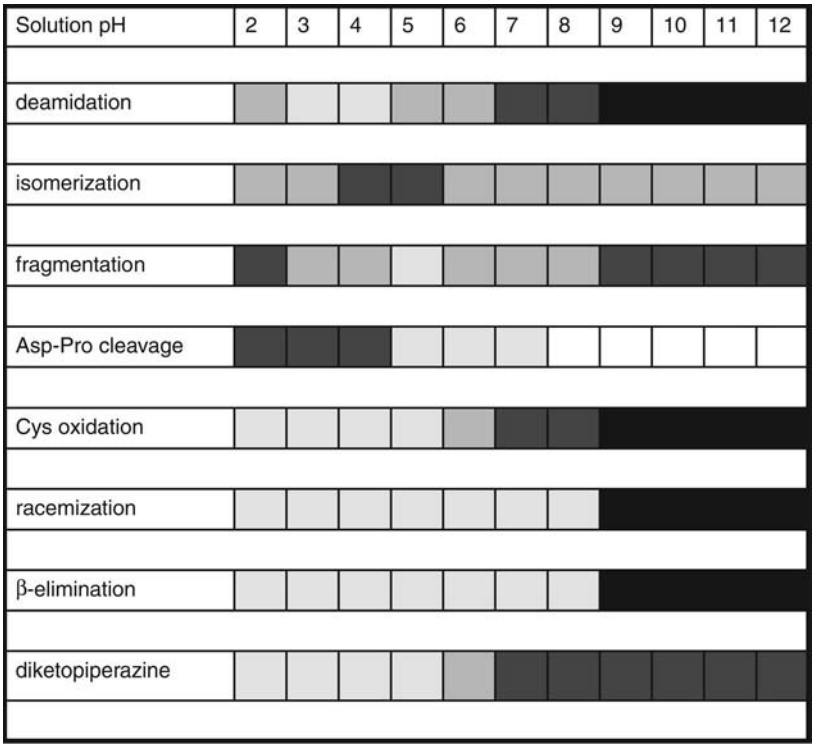

Figure 2 Reaction rates as a function of pH. (Darker color indicates a faster reaction rate at designated pH range.)

chemical stability. It is important to define an optimal pH that is a good compromise among all these characteristics.

- As chemical stability involves several different degradation pathways and each degradation pathway may have a different stability profile as a function of pH in solution (Fig. 2), it is important to balance all the degradation profiles before finalizing the optimal pH for the formulation. The degradation pathways that lead to significant loss in potency or biological activity or that cause immunoreactivity should be kept to a minimum when selecting the pH.
- The optimal pH selected in solution may not be optimal for proteins in solid dosage forms, so it is important to reassess the effect of pH when the protein dosage form changes from liquid to lyophilized form. For example, opposite trends in pH-dependent stability were observed for lyophilized and liquid formulations (80).
- The selected pH should not have any impact on the stability of other excipients in the formulation. Certain excipients may not be stable in a certain pH range under long-term storage conditions. For example, acidic pH has caused the hydrolysis of sucrose, a commonly used stabilizer in protein formulations (81,82).
- The selected pH should be compatible with product-contacting surfaces during manufacturing and storage. It has been reported that acidic pH caused corrosion of stainless steel in the presence of chloride ions, which generated iron ions that catalyzed methionine oxidation in a monoclonal antibody (83). Also, high or low pH may cause more leachables from stoppers, which serve as primary container closure.
- The selected pH needs to be safe to use for its intended administration route. A certain pH range may be suitable for IV injection but cause side effects when used for SC, IM, or IT injection.

For most protein formulations, the selected pH is in the range of 6 to 7, as this range is close to the physiological pH and also provides the optimal stability for most proteins. For

peptides, formulation pH is mostly in the acidic range of 4 to 5, as this pH range provides better aqueous solubility and less adsorption.

Buffers

Buffers are often used to control the formulation pH, keeping it within a narrow range to prevent small changes that can affect the stability and solubility of proteins. The selection of a proper buffer type and concentration for proteins should be based on the following considerations:

- The buffer species and concentration should not cause protein instability. For example, sodium phosphate buffer may result in a significant pH drop upon freezing, which has been found to cause instability of some proteins, particularly at high buffer concentration or low protein concentration conditions (84).
- The buffer species should have a pK_a near the target pH, preferably within one pH unit. Table 1 lists the pH control ranges for some commonly used buffer species.
- When buffer concentration has no effect on the protein stability, it should be kept to a minimum, but high enough to provide sufficient buffering capacity to control the formulation pH. High buffer concentration may cause some pain or discomfort upon SC or IM injection. In addition, for lyophilized product, a high concentration of buffer species such as sodium phosphate or citrate may lead to a lower glass transition temperature, which would require a longer lyophilization cycle.
- Buffers, like salts of ionic compounds, contribute to the overall ionic strength of the formulation solution. Therefore, buffer concentration also influences other properties that are dependent on ionic strength, such as protein solubility and stability.
- Certain buffer species, besides controlling solution pH, may also serve as a stabilizing agent in some protein formulations. For example, citrate may serve as a chelating agent to remove any heavy metals that potentially catalyze oxidation. Histidine also has an antioxidant effect (85).
- The buffer species and concentration should be safe to use for its intended administration route. Some buffer species and concentrations may be suitable for IV injection but may not be compatible when used for SC, IM, or IT injections. For example, citrate was found to cause more pain than histidine as a buffer when administered by SC injection (86).

It should be noted that as zwitterions, proteins have their own buffering capacity, especially at high concentration, so a buffer may not be required in formulations if the pH can be maintained by the protein itself. It has been reported that monoclonal antibodies at 60 mg/mL have a strong self-buffering capacity and that the long-term stability of self-buffered formulations is comparable to that of conventionally buffered formulations (87).

Ionic Strength/Salt Concentration

As proteins carry both negatively and positively charged groups, ionic strength in formulation solution may directly affect the solubility and stability. Ionic strength in parenteral formulation

Table 1 Buffers for Protein Formulations

Buffer	Acid	Base	pH range control	Examples
Phosphate	Monosodium phosphate	Disodium phosphate	5.8–7.8	Elaprase®, Remicade®
Acetate	Acetic acid	Sodium acetate	3.8–5.8	Avonex®, Neupogen®
Citrate	Citric acid	Sodium citrate	3.0–7.4	Amevive®, Rituxan®
Succinate	Succinic acid	Sodium succinate	3.3–6.6	Actimmune®
TRIS	TRIS HCl	TRIS	7.1–9.1	Wellferon®, Enbrel®
Histidine	Histidine HCl	Histidine	5.1–7.0	Xolair®, Raptiva®
Carbonate	Sodium bicarbonate	Sodium carbonate	5.4–7.4, 9.3–11.3	Fuzeon™

is mainly adjusted using NaCl. NaCl affects electrostatics in a protein either by nonspecific (Debye-Huckel) electrostatic shielding or by specific ion binding to the protein. At low concentration, salts affect electrostatic shielding and weaken ionic repulsion/attraction as counter-ions, so this shielding effect may be stabilizing when there are major repulsive interactions leading to protein unfolding, or could be destabilizing when there are major stabilizing salt bridges or ion pairs in the proteins. At high concentrations, electrostatic shielding is saturated. The dominant effect of salt, as of other additives, is on the solvent properties of the solution. The stabilizing salts seem to increase surface tension at the water-protein interface and strengthen hydrophobic interaction by keeping hydrophobic groups away from water molecules, inducing preferential hydration of proteins (13). Therefore, ionic strength or salt concentration affects both the solubility and the stability of proteins. The following areas should be assessed when selecting a proper ionic strength or salt concentration:

- Similar to the effect of pH, the profiles of solubility and stability as functions of ionic strength may not overlap each other for a specific protein, so the optimum ionic strength at maximum solubility may not be the same as at maximum stability. It is important to define an optimal ionic strength that is a good compromise between these two characteristics.
- Ionic strength or NaCl concentration has been reported to have an impact on the viscosity of formulations, especially at high protein concentrations (88). It may therefore serve an important factor in adjusting the viscosity of the product. Viscosity is an important parameter for syringeability of high–protein concentration formulations used in SC and IM administration.
- Ionic strength or NaCl concentration should be compatible with other excipients in the formulation and with product-contacting surfaces. It has been reported that high salt concentration combined with acidic pH may cause rusting of stainless steel, resulting in an elevated level of iron ions responsible for oxidation of the protein (83).
- Ionic strength or NaCl is certainly a key contributor to the overall tonicity of the formulation, and it is important to keep the concentration or tonicity suitable for the intended administration routes.

Stabilizers

When adjusting the parameters discussed above—protein concentration, pH, buffer, and ionic strength—still does not result in sufficient protein stability, the addition of stabilizers should be considered. Several types of stabilizers, listed in Table 2, are commonly used in protein formulations to stabilize proteins against various stresses. It is important to consider the following aspects when choosing a stabilizer:

- The choice of stabilizer type and concentration should be rational. An experimental laboratory-scale model should be developed to screen the stabilizer type and concentration for specific degradation against specific stress. For example, to identify a stabilizing excipient against shaking stress, surfactants and concentration ranges should first be tested through an established shaking model. To find a stabilizer against freezing/thawing stress, an appropriate freeze/thaw stress model should be used to screen various cryoprotectants and their concentration ranges.
- The number of stabilizers in a single formulation should be minimal and based on needs. An ideal stabilizer inhibits or minimizes multiple degradation pathways. For example, conformational stabilizers, such as sucrose, which enhance conformational stability, minimizes not only the aggregation but also other chemical degradations such as oxidation and fragmentation that occur when the reactive sites are exposed in absence of sucrose.
- The type and concentration of stabilizers should be compatible with other excipients in the formulation. For example, Ca^{2+} may be a good stabilizer, but if the buffer is phosphate and the pH is above neutral, precipitation of calcium phosphate may occur.
- Any stabilizers that may cause interference with protein assays should be avoided. For examples, polymers and albumins used as stabilizers in formulations may interfere

Table 2 Commonly Used Stabilizers in Protein Formulations

Type	Hypothesized stabilizing mechanism(s) (reference number)	Examples
Sugars	Stabilize proteins by preferential hydration in solution; serve as cryoprotectant and/or lyoprotectant; certain sugars such as glucose may chelate heavy metals, thus serving as antioxidants (89).	Sucrose: Follistim®, Panglubulin®, Ovidrel®, Xigris™ Trehalose: Advate, Herceptin® Lactose: Factrel®, Glucagon® Glucose: Gammagard® S/D Maltose: Bexxar®, Gamimune®
Polyols	Stabilize proteins by preferential hydration in solution; may serve as cryoprotectant and/or lyoprotectant; certain polyols such as mannitol may also serve as antioxidants by chelating metal ions (89).	Mannitol: DigiFab™, Fabrazyme®, Cerezyme® Sorbitol: Digibind®, Neulasta™, Neupogen® Glycerol: Humalog®, Humulin®R
Surfactants	Reduce agitation-induced aggregation by reducing surface tension; facilitate refolding by specific or nonspecific binding to protein; minimize adsorption and prevent other degradation by preferentially binding to interfaces (air-liquid, ice or solid surfaces).	Polysorbate 20: Neulasta™, Replagal®, Raptiva® Polysorbate 80: PEG-Intron, Remicade®, WinRho SDF® Poloxamer 188: Elitek™
Amino acids	Suppress protein aggregation and protein-protein or protein-surface interactions; arginine increases the surface tension of water, thus favorably interacting with most amino acid side chains and peptide bonds (90).	Glycine: Gamunex®, Synagis®, Neumega® Arginine: Activase®. TNKase® Cysteine: Acthrel®, SecreFlo® Histidine: BeneFIX®
Metal chelators	Chelate heavy metals to prevent metal-ion-catalyzed oxidation of cysteine and methionine residues in proteins.	EDTA: Kineret™, Ontak®
Divalent metal cations	Stabilize protein conformation by specific binding to certain sites of protein.	Ca^{++}: Pulmozyme®, ReFracto®, Kogenate® Zn^{++}: Nutropin Depot™, Aralast™
Polymers or proteins	Stabilize proteins by mechanisms similar to those of surfactants; serve as cryoprotectant or lyoprotectant.	Albumin: Intron®A, Rebif®, Zevalin™, Procrit® PEG: Autoplex®T, Hemofil®M, Monarc-M™, Prolastin® Carboxylmethylcellulose: Plenaxis™ Heparin: Thrombate III®, Autoplex®T Dextran: Mylotarg™

with protein assays, particularly in UV, HPLC methods and gel electrophoresis, creating complications for release and stability testing.

- Any stabilizers that may introduce potential contaminants, especially animal or human derived, should be avoided. Albumin, for example, is an excellent stabilizer for many therapeutic proteins and was widely used in early products; however, because of concerns about blood source contamination, it has seldom been used as a stabilizer in recent products. However, availability of pharmaceutical grade recombinant human serum albumin may change this dynamics.
- Stabilizers of high quality from a reputable vendor should be used for lab screening studies. Like any excipients, stabilizers may contain different levels and types of impurities when they are made from different sources or processes. These impurities

could cause inconclusive results from screening studies, as they may result in instability even while the stabilizer itself has a stabilizing effect. A case in point is the peroxide level in polysorbates: varying peroxide levels in polysorbates used in formulation studies often confound the study results.
- The type and concentration of stabilizers chosen should be safe to use for intended administration routes. It is important to assess the safety and toxicity of any new excipient prior to clinical studies.

As shown, choosing the proper type and concentration of stabilizer is a challenging process. It is important to consider all aspects, including solubility, stability, compliance, safety, and operational challenges (such as posing analytical difficulties).

Tonicity Modifiers

For parenteral administration, the final product is generally formulated to be isotonic or iso-osmotic, which is equivalent to 0.9% or 150 mM NaCl with an osmolality of 289 mOsm/kg. The following aspects should be considered when finalizing the type and concentration of tonicity modifiers in the final product:

- Commonly used tonicity modifiers in protein formulations include NaCl, mannitol, and sorbitol. An excipient already selected for the formulation, such as salt or stabilizer, is the preferred choice when increasing concentration does not have an effect on the overall properties of the formulation.
- The requirement of isotonicity depends on the administration route and dose preparation. If the product is diluted into IV fluid such as normal saline solution prior to administration, the formulation may not be required to be isotonic. However, if the product is directly injected without any dilution, isotonicity is preferred, particularly for SC, IM, and IT injections.
- For lyophilized product, the formulation prior to lyophilization may not be required to be isotonic even when the reconstituted solution is required to be isotonic. Isotonicity in final reconstituted solution can be achieved by choosing the proper type and volume of diluent to reconstitute the lyophilized product.

It should be noted that some recent studies have shown that infusion of solutions with iso-osmolality but hypotonicity may cause some adverse effects (91). This suggests that although the terms "isotonic" and "isoosmotic" have been used interchangeably, they may have different effects on safety, particularly for products that will be infused in large quantity.

Bulking Agents (for Lyophilized Product Only)

For lyophilized product, bulking agents are required to provide enough solids to maintain good cake structure during lyophilization and long-term storage. To choose the proper type and amount of bulking agent, the following aspects should be considered:

- The type and amount of bulking agent added to the formulation should be compatible with the protein and other excipients. It should not cause significant protein instability during lyophilization or storage. An excipient already selected for the formulation, such as a stabilizer (sucrose or trehalose), should be preferred as the bulking agent when increasing the concentration does not have an effect on the overall properties of the formulation (e.g., high sucrose concentration may require an extremely long lyophilization cycle or result in partially collapsed cakes).
- Mannitol and glycine are commonly used bulking agents as they provide better cake appearance and do not require a longer lyophilization cycle. However, because of their crystalline nature, they may cause phase separation during storage, leading to stability issues. In addition, a high content of mannitol may lead to vial breakage during freezing (92) due to volume expansion during crystallization. This vial breakage phenomenon has been also observed with NaCl crystallization during lyophilization (93).

Table 3 Preservatives for Protein Formulation

Type	Example(s)	Comments
Phenol	Antivenin Aplisol® Nutropin AQ®	Air and light sensitive; may act as a reducing agent.
Benzyl alcohol	Epogen® Nutropin® Pegasys® Factrel®	Usually in diluent for reconstitution of lyophilized product.
m-Cresol	Humatrope NovoLog®	Used in both liquid multiuse products and diluents for lyophilized products.
Thimerosal	Antivenin	Not commonly used for recent products because of mercury-related toxicity.
Chlorobutanol	Desmopressin	Widely used preservative in pharmaceuticals, including injectables. Typically used at 0.5%.

- Polymeric bulking agents [e.g., hydroxyethyl starch (HES) or dextran] and proteins (e.g., albumin) may significantly reduce the length of lyophilization cycle by raising T_g', however, some of them may interfere with certain protein assays. Therefore, potential complications in analytical testing should be considered when choosing this type of bulking agents.
- For high-protein-concentration formulations, bulking agents may not be required, as a protein itself serves as a bulking agent to provide good cake structure.

Preservatives (for Multidose Products)

Most injectable protein products are intended for single-dose injection, which does not require inclusion of antimicrobial preservatives in the formulation. However, some products are intended for multidose administration, which requires preservatives in the formulation to prevent any microbial growth from the time the product is opened for use to the time the last dose is administered.

Table 3 lists the preservatives that have been used for protein formulations. To choose the appropriate type and concentration of preservatives in a formulation, the following aspects should be considered:

- The type and concentration of preservatives selected for a formulation should make the final product meet the antimicrobial effectiveness testing required by USP and BP/EP at the time of product release and at the end of shelf-life and last dosing. One needs to be aware that requirements in BP/EP are more stringent than those in USP (94).
- Adding preservatives generally results in protein instability. This is not surprising, as the bactericidal or bacteriostatic effect is derived from the preservative's interaction with proteins or DNA in microorganisms. It is important to screen for a compatible type of preservative for specific formulations or proteins.
- Minimizing the contact time between preservative and protein is a general approach to reducing the preservative's stability impact. In this approach, preservatives are typically added to the diluent (for lyophilized product) or to the product upon preparation for the first dose.

General Strategies for Formulation Screening and Optimization

As discussed above, protein formulation has multiple parameters, including the protein itself, buffers, pH, stabilizers, and other excipients, and each parameter has its own functions. Some of these parameters may interact with each other; for example, pH and stabilizers both affect

Table 4 Formulation Parameters, Evaluation Models, and Critical Attributes for Design of Experiments

Formulation parameters	Evaluation models	Critical attributes
Protein concentration	Thermal stress	Appearance
pH	Freeze/thaw cycle	Content
Buffer type/concentration	Peroxides exposure	Aggregation
Stabilizer type/concentration	Light exposure	Fragmentation
Tonicity modifier type/concentration	Shaking/agitation	Oxidation
Preservative type/concentration		Deamidation
		Potency

the stability of the molecule. In addition, the product has to be exposed to multiple process-related stresses during manufacturing, storage, and handling, such as extreme temperatures, freeze/thaw cycles, agitation, and pressure. Therefore, it is challenging to develop a stable formulation containing many parameters against various process-related stresses.

Design of experiments (DOE) has proven an effective tool in dealing with such a complicated development process involving multiple variables. DOE, as part of the concept of quality by design (QbD) recently introduced by regulatory agencies for pharmaceutical development, is a tool to establish the design space through statistical analysis. The design space forms a link between development and manufacturing design (ICH Q8, Pharmaceutical Development). For formulation development, the design space refers to the defined range of formulation parameters and quality attributes that have been demonstrated to provide assurance of quality.

Formulation development using a DOE approach typically has two stages: formulation screening and optimization/robustness studies. The goal of the screening study is to identify the key formulation parameters, while the optimization/robustness study defines the optimal or robust range of the selected key parameters.

To design a proper space for statistical DOE studies, it is important to collect all the information from preformulation development activities and any prior knowledge on the protein. This information helps in identifying the critical formulation parameters and the key degradation pathways that potentially affect the critical quality attributes (CQAs) of the product for the design space, and in selecting a proper evaluation model that can be used for screening or optimization of formulations. In addition, the target product profile (TPP) should also be established prior to DOE studies.

Table 4 lists the parameters and attributes for DOE studies. Once the study is completed and data are collected, statistical analysis should be performed to establish the design space. This established design space can not only justify the choice of formulation ranges and help identify the robust region of the formulation, but can also enable study of the interactions between each formulation parameter. Several case studies using the DOE concept have been described in a book chapter written by Ng and Rajagopalan (95).

Choice of Container and Closure System

Because proteins may interact with the contact surfaces, the compatibility of immediate packaging material with protein product needs to be evaluated during selection of the container closure system.

As described in section "General Formulation Development Process in Industry," most protein products are formulated as drug substance and drug product, which are typically stored at frozen and refrigerated conditions, respectively. In choosing an appropriate container closure system for drug substance, the following aspects should be considered:

- Commonly used container closure systems for drug substance include plastic bottles or bags and stainless steel vessels. Various types of plastics have been used in packaging protein drug substance, including Teflon, polyolefin, glycol-modified polyethylene terephthalate (PETG), polypropylene (PP), polycarbonate (PC), polyvinylchloride (PVC), polyvinyl alcohol (PVA), and polyethylene (PE). It is important to

evaluate whether the product remains stable under intended storage conditions. For example, hydrophobic proteins tend to adsorb more on hydrophobic polymers such as Teflon. In addition, the material's gas permeability and leachables should also be evaluated. Plastics with high gas permeability may affect product stability during long-term storage if the product is oxygen sensitive. Plastics such as PVC that contain a substantial amount of plasticizer may generate more leachables than other plastics. When stored in a stainless steel vessel, acidic pH and chloride ions may cause corrosion. With an increased amount of dissolved metal ions, protein oxidation is a concern (83).

- The material should retain its function at the intended storage condition. Since most protein drug substances are stored frozen, it is important to assess the brittleness point of the plastics at the intended storage temperature. If the brittleness point is above the intended storage temperature, container closure integrity may be compromised. In addition, breakage of container closure may occur upon impact, such as an accidental drop. Therefore, PC and Teflon are preferred because of their lower brittleness points.
- The size of the containers should be selected on the basis of product stability, potential expansion of product, and cost effectiveness. For example, a sufficient amount of headspace should be allowed to accommodate volume expansion upon freezing for frozen drug substance. If the product is stored as a liquid, then minimal headspace should be considered to minimize the potential instability caused by agitation upon handling and shipping. With large containers, it may be difficult to control the freezing process. Stainless steel cryo-vessels with temperature-controlling systems have many advantages over plastic containers, such as controlled freezing and thawing rates and nonbreakable characteristics. However, they are expensive and need to undergo cleaning validation for multiuse purposes. In addition, they may need frequent passivation to retain resistance to potential corrosion.

To choose an appropriate container closure system for drug product, the following aspects should be considered:

- Unlike drug substance, most drug products are stored in glass vials with rubber stopper systems. Some products are also packaged in prefilled syringes, cartridges, and dual chamber Lyo-Ject® syringes, which all consist of glass barrels or tubing and rubber stoppers. Plastic vials or tubing have also recently been introduced. It is important to evaluate the product's compatibility with various types of glass, plastics, and rubber stoppers, as protein adsorption and other degradations may occur.
- Container closure integrity should be retained to ensure the product's sterility throughout its shelf-life.
- Most glass vials are washed and then depyrogenated prior to use. It has been reported that siliconized vials may minimize adsorption of the product (17). However, the possibility of silicone oil causing protein aggregation also needs to be examined. For prefilled syringes, the glass barrels should be siliconized for proper syringeability. It is important to recognize, however, this step may affect not only syringeability but also product stability. For lyophilized product, the stoppers may need to be dried in an oven or autoclave following steam sterilization, because retained moisture may be released to the lyophilized cake during long-term storage and cause instability issues, which are critical for moisture-sensitive products.
- The type of container closure system should be decided on the basis of the product's stability, the development stage, the intended use (indication and administration route), and marketing competitiveness. For an early clinical development stage, a vial and stopper system is often chosen, as it involves less technical complexity and thus requires less development time. For late development or commercial stages, the performance of a container closure system—such as improving ease of administration, minimizing drug wastage, and conforming with patient needs (e.g., self-administration)—becomes more important. Prefilled syringes, self-injection devices using cartridges, and dual chamber lyo-ject syringes may help the drug product to gain a greater market share because of their convenience of use, which leads to better patient compliance.

- The size of container closure should be decided on the basis of several factors, including dosing regimen, product stability, cost effectiveness, and compliance for patient use. For example, vial size affects the headspace, which is a critical parameter for most liquid products as it may impact product stability upon agitation during shipping and handling. For prefilled syringes, the size of the syringe also affects the headspace and the movement of the stoppers upon exposure to reduced pressure during airplane shipping. Cartridges tend to have less headspace.

For both drug substance and drug product, the suppliers of the container closure system should be reputable and well established. Suppliers that have established Drug Master Files (DMFs) for the packaging components should be preferred. In addition, for commercial products, a second source for the container closure system may need to be established in case issues arise with the primary source.

Manufacturing Process Development

The next step after selection of an appropriate formulation and container closure system is to develop a manufacturing process that maintains protein stability under process conditions such as mixing, filtration, filling, and lyophilization. Instability of proteins under these conditions is often observed, and therefore it is important to evaluate and define the optimal process conditions prior to cGMP manufacturing. From these studies, the critical process parameters and acceptable operation ranges should be defined using a DOE approach similar to that used in formulation development. The CQAs impacted by these process conditions should be evaluated. The following aspects should be considered when developing a robust and suitable manufacturing process:

- All product-contacting surfaces during manufacturing should be compatible with the formulation. Generally, the product-contacting surfaces during drug product manufacturing include silicone tubing, a Teflon-coated stir bar, a stainless steel tank or impeller mixer, rubber gaskets, plastic connectors, plastic housing and filter membranes, and other glass or plastic containers. It is important to evaluate the compatibility of the protein with these contact surfaces prior to the start of manufacturing using these materials.
- The container closure system should be compatible with the fill line at the manufacturing site. Typically, machinability needs to be conducted prior to cGMP manufacturing to ensure that the filling and stoppering operations run smoothly, with low rejection rates, and that the final container closure system meets the container closure integrity test criteria (integrity is typically tested by dye leak or vacuum decay method).
- The mixing condition should not result in product degradation. If mixing by a magnetic stir bar causes protein precipitation, alternative mixing methods such as an impeller should be considered.
- If the product is sensitive to dissolved oxygen in solution, several manufacturing process steps should be designed appropriately. Degassing the solutions and overlaying inert gas (nitrogen or argon) in the headspace of vials may be required to minimize oxidation due to dissolved oxygen in the product.
- The filter size for sterile filtration should be large enough not to give high back pressure during filtration. For aqueous protein formulations, a polyvinylidene fluoride (PVDF) membrane is the most commonly used. A filter sizing study should be performed to define the proper size of filter for cGMP manufacturing.
- The filling conditions should be compatible with the product. For solution filling, several filling machines are commonly used, including peristaltic pump, stainless steel piston syringe, ceramic piston syringe, rolling diaphragm, and rotary time pressure filling systems. While a syringe-filling system typically provides better accuracy, it applies high shear stress between the piston and barrel during movement, which could lead to precipitation of proteins. In addition, the filling speed needs to be controlled to avoid foaming or splashing during filling.

- The lyophilization parameters developed at laboratory scale should be robust enough to produce consistent product quality. Sometimes when the lyophilization cycle developed for the laboratory-scale lyophilizer is directly transferred to the production-scale lyophilizer, product quality may not be the same. This could be caused by the poor robustness of lyophilization cycle, as different lyophilizer designs may lead to different levels of heat and mass transfer, which could cause changes in product quality, such as cake collapse, if the selected lyophilization parameters are on the edge of the process design.
- Hold conditions (time and temperature) for all process intermediates should be established to meet the needs of routine production operations and to support potential excursions. An operation deviation may result in longer hold time for the process intermediate than during routine manufacturing; examples could be deviations prior to the formulation step or filling into final product, or after lyophilization. Supporting data for defining acceptable hold conditions need to address product quality from both chemical stability and microbiological perspective. Assessment of the microbiological acceptability of a process intermediate hold time is tied to manufacturing process operations and to the microbial growth potential of the intermediate composition. Support for extended or cumulative hold times generally comes from development-scale studies, while support for microbiological properties comes from manufacturing-scale studies.

Stability Studies

After the manufacture of drug substance and drug product, the following stability studies are generally conducted to support the shelf-life during long-term storage and the product quality during distribution and use at clinics:

- *Long-term stability studies to support shelf-life*. Several ICH guidelines outline the content and testing requirements for stability studies supporting shelf-life at long-term storage conditions (ICH Q1A, Q1C, Q1D, Q1E, and Q5C). Depending on the intended long-term storage condition, accelerated and stress conditions are often required for the clinical stability program and lots made during process validation. For postapproval commercial stability programs, one lot of drug substance and one lot of drug product are generally required to be placed on stability annually, and only at the long-term storage condition.
- *Temperature cycling studies to support excursions during distribution*. From completion of manufacturing to the time when the product is dosed into patients, the drug product experiences exposure to various temperatures, different from the intended long-term storage temperature range, which is typically the refrigerated temperature. It is important to conduct studies to evaluate the effect of these temperature variations on product stability. The design of these studies has been recommended in the Parenteral Drug Association (PDA) Technical Report No. 39 (96).
- *Shipping studies to support the exposure to vibration and reduced pressure during air and ground transportation*. It is important to recognize that shaking studies conducted at laboratory scale may not be representative of the actual shipping conditions to which the product is exposed, since the shaking studies may not have vibration amplitudes and frequencies similar to those generated during shipping. In addition, reduced pressure occurs during air shipment. This is a concern particularly for prefilled syringes, as stopper movement has been observed during multiple cycles of reduced pressure, which may affect the sterility of the product (97). Therefore, it is important to demonstrate that the product remains intact or within the designed space using actual or simulated shipping conditions, including representative secondary packaging and product orientation.
- *Confirmatory photostability studies to support exposure to light*. Sensitivity to light is highly dependent on the composition, structure, and formulation of the product. Most proteins and peptides are sensitive to intense light, particularly UV light, which

typically results in oxidation. However, they are relatively stable under normal indoor light. In addition, protein products are typically stored in refrigerated condition, in which there is no direct exposure to light. Commercial products normally have secondary packages, which prevent the product's exposure to light. To demonstrate that the product remains stable, it is important to conduct a confirmatory photostability study using representative commercial packaging per the ICH guideline Q1B.

- *In-use and compatibility studies with the administration system to support product stability and to assess compatibility with product-contacting surfaces and stresses during administration.* For IV infusion, some products may need to be diluted in an IV bag and then infused into patients through an IV infusion apparatus by a pump or other mechanism. It is critical to assess product stability after dilution prior to dosing, as well as the compatibility of the product in contact with the surfaces of the administration apparatus and infusion system, such as pumps. For lyophilized products, the stability of reconstituted solution should be evaluated to ensure the product remains stable during the ambient exposure period after reconstitution.

FORMULATIONS OF MARKETED PROTEIN PRODUCTS

In the United States, by law all marketed injectable products must disclose quantitative formulation, so the details on each marketed product are public. Sources on prescription information include the Physicians' Desk Reference (PDR) and numerous websites, including the FDA's http://www.accessdata.fda.gov/scripts/cder/drugsatfda and websites for specific products (which commonly take the form www.*tradename*.com—e.g., www.simponi.com). The formulations of discontinued products can be found in older editions of the PDR.

The excipients used in parenteral products were first reviewed and collated by Wang and Kowal in 1980 (98). Subsequently, there were reviews by Nema et al. (99) and Powell et al. (100). Specifically for biotech products, they were first reviewed by Wang and Hansen (101), and recently by Gokarn et al. (102).

The types of excipients used in protein formulations have evolved over time. For example, in the early years (1980s–1990s), human serum albumin (HSA) was commonly used as a stabilizer in many protein formulations, particularly low-concentration, high-potency products such as interferons, Factor VIII, and other growth factors. However, because of concerns about potentially contaminated blood that might compromise the quality of albumin, most products have been reformulated into HSA-free formulations. For example, Eprex® (epoetin α), originally containing albumin, was reformulated to use polysorbate 80 as an HSA-free product in prefilled syringes. Avonex® (interferon β), originally having albumin in lyophilized form, was reformulated to a polysorbate-20-containing liquid formulation in prefilled syringes.

Another example of evolving parenteral formulation excipients is the type of polysorbate. Because of concerns about potential auto-oxidation of the unsaturated double bond in polysorbate 80 (103), used predominantly in the early days of protein formulations, the trend seems to be moving toward the use of polysorbate 20. For example, Neupogen® is formulated with polysorbate 80, but the surfactant in its newer version, PEGylated protein (Neulasta®) was changed to polysorbate 20. Polysorbate 80 included in the Activase® formulation was also changed to polysorbate 20 in its newer variant TNKase®.

To show how formulations have evolved over time, examples of recombinant human growth hormones and monoclonal antibodies (including Fab, Fab-PEG, and Fc-fusion proteins) are listed in Tables 5 and 6, respectively. For human growth hormone, changes of formulation have been minimal since its first approval in 1985 to one recently approved in 2008. For lyophilized monoclonal antibodies, the buffer species used has changed from phosphate in early approved products to histidine in recently approved products. Sucrose is the most commonly used sugar in lyophilized formulations, and only a few products use trehalose or maltose. In addition, amino acids including arginine and glycine are used in both liquid and lyophilized formulations. For liquid monoclonal antibodies, sodium phosphate appears to be the most commonly used buffer, likely because of its good buffering capacity

Table 5 Formulations for Human Growth Hormone (in order of approval year)

Trade Name	Approval year	Dosing route	Presentation	Dosage form	Formulation
Protropin®	1985	SC	Vial/stopper	Lyophilized	5 mg vial[a]: 40 mg mannitol, 0.1 mg monobasic sodium phosphate, 1.6 mg dibasic sodium phosphate, pH 4.5–7.0
Humatrope®	1987	SC/IM	Vial/stopper	Lyophilized	5 mg/5 mL vial: 25 mg mannitol, 5 mg glycine, 1.13 mg dibasic sodium phosphate, pH 7.5
			Pen cartridge	Lyophilized	6 mg/2 mL cartridge[a]: same formulation as in vial/stopper
Norditropin®	1987	SC	Pen cartridge	Liquid	5 mg/1.5 mL cartridge[a]: 1 mg histidine, 4.5 mg Poloxamer 188, 4.5 mg phenol, 60 mg mannitol
Nutropin®	1993	SC	Vial/stopper	Lyophilized	5 mg/1 mL vial[a]: 45 mg mannitol, 0.4 mg sodium phosphate monobasic, 1.3 mg sodium phosphate dibasic, 1.7 mg glycine, pH 7.4
			Vial/stopper	Liquid	10 mg/2 mL vial: 17.4 mg sodium chloride, 5 mg phenol, 4 mg polysorbate 20, 10 mM sodium citrate, pH 6.0
			Pen cartridge	Liquid	5 mg/1 mL cartridge[a]: same formulation as in vial/stopper
Tev-Tropin®	1995	SC	Vial/stopper	Lyophilized	5 mg/5 mL vial: 30 mg mannitol, pH 7–9
Genotropin®	1995	SC	2-chamber cartridge	Lyophilized	5 mg/1 mL cartridge[a]: 5.8 mg somatropin, 2.2 mg glycine, 1.8 mg mannitol, 0.32 mg sodium dihydrogen phosphate anhydrous, 0.31 mg disodium phosphate anhydrous, pH 6.7
Saizen/Serostim®	1996	SC	Vial/stopper	Lyophilized	4 mg vial[a]: 27.3 mg sucrose, 0.9 mg phosphoric acid, pH 6.5–8.5
Zorbtive®	2003	SC	Vial/stopper	Lyophilized	4 mg vial[a]: 27.3 mg sucrose, 0.9 mg phosphoric acid, pH, 7.4–8.5
Omnitrope®	2006	SC	Vial/stopper	Lyophilized	1.5 mg/1.13 mL vial[a]: 0.88 mg disodium hydrogen phosphate heptahydrate, 0.21 mg sodium dihydrogen phosphate dihydrate, 27.6 mg glycine
			Pen cartridge	Liquid	5 mg/1.5 mL cartridge[a]: 1.3 mg disodium hydrogen phosphate heptahydrate, 1.6 mg sodium dihydrogen phosphate dihydrate, 3.0 mg poloxamer 188, 52.5 mg mannitol, 13.5 mg benzyl alcohol
Valtropin®	2007	SC	Vial/stopper	Lyophilized	5 mg vial/1 mL: 10 mg glycine, 45 mg mannitol, 0.22 mg monobasic sodium phosphate, 2.98 mg dibasic sodium phosphate, pH 6.0
Accretropin®	2008	SC	Vial/stopper	Liquid	5 mg vial: 0.75% NaCl, 0.34% phenol, 0.2% Pluronic F-68, 10 mM sodium phosphate, pH 6.0

[a]Indicates a product that has multiple dose strengths, but only the lowest strength is listed

Table 6 Formulations for Antibodies, Fc Fusion, and Fab Conjugates (in order of approval year)

Trade name	Nonproprietary name	Approval year	Dosing route	Presentation	Dosage form	Formulation
Orthoclone OKT-3®	Muromonab-CD3	1986	IV	Ampule	Liquid	5 mg/5 mL ampule: 2.25 mg monobasic sodium phosphate, 9.0 mg dibasic sodium phosphate, 43 mg sodium chloride, 1.0 mg polysorbate 80, pH 6.5–7.5
Reopro®	Abciximab (Chimeric Fab)	1994	IV	Vial/stopper	Liquid	10 mg/5 mL vial: 0.01 M sodium phosphate, 0.15 M sodium chloride, 0.001% polysorbate 80, pH 7.2
Rituxan®	Rituximab	1997	IV	Vial/stopper	Liquid	100 mg/10 mL vial[a]: 9 mg/mL sodium chloride, 7.35 mg/mL sodium citrate dihydrate, 0.7 mg/mL polysorbate 80, pH 6.5
Enbrel®	Etanercept	1998	SC	Vial/stopper	Lyophilized	25 mg/0.5 mL vial: 40 mg mannitol, 10 mg sucrose, 1.2 mg tromethamine, pH 7.1–7.7
				PFS	Liquid	25 mg/0.5 mL PFS[a]: 1% sucrose, 100 mM sodium chloride, 25 mM L-arginine hydrochloride, 25 mM sodium phosphate, pH 6.1–6.5
				Autoinjector	Liquid	Same as Liquid in PFS
Remicade®	Infliximab	1998	IV	Vial/stopper	Lyophilized	100 mg/10 mL vial: 500 mg sucrose, 0.5 mg polysorbate 80, 2.2 mg monobasic sodium phosphate monohydrate, 6.1 mg dibasic sodium phosphate dihydrate, pH 7.2
Simulect®	Basiliximab	1998	IV	Vial/stopper	Lyophilized	10 mg/2.5 mL vial[a]: 3.61 mg monobasic potassium phosphate, 0.5 mg disodium hydrogen phosphate (anhydrous), 0.80 mg sodium chloride, 10 mg sucrose, 40 mg mannitol, 20 mg glycine
Synagis®	Palivizumab	1998	IM	Vial/stopper	Liquid	50 mg/0.5 mL vial[a]: 1.9 mg histidine, 0.06 mg glycine, 0.2 mg chloride, pH 6.0
Herceptin®	Trastuzumab	1998	IV	Vial/stopper	Lyophilized	440 mg/20 mL vial: 400 mg α,α-trehalose dihydrate, 9.9 mg L-histidine HCl, 6.4 mg L-histidine, 1.8 mg polysorbate 20, pH 6.0
Zenapax®	Daclizumab	1999	IV	Vial/stopper	Liquid	25 mg/5 mL vial: 3.6 mg sodium phosphate monobasic monohydrate, 11 mg sodium phosphate dibasic heptahydrate, 4.6 mg sodium chloride, 0.2 mg polysorbate 80, pH 6.9
Mylotarg®	Gemtuzumab ozogamicin (calicheamicin)	2000	IV	Vial/stopper	Lyophilized	5 mg/5 mL vial: dextran 40, sucrose, sodium chloride, monobasic and dibasic sodium phosphate[b]
Campath®	Alemtuzumab	2001	IV	Vial/stopper	Liquid	30 mg/1 mL vial: 8.0 mg sodium chloride, 1.44 mg dibasic sodium phosphate, 0.2 mg potassium chloride, 0.2 mg monobasic sodium phosphate, 0.1 mg polysorbate 80, 0.0187 mg disodium edetate dihydrate, pH 6.8–7.4

Humira® (United Stataes) Trudexa (European Union)	Adalimumab	2002	SC	PFS	Liquid	40 mg/0.8 mL in PFS: 4.93 mg sodium chloride, 0.69 mg monobasic sodium phosphate dihydrate, 1.22 mg dibasic sodium phosphate dihydrate, 0.24 mg sodium citrate, 1.04 mg citric acid monohydrate, 9.6 mg mannitol, 0.8 mg polysorbate 80, pH 5.2
Zevalin®	Ibritumomab tiuxetan (^{90}Y)	2002	IV	Vial/stopper	Liquid	3.2 mg/2 mL vial: 0.9% NaCl, 50 mM sodium acetate
Amevive®	Alefacept	2003	IV/IM	Vial/stopper	Lyophilized	7.5 mg/0.5 mL vial[a]: 12.5 mg sucrose, 5 mg glycine, 3.6 mg sodium citrate dihydrate, 0.06 mg citric acid monohydrate, pH 6.9
Bexxar®	Tositumomab (IgG and IgG-^{131}I)	2003	IV	Vial/stopper	Liquid	35 mg/2.5 mL vial[a]: 10% maltose, 145 mM sodium chloride, 10 mM phosphate, pH 7.2
Xolair®	Omalizumab	2003	SC	Via/stopper	Lyophilized	202.5 mg/1.4 mL vial: 145.5 mg sucrose, 2.8 mg L-histidine hydrochloride monohydrate, 1.8 mg L-histidine, 0.5 mg polysorbate 20
Raptiva®	Efalizumab	2003	SC	Vial/stopper	Lyophilized	150 mg/1.3 mL vial: 123.2 mg sucrose, 6.8 mg L-histidine hydrochloride monohydrate, 4.3 mg L-histidine, 3 mg polysorbate 20, pH 6.2
Avastin®	Bevacizumab	2004	IV	Vial/stopper	Liquid	100 mg/4 mL vial[a]: 240 mg α,α-trehalose dihydrate, 23.3 mg sodium phosphate (monobasic, monohydrate), 4.8 mg sodium phosphate (dibasic, anhydrous), 1.6 mg polysorbate 20, pH 6.2
Erbitux®	Cetuximab	2004	IV	Vial/stopper	Liquid	100 mg/50 mL vial[a]: 8.48 mg/mL sodium chloride, 1.88 mg/mL sodium phosphate dibasic heptahydrate, 0.41 mg/mL sodium phosphate monobasic monohydrate, pH 7.0–7.4
Tysabri®	Natalizumab	2004	IV	Vial/stopper	Liquid	300 mg/15 mL vial: 123 mg sodium chloride, 17.0 mg sodium phosphate monobasic monohydrate, 7.24 mg sodium phosphate dibasic heptahydrate, 3.0 mg polysorbate 80, pH 6.1
Vectibix™	Panitumumab	2005	IV	Vial/stopper	Liquid	100 mg/50 mL vial[a]: 29 mg sodium chloride, 34 mg sodium acetate, pH 5.6–6.0
Orencia®	Abatacept	2005	IV	Vial/stopper	Lyophilized	250 mg/10 mL vial: 500 mg maltose, 17.2 mg monobasic sodium phosphate, 14.6 mg sodium chloride, pH 7.2–7.8
Lucentis®	Ranibizumab	2006	Intravitreal	Vial/stopper	Liquid	0.5 mg/0.05 mL vial: 10 mM histidine HCl, 10% α,α-trehalose dihydrate, 0.01% polysorbate 20, pH 5.5

(*continued*)

Table 6 Formulations for Antibodies, Fc Fusion, and Fab Conjugates (in order of approval year) (*continued*)

Trade name	Nonproprietary name	Approval year	Dosing route	Presentation	Dosage form	Formulation
Soliris®	Eculizumab	2007	IV	Vial/stopper	Liquid	300 mg/30 mL vial: 13.8 mg sodium phosphate monobasic, 53.4 mg sodium phosphate dibasic, 263.1 mg sodium chloride, 6.6 mg polysorbate 80, pH 7.0
Cimzia®	Certolizumab pegol	2008	SC	Vial/stopper	Lyophilized	200 mg/1 mL vial: 100 mg sucrose, 0.9 mg lactic acid, 0.1 mg polysorbate, pH 5.2
Arcalyst®	Rilonacept	2008	SC	Vial/stopper	Lyophilized	160 mg/2.3 mL vial: histidine, arginine, polyethylene glycol 3350, sucrose, glycine, pH 6.2–6.8[b]
Nplate®	Romiplostim	2008	SC	Vial/stopper	Lyophilized	250 μg/0.72 mL vial[a]: 30 mg mannitol, 15 mg sucrose, 1.2 mg L-histidine, 0.03 mg polysorbate 20, sufficient HCL to bring pH to 5.0
Simponi®	Golimumab	2009	SC	PFS	Liquid	50 mg/0.5 mL in PFS: 50 mg golimumab antibody, 0.44 mg L-histidine and L-histidine monohydrochloride monohydrate, 20.5 mg sorbitol, 0.08 mg polysorbate 80, pH 5.5
Ilaris®	Canakinumab	2009	SC	Vial/stopper	Lyophilized	180 mg/1 mL vial: sucrose, L-histidine, L-histidine HCL monohydrate, polysorbate 80[b]

[a]Indicates a product that has multiple dose strengths, but only the lowest strength is listed
[b]Quantitative formulation is not disclosed, not consistent with 21 CFR 201.100 (b) (5) iii.
Abbreviations: SC, subcutaneous; IM, intramuscular; IV, intravenous.

around pH 6 to 7, the pH at which most monoclonal antibodies are formulated. Polysorbate 80 or 20 is also present in many monoclonal antibody formulations.

CONCLUSION

In this book chapter we have attempted to provide an overview of formulation development for peptide- and protein-based therapeutics. For successful formulation development, it is important first of all to understand and characterize the unique characteristics of the protein or peptide, including molecular composition, structures, size, charge profile (pI), solubility, thermal transition midpoint, and key degradation pathways. Preformulation activities are also critical in identifying the key instability issues and potential pharmaceutically relevant sources responsible for specific degradation pathways. This chapter provides general principles and examples of major pharmaceutical development activities, including evaluation of critical formulation parameters, selection of container closure, development of the manufacturing process, and stability studies to support shelf-life and clinical use conditions. The results from these development activities are generally required in completing the pharmaceutical development sections of regulatory filings. Finally, trends in the evolution of formulation development since the early 1980s are described on the basis of several examples, including human growth hormone and monoclonal antibodies.

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10 | Development of ophthalmic formulations

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INTRODUCTION

Ophthalmic formulations are those that are intended for treating conditions of the eye; they may be intended to alleviate the signs or symptoms associated with a certain disease state, to provide relief from minor discomfort and irritation, or for treatment of the cause of a disease of the eye itself. In general, the best way to treat ophthalmic diseases is with a local treatment such as a topically administered eye drop. However, the biological design of the eye is optimized to keep the surface of the eye clear of all foreign substances and to provide a substantial barrier to transport of materials into or out of the eye. As a result, ophthalmic drug delivery presents a significant technical challenge. The ophthalmic formulator, therefore, must begin with a good understanding of the physiology of the eye and understand what ophthalmic drug delivery possibilities are available. The formulator must also understand the nature of the drug substance that needs to be delivered to the eye and its limitations. The ultimate job of the ophthalmic formulator is to discover the best way to bring the drug and the eye together in a fashion that will provide the optimal benefit to the patient.

This chapter will focus on the anatomy and physiology of the eye and the challenges in drug delivery to this organ. The goal will be to familiarize the reader with the biopharmaceutical aspects of drug delivery to the eye, the various strategies for targeting different tissues within the eye, and to provide a guide to a rational approach to formulation development for ophthalmic drug delivery. We will also provide a brief overview of ophthalmic formulation preservation, manufacturing and packaging, and regulatory pathways for bringing a formulation to market. Finally, the chapter will discuss some recent advances in drug delivery and the future of ophthalmic drug delivery.

STRUCTURE AND FLUID COMPOSITION OF THE EYE

The eye globe is continually cleansed and hydrated by the secretions of the nasolacrimal system. The eye globe can be divided into three concentric tunics: the fibrous tunic comprising the cornea and sclera, the vascular tunic (consisting of the iris, ciliary body, and choroid), and the retinal tunic. Other internal components of the eye include the lens, aqueous humor, and vitreous humor. Figure 1 illustrates the relative locations of these tissues in the eye. When a drug is delivered to the outer surface of the eye, it may need to diffuse through many of these tissues before it can reach the target tissue. The following is a very basic review of the main components of the eye relevant to the drug delivery:

Nasolacrimal System

The nasolacrimal system consists of three parts: the secretory system (lacrimal glands, meibomian glands, and goblet cells), the distributive system (eyelid movements and blinking), and the excretory system (lacrimal puncta; superior, inferior and common canaliculi; lacrimal sac; and nasolacrimal duct). The nasolacrimal system plays a major role in protecting and hydrating the eye surface. It also has significant impact on the amount of drug absorbed to the eye from topical administration.

Tear Fluid Secretion and Volume

Tears are continuously secreted by the lacrimal glands and the goblet cells. The normal (basal) secretion rate is about 1.2 mcL/min (1), however, under reflex tearing the secretion rate may increase to as high as 300 to 400 mcL/min (2). The normal volume of tear fluid on the eye is about 6 to 7 mcL. When additional fluid is added to the eye surface, the eye can hold about 25 mcL of fluid but will appear "watery" because of the added liquid. With greater additions of fluid, the excess tear fluid will immediately overflow at the lacrimal lake (1) or be splashed into the eyelashes by reflex blinking (3).

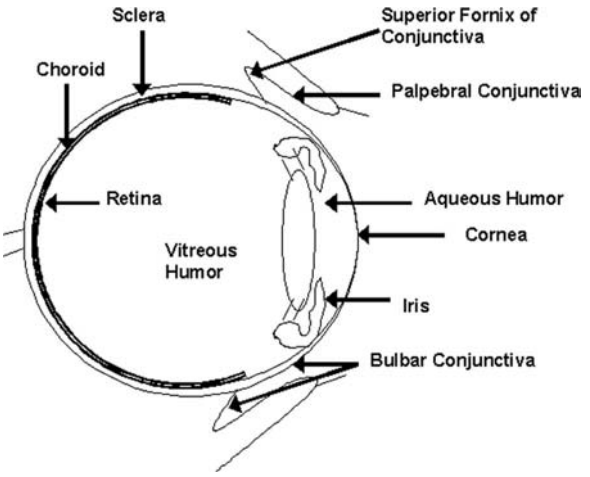

Figure 1 Schematic cross section of human eye.

Tear Fluid Lipid Content

The lipid layer of the tear film is secreted by the meibomian glands. The lipid layer of the tear film serves many functions including reducing evaporation from the ocular surface, lubricating the ocular surface, controlling the surface energy of the tear film, and providing a barrier function at the lid margin to inhibit the flow of skin lipids into the eye and tear fluid out of the eye (4,5). The meibomian secretions are primarily wax esters and sterol esters (about 59%), phospholipids (about 15%), and the remainder is diglycerides, triglycerides, free fatty acids, free sterols, and hydrocarbons (4,5). The polar lipids (phospholipids) are primarily phosphatidylcholine (40%) and phosphatidylethanolamine (18%) (6). The meibomian secretions are produced at a rate of about 400 mcg/hr and are excreted onto the lid margin and the anterior surface of the tear film by the normal blinking action. The thickness of the oil film on the tear fluid has been measured by various interference techniques giving values of 32 to 80 nm in thickness (5). From this thickness, the steady state amount of oil present on the surface of the tear fluid is calculated to be about 9 mcg per eye (5).

Tear Fluid Proteins and Enzymes

Tear fluid contains proteins in high concentration (about 8 μg/μL). Major components include lysozyme (an antibacterial enzyme), lactoferrin (which sequesters iron), secretory immunoglobin A (an antibody important for mucosal immunity), serum albumin, lipocalin and lipophilin (7). In addition, over 400 other proteins have been identified that serve various roles in the tear fluid (7).

Tear Fluid Mucus Layer

The mucus layer is secreted onto the eye surface by the goblet cells. Mucus consists of glycoproteins, proteins, lipids, electrolytes, enzymes, mucopolysaccharides, and water. The primary component of mucus is mucin, a high-molecular-weight glycoprotein that is negatively charged at physiological pH. The mucus layer forms a gel layer with viscoelastic properties which protects and lubricates the eye. The mucus gel traps bacteria, cell debris, and foreign bodies. The mucus layer may hinder drug delivery by forming a diffusional barrier to macromolecules, but it may also bind other substances (i.e., cationic or mucoadhesive) and prolong residence on the surface of the eye.

Tear Fluid pH and Buffer Capacity

The pH of normal tear fluid is 7.4 ± 0.2 (8). The primary buffering components present in the tear fluid are bicarbonate and proteins (8,9). The buffering capacity of the tear fluid is not symmetric around the mean pH. Rather, the tear fluid has more than twice the buffer capacity to resist drops in pH than it has to resist increases in pH (9). As a result of this asymmetric

buffering capacity, unbuffered solutions in the pH 4.0 to 8.5 range will cause minimal shift of the pH on the surface of the eye and will be easily neutralized by the tear fluid (9). Solutions with higher buffer capacities, particularly if greater than that of the tear fluid, may be uncomfortable to the eye if they result in a significant shift of the tear fluid pH.

Tear Fluid Osmolality

The tear fluid osmolality normally ranges from about 300 to 320 mOsm (3,8) and most of the osmolality in the tear fluid can be attributed to the salt content of the lacrimal fluid which is primarily sodium chloride, sodium bicarbonate, potassium chloride, calcium chloride, and magnesium chloride (3). Normally, there is about five times more sodium than potassium in the tear fluid and the levels of calcium and magnesium are less than 1/200th of the sodium levels (3). Higher than normal osmolality in the tear fluid is often seen in patients with dry eye syndrome. Abnormally high evaporation of tear fluid increases the salt levels and results in higher osmolality. As a result, many products for treatment of dry eye are often formulated with lower than normal osmolality.

Tear Fluid Viscosity and Surface Tension

The viscosity of the tear fluid would be expected to be primarily controlled by the higher molecular weight proteins dissolved in the lacrimal fluid. The viscosity of human tears has seldom been determined because of the difficulty of collecting enough sample for a determination. Schuller, et al., (10) found the viscosity of human tears ranges from 1.3 to 5.9 cps with a mean value of 2.9 cps. The viscosity of ophthalmic solutions may be increased in an effort to improve retention on the ocular surface. Hung, et al., (11) estimated that a painful sensation would be elicited if the tear fluid viscosity is increased above 300 cps at the shear conditions of the closing eyelid (shear rate of 20 000/sec). The surface tension of the tear fluid depends on the presence of soluble mucins, lipocalins, and lipids. The mean surface tension value is about 44 mN/min (12).

Fibrous Tunic

Cornea

The cornea is a transparent structure responsible for the refraction of light entering the eye. It forms the anterior one-sixth of the eyeball. The cornea is thinnest at its center (0.5–0.6 mm) and thicker in the periphery (1.2 mm) (13). The cornea is an avascular tissue that is supplied with oxygen and nutrients via the lacrimal fluid, aqueous humor, and the blood vessels at the cornea/sclera junction. The cornea is composed of five layers.

1. The epithelium—a stratified squamous epithelium made of 5 layers of cells (10 layers at the corneoscleral junction, i.e., the limbus) that has total thickness of around 50 to 100 μm. At the limbus, the corneal epithelium is continuous with the bulbar conjunctiva. The epithelial cells are connected through tight junctions which limit drug permeability significantly.
2. Bowman's membrane—lies between the basement membrane of the epithelium and the stroma, and is composed of acellular interwoven collagen fibers.
3. The stroma—accounts for 90% of the cornea thickness and is mainly composed of water and collagenous lamellae that gives the strength and structure for this layer and yet allows the penetration of light. Generally, it does not significantly limit drug permeability.
4. The Descemet's membrane—composed of collagen fibers, it lies between the stroma and the endothelium.
5. The endothelium—composed of a single layer of flattened cells that are connected via tight junctions. It controls the hydration of the cornea by limiting access of water from the aqueous humor and by active transport mechanisms.

The cornea provides a limited surface area of about 1 cm^2 for drug diffusion, and is a significant barrier to both hydrophilic and lipophilic compounds. Lipophilic molecules will

diffuse more easily through the epithelium and the endothelium, but hydrophilic molecules will diffuse more easily through the highly aqueous stroma.

Conjunctiva

The conjunctiva is a thin mucus secreting membrane that lines the posterior layer of the eyelids (palpebral conjunctiva), the anterior sclera (bulbar conjunctiva), and the superior and inferior conjunctival fornices (joining areas between the palpebral and bulbar conjunctiva). The conjunctiva is composed of two layers: an outer epithelium layer (which is continuous with the corneal epithelium) and an underlying stroma layer.

The conjunctival epithelium is made of 5 to 15 layers of stratified epithelial cells that are connected at the apical side with tight junctions and it plays a major role in limiting drug penetration (14). Nevertheless, the human conjunctiva is 2 to 30 times more permeable to drugs than the cornea (15).

The stroma layer of the conjunctiva contains the nerves, lymphatics, and blood vessels and it attaches loosely to the sclera. The conjunctiva contributes to the tear film formation by secreting electrolytes, fluid, and mucin (14).

Sclera

The sclera covers five-sixths of the eyeball surface and has a mean surface area of 16.3 cm^2 (16). It connects to the cornea anteriorly at the limbus. The sclera is mainly composed of collagen fibers with varying sizes and orientation that are embedded in a glycosaminoglycan matrix. Scleral thickness varies by location; the mean thickness is 0.53 mm near the limbus, is 0.39 mm near the equator, and is about 0.9 to 1.0 mm near the optic nerve (17). The sclera is composed of three main components.

1. Episclera—the outermost layer made of loosely arranged collagen fibers that is connected to the eyeball sheath (Tenon's capsule).
2. Stroma—composed of larger collagen fibers and elastic tissue.
3. Lamina fusca—the innermost layer of sclera that forms the uveal tract with the choroids. It is composed of loosely coherent collagen bundles and melanocytes.

Aqueous Humor

Aqueous humor is a clear fluid that is secreted by the ciliary body via the filtration of blood passing the through the ciliary body capillaries. It has several functions including maintaining the shape of the eye by controlling its pressure, providing nutrition to the cornea and lens, and providing transport of waste materials away from surrounding tissues. The aqueous humor is composed mainly of water, high concentrations of ascorbic acid, glucose, amino acids, and limited levels of proteins. Aqueous humor flows from the ciliary body in the posterior chamber (behind the iris) into the anterior chamber (between the iris and the cornea). Aqueous humor flows out of the eye through the trabecular meshwork (a network of collagen fibers and endothelial-like trabecular cells) into Schlemm's canal, and through the uveoscleral route (18). The entire volume of the aqueous humor is about 0.2 mL and is replaced every one to two hours (13). Maintaining the intraocular pressure (IOP) of 10 to 20 mmHg is a balancing act of production and drainage.

Vitreous Humor

Vitreous humor is a gel-like material that occupies the space between the lens and the retina. The vitreous humor is composed mainly of water (98–99.7%), collagen fibrils and hyaluronic acid (19). It supports the posterior surface of the lens and helps keep the neural part of the retina in place. The normal aging process can lead to liquefaction of the vitreous (>50% by age 80–90) and posterior vitreous detachment. The close proximity of the vitreous to the retina and choroid makes this cavity a direct place for drug delivery to the posterior tissues.

Vascular Tunic

Blood-Retina Barrier

The blood-retina barrier is composed of two parts which regulate the transport to the retina: the outer retina barrier formed by retinal pigment epithelium (RPE) and the inner retina barrier formed by the endothelial cells of the retinal vessels.

Two vascular beds supply the retina. Retinal vessels supply the inner two thirds, while the outer retina is avascular and receives oxygen and nutrients from the choriocapillaris. The choriocapillaris is fenestrated to enhance nutrients transport to the underlying retina. Plasma leaks from the choriocapillaris and diffuses through the Bruch's membrane and through the RPE to the outer retina. RPE tight junctions constitute the outer blood-retinal barrier.

Retinal vessels are supplied by the central retinal vessel. Retinal capillaries are composed of a single layer of endothelial cells surrounded by a basement membrane and pericytes. The endothelial cells are attached to each other by tight junctions forming the inner blood-retina barrier. These narrow tight junctions, similar to those present in the brain vessels, impair the paracellular transport of hydrophilic compounds and necessitate their passage through the intracellular routes (20).

Choroid

The choroid is a highly vascularized tissue between the retina and the sclera. It consists of: the vessel layer, the choriocapillaris, and Bruch's membrane (which is in direct contact with the RPE). Between the sclera and the choroid there is the suprachoroidal, or perichoroidal, space. This is a very thin space consisting of various connective tissue lamellae and is characterized as sponge tissue. Substantial amounts of the aqueous humor that leaves the eye via the uveoscleral route ends up in the suprachoroidal space and is finally drained out from the eye through porosites in the sclera.

The choriocapillaris is found in the inner portion just below the RPE and it provides nutrition to the RPE and the outer one third of the retina. Between the RPE and the choriocapillaris is the Bruch's membrane. Bruch's membrane is composed of five layers: the basement membrane of the RPE, an inner collagenous layer, the elastic layer, the outer collagenous zone, and the basement membrane of the capillary endothelial cells.

Retinal Tunic

The retina is composed of neural retina and RPE. The inner surface of the neural retinal is facing the vitreous humor while the outer border is next to the RPE. The neural retinal is composed of nine layers containing the nerve fibers and the photoreceptors responsible for light detection. The RPE is composed of a single layer of cells connected by tight junctions.

Biopharmaceutics and Routes of Administration

Drug penetration into the eye is a challenging task and can follow different pathways to reach the ocular target tissues depending on the route of administration and the drug's physicochemical properties. The target tissue within the eye is different for each drug and indication. In general, the focus of drug delivery to the eye can be divided into delivery to the anterior segment of the eye and to the posterior segment of the eye.

Delivery to the Anterior Segment of the Eye (Topical)

The anterior segment includes the conjunctiva, the cornea, the anterior sclera, the iris, the ciliary body, and the aqueous humor. Topical administration of drugs is considered the most common and acceptable route of administration for these target tissues. Drugs applied topically as an eye drop of a solution or suspension or as an ointment are easy to administer and noninvasive. However, drug penetration via this route is inefficient—bioavailability is generally less than 5% of the administered dose. Accordingly, the majority of the dose will end up in the systemic circulation and may have systemic effects (21,22). In certain conditions, particularly when sustained prolonged drug release is preferred, a subconjunctival injection or implant may be used to target these tissues. Drug penetration to the anterior tissues from topical administration faces significant barriers that limit its ocular bioavailability (Fig. 2).

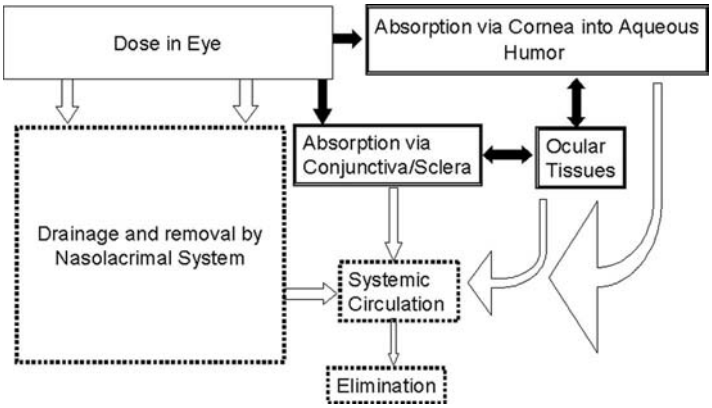

Figure 2 Schematic representation of compartmental drug penetration and elimination from topical administration.

Nasolacrimal Drainage

One of the most important attributes of a good ophthalmic topical formulation is that it needs to remain on the surface of the eye long enough to deliver a therapeutic amount of the medication. This necessarily means that the formulation needs to mix with or replace a portion of the natural tear fluid and should be as comfortable on the surface of the eye as the natural tear fluid. If the formulation evokes discomfort on the eye in any way (pH, osmolality, viscosity), it will lead to reflex tearing and blinking as the body attempts to flush the offending agent from the surface of the eye. Therefore the first step to understanding how to formulate an ophthalmic formulation is to understand the characteristics of the tear film (discussed above) that should be appropriately mimicked by the formulation. In addition, the formulation should be designed so that it does not adversely interact with the components in the natural tear fluid.

Most of the topical dose is lost through the nasolacrimal drainage before it can reach the eye. The limited volume that the eye surface can accommodate (30 μL), the high tear turnover rate (0.5–2.2 μL/min), and blinking rate are all natural ways of the eye to protect itself and limit penetration through its surface. The introduction of an eye drop (average volume of 39 μL), and possibly its composition will induce more tear secretion and increased blinking that will enhance the drainage out of the eye surface and reduce amount of drug available for absorption (23).

Corneal Absorption

The cornea offers the major site of drug diffusing into the anterior chamber of the eye, especially for small molecules. Drug penetration through the cornea can be by passive diffusion or by active transport mechanisms. The two main factors influencing the passive diffusion are lipophilicity and molecular size. Small lipophilic compounds generally penetrate through the epithelium via the intracellular route, while small hydrophilic compounds are limited to the paracellular route (partitioning of small lipophilic compounds into the cornea causes it to act as a depot). Large hydrophilic compounds (5000 Da) are generally excluded by the epithelium tight junctions (24). The fraction of a lipophilic compound penetrating through the cornea is 20 times more than a hydrophilic molecule of similar molecular size (25). A logD value of 2 to 3 for β-blockers was reported to provide optimal corneal permeation (26). Molecular size is also an important factor for small hydrophilic and lipophilic compounds. Increasing the molecular size from 0.35 nm to 0.95 nm reduces the permeability through the cornea, and conjunctiva significantly (25).

Active transport in the cornea can carry drug molecules from the eye surface into the aqueous humor and vice versa. However, saturation of the active transporter is possible and may limit the significance of this route during the limited residence time of the formulation on the surface of the eye. A prodrug approach targeting certain transporters in the cornea to enhance the permeation of acyclovir has been recently reported (27,28). Mannermaa, et al., (29) has provided a detailed review on the emerging role of transporters in ocular delivery.

Conjunctival and Scleral Absorption

Permeation through the conjunctival epithelium is limited by the tight junctions. However, the pore size of 5.5 nm in the conjunctiva allows larger molecular weights up to 38 600 Da to passively diffuse (30). The high surface area of diffusion of the conjunctiva compared with the cornea (17:1) contributes the importance of this route especially for hydrophilic compounds and large molecules (31). Compounds penetrating through the conjunctiva can continue the penetration into the eye through the sclera. Scleral permeation does not depend on the compound lipophilicity, but depends on the molecular radius (32). The presence of blood vessels in the conjunctiva can act as a sink condition that limits drug penetration to the sclera, carrying drug instead to the systemic circulation. As with the cornea, active transporters in the conjunctiva have been reported and reviewed (33).

Elimination from the Anterior Segment of the Eye

Drug molecules reach the aqueous humor though the corneal route or the iris/ciliary body through the conjunctiva/sclera route can be cleared through the aqueous humor drainage and through the blood vessels penetrating the eye to the systemic circulation.

Delivery to the Posterior Segment of the Eye

Posterior drug delivery may target the retina, choroids, and vitreous humor. Targeting the posterior tissues of the eye has gained significant interest in recent years with the advent of new agents for treatment of age-related macular degeneration and diabetic retinopathy.

Several routes can be used to direct drug molecules to the posterior tissue (Fig. 3). The following is a summary of these administration and possible penetration routes:

Topical

Several compounds have been reported to reach the posterior segment of the eye from topical administration (34,35). As with the delivery to the anterior segment of the eye, there are two main pathways for drugs to reach the posterior segment of the eye: the corneal route, and the conjunctival/sclera route. Once the drug molecules reach the anterior segment tissue it can penetrate to the rest of the ocular tissues via several routes as explained in Figure 4. Penetration through the lens into the vitreous is limited and generally observed with lipophilic compounds (36). Alternatively drug molecules can diffuse against the aqueous humor outflow to into the vitreous, or through the uveoscleral route. Drug penetration through the conjunctiva/sclera route is believed to be most significant in reaching the retina and choroids. Drugs reaching the sclera can diffuse laterally around the orbit and into the choroids and retina. Systemic recirculation plays a role in reintroducing the drug molecules lost to the systemic circulation back to the ocular tissue as observed with the effect of topical β-blockers on the contralateral eye (37).

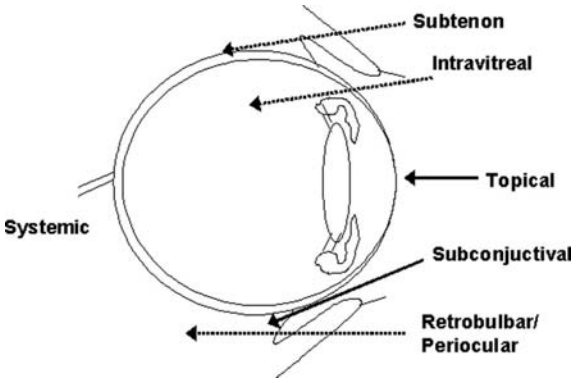

Figure 3 Different routes of ocular administration.

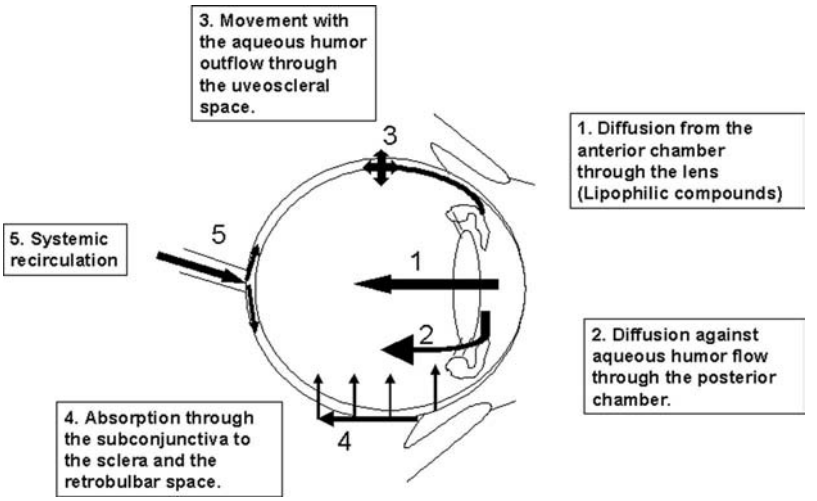

Figure 4 Schematic representation of drug penetration pathways to the posterior segment of the eye.

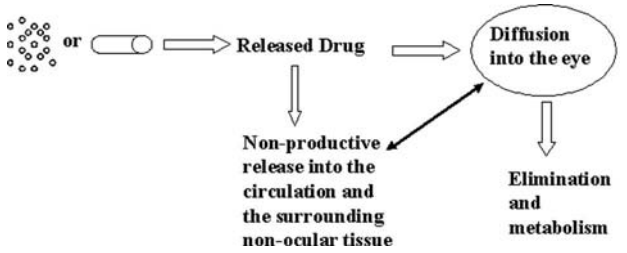

Figure 5 Schematic representation of drug release and diffusion into the eye from controlled delivery system (microsphere or implant) after periocular administration.

Subconjunctival/Transscleral Delivery

This is an important and promising route of delivering compounds to the back of the eye. It includes subconjunctival, peribulbar, retrobulbar, and subtenon injections. In all these injections the major permeability and loss to the systemic circulation limitations through the conjunctiva is avoided. Additionally, the drug has more time to diffuse through the sclera to the choroids and retina than that with topical administration. Scleral permeability, as discussed before, is not affected by lipophilicity of the compound but with the molecular radius. Large molecules up to 70 kDa are still able to penetrate the sclera (38). The large surface area of the sclera offers great potential for both small and large molecules to diffuse into the choroids, retina, and vitreous. In the periocular delivery, drug release from various delivery system and elimination can be depicted as in Figure 5. Once drug molecules diffuse through the sclera, it has to diffuse through the suprachoroidal space to the choriocapillaris, and then through Bruch's membrane to the RPE (outer retina-blood-barrier). The major limitation of drug to diffuse to the retina is the RPE. The majority of drug dissolved or released will be lost to nonocular tissue and eventually to the systemic circulation. Minimal loss to the choroidal circulation is expected (39). The ability of the nanoparticles to penetrate through the sclera-choroid-retina has been recently reported to have nonsignificant transport across these tissues with the majority of the nanoparticles being lost to periocular circulation and lymphatics (40). Differences between the various injections (subconjunctival, peribulbar, retrobulbar, and subtenon) exist with regard to penetration into posterior tissues (41). More drug is available in the vitreous and subretinal fluid when given as a subconjunctival injection compared with peribulbar injection (42,43). This can be due to the close proximity to the eye in case of subconjunctival injection. Subtenon injection also utilized for the delivery of active compounds behind the macula for effective delivery to the choroids and retina. The advantage of this

injection is the potential ability of Tenon's capsule to capture the delivery system (suspension, microspheres, or nanoparticles) in place where drug release/dissolution will continue for extended period of time. The transscleral route is most promising and less invasive route when compared with intravitreal delivery, especially with the advancement in the controlled release delivery systems.

Intravitreal Injection/Implant

Delivery through the intravitreal route is the most direct way to the retina. When delivered via this route, drug molecules only need to the diffuse through the vitreous to reach the retina, and through the RPE to reach the choroids. The low systemic exposure with intravitreal injection is a major advantage for this route. However, repeated injections of medication are often required which may lead to increased risk of endophthalmitis, damage to the lens, and retinal detachment. Intravitreal injections are typically administered in the inferotemporal quadrant, approximately 4 mm from the limbus (44). Controlled release formulations and implants can be used to decrease the frequency of administration required. Drug elimination and distribution from intravitreal delivery is controlled by the position of an intravitreal injection, and the lipophilicity and molecular size of the drug (45). Disposition from the vitreous humor can be through retinal absorption (retina/choroid/sclera) or via the posterior chamber (annular gap between lens and the ciliary body) then through the aqueous humor. Compounds with high lipophilicity are believed to be cleared via the retina pathway, while small hydrophilic and macromolecules are cleared anteriorly through the aqueous humor flow. Clearance and localization of polymeric nanoparticles after intravitreal injection was reported to depend on the size of the particles (46,47).

Ophthalmic Indications and Diseases

Table 1 lists several examples of marketed ophthalmic formulations used to target disease conditions in both the anterior and posterior tissues. While it is beyond the scope of this chapter to provide a comprehensive listing of ophthalmic diseases and indications, we will briefly discuss the most common indications.

Anti-infective Agents

There are many drugs available to treat bacterial, viral, and fungal infections of the eye. The antibiotic drugs that are available are generally broad spectrum. Ophthalmic formulations in this category are in the form of ointments and suspensions in addition to conventional solution eye drops. Many of the products are combinations of drugs and the relative efficacy of the formulations is judged on the frequency of instillation and duration of treatment.

Broadly the following are the major types of ocular infections that are treated by antibiotics or a combination of antibiotics and anti-inflammatory agents (48):

> Conjunctivitis (viral, bacterial, neonatal); episcleritis; keratitis (viral, bacterial, keratitis due to light exposure); uveitis (anterior, intermediate, posterior, and retinitis); hordeolum and chalazion; dacryocystitis; and periorbital and orbital cellulitis.

During the day, patients are usually treated using eye drops (sometimes up to several times a day) and at night they may be additionally directed to use an ophthalmic ointment (e.g., in the case of severe infections and blepharitis).

Anti-inflammatory Agents

Inflammation is the manifestation of vascular and cellular response of the host tissue to injury. Injury to the tissue may be inflicted by physical or chemical agents, invasion of pathogens, ischemia, and excessive (hypersensitivity) or inappropriate (autoimmunity) operation of immune mechanisms. In ocular tissues, inflammatory reactions are mediated by arachidonic acid cascade products formed via the cyclooxygenase pathway. There are two types of anti-inflammatory agents: corticosteroids and nonsteroidal anti-inflammatory drugs (NSAIDs). Both corticosteroids and NSAIDs may be administered orally as well as topically, but topical

Table 1 Examples of Marketed Ophthalmic Formulations for Treatment of Anterior and Posterior Indications

Indication	Active ingredient	Class/mechanism	Formulation type	Product example
Anterior drug delivery examples				
Acute infection—blepharitis	Bacitracin	Miscellaneous antibiotic	Ointment	Ciloxan (Alcon)
Acute infection—conjunctivitis	Tobramycin	Aminoglycoside	Suspension	Tobrex (Alcon)
Acute infection—conjunctivitis	Azithromycin	Macrolide	Mucoadhesive solution	AzaSite (Inspire Pharma)
Acute infection—keratitis	Ofloxacin	Quinolone	Solution	Ocuflox (Allergan)
Acute pain/inflammation	Prednisolone acetate	Corticosteroid	Suspension	Pred-Forte (Allergan)
Acute pain/inflammation	Diclofenac sodium	NSAID	Solution	Voltaren (Novartis)
Acute pain/inflammation	Flurbiprofen sodium	NSAID	Solution	Ocufen (Allergan)
Acute pain/inflammation	Loteprednol etabonate	Soft steroid	Suspension	Lotemax (Bausch & Lomb)
Allergy, OTC	Ketotifen fumarate	Antihistamine	Solution	Alaway (Bausch and Lomb)
Allergy, OTC	Naphazoline HCl, pheniramine maleate	Decongestant/vasoconstrictor	Solution	Opcon-A (Bausch & Lomb)
Allergy, Rx	Azelastine HCl	Antihistamine	Solution	Optivar (MedPointe)
Allergy, Rx	Olopatadine HCl	Antihistamine	Solution	Pataday (Alcon)
Allergy, Rx	Loteprednol etabonate	Soft steroid	Suspension	Alrex (Bausch & Lomb)
Dry eye, OTC	PEG400, propylene glycol	Aqueous tear-fluid replacement/ stabilizer	In situ gelling solution	Systane (Alcon)
Dry eye, OTC	Glycerin, propylene glycol	Aqueous tear-fluid replacement/ stabilizer	Mucoadhesive solution	Soothe (Bausch & Lomb)
Dry eye, OTC	Light mineral oil, mineral oil	Ocular lipid replacement	Emulsion	Soothe XP (Bausch & Lomb)
Dry eye, Rx	Cyclosporine	Immunomodulator/anti-inflammatory	Emulsion	Restasis (Allergan)
Glaucoma	Brimonidine tartrate	$\alpha 2$ adrenergic agonist	Mucoadhesive solution	Alphagan P (Allergan)
Glaucoma	Betaxolol HCl	β-blocker, $\beta 1$	Mucoadhesive, complexed solution	Betoptic S (Alcon)
Glaucoma	Timolol maleate	β-blocker, $\beta 1$ and $\beta 2$	In situ gelling solution	Timoptic XE (Merck)
Glaucoma	Dorzolamide HCl	Carbonic anhydrase inhibitor	Solution	Trusopt (Bausch & Lomb)
Glaucoma	Latanoprost	Prostaglandin	Solution	Xalatan (Pfizer)
Posterior drug delivery examples				
"Wet" AMD	Ranibizumab injection	Monoclonal antibody fragment/VEGF inhibitor	Solution, intravitreal injection	Lucentis (Genetech)
Wet AMD	Pegaptanib sodium	Oligonucleaotide/VEGF inhibitor	Solution, intravitreal injection	Macugen (Pfizer)
Chronic uveitis	Fluocinolone acetonide	Corticosteroid	Implant	Retisert (Bausch & Lomb)
Cytomegalovirus retinitis	Ganciclovir	Antiviral	Implant	Vitrasert (Bausch & Lomb)

Abbreviations: NSAID, nonsteroidal anti-inflammatory drug; VEGF, vascular endothelial growth factor.

administration is the preferred route for management of ocular inflammation as it provides high ocular drug concentrations and reduces the systemic side effects.

Corticosteroids work by blocking the enzyme phospholipase A2 to inhibit arachidonic acid production, thereby preventing the synthesis and release of prostoglandins, thromboxanes, and eicosanoids. Some concerning side effects of corticosteroid treatment are an increase in IOP, suppression of the immune system response to pathogens, slowed wound healing, and formation of cataracts. Steroids have been used extensively before and post surgery as a result of their broad effects and are generally more potent than NSAIDs for treatment of severe inflammation. Recently, soft steroids have been introduced in an effort to maintain the potent efficacy of the cortiosteroids while reducing the undesirable side effects. One of these soft steroids is loteprednol etabonate, which has less effect on IOP because of its rapid metabolic deactivation. Most of the steroids have low aqueous solubility and, hence, most are formulated as suspensions or emulsions.

NSAIDs exert their anti-inflammatory action by inhibiting the cyclooxygenase enzymes (viz., COX-1, COX-2, and COX-3). NSAIDs are commonly used to treat postoperative inflammation, in the prevention and treatment of cystoid macular edema and for relief in allergic conjunctivitis. Treatment with NSAIDs is preferred because of the lower occurrence of side effects associated with steroidal drugs; however, most of the NSAIDs for ophthalmic use are weakly acidic compounds (49) which have a tendency to lower the pH of the formulations making the formulations somewhat irritating. In addition, many of them have poor water solubility; thus, they are often used in the form of their more soluble salt forms (e.g., sodium, potassium, tromethamine, or lysine salts) or are formulated with solubilizers like surfactants or cyclodextrins.

Antiallergy Agents

Ocular allergic disorders include seasonal allergic conjunctivitis (SAC), perennial allergic conjunctivitis (PAC), vernal keratoconjunctivitis (VKC), giant papillary conjunctivitis (GPC) and atopic keratoconjunctivitis (AKC). The treatment of acute and more chronic forms of allergic conjunctivitis has been mainly focused on symptomatic relief of symptoms such as, redness, itching, and burning. They are primarily antihistamines, that is, H1 blockers. In some cases the allergic condition may require the use of topical corticosteroids as well. Some of the antiallergy eye drops are available OTC, but many are still only available as prescription medications.

Dry Eye Treatments

Dry-eye syndrome results from problems originating in the nasolacrimal system resulting in inadequate quantity and quality of tears or ocular surface abnormalities. Signs and symptoms of dry eye include itchiness, redness, foreign body sensation, and grittiness. Most treatments alleviate the signs and symptoms of dry eye rather than treating the cause. Most OTC dry-eye treatments are designed to replace and stabilize the aqueous portion of the tear film. A couple of OTC treatments are also available to replace the lipid portion of the tear film which may be inadequate to prevent evaporation of the aqueous portion of the tear film. A pharmaceutical approach to treating dry eye may involve the use hormones (or analogs) to increase the lipid production of the meibomian glands or to treat inflammation of the lacrimal glands to increase the secretion of the lacrimal glands (50). Regardless of the type of treatment, most dry-eye products are often dosed several times a day and therefore need to be mild and contain little or no preservatives or preservatives that are nonirritating.

Antiglaucoma Agents

Glaucoma is a sight-threatening optic neuropathy. The disease is characterized by increased IOP, excavation of the optic nerve head, reduction in the number of retinal ganglion cells, and a resultant progressive loss of visual field. Elevated IOP is a major risk factor and available antiglaucoma drugs treat this facet of the disease. The most common form of the disease is open-angle glaucoma in which IOP rises as a result of decreased outflow of aqueous humor through the trabecular meshwork and Schlemm's canal. Antiglaucoma drugs may act by decreasing aqueous humor production or increasing aqueous humor outflow (via the

trabecular meshwork or the uveoscleral pathway) (51). Drugs that affect aqueous humor production include β2-adrenergic receptor agonists, β1-adrenergic receptor agonists, α2 adrenergic receptor agonists and carbonic anhydrase inhibitors. The newest category of drugs used in the treatment of glaucoma is the prostaglandin analogs which affect aqueous humor outflow (52,53). Most of these products need to be dosed once or twice daily. The prostaglandin analogs however, have certain side effects associated with them namely, iris hyperpigmentation and change in the length, color and thickness of eyelashes, hyperemia and pruritis.

Posterior Indications

"Wet" age-related macular degeneration is a condition where blood vessels behind the retina start to grow and leak blood and fluid. This causes damage to the macula (the center of the retina) and results in central vision loss that can occur quickly. The medications available to treat wet AMD work by inhibiting the action of vascular endothelial growth factors (VEGF). Delivering these actives to the retina involves an intravitreal injection every 6 to 12 weeks.

Intravitreal implants are available for the treatment of posterior diseases as well and offer the ability to reduce the dosing frequency for these posterior treatments to once per year. However, there are two significant issues with intravitreal implants. Firstly, current intravitreal implants require a surgical procedure that is more invasive than an intravitreal injection. Secondly, a formulation that delivers drug over the course of a year requires a much longer (and, hence, more costly) clinical trial. Therefore, the use of intravitreal implants is limited.

Formulations for Ophthalmic Delivery

The following section discusses the various components and factors to be considered in the development of the different types of formulations for ophthalmic medications.

Excipients for Use in Ophthalmic Formulations

A suitable ophthalmic formulation must include excipients to control the osmolality, pH, and stability of the formulation. Control of the formulation stability includes chemical, physical, and antimicrobial stability. In addition, some excipients may be added to a formulation to enhance the drug delivery of the formulation by modifying the solubility of the active ingredient or increasing the retention of the active ingredient on the surface of the eye. When a formulator begins to design a new formulation for an active ingredient, they must be aware of the additional development effort or risk that might be associated with the use of novel formulation ingredients. In some cases, the risk of using novel ingredients will be warranted in an effort to gain patent protection or overcome difficult drug delivery issues. In other cases, the risk may not be warranted as it could lead to longer and more costly development programs. In either case, the formulator should always begin their formulation development efforts by selecting ingredients from those that have previously been used in ophthalmic formulations. In the U.S. market, the Food and Drug Administration (FDA) inactive ingredients database provides a convenient listing of these materials. A listing of these ophthalmic excipients and their potential use is provided in Table 2. No such database is currently available from the other primary regulatory agencies.

Solutions

An ophthalmic solution formulation is always the first choice if a drug substance has suitable aqueous solubility and stability in the range from pH 5 to 8. A good example of the simplest approach to a topical solution formulation is the marketed latanoprost formulation. The formulation is a neutral pH, phosphate-buffered saline preserved with 200 ppm benzalkonium chloride (BAK). This formulation example demonstrates that even the simplest formulation should provide control of pH and osmolality, and provide antimicrobial stability.

The osmolality and pH of the formulation should always be matched as closely as possible to that of the tear fluid; however, significant ranges for both of these variables have been found to be acceptable in practice. The eye is better able to tolerate pH excursions on the acidic side rather than on the basic side, hence, the range of acceptable pH values is skewed more to the acidic side of the mean tear fluid pH. There are many topical formulations in the pH 5.5 to 7.5 range, and a few that go as low as 4.0 and as high as 8.0. This asymmetry of the

Table 2 Excipients Listed in Food and Drug Administration Inactive Ingredients Database

Category of excipient	Ingredient name	Use level in database	Compendial listing(s)
Wetting and solubilizing Agents/emulsifying agents	Benzalkonium chloride	2%	NF, PhEur, JP
	Benzethonium chloride	–	USP, PhEur, JP
	Benzododecinium bromide	0.01%	–
	Carbomer 1342	0.05%	NF, PhEur, JPE
	Cetyl alcohol	0.5%	NF, PhEur, JP
	Cholesterol	–	NF, PhEur, JP
	Cocamidopropyl betaine	0.002%	–
	Glyceryl monostearate	0.5%	NF, PhEur, JP
	Lanolin alcohols	10%	NF, JPE
	Lauralkonium chloride	0.005%	–
	N-lauroylsarcosine	0.03%	–
	Nonoxynol 9	0.12%	USP, JPE
	Octoxynol 40	0.01%	JPE
	Poloxamer 188	0.1%	NF, PhEur, JPE
	Poloxamer 407	0.2%	NF, PhEur, JPE
	Polyoxyl 35 castor oil	5%	NF, PhEur, JPE
	Polyoxyl 40 hydrogenated castor oil	0.5%	NF, PhEur, JPE
	Polyoxyl 40 stearate	7%	NF, PhEur, JP
	Polysorbate 20	0.05%	NF, PhEur, JPE
	Polysorbate 60	15%	NF, PhEur, JPE
	Polysorbate 80	4%	NF, PhEur, JP
	Sorbitan monolaurate	–	NF, PhEur, JPE
	Tyloxapol	0.3%	USP
Suspending and/or viscosity-increasing agents	Carbomer 1342	0.05%	NF
	Carbomer 934P	0.45%	NF, JPE
	Carbomer 940	4%	NF, JPE
	Carbomer 974P	0.5%	NF, PhEur, JPE
	Carboxymethylcellulose sodium	0.5%	NF, PhEur, JP
	Gellan gum	0.6%	NF
	Hydroxyethyl cellulose	0.5%	NF, PhEur, JPE
	Hypromellose 2906	0.5%	USP, PhEur, JP
	Hypromellose 2910	0.5%	USP, PhEur, JP
	Methylcellulose	0.5%	USP, PhEur, JP
	Polycarbophil	0.9%	USP
	Polyethylene glycol 8000	2%	NF, PhEur
	Polyvinyl alcohol	1.4%	USP, PhEur, JPE
	Povidone K30	2%	USP, PhEur, JP
	Povidone K90	1.2%	USP, PhEur, JP
	Xanthan gum	0.6%	NF, PhEur, JPE
Acidfying agents/alkalizing agents (pH adjustment)	Acetic acid	0.2%	NF, PhEur, JP
	Ammonium hydroxide	–	–
	Citric acid	0.2%	USP, PhEur, JP
	Diethanolamine	–	NF, JPE
	Hydrochloric acid	1.06%	NF, PhEur, JP
	Nitric acid	–	NF, PhEur
	Phosphoric acid	–	NF, PhEur, JPE
	Sulfuric acid	0.02%	NF, JPE
		1.1%	NF, PhEur, JP
		–	–
		1%	NF, PhEur, JP
		0.1%	NF, PhEur, JP
	Sodium borate		
	Sodium bisulfate		
	Sodium carbonate		
	Sodium hydroxide		
Buffering agents (pH control)	Acetic acid	0.2%	NF, PhEur, JP
	Boric acid	37.2%	NF, PhEur, JP
	Citric acid	0.2%	USP, PhEur, JP

(*Continued*)

Table 2 Excipients Listed in Food and Drug Administration Inactive Ingredients Database (*Continued*)

Category of excipient	Ingredient name	Use level in database	Compendial listing(s)
	Phosphoric acid	–	NF, PhEur, JPE
	Potassium acetate	4%	USP, PhEur, JPE
	Potassium phosphate, monobasic	0.44%	NF
	Potassium sorbate	0.47%	NF, PhEur, JPE
	Sodium acetate	1.27%	USP, PhEur, JP
	Sodium borate	1.1%	NF, PhEur, JP
	Sodium carbonate	1%	NF, PhEur, JP
	Sodium citrate	2.2%	USP, PhEur, JP
	Sodium phosphate, dibasic	1.4%	USP, PhEur, JP
	Sodium phosphate, monobasic	0.78%	USP, PhEur, JPE
	Sorbic acid	0.2%	NF, PhEur, JPE
	Tromethamine	0.93%	USP
Humectants/tonicity agents/salts (ionic strength and osmolality control)	Calcium chloride	0.04%	USP, PhEur, JP
	Glycerin	2.6%	USP, PhEur, JP
	Magnesium chloride	0.03%	USP, PhEur
	Mannitol	23%	USP, PhEur, JP
	Polyethylene glycol 300	–	NF, PhEur, JPE
	Polyethylene glycol 400	4.99%	NF, PhEur, JP
	Potassium chloride	22.2%	USP, PhEur, JP
	Propylene glycol	10%	USP, PhEur, JP
	Sodium chloride	55%	USP, PhEur, JP
	Sodium nitrate	1.18%	–
	Sodium sulfate	1.2%	USP, PhEur, JPE
	Sorbitol	40%	NF, PhEur, JP
Ointment base	Lanolin	3%	USP, JP
	Light mineral oil	–	NF, PhEur, JP
	Mineral oil	59.5%	USP, PhEur, JP
	Petrolatum	85%	USP, PhEur, JP
	Petrolatum, white	89%	USP, PhEur, JP
Antioxidants/chelating agents/sequestering agents (chemical stability control)	Citric acid	0.2%	USP, PhEur, JP
	Creatinine	0.5%	NF, JPE
	Divinylbenzene styrene copolymer	0.75%	USP, JP
	Edetate sodium	10%	USP, PhEur, JP
	Sodium bisulfite	0.1%	JP
	Sodium citrate	2.2%	USP, PhEur, JP
	Sodium metabisulfite	0.25%	NF, PhEur
	Sodium sulfite	0.2%	NF, PhEur, JPE
	Sodium thiosulfate	5%	USP, PhEur, JP
	Tocophersolan (Vit E TPGS)	0.5%	NF
Antimicrobial Preservatives	*Quaternary ammonium compounds*		
	Benzalkonium chloride	8.8%	NF, PhEur, JP
		–	USP, PhEur, JP
		0.01%	–
	Benzethonium chloride	0.0005%	–
	Benzododecinium bromide		
	Polyquaternium-1	0.2%	NF, PhEur, JP
		37.2%	NF, PhEur, JP
		4%	USP, PhEur, JPE
		0.47%	NF, PhEur, JPE
		1.27%	USP, PhEur, JP
		1.1%	NF, PhEur, JP
		0.2%	NF, PhEur, JPE
		0.65%	NF, PhEur, JP
		0.5%	USP, JPE
		0.0008%	NF, PhEur
		0.002%	NF, PhEur
		1%	USP, PhEur
		0.05%	NF, JP
		0.01%	NF, JP

(*Continued*)

Table 2 Excipients Listed in Food and Drug Administration Inactive Ingredients Database (*Continued*)

Category of excipient	Ingredient name	Use level in database	Compendial listing(s)
		0.005%	–
		0.0025%	USP, PhEur, JP
	Acid/base compounds		
	Acetic acid		
	Boric acid		
	Potassium acetate		
	Potassium sorbate		
	Sodium acetate		
	Sodium borate		
	Sorbic acid		
	Alcohols		
	Chlorobutanol		
	Phenylethyl alcohol		
	Organic mercurial compounds		
	Phenylmercuric acetate		
	Phenylmercuric nitrate		
	Thimerosal		
	Parabens		
	Methylparaben		
	Propylparaben		
	Oxidizing agents		
	Sodium chlorite		
	Metal salts		
	Zinc chloride		

Note: Compendial listings for ingredients are also noted.

acceptable pH range is partially due to the buffering capacity of the tear fluid, but is also a result of the fact that excursions to high pH can result in saponification of lipids in the tissues and immediately compromise the barrier properties of the tissues (2). to minimize the discomfort caused by a formulation, at the extreme low or high end of this pH range, it is advisable to minimize the buffer capacity of a formulation. For example, a 50 mM pH 5 acetate buffer would be more easily neutralized by the tear fluid than a 50 mM pH 5 citrate, but a 5 mM pH 5 acetate buffer would be even better if it could provide sufficient pH stability for the formulation. For osmolality, reflex tearing is generally not seen in the range of 200 to 400 mOsm/Kg (2,3), but some studies suggest that hypotonic formulations may be better tolerated than hypertonic formulations and have been shown to enhance drug delivery in some instances (3). The osmolality of the formulation may be controlled equally well using electrolytes or nonelectrolytes and a comfortable formulation may be designed using either. In general, it may be preferable to rely on nonelectrolytes for osmolality control and target osmolalities slightly less than that of the tear fluid to avoid aggravating the hyperosmotic conditions that afflict the population of people with evaporative dry eye.

Additional formulation components that may be beneficial for solution formulations include surfactants and viscosity increasing agents. Even if not required for its solubilization, the addition of a small amount of surfactant may help with the wetting and spreading characteristics of the formulation on the surface of the eye. The addition of a viscoelastic polymer to the formulation can also be beneficial in prolonging the retention of the formulation on the surface of the eye. Increasing the viscosity of a solution formulation to 12 to 15 cps was shown to provide optimal benefit—higher viscosities show diminishing improvements in slowing the drainage rate (54).

Ointments

After solution formulations, the next most complicated formulation to design and manufacture is an ointment. Ointment formulations are generally suspensions of drugs in a base of mineral

oil and petrolatum. These formulations are generally suspensions because mineral oil and petrolatum are not good solvents for most drug compounds. The ointment base of petrolatum softens at body temperature and melts between 38°C to 60°C. The melting and softening behavior of the petrolatum base may be modified by the addition of mineral oil or light mineral oil. Ophthalmic ointments typically contain from 40% to 90% petrolatum with 60% to 10% mineral oil. The simplest ointment formulation will have only the active ingredient suspended in this mineral oil/petrolatum base. The active ingredient will need to be suitably controlled with respect to its particle size; hence, the drug substance is typically micronized before addition to the ointment base. The mineral oil/petrolatum base is not supportive of microbial growth (i.e., due to the low water activity), and does not affect the pH or osmolality of the eye, hence, additional excipients are not required.

This simple petrolatum and mineral oil base is the best option for drug substances that are hydrolytically unstable, but the drug delivery from such an ointment may suffer because of the fact that the ointment base is not readily miscible with the tear fluid. To improve the drug delivery characteristics of an ointment, a formulation may include a water-in-oil surfactant such as lanolin. This creates what is termed an absorption ointment base and is intended to improve the drug absorption from the ointment as well as improve the ability to incorporate hydrophilic drugs. The absorption ointment base is more likely to require the addition of a preservative such as chlorobutanol or parabens.

Suspensions

Suspensions are a necessary formulation option for cases where the aqueous solubility of the drug substance is extremely low or when the stability of the drug substance is significantly enhanced by keeping it as a suspended particle rather than dissolved (e.g., to reduce hydrolysis). Suspension formulations have the additional concerns of particle size distribution, sedimentation and resuspendability, and content uniformity of the delivered formulation.

Particle Size Distribution

The particle size distribution in an ophthalmic suspension must be controlled to assure the comfort of the formulation on the surface of the eye as well as assure that the drug delivery characteristics are consistent. Particle size of the active agent also plays a key role in physical stability of the drug product. The rate of sedimentation, agglomeration and resuspendability are affected by particle size. Table 3 lists the current compendial requirements for ophthalmic suspensions.

The most common method for controlling the particle size is mechanical comminution of previously formed larger, crystalline particles (e.g., by grinding with a mortar-pestle, air-jet micronization, or wet-milling with ceramic beads). Another method is the production of small particles using a controlled association process (e.g., spray drying, precipitation from supercritical fluid, or controlled crystallization). The process used to obtain the desired particle size distribution may have significant effects on the properties of the drug product. For example, comminution methods may generate heat that can create amorphous regions or polymorphic changes in the active pharmaceutical ingredient (API) particles which can, in turn, affect dissolution and drug delivery characteristics. In addition, if a change is made from one comminution method to another during the course of development, the API behavior may change significantly. For example, jet-air micronization can result in triboelectrification

Table 3 Summary of Compendial Requirements for Suspension Particles Size Distributions

USP	"It is imperative that such suspensions contain the drug in a micronized form to prevent irritation and/or scratching of the cornea. Ophthalmic suspensions should never be dispensed if there is evidence of caking or aggregation."
EP	NMT 20 particles $>$ 25 μm/10 mcg solid NMT 2 particles $>$ 50 μm/10 mcg solid No particles $>$ 90 μm/10 mcg solid
JP	No particles $>$ 75 μm

(i.e., charging) of the API particles. This charging of the particles may impact the aggregation and processing requirements of the formulation. Because is not practical to fully optimize the method for controlling the particle size independent of the formulation, a formulator must keep in mind how the process may change during the planned development and scale-up activities and be prepared for those necessary changes. The ultimate goal is to develop the formulation and particle size control method that will be used for manufacturing the marketed drug product and, therefore, the earlier this compatibility can be tested and verified, the better.

When formulation research is started, the formulation scientist typically has very little API available for evaluation of particle size methods. Some simple, small-scale experiments may help indicate what particle size control methods are viable options. For example, grinding a small amount of drug substance with a mortar-pestle to evaluate how easily a material can be ground (brittleness) and evaluation of the crystallinity of the drug substance before and after grinding may indicate if comminution methods are viable. Likewise, small-scale experiments with dissolving and precipitating the drug may indicate if a controlled precipitation process will produce a suitable crystalline particle.

Physical Stability (Sedimentation and Resuspendability)

It is important to understand that suspensions are kinetically stable but thermodynamically unstable systems. When left undisturbed for a long period of time the suspension particles will aggregate, sediment, and eventually cake. When a suspension is very well dispersed (i.e., deflocculated), the particles will settle as small individual particles. This settling will be very slow and will result in a low-volume, high-density sediment that may be difficult or impossible to redisperse. When the particles are held together in a loose open structure, the system is said to be in the state of flocculation. The flocculated particles will settle rapidly and form a large-volume, low-density sediment that is readily dispersible. Relative properties of flocculated and deflocculated particles in suspension are provided in Table 4.

The flocculation state of a suspension product is primarily controlled by the nature of the surface of the suspended particles. The surface charge (i.e., zeta potential) of the particle may be adjusted to move between a flocculated and deflocculated state. Also, adsorption of surface active polymers or surfactants can stabilize suspensions by preventing the removal of water from between the particles. A textbook example (55) illustrates how to modify the zeta potential of a suspension to switch between a deflocculated and a flocculated state. First, the adsorption of a cationic surfactant (e.g., BAK) to the surface of a suspended particle provides charge-charge repulsion resulting in a deflocculated suspension. Then, an oppositely charged flocculating agent (e.g., phosphate) is added at increasing levels to shield these surface charges and reduce the zeta potential close to zero, at which point flocculation is observed. A list of the formulation factors that can be adjusted to affect the physical stability of a suspension formulation includes the following:

- Flocculation/deflocculation: (*i*) add charged surface active polymer or surfactant, (*ii*) add an oppositely charged flocculation agent, (*iii*) add a nonionic surface active

Table 4 Relative Property of Flocculated and Deflocculated Particles in Suspension

Deflocculated	Flocculated
Little to no aggregation. Particles are present as primary particles.	Particles form loose aggregates (flocculants).
Sedimentation is slow.	Sedimentation is fast.
Sedimentation volume is small as particles may pack more efficiently.	Sedimentation volume is typically large.
Sediment may become a hard cake that is difficult or impossible to redisperse. Resuspendability is typically poor.	A dense cake does not form. The sediment is easy to redisperse, so as to reform the original suspension. Resuspendability is typically excellent.

polymer or surfactant, (*iv*) adjust the ionic strength of vehicle, and (*v*) if drug has a pKa, adjust pH to modify the surface charge.
- Sedimentation rate: (*i*) increase the viscosity of the vehicle, (*ii*) decrease the particle size of the drug, and (*iii*) develop a structured vehicle, which does not settle.

Content Uniformity in Delivery from the Selected Container/Closure

Another difference between a suspension formulation and a solution formulation is that when a suspension drop is delivered from the controlled-tip dropper bottle, it is not guaranteed to be uniform. Several factors, which may affect the uniformity of the drop delivered to the patient's eye, include compatibility between the formulation and the package, resuspendability of the formulation in the selected package, and the patient's ability to properly resuspend the formulation within the selected package. Typically, patients are not willing to vigorously shake a bottle of suspension for more than a few seconds. In addition, the resuspendability of a suspension formulation may be significantly affected by the material of the container (e.g., polyethylene vs. glass). It is advisable that careful, early evaluation of the resuspendability of suspension formulations be performed under simulated use conditions in the selected container/closure system to identify and fix physical stability issues as early as possible. An evaluation like this should indicate that a drop delivered from the selected package will have the appropriate potency (e.g., 90–110% of label) when delivered according to the label instructions.

Emulsions

Although emulsion formulations are not very novel and have been used extensively in topical (dermatological) and oral delivery routes, there are currently only two marketed formulations for ophthalmic use [Restasis® (cyclosporine emulsion in castor oil) and Durezol® (difluprednate emulsion in castor oil)]. The potential advantages of emulsions for ophthalmic drug delivery include being able to provide a greater driving force for drug delivery of low solubility compounds and being able to eliminate many of the quality control issues associated with suspended drug particles. The disadvantages of the emulsion formulations are that they have proven to be difficult formulations to preserve and difficult to manufacture under sterile conditions. These disadvantages are being overcome and there will undoubtedly be many new ophthalmic emulsions brought to the market over the next several years.

The emulsion formulation has an aqueous continuous phase that must comply with the same requirements as the solution formulations discussed above. In addition to the aqueous continuous phase, the emulsion formulation contains an oil (lipid) phase, which is dispersed in the continuous phase with suitable emulsifiers. The oil phase for an emulsion should be selected to provide adequate solubilization of the drug substance. The oil-in-water emulsifiers may include surfactants (e.g., polysorbate 80 or polyoxyl 35 castor oil), Carbomer 1342, or both.

The difficulty in preserving an emulsion formulation is evident from the fact that most of the antimicrobial preservatives readily available for ophthalmic use are incompatible with some aspect of the emulsion formulation. The emulsion formulation generally contains either high levels of surfactants or Carbomer 1342. High levels of surfactant can deactivate parabens, BAK, alcohols, and organic acids. Carbomer 1342 is an anionic polymer that may interact strongly with the quaternary amines. In addition, any surface active or lipophilic preservatives may partition into the oil phase and become unavailable for preservation of the aqueous phase. In the Restasis formulation, the preservative problem was solved by designing a preservative-free, single-dose formulation. For the Durezol formulation, the use of a combination of three water-soluble antimicrobial acids (sorbic, acetic, boric) provided sufficient preservative efficacy.

Enhanced Drug Delivery Systems

After topical administration, typically less than 5% of the applied drug penetrates the cornea and reaches intraocular tissues. The primary problem for topical delivery of ophthalmic drugs is the rapid and extensive precorneal loss caused by drainage and high tear fluid turnover. A major portion of the formulation efforts have been aimed at maximizing ocular drug absorption through prolongation of the drug residence time in the cornea and the conjunctival sac. Improved ocular residence of liquid formulations has been accomplished through the use

of viscosity-increasing and mucoadhesive agents, *in situ* gelation of the formulation, and use of charge-charge interactions between cationic components in the formulation and the anionic surface of the eye. Even greater residence improvements can be made by using polymeric inserts for drug delivery. Enhanced drug delivery from these formulations may allow the treatment of posterior indications with topical administration. In addition, various polymers may be used to produce prolonged-delivery systems which allow less frequent injections for posterior treatment.

High-Viscosity Liquid Formulations

A high-viscosity formulation can improve the retention of a drug substance on the surface of the eye, however, if the viscosity is too high under the shear conditions of the closing eyelid (about 20,000/sec) it may cause discomfort and reflex tearing (56). Many commonly used viscosity-increasing agents result in Newtonian viscoelastic behavior so that the viscosity increases similarly at both low and high shear. Polymers that thicken this way include hydroxypropylmethyl cellulose (HPMC), hydroxyethylcellulose (HEC), polyvinylpyrrolidone (PVP), and polyvinyl alcohol. Patton, et al., (54) found that increasing the viscosity to about 12 to 15 cps using either HPMC or PVA resulted in significant improvement in ocular retention whereas further increases in viscosity resulted in only small improvements. Other polymers may be used to produce non-Newtonian viscoelastic fluids that are either shear thinning or thixotropic. Polymers resulting in shear-thinning behavior include Carbomers and sodium carboxymethylcellulose. Polymers that shear thin more dramatically and can be considered thixotropic include polycarbophil and xanthan gum. In the AzaSite® (polycarbophil suspension of azithromycin) formulation, the polycarbophil creates a low-shear viscosity of over 2000 cps, but the formulation is still well tolerated in the eye because the viscosity of the formulation during the eye blink is much less (i.e., less than 300 cps).

Mucoadhesive Liquid Formulations

Mucoadhesion refers to the tendency of a polymer to specifically bind with the mucins of mucus membranes and lead to enhanced retention or viscosity as a result of the polymer-polymer interactions. The mucoadhesive performance of the ophthalmically-used polymers can be qualitatively ranked as follows (12): carbomers, polycarbophil > hyaluronan > carboxymethylcellulose sodium > sodium alginate > poloxamers, HPMC, methylcellulose, PVA, PVP.

Examples of formulations taking advantage of the ability of mucoadhesion to enhance the retention of a formulation include the Pilopine HS® (Carbomer 940 gel of pilocarpine HCl), and Alphagan® P (NaCMC solution of brimonidine tartrate).

In Situ Gelling Liquid Formulations

In situ gelation can be induced on the surface of the eye because of the change in pH, temperature, or ionic strength that occurs after the formulation is administered and mixes with the tear fluid. The change in pH can be used to induce *in situ* gelation between borates and polyol-containing polymers. The OTC dry-eye treatment Systane® takes advantage of the gelation between borates and HP-Guar as the pH is increased after administration. *In situ* thermal gelation with poloxamers has also been investigated, but is not currently applied in any marketed products. The gelation induced by interaction with the salt content of the tear fluid is used by the Timoptic-XE® (timolol maleate solution in gellan gum) product.

Cationic Liquid Formulations

Because the surface of the eye is generally anionic, the application of cationic drugs or drug delivery systems should interact electrostatically with the mucins on surface of the eye and lead to enhanced retention. Some formulations demonstrating this approach include cationic nanoparticles, cationic emulsions, and formulations using of cationic suspending or mucoadhesive agents. Nanoparticles may enhance delivery of poorly water-soluble drugs, but without improved retention on the eye nanoparticles are unlikely to result in delivery

superior to a solution. Preparation of cationic nanoparticles can be accomplished using either cationic Eudragit® polymers, chitosan polymers, or by incorporating cationic surfactants into solid-lipid nanoparticles (57). Chitosan polymers and cationic cellulosic polymers (e.g., polyquaternium-10) have also been used as cationic suspending agents and have been shown to provide good mucoadhesion properties (12). Cationic emulsions have been prepared by incorporating cationic surfactants at the solid-liquid interface of the emulsion to enhance drug delivery (58).

Prolonged-Delivery Polymeric Systems

Topical eye drop administration is mainly suitable for treatment of ocular conditions in the anterior segment of the eye. Targeting the posterior segment of the eye presents a far greater challenge and represents an area of unmet medical needs. Many of the newer drugs aimed at treating conditions such as diabetic retinopathy and age-related macular degeneration are administered via repeated intravitreal injections. Alternative approaches that would improve patient acceptance such as biodegradable inserts or micro- and nanoparticulate delivery systems present a growing field in the area of ophthalmic drug delivery.

Controlled release of drugs can be obtained by encapsulating the drug in micro-(1–10,000 μm) or nano(1–1000 nm)particles. These are usually given as intravitreal injections. They can provide sustained delivery over few weeks up to several months (59). However, the intravitreal injections of these particulates can cause vitreal clouding. Microparticles tend to sink to the lower part of the vitreal cavity, while nanoparticles are more susceptible to cause clouding in the vitreous (59).

Biodegradable and biocompatible polymers such as polylactide and PLGA [poly-(lactic-co-glycolic acid)] (both approved by the FDA) are typically used. In these materials, the drug is released by bulk erosion of the matrix following cleavage of the polymeric chains via autocatalytic acid/base and/or enzymatic hydrolysis; the products lactic and glycolic acids, are metabolized to carbon dioxide and water. Low molecular weight polymers tend to degrade rapidly; copolymers such as PLGA degrade faster than the corresponding homopolymers. Some microsphere formulations have shown promise in preclinical studies but have yet to undergo clinical trials. A microsphere formulation of PKC412 (protein kinase C inhibitor + receptors for VEGF) was administered via pericoular injection to treat choroid neovascularization. The studies showed a significant suppression of neovascularization using this delivery system.

Poly(anhydrides) and poly-(ortho-ester)s are also promising polymers for drug delivery; their release properties are regulated mainly by surface erosion rather than diffusion (60). Poly (orthoester)s have shown excellent ocular biocompatibility and have been used to demonstrate the sustained release of 5-fluorouracil (61).

Nanoparticulates are of importance since colloidal delivery systems are particularly suitable for poorly water-soluble drugs. However, the major impedence to the use of nanoparticles has been the availability of a universally acceptable method of making the nanoparticles especially on large scale and the stabilization and sterilization of the formulations. Some nanosytems based on surface-charge segregated particles containing chitosan or polyethylene glycol have been found to be stable and also in overcoming preclinical barriers.

Intraocular implants are usually placed intravitreally, at the pars plana of the eye and therefore, requires minor surgery. However, the use of implants have the benefit of by-passing the blood-ocular barriers to deliver constant therapeutic levels of drug at the site of action, avoidance of repeated administration and use of smaller doses of drugs (62). Implants may be nonbiodegradable or biodegradable depending on the material from which they are fabricated. Biodegradable implants of a poly (DL-lactic-co-glycolic acid) implant containing a novel aldose reductase inhibitor, fabricated with 50% drug loading have been shown to give sustained drug release in vitro and in vivo in rats (63). Nonbiodegradable implants provide more accurate/reproducible dosing lasting over longer periods of time than biodegradable inserts (62). The nonbiodegradable implants however, require surgical removal after completion of therapy. Vitrasert® and Retisert® (Bausch and Lomb) are two clinically used nonbiodegradable implants for the treatment of CMV retinitis (AIDS-related) and chronic uveitis, respectively.

Other implant systems in different phases of clinical trials include Medidur® (Alimera Sciences) for treatment of diabetic macular edema; Surodex® and Posurdex® (Allergan, U.S.A.) containing dexamethasone.

Transporter-Mediated Drug Delivery

Transporter-mediated drug delivery involves targeting of drug molecules to the membrane transporters to enable efficient passage across the cell membranes. Various transporters may be utilized to facilitate the passage of drugs across cell membranes (64); these include nutrient transporters for peptides, aminoacids, monocarboxylic acids, folates and organic anion and cation transporters, etc. Various peptide and amino acid transporters have been utilized for retinal drug delivery. Majumdar et. al. have studied the role of various dipeptide prodrugs of gancyclovir to improve its ocular bioavailability after topical administration and found good corneal permeability with a Val-Val dipeptide gancyclovir prodrug (65). The approach of using various transporter mechaninsms in the eye for improved intraocular delivery following topical administration, is interesting and provides newer opportunities for ophthalmic drug delivery.

Intraocular Irrigation Solutions

An ophthalmic irrigation solution is used for the application on the external surface of the eyes topically and in ocular surgeries to rinse, as well as to keep the operated ocular tissues moist. Replacement of the aqueous or vitreous humors with the irrigation solution occurs as the consequence of ocular surgeries including corneal transplant (penetrating keratoplasty), cataract extraction, intraocular lens implantation and vitrectomy. In these instances, the irrigation solution remains in the eyes after surgery until the components are either deprived by the surrounding tissues or the solution is eventually equilibrated with body fluids, with subsequent clearance through the circulation. Thus, it is essential that the irrigation solution used should be physiologically compatible, including tonicity and pH, and desirably should also contain components enabling the cells to sustain their viability and capability to perform physiological functions.

Irrigation solutions used during and after surgery are of particular importance to the cornea and the lens. Both organs are avascular. The cornea obtains its nourishment mainly from the fluid in the anterior chamber, and to a lesser extent, from the tear. The lens obtains its nourishment from fluids, both in the anterior chamber and in the vitreous. The retina, ciliary body and iris are vascularized tissues; they obtain their nourishment through the circulating plasma of the blood vessel network. Therefore, the components of the irrigation solution may not exert an effect on these tissues as significant as that on the cornea and the lens. A proper electrolyte balance as well as addition of certain nutrients such as glucose, amino acids, etc., may add to the beneficial nature of an irrigation solution. Often irrigation solutions are used to simply bathe and soothe the eye and help wash away impurities and contaminants from the environment. There are two intraocular irrigation solutions presently being used in ophthalmic surgeries. These two irrigation solutions are BSS and BSS Plus (both by Alcon Labs Inc.). BSS is a balanced salt solution that incorporates a sodium citrate of a balanced salt solution with a bicarbonate buffering system, with Dextrose added as an additional osmotic agent and energy source. An additional component, oxidized glutathione is reduced by the ocular cells and serves as an antioxidant. In addition, some intraocular irrigation solutions may contain viscoelastic components or viscosity enhancers such as sodium hyaluronate, chondroitin sulfate, hydroxypropylmethyl cellulose, and polyacrylamide. However, the use of these agents may lead to an elevation of IOP (66).

PRESERVATION OF OPHTHALMIC FORMULATIONS

Ophthalmic formulations must not only be sterile products but need to be adequately preserved from microbial contamination once the package is opened. Most ophthalmic products are multidose products packaged in semi-permeable containers. The repeated opening and closing of the containers as well as frequent contact with the ocular surface (e.g., for dropper tips) exposes the contents of the package to a variety of microorganisms from

the external environment. Many of the microorganisms can cause severe reactions (inflammation, itching, pain, loss of visual acuity, etc.) including, in the most severe cases, blindness. The choice of the preservative is dictated by the nature of the formulation itself, whether it is a suspension, solution or gel system. Often the choice of buffer/vehicle composition will also affect preservative efficacy. It is well known that the borate buffer system itself has good antimicrobial properties (67) and can help boost the antimicrobial efficacy of some preservatives. Additionally it is known that high salt concentrations can decrease preservative efficacy. The specific composition of the formulation not only affects the efficacy and stability of the preservative system but, may also alter the tolerability of the preservative system. For example, incorporation of viscosity-increasing agents can increase the irritation potential of a preservative because of increased residence on the eye—this has been demonstrated in BAK-containing systems with hydroxyethylcellulose (68). In addition the incorporation of surfactants and polymers that bind the preservative(s) will result in decreased antimicrobial efficacy. The use of preservatives in chronic-use products such as antiglaucoma and dry-eye medications is of concern because of the cumulative toxicity of certain agents on the corneal epithelium (69). Thus, such medications should ideally be preservative-free or contain preservatives that have little to no chance of accumulating in ocular tissues.

Antimicrobial Effectiveness Testing

Antimicrobial effectiveness testing (AET) is used to ensure that a product is adequately protected from microbial contamination during patient use. The AET method is described in the major compendia—the USP in chapter <51> (70), the PhEur in chapter 5.1.3, and the JP in chapter <19>. The bacterial challenge organisms used in the AET are *Escherichia coli* (ATCC8739), *Pseudomonas aeruginosa* (ATCC9027) and *Staphylococcus aureus* (ATCC6538), and the yeast/mold challenge organisms are *Candida albicans* (ATCC10231) and *Aspergillus brasiliensis* (ATCC16404). Criteria for the effectiveness of a preservative system are expressed as the percentage of reduction in viable cells in a specific amount of time. At this time, there is not one harmonized criteria that is accepted globally for product preservation. Table 5 summarizes the criteria that are most widely used for antimicrobial preservative testing—from the USP, PhEur, and JP. The PhEur criteria are the most stringent among the three and guide the development of globally-acceptable pharmaceutical formulations.

Preservatives Used in Ophthalmic Formulations

There are a wide variety of agents that alone or in combination with each other can act to effectively reduce the chances of contamination of a formulation by microbial growth. The section below addresses some of the more widely accepted ophthalmic preservatives that are used today. Many previously used preservatives such as the organic mercurial compounds (e.g., thimerosal) have seen a decline in use because of evidence of hypersensitivity and ocular toxicity upon long-term use (71,72).

Table 5 Criteria of Acceptance for Antimicrobial Effectiveness Testing (USP Category "1" Products, PhEur Parenteral and Ophthalmic Formulations, and JP Category IA Products)

		Log_{10} reduction				
	Innoculum (CFU/mL)	6 hr	24 hr	7 days	14 days	28 days
USP: bacteria	10^5–10^6	–	–	1.0	3.0	No increase[a]
JP: bacteria	10^5–10^6	–	–	–	3	No increase
PhEur-A: bacteria	10^5–10^6	2	3	–	–	No recovery
PhEur-B: bacteria	10^5–10^6	–	1	3	–	No increase
USP: yeast/mold	10^5–10^6	–	–	No increase	No increase	No increase
JP: yeast/mold	10^5–10^6	–	–	–	No increase	No increase
PhEur-A: yeast/mold	10^5–10^6	–	–	2	–	No increase
PhEur-B: yeast/mold	10^5–10^6	–	–	–	1	No increase

[a]No increase implies no decrease in the log reduction values for microbial growth from previous time point.

Quaternary Ammonium Compounds

Quaternary ammonium compounds are small, positively-charged molecules. It is believed that they act by perturbing the cell membrane of gram-positive and gram-negative bacteria specifically via intercalating into the lipid bilayers and displacing ions, such as calcium and magnesium, that play a crucial role in stabilization of the bacterial cytoplasmic membrane (73). These agents can interact with the teichoic acid and polysaccharide elements in gram positive bacteria and the lipopolysaccharide element in Gram-negative bacteria. It is believed that chelating agents such as ethylenediamine tetraacetic acid or EDTA and ethylene glycol tetraacetic acid or EGTA (used in concentrations from 0.01–0.1% w/w), further potentiate the antimicrobial effect of these agents. The most commonly used agent in this category is benzalkonium chloride (BAC or BAK) followed by cetyltrimethyl ammonium bromide (cetrimide). These agents are usually used in concentrations from 20 to 200 ppm, have good ocular tolerability profiles, good stability, and a long history of use. They are incompatible with high concentrations of anionic components or surfactants in a formulation. Although widely used, it is generally agreed that there may be concern regarding the cumulative toxicity of these agents when present in chronic use products such as dry-eye medications and antiglaucoma medications. Therefore, there is a growing preference for other, more gentle antimicrobials.

Polyquaternary Ammonium Compounds

Polyquaternium is the International Nomenclature for Cosmetic Ingredients designation for several polycationic polymers that are used in the personal care and pharmaceutical industry. Polyquaternium is a generic term used to emphasize the presence of multiple quaternary ammonium centers in the polymer. INCI has approved at least 37 different polymers under the polyquaternium designation. Because of their large size, they are generally thought to be less permeable across the corneal epithelium and, hence, pose less risk of accumulation in ocular tissues leading to chronic toxicity issues. Their mode of action is similar to the monoquaternary compounds in that they also destabilize the outer membrane of bacteria and cause leakage of intracellular components leading to cell death (73).

Two commonly used polyquaterniums are polyquaternium-1 (PQ-1) and polyquaternium-42 (PQ-42) and the molecular weight of these compounds can go up to several thousand Daltons. Chemically, PQ-1 is ethanol, 2,2′,2″-nitrilotris-, polymer with 1,4-dichloro-2-butene and *N*,*N*,*N*′,*N*′-tetramethyl-2-butene-1,4-diamine; typically, it has an average molecular weight of around 6 kDa. It can be used in concentrations of 1 to 10 ppm and its efficacy against yeast and fungi is improved at higher pHs. Chemically, PQ-42 is [polyoxyethylene(dimethylimino) ethylene-(dimethylimino)ethylene dichloride]. It has been used in ophthalmic formulations such as Freshkote, Dwelle, and Dakrina eye drops and Nutra-tear. It is also used in a lens care solution for rigid gas-permeable (RGP) lenses (Total Care CLS by AMO) at a concentration of 6 ppm by weight. PQ-1 is a more potent antimicrobial agent than PQ-42.

Biguanides and Polymeric Biguanides

Biguanides refer to the class of compounds that are derivatives of imidodicarbonimidic diamide. The most commonly known biguanide is chlorhexidine [1,6-bis(4′-chloro-phenyl-biguanide)hexane; usually used a its digluconate salt] which has a broad spectrum of activity. However, its action is pH dependent and greatly reduced by the presence of organic matter. It can only be used in very low concentrations in ophthalmic formulations because of its irritation potential. Chlorhexidine is believed to exert its action by membrane destabilization leading to the leakage of intracellular components; at high concentrations it can cause protein and nucleic acid precipitation (74). It is generally used at concentrations of 5 to 10 ppm by weight. Because of its weak activity against yeast, fungi, and *Serratia marcescens*, it is usually used in combination with other agents such as EDTA, BAK, etc.

Polymeric biguanides are also available, the most widely used one being polyaminopropyl biguanide or PAPB (also known as polyhexamethylene biguanide or PHMB, or polyhexanide) and is commercially available under the trade names of Cosmocil and Vantocil. PAPB has a broad spectrum of activity and can be used in concentrations as low as 0.5 ppm up

to 5 ppm. Lower concentrations may be used in combination with other antimicrobial agents. PAPB activity is reduced by anionic polymeric agents such as hyaluronic acid, carboxymethylcellulose, alginates, etc., and cellulosic polymers.

Alcohols

Phenylethyl alcohol and chlorobutanol are antimicrobial alcohols. Phenylethyl alcohol (up to 0.5%) is usually used in combination with another preservative but is limited in its application because of its volatility and tendency to permeate through plastic packaging. Chlorobutanol is a commonly used ophthalmic preservative and is generally considered to be quite safe (75). It is mostly used in ophthalmic ointments because it has good solubility in petrolatum. It can be used at concentrations up to 0.5%, but it is unstable at pH > 6, high temperature, susceptible to absorption into packaging components and may be lost through the headspace of semi-permeable packaging because of its volatility.

Parabens

Parabens are esters of p-hydroxybenzoic acid. They have been widely used in pharmaceuticals and as ophthalmic preservatives. They have a well established safety and tolerability profile. The useful concentration is typically limited by the water solubility, and therefore a combination of parabens can be used together to enhance their activity (e.g., 0.05% methylparaben + 0.01% propylparaben). Parabens are effective in the pH range of 4 to 8, but they are more susceptible to hydrolysis at high pH. Parabens may permeate into packaging components, and may be inactivated by high concentrations of surfactants or polymers.

Acids

Antimicrobial acids have a useful pH range around the pKa of the acid and the optimal antimicrobial activity will typically occur very close to this pKa. The most commonly used acid for preservation of ophthalmic formulations is sorbic acid (or potassium sorbate) which has a pKa of 4.76. Sorbic acid is primarily antifungal, but does have antibacterial activity. Sorbic acid is useful in the range of pH 4.5 to 6 and is usually combined with EDTA or other preservatives for broad-spectrum preservation. Sorbic acid is sensitive to oxidation, which results in discoloration of the product, and is more rapidly degraded at temperatures above 38°C. Boric acid is another useful acid for preservation in ophthalmic formulations; however, its activity is classified as bacteriostatic rather than biocidal.

Oxidizing Agents

Oxidizing agents are generally deemed much safer and well tolerated than most other preservatives because the preservatives "disappear" over time and pose little or no chance of accumulation in ocular tissues over repeated use. The two most widely used preservative systems in this category are stabilized hydrogen peroxide systems and hypochlorites. Hydrogen peroxide provides its antimicrobial action via generation of the hydroxyl radical which can readily attack bacterial cell membrane lipids and intracellular DNA (76). Hydrogen peroxide is effective against a wide variety of microorganisms and relatively unaffected by pH. Aside from hydrogen peroxide itself, other peroxide-generating compounds that are useful include sodium perborate, percarbonates and carbamate peroxide. The use of hypochlorites in ophthalmic formulations was introduced in 1996. The stabilized oxychloro complex (SOC) (i.e., Purite) is a hypochlorite preservative consisting of 99.5% chlorite; 0.5% chlorate and a trace amount of chlorine dioxide. The formation of chlorine dioxide in the microbial acidic environments leads to disruption of protein synthesis. However, the components of the preservative system dissipate readily in the eye into components already found in human tears (Na^+, Cl^-, O_2, and H_2O).

CONTACT LENS CARE SOLUTIONS AND REWETTING DROPS

Contact lenses may be rigid gas-permeable lenses (RGP) or soft contact lenses. to properly use contact lenses, they must be kept clean and free from microbial contamination when stored. Contact lens solutions are mainly multipurpose solutions (MPS) that achieve

cleaning, disinfection, and lubrication (for insertion comfort) all in one step. The development of new contact lens multipurpose solution compatible with an increasing array of soft contact lens materials on the market is very challenging. In addition to being able to effectively clean and disinfect the contact lenses, solutions are required to provide patient comfort when the cleaned lens is inserted back into the eye. The products must also maintain their ability to effectively clean and disinfect when stored in unopened containers over a period of 18 months to two years. An even greater challenge is designing a product that is robust enough to counter noncompliance of patients in their contact lens cleaning regimens (77) where compliance requires discarding opened solutions after three months; changing the contact lens cleaning case and never resoaking lens in previously used solution. The market needs are constantly evolving such that there is a continuous need for newer and better products. Biocidal efficacy is tested against five organisms (three bacteria: *Pseudomonas aeruginosa*, *S. aureus*, and *S. marcescens*; one yeast: *C. albicans*; and one fungus: *Fusarium solani*) in the presence of organic soil (required for United States and not for Europe) with a defined (e.g., four hours) exposure time. At least a 3 log reduction in CFUs per mililiter for the bacteria and 1 log reduction in CFUs per mililiter for the yeast and fungi are required (initially and throughout shelf-life) to be considered as passing the biocidal efficacy testing (78).

The key components of MPS are: surfactants or cleaning agents (such as the block copolymers Tetronic 1107 or 1304), lubricating agents (e.g., hyaluronic acid, hydroxypropyl guar, cellulosic polymers), disinfectants (viz., PQ-1, PAPB, SOC, sodium perborate, PQ-42), chelators (e.g., EDTA or hydroxyalkylphosphoates) and other agents that help in moisture retention (e.g., dextran, glycerin). In addition, buffers, electrolytes and stabilizers (e.g., antioxidants) are also included. The ionic strength of the formulations is of particular importance in the formulations because of the significant effect on lens shrinking and swelling. Preservatives can also be taken up by the lenses resulting in changes in lens dimensions.

Contact lens rewetting drops contain a suitable wetting agent (surfactant), an ocular demulcent, a preservative system in a suitable vehicle containing buffers, electrolytes and stabilizers. Rewetting drops help relieve symptoms of ocular discomfort (dryness, foreign body sensation, itching, blurry vision, etc.) in contact lens wearers during use.

MANUFACTURING AND PACKAGING OF OPHTHALMIC FORMULATIONS

Sterile Manufacturing

In 1953, the FDA announced that all ophthalmic products must be manufactured sterile (79). The sterility requirements for ophthalmic formulations first appeared in USP XVIII, third supplement, 1972. In general, ophthalmic formulations are described in the USP as "sterile dosage forms essentially free from foreign particles suitably compounded and packaged for instillation in the eye" (80). The formulations should be terminally sterilized by autoclaving whenever possible. As an alternative to steam sterilization, formulations may be sterilized by sterile filtration through 0.22-μm filters. If neither steam sterilization nor filtration is an option, then aseptic processing of presterilized components is required (81). The requirements and guidance for the compounding of sterile preparations is outlined in detail in USP <797>. In addition to the quality of raw materials and packaging components, and the condition of manufacturing components, a major factor in ensuring the quality of the final product is the environment in which it is manufactured and filled. For ophthalmic formulations, manufacturing must be carried out in an ISO class 5 (previously class 100) environment. There are also high standards that are described with regard to personnel garbing and gloving; personnel training and testing in aseptic manipulations, environmental quality specifications and monitoring and disinfection of gloves and surfaces. Formulation compounding may involve several steps rather than the simplified idea of putting all ingredients into a sterilized mixing vessel and mixing. In many cases the manufacturing may consist of a multistep process where the thermostable portion of the formulation is autoclaved and then the heat sensitive components are added aseptically (through a sterile filter) to the autoclaved portion (after cooling down to acceptable temperatures). When

developing a formulation it is very important to focus on the following general points to establish a manufacturing process that will be scaleable, reproducible, and cost and time efficient.

1. The order of addition of the components in the formulation.
2. The time (and temperature) required for mixing and type of mixing that may be desirable.
3. Possible interaction of formulation components with the manufacturing components, including tubing, filters, filter housing, cleaning agents that may be used to clean manufacturing components, sources of trace metal contamination, etc.
4. Filter choice is of particular importance when dealing with potent drugs and preservatives since these are prone to significant loss because of binding by the filter. In such cases several developmental batches may be necessary to determine the flush and discard volume (prior to beginning of the filling operation) to saturate the filters and minimize losses to the filter. Often a certain overage is included in the formulation to account for losses during manufacturing.

Other things to focus on are formulation specific, that is, different manufacturing requirements for suspensions/emulsions, regular solutions versus viscous formulations (gels), ointments, etc. Below are two examples of formulations requiring special compounding procedures for manufacturing and filling.

Manufacturing Example 1: an Aqueous Solution Sterilized by Filtration
The active ingredient, a lipid-soluble drug substance, was weighed into a glass vial with a calculated overage to compensate for loss to filters and the process surfaces during manufacturing. A cationicpreservative, which also serves as a solubilizer for the drug, was added as a concentrate solution to a glass beaker. The drug was transferred from the glass vial into the glass beaker with rinsing and this drug/preservartive concentrate was mixed thoroughly for a sufficient length of time. In a large stainless steel manufacturing vessel the other formulation ingredients were dissolved with constant stirring in $\sim$80% of the water for injection (WFI) for the batch. After all ingredients were dissolved, the concentrated premix solution was quantitatively transferred to the manufacturing vessel and the solution in the vessel was stirred continuously. In-process pH adjustment was performed and the formulation brought to its final weight with WFI. The filling operation was performed in an ISO class 5 environment. The filling line had a 5-μm pore size clarifying filter followed by two, serial, 0.22-μm sterilizing filters. Several liters of formulation were purged through the filling lines and filter assembly to saturate the filters with drug and preservative before the formulation was filled into presterilized plastic bottles. Presterilized tips were inserted and presterilized caps were applied in the ISO class 5 environment. Additional labeling and packaging was performed in an ISO class 7 manufacturing environment.

Manufacturing Example 2: Sterile Addition of a Drug Suspension to an Autoclaved Gel
A drug having very low solubility was suspended in a Carbomer gel to enhance drug delivery. The Carbomer was first dispersed, in an ISO class 7 manufacturing environment, at a high concentration in a clean compounding vessel using high speed homogenizers. The Carbomer phase was then transferred using a diaphragm pump to the manufacturing vessel in an ISO class 5 manufacturing environment. The Carbomer phase was autoclaved in the manufacturing vessel with continuous mixing and then cooled to $\sim 40^{\circ}$C. The micronized drug substance, which was sterilized by γ irradiation, was aseptically added, with continuous mixing, to the Carbomer phase in the ISO class 5 environment. Approximately 50% of the water in the formulation was used to dissolve the chlorobutanol preservative and this solution was sterile filtered through a 0.22-μm filter into the manufacturing vessel. The pH was adjusted to 4.5 using autoclaved sodium hydroxide to produce a thick gel. The final formulation was aseptically filled into presterilized tubes and closed. The final labeling and packaging operations were completed in an ISO class 7 manufacturing environment (82).

Table 6 Cap Color Coding for Ophthalmic Products

Therapeutic class	Color
β-blockers	Yellow, blue, or both
Mydriatics and cycloplegics	Red
Miotics	Green
Nonsteroidal anti-inflammatory drugs	Grey
Anti-infectives	Brown, tan
Carbonic anhydrase inhibitors	Orange
Prostoglandin analogs	Teal

Packaging

Packaging of ophthalmic formulations is very important since the shelf-life of a product is inherently tied in with packaging choice in many cases. The vast majority of ophthalmic formulations (except the injectable and specialized delivery systems) are packaged in polyolefin containers predominantly high-density polyethylene (HDPE), LDPE (low-density polyethylene), polypropylene (PP) and may also include materials such as polyethylene terephthalate (PET). Topical eye drops are typically packaged in 5 to 15 mL LDPE or HDPE bottles with tips that can be of linear low-density polyethylene (LLDPE) or HDPE or PP and caps that are usually HDPE or PP. LDPE is generally preferred for eye drop bottles because of their pliability which affects the ease with which a drop can be dispensed. The quality of the product may be affected by additives in the polymer which may interact with formulation components (e.g., binding of preservatives and actives, formation of insoluble complexes resulting in haze over time, etc.) or they may appear as contaminants in the form of extractables and leachables. Extractables and leachables may also be contributed by labels and secondary packaging components such as cartons and package inserts. The FDA is highly sensitive to the presence of extractables and leachables in ophthalmic products. To ensure the best quality of the product, bottles have some form of tamper evident seal. All primary packaging components must be sterile. Sterilization of plastics may be by ethylene oxide vapors (typically for LDPE and PP) or by γ irradiation (HDPE, LLDPE) and the sterilization method for packaging components must be validated. In blow-fill-seal (BFS), or form-fill-seal, operations product is filled into the bottle as it is being formed (in a sterile environment); because of the high temperature of the polymer as it is molded, it is assumed to be sterile and no further sterilization of the end product is generally required. The dropper tips may be molded as part of the operation or separate preformed, presterilized tips may be inserted followed by capping. There are specific color-coding requirements for different ophthalmic drugs as outlined in Table 6.

Preservative-free Multidose Devices

Although the great majority of eye drops are available as preserved multidose formulations in traditional LDPE dropper bottles, there are some patient populations that are sensitive to the presence of preservatives in formulations. That is the reason many formulations are also packaged as "unit-dose" or single-use vials. These are usually small volumes (0.5 mL or less) in LDPE form-fill-seal containers with twist off caps. Once opened these containers can not be stored beyond a single day of use and need to be discarded because of risk of contamination. The unit-dose presentations are more expensive to manufacture and as such are more costly for patients. As a result the market has seen the advent of preservative-free multidose devices (PFMD). The ABAK® system is a patented preservative free multidose eye drop dispenser (Fig. 6). It contains a 0.2 nylon fiber micro membrane that filters the solution. The pressure exerted causes the solution to pass through the antibacterial filter in the ABAK® system, forming a drop that falls from the tip of the dispenser. When pressure is released, the solution is reabsorbed and filtered from bacteria and air, ensuring the protection of the solution throughout its use. The ABAK® system filter provides a double protection: without using preservatives, it protects the solution inside the bottle from microbial contamination. The system has been used for the delivery of preservative-free timolol formulations to the eye and

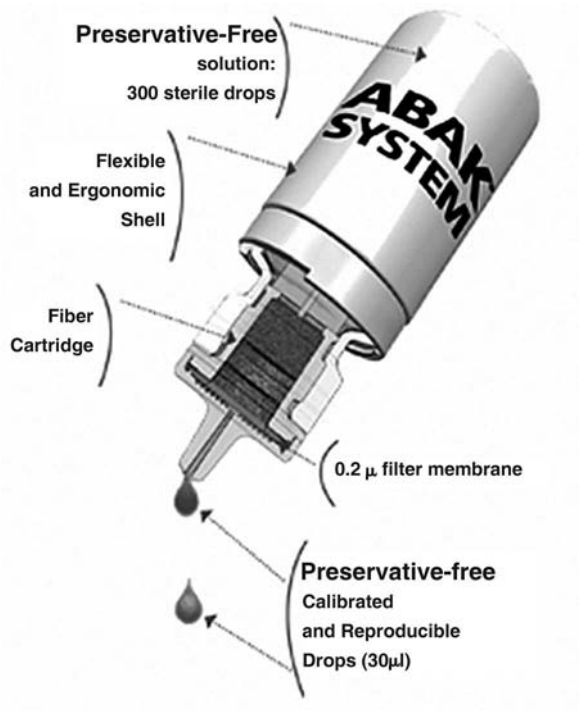

Figure 6 A preservative-free delivery device: ABAK®. *Source*: From Ref. 85.

is available in certain markets under the trade name of Timabak (Thea, France; Nitten Pharmaceutical Ltd., Japan). Administration of timolol via Timabak® showed marked decrease in cytotoxicity in human corneal epithelial cells when compared with preserved formulations containing 0.2% benzalkonium chloride (83). Alternatively, devices which employ a valve-mechanism that prevents the suction of air back into the container, which could contain bacteria, can also be employed for multiuse applications of preservative-free formulations. PFMDs have recently been used for several dry-eye therapies including Artelac-advanced-MDO and Hycosan® (both marketed by Bausch and Lomb) and Hylo-Comod® (Ursapharm). With growing regulatory and safety concerns regarding the use of preservatives in ophthalmic formulations, especially those intended for chronic use, PFMDs are likely to gain more popularity.

Regulatory Pathways for Ophthalmic Formulations

Ophthalmic New Drug Applications

The cost, monetarily and in time, for developing a new ophthalmic formulation will be determined primarily by the number and complexity of in vivo studies required. These regulatory requirements, as well as the potential market exclusivity of the new formulation, may influence whether a decision is made to develop a generic formulation, a new formulation, or to not develop a formulation at all. The benefit of developing a generic formulation is that a product may be developed without any in vivo studies, or, possibly, with one small in vivo study. Typical development time for a generic formulation is two to four years. Once approved, the generic formulation may then be prescribed for any indications for which the reference-listed drug (RLD) is approved. This pathway has very little risk with regards to safety/efficacy of the active, little risk with regards to clinical efficacy, and moderate risk with regards to regulatory approval. However, this pathway results in no market exclusivity except for the potential 180-day market exclusivity granted to the "first-to-file" generic developer when the patent has expired. Alternatively, if faced with the prospect of performing in vivo studies to demonstrate clinical efficacy of a new formulation in a particular indication, it may make more sense to produce a new, nonequivalent formulation of a

Table 7 Summary of Regulatory Pathways, Risks, and Costs for New Formulation Development

	Generics	Supergenerics	Proprietary
Filing	ANDA 505(j)	NDA 505(b)(2)	NDA 505(b)(1)
Exclusivity	None or 180 days	3 yr	Composition or application patent
Cost	Low	Medium	High
Time	2–4 yr	3–5 yr	10–12 yr
Development	0.5–1.5 yr	0.5–1.5 yr	1–3 yr
Animal safety	–	0–0.5 yr	3 yr
Human safety	–	0–1 yr	1 yr
Human efficacy	0–1.5 yr	0.5–1.5 yr	3 yr
Regulatory review	1–2.5 yr	1–2.5 yr	2.5 yr
Risks			
Safety/efficacy risk	Low	Low	High
Clinical risk	Low	Medium	High
Regulatory risk	Low	Medium	High
Revenue/margins	Low	Medium	High
Commercial advantage	Price	Benefit to patient/price	Benefit to patient
Required stability data	3 mo real time on 1 batch, accelerated 3 mo	12 mo real time on 3 batches, accelerated 6 mo	12 mo real time on 3 batches, accelerated 6 mo

previously approved drug. Typical development time for this pathway is three to four years, but involves more costly in vivo studies. This regulatory pathway also has little risk with regards to the safety/efficacy of the active, but does have moderate risk with regards to clinical efficacy and regulatory approval. The potential benefit of this development pathway is three-year market exclusivity for the new formulation or new indication. In addition, there is always the potential that the new formulation may be patentable and provide additional exclusivity for the formulation composition that is developed. The longest and most costly development pathway is for a new pharmaceutical ingredient. This pathway has the same clinical and regulatory risks as developing a new formulation for an approved drug, but this is compounded with significant risk with regards to the safety/efficacy of the active ingredient. The typical development time for this pathway is 10 to 12 years. Table 7 summarizes the differences between these regulatory/development pathways.

Ophthalmic Medical Devices [510(k)]

Section 510(k) of the Food, Drug and Cosmetic Act requires device manufacturers who must register to notify FDA, at least 90 days in advance, of their intent to market a medical device. A medical device, according to the U.S. FDA, is an instrument, apparatus, implement, machine, contrivance, implant, in vitro reagent or other similar or related article, including component part or accessory. Thus, certain nonmedicated OTC products (e.g., contact lens rewetting drops), irrigation solutions and contact lens solutions would generally be filed in this category. There are three classes of medical device class I (general controls are sufficient to show safety and efficacy); class II (general and special controls are required; special controls may include labeling requirements, requirements for postmarketing surveillance, etc.); and class III (general controls and premarket approval (PMA) are required to demonstrate safety and effectiveness). Most of the ophthalmic products that enter the market through the 510(k) filing fall into class II and III medical devices. The process involves detailed scientific review by FDA for PMA including clinical studies protocol that has been agreed by FDA. There is no regulatory time-limit for PMA review but, the FDA does target completion of approval within 180 working days of receipt, if it can be approved as received, or 320 days if additional information is required. The filing of a 510(k) requires identification of a "predicate" device (with very similar composition, packaging, and use) and a detailed comparison with the predicate device.

Stability Storage and Testing of Ophthalmic Formulations

The stability testing requirements for various types of ophthalmic products (eye drops, eye ointments, ophthalmic inserts, injections, irrigating solutions, lens care products, etc.) are not always straightforward. The International Conference on Harmonization (ICH) guidelines do not address all of the stability requirements for the diverse array of products. For the large number of ophthalmic formulations that are packaged in semi-permeable containers, "stress conditions" are present at high temperatures and low humidity. Thus, accelerated testing of these products is carried out under these conditions as per ICH guidelines. The specific conditions include long-term stability testing at 25°C/40%RH; intermediate accelerated (if 40°C fails) testing at 30°C/40% RH (FDA guidelines) or 30°C/60%RH (ICH guidelines) and accelerated testing at 40°C/15%RH (84). For specialized formulations and packaging systems, a well-planned, customized stability protocol will have to be written that properly addresses important product characteristics during use and storage. To increase the chances of product approval it is important to develop a well-defined stability protocol that aims to address all international, regional and local requirements that is approved by the regulatory authorities prior to start of stability studies.

FUTURE DIRECTIONS

Many new ophthalmic drug delivery technologies which offer potential advantages are currently available, and more will be discovered in the near future. However, the development of a new ophthalmic drug or new ophthalmic drug delivery technology is an expensive and time-consuming project. Therefore, the decision of whether or not to develop a new formulation will continue to be based on the added value that a new product will offer to the patient. A new technology may offer more comfortable, less invasive treatment of a disease, less frequent dosing of a product, or safer, more effective treatment of a particular indication.

The advances in drug delivery technology that promise to reduce the dosing frequency of a drug substance (e.g., moving from four times per day to twice per day), or offer more comfortable formulations will likely be developed for the treatment of chronic indications, such as glaucoma and dry eye. The benefits of lower dosing frequency and offering more comfortable treatment are not outweighed by the cost increase for treatment of acute indications, but over years of treatment, a patient will be willing to pay more for an improved formulation technology. It is also likely that combination products, which offer the convenience of delivering a single drop rather than multiple drops, will continue to be developed for chronic indications, but will not likely be developed for acute indications.

Although noninvasive methods such as use of an oral tablet or a topical formulation for posterior treatment (rather than an intravitreal injection) will always be of significant interest for any ophthalmic indication, they will most likely be of greatest use in the treatment of acute indications. Invasive technologies involving implants and intraocular injections will be of importance in treating chronic indications and where patient compliance is likely to be low because of a frequent dosing schedule (e.g., in elderly glaucoma patients).

Whenever feasible, new drug substances will continue to be brought to market, at least initially, in simple, low-risk formulations like solutions and suspensions.

In the future, the ophthalmic formulator will need to continue to have a firm understanding of the structure of the eye, the nature of the drug substance that needs to be delivered to treat the eye, and the options for how to bring the two together in the best way possible. The final choice of drug substance, formulation type, delivery method and manufacturing and packaging for the final product will need to take into account the overall market potential of the product as well as the cost and benefit to the patients.

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11 Glass containers for parenteral products

Robert Swift

INTRODUCTION

Glass containers have a long history as packaging materials for foods, beverages and medicinal products. Among other properties, glass compositions suitable for use as pharmaceutical containers offer sufficient inertness to minimize product interactions, impermeability to prevent ingress of contaminants, dimensional stability at temperatures needed for sterilization or depyrogenation as well as for lyophilization or frozen storage and transparency to allow product inspection. Where needed, coloration for light protection is possible. From the business side, a wide range of glass container styles and sizes is readily available in large quantities at reasonable cost. Increasingly, many manufacturers also offer preinspection, sterilization, barrier coatings or other specialized services needed for specific applications.

Despite general familiarity with glass in everyday life, detailed knowledge about the chemistry and manufacture of glass containers—and, specifically, glass containers used for parenteral medications—is limited. To provide basic information about glass, this chapter explores the characteristics of the glassy state, the broad range of industrial glass compositions and applications, the function of the various types of constituents that are included in commercial glasses and the manufacturing process steps that are common to the production of virtually all glass articles. This is followed by a more specific discussion of types of glass compositions used for pharmaceutical applications, how they are categorized and tested in the major pharmacopoeia, the various design families of containers used for parenteral products and the manufacturing processes by which they are produced. Some key aspects of quality control also are mentioned. The chapter concludes with a series of topics that are relevant to pharmaceutical formulation development, pharmaceutical filling, inspection and packaging operations and the quality of parenteral drug products that are filled into glass containers: the chemical, thermal and physical properties of containers and an overview of some quality blemishes and defects that can arise at various points throughout the supply chain.

When the first edition of this work was published in 1984, molded bottles for both small volume parenterals (SVPs) and large volume parenterals (LVPs) were in widespread use. By the time the second edition was published, in 1992, a significant proportion of LVPs had shifted to flexible containers. The LVP container information in the earlier editions is largely unchanged and still may be relevant in some markets. However, recent market trends for SVPs have increased interest in single dose vials, prefilled pens and prefilled syringes. This edition addresses these containers more fully.

THE GLASSY STATE

Glassy materials have been described or defined several ways by numerous authors and organizations. For example, Boyd (1) quotes Morey, ASTM C162 and Shelby, while Pfaender (2) provides three popular answers to the question "What is glass?" Some common themes can be summarized as follows:

- A supercooled liquid that has solidified or frozen without crystallization
- A solid material with amorphous, liquid-like structure
- A liquid with such high viscosity at room temperature that it behaves as a solid
- A material which lacks long-range molecular order but exhibits the stress-strain characteristics of a brittle, elastic solid.

While a wide range of materials, including organic molecules can be induced to form glasses, commercial container glasses are inorganic silicates produced by melting. With this

restriction, one can say that glass is an inorganic material or mixture of materials that has been heated to a molten liquid state then cooled without crystallization to a solid state.

The backbone of any glass formulation is a network former. There are several metallic oxides that readily cool without crystallization to form glasses. Special purpose glasses are produced using oxides of boron (B_2O_3), phosphorus (P_2O_5) or germanium (GeO_2) as the network former (3). However, the primary network former in glass formulations for commercial applications—including parenteral containers—is silica (silicon dioxide, SiO_2).

The basic network building block for silicate glasses is a tetrahedral form of silica, (SiO_4) (Fig. 1). Ideally, each silicon atom has shared bonds with four oxygen atoms and each oxygen atom has shared bonds with two silicon atoms. This configuration leads to a cross-linked, 3-D network (Fig. 2) of shared covalent bonds. The spatial interaction of these bonds causes viscosity to increase rapidly with decreasing temperature and inhibits the molecular reordering needed for the material to make the transition from a randomly ordered structure of the liquid state to the regular, long-range order of a crystalline solid. As a result, the network cools to rigidity in the glassy state. When processed under the appropriate conditions silica will crystallize as quartz (Fig. 3).

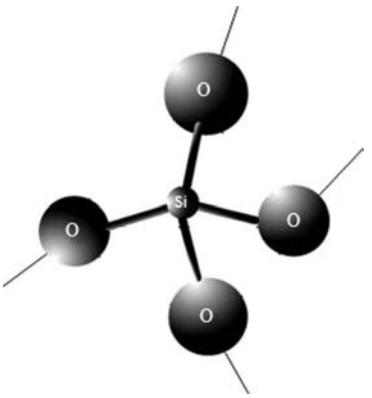

Figure 1 The SiO_4 tetrahedron is the basic network building block for silicate glasses. Each oxygen atom binds to the central silicon atom and either bridges to an adjacent silicon atom or exists as a nonbridged oxygen anion.

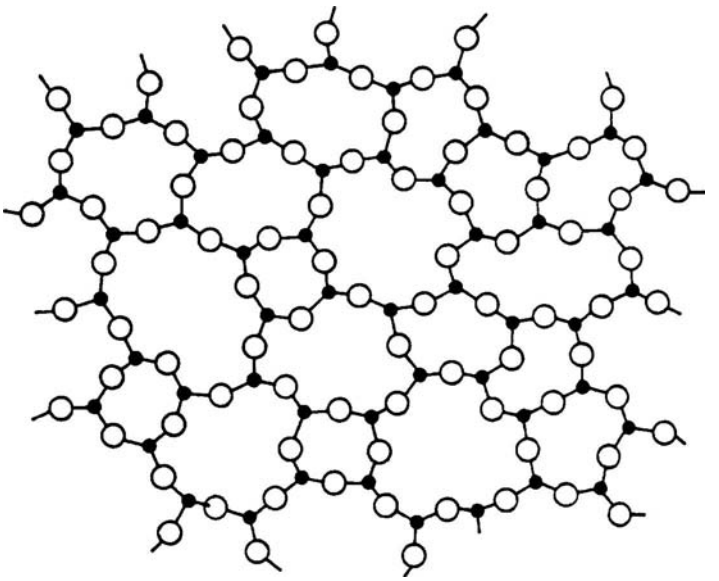

Figure 2 Two-dimensional schematic representation of glassy silicon dioxide in a random 3-D network of tetrahedral silica. *Source*: From Ref. 5.

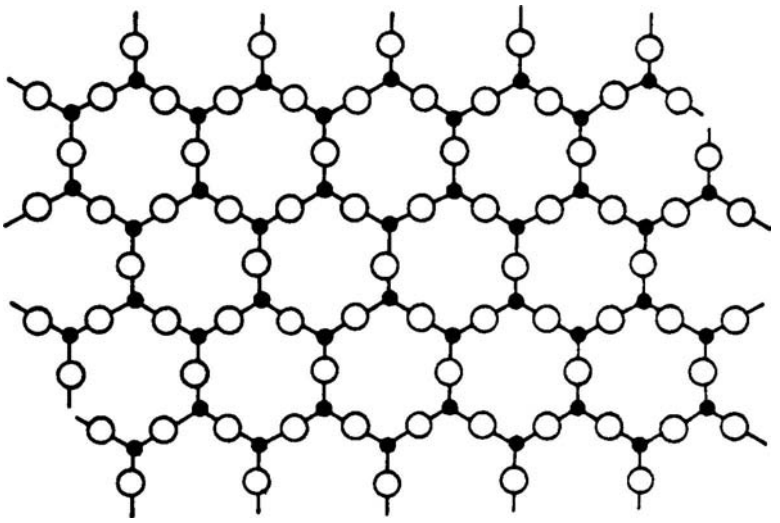

Figure 3 Two-dimensional schematic representation of 3-D crystalline quartz with long-range structure composed of tetrahedral silica. *Source*: From Ref. 5.

SILICATE GLASS FORMULATION FAMILIES

Both vitreous (glassy) silica and crystalline silica (quartz) are found in nature. However, commercial glass melting techniques require the viscosity of the melt to be in the range of 1 dPa-sec. For pure silica, this viscosity corresponds to about 2300°C, which is not practical for industrial production. Similarly, upon cooling, the viscosity of molten silica increases too quickly to be formed into containers using conventional production processes. As a result, practical glass formulations for containers are mixtures of silica and other minerals that lower the melting point and modify the properties of the glass to improve workability.

In fact, the network modifiers have the greatest influence on the physical and chemical properties of the glass and resulting finished glass articles. For this reason, glass formulations can be divided into broad families on the basis of the primary network modifiers used. The following sections describe the glass families used for containers and the role of the various network modifiers.

Soda-Lime-Silicate Glasses

The oldest and most widely melted glasses are known as soda-lime-silicate glasses. In the raw material mixture, or batch, these oxides typically are supplied as soda ash (sodium carbonate) and limestone (calcium carbonate)—hence, the common description "soda-lime" glass (4). In a glass formulation, soda and lime refer to sodium oxide and calcium oxide, which are the primary network modifiers and comprise roughly 25% of the composition by weight. Glasses in this family may include some magnesium oxide by the addition of dolomite (calcium magnesium carbonate). Potassium oxide, supplied as potash (potassium carbonate) may also be used. Within the silica matrix, the monovalent cations, sometimes called alkaline oxides, (Na^+ and K^+) satisfy the charges of nonbridged oxygen atoms (Modifier cation M_1 in Fig. 4). This reduces the extent of cross-linking in the silica backbone, which lowers the melting point. However, the sodium or potassium cations are relatively mobile and can be leached from the surface which limits chemical durability of the glass. The bivalent cations, also known as alkaline earth oxides, (Ca^{2+} and Mg^{2+}) interact with the silica matrix in a similar way occupying locations adjacent to two nonbridged oxygen atoms (Modifier cation M_2 in Fig. 4) and are more resistant to leaching. Usually, 2% to 3% aluminum oxide (Al_2O_3) is added to facilitate melting and to improve chemical durability. The aluminum cations (Al^{3+}) are able to form covalent bonds within the silica matrix (Modifier cation M_3 in Fig. 4) and, thus, are much more resistant to leaching. When light protection is needed, ferric oxide (Fe_2O_3) is added to produce amber glasses, which absorb ultraviolet wavelengths more effectively than colorless

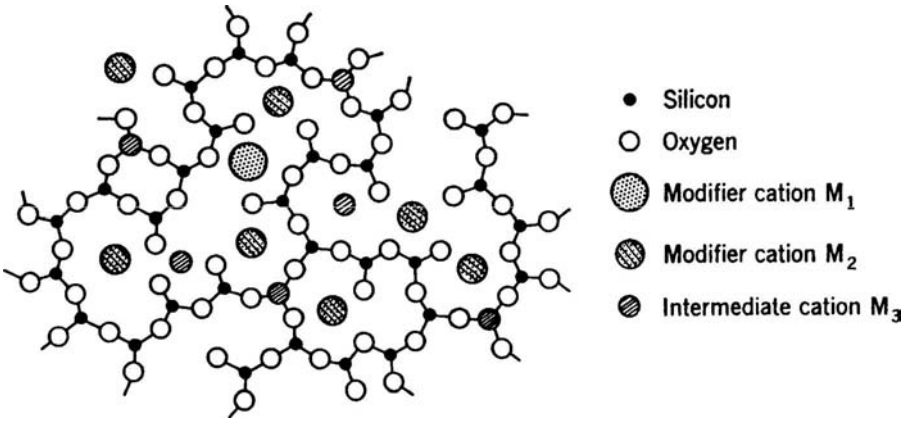

Figure 4 Two-dimensional schematic representation of the 3-D structure of a multicomponent glass. Monovalent and divalent cations exist in interstitial space and balance the negatively charged nonbridged oxygen atoms. Trivalent cations integrate into the silica network. At surfaces, nonbridging oxygen atoms are dominant and yield a net negative charge. *Source*: From Ref. 5.

glasses. The nominal compositions and properties of several soda-lime container glass formulations are shown in Table 1.

Borosilicate Glasses

In the late 19th century, Otto Schott, a German chemist and glass researcher, conducted systematic research to investigate the effects of various minerals and oxides on the optical, chemical and thermal properties of silicate glasses. He discovered that replacing some of the sodium and calcium with boron oxide (B_2O_3) resulted in glasses with exceptional chemical durability and heat resistance—including resistance to abrupt temperature changes, or thermal shock (7). Over time, a wide range of borosilicate glasses and other special glasses (8) have been developed for various applications including pharmaceutical containers as well as the familiar Duran®, Kimax®, and Pyrex® brands of laboratory glassware. Thermal shock and the related property of the thermal expansion coefficient will be developed more fully in the section Mechanical and Thermal Properties later in this chapter.

Borosilicate glasses require higher melting and forming temperatures than soda-lime glasses. However, with roughly ten-fold improvement in durability, as measured by extractable alkali, and high tolerance for thermal processes such as depyrogenation, lyophilization and terminal sterilization, borosilicate glasses now account for nearly all containers used for small volume parenterals. Ferric oxide (Fe_2O_3) and titanium oxide (Ti_2O_3) or manganese oxide (MnO) can be added to produce amber borosilicate glasses for protection from ultraviolet light. The nominal composition and properties of representative borosilicate container glasses also are shown in Table 1.

Compendial Classifications and Test Methods

Pharmacopoeias around the world acknowledge these two families of glass compositions as suitable materials for drug product containers. In fact, the compendia designate glass "types" based on these composition categories and reference the composition family in the general description of each type. For example, in the USP (9), type I containers have the description "Highly resistant, borosilicate glass" while type III containers are described as "soda-lime glass." Test methods used to differentiate between borosilicate and soda-lime containers and classify them according to type rely on the substantially lower quantity of alkaline ions that can be extracted from borosilicate glass or containers.

For example, the USP "powdered glass" test assesses the intrinsic chemical resistance of the glass formulation by crushing containers to obtain powder of a defined grain size and performing an extraction from the powdered glass into water by autoclaving. The alkali

Table 1 Nominal Compositions and Properties of Representative Soda-Lime and Borosilicate Glasses Used for Blow-Molded and Tubular Containers for Parenterals

Chemical composition (weight %)	Borosilicate							Soda-lime	
	Tubing					Molded		Molded	
	Clear			Amber		Clear	Amber	Clear	Amber
Silicon (SiO_2)	81	75	74.7	69	70	69	66	73	72
Boron (B_2O_3)	13	10.5	11.1	10	7	12	11		0.5
Sodium (Na_2O)	4	7	7.3	6	7	10	8	14	14.2
Aluminum (Al_2O_3)	2	5	6.1	6	6	6	6	2	2
Calcium (CaO)		1.5	0.4	0.5	< 1	1	1	10.5	10
Potassium (K_2O)			0.8	2	1				
Magnesium (MgO)				0.5					
Barium (BaO)				2		2	1		
Titanium (TiO_2)				3	5				
Manganese (MnO)							5		
Iron (Fe_2O_3)					1	<0.1	1	<0.05	0.3
Zinc (ZnO)				1					
Chloride (Cl^-) and fluoride (F^-)			0.4						
Sulfate (SO_3)				0.2				0.2	
Physical properties									
Thermal expansion 0–300°C ($\times 10^{-7}$)	33	49	51	54	55	60	62	88	91
Softening point (°C) ($10^{7.6}$ dPA-s)	825	785	783	765	770	739	745	729	713
Annealing point (°C) (10^{13} dPa-s)	560	565	566	558	560	567	530	548	536
Strain point (°C) ($10^{14.5}$ dPa-s)	525	Not published	518	520	Not published	528	515	510	496
Density (g/cm^3)	2.23	2.34	2.32	2.39	2.42	2.41	2.48	2.48	2.5

Source: From Ref. 6.

content of the resulting extract solution is determined by titration with acid. Although the test details differ, other compendia include similar methods. These methods can differentiate between type I borosilicate glass and type III soda-lime glass because of the significantly higher quantity of alkali that can be extracted from soda-lime glass. This is expected given the much higher levels of sodium, calcium and other alkaline and alkaline earth oxides present in soda-lime glass. The USP glass powder test and similar methods assume that the fresh surface exposed by crushing the container is representative of the inner surface of the container which will contact the drug product. This assumption is not always justified, as will be discussed in the section "Glass Chemistry" later in this chapter.

There are chemical treatments that can be applied to the inner surface of freshly formed containers to react with the alkaline ions at or near the surface. Sulfur dioxide (SO_2) or sulfur trioxide (SO_3) gas, or, more conveniently, ammonium sulfate [$(NH_4)_2SO_4$] pellets or solution may be injected into the containers before annealing. At elevated temperature and in the presence of water vapor, these substances produce sulfuric acid which reacts with alkaline ions on the glass surface to form various salt residues that are readily removed by rinsing prior to use. The removal of alkaline ions from the inner surface in this way significantly reduces the level of alkali available for leaching into the drug after filling. When type III containers made from soda-lime glass are treated in this way, the surface resistance is improved to such an extent that the pharmacopoeias recognize them separately as type II glass or containers. For example, USP <660> (9) designates Type II glass and provides the general description "Treated soda-lime glass." Similar classifications and descriptions are found in the European Pharmacopoeia (10).

Since only the surface resistance is improved by the treatment process, glass powder test methods cannot differentiate between treated or nontreated containers or assess the effectiveness of the treatment process. For treated containers, alternative test methods such as the USP Water Attack at 121° Test, the USP Surface Test, the Ph. Eur. Test for Surface Hydrolytic Resistance, or similar method must be used. In these tests, the extraction into water is performed using intact, filled containers rather than glass powder. As with glass powder methods, the results usually are determined by titration of the extract with acid. Some methods allow the use of spectroscopy to quantify directly the concentration of extracted alkaline ions.

If the composition family of the container glass is known (e.g., soda-lime glass), one may perform any of the surface test methods, apply the corresponding limit values and confirm the use and effectiveness of a chemical treatment process. When neither the glass formulation family nor use of chemical treatment is known, it may be necessary to perform both a surface test and a glass powder test to classify the containers correctly. However, many pharmaceutical companies confirm the container type on the basis of the supplier's test results and certificate of conformance.

As will be explained in section "Surface Chemistry," later in this chapter, there is another reason that the chemical resistance of the inner surface may be different from the intrinsic resistance of the glass formulation. The container forming process can cause degradation of the physical and chemical properties of the inner surface even when borosilicate glass is used. The Ph. Eur. test for surface hydrolytic resistance, the USP Surface Test or other similar methods may be used to evaluate residual surface alkalinity of containers made from borosilicate glass to confirm that the inner surface retains the level of chemical resistance expected in type I containers. The compendia are silent on test methods for and classification of containers made from borosilicate glass that are subsequently chemically treated to reduce alkaline surface residues deposited during forming. Users of "treated" borosilicate containers are advised to consult with their supplier to understand how the forming and "dealkalization" processes are controlled to ensure consistent results.

There is a tendency to assume that the terms soda-lime and borosilicate, especially as used in the pharmacopeias, refer to specific glass formulations. In fact, within the broad categories of soda-lime and borosilicate glasses, a wide range of glass formulations have been developed for specific applications. This point is especially relevant to borosilicates where two major subfamilies are important for parenteral containers. Within the industry, these subfamilies are often identified as "33 expansion" and "51 expansion." These terms are derived from the thermal expansion coefficient of some typical formulations in each group.

The American Society for Testing and Materials has published ASTM E-438-92 (11) defining nominal composition ranges and physical properties for glassware used in laboratory apparatus. This standard differentiates between the two borosilicate subfamilies by the designations "Type I, Class A" and "Type I, Class B." Although these designations are not used in the pharmacopeias, they are understood by glass manufacturers. Thus, for example, a specification defining the material requirement as ASTM E-438 Type I, Class A ensures that a 33-expansion borosilicate glass will be used.

GLASS PRODUCTION

Regardless of the glass composition, production of all glass containers begins with the transformation of inorganic raw materials into molten glass in large furnaces lined with refractory brick. A simplified cross section is shown in Figure 5.

The conversion of granular high purity silica sand, alumina, various carbonates and, for borosilicates, sodium borate into molten glass suitable for forming involves a series of complex physical and chemical reactions well beyond the scope of this chapter. However, the main process steps can be summarized as follows. The raw materials, or batch, are weighed, blended and conveyed continuously to the melting furnace. Typically, the batch includes a controlled percentage of internally recycled crushed glass known in the industry as cullet, which facilitates melting of the other batch materials. As the materials are heated and the melting process begins, carbon dioxide is liberated by decomposition of the carbonates and dissolved water is released. A substantial portion of the overall melting process is the refining process during which the CO_2, H_2O, and other gases coalesce, rise through the molten glass and escape into the furnace atmosphere. Bubbles that do not escape can be carried through to the forming process as seeds or blisters in molded bottles or as air lines in tubular containers. During refining, convection currents within the glass serve to homogenize the melt. Finally, the refined, homogenized molten glass must be slowly and uniformly cooled to reach the viscosity needed for the forming process which follows.

Glass composition is controlled primarily through careful monitoring of the raw material composition and corresponding minor adjustments to the batch proportions. Complete chemical analysis of glass composition is difficult and time consuming. Therefore, day-to-day monitoring of the melting process is accomplished by measuring physical properties such as density and thermal expansion coefficient that are extremely sensitive to changes in composition. Homogeneity and relative absence of bubbles are monitored by quality control inspections of the molded containers or tubing.

Figure 5 Longitudinal cross section of a large industrial glass melting furnace. Raw materials are added continuously at the batch feeder (1). Melting (2), refining (3), and homogenization (4) occur gradually as the melt progresses through the main furnace chamber. The molten glass flows under the bridge wall (5) into the conditioning section (6) before passing into the forehearth (7) for transfer to the blow-molding or tube draw process. *Source*: From Ref. 2.

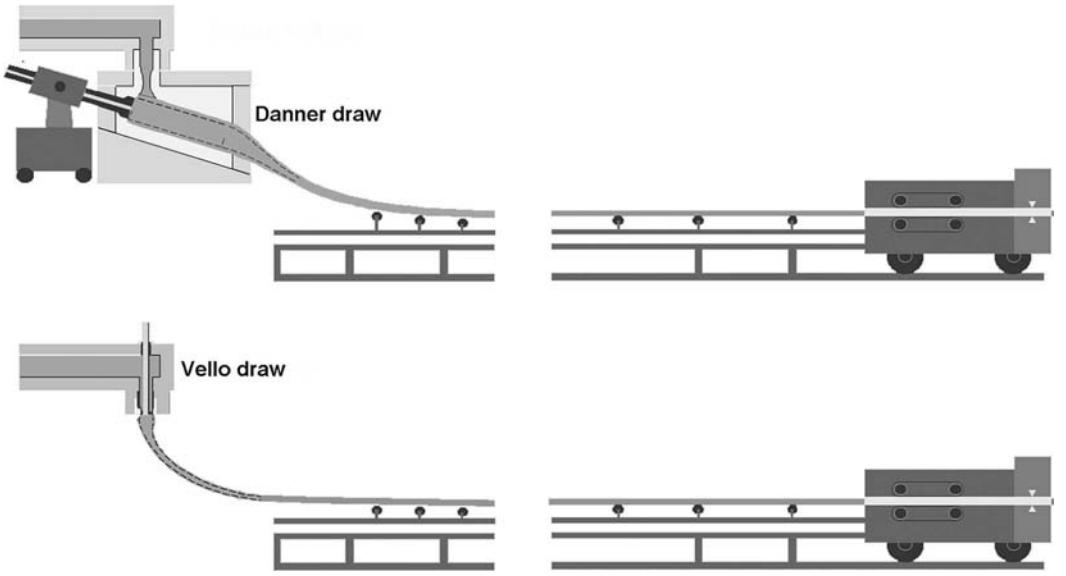

Figure 6 Glass tubing production for small volume parenteral containers uses either the Danner process of the Vello process. Both processes receive a continuous vertical stream of molten glass at the appropriate viscosity and transform it into discrete lengths of glass tubing with precisely controlled outer diameter and wall thickness. *Source*: From Ref. 4.

For both types of forming processes, the continuous output of the melting process is molten glass that has been cooled to reach a suitable viscosity. For blow-molded containers, a reciprocating plunger in the forehearth pumps the glass so that it can be cut into discrete charges, or "gobs," of molten glass that are guided through chutes to the forming machine. For all other styles of parenteral containers, the molten glass flows continuously from the furnace and is drawn into tubular form. The diameter and wall thickness of the tube are as needed for the body of the container into which the tube will later be formed. Glass tubing for parenteral containers is produced using either the Danner process or the Vello process (Fig. 6).

In the Vello process, a mandrel with the approximate shape of a bell is positioned in the stream of molten glass flowing from the orifice ring located in a bowl-shaped "drain" in the bottom of the forehearth. The molten glass flows out of the furnace and over the bell. Compressed air is blown through the center of the bell to form and maintain the stream as a tube.

The Danner process is similar except that the molten glass streams from the furnace onto a ceramic mandrel as a ribbon. The mandrel rotates slowly and is inclined slightly downward. As the glass flows down the length of the mandrel, it cools to the appropriate viscosity. Compressed air is blown through the center of the mandrel to form the tube.

With either process, the tractor belts of the drawing machine, located up to 120 m (~400 ft) downstream, provide a pulling motion which redirects the glass stream into a horizontal orientation. As the continuously moving glass tube cools and solidifies, it is supported on carbon rollers or air beds. The diameter and wall thickness are controlled by a delicate balance of the glass flow rate out of the furnace, the pressure of the blowing air and the speed of the drawing machine. The glass flow rate cannot be controlled directly but is the result of precise control of forehearth temperature, glass level within the furnace and, for Danner, the mandrel temperature or, for Vello, the relative dimensions and positions of the bell and ring. For either process, just after the drawing machine, the continuous tube is cracked off into discrete lengths, the ends are flame-smoothed or trimmed and fire-polished to prevent chipping and cracking and the tubes are packaged for shipment to the container producer.

In state of the art production facilities for pharmaceutical grade glass tubing, tubing diameter and melting defects such as knots, stones, and air lines are continuously inspected on

the tubing alley between the furnace and the drawing machine using laser or camera-based instruments. Additional checks of all other tubing dimensions and attributes are performed through automated or visual inspection of finished tubes. In general, acceptable quality level (AQL) sampling plans are used.

The next sections will discuss the various styles of container designs, some advantages and disadvantage of each and provide some details about the container forming processes.

CONTAINER DESIGNS AND MANUFACTURING PROCESSES

There are four main categories of container designs used for parenteral medications. Each is available in a range of sizes and shapes from multiple manufacturers.

Ampoules

An ampoule is a complete one-piece container system made entirely of glass and produced from tubing. The most common capacity range for ampoules is 0.5 to 2 mL. Capacities up to 20 mL or larger are possible for special applications. Some sizes and configurations have become de facto standards in certain markets. In addition, the International Organization for Standardization (ISO) has defined materials, dimensions, capacities, performance and packaging requirements for glass ampoules for injectable products in ISO 9187-1 (12).

The main steps of the forming process are illustrated in Figure 7. The wall of the tip or stem is thin and tightly controlled in the forming process. Similarly, the wall of the constriction is also tightly controlled. The constriction generally is preweakened by scoring or by the application of a color-break band to facilitate breaking the ampoule in the constriction at the time of use. Color-break bands are ceramic enamels with a slightly different thermal expansion coefficient. The mismatch prestresses the constriction to reduce the force needed to open the ampoule. An improved ampoule opening system called "One Point Cut" (OPC) has been developed. In this system, a small score of precise width and depth is cut at a single point of the constriction. OPC is claimed to provide more consistent opening force and fewer glass particles. ISO 9187-2 describes the requirements for ampoules using this design.

Quality control for ampoule manufacturing may include online 100% gauging of critical dimensions. State of the art producers use feedback control of the flames to maintain tight

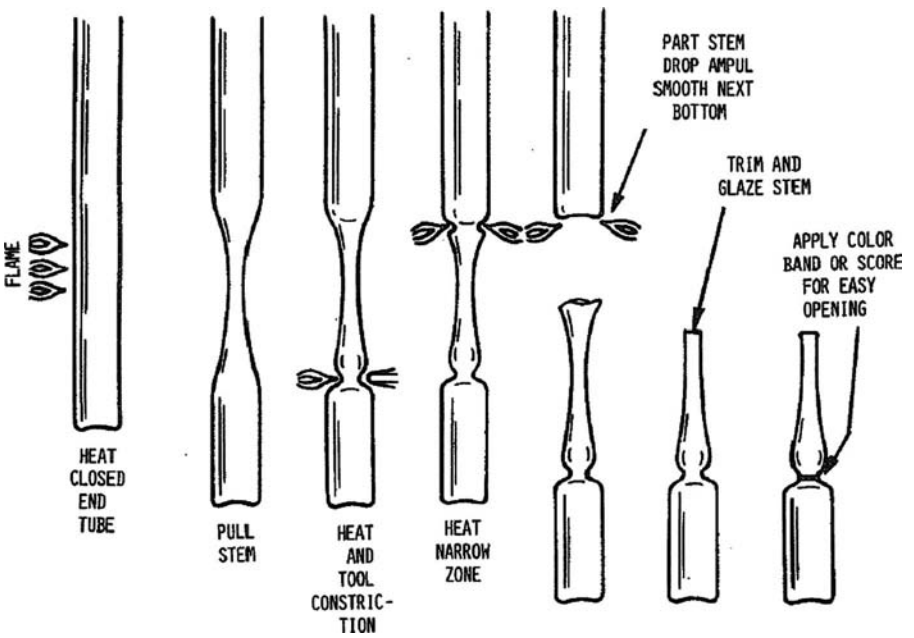

Figure 7 Typical process steps to form ampoules from glass tubing. *Source*: From Ref. 6.

control over the diameter and wall thickness of the stem. This minimizes variability in the sealing process during pharmaceutical processing. Some manufacturers offer supplemental 100% camera-based inspection to eliminate minor cosmetic blemishes at the point of manufacture to reduce container-related rejection of ampoules after filling and sealing. Additional quality control checks for dimensional and cosmetic attributes and breaking strength are performed periodically on finished ampoules. In general, AQL sampling plans are used.

After filling by the pharmaceutical producer, the stem of the ampoule is melted and usually pulled to seal the container. The combination of geometry and preweakening at the constriction allows the user to snap off the tip at the time of use and withdraw the contents of the ampoule into a disposable syringe so that the dose can be administered.

The main advantage of the ampoule container system is the simplicity of a single product contact material, highly inert borosilicate glass, throughout the shelf life of the drug product. However, breaking glass to gain access to the contents is not considered to be user-friendly. One also must consider the safety aspects of the sharp edges created when opening an ampoule and the possible need to use a filter when transferring the dose to a disposable syringe for administration. Therefore, while ampoules still are widely used for generic drugs and in developing countries, it is rare for new products to be developed in ampoule format.

Bottles and Vials

The most recognizable container system for parenteral products is a glass bottle or vial that has been closed with an elastomeric stopper and aluminum crimp seal. The glass container may be produced from glass tubing. Tubing vial capacities generally are limited to 30 mL. ISO has defined the materials, shape, dimensions, capacities and performance requirements for injection vials made from glass tubing up to 30 mL in ISO 8362-1 (13). A wide range of other heights, diameters and wall thickness also are produced. In addition, with specially designed forming machines and tubing up to 50 mm (~2 in) in diameter, it is possible to produce tubing vials up to 100 mL capacity or larger.

The production steps to form a vial from a glass tube are shown in Figure 8. The dimensions of the container body are unchanged by the forming process and retain the diameter and wall thickness of the original tube. As such, the wall thickness and diameter are uniform and well-controlled. This may allow higher filling and packaging line speeds and facilitate the use of high speed, camera-based inspection of filled containers. Forming the shoulder and bottom of the vial can cause occasional slight dimensional variation which may affect processing efficiency. In addition, the lighter weight of tubing vials may cause handling problems on lines orginally designed for heavier molded bottles.

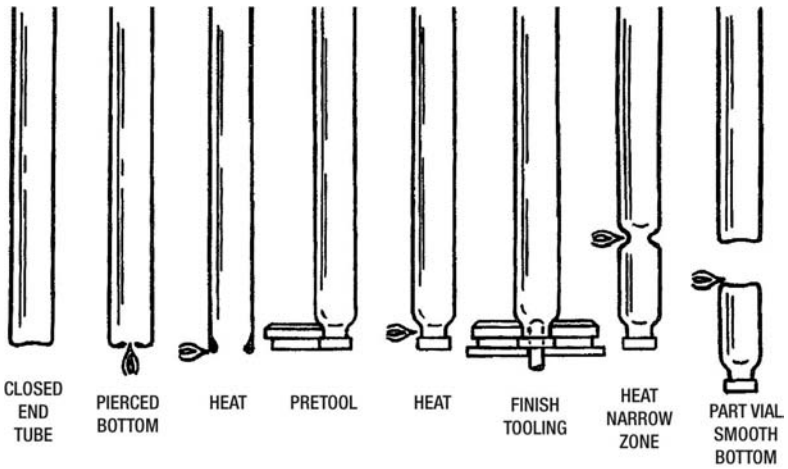

Figure 8 Typical process steps to form vials from glass tubing. *Source*: From Ref. 6.

Leading producers generally use camera-based systems directly after forming to perform 100% inspection of dimensions affecting the interface with stopper and seal. As with ampoules, some manufacturers offer supplemental 100% camera-based inspection to eliminate minor cosmetic blemishes at the point of manufacture to reduce container-related rejection of vials after filling and sealing. Additional quality control checks for dimensional and cosmetic attributes are performed periodically on finished vials using AQL-based sampling plans.

Containers for use with elastomer stoppers and aluminum seals also may be blow-molded bottles. Molded bottles for parenterals are available with capacities from 2 mL to 1 L or more. ISO has defined materials, shape, dimensions, capacities and performance requirements for injection vials made of molded glass in ISO 8362-4 (14). As with tubing vials, a wide range of other sizes and shapes are available. Typical process steps are shown in Figure 9. Compared with forming lines for tubular vials, molded bottle production lines have higher tooling costs longer changeover times and also must be located adjacent to the melting furnace. As such, production campaigns for molded bottles may be longer but less frequent. Production planning and inventory levels can be adjusted accordingly. Quality control steps for molded bottles are similar to those for tubular vials.

The nature of the blow-molding process is such that the wall thickness of a molded bottle will be heavier and more variable than the wall thickness for a tubing vial of similar capacity. Optical distortion caused by wall thickness variation can complicate inspection of the contents, especially when using automated, camera-based inspection systems. To accommodate the longer overall working time needed, borosilicate glass formulations suitable for blow-molding tend to have slightly higher sodium and boron content when compared with similar tubing glass formulations. On the other hand, heavier wall molded bottles may be more resistant to breakage caused by accidental abuse or mishandling.

For bottles and vials intended to be used with elastomeric stoppers and aluminum seals, the bottle or vial is only part of the overall container-closure system. Three-dimensional parameters of the mouth or finish are of particular functional importance at the interface with the stopper and seal. The neck inner diameter must ensure an appropriate interference fit with the plug of the stopper. Similarly, the outer diameter and thickness of the lip or finish must be suited to the diameter and skirt length of the aluminum seal. Other details of angles and radii also are important in matching the three components and the sealing equipment to create a robust container-closure system. While all of these parameters matter, by convention, the size designation is based on the nominal outer diameter of the finish. For small volume parenterals, typical container systems use finishes with either 13 mm or 20 mm nominal flange diameter.

ISO 8362-1 and ISO 8362-4 standards for injection vials provide dimensional details for the finish area as well as design parameters for complete containers, that is, diameter, total height, wall thickness, capacity, etc. ISO 8362—parts 2, 3, 5, and 6 are companion standards for elastomeric closures, aluminum caps and aluminum-plastic combination caps. This family of standards is intended to facilitate suitability of components from different suppliers in different but related industries. The roots of these ISO standards can be traced to German DIN standards. As such, the nominal dimensions and tolerances were developed in millimeters.

Historically, in the United States, container finish dimensions and matching closures have been based on the "2710 Biological Finish" standard developed in the 1940s by the Glass Container Manufacturer's Institute (GCMI), now known as the Glass Packaging Institute (GPI). The dimensions and tolerances of the GPI 2710 standard (15) are in inches but the size designations also are based on the nominal outer diameter of the finish in millimeters. As a consequence, both the ISO family of standards and the GPI 2710 standard include finish designs having finish outer diameters of about 13 and 20 mm. The important dimensions are similar but not identical. When selecting components, one must be aware, for example, that a "20-mm" stopper and seal from a U.S. producer may not be optimized for use with a 20-mm vial from a European producer. Discrepancies of this nature may also exist in published or de facto standards that may be widely used in other markets. Care must be taken to ensure the selected components are suitable for use as an integrated container-closure system.

As pharmaceutical filling line speeds have increased, container and closure manufacturers have worked with their customers to optimize processing efficiency. When stoppers are inserted into filled vials, the stopper plug often seals the neck of the vial before the stopper is

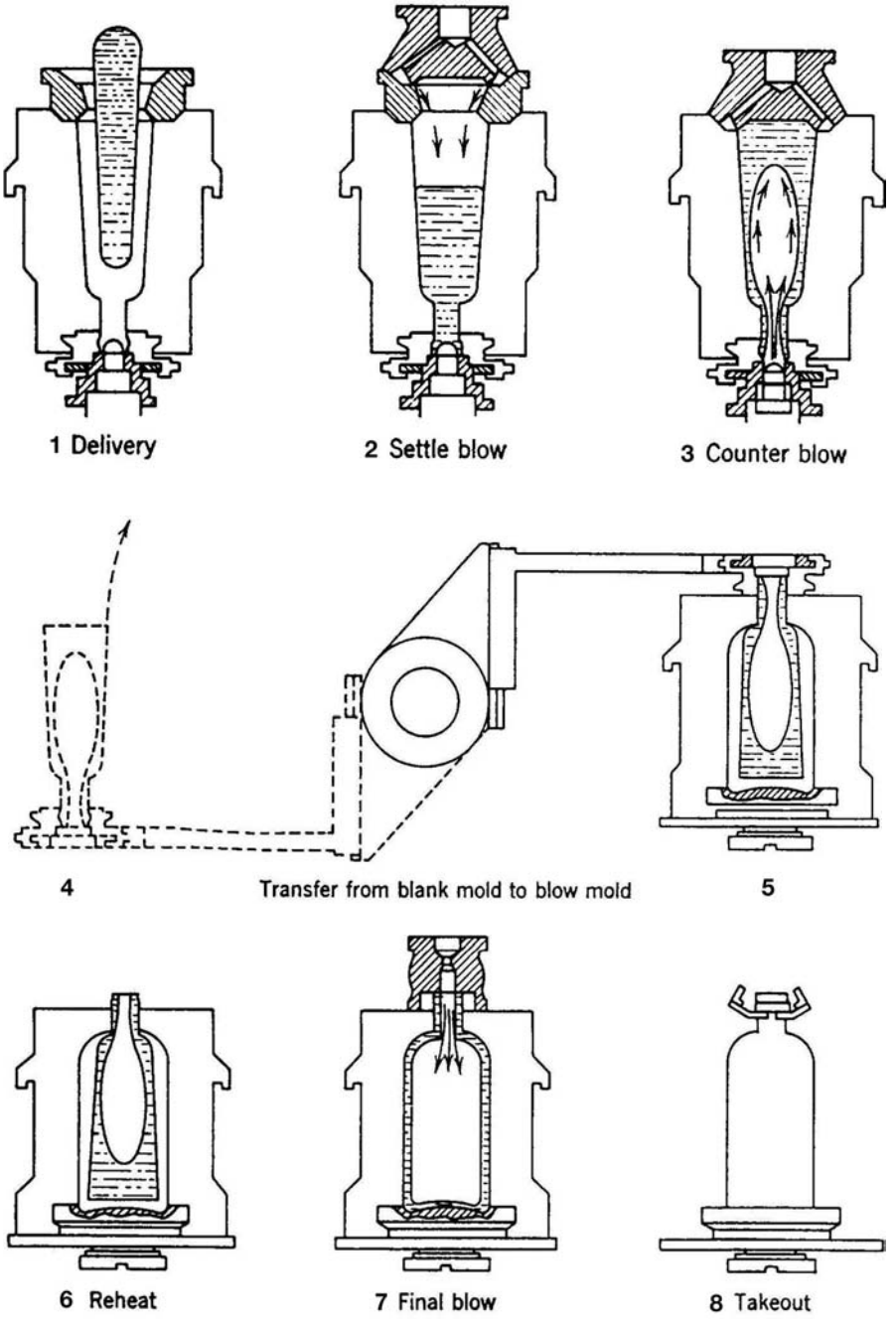

Figure 9 Typical process steps in the production of molded bottles by the blow-blow process. A charge or gob of molten glass is delivered by a chute from the furnace to the preform mold (**1**). Compressed air blows the glass into the mold to form the container mouth and neck (**2**). Compressed air then counterblows to shape the preform (**3**). The preform mold retracts (**4**), allowing preform to be transferred to the final mold which closes around it (**5**). The outer surface of preform that has been cooled by the preform mold reheats from residual heat in the molten core (**6**). Compressed air blows the glass out to the shape of the final mold (**7**). After some cooling time, the finished bottle is removed from the mold (**8**) and conveyed to the annealing (stress relief) tunnel. *Source*: From Ref. 5.

fully inserted. Completing the insertion creates a slight overpressure in the headspace resulting in a tendency for stoppers to "pop up" slightly after insertion. To address this, a "no-pop" ring can be molded into the stopper plug and a corresponding "blowback" ring can be formed into the neck of the vial. The intention is to provide additional mechanical interference to help retain the stopper in the seated position until the aluminum overseal is positioned and crimped. Here also, care is needed to ensure that the design details of each component are appropriately sized and positioned. The container system designer is advised to work closely with the component manufacturers to ensure compatibility.

The blowback feature originally was developed for smaller containers, for example, a vial with a nominal fill capacity of 2 cm^3 having a fill volume of 2 mL plus overage. In this situation, the volume of the stopper plug can be a significant percentage of the total headspace volume which increases the likelihood of pop-out because of pressurizing the headspace. Pharmaceutical companies producing lyophilized products also recognized the possibility for the blowback feature to improve the control over the position of the partially inserted stoppers during transfer of filled vials between the filling suite and the lyo chamber. Thus, vials and stoppers for lyophilization also often incorporate blowback rings.

Prefilled Cartridges

Glass cartridges are tubular glass containers that are open on one end to receive a suitable elastomeric plunger stopper. The opposite end has been tooled to form a neck and flange. After filling, the tooled end is closed with an aluminum cap which is lined with a suitable elastomeric septum. Just before use, a double-ended needle is attached. When the needle is attached, the end of the needle at the aluminum seal pierces the septum allowing the medication to be administered. Dental anesthetics and insulin therapy are two important markets for prefilled cartridge systems. For ease of use, the systems often are combined with reusable holders or, increasingly, adjustable multidose pen devices. Compared with a vial of equal capacity, a cartridge-based system will be longer, smaller in diameter and have little or no headspace gas. ISO has defined materials, dimensions, performance, and test methods for the product contact components of such systems in ISO 11040. Parts 1 and 4 (16,17) of the standard are glass cylinders, while parts 2, 3, and 5 address plungers, septa (disks) and aluminum caps. Additional requirements for components used in pen-injector systems are defined in ISO 13926 (18)—parts 1 through 3.

The glass forming process for the finish of a pen cartridge is similar to that used to form the neck and flange of a tubular vial. Online 100% inspection and off-line quality control checks also are similar. Cartridges are produced from tubing and can be formed using either one of two basic process concepts. The neck and flange may be formed, as with tubular vials, on the end of the tube. After forming the finish, the cartridge is separated from the tube using thermal shock and the open end is flame polished. Alternatively, full length tubes may be first cut into blanks using thermal shock and flame polished. On a separate forming line, the flange and neck are formed on one end of each blank. The smoothness and uniformity of the open end can have an important effect on the ability of the finished cartridge to endure the rigors of packaging and distribution.

In addition to its role as a drug product container during shelf life, at the time of use, the cartridge also plays a functional role as part of the drug delivery system. To fulfill this function, the body of the cartridge must be lubricated to reduce and control the static and dynamic friction between the glass cylinder and the elastomeric plunger. Generally, the lubricant is an emulsion of polydimethylsiloxane that is added to the final WFI rinse prior to depyrogenation using dry heat. The depyrogenation process drives off the residual water leaving behind the lubricating silicone layer. The interaction between the glass surface, the silicone fluid, the drug product and the elastomer plunger is complex. The processes affecting this interaction should be characterized thoroughly, validated and monitored to ensure consistent functional performance throughout shelf life. This is especially important for pen-injector systems where precise dosing is required. Cartridges for injection devices also may have additional dimensional requirements related to dose accuracy or to fit and function within the device.

Prefilled Syringes

In some ways, prefilled syringes can be considered an extension of the cartridge concept. Prefilled syringes also are formed from glass tubing. With a cartridge, one end is open to receive a suitable elastomeric plunger stopper. Unlike cartridges, the open end of a prefilled syringe is tooled to form a finger flange by which the syringe is held during administration of the dose. The opposite end of the syringe may be tooled to the shape of a male luer taper or to accept a plastic luer lok adapter or a small channel may be formed at the inner diameter of the tip into which a cannula is later inserted and glued. In each case, prior to filling, the syringe tip is fitted with a suitable elastomeric luer tip cap or needle shield. Prefillable syringes can be supplied as "bulk" (unprocessed) containers intended to be rinsed, siliconized and sterilized just prior to filling. Luer tip and Luer Lock syringe barrels can tolerate dry heat depyrogenation and the tip cap or tip cap and adapter are assembled under aseptic conditions in the filling suite. The adhesives typically used on syringes with glued in cannulae cannot tolerate dry heat. "Bulk" staked needle syringes are sterilized by autoclaving rather than by dry heat.

As with cartridges, prefilled syringes are produced from tubing and can be formed using either one of two basic process concepts. The tip may be formed, as with tubular vials, on the end of the tube. After forming the tip, the syringe body is separated from the tube using thermal shock and the open end is flared and tooled to form the finger flange. Alternatively, full length tubes may be first cut into blanks using thermal shock and flame polished. On a separate forming line, the finger flange is formed on one end of each blank and the tip is formed on the other end. The flange forming process may occasionally reduce the inner diameter at the flange opening. This may affect processing when mechanical plunger setting tubes are used.

Numerous dimensional and functional attributes of the glass barrels and various in-process assembly steps for prefilled syringes are 100% inspected using camera-based systems. Other process control and quality checks are performed at the appropriate stages of production using both time-based and AQL-based sampling plans.

In addition to bulk, unprocessed syringe barrels, there also is a significant and growing market for prefillable syringes that have been rinsed, siliconized, suitably packaged and then sterilized by the syringe manufacturer. These ready to fill systems are sterilized by ethylene oxide using validated cycles. Sterility testing is routinely performed on each sterilization batch.

As with pen cartridges, prefilled syringes serve double duty as the container-closure system during shelf storage of the drug product and as an integral part of the drug delivery system at the time of use. In prefillable syringes, the lubricant generally is applied as an aerosol mist of silicone fluid. The processes affecting this aspect of the syringe system should be well understood and controlled to ensure consistent functional performance.

For prefilled syringes, there is an additional level of complexity in that the tip cap or needle shield also serves a dual purpose. During shelf storage, this product contact interface is an integral part of the container-closure system. Yet, at the time of use, the tip cap or needle shield must be easily removed. And, for a luer tip or luer lok syringe, system performance requirements include the ability to form a leak-tight seal with the injection needle or delivery system adapter. Prefilled syringes also are increasingly being incorporated into automatic injection devices. Additional specification requirements and quality control tests may be required to ensure consistent drug delivery performance of prefilled syringes and auto-injectors.

While the focus of this chapter is on glass containers for parenterals, it is important to recognize that from the perspective of drug product compatibility, prefilled cartridges and prefilled syringes have added complexity compared with vial-stopper-seal systems. At a minimum, these systems include a second elastomer in the septum, tip cap or needle shield in addition to the plunger stopper. These systems also include the silicone fluid lubricant on the barrel and generally on the plunger stopper as well. Finally, for syringes with preattached needles, the stainless steel cannula and adhesive are in direct contact with the drug product throughout shelf life. The potential effects of each of these additional product contact materials needs to be assessed during qualification of the container-closure system.

Specialty Items

Other special purpose container systems, such as dual chamber vials, cartridges and syringes, threaded vials for infusion systems and high-strength capsules for needle-free injection systems also are available. An exhaustive review of these systems is beyond the scope of this chapter. The interested reader is encouraged to contact glass container manufacturers to learn about speciality products and new developments.

SURFACE CHEMISTRY

There are two fundamental mechanisms of chemical attack that can occur when an aqueous solution is in contact with the surface of a glass container (19). Through ion exchange, H_3O^+ ions in the solution can replace Na^+ ions in the glass. Once the sodium ions have been removed from the near surface layer, the rate of diffusion of sodium ions from within the bulk glass slows the process considerably. Ion exchange is the dominant mechanism of attack for most acidic and neutral formulations.

By contrast, hydroxyls and other alkaline species attack the silica network itself by breaking Si-O bonds. The rate of attack is highly dependent on the glass formulation and the solution pH. Surprisingly, several investigators (20–23) have shown that, at the same pH, different buffer systems can have markedly different rates of attack. It has been speculated that chelating agents are more aggressive toward glass because they are able to pull the various metal ions out of the surface. The resulting voids are then more susceptible to the other mechanisms of attack. Unfortunately, this means that simple formulation guidelines based on pH alone are not adequate.

In addition, the chemical resistance of the container surface also may vary. As mentioned earlier, the forming process can alter the composition, morphology and physicochemical characteristics of the container surface. During forming, especially when making the bottoms of ampoules and tubular vials, the temperature of the inner surface can exceed the boiling point of the more volatile ingredients of the formulation, primarily sodium and boron. These elements can vaporize from the hotter surface of the bottom and subsequently condense on the cooler sidewall as sodium borate. Then, as the finished container passes through the annealing oven, the deposits can be partially reintegrated into the underlying silica network. As a result, the alkaline deposits may not be completely removed by the pharmaceutical company's rinsing process but remain as less durable regions of the surface that is in contact with the drug product. This phenomenon will occur to some extent in the production of any container from glass tubing. For molded borosilicate glass bottles, vaporization and condensation of alkaline ingredients is generally not significant since the peak temperature of the glass is inherently lower. The resulting quantity of alkaline residue can be controlled by production speed, heating rate and maximum glass temperature. Residual alkalinity can be monitored by testing the surface resistance of the finished containers.

The alkaline residues can affect the drug product through three separate but related mechanisms. Firstly, the locally alkaline region or leached ions may react directly with the formulation. Secondly, by ion exchange with Na^+ ions in the glass, the loss of H_3O^+ ions from the solution can increase the pH of unbuffered or weakly buffered solutions. Thirdly, in extreme cases, the interaction can trigger the formation of an unstable layer of silica gel which can slough off as delaminated glassy particles.

Chemical dealkalization of borosilicate containers, for example, by the introduction of ammonium sulfate solution into the containers just before annealing, has been used, especially in the United States, as a means to control or minimize these effects. This process has been shown to be highly effective in reducing extractable alkali and the related effect on pH. Some users have found that the combination of controlled alkalinity in the forming process plus chemical dealkalization yields precise pH control for unbuffered products. However, studies by Ennis (24) showed that ammonium sulfate treatment without proper forming process controls did not eliminate delamination. In fact, in those studies, higher quantities and concentrations of treatment solution increased the formation of glass flakes.

Unpublished studies with which the author is familiar showed that delamination resulted from an interaction between excessive residual alkali on the vial surface, the parameters of the rinsing and depyrogenation processes, and the pH and composition of the

drug product vehicle. Anecdotally, acidic residues from excessive dealkalization also have been reported to have caused a reduction in drug product pH and long term damage to washers and deypryogenation tunnels.

Phenomena such as these highlight the importance of evaluating the chemical durability of the inner surface of the finished container using, for example, the USP Surface Test, the Ph. Eur. test for surface hydrolytic resistance, ISO 4802-1 (25) or similar quantitative spectroscopic surface extraction test methods such as ISO 4802-2 (26).

MECHANICAL AND THERMAL PROPERTIES

The preceding section addressed the chemical properties of the product contact surface, which can be of vital importance to the physical and chemical stability of drug products stored in the containers. Physical integrity of the container as a means to maintain product sterility is another equally important requirement of containers for parenterals. In this respect, the mechanical and thermal characteristics of glasses must be considered. Earlier in this chapter, glasses were described as amorphous materials exhibiting the stress-strain characteristics of a brittle, elastic solid. Describing glass as a "brittle" material is perhaps consistent with the general perception that glass is fragile. By contrast, the notion that glass is "elastic" seems contradictory. However, as material science terms, brittle and elastic have more precise meanings both of which apply to glasses.

In this context, brittle refers not to the strength of the material but to the failure mode when local stress exceeds local strength. Most metals, when overloaded, will deform in a permanent way, technically, "plastic deformation," before breaking. Brittle materials, such as glasses, are unable to undergo plastic deformation and therefore break abruptly (27). Intrinsically, glasses are very strong materials in response to compressive loads. However, surface damage significantly reduces the effective strength under tensile stress. A compressive load squeezes the margins of a surface flaw or discontinuity together and has little effect. By contrast, a tensile load pulls a surface flaw or discontinuity apart and concentrates the stress at the bottom of the discontinuity. Thus, the flaw or discontinuity significantly reduces the practical strength of the material as elucidated by Griffith (28).

Similarly, as a material science term, elastic refers to the response of a material to the application and removal of a mechanical load that does not exceed the strength of the material. Elastic materials deform when loaded then return to the original shape when the load is removed. The stiffness of a material can be characterized by its elastic modulus, also known as Young's modulus, which is the ratio between the applied unit load, or stress, and the resulting unit deformation, or strain. In this respect, glasses are relatively stiff. Typically, the elastic modulus of glass is about the same as aluminum (29). Jiang (30,31) attached strain gages to the outer surface of glass vials to observe in real time the physical deformations of and corresponding stresses in the vials during freezing, frozen storage and subsequent rewarming and thawing of various buffers and formulated drug products. Although it was not the objective of the studies, the work demonstrates the elastic deformation of the glass in response to the changing physical dimensions of the contents.

Because of the combination of stiffness, brittle behavior and reduction in strength at surface flaws, one does not usually observe directly the elastic deformation that occurs in glass containers before catastrophic brittle failure occurs. Indirectly, when failure occurs, the energy stored by elastic deformation may be observed in the form of rapid fracture propagation and dispersion of the glass fragments.

Stress in glass containers can result from forces exerted on the container, either externally or internally. Stress also can be the indirect result of nonhomogeneous composition or other imperfections from the melting process or from thermal effects. Thermally induced stresses may be either permanent artifacts from the glass forming process or a transient response to temperature gradients within the glass. Moreover, stress in the glass is additive. The total stress at a given point is the sum of the stresses at that point regardless of the source.

Silicate glasses have relatively low thermal conductivity. As a consequence, heating or cooling results in a steep temperature gradient between the heated or cooled surface and the underlying glass core. This is the reason that the coefficient of thermal expansion of the glass composition is important in determining the thermal resistance of a container. When a

container is cooled, the outer surface tries to contract. The contraction at the surface is resisted by the warmer core resulting in tensile stress at the outer surface. While this phenomenon is the principle behind "cutting" glass by thermal shock, it also can lead to unintended cracks during container production as well as during pharmaceutical processing.

For a given temperature difference, the stress level is proportional to the thermal expansion coefficient and the modulus of elasticity of the glass composition (32). Thus, all other conditions being equal, a 33-expansion borosilicate glass container can withstand a temperature difference on the order of three times larger than a container of identical size, shape and geometry made from a "90-expansion" soda-lime glass. It should be noted that, in addition to the properties of the glass, the cooling rate, the geometry of the container and the presence of surface flaws caused by handling all contribute to thermal resistance.

QUALITY ATTRIBUTES

Several aspects of quality control already have been mentioned in the discussions of the manufacturing processes. These described the process points where quality control checks are performed rather than the quality attributes being examined. A detailed discussion of the full range of possible container defects and cosmetic flaws is beyond the scope of this chapter. Nevertheless, it is worthwhile to point out that certain types of flaws can occur only in specific process steps. As such, some basic knowledge can be helpful when investigating container defects and failures. For example, glass flaws known as knots, stones, cord, seeds, blisters and airlines all originate in primary glass melting and tubing manufacture. Certain types of surface blemishes can occur only during blow-molding or conversion of tubing into ampoules, vials, cartridges or syringes. Finally, there are blemishes and defects that are more likely to be the result of interactions between containers and fill-finish equipment or processes. On the other hand, scratches, scuffs, bruises, and metal marks may occur at any process or handling step. Even in these cases, though, detailed examination may yield clues pointing to the root cause. For example, a scratch running the full length of the body of a tubing vial and fading into the heel and shoulder may indicate that the scratch was present on the tube prior to forming the container. Similarly, the location and orientation of a scuff or metal mark may eliminate most potential points of contact. The interested reader is advised to explore these topics with container producers. In addition, the Parenteral Drug Association (PDA) has published lexicons of attributes for tubular vials and molded bottles (33). Similar lexicons are being developed for ampoules, cartridges and prefilled syringes.

In some situations, the use of more sophisticated analytical tools may be warranted. Glass fracture analysis is the science of determining the origin of the breakage and the nature, direction and relative magnitude of the force that caused the breakage. Scanning electron microscopy with X-ray diffraction analysis or similar methods can be used to determine the elemental composition of surface flaws or of foreign materials that may be present.

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12 Plastic packaging for parenteral drug delivery

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INTRODUCTION

Driven by the development of biotechnology products, newer drug therapies, and reformulation of poorly soluble drugs, parenteral delivery is expected to provide strong growth in years to come. Routes of administration include subcutaneous, intramuscular, intradermal and intravenous injections. Drug products have been almost exclusively dispensed in glass containers, primarily because of the clarity, inertness, barrier property and thermal resistance of these containers. With the development of plastic polymer technology over the last 30 years, plastics have become logical alternatives for small-volume parenteral (SVP) and large-volume parenteral (LVP) packaging. Although plastic containers have become well-established as containers for LVP products, plastics have been, until recently, used on a limited scale for SVPs.

Glass vials are the primary container of choice because of their excellent gas and moisture barrier properties. More importantly, there is an extensive knowledge base on processing, filling, regulatory review and commercial availability of glass containers. Glass, however, may not be the best solution for all chemical or biological drug candidates. Glass contains free alkali oxides and traces of metals. Depending on the characteristics of the drug being packaged, it is likely that delamination could occur for high pH products over time, thereby affecting the shelf-life of the drug product. Proteins and peptides can be readily adsorbed onto the glass surface and can be denatured or become unavailable for treatment. With a glass prefillable syringe (PFS), potential leachables such as silicone, tungsten and adhesive can affect the stability of biopharmaceutical products. Glass may break during processing or transportation and when stored at low frozen temperatures. In these and other areas, plastic containers have made clear in-roads in the parenteral drug delivery market.

With the proliferation of new polymers and newer process technologies, most of the less-desirable characteristics of plastic containers have been overcome and the use of plastic packaging as vials and syringes is increasing. This chapter will discuss the role of plastic in pharmaceutical parenteral drug delivery. The discussion will provide insights on the following areas:

- Advances in plastic resins for SVP packaging with an emphasis on cyclic olefins as well as other plastics used: The properties of these plastics, applications and challenges will also be discussed.
- Plastic vial systems: This section will discuss in detail the development activities in this area including the use of plastic vials in lyophilization and the use of reconstitution devices.
- Plastic PFS systems: As more biopharmaceutical drugs and higher viscosity formulations are delivered in a PFS, there is the need for a break-resistant, high-quality, plastic PFS. Challenges with glass include breakage, reactivity of glass and leachables, such as silicone, tungsten and adhesive. Discussion will include how plastic PFS offer options to solve these challenges.
- IV bags and disposable bags: Following a brief overview of use of plastics for IV bags for LVPs, discussion will focus on new developments in the use of plastics for disposable bags in the packaging of biologics, including considerations for selection of disposable bags.
- Quality and regulatory considerations: U.S. Pharmacopeia (USP), European Pharmacopoeia (Ph.Eur.) and Japanese Pharmacopoeia (JP) compendial requirements will be discussed and referenced for plastic containers.

This chapter provides the reader with adequate information on recent developments, availability and use of various plastic packaging systems for pharmaceutical drug products, including suitable references to commercialized drugs products.

ADVANCES IN PLASTICS

Plastic resins are the most widely used raw materials in global pharmaceutical packaging, accounting for 61% of consumption compared with glass, paper products and aluminum foil. The worldwide demand for plastics for packaging was estimated at $25.8 billion or 2.3 billion lbs. of material consumed in 2006 (1). High-density polyethylene (PE) is the most widely used plastic with 1.2 billion lbs. consumed, followed by polypropylene (PP) at 0.4 billion lbs. However, the fastest growth is expected with the newer resins, the cyclic olefins growing at a compound annual growth rate of 5.5% by 2011 (Fig. 1). The growth is expected to penetrate specialty fields such as pharmaceutical drug delivery. This is driven by a need for clear, highly transparent, biocompatible packaging systems with improved quality and improved barrier protection.

Cyclic olefins: Compared with the traditional plastic resins, the development and application of cyclic olefins in parenteral drug delivery is relatively new. Cyclic olefins are prepared by additional polymerization of monocyclic olefins, cyclobutane or cyclopentane or bicyclic olefins such as norbornene. The resulting product has improved chemical and physical properties, such as glass-like transparency, excellent chemical resistance and improved moisture barrier. Mitsui Petrochemical Industries produced copolymers of ethylene and other cyclic olefins. Starting in the 1980s, Mitsui and Hoechst (2,3) began using single-sited metallocene catalysis in the polymerization of cyclic olefins that led to the development of the cyclic olefin copolymer (COC) Topas® by Ticona. In this process, 2-norbornene was reacted with ethylene in the presence of a metallocene catalyst to produce a series of copolymers whose properties can be modified by varying the norbornene percentage in the material. Another commercially viable route is through a two-step process based on the ring-opening metathesis polymerization (ROMP) of dicyclopentadiene followed by complete hydrogenation of the double bonds to form cyclic olefin polymers (COP) (Fig. 2). Using this process, the Zeon Corporation developed the Zeonex® and Zeonor® line of COP. A similar process also resulted in another clear COP plastic, called Daikyo Crystal Zenith® (CZ) that is available only in a finished container format from Daikyo Seiko, Ltd.

COP and copolymers (COC) possess many excellent properties, including glass-like transparency. This glass-like transparency of the polymers permits visual inspection of

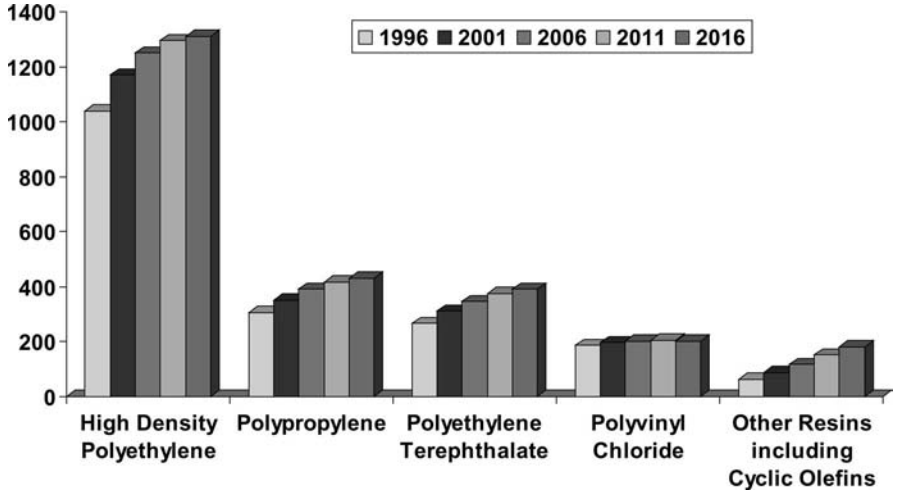

Figure 1 (*See color insert*) World pharmaceutical packaging plastics demand by resin (million pounds by weight).

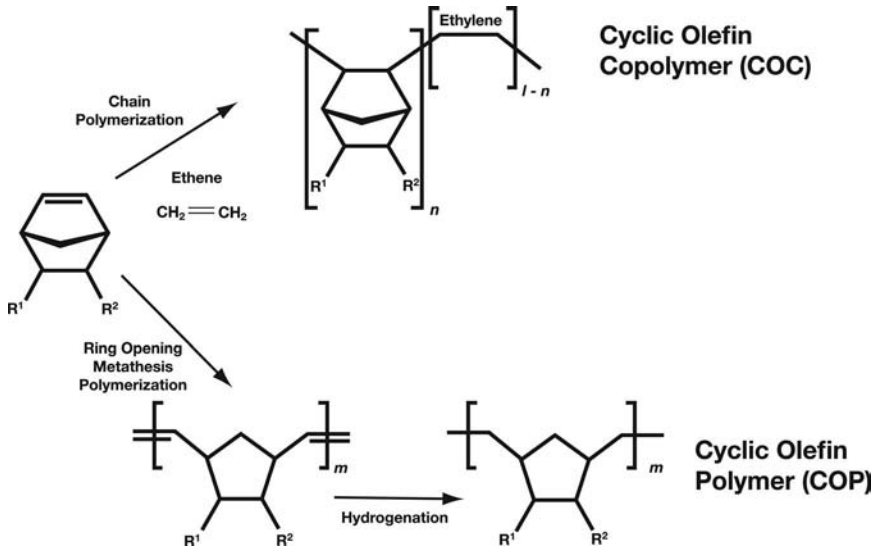

Figure 2 Process of polymerization in the development of cyclic olefin polymers/cyclic olefin copolymers. *Source*: Reproduced from Ref. 2.

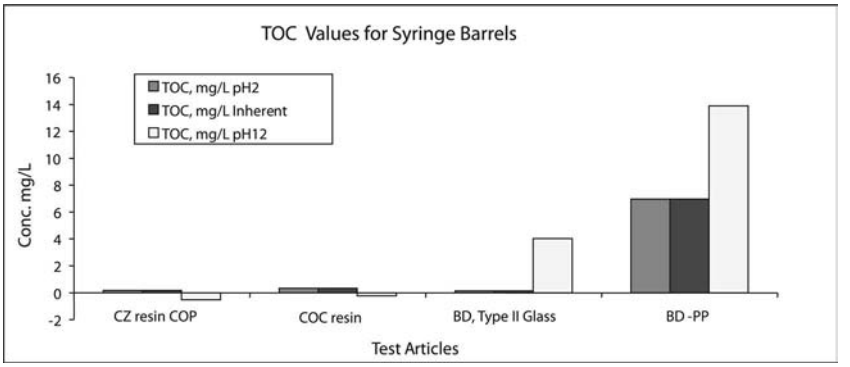

Figure 3 (*See color insert*) Comparison of total organic carbon as an extractable from syringe barrels. *Source*: Reproduced from Ref. 6.

the resultant manufactured components, as well as the parenteral products that are delivered to the end user. The polymers have good melt flow properties that readily lend themselves to plastics processing, for example, molding and thermal forming. The polymers exhibit a high impact and break resistance, and they form an excellent moisture barrier (2–5). Additionally, they possess good chemical resistance to acids, bases and alcohols. These polymers are sterilizable by autoclave, ethylene oxide and radiation sterilization processes. As with most plastics in comparison with glass, the number of potential compounds that may be an extractable or leachable is higher for plastic than for glass because the number of components in the formulation is higher. These compounds are organic, whereas glass potential extractables are inorganic. Plastic vendors can provide a list of potential extractables developed with suitable extracting solutions. A decision may then be made on which potential extractables should be studied as leachables during stability testing. Preliminary studies have shown that, when compared with other materials that are used for parenteral applications, COP and COC exhibit very low extractables (Fig. 3). When studied for total organic carbon (TOC) extracted from syringe barrels at various pH levels, the

Table 1 Features of Cyclic Olefins for Parenteral Drug Delivery

Key benefits	Drawbacks
Glass-like transparency	Gas and moisture barrier properties are less than glass but better than other plastics
Sterilizable (via autoclave, radiation and ethylene oxide)	Sensitivity to scratches
High break resistance	Short-term discoloration due to radiation
Excellent moisture barrier	
Biocompatible (inert, low binding, and ion extractables)	
Design flexibility and excellent dimensional tolerances	
Good chemical resistance	

data shows very low extractable for COP (CZ) and COC compared with PP and glass (6,7). On the basis of this data and other information available, COP and COC are considered to be ideal plastic packaging containers for SVP. There are some drawbacks, however. Understanding these drawbacks will be important in the selection of cyclic olefins as a packaging system (Table 1). These plastic containers cannot match the barrier properties of glass to oxygen and moisture ingress, although they are much superior to other plastics, including PP, polystyrene and polycarbonate (PC). For oxygen-sensitive compounds, this may be a concern. Suitable secondary packaging can prevent moisture loss or oxidation, with the addition of a moisture absorbent or oxygen scavenger material.

High-density polyethylene (HDPE): The polymer is based on a simple repeating carbon/hydrogen molecule that branches out during polymerization to form a polymer with a high degree of regularity. This regularity creates the formation of crystal lattice structures. Polyethylene (PE) is recognized as having a high degree of crystallinity. During polymerization, the amount of branching that occurs during the process will determine the overall density and crystallinity of the resulting PE. As a result of their relatively high degree of crystallinity as compared with lower-density PEs, HDPEs have greater tensile strength and stiffness and have a higher melting point than the low-density polyethylene (LDPE) resins. Another important property is excellent chemical resistance, a characteristic of all polyethylene grades. HDPE is typically used for low-to-medium barrier medications, such as bottles, closures and in some cases, secondary packaging of parenterals and blister packs for solid dosage forms. The material is characterized by strong impact resistance, chemical resistance, drug compatibility for oral dosage forms and temperature tolerance. Both HDPE and LDPE are used to form containers by blow-fill-seal technology, primarily for ophthalmic and nasal/respiratory drugs, but also have been used for both SVP and LVP products.

Polypropylene (PP): PP is the leading plastic employed in containers, disposable syringes, PFS and closures. PP is a linear, high crystalline polymer, made of carbon and hydrogen in a very orderly fashion. The regularity of its structure imparts the high degree of crystallinity found in most commercially available PP. Within the crystal array, the methyl groups impart stiffness to the polymer, making it different from its close relative, polyethylene. PP exhibits a high tensile strength, which is the ability to withstand forces tending to pull apart or distort the material, and is more rigid than HDPE. High tensile strength, in conjunction with a high melting point of 165°C, is particularly important for packaging drugs. Consequently, the material has the ability to withstand higher temperatures of autoclave sterilization for a limited number of cycles. PP is also resistant to chemical attack from organic solvents and strong acids and bases at room temperature. Because of the level of crystallinity present, it is not possible to achieve the optical clarity found with cyclic olefins: the crystal lattice sites tend to refract light, which imparts haze. The resin generates significant demand for the manufacture of blow molded bottles, pouches, laminates and plastic containers. Because of its improved moisture resistance and effective chemical resistance, PP is

typically used in disposable containers or delivery systems. It may have poor impact resistance at lower temperatures and increased extractables, and its translucency limits its role in the storage of parenteral drug and biological products for long duration.

Polyethylene terephthalate (PET): PET is a high-quality thermoplastic polyester that offers good barrier protection, chemical resistance and processing properties. It is typically used in packaging drugs that may require barrier protection as in blister packs and blow molded containers. It is cost competitive with HDPE and PVC and is used in development of bottles and blister sheeting. PET is polyester that is a condensed polymer prepared from ethylene glycol (EG) and either terephthalic acid (TPA) or the dimethyl ester of terephthalic acid (DMT). The EG monomer is prepared using ethane as feedstock and the TPA is manufactured using paraxylene as feedstock. TPA can then be purified by reaction with methanol to form the DMT. PET can exist in an amorphous state, an oriented and partially crystalline state and a highly crystalline state. Because of its low glass transition temperature, PET cannot tolerate autoclave sterilization. The material does hold up well to gamma radiation, making it the preferred method for sterilization. Ethylene oxide sterilization is also acceptable with PET resins. PET film may potentially be used as a coextruded layer of LVP bags (replacing use of PVC resins).

Polycarbonate (PC): PC is known for its mechanical properties and higher clarity with poor barrier properties. PC-based polymers are aliphatic molecules and are synthesized in various forms. These aliphatic PCs become extremely soft in the 40°C to 60°C temperature range. Bisphenol A PC is extremely stable and virtually nondegradable under physiological conditions. PC can be processed readily, possesses high mechanical strength and is very shatter resistant. PCs are used extensively as bottles and containers for parenteral applications. PC resin contains repeating aromatic rings in its main chain structure. The material is a polyester of carbonic acid and is generally produced using an interfacial reaction between dihydric or polyhydric phenols and a suitable carbonate precursor such as dichlorocarbonate. Currently most PCs are produced with a reaction between bisphenol A and carbonyl chloride in an interfacial process. Other polyhydric phenols are sometimes used to form copolymers for special end uses. The material is well suited for the injection molding process. PC shows excellent creep resistance over a broad temperature range, enabling its use in applications previously open only to thermoset materials. There are, however, some areas where PC resins are inferior. PC materials have limited chemical and scratch resistance and a very high water transmission rate when compared with other plastics. The resin also has a tendency to yellow with light exposure and with exposure to radiation sterilization.

Polyvinyl chloride (PVC): Less popular in parenteral packaging, PVC is prepared by polymerizing a gas, vinyl chloride or monochloroethylene, in the presence of organic peroxides or inorganic persulfates as initiators. The length of the molecular chain and the structure of the side chains are altered by the temperature, pressure and the nature of the initiator. PVC's growth in pharmaceutical packaging is much slower compared with its peers because of environmental concerns. This includes the formation of dioxin when PVC is incinerated. Additionally, di(2-ethylhexyl) phthalate (DEHP) plasticizers are used in the production of many PVC materials. These types of phthalates, which are known to leach out of PVC containers, may have potential health risks. Growth has slowed in this area, which probably reflects preferences for better performing and safer plastics.

Multilayer plastics: Plastic bags commonly used for LVP generally consist of between three and five layers of plastic film consisting of two or more different resins. Similarly, plastic film used for blister packaging of tablets is also multilayered. The purpose is to produce a plastic film that combines the best properties of each film including good clarity, excellent flexibility and durability, which also is a strong barrier to water vapor transmission.

Plastics fabrication: There are many processes used to convert plastic resins from pellets into desired shapes or configurations. This is a brief description of the plastic molding

processes. All plastic processes are similar in the use of three basic elements to convert the resin from a pellet to its processed shape.

1. *Heat*: excites the molecular structure to allow free movement of molecules
2. *Pressure*: forms the free-flowing polymer into a desired shape
3. *Time*: allows the transfer of heat into the plastic followed by time for removal of heat (cooling)

Extrusion of plastics: The process of extrusion involves melting a plastic and forcing it through a die under pressure to form a desired shape. There are several types of extrusion, depending on the die arrangement used to form the plastic. The three most widely used for parenteral packaging are flat-sheet extrusion, profile-tubing and blown-film extrusion. Flat-sheet extrusion is a versatile process, with the capability to produce sheet stock over a wide range of thicknesses from a wide range of resins. The process may also be used to produce coextruded sheeting where two or more different resins are brought together in the die manifold from two or more extruders. Flat-sheet extrusions can be used for blister packaging and form, fill and seal packaging. Clear grades of plastic that have a high degree of stiffness are generally preferred for extrusion processing. Another application for this process is the production of LVP containers.

Injection molding: Injection molding is a process used to convert resin from a melt into a molded shape using a mold pattern to form the part. Injection-molded products are replacing materials such as glass, metals and paper in many areas of parenteral drug packaging. The development of newer plastic resins, combined with improvements in the injection molding process, is setting the stage for these changes. For example, materials such as CZ resin have been used to develop larger containers such as the 1-L bottle by injection molding. Many of these newer resins are used for drug delivery systems that are replacing products traditionally made from glass. In this process, plastic resin is melted using the extrusion process and is injected into a mold where the resin is cooled enough to be removed in a solid state. Like the other plastic processes, heat, pressure and time are used in each of the steps to produce a molded product. Injection-molded items are finding many uses in parenteral drug packaging. The injection molding process is also used to produce components such as IV spikes and IV administration sets.

Blow molding: The blow molding process has grown rapidly over the past three decades. The two types of blow molding in use are extrusion blow molding and injection blow molding. A uniform tube of heated resin with one end closed is formed during the extrusion blow molding process and is moved into a mold where the two ends are pinched off, and the material is blown outward into the shape of the mold. The injection blow molding process is similar in concept except that is a two-step process. A preform is molded using a first-stage mold and the principles of injection molding. The form is then transferred into a second mold, and blown outward using pressurized air to form the container. Containers produced for health care applications, such as tablet bottles, are made primarily using the injection blow mold process. With small containers, this process is more cost effective than extrusion blow molding because it is capable of handling a large row of preforms at one time. Extrusion blow molding lends itself to larger containers where it becomes more economical and practical to eliminate the preform step. The blow molding process enhances the physical, chemical and barrier properties of certain materials, for example, PET, because it creates a high level of bi-axial orientation of the polymer. CZ, Zeonex and Topas resins also use the blow molding process to manufacture vials.

VIAL SYSTEMS

Market considerations: A vial is a SVP container with a stopper and a seal, intended to package liquid or a dry powder formulation for either single or multiple doses. Glass vials, typically made of type I glass, are most commonly used as vials for parenteral

applications. Recently there is increased interest in the use of newer plastics, particularly the cyclic olefins, as parenteral vials as they provide clarity and inert surfaces for biopharmaceutical and biological applications. When combined with plastic's inherent break-resistant attribute and the need for biologics to be stored and transported at lower temperatures, the future of cyclic olefin based plastics appears bright. Cyclic olefin polymers (COP) and copolymers (COC) are considered to be an ideal plastic for vial systems because they have glass-like clarity and suitable physicochemical properties and the ability to be sterilized.

The vendors in this area may be divided into those that manufacture the COP and COC resins such as the Zeon Corporation and Topas Advanced Polymers and companies that convert the resin into parenteral containers such as Schott Forma Vitrum that offers a range of sizes of both syringes and vials made out of COC under the brand name Schott TopPac®. Daikyo Seiko, Ltd. of Japan has used a proprietary COP resin to produce a range of sizes of conical, flat-bottom vials and larger screw-top containers, under the brand name of Crystal Zenith. West Pharmaceutical Services, Inc. (West) partners with Daikyo to codevelop, market and sell sterile and nonsterile CZ vials. As a result of the anticipated growth, the suppliers of resins and products have made significant investments to their supply chain to maintain continuity of supply. Rexam offers a new generation of multilayered plastic vials called MLx that are being used as a container with improved barrier properties. The COC vials produced by Aseptic Technologies represent a newer approach to vial handling and filling called the Crystal® technology, licensed from Medical Instill Technologies (Table 2). The vials and stoppers are molded and assembled immediately under clean conditions and gamma sterilized. Filling is achieved by piercing the thermoplastic closure and then immediately resealing the puncture with a laser. COP and COC vials have been tested and used to replace glass in various pharmaceutical parenteral applications. This is because glass contains free alkali oxides and traces of metals and, at higher pH conditions, can undergo delamination, thus affecting the stability of the drug product (8,9). Proteins and peptides can be adsorbed on a glass surface and can either be denatured or become unavailable for treatment (10,11). Glass particles can promote protein particulate formation, and glass is also more likely to break under processing, storage or transportation of biopharmaceutical products, especially at lower temperatures. In these areas and more, plastic vials have made clear in-roads in the pharmaceutical drug delivery market.

Protein and peptide adsorption: Numerous studies have addressed the adsorption of proteins to packaging containers. This interaction of proteins and peptides with the surfaces of storage containers can result in their loss and destabilization (12–14). Although the amount bound is typically low, this problem can be acute at low protein concentration where a substantial portion of what is usually assumed to be solution-state protein may actually be adsorbed to the container walls. Although protein

Table 2 COP/COC Packaging Systems for Parenteral Delivery

Company	Trade-name/type of cyclic olefin	Delivery system/sizes
Amcor/Alcan Packaging	COC	Vials 2 mL and 5 mL
Aseptic Technologies/Rexam	Crystal®/COC	Closed vials[a] 1–50 mL
Becton Dickinson	Sterifill®/Crystal Clear Polymer	PFS[b] 5–50 mL
Daikyo/West	Daikyo Crystal Zenith®/COP	PFS[b] 0.5–100 mL Vials[a] 0.5–1000 mL
Gerresheimer/Taisei Kako	Clearject®/COP	PFS[b] 1–20 mL
Rexam	MLx/COC, COP	Multilayer vials & bottles
Schott	Schott TopPac®/COC	PFS[b] 0.5–50 mL Vials 2–100 mL

[a]Presterilized vials and containers available
[b]Presterilized formats available
Abbreviations: COP, cyclic olefin polymer; COC, cyclic olefin copolymer; PFS, prefillable syringe.

binding is protein and formulation dependent, studies have shown a trend toward less protein adsorption to cyclic olefin containers. Burke et al. (15) compared glass vials with plastic vials made of polyester, PP and nylon for protein binding. Although no clear conclusion could be drawn on the binding characteristics of these primary packaging materials, it was observed that the degree of binding was highly protein-dependent. Qadry et al. (11) showed less protein binding to plastic CZ vials compared with type I glass, suggesting that the CZ vial is a potential candidate for an alternative material to the glass vial because of low affinity of proteins to bind to its surface. Eu et al. (16) compared the level of adsorption between glass and CZ vials and showed that a model protein preferentially adsorbed to glass vials compared with CZ vials. The authors used gold nano-particle staining techniques for a visual comparison of protein adsorbed to vial surfaces, but this technique does not permit quantitation of the amount of protein adsorbed to the surface. Waxman et al. (17) developed methods to quantitate protein adsorption on vial surfaces. One method uses the protein stain colloidal coomassie, which binds to protein adsorbed to vial surfaces and can be eluted and quantitated spectrophotometrically; the other method involves hydrolyzing the protein adsorbed *in situ* and quantitating the peptides released fluorometrically after reaction with fluorescamine. These approaches allow testing over a much broader range of protein concentrations without the use of radiolabeling. Using these methods, the authors confirmed that binding occurs rapidly and the amount of protein adsorbed per SVP vial is typically in microgram quantities. Protein adsorption to CZ vials was found to be independent of ionic strength, likely because of its hydrophobicity; in contrast adsorption to glass vials was inhibited with increasing ionic strength, indicating the effect of electrostatic interaction with glass containers. In our opinion, protein adsorption is clearly protein dependent, and testing needs to include glass and plastic containers with elastomer influence, before optimizing the drug formulation and container closure system.

Storage and transport at low temperatures: In the area of cell therapy, stem cell research holds significant promise for development of innovative therapies for many unmet or partially met disease treatments. As products enter clinical development stages, there is need for clean, clear, biocompatible, low extractables containers. The ideal vial-based system should be a suitable package to store and transport cell therapy products at lower temperatures; it should be suitable for commercial filling and meet pharmaceutical quality requirements. PP is a plastic resin that has been used for decades for various packaging applications including bottles, pouches, prefilled syringes, tubes and containers. Plastic resins have made minimal headway in the area of parenteral vials because of various quality attributes. A study investigated the use of CZ plastic vials for storing and shipping cell therapy products at low temperature ($-85^{\circ}C$) or cryopreserved ($-196^{\circ}C$) for six months using 0.5, 5.0 and 30 mL volume vials (18). Vials were tested for durability and integrity of a filled vial using a 1-m drop test, and for the ability to maintain viability and functionality of stem cells over the time of storage. No evidence of external damage was found on vial surfaces in the 1-m drop test. Post-thaw viability using dye exclusion assay was $>95\%$ and stored cells exhibited rapid recovery two hours post-thaw. Cultures were $\sim 70\%$ confluent within five to seven days, consistent with nonfrozen controls and indicative of functional recovery. CZ vials were durable and allowed for preservation and maintenance of cell viability and functionality, showing that these vials offer significant benefits to storing and transporting biological and biopharmaceutical products for storage, clinical and commercial applications.

Lyophilization and reconstitution: Cyclic olefin based plastics COC and CZ vials have been extensively studied for packaging lyophilized products. Freeze-drying in a plastic vial brings added advantages, especially when cytotoxic and biohazard products need to be packaged. Crystal technology, developed by Aseptic Technologies, applies the closed-vial technology for lyophilization (19) and for liquid fills. After filling closed vials using a piercing needle, a small disposable device called the penetrator reopens the orifice and, when the lyophilization chamber shelves move, the penetrator is

pushed down, releasing the water vapor. Lyophilization of mannitol and arginine was studied in Daikyo's CZ vials and compared with molded glass and tubing glass vials. The crystallinity of mannitol in CZ vials was either greater or comparable to glass vials. There was thermal homogeneity within the CZ vial during the lyophilization cycle, providing more uniformity within the cake (20). Despite the fact that COC and CZ plastics provide a high moisture vapor barrier, it is always recommended that a secondary packaging barrier such as an aluminum pouch or a blister pouch with aluminum lidding and very low water vapor thermoformable film be used to assure adequate shelf-life protection for lyophilized products. For liquid fills in COC or COP, additional barriers are not necessary because of the low moisture vapor transmission rate of cyclic olefins.

Many drug candidates are marketed in lyophilized form to maintain shelf-life stability and require reconstitution prior to administration. Some of these products, including treatments for hemophilia, multiple sclerosis and autoimmune diseases, may be administered in a home environment. Traditional reconstitution requires the use of multiple vials and needles, which can prove to be complicated for patients or untrained personnel, and may increase chances for needle stick injuries. In recent years, there has been an increasing use of safer and more convenient reconstitution devices made out of plastics. These provide simple methods to reconstitute products without the use of needles and may also improve the effectiveness of the reconstitution process and compliance with the dosing regimen. There are several types of reconstitution systems designed to connect the drug container (typically a vial) to a diluent container (either a vial or a prefilled syringe). Plastic reconstitution devices are sterile, nonpyrogenic, biocompatible and fully supported by appropriate regulatory filings (21). They are designed for short-term contact with the drug product, and can be manufactured from a variety of medical grade plastic materials, such as PC and polyolefins, with the precise material selected on the basis of functional requirements. An example of a plastic reconstitution device, a vial adapter, is shown in Figure 4. For most vial adapters, and other components where a plastic spike is required, PC is used as it provides the appropriate balance of rigidity and sharpness to optimize spiking performance and attachment to the vial. Other materials, such as HDPE, can be used for components within the system where a stopcock system is required. These component devices are packaged in a rigid blister, often made from PET, to maintain sterility and to enable ease of handling and protection of the device during use. Plastic vial adapters can provide safe, easy to use and cost-effective diluent transfer to a lyophilized drug vial. The adapter snaps to the neck of the standard vial after the plastic button has been removed. A plastic spike pierces the stopper; needles are not

Figure 4 Vial adapter. *Source*: From Medimop Medical Projects Ltd.

used. Plastic vial-to-vial transfer systems also offer a similar level of simplicity and cost-effectiveness through a double-spike adapter that connects to the top of each vial (lyophilized drug and diluent). This is an ideal solution for connecting vials of different sizes. These advanced plastic reconstitution systems offer several benefits, including ease of use by patients and caregivers; protection against drug spray-back and accidental needle stick injuries; needleless reconstitution and transfer. They may also help drug manufacturers reduce the amount of overfill in the drug vial (22).

Process considerations: Glass vials are washed, depyrogenated and sterilized by heat before they are filled. Plastic containers cannot be heated to high temperatures for depyrogenation, therefore alternative methods are used. Plastic molding and packaging in environmentally controlled clean rooms usually produce products that have very low bioburden and low particulate level. Nonsterilized vials undergo water-for-injection rinses for depyrogenation, followed by sterilization using autoclave, radiation (gamma or e-beam) or ethylene oxide. All handling operations are designed to avoid scratching the vials' outer surfaces, as plastics have a tendency to scratch. To minimize scratching, care is usually taken not to stack vials too tightly in processing. During autoclave sterilization processing, hazing of the plastic walls is known to occur. This is where moisture gets trapped during processing and may take a few days to diffuse out, but the clarity and integrity of the vial is not compromised. Vial spacing during the autoclave sterilization process may help mitigate this effect. For vials in a ready-to-use format, vendors offer sterile vials and containers. Sterile vials or containers are nonpyrogenic and have a very low particulate level, and could be used to store and transport drug products as early as first-in-human studies. Most commercial filling companies can accommodate filling of COP and COC plastic vials if care is taken to accommodate the characteristics of plastic vials. During filling of plastic vials, guide rails and vial handling change parts should be covered with a material that will limit scratching of the vials. The speed of the filling line may also need to be adjusted to accommodate filling of the lighter plastic vials.

PREFILLABLE SYRINGE SYSTEMS

Market considerations: In the current global market, PFS comprise more than 2.0 billion syringes per year in development and use. The origin of the prefilled syringes' rise as the preferred container was an extremely successful market introduction of syringes for heparins by Sanofi and Rhone Poulene-Rorer (Sanofi-Aventis) in Europe in the early 1980s. The PFS market has now exploded because of several factors: the growth of biopharmaceuticals; the need to eliminate overfills; precision of delivery volume; convenience of delivery, cost-effectiveness; elimination of dosage errors or a combination of these factors (23–26). Glass continues to dominate the PFS markets with a significant market share; however, plastic PFS are beginning to make advances, especially where glass has been unsuitable as a delivery system. PFS have been in use as larger volume containers for x-ray contrast media or medical devices such as hyaluronic acid derivatives (23). In the last decade, however, pharmaceutical drug products have been approved for use with prefillable plastic syringes, including a new chemical entity for oncology and a peptide drug product for the treatment of osteoporosis (Table 3).

Table 3 Global Regulatory Approvals of Drug Products in Cyclic Olefin Polymers/Cyclic Olefin Copolymers

Therapeutic area	Plastic packaging	Approvals
Anemia	Cyclic Olefin	Japan
Osteoporosis/Oncology	CZ vials	United States, Europe, Japan
Antifungal	CZ vial	Japan
Osteoporosis	CZ syringe	Japan
Radiology	CZ syringe	Japan
WFI product (for thrombolytic drug)	TopPac® syringes	Europe

Abbreviation: CZ, Daikyo Crystal Zenith®.

Although not reaching the adoption level of glass syringes, plastics syringe systems continue to gain strong acceptance from pharmaceutical manufacturers because of recent improvements in design, composition and manufacture. Plastic syringes were historically made out of PP, however, recent developments in the area of thermo-elastic polymers, such as cyclic olefins, have made substantial headway in the use of plastics as a PFS system. COP is as clear as glass, has low extractables, is less reactive and has better barrier properties compared with PP. Multiple vendors offer different sizes of syringes in sterile nested configuration or as nonsterile bulk syringes. Cyclic olefin plastic barrels are formed by injection molding under clean conditions and assembled in similar conditions, primarily to maintain a high level of cleanliness. Plastic syringes are sterilized either by autoclave, radiation (gamma or electron beam) or by ethylene oxide, but not by dry heat, and are offered as assembled sterile syringes that are ready for filling. The molding process also provides a greater degree of flexibility to include design features such as a plastic finger grip that can be combined with a back stop to prevent the piston being pulled out of the barrel.

To meet the need for lubricity and sealability, syringe manufacturers use silicone to coat the glass barrels and elastomer components. Silicone facilitates ease of movement of pistons in filling and stoppering equipment, and allows pistons to glide smoothly on activation of syringes. Silicone, however, can interact with drug formulation components (27,28). Recent developments to minimize free silicone include baking silicone at high heat onto the glass barrels, thereby minimizing the amount of free silicone that can interact with drug product. Advances in elastomer closure technologies have produced closures that do not require siliconization because of a special polymer lamination applied to the outer surface of the piston, thereby offering a silicone oil–free PFS system such as the Daikyo CZ syringe system. The syringe system includes a plastic COP barrel, nozzle cap and piston laminated with a fluoro-polymer lamination, Flurotec®, and requires no silicone for consistent functionality. Flurotec is a lamination technology using copolymer film of polyethylene tetrafluoro-ethylene (PTFE) or ethylene tetrafluoroethylene (ETFE). Helvoet (Omniflex® 3G) pistons also have a fluoro-polymer coating, however, these typically are coated with a sprayed-on polyvinylidene fluoride (PVDF) and will need siliconization for use with glass or plastic barrels. Use of these coated stoppers provides lubricity for machinability and reduces piston clumping in feeder bowls. Additional benefits, depending on the coating used, include a decrease in particle generation and a reduction of extractables from the elastomer (27,29).

Improving protein stability: Growth in the pharmaceutical industry is expected to be driven by biotechnology products and vaccines. This will be associated with significant challenges in the formulation development of proteins such as monoclonal antibodies, as they are typically administered in high doses. High-concentration proteins have a propensity to interact with each other and with the packaging components and cause protein instability, especially when the volume of delivery is approximately 1 mL. Challenges with glass PFS typically encompass breakage, presence of particulates, glass reactivity to the drug product and potential leachables including silicone, tungsten and adhesive. A plastic PFS offers options to solve such challenges. A plastic PFS can eliminate silicone, tungsten and adhesive, depending on the quality attributes of the entire prefillable system. For instance, the CZ insert needle system uses no silicone for syringe functionality, no tungsten (commonly used during the glass syringe forming process) and no adhesive (commonly used to hold the staked needle in place).

There are reports that the detachment of silicone oil in water-filled syringes is possible (30) and can result in particulate matter and clouding phenomenon. Silicone oil interaction has been suspected as being responsible for aggregation in protein pharmaceuticals. Several publications in the 1980s have discussed this issue, especially with regard to the aggregation of insulin in disposable siliconized plastic syringes (31–33). Surfactants such as polysorbates have been used extensively to prevent/inhibit protein surface adsorption and aggregation under various processing conditions (34,35). One consequence with using polysorbates in protein preparations

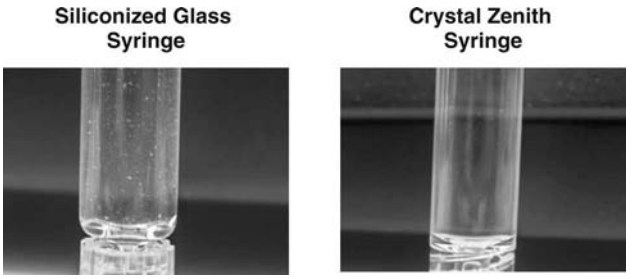

Figure 5 Aggregates in siliconized syringes and silicone-free syringes.

is their potentially adverse effect on protein stability, including the oxidative damage of the residual peroxides in Tweens, which are generated during processing or storage (36). This can pose a serious problem affecting the shelf-life of products. Polysorbates and their concentration should be selected carefully. In addition, the choice of a suitable container will help mitigate significant risks of protein aggregation caused by silicone oil. The propensity of proteins to aggregate when silicone oil is present in formulation was further investigated by Esfandairy et al. (37). Silicone oil–induced aggregation of proteins was studied on silicone oil–free plastic syringe systems and siliconized glass PFS systems. The study included model proteins at low concentrations of 0.35 mg/mL to as high as 25 mg/mL. Although no unambiguous generalization was drawn at lower concentration, there was a clear effect at protein concentrations as high as 25 mg/mL. Effects on protein aggregation with silicone oil were observed during air shipment of samples, caused by effects of agitation and vibration. The study showed that the extent of aggregation in silicone oil–free CZ syringes was less compared with siliconized glass syringes under the conditions examined (Fig. 5). The study recommended that the susceptibility of therapeutic proteins to silicone oil–induced aggregation be investigated on a suitable container closure system before finalizing stabilized formulations and container selection.

Various methods are used to siliconize syringes, including stationary nozzles and diving nozzles. Recent studies have shown that (16) silicone oil distribution is often nonuniform, leaving certain areas of the syringe surface without any silicone oil. The low or inconsistent silicone oil coating can have a significant impact on the piston travel/glide forces, especially in the use of autoinjectors. In 2006, lots of Neulasta® delivered by an autoinjector containing a glass PFS were recalled in a number of European countries because of problems with slow or incomplete delivery of the drug (38). Areas of nonuniformity cause travel forces to increase, causing failure or incomplete injection. In addition, there has been significant attention to tungsten as a leachable present in glass PFS. These reports discuss tungsten-based particulate matter leaching and interacting with the protein drug product (39). Tungsten pins are typically used to keep the fluid path open at the nozzle end of the syringe at around 1200°C during the glass syringe forming process. Upon cooling, a needle is staked-in with adhesive, to make a glass PFS with a staked needle. The residual tungsten had migrated into the drug product and caused the protein to form protein-tungsten aggregates. Although this appears to be protein specific, it is important to test for protein-tungsten interaction at an early stage of drug development. In another case, a residue was observed in a PFS during the manufacturer's inspection. Upon investigation, the material was identified as poly (metaxylylene adipamide), a component of the glass fiber pin use by the syringe developer during the needle assembly and curing process (40). Such concerns may be mitigated with the use of COP/COC syringes. Silicone oil–free CZ syringes have been shown to have consistent travel forces over time and temperature. The dimensional tolerance of plastic syringes and consistency of syringe functionality will provide a predictable operation of a drug product–filled autoinjector. CZ syringe systems have no tungsten as a leachable because the needle is insert-molded, avoiding the need for tungsten pins and adhesive, which

are typically used with glass staked-needle syringes. The manufacturer has developed a PFS system intended for biopharmaceutical drug delivery that is free of silicone oil, tungsten and adhesive (41).

Process considerations: In the current market environment, presterilized, ready-to-fill syringes are increasingly more prevalent. PFS are now available in sterile and ready-to-used formats. As glass PFS are already being filled using tubs, a switch to PFS in a similar tub and nest configuration has been achieved using the same filling machines, with minor modification and change parts to accommodate plastics. Most commercial filling companies can accommodate plastic syringes. The control of dimensional tolerances of plastic syringes far exceeds that of glass syringes and, because they are less prone to breakage and shattering, plastic prefilled syringes are generally easy substitutions for glass PFS on modern filling/processing equipment. There are, however, some physical differences between glass and plastic that should be considered before running plastic PFS on a filling/processing line designed for glass PFS. Plastic syringes are prone to scratches and cosmetic defects from contact with metal surfaces in processing equipment and the weight of plastic PFS is less than their glass equivalents. Scratching may create an unacceptable level of cosmetic defects. Lighter weight syringes can cause problems when gravity is responsible for syringes settling into place in processing equipment. The issues of weight and scratching often manifest themselves when metal centering devices are used to hold and center PFS during filling and stoppering processes. These problems can be overcome by reengineering some parts of filling and processing equipment or by running equipment at slower speeds. It is expected that, as the use of plastic PFS becomes more prevalent, manufacturers of filling/processing equipment will design equipment that performs equally well with both glass and plastic PFS.

There are various processes for filling and stoppering PFS. These include filling and stoppering using vent-tube, or vacuum fill or/and stopper placement. Vent-tube is more commonly used for uncoated or partially coated pistons intended for glass and plastic PFS. For coated pistons, vacuum placement works well as the procedure uses differential pressure rather than force to eliminate wrinkling of the lamination. Vacuum placement is particularly important for laminated pistons, especially in CZ syringe systems, which use a piston that is coated on the drug product contact and syringe barrel contact surfaces. The piston provides lubricity for efficient piston release and consistency of travel forces for a silicone oil–free system. An option offered at Hyaluron Contract Manufacturing, Burlington, Massachusetts, for filling PFS, BUBBLE-FREE FILLING®, uses online vacuum filling and online vacuum stoppering (42). The primary advantage is the reduction of the air bubbles that exist between the product and the stopper in traditionally filled syringes. This may help mitigate concerns regarding oxidation of the product.

LARGE-VOLUME PARENTERALS

LVP refer to sterile diluents, electrolytes, irrigating fluids, blood derivatives, nutritional preparations and premixed injectable drugs administered in quantities of over 100 mL. LVP are packaged in semi-rigid plastic containers, flexible minibags and, to a lesser extent, glass containers. Three major global manufacturers of LVP include Baxter, B. Braun and Hospira. The sterile formulation of LVP necessitates the use of containers with good barrier properties and sizes of semi-rigid plastic IV containers range from 250 mL for biologicals and nutritionals up to 4 L for standard diluents (such as sodium chloride and dextrose).

Minibags are used for administering lower-volume parenteral admixtures, and most premixed IV solutions are packaged in specially designed minibags. IV minibags usually contain 50- or 100-mL volumes of solution and are made of PETG, PP and various polyethylene-based coextrusion. These containers provide a sterile format consisting of a drug mixed with an appropriate diluent solution. Premixed minibags eliminate the need for independent admixture preparation and provide significant time, labor saving and waste

reduction advantages. Most major parenteral drugs are now available in this format, including drugs for antibiotic, analgesic, anticonvulsant, cardiovascular, psychotherapeutic and respiratory preparations. Some solutions packaged in the container must be stored frozen and thawed no more than 24 hours prior to use.

Historically, PVC was the leading material employed in the production of LVP configurations. However, this trend has changed because of potentially adverse patient reactions to a plasticizer used to stabilize the resin. Known as DEHP, the plasticizer has been linked to infertility and hormonal imbalances in laboratory animals. Regulatory authorities have recommended that all medical products based on PVC and DEHP be either adapted to alternative materials or include a label warning about the plasticizer. In response, the producers of IV solutions have adopted newer plastics for their containers. B. Braun Medical eliminated the used of PVC in IV packaging. The company's Excel® and PAB® IV containers now include specialized PP materials. Newer, higher-grade plastics, such as PETG copolyester, are being used for minibag applications to keep solutions stable, including Baxter's and Hospira's products. Baxter International recently introduced Buminate® human albumin solution in a Galaxy® minibag that is composed of proprietary, high-barrier plastic film. The new Galaxy package can provide a shelf-life of two years and eliminates the need for preparing admixtures in hospital pharmacies. Hospira's ADD-Vantage® system is a specially designed diluent container that connects to a vial. Once the vial is affixed to the container, the active drug blends with the diluent and creates the finished IV solution. The ADD-Vantage system allows the IV solution to be mixed directly at the site of administration. Another innovative IV minibag system is the Duplex® Drug Delivery System developed by B. Braun. Duplex is a dual-compartment flexible plastic IV bag that stores unit dosages of drug powder and diluent separately in the same container. The health care professional squeezes the bag to break the quick-release seal, mixing the drug and diluent just prior to administration. Designed to simplify the intravenous delivery of antibiotics, the Duplex container reduces product waste, eliminates the use of vials from the preparatory process, and is equipped with a standard linear bar code to reduce dosage errors and track inventory.

X-ray contrast media is also packaged in a range of volumes from 50 to 500 mL in both plastic and glass containers, with the 500 mL containers labeled as pharmacy bulk packages. PP prefilled syringes and prefilled PP cartridges designed to fit a specific range of power injectors for computed tomography are available (43). Another design of a prefilled cartridge called REDIFLOW™ is available in a clear plastic to fit a second range of power injectors for computed tomography as well as PP bottles (44).

PLASTICS AS DISPOSABLE SYSTEMS FOR BIOPROCESSING

Market considerations: Plastic packaging systems for LVP drugs are facing increasing scrutiny. All packaging systems, stainless steel or plastic, need to provide and meet the same requirement for protection, compatibility and safety as those used for SVP (45). This section addresses the use of plastics as disposable bags in packaging large-volume drug substances or drug products in bioprocess development and fill/finish operations (46,47).

According to the report released by the Tufts Center for the Study of Drug Development, Outlook 2009 (48), there are more than 200 new monoclonal antibodies in development worldwide, and the FDA has approved 22 monoclonal antibodies. To support development of these biologics, the biopharmaceutical manufacturing industry is rapidly adapting to disposable systems. Single-use bioprocess systems referred to as disposables have become common in the industry. Disposable systems have gained increased acceptance for manufacturing-scale storage and processing of recombinant proteins and monoclonal antibodies in liquid and frozen forms (49,50). This is driven primarily by the key benefits plastic disposable containers offer over stainless steel containers. These include reduced capital expenses (stainless steel vessels, cleaning and sterilization validations), minimizing cross contamination, flexibility in manufacturing and easier scale up (51,52).

Disposable technology employs a multitude of plastics to customize processing and may include bags, filters and tubing. Plastic materials that make up the critical

Table 4 Commonly Used Plastics in Disposable Systems

Disposable bags	Polyethylene, ethylvinyl acetate, PVDF
Filters	PTFE, polypropylene, PVDF
Tubing	Silicone, PTFE, PVDF

Abbreviations: PVDF, polyvinylidene fluoride; PTFE, polyethylene tetrafluoroethylene.

components of a disposable system include filters (e.g., Millipore, Sartorius, Pall, GE Healthcare), tubing (e.g., Amesil, Saint Gobain), and disposable bags (e.g., Hyclone, Stedim, TCTech, Pall). Disposable bags are larger volume containers that are used for large volumes of drug substances or products and have the greatest dwell time of product exposure. These bags are used in upstream and downstream bioprocessing and in fill/finish operations, examples include media preparation, bioreactor, storage and transportation. Multilayer bags are typically used and are intended to maintain product integrity. These bags provide gas and moisture barrier properties, functionality after sterilization, durability and biocompatibility (Table 4). Very few materials possess a balance of properties in one layer and PVDF film may be the best solution (47). The outer layer of a multilayer bag provides durability, and many materials are used with varying thickness. These materials are made up of nylon, polyesters, ethylvinyl acetate (EVA) and polyethylenes. As a sandwich layer, ethyvinyl alcohol (EVOH) is commonly used. EVOH has extremely low gas permeation and excellent barrier characteristics. Because it has a propensity to absorb moisture and lose its barrier property, it is sandwiched in a multilayer bag. LDPE is commonly used as the drug contact layer because of its good chemical compatibility profile. While EVA films are typically considered superior as the product contact layer, there are limitations to large-scale manufacturing of EVA film, and, consequently, LDPE becomes a good alternative, especially with three-dimensional bags such as those used in disposable mixing applications.

Many factors are usually considered during the design phase when choosing a disposable bag. Two important questions to be addressed are: Is the plastic polymer safe and is it compatible with the solution it is in contact with? Several facets related to the qualification and selection of a disposable container must be considered to address these questions. This includes a validation package from the vendor with information related to the materials of construction, sterility, USP plastic class VI data, extractables, heavy metals, particulates, pyrogens and cytotoxicity testing from the vendor. This information—in combination with knowledge of the drug substance or drug product processing that may include processing volumes, chemical stability, compatibility, number of campaigns, formulation components, processing conditions such as temperature, pressure and, most importantly, extractable and leachable considerations—can provide insights into the choice of disposable bag for bioprocessing. The primary considerations should include:

Chemical resistance study: Chemical compatibility studies should be conducted to evaluate the choice of a single-use container prior to its selection. The tests can include weight loss, clarity, visual inspection, drop test, tensile strength, thickness of the film and testing using various solvent systems including buffers, organic solvents or other components that may be intended for drug product development. For most aqueous formulations, the plastics (e.g., LDPE, HDPE, PP, etc.) have an acceptable compatibility profile. However, organic solvent usage may cause incompatibility issues. Emerging disposable systems bags such as PVDF, which has a chemical compatibility profile similar to Teflon®, may offer options for accommodating formulations based on organic solvents.

Protein adsorption: Single-use systems are increasingly prevalent in downstream processing, final formulation development and in fill/finish of protein solutions. These systems gained acceptance for storage and processing at manufacturing scale of recombinant proteins and monoclonal antibodies in liquid or frozen forms. The

container-protein interactions may include protein adsorption onto the plastic container surfaces. The major driving forces influencing adsorption of protein are hydrophobic and electrostatic interactions. These interactions are responsible for nonspecific protein binding on a variety of surfaces. Interaction factors between plastic surface and protein could be affected by the physical nature of the surface (material surface or any coating), product formulation (pH, ionic strength, surfactant, etc.), storage conditions (temperature and contact time) and the concentration and conformational properties of the protein. Studies have shown a low binding level of model proteins on plastic polymeric surfaces compared with borosilicate glass surfaces. It is important to evaluate plastics using specific protein binding assays under various processing conditions, using large surface-to-volume ratios to determine their acceptability (53).

Extractables and leachables: The release of compounds from the plastic may affect product quality such as plasticizers, stabilizers or solvents. Regulations mandate that the equipment and materials used in the manufacture of pharmaceuticals should not alter the safety, efficacy and potency of the final drug product. An evaluation of potential extractables is required for plastic disposable bags to ensure compliance. Extractables are substances that can be extracted from a plastic using solvent or extraction conditions that are expected to be more aggressive than the processing conditions intended. Leachables are substances that could be present in the finished product because of interactions between plastics and the drug product during the products shelf-life. The suppliers of the plastic bags or components should provide a full and complete potential extractables list which could be used to evaluate product suitability with the plastic disposable bag (54).

Sterile barrier integrity: Maintaining integrity of a disposable device is critical to protect the product from microbial contamination. When plastic bags or components are provided as sterile, the integrity of these products must be demonstrated. Container closure validation can be performed to reduce the risk of compromise. These tests may include helium leak testing, pressure testing, dye ingress or microbial ingress challenges. Guidance documents from the FDA and European Medicines Agency (EMEA) can help to define the level of validation and qualification necessary for the safety of the single-use systems. These include the FDA's guidance document issued in May 1999, "Container-Closure Systems for Packaging Human Drugs and Biologics" (45) and EMEA's guidelines on plastic primary packaging materials (55).

QUALITY AND REGULATORY CONSIDERATIONS

There are numerous plastic containers that have been used for parenteral applications, including drug products in cyclic olefin containers that have been approved for marketing in the United States, Europe and Japan (Table 3). Guidance documents from FDA and EMEA help define requirements and the level of validation and qualification needed. This guidance has been universal to encompass all plastic containers for SVP or LVP, including vials, PFS or flexible bags. The FDA document "Container Closure Systems for Packaging Human Drugs and Biologics" provides the fundamental guidance on container closure systems, including plastic materials (45). The United States has a drug master file system (DMF) in which companies provide confidential information on the manufacturing and the composition of the plastic in a type III packaging material DMF and is incorporated into a letter of authorization for referencing the DMF upon FDA review. Canada has a similar DMF system, except that packaging materials are listed in a type II DMF. In Europe, the EMEA limits the information contained in a DMF to drug substances; therefore, the drug manufacturer will usually provide the required information on the packaging system. Guidelines for plastic containers can be found in the newly revised EMEA's Guideline on Plastic Primary Packaging (55). Both Ph.Eur. and USP have chapters referencing plastic materials and plastic packaging. Ph.Eur. section 3.1 has detailed chapters on various plastics including "polyolefins," and Ph.Eur. 3.2 specifically focuses on plastic containers (56,57). USP combines guidelines for plastic containers and plastic materials in chapter <661> (58). With respect to biocompatibility, both in vitro and in vivo

biological reactivity needs to be performed on plastic containers (59,60). The quality-conscious Japanese market has seen the plastic market grow significantly for SVP. Mitigation or elimination of particulates or defects, safety, break resistance and clarity are clearly the drivers for using plastics in Japan. Key JP guidance is described under General Tests Processes and Apparatus, 7.02 Test Methods for Plastic Containers and General Information 17, Plastic Containers for Pharmaceutical Products (61).

SUMMARY

Application of plastics for parenteral delivery is expected to grow in years to come. Although PP material is more commonly used because of its availability and cost-effectiveness, there has been a recent surge in the use of superior plastics, the cyclic olefins, for parenteral delivery. The features of cyclic olefins are seen very favorable when packaging SVPs, highlighted by properties such as break resistance, glass-like transparency, better barrier properties compared with other plastics and its biocompatibility. However these features need to be balanced with the needs of a drug product, especially in the areas of oxygen or moisture sensitivities, where secondary packaging may help reduce such risks. Plastics are also favored because of their moldability and tight dimensional tolerance and can lead to newer design integrations. Examples include front finger grips, larger flanges and back stops for syringes. This capability is especially important because the home health care market is a growing segment. Many drug products are produced with the intention of being used in a home setting. Material flexibility also allows the same resin to be used in an assortment of designs, from vials through PFS systems, without substantial chemistry differences. Recently cyclic olefin syringes have become available in sterile assembled formats for ease of filling, similar to that of glass syringe packaging, making it easier for drug manufacturers to switch to plastics. Similarly sterile and nonsterile plastic vials and containers are also available.

Plastic container systems can also play a significant role in influencing the stability of a drug product. For example, they are used with drug products that would otherwise delaminate glass or with water-for-injection products to maintain pH. Recent advances in plastic PFS systems include developments in silicone-oil free and tungsten-free syringe systems that can help mitigate or eliminate any potential interaction of leachables from a packaging system. Formulators and package engineers now have more options to evaluate and optimize drug formulation with suitable packaging components at early stages of drug development. Protecting the drug product in a package that does not break or crack is a substantial benefit, especially with biological products that need low temperature storage and transport. Plastic vials are now considered in these areas. In addition, availability of plastic cartridges and plastic dual chambered syringe systems for liquid-liquid or lyophilized powder-liquid systems clearly illustrates the ability of vendors to offer such designs for various drug delivery applications. For large-volume packaging and processing of bulk drug products, plastic disposable bags are being considered. Clearly plastic disposable bags offer many benefits over stainless steel containers in downstream bioprocessing, including fill/finish operations; however, due diligence is a must for the right choice of plastic for the product. Plastics will increasingly be utilized throughout the entire total supply chain of pharmaceuticals and provide opportunities for total life cycle containment of pharmaceutical products. These opportunities can allow for the lowest total cost of ownership to be provided with plastic packaging materials.

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13 Elastomeric closures for parenterals

Renaud Janssen

SUMMARY

The present chapter in this review work intends to give insight into elastomeric closures that are used for parenterals. The single most important reason why elastomeric materials are used for closures for parenterals is that the elasticity of such materials allows for preservation of the sterility of the packaged drug, by ensuring a tight seal between the closure and the container, and by ensuring adequate resealing of the closure after penetration with a needle or with a spike in cases where this is applicable.

Of course sealing and resealing are not the only features that characterize elastomeric materials. Elastomeric closures also have benefits in that they are able to give a property profile that is an ideal combination of physical, chemical, functional and biological performance, combined with microbiological and particulate cleanliness.

This chapter is an endeavor to give the reader insight into this complex system of properties and requirements.

THE MANUFACTURING PROCESS FOR ELASTOMERIC CLOSURES

The text below describes the operation of a typical modern elastomeric closure manufacturing plant. In any such plant, irrespective of the name of the company, the major steps in pharmaceutical rubber stopper manufacturing will consist of weighing according to a recipe, mixing, preforming, molding, die-trimming, washing, drying and packing (Fig. 1).

Raw Materials

The basis for the manufacturing of rubber closures is a so-called rubber compound. It is composed of a number of raw materials.

Raw materials are quarantined upon receipt and there is a system in place for testing of raw materials for identity and purity according to specific procedures and specifications.

Upon acceptance by the control laboratory, raw materials are released for production and a raw material lot number is assigned. All relevant data are stored in a computerized raw material lot file. There are provisions in the manufacturer's quality system to protect against inadvertent use of nonreleased raw material lots.

Mixing and Preforming

Individual rubber compound batches are composed by combining the required amounts of each rubber ingredient in accordance with a formulation sheet ("recipe"). The ingredient's weight accuracy and lot numbers are stored in the compound batch file. Each weighed quantity is duly identified.

Weighing of the ingredients and composition of the individual compound batches take place in specially equipped rooms, designed for cleanliness and logical material flow. Large-volume ingredients such as fillers may be stored in silos in which case they are automatically weighed and delivered directly to the mixer, thus largely reducing the potential for dust and contributing to cleanliness of the manufacturing environment.

The compound ingredients are mixed in a Banbury type mixer. A Banbury type of mixer consists of an extremely robust mechanical chamber in which the rubber ingredients are mixed by the action of cooled cylindrical rolls that rotate into each other. Prior to introducing the ingredients into the mixer, their identity is verified.

The mixing process is highly automated and entirely computer controlled, as it functions according to a predetermined "mixing recipe." The mixer parameters that are important for the quality and the properties of the mixed material typically are constantly monitored and recorded.

At the end of the mixing cycle, the rubber compound batch is transferred onto an open mill where it is cooled and further homogenized. Next the rubber compound batches are

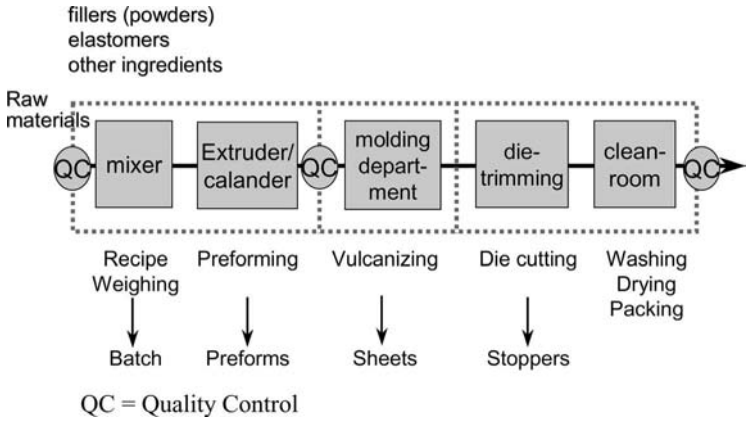


Figure 1 Elastomeric closure manufacturing process.

shaped into "preforms" with the size and weight required for molding in a particular mold. The preforming operation may have different forms. It may consist of passing the mixed and milled rubber through an extruder and cutting the extrudate into bricks of a well-defined form and weight. Alternatively it may consist of a calandering operation where the rubber coming from the calendar is cut into slabs that again have a well-defined shape and weight. At the stage of mixing or preforming typically a sample of each compound batch is checked for correct vulcanization properties by means of a rheometer test. Furthermore, a sample is sent to the laboratory for testing of physical and chemical properties. All data are stored in the compound batch file and are fully traceable.

Molding

Both injection and compression technologies may be used for molding rubber closures. The choice depends on the technical requirements and characteristics of the products.

The rubber preforms are heated under high pressure in multicavity molds. During this process the rubber vulcanizes. In the vulcanization process, by the use of cross-linking agents that are contained in the rubber compound, chemical bonds are formed between individual polymer molecules that form the elastomeric base of the rubber. It is only at the stage of molding that the rubber turns from a plastic into an elastic material, and that it acquires its required shape in the form of a vial stopper, of a plunger for a cartridge or a prefilled syringe, or of any other geometrical form that is intended to shape the rubber in.

The products leave the molds in the form of "sheets," each carrying many closures. The operators performing the molding operation typically examine the quality of the molded sheets at this stage, which marks the first quality check of the elastomeric components.

The use of modern, proprietary compression and injection molding technology, combined with proprietary mold construction technology, results in rubber closures with narrow tolerances and stable nominal dimensions.

Die-Trimming

The sheets with the products are then die-trimmed to result in individualized stoppers. This operation may take place in the immediate vicinity of the molding press or in a separate area that is designed for higher cleanliness. Die-trimming of elastomeric closures requires a trimming agent, which is typically a silicone emulsion, that is then removed by rinsing the freshly die-trimmed stoppers or, in case this is not present, in the next manufacturing step, which is washing.

Washing Process for Elastomeric Closures

The die-trimmed closures are transferred to the washing and posttreatment area. At present time rubber closures for parenteral applications are always washed, regardless of the closure manufacturer.

Washing of rubber closures typically is combined with siliconization. Siliconization of rubber closures is necessary to overcome the stickiness that is inherent to typical rubber formulations that are used for parenteral stoppers. Washing is performed to improve the state of microbiological and particulate cleanliness of the stoppers. Washing and siliconization may take place in washing equipment of various types. Very often, rotating drum type equipment is used for washing, siliconization and drying. However, the state of the art practice is that closures are washed in a pass-through machine. Loading of the closures takes place at the "dirty" side of the machine, while unloading is foreseen at the "clean" side in a room with a controlled state of cleanliness.

Various procedures exist for washing of parenteral closures. Every stopper manufacturer has its own process. More on stopper washing and siliconization can be found in a later paragraph of this chapter. At any rate washing is followed by drying with air of controlled cleanliness.

Packaging

After drying, the rubber closures are immediately packed in clean polyethylene (PE) bags, and sent out of the washing area into the packaging area where the bags are put into cardboard or plastic boxes. The plastic bags and the boxes are labeled with identification data such as product and compound code, lot number, packaging date and information on the final treatment.

In case the closures are manufactured "ready for sterilization" or "ready to use," packing takes place in dedicated functional ready-for-sterilization (RfS) or ready-to-use (RtU) bags. RfS and RtU bags are overwrapped with protective plastic bags before putting them into the cardboard or plastic boxes.

Classification of Manufacturing Environment and Environmental Controls

The manufacturing of rubber still to a large extent is an industrial process, especially in the first steps of mixing and to a lesser extent in molding. Throughout the manufacturing process it is usual that the closure manufacturer progressively implements measures to work in cleaner areas and to protect the products or intermediates from contact with the environment, including the manufacturing personnel.

In practice this comes down to implementing systematic cleaning programs in all areas, sound gowning procedures for operators, for their supervisory personnel and for plant visitors, and appropriate measures to protect products from environmental contamination. In the initial manufacturing steps of mixing, molding, and die-trimming it is not common that a closure manufacturer will classify the manufacturing areas. Exceptions to this are for new plants that are built from scratch. For washing and packaging areas, though, it is common that these areas are classified.

Classification may be done in various ways. Whereas in the past it was most common to speak of class 100 or class 1000 or class 10,000 or . . . in terms of the U.S. Federal Standard 209, today classification is mostly done in terms of International Standardization Organisation (ISO) 14644-1, "Cleanrooms and associated controlled environments—Part 1: Classification of air cleanliness" and/or in terms of the FDA Guidance for Industry, "Sterile Drug Products Produced by Aseptic Processing—Current Good Manufacturing Practice" or the EU Guidelines to Good Manufacturing Practice, Annex 1, "Manufacture of Sterile Medicinal Products" Grade A/B/C/D classification. It may be noteworthy to verify whether a manufacturer claims a classification for his manufacturing areas "at rest" or "in operation."

Classification of manufacturing areas needs to go hand in hand with the implementation of a monitoring system to demonstrate not only initial compliance but also continuous compliance. This system to demonstrate continuous compliance is then based on a sound rationale for measuring nonviable and viable airborne particulates, complemented by measurements of surface microbiological cleanliness, and in the highest degree of sophistication also of contamination of personnel gowning. Since in the final washing of closures for parenteral application, modern standards require that water of defined purity such as purified water and water for injection is used, also monitoring of the compliance of the various water types will need to be part of the manufacturer's total monitoring system.

PARENTERAL CLOSURE TYPES AND DESIGNS

The present part of this chapter gives an overview of the most important and common types and designs of closures that are used as *primary* packaging in parenteral applications. No attempt is made to review components that are used as secondary packaging such as aluminum or aluminum/plastic crimp caps. Since some closure *designs* may be proprietary to the closure manufacturer or end-user, it is impossible to put together an exhaustive listing here. This will not preclude though that the overview below is as complete as possible pertaining to closure *types*.

Stoppers for Vials and Bottles

Closures for Serum Vials

These closures are the rubber stoppers that are used for closing glass or plastic vials or bottles stemming from liquid or dry powder fills (Fig. 2).

These closures consist of a flange having a larger diameter and a plug part having a smaller diameter. The plug part fits into the vial neck while the flange part rests on the rim of the vial.

Closures in this category are usually subdivided by their size. These subdivisions include 13-mm stoppers and 20-mm stoppers for small-volume parenterals (SVP), and 28-, 29-, and 32-mm stoppers for large-volume parenterals (LVP). These sizes do not correspond with any diameter of the closure itself, however they indicate the largest diameter of the vial neck. For example, a 20-mm stopper is used for closing a vial with 20 mm as the outer diameter of the vial neck, while the flange diameter of the stopper typically is between 18.8 and 19.1 mm.

Stoppers in this category have two further features.

1. On the top of the flange there is an antistick marking. Rubber always has a tendency to stick, especially the type of rubbers that most parenteral stoppers are made of. The purpose of the antistick marking is to prevent the two large flat flange surfaces of two different stoppers from sticking together during storage of the stoppers, during steam sterilization and during filling operations at the pharmaceutical company. A well-studied design of antistick markings greatly helps in preventing clumping of stoppers in all these stages.

   The antistick markings also often delineate the target area of the stopper, that is, the area that is intended to be pierced with a needle or a spike.
2. The presence or absence of a constriction just underneath the flange. This constriction is called "blowback." Its role is to fit into a corresponding protruding part of the inner rim of the vial mouth so as to prevent the stopper after placement from rising and popping out of the vial neck. In this respect one also speaks of a "no-pop" feature or a no-pop stopper.

Significant dimensions of this type of stopper to consider are as follows:

1. Flange diameter: obviously this diameter has to be compatible with the outer diameter of the vial neck.
2. Plug diameter: obviously it has to adequately match the inner diameter of the vial neck and in forthcoming case its blowback.

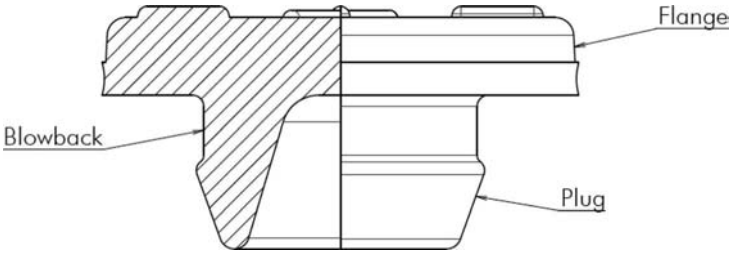


Figure 2 Section of a serum stopper with blowback.

3. Flange thickness: this dimension may be of primary importance for machineability of the stoppers on filling lines. Flange thickness should be well controlled by the stopper manufacturer.
4. Total stopper height: depending on the filling line this dimension can play an important role in stopper machineability.
5. Penetration thickness (the thickness of the stopper in the penetration area): this thickness is one of the contributing factors in determining the coring, the resealing and the penetration behavior of the stopper. Additionally, this thickness after capping of the vial determines the permeability to gases of the stopper/vial/cap combination. Given a certain rubber material, higher penetration thicknesses can lead to higher resistance to permeation of air and moisture into the vial and thus into the drug.

All these dimensions are expressed as nominal values and respective tolerances. Both are partly normalized in ISO standards such as ISO 8362-2 (closures for injection vials) and ISO 8536-2 (closures for infusion bottles).

For design purposes it is necessary to understand that tolerances of rubber parts cannot be as tight as for plastic parts. Dimensions on rubber parts as per ISO 3302-1 can be subdivided into dimensions that are determined by the rubber mold and dimensions that are determined by the rubber molding process. The former ones are tighter than the process related dimensional tolerances, however they are still larger in comparison with what is usual for plastics. With respect to serum stoppers, diameters are mold related, while dimensions such as flange thickness, penetration thickness and total height are process related.

A frequently asked question is where the effective seal between stopper and vial takes place, or which matching surfaces of stopper and vial are responsible for container/closure integrity.

For capped vials, or where under the influence of a crimp cap the underside of the stopper flange exerts a force on the top of the rim of the vial neck, it is this interface (underside flange / top of vial neck) that constitutes the seal. The permanent seal thus is not formed by the sidewall of the stopper plug pressing into the inner diameter of the vial neck. Such a seal can only be effective until the moment the vial is crimped. More on this can be found in the separate chapter of this book on container-closure integrity.

Freeze-Drying Closures

Obviously these closures are not used in powder or liquid fills but in lyophilization, or freeze-dry, applications. In the lyophilization process, the drug in its liquid state is filled into the vials. The freeze-drying closure is put on the vial in a halfway down position, so that there is a vent opening between the inside of the vial and the area around the vial. Through this opening, sublimation of the liquid takes place under the influence of underpressure in the lyophilization chamber and heat that is transmitted by the plates of the freeze-dryer. At the end of the lyophilization cycle the stoppers then are fully pressed down into the vials by the shelves of the freeze-dryer (Figs. 3 and 4).

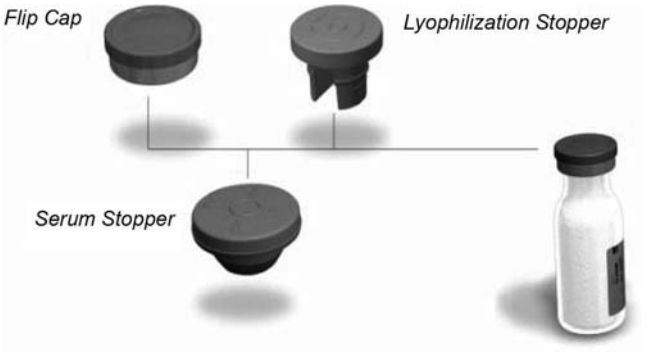


Figure 3 Various closures for serum and for freeze-drying vials.

Figure 4 Lyophilization vials and their stoppers. The vial on the right hand side has a stopper in its halfway down position before freeze-drying; the vial on the left has its stopper fully pressed down after freeze-drying. In front of the vials are stoppers showing their lyophilization opening.

Lyophilization stoppers need to be stable in the halfway down position, to allow for proper mass transfer (sublimation!), and to prevent falling off the vials during the transport between the liquid fill station and the lyophilization chamber.

The dimensions of the closure plug, including diameter and height of the zone underneath the flange to the vent opening, must provide enough surface area to contact the vial in such a way that seal integrity is not jeopardized, from the time between unloading of the freeze-drying vials from the lyophilization chamber to the moment of crimping the vials. In practice several hours may develop between these two time points. However, if the closure dimensions are too large, then interference during initial insertion and during full insertion of the lyophilization closure may pose a problem.

Antistick markings in general are designed as part of the closure to prevent sticking/mating of stoppers during bulk transportation and within feeding lines. Another primary function of these markings with respect to lyophilization closures is to prevent closure adhesion to lyophilizer shelves upon full insertion of the stoppers. If stoppers at this stage adhere to the shelves, vials containing the freeze-dried product remain stuck to the shelves when they retract after pushing down the stoppers. This leads to undesired problems when the freeze-dryer is unloaded and to unacceptable product loss (Fig. 5).

In view of the moisture sensitivity of many freeze-dried drugs, it is clear that for lyophilization closures, penetration thickness and good control of it is of even higher importance than for serum closures.

Like serum stoppers, freeze-drying stoppers can be subdivided by their size. Most commonly found are 13- and 20-mm stoppers. Standards on freeze-drying closure design can be retrieved and ISO 8536-6 (infusion stoppers for freeze-drying) and ISO 8362-6 (infusion stoppers). Notwithstanding these standards, the market offers freeze-drying closures in a broad variety of designs, especially with respect to the design of the plug part. Each of these designs ("igloo design," "two-leg design," "three-leg design," etc.) has specific benefits in areas such as stopper stability, behavior upon reconstitution of the vial contents, and ease of withdrawal of the reconstituted from the vial (Fig. 6).

Components for Prefillable Syringes and for Cartridges

More and more drugs are packaged in prefillable syringes or cartridges, in addition to or instead of a vial presentation. Prefillable syringes are claimed to have distinct advantages over vials, including ease of use, dose accuracy and minimization of product loss in the emptied packaging.

The market offers many different presentations of prefillable syringes and it is impossible to list them all here. They consist of a series of components of various natures, but at a minimum have a barrel in glass or plastic, plus (at least) two different elastomeric sealing components.

Figure 5 On the left, a picture of a pilot scale lyophilization chamber. Vials are placed on the shelves. The shelves can move so that they can bring the stoppers from their halfway down into their fully pressed down position. On the right, a picture of a shelf with vials after unsuccessful insertion of the stoppers. Stoppers got stuck to the shelf that pressed them down!

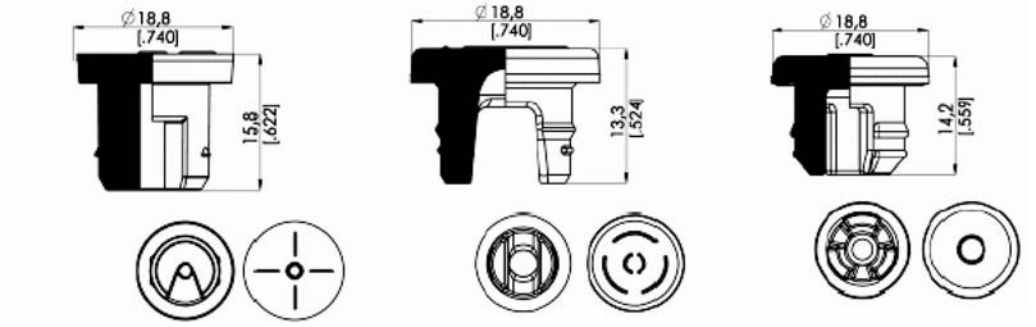


Figure 6 20-mm lyophilization stoppers in various product designs.

- An "internal" component that makes a seal on the internal diameter of the barrel. This component most commonly is called a "rubber plunger," sometimes also a "plunger stopper." After filling of the syringe this plunger is in long-term "intimate" contact with the drug, just as the cavity of the stopper plug is in case of a vial application. During the drug shelf life the plunger must maintain an adequate seal on the inner side of the barrel. However, at the time of administration of the drug to the patient, the plunger also must exhibit efficient gliding behavior in the barrel to adequately transfer the syringe contents into the patient.
- An "external" component that makes a seal between the inside of the syringe and the outer world. Basically the syringe is delivered with either a needle already being present ("staked needle") or with a prevision to place a needle at the time of administration. In the first case the needle will be protected by a rubber needle shield, also called "cover" or "sheath." The tip of the preassembled needle will stick into rubber at the interior of the needle shield, while the opening of the needle shield forms a seal on the tip of the syringe.
- In syringes without staked needle, the latter function is taken over by another rubber component, called "tip cap." The inside of the tip cap takes care of forming a seal on the tip of the syringe.

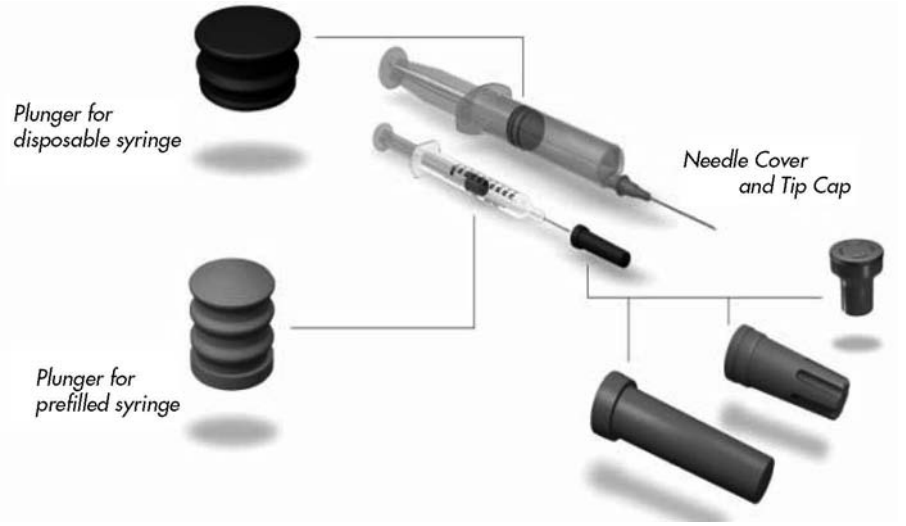


Figure 7 Elastomeric components for prefillable and for disposable syringes. Plungers on the left, needle shield and tip caps on the right.

- Even if the contact area between the syringe contents and the external rubber component may not be claimed to be zero, it is clear that this contact is less "intimate" in comparison with the contact the elastomeric plunger has (Fig. 7).

Whereas needle shields and tip caps in the past were found as components made purely out of rubber, today's tendency is to put these items into a plastic cover and, assembled in this way, to mount them onto the syringe barrel. In this case the market speaks of "rigid needle shields" and "rigid tip caps." Rigid needle shields and rigid tip caps offer or can be designed to offer enhanced product features, including tamper evidence for the syringe and extra protection against needle-stick at the time of drug administration.

Plungers for prefillable syringes are standardized by ISO 11040-5. At the time of writing there is no standard for elastomeric needle covers or tip caps.

Another prominent tendency at this time is to package drugs in cartridges. These cartridges may be intended to be used in self-administration devices, like insulin pens or growth hormone pens, or may be intended for administration by medical staff. The most well-known example in this class is a cartridge with a dental anesthetic. Like prefillable syringes, cartridges are equipped with rubber plungers. However, the second sealing element most frequently consists of a rubber disk being assembled in an aluminum cap. The cap with assembled elastomeric liner is crimped onto the front end of the cartridge. In this case, two rubber components (plunger and disk) are in long-term contact with the drug. At the time of administration the disk is perforated by a double-ended needle, one end making contact with the cartridge contents and the other end being the patient end (Fig. 8).

Typical for nondental applications, such as insulin and growth hormone cartridges, is that the cartridge contains multiple drug dosages. After administration of each dose, the rubber disk must adequately reseal so as to preserve drug sterility, and at every next dose the plunger must again smoothly move over a small distance.

Information on standardization of plungers for dental cartridges and plungers for pen systems can be found in ISO 11040-2 and ISO 13926-2, respectively.

Components for Disposable Syringes

Apart from prefillable syringes and cartridges a very large amount of rubber plungers, sometimes also called "gaskets," are used in disposable syringes that are used to administer parenteral products to patients.

Figure 8 Dental cartridge components.

A similarity between a disposable and a prefillable syringe is that in both cases the plunger must be able to move smoothly, with a well-controlled force to start the movement and with a well-controlled force to sustain the movement as long as this is needed. A very important difference between the plungers in prefillable and in disposable syringes however is the contact time with the drug. For a prefillable syringe this time is expressed in years, whereas for a disposable syringe plunger it will be minutes or hours. This difference has a large impact on the type of material that the plunger is made of. A prefillable syringe plunger will be designed to ensure adequate gliding behavior as well as to aim for low levels of material that could be extracted from the rubber into the drug product as a leachable, while disposable syringe plungers will be designed primarily to ensure acceptable administration behavior.

Plungers for disposable syringes are standardized to some extent by ISO 7886-1.

Other Components

There are many other elastomeric components used in parenteral products, other than the ones listed so far. Among the products that are in long-term contact with parenteral drugs it is worth mentioning here parts that are used in special systems such as dual chamber syringes or vials with two compartments. In the category of short-term contact products certainly components for injection ports on flexible bags and parts used in blow-fill-seal applications take a large part.

RUBBER COMPOUNDS FOR APPLICATION IN CLOSURES FOR PARENTERALS

This part of the chapter contains information on the composition of elastomeric closures for parenterals and explains which rubber compounds are suitable in the various applications.

General Outline

The main characteristic of an elastomeric material is its elasticity. Elasticity is introduced by cross-linking the polymer chains of the elastomer base of the material by using cross-linking agents. This cross-linking process, also called "vulcanization" or "curing," uses curing agents that make chemical bonds between polymer molecules. The vulcanization takes place under the influence of temperature and pressure in a heated mold. During the vulcanization the rubber will adopt the shape of the cavities of the mold in which it is being cured. In this sense one speaks of "thermoset" rubbers.

Before vulcanization, the elastomer behaves in a plastic way, as mechanical deformation will result in a permanent deformation. By cross-linking, the elastomer turns into a rubber. After vulcanization the resulting rubber material behaves in an elastic way, and as such after imposing and taking away a mechanical deformation the material will regain its original shape.

The total set of materials that are used in rubber compounds can be listed as follows:

- *The elastomer*: It is the polymer base of the compound. A rubber compound may either use one single elastomer or a blend of different elastomers. The type of polymer(s) will heavily influence a number of characteristics of the resulting rubber.

- *The cure system*: It consists of a defined set of chemicals that take care of the cross-linking reaction. This set not only comprises the actual cross-linking agent that makes the chemical bonds, but also other chemicals that activate or accelerate the cross-linking reaction.

  There are many types of cross-linking agents of which sulfur for sure historically is the best known. Other types are phenol-formaldehyde resins, peroxides and amines. A well-known activation system is zinc stearate or zinc oxyde in combination with stearic acid. The zinc ion therefore may be readily found in aqueous extracts of quite a number of rubber materials.

  Special caution shall be given to the use of accelerators in rubber compounds for parenteral applications. In fact, these accelerators typically are organic molecules like thiurams, sulfenamides and thiazoles that are relatively easily extractable and some of which, like 2-mercaptobenzothiazole, are directly linked to health hazards, while others may give rise to the formation of hazardous reaction products as nitrosamines. Modern, unconventional curing systems for parenteral rubber compounds therefore will avoid the use of such accelerators.
- *The filler*: It attributes mechanical strength to the rubber compound. In modern parenteral applications the fillers that are used most commonly are inorganic mineral materials like aluminum silicate (clay) and magnesium silicate (talc). Carbon black, which is commonly used in other rubber applications, is avoided for use as filler for parenteral applications. This is due to the potential link with polynuclear aromatics (PNAs) that may pose a health hazard.
- *The pigment*: It attributes a color to the compound. In parenteral applications most components are gray, red, or black. The gray color is obtained by incorporating titanium oxide (white) and minor amounts of well-defined carbon blacks. The red color comes from the use of red iron oxide. Pigments for rubbers for parenteral application preferentially are not of organic nature, again because they may be extractable.
- *Other rubber ingredients*: In this class are various materials that either influence the physical properties of the rubber, like plasticizers, or the physicochemical stability of the rubber compound, like antioxidants and antiozonants, or the surface state of molded products, like migrating plasticizers or waxes. Modern parenteral rubber formulations will use these ingredients only if really needed and at any rate their extractability will be a design factor in the development of the compound.

Halobutyl Compounds

For parenteral applications, the most widely used compounds for long-term contact applications (vial stoppers and plungers for prefillable syringes and cartridges) are pure halobutyl compounds or are blended compounds where the halobutyl polymer is the main elastomer.

There are three major reasons for this. First, halobutyl elastomers allow for the lowest possible gas permeability of polymers that are available worldwide on an industrial scale. For sure in parenteral applications, where oxygen and moisture permeability are an issue, this is of the highest importance. Also, even if it cannot be linked one to one, low gas permeabilities are linked to lower absorption characteristics, especially with respect to preservatives that are present in parenteral formulations, and with lower leaching characteristics into the drug.

Secondly, halobutyl compounds allow using the cleanest curing systems. Accordingly, vulcanization can be obtained using the smallest possible set of curing agents with low extractable potential.

Thirdly, halobutyl elastomers, thanks to their low level of unsaturation, have extremely good ageing characteristics. This allows working with the lowest possible antioxydant levels, thus again preventing extractable and leachable issues, and still achieving a shelf life of multiple years.

Traditional halobutyl elastomers are obtained by polymerization of isobutylene and isoprene, followed by chlorination or bromination of the resulting copolymer. In the mid-1990s

an even more stable brominated copolymer of isobutylene and para-methylstyrene was brought to the market. This new elastomer at present time is used in a small number of parenteral rubber compounds only.

It is to be noted that nonhalogenated copolymer of isobutylene and isoprene, named butyl elastomer, equally may be in use for parenteral applications. Little or no new rubber compounds based on butyl elastomer are however offered to the market anymore.

A frequently asked question is whether bromo- or chlorobutyl is to be preferred. The answer is that principally bromobutyl cross-linking can still be achieved in a "cleaner" way, however the difference with chlorobutyl cross-linking is not of practical relevance. In fact, the use of bromobutyl or chlorobutyl compounds can be linked to a historical or even geographical background. Furthermore, it is very often forgotten that it is not so much the elastomer that is responsible for the chemical cleanliness of a parenteral rubber compound, but rather the rest of the compound recipe!

Poly-isoprene Compounds

Whereas halobutyl compounds stand for impermeability, chemical cleanliness and high stability, it is difficult to achieve with these materials the levels of elasticity that are required in some parenteral applications. Notorious in this respect are multipuncture applications, as encountered for instance with stoppers on insulin vials or with rubber seals on cartridges containing insulin or growth hormone. If the number of penetrations with a needle is tens of times—design specifications sometimes are over 100 times—it is not possible to ensure proper functionality in the sense of adequate resealing and of absence of coring with a pure halobutyl compound. For these applications historically natural rubber compounds or blends of halobutyl and natural rubber or laminates of these two materials were used. Since the last decade of the 20th century however, natural rubber has been largely phased out for use in pharmaceutical and medical rubber since, justifiably or not, it is associated with the risk of "latex allergy." Synthetic poly-isoprene has replaced natural rubber in most applications.

While mechanically superior to halobutyl compounds, poly-isoprene compounds fall short in other areas that make halobutyls so performant for pharmaceutical applications. Oxygen and water vapor permeability of poly-isoprene compounds are one to two orders of magnitude larger than for halobutyl materials. Poly-isoprene compounds also require more complex cure systems, which often means less pure and / or higher concentrated cross-linking agents. Residuals of the cure system in a number of cases may migrate to the surface of poly-isoprene components ("blooming"). Ageing characteristics of poly-isoprene compounds need to be improved by incorporating higher levels of antioxidants and in forthcoming case by including antiozonants.

In a number of applications components made of distinct layers of a halobutyl compound and of a poly-isoprene compound are able to bring a solution that offers the best of both worlds. This type of solution can be applied in the case of seals on insulin cartridges, where the rubber disk may be a laminate consisting of halobutyl material facing the drug and with a poly-isoprene side not in contact with the drug, however ensuring perfect resealability upon multiple puncturing. Unfortunately, such a laminate solution is not industrially feasible for vial stoppers.

Other Compounds

Whereas most parenteral applications call for low permeability compounds, some do just the opposite. The most important example is that of an elastomeric needle shield for a prefillable syringe. In a lot of cases these needle shields are preassembled on the cleaned and siliconized barrels of prefillable syringes with staked-in needles, packaged in gas permeable tubs and then subjected to ethylene oxide sterilization. Since the open end of the needle shield forms a hermetic seal on the hub of the syringe, the ethylene oxide must be able to permeate through the wall of the rubber shield to have its sterilizing effect on the needle that is covered by it. The needle cover thus must have a high instead of a low gas permeability. Rubber compounds used for these needle covers, and partly also for tip caps for prefillable syringes, therefore are made of poly-isoprene compounds, or alternatively of a compound based on a styrene-butadiene elastomer [styrene-butadiene rubber (SBR)]. The latter also displays a suitable gas permeability for this application.

The use of compounds other than halobutyl, poly-isoprene and SBR on the parenteral scene is for the most part restricted to niche applications. Examples are nitrile rubber for use in combination with mineral oil based drug formulations, which is often seen in veterinary applications, and silicone rubber in ophthalmic applications.

COATED CLOSURES

The compounds for elastomeric components for long-term contact with parenterals are designed to have no or the smallest possible level of interaction with the drug. For most applications, halobutyl formulations are able to achieve this goal. However, in a number of cases requirements are so high that halobutyl compounds are not adequate. Worth mentioning in this respect are biotech drugs that are used in very small quantities per dose and where no absorption by the vial stopper is allowed. Another example is cephalosporins, which in contact with halobutyl stoppers always tend to develop a measurable level of turbidity that in a number of cases is not deemed to be acceptable.

For such applications, solutions are offered to the market in the form of coated vial stoppers and coated syringe plungers. The two products that have established an accepted market position utilize fluoropolymer coatings, at least in the contact zone with the drug. Depending on the manufacturer of these coated components, the coating may have a different level of fluorination, but always will be high. Also, in all cases the coating will exhibit barrier behavior between the rubber component and the drug. This means that leaching of materials from the stopper into the drug and from the drug into the stopper is further suppressed. This in combination with the inert nature of the fluoropolymers that are used leads to better stopper/drug compatibility.

It is important to point here to the fact that the barrier function of coatings is not absolute. While extractables and leachables will be reduced, this will not be to a level of zero. The level of extraction will in part be dependent on which extractable is involved, as to whether the barrier function of the coating will be stronger or weaker. Where fluoropolymer coatings are not barriers is against water vapor. Fluoropolymer coatings thus are not suitable for preventing uptake of moisture during steam sterilization.

A difference between the two types of coated closures in the market, apart from the identity of the fluoropolymers, is the area in which the barrier coating is applied and how it is applied.

The first type starts from a fluoropolymer film that in a special type of molding process is applied to the closure in the contact area with the drug only (the largest part of the plug for a vial stopper). Other parts of the stoppers, including the topside, sidewall and underside of the flange and the part of the plug immediately underneath the flange, are left uncoated. This allows for achieving compatibility improvement with the drug with a thicker film of fluoropolymer material. The top part of the flange of these stoppers still needs some sort of siliconization to avoid stopper clumping during transport and machining. Equally it is debatable whether the entire drug contact area is coated or not.

The second type of coated closures uses fluoropolymer that is deposited on the closures in a proprietary type of spray coating process. The coating in this case is thinner, however still clearly exhibits a barrier function. This process enables coating of the entire closure, not only in the drug contact zone but also in all other areas. Since the coating is nontacky in itself, these closures do not require any surface siliconization, which in applications where the drug is sensitive to silicone of course is of highest value. Also coating of the sidewall of the flange is of help in prevention of formation of particulates during machining of the stoppers in feeding bowls and in chutes.

Fluoropolymer coated closures are available as vial stoppers and more recently also as plungers for prefillable syringes. Coated vial stoppers may require minor adaptations to the settings of filling machine but for the rest do not require too much attention in terms of machineability. This is different for coated plungers, especially when they are strongly mechanically stressed when they are inserted into the barrels of the syringe. At this stage the coating may start to exhibit wrinkling which worst case may lead to marginal sealing behavior on the inner diameter of the syringe barrel. Precautions to prevent this are indicated, either by using a suitable filling technique or by using adapted machine parts.

Coated closures mostly are encountered in high value applications, like biotech drugs, or for silicone sensitive drugs like some proteins. Since these closures require the use of costly fluoropolymers plus the use of extra process steps to apply the coating, the cost of coated closures is considerably higher than for uncoated closures. In spite of their superior product properties this high cost precludes their more widespread use, especially in cases where the cost of the component is not negligible compared with the cost of the drug.

PROPERTIES OF PHARMACEUTICAL RUBBER AND OF CLOSURES

This part of the chapter gives an overview of the most important properties that are or can be of interest for closures for parenteral application. The overview lists both properties of the elastomeric material itself and properties of components made thereof.

Physical Properties

Hardness

Hardness is the physical property of a rubber that is most apparent to the user since manipulating the closure or penetrating it with a needle gives an idea of its hardness. The hardness of a rubber is determined by a number of factors. The most important ones are the ratio of filler to elastomer and the presence or absence of a plasticizer. For a given compound system hardness will increase with increasing the amount of filler relative to the elastomer. Hardness of closures for parenteral applications is usually in three ranges: soft, hard and intermediate. The softest formulations can be found in applications where resealing is of critical importance, such as in injection points for flexible bags. These formulations tend to have no or only a low amount of filler. Most vial stoppers on the other hand are in an intermediate range. Softer stoppers, in as far as they do not contain a plasticizer, are made of formulations with relatively little filler, while in harder stoppers the ratio of filler to elastomer is higher. The hardest formulations for parenteral applications will be found in syringe plungers. The background for that is that gliding forces for harder formulations are more favorable than for softer ones.

There are numerous scales in which hardness of materials is expressed. Hardness for rubber formulations for parenteral closures though is expressed in Shore A. Values that are encountered in practice are in a range of grossly between 30 and 55, with exceptionally numbers up to 65° to 70° Shore A.

Hardness of rubber formulations is measured according to standardized methods on test buttons of standardized dimensions. ISO 7619-1, "Rubber, vulcanized or thermoplastic—Determination of indentation hardness—Part 1: Durometer method (Shore hardness)" is such a method. As the title already indicates the hardness of a rubber is determined by measuring the indentation depth of a standardized "pin" into the test button. There is often confusion about the fact that the value that results in this way cannot be reproduced by measuring on the rubber product (stopper or plunger) itself. Values measured on closures therefore will often be out of the hardness range that the closure manufacturer specifies on their data sheets.

Ash Percentage

Ash percentage measures the portion of noncombustible material in a rubber compound. This comes down to measuring the portion of material of inorganic nature to material of organic nature in the rubber material. Inorganic materials in rubber compounds for parenterals are primarily fillers, and to a lesser extent the pigment and potentially a portion of the cross-linking system. Materials of organic nature in rubber compounds are of course the elastomer, and also potentially a plasticizer. Since the primary inorganic and organic constituents are filler and elastomer, respectively, and since hardness is primarily determined by the ratio of these two, it is not surprising that hardness and ash percentage are linked to each other. Basically they yield the same information about the rubber formulation. Hardness though is less laborious and less cumbersome to measure in comparison with ash percentage. A standardized method to measure ash in rubber is ISO 247, "Rubber—Determination of ash."

Compression Set

Rubber is used for parenteral closures because of its elasticity, or its ability to return to its original form after being mechanically compressed. Yet, rubber is not perfectly elastic. This means amongst others that if a mechanical compression is being exerted for a long time on an elastomeric component, that it will not 100% return to its original form again. The difference between the original and the final form is called "permanent deformation." There is a standardized test (ISO 815) that measures permanent deformation of rubber under standardized conditions. It expresses the permanent deformation of a test part as a percentage of the deformation that the part was subjected to. This percentage is called "compression set."

The higher compression set of a rubber is, the higher thus is its permanent deformation under influence of a mechanical load. Expressed differently, the higher is the tendency of the rubber to adapt to the shape of its environment. Translating this into practical terms for prefillable syringe plungers that are compressed for a long time into a barrel, it means that the outer diameter of plungers made from a rubber with a high compression set tends to adapt to the inner diameter of the barrel. Of course, this is not desired or at least must be under control, since the plunger is expected to yield over time a high enough force on the inside of the barrel to guarantee seal integrity before and at the time of activation of the syringe.

Parenteral applications thus call for elastomeric materials with low enough compression sets. When measured according to ISO 815 (24 hours at 70°C) compression sets for rubbers for parenteral applications will be found to be in a large range between 10% and 50%. Depending on the application this may or may not be acceptable. A typical compression set for a halobutyl compound is in the range of 15% to 40%.

It is worth mentioning here that γ irradiation has a significant impact on the permanent deformation of rubber. This means that when rubber is subjected to the simultaneous action of mechanical compression and of γ irradiation its permanent deformation will be larger than when subjected to compression alone. The difference between the two, which is also function of the irradiation dose, can, depending on the rubber, range from significant to very significant. There are rubber formulations that have an acceptable compression set but an unacceptable "irradiation set," which means that under the combined action of compression and irradiation their permanent deformation is too large to still guarantee functionality. This aspect must be taken into consideration when making selections like that of an elastomeric part for a syringe that is irradiated with the plunger being assembled.

Gas Permeability

It has been pointed out in paragraph 3 of this chapter that gas permeability is a property of major importance for elastomeric closures used for parenterals. The majority of parenteral applications call for low permeability of the rubber closure (vial stoppers and prefillable syringe plungers), however as explained in a previous part of this chapter some applications require just the opposite (needle shields and tip caps for prefillable syringes).

The two extremes of permeability in the parenteral area are formed by halobutyl rubber (low permeability) on one hand and poly-isoprene or natural rubber (high permeability) on the other hand. In between are rubbers like SBR. Relative oxygen permeabilities at 40°C for different rubber compounds as cited by literature and confirmed by own measurements are approximately 1 for halobutyls to about 10 for SBR to 20 to 30 for poly-isoprenes. Similar relative rankings apply for moisture vapor permeability measured at the same temperature. Gas permeability of a rubber primarily depends on the type of polymer, but also on other factors as the type and degree of filler. Among external factors that influence gas permeability certainly temperature needs to be mentioned, with higher temperatures causing higher gas permeabilities.

The ISO standard to measure gas permeability is ISO 2782. For pharmaceutical rubber it is however more common to refer to ASTM standards ASTM D3985 (oxygen) and ASTM F1249 (water vapor).

It is worth mentioning here that recently instruments have been introduced into the market to nondestructively measure moisture or oxygen in the headspace of individual vials. The technique is based on laser absorption spectroscopy.

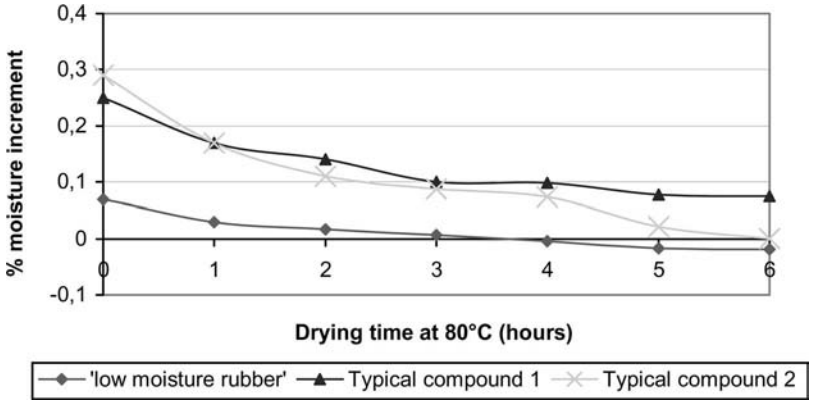


Figure 9 Moisture uptake and release of three different rubber formulations. The curves show moisture uptake at initial steam sterilization (30 minutes at 121°C) and subsequent release at drying at 80°C.

Moisture Absorption/Desorption

Water vapor permeability of the rubber compound influences the amount of water that over time will permeate through the rubber closure into a vial with medicinal product. Another factor that influences the amount of water that will permeate through the rubber closure is the amount of water that is withheld in the stopper itself at the moment it is placed on the vial. This moisture over time will partly end up in the drug product. Whereas for aqueous solutions this will not be of an issue, it can be for moisture sensitive products that are filled as powder or are freeze-dried. The lower the amount of active pharmaceutical product contained in the vial is, the more critical the situation can get. Therefore, in cases where moisture sensitivity of the drug formulation is an issue it is indicated to monitor the moisture content of the elastomeric closure at the moment of filling.

The moisture content of halobutyl stoppers in the state as they are supplied to pharmaceutical companies typically is in the range of 0.3% to 1%. It must be stressed though that by steam sterilizing the stoppers, as is usual for aseptic filling, a significant amount of extra water is "pumped" into the closures. This extra moisture needs to be dried to a level that is compatible with the moisture sensitivity of the drug application. Recently "dry" halobutyl compounds have been offered to the market, or compounds that take up significantly less water during steam sterilization while maintaining the typical drying behavior of halobutyl materials. These dry compounds target specifically lyophilization applications. Figure 9 depicts the moisture absorption/desorption behavior of such a dry compound in comparison with two "traditional" halobutyl formulations. The time point $t = 0$ represents the percentage weight increase of the stoppers as noted during a steam sterilization of 30 minutes at 121°C. The other time points represent the drying behavior at 80°C as found during laboratory drying. It should be noted that since the stoppers before autoclaving also contain moisture, negative values for the drying part of the curve are possible.

A standardized method for measuring moisture of elastomeric closures can be found in ISO 8362-5, "Injection containers for injectables and accessories—Part 5: Freeze-drying closures for injection vials." The principle of the method that is outlined there is a coulometric Karl-Fisher titration of the moisture that is dried off from a part of the stopper. The advantage of this method obviously is that it specifically measures moisture. Simple weight change methods to measure moisture absorption/desorption of elastomeric closures are also frequently used.

Absorption of Preservatives

Many drug formulations are stabilized by the use of preservatives like parabens, m-cresol, or benzalkonium chloride. These preservatives are added in low concentrations, however they have a tendency of being absorbed by rubber, thus loosing their effect in the drug solution. Depending on the combination of type and concentration of preservative and type of rubber

compound this absorption can be more or less pronounced. Development of new rubber compounds and development of drug formulations must take this absorption into account.

Swelling

Many drug formulations are aqueous solutions. Water to a certain extent is absorbed by the rubber closure. Where water is in contact with the closure it may cause a local discoloration, for example, a dark gray stopper may discolor to a lighter gray. This discoloration is not of any functional concern and can be reversed by drying the stopper.

In contrast with water, other drug diluents may display a higher amount of absorption into the rubber closures. They cause a clearly measurable increase in the weight and, in forthcoming cases, in the dimensions of rubber closures. In this case one speaks of "swelling" of the stopper. Swelling usually is expressed as a percentage of weight gain of the stopper.

Oils are known to make rubber swell. For example, vegetable oils over one month will typically cause a 3% to 4% weight increase in halobutyl stoppers. Usually this will not hinder the functionality of the closure. Mineral oils on the other hand will cause a much higher swelling in halobutyl stoppers and therefore are incompatible with them. In such cases either the use of special rubber formulations (nitrile rubber) or of coated closures is indicated.

Apart from the physical effect of swelling, the diluent that penetrates the closure and is absorbed there also may dissolve rubber chemicals and act as carrier for leachables into the drug solution.

Chemical Properties

Extractables According to Pharmacopeial Methods

As set out earlier, rubber compounds are composed of different materials that have been vulcanized through a curing step at elevated temperature. In contact with a drug solution some of these materials, their impurities, their reaction products or their thermal breakdown products may be extracted from the rubber closure.

A common way to make an assessment of extractables from pharmaceutical rubber is to prepare an extract of the rubber under well-defined model conditions and then, by using primarily wet chemistry methods, to measure for extractables. Such methods can be found in all major pharmacopeia, specifically in U.S. Pharmacopeia (USP) <381>, "Elastomeric Closures for Injections," in European Pharmacopeia (Pharm. Eur.) 3.2.9, "Rubber closures for containers for aqueous parenteral preparations, for powders and freeze-dried powders" and in Japanese Pharmacopeia (Pharm. Jap.) 7.03, "Rubber Closures for Aqueous Infusions." Also ISO 8871-1, "Elastomeric parts for parenterals and for devices for pharmaceutical use—Part 1: Extractables in aqueous autoclavates" is such a method.

The methods for measuring extractables in USP <381> as from 2009 on are extremely close to the methods in Pharm. Eur. 3.2.9 and in ISO 8871-1.

All three aforementioned methods use water as a model solvent and extract rubber by autoclaving it for 30 minutes at 121°C in a ratio of 1 cm^2 of rubber surface area exposed per 2 mL of water. In the aqueous extract that is obtained in this way, a number of determinations are done, including measurement of acidic or alkaline substances, measurement of reducing substances, assessment of the UV absorbance spectrum of the extract, and measurement of volatile sulfides and of zinc (both are common rubber chemicals). The results of the testing have to be within certain "type I" limits or within more loosely set "type II" limits as "fallback position." The idea behind this is that rubber for parenteral applications should be as clean as possible and thus meet the type I requirements. However for rubber articles where the mechanical requirements are so high that they cannot be met by using the cleanest cross-linking systems, the less strict type II limits allow these compounds still to qualify as "pharmacopeia compliant" or "ISO compliant."

In view of the fact that the ratio of surface area of rubber per volumetric unit of water is constant, the results for chemical testing of USP <381>, Pharm. Eur. 3.2.9 and ISO 8871-1 are independent of the size of rubber product that is extracted. Pharm. Jap. 7.03 is different. It also uses water as model solvent, however it extracts rubber in a fixed ratio of 1 g of rubber per

10 mL of water. As a consequence, for smaller rubber parts that are lighter in weight, relatively more surface area will be exposed to the extraction medium. Therefore for such small parts it is relatively more difficult to comply with Pharm. Jap. 7.03. Also the list of tests in Pharm. Jap. 7.03 is quite different from the other pharmacopeia and there is only one single set of limits.

Extractables and Leachables

No doubt the most discussed topic in the area of elastomeric closures for parenterals in the last decade has been the subject of extractables and leachables.

It has become clear that whereas pharmacopeial extractable methods are able to discriminate between cleaner type I formulations and less clean type II rubber compounds, they are not appropriate to distinguish between rubber formulations that have a general low extractable profile and compounds that are especially developed to release as little as possible to drug formulations. Also pharmaceutical companies and health authorities definitely want to know more about the specific identity of species that are released by packaging materials so that appropriate toxicological assessments can be performed.

Pharmacopeial extraction methods, with the exception of the determination of zinc, are not able to offer this. Therefore, more and more they are considered as a base level of extractable documentation that must be supplemented with more and more specific information. At the time of writing there are no standardized methods yet that describe how such additional extractable data can be obtained. However, initiatives such as the Product Quality Research Institute (PQRI) Working Group on Extractables and Leachables are underway. These initiatives no doubt over time will generate standardized methods for determining extractables under model conditions in model solvents and most likely will introduce concepts of threshold values below which extractables are accepted as safe, and above which toxicological assessments will be needed. What is then still left is the task to describe and ideally standardize the way to assess compounds from packaging materials that end up in real drug products, not in model solvents, in other words: how to assess leachables, not extractables.

A far more elaborate discussion about extractables and leachables is offered in a separate chapter in volume 3 of this reference work.

Functional Properties

Container/Closure Seal Integrity

The ultimate function of a parenteral closure is that it is able to guarantee integrity of the seal that it is forming with the container on which it is placed. Only in this way it is assured that sterility of the vial contents is preserved and that label claim specifications are met. USP <1>, "Injections," in this respect states that "containers are closed or sealed in such a manner as to prevent contamination or loss of contents." For a stopper sitting on a vial, the seal, after capping of the vial, is formed between the underside of the flange of the stopper and the top part of the vial neck. For a plunger for a prefilled syringe the seal is formed between the ribs of the elastomeric plunger and the inside surface of the glass or plastic barrel. For prefilled syringe needle covers and tip caps the seal of the elastomeric part with the cone of the syringe barrel must exhibit integrity.

USP's general chapter <1207>, "Sterile Product Packaging—Integrity Evaluation" discusses the maintenance of microbiological integrity of sterile product packaging over the life cycle of the medicinal product. Integrity testing should take place during three phases: product package development phase, routine manufacturing phase and marketed product stability phase.

Closure/vial seal integrity testing methods fall into two classes: microbiological methods and physical methods. Microbial methods include liquid immersion challenge tests and airborne microbial challenge tests. Under the physical methods there is a whole array including generally accepted dye ingression methods, gas leak methods, vacuum or pressure decay or retention methods, and relatively simple weight loss/weight gain methods.

Since closure/vial seal integrity is so intimately linked to microbial integrity and preservation of sterility, one would expect that standardized microbiological challenge test methods would have developed and could be found in the major pharmacopoeia and in international standards. This however is not the case. In none of the pharmacopeia are any microbial ingression test methods described in concrete wording, while in existing ISO standards all closure/vial seal integrity testing methods to date are physical methods, notably dye ingression methods.

At this place no extensive overview of closure/vial seal integrity methods will be given. An extensive discussion of the topic is given in a separate chapter of this volume. Also PDA's technical report no. 27, "Pharmaceutical Package Integrity," 1998, is a very useful review document.

Coring

Functional test methods for elastomeric closures that are well described in pharmacopeia are coring, penetration and resealing after puncturing. A description of test methods for closures intended to be pierced with a hypodermic needle is available in Pharm. Eur. 3.2.9, as well as in USP <381>. The test methods are the same as in ISO 8871-5, "Elastomeric parts for parenterals and for devices for pharmaceutical use—Part 5: Functional requirements and testing."

Coring, sometimes also termed "fragmentation," is the phenomenon whereby upon puncturing a stopper, small parts of the closure are dislodged by piercing or by abrasion. These small particles risk eventually being injected into patients. The latter of course is undesired.

Looking at vial closures for SVP and hypodermic needles, coring test methods consist of piercing a fixed number of closures a fixed number of times and collecting on a filter the particles that are formed by these penetrations. The number of particles that is visible with the naked eye must not be larger than a certain limit value.

Factors that influence the result of the coring test for SVP closures are multiple. A perhaps still nonexhaustive list is the following:

- *Physical properties of the closure*: Most important in this respect are the closure's hardness and tear strength which are both linked to the closure composition. In general softer closures tend to be less prone to coring. So are closures made from elastomeric formulations with high tear strength. The link between these properties and coring results however is not unique, as there are formulations with more elevated hardness that still are acceptable in terms of coring behavior.
- *Penetration thickness of the closure*: All other things remaining the same, higher piercing thicknesses increase coring tendency.
- *Single versus multiple piercing*: Clearly multiple piercing of the same closure increases the risk for coring. For closures that are intended to be pierced a high number of times, using special rubber compound formulations may be indicated.
- *Irradiation sterilization of the closure*: With quite many elastomeric closure formulations an increase in coring is seen after γ irradiation. The increase is higher with higher irradiation dose. However, typical doses of 25 kGy for various closure formulations are enough to cause coring results to go out of compliance with compendial limits. Use of specially developed compound formulations is indicated in these cases.
- *Quality and size of the needle*: Especially the finishing of the tip and of the sharp edges of the canula and the surface state of the needle are important. Dull needle tips and sharp edges that have a rough finish increase coring. The outer surface of the needle should have an adequate finish, meaning a surface that is not too rough and that is adequately siliconized, not to cause abrasion when penetrating the stopper. Thicker needles tend to yield higher coring results.
- *Surface state of the closure*: Also the surface state of the closure must be sufficiently lubricious. This can be achieved by adequately siliconizing the closure, or, in case of totally coated closures, by taking care that the coating displays enough lubricity.

- *The way the closure is pierced*: When piercing the closure out of its target area, or when penetrating it with the canula nonperpendicular to the closure surface, or when penetrating it with too high speed, the risk of coring increases.

Of course also for LVP coring is an issue. In case the LVP is contained in a glass bottle or in a Blow-Fill-Seal package ("bottelpack") the elastomeric closure will be pierced with a spike of considerably larger outside diameter than a hypodermic needle. Spikes of this type, unlike hypodermic needles, are made out of plastic. The same list of factors influencing coring as for SVPs is valid. Coring of LVP closures that are penetrated with a plastic spike is not described in any pharmacopeia. Test methods can be found in standards ISO 8536-2, "Infusion equipment for medical use—Part 2: Closures for infusion bottles" and ISO 15759, "Medical infusion equipment—Plastics caps with inserted elastomeric liner for containers manufactured by the blow-fill-seal (BFS) process." These test methods use steel spikes with specified dimensions.

Penetration Force

Elastomeric closures for parenterals must have an adequate penetration force, or a force high enough to feel some resistance upon puncturing but more importantly not too high. With respect to factors influencing penetration force again the same list as above can be used, although single/multiple piercing is not relevant for this property. Penetration force testing for SVP and LVP closures is described in the same pharmacopeial paragraphs and the same ISO standards as for coring.

Typical penetration forces for SVP elastomeric closures are between 2 and 3 N.

Resealing

Resealing of an elastomeric closure concerns its ability to perfectly reseal after being punctured and after withdrawal of the needle (or in forthcoming case the spike). Resealing must be guaranteed to preserve sterility of the vial contents before the next penetration of the closure. It is clear that resealing is only relevant for closures that are intended to be pierced more than once. Resealing of elastomeric vial stoppers for SVP's again is described in the same pharmacopeia and standards as where coring and penetration force are described. The type of test method that is found in standards always is a physical dye ingress method. A number of penetrations equal to 10 is assumed. In practice, for some drug products the number of penetrations can still be higher. In the development stage of such products this must be taken into account. SVP stoppers that are crimped on vials are pierced 10 times. Thereafter the vials + stoppers are put in a dye bath where they are subjected to an underpressure for a certain time. After atmospheric pressure has been restored it is observed that no dye has ingressed through the stopper area where the multiple piercing took place.

Applications where the number of penetrations definitely is higher than 10 are cartridges, an example of which is those that contain insulin or human growth hormone. Such cartridges are intended to be used in pen systems for self-administration by the patient. They consist of a glass barrel that is sealed at one end by a rubber plunger and at the other end is crimped with an aluminum cap containing an elastomeric liner of thickness 1.5 to 2 mm typically. At every activation of the pen system a new double-ended needle is to be used. One end of the needle penetrates the rubber liner, the other end penetrates the patient's skin. The number of activations for such pen cartridges may go up to 50 or more times. In the development stage of such products a safety factor concerning number of penetrations is taken into account, as even if the cartridge is developed to contain 50 doses, testing of resealing during system development will take place at two to even three times this number of penetrations. A perfect reseal of the elastomeric liner is difficult to realize. Substantial improvement can be achieved by using a laminate liner, or a liner that consists of two layers of nonidentical elastomeric formulations. The layer that is not in contact with the drug is made of a formulation that is specially developed with a view to multiple piercing and perfect reseal while the layer in contact with the drug is made of a cleaner rubber formulation. In practice the layer that promotes resealing (and at the same time also improves the coring behavior of the

seal) will be a poly-isoprene formulation, while the contact layer with the drug will be a halobutyl formulation.

Spike Retention Force

LVP closures are pierced with a spike. This spike is part of an infusion set that makes the connection between the contents of the LVP package and the patient. The spike will be sitting in the closure for the entire duration of the administration of the LVP to the patient. Since the LVP package itself during administration will be hung up, the spike will be remaining in a hanging position in the closure for potentially several hours. During this time the closure should exert sufficient force on the spike, so that it does not slip out of its position, also not when the patient is transported between different locations in the hospital. This force is called retention force.

Retention force testing may take place in two ways, a static way and a dynamic way. In the static testing mode a well-known weight is attached to the spike for a well-known time. During this time the spike shall not slip out of the closure, nor shall any leakage of liquid be observed in the seal area between the spike and the closure. In the dynamic testing mode the force needed to pull the spike out of the closure is measured on a force testing machine.

Methods for testing spike retention can be found in ISO 8536-2 and in ISO 15759, both of which were previously mentioned in the paragraph "coring."

Gliding Behavior

Vial stoppers take care of closure/vial seal integrity during the shelf life of the medicinal product and play their functional role when at the time of administration they are pierced with a needle or a spike. Syringe plungers partly have a different functionality. Clearly they assure closure/vial seal integrity, but obviously they are not pierced. Instead at the time of administration to the patient of the drug in the syringe they must be able to assure a smooth gliding in the syringe barrel.

When looking at the gliding behavior of syringe plungers one makes distinction between the force that is needed to make the plunger start moving and the force that is needed to sustain movement of the plunger. The former is typically called "activation force" or "break-loose force," while for the latter the names "gliding force" or "extrusion force" or "propagation force" are used.

A typical force curve for the gliding of a plunger in a prefilled syringe is given below. The curve displays the force that is needed to move the plunger as a function of the distance that the plunger travels into the syringe barrel. From this curve it follows that it needs a certain build-up of force to start the movement of the plunger. Thereafter the force to keep the plunger moving decreases. Gliding forces thus are typically lower than break-loose forces. Break-loose forces must be low enough to guarantee smooth activation of the syringe. Gliding forces equally must be at an acceptably low level. Moreover gliding forces must be continuous, or without increases and decreases. Should the movement be "interrupted," then one speaks of shattering of the syringe. Shattering obviously for the comfort of the patient must be avoided (Fig. 10).

There are many factors that have an impact on gliding behavior of plungers in a syringe. One variable for sure is the design of the plunger. Forces are higher the more surface area of the rubber part is in contact with the inside of the barrel. The number of sealing ribs of the plunger and the way they are dimensioned thus play a role. Next there are the physical properties of the plunger. Harder plunger materials tend to yield lower gliding forces. Also the barrel material has an impact. Glass and plastic barrels of the same dimensions will give rise to different gliding behavior of the same plungers. Furthermore there is the surface state of the elastomeric plunger and of the inside of the barrel. This surface apart from exceptional cases is always siliconized. The degree and way of siliconization of the plunger, the degree and way of siliconization of the inside of the barrel and the homogeneity of siliconization of the inside of the barrel over the total path length of the plunger strongly influence break-loose and gliding forces. More sophisticated application methods that guarantee better homogeneity of silicone distribution in barrels as well as methods to verify this distribution recently have emerged.

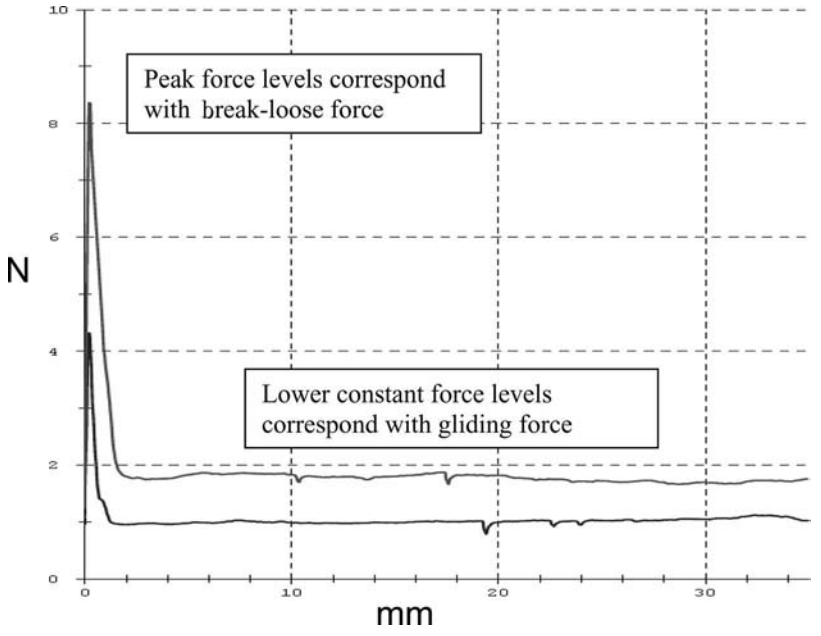


Figure 10 Gliding curves of two different plungers in the same type of barrel. The curves display gliding force as a function of the pathway of the plunger. At the left hand side, peaks correspond with break-loose (or activation force). The lower part of the curves corresponds with the gliding force for the two different plungers.

Biological Properties

In this paragraph the biological properties of materials for elastomeric closures are discussed. Discussion of the state of biological cleanliness of elastomeric closures themselves in terms of presence/absence of endotoxins and colony-forming units will take place in the next chapter.

The leading reference about biological properties of elastomeric closure materials is USP. USP <1031>, "The Biocompatibility of Materials Used in Drug Containers," spends a separate paragraph on elastomeric closures. There it is stipulated that the biocompatibility of an elastomeric material is evaluated according to a two-stage testing protocol specified in section "Biological Test Procedures" of USP <381>. Unlike plastics thus no class I-VI designations are assigned to elastomeric materials.

USP <381>, "Elastomeric Closures for Injections" in turn refers to USP <87>, "Biological Reactivity Tests, In Vitro" as the first-stage test to be performed. The tests in USP <87> are designed to measure the response of mammalian cells to specific extracts prepared from the closure material. If the requirements of USP <87> are met, then no further testing is required. If however the elastomeric material does not meet the requirements of the first-stage testing as per USP <87>, then it may still qualify as a biocompatible material by passing the "more forgiving" second-stage testing as per USP <88>, "Biological Reactivity Tests, In Vivo." USP <88> tests are designed to measure the response of animals to the injection of specific extracts prepared from the elastomeric material under test. Unlike the situation with chemical properties of elastomeric closures no class or type distinction is made between elastomeric materials that meet the requirements of first-stage testing and those that qualify as biocompatible meeting the second-stage requirements only.

USP <87> lists three possible test methods: the agar diffusion test, the direct contact test, and the elution test. In practice however it is always the Elution Test that is carried out.

USP <88> equally lists three possible test methods: the systemic injection test, the intracutaneous test, and the implantation test. Since the latter is not of relevance to elastomeric closures only the first two are carried out in practice.

Not meeting the requirements of USP <87> but still passing USP <88> is typical for elastomeric materials that use certain rubber chemicals, notably accelerators, that have a cytotoxic effect on mammalian cells as per the test conditions of the "Elution Test" in USP <87>.

The relevant ISO standard on biological material properties of elastomeric closures is ISO 8871-4, "Elastomeric parts for parenterals and for devices for pharmaceutical use—Part 4: Biological requirements and test methods." In essence however this is a copy of what is described in USP. At some places ISO 8871-4 refers to the ISO 10993 series of standards, "Biological evaluation of medical devices." Also this reference however does not preclude that ISO 8871-4 and USP come to the same result regarding biological properties of elastomeric closure materials.

Compatibility Behavior

The term compatibility behavior in the case of an elastomeric closure refers to its capability to preserve identity, strength, purity and stability of the drug product that it is in contact with. A closure that is compatible thus will not interact with the dosage form in such a way as to cause unacceptable changes in the quality of either the dosage form or the closure itself, an example of which would be by an unacceptable degree of swelling.

FDA's 1999 Guidance for Industry, "Container Closure Systems for Packaging Human Drugs and Biologics—Chemistry, Manufacturing and Controls Documentation" is the most prominent document that further discusses the subject of compatibility of primary packaging components including elastomeric closures with pharmaceutical dosage forms. This document, amongst others, lists examples of interactions, such as "loss of potency due to absorption or adsorption of the active drug substance, or degradation of the active drug substance induced by a chemical entity leached from a packaging component; reduction in the concentration of an excipient due to absorption, adsorption or leachable-induced degradation; precipitation; changes in drug product pH; discoloration of either the dosage form or the packaging component; or increase in brittleness of the packaging component."

Investigating compatibility of the elastomeric closure with the dosage form is the responsibility of the pharmaceutical company that is qualifying the closure. Changes noted during pre or postapproval stability studies thus shall be adequately addressed.

Ageing Behavior

The ageing behavior of an elastomeric closure refers to the evolution of the property profile of that closure over time. Closures that are affected by ageing will show a deterioration of some of their properties over time. By adequate studies it must be assured that this deterioration is not in conflict with the shelf life of the dosage form that uses that particular closure.

When ageing has an effect on an elastomeric closure, then that will most likely be seen in either the surface properties or the functional properties of the closure.

In terms of surface properties various effects are possible. One of those effects is that over time ingredients of the rubber migrate to the surface and form a layer there that is different in composition compared with the bulk of the article. The phenomenon is also known as "blooming." Blooming ingredients typically are low molecular weight ingredients like accelerators, oils and waxes, and fatty acids and their salts, like zinc stearate. Blooming will have an effect on the chemical properties of the closure. Blooming clearly can only occur with rubber formulations that contain certain rubber ingredients. Avoidance of these ingredients is indicated. If this is not possible, then only storage under well-controlled conditions can help to suppress surface migration.

Another ageing effect is the change of the skin of the elastomeric closure as a result of the attack of oxygen or of ozone. Particularly ozone attack is able to induce cracks at the surface of some rubber formulations. Those cracks however may penetrate further into the body of the elastomeric part, especially in components that are mechanically stressed when they are in use. Cases have been reported of ozone cracks in tip caps for prefilled syringes that resulted in splits of the entire sidewall of the tip cap. Consequently the integrity of the seal of the cap on the tip of the syringe barrel was at stake. Ageing as a result of oxygen or ozone attack is typical for particular elastomeric formulations based on natural rubber, poly-isoprene rubber and SBR that have not been adequately formulated, or those that do not contain enough antioxydant and antiozonant of the correct type. With halobutyl formulations in general there is no issue with neither oxidation nor ozone attack.

Still another ageing effect involving the surface of the elastomeric component has to do with surface siliconization. Surface siliconization of elastomeric parts is necessary to prevent clumping of the parts during storage and transport before use and to enable processing of the parts on filling or assembly lines. Surface silicone however, depending on the type of silicone and on the type of the rubber formulation, over time can be absorbed by the closure. Hereby the silicone becomes inactive at the surface. Stickiness, clumping and in the worst of cases deformation of the parts will develop. Absorption of silicone can be countered by choosing higher molecular weight silicones or by choosing silicones that are able to crosslink and so increase in molecular weight. Silicone absorption will take place earlier in rubber formulations with high permeability such as poly-isoprene. Again, in halobutyl formulations, depending on the molecular weight of the silicone, adsorption will not be or at least will be less of an issue.

Finally, also functional properties of elastomeric closures may be affected by ageing. Particularly coring, sealing and resealing behavior are to be mentioned in this respect. Again, in halobutyls, worsening of these properties over time at most is a slow process. Yet it is indicated to check as closures before they are assembled on vials may already have some age—practice shows that this can go up to two to three years—and to this the shelf life of the pharmaceutical product still has to be added.

At present there is no standard that is dedicated to ageing of pharmaceutical rubber parts. General guidance is given by ISO 2230, "Rubber products—Guidelines for storage." For halobutyl products, at least when stored under appropriate conditions of light and temperature, an indicative shelf life of seven years is given. For poly-isoprene articles this is less. Indicative shelf lives for such articles are three to five years.

Machineability

Machineability of elastomeric closures refers to the processes at pharmaceutical or at medical device companies that are used to bring closures into their final position on vials or in syringes or cartridges. Therefore machines will be used that are designed to have a certain capacity. Such machines typically involve feeding bowls in which the elastomeric parts, mostly after sterilization, are brought in, then feeding lines or chutes that bring the closure in the vicinity of the vial or syringe and next a pick-up and positioning mechanism that assembles individual closures onto or into individual vials or syringes.

A first prerequisite is that elastomeric parts do not clump when they are brought into a feeding bowl. Clumping is very typical for halobutyl components. Clumping behavior can largely be prevented by giving an appropriate surface state to the closures. For nonpolymer coated closures this means that the surface of the closure must be designed so as to maximally prevent sticking of individual parts by including antisticking dots or bars, that the surface of the closure has an adequate roughness that is "copied" from the roughness of the mold out of which it is produced, and that the closures have an adequate degree of surface siliconization. Furthermore care shall be taken so that closures are put into their transport packaging when they are at or close to room temperature, that they are not packed too tightly and that their shelf life for storage is taken into consideration.

Feeding behavior of closures in feeder bowls and chutes mostly is a matter of adequate surface states and of adequate dimensioning of closures and machine parts, however minute details in design may have an unexpected impact here.

Insertion behavior of stoppers into vials and of plungers into syringes or cartridges also primarily is a matter of assuring the dimensions and the surface state of the closures, vials, syringes and machine parts are well adapted to each other and are well controlled.

CLOSURE WASHING AND SILICONIZATION

Elastomeric closures for parenterals are manufactured under industrial circumstances with still a lot of manual operator intervention and using industrially available materials. Closure manufacturers spend a great deal of effort to improve the cleanliness of their plants and to tighten their procedures and quality systems so as to guarantee the quality and the cleanliness of their products. Yet, unlike with plastic products, it is not possible to collect at the end of the molding and die-trimming process the resulting products and to pack them without first subjecting them to a washing process.

There are several reasons for this.

1. Before washing, the products are not in a controlled state of cleanliness. After molding most closures are die-trimmed. Silicone in some form is used as a die-cutting agent that prevents the trimming die from getting dull. This silicone, together with the whole manufacturing history of the closures that precedes die-trimming, brings the closures in an undefined state of particulate and microbiological cleanliness. Washing of the closures is necessary to bring the closures within clear specifications, therefore to bring them in a certifiable state of cleanliness, both from the point of view of microbiological and of particulate cleanliness.
2. Closures have not been subjected to a depyrogenation process as required by regulations. FDA's 2004 "Guidance for Industry—Sterile Drug Products Produced by Aseptic Processing—Current Good Manufacturing Practice" states that "containers and closures shall be rendered sterile and, for parenteral drug products, nonpyrogenic." Nonpyrogenicity is obtained by subjecting the closures to well-defined washing, rinsing and drying processes. More and more this washing is delegated to the closure manufacturer who therefore needs to develop validated washing programs.
3. If not siliconized, closures will clump and machineability cannot be guaranteed. As indicated in the previous chapter uncoated closures need siliconization in order not to develop clumping during storage and to be machineable on filling or assembling lines. Closure siliconization typically is combined with the final washing and drying at the closure manufacturer.

Washing Procedures for Elastomeric Closures

The washing of elastomeric closures can be performed in different types of washing machines. Most often encountered are machines of the rotating drum type and, alternatively, machines that are based on an "overflow" principle. The former ones consist of a rotating drum with a perforated wall through which contamination can be removed. It is necessary for the machine to supply water of different types and the necessary auxiliaries, including silicone in forthcoming case. The drum can be partitioned or not, as it can consist of a number of smaller segments that each contain a smaller number of products. Washing and drying either take place in the same machine, or the washer is combined with the necessary dryers, equally of the rotating drum type. In overflow machines the flow of water is from the bottom of the machine through the stopper bed to the overflow. The closures are in a kind of fluidized bed state and contamination is continuously removed via the overflow. In some machines of both types apart from washing and drying also steam sterilization of the closures can be performed.

Washing programs for elastomeric closures vary from company to company, irrespective of whether it concerns a pharmaceutical company that still washes the closures or the closure manufacturer. A typical washing and drying program of elastomeric closures consists of the following steps:

- A washing step with water of a specified grade plus a detergent.
- A number of rinsing steps with water of specified grades. One of the rinsing steps may be combined with siliconization of the closures.
- A drying step with hot filtered air.

As to the types of water used for the washing of elastomeric closures it is worth pointing to two documents. The first of these documents is the 2004 FDA Guidance for Industry that was cited already earlier. This guidance mentions that "at minimum the initial rinses for the washing process should employ at least Purified Water, USP, of minimal endotoxin content, followed by final rinse(s) with WFI (water for injection) for parenteral products." The second document is the European Agency for the Evaluation of Medicinal Products (EMA)'s 2002 "Note for Guidance on Quality of Water for Pharmaceutical Use." For closures that are used for sterile parenterals this document equally speaks of purified water for initial rinses and water for injection for the final rinse. The major closure manufacturers therefore have invested in

water plants and control systems for these plants so that they are able to guarantee the quality of the water that is used in the various stages of closure washing. What they have also invested in is the installation of clean rooms in which the washing and final packing of closures is performed and in developing monitoring schemes to demonstrate that these rooms are in compliance with standards for biological and particulate cleanliness.

Microbiological Cleanliness

The microbiological state of cleanliness of elastomeric closures relates to the presence or absence of microbiological contamination at their surface. This contamination may be present either in the form of bioburden that can be expressed as colony-forming units, and/or as endotoxins, expressed as endotoxin units.

Bioburden

In the majority of cases closure manufacturers do not sell their product sterile (or even "sterilized"). Alternatively, they sell their products with a defined state of high microbiological cleanliness, or low bioburden levels. This is particularly the case when closures are not rewashed at the pharmaceutical company itself. Closures in case of aseptic manufacturing at the pharmaceutical company then are rendered sterile prior to filling, mostly by steam sterilization.

Bioburden on elastomeric closures can be determined with a method as described in ISO 8871-4, "Elastomeric parts for parenterals and for devices for pharmaceutical use—Part 4: Biological requirements and test methods." Such method consists of an "extraction" or "rinsing" phase where bioburden is transferred from the stopper surface to the extracting liquid, followed by determination of the number of colony-forming units in the rinsing liquid. The latter typically is done by filtration on a filter with a suitable growth medium and incubation of the filter. From the result the number of colony-forming units per square centimeter of stopper surface area or per stopper then can be calculated. Methods for bioburden determination on elastomeric closures need to be validated.

Endotoxins

In case of elastomeric closures the absence of bacterial endotoxins is taken as a synonym for the absence of pyrogenic components. As with bioburden closure manufacturers will sell their product with a defined state of endotoxin cleanliness. Determination of endotoxins equally is described in ISO 8871-4. Methods are similar to bioburden determination methods in that they consist of an extraction or rinsing step, followed by a determination step. Current practice is that most often determination is performed using an instrumental LAL method, or alternatively the LAL gel clot method. Also methods for endotoxin determination on elastomeric closures need to be validated.

Particulate Cleanliness

Elastomeric closures like vial stoppers and prefilled syringe plungers are part of a packaging system for injectables. Injectables are subjected to requirements on the presence/absence of particulate matter, including USP <788>, "Particulate Matter in Injections." Elastomeric closures thus are linked, be it indirectly, to the particulate cleanliness of parenteral products.

Particulate cleanliness of elastomeric closures can be approached from various sides. As explained, rubbers are composed of various raw materials that are mixed. If mixing is not perfectly homogeneous this may lead to imperfect dispersion of ingredients like fillers or pigments. This may be visible by a trained eye or under magnification as small particulates of ingredients like filler particles that are different in color from the rest of the stopper. These particles however are still firmly embedded in the rubber matrix and they will not be dislodged from this matrix. Thus they will never compromise particulate cleanliness of the parenteral product.

For particulate contamination that is present at the stopper surface in loose form this is different. These particles effectively may be transferred from the closure into the medicinal product without particular effort. Particulate contamination on elastomeric closures may still have the same material identity as the closure itself, may be part of the ingredients of that closure formulation (endogeneous particles), or may be contamination from the manufacturing

environment that either has not been removed by washing or that is the effect of or a recontamination after washing (exogeneous particles).

USP <788> refers to microscopic methods and to light obscuration methods for the determination of particulate contamination in injections. For the determination of the particulate state of cleanliness of stoppers methods of the same types are standardized in ISO 8871-3, "Elastomeric parts for parenterals and for devices for pharmaceutical use—Part 3: Determination of released-particle count." The methods consist again of two steps. In the first step the particulate contamination is transferred from the stopper surface into an extraction or rinsing liquid and in a second step the contamination that is transferred is sized and counted. For subvisible particulates a light obscuration technique is used. Particles typically are sized in classes 2 to 10 μm, 10 to 25 μm, and >25 μm. For visible particulate contamination particles are collected on a membrane filter where they are sized and counted, either by an operator or by a microscope that is connected to a suitable software system for sizing and counting of particles. Visible particles are typically sized in classes 25 to 50 μm, 50 to 100 μm, and >100 μm.

At present time there are no limit values for subvisible or visible particulate contamination of elastomeric closures, neither in any pharmacopeia, nor in the aforementioned ISO 8871-3. Limit values may be present in quality agreements between manufacturer and customer, but this is on a voluntary basis. The same holds for biological cleanliness of closures.

In case limit values for particulate cleanliness are agreed on, it must be assured that determinations at the closure manufacturer and at the user yield sufficiently comparable results. Although it seems logical that a determination method yields a result with a certain precision and accuracy, intralaboratory repeatability and interlaboratory comparability of particulate cleanliness determinations on elastomeric closures is known to be poor in comparison with other analytical methods.

Closure Siliconization

The purpose of closure siliconization has been explained before. Siliconization of closures usually is part of the final washing of the parts. In one of the rinsing steps silicone is added to the rinsing water. Closures pick up some of the silicone. The water that at the same time is picked up is removed in the drying step of the washing/drying program.

There are various types of silicone that are used for closure siliconization and there are various ways to introduce these silicones into the closure washing machine. Silicone (polydimethylsiloxane) may be introduced as pure silicone or as a silicone emulsion that makes uses of an emulsifier to hold the silicone in an emulsion. The former method is preferred since the emulsifier is not removed by drying. This means that it stays on the closure and, in case of renewed contact with an aqueous medium, as often is the case with a drug product in a vial or a syringe, it will bring the silicone in emulsion again. This emulsified silicone is detectable as subvisible particulate matter. Silicone thus acts as an important source of particulate matter in parenteral products. Also in case no emulsifier is used it deserves attention to bind the silicone as well as possible to the rubber surface. A way to achieve this is to use silicone of higher viscosity, or of higher molecular weight. The longer polydimethylsiloxane chains have lower mobility and attach better to the stopper surface. An alternative way to immobilize silicone at the closure surface is to use a crosslinkable silicone. Such silicone typically is not added in the washing stage of the stoppers but in an earlier stage when the stoppers have not yet been die-trimmed from the sheets in which they are molded. Crosslinkable silicone may be sprayed on the sheets that subsequently are subjected to a silicone curing reaction.

Silicones used for siliconization of elastomeric closures are subjected to the requirements of the USP chapter "Dimethicon" and to Pharm. Eur. 3.8.1, "Silicone used as a lubricant." The viscosity ranges of silicone in these two documents do not perfectly match. The lower limit for Dimethicon is 350 cSt (centistokes) while the lower limit as per Pharm. Eur. is 1000 cSt.

Validation of Stopper Washing

FDA's 2004 Guidance for Industry "Sterile Drug Products Produced by Aseptic Processing—cGMP" mentions that "containers and closures should be rendered sterile and, for parenteral drug products, nonpyrogenic" and that "the validation study for such a process should be adequate to demonstrate its ability to render materials sterile and nonpyrogenic." For

pharmaceutical companies who wash elastomeric closures themselves and then sterilize them, this implies that they develop validation programs for closure washing and sterilization. At many occasions however it is closure manufacturers who perform the last washing of elastomeric closures. In this situation, closures are not rewashed by the pharmaceutical end-users, and only the sterilization is taken care of by them. This practice implies that the depyrogenation process of the closures is delegated to the closure manufacturer who consequently must avail of a validation package for their washing program. The core of such validation studies is inspired by the statement in the Guidance that "the adequacy of the depyrogenation process can be assessed by spiking containers and closures with known quantities of endotoxin, followed by measuring endotoxin content after depyrogenation.... Validation study data should demonstrate that the process reduces the endotoxin content by at least 99.9% (3 logs)." The closure manufacturers will therefore have to develop rationales for the closures to be included in their studies so as to bracket the relevant product portfolio and for which (worst case) conditions are going to be adopted in validation experiments. Not all closures are equally easy to wash. It is accepted in the industry that the ease with which endotoxin can be removed from closures is related to the ease with which the washing and rinsing water have access to the concave parts ("cavities") of the closures. Endotoxin spiking thus for validation purposes shall be done at these parts of the stoppers. For larger stoppers with shallow cavities it will prove to be easier to demonstrate a log 3 endotoxin reduction than for smaller closures with deeper cavities.

Validation of closure washing, apart from the essential part of endotoxin reduction, will also contain validation data about the microbiological cleanliness of the parts after the depyrogenation process. Other properties such as reduction of particulate burden by washing, particulate cleanliness of washed and dried parts, siliconization and presence/absence of washing detergent may form part of washing validation, also when not required by the aforementioned Guidance.

STERILIZATION OF PARENTERAL CLOSURES

Sterilization of parenteral closures may take different forms. The contact area of the parenteral closure with the drug product must be sterile at the time of use. This is achieved by either terminal sterilization of the packaged drug or by aseptic filling where all packaging materials are sterilized prior to filling. In case of plungers for disposable syringes sterilization takes place on the assembled and packaged syringe.

Steam Sterilization

The most common method to sterilize closures for parenteral applications is by steam sterilization, either prior to aseptic filling or by terminal sterilization whereby the packaging components are already assembled. The most typical sterilization temperature that is used for sterilization of elastomeric closures is 121°C, the most typical length of the cycle is 30 minutes. Only in seldom cases higher steam sterilization temperatures such as 134°C are used. For some applications such as blow-fill-seal packages lower temperatures of 106°C or 110°C are applied. Of course every sterilization process of packaging components shall be validated.

As mentioned before steam sterilization puts a considerable amount of moisture into elastomeric closures. Therefore closures after steam sterilization shall be dried again using appropriate procedures that take into account the sensitivity of the drug product to residual moisture in the closure. Closures for lyophilization applications therefore often will be dried to lower residual moisture than closures for liquid fills. Typical drying temperatures for elastomeric closures range from 80°C to 110°C. In a number of cases drying times of only one hour are applied, in other cases drying cycles of up to sixteen hours are qualified.

Other than the moisture uptake, steam sterilization of elastomeric closures, followed by drying, will not affect their functional properties. This still holds when the cycle is applied more than one time on the closures, albeit that this shall not be encouraged and that for multiple sterilizations a check on closure functionality may be indicated, depending on the exact use of the closure in question.

Notes

1. Whereas drying at temperatures of 80°C to 110°C will not affect elastomeric closure functionality, the same does not hold for substantially higher dry heat temperatures. Depending on the elastomeric formulation in question dry heat treatments where closures are exposed to temperatures of approximately 150°C or higher for longer times (15, 30, ... min) are to be avoided. Dry heat sterilization of elastomeric closures is to be totally advised again.
2. It is worth mentioning here that steam sterilization obviously has a sterilizing effect on elastomeric closures, however it cannot serve as depyrogenation process.

Sterilization by Irradiation

Of increasing importance is the use of irradiation sterilization for elastomeric closures. In such cases the pharmaceutical user will choose to be supplied with closures that have been washed by the closure manufacturer and that then have been subjected to a γ irradiation treatment at a sterilization contractor (see also later under "Packaging Ready to Use").

Sterilization by β irradiation of elastomeric closures is not excluded, however γ irradiation because of its much higher penetration capability is preferred. γ Sterilization of elastomeric closures can take place on entire pallets with closures packed in cartons, but more often is carried out with a more limited number of cartons, typically six or eight, being put together in sterilization "totes." One of the advantages of tote sterilization is that the dose distribution over the different cartons with closures will be more homogeneous, as the ratio of maximum to minimum dose achieved over the entire tote is smaller than the same ratio in an irradiated pallet. Since in case of irradiation sterilization the objective is to reach a validated minimum dose, the maximum dose in the case of tote sterilization therefore will be smaller compared with the case of pallet sterilization.

This is of significant importance, since unlike steam sterilization, γ irradiation is more likely to have an effect on the functional properties of the closures. γ Irradiation may have different effects in elastomeric closures. Depending on the formulation of some rubbers, additional cross-linking may take place. In others just the opposite occurs, or the rubber is de-crosslinked to a certain extent. Because of these effects in some rubbers loss of elasticity is found, resulting in a certain "hardening," "stiffening," increase in coring rate and, worst case, inadequate resealing behavior. In other cases closures after γ irradiation exhibit increased tackiness. All of these effects are more pronounced with increasing γ dose. For every individual application it shall therefore be investigated whether the applied irradiation dose does not affect the closure performance up to a level that it is no longer compatible with the requirements of the application. Especially attention has to be given to multidose applications where the closure by the nature of the application is penetrated multiple times. If there is an effect of γ irradiation on the functional properties of elastomeric closures, it will be noticed immediately after the irradiation, unlike with plastics where the effect may be delayed and become apparent only longer time after irradiation.

The most encountered γ dose applied for elastomeric closure sterilization in the past was 25 kGy. As a result of the publication of ISO 11137 on radiation sterilization of health care products newer applications use lower doses that are friendlier to elastomeric components. Of course such lower doses must be demonstrated to be efficient, therefore capable of guaranteeing a certain sterility assurance level. Information and instructions on how to achieve this are given in the same standard.

The effect of γ irradiation is most prominent with respect to the mechanical and functional properties of elastomeric closures. The effect on chemical properties is less evident. On the level of pharmacopeial compliance no effects will be noticed that would turn a compliant elastomeric formulation into a noncompliant one. On a more detailed level of extractables effects are not excluded, certainly not at higher irradiation doses.

Ethylene Oxyde Sterilization

Ethylene oxyde sterilization is very commonly used for the sterilization of disposable medical devices. In the area of elastomeric components for parenteral closures the most important case

is the sterilization of disposable syringes that very often contain an elastomeric rubber plunger. Sterilization is achieved by the action of ethylene oxyde gas on the biocontamination that is present on the plunger surface. To make this action possible the syringes will be packed in gas permeable packing that allows the gas to enter into the syringe. It is well known that ethylene oxyde sterilization leaves chemical residues in the form of residual ethylene oxyde and of ethylene chlorohydrine. Suitable aeration times that allow these residues to decrease below certain levels that are considered as safe must be established.

Apart from disposable devices ethylene oxyde sterilization is very common in one other application in the parenteral field, namely in the sterilization of assemblies of needle covers and tip caps on the barrels of prefillable syringes. One way to come to a presentation of a drug in a prefilled syringe is that pharmaceutical companies purchase syringe barrels with needle covers of tip caps already assembled on them at syringe system manufacturers. The system manufacturer performs the assembly of needle covers on syringes with a staked needle or of tip caps on syringes without needles. The assemblies are then put into tubs that carry a gas permeable plastic film. The tubs next are subjected to ethylene oxyde sterilization. In the case of needle covers the ethylene oxyde has to permeate through the wall of the needle cover to reach the needle surface where the ethylene oxyde has its sterilizing effect. Also these processes of course include suitable aeration or "degassing" cycles. The sterilized barrels may then be directly aseptically filled by the pharmaceutical company and subsequently stoppered with elastomeric plungers that are sterilized prior to aseptic filling.

PACKAGING FOR ELASTOMERIC CLOSURES

The last step in the manufacturing of elastomeric closures is a packaging step. The packaging for closures may just be a transport packaging or may have enhanced features.

Nonfunctional Packaging

In case of nonfunctional packaging the closures are put in single or multiple bags and the bags then are placed into cartons or some type of bulk packaging. Other than just the containment of the closures the bags also take care of preserving their state of particulate cleanliness. Bags of this type are simple polyethylene bags that themselves of course should not shed particles or fibers.

The pharmaceutical user will unpack the closures from the bags and, in case of aseptic filling, transfer them to containers that are compatible with their own sterilization process. These may be containers that are placed in an autoclave. Alternatively the pharmaceutical user may decide to rewash the closures.

Functional Packaging

In case of functional packaging the bags that contain the closures have an additional function at the time of sterilization of the closures. In case of steam sterilization one speaks of packaging "ready for sterilization," in case of irradiation sterilization the term RtU packaging is used.

Packaging Ready for Sterilization

The function of "RfS" bags is that the same bags are used to contain the closures during transport and during steam sterilization. In this case the pharmaceutical user will unpack the RfS bags with the closures from their protective wrapping and transfer them directly into his autoclave for steam sterilization. No rewashing of closures is undertaken.

RfS bags thus must have the following properties:

- They must resist autoclave conditions. RfS bags that currently are in the market resist to temperatures up to 125°C. They are compatible with steam sterilization at 121°C, but not at 134°C. Above 125°C they start to weaken and eventually melt.
- They must be permeable to gases. They must allow air to be evacuated during the vacuum phase at the beginning of the steam sterilization process. Then they must permit steam to enter into the bag to have its sterilizing action. During the drying phase at the end of the autoclave cycle they must allow water vapor to be evaporated.

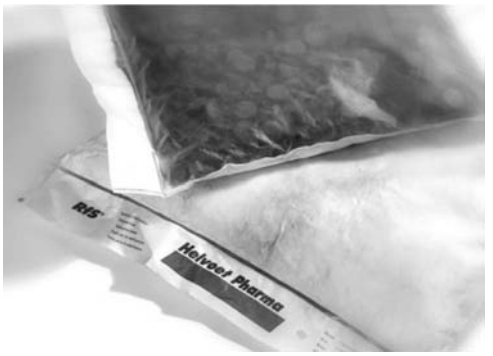


Figure 11 Picture of a ready-for-sterilization bag. The bag on the bottom has its Tyvek side up; the bag on top has its non-Tyvek side up.

- They must be impermeable to microbiological contamination. At the end of the sterilization cycle the closures in the bags are sterile. The bag must be able to guarantee that no microbiological recontamination takes place.

The market offers many types of RfS bags. The ones that are used for steam sterilization of elastomeric closures are composed of two layers of polyethylene in different physical form that are welded together. The welding must be very solid since the weight of the closures in the bag is considerable. One layer of the RfS bag consists of a nonwoven form of polyethylene that is known in the market as "Tyvek." Tyvek has the unique property of being permeable to gases, but not to microbial contamination. The second layer of the bag consists of a regular form of polyethylene that has high enough temperature resistance. This layer is not permeable to gases, nor to microbiological contamination.

It is clear that RfS bags need to have a defined level of particulate cleanliness (Fig. 11).

Packaging Ready to Use

RtU bags are suitable for γ irradiation of elastomeric closures. The closure manufacturer will after washing and drying pack the closures in the RtU bags and provide these bags with protective overwrapping in the form of one or more regular polyethylene bags. From there the closures are transported to an irradiator contractor who performs the γ sterilization of the closures. The pharmaceutical user who is the last in the chain will take off the protective wrapping from the RtU bags and transfer the closures directly to the filling lines in their sterile area. No rewashing nor sterilization of closures is undertaken. As such, RtU bags must be impermeable to microbial contamination.

RtU bags may be made of different types of polymers. Polyethylene can be sufficient since γ irradiation does not have a destructive effect on it. Other types of bags are however possible.

Rapid Transfer Port Packaging

A special case of functional packaging that is gaining more and more attention is rapid transfer port (RTP) packaging. Such packaging is designed to be easily connectable to dedicated ports on isolators or "restricted access barrier systems" (RABS). RTP packaging for elastomeric closures exists in both irradiation sterilization and in steam sterilization compatible forms. RTP bags will always have a "collar" integrated into them. This collar is the mobile part of a two-component system of which the port on the isolator or RABS is the fixed part. When the collar is docked onto the port a system is created that allows aseptic transfer of the sterilized components contained in the RTP packaging into the isolator or RABS (Fig. 12).

Packaging Validation

Validation of the packaging of elastomeric closures in particular is of relevance for functional packaging. At some point in their life cycle such packaging will contain sterile products. The validation of functional packaging comes down to yielding evidence that this packaging is

Figure 12 Picture of two different rapid transfer port bags. The collars are intended to be docked onto a restricted access barrier system or isolator port.

"tight and strong," both before and after sterilization. Microbial tightness of the packaging is important because ingress of microbiological contamination must be avoided before sterilization and of course recontamination after sterilization must be avoided at all times. Apart from choosing the correct materials for construction of the bag, assuring bag tightness can be obtained by developing suitable sealing processes after packing of the closures. The heat-sealing process for the bag shall be capable of generating a seal that is tight before sterilization and that is not affected by the steam sterilization or γ irradiation process. Demonstration of tightness of the seal can be done using microbiological methods or physical methods as a dye ingress method. Equally the sealing process shall generate a seal that is sufficiently strong to resist the weight of the closures, the stress during the steam sterilization process and the handling that inevitably is associated with the bags. Demonstration of the strength of the seals can be given by measuring tear strength of the seals. In the case of γ irradiation it shall equally be demonstrated that there is no effect of time after irradiation on seal strength. Validation of RTP packaging involves demonstration of tightness and strength of yet another seal, namely that of the collar on the bag material. Other points in validation of functional packaging may relate to particulate cleanliness of the bags and in case of γ irradiation to yielding data about discoloration of the bags after irradiation.

QUALITY CONTROL AND QUALITY ASSURANCE IN ELASTOMERIC CLOSURE MANUFACTURING

In-Process Control

Many controls can and will be executed during the manufacturing of elastomeric closures. They range for instance from checking weight on preforms to in-process monitoring of component height, to a visual check of the trimming edge of freshly trimmed stoppers. It is up to the closure manufacturer to determine which particular controls are deemed to be significant and should consequently be performed and documented.

The present paragraph does not intend to discuss further the aforementioned types of controls. Instead a further discussion will be made on controls that generally are formally carried out and documented by qualified people from a quality department.

Included in the category of in-process-controls are tests that serve to confirm the identity of the material that is being processed. Particularly after mixing or preforming, the manufacturer wants to confirm by testing that the material displays all the intended identity characteristics. This is possible by taking samples of the mixed or preformed material and by verifying physical and chemical properties on appropriate test plates made from it.

Physical properties may include a selection or the totality of the following tests:

- Specific gravity
- Ash percentage
- Hardness
- Aspect (assessment of color and homogeneity)
- Rheometry

It is to be noted that the aforementioned tests include only properties that can be affected by the weighing, mixing and preforming operations and do not relate to pure material properties such as gas permeability.

Chemical properties may include a selection or the totality of tests performed according to a standardized method such as USP <381>, Pharm. Eur. 3.2.9 or ISO 8871-1.

None of the aforementioned determinations is capable of confirming on its own the identity of the rubber material. However, every determination leaves its fingerprint and by combining the results of all tests the identity of a rubber compound can undisputedly be confirmed.

In addition to confirming the identity of the material, by carrying out these tests, data are generated that may be used for compiling the Certificate of Analysis or Certificate of Conformity of the product batches that result from the material.

Finished Product Inspection

The term finished product inspection describes the activities that are carried out on closures at the end of the manufacturing process. The tests at this stage comprise a selection, or if applicable the totality, of the following tests:

- Visual inspection of a sample of the inspected batch for the presence of *cosmetic defects*. Included in the category of cosmetic defects are only those defects that constitute a cosmetic failure and that will not influence the functional performance of the part. Cosmetic defects may be further subdivided into critical, major or minor, usually on the basis of their size. At any rate, if such subdivision is made an appropriate definition of the different classes needs to be made.
- Visual inspection of a sample of the inspected batch for the presence of *functional defects*. Functional defects are those defects that with a certain likelihood could lead to inadequate functional performance of the part. They may also be subdivided into critical, major and minor. Again, definitions of "critical," "major," and "minor" need to be established, whereby it is logical that critical defects must not be present in the sample.
- Check on a sample of the inspected batch for *dimensional compliance* with the product drawing. A distinction can be made here between product dimensions that are affected by the manufacturing operations of the part or those that are not affected by the manufacturing process. A typical example of the former class is the total height of a part; a typical example of the latter class is the depth of a product cavity that is determined by the mold dimensions only, and not by the molding operation. Finished product inspection will at least check a dimension that is affected by the manufacturing process, typically total height or flange thickness.
- Check on *functional performance*. In the case of a stopper, such tests can consist of determining coring, self-sealing, and penetration characteristics. Product specific testing may also be introduced under this heading, such as the determination of the holding force of needle shields on prefilled syringe barrels.
- Check on *surface siliconization* (for siliconized parts). This check may be carried out using a chemical analytical technique or may just consist of an assessment based on comparison with parts of known siliconization degree.
- Check on *particulate cleanliness*. Such a check includes the determination of visible and/or subvisible particulate cleanliness on a sample of the batch.
- Check on *microbiological cleanliness*. This check entails the determination of the bioburden and/or endotoxin load on a sample of the batch.
- *Chemical testing*. The manufacturer may decide to document chemical cleanliness of the material on finished product and not in-process. For coated parts, incorporating chemical cleanliness testing as part of finished product inspection testing is most logical.

Finished product inspection levels are usually taken from standards such as ISO 2859-1, "Sampling procedures for inspection by attributes—Part 1: Sampling schemes indexed by acceptance quality limit (AQL) for lot-by-lot inspection," for which the still much cited Military

Standard MIL-STD-105E has served as a basis. Both standards use the concept of "AQL" or "acceptable quality limit." The basis for the acceptance of a product batch is the occurrence of an acceptable number of defects in a statistical sample of the batch, whereas the rejection of a product batch is based on the occurrence of a number of defects that exceeds the acceptable limit. Sampling schemes, sample sizes, number of accepted defects, etc., are regulated by the standard.

Every user of elastomeric closures of course is permitted to make his own listing of defects to which he attributes acceptability or nonacceptability. A potentially useful, although not in all aspects up-to-date, reference that may be helpful in this respect is the "Defect Evaluation List for Rubber Parts," edited by Editio Cantor in Germany. This list has been compiled by a consortium of major German pharmaceutical companies that are active in parenterals.

Quality Systems

It is typical for elastomeric closure manufacturers to maintain a Quality System as per ISO 9001, "Quality management systems—Requirements." This system will usually cover their manufacturing, testing, sales and R&D activities. Apart from the normative aspects of ISO 9001, the Quality System will contain elements of current Good Manufacturing Practice (cGMP) that are typical for the pharmaceutical industry and that many times go beyond the scope of ISO 9001. Until recently every manufacturer at its own discretion included those elements that he thought were pertinent. An emphasis thereby typically was on traceability and on disposition status (released/rejected/quarantined) of raw materials, in-process materials and finished materials. A more comprehensive guideline in this respect has been offered by ISO 15378, "Primary packaging materials for medicinal products—Particular requirements for the application of ISO 9001:2000, with reference to Good Manufacturing Practice (GMP)." Certification against this relatively new standard is finding acceptance with elastomeric closure manufacturers.

STANDARDS FOR ELASTOMERIC CLOSURES FOR PARENTERALS

There are many standards that relate to elastomeric closures for parenteral use. In some cases this relation is very explicit as in pharmacopeia and ISO standards, however in some cases as FDA Guidances the relation can be less explicit. In this paragraph only a discussion of pharmacopeial sections related to elastomeric closure testing is given, as well as a listing of the most relevant ISO standards.

Pharmacopeia

There are three major pharmacopeia that impose requirements on elastomeric closures for parenterals: USP, Pharm. Eur., and Pharm. Jap. The relevant sections are USP <381>, Pharm. Eur. 3.2.9 and Pharm. Jap. 7.03. The types of tests that are contained are as listed in the table below.

	Chemical (extractables)	Functional	Biological
USP <381>	Yes As from May 1, 2009 on aqueous extract only and large degree of alignment with Pharm. Eur.	Yes As from May 1, 2009 on fully harmonized with Pharm. Eur.	Yes, through reference to USP <87> and USP <88>
Pharm. Eur. 3.2.9	Yes	Yes	No
Japanese Pharmacopeia **7.03**	Yes No harmonization with USP and Pharm. Eur.	No	Yes (hemolysis and pyrogens)

Abbreviations: USP, U.S. Pharmacopeia; Pharm. Eur., European Pharmacopeia.

ISO Standards

- ISO 247: Rubber—Determination of ash
- ISO 2230: Rubber products—Guidelines for storage
- ISO 2859-1**:** Sampling procedures for inspection by attributes—Part 1: Sampling schemes indexed by acceptance quality limit (AQL) for lot-by-lot inspection

- ISO 7619-1: Rubber, vulcanized or thermoplastic—Determination of indentation hardness—Part 1: Durometer method (Shore hardness)
- ISO 8362-2: Injection containers for injectables and accessories—Part 2: Closures for injection vials
- ISO 8362-5: Injection containers for injectables and accessories—Part 5: Freeze drying closures for injection vials
- ISO 8536-2: Infusion equipment for medical use—Part 2: Closures for infusion bottles
- ISO 8536-6: Infusion equipment for medical use—Part 6: Freeze drying closures for infusion bottles
- ISO 8871-1: Elastomeric parts for parenterals and for devices for pharmaceutical use—Part 1: Extractables in aqueous autoclavates
- ISO 8871-2: Elastomeric parts for parenterals and for devices for pharmaceutical use—Part 2: Identification and characterization
- ISO 8871-3: Elastomeric parts for parenterals and for devices for pharmaceutical use—Part 3: Determination of released-particle count
- ISO 8871-4: Elastomeric parts for parenterals and for devices for pharmaceutical use—Part 4: Biological requirements and test methods
- ISO 8871-5: Elastomeric parts for parenterals and for devices for pharmaceutical use—Part 5: Functional requirements and testing
- ISO 9001: Quality management systems—Requirements
- ISO 11040-2: Prefilled syringes—Part 2: Plungers and discs for dental local anaesthetic cartridges
- ISO 11040-5: Prefilled syringes—Part 5: Plungers for injectables
- ISO 11137: Sterilization of health care products—Radiation (3 parts)
- ISO 11608: Pen-injectors for medical use (3 parts)
- ISO 13926-2: Pen systems—Part 2: Plungers and discs for pen-injectors for medical use
- ISO 14644-1: Cleanrooms and associated controlled environments—Part 1: Classification of air cleanliness
- ISO 15378: Primary packaging materials for medicinal products—Particular requirements for the application of ISO 9001:2000, with reference to Good Manufacturing Practice (GMP)
- ISO 15759: Medical infusion equipment—Plastics caps with inserted elastomeric liner for containers manufactured by the blow-fill-seal (BFS) process

ACKNOWLEDGMENT

Thanks, Lisa, for reviewing this text!

14 Parenteral product container closure integrity testing

Dana Morton Guazzo

INTRODUCTION

The definition of container closure integrity is simply, the ability of a package to adequately contain its contents by preventing content loss or contamination. This basic description is clear and straightforward. But the concept of container closure integrity is surprisingly complicated given the variety and complexity of parenteral product dosage forms and their packaging.

The demands placed on parenteral product packaging often exceed the requirements of other dosage form containers. Clearly, all pharmaceutical product package systems must prevent content leakage or spillage. But for some parenteral product packages, product loss includes vacuum loss or escape of inert gases or solvent vapors. All pharmaceutical packages must prevent contamination from environmental dirt or debris. However, parenteral product packages must also preclude microorganism contamination. And for some parenteral products, contamination may include unwanted chemicals, even moisture, originating from the outside environment or leaching from the package components themselves.

Another complicating factor of parenteral container closure integrity is the multiplicity of parenteral package designs. For instance, many products are contained in vial package systems. A typical vial package is comprised of a glass or plastic vial or bottle stoppered with a viscoelastic closure compressed against the vial mouth and held in place via a crimped aluminum cap. Prefilled syringes and cartridges, made of either glass or plastic, are becoming increasingly popular. Such systems include a closure or plunger that must adequately contain and protect the contents but must still glide smoothly along the barrel wall at time of drug delivery. The delivery port for cartridges and syringes consists of either an adhesively bonded needle covered with an elastomeric shield, or a luer tip protected with an elastomeric or plastic closure. Flame-sealed glass ampoules were once very common, but are infrequently used for today's new products. On the other hand, plastic blow-fill-seal (BFS) ampoules often package nebulizer solution preparations. Ophthalmic solution products are primarily contained in plastic bottles with uniquely designed plastic caps for easy product use. The closure mechanisms of such bottle/cap systems often include screw-threaded closures and plug- or compression-fitted components. Larger volume intravenous infusion solutions are typically packaged in plastic bags with elastomeric ports for spike access, held together via heat seals and/or ultrasonic welds.

Taking one step back, many parenteral product formulations, and even active ingredients, must be aseptically stored prior to filling into the final product package system. Such bulk storage systems must meet critical package integrity criteria. To make matters even more challenging, finished product, bulk formulation and active substance package systems vary extensively in design and materials of construction.

Given the diversity of packages, products, and integrity requirements, it is no surprise that a universally acceptable container closure integrity test method is nonexistent. Even selecting one appropriate method for any given product package system can be daunting. Much discussion and research over the last three decades has focused on identifying and validating suitable parenteral product container closure integrity test methods for some of the more common packages. Microbial challenge tests continue to be used, although a growing number of approaches for leak testing packages by physicochemical methods are available. When validating a physicochemical container closure integrity method, debate continues on the need for a comparison study against a more traditional microorganism challenge test, how to perform such a comparison, and what should be the acceptance criterion.

Fortunately, consensus on how to evaluate the integrity of at least some parenteral product packages appears to be evolving. This chapter will attempt to introduce container

closure integrity concepts as they relate to some of the more widely used parenteral product packages, and to share new directions in finished product parenteral package integrity verification.

PACKAGE SEAL CHARACTERIZATION AND OPTIMIZATION

Package closure is effected either by physically mating package components or by chemically bonding them together. To ensure adequate container closure integrity, package design and development should include both theoretical and practical closure characterization and optimization studies. A clear understanding of critical component dimensions, materials of construction, and design enables the establishment of appropriate component purchasing specifications and quality controls. Package integrity studies during later development stages should also incorporate packages assembled according to actual or simulated manufacturing operation conditions. Containers assembled by hand or using laboratory scale equipment may not perform comparably to those assembled on automated, high speed manufacturing lines.

Mechanically Fitted Seals

Mechanically fitted components rely on precise dimensional fit, adequate compression, and/or tortuous paths for seal integrity. Therefore, component dimensions and tolerances should ensure the worse case "loosest" fit will still preclude leakage gaps, while the worse case "tightest" fit will permit successful, damage-free package assembly. Checking component dimensional specifications and tolerances provides a theoretical analysis of worse case component fit. However, package assembly line trials performed under anticipated manufacturing conditions play an important role in package integrity validation.

The vial/elastomeric closure/aluminum seal parenteral package (vial package) is an excellent example of a mechanically sealed package. The plug dimension of an elastomeric closure for a vial package should be sufficiently narrow to allow easy insertion into the vial neck, and so minimize vial breakage or closure "pop-up." Then again, some compression is necessary if the package must maintain an inert gas or vacuum atmosphere prior to aluminum seal capping. Elastomeric closure design, formulation, lubrication and polymer coatings all influence stopper insertion and closure-plug/vial-neck seal integrity. The vial throat dimension and design (i.e., absence or presence of a locking ring or "blow-back" feature) also significantly impact stoppered vial integrity and machinability. Finally, the aluminum seal height should be long enough to allow proper seal tuck under the stoppered vial flange, but not be so long that assembled packages exhibit inadequate closure flange compression. All these factors make a purely theoretical evaluation of such a package's closure mechanisms nearly impossible. Often vial, closure, and seal components are sourced from multiple suppliers making it difficult to ensure an optimally designed fit given all possible component combinations. Some pharmaceutical firms use computer modeling software to simulate closure compression during vial-neck insertion and seal capping. Certainly, such tools are useful, but the only way to be confident of a package's leak tightness is to integrity test finished containers, representing multiple component lots assembled at manufacturing line operational limits, using appropriately sensitive test methods. Reportedly, a few firms have gone so far as use vials made to worst case dimensions, and closures lubricated to either extreme for such studies.

Another example of a mechanically sealed system is the ophthalmic dropper-tip bottle with a screw-cap closure. Typically, the dropper-tip base snaps into the bottle neck creating a valve seal fitting. The other critical seal occurs where the inner top surface of the torqued cap presses down against the dropper-tip opening. Small shifts from optimum component designs or dimensions at these critical locations can have disastrous results. Plastic resin changes may affect component viscoelasticity which ultimately can also impact package integrity. For example, the screw cap may back off and/or component polymer creep may occur over time, especially upon exposure to temperature swings, shock or vibration. To ensure package integrity, assembled container leak test methods should identify leakage from these critical sealing locations. Supplier specifications and controls should be in place to ensure that molded components are made from approved materials, and that they conform to dimensional tolerance limits and to absence of defects specifications. Ophthalmic package production line

assembly trials prior to product launch can help identify unanticipated problems. For instance, marketed product-package integrity failures have resulted from incomplete insertion of the dropper tip into the bottle neck, insufficient or excessive screw-cap torque force, and gaps at the dropper-tip/torqued-cap sealing interface.

A syringe or cartridge has a mechanically fitted closure (also called a "plunger") positioned inside the syringe/cartridge barrel to prevent content leakage, yet is designed to glide smoothly with minimal resistance at time of drug delivery. The dimensions of the closure and barrel, and the closure's viscoelastic properties determine this mechanical seal's effectiveness. The amount of lubrication on the barrel wall and the closure also impacts closure performance. For this reason, studies to evaluate both syringe leakage and functionality may use components made to simulate tightest and loosest fit, lubricated and sterilized under the most challenging anticipated conditions.

Chemically Bonded Seals

Chemical bonding techniques are used for sealing various pharmaceutical packages. Heat sealing using thermal impulse or conductive heat sealers is one such technique. Examples of packages sealed in this manner include plastic bags for sterile powder storage, and barrier laminate pouches for protecting semi-permeable plastic BFS ampoules. Consistent seal strength and barrier properties rely on proper characterization and control of heat seal layer polymer composition, molecular structure, and laminate thickness. In addition, the heat sealing process critical parameters of heating, cooling, pressure and time should be controlled and monitored within optimized ranges along the entire length of the seal.

Ultrasonic welding is another well-known process used to create polymer-polymer seals for pharmaceutical packages, although other industries use this technique to bond metals to plastics or even metals to metals. Ultrasonic welding is very fast and usually produces welds relatively free of flash making it attractive in clean room settings. A welding tool transmits ultrasonic energy to the part to be bonded, causing mechanical vibration and frictional heat at the sealing interface. Rapid melting and bonding occurs at the connecting surfaces statically pressed together. Effective ultrasonic welding requires that the bonded polymer materials exhibit nearly equivalent melting points. Amorphous thermoplastics weld more efficiently than semicrystalline materials, harder materials with high modulus are also easier to weld. Thus, consistent welding requires proper characterization and control of polymer layers' thickness, composition and molecular structure. Optimization and control of ultrasonic frequency, oscillation amplitude, power level and pressures are vital, as well as the tool design used to direct energy between the welded parts.

Adhesives can also accomplish a chemical bond between package surfaces. For example, UV and visible light curing adhesives effect the bond between stainless steel needles and the tips of glass or plastic syringe barrels. Semi-rigid plastic trays used for many medical devices or drug-device combination kits often incorporate porous barrier lidding materials, such as Tyvek® or low-linting papers, bonded to the tray with a heat-activated adhesive. Well-sealed bonds depend on the adhesive's chemical composition and quality, the adhesive application process, and the curing process, as well as the nature and quality of the bonding surfaces.

Contiguous containers, such as flame-sealed glass ampoules, represent another chemical bonding process. Glass ampoules filled with product are sealed by one of two methods. In the first case, the ampoule's stem is flame-heated at the intended point of closure. As the distal tip is pulled away the stem narrows and closes. The second glass ampoule sealing process involves heating the ampoule's open end until the glass softens and closes under gravity. Ampoule seal integrity and quality is a function of several factors, including glass formulation, ampoule wall thickness, line speed, ampoule rotation speed, ampoule tip "draw" speed (if applicable), and flame heat. Typical glass ampoule defects include cracks, as well as pinholes, channels, and weak, thin-wall areas usually located at the sealed tip.

Plastic BFS ampoules, another type of contiguous container, are created, filled and sealed in one continuous, aseptic manufacturing process. Dosage forms packaged in BFS ampoules include unit-dose sterile solution products, such as nebulizer solutions and intravenous line flushing solutions. Integrity of these packages is a function of the plastic formulation and the forming/sealing parameters of time, pressure and temperature. Defects that can result in

package leakage include pinholes, thin-wall areas, and burrs or other contaminants trapped in the plastic wall.

LEAKAGE THEORY

Leakage occurs when a discontinuity or gap exists in the wall of a package that allows the passage of gas under the action of a pressure or concentration differential existing across the package wall. Leakage differs from permeation, which is the flow of matter through the barrier itself. Both leakage and permeation play vital roles in the study of parenteral product package integrity.

Permeation

Permeation is passage of a fluid into, through and out of a solid barrier having no holes large enough to permit more than a small fraction of the molecules to pass through any one hole. The process always involves diffusion through a solid, and may involve other phenomena such as adsorption, migration, solution, dissociation, and desorption. Permeation rate is a function of the permeant's concentration, its solubility in the barrier material, as well as the molecule's physical ability to migrate through the barrier.

The general equation for permeation is given by equation (1), where Q, the mass flow rate (Pa m^3/sec m^2) is a function of the permeation rate constant (K_P), which is a product of the solubility coefficient (S), and the diffusion coefficient (D). Permeation is directly proportional to A, the area normal to permeation flow (m^2), and ΔP, the partial pressure drop across the flow path (Pa), while inversely proportional to l, the path flow length (m) (1).

$$Q = K_P A(\Delta P/l) = (SD)A(\Delta P/l) \tag{1}$$

Permeation plays a role in package integrity assurance if the package must prevent loss of critical headspace gases or vacuum, restrict loss of product solvents or other permeable ingredients, or limit migration of external gases or vapors into the package. For example, small volume plastic BFS ampoules containing nebulizer solution are generally semi-permeable containers requiring a barrier laminate pouch secondary package to prevent the product from drying out over shelf life. Packages for hygroscopic lyophilized products or aseptically filled powders must limit moisture ingress from the outside environment or even from the package components themselves. Pharmaceutical products subject to oxidative degradation must be contained in packages that limit oxygen permeation. Some lyophilized products in vial packages require a vacuum headspace to help draw diluent into the vial upon reconstitution. Therefore, atmospheric gas permeation leading to loss of vacuum can make product use difficult and may cause end-users to question product quality.

Leakage Flux

Diffusion

Leakage is defined as the movement of molecules by convection plus diffusion through one or more gaps in the package barrier wall. The driving force for gas or liquid convective flow through a leak path is the pressure differential that exists across the barrier. If no pressure differential exists, only the concentration gradient of the leaking molecule existing across the barrier drives molecular flux according to diffusional flow kinetics.

Gas diffusion follows Fick's laws of diffusion (2). Fick's first law defines diffusion assuming a plane of infinitely small thickness [eq. (2)]. The negative sign means that when $\delta C/\delta x$ is positive, flux is in the direction of decreasing x or decreasing concentration.

$$J = -D(\delta C/\delta x)_t \tag{2}$$

where

J = amount of diffusion	$g/m^2 \cdot sec$
D = diffusion constant	$m^2 \cdot sec$
C = diffusant concentration	g/m^3
x = barrier thickness	m
t = time	sec

Fick's second law takes into consideration a barrier of measurable thickness, where the diffusant concentration varies across the barrier thickness and changes continually over time, thus changing the rate of flux.

$$\delta C/\delta t = D(\delta^2 C/\delta x^2) \tag{3}$$

An example of diffusional flux occurs in a parenteral vial package sealed under a nitrogen blanket. In this case, the vial interior contains a higher concentration of nitrogen and a lower concentration of oxygen than exist outside. Thus, nitrogen gas will tend to diffuse out of the vial, while oxygen will tend to leak into the vial. This tendency is especially true for stoppered vials prior to aluminum seal capping. While studies may show a stoppered vial capable of preventing ingress of relatively large air-borne microorganisms, gas molecules will readily diffuse across the tiniest leak paths.

Convection

For the most part, parenteral package integrity is concerned with fully assembled container closure systems, where measurable leakage linked to either dosage form loss or microbial ingress is chiefly convective, with little or no diffusional flow. So for the remaining discussion, unless otherwise specified, the term "leakage" refers to convective flow of gases moving from higher to lower pressure sides of a package boundary, without diffusional flux or permeation components.

Different physical laws relate leakage rate to the differential pressure gradient across the leak, the range of absolute pressure involved, and the nature of the gas moving through the leak. The five main types of pneumatic gas leak flow are turbulent, laminar, molecular, transitional, and choked flow. Approximate gas flow rates for these pneumatic modes are as follows (1):

1.	Turbulent flow	$>10^{-3}$	Pa m^3/sec
2.	Laminar flow	10^{-2}–10^{-7}	Pa m^3/sec
3.	Molecular	$<10^{-6}$	Pa m^3/sec
4.	Transitional	Between molecular and laminar	
5.	Choked	When flow velocity approaches the speed of sound in the gas	

Laminar and turbulent flow are both classes of viscous flow. Because turbulent flow is rarely encountered in leaks, the term viscous flow is sometimes incorrectly used to describe laminar flow. This chapter focuses on leakage ranging from turbulent to molecular flow—the leak rates of greatest concern for most nonporous parenteral packages. Laminar flow occurs when the mean free path length of the gas (λ) is significantly smaller than the leak path's cross-sectional diameter ($\lambda/d < 0.01$). The mean free path length is that at the average pressure within the leaking system. The leak rate (Q) follows Poiseuille's law for laminar flow through a cylindrical tube (1).

$$Q = [(\pi r^4)/(8nl)][P_a(P_1 - P_2)] \tag{4}$$

or

$$Q = [(\pi r^4)/(16nl)][(P_1^2 - P_2^2)] \tag{5}$$

where

Q = gas flow rate	Pa m^3/sec
r = leak path radius	m
l = leak path length	m
n = leaking gas viscosity	Pa sec
P_1 = upstream pressure	Pa
P_2 = downstream pressure	Pa
P_a = average leak path pressure, $\frac{(P_1+P_2)}{2}$	Pa

Molecular flow occurs when the mean free path length of the gas is greater than the cross-sectional diameter of the leak path ($\lambda/d > 1.00$). Molecular flow leak rates are defined according to Knudsen's law for molecular flow through a cylindrical tube, neglecting the end effect, as per equation (6) (2). By comparing equation (6) with equations (4) and (5), it is evident that laminar flow is a function of the leaking gas's viscosity, whereas molecular flow is a function of the gas's molecular mass.

$$Q = (3.342)(r^3/l)(RT/M)^{1/2}(P_1 - P_2) \tag{6}$$

where

Q = gas flow rate	Pa m^3/sec
r = leak path radius	m
l = leak path length	m
M = molecular weight of leaking gas	(kg/mol)
T = absolute temperature	Kelvin
R = gas constant, 8.315	J/(mol K)
P_1 = upstream pressure	Pa
P_2 = downstream pressure	Pa

Transitional flow occurs when the mean free path length is about equal to the leak's cross-sectional diameter ($\lambda/d = 0.01 - 1.00$). The equations for transitional flow can be quite complex. For further discussion on convective flux, refer to *The Nondestructive Testing Handbook* (1).

Practical Application

Package integrity research studies utilize the above equations and concepts in a variety of useful ways. For example, a leak path's nominal width can be calculated by measuring the gas flow rate through the leak (the leak rate), assuming either molecular or laminar gas flow behavior. University of Iowa researchers measured the helium leak rate through various capillary tubes embedded in the walls of glass vials to estimate these artificial defects' diameters (3).

In another example, package leakage through a hypothetical defect can be calculated and compared with actual package leakage, thus confirming the defect's absence or presence. For instance, consider a lyophilized product sealed under vacuum conditions in a stoppered/capped vial. The lower pressure conditions in the vial act to draw air into the package through any gaps present. By knowing the vial headspace volume and the absolute pressure in the package at time of capping, the theoretical vacuum loss over time due to a given-size leak can be modeled using convective flux equations. Actual headspace pressure readings below modeled predictions confirm the vial's integrity. Similarly, Fick's laws of diffusion can predict the rate of oxygen ingress into an inert gas flushed, stoppered vial as a function of a hypothetical leak. Both of these predictive models are explored more fully later in this chapter.

Leakage Units of Measure

Leakage rate is the amount of gas (mass or volume) which passes through a leak path under specific conditions of temperature and pressure. Therefore, leakage rate has dimensions of pressure multiplied by volume, divided by time. Table 1 lists several common leak rate units of

Table 1 Mass Flow Conversion Factors for Common Leak Rate Units

Pascal cubic meter per second	Standard cubic centimeter per second	Mol per second	Millibar liter per second	Torr liter per second
Pa m^3/sec	Std cm^3/sec Alternatively, sccs	mol/sec	mbar L/sec	torr L/sec
1	9.87 ($\simeq$10)	4.4×10^{-4}	1.00×10^{1}	7.50

Source: From Ref. 4.

measure. The international standard SI nomenclature is pascal cubic meter per second (Pa m^3/sec). To express leak rate in mass flow units, rather than volumetric flow units, the results must be converted to standard conditions of 101 kPa (760 torr) and 0°C (32°F). When expressing leakage volumetrically, test pressure and temperature conditions are specified.

PACKAGE LEAKAGE ACCEPTANCE LIMITS

Since leakage is the rate of gas flow through a leak path, it is meaningless to say that a package has zero leakage, or is leak-free without reference to a leak rate specification. This is similar to saying that a pharmaceutical ingredient is pure or a dinner plate is clean. These expressions are only meaningful when compared with some purity or cleanliness standard. In the same way, a leak-free package simply means the package does not leak above some acceptable leakage limit. The key to setting leak rate specifications is to select meaningful limits, while avoiding unreasonable, and costly requirements. Unnecessarily small leak rates limits will result in expensive instrumentation, increased test time, and rejection of otherwise acceptable product.

Setting realistic and useful leak rate specifications for parenteral products requires characterization of the package sealing mechanisms as well as an understanding of finished product dosage form specifications and the package's performance requirements. This enables logical and practical integrity test method selection. For example, all parenteral products must be sterile; therefore, all packages must be able to prevent liquid- and/or air-borne microbial ingress. All parenteral product packages must also contain the product, preventing loss. Thus, for liquid dosage forms the packaging must also prevent liquid leakage. Studies have shown that leaks that allow liquid flow are also at risk of microbial ingress; the larger the leak, the greater the risk. Conversely, when liquid cannot pass through a leak, microbes cannot (5–7). For this reason, leak tests capable of identifying the smallest leak paths able to contain liquid or permit liquid flow may serve to verify a package's microbial integrity. This microbial ingress/liquid leakage relationship, briefly introduced at this point, is a topic explored extensively throughout this chapter.

Some leak tests, such as helium mass spectrometry, provide test results in quantitative gas flow rate terms. Therefore, when using such methods it is important to know how gas leak rates correlate to critical package performance requirements. For example, helium trace gas leak test studies have linked gas flow rates as small as about 10^{-6} Pa m^3/sec to the smallest leaks able to permit liquid leakage plus microbial ingress (8). Leak detection texts define water-tight seals as meeting limits of about 10^{-4} Pa m^3/sec, whereas, relatively large leaks from misassembled, misshapen or damaged packages are most often above 10^{-4} Pa m^3/sec (9).

Gas headspace preservation is a practical package performance requirement linked to leakage acceptance criteria. For instance, if the product requires low oxygen container headspace content, then oxygen permeation plus air leakage must remain below a specified limit. Similarly, hydroscopic product packages must limit moisture ingress. Integrity tests that specifically monitor gas or vapor migration are reasonable options in such cases. For packages sealed under negative pressure, instruments to monitor headspace pressure are preferred.

LEAK TEST METHODS

Many leak test methods exist for testing everything from soft drink cans to vacuum pumps to heart pacemakers. Even within the relatively small world of parenteral packaging, numerous leak test methods apply (10). Rather than provide an exhaustive survey of all potentially useful leak test methods, this chapter will focus on those testing techniques having the broadest application for the most common parenteral packages, namely, vial packages, prefilled syringes, ophthalmic dropper bottles, and plastic or glass ampoules.

Microbial Challenge Methods

A microbial challenge test procedure includes filling containers with either growth-supporting media or product, followed by closed container immersion in a bacterial suspension or exposure to aerosolized bacteria or bacterial spores. Test containers are incubated at conditions that promote microbial growth, and container contents are then inspected for evidence of microbial growth. Positive challenge organism growth is indicative of package leakage.

Currently, no standard microbial challenge test method exists (10). In reality, any one of many possible microbial challenge methods may prove satisfactory as long as it is scientifically sound, given the package type and its protective function, and the product's anticipated exposure to conditions of processing, distribution, and storage. The following discussion explores factors to consider when designing a microbial challenge test.

1. Challenge mode. If a package is able to tolerate liquid immersion, then this approach is generally favored for parenteral package system testing, as it presents the greatest challenge to package seals. Aerosol challenge testing is most appropriate for packages that rely on tortuous paths, or seals not intended to prevent liquid leakage. Aerosolized challenges are frequently used in the food and medical device industries. Static testing, where packages filled with media are simply stored under normal warehouse conditions or in stability storage chambers, affords no definitive bacterial challenge and no significant pressure differential to the seals. If such long term storage of media-filled units is part of an integrity verification program, then some known bacterial challenge to the packages at the end of the storage period is appropriate.
2. Challenge parameters. Liquid immersion challenge tests preferably include vacuum/pressure cycling simulating pressure variations anticipated during product life processing, distribution and storage. These cycles will enhance flow of packaged media into any leak paths present, thus encouraging potential microbial ingress. For this reason, package position during the challenge test should ensure packaged media contact with seal areas. An aerosol challenge test chamber size and design should guarantee uniform distribution of viable aerosolized bacteria or spores around the test packages, considering factors such as chamber temperature and humidity, as well as airflow patterns and speed.
3. Challenge microorganism. Liquid challenge organism size, mobility and viability in the packaged media are important factors for consideration. Bacteria concentration in the challenge media at the initial time point should ensure a high concentration of viable organisms at the test's conclusion (e.g., $\geq 10^5$ CFUs/mL at end of test). Bacteria used in published immersion challenge studies include, but are not limited to *Escherichia coli*, *Serratia marcescens*, *Clostiridium sporogenes*, *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, and *Brevundimonas diminuta*. When performing aerosol challenge tests, aerosolized microorganism concentration and uniformity are important factors, as well as viability in the packaged media. Reportedly, aerosol challenge testing commonly uses *Bacillus atrophaeus* spores and *Pseudomonas fragi* microbes.
4. Growth promotion media. All challenge tests require test containers filled either with growth-promoting media or product that supports microbial growth. The product formulation itself or a product placebo is preferred as it most closely simulates the product package system. However, this may not be practical if the intention is to validate a variety of products in similar packaging. Verification of the media's growth promotion capability at the completion of the package integrity test is important, especially if the test sample holding time is lengthy.
5. Test package preparation. Two approaches are possible for preparing sterile packages for testing. Either previously sterilized package components are aseptically filled with the growth-promoting vehicle, or media-filled packages are terminally sterilized. If feasible, the sterilization procedures and package assembly processes chosen should mirror those used for the actual product. Otherwise, the test package and seal may differ in some respect from the marketed product package system. For example, vial package capped closures exhibit a certain amount of sealing force on the vial land seal surface. This residual seal force will noticeably decay upon terminal steam sterilization, thus potentially changing the seal quality (11,12). Similarly, plastic bag test samples exposed to gamma irradiation post heat sealing may not represent product bags normally sealed using ethylene oxide sterilized materials.
6. Test package quantity. There is no guarantee of microbial ingress even in the presence of relatively large defects. Microbial ingress is a notoriously probabilistic

phenomenon. For this reason, a valid test requires a relatively large population of test samples and positive controls.

7. Positive and negative controls. All leak test validation protocols, including microbial challenge tests, require positive control or known-leaking packaging in the test package population to demonstrate the test's leak detection ability. Negative controls, or so-called good packages, are also important to establish a baseline of intact package performance. Additional information on positive controls is included under a separate heading.

Microbial challenge tests have been used to verify container closure integrity for decades. However, there are problems with solely relying on this approach. First, microbial challenges, especially immersion tests, do not simulate real life, product bio-exposure conditions. Simply put, package seals are not typically soaked in media highly concentrated with microbes, while differential pressures promote liquid and microbial entrance. Yet, even under these extreme challenge conditions, the highly probabilistic nature of any microbial challenge test makes results difficult to interpret. Leak paths several fold wider than a microorganism will not guarantee microbial ingress, as numerous studies have shown (5,7,8,13). On the other hand, the rare occurrence of microbial grow-through across a package's fitted seam during an exceptionally severe biochallenge may negate the use of an otherwise acceptable container closure system, even though such a challenge does not realistically portray naturally occurring phenomena.

Conversely, inappropriately designed microbial challenge tests can easily make bad packages look good. Short exposure times; minimal or no differential pressure application; small test sample populations; and positive control packages with very large leaks all help samples with questionable seals pass a microbial challenge test, thereby falsely implying package integrity. In some cases, reliance on such tests has kept leery companies from adopting more reliable, physicochemical leak test methods, despite known product package integrity problems.

Suitably designed and executed microbial challenge tests, if used, are of greatest value during package development and early clinical research programs. Microbial challenge tests are one of the few appropriate tests for integrity verification of porous barrier materials and tortuous path closure systems. However, reliance on microbial challenge tests for most package types throughout a product's life cycle has disadvantages. Results are prone to error and the test itself consumes resources of time, space, equipment, and staff, making it much more expensive than cost of materials implies. Microbial challenge tests are not practical, for instance, for routine production lot integrity testing, for forensic investigations of recalled product, or when studying package component and assembly process variables. In addition, unless the product formulation supports microbial growth, the test cannot definitively validate the integrity of the actual product package system. Nevertheless, because parenteral packages must prevent sterility loss, microbial challenge tests will likely remain part of the package leak testing arsenal for some time to come.

Dye and Liquid Tracer Methods

A liquid tracer leak test consists of immersing test packages in a solution of either dye or other chemical tracer, then allowing time for liquid to migrate through any leaks present while pressure and/or vacuum are applied. After the liquid challenge, test packages' contents are checked for liquid leakage as evidenced by visual inspection or other appropriate analytical method. Liquid leak tests are relatively inexpensive, simple to perform and conceptually easy to understand. However, the test is destructive to the package, and results may vary considerably on the basis of several factors.

Test method parameters that promote greater liquid tracer test sensitivity include longer immersion times, increased pressure and vacuum conditions, smaller volumes inside the test package, and lower surface tension challenge liquids. On the other hand, debris in the challenge liquid may clog small leaks, and airlocks in leak paths may prevent liquid ingress. Restraining package part movement (e.g., partially filled syringes), or package expansion (e.g., flexible pouches) during vacuum exposure helps keep package internal pressure constant, thus ensuring consistent leakage driving forces.

The compatibility of the dye or tracer element with the package and its contents should be verified. Dyes may quickly fade or adsorb onto package surfaces shortly after leak testing; therefore, time gaps between testing and inspection or analysis should be limited and specified. Analytical methods for dye or tracer detection require appropriate validation. For the most reliable visual inspection results, qualified inspectors following defined inspection procedures in well-lit, controlled inspection environments are called for. Inspection procedures should dictate lighting intensity and color, inspection angle, background color(s), background luster, inspection pacing, and any comparator negative control package(s) used. Inspector qualification protocols should entail accurate segregation of packages containing trace amounts of dye from negative controls in a randomly mixed, blinded test sample population. A multisite study lead by H. Wolf demonstrated how differences in inspector capabilities and inspection environments play a significant role in interpreting dye ingress test results (14).

Numerous published leak test studies incorporate dye or liquid tracer test methods, some of which are described in section "Test Method Validation" (5,6,13). U.S. compendia (15), EU compendia (16), and ISO international standards (17) all specify methylene blue dye ingress tests for demonstrating punctured closure reseal properties. But before using such closure reseal methods for whole-package integrity testing, test parameters should be optimized and the methods validated using known positive and negative control packages. The importance of this was demonstrated in the previously cited study by Wolf et al., in which 1-mL water-filled syringes with laser-drilled defects in the barrel wall ranging in nominal diameter from 5 to 15 μm were leak tested according to the closure resealability dye ingress tests described in the U.S. and EU compendia and in ISO standards. None of these standard test methods permitted accurate identification of all defective syringes (14).

Vacuum Decay Leak Test Method

A vacuum decay leak test is a whole-package, nondestructive leak test method. Vacuum decay methods relate pressure rise, or vacuum loss, in an evacuated test chamber containing the test package to package leakage. A typical test cycle consists of placing the subject container in a test chamber, then closing the chamber and evacuating it to a predetermined vacuum level. Upon reaching this target vacuum within an allotted time segment, the test system is isolated from the vacuum source, and a short time for system equalization elapses. A defined test time segment follows for monitoring any subsequent pressure rise (vacuum decay) inside the test chamber. Rise in pressure above baseline, or background noise level, signifies package headspace gas leakage, and/or vaporization of product liquid plugging leak path(s). Total test cycle time is normally less than 30 seconds, but may vary with the test system, the product package tested, and the desired sensitivity level.

A package "fails" or "leaks" if any one of several events occurs during the vacuum decay leak test cycle. Failure modes include (*i*) failure to achieve initial target vacuum, indicative of largest leaks, (*ii*) rise in pressure above a defined reference pressure at any time throughout the test cycle, indicative of medium size leaks, or (*iii*) rise in pressure above a defined differential pressure value during the final test time segment, indicative of smallest leaks. Figure 1 illustrates these various failure modes.

The combination of test equipment, package test chamber, and testing cycle is unique to each product package system, and is identified on the basis of the package's contents (liquid or solid, with significant or little gas headspace), and the nature of the package (flexible or rigid, porous or nonporous).

Uniquely designed test chambers snugly enclose the test package, minimizing test chamber deadspace for maximum test sensitivity. Added features may be required to limit package movement or expansion during the test. For example, prefilled syringes require special fixtures to restrict plunger movement. Test chambers for flexible packages, such as bags or pouches, include flexible surfaces that conform to the package and prevent expansion that may stress package seals. Test chambers designed to test trays with porous barrier lidding have a single flexible bladder that masks gas flow through the porous barrier, allowing detection of leaks located around the seal perimeter or through the nonporous tray (19).

Test method reference parameters maximize test method sensitivity for each product package. These parameters include: Time to reach initial target vacuum, equalizing time,

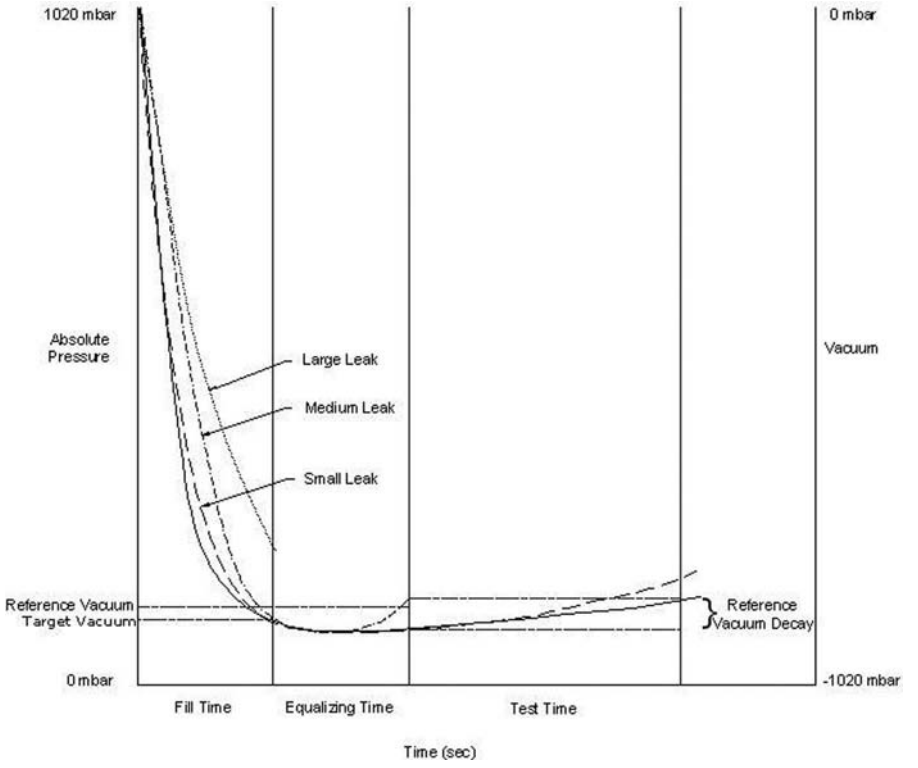


Figure 1 Pressure readings as a function of time during a vacuum decay leak test method for packages with and without leaks, according to ASTM F2338-09 *Standard Test Method for Nondestructive Detection of Leaks in Packages by Vacuum Decay Method*. *Source*: From Ref. 18.

vacuum loss test time, target vacuum level, and pressure loss limits. For instance, leaks plugged by liquid require target vacuum below the liquid's vaporization pressure, so that vaporized liquid yields a measurable rise in pressure. On the other hand, gas leaks are detectable at less severe vacuum settings. Pressure loss limits close to baseline make the test more sensitive, but run the risk of false positive test results. Generally, longer total test cycles improve test sensitivity, especially for gas leaks.

Vacuum decay leak tester designs vary among instrument manufacturers. While most models rely on a single 1000-torr gauge transducer, some instruments use a dual transducer system with either a 1000-torr gauge or absolute transducer coupled with a more sensitive, higher resolution 10-torr gauge transducer. One manufacturer that relies on the single gauge transducer approach also incorporates special software that continually readjusts the no-leak baseline to account for atmospheric pressure changes and no-leak noise variations that can affect test sensitivity. Another manufacturer is able to eliminate atmospheric pressure variation concerns and the need for calculated baseline adjustments by utilizing an absolute pressure transducer as part of their dual transducer test system (19). Automated multistation linear or rotary-style equipment enables 100% on-line testing; semi-automated or manually operated test systems with either single- or multiple-package test stations are useful for testing one or several packages simultaneously. In general, longer tests possible with off-line testers enable smaller leak detection. Thus any given vacuum decay leak test method is not only specific to the product package system, but also to the leak test instrument and its manufacturer.

Test method development and instrument functionality checks often utilize a calibrated airflow meter for artificially introducing leaks into the test chamber containing a negative, no-leak control package. Airflow meters certified by the National Institutes of Standards and Technology (NIST) or other recognized certification bodies are recommended for such

purposes. The smallest rate of airflow that triggers a significantly greater rise in pressure above background noise level is the limit of detection for the leak test. However, use of calibrated airflow standards alone is not sufficient for complete test method development and validation.

For instance, consider a grossly leaking package with very small gas headspace volume. If the time allotted for reaching initial target vacuum is too long, the headspace will be rapidly lost, preventing leak detection during the pressure rise test phase. Whereas, the same test performed using a flowmeter with unlimited gas supply will still yield test phase pressure rise despite the longest chamber evacuation times. In another example, consider a plastic bottle with a pinhole-size leak in the induction seal, beneath the torqued screw-thread cap. A proper test cycle may require additional time to draw out trapped air in the cap's threads, before leakage from the induction seal hole can be observed. This phenomenon would likely be missed if test method development only used a flowmeter for leakage simulation. Further, consider the fact that leaks simulated using a calibrated flowmeter only represent gaseous leakage and not leakage from liquid-plugged leak paths. Generally, liquids clogging leaks quickly volatilize once test pressure falls below the liquid's vaporization pressure. At this point, solvent volatilization causes a rapid rise in test system pressure, which quickly stops or perhaps fluctuates once saturation partial pressure is reached. This difference in leak behavior often requires different testing parameters when checking for gas versus liquid leaks, or some combination of both.

Negative controls used for vacuum decay test method development and validation may consist of actual no-leak packages, or they may be solid material, package-shaped models. However, at some point, tests using larger populations of actual, filled, no-leak packages will ensure the baseline represents all possible package-to-package variations. Actual leaking packages filled with placebo or product are also very useful to verify the test method's ability to find various types of leaks located at various seal locations. Prior to testing actual product packages, cleaning procedures should be in place in anticipation of test equipment contamination from leaking containers.

Two vacuum decay leak test research studies reported in the literature used Wilco AG leak test systems. For both studies test samples consisted of glass vials with micropipettes affixed into the glass vials to simulate leaks. Test package leakage was quantified using helium mass spectrometry, a leak test method previously compared with liquid-borne microbial challenge tests. In the first study, air-filled vials were vacuum decay leak tested (20). The second study evaluated vials filled with various solvents that plugged the leak paths using a so-called LFC pressure rise or vacuum decay approach. This concept required the test pressure to be substantially lower than the vapor pressure of the packaged liquid (21). LFC method test results indicated potentially greater sensitivity when testing liquid-filled vials.

ASTM F2338-09 *Standard Test Method for Nondestructive Detection of Leaks in Packages by Vacuum Decay Method* (22) is a recognized consensus standard by the U.S. Food and Drug Administration (FDA), Center for Devices and Radiological Health (CDRH), effective from March 31, 2006 (22). According to the FDA Consensus Standard Recognition Notice, devices that are affected include any devices that are sterilized and packaged. Packages that may be nondestructively tested by this method include: Rigid and semi-rigid nonlidded trays; trays or cups sealed with porous barrier lidding materials; rigid, nonporous packages; and flexible, nonporous packages.

The ASTM method includes precision and bias (P&B) statements for various types of packages based on round robin studies performed at multiple test sites with multiple instruments. P&B studies have looked at porous lidded plastic trays, unlidded trays and induction-sealed plastic bottles with screw caps. The most recent P&B studies used glass prefilled syringes. Test packages included empty syringes, simulating gas leaks; and water-filled syringes, simulating leaks plugged with liquid (liquid leaks). Laser-drilled holes in the syringes' glass barrel walls ranging from 5 to 15 μm in nominal diameter served as positive control leaks. The leak testers used incorporated an absolute 1000-torr transducer coupled with a 10-torr differential transducer, manufactured by Packaging Technologies & Inspection, LLC of Tuckahoe (New York, U.S.). Two different test cycles were explored; one with a target vacuum of 250 mbar absolute for testing gas leaks only, and another with a target vacuum of about 1 mbar absolute for testing both gas and liquid leaks. Results showed the leak tests reliably identified holes as small as 5 μm in both air-filled and water-filled syringes (23).

In summary, vacuum decay is a rapid, noninvasive and nondestructive leak test method. Depending on the test system, holes as small as 5 μm in a variety of nonporous, rigid packages are reliably detected. Vacuum decay is a practical tool for optimizing package-sealing parameters and for comparatively evaluating various packages and materials. Test methods are suitable as a stability program integrity test or as an in-process check of clinical or commercial manufacturing lots. Larger scale, on-line equipment may be used for 100% production lot testing, although leak test sensitivity is considerably less than for the most sensitive off-line instruments.

Electrical Conductivity Leak Test

Electrical conductivity testing relies on the application of a high frequency electrical current near the test package. Any liquid of greater conductivity than the package material present in or near a leak path located near the detector will trigger a spike in measured conductivity (Fig. 2). Conductivity spikes occur even if leak paths are clogged with dried product—an advantage not shared with other test methods that require an open leak path. This approach for testing liquid-filled packages has the added benefits of being extremely rapid, nondestructive and clean.

Electrical conductivity testing is appropriate for a wide variety of container closure systems, including plastic or glass ampoules, vial packages, prefilled syringes, and liquid-filled pouches. Electrical conductivity is not appropriate for testing flammable liquid products. In addition, only leak paths near detectors are identifiable; therefore, either package surfaces are checked using multiple detectors, or only the areas of greatest risk for leakage are monitored. Package rotation during testing may be required to capture defects around a package's circumference. Test method validation for a given product package requires demonstration of the test's ability to detect leaks at all likely package locations.

The electrical conductivity test, also known as high-voltage leak detection (HVLD), is widely employed for 100% on-line testing of plastic BFS ampoules and glass ampoules. Möll and colleagues described test method development and validation of an electrical conductivity test used for gel-filled low density polyethylene ampoules (24). Positive controls consisted of ampoules with laser-drilled holes positioned at the most likely zones for leaks to occur: the sealing zone at the ampoule bottom, and the top tear-off area. The voltage setting and the sensitivity or "gain" setting were the two parameters optimized to establish a window of operation that finds all defective ampoules and rejects few, if any, good ampoules. Replicate testing of a randomized population of negative and positive control test samples took place over three days. Each day of operation the HVLD test successfully "failed" all 210 positive control ampoules (150: 5–10 μm; 60: 10–20 μm), and "passed" 3830 negative controls. A dye ingress test confirmed the presence of defects in two of three so-called negative controls consistently rejected by HVLD. Therefore, the electrical conductivity test correctly identified all defective units and falsely rejected only one negative control sample.

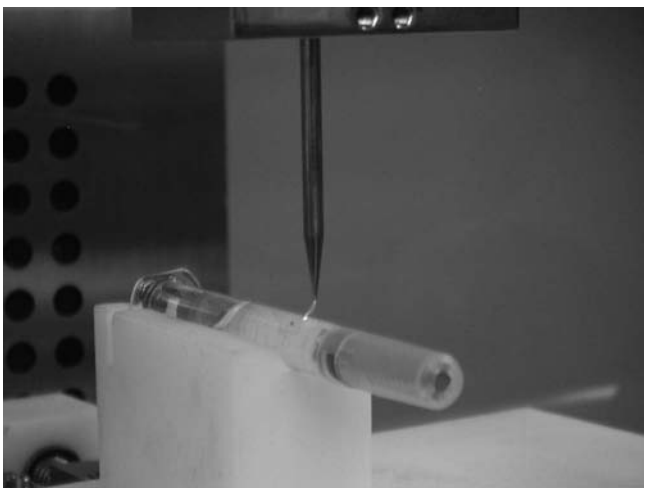


Figure 2 A glass prefilled syringe containing an aqueous liquid being tested using Nikka Densok's electrical conductivity method. Positive electrical current occurred near a laser-drilled hole in the glass barrel wall. *Source*: Courtesy of Nikka Densok, Inc., Lakewood, Colorado, U.S.

Frequency Modulation Spectroscopy

Frequency-modulated spectroscopy (FMS) is a rapid, nondestructive analytical method suitable for monitoring oxygen and water vapor concentrations as well as evacuated pressure levels in the headspace of sterile product containers. Frequency modulation spectroscopy was developed in academic and industrial laboratories in the 1980s and 1990s. Over the last 10 years, the technology has found commercial application in the pharmaceutical industry for leak detection (25), moisture monitoring (26) and oxygen monitoring (27). Systems for rapid nondestructive headspace analysis were first introduced to the pharmaceutical industry in 2000 (28), and are now routinely used in product development, process development and commercial manufacturing.

The key to these test systems are diode laser devices fabricated to emit wavelengths in the red and near-infrared regions of the electromagnetic spectrum where molecules such as oxygen and moisture absorb light. Containers made of glass (amber or colorless) as well as translucent plastics allow the transmission of near IR diode laser light and are compatible with FMS test methods.

The underlying principle of laser absorption spectroscopy is that the amount of light absorbed by a molecule at a particular wavelength is proportional to the gas concentration and the gas pressure. Therefore, FMS technology works by tuning the wavelength of light to match the internal absorption wavelength of a molecule and recovering a signal where the amplitude is linearly proportional to gas density (e.g., headspace oxygen and moisture) and the signal width is linearly proportional to gas pressure (e.g., vacuum level in the headspace of a sealed vial). Figure 3 presents a simple schematic of the FMS technique. Laser passes through the gas headspace region of a sealed package; light is absorbed as a function of gas concentration and pressure; the absorption information is processed using phase sensitive detection techniques; a mixer demodulates the radio frequency signal; the output voltage, proportional to the absorption lineshape, is digitally converted and further analyzed by a microprocessor, yielding final test results.

Examples of demodulated absorption signals for headspace oxygen, moisture and total pressure are shown in Figures 4 to 6. Figure 4 shows how the oxygen concentration in the headspace of a sterile product vial varies linearly with the peak to peak amplitude of the FMS signal. Figure 5 compares frequency modulation signals from vials filled with varying amounts of moisture. The total area is proportional to the moisture partial pressure and concentration. Figure 6 shows how the moisture laser absorption signal measures the total headspace pressure in a sealed container. As described above the moisture absorption signal width is linearly proportional to the total headspace pressure. As the total pressure rises because of a leak, the absorption signal broadens proportionately because of an increase in the collision frequency between moisture molecules and other gases. In general, measurements of higher headspace pressure require higher levels of moisture in the vial headspace.

A variety of diode laser-based system configurations can accommodate process monitoring and control and/or inspection of individual containers for oxygen, moisture or vacuum. Lighthouse Instruments, Inc., of Charlottesville, Virginia provides benchtop systems for laboratory use, as well as at-line, fully automated systems for 100% monitoring, control and

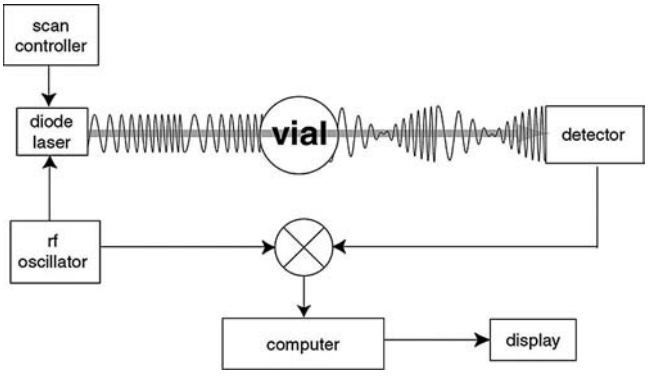


Figure 3 A schematic diagram of the frequency modulation spectroscopy technique. The frequency-modulated diode laser output is converted to an amplitude modulation after passing through a gas sample, which absorbs at a particular wavelength. The amplitude modulation is proportional to gas concentration and can be phase sensitively detected. *Source*: Courtesy of Lighthouse Instruments, Inc., Charlottesville, Virginia, U.S.

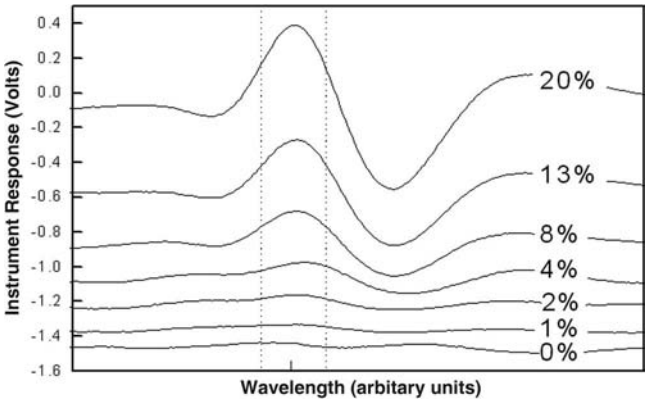


Figure 4 Frequency modulation signals from oxygen absorption. The peak-to-peak amplitude of each spectrum is proportional to oxygen concentration. *Source*: Courtesy of Lighthouse Instruments, Inc., Charlottesville, Virginia, U.S.

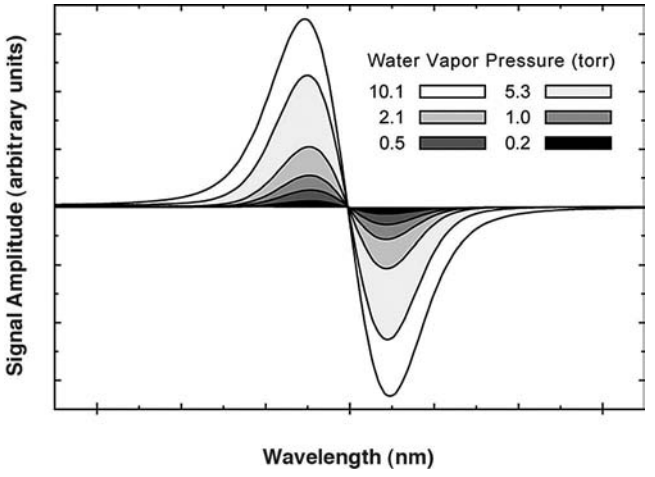


Figure 5 Frequency modulation signals from moisture absorption using 10-mL vials filled with certified amounts of moisture. Since the absorption strength of water vapor is 1000× stronger than oxygen in the near infrared, the total area of the absorption profile can be used to determine water vapor concentration. In these scans, the total area is proportional to the moisture partial pressure and concentration. *Source*: Courtesy of Lighthouse Instruments, Inc., Charlottesville, Virginia, U.S.

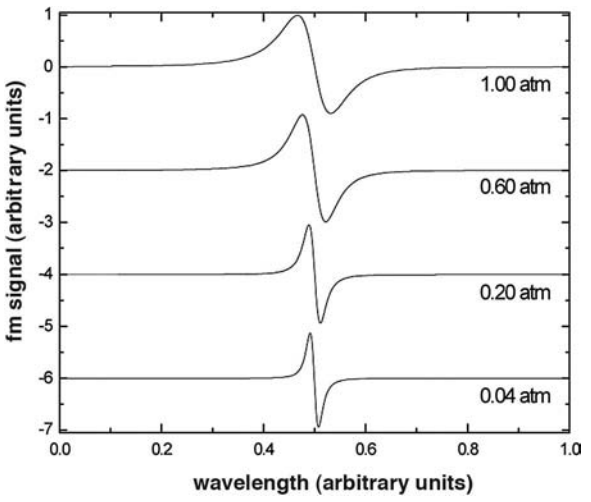


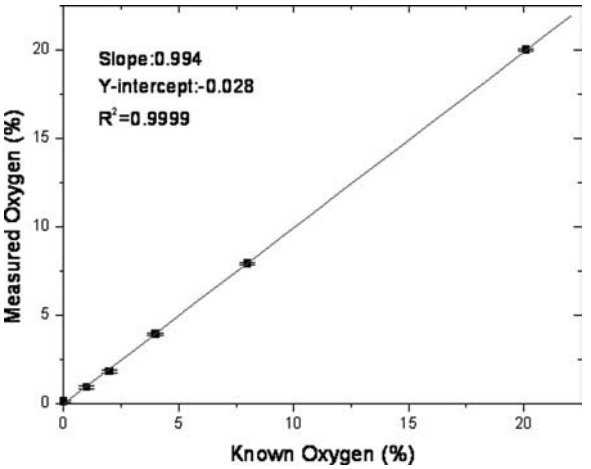
Figure 6 Frequency modulation signals due to pressure broadening of a moisture absorption signal. In principle, any molecule undergoes pressure broadening and can be used for measuring the gas pressure in a sealed container. These scans show how the absorption signal broadens as the total gas pressure increases from full vacuum to an intermediate pressure and finally to atmosphere. *Source*: Courtesy of Lighthouse Instruments, Inc., Charlottesville, Virginia, U.S.

inspection. Typical measurement times can be varied from 0.1 to 1 second corresponding to line speed throughput of 60 to 600 vials per minute. Maximum machine speeds will depend on the details of a particular application. Key parameters that impact maximum speed are container diameter and reject specification. Both faster speeds and smaller diameter packages increase measurement standard deviation.

Test systems are calibrated using NIST traceable standards of known gas concentration or pressure. Standards are constructed from the same containers used to package the pharmaceutical product, so that calibration represents containers identical to the test sample containers. For example, an oxygen-monitoring instrument would utilize standards of known oxygen concentration in containers of the same type and diameter as test sample containers. Datasets of standards measurements versus certified values enable calibration constant or calibration function generation. Subsequent measurements of unknown samples use this calibration information to convert measured absorption signals into meaningful values of headspace gas concentration and/or gas pressure. System measurement performance (method validation) is demonstrated by repeatedly testing a set of gas or pressure standards, evaluating the data following guidance in the U.S. Pharmacopeia, General Information <1225> for accuracy, precision, linearity and limit of detection (29). Figure 7 illustrates system performance data generated from 100 measurements of NIST oxygen concentration standards.

FMS offers invaluable insight for monitoring and controlling aseptic manufacturing processes. Oxygen sensitive products typically require an inert gas headspace, and lyophilized products often require either vacuum or inert gas headspace. Vial package systems, typically used for such products, cannot guarantee maintenance of inert gas or vacuum content post stoppering, prior to capping. Variations in component dimension, elastomer lubrication, gas flushing, stopper insertion, even handling, are only some of the factors that may influence the outcome. Upstream processing controls and monitors give some assurance of success, but a strong likelihood exists that some small percentage of the lot will not meet specifications. Destructive testing for either oxygen content or vacuum level using other off-line test methods is costly in terms of loss of product, and cannot provide timely information to correct a manufacturing deviation. And such test results cannot differentiate between a random glitch in the process versus system-wide failure. In contrast, FMS can be incorporated at-line for 100% automatic headspace content testing. Thus, FMS provides real-time headspace verification, enabling every unit not meeting specifications to be culled.

By testing sealed product some time post packaging, FMS technology can also verify container closure integrity, or absence of leakage. In the case of product sealed with an inert gas overlay, leakage of oxygen into the container will be a function of diffusive flow, driven by



Accuracy	0.2%
Precision	±0.08%
Linearity (R^2)	0.9999
Limit of detection	0.3%

Figure 7 Frequency modulation spectroscopy method linearity for oxygen measurement in a 10-mL vial. *Source*: Courtesy of Lighthouse Instruments, Inc., Charlottesville, Virginia, U.S.

Table 2 Time for Oxygen to Diffuse into a 10-mL Vial Container Through Holes 2 and 5 μm in Nominal Diameter

Predicted rise in package oxygen content		Time to reach predicted oxygen levels	
Partial pressure (atm)	Oxygen concentration (% atm)	5-μm hole (days)	2-μm hole (days)
0	0	0	0
0.005	0.5	<1	4
0.01	1	1	8
0.02	2	3	17
0.04	4	6	36
0.08	8	13	81

Note: Initial oxygen partial pressure is 0 torr. The defect length is assumed to be 0.1 mm. *Source*: Courtesy of Lighthouse Instruments, Inc., Charlottesville, Virginia, U.S.

Table 3 Predicted Vacuum Loss in a Leaking 10-mL Vial, Fully Evacuated Prior to Stoppering and Capping

	Package headspace pressure assuming stated leak size and laminar flow kinetics (torr)	
Time post package closing	5-μm diameter leak	2-μm diameter leak
0 min	0	0
1 min	13	2.4
5 min	63	12
10 min	126	24
60 min	756	144
5 hr	760	720
8 hr	760	760

Note: Laminar flow kinetics were modeled assuming a leak path length of 1.5 mm and air viscosity of 1.8×10^{-7} Pa·sec.

the greater oxygen partial pressure outside the container. Following Fick's laws of diffusion [eqs. (2) and (3)], assuming a 10-mL vial with initial oxygen partial pressure of 0 torr, and a length of 0.1 mm separating the vial headspace and the outside environment, oxygen ingress as a function of time can be predicted (Table 2). The results show that holes $\geq$5 μm will permit oxygen levels to rise above 1% within one day; 2-μm holes will bring about oxygen content greater than 1% after about eight days. Caution is advised, however, when attempting to predict package integrity for longer periods according to diffusion kinetics. Over time, packages are exposed to pressure differentials from changes in altitude or weather, or even by doors opening and closing, all of which drive faster, convective flux leakage, thus complicating such projections.

Consider a second scenario, in which a 10-mL vial containing lyophilized product is stoppered under vacuum. In this case, the differential pressure between the evacuated container and the atmosphere will drive air into the package according to either molecular or laminar flow kinetics, depending on the leak path diameter, the mean free path length of the leaking gas, and the package internal pressure. Table 3 presents the projected vacuum loss that will occur for a 10-mL vial initially stoppered under full vacuum (0 torr), assuming a leak path length (vial wall thickness) of 1.5 mm, and laminar gas flow leakage. Calculations assumed laminar flow [eq. (5)] and air viscosity at 15°C (1.8×10^{-7} Pa sec). Tabulated predictions show that leakage through a hole as small as 2 μm wide is evident within several minutes after package closing; vacuum is completely lost in less than eight hours. Therefore, FMR spectroscopy is reliable and sensitive approach for verifying the integrity of every evacuated container unit both upon package sealing and as a function of stability.

Trace Gas Leak Test Methods

Leak detection by trace gas analysis is the most sensitive leak test method available. Helium is the most common trace gas used for package integrity testing, although hydrogen is also used

(30,31). Detection of helium by mass spectrometry is capable of detecting large leaks of 10^{-2} Pa m^3/sec down to ultrafine leaks as small as 10^{-11} Pa m^3/sec. Helium trace gas testing is most useful for testing leaks in the moderate to ultrafine leak range. Greatest sensitivity is possible using the vacuum mode, in which a helium-flooded sealed package is exposed to vacuum conditions while inside a closed test fixture. Mass spectrometry detects helium drawn into the fixture from the leaking package. Alternatively, the sniffer mode works by scanning the test package's exterior surfaces checking for helium leakage into the atmosphere or into a special scanning fixture. The sniffer mode can pinpoint leakage location, and is especially suited for packages that cannot tolerate test vacuum conditions. ASTM F2391-05 *Standard Test Method for Measuring Package and Seal Integrity Using Helium as the Tracer Gas* describes both vacuum mode and sniffer mode techniques (32). The ASTM method text includes P&B data demonstrating the vacuum mode's ability to differentiate between cold-form aluminum foil blister packages punctured with a needle and covered with aluminum foil laminate tape (leak rate approximately 10^{-8} cc/sec/atm), to those punctured but masked with more permeable Scotch™ tape (leak rate approximately 10^{-6} cc/sec/atm).

There are possible sources of error or method interferences unique to helium mass spectrometry. Background helium present in the testing environment can mask package leaks. Steps to prevent elevated helium levels in the test area include proper ventilation, remote helium cylinder location, and proper sample isolation fixturing. "Virtual" leaks resulting from helium adsorbed onto package surfaces or trapped in seal areas can be mistaken for true leakage. "Washing" surfaces free of helium using an inert gas, or drawing off adsorbed helium by adding a preliminary vacuum cycle to the leak test are sometimes used to avoid virtual leaks. Helium easily permeates through many materials, especially plastics and some elastomers. Thus, helium permeation through the test package should be known to prevent misinterpretation of results. Care should be exercised when large leaks are suspected, as helium can be quickly lost even prior to conducting the test. Finally, sensor calibration using helium reference leaks is required to ensure accurate results.

Research teams lead by Kirsch (3,8,21) and Nguygn (20) used the helium mass spectrometry vacuum mode to measure the leak rates of positive control vials prior to microbial challenge and vacuum decay leak testing. More recently, Miyako and colleagues (33) used helium mass spectrometry for verifying the integrity of a double-bag system used for holding and transporting sterile freeze-dried powder from the bulk manufacturing site to the finished product packaging site. The bulk powder was bagged in a sterilized aluminum laminate bag which was flooded with sterile-filtered helium and subsequently sealed. This inner bag was then placed in a sterile polyethylene bag which was also sealed. The helium leak test was performed by placing the double-bagged package in a vacuum chamber. After target vacuum was reached, the vacuum source was isolated from the chamber and the double-bagged package remained under vacuum for up to one hour, allowing helium leakage to occur. The chamber was then flooded with sterile-filtered nitrogen, and a sniffer probe connected to the test chamber was used to collect a gas sample for helium detection. The helium leak test was able to find pinholes present in both bags between 20 and 500 μm in size. The size of the bag and the location of the sniffer probe inserted into the test fixture influenced leak detection.

Helium leak detection is a very useful tool for container closure integrity evaluation of packages in the research and development stages of a product's life cycle. Because some expertise is required to design and conduct leak tests by helium mass spectrometry, this technology is best performed in a laboratory setting by skilled workers. When properly performed, helium mass spectrometry provides valuable information on the quantitative leak rate of a package, as well as the package's leak location.

INTEGRITY TESTING THROUGH PRODUCT LIFE CYCLE STAGES

Changing Demands Through the Life Cycle

The scope of leak tests performed may change as a product moves through the various life cycle phases of product development, marketed product manufacturing, and marketed product stability (34). Package design and development involving seal characterization and optimization demand the most package integrity support, and may in some cases, require

multiple leak tests for verifying different performance criteria of individual seals. Once the package system and the assembly processes are well defined and controlled, leak tests used to support manufacturing practices may be able to focus on detecting larger leaks resulting from defective components or poor assembly.

For example, highly sensitive and quantititative helium mass spectrometry tests can be quite useful when characterizing a vial package system during package design and development. Helium leak test methods readily detect leaks at or below liquid leakage cut-off specifications. However, helium tracer tests take time to perform, are destructive to the package, may miss larger defects, and require considerable operator expertise, making this approach impractical during routine manufacturing. At the manufacturing stage, more rapid, nondestructive vacuum decay leak tests or electrical conductivity tests may make more sense for identifying leaks resulting from damage or misassembly.

While gas tracer or vacuum decay leak test methods are generically used for many container closure systems, other test methods are more product package specific. For example, electrical conductivity leak detection rapidly detects defects in liquid-filled glass or plastic packages, and is most useful in production environments for testing entire lots. Frequency modulation spectroscopy is ideally suited for testing vial package systems intended to maintain a low-oxygen or low-pressure headspace. This method is very rapid, highly sensitive, and nondestructive making it useful throughout all product life cycle phases, from research through 100% on-line production lot testing.

Integrity as a Function of Product Stability

Regulatory agencies around the world either imply or require product container closure system integrity verification as a function of stability to support new product market applications and to provide on-going postmarket product quality data. The U.S. FDA has issued several Guidances to Industry on this topic, discussed below.

The U.S. FDA Guidance of 1999 regarding container and closure systems for packaging human drugs and biologics (35) indicates the need for all pharmaceutical packaging to be suitable for its intended use. One aspect of suitability is protection—the ability of the container closure system "to provide the dosage form with adequate protection from factors (e.g., temperature, light) that can cause degradation in the quality of that dosage form over its shelf life." Common causes of degradation linked to package integrity cited in this Guidance include loss of solvent, exposure to reactive gases (e.g., oxygen), absorption of water vapor, microbial contamination, and contamination by filth. Package suitability verification provided in any new product submission must therefore include package integrity study results. As stated in the Guidance, "... the ultimate proof of suitability of the container-closure system and the packaging process is established by full shelf life stability studies." And later, "Stability testing of the drug product should be conducted using the container-closure systems provided in the application ... The container-closure system should be monitored for signs of instability. Where appropriate, an evaluation of the packaging system should be included in the stability protocol." Thus, integrity testing as part of stability protocols is strongly encouraged.

The U.S. FDA Guidance for Industry describing sterilization process validation submission documentation directly communicates the need to demonstrate the ability of a container closure system to maintain the integrity of its microbial barrier, and, hence, the sterility of a drug product through its shelf life (36).

More recently, an FDA Guidance for Industry addresses the issue of integrity as part of pre- and postapproval stability protocols for sterile biological products, human and animal drugs, including investigational and bulk drugs (37). As noted, manufacturers of drugs and biologics purporting to be sterile must test each lot or batch prior to release to ensure that the product conforms to sterility requirements. While stability testing must provide evidence on how the quality of a substance or product varies with time and under specific storage conditions. Stability protocols must therefore include a method(s) that supports the continued capability of containers to maintain sterility. Sterility testing satisfies this requirement; however, this newer Guidance acknowledges practical and scientific limitations for the sterility testing approach. Therefore, this Guidance allows the substitution of other integrity tests in stability protocols according to the information and recommendations spelled out.

The FDA Guidance of 2008 does not suggest specific test methods and acceptance criteria, nor does the agency provide comprehensive lists of tests. Instead, good scientific principles are recommended, taking into consideration the container closure system, product formulations, and, where applicable, routes of administration. The Guidance states, "Any validated container and closure system integrity test method should be acceptable provided the method uses analytical detection techniques appropriate to the method and is compatible with the specific product being tested. Innovative methodology is encouraged. Information submitted to the agency should detail what the test method evaluates and how it is applicable to microbial integrity. A test method is adequately validated if it has been proven through scientifically accepted studies to be capable of detecting a breach in container and closure system integrity." The selected integrity test should be "conducted annually and at expiry, or as otherwise required by applicable regulations." Both physicochemical and microbiological challenge methods are mentioned, but the onus for proper test method selection and validation lies with the product manufacturer.

Integrity as a Function of Distribution and Use

A complete package development program should include package integrity tests performed in conjunction with distribution and end-user handling challenges. Ship testing, whether simulated in a laboratory or performed in the field, provides much more meaningful data if packages are integrity tested before and after exposure to the distribution conditions. Otherwise, it becomes difficult to ascribe package damage discovered at the end of a study to the distribution challenge. Therefore, a nondestructive leak test method is best able to detect damaged product both before and after shipping.

Use testing provides valuable insight into the functionality and integrity of packages placed in the hands of the end-user. Studies comparing package use by subjects provided with careful product package usage instructions to those given no direction provide interesting and practical information that can help in final package optimization and product literature preparation. End-user populations should vary in age, sex, education, and skill level as appropriate. This is especially important for products intended for homecare administration, or for use by the elderly or physically impaired.

Production Lot Integrity Testing: 100% Vs. Statistical Process Control

The 2008 revision to Annex 1 of the European Union Good Manufacturing Practices (GMPs) for sterile products states that "Containers closed by fusion, e.g., glass or plastic ampoules should be subject to 100% integrity testing. Samples of other containers should be checked for integrity according to appropriate procedures" (38). Additionally, "Containers sealed under vacuum should be tested for maintenance of that vacuum after an appropriate, pre-determined period." Concerning stoppered vials, "Vials with missing or displaced stoppers should be rejected prior to capping." Another reference to integrity testing in the EU GMPs states: "Filled containers of parenteral products should be inspected individually for extraneous contamination or other defects." Direction is given for human inspection, and "where other methods of inspection are used, the process should be validated and the performance of the equipment checked at intervals."

The 2004 U.S. FDA Sterile Drug Products Aseptic Processing GMPs delineate similar standards (39). Referring to inspection of container closure systems, "Any damaged or defective units should be detected, and removed, during inspection of the final sealed product. Safeguards should be implemented to strictly preclude shipment of product that may lack container-closure integrity and lead to nonsterility. Equipment suitability problems or incoming container or closure deficiencies can cause loss of container-closure system integrity. For example, failure to detect vials fractured by faulty machinery as well as by mishandling of bulk finished stock has led to drug recalls. If damage that is not readily detected leads to loss of container-closure integrity, improved procedures should be rapidly implemented to prevent and detect such defects." Appendix 2 *Blow-Fill-Seal Technology* states the following: "Container closure defects can be a major problem in control of a BFS operation. It is critical that the operation be designed and set-up to uniformly manufacture integral units. As a final measure, the inspection of each unit of a batch should include a reliable, sensitive, final product examination that is capable of identifying defective units (e.g., *leakers*). Significant defects due

to heat or mechanical problems, such as wall thickness, container or closure interface deficiencies, poorly formed closures, or other deviations should be investigated in accordance with §§ 211.100 and 211.192."

USP <1207> *Sterile Product Packaging—Integrity Evaluation* discusses the issue of 100% testing versus sample testing. This general information chapter emphasizes that control of critical production processes is paramount to integrity assurance, regardless of the integrity testing approach used (34).

To summarize, mandates to leak test every product package unit released for market currently exist only for glass and plastic BFS containers. Still, the pharmaceutical manufacturer is responsible if defective, leaking containers of any type enter the marketplace. Component quality and manufacturing process control are keys to ensuring integral packaged product, but experience says that defects still occur even under the best circumstances. For this reason, it is sensible to integrity test every production lot at least on a statistical sampling basis. Upon finding leaking packages, further lot testing and a full investigation to determine and correct the cause of the defect and to eliminate other defective units are called for. As leak test methods become available for rapid and nondestructive detection of leaks in various product package systems, it is logical to expect their implementation will become standard practice.

TEST METHOD SELECTION

Integrity test method selection is based on many factors largely addressed elsewhere in this chapter. The following brief listing summarizes major selection criteria, along with a few examples.

1. Package design and construction. Rigid, nonporous packages best tolerate test methods requiring vacuum or pressure challenge conditions, such as dye ingress tests, vacuum decay tests, or the helium mass spectroscopy vacuum mode test. Flexible packages tested by such methods require special tooling to restrict significant package expansion that may damage seals or negatively influence test method sensitivity. Packages with a porous component, such as a Tyvek lidded tray, can be tested by vacuum decay as long as a test chamber fixture or other means is used to mask the porous lidding material. Packages made of permeable materials, for example, plastics or elastomers, may not accommodate trace gas testing using gases such as helium. Electrical conductivity leak detection is able to find defects in liquid-filled packages if the liquid is more conductive than the package material.
2. Seal type and location. Package seal type and location can influence test method selection. For example, ophthalmic dropper bottles have two main seals: the dropper-tip/bottle-neck valve seal and the dropper-tip/screw-cap seal. Both seals are hidden from view under a screw-thread cap making it impossible to inspect for evidence of liquid leakage at the actual seal locations. Thus, a whole-package test able to detect gas leakage, such as vacuum decay, makes more sense in this case. On the other hand, a translucent plastic bag is easily inspected for evidence of dye migration through heat sealed areas. Electrical conductivity leak detection is an excellent choice when checking physically accessible locations at higher risk for leaks, such as the seal tip end of a plastic BFS ampoule. If a seal relies strictly on a tortuous path or the quality of a porous barrier material, then microbial challenge testing may prove necessary.
3. Critical leakage rate. Seals made to prevent liquid leakage and microbial ingress require less stringent leak rate criteria than seals meant to prevent loss of vacuum or inert gas. When verifying absence of leaks $\geq$5 μm in a nonporous, rigid package to minimize risk of liquid loss and/or microbial ingress, viable options include electrical conductivity, vacuum decay, and liquid tracer tests, assuming appropriate method optimization. Frequency modulation spectroscopy is very appropriate for headspace content verification of clear or translucent packages, both upon initial sealing and over product shelf life. With appropriate fixturing and instrumentation, helium mass spectrometry is able to quantitatively measure package leaks ranging from 10^{-2} down to 10^{-11} Pa m^3/sec. However, such trace gas methods are perhaps most useful when detecting leaks not easily found with other leak test methods, namely, below about 10^{-5} Pa m^3/sec.

4. Product life cycle phase. Tests to prove a package's most critical leakage rate of concern are commonly performed during package design and development phases. Early research may also include a wide variety of tests to satisfy particular study objectives. Once package components and assembly are optimally defined, fewer test methods may be implemented to verify absence of larger, random defects or package misassembly. For example, early development of a vial package for a liquid formulation may incorporate helium mass spectrometry to verify the critical leak rate specification; a dye ingress test as a visual aid for finding package defects; and a vacuum decay test for supporting distribution and stability studies. Later in production, an on-line electrical conductivity test may check for package defects or improper assembly.
5. Regulatory and validation requirements. Region- or country-specific regulatory requirements influence leak test method selection. A parenteral product approval to market application often includes microbial challenge test data, along with sterility tests performed as a function of product stability. However, this trend is changing. A nonmicrobial method may successfully substitute for microbial challenge tests, or replace the sterility test performed through product expiry, if strong scientific rationale and validation data supporting the alternative method are provided. A study correlating the sensitivity of the alternative method to a microbial ingress test is helpful; such comparison may be theoretical or practical. Regardless, it is important to use validated test methods to support a product approval to market application or marketed product lot release. It is not adequate simply to follow an internationally recognized ISO, ASTM or compendial method. (ASTM methods typically include P&B statements based on round robin studies. These data provide a useful starting point for test method development and validation.) Even these methods require validation studies specific to the product package system, the test equipment and the test method parameters. Validation should include verification of method robustness, reliability, accuracy and range of leak sizes detected (sensitivity). Therefore, ease of method validation is also a factor in test method selection.
6. Cost versus benefit. The costs of package integrity test methods range from a few thousand to a several hundred thousand dollars, depending on the test method and its implementation. The least expensive tests include dye, liquid tracer, and microbial challenge tests, and are therefore often preferred. However, these probabilistic tests require the destruction of large test sample populations to generate the most reliable data. Conducting such tests expends resources of time, staff, equipment, and space. Human inspection processes for detecting dye or microbial ingress are especially costly, and results are prone to error. Numerous other challenges face microbial challenge test methods, as discussed in section "Test Method Validation."

   Sometimes a given test method may vary in expense as a function of the equipment manufacturer and the method's manner of application. For example, vacuum decay leak testers come as single-chamber, manually operated test systems costing tens of thousands of dollars, or as multichamber, rotary, 100% on-line systems costing hundreds of thousands of dollars, or more. The single-chamber manual systems are not well-suited for 100% testing of large lots, but they are less costly, easier to validate, and are capable of detecting smaller leaks. Each vacuum decay equipment manufacturer uses a different methodology for detecting leakage pressure rise, which then influences the validation approach and related costs. Which test system and manufacturer is most appropriate depends on many factors, including the product, the pharmaceutical manufacturer's philosophy, the nature and size of the leaks anticipated, and the quality control systems in place for incoming package components and product manufacture. Regardless, some significant investment in integrity test method selection, validation and implementation should be expected.

TEST METHOD VALIDATION

Package integrity test methods should be validated for robustness, reliability, accuracy, and range of leak sizes detected. Quantitative analytical methodology routinely relies on these test method validation concepts. But in the case of parenteral product package physicochemical leak

tests, often some assessment of the method's sensitivity to risk of microbial ingress is presumed, whether on the basis of scientific rationale or on the basis of actual laboratory studies.

Leak Test Sensitivity by Direct Comparison with Microbial Challenge Tests

How physicochemical integrity tests compare with microbial ingress tests is a topic frequently explored in publications from the food, pharmaceutical and medical device industries and academia. Generally, a population of both good and defective package units tested by both microbial ingress and the alternative container closure integrity method provide a direct comparison of the two approaches. The studies summarized below provide interesting insight on how to perform direct comparison studies, and perhaps, whether such comparisons are warranted.

About 20 years ago, the author and a team of researchers compared gas leak rates with liquid and microbial ingress from vial packages (5,40). Vials were made of stainless steel, electropolished to ensure exceptionally smooth sealing surfaces. Disc-shaped closures made of various elastomers, either uncoated or laminated with a variety of fluorocarbon- or propylene-based polymeric materials, were capped onto the metal vials at various seal forces. Test packages were mounted onto a manifold enabling them to be internally pressurized with filtered nitrogen. Package leak rates were determined by pressurizing the manifold-vial test system to target pressure, then monitoring the system's pressure drop over time. Measured gas flow rates ranged from 10^{-3} to 10^{-7} Pa m^3/sec, at 3 pounds per square inch gauge differential pressure test conditions. For the comparative microbial challenge test, each sterilized, manifold-mounted vial was filled with a suspension of *P. aeruginosa* ($\geq 3 \times 10^8$ CFUs/mL). The vial packages were submerged closure-end-down in sterile saline while being internally pressurized via the manifold. Microbial leakage into the saline was determined using a filter plate count method. In like manner, the liquid leakage test was performed by filling the vials with an aqueous solution of copper sulfate, and testing for copper ion presence in distilled water collection fluid by atomic absorption. No packages of gas leak rates less than 10^{-5} Pa m^3/sec demonstrated microbial or liquid tracer leakage. Interestingly, liquid passage occurred for every package exhibiting gas leakage at or above this rate limit, while microbial leakage only occurred sporadically, with the number of colony forming units moving across the seal bearing no relation to the gas flow rate.

In the 1990s, a team led by Lee Kirsch at the University of Iowa correlated helium leak flow rate from glass vial packages to microbial ingress and liquid leakage (8). Positive controls were made by imbedding glass micropipettes of various nominal diameters (0.1–10 μm) into the walls of glass vials. Vial package leakage was quantified by flooding open vials with helium just prior to stoppering and capping, then testing the packages using helium mass spectrometry according to the vacuum mode method. Microbial and liquid leakage through these same leak paths was determined by first filling each vial with sterile saline lactose broth. Broth-filled packages were immersed in a 60°C water bath for one hour, followed by immersion in a 25°C saline lactose broth, spiked with magnesium ion trace element, for another hour to allow the vial content's temperature to equilibrate to 25°C. The purpose of this procedure was to eliminate airlocks in the leak path. Next, the bath was spiked with 10^8 to 10^{10} viable *B. diminuta* and *E. coli* organisms/mL, and the vials continued to be immersed for 24 hours at 35°C. Post 13 days of incubation, vials were inspected for evidence of microbial growth, and vial contents were assayed for presence of magnesium tracer using atomic absorption spectroscopy.

Initially, the University of Iowa researchers only reported microbial ingress data for those test packages confirmed to contain magnesium; units failing to demonstrate a liquid pathway were eliminated from the analysis. Given these criteria, the probability of microbial ingress was near 100% at helium leak rates of about $10^{-1.9}$ std cm^3/sec (sccs), which was equivalent to about an 8-μm nominal diameter leak. An 80% probability of ingress corresponded to a leak rate of about $10^{-2.5}$ sccs (about 5 μm), and a 50% probability of ingress corresponded to a leak rate of about $10^{-3.7}$ sccs (about 0.7 μm). The likelihood of microbial failure at leak rates $\leq 10^{-5}$ sccs was remote; of the 66 test units with leak rates less than $10^{-4.5}$ sccs, only three failed the microbial ingress challenge.

Later, Kirsch used this same body of research to explore the relationship between liquid leakage verified by magnesium tracer and the likelihood of microbial ingress (6). He concluded

that both liquid leakage and microbial ingress are probabilistic occurrences. For any given leak, liquid passage was more likely to occur than microbial ingress. However, even at relatively large gas leak rates greater than 10^{-4} sccs liquid leakage at times failed to occur. Microbial ingress only occurred when liquid leakage was also present, but liquid leakage did not guarantee microbial ingress. Thus, it was concluded that microbial ingress through a leak sized at $<10^{-2}$ sccs requires liquid penetration through the leak path. And liquid leakage likely depends on variables such as liquid surface tension, defect diameter, leak morphology, leak surface conditions, environmental contaminants blocking the leak, and procedural technique.

Burrell et al. compared an ISO dye ingress method with a liquid immersion microbial challenge integrity test using vial packages (13). Positive controls were created by inserting polyimide-coated glass microtubes ranging in internal diameter from 2 to 75 μm through the elastomeric closures of 5-mL vial packages. Vials were challenged with dye solution (1% FD&C Red No. 40% and 0.25% sodium dodecyl sulfate) following procedures described in ISO 8362-2 Annex C (41). Exceptions to the ISO procedure included use of red dye, rather than methylene blue, and analysis by spectrophotometry, rather than by visual inspection. Challenge conditions included package immersion in dye solution for 30 minutes at 22 in Hg (75 kPa) vacuum, followed by rapid vacuum release and 30 minutes of dye immersion at ambient pressure. There was no attempt to eliminate airlocks in the microtubes. The microbial challenge test used positive and negative control packages, filled with saline lactose broth and immersed in an *E. coli* suspension ($\geq 10^8$ CFUs/mL), challenged according to the same ISO procedure. Results showed the dye ingress test and the microbial challenge test were equally sensitive. Dye and microbial ingress occurred in at least half the units with microtubes 10 μm in diameter. No leakage of any kind was detected in packages with smaller defects (2 and 5 μm). All units of microtubes ≥20 μm demonstrated dye leakage and microbial ingress. Therefore, the ISO dye ingress method was equally sensitive to a microbial challenge test performed according to identical challenge conditions.

Keller and team published an interesting study in 2006, further exploring the relationship between critical leak size and package sterility (7). Leaking package models were created using nickel microtubes, 7 mm long, with inner diameters of 2, 5, 7, 10, 20, and 50 μm, each placed through the elastomeric septa of a small glass cell encased in a glass water jacket. Negative controls utilized solid tubes. Sterilized test cells filled with nutrient broth were placed in an aerosol chamber with tube-end down to ensure liquid broth contact with the microtube opening. Motile *P. fragi* microorganisms were aerosolized to establish a concentration of approximately 10^6 CFUs/cm^3 during the 30-minute come-up period; static conditions followed for an additional 5 minutes. Post exposure incubation continued for 72 hours at 25°C. Test cell media turbidity was indicative of microbial growth. Special ports added to each test cell enabled the simulated packages to be exposed to various controlled pressure/vacuum/temperature conditions during the biochallenge. A randomized block design allowed independent measurement of each test variable's influence on test package sterility. Considering all test variables, results showed microbial ingress can occur through microtubes as small as 5 μm in diameter; 2-μm tubes and negative controls showed no growth in any case. Test conditions that promoted broth flow into or through the tubes correlated to higher risk of microbial ingress; the greater likelihood for liquid flow, the greater the sterility loss risk. For instance, static conditions in which no differential pressure was applied only triggered microbial ingress through two of nine tubes sized 50 μm wide. Factors that promote product liquid flow and therefore increase risk of packaged product sterility loss include defect size, liquid product surface tension and the pressures imposed on the package during processing, distribution and storage.

In conclusion, all studies described illustrate the probabilistic nature of microbial ingress through package defects. Microbial challenge tests require carefully designed and conducted procedures using relatively large test sample populations to support convincing conclusions. Numerous studies have attempted to pinpoint the critical leak size that corresponds to risk of product sterility loss. Results vary, with some studies implicating leaks as small as 0.2 μm, while others imply leak paths 10 μm and larger. Regardless, and perhaps most importantly, all research shows that liquid presence in the smallest defects is required for microbes to enter. Therefore, it seems logical that industry should move away from directly correlating

physicochemical leak tests to microbial challenge tests, to examining the leak test method's ability to detect defects capable of liquid passage—a less stochastic and more easily verified parameter.

Leak Test Sensitivity by Indirect Comparison with Microbial Challenge Tests

Literature studies describe indirect means of correlating physicochemical leak tests to risk of microbial ingress. In two publications, vacuum decay leak tests results were compared with helium trace gas detection by mass spectroscopy. Previously, the helium mass spec method had been judged against a microbial ingress test using the same test sample population type; thus establishing an indirect relationship between vacuum decay test results to risk of microbial ingress (20,21).

Another indirect comparison approach, explained under test method "*Frequency Modulation Spectroscopy*," is based entirely on gas leak rate predictions through a theoretical defect into an evacuated vial package. In the example cited, laminar gas flow theory was used to predict the pressure rise in 10-mL vial packages, initially sealed under vacuum, with leaks 2 and 5 μm wide. The text noted that as long as the actual vial package in question maintains an internal pressure at or below leaking package predictions, then no leaks of that equivalent size are present.

The works described in the previous subsection, "Leak Test Sensitivity by Direct Comparison with Microbial Challenge Tests," suggest that the presence of liquid in or moving through a leak path provides a better indication of the risk to package sterility afforded by the defect than a biological challenge test performed under the same test conditions. In fact, without liquid presence, microbial ingress through very small defects less than about 10 μm in nominal diameter appears improbable. With liquid presence or passage, sterility loss risk increases significantly. Therefore, a leak test reliably able to detect liquid passage can be indirectly assumed as good as, or better than, a microbial challenge test performed under the same test conditions.

Leak Test Sensitivity Based on Leak Rate Standards

Leak test method sensitivity may also be determined quantitatively using calibrated reference leak standards. Calibrated physical leaks are designed to deliver gas at a known flow rate. There are many types of standard leaks, falling into two main categories: (*i*) reservoir leaks that contain their own tracer gas supply and (*ii*) nonreservoir leaks that rely on tracer gas addition during testing. Calibrated gas leaks perform by one of two methods. Either the leakage rate depends on the permeation of specified materials by certain gases, or an orifice is present allowing specified gas flow rates under prescribed differential pressure conditions. Some leak test instruments, for example, helium mass spectrometry, incorporate internal reference standards to verify test system functionality.

Other leak test instruments that rely on air movement for leak detection, for example, vacuum decay testers, may utilize a calibrated variable rate flowmeter or a fixed size orifice to artificially introduce leakage into a test chamber during equipment qualification or start-up.

Whenever possible, leak test instrument performance should be challenged using such calibrated standards. The *Nondestructive Testing Handbook*, Volume 1 *Leak Testing* (42) is an excellent resource for precautions and limitations regarding calibrated leak usage. While calibrated leak standards provide valuable instrument functionality and sensitivity information, it is still important to challenge a leak test method using known positive and negative control package samples.

Positive Control Test Samples

Defect Types

Leak test sensitivity verification is not complete without a demonstration of successful leak detection using a randomized population of negative and positive control test samples. A positive control is a known-leaking test package. A common misconception is that a media-filled package used for a growth promotion check in a microbial challenge test is equivalent to a positive control test sample. A growth promotion test only proves that the packaged media can support microbial growth; it does not prove that bacteria would or could actually enter the package. Another false perception is that a calibration standard, such as a calibrated airflow

introduced into a vacuum decay leak test chamber, satisfies the need for a positive control test. Certainly, such a test is important as it correlates equipment response (pressure rise) to a known challenge (airflow rate). However, it does not prove that the method can detect leaks of various sizes or types at various locations on the package.

Simple ways commonly used to create positive control test samples involve inserting microtubes or needles through package walls, placing wires or film between sealing surfaces, or adhering thin metal plates with microholes over package surface openings. These types of defects are inexpensive, simple to create, and give a quick assessment of a leak test's capabilities. Because microtubes, microholes and needles have fixed diameters, test results infer detectable leak path sizes. On the other hand, such positive controls do not truly represent defects most likely to occur in actual product packages. Liquid or microbial migration around or through an item foreign to the package (e.g., needle, film, microhole, or microtube) may be very different from leakage through an actual defect located in or between package components.

A study by Morrical and associates illustrated this very point, by comparing helium leakage and microbial ingress through two types of defects in glass vial packages (43). One defect type consisted of a laser-drilled microhole in a thin metal plate mounted on a holed-stopper, capped on each test vial. Microholes ranged in diameter from 0.5 to 15 μm. The other leak type was a copper wire placed along the sealing surface between the elastomeric closure and the glass vial. Wire thicknesses ranged from 10 to 120 μm. Helium trace gas leakage was detected using mass spectrometry. The microbial challenge test included a suspension of *S. marcescens* ($\geq 10^8$ CFUs/mL). Challenge conditions consisted of one hour at 0.4 bar vacuum followed by one hour at 0.4 bar overpressure. Both test methods showed different leakage behavior for the two positive control types. Helium leak rates through the microholes matched theoretical predictions for gas moving through an orifice, whereas helium flow rates through the wired samples displayed complex, less predictable, gas flow dynamics. Microbial ingress occurred in at least a portion of the samples with microholes $\geq$4 μm (helium leakage rate $\geq 6.1 \times 10^{-3}$ mbar L/sec), while units with holes $\leq$2 μm ($\leq 1.4 \times 10^{-3}$ mbar L/sec) saw no microbial leakage. Microbial challenge results for hand-capped vials with wire defects demonstrated microbial leakage for wire diameters $\geq$20 μm (helium leakage rate $\geq 2.2 \times 10^{-5}$ mbar L/sec).

Whenever possible, positive control test samples should incorporate defects simulating actual leaks likely to occur. For example, typical vial package defects may include glass cracks or breaks (Fig. 8), misaligned or misshapen closures, and poorly crimped seals. Therefore, a laser-drilled hole in a glass vial wall could simulate vial breakage. Including defects positioned above and below the liquid fill level is important if the leak test method's performance is a function of liquid or gas presence in the leak path. Scoring the vial finish might represent another type of glass defect (Fig. 8). Removing slices along a closure's sealing surface, or loosely capping seals can replicate closure and seal defects, respectively. Pouch or bag positive control samples might include pinholes, open seals, channeled or wrinkled seals, weak seals, "burned" seals, and seals with trapped product inclusions. Ophthalmic dropper bottle positive controls could include loose caps, missing or poorly inserted dropper tips, defective tips or caps, and pinholes in the bottle.

With the exception of laser-drilled hole defects, the positive controls described will not necessarily provide information about the exact sizes of detectable leaks, but they will help define detectable leak locations and types. Risks inherent in this approach include the possibility that the leak test would not find all nonhole positive controls, and that the irregularities in defects' shapes or sizes may not permit statistically sound method reliability and sensitivity assessments. Nevertheless, including such positive controls in leak test method feasibility and optimization studies can provide invaluable information on the method's capabilities. Knowing this may give insight into ways of limiting the occurrence of actual defects not readily found by the chosen leak test method.

Defect Sizes

Published studies using microtubes or other artificial means to create leaks have unfortunately resulted in an expectation that all leak test methods need to detect defects as small as 0.2 μm in diameter, otherwise, the test method cannot compare to microbial ingress.

Figure 8 Defects found in glass vials. *Top row*: Line-over defect likely created during vial manufacturing process. *Middle row*: Crack in vial finish likely created during vial manufacturing or distribution to end-user. *Bottom row*: Crack in vial shoulder (*left*) and vial neck (*right*) likely created at the end-user manufacturing site. *Source*: Anonymous upon request.

The first problem with this premise is creating defects 0.2 μm in size. Experience says naturally occurring leaks in packages below a few micrometers wide are extremely rare, if they occur at all. Also, defects are not hole-shaped, but are complex tortuous paths. Even artificial laser-drilled holes through the walls of glass vials or syringes are really a convoluted matrix of capillaries and chambers (Fig. 9). Companies that laser drill holes certify their size by comparing the rate of pressurized gas flow through each hole with flow rates through standard orifices in thin metal plates. Generally, the smallest possible laser-drilled holes through small volume glass or plastic containers range from about 3 to 5 μm in nominal

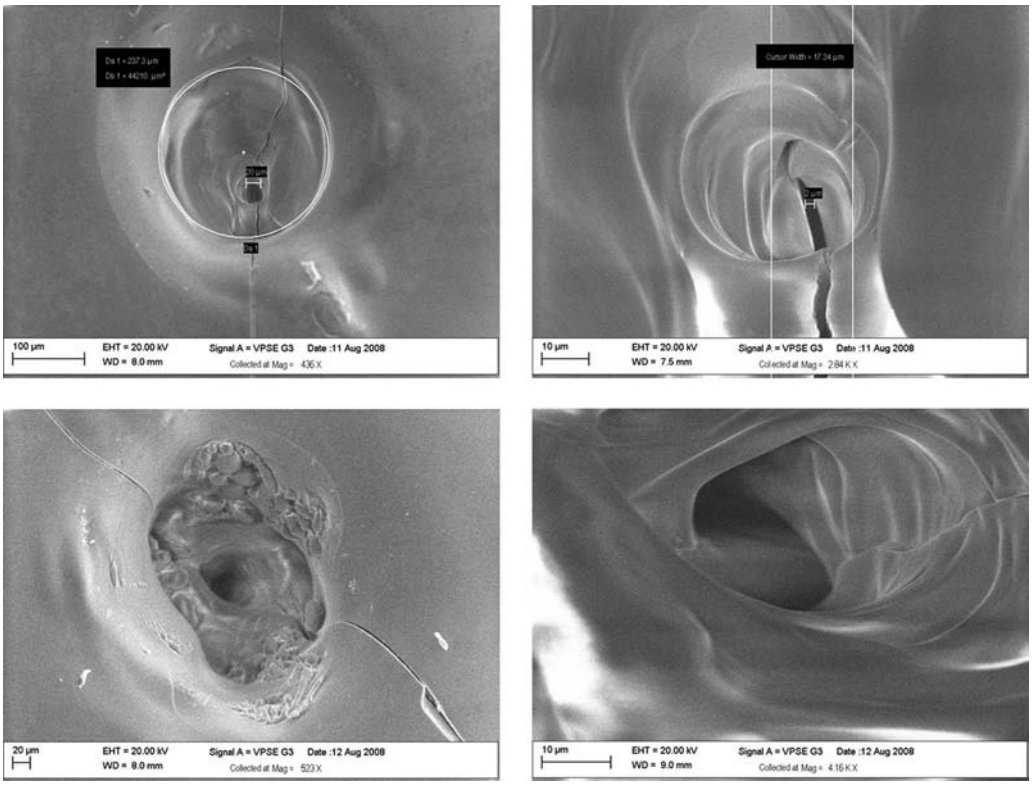


Figure 9 Scanning electron micrographs of laser-drilled holes through the glass barrels of 1-mL prefillable syringes. Each hole was nominally sized by comparing the rate of pressurized airflow passing through each hole with the flow rate through precisely formed, standard holes in thin metal plates. Nominal hole sizes are 10 µm (*top row*) and 15 µm (*bottom row*). *Source*: Reprinted from Amgen, Inc., Thousand Oaks, California, U.S.

diameter; smaller holes are difficult to make and readily clog. The smallest feasible holes through flexible laminates or films may vary from about 2 to 10 µm in diameter depending on the packaging material. Without a way of creating and sustaining holes sized below these practical limits, positive control test samples with smaller defects are not possible.

The other factor complicating this requirement is even typical microbial ingress tests cannot find 0.2-µm defects. Microbial ingress tests by Kirsch et al. (8) only found submicron-sized defects in a very small fraction of samples, under extreme challenge conditions, after meticulous measures to eliminate leak path plugs and airlocks. The risk of microbial ingress rose significantly for defects >1 µm, exceeding 80% probability for defects about 5 µm, and approached 100% probability for 8-µm defects. All defects considered in this analysis where those already confirmed as allowing liquid passage. In the absence of liquid passage, no microbial ingress occurred with any size defect (6). Research by Burrell et al. linked microtube defects ≥10 µm to a significant chance of dye and microbial ingress (13), while Keller's work using aerosolized microorganisms implicated microtube leaks ≥5 µm (7). Morrical detected microbial ingress in a portion of vial packages topped with thin metal plates having microholes ≥4 µm (43).

Therefore, positive control leaks should be as small as reasonably possible, given the type of package, the package dimensions, and the materials of construction. Parenteral product package positive control test units used for checking the lower limit of sensitivity of physicochemical leak test methods generally include defects ≥5 µm in diameter. Positive control sample populations should include larger defects as well as smallest defects, to represent the full range of anticipated leak sizes.

CONCLUSION

Container closure integrity is an easy concept to grasp. Simply put, packages must contain and protect their contents, preventing leakage in or out. However, the many parenteral product types and package integrity requirements make leak test method selection and leakage measurement anything but a simple process. First, leakage is not a straightforward, yes-or-no phenomenon. All package seals have the potential to leak gases to some extent; therefore, an understanding of leakage flux and critical leak rate specifications is necessary. When selecting leak test methods, microbial challenge tests are the traditional choice, despite their cumbersome application and demonstrated lack of reliability and sensitivity. Alternative physicochemical leak test methods are increasingly popular, including dye or liquid tracer methods, vacuum decay leak tests, electrical conductivity tests, frequency modulation spectroscopy, and trace gas detection. Each approach has unique advantages and disadvantages. Often more than one test may be necessary to provide full product support through all product life cycle phases. Any test selected must be appropriately developed, optimized and validated prior to use. Tools necessary for this process include calibrated reference leak standards, and positive and negative control test samples. The technique used to create leaks in positive control packages, and the size of these leaks, are significant factors in leak test sensitivity interpretation. Traditionally, final definition of leak test sensitivity requires some indirect or direct correlation to risk of sterility loss. Debate continues on the best approach to address this expectation, but mounting evidence supports a shift away from microbial ingress direct comparison studies. In summary, the last three decades have seen parenteral product container closure integrity move from a package testing afterthought to a major feature of product quality assessment. This evolution will likely drive the development of more reliable and sensitive package integrity test methods for future parenteral products.

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Index

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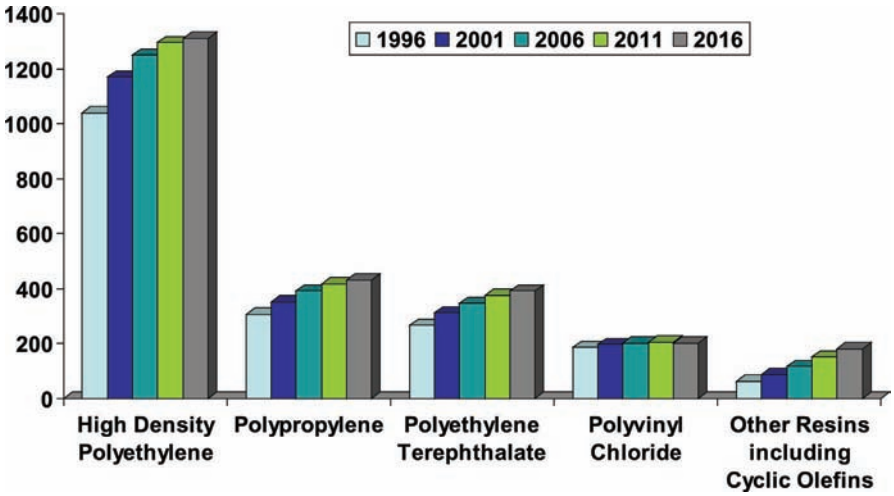

Figure 12.1 World pharmaceutical packaging plastics demand by resin (million pounds) (*see page 306*).

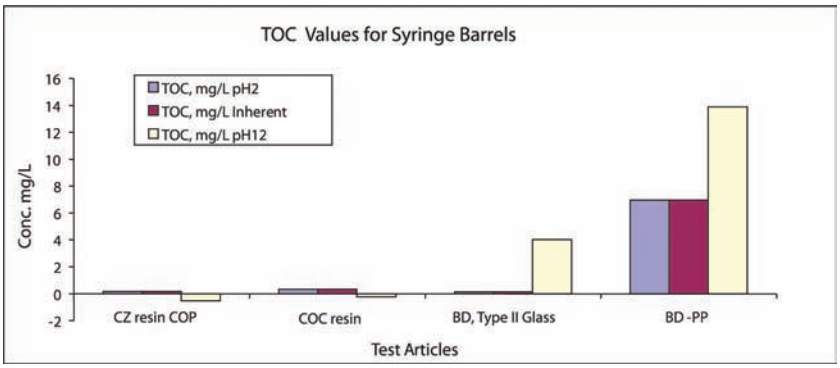

Figure 12.3 Comparison of total organic carbon as an extractable from syringe barrels. *Source*: Reproduced from Ref. 6 (*see page 307*).

Pharmaceutical Dosage Forms

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Pharmaceutical Dosage Forms

Parenteral Medications
Third Edition

Volume 2
Facility Design, Sterilization and Processing

Edited by

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We dedicate this work to those who have inspired us.
To my parents Walter and Ruth Ludwig and my wife Sue Ludwig
To my parents Hari and Pratibha Nema and my wife Tina Busch-Nema

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Foreword

I was a faculty member at the University of Tennessee and a colleague of Dr. Kenneth Avis when he conceived, organized, and edited (along with H.A. Lieberman and L. Lachman) the first edition of this book series that was published in 1984. It was so well received by the pharmaceutical science community that an expanded three-volume second edition was published in 1992. Dr. Avis did not survive long enough to oversee a third edition, and it was questionable whether a third edition would ever be published until two of his graduate students, Drs. Nema and Ludwig, took it upon themselves to carry on Dr. Avis' tradition.

Their oversight of this third edition is work that their mentor would be highly pleased and proud of. From 29 chapters in the second edition to 43 chapters in this new edition, this three-volume series comprehensively covers both the traditional subjects in parenteral science and technology as well as new and expanded subjects. For example, separate chapter topics in this edition not found in previous editions include solubility and solubilization, depot delivery systems, biophysical and biochemical characterization of peptides and proteins, container-closure integrity testing, water systems, endotoxin testing, focused chapters on different sterilization methods, risk assessment in aseptic processing, visual inspection, advances in injection devices, RNAi delivery, regulatory considerations for excipients, techniques to evaluate pain on injection, product specifications, extractables and leachables, process analytical technology, and quality by design.

The editors have done an outstanding job of convincing so many top experts in their fields to author these 43 chapters. The excellent reputations of the authors and editors of this book will guarantee superb content of each chapter. There is no other book in the world that covers the breadth and depth of parenteral science and technology better than this one. In my opinion, the editors have achieved their primary objectives—publishing a book that contains current and emerging sterile product development and manufacturing information, and maintaining the high standard of quality that readers would expect.

Michael J. Akers
Baxter BioPharma Solutions
Bloomington, Indiana, U.S.A.

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Preface

Pharmaceutical Dosage Forms: Parenteral Medications was originally published in 1984 and immediately accepted as a definitive reference in academic institutions and the pharmaceutical industry. The second edition was published in 1993. The ensuing years have produced incredible technological advancement. Classic small-molecule drugs are now complemented by complex molecules such as monoclonal antibodies, antibody fragments, aptamers, antisense, RNAi therapeutics, and DNA vaccines. There have been significant innovations in delivery devices, analytical techniques, in-silico modeling, and manufacturing and control technologies. In addition, the global regulatory environment has shifted toward greater emphasis on science-based risk assessment as evidenced by the evolving cGMPs, quality by design (QbD), process analytical technology (PAT), continuous processing, real time release, and other initiatives. The rapidly changing landscape in the parenteral field was the primary reason we undertook the challenging task of updating the three volumes. Our objectives were to (*i*) revise the text with current and emerging sterile product development and manufacturing science and (*ii*) maintain the high standard of quality the readers expect.

The third edition not only reflects enhanced content in all the chapters, but also more than half of the chapters are new underscoring the rapidly advancing technology. We have divided the volumes into logical subunits—volume 1 addresses formulation and packaging aspects; volume 2, facility design, sterilization and processing; and volume 3, regulations, validation and future directions. The authors invited to contribute chapters are established leaders with proven track records in their specialty areas. Hence, the textbook is authoritative and contains much of the collective experience gained in the (bio)pharmaceutical industry over the last two decades. *We are deeply grateful to all the authors who made this work possible.*

Volume 1 begins with a historical perspective of injectable drug therapy and common routes of administration. Formulation of small molecules and large molecules is presented in depth, including ophthalmic dosage forms. Parenteral packaging options are discussed relative to glass and plastic containers, as well as elastomeric closures. A definitive chapter is provided on container closure integrity.

Volume 2 presents chapters on facility design, cleanroom operations, and control of the environment. A chapter discussing pharmaceutical water systems is included. Key quality attributes of sterile dosage forms are discussed, including particulate matter, endotoxin, and sterility testing. The most widely used sterilization techniques as well as processing technologies are presented. Volume 2 concludes with an in-depth chapter on lyophilization.

Volume 3 focuses on regulatory requirements, risk-based process design, specifications, QbD, and extractables/leachables. In addition, we have included chapters on parenteral administration devices, siRNA delivery systems, injection site pain assessment, and control, PAT, and rapid microbiology test methods. Volume 3 concludes with a forward-looking chapter discussing the future of parenteral product manufacturing.

These three volumes differ from other textbooks in that they provide a learned review on developing parenteral dosage forms for *both* small molecules and biologics. Practical guidance is provided, in addition to theoretical aspects, for how to bring a drug candidate forward from discovery, through preclinical and clinical development, manufacturing, validation, and eventual registration.

The editors wish to thank Judy Clarkston and Lynn O'Toole-Bird (Pfizer, Inc.) for their invaluable assistance and organizational support during this project, and Sherri Niziolek and Bianca Turnbull (Informa Healthcare) for patiently leading us through the publishing process.

We also acknowledge the assistance of Pfizer, Inc. colleagues Lin Chen and Min Huang for reviewing several of the chapters.

We would like to express special gratitude to the late Kenneth E. Avis (University of Tennessee College of Pharmacy) for his dedication to teaching and sharing practical knowledge in the area of parenteral medications to so many students over the years, including us. Finally, we acknowledge the contributions of Dr Avis, Leon Lachman, and Herbert A. Lieberman who edited the earlier editions of this book series.

Sandeep Nema
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1 Aseptic manufacturing facility design

Mark Caldwell, Bob Helt, Beth Holden, Francesca McBride, and Kevin Schreier

INTRODUCTION

Sterile products may be in liquid or powder form (among others) as drug products and may be presented in formats including ampoules, vials, prefilled syringes, presterilized bottles, and blow-fill-seal containers. Product form and presentation influence processing conditions, equipment selection, and therefore, facility design. The sterile envelope refers to all the steps carried out during and following the final sterile filtration step through process completion, which occurs after filled product containers are sealed and a risk of environmental contamination to the product is eliminated. These steps include:

- Adjuvant, buffer and media formulation
- Addition of excipients
- Adjustment of concentration to achieve target potency
- Sterile filtration
- Component preparation
- Filling, stoppering/plugging, and sealing of product in final dosage containers

The design of the facility must meet all applicable regulatory guidelines, and meet GMP and safety guidelines. Current Good Manufacturing Practice (GMP) requires that areas of operation used for aseptic processing must prevent contamination from particles and microbes that may be present in the air, on product contact surfaces, or shed from personnel (1–5).

When processing biological products, such as live virus vaccines, attenuated vaccines and viral vectors, the biohazard nature of these products place extra demands on the facility. Potent compounds, like some biological products, also pose a risk to the operator and environment. Therefore, the facility and process design must also ensure both product and personnel safety.

This chapter establishes a basis for compliance with the global regulatory expectations for facility design, equipment interfaces, and utility requirements applicable to sterile processing and the manufacture of sterile products (6–10).

FACILITY DESIGN DRIVERS

As each facility is being designed, process requirements specific to each product must be considered. Each different type of product has different facility needs. Also, the number of products to be manufactured and the production campaign strategy will impact the facility design.

Product Types

Chemical Bulk Drug Substances (API)

Sterile chemical bulk drug substances are derived from chemical reactions. Facilities producing sterile API will be required to provide protection of the product during synthesis, isolation, and bulk filling. An adjuvant produced by precipitation is an example of a sterile API.

Potent Compounds

Potent compounds are classified as those chemical drug substances that are considered to be toxic to humans when exposure limits are exceeded, and may cause allergic reactions, birth defects, cancer, or other conditions. For this reason, it is required to ensure protection of operators working with potent compounds, ensure containment of all operations, and prevent release of products into the environment. It is acceptable to permit production of potent compounds in multiproduct facilities, provided the suite is segregated from other operations.

Following filling, it is recommended to wash the exterior of vials produced in potent compound facilities to limit uncontrolled exposure to the product during downstream operations.

Antibiotics

Antibiotics are drugs produced to treat bacterial or fungal infections. Antibiotics are considered to be sensitizers and can generate mild to severe allergic reactions in patients and operators. It is required to segregate production operations from personnel outside the production area. Campaigns of antibiotic must be segregated from other products, as the potential for cross-contamination between products can occur. In addition, β-lactam (penicillin) and nonpenicillin–based (cephalosporin) antibiotic products are not permitted to be produced in the same facility, as there is evidence that intolerance can occur for one antibiotic type, and not another.

To accomplish this segregation, it is a requirement that a separate dedicated suite be constructed for each antibiotic family. This suite can be housed inside a common structure with other functions. At no time should antibiotic production personnel come into contact with personnel operating in media or fermentation areas while gowned.

Following filling, it is recommended to wash the exterior of vials produced in antibiotic facilities to limit uncontrolled exposure to the product during downstream operations.

Biological Product

This category includes therapeutic proteins generated by fermentation or cell culture and inactivated vaccines. The facility is to be designed in same way as API production, except that terminal sterilization is often not feasible, due to the fragility of the product.

Live Virus Vaccines

Vaccines containing a live virus, or viral vector, must be designed to provide containment of the organism to protect both operators and the environment. Viral vectors are virus-like particles that inject genetic material into the cells of the organism being treated and are treated in a similar manner to live viruses. The biosafety level designated for the organism will drive decisions regarding the level of environmental controls that is required for the facility. Typically, these products require the use of adjuvants and will be suspension products, incapable of being filter sterilized. Products cannot be terminally sterilized. Live virus vaccine products can be campaigned in a multiproduct facility, as long as the vaccine production area is segregated from the remainder of the facility. Following the completion of the live virus campaign, the suite will need to be completely decontaminated prior to use for another product.

Facility Types

Single Product, Dedicated

This facility is designed to produce a single product at any one time, throughout the year, without concern for cross-contamination with a second product. The facility can be operated to produce multiple products in a series of campaigns, converting between products.

Multiproduct Multisuite

This facility is designed to produce multiple products simultaneously in multiple sterile suites. Sterile operations in each suite are to be segregated from one another to ensure that cross-contamination is prevented. It is a recommendation to clean and decontaminate any used components or equipment prior to exiting the suite and entering the return corridor.

Production Area Description

Conventional Aseptic Technology: Open

In conventional aseptic processing, the product is exposed to the room environment during operation. For this reason, aseptic operations are required to be performed under ISO 5

conditions, by sufficiently gowned operators, trained in aseptic technique. Sterility assurance levels for aseptic operations, including filling of vials or syringes, can be maximized through the use of barriers such as restricted access barrier systems or isolators to limit the size of the aseptic environment and remove operators from the ISO 5 fill area.

Terminally Sterilized Product

Whenever possible, it is required to terminally sterilize filled units of product. Application of an "overkill" sterilization methodology can provide a sterility assurance level of 10^{-6}, or better. Sterilization can be by steam, dry heat, gas, or radiation.

Restricted Access Barrier: Open and Closed

As defined by ISPE: A restricted access barrier system (RABS) is an advanced aseptic processing system that can be utilized in many applications in a fill-finish area. RABS provides an enclosed environment to reduce the risk of contamination to product, containers, closures, and product contact surfaces compared to the risks associated with conventional clean room operations. RABS can operate as "doors closed" for processing with very low risk of contamination similar to isolators, or permit rare "open door interventions" provided appropriate measures are taken (11).

In representative installations recently constructed, there is a wide variety of equipment configurations referred to as RABSs. In construction detail, some approximate isolators in their level of segregation of the clean environment from the background environment, while other examples employ less rigorous segregation between the clean environment and the background room.

When barrier doors are required to be opened, the opening must be protected within an ISO 5 zone, to the extent necessary to ensure continuous ISO 5 coverage. The door must be permitted to be opened without leaving the ISO 5 zone.

Isolation Technology

As defined by ISPE: An isolator is a leak-tight enclosure designed to protect operators from hazardous/potent processes or protect processes from people or detrimental external environments or both. A basic enclosure consists of a shell, viewing window, glove/sleeve assemblies, supply and exhaust filters, light (s), gauge (s), input and output openings (equipment door airlocks, RTPs, etc.), and various other penetrations. There are two types of isolators:

Closed isolators. Isolators operated as closed systems do not exchange unfiltered air or contaminants with adjacent environments. Their ability to operate without personnel access to the critical zone makes isolators capable of levels of separation between the internal and external environment unattainable with other technologies. Because the effectiveness of this separation, closed isolators are ideally suited for application in the preparation of sterile and/or toxic material. Aseptic and containment isolators are two types of closed isolators.

Open isolators. Open isolators differ from closed isolators in that they are designed to allow for the continuous or semicontinuous egress of materials during operation while maintaining a level of protection over the internal environment. Open isolators are decontaminated while closed, and then opened during manufacturing. Open isolators typically are used for the aseptic filling of finished pharmaceuticals (11).

Closed Processing

Closed system sterile processing. A closed system is one that does not contain any open aseptic manipulations or interventions by design or operation and does not allow microbial ingress. Validated sterilization cycles must be provided. The product is separated from the surrounding room environment by a sterilizing grade vent filters. Leak testing must occur pre- and post use to demonstrate the system integrity.

EQUIPMENT AND PROCESS SYSTEM IMPACT ON FACILITY

It is important to start the facility design with an understanding of each process step involved with the manufacture of a sterile product. In this section, an overview is provided for some of the more common process steps, including a description of the major equipment, material flows, and facility impacts.

Nonactive Materials

Nonactive materials are transported from the warehouse to the weigh/dispense area, where they are dispensed into containers under a hood with high efficiency particulate air (HEPA) filtration. The hoods used in the dispensing operation shall be designed for protection of the product and may also need to protect the operator from exposure to potentially hazardous materials used in the formulation process.

The number of hoods, and type, will be determined by evaluating the number of weighing operations that must be performed, the size of the weighing operations, the compatibility of materials that must be weighed, and any special ergonomic or personnel safety concerns. Materials that are not compatible may need to be dispensed in separate hoods to prevent any cross-contamination concerns. Also, dispensing operations involving large bulk containers will require either a lift assist or pallet jack access to prevent operator injury, requiring the hood to be designed as a walk-in type.

Hoods being designed for product protection typically recirculate air back to the dispensing room to reduce the HVAC consumption for the area. However, when weighing hazardous materials, the air from the hood may need to be captured and exhausted to the roof through some type of environmental control device. Hoods of this type are typically designed to be negatively pressurized with respect to the dispensing room.

Containers may be either single-use or reusable. Single-use containers are disposable. Reusable containers are required to be tracked and controlled to prevent cross-contamination of clean and used containers. Prior to reuse, used containers should be brought to a parts preparation washroom for cleaning and then placed in controlled storage.

Nonactive materials are dispensed into bags or plastic bottles and can be placed into plastic bins as part of preassembled kits. Preweighed nonactive materials are stored as kits and staged until they are ready to be transferred to the formulation area. Identifying labels should be placed on the containers.

It is typical to provide a local WFI drop feeding a sink in the weigh/dispense area, with WFI temperature controlled by a local WFI drop cooler. The WFI drop is periodically flushed and sampled per SOP. WFI is used to prepare solutions of nonactive materials in bottles. This operation may be performed with equipment such as a laboratory agitator.

Bench scales are used for dispensing of smaller-scale materials. Floor scales are used for larger quantities. Balances and measuring equipment should be of an appropriate range and precision.

Active Materials

Receipt/Storage

Active materials, API or drug substance (DS), may be received in a wide variety of container types, in either frozen or liquid form, or as a solid. Careful consideration should be given to the form and container type, since this affects the storage, transport, preparation, and handling of API. The following list is provided to indicate the diversity of some common examples.

1. Frozen in cryovessels, ranging in size from 50 to 300 L
2. Frozen in small containers, ranging in size from 1 to 20 L
3. As liquid in small containers, ranging in size from <1 to 20 L
4. As powder in canisters, ranging in size from <1 to 50 kg

Weighing and dispensing can occur for either sterile or nonsterile material.

It is recommended that sterile material be dispensed in an isolator to prevent contamination. The isolator should be fitted with a rapid decon antechamber to facilitate the addition of product and containers to be dispensed.

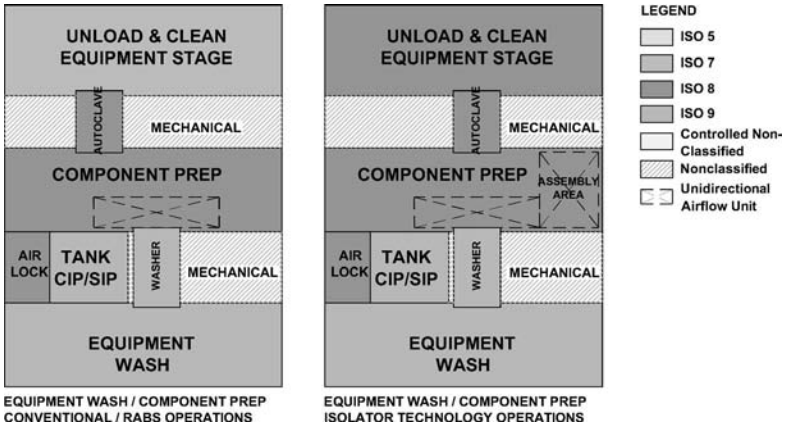

Figure 1 (*See color insert*) Weigh and dispense.

Nonsterile material should be protected by HEPA filtration during dispensing operations to prevent addition of particulate.

Containers of API should not be opened for sampling prior to use in the formulation area. Therefore, it is recommended that all lot of API containers be received at the warehouse facility with a tag-along container of sample volume, or with a material certificate of analysis from the supplier.

If there are any concerns regarding the cumulative time out of refrigeration for an API container, it should not be removed from cold storage in the warehouse area until it is required for use (Fig. 1).

Cryovessels

For transporting large volumes of bulk API, cryovessels are preferred over small containers. However, in addition to the added cost of the cryovessels, special consideration should be given to their storage and transport. Cryovessels can weigh approximately 1000 kg, before being filled with product. For this reason, it is recommended to avoid lifting them onto rack storage. This may impact the size of the storage space, depending on the quantity of material to be stored. This space can be either located within a walk-in freezer or in a special area equipped with a heat transfer system and ample docking stations for the required number of cryovessels.

It is recommended that cryovessels have wheels for portability and ease of movement, although moving them by hand would prove difficult. For long distances, such as transport from warehouse storage, fork trucks should be used. Once inside the formulation area, a tank manipulator can be used, which allows the cryovessel to be pulled or pushed into position.

Used cryovessels need to be cleaned prior to being returned to the supplier. This requires a designated area for the removal of the top head of the cryovessel for inspection and insertion of a CIP ring to properly clean the fin-shaped baffle inside the vessel.

Frozen or Refrigerated API Containers

While smaller containers of frozen or liquid API (up to 20 L) can be easier to transport and handle in the refrigerated warehouse, such containers may pose ergonomic concerns for the operator in the formulation area who must handle, manipulate, and lift them. A 20-L container weighs in excess of 40 pounds and may exceed the upper limit deemed acceptable for manual lifting. Therefore, a lift assist device may be required.

Small quantities of API may be received in either reusable or single use containers. While reusable containers must be cleaned and returned, single use containers pose a different set of concerns. Typically, disposable bags and bottles are not designed for either pumped transfer or manipulation by a lift assist. Therefore, disposable containers should be limited to a volume deemed manageable for handling, lifting, and pouring.

Thawing

If API is received in frozen form, an area adjacent to the formulation area should be designated as an API thaw room. Sufficient space shall be provided, depending on the API production requirements and duration of the thaw cycle.

If frozen in cryovessels, ample thaw/shaker stations shall be provided for the required number of cryovessels. At each station, a shaker mechanism is provided to gently mix the API during the thawing process. The thaw module will allow for precise control of the temperature profile during the process.

If frozen in small containers, consideration will be given to the selection of the thawing equipment. A shaker thaw bath will thaw several containers quickly, but needs a water supply. An environmental chamber will also thaw several bottle, but the operation will be performed at a much slower rate, due to the lower heat transfer rate of the air. The number of units will be determined by the quantity of bottles to be thawed for each batch, and the time allotted to perform the operation. If there are concerns regarding the accumulated time out of refrigeration, more units may be required.

Local freezers and refrigerators should be provided in the thaw area to provide staging space for material that is awaiting the thaw process as well as thawed material that is awaiting transport to the formulation room. The refrigerators and freezers should employ some system for segregation to maintain lot-to-lot integrity of the same material as well as containers of different products, avoiding any potential cross-contamination and product loss.

If live virus product is thawed, secondary containment is required during the thawing operation in the event of a container rupture. This can be accomplished by use of an overwrap or use of the thaw bath as secondary containment. Any use of water as a thawing medium for live virus should be routed to biowaste collection.

Formulation

The typical formulation module includes one formulation tank, positioned inside the formulation room, adjacent to a utility panel on the wall. Piping, electrical and instrumentation for the tank are connected to the utility panel. This tank has been cleaned via CIP and has been sanitized by clean steam. Following the sanitization operation, it has been maintained under positive pressurize with filtered process air.

Room height should account for elevation of equipment, including open charging operations, agitator clearance, and vent filters. Portable equipment must be able to fit under the door frame without disassembly. Room layout must account for any work platform required for equipment access.

WFI is supplied to this vessel through the utility panel from a local WFI drop, with WFI temperature controlled by a WFI distribution loop cooler. The addition of WFI can be made either manually or automatically. The WFI drop is periodically flushed and sampled per SOP. If the WFI is to be added after the API, it should be added through a dip tube to prevent the development of foam.

Large volume liquid drug substance in cryovessels or portable vessels is pressure transferred into the formulation vessel via a dip tube and can be considered a closed addition.

Solid additions are made through the open manhole of the formulation tank under the protection of a HEPA filtered air supply register. In cases where dust control is important, solid materials can be manually added through a tank nozzle using a lipseal for dust control to protect both the operator and surrounding environment. In cases where hazardous materials are being added to the formulation vessel, the use of an isolator or glove box may be considered.

Small quantity API and nonactive solutions are likewise manually poured into the vessel through a funnel that diverts toward the vessel wall. If necessary, a lift assist may be used due to the weight or awkwardness of the operation. As an alternate design, small quantities of liquid solution can be pumped or pressure transferred.

If quantitative transfers are required for all additions, each container may need to be rinsed with WFI following the transfer operation. This rinse bottle must be made up as part of the formulation recipe and included in the total mass balance.

The agitation in the formulation vessel must be sufficient to dissolve materials that could either sink to the bottom or float on the top of the vessel. For suspension products, vigorous

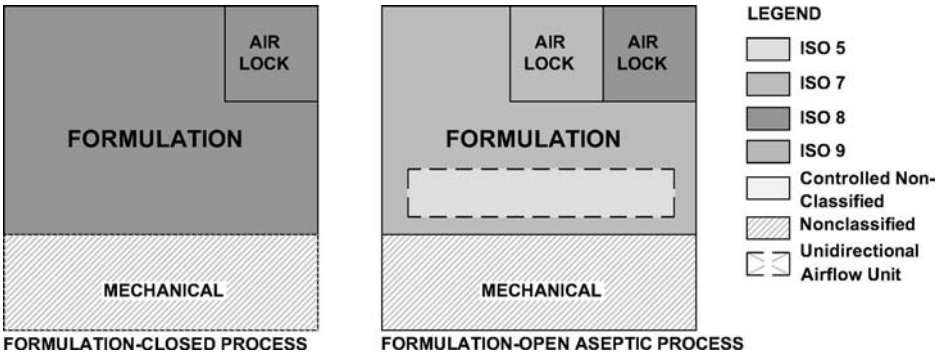

Figure 2 (*See color insert*) Formulation.

agitation may be essential to keep the disperse system homogenous. However, should crystals be present, the agitation must be gentle enough to avoid breakage.

In certain instances, if the product is oxygen sensitive, the contents of the tank may be sparged with nitrogen. It is also possible that only the gas in the headspace may need to be replaced. The requirement for this feature needs to be evaluated during design of the formulation tank. If nitrogen is used in the facility, it is important to provide oxygen monitoring to ensure that a safe working area exists for operators.

After the additions have been completed and the contents mixed, the pH is typically adjusted. Depending on the process requirements, this operation could be performed by adding predetermined quantities of acid or base to shift the pH. This can be accomplished easily as part of the formulation in a buffered system. In other formulation recipes, the pH may drift and require a titration process at each step in the process.

Once the material has been added, mixed, and at the proper pH, the batch is typically sampled for in-process testing. The in-process material is then pressure transferred through sterile filters to a holding tank or direct to the filling line (Fig. 2).

Sterile Filtration

As the in-process material is transferred from the formulation tank to the hold tank or the filling machine, it passes through a set of sterilizing grade filters which, when appropriately validated, will remove bioburden from the fluid stream, producing a sterile effluent. The sterile filtration must be redundant and must be located as close to the point of fill as possible.

Generally, sterile filtration occurs through two 0.2 μm filters configured in series. Both filters are identical in size, area, and porosity. Filter elements are used one time only.

Prior to use, the clean filter housings are fitted with new filter elements. The housings and elements are then steamed in place (SIP). After SIP, the new filter elements are integrity tested to verify the elements are intact prior to product filtration. Integrity testing requires wetting of the filter elements with either product or WFI. The system must be designed to test the second filter without compromising the sterility of the first filter. When WFI is used, if a filter requires replacement, only the SIP must be repeated. A commercially purchased filter integrity tester unit, supplied with process compressed air, shall be used to perform the bubble point test method. Post use, the sterile filters are once again integrity tested in place, or remotely at a bench, to verify the filter elements were intact during filtration.

Product Filling

Facilities and equipment for the manufacture of sterile products must be located, designed, constructed, qualified, operated, and maintained to suit the manufacturing process to

- minimize the risk of errors,
- avoid cross-contamination, and
- permit effective cleaning and sanitization.

Assuming the manufacturing process is similar for various products, a new aseptic filling facility should incorporate a degree of flexibility into the design to accommodate changing requirements for product mix and capacity.

Product filling can occur in facilities designed as conventional aseptic operations or in facilities designed to accommodate RABS or isolator technology. Figures 4 and 13 provide illustrations of the impact that technology selection makes on the facility design for vial and syringe filling suites, respectively.

It is recommended to transport presterilized wetted path components from the autoclave to the fill line, using bags for conventional lines, or rapid transfer port (RTP) containers for isolators. Proper space should be provided for the docking and lifting of these devices.

Proper space should also be provided for any lift assist required for operations such as docking of stopper or plunger bags to the RABS or isolator wall.

If portable vessels are used, the position of the vessel needs to be addressed. In a conventional or RABS filling line, the vessel should be maintained outside the ISO 7 fill room to avoid the need for sanitization of the vessel wheels and shell. In an isolator facility, the vessel would be moved adjacent to the filling machine and docked to a pedestal mounted utility panel.

Only one product should be manufactured on a filling line at one time.

Vial and Ampoule Filling

Sterile products including liquids, lyophilized liquids, and powder can be filled into vials or ampoules. It is recommended to load the glass onto the filling line in an ISO 9 space to avoid additional manipulation of pallets containing glass into ISO 8 areas (Fig. 3).

Figure 4 represents examples of vial filling operations utilizing conventional filling, RABS, and isolators:

Vial washing. Cardboard and other packing materials are to be removed prior to entry of vials/ampoules into the ISO 9 infeed area, which remain on pallets. Sufficient room is required to stage pallets of glass and provide space for maneuvering pallets to the filling line. Provisions need to be made for ergonomic assists during loading to prevent operator injury.

The vials are transported to the washer via elevating-type carts. The vials are received in trays on elevating carts or in a brick configuration wrapped where they are manually unwrapped and loaded onto the infeed tray-on station. It is recommended to evaluate component configuration and all load assist systems available for loading vials into the washer. After the vials are fed on the line they are conveyed into the washer for processing. Provisions for plastic waste removal need to be incorporated into the design of the syringe loading area.

Two common types of tray-on stations are linear (declining or fixed-elevation) or rotary, additionally the tray-on station can include a turntable to act as a buffer and/or accumulating device prior to the washing step.

The vials are singularized via a turntable with a star wheel, a turntable with nose guide, or a star wheel alone if the containers are not accumulated prior to rinsing. The vials are inverted at the first processing station before they are rinsed externally and internally.

The washer may incorporate as many as six (6) processing stations consisting of both WFI rinse and air blow nozzle manifolds of sanitary design and construction. The manifolds should be designed to allow WFI and clean dry air sampling. The design of the washer should permit flushing of the WFI use points of the washer.

First the vials are rinsed externally with recycled WFI collected from the final rinsing station. The vials can be cleaned with or without cleaning agents. The surfaces of the vials can be rinsed one to two times. After external rinse and before internal rinse(s) vial externals receive an air blow using sterile filtered dry (process-grade) air.

Following the external rinses, the interior surfaces of the vials are rinsed using hot virgin WFI, feed from the header directly; not heated recycled WFI. After internally rinsing the vials, the final station performs an internal air blow then the vials are inverted and finally transported to the tunnel infeed interface in single file.

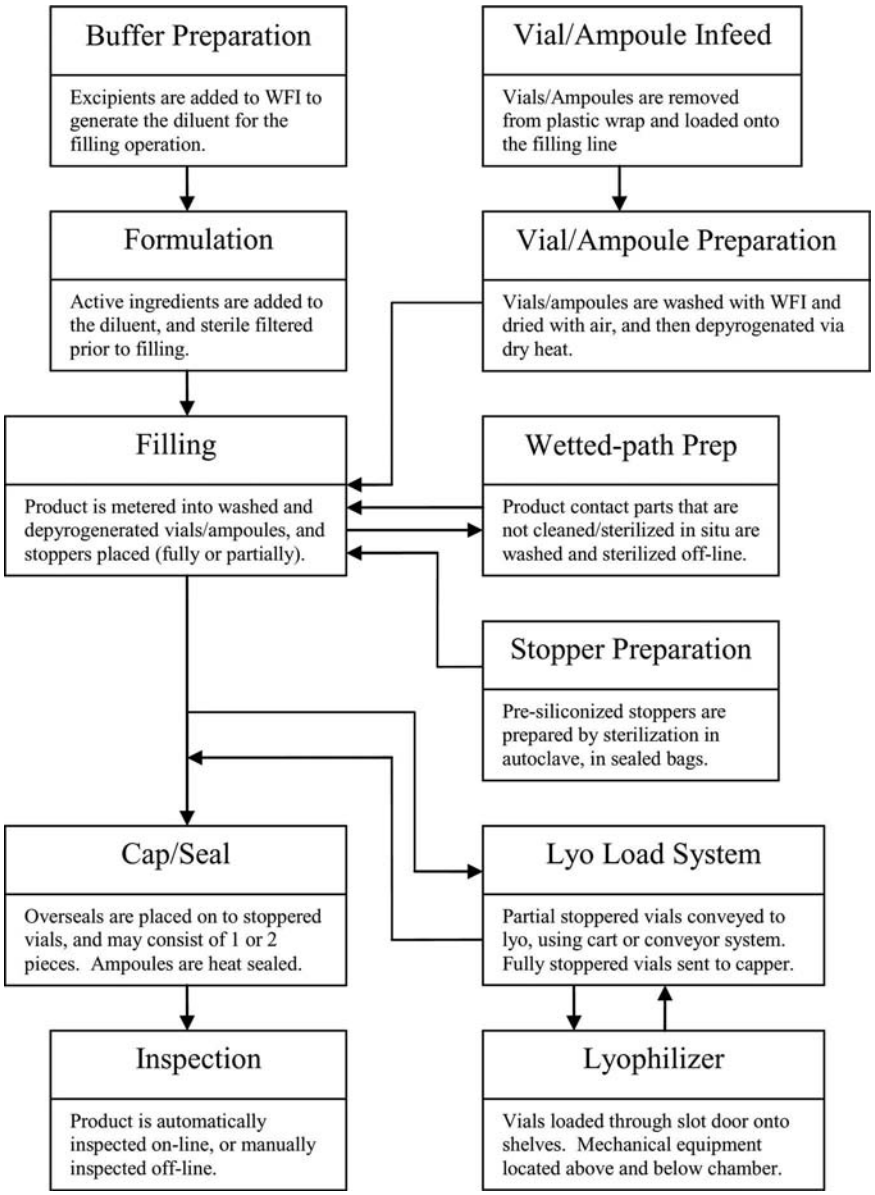

Figure 3 Sample process flow for manufacture of drug products into vials or ampoules.

It is recommended that an exhaust hose be attached to the cover to draw moisture and air away from the processing space to an exhaust fan located above the washroom.

Figure 5 shows a picture of a typical vial washer:

Depyrogenation. The vials are conveyed from the washer outfeed under ISO 5 unidirectional airflow to the tunnel infeed zone. The tunnel (Fig. 6) shall be provided with HEPA-filtered air, whether coming directly from the air supply duct or recirculated. The filter over the infeed zone of the tunnel should be sterilizing-grade but as an option can be specified as HEPA-grade.

The vials are accumulated and are desingularized (massed) at the infeed of the tunnel and enter the tunnel in a "bulk" configuration. It is recommended that the control of the bulking process is controlled by the tunnel as this process is critical to control vial back

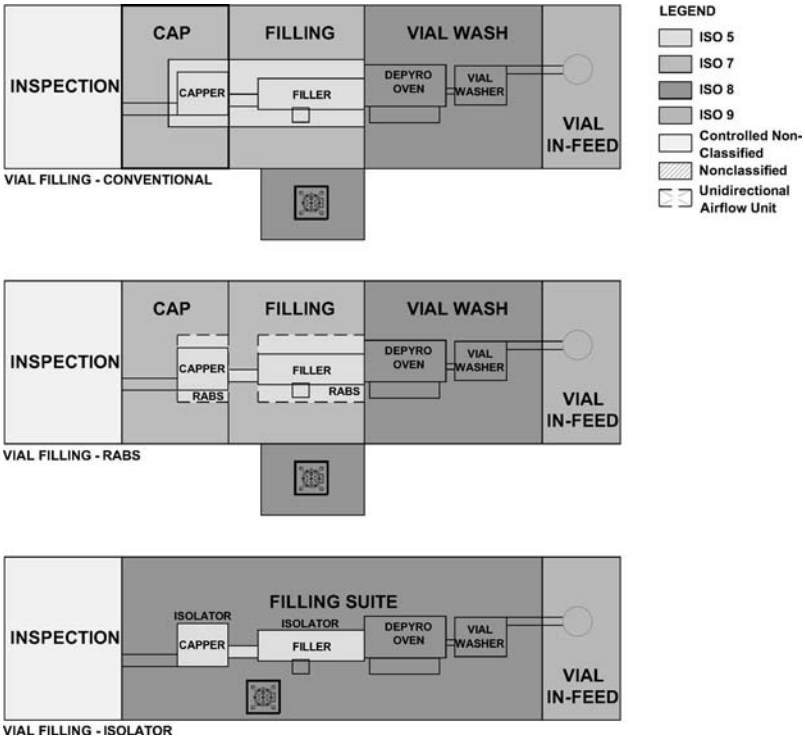

Figure 4 (*See color insert*) Vial filling.

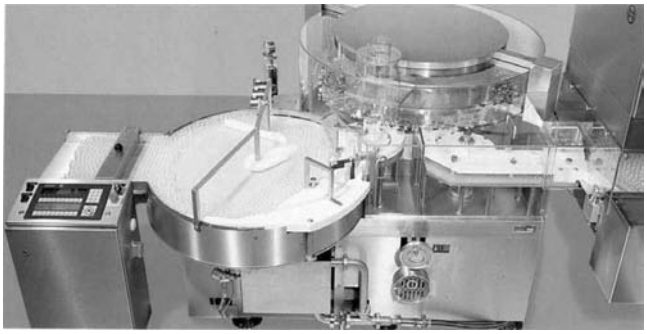

Figure 5 Vial washer (12).

pressure and load configuration to minimize vial damage (scratching), breakage, and falling. As the vials enter the infeed zone, they are gradually heated up before entering the sterilizing zone.

The sterilizing zone shall be designed to provide uniform load temperature distribution within $\pm 1.8^{\circ}F$ ($1^{\circ}C$) across the tunnel belt. The filters in the sterilizing zone shall be HEPA-grade filters and nonparticle generating to maintain air quality to ISO 5 requirements during all operating modes.

The vials are cooled to ambient prior to exiting the tunnel. The cooling zone should be sterilizable and will incorporate HEPA filtration to maintain ISO 5 air quality in all operating modes.

The sterilizable cooling zone is cooled via cooling coils located under the tunnel belt. At the end of the tunnel, a door should be incorporated to seal the tunnel at the cooling zone from

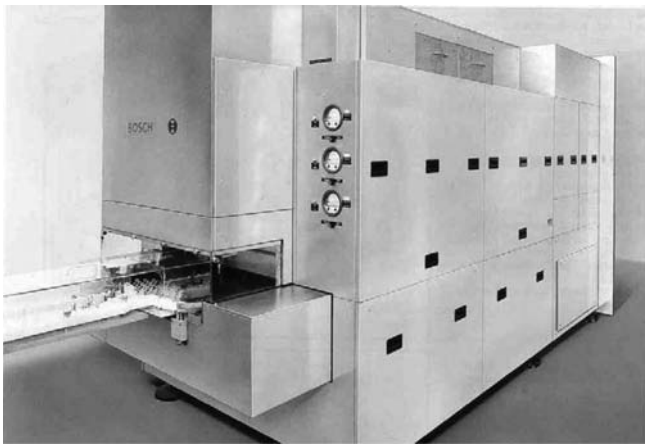

Figure 6 Depyrogenation tunnel (12).

the filler to allow the tunnel to be cooled or taken out of service while maintaining the controlled environmental conditions of the filling room.

The tunnel should incorporate filter integrity test ports for HEPA filter testing with POA (polyalphaolefin) or other equivalent challenge testing material in place of DOP (dioctyl phthalate aerosols). Isokinetic sample ports (per ISO 14644-3.) should also be located such that connection of probes allow for simple setup for particulate monitoring during tunnel operation.

Adjustable height gates to allow control of differential pressures between zones and the wash and fill rooms should separate each zone. The gates are adjusted statically, during the tunnel qualification process to the appropriate heights for achieving the proper pressure and airflow velocity balance for each vial profile. The gates between the sterilizing zone and the cooling zone and the cooling zone and the filler serve to isolate the cooling zone from these other areas during the cooling zone sterilization cycle.

The frame of the tunnel must allow expansion during the heating in the direction of the washer. It is recommended that the tunnel feature a presterilization mode to permit the heating up of the tunnel prior to production.

It is important to consider the installation details for the tunnel, including any need for shrouds to cover openings from the equipment to the floor or ceiling of the clean room.

An alternate to a depyrogenation tunnel is a batch oven. Batch ovens should be considered if the batch is small (max approximately 20,000 vials for the 2 mL) and infrequent. If a batch oven is considered evaluation of vial washing and handling, tray handling, staging, carts, and pass through oven configuration is required.

Sufficient space is required around the unit for maintenance of the depyrogenation oven such as replacement of terminal filters. In addition, sufficient exhaust is required to remove humidity and heat that is generated in the room while maintaining the pressurization profile of the filling line.

Vial filling, stopper placement and capping. The vial filler should be designed as a monoblock filler (Fig. 7) and specified to aseptically fill, stopper, and cap vials with product at the predetermined fill line speed, fill accuracy, and allowable fill velocity limits. The filler should be capable of operating in both a run mode and a maintenance mode.

Particular attention should be paid to the design of the filler and its stations for unidirectional airflow (UAF) via HEPA-filtration provided by the room HVAC system or a dedicated HVAC system.

Filler infeed As the vials exit the tunnel, they are singularized and transitioned into the filler by transport conveyor. The vials are positively conveyed and held by a belt designed to transport vials of the predetermined dimensions and mass of the specific glass vial.

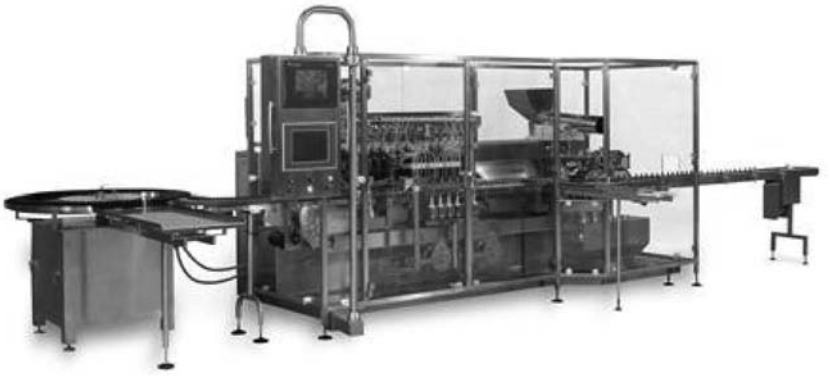

Figure 7 Monoblock vial filler (12).

The number of filling pumps and dispensing nozzles will be determined by the required line speed and the diameter (bore) of the filling nozzles as the diameter relates to the fluid properties of the particular product being filled. The characteristics of the product will affect the maximum fluid velocity allowable, thus determining the required fill nozzle bore and the number of pumps and nozzles to achieve the line speed.

Stopper placement After the vials are filled, the transport system conveys them to the stopper placement station. The preprocessed (cleaned) and sterilized stoppers are supplied to the filling line in disposable bags that docked at the plunger placement station RTP and are feed into a supply hopper through that RTP and are feed into a supply hopper sized according to the filling throughput. The size of the hopper should be dictated by

- the maximum dimensions permissible for sterilization of the hopper in an autoclave
- required unattended run time
- line speed
- maximum allowable load height the stoppers can withstand without being damaged and/or generate particulate.

The hopper will feed stoppers to the sorting bowl where the stoppers are orientated for accurate repeatable placement into the vials. The sorting bowl should be sized to supply stoppers to support the filling line speed and be limited such to permit sterilization in an autoclave.

Capping After the vials have a stopper placed the filled and stoppered vials are fitted with clean aluminum caps and then crimped such that the vials are designated as "closed containers." Care and consideration should be given to the design and operation of the capper to ensure that stoppers do not rise during transport, caps are not damaged during crimping, and particulate generation is minimized. The predetermined stopper placement criteria and capping requirements will be verified by the inspection system located in the packaging area.

Capping operations are to occur under ISO 5 protection. As the operation generates aluminum particulate, continuous monitoring at the capper head is not recommended, although viable monitoring can occur.

It is recommended to provide a transport path for presterilized cap-contact components from the autoclave to the capper. In a conventional or RABS filling line, this path should remain in ISO 7 conditions or provide additional wrapping. In an isolator facility, the components can be conveyed through ISO 8 and resterilized by vapor phase hydrogen peroxide after installation.

In a conventional or RABS facility, it is required to provide a method for feeding presterilized caps, in bags, into the ISO 7 room. It is important to note that not all aseptic operations require the use of sterile caps.

Sufficient space needs to be provided to stage caps. Proper space should also be provided for any lift assist required for operations such as docking of cap bags to the RABS or isolator wall.

In the ampoule sealing operation, it is required to supply natural gas to the sealing mechanism. It is recommended that a gas bottle manifold be provided outside classified space. In addition, gas detectors, tied to an alarm system, are required at the sealing area for safety.

Vial check weighing To minimize product loss (low fills rejected and required % over fill), it is recommended that the filler incorporates a check weighing system to verify and fine tune the fill volume during setup and as verification during the course of a run. A reject bin should be located for fallen or misaligned vials prior to tare weighing. At low speeds, 100% check weighed in line is possible; however, at higher line speeds only 4% of the vials can be check weighed due to the mechanical limitations of removing vials from the line to be check weighed out of place and then replacing them in the line. Typically, for most vendors, the line speed cut off for 100% check weighing is 200 VPM (vials per minute).

On the basis of net weight, the control system will correct the volume of product that is dispensed by the nozzles. The tare and gross weigh verification system should be calibrated prior to each fill operation.

The filler check weigh control system should be programmable to reject vials whose gross product volume does not meet the acceptable minimum and maximum volumes and to alarm after repeated low volume parameters programmed in the controller are exceeded.

Inspection Inspection operations can be manual, semiautomatic, or automatic. Tables utilized for manual inspection and sorting of rejects should be designed to permit segregation of pass and nonpass product. Lighting must be adjustable to permit optimal conditions for manual inspection.

Adequate space is to be provided for product to be accumulated onto trays/tubs/containers, covered, labeled, and manually palletized. A staging area for empty pallets needs to be included in the room design.

Trayloader. After the vials are capped/closed they are conveyed to the tray-off station where the vials are automatically trayed off and then the full trays are manually stacked on carts or pallets for transport to the packaging area or cold storage. The number of tray stations is determined based on line speed and size of tray.

Figure 8 is a picture of a commonly used tray loader:

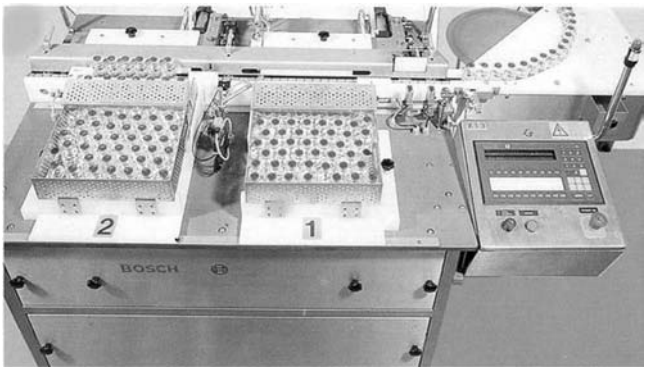

Figure 8 Tray loader (12).

Lyophilization
A lyophilization system generally consists of the following components:

- Drying chamber
- Movable shelves, inside the chamber
- Stoppering mechanism
- Vapor condenser
- Refrigeration system(s)
- Vacuum pumping system(s)

Depending on schedule and batch size requirements, there may also be equipment for automatic loading and unloading of the vials into and out of the chamber.

Typically, the lyophilization process consists of the following events:

- Transporting of liquid product filled, partially stoppered glass vials from the filling room to the lyophilization area.
- Loading of the vials onto the lyophilizer chamber shelves, under aseptic conditions. This may be done automatically, via a conveyor or a loading cart, or manually with the use of HEPA-filtered carts with trays.
- Freezing of the product in the vials by cooling the lyophilizer shelves
- Sublimation of the water by pulling vacuum in the lyophilizer chamber, and then heating the shelves. This vapor leaves the chamber, travels through a duct, and enters into a condenser, where it collects onto cooling coils in the form of ice.
- Once the product has completely dried, full stoppering of the vials by stoppering mechanisms inside the lyophilizer. This is typically performed after a partial release of vacuum.
- Relieving the vacuum in the lyophilizer chamber.
- Unloading of the vials from the chamber.
- Defrosting of the ice accumulated on the condenser coils.
- CIP of the chamber, shelves, and condenser.
- Leak testing of the chamber. This test can be performed either before or after the SIP operation.
- SIP of the chamber.

Lyophilization equipment should not be product specific, but production (i.e., throughput) specific. An overall production schedule must be created, illustrating the maximum production level possible for the facility. This schedule should include all the aspects of production, including preparation of buffers, product formulation, filling and freeze-drying times, equipment cleaning, and sterilization, etc.

Typically, the overall production schedule will be dictated by the most time consuming steps, such as filling and freeze-drying. It is desirable to maximize use of this equipment over the hours of operation. The overall schedule then shall evolve from a balance of targeted batch sizes and formulation activities required to support maximum use of the filling line and the lyophilizers.

When developing a production schedule that includes lyophilization, the following key elements shall be considered, in addition to the other factors related to the formulation and filling operations:

- Number of lyophilizers
- Size of the lyophilizer chamber
- Duration of the lyophilization cycles
- Equipment cleaning and sterilization
- Speed of the lyophilizer loading/unloading system

Consideration should be given for the length of the freeze-drying cycle. As the cycle time is reduced, a larger percentage of time is required for loading, unloading, and equipment turnaround between batches.

Sufficient space is required in the lyophilizer mechanical chase to provide maintenance access to the chamber, condenser, stoppering ram, and refrigeration systems. Platforms and ladders must be provided for safe access. Safety rails need to be provided at the edge of the chamber and condenser to prevent falls. Ladders should be provided with safety cages, if required.

The rear door of the chamber must be opened for inspection and must have a controlled environment.

It is recommended to locate the condenser below the chamber to limit the size of the lyophilizer aisle while maintaining access to the rear door.

Chamber. The drying chamber contains the shelves where the product vials are placed for the freeze-drying cycle.

The vials are only partially stoppered when they are introduced in the chamber. To reduce potential of product contamination during loading, there shall be a laminar airflow (LAF) curtain in front of the chamber door.

The chamber will have pressure proof door(s), fabricated of the same material as the chamber. A minimum of one door is required for vial loading, unloading, and maintenance. Typically, the chamber will also have a slot-type door if the vials will be loaded automatically (Fig. 9). The automated loading system will be designed to lock onto to this door prior to the vial transfer. The number of doors will also depend on the facility layout selected for the manufacturing processes: the chamber then can be designed as single sided, with vial loading and unloading from the same side, the clean area, or pass through, with vial loading from one room, and unloading from another room. Mechanical configuration options will be discussed further ahead, in section "Gowning Philosophy."

Shelves. Shelves are located inside the lyophilizer chamber and stacked in a vertical arrangement. They are hollow, containing channels through which diathermic heat transfer fluid (HTF) circulates to either cool or heat the shelves. They shall be constructed of 316L stainless steel.

Vial loading can be automatic, with the use of loading equipment, which is discussed in more detail in section "Product Filling." Loading can also be manual, using bottomless type

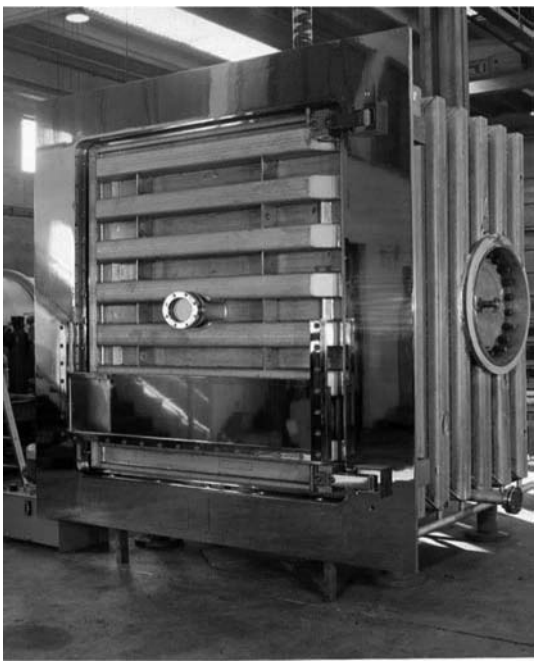

Figure 9 Lyophilizer chamber (13).

trays. With these, the vial pack is placed on the tray and tightly banded together with a ring. The operator places the tray onto the shelf, and then slides the tray off, leaving the vials on the shelf by holding onto the ring. This method places the vials directly onto the shelf surface, which is the preferred setup. The direct contact between shelf and vial promotes optimum heat transfer.

Alternatively, the tray could be left in with the vials for the entire length of the lyophilization cycle. The added material between the shelf and the vial will cause a decrease of heat transfer rate. Although it is possible that this could be a requirement for some products, typically, it is not recommended. With time, the tray could become deformed due to the constant temperature changes it is exposed to. A slight deformation will cause the tray not to be in complete contact with the shelf, compounding the heat transfer issue.

Prior to vial loading, it shall be possible to lower the shelf temperature prior to placement of the vials on the shelves. This is typically done for products that require constant refrigeration. It is recommended that this temperature be above the product freezing point. When this process is employed, it is recommended to provide a low humidity zone at the door to the lyophilization to minimize condensation on the shelves.

The shelf stack is sandwiched between two upper and bottom pressure plates. The plates are connected to a mechanism that moves the shelves up and down. It is this mechanism that allows the complete stoppering of the vials mid-cycle, after the product has dried. As the shelves move closer together, the stoppers are fully inserted in the vials, pushed by the underside of the shelf above.

Shelves must be fully loaded with vials during stoppering. If a shelf remains partially empty after loading all the product vials, empty vials will be loaded to evenly fill the shelf.

The moving mechanism can also be used to facilitate the loading and unloading of vials. For example, initially, the shelves can be collapsed at the bottom of the chamber on top of each other. At the beginning of loading, the top shelf aligns with the door and is loaded first. When it is full, the shelf moves upward one space, allowing the second shelf to be loaded. The process repeats until all shelves are full.

Condenser. The liquid removed from the vials during the drying process will accumulate and form ice onto coils containing HTF fluid. In smaller units, these coils can be internal to the chamber. However, more commonly, they are housed in a separate condenser vessel. Both the condenser and the coils shall be made of 316L stainless steel.

The condenser can either be located adjacent to the chamber or at a different elevation (different floors). Selection of the configuration is dictated by the facility layout. It is recommended that the condenser be installed below the chamber to facilitate cleaning and to reduce the facility footprint.

Refrigeration system. The refrigeration system(s) cool the HTF that is circulated through the shelves and through the condenser coils. A lyophilizer can have separate, dedicated refrigeration systems, servicing the shelves and condenser circuits independently from each other. Alternatively, there can be one all-encompassing refrigeration system, servicing both circuits. Typically, all condensers would be used for shelf cooling during freezing, and then some of them would be switched to the condenser coils circuit during product drying. This last option of dual purpose compressors is generally more space efficient.

It may be desired to have redundant backup compressors to ensure cycle continuation during the critical drying sequence, even in the event of principal compressor failure. The backup compressor would be sized for cycle continuation only, and not for the larger initial freezing load. Another feature that would support cycle continuation would be automatically switching the compressor cooling media from chilled water to city water in the event of power failure. This would be done because, typically, a plant's chilled water system is not on emergency power. Product value and schedule are principal drivers of these options.

Liquid nitrogen is used for applications requiring a condenser temperature lower than typical compressors can produce. It can also be used as a backup in the event of power loss.

Vacuum system. The chamber can be evacuated at different points of the operation (product drying cycle, chamber SIP) to a determined vacuum set point with the use of vacuum pumps.

To achieve a quality high vacuum, it is common practice to use more than one type of pump. An example would be a combination of a rotary vacuum pump plus a mechanical booster pump, in series. As with the condenser, depending on product value and schedule, requirements for equipment redundancy and emergency power must be evaluated.

Additionally, a liquid ring pump may often be used in the chamber and condenser drain lines to promote effective draining of cleaning solutions or steam condensate following CIP and SIP.

Shelf movement and stoppering mechanism. The lyophilizer is equipped with a mechanism enabling the movement of the shelves.

As previously mentioned, the vials are stoppered at the end of the drying cycle. As the shelves are moved together, the rubber stoppers are fully inserted into the vials, pushed by the underside of the shelf above.

In hydraulic stoppering, a hydraulic cylinder is mounted on top of the chamber. A hydraulic piston is introduced into the chamber through a seal and is attached to the pressure plate that is positioned above the shelves. The top shelf is connected to the pressure plate, and the shelves are connected to one another. Movement of the plate, thus, will move the shelves.

In hydraulic stoppering, the ram that moves the pressure plate positioned above the shelves can be introduced in the chamber as the shelves move together. To avoid contamination from this ram, which normally resides in the mechanical area of the lyophilizer, it is customary to fit it with protective bellows constructed from stainless steel or other suitable material. The bellows shall be designed to cover the full extension of the ram. The surface exposed to the chamber shall also be able fully cleanable and sterilizable. It is advisable to perform bellows integrity tests prior to SIP to ensure that contamination shall not leak into the chamber.

Gas system. After pulling and holding vacuum, the chamber is restored from vacuum back to atmospheric pressure by introducing air or other inert gases, such as nitrogen. The process is also referred to as backfilling.

The gases should pass through sterilizing filters prior to entering the chamber.

It is possible to vary the pressure set point up to which the chamber is backfilled to. For instance, some products require that stoppering occur at a pressure below atmospheric. Upon completion of the vial stoppering step, the chamber is then completely backfilled to atmospheric pressure.

Requirement to backfill the chamber with inert gases is largely dependent on the product being processed. The inert gas would be introduced during the stoppering operation to fill the vial head space. The inert gas is then purged from the chamber, and process air is introduced for complete backfilling prior to vial unloading. It is important to provide oxygen level sensors in inhabited areas adjacent to the lyophilizer both on the clean room side and on the technical chase to notify personnel in the event of a gas leak.

Load System Design

After the vials have been filled at the filling line, the product needs to be transferred into lyophilizers; the options range from completely manual to completely automatic. This can be done manually with carts or with a fully automated loading system, either cart based or conveyor based. In the cart-based approach, safety concerns with the use of robotic transfer can be accommodated by properly designing the lyophilizers aisle. In the conveyor-based design, personnel access to both sides of the filling line needs to be taken into consideration (Fig. 10).

In cart-based facility designs, it is required to provide barriers in place to prevent operators from entering the travel path of the robotic cart. These barriers can consist of safety rails or walls around the lyophilizer aisle area. Any doors that permit entry must be interlocked to prevent movement of the cart. In addition, operators must be protected from

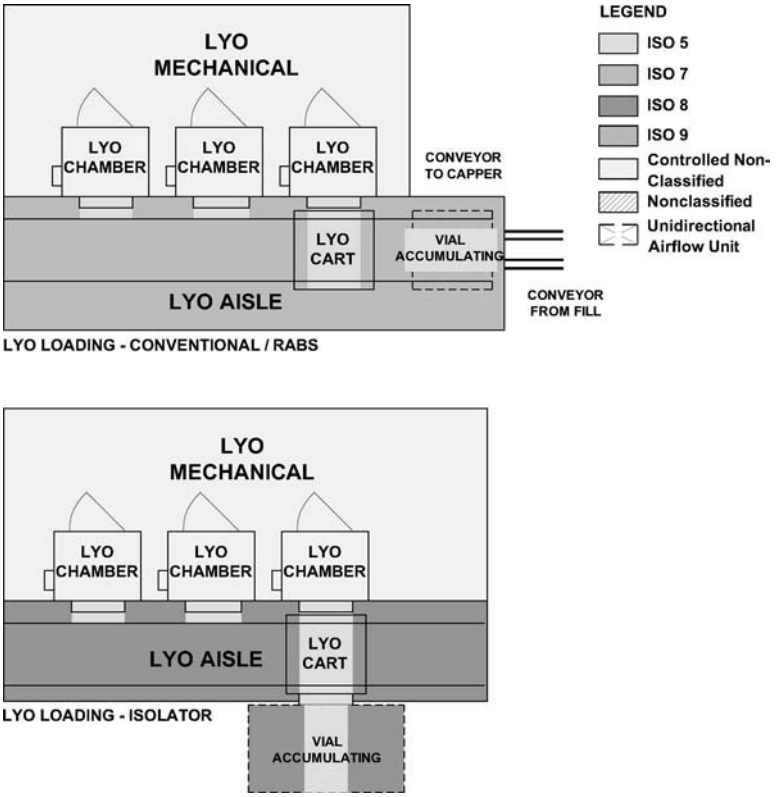

Figure 10 (*See color insert*) LYO load/unload.

contacting the powered rail during cleaning of the area. Alternatively, if the power rail is not sufficiently protected, power should be disabled during cleaning of the rail.

In conventional or RABS fill lines, sufficient ISO 5 coverage is to be supplied to permit operator access to the cart for sampling or cleaning, with barrier doors opened. In addition, a row of HEPA filters is required above the slot door to cover any gap during docking of the cart to the lyophilizer. The air is required to be dehumidified to prevent condensation on shelves, if cold loading is required.

Ceiling heights for isolator facilities should account for locations of terminal HEPA filters. Adequate space should be provided to permit routine testing and replacement.

In isolator facilities, space for a docking station is required to permit automated cleaning and sterilization of the cart.

Facility design for cart-based systems need to provide an area for maintenance, permitting full access around the cart.

The loading system must be capable of providing the ability to control vial movement from the filler to the lyophilizer, and from the lyophilizer to downstream operations of capping and inspection. Positive control must be maintained over the vials to prevent tipping. Depending on the layout and production demands, the design may need to provide flexibility to convey different vial sizes for products with different schedules. The loading system must provide ISO 5 laminar flow air above the open vials at all times. It also must be capable of being cleaned and sanitized. The following is a selection of load system options.

Manual loading with HEPA carts. This is the most basic, flexible, and cheapest option to load the lyophilizers. It would require the use of manual HEPA filtered transfer carts. Ten to twenty trays are manually loaded into the cart and transferred manually into each lyophilizers through the slot door. This operation requires an ISO 7 lyo aisle with localized ISO 5 at the

lyophilizer doors. Carts are charged at 120 V power outlets and can hold their charge for duration of batch. It can process both vials and syringes. Trays must be used to perform this transfer. The trays must be cleaned and sterilized separately and stored in an ISO 7 environment.

Manual loading with pusher mechanism. This option consists of a pushcart type device that rides on rails down the lyo aisle. Vials are manually loaded on to the cart then wheeled down to the lyophilizer. Vials are pushed with a mechanical assist on the cart into the lyophilizer through the slot door. There is much better vial handling with this option, but it may require a slower line speed than targeted. It will require ISO 5 space in the lyo aisle. Although this approach has been successfully implemented, it has not been done recently. This option cannot process syringes. No containment can be offered with this option.

Conveyor. This option consists of a conveyor that runs down the lyo aisle from the filler to an auto loader stationed in from of each lyophilizer. A pusher mechanism pushes vials onto each shelf, row by row. After the lyophilization process is completed, the vials are removed by using a pusher in the back of the chamber or a sweep arm to pull vials out through the slot door. Vials are then descrambled and sent by conveyor to the capper. This process eliminates the manual handling of vials. It will require ISO 5 space above the entire length of the conveyor. The use of a buffer table permits continuous operation of the filler during the actuation of the pusher mechanism.

Transfer cart. This option consists of an auto loader cart, which shuttles between an accumulating table and each of the lyophilizers (Fig. 11). The system is designed to accumulate enough vials to fill either a full lyo shelf or half of a shelf only. It would not be able to handle syringes. This option would require ISO 7 space in the lyo aisle with at least a localized ISO 5 hood above the cart. If an isolator is used, the lyo aisle environmental grade can be lowered to ISO 8.

Integration of loading system into the facility. In a cart-based design, the isolated cart will be required to dock with a cleaning and sterilization station. The station is capable of performing CIP and sterilization by VHP. For a RABS design, the cart would be parked in a maintenance area, where a manual cleaning and sanitization would occur.

Figure 11 Lyophilizer transfer cart with loader and unloader (13).

Depending on the facility design and the production requirements, the lyophilizer load system may interface with different unit operations, including but not limited to the following:

- Filler outlet conveyor
- Capper inlet conveyor
- Lyophilizer chamber slot door
- Buffer accumulator, if cart, to facilitate the unload of the lyo chamber
- Cleaning/sterilization docking area, if cart and designed as an isolator
- Maintenance area, if cart

Aseptic Syringe Filling Line Process Overview

There are various options and configurations available for a syringe filling line, including use of presterilized syringes, material of construction, number of chambers, and filling technology. The configuration choice should be based on requirements for the following:

- Product: A specific requirement of the product for one filling technology, due to material incompatibility, product stability, or marketing requirement.
- Component selection: Selection of filling technology must be based on product needs.
- Level of sterility assurance
- Delivery technology: Delivery method affect the selection of the filling technology
- Number of units: Selection of the filling technology must be based on the annual production requirement and batch sizes
- Unit cost: The cost to produce each syringe
- Capital cost: The actual cost to purchase equipment, not including engineering, construction, validation, and commissioning.
- Operational cost: The cost to operated equipment and supporting facility.
- Preference: Is there a clear preference for a specific filling technology due to corporate experience, level of comfort with a given technology, or market demands.

The following figure (Fig. 12) maps the decision process for the appropriate line configuration.

Although alternate technology selections will be mentioned, for this chapter, focus will be on filling of single-chamber, presterilized glass syringes in a nested format.

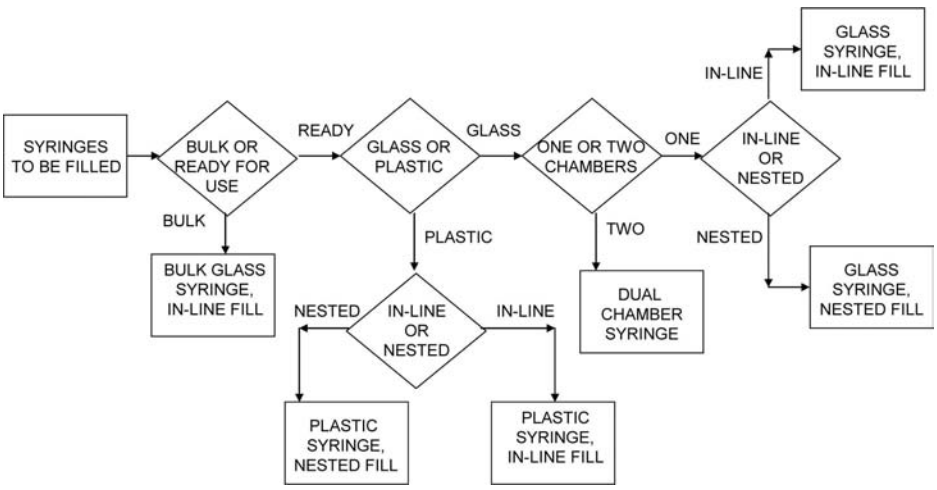

Figure 12 Syringe filling options.

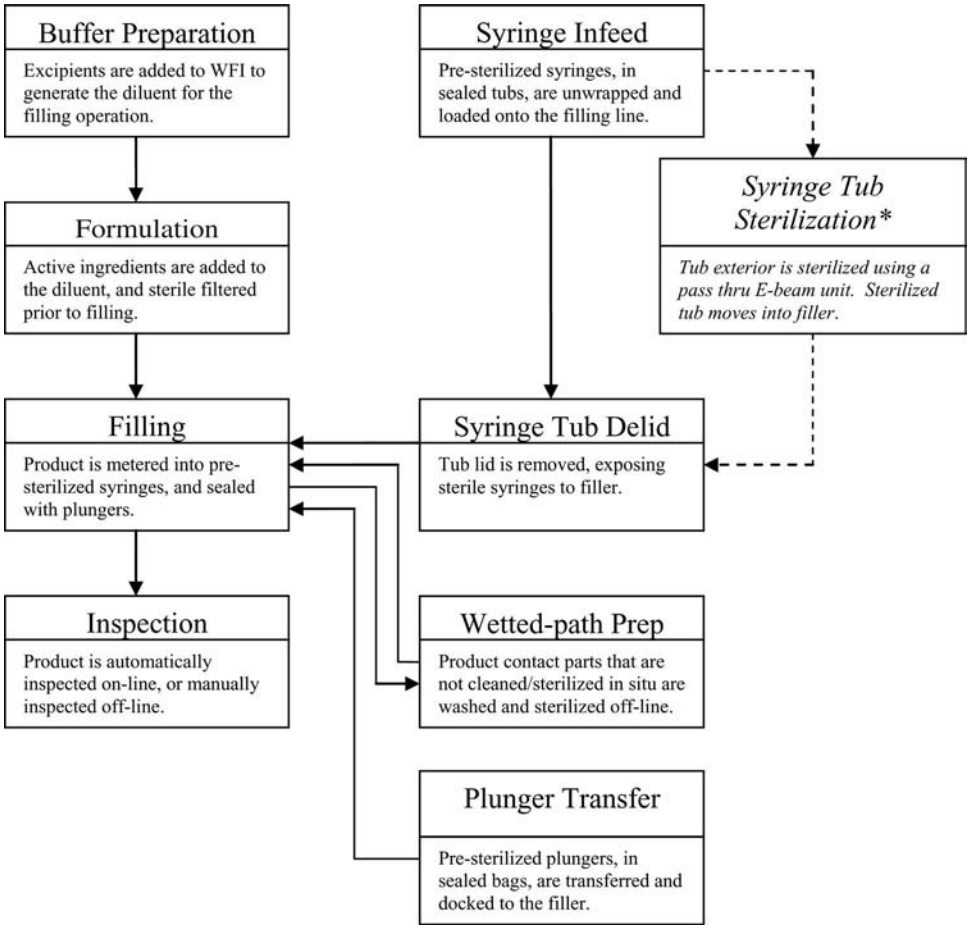

Figure 13 Process flow, nested syringe.

Figure 14 Wrapped tub (14).

Nested syringe filling. Typically, the nested syringe filling line process consists of the following events (Fig. 13):

- Transporting empty glass syringes (precleaned and presterilized) in bagged tubs to the nested syringe filler (Fig. 14)
- Sanitizing the bag under unidirectional HEPA-filtered airflow.
- Unwrapping tubs of syringes from their protective bag(s) under unidirectional HEPA-filtered airflow
- Removing the lids of the tubs (delidding) under unidirectional HEPA-filtered airflow
- Automatically conveying the syringes into the syringe filler infeed
- Removing nests from tubs

- Aseptically filling the syringes with sterile filtered product feed from the product hold tank(s)
- Inserting the plunger to the product-filled syringes
- Replacing nests in tubs and conveying to inspection
- Removing syringes from tubs and nests
- Inspecting syringes. Addition of ID code
- Placing syringes into tubs or Rondo trays
- Transferring the closed syringes for downstream processing to the packaging area or cold storage

Figure 15 represents examples of syringe-filling operations utilizing conventional filling, RABS, and isolators.

Tub sterilization station. Cardboard and other packing materials are to be removed prior to entry of syringe tubs into the ISO 8 infeed area. In the material airlock, tubs should be transferred onto captured pallets or carts to limit particulate or bioburden. The nested syringe tubs are transported to the filling line and placed onto the infeed at the tub wipe down station, which is under unidirectional HEPA-filtered airflow. The outer bag of the double-bagged tubs is sprayed and wiped down with disinfectant and then removed and disposed. The inner sterilized bag is then removed and the tub is feed forward to the lid removal station.

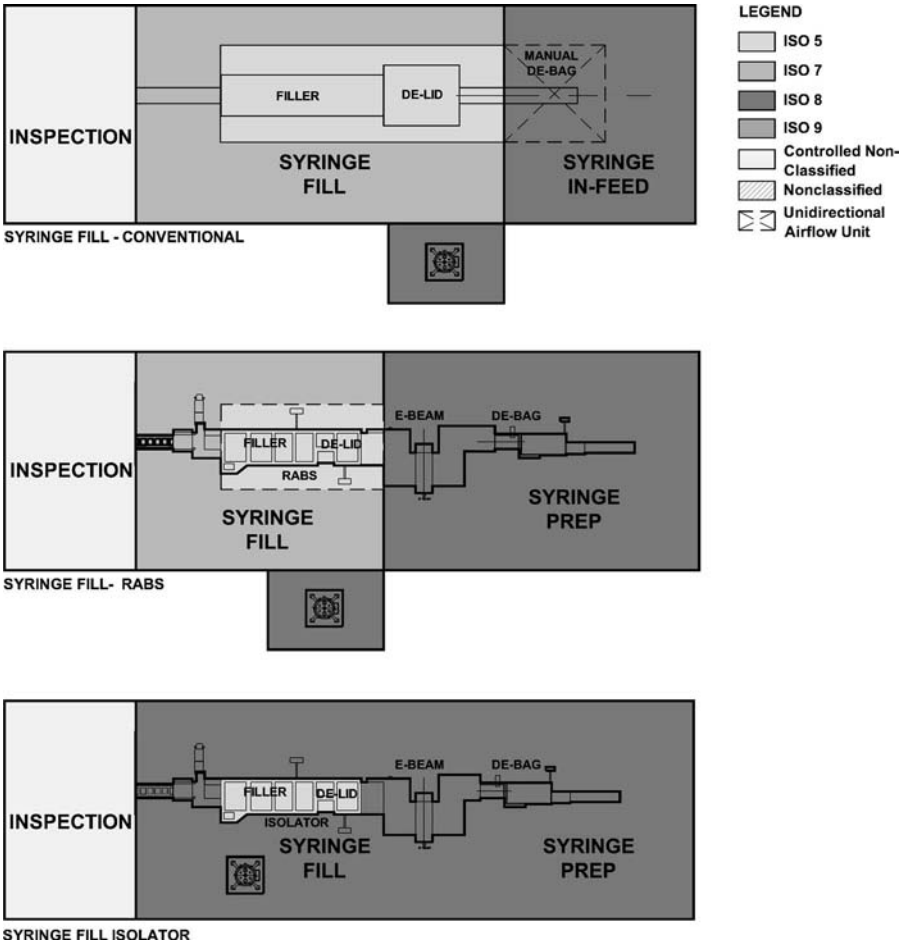

Figure 15 (*See color insert*) Syringe fill.

Provisions for plastic waste removal need to be incorporated into the design of the syringe loading area.

As there are large quantities of presterilized tubs that must be loaded onto the filling line, the aseptic handling and manipulation of tubs is critical to the process, in a conventional clean room facility. In a conventional facility, plastic overwrap is removed under an HEPA-filtered hood to protect the tubs during the operation. Plastic is peeled back using aseptic technique. Unwrapped tubs are passed through from the loading area into the ISO 7 filling room, utilizing a gravity conveyor. In a RABS or an isolator facility, it is recommended to remove the plastic overwrap and sterilize the exterior surface of the tub prior to entering the fill zone, using technology such as e-beam. The plastic bag can be removed either manually or automatically. Alternatively, for small-scale facilities, vapor-phase hydrogen peroxide (VPHP) chambers can be used to sterilize the bag, with tub inside, prior to entering the ISO 5 fill area.

An "e-beam" can be implemented in lieu of manual disinfection of tubs prior to lid removal station. An e-beam sterilization unit provides repeatable, monitored, validatable sterilization of the tubs and minimizes manual manipulations of the bags and tubs and operator contact prior to entering the ISO 5 filling space. The addition of e-beam sterilization technology, however, adds significant equipment cost and additional facility space requirements to accommodate the footprint required for this type of system.

Sufficient space is required around the unit for maintenance operations such as removal of lead shields, using ergonomic assist. In addition, a maximum distance is allowable from the e-beam generator units to the sterilizer, requiring location of generators in close proximity above room.

When an e-beam sterilizer is used, monitors for ozone and X-ray levels must be provided to ensure a safe working area for operators.

Lid removal station. At the lid removal station, the lid, Tyvek cover, and liners are removed from the tub and disposed. The tub is moved from the work space receiving unidirectional HEPA-filtered airflow through a wall partition (pass through) into the ISO 5 filling and plunger insertion machine as the lid is removed.

An automatic lid removal machine can be implemented in lieu of manual lid removal the tubs. An automated lid removal operation provides repeatable, monitored, validatable removal of lids and minimizes manual manipulations and operator contact. The automated system is required when utilizing an e-beam machine.

Adequate space is to be provided for operators to manually remove the lids and liners from tubs in a conventional line, without introduction of particulate or bioburden. It is recommended to provide a barrier for operator segregation. In a RABS or isolator line, this operation is to occur automatically inside a barrier.

Provisions for waste removal needs to be incorporated into the design of the fill room, where the delid operation occurs.

Nested syringe filling and plunge insertion station. The syringe filler should be designed as a monoblock filler and specified to aseptically fill and insert a plunger at the predetermined fill line speed, fill accuracy, and allowable fill velocity limits. The filler controller should be capable of operating in both a run mode and a maintenance mode.

Particular attention should be paid to designing the filler and its stations for UAF via HEPA-filtration provided by the room HVAC system or a dedicated HVAC system.

The nested syringes are automatically conveyed from the lid removal station, automatically removed from their nests (Fig. 16), and filled (Fig. 17). The syringe filler should be specified to aseptically fill and insert a plunger into the syringes on a nested-syringe filling system. As they are transitioned into the filler the nested syringes are conveyed and held by a belt designed to transport syringes in nests.

Singularized syringe filling and plunge insertion station. The singularized syringes are automatically conveyed from the depyrogenation tunnel to the filler and filled. The syringe filler should be designed and specified to aseptically fill and insert a plunger into the singularized syringes (Fig. 18).

Figure 16 De-nesting (15).

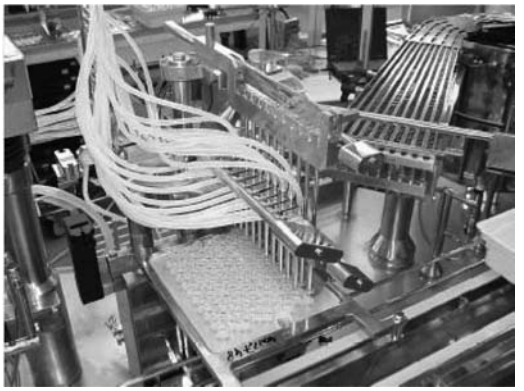

Figure 17 Nested filling (15).

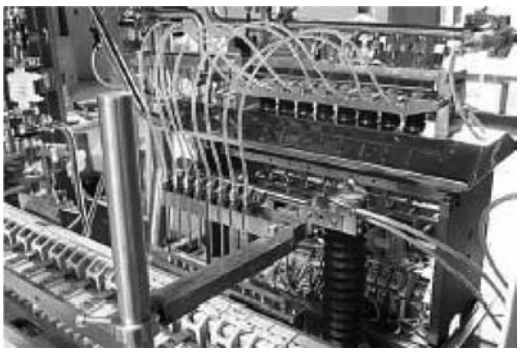

Figure 18 Singularized filling (15).

The syringes will be filled via the product dispensing fill nozzles with the volume of product programmed into the control system. To provide sterile-filtered product, the product is fed via an RTP from the hold tank positioned at the filler to rotary piston filling pumps and sent to the product dispensing nozzles. The product tank/filler docking station with RTP will be subject to unidirectional airflow.

Plunger insertion. After the syringes are filled, the syringes will have a plunger inserted. The plunger insertion rams are located immediately after the filling nozzles (Fig. 19). Flushing with gas during the insertion of the plunger and/or the addition of vacuum assist for plunger placement is to be evaluated on a project/product specific level where product protection is

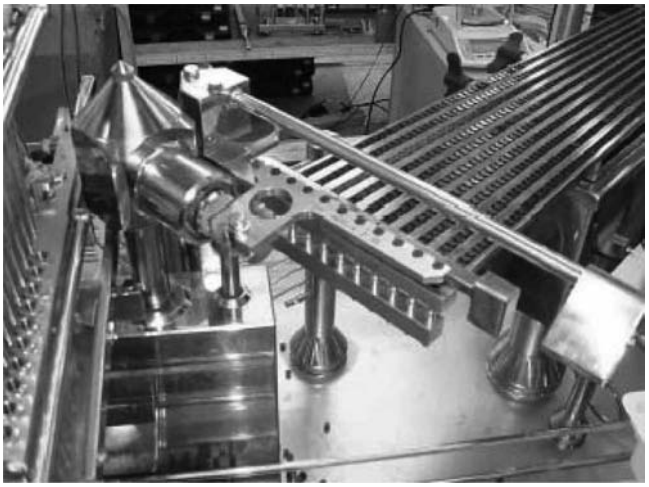

Figure 19 Plunger insertion (15).

required. The preprocessed (cleaned) and sterilized syringe plungers are supplied to the filling line, in disposable bags that docked at the plunger placement station RTP and are feed into a supply hopper through that RTP. (Note: Plungers supplied in matrix form could be considered as an alternate; however, this is an open issue in regards to the RABS design.) The supply hopper is sized according to the filling throughput. The size of the hopper should be dictated by

- the maximum dimensions permissible for sterilization of the hopper in an autoclave
- required unattended run time
- line speed
- maximum allowable load height the plungers can withstand without being damaged and/or generate particulate.

The hopper will feed plungers to the plunger guide track where plungers are oriented for accurate repeatable placement into the syringes by the plunger insertion rams. When the plungers are placed, the containers are considered sealed. The plunger insertion depth is programmable by the control system and will be verified by a downstream inspection.

Syringe check weighing. To minimize product loss (low fills rejected and required % over fill) it is recommended that the filler incorporates a check weighing system. The singularized syringe presentation allows individual verification and fine tuning of the fill volume during setup and statistical verification during the course of a run. At low speeds, 100% check weighed in line is possible; however, at higher line speeds approximately 3% of the syringes can be check weighed, due to mechanical limitations of removing syringes from the line to be check weighed out of place and then replace in the line. Typically, for most Vendors, the line speed cut off for 100% check weighing is 200 syringes per minute (SPM).

On the basis of net weight, the control system will correct the volume of product that is dispensed by the nozzles. The tare and gross weigh verification system should be calibrated prior to each fill operation.

The filler check weigh control system should be programmable to reject syringes whose gross product volume does not meet the acceptable minimum and maximum volumes and to alarm after repeated low volumes parameters programmed in the controller are exceeded.

De-nesting (nested). After the syringes are closed they are conveyed to the denest station where the syringes are removed from the nests with a 10-syringe manipulator.

At the exit of the machine, it may be desirable to install a paternoster, or elevator, to lift the syringe tubs prior to exiting the room. This permits operators to have access to both sides

of the filling line, improving the personnel flow in the room. The paternoster can also be used to transport tubs to another downstream operations taking place on another level of the building.

The syringes are then singularized for inspection, labeling, or repackaging into Rondo trays. The syringes are removed from the line, placed into tubs with lids, and manually stacked on syringe carts or pallets for transport to the packaging area or cold storage. In the event Rondo trays are used, the tubs and nests are removed from the station for disposal.

Alternate filling technologies

Blow-fill-seal. This fill technology can be considered a closed process as the bottle is formed immediately prior to filling and then sealed under ISO 5 conditions (Fig. 20). Following filling, the plastic ribbon generated in the filler, containing the bottles, needs to be cut away. Strips of bottles are then inspected, using destructive methods. If the product requires containment, additional segregation is required for the workstation.

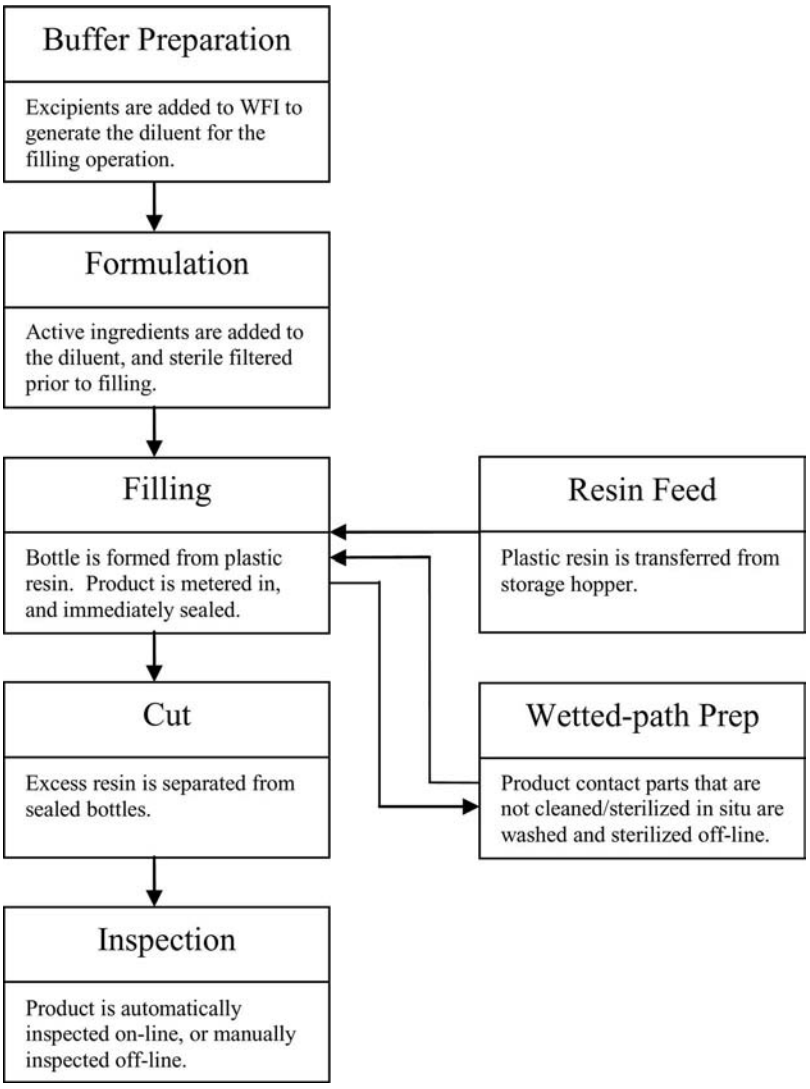

Figure 20 Process flow, blow-fill-seal.

Sufficient space needs to be provided for bulk containers of bulk plastic resin. Sufficient ceiling height is required to house the resin feed system. Exhaust ventilation is required to ensure that plastic dust particles are not permitted to migrate from the space and be carried into clean rooms.

As the filler is a closed system, the filling machine can be located in an ISO 8 room. Sufficient room height is required for the resin hopper. Sufficient clearance is required for maintenance of the filler. A flow path, including material air lock, for removal and replacement of filler parts is required.

Provisions for plastic waste removal need to be incorporated into the design of the ribbon cutting area.

As fill check is typically destructive and requires product to be expelled from the filled units, provisions need to be made for a workstation, with vacuum. If product requires containment, additional segregation is required for the workstation.

Plastic bottle. The infeed process in presterilized bottle filling is similar to that of the presterilized syringe. As there are large quantities of presterilized bottles, tips, and caps that must be loaded onto the filling line, the aseptic handling and manipulation of components is critical to the process. Cardboard and other packing materials are to be removed prior to conveying bags containing presterilized bottles into the ISO 8 infeed area. In the material airlock, bags of bottles should be transferred onto captured pallets or carts to limit particulate or bioburden.

In a conventional or RABS filling line, outer bags are removed, exposing inner bag. Bags should be unwrapped under HEPA filtration. Bags are handled via aseptic technique and passed into the ISO 7 filling room. In isolator facilities, it is recommended to permit the bags to dock to the isolator for transfer (Fig. 21).

Provisions for plastic waste removal need to be incorporated into the design of the bottle infeed area.

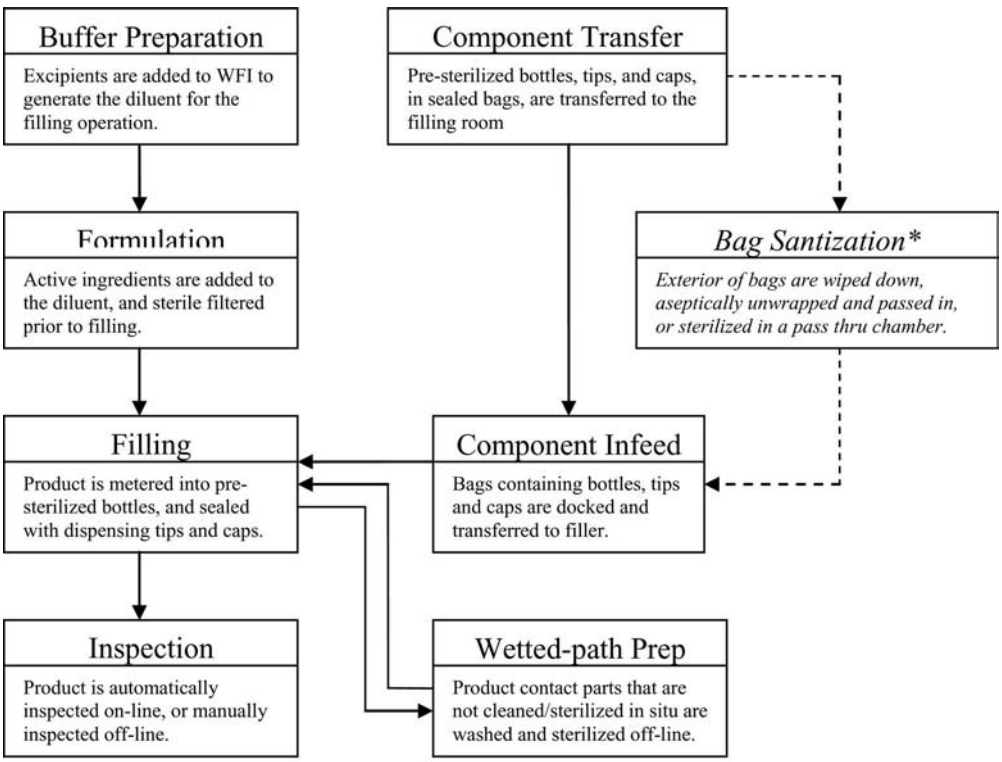

Figure 21 Process flow, plastic bottle. *Not required if isolation fill technology is used.

Component Prep

In all sterile facilities, it is important to properly prepare new components for use in the filling operation. It is also required to adequately decontaminate, clean, and sterilize all reusable parts. This section describes the various steps in the component preparation process (Fig. 22).

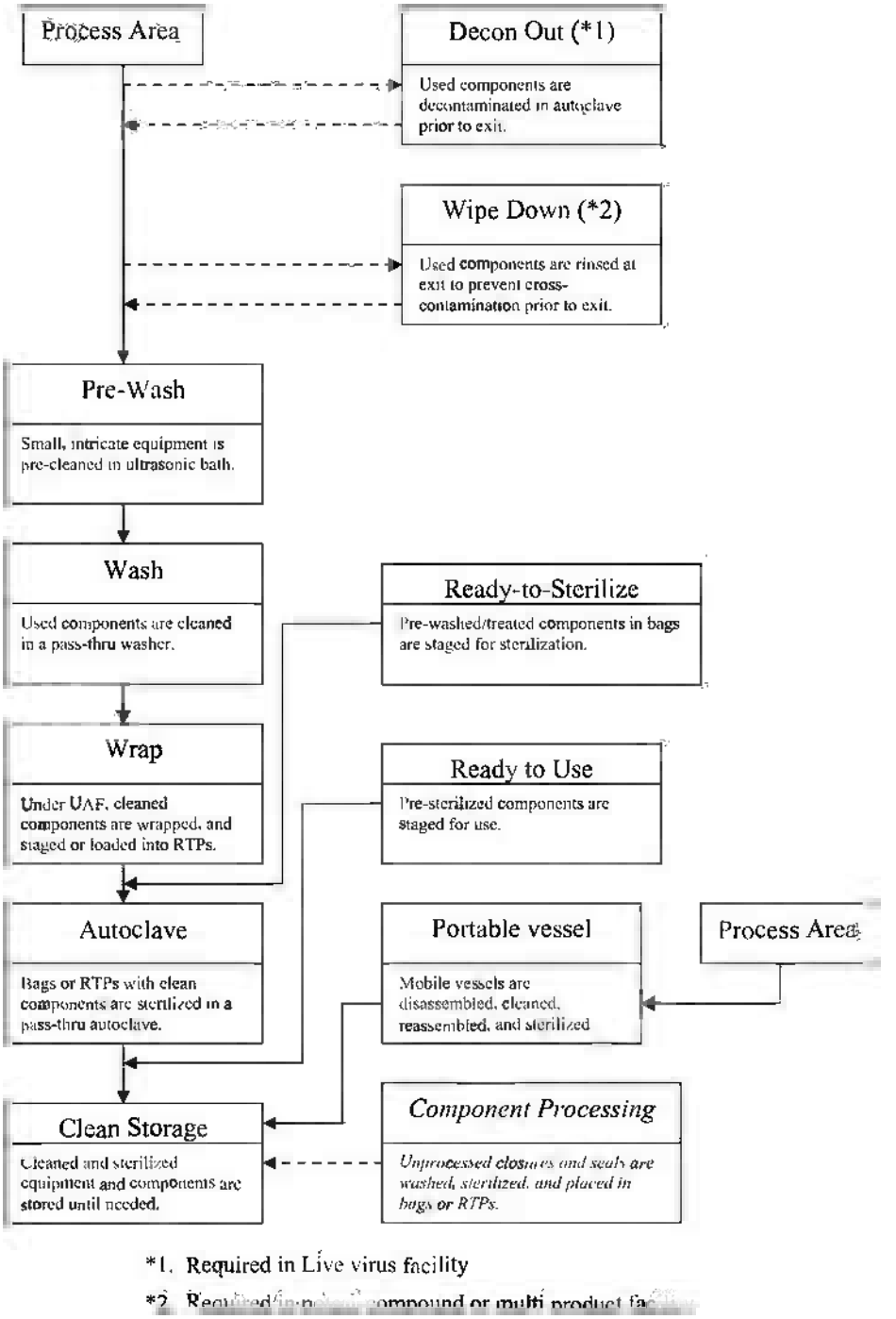

Figure 22 Process flow, component prep. *1. Required in live virus facility. *2. Required in potent compound or multi product facility.

If live virus or viral vectors are in use, it is required to decontaminate any used components or waste prior to exiting the suite. A decontamination autoclave is to be provided for safe disposal of solid materials. For liquid waste, chemical or heat decontamination is required prior to discharge to sewer.

In a multiproduct multisuite facility, it is recommended to place all used components into a secondary containment device, such as a bag or a cart, seal all openings, and wipe down the exterior of all used components prior to exiting the suite and entering the common return corridor.

Once components are returned to the washroom, it is required to disassemble components, discard trash, and wash reusable components. Small intricate components are to be prewashed in an ultrasonic sink prior to placing them through a parts washer.

Adequate space is to be provided for operators to manually wash components and place on drying racks. Exhaust ventilation is required to remove humidity buildup during washing.

It is recommended to provide space for post-use integrity testing of vent filters prior to disassembly at this location.

Provisions for waste removal need to be incorporated into the design of the room.

Pass-through washer shall be provided with specialized racks to contact all internal and external surfaces of components being washed and depyrogenated. Following the washing operation, the washer shall be designed to completely dry all components prior to unload. External ventilation over the unload door is not required for this reason.

Adequate space is to be provided for operators to load, unload, and change racks for different load patterns. A staging area is required for racks that are not in use.

Sufficient space is required for staging of parts to be washed as well as space for washed parts following unload. Unload should be conducted under HEPA filter protection.

When components such as RTPs or small vessels are cleaned in the parts washer, loading and unloading operations are to occur with the assistance of an ergonomic lift to avoid operator injury.

Components that have been washed shall be wrapped under HEPA filter protection to avoid redepositing particulate, including endotoxins. Once bags are sealed, they can be stored on racks. Adequate space is to be provided for the wrapping operation followed by heat sealing of the autoclave bags.

In an isolator facility, it is also required to load the RTP under HEPA filter protection to avoid depositing particulate inside the RTP.

Once bags and RTPs are sealed they can be stored on racks to wait for loading into an autoclave.

Autoclaves are designed to sterilize dry porous (stopper bags) and nonporous loads (steel components). Following sterilization, if components have been placed into sealed containers and cooled to ambient conditions, an ISO 5 area is not required at the exit of the autoclave. If components are not completely sealed, an ISO 5 area is required to complete any reassembly or sealing.

Pass-through autoclave is designed to sterilize all components, porous and nonporous, with clean steam. The autoclave is to be designed with ventilation to provide cooling to components prior to unload. As components are cooled to ambient conditions prior to unload, exhaust ventilation is not required at the exit.

A sufficient space is to be provided for staging of autoclave racks not in use.

Adequate space is to be provided for operators to stage racks of cleaned and sterilized materials required for production operations.

Space is also required for staging mobile vessels. Docking stations are required for this purpose, permitting pressurization with process air or nitrogen and connection to process control for monitoring the vessel pressure. Sufficient space is required for moving vessels in and out of the staging area.

If raw components, including stoppers, caps, and plungers, are to be used in the process, it is recommended to design the facility to accommodate processing on-site. Processing consists of washing, drying, siliconization, and sterilization.

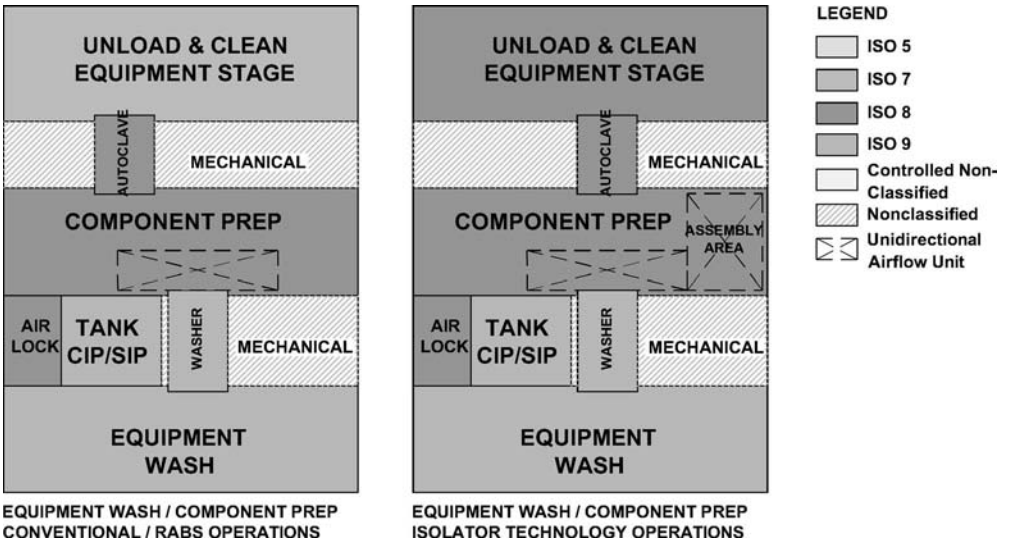

Figure 23 (*See color insert*) Equipment wash/component prep.

Components can be loaded into a pass-through processor or into a single-sided processing vessel. The facility will be designed around one technology.

Processed components can be discharged into stainless steel RTP vessels or bags. Adequate space is required to stage empty and full vessels.

Figure 23 represents examples of component preparation operations utilizing conventional filling, RABS, and isolators:

Use of Barrier or Isolation Technology

It is recommended that a filling line incorporate a RABS as a minimum. Barrier systems like RABS or isolators protect the product (active ingredients, etc.) from contamination, the environment, or personnel. They also protect the personnel from the product in the case of potent ingredients and compounds. The degree that the operator must be separated from the process or the operator protected from the product in part determines the type of barrier system, RABS, or isolator.

The operation of RABS fillers and isolated fillers require strict adherence to standard operating procedures (SOPs) in regard to material introduction and the use of the gloveports for manipulations and other operator interventions.

Automation and interlocks integrated into the overall design of the RABS or isolator can support the aseptic integrity of the barrier system in place.

Isolator facilities should be designed to include space for adjustable height work platforms at routinely accessed glove ports for ergonomics. Ceiling heights for isolator facilities should account for locations of terminal HEPA filters. Adequate space should be provided to permit routine testing and replacement.

RABS

RABS can be designated as passive, active, and closed. The air classification for any type of RABS should be ISO 5 within the critical zone and the background environment should be ISO 7. All RABS provide the enclosed environment with unidirectional airflow positive to the surrounding space or room. All RABS incorporate gloveports for making interventions into the filler while the doors are closed for production. All RABS provide material transfer through RTPs for the introduction and exit of components, tools, trash, environmental monitoring (EM) materials, etc (Fig. 24).

Figure 24 RABS filling (15).

A passive RABS provides HEPA-filtered air over the critical areas and exhausts the airflow at the bottom of the barrier into the surrounding room returns. For a passive RABS, the RTPs should include HEPA coverage. The vials leave the filler through a small cut out in the barrier commonly referred to a "mouse-hole." Passive RABS designs are decontaminated via manual disinfection.

An active RABS is designed like a passive RABS but incorporates an integral dedicated HVAC system to supply HEPA-filtered air to the enclosed work environment and exhaust air to the room returns and the HEPA unit. The vials leave the filler through a "mouse-hole," which can be designed with an exit tunnel. An active RABS is decontaminated via manual disinfection.

A closed RABS is very similar to an isolator and features a dedicated HVAC system to recirculate the airflow within the barrier work environment. The closed RABS features integrated returns ducted directly back to the dedicated HVAC system. The tunnel interface where glassware is introduced into the filler will require an air curtain with integral air return ducts. The vials leave the filler through a "mouse-hole," which can be designed with an exit tunnel or include HEPA coverage.

Closed RABS and isolators are similarly complex in their design, construction, and operation; however, the decontamination processes between the two different systems should be noted and carefully considered. A closed RABS is decontaminated via manual disinfection; however, an isolator is decontaminated in a highly controlled process utilizing sterilants such as VHP.

Isolator

An isolator is a closed barrier system incorporating hard walls and utilizing HEPA-filtered air to maintain positive or negative pressure within the critical work environment. Manipulations or interventions into the isolator are performed through gloveports, half-suits, and RTPs to isolate the operations personnel from the critical process. The interior of the isolator must maintain ISO 5 conditions with a background of ISO 8. Critical airflow velocities and pressures within the isolator are maintained by the control system (Fig. 25).

Figure 25 Isolator filling (16).

General Design Considerations for Transfer Between Machines

It is essential to maintain a break from one room environment to another when designing the conveyors and transfer mechanisms of a filling line. From one grade to another, the transfers cannot "cross" over; segregation must be maintained whether the conveyor or transfer mechanism is side-to-side or a dead plate (the type of dead plate would depend on the vendor and location of transfer required). The conveyors and transfer mechanisms should be designed to avoid damage to the vials. No catch points should exist on the conveyors or side rails that can tip or scratch the product or damage the identification markings. Mechanical pressure transfers and accumulation that require hard mechanical contact shall be avoided.

Accumulation allows for slight decoupling of the machines and helps smooth out line flow. It is recommended to obtain sufficient accumulation capacity to keep the upstream equipment operating for five minutes minimum (starting with an empty accumulation) with the downstream equipment stopped for all vial sizes. The design, requirements, type, and locations of accumulation should be reviewed prior to final design. Additionally, when considering accumulation, time spent in accumulation and applications of "first in, first out" (FIFO) or periodic accumulation clearance must be addressed.

Stainless Steel Vessels

Vessels are used for formulation, homogenization, temperature control, and storage of drug products. Vessels shall be of a sanitary design and construction and shall be designed in accordance with current edition of the ASME Boiler and Pressure Vessel Code (17) and the ASME Bioprocessing Equipment (BPE) standards (18).

It is recommended that vessels shall be considered portable when the volume is less than or equal to 300 L and movable when the volume is greater than 300 L but less than 500 L. At or above 500 L, vessels should be considered fixed or stationary. Rooms should be configured to enable the maneuvering of equipment while utilizing ergonomic assists.

Vessels that are exposed to temperatures above 176°F (80°C) through the use of hot WFI, hot CIP solutions or SIP, should be designed for full vacuum service.

All vessels should be jacketed for temperature control, using dimple jackets, which allow for the greatest amount of heat transfer with the least amount of holdup in the jacket. If

required, the jacket fluid should be designed for maintaining the contents at 2°C to 8°C with a propylene glycol/water mixture supplied by a temperature control module.

A single vent filter serves to filter bidirectional flow for both tank venting and process compressed air into the vessel. When designing facilities with portable vessels, all door heights should be set on the basis of the total vessel height, including the vent filter and associated piping, in addition to the dimensions of the vessel.

Weight, a nonintrusive measurement, should be used to determine level rather than other types of intrusive level measurement. If stationary, the formulation tank is mounted on weigh cells for level control. If portable, the vessel can be placed on a floor scale. Prior to use, the empty vessel can be measured and tared. This allows for an accurate measurement of the fluid inside the vessel. Care must be taken during piping design to avoid introducing error in the measurements through pipe stress, which can occur with temperature fluctuations. With proper design (sufficient length and/or bends) sanitary tubing provides enough flexibility to avoid installation of flexible hoses. Schedule 10 or 40 piping will require flexible hoses to be placed in line. Scales can be designed to be either installed in pits or placed on the floor. While pit-mounted scales provide greater access for vessels, they are more difficult to clean and must be located in the exact position they will be needed in. Floor-mounted scales provide more flexibility during design, are easier to clean, but require a ramp from the floor to the scale platform. This ramp will typically require that a tank manipulator be provided to assist the operator.

Following production operations, equipment requiring washing in parts washer are removed. The vessel is fitted with a sprayball. The vessel is connected to a CIP skid at a washing station, designed as a pass-through. This station is provided with a safety curtain to prevent accidental contact of chemical solutions, hot WFI, or clean steam. A sealed floor drain is required in the area. Exhaust ventilation is required to prevent buildup of humidity in the event of an accidental release.

Adequate space is required for operators to make and break connections easily without risking injury including burns.

If local recirculation is required at the vessel station, space is required for a floor-mounted pump.

Proper space should be provided to operate and maintain equipment. All nonessential devices associated with the media or buffer preparation process should be removed from the clean room and located in a technical chase, where they can be routinely inspected. Piping penetrations from the chase into the clean room should be grouped into a single stainless steel wall panel for rigidity and cleanliness.

Following cleaning, but prior to steaming, the sprayball is removed. The vessel is reassembled with all components required for operation and sterilized at the station. Following sterilization, positive pressure is blocked in, the vessel is disconnected, and then moved to staging.

Use of Disposables

It is possible to replace stainless steel equipment with disposable components, including tanks and piping. Disposable bags have been specially designed to hold liquid, although they will still require stainless steel frames. Liquid transfers can be performed by using presterilized disposable tubing, coupled together by sterile tubing connectors. Solids charges can be conducted via specially designed bags.

When disposable components are used, the requirements for cleaning and sterilization are eliminated, including all piping and automation, which greatly simplifies the construction of the facility. However, product compatibility studies are required to confirm that no issues with product degradation can be anticipated.

FACILITY CLEANING AND SANITIZATION

Filling Line Cleaning and Product Pathway Decontamination

The filling line should be constructed and designed for surface sanitization with an approved cleaning agent on a periodic basis. The product contact parts, such as the stopper hopper, stopper sorting bowl, change parts, should be cleaned and sterilized. The nonproduct contact

parts, such as starwheels, feed screws, guide discs, should be manually cleaned or disassembled and cleaned out of place in a parts washer.

Isolators are decontaminated with sterilants, such as vapor-phase hydrogen peroxide (VPHP), chlorine dioxide, or peracetic acid. Decontamination cycles for isolators require multiple steps to prepare, introduce, circulate, and exhaust the sterilizing material prior to use in filling, compounding, etc.; the cycle is designed such that it is considered reproducible. VPHP, which is very widely utilized in the industry as a sterilant, is toxic if released into the outside air when concentrated. This issue is among a number of additional details that must be addressed in implementing an isolator for filling line. An isolator decontamination cycle requires adequate time in the operational time cycle for both setup and execution.

Location and Usages of Drains

Drains are typically required for equipment and facilities housed inside clean rooms. Special considerations are required to ensure that drains do not become sources of contamination. Floor drains are not permitted within classified spaces for aseptic facilities. If spillage occurs from a nonroutine event, it must be mopped up instead. For equipment drains, it is required to separate the equipment from the drain line with the use of an air break. As it is not permitted to have an open drain inside a clean space, the air break should be located in a technical chase.

If it is not possible to locate the air break in a technical chase, the use of a double air break is permitted, allowing an air break locally at the equipment and providing a second break inside a technical chase. Room air from the pressurized clean room flows through the drainpipe into the technical chase. This creates a barrier against migration of contamination into the clean room. In addition, all drain hubs should be sanitized regularly.

At local drains for sample connections, it is typical to provide a funnel at the air break to catch rinses during sampling routines.

It is permitted to use a common drain header for several drain points, provided that cross-contamination cannot occur. For this reason, it is acceptable for drains from a common system to be piped to drain using a common header, but not for drains from multiple systems.

At drain hubs located at floor level in technical chases, it is recommended to install a curb to prevent floor sweepings from becoming inadvertently entering the drain. At each drain hub where steam condensate is routinely encountered, it is typical to provide an HVAC exhaust trunk to extract steam vapors. This practice provides ventilation in technical chases, eliminates buildup of humidity, and inhibits growth of mold. As the steam vapor condenses to form WFI, an appropriate material of construction for the exhaust trunk should be selected.

It is good practice to reduce the temperature of liquid waste entering the process drain system. In low-flow streams that experience high temperatures infrequently, drain coolers using dilution with potable water can be installed. For high volume drain lines that frequently see high temperatures, permanently mounted drain heat exchangers are recommended.

Prior to discharge to municipal sewer, it is typically required to reduce the temperature to below 60°C and adjust the pH to neutralize extremely acidic or basic streams. Collection and treatment equipment can be provided for this purpose, either inside the facility or outside on site.

Waste Decontamination

If live virus or vectors are in use, it is required to decontaminate any used components or waste prior to exiting the suite. The facility should be configured to permit the use of a pass-through autoclave at the exit of the sterile suite. Used materials should be bagged to prevent contact with operators during transfer.

Liquid waste generated during facility cleaning is also required to be decontaminated chemically prior to discharge to the biowaste collection system. Adequate facilities are to be provided for this operation, including installation of a biowaste discharge point.

Adequate staging space is required to temporarily store material ready for decontamination operations. Provisions for waste removal following decontamination need to be incorporated into the design.

The requirements for collection of waste and the recommended treatment methods are dependent on the biowaste level for the specific product. It is typical to segregate waste streams containing biowaste from waste streams containing process waste.

If possible, it is recommended to inactivate the waste in-process prior to discharge into the biowaste collection system. Inactivation can be either by thermal or by chemical methods. Floor washings from rooms in contact with live virus products can be chemically inactivated prior to discharge to the biowaste system. Waste from equipment such as autoclaves or washers can either be chemically or thermally inactivated prior to discharge.

If containment is required for the collection system, special attention is required for the air break between the equipment drain and the collection system. In this case, any vents on the biowaste collection system would be required to have 0.22 μm vent filters. Air breaks would be provided in vented enclosures, providing containment while providing a break in the system.

The biowaste collection system should be designed to permit periodic decontamination, either by chemical flooding or by steam. Special attention must be paid to all drain connections to ensure that air is not entrapped in lines during decontamination procedures.

Waste Containment and Disposal

In facilities handling potent compounds, it is required to segregate waste streams that have product contact and treat onsite to isolate or inactivate the compound, or ship off-site for disposal. A segregated waste collection piping system, with collection tank, is required for this purpose.

If the product is insoluble in water, it is possible to filter the waste stream and concentrate the waste to be disposed. If the compound is well characterized, it can be converted by chemical reaction to render it inactive. Equipment and facilities need to be provided for these purposes.

If the process is localized, and at sufficiently small volume, the waste can be drummed up locally rather than providing a segregated drain piping system.

ARCHITECTURAL REQUIREMENTS

Gowning Philosophy

CGMP requires that areas of operation used for aseptic processing should prevent contamination from particles and microorganisms that may be present in the air, on product contact surfaces, or shed from personnel. Classified pharmaceutical manufacturing areas are defined by their low levels of viable and nonviable particulates that need to be monitored regularly to demonstrate that they are being kept under control. Personnel are one of the greatest sources of particulate contamination in the clean room. Therefore, it is imperative that the shedding of particulates from personnel required to enter a clean room environment is kept to a minimum.

In multiproduct facilities and in facilities where multiple stages of production occur (i.e., pre- and post-viral inactivation) in the same building, segregation and separation of the products, processes, and personnel are critical to avoid cross-contamination. Successful prevention of cross-contamination of products and stages of the process is supported through the control of personnel flow and the gowning program that is implemented.

This is accomplished by establishing a robust personnel gowning program that is based on defining:

- The functional areas and activities performed in the sterile processing areas.
- The quality of the environments of the functional areas.
- Personnel access to the manufacturing areas.
- Personnel hygiene practices.

- The gowning requirements for each functional area.
- The procedures for personnel gowning and degowning.
- The procedures for control and maintenance of gowning supplies.

Written and approved procedures must be implemented for the personnel gowning program that address all aspects of the program, including personnel flows and movements through the facility, gowning procedure training, good aseptic gowning technique, instructions for maintaining the garments' cleanliness and integrity after donning, and the requirements for gowning-qualification.

The gowning protocol will impact the design of the facility by defining the requirements for

- locker room design and layout
- gowning airlock design and layout
- clean gowning supply and storage
- soiled gowning staging and retrieval

Architectural and Layout Requirements

This section focuses on the architectural aspects attributing to the successful design of a compliant and operational sterile process and product manufacturing facility. The primary driver in the design of facilities for this purpose is contamination prevention and the protection of the product.

A compliant facility takes full account of GMP requirements as well as safety, health, and environmental requirements. An operationally successful facility satisfies process requirements; accommodates equipment layout, ergonomics, and maintenance access requirements; and allows for the proper flow of personnel, materials, and waste. Essential aspects of contamination prevention are the adequate segregation of operations, the proper gowning of personnel, and selection of appropriate finish materials.

The following is an overview of the architectural design characteristics, addressing these expectations.

Classification of Spaces

Critical processes and clean support operations occupy areas of a facility identified as classified GMP space. Classified spaces are designed, operated, and controlled to effectively control risk of contamination from particulates, including microorganisms and endotoxins, having potential direct impact on product quality. Classification designations for classified GMP space include ISO 5 (A), ISO 7 (B), ISO 8 (C), and ISO 9 (D), which are assigned to spaces on the basis of specific operational characteristics, product type, and/or technology used. Refer to section "HVAC Systems and Requirements" for further discussion on the specific criteria for the various classification levels.

ISO 5 is attributed to the critical zone where sterilized product, components, or product-contact equipment are exposed. The ISO 5 environment is achieved within RABS or isolator, or, in conventional facilities, within an LAF zone surrounding critical processes.

ISO 7, ISO 8, and ISO 9 room classifications are assigned depending on operational characteristics and the type of aseptic technology implemented. ISO 7 is required as the background room condition for open aseptic process operations and final product filling using conventional or RABS technology. ISO 8 is required as the background condition when isolators are used. Clean support operations typically occur under ISO 8 and/or ISO 9 conditions.

In certain instances at particular process steps, local protection is required to provide an enhanced operating environment. These enhancements include UAF units, LAF cabinets, and biosafety cabinets (BSC). An additional designation of controlled nonclassified (CNC) is attributed to areas directly related to and supporting classified GMP operations but physically separate.

Tables 1 to 4 list the appropriate minimal classification level for the various process operations and support functions attributed to sterile operations:

Table 1 Sterile and Nonsterile Material Prep/Sampling

Operation	Sterile Materile		Non-Sterile Material		Remarks
	Contained	Non-Contained	Contained	Non-Contained	
Sampling	BSC or Isolator **ISO 8**	Isolator **ISO 8**	LAF Cabinet **ISO 9**	Downflow Booth ***ISO 9**	*Non-sterile components (e.g., vials) in ISO 9 room
Weigh & Dispense	BSC or Isolator ISO 8	Isolator ISO 8	LAF Cabinet ISO 9	Downflow Booth ISO 9	
Buffer Prep	NA	NA	NA	UAP *ISO 8	*Open low risk operations or closed operations in ISO 9 room
Buffer Hold	NA	ISO 8	NA	*CNC	*Prior to final sterile filtration
Media	NA	NA	NA	UAP *ISO 8	*Closed operations in ISO 9 room

Table 2 Opened or Closed Processes

Operations	Process System			Remarks
	Open Aseptic	Open Non-Aseptic	Closed	
Bulk Biological Synthesis:				
Cell Culture - Seed Preparation	ISO 5 ISO 7	UAP ISO 8	ISO 8	
Cell Culture - Bioreactors	ISO 8	ISO 8	CNC	
Recovery	ISO 5 ISO 7	UAP ISO 8	ISO 9	
Purification	ISO 5 ISO 7	UAP ISO 8	*ISO 9	*Final purification occurs in ISO 8
Bulk Filling	ISO 5 ISO 7	UAP ISO 8	ISO 8	
Bulk Chemical Synthesis:				
Final Purification	ISO 5 ISO 7	UAP ISO 8	ISO 8	
Formulation	ISO 5 *ISO 7	UAP ISO 8	ISO 8	*ISO 8 if isolator is used
Sanitization Prep	ISO 5 ISO 7	UAP ISO 8	ISO 8	Preparation of sterile sanitizing agents for wipe down

Table 3 Aseptic Technologies Application—Filling

Operation	Aseptic Technologies			Terminally Sterilized Product	Remarks
	Conventional Technology	RABS Technology	Isolator Technology		
Vial / Ampoule Filling:					
Vial / Ampoule In-Feed	ISO 9	ISO 9	ISO 9	ISO 9	
Vial Wash / Depyrogenation	ISO 8	ISO 8	ISO 8	ISO 8	
Vial Fill & Stopper / Ampoule Fill & Seal	ISO 5 ISO 7	RABS ISO 5 ISO 7	Isolator ISO 5 ISO 8	ISO 8	Non-lyophilized product in vials will move directly to capping, ampoules no further action
Lyo Loading & Unloading	ISO 5 ISO 7	RABS ISO 5 ISO 7	Isolator ISO 5 ISO 8	NA	
Capping (pre-sterilized and specialized caps)	ISO 5 ISO 7	RABS ISO 5 ISO 7	Isolator ISO 5 ISO 8	ISO 8	*e.g., ADD Vantage®, BIO Set®
Capping (non-sterile caps)	UAP ISO 8	UAP ISO 8	UAP ISO 8	ISO 9	
External Vial Wash	CNC	CNC	CNC	CNC	
Syringe Filling:					
Syringe In-Feed	UAP ISO 8	UAP ISO 8	UAP ISO 8	NA	
Tub Exterior Sterilization	UAP ISO 8	ISO 8	ISO 8	NA	
Syringe Fill & Plunger Insert	ISO 5 ISO 7	RABS ISO 5 ISO 7	Isolator ISO 5 ISO 8	NA	
Blow Fill Seal:					
Resin Feed	NA	NA	CNC	NA	
Blow / Fill / Seal	NA	NA	BFS* ISO 5 ISO 8	NA	*BFS machine integral ISO 5 zone
Cut	NA	NA	CNC	NA	
Resin Waste	NA	NA	NC	NA	
Pre-Sterilzed Bottles:					
Bottle In-Feed	UAP ISO 8	UAP ISO 8	UAP ISO 8	NA	
Bottle Exterior Sterilization	UAP ISO 8	ISO 8	ISO 8	NA	
Bottle Fill & Cap	ISO 5 ISO 7	RABS ISO 5 ISO 7	Isolator ISO 5 ISO 8	NA	

Table 4 Aseptic Technologies Application—Filling Direct Support

Operation	Aseptic Technologies			Terminally Sterilized Product	Remarks
	Conventional Technology	RABS Technology	Isolator Technology		
Pre-Wash / Equipment Wash	ISO 9	ISO 9	ISO 9	ISO 9	
Component Prep	~~UAF~~ ISO 8	~~UAF~~ ISO 8	~~UAF~~ ISO 8	~~UAF~~ ISO 8	Washer unload & parts wrapping occur under UAF; refer to Stopper Processing for stopper prep
Autoclave Unload / Staging	ISO 7	ISO 7	ISO 8	ISO 8	Provide UAF zone if cooldown occurs outside of autoclave
Stopper Processing	~~UAF~~ ISO 9	~~UAF~~ ISO 9	~~UAF~~ ISO 9	~~UAF~~ ISO 9	Processor vessel charging occurs under UAF; Prewashed stoppers brought directlly to Component Prep
Tank CIP / SIP	ISO 9	ISO 9	ISO 9	ISO 9	May occur in dedicated room or in Equipment Wash
Lyo Mechanical Space	*CNC	*CNC	*CNC	NA	*Classification depends upon lyo type & access to lyo chamber-unclassified space may be acceptable
Inspection	CNC	CNC	CNC	CNC	

Transition Zones and Airlocks

Transition zones and airlocks are necessary to maintain the integrity of the environmental classifications of spaces by controlling the dispersion of particulates between areas of different classification and to control the movement of personnel and materials into and out of GMP areas.

Transition zones and airlocks support the control of particulates by maintaining air pressure differentials between areas of differing classifications, or, in some instances, by separating adjacent operations occurring at the same level of classification. Airlocks are also utilized to establish a barrier zone ("bubble" or "sink") when containment is a criteria.

Movement of people and the staging and movement of materials must be designed to minimize errors, maintain gowning room hygiene, and minimize the risk of cross-contamination. Transition zones and airlocks provide a clean space for personnel gowning and a controlled environment for the transfer of materials into GMP areas and between classified areas.

General airlock expectations.

- Airlocks are required between areas of different classifications.
- Airlocks must be appropriately sized and environmentally controlled depending on the activity occurring within the airlock (e.g., number of people gowning at one time, or material wipe-down or staging).
- The number of airlocks in sequence is dependent on the number of grade levels between the start and final destination, the operations occurring during the transition (e.g., gowning stages requiring segregation) and containment requirements (hazardous/biohazardous).
- Separate entry airlocks for personnel and materials are typically utilized for entry from nonclassified areas into GMP areas and when entering into areas of higher classification from areas of lesser classification (exception: combined personnel/material airlock may be considered for small scale operations with infrequent material transitions). ISO 5 zones are typically a segregated part of an ISO 7 area with limited direct access.

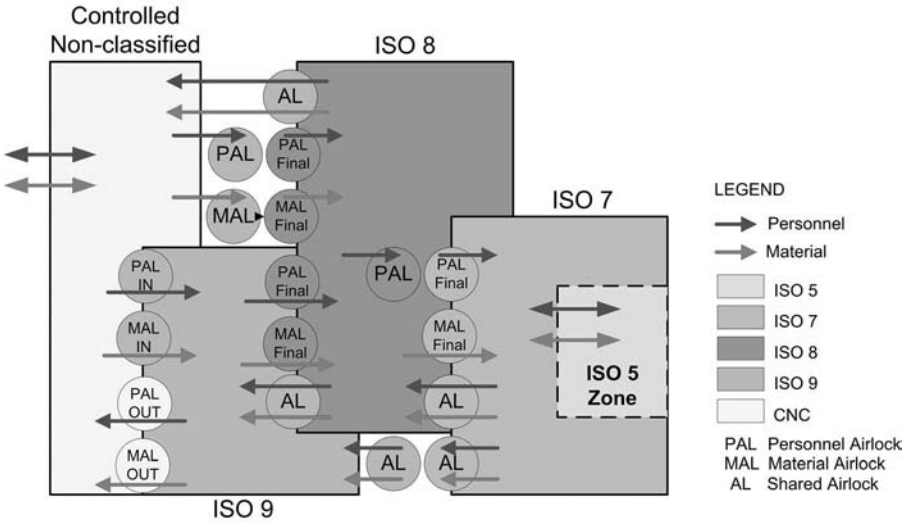

Figure 26 (*See color insert*) General airlock concept by classification.

- Combined personnel and material entry airlocks are permitted, within a process suite between areas of the same classification.
- Combined personnel and material airlocks are permitted for exiting all classification levels.
- Gowning and degowning typically occur in dedicated airlocks (exception: combined entry/exit personnel airlock may be considered for small scale operations).
- Entry and exit via the same airlock is permissible for areas supporting preparation of nontherapeutic products (e.g., buffer prep).
- Airlock doors are required to be fitted with either physical interlocks to prevent the possibility of more than a single door being open at a time or visible and/or audible warnings such that multiple doors are not simultaneously open; *note: physical interlocks must be connected to a centralized alarm system so that the interlocks automatically disengage in an alarm situation*.

General airlocking and flows are captured in Figure 26.

General facility layout expectations.

- Develop a program of spaces based on process, operational and project requirements
- Identify critical adjacencies, recognizing the interdependence of support operations and core activities
- Establish a hierarchy of operational and transition zones
- Identify appropriate classification of spaces based on project drivers (product type, process systems, and technologies utilized, dosage form)
- Properly address the movement of people, materials, and equipment:
  - Provide adequate space to allow orderly movement and staging
  - Provide adequate protection against contamination risks by incorporating unidirectional flow into and out of ISO 7 areas
  - Provide an adequate number of airlocks, gowning rooms and transition zones to accommodate flows, and ensure they are properly sized for the expected level of activity

- Provide adequate space to accommodate process operations, equipment, maintenance operations and storage
- Locate personnel locker rooms and toilets outside the classified manufacturing areas
- Properly address cleaning, sanitizing and housekeeping procedures:
  - Provide adequate facilities for preparation and storage of cleaning supplies in dedicated rooms.
  - Locate cleaning supply rooms in areas outside of ISO 7 classified areas.
  - Segregate areas requiring disinfection with fumigants from areas not intended to be fumigated.
- Minimize activity within classified areas:
  - Provide glazed viewing panels (windows), where appropriate, to allow observation of processes—for visual communication for operators within the area and from outside of the process area for visitors and supervisors.
  - Install phones or telecoms where appropriate for audible communication between process areas.
- In addition to CGMP requirements, facilities must be designed to meet the requirements and expectations of other agencies and regulators, including:
  - Local regulatory agency codes and standards.
  - Loss prevention provider standards.
  - Americans with Disabilities Act (for facilities built within the United States) (19).

Facility types. Figures 27 and 28 demonstrate the basic layout principles for single product and multiproduct facilities, respectively:

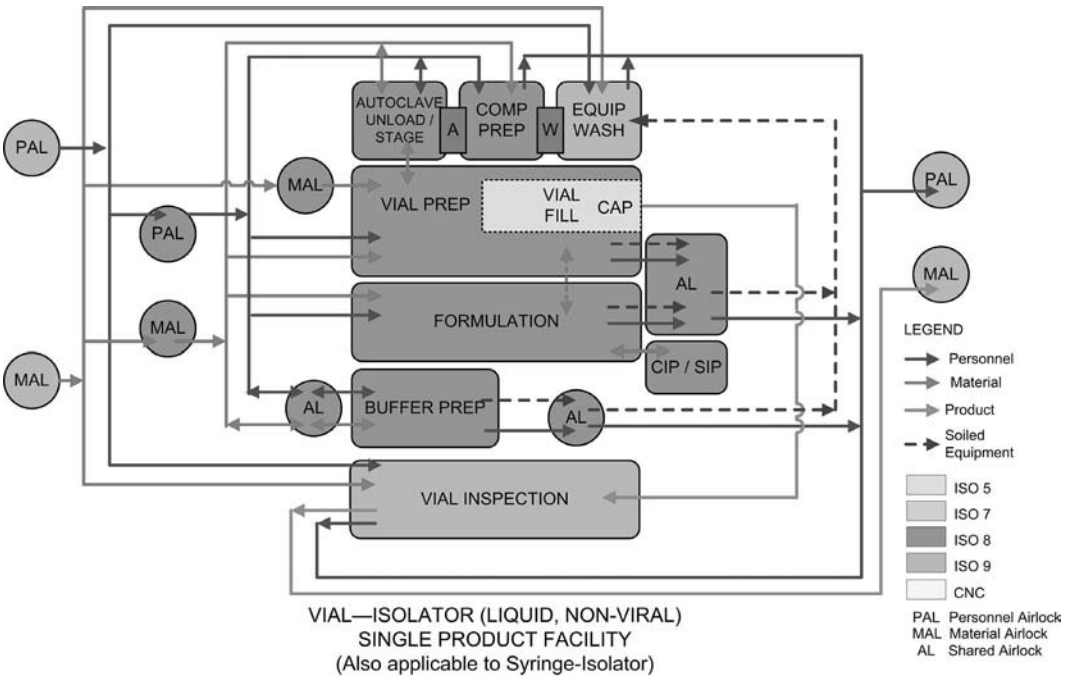

Figure 27 (*See color insert*) Single product—single-suite module.

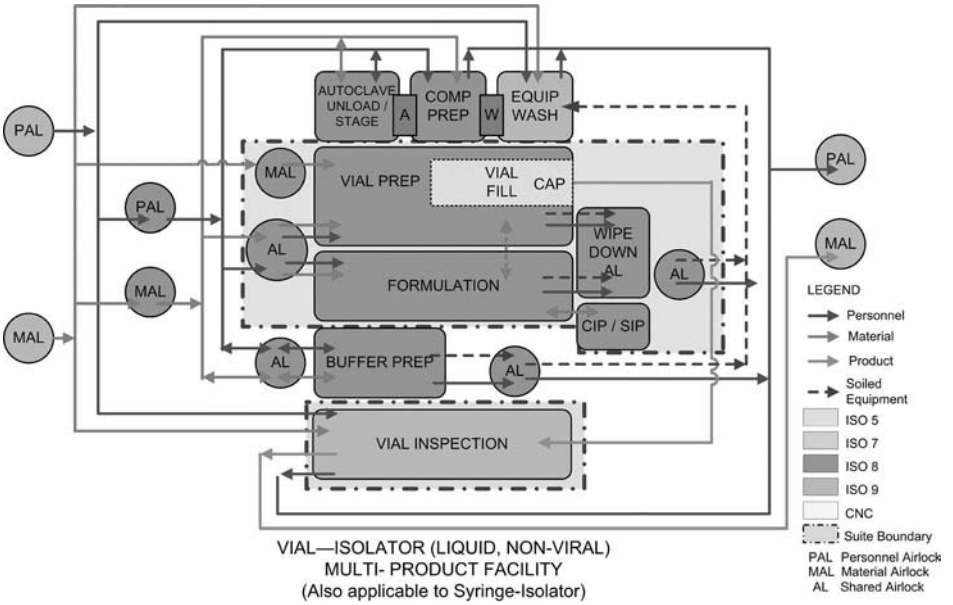

Figure 28 (*See color insert*) Multiproduct—multi-suite module.

Special conditions. Certain product types including pathogens or genetically modified microorganisms (e.g., some vaccines), and potent compounds (e.g., cytotoxics and steroids) and sensitizing compounds (e.g., β-lactam antibiotics) present particular issues and special conditions that further impact facility design. Each of these product types requires special accommodation for the containment of the product and protection of the operators working directly with the product.

Special design conditions include:

- Isolators should be considered for all open processes and filling.
- Process areas where open operations occur should be segregated from adjoining areas with a "barrier zone" (bubble or sink airlocks)
- Segregation must be provided for facilities processing potent and sensitizing compounds and for pathogens and genetically modified microorganisms. However, the facility is not required to be constructed as a separate building.
- For potent and sensitizing compounds and for pathogens and genetically modified microorganisms, decontamination is required for all product contact equipment.
- For processes involving the potential generation of aerosols of live cells, special provisions must be made for the decontamination of operator gowns.
- If personnel protective equipment (PPE) is required, the gown and degown airlocks should be configured to accommodate shared equipment (e.g., pass-through cabinets).
- For potent compound products, a misting shower should be included in the degown airlock for use in the event of a mishap.
- Depending on the type of product and the requirements/recommendations of applicable building and fire codes, and regulators (e.g., NIH or CDC), containment of firewater may be necessary (20).

Facility Finish Materials

Interior finishes and materials of construction should be appropriate for the type of activity occurring in the area and the recommended level of CGMP compliance—ease of cleaning is always of utmost importance.

Finish materials basic criteria. All exposed surfaces and finish materials in classified areas should be smooth, nonporous, and

- free from cracks and open joints
- resistant to shedding of particles
- resistant to sustaining microbial growth
- resistant to damage from normal mechanical abrasions and impacts
- resistant to damage from repeated application of cleaning agents, disinfectants, sterilants, and sanitizers; note: it is important to identify the cleaning agents and sanitizers used as well as the cleaning protocol. Prior to specifying materials and finishes, confirmation should be obtained from the manufacturer that the materials can withstand exposure to the agents used.

Configuration of the surfaces and their method of interface are also important. Horizontal ledges should be avoided as these are areas where particles and microorganisms could accumulate. Materials should align in the vertical plane, and joints between dissimilar materials should be caulked with sanitary silicone sealant. Coved transitions should be provided between walls and floors and between walls and ceilings.

The selection criteria for appropriate materials should include:

- Constructability and maintainability (local labor capability, difficulty of repair, ease of cleaning)
- Desired appearance (patterns, solid colors)
- Cost (both first cost and lifecycle costs)

Floor systems. Floor systems for critical and primary support areas of aseptic manufacturing facilities can be divided into two categories: sheet systems (PVC, rubber) and resin-based multi-layer systems. The appropriate selection of either system is dependent on the following criteria among others:

- Substrate conditions (new or existing concrete slabs)
- Expected frequency of traffic (material loads)
- Expected loading of traffic (heavy rolling loads such as tanks, carts, forklifts, etc.)

In controlled, nonclassified CGMP areas, floor systems such as pigmented concrete sealer, sheet vinyl and thinner resin-based systems may be considered for both cost and functional reasons.

Wall systems. Wall systems for critical and primary support areas of fill finish facilities can be composed of site fabricated assemblies (concrete block or metal stud/gypsum board walls with applied coatings), premanufactured assemblies (modular clean room partition systems), or a hybrid of the two.

Wall systems should also be evaluated on the basis of the following additional criteria:

- Expected frequency of reconfiguration/relocation
- Ease of modification (future installation/removal of panels, doors, windows, etc.)
- Design and construction schedule
- Regulations set by building code authorities and recommendations by insurance underwriters (e.g., FM Global)

Coatings, on site-fabricated walls in classified areas, can range from high performance epoxy paint systems to multilayer resin-based systems depending on budget, schedule, and availability of skilled labor.

In controlled, nonclassified CGMP areas, concrete masonry units or metal stud/gypsum board walls are typically specified and finished with high-quality epoxy paint unless there are other factors present that would require more robust systems.

Ceiling systems. Ceiling systems are similar to wall systems in that they may be site-fabricated assemblies (suspended or wall-supported gypsum board with applied finish coating or material), premanufactured assemblies (clean room ceiling system), or a hybrid of the two. In addition to the criteria used for wall systems, the selection of the ceiling system is influenced by

- requirement for walkable ceiling surface
- accessibility needs (to controls and devices located above the ceiling); note: care should be taken to locating access points outside of critical areas

The appropriate ceiling finish material is dependent on the system chosen and includes epoxy paint, PVC rigid seamless sheet, high-build surfacing, or PVC-coated composite panel.

Ceilings in controlled nonclassified areas may be suspended clean room type with gypsum or composite panels, hold-down clips, and gaskets or sealant at panel perimeter.

Doors. Doors should be seamless, sealed, flush, and box-type without recesses.

- Doors in ISO 5, ISO 7, and ISO 8 areas: frames should be installed flush with adjacent wall surfaces; vision panels should be double glazed and flush with door face.
- Fully glazed doors may be used as an alternative in all areas.
- Door hardware shall be heavy duty commercial grade, and hinges shall be sealed, nonparticle generating.
- Swinging doors should typically swing closed in the direction of airflow to maintain sealing at the jamb and minimize air leakage.
- Sliding doors are not recommended in ISO 5 and ISO 7 areas.
- Doors at material airlocks and otherwise accessed for moving equipment and materials are recommended to have automatic operators.
- Powered (electrical or pneumatic) doors shall be appropriate for the level of classification, taking into account cleanability, exposure to sterilizing agents (including fumigants, if applicable), and electrical classification of the room.

Windows

- Windows in classified areas shall be of double pane glazing and flush with the wall finish on both sides.
- Windows between classified and controlled nonclassified areas shall have single pane glazing, be flush with the wall finish on the classified side and may have a sloped sill on the unclassified side.
- Windows in controlled nonclassified areas shall have a single glazing pane; the frame shall be epoxy-painted galvanized steel.
- Exterior window are not recommended in classified areas.

Room fixtures

- All fixtures installed in classified areas must be constructed of materials meeting the same basic criteria for all finish materials.
- All fixtures in classified areas should be designed and fabricated in a manner to minimize joints, ledges, and seams.
- All panels (utility, access) in classified areas shall be installed flush with the wall or ceiling surface.
- Recessed sprinkler heads are to be provided where permitted by building code and underwriters (factory mutual) requirements.
- All penetrations in the wall or ceiling surface in classified spaces must be sealed and gasketed, as required, to prevent air leakage; sealants and gasket materials must meet the same basic criteria for all finish materials.

Table 5 Comparison of FDA, EU, ISO Requirements

Grade	At rest		In operation	
	EU Max permitted number of particles per m^3 (particles per ft^3, former Federal STD 209) [ISO Class]			
	0.5 μm	5.0 μm	0.5 μm	5.0 μm
A	3520 (100) [ISO 5]	20 (Note 1) [<ISO 5]	3520 (100) [ISO 5]	20 (Note 1) [<ISO 5]
B	3520 (100) [ISO 5]	29 (Note 1) [ISO 5]	352,000 (10,000) [ISO 7]	2900 (Note 1) [ISO 7]
C	352,000 (10,000) [ISO 7]	2900 (Note 1) [ISO 7]	3,520,000 (100,000) [ISO 8]	29,000 (Note 1) [ISO 8]
D	3,520,000 (100,000) [ISO 8]	29,000 (Note 1) [ISO 8]	Not defined	Not defined

Note 1: FDA traditionally monitors at 0.5 μm and above. EU also monitors 5.0 μm and above.

Table 6 Comparison of FDA, EU, ISO Classifications

Former US class (particles per ft^3 ≥0.5 μm)	EU "at rest"	EU "in operation"	ISO designation
100	A and B	A	5
10,000	C	B	7
100,000	D	C	8

HVAC SYSTEMS AND REQUIREMENTS

Definition of a Clean Room

A clean room is a room that is designed and operated to control internal particulate levels. To do this usually requires control of appropriate environmental parameters such as temperature, relative humidity, and pressure level. In the pharmaceutical industry, allowable particulate levels are separated into viable (living, i.e., bacteria) and nonviable categories. The remainder of the section focuses on the nonviable category.

Tables 5 and 6 provide an approximate comparison between the EU, ISO, and the now retired Federal Standard 209 (21).

Air Change Rates

Minimum supply air change rates are based on industry general practice and benchmarks. Higher airflow rates may be needed for a given classification when unique operations occur within a room such as open powder–handling operations. In addition, actual airflow rates may need to be higher on the basis of equipment heat load, exhaust rates, and pressurization requirements.

Minimum air changes per hour commonly used in industry are:

Grade A (ISO 5):	0.45 m/sec (90 FPM)
Grade B (ISO 5):	30–40 air changes per hour
Grade C (ISO 8):	20–25 air changes per hour
Grade D (ISO 9):	20–22 air changes per hour
Unclassified:	As required to maintain cooling load, ventilation or pressurization.

Additional considerations: Provide an additional 10 to 15 air changes per hour in airlocks and gowning rooms for the listed grade. The instantaneous particulate gain due to people is generally higher in these small spaces.

Certain applications for ISO 5 (grade A) areas and unidirectional flow hoods may require computational fluid dynamic (CFD) modeling to ensure complete coverage of critical area and acceptable airflow patterns.

Low Wall Returns, Balancing Dampers, and Other Duct Considerations

ISO 5 and ISO 8 areas should have low wall returns located within the room to provide even air distribution throughout the space. To assure adequate airflow patterns, the location of the low wall returns involves a coordinated effort between the process equipment engineer, the architect, and the HVAC engineer.

Low returns should be cleanable and constructed of stainless steel to a point 48 in. above finished floor to allow for cleaning and wash down activities. Design the return grille to be removable and locate 6 to 12 in. above finished floor. Stainless steel type used for return grilles and for low return ducts to be determined on the basis of specific room cleaning requirements and the cleaning agents that will be used. In most cases stainless steel type 304 is adequate.

Always install room return duct and grille even if air balance does not indicate any return air quantity requirement. Pressurization values are estimated via door and various crack calculations. In practice, the calculated pressurization air may be much lower than expected and return air may be required.

Manual volume balancing dampers with double setscrew locking quadrants should be provided for each low air return grille and supply air branch duct. Low air returns within a room combine to a single duct per room where a main room return air balancing damper is to be provided. The main room return air balancing damper must be easily accessible for routine balancing. The accessibility of the other dampers is less critical since they will not be adjusted after start-up but must be available for access during initial balancing.

The room main supply air-balancing damper must be easily accessible. Each terminal HEPA filter should have an integral trim balancing damper in the terminal HEPA filter. This trim damper should be accessible from the process room. Whenever possible, it is recommended to install a damper in the branch duct to each individual terminal HEPA filter.

For areas with tank platforms, low-return grilles should be added at the platform level as well as under the platform to achieve adequate air circulation in support of cleanliness levels.

Ductwork from washer machines or any other equipment that generate moisture should be sloped away from the equipment to a separate moisture collection point and then drained. Consideration should be given to proper duct construction materials and installation methods to ensure liquid tight construction.

Room Pressurization

The space pressure cascade scheme should be from cleanest room to least clean room. The design differential pressure as measured between different classified rooms to be a minimum 10 to 15 Pa (0.04–0.06 in. water gauge, w.g.), with all doors in their normal closed positions. It is also good practice to design for 5 to 15 Pa (0.02–0.06 in. w.g.) pressure differential between areas of same classification with the more critical space at a higher pressure.

When containment is also required, design a combination of a pressure "sink" and pressure "bubble" to achieve containment and cleanliness cascade.

Coordinate with clean room manufacturer supplying walls and doors to evaluate anticipated door cracks and associated leakage values to maintain intended pressure differentials.

Room Temperature and Relative Humidity

Set points for classified spaces (ISO 5 through ISO 9) are typically 68°F with 35% to 50% relative humidity. The normal control tolerance for temperature is ±2°F and ±5% for relative humidity. Relative humidity controls should be arranged to prevent the humidity from falling below the low limit in the winter and from rising above the high limit in the summer. Some

operating companies prefer to maintain ISO 5 spaces at 66°F with 35% to 45% relative humidity to increase comfort to heavily gowned personnel.

Additional considerations: Individual process rooms should have separate thermostatic control due to variation in cooling loads from room to room. Areas of similar use and internal heat gain (i.e., airlocks) can be combined on a single HVAC zone.

Humidification must be controlled to maintain a stable environment. Where room loads are similar and stable, this may be accomplished centrally through a unit-mounted or duct-mounted humidifier. Plant steam may be used for humidification provided that only acceptable boiler water additives are utilized. However, it is recommended to use a humidification source that does not have additional chemical additives.

Steam for humidification must be discussed early in the project to ensure that plant steam is suitable for use and that the system does not require a costly clean steam supply system. Humidifiers must be installed to be easily accessible to perform routine maintenance.

Proper humidifier selection along with proper ductwork design procedures should be followed to ensure humidifiers are located to allow proper steam absorption. Ductwork materials should be selected for a wet application.

It is preferable to locate temperature and humidity sensors in the return and exhaust air ducts where they can be accessed and maintained without requiring entry into the classified space.

It is recommended to maintain a room criteria chart to assure that all stake holders are aligned regarding room design parameters.

Air Filtration Requirements

Air-handling systems serving classified areas should be provided with ASHRAE MERV 8 (formerly 30% ASHRAE) and ASHRAE MERV 14 (formerly ASHRAE 95%) upstream of the heating and cooling coils.

ISO 5 through ISO 8 spaces require terminal HEPA filtration of supply air. This means the HEPA filters are located in the ceiling of the room served.

ISO 9 spaces require HEPA filtration, but the HEPA filters can be centrally located in the air-handling unit (AHU).

Assure the duct material does not shed particulate and is kept clean during construction.

In rooms where potent compounds are handled or if the room has a biosafety rating extract air HEPA filtration may be required.

Terminal HEPA filtration can be substituted for central filters for ISO 9 areas when the system serves ISO 9 spaces and spaces of higher classification.

The HEPA filter installation to provide for:

- Injection of a filter challenge aerosol upstream of the filter.
- Sampling to confirm concentration of upstream filter challenge aerosol.
- Scanning the face of the filter when necessary or taking a downstream air sample at a single point. Generally, HEPA filters serving ISO 5 through ISO 8 spaces must be scanned to prove leaks are not present. Centrally located HEPA filters serving ISO 9 spaces can be tested at a single point downstream of the filter. HEPA filters serving ISO 5 spaces are generally scanned twice a year and HEPA filters serving ISO 8 and 9 spaces are tested at least annually.
- Measuring static pressure drop across the filter.
- Assure the HEPA filter media is accessible to test and replace.

Zoning

AHUs should be dedicated to serve areas of similar use and same classification. Usually, systems should be zoned to reduce the number of AHUs in the facility.

General AHU zoning considerations: Nonclassified spaces should be on a different AHU system than ISO 5 through ISO 9 areas. It is acceptable to place ISO 9 areas on the same system as ISO 5 and ISO 8 areas provided that the ISO 9 area is fitted with terminal HEPA filters. In applications with large ISO 9 supply air requirements, it may be economically advantagious to use a dedicated AHU system for the ISO 9 areas.

Airlocks leading to a process suite should be zoned with the suite.

Nonpathogen and pathogen areas must be served by separate AHU systems. Try to group different pathogen areas according to their biological risk level. It is preferable to zone airlocks leading to an area with a suite with a biological level with the suite even if the air lock does not have a biological rating.

Air from potent, antibiotic, and virus areas should not be circulated to other areas unless a risk analysis proves that HEPA filtration of the return air would be an acceptable strategy.

Try to directly exhaust air from wet areas such as washrooms. It is acceptable to share supply air to these spaces with other spaces.

Special considerations for biological rated areas include:

- Rated area to be negative pressure to surrounding areas with airlocks/vestibules configured for containment.
- Supply/return/exhaust systems to be interlocked to prevent sustained positive pressure in the room.
- must be designed to eliminate any possibility of reversal of airflow upon loss of building power.

HVAC System Testing, Adjusting, and Balancing

The Testing and Balancing Company Requirements

- A member of either the Associated Air Balance Council (AABC) or National Environmental Balancing Bureau (NEBB), or equivalent, certifying organization.
- In good standing with the certifying organization.
- Listed in the latest certifying organization's directory of certified firms.

Instrumentation

- Instrumentation shall be calibrated, including field calibration in same environment being tested to properly perform specified TAB work. Instruments shall be recalibrated and certified by approved test agency every 12 months or less depending on usage. TAB reports shall include type of instruments used and last date of calibration and certification.

Prior to Start of Building Construction

- Report on conditions found that will impede or prevent proper testing, adjusting, and balancing of systems include suggested corrective measures. Report shall also identify additional balancing and measuring devices required in air distribution and piping systems where absolutely essential to system adjusting and balancing. Include locations and sizes of each balancing device.

General Testing, Adjusting, and Balancing

- Test, adjust, and balance supply and specific exhaust systems within plus 10%/minus 5% of air outlet and inlet quantities and water quantities shown on the drawings except where shown or specified otherwise. Air outlets and inlets include diffusers, registers, grilles, laminar flow modules, and terminal air filter modules. Balance return and general exhaust systems to achieve space pressure relationships, not values indicated on the design drawings.
- Permanently mark air distribution and piping system–balancing devices after balancing is complete. Set memory stops where installed.

ELECTRICAL

Electrical installations shall be designed for code compliance by the authority having jurisdiction. This includes domestic and international installations. U.S. applicable standards shall be enforced as a minimum for international locations where relevant standards do not exist.

Impact of Site Location on Design

Consideration shall be given to the differences that exist between locations with regard to locally enforced electrical codes. This is particularly true when transferring an existing design from one geographic location to another. Areas that are impacted include

- equipment-labeling requirements, especially in hazardous area locations
- acceptable raceway methods
- hazardous area classification
- wire identification
- materials of construction

Cord Connected Equipment

Outlets for cord-connected mobile equipment shall have a dedicated purpose. Consideration should be given to the operation of the facility with respect to mobile equipment (e.g., portable pumps or agitators). The quantity of outlets shall be minimized in classified GMP areas. The location of these devices shall be reviewed to assure that they minimize the impact on production personnel and material movement.

Outlets and Enclosures Within Classified GMP Areas

- Surface-mounted receptacles should be weather proof (Nema 4X) and installed in recessed stainless steel box (22).
- Receptacles with corrosion-resistant, spring-loaded gasketed covers suitable for washdown.
- Electrical enclosures, panels, and boxes shall be flush mounted and be constructed of stainless steel. The internal bottom surface of the panel shall be sloped down. All efforts shall be made to minimize the number of enclosures in ISO 5 areas.
- Electrical utility stations housing outlets for process use shall not have doors.
- Locate any code required safety switches outside of the manufacturing space. The safety switch shall be lockable with shunt trip provisions. Locate an approved pushbutton device within the manufacturing space local to the equipment connected to the safety switch. The pushbutton shall de-energize the equipment by activating the shunt trip device in the safety switch.
- If a surface-mounted electrical panel is required, the panel shall be constructed of stainless steel and have a sloped top and internal bottom surface so as not to accumulate dust. The enclosure must be sealed in such a manner to ensure that dust cannot accumulate between the wall and the box.

Lighting Fixture Requirements

- In all cases light fixtures shall be sealed to maintain room pressurization.
- Lighting fixtures may be accessible from above or below.
- Grade A areas shall use clean room fixtures that do not interfere with the laminar flow ventilation yet provide uniform distribution of lighting to minimize shadows.
- Other classified GMP areas shall utilize sealed lay-in fluorescent fixtures with clean room type stainless steel covers and smooth lenses for ceilings no more than 12 feet above finished floor. Fixture covers shall be attached with wire to protect from falling to the floor when opened.
- For higher ceilings, consider pendent-mounted metal halide fixtures suspended above clean room ceiling. Clean room type stainless steel frames and smooth lenses shall be provided to permit light into room. Maintenance access must be considered with this installation. If lamps are to be serviced from within the room, these lens covers shall be attached to the fixed frame with wire to protect from falling to the floor when opened.
- If perimeter wall–mounted lighting is required, utilize wall-mounted fluorescent fixtures. Avoid horizontal surfaces created on the top of these fixtures such that dust and dirt will not accumulate.

Illumination

The quality of illumination is a critical component of a safe and efficient manufacturing area work environment. Furthermore, adequate illumination contributes significantly to the minimization of errors attributable to misreading labels, controls, gauges, etc.

- Illumination levels shall be sufficient for each task. Generally speaking, 60 to 75 foot candles (fc, or 600–750 lux) is adequate for classified areas; however, locally higher levels may be required in areas of inspection and label reading. Note that certain jurisdictions require enhanced illumination requirements such as natural light for quality of worker environment and/or improved efficiency. It is the responsibility of the design engineer to familiarize themselves with these local requirements.
- Provide even light distribution and shadow reduction while minimizing the number of fixtures to facilitate the cleaning of the room.
- Light switches shall not be installed in ISO 5 areas unless required by a specific process criteria.

Hazardous (Classified) Areas

It should be recognized that electrical equipment labeled for use in hazardous areas is, generally speaking, less compatible with the cleanability requirements of classified GMP areas. It is recommended to minimize or reduce hazardous areas within cGMP manufacturing spaces. Where hazardous environments cannot be avoided, use materials and installation methods approved for the environment.

Access Control

Card readers shall be required on the gowning airlocks providing ingress from controlled unclassified to grade C and from grade C to grade B. No card readers shall be provided for the reverse direction.

- Self-actuated doors shall be held open for a preset minimum time and initiated by a motion sensor. Optical sensors shall not be used to detect motion as a static object, such as a cart left in the doorway, will prohibit the door from closing.
- Airlock engineering controls (interlocks) shall be provided for all gowning, degowning, and material airlocks. At a minimum, this interlock should monitor the correct logic, which is only one door of an airlock open at any time. If proper logic is not followed, a significantly audible alert tone shall initiate for a predetermined amount of time. This tone is intended to alert supervision of a violation of SOP.

Emergency and UPS Power

Electrical power is a critical utility for sterile process and product manufacturing sites. Therefore, the power system reliability must be studied to determine appropriate system designs for these facilities.

Product loss prevention is directly proportional to electrical power reliability. Many levels of system redundancy and reliability may be built into the electrical distribution system. Power distribution system costs must be weighed against product value to determine the appropriate design. Table 7 identifies a three-level guideline for identifying power system designs according to product loss prevention.

Means to prevent loss of electricity to critical and specialized systems associated with the process should be evaluated. For example, equipment shall be considered for stand-by power if power loss to the equipment will cause the following to occur:

- Product loss
- Reduced product production
- Equipment damage
- Increased personnel exposure risk

Table 7 Three-Level Power Distribution System

Reliability level	Product value	Normal power system design	UPS/stand-by power system design
Level 1	Low—shutdown acceptable	No redundancy required. Simple radial system design is acceptable	Life safety systems shall meet applicable code requirements
Level 2	Moderate—shutdown acceptable	Looped primary, primary and secondary selective system design recommended	Life safety systems, stand-by generation system required for selected equipment
Level 3	High—shutdown not acceptable	Primary and secondary selective system design recommended	Life safety systems, stand-by generation system, and uninterruptible power system required for selected equipment

SYSTEM INSTALLATION

Automation, Instrumentation, and Controls

The process control systems will be designed for safe and efficient monitoring and operation of the manufacturing, quality, and facility processes. Automated, semi-automated, and manual controls shall be implemented where necessary to achieve this goal.

The level of automation will be defined for each manufacturing, quality, and facility process. On the basis of the required level of automation and the type of process, several control system architectures can be used, including:

- Programmable logic controller (PLC)/human-machine interface (HMI)
- PLC/supervisory control and data acquisition (SCADA)
- Distributed control system (DCS)

The process control system shall provide for recipe control of the unit operations associated with the formulation area. The process control system shall provide the necessary regulatory, sequential, and batch controls to perform equipment functions such as liquid charging, solids charging, mixing, heating/cooling, purging, inerting, CIP, SIP, and transferring of materials. The process control system shall provide means to alarm the operator for abnormal conditions and provide the necessary safety interlocks.

Although not mandatory, the use of an electronic batch record (EBR) system is preferred. All equipment and systems must be capable of transferring data and communicating with an EBR system, regardless of whether the use of an EBR system is an immediate requirement or a future consideration. If an EBR system is used, the system shall provide a printed report of the formulation operations on a per batch basis.

All process control systems that operate a cGMP equipment or process will be connected to a Data Historian. Quality data to support the EBR will be collected at the Data Historian.

Control Systems Hardware and Network Design

All process control systems shall provide adequate alarming and alarm log capabilities to meet all GMP requirements.

All process control systems shall provide interlocking capabilities to protect the people, equipment, and process.

All process control systems shall provide event logging capabilities to meet all GMP requirements.

Where required, process control systems shall provide continuous and historical trending of operating parameters.

Process control systems operating on cGMP equipment and processes shall provide a stand-alone report to support compliance or operational requirements. Wherever possible, report shall use a common reporting format.

All process control systems shall provide the necessary operator interface to monitor and operate the equipment in an efficient and safe manner. Where appropriate, operating parameter with the appropriate security access shall be made available to the operator for manipulation.

All process control systems shall provide the necessary hardware to communicate to an Ethernet manufacturing system network.

Filling Line Control

The filling line will operate as "island(s) of automation" by which the control of each major equipment component shall operate as stand-alone system not requiring information or communication to operate.

The line components control systems should be capable of operating in both a run mode and a maintenance mode.

All filling line components shall be designed such that a SCADA data collection and monitoring could be implemented at a later date from a control level above the individual controllers.

As a minimum all filling equipment controls shall contain the ability to keep count of consumed components and work-in-progress inventory to facilitate integration with an enterprise resource planning production management system.

Process Analytical Technology

Gains in quality, safety, and/or efficiency from the application of process analytical technology (PAT) will vary depending on the product but are likely to come from

- Reducing production cycle times by using on-, in-, and/or at-line evaluations and controls.
- Minimizing the risk of rejects, scrap, and reprocessing.
- Considering the possibility of near-real-time release.
- Increasing automation to improve operator safety and reduce human error.
- Facilitating continuous processing to improve efficiency and manage variability

  -Using small-scale equipment to eliminate or minimize certain scale-up issues and dedicated manufacturing facilities to minimize setup, changeover, and cleaning disruptions.

  -Improving energy and material use and increasing throughput.

The application of PAT is project specific and shall be considered on a project basis. As an example, Pat should be evaluated for the following parameters for filling lines:

- Temperature
- Speed
- Pressure
- Flow rate
- Weight
- Number of rejects

Piping Design

All product-contact piping shall be constructed of hygienic, orbitally welded stainless steel piping and tubing. Electropolishing is not required. The piping system shall be passivated after installation. The materials and installation shall meet ASME BPE standards for high purity piping, including absence of dead legs, the use of sanitary connections, material traceability, welding techniques, and documentation.

Joints in the piping system, including valves, shall be welded whenever possible. When welding is not possible or practical (e.g., connections to equipment or instrumentation), sanitary clamp fittings shall be used with bolted clamps for increased integrity and safety. The use of threaded joints is not permitted.

Proper space should be provided to operate, clean, and maintain equipment. All nonessential devices associated with the formulation process should be removed from the formulation room, and located in a technical chase, where they can be routinely inspected. Piping penetrations from the chase into the clean room should be grouped into a single stainless steel wall panel for rigidity and cleanliness.

Because the entire system is steam sanitized and clean steam condensate is highly corrosive, drain piping shall be constructed of orbitally welded stainless steel tubing. The condensate collection system shall be passivated after installation. Flanged connections are permitted for condensate collection.

All product-contact piping, clean steam distribution lines, and clean steam condensate collection piping shall be insulated and sheathed for personnel safety.

Isolation Valves

Diaphragm-type valves are specifically preferred for bioprocessing fluid applications. Valves will be designed so that complete drainage of fluid from inlet to outlet is optimized when mounted in the position specified by the manufacturer. All valves shall be capable of being fully opened or exposed during CIP/SIP.

Pressure Safety Valves and Rupture Discs

Rupture discs on pressure vessels should be installed as close as possible to the system's highest point; however installation shall comply with a length/diameter (L/D) ratio of 2:1 or less. The cleaning system design should ensure that the rupture disc will not be damaged by cleaning media impact.

Steam Traps

Steam traps installed on process systems shall be capable of effectively venting air. Traps shall be sized and installed such that there is no backup of condensate into the process equipment. For these reasons, balanced pressure thermostatic steam traps are preferred. The advantages of using a thermostatic steam trap include complete drainability and the ability to remove noncondensable gases (such as air during start-up) at a high flow rate.

Steam traps in process systems shall be maintainable to allow easy inspection and cleaning. Bolted sanitary clamps should be used for steam trap installation to allow removal for maintenance or replacement.

Materials of Construction

All components in contact with the in-process materials shall be 316L stainless steel, with an internal surface finish of 25 μmRa max. Electropolishing is recommended, but not required. 304L stainless steel is recommended for other components including the equipment supports, skid framing, and paneling.

Equipment Details

Heat Exchangers

The use of heat exchangers in direct product contact applications should be avoided because of the product losses that could ensue. Heat exchangers are typically used in temperature control modules, which provide heating and cooling of noncontact heat transfer fluid used in formulation tank jackets. They are also used to provide cooling of WFI, either in the formulation area WFI sub-loop or at the WFI use point.

All heat exchangers shall be shell and tube type exchangers. Heat exchangers shall be of a sanitary design and construction, including use of double tube sheets, and shall be designed in accordance with the current edition of the ASME Boiler and Pressure Vessel Code and the ASME BPE standards.

Transfer Panels

Transfer panels provide a method of connecting multiple fluid paths, without costly divert valves, automation, and cleaning requirements. The use of transfer panels also accomplishes

complete physical separation of a system with product from cleaning fluids or steam, preventing any concerns of cross-contamination.

Transfer panels shall be self-draining and pitched to a draining point. If the design of the panel is such that it is not physically possible to completely self-drain, then a tray shall be positioned under the panel to collect any material leakage as jumpers are disconnected. The tray, if required, shall be sloped to a drain line equipped with a shut-off valve.

The number of jumper sizes should be minimized to provide the most possible combinations of connections. Conversely, jumpers of different sizes may purposely be used to avoid accidental, undesired connections. The simultaneous crossing of multiple jumpers should be avoided.

Proximity switches shall be provided for each possible jumper position. The automation system can then be used to verify the proper set-up of connections and provide interlocks to prevent loss of containment from open-ended fluid pathways.

The transfer panel or piping system design should provide a means to verify the release of line pressure prior to manually disconnecting a jumper. This can be achieved either though the automation system with in-line pressure indicating transmitters or by physically mounting pressure indicators within the operator's view on the panel or jumper.

Filters

Filter housings shall be designed to allow for complete venting and draining. Liquid tee-type filter housings should be installed vertically and vent type in-line filter housings should be installed vertically with the condensate/drain port directed downward. All nozzle connections should be of a hygienic design.

Vent filters for hot process services should be heat traced or steam jacketed to prevent the accumulation of moisture in the vent filter.

SUMMARY

Recently, the trend has been to simplify the complexity of the facility, by requiring additional sterility assurance from the equipment. Use of isolation technology or blow-fill-seal permit more simple facilities to be constructed, as fewer aseptic operations are conducted.

As demonstrated by the preceding chapter, many factors influence the design of a sterile facility. The challenge for any design is to blend the needs for the known processes, select a technology platform, meet known regulatory requirements, and then add the proper level of preinvestment for facility flexibility that may be required to meet future changes in technology and regulations.

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2 Personnel and their impact on clean room operations

Jeanne Moldenhauer

INTRODUCTION

In pharmaceutical environments, the presence of contamination is of key concern to the quality of the product. In addition to the presence of contamination, some contaminants are more serious than others depending on the type of product being manufactured. There are a variety of potential sources for contamination in the clean room; for example, the supplies used, transport of the supplies into or out of the area, the utilities used in the manufacturing process, the ingredients used, and the personnel operating in the area. It is critical to use appropriate contamination control procedures to ensure that the final product is safe and effective for its final use.

Pharmaceutical clean rooms may be used to manufacture products for both human and veterinary use. It is very important in these environments to prevent or minimize the risk of contamination. In an aseptic environment, using current technology, it is widely believed and accepted that the involvement of humans in the process is the greatest risk to the sterility of the product (1). The Food and Drug Administration (FDA) indicated that the presence and activity of personnel in manufacturing areas where sterile dosage forms are manufactured, should be considered risk areas in an aseptic process and are necessary components of a process simulation evaluation to ensure that they are not adversely impacting the product manufacture (2).

The risks associated with the presence of contamination vary depending on the type of pharmaceutical product being manufactured; for example, some types of contamination may be allowed for nonsterile products providing they are within specified acceptance criteria. Other products labeled sterile may have more stringent requirements for the allowable levels of contamination, as well as the steps in the manufacturing process where the contamination may be present. A product that is terminally sterilized, meaning that after filling and sealing the product it is subjected to a sterilization cycle in its final container, may allow more contamination throughout the process than one that is aseptically filled. Aseptically filled products are manufactured from presterilized components that are handled aseptically to fill and seal. Since these types of products are not subjected to a final sterilization cycle, the risk of contaminating the product in the event that contamination is present is higher.

It is also widely accepted that the people working in clean room environments are the greatest source of contamination (3). The personnel present contribute contamination to the area by releasing or shedding of viable and nonviable particulates in the area. This happens in varying amounts depending on the personnel activity in the area.

In controlling contamination, one of the key axioms is that you can not contaminate the area if you don't bring contaminants into the room. As such, controlling those operations that can contribute contamination to the area are critical. Since people are a significant source of contamination, it is important to control their activities. Controls should be designed and established that include requirements for hiring new employees, training, monitoring during the working periods, and continuing until the person is no longer working in the area. These controls should include appropriate supervisory observation, testing, and programs to motivate the personnel to correctly perform their assigned activities.

In those cases where contamination events have occurred in manufacturing environments that exceed established allowable limits, one of the common tests that occur is the identification of the microorganism. It is common to use sources like *Bergey's Manual*, which is like an encyclopedia and dictionary of microorganisms, to determine the origin of the type of contamination. The great majority of all organisms found are stated to be human borne. As such, the focus of many investigations does not include the impact the personnel may have on other equipment and items within the clean room.

This chapter describes some of the personnel-related sources of contamination in the manufacturing environment, the impact of these types of contamination, and the types of measures that can be established to control these processes. While other sources of contamination are important, they are not discussed in this chapter as many are discussed elsewhere in this text.

WHAT IS CLEAN?

There is no one set definition of clean when it comes to clean rooms. In fact, the attributes that may make a room clean could also be the same factors that make it dirty for another attribute. As such, the term clean is relative and can be associated with the specifications of the associated products. For example, you can classify on the basis of various criteria such as chemical contamination, bacterial contamination, nonviable particulates. Looking with the naked eye, a room may appear to be free of particles since we can only see contaminants down to a size of about 25 μm (assuming a strong beam of light is present). This size is about a quarter of the diameter of a piece of hair, which on average is about 100 μm in diameter (4). In reality, there may be a wealth of particles that are present; they just were not detectable with the naked eye.

There are various regulatory and industry guidelines that provide standards for the classification of clean rooms; that is, they describe the requirements that must be met to be "clean" to a specified level. Clean rooms are used for a variety of purposes including both electronics and pharmaceuticals for example. There are specific requirements specified in several documents, with one of the most commonly used designations in the International Standards Organization (ISO) Guidance 14644-1 (5). This guidance describes air cleanliness classifications in terms of the concentration of airborne particles present. The range of particle sizes considered in this evaluation is from 0.1 to 5 μm. It does not look at classification in terms of physical, chemical, or viability of the airborne particles. The classifications in this system are whole numbers starting with 1 and going through 9, with 1 being the cleanest (Fig. 1).

Another commonly used classification system is described in the European GMPs Annex 1 (6). This system uses a letter designation (A through D), with grade A being the cleanest. In the FDA's aseptic guidance, the ISO classification system is supplemented with requirements for microbiological control (2).

TYPES OF CONTAMINATION

Many different items can cause contamination in a clean room, for example, microorganisms, viruses, dirt (soil), organic matter, animal excrement, pollen, and so forth. In addition, there are man-made contaminants such as tobacco smoke, unburned hydrocarbons, fly ash, dust from things such as construction, engine exhausts, and the like. During manufacturing operations, one can generate contaminants through the garments being worn, the packaging, and other similar operations. People themselves are also a source of contamination. They can shed particles such as skin flakes, dandruff, cosmetics, hair, and fibers. Regardless of the type of contaminant generated, it can contribute to undesirable conditions in a clean room (4). The discussion for this chapter is limited to those contaminants that are or can be emitted from personnel.

Airborne Particulate Cleanliness Classes From ISO 14644-1						
ISO Class	0.1 μm	0.2 μm	0.3 μm	0.5 μm	1 μm	5 μm
1	10	2				
2	100	24	10	4		
3	1 000	237	102	35	8	
4	10 000	2 370	1 020	352	83	
5	100 000	23 700	1 020	3 520	832	29
6	1 000 000	237 000	102 000	35 200	8 320	293
7				352 000	83 200	2 930
8				3 520 000	832 000	29 300
9				35 200 000	8 320 000	293 000

Figure 1 ISO classification scheme.

Two types of particulates may be shed into the environment by the personnel working in an area, viable microorganisms, and nonviable particulates. Some of the types of particulates shed by humans include skin cells and flakes; human hair; moisture droplets from sweat, breathing, and speaking; cosmetics (make-up, hair spray, and deodorants); lint; starch and other particles from fabrics; and dirt flakes. These particles can be dispersed into the air. Once in the air they can either stay in the air, or land on other items and surfaces in the area. Test data indicates that just conducting normal activities, a person could release several hundred colony forming units (CFUs) per hour, even wearing clean clothing (4).

Typically, people give off one outermost layer of epithelial cells every 24 hours, or about 10^9 skin cells per day. Skin cells average 20 μm in size. Approximately 5% to 10% of the cells are less than 10 μm in size. A portion of these cells is released into the environment and acts as a carrier or a raft for viable microorganisms. These cells may be whole or fragmented. The amount of airborne dispersion varies for each person, for the activities being performed, and over time. However, it is commonly stated that people disperse approximately 1000 cells per minute that are carriers of viable contamination. Comfort levels (e.g., temperature and humidity) for personnel in the clean room also contribute to the amount of airborne dispersion. For example, when the temperature is elevated, some people become more uncomfortable and may perspire. The more they perspire, the more likely they are to more airborne dispersion (7).

In addition to skin fragments, people tend to carry contamination on their clothing and shoes. Shoes and clothing that are worn both inside and outside of the building carry larger populations of microorganisms. This is true for shoes and clothing used in different room classifications as well (7).

Activities conducted outside of the clean room can also contribute to contaminants being brought into the room. Smoking tobacco products can result in tobacco smoke being detected in exhaled air for hours after the smoking occurred. Medicines that have been taken can be found in the skin and hair fragments for days after use. Washing and gowning activities are not always sufficient in totally removing cosmetics that have been used. As such, these external activities can also contribute to the contaminants present (4).

The following sections describe the types and levels of contamination in more detail.

Nonviable Particulate Contamination

Analysis of the particles present on a person is useful in describing the living conditions and habits of the individual. For example, various contaminants can be deposited on a person during the course of a day. Our everyday operations are not conducted in sterile environments. If you have a pet, there is probably pet dander or other contaminants that are present. Walking around generates millions of particles into the environment. These particles are deposited on other surfaces in the surrounding areas, including other people. Walking itself causes particles that are on the floor to be aerosolized (4). Many of the determinations of forensic scientists are made on the basis of the particulates left on people.

The shedding of particulates gets more complicated because all employees do not shed particulates at the same rate. The rate can be affected by a variety of factors, the activities being conducted, the behavior of the operator, and even just the personnel themselves. For example, some individuals sweat profusely and shed many more particulates than someone who does not sweat at all. Rates at which particulates are shed are typically reported as particles per minute.

Cosmetics are a concern for generation of nonviable particulates. The base of most cosmetics is ground talc. It is used in after shave as well as in the foundation makeup used. When the cheek muscles move, while speaking or coughing, the talc falls off the surface of the skin. Since skin also harbors bacteria, they are frequently attached to the cosmetic material (4). In addition to cosmetics, newer trends like the use of nail wraps and nail extensions have the potential to generate additional levels of contamination.

Regulators have been concerned with particulate levels for many years, as the values could be collected in real time. Since it has historically been impossible to collect viable microbial counts in real time, the particulate level was indicative of the number of total viable microorganisms present in the environment and this provided information on the quality of product currently being manufactured (8).

Data was generated by several different scientists that also gave weight to the measuring of particulate counts for assessing potential contaminants. Scientific studies conducted by Whyte indicated that particles less than 11 μm in size could not contain viable contaminants because of the desiccating nature of the dry clean room environment. Ljungqvist and Reinmuller generated compelling data to show a correlation between particulates in the 10 to 20 μm size and the risk of viable microorganisms as a risk to the manufacturing process (8). The data generated in these studies strengthened the belief that controlling particulate levels can aid in the control of viable microorganisms in an area.

Contamination with Viable Microorganisms

Skin surfaces give off squamous cells that can serve as sources of contamination for the pharmaceutical environment. This contamination can be composed of both viable and nonviable particulates. The particulates are unique to each individual and they may or may not include viable microorganisms. When the particulates include viable microorganisms they can be a source of contamination in the area.

The contaminants present on humans can also be transferred to the surfaces they touch or with which they come into contact. It is important that the procedures used in the manufacturing operation are able to eliminate or greatly reduce the contamination that has the likelihood to affect the product quality. Touching the surface however is not the only potential source of contamination. Actions such as sneezing, coughing, exhaling, perspiring are also potential sources. Even the dust particles present can carry viable microorganisms (4).

When viable microorganisms are deposited onto surfaces they can replicate if nutrients are available and they are not disturbed. Given sufficient time, the organisms can be emitted from this site for long periods of time. While much concern is given to the single cell present in an aseptic environment, microbes seldom exist in a clean room environment as a single cell. Frequently, they are found riding on dust particles or water droplets. While a bacterial cell may be about 1 to 3 μm in size, the larger dust particle may range 15 to 20 μm (4).

The viable contaminants present in a clean room can be a significant concern. It is possible for them to grow on and in the product being manufactured. Particles can even spread through liquids and powders being used or manufactured in the process. As such, surfaces where particles may have landed should be routinely cleaned and sanitized or sterilized to reduce the risk of subsequent contamination. Since a single microbial cell can replicate itself in about 20 minutes, doubling the initial population, it only takes 10 hours to generate a million cells (4).

Speaking and coughing in the clean room can result in large numbers of microorganisms being released into the room. Use of consonants when speaking generates a pulse sound that results in emission and ejection of material from the back of the mouth. It could be considered a spitting function. If the individual is not wearing a mask or wearing a mask incorrectly, this person is literally spitting into the area (4). Even wearing a mask correctly does not ensure that contamination is prevented. Once the mask gets wet, it only takes about 20 minutes for bacteria to be able to traverse the wet mask and get released into the area. Coughing is described as a mini hurricane by Dr Munzer, president of the American Lung Association (in *USA Today* October 4, 1993). He indicated that a cough generates a 125 mph blast, intended to eliminate an intruder from the lungs (4).

Personnel generate moisture droplets that may contain microorganisms from other activities such as yawning, coughing, sneezing, speaking, and shouting. Coughing may generate 600,000 droplets, while sneezing doubles that amount. The droplets generated from speaking vary by person and activity (4).

The risk of contamination increases if the person is not healthy, or has open or exposed skin wounds.

While traditionally nonviable particulate monitoring has been used as a real-time indirect measure of the level of viable microorganisms in the area, newer technologies have been developed to allow for real-time or near-real-time detection of viable microorganisms. The ScanRDI system (aka ChemScan in Europe) manufactured by AES-Chemunex is able to be used for air (Sampl'air method), surface (ChemSwab), and product monitoring. Surface monitoring methods can also be used for personnel monitoring. With this test methodology one can determine if viable microorganisms are present within a few hours (90 minutes to 4 hours,

depending on the type of test method selected). This system allows for detection of cells without any requirement for the cells to grow prior to detection (9).

The new SMA air sampler, manufactured by Veltek Associates, Inc., allows for the collection of a particulate count and an option to collect viable microorganisms in either a liquid solution or on an agar plate. The liquid collection option allows for the sample to be evaluated with a variety of liquid-based rapid microbiological methods for analysis (10).

Another useful tool for microbial detection has been the IMD-200-1 and the IMD-220-4 manufactured by BioVigilant, Inc. This technology allows for instantaneous microbial detection (real time). It looks like a particle counting probe and uses optical detection and sizing coupled with riboflavin metabolism to determine whether microorganisms are present and how many are present. Using this technology, counts can be made that directly compare the number of viable and nonviable microorganisms that are present. Additionally, because of the sizing, it is also possible to use this instrument to distinguish the number of molds present (separate from the other microorganisms). This system can be used to determine the level of contamination present. It also can aid in studying sources of contamination in an investigation. For example, in a facility with mold contaminants it is typical to spend weeks trying to find the source of the contamination. Using this unit, one can frequently determine the source(s) of contamination quickly. In the area of personnel, this type of system can be useful in training of clean room personnel. As an example, one might monitor the area near a specific individual while they are performing different operations to determine which method of performing an activity has the lowest risk of subsequent contamination. Another use is to evaluate the effectiveness of the gowning procedure. These are just a few ways in which newer technologies can be used to reduce the overall risks of product contamination (11).

The Rapid Enumerated Bioidentification System (REBS) was developed by Battelle. The technology is based on Raman optical spectroscopy. It provides for the detection, identification, and enumeration of particulates and microbial materials without the need for expensive reagents or consumables. The system provides results in approximately 15 minutes. (The timing is targeted to process at a rate of 5 minutes $\pm$ 20 seconds per particle detected.) This system has several features that make it attractive for monitoring for contamination, whether it is in a dry or liquid sample. Some of these features include (12):

- The ability to detect, identify, and enumerate particles that are larger than 300 nm in diameter.
- The consumables are less than $10/sample.
- Ability to identify molds, yeasts, fungi, gram-positive and gram-negative cells.
- The ability for single-cell, single-particle detection.
- There is minimal sample preparation required.
- Staining is required, only if there is a need to determine if the cell is viable. There are no steps for amplification or lysing required.
- The system is nondestructive, allowing for additional identification systems (like nucleic acid methods) to be used, if necessary.

With the availability and implementation of some of these newer systems, our knowledge base on the types and amounts of contamination present in our clean room environments may increase dramatically. They also aid in identifying and remeditating contamination events in a timely fashion.

PERSONNEL CHARACTERISTICS

The personnel present in the clean room can have a significant impact on the contamination present in the environment. The amount of contamination that can be contributed is affected by many things including (13): the amount of microbes present on the skin, those present in the person, the types of microbes present, where they are located, and how they are dispersed (13).

Microbial Load

The amount of microbial load present on a person's skin varies across time. It is significantly influenced by the personal hygiene habits of the individual. The level initially present on the

person increases as the person is confined in an area, where other personnel are not able to practice their hygiene habits. Additionally, the organisms are not evenly distributed on the body surface. Rather than existing as single cells, microbes are more likely to grow and be in clumps of cells (micro or macro colonies) on the skin surface. To be visible to the naked eye, there must be greater than 10^6 cells/mL. The skin microcolonies are typically in the range of 10^2 to 10^5 cells (13).

Types of Microorganisms

The predominant types of microorganisms present as contaminants in pharmaceutical products are bacteria and fungi. While viruses may be present, they are typically part of biological products and since they are obligate parasites they do not replicate outside of a cell. The bacteria present are either gram negative or gram positive. Gram reactions are a type of differential stain that provides information regarding the type of cell wall structure. Most of the gram-negative cultures in pharmaceutical applications are bacilli (rod shaped), while most of the gram-positives are bacilli (rod shaped) or cocci (sphere shaped) (13).

In contamination events, *Propionibacterium acnes* is a frequent gram-negative contaminant. Other typical gram-negative contaminants are water contaminants rather than human borne. For gram-positive contaminants, various forms of *Bacillus* are common as are staphylococci, micrococci, and streptococci, the bulk of which are human borne. In the production of biological products, many of the microbes recovered show resistance to antibiotics (usually the ones used in the production process), for example, Gentamicin-resistant *Bacillus cereus*.

The fungi are divided into yeasts and molds. *Candida albicans* and other species are frequent yeast contaminants and can be human borne. Most of the molds in the clean room come from a variety of species and may occasionally be human borne, but most often are a result of poor cleaning in the area.

Body Areas Shedding Organisms

Figure 2 describes the relative amounts of surface area found in the body.

The body surface is the source of most of the microorganisms shed by humans. While each person maintains a flora unique to himself/herself, there are specific organisms associated with different areas of the body. The amount of microorganism shed from the human into the environment is dependent on specific factors including the amount of microorganisms present on the person and how active the person is, including which body parts are encompassed in the activity (13).

The contaminants given off from the skin, nose, ear, mouth, respiratory track, and intestinal tract tend to be viable microbes. Diseased skin, as seen with eczema and psoriasis, show an increased level of *Staphylococcus aureus* and *Streptococcus pyogenes* as shed organisms. When individuals showing these diseases are working in the clean room, shedding these pathogenic organisms can be a significant risk (13). Another common skin contaminant is *Propionobacter acnes*.

Pathogenic cocci are frequently found in the nose and ear, as are diplococci and *Haemophilus*. Other microorganisms are commonly found in the oral cavity, such as *Streptococcus salivarius*, *Lactobacillus* spp., and *Candida albicans*. The contamination levels present in saliva are about 10^8 microbes/mL. Organisms originating in the intestinal tract include anaerobic, nonsporulating rods (putrefactive bacteria) and gram-positive lactobacilli. While aerobic microorganisms can be present, they tend to be much fewer. When present, typical organisms are coliforms, *Proteus*, enterococci, and staphylococci (13).

Portion of Body	Relative Percentage of Body Surface Area
Arm, Left	9
Arm, Right	9
Axilla	<2
Head	9
Leg, Left	18
Leg, Right	18
Perineum	<2
Trunk	37

Figure 2 Relative amounts of body surface area. *Source*: Adapted from Ref. 13.

Individuals having diseases that result in excessive bacterial oral and nasal discharges can emit these organisms when they cough, sneeze, or blow their noses. Having this type of individual in a clean room with horizontal laminar flow is a greater threat of contamination than having a vertical laminar air flow system, due to the high velocity of material released during sneezing. If horizontal laminar air flow is used, a barrier can be an effective method of eliminating the potential for contamination (13).

Differences in Shedding Rates (Male Versus Female)

Data generated in studies of shedding rates indicated that males shed microorganisms at a much higher rate (approximately 2500 microbes/contact plate) than corresponding females (approximately 700 microbes/contact plate). Other studies conducted did not show this level of difference in shed rates (13). As new individuals are added to clean room staff, environmental data should be trended and evaluated for potential shedder employees.

The Carrier Concept

Individuals carry specific microorganisms on their skin surface. Depending on the changes to which the microbes are subjected; it is possible for them to undergo growth and colonization, different from or exceeding the normal flora present. When this happens, the person is described as a carrier. This term was defined by McDade as "an individual in or on whom pathogens reside and multiply, without producing demonstrable disease or ill effect on him." Carriers are classified as temporary and permanent. Temporary carriers only carry the microbes for a short period of time, while permanent carriers carry the microbes for long periods of time. Another term used to describe these types of individuals is shedders or disseminators. Many people may be described as carriers, but few are considered to be disseminators. The importance, however, is that they can have a significant impact on the environment (13).

SELECTION OF CLEAN ROOM PERSONNEL

The operations conducted by clean room personnel are so important and critical to the overall operation. As such, great care should be taken to ensure that the personnel selected do not inherently adversely affect the environment as well as providing appropriate training on the acceptable behavior to use in the manufacturing environment.

There are four key factors in selecting personnel for clean room operations: physical requirements, skills, job performance, and psychological characteristics (13).

Characteristics of Clean Room Employees

Individuals that have been selected to work in a clean room environment should be neat and clean. It is important that their hygiene habits will minimize rather than maximize the risk of contamination of the environment. Even their hair is an issue, as it can contribute contamination to the environment. As such, it should be kept clean and dry during the operation. Excessive dandruff or skin flaking also has the potential for significant adverse impact on the environment. Hair issues also apply to males with facial hair (13).

In some companies, evaluation of clean room personnel can include evaluation of the individual's personal health, degree or dry skin/shedding, determinations of whether skin diseases are present which may increase the risk of shedding (like eczema) whether the person has asthma, emphysema, chronic obstructive pulmonary disorders, or other diseases/allergies that cause the individual to breathe through their mouth, cough, or sneeze (13).

The ideal workers in a clean room operation have several common characteristics. Some of these characteristics are as follows:

- Specified level of education (typically at least completion of high school)
- Good manual dexterity
- Good personnel hygiene
- High level of attention to detail (so that they will carefully think through how to perform each activity, and once instructed they will always work in the same way)
- Have a basic understanding of the need to work in a specific way at all times
- Have a basic understanding of the work to be performed.

- Recognize that following the "rules" of behavior is critical for safe manufacture of the product
- Do not shed or give off high levels of particulates (This may be uncontrollable on their part, but it may also make it inappropriate for them to work in this type of area. For example, excessive perspiration or excessive dandruff or flaking of skin could routinely lead to higher than expected counts.)

The clean room is an inappropriate place to select workers only on the basis of their seniority in the company. While satisfactory performance of one's job is important, so is the concern of cleanliness in performing these tasks. They need to be aware of the risk of contamination and how they can avoid or minimize these risks (13).

The details and work required to operate in a clean room environment can be stressful to the point of making some workers unsuitable for this type of practice. Continued violations of procedures by clean room personnel can contaminate the area and significantly impact the quality of the products produced (13).

Studies conducted by psychologists indicate that good workers have emotionally stable characteristics; average active and social characteristics; and low scores in impulsive, dominant, and reflective characteristics. It is good for them to have even dispositions. Very nervous or emotional personal do not do well in clean room environments (13).

There are some other traits that also are important for clean room personnel including (13):

- They are highly motivated.
- They take pride in performing a good job.
- They have an above average attitude about their job.
- They are willing to endure the inconveniences of working in a clean room, like the aseptic behaviors and gowning that must be performed.
- They maintain cleanliness.
- They are conscientious.
- They want to manufacture a quality product.
- They are orderly and reliable.
- The concept of repetitive operations does not cause them mental stress.
- They pay attention to details.
- They are punctual.
- They are good listeners.
- They are truthful!
- They have a sense of duty, that is, they know what they should do and the importance of performing these tasks as directed.

Working together and having a sense of pride of accomplishment makes for a good operation in the clean room (13).

PERSONNEL FACTORS REQUIRED TO CONTROL CONTAMINATION

There are many reasons that contamination risks should be controlled in the clean room environment including maintaining product sterility (for sterile products), maintaining the allowable microbial level (for nonsterile products), and preventing pyrogenicity (for products purported to be nonpyrogenic).

Since personnel have a high risk of contaminating the product and process, clean room clothing is used to protect the environment from human-borne contamination. Airborne microorganisms are normally dispersed into the clean room from people on skin cells. Properly designed and used clean room clothing will cover or envelope the person and minimize or eliminate the dispersion of contaminants into the clean room environment. Some clean room clothing may eliminate the dispersion of contaminants, for example, a sealed, water-proof surface, but may be so retentive that the person wearing the clothing becomes overheated and extremely uncomfortable. Being uncomfortable in the clean room can result in poor clean room behavior and subsequent contamination.

The fabrics used for clean room clothing should be tested for various properties, for example, air permeability, the retention of particles, the generation of particles, and the pore size. Other considerations for clothing include the durability of the fabric (e.g., how prone the material is to tearing), effects of aging, washing, drying, sterilization, and flexibility (14–16).

Some companies have shoes that are dedicated to use only when within the manufacturing facility. Another approach is to have shoes dedicated to areas with specific room classifications. A more common approach is to have dedicated shoes for the class 100/grade A/ISO 8 areas and to use shoe covers for the shoes used in other areas. For companies that allow the employees to go outside, for example, smoking, or walking between buildings; special precautions should be taken to ensure that shoes and clothing are appropriately protected during these excursions.

Gowning Purposes

The clean room gown is frequently talked about as the first line of defense in human contamination control. While it is designed to provide a barrier between the individual and the manufacturing or laboratory operation, it does not completely eliminate particles from being shed by the individual. In reality, particulates are shed through several areas of the gown including (13) the seams, zipper or closure areas, openings at the wrist, foot, neck, and around the eyes, and even from the surface of the gown. The amount of dispersion is affected by the type of gown selected, the material, and the environment in which it is used (13).

The clean room gown functions as a filter around the individual. Consider it to be packaging around your body. Packaging engineers will state that "all packages leak." The importance is the rate at which they leak and how much they leak. The same can be said for many of the clean room garments currently worn. Most of the particle sizes important to regulators for their risk of either being contamination or carrying contamination are too small to be seen by the naked eye. On the other hand, many of the openings, for example, the space between threads that are woven together on the gown are very large in the range of 60 to 80 μm. The very small particulates shed can get through the gown. The more tightly the fabric is woven, the smaller the hole, corresponding to reduced dispersion of particulates. The particulates shed through the gown typically come from the street clothes or uniforms worn under the clean room gown and any exposed skin or hair underneath the gown (4).

Testing of some garments that are very poor versus those that are very good have shown differences in shed rate of a million or more particles. Since the various components of the clean room garb may consist of different the amount that can be dispersed may vary. In recent years, one-piece garments have been developed for use (4). One of the newer uniforms actually incorporates the mask and eye shield into the one-piece design, requiring only gloves to be added to complete the uniform.

In class 10,000/grade C/ISO 7 areas coverall uniforms are routinely used. It is also possible to wear knee-length lab coats or smocks depending on the operation being conducted.

Clean room garments should have several important properties: nonflammable, limited linting properties, a fiber or weave that does not fuzz, low or no electrostatic generating properties, cleanable without causing linting (if it is reusable), and so forth (4).

Many companies have separate gowning systems for workers and visitors to an area. For example, company employees may wear reusable plant uniforms and reusable clean room garb while visitors (who may not come into direct contact with the product) are allowed to wear a disposable uniform over their street clothing.

In those situations where an individual's personal clothing is worn under the clean room garb, it is important to ensure that they are restricted from wearing fabrics with high linting or shedding properties, for example, mohair sweaters (4).

Some companies use color coded uniforms that make it easy to quickly determine if an employee belongs in an area, and/or their job function.

Gown Fabrics

An important feature of clean room clothing is the material or fabric chosen for each item. One wants to ensure that minimal dispersion of particulates occurs both from under the garment (the plant uniform, skin, or personal clothing) and from the garment itself. The choice

of fabric can dramatically change the amount of particulates that are allowed passage and dispersion (4).

There are several characteristics common to clean room garment design including (4):

- They should be manufactured from synthetic fabrics.
- There should be a minimal number of seams. If seams are present, the raw edges should be enveloped to prevent shedding of lint or opening of the edge.
- There should not be any pockets, belts, pleats, or tucked areas.
- The fabric material used should have filaments that are strong material. They should not be easy to break down.
- The weave of the fabric should be such that the openings are very small, reducing the person's particulates to be dispersed into the environment.
- The sewing threads should be monofilament materials.
- The construction should be such that the body is covered, with closures at the wrist and neck preventing easy release of particulates from these areas.

A variety of fabrics can be used for the construction of clean room garments. The following describes some of the materials that are available for use, although new materials are constantly being developed.

Antistatic Garments

Most of the materials used with these claims and are rendered antistatic by dipping the material in a topical antistat. When this dipping procedure is properly performed, the materials are about equal in controlling electrostatic properties (4).

Cotton

This material has low static properties. Unfortunately, it does generate large amounts of particulates. It can harbor microorganisms and requires rigorous cleaning. It is not considered desirable for outer clean room garments (4).

Dacron®

Dacron is made up of polyester fiber. It is considered to be an improvement over Nylon as it is softer and drapes in a smoother fashion. The color is whiter than Nylon and stays white after proper washing. It is also very wrinkle-free unless it is subjected to excessive heat. In the presence of excessive heat, it is likely to cause permanent wrinkles. A fire will melt the polyester rather than cause it to go up in flames. Moisture is absorbed at a very low rate of 0.2% to 0.85%. The long wearability of these garments makes them favorable for clean room wear (4).

Gore-Tex®

This type of garment is manufactured from a laminate of Gore-Tex membranes, which are expanded polytetrafluoroethylene or PRFE, bonded to a monofilament polyester knit. The outer membrane is designed to reduce the cling of particles and contains the particles from inside the garment, for example, on the individual using the garment. It is reported that this type of garment releases about 50% fewer particles that polyester garments. It is also very effective at capture particles approximately 0.1 μm in size (4).

This type of garment allows penetration of moisture into the garment (also called more breathable). Typically, this is associated with a garment that is more comfortable to the wearer (4).

Gore-Tex garments are manufactured as a two-layer fabric and a three-layer fabric (like a sandwich with the PTFE between two polyester layers, one of which may be antistatic). There are special rules for how seams are stitched and also precautions are taken to prevent fraying. It is available with different types of face masks (4).

Membrane Garments

Membrane garments are manufactured using a membrane film laminated to a base material; sometimes in a sandwich format with two base materials. They are considered the best barrier

for particulate pass-through. If the membrane is damaged or comprised, the barrier properties are compromised (4).

Nylon®

This type of material, a synthetic hydrophobic fiber, can be used for clean room garments, providing static is not an issue. Nylon garments are crisp and firm. They can look silk-like. Typically, they are very durable and stain resistant. Nylon can be easily washed and dries quickly; however, it has a tendency to yellow with age and uncontrolled conditions. It has a low moisture absorbance rate of 4% to 5% (4).

Polyester Garments

Garments manufactured with polyester fabrics, with continuous filament synthetic yarns, have fewer emissions of particulates through the garments. Polyester fiber is considered strong, nonabsorbent, and may be treated to reduce static charges. In the event that the garment is damaged, worn or tears, it can become a generator of particulates. Polyester garments are selected most often as reusable clean room garments (4).

A tightly woven polyester garment was developed for use in sterile manufacturing operations. This material has the benefits of polyester garments and also increased filtration efficiency. The efficiency of this polyester is similar to that of spun bonded olefin. It is comfortable, easy to wear, and reusable. The moisture vapor transmission rate is similar to standard clean room garments (i.e., better than spun olefin). This material can be sterilized via gamma irradiation (4).

Silvertech® Garments

This type of garment is manufactured using a coated polyester/carbon-suffused nylon monofilament fabric to achieve its barrier properties. The unique coating used makes the garment flexible. It also provides a hygroscopic moisture vapor transmission, keeping the user cool and dry (4).

Tyvek®

This material has been used for clean room garments for decades. They have very small pore openings (about 1/10 the size of reusable garments). Tyvek is usually a single-use, one-time wear garment. It is manufactured out of spun-bonded olefin, which is not a woven fabric. The material is manufactured by laying down fibers of the material to form a sheet and passing them through hot rollers under pressure, fusing them together. The resultant pore sizes are about 10 μm (4).

Tyvek garments are manufactured and sold under various trade names.

Gown Types

Ljungqvist and Reinmuller (17) executed several studies on the impact of human contamination sources and different clean room clothing systems. These studies were conducted using a dispersal chamber and individuals dressed in modern clean room garments. In these studies, they found that the values of released airborne microbial particulates were not significantly different with the minor variations in gowning styles; for example those with and without goggles, different types of face masks, and different types of hoods. They also found lower values for long-sleeved undershirts that were worn with long-legged clean room pants, when they considered different.

Additional studies were performed to compare the results of garments that had been repeated washed (25 or 50 times) versus new clean room garments. When combined with appropriate clean room undergarments, garments washed and sterilized 50 times were effective in protecting the environment from the human inside the garment (17).

They also indicated that it would be beneficial if the designs of zippers and snap fasteners could be improved, as they were a source of defects throughout their studies (17).

An interesting finding in the same clothing studies was that the coverall systems manufactured for use in the United States were more effective.

REGULATORY REQUIREMENTS FOR GOWNING

The FDA current Good Manufacturing Practices (cGMPs) includes requirements for products manufactured for marketing in the United States. The EU GMPs are included in Annex 1 of Eudralex Volume 4. Both of these documents include requirements for personnel gowning practices.

EU Requirements

The "Personnel" section of Annex 1 (6) indicates the following:

19. The clothing and its quality should be appropriate for the process and the grade of the working area. It should be worn in such a way as to protect the product from contamination.

    Descriptions are also provided for the required clothing in each grade:

    Grade A/B: Headgear should totally enclose hair, and where relevant, beard and moustache; it should be tucked into the neck of the suit; a face mask should be worn to prevent the shedding of droplets. Appropriate sterilized, nonpowdered rubber or plastic gloves and sterilized or disinfected footwear should be worn. Trouser-legs should be tucked inside the footwear and garment sleeves into the gloves. The protective clothing should shed virtually no fibers or particulate matters and retain particles shed by the body.

    Grade C: Hair and, where relevant, beard and moustache should be covered. A single- or two-piece trouser suit, gathered at the wrists and with high neck and appropriate shoes or overshoes should be worn. They should shed virtually no fibers or particulate matters.

    Grade D: Hair and, where relevant, beard should be covered. A general protective suit and appropriate shoes or overshoes should be worn. Appropriate measures should be taken to avoid any contamination coming from outside the clean area.
20. Outdoor clothing should not be brought into changing rooms leading to grade B and C rooms. For every worker in grade A/B area, clean sterile (sterilized or adequately sanitized) protective garments should be provided at each work sessions. Gloves should be regularly disinfected during operations. Masks and gloves changed at least for every working session.
21. Clean area clothing should be cleaned and handled in such a way that it does not gather additional contaminants that can later be shed. These operations should follow written procedures. Separate laundry facilities for such clothing are desirable. Inappropriate treatment of clothing will damage fibers and may increase the risk of shedding particles.

Additional requirements are included in the "Premises" section of the GMPs (18).

27. Changing room should be designed as airlocks and used to provide physical separation of the different stages of changing and so minimize microbial and particulate contamination of protective clothing. They should be flushed effectively with filtered air. The final stage of the changing room should, in the at rest state, be the same grade as the area into which it leads. The use of separate changing rooms for entering and leaving clean areas is sometimes desirable. In general, hand washing facilities should be provided only in the first stage of the changing rooms.

FDA cGMP Requirements

Several mandatory requirements are included in §211.28, "Personnel Responsibilities" (19):

(a). Personnel engaged in the manufacture, processing, packing, or holding of a drug product shall wear clean clothing appropriate for the duties they perform. Protective apparel, such as head, face, hand, and arm coverings, shall be worn as necessary to protect drug products from contamination.
(b). Personnel shall practice good sanitation and health habits.

(c). Only personnel authorized by supervisory personnel shall enter those areas of the building and facilities designated as limited-access areas.
(d). Any person shown at any time (either by medical examination of supervisory observation) to have an apparent illness or open lesions that may adversely affect the safety or quality of drug products shall be excluded from direct contact with components, drug product containers, closures, in-process materials, and drug products until the condition is corrected or determined by competent medical personnel not to jeopardize the safety or qualify of drug products. All personnel shall be instructed to report to supervisory personnel any health condition that my have an adverse effect on drug products.

The FDA's guidance on aseptic processing (2), which is labeled pharmaceutical cGMPs, includes expectations for personnel gowning in the "Manufacturing Personnel" section of the document.

> Personnel who have been qualified and permitted access to the aseptic processing area should be appropriately gowned. An aseptic processing area gown should provide a barrier between the body and the exposed sterilized materials, and prevent contamination from particles generated by, and microorganisms shed from, the body. Gowns need to be sterile and nonshedding and should cover the skin and the hair. Face masks, hoods, beard/moustache covers, protective goggles, elastic gloves, clean room boots, and shoe overcovers are examples of common elements of gowns. An adequate barrier should be created by the overlapping of gown components (e.g., gloves overlapping sleeves). If an element of the gown is found to be torn or defective, it should be changed immediately.

ISO Guidance

Documents from the ISO are not mandatory requirements for all countries; however there is useful information in many of these documents. It should be noted that these documents are not restricted to only pharmaceutical applications. In ISO 14644-5 (20), the Clean room Operations notes in part 4.2 indicates that "the environment and the product shall be protected from contamination generated by the personnel and their clothing. To maximize the containment, the choice of barrier fabric, the clothing style, and the extent of coverage of personnel by the ferment shall be established." It also points out that "clean room clothing shall be made of minimal linting fabrics and materials resisting breakdown and not shedding additional contamination. The necessary cleaning, processing, and packaging shall be defined."

There are several annexes to this document. Annex B discusses the requirements for clean rooms and how the clothing protects the environment from contamination by personnel. It provides information on the types of clothing suggested, for example, hoods, caps, helmets, coveralls, overboots, gloves, facemasks, and goggles or safety glasses. Information regarding the design and construction of clean room garments is also included.

Another addition is Annex C, which discusses the training, access, clothing, and personal items. It includes an example procedure for how to don clean room clothing (17).

Other Industry Guidance

Clean room clothing systems (17) provide comprehensive information and testing data regarding how people can be a source of contamination in clean rooms. A summary of data is provided on a variety of clean room garments and their effectiveness in preventing contamination. Some of the types of considerations discussed are

- The system used for clothing (clean room and surgical) and how different components contribute to the overall effectiveness.
- The effectiveness of clothing after repetitive washes such as the differences between 25 and 50 washes.
- Case studies evaluating clothing and particulate generation.

Many regulators have critically reviewed and accepted this data.

PERSONNEL GOWNING PRACTICES AND PROCEDURES

Since humans have such a great potential to contaminate the environment, appropriate protective clothing is used to provide a barrier between the human (and its associated contamination) and the environment.

Practices Related to Gowning in Noncritical Processing Zones

The typical gowning requirements for noncritical processing zones include wearing a plant uniform, with a disposable Tyvek® laboratory coat over the uniform. The laboratory coat should be snapped closed all the way from the bottom to the top of the neck. Each time the individual enters the controlled area, clean hair and shoe covers should be used. It is also appropriate to use safety glasses or goggles to minimize the contamination that can originate from the eyebrows and eye area. If these individuals also work in laminar air flow benches or cabinets, latex gloves should also be used. It may be prudent to use a disposable sterile sleeve also (21).

Practices Related to Gowning in Areas

The gowning selected for personnel that are working in aseptic areas should be sterile and enclose the whole body. It should provide a barrier between the operator and the environment. It is important for the operator to understand how personnel can contribute contamination to the environment. The personnel must be educated to understand that the gown is the key mechanism used to prevent product contamination. The selected gowning should cover the person from head to foot. Ideally, there should be no exposed areas, for example, skin, hair or eyes. These gowns come in both disposable and reusable formats.

The Institute for Environmental Sciences has established test procedures for reusable gowns to ensure that the gown is able to keep particulate matter and maintains a microbial barrier. This testing is performed at the initiation of use and periodically through the life cycle of the gown. When sufficient data has been established, expiration dates should be defined for the gown materials. Many times they are based on the number of times the uniform has been washed and/or sterilized. It is appropriate to qualify or certify vendors of aseptic gowning materials. These procedures should ensure the vendors ability to sterilize the gowning, maintain sterility post sterilization, and so forth (21).

Loss of Protection Using Gowning

The gowning barrier can be breached. It is important that personnel be aware of the ways that the system can be breached and the associated consequences. The gowning system used is capable of losing its barrier properties in a number of ways, including rips or tears in the gown, gaps in how the sterile mask covers the face, use of glasses or goggles that do not completely cover the eye area, and hoods that have been designed with an opening that extends from the forehead to the chin. All of these examples can result in areas where microorganisms and particulates can be shed and potentially contaminate the environment (21).

Once the uniform or mask gets wet, it also can loose its barrier properties. It has been reported that when personnel talk excessively in the clean room the mask can get wet. Once it gets wet, it only takes about 20 minutes for microorganisms to travel through the moisture barrier. This same type of loss of barrier protection can occur due to excessive perspiration.

Typical Gowning Procedure for Aseptic Areas

The following description of gowning procedures is utilized by some pharmaceutical manufacturers (21,22).

- Upon entry into the facility, the employee changes into a plant uniform and dedicated plant shoes.
- Clean head and foot covers are donned prior to entry into a controlled area.
- When an employee moves from a noncritical area to a critical area, for example, from a grade C to a grade B area, the operator dons aseptic gowning and enters via a gowning room and airlock.
- As an operator is to enter the gowning area or earlier in the process (e.g., when changing into a plant uniform), all make-up and jewelry are removed.

- The operator washes their hands with soap and water, followed by a sanitizing agent. The agent used should be qualified for effectiveness, with a specified contact time. A common sanitizer is ethanol foam.
- A pair of sterile gloves is donned. The glove is positioned on one hand by grasping the glove using the inside fold of the glove. The second glove is picked up with the first glove using the folded cuff. The bare skin of each hand is never in contact with the exterior of either glove.
- Next the operator places the sterile mask onto their face. It is important to make sure that the exterior of the mask does not come in contact with the face. It should fit tightly on the nose and face, so that there are no gaps with exposed skin or hair. If necessary, the nose bridge should be molded to fit the nose firmly.
- The operator then dons their sterile boots. As the boot is put on, the operator moves from the dirty side of the gowning room to the clean side, typically by stepping over the bench. The sterile boot is not allowed to touch any surface on the dirty side of the gowning room, if ties or clasps are used on the boot, they should be fastened prior to crossing over to the clean side.
- A sterile hood is removed from the package and is placed on the head, over the hair cover. The hood should only be touched on the inside surfaces.
- The sterile gown is removed from the packaging and is donned, only touching the inside of the uniform. Care must be taken to ensure that none of the uniform comes in contact with the floor while gowning. Several different ways to hold and don the gown are available, without contaminating the uniform.
- The leg portion of the sterile boots should be arranged to be over the bottom of the legs, with no exposed areas.
- The sterile goggles or glasses are obtained from the sterile storage area and may be disinfected prior to use.
- A second pair of sterile gloves is donned, using the same technique described previously.
- It is useful to check the gowning in a mirror prior to entering the aseptic area.

Training for operators should include awareness that if any tears, cracks, or excessive dirt is present on the uniform, the gown should be changed in the gowning room (Fig. 3). If the operator's hands go outside of the grade A/class 100/ISO 5 area, the sterile gloves should be changed; this may be performed in the room if double gloving was used (22).

TRAINING PROGRAMS FOR CLEAN ROOM EMPLOYEES

Since personnel are reported to be the biggest source of contamination in a clean room environment, it is very important that personnel working in these areas be appropriately trained in aseptic technique. They must also have training in the importance of personnel hygiene. For example, while it may be acceptable for a worker in an office setting to come to work when not feeling well, this may not be appropriate in the clean room. Working in aseptic environments requires a high level of attention to detail even in matters of personal cleanliness (3). A comprehensive training program is an essential ingredient of successful clean room operations.

Development of the Training Program

The training program should be developed by a team of experts, including members of the operations unit, microbiology, quality, and training personnel. This program should at minimum include the following (3):

- Discussion of the basic concepts of microbiology, and how they apply to clean room environments.
- Description of the acceptable types of behavior when working in clean rooms and aseptic areas.
- The regulations (and/or industry guidances) applicable to clean room classification systems, clean room operations, and aseptic processing requirements (including process simulations).
- Discussion of the applicable cGMPs.

Figure 3 Example of a gowned clean room person.

- Gowning requirements and procedures.
- The aseptic techniques that should be used in the clean room, including how to handle materials and transfer items from one area to another.
- The methods used for cleaning and sanitization.

It is important that the finished product be comprehensive, but also something that keeps the interest of the attendee. Use of multidiscipline approach to the development of the program can significantly aid in this process.

Conducting the Training Program

Training of personnel cannot be completed by only having the individuals read standard procedures and policies. This type of training must also include methods to evaluate whether the personnel have absorbed and understood the material presented. Typical training programs may include classroom instruction, reading of applicable procedures and/or regulations, examinations for the classroom portion, time to practice the various procedures used (with supervision to aid in understanding), and an evaluation of the person's proficiency to accomplish these tasks.

Proficiency is frequently evaluated using supervised operations, and in most cases microbiological qualification as well. The type of microbiological qualification may differ by company, but most require the ability to gown without contaminating the gowning above acceptable limits. Another trend is to have either a complete or "mini" media fill/process simulation as part of the training. The intent is to show that the person can successfully integrate all of the aspects of the training and use it to successfully manufacture product, or simulated tasks.

The regulations in most countries also require that personnel in aseptic areas be qualified in as part of a media fill (process simulation study) on a periodic basis. The time period varies depending on the country's regulations.

Management Philosophy and Commitment to Regulatory Compliance

Training programs within a company are only as successful as the level of management support and commitment provided. The most comprehensive training program will not be successful if management fails to provide support to the system. Employees need to know intuitively that their management believes in this material, its importance, and wants to be compliant to all regulatory requirements.

Development of a Positive Attitude

While it is very important to communicate to personnel, the requirements to act appropriately in gowning and behavior in the clean room environment, it is also important that they do not operate in a constant state of fear. It is important for them to believe that the rules established can be followed and be effective in maintaining the appropriate level of product quality.

PROTECTION OF THE ENVIRONMENT FROM PERSONNEL

There are many types of precautions that can be taken to reduce the risk of personnel contaminating the clean room environment. The three main types are facility designs, gowning, and cleaning and disinfection programs.

Facilities

Current engineering designs provide several opportunities to protect the environment from the contamination that could occur because of the person working in the clean room. Among the most effective methods are the incorporation of isolators and barrier systems.

Protective barrier systems are used as a generic term to describe the various types of systems available to aid in the prevention of contamination risks to the product and work area. There are two major types of systems: isolators and barriers. They are distinguished by the level of isolation and protection they provide for the area. After this initial classification, there are subgroups or levels within each main category (23).

The definition of a barrier describes it as any physical obstacle to contamination that, for example, separates, demarcates, or serves as a barricade. In other words, one might say that it is anything that separates or holds contamination away from the area (24). It does not offer the same level of isolation and protection as an isolator. Examples of protection apparatus include biosafety cabinets, goggles, gloves, and face shields. Barriers do not typically have complete isolation of humans—product/test item interaction (24). In some cases the barrier may include a rapid transfer port, but it is not typical. Barriers (24):

- May serve as personnel protection, for example, cabinets and glove boxes
- May allow human interaction with the product or test area
- Typically do not include sterile transfer capabilities
- Once sterilized, the system cannot maintain sterility
- May be designed to include HEPA filtered air
- Capable to have laminar or unidirectional air flow
- Possible to allow contaminants to penetrate the area
- Allow air exchange with the external environment

An isolator is different from a barrier in that it provides complete separation between the areas, that is, the defined area is completely protected from the external environment. They provide the highest level of separation from environment. Typical construction includes the ability to maintain a germ-free or contained environment, no personnel direct contact with the contained area. Some designs include pressure differentials to ensure that the area containment and sterility are maintained. Isolators come in both a closed and open configuration. Features of isolators include (24):

- They are designed to provide a closed environment
- Environment is fully contained and can be sterilized
- They separate and protect the product/test area from the personnel
- They limit likelihood of human-borne contamination
- They are capable of maintaining their sterile state

- They can be designed or customized to meet user's requirements or may be purchased in an off-the-shelf configuration
- They may also be considered as a controlled size clean room

Assigning levels to the various isolators and barriers might result in the following list (24):

- Level 1—Partial barrier
  This type of system provides a minimum level of protection. There is no capability for sterile transfers. The work area cannot be ensured to be sterile during operation. Examples of this type of system include curtains, conventional clean rooms, and personnel protective equipment.
- Level 2—Closed barrier
  This type of system has limited the opening and handling area. It provides more protection than a partial barrier. Typically, they do not include sterile transfer capability. Some current systems have added transfer ports to reduce the risks of contamination. The work area for this design cannot be ensured to be sterile during use. It does not provide complete isolation of the personnel from the product/test area. Examples of this type of system include restricted access barriers and glove boxes.
- Level 3—Open isolator
  An open isolator provides a high level of isolation for the process and transfer of materials. In these systems, one can maintain the sterility of the area during activity. Overpressure in this area can aid in the integrity of the system, in spite of areas where product or materials exit the isolator, for example, through a mouse hole. Most have special methods or devices for the transfer of materials in and out of the isolator. An example of this type of system is a production isolator that incorporates openings, for example, mouse holes to allow product to exit the area.
- Level 4—Closed isolator
  This system provides a totally contained process and associated transfer capabilities. It provides the highest level of protection. The advantage of these systems is that it is a closed system. It can maintain the sterility of the work area during activity. These systems provide specialized methodologies for transfer of materials into and out of the closed environment. Examples of this type of system include closed, leak-tight isolators used for batch production or quality control testing.

The trend to utilize isolators is increasing. This is especially prevalent in aseptic processing of pharmaceutical products. Typically, they use an open isolator where the containers exit through a mouse hole. Isolators are routinely used for sterility testing in the quality control laboratory. Many units are available for sterility testing that can be purchased in an off-the-shelf configuration (23).

For those companies that do not have isolator or barrier systems, the laminar air flow system should have air flow patterns that minimize the risk of personnel contaminating the product and product contact areas. It becomes very important to evaluate the various types of manipulations and interventions that personnel might conduct during the process to demonstrate that these actions do not adversely affect the surrounding environment.

Gowning

Clean room garments, when worn properly, are designed to provide a barrier between the individual working in the clean room and the clean room environment. The effectiveness of this barrier is dependent on the garments selected, the materials used for construction of the garments, and how well the established rules for gowning are followed.

Another concern with gowning effectiveness is the level of particulates present on the individual and their personal hygiene practices.

Cleaning and Disinfection Programs

Cleaning and disinfection programs are key components to maintenance of a controlled clean room environment. Typical components of this type of program are:

- Determining the type of flora routinely present in the environment.
- Finding a cleaning/disinfecting agent(s) that is effective for the types of flora routinely present. Note: While some individuals imply that microorganisms develop a resistance to cleaning agents, similar to bacterial resistance to antibiotics, there is no sufficient data to support this premise. Rather, it is a company choice to use a single disinfectant or a rotation of several disinfectants. It is also common to have different sanitizing agents depending on what is being sanitized, for example, foam agents or spray bottles of isopropyl alcohol for hands and other agents for floors and surfaces. For aseptic processes, it is a regulatory expectation that a sporicidal agent, like bleach, is incorporated into the cleaning and disinfection program.
- In vitro laboratory studies to show the effectiveness of the cleaning/disinfecting agent.
- Validation of the cleaning and disinfection program using at least three replicate studies of performing the cleaning using the specified cleaning procedures and collecting environmental samples both before and after the cleaning regime (in situ studies). For very clean areas, such as aseptic processing areas, it may not be possible to show a reduction in bioburden. If you have a room where zero is the expected count and one is an excursion, it will be difficult if not impossible to show reductions. For these types of areas it may be appropriate to just indicate that the cleaning program does not cause a significant increase in counts.
- After the initial validation, on-going monitoring of the environmental quality is performed to ensure that the cleaning program continues to be effective.

QUALIFICATION OR CERTIFICATION OF PERSONNEL

Qualification or certification of personnel should be conducted to show that they have correctly gowned for aseptic operations prior to allowing individuals to work in aseptic areas. Demonstration and training to show the proper gowning procedures and aseptic behavior should be conducted prior to the certification. The intent is to ensure that all of the necessary skills and techniques have been learned prior to the individual working with product. When the training has been completed, a series of evaluations should be conducted to ensure that the gowning procedure used does not contribute to the risk of contamination to the environment or product in an aseptic operation. Certification programs vary across companies (7).

A typical certification, or qualification program for personnel gowning typically includes (7):

- Provision of an overview of basic microbiology, and how it is applicable to clean room operations
- Description of the GMP requirements for personnel hygiene and clean room operations
- Description and example of the appropriate behavior for working in aseptic areas
- Discussion and explanation of laminar air flow properties, for example, impact of disruptions, good ways to perform interventions, how to protect the environment
- A description of the requirements for environmental monitoring and sampling methods/techniques and requirements to be used
- Discussion of important regulatory information on aseptic processing (although some companies choose to do this separately from the gowning certification), for example, applicable requirements from the aseptic processing guidance
- Discussion and review of the applicable SOPs for the process
- A physical demonstration of how to perform gowning by an individual who is certified and has demonstrated good techniques. (It is useful to routinely examine gowning techniques used within a facility and across facilities and evaluate what are the best demonstrated practices.)
- Opportunities for the personnel to attempt to gown correctly before going to the aseptic area, for example, in the classroom, with a trainer present to identify any potential concerns
- Employee must perform the gowning correctly in the gowning room with the trainer present

- Employee must gown and be microbiologically sampled at least three separate times. The results of this sampling must meet all established levels or limits. Note: Since this sampling is conducted prior to working in the aseptic area, some companies establish lower levels as acceptable for certification. Typically, an increased number of sites are sampled for qualification. Common programs include 12 to 18 sampling sites on the individual.
- An established procedure for how a person is decertified for gowning, for example, expiration of allotted time period since last certification, maximum amount of time a person can be absent from the aseptic clean room operations (e.g., an employee that has a major medical problem or maternity leave and does not work for several weeks), or what actions, trends, or monitoring results necessitate decertification. For example, if a person routinely fails to meet microbiological monitoring results, does this disqualify them from working in the area?

The requirements for certification are frequently documented on a checklist.

It is also important to ensure that this certification or qualification is updated on a routine basis. There are several types of recertification programs. Limited recertification may include reading the SOP, gowning with a trainer and replicate microbiological monitoring of the gowned individual. This is typically considered for employees that have been previously certified and may not have been working in the aseptic area recently. Periodic recertifications may include review of the entire certification program (7).

These types of programs are frequently used in part or in full, when retraining operators following results that exceed established levels or when adverse trends are observed (7).

Some certification programs only look at absolute numbers, for example, levels to determine if the results are acceptable and they do not look at how the baseline data for the operator changes as a result of gowning and operating in an aseptic condition. For example, trended data may show that an employee has an extremely high percentage of samples that are over the baseline results, 41%, indicating that a significant change has occurred and remedial action should be warranted. There is no regulatory requirement to perform this type of analysis (i.e., comparison to baseline data), but it provides useful information on the skills and training of specific operators (7).

MONITORING OF PERSONNEL—DEMONSTRATION THAT YOUR SYSTEM IS OPERATING IN A STATE OF CONTROL

It is important to establish programs that demonstrate whether the contamination control systems established are sufficient. Personnel monitoring is typically performed as part of the facility's environmental control program.

Types of Monitoring Methods Used

There are two predominant types of monitoring used routinely in personnel monitoring, surface sampling with a solid agar medium [e.g., replicate organism and counting (RODAC) plates] and surface sampling using swab sampling. Swab sampling is used less frequently, with traditional methods. With the advance of some of the viability based technologies, there is an increase in the number of people using swab methods coordinated with rapid microbiological methods for sampling. Surface sampling with RODACs and touch plates are the most widely used traditional sampling methods (22).

RODACs are used to sample various sites on the clean room clothing. Touch plates are used for fingerprint impressions [frequently referred to as fingerprint impression sample (FIPS)] of the gloved hand. Some companies use swab samples, especially in their isolator applications to sample the isolator gloves in a more comprehensive manner. The samples are recovered, incubated, and enumerated to determine the number of microorganisms recovered (22).

Sampling Sites

The number and location of personnel sampling sites used vary. The common thread for all of the sampling methods is the FIPSs. All of the regulatory agencies expect to see this type of sampling for aseptic processing. A variety of other sampling locations may be used including

hoods, masks, shoulder, forearms (either frontal, rear, or both sides), wrists, chests, legs or boots (22).

Selection of sampling locations should include consideration of the potential for microbiological risk to the product and/or the environment. For example, in an isolator environment, the gloves and forearms are a likely source of contamination and are usually tested. In many operations, personnel tend to rest the back/bottom of their forearms against surfaces and as such, this might be an appropriate sampling location. If other body parts come into contact with clean room surfaces, these sites should be sampled; for example, people who use their shoulders to push open clean room doors (22).

Some company sampling plans include masks. High counts on mask samples may indicate that the personnel are talking excessively. When the operator is talking frequently, using a mask, the talking results in a wet/moist mask. The wet path allows for microbial passage through the mask. It is postulated that the microorganisms can ingress through a wet mask in about 20 minutes (22).

Goggles and glasses are not typically monitored.

It is not necessary for the same number and type of samples be taken for all employees in the clean room. For example, if a person is responsible for setting up the filling line needle equipment, the criticality of the operation may justify that an increased number of samples are taken and/or that samples be taken at additional locations. Material handlers may also be prone to contamination at different sites (e.g., legs or chest areas) because of how they move or lift the affected materials (22).

The required number of sites, sampling locations, sampling frequencies, and the rationale for how these were determined should be documented, typically in a standard operating procedures or policy. Typically, each person is sampled for two to six different sites (22).

When Should Monitoring Be Performed?

Use of surface sampling plates, either RODACs or touch plates, results in the potential to leave behind residues of the media on the sample location. This media can then become a harbor for microbial contamination. For this reason, it is appropriate to perform personnel monitoring at the end of production activities, for example, end of shift, before changing out of uniforms, etc. Sanitizing agents may reduce the likelihood of recovery organisms present on gloves. Accordingly, sampling should not be performed immediately following sanitization of gloves (22).

For some operations, for example, critical aseptic connections, it may be appropriate to take the samples at the end of the activity. This may necessitate that the operator changes gloves or uniforms prior to returning to work in the aseptic area (22).

Who Performs the Sampling?

A common question at manufacturing facilities is who should be responsible for taking the samples? In most cases, the debate is whether microbiologists or manufacturing personnel should be used to perform the sampling. Some companies believe that the quality control (Assurance) microbiologists should perform the sampling, as QC has responsibility for oversight of manufacturing operations. Other companies believe that manufacturing personnel can perform the sampling, if properly trained and qualified. Many times the microbiologists do not want to participate in off-shift sampling (22).

All personnel who are responsible for sampling should be trained in how to perform the sampling, when to perform the sampling, what to do with the samples once they have been taken, concerns/limitations of testing and the basic microbiology associated with the testing methods (22).

For those companies where manufacturing personnel are responsible for sampling, safeguards are established to protect the validity of the sampling methods and results. Typical types of safeguards include (22):

- A qualification program for samplers
- Periodic auditing of sampling methods by qualified microbiologists
- Sampling “booths” or rooms, where auditors routinely monitor or audit the sampling procedures as they are conducted for each batch of product

It is important to also train and qualify the microbiologists who perform sampling in the proper methods and techniques to be used.

What Should be Done with the Data Collected?

Data obtained from sampling of personnel should be compared to established levels to determine whether these levels are exceeded. It should also be maintained and trended using an appropriate environmental monitoring trending application. If levels are exceeded or adverse trends occur, appropriate corrective and preventative actions (CAPAs) should be taken (22).

Establishing Levels or Limits for Personnel Monitoring Results

Personnel monitoring levels or limits are specified in some regulatory documents, for example, EU Annex 1 to the GMPs, FDA's Aseptic Processing Guidance. If products are manufactured for countries that have defined regulatory limits, then the established requirements should fall within those limits (22).

For systems where regulatory limits do not exist, the data should be trended and evaluated to determine an appropriate baseline of counts, which are used to establish the acceptable alert and action levels. Keep in mind, however, that environmental data may not be normally distributed, so appropriate evaluations should be conducted to determine if the statistical methods used are appropriate for the data. Another consideration in setting levels is that cells or CFUs should typically be integers. One does not have ½ or ¼ of a microbial cell present in the environment. Long-term use of statistics where very low levels of contamination are present can result in statistical values for results that make little or no sense.

It may be necessary to have different monitoring levels established for production personnel and cleaning personnel. The nature of the cleaning operations may make them prone to higher levels of contamination while correctly performing their work assignments (22).

ON-GOING EVALUATION AND RISK ASSESSMENTS

In addition to the initial certification of personnel working in clean rooms, it is important to have on-going programs to evaluate the state of control during operation. The most prevalent way to do this is to incorporate personnel monitoring into the environmental control program.

The levels or limits established during qualification or via regulatory documents are used to standardize acceptable levels of control. These levels or limits should also take into account the risk associated with the procedure, in terms of both the product and the personnel.

CAPAs for Numbers Exceeding Established Levels

Data should be routinely generated to evaluate the effectiveness of personnel monitoring programs. In the event of the established levels are exceeded or an adverse trend in monitoring results is observed an investigation should be conducted. It is important that the investigation occur in a timely manner. Typical types of follow-up actions may include (7):

- A increase in sampling frequency
- Increased observation of personnel behavior and gowning by supervisory personnel
- Retraining of the operator
- Gowning requalification performed earlier than routinely required
- Decertification of the person for the aseptic area until appropriate qualification or certification requirements and training are met
- Reassignment of the individual to another activity outside the clean room.

There are several areas of concern that should be taken into account when dealing with corrective actions for personnel monitoring results. The methods used for personnel monitoring often lack sensitivity, and like all manual operations are prone to human contamination, that is, false positives. The low levels of contamination are allowed in some regulatory guidance documents, for example, a count of one may be an issue. One must remember that microbiology is a logarithmic science, and scientifically there is no real

difference between counts of one and nine. Conversely, the intention or goal of aseptic practices is to operate throughout the manufacturing process contamination free (7).

Some of the typical preventative actions taken by companies include increased training and emphasis on the gowning certification and recertification program (7).

NOTE

Throughout this chapter, the identification of a genus or species of microorganism has been given. In recent years, the American Type Culture Collection has been actively sequencing their collection. This has resulted in many genus and/or species names changing. It is important when making decisions about a risk of contamination from an organism to know its current name, any previous names, and any previous similarities/links to other organisms. While every effort has been made to keep these names current, it can be impossible to do so. For the most current information, contact the American Type Culture Collection, or the agency in your country.

CONCLUSION

The importance of personnel in clean room operations should not be underestimated. Effective operations require that control of contamination is demonstrated. Personnel monitoring and control, for example, certification are critical components of a control program for aseptic areas. Effective training programs communicate all of the necessary requirements for aseptic gowning and behavior and have definable milestones to show that the operator understands and has effectively implemented these procedures.

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3 The fundamentals of an environmental control program

William H. Miele

INTRODUCTION

This chapter is intended to assist the reader in developing an understanding of current thinking regarding environmental monitoring and control and its application in aseptic processing. Some background historical information will be discussed to describe the journey from where we came and where we are in an effort to appreciate the nuances of the journey forward. Of course it is our present situation that dictates how we function on a day to day basis and it is the vision of where we want to be that drives our thoughts and processes through the challenges presented by continual change.

Continual change has not always been the accepted mode of operation. A minimalist mind set was once pervasive throughout the industry. The development of technologies applied to aseptic processing from the HVAC (Heating, Ventilation, and Air Conditioning) systems and facility engineering to the technologies directed at the detection and identification of contamination have changed the way we approach our business. This along with the ever-escalating industry standards and the evolution of risk- and science-based approaches to regulatory compliance have driven environmental monitoring and control practices to a new level of performance and expectation. It is this combination of events that requires us as leaders in aseptic manufacturing to know and understand the continuum to scope out the future.

TRADITIONAL APPROACH TO MONITORING, PRESCRIPTIVE CONTROL

Origins of Monitoring

Looking at the history of drug control and enforcement in the United States, it is evident that sterile drug product review was superficial at best and lacking in the details required to minimize risk to the public prior to 1970s. Prior to this era drug applications were not sufficiently detailed for government reviewers to determine exactly how sterile products were manufactured. From this was spawned the 1976 CFR rule on LVPs and Good Manufacturing Practices for sterile drugs that gradually gave way to the FDA guidance on sterile drug manufacture of 1987. We are all familiar with the 2004 update of that document. In the period of the 1990s much attention would be given to aseptic processing because of events that transpired in the generic drug industry, which flourished after the Hatch-Waxman Act of 1884. As a result there was a corresponding response at regulation and enforcement by the U.S. Food and Drug Administration through the Office of Generic Drugs and Center for Drug Evaluation and Research. A plethora of guidance documents flooded the industry covering how to do and what to do in various aspects of sterile drug manufacturing. The frame work of aseptic processing was being cast including the support of documents such as Federal Standard 209E addressing airborne particulate cleanliness in clean rooms. At the same time there was the explosion of contributions from industry organizations as well, such as the Parenteral Drug Association Technical Report 13 and from the United States Pharmacopeial Convention with the advent of Informational Chapter <1116> (14).

Reliance on Numbers

During this time period, pursuing their missions to protect the safety of the public, the regulatory bodies produced more and more documents to fulfill their role. These documents may have taken the form of guidances directed to their own inspectional efforts or to the industry, companies under their inspection. The USP established General Information Chapter <1116> USP 26. This offering referenced U.S. Federal Standard 209E (September 11, 1992) (1) for airborne particulate classes and offered frequencies for sampling and limits for classes to the decimal place in particles per cubic foot. Microbial considerations were listed in colony-forming

units (CFUs) for equipment surfaces and floors in class 100 and class 10,000 including a prescribed limit for floors. One can only speculate that the microbial data presented originated with a 1967 NASA (2) document referenced in the 1984 USFDA aseptic guidance and promulgated through various regulatory documents and guidances in the United States and in Europe. With each iteration or unique perspective greater and greater specificity based on "what is considered attainable and desirable" emerged. Rather than emphasizing how processes were being engineered and managed to be compliant and gain new product approvals to the marketplace, the industry responded by producing an avalanche of data for drug submissions and review based on the flood of often confusing and sometimes conflicting guidance information available. The response by industry was not one of integration of best practices into processes but the response was additive to the processes, not the intent of the regulatory agencies but the result of the response by industry.

Simultaneously, there was an explosion of commerce taking place on a global level that few envisioned or were prepared to address regarding approaches to regulatory compliance. This further exacerbated the situation by overlaying geographically dispersed regulatory requirements upon already burgeoning seemingly endless amounts of descriptive information to assimilate and put into practice. The c in cGMP had figuratively moved from a lower case c to an uppercase C. Company's marketing their products globally were forced to do a "juggling act" to balance manufacturing operations to encompass all the variables for the intended areas of marketing. One example of a regulatory document of geographical origin but global significance was the "EC Guide to Manufacturing Practice for Medicinal Products and Active Pharmaceutical Ingredients" and in particular the supplementary guideline known as Annex 1, which addresses sterile product manufacture. It appeared that each regulatory body interpreted regulations in an independent manner as if drafted with little consideration of the other. This included the USFDA aseptic guidance document of 1987.

All this was happening in a rapidly changing environment. The knowledge of quality management principles as developed and fostered by Demming, Juran, and others were taking hold and producing significant gains in product reliability and manufacturing efficiencies in other industries. But the perception of quality and how to go about integrating the "new" quality management principles into processes long driven primarily by law/regulation seemed out of the grasp of the pharmaceutical industry. As a result the industry found itself behind the learning curve compared to other highly focused engineering and technology concentrated industries. It appeared the pharmaceutical industry had its feet cemented in the detail and seemingly inflexible mindset of the past and had not embraced the changes that were occurring all around it. What the industry did do was mire itself in detail and redundancy to try and attach quality to its terminal processes. This apparent conundrum led the industry to claim the regulations restricted their ability to change, while regulators claimed the industry had not adopted change, as had been the case in other industries also heavily technology and engineering oriented. For some period of time the reliance on details and numbers stuck and what appeared to be a standoff persisted.

A CALL FOR CHANGE

New Paradigm of Control

The global nature of the rapidly developing industry stimulated the pursuit and expansion of global harmonization. The need to harmonize was recognized because of the growth of the European Union and the establishment and contributions from organizations such as the International Congress on Harmonisation (formed in 1990) and the American National Standards Institute to name but a couple. The diversity of interests and the global development of the industry emphasized the need for geographically different approaches to embrace regulatory initiatives and harmonize.

In the United States things were not standing still. Great change was occurring because of the tremendous growth of pharmaceutical manufacturing in the areas of drugs, devices, biologicals, and the new and exploding biotech industry. Events brought on by the apparent inability of the industry to adapt the current regulations to their day-to-day operations resulted in significant encounters between government regulators and certain segments of the industry. Two such examples might exemplify these activities, the "Barr decision" (1993) (3) and the

resulting drafting of the guidance for industry on out of specification results and the advent of Team Biologics (1997). The latter was created in an effort to more broadly harmonize regulation and compliance between CDER and CBER. A revolution, if you wish, was developing. The seeds of change were being planted and were fertilized by documents such as 21 CFR Part 820, Quality System Regulation (1997), and the FDA Guide to Inspections of Quality Systems (1999). Then in 2002 the USFDA published "Pharmaceutical cGMPs for the 21st Century: A Risk-Based Approach, A science- and risk-based approach to product quality regulation incorporating an integrated quality systems approach" (4). It represented a giant departure from an approach to regulation that could have been described as inflexible and protracted, while now advocating a systematic, integrated approach with science and risk as the basis for decision making. This was not introduced to replace regulation as published but was intended to augment, supplement, or facilitate the attainment of compliance to the regulations as written. This new holistic approach supported communication and harmonization across areas both geographic and technical never before communicated no less previously supported. The document endorsed the concept of collaboration with regulatory authorities in a variety of unofficial venues utilizing industry organizations and collaborative initiatives such as ICH. With this came the support of the trilogy, ICH, Q8 Pharmaceutical Development, Q9 Quality Risk Management, and Q10 Pharmaceutical Quality System, and a new paradigm was borne (5).

Initially on publication there were more questions than answers and more naysayers than advocates. Change takes time but through what appears to be a remarkably well-orchestrated initiative, the word went out to both agency and industry organizations and then collaboratively through industry participation. Selected salient publications authored or coauthored by both agency and industry professionals included Friedman et al., "Risk Factors in Aseptic Processing" (6) and Hussong, "Environmental Monitoring for Aseptic Processing" (7). In these articles both the myths and truths in aseptic processing and control of aseptic environments were discussed in an unprecedented manner. As the discussions surrounding the 21st century risk- and science-based approach continued the support for the ICH Q8, 9, and the draft ICH Q10 became the topics of the day and the dialogue still continues. Just as important a path forward and a vision for the future were being characterized. These publications were written during the drafting and comments stage of the 2004 revision of the USFDA 1987 "Guideline on Sterile Drug Products Produced by Aseptic Processing" (8,9). Parallel developments occurring in the European Union involved the revision of Annex 1, Manufacture of Sterile Medicinal Products, of the EC Guide to Good Manufacturing Practice for Medicinal Products and Active Pharmaceutical Ingredients in 2008 (10). This document also emphasized the theme of good science and assessment of risk. The European Union went as far as adopting ICH Q9 as an informational annex, Annex 20. The recognized need and willingness for harmonization was spreading through organizations addressing global development of harmonization. The Pharmaceutical Inspection Convention, Pharmaceutical Inspection Co-operation Scheme, PIC/S, is an organization whose mission is to facilitate international harmonization of good manufacturing standards. It is composed of regulatory bodies for their benefit and the countries and patients they serve. The USFDA is not currently a member but has applied for membership.

There has been a shift in philosophy on how to achieve compliance with the regulations. It has not been "out with the old and in with the new," but it has encompassed a change in how to respond to an environment that is in continual change. This is an acknowledgement of how one goes about the process of compliance and that compliance with the regulations is an evolutionary endeavor due to the introduction of new technologies and new management approaches. This represents a significant change and a lot of change in a relatively short time span for many stakeholders. Each stakeholder trying to understand the changes and at the same time formulate implementation plans to cover the perceived change.

The mode of operation is new and challenging, but not impossible. It emphasizes a "holistic" or systems approach rather than a prescribed approach to compliance. In saying that there must be emphasis on the underlying intent of the regulations rather than the absolute adoption of the prescriptive requirements, there needs to be some clarification. Historically, the "black or white" approach to compliance is thought by some to be the cause of compliance

problems rather than a solution and thought to be a contributor to the lack technological innovation by the industry. With the advent of modern theories for the integration of quality into manufacturing processes in conjunction with the advent of new technologies, a shift to equivalent or better is immerging as the "new paradigm."

The Uncertainty Principle and Microbiological Control

Akers and Moore (11) published an article about 10 years ago, which turned out to be quite prophetic and framed the dilemma that is currently developing. It is risky to cite the famous Heisenberg Principle to this application but it does represent a scenario that suggests a parallel. The changes occurring in aseptic processing control do have implications to the uncertainty of the situation. The inability to measure accurately will limit our ability to predict or document an event as it is occurring and to determine its impact. Currently, the engineering and clean room technology used in the 21st century to control manufacturing processing is far superior to the environmental monitoring/microbiological technology and methods in use and their abilities to detect and quantify microbiological contamination in a relevant time frame. This disparity between engineering technology and microbiological measurement and control has highlighted the lack of our current capabilities and forced the decision making process to focus on a "holistic" approach to control, most probably where it belonged in the first place. Not to detract from the holistic as being appropriate and correct but the identified disparity places a greater reliance on that approach because of the paucity of exact on the spot data.

As microbiologists supporting aseptic control and environmental monitoring in aseptic processing we are using 200-year-old technology and trying to keep pace with processing in the 21st century. Add to that the disparity are the myths we as people harbor for considering and assessing microorganisms we cannot even see and are only capable of detecting by relatively crude and inaccurate means. As scientists we thrive on our ability to measure, quantify, qualify, and describe, and we respond to those expectations. As a result many of the quantitative assumptions and qualitative descriptions we have embedded in our guidance documents have become part of our "body of knowledge" and are laced with notions that may be inaccurate and only serve to mislead us. Although we would like to take numbers of CFUs generated from microbiological media as representative of a defined microbial population, it can not categorically be stated that a microbial event(s) captured on microbiological media actually represents the status of product quality or for that matter process quality. Some firms have positioned themselves in such a manner as to have no recourse but to use EM data for the release of product. It is probable that the regulations as written were never intended to force companies into such a position.

A Sound Approach

Environmental control of any production operation is the objective, while environmental monitoring is just one set of tools in the arsenal employed. There is no one correct approach, but the consensus says best results can be obtained when you approach environmental control from a holistic perspective starting with the general and working toward the specific, essentially building quality into the process. This approach is taken in our more ardently followed guidances and regulations. Both the FDA guidance for industry and the EU guide to GMP practice, including Annex 1 offer that approach, each includes buildings and facilities for grounding of their recommendations for necessary control measures. Prior to discussion of the utility of microbiological monitoring and counting colonies as being effective for its intended use, we would be remiss if we did not stress the fundamentals of a control program.

There are specific circumstances or situations within the manufacturing process that may allow for a microbial hazard to occur. A review of such areas would be essential to identifying issues and maintaining control such as

- Facility design
- Warehousing/storage of raw materials (API and excipients)
- Pre/post manufacturing storage and transport conditions
- Manufacturing equipment

- Cleaning and sanitization methods
- Water/utilities
- Processing conditions
- Personnel behaviors

Monitoring programs as outlined in regulatory and industry documents were designed for use in clean environments (aseptic processing). However, monitoring, as such, has been taken by some and applied to every thing from assessing microbial levels in uncontrolled nonsterile manufacturing areas to monitoring warehouses. There have even been suggestions, presumably seriously offered, that request the outside environment be monitored to determine the local microflora. In my opinion this exemplifies our apparent need to try and quantify, qualify or describe without consideration to the value of the information. The monitoring programs or there components described in guidances and regulation for aseptic manufacturing operations are not intended for use to control microbial contamination in a nonsterile setting or nonsterile manufacturing process. A program of contamination control for nonsterile application can be developed through a documented risk assessment that incorporates an evaluation of the steps in the nonsterile process. Similarly a risk assessment process should also be applied to aseptic program development to fill in and connect the prescribed portions of an aseptic program. From the risk assessment process potential microbial hazards can be identified. After the microbial hazards are identified, the existing control measures in the process, if any, are evaluated to determine their efficacy. If adequate controls are not present, controls are put in place to prevent or minimize the introduction of microbial contamination. These controls may be physical (e.g., temperature, holding times), chemical (e.g., pH), procedural (e.g., cleanliness, dryness), or microbiological (raw material acceptability, microbial reduction steps). A program can be established that will monitor (by observation or by measurement, physical or microbiological) the controls to assess the process and subsequently reduce risk of microbial contamination.

Without presenting a primer on quality risk management it might be useful to define a few basic terms for understanding. Risk is defined in ICH Q9 as the combination of the probability of occurrence of harm and the severity of that harm. A hazard being defined in ICH Q9 as "a potential source of harm" and harm being defined as "damage to health, including the damage that can occur from loss of product quality or availability." There are specific circumstances or situations within the manufacturing process that may allow for a microbial hazard to occur. Microbial hazards may originate from improper facility design. Such hazards could include deficient control of humidity and temperature in manufacturing areas, improperly balanced or maintained air cleanliness levels both at rest and in operation, and proper room construction and design to permit effective cleaning and maintenance. Inappropriate layout of rooms could facilitate cross-contamination or recontamination of an area due to personnel, materials, or equipment flow. Inappropriately maintained and controlled warehouse and storage areas may precipitate potential microbial hazards. Some raw materials are very hygroscopic; high humidity and/or improper container closure could cause microbial ingress and/or proliferation. Microbial hazards may be introduced into a manufacturing process because of improper sanitary design of the manufacturing equipment, especially equipment used for aqueous processing steps. For example, microbial contamination can arise from entrapped water drainage or product residues that remain hidden from procedural cleaning processes due to threaded pipe fittings, nonsanitary valves, piping dead legs, nonsloping pipes, equipment crevices, recessed access ports, bottom discharge valves, and pocket flow meters. Inadequate equipment maintenance may also serve as a potential hazard. For example, misaligned, damaged, or over torqued gaskets between piping connections may harbor a reservoir of trapped microorganisms.

Inadequate or inappropriate cleaning and sanitization of the equipment and manufacturing areas can potentially serve as a major source of microbial hazards. Other examples of potential microbial hazards could include the following:

- Cleaned equipment that is not properly dried and stored wet.
- Cleaned equipment that is not properly stored.

- Manufacturing areas that are not adequately or routinely cleaned before use (e.g., standing pools of water, construction materials, cardboard, or other debris).
- Cleaning utensils such as mops, buckets, and brushes that are not stored dry or clean.
- Intervals between manufacturing runs with no cleaning or variable time limits before cleaning with no consideration for the potential for microbial contamination.

Water, a major component of many dosage forms or for equipment or facility cleaning, is a potential source of contamination. It is essential to use water of the proper purity standard for its intended use. Unless sterile and used in a sterile environment, a lesser quality of water will foster microbial contamination with time and exposure. For example, if maximum hold times for in-process materials with appreciable water content are not properly validated and used within that validated period, microbial proliferation could occur.

Personnel may be the single most significant contributor to contamination, particularly as the environmental and microbial control of an area becomes more stringent. Putting people in an aseptically controlled area is the greatest challenge to contamination control to process and product. Not only the direct involvement of people within the manufacturing process is important but also the movement of people between various stages of the work processes. People flow between areas of differing microbial control, including nonclassified areas into controlled and classified areas is critical. There is also acknowledgement that personnel activities outside of work do influence control efforts within the plant environs, and it can also be influenced by seasonal variations.

This is just an overview of areas to be considered as a foundation for a total environmental control program. It is recommended that a risk assessment of the work flow, locations and movements of materials, equipment and personnel be performed to identify the potential hazards encountered and assess existing controls and their levels of effectiveness. An action plan can be formulated with a rationale supporting the decisions and changes. Without this strong foundation the efforts of more focused initiatives for environmental control will face a greater challenge and may not be sustainable.

Aseptic Control, The Devil Is in the Details

It is well documented and accepted that aseptic processing presents the highest safety risk, that is, risk to the patient, than any form of commercial drug manufacture. For the manufacture of sterile parenteral products terminal sterilization is the process of choice, and aseptic processing is only acceptable when there is no other means to process the particular drug product. Given the complexity of the aseptic manufacturing process, individually sterilizing component parts and then assembling and dosing product in an aseptic "clean," but yet not sterile environment, presents a challenge to the manufacturer to establish controls and develop a monitoring program in response to the risk level. The regiment is a fine-tuned array of engineering prowess and management oversight, and diligent operation of the program to ensure all established parameters and acceptance criteria are met on a continual basis. This necessitates a rigorous program of planning, actions, checks, and rechecks. As indicated earlier, this pursuit is made more arduous because of the limited precision and accuracy of the monitoring methods we employ today.

ENVIRONMENTAL CONTROL AND MONITORING, A HOLISTIC APPROACH

Facility Design, HVAC Engineering, Layout, and Process Flow

The design of the aseptic areas and the supporting rooms is of utmost importance. For most applications there is a core area, positioned with supporting rooms leading to the core and allowing for exit. The most critical activities take place in the core that is the most "clean," and supporting areas to the core being held to lesser cleanliness standards. The core areas where the most critical activities take place include filling, lyophilization when done, and supporting operations to prepare and make available to the process sterile containers and stoppers, etc. The location/positioning of crimping equipment when applicable is flexible based on regulatory requirements and operational setup. This basic layout provides for sterile components, glass, drug substance, and stoppers to be fed directly into the core. Other "stuff" to enter the core not capable of being heat or filter sterilized such as some equipment,

and materials are disinfected in stages or encapsulated in previously sterilized gowns, gloves, hoods as attempted with personnel for entry. Their level of disinfection and aseptic condition should be equal or better than the environment in which they are used. From the core to the supporting rooms there is a cascading air overpressure for facilities engaged in filling nontoxic (to filling personnel) drug products. In conjunction with the cascading pressure differentials from room to room, there must be sufficient air changes per room to ensure nonviable and viable particulates are controlled to specified levels. The air is supplied through HEPA filters and the air flows, and exchange rates must be justifiable and aligned with regulatory requirements. Air flows from higher graded or classified areas to lower graded areas, that is, class 100, class 10,000, class 100,000 depending on requirements (USFDA guidance classification cited). Rooms themselves must be composed of smooth, nonshedding materials impervious to disinfectants or the exposure to ultraviolet radiation that may be used in the disinfection process.

Using this foot print as a supporting backdrop it is then necessary to superimpose the process flow dynamics to the facility. Drug substance in and drug product out. Materials and clean and disinfected equipment in and "dirty" equipment and wastes out. If the manufacturing process requires support by people necessary personnel must enter and leave. This is to be accomplished without compromising clean with "dirty" and within the confines of the physical plant and established room classifications. It represents the challenge to maintain the aseptic core and core supporting areas as contamination free. This is the complexity of aseptic processing that gives it the high risk status it is known for.

Sanitization and Disinfection

An integral part of any contamination control program is facility cleaning and disinfection practices. The program must be sufficiently robust to maintain the environment in a steady state of microbial control on the basis of criticality of operations and classification designation. The program must be designed to account for any routine variability to the day-to-day function and be flexible enough to respond to the nonroutine perturbations that may challenge the program. Trending and interpretation of both viable and nonviable control data will provide the evidence to make "mid-course" corrections in a well designed and implemented program. Fortunately there are guides available and experience to help determine anticipated frequency and concentrations of cleaning and disinfecting solutions to attain validation goals and maintain control. Adjustments can be made on the basis of data. Selection of agents should be made on the basis of usual, "normal," flora and control of biotypes. Application of disinfectants is typically done on a manual basis with regiments and schemes available from industry benchmarks and vendor information.

Much discussion has been devoted to the topic of rotation of disinfectants and the "need" to control the adaptation of microbes to the mode of action of a disinfectant. It is now generally understood and agreed that the ability of microbes to adapt to the mode of action of a disinfectant and gain "resistance" is negligible or nonexistent. However, it is acknowledged that by not rotating disinfectants there can be a "selection" process for those microbes not susceptible to the mode of action of the chemical of use. It has been shown and is logical that naturally resistant microbes can persist and potentially increase in number by not rotating disinfectants. This has been demonstrated with in vivo data of endospore formers in the presence of quaternary ammonium compounds versus sporicidal agents to which they are susceptible.

Environmental Monitoring System

Evaluating the quality of air and surfaces in a clean room environment should start with a well-defined and written program. PDA TR #13 revised (2001) (12) as a starting point will provide a variety of information to help develop a program suitable to your facility and aligned with industry practices. Other reference documents such as ISO 14644, clean rooms, and associated controlled environments should be considered (13). Methods employed should be qualified/validated prior to implementation. The number of sampling sites will vary depending on the design of the area and the clean room technology employed (conventional filling line, RABS, or isolator). An aseptic core utilizing a conventional filling line would

present the greatest control risk due to much more operator interaction and intervention. With employment of more restrictive technologies such as RABS or isolators, human contact would be reduced or virtually eliminated and hence the number of sampling sites could be reduced accordingly. The program should address all shifts and include all exposed surfaces of the room and equipment and include critical surfaces that come in contact with the product, containers, and stoppers. Sample timing, frequency, and location should be carefully selected on the basis of their relationship to the operations performed. Recent application of risk-based approaches to site selection and decision making has gained acceptance.

It is useful to utilize current approaches emphasizing good science and risk-based approaches to contamination control. Application of Quality Risk Management (QRM) approaches as delimited in documents such as ICH Q9, and tools that support such approaches can be utilized effectively for such purposes. Identifying contamination/risk points in your process stream and applying a QRM mindset will document the thought and support the process for establishing your program. Emphasis should be placed on contamination caused by operator interaction/intervention and at intervals in the process where there is the intersection of inputs and outputs into and from the core area. These transition points are most vulnerable to contamination introduction. These assessment points will be indicative of your operation, and there can be variability from line to line or between facilities. Each will exhibit its own "fingerprint" for usual microbial flora, their numbers, and location of recoveries, and is influenced by disinfection program, facility layout, work flow, that is, the total control program. This fingerprint is developed from trended data. Although some variability of such data is normal the level of control should be sufficiently robust and sufficiently detailed to recognize trends indicating a perturbation in control and potential risk. Implementation of a total control program provides sufficient control to maintain operations within guidance or regulatory levels.

Air Monitoring, Nonviable and Viable

Any comprehensive environmental control program should include both nonviable and viable monitoring. There has always been some discussion about the potential interrelatedness of the data generated from each activity; the position microorganisms need carriers such as nonviable particles for dispersal has won both skeptics and advocates. On that topic the evidence seems to support the premise that you must have particles for microbial transfer but not all particles necessarily carry microbes. This premise has also been reflected in the argument supporting both 0.5 and 5.0 μm particle monitoring. I do not think any one would refute the basic premise of microbes "piggy backing" on particles but to say HEPA (high efficiency particulate filters) filtered air is a significant source of microbes is questionable. In any case it is generally recognized that some level of interrelatedness is apparent and warrants the pursuit of control with both approaches, nonviable and viable contributing to the total program. A great injustice would be done by looking at the data so prescriptively that the value of the information is lost in the argument over the interpretation of the details. Examining the inside out approach to environmental control described earlier, core to lesser controlled areas, it is evident that monitoring data generated in areas of lesser control and consequently "closer" to an uncontrolled state are more difficult to interpret than data generated in more highly controlled areas, e.g., class 100 compared to class 100,000 since the frequency of testing tails off sharply as we move out from the class 100 area.

Data Interpretation

Environmental monitoring is for the most part not an exact science but it represents our best attempt, given the tools currently available to us, to help characterize an environmental control program. Individual data represents a static point at which the data is collected, a snapshot in time is the terminology sometimes used. The totality of that data, all the data points collected, typifies the level of control during the operation on any given day at any given time. No one data point absolutely defines the conditions or level of control of a fill or of the total program. If the total control program is designed and implemented appropriately no single data point would indicate a process breakdown unless there was a catastrophic failure. That scenario would be the best case situation verifying the program as implemented is working as intended.

Single events should typify an isolated incidence that could be assessed through investigation and risk assessment to determine impact. Since the level of control often varies on the basis of the type of technologies employed and operator interaction with that technology, some variability in data can be expected. Each data point and the collective array of data should be evaluated against the data history for the line, area, or process to determine consistency in control levels. Alert and action levels should be established and a mechanism to identify deviations and formulate responses should be clearly elaborated. Responses should be science-based and appropriate to the potential risk presented by the deviation. Responses should be holistic in approach as well as mitigating the immediate risk posed by any event keeping in mind the lag time between the event and the results.

Aseptic processing is based on the separation of the filling environment from potential contamination and any inputs to the process stream including operators. With the technologies available, and considering the conglomeration of life on this planet, this is essentially done in a somewhat arduous and cascading manner. The least areas of control are furthest from but connect to the greatest areas of control. The areas of greatest control are the class 100/class 10,000 core area, using USFDA terminology, which are the cleanest and the most free of contamination and furthest from the natural uncontrolled world. Through that connection and by devising increasing levels of environmental control outside to inside, from natural uncontrolled areas to highly controlled core area, maintenance of a class 100 area can be accomplished. Much attention is given to the control of that class 100 area and understandably so, but it is often overlooked or underestimated that inputs in your process stream including operators must traverse the lesser controlled areas to perform the end functions of filling and stoppering of the product. What happens upstream will decidedly have an impact on operations downstream. By sequentially cascading areas, from nature outside the physical plant to unclassified area inside the plant to various levels of classification, we achieve what is regarded as a suitable level of control of the area to accomplish aseptic processing. It is not considered the best of situations by regulators for product manufacture, but when the nature of the drug product does not permit sterilization by other accepted technologies, aseptic processing is permitted for manufacture of sterile products.

When something happens that impacts our control it should be detected. If it is not detected it may indicate we are not "measuring/monitoring" the appropriate indicators of change for the area. Since major control parameters can be more readily measured, it is usually the subtle events, those not directly measured or detectable, that impact control. That underscores the importance of looking for trends. Often our concentrated efforts are at the immediate point of focus and do not consider the control of areas and operations cascading from areas of lesser control, and as a result we struggle with appropriate corrective actions. Having said that, it is not reasonable that we monitor less critically controlled areas with the same rigor that we monitor class 100 areas but in an effort to get meaningful data from less controlled areas we must have greater assurance that what we do monitor from those areas adds value to the program. In many instances decisions of when and where and even possibly who we monitor in lesser controlled areas are not scientifically sound and not as well conceived as what we do for class 100 areas. Of course there is more prescriptive guidance on what to do in the class 100 area and frankly in some instances we have adopted such guidance to lesser controlled areas without thinking through our actions. Here is where the QRM process and a risk-based assessment can greatly enhance the value of our monitoring activity and make our control programs more robust. In numerous instances, using a QRM approach to improve control in our lesser controlled areas has decreased costs through reduced monitoring and manpower expenditures and at the same time increased its value because of acquisition of more meaningful data.

If data generated has greater capability to indicate perturbations in microbial control we should be able to respond in a more deliberate and effective manner. To do so this requires a more in depth review of data and a more frequent review of data. Not only is a response required at the point of deviation but one should also look at the potential impact upstream and downstream of that event. This expanded look and response could be proactive as well as reactive even if further contamination is not detected. We tend to be too focused in our reviews and responses on specific events and as a result subtle irregularities were not seen until they

became events and were widespread and problematic. This has been exacerbated due to the delay in getting data from monitoring to detect them they must grow. Reaction time is the cornerstone of control.

With our current capabilities even for the most controlled environments it is broadly recognized that interpretation of data in the vast amount of cases can not be directly extrapolated to product risk. To deliver value in most instances the entire control picture must be formatted and analyzed to get an understanding of what if anything is going on microbiologically, knowledge versus information. This necessitates looking at data in other ways then the daily format associated with the collection of information. Trends, departure from routine or expected data, can best be recognized by grouping information into related categories and looking for changes. Multiple approaches to grouping information can be used to assist with change identification. Groupings focused by room, by classified area, and by microbial characteristics are but a few. Microbial characteristics such as Gram reaction, Genus and species identification where and when appropriate, and numbers isolated in CFUs are common approaches. The transition between classified areas, airlocks, and pass-throughs are excellent areas where potential issues can be identified as they are developing. Changes in any one of these parameters may have significance in the overall microbiological maintenance/control of the facility. Frequency of review is paramount to identification of trends, events, or perturbations in the control program. Generally, the more data the better the capability of analysis but more is only useful unless it is relevant and if it is analyzed. Data collection for data's sake is not productive. If you have information then you must do something with it and that it adds value to the program or it is not worth collecting. If it does not add value to your control program then do not pursue it.

Where or when does environmental monitoring not add value? There is a movement of late to monitor areas remote from the classified areas. The data generated may be taken at long/infrequent intervals. Such data adds little knowledge to the maintenance of the control process and is likely to expend significant manpower and cost to generate the data. A well-devised and implemented facility cleaning and control program as described earlier will contribute more to maintenance of facility control than environmental monitoring. If microbial monitoring is to be done in support of unclassified area control, the application of microbial acceptance criteria to cleaning and sanitization validation would be appropriate. Routine monitoring at long intervals is not scientifically sound and adds little value. The best approach is to incorporate a risk-based approach to decision making when devising a total program. Use of tools like HACCP has been shown to be effective in such applications.

There are multiple regulatory or guidance recommendations for periodicity of data collection. Recommended frequency of data collection is generally higher in more controlled areas and less in lesser controlled areas. However, since facility equipment and personnel control is basically from the outside in, class 100,000 → 10,000 → 100 or grade D > C > B > A, whatever scheme you choose to follow, it might be advisable to monitor or review data with greater frequency in the outer areas to give a more dynamic picture and information that can be the key to response and subsequent control. What appears outside by design generally works its way to the inside. Actually frequencies of data collection and review should be flexible based on the operations they are intended to control and the data recovered for analysis. To maintain control in a cascading system, it is most effective to put an emphasis on the upstream process to mitigate issues before they get to your critical areas. This is a position that is now being stressed in guidance information but is not always heeded by QA/QC units. Regulatory requirements or guidance recommendations should be regarded as minimums and adjusted to fit your circumstances and total control program.

How to Handle Excursions

Like any deviation to requirements an investigation into the cause is expected. The extent of the investigation should be commensurate with the event and its proximity to the core A/B classified area or potential impact on the process. Investigations should have some consistency in approach or scope again based on criticality. Identified trends, alert level excursions, and action level excursions can be treated differently but must be defined by procedure. A rationale based on science and risk should support the position. Investigation should be systematic in

approach and of sufficient scope to determine potential impact both upstream and downstream of the event and between recently manufactured batches. All investigations having the potential for batch implication should be closed and reviewed by the quality unit prior to batch release.

Process Simulation—Media Fills

By definition media fills are designed to mimic the manufacturing process. They also represent an opportunity to collect additional data and knowledge about aseptic control mechanisms in your process. During media fills we are qualifying operators and stressing our processing environment by running our processes to their defined limits both upper and lower, both mechanically and by simulating in a condensed format the extremes of anticipated operating conditions. By doing added environmental and personnel monitoring during these exercises we can determine when and where risks are elevated due to the added interactions of personnel with the equipment. Not only do these activities test the robustness of your aseptic process, but the information obtained can give additional insight into where and when microbial risk arises with activities. From such data modification of location or timing of EM can be enhanced. Recall, however, that media fills are to represent the normal activities during the process and are not meant to justify practices that pose an unnecessary contamination risk. During routine aseptic manufacturing conditions you would want to do the minimum amount of monitoring since the activity in and of itself introduces a level of risk. Media fills give the latitude to explore that arena and apply leanings.

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4 Water systems for parenteral facilities

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INTRODUCTION

The most challenging pharmaceutical water applications are typically associated with parenteral products since, by their nature, parenterals are uniquely able to access bodily fluids and tissues. In this aqueous environment, water is uniquely suited to serve in a multitude of roles associated with drug development, testing, manufacture, and delivery.

Because of their unparalleled access to critical areas of the body, and compounded by their irretrievable nature, parenterals must meet extremely stringent requirements both in the United States and around the globe. Injectables necessitate the use of water that is chemically and microbiologically pure, to exacting standards, both from a practical perspective and based on regulatory dictates to avoid patient risk and to ensure product and treatment efficacy. As a product, excipient, cleaning agent, solvent, etc., water is used in significant quantities and, in many cases, it is the single largest volumetric commodity associated with any finished product.

This chapter will discuss the uniqueness of parenteral water applications including their current regulatory requirements. Discussion will focus on injectable risks, compendial limits for chemical purity, viable and nonviable microbial contamination, and added substances. Approved water treatment methods vary significantly throughout the world in spite of harmonization efforts and pose a significant hurdle for global firms wishing to reduce costs, consolidate manufacture, and standardize operations. As part of the discussion associated with approved methods of producing parenteral waters, common equipment types, basic system designs, operational challenges, and delivery/utilization issues will be reviewed. Of paramount concern is the ability to design, install, operate, and maintain a system that will consistently produce suitable quality water. Sanitization, testing, and monitoring are a few of the other key items that will also be addressed.

WATER GRADES

There is a considerable number of water grades used for pharmaceutical applications with varying regulatory requirements. The most significant of these are tabularized with their primary criteria in Table 1. These various grades are best characterized by their use, falling primarily into two groups, within which both bulk and packaged waters are defined. It should be noted that these two primary groups are identified by their role relative to parenteral products, such that one is specifically designated *for* parenteral use while the other is designated *not for* parenteral use. Hence, it will be most practical to begin the discussion of water types and their application by reviewing the two primary types of bulk water, followed by discussion of each individual packaged grade including the requirements that make each unique and specialized.

BULK PHARMACEUTICAL WATERS

Bulk waters are those waters produced by pharmaceutical manufacturers for use in or during production of their products and usually within their facility, while packaged waters are waters typically produced for incorporation into limited sized containers, most often one liter or less, and sold as a finished product for use in a multitude of applications ranging from extemporaneous compounding to laboratory testing. Packaged waters are most often sterilized to ensure that any residual microbial contamination does not multiply out of control, resulting in a compromised product or injured patient and owing to a general avoidance of preservatives.

Sterility is not required for bulk water, including Water for Injection (WFI); however, responsibility is placed on the drug manufacturer to ensure the safety and efficacy of their products. This reliance is monitored by regulatory bodies tasked with protection of the public health. The lack of a sterility requirement for bulk water is not based on a lack of concern but instead on the recognition that WFI in bulk form will often require additional processing, and

Table 1 Water Grades with Monograph Designation of Applicable Standard (Water for Hemodialysis Has Been Omitted)

	Water for Injection	Water, Highly Purified[a]	Bacteriostatic Water for Injection[b]	Sterile water for Inhalation[b]	Sterile water for Injection[b]	Sterile water for Irrigation[b]	Purified Water	Sterile Purified Water
Bacterial endotoxins <85>	<0.25 EU/mL	<0.25 IU/mL	<0.5 EU/mL	<0.5 EU/mL	<0.25 EU/mL	≤0.25 EU/mL	NA	NA
Total organic carbon <643>	Meets the requirements	Meets 2.2.44	NA	NA	NA	NA	Meets the requirements	NA
Water conductivity <645>	Meets the requirements	Meets the requirements	NA	Meets the requirements	NA	Meets the requirements	Meets the requirements	Meets the requirements
Packaging and storage	NA	NA	Specific requirements per monograph	Specific requirements per monograph	Specific requirements per monograph	Specific requirements per monograph	NA	Specific requirements per monograph
Labeling	NA	NA	Specific requirements per monograph	Specific requirements per monograph	Specific requirements per monograph	Specific requirements per monograph	NA	Specific requirements per monograph
Antimicrobial agents <51> & <341>	NA	NA	Meets effectiveness and Content	NA	NA	NA	NA	NA
Particulate matter <788>	NA	Clear and colorless	Meets the requirements	NA	Meets the requirements	NA	NA	NA
pH <791>	NA	NA	Specific requirements per monograph	NA	Specific requirements per monograph	NA	NA	NA
Sterility <71>	NA	NA	Meets the requirements	Meets the requirements	Meets the requirements	Meets the requirements	NA	Meets the requirements
Other requirements	NA	Specific Requirements per EP	Specific requirements per monograph	Specific requirements per monograph	Specific requirements per monograph	Specific requirements per monograph	NA	Specific Requirements per monograph
Microbial guidance <1231>	≤10 CFU/ 100 mL	≤10 CFU/ 100 mL	Meets the requirements	Meets the requirements	Meets the requirements	Meets the requirements	≤100 CFU/mL	Meets the requirements

[a]Column is only applicable to the European Pharmacopoeia 6.3 (USP designations are not applicable)
[b]Products produced from bulk Water for Injection (WFI)

sterility on such a large scale is difficult, if not impossible, to achieve and/or prove. This situation may be compounded by testing, often in uncontrolled environments. Hence, it is frequently more practical to control final sterility using one or more alternative methods such as portion sized terminal sterilization or sterilization via filtration, all of which can be assured at a higher level of reliability than the bulk counterpart.

Sterilization reliability is primarily based on statistical methods as absolute testing for sterility is effectively impossible with the technology currently available. As a result, sterility can be achieved but cannot be practically measured on a direct quantitative basis without compromising the sterility itself or destruction of the product. This is complicated additionally by the nature of microorganisms as living entities and their lifecycle tendencies including their reproductive processes, their resilience, their potentially unique and individual response to stressed conditions, and their ability to colonize, just to name a few of the traits that improve their survivability and make sterilization quite challenging.

Discussion of microorganisms and biofilm will occur further on in this chapter to provide a basic understanding of those challenges associated with biologic control of water used in, and for, pharmaceutical product manufacture, as well as the testing required to ensure the suitability of water intended for use in parenteral products. Traditional cultivative pour-plate methods that are typically employed result in lengthy delays before data is available, causing slower than optimal reaction to failures or anomalies and implementation of less than efficient quarantine practices to ensure safety and efficacy prior to product release. System configuration can affect the ability to obtain valid cultivative data compounding an already difficult situation.

Microbiologists familiar with pharmaceutical water system design, operation, sanitization, and, most importantly, the flora likely to exist in a particular system should be consulted when methods and practices are established for a new system and also throughout the life of the system, as changes can occur over time and as the system, and its resident biofilm, change.

Ultimately, controlling biofilm formation and growth are the most critical aspects of water system microbiologic performance, and certain designs are better than others in this regard. Only through knowledge and understanding can system designers, operators, and quality personnel assure that a system is suitable and functional for the intended purpose. In spite of what many may think, water isn't "just water" as it comes from the faucet, water is a critical utility without which pharmaceutical manufacturing might quickly grind to a halt, resulting in significant financial impact. Water may be designated as a critical raw material essentially manufactured on site and often used without the review and approval of Quality Control (QC), unlike that required for virtually all other raw materials. It is extremely important that adequate water is available for manufacturing but it is even more important that the water is of the required quality.

As mentioned, bulk water is primarily used during product manufacture; however, it is important not to overlook the fact that both WFI and Purified grades of water are also available in packaged form for extemporaneous and other uses. As such, there must be recognition and understanding of the difficulties associated with packaging, storage, and use beyond the confines of a controlled manufacturing environment.

Using the current US Pharmacopeia Revision 32 as our compendial reference, the two primary types of bulk pharmaceutical water are designated; Purified Water, USP and Water for Injection, USP. Each of these water grades is defined monographically and must meet specifications for quality and purity; however, certain critical aspects are left somewhat to the discretion of the drug product manufacturer, based on guidance provided in informational sections of the compendium. Often this guidance, which includes recommended standards, is enforced by regulatory agencies along with those mandatory specifications listed in the monographs themselves. Such is the case for microbial action levels (and alert levels although no numeric value is delineated), included in information section <1231> of the USP, as FDA enforces these at least as strictly as the actual requirements presented in the monographs.

It is worthy of note that the US Pharmacopeia is prepared and published by an independent not-for-profit corporation that is not affiliated with the United States government, yet the resulting specifications, called monographs, are recognized as law, based on action taken by the US Congress beginning with the Food, Drug & Cosmetic Act of 1938. The US Food

and Drug Administration (FDA), part of the Department of Health and Human Services, is tasked with enforcement of these specifications. However, the FDA does not have authority to make changes to these regulations or to promulgate new ones.

Purified Water, USP is defined within the US Pharmacopeia as "... water obtained by a suitable process." and "...prepared from water complying with the U.S. Environmental Protection Agency National Primary Drinking Water Regulations (EPA-NPDWR) or with the drinking water regulations of the European Union, Japan or with the World Health Organization's Guidelines for Drinking Water Quality (1)." This water must contain no added substances and must meet limits for total organic carbon per USP <643> and conductivity per USP <645>. As well, Purified Water must be protected from microbial proliferation and monitored using suitable action and alert levels to ensure control and use of only suitable quality water. Purified Water *is not* to be used in preparations intended for parenteral administration but rather, it is intended for use as an ingredient and in tests and assays as appropriate. The USP monograph for Purified Water does not include limits for endotoxin; however, the European Pharmacopoeia (EP) has recently added a grade of water designated as "Water, Highly Purified (2)" (Aqua valde purificata) that does include an endotoxin specification limit; however this new grade is not intended to replace WFI, but rather to create a grade between Purified and WFI. In addition, a number of companies, based on their product requirements, have implemented internal specifications that effectively add endotoxin limits to Purified Water specifications. Some of these Purified Water applications, such as for ophthalmic solutions (used during surgery for cataract removal or Lasik) and inhaled products, may require endotoxin control, based on the nature of the use.

WFI, USP is defined within the US Pharmacopeia as "... water purified by distillation or a purification process that is equivalent or superior to distillation in the removal of chemicals and microorganisms (3)." and, "... prepared from water complying with the U.S. Environmental Protection Agency National Primary Drinking Water Regulations or with the drinking water regulations of the European Union, Japan or with the World Health Organization's Guidelines for Drinking Water Quality (4)." This water can contain no added substances and must meet limits for total organic carbon per USP <643>, conductivity per USP <645>, and Bacterial endotoxin per USP <85>. WFI must be protected from microbial proliferation and monitored using suitable action and alert levels to ensure control and use of only suitable quality water. WFI is intended for use in the preparation of parenteral solutions. The chemical purity requirements (both conductivity and total organic carbon) for WFI are the same as those for Purified Water; however, WFI must meet the added requirement for bacterial endotoxin. In addition, the recommended microbial action and alert levels for WFI are 1000-fold more stringent than for Purified Water.

Table 2 summarizes the expectations both monographic (in green) and enforced guidance (in blue) for the bulk grades of pharmaceutical water per the current USP.

Note: USP <645> for conductivity is a multistage testing methodology that allows for compliance under three possible scenarios with the most preferred being stage 1; associated with on-line testing. Off-line testing can also be accomplished using stage 2 and 3 methods; however there is greater chance for sample error using these options. Off-line tests are designed to account for the effects of carbon dioxide and its resultant impact on water quality. Further details can be obtained by referring to the USP or, if appropriate, the applicable regulatory document from another locale.

Endotoxin is, more specifically, a component of the cell wall of gram negative bacteria and properly referred to as a lipopolysaccharide (LPS). These compounds are toxic, eliciting a fever when injected into a patient's tissue or bloodstream, hence the term pyrogen. Patient response can range from a rise in body temperature to a state of shock and even death. "The

Table 2 Summary of Requirements for Bulk Pharmaceutical Waters

	Conductivity	Total organic carbon	Endotoxin	Microbial (NMT)
Purified Water	Per <645>	Per <643> (≤500 ppb)	NA	100 CFU/mL
Water for Injection	Per <645>	Per <643> (≤500 ppb)	< 0.25 EU/mL	10 CFU/100 mL

term "endotoxin" is usually interchangeable with the term "pyrogen," although not all pyrogens are endotoxins, and pyrogen testing alone cannot be used entirely for detection and characterization of microbial endotoxins (5)." Hence, endotoxin must be controlled to the lowest levels possible or removed entirely from any water used in parenteral preparation to ensure patient health and safety. There are two primary test methods suitable for detecting these materials; the USP Pyrogen Test <151>, which is based on a rise in body temperature in rabbits, and the USP Bacterial Endotoxin Test <85>, also known as the *Limulus Amoebocyte Lysate* (LAL) test. LAL options include gel-clotting, turbidimetric, and chromagenic versions all of which rely on the reactivity of horseshoe crab blood cells. All of these test protocols must be performed off-line in a laboratory and require considerable time to produce results, making biologic control in a water system all the more critical.

In summary, Purified Water that complies with the USP can be manufactured using any suitable process and must meet the summary requirements listed above. On the other hand, WFI can be produced by distillation or alternatively, in the United States, the producer must be able to prove, via scientific methods and testing, that an alternate method is equal to, or better than, distillation when employed in a well designed and operated system. Coupled with the requirement by the EP to produce WFI only by distillation, these confines have dissuaded most from the challenge, rendering almost all WFI, produced for use in the United States, distilled, as well as, all WFI regulated by the EP. Notwithstanding, WFI must meet all of the summary requirements listed above for chemical and biologic purity as well as the inferred microbiologic levels enforced by FDA.

PACKAGED PHARMACEUTICAL WATERS

Packaged grades of water, including Bacteriostatic WFI, Sterile Water for Inhalation, Sterile WFI, Sterile Water for Irrigation, and Sterile Purified Water, are all sterile packaged waters typically produced from their bulk counterpart with further processing to meet the requisite monographic requirements and then packaged for subsequent use.

Packaged water can be contained in glass or plastic containers. The container configuration can be rigid as might be the case for vials, syringes, or bottles, or can be flexible as in the case of intravenous injection (IV) bags. Packaged water can be configured for single dose or for multiple dose applications provided suitable preservatives and labeling are employed. Container size, as mentioned, may be limited as is the case for Bacteriostatic WFI (30 mL max.) and Sterile WFI (1 L max.). Other packaged waters such as Sterile Water for Inhalation, Sterile Water for Irrigation, and Sterile Purified Water may be available in larger than 1 L containers based on their use (i.e., Sterile Water for Inhalation used for humidification or Sterile Water for Irrigation used in large volumes during surgery and often designed for rapid emptying). In spite of its sterile condition, Sterile Purified Water is not suitable for preparations intended for parenteral administration.

Glass packages are typically more inert and are traditionally considered to be more pharmaceutically elegant; however it must be understood that only certain grades of glass are suitable for parenterals. Like water, glass is also often designated on the basis of its relation to parenterals such that borosilicate glass is typically most preferred and Type III soda-lime glass is typically identified with the label NP, meaning "not for parenterals." Notwithstanding its benefits and image, glass is often more susceptible to breakage, is usually more costly and weighs considerably more than its plastic counterpart resulting in added cost for shipping, handling and losses from breakage. Plastic, on the other hand, although lighter and less expensive to produce, typically requires higher levels of testing to ensure the product will not be contaminated by, or absorbed by, the package material. Concerns relative to plastics often dictate extended testing for leachables and extractables and to verify product integrity.

There are two methods of producing a sterile packaged product. These methods vary significantly based on their primary techniques. The more highly preferred of the two is terminal sterilization because the product is first sealed in its container and then the entire unit (product and container) is sterilized. This is preferred since completion of the sterilization process produces a higher reliability of final unit integrity. Alternatively, products can be manufactured aseptically whereby a sterile product (e.g., water) and sterile components (e.g., container) are brought together in a controlled environment such as a Class 100 clean space for

final filling and sealing. Both of these methods are effective when properly implemented recognizing the product, container, and any other limiting variables that might be applicable; however, aseptic filling of water products is typically only utilized when terminal sterilization is impractical.

For terminal sterilization, there are five methods listed in USP section <1211>, including steam sterilization, dry-heat sterilization, gas sterilization, sterilization by ionizing radiation, and sterilization by filtration. Although this chapter is not intended to address each of these in detail, the suitability of sterilization method relative to water and its package becomes readily apparent. Dry heat sterilization that results in vaporization of the water or destruction of the container would obviously be inappropriate. As well, gas sterilization relies on permeation through the package with diffusion of the gas into the product. Hence, it is not suitable for all applications, possibly due to residual sterilant in the product or where the container would not allow this to occur, such as with glass vials or bottles or with liquid products such as water.

Alternatively, aseptic processing allows the water to be sterilized by a suitable process (i.e., filtration) and the container, be it glass or plastic, to also be appropriately sterilized before entering the classified space (ISO Class 5, EU Grade A, SI (Metric) Grade M3.5, or previously applicable FS-209E Class 100). The difficulty of aseptic processing remains in the necessity to design and maintain the conditions adequately to ensure a sterile environment.

It is important to note that absolute sterility cannot be tested without complete destruction of the product. Hence, statistical methods are required for determination of sterility and sterilization reliability. The subject of sterility and sterility testing is covered in the USP in detail as is also the case with other regulatory volumes throughout the world. Sterility will not be covered in extensive detail in this chapter.

PHARMACEUTICAL WATER SYSTEM DESIGN

Water used for parenterals (WFI) is produced by distillation in most cases, as previously noted. Whether used in bulk or further processed to create packaged versions, there are basic requirements for the systems that produce this water to ensure that it is of the proper quality and that it can be delivered to suitable locations for subsequent processing as part of a finished product, as a packaged water, for cleaning, for laboratory testing applications, or for other suitable purpose.

Regulatory requirements demand that water complying with EPA NPDWR standards serve as feed water for WFI applications. This requirement is typically considered to be a minimum such that often pharmaceutical manufacturers will include additional treatment steps or will use even higher quality feed water (including Purified Water) to ensure the highest chemical, microbial, and mechanical reliability for the systems that produce WFI. Hence, there are a number of system configurations that are able to reliably produce WFI, a few of which will be discussed in detail, further on in this chapter.

Design of a water treatment system typically requires an analysis of the feed water that will be employed. Although EPA NPDWR regulations are prescriptive, it is important to recognize that virtually no two water supplies are identical in the amount and types of chemical contaminants present. Water supplied from a surface source in the southeast may have significantly higher levels of organic contaminants and suspended solids than water from a deep well in the northwest. Alternatively, deep wells may carry higher levels of dissolved minerals than their shallow or surface counterparts elsewhere. All of these variations still fall within the acceptable confines of potable water meeting the standards for EPA NPDWR and are suitable as feed water.

As a result, the task of the water system designer is far more complex than simply selecting a single treatment regime based solely on flow rate or locale. Analysis of the water contaminants present in the feed stream will dictate the type of pretreatment required and may include components such as filters, softeners, reverse osmosis (RO) membranes, and even chemical injection units for applications ranging from the introduction of sanitants, such as chlorine, to injection of flocculants to improve the effectiveness of other unit operations in their contaminant removal.

Each of the pretreatment steps is typically employed to improve the functionality of subsequent downstream components with distillation typically as the ultimate final unit

Table 3 Contaminant Tests Commonly Applied to Evaluate Feed Water Supplies

1. Dissolved inorganics (cation and anion)
2. Resistivity/conductivity
3. Total dissolved solids
4. Silica and iron (reactive and nonreactive)
5. Barium
6. Strontium
7. Hardness
8. Alkalinity
9. Chlorine and chloramine
10. Total organic carbon
11. PH
12. Temperature
13. Silt density index
14. Particle counts
15. Bacteria level
16. Pyrogens
17. Dissolved gases

operation. For example, a coarse filter may be included prior to an RO membrane. Although the RO is capable of removing coarse suspended contaminants, it is far more cost effective to include a prefilter than to clean or replace the RO membrane on a frequent basis. It is these issues, relating to reliability and cost effectiveness, that often drives the component and unit operation selection process in a water system design.

Discussion of the details relating to feed water analysis is beyond the scope of this volume; however Table 3 provides a list of many of the more common contaminants evaluated during system design and upon which a treatment regimen is based. Not all of these may exist in every Feed stock but if present at, or above, certain threshold levels, these constituents can have a significant impact (usually negative) on the operation of a system inadequately designed for their removal or mitigation. That impact can range from unsuitable water being produced to a need for frequent service or cleaning that results in system outage. In either case product manufacturing can be severely restricted as a result.

On the basis of analysis of the feed water and with consideration given to the experiences and preferences of those at the manufacturing site, certain initial decisions will set the basis for the overall system design. For example, if the manufacturing site is located in the United States but the firm plans to produce products for distribution in the European Union, it is very likely that a single system, based on distillation, will be selected for both. Also, if the feed water contains chlorine (or chloramines) the system pretreatment must address its removal using appropriate methods. Assuming the use of cost prohibitive exotic alloys is inappropriate, most likely either granular activated carbon (GAC) or chemical reaction, possibly using a variant of sodium sulfite, will be most suitable. Carbon is excellent for chlorine removal and is also reliable for removing dissolved organics; however carbon is also notoriously difficult to control microbiologically because of the large surface area, resident nutrients, and sanitization difficulty. Alternatively, sodium sulfite can be successfully applied recognizing it is not a panacea and its use includes both advantages and disadvantages when compared to activated carbon. Therefore, the designer must evaluate all appropriate options, choosing the most suitable selection for a specific application. As well, other factors may play a significant role in the decision. For example, high organic contaminant loading in the feed water might indicate a preference for the use of GAC versus injection unless other factors are controlling. Hence, the use of a specific treatment technology may be more or less desirable based on individual system circumstances rather than being based on an ideal theoretical design model. Above all it must be remembered that there are almost always multiple approaches that achieve the same end result. For example, a GAC filter may be used when high organic levels are present; however, sodium sulfite combined with an organic scavenger resin may prove equally effective and more desirable under certain circumstances.

These types of process decisions are present at every step of system design; yet in many cases a suitable basic composite design can still be considered provided the selection is supported by technical review and confirmation for applicability. The following sample system designs are included below to depict common configurations that can be suitable for production of WFI. These are not meant to be universally applicable models but rather to offer examples of frequently used frameworks on which many successful designs have been based. The schematics have been simplified for ease of review and do not contain the level of detail necessary to configure a system properly. These options provide typical design scenarios ranging from very basic to relatively complex acknowledging a plethora of options in between and beyond.

Implementation of these designs can be additionally complicated by site requirements for automation, including data acquisition and trending. It should not be inferred that simpler designs are less costly, as again this may be affected by any number of variables associated with capital acquisition, installation, and system operation. Additional reference material is available in volumes written by authors such as Meltzer et al. (6), as well as in guides such as those produced by the International Society for Pharmaceutical Engineering (7) (ISPE).

Additionally, the cost to validate any pharmaceutical water system is significant, accounting for a large portion of the budget necessary for project completion. Validation, which more recently has been referred to as commissioning and qualification (C&Q), is the verification that a system can and does produce water of the proper quality and may include activities such as design qualification (DQ), installation qualification (IQ), operational qualification (OQ), and performance qualification (PQ), using tools such as commissioning, factory acceptance tests (FATs), and site acceptance tests (SATs).

The design depicted in Figure 1 would be best described as one of the more complex conceptual arrangements, as it provides feed water to the still that will usually meet Purified Water quality requirements. Hence, there is greater complexity with the inclusion of additional unit operations prior to distillation. This format uses RO to prepurify the water fed to the still, reducing the load on the distiller. This design is common when the facility requires both grades of water, based on feed water characteristics, or when dictated by economic or other factors. The common result of this design is a more robust pretreatment system with less challenging demands placed on the still. This robustness is only available based on added cost associated with the purchase and proper operation of additional equipment, such as the RO. Considering the high quality of the feed water presented to the still, in most cases, any type of distiller would function although it would be more likely for traditional distillation technology (i.e., single effect or multiple effect types) to be implemented based on the possible added cost

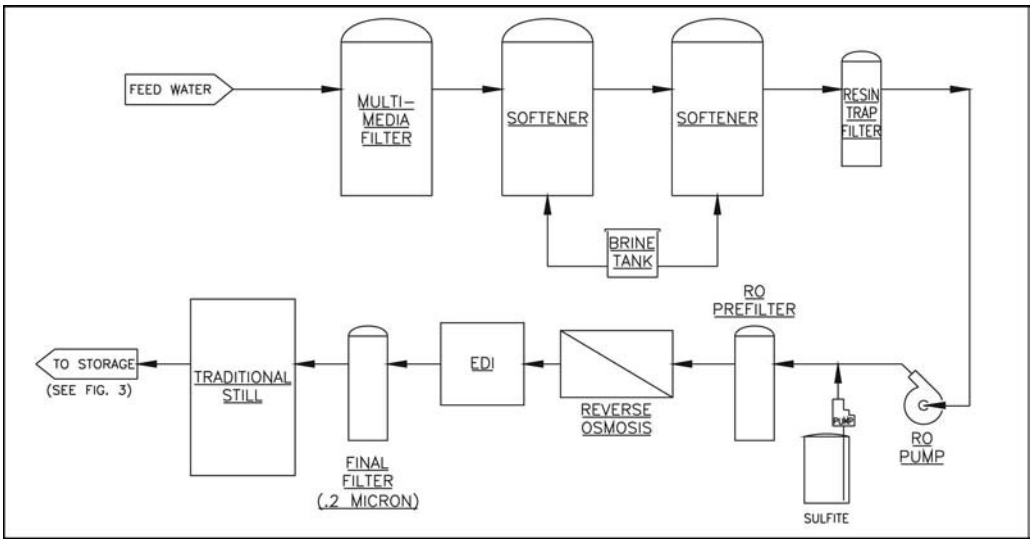

Figure 1 Schematic of a typical traditional still application with Purified Water feed.

associated with a vapor compression still. However, it should be noted that traditional distillation technology typically requires higher quality feed than vapor compression (VC) style stills, to ensure reliable operation, on the basis of operating temperature. For the design shown in Figure 1, the cost of a more complex pretreatment system with its associated higher operating cost partially offsets the lower purchase and operating costs associated with a traditional still design.

In summary, a multimedia depth filter removes coarse contaminants (typically an industrial version of the sand filter used for a backyard swimming pool) and supplies water to dual series softeners that in turn ensure hardness, which would foul the RO, and ammonia, which would pass through the RO are eliminated. The duplexing of this unit operation allows regeneration of one column without the risk of passing such contaminants to the RO. A common brine system, used for regeneration of the ionic softener resin, alternates between columns. A cartridge prefilter protects the RO feed pump from resin and fines while a pre-RO filter protects the membranes and provides additional filtration. The RO feed pump generates the pressure necessary for RO membrane operation with pressures typically in the range of 200 to 600 psig. Sodium sulfite is injected prior to the RO, to remove residual chlorine from the municipal source, which was allowed to remain since it assisted with microbial control in the pre-RO equipment. Most RO membranes are extremely sensitive to miniscule amounts of chlorine making its removal of significant concern. In addition, although not shown, pH adjustment may be required, or desirable, prior to an RO to improve overall performance. The RO produces permeate, or product water, and a reject or waste stream. Permeate is then treated by a continuous electro-deionization (CEDI) module that further improves the water's chemical quality. Since CEDI technology does not purport to control microorganisms, it is common to place a sterilizing grade filter (0.2 or 0.22 μm) after the CEDI module and prior to the distillation unit. In addition, although not shown, an optional ultraviolet sanitizer may also be present prior to the final filter to reduce the number, and likelihood viable organisms will populate the filter surface and eventually pass through it. Used in combination, UV prior to filtration typically lengthens the usable bacteria-retentive life of the filter. The final step in creating WFI using this configuration is a single-effect or a multieffect distillation unit from which WFI is supplied to a storage tank for distribution throughout the facility (Fig. 3).

The design depicted in Figure 2 is generally regarded as one of the more simple conceptual arrangements based on the inclusion of limited unit operations prior to distillation.

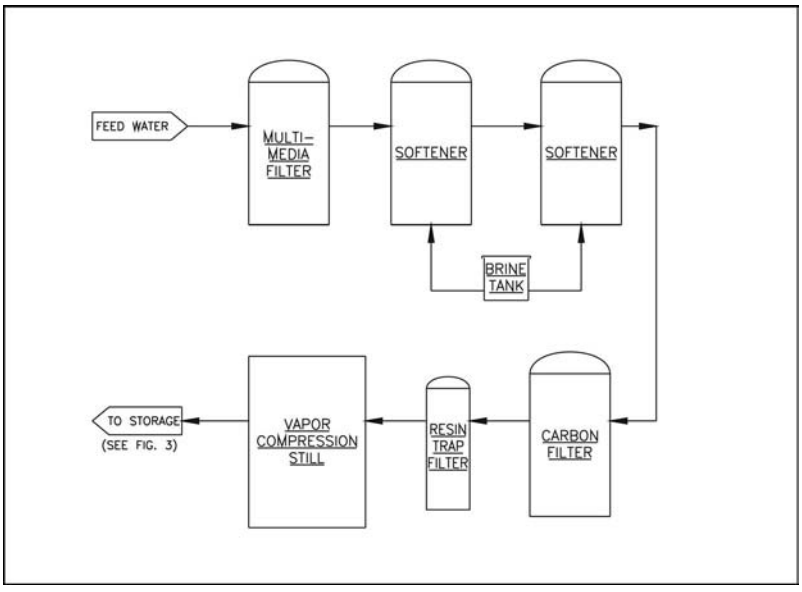

Figure 2 Schematic of a typical vapor compression still application with softened water feed.

The ability to limit pretreatment is driven primarily by the selection of a vapor compression distiller, which is less likely to become fouled by certain ionic constituents in the feed water than a traditional distiller, on the basis of operating temperature. However, it should be recognized that this equipment may be associated with a cost premium possibly based on purchase price and/or operating cost. Hence, like the previous example, the savings associated with the purchase and operation of less complex pretreatment may be partially offset against increased capital and/or operating cost associated with a more complex still design.

These simpler WFI system designs (Fig. 2) include only basic pretreatment, usually consisting of back-washable media filtration followed by dual series water softeners, similar to that of the previous example used for pretreatment prior to a traditional distiller. The media filter, as in the previous example, is used to remove suspended contaminants, common in potable water, in the size range of 10 μm and larger. The subsequent softeners are resin-based exchange type devices used to remove hardness, including calcium, magnesium, etc., to minimize the potential for downstream scaling of the still. The softeners in both examples exchange hardness for sodium ions that are less likely to impact subsequent downstream components. If hardness were not removed, components such as the RO and still would experience the residue buildup similar to that occurs on residential plumbing fixtures such as sinks, tubs, and showers. Again a post-softener cartridge filter is commonly included for removal of any resin fines that might be generated as the resin degrades and to protect in the event of a catastrophic resin release resulting from softener failure. Unlike the first example, RO and CEDI are usually not required to protect a VC distiller; however, this is confirmed during feed water analysis.

An important situation that cannot be overlooked relates to chlorine and/or chloramines that are commonly present in the feed water as part of a sanitization regime implemented by a municipality (or other supplier). Chlorine has been used extensively since the early 1900s; however, the carcinogenic effects of resulting trihalomethane by-products and other issues have resulted in increased use of chloramine (primarily chlorine and ammonia) by municipalities and other organizations for sanitization of potable water. It should be further noted that chloramines may be more difficult to remove than free chlorine. Chlorine, especially at the elevated temperatures within a still, will result in corrosive attack of the stainless steel construction hence it must be removed prior to distillation. Whereas the chlorine in the first example is addressed by sodium sulfite injection this example includes granular activated carbon (GAC). However if chloramine is present, it should be noted that ammonia is a by-product of chloramine removal in a GAC, which is not removed during distillation. Hence positioning of the GAC upstream of softening eliminates this concern as ammonia will be removed by softening, although at the expense of dechlorinated water in the softener. Softener bed capacity must be carefully considered when ammonia removal is important based on ion removal order relative to hardness and ammonia. The addition of a GAC (regardless of placement) makes the system slightly more complex and must be considered during capital and operational cost comparison/evaluation.

Product water from the still is again fed to a distribution storage tank which in turn feeds distribution sites throughout the manufacturing area(s) where WFI is further processed or used for formulation, cleaning, etc. This distribution gives rise to additional significant concerns relating to both chemical and microbial quality maintenance and will be discussed further below.

The design examples presented above do not include schematics for distribution, as distribution for either design would probably be very similar for the same application. Notwithstanding, distribution is a critical part of any bulk system design as it requires its own specialized features and poses its own challenges to the designer. Not only must it comply with all the appropriate good practices, but it must be able to blend into the building structure seamlessly to avoid conflicts with functional utilization including process and occupancy-related issues. Distribution design often makes it very difficult to interface easily with other utilities such as heating, ventilation, and air conditioning in a three-dimensional setting such that the cubic volume of the facility is not unreasonably reduced or compromised. Distribution must efficiently and effectively deliver suitable quality water at appropriate pressure and temperature and in volumes commensurate with manufacturing or process requirements.

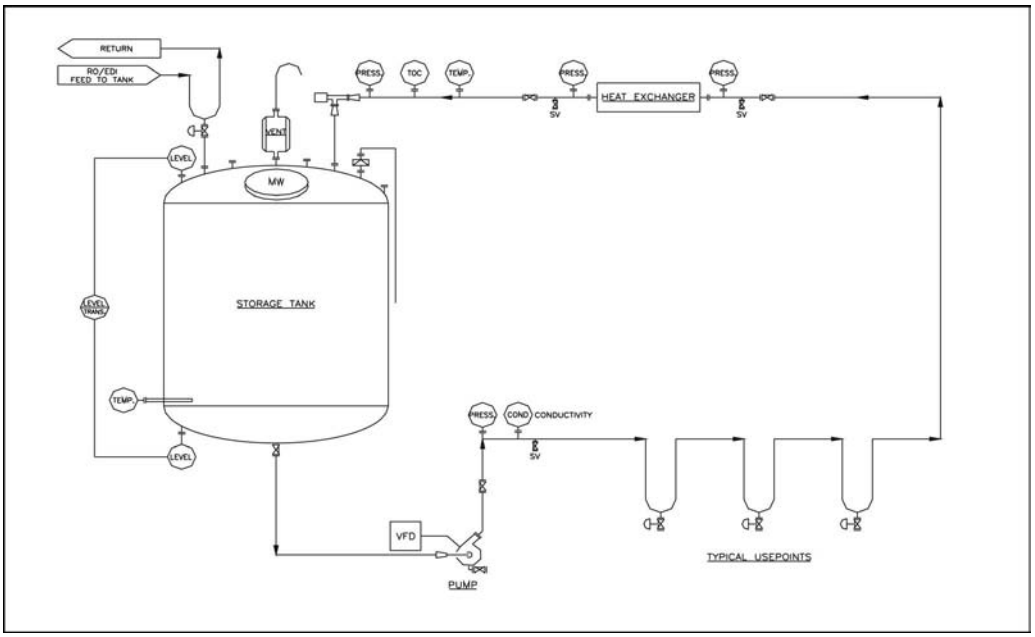

Figure 3 Schematic of a typical WFI distribution system.

Distribution must allow for monitoring of the water so that quality attributes can be ensured and as well practical controls must be available to operations personnel to avoid the use of water that does not conform to necessary requirements. This necessitates the inclusion of monitoring devices and instruments suitable for determining the water quality, comparing those attributes to an accepted value and controlling various features or operations to ensure unsuitable water quality is not delivered for use in production-related activities. Certain components must be strategically located for access and for ease of service and calibration while minimizing downtime that would result in lost productivity. A typical WFI distribution schematic is shown above in Figure 3. It is important to recognize that typical distribution systems contain no provisions for correcting WFI deficiencies (retreating the water) hence the distribution must be configured such that it does not reduce water quality below acceptable levels either as the result of poor design or because operation and/or maintenance is inappropriate.

Schematically, certain unique features in a pharmaceutical water system may be readily apparent. For example, user points are not shown as branches from a main header as is common in traditional industrial, residential, or commercial piping systems. They are represented as typically installed in a WFI system where the piping is looped and water recirculates as close to the actual delivery point as possible with continuously flowing water, often only fractions of an inch away from the actual point of utilization (see Fig. 3). This practice reduces the potential for water stagnation but significantly increases both the size and complexity of most WFI distribution systems based on increased pipe length and often as a result of increased pipe diameter. Other unique aspects of WFI distribution include complete drainablility based on pipe pitch, targeted flow velocities, and specialized materials, connectors, and finishes as well as design that is suitable for sanitization.

Some of these "best practice" aspects of water system design, including materials and finish, will be discussed further. Flow velocity will be partially addressed adjunct to the following biofilm discussion; however, it should be noted that target design velocities range from above five feet per second (FPS), measured directly as velocity to simple establishment of turbulent flow based on a calculated Reynolds Number. The use of Reynolds Numbers, a dimensionless numeric, has recently become popular in spite of the lack of agreement as to exactly what value represents turbulent flow (figures often used range from 2500–5000).

Notwithstanding, flow velocities using Reynolds Numbers result in significantly lower system flow rates than the previously applied velocities ranging from 3 to 5 FPS. For example, a 3" OD sanitary distribution network designed for 5 FPS velocity results in water flow exceeding 101 GPM. Alternatively, use of a Reynolds Number of 3000 results in water velocity of less than 0.136 FPS and flow of 2.7 GPM; more than a 35-fold variation in design flows.

In spite of these changing trends, designers must consider all facets of good design to ensure the piping remains flooded, is capable of sanitization and minimizes biofilm formation, all while not adversely affecting water quality, either chemically or microbiologically, and while remaining capable of delivering proper water volumes, temperature, and pressures as required. Good piping practices usually dictate the inclusion of pressure gauges, sample valves and isolation valves before and after each major system component to enable rapid diagnosis and remediation of any system anomalies that occur.

Sampling can also have a profound effect on distribution system design based on facility design and operation as it is commonly accepted that water used in manufacture should be sampled in the same fashion as it is used. Hence, piping that is direct connected to vessels, tanks, or equipment poses an additional challenge for sampling that must be overcome. Sampling for process control will often result in procedures that are different than those used as part of a quality program related to ensuring products meet requisite standards and specifications.

Best practices for sanitization design are also the subject of debate; however, it is never inappropriate to include the flexibility to utilize multiple methods of sanitization in the event one method fails to deliver acceptable results or alternative methods are required. The primary methods of water system sanitization include periodic or continuous application of heat using hot water or steam (most popular for WFI Systems), periodic chemical sanitization (less favorable), and the use of ozone where the main volume of stored water is continuously ozonated and the distribution network is periodically ozonated (not currently applied to WFI). It should be noted that WFI systems designed primarily for heat sanitization can also be sanitized with chemicals including ozone provided the design is appropriate and proper controls are implemented to assure no residual that could adversely affect WFI quality remains after completion. Both chemicals and ozone can also be implemented on an ad-hoc basis in suitable locations provided the system hardware is capable of contact with these materials.

Heat sanitization is regarded as the most reliable as heat is able to penetrate through even a substantial biofilm; however, heat is typically unable to remove the dead biomass leaving a readily colonizable nutrient rich base for future generations of organisms. Hence, the use of supplemental methods may be required as systems age and biofilm develops and evolves.

Finally, the issue of system drainability is often misunderstood as the reasons for draining can vary significantly. In some systems, draining occurs on the basis of intermittent use and the speed, and ease of draining results in lower cost and higher process reliability. Alternatively, systems that undergo periodic sanitization with chemicals must be drained to remove residual sanitant with drainability again lowering cost and improving reliability. However, WFI systems that are steam sanitized require drainability to assure removal of steam condensate that may be below sanitization temperature. Trapped condensate within the system may compromise the sanitization effectiveness by creating "cold spots" that are not fully sanitized and can result in system microbial recontamination or rebound based on system operational characteristics. For systems that operate continuously heated, the requirement for drainability is less critical as only during periods of outage for service or maintenance does drainability become a concern.

As previously indicated, in addition to the primary (pretreatment) and secondary (distillation) treatment steps to bring the water quality to acceptable levels, other important functions must be performed, such as maintaining the quality of the water after it is produced and testing to ensure compliance. These additional requirements increase the complexity of any bulk system dramatically. Maintaining large volumes of water to precise chemical and microbiologic standards can be very difficult as contact with air, piping, equipment, etc. will result in rapid deterioration of the water quality. As a result, specially designed components, expensive materials, and costly processes are usually required to ensure maintenance of the water quality.

For example, WFI is usually maintained at 165°F (equivalent to approximately 74°C) or even higher to reduce microbial proliferation and to maintain suitable microbiologic control. This method of bacteria control is costly from both an operating perspective and because it limits the materials of construction, increasing the system capital cost. Designs usually include a recirculated loop piping configuration, which helps to maintain temperature, for the purpose of minimizing bacteria and biofilm-related problems, but with a resulting cost increase associated with greater energy use, increased piping length and size, and higher installation costs. These and many other specific design practices associated with WFI systems increase complexity, cost, and maintenance, and unfortunately, few options are currently available to the designer to eliminate or even reduce these common "good practice" requirements.

Costs associated with WFI systems are extremely high when compared to comparable commercial or industrial systems of similar size. This is not the result of a single expensive component or technology but rather is the result of a multitude of contributing factors that build incrementally on each other. For example, valves cannot just be stainless steel (SS), nor can they be simply 316SS, they must be 316LSS. In addition, they cannot have just sanitary end fittings; they must be sanitary throughout often including areas of nonproduct contact. Furthermore, they must be certified with support documentation that traces each subcomponent to its original source, including verifications of manufacture, finish, and any other pertinent details. These requirements are necessary for virtually every component in the system driving the cost as a result. As well, the installation of WFI systems usually involves a more highly skilled work force and the use of specialized tools is often mandated to assure the quality of the completed installation.

In addition, it is commonplace for WFI systems to be constructed of sanitary stainless steel beginning with portions of the still and including components such as vessels, pumps, valves, instruments, and all other WFI contact components. Sanitary materials typically include special ends for joining components that are more hygienic than industrial joining methods such as threads or flanges. These components are usually polished to finishes of 32 μin. roughness average (Ra) or better with some designers specifying better than 10 μin. Ra finishes, supplemented by electrochemical polishing to obtain mirrorlike reflective surface conditions. Sanitary piping is, in actuality, not piping at all but rather sanitary tubing. Piping is simply the generic reference term used based on common parlance. In simple terms, pipe is industrial quality cylindrical material used to transport many fluids. It is measured nominally and designated by its interior diameter such that 1 in. diameter pipe has an inner diameter of approximately 1". Conversely, based primarily on the precision required for polishing, sanitary tubing is used for WFI applications and indeed for most sanitary applications. Sanitary tubing that is designated as 1" in diameter has an actual exterior diameter of 1" as opposed to the approximate inner diameter used to measure pipe. Sanitary fittings are typically fabricated from the same precision tubing to ensure exacting alignment during assembly, which further results in smooth interior surfaces that are cleanable and drainable with fewer large gaps into which microbial growth can extend.

Stainless steel finishing itself is an extensive subject and is beyond the scope that can be effectively covered within this chapter. However, it should be noted that most finishes are mechanically applied, progressively, using increasingly finer abrasives, similar in concept to that used for wood sanding. Higher quality finishes typically require application of electrochemical polishing, or electropolishing usually over a high quality mechanical finish. The reverse of plating, electropolishing, also known as chemical machining, removes surface material to create an extremely smooth and attractive finish that may be easier to clean based on the materials in contact with the surface. In addition, electropolishing creates a passive layer on the surface of the stainless steel, increasing its corrosion resistance. Passivation will be discussed in more detail below.

Another aspect of WFI system complexity is that of the specialized welding required, which serves to reduce the number of mechanical joints and as a result reduces maintenance costs and minimizes sites for possible leaks. Unlike the traditional welding methods with which most of us are familiar (manual welder wearing a welding mask or shield as protection from an exposed arc), the process employed for WFI piping is automated and typically enclosed. The computer controlled welding power source works in conjunction with a

precision automated welding torch that encloses the area around the weld joint. The equipment precisely controls all facets of the welding process, including voltage, current, rotational speed, arc gap, and time, at a level unmatched by a manual operation. When supplemented by specialized pipe cutting and end preparation tools, proper procedures and inspection equipment, virtually flawless conditions can be repetitively achieved.

Orbital welding, as this process is known, removes the variability associated with manual welding but requires skilled labor that is trained in all facets of sanitary installation to ensure system integrity. The cost of the welding equipment is substantial, far higher than similar industrial machinery; however, the welding machine is only a portion of the complement of related tools needed to complete the work. Additional specialized tools are usually required such as precision cutting and facing machines, gas analyzers, tungsten grinders, and specialized inspection devices used to view the interior of the tube after it has been welded. These ancillary components can easily exceed the cost of the welding system by two to four times.

Inspection devices, also known as borescopes or videoscopes, are similar to the endoscopes used by medical professionals. A miniature video camera is attached to the end of a fiber-optic cable that in turn is attached to a video processor with recording capability. The scope can be inserted into the tube, prior to installation, to confirm the interior finish and can also be used to view the completed weld for conformance to the specification. These devices are often in the range of 25 ft in length, based on the length of standard tube sections, and to negotiate bends (elbows or tees) many scopes are equipped with articulation. Articulated movement allows the operator to navigate sections of installed tube with minimal potential for damaging the interior finish.

It is also noteworthy that continuous quality monitoring has become the norm for WFI production relying heavily on electronic instrumentation and controls that are typically integrated into the system. These devices, although typically not mandated by regulation, allow for more consistent and reliable quality tracking and may in the long run be more cost-effective than other alternatives. Continuous monitoring can be accomplished for a significant number of attributes including conductivity, TOC, temperature, pressure, flow, and level using in-line, at-line, or on-line equipment. Other data important for ensuring the proper operation of individual unit operations may be necessary or desirable. Hence, it is not uncommon to find pH, chlorine, oxidation reduction potential (ORP), and ultraviolet intensity monitors as part of the water treatment monitoring regimen, or even to find dissolved ozone monitors as part of a feed water sanitization program. Unfortunately, instrumentation for continuous microbial monitoring (enumeration, detection and/or speciation) is not currently available although a number of rapid microbial detection systems have evolved that can speed the traditional and laborious work associated with pour plates, incubation, and colony counting. Laser detection systems, based on light scattering technology, currently in development and testing and may eventually be capable of performing these functions and may prove to be viable options in the future. Interestingly, process analytical technology (PAT) was in use for pharmaceutical water systems long before the FDA's risk-based initiative with PAT was implemented.

MICROBIAL CONSIDERATIONS AND SANITIZATION

It has been said that the chemical purification of water is by far the easier part of producing WFI, while the microbial control aspect is far more difficult. The reason for this is primarily because the technologies used to chemically purify water are well known and understood, proven through years of application and use, and are mechanically and operationally reliable. Coupled with limited sources for chemical recontamination that are relatively easy to control, the chemical purity of water can be readily achieved and maintained. Alternatively, microbial contamination is mostly unseen, monitored using random grab-samples that are typically not representative of actual conditions and often misunderstood by engineers responsible for water system design. Organisms can react to their environments, such that many can survive in low-nutrient environments and under stressful conditions. Organisms can exist as planktonic entities floating unprotected in a water stream, and they also have the ability to attach themselves to surfaces, no matter how smooth, in search of nutrition. Attachment results in the creation of a biofilm that serves to protect and insulate the organism from sanitants

while providing a relatively nutrient-rich environment in which reproduction can safely occur and from which further colonization can originate.

Initially organism attachment to a surface is relatively weak; however, once stationary (sessile), the microbe quickly begins to produce a sticky polymeric material known as glycocalyx, which more firmly attaches the organism to the surface and helps it adapt to sessile existence. This conversion will occur fairly rapidly, primarily dependent on the species present and the level of nutrients, such that transition can take hours in some cases and days in others. As reproduction occurs, additional strongly attached cells develop forming a community that provides further protection and benefits for the occupants as the sticky extracellular glycocalyx snags floating nutrient particles, other planktonic organisms, and even flocs of biofilm that may have come loose and become free-floating.

Much like the water itself, biofilm development is seldom similar from site to site. The biomass that develops is based on the nutrients present and the affinity or adaptability individual species have for that available nutrient base. Biofilm will be somewhat the product of local conditions; for example, in stagnant or slow-moving water, biofilm will tend to grow taller and further into the water stream exposing greater surface area. However, this growth is far more susceptible to shear forces that might occur during water use, sampling, flushing, or as the result of other system events such as water hammer. Alternatively, in fast-flowing turbulent water biofilm will tend to be denser, with less exposed surface that would be susceptible to disturbance from the action of the water movement. Biofilm provides a secure stationary base for organisms and is not directly monitored by traditional sampling. The results of such water sampling typically only represent a small portion of the actual microbial content in a system. In other words, traditional sampling is based on "grab" samples of water rather than sampling of the resident biomass. The reason for this dichotomy may be the result of misguided thinking or may be reaction to the difficulties associated with more suitable sampling techniques and test methods. However, regardless of the reason, the fact is that far more organisms are likely to be resident in biofilm than floating in the water stream. This will be true of most systems except those that are under continuous sanitization conditions such as systems that are operated at elevated temperatures (heated) constantly. Various rationales have been offered for the current testing methodologies, with the only appropriate justification based on a continuously sanitizing environment, where biofilm would expect to be virtually nonexistent as a result of the hostile conditions.

In fact, biofilm development in most systems undoubtedly complicates sampling methods such as might be the case when dense tightly adhered biofilm results in few, if any, planktonic counts during routine testing, incorrectly interpreted as very low levels of microbial activity. Other anomalies in the same system may additionally confuse and cloud analysis, when sudden and unpredictable release of sections or flocs of biofilm into a sample indicate significant counts far above those seen during routine tests.

Excellent and concise summaries of water system microbiology have been written by T.C. Soli; as part of *Microbiology in Pharmaceutical Manufacturing* (8) and for the upcoming revision to the *ISPE Water and Steam Baseline Guide* expected to be released in 2010, subject to FDA review and final approval. These summaries provide nonmicrobiologists with a clearly written, simple, down-to-earth explanation of microbial concerns relative to water systems written by an expert with years of practical experience in pharmaceutical manufacturing, consulting, and as a member and vice chair of the USP Expert Committee on Pharmaceutical Waters.

As noted, heat is the sanitization method most commonly employed during WFI production, storage, and distribution for microbial control. Those wishing to either employ RO or to operate their systems below 65°C must address difficult issues, such as the limited ability of RO membranes and the most common RO equipment to operate hot continuously. As well there is a perceived regulatory expectation to dump unused WFI after 24 hours if it is not heated above 65°C for sanitization.

Other common methods of sanitization that may be appropriate for less critical applications are usually not deemed acceptable for WFI. However, industry convention and regulatory interpretation that has banned the use of ozone for sanitization of WFI, even though it has become common for Purified Water application, is currently being reevaluated, and

changes may ultimately result. If this is indeed the case, the cost of producing WFI will most likely drop, based primarily on energy savings and supported further by "green" initiatives and "carbon footprint" reduction.

Previous concern relating to ozone use was based on the dictate in the WFI monograph that precludes added substances. In the past, this had been inappropriately interpreted to mean that no chemicals or materials could be added to water destined to become WFI. This has since been clarified so that it is understood that any substance added to the water to facilitate its treatment must be removed prior to use and that adequate monitoring and documentation is required for confirmation of its removal. On the basis of ozone monitoring limitations, controversy still remains regarding whether detection limits for ozone are adequate to ensure WFI safety. There has also been previous concern since earlier, now superseded, USP language existed stating that distillation (or RO if approved) must be the final treatment step to produce WFI. Hence, since ozone is a chemical oxidizing agent that would be added to WFI, after production, for the express purpose of maintaining biologic control; many believe that ozone addition would not meet the intent of having distillation as the final process step. These issues are currently the subject of many industry discussion groups as they try to come to grips with drug manufacturing cost and the FDA's Pharmaceutical cGMP's for the 21st Century: A Risk-Based Approach, and PAT initiatives.

MATERIALS OF CONSTRUCTION

The primary material of construction for the vast majority of WFI systems is stainless steel of the 300 series, typically 316SS. This austenitic alloy is resistant to rusting and many other forms of corrosion associated with water applications provided chlorine and chlorides are not present. Stainless steel is generally considered to be corrosion resistant and easy to fabricate. It can be polished to present a uniform, smooth, and pleasant-looking reflective surface that is considered cleanable and hygienic. Stainless steel is relatively inexpensive when compared to more exotic alloys and is readily available in the 316L alloy configuration most suitable and accepted. When produced and utilized in this low carbon "L" grade version (316LSS), it is additionally resistant to forms of corrosion that may occur as a result of field construction such as that which might be required to build or assemble a WFI system within a pharmaceutical manufacturing facility, making 316LSS by far the material of choice for WFI-related applications.

Stainless steel is a unique alloy with iron as the predominant component, yet it is corrosion and rust resistant based primarily on the alloying constituents added that instill its special properties. The mechanism that makes stainless steel suitable where other iron-based alloys would fail is termed passivity. Passivity is a naturally occurring surface oxide resulting from chromium used as an alloying material. The chrome oxide layer that develops is extremely thin, typically in the range of 5 to 50 Å (1 Å equals 1 ten-billionth of a meter) yet, except for extremely corrosive environments (including chloride attack), this very thin covering is adequate to protect the material from many common corrodants.

The passive layer that protects stainless from corrosion forms in contact with air and can easily be disturbed during the manufacturing process (by tools and abrasives), during the installation process (by welding and handling), and during use (as a result of high-temperature operation, high flow velocity, and due to the chemically aggressive nature of WFI). Hence, it is common for materials, equipment, and systems to undergo passivation procedures to expedite and enhance naturally occurring passivity. These procedures can take any number of forms; however the goal is to recreate or strengthen the natural passive layer and to reduce the time required before the material is suitable for use. Passivation or repassivation procedures typically involve either submerging the parts or, as may be the case for large systems, filling the components and recirculating the required solutions, both for a suitable time and at a suitable temperature to achieve the desired result. Procedures usually include a caustic cleaning step to remove oils or other contaminants, followed by contact with an acidic solution to remove surface iron. Rinsing with purified water ensures no residual chemicals remain, which might impact either the stainless or the WFI. This procedure allows the passive layer to form more quickly and to be more robust.

Finishing or polishing of stainless steel, as discussed in the preceding text, is often a hotly debated topic relative to pharmaceutical application. Some believe that the smoother surface results in slower and less tenacious biofilm development; however, this is not supported by microbiologists who believe that even the smoothest finishes available only minimally delay biofilm development and have minimal impact on organism attachment. Notwithstanding, most pharmaceutical and biopharmaceutical equipment receives some degree of polish to improve the appearance and ostensibly to improve cleanability. It is commonly agreed that large-scale surface imperfections either in material surfaces or at connection points afford microbes a safer haven from sanitants and hence should be avoided. The disagreement typically resides in the definition of "large" such that one school of thought is to avoid crevices larger than the organism itself, dictating extremely smooth surfaces, while the alternative posits that attachment will occur regardless of the surface finish inferring that only minimal surface preparation is needed. Both schools of thought agree that minimizing mechanical joints is prudent and where necessary the use of sanitary connections is recommended.

TECHNOLOGIES SUITABLE FOR PRODUCTION OF WFI

As mentioned, there are a limited number of production options currently available for WFI. For example, in certain regions of the world, RO technology is permitted for use in producing WFI. However, as noted, only the use of distillation is permitted by the EP for WFI production. Hence, any firm wishing to employ RO to make their WFI will find it either more challenging or even impossible where regulations insist that alternatives to distillation be proven technologically for each and every application.

When produced by distillation, there are two primary technologies used for the manufacture of WFI. The first is traditional distillation wherein an evaporator and a condenser are connected in series to first evaporate the feed water to steam, leaving behind any contaminants, and then to condense the pure steam that has formed to water of WFI quality. This simplified explanation is intended to offer the reader only a conceptual understanding of a far more complex process that requires separation of contaminants from the pure steam and will include either the use of rising film or falling film evaporation, as well as other technologies appropriate to the design.

A variation of this design includes the connection of multiple evaporators to increase efficiency. These additional evaporators or "effects" use the pure steam that is produced to generate additional capacity, thus reclaiming energy that might otherwise be wasted. Units ranging from 3 to 6 effects are common based on utility steam pressure/temperature; however stills with 7 or more effects are possible although often the savings cannot offset the added cost of the required equipment. It is critical to note that this process, termed "multieffect" distillation, is not a multidistillation process as the water is evaporated to steam and condensed only once, no matter how many effects are employed (Fig. 4). Traditional distillation typically involves no moving parts (with the exception of valves, etc.) and is driven most often by utility steam although electric and superheated water driven units are possible. Product from this type of distiller is typically at/or above 185°F and near ambient (or atmospheric) pressure.

Traditional multiple effect distillers typically require greater levels of pretreatment, owing to the higher operating temperature; however, this higher temperature is an advantage when it comes to pyrogen destruction.

Alternatively, VC distillation is a technology that includes an electrically driven compressor within the still to increase the initial pure steam pressure (by 1–3 psig) and temperature and the resulting higher-energy steam is used to generate additional capacity (Fig. 5). This still technology is typically configured to produce WFI between 80 and 85°C but can produce water at lower temperatures more efficiently than traditional distillation with outlet temperatures for "cold" WFI normally 6 to 15°C above the feed water temperature. Noncondensable gases are of greater concern for VC stills and as a result most are equipped with either a feed water deaerator (decarbonator) or a vent condenser. VC distillation, however, typically requires significant amounts of electricity to power the compressor, which partially offsets the lower steam consumption common for this type of equipment. As well, the compressor adds a level of mechanical complexity and is a source of added maintenance beyond that required for traditional distillation.

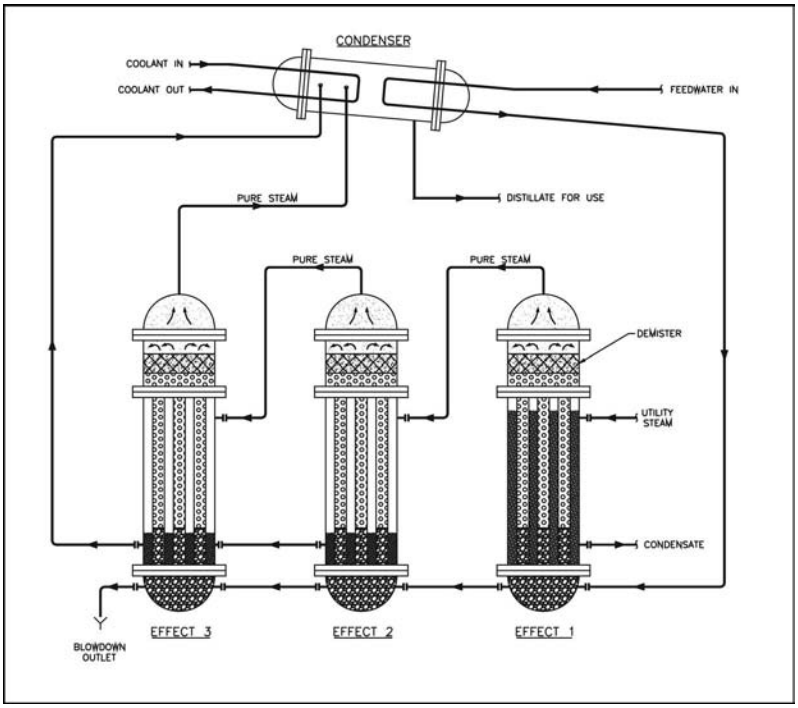

Figure 4 Schematic of traditional distiller (multieffect type).

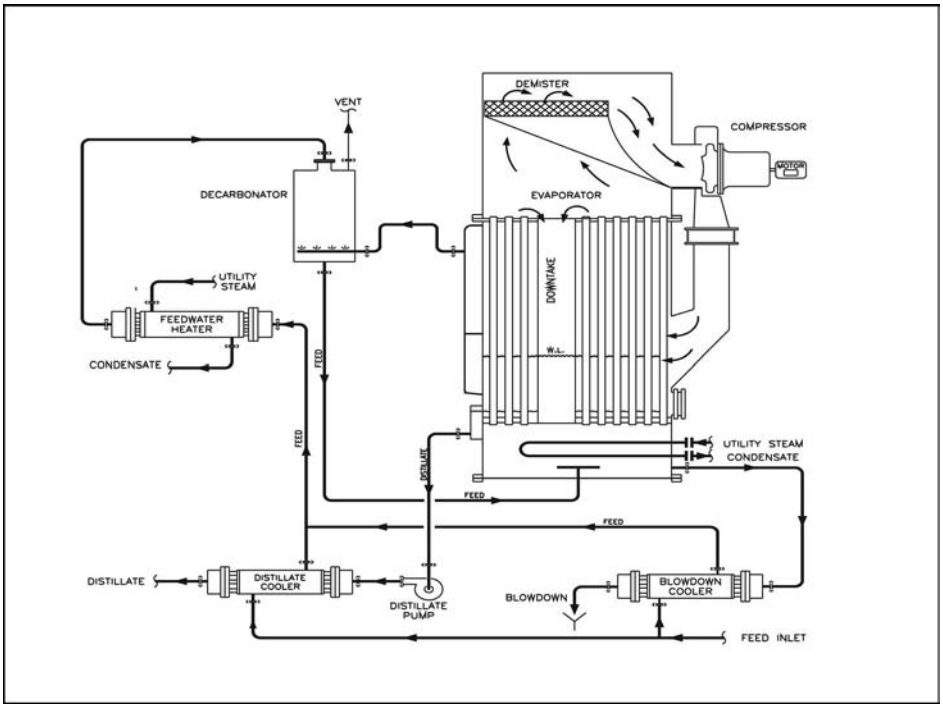

Figure 5 Schematic of vapor compression distiller.

For either distiller, design is critical such that steam velocity through the unit cannot allow entrainment of water droplets that would carry contaminants along with the steam into the final WFI. Therefore, mist eliminators/separators and other options including centrifugal force are usually employed to ensure final WFI quality and suitability. For both unit designs, water in the evaporator section requires periodic purging to eliminate the concentration of contaminants that result from the continuous evaporation. This process is termed "blow-down," and is initiated typically by the equipment controls (usually at a fixed rate) based primarily on the volume of WFI produced, but may also activate during start-up and/or shut-down of the equipment. Silica scale is of concern for all stills and the rate of blowdown is one mechanism used for its control. Stills are typically capable of at least a 4 $\log^{10}$ (99.99%) reduction in endotoxin making it imperative that feed water is of suitable quality so as to not over tax the unit's capability.

Instrumentation and controls for either design must be adequate and capable of ensuring the quality of WFI produced. Any control failure could result in contaminated WFI with the associated rejection of product, production delays, and possible patient injury if undetected. Overall still quality, including design, materials, finishes, components, and assembly, is imperative to WFI quality, as is the requirement for reasonable and sufficient distiller maintenance. Monitors and controls within the WFI distribution are also required to ensure that the WFI quality has not been compromised and that delivery system integrity is uncompromised.

Alternative technologies can be implemented to produce WFI when not constrained by regulation or practicality. These options may include RO and ultrafiltration (UF) that are both based on the use of a barrier to limit the passage of contaminants. RO, which is commonly used for producing Purified Water, employs a semipermeable membrane capable of passing water molecules but which does not allow the passage of contaminants that are typically larger in size (Fig. 6). This pressure driven tangential flow filter works in reverse of normal osmosis, hence the name. RO equipment produces a continuous waste stream of water, when in operation, that is typically in the range of 15% to 30% of the influent. This "reject" water continually flushes the membrane surface removing contaminants that would otherwise clog the membrane. The waste stream can amount to a significant volume of water, discarded as waste to a local sewer, and often necessitates creative thinking to develop alternative uses, including cooling tower or boiler makeup, vessel prerinsing, lawn sprinklers, etc. Product water, also termed permeate, from a single pass RO is usually unable to meet the conductivity requirements of Purified Water and is often supplemented by subsequent deionization, possibly in the form of CEDI, to ensure that the water produced is of adequate chemical quality. Since deionization is considered by most regulatory agencies as unacceptable for the final treatment of WFI, those considering RO as a means of production should only consider double pass (2-pass) RO, also known as product-staged RO, which must be operated meticulously, possibly with optional features, to ensure chemically suitable effluent quality on a continuous operating basis.

RO membranes are capable of the finest filtration commercially available with removal rates typically below 1000 molecular weight (Daltons) and often specified with a molecular weight cutoff (MWCO) of approximately 100, meaning molecules of 100 molecular weight or greater are rejected at greater than 90%. Recent developments allow for some membranes to be heat sanitized, providing a significant improvement over previous products that experienced the drawbacks associated with chemical sanitization. New style membranes, capable of continuous operation at or near 185°F, are becoming commercially more viable, eliminating the possible downstream growth that has plagued RO technology for decades because of the inability of chemicals to sanitize the permeate side of the membrane.

UF is capable of particulate removal typically between 10,000 and 300,000 Da, and is not nearly as fine as RO membranes. However, UF under certain circumstances may have the

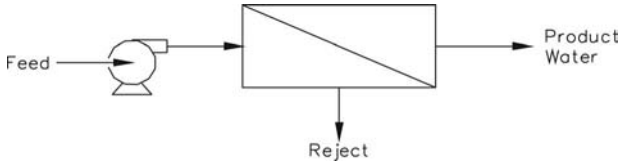

Figure 6 Schematic of reverse osmosis.

ability to operate at significantly lower pressures than RO such that UFs can be utilized at pressures more common to WFI systems and without special high-pressure ratings. Whereas the majority of RO membranes are sheet material in a spiral wound configuration, UFs can be constructed in alternate forms, including hollow fiber and in various materials such as porous solid ceramic, making some more readily sanitizable. Although UF technology may offer options in the future, these designs are currently the least accepted for manufacture of WFI, since UF technology is only specifically listed and approved by name for WFI production in the current Japanese Pharmacopeia (9) and no other compendia.

There are no definitive published statistics regarding WFI production methodologies; however, it is estimated that more than 98% of the WFI created worldwide is made using distillation of one type or another, with the remaining, less than 2%, made using alternate technologies such as RO and UF. In fact, this author was unable to confirm even one system officially validated for WFI manufacture using UF technology anywhere in the world.

Once produced, WFI must be protected against contamination and maintained at suitable quality levels to ensure its safety. Accomplishing this task is far harder than often realized and some system designs have been flawed because inexperienced designers failed to recognize the rigors associated with WFI storage and distribution. Storing of WFI at/or above 165°F is acknowledged to be the most secure and robust method; however even simple related tasks such as delivering the water to manufacturing or packaging sites within a facility can prove extremely challenging. For example, piping that might be used to carry the WFI to its point of intended use could become problematic based on cooling below sanitization temperature if use were not continuous. As a result WFI piping is almost always sanitary stainless steel, recirculated as close to use-points as possible and continuously reheated to ensure a suitable temperature is maintained. There can be no deadlegs at lab or work benches, sinks, or equipment that would cool sufficiently to allow microbial growth. Dead-legs, piping sections that cannot be circulated, of even short lengths often result in contamination that can disable an entire WFI system. Dead-legs can occur as the result of closed valves, improper piping techniques, or improperly mounted accessories such as instruments. Dead-legs are a continuing topic of debate relative to tolerable length. Suffice it to say that dead-legs should be minimized as opposed to application of a maximum length "rule." Common industrial or commercial piping practices cannot be employed in WFI systems as they would undoubtedly result in long-term system quality issues based on their nonsanitary nature.

WFI piping that operates at an elevated temperature will commonly require insulation to reduce heat loss, as well as for personnel protection and to reduce operating expense. Suitable insulation must be chloride free to reduce the risk of corroding the stainless steel piping system and components. Insulation should be continuous to avoid cold spots that might harbor bacteria. This situation results in a conundrum since continuous insulation would hamper the maintenance, service, and calibration efforts that are required to maintain the system in good operating condition and in a validated state.

WFI distribution systems often must traverse hundreds, if not thousands of feet of distance within a manufacturing facility to service users in varied locations. This piping often rises up or drops down through multiple floors within a building. High flow rates, compounded by the need for recirculation, often result in the requirement for relatively large diameter piping. This piping within a facility may transit through unconditioned spaces such as attics or on roofs or through minimally conditioned spaces such as ceilings or warehouses. These situations compound the difficulty of maintaining a system's integrity and pose additional challenges relative to temperature maintenance and as a result may also influence sanitization efforts and validation.

Stainless steel in the 300 series, although extremely corrosion resistant, is susceptible in water systems to a phenomenon termed "rouging" where deposits of metal oxides (mostly of iron-based origin) form on the surface of the stainless steel materials and eventually migrate throughout the entire system. This condition is most prevalent in WFI systems at temperatures in the range of 165 to 185°F (or higher, as in the distiller) and becomes progressively worse as water purity and/or temperatures increase. This situation has been documented extensively over the years at virtually all facilities operating within these parameters, although the degree of rouge that develops is often inconsistent from site to site.

Three primary types of rouge have been defined and cataloged by Tverberg (10) of which all are frequently found in pharmaceutical and biopharmaceutical environments. Interestingly, rouge that develops typically does not affect water quality based on current standards, resulting in many operators ignoring rouge as they consider it to be irrelevant. However, rouge that continues unchecked can result in particulate generation, which is certainly unacceptable in WFI. Also, in extreme cases excessive rouge results in pitting of the base metal that damages the surface finish reducing the hygienic effect and can, in severe cases, result in leaks around seals and gaskets.

Rouge can be orange-red in color and loosely adhered such that it could be removed by wiping with a clean soft cloth. This type of rouge may develop quickly in elevated temperature systems or more slowly in systems operating at lower temperatures. Rouge can also occur in darker shades (red-brown or bluish), which are usually more tightly attached as the result of higher temperatures or extended periods. Both of these rouge types are common in WFI systems and can usually be removed by chemical treatment. Unfortunately, even after removal, it is common for the condition to recur requiring periodic derouging and repassivation of most WFI systems that operate at or above sanitization temperatures. Rouge is often first detected in areas of high velocity such as in pump volutes and spray devices presumably the result of erosive action and/or microcavitation.

A third type of rouge is more common to systems operating above 212°F with characteristic dark blue-black color that is so tightly adhered that removal may damage the base metal making it unsuitable for continued use. This type of rouge is most common in Pure Steam systems or WFI systems that are regularly/frequently steam sanitized.

Derouging of water systems can be performed using various procedures; however, passivation chemicals are not typically effective for rouge removal and the effectiveness of a passivation procedure is often compromised if rouge is present and not removed prior. On the basis of the variety of rouge types, it is prudent to design equipment and systems with removable/replaceable coupons (a fitting or small tube section is sufficient) that can be utilized off-line to test a proposed derouging procedure.

Because of the critical nature of the effluent produced by a WFI system, validation, or more recently commissioning and qualification, activities are critical to system acceptance. As well, ongoing acceptance is predicated upon suitable system operation and upkeep. Effectively, all components must be maintained properly and must operate as intended, such that the system must produce WFI consistently. Any operational trends that provide indication that performance is deteriorating must be addressed and control reestablished to ensure water quality is not out of specification.

SUMMARY

It is surprising to many that water deemed acceptable for human consumption, and termed potable, requires significant additional treatment to allow its use in drug manufacture. As a minimum, filtration and softening are commonly employed as pretreatment and when required, additional unit operations are included as necessary prior to distillation to ensure water quality and system reliability. In addition, microbial control is required throughout the entire process impacting the selection of components, their arrangement, and method of operation. As well, sanitants and sanitization procedures cannot be universally applied on the basis of compatibility with individual unit operations overall system suitability.

WFI, whether injected into a patient directly or as part of a parenteral preparation, must meet exacting standards. Chemical and biologic quality must be unquestionable as many patients that receive treatment are already in a compromised state such that any quality imperfection might prove to be detrimental.

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5 Particulate matter: subvisible

D. Scott Aldrich

OVERVIEW

Definitions

Pharmaceutical Dose Forms

United States Pharmacopeia (1) defines the following parenteral dose forms:

a. *Injection*—Liquid preparations that are drug substances or solutions thereof,
b. *For injection*—Dry solids that, upon the addition of suitable vehicles, yield solutions conforming in all respects to the requirements for *injections*,
c. *Injectable emulsion*—Liquid preparations of drug substances dissolved or dispersed in a suitable emulsion medium,
d. *Injectable suspension*—Liquid preparations of solids suspended in a suitable liquid medium, and
e. *For injectable suspension*—Dry solids that, upon the addition of suitable vehicles, yield preparations conforming in all respects to the requirements for *injectable suspensions*

This chapter will address the presence and nature of subvisible particulate matter in regard to these dosage forms, and in a general aspect for any medical product.

Particulate Matter

The primary subjects of this chapter are particles and their size. The following discussion explores the lack of specificity for these subjects; to be comprehensive in our evaluation, we must not consider only particles that are one entity or characteristic, nor may visibility be described by a single "line-in-the-sand" size threshold or definition. In practice, the ultimate definition(s) of particulate matter must be suitably broad to allow us to consider many different aspects of character and size to make the products we develop most robust. Our discussion concerns particles and specifically, those we are unable to see. Our ability or inability to see them is not a simple matter, and dependent on many factors. Overall, our intent is to find the particle(s), understand them, and change something to alleviate their presence, inclusion, growth, or change. But first let us discuss the primary topics.

Particulate Matter—What Is It?

Particle: a body having finite mass and internal structure; a minute portion, piece, fragment, or amount; a tiny or very small bit; a grain, speck

Particulate matter (PM) is a broad term to include many varieties of conditions, sizes, and associations of particles in the product fluid. In an ideal state, a single particle is a single type of material, in solid form present in the pharmaceutical product and detected by a human observer or an electronic counting device. Generally, if detected by human vision, it is visible. However, certain attributes of the PM may yield "visibility" of material when in a size far below human resolution. Human detection of PM is probabilistic, not deterministic. PM is not an intended component of the formulation, such as active ingredient or suspension agents. The current USP definition (2) is "*particulate matter in injections and parenteral infusions consists of mobile undissolved particles, other than gas bubbles, unintentionally present in the solutions.*" The intent of this definition is interpreted by some as warning to exclude only extraneous matter or contamination from the final product.

Just what is contamination? Is it the presence of unwanted and foreign material in the product? Is it a noxious substance that may cause harm, such as microbial colonies, spores, pyrogens, chemical substances extraneous to the formulation, particles of vermin (flies, insects, etc.), environmental debris, package fragments? Is it a form or derivation of the active ingredient or excipient mix that has now appeared? Yes to all. Some are certainly worse than

others and cannot be tolerated, such as filth (insect parts) and pyrogens. But that should not be the limit of our concern. Other PM, while not contamination in a filth sense, is deleterious to the physical integrity of the formulation.

A more comprehensive and quality-relevant definition of PM is to include all forms of nonformulation substances that may be seen or detected by analytical means, at release and for the shelf life of the product. Careful and comprehensive investigation of the PM content, type, and origin is the only means to true high-quality products in this category.

Certainly, PM is an unintended consequence of product manufacture. Only extraneous forms should be unexpected, that is, PM forms that may arise from within the product are well understood and excluded. In the worst case, PM appears after batch release and while the product is being distributed, stored, and used. PM may cause further problems such as formulation change, appearance, dose performance change, dose delivery effects, and medical impact on the patient. The pharmaceutical product is expected to be an example of careful planning and utmost control. Occurrence of PM and even worse, unexpected change in the nature of the formulation or package because of its presence is a failure. Thus, USP Chapter <1> Injections definition of foreign and PM uses *visible particulates* to define all, so as not to discriminate between types or allow one category over another (3).

Particle Categories

PM implies an ideal; a solid or collection of solids, observed as a single solid. Solid PM or a collection of solids are certainly the most prevalent nature of PM; however, many other and alternate conditions (nonideal) may be considered as PM by the observer, and may affect the quality of the formulation. If we are to comprehensively explore the content of PM in the final package, we must consider the following as well:

- Immiscible liquids
- Immiscible semisolids
- Microscopic solids, in sufficient number to produce observable light scatter(haze)
- Microscopic solids joined by a matrix to form larger entities
- Thin solids, even in large sizes, invisible to normal observation (require high-intensity light and reflectance necessary for visualization)
- Poorly dissolving product, from dry powder or lyophilization reconstitution
- Package-attached material, of any size
- Combinations of any one or more of the above, and with single solids

Particulate Matter Size

Visible: The greatest distance at which a person with normal eyesight can see and identify an object. Or, the smallest object (characteristic) a person may readily detect with unmagnified 20/20 vision.

Particle size: x:y:z dimensional size in micrometers, Feret's diameter, Martin's diameter, chord, longest dimension, geometric volume mean, projected area diameter (equivalent circular diameter), area.

Size is an absolute measure, described in three dimensions. The x, y, and z axes of the solid entity describe its volume, are the basis of its shape, or habit, and provide a seemingly boundless number of descriptive terminology. The size of a sphere can be reported unambiguously by radius or diameter. Cubic or otherwise equant ($x \sim y \sim z$) particles may also be well described by edge length or diameter. However, consider other measures, such as minimum length, maximum length, same sedimentation rate, volumes, areas, weight. Which categorical measure would we use and report? Just as for determinations of powder character in regard to fine, coarse, large, and small, the method and analysis one employs affect the outcome and must be relevant to the industry.

When thinking of PM in terms of a (single) dimension or size, the correlation of x-y-z dimensionality to a single factor or equivalency can be confusing. For spheres, we can state a single and relevant dimension, diameter. No matter what orientation, we still observe a size of diameter. For all other (and more common) particle shapes, which dimension shall we use? Correlating the subvisible population to a single index or area under the curve from a range of

Table 1 Sizes of Common Materials

Common material	Sizes
Flu virus	0.07 μm
Pollen	7–100 μm
Diameter of human hair	50–150 μm
Sneeze particles	10–300 μm
General size descriptions	
Molecular	<1 nm
Colloidal	1 nm–0.5 μm (protein formulators consider >500 nm to be coarse)
Floc or flocculate	Size: sub-10 μm, but more importantly as character: suspended, aggregated material held together by weak physical forces such as surface tension or adsorption
Fines	up to 10 μm
Powder, particles	up to 100 μm
Coarse	up to 500 μm
Granular	>500 μm
Chunk	Millimeters
Mass	Really big!

Source: From Ref. 7.

the particle population would be another form of limits test, but has not been generally accepted (4,5). Particle load in the product and subsequent impact on the patient, especially in chronic administration of medication, is quite important. Setting the appropriate limits within constraints of manufacturing and across all product forms is not accomplished by a single threshold size or load limit (6). The emphasis is one of continual product improvement to ensure patient safety and robust final packaged product.

Some common particle types and sizes are shown in Table 1.

What truly is subvisible? There are a number of conflicting reports for the lower threshold of visibility, ranging from ~11 to ~150 μm. Delly (8) reported an absolute minimum detection of 11 μm on the basis of the minimum arc of view at reasonable near vision, given the optical lens system of the human eye. Literature often (9,10) defines 30 to 50 μm as the distinction dimension between the visible size for human visual inspection and truly subvisible PM size. Review of visual inspection threshold at the 1995 PDA meeting (11) reported 150 μm as a reasonable, probabilistic threshold of detection (70%) for trained inspectors in defined commercial release assays for a single particle in a clear liquid formulation, within a transparent uncolored glass ampoule. On the other end of the size range, the fundamental range for protein formulations is defined as those particles too large for size-exclusion chromatography (>0.1 μm), yet below visibility (<100 μm) and for protein active ingredient, the 0.1 to 10 μm size range is quite important for monitoring formulation stability (12). The size domain below 10 μm is not addressed by the current compendia.

You see from these examples disagreement from established and reliable scientists determining visibility dimension in a variety of applications. I believe they are all correct, in the given application and at the point in time of measurement. I will not set a specific, hard threshold for the subvisible zone because of a number of factors. One must consider and accept the concept that the visible dimension and subvisible dimensions meet in a gray zone of detection, one of probability not certainty. We can agree that a 10 μm particle would be very difficult to observe visually, whereas, a 300 μm particle should be seen quite readily under reasonable inspection conditions.

Certainly, the training of the observer, the method of detection, and the attributes of the PM affect visibility. Consider the properties of PM in Table 2 that may enhance or diminish detection. Greater visibility of PM in the fill solution is given by increased size, distinctive color, extremes of buoyancy, and high reflectivity. These properties also enhance the visibility of small particles that would otherwise go unnoticed. Further, the significance of the PM in the pharmaceutical product has not so much to do with its size and hence visibility, but its effect or potential effect on the quality and integrity of the medicinal agent. Pharmaceutical aerosols are

Table 2 Properties of Particulate Matter

	Detection probability	
Property	Enhanced	Diminished
Size	>100 μm	<100 μm
Spatiality	In foreground	In background
Color	Bold	Weak, pastel
Contrast with matrix	High	Low
Reflectivity	High	Low, none
Buoyancy	Neutral	Extremes

preferentially 2 to 5 μm (13). Pharmaceutical suspensions are formulated in near micrometer to ~30 μm crystals, dependent on the requirements of the API. Microsuspensions and nanoparticle formulations are by definition submicrometer in nominal size. What is the material, how is it intended to be included in the formulation or formed, what is its stability, and what is its nature? While many of the above descriptive categories relate to dispersed powders and dry solids, it is important to use consistent and common terminology for description.

PM is a cause of instability in the pharmaceutical liquid and is signaled by observation of PM or change in the filled product package, by excessive PM load determined by quantitative assay and by an upward trend of PM content even if below compendial limit. USP and major compendia sets limits for subvisible particles, dependent on container nominal fill volume, at ≥10 μm and ≥25 μm size thresholds, certainly below the lower end of the visible range (14).

PARTICULATE MATTER CHARACTER

Particulate Matter Category

Extrinsic: outside, from the exterior
Intrinsic: inherent, part of the whole

An important concept, discussed in many reviews of PM, is the categorical origin of particulate matter. Two general categories describe all sources; first, *extrinsic* or truly foreign matter, introduced during batch fabrication (formulation assembly, package preparation, or filling operations) and resident in the package. This added particle load does not change unless due to fragmentation, swelling, (hydration), or degradation. The second, *intrinsic* particulate matter derives from product-related sources such as formulation-container incompatibility, component impurities, formulation degradation, substance extraction in points of contact, component precipitation, nucleation, sedimentation, etc. All are likely to change over time and may not be detected until months after release. These categorical types cover all particle populations.

Particulate Matter Nature

Table 3 shows the nature of the particulate matter as the state or degree of complexity, the fundamental identity of the substance(s). We consider the extremes: from simplest (single crystal) to the most complex [multiple species, varying crystallinity and combination of physical state (liquid and solid with entrained gas)]. These conditions provide the boundary of PM nature and from simplest to complex, least to most challenge for analytical detection and characterization. Both extremes may occur in the developing formulation and may cause deleterious effect. Are they detected and identified soon enough to be removed prior to late-stage trials?

Table 4 shows crystallinity states; considerations, which may apply to all, a portion, or in a continuum state of the solid particle. All of these properties may be used to identify the material.

Certain terms are the basis for common descriptions and are important to understand for subsequent identification of the unknown material, a shown in Table 5.

Table 3 Common Associations of Particulate Matter

- Singular
  - Liquid
  - Solid
  - Combinations
- Multiple
  - Aggregate/agglomerate
    - Boundaries (matrix evident?)
    - No boundaries
    - With similar material, foreign material
    - Groups of groups
    - Homogeneous heterogeneity
  - Crystal grouping
    - Polycrystalline—many large individual crystals
    - Microcrystalline—many small individual crystals
    - Cryptocrystalline—many nucleation sites apparent, or their effects evident, without resolution of discrete particles
- Layered, banded, lamellar
- Coated
- Foliated—platy aggregate
- Suboptimal and continuum states

Table 4 Crystallinity States

Nonevident—Amorphous, glassy
Evident or continuum
- Liquid crystals: 2D order (mobile, yet ordered)
  - Nematic—parallel molecules with at least one rotation axis: thread or cable-like arrangement
  - Smectic—molecules arranged in layers, with long axis perpendicular to the layers (planes)
  - Cholesteric—nematic layers in a helical arrangement
- Solid crystals: 3D order
  - Isometric (1 ref. ind.)
    - Three equivalent axes, intersect at right angles
- Uniaxial (2 ref. ind.)
- Tetragonal
  - Three axes, two of which identical in length, intersecting at right angle. Third axis is longer aligned at right angle to others.
- Hexagonal (trigonal)
  - Four axes, three of which lie in the same horizontal plane, at 120° angle. Fourth axis is perpendicular to the plane formed by the others, and of different length
  - Biaxial (3 ref. in)
- Orthorhombic
  - Three axes of different lengths, intersecting at right angles.
- Monoclinic
  - Three axes of different lengths intersecting such that a and c form an oblique angle, and b is perpendicular to the plane formed by the other two.
- Triclinic
  - Three axes of different lengths all intersecting at oblique angles.

Table 5 Common Descriptive Terminology

1. Clustered—observed as a group, but without bonds or matrices holding the PM together
2. Aggregated—particles touching, joined at edges, fragile collection easily broken apart
3. Agglomerated—particles joined at faces, fused, in a tenacious collection; not easily broken apart per ASTM (15). Others note that all assemblages are agglomerates and that an aggregate must be confined to prenucleation association of molecules that may crystallize (16).
4. Cemented—particles held in a solid matrix by another material, such as stones in a cement sidewalk.
5. Inclusions—particles, liquid, or gas entrained in another solid or semisolid
6. Occlusions—PM held on the exterior of a solid or semisolid, such as “sugar on a doughnut.”
7. Material is *sectile* if it sections into plates.
8. Spherulitic—three-dimensional, radial association of material; a ball of needles all with one end in a nucleus. Also called a rosette.
9. Splintered—fragmented by force into thin pieces, not necessarily with consistent dimension or habit, which would indicate crystalline subunits.

Table 6 Appearance—Shapes/Habit and Axial Ratios

Name	Description
Equant	$x \sim y \sim z$ are roughly equivalent; cube, sphere
Rod	$x \sim y < 3$ and z: up to 50
Column	Thicker rod—$x \sim y$: 2–6 and z: 10–50
Acicular/needle	Very thin rod—$x \sim y < 1$: $z > 10$, often >100
Plate	x: 7–10; y: 1–5; z: 7–10
Flake	Thinner plate—x: 7–10; y: <1; z: 7–10
Blade	x: 7–10; y: 3–5; z: 10–50
Lath	Thinner blade—x: 7–10; y: <3; z: >10
Ribbon	Thinner lath—x: 5; y: <1; z: >50

Table 7 Luster and Hardness Scales

Luster (relectivity: 10 = high)	Mohs hardness scale (10 = high)
Absorbing – 0	Talc – 1
Earthy – 1	Gypsum – 2
	Calcite – 3
Silky – 4	Fluorite – 4
Pearly – 5	Apatite – 5
Greasy – 6	Orthoclase – 6
Waxy – 7	Quartz – 7
Vitreous (glassy) – 8	Topaz – 8
Flash – 9	Corundum – 9
Adamantine – 10	Diamond – 10

Appearance

Descriptive terms for the particle exterior may offer insight to the formation and exposure. Shape or habit description of the solid may seem difficult at first, but in reality, only three basic shapes need consideration, with subsets of dimensional variation. The boundaries of the axial dimensions may be arguable, but need to be defined and then consistently followed or best communication. These are shown in Table 6. Opacity of the material may be described opaque, translucent, transparent, and possibly variations thereof. Upon close examination, surface characteristics may also offer insight to identity, formation, exposure, and condition.

In the collection of material properties, one cannot overlook *luster*, a reflective property that may indicate PM origin or even identity and is often correlated to hardness. Note the series of hardness values with representative minerals, and the correlated luster examples shown in Table 7 (17).

Common Particulate Matter Types

The common types of PM encountered in pharmaceutical injections, primarily from earlier and less-refined manufacturing designs that allowed too much of the natural world to creep into the liquid fill, was first addressed by Australian researchers Garvan and Gunner (18,19). This impact is well documented in the literature (20–24) and discussed in topical lectures (25,26). Table 8 contains a listing of prevalent categories. While less common in modern parenteral products, contamination by natural materials is always a threat to the sterility and purity of the product.

The commonality of particle types has much to do with the sources and ability of the assembly and filling arena to exclude them. These types are (*i*) the commercial assembly equipment (metals, polymers, resins); (*ii*) assembly arena design and use (airborne and personnel-borne PM—pollens, molds, bacteria, epithelial cells, hair, fibers, soil minerals, insects); (*iii*) packaging (olefins, glass, silicones, rubber) and processing (silicones, lubricants, phthalates); and (*iv*) formulation components. Thus, a more comprehensive and categorical listing is appropriate.

Table 8 Common Particle Types

Filth	Common extraneous matter	Intrinsic matter
Insect parts	Glass	Crystalline material
Bacteria	Rubber	Diatoms
Fungi	Metal	Extracts
Biological debris	Paper and cellulose	Leachates
Human epithelial cells	Starch	Drug changes
	Talc	Package degradation
	Facility contributions	Process contributions

Minerals and inorganic species such as titanium dioxide, iron oxide, zinc oxide, calcium sulfate (carbon black), clays, calcite, etc., added intrinsically from septum systems and environmentally from soil and the processing arenas.

Fibers and fragments:
Cellulose in all forms, natural and processed—cotton, rayon, wood, paper, clothing dust.
Man made—nylon, polyester, Aramide, olefins, Teflon, Nylon, Orlon, Lycra, etc.

Hairs: Human, anima

Metals: Primarily stainless varieties; however, the effects of dissolved metallic contamination from excipients, active ingredient, formulation processing, and storage are significant. Minimal content of Al, S, Ca, K, Ba, Cr, Fe, Ni, Cu, Zn, V, Sn, Ag, and others may be significant enough to induce a cascade of change on the otherwise stable dissolved active. Instability of β-lactam antibiotics cascades from leached divalent ions from plastic and glass containers, rubber stoppers, and tubing. In particular, leached Zn catalyzes the opening of the β-lactam ring leading to API degradation and insolubility and thus nucleation of subvisible particles (27).

Biological matter: Seeds, trichomes, pollens, vegetation fragments (cell wall, structural components, leaves), and insect fragments.

Processing: Extracted organic moieties such as phthalates, emulsifiers, silicones.

Minerals: (*i*) filler components of septum (closure) and other modified elastomeric systems and (*ii*) local and trafficked soil-related minerals not removed during preparation and added during filling.

Building: Paint and components (titanium dioxide, calcite, mica), gypsum (wallboard), calcite, rust, polymers, epoxies.

PARTICULATE MATTER SOURCES

Formulation/Package, Manufacturing, Administration Practices

All points of contact, residence, manipulation, and storage may contribute to the particulate matter load in the final product. A large but not exhaustive list of these sources includes:

1. Manufacture of the bulk active ingredient (processing hardware-extracted moieties, nanoparticle content), all of which will pass through finishing and sterilization media;
2. Sterile manufacturing operations (residence in sterilized stainless vessels, air exposure, HVAC quality, personnel, transfer tubing/filters/gaskets exposure), sterilization media extractable moieties;
3. Filling arena stainless exposure, valves, gaskets, tubing, in-line filters,
4. Container cleanliness and residence;
5. Pharmacy operations of withdrawal/transfer, reconstitution/transfer, exposure to IV sets, dilution with and in sterile vehicles (D5W, normal saline, Ringer's solution, etc.), temporary receiving-transfer vessels; and
6. Container use contributions. These include piercing of the septum, connection of IV sets, activation of sliding septae, multiple product chamber mixing, needle quality and handling.

In review of the literature, one is drawn to the conclusion that much of the gross and obvious sources of particulate matter have been identified, verified, and removed from the systems and packaging that accommodates parenteral medications. This is generally true for established companies and their legacy product processes in which constant analytical vigilance is maintained for particle content and variation. Much is known about the entire process stream, and strict control is imparted for microbiological, chemical, and physical insult.

The trending of batch results and facility performance for well-maintained visual inspection and subvisible particulate matter assays is the key operation for controlling particulate matter content. Maintenance of these operations is an important modifier, since all may be run as discrete operations, without proper quality control, for example, use of targets and action limits that precede specification performance; investigation of variation and out-of-target results to identification of rejection or out-of-limit, incident cause, and remediation. Connection of the detection and quantitation assay units with the organizational units responsible for batch investigation is an important process improvement factor.

However, in consideration of new facility operations, new product lines, new equipment, site changes, component vendor alteration or new vendor, new package, new therapeutic line to name a few, increased particulate matter inclusion in the final product is more likely until these parameters are understood and controlled.

PARTICLE DETECTION

Human Inspection

No discussion of subvisible particulate matter is complete without consideration of visible particles, for two reasons:

a. The division between visible and subvisible is not a hard line at a single particle size.
b. Visible and subvisible particles are bound together regardless of size (at the moment) since there is often a relationship if not origin from time of fill to final shelf age.

The USP defines visible particulate matter to consist of mobile undissolved particles, other than gas bubbles, unintentionally present in the solutions. This definition has been interpreted by some as meaning extrinsic or foreign material only. This is not true since the methods detect all forms of solid matter. We should add *. . .that may originate from extrinsic sources or from changes due to instability of the product and in some instances may be seen as immobile due to storage conditions or aging.*

Visual inspection provides an effective means of evaluating the physical integrity of product candidates and is required, in some manner, by all major compendial organizations for final package presentation. The pristine appearance and high integrity of the product is the result of a complex R&D effort to limit particulate matter load in the final package, contributed or formed from active or excipient ingredient, formulation pH/tonicity/concentration, container quality and cleaning and the manufacturing processes to assemble the final product package. The change of particle load or presence of visible "particles" in the final package necessitates their removal from the batch or, if not detected, could cause recall of the distributed product.

Typical Visible Particle Pharmaceutical Tests

Appearance Test Under Ambient Condition

The most prevalent means of inspecting a parenteral container, and any retail object, for that matter, is visual examination with normal vision in ambient light. Now, to give the object a good scrutiny, one takes a little time to look on and through the package in good lighting (500–1000 lux) with normal reasonable vision (at least 20 ft/40 ft where 20 ft/20 ft is standard visual acuity). Background is not an important issue, just a reasonable examination in good lighting. We should see obvious package problems such as label type and placement (presence?), fill volume, color and gross condition, package integrity (intact, cracks, soiling?). A few seconds duration suffices to observe these conditions, depending on the level of scrutiny and familiarity with the package. This is an obvious step, not often used within the laboratory.

Visual Inspection in Light Box

Compendial procedures ordain the use of a final visual inspection step to release parenteral packages. All units exhibiting particulate matter, package defects, or cosmetic anomalies must be rejected and culled from the batch. Pace, light intensity, and background are all controlled and important assay parameters, with maintenance of trained and adept inspectors within a quality assurance program also necessary. It is in this application that we experience "what is visible?" Much variation of opinion is seen in the literature, with many definitions of 50 μm as lower detection limit. However, the true detection limit depends on many factors, including number and type of particles, package characteristics, method of inspection, and capability of the inspectors; however, one can rely on the compilation by Parenteral Drug Association (28) for visual inspection detection of one particle in a simple package (clear solution, glass ampoule), as 1% probability of detection at 50 μm, 50% probability of detection at 100 μm, and 70% probability of detection at 150 μm. The importance of analytical particle detection and counting to cover the region below 200 μm is obvious.

Visual Inspection with Tyndall Beam Light in Light Box

A strong, collimated light beam will scatter when encountering dispersed, small solids—a Tyndall effect. The size of the solids that will yield scatter effects ranges from colloidal (500 nm) to about one micrometer. Above that, one may observe "twinkling" reflection from individual, otherwise invisible particles. Use of qualitative Tyndall inspection is insufficient alone, being a complementary method for over-lighting inspection, revealing settled solids and otherwise invisible but large populations of submicron particles.

EP 2.2.1 Clarity and Degree of Opalescence of Liquids

Nephelometry as a measure of haze or cloudiness per *EP 2.2.1* is a comprehensive and quantitative development tool. While not a requirement in U.S. formulations, it is a prudent measure of formulation stability and acceptability. Often, it is not the absolute measure of formulation clarity that is important, but the variation unit-unit. Comparison to four levels of haze allows grading of the parenteral formulation.

PARTICLE QUANTITATION

Particle Quantitation Methods—Compendial

Light Obscuration and Membrane Filtration—Microscopic Quantitation Methods

PM quantitation for discrete size thresholds may be accomplished by many methods, such as electrozone, light blockage, light diffraction, light scattering, and by filtration of the liquid and counting of the retained solids. All are quantitative, sensitive, and linear, with low variation and are easily calibrated. Light obscuration (LO) methods are preferable in one sense, for all the reasons one uses controlled instrumentation, and large populations of particles are sampled. Controlled and repetitive testing may be performed with a minimum of time, cost, expenditure. Trending of pharmaceutical liquid particle populations is routine and preferred by most analytical groups. The method offers defined instrumental parameters, calibration, and good correlation between labs with minimal variation. USP maintains a particle count reference standard (PCRS) and solicits participation from industrial, academic, and government labs to validate each generation of the standard. The USP promotes collaboration of pharmaceutical users and subsequent consensus on calibration approach.

However, LO is not preferable in one way: it is nondiagnostic (one does not visualize or capture the particles causing counts), is prone to artifact counting (air, immiscible oils), and size derivation is dependent on the nature of the particle. Instrumental methods provide some vector or derivation of true size, for example, equivalent circular diameter (ECD), geometric volume mean, or chord for example. For a spherical particle, the diameter will very closely equal the reported size, but for particles with nonequivalent axes ($x = y \neq z$) such as a rod or flake, the reported size is much different than the actual dimensions one would view or measure microscopically.

Membrane microscopy (MM) is the secondary or Tier 2 method endorsed by USP for subvisible particulate matter. Although microscopy is a preferable diagnostic tool, its

application is more time-intensive and tedious, may be more subjective in particle size evaluation, and not as well known, thus the method is not commonly used for routine release or stability assay. However, in a well-controlled laboratory with experienced microscopists, the membrane isolation and microscopical counting method provides diagnostic insight to the level and type of particulate matter occurring in the stability batch. Are the particles a variety of types, or more indicative of a single, or point source? Are there singular or aggregated, amorphous or crystalline, package or formulation particles present? Is the particle load low or trending higher? These are all good questions and necessary for process improvement. One cannot pursue the elimination of particulate matter without an understanding of their origin(s). Recent harmonization of these two methods among the USP, Ph. Eur., and JP compendia has occurred (29). The tests for subvisible particle content may be used interchangeably in the ICH regions, given suitable instrument calibration and system suitability requirement satisfaction.

The two-stage determination of particulate matter load for these products utilizes the LO method for routine lot release and stability evaluation, supplemented by the historical membrane microscopic method. The current methods have been revised from previous compendial procedures and the revised particle content limits official in USP XXV. LO (light extinction, light blockage) electronic particle count methodology is the desired stage I method, using tabulation from electronic detection of light blocked by particulate matter in the sample fluid streaming past a fixed photodiode detector.

The membrane method is provided for particle counting by microscopical examination of a membrane isolate, as a stage II method. The membrane microscopic method was described as "referee" by some researchers due to retention and examination of the particles after counting. One can reexamine the results, to determine the nature of the particles, and verify the count. LO samples are lost, or if captured during testing are at least corrupted and may not be useful in a retrospective analysis. The LO method is certainly more reliable in regard to standardization and precision; however, it will suffer influence from immiscible liquids, air, and particles with nonequant (1:1:1) habit. Both methods have their attributes and drawbacks, and are best used in conjunction with other analytical equipment configured for small particle analysis.

In the process of conventional microscopical particle sizing, the operator utilizes a calibrated linear scale to compare particle dimensions and render judgment according to desired criteria1. The USP graticule design, Figure 1, provides a defined "graticule field of

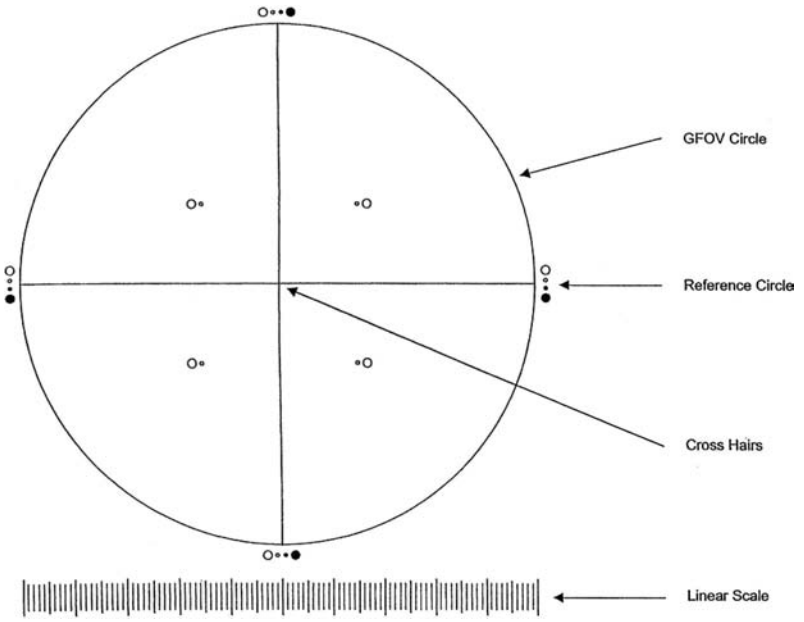

Figure 1 USP graticule. Reprinted with permission. Copyright 2009 United States Pharmacopeial Convention. All rights reserved.

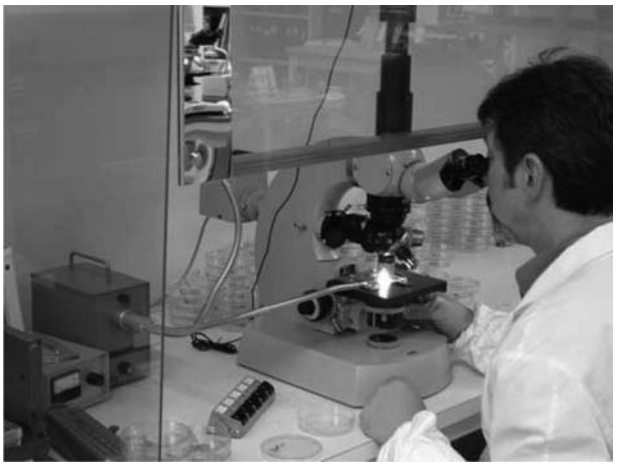

Figure 2 Operator examining a membrane isolate within an UAFW.

view," comparison circles and a linear scale. The design facilitates the estimation of particle size on the membrane through comparison to open and filled circular areas with 10 and 25 μm diameters or to the linear scale in 10 μm divisions. The operator, Figure 2, scans the entire filtration area of the membrane, determining size against the calibrated scales and tabulation particle counts in the threshold bins.

The tests contained in USP Chapter <788> are physical limits tests performed for the purpose of counting or enumerating subvisible extraneous particles within specific size ranges. There is also a guideline for liquid ophthalmic products, USP Chapter <789>, that follows the chapters <1> and <788> direction. USP Chapter <788> testing applies to the following dose forms: all large-volume injections for single-dose infusion (large IV bag or bottle) and small-volume injections (smaller vials and ampoules), unless otherwise specified in an individual compendial monograph.

USP Chapter <788> is now harmonized with the Japanese Pharmacopeia and European Pharmacopeia (EP), providing the test approach for counting particles by two methods and relevant PM limits. The injectable product is first tested by the LO procedure or method 1, having a specific set of particle content limits. If the test article fails to meet the limits, it may be tested by and must pass a second method, known as the membrane microscopic procedure, also referred to as method 2, with its own set of limits. The limits are defined dependent on parenteral container volume and method used: count per mL for large volume injectable products (1.A. LO and 2.A. MM, >100 mL) and per container for small volume injectable (SVI) products (1.B. LO and 2.B. MM, 100 mL and less) as shown in Table 9. For nominal 100 mL volumes, the 1.A and 2.A. limits are used in the United States.

There may be technical reasons the injection product cannot be tested by LO, and so microscopic testing may be used exclusively. Documentation will be necessary by the company to demonstrate that the LO procedure is incapable of testing the injection or produces invalid results. Finally, while it is expected that most products will meet the requirements on the basis of the LO test alone, it may be necessary to also test some by both methods to (*i*) prepare for any future use of the membrane microscopic method and (*ii*) reach a conclusion on conformance to requirements. An example may be a formulation that consistently yields

Table 9 USP Chapter <788> Particulate Matter Limits for Parenteral Products

	Method 1—light obscuration		Method 2—membrane microscope	
Parenteral volume	≥10 μm	≥25 μm	≥10 μm	≥25 μm
A: LVI: >100 mL	25/mL	3/mL	12/mL	2/mL
B: SVI: 100 mL and lower	6000/container	600/container	3000/container	300/container

higher counts by LO, and the membrane method verifies lower counts, possibly due to air or immiscible liquid artifacts.

The membrane one selects for the MM assay may be any porosity 1.0 μm and less, gray-black-color contrast; however, in practice the 0.45 μm and less presents a much more flat and homogeneous background. Avoid gridded membrane varieties. They have marginal value in when partial counting and just get in the way when scanning across the membrane.

Chapter <788> states "... do not attempt to size or enumerate amorphous, semiliquid, or otherwise morphologically indistinct materials that have the appearance of a stain or discoloration on the membrane surface." These materials will show little or no surface relief and present a gelatinous or film-like appearance. The historical precedent for this caveat was specifically for large volume parenteral diluent products, especially terminally sterilized varieties that contained a small amount of degraded, gelatinous material in the fill. In development of this method, participating LVP firms producing terminally sterilized dextrose solutions encountered degradation products that were nondiscrete particles, consisting of 5-hydroxymethyl furfural, a known impurity in the products (30). Reactions of proteins with 5-HMF and the presence of further breakdown products such as levulinic acid and so-called "humins" all contribute to the browning of the solutions and presence of gelatinous material (31). If excluding all gelatinous substances during the count, conducting and interpreting the count results will clearly reflect only solid particles; however interpreting the final solution quality may be difficult. If any gelatinous material or any material eluding particle definition is a persistent isolate, this sort of retained material may be indicative of product change or instability and should be investigated during development. It is prudent to investigate the consistent presence of an unknown and uncontrolled material.

High molecular weight and proteinaceous formulations are prone to active ingredient aggregates, ensembles, semisolids, casts and skins that seem ever-present and not rinsed away. In development, it is prudent to investigate these materials, especially when observed only occasionally, as they may indicate a system or condition out of control. If a particle appears to have three dimensions as viewed under the microscope, then it is a particle. However, when using membrane methodology in development, if persistent or significant forms are not classified to be countable particles, it is necessary to pursue their identity and cause to avoid lot failure later.

There is a counter-intuitive part of the size determination in MM method 2 for classic analytical microscopy. In the process of conventional microscopical particle sizing, the operator utilizes a calibrated linear scale to compare particle dimensions and render judgment according to desired criteria1. The USP graticule design provides a defined "graticule field of view," comparison circles and a linear scale. The design facilitates the estimation of particle size on the membrane through comparison to open and filled circular areas with 10 and 25 μm diameters, or to the linear scale in 10 μm divisions.

One does not need to directly superimpose circle or scale over the particle, but can estimate particle size in the field of view by comparison to the circles on the graticule. USP Chapter <788> states ... "transforming mentally the image of each particle into a circle." Thus, rather than using the more direct and simple longest dimension or maximum chord, the intent of the committee and the revision to "Improved Microscopical Assay" (IMA), the particles are to be counted after classifying them in regard to an ECD. Barber (24) provides the historical reference for this conversion, with work by the HIMA USP advisory committee (32). This research revised the original MM assay and provided the basis to fit microscopical counts to the instrumental count paradigm. One converts the area of nonspherical particles into the area of an equivalent circle. This is a problematic conversion, since it can only be performed "mentally" and by visual comparison to the 10 and 25 μm circular features. Without image analysis and accurate calculation of the observed area, one must process this decision on an estimate of the diameter or radius and convert to area. Since area $= \pi r^2$, the shape of the particle can be converted to area by deriving the radius from the x-y dimensions of the resident particle. If we first find the equivalent radius (ER) of the x-y dimensions by the square root of $(X \times Y) \div 2$, then equivalent circle diameter $= \pi(ER)^2$. This conversion is the basis for the mental conversion of the observed particle to an area and would bring the count of nonspherical particles more in line with that produced by the LO assay. Mentally converting the actual size of the visualized particle into an equivalent size category generated by the

Table 10 Comparison of Axial Dimensions to Calculated Equivalent Circular Diameter and Resultant USP <788> Category

Table Size. Conversion of particle habit size to equivalent circle diameter

Shape	*x* axis	*y* axis	*z* axis	ECD	ED	USP size
Sphere	10 μm	10 μm	10 μm	79 μm^2	10 μm	≥10 μm <25 μm
	25 μm	25 μm	25 μm	491 μm^2	25 μm	≥25 μm
Equant	30 μm	35 μm	25 μm	824 μm^2	32 μm	≥25 μm
Flake	10 μm	10 μm	4 μm	79 μm^2	10 μm	≥10 μm <25 μm
Rod	50 μm	5 μm	5 μm	196 μm^2	16 μm	≥10 μm <25 μm
	150 μm	2 μm	1 μm	236 μm^2	17 μm	≥10 μm <25 μm
	200 μm	4 μm	4 μm	626 μm^2	28 μm	≥25 μm
Needle	70 μm	1 μm	1 μm	55 μm^2	8 μm	No (<10 μm)
	200 μm	1 μm	1 μm	157 μm^2	14 μm	≥10 μm <25 μm
	300 μm	3 μm	1 μm	707 μm^2	30 μm	≥25 μm

ER = equivalent radius = square root of $(X \times Y) \div 2$
ED = equivalent diameter = $ER \times 2$
ECD = equivalent circular diameter = $\pi(ER)^2$

preferred analysis is quite cumbersome when confronted with highly aspherical particles. Considering the reference USP circles, the 10 μm circular area is 78.5 μm^2 and the 25 μm circular area is 490.9 μm^2. Observed particle area would be judged against these comparators, shown in Table 10. The comparison is quite revealing; roughly spherical or equant, flake, and tablet-shaped particles will be sized quite directly against the linear scale or the circles, since the two largest axes present nearly equivalent circle area dimension. For particles with an elongated shape axis, such as long laths, rods, fibers, or needles, however, where one or two axes are quite minor, the estimate of size according to an equivalent circular area will yield significantly different values from longest dimension.

Thus, rod and needle shapes, while quite long, would be counted in much smaller categories (if at all) than the apparent lengths. Many companies have converted the maximum length of particulate rods and needles to the ECD for years, and maintaining this historical database is important to them. An alternate practice is to use maximum length estimates as a conservative, worst-case stance, thereby generating counts for particles as if they were all diameters of spheres. This conservative approach raises a warning flag for formulations laden with thin crystals as "acceptable" by USP definition, when objectionable by practicality. Use the microscopical evidence for its true value, seeing particles resident and dry on the membrane, as a complement to the LO data.

Ultimately, all sterile injectable products and certain topical (ophthalmic) products must meet compendial visible and subvisible particulate guidelines. The requirements for visible extraneous particulate matter are simple and are typified by the USP description:

> **USP <788>** "*... injectable solutions ... are essentially free from particles that can be observed on visual inspection...*

The USP has stringent rules regarding Foreign Matter, "...every care...to prevent contamination with microorganisms and foreign matter...each final container be subjected individually to physical inspection (whenever the nature of the container permits)...every container whose contents show evidence of contamination with visible foreign material be rejected" (33); however, USP makes no attempt to describe inspection conditions. Both Japanese (34) and European (35) guidance are more explicit in regard to light intensity, type, and method. The guidelines apply to reconstituted solutions as well.

Dry solids for reconstitution are constituted at the time of use and must comply for:

Completeness and clarity of solution

a. The solid dissolves completely, leaving no visible residue as undissolved matter.
b. The constituted solution is not significantly less clear than an equal volume of the diluent or of Purified Water contained in a similar vessel and examined similarly.

Particulate matter—constitute the solution as directed: the solution is essentially free from particles of foreign matter that can be observed on visual inspection.

Therefore, for all forms of parenteral products, in a development stability matrix, parameters we would routinely evaluate in sequence include

a. color of cake (sterile powder)
b. reconstitution time (sterile powder)
c. color of reconstituted/all solution (all)
d. clarity of solution (all)
e. particulate matter evident by inspection (all)
f. pH (all)

Particle Quantitation Methods

Coulter Counter—Electrical Sensing Zone Method, Size Range 0.4–1200 μm

The sample is diluted into a weak electrolyte solution and drawn through a small aperture, passing between active electrodes, interrupting an electric field. Response is based on the displacement volume of the electrolyte, and thus sizing is in geometric volume means. The response is unaffected by particle color, shape, composition, or refractive index. Suspending all but simple formulations in the saline buffer electrolyte, however, may cause a number of unwanted assay artifacts because of formulation instability or particle changes. Coulter is an excellent choice for dispersed powders, simple solutions, and suspensions (36).

Laser Diffraction—Size Range, 0.1–600 μm

Operating in a wide size range, these high-performance instruments are most useful for dispersed systems and evaluation of bulk powders. In laser diffraction particle size analysis, a representative "ensemble" of particles passes through a beam of laser light, which scatters the incident light onto a Fourier lens that focuses the light onto a detector array. With specific algorithm, particle size distribution is inferred from the collected diffracted light data.

Photon Correlation Spectroscopy, Dynamic Light Scattering, Static Light Scattering Size Range <1 nm to 6 μm

Particle size is determined utilizing fundamental light properties. In diffraction instruments, the angle at which the light is diffracted depends on the wavelength of the light and the particle size. The angle of diffraction is measured to determine size. For a particular particle size, should the wavelength change, the angle will change. Using light frequency, the number of waves pass through a given point per unit time. Higher frequency is recorded as more waves cross the point or as the distance between waves shortens. Frequency change or shift information is used in dynamic light scattering. These instruments are essential in probing the submicron and nanodomains of the product fluid, with formulation character and stability as primary goals (37).

Image Analysis—Static: 0.2 μm to 100s of μm Range

How can one improve on optical microscopical examination of material? By retaining the realistic views of optical microscopy in the optimal illumination, digital images are captured and further deconvolved using the software systems in image analysis. Although a quite powerful technique because of operator control and manipulation of the data, it is also problematic for primary records and product release for the same reasons. One may ask, what has been done to the primary particle(s) in question? To facilitate particle study, one may select filters to screen out unwanted artifacts or particle populations (circularity, aspect ratio, etc.) and then commence study of the selected population set.

Image Analysis—Dynamic: 0.5 μm to 100s of μm Range

As in the static systems above, but utilizing image capture in dynamic, flowing systems, such as undiluted product fluid. Dynamic image analysis utilizes the microscopical components

(*i*) illumination, objective lens, and focusing lens elements, and adds fluid pathway and flow cell, plus camera, and processor for acquisition and analysis. The realistic views of optical microscopy for particles in situ augment interpretation of other in-suspension measurements that cannot visualize the particles in study.

The advantage of dynamic flow microscopy is size and feature analysis of the actual particle image, not calibration against a response curve for derived properties such as equivalent circular diameter (ECD) or geometric volume mean (GVM) or similar indirect measurements.

Sterile Injections, Suspensions, Emulsions

This discussion concerns the evaluation of parenteral formulations in a candidate product development program.

Compendial Methods for Parenteral Products

Global compendial organizations provide guidance for the local use of the pharmaceutical product. This guidance includes (*i*) definitions of products and uses, (*ii*) monographs containing medication-specific criteria, (*iii*) general rules for the use of pharmaceutical products, (*iv*) guidance for manufacturing, (*v*) guidance for physical facilities, (*vi*) specific test methods, and (*vii*) reference standards.

Compendial Considerations for Pharmaceutical Parenteral Products

USP

Parenteral products are defined by the USP in General Chapter <1> *Injections* as products intended for injection through the skin or other external boundary tissue in single-dose or multiple-unit containers, with careful control of sterility, pathogens, particulate matter, and other contaminants (38). The definition is shared by other compendia and by major compendia JP and EP. The particulate matter requirements are applicable to all liquid formulations and certain emulsion, liposomal, microparticulate candidates, regardless of container type. The concepts of sterile parenteral parameters have been applied to ophthalmic formulations recently, with guidelines for particulate matter quality of ophthalmic products are similar to parenteral products (39).

Global Particulate Matter Guidelines

The impetus for harmonization of the particulate matter determination methods came from the Pharmacopeial Discussion Group (PDG). Harmonization of compatible procedures is a primary goal of PDG. All three compendia have the same version, except for national text and the slight difference in SVI definition by JP, to exclude 100 mL nominal fill.

World Health Organization

The World Health Organization (WHO) maintains the International Pharmacopeia (Ph. Int.) in a broad guidance reference that does not specifically address particulate matter in injectable products (40). The WHO philosophy considers the first-tier issues of medication identity, safety, and efficacy. Injection safety is of utmost importance, followed by the necessity of injection therapies.

Major compendia include European Pharmacopeia (Ph. Eur.) and Japan (JP). Two methods for particle numeration have been harmonized through the efforts of the Pharmacopeial Discussion Group principally for USP, EP, and JP. These are the methods described earlier, LO and MM. The related particle content limits are contained in USP Chapters <788>, Ph. Eur 2.9.19 (41), and JP 6.07 (42). Both methods are suitable for the evaluation of subvisible particles in a variety of parenteral product formulations and presentations and may be used in development of formulae for other product forms, with appropriate validation. The same methods and product limits are endorsed by all. The intent of both LO and membrane methods is to provide robust means to assess total subvisible particle content in the commercial product.

PM that may be seen visually, USP Chapter <1> and in subvisible range, USP Chapter <788> are addressed. There has been an evolution of particulate matter measurement methodology and acceptance criteria since the inception of public domain limits for the content of particulate matter in parenteral products. In 1975, with USP XIX (43), particle matter in large volume injection (>100 mL nominal fill) parenteral products was determined by membrane-based testing with particle limits of not more than (NMT) 50 particles per mL $\geq$ 10 μm and NMT 5 particles per mL $\geq$ 25 μm. Since this initial membrane microscopical method was applied for LVI products, much investigation and method revision has occurred in USP guidance. LO was developed and implemented as the preferred or primary method, largely because of method control and efficiency.

The current methods and their reliability have been continually reviewed and improved through the efforts of USP expert committees, contribution from industry, and assistance by industry specialists overseen by the USP-PPI (Parenteral Products—Industry) expert committee. Certain formulations cannot be tested by either method, such as extraneous matter in sterile suspensions. The current methods are now also applied to ophthalmic solutions in the United States (44).

Compendial Considerations for Pharmaceutical Ophthalmic Products

A new U.S. guideline for ophthalmic products was official in 2004. The evaluation of ophthalmic formulations is conducted quite similarly to that of the parenteral candidate product development program. Ophthalmic products are defined by the USP as products intended for topical application on the eye; however, they have been applied for intravitreal, conjunctival, and subtenon injection for eye therapies. Careful control of sterility, pathogens, particulate matter, and other contaminants apply.

Certain ophthalmic products must meet compendial visible and subvisible particulate guidelines, as defined in USP Chapter <789>. The guideline refers to the parenteral chapter <788> for methodology as well:

> **USP <789>** *"... topical preparations ... are essentially free from particles that can be observed on visual inspection ..." "... particle content is limited ..."*

For ophthalmic products particulate matter determination, note that only per milliliter limits are used, and that if the MM method is run, an additional size threshold must be reported as shown in Table 11. Thus, visual inspection and subvisible particle load evaluation is prudent and necessary for proper development of both parenteral and ophthalmic products.

PARTICULATE MATTER CONTAMINATION

Medical Impact—Physical Blockage

The presence of particulate matter is certainly unwanted in parenteral ophthalmic products, and care must be taken to minimize the content. What content or level matters? The answer has never been simple or dogmatic, since the "insult" must be measured in regard to absolute number, at given size, mode of entry (pulmonary, venous, etc.), duration (chronic or acute?), patient health and patient resilience. These last two factors are real but do not present reasonable consideration; we do not grade products in regard to the capacity of the patient to accommodate them. Medical effects from particle "insult" include infusion phlebitis, pulmonary granulomas, pulmonary arterial lesions, severe pulmonary dysfunction, and loss of functional capillary density of post-ischemic striated muscle to death (45).

Table 11 USP Chapter <789> Subvisible Particle Limits for Ophthalmic Solutions

Method 1—LO		Method 2—microscope		
$\geq$10 μm	$\geq$25 μm	$\geq$10 μm	$\geq$25 μm	$\geq$50 μm
50/mL	5/mL	50/mL	5/mL	2/mL

Pathophysiologic Mechanisms

The obvious and direct effect of undissolved solids in the injected liquid is mechanical blockage of small caliber arterioles. Other effects include activation of platelets and neutrophils, generation of occlusive microthrombi (46).

Why has there been so much work and concern regarding particulate matter in the pharmaceutical industry and regulatory agencies? Intuitively, one may conclude that injecting solid extraneous matter into human arteries is an objectionable practice. Indeed it is. Certainly the foremost concern for the population receiving the injectable product is sterility, and extraneous matter is a known vector for the transmission of microbial contamination. Secondarily, minimizing the content of foreign matter delivered to the patient is certainly important. Finally, counting and tracking the content of extraneous particulate matter is an important process control tool. The "Holy Grail" of particulate matter control is a product with zero extraneous matter content. Are there known levels of particulate matter that cause physiological problems?

Among the many reports that detail the harmful effects of extraneous solids injected into the human arterial system, all agree that the content of particulate matter must be minimal and constantly sought to be minimized. In comprehensive reviews of pharmaceutical product quality, the evidence of human systemic contamination and physiological damage in high-contamination doses is obvious. Injected particulate matter is medically objectionable because of the potential for capillary emboli, for example, sudden obstruction of a blood vessel by debris. Recently, Carpenter et al. (47) proposed that subvisible particles below the current compendial limit (<10 μm) promote protein aggregation and must be monitored and controlled in therapeutic protein products. Just where do agree the line in the sand should be? I'm not sure we will ever have a single load and size limit.

The concern does not end at the therapeutic agent alone; product use and combination are also issues. For example, multicomponent admixtures aseptically compounded from multiple source containers that individually pass particle size limits of <788> may collectively contribute a large particle load. Evidence of this effect has been shown with physical confirmation of glass fragments and cotton fibers in the pulmonary arterial system during the *post mortem* examination of infants receiving parenteral therapy (PN) (48).

Thus, the primary medical risk from elevated particle content and patient insult is capillary embolism, causing interruption of blood flow that provides oxygen and nutrients to cells (49).

Utilizing these methods is an important measure for reducing these risks to the ultimate consumer, the ill patient. While it is doubtful any commercial product presents an extreme level of risk, we assay and track the particle level because particulate matter may present a physiological problem for the patient, especially when administered to certain patient populations at high levels and over time. The fundamental reasons to measure particle load content and evaluate process trends provide the means to understand control and improve product quality. Indeed, researchers have argued that pursuit of reduced contamination is less one of physiological impact to the patient, but more an indicator of improved process quality control (50).

STABILITY—DESIGNING ROBUST FORMULATIONS

We mentioned that investigation and optimization of the formulation and the subsequent production process occur during development. The particle determination methods are key to this development. Table 12 shows the categorical types of particles that will form significant particle species.

Both compendial particle counting methods tabulate solid matter and may be skewed by certain artifacts. While it is the intent of these guideline methods to measure the content of low levels of extraneous matter, particulates arising from other phenomena and product component interactions will also be detected. Even a low level content of extraneous matter at time of release may be from a single-event addition, a point source. Also present may be intrinsic-sourced material, which must be rigorously detected, examined, and removed during

Table 12 Varieties of Particles Within Two General Categories of Source

Extrinsic—external	Intrinsic—internal
Natural	Product-package interaction
Vegetative	Hydrolysis
Anthropogenic	Leachates
	Corrosion
Manufacturing	Active ingredient/component change:
Metals	Degradation
Polymers	Hydrolysis
Corrosion products contributed	Salt forms
Extracts from points of contact	Oligomer forms
Cleaning processes	Nucleation/crystallization
Filling arena	Coalescence
Water	Sedimentation
Materials	
Air quality	
Personnel	
Equipment	
Package cleanliness	Sedimentation
	Impurity content and growth
	Process-contributed
	Extracts/leachables, primarily from package

evolution of the product form. These particle sources may represent significant formulation instability and may result from one or more of the following:

- Process control failure,
- Poor formulation design in regard to use, storage, compatibility,
- Special concerns of biomolecule formulation stability, such as protein aggregation
- Adverse interaction between the formulation and the container/closure system,
- A package system that is archaic or unsuitable for the fill
- Leaking or excess vapor loss,
- Uncontrolled or unknown excipient quality,
- Active ingredient quality.

Robust and stable formulations do not occur by chance. Utilizing a comprehensive development program of formulation, physical and chemical stability evaluation is a prudent step in the product development process, and also yields assessment of the product appearance and measurement of particulate matter content. Development stability programs must include USP <1> product appearance (package aesthetics and verification of particle-free final package) and USP <788/789> particulate matter content, in statistically relevant sampling schemes that probe the batch population and indicate the appropriate sampling levels for the product process. These measures conducted during development help ensure that commercial product quality will remain on track through shelf life.

While it is not the purpose of this program to train you in chapter <1> guidelines, one must appreciate the connection between chapter <1> Injections, Foreign and Particulate Matter, the particulate matter determination described in <788>, and accordingly, <789>. Analytical approaches and sensitivity and manufacturing controls and equipment continue to be refined, and the improved physical and chemical quality in modern pharmaceutical products has been remarkable. Simple product appearance methods and more sensitive visual inspection methods must be appreciated for their ability to detect low levels of heterogeneous, insoluble, nonvolatile substances not detected by instrumental means. Beginning in early development, the use of visual inspection and human evaluation of product stability sets and a variety of illumination configurations must not be underestimated. Collimated light beam

inspection of the liquid formulae reveals light-scattering phenomena or Tyndall from submicron particle populations. More quantitative techniques such as nephelometry, turbidimetry, and color determinations provide early detection of physical change and instability in product forms.

But where does the boundary between visible and subvisible particle size (and type) exist? One cannot utilize visual inspection alone for the larger particles, just as assays for subvisible particle content also reveal particles in the near-visible sizes. Fundamental to the development of robust formulae in stable packages is utilization of many methods of overlapping detection to reveal all variations of contamination. Most of the R&D methods do not carry through to commercial target release assays, having established the fundamental properties of the product and direction for improvement in early phase operations.

In this approach, R&D and production understand each others' goals and needs and work in concert to attain robust final product within the design of production systems. Rigorous investigation of API, excipients, vehicle, container/closure, their process streams, and compatibility are essential in effects such as color, haze, precipitation, aggregation, crystal formation. The integration of visual inspection and particulate matter level determination on stability provide good sensitivity for the detection of physical changes, such as color, haze, precipitation, aggregation, crystal formation, and resultant particulate matter increase. In this design, any appearance change or particle content increase because of one or more of these factors allows their detection, isolation, identification, and data used to improve the formulation-package design.

Discussion of Particle Origin and Nature

Particles will always be present, and therefore it is the effects on the recipient and the indication of formulation integrity that are most important, in that order. With pharmaceutical cleaning, fill arena preparation and maintenance, and solution filtration to 0.22 μm nominal porosity, the inclusion of significant particle numbers is much reduced. Even single organisms included in the fill could bloom to a large number or pose pyrogenicity concerns.

Point Source

Limiting the discussion to number and size of particles is insufficient. Certainly the goal of pharmaceutical processing is a product that is consistent, efficacious, low impurity, high potency, sterile, low particle-load fill volumes that remain that way for their shelf life. But should we deem formulation meeting the public limits, or even fractional target limits, sufficient? In certain situations, even low numbers of particles may be objectionable, if from toxic sources, from known adulterants, and even from a singular or point sources. Toxicity and adulteration events are obvious; however, consideration of point sources is important. Consider the following case history:

> A batch of 87,000 ampoules were filled over a one-day sequence with in-line processing for washing, drying, and sterilizing the open ampoules just prior to filling. In-process mil std. inspection and terminal 100% inspections showed <1% rejection for all defects, with much <0.5% rejected due to particles. Assay for subvisible particle load by LO of 10-unit pools from each of the 10 sublots resulted in no individual units and no pool average 100 particles $\geq$ 10 μm and 16 particles $\geq$ 25 μm. The lot was released, but prior to labeling and shipment, samples of the released ampoules were used for microscopical assay training exercises. The resultant counts by MM were well correlated to LO (no silicones in an ampoule to skew the LO count) at 76 particles $\geq$ 10 μm and 23 particles $\geq$ 25 μm. However, the microscopist counting the samples asked why all the particles were the same and so shiny? Examination of the particle isolate membranes by lab supervision revealed a nearly singular occurrence of one particle type, metallic flaky solids. Upon further investigation, identification of the particles confirmed them to be stainless 316L and tracked to a particular (new) valving system being used on the filler (without in-line fill filter). The lot was retrieved from the distribution centers and rejected.

Why was the lot quarantined and rejected? All tests met particle specifications, for example, particulate matter content was far below the SVI products limit of 6000 particles $\geq$ 10 μm and 600 particles $\geq$ 25 μm limits per container set by USP <788> for analysis by LO

and the SVI limit of 3000 particles $\geq$ 10 μm and 300 particles $\geq$ 25 μm limits set by USP <788> for analysis by MM. Rejection was selected because the particle load was uncontrolled, difficult to observe by the 100% release inspection (too small and settled quickly) and so reinspection by any method was unlikely to remove isolated metallic contamination, and most objectionable as a particle type. The event was isolated, since this was the first use of this type of valving system. The finding necessitated the valve removal from any future processing step, launched an engineering investigation into the effects of different valve metal components, and thus isolated this incident from related manufacturing.

PROCESS CONTROL—MEASURE OF INDUSTRIAL COMPETENCE

Manufacturing Arena

Federal Air Standards

No discussion of particulate matter in parenteral medications is complete without reference to the expectation for air quality in the assembly and filling zones curtaining our package preparation, setup, filling, and capping. The air quality and personnel gowning and movement within these zones has significant impact on the sub-10 μm particulate content in the final product. Historically guided by federal standard 209E and currently by corollary ISO 14644 classification, ISO class 5 is equivalent to historical Federal Std. 209E class 100 (51). Filling arenas operating at this level will sample three locations, and minimal 19.6 L volume and require the following airborne particulate matter level limits:

Count	Size (μm)
100,000	0.1
23,700	0.2
10,200	0.3
3,520	0.5
832	1
29	5

While not directly contributing to the >10 μm population, the sub-5 μm population is a direct indicator of the most transmissive and contamination-bearing particle population. Spikes in the sub-5 μm population are most certainly a warning that larger particles may also be increasingly present.

Area Control

We have been discussing minimization of particles in regard to extraneous matter. Elevated levels, extraneous types, and variable content of particulate matter are all significant indications of preparation and assembly processes that are not in control. We must appreciate the fundamental requirements for pharmaceutical products in regard to safety, sterility, stability, efficacy, and purity as the essential foundation of commercial pharmaceutical products and often relating to the assembly process. The selection of raw materials, storage, assembly, and packaging of the final product must be derived from the development process and well controlled for commercial production. Modern pharmaceutical filling arenas are designed such that visibly sized, environmentally related particles are minimized in the final package, since equipment and processes have been configured to minimize exposure of the product fill and package interior to extraneous matter. Form-fill-seal processes are good examples of the utmost commercial filling design refinement. Process streams starting with the vendor are often configured specifically for the product, and at a minimum, components are selected and optimized on the basis of the properties of the formulation.

At the end of the process, normally prior to labeling, 100% final package inspection is conducted to reveal the presence of any visible particulate matter that may have been included in the product. Inspection systems and their reliability are important because of the relatively insensitive detection at particle sizes approaching the lower detection limit (which we stated as 100 μm) and the upper end of the detectable size ranges by the primary method, LO. Although

visual inspection is based on human detection capability, many pharmaceutical firms utilize machine-vision inspection systems calibrated as equivalent to, or more sensitive than, the human inspection methods.

Visibility of particulate matter is a function of several parameters but can be grouped in three categories: (*i*) operator acuity/machine sensitivity, (*ii*) particle physical properties, and (*iii*) inspection conditions. Many companies use a combination of (*i*) machine and human systems, (*ii*) serial inspections, and (*iii*) trained quality control auditing to increase the reliability of detection.

While product batch acceptance is dependent on subvisible particle content determination by these USP <788> methods, most new drug applications (NDAs) are filed with LO test data that also provide an excellent means after market launch to survey the batch quality through count levels and trending, minimizing the risk of defective packages from entering the marketplace (24,52).

In addition to verifying the batch meets compendial expectations, quantitation of particulate matter by either method provides a means to study process variations that may affect the product. Full understanding of the particle population by visual inspection alone is not realistic because of the limits of visual resolution (i.e., variability in visual acuity between persons and observations by the same person, as well as failure to observe specimens appropriately and the probabilistic nature of detection. Quantitation of particles is essential for trending and setting realistic (and improving) internal limits for batch quality, which will vary somewhat on the basis of the type of parenteral or ophthalmic formula and the packaging system. Membrane testing for quantitation is also an appropriate prelude to more extensive methods for the characterization and identification of particulate matter. Not only will the particle types isolated indicate potential stability and assembly issues, even small populations of single-type contaminants indicate a point source of particulates to be eliminated. Insight to the identity and thus cause(s) of elevated particulate matter will facilitate their control and minimization (53).

Nath et al. (54) has written a stimulus to the revision process to comment on the level of particulate matter in Office of Generic Drugs review of 295 ANDAs. Included were liquids and lyophilized and powder forms of parenterals, filed from 1998 to 2002, covering 51 firms and 110 drug substances. The data was determined primarily by LO. In these files, batches contain significantly less particle load than the current compendial limits, and the paper position is that compendial limits are far too high. Trends in mean counts were evident for aseptic versus terminal sterilization in package forms and in product forms. Nath and his coworkers detailed the "wide gap" between the current USP limits and the performance of freshly filled drug products. Mean counts for particles $\geq$10 μm per container were 27× and 40× lower than USP limits of 6000 $\geq$10 μm and 600 $\geq$25 μm, respectively.

Pharmaceutical and Delivery Systems

Devices and Delivery (IV) Sets

To maintain the integrity of the product, we have promoted the concept that one must carefully design and control the emerging formulation design with a carefully selected package to ensure continued stability. This is a difficult and complex task in pharmaceutical development, and especially so with shortened time frames, increasing use of innovative devices, which may be developed without good appreciation for the effects of contamination sources, and increasing use of non-core company resources of material and supply, which expands the "universe" the developer must understand and control.

The key element of the new formulation may be a one-of-a-kind device or process that is being licensed to good market advantage. Does the formulator understand the mechanistic design, the supply and control of components, and the process? Has it changed recently. . ..will it change without much control? If essential for the product and proprietary, can the formulator review for the innovator/supplier the concerns regarding particle addition and formation pathways, so as to prevent them? Are there details of the industry known to the practitioner, but not apparent to the user that may present a PM challenge? Sorting out answers to these questions early in development is advised, and use of stability set review is essential to predict final product stability and use issues.

PARTICLE ORIGINS

Additive/Extrinsic

Extrinsic particle types originate by *direct contamination or addition*. These are often singular events, although may occur repetitively and with multiple particles per event. The key is that extrinsic particles are often an event and are unchanging; the added particle(s) will not generally change over time unless there is fragmentation into smaller bits because of mechanical agitation or chemical activity (corrosion). PM typical of extrinsic origin will include paper, fibers, geological matter (soil minerals), biological matter (vegetative, insect), and airborne particles <10 μm.

The types of particles in extrinsic contamination events are environmental, machine-related (unless representing product effect on the machine), personnel-borne, as a result of inadequate preparation or cleaning.

Multiple Event/Multiuse Package

In certain cases, the extrinsic single event reoccurs because of reuse of the product, especially in regard to the closure integrity to resist coring (single large particle) and to resist shredding or otherwise losing filler material so common in elastomers. The source of particulate matter is finite; however, very little is needed to cause a defect.

Growth/Intrinsic/Changing

In considering the entire pharmaceutical package, the level of particulate matter that may be seen or may cause excessive particle load or even failure to meet compendial subvisible count limits is exceedingly low. Consider the example of particle number versus concentration in Table 13.

In the following examples, consider from the above table just how little content or change may promote an undesired particle event.

Package Change

Leaks and evaporative events may initiate change for actives that readily form crystalline hydrates. As the formulation loses water vapor, the elevated potency may be detected through stability assay and addressed. If the change is occurring for only a few containers or not sampled, the vapor loss may result in the nucleation and crystallization of the API to a less-soluble hydrate form, inducing a cascade of many more crystals. Package defects and other (extrinsic) particles present in the fill volume would serve as potential nucleation sites for this event.

Ingredient or Active Purity/Change

Particles will form as chemical changes in the active ingredient or formulation excipient produce less-soluble moieties over time. Any insoluble forms >0.2 μm are removed by sterilization filtration and the remainder <0.2 μm if unchanging, remain as trace levels of

Table 13 Relationship of Particle Count, Size, and Concentration for Selected Cases in a 10 mL Fill Volume

Particle count	Particle size	Particle concentration[a]
10,000	2 μm	0.004 ppm. . .(subvisible, not reported)
10,000	10 μm	0.52 ppm. . .. . . (subvisible, at threshold)
1000	25 μm	0.82 ppm. . .. . .subvisible, but visible if viewed after settling, then swirling container
1	100 μm	0.05 ppm. . .. . .just visible
1000	2 μm equant	8 ppb. . .. .visible due to light scatter
Subvisible		
100	5 μm particles = 0.125 μg mass = 12 ppb in 1 mL	
50	10 μm particles = 0.5 μg mass = 50 ppb in 1 mL	
5	25 μm particles = 0.781 μg mass = 41 ppb in 1 mL	

[a]Density = 1 g/cm^3.

formulation impurities. These forms may appear (in chemical assays, and as subvisible particles, and even to visible sizes at higher concentrations) as their concentration builds above their solubility product in the formulation, in storage, over the shelf life of the medication. Very little material is needed to yield a single, visible particle, or significant amounts of subvisible particles >10μm to yield elevated PM assay counts (Table 13).

Product-Package Interaction

The integrity of the formulation has much to do with the lack of product-package interaction. We desire an inert package with the product fill. Ions will seek equilibration in the product fill from all product contact surfaces and dissolved ions from the formulation constitute the new formulation "stew" from which new more insoluble particle may form. Drug ingredient with low solubility product (K_{sp}) for any available ion will readily form solids, and often well-formed crystals, especially at promoting temperatures during storage.

Glass corrosion occurs in borosilicate tubing vials on exposure to base and elevated temperature. The effect is 25 times more severe in mild base around pH 8 than in mild acid around pH 4 (55). The inner glass wall corrosion is pronounced at sites of extreme container formation temperatures, such as the base. Glass borosilicate ampoules with much thinner walls have not shown the same effects, because of the much lower level of finishing heat needed to form the container. Similar corrosive effects have also been promoted by so-called aggressive solutions, such as high concentration solutions with counter ion, such as sodium bicarbonate and drug phosphates. Sulfate-containing formulations have shown white ring deposits on the inner glass sidewall, at areas of greatest heat influx, and correlated barium ion migration, forming highly insoluble Ba_2SO_4 crystals on the glass reaction surface (56).

The much more reactive and rich formulae of closures can have a significant impact on the formulation as material physically falls from the elastomer and extractables exchange with the formulation fluid. As in the above examples, the chemical equilibrium drives the inclusion of small amounts of elastomer formula components in the fill, especially the volatile, low melting, small mass, and high vapor pressure moieties. If the substances have limited solubility in the fill, solid PM will form and grow and may reach detectable size ranges.

CHANGE MECHANISMS

Coalescence/Aggregation

Direct

The affinity of separate <50 μm solids to cluster group in fragile aggregates will be the fundamental cause of size change for microparticulate matter. The adhesion of particulate matter, whether homogeneous or heterogeneous, is driven by weak yet effective forces (57):

1. van der Waals forces between particles
2. Capillary force of hydrophobic liquid between particles in hydrophilic matrix
3. Electrostatic forces—for particles >5 μm may only be a concern in airborne and dry surface situations, as in delivery of the foreign matter to the pharmaceutical container.
4. Adsorption at point of contact, promoted by elevated temperature and time, resulting in chemisorption between particles and stronger adhesion.

The consideration of these forces is most important for preparation and cleaning of the pharmaceutical package, since in the fill solution, much of the weak force is overcome by the aqueous or oil solution.

Aided

Oils, liquids, polarity groups (friends) within matrices of opposite polarity all serve to enhance the grouping of separate solids. Silicone oil is one of the most common coalescence aids in aqueous media due to its ubiquitous use, relative insolubility, and stability. Water in hydrophobic media would have a similar grouping or adhesive effect.

Sedimentation

The smaller the particles, the less likely one will see them visually and count them analytically. In certain instances, one may visualize settled particles that were never observed when dispersed throughout the fill. Thus, it is prudent to allow parenteral fill solution to settle for a day prior to first visual examination. Centrifugation may hasten the process. Examination on a gentle swirl of the container resuspends particles and is preferable to vigorous inversion mixing and mechanical axial rotation to cavitate the fill. Slow axial rotation (a swirl by hand, maybe?) will reveal even the most light dusting of very small particles, especially if assisted by a strong light beam to impart a Tyndall effect.

Degradation

Active ingredient and to a much lesser extent due to concentration excipient components will degrade. Many factors can augment the process and may yield sufficient less-soluble product to yield particulate matter. Hydrolysis and oxidative processes are most likely; however, the change imparted by physical effects of cold, heat, shear, and light cannot be ignored in the change event.

Precipitation

This phenomenon is most probable for formulations near or above the solubility limits of the formulation components, especially the active ingredient. Ideal conditions are those with no nucleation site potential (PM and package defects), at target pH and held at insert temperature; however, changes in the formulation may promote active ingredient precipitation. The most common and likely event is for active ingredient with significant insolubility for certain common counterions, such as cations Al, Mg, Ca, K, Ba, and all transition metals. Ionic extracts from the package must be considered, and while bulk content is important, surface concentration for all formulation contact is very significant. For inorganic substances with very low solubility products, storage contact with formulations containing simple buffering systems of sulfate, phosphate, acetate, and even higher mw counter anions may promote precipitation to yield elevated particle count in a wide size range. Precipitation of barium sulfate crystals was evident on the glass surface of a container with available (extractable) Ba^{2+} in contact with the high concentration of drug anion and bisulfite ion of the antioxidant (58,59). The aforementioned example was from a glass package with BaO content, but at a minor constituent concentration of 2%.

Closure systems were common sources of extracted trace ions promoting particle formation with actives and buffering systems until reduced and alleviated in some cases by improvements in elastomeric formulation to remove highly mobile and components with high counterion capacity. Barrier systems of Teflon and proprietary polymer systems on the formulation face of the closure have been very successful.

Nucleation

Consider the minute sites responsible for the promotion of growth of a singular species to be a nucleation event. In our consideration, the heterogeneous nucleation phenomenon is most important; for example, a foreign body nucleating the growth of drug crystals from solution. Certainly, any material may provide a sufficient platform; however, small inert solids such as glass or minerals are ideal nucleators. Defect zones consisting of rough surfaces on the package walls also provide good sites for particle formation. One can also consider silicone oil or immiscible liquids in the pharmaceutical fill to be potential nucleators, although their promotion of crystal growth is much less likely than as formers or gatherers of material.

Crystallization

One of the most dramatic events one may encounter in formulation stability is the generation of crystalline solids, significant as stability failure and in a dynamic process, crystal generation indicates a serious challenge for the formulation. PM may be present or form from API impurities and degradation products. Larger (visible) PM may be present even from ppm concentrations. Most critical factors are relatively insolubility in the product solution -pH, ion content, temperature, and dilution. In situ salt formation is sometimes performed during

manufacturing process, acid or basic impurities dissolve in the product during the titration process even after the pH is uniform throughout the solution, those dissolved impurities could still exist in solution in a supersaturated state, not removed by filtration. The dissolved impurities precipitate over time, leading to product failure. API may form a gel after dissolving in solution and take a long time to solidify, especially at low concentrations of API. The gelling process typically is preceded by haze formation, with detection aided by Tyndall beam light. Finally, incompatibility between the active ingredient and diluents used in admixing and preparation can be another source for PM in the final dosing solution.

Nucleation
Insoluble impurities may be present in a more soluble form (amorphous) and precipitate in final product in a more stable form (crystalline).

Hydrate or Solvate Formation
These forms are more thermodynamically stable than the parent molecule and often less soluble than the anhydrous form, thus constitute significant threat to formulation stability in high concentration formulations experiencing dehydration, and volume loss. Accordingly, solvate crystal forms may also occur, though rarely in aqueous formulation. Hydration of proteins is essential, often occurring at high percentage of the drug volume and so hydration effects are more pronounced in these formualtions.

Polymorphism
If conditions allow, especially in high concentration formulations, alternate physical structures may nucleate and reside in solid form. Chemically identical, however, different physically, the formed polymorph is the end of the stable formulation as intended, since return to the original polymorph is quite unlikely energetically. Again, the key for remediation of any crystallization event is full understanding of the solubility and stability parameters for the form and any causative events, such nucleation seeds or surfaces, pH change.

Salt Formation
Much more likely than solvates and polymorphs, newly formed, less soluble salt forms are a danger in packages with potential for ion exchange, such as (*i*) sealing with elastomeric septae, (*ii*) formulation components that may contribute ionic character, (*iii*) pharmaceutical water, (*iv*) insufficient cleaning of components, and (*v*) ionic species from the glass container.

Impurities
The presence of trace to low concentration impurities is common and more pronounced upon storage. Formulation physical stability may be compromised by the growth of impurity-related particles, especially for relatively insoluble moieties, in the presence of foreign material, immiscible liquids, and a growing population of other impurities, soluble or insoluble.

Micellar Change
Many drug moieties are amphophilic, for example, drug molecules with a polar (hydrophilic) and nonpolar (hydrophobic) characteristics, primarily due to functional group characteristics. This is characteristic of detergent molecules, and hence their desired capability to "bridge" grease in water, and of many pharmaceutical compounds as well. The hydrophilic and hydrophobic ends of the molecule align in solution, forming micelles, or associations of molecular polarity with nonpolar cores in the aqueous formulation. Certainly, more complex variations of the micelle in nonaqueous systems and in conjunction with selected hydrophobic materials allow micelles to "carry" the relatively water-insoluble substances in an aqueous medium. The associated groups are small in number (up to 100 molecules is typical) and in size, ranging up to 10 nm. The micellar group can solubilize other hydrophobic substances, as a function of surfactant concentration. There is a critical concentration above which the

surfactant enters the micelle and the monomeric surfactant concentration remains constant. This is called critical micelle concentration (CMC). This phenomenon is particularly evident when excipients held in the micelle are subsequently released upon dilution of the formulation, brought about by dilution of the surfactant concentration below the CMC.

Micellar formulation stability depends on the stability of the small micellar groups. Imagine the effect on the molecular group where another hydrophobic material has been entrained in the hydrophobic core or is competing with the parent molecule. This competition is most significant in the cases of extracted organic moieties, such as plasticizers, for drug analogs, impurities, and degradation products that are more hydrophobic than the parent and alter the solubilizing activity of the parent micellar groups. The importance of the micellar activity in particle formation or, more realistically, particle appearance, is that seemingly stable, clear formulations can suddenly appear cloudy with the onset of nucleation, aggregation of material previously held in solution, and now falling out of the micellar groups due to competition with more amenable substances.

Oligomers

Monomers or single molecules will join through chemical processes to form dimers, trimers, and oligomers (a limited assemblage of monomers, short of polymerization). The importance of the larger molecules to the solution integrity is their inherent solubility, especially in regard to micellar active ingredient formulations and with specific emphasis for subsequent dilution of the formulation. How can a longer chain of the drug or even drug analog be a problem as particulate matter? It is directly related to solubility, and thus most prevalent in high concentration and saturated formulations, and those requiring micellar association for stability (solubility).

Leaching/Extraction

Extractables: "Substances that can be extracted from plastic materials/systems using extraction solvents and/or extraction conditions that are expected to be more aggressive than the conditions of contact between the material/system and a finished drug product."

Leachables: "Substances that are present in the final drug product because of its interaction with a plastic material or system (60)*."*

PM may not be evident until well into the shelf life of the drug product due to slow changes in the formulation and trace levels of leached substances. At each point of contact for components of the formulation, potential for including extracted substances must be investigated. A case history describing the bloom of an extracted substance in a concentrated active formulation occurred because of the extraction of a phthalate ester from a process filter-housing into the active moiety mother liquor during synthetic process yielded a significant visual cloud in the otherwise acceptable ampoule injectable product (61).

Particle Detection—Inspection

One must appreciate the connection between USP guidelines for packages, visual guidelines, and subvisible testing. Analytical approaches and sensitivity and manufacturing controls and equipment continue to be refined, and the improved physical and chemical quality in modern pharmaceutical products has been remarkable. Simple product appearance methods and more sensitive visual inspection methods must be appreciated for their ability to detect low levels of heterogeneous, insoluble, nonvolatile substances not detected by instrumental means. Beginning in early development, the use of visual inspection and human evaluation of product stability sets and a variety of illumination configurations must not be underestimated. Collimated light beam inspection of the liquid formulae reveals light-scattering phenomena or Tyndall from submicron particle populations. More quantitative techniques such as nephelometry, turbidimetry, and color determinations provide early detection of physical change and instability in product forms.

But where does the boundary between visible and subvisible particle size (and type) exist? One cannot utilize visual inspection alone for the larger particles, just as assays for subvisible particle content also reveal particles in the near-visible sizes. Fundamental to the

development of robust formulae in stable packages is utilization of many methods of overlapping detection to reveal all variations of contamination. Most of the R&D methods do not carry through to commercial Target release assays, having established the fundamental properties of the product and direction for improvement in early phase operations.

In this approach, R&D and production understand each others' goals and needs and work in concert to attain robust final product within the design of production systems. Rigorous investigation of API, excipients, vehicle, container/closure, their process streams, and compatibility are essential. The integration of visual inspection and particulate matter level determination on stability provide good sensitivity for the detection of physical changes, such as color, haze, precipitation, aggregation, crystal formation, and resultant particulate matter increase. In this design, any appearance change or particle content increase because of one or more of these factors allows their detection, isolation, identification and data used to improve the formulation-package design.

Light Obscuration Vs. Microscopy

The preference for the LO analytical method resides in its general availability, common use, and experience among the manufacturers. Instrumentation, calibration schemes, and vendor support make this method a far more routine and regimented application. The modular aspects of the instrument with strong vendor support, especially in calibration, make it the logical first-pass for particulate matter determination. The negative aspects of LO include the counting of artifacts (oils, air) and a volume-based sizing that often renders a low-bias size determination. Indeed, there are many parameters of LO operation one must control, which are shown in Table 14 (62).

MM has high value in revealing the nature of the retained particles and the type; however, it requires much more specialized training and may be considered imprecise without careful lab control. Membrane methodology is time consuming due to its labor-intensive nature and has several operational parameters that must be controlled or will add to counting

Table 14 Light Obscuration Assay Defects

Inherent (instrumental)	Applications
Size error:	**Calibration error:**
Pulse height $\sim$ projected area (sphere is best) Particle nature Under-sizing of transparent vs. opaque/color	All calibrations are secondary because they do not match the particle size parameter they are used to measure; e.g., how many polystyrene latex beads are in your solution?
Between-system *variation*: sensor type	**Coincidence effects** *Undercounting*—hidden particles *Oversizing*—two or more smaller particles counted as larger particle
Optical considerations: $\uparrow$ Refractive index difference $\sim \uparrow$ size	Artifacts: • Air bubbles counted, • Release of dissolved air (negative pressure sampling), • Immiscible oils – light affected by subpopulation, shift of baseline (added pulses) • Incompletely dissolved drug
Flow too slow: $\downarrow$ S/N	Sampling variability Stratification of natural particle types Small sips in large volumes LO sample relative to batch Inadequate mixing
Focused sampling: sips of 5 mL but representing what volume?	Counting efficiency Serious undercounting above 25 μm
Sample bias: large, dense particles settle	Sample volume LO samples 4 $\times$ 5 mL volumes If the sample pool is large (250–1000 mL) the coefficient of variation will be much larger than that from a 100 mL pool

Source: From Ref. 62.

Table 15 Improved Microscopical Assay—Membrane Microscopy Assay Defects

Inherent (instrumental)	Applications
Artifacts: membrane defects and preparation damage	Sizing: Graticule or linear scale used? Determining ECD by "mental" comparison, or by max. chord
Illumination: may be too low and/or misaligned Magnification : 100× ± 10×? USP graticule ± 2%?	Counting: Manual tabulation errors Blank: low and controlled enough?

Source: From Ref. 63.

and sizing error (Table 15) (63). Both tests are valuable and practitioner labs must have proficiency in each method. Key to these methods is the concept that there is no single ideal method, and development units must be adept at all compendial and alternate methods of assay (64). Strong formulation development and new product testing laboratories will maintain both methods.

PARTICLE IDENTIFICATION

Introduction

To control and eliminate particulate matter in the final product, the source must be controlled or removed from the assembly system. The ideal is to identify each particle or at least categorize in pursuit of the source. The manner in which the identification is pursued follows a stepwise and logical path, mediated by microscopical methods. Why microscopy? Advantages are that it (*i*) gives us the direct view into specimen state, (*ii*) requires minimal sample—ng-mg, (*iii*) instant recognition for many materials, (*iv*) context is evident, (*v*) associated and extraneous materials are identified, (*vi*) allows one to make the best judgment about next steps as a triage function. Disadvantages of this application are that it (*i*) requires extensive training and (*ii*) is quite labor-intensive.

Stepwise Process

A problem is usually manifested by incidence of visible particles and/or elevated subvisible particle content. The first of many steps begins with confirmation of the incident and data-mining with the observer. Clues to the identity and changes associated with the incident may reside with the initiator or owner of the process, assay, or system that discovered the particles. Representative reject containers are evaluated and confirmed present in the rejected units. Typical categories of particles by visual appearance or microscopical appearance and the nature of these isolates explored for fundamental properties and clues to their presence. In this first sequence, visual examination and low magnification stereomicroscopical evaluation aids categorization of the particles into size, physical features, solubility, hardness, reaction to chemical reagent groups. The properties are further determined to an identification using low to high magnification polarized light microscopy (PLM), wherein, refractive index, birefringence, extinction, pleochroism, crystal system, association (anything else there?), condition (what's happened to the particles?), crystallinity, more physical/chemical properties that may be conducted at the microlevel to reveal surface character, size, shape, degree of transparency, dispersion of refractive index, confirm functional groups, predict solubility (helpful for standard assays—chromatography, mass spectrometry, NMR). Microfurnaces or hot stages may be utilized with PLM to determine thermal effects, showing dehydration, melt, glass transition, crystallization, nucleation, crystal transition. Obvious particulate species may be identified by this stage – common to the microscopist, and often unknown by conventional laboratory assay, such as cellulosics, all forms or biological solids, fibers, hairs, insect-related matter, for example, commonly extrinsic materials. Metals, polymers, paint, glass, rubber, oils, inks may all be categorized but not identified by this time. More importantly for long-term stability, what forms of intrinsic species are present? Drug and formulation component species may be identified by crystallographic properties alone. Others may require preparation

mediated by microscopy and analysis by infrared microspectroscopy, or (Fourier transform or Dispersive) Raman microscopy, bulk X ray, examination by scanning electron microscopy with energy-dispersive X-ray spectrometry, and mass spectrometry (65).

Particle Evaluation

At this point in the analysis you may know the types of particles involved, as contamination, process equipment, formulation, package, or true active ingredient change. Is the event due to single or multiple incidents? Often an inspection method may alert the lab to the largest or most distinctive particles, but not the true cause of instability. Careful investigation of the subvisible content often reveal those species responsible for the visible event. Are the particles representative of a chemical or physical change? Are they extrinsic or intrinsic? What level is occurring, and has it changed with time? Is the origin obvious in the primary particle or indicated by secondary, low level particle load?

Particle Characterization—Process Control

Minimization of particulate matter content is an important facet of the pharmaceutical assembly process. Modern filling arenas are designed such that visibly sized, environmentally related particles are rare in the final package. 100% final package inspection is conducted to reveal the presence of any visible particulate matter that may have been included in the product. Although this inspection is based on human detection capability, many companies utilize machine-vision inspection systems calibrated equivalent to or more sensitive than its human counterpart. Visibility of particulate matter is a function of several parameters but can be grouped in three categories (*i*) operator acuity/machine sensitivity, (*ii*) particle physical properties, and (*iii*) inspection conditions. Many companies use a combination of machine and human systems to detect visible particle content in the parenteral product. Requiring product batch acceptance by visual inspection and subvisible particle content by USP methods such as stage I (LO) or stage II (membrane) testing provides an excellent means to survey the batch quality and prevent defective packages from entering the marketplace (24).

In addition to verifying the batch meets compendial expectations, quantitation of particulate matter by either method provides a means to study process variations that may affect the product. Quantitation of particles by visual inspection is not realistic due to the probabilistic nature of detection. Membrane testing is an appropriate prelude to more extensive methods for the characterization and identification of particulate matter that provide insight to the cause of elevated particulate matter and thus allow its control.

Investigation of Change

Several categories of change and events are most helpful to consider in particle-generation investigations:

- Process control failure,
- Poor formulation design in regard to use, storage, component/package compatibility,
- Special concerns of biomolecule formulation stability, such as protein aggregation induced by heat, light, salts, etc.
- Inability to maintain formulation integrity because of solubility product
- Adverse interaction between the formulation and the container/closure system,
- A package system that is archaic or unsuitable for the fill
- Leaking or excess vapor loss,
- Uncontrolled or unknown excipient quality,
- Active ingredient quality

SUMMARY AND WRAP

PM content in the final package pharmaceutical product is inversely proportional to the degree of development, investigation of robustness, and the quality of commercial preparation at product assembly. Investigation and optimization of the formulation and the subsequent

production process occurs during development and is continued in the commercial arena. Particle determination methods are key to this development. Many analytical methods are utilized for particle determination, often most appropriate within specific size ranges, such as the submicron, sub-10 μm, and 10 μm to visible ranges. Both USP compendial particle counting methods tabulate solid matter but may be skewed by certain artifacts. While it is the intent of the public guideline methods to measure the content of low levels of extraneous matter, particulates arising from other phenomena and product component interactions will also be detected. Even a low level content of extraneous matter at time of release may be from a single-event addition, a point source. Also present may be intrinsic-sourced material, which must be rigorously detected, examined, and removed during evolution of the product form. More critically, what investigational methods are most pertinent to the dose form, formulation, and stability-indicating properties of the active ingredient?

In development and even for commercial testing and release, the public domain methods and relevant limits may be insufficient for full analysis and control of the final product. Deep understanding of the formulation integrity, in the selected package over time is necessary for continued high quality production. Investigation and use of additional analytical and performance methods will aid the ongoing effort. Integration of the efforts of key pharmaceutical teams in analytical, formulation, packaging, clinical, quality assurance, and regulatory will keep the products clean and stable.

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6 Endotoxin testing

Michael E. Dawson

INTRODUCTION

Endotoxin is a potent biological response modifier, with a wide range of effects including fever, shock, and death. Endotoxin contamination of parenteral medications is of particular concern because these products are administered directly into the body, bypassing the protective barriers of the skin and the intestinal wall. Consequently, endotoxin contamination of parenteral medications must be strictly controlled to prevent deleterious effects on recipients of the products. By contrast, endotoxin contamination of topical and orally administered therapies is generally not a concern because of the effectiveness of the skin and intestine wall in preventing the entry of endotoxin into the blood and other tissues.

The bacterial endotoxins test (BET) is one of the two critical microbiological release tests common to parenteral products, the other being the sterility test. The test is imperative because of the harmful effects caused by endotoxin. The high potency of endotoxin is illustrated by a comparison with therapeutic products. The minimum endotoxin dose expected to elicit a fever response in humans in a single administration (the threshold pyrogenic dose) is five endotoxin units per kilogram body weight (5 EU/kg) (1), which is equivalent to approximately 0.5 ng/kg of *Escherichia coli* endotoxin. This is orders of magnitude less than the doses of most drug products, which typically have doses in the milligram per kilogram range. Endotoxin is clearly a very potent substance and its effects are predominantly deleterious.

Endotoxin is almost universally present in the natural environment, and it is resilient and persistent. Stringent and specific steps must be taken to ensure that parenteral products are not significantly contaminated with endotoxin, either from component materials or by introduction during the manufacturing process. Finished parenteral products must not contain quantities of endotoxin in excess of the limits specified in either compendial monographs or approved submissions. Low levels of endotoxin, below these limits, are tolerated by humans (and other species).

This chapter begins with a review of the nature of endotoxin and its role in bacterial cells. The biochemistry of endotoxin; its effects and properties; the practical implications of the properties of endotoxin for parenteral products; and standard endotoxin preparations are then discussed. The *Limulus* amebocyte lysate (LAL) reagent is introduced and the various LAL test methodologies for the detection of endotoxin are described. The regulatory context for the endotoxin testing is addressed with particular emphasis on the harmonized pharmacopeial endotoxins test chapters and the United States Food and Drug Administration (FDA) Guideline on the *Limulus* amebocyte lysate test (2). Practical information is presented on testing parenteral products and a brief section on testing medical devices is included. The chapter finishes with an overview of depyrogenation.

ENDOTOXIN

Endotoxin Structure

Endotoxin is a structural component of the cell envelope of gram-negative bacteria, which consists of an inner and an outer membrane. The inner (cytoplasmic) membrane is a typical biological membrane consisting of a phospholipid bilayer with embedded proteins and is similar in structure to the cytoplasmic membrane of gram-positive cells. The outer membrane comprises an inner phospholipid leaflet (layer) and an outer leaflet, of which the principal component is lipopolysaccharide (LPS), not phospholipid. Essentially, LPS is endotoxin. The structure of the gram-negative cell envelope is shown in Figure 1.

Because of differences in details of their structure, some LPSs have greatly reduced biological activity and toxicity. Consequently, it has been suggested that such LPSs are not endotoxins. Naturally occurring fragments of gram-negative bacterial outer membrane may include membrane proteins associated with the LPS. Others have suggested that the term

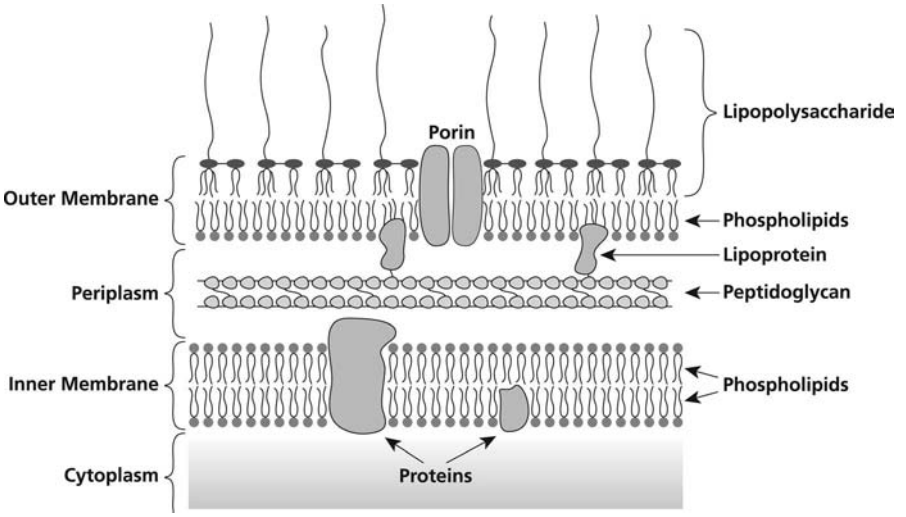

Figure 1 The cell envelope of gram-negative bacteria.

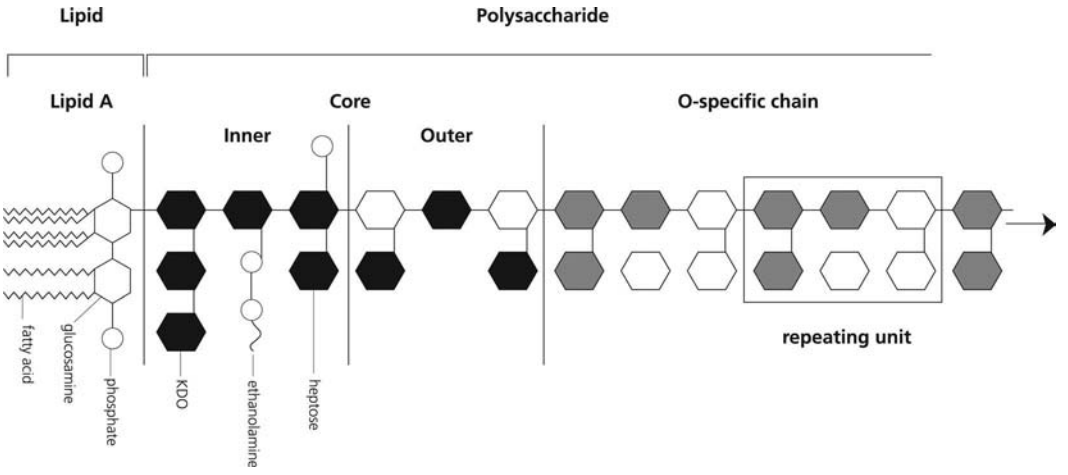

Figure 2 Schematic representation of the structure of LPS (*Salmonella typhimurium*). *Source*: From Ref. 3.

endotoxin be reserved for naturally occurring membrane fragment material and that purified material should be referred to as LPS. However, this distinction is not observed by those testing for endotoxins and the purified LPS preparations used as standards for LAL tests are generally referred to as endotoxin standards.

As the name suggests, LPS consists of a lipid and a polysaccharide moiety. More commonly, LPS is considered to consist of three regions, one lipid and two saccharide (Fig. 2). The lipid region is termed lipid A. The saccharide regions are the core and the repeating oligosaccharide. Lipid A is hydrophobic and is negatively charged. It anchors the LPS in the outer membrane. The core connects the lipid A to the repeating oligosaccharide chain, the hydrophilic, antigenic region of the structure that is presented to the environment of the bacterial cell. As a general principle, the uniformity of structure between the different groups of gram-negative bacteria decreases outward from the more conserved lipid A, to the core, to the highly variable repeating oligosaccharide.

Lipid A

Classic enterobacterial lipid A, represented by that of *E. coli*, consists of a disaccharide of glucosamine with a phosphoryl group on each of the two sugar residues. The glucosamines are substituted with fatty acid chains (generally between 10 and 28 carbons in length). The numbers of fatty acid chains are variable and may be asymmetrically or symmetrically distributed between the two glucosamine residues. The fatty acid chains are important to the toxicity of lipid A and deacylation of lipid A reduces the toxicity. Six chains in the specific asymmetric distribution found in *E. coli* gives greater activity. Similarly the presence of two phosphoryl groups is critical to LPS toxicity. Monophosphoryl lipid A (MPLA), produced by controlled acid hydrolysis of lipid A, is substantially less toxic than diphosphoryl lipid A. (Regulatory approval is being sought for MPLA as an additive, or adjuvant, to vaccines because it retains many immunostimulatory properties of LPS without the toxicity (4).)

Structure is critical to the toxicity of lipid A. The details of structure of lipid A and its toxicity vary between species of gram-negative bacteria; consequently the toxicity of lipid A varies depending on its source. Full endotoxic activity requires two phosphoryl groups and six fatty acid chains of appropriate length in the locations found in *E. coli*. Despite the importance of differences in the details of structure, lipid A is the least variable of the three regions of LPS.

Core

The polysaccharide core region of LPS is subdivided into an inner and an outer region. Working out from the lipid A, the inner core includes the linking structure common to all LPSs. The unusual sugar providing this linkage is characteristic of LPS, 3-deoxy-D-*manno*-oculosonic acid, which is commonly abbreviated to KDO (reflecting the older name of 2-keto-3-deoxyoctonic acid). In most (but not all) gram-negative bacteria KDO is linked to another unusual sugar, heptose, which is in turn linked to the outer core. A second and sometimes a third KDO may be linked to the first KDO as a side branch. The sugars of the outer core are typically common hexoses, including glucose, galactose, glucosamine, and galactosamine.

The core influences the properties of the lipid A. Lipid A separated from the core is generally less toxic than that attached to the core. Some species of bacteria contain only a core polysaccharide and no repeating oligosaccharide region. These include the highly pathogenic *Bordetella pertussis, Neisseria meningitidis*, and *N. gonorrhoeae*. Rough mutants (so called because of the rough appearance of their colonies) of other species, notably *Salmonella*, are unable to synthesize the repeating oligosaccharide (Ra mutants). Other mutants are unable to synthesize not only the oligosaccharide, but also increasing portions of the core (Rb to Re mutants). These mutants have proved to be invaluable tools in the elucidation of structure of the core and the roles of its components.

Repeating Oligosaccharide

The repeating oligosaccharide is also referred to as the O-specific chain (O for oligosaccharide) and is the principal antigenic region of LPS. The repeating oligosaccharide region is highly variable, even between strains of the same species. The various serotypes of a species are typically determined by the different antigenic properties of their repeating oligosaccharides. The region consists of repeating groups of a small number of sugars; some strains only have one sugar represented, others have up to eight. The number of repeats of the oligosaccharide may be as high as fifty. There are many different sugars that may make up the oligosaccharide and multiple ways in which they may be linked. Consequently, there are an enormous number of potential conformations and a great deal of variability is observed in nature.

The repeating oligosaccharide, together with the core region, is hydrophilic and serves as the solute carrier of LPS. It is also reported that the repeating oligosaccharide downregulates the endotoxicity of LPS (5). Free LPS is not part of a living cell membrane. It was derived from a dead cell, or released during cell growth and division, or because the LPS has been purified, as in the case of a standard endotoxin preparation. Some free, purified LPS preparations are rather insoluble and must be solubilized to exhibit full toxicity (6).

Properties of LPS

The size or molecular weight of individual subunit of a particular LPS depends on its structure. LPSs with long repeating oligosaccharide chains are significantly larger than those with shorter chains or no oligosaccharides. Having said this, the molecular weight of a typical LPS subunit is about 10,000 Da, but may be less (down to a few thousand daltons) or more, depending on the length of the repeating oligosaccharide chain. However, endotoxin rarely occurs as individual subunit. From the discussion of structure, it is clear that LPS is amphiphilic. That is, one end of the structure is hydrophobic (the lipid A) and the other (the polysaccharide) is hydrophilic. Consequently, in aqueous solution the tendency is for the lipid A to aggregate while the repeating oligosaccharide is exposed to the aqueous medium. The conformation of aggregations of LPS depends on the details of the structure of the lipid and on the chemical nature of the medium. Structures include micellar, hexagonal, lamellar bilayers and ribbons, and cubic arrangements. Native (naturally occurring) endotoxin, which is the potential contaminant of healthcare products, is not a highly purified LPS preparation. The conformation of native endotoxin, with its associated proteins and other non-LPS membrane fragments, differs again and tends to be less ordered, but it will typically be present as aggregates.

Environmental factors that influence the aggregation state of endotoxin include temperature, pH, salts (particularly divalent cations), surfactants, bile salts, and proteins. Changes in the bioactivity of endotoxin have been reported with accompanying changes in aggregation state (as measured by sedimentation coefficient) (6). The notion that aggregation of endotoxin is required for its activity, including endotoxicity, has been advanced (7,8), while others have maintained that it is the individual subunits that are the active form. Suffice it to say that neither the aggregation state of endotoxin nor its activity is fixed. Both can change with chemical conditions and the input or the removal of energy. It is as well for those performing BETs to bear this in mind. Further, different endotoxins may not behave in the same way when conditions change. Those performing endotoxin tests may encounter differences between the behavior of native endotoxin in a sample and that of the purified endotoxin (LPS) of the standard preparation. When this occurs, it will be necessary to determine test conditions under which both endotoxins behave in a similar manner.

In addition to being amphiphilic, endotoxin carries a net negative charge, at least in aqueous solutions of pH 4 or higher. The negative charge prevents the degree of aggregations that might otherwise occur as like charges repel each other, countering the tendency of the hydrophobic lipid moiety of the structure to aggregate. This explains, at least in part, the mechanism by which cations in the medium enable formations of greater aggregations. Cations neutralize the negative charges and LPS tendency to repel one another. Divalent cations can neutralize the negative charges of two adjacent subunits and form cationic bridges linking the two, further promoting aggregation.

Finally, endotoxin is very stable and is not readily destroyed. Normal sterilizing conditions are generally not sufficient to destroy endotoxin. This applies not only to dry heat and steam sterilization, but also to ethylene oxide (EtO) and γ-radiation. In the late 19th and particularly the early 20th century, after the importance of sterility was appreciated and the potential of injectable therapies was being investigated, the phenomenon of injection fever was commonly reported. The nature of the fever-causing agent (or pyrogen) was not known, but it was recognized that it withstood sterilization (9). This pyrogen was endotoxin.

Effects of LPS

The toxic effects of LPS (or endotoxin) include fever (a pyrogenic response), local Schwartzman reactivity (following subcutaneous injection of a small, relatively nontoxic dose of endotoxin, a second dose injected intravenously results in bleeding at the site of the original injection), disseminated intravascular coagulation, hypotension, tachycardia, shock, and lethal toxicity. It is the concern about these effects that has led to endotoxin limits for injectable products and medical devices that contact the blood or cerebrospinal fluid (CSF).

The effects of LPS on mammals are frequently mediated by cytokines. Upon exposure to LPS, macrophages produce a range of cytokines, notably interleukin (IL-) 1, IL-2, IL-6, and tumor necrosis factor. The cytokines in turn elicit a range of responses, the severity of which depends on the dose of LPS and the nature of the response. As seen in the Schwartzman

reaction, multiple exposures can lead to sensitization to LPS, but with appropriate doses and timing can also result in increased tolerance. In the case of the pyrogenic response, cytokines reach the thermoregulatory center in the hypothalamus region of the brain and stimulate production prostaglandin E_2 (PGE_2), which activates the hypothalamus and resets the body's thermostat resulting in elevated temperature, or fever. More recently, it has been reported that endotoxin binding with Toll-like receptor (TLR) 4 in the hypothalamus can result in PGE_2 production, eliciting a fever response without (or in parallel with) cytokines (10).

The pyrogenic response is usually the initial effect of exposure of humans to lower, but physiologically significant, doses of endotoxin. Thus, endotoxin is a pyrogen, a substance capable of inducing fever. The need for a test for pyrogens was initially met by injecting rabbits with the substance in question and monitoring the rabbit for a temperature rise, that is, for fever. The test was developed in the early 1900s by Hort and Penfold (9) and refined and publicized by Florence Seibert in the 1920s (11). It was not until 1942, spurred by the entry of the United States into World War II and anticipation of the need for intravenous solutions in the battlefield, that the Pyrogen test was included in United States Pharmacopeia XII. In the pyrogen test, rabbits are injected with the test solution and monitored for temperature rise. Until the introduction of the LAL test in the 1970s, the pyrogen test was used to safeguard injectable therapies and critical medical devices.

The pyrogen test detects pyrogens from any source, not just bacterial endotoxins. This is in contrast with the LAL test, which is (generally) specific to endotoxin. Acceptance of the LAL test in place of the pyrogen test required a high degree of confidence that endotoxin was (and is) the pyrogen of greatest concern and the most likely pyrogen to occur in injectable therapies and medical devices. This illustrates the fact that the terms pyrogen and endotoxin are not synonymous.

Endotoxin exerts a direct influence in vitro on a wide range of cells other than macrophages. The effects include mitogenicity, inhibition of mitosis, morphological changes, and cytotoxicity. It is important that tissue culture media are not contaminated with an endotoxin concentration that is significant to the cells to be grown. The critical endotoxin concentration is very variable because the susceptibility of different cell types and cell lines to endotoxin varies substantially. In vitro fertilization is a special case of cell culture in which it is important to assure the absence of significant concentrations of endotoxin. In the presence of endotoxin, the success rates of fertilization, embryo implantation, and pregnancy are significantly reduced (12,13).

Because of the wide range of effects of endotoxin, it is important that the properties of a substance, such as a protein, be determined in the absence of endotoxin. Otherwise there is the danger that properties attributed to the substance are actually those of endotoxin.

Endotoxin Heterogeneity

The heterogeneity of endotoxin is apparent from the discussion above. The properties of endotoxin that have been discussed are generalizations and differ in degree between species or strains. They can also differ between cultures of the same organism grown in different media. Also, the properties of LPS preparations can be influenced by the extraction method and the degree of purification. Users of control standard endotoxin (CSE) for LAL testing may be aware that different batches of the same endotoxin preparation can have different potencies when measured with a given lot of LAL reagent. Potencies of CSEs are discussed in more detail later in this chapter.

Differences in chain length of the repeating oligosaccharide affect the solubility of the endotoxin in water. Solubility has been shown to influence the pyrogenicity of the endotoxin (6). Also, different endotoxins vary in potency in the pyrogen test and especially in LAL test (14). The differences in pyrogenicity in humans between endotoxins from different species are well illustrated by the classic studies from Greisman and Hornick (15), data from which are presented in Table 1. Similar differences in potency between species are evident in the LAL test.

Pearson et al. (16) report that native endotoxins are less pyrogenic than purified standard endotoxins of equal LAL reactivity. These authors noted that this phenomenon serves as a safety factor for endotoxin tests conducted using the LAL reagent. Similarly, LPS extracted from *Pseudomonas aeruginosa* has been found to be half as potent as LPS from *E. coli* in a

Table 1 Human Threshold Pyrogenic Doses of Three Endotoxin Preparations

Source of endotoxin (species)	Threshold pyrogenic dose (ng/kg)
Salmonella typhosa	0.1–0.14
Escherichia coli	1.0
Pseudomonas sp.	50–70

Note: The threshold pyrogenic dose is the minimum dose of endotoxin required to elicit a fever. The smaller the dose required the greater the potency of the endotoxin preparation.

LAL test. In contrast, in a whole blood pyrogen test, *E. coli* LPS was approximately 1000 more potent than that of *P. aeruginosa*, which occurs commonly in water and is much more likely to contaminate parenteral products than *E. coli* LPS (17). Thus, it appears that LAL reagent might overreport the threat posed by some endotoxins.

Endotoxin Standards and Units

In the early days of endotoxin testing using LAL, results were initially reported in units of weight of endotoxin. However, results in units of weight are often not comparable because different endotoxins have different potencies. The activity 1 ng of one endotoxin preparation is not necessarily equivalent to that of 1 ng of a different preparation. Also, results reported in units of weight imply that the test is measuring the absolute amount (mass) of endotoxin present. This is misleading. A result reported in ng/mL means that the endotoxin present has a reactivity equivalent to the stated number of ng of the standard endotoxin preparation being used in the assay. It is not a measure of the mass of endotoxin detected.

Primary Standards

The variability of activity between different LPS preparations resulted in recognition of the need for endotoxin standards. To meet this need, the USFDA commissioned the preparation of a quantity of LPS, which was designated EC, from *E. coli* O113:H10 K negative (18). From this stock material a series of standard endotoxin preparations were produced and FDA standard EC-2 was the first to be disseminated. Importantly, the endotoxin unit was introduced with EC-2 and defined as 5 EU/ng, or 5000 EU per 1 μg vial. The endotoxin unit is a measure of activity (or potency) of endotoxin as measured in a LAL test, *not* a measure of mass. Two subsequent batches, EC-3 and EC-4, did not become established standards, but EC-5 did and was made publicly available as United States Pharmacopoeia (USP) Endotoxin reference standard (RS) lot F. The potency of EC-5 was double that of EC-2, at 10,000 EU/vial and the mass of LPS was not stated for this standard.

More recently, the USP was responsible for the production of USP Endotoxin RS lot G, which was filled by the National Institute for Biological Standards and Control in the United Kingdom. This standard was prepared from the same bulk EC preparation as the previous FDA standards and, like EC-5, contains polyethylene glycol and lactose as excipients. The USP has made some of this lot available to the FDA, who designated the standard EC-6. In addition, some of this standard was given to the World Health Organization (WHO), who established it as the second International Standard (IS) and assigned the potency in International Units (IU) of endotoxin, with 1 IU being equivalent 1 EU. Another portion of this material was given to the European Pharmacopoeia (EP) who designated it Biological Reference Preparation 3 (BRP-3), also with potency expressed in IU.

As consequence of work by a number of national and international bodies, there is now a single reference standard endotoxin (RSE) for the US, WHO, and EP. Interestingly, and sometimes confusingly, vials of this single standard bear four different labels and two different units. These are summarized in Table 2. These standards may be referred to as primary standards as they do not derive their activity from any other current standard (though USP lot G was compared with EC-5 in an unpublished collaborative study).

Table 2 The Four Presentations of the Current Primary Reference Standard Endotoxin

Organization	Designation	Units	Comments
USP	Lot G	EU	Available from the USP
US FDA	EC-6	EU	EC-6 is only available to licensed manufacturers for standardization LAL reagents.
WHO	IS-2	IU	Accepted by WHO in October 1996
EP	BRP−3	IU	Available from the EP

Given the harmonization of the US, WHO, and EP endotoxin standards, the question arises how to take advantage of this so that test results can be expressed in either EU or IU. Firstly, the USP, EP, and Japanese Pharmacopoeia (JP) endotoxins test chapters state that 1 EU = 1 IU of endotoxin. Thus results in EU and IU can be considered equivalent. Secondly, the LAL and endotoxin manufacturer's certificates of analysis commonly give the potency of the CSE in both EU/ng and IU/ng.

The primary endotoxin standard in Japan is a different preparation from that used for the standards in Table 2. The (Japanese) endotoxin unit has been set to be equivalent to 1 (US) EU. A case can be made for calling this a secondary standard as the potency was set with reference to the USP standard. However, as far as the JP is concerned, this is the primary standard. Unfortunately, as was the case with WHO first International Standard and the European BRP-2 before harmonization of these standards with the US standards, sometimes it has not been possible for users of this standard to confirm the labeled sensitivity of a given lot of gel-clot LAL reagent. The reason for this is that the assigned equivalent potency for the Japanese standard may be significantly different from the actual potency as measured with the particular lot of LAL reagent, even though the *average* potency for multiple determinations made with multiple lots of reagent indicates a one-to-one equivalency. Thus the sensitivity of a gel-clot reagent (which is labeled on the vial of reagent produced by FDA-licensed LAL manufacturers and is determined with US endotoxin reference standard) differs from the sensitivity measured with the Japanese standard.

If the labeled sensitivity of a LAL lot cannot be confirmed with the Japanese RSE (or with a CSE of known potency), the potency of the standard can be determined with reference to the USP RSE. Because the RSE is used to do this, sensitivity of the LAL reagent can be verified against the primary standard, and the procedure will indicate whether there is truly a potency discrepancy or some other problem with the test or reagents. Potency determination is discussed in detail in the section on essentials of LAL testing later in this chapter.

Secondary Standards (CSE)

The term secondary standard is used here to denote one that has been standardized with reference to a primary standard. The term control standard endotoxin is commonly used for such secondary endotoxin standards. A CSE may be defined as "an endotoxin preparation other than the RSE that has been standardized against the RSE." This definition is taken from the USP BET chapter prior to harmonization of the chapter with the JP and EP [which can be found in Appendix B to the 1987 FDA Guideline (2)]. This definition is still applicable. The most widely used CSEs are those supplied by the LAL reagent manufacturers. These are usually preparations of LPS from *E. coli*, such as *E. coli* O113:H10 or *E. coli* O55:B5, with or without additives or fillers.

To use a CSE, its potency (or activity) must be determined with reference to a primary standard. Such determinations are specific to the lot of LAL reagent used for the determination. The importance of determining a specific potency for each combination of CSE lot and LAL lot was recognized in the pre-harmonization BET, which stated "Calibration of a CSE in terms of the RSE must be with the specific lot of LAL reagent and the test procedure with which it is being used." The procedures for determining potency are discussed in detail in the section "CSE Potency Determination" later in this chapter.

LAL REAGENT: SOURCE AND DISCOVERY

LAL is an extract from the blood cells (amebocytes) of the horseshoe crab, *Limulus polyphemus*. LAL contains the proteins of the blood clotting mechanism and clots in the presence of endotoxins (and also (1→3)-β-D-glucans). Unlike the blood of mammals, which contains all of the components required for clotting, horseshoe crab blood requires the external stimulus from endotoxin (or glucans) to clot.

The proteins of the clotting mechanism are located in granules in the amebocytes and can be extracted in a lysate obtained from disrupted cells. The raw lysate obtained from the cells is then formulated into a LAL reagent for one of the endotoxin test methods. A LAL test is any method that uses LAL to detect endotoxins. The gel-clot method mimics in vitro the response of the blood of a horseshoe crab in vivo when it is exposed to endotoxin (either by injury or by gram-negative bacterial infection).

Discovery and History of LAL

The beginnings of the LAL test for bacterial endotoxins lie in the work of Frederick Bang in the 1950s with descriptions of blood clotting and a bacterial disease of the horseshoe crab and the observation that clotting was caused by gram-negative bacteria (19,20). In the 1960s Levin and Bang demonstrated that clotting is initiated by endotoxin and that the clotting components of blood are located in amebocytes (21–23). In demonstrating that the source of the clotting mechanism is the white blood cells (amebocytes), Levin and Bang described the method of producing LAL. They also described gel-clot and kinetic turbidimetric test methodologies. Their work led to interest in the potential of LAL reagent as a diagnostic tool. However, the clinical value of testing for endotoxin has not been clearly demonstrated and the LAL test is not widely accepted as a diagnostic for gram-negative sepsis (24). However, more recently, a glucan-specific LAL reagent–based test of human serum has been introduced as an aid in the diagnosis of invasive fungal infections (25).

While the clinical utility of LAL reagents was first being studied, the potential of endotoxin testing with LAL as an alternative to the pyrogen test was also being investigated. The first proposal for use of LAL test as an alternative to the pyrogen test for injectable products (radiopharmaceuticals) was published in 1970 by Cooper and coworkers (26). (One of whom was Levin, who was simultaneously investigating clinical applications of the reagent he had discovered.) Other studies followed and the range of products to which the test was applied expanded to include biologicals, parenteral solutions, and drugs (27–30).

In contrast with the mixed results obtained in clinical studies, endotoxin testing of healthcare products with LAL reagents showed considerable promise. However, if it was to replace the pyrogen test, a major obstacle had to be surmounted. By definition, the pyrogen test detects any pyrogen. In contrast, the LAL test is only sensitive to one significant pyrogen, endotoxin. This raises the obvious question, how significant are pyrogens other than endotoxin? The answer is hardly at all, which has allowed the LAL test to replace the pyrogen test in the great majority of applications. However, the question has not gone away. Pyrogen tests are still used in some cases. The EP Guidelines for using the BET (31) recommend parallel testing by the (pyrogen) test and the BET for manufacturers seeking to replace a pyrogen test with the BET for a product for which the pharmacopeial monograph specifies the pyrogen test.

Various in vitro pyrogen tests have been described in which release of cytokines by various cell types (both natural and cultured) is measured by ELISA. Five of these methods were evaluated in a study conducted under the auspices of the US National Institutes for Health (32). It was concluded that while none of the methods can be considered a complete replacement for the rabbit pyrogen test for the detection of gram-negative endotoxin, they can be considered for detection of endotoxin in human parenteral drugs on a case-by-case basis, subject to validation for each specific product to demonstrate equivalence to the pyrogen test.

Important work establishing the LAL test as a valid alternative to the pyrogen test was conducted by a group of workers from Travenol Laboratories (the forerunner of Baxter) in the late 1970s and early 1980s. Mascoli and Weary (33) described the substantial amount of comparative data collected on LAL and pyrogen tests. As early as 1977 this group conducted over 28,000 rabbit tests and more than 143,000 LAL tests. In these tests, 37 samples failed to meet the endotoxin specification and four of these samples also failed the rabbit pyrogen test.

None of the samples that passed the LAL test failed the pyrogen test, that is, no false-negative LAL test results were obtained. It is worth noting that there were no official (USP) endotoxin limits at this time and there was no standard endotoxin. The Travenol researchers had to set their own limits and identify an appropriate endotoxin standard.

In addition to reporting on the test data of parenteral products, the Travenol group examined the results of seven marginally failing pyrogen tests (cumulative temperature rises ranged from 3.7 to 4.55°C). In a positive eight-rabbit pyrogen test not all of the rabbits necessarily show a fever. The authors considered all of the possible combinations of the 56 rabbits from the seven tests. They showed that, given the worst case grouping of rabbits, approximately 30% of the tests could have passed the pyrogen test. This served to illustrate the variability of response of the test animal, one of the disadvantages of the pyrogen test.

The Travenol authors also described an incident of human serum albumin (HSA) contamination. Batches of HSA, which had been tested and released by the pyrogen test, were found to be pyrogenic in humans. This led to a program to develop a LAL test for HSA. To do this, it was necessary to resolve an interference problem. (A LAL test was also desirable because HSA is antigenic and rabbits must be destroyed after a short test life.) Once a test had been developed, Travenol screened HSA lot retentions and two more potentially pyrogenic lots were identified and withdrawn from the market. As a result of this experience in which the pyrogen test had failed to fully protect public health, FDA required LAL tests on future lots of HSA and prohibited release of batches that tested positive in the LAL test, even if negative in the official (i.e., pharmacopeial) rabbit pyrogen test.

Mascoli and Weary concluded the following: endotoxins are the primary pyrogen of concern in parenteral products; a negative LAL test had never been observed when the pyrogen test was positive; the LAL test is more sensitive than the pyrogen test (the USP pyrogen test had allowed the release of some materials proved to be pyrogenic in humans and were subsequently shown to contain endotoxin in LAL tests). The considerable variability of the pyrogen test was noted in this work and in other publications from the Travenol group (34,35).

In 1988 the USP held an open conference on alternatives to in vivo tests. At this time many manufacturers of parenteral products and medical devices were routinely using LAL tests to either supplement pyrogen tests (primarily by in-process testing) or replace the pyrogen test as a release test. This was despite the fact that the great majority of monographs in the USP specified the pyrogen test for most injectable products. The FDA Guideline on the LAL test (2) had been published in 1987, and by the time of the 1988 conference the LAL test had proven itself be superior to the pyrogen test in most applications. The LAL test for endotoxins was a prime example of an in vitro replacement for an in vivo test. In the succeeding few years after the conference requirements for the pyrogen test in USP monographs for injectable products were changed to a LAL test as described in the USP BET chapter (36). The great majority of monographs for injectable products now specify the BET, not the pyrogen test.

The LAL Clotting Reaction

Levin and Bang first proposed a mechanism for the LAL test (21) and postulated that endotoxin activated an extract from the blood of *Limulus* (which they termed "pre-gel") and formed a gel-clot. A simple model for the clotting reaction was subsequently proposed (Fig. 3).

This model proved to be essentially correct but the clotting mechanism is actually more complex in two regards. First, it has been clearly demonstrated that the activation of clotting enzyme by endotoxin is not direct. Endotoxin activates factor C, the first in a series of serine protease zymogens, which in turn activates factor B. Active factor B then acts on the proclotting enzyme, which cleaves the substrate in a classic cascade type of reaction. The intermediate enzymes are important as they amplify the initial signal (the recognition of

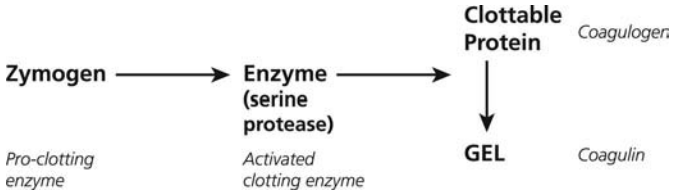

Figure 3 Early model for activation of the LAL clotting mechanism by endotoxin. *Source*: From Ref. 37. (Current names of components added in italics.)

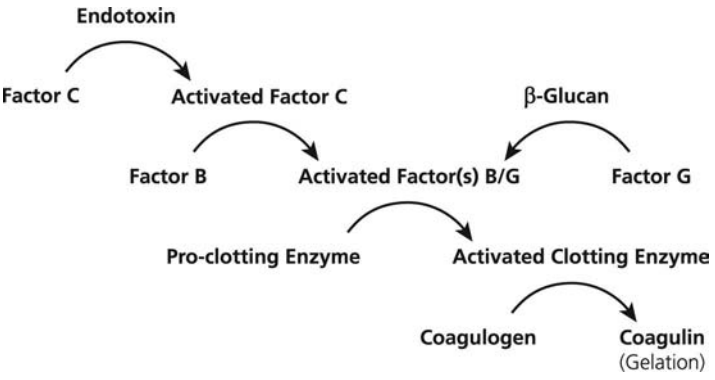

Figure 4 Clotting mechanism of *Limulus* amebocyte lysate. *Source*: From Ref. 38.

endotoxin by factor C) in a process analogous to a chain letter (if unbroken). The cascade reaction is the key to the extraordinary sensitivity of the LAL test. The activated clotting enzyme cleaves a peptide (peptide C) from the middle of the substrate, coagulogen (the accepted name for Levin and Bang's pre-gel). The two remaining peptides (A and B) remain linked by two sulfide bridges and reconfigure as the clotting protein, coagulin. Particles of coagulin coalesce and, when a sufficient concentration is reached, they coagulate to form a gel.

Second, the endotoxin initiated pathway is not the only route by which the clotting enzyme is activated. In a second pathway, (1→3)-β-D-glucan activates factor G, which acts directly on the proclotting enzyme (38). Activated clotting enzyme then cleaves coagulogen as described in the preceding text for the endotoxin pathway. LAL reagent is approximately 10-fold less sensitive to glucan than to endotoxin on a mass basis. This is attributable, at least in part, to the additional step in the endotoxin pathway that amplifies that reaction. The complete pathway is shown in Figure 4.

LAL Test Methodologies

Gel-Clot Method

The gel-clot LAL test essentially mimics the clotting of *Limulus* blood in vivo. The end point of the test is a gel-clot, formed in a small reaction tube as opposed to a wound site on a horseshoe crab. The test is typically performed by mixing equal volumes of sample and LAL reagent and incubating the mixture under standard conditions of time and temperature ($37 \pm 1^{\circ}C$ for 60 minutes $\pm$ 2 minutes). The sensitivity of gel-clot reagent is determined by the manufacturer using RSE and is labeled on the packaging. It is the minimum endotoxin concentration required to cause clotting. The term λ (lambda) is used to generically denote test sensitivity. Sensitivity is verified using dilutions of standard endotoxin, or at least by running a single positive control at a concentration that should always give a positive test result. Negative controls are also included with each test.

A gel-clot test is scored as positive if a clot forms, which withstands a smooth 180° inversion of the reaction tube. Otherwise it is negative, even if there is evidence of gel formation in the tube. A negative result means that the sample contains no detectable endotoxin, which is reported as a concentration of less than the labeled sensitivity of the reagent. A positive result means that the sample (or sample dilution) tested contains an endotoxin concentration of equal to or greater than the sensitivity of the reagent. The concentration of endotoxin in a sample can be quantified in a gel-clot assay by testing a series of twofold dilutions of sample. The dilutions are prepared in water that is free of detectable endotoxin. The greatest dilution at which a clot is formed is termed the *end point*. The endotoxin concentration in the sample is calculated by multiplying the dilution factor of the end point by the labeled sensitivity of the LAL reagent.

By convention, results of gel-clot assays are reported as single values, not ranges of $\geq X$ EU/mL but $< \frac{1}{2}X$ EU/mL. This is because of the error of the test, which includes the fact that the labeled sensitivity of the LAL reagent itself is determined by testing a series of twofold dilutions. No attempt is made to achieve resolution between twofold dilutions; such attempts

at greater precision are meaningless. It is generally accepted that the error of the test plus or minus a factor of two. Consequently, a result of 0.5 EU/mL should not be regarded as significantly different from either 1 EU/mL or 0.25 EU/mL. Acceptance of this variability is incorporated in the pharmacopeial endotoxins test chapters. When verifying the performance of a gel-clot reagent, the labeled sensitivity must be verified within a factor of two using standard endotoxin. Similarly, for positive and positive product controls (PPCs) a concentration of double the label claim sensitivity is used because it is recognized that the clotting may not occur at the sensitivity labeled on the reagent (though it may also clot at half the concentration of that labeled as the sensitivity). However, the reagent should always clot at an endotoxin concentration of double the labeled sensitivity. If it does not, the test is invalid and the reason should be determined.

Finally, in the pharmacopeial endotoxins test chapters the gel-clot method is the referee test, or the default compendial test, in the event of a dispute. In this context a "dispute" is typically an action initiated by a regulatory authority. Thus, resolution of a dispute is based on results from the gel-clot method unless another method is specified in the monograph for the product in question. Generally product monographs do not specify a test method (or "technique," to use the terminology of the pharmacopeia).

Photometric Methods

The harmonized USP, EP, and JP endotoxin test chapters address the turbidimetric and chromogenic LAL test methods under the common heading of Photometric Methods. This is a logical move because the fundamental principles of these methods are the same. There is no need to address them separately, as they are in the FDA Guideline. However, in the present chapter the methods are described separately because of their different chemistries. They are addressed together as photometric methods in the discussion of regulatory requirements.

In the LAL reaction, as the concentration of insoluble coagulin (the clotting protein) particles increases the reaction mixture becomes turbid. Reduction of light transmission due to the turbidity can be detected at a wide range of wavelengths, but is greater at shorter wavelengths. Because the initial steps of the cascade do not result in the production of insoluble coalescent protein, there is a time lag before turbidity start to increase. Levin and Bang (23) described how the rate of increase in turbidity [as measured by optical density (OD)] increased with endotoxin concentration. This phenomenon is the basis of the turbidimetric LAL test methods. Because turbidity development begins well before a solid gel is formed, this is a much more sensitive test than the gel-clot method.

The kinetic method is by far the most widely used turbidimetric technique. In it, typically the time taken to reach a specified level or threshold of turbidity is determined. This time is commonly called the "onset time." The higher the endotoxin concentration is in the sample, the faster the reaction and the shorter the onset time. There is an inverse log/log relationship between endotoxin concentration and onset time. Consequently, the sign of the slope of a plot of the log onset time against the log endotoxin concentration is negative (and so is the sign of correlation coefficient). Alternatively, the rate of the increase in turbidity may be determined, either as the V_{max} (the maximum rate of increase of OD calculated as a moving "boxcar" average over a relatively short time throughout the test) or V_{mean} (the mean rate of increase of OD over the test period). The slopes of standard lines for both V_{max} and V_{mean} are positive because these two parameters increase in value as the endotoxin concentration increases.

To perform a kinetic turbidimetric test, it is necessary to record the turbidity of the reaction mixture of each sample at regular intervals (no longer than about 1 minute) throughout the test while maintaining the reaction mixture at a stable temperature, typically 37°C. Prior to the availability of incubating, computerized instrumentation, this was a laborious procedure and not practicable for a routine assay, though Levin and Bang (23) did so. Since the 1980s and the advent of incubating tube and plate readers, kinetic test methods have become a practical methodology. A major advantage of the method is the wide range of endotoxin concentration that can be detected.

An alternative approach is to incubate the sample plus LAL reagent for an appropriate period and then to read the OD once at that end point. In the end point method, there is a linear relationship between endotoxin concentration and OD. There are three disadvantages of

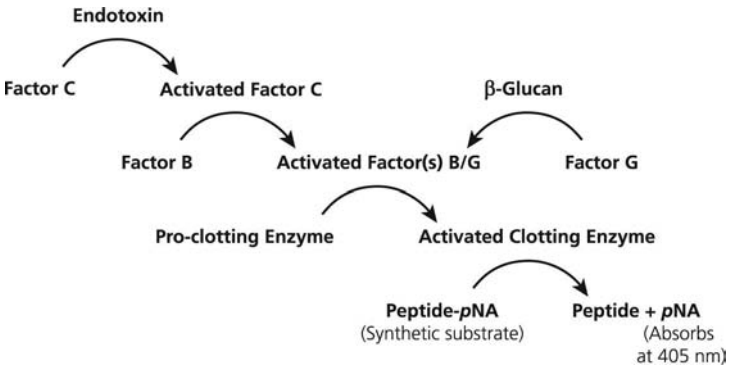

Figure 5 The chromogenic LAL reaction. *Source*: Modified from Ref. 39.

this method. First, for a given incubation time only a relatively narrow range of endotoxin concentrations can be detected; this range is generally about a factor of 10, commonly referred to as 1 log (base 10 assumed), such as 0.1 to 1 EU/mL. Higher endotoxin concentrations cannot be discriminated from one another because the reaction is largely complete and they are all approaching the same saturation point (i.e., the substrate is limiting). For lower endotoxin concentrations, the turbidity of the sample has not yet begun to increase significantly. Detection of higher or lower endotoxin concentrations requires shorter or longer incubations, respectively. Second, the development of turbidity cannot be stopped, so there is only one chance to read turbidity at the end point. The only advantage of this method is that an incubating reader is not required. The incubation can be performed using a heat block prior to reading the OD. However, this points to the third disadvantage, the operator must be available to read the test at the conclusion of the incubation (unless an incubating reader can be set to take the reading).

Kinetic turbidimetric assays are performed in either microtiter plates or in reaction tubes in a tube reader. A sensitivity of 0.001 EU/mL can be achieved in a tube reader, as opposed to 0.005 EU/mL in a microtiter plate reader. The greater sensitivity is due to the longer path length in a tube. Assuming a typical standard endotoxin potency of 10 EU/ng, 0.001 EU/mL is equivalent to 0.0001 ng/mL, or 0.1 pg/mL. This is 0.1 ppb, a high sensitivity for any assay.

In contrast with the turbidimetric method, which utilizes the endogenous chemistry of native LAL, in the chromogenic methods a synthetic substrate is added to the LAL reagent, either in the formulation of the reagent or by the user as the test is performed. The substrate is colorless and consists of a peptide to which a terminal chromogen, *para*-nitroanilide (*p*NA) is attached. The amino acid sequence of the peptide is recognized and cleaved by the clotting enzyme to release the *p*NA chromophore (now *para*-nitroaniline), producing a yellow-colored solution that, unlike the intact chromogen, absorbs light at a wavelength of 405 nm (Fig. 5).

In the chromogenic LAL test, the absorbance may be monitored in a kinetic mode and the onset time determined, just as it is in the kinetic turbidimetric method. Alternatively, an end point test may be performed. Unlike the turbidimetric reaction that cannot be conveniently stopped, the chromogenic reaction can be stopped by the addition of acetic acid and the absorbance of the chromogen measured. However, the end point method still suffers from two drawbacks: the limited detection range and the requirement of an operator to stop and read the test. Chromogenic assays are frequently performed in microtiter plates and achieve a sensitivity of 0.005 EU/mL, the same as turbidimetric assays in plates.

REGULATION OF ENDOTOXIN TESTING

The regulatory requirements for endotoxin testing are relatively straightforward, at least for those concerned with compliance in the United States, Europe, Japan, and countries that adhere to the regulations in these countries. The principal reference documents are the endotoxins test chapters in the USP, the EP, and the JP. Second to these documents is the US FDA Guideline on the LAL test, published in 1987. (This document includes an Appendix B that is the BET in USP XXI, which was the current USP at the time the Guideline was issued.) In 1991 FDA issued a follow-up guidance document that addressed kinetic turbidimetric testing of drugs and biologics. This document is incorporated in the version of the 1987 Guideline document available on the FDA website. In addition, in the EP under the section on General Texts, there is a chapter Guidelines (number 5.1.10) (31). The Guideline(s) and Guidance are

what the titles imply, guides, and they do not have the force of law. The pharmacopeial chapters do have the force of law, at least as far as release testing of products with monographs that specify an endotoxins test. Finally, there is a standard published jointly by the American National Standards Institute (ANSI) and the Association for the Advancement of Medical Instrumentation (AAMI) (40) titled "Bacterial Endotoxins—Test Methodologies, Routine Monitoring and Alternatives to Batch Testing." This standard contains general information on endotoxin testing but is primarily aimed at testing of medical devices.

There are three core elements of regulatory compliance that are addressed in the pharmacopeial endotoxins test chapter and the FDA guidance documents. These elements are:

1. Verification of reagent performance/technician certification/laboratory qualification. Prior to testing product it must be demonstrated that the test is working and that it can be consistently performed by appropriately trained, certified technicians using endotoxin standards diluted in water.
2. Inhibition/enhancement Testing—validation. Before testing a product to determine whether it can be released for use, it must be demonstrated that the test method will detect endotoxin in the product and, very importantly, that the test sample does not interfere with the ability to detect endotoxin.
3. Routine testing. Testing is conducted according to the method validated in step 2 and includes controls to once again assure that the test sample does not interfere with the ability to detect endotoxin. To meet the requirements of the test, the sample must contain less than the endotoxin limit.

These core elements should be borne in mind during the following review of the principal regulatory documents.

Pharmacopeial Endotoxins Test Chapters

As a result of a harmonization effort led by the JP, the USP and the EP published major revisions to the endotoxins test chapters in 2000. The JP revision was published in 2001. The EP and USP chapters became official on January 1, 2001, and the JP chapter became official on April 1 of the same year. (The English language version of the JP chapter became available in 2002 and is the only pharmacopeia available freely on the Internet (41).) The USP first published proposed changes to the BET in 2007. The final changes were published in USP 33 with an official (effective) date of October 1, 2010. Similar changes to the EP chapter were also published in 2007 and became effective on January 1, 2010 (42). The revisions did not alter the fundamental principles of the chapters.

The harmonized bacterial endotoxins chapters in the three pharmacopeia are very similar. There are some wording differences and some concepts are addressed in one document but not others. The introduction to the USP BET notes that portions of the chapter have been harmonized with the EP and/or JP and nonharmonized portions are marked. A summary of the content of the harmonized chapters follows.

***Important**: this summary is presented as a guide to the pharmacopeial chapters and as a basis for further discussion. It is not intended to be, and it should not be used as, an alternative to the pharmacopeial chapters. The chapter is always subject to revision and a summary will always omit some points and nuances that are present in the original document. The current version of the chapters should always be consulted when making compliance decisions.*

The chapter states that LAL is obtained from *Limulus* or *Tachypleus* and "manufactured in accordance with the regulations of the competent authority." The BET chapter cautions that "LAL reacts with some β-glucans in addition to endotoxins. Some preparations that are treated will not react with β-glucans and must be used for samples that contain glucans."

Three techniques (the gel-clot, turbidimetric technique, and chromogenic) are described in the initial, general section, not two (gel-clot and photometric). Testing may be conducted using any one of these techniques unless otherwise specified in the monograph. As noted in the discussion of the gel-clot method, in case of a dispute, the final decision is based on the gel-clot techniques unless otherwise specified in the monograph.

In the gel-clot methods, samples are tested in parallel with dilutions of an RSE. Endotoxin concentrations are determined using a sensitivity assigned to the reagent by the manufacturer, which is verified using the RSE. For the photometric methods, samples are tested and the endotoxin concentration is determined using a standard line constructed using a series of endotoxin RSE concentrations. The reagents do not have an assigned sensitivity. The chapter states the equivalence of the endotoxin unit and the international unit of endotoxin.

Regarding Apparatus and Glassware, the chapter states that glassware and materials should be depyrogenated and that commonly used minimum time and temperature settings are 30 minutes at 250°C for dry heat. Another footnote in the BET addresses validation and references USP chapter <1211> (43). An important caution states that plastics should be tested for contamination and interference.

The BET specifies use of USP Endotoxin reference standard (RS). (Since it was harmonized with the EP and JP chapters, the BET no longer mentions CSE. This is discussed further later in this chapter.) For details of reconstitution, use and storage of USP Endotoxin Reference Standard (Endotoxin RS) the user is referred to the "package leaflet" (i.e. instructions). Dilutions of RSE are prepared with Water for BET and should be used as soon as possible to avoid loss of activity by adsorption.

Under the heading "Preparatory Testing," two requirements are stated that must be met before official test can be performed. These are the first two essentials of LAL testing stated above, the first of which is the confirmation of LAL reagent sensitivity. It should be noted that this actually only applies to the gel-clot method as photometric reagents do not have assigned sensitivities. However, later in the chapter the requirements for photometric reagents are given. Secondly, the test performance must be validated in the presence of the sample under test by performing the test for interfering factors to demonstrate that the sample does not interfere with the detection of endotoxin.

Preparation of sample solutions is discussed. Drugs are dissolved or diluted while medical devices are extracted, both in Water for BET. The chapter states that other solutions may be used, but gives no further guidance as to what type of solutions these might be or what type of validation may be required for their use. The pH of the reaction mixture (sample + LAL) must be in the range specified by the LAL manufacturer, which the chapter notes is usually 6.0 to 8.0. This is an important point. If out of range, pH can be adjusted with acid, base, or suitable buffer as recommended by the lysate manufacturer. Buffers should be validated as free of detectable endotoxin and interfering factors.

An important parameter discussed is the maximum valid dilution (MVD). The MVD is the greatest dilution of the product at which the endotoxin limit can be detected. If a test is conducted at a dilution greater than the MVD, it cannot be stated that the sample passes the test; it clearly fails if any endotoxin is detected. The equation for calculation of the MVD is given:

$$\text{MVD} = \frac{\text{Endotoxin limit} \times \text{conc. of sample}}{\lambda}$$

where λ is the "sensitivity of the test".

The sensitivity of gel-clot reagents is labeled on the packaging. For photometric methods the sensitivity is the lowest concentration on the standard curve and is therefore flexible, within limits, and determined by the user when the range of the standard curve is selected. MVDs are discussed more fully in the section of this chapter on testing drug products.

The method by which endotoxin limits are established for drug products is described in the BET, and an equation is given for the calculation of the endotoxin limit for an individual drug product. Details regarding endotoxin limits are given in a footnote in the USP BET. The equation is discussed in detail in the section on testing drugs and biologics. Endotoxin limits for medical devices are not addressed in the pharmacopeial endotoxins test chapters, but are given in USP chapter <161>, "Transfusion and Infusion Assemblies and Similar Medical Devices" (44).

Requirements for the Gel-Clot Technique

The section on the Gel-Clot Technique begins by stating that the endotoxin concentration required to cause clotting of the reagent under standard conditions is the labeled sensitivity of the LAL reagent, which is determined by the manufacturer. In the preparatory testing section

specific to gel-clot techniques, the first requirement is confirmation of labeled sensitivity (λ). This is performed using at least one vial of reagent for each new LAL lot or when test conditions change. Four standard endotoxin concentrations are tested in quadruplicate with negative controls. The endotoxin concentrations are 2λ, λ, $1/2\lambda$, and $1/4\lambda$. The test is performed by mixing equal volumes of standard endotoxin and LAL reagent (such as 0.10 mL aliquots). The reaction mixture is incubated according to the LAL reagent manufacturer's directions (usually at $37 \pm 1°C$ for 60 ± 2 min). Reading of the test is described, notably the requirement to smoothly inverting each test tube through 180° to determine whether a firm gel-clot has formed that withstands inversion, which defines a positive test. The lowest standard concentration ($¼\lambda$) must test negative for the test to be valid. The end point is the last positive test in a series of decreasing concentrations of endotoxin standard (or sample). The geometric mean of the four end points must confirm labeled sensitivity, expressed in EU/mL, within a factor of two.

The second requirement for preparatory testing is the test for interfering factors. This requires confirmation of label claim in quadruplicate using endotoxin RS added to test sample at a dilution less than the MVD that contains no detectable endotoxin. In parallel with this, label claim is verified by testing in at least duplicate a series of standard endotoxin concentrations diluted in water. Though not stated explicitly in the chapter, the purpose of this is to demonstrate that the ability of the test to detect endotoxin in product (or a specified dilution thereof) is not significantly different from that in water. The pharmacopeial endotoxins test chapters use a table to explain test setup. If interference cannot be overcome at a product dilution less than the MVD, the MVD may be used to allow further dilution to overcome the interference.

In order for the test to be valid, the sample dilution to which no endotoxin has been added and the negative controls must test negative. The geometric means of the standard endotoxin concentrations in both water and in the product must confirm the labeled sensitivity of the LAL reagent.

The BET allows for interference to be overcome by treatment such as filtration, neutralization, dialysis, or heating. Such treatments must be validated by adding endotoxin to the sample, performing the treatment and then demonstrating that endotoxin can be recovered and is not removed by the treatment.

After the preparatory testing section, the pharmacopeial chapters describe the procedure for a gel-clot limits test, which is a positive/negative test at a particular endotoxin concentration. A positive limits test indicates that the sample being tested contains an endotoxin concentration of at least the sensitivity of the test. It gives no information about what that concentration might be. (Endotoxin concentrations are determined by performing an assay, which is described in the next section of the BET.) It is implicit in the wording of limits test that, when performed at the MVD, it is a pass/fail test at the endotoxin limit.

The procedure for the limits test is described in a table and may be summarized as follows. The specimen is tested at the dilution (not greater than the MVD) used in the test for interfering factors (and as treated in that test if applicable). The following controls are required: negative controls, positive controls consisting of an endotoxin concentration of 2λ made up in Water for BET and positive PPCs, which consist of product at the test concentration containing a standard endotoxin concentration of 2λ. All conditions (sample and controls) are tested in duplicate. A full series of standard endotoxin concentrations is not required.

In order for the test to be valid, both replicates of the negative controls must test negative (not clot) and both replicates of 2λ positive control and of the 2λ PPC must clot. To meet the requirements of the test, the test must be valid and the two replicates of the sample (or sample dilution) must test negative. The BET states that the test should be repeated if one replicate of the sample tests positive and the other negative. The sample does not meet the requirements of the test if any of the replicates test positive in the retest. If a positive result is obtained for the product at a dilution less than the MVD, the test may be performed at a dilution not to exceed the MVD.

The quantitative gel-clot test is used to assay the endotoxin concentration in a sample. Like the limits test, the test setup is described in a table. A series of sample dilutions of sample "not to exceed the MVD" are tested. A PPC, the same as described for the limits test, is included for the first dilution tested only. The BET requires inclusion of a full series of standard endotoxin concentrations and a negative control. Like the limits test, the assay is conducted in duplicate.

For a test to be valid, the negative controls must be negative, the end point of the series of endotoxin standards must confirm labeled sensitivity within a factor of two and the PPC must test positive. To calculate the endotoxin concentration for each replicate series of dilutions of the sample, the end point dilution factor is multiplied by the lysate sensitivity. The geometric mean of the endpoint endotoxin concentrations for the two replicate series is then calculated. The sample meets the requirements if the geometric mean endotoxin concentration of the sample is less than the limit in the monograph. If none of the dilutions of the sample test positive, the result is given as less than the concentration that would have been reported if the end point had been at the first (lowest or least) dilution of product.

There is a difference between the wording of BET and the wording for endotoxin limits in the great majority of product monographs in the USP. Most monographs state that the substance shall contain *not more than* a particular endotoxin limit. The BET states that the article (i.e., the product sample) meets the requirements of the test if it contains *less than* the endotoxin limit. This is consistent with the requirement for the limits test in which the sample must test negative at the MVD. The more conservative (stringent) specification (which is that in the endotoxins test chapter) should be adopted.

Requirements for the Photometric Quantitative Techniques

Photometric test methods are outlined in the introduction to the "Photometric Quantitative Techniques" section of the pharmacopeial chapters; both end point and kinetic approaches to the turbidimetric and chromogenic assays are addressed. Tests are carried out at the incubation temperature recommended by the LAL manufacturer, usually $37 \pm 1^{\circ}C$.

Preparatory testing for the photometric techniques serves essentially the same purposes described for the gel-clot method: first, to verify that the test is performing properly and, second, to demonstrate that sample material being tested does not interfere with the detection of endotoxin. Unlike the gel-clot method, there is no labeled sensitivity to confirm for the first of these two requirements. The test is performed for each lot of LAL reagent and if there is any change in conditions that are likely to influence the test result.

To demonstrate that the test is performing properly, standard curve criteria are verified by testing at least three standard endotoxin concentrations in at least triplicate according to the recommendations of the LAL manufacturer. If the range of endotoxin concentrations exceeds two logs (i.e., a factor of 100–base 10 is assumed), additional standards are required to bracket each log increase in range. A standard line is constructed and the absolute value of the correlation coefficient (lrl) must be at least 0.980. The absolute value of the correlation coefficient is specified because of the negative slope and r value of the standard lines for most kinetic assays. It is notable that, unlike the gel-clot method, there is no check against manufacturer's criteria or other external standard.

The interfering factors test for the photometric techniques is described in another table. A series of at least three standard endotoxin concentrations are tested to construct the standard curve as was described in the verification standard curve above. The same considerations regarding the range of the curve applies, but a minimum of two replicates are required, not three. The lowest of the standard endotoxin concentrations in the series is designated λ. This is the lowest concentration that can be quantified and is therefore the sensitivity of the test; it is thus analogous to λ in the gel-clot method. Negative controls are also required. Samples are tested at a dilution not to exceed the MVD, "unspiked" and "spiked" with added endotoxin to give a PPC with a concentration at or near the middle concentration of the standard curve. (The terms "unspiked" and "spiked" are not used in the pharmacopeial endotoxins test chapters, but are commonly used in LAL testing.) Like the standard series, the negative control, sample, and PPC are all tested in at least duplicate.

Two requirements for test validity are described. First, the absolute value of the correlation coefficient of the standard curve generated must be greater than or equal to 0.980. Second, the endotoxin concentration of the negative control must not exceed the limit described in the instructions for the lysate reagent (or it is less than the endotoxin detection limit of the lysate reagent employed). The mean measured concentration of endotoxin in the PPC must be quantified within 50% to 200% of the known concentration after subtraction of any endotoxin in the unspiked sample. When the endotoxin recovery is out of the specified

range, the interfering factors must be removed as described for the gel-clot method. The treatment must be validated and shown to eliminate interference without loss of endotoxins. This is achieved by performing the assay described above (the test for interfering factors) on sample to which a known concentration of standard endotoxin has been added and which has then been submitted to the treatment.

One of the pleasures of the pharmacopeial endotoxins test chapters is the section on the procedure for testing by the photometric techniques, which consists of a single sentence that refers to the section on the interfering factors test. There is elegant simplicity to the procedure for the test being the same as that used for its validation.

The endotoxin concentrations are determined for each replicate of unspiked sample (i.e., with no added endotoxin, which is solution A in the table presented in the chapter) using the standard curve. In order for the test to be valid,

- The standard series must meet the requirements described under Verification Criteria for the Standard Curve, that is, the correlation coefficient, lrl, must be at least ≥ 0.980.
- The added endotoxin spike must be quantified within 50% to 200%
- Negative controls should not exceed the limit in the description of the LAL reagent used (i.e., as described in the instructions for use in the product insert).

The final section of the chapters, "Interpretation" states that to meet the requirements of the test the mean of replicates of the sample must contain less than the endotoxin limit when corrected for dilution and concentration. However, this statement should be interpreted in the context of current good manufacturing practice, which is discussed in the section on retesting later in this chapter.

It should be noted that if a standard operating procedure states that testing is performed according to the pharmacopeial method, close attention should be paid to the details of the procedure. For example, use of RSE (not CSE) is specified in the USP. Also, a full series of standards is required for every quantitative test, but not for the gel-clot limits test. Any deviations from the specifics of the BET should be stated.

The FDA Guidance Documents

While the harmonized endotoxins test chapters serve as the primary reference for LAL testing, the 1987 FDA "Guideline on Validation of the *Limulus* Amebocyte Lysate Test as an End-Product Endotoxin Test for Human and Animal Parenteral Drugs, Biological Products and Medical Devices" (2) ("the Guideline") is a valuable reference, despite its age. The Guideline contains additional information on points not addressed in the pharmacopeial chapters. The whole document is not summarized here, but key points are noted.

The Guideline states that a CBER (the FDA Center for Biologics Evaluation and Research) licensed reagent shall be used for validation, release, and in-process testing (the latter despite the title of the Guideline, which refers to end-product testing). Variability of the test laboratory should be assessed by having each analyst qualify the reagent and Appendix A is referenced for details. However, Appendix A is missing from the version of Guideline available on the FDA website. The USP BET preparatory testing sections on confirmation of sensitivity or Verification Criteria for the Standard Curve can be used for the gel-clot and photometric methods, respectively.

The prescribed limit for positive product control (PPC or spike) recovery for photometric methods is $\pm 25\%$. This is now superseded by harmonized pharmacopeial endotoxins test chapters, which give a range of 50% to 200%. Similarly the endotoxin "spike" concentration in PPCs of 4λ is also superseded by endotoxins test chapters, which specify that the PPC contain a concentration equal to or near the middle of the standard endotoxin series.

The Guideline refers to the BET test for interfering factors as "inhibition and enhancement testing" and "validation." It states that at least three batches of each finished product should be tested for inhibition and enhancement and it gives guidance on when to repeat validation. When the LAL reagent manufacturer is changed, but not the test method, the inhibition and enhancement test should be repeated for one lot of product. What is not stated, but should be assumed, is that this recommendation is based on the assumption that the

degree of interference is likely to be similar with the two manufacturers reagents and that the test on a single lot will serve to verify this. However, it may not be true that interference is similar as reagents from different manufactures may have quite different formulations and are unlikely to have the same interference characteristics (45). Consequently, if the reagent manufacturer is changed and the requirements for the test for inhibition and enhancement are not met, appropriate steps should be undertaken to overcome the interference. As a new test method has been developed, it would be prudent to conduct inhibition and enhancement testing on three lots of product. The Guideline states that when the test method is changed, regardless of whether the manufacturer remains the same, validation should be performed on at least three lots.

Interestingly, the Guideline does not recommend revalidation for changes to the manufacturing process, formulation, source of an ingredient, or LAL reagent lot change. It is stated that the PPCs can be used to reverify validity. This is reasonable for changes in lot of LAL reagent (from a single manufacturer) and perhaps for changes in source of well-characterized chemical components. However, changes in formulation and manufacturing process have the potential for changing the chemistry of the sample and consequently its interference characteristics. The pharmacopeial endotoxins test chapters require validation for such changes, so they should be evaluated with the need for revalidation in mind, at least on a single lot of product. For the photometric methods, since the procedure for the test is the same as that for the test for interfering factors, this is quite straightforward and revalidation is a matter of appropriately documenting the procedure. The actual test is unchanged. For the gel-clot method, consideration should be given to performing the test for interfering factors since the limits test and the assay do not indicate enhancement.

For photometric methods, the Guideline includes provision for use of product standard curves that are addressed in the section "Test Method Development" later in this chapter.

For routine testing, the Guideline states that samples, standards, PPCs, and negative controls should be tested in at least duplicate, as do the pharmacopeial endotoxins test chapters. Sampling should be based on the manufacturing procedures and the batch size. A minimum of three units, taken from at least the beginning, middle, and end of the manufacturing run is recommended. Samples may be pooled for testing. (The same can be applied to samples for validation.)

For the gel-clot method, the Guideline allows that, once consistency has been demonstrated in the test laboratory, it is not necessary to run a standard series with every test. A full series should be included with the first test of the day and repeated if LAL reagent or endotoxin lot, or other test conditions change. This is different from the pharmacopeial chapters, which specify a positive control only for limits tests and a full standard series with assays. Negative controls are required for all tests.

The Guideline provides for the use of archived (or stored) standard curves for the kinetic turbidimetric method. An archived standard curve is a set of previously determined parameters (usually the slope and y intercept) that define the standard line used to calculate the endotoxin concentrations of unknowns. The curve is valid over the range of standard endotoxin concentrations used in its construction. Prior to using archived curves, the Guideline states that consistency of standard curves should first be demonstrated. For each test in which an archived curve is used, the Guideline specifies inclusion of a standard control and that the (presumably mean) measured concentration of the control should be within $\pm 25\%$ of the nominal concentration. This serves to confirm the validity of the archived standard curve. This same control should be run for cartridge test methods that rely on an archived standard curve.

Like the pharmacopeial endotoxins test chapters, the Guideline includes some provisions for retests and these are discussed below in the context of the FDA Guidance on Out-of-Specification (OOS) results of 2006 (46).

The FDA Interim Guidance of 1991 is specific to Human and Veterinary Drug Products and Biologicals (but apparently not to medical devices) and addresses Kinetic LAL techniques. This document is inserted in the middle of the online version of the 1987 Guideline. In the Guidance the requirement for spike recovery for PPCs is increased to $\pm 50\%$, but, as stated for the Guideline, this is now superseded by the pharmacopeial endotoxins test chapters. In addition, a PPC concentration of 0.5 or 5 EU/mL is specified, depending on the endotoxin limit for in the dilution being tested. Again this is superseded by the pharmacopeial chapters.

The document also includes additional guidance on archived standard curves. It specifies that an archived curve should be constructed from the data points of a minimum of three standard series tested over three consecutive days.

Control Standard Endotoxins

Finally, before leaving this review of regulatory documents, it is notable that there is no longer any mention of CSE in the BET. CSEs are addressed in Appendix C to the FDA Guideline (as well as in Appendix B, which is the pre-harmonization BET from USP XXI). The removal of mention of CSE from the USP caused some concern among LAL users. However, the issue of CSEs was addressed in Pharmacopeial Forum 26(1), Jan–Feb, 2000, when the proposed text of the harmonized chapter was published. The preamble to the proposed new BET chapter stated:

"The use of in-house standards shown to be equivalent to USP Reference Standards is permitted under the requirements for alternate methods in the General Notices. The CSE has thus been deleted because in-house standards have to be shown to be equivalent to the USP Endotoxin RS."

Thus, it is clear that the USP did not intend to change the status of CSEs or prevent their use. However, in a referee test, the USP reference standard should be used to assure full compliance with the BET. The term "in-house standards" used in Pharmacopeial Forum suggests standards that are made up in individual laboratories. CSEs provided by the LAL manufacturers are widely used throughout the industry. They are not made and used by a single laboratory or one organization. They are more than in-house standards, but they should be validated as alternatives to the USP Endotoxin Reference Standard. Consequently, alternate endotoxin standards shown to be equivalent to the RSE can be used for routine tests and their validation.

Documentation of the equivalence of the CSE to RSE is provided by LAL reagent manufacturer's certificates of analysis (Cs of A) that state the potency of the CSE relative to RSE. There is a long history of the acceptance of Cs of A by regulatory agencies of many countries. One option is to accept the certificate of analysis at face value. (It should be noted that for the gel-clot method, every successful confirmation of label claim using a CSE supports the potency stated on the C of A.) Another option is to perform testing on a limited number of lots of CSE to confirm that potencies given on certificates of analysis are correct and verifiable in the user's laboratory and thus justify accepting the potencies stated on Cs of A. Finally, the potency determination can be performed for every CSE/LAL lot combination, either in place of the potency on the C of A or to verify each C of A received.

TESTING DRUGS AND BIOLOGICALS

Endotoxin Limits, Maximum Valid Dilution, and Minimum Valid Concentration

Endotoxin limits are generally based on the threshold pyrogenic dose for endotoxin. This is the minimum amount of endotoxin that can be expected to elicit a pyrogenic response. The term K is used for generic endotoxin limits. The threshold pyrogenic dose and the value of K is 5 EU/kg of patient body weight per hour for parenterally administered drugs and therapies, other than those administered intrathecally [into the cerebrospinal fluid (CSF)]. Both a controlled clinical study (1) and field experience (47) demonstrate that a pyrogenic response, may be elicited by endotoxin at this very low dose. The endotoxin limit is more stringent for products that are administered intrathecally and the value of K is 0.2 EU/kg/hr. (Note: None of the pharmacopeial chapters state that K is expressed per hour. It is necessary to express the limit per hour if the maximum dose of the drug product is expressed per hour in order for the units to cancel out in the endotoxin limit equation.) From the generic values for K, product specific limits are calculated and these are given in product monographs in the USP. For products that have a dose expressed per person, the average human adult body weight is assumed to be 70 kg. (Note: An average human body mass of 60 kg is assumed in the JP (41).) Thus, the endotoxin limit on a whole body basis is 350 EU/hr for parenteral products that are not administered intrathecally.

The product-specific endotoxin limit for a parenteral product is based on the maximum dose of the product. The greater the dose of the product, the lower (or more stringent) the endotoxin limit per unit of product. In the pharmacopeial endotoxins testing chapters and in

the FDA Guideline, the formula given for calculating the endotoxin limit for a product is Limit $= K/M$

where,

K is the generic endotoxin limit in EU/kg/hr (5 EU/kg/hr for most products; 0.2 EU/kg/hr for intrathecally administered products)

M is the maximum bolus dose of product/kg. If the product is injected at frequent intervals or administered continuously, M is the maximum total dose administered in a single hour period. If the dose is expressed for whole (adult) body, divide it by 70 kg to give the dose per kg.

Note 1: The maximum dose, M, excludes the heroic dose that might be used under extraordinary circumstances.

Note 2: The 1987 Guideline states that the dose used should be the rabbit dose (as used in the USP pyrogen test) or the maximum human dose, whichever is greater. USP has changed the basis of calculation of endotoxin limits to human doses only.

The equation gives the endotoxin limit expressed per unit of product, where the units are those in which the dose is expressed (weight, volume, international unit, equivalents, etc.).

In the case of drugs administered per m^2 of body surface area, calculations of endotoxin limits are based on an average human adult surface area of 1.8 m^2. Given the whole body limit of 350 EU and an average human surface area of 1.8 m^2, the endotoxin limit expressed per square meter is 350 EU/1.8 m^2 = 194 EU/m^2. This value of 194 EU/m^2 is an absolute and is equivalent to a value of 5 EU/kg for K for products administered per kg, but it is not currently stated as such in any regulatory document. To calculate the endotoxin limit of a product with a dose expressed per square meter, divide 194 EU/m^2 by the maximum dose (M) to give an endotoxin limit per unit of product. This is a simplification of the approach described in the BET, which states that the dose per m^2 should be multiplied by 1.8 m^2 and divided by 70 kg to convert it into a dose per kg. The product-specific limit is then calculated as described above by dividing the 5 EU/kg/hr by the dose/kg/hr.

For radiopharmaceuticals, the BET and the FDA Guideline state that endotoxin limits are calculated using a variant of the K/M equation whereby the limit $= 175/V$ (and for intrathecally administered radiopharmaceuticals the equation is $14/V$). In these equations, V is the maximum dose (on a whole body basis) in mL at expiration of the product. It should be noted that there is an error in these equations in that the units have been omitted. To give limits in EU/mL, the equation must be expressed as:

$$\text{Limit} = \frac{175\,\text{EU}}{V}$$

(and 14 EU/V for intrathecally administered radiopharmaceuticals).

The endotoxin limits for health care products do not account for a patient being given several drugs and/or solutions at once, and it had already been noted that the limit of 5 EU/kg/hr does not give a significant safety margin. Fortunately, most drug products and solutions are considerably cleaner than the allowable limit. Many companies have internal specifications that are tighter than the limit, particularly for in-process samples and materials, and these provide a safety margin. If a more stringent limit is set for a product and stated as a specification, then that is the specification against which the product must be judged. A product that exceeds that limit fails to meet specification, even if it contains less endotoxin than the pharmacopeial limit.

For some products, there are compendial limits that are not based directly on the K/M equation. The endotoxin limit for large volume parenterals (dose of at least 100 mL) is generally not more than 0.5 EU/mL. This limit has its origins in a rabbit dose of 10 mL/kg. The limit for water for injection (WFI) and sterile WFI (SWFI) is less than 0.25 EU/mL (USP/EP).

Product-Specific Endotoxin Limits

When faced with an unfamiliar product, the product monograph in the appropriate pharmacopeia should be consulted. The maximum dose for the product should be determined from the product insert and the limit should be calculated for that dose. It is possible that the limit in the monograph was calculated from a different or incorrect dose, or that a mistake has

been made in the calculation. As a general rule, in such cases the more conservative (more stringent or lower) endotoxin limit should be adopted.

Endotoxin limits in pharmacopeial monographs are stated per unit of active pharmaceutical ingredient (API). It is important to understand that this is actually the limit for the whole finished product. Similarly, endotoxin limits calculated from the dose of API is the limit applied to the whole finished product.

Calculating Endotoxin Limits

Consider a parenteral product (with a nonintrathecal route of administration) with a maximum human (whole body) dose of 1.0 g. Using the equation given in the pharmacopeia and in the FDA Guideline,

$$\text{Limit} = \frac{K}{M}$$

First the dose must be converted into a dose per kilogram of body weight:

The dose per kg = 1 g/70 kg = 0.0143 g/kg = 14.3 mg/kg.
The endotoxin limit = K/M = 5 EU/kg/14.3 mg/kg = 0.35 EU/mg

The sensitivity of the LAL test used to test the product is expressed in EU/mL. To give an endotoxin limit in the same units as the test (unless the limit is already expressed in EU/mL), the endotoxin limit per unit of product is multiplied by the concentration of the product (sometimes referred to as the potency). To continue with the above example, if the concentration of product is 100 mg/mL, or if a solution of product is prepared to give 100 mg/mL, the limit is 0.35 EU/mg × 100 mg/mL or 35 EU/mL. This limit is specific to a product concentration of 100 mg/mL.

For a product with a dose of 1 g/m^2 and a concentration of 50 mg/mL, the endotoxin limit = 194 EU/m^2/1 g/m^2 = 194 EU/g (or 0.194 EU/mg). (Using the equation presented in the USP is a little less straightforward but gives the same result.) The limit expressed per mL = 0.194 EU/mg × 50 mg/mL = 9.7 EU/mL.

For a radiopharmaceutical product with a (whole body) dose at expiration of 7 mL, the endotoxin limit = 175 EU/7 mL = 25 EU/mL.

Calculating the MVD

Many drug products interfere with the LAL test. However, the fact that the LAL test is usually more sensitive than is necessary to detect the endotoxin limit (and sometimes much more sensitive) allows products to be diluted to overcome interference. In an early study conducted by FDA (30), of 333 products test dilution was effective in overcoming the interference for 236 products. Dilution is the most widely used means of overcoming interference. However, there is a limit to the amount by which a product can be diluted and the endotoxin limit can be detected. That limit is the MVD, a concept addressed in the pharmacopeial endotoxins test chapters and introduced in that section above. At the MVD, the endotoxin limit for the product dilution is equal to the sensitivity of the test. Valid testing cannot be conducted at dilutions greater than the MVD because in a sample contaminated at the limit concentration the endotoxin cannot be detected.

The concept of the MVD is quite intuitive. Consider a product with an endotoxin limit concentration of 25 EU/mL that is tested with a gel-clot reagent with a sensitivity of 0.25 EU/mL. Clearly the reagent is 100× more sensitive than is necessary to detect the limit concentration. Consequently, the product can be diluted by a factor of 100 and endotoxin at the limit concentration can still be detected. The MVD is 100.

The formula for calculation of the MVD as given in the section on the Pharmacopeial Endotoxins Test Chapters above is:

$$\text{MVD} = \frac{\text{Endotoxin limit product concentration}}{\lambda}$$

where λ is LAL test sensitivity.

If the endotoxin limit is already expressed in EU/mL, the equation may be simplified to

$$\text{MVD} = \frac{\text{Endotoxin limit (EU/mL)}}{\lambda}$$

The MVD is a unitless dilution factor.

As an example, for the hypothetical product with a limit expressed as 0.35 EU/mg tested using a LAL reagent with a sensitivity (λ) of 0.25 EU/mL:

$$\text{MVD} = \frac{0.35\,\text{EU/mg} \times 100\,\text{mg/mL}}{0.25\,\text{EU/mL}}$$

If the limit is expressed as 35 EU/mL (see above):

$$\text{MVD} = \frac{35\,\text{EU/mL}}{0.25\,\text{EU/mL}} = 140$$

Note that MVD increases as the sensitivity of the method/reagent increases. If the sensitivity of the LAL test is increased to 0.125 EU/mL for the above product:

$$\text{MVD} = \frac{35\,\text{EU/mL}}{0.125\,\text{EU/mL}} = 280$$

Thus, the MVD of a product can be increased by using a more sensitive gel-clot reagent or by using a lower range standard curve for photometric methods.

Calculating the MVC

A parameter directly related to the MVD is the minimum valid concentration or MVC and is discussed in Appendix D to the FDA Guideline, but not in the pharmacopeial endotoxins test chapters. The MVC is the product concentration at the MVD. It is the lowest concentration of product at which the endotoxin limit can be detected. The MVC is inversely related to the MVD because the greater the dilution of the product, the lower the concentration.

The formula for calculation of MVC is

$$\text{MVC} = \frac{\lambda}{\text{Endotoxin limit}}$$

For example, for the product with an endotoxin limit of 0.35 EU/mg, if the LAL sensitivity is 0.25 EU/mL:

$$\text{MVC} = \frac{0.25\,\text{EU/mL}}{0.35\,\text{EU/mg}} = 0.71\,\text{mg/mL}$$

Unlike the MVD calculation, the initial concentration of the product is not a parameter in the equation. Thus, the MVC is constant for a given LAL test sensitivity, while the MVD changes with the product concentration.

The conversion of MVC to MVD is described in Appendix D of the FDA Guideline and is accomplished by dividing the product concentration by the MVC. For example, continuing with the example from above,

$$\text{MVD} = \frac{\text{Product concentration}}{\text{MVC}} = \frac{100\,\text{mg/mL}}{0.71\,\text{mg/mL}} = 140$$

MEDICAL DEVICES

Introduction

To test a medical device, an aqueous extract of the medical device must be prepared because endotoxin tests can only be performed on aqueous samples. The USP BET states that extracts of medical devices should be prepared using Water for BET (formerly and commonly known as

LAL Reagent Water, or (LRW)) or other aqueous solutions. In the EP and JP bacterial endotoxins chapters, there is no mention of medical devices. Procedures for preparing extracts of medical devices are given in USP chapter <161>, "Transfusion and Infusion Assemblies and Similar Medical Devices." This chapter applies to a wide range of devices but orthopedic products, latex gloves, and wound dressings are explicitly excluded from the scope. Slightly different extraction procedures are given in the FDA Guideline on LAL testing and these are echoed in the standard ANSI/AAMI ST72:2002.

Endotoxin Limits for Medical Device Extracts

Generic endotoxin limits specified in USP chapter <161> are 20 EU/device for most devices labeled as nonpyrogenic and 2.15 EU/device for devices that contact the CSF. The formula given for the calculation of a limit specific to the extraction volumes in EU/mL is

$$\text{Endotoxin limit} = \frac{K \times N}{V}$$

where,

K is the endotoxin limit per device (e.g., 20 EU/device for devices that do not contact CSF)
N is the number of devices to be tested
V is the total volume of extract or rinse (i.e., extract volume per device multiplied by the number of extracts pooled).

Once an endotoxin limit has been determined, the concept of the MVD can be applied as described for drug products. The MVC is less useful for medical devices and is rarely used.

The formula for endotoxin limits does not account for unequal distribution of endotoxin contamination between the devices. For example, if 10 extracts of 40 mL are pooled to give 400 mL, the endotoxin limit is 0.5 EU/mL per the above equation. The total amount of endotoxin in the extract must reach 200 EU before a test failure is recorded at 0.5 EU/mL. It is quite possible that all of this endotoxin could have come from a single device. Consequently, there is a discrepancy between the stated limit 20 EU/device for a single device and a de facto limit of 200 EU/device when 10 extracts are pooled, a point that has not been widely acknowledged. In the introductory background section, the FDA Guideline recognizes the potential for unequal distribution of endotoxin on medical devices and that extraction procedures are unlikely to be 100% efficient. The endotoxin limit for medical devices is apparently more stringent than that for drugs (200 EU/person vs. 350 EU/person) for this reason.

Sampling Medical Devices for Testing

Sampling procedures for medical devices are based on lot size but there are slight differences between the FDA Guideline and USP <161>. The USP specifies testing 3% of the lot up to a maximum of 10 devices and no fewer than three devices. The FDA Guideline states that for lot sizes of less than 30 devices, 2 devices should be tested. For lot sizes of between 30 and 100 units, test three devices, and for lot sizes of greater than 100, test 3% of the lot up to a maximum of 10 devices.

Extraction Procedures

Extraction procedures in USP chapter <161> are simple and clear. Not less than three and not more than 10 devices are rinsed or soaked at controlled room temperature for 1 hour in LRW that has been heated to 37 $\pm$ 1°C. For devices labeled "nonpyrogenic fluid pathway," the pathway is flushed for 1 hour at controlled room temperature, again using the extraction solution that has been heated to 37 $\pm$ 1°C. It may be necessary to cut up or dissemble devices before extraction but this is not stated in chapter <161>. It is stated that "Extracts may be combined, where appropriate"; combining extracts is usual for testing medical devices.

Extraction procedures in the Guideline are the same as those in the AAMI/ANSI standard ST72 but are different from the USP. For devices being flushed, the rinse solution should be held in the fluid path for one hour at room temperature (>18°C). To perform extraction, the minimum extraction time should be 15 minutes at 37°C or one hour at room temperature (>18°C). Of these procedures, the one given in USP Chapter <161> includes both the maximum time and temperature. It also has the advantage of being a compendial procedure and therefore does not require validation.

The question of validation of the effectiveness of extraction procedures was addressed by the AAMI Microbiological Methods Committee, a group that included a representative from FDA. Validation of an extraction procedure requires adding a known amount of endotoxin to a device (usually drying it) and then demonstrating recovery using the procedure. The task group's report (48) concluded that validation of efficiency of endotoxin recovery should not be included in the ANSI/AAMI ST72 (40) standard.

The Guideline allows different devices of similar chemical (i.e., material) composition to be grouped for inhibition or enhancement testing. The devices selected for validation should be those with the largest surface area exposed to the body or the fluid for administration to a patient.

Manufacturers of pharmaceutical product can draw from the provisions for testing medical devices to meet the pharmacopeial endotoxins test chapter requirement that plastics be tested for contamination and interference. These must be extracted prior to testing in a manner similar to medical devices. However, appropriate limits should be set. The equation given for medical devices above is usually not applicable and could allow for unacceptable levels of endotoxin contamination. Finally, the Guideline states that "liquid devices should be more appropriately validated and tested according to the requirements for drugs by taking the maximum dose per kilogram of body weight into consideration."

ESSENTIALS OF ENDOTOXIN TESTING—A PRACTICAL APPROACH

In the introduction to the discussion of the pharmacopeial endotoxins test chapters, three essentials of endotoxin testing were identified. In addition to these, there are two other important elements. One of these (the second in the list below) is determination of CSE potency. This topic was introduced in the section on secondary standards (CSEs) and discussed in the section on the FDA Guideline.

The second additional element (number three on the list below) is preliminary testing of samples for test method development. This is a very important element, but it is not addressed in any of the regulatory documents. If method development is performed correctly, validation (the test for interfering factors) and subsequent routine testing should be trouble free.

Together, the five elements are:

1. Verification of reagent performance, technician certification, laboratory qualification
2. CSE potency determination, if necessary (not addressed in the pharmacopeia)
3. Preliminary testing (not addressed in any regulatory document)
4. Inhibition/enhancement testing - validation
5. Routine testing

Practical aspects of the application of these essentials are discussed in turn.

Verification of Reagent Performance, Technician Certification, and Laboratory Qualification

As was stated in the review of the regulatory requirements, it must be demonstrated that each lot of reagent is performing to specification prior to performing any tests of product. Also, the Guideline states that analysts must be qualified, which is a requirement of the good manufacturing practice (GMP) regulations, and that the variability of the testing laboratory should be assessed. The results for technician qualification can be compared to determine the variability of the test laboratory. These qualifying tests are all performed using the same procedure.

For the gel-clot method, this is achieved by confirming the labeled sensitivity of the LAL reagent by testing a series of four standard endotoxin concentrations with concentrations of 2λ, λ, $1/2\lambda$, and $1/4\lambda$ (and negative controls) in quadruplicate. For example,

Standards		2λ	λ	$1/2\lambda$	$1/4\lambda$	Negative controls
Replicate series	1:	+	+	−	−	−
	2:	+	+	−	−	−
	3:	+	+	−	−	−
	4:	+	+	−	−	−

The result in this example is the ideal one in which all four replicates give an end point at the labeled sensitivity of λ. The requirements are met provided that the geometric mean end point is between $1/2\lambda$ and 2λ. An implicit (but not explicit) requirement is that all replicates should clot at the 2λ concentration and none should clot at $1/4\lambda$. In addition, while not a requirement, it is reasonable to expect that the end points of any two replicate series should not differ by more than a factor of two.

For the photometric methods, at least three standard endotoxin concentrations are tested in at least triplicate, and the different standard concentrations should not differ by more than a factor of ten. A standard line is constructed and the absolute value of the correlation coefficient (lrl) must be at least 0.980. As was noted above in the discussion of regulatory requirements, for the photometric methods there is no check against manufacturer's criteria or other external standard, unlike the gel-clot method. Consequently, it is quite possible to make a dilution error and still meet the requirement for linearity. It is therefore recommended here, but not in any regulatory document, that other parameters such as OD values, onset time, and y intercept be routinely checked for atypical values (slope is not a good indicator of such errors).

CSE Potency Determination

The only endotoxin standard referred to in the pharmacopeial endotoxins test chapters is the primary standard endotoxin (such as the USP endotoxin RS). In practice, for most testing, a CSE that has been standardized against a primary standard is used. The quantity of CSE is typically expressed in units of mass. To convert the units of mass to endotoxin units (EU) of international units of endotoxin (IU), the potency of the CSE must be determined.

It is important to remember that a CSE potency is only applicable for the lot of LAL reagent and CSE with which it was determined. A potency determined with one reagent lot combination cannot be assumed to apply to any other combination. Consequently, only a CSE provided with a kit should be labeled in EU and the potency given on an accompanying certificate of analysis. The potency of this CSE should not be assumed to apply to other lots of LAL reagent. CSE provided for general use should be accompanied by a certificate of analysis specific to the lot of CSE and the particular lot of LAL reagent with which it will be used. Alternatively, the potency of the CSE can be determined by the user, again with a specific lot of LAL reagent.

Regardless of the test method, the potency of a CSE is determined by testing parallel series of dilutions of RSE and the CSE. The units are EU/mL for the RSE and units of mass (often ng/mL) for the CSE. The range of CSE concentrations to give equivalent activity to RSE has to be estimated. A good working assumption is 10 EU/ng for most CSEs, but it is wise to add an extra CSE concentration to the upper and lower ends of the series in case the potency of the CSE is higher or lower than this.

Determining CSE Potency by the Gel-Clot Method

For the gel-clot method, a series of dilutions is prepared for both RSE (2λ, λ, $1/2\lambda$, and $1/4\lambda$) and CSE. If the test is being conducted to verify the CSE potency given on a certificate of analysis, that potency can be used to determine the appropriate concentrations of CSE to be tested. Divide the 2λ, λ, $1/2\lambda$, and $1/4\lambda$ concentrations by the stated potency to give the concentrations of CSE to be tested.

The dilutions of the two standards are tested in quadruplicate. From the end points of the four replicates series, the geometric mean end point endotoxin concentration is determined for

each standard, in EU/mL for the RSE and ng/mL for the CSE. The geometric mean of the RSE (which must be within a factor of two of the labeled sensitivity) is then divided by that of the CSE to give a potency of the CSE in EU/ng.

The procedure is described in Appendix B to the FDA Guideline, which is the BET from the first supplement to USP XXI and was current at the time the Guideline was issued in 1987. It calls for testing one vial of RSE and four vials of CSE. In a subsequent revision of the BET, but before the chapter was harmonized and reference to CSE was removed, the requirement was changed to a single vial of CSE.

The following is an example of a CSE potency determination using a LAL reagent with a sensitivity of 0.06 EU/mL:

RSE	2λ	Λ	$1/2\lambda$	$1/4\lambda$	Negative controls
(EU/mL)	0.125	0.06	0.03	0.016	0
	+	+	−	−	−
	+	+	−	−	−
	+	+	−	−	−
	+	+	−	−	−

$$\text{GM RSE endpoint} = \text{antilog}\frac{-4.89}{4} = 0.06\,\text{EU/mL}$$

CSE (ng/mL)	0.025	0.0125	0.006	0.003	0.0016	0.0008	Negative controls
	+	+	+	−	−	−	−
	+	+	+	−	−	−	−
	+	+	+	−	−	−	−
	+	+	+	−	−	−	−

$$\text{GM CSE endpoint} = \text{antilog}\frac{-8.89}{4} = 0.006\,\text{ng/mL}$$

$$\text{CSE potency} = \frac{\text{GM RSE endpoint}}{\text{GM CSE endpoint}} = \frac{0.06\,\text{EU/mL}}{0.006\,\text{ng/mL}} = 10\,\text{EU/ng}$$

As is generally true for the gel-clot assay, the method does not have the resolution to determine potencies between twofold dilutions. If the "true" potency is 7 EU/ng, with some LAL reagents the potency may be determined to be 10 EU/ng, with others it could be 5 EU/ng. One reason for this is that reagent sensitivity is determined using a twofold series of endotoxin dilutions, so the labeled sensitivity is an approximation. This lack of precision of the method is one reason for the potency of a CSE determined using one lot of LAL reagent may differ from that obtained using another lot.

Determining CSE Potency by the Photometric Methods

The procedure for potency determination by photometric methods is given in Appendix C to the FDA Guideline. Slight variations are described for different test methodologies but the principles are the same. A series of RSE concentrations are prepared to enable construction of a standard curve. These are tested in parallel with four series of known concentrations of CSE (in units of weight, e.g., ng/mL) prepared from four vials of CSE. The CSE concentrations are treated as unknowns and their endotoxin concentrations determined against the RSE standard curve. For each measured CSE concentration that falls within the range of the RSE standard curve, the measured concentration (in EU/mL) is divided by the known concentrations (in ng/mL) to give a potency in EU/ng. Then the mean potency of the individual CSE concentrations is determined.

Test Method Development

Test method development is conducted to determine the interference characteristics of the product/sample and is sometimes called product characterization. The goal is to develop a method to overcome interference with the endotoxin/LAL reaction caused by the product and to determine whether the product contains contaminant endotoxin (or glucan) at a concentration that might interfere with the test for interfering factors, which is conducted to validate the test. If it is necessary to treat the sample in any way other than by dilution, it must be demonstrated that the selected treatment does not mask or remove endotoxin in the sample. Test method development is the foundation on which validation is built (where validation consists of successfully performing the USP BET test for interfering factors described in the next section).

As dilution is the simplest and most common means of overcoming interference, the first step in the method development process is to determine the MVD as has already been described. To determine the greatest possible MVD, the maximum possible test sensitivity should be used in the calculation, even though it may not be necessary (or desirable) to use this sensitivity.

The pH of the sample/LAL reaction mixture should be measured to assure that it is within the range specified in the package insert for the LAL reagent. It is the pH at the concentration/dilution at which the test is conducted that is important. If the MVD is 500 and the pH of the reaction mixture with the undiluted sample is out of range, but it is in range at a 10-fold (10×) dilution, then test the product at dilutions made from the 10× dilution. There is no need to adjust the pH of the undiluted sample or to use additional buffer. If necessary, pH can be adjusted with endotoxin-free NaOH or HCl. A sample with a pH outside this range does not always need adjustment. WFI tends to be slightly acidic, typically having a pH of about 5.8, but it is unbuffered and should not need adjustment prior to testing. The buffering capacity of the LAL reagent may be sufficient to bring the pH of the reaction mixture (sample plus LAL reagent) into range, and this should be verified by measurement. Alternatively, a LAL reagent reconstituted in a suitable buffer may be used.

The initial objective of preliminary testing for method development is to determine whether dilution alone is sufficient to overcome interference, without exceeding the MVD. The goal is to determine the dilution required to overcome interference (DROI). This dilution contains the highest noninterfering concentration of product (sometimes referred to as the NIC) and no significant endotoxin contamination. For the gel-clot method, the DROI is the least dilution at which the PPC (sample "spiked" with a known concentration of added endotoxin) clots but the sample does not. For photometric methods, it is the lowest dilution at which a valid PPC recovery is achieved (i.e., quantified within the range of 50% to 200% of the nominal concentration).

Regardless of methodology (gel-clot or a photometric), the usual procedure is to test a series of dilutions of product. The greatest dilution tested might be the MVD or it might be the last two dilutions might bracket the MVD. At each dilution, the product is tested both "unspiked" (no endotoxin added) and "spiked" with added endotoxin. The spike concentration is the same at each dilution, but the product concentration declines down the dilution series.

The appropriate endotoxin spike concentrations depend on the test methodology and are generally the same as those specified in the pharmacopeial endotoxins test chapters for the test for interfering factors. For the gel-clot method the final concentration of added endotoxin is 2λ (which is double the label claim sensitivity). This is the lowest endotoxin concentration that should always clot. For the photometric methods, the endotoxin concentration of PPCs should be equal to or near the middle concentration of the standard curve.

Spiking Methods

There are three different methods of achieving the desired endotoxin spike concentration. First is the addition of a small volume ($\leq$10% of the sample volume) of concentrated endotoxin to sample to give the desired final concentration. The volume added is small enough so that it does not dilute the sample significantly. For example, to achieve a final endotoxin concentration of 0.125 EU/mL, 0.01 mL (10 μL) of 1.25 EU/mL endotoxin

standard can be added to 0.1 mL of sample in the reaction tube. This is sometimes referred to as the hot spike method. It is the only way to spike an undiluted sample without significantly diluting it.

For the second method, a volume of standard endotoxin at double the desired final spike concentration is mixed with an equal volume of sample at double the desired final concentration. The endotoxin solution and the sample dilute each other to the final desired concentrations. The disadvantage of this method is that it cannot be used to test undiluted sample; the minimum dilution is a twofold. The advantage of the method is that the same endotoxin dilutions can be used to prepare endotoxin standards and to prepare spiked product.

The third method is for preparing a series of dilutions of sample spiked at particular endotoxin concentration and is used after preparing an initial volume of spiked sample by one of the first two methods. The spiked sample is then diluted with diluent (usually LRW) containing endotoxin at the spike concentration. The product concentration is reduced at each dilution but the endotoxin concentration remains constant. This procedure is ideally suited to preliminary testing for method development.

In the gel-clot test, the unspiked sample should not clot. If it does, it either contains contaminant endotoxin (the most likely possibility), or there is an interference that causes a false positive such as (1→3)-β-D-glucan (unless an endotoxin specific test is being run) or a serine protease that mimics the activated clotting enzyme and cleaves the clotting protein, coagulogen. The spiked sample should clot. If it does not, the sample is inhibiting the clotting reaction. The +/− twofold error in the gel-clot test may "hide" interference. PPCs may still clot in the event of 50% inhibition. This is one of several reasons not to validate the test at the DROI. It should be noted that, in the case of the gel-clot method, this test is only capable of detecting inhibition, not enhancement.

For photometric methods, in order for the test to be valid the measured endotoxin concentration of PPCs should be within 50% and 200% of the known concentration and ideally well within that range. Spike recoveries are determined relative to the nominal (known or theoretical) concentration of the PPC. However, it is often useful to compare recovery with the measured concentration of the equivalent standard (or to a positive control consisting of water spiked in the same way as the PPC if PPCs and standards were not prepared in the same way). Greater or lesser spike recoveries indicate enhancement or inhibition, respectively.

It is not always possible to overcome interference by dilution of the sample, even after increasing the test sensitivity, which increases the MVD and the scope for dilution. In this case one strategy is to try a different test method. The formulation of the reagent for another method may be better suited to the chemistry of the product and enable interference to be overcome at a product dilution less than the MVD. Or, changing to a more sensitive method will result in a greater MVD, and interference may be overcome at a greater dilution.

For some samples, neither dilution nor a change of test method is sufficient to overcome interference and some type of treatment is required. The goal of treatment is to eliminate the interference without removing any endotoxin that might be present in the sample. When a treatment other than dilution is used or when buffers are used in a manner not described in the LAL reagent manufacturer's instructions, it is necessary to validate the treatment to demonstrate that it removes interference but not endotoxin. Use of a LAL reagent manufacturer's buffer in accordance with the package insert does not constitute a "treatment" requiring special validation. To validate a treatment, the BET states "... perform the assay ... described above using the preparation to be examined to which USP Endotoxin RS has been added and which has then been subjected to the selected treatment," where the "assay described above" is the test for interfering factors. This means that standard endotoxin must be added to the sample prior to treating it. An appropriate addition would result in a concentration in the product of twice the endotoxin limit. The addition should either be a small volume of concentrated endotoxin that will not significantly dilute the product or the dilution should be taken into account. The "spiked" product should be subjected to the selected treatment and then tested. The pharmacopeia do not state what an acceptable recovery is but a recovery between 50% and 200% is reasonable and is consistent with the recovery specified for PPCs.

One option for addressing interference is described for photometric methods in the FDA Guideline. This is the use of a "product standard curve." This involves preparing the standard endotoxin concentrations in the product to be tested (or in a dilution of product). The advantage is that any degree of interference in the sample is also experienced by the standard endotoxin concentrations. The product (or dilution) cannot contain a significant amount of endotoxin (i.e., the endotoxin concentration must be less than that of the lowest standard endotoxin concentration), otherwise the standard curve will be distorted at the lower concentrations. Product standard curves should be considered a method of last resort because of the influence of contaminant endotoxin on the standard line and the possibility of different degrees of interference between lots of product.

From the results of the preliminary characterization tests performed for method development, a product dilution/concentration is selected for validation (the test for interfering factors) and subsequent routine testing. The criteria for this selection process are as follows:

- The product dilution should contain significantly less endotoxin than the endotoxin limit.
- The product dilution selected for validation should be at least one- to twofold greater than DROI (the first dilution in which no interference is evident).
- The dilution selected must not exceed the MVD and, if possible, should be less than the MVD.

The method development process should be documented, including the rationale for decisions made. This documentation can then be referenced to explain the dilution selected for the test for interfering factors and the need for any sample treatments.

The Test for Interfering Factors

Provided that the method development process has been properly executed, performing the test for interfering factors should be straightforward. However, it is important that it be done correctly; if it is not, interferences may occur during the test for interfering factors or subsequent routine testing of the product. The purpose of the test is to demonstrate that the ability to detect endotoxin in a particular product or sample type is unaffected by the presence of the product. To have confidence in the test, it must be clear that negative test results are due to the absence of endotoxin, not to inhibition of the reaction that is preventing detection of endotoxin in the sample.

To validate a test method for a particular product, the FDA Guideline states that the test for interfering factors should be performed on three separate lots of product. The test is conducted at single dilution (i.e., a single concentration) of product. Upon successful completion, the method is considered validated for the product dilution/concentration at which it was conducted and also at greater dilutions. Testing should not be performed at a lesser dilution (higher concentration) without revalidation. The test should be validated for each sample type, regardless of whether the sample is finished drug product, medical device extract, in-process sample, component, raw material, WFI, or any other type of sample.

The number of units tested per lot is not specified in any of the regulatory documents. Drawing from the recommendations for routine testing of drug products in the FDA Guideline, it is reasonable to test a minimum of three units taken from the beginning, middle, and end of the lot. It is also reasonable to assume that these units may be pooled for testing. For medical devices, the number of extracts and their handling (e.g., pooling) should be the same as is planned for routine testing.

Gel-Clot Method

For the gel-clot method, parallel label claim verifications are performed in water and in product (or product dilution). One series of standard endotoxin concentrations is prepared by diluting with water; the second series is prepared with the same endotoxin concentrations diluted in the selected concentration of product. The standards in water (the control series) are

tested in duplicate and the series of standards in product are tested in quadruplicate. Duplicate negative controls are included with quadruplicate product negative controls consisting of unspiked product at the selected test dilution.

For both the series of standards in water and the series in product, the geometric mean end point must be within twofold of the labeled sensitivity of the LAL reagent. There is no requirement regarding agreement of the end points of the two series with each other. Prior to harmonization of the USP, EP, and JP endotoxins test chapters, the EP required that the geometric mean end points be within a factor of two of each other, and this is a reasonable goal, but it is not a requirement. End points differing by more than a factor of two in parallel tests suggest that there might be interference that needs investigation.

Example of the test for interfering factors by the gel-clot method:

Assume a label claim sensitivity (λ) of 0.125 EU/mL and a product with an MVD of 100. (The MVD could be increased to 400 by switching to a LAL sensitivity of 0.03 EU/mL.) Also assume that preliminary testing indicated inhibition at dilutions down to 1:4, and that this product is to be validated at a 1:25 dilution.

Standards in water (EU/mL):

0.25	0.125	0.06	0.03	Negative Controls
+	+	−	−	−
+	+	−	−	−

Geometric mean end point 0.125 EU/mL.

Standards in sample (EU/mL) at a 1/25 dilution:

0.25	0.125	0.06	0.03	Sample negative control
+	+	−	−	−
+	+	−	−	−
+	+	+	−	−
+	+	−	−	−

Geometric mean end point 0.11 EU/mL

The geometric mean end points for both the series of standards in water and in product are within twofold of the labeled sensitivity of the LAL reagent. (Also, the end points are within a twofold of each other.)

Photometric Methods

For the photometric methods, just as with the gel-clot method, the sample of product is tested at a dilution not to exceed the MVD (usually as determined during method development). Like the gel-clot method, the sample is tested unspiked and spiked with added endotoxin. However, unlike the gel-clot method, which requires that the sample be tested in quadruplicate, the test is conduct in duplicate. According to the pharmacopeial endotoxins test chapters, the concentration of endotoxin in the PPC should equal one of the concentrations from or near the middle of the standard curve. (There are different recommendations for PPC concentrations in the 1987 FDA Guideline and the 1991 Guidance, depending on the specific test method, but these have been superseded by the pharmacopeial chapters.)

In order for the test to be valid, the harmonized pharmacopeial endotoxins test chapters require that the negative control must contain less endotoxin than the lowest standard concentration, λ. (The negative controls should actually contain significantly less endotoxin than the lowest standard because concentrations of contaminant endotoxin approaching λ distort the standard curve.) The mean measured concentration of added endotoxin in the spiked product is required to be within 50% and 200% of the known or nominal concentration. If any endotoxin is detected in the unspiked product, this is subtracted from the total measured in the spiked product prior to determining the percentage recovery of the added endotoxin.

Although the endotoxins test chapters allow for a recovery range of 50% and 200%, it is recommended that recoveries closer to 100% be expected. If recoveries near the extremes of the range are accepted for the test for interfering factors, it is quite possible that a subsequent batch of product may have slightly different interference characteristics and cause the PPC to fall outside the allowed range, resulting in an invalid test result.

Routine Testing

Once the BET has been validated for a particular product, routine testing may be conducted. For finished product, every lot of product should be tested. (The AAMI/ANSI Standard, ST72, "Bacterial Endotoxins—Test Methodologies, Routine monitoring, and Alternatives to Batch Testing" does make provision for not testing every batch of finished product. This document is included in the recognized standards listed by the FDA Center for Devices and Radiological Health. The scope statement limits the standard to medical devices but notes that the requirements and guidance given may be applicable to other health care products.)

Regardless of test method, all routine tests should be conducted in at least duplicate; negative controls must be included with all tests, and standards or appropriate positive controls must also be included.

Routine Testing by the Gel-Clot Method

As previously stated, the FDA Guideline states that a standard series need not be included with every gel-clot test once consistency of standard end points has been demonstrated in the test laboratory. In that case, a standard series should be run for at least the first test of the day. If the lysate lot, the CSE lot or the test conditions change, a new standard series should be run for the new conditions. For the limits test described in the BET, the required controls are negative controls (LRW) and positive controls (endotoxin diluted in water) at a concentration of 2λ. However, to meet the FDA Guideline recommendations, a full series of standards should be included with the first test of the day. The sample is tested at the validated dilution with and without added endotoxin at a final concentration of 2λ, the latter being the PPC.

In order for the test to be valid, the negative controls must not clot and both the positive control (in water) and the PPC (in product) must clot. If these conditions are met and the sample tests negative, it meets the requirements of the test. If the sample tests positive at a dilution less than the MVD, it may be diluted not to exceed the MVD and retested.

Example of the results of a gel-clot limits test:

Negative controls	2λ positive controls
−	+
−	+
Sample (unspiked)	**PPC (2λ)**
−	+
−	+

In this test, all controls meet specification indicating that the test is valid. The sample tests negative and therefore meets the requirements of the test.

For the gel-clot assay described in the pharmacopeia, in addition to negative controls, a full series of standards (at concentrations of 2λ, λ, $1/2\lambda$, $1/4\lambda$) is specified. (This is in contrast to the Guideline allowance for only testing a full series of standards with the first test of the day. In the absence of a full standard series, a 2λ positive control is included.) A series of dilutions of the sample, not exceeding the MVD, is tested. While not stated explicitly, the first dilution should be that at which the test was validated. Also, a PPC at a concentration of 2λ is included for the first dilution of the sample.

For an assay to be valid: the negative controls must be negative, the geometric mean end point of the series of standards must confirm labeled sensitivity of the LAL reagent within a factor of two, and the PPC must test positive. If the geometric mean of the endotoxin concentration in the sample is less than the limit stated in the monograph in a valid test, the sample meets the requirements.

The limits test is typically used for routine testing of product. The assay procedure is used to quantify the endotoxin concentration in a sample and might only be run if the sample tested positive in the limits test. The assay would serve to determine whether the sample meets the endotoxin limit specification at a dilution no greater than the MVD. In this case the assay should be designed so that the series of dilutions tested includes the MVD. If necessary, the assay can be run using dilutions beyond the MVD for informational purposes to determine the endotoxin concentration of a sample that fails to meet the endotoxin specification (though this is beyond the scope of the pharmacopeial endotoxins test chapters).

Routine Testing by the Photometric Methods

The procedure in the pharmacopeia for routine testing by the photometric methods is the same as that used for the test for interfering factors described above. There are other procedures in the FDA Guidance documents, but as these precede the current BET, and as they are less straightforward than the BET, adherence to the BET is recommended.

As was described in the discussion of the Guideline and/or the Guidance above, these documents provide for use of an archived (stored) standard curve for routine testing if consistency has been demonstrated in the user's laboratory. The standard curve may be omitted provided a standard control (a spike in water at the same concentration as the PPC) is included. Endotoxin concentrations are then determined relative to an archived standard curve. In order for the test to be valid, the determined value of the standard control should be within 25% or the nominal concentration. Unless it can be demonstrated that standard controls can be routinely detected within 25% using the archived curve, this approach is not recommended. However, archived curves have been adopted in commercially available readers in which the LAL reagent is presented in cartridges. Meeting the recommendation for inclusion of standard controls may not be possible if the system capacity is limited.

For drug products, the Guideline calls for testing at least three samples from each lot of product. The samples should represent the beginning, middle, and end of the production run. The Guideline allows for samples to be pooled for testing. However, if samples are pooled they should not be tested at the MVD. This is in case one vial is contaminated but the others are not and endotoxin in the contaminated vial is diluted below the limit by the clean product when the samples are pooled. The MVD should be reduced by a factor equal to the number of vials pooled. This means that the endotoxin limit for the pooled samples is reduced by the same factor. This is not written in any regulation, but it is necessary to assure that all of the units tested meet specification and has been stated in public meetings by US FDA officials.

RETESTING

Both the pharmacopeial endotoxins test chapters and the FDA Guideline include provisions for retesting samples that contain endotoxin at or above the endotoxin limit. A test result in which the endotoxin concentration of the sample fails to meet the specification for the product is an OOS result. Before considering procedures for retesting, it is important to note that an invalid test is not a test failure, that is, it is not an OOS result. If the requirements for the controls are not met, the test should be invalidated and the reason for invalidating the test should be documented. The test may be repeated. A clear distinction should be made between repeating a test because the initial test was invalid because controls did not meet the requirements for a valid test and repeat testing following an OOS result in a valid test. A product cannot be said to fail to meet specification as a result of an invalid test (though the result might indicate that it would likely fail to meet specification), and product cannot be released on the basis of an invalid test. It should be noted that for the gel-clot method, a product has not failed to meet specification until it tests positive in a valid test conducted at the MVD. Performing an assay after a positive result in a limits test at a dilution less than the MVD does not constitute a retest; it is additional testing to determine whether the sample meets the endotoxin specification.

The USP and EP specify a retest of the gel-clot limits test if one of the two replicates clots and the other does not. The USP medical devices chapter (44) permits a retest of medical devices that fail to meet specification. There is provision for two retests in the FDA Guideline, which requires the inclusion of standards "when confirming end-product contamination," that is, when retesting. The first retest is to ensure that the test itself was not contaminated and is a repeat of the original test of the sample in question (pooled or not) but with double the original number of replicates. The second retest only applies to pooled samples and is to check for the possibility that the samples of the article were contaminated after they have been taken and pooled. For the second retest of 10 new units of the product tested individually (not pooled) and all must pass the test.

The US FDA has issued a Guidance document (46) on OOS results, which provides the context in which retests of samples giving OOS results should be performed. One of the essential elements of this document is that retests of OOS results should not be conducted before first conducting an investigation. The investigation should be conducted in accordance with an approved OOS SOP, and justification for the retest should be documented. A distinction should be drawn between testing for investigational purposes and a repeat of a release test. As was stated above, the distinction between invalid tests (controls not all valid) and OOS results for product should be clear. One concern that must be addressed is that if a product fails an initial test, why is the repeat test any more valid than the initial test? Unless there is a reason to invalidate the initial test, both the initial OOS result and any subsequent results should be taken into account by the quality unit (typically quality assurance) when the decision whether to release a batch of product is being made. Finally, the guidance addresses the issue of averaging; caution should be exercised regarding "averaging into compliance." This is not a concern if all replicates contain less than the endotoxin limit concentration (as they must for the gel-clot method).

MATERIALS AND IN-PROCESS TESTING

For materials (including excipients) and in-process samples, the same principles apply as for release testing of finished products. Preliminary testing should be conducted to develop an appropriate test method, and the test should be validated by performing the test for interfering factors. A major difference between testing finished products and excipients, materials, and in-process samples concerns endotoxin limits. For finished products, limits are clearly specified in product monographs; for excipients, materials, in-process samples, and API, appropriate limits have to be set by the manufacturer.

Raw materials should first be assessed for the need for an endotoxin specification. This can be done by compiling a list of materials and supplies and then identifying those for which an endotoxin specification is clearly not required. For example, there is no need for an endotoxin limit for glass vials that will be depyrogenated using a validated process. There is a very low risk that the vials will contribute endotoxin to the finished product. Materials that may require an endotoxin specification can then be assessed on the basis of the risk that they might be contaminated and contribute endotoxin to the finished product.

When setting endotoxin limits for excipients and other components, it should be noted that the risk of endotoxin contamination is much greater for some materials than for others. Materials derived from natural (plant and animal) sources are more likely to be contaminated with endotoxin. Other materials may contain little or no endotoxin because of their origin or manufacturing procedure. Inorganic salts typically have a low level of contamination. Some materials may be depyrogenated prior to incorporation in the formulation.

It was noted above that endotoxin limits in pharmacopeial monographs (and those calculated from the dose of API) are stated per unit of API, but that the stated limit applies to the whole finished dosage form. If an in-process endotoxin limit for the API is set at the limit stated in the monograph, there is no allowance for the occurrence of endotoxin in the excipients, containers, and closures, etc. Appropriate in-process limits should be set for the API and other components of the product. It is important *not* to calculate the endotoxin limit for each component as if it were a separate product. This would allow each component to contain

passing amounts of endotoxin based on individual doses, but the cumulative total in the formulated product could exceed the endotoxin limit for the product.

The logical approach to determining endotoxin limits for excipients is to work back from the endotoxin limit for the finished product, which can be calculated as previously described on the basis of the dose of the API. Then the decision can be made as to whether it is necessary to apply a "safety factor," such as dividing the limit by two. The endotoxin limit must then be divided between the various components of the product. A simple and unbiased approach is to assign endotoxin limits on the basis of mass. This is accomplished by calculating the total mass of all of the components in the product (including the API and all the excipients) in a given unit of product, such as one milliliter. Then the limit per unit of product (e.g., EU/mL) is divided by the total mass per unit (e.g., mg/mL). This gives a limit per unit mass (e.g., EU/mg) that applies to all of the components, not just the API.

A more sophisticated approach applies knowledge of the nature and origin of the components and takes account of any historical data from past endotoxin testing. This information is used to modify the limit calculated on the basis of mass and set limits for the individual components. Thus, more stringent limits can be set for components that are unlikely to be contaminated with endotoxin, allowing higher limits to be set for those that are likely to be contaminated, notably those of natural origin.

To put limits calculated for an individual excipient into a larger context, the limit can be determined for each of the products of which it is a constituent. The most stringent of the limits can then be selected as the limit for that material, allowing it to be used in any of the product of which it is a component. At the time of writing, the USP is in the process of developing endotoxin limits for excipients. When such limits are published, they should be used as an upper limit. If the limit determined as described above for a specific products result in a more stringent value, then the more stringent limit should be adopted.

DEPYROGENATION AND ITS VALIDATION

Depyrogenation is the removal or destruction of pyrogens (and particularly endotoxin) from an article. Endotoxin is not only the most significant pyrogen in most situations, it is also the most refractory to degradation. Consequently, conditions required to destroy or remove endotoxin will also destroy/remove the great majority of other pyrogens, so the term depyrogenation is not inappropriate, even though it is generally endotoxin removal that is of concern. Depyrogenation processes should be validated to show a minimum level of endotoxin removal or destruction. The degree of removal/destruction is typically expressed as a multilog reduction from an initial concentration.

Depyrogenation can be accomplished in a number of ways with thermal destruction of endotoxin by dry heat being the most common. Other methods of destruction may also be effective, or an endotoxin removal process may be used. The latter is best illustrated by rubber stoppers as they are not suitable candidates for dry heat treatment. In the discussion that follows, depyrogenation by endotoxin removal is considered first, followed by destruction of endotoxin.

Methods of Depyrogenation

Depyrogenation by Removal of Endotoxin

Distillation. This removes the solvent, typically water, from the endotoxin and other impurities and is very effective (49). It is possible to overwhelm a still if the endotoxin load or "pyroburden" is too high.

Reverse osmosis. In the depyrogenation of water by reverse osmosis (RO), water is forced through very small pores in a membrane against the osmotic gradient; water passes through the membrane but endotoxin does not. The molecular weight cutoff (MWCO) for RO membranes is generally not specified as it is for ultrafilters. However, as an RO membrane is generally one that will remove salts (50) and as the molecular weight of sodium chloride is 58.44, the size exclusion of RO membranes is clearly less than 100 Da.

Ultrafiltration. Ultrafiltration is an effective process scale treatment that relies on the solution or molecules to be depyrogenated being significantly smaller than endotoxin (51,52). A membrane rated to retain molecules (or aggregations thereof) of 100,000 Da will generally remove endotoxin from aqueous solutions because the LPS is aggregated. However, if the physicochemical conditions of the solution cause the LPS to disaggregate, a lower MWCO ultrafilter is needed. A membrane with a 10,000 Da MWCO can be expected to remove endotoxin from most solutions.

Ion exchange resins. An anion exchange resin will remove the negatively charged endotoxin. Use of this technology to remove endotoxin and purify an enzyme has been described by Belanich et al. (53). In common with activated carbon (discussed in the following text), ion exchange resin is best suited to batch processing. Unless carefully sanitized, an ion exchange resin can become fouled by bacteria and might contribute endotoxin to the system rather than remove it. This is true in water systems as well as other applications.

Activated carbon. Activated carbon binds and removes organic molecules, including endotoxin, and can be effective in the depyrogenation of solutions (54). Activated carbon can be depyrogenated by dry heat prior to use for depyrogenation. It can be added to the solution to be depyrogenated or the solution can be passed through a column or cartridge containing activated carbon. As is the case for ion exchange resins, it is better suited to batch processes but it readily becomes fouled and can become a source of endotoxin if resident in a system for an extended period. The effectiveness of activated carbon is reportedly increased when combined with autoclaving 121°C for 90 minutes (55). Activated carbon can be removed by settling, filtration, or centrifugation, or by a combination of these approaches. For injectable products or their components, care must be taken to assure removal of particulates.

Charge modified media. Positively charged filter media can remove endotoxin from aqueous and salt solutions, though may not be effective for protein solutions. Historically asbestos was used in this application (56), but this is now specifically prohibited for pharmaceuticals in the revisions to cGMPs for pharmaceuticals (21 CFR Part 211.72) that became effective on December 8, 2008. Filters with a positive zeta potential (i.e., a positive charge), commonly nylon, are now used (e.g., Blanden et al., 1991) (57).

Affinity devices. Devices with a specific affinity for endotoxin are available for endotoxin removal. These include Polymyxin B on sepharose or agarose chromatography columns, heparin affinity devices, and histamine sepharose. These may not be effective in certain protein solutions, and it may be necessary to adjust salt concentration and pH to obtain maximum selective binding of endotoxin.

Washing/rinsing. Washing or rinsing can used to remove endotoxin from solid articles that cannot be depyrogenated by dry heat. This may be a hot WFI rinse or may involve use of a chemical agent followed by rinsing. Berzofsky et al. discuss depyrogenation of rubber stoppers (58); Feldsine et al. (59) and Berman et al. (60) address rinsing of containers. Chemical agents to aid the process include surfactants, NaOH (typically in the range of 0.05–0.5 M), or commercial cleaning agents.

Depyrogenation by Chemical Destruction of Endotoxin

Acid hydrolysis. Mild acid hydrolysis severs the ketosidic linkage between the lipid A and the sugar moiety of the structure (61). The free lipid A then aggregates and is relatively nonpyrogenic until it is solubilized. Further hydrolysis of the lipid A by acid may truly reduce the pyrogenicity. Reported acid treatments include 0.12 M HCl for 30 minutes followed by extensive rinsing (62); 0.05 M HCl for 30 minutes at 100°C (63); 1% glacial acetic acid for 2 to 3 hours at 100°C (61).

Base hydrolysis. Treatment with alkaline solution is used in cleaning and treatment to reduce endotoxin contamination, primarily of equipment such as tanks and tubing. The mechanism of

depyrogenation is by saponification of the fatty acids of lipid A. Sodium hydroxide is commonly used at concentrations between 0.05 and 0.5 M. In addition to destruction of endotoxin by hydrolysis, at high pH the surface affinity of endotoxin is reduced and solubilization is increased, allowing it to be rinsed away. In many instances, particularly if elevated temperatures are not used, this is probably as important as destruction.

Sodium hydroxide requires substantial amounts of water (usually WFI or other water of low endotoxin concentration) to rinse it away (but it is easy to determine when the residue has been removed by monitoring pH of the water). Rinsing with copious volumes of water will aid depyrogenation. KOH is also effective and is more readily removed by rinsing.

The rate of depyrogenation by alkaline hydrolysis is increased by even moderate heating. For example, treatment with 0.25 M NaOH at 56°C for 1 hour has been shown to be effective (64). Depyrogenation by 0.1 M NaOH is also reportedly enhanced in the presence of 95% ethanol or 80% dimethylsulfoxide (65).

Oxidation. Hydrogen peroxide has been shown to be an effective agent for depyrogenation at high concentrations at elevated temperatures (27% at 100°C). It showed some effectiveness at 2.7% and 37°C (66), and it has been suggested that it is probably more effective at higher pH. Ozone has been used to produce water of low endotoxin concentration (67), and effectiveness is reportedly increased in the presence of ultraviolet light (68). Sodium hypochlorite (bleach), an oxidizing agent commonly used in sanitization and cleaning, is not generally recognized as being effective in depyrogenation.

EtO is another oxidizing agent. It is widely used to sterilize material, particularly medical devices. EtO has been reported to inactivate endotoxin but it is not sufficiently effective to achieve a greater than 3 log reduction (69,70). However, it should be noted that it is inhibitory in LAL tests (71), and articles must be allowed to outgas before being tested for endotoxins.

Depyrogenation by Physical Destruction of Endotoxin

Radiation. Sterilizing doses of radiation (γ-radiation) are not sufficient to significantly reduce endotoxin concentration (70). High doses will destroy endotoxin but may affect articles being treated, particularly plastics.

Moist heat. Autoclaving is not generally regarded as an effective means of depyrogenation, but it does have some effect. Long cycles, especially at elevated temperature and pressure, will destroy endotoxin, for example, 5 hours at 20 p.s.i. and pH 8.2, or 2 hours at pH 3.8 (72). At 15 p.s.i. (the pressure typically used in autoclaves), significant reduction in endotoxin concentration has been reported after three hours (55). As stated above, the combination of autoclaving and activated carbon has been found effective. Also, Bamba has demonstrated endotoxin destruction by autoclaving in the presence of a nonionic surfactant as well as some level of depyrogenation by autoclaving alone (73).

Dry heat. Dry heat is the most effective and commonly used method for depyrogenating many articles. Provided that the articles can tolerate the heat exposure, it is the method of choice. It is widely used for glass and stainless steel and can also be used for Teflon®. Silicone (e.g., tubing) can be depyrogenated by dry heat, but temperatures of 250°C render tubing brittle and prone to cracking and breakage (unpublished observation).

Temperatures in excess of 180°C effectively destroy endotoxin (74,75) with the time required decreasing as temperature increases. When discussing depyrogenation, the USP BET states "Commonly used time and temperature settings are 30 minutes at 250°" and references the informational chapter <1211> (43) "Sterilization and Sterility Assurance of Compendial Articles."

Some terminology, which has been adopted from theory of sterilization, has been applied to depyrogenation. The model for sterilization is one of log linear kinetics of lethality over time under a given set of sterilizing conditions. This is illustrated by the D value, which is the time required to give a 1 log (90%) reduction in the number of viable organisms at a given temperature/pressure—thus a time of $3 \times D$ will give a 3 log reduction. For depyrogenation,

the D value is the time required for a 1 log destruction of endotoxin at a given temperature. The z value is the temperature change required to change the D value by 1 log. It requires D value to be known at two temperatures and assumes linear destruction kinetics. The F value is the time required to give equivalent lethality (or destruction of endotoxin) at different temperatures; the time depends on the degree of lethality or destruction specified (2 logs or 3 log, etc.).

Unfortunately, destruction of endotoxin by dry heat does not follow log linear kinetics like sterilization (74,75). Consequently, the value of D, z, and F in discussions of depyrogenation is limited. This may explain the lack of agreement in the literature for the times at a given temperature required for given destruction of endotoxin (F values). Avis et al. (76) compared other depyrogenation studies with their own and cite F values of 10, 500, and 130 minutes for equivalent destruction of endotoxin at 250°C in three different studies.

While this may appear to complicate the selection of a time and temperature regime to be used for a dry heat depyrogenation process, at least 30 minutes at a temperature of at least 250°C is commonly used. This is well supported by the work of Hecker et al. (75), and these are the conditions cited in the pharmacopeial endotoxins test chapters.

Validation of Dry Heat Depyrogenation

Prior to starting the validation of a depyrogenation process, installation qualification (IQ) of the depyrogenation oven should be conducted. This should be followed by operational qualification (OQ) of the oven in which the heat distribution of the empty oven is mapped using temperature probes (typically thermocouples). All stages of the qualification process should be conducted according to preapproved validation protocols or procedures. The aim of the OQ is to ensure that temperature is suitably uniform in the oven and that there are no cold spots that may indicate either poor design (such as poor air circulation within the oven) or a defect, such as a leaking door seal. The time for the oven to reach operational temperature should be noted during this phase. This is useful as a reference and for comparison in future qualifications to determine whether oven performance has changed. Ideally the oven timer will not start until a specified temperature set point has been reached.

Once a functional oven is qualified and available, it may be necessary to conduct experimental work to determine the heating times of the various oven loads to be processed. Then, for performance qualification (PQ) of the oven, the heat distribution of each specific oven load configuration should be mapped with temperature probes in vials, vessels, or materials throughout the oven, including the middle of the load, to assure temperature uniformity and to identify the last point in each load to reach the minimum process temperature and the time taken for that to occur. The load configuration should be documented using diagrams and/or photographs. The lag time between the point at which the oven controller reaches temperature and the time at which the last point of the load reaches temperature should be identified and added to the cycle time. The lag time can be reduced somewhat by setting the oven temperature higher than the selected process temperature, such as 260°C for a process temperature of 250°C.

In addition, it must be demonstrated that at least a 3 log reduction in endotoxin is achieved throughout the oven load. This is done by placing vessels containing endotoxin in the oven and then assaying for endotoxin after they have been exposed to the depyrogenation cycle.

Endotoxin challenge articles should contain or carry at least 1000 EU endotoxin (per USP General Information chapter <1211> (43)), and it is recommended that this be interpreted to mean recoverable 1000 EU. Thus, if 20% is the lowest acceptable recovery, an addition of 5000 EU is appropriate. A new monograph titled "Endotoxin Indicator for Depyrogenation" (77) has been added USP 33. Challenge articles (endotoxin indicators) may be vials of endotoxin purchased for the purpose of performing depyrogenation studies. Alternatively, challenge articles may be prepared by adding a small volume of high-potency endotoxin to the article and then drying the added endotoxin on the articles by air drying or lyophilization (the former is more common and perfectly acceptable) (78). The recovery of added endotoxin from the challenge articles is then tested. Ideally, at least 20% of the added endotoxin should be recovered, but there is no specification for recovery.

Regardless of whether commercially prepared vials or indicators prepared in-house are used, the challenge articles are distributed throughout the oven, including the cold spot, in the load configuration being validated. Five vials per shelf in an X pattern, with a minimum of three shelves, top, middle and bottom, is a common arrangement, except in the smallest ovens. A number of articles/vials are left out of the oven to serve as untreated controls. The depyrogenation cycle is then executed and challenge articles are recovered for testing.

To extract endotoxin (or any remaining endotoxin) from the untreated and heat treated articles, a minimal volume of LRW is added to the article (or if the article is not a vessel, it must be extracted like a medical device). A small volume of water is used to minimize dilution of any endotoxin and maximize the chance of detecting it. The endotoxin concentration in the extracts is then assayed. The test method used must be capable of detecting at least a 3 log (1000-fold) reduction from the measured endotoxin concentration of the untreated articles. The endotoxin detected in the extract of the treated (or processed) article should be at least 3 logs less than that in the extract from the untreated controls. A 4 log reduction is desirable (but not required) as it will provide a high level of assurance that depyrogenation is effective.

The standard operating procedure for depyrogenation should state the conditions that will necessitate revalidation. Verification that the validated conditions are being maintained by the equipment and process should be conducted at least annually. If the physical data (temperature and time) assure that specified minimum conditions are met or exceeded, and show that the rate of heating is not significantly different from the previous validation, it may not be considered necessary to repeat the endotoxin destruction studies every year. Temperature probes and chart recorders must be calibrated regularly and not used when out of calibration. The specified, validated loading condition must not be exceeded and each individual oven should be validated.

CONCLUSION

Endotoxin is highly biologically active and ubiquitous in the natural environment. Living organisms have evolved effective defenses against exposure to it. Parenteral products bypass the protective barriers of the skin and the gut wall and have the potential to introduce endotoxin into the body where it can elicit a wide range of deleterious effects. Consequently, parenteral products are manufactured in a manner that controls and minimizes endotoxin contamination. Because of the risk of contamination and the severity of the effects of endotoxin, parenteral products must be tested according to a validated procedure, and they must meet endotoxin specifications to ensure that they are safe to use. This chapter provides an overview of the nature of endotoxin and its effects on biological systems, of the regulatory requirements that apply to parenteral products, and of some practical considerations regarding testing for and removal or destruction of endotoxin. An understanding of these issues will help ensure that the potential for endotoxin contamination is recognized and will help to identify and eliminate contamination when it is identified. Similarly, an understanding of how endotoxin interacts with different types of sample under various physicochemical conditions can assist in overcoming interference in endotoxin testing and ensure that rugged test methods are developed and employed. A goal in manufacturing of parenteral products is to ensure that endotoxin is kept out of the process, removed from it, or is maintained at subcritical levels so that the final release test of product is almost a formality.

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7 The compendial sterility tests

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BACKGROUND

The compendial sterility test is frequently presented as a flawed test for its stated purpose. This statement, of course, begs the question as to what exactly is the purpose of the sterility test as described in the compendia? The test first appeared in 1932 (1) and included the basic features of the modern test—two media, prescribed dilution scheme (for bacteriostasis/fungistasis or method suitability) and a defined incubation time. The original test differed from the contemporary method in that it had the media incubated for five days rather than 14 and allowed two retests (all three had to fail to fail the test). However, the basic structure of the test is present.

This test has generated controversy as to its role in product quality testing for decades. Part of the problem is in understanding the role of the compendial tests. Those chapters in USP numbered less than 1000 (for example, the Sterility Test is USP chapter <71>) are referee tests—in other words they are in place solely to demonstrate conformance to qualities specified in the product monograph as described in the current National Formulary (the other part of the book). A rigid interpretation would have it that if the product is not described by NF monograph, the test does not directly apply. In fact, the preface to the internationally harmonized sterility tests reads:

> The following procedures are applicable for determining whether a Pharmacopeial article purporting to be sterile complies with the requirements set forth in the individual monograph with respect to the test for sterility.

In a similar vein, sterile finished dosage forms have the following requirement in USP (from <1> *Injections*):

> "**Sterility tests:** Preparations for injection meet the requirements under *Sterility Tests* <71>"

This has a nice symmetry—the test states that it is applicable for meeting the requirements set forth in the monograph, the requirement being that the material meets the requirements of the test.

So, one would have to conclude that the test is not flawed for its intended purpose, that purpose being to show that the material tested meets the requirements of the test. How did we come to think that this test was designed to show the sterility of the product?

We need something to demonstrate product sterility. 21 CFR 211 states the requirement:

> "211.167 Special testing requirements.
> (a) For each batch of drug product purporting to be sterile and/or pyrogen-free, there shall be appropriate laboratory testing to determine conformance to such requirements. The test procedures shall be in writing and shall be followed."

The difficulty, of course, is that there is really no way, given current technology, to demonstrate sterility of a batch. This imposes significant validation issues as the most direct and persuasive documentation of product sterility.

However, there is an expectation in the GMP that a sterile finished product will have a release test. How are we to determine a suitable, "validated" release test for a characteristic that cannot be measured? A way to satisfy this requirement is provided in:

> "211.194 Laboratory records.
> (a) Laboratory records shall include complete data derived from all tests necessary to assure compliance with established specifications and standards, including examinations and assays, as follows: . . .
> (2) A statement of each method used in the testing of the sample. The statement shall indicate the location of data that establish that the methods used in the testing of the sample meet proper standards of accuracy and reliability as applied to the product tested. (If the method employed is in the current revision of the United States Pharmacopeia, National Formulary, AOAC INTERNATIONAL, Book of Methods,{1} or in other recognized standard references, or

is detailed in an approved new drug application and the referenced method is not modified, a statement indicating the method and reference will suffice). The suitability of all testing methods used shall be verified under actual conditions of use."

So if we can cite a "validated" test we do not need to develop one ourselves. Thus, the internationally harmonized Sterility Test is pressed into service as a product quality test, even though that is not its design nor its purpose.

THE STERILITY TESTS

There are two different GMPs describing sterility in the United States. The first is 21 CFR 211 and the second is the "Biologics" 21 CFR 610 and 612. By common consensus, the 21 CFR 211 cGMP looks to the compendial sterility tests, while 21 CFR 610 describes a separate test in 21 CFR 610.12. The Biologics test is similar in fundamental aspects to the compendial sterility tests. There is a finite (and small) sample size and two recovery media are used, each with specified incubation conditions. So both types (compendial and Biologics) share some common limitations (see the following text).

The compendial sterility tests describe two separate types of tests, the membrane filtration and the direct transfer methods. In the first, solution from a specified number of containers (volume and number determined by batch size and unit fill volume) is filtered through a filter of nominal pore size 0.45 um. Recovery of viable cells from the filter(s) is performed by submerging the filter in one of two recovery media followed by incubation as specified temperatures for 14 days. The second test is a direct immersion of the product or suspensions into a suitable volume of the two media to allow growth. The media are designed to support growth in aerobic, or growth in an environment of limited oxygen availability. Both types of tests require demonstration that the specific method used is suitable for that product.

As early as 1956 Bryce published an article describing the two critical limitations of this test. He put forward that the test was limited in that it can only recognize organisms able to grow under the conditions of the test, and that the sample size is so restricted that it provides only a gross estimate of the state of "sterility" of the product lot (2). Other concerns about the Sterility Test (e.g., choice of sample size, choice of media, time and temperature of incubation) were extensively reviewed in an article by Bowman (3).

There have been several changes in the compendial Sterility Test since that time, culminating in the internationally harmonized test (4). However, the two basic problems outlined in 1956 by Bryce remain today.

Limitations to the Sterility Tests

Sample Size

The sample size is set arbitrarily and does not provide a statistically significant population to estimate sterility (5). This is indisputable and unavoidable with a test of this type, which is destructive in nature. Let's look at some of the numbers:

Let the likelihood of a contaminated unit $= \lambda$

By the Poisson distribution, the probability of picking a sterile unit from the fill (denoted P) is $e^{-\lambda}$, or $2.7182818^{-\lambda}$

Then, if you are picking 20 samples from an infinite supply (or for this discussion, from a pharmaceutical batch),The probability of passing the sterility test is P^{20}.

Conversely, the probability of failing the sterility test is $1-P^{20}$

Therefore, given a known frequency of contaminated units in the batch:

Frequency of contaminated units in the batch	Probability of failing sterility test with the current sample size
0.001	0.0198–2%
0.005	0.0952–9.5%
0.01	0.1813–18%
0.05	0.6321–63.2%
0.1	0.8647–86.5%
0.5	1.0000–100%

The only way to modify this limitation would be to degrade the media (resulting in lesser recovery and therefore false negatives) or to increase the number of samples. Changes of this sort seem unlikely in the compendial sterility tests at this point in time. A discussion of different sampling plans that might be used is presented in Bryce (2), and a more full discussion of the controversy over the final resolution of the current procedure is provided in Bowman (3). After extensive review, all of the proposed sampling plans were found wanting for one reason or another.

One frequently overlooked aspect of discussions of sampling plans is that the statistical analyses all assume that the test system would recover even a single microorganism if it were present in the sample. In other words, one contaminating cell would result in media turbidity. This (unverified and unlikely) assumption leads us to the next topic.

Recovery Conditions

The harmonized test utilizes Trypticase Soy Casein Digest Broth and Fluid Thyioglycollate Medium. These media and their corresponding incubation temperatures were chosen to maximize recovery of potential contaminants early in the development of the tests. However, some authors have questioned the choice of media (6), while others have suggested the use of solid media rather than liquid media would be appropriate (7). The choices in the current harmonized procedure reflect those media to which all parties in the harmonization process could agree.

Then there was a concern about incubation duration. USP 23 (8) allowed a 7-day incubation period for products tested by membrane filtration; 14 days for those tested by the direct transfer method. This requirement changed in USP 24 (9) to include a 14-day incubation period for both types of tests with the exception of products sterilized by terminal sterilization (this exception was removed by USP 27 (10)). Similarly, the *Pharm Eur* 3rd Edition (1997) allowed a 7-day incubation period (unless mandated by local authorities). This allowance was amended in 1998 with the 4th edition to 14-day incubation. This extension was the result of concerns that the methodology might not be able to detect "slow-growing" microorganisms.

The incubation period was identified as a concern by Ernst et al. (11) who recommended a longer period of incubation time than 7 days might be necessary, perhaps as long as 30 days. More recently this position was repeated with retrospective data provided by German and Australian workers who wished to ensure that a harmonized procedure included an incubation period of at least 14 days (12,13).

However, even with the longer incubation period there is no assurance that all microorganisms can grow under these conditions, but are metabolically active. In fact a growing body of evidence suggests that there are a large number of microorganisms that are unable to replicate under standard laboratory conditions (viable but not culturable—VBNC) (14–16).

CLARIFICATIONS AND ENHANCEMENTS TO THE HARMONIZED STERILITY TEST

There have been quite a few clarifications offered by different regulatory agencies to the compendial sterility tests. This section will not be a review of the genesis of the sterility tests; that discussion is outside the scope of this chapter. We will, however, take a look at a few of the clarifications offered by different regulatory agencies on the implementation of the harmonized test.

US FDA/CBER

US FDA/CBER (the Center for Biologics Evaluation and Research) has a section of the GMP under section 21 CFR 610. In this section, 610.12 describes a separate sterility test to be used with those products under CBER purview. There are several differences in the test from the internationally harmonized tests that include controls, method suitability requirements, media growth promotion procedures, etc. A major difference between the tests is that the CBER test allows a retest if the original sterility test fails. This retest must also fail for the product lot to be out of specification. While the manufacturer is urged not to attempt this approach by the author of this chapter, this is still technically allowed in the Biologics sterility test.

As an aside, the pharmacopeias and 21 CFR 610.12 do not reference or provide sterility guidelines for unprocessed bulk samples for protein and virus products, although the FDA guidance documents "Points to Consider in the Manufacture and Testing of Monoclonal Antibody Products for Human Use" (17) and "Points to Consider in the Characterization of Cell lines Used to Produce Biologicals" (18) require this testing. Common practice is to use 10 mL/media (for a total of 20 mL) for this testing.

USP

The USP introduced clarification in 2007 with a new chapter <1208> "Sterility Testing—Validation of Isolator Systems" (19). This informational chapter provides background in isolator design and construction, the equipment qualification considerations for the isolator, validation of the decontamination cycle (this would include the internal environment, the exterior of the product containers entering for testing and the protection of the product from the decontamination cycle), and the maintenance of asepsis within the isolator environment. The reader is also instructed that the sterility test performed in a properly functioning isolator is very unlikely to result in a false-positive result. Finally, instruction is provided on the training and safety aspects of the isolator operation.

Pharm Eur

The European Pharmacopeia have published a nonmandatory chapter "5.1.9 Guidelines for Using the Test for Sterility" (20) in which further information on the sterility tests is provided. The user is instructed that the test can be performed in a class A laminar air flow cabinet located in a class B room, or an isolator. The reader is also reminded that this test cannot demonstrate sterility of a batch, and that it is the manufacturer's responsibility to adopt a representative sampling plan. Finally, elaboration is provided on "Observation and Interpretation of Results" in that during an investigation,

> "... if a manufacturer wishes to use condition (d) as the sole criterion for invalidating a sterility test, it may be necessary to employ sensitive typing techniques to demonstrate that a microorganism isolated from the product test is identical to a microorganism isolated from the test materials and/or the testing environment. While routine microbiological/biochemical identification techniques can demonstrate that 2 isolates are not identical, these methods may not be sufficiently sensitive or reliable enough to provide unequivocal evidence that 2 isolates are from the same source. More sensitive tests, for example, molecular typing with RNA/DNA homology, may be necessary to determine that microorganisms are clonally related and have a common origin."

TGA

The Australian Therapeutic Goods Administration (TGA) has published a 33-page document entitled *TGA Guidelines on Sterility Testing of Therapeutic Goods* (21) to explain how the harmonized sterility tests are to be interpreted when submitting a product into Australia while noting that the *British Pharmacopeia* (and therefore *Pharm Eur*) is the official test. This document is extensive and expands the details provided on controls recommended in the harmonized Sterility Test.

The Stasis Test is an additional control recommended here. In this test, spent media from a negative Sterility Test (media that has seen the membrane that filtered product and 14 days of incubation) is subjected to an additional growth promotion test to demonstrate its continuing nutritive properties.

There is also a great deal of discussion in this document on the interpretation of the test results and on how to investigate Sterility Test failures (see below).

PIC/S

The Pharmaceutical Inspection Convention and Pharmaceutical Inspection Co-operation Scheme (jointly referred to as PIC/S) has as its mission, "... to lead the international development, implementation and maintenance of harmonized Good Manufacturing Practice

(GMP) standards and quality systems of inspectorates in the field of medicinal products." There are currently 37 Participating Authorities in PIC/S (as of October 2009—see http://www.picsscheme.org for current information). The US FDA has applied for membership several years ago and awaits disposition of its application (22).

PI 012-2 "Recommendations on Sterility Testing"

PI 012-2 "Recommendations on Sterility Testing" provides a great deal of additional information that the inspectors are instructed to ask about. This includes direction on acceptable training of personnel, the sterility test facilities (including clean room design, airlocks, aseptic gowning, and clean room fittings), cleaning and sanitization, as well as environmental monitoring of the sterility test area. Additional detail is also provided on the test method.

The Sterility Test controls are also provided some attention in this document. In addition to their execution, the inspector is instructed to require a table of negative control failures and positive control failures.

The instruction provided for "validation" (or bacteriostasis/fungistasis) by PIC/S in this document is in conflict with the harmonized chapter. Where the harmonized chapter informs the user to add the inoculum to the final rinse, the PIC/S document states that the product should be inoculated unless it is not practical due to product interference (such interference, presumably, would have to be documented). In addition, the PIC/S document asserts that it is good pharmaceutical practice to revalidate all products every 12 months. The author is unaware of this practice outside this document for the pharmaceutical industry. The Stasis Test is also recommended in the PIC/S document. This test is also recommended to be repeated at least every 12 months.

Finally, there is a good deal of discussion on investigations (as in the TGA guidance). This will be discussed below.

PI 014-3 "Recommendation: Isolators Used for Aseptic Processing and Sterility Testing"

This guidance document covers the same basic material as described in the preceding text for USP chapter <1208> with some significant expansion on validation considerations, the nature of the sporicidal decontaminant, and the logistics of the isolator's operation. While this guidance is directed primarily to the use of isolators in manufacturing, it also claims sterility testing to be within its scope.

RMM AND THE STERILITY TESTS

A frequently discussed option for the sterility testing of finished dosage forms is to use a "rapid" method (23). Currently marketed rapid microbiological methods (RMM) can be grouped into two types—those that require amplification (growth) to show low-level contamination and those that do not. In the first group would be technologies such as ATP bioluminescence, head-space analysis, and others. Examples of the second type might be technologies such as PCR and vital dye/chromatography methods. Why is this distinction important?

The concern with recovery conditions is that we do not know how to grow all microorganisms that might contaminate pharmaceutical products. Applying an alternate technology that requires growth does not result in an improvement in the sterility test method, since organisms that currently do not grow would not grow in the new method either (24). In addition, there is the continuing concern about the duration of the incubation period.

The currently required 14-day incubation period imposes a significant burden on the manufacturer who must quarantine product until successful completion of the test. Can this be shortened in an alternate test? The time required for microbial growth to turbidity can be thought of as the sum of two stages: a lag phase where the microorganism prepares to grow and the generation time requirements for a low level of microorganisms to grow to a concentration where they are visible using human vision, that is, approximately 10^7 cfu/mL. This separation of stages is important, as it seems that the lag phase is the most significant portion of time required for turbidity (25). Therefore, any alternate methodology that requires growth to amplify the microorganism will likely be required to incorporate a lengthy incubation period to ensure the recovery of "slow-growing" microorganisms.

Duguid and du Moulin (26) describe one approach to overcoming this issue. Using an amplification stage for an ATP bioluminescence technology, they started in 1999 to validate a sterility test for an autologous cell therapy product. This sterility test, which provided for product release in 72 hours with confirmatory results at the standard 14 days, was approved by FDA/CBER in 2004. In the time since they report almost 6000 sterility test results (samples included primary, expansion, and final product from this process) were collected including four positives detected. The alternate method detected them, on average, approximately 35 hours earlier than the confirmatory test (19 vs. 54 hours incubation).

Interestingly, US FDA/CBER (the Biologics group) has issued a draft guidance document on the validation of growth-based rapid methods for use in sterility testing (27). This CBER document is remarkable in its complete avoidance of any mention or consideration of the previous work done in validation of RMM by FDA/CDER, Pharm Eur, USP, or PDA.

The limiting aspects of growth-based methods as an alternative for the sterility test can be avoided by use of a rapid microbiological method (RMM) technique that does not require growth (24). The use of a method that avoids growth requirements offers an additional advantage in that the question of VBNC organisms is completely side-stepped. As no culturing is required, the recovery phase of the sterility tests can be optimized to all microorganisms regardless of growth requirements. This approach is described by Gressett et al. (28).

INVESTIGATIONS IN THE STERILITY TEST

There is a significant amount of literature written on OOS and investigations. Most of this concern, of course, stems from the 1993 Barr Decision (29). Barr Laboratories had a history of repeated current good manufacturing practice (cGMP) deficiencies, including repeated retesting and resampling of product as well as reprocessing of defective product without adequate justification in a practice that has come to be known as "testing to compliance." This is not good practice—the out-of-specification (OOS) data is telling the manufacturer important information about the product and must be resolved. Unfortunately for the microbiology community, this initial situation, as well as most of the subsequent writing on this topic, has focused on OOS from an analytical chemistry perspective. The Food and Drug Administration (FDA) has provided guidance following the Barr decision, and drafted the "Guidance for Industry—Investigating Out of Specification (OOS) Test Results for Pharmaceutical Production" (30). Interestingly, this guidance document only briefly touches upon microbiological data, stating that "the USP prefers the use of averages because of the innate variability of the biological test system." In addition, this guidance document specifically excludes microbiology from its scope in footnote 3.

A PDA task force that was assembled to look into this issue recommended the use of the phrase "Microbial Data Deviation" (MDD) in the investigation of issues in microbiology, at least until it is clear that the issue is a true product specification failure, as opposed to a lab error or process monitoring concern (reviewed in Ref. 31).

The harmonized Sterility Tests provide some guidance on MDD investigations:

> If evidence of microbial growth is found, the product to be examined does not comply with the test for sterility, unless it can be clearly demonstrated that the test was invalid for causes unrelated to the product to be examined. The test may be considered invalid only if one or more of the following conditions are fulfilled:
>
> a. The data of the microbiological monitoring of the sterility testing facility show a fault.
> b. A review of the testing procedure used during the test in question reveals a fault.
> c. Microbial growth is found in the negative controls.
> d. After determination of the identity of the microorganisms isolated from the test, the growth of this species (or these species) may be ascribed unequivocally to faults with respect to the material and or the technique used in conducting the sterility test procedure.
>
> If the test is declared to be invalid, it is repeated with the same number of units as in the original test. If no evidence of microbial growth is found in the repeat test, the product examined complies with the test for sterility."

Conditions "a" and "b" basically refer to a catastrophic failure of control. If it can be demonstrated that either the technique or the environment was not in control at the time of the test, the test can be declared invalid.

Condition "c" is interesting in its own right. The assumption when running a control is that the effort to run that control is justified by the information provided by the test. However, many labs will only consider the results from the negative control if the test fails. In other words, although the negative control is supposed to demonstrate the adequacy of the test conditions and performance, if the test samples pass, then a failing negative control is ignored. If the test samples fail, a failing negative control is used to invalidate the test. The author of this chapter urges that a consistent interpretation of controls be used in all testing.

Condition "d" is one that has received a great deal of attention. Additional detail is provided the previously cited Pharm Eur 5.1.6, the PIC/S guidance on sterility test, and the TGA document. This topic is also discussed in FDA's Aseptic Manufacturing Guide (32). Reduced to its essentials, the user is urged in these documents to use methods sensitive enough to demonstrate that the microorganism is not only of the same species, but also of the same strain or substrain of that species. It should be noted that even with this detail the best that can be done is to show a correlation between the presence of the strain from the two sources rather than a causal relationship. In other words, finding the same strain of *Staphylococcus aureus* on the testing technician and in the sterility test does not prove that the only possible source of that was the technician (the strain could also be present in the aseptic core), but it is accepted as sufficient proof in regulatory guidance that the test was compromised and so invalid.

The pharmaceutical literature provides some examples of Sterility test investigations that can be used as guides. Lee (33) described a detailed sterility investigation that included the identification of the contaminant, reviews of documents, training records, gowning practices, environmental monitoring records, lab procedures, and other critical controls. It should be stressed here that most of the work in an investigation occurs reviewing records. The practice of complete proactive documentation is critical to the success of any investigation. The likelihood of an inconclusive investigation (and therefore surety of failing product) is assured if the associated records do not support a definitive finding.

Schroeder (34) published a thoughtful review of considerations for a sterility failure investigation. He argues that for products sterilized by filtration filter failure must also be considered in addition to the other commonly cited areas of investigation.

CONCLUSIONS

The current, harmonized Sterility Test has two fundamental weaknesses, both of which have been obvious from its inception. The first is that the sampling plan is insufficient to meet the requirements implied by the title of the test. This weakness is not solvable in the current regulatory climate (nor has it been for over 70 years). The second weakness of the test involves recovery and recognition of microbial contamination in the sample, should it exist. There are several different varieties of the Sterility Test, and even when citing the harmonized test the user must be sensitive to regional expectations for that test. While there is great promise in finding a rapid method for conducting sterility tests, few examples exist of this having been successfully accomplished. Finally, there are clear expectations on the investigations to conduct into a failed Sterility Test, and the user is urged to be familiar with these expectations.

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8 Industrial sterilization technologies: principles and overview

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INTRODUCTION

A sterile medical product is one that is free of viable microorganisms. Most medical products produced under standard manufacturing conditions according to the FDA requirements (21 CFR 820 and 21 CFR Part 210/211) (1) have microorganisms on them, even though the numbers may be low. Such medical products are nonsterile. The purpose of sterilization then is to inactivate the microbiological contaminants and thereby transform the nonsterile medical products into sterile one. Also, the sterilization treatment must not render the medical product materials or functions unacceptable. Basic to the comprehension of a sterilization process is an understanding that the kinetics of inactivation of a pure culture of microorganisms by physical and/or chemical can be expressed by an exponential relationship between the numbers of microorganisms surviving and the extent of treatment with the sterilant. This means that there is always a finite probability that a microorganism may survive regardless of the extent of treatment. Therefore, for a given treatment, the probability of survival is determined by the number and resistance of microorganisms on or in the product and by the conditions used during the sterilization treatment. It follows that the sterility of any one medical product in a population subjected to sterilization processing cannot be guaranteed and the sterility of a processed population is defined in terms of the probability of there being a viable microorganism present on a medical product.

The requirements for the validation and routine operation of sterilization methods are given in a series of ISO (International Standards Organization) sterilization standards and guidelines published in the United States by the Association of the Advancement of Medical Instrumentation (AAMI). The requirements are the normative parts of the standard with which compliance is claimed. The guidance given in the informative annexes is not normative and is not provided as a checklist for auditors. The guidance provides explanations and methods that are regarded as being suitable means for complying with the requirements. Other methods may be used if they are effective in achieving compliance with the requirements of the standard. The development, validation, and routine control of a sterilization process comprises a number of discrete but interrelated activities; for example, calibration, maintenance, product definition, process definition, installation qualification, operational qualification, and performance qualification. There is generally a prescribed sequence of events outlined in each applicable standard that will expedite the validation process.

The standards for quality management systems recognize that, for certain processes used in manufacturing, the effectiveness of the process cannot be fully verified by subsequent inspection and testing of the product. Sterilization is an example of such a process. For this reason, sterilization processes are validated prior to use, the performance of the sterilization process monitored routinely, and the equipment maintained. Exposure to a properly validated, accurately controlled sterilization process is not the only factor associated with the provision of reliable assurance that the product is sterile and, in this regard, suitable for its intended use. Attention is therefore given to a number of considerations including

1. the microbiological status of incoming raw materials and/or components;
2. the validation and routine control of any cleaning and disinfection procedures used on the product;
3. the control of the environment in which the product is manufactured or reprocessed, assembled, and packaged;
4. the equipment and processes validated, calibrated, and controlled;
5. the control of personnel and their hygiene;

6. the packaging process and materials;
7. the product storage conditions.

There are three (3) main industrial sterilization technologies that are used to sterilize medical products: ethylene oxide (EO) gas, irradiation (either gamma or E-beam), and moist steam and three (3) less commonly used methods: dry heat, filtration, and liquid chemical. The most efficient of these sterilants is moist steam under pressure or autoclaving. The next most effective are dry heat and ionizing radiation. Radiation sterilization requires close monitoring with chemical and/or physical dosimeters. Next in efficacy are gaseous sterilants, such as EO, that are usually monitored with biological indicators (BIs) in every lot. The least effective sterilants are liquid chemicals that cannot penetrate to as many sites inside the product. Filtration procedures also fall in this last category. However, they all share two common characteristics:

- Providing sterility, as defined by compendial tests
- Require validation and monitoring to prove their effectiveness

The selection of the appropriate method depends on the product materials, design features, and contamination levels. The type of contamination on a product varies and this ultimately impacts the effectiveness of a sterilization process. So during this process of defining the sterilizing agent (Table 1), one must also demonstrate its microbicidal effectiveness, identify the factors that influence microbicidal effectiveness, assess the effects that exposure to the sterilizing agent have on materials, and identify requirements for safety of personnel and protection of the environment. This activity may be undertaken in a test or prototype system; the final equipment specification should be capable of being related to the experimental studies. Table 1 contains some significant considerations, but certainly not all, that will help with this decision.

The basic requirement to validate manufacturing processes, of which sterilization is one, is defined in the Food and Drug Administrations' Quality System Regulation 21 CFR Part 820, Sec. 820.75 (2), "Where the results of a process cannot be fully verified by subsequent inspection and test, the process shall be validated with a high degree if assurance and approved according to established procedures. The validation activities and results, including

Table 1 Considerations in the Selection of Appropriate Sterilization Method

Consideration	Ethylene oxide	Radiation	Moist steam
Product materials	Compatible with most materials; maximum temperature tolerance of 130°F; can use 100–120°F, but less effective (cycle time will be longer)	Selection of suitable grades of plastics to prevent degradation over time after exposure to maximum dose ranges	Very high heat (121°C) may destroy plastics or fabrics
Product design	Must allow penetration of gas and humidity into interior spaces	No restrictions	Few restrictions
Product package	Must be permeable to gas and humidity and allow aeration after cycle completion	No restrictions	Must be permeable and withstand high heat
Time from start to product release	9 days for BI release 4–7 days with BI incubation reduction 1–2 day with parametric release	2–3 days for E-beam 2–5 days with gamma	1–2 days
Post sterile time	3–7 day quarantine for BI release and EO gas dissipation Parametric release is possible but requires additional validation testing	Dosimetric release No hold time	Dry time may be needed Parametric release
Typical products	Custom trays with multiple components	Liquids in impermeable package	Metal products Contacts

the date and signature of the individual(s) approving the validation and where appropriate the major equipment validated, shall be documented." Recent revision of 21 CFR Part 211 has included the following language in section 211.113(b) "...Such procedures shall include validation of all aseptic and sterilization processes."

While following this mandate in the validation of a sterilization process, additional assurance that the product is sterile and suitable for its intended use is obtained during the process by

- establishing, documenting, and following procedures to prevent microbiological contamination of products purported to be sterile;
- Defining, documenting, and validating the hardware and software used in the process and the operating characteristics of each piece of equipment;
- verifying the microbial kill (sterility assurance level, SAL) in the production vessel;
- ensuring by extra monitoring and sampling locations that the process is uniform and reproducible from cycle to cycle;
- confirming that the routine monitoring positions and the data obtained from these locations is sufficient to control the process.

Exposure to a properly validated and controlled sterilization process is not the only factor that provides a reliable assurance that the product is sterile. Medical products should be manufactured under conditions in agreement with requirements of a defined quality system defined in approved procedures.

As such, attention should also be given to several other factors including

- the microbiological status (bioburden) of raw materials;
- the resistance of the bioburden to the sterilizing agent;
- validation and control of any cleaning or disinfection methods used in the manufacture of the product;
- control of the manufacturing environment and personnel working therein;
- packaging of the product and configuration of the load;
- maintenance and calibration of the equipment;
- appropriateness of the cycle.

The validation process must be documented, monitored at a higher level than routine production cycles, and repeated to show consistency of operation and microbial kill. The validation will serve to define the limits of routine processing.

The entire sterilization system outlined in Figure 1 consists of multiple components, all of which require application of quality procedures, operator training, continuous monitoring, and failure investigation when necessary. The elements of the system are illustrated and will be discussed in this chapter.

MICROBIOLOGICAL CONSIDERATIONS

Microbiologists are familiar with the concept that a homogeneous population of bacteria subjected to a sterilizing agent will, in theory, die exponentially with time at a uniform rate. A constant percentage of the microbial population is inactivated with each successive time interval. The exposure time required to destroy 90%, or one (1) log, of the microbial population is defined as the *D* value, or decimal reduction value. Therefore, a semi-log plot (Fig. 2) will yield a straight-line relationship. Note that when the line crosses below 10^0, resulting in less than one survivor, it is expressed as a probability of survival. Thus, the 10^{-6} survivor level or SAL or a 12-spore log reduction (SLR) represents a one-in-one million probability of one microorganism surviving the process. Products intended to come in contact with compromised tissue, those with a sterile fluid pathway claim or those that are surgically implanted generally are validated to a 10^{-6} SAL. For other products not intended to come in contact with compromised tissue, for topicals, mucosal contact products or nonfluid pathway surfaces of sterile products an SAL of 10^{-3} can be used. It should be pointed out, however, that for sale of product labeled "sterile" in Europe an SAL of 10^{-6} is required used.

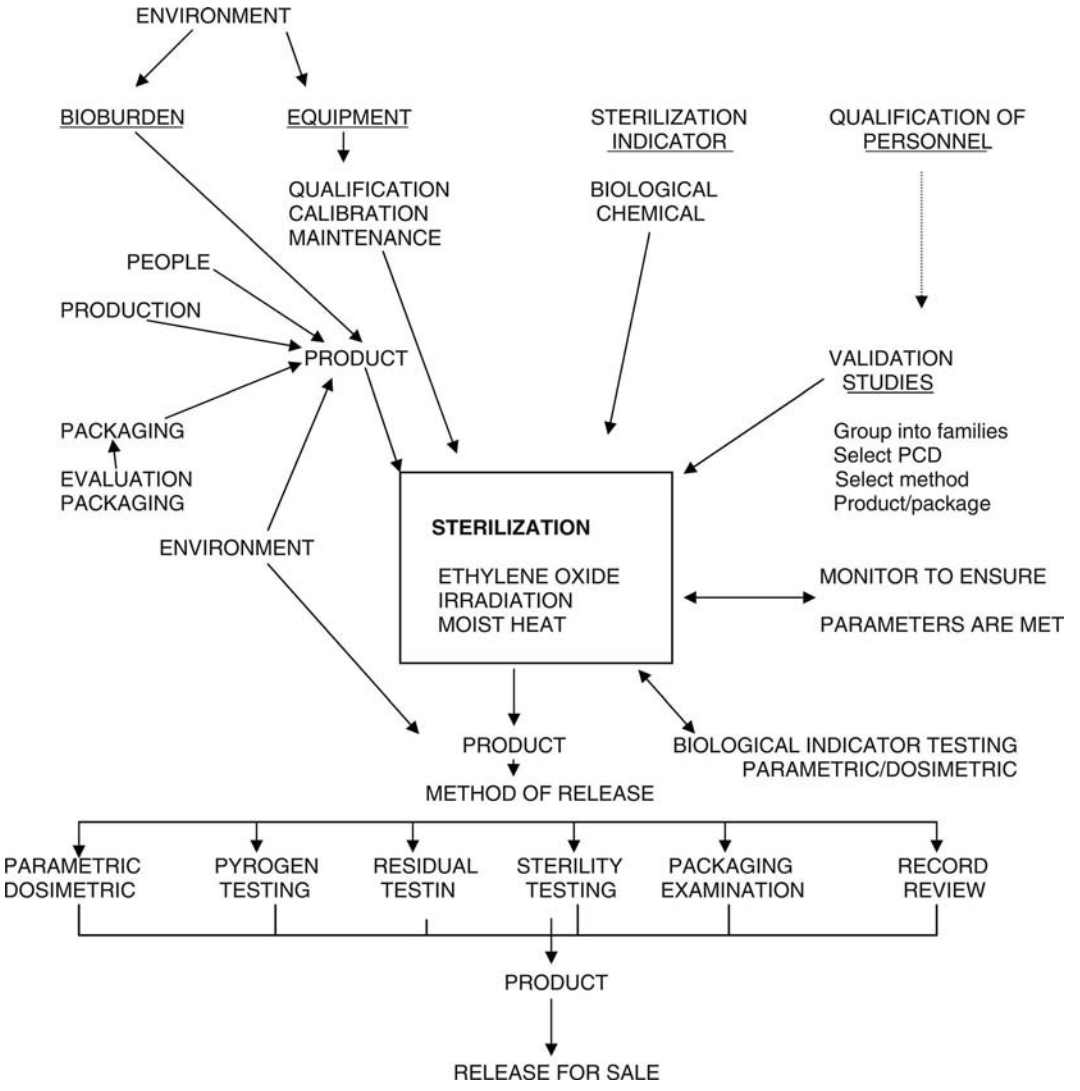

Figure 1 The sterilization system.

PRODUCT BIOBURDEN

An understanding of the viable microorganisms on a finished product is necessary and required to support the validation process. Recently, FDA has added bioburden testing in the list of control procedures in section 21 CFR Part 211.110. Bioburden data are important because the extent of the treatment of a sterilization process is a function of the bioburden on the product, the resistant of the bioburden, and the SAL required. The assessment of the bioburden needs to include the number of microorganisms with their identities. The identification need not be exhaustive, but confirmation of Gram stain characteristics and genus provide useful information and can be used to monitor changes over time and as a comparison to organisms recovered during environmental monitoring. In fact, by combining the simple information of cell arrangements (e.g., single, in clumps, chains), cell shape (e.g., sphere, rod), and Gram stain reaction, much can be deduced about the source and thereby, the control of the specific organism (Table 2). Some bacteria *(Bacillus* sp.) can form spores, a dormant form that is very

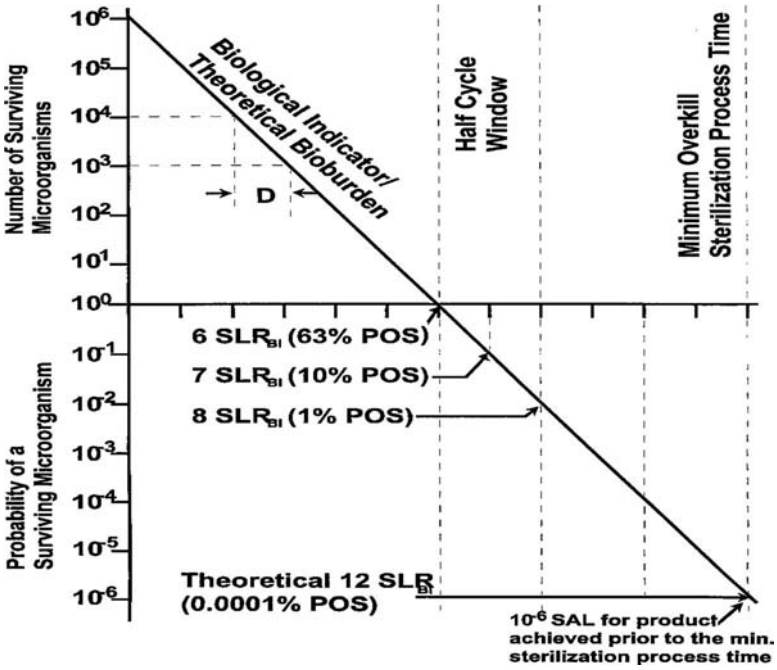

Figure 2 Semi-log plot of theoretical microbial inactivation. *Source*: Courtesy of PDA/DHI Publishing.

Table 2 Characterization of Organisms Extracted from Medical Products

Microorganism	Characterization	Source
Acidovorax	Gram −ve rod	Soil
Acinetobacter	Gram −ve rod	Skin
Arthrobacter	Gram +ve rod, nonspore	Soil
Aspergillus	Mold	Soil, packaging
Geobacillus	Gram +ve spore former	Soil, water
Brevibacterium	Gram +ve rod, nonspore	Skin
Burkholderia	Gram −ve rod	Water
Candida	yeast	Environment
Cellulomonas	Gram +ve rod, nonspore	Soil
Cladosporium	Mold	Soil, packaging
Chrysosporium		
Cochliobolus	Fungi	Packaging
Clostridium sp.	Gram −ve rod, anaerobe	Environment
Corynebacterium	Gram +ve, nonspore rod	Mucous membrane, skin
Cryptococcus	Mold	Soil, packaging
Deinococcus	Cocci	Human
E. coli	Cocci, Gram −ve rod	Human and animal colon
Flavimonas	Gram −ve rod, nonspore	Human
Fusarium	Mold	Soil
Kocuria/Micrococcus	Gram +ve cocci	Human
Microbacterium	Gram +ve	Skin
Micrococcus	Gram +ve cocci	Water, dust, soil
Moraxella	Gram −ve rod	Human
Paenibacillus	Gram +ve rod	Soil
Penicillium	Mold	Soil, packaging
Propionibacteria	Gram +ve, nonspore	Human skin
Pseudomonas	Gram −ve rod	Water, packaging
Staphylococcus	Gram +ve cocci	Skin, mucous membrane
Streptococcus	Gram +ve cocci	Human
Streptomycetes	Mold	Soil
Trichoderma	Mold	Soil

resistant to adverse conditions. This renders them more difficult to sterilize than organisms existing in the vegetative stat.

Bioburden data exhibits significant variability with a skewed distribution. Contributors to bioburden levels may include one or more of the following:

- Raw materials
- Manufacturing components
- Assembly process (especially manual cutting and assembly)
- Manufacturing environment
- Product handling by manufacturing and inspection personnel
- Assembly aids, such as compressed air, water, lubricants, etc.
- Residue from cleaning processes
- Packaging

Detection of bioburden on and/or in products is performed by selecting 3 to 10 packaged products randomly from one (1) lot of recently manufactured product. Sample size can depend on

- magnitude of change in bioburden to be detected (for early detection of small changes, a larger number is recommended),
- variations in estimates of numbers present on individual products

If products are costly, the number sampled can be reduced to three to five items. A simulated product can be used but must be made from the same materials and in the same manufacturing process. Products rejected during the manufacturing process can also be used as long as they were exposed to all process steps. Do not use expired or "old" product for bioburden evaluation because the organisms on such products may not represent those present on recently manufactured products.

The frequency of the bioburden estimations, supported by documented evidence or rationale, should be established on the basis of several factors including

- data from previous bioburden estimates—if historical data is consistent, less frequent testing is indicated (e.g., shift from monthly to quarterly or semiannually);
- use to be made of the bioburden data;
- manufacturing processes;
- batch size;
- production frequency for the product;
- materials used—change in materials may trigger new bioburden estimate;
- variations in the bioburden estimates—spikes or swings in data could signal more frequent testing.

The test method used only produces an estimate of the number of microorganisms. The method can be validated to establish the relationship between the estimate and the true number of microorganisms on the product. Whatever method is used must be reproducible so that the results generated on one occasion can be compared to data generated subsequently. The method of extraction most effective for bioburden recovery varies according to the substrate; therefore individual products may require different extraction methods to optimize organism removal. All treatments should avoid conditions that are likely to affect the viability of microorganism, such as excessive cavitation, shear forces, temperature rises, or osmotic shock. Acceptable bioburden recovery methods are available in ANSI/AAMI/ISO 11737-1:2006 (3), *Sterilization of medical products—Microbiological methods—Part 1: Determination of a population of microorganisms on products*. In addition, ISO 11737-3:2004, *Sterilization of medical products—Microbiological methods—Part 3: Guidance on evaluation and interpretation of bioburden data*, provides guidance on evaluating and interpreting the data generated during routine monitoring of the microbiological quality of medical products.

The estimation of the bioburden can be divided into three phases, all of which may affect the final results and therefore should be considered in the validation:

1. The removal of the microorganism from the product—extraction techniques could include use of ultrasonication, mechanical agitation with or without glass beads, vortex mixing, flushing, blending, swabbing, and contact plating and stomaching. A surfactant may be used in the extraction fluid to facilitate removal of organisms.
2. Transfer of the organisms to the cultural conditions quickly—methods could include membrane filtration, pour plating, spread plates, and/or serial dilution if large numbers of organisms are expected. Use proper incubation conditions for aerobic bacteria at 30 to 35°C for two to five days; yeasts and molds at 20 to 25°C for five to seven days and anaerobic bacteria at 30 to 35°C for three to five days.
3. Enumeration of the microorganisms—colony counting is most commonly used.

Bioburden should be evaluated at least annually prior to the validation or requalification; it is recommended, however, to test product quarterly or semiannually to trend history and help monitor the component suppliers and the manufacturing environment. The resistance of the bioburden must be equal to or less than that of the BI used during validation of a sterilization process (as in EO, moist steam, and dry heat).

The following methods have been used to evaluate the resistance of the bioburden:

1. When the bioburden estimate is accompanied by microbial identifications, the *D* values can be determined or obtained from the literature for the resistant portion of the population. The time required to inactivate the bioburden can be compared to that of the BI. If the bioburden population consists mainly of vegetative organisms, physical determination of the *D* value may be impossible due to the rapid death rate of these organism.
2. When microbial identifications are not performed and the bioburden is low (<100), the appropriateness of the BI can be shown by inspection, in that the entire bioburden population would need to have a *D* value which is 1.5 to 2 times that of the BI to present a greater challenge. Resistance of this magnitude for naturally occurring bioburden is not supported by the literature.
3. When the microbial identifications are not performed and the bioburden is high, the appropriateness of the BI should be determined by exposure to sublethal cycles, as described in Cycle Development.

PRODUCT STERILITY TESTING

Crucial to the validation of any radiation process is product sterility testing of products subjected to sublethal dosing. Guidance for appropriate sterility testing can be found in AAMI/ISO 11737-2:2000, *Sterilization of medical products—Microbiological methods—Part 2: Tests of sterility performed in the validation of a sterilization process*.

There are two (2) general approaches in the performance of product sterility tests. These are as follows:

1. Direct immersion of the product into growth medium or by placing growth medium into the product followed by incubation for 14 days.
   - The product may be disassembled prior to exposure to facilitate transfer or aseptically subdivided prior to transfer to medium container.
   - Sufficient growth media should be used to cover the product or to achieve contact between the growth medium and the whole product
   - Agitate after placement in growth medium
   - Maintain contact between medium and product for the duration of the incubation. If the product is large, medium can be swirled daily to contact all product surfaces

Table 3 Quantities of Product for Sterility Testing

Quantity per product container	Minimum quantity for test
Liquids	
<1 mL	Whole contents of container
1–40 mL	Half of contents, but not less than 1 mL
>40 mL, <100 mL	20 mL
>100 mL	10% of contents, but not less than 20%
Antibiotic	1 mL
Product soluble in water or isopropyl myristate	Whole contents of each container, not less than 200 mg
Insoluble products	Whole contents of each container, not less than 200 mg
Solids	
<50 mg	Whole contents
>50 mg, <300 mg	Half, but not less than 50 mg
>300 mg up to 5 g	150 mg
>5 g	500 mg

Removal of microorganisms from the product by elution and either filtration of or transfer of the removed microorganisms to culture conditions.

- Use elution techniques similar to those used in bioburden estimation
- Addition of a surfactant may be required to improve removal of organisms by moistening the product surfaces
- Membrane filter should be rated 0.45 μm
- Aseptically transfer filter to growth medium or use Steritest system and add media after filtration.

Generally, a single culture medium is used that is optimal for the culturing of aerobic and facultative microorganisms during radiation dose verification studies. Soybean-casein digest medium (tryptic soy broth, TSB) is commonly used and the test samples incubated at 30 to 35°C for 14 days. Samples should be checked daily and growth, if any, recorded. During validation of EtO processes or in conjunction with aseptic fill validations, sterility testing follows USP <71> requirements. For devices 40 product samples and 2 media are used: 20 products are immersed in TSB and incubated at 20 to 25°C and 20 products immersed in thioglucolate (THIO) and incubated at 30 to 35°C. These tests are both incubated for 14 days. To ensure the test results are not adversely affected by the product or any leachable substance from the product, a bacteriostasis/fungistasis test is performed by inoculating 10 to 100 selected organisms into test samples containing the product. Quantities for sterility testing of other types of medical products are listed below in Table 3 excerpted from USP <71>, Vol 30.

DEFINITION OF TERMS

The inactivation of microorganisms that occurs during a sterilization process can be described using the following terms:

D Value (decimal reduction value) is the time, or radiation dose, under a given set of sterilizing conditions required to kill 90% (or one log) of a homogenous microbial population (see example below).

Time minutes	Population at start of new minute	Population killed in one minute
First	1,000,000	900,000
Second	100,00	90,000
Third	10,000	9000
Fourth	1000	900
Fifth	100	90
Sixth	10	9

Table 4 F_0 Lethality Equivalents

Equivalent Minutes	
Temperature (°C)	At 121.1°C (F sub 0)
100	0.0077
110	0.079
115	0.251
116	0.316
117	0.398
118	0.501
119	0.631
120	0.794
121	0.977
121.1	1.0
122	1.23
123	1.59
124	2.0
125	2.82

F sub 0: In steam sterilization, the equivalent time in minutes (F value) to produce a given sterilization effect at 121.1°C (250°F) when $Z = 10$°C (18°F) and D value = 1 minute F_0 of 12 to 15 minutes is usually regarded as adequate (Table 4)

- 60′ at 115°C
- 15′ at 121°C
- 4′ at 127°C

SLR is the lethality observed in a full or fractional sterilization cycle. SLR can be calculated as the log of the initial population minus the log of the final population. SLR = $\log N_o - \log N_f$. If there are no survivors, the true SLR cannot be calculated. If one positive is assumed for the purposes of calculation, the SLR should be reported as "greater than." 10^{-6} for most terminally sterilized products.

SAL is the statistical probability that a microorganism will survive the sterilization process (see example below). At any givenminute, one log or 90% of the microbialpopulation is killed. Theoretically, complete kill is never achieved.

Organisms surviving at each minute	Time (min)	Logarithm survivors
1,000,000	0	
100,000	1	5
10,000	2	4
1000	3	3
100	4	2
10	5	1
1	6	0
0.1	7	−1
0.01	8	−2
0.001	9	−3
0.0001	10	−4
0.00001	11	−5
0.000001	12	−6

Most probable number (fractional negative) or Stumbo-Cochran-Murphy method is used to calculate the D value under specified conditions. At sterilization doses where a fraction of the samples may contain survivors, the most probable number (fractional negative) or

Stumbo-Cochran-Murphy method is used to calculate D value

$$D \text{ value} = \frac{U}{\text{Log}\, N_o - \text{Log}\, N_u}$$

where U is sterilant exposure time (or radiation dose); N_o, initial bacterial population; $N_u = 2.303$ (ln n/r); N, total number of tests; R, number of sterile tests.

For example, 20 BI test samples within load subjected to EtO sterilization dwell of 20 minutes. Eighteen sterility samples are negative. Use equation to determine D value and predict the dwell time required to produce a 10^{-6} SAL.

REVIEW OF STERILIZATION TECHNOLOGIES

Ethylene oxide is an organic compound with the formula C_2H_4O. This colorless flammable gas with a faintly sweet odor is the simplest epoxide, a three-membered ring consisting of two carbon and one oxygen atom. Ethylene oxide is an alkylating agent that disrupts the DNA of microorganisms, which prevents them from reproducing.

EO sterilization is a chemical process consisting of four primary variables: gas concentration, humidity, temperature, and time. The sterilization process consists of several distinct phases as described below.

Preconditioning—The Preliminary Portion of the Process

Humidity is the most complex and critical of the controllable variables. Levels of humidity above 30% have been shown to be necessary for effective EO sterilization. The purpose of prehumidification both prior to entrance into the sterilization chamber is to drive the moisture deep into and through the materials within the sterilization load The most commonly used level is 50% to 60% RH at 130°F. The effect of humidification of kill time is shown in Figure 3.

Conditioning

If used, in-chamber heating and humidification should be shown to achieve minimum required product humidity and temperature before the gas exposure time. It is important that the humidity be added before the gas, so that the moisture will be carried in front of and with the sterilant. This way, the moisture will be forced into the inner most areas of the products and will not be left behind by the faster-diffusing EO. Addition of steam during the conditioning phase can take place in several different ways (Fig. 4):

- Dynamic environmental conditioning (DEC)
- Incremental addition
- Pulsed addition

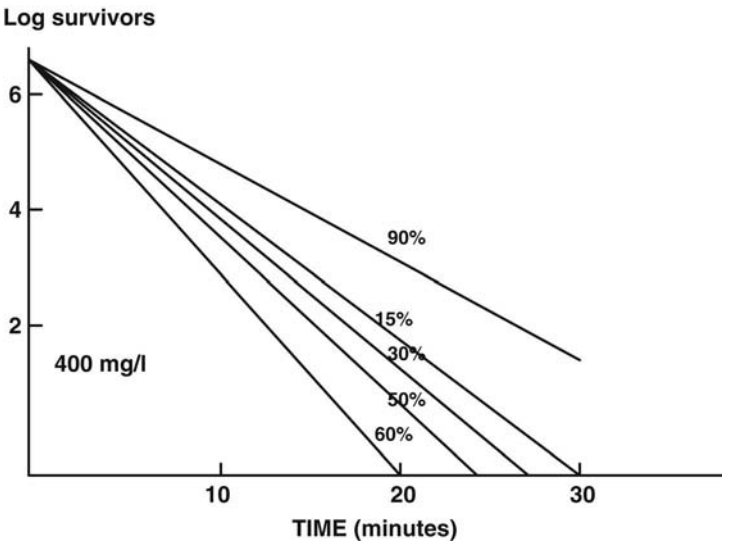

Figure 3 Effect of relative humidity of inactivation of *B. atropheous* spores. *Source*: Courtesy of PDA/DHI Publishing.

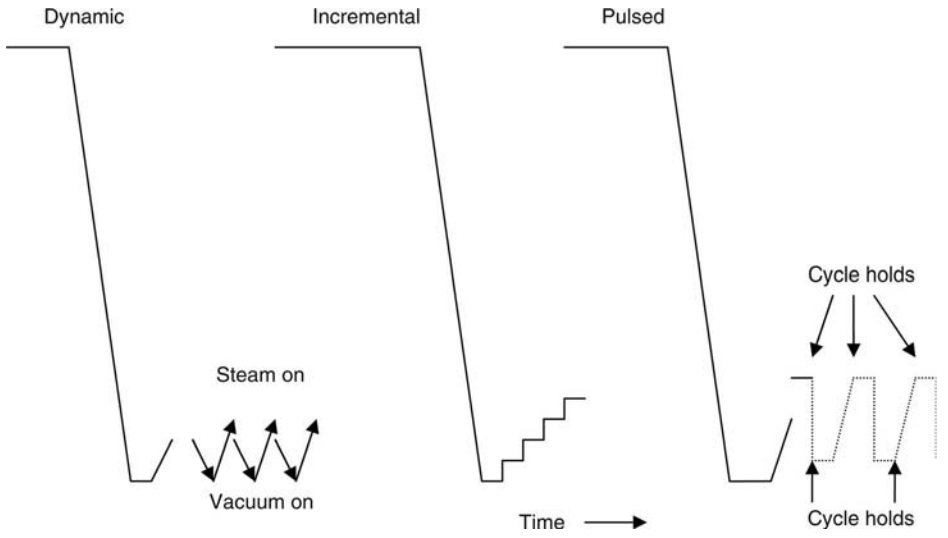

Figure 4 Three chamber conditioning methods for EO. *Source*: Courtesy of PDA/DHI Publishing.

The first method—DEC—consumes vast amounts of steam, which results in rapid and deep penetration of the steam into the product interiors. The second method—incremental—adds steam in increments until a preset vacuum point is reached. This can be repeated a number of times or can be the prelude to a static dwell period. The third method—pulsed—alternates between the deepest vacuum set point and steam rise set point, thereby pumping steam into packaging while maintaining the gentler dynamics. It can be used alone or with static dwell.

Sterilization—Addition of Ethylene Oxide

The temperature of the load influences microbial kill rate (Fig. 5). This effect is expressed as the Q_{10} value or the factor by which microbial death rates change as a result of a 10°C or 18°F change in temperature, and has been reported to range between 1.8 and 2.7 depending on the substrate. Thus for an 18°F difference below a designated sterilization temperature, the *D* value should approximately double. Thus, measures should be taken to minimize the temperature range within the sterilization load.

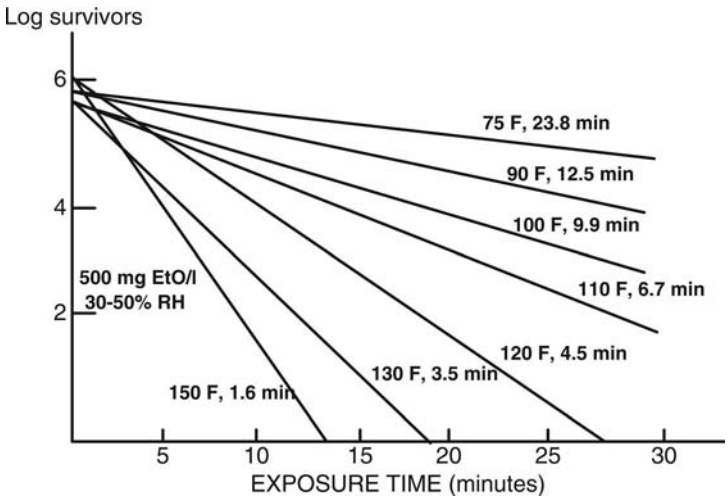

Figure 5 Effect of temperature on the *D* value of *Bacillus subtilis* var. *niger*. *Source*: From Ref. 5 (Courtesy of PDA/DHI Publishing).

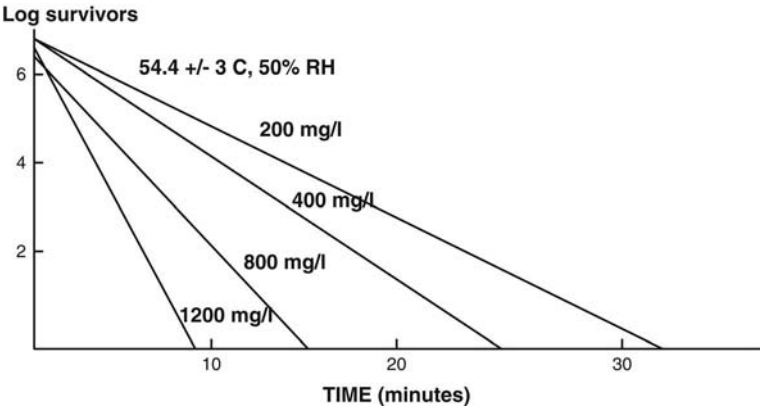

Figure 6 Effect of EO concentration on microbial inactivation rate.

The temperature range should be specified as the minimum range for routine sterilization. Product temperature should be measured during validation. It is common practice that the temperature range within a load during gas exposure be $\leq 10^{\circ}C$ ($18^{\circ}F$). If this cannot be achieved, a minimum temperature at the end of conditioning should be specified.

The sterilization chamber should have the following capabilities:

1. Independent systems for recording and controlling pressure, chamber temperature, and chamber humidity. Place at least one (2) probe at the coolest location,
2. Instrumentation for direct analysis of humidity during conditioning and EO concentration during exposure (parametric release requirement),
3. An adequate gas recirculation system to ensure uniformity of temperature, humidity, and gas concentration within the chamber,
4. Airflow detection alarms on the air recirculation system to ensure it operates within specification,
5. An instrument to monitor gas inlet temperature to ensure gaseous EO enters the chamber,
6. Recirculation system,
7. If software is used to run the cycle, it should be validated.

The EO concentration has a dramatic effect on microbial kill. As the EO concentration increases from 50 to 500 mg/L, there is a significant increase in the microbial death rate (Fig. 6). At concentrations above 800 mg/L, the rates do not increase significantly. Concentrations between 400 and 650 mg/L are recommended for effective microbial inactivation and more efficient gas removal from product at completion of the sterilant exposure. As EO is added to the chamber, it may be absorbed by the product and packaging materials in the load, and, subsequently, the pressure within the chamber will decline. Pressure can be maintained throughout the exposure phase by adding additional EO as the pressure drops. The use of inert gasses to maintain the pressure may result in reduction of EO concentration over time.

Aeration

Residuals of EO and its reaction products may be hazardous. Elevated temperature, dwell time, forced air circulation, and loading characteristics will all affect the rate at which gaseous EO diffuses out of the product load. Optimal aeration occurs at elevated temperatures in chambers or rooms (Fig. 7) with forced outside air circulation and product loading with adequate spacing between pallets. Also, some additional microbial kill can occur during aeration so it is recommended that the aeration time be minimized prior to removal of BI test samples during half-cycle performance runs.

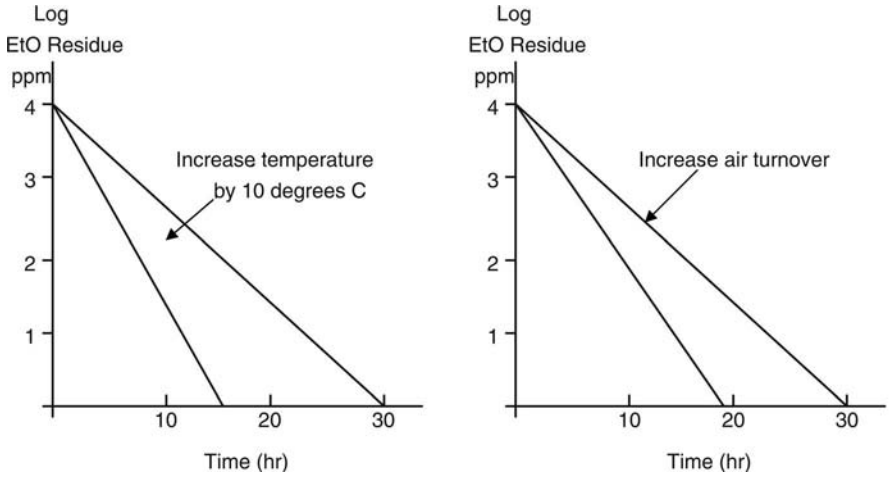

Figure 7 Effect of temperature and air exchange rates on reduction of EO.

The aeration areas should have the following capabilities:

- Airflow detection alarms or indicators on the air handling system to ensure continuous operation,
- Recirculation

Typical cycle parameters for an EO process is shown below:

Parameter	Fractional cycle	Full cycle
Preconditioning	90–125°F, 45–75% RH Minimum 18–20 hr	90–125°F, 45–75% RH 24–96 hr
Initial evacuation	2.0″ HgA ± 0.5″ HgA	2.0″ HgA ± 0.5" HgA
Humidity inject	Inject 1.0″ rise to 3.0″ HgA ± 0.5″ HgA	Inject 1.0″ rise to 3.0″ HgA ± 0.5″ HgA
Humidity dwell	40 min ±5 min	45 min −0, ±20 min
Gas inject	14.5″ HgA ± 0.5″ HgA	15″ HgA ± 0.5″ HgA
Nitrogen	27″ HgA ± 0.5″ HgA	27″ HgA ± 0.5″ HgA
Gas dwell	45 min −5, +0	4 hours, −0, +30 min
Evacuation	2.0″ HgA ± 0.5″ HgA	2.0″ HgA ± 0.5″ HgA
Nitrogen washes	HIGH 27.5″ HgA ± 0.5″ HgA	HIGH 27.5″ HgA ± 0.5″ HgA
(2 repeats)	LOW +2.0″ HgA ± 0.5″ HgA	LOW +2.0″ HgA ± 0.5″ HgA
Dwell temperature	120°F ± 5°F	125°F ± 5°F
Aeration	90–130°F ≥ 24 hr	90–130°F ≥ 24 hr

IRRADIATION TECHNOLOGIES

Two different types of irradiation processes are used in industrial radiation processing of medical products, that is, gamma rays and electron beam. A third type, X rays, have been shown to have microbicidal effects, but this method is not currently available for industrial sterilization. The microbial lethality of gamma rays and electrons is accomplished by ionization; electrons are direct ionizing radiation whereas photons are indirect ionizing radiation. The energy transferred by these radiations during the sterilization process produces chemical and/or physical changes at the molecular level resulting in chain scission, polymerization, cross-linking, sterilization, and disinfection.

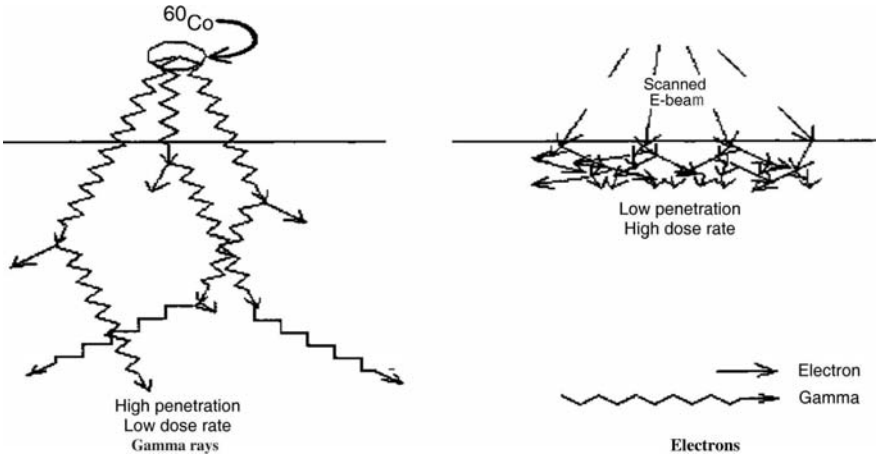

Figure 8 Penetration pattern of gamma and electron beam radiation.

PHYSICAL CHARACTERISTICS OF RADIATION

By far the most commonly used of the three methods is gamma radiation. Gamma rays are emitted from radioactive isotope source materials, the most common being cobalt 60 (^{60}Co). Gamma rays are electromagnetic waves frequently referred to as photons. Having no electric charge or mass, photons transfer energy to materials mainly through Compton scattering collisions with atomic electrons resulting in a uniform, exponentially decreasing depth dose distribution. The photon strikes free electrons in the material and pass part of their energy to the electron as kinetic energy. These displaced electrons continue on their way, deflected from their original path. The scattered gamma ray carries the balance of the energy as it moves off through the material, possibly to interact again with another electron. In the place of the incident photon, there are now a number of fast electrons and photons of reduced energy that may go on to take part in further reactions (Fig. 8).

It is the cascade of electrons that result in the physical and chemical changes in the material as well as the destruction of microorganisms. Because the probability of Compton scattering is low, the primary beam of gamma rays will penetrate long distances in material before the scattering occurs. This means that the gamma rays deposit energy over a relatively large area so that penetration is high (up to 50 cm) but the dose rate is low (Table 4).

By contrast to gamma, electrons focused into a beam generated by a linear accelerator with beam energies of 5 to 10 MeV have both mass and charge, so they interact readily with other charged particles, transferring their kinetic energy to materials by numerous elastic and inelastic collisions. In fact, as soon as charged particles penetrate solid materials, they are subject to the Coulomb force exerted by the atomic nuclei and are therefore in almost constant interaction with the material. These interactions result in many directional changes, ionizations, and radioactive processes that slow the electrons and ultimately limit their penetration to only 5 cm into material with a density of 1.0 g/cm^3 using a 10 MeV beam. E-beam energy is therefore deposited within materials over a short distance, making the dose rate very high (22,000 kGy/hr for a 50-kW beam) and allowing sterilization to take place in less than one minute.

The parameter measuring the energy transferred from the radiation source to the product is called the absorbed dose. The dose can be translated in terms of power requirements (i.e., intensity and energy of the beam) by taking into account the product characteristics (shape, size, and density) and the process parameters (i.e., throughput, scanning length). The penetration of gamma rays and electrons is inversely proportional to product density. The absorbed dose is the quantity of ionizing radiation energy imparted per unit mass of a specified material and is expressed as the gray (Gy) where 1 Gy = 100 rads or 1 kGy = 0.1 megarad. When a population of microbial cells is irradiated, the number of living units diminishes exponentially as the dose

increases, until no viable cells remain. Sterility is obtained in living organisms in two ways: directly through DNA strand rupture or through cell destruction related to chemical reactions in the organism or in its environment. Energy can be directly deposited in a bond of a macromolecule (protein, DNA, RNA) causing a rearrangement of its structure or free radicals generated from the water contained within the cell. The free radicals then react with the macromolecule altering its normal cellular metabolism that leads to loss of the reproductive capacity of the microorganism. In a nonaqueous environment as found in sterilization of most medical products, the principal sterilization mechanism is ionization of cellular material altering molecular structure or spatial configuration of biologically active molecules.

The parameters used to determine acceptable dose delivery of gamma sterilization are:

- Cycle time
- Product density
- Loading pattern
- Density mix

Process reliability and consistency are guaranteed by the well-known decay rate of the radioisotope. When the source and product are positioned correctly, small incremental changes are automatically programmed into the timer setting to account for the decay, thereby allowing products to be processed consistently. If product configurations remain the same as validated, the only difference in measured dose will be related to the variability in positioning product and uncertainties in dose measurement.

The parameters used to determine acceptable dose delivery of electron beam sterilization are:

- Beam energy
- Beam current
- Conveyor speed
- Scan width
- Product geometry
- Product density

Process reliability and consistency are guaranteed by control and monitoring of the beam, conveyor, and process parameters. Once parameters are established, products will receive the specified dose as long as product density, product packaging, and orientation are unchanged. The change from one product to another is relatively simple since the effect of the adjacent product are minimal.

STERILIZATION BY HEAT

Heat can be applied in either of the two forms: dry heat or moist heat. Dry heat kills the organisms by destructive oxidation of essential cell constituents. Inactivation of the most resistant spores by dry heat requires a temperature of about 160°C for 60 minutes. Dry heat is employed for glassware, syringes, metal instruments, and paper wrapped goods, which are not spoiled by high temperatures. It is also used for anhydrous fats, oils, and powders that are impermeable to moisture.

Moist heat kills organisms by coagulating and denaturing their enzymes and structural protein. Sterilization by moist heat of the most resistant spores generally requires 121°C for 15 to 30 minutes. Moist heat is used for the sterilization of culture media, and all other materials through which steam can penetrate. Moist heat is much more effective than dry heat. Sterilization can be done at lower temperatures in a given time at a shorter duration at the same temperature. Many sterilization cycles have been developed for use in a moist heat environment with calibrated equipment that has been properly installed and validated. Among the processes commonly used in industrial moist heat sterilization are the following:

1. Gravity air displacement: Sterilizers use gravity to remove air from their chambers. Steam introduced into the chamber creates a layer above the air, which increases until the air is pushed down through a drain at the bottom of the unit. After the air is

removed, steam temperature and pressure builds, and exposure time begins when the sterilization temperature is reached. Gravity sterilizers are used to sterilize surgical instrumentation, liquids, and linen.

2. Dynamic air removal (Prevac): This process is intended to sterilize products consisting of porous materials and/or items having cavities where air is difficult to remove. Prevacuum sterilizers use a pump to remove air from the chamber before steam is introduced. Dynamic air removal units are, therefore, more efficient than gravity air displacement sterilizers because air is pumped out before steam enters the chamber, so the steam can immediately penetrate packages.
3. Air pressure systems: Some product packaging cannot withstand the vapor pressure changes associated with moist heat sterilization. There are a number of available processes in which filtered compressed air is used to ensure that, for part or for the duration of the sterilization cycle, the pressure on the outside of the product equals or exceeds the inside pressure. These processes include cycles using air/steam mixtures, water spray, and water immersion.

Steam sterilization requires four critical parameters: steam, temperature, pressure, and time. Steam must be of high quality and contain no more than 3% moisture and a relative humidity (the amount of water vapor) of 97%. The appropriate temperature depends on the type of sterilizer being used. Gravity air displacement sterilizers require a temperature of 250°F (121°C). Dynamic air removal, washer sterilizers, and flash sterilizers require a temperature of 270–275°F (132–135°C). To achieve these temperatures, the pressure must reach 15 pounds per square inch (psi) for the 250°F (121°C) setting, and 27 psi to sterilize at 270°F. Note: Because the psi required to reach sterilization temperatures is related directly to the altitude, the exact psi required may vary slightly by geographical location. It is always best to consult the sterilizer's manufacturer for requirements in your area.

A typical steam cycle is outlined below:

Parameters for steam sterilization process (full cycle)

Phase	Parameter	Set point	Allowable tolerances
Pre-heat (Jacket temp. at 110°C)	Duration	35 min	±5 min
Conditioning	Vacuum pulses	3 with 6.50 psia delta	N/A
	Final pressure	2.0 psia reference (not a set point)	N/A
	Final temperature	114°C	±1°C
Steam injection	Rate (setting)	2°C/min after 112°C is reached	N/A
	Final pressure	31 psia (reference)	±3 psi
Exposure	Time	40 min	+1 min −0 min
	Temperature	122.0°C	+3.0°C −1.0°C
Exhaust	Exhaust rate	−3 psi/min	±.2 psi/min
	Final pressure	2 psia	±1 psi
Drying time (under vacuum)	Time	20 min	+5 min −0 min

VALIDATION TECHNIQUES

A combination of biological and physical methods can be used to determine the optimal sterilization parameters for moist steam and EO. But for either gamma or E-beam irradiation, only the bioburden method is used. The selection of the appropriate approach is based on the nature of the product, bioburden, and packaging, manufacturing conditions, and type of sterilization equipment.

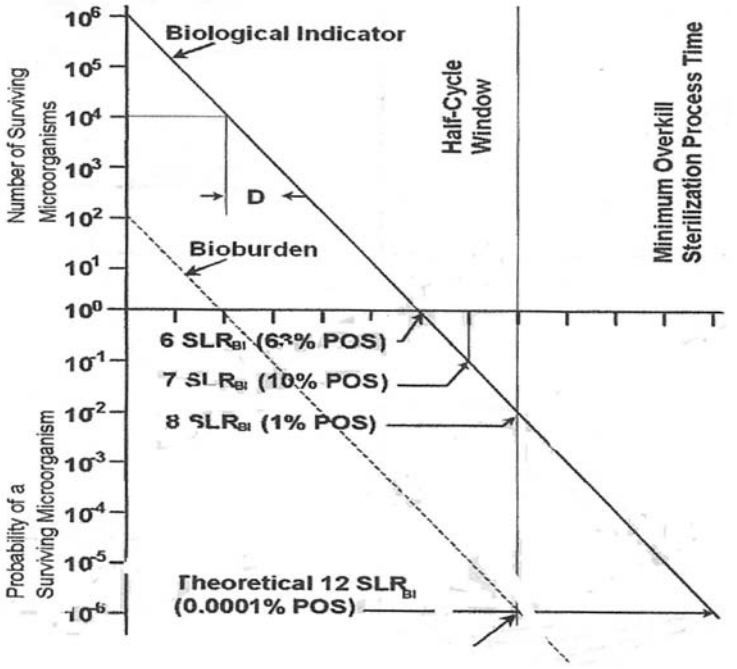

Figure 9 Example of an overkill cycle. *Source*: Adapted from ISO 11135.

Several methods can be used to develop effective cycles including:

- The overkill approach (Fig. 9) is the most widely used method for validation of EO or steam processes because it produces an overkill based on conditions causing inactivation of one million resistant bacterial spores that are more severe than those required to kill the bioburden. Three methods may be used in this approach:
  1. Minimally a 6-SLR at a half-cycle exposure time is demonstrated. This theoretically results in 10^0 survivors. When exposure is doubled, a 12 SLR is delivered and the product is considered sterile (has a sterility assurance level – SAL-of 10^{-6}).
  2. Use a BI that has a greater resistance than required by a smaller and less resistant microbial population (Fig. 10).

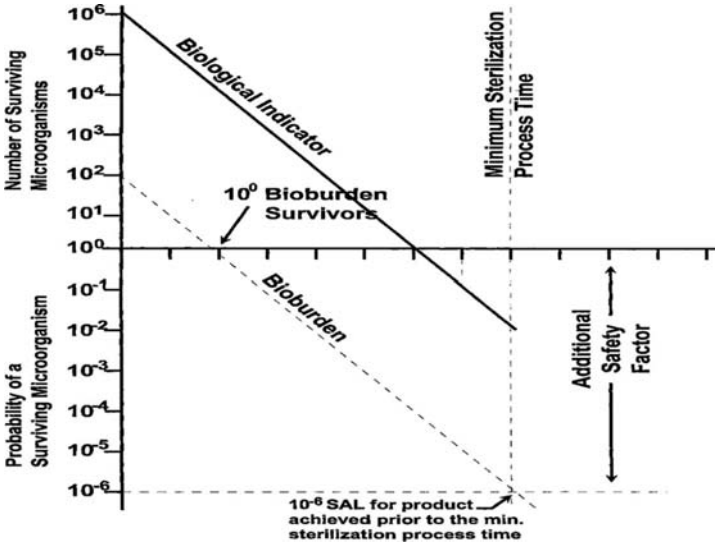

Figure 10 Relationship between bioburden and BI Survival.

For a steam process, this safety factor represents the inactivation of 12 logarithms of microorganism with a D121°C of approximately 1.0 minutes and a z value of 10°C. For challenge microorganisms having different D values, the population can be adjusted to achieve equivalent lethality; that is, the more resistant the challenge microorganism, the lower the population that can be used. For example, the challenge characteristics of a 10^3 population having a D value of 2.0 minutes, or a 10^5 population with a D value of 1.2 minutes are equivalent to those of a 10^6 population with a D value of 1.0 minute. It is important to remember that the D values and z values of microbiological challenges and product bioburden can vary in different environments (e.g., solutions and different manufacturing sites) and in different containers and closures.

- The bioburden approach—this approach is used to validate either gamma or E-beam radiation processes. Sometimes natural bioburden may have a resistance greater than the BI system because of very high bioburden levels, high bioburden resistance to the sterilant, or the location of the bioburden on/in the product. Representative product samples should be subjected to incremental exposures (doses), sterility tested, or enumerated to generate a kill curve. Sometimes products may have very low bioburden or be made of temperature sensitive materials. Being able to validate a shorter exposure cycle or dose should be beneficial since the bioburden is usually much less resistant than a BI. Very tight control of bioburden is necessary for this approach.
- A combination BI/bioburden approach—this approach is used when sufficient bioburden data is available to demonstrate that a BI challenge lower than 10^6 per carrier can be used. This method usually results in shorter cycle times and is gaining acceptance in efforts to optimize cycles.

VALIDATION OF A STEAM OR EO CYCLE

The sterilization validation program is conducted to demonstrate that the designed process can reproducibly sterilize specified products or product families to a defined SAL without damage to the product or package. The overkill method is commonly used. The BI for steam is usually 10^5 to 10^6 of heat-resistant spores of *Geobacillus stearothermophilus* with a D value of 1 to 1.5 minutes deposited on a carrier material or inoculated into a liquid-filled vial. For EO, the BI is a 10^6 population of *Bacillus aetrophaeus* spores with a minimum D value of three minutes inoculated on a carrier material, such as a filter strip, thread, or suture. A six (6) SLR of 10^6 BIs at a half-exposure cycle time is demonstrated, which correlates to 10^0 survivors. When doubled, the exposure time delivers a 12 SLR and an SAL of 10^{-6}. In addition, the process must demonstrate a microbial SAL appropriate for the product being sterilized. If products will be sold in Europe, an SAL of 10^{-6} is required for all products labeled "sterile." In the United States, some products that contact uncompromised tissue, such as drapes and gowns, can be validated to an SAL of 10^{-3}.

For efficient and cost-effective validation performance, prior product and process evaluation is suggested. If your company produces a wide range of sterile products, similar products can be grouped into families. A family of products can be considered to be all those products of similar design and materials of construction, but consisting of different sizes, that is, all Foley catheters, sized 8 French to 16 French, and similar bioburden levels. After family groups are determined, select the most difficult-to-sterilize representative product in the family to represent all the products in the group. Generally this product will have the highest and most resistant bioburden population (radiation) or have the most challenging design configuration and packaging that renders permeation a gas and steam into the product (EO and steam). If your evaluation results in multiple product families, it is advisable to select from the representative products, a single most-difficult-to-sterilize product that will be used as the master process challenge device (PCD).

SELECTION OF FAMILY REPRESENTATIVE

Each family of products will contain a number of products. From these products, the representative challenge product is selected. The selected product then will be the most

difficult to sterilize product in the family group and will be used in verification dose experiments. A simulated product not intended for sale can be used as long as it is made of similar materials and uses similar manufacturing processes as the actual product. The establishment and continued validity of the sterilization dose are related to both numbers and resistances of organisms on or in the product. This is the basic characteristic used to select the representative product. Other criteria that should be considered by a knowledgeable person to select the challenge product are:

- Number of microorganisms
- Types of microorganisms
- Size of product
- Number of components
- Complexity of product
- Degree of automation during manufacture (manually assembled products will generally have higher bioburden levels)
- Manufacturing environment

Modifications to products, such as raw materials, components or product design, changes to the manufacturing process, facility or environment should be formally evaluated and documented to assess their effects on bioburden levels and dose validation. Bioburden data should be collected on an establish timeframe for all products within the family to ensure that the selected representative product continues to be the most difficult to sterilize item in the group.

PREPARE THE PCDS BY PLACING THE BI STRIP (OR DOT OR THREAD) WITHIN THE PRODUCT

The BI should not occlude any passageway or limit the diffusion of the gas, but should be placed in the most interior location. Sometimes a simulated PCD is used, for example, a long length of tubing can be cut in half, the BI placed in a plastic connector and the two lengths of tubing attached to the connector. When making the simulated carrier be sure to seal the cut edges with adhesive to ensure that the gas cannot penetrate through the cut. This is usual done when the BI cannot physically be placed within the finished product. In steam validation, a small volume (10 μm) of a liquid suspension containing the challenge organism can be directly inoculated onto or in products. Sometimes the BI is placed within a product during manufacturing process before the product manufacturing process is completed. BIs of different physical shapes, including strips, dots, or threads, can be obtained from manufacturers to facilitate placement into small spaces.

The basic elements of the validation program are described below.

1. Installation qualification (commissioning): Equipment-oriented evaluation consisting of establishing and implementing the ancillary equipment programs and documenting the equipment present. The system must be defined by the operator (contract sterilizer or in-house) of the equipment and reviewed by the manufacturer.

   The minimum documentation required should include

   - As-built drawings and blueprints of the equipment and facility including sterilizer, all processing equipment, precondition rooms, aeration rooms and any ancillary systems for air, steam, EO, and water;
   - model and serial numbers of all individual components including gauges, timers, etc.;
   - calibration of all instrumentation used for monitoring, controlling, and recording;
   - operating instructions/procedures, calibration, and preventive maintenance procedures in place;
   - piping and electrical schematics and drawings;
   - copy of computer software and its validation;
   - utilities including adequacy and proper operation, proper materials of construction, sufficiency of supply, presence and location of filters, absence of dead legs in water and steam supply;

- safety procedures;
- operating procedures for all the equipment;
- step-by-step operating instructions.

2. Operation qualification: Cycle exposure tests necessary to assure the equipment operates as designed and is capable of delivering the specified process within tolerances. The tests are conducted without product.
3. Performance qualification: A series of planned runs with product, microbial challenge (PCD), and measurement of load temperatures (RH in EO) that confirm that the cycle parameters from the cycle development program produce the required microbial lethality and do not compromise product or package functionality. Any change to loading pattern, packaging, equipment or process parameters, or on the addition of new or altered products shall result in an evaluation of their effect on the validation. Loading patterns shall be specified and a representative load based on the most difficult to sterilize load shall be used. During design of a steam cycle, F_0 calculations can be considered because lethality is occurring at temperatures above 100°C (Table 4).

At a minimum, the following runs shall be performed in an EO validation only:

1. Fractional cycle: A minimum of one (1) run with all critical parameters at a minimum and the gas exposure time set at 1/4 or 1/6 of the predicted full cycle exposure time. This cycle should contain high bioburden product(s) of product family representatives and BIs placed within the process challenge device(s) (PCD) to demonstrate that the resistance of the product bioburden is less than or equal to that of the BI.
   - Sterility tests results should indicate total kill of product bioburden and survival of some or all of the BIs. If product bioburden is not entirely inactivated, but fewer tests are positive compared to the BI, the run is acceptable. Another fractional cycle with increased gas exposure time can be performed to demonstrate total kill of the bioburden.
   - An external PCD can also be included to determine the relationship to the internal PCD. If the BI growth from the external BI is equal to or greater than the BI growth from the internal PCD, then the external PCD can be used for monitoring routine loads.
2. Half cycles in both steam and EO: A minimum of three (3) consecutive acceptable runs with all critical parameters at a minimum and the dwell time set at 1/2 of the predicted full cycle time.
   - All BIs placed within the PCD shall be inactivated in these cycles. All process parameters shall operate with defined specifications and tolerances. If unacceptable data from any of the three (3) runs is found (e.g., BI positive, cycle parameter not met), an investigation is performed. A cause related to cycle lethality may result in restarting the validation.
   - BIs in the external PCD used only in EO can be all negative or some positive since this BI is merely an indicator of lethality and is shown to be more difficult than internal PCD.
3. Full cycles: A minimum of one (1), but three (3) suggested, with all critical parameters at nominal settings. One (1) additional run may be performed with critical parameters set at maximum for evaluation of product residuals and/or functionality.
   - Product samples are evaluated for residuals and functionality; packaging for maintenance of sterile barrier.
   - EO residuals are evaluated in one (1) or more products for EO and ECH.
   - If more than one resterilization is contemplated, some product samples can be exposed to more than one full cycle to ensure functionality when resterilized.
4. Certification: Formal review of the data and documentation with an approval by the appropriate organizations within the company (final report).

   The PQ shall confirm for an EO validation:

   a. At the end of preconditioning, the load is within the temperature and humidity ranges document in protocol cycle parameters;

b. The maximum transfer time of the load from preconditioning to the sterilization chamber is not exceeded;
c. Gaseous EO has been admitted to the chamber (pressure rise, loss of weight of the gas cylinders);
d. Quantity of gas used is within the specified range;
e. All BIs are inactivated in the half cycles
f. The temperature and humidity in the chamber, and other process parameters, are within ranges documented in the protocol;
g. During aeration, the load is within specified range.

The PQ shall confirm for a steam validation:

a. All BIs are inactivated in the half cycle
b. Product and packaging remain functional after a full cycle
c. Dry time is sufficient

5. Requalification timeframes: A statement on the frequency of the requalification; the industry average is annually.
   - It is acceptable to perform a paper work review at the first annual term. If this review shows that no changes have been made to the cycle or product that the cycles run during the previous year were without major deviations and the contractors equipment testing is acceptable, the cycle can be considered requalified upon acceptance of review documentation.
   - At the next annual review, a minimum of one (1) half cycle should be run under protocol to document the continued process lethality.

VALIDATION OF AN IRRADIATION PROCESS

Four approaches to selection of the dose can be used depending on the batch size and product bioburden level:

1. Method 1: Determination of the bioburden then used to select and test a 10^{-2} verification dose based on population C,
2. Method 2A and 2B: Incremental dosing of product samples,
3. Method VD_{max}: Substantiation of 25 kGy as a sterilization dose; appropriate for products with <1000 colony forming units (CFU)/product.

GROUPING INTO PRODUCT FAMILIES

Product families for radiation processing are based on bioburden. Bioburden histories for individual products should be maintained over time. In addition, assessment of individual products and their similarities should be considered as well as the impact of the variables shown below on the bioburden. Document the review and the rationale for placement of products into families and create a final family listing including the product name and catalog (part) number. This can become part of the protocol or incorporated into a standard operating procedure (SOP). Additional sterilization doses at 15, 17.5, 20.0, 22.5, 27.5, 30.0, and 32.5 kGy can be validated as outlined in AAMI TIR 33: 2005 (5).

After evaluation of bioburden populations, examples of product-related variables to consider are:

- Raw materials
- Components
- Product design and size
- Manufacturing process
- Manufacturing equipment
- Manufacturing environment
- Manufacturing location

DOSE SETTING USING METHOD 1 (BIOBURDEN METHOD)

The methods of selection of the sterilization dose use data derived from the inactivation of the microbial population in its natural state and are based on a probability model for the inactivation of microbial populations. The selection depends up experimental verification that the response to radiation of the product bioburden is greater than that of a microbial population having a standard resistance. Using computational methods and the standard distribution of resistances (SDR) shown below, individual doses required to achieve stipulated SALs have been calculated for levels of bioburden on product just prior to irradiation. These values are the basis of the dose table documented in ISO 11137-2:2006 (6).

Standard Distribution of Resistances D_{10} Values

D_{10} kGy	1	1.5	2	2.5	2.8	3.1	3.4	3.7	4	4.2
Probability	0.6549	0.2249	0.063	0.0318	0.0121	0.0079	0.0035	0.0011	0.0007	0.00007

This method depends on experimental verification that the response to radiation of the product bioburden is equal to or less than that based on historical data of microbial population having a standard resistance. In other words, the probability model used to develop Table 5 in ANSI/AAMI/ISO 11137-2:2006 assumes that the in situ bioburden is a mixture of homogeneous populations, each having its own unique susceptibility to radiation and its own rate of inactivation (Fig. 11), which presents a lesser challenge than the model. Testing is performed at a dose calculated to give an SAL of 10^{-2}. This is called the verification dose and represents the probability that a unit of product contains one or more viable organisms. Sterility testing of products subjected to the verification dose should produce 1% positives. If a larger than expected number of units test positive, then either the resistance of the bioburden is higher than expected or the bioburden has been underestimated. Method 1 is preferred in most situations because of its reasonable cost and study time. Sample requirements initially total 136 (100 for the dose experiment, 30 for bioburden determination, and 6 for bacteriostasis/fungistasis testing) and 110 (100 for the dose experiment and 10 for bioburden determination) thereafter on each quarterly dose audit.

The sequence of steps required to validate a radiation process using method 1 as follows:

1. Select the appropriate SAL and obtain samples of product units.
2. Determine the bioburden levels using 10 final packaged products from 3 different batches. Apply correction factor. Even though validation of bioburden recovery

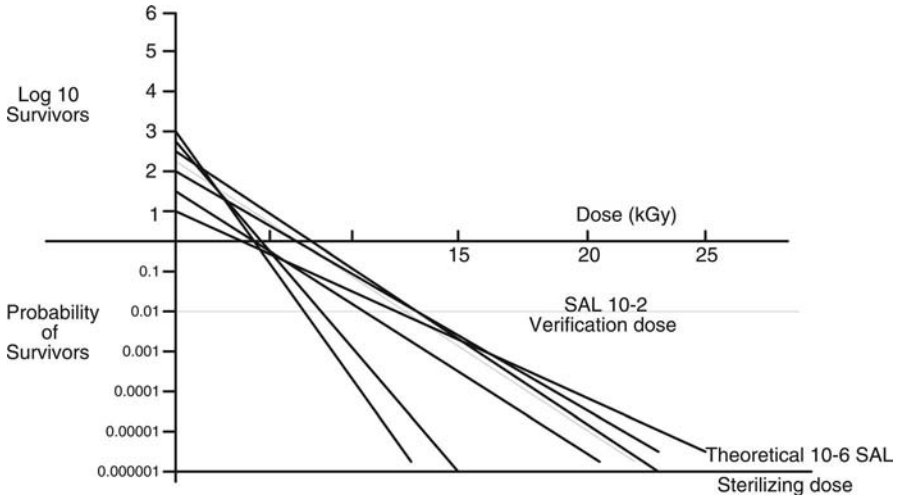

Figure 11 Theoretical survivor curves for method 1 population (1000 CFU). *Source*: Courtesy of PDA/DHI Publishing.

Table 5 Material Penetration Depth of Three Types of Radiation

		Penetration (cm)	
Type	Source	Irradiating 1 side	Irradiating 2 sides
Gamma	CO^{60}	10.2	40.6
X ray	50 KeV	<0.1	0.5
	10 MeV	12.7	61
E-beam	5 MeV	1.8	4.3
	10 MeV	3.8	8.6

method is not required, it is recommended to have a better understanding of the actual numbers of organisms that will be subjected to the verification dose.

3. Determine the batch average of each of the three batches.
4. Calculate the overall batch average.
5. Select the verification dose from dose Table 5 of ISO 11137-2 using either the highest batch average (if one or more batch average is greater than the overall batch average) or the overall batch average.
6. Perform the verification dose using 100 final packaged products from a single batch. The samples can be selected from any of the three batches from which the bioburden samples were taken or from a fourth batch. Send the packaged samples to the irradiator and indicate the purpose and the dose. The actual dose delivered can vary by +10%. If the dose does not meet the specification, do not proceed to the sterility test. Repeat the verification dose using fresh samples.
7. Sterility test the 100 units by incubating the dosed products in soybean/casein broth at 30°C ± 2°C for 14 days. Bacteriostasis/fungistasis testing should also be performed if this is the first time the product has been subjected to a sterility test.
8. Review results to assess the acceptability of the experiments:
   - 1 or 2 positive tests = acceptable.
   - >2 positives with no deviations in the testing or dose delivery = dose method is not valid for the product and the alternative method should be used (method 2)
9. Establish sterilization dose if test is acceptable by finding the closest bioburden number in dose Table 5 equal to or greater than the average bioburden and the selected SAL level.

Recently, a new validation approach called VD_{max} was developed. This method based on the SDR of the method 1 population can be used for any size production batches with average bioburden of less than 1000 CFU per product. The method preserves the conservative aspects of the resistance characteristics of the SDR, but is more accurate for low bioburden products. It is not limited to batch size or production frequency and the number of product samples (10) needed for the verification experiment is constant. The VD_{max} method can be used for selected sterilization dose of 15 and 25 kGy as outlined in ISO 11137-2:2006, *Sterilization of health care products—Radiation—Part 2: Establishing the sterilization dose.*

The following steps are followed for substantiation of a 25 kGy sterilization dose:

1. Obtain at least 10 product units from each of three production batches immediately prior to sterilization.
2. Determine the average bioburden on each product as outlined in ISO 11737-1:2006 and average the bioburden values for each batch. Apply the correction factor on the basis of the validation of bioburden recovery. Compare the three batch averages and select the grand average or one average if two or more times the overall average.
3. Obtain verification dose. Find the closest bioburden value greater than or equal to the average in Table 9 in ISO 11137-2. Obtain the corresponding verification dose.
4. Irradiate 10 product units from a single batch at the VD_{max} obtained in Table 9. These may be selected from any one of the bioburden batches or a fourth batch. The actual

dose may vary from the calculated dose by not more than +10%. If the delivered dose is less than 90% of the verification dose, the experiment may be repeated.

5. Sterility test the product units according to ISO 11737-2 (7) using soybean-casein digest broth incubated at 30 ± 2°C for 14 days. Record the number of positive tests.
6. Interpretation of results. If no more than one positive test is observed in the 10 tests, 25 kGy is substantiated as the sterilization dose to achieve at least a 10^{-6} SAL. If 2+/10 tests are observed, a confirmatory verification dose experiment shall be conducted. If 3+/10 tests are observed, 25 kGy is NOT substantiated and another dose setting method must be used.
7. Confirmatory verification dose experiment (if required)

   Randomly select 10 product units from a single batch (can be from the batches previously sampled or from a new batch). Use the same dose as determined initially and irradiate the 10 product units at the confirmatory verification dose. The same dose tolerances apply. Sterility testing results are evaluated as follows:
   - 0+/10–25 kGy is substantiated
   - 1−10+/10–25 kGy is not substantiated.

ROUTINE MONITORING FOR EO AND STEAM

After successful completion of the sterilization validation, a process specification must be written, which explains the proper procedures to be followed routinely. The process specification must describe the aspects of the sterilization process necessary to assure conformance with the validated cycle and be maintained with an established change control procedure. All specified process parameter minimum values must be met or product cannot be released as sterile regardless of the microbial test results. All BIs must test sterile for the indicator organism and results of product sample (if used) testing must be acceptable.

The process specification should include

1. identity of equipment qualified for sterilization;
2. list of the items approved for sterilization in the process covered by the specification, that is, the product listing;
3. written procedures for sterilization process operations, or reference to specific operator manuals;
4. sterilizer loading configurations and pallet patterns (EO);
5. descriptions and diagram of the placement of BIs and other test samples;
6. list of all process parameters with set points and minimums and maximum tolerances, and reference to the recording and controlling instruments for each;
7. requirements for routine quality control tests and periodic audits related to sterilization;
8. written criteria for sterile product acceptance, reprocessing, rejection, and release for distribution, including instructions for selection, handling, and testing of samples.

Routinely, the process is monitored with the same resistant BI used to qualify the cycle. Routine use of product sterility testing is not required. A minimum number of BIs must be included in each cycle. The recommended number for EO is based on the load volume as defined in ISO 11135-2:2006. If an external PCD has been validated, no internal BIs are required. EtO cycles can be validated for parametric release as outlined in AAMI TIR 20: 2001. (8).

The quality function is usually responsible for reviewing sterilization documentation. Even if the process is performed by a contractor, the manufacturer is responsible for assuring that the appropriate cycle was performed and that the cycle parameters were within acceptable tolerances established during the validation, as follows:

1. Minimum product temperature was met before entering preconditioning
2. Temperature and humidity in preconditioning met specification
3. Transfer time from preconditioning to the sterilization chamber
4. Temperature and pressure throughout the cycle
5. Secondary record of gas admission to the chamber (usually cylinder weight)
6. Exposure time

7. BIs used were sterile and within expiration date
8. Time and temperature within aeration room

Failure to meet the physical specification or BI sterility should result in quarantine of the sterilization load and in an investigation. The investigation should be documented. If the physical process variables are below the minimum tolerances of the specification or growth of the test organisms is observed, the sterilization load should not be released; product should be either resterilized or scrapped. If one (1) or more of the BIs test positive and the growth is identified as the indicator organism, an investigation should be performed and the load resterilized. If the BI on a validated full routine cycle tests positive, then a major problem has occurred that just be identified and rectified.

ROUTINE MONITORING FOR RADIATION

After successful completion of the irradiation sterilization validation, a process specification must be written, which explains the proper procedures to be followed routinely. The process specification must describe the aspects of the sterilization process necessary to assure conformance with the validated dose and dose mapping and be maintained with an established change control procedure. All specified process parameter values must be met or product cannot be released as sterile. The process specification should include

1. identity of radiation modality qualified for sterilization;
2. list of the items approved for sterilization in the process covered by the specification, that is, the product listing;
3. the maximum dose allowed and the sterilization dose;
4. written procedures for sterilization process operations, or reference to specific operator manuals;
5. sterilizer tote loading configurations and dose mapping showing relationship between the reference point and the maximum and minimum dose positions;
6. descriptions and diagram of the placement of dosimeters and other test samples;
7. specified minimum dose and minimums and maximum tolerances, and reference to the dosimeter system used routinely;
8. requirements for routine quality control tests and periodic audits related to sterilization;
9. written criteria for sterile product acceptance, reprocessing, rejection, and release for distribution, including instructions for selection, handling and testing of samples.

Failure to meet the physical specification should result in quarantine of the sterilization load and in an investigation. The investigation should be documented. If the delivered dose is below the validated dose, the sterilization load should not be released; product should be either resterilized or scrapped. Since radiation effects on materials are cumulative, any decision to resterilize must be based on acceptable product aging test data after multiple sterilizations. Process interruptions or delays should be evaluated to determine the effect on the microbiological quality of the product and on the dosimetry systems.

In addition, to ensure the numbers and resistance of the bioburden remains steady, a verification dose audit is preformed each quarter. This is essentially a repeat of the initial validation dose experiment but only 10 bioburden samples are pulled from a single lot. The result of the bioburden test is for information only because an additional 10 products (for VDmax) or 100 products (Method 1) are dosed at the original validated verification dose no matter the current bioburden levels. If audit results fail ($>1+/10$ or $>2+/100$) augmentation or revalidation is required.

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9 Steam sterilization

James Agalloco

INTRODUCTION

Moist heat sterilization in an autoclave is certainly the most widely utilized means of sterilization within the healthcare industry. It may also be the oldest method in use with the first practical sterilizer dating to the late 19th century (1). The vast industry experience with steam sterilization has resulted in the development of a variety of steam processes adapted for specific applications. In each of these, the sterilization process is accomplished by the presence of liquid water on the surface of the microorganism at elevated temperatures (2). The liquid water is necessary for coagulation of proteins within the microbe that result in its death and is implicit in "moist heat" sterilization.[a] In steam processes, the liquid water can be provided by saturated steam in contact with the surface (as utilized in hard goods sterilization) or via water content in a liquid filled container (as in media preparation or terminal sterilization of finished products). The requirement for liquid water must not be overlooked; sterilization with superheated steam (where no liquid is present) has far more in common with dry heat processes than it does with moist heat. A graph of the saturation curve for steam is shown in Figure 1.

Moist heat sterilization can be accomplished along the saturation curve itself where water is present both as liquid and as a gas (steam), or in the liquid region (above the saturation curve) where the pressure exceeds saturation and only the liquid phase is present. Sterilization with saturated steam is preferable to water, due to the additional heat available when the saturated steam condenses and releases its heat of condensation.[b] The rapid transfer of heat to the items to be sterilized by condensing steam is essential to rapid destruction of microorganisms and a major reason why moist heat is preferred over other sterilization methods.

MICROBIOLOGY OF STERILIZATION

The death of microorganisms by all sterilization methods shares a common phenomenon (3). The log number of surviving microbes when plotted against the exposure time provides an essentially straight line (Fig. 2).

The steeper the slope of this line, the less resistant the organism is to sterilization process. The inverse of this slope is known as the *D*-value and is usually expressed in minutes. The *D*-value can be influenced by a number of factors aside from the microbial identity including recovery media, age of the microbe, recovery methods, substrate on which the microbe is exposed, etc. (2). *D*-values are determined through the use of a specially designed sterilized vessel called a biological indicator evaluation resistometer (BIER) (4). BIER vessels provide precise control over exposure temperature and process time to allow for determination of the microbial death curve. Bioburden microorganisms are destroyed far more rapidly than the moist heat-resistant spore formers customarily utilized as biological indicators (Fig. 3). Nearly all sterilization processes are validated using biological indicator with higher resistance whose death in the validation exercise provides added assurance that the bioburden microorganisms with lower resistance are destroyed in routine usage of the sterilizer (when the biological indicator is not present).

The preferred biological indicators for steam sterilization are spore-forming microorganisms from the *Bacilli* or *Clostridia* sp. whose resistance is several orders of magnitude higher than vegetative cells under the typical moist heat process conditions. The body of

[a]Sterilization in the absence of liquid water requires substantially higher temperatures (typically >150°C) and impacts the microorganisms differently.

[b]A 1°C drop in temperature for 1 g of liquid water releases 1 calorie. The condensation of 1 g of saturated steam to liquid water at 121°C releases approximately 525 calories.

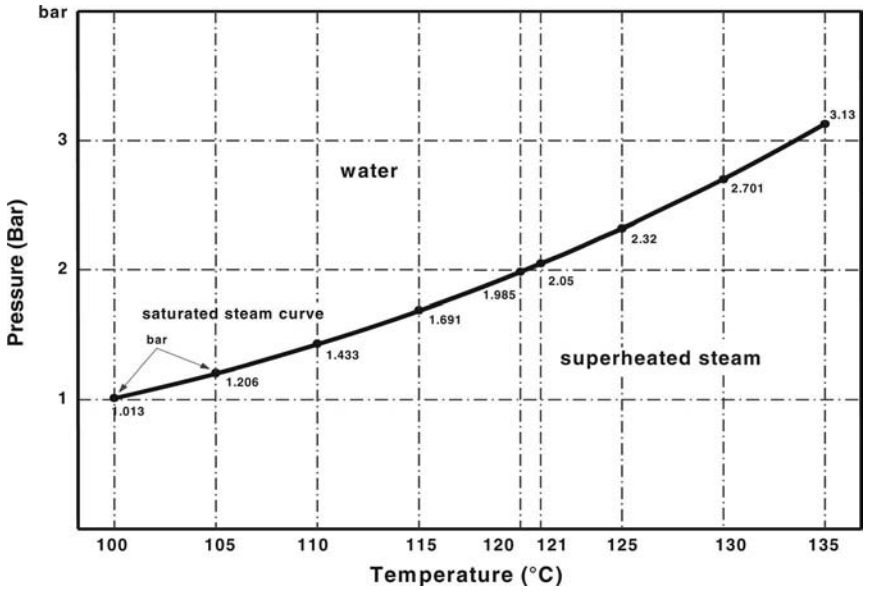

Figure 1 Saturated steam curve. *Source*: Courtesy of Fedegari Autoclavi.

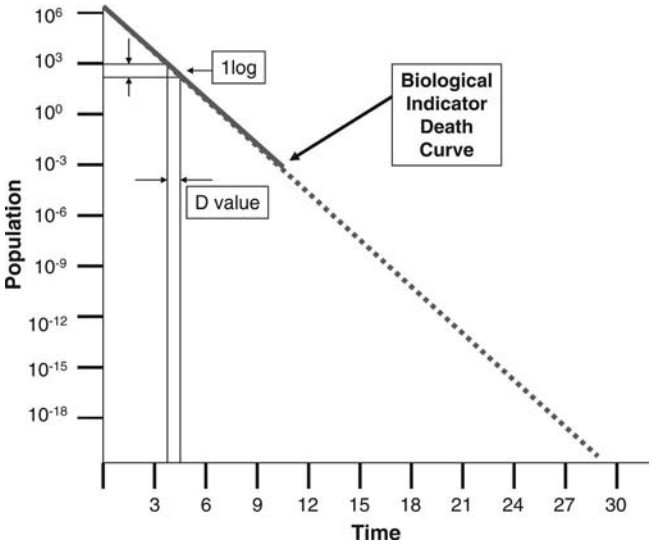

Figure 2 *D*-value.

knowledge regarding steam sterilization and consistency of the results is such that mathematical correlations between the physical and microbial data are utilized to provide appropriate process control (2). These correlations are necessary to assure product safety (sterility) to the desired level. A closer look at Figure 3 provides some clarification as to why these correlations are necessary. In each death curve, determining the number of microorganisms is only possible when there are viable microorganisms present (the solid portion of the biological indicator death curve). The dotted line portion of the death curve represents the probability of surviving microbes where their number is too low to count. Where the desired level of confidence in the sterilization process on the vertical scale intersects the death curve defines the minimum process time required (Fig. 4).

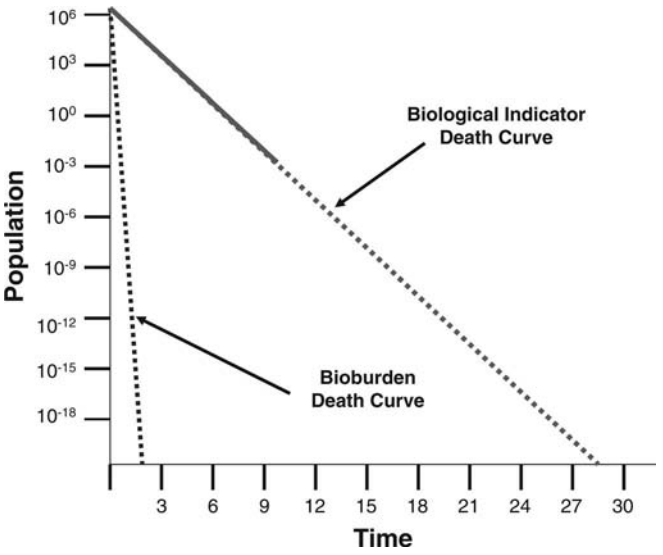

Figure 3 Microbial death curves—relative resistance of bioindicator and bioburden organisms.

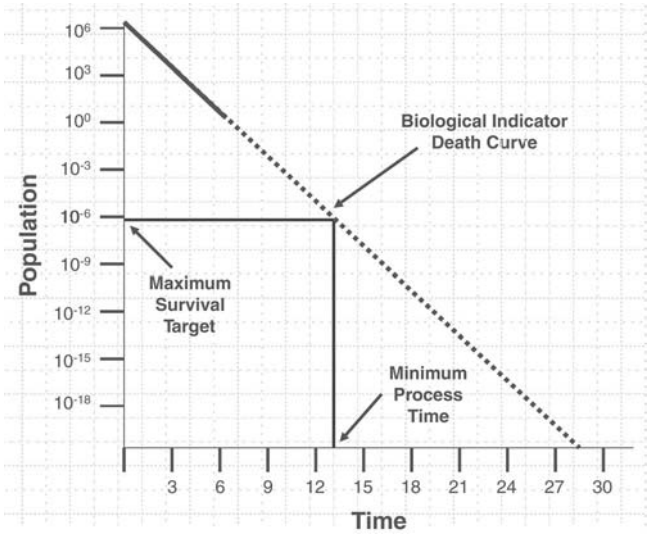

Figure 4 Setting process target for 12 log reduction of biological indicator population.

Were the process to be operated at precisely the conditions where the *D*-value has been determined, initial validation and day-to-day process control would be greatly simplified. Unfortunately, it is extremely difficult to provide essentially constant process conditions in routine sterilization process on a commercial scale. There are many real world factors that prevent constant conditions in production settings including chamber size, load size, item complexity, item wrapping materials, and item orientation. To accommodate these elements, a means for relating physical conditions at varying temperatures to microbial destruction is necessary. The *D*-value, which is essentially the rate at which microorganisms are killed, is largely a function of the temperature—the higher the process temperature, the more rapid their destruction. The general method for sterilization process evaluation uses this temperature dependency to allow for the estimation of the lethal effect on microbes at a range of temperatures near the *D*-value (5,6). A plot of the *D*-value against process temperature provides a straight line for many organisms and the slope of the line is termed the *z*-value (Fig. 5).

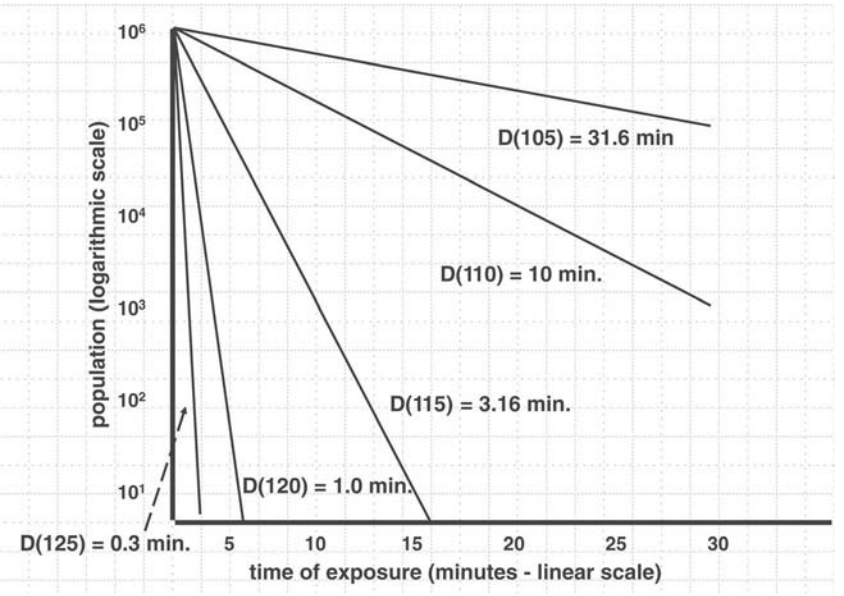

Figure 5 Effect of temperature on *D*-value.

The general method estimates the lethality over the process duration by calculating the kill rate for microorganisms as the temperature progresses through the sterilization cycle. The shorter the time interval at which the temperature is measured the more accurate the estimate will be (Fig. 6).

To compare the effectiveness of sterilization processes, a standard means of lethality estimation was defined. When first developed, the concern was for food safety and the survival of *Clostridium botulinum* in canned foods. A process temperature of 250°F was found to be effective for this process, and this condition was established as the standard base temperature for estimation of sterilization process lethality. The Celsius temperature scale equivalent of 250°F is 121.1111...11°C.[c] To calculate lethality relative to a base temperature a correction is applied employing the *z*-value. At 121.1°C (250°F) a *z*-value of 10°C (18°F) is customarily assumed.[d] One minute at this temperature has been defined as F_0. Simple mathematics can be utilized to calculate estimated lethality at other temperatures using the lethality equation. For the specific reference temperature of 121°C and a *z*-value of 10.0°C, the lethal-rate equation is:

$$L = 10^{\frac{T-121.1°C}{10.0°C}}.$$

Summing the instantaneous lethality over the sterilization process allows for the calculation of the overall process lethality delivered at those varying conditions. Table 1 indicates how this might be determined for an arbitrary steam sterilization process. The accumulation of lethality is only possible when the steam is saturated. For terminal sterilization, this occurs throughout the process. For parts sterilization it begins during the come-up and ends when evacuation of the chamber begins at the conclusion of the dwell period. It is customary to only consider lethality contributions at temperatures above 100°C because the contribution below that temperature is miniscule (7).

[c]For the sake of convenience, the temperature value is truncated to 121.1°C, but 121°C or even 120°C could be used, allowing for easier calculation, albeit with slightly different values. The arbitrary nature of the lethality standard must be recognized, given that the original choice of 250°F was equally arbitrary.

[d]The *z* value chosen 10°C is another arbitrary choice that facilitates calculation. The z values for moist heat resistance biological indicators are typically between 7 and 12 minutes at 121°C.

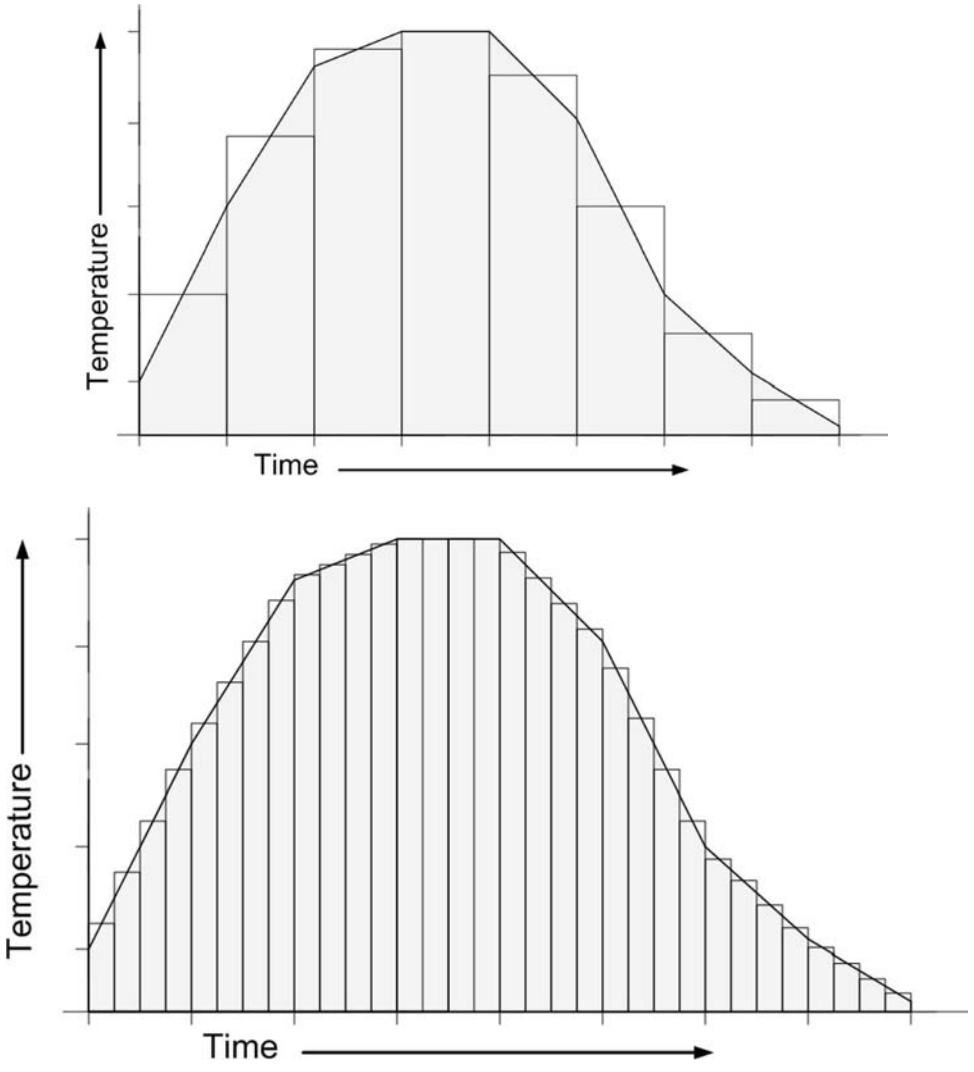

Figure 6 F_0 graphical calculation.

Table 1 Calculating Process Lethality

Calculating lethality $L = 10^{\frac{(T_0 - T_b)}{z}}$				
T	$T - T_b$	$\frac{T - T_b}{z}$	Incremental lethality	Cumulative lethality
101	−20	−2	0.01	0.01
111	−10	−1	0.1	0.11
121	0	0	1	1.11
124	3	0.3	1.995	3.006
131	10	1	10	13.006
121	0	0	1	14.006
111	−10	−1	0.1	14.106

$T_b = 121^\circ C$, $z = 10^\circ C$, $\Sigma L = F_0$.

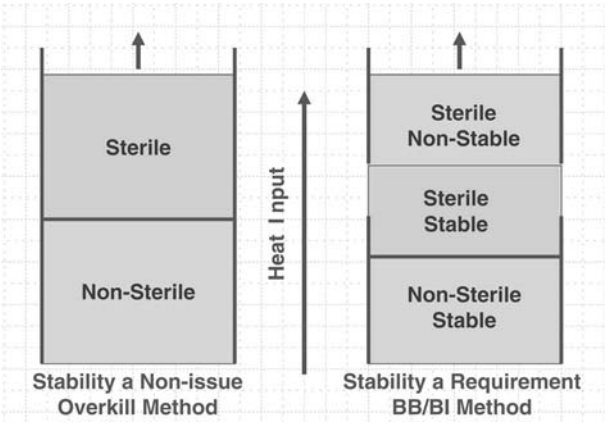

Figure 7 Comparison of parts and terminal sterilization.

STERILIZATION AND MATERIALS

The effects of the sterilization process must be considered on more than just the microorganisms present on or in the materials. Sterilization processes by their very nature incorporate conditions that have a deleterious impact on the materials being sterilized. The extreme temperature and moisture conditions that are microbiologically lethal can readily alter the chemical and physical properties of many materials. In the sterilization of stainless steel, glass or most other equipment items the adverse material effect is minor or nonexistent. The time-temperature conditions required for microbial inactivation can be substantially exceeded without concern for the material consequences. This can be compared to jumping over a hurdle; clearing the hurdle by an excessive height has no measurable adverse effect. Where the items being sterilized are subject to damage by the sterilization process, an upper limit to the process should also be defined. This might be considered equivalent to jumping through an open window (Fig. 7). Maximum conditions that must not be exceeded if material properties are not to be altered should be defined in addition to those required minimally to achieve sterilization. Having upper and lower constraints on the process may require changes in the process design, process equipment, and validation methodology. Each of these will be addressed later in this chapter.

The division between the simpler process requirements necessary for heat stable items and the more complex needs of materials that are potentially affected by heat has resulted in increasing differences in the sterilization cycles. Loads of heat-resistant items are typically called hard good loads or porous item loads in Europe and parts loads in North America. The items in these loads are sterilized by direct steam contact with the items on the surface. The removal of air (and condensate) from the sterilizer chamber assures a more consistent process across the chamber. For items that are sensitive to excessive heat such as finished products in their final product containers, in-process solutions, and laboratory media, care must be taken to avoid overprocessing. The internal water content of the materials in the containers provides the means for sterilization of the contents. The heat necessary to reach sterilizing conditions is provided by heat sources external to the product container by steam, steam-air mixtures, steam-water-air mixtures, or superheated water. In these systems the removal of air (and condensate) from the chamber may not be necessary. Loads for liquid filled containers are termed nonporous in Europe. Consistent with the differences in the sterilization cycle being performed, the sterilizer equipment may be adapted to better suit the specific process being executed.

STEAM STERILIZATION CYCLES AND EQUIPMENT

The first steam sterilizers originally built in the later 1800s were relatively simple pieces of equipment in which saturated steam was supplied to a pressure vessel (1). Air and condensate (formed by the steam as it heated the load items) left the chamber at the bottom through the actions of gravity (cold air and condensate are both denser than hot steam). This type of

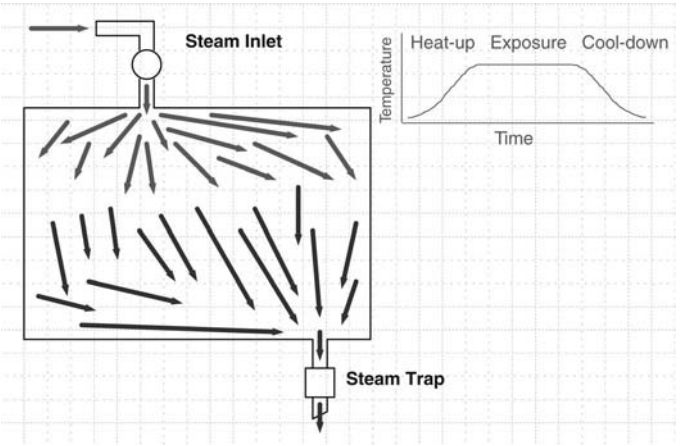

Figure 8 Gravity displacement cycle.

process was termed a gravity displacement cycle and is still in common use in smaller sterilizers utilized in medical and dental offices. The cycle profile is shown in Figure 8 and shows a slow ramp-up of temperature to the desired process condition.

One of the primary limitations of the gravity cycle is the time required for steam to penetrate all of the loads items. If sufficient time is not allowed during the dwell portion of the cycle, residual air and/or condensate could be retained that might prevent the cycle from achieving sterility of the load items. This cycle can be performed in the simplest of sterilizer designs: a simple chamber with a steam inlet at the top and a drain at the bottom.

The prevacuum cycle was developed as a means for improving process efficiency and effectiveness through the mechanical removal of air and condensate. Initially, only single prevacuum was drawn on the chamber, but it was quickly recognized that multiple alternating vacuum and steam pulses would be substantially more effective (Fig. 9).

Adapting the sterilizer design to accommodate the prevacuum cycles requires only the addition of a vacuum pump to the chamber (Fig. 10). The vacuum pump provides a means for improved drying of load items post exposure by lowering of the chamber pressure aiding in steam and condensate removal by evaporation.

The gravity displacement and multiple prevacuum cycles are commonly utilized for sterilization of hard goods, and while they can be utilized for the more complex processes needed for liquid filled containers, sterilizer designs specifically intended for nonporous loads are employed where those processes are in routine use. Steam-air (Fig. 11) and steam-water-air (Fig. 12) sterilizers are in common use for liquid containers in the pharmaceutical industry. The water utilized in steam-water-air units is customarily sterilized with the load, so its initial microbial quality is of little consequence. Air utilized for overpressure (or for breaking of vacuums at the conclusion of post-cycle drying) is 0.2 μm filtered just prior to entering the chamber. The cycle performed in these units are somewhat similar, and many employ an increase in air pressure during the latter stages of the cycle to prevent container damage due to pressure differences between the container interior and exterior (Fig. 13).

Firms producing larger volumes may employ continuous sterilizers in which a belt system moves containers through heating and cooling chamber in series. These types of designs are also commonplace in the food industry for canned goods. Immersion sterilizers where the load is sterilized by superheated water are utilized for smaller volumes in the food industry, but have not seen widespread use in the global healthcare industry.

Terminal sterilization is frequently associated with parametric release, especially for those firms that produce large volume parenterals. Parametric release replaces the end-product sterility test with controls that focus on successful execution of the sterilization process within restrictive requirements derived from the validation effort. Parametric release requirements are defined by the regulators to assure that the firm's product release approach adequately assures patient safety (8–11).

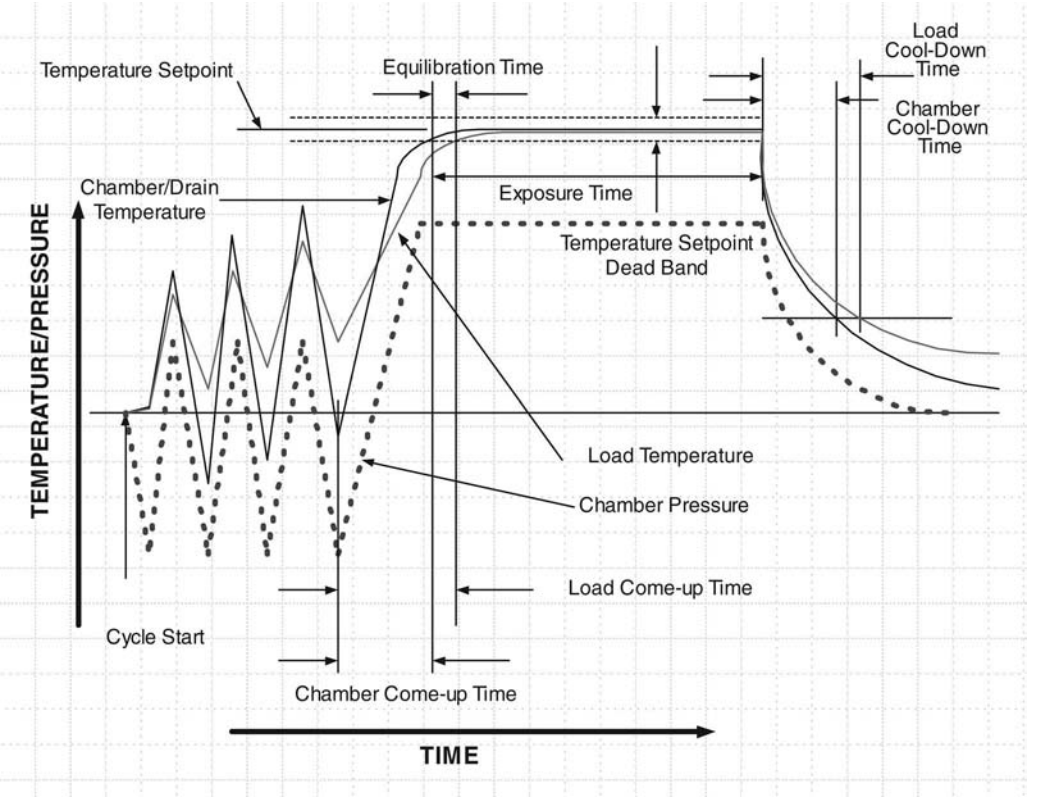

Figure 9 Multiple prevacuum cycle.

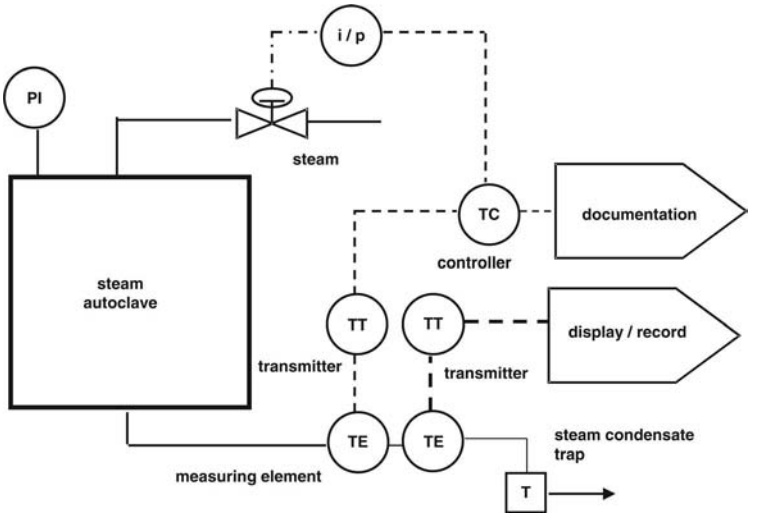

Figure 10 Typical sterilizer with vacuum pump.

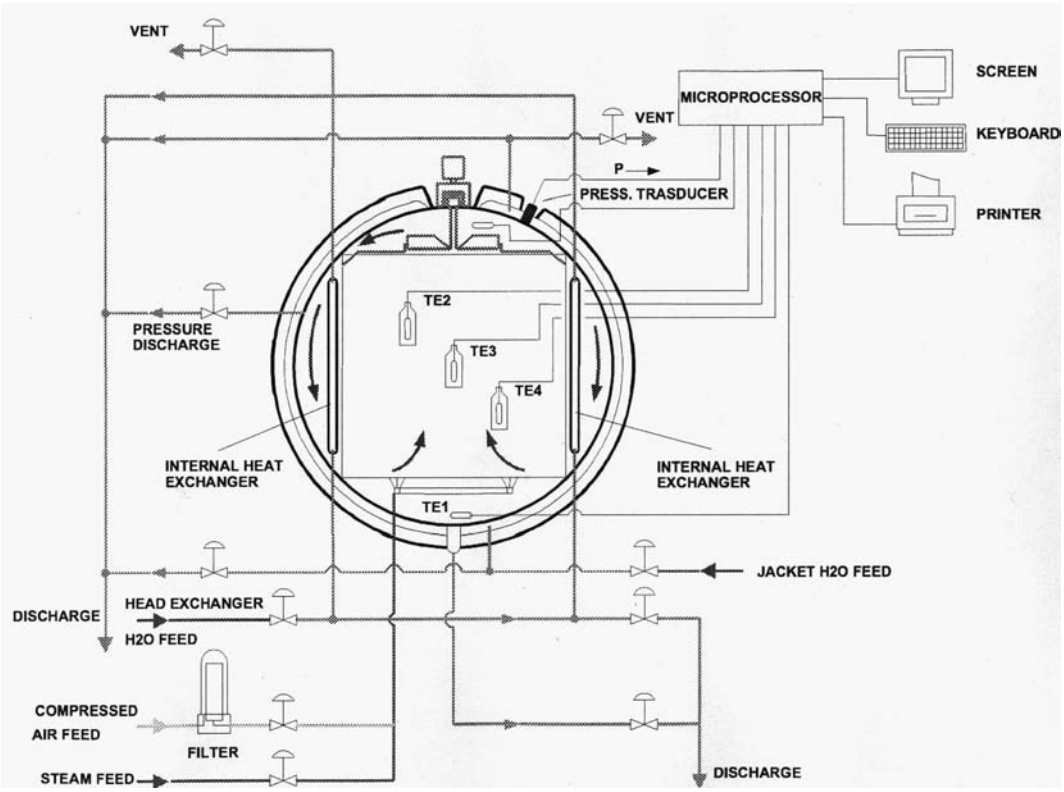

Figure 11 Steam-air sterilizer. *Source*: Courtesy of Fedegari Autoclavi.

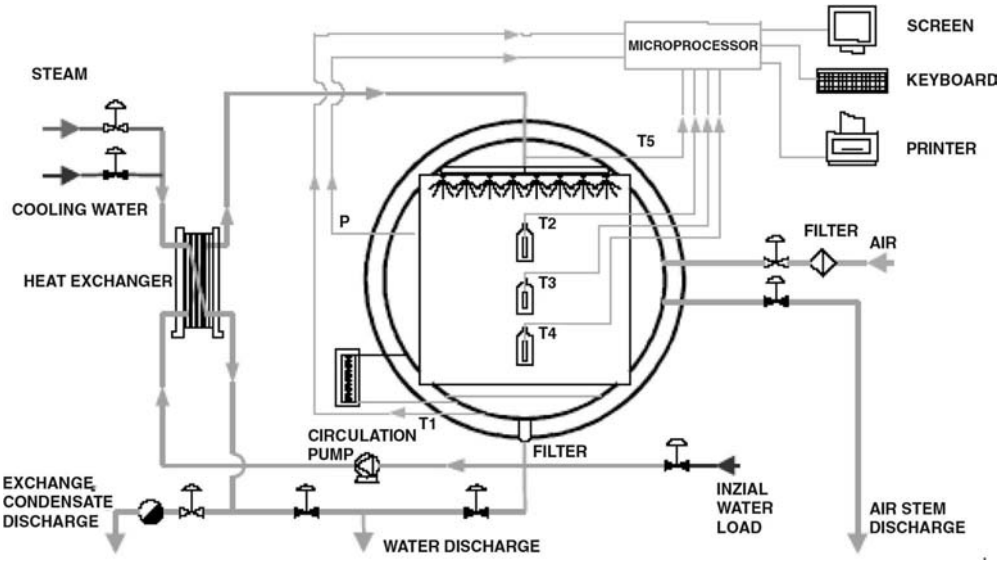

Figure 12 Steam air-water sterilizer. *Source*: Courtesy of Fedegari Autoclavi.

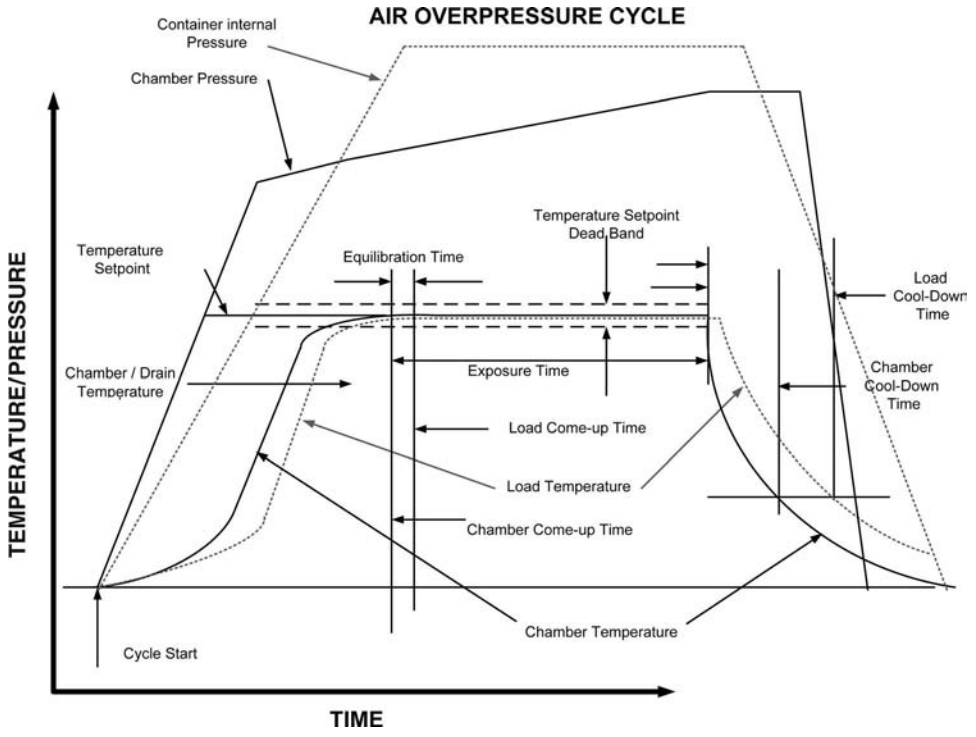

Figure 13 Air overpressure cycle.

APPROACHES TO STERILIZATION CYCLE DEVELOPMENT AND VALIDATION

There are three methods for the design/development and validation of a steam sterilization process, and it is essential the same approach be utilized for both activities (7). The different approaches exist in large part because of the differences in heat resistance of the items being sterilized. The overkill approach is the simplest, and inherent in its selection is the recognition that the load items will be subjected to a larger amount of heat than with the other methods (12). The bioburden approach requires the most effort initially and on an ongoing basis, but subjects the materials to the least amount of heat.[e] The bioburden/biological indicator [BB/BI or combination] method falls between these extremes with regard to both ease of development/validation and the amount of heat applied to the materials. Figure 14 provides a pictorial representation of how the various sterilization validation approaches compare.

The choice of sterilization approach is largely defined by the types of items being sterilized. While the overkill method is always the method of choice due to its relative ease of use, the BB/BI may be more appropriate for heat sensitive materials. The terminal sterilization of liquids in their final containers follows the BB/BI approach as it results in shorter cycles more conducive to maintaining product shelf life. Hard goods by virtue of their heat stability are almost always validated using the overkill method.

Overkill Method

This method despite its almost universal usage across the industry suffers from a lack of clarity. There are a number of conflicting definitions for this method. A recent definition was

[e]The bioburden method is little used for steam sterilization in industrial settings, as the bioburden/biological indicator method is substantially easier to manage and subjects the materials to only slightly more heat input.

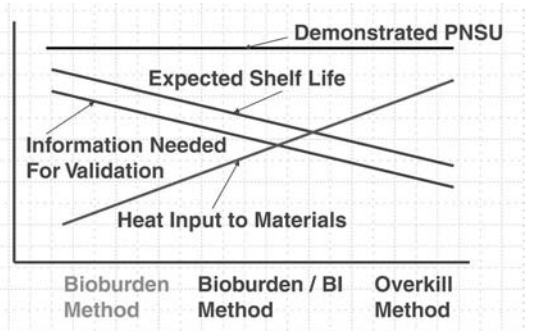

Figure 14 Sterilization approaches.

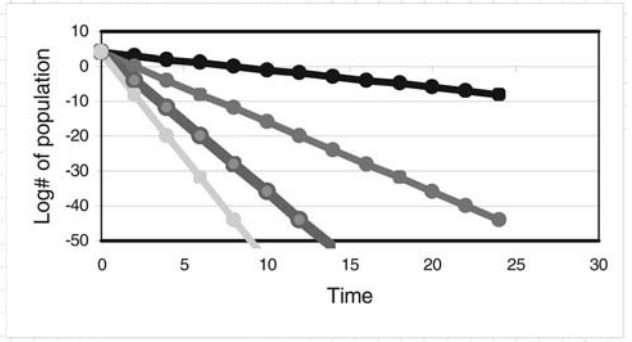

Figure 15 *(See color insert)* Overkill approach.

developed with the goal of reaffirming the original intent of the overkill treatment (overwhelming destruction of the bioburden):

> "Overkill sterilization is a process where the destruction of a high concentration of a resistant microorganism supports the elimination of bioburden that might be present in routine processing. That objective can be demonstrated by attaining any of the following: a defined minimum F_0; a defined time-temperature condition or a defined log reduction of a biological indicator." (12)

The overkill approach relies on the substantial difference in the relative resistance of biological indicator as compared to the bioburden (as is the case for the other methods as well). Figure 15 shows how this might be accomplished in a real world validation study.

The universal assumption made when using the overkill method is that any bioburden present will have substantially less resistance than the biological indicator, and that destruction of the large numbers of the resistant indicator organism (customarily replicate studies with multiple biological indicators with a population of 10^4 or more per strip) supports a greater reduction (1,000,000 or more times given the differences in relative resistance) of the bioburden. Cycle times are established by estimating the time required to inactivate the bioindicator (typically 8–9 times its *D*-value) and adjusting the cycle dwell time accordingly. The biological indicator is placed within the load items at locations expected to be slow to heat, that is, center of filters, inside tubing, etc. In this approach, it is required that all of the indicators are killed during the cycle. Since the number of biological challenges placed in the load is at least 10, a 6 log reduction in the microbial population is obtained when all of the indicators are dead when a minimum population of 10^4 spores is used and greater reduction can be achieved with a higher challenge level. The kill of the biological indicator demonstrates a greater than 6 log reduction of spores of *Geobacillus stearothermophilus*. The reduction in bioburden population assuming a biological indicator D_{121} value of 1 minute and D_{121} value of the bioburden of 0.00001 minutes would be 100,000 times greater.

Clearly, this approach provides levels of sterility assurance for bioburden organisms that provide substantial confidence in the effectiveness of the cycle. The bioburden organisms are killed in such excess that the process is deemed an "overkill" process. Given this degree of lethality that is provided by the process, little consideration is given to identification, quantification, or resistance determination for the bioburden. Some inspectors have made absence of information on the bioburden an issue, but there is little rationale for this concern.

Bioburden/Biological Indicator Method

The BB/BI method also relies on the differences in relative resistance of the bioburden and biological indicator. Destroying even a modest population of the biological indicator requires heat input to the materials that may alter its properties adversely. The enormous difference in relative resistance means that a robust process can be defined in which the biological indicator used has a lower population or is not fully inactivated, but still confidently supports bioburden death in all instances.

The BB/BI process requires information on the population and moist heat resistance of the bioburden and ongoing monitoring/control over the bioburden (Figs. 16 and 17).

In this model, the initial biological indicator population of 10^6 is reduced over an 8-minute cycle to a population of 10^2 (a 4 log reduction). Over the same 8-minute period, the bioburden population is reduced from 10^4 to 10^{-12} (a 16 log reduction). In this example, the biological indicator D_{121} is 2 minutes while the bioburden D_{121} is 0.5 minutes (a resistance and population much higher than might be anticipated in a typical pharmaceutical solution). The only difficulty with this experiment is that the biological indicator population must be accurately determined at the 8-minute time interval.

The second example has an initial biological indicator population of 10^2 that is reduced over the same 8-minute cycle to a population of 10^{-2} (also a 4 log reduction). Over that same 8-minute period the bioburden population is reduced from 10^4 to 10^{-12} (a 16 log reduction). In this example, the biological indicator D_{121} is 2 minutes, while the bioburden D_{121} is 0.5 minutes (a resistance and population much higher than might be anticipated in a typical

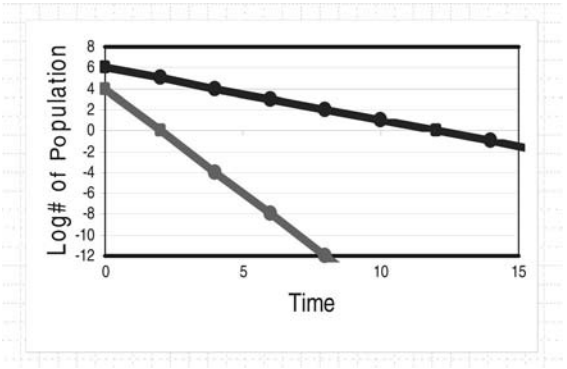

Figure 16 *(See color insert)* BB/BI method with survival count.

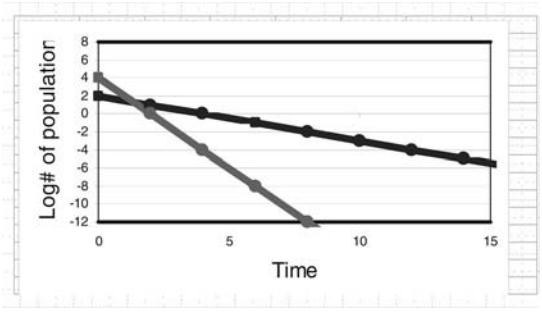

Figure 17 *(See color insert)* BB/BI method with total BI kill.

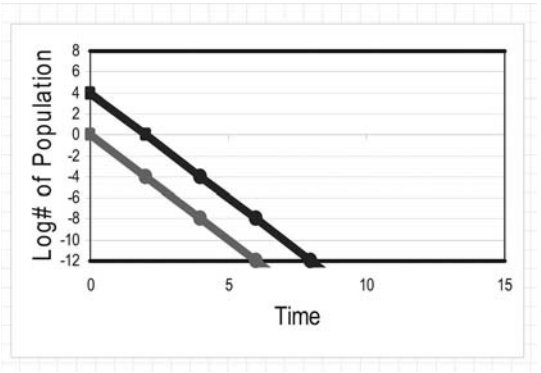

Figure 18 *(See color insert)* Bioburden approach.

pharmaceutical solution). In this experiment, the biological indicator is fully inactivated in the 8-minute process dwell easing execution of the study.

Bioburden Sterilization

For bioburden sterilization, a "worst case" bioburden isolate is utilized as the biological indicator. It requires knowledge of the bioburden present in every lot produced. Initial screening of the bioburden is made to identify the most resistant strain of microorganism present. Once determined this organism is used as the biological indicator, following methods similar to that described in the preceding text for the BB/BI method. If the proper organism is initially selected, then its destruction in the process confirms that any of the other bioburden organisms, presumably of lesser resistance and lower number [the chosen organism should be cultivated such that when used as a challenge the number present will be substantially higher than the lot bioburden] (Fig. 18). Monitoring of the bioburden for each load is required to confirm that the population and resistance have not increased to the point where the cycle is no longer effective. Inherent in the use of this method are defined limits for the bioburden number and resistance applied to each lot prior to sterilization. Any lot not meeting the limits cannot be accepted as adequately sterilized by the process cycle.

Obviously, a bioburden cycle will require substantially less time at temperature to inactivate the typical organism, which might be used as the challenge. It appears to be the ideal choice for the terminal sterilization of filled containers as a consequence of the reduced heat input that the filled units must receive to inactivate the nonspore forming organism used in this method. Less heat required to achieve sterilization should mean that products sterilized using this approach will have greater chemical stability post process as a result when compared to the same product sterilized by the other methods. This advantage is largely offset by the intensive bioburden monitoring required, initially to establish and on an ongoing basis to maintain confidence in the sterilization process. Appealing as this process might appear, it is the least widely used of all approaches because of the extensive microbial testing support required.[f]

QUALIFICATION/VALIDATION OF STEAM STERILIZATION

The terminal sterilization of liquid-filled LVP containers was perhaps the first process subjected to validation in the pharmaceutical industry. Global practices for all validation activities have their roots in the early 1970s, when microbial contamination in LVPs in the United States and hospital infections in the United Kingdom led to the introduction of a regulatory expectation for sterilization validation (13). The speed with which sterilization validation was introduced into the global industry led to some unfortunate simplifying assumptions that have had long-term consequence. Sterilization processes of all types, but

[f]The bioburden method forms the basis for all forms of radiation sterilization, and only occasionally with other sterilization methods.

most importantly in the context of this chapter, parts sterilization had their validation requirements defined by the more rigorous requirements of terminal sterilization. Much of what is considered essential for parts sterilization has never been evaluated objectively against the simpler needs of their sterilization.

The first efforts to codify the requirements of a validation program were found in the U.S. FDAs Proposed Good Manufacturing Practices for Large Volume Parenterals (14). An essentially parallel, but quite differently focused effort in the United Kingdom resulted in HTM-10, which did not appear in print until 1980 (15). These documents were the first regulatory efforts to outline validation practice for moist heat sterilization. Soon after FDA published its proposed regulation, PDA (at that time a predominantly U.S. Association) developed Technical Monograph #1, Validation of Steam Sterilization (16). PDA's effort focused on FDA's proposed regulation and thus the common practices of the U.S. LVP industry formed the basis for the steam sterilization validation across U.S. firms. The PDA's document relied heavily on biological indicators as the principal means to establish sterilization cycle efficacy. The practices outlined focus on biological challenges using resistant microorganisms as the most appropriate means to establish cycle effectiveness.

By the time UK's HTM-10 appeared, the global pharmaceutical industry had already begun to adopt practices following PDA's Monograph. It was not until the formation of the EU along with the emergence of ISO standards and establishment of EMEA that the precepts of HTM-10 were brought into greater prominence. The original HTM-10 and its many derivative standards have a completely different focus to steam sterilization validation that what was derived from FDA expectations and PDA's initial efforts (17,18). These standards place substantially greater emphasis on physical measurements of process parameters, especially those that relate to steam quality and equilibration time. The points of contention between European regulatory expectations and U.S. style validation practices persist; practitioners are forced to satisfy regulatory communities with quite different perspectives (19,20).

Over the years the differences in validation emphasis have endured to the point where the validation of steam sterilization, especially as it relates to parts sterilization, is one of the more contentious subjects within the global healthcare industry. The chapter will review the areas of agreement and difference with respect to the validation of both terminal and parts sterilization.

EXECUTION OF PERFORMANCE QUALIFICATION STUDIES

The validation of any process commences with the qualification of the process equipment and steam sterilization is no exception. This is a subject that has been treated extensively in the literature and is largely without any confusion or contention. The reader is encouraged to follow the well-documented practices in this area (21).

Empty Chamber Studies

Performance qualification of steam sterilization ordinarily begins with evaluation of empty chamber temperature distribution. This entails the placement of thermocouples (type T thermocouples are most commonly used) across the chamber, with the most important locations in the eight corners of the autoclave chamber, and at the location of the autoclave's controlling temperature sensor. Other locations can be monitored if additional probes are available. Thermocouple access for conducting these studies must not obstruct the steam inlet, drain valve, or any safety release access. The autoclave cart can be used as a support structure for this assessment to provide greater reproducibly of thermocouple location. The thermocouples should be positioned so that they do not contact any internal surface and are measuring steam temperature (Fig. 19).

The customary criterion ($\pm 0.5^{\circ}C$) for the empty chamber temperature distribution is derived from FDA's Proposed LVP CGMP's from 1976 (14). Originally established for heat sensitive materials where a tight control is required, it was adopted as an appropriate criterion for all steam sterilizers. Its application without alteration for parts sterilization is excessive, given that there is no reason to limit the temperature provided it exceeds the desired set point. For a comparatively simple requirement, it is subject to some interpretation. Consider that

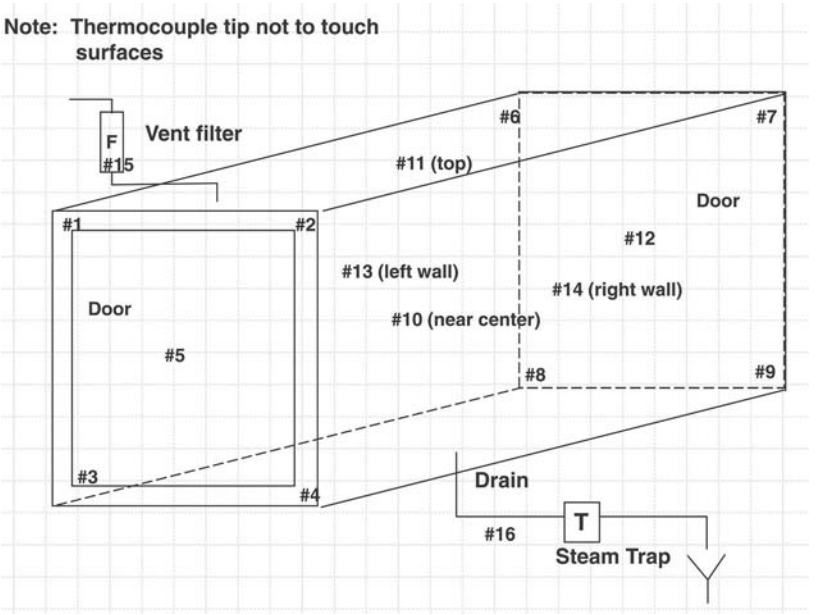

Figure 19 Empty chamber study thermocouple locations.

there is no defined method for interpreting the temperature data. The criterion can be applied in a variety of ways:[g]

- All thermocouples over entire cycle duration
- All thermocouples excluding the first few minutes
- All thermocouples over a shorter period
- All thermocouples over a single time period

Conducting the evaluation omitting the first few minutes of exposure is perhaps most appropriate; it ignores only the very beginning of the dwell when steady state might not have been reached at all locations.

Regardless of the criterion and data set utilized, the most important consideration is the frequency of execution. Empty chamber studies should be conducted on each cycle the sterilizer can execute (cycles differing only in the duration of the dwell period can be evaluated in only the shortest duration cycle) during initial qualification. It may also be useful in the evaluation of changes to the sterilizer that are primarily mechanical or control system related. Its utility for the periodic requalification of the sterilizer is extremely limited as it cannot evaluate steam penetration (the most important consideration in cycle effectiveness).

Container/Component Mapping

Before inserting any container or object into a sterilization load, it should be evaluated for its steam penetration. Complex items of hose, stainless steel parts and filters with wrappings, and containers larger than 50 mL may have a discernable cold spot where the temperature reaches the set point temperature last (7). Smaller containers and simple geometry hard good items can ordinarily be ignored in these studies as it will be virtually impossible to identify a discernable cold spot.

Mapping studies should be conducted to determine where in the item the temperature probe and biological challenge should be placed. These studies can be performed in a laboratory setting provided that prevacuums and steam introduction is comparable to that of

[g]There is no broadly accepted method for this test in the regulatory literature or compendia.

the sterilizer the item is being introduced into. Orientation and wrapping for these studies should be identical to that used in routine sterilization. Care must be taken in these studies not to impede or assist air/condensate removal and steam penetration as this will lead to location errors. Special fittings should be employed to provide thermocouple access without alteration of the results (these fitting are also necessary for steam penetration studies in the sterilizer). Once these locations with the items have been established, they should be monitored in all subsequent studies.

Loaded Chamber Temperature Distribution Studies

This activity is largely associated with terminal sterilization processes, where excessive variation in temperature across the chamber could result in localized under- or overprocessing. While the true demonstration of cycle effectiveness is the subsequent heat penetration studies, difficulties with temperature distribution may predict later problems with that activity. Where all of the items in the load are identical as is customary in terminal sterilization and may also be prevalent in component sterilization for stoppers and other items, these studies can be of some benefit in identifying whether uniform conditions can be attained. Difficulties with temperature distribution can ordinarily be resolved by altering load density, positioning, and/or arrangement. Other possible corrections would entail changes in process parameters, physical location of temperature probes, steam entry, cooling water introduction, etc. Criteria for this study are not defined; the only expectation is that conditions across the load be reasonably constant at steady state. In the course of these studies, load cool and hot zone or spots may be identified. This knowledge is essential for the subsequent steam penetration/biochallenge studies to follow. The objective of this study is to establish the uniformity of process conditions across the sterilization chamber that is essential to a consistently lethal sterilization process.

Where the load is composed of mixed items of differing size, dimension, and mass, heat distribution studies are of substantially less value. The difference in the items is of far greater consequence than any chamber variation and thus evaluation of loaded chamber temperature distribution can be omitted in the validation of parts sterilization loads (22).

Load Mapping

When sterilizing identical items, whether for part or terminal sterilization, a definable cold spot in the sterilizer can be located where probed items demonstrate the lowest overall F_0. Identification of this region is of greater importance than loaded chamber heat distribution as it focuses on the sterilizing effect. Load items in this area are those that are at greatest risk for underprocessing. In most loading patterns, this is usually a point near the bottom center of the load. When performing the biochallenge studies, the preponderance of challenge units should be in or near this zone.

In terminal sterilization efforts, it is also necessary to identify the hottest portions of the load where the maximum F_0 is delivered. Product stability at these conditions may be adversely affected and when collecting samples for stability studies, preference should be given to this region of the load.

Load mapping must address variable loads if that is the expected operational practice for sterilization. The "cold" and "hot" spots should be identified in both minimum and maximum loads. As noted in the prior section, where the load is comprised of mixed items, "cold spots" are ordinarily associated with the item and not with sterilizer performance. In these situations, the load should be rearranged between repetitive runs to support that cold spots are item dependent. This can be accomplished in separate load mapping studies specific to that purpose (and then repeated in the biochallenge runs) or directly in the biochallenge runs (22).

The process control of many terminal (and even a few parts) sterilizers may be supported by load temperature probes positioned with the load. It might seem appropriate to place these temperature sensors in the coldest parts of the load, and thus assure that minimum sterilizing conditions have been delivered. From an operations perspective, this has proved somewhat impractical. These probes are quite large and difficult to place and remove from the middle of the load where the coldest items are located, especially when the load arrives at the sterilizer as a complete pallet. These probes are best placed in convenient units near the top of the load,

with the lethality delivered there correlated to what is attained at the load cold spot. This practice accommodates such aspects as: container size; fill volume; viscosity, and heat capacity differences across the various products the sterilizer will process. In parts sterilization, load probes serve little purpose and they can be either removed or placed in a standard location in all cycles (22).

Loaded Chamber Heat/Steam Penetration and Biological Challenge Studies

The core of all sterilization validation efforts is the challenge study in which biological indicators are distributed throughout the load to confirm the lethality of the process. For steam sterilization, this is accomplished simultaneously with heat/steam penetration using temperature measurements within the load items.

Parts Sterilization

In part sterilization studies, these studies are relatively easy to perform. Biological indicators and thermocouples are placed within the load items and customarily exposed to conditions only slightly less lethal than the routine sterilization process (a 1°C set point reduction and a 1-minute shorter dwell period is sufficient) (22). Control of sterilization cycles for parts loads is customarily accomplished by temperature measurements in the drain line where temperatures are the coldest. Destruction of the biological indicator (ordinarily spores of *G. stearothermophilus*) coupled with comparable physical lethality (as established by the temperature probes) in this worst case cycle supports the efficacy of the sterilization process. In some firms the load arrangement for these studies is fixed, however more progressive efforts can support changes in load positioning, provided wrapping and orientation are maintained. This is accomplished by performing triplicate studies (as is customary in the validation of all loads in a new sterilizer or a new load in an existing sterilizer) in which the load is reconfigured between the individual runs.

The biological indicator is customarily a spore strip of *G. stearothermophilus* inserted in the item at the location previously determined to be slowest to heat. Custom biological indicators in the form of inoculated wires or strings can be used in smaller items. Inoculation of the spores on the surface of the item is the method of choice, as there is a regulatory belief that the resistance of the microorganism will change dramatically relative to a spore strip. While there is a change in resistance of spores on the surface relative to a spore strip, the difference is ordinarily within a single order of magnitude. As the confidence in the sterilization cycle is actually obtained from the difference in resistance between the biological indicator and any bioburden present on the item (which is minimally 6–7 orders of magnitude different), requiring inoculation of surfaces provides minimal additional confidence in the sterilization process.

Temperature measurements are typically performed using thermocouples positioned in slow to heat zones with the load items. The use of specialized fittings to permit thermocouple access without compromising the integrity of the item and any wrapping material is strongly recommended. Where this is not the case, the physical data should be considered suspect as air removal, and steam penetration may be improved relative to unprobed load items. If there is any question regarding the integrity of temperature probed units, biological indicators should be located in an adjacent identical item without penetrations for a temperature probe. In evaluating the physical data, the location with the lowest overall F_0 is considered of greatest concern. It represents the item(s) where the delivered lethality is the lowest. That knowledge is essential to understanding the sterilizers' performance. The load in which the lowest F_0 is demonstrated is conventionally utilized in annual reevaluation of the sterilizer.

In considering the loads to evaluate, the maximum load of mixed items is most appropriate as a worst case challenge for each unique sterilization process. The large mass of the maximum load will entail greater steam to bring the items to sterilizing conditions resulting in more condensate than would be encountered with smaller loads. The choice of the largest load would ordinarily include items where air removal might be difficult to accomplish. Where a smaller load includes a unique item with potential air removal issues, it should be validated as well. For loads comprised of many identical items such as stoppers or containers, the evaluation of both minimum and maximum loads affords the greatest flexibility in routine operation. Where air removal and steam introduction differ for loads in a sterilizer,

that is, gravity displacement and prevacuum cycles are both utilized, then the loads for each type should be considered separately. As noted above, rearrangement of the loads between repetitive runs is recommended to ease operational loading of the sterilizer.

Terminal Sterilization

Sterilization of products entails consideration of both sterility and stability; a two-sided concern that essentially doubles the work required relative to parts sterilization.[h] Biological challenges must consider the effect of the fluid on the moist heat resistance of the microorganism: the effect can be either protective or destructive and must be determined precisely in specifically designed laboratory studies. Once determined the *D*-value in the fluid will define the minimum lethality to be delivered across the sterilizer (usually a Probability of a Non-Sterile Unit (PNSU) of not greater than 1 in 1,000,000 units). The fluid must have available water content (values as low as 5% water appear to be adequate, but must be confirmed experimentally for any fluid with low aqueous content) that serves to sterilize the fluid and when converted to steam the headspace above the liquid. The biological challenge for terminal sterilization must be considered with some caution. *G. stearothermophilus*, the preferred challenge organism for steam sterilization of hard goods is often inappropriate for use with terminal sterilization. Its resistance to steam sterilization is such that the minimum F_0 with which it can be comfortably used (assuming a D_{121} of 2 minutes and a challenge level of 10^6 spores per container) is 18 minutes.[i] As that amount of heat input is excessive for many materials, alternative indicator spore forming microorganisms are often chosen. Among the organism that can be used as biological indicators are *B. coagulans*, *C. sporogenes*, and *B. subtilis.* Those organisms and others are appropriate choices provided the resistance of the chosen spore is evaluated in the product.

Where either the containers or closures are not sterilized prior to filling, a further complication ensues. The process must be able to demonstrate sterilization at the container-closure interface where steam from the fluid may not penetrate. This is accomplished by inoculation of the interface with spores of *B. atrophaeus* (a dry heat biological challenge indicator microorganism) and confirming their destruction in the intended process. The challenge level may be reduced provided bioburden controls on the components are in place. In some cases, the time-temperature conditions to inactivate the spores in the interface may exceed those necessary for the sterilization of the fluid in the container (23).

Temperature measure inside the liquid filled containers is accomplished by positioning thermocouples through the stopper in the container[j]. Syringes and ampoules are customarily monitored using thermocouples external to the container, which given their typically smaller size and thinner walls rarely creates significant difficulties in cycle confirmation. The use of self-contained probes that can individually record data can be used in very large sterilizers or continuous sterilizers where the use of wired thermocouples is problematic.

The studies should be conducted at the intended cycle conditions as the "window" for attaining sterilization while maintaining product stability rarely allows adjustment of parameters without adverse impact (Fig. 7). Biological challenge units in product-filled containers are positioned across the load pattern, with emphasis on the cool point determined during the load mapping studies. Thermocouples are positioned in separate containers next to those with the biological challenge. The entire sterilizer load for validation need not utilize product containers; the use of placebo filled containers is commonplace, provided that the placebo units approach the tested product in fill volume, viscosity, and heat capacity.

Minimum and maximum loads should be evaluated in triplicate studies. In each load size, consistency of minimum and maximum delivered F_0 is the key requirement. Biological challenge results must perform as intended.[k]

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[h]Laboratory media and in-process fluid sterilization can be validated in a similar manner.

[i]Assumes a 9 log reduction is required to provide a 1 in 1000 chance of a survivor in the validation studies.

[j]The location should have been determined in the mapping studies described earlier.

[k]In cycles defined by the bioburden/biological indicator sterilization method, bioindicator count reduction rather than destruction may be the intent.

ONGOING CONTROL

Steam sterilizers share many considerations as other pieces of pharmaceutical process equipment. To be utilized they must adhere to common CGMP practices intended to support commercial use. These practices include requirements for instrument calibration, maintenance (preventive and corrective), review of records, and bioburden monitoring.

Proper calibration of the steam sterilizers' instrumentation on a periodic basis is essential for maintaining process effectiveness. As microbiological kill is logarithmically related to the sterilizing temperature, slight variations in temperature can have a substantial effect on process lethality. This must consider the entire control system from point of measurement to the process recorder (24). The pressure and any other instrumentation on the unit should also be calibrated. Calibration must include any instruments that do not record or display. Instrumentation utilized for the validation of the process must be calibrated as well.

Keeping the equipment in proper working order is an essential requirement. Preventive maintenance as defined by the sterilizer manufacturer is intended to keep the sterilizer in proper working condition. There should be a defined schedule for its execution using methods and parts provided by the vendor. This form of maintenance is presumed to have no adverse impact on the sterilization process, and while records of it must be maintained evaluation of the change is normally not indicated. Corrective change that repairs malfunctions of the equipment presents quite a different situation. Each repair whether planned or unplanned must be formally evaluated for its potential impact on the performance of the system. The review must consider the extent to which the repair and/or the condition prior to the repair could alter the effectiveness of the cycle. In some instance, there will be little or no impact from minor changes to the system, while more extensive changes will likely result in a formalized evaluation of the equipments performance. The evaluation might require a repetition of one of more of the elements of the equipment qualification, or, in extreme cases, the performance qualification of the sterilizer itself.

Record review is a requirement for the release of materials produced by any process. In steam sterilization, the records of individual cycles must be carefully reviewed to determine their conformance to process requirements. Many firms establish formalized review sheets defining the expected conditions to be attained and the tolerance around them for ease of record review.

Where the sterilizing approach mandates that the presterilization bioburden conforms to specified limits, it is essential that routine testing be performed. For parametric release this is an every lot requirement.

CONCLUSION

Steam sterilization is a relatively simple process; its criticality and universal use suggest that individuals working in this industry must have a thorough understanding of the principles associated with its use and validation. There is perhaps more information available on this process than any other in our industry. The reader is encouraged to explore that information if the information provided within this effort proves inadequate.

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10 Gas, vapor, and liquid chemical sterilization

James Agalloco

INTRODUCTION

There are instances in the manufacture of pharmaceutical products and medical devices where an item must be sterilized, yet its properties eliminate methods of sterilization based on moist or dry heat or radiation. The simplicity and speed of heat and radiation sterilization makes them the methods of choice in most instances; however, the effects of these sterilization processes on many materials are detrimental to essential material properties. When faced with these circumstances, the practitioner often turns to chemical methods where microorganisms are destroyed by exposure to chemical agents in gas, vapor, or liquid form. This chapter will review the available processes, outline their development, describe suitable validation approaches, and delineate the necessary routine process control requirements.

While all of these processes rely on a chemical action against microorganisms, there are meaningful differences in their application that must be understood to use them effectively. The same chemical agent will likely require differing controls when delivered in a different manner. The processes for sterilization by the varying agents that operate in a particular phase are all similar and resemble each other more than the processes for a single agent applied in different phases. This can perhaps be better understood by a rapid review of the relevant aspects of physical chemistry. The basic definitions of a gas and liquid are presented below:

> Gas: Matter in a state intermediate between liquid and plasma that can be contained only if fully surrounded by a solid; it can condense to form a liquid (1).
> Liquid: A state of matter between solid and gaseous. A liquid can evaporate into a gas (1).

All materials in a liquid state have some tendency to evaporate into the gaseous form. At any fixed temperature of a liquid, there is a vapor pressure created by the gas in equilibrium with that liquid. As the temperature increases, so does the vapor pressure, corresponding to a higher concentration of the material in the gas phase above the liquid. As gases cool, they may reach their dew point at which temperature a portion of the gas reverts (condenses) to the liquid state. Chemical agents such as hydrogen peroxide and peracetic acid are utilized for sterilization in ways where both liquid and gas phase may be present simultaneously and is often called a vapor. Gaseous agents such as chlorine dioxide and ozone are also effective in aqueous solution under very different conditions from those used for gas sterilization.

> Vapor: "Diffused matter (as smoke or fog) suspended floating in air and impairing its transparency (2)." When large amounts of liquid are suspended in the gas it has the appearance of a fog or cloud (Fig. 1A).

The laws of physics mandate that both gases and liquids be uniform in the concentration of all components present in each. As a consequence these processes are relatively simple to develop, validate, and operate. The biphasic nature of vapor presents several challenges to the scientist. The premise behind most vapor sterilization processes is that by increasing the temperature of the liquid it can be converted into a gas and maintain the same high concentration despite the phase change. This may result in a meta-stable situation with localized condensation of the material at locations where the surface temperature is less than the dew point temperature of the material. Variations in temperature across a chamber will result in different amounts of condensation at each location. Locations where the temperature is higher may not have any condensation. All of this tends to make sterilization using vapors far more problematic than either gas or liquid sterilization. The situation is actually even more complex, as introducing a hot vapor into an ambient temperature chamber will result in a gradual temperature rise over the course of the process.

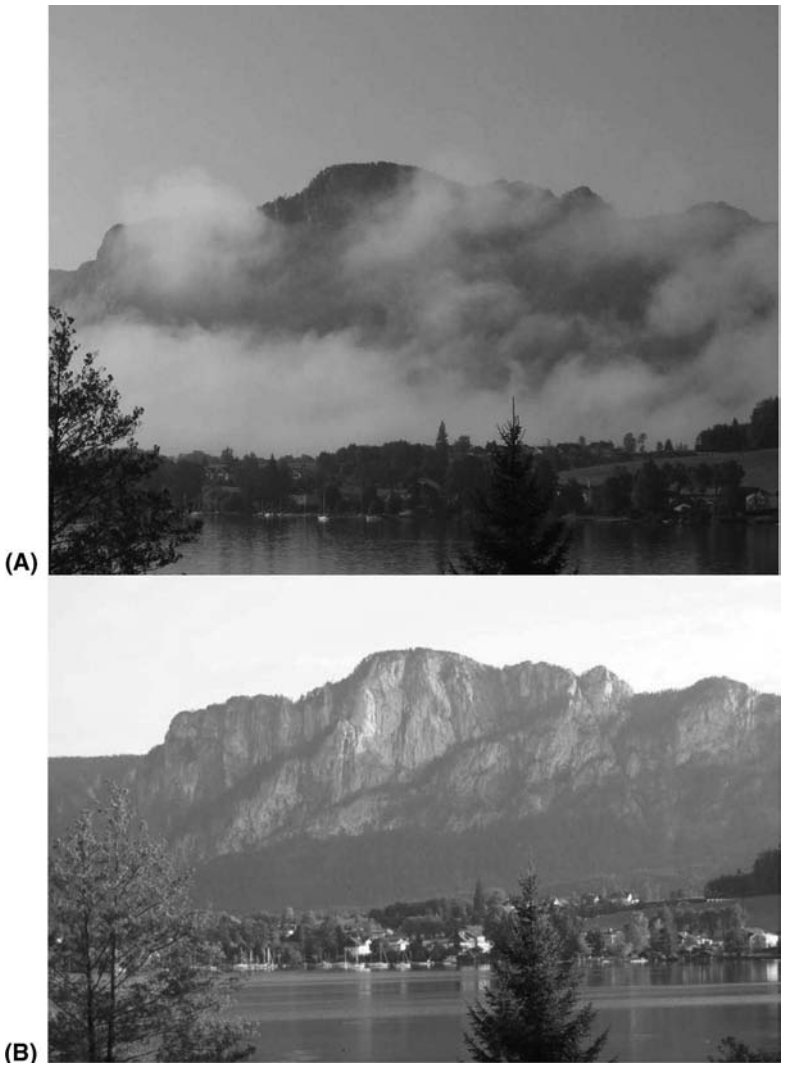

Figure 1 (**A**,**B**) The Mondsee & Schafberg with and without "vapor."

The chemical effect of the gas, liquid, and vapor agents against microorganisms is believed identical regardless of the phase in which the agent is present when exposed to the agent. The concentration of the chemical agent has the greatest impact on the effectiveness of the sterilization process regardless of the phase. Of course, substantially higher concentrations are possible in the liquid phase relative to the gas phase. Attaining the same concentration in each phase for a vapor agent is virtually impossible. Therefore in vapor processes, the sterilizing effect on the microorganisms will differ in the vapor and liquid phases due to localized differences in concentration, and adsorption potential of the agent from each phase to the microorganism. Further difficulties result from the solid nature of the target microorganisms (whether biological indicator or bioburden) and the potentially varying nature of the agent at the point of exposure to the microbe.

There are other important factors essential for effective sterilization of microorganisms by chemical agents. Moisture must be present as well for effective sterilization to assist in penetration of the agent through the spore coat (3). In liquid sterilization, the presence of liquid water is assured. In gas sterilization, moisture is provided by the humidity present in the gas phase. For vapor sterilization the moisture necessary for effective sterilization is present as

either a gas or liquid depending on the temperature at the location. Vapors present additional problems for determination of moisture levels as the amount of water will be different in each phase. Temperature for the gas and vapor process is important, predominantly as it influences the relative humidity level with gases and vapors.

In the context of gas, liquid, and vapor sterilization, the essential factors are comparatively easily determined in gases and liquids, whereas vapor processes present all manner of measurement uncertainties. The agent concentration and humidity levels are neither constant across the processing environment (and process cycle), and measurement of one phase to calculate the equilibrium concentration in the other is only useful where the entire process is isothermal given that the antimicrobial agent and water are present in both phases. As vapors are introduced as either hot gases or derived from hot liquids, temperature is rarely constant and thus concentration measurement is at best a locally correct number, and at worst near useless in establishing process conditions and relating them to lethality.

STERILIZATION BASICS

Sterilization is a process that completely destroys or removes microorganisms. In the context of this chapter, the emphasis is on completeness of the treatment. The agents described in this chapter when applied without adequate control measures should not be considered sterilizing. In sterilization processes, microbiological death curve can be graphically described by the logarithmic number of microorganisms remaining alive (4). When plotted against time, a straight line results. This line can be extrapolated to estimate the number of possible survivors in a large number of units (Fig. 2). This is termed the Probability of a Non-Sterile Unit (PNSU). An acceptable PNSU has been defined as 1 positive unit in 1,000,000 units (a risk value originally developed for food safety).

The slope (the inverse of which defines the *D*-value) of the microbial death curve is an inherent property of the microorganism and the conditions of the sterilization treatment itself. The slope of the curve is the time in minutes for the microbial population to be reduced by 90% (or 1 logarithm) and is commonly termed the *D*-value (4). Accurate determination of the *D*-value requires precise measurement of the lethal conditions to which the microorganism is exposed. As noted earlier, the determination of sterilizing conditions for gases and liquids is relatively simple. Establishing the conditions for vapors sterilization is problematic; however, the principles for establishing cycle efficacy for vapors are essentially identical to that for gases and liquids. Validating the physical destruction of microorganisms relies in part on differences in the relative resistance of a biological indicator and bioburden organisms (Fig. 3) (5).

The validation exercise supports the efficacy of the sterilization process against the microorganisms present during routine processing. Depending on whether the sterilization process is gaseous, liquid, or vapor, the details of the validation will vary; however, the basic principles described in the preceding text remain the same. Each of these will be discussed

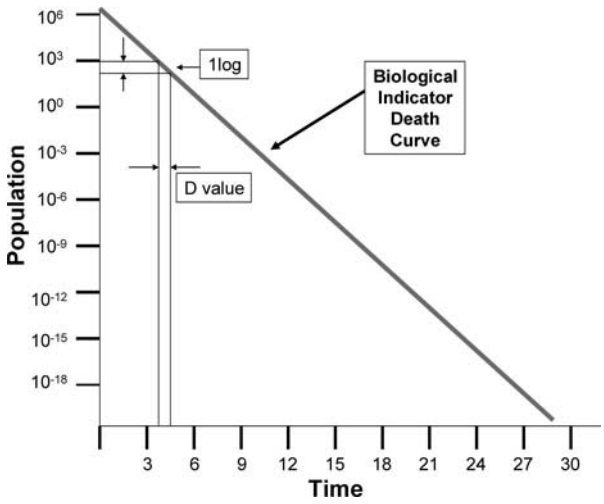

Figure 2 Microbial death curve.

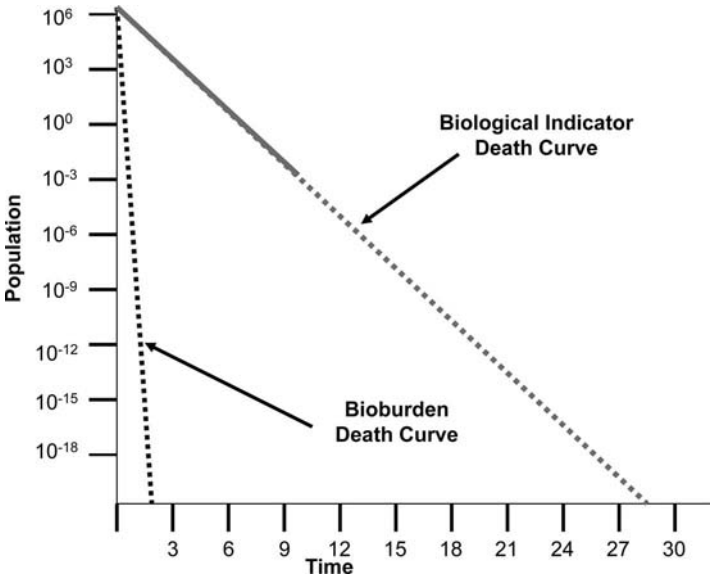

Figure 3 Relative resistance of bioburden and biological indicator organisms.

separately accommodating the differences in them. A sterilizing agent will require different instrumentation, equipment, and controls for effective usage depending on the phase(s) in which it is delivered. All of the validation methods exploit the difference in resistance of the biological indicator relative to that of the natural bioburden as depicted in Figure 3 regardless of whether the sterilizing agent is gaseous, liquid, or vapor.

GAS, VAPOR, AND LIQUID STERILIZATION FUNDAMENTALS

Material Effects

Sterilization processes are designed to kill microorganisms and as such they utilize conditions that may be destructive of essential material properties. Moist and dry heat sterilizers employ extremes of temperature, while radiation processes expose the materials to various forms of radioactivity. These processes have potentially adverse effects on the materials being processed, and the development of sterilization treatments must always consider that effect. Gas, vapor, and liquid sterilization processes are not exempt from this phenomenon and material evaluation required. The strong oxidative powers of many chemical agents, pH extremes of acids and bases, and the presence of substantial moisture can all lead to significant changes in the materials being sterilized. Some agents, especially ethylene oxide (ETO), are known for allowing degradants to remain on the materials post processing, presenting a different adverse effect, and the amounts of these residuals is closely regulated (6). Lastly, the effect of the agent on the processing equipment must be considered. The typical sterilizing chamber is comprised of many different materials all of which must be tolerant of the sterilizing conditions. Consideration of each of these possible adverse consequences must be an integral part of process selection, equipment design, cycle development, and process validation.

Process Equipment

Gas and vapor sterilizations are ordinarily carried out in jacketed chambers much like those utilized for steam sterilization. To assure greater process reliability, external and/or internal mixing is utilized to enhance uniformity of the lethal agent and relative humidity throughout the chamber. The jacket provides for temperature control, while the pressure (and vacuum rated) chamber serves to contain the potent chemicals employed for the sterilization process. The process is executed by a control system that provides sequencing, regulation of process parameters, and documentation on the process.

Contemporary control systems for sterilization systems are electronic, either programmable logic controllers (PLCs) or minicomputers. These systems include various features including operator interface, recipe management, process execution and control capability, documentation, and interfaces with surrounding systems. The control system is vital to sterilization success. A well-designed control system facilitates operation of the system and is essential to maintaining a compliant sterilization process. Its importance cannot be overstated. It is the critical for providing the control necessary to support and maintain a validated sterilization process.

There are vendors that supply stand-alone control systems that can be used to supply and, in some instances, exhaust simple vessels with gases or vapors for sterilization. In these instances, the end-user is responsible for interfacing their process equipment with the freestanding control system. Temperature regulation, pressure/vacuum capabilities, and other operational features must be provided independent of the vendor-provided controller. The process equipment that can be sterilized with these units varies from the complexity of a freeze dryer to the simplicity of a stirred tank.

Equipment for liquid sterilization varies with the scale of the operation. Large commercial systems might use a jacketed stirred tank, the liquid counterpart of the sterilizing chamber used for gases and vapors. Process control would be provided by a PLC, distributed control system (DCS), or minicomputer. For smaller scale processes, the equipment might be as simple as a nonpressure rated container where the items to be sterilized are submerged. Agitation, temperature control, and sequencing would be provided by the operator using laboratory apparatus and/or room environmental controls.

GAS STERILIZATION

Gas sterilization is widely used for materials and equipment liable to damage by moist heat, dry heat, or radiation processes. Many of the common polymeric materials used in medical devices are difficult to sterilize by any other means. When finally packaged for delivery into operating and other critical settings, the medical device packaging must be sterile as well. The most prevalent gas utilized for sterilization is ETO, and sterilization using other agents is based on methods used for ETO. Other commercially available gas agents for sterilization are ozone and chlorine dioxide. While their use is not widespread, they offer the user alternates to ETO. Other gases that have demonstrated sterilization capability but almost no commercial support include methyl bromide, propylene oxide, helium/oxygen plasma, and sulfur dioxide (7,8).

Gases will not condense under typical sterilizing conditions and are highly penetrating. The penetrating abilities of the more common gases vary: ETO is superior to ozone, which is in turn superior to chlorine dioxide (9). Sterilization efficacy is enhanced when prehumidification is provided prior to sterilization. Optimum temperatures vary: ozone and chlorine dioxide are typically performed at room temperature, while ETO can be effective from ambient temperature to 60°C (9).

Gas sterilization process equipment must properly control gas concentration, relative humidity, and temperature throughout the process to provide consistent process lethality, as changes in the essential process parameters can alter the effectiveness of the gases ability to penetrate and react with the microorganisms and thus lethality. Humidification is typically accomplished using clean steam injection directly to the sterilizing chamber. ETO is highly penetrating through corrugate, polymers, and paper materials, which make it well suited for sterilization of medical devices in their final packaging. Ozone and chlorine dioxide are less penetrating, and their application for medical devices must be considered with some caution. Because each of these agents is a gas and the chamber is well mixed, single-point monitoring of gas concentration and RH provides adequate process control over the sterilization process. Despite this seemingly minimal monitoring, regulatory approval for parametric release for ETO sterilization is widespread.

ETHYLENE OXIDE

ETO is a powerful oxidizing gas that kills microbes primarily by chemical reaction with various sites in microorganisms primarily those with –NH2, –SH, –COOH, and $–CH_2OH$ groups (10). Microbial kill with ETO approximates first-order kinetics and is directly related to

gas concentration, relative humidity, and process temperature (10). ETO is widely used for terminal sterilization of medical devices in final packaging. Sterilization methods for ETO (and essentially all other sterilizing gases) for the pharmaceutical processes follow medical devices practices because of the extensive experience with ETO for that application. ETO sterilization is effective across a wide range of conditions: gas concentration (300–1000 mg/L); relative humidity (35–85%), and temperature (20–65°C), although the usual processing ranges are somewhat narrower (10). ETO is an extremely potent material, has been identified as a mutagenic, carcinogenic, neurotoxic, and highly explosive (11). Trace residuals from ETO sterilization are also associated with adverse effects, so effective aeration of this is essential for safe use. For these reasons, internal usage within pharmaceutical operating companies has decreased. There are a number of firms providing contract ETO sterilization that have invested in the necessary controls to assure both worker and patient safety, and these offer most of the available industrial capacity for ETO sterilization.

As ETO processes are so extensively utilized for medical devices, the typical process is largely tailored to the specific requirements of their sterilization. The typical ETO process sequence includes

- pre-humidification (to raise internal humidity and performed in a room dedicated for that purpose);
- transfer to the sterilizer (with minimal delay);
- reconditioning in the chamber (to replace humidity lost in transit);
- air removal (to enhance gas/humidity penetration);
- exposure to ETO with humidity adjustment;
- initial aeration in the sterilizing chamber;
- transfer to a post-exposure aeration location; and
- post-conditioning (final aeration to remove residual ETO, ethylene chlorhydrin, and ethylene glycol) (6).

The preprocess treatments ensure adequate moisture is present on the surface of the materials for effective kill. The use of pre-humidification chambers/rooms to raise the internal moisture content of medical devices is almost universal for ETO sterilization. Post-processing aeration chambers are utilized with ETO to reduce residuals to safe levels after exposure. ETO sterilization processes introduce essentially all of the gas at the start of the process and minor adjustment during the exposure may be performed to maintain pressure. Humidity is commonly introduced using clean steam to the chamber preexposure for reconditioning after transfer, and adjustment may be required through the end of the exposure period.

ETO process control, like all sterilization processes, relies on a combination of physical measurements and biological assessments. Biological indicator kill in conjunction with data from the sterilizer instrumentation are utilized in evaluating process effectiveness. Recently, a lethality model has been proposed that mimics those utilized for steam and dry heat (12). Its broader adoption by ETO practitioners is anticipated as it simplifies lethality confirmation.

The extensive experience with ETO in medical devices has allowed many firms to implement parametric release in lieu of sterility or biological indicator testing of ETO-sterilized materials. Parametric release replaces sterility testing with a defined set of requirements derived from the initial validation exercise that must be satisfied in conjunction with the execution of each subsequent sterilization cycle (13,14). Submission to regulatory agencies is required prior to implementation and must be supported by comprehensive data derived from prior practice. Once implemented, the user is obligated to utilize parameter evaluation exclusively.

OZONE

The simplest of all gas sterilization processes uses ozone. The electrical field generation (starting with pure oxygen) and destruction (using a platinum catalytic converter) of ozone requires no moving parts, and the only required utilities are oxygen and steam (for humidification of the load) (15). Ozone has a half-life of several hours in the gas phase at ambient temperature (16). Ozone is microbially lethal at concentrations ranging from 2% to

10% at humidity levels of approximately 80% at room temperature. Ozone is less penetrating than ETO, and due to its reasonably short half-life it does not require post-cycle aeration. Ozone sterilization processes follow a sequence of humidification, injection, exposure (without added O_3), and exhaust. Pre-humidification of the materials may be beneficial prior to introduction into the sterilizing chamber. The preferred biological indicator for ozone is *Geobacillus stearothermophilus*. Process control requirements are essentially identical to those indicated for ETO. There are no reports of parametric release for ozone sterilization.

CHLORINE DIOXIDE

Chlorine dioxide is one of the newer sterilization methods available. Chlorine dioxide is a relatively unstable gas and must be generated in situ. It has none of the safety or environmental limitations of ETO. It is less penetrating than ETO, and because of its limited penetration and low absorption, aeration is relatively easily accomplished. Chlorine dioxide cycles incorporate preconditioning (outside the chamber), humidity stabilization, ClO_2 injection, exposure dwell period, and aeration (17). Chlorine dioxide concentrations required for sterilization range from 5 to 30 mg/L with humidity levels in the 60% to 75% range at ambient temperature. Biological indicators utilize spores of *Bacillus atrophaeus*. Chlorine dioxide levels can be measured using UV sensors, facilitating routine process control. Process control mirrors the practices for ETO described previously with only minor adaptation. The limited industrial experience with chlorine dioxide is such that it is premature to consider parametric release.

VAPOR STERILIZATION

Sterilization using vapors presents a substantial difficulty to the practitioner because of potential condensation of the agent (and perhaps water vapor as well). The most commonly utilized vapor agent is hydrogen peroxide, although materials such as peracetic acid or formaldehyde can also be utilized. These materials are supplied in aqueous solution and are always introduced into the process with substantial amounts of water vapor. Vapors are delivered to the sterilizing chamber as either an elevated temperature gas (vapor) or an atomized mist of liquid. In either case, the injection will result in temperature and relative humidity variation across the chamber initially and throughout the process. Attaining a consistent uniform process with vapors is substantially more difficult than for gases or liquids and requires constant mixing.

The addition of heat converts the solution components to the vapor phase as it is introduced into the sterilization chamber. On entry into the chamber that is generally at a lower temperature than the inlet gas stream, some portion of the vaporous material will revert to the liquid (or solid in the case of formaldehyde) phase. Vapor processes differ from gas sterilization in that vapors always have two distinct phases present inside the sterilizing chamber. Vapor sterilization processes typically operate at or near room temperature and are thus appropriate for heat sensitive materials. Depending on the temperature within the chamber, agent concentration and humidity level within the sterilizing chamber, some quantity of the agent will revert to its initial liquid (or solid) state. As the water vapor is also subject to condensation, it too can be in either phase. The concentration of agent and water condensed at each location may be variable based on the temperature at that location. The concentration in the gas phase will be uniform to the extent that the internal chamber is well mixed. Penetration by vapor agents through permeable materials as gases is certainly possible; however it is unlikely to occur once they have condensed. As a consequence, these agents are rarely utilized where penetration through layers or wrapping is required.

Vapor sterilization requires appropriate agent concentration and relative humidity. The difficulty created by the presence of two phases in the sterilizing chamber is that concentrations of the agent and relative humidity will not be constant across the entire chamber. Concentration determinations in the gas phase (where concentration can typically be measured rather easily, if not inexpensively) may not correlate with concentration in the liquid phase. This substantially complicates precise control of the sterilization process, as the target microorganisms are solids, and presumably at a lower temperature than the vapor. Kill rates of microorganisms by these agents differs with concentration and the phase present (35% liquid

kills at a different rate than 35% gas), and is further complicated by temperature variation across the chamber that creates localized concentration and relative humidity differences. Nevertheless, provided the system maintains reasonable temperature control, and the vapor within the chamber is well mixed, the process uncertainties can be minimized and effective sterilization demonstrated across the entire chamber and load.

Biological indicators for vapor systems cannot have defined resistance in the form of *D*-values as the effective concentration of the agent in contact with the microorganism cannot be determined with precision because of the condensation potential. Gas phase concentration (which can be measured) cannot correlate directly to surface concentration (where condensation might be present) unless the temperature throughout the chamber is constant. Thus, while microbial destruction is certainly evidenced by vapor processes, the rate of kill is unfortunately inexact. As the process parameters cannot be accurately determined, *D*-value determination is problematic and reported values are likely inaccurate.

HYDROGEN PEROXIDE

Hydrogen peroxide effectiveness as a sterilizing agent is well established (18). Hydrogen peroxide is available commercially in aqueous mixtures. Solutions of hydrogen peroxide should be kept away from flammable materials and reducing agents for safety reasons. Solutions of H_2O_2 should also be protected from light. Delivery to sterilization chambers is accomplished by heating the solution (30–50% H_2O_2 in H_2O mixtures have been used) above the boiling point ($\sim 100^{\circ}C$), simultaneously supplying the sterilizing agent (H_2O_2) and required humidity (H_2O). The sterilization process may incorporate an evacuation (or drying) step to allow for increased H_2O_2 concentration without condensation. Thorough mixing of the chamber is recommended as it increases uniformity of all process variables. Penetration of H_2O_2 in the gas phase is likely comparable to that of H_2O, while liquid penetration is minimal. After exposure the chamber is aerated/evacuated to remove H_2O_2 from the materials. This portion of the cycle may be the longest as re-evaporation of any condensed H_2O_2 typically requires more time than the rest of the process. A slight modification of the more common process includes the addition of electrical energy to an H_2O_2-filled chamber, which increases the process effectiveness by the creation of short-lived free radicals (19).

PERACETIC ACID

Peracetic acid, which is typically supplied as a mixture with H_2O_2, is an effective sterilant because of its strong oxidizing potential (9). It is explosive at temperatures above $110^{\circ}C$, and thus is introduced into sterilization processes as a liquid mist at ambient temperature. A small amount of the peracetic acid may evaporate into the gas phase. Surfaces to be sterilized must be exposed directly to the liquid because concentration in the gas phase is generally low. It is a strong oxidizing agent and corrosive to many materials, and thus presents considerable handling/safety issues.

LIQUID STERILIZATION

There are many available liquid materials that are effective for microbial destruction by a variety of chemical reactions. These chemically active agents are capable of rapid kill of vegetative cells and spores. Acids, bases, aldehydes, halides, and strong oxidants are all effective liquid sterilants (20). The item to be sterilized is immersed in the chemical, allowed to remain (with or without mixing) for the required time period. Following the dwell period, the item is either removed from the agent and treated to remove the agent or the agent is chemically neutralized in situ. The steps that follow the sterilization dwell proper must be performed in a aseptic manner that preserves sterility of the object. Removing the item from the chemical agent from the object mimics the removal of a previously sterilized object from its protective wrap. That is it is not a part of the sterilization process, but essential to proper use of liquid sterilization. Depending on the end use of the items and the chemical activity of the materials used, neutralization may have its own adverse material effects to consider. In validation of liquid chemical sterilization, agent removal (whether accomplished by physical or chemical means) is an important part of the overall sterilization process.

Liquid chemicals in aqueous solution capable of sterilizing physical objects as described above include:

- Aldehydes—glutaraldehyde, formaldehyde, etc.;
- Acids—peracetic, nitric, sulfuric, etc.;
- Bases—sodium hydroxide, potassium hydroxide;
- Oxygenating compounds—hydrogen peroxide, ozone, chlorine dioxide; and
- Halides—sodium hypochlorite, chlorine.

In the simplest of systems, the process is executed in open vessels positioned within an aseptic environment (ISO 5). The process is executed by gowned personnel following a detailed process record providing chemical addition, agitation, and neutralization. The aseptic environment allows for the removal/neutralization of the agent with reduced opportunity for recontamination of the items. In its most evolved form, the process can be carried out in a closed and agitated vessel with considerable automation.

Identical to gas sterilization, liquid sterilant effectiveness varies with concentration and temperature (humidity is provided by the water in the solution); however, because of the mixing it can be considered uniform throughout the vessel and constant over the course of the process. Other factors impacting antimicrobial activity include pH, agitation (if utilized), and presence of soil or other contaminants that might protect the microorganism. Assuring effective liquid chemical sterilization processes is straightforward, due to its simplicity.

As with other forms of sterilization, the effect of the sterilization on the materials must be thoroughly evaluated. The chemical activity of these agents is such that their effect on the items being sterilized can be substantial. Extreme pHs, significant oxidation, and reaction potential, all of which make the agent effective against microorganisms can play havoc on materials (and processing equipment) as well. Chemical activity in the form of materials compatibility is widely available in the literature (21).

VALIDATION OF GAS, VAPOR, AND LIQUID STERILIZATION METHODS

The performance qualification or "validation" activity has been described as documentation that the process or product conforms to expectations as determined through independent parameter measurement and/or intensive sampling or challenge. It is the focus of regulatory attention for any sterilization process. It is common practice in performance qualification to utilize "worst case" challenges in validation, and that is most prevalent with sterilization processes. Typical worst case challenges for gas, vapor, and liquid sterilization include reducing the process (set-point) temperature; reducing cycle dwell time; reduction of both time and temperature; reduction of agent concentration, and the use of resistant biological challenges as bioburden surrogates. More detailed information on the expected practices can be found in the myriad of industry and regulatory publications on this subject (22,23).

Historically, gas sterilization processes have been validated using the half-cycle approach, which uses conservative assumptions about the microbial resistance and number of bioburden microorganisms and was originally developed for use with ETO (24). Prior to the development of parametric lethality calculation for ETO, accurate information on gas concentration, relatively humidity, and temperature was largely unavailable, so the half-cycle method was utilized as a worst case approach. The half-cycle approach mandates a sterilization dwell period that destroys not less than 10^6 spores of a resistant biological indicator. In routine operation, the process dwell period is doubled (thus the term half-cycle) and supports a PNSU of 10^{-6} (5). The half-cycle method as utilized for gas sterilization is graphically depicted in Figure 4. The half-cycle method does not rely substantially on the resistance of biological indicator (as surrogate for the bioburden), because complete destruction of the indicator is required in the "half-cycle". Actual determination of the indicators *D*-value at the chosen parameters requires substantially more effort, and has been ignored by some practitioners. Half-cycle approaches are inherently conservative, and little effort is made to optimize the process dwell period, when it will be arbitrarily doubled in routine use anyway. The half-cycle method evaluates only the effect of time, assuming that the

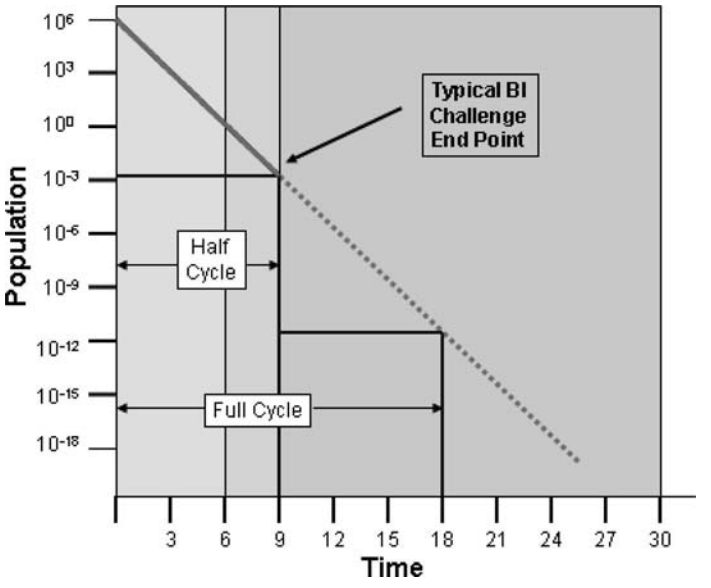

Figure 4 Half-cycle sterilization validation.

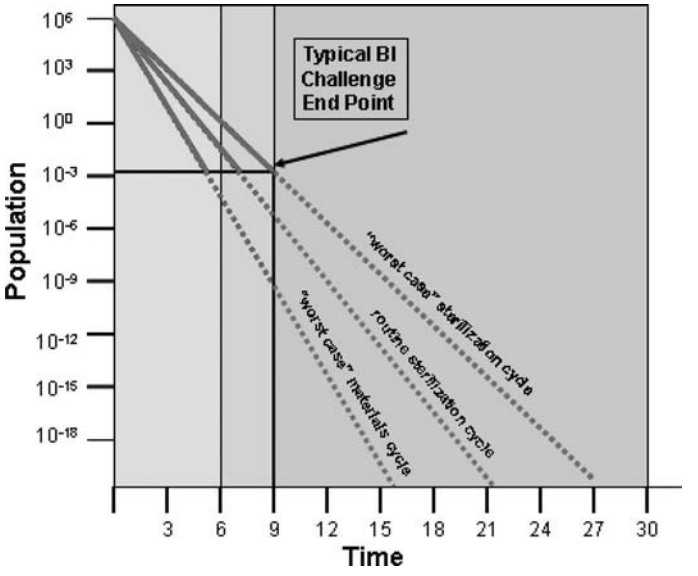

Figure 5 Sterilization validation using bracketing.

effect of lethality of variations in the other essential parameters, gas concentration, relative humidity, and temperature can be ignored. This is a severe limitation of the method.

Another method suited for sterilization validation is a bracketing approach that better supports the extremes of the operating ranges for the critical process parameters (25). In the bracketing approach, a cycle with lower concentration, lower relative humidity, and a shorter dwell period is confirmed by microbial indicator destruction using what are less lethal conditions. Material effects are evaluated in a cycle employing a higher concentration, higher relative humidity, and a longer dwell period where the adverse impact is believed to be greater. Routine operation of the system utilizes conditions that fall between the process extremes that have been evaluated (Fig. 5). This method does not require a precise *D*-value for the biological indicator, because this method supports all of the critical sterilization parameters it is readily defendable without that information.

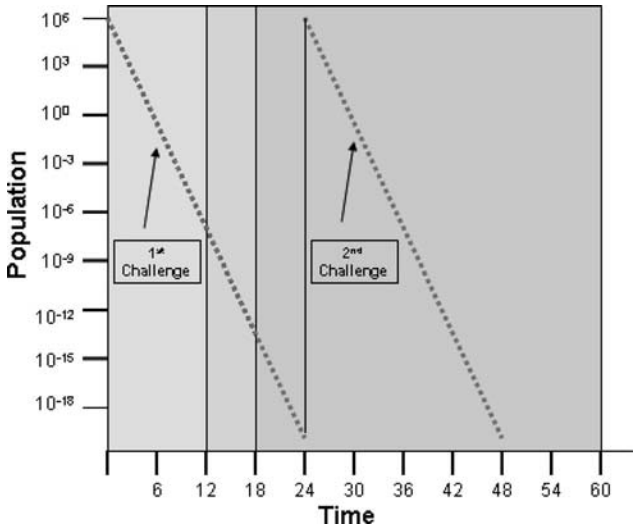

Figure 6 Double-spike validation method.

The half-cycle and bracketing approach are fully compatible with sterilization using any of the gas or liquid agents giving the practitioner a choice of methods. As precise *D*-value determination is not required for either of these methods, their use for vapors is also rather simple. The choice between them is between the simple, but less certain efficacy of the half-cycle method versus the more complex, but perhaps more defensible bracketing approach. Where the *D*-value for the agent has been determined at the operating conditions for either gas or liquid sterilization, the process dwell period can be defined more precisely, and a somewhat shorter cycle time established.

Liquid sterilization can also be validated using a double-spike method that has been specifically developed for it (26). It is an adaptation of the half-cycle method in which a second microbial challenge is introduced at the mid-point of the cycle. The microbial challenge is introduced both at the start of the process and again at the same concentration mid-way through the process dwell. Samples are taken and neutralized at intervals after each inoculum to demonstrate microbial kill rates are essentially constant throughout the sterilization process (Fig. 6). The premise in this approach is that the agent might not have sufficient antimicrobial activity over a lengthy process, and thus destruction in the second half of the process might not occur at the same rate. If this is not the case, then the bracketing approach described early can be utilized for liquid sterilization.

Regardless of the validation method utilized, there are common elements in all validation efforts.

- Equipment qualification: The equipment utilized for the sterilization process (pressure vessel or stirred tank) as well as any rooms utilized for pre- or post-cycle processing must be fully documented with respect to installation details and operational characteristics. Equipment qualification serves as the basis for change control for the physical equipment. This effort must of course include calibration of instrumentation and qualification of the control system.
- Empty chamber/vessel parameter distribution: Parameter measurement within the sterilization chamber/vessel is appropriate. Depending on the agent used this may be single or multiple point, with the cost of measurement an important consideration. For vapor systems, the real utility of concentration determination can be questioned as gas phase values will not correspond to liquid phase conditions. The goal in this exercise is to be able to correlate the values obtained during this study to the routine monitoring location(s). Where the vessel is mixed during the process (as is almost universally desirable), this study confirms the effects of that mixing. Overmixing in these processes is not a consideration, as additional mixing can only improve uniformity of

Table 1 Biological Indicators for Common Chemical Agents

Gases
Ethylene oxide—*Bacillus atrophaeus*
Chlorine dioxide—*Bacillus atrophaeus*
Ozone—*Geobacillus stearothermophilus*
Vapors
Hydrogen peroxide—*Geobacillus stearothermophilus*
Peracetic acid—*Geobacillus stearothermophilus* or *Bacillus atrophaeus*
Liquids
All—none established—*Bacillus atrophaeus* is perhaps the most appropriate

the process parameters. Biological indicators are not required in the evaluation of the empty chamber/vessel uniformity. The limitations of gas phase measurements in vapor processes must be understood.

- Component and load mapping: These activities are not a part of gas, liquid, or vapor sterilization, because sampling systems placed within the load items would alter agent penetration. This evaluation is best provided by passive biological indicators placed within the load items. The use of physical/chemical indicators placed within the items can be used to support this effort, but as there are no available chemical integrators, this practice is of limited use.
- Biological indicators: The use of a biological indicator for initial validation and routine process control is an integral part of many validation efforts for gases and vapors.[a] The principal exception to that general situation is ETO, where parametric release has been successfully accomplished by numerous practitioners. For all of the other sterilization methods described in this chapter, biological indicators are essential. The biological indicator serves as a worst case surrogate for the bioburden present in routine operations. Biological indicators are conventionally spores of a microorganism (most often a *Bacillus* or *Geobacillus* species) chosen specifically for its greater resistance to the sterilizing process than the expected bioburden. Inactivation of the biological indicator during the validation establishes the lethality of the process across the items being sterilized. The measurement of physical conditions during the validation exercise and routine operation allows for estimations of process lethality. The biological indicators of choice for the various sterilizing agents are listed in Table 1.

  Spore challenges may be either a strip or a coupon positioned within the load or spores inoculated on a load item. Inoculated items should have their population determined by the end user, and where possible their resistance to the sterilization process confirmed. Indicators are placed among the load items at locations believed to be hardest for the agent/humidity. The use of biological challenges for liquid sterilization is limited to the initial validation of the process, as the materials must be in direct contact with the liquid agent making placement and recovery of suitable biological indicators problematic in routine processing. Liquid sterilization processes are customarily established as parametrically released from the onset (a typical situation with many sterilization processes that are utilized in-process).
- Process confirmation/microbiological challenge: The core of the validation activity is the confirmation of acceptable process parameters and inactivation of the microbial challenge. Proof of cycle efficacy is provided in replicate studies in which the biological indicators are killed, and physical measurements are taken as documentation. Differences in resistance are exploited in the validation of these sterilization methods for ease of validation and routine process control.

[a]Where parametric release has been attained, the routine use of biological indicators may not be required.

ROUTINE PROCESS CONTROL

Sterilization processes must be subject to routine controls that support the efficacy of the cycle over time. Validation is not a one-time activity project, but an integral part of a CGMP compliant facility that must be sustained over the useful life of the facility and its products (27). Control over sterilization processes is commonly achieved through practices defined specifically for that purpose including: calibration of instruments, physical measurements of process parameters, use of physical integrators/indicators (and in some cases biological indicators), change control, preventive maintenance and periodic reassessment. In the absence of approvals for parametric release, biological indicators are utilized for routine release of each sterilization load along with documentation from the control system.

ISOLATOR/ROOM DECONTAMINATION

When isolators were first introduced in the health care industry, their internals were decontaminated using a liquid/vapor process using peracetic acid (J. Agalloco and D. Meyer, personal communications, 2002). The corrosive nature of this material and the time required to remove it (via evaporation and air exchange) led to interest in alternative agents for isolator preparation. The first of these to come into widespread use was hydrogen peroxide, as commercialized by Steris Corporation in the late 1980s.[b] The initial VHP-1000 systems that were offered for sale utilized the term "sterilization" in much of the documentation provided. This led directly to the assumption that these systems could readily sterilize the enclosures to which they were connected. Sterilization with these vapor delivery systems is certainly possible; however, considerable care must be taken to establish a process it that will "sterilize" the entire treated volume. Sterilization using H_2O_2 requires careful attention to the details outlined in the preceding text; it is more appropriate to consider the H_2O_2 process as a decontamination that prepares the isolator for use in processing in much the same manner as a manned clean room. Where decontamination is the process objective, the treatment needs be less aggressive, shorter, and thus less harmful to the isolator materials (especially the gloves and gaskets) and is more in line with the real objective of the treatment.[c] In more recent years, the notion that isolators need only be decontaminated rather than sterilized has eased the implementation of this technology. Chlorine dioxide has been applied to isolator treatment as well, and because it is a true gas it offers a simpler methodology; nevertheless while again capable of sterilization, decontamination is the more appropriate target.

The treatment of isolators using gases and vapors has reinvigorated the means by which clean rooms are prepared for use. Hydrogen peroxide, chlorine dioxide, and ozone have all been successfully used in the decontamination of processing environments achieving a degree of consistency and lethality unattainable with manual decontamination (28). The applications have included buildings contaminated with *Bacillus anthraces*, facilities with mold and other microbial infestations, and health care processing environments. The gassing/fogging processes utilized are substantially more effective than the manual practices they replaced, nevertheless the temptation to consider these sterilization processes should be resisted, as that is rarely the goal of these treatments.

CONCLUSION

This chapter provides an overview of the prevalent gas, vapor, and liquid sterilization methods and their validation. This chapter has broadly outlined the primary considerations with respect to each of these sterilization processes. The reader is encouraged to review the substantially larger body of knowledge available on these processes before their implementation. The accompanying bibliography outlines some recommended sources on this topic.

[b] Steris Corporation acquired AMSCO which had introduced Vapor Phase Hydrogen Peroxide as a means for isolator treatment in 1990.

[c] The isolator is not a drug or medical device and will not be injected into a human, thus its sterility is not essential for use as a processing environment.

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11 Dry heat depyrogenation and sterilization

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INTRODUCTION

Dry heat is one of the oldest methods of sterilization. Dry heat is used in the pharmaceutical industry mainly for depyrogenation of glassware and equipment going into an aseptic processing area. Because of the high heat required for the depyrogenation process, products that have been validated for depyrogenation are considered to be validated for sterilization without additional work (1). More limited use of dry heat processes are for sterilization alone.

This chapter includes information derived from a review of existing literature and publications on dry heat sterilization and depyrogenation, as well as additional information gained from practical experience. A technical information report was published by the Parenteral Drug Association (PDA) in 1981, on validating dry heat sterilization and depyrogenation processes (2). The technical report is currently being updated and is due for publication shortly. Regulatory standards have also been published on the subject; most notably ANSI/AAMI ST63, published in 2002 on validation of dry heat processes used in the healthcare industry (1). This standard is currently undergoing global harmonization and is expected to be published soon as an ISO standard. Although the focuses of the standards are sterilization in the health care industry, the basic premises are applicable to the pharmaceutical industry and to development of depyrogenation processes using dry heat. Because the standards are developed as consensus documents with the input of regulatory authorities, they represent the current best practices in the industry.

DEPYROGENATION BY DRY HEAT

One of the most effective ways to inactivate endotoxin, or depyrogenate, is by a dry heat process. This occurs basically through an incineration process. The materials being treated must be heat stabile due to the high temperatures required for an effective process.

The ability to depyrogenate by dry heat is achieved by inactivating known challenges of purified endotoxin, resulting in the demonstration of a 3 log reduction of endotoxin. Purified endotoxin, consisting only of lipopolysaccharide, is pyrogenic in lower doses than naturally occurring endotoxins, where associated proteins and phospholipids are a factor in mediating pyrogenicity (3). Various studies have been conducted, and publications issued, on the time and temperatures necessary for the inactivation of endotoxin.

The United States Pharmacopeia (USP) specifies to inoculate with 1000 or more USP units of bacterial endotoxin; endotoxic substance should be reduced to not more than 1/1000 of the original amount (3 log reduction) (4). The European Pharmacopeia, the British Pharmacopeia, and the Japanese Pharmacopeia are harmonized on their depyrogenation chapters and state temperatures greater than 220°C without specifying a time, resulting in a 3 log reduction in heat resistant endotoxin (5–7).

In the specific chapters in each of these compendia on performing the pyrogen test or the endotoxin test, the temperature of not less than 250°C for 30 minutes is noted as the commonly used minimum time and temperature settings for depyrogenating glassware and apparatus used in laboratory testing. The USP is also more specific in both the pyrogen and the endotoxin test chapters and says to depyrogenate all glassware and other heat-stable materials in a hot-air oven using a validated process with the commonly used minimum time and temperature settings of 30 minutes at 250°C.

Several studies evaluating temperature and time for endotoxin inactivation are noted in the literature. Tsuji and Lewis looked at the destruction kinetics of lipopolysaccharides from *E. coli*, S. *marcescens*, and *S. typhosa* at temperatures ranging from 170 to 250°C, and postulated that the destruction kinetics were 2nd order, with a *z*-value of 46.4 (8). In this study, at 250°C, a *D*-value of 4.99 minutes was identified for *E. coli* lipopolysaccharide. The destruction curve for lipopolysaccharide in studies since has been postulated to consist of two distinct linear phases;

the first occurring at a rapid rate and the second phase of the curve flattening out, with the reduction of LPS occurring at a much slower rate (9,10).

Nonlinearity of destruction curves of bacterial spores has been attributed to the lack of homogeneity of the spore population (8); a similar principle may be at work here. Because of the variety of responses in development of inactivation curves, it has been difficult to apply standard F_H calculations to depyrogenation studies, since an accurate *z*-value is necessary for these calculations (11). It has also been noted that *D*-value calculations rely on first-order kinetics throughout the entire process (12); the biphasic model for endotoxin inactivation would indicate that estimation of a *D*-value is not appropriate.

Because of the variability in endotoxin inactivation and recovery, the efficacy of the depyrogenation process is demonstrated by inactivation of the endotoxin indicator, rather than relying on empirical calculations of time and temperature. That being said, the measurement of heat input remains an important factor in monitoring the process to ensure an ongoing state of control.

Developmental studies are conducted to evaluate endotoxin reduction or inactivation. The items to be depyrogenated are inoculated with endotoxin, exposed to the desired temperature for various times, and then tested to measure endotoxin inactivation. The purpose of the developmental studies is to determine the minimum time and temperature necessary to demonstrate the required 3 log reduction of endotoxin using the *Limulus* amebocyte lysate (LAL) assay.

Factors affecting the inactivation of endotoxin are the particular endotoxin formulation, the purity of the concentration, the method of application, and the concentration used (11). Endotoxin has a tendency to bind tenaciously to surfaces, which factors in the removal and recovery of the endotoxin. Most often, surfaces are inoculated at greater than three logs, often at five to six logs to facilitate removal of endotoxin. However, with inoculum levels that high, an issue of concern would be whether aggregated endotoxin is being removed, or whether the endotoxin in contact with the surface of the material being evaluated is being removed (13).

Articles to be processed can be directly inoculated with endotoxin, smaller portions of a similar surface material can be inoculated, or a commercially available endotoxin indicator (EI) can be used. For materials that are directly inoculated with endotoxin, inoculate at the desired concentration, determine the recoverable amount of endotoxin, and perform inhibition/enhancement (I/E) testing to ensure that the surface or carrier does not leach any materials that will interfere with endotoxin recovery. Recovery can be performed by using LAL reagent water, generally in the smallest amount that will cover the endotoxin-spiked area, and shaking, vortexing, or sonicating to extract the spike. In some cases, depending on the process being analyzed, a surfactant or additive can be used to assist endotoxin recovery.

The LAL assay (I/E testing) is then performed to determine recoverable endotoxin in the preliminary phase and inactivated endotoxin following exposure to dry heat. Log reduction is calculated by determining the log of the recoverable endotoxin units (EUs) in the positive control minus the log of the EUs remaining in the processed article, carrier, or commercial EI.

STERILIZATION BY DRY HEAT

Dry heat is not widely used as a mode of sterilization because of the inefficiency of the process. Air is a good insulator, causing slow heat transfer from air to the product/items in dry heat processes. The heat conductivity of the items themselves can be somewhat slow, and stratification of air in the chamber can occur. However, for select, heat stable products, dry heat is the mode of choice for implementing the sterilization process.

Dry heat kills microorganisms primarily by reacting with, and oxidizing, their proteins, although other factors such as the depurination of DNA may play a secondary role (14). The effectiveness of dry heat as a microbicidal agent with the ability to kill a wide range of microorganisms has been well established (15).

The microorganism generally selected as a biological indicator for use in dry heat sterilization validations is *Bacillus atrophaeus* (formerly *B. subtilis*). This microorganism has been chosen for its known resistance to dry heat. Depending on the approach taken to the development of sterilization processes, i.e., if a product specific approach is taken, supplemental studies may be needed to evaluate the resistance of the naturally occurring bioburden on the product/items.

PROCESS DEVELOPMENT—DEPYROGENATION

The initial step in process development is to determine the heat stability of the items to be depyrogenated. The higher the temperature used for processing, the more efficient the process will be. The considerations that apply during process development are variations in load density, initial load temperature, and specific heat of the load components.

Temperature distribution studies are performed to understand the dynamics of temperature in the oven or tunnel in which the dry heat processing will occur. Temperature penetration studies are then (or concurrently) performed to understand the dynamics of the particular load being processed, especially to identify the cool spots in the load. Evaluation of the worst case load in terms of thermal mass should be carried out, and the studies or evaluation should be designed to encompass loads with less mass. Decision must be taken whether to utilize a single process for all materials to be processed or if there is a wide variation in terms of thermal mass, then it may be more efficient to design more than one process.

For glass loads, the smallest vial size with the greatest mass is often the worst case because of the density of the load to be processed, and with the greatest heating lag time. However, in some cases, a small load may be the worst case due to quick heat up and cool down times, thus decreasing total heat input. In dry heat tunnels, with various loads and belt speeds, the different combinations should be challenged to identify the combination that results in the lowest heat input. This combination would then be used during the biological validation study.

Critical operating parameters should be defined during process development studies. These are temperature set point and exposure time for an oven, and temperature set point and belt speed for a tunnel. Perform temperature-mapping studies using qualified and calibrated temperature sensing devices.

Temperature studies may be conducted as separate studies or in combination with biological studies using the endotoxin indicators. Place the inoculated materials or endotoxin challenge vials adjacent to duplicate materials or vials with temperature sensors. Determine the temperature and time necessary to achieve the required level of endotoxin inactivation. This data will then be utilized during the temperature penetration and distribution studies to evaluate the conditions required to achieve the minimum temperature in the coldest portion of the load that is necessary to achieve inactivation of endotoxin in the loaded chamber or tunnel.

PROCESS DEVELOPMENT—STERILIZATION

Following are the pieces of information that need to be understood or identified to develop a sterilization process:

- Dry heat resistance of the biological indicator (BI) organism and/or bioburden on the product
- Heat stability of the product being processed
- Temperature distribution in the chamber or tunnel
- Temperature penetration into the product or load
- Identifying the reference temperature for routine processing
- Equating heat input with delivered lethality, and you can calculate the process (time and temperature) necessary to deliver the desired sterility assurance level (SAL).

The intent of the process development studies for dry heat sterilization is to identify the minimum time and temperature necessary to achieve the desired level of lethality for the items being processed. It is necessary to understand the resistance of the microorganisms on the load to be processed, and the amount of heat delivered to the load being processed, to determine the time and temperature necessary for sterilization. The *D*-value is a measure of resistance of the microorganism and is defined as the time required to achieve inactivation of 90% of a population of the microorganism under stated exposure conditions. As noted previously, the microorganism used as a BI in dry heat processes is *Bacillus atrophaeus*.

Process development follows traditional sterilization concepts, as applied in moist heat sterilization. The F_H replaces the F_0 terminology and is a measure of heat input. Historically 170°C was used as a reference temperature with a z of 20°C (2). More recent publications of international standards and compendial references to BIs for dry heat sterilization have used

160°C as the reference temperature for *D*-value analyses. Whether 160°C or 170°C is used as the reference temperature is somewhat arbitrary when calculating equivalent heat input, since the value is used as a reference, and not an absolute, temperature. The *z*-value of a microorganism is a measure of how heat resistance changes with changes in temperature. The *z*-value is the number of degrees that are required to change the *D*-value by one logarithm, or a factor of 10 (1,2).

Process lethality as measured by BIs is determined by the following equation:

$$F_{\mathrm{H}} = D_{170^{\circ}\mathrm{C}}(\mathrm{Log}a - \mathrm{Log}b)(2)$$

where F_{H} is the minimum lethality required (assume z = 20°C), expressed as the number of minutes equivalent time at 170°C that the slowest to heat item in the load should be heated; $D_{170^{\circ}\mathrm{C}}$, the resistance of the BI (this could also be calculated at 160°C, as appropriate); A, the initial population of the BI prior to exposure; and b, the natural log of the total number of BIs tested divided by the number of negative BIs.

Use of the F_{H} concept can also be used to determine equivalent times and temperatures; for example, if a process is qualified at a certain time and temperature, then the equivalent time in terms of heat input can be determined on the basis of the following equation:

$$F_t^z = \frac{F_{\mathrm{H}}}{L}$$

where F_t^z is the equivalent time at temperature t delivered to an item for the purpose of sterilization with a specific value of z (e.g., 20°C); F_{H}, the equivalent time of 170°C delivered to an item for the purpose of sterilization; a z-value of 20°C is used.

The use of the F_{H} concept helps to integrate the lethality that occurs during the heat-up and cool-down phases of the sterilization process. Physical measurements of heat input can then be correlated to the rate and extent of microbiological kill achieved by a dry heat process.

The time and temperature required to deliver the desired lethality are determined through establishment of a lethality curve or kill time, using subminimal conditions of time and/or temperature to determine the rate of microbial inactivation.

Lethal rate is

$$L = \mathrm{Log}^{-1}\frac{T_{\mathrm{o}} - T_{\mathrm{b}}}{z} = 10^{(T_{\mathrm{o}} - T_{\mathrm{b}})/z}$$

where T_{o} is the temperature within the commodity; T_{b}, the reference temperature (i.e., 170°C); Z, the temperature increment required to change the *D*-value by a factor of 10. A *D*-value of 20°C is commonly used as the dry heat *z*-value.

The ultimate purpose of the developmental studies is to determine the rate of kill and the minimum conditions required to achieve the desired SAL.

There are three distinct microbiological approaches that can be used for dry heat sterilization—they are the bioburden method, the bioburden-biological indicator combined method, and the overkill method, using the biological indicator itself.

The absolute bioburden method evaluates the resistance of the naturally occurring bioburden on the product or items to be processed, and calibrates the sterilization process based on that information. This approach is appropriate when the bioburden and its resistance to dry heat are well understood, and the manufacturing environment is maintained in a good state of control. It may also be appropriate for materials that are more heat sensitive, but for which dry heat is the best choice as a sterilant.

The bioburden-biological indicator approach evaluates the resistance and amount (quantity) of bioburden and combines that information with use of a resistant BI to demonstrate the inactivation of the BI that gives the theoretical kill of the microorganism.

The BI approach evaluates inactivation of a resistant BI at an appropriate population level to demonstrate the desired SAL without necessarily correlating the population level to the product or commodity bioburden.

Both the PDA Technical Report 3 and ANSI/AAMI ST63 have information and detail on these three approaches.

EQUIPMENT/HARDWARE CONSIDERATIONS

One of the primary considerations for the equipment will be to determine if an oven or a tunnel should be utilized for the application. An oven can be utilized for a wide range of applications, but it is a batch process and the loads must be manually transferred from the oven to the downstream processing equipment. In many applications, this transfer would need to be done aseptically in an ISO class 5 (or equivalent) environment. An oven is more simple from an equipment design standpoint because the load is fixed. Thus, the environment/load is heated to the desired temperature, then held at temperature for the specified time and then cooled to a user-defined temperature.

Tunnels are more appropriate for continuous feeding of glassware directly into a unit operation such as an aseptic filler. When used for continuous operations, tunnels are typically fed continuously with vials from a glassware washer. Tunnels generally have at least three temperature-controlled zones to heat-up and cool-down the glassware. The cooling section must be sized to cool the glassware to a user-defined level, and provisions must be in place to sanitize the cooling section after system maintenance or after other events that may have contaminated the cooling section. Many of the newer tunnels are equipped with heating elements so that the cooling section can be hot air sanitized.

Other equipment/hardware considerations include the following:

- Load sizes and throughput requirements
- Cooling requirements: these requirements should be well defined by the user as the specific cooling requirement can impact the cooling system design and can impact the length of the cooling section for tunnels
- Air flow and differential pressure requirements: user requirements should specify the direction of air flows and differential pressure requirements of the oven/tunnel in relation to the load and unload sides of the tunnel/oven
- HEPA filter testing and other validation provisions
- Particulate control and particulate monitoring provisions
- Filter burn-in provisions (as applicable)
- Filter selection
- Sanitization of tunnel cooling sections
- Energy conservation (especially for tunnels during non-production hours)
- Fire safety provisions (especially during a power outage)

INSTRUMENT AND CONTROL CONSIDERATIONS

A key to effective oven/tunnel operation lies in the automated process control system. By eliminating the dependence on operator intervention and data recording, automatic temperature and sequential control provides assurance that the "validated" sterilization and/or depyrogenation cycle is consistently and repeatedly delivered. A typical control system for a new oven/tunnel includes the following hardware components:

- PLC (programmable logic controller)
- Operator interface panel(s)
- Data recorder/data collection system
- Process variable sensors
- Input/output (I/O) devices

The PLC is most commonly used as the primary component of the automated process control system as it provides sequential control of the process, provides control of all analog devices used for temperature and pressure control, controls all digital devices, receives operator input via the operator interface panels, and provides process information (such as process variable information and alarms) to the operator via displays and/or operator interface panels. The PLC typically contains specific recipe information for the various cycles to be utilized. In some cases, the PLC can be used for data collection, but it is much more common to use a separate data recorder/data collection system.

The operator interface panel can be as simple as switches and displays or as complex as a stand-alone PC running a supervisory control and data acquisition (SCADA) with a human machine interface (HMI) software package. These devices are typically used to select the recipe, start the cycle, and display process information during the cycle. The higher level PC-based SCADA type operator interface panels can provide detailed cycle reports and trending information.

The data recorder/data collection system can range from a simple strip chart recorder to a full-blown Manufacturing Execution System (MES) type data collection system. In many cases, the PLC can also provide batch data logging functionality. The minimum variables to record for dry heat sterilization/depyrogenation processes are typically temperatures, exposure times (including belt speeds for tunnels), and differential pressures.

Typical sensors include temperature measurement devices (or thermocouples), pressure measurement devices, and, where applicable, belt speed measurement devices. It is customary that the temperature sensor used to control the process temperature not be used to provide the batch record process data. An independent/secondary temperature sensor for batch reporting provides a high degree of assurance that the cycle actually ran within its defined limits.

The pressure transmitters need to be appropriately placed to maintain the manufacturer's recommended exposure conditions as the ambient conditions can impact the accuracy of the measured pressures.

Newer tunnels and ovens typically utilize variable frequency drives (VFDs) to control the tunnel/oven pressures (internal and differential) as these drives can adjust to changing room pressures.

For input/output devices, there are analog types and discrete types. The analog inputs are typically from process sensors and the analog outputs are typically for control of proportional valves, heaters, and VFDs. The discrete inputs are typically from switch type (operator and process) devices, and the discrete outputs are typically for activating hardware such as valves, lights, etc.

The design and development of the oven/tunnel control system software should follow the principles of ISPE GAMP (Good Automated Manufacturing Practice) 5, a risk-based approach to compliant GXP computerized systems (16). This guideline details a software lifecycle from conception through decommissioning.

VALIDATION OF DRY HEAT PROCESSES

Validation consists of the documented installation and operational qualification of the equipment used to deliver the dry heat process, followed by the documented performance qualification of the physical and biological aspects of the dry heat process.

Installation Qualification

The purpose of the installation qualification (IQ) is to demonstrate that the equipment is suitable for its intended use as installed and that it has been appropriately built according to the user's requirement specifications. It is the documented verification that the facilities, systems, and equipment, as installed or modified, comply with the approved design and the manufacturer's recommendations (17). The IQ also verifies that the documentation required for the equipment's operation, maintenance, calibration, and cleaning/sanitization is provided, and that programs are in place to maintain the equipment in a continued qualified state for operation.

The equipment should have available the appropriate utilities including air supply, electrical, exhausts, cooling water and HVAC (heating, ventilating and air conditioning) provisions to maintain the desired environmental temperatures and differential pressures. Items to be considered during the qualification testing are as follows:

- Safety and alarm features should be installed correctly and tested
- Operations and maintenance manuals should be available for the equipment and chart recorders
- Software should be validated and compliant for appropriate regulatory authorities

- Program logic control manual should be available
- Wiring and "as built" diagrams for equipment should be available
- HEPA filters should be qualified and tested to meet current standards
- Accuracy of temperature, time, airflow, pressure and belt speed (as applicable) monitoring devices should be established and documented
- Calibration certificates should be available for controlling instruments, such as timers, pressure gauges, anemometers, thermocouples, and recording charts
- Sensors and equipment should be placed on a calibration and preventive maintenance schedules
- Details of cycle programming should be available
- Any necessary inventory of spare parts should be considered at this time

IQ testing of the control systems for computerized equipment and systems will be determined by and specific to the type of computer system.

Drawings of equipment and instrumentation are generally developed during the design phase of the project and are used to build the system. These become an important historical document to track the equipment and subsequent changes to the equipment. Items to be considered at this time for documentation and verification include the following:

- HEPA filters
- Validation ports
- Instruments
- Conveyor systems
  - Nominal size (length and width)
  - Materials of construction
  - Drive motor
- Fans
  - Type
  - Rated capacity
  - Motor horse power/revolutions per minute (RPM)/volts/amps/phase
- Motors
- Gates
  - Numbers and settings of gates
  - MOC of the tunnel and gates
- Heaters
  - Type
  - Rated capacity
- Cooling elements
  - Type
  - Rated capacity

Any inconsistencies between the drawings and specifications and the system and componentry as installed should be resolved at this time to ensure that the documentation on file accurately represents the installed system.

HEPA Filter Integrity Testing

Each HEPA filter installed must pass integrity testing in situ to verify the integrity of the filter frame seals and the proper seating of the filter in the frame or grid. Filter testing includes flow rate and integrity testing, and air testing downstream for particulates to ensure that filters do not leak or shed particles. Items to be considered and documented include the following:

- Procedures for HEPA filter integrity testing and repair
- Serial numbers and locations of filters

- Testing medium used for integrity testing
- Upstream concentration of testing solution
- Integrity and leak test report
- Repair and retesting report
- Surface area of repairs
- Velocity of air
- Grid location of repairs.

Support Utilities
Verify any of the critical utilities necessary to support the dry heat oven or tunnel. These will include electrical power, cooling water, and instrument air. Any discharge connections from the equipment should also be verified.

Critical Instrumentation Installation
Verify the installation of any critical instruments, which are those used to make operational decisions or which are a part of the production or maintenance records. These may include the following:

- Temperature sensors, recorders, or display systems
- Timers, recorders, or display systems
- Differential pressure sensors, recorders or display systems
- Belt speed sensors, recorders, or display systems

All of the IQ documents should be reviewed and approved by the appropriately designated individuals responsible for the quality of the installation process.

Operational Qualification

Operational qualification is the documented verification that the facilities, systems, and equipment, as installed or modified, perform as intended throughout the anticipated operating ranges (17). The operational qualification also demonstrates that all controls function properly and that temperature control and uniformity meet functional specifications.

Items for consideration during the operational qualification are as follows:

- Programmable logic reliability—testing each stepped sequence
- Door interlock (ovens)
- Gasket integrity (ovens)
- Blower rotation—RPM and direction of rotation
- Heater elements—ensure that all are working
- Room balance—ensure positive pressure to retain the integrity of clean areas
- Air filtration—integrity of the air supply, recirculation and exhaust HEPA filters that supply air for ovens. Verify the integrity of in-feed, hot, and cooling zone HEPA filters for tunnels
- Belt speed and speed recorder for tunnels
- Air velocity profiles across the unit
- Monitoring of nonviable particles required to demonstrate the appropriate clean area classification
- Sanitization of cooling section (for tunnels equipped with heating coils)

An operating procedure for the operation of the dry heat oven or tunnel should be written and available at this stage, with documentation of operator training considered for inclusion as a part of the operational qualification protocol.

Any operating controls on the control panel for the oven or tunnel should be tested to ensure that they function according to manufacturer's or system specification. These include any switches, pushbuttons, indicators, controllers, recorders, etc.

Safety and alarm testing should be conducted and include any features necessary to ensure personnel and equipment safety. Some typical alarms and interlocks may include the following:

- High/low temperature alarms
- High/low pressure alarms
- Airflow alarms
- Belt speed alarms
- Belt/temperature interlocks
- Fan/heater interlock alarms
- Emergency stop button
- Differential pressure alarms (across the HEPA filters)
- Gate interlocks
- Abort alarms

Loss of utility testing should be conducted to verify the response of the equipment to loss of electrical power or air supply. It is important that the critical data not be lost, and also to verify that the response of equipment is appropriate upon resumption of power or air supply.

Airflow Velocity Testing
Testing is conducted in critical zones (class 100, ISO 5) to verify and document sufficient airflow velocity across the face of the HEPA filter. This testing is generally conducted at ambient temperature.

Airflow Pattern Testing
Airflow testing is conducted in tunnels to ensure that integrity of clean areas or zones is maintained, and that turbulence does not cause any clean areas to be compromised. Testing should verify unidirectional flow from higher pressure or clean zones to lower pressure or less clean zones. Consideration should be given to performing airflow testing with gates at both maximum and minimum settings. It is preferable to perform any visual verification of airflow patterns through use of a vapor generated in a manner that leaves no residue on the surface of equipment being tested.

Nonviable Particulate Testing
Nonviable particulate testing is applicable in ovens and tunnels where open containers or items are being processed. Testing is generally conducted at processing temperature with the particle sampling probe placed at representative locations.

Empty Chamber Studies
Empty chamber temperature distribution studies are performed to show temperature uniformity across the chamber or tunnel and to identify any cold and hot spots. Temperature sensors should be placed to give the greatest amount of information about the space to be occupied by the load being processed. For an oven, the temperature sensors are often place in a three-dimensional "X" pattern to ensure that the top, middle, bottom, front, and back of the sterilizer are being evaluated.

In a tunnel, if different temperature zones are used, each zone should be monitored with temperature sensors. Sensors can be mounted on a metal bar (usually stainless steel) above the conveyor belt to map the temperature within the tunnel. Temperature sensors should also be placed next to the fixed sensors that will be used to monitor and control during routine processing. The critical parameters should be recorded, and process variability established at this time.

It should be noted that some people forego the empty chamber studies and use the heat penetration and distribution data established during the loaded chamber studies (see below) to establish temperature uniformity data used in validation.

Process Validation

Process validation is the documented evidence that the process, operated within established parameters, can perform effectively and reproducibly to produce a medicinal product meeting its predetermined specifications and quality attributes (17). The performance qualification involves studies of temperature distribution, heat penetration, and endotoxin or BI (microbial) challenges.

Loaded Chamber Studies

Loaded chamber studies are conducted on the worst case loading pattern, utilizing the information gained from the thermal-mapping studies of the empty chamber or tunnel, if applicable. The intent of the loaded chamber studies is to obtain and document the temperature distribution and penetration data with the actual items to be processed. Temperature sensors are placed across the width of the belt in a tunnel, and are placed at the front, middle, and rear of the load passing through the tunnel. As noted previously, temperature sensors in an oven are placed in a three-dimensional pattern that ensures that the top, middle, bottom, front, and back of the oven are being evaluated.

The purpose of monitoring the loaded chamber or tunnel is to ensure that the coolest location in the load reaches the required temperature for the required length of time as identified during the developmental studies. The spread of temperatures throughout the chamber or tunnel is measured, and ability to achieve the desired biological inactivation is demonstrated in the coolest portion of the load. Endotoxin reduction studies could be conducted at this point.

Temperature distribution thermocouples or sensors are intended to monitor the air temperature within the oven or tunnel and should not be in contact with any surface. Temperature penetration thermocouples or sensors are intended to measure the temperature of the items being processed and should be in contact with the surface of the item itself. The locations of all temperature sensors should be documented, showing the location of the sensor within the chamber or tunnel, within the individual items and within the load itself. Temperature sensors should also be placed next to the recording and controlling temperature sensors in the oven or tunnel where possible.

The biological inactivation portion of validation studies demonstrate that the delivered endotoxin or microbial inactivation has been delivered to the product or items being processed, and that the process is repeatable and reproducible. Using the data obtained from the developmental studies, and the heat penetration and distribution studies, the process is run three times, most often at reduced time or temperature for a batch oven and reduced temperature or increased belt speed for a tunnel. Laboratory testing is conducted to evaluate endotoxin inactivation or microbial lethality, and the studies are documented.

Documentation

Items to be considered in the documentation of the qualification studies include the following:

- A conclusion stating whether the objective of the study has been achieved
- Confirmation that all data collection instruments and equipment were within calibration tolerances during the interval comprising the validation studies
- Testing performed during the studies was properly documented and that test methods were validated where applicable
- Certification for the endotoxin and/or biological indicator used in the studies, i.e., manufacturer, origin of endotoxin/microorganism, inoculum level, etc.
- Sampling and numbers of replicate trials are rational and supported
- Operating parameters, process parameters, and environmental parameters have been met as required
- Any nonconformances, their cause, and resolution have been addressed

- Equipment, processes, and products covered by the qualification studies are identified, whether as a result of inclusion and direct testing in the studies, or through use of a bracketing or matrixing approach, or equivalent determination.

POST-VALIDATION ACTIVITIES

Because of the operational importance of depyrogenation/sterilization processes and the potential for adverse consequences to product quality, continuing evaluation, control, and maintenance of depyrogenation/sterilization cycle performance is critical. Evaluation of depyrogenation/sterilization cycle performance is typically accomplished through data monitoring and periodic requalification. Control is achieved through investigation and resolution of cycle deviations and equipment/process change control. Finally, to ensure maintenance of performance, effective preventative maintenance and calibration programs are essential.

Use of Risk Management Postvalidation

Post-validation activities ensure that the system and processes supporting depyrogenation/sterilization continue to operate as intended and achieve desired levels as required by the production process requirements. These activities encompass requalification and revalidation, which have traditionally been executed on a periodic basis, regardless of historical depyrogenation/sterilization process performance or potential impact to product quality. Many in industry have begun to make use of risk management and statistical process control methodologies to identify those systems that pose the greatest risk based on inherent variability or process capability and concentrate post-validation efforts accordingly. For very capable processes, post-validation activities may be limited to periodic or continual monitoring, depending on the level of automation, with revalidation conducted as an event-driven activity.

Routine Monitoring

Following completion of the cycle development and performance qualification exercises, monitoring of the routine operational cycles should be performed to assure an ongoing state of control. Critical parameters should be documented and data recorded (critical data) for each cycle. Routine monitoring data should be analyzed to ensure the system has remained in a state of control as demonstrated by the qualification data. The routine operational cycle is typically controlled to produce additional lethality over the qualified minimum acceptable cycle to provide increased sterility assurance. Cycles that have not met minimum defined critical cycle parameters should be rejected. Deviations from key parameters should be investigated and their impact assessed to consider whether the cycle is acceptable.

An alarm system for temperature and/or pressure may be used to facilitate the detection of any deviation from the defined process parameters.

OPERATIONAL PARAMETERS

Critical operational parameters may include the following:

- **Temperature**
  Temperature should be monitored using calibrated, redundant, independent monitoring devices with defined accuracy.
  Temperature and pressure profiles for the depyrogenation/sterilization cycles should be recorded and assessed on a periodic basis to confirm that no significant change in the qualified state has occurred.
- **Pressure**
  The system differential pressures should be continuously monitored at appropriate locations.
- **Time**
  Time duration of cycle phases should be monitored to ensure the depyrogenation/sterilization cycle remains within the qualified state.
- **Belt speed (for tunnels)**
  The belt speed should be continuously monitored and recorded.

Strategies for the monitoring of depyrogenation/sterilization parameters and their associated alarms should be designed to provide the appropriate data to demonstrate that the depyrogenation/sterilization process was performed successfully. System monitoring may be automated, manual, or a combination of both, provided that the data obtained is accurate and easily retrieved. The information recorded for each run should be linked to the validation of the cycle. Resumption of a depyrogenation/sterilization cycle following resolution of an alarm condition should ensure that the minimum exposure time is achieved.

Change Control/Revalidation

A robust change control system should be in place to maintain the validated state of the depyrogenation/sterilization process.

Any proposed changes to the depyrogenation/sterilization process (including procedures, hardware, software, cycle configuration, supply utilities, filter types/sizes) should be evaluated to determine the potential effects of those changes on the depyrogenation/sterilization cycle and the extent of requalification/revalidation required to demonstrate that the modified process performs as intended and still meets the applicable acceptance criteria.

Periodic Requalification/Revalidation

A periodic review of the system should be performed to ensure the state of control is maintained and to evaluate the impact of cumulative "minor changes" over the review period.

This review should also include review of performance data from various monitoring sources (e.g., engineering, maintenance, and calibration data) to verify that there have been no adverse trends or drifts away from the baseline performance established during validation. A review of change control documentation should be conducted as part of the requalification/revalidation.

Review frequency should be based on the system's intended use and applicable regulatory expectations. For systems claiming sterilization, requalification may include supplemental thermal and/or biological testing.

Preventative Maintenance Strategy

To ensure consistent system performance, a maintenance strategy should be in place that addresses potential changes in material and component performance because of operation, exposure, and time. In particular, the strategy should take into account how thermal and pressure cycles associated with heat-up, exposure, and cool-down may impact the service life of various components, particularly HEPA filters.

During development of a maintenance strategy, special consideration should be given to polymer replacement practices because of their criticality in maintaining system integrity and their limited lifetime. In general, polymer service life is affected by various operational stresses such as thermal conditions, process frequency, product chemistry, and cleaning frequency.

Within the preventative maintenance program, components that are critical to depyrogenation or sterilization performance should be periodically inspected and/or replaced. The frequency of the preventative maintenance may be determined on the basis of component maintenance history, manufacturer recommendations, or risk evaluation and mitigation.

CALIBRATION STRATEGY

The calibration program should include instruments that are used to control and monitor the cycle. Both the control of the depyrogenation/sterilization cycle and the confirmation of successful cycle completion are dependent on the proper indication and recording of critical operational parameters. Calibration serves as both the means to maintain instrument performance as well as to document proof of performance.

Determination of calibration tolerances and periodicity is determined by instrument capability, history, manufacturer recommendations, and process risk. The impact of instruments found outside calibration tolerances during periodic recalibration evaluations should be investigated. A risk assessment can be used to establish instrument calibration requirements.

CONCLUSION

This chapter has provided a brief review of historical literature and current practices in dry heat depyrogenation and sterilization processes. While not as widely used as other modes of sterilization, dry heat does provide a very effective and reproducible means of sterilization and is a very effective process for inactivating endotoxin.

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12 | Radiation sterilization

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INTRODUCTION

The radiation sterilization industry traces its origin back to over 50 years ago. The first irradiator for commercial sterilization of medical device products came on-line in the United States in the 1950s. Over the intervening 50 years, radiation in the form of high-energy electrons produced by high-power accelerators or gamma rays produced by radioisotopes has been used to terminally sterilize a broad spectrum of medical devices and different types of pharmaceutical products. More recently a third source of radiation; that is, X-ray (bremsstrahlung) radiation has been introduced to the radiation sterilization industry. The proven efficacy of the process and available methodologies to validate a sterility assurance level (SAL) of 10^{-6} has made radiation an attractive alternative for terminal sterilization of many types of products. Because radiation sterilization is classified as a cold process, it also can be used to sterilize heat-labile products. As a final attribute, products that have been radiation sterilized can be released on the basis of certification of the absorbed dose of radiation delivered to the product, that is, dosimetric release. There is no need to conduct post-irradiation sterility testing of the product, thereby expediting time to market for critical products and cost for the sterility tests.

This chapter begins with a discussion of the fundamentals associated with the interaction of radiation with materials. A section is devoted to dosimetry, a critical part of the process, which forms the basis for dosimetric release of product. All three modalities that are presently used for radiation sterilization of products are covered including design and operation of irradiators and control of the irradiation environment. The chapter addresses available methods for setting an acceptable minimum dose to achieve the desired SAL as well as an acceptable maximum dose that ensures the safety and performance of the irradiated product over its lifetime. A final step before routine processing of product, termed "performance qualification," completes the discussion of the radiation sterilization process. The last sections of the chapter address radiation chemistry of liquids and solids, radiation effects, and a final section on irradiation of specific drug products. A more comprehensive discussion of the radiation sterilization process can be found elsewhere (1).

INTERACTION OF RADIATION WITH MATERIAL

Sources of Radiation

Three sources of radiation are used in the radiation sterilization process. The first source of radiation is gamma ray–emitting radioactive isotopes. Gamma rays are pure electromagnetic energy in the form of quanta of radiant energy called photons. The energy of the photons is dictated by the radioisotopic source. Two isotopes are used in the radiation sterilization industry with the principal isotope being cobalt-60, which emits two photons per disintegration of the nucleus with energies of 1.17 and 1.33 MeV. The other isotope cesium-137 emits one photon per disintegration at an energy of 0.662 MeV. Cobalt-60 is usually the isotope of choice for commercial applications of radiation processing. It can be manufactured in a metallic form, which is inherently stable and produced in much higher specific activities, that is, approximately 100 curies/g, than cesium-137. Because of its high specific activity; cobalt-60 can be fabricated in compact-energy efficient sources. For example, a single source of about 10,000 curies can be fabricated in a geometry that is about 18 in. in length by less than 0.5 in. in diameter. To achieve the megacurie levels of activity that are used in commercial gamma irradiators, literally hundreds of these sources are used to build the source plane(s) in a commercial irradiator. Cesium-137 has one advantage over cobalt-60 in that its half-life, which is a measure of the rate of decay of the radioactive isotope, is much longer than that of cobalt-60. The half-life of cobalt-60 is 5.27 years and of cesium-137 is 30.17 years. Because of its shorter

half-life, cobalt-60 loses about 12% of its activity in a year, whereas cesium-137 only loses about 2% of its activity in a year. For this reason, isotope replacement in a cesium-137 irradiator can be done on a much less frequent basis than in cobalt-60. This fact favors cesium-137 for use in what are referred to as self-contained irradiators such as blood irradiators that are used to prevent transfusion-induced graft-versus-host disease.

The second source of radiation consists of high-power accelerators that generate high-energy electrons. As we will see these high power accelerators are capable of producing output powers up to several hundred kilowatts. Dependent on the accelerator design, electron energies can range from less than 1 MeV up to about 10 MeV. The third source of radiation occurs when the high-energy electrons from a high-power accelerator impinge on a conversion target. Because the conversion efficiency increases as the square of the atomic number of the target material, conversion targets are fabricated from high atomic number materials, for example, tantalum. The high-energy electrons that impinge on the conversion target are deflected in the field of the nucleus of the atoms in the conversion target and in the process of being accelerated emit electromagnetic radiation in the form of X-rays. This form of electromagnetic radiation is referred to as bremsstrahlung radiation, which translated literally means "braking radiation." The radiation appears as a continuous spectrum of energies with a maximum energy equal to the energy of the incident electrons. The maximum energy for this source of electromagnetic radiation is limited to 7.5 MeV, which is dictated by the need to avoid unwanted radioactivity that could potentially be induced in the irradiated materials via photonuclear reactions at higher photon energies (2).

A common attribute of the three sources of radiation that are employed in the radiation sterilization process resides in the fact that the energy of the incident radiation is sufficient to ionize the atoms that make up the molecules of the materials that are irradiated. In the ionization process, sufficient energy is imparted to the orbital electrons of an atom to remove the electrons from their bound state around the atom. Dependent on the specific element, the energy to remove the outer most electrons, that is, first ionization potential, from the atom ranges from a few eV up to approximately 20 eV. Because the energy of the incident radiation is measured in MeV or millions of eV, sufficient energy is obviously available to initiate the ionization process. For this reason, these radiations are referred to as ionizing radiations and are differentiated from nonionizing radiation such as optical radiation, that is, light, and infrared radiation, that is, heat. The energy of the photons from these two sources of electromagnetic radiation is less than a few eV, which is insufficient to ionize an atom. There is no mystique to the ionization process; it is simply a matter of energetics.

Interaction of High-Energy Photons with Materials

At intermediate photon energies that characterize the gamma ray and X-ray (bremsstrahlung) sources of radiation used in the radiation sterilization process, the dominant channel for interaction of the photons with the orbital electrons occurs via a process called Compton inelastic scattering. This method of energy transfer is named after the person that first described the quantum mechanical relationships governing the scattering process (3). A photon undergoing Compton scattering, transfers part of its energy to the orbital electron. The amount of energy transferred to the electron will depend on the quantum mechanical relationships governing the scattering event, but is usually sufficient to not only ionize the atom but also leave the electron with significant kinetic energy. In fact, the most probable Compton scattering event is a backscatter of the photon, which transfers maximum energy to the electron. For gamma rays emitted by a cobalt-60 source, a backscattered photon will deliver about 1 MeV to the orbital electron. These high-energy electrons are referred to as primary electrons. The scattered photon continues to undergo scattering events and generate additional primary electrons until its energy is dissipated. The primary electrons have sufficient energy to ionize other atoms via an electron-electron inelastic scattering process. A whole cascade of secondary electrons can be produced in this manner. From a numerical standpoint, it is these secondary electrons that are the source of the physical and chemical events that lead to the radiation-induced changes in materials and sterilization of the drug product. The photons function only as an initiator of the process that leads to radiation sterilization throughout the bulk of the drug product.

Interaction of High-Energy Electrons with Materials

For most pharmaceutical products and low atomic number materials that make up the bulk of medical device products, high-energy electrons from an accelerator mainly lose energy in the material via a large number of inelastic scattering events with the orbital electrons. The energy loss per collision is relatively low compared to the energy of the incident electron. For example, the average energy loss per scattering event for a 10 MeV electron is on the order of 100 eV per collision or less (4). It is of interest to note that the energy lost by primary electrons that are produced by Compton scattering of photons occurs in the same manner. Therefore, regardless of the modality of the incident radiation, the energy transfer mechanisms that lead to the sterilization of pharmaceuticals and changes in material properties are the same. The effects of high-energy photons are indistinguishable from those produced by the same amount of energy per unit mass (absorbed dose) imparted by high-energy electrons. This equivalence of effects is the basis for the use of both forms of radiation in radiation processing. However, the rate of energy deposition for the different sources of radiation can be quite different, and this parameter may play an important role in the resultant effect on materials that are irradiated. This topic will be discussed in a subsequent section of the chapter.

RADIATION-ABSORBED DOSE AND MEASUREMENT

Definition of Absorbed Dose

Energy must be absorbed by a material to cause change be it sterilization of a drug product or change in a material property. Energy from the incident radiation is transferred to the material by various pathways that are discussed in the previous section. The energy that is absorbed in a material from radiation exposure is termed absorbed dose. It is defined as the quantity of ionizing radiation energy imparted per unit mass of a specified material (5). The SI unit of absorbed dose is the gray (Gy), where 1 gray is equivalent to the absorption of one joule per kilogram of the specified material (1 Gy = 1 J/kg). The previous unit that was used to measure absorbed dose was the rad, which is no longer in use nor recommended (100 rads = 1 Gy). It is of interest to note that absorbed dose is defined in terms of a specified material. For example, two different materials could be exposed to the same incident radiation field yet receive different absorbed doses. Absorbed dose is measured with well-characterized devices called dosimeters and dose is normally recorded as dose delivered to the dosimeter. The standard material in which absorbed dose is usually expressed is water. Many dosimeters that are commonly used to measure absorbed dose have energy absorption characteristics that are water equivalent so absorbed doses are effectively reported in terms of absorbed dose in water. In radiation sterilization applications that involve the biocidal action of radiation on microorganisms, the difference in absorbed dose between microorganisms and water is relatively small. However, this may be a mute point because the same dosimeters that are used to measure absorbed dose during routine processing of a product are oftentimes used to validate the acceptable minimum and maximum doses for irradiation of the product.

Dosimetry—A Critical Part of The Process

Absorbed dose is a critical parameter that impacts the radiation process from its beginning to its end. Dosimetry, that is, measurement of absorbed dose, enters the radiation process during operational qualification (OQ) of an irradiator, which occurs before the pharmaceutical product is irradiated (6). Studies conducted during OQ demonstrate the capability of the irradiator to deliver the range of doses required for the sterilization process that has been previously specified. OQ demonstrates that the irradiator, as installed, is capable of operating and delivering appropriate doses within defined acceptance criteria. As a first step in the radiation sterilization of a pharmaceutical product you need to determine an acceptable minimum dose that ensures the specified SAL is satisfied and an acceptable maximum dose that ensures the safety and performance of the drug product over its lifetime. Established methodologies that involve a matrix of test irradiations are used to validate an acceptable minimum dose. Accurate measurement of absorbed dose delivered to test samples is a critical part of this validation program. An acceptable maximum dose is determined by irradiation of test samples at specified absorbed doses and post-irradiation analysis of the test samples. Doses need to be delivered to the test samples in a precise manner, which requires accurate

measurement of the absorbed doses. Following validation of acceptable minimum and maximum doses, the pharmaceutical product goes through another step before routine processing, which is called performance qualification (PQ) (6). In PQ, the product is loaded into the irradiation containers in accordance with a specified loading pattern, and absorbed dose is measured at prescribed locations in the product load. The information from this dose map is used to identify the location and magnitude of the minimum and maximum doses. Upon completion of the PQ study, the product is ready for routine processing. During routine processing, absorbed dose is measured at various locations in the run to confirm that all product in the run received the minimum absorbed dose and no product in the run exceeded the established maximum absorbed dose. As a final step, the absorbed dose delivered to product along with its certification is used to release the product. This process is referred to as dosimetric release. With dosimetric release there is no requirement or need to perform post-irradiation sterility testing. This topic is discussed later in the chapter.

Because of the importance of absorbed dose in the overall radiation sterilization process, we obviously need to have a quantitative tool for its measurement. Furthermore, the measurements need to be accurate and we must be confident in the measurement results. The quantitative tool that meets these requirements is called a dosimeter and is defined as a device that, when irradiated, exhibits a quantifiable change that can be related to the absorbed dose in a given material using appropriate measurement instruments and procedures. A key word in the definition of dosimeter is "quantifiable." Dosimeters are highly characterized and calibrated devices. Dosimeters are only one part of the measurement system, which is referred to as the dosimetry system. In addition to the dosimeters, you require a calibrated instrument for measuring the dosimeter response as well as standards and procedures. A dosimetry system is defined as a system used to measure absorbed dose, consisting of dosimeters, measurement instruments with associated reference standards, and procedures for the system's use.

Method of Measurement

The dosimetry systems that are used in the radiation sterilization industry are divided into various classes dependent on where they fit in the metrological hierarchy and field of application. Reference standard dosimetry systems are of high metrological quality and are used to calibrate the dosimetry systems that are used for routine measurements of absorbed dose at an irradiator. This class of dosimetry systems may be held at a given location, that is, irradiator site, or take the form of transfer standard dosimetry systems operated by a national standards laboratory or an accredited dosimetry calibration laboratory. Transfer standard dosimetry systems are sent to an irradiator for irradiation and then returned to the calibration laboratory for measurement. The concept of high metrological quality implies a dosimetry system with low uncertainty and traceability to appropriate national or international standards. A routine dosimetry system, which is used for routine measurements of absorbed dose at an irradiation facility, is calibrated against a reference standard dosimetry system. The dosimeters that are used for calibration purposes have high metrological quality and form a separate class of dosimeters from routine dosimeters that are used to measure absorbed dose at an irradiator. Routine dosimeters are still highly characterized and calibrated devices that provide accurate measurements of absorbed dose. Tables 1 and 2 provide examples of dosimeters that are used to calibrate other dosimeters and for routine measurement of absorbed dose (7).

Table 1 Dosimeters for Calibration Applications

Dosimeter	Description	Radiation-induced effect	Method of analysis
Alanine	Pellet or film containing alanine, an amino acid	Generation specific stable free radicals	Analysis of radiation-induced free radicals using electron paramagnetic resonance (EPR)
Ceric-cerous sulphate	Ceric sulphate and cerous sulphate in sulphuric acid	Change in optical absorbance or electropotential	Spectrophotometry or potentiometry

Note: The cited dosimeters are also used for routine measurement of absorbed dose.

Table 2 Dosimeters for Routine Measurement of Absorbed Dose

Dosimeter	Description	Radiation-induced effect	Method of analysis
Calorimeter	Mass of energy absorbing material, thermal insulation and calibrated temperature sensor	Increase in temperature	Temperature measurement
PMMA (Perspex)	Calibrated chip of PMMA in sealed sachet	Color, darkening of dyes	Spectrophotometry
Radiochromic film	Thin film containing special dye precursors	Dyes becoming colored	Spectrophotometry

IRRADIATOR ENVIRONMENTS

Gamma Irradiators

Irradiator Categories

Nuclear regulatory agencies have divided gamma irradiators into four categories according to their design and operation. Because only two of the four categories may find significant application for irradiation of pharmaceuticals, the discussion is limited to these categories. Category I irradiators are self-contained, dry source storage irradiators. This category irradiator was noted in the section on the interaction of radiation with material as a possible source for blood irradiation. The design of category I irradiators typically does not allow a large volume of product to be irradiated over a given period of time. Irradiation of blood as well as some types of drug products fit that product profile. Other possible applications for category I irradiators may include irradiation of test product, clinical studies, research, dose validation, and calibration. The radioactive source in category I irradiators remains shielded inside a biological shield at all times, and it is not possible for an individual to come in contact with the source at any time. For this reason, the regulatory agencies treat this category irradiator differently from the other category irradiators. Category I irradiators are relatively small, that is, typically less than several feet in diameter and several feet in height, and could easily fit in the space that normally serves as a room in a laboratory. In fact, if you were to visit a national calibration laboratory such as the one at the National Institute of Standards and Technology (NIST) in Washington, DC, you could find gamma cells, which are a type of category I irradiator, sitting in a laboratory and being used to calibrate dosimetry systems. Category IV irradiators are panoramic, wet source storage irradiators, which are designed for high-throughput operation. When not in use, the sealed gamma source is stored in a large pool of water within a shielded room that is referred to as the cell. When all personnel have safely exited the cell and a safety system is activated, the sources are automatically removed from the pool of water into the room and irradiate product that is within the cell. Because people can enter the room where the sources are stored and used, regulations for operation of category IV irradiators are more stringent than for category I irradiators.

Dependent on the mission, the design and operation of category IV irradiators can vary significantly. However, there are several common features that will be found in all category IV irradiators. First you need a source of ionizing radiation that comes in the form of a radioisotope, usually cobalt-60 that is doubly encapsulated to form sealed sources. Sources of this type are grouped into racks that are stored in a pool of water inside a shielded room called the cell. Because the radiation levels to kill microorganisms are typically 1000 times greater than the levels to kill individuals, you need a biological shield that surrounds the cell. The shield typically comes in the form of concrete walls and ceiling approximately 6 ft in thickness. However, as a means to reduce the size of the cell, the biological shield is sometimes metallic in nature. Of course, you need a redundant safety system to protect personnel and preclude entry to the cell when the sources are exposed. Category IV irradiators are typically high-throughput systems; that is, some are capable of processing several million cubic feet of product per year, and for this reason you need a conveyance system that is capable of moving large volumes of product into and out the cell on a routine basis. A control system that usually takes the form of a programmable logic controller is required and lastly an air exchange system is required to

remove ozone from the cell that is produced from interaction of the gamma rays with oxygen molecules in the air.

Operation of Category IV Irradiators

A conveyance system moves product through the irradiator in various size containers that depend on the design and mission of the irradiator. For example, these containers may consist of aluminum or stainless steel boxes called totes, carriers, or possibly entire pallets of product. Dependent on the irradiator design, totes may vary in length and height from a few feet up to several feet. The width of the tote, which is the dimension through which the gamma rays must penetrate, is typically 2 ft or less in size. A carrier may have a footprint similar to a tote but be several feet in height. Some carriers have a single shelf with a limited volume for irradiation. In some irradiators, an entire pallet of product is loaded onto the conveyance system as an entity. Regardless of the size and design of the irradiation container, most gamma irradiators move product through the cell in what is referred to as a "shuffle-dwell" principle. In a shuffle-dwell operation, the irradiation containers shuffle to a location in the cell where they accumulate in rows that surround the source plane. They dwell at that location for a preset time called the cycle time after which they shuffle to the next location and repeat the operation until the container has fully traversed the cell.

Category IV irradiators are designed to operate in a batch mode or continuous mode. In a batch mode, the irradiation containers are loaded with product and moved into the cell, where they are positioned around the source location. After this operation is completed, the cell is exited, the safety system set, and the source raised into the cell room. The irradiation containers then proceed to increment around the source in a shuffle-dwell mode until the product has received the required dose. The source is then lowered into the pool of water, and the irradiation containers removed from the cell. In a continuous mode of operation, the irradiation containers continuously move into and out of the cell while the source(s) is in the exposed position. This mode of operation can be accomplished by moving the irradiation containers through a maze before entry to the cell. Figure 1 shows a tote box irradiator that operates in a continuous mode. Up to 5 MCi of cobalt-60 can be loaded into this irradiator, so it is a high-throughput system. The irradiation container is approximately 3.5 ft in length by 6 ft in height by 2 ft in width. The irradiation containers are moved into the cell through a maze via a floor conveyer. Once in the cell, the totes accumulate around the source plane and proceed to increment around the source in a shuffle-dwell mode. A cut-a-way of the biological shield and air exchange system also can be seen in Figure 1.

It is of interest to note that in Figure 1 the totes totally surround the cobalt-60 source. This is due to the fact that the radiation field is isotropic in nature; that is, the gamma rays are emitted in all directions from the cobalt-60 source. For this reason, it is important to surround the cobalt-60 source with product containers thereby capturing as many of the source photons as possible and in the process increase the intrinsic efficiency of the irradiator. Because of the size of the tote and volume of product in the tote, not all product in the tote will receive the

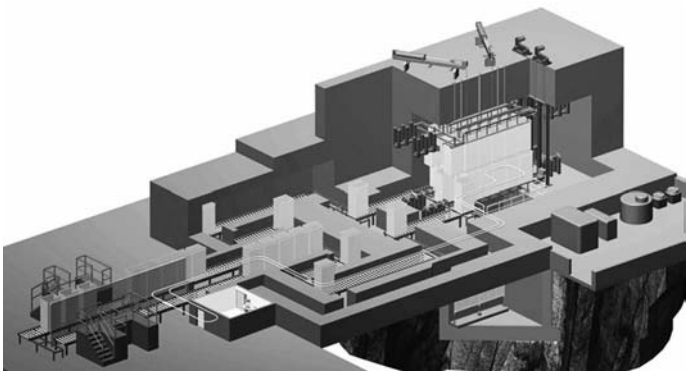

Figure 1 Gamma tote box irradiator.

same absorbed dose. This is due to shielding by the product, that is, absorption of gamma rays by the product, and geometric attenuation, which is due to the fact that the radiation field is isotropic. Even in an empty tote, the absorbed doses are different at various locations within the tote due to the different distances from the source. A similar effect can be noted from the isotropic emission of optical radiation from a light bulb. The ratio of the maximum absorbed dose to minimum absorbed dose delivered to product in the tote is referred to as the dose uniformity ratio (DUR). There are obvious advantages to keeping this ratio as close to one as possible. Techniques for doing so are discussed in the section on methods of control.

Electron Beam Irradiators

Design

A high-power electron beam accelerator is at the heart of an electron beam irradiator. The accelerator serves as the source of radiation analogous to gamma rays in gamma irradiators. The different types of electron beam accelerators that are used in the radiation sterilization share one common attribute, which is high-output power. Power equates to throughput, and electron beam irradiators similar to gamma irradiators are capable of processing millions of cubic feet of product per year. Electron beam irradiators share many of the same design features as gamma irradiators. You need a biological shield to protect individuals from the high levels of radiation that exist in the cell when the accelerator is operational, a conveyance system to transport product in front of the beam of electrons and a safety system that precludes entry to the cell when the accelerator is operational. In addition you need a system for controlling the irradiator and an air recirculation system to remove ozone from the cell. An example of an electron beam irradiator is shown in Figure 2. In this type of accelerator, electrons are accelerated in a resonant cavity that is cylindrical in geometry. This accelerator design is capable of very high-output powers and dependent on the port from which the electrons are extracted can deliver different energy electrons up to 10 MeV. A cut-a-way of the biological shielding is shown in Figure 2 along with the conveyance system that moves product via a floor conveyor under the electron beam. Because electrons are charged particles, they can be steered and directed using magnetic fields. In Figure 2, the electrons exiting the accelerator are defected 90° and steered to the product that is moving on a floor conveyor in a room below the accelerator. Because the electron beam exiting an accelerator is typically only a few centimeters in diameter, the beam needs to be scanned in a transverse direction to the motion of the product on the conveyance system thereby ensuring high-energy electrons uniformly irradiate the entire product surface. Magnetic fields can be used to deflect the beam using a device called a scan horn. Beam scan and conveyor motion need to be synchronized to ensure all parts of the product are irradiated. The irradiator shown in Figure 2 only represents one type of electron beam accelerator and conveyance system that is used to irradiate product. For example, linear accelerators called Linacs may be used as the source of high-energy electrons and product conveyed in carriers horizontally in front of the beam of electrons. Dependent on the application and mission of the irradiator, other configurations are also possible.

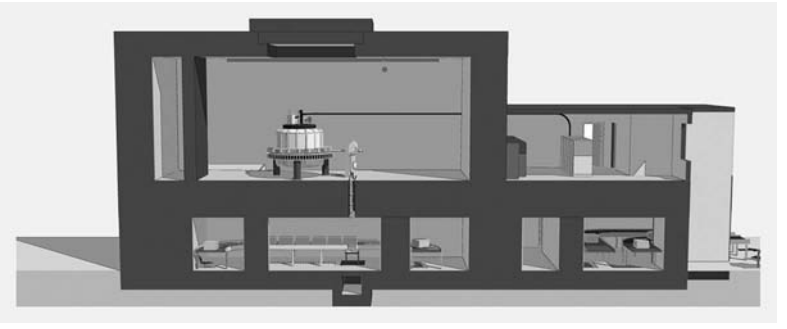

Figure 2 Rhodotron electron beam irradiator.

Operation of Electron Beam Irradiators

Unlike the isotropic radiation environment in a gamma irradiator, the radiation environment in an electron beam irradiator is in the form of a beam that can be steered and directed using magnetic fields. These are nice attributes that can be used to optimize the intrinsic efficiency of the irradiator. Because the radiation emitted by an accelerator is in the form of a beam of radiation, it is only necessary to move the product in a controlled manner in front of the beam; that is, you don't need to surround the accelerator with product as is done in a gamma irradiator. The unit of product that is moved in front of the beam may be in the form of a single box or possibly an entire carrier of product. It has sometimes been noted in the literature that processing time in an electron beam irradiator is much faster than that in a gamma irradiator. This may be true for a single box of product because of the method that is used to convey product through the irradiator. In an electron beam irradiator the box of product is effectively scanned in front of the beam, whereas in a gamma irradiator it needs to be placed in an irradiation container and incrementally moved in a shuffle-dwell method through the entire cell. In one case, the box of product can be literally processed in seconds while in the other case process time can take up to a few hours. However, for large volumes of product, that is, truckload quantities, the output power of the irradiator is the cogent parameter. In any event, it may be somewhat of a mute point given the fact that ship time and queue time often control turn time.

Penetration of the radiation into the target material is more of a concern with high-energy electron than photon irradiation. The mean-free path for the gamma rays from cobalt-60 are more than an order of magnitude longer than the equivalent mean-free path for 10 MeV electrons. The photons produced in an X-ray (bremsstrahlung) irradiator have a mean-free path at least equivalent to cobalt-60 photons. In a gamma or X-ray (bremsstrahlung) irradiator, the high-energy electrons are created internally within the target material via a Compton scattering process whereas in the case of high-energy electrons from an accelerator the electrons effectively need to be driven into the target from the outside. A technique that is frequently used to increase the penetration depth is referred to as two-sided irradiation. In this method, the product is first scanned in front of the beam from one side and scanned from the other side in a subsequent pass. This method has been effectively used for processing a variety of products with bulk densities less than a few g/cc. For higher bulk densities or heterogeneous products that may contain localized high-density regions, special processing techniques may be required.

X-Ray Irradiators

Design

X-ray irradiators contain all the features of a high-power electron beam irradiator and in addition have a target that converts the high-energy electrons into photons (bremsstrahlung radiation). As is the case in gamma irradiators and electron beam irradiators, X-ray irradiators require a biological shield, a conveyance system for transporting the product in front of the beam of X-rays, a control and safety system as well as an air recirculation system. An example of an X-ray irradiator, which is the only X-ray irradiator presently operational in the United States, is shown in Figure 3. Because the conversion efficiency of high-energy electrons to X-ray (bremsstrahlung) radiation is relatively low, that is, 8% for 5 MeV electrons on a Tantalum target and 12% for 7.5 MeV electrons on the same target, very high power electron beam accelerators are required to generate sufficient X-ray output for commercial applications. In Figure 3, the source of electrons is a 190-kW Rhodotron. Dependent on the exit port from which the electrons are extracted from the accelerator, the energy could be 5 MeV or 7.5 MeV, which is the reason two scan horns are shown in Figure 3.

Operation of X-Ray Irradiators

As in the case of electron beam irradiators, in X-ray irradiators we are dealing with a beam of radiation rather than an isotropic radiation environment. However, unlike electrons, the forward directed beam of X-rays emanating from the conversion target cannot be steered or directed. In addition, the beam of X-rays has a small angular divergence, which is dependent

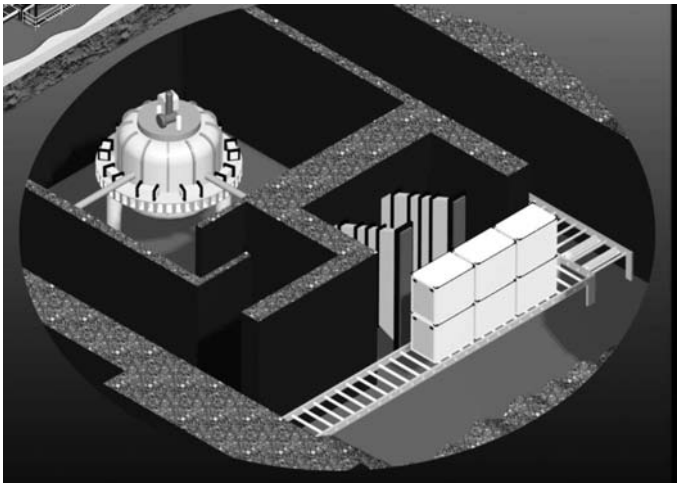

Figure 3 X-ray irradiator.

on the energy of the photons and needs to be taken into account in the design of the conveyance system that moves product in front of the beam. Because the radiation environment consists of high-energy photons, penetration of the radiation into the target is not the issue that it is in electron beam irradiators. In fact, the radiation in an X-ray irradiator is effectively more penetrating than the photon radiation in a gamma irradiator. For this reason, as seen in Figure 3, product may be transported in large carriers in front of the beam of radiation. In fact, because of the highly penetrating nature of the X-ray (bremsstrahlung) radiation, X-ray irradiators have been designed to process entire pallet loads of high-density product. Because of the directional nature of the radiation field in an X-ray irradiator, it is not necessary to surround the source with product, as is the case in gamma irradiators, but to increase the intrinsic efficiency of the irradiator; additional rows of carriers may be conveyed in front of the beam.

CONTROL OF THE IRRADIATION ENVIRONMENT

Absorbed Dose and Dose Rate

Absorbed Dose

Absorbed dose is the amount of energy absorbed per unit mass of material. It controls how a material will respond to being irradiated. In gamma irradiators, product is loaded in irradiation containers and moved through the cell in a shuffle-dwell mode of operation. The dwell period is controlled by a preset cycle time. Increasing the cycle time increases the time the irradiation container remains in the cell and is exposed to gamma rays from the source. All other things being equal, a longer resident time in the cell will obviously lead to a higher absorbed in the product. Changing the cycle time is a standard method for changing the absorbed dose delivered to product. Some irradiators offer more than one product path through the irradiator, which allows different absorbed doses to be delivered to the product even at the same cycle time. For example, the tote box irradiator in Figure 1 offers the user an option of using only the outer two passes for incrementing the totes through the cell rather than using all of the four passes that are available. The amount of isotope loaded into the source plane(s) will also dictate the amount of absorbed dose delivered to product at a given cycle time. For electron beam irradiators and X-ray (bremsstrahlung) irradiators, adjustment in the speed of the conveyor system that moves product in front of the beam is a principal method for controlling the amount of absorbed dose delivered to product. The amount of absorbed dose delivered to product also can be adjusted by simply changing the output current of the accelerator. The previous methods allow a wide range of absorbed doses to be delivered to products even within the same irradiator.

Dose Rate

In addition to the amount of absorbed dose delivered to a product, the rate at which energy is delivered to the product may play an important role in its response to the incident radiation.

For this reason, it is important to understand the key parameters affecting dose rate and methods for controlling the dose rate. Dose rate is a function of two parameters. The first of these parameters is the incident power density that is given in units of w/cm^2. In a gamma irradiator, output power is dictated by the amount of isotope that is loaded into the source plane(s) where one megacurie of coblalt-60 equals 14.7 kW of power. Because of the isotropic nature of the radiation field in a gamma irradiator, the power density in w/cm^2 will depend on the distance from the source; that is, the w/cm^2 decreases with distance from the source. In electron beam irradiators and X-ray irradiators, where the radiant energy is delivered in the form of a beam of radiation, the power density will depend on the output power of the accelerator or conversion target and the area over which the power is delivered to the target. The second parameter that controls dose rate is the mass absorption coefficient of the target material. If the mass absorption coefficient is very high, all of the incident radiation will be absorbed in a relatively thin layer of material versus a much thicker layer of material for a low mass absorption coefficient. At a given incident power density, a high mass absorption coefficient will lead to a higher dose rate than a low mass absorption coefficient. The equation for dose rate is given as the product of the preceding two variables.

$$\text{Dose rate} = D(t) = 3.6 \times 10^3 P_A \times \mu_\rho (\text{kGy/hr}) \quad (1)$$

In equation (1), the numerical factor of 3.6×10^3 converts w/g to kGy/hr, P_A is the incident power density in w/cm^2 and μ_ρ is the mass absorption coefficient in cm^2/g.

At equivalent output powers, gamma irradiators have the lowest dose rates, X-ray irradiators higher dose rates, and electron beam irradiators the highest dose rates. By way of comparison, if the dose rate in a gamma irradiator were normalized to 1, dose rate in an X-ray irradiator would be approximately 10 or more, and dose rate in an electron beam irradiator would be greater than 100. For a given modality of irradiation, various methods are available for controlling the dose rates that are delivered to a product. For example, decreasing the output power of the irradiator offers one method for reducing the dose rate. In the case of a gamma irradiator, this would entail loading less isotope in the source plane(s), and for electron beam and X-ray irradiators it could be accomplished by simply dialing down the current of the accelerator. Placement of a shield between the source and target is another method for reducing the dose rate. In a gamma irradiator one can take advantage of the isotropic nature of the radiation field and simply move the product further from the source, which will reduce the power density incident on the target. If dose rate is considered an important parameter in the irradiation of a specific pharmaceutical product, selection of the modality of radiation that best meets the dose rate requirements should be taken into account at an early point in the sterilization project.

Dose Range—DUR

Because of the finite size of a product unit, that is, individual box, tote or carrier, that is transported through an irradiator and shielding by the product itself, all product within a product unit will not receive the same absorbed dose. The product unit will receive different absorbed doses ranging from a minimum absorbed dose up to a maximum absorbed dose. The ratio of maximum to minimum dose is referred to as the dose uniformity ratio or DUR. To satisfy technical criteria for irradiation of the product, no less than the minimum dose must be delivered to the product unit. However, absorbed doses in excess of the minimum dose are not required and in fact if the maximum dose is too high, it may lead to unacceptable degradation of the product. Therefore, it is desirable to keep the DUR as close to one as possible while still allowing product to be processed in an efficient manner. There are various methods for controlling the DUR and range of absorbed doses delivered to product. In this regard, selection of the modality for irradiation, that is, gamma, electron beam, X-ray, should be taken into account during the initial evaluation of the methodology for irradiation of your product. For high-density products and those products that are highly heterogeneous in nature, photon radiation whether gamma or X-ray may be preferred to high-energy electrons. As previously noted, the radiation mean-free path for the photon energies used in the irradiation industry are more than an order of magnitude greater than the radiation mean-free path of 10 MeV

electrons; that is penetration of gamma and X-ray radiation into the product unit is of less concern than for high-energy electrons.

DUR and Gamma Sources

Because of the isotropic nature of the radiation field in gamma irradiators, the DUR depends not only on product shielding but also the geometry of the product unit. Gamma rays are a highly penetrating source of radiation. The penetration of high-energy photons in materials is described by the product of an exponential factor and a semiempirical buildup factor that accounts for scattering of the photons. In lower density materials, gamma rays easily penetrate through a large thickness of material, and shielding is not a dominant factor in the resultant DUR rather it is geometric attenuation. One method of decreasing the effect of geometric attenuation on dose distribution and the resultant DUR is to increase the standoff distance of the product unit from the source. As noted earlier, this option is available for the tote box irradiator shown in Figure 1. Product can be transported around the source plane in the outer two passes only, which significantly increases the standoff distance of the totes from the source plane, thus reducing the impact of geometric attenuation on dose distribution. In irradiators that are designed for precision dosing of product, such as those used in dose validation studies, the irradiation containers are typically offset further from the source than the standoff distance found in production irradiators. Use of lightweight metal framing for the carrier structure also can be used to reduce the effect of shielding by the carrier and resultant effect on the DUR. In category IV irradiators, it is standard practice to equally expose both sides of the irradiation container to the source(s). Figure 4 shows the four-pass product path of a tote though the irradiator shown in Figure 1. As seen from this figure, at the completion of the process cycle both sides of the tote have been exposed to equal amounts of radiation. The symmetric pattern of irradiation shown in Figure 4 also allows the DUR to be decreased by a method called center loading. On the basis of this method, the product is not loaded over the entire width of the irradiation container, rather the width of the product is reduced to a dimension less than the irradiation container width and the product is centered in the irradiation container along its mid-plane. This serves two purposes, first it reduces the amount of shielding due to the reduced target width and second you move the outer surfaces of the target further from the source, which reduces the effect of geometric attenuation on the DUR. Center loading and increasing the standoff distance from the source are but two methods that can be used to reduce the dose spread in the product unit and control the DUR.

DUR—Electron Beam and X-Ray Sources

Because of the directional nature of the radiation environment in an electron beam irradiator, the effect of geometry on the DUR is less important than in gamma irradiators. However, because of the much higher mass absorption coefficient of high-energy electrons in materials than that of high-energy photons, shielding and its effect on the DUR is a more important

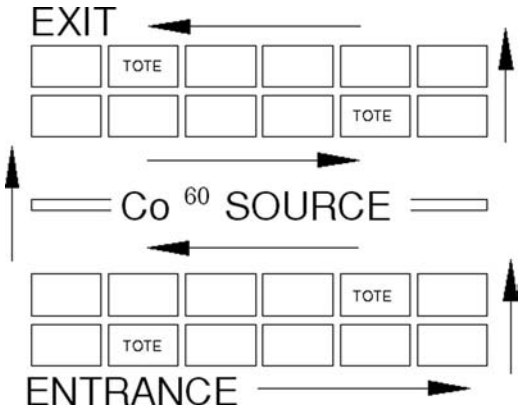

Figure 4 Product flow-tote box irradiator.

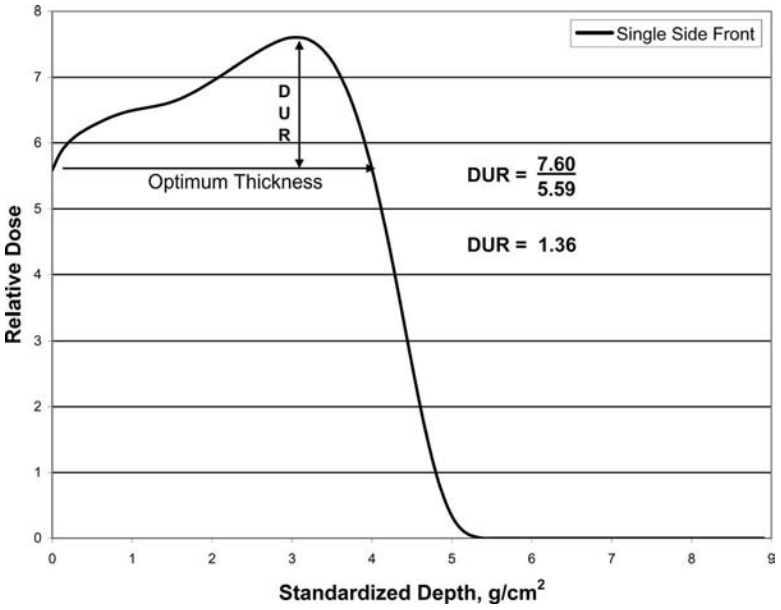

Figure 5 Depth-dose profile—single-sided irradiation.

consideration in electron beam irradiators than in gamma or X-ray irradiators. In homogeneous materials, the depth-dose profile of high-energy electrons in materials is a well-characterized parameter. An example of the depth-dose profile for a beam of 10 MeV electrons incident on a plastic target is shown in Figure 5. The fact that the absorbed dose is greater inside the target than at the surface where the electrons are incident is due to scattering of the electrons as they penetrate deeper into the target. The precipitous falloff in absorbed dose at deeper penetrations into the target occurs after the electrons have given up most of their energy in inelastic scattering collisions. The target thickness is clearly limited by the need to maintain an acceptable DUR. The optimum thickness occurs when the exit dose equals the entrance dose. The horizontal arrow in Figure 5 indicates this thickness and the vertical arrow represents the DUR for single-sided irradiation at the optimum thickness. A standard method for significantly increasing target thickness while maintaining an acceptable DUR is to equally irradiate both exterior surfaces of the target to the beam of high-energy electrons. The result of this two-sided irradiation is shown in Figure 6. Superposition of the dose profiles from irradiation of the two exterior surfaces of the target leads to a DUR that is actually the same as the DUR for the optimum thickness and single-sided irradiation. Other more imaginative methods may be used to reduce the DUR. For example, the product can be displayed in a planar geometry, and the DUR further reduced using metal scatter plates (8).

Of the three modalities for irradiation, an X-ray irradiator offers the potential of delivering the most uniform dosing to product. Dependent on the maximum energy of the X-rays, the mass absorption coefficient can be less than that of cobalt-60 photons. For this reason, shielding is less a concern in X-ray irradiators than in gamma irradiators. In addition, because of the directional nature of the radiation field, geometry is not as important as in gamma irradiators. However, geometrical effects will come into play at boundaries of the product unit due to the beam properties of the X-ray radiation. These so-called edge effects need to be taken into account in controlling the DUR. One method for reducing the effect of edge effects on DUR is to add scatter material at the product unit boundaries. In this manner, photons can also scatter into the material as well as out of the material.

Temperature

The increase in temperature of irradiated products is dependent on three basic parameters. The first of these parameters is the energy absorbed per unit mass of material. Because the energy

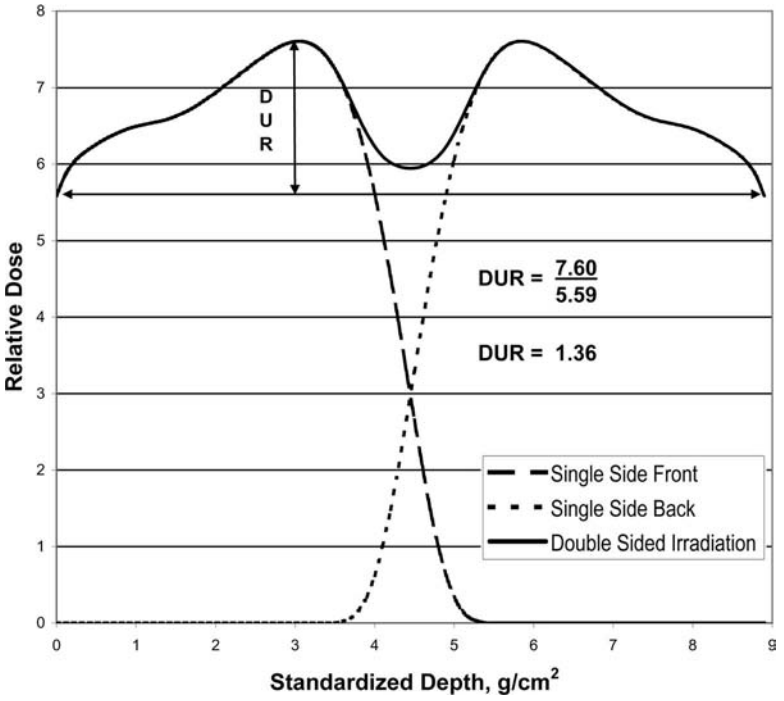

Figure 6 Depth-dose profile—two-sided irradiation.

absorbed per unit mass of material is equal to absorbed dose, it follows that higher absorbed doses should lead to higher excursions in temperature. The second parameter that has an effect on the change in temperature is the rate at which the energy is deposited, that is, dose rate. At high dose rates the material may not have sufficient time to thermally relax, thus leading to higher temperatures in the irradiated product. The last parameter that can significantly affect product temperature is related to the thermal properties of the irradiated material. For equivalent irradiation conditions, materials that are good conductors of heat with similar specific heats should experience a smaller increase in temperature than materials with high thermal resistance. Let's consider each of these parameters and its potential effect on product temperature.

Dose and Dose Rate

The absorbed dose delivered to a product will depend on the minimum dose that is required to achieve the desired SAL and the DUR, which controls the maximum dose delivered to product. As we will see in the section on establishing the sterilization dose, the different methodologies for establishing a minimum dose are bioburden driven, that is, dependent on the initial bioburden. For this reason, a lower initial bioburden on a unit of product translates into a lower minimum dose to achieve the desired SAL. A lower absorbed dose equates to a lower amount of energy absorbed per unit mass and a smaller increase in temperature. At high dose rates the irradiated material does not have an opportunity to thermally relax and essentially behaves in an adiabatic manner. In adiabatic heating the change in temperature is given by the following relationship:

$$\Delta T = \frac{D}{c} \tag{2}$$

In equation (2), D is the absorbed dose and c is the specific heat of the irradiated material.

As an example, consider the case where a minimum dose of 25 kGy is delivered to a product and the DUR is 2, which equates to a maximum dose of 50 kGy or 12 calories/g. If the specific heat of the irradiated material is approximately 0.5 calories/g-°C, which is a

representative number for the types of materials being irradiated, the radiation-induced increase in temperature is 24°C. If the ambient temperature is approximately 30°C, the product temperature in this example could reach 54°C. Therefore, given the right conditions, significant increases in temperature can occur in irradiated products. For some products such as proteins where temperature could have significant impact on the process, an increase in temperature of this magnitude could have an important effect on the response of the protein. The possibility of adiabatic heating is greater in high dose rate environments, for example, electron beam, than low-dose rate environments, for example, gamma.

There are various ways to control the temperature and mitigate its effect on the irradiated product. The first is to irradiate the product at a lower dose while still achieving the desired SAL. The second method of control is to irradiate the product in a low dose rate environment. If it is necessary to irradiate the product in a high dose rate environment, delivering the dose in segments; thus allowing the material to thermally relax between dose deliveries offers one possibility for reducing the increase in product temperature. An additional consideration would involve refrigeration of the product so the initial temperature is below the ambient value.

Thermal Properties

A key thermal property that controls the temperature of irradiated materials is the thermal diffusivity of the material. This parameter, which can be extracted from the heat conduction equation, is

$$\alpha = \frac{k}{\rho c} \tag{3}$$

In equation (3), k is the thermal conductivity of the material, ρ the materials density, and c its specific heat. The unit of thermal diffusivity is cm^2/sec, which is a measure of the rate at which a heat front moves through a material. Because the thermal diffusivities of typical pharmaceutical products and medical devices are relatively low; that is, they are not good conductors of heat such as metals, the rate of diffusion of thermal energy from the region being irradiated is normally quite low. Although little can be done to remedy this condition, it may be possible to enhance heat flow by appropriate selection of packaging materials and other materials that may surround the product unit. In this regard, removal of packing material such as Styrofoam that may encase the product or replacement with a more efficient heat conducting material may be beneficial.

ESTABLISHING THE STERILIZATION DOSE AND MAXIMUM DOSE

Inactivation of Microorganisms

The biocidal effect on microorganisms from exposure to radiation is well documented (9,10). The number of surviving microorganisms decreases with increase in the absorbed dose. The dose survivor curve, which plots survivors versus dose, can take on different shapes, but a common shape obeys first-order kinetics and follows an exponential decrease in surviving microorganisms with dose (11). A key parameter that characterizes the dose-survivor curve is the incremental dose that is required to reduce the number of survivors by one log or a factor of 10. This parameter is referred to as the D_{10} value, and for microorganisms that typically reside on pharmaceutical products and medical devices, D_{10} values range from less than 1 kGy up to several kGy. It is of interest to note that because of the exponential behavior of the dose-survivor curve, absolute sterility is not achievable; that is, no survivors can only be approached in an asymptotic manner. For this reason, sterility is expressed in terms of a sterility assurance level or SAL, which is defined as the probability of a single viable microorganism occurring on a product after sterilization. A commonly accepted SAL that defines a sterile product is 10^{-6} or one chance in a million of finding a viable microorganism on a unit. The total absorbed dose to achieve an SAL of 10^{-6} is a function of the D_{10} value, which defines the slope of the dose-survivor curve, and the initial bioburden that is present on the product unit. Both of these parameters depend on the manufacturing conditions, which define the type and level of microbial contaminants on the manufactured unit. As we will see in the section on establishing

the sterilization dose, the methodologies for setting minimum dose are bioburden driven and for this reason a product that is manufactured under clean conditions with low initial bioburden may be terminally sterilized at a lower minimum dose than products with higher initial bioburdens.

Establishing the Sterilization Dose

During the early years of the radiation sterilization industry, a minimum dose of 25 kGy was generally considered sufficient to achieve an SAL of 10^{-6} (12). The adequacy of this minimum dose was contingent on adherence to good manufacturing procedures. Selection of a 25-kGy minimum dose is still used in some instances (13). In the late1970s, a North American Working Group was convened under the auspices of the Association for Advancement of Medical Instrumentation (AAMI) to develop guidelines for controlling the sterilization of medical devices by radiation. Part of the work of this group included the development of methods for establishing the sterilization dose. Two methodologies, subsequently referred to as Method 1 and Method 2, flowed out of the work of this group. These methods are bioburden driven, that is, dependent on the initial bioburden and, dependent on the level of bioburden present on the unit of product, allow sterilization doses less than 25 kGy, which may be beneficial for terminal sterilization of drug products. A discussion of these methods was first included in the Proceedings of the Second International Kilmer Memorial Conference on the Sterilization of Medical Products (14). These methods are now embodied in ISO standards (15). Over the nearly 30 years since they were first developed, these dose-setting methods have been successfully used to terminally sterilize a broad spectrum of medical device and pharmaceutical products. A more recent method referred to as Method, VD_{max} also has been successfully used over the past several years to terminally sterilize many types of medical device and pharmaceutical products (15,16). Because establishing the minimum dose using Method VD_{max} requires fewer sacrificial samples than Method 1 and Method 2, it offers potential advantages in the case of high-unit value products. Method 1 and Method VD_{max} are both based on testing against a challenge population that is considered to be more resistant to radiation than the natural bioburden that may be present on the product. Experimental verification is required. In Method 2, information is obtained about the resistance to radiation of the natural bioburden present on the product. This information is used in setting the minimum dose for irradiation. The key features of all three of these methods are discussed here. Additional details that pertain to the challenge populations, selection of the SALs for verification dose testing and pass/fail criteria can be found in ANSI/ISO/AAMI 11137-2:2006.

Method 1

Following selection of the desired SAL, the first step in the application of Method 1 is determination of the average bioburden on a product item where a product item is defined in terms of how it is used in clinical practice. A total of 10 product items are selected from each of three independent product batches, and these 30 product items are tested for bioburden. In those cases where manufacturing is limited to a single batch, only 10 product items need to be tested for bioburden. Rather than sampling the entire product item, it is sometimes possible to sample a portion of the item, which is referred to as a sample item portion or SIP. Procedures for using an SIP are defined in the ISO standard on methods for establishing the sterilization dose (6). After the average bioburden has been quantified, the next step is to perform a verification dose experiment at an SAL of 10^{-2}. Selection of the verification dose is based on the average bioburden number, and a standard distribution of resistances (SDR population) that represents a greater challenge than the natural bioburden present on the product. The challenge population used for dose verification in Method 1 was based on testing of several hundred isolate resistances from some 70,000 microbes (17). The SDR population consists of a superposition of different D_{10} values in percentages that were derived from this experimental study. From a comparison of the resultant challenge population with other proposed populations, it was concluded that the SDR population provided a conservative presterilization microbial resistance reference profile. The basis for selection of the SDR as the microbial challenge population is discussed in reference (15). Because the verification dose experiment is

performed at an SAL of 10^{-2}, 100 samples need to be selected from a manufacturing batch for testing. Following irradiation at the verification dose, the 100 samples undergo sterility testing and if the number of positives is less than a preset number, a sterilization dose that is based on the challenge population may be selected. It is important to note that the sterilization dose is dependent on the initial bioburden that is present on a product item. At an average bioburden of 1000 colony forming units (CFU), the 10^{-6} SAL dose is approximately 25 kGy, that is, a sterilization dose commonly used during the early years of the radiation sterilization industry, but for an average bioburden of 0.1 CFU, the 10^{-6} sterilization dose can be as low as 11 kGy. However, the number of sacrificial samples that is needed to complete the test matrix may be problematical for high unit value products. Method 1 may be better suited for products that are manufactured in relatively large product lots, and unit costs are not extremely high.

Method 2

Method 2 actually consists of two methods that are referred to as Method 2A and Method 2B. Method 2A applies to products with average bioburdens per product item greater than about 10 CFU and Method 2B applies to products with consistent and very low bioburdens. In both methods, information is obtained about the resistance to radiation of the natural bioburden present on the product. This is accomplished by exposing product items to a series of incremental doses to estimate the dose at which one in 100 product units are expected to be nonsterile, that is, 10^{-2} SAL dose. The data from the incremental dosing is also used to estimate the D_{10} value of the natural bioburden present on the product. In effect Method 2 provides an estimate of the 10^{-2} SAL dose and slope of the dose-survivor curve, which allows extrapolation to an SAL of 10^{-6}. The number of sacrificial samples that are required for execution of Method 2A or 2B is quite large, that is, at least several hundred, which probably would make application of Method 2 inappropriate for high unit value products or products that are manufactured in small batches. However, for products with low bioburdens and/or products contaminated by microorganisms with low radiation resistance, it may be possible to validate a 10^{-6} SAL dose that is less than 10 kGy.

Method VD_{max}

Method VD_{max} was initially developed for irradiation of product at a sterilization dose of 25 kGy, but subsequently the method was extended to doses down to 15 kGy (15). From an operational standpoint, VD_{max} is similar to Method 1 in that it requires determination of bioburden and performance of a verification dose experiment. However, VD_{max} differs from Method 1 in two basic respects. First, the sterilization dose is fixed to a maximum bioburden number and the sterilization dose does not scale to lower doses with a decrease in bioburden below the maximum value, as is the case for Method 1. The sterilization dose, however, will change with selection of different values for the maximum bioburden. For example, at a maximum bioburden of 1000 CFU, the sterilization dose is 25 kGy and for a maximum bioburden of 1.5 CFU, the sterilization dose is 15 kGy. The second difference between Method VD_{max} and Method 1 involves the number of samples that are required for the verification dose experiment. In Method VD_{max} the verification dose experiment is performed at an SAL of 10^{-1} rather than 10^{-2}, which reduces the number of samples that are needed for testing from 100 to 10. The number of samples that are required for bioburden testing is the same as Method 1, but due to the reduced number of samples that are required for the verification dose experiment, the total number of samples that are sacrificed using Method VD_{max} is only 40 compared to 110 for Method 1. At the maximum bioburden, the VD_{max} sterilization dose converges to the Method 1 dose for the same bioburden. Also, Method VD_{max} rigorously preserves the conservative aspects of the SDRs that represent the challenge population for Method 1.

Establishing the Maximum Acceptable Dose

In addition to establishing a minimum dose that ensures a specified SAL is satisfied, a maximum dose that ensures the safety and performance of the product over its lifetime also needs to be established. The procedure for determining an acceptable maximum dose involves

irradiation of product samples at precise doses followed by post-irradiation testing of the product. In selecting the radiation environment for the tests, it is important to consider the temperature and dose rate in which the product will be irradiated on a routine basis. Dependent on the type of product that will be irradiated, both of these parameters could significantly impact the test results. The test matrix not only includes the drug product and medical device if you are dealing with a combination product, but also any closure system and packaging. Both functionality and biocompatibility are included in the test matrix. The doses that are selected for the tests should take into account a range of doses above the minimum dose that would allow the product to be processed on a routine basis without significant constraints. As we have seen, dependent on the size and density of the product unit as well as the irradiator environment, the product will be exposed to a range of doses that is characterized by a DUR. For example, if the minimum acceptable dose is 15 kGy, initial testing to establish an acceptable maximum dose could begin at a dose of 30 kGy, which would allow for a DUR of 2 during routine processing. If the initial results of post-irradiation testing of the product are acceptable, further testing probably will not be required. However, any negative results would require repeating the tests at a lower dose, for example, 25 kGy. It should be remembered that a tighter allowable dose range could place constraints on how the product can be processed on a routine basis. For example, in the case of gamma irradiation, this may require center loading of the product in a tote, which would reduce the tote load efficiency.

Dosimetric Release

Dosimetric release is an important aspect of the radiation sterilization process. In essence, dosimeteric release allows product to be released following irradiation based on certification that all product in the run received an acceptable minimum dose and no product in the run exceeded an acceptable maximum dose. Post-irradiation sterility testing of product samples is not required and use of biological indicators is no longer a recommended practice. The efficacy of the dosimetric release process rests on the use of an established methodology for selecting an acceptable minimum dose to achieve the desired SAL and certification that no dose exceeded a maximum acceptable value, which was based on a matrix of tests involving functionality and biocompatibility. In addition to establishing acceptable minimum and maximum doses, routine monitoring and control of the irradiation process and maintenance activities that include controls on the manufacturing process and periodic dose audits are important aspects of the radiation sterilization process.

PERFORMANCE QUALIFICATION

Performance qualification (PQ) is the final step in the radiation sterilization process prior to routine irradiation of the product. PQ involves two activities that include loading the product into the irradiation containers in accordance with a specified loading pattern and dose mapping of the product to determine the distribution of dose within the product load and identify the location and magnitude of the minimum and maximum doses. As we have seen, product may be loaded into an irradiation container in different configurations dependent on the carton size, product density, and possible constraints that are dictated by the DUR. Regardless of the load configuration, once established it must be maintained during the irradiation process. The dose distribution in this established load configuration must be measured, and the zones where the dose extremes occur must be identified.

Product Loading Pattern

Dependent on the modality of radiation that is used to sterilize the product, different constraints may be imposed on possible loading patterns. Because of the much longer radiation mean-free path of high-energy photons in materials than energetic electrons, there are typically fewer constraints on loading geometries in gamma and X-ray irradiators than in electron beam irradiators. For many types of health care products that are processed in a gamma or X-ray irradiator, the product within a carton may be treated as effectively homogeneous in nature and only carton size and weight are measured to determine the bulk density. However, some products may contain localized regions of high density that could affect the distribution in dose within the product load and therefore need to be taken into

account. For example, glass vials may be filled with an API powder and surrounded by low-density packing material. In such cases, a measurement of only bulk density may not suffice. For these types of products, it may be necessary to take into account the orientation of a product item within a carton and how the carton may be loaded into the irradiation container. Additional factors other than optimization of the fill efficiency may affect the final load pattern. For example, the final load configuration may take into account dose uniformity requirements, ease of loading, and compatibility with other product runs.

Some pharmaceutical products that are in an aqueous form respond more favorably when frozen and should to be irradiated in a refrigerated state. The loading pattern for refrigerated products requires special consideration. Refrigerants, be it wet ice or dry ice, are high-density materials that can significantly affect the dose delivered to product. For this reason, it is important that the product packaging be properly designed and the refrigerant confined to a specified location within the carton. In selecting the location of the refrigerant within the carton, it is important to consider the geometric relationship between the source of radiation and the product. Whether it is a beam of radiation as is the case for electron beam and X-ray sources or the isotropic radiation environment in a gamma irradiator, placement of the refrigerant between the source and product is generally not a good option. At this location, the refrigerant will significantly attenuate the incident radiation. The situation is further complicated in the case of dry ice, which sublimes, that is, the effect of the refrigerant on dose delivery is time dependent. Of the different possible locations of the refrigerant, perhaps the best place to locate the refrigerant to minimize its effect on dose delivery is in the lead and trail regions of the irradiation container.

Because of the much shorter radiation-free path of energetic electrons in materials compared to high-energy photons, additional considerations should be taken into account in selecting the loading pattern for electron beam irradiation. Loading patterns should be established for each product type. For this modality of irradiation, the loading pattern should take into account the orientation of the product items within the package material as well as any secondary packaging and orientation of the product item with respect to the incident beam of electrons.

Dose Mapping Gamma and X-Ray

For many types of lower bulk density materials and those that are reasonably homogeneous in make-up, the dose map may consist of a standard three-dimensional grid of dosimeters that are placed throughout the product load. For gamma irradiation, geometric attenuation may play an important role in the distribution of dose and for X-ray irradiation, edge effects at product boundaries may be important. These factors need to be taken into account in the placement of dosimeters within the product load. An additional consideration is the region of the product load furthest from the source of radiation. For gamma and X-ray irradiators, this occurs at the mid-plane of the irradiation container. Dependent on how the cartons of product are loaded into the irradiation container, it is frequently possible to place dosimeters only on the outside surfaces of the cartons; that is, it is not necessary to go inside a carton to locate dosimeters.

In those cases, where product may contain localized high-density regions within a carton, it may be necessary to place dosimeters inside the carton and even within a localized high-density region itself. This situation may occur more frequently in the irradiation of pharmaceutical products that are oftentimes formulated in a high-density configuration. Because the maximum dose zone is usually found on an outside surface of the product load, the presence of localized high-density regions usually only affects the location of the minimum dose zone. A customized dose map grid is required whenever it is deemed necessary to place dosimeters inside a carton of product. Whenever, a dosimeter needs to be placed inside a carton or within a product item, it normally is not practical to place a dosimeter at that location during routine processing of the product. In these cases, it is standard practice to measure dose at a reference location, which typically is on an exterior surface in the product load or standard monitoring location and relate the dose measured at the reference location to the dose measured inside the product. The relationship between the dose measured at the reference location and dose at the interior location, is commonly called an adjustment factor (AF) (18).

The AF for minimum dose, which oftentimes is the dose measured at an interior location, is given by

$$\mathrm{AF}_{\min} = \frac{D_{\mathrm{ref}}}{D_{\min}} \tag{4}$$

In equation (4), D_{ref} is the dose measured at the reference location and $D_{\min}$ is the dose measured at the minimum dose location.

It is important to note that when reference location dosimetry is used to monitor dose during routine processing of product, the minimum dose at an interior location is not measured rather it is calculated on the basis of a statistical relationship given by the AF. For this reason, it is standard practice to measure the dose distribution in more than one product load under the same processing conditions with three product loads considered the minimum number to be dose mapped. Statistical analysis of the data from three dose maps is used to evaluate reproducibility in the measured dose and uncertainty in the statistical relationship that is used to calculate the minimum dose. This estimate of statistical uncertainty in the calculated value of dose can be used to set process parameters for routine irradiation of the product.

Dose Mapping Electron Beam

Because of the much shorter radiation mean-free path of high-energy electrons in materials than high-energy photons and the fact that we are dealing with a beam of electrons, shielding and scattering effects introduced by localized heterogeneities within a carton of product or even within a unit of product in the carton can significantly affect the dose delivered to the product. For example, the range of 10 MeV electrons is approximately 5 cm in water and polymers that commonly serve as packaging materials and closure systems for pharmaceutical products. In a metal such as stainless steel, the range of 10 MeV electrons is less than 1 cm. Therefore, localized high-density regions can result in significant dose gradients within a small volume and shadowing of other regions in the carton of product. These factors need to be taken into account in the selection of the locations of dosimeters within the product load. There are no standard dose map grids as is sometimes the case for gamma or X-ray irradiation. Dose map grids in high-energy electron beam irradiation are unique to each product type. In electron beam irradiation, it is common practice to use reference location dosimetry for monitoring dose during routine processing of product. An external surface such as the surface where the electron beam is incident on the product load may sometimes serve as the reference location or it may be at a fixed location adjacent to the product load and simply referred to as the monitoring location. In the case where the reference location is on an external surface, it sometimes may also represent the minimum dose zone, which would only require use of an AF to calculate the maximum dose delivered to the product load. To establish the reproducibility in dose delivered to the product load and estimate the uncertainty in the AF(s) that is used to calculate dose, multiple product loads, that is, typically three, are dose mapped. The uncertainty in the dose measurement process should be taken into account when setting process parameters.

RADIATION CHEMISTRY

Radiation Interactions with Parenteral Drug Products

As we have seen, high-energy electrons injected into a drug product from a high-power accelerator or generated within the medium from Compton scattering of energetic photons are responsible for the changes in the properties of the drug product and its sterilization. These high-energy electrons, which typically have energies in the 1 to 10 MeV range, suddenly find themselves embedded in the surrounding medium. Atomic electrons of the atoms in the medium effectively shield the attractive force of the positive charges of the nuclei, and the high-energy electrons experience only the repulsive Coulombic force that is instantly established between them. The velocity of a 1 MeV electron is of the order of magnitude 10^{10} cm/sec, which is close to the speed of light. The velocity of atomic electrons is on the order of 100 times less. It takes about 10^{-17} seconds for a 1 MeV electron to cross a diameter of an atom. During that time an atomic electron remains practically stationary and "feels" the rising and falling action of the repulsive Coulombic force created by the approaching and

leaving of the high-energy electron passing by. The momentum exchanged between the two electrons (the product of the electrostatic force and duration of the collision) is small in comparison with the kinetic energy of the incident electron but may be large in comparison with the binding energy of the orbital electron. If the exchanged energy exceeds the energy that binds the electron to an atom (ionization potential), ionization of that atom will occur, whereas the exchange of a smaller amount of energy will result in its excitation.

Studies have shown that the energy exchange events in liquids and solids involve energy packets between 6 and 100 eV, the most probable being around 25 eV. This is true in simple molecules such as water and cyclohexane (19), as well as in macromolecules such as DNA (20). Obviously all materials consisting of low-Z elements, including biological materials and APIs, absorb energy by similar mechanisms that occur with similar probabilities. The energy of 25 eV is sufficient for the creation of one or two ion pairs and one or two excited molecules in liquid water. The small element of volume within which energy deposition occurs and within which newly formed species are confined for a limited time is called a spur. Occasionally, a larger package of energy is absorbed forming a blob (100–500 eV) or a short sidetrack (500 eV–5 keV). Spurs outnumber blobs by about 50:1 and short tracks by about 500:1. For cobalt-60 gamma rays and 1 MeV electrons in water, the partition of absorbed energy is approximately spurs: 75%, blobs: 12%, and short tracks: 13% (21). Essentially the same distribution of probabilities exists in water vapor and ice underscoring the random character of primary interactions, irrespective of the phase. This leads to the estimate that the absorption of a 1 MeV electron creates about 25,000 spurs, 500 blobs, and 50 short tracks.

The initial volume of a spur in water may be about 1 nm^3 (22), and the volumes of blobs and short tracks may be orders of magnitude larger, 10 and 100 nm^3, respectively. Together they may occupy the volume of the order 10^5 nm^3 containing about 10^6 molecules of water. Sterilization dose of 25 kGy is equivalent to the absorption of 1.56×10^{20} eV/g requiring total absorption of 1.56×10^{14} 1-MeV electrons in 1 g of water. The absorption of this amount of energy would initially affect 1.56×10^{20} molecules/g out of 3.3×10^{22} molecules present in 1 g of water, or 1 in about 200. Allowing that more than 10 water molecules may be contained within a 1 nm^3 spur reduces this estimate to less than one in 2000.

The above picture is oversimplified: there is a distribution of spur sizes and some overlapping of spurs. Nevertheless, it teaches us that precursors of chemical change are initially inhomogeneously distributed only along the tracks of fast electrons while the rest of the volume remains unaffected. It also teaches us that a significant fraction of small molecules may initially escape ionization or excitation, but that larger molecules will not be spared of radiation acting directly. It is also obvious that in solutions, it is mostly solvent molecules that absorb radiation energy resulting in the creation of reactive species. The initially inhomogeneous distribution of primary products: electrons, positive ions, and excited molecules throughout the irradiated medium is one of the key features of radiation chemistry.

Spatial inhomogeneity determines the earliest stage of radiation action, which is termed physical stage. It starts at 10^{-17} seconds with the absorption of energy and extends to approximately 10^{-13} seconds until thermal equilibrium has been reached. The probability of interactions of electronic systems of atoms with photons and electrons during that stage is perfectly random, and nothing can be done to reduce it or to decrease the amount of ionization and excitation. The energy required for the creation of one ion pair in gas (W) is similar (25–30 eV) for a wide range of compounds (23), which forms the basis for the expectation that approximately the same number of ion pairs would initially be created, irrespective of the chemical nature of the substance. However, the amounts of radiation-induced changes that become measurable at later stages greatly differ depending on the medium.

Radiation Chemical Yield

In an empirical approach to quantify and compare chemical effects of irradiation, the measured amounts of radiation-induced chemical changes have been normalized to dose. The quantity obtained in this way is called radiation chemical yield (G):

$$G(X) = \frac{C(X)}{\rho D} \tag{5}$$

where $G(X)$ is the radiation chemical yield of substance X created, destroyed, or altered; $C(X)$ is the concentration of substance X created, destroyed, or altered; ρ, the density; and D, the dose.

The unit of $G(X)$ is mol/J but an older unit (molecules/100 eV) is still sometimes used (1 mol/J = 9.65×10^6 molecules/100 eV). The knowledge of G values allows the fraction of molecules affected by irradiation of 1 kg of some substance to be estimated as:

$$\frac{C(X)}{C} = 10^{-3} \times G(X) \times D \times M \tag{6}$$

where C is molar concentration of the neat substance and M is its molecular mass. The larger fraction of molecules will be affected by the larger dose and the larger is the molecule. In water, $G(X)$ accounting for all interactions could be on the order of 1 μmol/J, which, for the dose of 25 kGy, gives $C(X)/C = 4.5 \times 10^{-4}$, or about one out of 2000 molecules, which fortuitously well compares with the previous estimate.

If there were no influence of the medium on the initially produced ion pairs, G(ions) in all media would be 100/W, that is $3 - 4/100$ eV ($\sim$0.3–0.4 μmol/J). However, measured values of radiation chemical yields of primary species electrons, ions, and excited molecules strongly depend on the time of measurement and the nature of the medium. This means that they are modified by the medium during the intervening interval of temporal evolution called physicochemical stage that extends from 10^{-13} to 10^{-10} seconds.

Liquid Formulations—Radiolysis of Water

The understanding of physicochemical processes occurring at early stages of radiation action helps in devising meaningful ways to mitigate radiation-induced damage to the parenteral drug product. Parenteral drugs in solid form or a dry state respond rather favorably to radiation. However, liquid formulations particularly those aqueous in nature present more challenges. The peculiarities of aqueous radiation chemistry are discussed in this section.

An important reaction occurring during physicochemical stage in liquid water is the fastest known chemical reaction:

$$H_2O^+ + H_2O \rightarrow H_3O^+ + {}^\bullet OH \tag{7}$$

which generates the strongest known oxidizing species, hydroxyl radical. It can oxidize any molecule with which it comes in contact and is mainly responsible for the radiation-induced damage of solutes in irradiated aqueous solutions. Another route for the formation of hydroxyl radical is the dissociation of excited water molecules that becomes possible in the same time window with the onset of molecular vibrations:

$$H_2O^* \rightarrow H^\bullet + {}^\bullet OH \tag{8}$$

On the same timescale, the reorientation of dipolar molecules leads to the solvation of charged species, notably the free electron becomes hydrated in water, which, as the strongest reducing species known, can affect radiation sterilization of aqueous solutions of reducible substances.

During that time frame radiation-induced species react within spurs or escape from the spurs by diffusion into the bulk where homogeneous distribution of reactive species is eventually established. The recombination of radical species gives stable molecular products:

$$H^\bullet + H^\bullet \rightarrow H_2 \tag{9}$$

$$^\bullet OH + {}^\bullet OH \rightarrow H_2O_2 \tag{10}$$

which, however, are of little concern for radiation sterilization of solutions.

During the physicochemical stage, dielectric properties of the medium have the strongest modifying effect on radiation chemical yields of charged species. Dielectric constant of the medium determines the critical distance at which the Coulombic attractive force of the ion pair equals the thermal energy that drives them apart. Only those electrons that escape the recombination with the parent ion become solvated and eventually participate in the bulk reactions. In a polar liquid like water the probability that an electron will escape the recombination with its parent ion steeply increases with the increase of the initial electron-ion

separation distance. Therefore, free ion yield is high in water and polar liquids and low in nonpolar liquids.

At the beginning of the chemical stage radiation chemical yields (in μmol/J) are as follows: $G(^{\bullet}OH) = 0.28$, $G(^{\bullet}H) = 0.06$ and $G(e_{aq}^{-}) = 0.27$. Until this moment, the only modifying action on these yields was that of the medium itself, and no additives could have altered them. As it now comes to chemical reactions with the components of the medium, the complex interplay of ionization potentials, electron affinities, bond dissociation energies, and chemical reactivities of the involved species finally determine the outcome of the chemical stage on nanosecond to micro- and millisecond timescales.

The extremely high rate constant of the reaction given by equation (7) and the high molarity of neat water even in concentrated solutions make the reactions given by equations (7) and (8) unavoidable. Any attempts to mitigate in advance ill effects of hydroxyl radical–induced oxidations must admit the impossibility to prevent its formation and recognize that the first opportunity to convert it into a more innocuous species occurs only after it has been already formed.

The hydroxyl radical can oxidize any molecule with which it comes in contact and is mainly responsible for radiation-induced damage of solutes in irradiated aqueous solutions. If the substance of interest, an API, reacts with $^{\bullet}OH$ radical with the rate constant k_{API} giving an unwanted product P, it is possible to find a compound S with a preferably higher reactivity with $^{\bullet}OH$ (rate constant k_S), which acts as a scavenger and which does not give P. The hydroxyl radical is thus given two channels to react:

$$\mathrm{API} + {}^{\bullet}\mathrm{OH} \rightarrow \mathrm{P} \tag{11}$$

$$\mathrm{S} + {}^{\bullet}\mathrm{OH} \rightarrow \text{no P} \tag{12}$$

Radiation chemical yield of unwanted product P, G(P) is given by the ratio of probabilities of $^{\bullet}OH$ reacting in the channel giving P to the overall probability of $^{\bullet}OH$ reaction:

$$G(\mathrm{P}) = G({}^{\bullet}\mathrm{OH})k_{API}[\mathrm{API}]/(k_{API}[\mathrm{API}] + k_S[\mathrm{S}]) \tag{13}$$

G(P) will be at minimum the higher the product k_S[S], that is, the more reactive scavenger and the higher its concentration. The same formalism is applicable to all other reactive species.

The hydrated electron and hydrogen atom may be considered a basic and an acidic form, respectively, of a reducing species in the radiolysis of water. Their interconversion is possible because the respective chemical equilibria are strongly shifted to the right. In acidic media, hydrated electrons are converted into $H^{\bullet}$ atoms:

$$e_{aq}^{-} + H_3O^{+} \rightarrow H^{\bullet} + H_2O \tag{14}$$

whereas in basic media all $H^{\bullet}$ become e_{aq}^{-}:

$$H^{\bullet} + OH^{-} \rightarrow e_{aq}^{-} + H_2O \tag{15}$$

Using scavengers that specifically react only with the oxidizing or the reducing radicals, it is possible to achieve the presence of only one kind of radicals. In a reducing medium hydroxyl radicals are converted into $H^{\bullet}$ atoms:

$$^{\bullet}OH + H_2 \rightarrow H^{\bullet} + H_2O \tag{16}$$

while in an aqueous solution saturated with N_2O (0.02 mole/L), e_{aq}^{-} are converted into $^{\bullet}OH$:

$$e_{aq}^{-} + N_2O + H_2O \rightarrow {}^{\bullet}OH + OH^{-} + N_2 \tag{17}$$

Tertiary butanol efficiently removes $^{\bullet}OH$ and slowly reacts with $H^{\bullet}$, while other alcohols (e.g., isopropanol) remove both $H^{\bullet}$ and $^{\bullet}OH$. At the same time alcohols do not react with e_{aq}^{-}.

Aqueous (Liquid and Frozen) Parenterals

The absorption of radiation energy in a crystalline solid is not focused on a single atom, but a collective excitation involving many electrons spread throughout the crystal lattice is induced.

The energy that would have been localized on an individual chemical bond in an isolated molecule in gas or in a molecule in solution is distributed over many bonds in a crystal. Consequently, radiation chemical yield of decomposition in a crystalline matrix is lower than in solution, which is in turn lower than that in gas, $G_{gas} > G_{liquid} > G_{solid}$.

The buildup of free radicals in solids at low doses proceeds proportionally to dose, then the rate of their accumulation decreases until the concentration reaches the limiting value. The limiting concentration is reached when sufficient free radicals are produced within each other's migration volume so that they can recombine. The upper value of the recombination radius critical for permanent trapping in a solid is considered to be about 1 nm (24).

The uptake of radiation energy by a medium is essentially proportional to the total number of electrons (valence and bound) present in a unit volume, that is, proportional to the mass of material exposed to irradiation. On irradiation of solutions most energy is deposited in the solvent. In irradiated aqueous solutions, reactive species e_{aq}^-, $H^\bullet$, and $^\bullet OH$ produced by radiolysis of water react with any dissolved substances that act as their scavengers and consequently suffer chemical changes. Radiation-induced effects that occur as a consequence of the absorption of energy in the target compound are termed direct effects, whereas those that occur in the reactions between a target compound and reactive species produced in a solvent are termed indirect effects.

Effect of Temperature

Direct effects are not expected to depend on temperature. The effects of elevated temperature on chemical reactions of reactive species in solution that are responsible for the indirect effect can be described by the Arrhenius equation. As the activation energies are rather small (6–30 kJ/mol), the effects on reaction rate constants are also not large. The effects of reduced temperature are more dramatic because a significant increase of solution viscosity impedes the diffusion of reactive species, which leads to their spending more time close to their respective places of origin and ultimately, to their enhanced recombination. For example, radiation chemical yield of e_{aq}^- is reduced by a factor of 10 on reducing the temperature from -5 to $-55^\circ C$ (25) and that of the hydroxyl radical by a factor of 60 on reducing the temperature from 20 to $-40^\circ C$ (26). The yields of products derived from electron or hydroxyl radical attack at these temperatures in ice would be reduced by about 90% and 99.7%, respectively, compared to fluid solutions. Because of the reduced mobility at low-temperature reactions, damaging to solute would be possible only at solute concentrations high enough to have solute molecules in a region of reactive species formation, which we have estimated to be one in 2000 water molecules. However, even at low temperature, larger molecules such as proteins cannot escape direct effects.

Effect of Oxygen

Oxygen normally does not react with stable compounds at room temperature, but its paramagnetic properties make it reactive with free radicals, which are also paramagnetic species created by irradiation of APIs, excipients, or solvents:

$$R^\bullet + O_2 \rightarrow ROO^\bullet \tag{18}$$

The most simple route for creating free radicals directly is the dissociation of an excited molecule R-H yielding a hydrogen atom and a free radical residue $R^\bullet$:

$$(R-H)^* \rightarrow R^\bullet + H^\bullet \tag{19}$$

In an indirect radiation action, the abstraction of a hydrogen atom by $H^\bullet$ or $^\bullet OH$ radicals formed in the radiolysis of water or dissociative electron attachment by a molecule R–X, containing a strongly electronegative substituent X, also yield free radicals:

$$R-H + {}^\bullet OH \rightarrow R^\bullet + H_2O \tag{20}$$

$$R-X + e_{aq}^- \rightarrow R^\bullet + X^- \tag{21}$$

Doubly allylic hydrogen atoms, such as found in polyunsaturated fatty acids, are particularly weakly bound to the backbone of a molecule, which makes these locations especially vulnerable to oxidation. Peroxyl-free radicals formed by the reaction given by equation (18) propagate a chain reaction:

$$ROO^{\bullet} + R-H \rightarrow ROOH + R^{\bullet} \quad (22)$$

which continue to produce damage of an oxidizable substance as long as there is a steady supply of oxygen.

Oxidation is one of the major causes of drug instability, even without radiation. The ill effects of oxidation can be avoided by the exclusion of oxygen that underscores the importance of packaging and closure systems. It can also be prevented by the use of compounds that interfere with the propagation of radical chains by competing with the reaction given by equation (22), which are known as antioxidants. An antioxidant molecule A–H itself possesses a weakly bound hydrogen atom, the abstraction of which produces free radical $A^{\bullet}$, that is more stable (less reactive) than $R^{\bullet}$ and that therefore cannot further propagate the chain reaction:

$$ROO^{\bullet} + A-H \rightarrow ROOH + A^{\bullet} \quad (23)$$

More detailed aspects of stabilization of pharmaceuticals to oxidative degradation can be found in (27).

RADIATION EFFECTS

When considering the effects of radiation on a parenteral drug product, it is important to take into account all elements of the drug product that may be exposed to the radiation environment. This includes the container, closure systems, and packaging materials. If the drug product was previously sterilized using a modality other than radiation, some materials that were selected because of physical-chemical features or tribological attributes may not be radiation compatible, which would entail selection of different materials for the radiation sterilization process. Therefore, whenever possible it is important to select the modality of sterilization early in the development of a new drug product.

Container/Closure Systems and Packaging

Most materials that are found in container/closure systems and packaging consist of different types of polymers and glass. In the evaluation of the effects of radiation on these materials, it is important to take into account possible changes in mechanical properties, radiation-induced discoloration, and biocompatibility. Because glass is amorphous, its mechanical properties are unchanged when exposed to radiation. However, most glass materials discolor in varying degrees when exposed to radiation, which may not be acceptable from the standpoint of aesthetics or possibly functional reasons. The degree of discoloration depends on the type and amount of impurities in the glass, which are a source for radiation-induced stable conjugated chromophores. Some types of glass such as cerium oxide glass show less discoloration than borosilicate glass when exposed to radiation (8). A very high purity glass material such as synthetic fused silica also will not discolor when irradiated. Polymers fall into three general classes that include thermoplastics, thermosets, and elastomers. Thermoplastics are the class of polymers that are commonly selected for containment of a drug product, and closure systems are usually elastomeric in nature. A large compendium of information on the effects of radiation on these classes of polymers can be found in published references and from the manufacturers of the polymers themselves (28,29). Only a few polymers are not radiation compatible and should not be used if radiation is the choice for sterilization. Polyacetals, for example, Delrin and Celcon, polytetrafluoroethylene, that is, Teflon, and natural polypropylene are not radiation tolerant and should be avoided. Polypropylene auto-oxidizes and will continue to degrade following irradiation. A radiation-stabilized polypropylene with antioxidants may be used in some applications. Two elastomers that are not radiation tolerant and should be avoided are butyl rubber and a fluoroelastomer. For example, butyl rubber is friable and will shed particulates. It is important to note that a poor choice in the selection of the polymer is not the only reason a part may fail when it is exposed to radiation. Improper

processing of a polymer or incorrect design may lead to failure of a part that is irradiated even though the polymer is considered radiation compatible. For example, thermoplastics are often fabricated using an injection molding process. If the conditions for fabrication are not optimum, for example, temperature during the mold process, the final part may contain residual tensile stresses. Irradiation leads to breakage of molecular bonds in the polymer. Because of the presence of residual tensile stresses, crazing and microcracking of the polymer may occur. In the design of a part, stress raisers should also be avoided, for example, avoid sharp corners in design of the part.

Radiation Effects—Excipients, Biopolymers, and APIs

Excipients are used to promote pharmacological action of an API by formulation of the drug product in a viable delivery system. Examples of excipients, some of which may appear in parenteral medications, include gum Arabic, talc, starch, and paraffin. The principal effects of radiation that need to be taken into account are change in color, change in pH, and lowering of viscosity. Past studies have shown that excipients should respond favorably up to doses required to sterilize the drug product, that is, 25 kGy or less (30). Loss of viscosity may be of some concern in some cases. In particular, some thickening agents may suffer a significant loss in viscosity at relatively low doses of radiation. Radiation-induced chain scissions in the aliphatic molecular structure of the cellulose component significantly lowers its molecular weight with a concomitant decrease in the viscosity of the thickening agent. Addition of a radical scavenger may significantly improve the radiation stability of the thickening agent.

Biopolymers are used for controlled drug release (CDR) and controlled drug delivery (CDD) of APIs following parentral administration (31). Biopolymers react to radiation in a manner similar to other polymers. There is a possibility of chain scissions, cross-linking, and formation of free radicals. The principal changes of concern from irradiation of biopolymers include change in color and physical properties, which may lead to a change in the drug release characteristics of the biopolymer. For example, polyester polymers such as poly(lactic acid) (PLA) and copolymer poly(lactic acid-co-glycolic acid) (PLGA) are routinely used in CDR/CDD applications. Radiation will reduce the molecular weight of these polymers, with the percentage reduction increasing with increase in absorbed dose. For drug products that have low levels of microbiological contamination, it is possible to set an acceptable minimum dose that satisfies the desired SAL while maintaining a maximum dose that keeps the reduction in molecular weight within acceptable limits.

The principal effects of radiation on an API are formation of small amounts of degradation by-products and possible changes in the chemical-physical properties of the API including pH, color, and viscosity. The radiation-induced degradation by-products may produce toxic extractables that need to be taken into account in the evaluation of the biocompatibility of the API. Changes in the chemical-physical properties of the API could affect the efficacy of the drug product, that is, its potency. Because a vast variety of chemical entities may appear as the APIs, it is almost impossible to accurately predict radiation sensitivity of individual compounds. Previous work on particular or related molecules may inform and guide the assessment of radiation stability of an API.

The effects of irradiation on drugs have been attracting the attention of researchers over the past 60 years. Bibliometric count finds about 1400 references until the year 2000, peaking in the seventies. This literature has been periodically reviewed and a compilation of results from the selection of 217 papers on some 380 APIs has recently been published in form of an encyclopedia (32). Most of the included drugs and excipients are used in sterile product formulations suitable for parenteral administration. The material included in another more recent review (33) is partially overlapping with the former one giving, in addition, an insight into the more recent work, mainly originating from the authors' group. These data may provide clues to the parameters affecting the radiation stability of a drug, the types of possible radiolytic damage, and radiation chemical yields of stable radiolytic products under a variety of irradiation conditions. Together with radiation chemistry principles expounded in the previous section, these data can help the optimization of key parameters to reduce the radiolytic degradation of water-based parenteral drug products. API's in a dry formulation, for

example, powder or freeze dried, are being successfully terminally sterilized on a commercial level using radiation. Parenteral medications in a liquid form present a greater challenge.

IRRADIATION OF SPECIFIC DRUG PRODUCTS

Vaccines

The use of radiation to inactivate a pathogen in the preparation of a vaccine was explored at an early point in the evolution of the radiation sterilization industry (34). These early studies were typically conducted at relatively high doses of radiation, that is, >25 kGy, which was considered necessary to inactivate the pathogen. Even so, some successes were observed wherein sterility was achieved while the antigenic properties of the vaccine were preserved. Most of these studies appear to only have advanced to a preclinical stage. Over the past several years, there has been a renewed interest in the use of radiation in the preparation of vaccines. The reemergence of certain infectious diseases such as tuberculosis may have stimulated this renewed interest in vaccines that are prepared using irradiation. Dependent on the microorganism, the dose of radiation to inactivate the pathogen may be relatively low. For example, researchers at the University of California, San Diego, have shown that *Listeria monocytogenes*, a bacterial pathogen, was inactivated at doses as low as 6 kGy and the irradiated vaccine still triggered long-term immunity in the vaccinated animals (35). However, viral pathogens, which typically have significantly higher D_{10} values than bacterial pathogens, may require much higher doses of radiation, that is, greater than 25 kGy, to inactivate the pathogen. On the basis of studies that have been conducted over the past several years, a significant advantage of radiation in the preparation of vaccines may reside in the possible formulation of vaccines in a dry state, for example, freeze dried (36). A vaccine that is prepared in this manner could possibly be stored for long periods of time in an unrefrigerated state, shipped world wide to a location of need, and reconstituted on site.

Proteins

Protein drugs are specific, exert their effects at low concentrations, and their virtually limitless number enables their use to influence a large variety of biological processes. Therapeutic proteins include monoclonal antibodies, growth factors, cytokines, soluble receptors, hormones, and proteins that block the function of a variety of infectious agents. Specific functions of proteins in the body strongly depend on their structures.

Proteins are characterized by four levels of structural organization. Primary structure of proteins is defined by the amino acid sequence. The ability of antigenic structures to elicit immune response is mostly a sequence-dependent property. At this (primary) level of structural organization, proteins are rather stable to irradiation. Together with the fact that a considerable degree of denaturation can be tolerated in vaccines, this enables the use of radiation in the preparation of vaccines.

Increasing complexity of structures generally brings about their increased susceptibility to mechanical, thermal, and chemical stresses. Consequently, terminal sterilization techniques, including heat, gas, and radiation, have traditionally not been considered suitable for parenteral solutions of proteins (37). Irradiation of proteins in aqueous solution in the presence of oxygen should be avoided on the basis of the first principles of radiation chemistry because it results in the formation of OH radicals and their subsequent addition to C–H bonds along the protein chains, which ultimately leads to oxidative degradation. Irradiation in deoxygenated solutions, on the other hand, favors the reactions of hydrated electrons with peptide bonds and protonated end amino groups. The former reaction also leads to fragmentation and the latter to deamination, and both are unacceptable.

Unique three-dimensional conformation of proteins (tertiary structure) is maintained by the interactions between amino acid residues that are distant from each other in the primary structure. These interactions include hydrophobic and electrostatic interactions, salt bridges, and covalent and hydrogen bonds. They are sensitive to the presence of water, pH, ionic strength and temperature effects, radiation-induced modifications of interacting groups, and dissociation of bonds. For example, an electron adduct radicals formed by irradiation may transfer the electron to a disulphide bond causing its reduction and eventual collapse of the tertiary structure maintained by that bond.

The weakening of the interactions maintaining tertiary structure at an elevated temperature leads to the loss of the tertiary structure (known as denaturation) of proteins on the one hand and to their increased susceptibility to irradiation on the other hand. For example, a three-time larger reactivity of ribonuclease with the hydrated electron has been observed at 65°C as compared to the reactivity at 55°C (38).

It has been demonstrated that radiation-induced degradation of functional properties of proteins (enzyme activity) in solution can be reduced by reducing the irradiation temperature and by additives. For example, the characteristic e-folding values of doses required for the reduction of an enzyme activity to 37% of its initial value (D_{37} values) could be increased by a factor of 4 if enzymes were irradiated in frozen solutions at −200°C, as compared to irradiation at 30°C (39). Other studies have shown that freezing alone may not be sufficient and addition of antioxidants in combination with irradiation in the frozen state was needed to maintain the integrity of the protein at high doses, for example, 50 kGy (40). Even at low doses, for example, 10 kGy, in a low pH solution, irradiated insulin suffered significant cleavage, dimerization, and oxidation (41). Addition of scavengers such as ascorbic acid or oxidized glutathione along with processing at dry ice temperature provided sufficient protection to enable recovery of more than 90% activity. It should be possible to irradiate proteins at dry ice temperature on a commercial level without major constraints. For example, tissue products are being routinely irradiated at dry ice temperature. Irradiation at lower temperatures, for example, liquid nitrogen temperature, would prove more difficult.

However, lyophilization with a well-designed formulation should enable irradiation sterilization to be utilized for terminal sterilization of the drug product. Drying reduces the secondary or indirect damaging effects from radiation while allowing the primary effect to inactivate the pathogens for the desired SAL. Suppression of secondary effects requires the addition of radical scavengers, for example, hindered phenols, ascorbic acid, cysteine, and glutathione (42–45).

Published information on the effects of dose rate on response of irradiated proteins does not provide a clear answer as to whether high-dose rates or low-dose rates are preferred. It is possible that dependence of temperature change on dose rate has clouded some of the results. All other factors being equal, higher dose rates will typically lead to a larger increase in the temperature of the irradiated product. Because temperature can play an important role on the response of proteins, it may be responsible for observed deleterious effects on irradiated proteins rather than dose rate.

CONCLUSIONS

Since the 1950s, radiation has been used to terminally sterilize a whole host of health care products and many types of pharmaceutical products including those used in parenteral medications. Radiation sterilization is an efficacious process that is simple to apply and can be validated in a straightforward manner using existing methodologies. International standards offer guidance on execution of the process. Today 170 gamma irradiators and 41 electron beam irradiators are being used around the world for commercial sterilization applications. Only a few X-ray irradiators are presently operational, but that will change with time. High-energy electrons from high-power accelerators, gamma rays from radioisotopic sources, and X-rays from accelerator-initiated sources are all capable of penetrating deeply into most materials, thus effectively sterilizing all elements of the product. Investigations have shown no evidence of nosocomial infections that are traceable to the sterilization process, for example, Epidemiologic Investigations by CDC/Hospital Infections Program, 1980 to 1990.

This chapter has attempted to elucidate all the key features of the radiation sterilization process including methods of controlling the environment to the benefit of the product that is being irradiated. The section on radiation chemistry focused on liquids, which represent a greater challenge to the radiation sterilization process than drug products that are formulated in a dry state. Dry formulations of parenteral drug products are presently being successfully radiation sterilized on a commercial basis. Methods for improving the tolerance of liquid-based parenteral medications to radiation including addition of anti-oxidants or scavengers and irradiation in a frozen state are presented in this chapter. Because the radiation dose to achieve a desired SAL is bioburden based, the anticipated very low bioburden of liquid-based

parenteral drug products should allow their sterilization at low doses of radiation, thereby enhancing the probability of success. With the development of new biologically derived drugs and combination drug-device products, there will be challenges for effective sterilization of these products. Radiation may become a preferred modality for terminal sterilization of many of these complex products.

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13 Filters and filtration

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FILTRATIVE SEPARATION

Sterile filtration is widely used in the biopharmaceutical industry to remove contaminants, especially microorganisms from liquids and gases. Microorganism removal is required either to achieve a sterile filtrate or to reduce bioburden levels, which in turn decreases endotoxin threats. Sterilizing grade membrane filters are defined by the FDA Guideline on Sterile Drug Products Produced by Aseptic Processing, 2004 by being able to retain more than 10^7 *Brevundimonas diminuta* (formerly *Pseudomonas diminuta*) organisms per cm^2 of filtration area at a differential pressure of 29 psi (2 bar). The retention efficiency has to be validated, using the actual drug product and the process parameters, because of the possibility of an effect to the filters compatibility and stability and/or the microorganism size and survival rate. Performing product bacteria challenge tests became a regulatory demand in 1995 and is now a part of standard filter validation (1). Prior to performing challenge tests, the appropriate challenge methodology has to be evaluated via viability tests. These tests determine the mortality rate of the challenge organisms due to product or process parameters. PDA Technical Report No. 26, 2008, describes the individual parameters, the possible effects, and mechanisms to be used to perform challenge tests. Additionally, the report discusses filtration modes, sterilization, and integrity testing. Multiple parts of this document have also been adopted by ISO (2) and the FDA Aseptic Guideline (3).

FILTRATION GOALS

Contamination Removal

Prime purpose of filtration is the removal of contaminants, which however can vary either being particulates, microbial, viral, colloidal, or gels, etc. The first essential question to be asked when filtration steps are developed or implemented would be what the retention purpose is? The answer to this question is the basis for any step and decision that follows. For every application and removal purpose filter types and designs are required to reach an optimal result. Retention mechanisms of the various contaminants differ, the filter construction can be affected by the contamination type, and the performance of a filter is determined by the form and load of the contaminant (4).

For example, colloidal contaminants, haze, or lipids are retained best by adsorptive filter forms (see adsorptive separation). Microbial retentive filters, especially sterilizing grade filters, should preferably be sieve retentive to assure appropriate organism removal and filtrate sterility. Pore-size specification depends on the contaminant removal purpose, as a filter that is too tight could result in performance losses or oversized systems. Most of the time the purpose of filtration is to remove the contaminant but pass the drug of interest through the filter. Again, a membrane that would be sized too tight could jeopardize the yield outcome. The removal need requires to be well defined and should fit the filtrate quality necessity without dismissal of filtration performance criteria (5).

The design or construction of the filter is determined by the removal need and contamination load. If the load is high and the particulate matter size distribution widespread, the filter should be designed to gain fractionate retention, meaning larger particles are retained first and smaller gradually within the depth of the filter. Such filter would have a multilayer construction to cope with the load and spectrum. If the contaminant is well defined, a sharper retentivity can be utilized, and it may well be that only a single layer membrane will be able to separate the target contaminant. The design of a filter element and/or a filter combination depends on the contamination form and load.

Process parameters, especially differential pressure conditions, often require careful observation, as an elevated starting differential pressure could block the filter prematurely, especially with gel-like contaminations. Filter cake compaction in such instances has to be

avoided as the total throughput would be restricted. The pressure conditions in these contamination occurrences require being as low as possible to avoid any premature blocking.

Rate of Flow

Flow rate becomes a main focus when the fluid to be filtered has a limited amount of contaminants or fouling components. In this instance, a particular fluid volume must be filtered in the fastest time possible. Important here is the timeframe of the filtration process to make the equipment available again for reuse as fast as possible, as it determines parts of the downtime and therefore the capacity available within a production facility. For example, a low flow rate 0.2 μm filter (2500 L/hr) would require 48 minutes to filter a 2000-L volume versus only 20 minutes for a high-flow filter (6000 L/hr). This would reduce equipment use time by half or the effective filtration area (EFA) could be reduced, which would cut filter costs. High flow rates are most commonly required in the filtration of buffers or large volume parenterals. To gain optimal flow rates from membrane filters, there are limited parameters that can be controlled within the filtration process. Either the differential pressure, which is limited, can be raised or larger filter surface can be applied, with the disadvantage of increases in consumable and capital investment costs.

Flow rate depends on the whole filter cartridge design and not solely on the membrane's porosity, thickness, and construction. If a membrane, with an exceptional flow rate, cannot be pleated, it is of no use within a filter cartridge construction. The flow rate optimization of filtration processes requires tests using comparable filter elements, commonly 10 in. filter cartridges. A side-by-side trial can be performed using such comparable filter units, as only this test method would evaluated the entire design of the filter and membrane, in addition to the EFA, flow distribution due to pleat densities, and the fleece thickness. The test would be performed under the specified process conditions, commonly using a set inlet pressure, while the time to filter the fixed fluid volume will be measured. Important is that the process parameters are kept constant, meaning the same buffer composition, pressure, and temperature settings must be applied.

The use of 47 mm disks as an indicator trial for flow rate is meaningless, as these tests do not take constructive designs into consideration. Forty-seven millimeter flow rate results differ greatly from the 10 in. element flows and are of no use in determining an appropriate filter type and scale. Only large-scale trials can determine the best flow rate filter (6).

Total Throughput

Total throughput, meaning the total volume filtered before the filter element blocks, is probably the most widely required performance criteria in most filtrative applications. It is directly proportional to the filter design, surface area, system size, and prefilter combinations. Total throughput has a major impact on filtration costs, and what might appear to be a less expensive filter may actually significantly increase the filtration costs.

The total throughput of a filter cartridge depends on the membrane filter polymer, pore structure, and filter design. Some membrane polymers are adsorptive and higher adsorptivity is commonly associated with a higher fouling rate and therefore lower total throughput. Membranes with a higher asymmetric proportion, meaning a larger pore structure on the upstream side than filtrate side, commonly have a larger total throughput than a symmetric membrane structure due to fractionate retention. Another design improvement is the membrane combination within the filter element. A coarser prefilter membrane layer in front of the final filter membrane, the so-called heterogeneous double-layer membrane, has a distinctly higher total throughput.

Throughput is also referred to as the filter's capacity, meaning the filter's capacity for dirt-holding. It can be expressed as the mass of particulate matter held by a filter, or by the volume of fluid supplied to a filter, it being assumed that the dirt concentration of the suspension is homogenous and constant. The capacity of a filter is a measure of the total volume of fluid that can be processed before a pressure drop develops to decrease the flow to an unacceptably low rate. Capacity may vary dramatically depending on the particles' types and sizes, whether hard and incompressible or gel-like and deformable. The goal aimed for is

the complete and timely processing of a production run; timely being defined in terms of practical and economic significance (6,7).

Throughputs can be judged insufficient; if given the selected conditions of available filter area and differential pressure, the quantity of effluent produced is at too slow a rate to meet the time requirements of the operation. The correction of the condition can be managed by increasing the filter's available area, the differential pressure's (Δp) driving pressure, or both. The larger ratio of EFA to TSS (total suspended solids) thus contrived may limit the filter cake build-up to more modest depths over the filter's now-larger surface. Less blockage would result per unit time. Increasing the filter's available area, or the Δp's driving pressure, or both should correct this condition. If the filtration is already under way, increasing the filter area will be the more difficult alternative. Raising the differential pressure, although easier to accomplish, deserves judicious application. It risks compaction of the retained particles and reduction of the flow rate.

The total throughput can be further advanced by evaluations of appropriate pre- and final-filter combinations, if required. A lower-cost prefilter might be used to protect the final filter and reduce the required final filter size.

Total throughput tests to determine the appropriate final filter and/or combination of pre- and final filter are performed with 47-mm flat filter composites. These composites have to have the same fleece and filter combination as the filter element to be used later. Commonly, multiple composites are tested to determine the appropriate final filter and to be able to test multiple prefilter options. These tests will determine the optimal combination that achieves the highest fluid throughput per EFA.

Nevertheless, 47 mm tests can only suggest the best filter combination. To define the proper filter size required within the production process, small-scale pleated devices of the predetermined filter combination should be utilized.

Unspecific Adsorption

Unspecific adsorption is the second leading cause of yield loss within biopharmaceutical processes after protein degradation due to slow process flows. Any yield loss is proportional to loss of production capacity and market value. Therefore, unspecific adsorption testing must be a priority within applications, which might be adsorption sensitive. Applications encompassing drug products containing preservatives and therapeutic proteins are common examples of adsorption-sensitive processes.

Protein adsorption is a many-faceted phenomenon. It is difficult to predict. Protein surfaces can contain different hydrophobicity, charge, and degree of hydration, and can change with protein conformation and with solution characteristics (8). The filter surface has similar differences. Neither surface is uniform regarding charge or composition. Both hydrophilic and hydrophobic adsorptions may occur. The interaction of protein and surface increases with the hydrophobicity of each. Therefore, hydrophobic adsorptions are believed to reflect protein-filter interactions.

Truskey et al. (9) measured protein adsorption, circular dichroism, and the biological activity of protein solutions. Shifts in circular dichroism and decreases in enzyme activity resulted from conformational changes of the protein structure. Protein-membrane interaction caused the protein to expose its hydrophobic sites, which were folded within its structure during its exposure to aqueous solution. This shows a connection between protein shape and function. Also, shearing of protein molecules and loss of protein properties may result from passage through a filter's tortuous pores. But shearing is seen as causing fewer functional losses via denaturation than do adsorptive conformations.

As described in testing for total throughput, commonly unspecific adsorption assays are performed during small-scale trials or within the process validation procedures of a filter into the specific product and process specifications. Small-scale trials should be performed as early as possible to avoid any surprises or possible validation delays further down the development process. As these trials commonly utilize a small volume of the actual drug product, optimization trials can also be performed. For example, in certain applications it has been found that buffer flush, specific pH, or temperature conditions can minimize the unspecific adsorption into the filter membrane. These conditions require evaluation besides the actual

membrane filter polymer and composite. Forty-seven millimeter disk trials, as in total throughput evaluation, are the best evaluation tool to find the most favorable process parameters and membrane polymers, but the unspecific adsorption of a filter element is also directly proportional to the EFA and the design. The larger the area or the more membrane layers that are utilized the higher the adsorption.

TYPES OF FILTERS

Membrane Filters

Membrane filters commonly have a defined pore structure and porosity band. The narrower the porosity band the more defined the retention rate of such membrane is. The filtration obtained by the use of such membrane filters is often referred to as microfiltration, or MF. Microporous membrane filters have a much-defined porosity than is available within prefilter matrixes. Depth filters have a randomness of the fibrous material that does not allow producing a defined porous structure as within membrane filtration. Membranes are produced by an evaporation, quenching, stretching, or track-etched process. In the evaporation process, the casting solution is applied onto a belt. Because of defined temperature, belt speed, and air conditions, the solvent from the casting solution starts evaporating, this leads ultimately to the formation of the wet-gel form of the microporous membrane (10). Changes in the described conditions and the casting solution mix will create different pore structures, porosities, and membrane structures. In the quenching process, the polymer/solvent mix is applied onto a drum or belt, which immerses into a solvent or extraction bath. The polymer dope starts precipitating and forms a porous membrane. Stretching production process to form membrane is mainly used for polytetrafluoroethylene membranes. Melt extruded films are stretched under defined process conditions to create a thin membrane. The thinnest (10–20 μm) membrane films are created by track-etched manufacturing process. Commonly polycarbonate is subjected to a barrage of high-energy particles. The membrane polymer is damaged at the particle track, and after the submittal to an etching bath pores are formed along the damage. The pore structure of track edge membranes is very defined, but due to the avoidance of particle track overlaps, the porosity is low.

Membrane filters can be formed in a variety of structures for specific application purposes. For example, the formation of asymmetric membrane structures, the pore size on the upstream side is larger than the downstream side of the membrane, can enhance the dirt load capacity of such filter. Membrane filters are the most common filtration devices used in aseptic processing to remove organisms from liquids or gases. Because of the defined structure, these filters are highly reliable with respect to the retention requirements and furthermore can be integrity tested.

Depth Filters

Lenticular filter designs are mainly used as clarifying filters. Highly adsorptive cellulosic or kieselguhr-containing depth filter pads are welded together in a plate format. These plate formats commonly have a diameter of 12 or 16 in. and contain stacks of 4 to 16 to create a depth filter unit. The benefit of lenticular filter types is the high dirt load capacity. The adsorptive depth filter material is ideal to separated colloidal substances and lipids; as a result these filters are very often used in plasma and serum applications. Nowadays lenticular filters are most often used in cell harvest applications after the fermentation process.

When lenticular filter combinations are tested, the tests do not only involve the total throughput of the filter element as it is commonly the case with pleated prefilter cartridges, but an important factor is the turbidity measurement of the filtrate. The turbidity measurement will create an indication of the protective properties of the lenticular filter retention rating used and how much of the contaminants are separated by the particular filter rating.

Test methods, validation requirements, and design specifics have recently been elaborated in the PDA Technical Report 45, Filtration of Liquids Using Cellulose-Based Depth Filters (11).

Prefilters

Prefilters are most commonly depth filter types and are generally constructed of nonwoven or melt-blown fiber materials such as polypropylene, polyamide, cellulosic, glass fiber, metal

fibers, and sintered stainless steel (12). Most commonly, prefilter materials are constructed into mats by the random deposition of either individual or continuous fibers whose fixation is accomplished by pressing, heating, gluing, entanglements, or other forms. The pores of such filter constructions are rather random interstices among the fibers. Such pore-size distribution can be influenced by the thickness of the individual fiber or the compactness of the matrix. Therefore, prefilter types have a large variety and can be selected for many kinds of application.

A major advance in depth filter design technology was the construction of melt-spun depth filter types and the introduction of heat stabilization of fiber fleeces. These treatments avoided the release of particulate matter and were utilized to stabilize the final filter fleece. Additionally, these technologies allowed producing fleece construction of different fiber diameters within a filter matrix. This allowed improving the total throughput performance of these filters due to fractionate retention of a large spectrum of particle sizes. A further advance in depth filter technology occurred with the advent of the first melt-blown type of cartridge that incorporated various fiber diameters, as the filter was manufactured, to achieve a graded pore design by means other than varying the fiber packing density. This design is based on using a variation of standard melt-blowing equipment. In this process, the polymer is extruded through a multihole die and the polymer stream is stretched and attenuated by a high-velocity heated air stream. The mean fiber diameter is changed as the filter is being made by adjusting the air velocity or one of the other variables that contribute to the formation of the fiber sizes, for example, temperature or polymer pumping rate. This technology is becoming more advanced, with some manufacturers naming the fibrous fleece constructions as nanofiber fleeces.

The concept of using a graded or changing pore size to enhance filtration performance is a desirable one. This technique involves incorporating a series of prefilters into a single stage to maximize the use of the entire filter and extend filter life (dirt-holding capacity). The factor of fractionate retention is especially important for applications with a wide particulate spectrum, as for example water pretreatment.

Prefilters can also contain membranes, porous or fibrous, commonly from cellulose, mixesters, or borosilicate. These prefilter types are utilized to remove a very fine band of particulate or contaminants from the fluid to specifically protect sterilizing grade membrane filters. The retention rating of a prefilter is not defined by pore size but by nominal retentivity, commonly a particle-size retention of more than 99%.

Nanofilters

Most commonly, nanofilters are designed to separate viruses, using size exclusion as the predominate mechanism of removal. Since nanofilters are extremely tight filters, the water bubble point is commonly higher than the maximum allowable operating pressure. Therefore, integrity testing of these filters requires special test methodologies, such as liquid porosimetry (13,14). This test method uses two immiscible liquids that are successively intruded by pressure into largest pores of the membrane. These porosimetry measurements may be correlated to viral removal post filtration, which allows the test to be used to validate viral removal in actual practice. Nanofilters or viral retentive filters are an essential contaminant removal step especially in bioprocesses. A multitude of nanofilters are available for different applications and target contaminants. Most common retention ratings are 20 and 50 nm, also known to separate parvo- and retroviruses.

GENESIS OF PORES

Pore Structure

Microporous membrane's analogy is that of a polymeric sponge (Fig. 1). The oversimplified picture of the filter pores is that of irregular and tortuous capillaries composed of the interconnected spaces within the polymer matrix. The structure derives from a polymer solution and the chain segments are separated from one another by distances that reflect the polymer dilution. It is the final interstitial distances that in their interconnections prefigure the pores of the finished membrane. Formulae of different polymer concentrations give rise to different intersegmental separations, ultimately to different porosities (15).

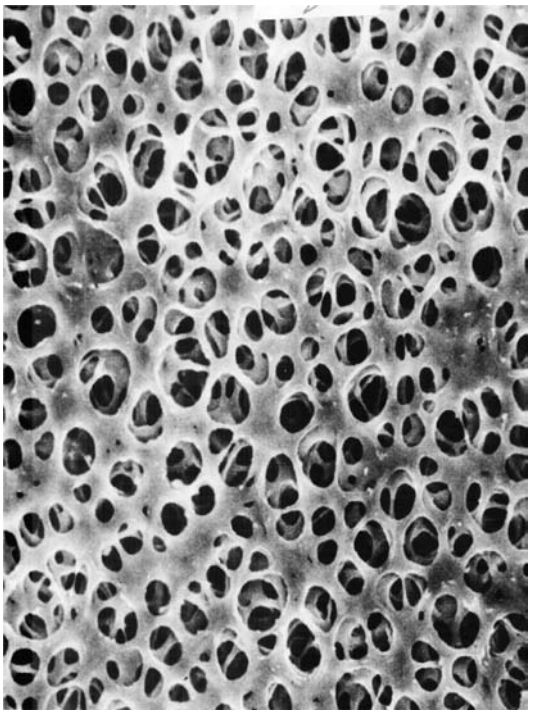

Figure 1 Microporous membrane structure. *Source*: Courtesy of Sartorius Stedim GmbH.

The casting solution consists of polymer dissolved in a mixture of solvent and high-boiling nonsolvent. Pore formation occurs as follows: As solvents progressively evaporate from the casting solution, the nonsolvent increases in content to the point where phase separation takes place. Nonsolvent droplets separate within the polymer/solvent phase, and polymer comes out of solution to concentrate at the droplet interfaces. The swollen polymer shells surrounding the nonsolvent droplets thicken as continuing solvent loss causes more polymer deposition. The eventual disappearance of the polymer/solvent phase brings the polymer-surrounded droplets into mutual contact. They consolidate into clusters, and distort into polyhedral cells filled with nonsolvent under the impetus of the area minimizing forces. Finally, the edges of the cells accumulate polymer at the expense of the cell walls. Thinning of the walls of the polyhedra leads to their rupture and interconnection. The reticulation of the discrete cells of the polymeric matrix permits the removal of the nonsolvent, as by washing. Not the polyhedral cells, but their interconnecting openings, thus formed, comprise the metering pores of the membrane (15).

Polymeric Types and Properties

As one can expect, there are distinct differences between the individual membrane and prefilter polymers. Table 1 lists the different membrane polymers available and the advantages and disadvantages, which depend on the properties of the polymer. The table shows that there is no such thing as a membrane polymer for every application. Therefore, filter membranes and the filter performance have to be tested before choosing the appropriate filter element.

PORES SIZE

Ratings

Where sieve retention of particles is the only consideration, the size of the largest pore, present in the filter is ultimate concern. Particularly in the filtrative sterilizations of pharmaceutical preparations, there is an emphasis on achievement of that particle size/pore size relationship that can produce organism removal solely by sieve retention. However, in theory, complete organism (particle) removal does not require the exercise of sieve retention. Adsorptive particle capture may also be utilized. Microporous membranes are used in filtration sterilization because there is considerable surety of particle retention that can be in most

Table 1 Properties of Different Membrane Polymers

Membrane material	Advantage	Disadvantage
Cellulose acetate	• Very low nonspecific adsorption (nonfouling) • High flow rates and total throughputs • Low environmental impact after disposal	• Limited pH compatibility • Not dry autoclavable
Cellulose nitrate (nitrocellulose)	• Good flow rate and total throughputs • Capture of smaller particles than the pore size	• High nonspecific adsorption • Limited pH compatibility • Not dry autoclavable
Regenerated cellulose	• Very low nonspecific adsorption (nonfouling) • Very high flow rates and total throughputs	• Limited pH compatibility • Not dry autoclavable
Modified regenerated cellulose	• Very low nonspecific adsorption (nonfouling) • Moderate flow rates and total throughputs especially with difficult to filter solutions • Broad pH compatibility • Easily cleanable (in cross-flow applications required)	• Ultrafilters not dry autoclavable
Polyamide	• Good solvent compatibility • Good mechanical strength • Broad pH compatibility • Dry autoclavable	• High nonspecific protein adsorption • Low hot-water resistance • Moderate flow rate and total throughput • Vacuole formation during casting can result in exaggerated pore sizes
Polycarbonate	• Good chemical compatibility	• Moderate flow rates • Low total throughputs • Difficult to produce
Polyethersulfon	• High flow rates and total throughputs • Broad pH compatibility • Highest versatility • Mainly found as asymmetric membrane structure	• Low to moderate unspecific adsorption depending on surface modifications • Limited solvent compatibility
Polypropylene	• Excellent chemical resistance • High mechanical resistance	• Hydrophobic material • High nonspecific adsorption due to hydrophobic interactions
Polysulfone	• High flow rates and total throughputs • Broad pH compatibility	• Moderate to high nonspecific adsorption • Limited solvent compatibility
Polytetrafluorethylene	• Excellent chemical resistance • High mechanical resistance • High hydrophobicity (used for air filtration)	• Hydrophobic material • High nonspecific adsorption due to hydrophobic interactions • High-cost filter material
Polyvinylidene difluoride	• Low nonspecific adsorption • Dry autoclavable • Good solvent compatibility	• Moderate flow rate and total throughput • Hydrophobic base, made hydrophilic by chemical surface treatment; may lose hydrophilic modification due to chemical attack • High-cost filter material

cases demonstrated to be independent of operating conditions. Sterilizing grade membranes are expected to have a pore-size distribution pattern wherein the largest pore is smaller than the smallest microbe whose retention is being required. Sieve retention is consequently assumed to be the sole particle-capture mechanism operational. This is the intended situation, for the dependability of sieve retention is seen in its freedom from the operational factors that

influence the efficiencies of adsorptive removals, such as the organism challenge level, the magnitude of the applied differential pressure, and even such parameters as fluid temperature, viscosity, ionic strengths, the presence of wetting agents, etc., that constitute the contribution of the liquid vehicle (16,17). In fact, filter reliability, involving whatever mechanisms of particle removal, is demonstrated beyond doubt by the exercise of filter validation.

Semantics enter the picture of the largest pore. As commonly considered, a penetrating particle encountering the filter enters by way of a large enough pore and completes its penetration unhindered. In this scenario, the large inviting pore maintains its generous dimensions clear through the filter. In this sense, the bubble point assay measures the diameter of the entire pore passageway; no distinction is made between the "largest pore" and any particle-restraining portion of the pore. Actually, the pore diameter not being uniform throughout the bubble point measures the narrowest point of the overall widest pore.

Regrettably, the current use of the word "pore" is undifferentiated with regard to its meaning. Its use covers both the polyhedral chambers and their connecting, restrictive, smaller apertures. The pore passageways consist of an assemblage of larger and smaller apertures interconnecting the polyhedra. Overall, certain of these passageways are the largest in the sense that they are least restrictive. However large the passageways, it is their restrictive dimension that is measured by the bubble point. In this sense it is not the largest pore, the largest aperture leading from the polyhedron, but the narrowest of those comprising the largest pore path overall that comes to be measured. Strictly speaking, therefore, it is not the largest pores that are revealed in the bubble point measurement but the most restrictive ones associated with them in the overall largest pore path.

Distribution

Pore-size distribution means the spread of different pore sizes within the membrane structure. The membrane structure being sponge like, one finds a pore size spread over the entire membrane structure, width as well as depth. The efficiency of particle removal varies inversely with the challenge density. This can be explained on the basis of a pore-size distribution wherein the number of smaller pores far outweighs the fewer large pores. Only when so great a number of organisms are present as to enable confrontations with the few larger pores, might organisms escape capture. The attention, especially in sterilizing filtrations, is so focused on restraining bacterial passage that only the largest pores, those that the organisms can negotiate on a size basis, are a matter of concern. Hence, the emphasis is on the bubble point measurement of the set of largest pores. There is reason to believe that, despite their relative paucity, the larger pores are early on engaged by the hydrodynamic flow when diluter organism suspensions are fed to the filter (18–20). One factor that had delayed explanation of the dependence of organism retention on the challenge density was the de-emphasis of the pore-size distribution. The pore-size distribution of membranes had early on been explored by mercury porosimetry.

When mercury is forced into a pore, the pressure required to fill that pore completely is in inverse proportion to its size. The relationship is, as for the capillary rise equation,

$$D = -\frac{4\gamma \cos\theta}{P}$$

except that the minus sign is required by the nonwetting nature of mercury relative to membrane surfaces. Here P is the pressure; D, the pore radius; γ, the surface tension of mercury; and θ, the contact angle of mercury with the solid pore surfaces.

Assuming that $\theta = 130^\circ$, γ has a value of 485 dynes/cm. Converting dynes per centimeter to psi yields $D = 181/P$ when the pore diameter in micrometers is inversely proportional to the mercury intrusion pressure in psi. In this procedure, the precise measurement of the mercury volume at any pressure, and hence a means of gauging the volumes intruded into the filter, is assessed dilatometrically, a method offering great accuracy.

Whatever its virtues, the method has serious shortcomings. Badenhop (21) concludes that mercury porosimetry is unsuited to the pore-size measurement of microporous membranes, and Williams (22) states that, in principle, fewer than 20% of the largest apertures (pores) need be breached by the intrusion of mercury to fill the membrane entirely. The chief objection to

mercury porosimetry arises from the artificialities its manipulations bear to the filtrative process, an operation that usually involves aqueous flow through a filter under rather moderate pressures, the very essence of the flow-pore regimen. In any case, using this procedure, measurements can be made of the cumulative volume of mercury introduced into a filter at different pressure levels. From this, the percentages of the various pore sizes become available, and also the pore-size distribution curve.

Early work was taken to suggest that membrane filters had a pore-size distribution of ± 0.02 μm about their mean pore-size rating. This narrow distribution had significance, as it was suggested that these filters would be expected to exhibit "absolute retentions," and this was further supported by the successful use of such membranes in filter sterilizations. However, examination of four commercially available 0.45 μm-rated membranes, each from a different manufacturer, by mercury porosimetry demonstrated that none of the tested filters had pore-size distributions as restrictive as ± 0.02 μm. Therefore, it has been stated that the high reliability of their 0.2 μm-rated membranes for filtration sterilization applications is, therefore, derived from one or a combination of other physical and/or physiochemical considerations (23).

FILTER DESIGN AND CONSTRUCTION

Filter Styles

Disk or flat filters were the first filter configuration used in the pharmaceutical industry, mainly as 293 mm disks within multistack stainless steel–holding devices. The assembly of such housing was/is difficult as one works with wetted flat filters and has to be extremely careful not to damage the filter membrane. Also wrinkles or bends during assembly might cause problems during the filtration process. Disk filters are cut from the casted membrane sheet and are available in a large variety of size, either builds into a disposable plastic housing or placed into a filter holder with diameters from 4 to 293 mm. The most common 47 and 50 mm are utilized as microbial (analytical) assessment filter and can have different colors or colored grids printed on the membrane. Such analytical filters commonly have a pore size of 0.45 μm and utilize adsorptive polymeric materials, for example, nylon or cellulose nitrate (21,24). The reason for the material choice is the requirement of adsorptive capture of the organisms. The pore size is chosen to be 0.45 μm to assure the nutrient, on which the membrane is placed, penetrates through to the membrane surface to feed the captured organisms.

Since disk filters are restricted within its EFA, pleated filter cartridge designs were developed to increase the filtration area without increasing the footprint of the filtration system or filter holder (Fig. 2).

The primary stimulus to develop pleated membrane cartridges was the need of an increase in the filter area sufficient to secure the engineering advantages of lower applied differential pressures and larger volume flows. Achieving this goal in the pleated filter cartridge form meant, moreover, that less floor space needed to be allocated for filter installations. To replace a common 10 in. filter cartridge and to achieve its same EFA, fifteen 293 mm disks would be needed. Therefore, the footprint of such system is by far larger than the need of a 10 in. filter housing. Moreover, every disk filter required O-ring sealing, therefore the assembly was time consuming and insecure.

The first pleated membrane materials were cellulose acetate, cellulose mixesters, polyamide, and polyvinylidene fluoride. Commonly, these membrane materials were surface treated to achieve pleatability, wetability, and stability of the membrane, which required large water flush volumes before the filter could be used. Nowadays available pleated filters are composed of cellulose acetates, Teflons, polyvinylidene fluoride, polysulfone, polyethersulfon, nylon, etc. The pleating arrangement, the back-and-forth folding of the flat membrane filter on itself, permits the presentation of a large filter surface area within a small volume. A pleated membrane cartridge of some 2.75 in. (70 mm) plus in diameter and 10 in. (254 mm) in length can contain from 5 to 8 ft^2 (0.5–0.8 m^2) of filter surface, depending on the membrane thickness, prefiltration layers, and construction detail. Pleated membrane cartridges are also offered in various lengths from 2 to 40 in. and EFA s from 0.015 to 36 m^2. This range of sizes and EFAs are

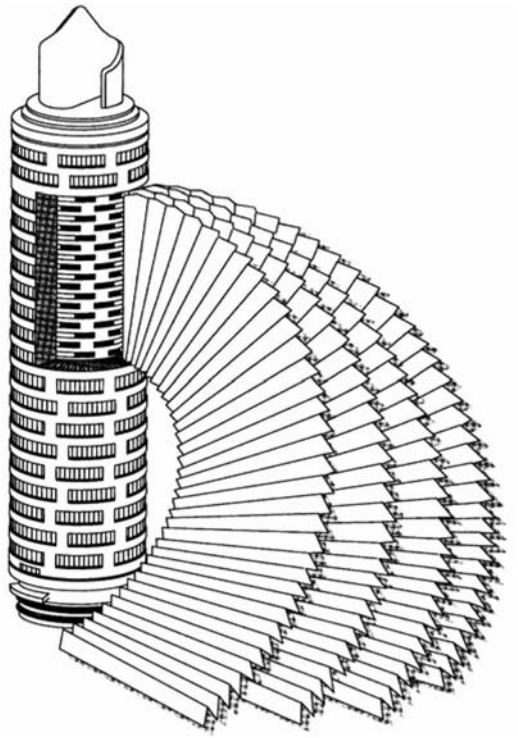

Figure 2 Typical pleated filter cartridge design. *Source*: Courtesy of Sartorius Stedim GmbH.

required for scale-up and down within the process and development steps. A pleated filter device should be able to scale-up linear from the preclinical volume size to process scale (11).

Typical construction components of the pleated filter cartridge are as follows:

The end caps are the terminals for the cartridge pleat pack and are responsible for holding the cartridge contents together. The end caps are also responsible for providing the seal between the cartridge and the O-ring recess on the cartridge-housing outlet plate or a base for the filter cartridge adapter, which can be of different shape and form. The adapter would be used to create a reliable seal between the filter and the filter housing. Polypropylene end caps are frequently adhered to the membrane pleat pack, by the use of a polypropylene melt softened preferably by fusion welding. In the past, stainless steel rings in the cartridge adapter stabilized the cartridge adapter against steam-induced dimensional changes and so preserved the integrity of the O-ring seal against bypass. The use of such dimension-stabilizing rings is made in the construction of pharmaceutical-grade cartridges intended for sterilization(s), especially when polypropylene end caps are involved. Nevertheless, it has been also found that such stainless steel ring, with different expansion rates during temperature changes, can also cause problems with respect to hairline cracks and fissures within the adapter polymer or the welding sites. This could go so far that the adapter damage does not allow proper O-ring sealing. This effect often has been seen with adapter, which has not been molded from one piece. The welding starts cracking, liquid penetrates into the stainless steel ring cavity, and expand during the next steaming (25). To avoid the differences in expansion of the support ring and the adapter polymer, most of the adapters are constructed with a polymer support ring.

The outer support cage is responsible for forming the outer cylinder of the cartridge and for holding the pleated internal contents together. The outer support cage also provides for a backpressure guard in preventing loss of filter medium integrity as a result of fluid flowing in the opposite direction under excessive backpressure. Additionally, it eases the handling of the filter cartridge during installation. The user does not come in direct contact with the pleats and damage can be avoided.

The upstream nonwoven support layer serves as a multipurpose component. Pleating, and the assembly of the membrane into cartridge form, requires its inclusion in the cartridge. The supportive outer pleated layer aids in protecting the filter medium throughout the cartridge pleating and assembly operation. The material also serves as a prefilter to extend the useful service life of the final membrane that lies beneath it. Lastly, the support maintains the structure throughout fluid processing. Without this layer, the pleats under pressure might be compressed, limiting the filter area available to the fluid processing.

The drainage or downstream nonwoven support screen, similar to the upstream filter pleat support, stabilizes the pleating of the pleat pack. Moreover, it keeps the filter medium pleats separated during fluid processing to assure that maximum filtration area is open for optimum flow rates and drainage of remaining filtrate, that is, reducing the dead volume or otherwise trapped fluids. The filter arrangement of the microporous membrane sandwiched between the support and drainage layers, all simultaneously pleated, is often called "the filter pack" or the "pleat pack."

The filter cartridge inner core serves as the inner hollow tube on which the pleated pack is supported. It confers strength on the cartridge assembly. This component also determines the final assembly length of the cartridge. Lastly, the core is the outlet port of the cartridge. Through its perforations, the filtered fluid passes to be guided to the outlet plate of the filter housing. The cartridge core should not be flow limiting but can be in high-flow applications, that is, air filtration or water filtration with prefilter cartridges.

The filter membrane is the center of the filter cartridge, responsible for removal of the contaminants. Solutions permeate into and through the filter medium and into the cartridge inner core, then proceed through the adapter assembly and effluent piping. Once the filter medium has become fully wetted, processing can be continued until one of several flow decay indicators signals the need for cartridge replacement, as customer preference dictates.

Cartridge designs can be manifold and fit for the application. Not only size differences are applicable, but also cartridge adapters, that is, plug-ins, which fit into filter housings sockets and recesses. Single open-ended filter cartridges with bayonet locking are mainly used for sterilizing grade filter cartridges due to the reliability of the fit into the housing. Bypass situations have to be avoided, which can only be accomplished, if the sealing between the filter cartridge and its holder is snug. In the case of the string-wound cartridges, no end caps are used, because the avoidance of product bypass is not as critical as in sterilizing grade filtration (11); only the double open-end cartridges and the adapter pieces need be stocked.

In microporous membrane applications, frequent use is made of the single open-end 10 in. cartridge, usually in T-type housings. Therefore, such a unit is manufactured with an integral end cap. Such cartridges are also constructed in 20 and 30 in. lengths. Attempts have been made to offer pharmaceutical manufacturers the versatility of 10 in. single and double open-end units to be assembled via adapters with O-rings. Since such an arrangement increases the critical sealing area, its acceptance has been limited. The more widespread use in critical pharmaceutical manufacture is of single open-end 10, 20, and 30 in. cartridges.

The O-ring materials used are also of critical importance, as the chemical compatibility of the O-ring material has to be determined toward the fluid to be filtered. The O-ring is the critical area of the separation between up- and downstream side, therefore any incompatibility might be a hazard to the filtrate quality. Furthermore, in instances of multiple steam sterilization, the O-ring material has to be checked for so called heat set. The O-ring experiences the pressure points from the housing wall and the cartridge adapter. When the temperature is elevated, as in the steaming process, the O-ring starts deforming at the pressure points. If the O-ring material is not flexible enough, the deformation (heat set) will be maintained. The O-ring will commonly show an oval shape. It is important that O-rings are visually inspected on a routine basis to see whether the O-ring is deformed. Any heat set might result into a bypass situation. Ethylene propylene diene monomer (EPDM) O-ring materials showed so far the highest heat set tendency, nevertheless, are very compatible to chemicals. Silicone has commonly a high flexibility and low heat set (5).

The resulting increase in the EFA reflects two factors in addition to the cartridge diameter. The first consideration is the diameter of the center core of the cartridge. Each pleat consists of a membrane layer or of multiple membrane layers, sandwiched between two

protective layers whose presence is necessary to avoid damage to the membrane in the pleating process, and which serve usefully in the finished cartridge as pleat separation and drainage layers. As a consequence of this sandwich construction, each pleat, naturally, has a certain thickness. Fewer of these thicknesses can be arranged around a center core of narrower diameter. Therefore, increasing the diameter of the center core increases the extent of its perimeter and the number of pleats that can surround it. This governs the number of pleats possible in the pleat pack that can comprise the membrane cartridge, thus increasing its EFA.

To define a cartridge, designations must be made of such considerations as its pore-size label (3), its diameter, its length, the type of outlet, for example, the O-ring(s) sizes, the configuration of the outer end, for example, open or closed, with or without fin, the type of O-ring or gasket seal, for example, silicone rubber, EPDM rubber, and any nonstandard features. Manufacturer product numbers serve as shorthand substitutes for the detailed specifications.

The optimum number of pleats to be arranged about a center core of a filter cartridge may reflect the filtrative function for which it is intended (25,26). In the handling of rather clean, prefiltered liquids, as in most pharmaceutical final filtrations, relatively few particles require removal. A crowding of as large a number of pleats as possible to enhance the filter area may be acceptable because the pleat separation layers will operate to make even the crowded surfaces individually available to the liquid being filtered. Where there are high solids loadings in the liquid, or a viscous fluid, a different situation may result however. The particles being removed may be large enough to bridge across a pleat to block the interval between two adjacent pleat peaks. Or, being small, they may, after their individual deposition on the filter, secrete and grow large enough to cause bridging. Whatever the mechanism, the bridging serves to deny the liquid, being processed, access to useful flow channels bordered by membrane.

In practice, pleated cartridges are built for general usage in what is still an artful construction (5,25,27). Nevertheless, there is said to be available an empirically developed formula that relates the outer cartridge diameter to the maximum core diameter, and to the number of pleats of given height that should be used.

Care must be taken to protect the surface of the membrane during the pleating operation and to avoid damage to the filter structure. Both these objectives are furthered by sandwiching the membrane between two support layers and feeding the combination to the pleater. The outlying support layers protect the membrane surfaces. Nevertheless, the fleeces have to be chosen properly; for example, a fleece too coarse could press too much on the membrane at the pleating curvation and starts pressing into the membrane. In Figure 3, one can see the result of

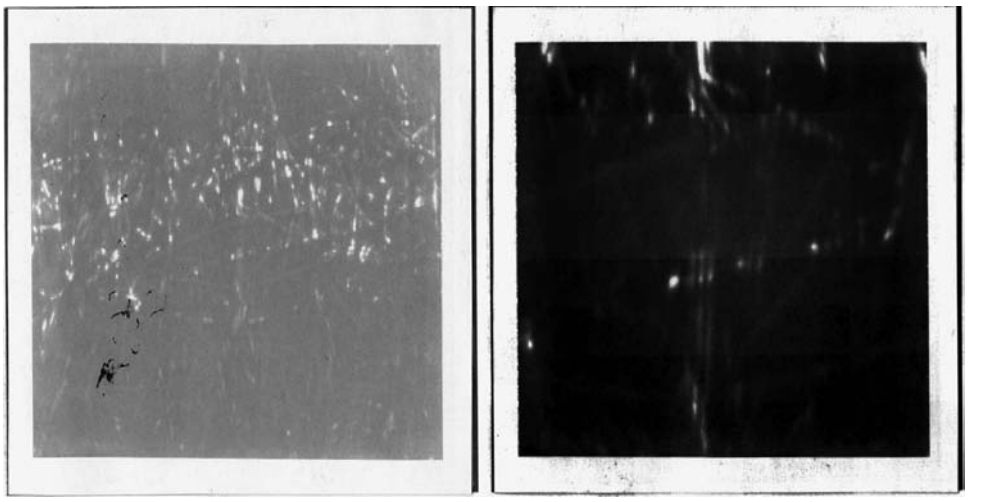

Figure 3 Prefilter impressions on a PTFE membrane filter membrane. *Source*: Courtesy Sartorius Stedim GmbH.

coarse fleece compression on a PTFE membrane, which weakens the membrane and might be detrimental in long-term use of the filter. Air filters are used over a long period and experience multiple in-line steam sterilization. If the membrane shows impressions by the coarse filter fleece, this commonly means that the filter membrane in this area is thinning. Multiple steam sterilization could exaggerate this thinning and flaws can develop. On the other hand a fleece, which is too soft will not support the membrane sufficiently. Usually soft fleeces have a high-fiber density and a small-fiber diameter, which means liquid, would be bound within the fiber structure. Such phenomenon needs to be avoided, for example, in air filtration, because it could cause water logging.

Additionally, the sandwich in its thickness minimizes opportunities for the membrane to be too strongly compressed at the pleat. What is required is a pleat having some radius of curvature rather than a sharp, acute angle of fold. This prevents the membrane from being subjected, at the pleat line, to forces in excess of its mechanical properties as expressed in the magnitude of its tensile and elongation values. Different polymeric materials will, of course, have different tensile and elongation qualities; various materials differ in their brittleness. Additionally, sharp pleat edges or pleatings with a high pleat density will have a gap in between the pleats, which would result into capillary activity; that is, in air filtration condensate could potentially be trapped in between the pleats and the air filter might experience water blockage. Therefore, filter designs and construction require thorough investigation in development to achieve the best performance ratios. In instances the highest effective filtration are in the confined construction of a filter cartridge might not be the optimal solution, as the pleat density becomes too high. Nevertheless, EFA should also not be too low as it will influence the flow rate and total throughput. Decreasing the diameter of the center core will serve to lessen the number of pleats, although in applications that require a high flow; for example, air, the inner core becomes the flow restrictor. Therefore, the inner core again needs to be optimized to the filter cartridge utilization. For example, a 28-mm core diameter will require a 40% to 50% higher differential pressure than a 35-mm inner core to achieve an air flow rate of 100 scbm. This differential pressure increase might not seem to be high, but the costs involved running such pressure difference is substantial.

The disk and cartridge filters of commerce are usually disposables. It is their housings and holders, usually of metal, that are permanent. However, filters encapsulated into plastic housings have been devised wherein the entire unit is disposable (Fig. 4). There are advantages to these devices. Among them is that many are available in presterilized conditions, by gamma irradiation, steam, or ethylene oxide. Another advantage, therefore, is their ready availability. That they are disposables does not necessarily militate against the economics of their usage.

Figure 4 Different filter capsule devices. *Source*: Courtesy of Sartorius Stedim GmbH.

Calculations show that where labor costs are reckoned, the installation of a single 293-mm filter disk in its housing is more costly than the equivalent filtration area in the form of a disposable filter device. The use of the disposables entails very little setup time and no cleanup time. There is no need to sterilize the already presterilized units. Disposal after the single usage eliminates risks of cross-contamination. Pleated disposable device show commonly better performance due to the prefilter fleeces and sometimes prefilter membrane in front of the final filter membrane. Therefore, 293 mm disk filters could potentially also be replaced by 150 or 300 cm^2 disposable devices, even when such have a smaller EFA.

The venting of disposable filter devices has been the subject of good design considerations. One disposable-capsule manufacturer has positioned the vents at the highest point of the containing shell, exactly where they are most effective. Another design utilizes a self-venting device in the form of a hydrophobic membrane. This permits the self-venting of air while safeguarding against the passage of liquid or contaminants (in either direction).

There are often ancillary advantages to the use of disposable filter devices. Some manufacturers construct their shells of transparent polymers so that the filtration process is observable. The instruments are compact and relatively lightweight, hence, easy to handle; also their construction does not lack the sophistication of their metal housing–contained counterparts. Many of the disposable units are equipped with vent plugs and drain plugs. The identifying description they bear on their outer casings, make their traceability, in accordance with FDA record requirements rather certain (3). Product and batch numbers become part of the permanent operational record. Above all, the use of these disposables obviates the need to expense or amortize stainless steel filter holders. No capital expenditures are involved.

The use of disposable filters can reduce costs in respect of cleaning, which would occur with stainless steel filter housings after every use. Cleaning validation, which needs to be performed with fixed equipment like filter housings, will be greatly reduced. The disposable filters do not go through such cleaning regime and therefore the validation of cleaning exercises is avoided. For this reason and the convenience of the use of disposable filters, the biopharmaceutical industry has switched more and more to capsule filters instead of filter housings. Commonly, a disposable capsule filter is connected to a disposable bag, both are available in different sizes for the individual purpose. Once the capsule filter is connected to the bag the entire setup is gamma irradiated to sterilize. Certainly the filter material and polymers need to be gamma stabile otherwise particle shedding or an excess amount of extractable can occur.

Another advantage of disposable filter capsules is the fact that the user will not encounter the product filtered. This certainly could be the case when using cartridge filters within a housing. The cartridge has to be removed from the housing at the end of the filtration run, that is, the user probably comes in contact with the filtered product remaining on the filter cartridge and housing, which may need to be avoided due to health hazards or biological activity. Disposable filters create the opportunity to replace a filter without being in contact with the product.

The disposable filter devices are available in a variety of constructions, whether disk, multidisk, pleated cylinders of various lengths and of different EFA s. Their expanse of filter surface runs from 4 mm disks suitable for affixing to hypodermic needles to 30 in. capsules of about 180 ft^2 (1.8 m^2). The filters are made of a variety of polymeric filter materials, both hydrophilic and hydrophobic, namely, cellulose esters, polyvinylidene fluoride, polysulfone polyethersulfone (26), nylon, polyethylene, Teflon, etc. Their shells can be composed variously of polycarbonate, polyethylene, but most often polypropylene.

The use of most cartridge filters accords with FDA emphasis on record keeping. Despite all the care with which filter manufacturers pack flat disk filters, the membranes themselves are unlabeled. Cartridge filters are, however, available with identifying data (28). Most are identified with some code, if not on the cartridge itself then on its container. Some manufacturers stamp the cartridge end cap with the part number, its pore-size identity, and its lot number as well. Indeed, some manufacturers even number each cartridge consecutively within each lot. Should the need ever arise to trace the components and history of these filters, and of their components, the ability to do so exists. Batch records in concert with the appropriate manufacturing QC records make this possible.

Because of the fragility of most membrane filters, appropriate and even extreme care is to be used in their handling. In the case of cartridge filters, this practice continues. However, the actual membrane surface of these instruments is out of reach ordinary handling. There is, therefore, far less possibility of damage to the filters. Overall, cartridges are used mostly for the more rapid flow rates and/or the large-volume filtration productions they enable, a consequence of their aggrandized EFAs.

Membrane Configurations

A homogenous membrane is usually a dense film ranging from 10 to 200 μm thickness. A porous membrane understandably has a porous structure (29–31,70). The size and shapes of the pores largely determine the separation characteristics. As the pore size increases, the separation become more similar to that of a filter, where compounds are allowed to pass on the basis of size. The intrinsic nature of the material can still have an effect on the separation by, for instance, slowing the passage of one compound due to molecular attractions. The pores in a porous membrane can be cylindrical. However, it is more common that the pores have a range of size and are tortuous path. The separation mechanism for porous membranes is more similar to conventional filtration—larger particles or compounds cannot pass through the pores and are therefore retained.

In addition to porous and homogenous, membranes can be classified as symmetric and asymmetric. Symmetric membranes have a structure that is consistent throughout. Homogenous membranes are symmetric. Porous membranes can also be symmetric with pore sizes and pore shapes consistent throughout. Nevertheless, there is no general understanding, defined parameters, or equation to classify a membrane as asymmetric or symmetric. Therefore, each membrane manufacturer and user have their own approach to the definition of this membrane parameter.

In general, an asymmetric membrane has a structure that is different on the surface compared to the interior. In one case, the surface, or skin, may be dense and the rest of the membrane is porous (Fig. 5). Or the surface may have different-sized pores compared to the membrane interior. Since most of the separation characteristics result from the surface, the surface can be tailored according to the application. For instance, a porous membrane could have an integral dense skin on the surface. The dense skin is much thinner (0.1–1.5 μm) than a comparable homogenous membrane and therefore has higher permeability. This sort of membrane is usually more effective for gas separations and for reverse osmosis than a dense homogenous membrane made of the same material. The porous substructure of the membrane gives the membrane strength without adding resistance to mass transfer.

The process to create a dense membrane skin on a porous support from a single material is difficult. It is often simpler to deposit a coating on a porous membrane surface that acts as the dense, highly selective membrane. The coating can be a different polymer that is more

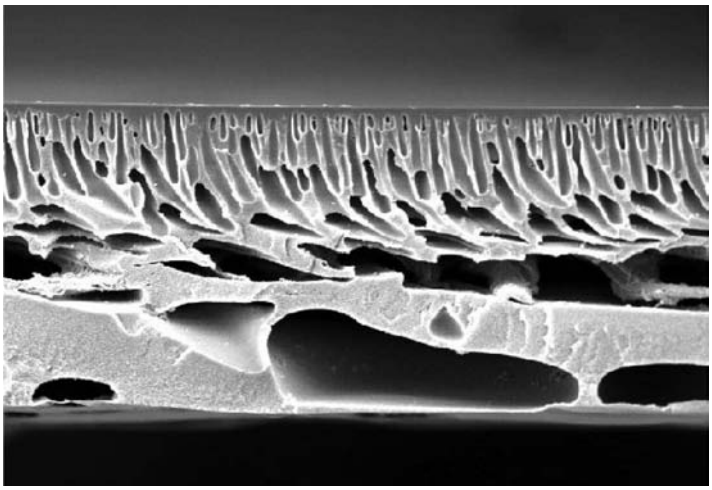

Figure 5 Skin layer membrane. *Source*: Courtesy of Sartorius Stedim GmbH.

selective for the application than the intrinsic properties of the polymer support layer. The polymer can be applied by many techniques, the most important of which are dip coating and interfacial polymerization.

SEPARATION MECHANISMS

Sieve Retention

Sieve retention of particle capture is the one most evident in common filtration experiences. It occurs whenever a particle is too large to pass through a filter pore. It is a geometric or spatial restraint. This type of particle arrest is considered "absolute" (but only for the defined size of the particle) in that it is independent of the filtration conditions. The applied differential pressure does not influence it, unless the level is so high as to deform either the particle or the filter pore, an occurrence not alleged in pharmaceutical filtrations. Sieve retention is also free of the influences of the particle challenge level. Regardless of the number of particles confronting the filter, if each is too large to pass the filter pores then none will be able to do so, and all the particles, regardless of number, will be retained. Additionally, the particle retention will be independent of the suspending liquid vehicle as defined by its ionic strength, pH, surface tension, temperature, viscosity, and presence or absence of surfactant, etc. (17,71).

Adsorptive Retention

Zsigmondy (32) pointed out that the filter surface has a certain adsorbing capacity whose affinity must first be satisfied before unhindered passage of the dispersed phase through the filter may occur. Numerous investigators have since noted specific adsorptions of many entities. Elford (29) reported that dyes could adsorptively be removed from true solutions by collodion membranes (cellulose nitrate, one of the most adsorptive materials). The strong adsorption tendencies of the cellulose nitrate polymer had also been noted by Elford (13) in the case of viruses. The use of membrane filters adsorptively to collect and isolate nucleic acids, enzymes, single-strand DNA, ribosomes, and proteinaceous materials in scintillation counting operations is well established. Moreover, such adsorptive retentivity is utilized nowadays in chromatography and membrane adsorber steps of the downstream purification process. Bovine serum albumin, antigen/antibody, and antibody complex, and specific binding and receptor protein adsorption to cellulose nitrate has been shown to occur. Berg et al. (33) investigated the adsorption of both inorganic and organic compounds on polymers such as cellulosic filter papers, nylon, polyethylene, and cellulose diacetate dialysis membranes. That water-soluble organics could adsorptively be removed from aqueous solutions by filters was observed by Chiou and Smith (34). These investigators were thus led into a rather thorough study of such adsorptions by filters. Undani (35) and Brose et al. (36) studied the adsorptive sequestration of such preservatives as benzalkonium chloride, chlorocresol, and chlorhexidine acetate from their solutions by membrane filters. The adsorptive removal of flu vaccine impurities and antibodies onto membrane filters has been reported (37,38). Inorganic particulate matter can be removed filtratively through the adsorption mechanism. It is thus well documented that molecules and materials can be adsorbed onto filters to become filtratively removed thereby.

There are several references in the literature pertaining to the retention of organisms by contact with filter surfaces. Pertsovskaya and Zvyagintsev (39) report that films of such polymeric as polyamide, polyacrylate, polyethylene, and cellulose triacetate adsorb different groups of different bacteria. Zierdt (40) and Tanny et al. (41) demonstrated that bacterial adsorption could take place on the surfaces of membrane filters whose pores are many times larger than the organisms. During the laboratory development of a lyses-fractionation blood culture technique, Zierdt et al. (42) at the National Institutes of Health noted that both gram-negative and gram-positive organisms were attracted to the membrane materials during filtrations. The filters were composed of polycarbonate and cellulose mixesters. Furthermore, the arrested organisms resisted removal by the mechanical or adsorptive action of backwashing with buffer. These investigators were therefore enabled to use filter membranes with porosities much larger than would normally be expected to arrest the bacteria whose retention they wished. The organisms involved were *Escherichia coli* and *Staphylococcus aureus*.

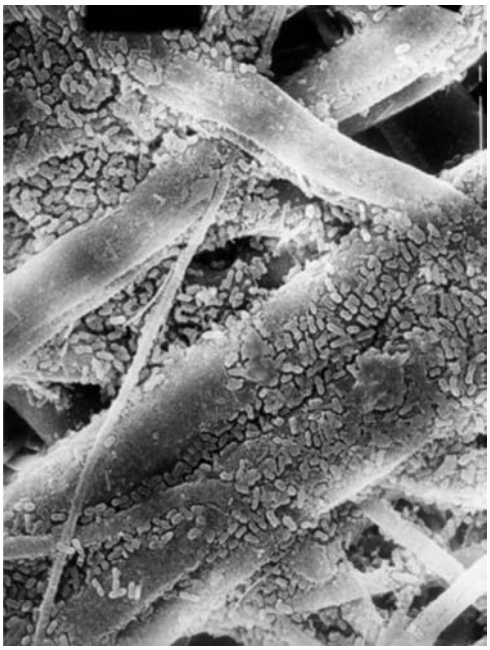

Figure 6 Microorganisms captured on glass fiber. *Source*: Courtesy of Sartorius Stedim GmbH.

Sterility was neither sought nor obtained. Beyond doubt, however, the bacterial capture by the membrane filters involved adsorptive arrest.

Zierdt et al. (42) found that a higher percentage of bacterial retention occurs at low organism concentrations, about 500 to 100 CFU/mL. At higher levels of 10^8 to 10^9 CFU/mL, increasing percentages of *E. coli* pass through the membranes, although a larger total number is retained. These phenomena accord with adsorption. Retention was investigated as a function of the filter pore-size ratings. As expected, the larger the pore-size ratings of the filters, the greater the amount of bacterial passage. At low bacterial numbers, 6.2×10^2 CFU for *E. coli* and 7.3×10^2 CFU for *S. aureus*, apparently no *E. coli* pass a 3.0-μm filter nor *S. aureus* a 5.0-μm filter. All of the above reflect the influence that organism concentration exerts on filter capture efficiency during adsorptive sequestrations.

It also has been shown that *B. diminuta* (formerly *P.*) can be retained by adsorptive glass fiber filters (Fig. 6). It is evident that many of the organisms are retained by contact capture rather than by sieve arrest; the filter pores, the spaces among the fibers, obviously are often too large to serve as retaining orifices.

Surface phenomena, such as adsorptions, can be related to forces between molecules, especially to an asymmetry or unbalance of forces at an interface. The hydrogen bond is an example of an asymmetric force caused by the presence of unequally shared electrons within the water molecule. This creates partial charges, electrical in nature, on atoms of the water molecules. The oxygen atom, retaining more than its share of electrons, becomes negatively charged. The hydrogen atoms of the water molecule, possessing a smaller portion of electrons, become positively charged. Opposite charges attract one another; similar charges repel one another. The opposite electrical signs on separate water molecules result in adsorptive interactions called the hydrogen bond. The electrical forces between ions are full charges. The electrons composing them are completely donated by one atom of a molecule and are fully received by another atom. They are not partial charges. The attractive forces resulting from partial charges are short range and electrostatic, and are usually characterized as van der Waals forces, such as govern the condensation of a vapor into a liquid.

Energy is required to effect the separation of a bacterium from a surface to which it is adsorbed. The energy level is an expression of the bonding strength, the adsorption, between the organism and the polymer surface. This, in turn, depends on the contributions made to the bond by the membrane surface and by the organism. It is not surprising, therefore, that

different filter surfaces bond differently with a given organism, and that different organisms adsorb differently to a given filter surface. Additionally, product parameters, that is, the filtrate properties do influence the adsorptive capture or attractiveness of and to a surface tremendously.

Ridgway (43) found that mycobacterial adhesions to polyamide type reverse osmosis membranes showed a 5- to 10-fold greater affinity than did their adsorptions to cellulose ester RO membranes. It may be speculated on the basis of this finding that strong bacterial adsorptions to polyamide (nylon) membranes account for the sterilizing effects of such 0.2 μm-rated membranes, even when they are more open than their counterparts not composed of this polymer. Ridgway also found that different organisms had different propensities to adsorb to surfaces, as gauged by biofilm formation. It is possible, however, that this adsorptive phenomenon reflected particular morphological features of the different organisms rather than their molecular makeup.

An interesting example of adsorptive interconnections formed between molecules of entirely different compositions is given by the actions of surfactant molecules. These compounds reduce the high interfacial surface tensions that separate nonpolar hydrocarbons, such as oils, from polar liquids such as water. Emulsifications in particular characterize the forces at play; for example in liquid-liquid contacts. As stated, the interfacial tensions are highest between those of different polarity and structure. High interfacial tensions are a negative for interactions among different molecular structures. Reduced interfacial tensions favor such interconnections. Wetting agents or surface acting agents perform their functions by reducing the interfacial tensions. Consider the immiscibility of oil and water, one a hydrophobic, nonpolar compound and the other a molecular structure so polar as to be importantly significant in hydrogen bonding interactions. Tobolsky (44) points out that the molecular structure of sodium oleate, a surface tension reducer for water, has a "strongly polar head and a long nonpolar tail." The nonpolar molecular group of the surfactant attaches to a nonpolar oil molecule. Its polar group attaches to the polar water molecules. The sodium oleate molecule in so doing bridges the polarity difference between the oil and water. The result is an aqueous emulsification of the oil; the one wetting the surface of the other. The bonding versatility of the surfactant molecules bridges the differences in polarity between polar and nonpolar compounds. An oil in water emulsion results.

In specific applications the adsorptive sequestration mechanism is sought. Its application to pharmaceutical filtrations will certainly require in-depth validation. If adsorptive sequestration is a major function of the retentivity of a filter, such retentive effectiveness needs to be analyzed utilizing process conditions and the actual product to be filtered. Under no circumstances can filtrative efficiency be assumed, if not documented by bacteria challenge test results. This also is valid for claims of endotoxins removal by filtration. Such removal requires qualification over the filtration period at very defined process conditions. Any changes in the process conditions can alter the filtration result. For this reason, any sterilizing grade filter needs to be validated using the product as the challenge test carrier and the actual process conditions.

FILTRATION CONSIDERATIONS

Pressure/Flow

The differential pressure is the prime motivator of a liquid's flow and determinant of its rates. Most of the aqueous preparations dealt within the pharmaceutical industry are Newtonian fluids. By definition, a direct and linear relationship exists between their clean (absent particles) flow rates and the differential pressure (Δp) for a given EFA. To overcome the resistance to flow caused by placing a filter in the path of a clean fluid stream, a higher differential pressure or a more extensive EFA is required. The difference in the pressures, upstream and down, determines the rate of flow. Alternatively, an increase in the available filter area will compensate for the decrease in the flow rate. Maintaining a constant flow while changing one of these parameters by some percentage or multiple necessitates a balancing of its influence by compelling an opposite change in the second parameter by the same percentage or multiple.

The flow rate of clean liquids, those not encumbered with particles, is also affected by viscosity and by temperature, its reciprocal, as also by its degree of adsorptive interactions, if

any, with the filter. The latter may result in a plasticization of the polymeric matrix. This may manifest itself by a swelling of the matrix into its open spaces, the pores, which, in response, may be accompanied by their shrinkage. This would impede the liquid flow (45).

The initial rate of flow of "real fluids," defined as those containing particles, will progressively undergo reductions proportional to the pore blocking occasioned by the filter's ongoing particle retentions. Moreover, decreases in flow rates may result due to the differential pressure's compaction of filter cakes that may form on the filter's surface. This contaminated rate of flow reflects the rate of flow decay. The total throughput, an important goal of the filtration as measured by weight or volume, will depend on the sizes and shapes and numbers of the total suspended solids' components (TSS), and on the particle size/pore size relationships governing particle retentions and pore blockage. Throughput is flow rate dependent over time. Its utilitarian value is self-evident in terms of yield.

The filter system's design, whether of the membrane combinations or of other structural features, may vary the resistance to flow. For example, a single membrane layer will have a higher flow rate than the same filter area for each of a double-layer combination.

On the basis of the extrapolation of graphs from filter makers catalogues, it may appear that a high-flow-rate system (e.g., 400 L/min) can be designed with one or two 10 in. (25.4 cm) cartridges. This design, however, would not take into account the resistance to flow of the filter housings. Filter manufacturers publish graphs of flow rates that are fairly linear within a range. Within this range, most of the differential pressure is used to drive the fluid through the filter and only a small portion is involved in overcoming the flow resistance of the housing. Filter manufacturers generally supply data concerning the rates of flow through their various filters (and housings) as a function of incremental pressure differential, for example, 4 L/min/psi for a 0.2 μm-rated membrane. One must choose as a flow limit that differential pressure that will not cause the flow capabilities of the filter housing to be exceeded.

Viscosity/Temperature Effects

Flow rate is the easiest to measure from among the filter properties of interest: flow rate, throughput, and extent of particle removal. Flow is, for most fluids, a product directly defined by the differential pressure and is inversely moderated by viscosity. Viscosity, in turn, is reciprocal to temperature. Rates of flow can be varied by manipulating the differential pressure and the temperature/viscosity relationship.

Flow rate information is normally given for water. Since the rate of flow varies inversely with viscosity, the flow rates for more viscous liquid media will be reduced proportionately and must be corrected for. Water, the standard, has an assigned numerical viscosity value of 1 centipoise (cP). A liquid having a viscosity of 3 cP will flow one-third as fast; a liquid whose viscosity is 36 cP will flow 1/36th as rapidly, etc. The viscosity effect on rates of flow is not exact, as it ignores liquid/filter interactions that in their extreme manifest themselves in filter swelling and other expressions of incompatibility. Fortunately, substituting other liquids for water generally minimizes these aberrations.

Viscosity may also affect particle retentions. Higher viscosities exert greater "drag" forces on a suspended particle. The "drag" is the partial charge attractive force manifest between particle and the liquid molecules that are themselves interconnected by hydrogen bonding. It is hypothesized that the effect on a particle could be tantamount to a higher delta pressure in that its "drag" may exert a force sufficient to frustrate its adsorption onto a filter's surface or even be strong enough to cause the particle's desorption.

Generally, liquids tend to be less viscous at elevated temperatures and filter more rapidly. The heating of liquids to effect more rapid filtration is usually not used however. In particular, the thermal denaturation of protein poses a threat, and certain ranges of temperature over time are encouraging to organism growths. In studying liquid behavior, note should be made of the liquid's temperature.

Contamination Load

There are threats posed by contaminants to drug preparations. None is more significant than the presence of organisms. They are the contaminants whose presence, if tolerable, is least desirable. To make certain that the filters employed for their removal are sufficiently efficient for the task,

high standards are set by the governmental authorities for confronting the filters with large quantities of live organisms. From tests performed on the filter effluents, microbiological assaying can determine the extent of completeness with which they were filtratively removed. In instances where sterility is the goal, the complete retention of the organisms must be ascertained. In processing operations, it is necessary to use methods, techniques, all equipment, and appurtenances, etc., that have been validated to attain that accomplishment.

The FDA sets the standard microbial challenge at 1×10^7 CFU/cm^2 of EFA. The thinking is that if a filter can perform against so large an amount of organisms, it can surely manage the removal of a lesser number. As logical as this standard may seem, it does not meet with universal agreement. The EMEA requires that the final filter in the filter train should not be confronted with more than 10 CFU/100 mL organism level (46). Larger numbers than that will require a "sterilizing grade" filter downstream of it. Apparently, the thinking is that the fewer the number of organisms that confront a filter, the less likely is one to penetrate it.

Complicating the situation is the myriad number of different organisms that are extant. Dealing with this complication is made easier by designating a particular microbe to serve as a model for those most likely to be encountered in pharmaceutical settings. For this purpose, *B. diminuta* ATCC 19146 is used. It usually serves adequately, but with exceptions. *B. diminuta* is of a size that suits it to be sieve retained by 0.2 μm-rated membrane filters. There are, however, organisms that undergo changes in their size on exposure to certain liquids. Other organisms are known to be alive, but whose existence cannot be verified because they are not amenable to cultivation.

Compatibility

The filter must be compatible with the liquid it is to filter. It should not undergo chemical attack, nor should its pore structure become modified lest its retention capabilities become altered. Chemical degradation is usually obvious in the filter's physical property changes it causes. Color changes in the filter or its embrittlement may signal oxidative free radical attacks (Fig. 7). Hydrolytic actions by strong acids or bases may partially destroy the filter or permit it to swell in water. Solvents will either gradually dissolve the filter or cause it to soften noticeably. In rarer instances, it will show distortive shrinkage or develop scalloped edges or wrinkles. Gross incompatibilities are not difficult to detect. More subtle effects are of greater concern because they may be overlooked.

Since the chief filter action is the removal of particles from their suspensions, changes in their pore structures are to be guarded against. Their occurrence can be detected by comparing filter's bubble point values before and after its exposure to the liquid for at least the time

Figure 7 Oxidative coloration and disintegration of an air filter. *Source*: Courtesy of Sartorius Stedim GmbH.

period from their initial contact through to the filtration's completion. Determining their diffusive airflow rates before and after contact will be an even more sensitive gauge. The bubble point test will disclose enlargements of the largest pores, if any. The diffusive airflow will reveal alterations in pores of any size. While the former test will be more pertinent to the implications of particle passage, the latter, being more inclusive of all size pores, may by its sensitivity indicate a potential for unwanted pore-size mutations.

Incompatibilities that may alter pore shapes or sizes or otherwise compromise microbial retentions are of prime importance. A lack of compatibility can also serve to weaken the mechanical strengths of a filter by a plasticizing action making it less able to withstand its former differential pressure. This most likely will also reduce the filter's density. A larger or faster leaching of compounds from within the polymeric filter may result. The quantity of extractables and the speed of their transfer from within the filter body will also be expedited.

INTEGRITY TESTING

Sterilizing grade membrane filters are required to be tested to assure the filters are integral and fulfill the purpose. Such filter tests are called integrity test and may be performed before, but must be performed after the filtration process. Sterilizing grade filtration would not be admitted to a process, if the filter would not be integrity tested in the course of the process. This fact is also established in several guidelines, recommending the use of integrity testing, pre- and post filtration. This is not only valid for liquid, but also air filters.

Integrity tests, such as the diffusive flow, pressure hold, bubble point, or water intrusion test, are nondestructive tests, which are correlated to the destructive bacteria challenge test with 10^7 CFU/cm^2 *B. diminuta* (47–49). Derived from these challenge tests specific integrity test limits are established, which are described and documented within the filter manufacturers literature. The limits are water based, that is, the integrity test correlations are performed using water as a wetting medium. If a different wetting fluid, or filter, or membrane configuration is used, the integrity test limits may vary. Integrity test measurements depend on the surface area of the filter, the polymer of the membrane, the wetting fluid, the pore size of the membrane, and the gas used to perform the test. Wetting fluids may have different surface tensions, which can depress or elevate the bubble point pressure. The use of different test gases may elevate the diffusive gas flow. Therefore, appropriate filter validation has to be established to determine the appropriate integrity test limits for the individual process.

Bubble Point

Microporous membranes pores, when wetted out properly, fill the pores with wetting fluids by imbibing that fluid in accordance with the laws of capillary rise. The retained fluid can be forced from the filter pores by air pressure applied from the upstream side to the degree that the capillary action of that particular pore is overcome (Fig. 8). During the bubble point test, the pressure is increased gradually in small increments. At a certain pressure level, liquid will be forced first from the set of largest pores, in keeping with the inverse relationship of the applied air pressure P and the diameter of the pore, d, described in the bubble point equation:

$$P = \frac{4\gamma \cos \theta}{d}$$

where γ is the surface tension of the fluid; θ, the wetting angle; P, the upstream pressure at which the largest pore will be freed of liquid; d, the diameter of the largest pore.

When the wetting fluid is expelled from the largest pore, a bulk gas flow will be evaluated on the downstream side of the filter system during a manual test. The bubble point measurement determines (to a certain degree) the pore size of the filter membrane, that is, the larger the pore the lower the bubble point pressure. Therefore, filter manufacturers specify the bubble point limits as the minimum allowable bubble point and correlate the bubble point test procedure to the bacteria challenge test. During an integrity test, the bubble point test has to exceed the set minimum bubble point for it to pass.

Key for a successful bubble point test is the qualified wetting fluid and its surface tension. The bubble point will be highly influenced by surface tension changes within the

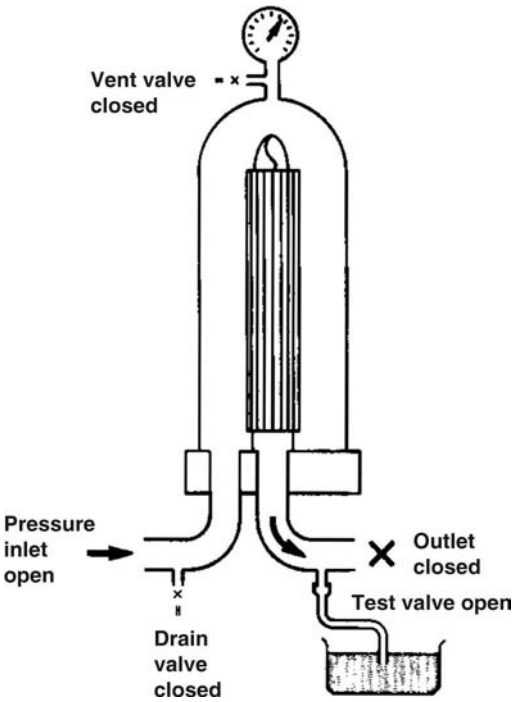

Figure 8 Manual bubble point test setup. *Source*: Courtesy of Sartorius Stedim GmbH.

Table 2 Bubble Point Values for Different Wetting Agents Using Cellulose Acetate 0.2 μm

Product	Bubble point value (bar)
Water	3.20
Mineral oil	1.24
White petrolatum	1.45
Vitamin B complex in oil	2.48
Procainamide HCl	2.76
Oxytetracyline in PEG base	1.72
Vitamin in aqueous vehicle	2.07
Vitamin in aqueous vehicle	2.69

Source: Courtesy of Sartorius Stedim GmbH.

wetting fluid. Table 2 shows different possible wetting fluids and the bubble point changes of such, utilizing the same membrane.

Yet, the surface tension of the wetting liquid, as also its viscosity, diminishes with rising temperature, while the angle of wetting increases, and its cosine decreases with the hydrophobicity of the filter polymer. The less hydrophilic the polymer, the less perfectly does it wet, particularly with aqueous liquids. Therefore, the bubble point is a specific product of the each particular filter/liquid couple. It varies from one polymer to the other and therefore bubble point values given and obtained are not equal, even for the same pore-size rating. That the bubble point of a filter differs for different wetting liquids is commonly known. That it differs also for polymeric materials is less appreciated.

The bubble point test can only be used up to a certain filter size. The larger the filter surface, the larger the influence of the diffusive flow through the membrane. The diffusive flow would cover the actual bubble point due to the extensive air flow. Therefore, the bubble point finds its ideal use with very small system to medium size systems (some mention the critical borderline to use the bubble point is a 3 × 20 in. filter housing, depending on the pore size).

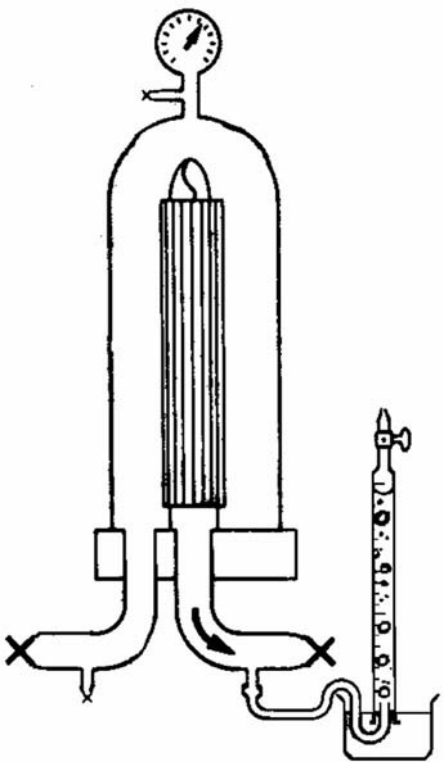

Figure 9 Manual diffusive flow test setup.

Diffusive Flow

A completely wetted filter membrane provides a liquid layer across which, when a differential pressure is applied, the diffusive airflow occurs in accordance with Fick's law of diffusion (Fig. 9). This pressure is called test pressure and commonly specified at 80% of the bubble point pressure. In an experimental elucidation of the factors involved in the process, Reti simplified the integrated form of Fick's law to read

$$N = \frac{DH(p_1 - p_2)\rho}{L}$$

where N is the permeation rate (moles of gas per unit time); D, the diffusivity of the gas in the liquid; H, the solubility coefficient of the gas; L, the thickness of liquid in the membrane (equal to the membrane thickness if the membrane pores are completely filled with liquid); P $(p_1 - p_2)$ is the differential pressure; and ρ, the void volume of the membrane, its membrane porosity, commonly around 80%.

The size of pores does only enter indirectly into the equation; in their combination they comprise L, the thickness of the liquid layer, the membrane being some 80% porous. The critical measurement is the thickness of the liquid layer. Therefore, a flaw or an oversized pore would be measured by the thinning of the liquid layer due to the elevated test pressure on the upstream side. The pore or defect may not be large enough that the bubble point comes into effect, but the test pressure thins the liquid layer enough to result into an elevated gas flow. Therefore, filter manufacturer specify the diffusive flow integrity test limits as maximum allowable diffusion value. The larger the flaw or a combination of flaw, the higher the diffusive flow.

The diffusive flow cannot be used for small filter surface, due to the low diffusive flow with such surfaces. The test time would be far too extensive, and the measured test value too unreliable to be utilized. Nevertheless, the diffusive flow as well as the pressure drop test are best used for larger filtration surfaces, where the bubble point test finds its limitations (50).

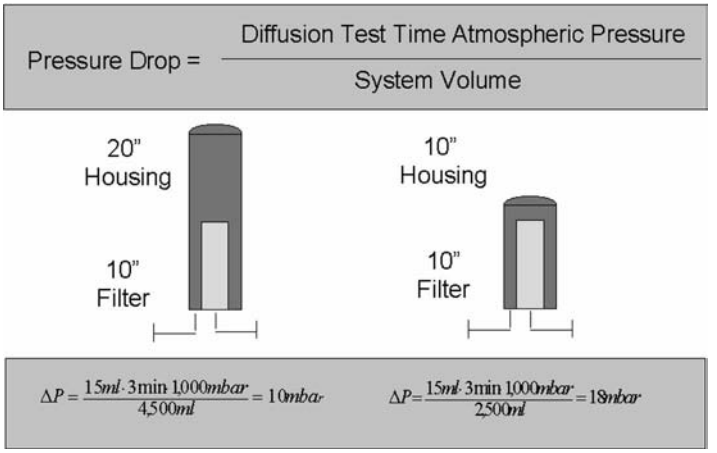

Figure 10 Influence of the upstream volume to the pressure decay measurement.

Pressure Hold

The pressure hold test is a variant of the diffusive airflow test. The test setup is arranged as in the diffusion test except that when the stipulated applied pressure is reached, the pressure source is valved off. The decay of pressure within the holder is then observed as a function of time by using a precision pressure gauge or pressure transducer.

The decrease in pressure can come from two sources: (*i*) the diffusive loss across the wetted filter. Since the upstream side pressure in the holder is constant, it decreases progressively all the while diffusion takes place through the wetted membrane and (*ii*) source of pressure decay could be a leak of the filter system setup.

An important influence on the measurement of the pressure hold test is the upstream air volume within the filter system. This volume has to be determined first to specify the maximum allowable pressure drop value. The larger the upstream volume, the lower the pressure drop (Fig. 10). The smaller the upstream volume, the larger the pressure drop. This means an increase in sensitivity of the test, but also an increase of temperature influences, if changes occur. Filter manufacturers specify maximum allowable pressure drop values, utilizing their maximum allowable and correlated diffusive flow value and convert this diffusive flow maximum with the upstream volume into a maximum allowable pressure drop.

Another major influence on pressure decay is temperature. Any temperature change during the test will distort the true result, as an increase in the temperature will lower the pressure drop and a decrease will artificially elevate the pressure drop. Therefore, the temperature conditions during the test should only vary slightly. This also means that the wetting agents used should have a similar temperature as the environmental temperature surrounding the test setup. Temperature differences between the wetting solution and the test gas and the temperature of the environment will influence the true test result. The pressure hold test is an upstream test, even when performed manually.

Multipoint Diffusion Test

In single-point diffusive flow testing, the test is performed at a defined test pressure, which is commonly around 80% of the bubble point value. Therefore, the area between the diffusive flow test pressure and the bubble point value is not tested and stays undefined. In comparison, the multipoint diffusive airflow test is performed at a multitude of test pressures. Usually, this test is performed with an automated test machine, which allows defining the individual test pressure points with high-test accuracy. Moreover, once the pressure points are defined the machine performs the test without the need of supervision. Therefore, valuable time and resources are not bound. To the benefit of data storage, the test machines also print an exact graph of the test performed, therefore any irregularities will be detected.

Multipoint diffusion testing has advantages over single-point diffusive testing, because it can more rapidly detect a pending product failure due to gradual filter degradation (51,52). A

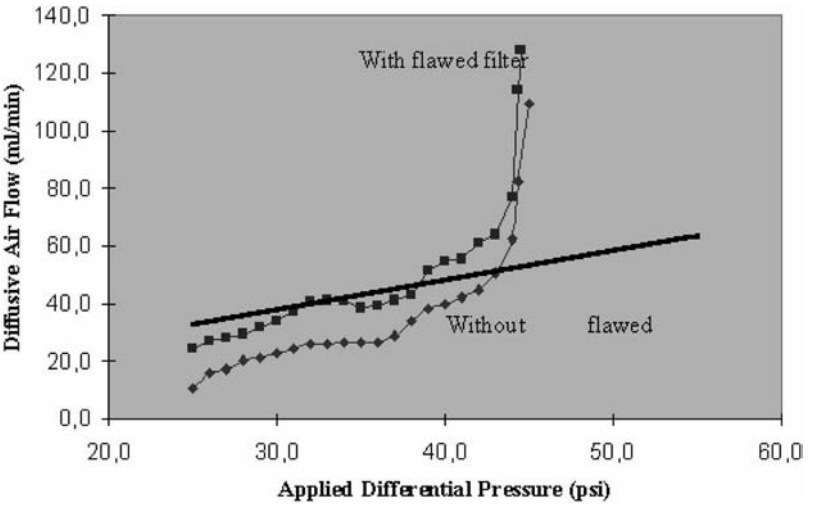

Figure 11 Multipoint diffusive flow testing to detect a flawed filter in a multiround filter housing.

multipoint integrity test could indicate a trend of increasing diffusion over time that might be overlooked with single-point diffusion testing and even through bubble point testing. Furthermore, the multipoint diffusion test seem to have the ability to test multiround housings reliably (Fig. 11). As described in the bubble point and diffusive flow test section, both tests have their limitations accurately integrity testing multiround filter housings. A single-point diffusive flow test may not be able to find a flawed filter within the multitude of filters. The bubble point may be covered by an excessive diffusive flow.

In any case, the multipoint diffusive flow test seems to be able to find a flawed filter due to the change of the slope of the linear section of the diffusive flow. A single flawed filter cartridge can be detected within a three round filter housing, where a single-point test would not have determined the defect. Such test may take longer in its test time, but will add to the overall accuracy of integrity testing multiround housings.

In instances, the multipoint diffusion test finds also its usefulness in the analysis of failed filter integrity tests. For instance, when a filter failed the single-point diffusive flow test or bubble point test, one should aim for testing the filter with a multipoint diffusion test to see the entire graphic. This result could be compared to the graphs established during the performance qualification phase. Commonly, there are distinct test graphics, which show whether the filter has a flaw and if so what the cause of the flaw could be.

Water Intrusion Test

The water intrusion (also known as water pressure hold) test is used for hydrophobic vent and air membrane filters only (53–55,73). The upstream side of the hydrophobic filter cartridge housing is flooded with water. The water will not flow through the hydrophobic membrane. A specified gas pressure is then applied to the upstream side of the filter housing above the water level. This is done by way of an automatic integrity tester. A period of pressure stabilization takes place over a specified timeframe, recommended by the filter manufacturer, during which the cartridge pleats adjust their positions under imposed pressures. After the pressure drop stabilizes, the test time starts and any further pressure drop in the upstream pressurized gas volume, measured by the automatic tester, signifies a beginning of water intrusion. The automated integrity tester is sensitive enough to detect the pressure drop. This measured pressure drop is converted into a measured intrusion value, which is compared to a set intrusion limit, which has been correlated to the bacteria challenge test. As with the diffusive flow test, filter manufacturers specify a maximum allowable water intrusion value. Above this value a hydrophobic membrane filter is classified as nonintegral.

FILTER VALIDATION

The probably most thorough guidance (recommending) document is the PDA Technical Report No. 26. It describes filter structures, usage, purpose, and integrity testing. Most important is the description of the filter validation needs within the actual filtration process (2,3,11,56,72). The document defines the needs for viability, product bacteria challenge, extractable, particulate, and adsorption testing. Before the PDA Technical Report has been accomplished, FDA's Guideline on Sterile Drug Products Produced by Aseptic Processing has been the guidance document of choice. The 1987 guidance has been replaced by a new guidance document of September 2004, which adopted multiple descriptions of Technical Report #26. Similarly, the ISO 13408 leans very much toward Technical Report 26 and describes appropriate filter validation very much in the fashion of the mentioned report.

The United States Pharmacopeia (USP) as well as any other pharmacopeia should be closely monitored, due to the descriptions of required limits for particulate, endotoxins, and biocompatibility testing. Within the filter manufacturers filter qualification tests, pharmacopeial limits are analyzed and need to be met by the filter products distributed. These tests commonly cover toxicological, endotoxins, extractable and particulate tests, which are well defined with the , and any filter utilized within the biopharmaceutical industry requires being compliant. These tests are the basic requirements to be fulfilled and should not be misinterpreted as appropriate filter validation studies. Filter validation requires to be performed with the actual drug product to be filtered under process conditions. Most of the pharmacopeial tests are performed with water or other pure solvents.

A guideline of considerable importance, especially in regard to revalidation or second filter vendor implementation, is the FDA Guidance for the Industry—Changes to an Approved NDA or ANDA, section VII, Manufacturing Process (1999). This guideline describes distinctively the different needs of prior approvals, if changes have been made to the actual processes. It defines what is a minor, moderate, or major change with respect to filtration devices and changes to sterilizing grade filters and what are the consequences.

A guideline that causes confusion and insecurities with respect to redundant 0.2 μm filtration is the EMEA CPMP/QWP/486/95 Guideline (46). This guidance document defines a maximum allowable bioburden level of 10 CFU/100 mL in front of a 0.2-μm sterilizing grade filter. If this level is exceeded, a bioburden reducing filter has to be used in front of the sterilizing grade filter. Although, the guidance leaves room for interpretation in respect to what type of filter this could be, it also states that the use of a second 0.2 μm in front of the final 0.2 μm filter does not required additional validation. It is now debatable whether the bioburden limit defined is reasonable, as well as the excessive reliance on pore size.

Bacteria Challenge Test

Before performing a product bacteria challenge test, it has to be assured that the liquid product does not have any detrimental, bactericidal, or bacteriostatic effects on the challenge organisms, commonly *B. diminuta*. This is done utilizing viability tests. The organism is inoculated into the product to be filtered at a certain bioburden level. At specified times, defined by the actual filtration process, the log value of this bioburden is tested. If the bioburden is reduced due to the fluid properties different bacteria challenge test modes become applicable. There are three bacteria challenge methodologies described within the PDA Technical Report No. 26; high organisms challenge, placebo (modified product) challenge, and product recirculation with a challenge after recirculation. If the mortality rate is low, the challenge test will be performed with a higher bioburden, bearing in mind that the challenge level has to reach $10^7/cm^2$ at the end of the processing time. If the mortality rate is too high, common definition is more than 1 log during processing time, the toxic substance is either removed or product properties, for example, pH, temperature, etc., are modified. This challenge fluid is called a placebo. The third methodology would be to circulate the fluid product through the filter at the specific process parameters as long as the actual processing time would be. Afterward the filter is flushed extensively with water and the challenge test, as described in ASTM F838-05 (57) performed. Nevertheless such challenge test procedure would be more or less a filter compatibility test.

Sterilizing grade filters are determined by the bacteria challenge tests. This test is performed under strict parameters using a defined solution (57). In any case, FDA nowadays also requires evidence that the sterilizing grade filter will create a sterile filtration, based on the actual process parameters, fluid properties, or bioburden found. This means that bacteria challenge tests have to be performed with the actual drug product, bioburden, if different or known to be smaller than *B. diminuta* and the process parameters. The reason for the requirement of a product bacteria challenge test is threefold. First of all the influence of the product and process parameters to the microorganism has to be tested. There may be cases of either shrinkage of organisms due to a higher osmolarity of the product or prolonged processing times or starvation due to the extreme low organic properties of the fluid. Secondly, the filter's compatibility with the product and the process parameters has to be tested. The filter should not show any sign of degradation due to the product filtered. Additional assurance is required that the filter used will withstand the process parameters, for example, pressure pulses, if they occur, without influencing the filter's performance. Thirdly, there are two separation mechanisms involved in liquid filtration: sieve retention and retention by adsorptive sequestration. In sieve retention, the smallest particle or organism size is retained by the biggest pore within the membrane structure. The contaminant will be retained, irrespective of the process parameters. This is the ideal situation. Retention by adsorptive sequestration depends on the filtration conditions. Contaminants smaller than the actual pore size penetrate such and may be captured by adsorptive attachment to the pore wall. This effect is enhanced using highly adsorptive filter materials, for example, glass fiber as a prefilter or polyamide as a membrane. Nevertheless, certain liquid properties can minimize the adsorptive effect, which could mean penetration of organisms. Whether the fluid has such properties, which will lower the effect of adsorptive sequestration, and may eventually cause penetration has to be evaluated in specific product bacteria challenge tests.

Extractable/Leachable Test

Besides the product bacteria challenge test, tests of extractable or leachables substances have to be performed. Previous reliance on nonvolatile residue (NVR) testing as a method of investigating extractable levels have been dismissed by the regulators in 1994 (58). Since then extractable/leachables analysis from filters and other components are routinely done by appropriate separation and detection methodologies. Extractable measurements and the resulting data are available from filter manufacturers for their individual filters.

These tests are performed with a specific solvent, for example, ethanol and water at "worst case" conditions. Such conditions do not represent true process realities Therefore, depending on the process conditions and the solvents used, explicit extractable tests have to be performed. Formerly, these tests were done only with the solvent used in the drug product formulation, but not with the drug ingredients themselves, because the drug product usually covers any extractable during measurement. Nevertheless, recent findings have been presented, which reported the possibility to evaluate extractable utilizing the actual drug product as the extraction medium. Such tests are conducted by the validation services of the filter manufacturers using sophisticated separation and detection methodologies such as GC-MS, FTIR, RP-HPLC, UV-VIS, GPC-RI, HPCE, and SFC (59,60). These methodologies are required due to the fact that the individual components possibly released from the filter have to be identified and quantified. Elaborate studies on sterilizing grade filters, performed by filter manufacturers, showed that there is neither a release of high quantities of extractable (the range is ppb to max. ppm per 10 in. element) nor have toxic substances been found.

Authorities and organizations nowadays seem to have changed their focus to other equipment used within the industry, for example, disposable media bags, plastic vials, tubing, or stoppers. Prefilters also have become a target. There are already extractable studies performed on a variety of pleated prefilter types of polypropylene and glass fiber. Nevertheless, lenticular and string wound prefilters, widely used within the biopharmaceutical industry still, have to undergo such investigation.

Chemical Compatibility Test

The PDA Technical Report No. 26 describes very specifically "A simple chemical compatibility chart will often not provide enough information for predicting filter system compatibility, thereby requiring additional testing." Chemical compatibility has been underestimated in the past and reliance has been focused on chemical chart of pure solutions. The aim of chemical compatibility testing is to find subtle incompatibilities, which may happen due to a mix of chemical components and entities or specific process conditions. Elevated temperatures or prolonged filtration times may result in a filter incompatibility, which has to be investigated.

Even though the filter membrane is not compromised in respect to its retentivity, it can add extractable/leachables. Therefore, appropriate compatibility tests have to be performed with the actual drug product at the process conditions. Commonly, integrity tests before and after the submersion of the filter in the product will show whether an incompatibility exists. Sole reliance, though, should not be on integrity testing. NVR testing parallel to integrity testing may be the procedure of choice, in case the filter is integral but shows elevated extractable levels. Scanning electron microscopy may be utilized to see any chemical attacks on the membrane surface. Above-mentioned bacteria challenge tests and extractable analysis also contribute valuable information with respect to the filters compatibility.

Other Requirements

Particulates are critical in sterile filtration, specifically injectables. The USP (and BP (British Pharmacopeia) quote specific limits of particulate level contaminations for defined particle sizes. These limits have to be met and therefore the particulate release, if any, from sterilizing grade filters has to meet these requirements. Filters are routinely tested, evaluating the filtrate with laser particle counters. Such tests are also performed with the actual product under process conditions to prove that the product and especially process conditions do not result in an increased level of particulates within the filtrate. Specific flushing protocol, if necessary, can be established for the filters used. These tests are also useful for any prefilter as it reduces the possibility of a particulate contamination within the process.

Additionally with certain products loss of yield or product ingredients due to adsorption shall be determined. Specific filter membranes can adsorb, for example, preservatives, like benzalkonium chloride or chlorhexidine. Such membranes need to be saturated by the preservative to avoid preservative loss within the actual product. This preservative loss, for example, in contact lenses solutions, can be detrimental due to long-term use of such solutions. Similarly problematic would be the adsorption of required proteins within a biological solution. To optimize the yield of such proteins within an application, adsorption trials have to be performed to find the optimal membrane material and filter construction, but also flow conditions and prerinsing procedures. Any yield losses by unspecific adsorption can cost millions due to lost product and its market value. Adsorption studies are helpful to optimize downstream process in regard to any yield loss that in turn can influence production capacity.

To summarize, most of the described validation effort have to be performed and are part of the validation master file of a particular process and drug product. Interestingly enough, validation receives emphasis and attention, but one should also never forget training. Without appropriate personnel training any validation effort done is in vain. Filter users should also test their staff to be able to handle filtration, the sterilization, and integrity test of such installation and sanitization. Training has to be the focus of all operations to deliver a reliable and sustainable process.

Product Wet Integrity Test

More often, postfiltration integrity testing is performed by using the product filtered as the wetting agent, due to the fact that a flush with water may need a copious amount of such. Certainly, the contact between certain membranes and various pharmaceutical preparations can produce depressed bubble points compared with the values for water (Table 2). The depressed bubble point can be restored, more or less, but mostly less, by abundant washing of the filter with water, depending on the filter material and/or product ingredients used. Some subtle wetting effects, adsorption or fouling involving product ingredients, may be at work

here whose surface physics is not comprehended. In addition, the surface tension differences between the product and water are contributory to the anomaly.

Often, efforts are made to flush the filter with water before running the final integrity test so that pre- and postfiltration bubble point tests using water are obtained for comparison. However, even copious water flushing may not restore the water bubble point. For example, it was reported that nylon membranes became fouled by proteins in an albumin filtration process that resulted in filters not being wetted with water leading to false-negative results. Same was found with products containing Tween. Even after large water flush volumes, the surface tension reducing properties were seen. In such cases, pre- and postfiltration comparisons may usefully be performed using product as wetting agent for the filters. The displacements in bubble values being ascribed to unknown wetting effects, but largely to the influences of the surface tension values of the product, are assumed not to reflect on the organism removal capabilities of the membrane.

However, regulatory authorities also advocate to perform bacteria challenge tests with the actual product under process conditions. Such challenge tests, involving also viability testing, confirm the filter's retentivity; moreover they reveal any negative influences of the product toward the challenge organism (17).

Parker (61) determined the acceptable minimum bubble point for a given type of filter using product as wetting medium in accordance with the formula

$$P_{\mathrm{p}} = \frac{P_{\mathrm{o}} \cdot P_{\mathrm{m}}}{P_{\mathrm{w}}}$$

where P_{p} is the minimum acceptable product bubble point; P_{o}, the observed bubble point using product; P_{w}, the average of the water bubble points observed for samples of the filters (commonly 3 filters from 3 different batches); and P_{m}, the filter manufacturer's stated minimum allowable bubble point. Enough filters or filter devices are secured from each lot of the subject filter type to yield an acceptable average value. Testing is performed for each product being filtered using 47 mm disk filters or small-scale pleated filter devices.

Desaulnier and Fey (62) confirmed Parker's findings. Parker and Desaulnier and Fey describe the exact protocols by means of which the product bubble point may be determined. The latter authors also describe an apparatus suitable for the purpose.

Usually, the evaluation of the so-called product integrity test values requires three filter membranes or devices of three different lots, that is, nine tests in total. At one point, it was recommended that one of these filter lots must be close to the minimum allowable water bubble point value given by the filter manufacturer to ensure retentive capability at the established limit values. This factor is now included within the corrected product-wetted bubble point value evaluation.

Commonly the diffusive flow is measured at around 80% of the bubble point pressure as the test pressure. A drug product, which is used to wet the filter membrane, can shift the bubble point value and therefore one has to determine the test pressure to be used to perform a product-wetted diffusive flow test. This determination of the test pressure is commonly done by a series of product and water-wetted bubble point tests. The values of these tests will then be used to calculate the product-wetted test pressure for the diffusive flow test. The PDA Technical Report 26, 1998, describes the formula in detail as other have done it before (61–63).

$$\mathrm{TP_{PW}} = \mathrm{MTP_{WW}} \frac{\mathrm{PBPavg}}{\mathrm{WBPavg}}$$

where $\mathrm{TP_{PW}}$ is the product-wetted test pressure; $\mathrm{MTP_{WW}}$, the water-wetted test pressure specified by the filter manufacturer; PBPavg, the average product-wetted bubble point; and WBPavg, the average water-wetted bubble point both evaluated during the test series described earlier.

Once the product-wetted test pressure is evaluated, then the product-wetted diffusive flow limit will be determined. For this determination one will first water wet the membrane filters and perform repeated test of minimum of three different filter lots. After this is done, the filters should be dried or rinsed sufficiently with the product. Again the filters will

undergo several, previously defined diffusive flow tests. The values of all tests can then be used to calculate the maximum allowable diffusion limit for a product-wetted filter using the formula, described by the PDA Technical Report 26, 1998:

$$\mathrm{DFL_{PW}} = \mathrm{DFL_{WW}} \frac{\mathrm{DF_{PW}}}{\mathrm{DF_{WW}}}$$

$\mathrm{DFL_{PW}}$ is the maximum allowable product-wetted diffusive flow limit; $\mathrm{DFL_{WW}}$, the water-wetted diffusive flow limit defined by the filter manufacturers correlation; $\mathrm{DF_{PW}}$, the product-wetted diffusive flow, and $\mathrm{DF_{WW}}$, the water-wetted measured diffusive flow value. This test restricts itself to the single-point diffusion test, and one can argue about its accuracy (50). In any case, the more accurate test would be a multipoint diffusion test, evaluating the slope of the diffusive flow at the test pressure with different wetting media. Such test purely from a statistical point of view is more accurate besides plotting the entire diffusion graph. The plot of the graph will not only show the slope of the linear section of the diffusive flow, but also a shift of the exponential, bubble point, sections. Further details can be found in the multipoint diffusion test paragraph.

APPLICATIONS

Liquids

An ideal liquid filter would have following attributes:

- The filter should have a high flow rate at low differential pressures
- The filter should have a high total throughput performance
- The filter must retain contaminants, especially microbial, as defined and desired
- The filter membrane polymer should be low adsorptive, if used in specific applications, which do not have the need of adsorptive retention
- The filter requires to have a high mechanical robustness to withstand possible differential pressure surges
- The filter requires to withstand up to 134°C steam sterilization temperatures or be able to be gamma irradiated

Such filter represents the "perfect world." Most commonly one has to settle for a compromise between the listed attributes. There is not such filter that is optimal for every application. Liquid filters are commonly developed and designed to work best within specific applications.

Solvent (API) Filtration

Filters within this type of application require being highly compatible to aggressive solutions or process parameters. The fluids are highly aggressive and the best filters to be found for such applications are polyamide or polytetrafluoroethylene membrane polymers. Before the filters can be used within such application, appropriate performance qualification trials should be completed to assure the filter is compatible with the fluid and process parameters. Especially subtle incompatibilities can cause major problems if not determined early enough. The liquid filters used in these applications are polishing or bioburden reducing filters. The bioburden in aggressive solutions may mainly be spores as the fluids are commonly bactericidal. However, any potential contaminant requires to be removed to avoid microbial contamination in the downstream process.

Ophthalmics Filtration

Ophthalmic solutions require two main attributes, besides the obvious microbial retentivity: (*i*) high total throughputs for cellulosic-based complex solutions with high viscosities and (*ii*) low unspecific adsorption for solutions containing preservatives like benzalkonium chloride or chlorhexidine. The total throughput determination can happen via 47 mm disk trials followed by verification trials with small-scale pleated devices. The solutions are complex and may require prefilter/final filter combinations. During the filterability trial work

it is of importance to sample the filtrate in specific volume or time intervals and check the filtrate in regard to the preservative concentration, if applicable. Preservative adsorption to the membrane filter polymer is not uncommon and requires to be established to avoid an out-of-specification event in terms of the preservative level within the final container. Low adsorptive polymeric membranes should be used, for example, polyvinylidene fluoride, cellulose acetate, or modified polyethersulfone. It might be that the solution requires to be recirculated over the membrane to saturate the adsorptive sites, before the solution is redirected to the fill line. Often ophthalmic solutions are filled utilizing blow-fill-seal equipment, which could mean a prolonged filling period. If this is the case, the filter validation (retention study) requires including such prolonged filling period.

Cell Culture Media

Media are available in a large variety from different raw material sources and of different compositions. Moreover, the raw material quality experiences seasonal, dietary, growth, and regional variations, which makes it in instances difficult to define the exact performance of a raw material. This factor can be challenging when filtration systems have to be determined and sized. Therefore, the main performance criterion for filtration systems for media is total throughput or filter capacity, the total amount of fluid that can be filtered through a specified filtration area. Filters used in media filtration should be optimized to achieve the highest total throughput and will be tested accordingly. To achieve reliable data, it is always of advantage when the test batch is at the lower end of the quality specification to gain a worse case scenario. Temperature, differential pressure, and pretreatment of the filter play an important role in performance enhancement of the filter system (64,65). For example, it has been experienced that lower temperature of the media filtered and even the filter system might enhance the total throughput by 30%. The flow rate will be affected by the higher viscosity, but again the essential performance part is not flow but total throughput. Too high flow rates in the filtration of biological solutions showed the negative side effect of gel formation on the membrane and therefore premature blockage. To start with lower differential pressure has been seen advantageous, as again gel formation and/or cake compaction will be avoided. The lower the differential pressure at start of the filtration, the better the performance. A preflush of the filter system with preferably cold buffer will also enhance the total throughput. Hitting the filter with just the media has been found to foul the filter faster and therefore reduce the filter's capacity. In instances it is necessary to utilize prefilter combinations to avoid fouling or blocking of the sterilizing grade or 0.1 μm final filter element. These combinations need to be determined in filterability trials to gain the most optimal combination to filter the particular media and to size the system appropriately.

Another important, but often overlooked factor of media filtration is the influence of unspecific adsorption of the filter material. To separate lipids in the media raw material adsorptive filter media are desired. However, in cell culture media, especially containing growth promoters, unspecific adsorption has to be avoided. Certain membrane polymers do have a higher unspecific adsorption. Sometimes, the membrane polymer can be of similar type, but the surface treatment of the polymer is different or the design of the filter device is different. In any case, high unspecific adsorption can have an influence on growth promoters like IGF.

Buffer Filtration

Since buffers are commonly of high purity the filter performance criteria focuses on flow rate and not total throughput. A premature blocking of the filter is often not experienced. Flow though is the determining factor of process time within the buffer preparation process. The faster the flow rate of the filter the higher the equipment utilization. The better the flow rate of the filter the lower the required EFA, respectively the cost per liter will be reduced. For example, a low flow rate (2500 L/hr), 0.2 μm-rated filter would require 48 minutes to filter a 2000-L volume versus only 20 minutes for a high-flow filter (6000 L/hr). This would reduce equipment's used time by half or the EFA could be reduced, which would cut filter costs.

Another important factor to consider is the buffer's pH range or the variety of buffers used. One can find certain pharmaceutical processes where the pH ranges from 1 to 14, which in some polymers are capable to withstand and others not. Again filter vendors are aware about this fact and developed high-flow filters most often with a polyethersulfone base polymer as this material is compatible over the entire pH range.

Gases

An ideal gas filter requires listed attributes:

- The filter must retain microorganisms and other contaminants, even under unfavorable conditions such as high humidity
- The filter must have high thermal and mechanical resistance
- The filter ought to withstand multiple steam sterilization cycles
- The filter should allow high gas flow rates at low differential pressures
- The membrane should be hydrophobic to resist blockage by elevated humidity, condensate, or water remaining from a water intrusion test
- The filter must not release fibers
- The filter must be integrity testable with a test correlated to removal efficiency with various contaminants.

An optimized air filter can be described as a perfected recipe, as all components utilized, the design of the filter fulfilling the listed attributes. If only one of the attributes is focused on, it might be that the filter is imbalanced and does not meet other criteria of importance.

Fermentor Inlet Air

Air volume requirements vary during the different stages of fermentation and therefore the filter system used in large volume fermentation are of different sizes. For example, filter systems size used for seed fermentors are usually single 10 or 20 in. filter cartridges, whereby filter systems for large-scale fermentation may utilize a multifilter housing of 96 round 30 in. cartridge elements, depending on the product and fermentor volume. Such filter systems are used on a long-term basis and could be used for over a year; that is, these kind of filters require a high mechanical and thermal stability. These filters withstand sterilizing cycles of up to 200 cycles at temperatures up to 134°C. The filter manufacturers optimize membrane filter cartridges to create high flow rates at very low differential pressures. Membrane materials are chosen to achieve high pore volumes, hydrophobicities, and sterile filtration capability. Construction of the filter cartridges is optimized to avoid water logging and high velocities and the resulting pressure losses.

Fermentation can last up to 1 to 20 days therefore high security is required. It would be disastrous in terms of the product intake costs and running costs, if such large-scale fermentor became infected after several days of fermentation.

Fermentor Off-Gas

Off-gas filtration becomes a major concern and requirement, especially in the biotech industry. In the past, most of the fermentation sites did not use any exhaust filter system, because the head pressure in the fermentor eliminated the risk of contamination from the off-gas side. Because of new restrictions and an environmental awareness, more and more facilities employ exhaust filter systems. The aim here is not to protect the fermentor content, but rather the environment to microbial contamination. For this reason different separation methods were evaluated, for example, cyclones in combination with depth filter types or heat. Both methods do not create the assurance level needed, beside one is very costly, therefore the use of membrane filter system becomes common practice.

The filtration of exhaust gases creates some major problems due to the moisture content that the gas carries. The gas is usually warm and saturated with moisture due to the contact with the fermentation medium. When the exhaust gas cools down, large amount of condensate will be the result, which could water block the sterilizing grade filter and increase the pressure

drop over the filter. An increase in pressure drop means an immediate rise of the head pressure of the fermentor, which needs to be avoided. Particles and microbial contamination carried over from the fermentor into the exhaust stream could block the filter device. The retentive ability of such filter needs to be high, otherwise organisms will penetrate through the filter element. In some instances the microbial load of such filter can be up to 10^{11} organisms in a seven-day fermentation (66). Often enough, when the fermentor runs at the highest rate, foaming of the fermentor broth happens and can blind the filter.

Heating by steam and electrical tracing of the filter housings or pipework will avoid condensation due to the fact that the system temperature is held above the dew point of the air. If condensation occurs, the filter needs to be able to achieve required flow rates due its hydrophobicity. Condensate will be repelled and drained from the system. To assure that the filter will not loose its performance due to foam reaching the membrane either antifoam agents or mechanical foam breakers like demisters and baffles or cyclones can be used. Antifoam agents can have the disadvantage of fouling downstream processing filter devices rapidly, besides the antifoam agent needs to be sterile filtered. Mechanical foam breakers and cyclones (67) avoid the mentioned disadvantages, but usually work only effectively at specific air flow rates that vary from phase to phase of the fermentation process. Fine aerosol carried over from demisters or cyclones can be separated by tight depth filter cartridges containing polypropylene fleeces. These filters are very sufficiently protecting the costly sterilizing grade filter, due to the high dirt load capacity and a certain hydrophobicity, which avoids blocking of the depth filter fleeces. The void volume of these, filter is very high, therefore the pressure losses are minimal. Particles and microbial contamination will be greatly reduced and the lifetime of the sterilizing grade filter prolonged.

Vent Filters on Tanks

Every pharmaceutical application uses tanks, containers, and/or bags for a wide variety of purposes, for example, storage tanks for intermediate or final products, water storage tanks, transport vessels, or mixing tanks. Some applications only require a depth filter type, due to the product or medium stored in the tanks, which is unsuitable for any microorganism growth. Nevertheless, most of the tank-venting applications have in common that the air supplied into these tanks needs to be sterile and free of contaminations, usually achieved via a sterilizing grade, hydrophobic membrane filter.

When liquid is drawn from the tank or added to the tank, the air needs to be vented into or from the tank. Open to the atmosphere, the air needs to filtered through a sterilizing grade vent filter to avoid any contaminations, which could spoil the product stored in the tank. Often, the product fed into the tank is sterile filtered and the tank steam sterilized, therefore the vent filter needs to perform with highest security to ensure sterility. The filter needs to be and remain hydrophobic to avoid any condensate blockage and microbial growth on or within the filter matrix, especially when the vent filter is used over a long period of time without steam sterilization. This is the case on water storage tanks, which hold water of lower quality than Water for Injection (WFI), which is stored at around 80°C. The water temperature of WFI avoids or restricts microbial growth, but has the side effect of a high condensate rate, due to the high humidity of the air overlaying the hot water. A condensation of water on the filter cartridge can be avoided by using heat-jacketed filter housings, preferably an electrical heater. When using such heat-jacketed housing the filter cartridge must be visually checked on a routine basis, some manufacturers quote around every three months, to see whether parts of the filter are damaged by oxidization.

Nonvacuum resistant tanks, which are steam sterilized, need to be equipped with an appropriately sized vent filter system to overcome the condensation vacuum, created by the collapsing steam when the tank cools down (64,68). If the filter system is not correctly sized or the vent filter blocks due to a low hydrophobicity, the created vacuum could cause an implosion of the tank. Therefore, sizing of such vent filter systems is done by experienced and trained professionals.

The volume of some tanks is too vast to use a static vent filter system, at that point compressed air is pushed via a sterilizing filter into the tank to break the vacuum in the tank.

Implosion of the tank can also be avoided by using burst disks or pressure relief valves, which open up when the vacuum in the tank reaches the allowable limit. Unfiltered air rushes into the tank and breaks the increasing vacuum, which means burst disks and pressure relief valves are just precautions in case of an insufficient working vent filter.

Vent filters on tanks and vessels are generally steamed from the reverse flow direction. In this instance, the differential pressure over the filter device during steaming needs to be operated carefully. Most of the filter manufacturers allow a maximum differential of 0.2 to 0.5 bar at around 134°C steam temperature. Steaming in reverse direction is usually more stressful to the filter construction. It is therefore advisable to integrity test the filter system after steam sterilization.

Autoclave and Lyophilizer Vent Filter

In the past, the vent filters used for autoclaves and lyophilizers were depth filter type cartridges, sometimes even coalescing type filters. Because of stringent quality standards and demands of the regulatory agencies, these filter were replaced by sterilizing grade membrane filters. When breaking the vacuum created in these machines, the air vented into the chambers can come in direct contact with the product. Therefore, it is of great importance that these filters stand up to the requirements set.

Main demand is the sterile filtration ability of the filters, which is achieved by several different sterilizing grade, 0.2 μm rated, membrane filters, available in the market. These filters are usually correlated to challenge tests, like the ASTM Bacteria Challenge test (57) or aerosol challenge tests, performed by the individual filter manufacturer or independent institutes. Having the ability to create a sterile filtrate does not mean that the individual filter will be the right choice for this type of application. Another important aspect is the hydrophobicity of the filter membrane and the construction of the cartridge, as pointed out in the section on sterilizing grade filters. If the hydrophobicity of the membrane material used is of lower value, the pore structure could be blocked by condensate, which is possible after steam sterilization. At this point, the vacuum in the chamber cannot be broken and the filter needs to be bypassed, which means the chamber is unsterile and the process will have to be repeated. It goes without saying that hydrophobicity is of major importance, yet in the field some filter users still utilize with filters of lower hydrophobicity. Some users were even advised to use hydrophilic sterilizing grade filters to overcome the use of wetting media like solvent/water mixtures, so they can use water to integrity test the hydrophilic filters. To create airflow through this type of filter the bubble point needs to be exceeded, even when heat-jacketed housings are in use. This not only creates insecurities, but process failures. The construction of the filter cartridge needs to be optimized so that condensate can run into the condensate chamber and drain. The size of the filter system used on these units is usually bigger, due to the amount of condensation and the low differential pressures, down to 10 mbar, especially close to the end of the venting process.

These filters must withstand a high amount of steaming cycles. Some large volume hospital sterilizers are used up to five times a day and more; that is, the filter will be steam sterilized five times. Certainly, these filters are not changed every time. The number of steaming cycles can be as high as 250 cycles. Often enough the steaming happens to be in reverse direction of the filter cartridge, which is a higher stress factor to the material and construction of the filter cartridge. The maximum differential pressure over the filter must be checked carefully, otherwise the filter could be damaged. Filter manufacturers quote maximum allowable differential pressure at elevated temperature of 134°C from 0.2 to 0.5 bar. As one recognizes there is a higher-risk factor of damage of these filter cartridges due to mentioned stress factors, and therefore these filters should be integrity tested on a routine basis.

In the past, the filters were either not tested and discarded after a certain period of time or tested off-line before steam sterilization. These days, filter manufacturers offer integrity test methods, which are able to integrity test the filter in-place, even after steam sterilization. These tests methods either accommodate the common solvent/water mixture to integrity test the filter via diffusion or bubble point test or just water for the water intrusion test. Moreover, manufacturers of autoclaves and lyophilizers have either incorporated fully automatic

integrity tests methods in their equipment or advised their clients to install additional test equipment subsequently.

Filtration of Service Gases

Service gases, usually air and nitrogen, are used for pneumatic actuated valves and switches, head pressures of tank, transfer gases, drying purposes, and filling machines. These gases need to be sterile, because they are commonly supplied into clean room or sterile areas and come in contact with the product or the container, like vials, flasks, bottles, and tanks. Unfortunately, often enough these filters are overlooked, because there are so many in a standard pharmaceutical facility and sometimes not easily accessible or not obvious. This usually means that these filters are not integrity tested on a routine basis or not exchanged for a long period of time. Because of the more stringent requirements of the regulatory bodies, the awareness level for those filter units has increased and maintenance and quality assurance departments enforce checks on a regular basis.

With some exceptions, service gas filters are either not easily accessible or in pipe work, which is not steam sterilized. One major exception is blow-fill-seal filling machines. These filling machines mold the required containers, sterile fill them, and seal the containers. The need of an excessive amount of sterile air for extrusion, cooling, and overlaying purposes is obvious. Often these filling machines are equipped with up to four different air filtration units, for their different functions. Important here is that the air comes into direct contact with the plastic container and is introduced into the filling area (69). Therefore, the emphasis of routine steam sterilization and integrity testing of the filters is evident.

Integrity testing of such filters is done off-line, otherwise the solvent/water mixture used to wet the hydrophobic filter and perform the diffusion or bubble point test may contaminate the process. Tested off-line the filter is then flushed and dried, afterward installed and steam sterilized. This certainly created insecurity, because there was no assurance that the filter was integral after steam sterilization. Nowadays water-based tests, like the water intrusion or water flow integrity test are used to integrity test the filters in place after steam sterilization. As with the autoclave and lyophilizer vent filters, the filter elements can be tested fully automatically on a routine basis, preferably after every sterilization cycle.

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14 Processing of small volume parenterals and large volume parenterals

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DEFINITION OF SVPs AND LVPs

Parenterals are defined as preparations intended for injection through the skin or other external boundary tissue so that the active ingredients contained are delivered directly into the blood stream or body tissue. Parenterals are manufactured with extreme care by procedures designed to ensure that pharmacopeial requirements such as sterility, pyrogens, and particulate matter are met. Two categories of parenterals are the small volume parenterals (SVPs) and the large volume parenterals (LVPs). The term small volume parenterals applies to injections or preparations that are packaged in containers of 100 mL or less, whereas LVPs are usually intended for intravenous use and are packaged in containers of 100 mL or more (1).

CATEGORIES OF SVPs AND LVPs

Today's business world for the pharmaceutical industry shows an ever-increasing emphasis on producing products acceptable for world markets. Requirements are becoming more unified and are tending to reduce into three major pharmacopeias—the United States Pharmacopeia (USP), the European Pharmacopeia (EP), and the Japanese Pharmacopeia (JP). Although fundamentally equivalent, the USP and EP define, measure, and specify different requirements at the detail level (2–4). Requirements for today's processing of parenteral products are based on validated procedures and maintained under guidelines of current Good Manufacturing Practices (cGMPs). The validated methods encompass strict controls to assure products meet pharmacopeial requirements for sterility, pyrogens, particulate matter, and other contaminants. Water used in the manufacturing of parenteral products is strictly tested, controlled, and specified to meet critical requirements for microorganism and chemical contaminants (5).

The pharmacopeia categorizes parenteral products into small and large volume parenterals based on their fill volume or their use. For example, the USP designates SVPs as containers labeled as holding a 100 mL volume or less, whereas the EP views LVPs in terms of intended use, such as infusions, solutions for irrigation, and so forth. The importance in separating definitions between large and small volume products is for purposes of specifying impurity levels associated with dosing and the sampling of individual containers for product consistency, such as particulate matter and requirements for sterilization consistency (2).

The USP further describes the definition of the Pharmacy Bulk Pack as a separate category of sterile preparation for parenteral use that contains many single doses for the specific use in a pharmacy admixture program.

Drug product form determines the next higher level of categorization. The EP lists the several categories as injections, infusions, concentrates for injections or infusions, powders for injections or infusions, gels for injections, and implants. The EP defines injections as sterile solutions, emulsions, or suspensions prepared by dissolving, emulsifying, or suspending the active substance(s) and adding excipients in water, or a suitable nonaqueous liquid or in a mixture of these vehicles (3).

Control and measurement of sterility, bacterial endotoxins-pyrogens, and uniformity of units and contents are critical quality parameters.

DOCUMENTATION MANAGEMENT

A cornerstone of good manufacturing practices (cGMPs) in the pharmaceutical industry has been good documentation practices. As stated in Title 21 CFR sections 211.100 and 211.192, "There shall be written procedures for production and process control designed to assure that the drug products have the identity, strength, quality, and purity they purport or are represented to possess. Written production and process control procedures shall be followed ... and shall be

documented at the time of performance. Any deviation from the written procedures shall be recorded and justified. All drug product production and control records, including those for packaging and labeling, shall be reviewed and approved by the quality control unit to determine compliance with established, approved written procedures before a batch is released or distributed."

All process and environmental control activities must be maintained and documented on a daily basis for aseptic processing operations. Review of all batch records and data is required to assure compliance with written procedures, operating parameters, and product specifications before final release of product for a given manufacturing cycle. Information in the batch record documentation includes data relating to in-process testing, environmental control, personnel monitoring, utilities [e.g., HVAC, water for injections (WFI), and steam], equipment functioning (e.g., alarms, integrity of filters), and deviations (5).

Especially relevant to aseptic processing of parenterals are the documentation practices for interventions and/or stoppages. Filling line stoppages and unplanned interventions should be recorded in the batch record noting the time and duration of the event. Interventions can increase contamination risk, and their frequency may indicate a process requiring additional controls. Written line clearance procedures, such as machine adjustments and repairs, must be established. Interventions that require substantial activity near exposed product or container closures to correct usually involve local or full line clearance. A power outage, even though brief, may affect product quality and is considered a manufacturing deviation (1).

Validation Documentation

Validation documentations are mandatory in the qualification of equipment and processes (6). These documents include user requirements specification (URS), design qualification (DQ), installation qualification/operational qualification/performance qualification (IQ/OQ/PQ), validation master plan, process validation protocols/reports, test method validation, cleaning validation, technology transfer plan, facility/process risk management assessment, and media fills/smoke studies where appropriate (5).

The URS is a critical document. For mechanical systems and software programs, the successful execution of the IQ/OQ/PQ depends upon the system expectations defined in the URS. The scope of the URS should include full details of end user operability, full details of functionality, software functionality interface, description of required system performance, performance criteria (critical parameters and operating ranges), cleaning requirements, calibration schedule, maintenance requirements, and training/documentation requirements (7). Quality must review the final set of requirements and must approve changes to any requirements that may affect the product attributes (8).

For the parenteral facility with aseptic processing, complete and rigorous validation packages are the expectation to address particle monitoring systems, isolators (media fills, smoke studies), sterilization processes (autoclavation, ETO), cleaning processes, air handling, and utilities (WFI, steam).

Electronic Document Management Systems

The implementation of compliant electronic document management (EDM) systems with process monitoring functionality, fully automated work flow, electronic batch records (EBRs) and signatures, and environmental monitoring has been slow for the pharmaceutical industry. The combination of vague regulatory agency guidance, lack of significant financial investment, extensive training, and poorly aligned technologies has impeded companies from implementing compliant EDM systems. The most recent FDA guidance for electronic records and signatures is contained in the 21 CFR Part 11 (9). In this 2003 guidance, the FDA's goal was to alleviate concerns that have been raised that Part 11 requirements would (*i*) restrict the use of electronic technology that is inconsistent with the agency's intent in issuing the ruling, (*ii*) significantly increase the costs of compliance, and (*iii*) discourage innovation and technological advances. These concerns have been raised particularly in the areas of Part 11 requirements for validation, audit trails, record retention, record copying, and legacy systems.

Pharmaceutical manufacturing facilities mostly remain on a mid-20th century platform (10). The spread of computer technology has stalled at machine-level data collection for

tracking basic processing information. In-process samples are still taken and delivered to support laboratories and test results are delivered hours to days later. The pharmaceutical industry has lagged behind in implementing the use of IT on a large scale to automate and streamline manufacturing steps, specifically its batch record systems and for maintaining process quality control. Limited automation, paper record keeping, poor process understanding and controls, and outdated information archiving practices contribute to the industry's poor manufacturing efficiency record (11). EBR and process analytical technologies (PATs) are systems available to eliminate these inefficiencies.

EBR and PAT can create a database that can be translated into process knowledge, increased yields, and strategic-planning tools. In fairness to pharmaceutical manufacturing, reporting requirements and public safety regulations for drugs make the reliability of IT systems a critical priority. IT system crashes or glitches would likely result in lost data or process verification that could turn a million-dollar batch of medication into a company's largest quarterly loss. Laboratory information management systems (LIMSs) have emerged that are capable of addressing the complexity of the regulatory compliance and industry's best practices (12). With LIMS, data generated from an instrument electronically and then captured as a direct computer input can be identified at the time of the input by the analyst responsible for direct data entries. LIMS provides retention of full audit trails to show all changes to the data and uses timed and dated electronic signatures. The justification of changes are recorded and saved with each entry. LIMS generates final reports that automatically provide a description of the methods and materials used and a presentation of the results including calculations and statistical analysis.

MATERIALS MANAGEMENT

Materials management is the logistical planning required to ensure that sufficient raw materials, commodities, packaging components, and warehousing are available to manufacture the product and to satisfy the supply chain (13). Materials management also ensures the materials used to manufacture, package, and ship the product meet minimum quality requirements and are compliant with international standards and relevant regulatory guidelines (6).

Quality assurance for finished pharmaceuticals and medical devices includes the specification and control of those components that have product contact during manufacturing. Product contact with commodities and equipment may result in the extraction of foreign substances that may impact patient safety or compromise product potency and stability. This also includes the quality and consistency of the raw materials (excipients including salts, sugars, stabilizers, and surfactants) in the product formulation (14).

Many principles in this section are derived from international guidance for the application of appropriate GMP. This section combines existing governmental regulatory GMP principles and international quality management system requirements as developed by the International Organization for Standardization (ISO). In view of the increasing globalization of the pharmaceutical industry and the harmonization of pharmaceutical registration requirements, deference to both schemes is becoming necessary. The reach of the requirement for cGMP is moving upstream in the overall manufacturing process and in today's environment is touching on the fringe of control of excipients.

The ISO 9000 series is a quality system standard of general application that can be applied to cover every aspect of manufacturing to the benefit of both the manufacturer and the customer. It has taken several years since its introduction in 1987 for the ISO 9000 series to be utilized worldwide. Obtaining certification is a business decision as there is no current regulatory requirement in Europe, Japan, or the United States for third-party certification.

A manufacturer may apply the standard with or without certification. However, certification has the benefit of providing assurance to customers that conformance to this quality system has been independently confirmed. Incorporation of GMP requirements into the ISO 9000 quality system enhances not only the quality system, but a company's operational procedures as well. Finally, there is an increasing expectation worldwide for compliance with ISO 9002 as an essential element to qualify suppliers.

A material's qualification and control program (Fig. 1) is key to assuring drug quality, yet it is often viewed as a burdensome requirement in a busy firm and not an activity

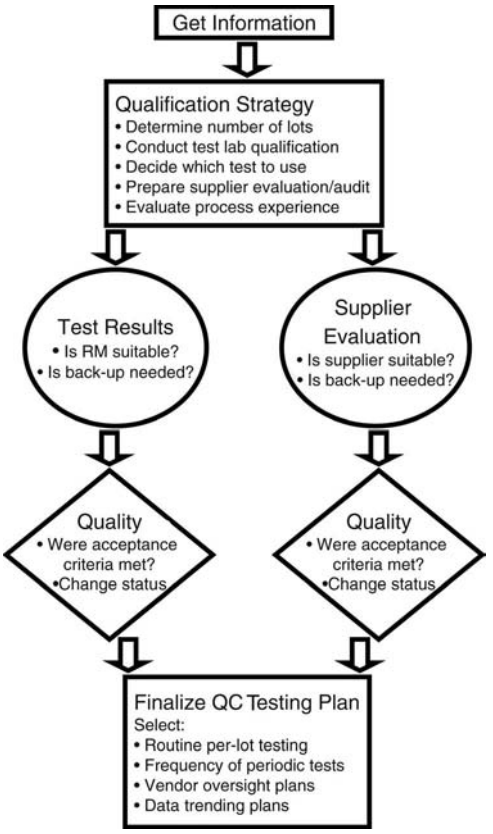


Figure 1 Material qualification and control program.

that brings process control to the product until the supply chain is derailed by a single failure (15).

GMP regulations require that pharmaceutical raw materials and their suppliers be qualified both initially and periodically (5). Similar requirements can be found in the U.S. Code of Federal Regulations (CFR), ICH guidance documents, European GMP regulations, and within ISO. Patient safety drives this requirement, dating back to several events within the pharmaceutical and food industries where unsuitable raw material led to toxicity, resulting in hallucinations and other severe symptoms (16). Mix-ups and errors of identity have also occurred.

Attention should also be paid not only to the manufacturing operation but also to how the raw material is packaged. Laboratory animal studies have reported bisphenol A to be a potential carcinogen. This leachate comes from plastic containers used in the food industry.

A pharmaceutical firm is legally responsible for the quality of the product contact materials that it purchases and uses in a cGMP manufacturing process. Consequently, it is good a business practice for a firm to oversee suppliers and test laboratories and to characterize materials appropriately (5).

Control of Excipients

It is important that manufacturers identify and set appropriate limits for impurities in excipients based on appropriate toxicological data, or limits described in national compendia as requirements, as well as sound manufacturing practice considerations. Most excipients are used in the final dosage form without further processing so all impurities obtained in the excipient generally remain in the final dosage form.

Excipients in Finished Dosage Forms

Bulk pharmaceutical excipients are required to be uniform in chemical and physical composition to assure consistent and continued final dosage-form products.

The excipients used to manufacture commercial lots should not significantly differ from those used in clinical lot manufacture. Where significant differences do occur, additional testing by the manufacturer of finished dosage forms may be required to establish that the bioequivalence of the finished product is not adversely affected over time.

QUALITY POLICY AND CONTROL

Management should demonstrate commitment to a quality policy that should be implemented within the operational unit. Management should also participate in the development of the company's quality policy and should provide the resources necessary for development, maintenance, and review of such policy and quality systems at least annually. Management should be committed to this policy and should appoint appropriate company personnel to be responsible for coordination and implementation of the quality systems (17).

Organization

There should be a quality unit, independent of production, with the responsibility and authority to approve or reject all components, excipients, in-process materials, packaging materials, and finished drug product. The quality unit should have the authority to review production records to ensure that no errors have occurred or, if errors have occurred, that they have been fully investigated. The quality unit should be responsible for approving or rejecting product manufactured, processed, packaged, or held under contract by another company. The quality unit can delegate these responsibilities if proper controls, such as periodic audits and documentation of training, are in place. Adequate laboratory facilities for the testing and approval or rejection of raw materials, packaging materials, in-process materials, and finished dosage form should be available to the quality control unit.

It is the responsibility of an independent unit, usually the quality assurance group, which is independent of production, to participate in issuing procedures; authorizing changes to processes, specifications, procedures, and test methods; and investigating failure and complaints.

Manufacturer and User Responsibilities

Contract Review

The manufacturer and user should mutually agree upon the specifications. The manufacturer must have the facility and process capability to consistently meet the mutually agreed-upon specifications of the product(s). Subcontracting or significant changes to a supplier's audited process that could affect the physical properties, chemistry, or functionality of the excipient in a final dosage form should be immediately communicated or preapproved as mutually agreed upon between customer and supplier.

Document and Data Control

The excipient manufacturer should have a system to control all documents and data that relate to the requirements of the quality system. Date of issue and location of these documents should be recorded. Each document should include a unique identifier, date of issue, revision number on each page, and the issuing department. All changes and the reasons for the changes should be documented. Documents and subsequent changes to the documents should be reviewed and approved by designated qualified personnel before issuance to the appropriate areas identified in the documents.

Purchasing

The purchaser should verify that the supplier of raw materials, components, and services for the manufacture of excipients has the capability to consistently meet the agreed-upon requirements. This may include periodic audits of the vendor's plant, if deemed necessary. Purchasing agreements should contain data clearly describing the product ordered, including where applicable, the following:

- The name, type, class, style, grade, item code number, or other precise identification traceable to the raw material specification

- Drawings, process requirements, inspection instructions, and other relevant technical data, including requirements for approval or qualification of product, procedures, process equipment, and personnel

These requirements also apply to selection and control of subcontractors, which include toll manufacturers and contract laboratories.

Product Identification and Traceability

All items should be clearly identified and traceable through a documented system. The system should allow the traceability of product upstream and downstream. Identification of raw materials used in the production of processed materials should be traceable using a batch numbering system or any other appropriate system. The finished product should be traceable to the customer and retrievable in case of the need for a product recall.

Labeling

Labeling requirements for excipient packages are subject to applicable national and international regulatory requirements that may include transportation and safety measures. Procedures should be employed to protect the quality and purity of the excipient when it is packaged and to ensure that the correct label is applied to all containers. At a minimum, a good system of labeling should have the following features: the name of product, the manufacturer and distributor, a lot or batch number from which the complete lot or batch history can be determined, a file of master labels (*Note:* A designated individual should review incoming labels or labels printed on demand against the appropriate master labels), storage of labels in separate containers or compartments to prevent mix-ups, formal issuance of labels by requisition or other document, issuance of an exact number of labels sufficient for the number of containers to be labeled, retention copies, and calculated excesses, if any; reconciliation of the number of labels issued with the number of unit packages and retention labels together with the destruction of excess labels bearing lot or batch numbers; and avoidance of labeling more than one lot or batch at a time without adequate separation and controls.

There should be documentation of the system used to satisfy the intent of the previously mentioned requirements in all instances whether excipients are labeled on the packaging line, packaged in preprinted bags, or bulk shipped in tank cars.

If the need for special storage conditions exists (e.g., protection from light, heat, etc.), such restrictions should be placed on the labeling.

Retained Samples

Reserve samples of an excipient should be retained for one year after the expiration or reevaluation date or for one year after distribution is complete, whichever is longer. Sample size should be twice the amount required to perform specification testing.

EQUIPMENT CONTROL

Multipurpose Equipment

Equipment used in the manufacture, processing, packaging, or holding of a product should be of appropriate design, adequate size, and in a suitable location to facilitate its operation, cleaning, and maintenance.

Many parenterals are produced using multipurpose equipment. With few exceptions, such multiple usages are satisfactory provided the equipment can be adequately cleaned according to validated written procedures. The cleaning program should take into consideration the need for different cleaning procedures, depending on the safety considerations of the product or intermediate and what product or intermediate was previously produced. Products that leave residues that cannot be easily removed should be produced in dedicated equipment.

Where multipurpose equipment is in use, it is important to be able to determine previous usage when investigating cross-contamination or the possibility of such contamination. Methods of determining prior use include any documentation system that clearly identifies the

previous lot or batch and shows that the equipment was cleaned. An equipment cleaning log is perhaps the most desirable and preferred method of determining prior use.

The cleaning and disinfection procedures should be properly established by competent personnel using the model product approach. These procedures should be designed to meet or exceed the particular needs of the product and process involved and should be set down in a written schedule available for the guidance of employees and management. An effective and regular cleaning program should be put in place to remove product residues and dirt that may also contain microorganisms and act as a source of contamination.

The manufacturer should demonstrate the effectiveness and efficiency of the cleaning and disinfection procedures for each piece of equipment, and the cleaning status of equipment should be recorded. Validation data ought to prove that the cleaning procedure is acceptable. An evaluation should consider the potential impact that traces of contaminant may have on the product supplied to the customer. All equipment that has been in contact with contaminated material must be thoroughly cleaned and disinfected before coming in contact with product.

Single-Use Technologies for Multiuse Production Facilities

Disposables are growing in popularity due to the large numbers of biological drugs being developed that require aseptic processes. Disposables are able to minimize cross-contamination, cleaning, start-up timeline, capital investment, production cycle, and assurance of sterility. The time saved when substituting a disposable capsule filter for a cartridge filter in stainless steel housing is presented in Table 1. When implemented, disposables, also known as single-use systems, simplify the transfer of processes across multiple sites because single-use systems are flexible, modular, and customizable. If disposables have already been designed into the biopharmaceutical process, qualification and validation are simple during technology transfers.

So what are the implications for facility design? One example is presented by considering the amount of water used for cleaning a stainless steel facility. This traditional facility design is composed of complex piping and controls for steam-in-place (SIP) and clean-in-place (CIP). If one considers a model 500 kg bulk monoclonal antibody facility of stainless steel, approximately 155,000 L of solution would be required annually for cleaning (18). However, in a disposable facility, one could:

- remove most CIP and SIP infrastructure
- remove the autoclave and washing areas
- remove process pipework between the unit operations by replacing it with physical movement and disposable tubing

The result is a facility containing clean rooms with little process infrastructure. The process is configured by setting up process operations at designated workstations that are minimally equipped with power, data links, and gases. Therefore, the operational space becomes flexible and can be easily reconfigured as desired in multiuse facilities.

Table 1 Time Comparison to Perform Filtration

Step	Presterilized capsule filter	Cartridge filter in SS housing (min)
Remove filter from packaging	5 min	5
Collect components/assemble housing	N/A	15
Autoclave filter assembly	N/A	60
Cool filter to room temperature	N/A	60
Transport assembly to process area	N/A	5
Aseptically connect filter to process train	15 min	15
Total time required	20 min	160
Time saved with presterilized filter	140 min	

However, disposables do pose challenges. The procurement and quality teams need to perform the vendor/supplier assessments in terms of pricing, operational risk, product/chemical compatibility, and security of supply. Production will rely more on manual handling rather than pipework to move product, solutions, and materials throughout the facility. Companies will also have to address disposal options for the large volume of disposables produced by the single-use technology to be environmentally friendly as well as cost-effective.

Product Contact Material

In the course of establishing a manufacturing process, it is mandatory that the impact of materials used in the manufacturing equipment on drug product is well understood, which is also reflected in regulatory requirements (e.g., CFR Title 21, Part 211.65) (5).

At the time of implementing a manufacturing process, a complete product contact material assessment should be available. Materials used in the manufacturing process typically comprise various types of stainless steel, plastics, rubber, lubricants, and glass in the form of stainless steel tanks, plastic containers, tubing, stirrers, gaskets, valves, rings, filters, sampling devices, pumps, or fill needles. The main factors to be examined in such assessment are extractables and leachables, sorption, and chemical and physical compatibility. Consequently, a compatibility assessment of the drug and a comprehensive set of product contact materials involve exposure studies at relevant conditions where stability and sorption properties of the drug substance are monitored, along with detection of leachables from the exposed materials. In addition, extractable studies are required according to relevant guidance provided (e.g., in the USP monographs) (2). Information from prior experience, published literature, and vendors initially may be used for an assessment. However, in most cases corroboration is needed in the form of experimental data. This is especially important in the cases of biologics. The considerable complexity regarding molecule size, number of potential interaction sites, and sensitive structure/function dependence of this molecule class poses a high potential for being impacted by product contact material interactions (e.g., by leachable substance mixtures of diverse chemical nature). Therefore, it is recommended that every biologic product and its contact materials be assessed on an individual basis.

Various case studies of incidents involving leachables originating from processing equipment (i.e., stainless steel tanks, rubber gaskets, silicone tubing, and filter membranes) in commercial manufacturing have been reported in the literature (19). However, in many cases, the focus of development activities regarding material compatibility, especially in terms of leachables and extractables, is still solely on long-term storage in primary and secondary packaging containers (20,21).

Plastic Containers

Today, plastic containers are commonly used for the following types of products: LVPs, ophthalmics, otics, and inhalation therapy. The replacement of the glass container for these products has been gradual over the last few decades. A high degree of caution was based on data that showed that glass was inert and that the glass container provided a better barrier to the environment, for example, better water vapor transmission protection and better protection against intrusion of gas (22).

This early perception regarding plastics has been changing. In the case of LVPs, durability and weight savings were obvious advantages for using plastic instead of glass. However, the flexibility of plastic was also an important consideration. Plastic bags that are used to package LVP products will collapse as liquid drains out. This occurs because of the fact that the system is completely closed. Glass bottles, on the other hand, do not collapse as the fluid drains from the container and a venting system must be provided to replace the evacuated liquid with air. The venting systems have gone through a series of improvements, all of which are not as effective as the closed system provided by a plastic bag.

The blow/fill/seal technology, also known as form/fill/seal technology, is used to manufacture and fill plastic containers. This technology involves forming the container via a process known as blow molding (i.e., forming the molten plastic into a container of the desired shape) while simultaneously sterilizing the container, filling the formed sterile container with a sterile product, and then sealing the container. All of the operations are completed aseptically

on one machine and the entire process is completed uninterrupted and in sequence. The formulated product within the container is not exposed to the surrounding environment. This eliminates the need for container inventory, washing, sterilization, and in some cases, labeling. This new technology is largely responsible for making plastic a more attractive alternative to glass for packaging SVP products.

However, the manufacturers of SVP products have been reluctant to switch from glass to plastic because plastic containers still need to be washed and sterilized like glass. In addition, the chemical sterilization methods that are required for some plastics can be more difficult to perform and less reliable than the thermal methods used to sterilize glass containers.

Nonetheless, plastic materials have some advantages for SVPs. Their higher mechanical strength can be beneficial when developing a container for the use in a device. Additionally, their modern appearance might be a marketing advantage.

LVP Films

LVPs are packaged either in glass vials with rubber stoppers or in plastic bags. Plastic bags are either delivered presterilized and ready to use with no further pretreatment before usage required, or the blow/fill/seal technology is used for manufacturing. An advantage of plastic bags over glass vials is the number of different sizes that are available and the flexible, hardly breakable properties of the materials. A high variety of assemblies with multiple connectors is available and can be customized to the special requirements of a certain application.

These plastic bags are sterilized with gamma irradiation and consist of multiple film layers of different materials such as ethylene vinyl acetate (EVA), ethyl vinyl alcohol (EVOH), and with contact to the fluid ethylene vinyl acetate monomaterial (EVAM) or layers of polyamide (PA), polyethylene terephthalate (PET), ultralow density polyethylene (ULDPE), or EVOH. The plastic film materials must comply with relevant compendial monographs—for example, the monographs for "Physico-chemical test for plastics" (USP 661) or "Ethyl Vinyl Acetate Copolymer (EVA) for containers and closures" (EP 3.1.7).

For the validation of bags, testing of the films and their properties, integrity and biocompatibility, physicochemical tests, stability, chemical compatibility, bioburden, and shelf life are investigated. For example, the bags are filled with WFI, and parameters such as total organic carbon (TOC) are checked at various time points over certain time periods. Physicochemical testing according to USP 661 involves extractions with water at 70°C for 24 hours (nonvolatile residues, residue on ignition, heavy metals, and buffering capacity) or extractions with isopropanol (nonvolatile residues, residue on ignition, turbidity, and UV absorption) are performed.

For the investigation of extractables, the bag materials are "extracted" with model solvents such as water, ethanol, hydrochloric acid, or sodium hydroxide solution. The extracts are then analyzed for pH, conductivity, volatile GC-MS, nonvolatile LC-MS, and metal ICP. Additional product-specific leachable studies must be conducted and can be performed either by the bag user or by the bag manufacturer.

For the determination of protein adsorption, the bags are tested with a model protein-like bovine serum albumin or bovine IgG at different temperatures with contact times up to several days.

The evaluation of the permeation of microorganisms is performed with bags that have been aseptically filled with culture media (e.g., soybean digest casein broth) and preincubated for seven days. The bags are then immersed in challenge suspensions containing defined types and amounts of microorganisms and are afterwards incubated.

MANUFACTURING CONTROLS

Aseptic and Sterile Manufacturing

The manufacture of sterile products presents technical challenges. Since humans are the primary source of contamination in an aseptic operation, the process should be designed to eliminate this direct contact. Those aseptic operations that utilize considerable operator involvement should have adequate controls.

The manufacturer should document the sanitizing of critical processing equipment. Processes used for the sterilization of equipment should be validated. The manufacturer also should verify that no chemical interaction with the product occurs.

There are guidelines and compliance programs that provide detailed guidance for the manufacture of sterile products (1).

Validation of Process and Control Procedures

Parenteral manufacturers are expected to adequately determine and document that all significant processing steps are performed consistently. The type of drug product, the breadth of the specification relative to the degree of process control, and the other factors determine the extent of the process development and documentation required.

An important factor in the assurance of product quality includes the adequate design and control of the manufacturing process because product testing alone is not sufficient to reveal variations that may have occurred. Each step of the manufacturing process should be controlled to the extent necessary to ensure that the product meets established specifications. The concept of process validation is a key element in ensuring that these quality assurance goals are met. Documentation describing the process reactions, operating parameters, purifications, impurities, and key tests needed for process control should be written to provide the basis for validation.

Many manufacturers already possess the data necessary to validate that their processes perform in a consistent manner. For example, limitations of a reaction or purification step are usually identified in the development phase. Known impurities and tests used to determine their levels are also established at this phase. Thus, when the process is scaled up to production of a lot or batch size, a comparison can be made with development lots or batches. Scale-up and development reports, along with purity profiles, would constitute an appropriate validation report.

In-Process Testing

Parenteral products are normally subject to various in-process tests to show that a manufacturing process is proceeding satisfactorily. Such tests often are performed by production personnel in production laboratory facilities. Approval to continue with the process is often issued within the production department. The important considerations are that specified tests be performed and recorded by trained personnel and that the results are within specified limits.

In-process inspection and testing should be performed based on monitoring the process or actual sample analysis at defined locations and times. The results should conform to established process parameters. Work instructions should delineate the procedure to follow and how to utilize the inspection and test data to control the process.

Finished Product Testing and Release

Finished product testing should be performed by the quality unit and should conform to written specification. There should be a procedure that ensures prior to release that the evaluation of the appropriate manufacturing documentation and test data occurs.

All appropriate records relating to inspection and testing must be available for review. Where the process is continuously monitored, acknowledgment that the process was monitored and the results of the monitoring should be available.

Control of Nonconforming Product

Any raw material, intermediate, or finished product found not to meet specifications should be clearly identified and segregated to prevent inadvertent use or release for sale. A record of nonconforming product should be maintained. All incidence of nonconformance should be investigated to identify the root cause. This investigation should be documented and corrections made to prevent recurrence of the problem.

Procedures should exist for the evaluation and fate of nonconforming products. Nonconforming products should be reviewed in accordance with documented procedures to determine final outcome.

Inspection, Measuring, and Test Equipment

Calibration of all in-process and laboratory instruments, identified as quality instrumentation, should be traceable to recognized standards. The control program needs to include the

calibration of reagents, instruments, apparatus, gauges, and recording devices at suitable intervals in accordance with an established written program containing specific directions, schedules, limits for accuracy and precision, and provisions for action in the event accuracy or precision limits are not met. Equipment not meeting established specifications should not be used.

Computer systems used to verify that the product conforms to specifications should be audited to ensure satisfactory performance.

Quality Record Control

The manufacturer should establish and maintain procedures for identification, collection, indexing, filing, storage, maintenance, and disposition of quality records. Quality records should be maintained to demonstrate achievement of the required quality and the effective operation of the quality system. Pertinent subcontractor quality records should be an element of the data.

Quality records should be kept for at least as long as samples are retained or in accordance with legislative requirements. These records should be stored in facilities that provide a suitable environment to minimize deterioration or damage and to prevent loss and should be maintained in such a way that they are readily retrievable.

Batch production and control records should be prepared for each batch of drug product produced and should include complete information relating to the production and control of each batch. These records should include an accurate reproduction of the appropriate master production record, checked for accuracy, dated, and signed as well as documentation that each significant step in the manufacture, processing, packing, or holding of the batch was accomplished.

Internal Quality Audits

The parenteral manufacturer should carry out a comprehensive system of planned and documented internal quality audits to verify whether quality activities comply with planned arrangements and to determine the effectiveness of the quality system. Audits should be scheduled on the basis of the status and importance of the activity. The audits and follow-up actions must be carried out in accordance with documented procedures. The results of the audits should be documented and brought to the attention of the management personnel having responsibility in the area audited. Personnel responsible for the area should take corrective action on the deficiencies found by the audit. Quality risk management (e.g., FMEA analysis) should be implemented to reduce future risks, focus validation efforts, and maximize the business value of the manufacturing process (23).

Training

The parenteral manufacturer should establish and maintain procedures for identifying and providing the training needs of all personnel performing activities affecting quality. Appropriate records of training should be maintained. Training should directly relate to the employee's function or performance of specific operations and to GMP. This training should be conducted by qualified individuals on a continuing basis and with sufficient frequency to ensure that employees remain familiar with any applicable manufacturing practice requirements.

PROCESSING OF COMPONENTS

Siliconization

When manufacturing parenteral products, various parts of the primary packaging materials can be siliconized with polydimethylsiloxane (PDMS, e.g., Dow Corning DC-360, Medical Fluid). Examples are as follows:

- Vials
- Syringe barrels
- Stoppers
- Syringe needles

Silicone oil is applied on glass containers to mask glass surfaces by sealing microcracks. Also, siliconization facilitates complete emptying of syringes or vials, which in turn will decrease the loss of drug due to residual volumes in the containers and leads to a reduction of the required overfill volumes.

For syringes as primary packaging material, siliconization is needed to enable stopper movement in the syringe. Unsiliconized syringe barrels cannot be emptied due to high gliding forces. The quality and quantity of siliconization determines the ejection forces and are therefore crucial for the functionality of the syringe, which is especially important in autoinjector devices. Syringe forces are divided into categories of the breakout force and the gliding force. The breakout force is the initial force needed to start the stopper moving, while the gliding force is defined as the force needed to keep the stopper traveling to the end of the syringe barrel.

Syringe forces can be controlled by defining the type and amount of silicone oil applied. The higher the viscosity of the oil, the higher the forces. Forces can be decreased by decreasing oil viscosity and/or increasing the amount of silicone oil per syringe. The amount per syringe must be chosen so that it is compatible with the respective drug and does not detach from the syringe walls over time within the chosen storage time and temperature. In general, lower viscosities are preferred since the distribution is more even, resulting in smoother gliding forces and fewer areas with insufficient siliconization.

Stopper siliconization mainly improves processability during manufacturing by supporting stopper insertion and by preventing the stoppers from sticking together due to the high friction of the rubber. Syringe needles are siliconized to facilitate needle insertion into the skin and to reduce the sensation of pain.

PDMS has a molecular weight of 1000 to 150,000 Da and a viscosity of 10 to 107 mPa·s and can be applied to the glass surfaces as pure oil or as an emulsion. As an oil, it is applied via two methods, either as a wipe-on siliconization with silicone-soaked fabric or O-rings or with spray-on siliconization through nozzles. Under standard conditions no binding or polymerizations of the silicone occurs. For the emulsion, 1% to 3% PDMS with WFI and an emulsifier (e.g., polysorbate 20) are stirred until a stable, homogenous emulsion is obtained. Vials or syringes are immersed into the oil-water emulsion and are then heated to 250 to 300°C to generate covalent bindings (Si–O–Si), and 1 to 10 layers are fixed to the surface mostly as free silicone. Water and Emulsifier are removed with heat during that procedure, which is called "baking" of the silicone onto the glass surfaces.

The applied amount of silicone can be controlled by reading the scale of the tank display (consumed amount of oil per batch) or by analysis of the individual syringe or vial: control of pump movement and compressed air during application of the oil per unit; weighing before and after silicone application; extraction with Toluol (destructive); or via FTIR, Raman, or refractometry as nondestructive tests. For prefillable syringes, the quality and quantity of siliconization can be determined indirectly by force measurement (destructive). In general, the maximum breakout forces and gliding forces should be specified on the level of the empty syringes to avoid product failures with filled product. The average gliding force is a measure for the amount of silicone oil applied, and the profile is a measure of the uniformity of the silicone film. The more uneven the gliding force, the less uniform the distribution. Uniformity of siliconization is especially important when using an autoinjection device since these are usually spring driven and can only deliver a defined force over the barrel length.

Washing of Vials, Stoppers, Hoses, Pump Assembly, and Tanks

For the aseptic manufacturing of SVPs, all manufacturing equipment and primary packaging materials must be clean and sterile. Presterilization preparation of manufacturing materials usually involves a series of wash and rinse cycles to remove foreign particulate matter and to reduce bioburden as well as endotoxin load. The quality of water to be used is defined in FDA and European Medicines Agency (EMEA) guidelines and must comply with the monograph for "Purified Water" for first rinsing and washing and the monograph "Water for Injectable Products" for the final rinsing step for parenteral product equipment.

The use of detergents should be avoided, if possible, since residues could be hard to eliminate and as a result may contaminate the product.

The FDA recommends an area classified with a Class 100000 (ISO 8) air cleanliness level appropriate for less critical activities such as equipment cleaning.

The time between washing, drying, and sterilizing should be minimized since residual moisture can support microbial growth and the generation of endotoxins.

In addition, equipment should be designed to be easily assembled, disassembled, cleaned, sanitized, and/or sterilized. Pieces of equipment that are hard to disassemble or clean, such as tubings and fill needles, might be defined as single use to avoid costly cleaning validation.

Clean-in-Place

CIP is a method of cleaning the interior surfaces of pipes, vessels, process equipment, and associated fittings, without disassembly. Some CIP procedures employ initial rinses with appropriate high-purity water and/or cleaning agents followed by final rinses with heated WFI. The washing process consists of several cycles in which rinsing material is recycled through the vessels, pumps, valves, and other process equipment in a flow system. The cleaning end point is measured by analytical instruments that monitor the composition of rinse water.

Sterile-in-Place

Using SIP technology, the amount of aseptic manipulations can be reduced by sterilizing the preassembled connection of hoses, pipes, and tanks after CIP. The installation must be capable of withstanding steam pressure up to approximately 20 psi and sterilizing temperatures of 121 to 125°C. Furthermore, the whole system must be validated. Steam must be able to reach all parts of the equipment that have product contact for sufficient duration. Temperature sensors and pressure must be installed to monitor data during the sterilization cycle.

Some materials (e.g., rubber stoppers) are also available prewashed "ready to sterilize" or even already sterile "ready to use." This reduces the number of operations and risk of contaminations during the preparation steps. In addition, the components may be used immediately without additional operations, and investments and validations for washing and sterilizing equipment are decreased. Filling equipment can be different if "ready to use" materials are employed.

Depyrogenation and Sterilization

Depyrogenation of equipment surfaces, glass, and metal parts can be attained by high-temperature dry heat. For temperature-sensitive parts such as rubber stoppers and hoses, depyrogenation is achieved by multiple cycles of washing and multiple rinses of hot WFI prior to final steam sterilization (autoclaving), gas sterilization by ethylene oxide, or gamma irradiation sterilization.

COMPOUNDING SOLUTION

For the majority of LVPs and many SVPs, the compounding of parenteral bulk drug product involves simple dissolution of soluble ingredients in WFI. This generally straightforward process, however, is complicated by the high level of cleanliness that must be imposed to minimize the risk of product contamination by extraneous particulate matter, viable organisms, or pyrogenic substances.

Parenteral solutions typically contain soluble active ingredients next to several excipients such as osmotic adjusters, buffering agents, and (if required) bacteriostatic agents. The usual practice for compounding is to fill the tank with the larger part of the required volume of WFI and then to add the ingredients with agitation.

To ensure complete dissolution of even hard-to-dissolve ingredients or very concentrated solutes within a practical period of time, high temperatures and high mixing shear may be applied if the stability profiles of components allow. Special preparations for parenterals (e.g., suspensions, oil-in-water emulsions, cosolvent systems, and nonaqueous systems) may require shear-intensive dispersion and homogenization operations. Jacketed mixing tanks with both an inner and an outer wall are used for heating and subsequent cooling of the product solution. For heating and cooling, steam and cooling liquid, respectively, are admitted into the

space between the tank walls. The mixing process and mixing pattern in a stirred tank is defined by a number of parameters, such as tank geometry, mixing speed, eccentricity of the mixer, and mixer type. On the basis of required mixing efficiency and allowable shear, a mixer geometry producing a radial, axial, and tangential flow pattern, respectively, is chosen. Commonly used systems include top-mounted impeller or paddle, magnetically coupled bottom impeller, or stir bar. To further increase mixing efficiency and avoid vortex formation, baffles (i.e., static elements mounted radially at the tank wall) can be added. In general, the formation of a vortex in the liquid is to be minimized during mixing because it may lead to centrifugation with minimal mixing efficiency and potentially, to severe air entrainment.

After dissolution is complete, the preparation pH is checked and adjusted if required. The bulk preparation is brought to final volume with WFI and is mixed.

An increasingly important exception to this general process is represented by the group of biotech parenteral drugs (i.e., monoclonal antibodies). Here the bulk drug substance is commonly produced as aqueous liquid solution that has the same composition as the final product. The liquid state may be suitable for short-term holding. However, due to the benefits of increasing product stability, extending shelf life, and decreasing potential for microbial growth, the biologic bulk drug substance is preferably stored long term and shipped in a frozen state. Several platform technologies based on stainless steel tanks or disposable containers (e.g., bottles, carboys, and bags) have been developed for this purpose. All systems require a thawing step for the bulk drug substance before subsequent unit operations of the compounding process occur. Depending on the selected freeze and thaw system, additional low-shear mixing and dilution with a compounded excipient solution may be necessary.

FILTRATION

Filtration is a common method of sterilizing drug product solutions. A sterilizing-grade filter should be validated to reproducibly remove viable microorganisms from the process stream, producing a sterile effluent. Currently, such filters usually have a rated pore size of 0.22 μm or smaller. Use of redundant sterilizing filters should be considered in many cases. Whatever filter or combination of filters is used, validation should include microbiological challenges to simulate worst-case production conditions for the material to be filtered and integrity test results of the filters used for the study.

Factors that can affect filter performance generally include (*i*) viscosity and surface tension of the material to be filtered, (*ii*) pH, (*iii*) compatibility of the material or formulation components with the filter itself, (*iv*) pressures, (*v*) flow rates, (*vi*) maximum use time, (*vii*) temperature, (*viii*) osmolality, and (*ix*) the effects of hydraulic shock. When designing the filter validation protocol, it is important to address the effect of the extremes of processing factors on the filter capability to produce sterile effluent. Filter validation should be conducted at maximum filter use time and pressure.

It is essential that laboratory experiments model actual production conditions. A production filter's integrity test specification should be consistent with data generated during microbial retention validation studies. Sterilizing filters should be discarded after processing of a single lot (1).

A filter validation package should be updated when modifications are implemented that impact the filtration step(s) in the manufacturing process. These may include changes to filter device or membrane composition, filter contact time, batch size, solution formulation, temperature, flow rate, and pressure. A careful review of the microbial retention challenge filtration process conditions and the solution volume and properties is required to determine any gaps created by the change(s). A typical assessment is presented below.

Case Study: Batch Size Scale-Up Rationale for Filter Revalidation of Microbial Retention Challenge

Issue

The current filter validation for bulk solution supported 32L batch size filtered through 1000 cm^2 filter device based on microbial retention and filter device extractables. Production plan was to increase batch size to 130L while maintaining 1000 cm^2 filter device. No changes were made to the filter membrane, pore size, or product contact materials. Is microbial retention challenge revalidation recommended for the filtration step?

Rationale
A scaled-down batch volume of approximately 700 mL was filtered in the previous microbial retention challenge through a 13.8-cm^2 effective surface area membrane. Upon meeting the acceptance criteria of no detected bacterial growth in the filtrates (n = 3), the 700-mL scaled-down volume justified the filtration of bulk solution up to a maximum 50L batch size when using the 1000-cm^2 membrane surface area.

$$\text{Scaled-down batch volume (mL)} = \frac{\text{Maximum process batch volume}}{\text{Surface area of process filter (cm}^2\text{)}} \times \text{Surface area test filter (cm}^2\text{)}$$

$$700\,\text{mL} = \frac{\text{Maximum process batch volume}}{1000\,\text{cm}^2} \times 13.8\,\text{cm}^2 = 50,700\,\text{mL}$$

A scaled-down batch volume of 1794 mL is required for a 130L batch size using a 1000-cm^2 membrane surface area. The minimum normal flow volume processed during the revalidation microbial retention test for bulk solution was 2281 mL, which exceeded the scaled-down batch volume of 1794 mL required by calculation for the 130L batch size when using a filter device with 1000 cm^2 membrane surface area.

Therefore, the future 130L batch size processed using a 1000-cm^2 filter device will satisfy the quality requirements and objectives of the filter microbial retention challenge revalidation if the excipients and potency of the formulation, the flow rate, the pressure, the filter contact time, and the process temperature remain within current operating ranges.

DISPENSING/FILLING

The purpose of the dispensing step is to subdivide the bulk drug solution into individual container fills and to transfer these doses into the individual primary container. Commonly, the dispensing is performed in-line just after the final filtration.

Many fill systems rely on volumetric displacement pumps consisting of a cylinder and piston assembly. A fixed volume of fluid is hereby drawn into the pump chamber and then discharged into the primary container. The adjustable piston stroke determines the dispensed volume. However, in recent years, alternative pump technologies (e.g., time pressure filling, rolling diaphragm pumps, mass flow filling, and peristaltic pumps) have become more popular. This trend has appeared partly because of certain incompatibilities of the piston pumps with biotech products, which in some reported cases led to protein particle contamination in the filled containers.

In general, the dispensing step is to be considered critical for parenteral product quality and safety. This is mainly due to the potential impact of the dispensing step on fill volume precision and the increased contamination risk associated with procedures handling open product and open containers.

The fill volume precision of the dispensing step is critical to the dosing accuracy at delivery. The required precision and allowable variability of the delivered dose is generally determined by the clinical safety and efficacy data, that is, the therapeutic window of the drug, regulatory filings, specifications, and the level of required and feasible process control. The latter, in many cases, provides the most stringent requirements to fill parameters. The challenge for the manufacturer might be to find a realistic balance between manufacturing throughput for an effective use of production time and the level of fill volume precision and variability that exceeds the baseline of clinical, quality, and regulatory requirements.

> It must be noted that only the total nominal fill volume can be controlled by the dispensing step. However, the therapeutically relevant parameter is the extractable volume out of a given container according to compendial methods.

To ensure delivery of the labeled dose, overfill in addition to the target dose volume is required in the primary container. The excess fill accounts for nominal volume losses due to dead volumes in the container and delivery system (i.e., liquid volume that will remain inside

the system after the application is completed). For example, a prefilled syringe has only minimal dead volume between the stopper end position and the needle tip (24). For an infusion, the overfill needs to be sufficient to fill the intravenous set and provide for the undrained residue in the container. General guidance regarding the amount of excess volume is provided in the pharmacopeias.

In addition to system losses, the overfill volume is also influenced by the statistical variability of the fill process. In general, the more variable the fill volume, the larger the necessary overfill. The manufacturer might be tempted to focus solely on the optimization of the average fill volume, which is accessible more easily than the distribution. However, by means of statistics, the fill volumes of the individual containers will be more or less broadly distributed around the average fill volume. The extractable volume requirement is directed toward individual fills and not the average volume over a number of containers. Therefore, the added overfill needs to not only ensure sufficient extractable volume from a container filled with average volume but also for individual container fills that reside at the lower end of the fill volume distribution.

The filling into primary containers also presents an elevated potential for extraneous product contamination. In most cases, the dispensing requires the handling of open primary containers and open product solution. This increased exposure risk is aggravated by the fact that contamination originating from this part of the process cannot be removed by subsequent filtration downstream because final bulk filtration is usually performed before dispensing. From a process capability and risk management standpoint, this scenario is especially undesirable because, to avoid administration to the patient, the manufacturer has to solely rely on inspection and quality control mechanisms that will be able to function as safeguards to identify and reject the contaminated units or batches.

Nonetheless, the most stringent precautions with regards to risk of microbial contamination are to be applied to the manufacturing of aseptically filled preparations that are not terminally sterilized. Liquid formulations of biologics are typically sterilized by filtration and aseptically filled into vials or syringes. In a worst-case scenario, contamination of this type of product after sterile filtration can potentially lead to viable organisms residing and under circumstances growing in the product container, which if undetected, is to be considered a critical situation for patient safety.

For the dispensing of various parenteral products, special considerations for the fill process may be necessary. Colloidal disperse systems (e.g., suspensions) require additional attention to maintenance of uniformity through adequate mixing and/or recirculation during subdivision.

Lyophilization

Freeze-drying (lyophilization) is a drying process used for the manufacture of pharmaceuticals, biologicals, serums, and hormones that are thermolabile or otherwise unstable in aqueous solution for prolonged storage periods, but that are stable in the dry state (17). Additionally, lyophilization can improve the dissolution properties of hardly soluble compounds.

By removing the solvent by the physical process of sublimation, heat-sensitive drugs or biologicals can be dried with a minimum of degradation of product. Degradation is minimized by (*i*) reduction of heat input during drying and (*ii*) avoidance of prolonged solution of the drug in liquid solvent during the solvent removal phase. By comparison, an evaporative process requires heat to remove the solvent. The evaporative process continuously makes a more concentrated solution. These two factors can accelerate the degradation kinetics.

Drug product bulk materials for freeze-drying are prepared and sterilized as sterile solutions or sterile suspensions and are filled into containers. Most commonly, glass vials are used, but other containers such as bags are also available. Prior to placing the vials into the chamber, special closures (Fig. 2) are loosely placed into the necks of the vials. The slots in the closures allow solvent vapor to escape from the vials during the drying cycle.

Freeze-drying consists of the three steps: (*i*) freezing, (*ii*) primary drying, and (*iii*) secondary drying. After the desired amount of material is filled into a container, the container is subjected to freezing, and then the drying process is commenced. The product should be cooled to a temperature below its eutectic point (18) and is subjected to extremely

Figure 2 An example of a special closure on the neck of a vial with lyophilized drug.

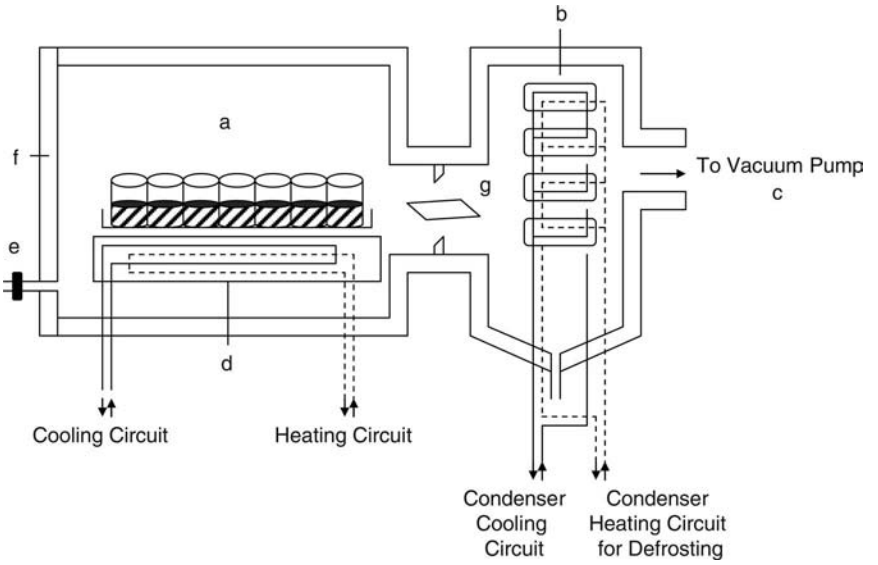


Figure 3 Schematic of a freeze dryer: (a) Drying chamber, (b) condenser, (c) vacuum pump, (d) heating and cooling shelving, (e) aeration valve, (f) loading/unloading door, (g) isolation valve.

low pressures. Under these conditions, the frozen solvent sublimates from the solid directly to the gaseous state. During primary drying, most of the solvent is removed and a "cake" is formed. During secondary drying, which usually requires a small amount of external heat energy input, vestigial solvent is eliminated.

A freeze dryer consists of a drying chamber with shelf space for the vials, a condenser for the sublimation of solvent, a pump for vacuum generation, and an electronic controller equipment (Fig. 3). For the manufacturing of parenteral formulations, the drying chamber is usually accessible from the aseptic working area.

The shelf space consists of metal plates with integrated cooling and heating circuits for freezing, cooling, and heating of the product.

Production-sized freeze driers are usually operated by an automatic control system. The temperature of a sample of the product is continuously monitored throughout the process. The temperature of the sample will steadily drop if no heat is introduced into the system because the vaporization of the solvent results in a removal of heat from the product. Therefore, after equilibrium has been reached, it becomes necessary to introduce heat into the system at a controlled rate. By monitoring the temperature of the sample, the rate of introduction of heat into the system is controlled in comparison with the rate experimentally found to produce a satisfactory product.

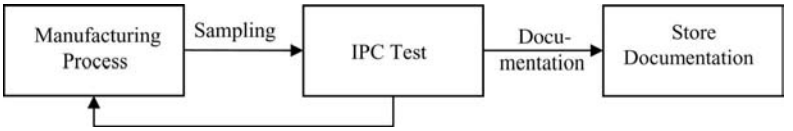
After the drying process is completed, the vials are sealed as rapidly as possible to prevent any sorption of moisture. Some freeze driers are equipped with a mechanism to press the rubber closures firmly into the neck of the vials prior to removal from the chamber.

In-Process Testing

In-process controls comprise all controls performed during a manufacturing process to monitor and control the process to obtain a product within its predefined specification. A sampling plan with the sampling points, number of samples, sampling frequency, place of sample analysis, sampling containers, acceptance criteria, and purpose of the test must be approved and in place before any manufacturing campaign. Samples should be statistically and/or scientifically representative for the manufacturing process.

Sample pull is performed as defined, and samples are either delivered to the appropriate departments or analytics are performed in the manufacturing area and results are documented as described in the sampling plan and SOPs. The results are then checked and the process is either followed as before, if the results are within the specifications, or adapted accordingly. Manufacturing processes can either be interrupted for IPC testing or testing can be performed in parallel and must be defined in the respective manufacturing instructions. Sampling equipment must be suitable and clean.

All steps of the manufacturing process are monitored with in-process control samples: preparation of excipient or bulk solution (e.g., temperature of WFI, pH, density, and osmolality of the solution), filtration pressure, hold times, and filling parameters such as fill volumes (vials and prefilled syringes) and stopper positions (for prefilled syringes). Parameters investigated also include environmental monitoring, for example, room temperature, pressure, humidity, and status of particles in the filling areas. In addition, microbial monitoring of the personnel, manufacturing area, and filling equipment is conducted.



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15 Freeze-drying: principles and practice

Steven L. Nail and Larry A. Gatlin

INTRODUCTION

Freeze-drying, or lyophilization, is a critical unit operation in pharmaceutical development and manufacturing because it allows removal of water from heat sensitive materials at low temperature, thereby avoiding thermal damage caused by more traditional drying operations. Freeze-drying is most important in production of injectable pharmaceuticals, but also finds application in manufacture of diagnostics and in certain solid oral dosage forms where rapid disintegration and dissolution is critical. In addition to allowing removal of water at low temperature, freeze-drying under appropriate conditions generally results in a solid material with a relatively high specific surface area, which facilitates rapid, complete dissolution. This is a critical quality attribute for drugs administered in emergency situations. Finally, freeze-drying is more compatible with sterile operations than filling a solid powder into vials. The solution can be sterile filtered immediately before filling, and fill weights of liquids dispensed into the primary container can be more consistently controlled than filling of dry powders. Filling of a solution into vials also avoids potential problems with cross-contamination through airborne particulate matter, as well as exposure of workers to potentially hazardous drugs.

Freeze-drying as an industrial process was introduced at around the time of World War II for production of freeze-dried human plasma, followed by manufacture of antibiotics, steroids, and injectable vitamins. The application of freeze-drying to manufacture of injectable products has continued to grow, particularly with the advent of biotechnology-based therapeutics. According to data from the Pharmaceutical Research and Manufacturer's Association, at least 165 biotechnology-derived therapeutic agents have been approved since the first such product, Humulin®, was approved in 1982. As of the end of 2008, there were 663 new medicines in development. Of these, 223 were vaccines, 192 were monoclonal antibodies, and 66 were recombinant proteins. While certainly not all of these dosage forms will be freeze dried, proteins are often either chemically or physically unstable in solution. This makes freeze-drying an essential step in the manufacture of many of these products.

Freeze-drying takes place because of sublimation, where water converts from the solid state to the vapor state without first becoming a liquid. This can only occur below the *triple point* of ice, which is at a temperature of about 0°C and a pressure of about 4.5 mmHg, or 4.5 torr. This pressure refers to the partial pressure of water vapor, not the total system pressure, so sublimation of ice can take place at atmospheric pressure as long as the partial pressure of water vapor is less than about 4.5 torr. Everyday examples of sublimation of ice include ice cubes shrinking over time in the home freezer, as well as "freezer burn" caused by local sublimation in frozen food products. However, these processes are very slow. To be a practical process commercially, the system pressure must be maintained below the vapor pressure of ice, so that water vapor is transported by bulk vapor flow from a region of high pressure (the ice surface) to a lower pressure maintained in the freeze dry chamber by means of a condenser operating at temperatures in the range of −60 to −80°C.

Freeze-drying has some important limitations. Often the physical state of a drug; that is, whether it is crystalline or amorphous, is critical in determining its stability as a solid. If a drug does not crystallize from a freezing system, and the amorphous solid is too unstable to provide an acceptable shelf life, then freeze-drying may not be feasible. In addition, freeze-drying is an inefficient and expensive process, both in terms of capital cost and operating expenses. This arises primarily from the high heat input required to sublime the ice (about 2800 J/g) and the fact that heat must be applied to an evacuated system, making for very poor heat transfer. Therefore, it is important for pharmaceutical development scientists and engineers to develop processing conditions that maximize efficiency and avoid consuming freeze-drying plant capacity unnecessarily.

The purpose of this chapter is to review the scientific and engineering principles important to freeze-drying and to present an overview of practical considerations important to both formulation development and manufacture of freeze-dried parenteral products.

PROCESS OVERVIEW

Nearly all freeze-dried injectables are aseptically processed (as opposed to being terminally sterilized), where the required volume of liquid is filled into previously washed, sterilized, and depyrogenated glass vials. Special elastomeric closures with slots allowing the escape of water vapor (called a "lyostopper", see Fig. 1) are then partially inserted into the neck of the vials, and the vials are transported from the filling/stoppering line to the freeze dryer. Many modern production operations avoid placing vials in trays with a bottom because this introduces variability in heat transfer. Instead, either removable tray bottoms are used when trays of filled vials are transported manually or an automatic loading/unloading system is used. In either case, vials sit directly on the shelf of the freeze dryer.

The basic features of a freeze dryer are shown schematically in Figure 2. The basic components of the freeze dryer are a heat transfer system for removing and applying heat to the product, a condenser to collect the water vapor from the product, and a vacuum system. The shelves of the freeze dryer contain internal channels allowing the flow of a heat transfer fluid, usually silicone oil, to control the temperature of the shelf. Freeze dryers for injectable products also have sterilization systems for the chamber and condenser, and commonly have internal spray nozzles to clean the system in place.

It is general practice during development of a freeze-dried injectable to place a temperature-measuring device (usually a thermocouple) in several vials of product to monitor the status of the product throughout the process (Fig. 3). The product is first frozen to a low enough temperature to allow complete solidification of the product. The chamber is then evacuated to a pressure lower than the vapor pressure of ice (Table 1). For example, the vapor pressure of ice at $-40^{\circ}C$ is about 96 millitorr (mT). In order for sublimation to take place at an appreciable rate, the chamber pressure must be lower than this pressure. After the required pressure is reached and the condenser is cooled, heat is applied to the shelves to provide the heat of sublimation of ice. This phase is called *primary drying*, where ice in the frozen material sublimes and flows through the porous bed of partially dried product into the headspace of the vial, out the open slot in the lyostopper, and ultimately collecting on the condenser. Primary drying is characterized by a visible sublimation front that recedes from the top to the bottom of the frozen layer. Product temperature usually increases at a slow rate during primary drying, since the heat supplied by the shelf provides the heat of sublimation of ice. When primary drying is complete, the process is usually not over because, in most real formulations, not all of

Figure 1 Freeze-dried products use a special stopper with a slot that is open when the stopper is in the partially seated position to allow escape of water vapor. The stack of shelves in the freeze dryer is compressed at the end of the cycle to force the stopper into its fully seated position.

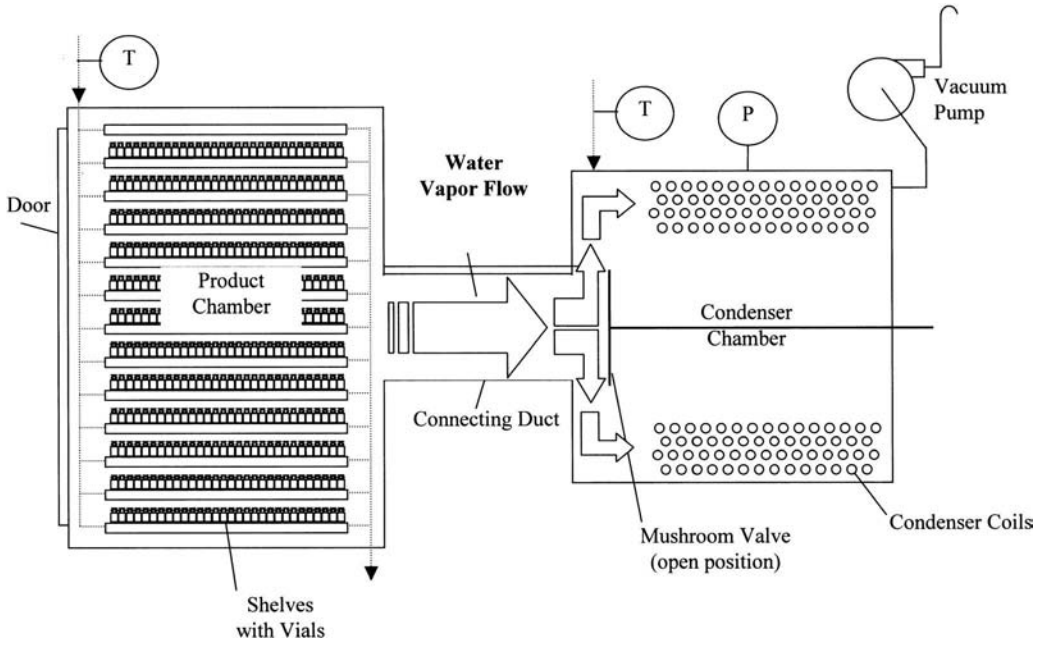

Figure 2 Schematic of a pharmaceutical freeze dryer.

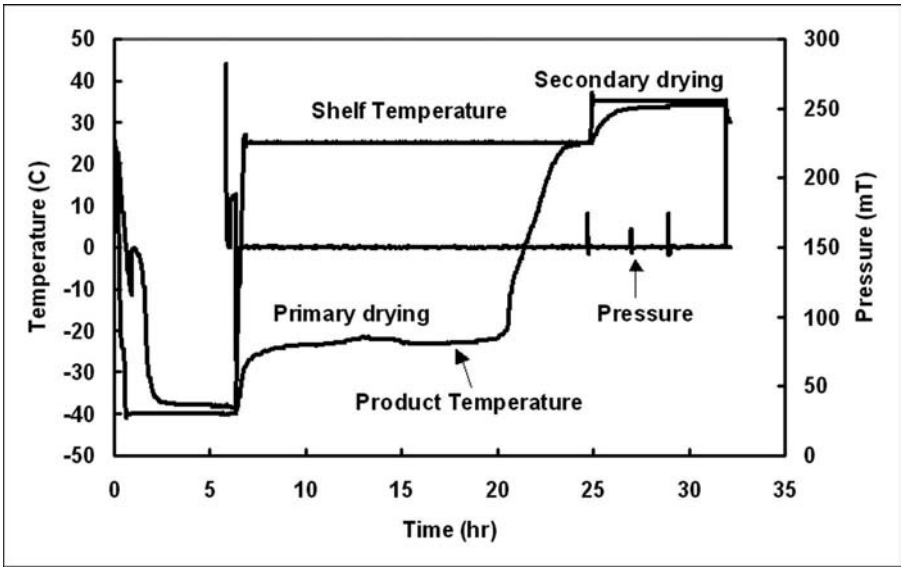

Figure 3 Primary and secondary drying stages of a freeze dry cycle as indicated by the product temperature.

the water freezes. This unfrozen water is removed during *secondary drying*. In secondary drying, ice is no longer present to use the energy provided by the shelf, and the product temperature increases relatively rapidly toward the shelf temperature. As the secondary drying process ends, the product temperature approaches a steady-state value near the shelf temperature.

When the product is sufficiently dry, the stoppers, which were partially inserted after filling the vials, are inserted into the completely seated position by means of a hydraulic

Table 1 Vapor Pressure of Ice

Temperature (°C)	Pressure (torr)	Temperature (°C)	Pressure (torr)
−2	4.58	−26	0.430
−4	3.86	−28	0.351
−6	2.76	−30	0.286
−8	2.33	−32	0.232
−10	1.95	−34	0.187
−12	1.63	−36	0.151
−14	1.36	−38	0.121
−16	1.13	−40	0.096
−18	0.939	−50	0.029
−20	0.776	−60	0.009
−22	0.640	−70	0.002
−24	0.526		

system that compresses the stack of shelves. It is common practice to stopper the vials while the chamber is still under at least partial vacuum, which aids in seating the stoppers and facilitates reconstitution. It is common to "backfill" with nitrogen or another inert gas prior to fully seating the stoppers.

The most important objective in developing a freeze-dried product is to assure the quality requirements are met not only initially but throughout the shelf life of the product. These quality attributes include complete recovery of the activity of the product after addition of water (called *reconstitution*), reconstitution time, freedom from extraneous particulate matter, sterility, the absence of pyrogens, and residual water content. In addition, however, process conditions should be chosen to maximize process efficiency. Freeze-drying often requires two to three days from the start of freezing to the completion of secondary drying. Success in meeting the quality requirements of the product as well as minimizing inefficiencies in the process requires a good understanding of formulation of freeze-dried products, physical chemistry of freezing, the principles of heat and mass transfer, and process monitoring. These topics will be covered in more detail below.

FORMULATION OF FREEZE-DRIED PRODUCTS

A good general rule in developing a formulation of a freeze-dried pharmaceutical product, or any product for that matter, is to keep the formulation as simple as possible, and to not include any component without a clear rationale for doing so along with supporting data. It is important to have a clear idea of the critical quality attributes of the product before beginning. Some attributes are obvious, such as sterile, nonpyrogenic, and compliant with compendial requirements for visible and subvisible particulate matter. Complete recovery of the activity present in the formulation prior to freeze-drying is always desired but may not always be possible. Vaccines, for example, tend to lose some potency as a result of freeze-drying, but the critical factor here is consistency of activity in the reconstituted solution. Dissolution of the freeze-dried cake should be complete, and the reconstitution time should be as fast as possible. Some quality attributes may, or may not, be critical depending on the intended route of injection. For example, it is always desirable for a formulation to be isotonic (the same osmotic concentration as normal physiological fluid). However, this attribute is only critical for certain routes of administration such as intraspinal, intraocular, or into any part of the brain. The same consideration applies to the pH of the formulation, where it is always desirable to have the formulation pH the same as normal plasma, but the reality is that the pH of injectable formulations varies widely as required to achieve suitable solubility and stability in solution. This applies to antimicrobial preservatives as well, but their presence in freeze-dried formulations is rarely justified.

Preformulation Considerations

The amount of drug per vial for freeze-dried products may vary from a few micrograms to two or more grams. At low doses, bulking agents are used so that the drug is uniformly dispersed

in a pharmaceutically acceptable solid matrix, in which case the freeze-drying characteristics of the formulation can be determined by the bulking agent. High doses can be more challenging, since the drug will probably determine the freeze-drying characteristics of the formulation. The total amount of dissolved solids should not be too low, or too high. If the dissolved solids content in the pre-freeze-dried solution is too low, the appearance of the cake may not be acceptable and, more importantly, the dried solids may be so friable that powder is ejected from the vial during primary drying. If the dissolved solids concentration is too high, this may lead to difficulty in process control if the resistance of the dried product layer to the flow of water vapor is too high, where a high resistance is indicated by a rapid increase in product temperature during primary drying. As a very rough guideline, the formulation scientist should aim for a total dissolved solids concentration somewhere in the range of 25 to 150 mg/mL.

The quantity of drug, along with its solubility, determines the feasibility of administering the required dose in the appropriate volume of solution, and the required volume is determined by the intended route of injection. For continuous IV administration, there is no upper volume limit as long as the volumetric rate of infusion does not exceed the ability of the kidneys to eliminate the excess volume of water. For IV bolus administration, the injected volume is generally 10 mL or less. For intramuscular administration, up to about 5 mL is injected and for subcutaneous administration, the injected volume is up to about 1.5 mL. Of course, many drugs are weak acids and bases, where the solubility (and often solution stability) is strongly influenced by pH, so both a solubility versus pH profile and a stability versus pH profile are needed over a reasonable formulation pH range.

Some information is necessary on both the routes, and the rates, of chemical degradation in solution. If this were not an issue, then there would be no need for freeze-drying. If the drug degrades too rapidly in solution, then degradation during compounding, sterile filtration, filling, and transfer to a freeze dryer can present a significant challenge. For protein therapeutic agents, physical stability must be examined in solution as well, where physical stability generally refers to the tendency of proteins to form aggregates, either soluble or insoluble. This can occur either spontaneously in solution or as a result of denaturation in response to adsorption to solid surfaces such as tubing or filters or adsorption to the air-water interface present during processing.

EXCIPIENTS IN FREEZE-DRIED PRODUCTS

No formulation scientist wants to risk delay of an NDA submission by using unprecedented excipients, so the choices of excipients, particularly relative to those available for solid oral dosage forms, is limited (1). Below is a brief survey.

Buffers

By far the most common buffer system in freeze-dried parenterals is sodium phosphate, since it is present physiologically and has a pK near the pH of normal plasma. A risk associated with freeze-drying of solutions containing sodium phosphate is pH shifts with freezing, discussed in the following text. Other buffer systems used in approved products include acetate, citrate, arginine, histidine, succinate, and Tris (tris-hydroxymethyl aminomethane).

There is no "rule" that buffers must be included in a formulation. If no buffer is needed, then it is appropriate not to use one.

Bulking Agents

Bulking agents, mentioned earlier, are needed when the drug quantity is insufficient to form a pharmaceutically acceptable freeze-dried solid, and the drug is dispersed in an inert matrix that has appropriate freeze-drying characteristics. Bulking agents fall into two general categories—those that tend to crystallize from a frozen system and those that remain amorphous. The most common crystallizing excipients are mannitol and glycine. Polyethylene glycols, which are less common, also tend to crystallize from freezing solutions. Whether these excipients actually crystallize depends largely on their concentration relative to other formulation components and, to a lesser extent, on the thermal history of freezing. Crystallizing excipients

have the advantage of allowing freeze-drying at relatively high product temperatures (see discussion later in this chapter), which provides more efficient processing.

Mannitol is known to, in some cases, promote vial breakage during freeze-drying (2). In addition to causing loss of yield of acceptable product, this can create problems with containment of cytotoxic compounds. The detailed mechanism by which this happens is not well understood, but important contributing causes include the relative concentration of mannitol in the formulation and the relative fill depth. Rates of breakage increase significantly when the relative fill volume exceeds about one-third of the capacity of the vial. Thermal history of freezing has been shown to influence vial breakage as well. The vial specifications may also play a role—particularly the heel radius, where the side wall joins with the bottom of the vial. The smaller the heel radius, the more the stress associated with expansion of the frozen system during freezing is concentrated, which in turn promotes vial breakage.

Amorphous excipients include disaccharides such as sucrose, trehalose, and, occasionally, lactose or maltose. These excipients may play a double role in a formulation both as a bulking agent and, for proteins and other biologicals, as a stabilizer. To be effective, the stabilizer must be amorphous and remain so throughout the shelf life of the product. Human serum albumin is used in several protein formulations both as a stabilizer of the protein in the solid state and as a competitive surfactant to inhibit loss of the active protein by adsorption to surfaces. A detailed discussion of mechanisms of stabilization of biological formulations is outside the scope of this chapter, and the reader is referred to publications by Carpenter et al. (3), Arakawa et al. (4), Cleland et al. (5), and Chang et al. (6).

Added Salts

Salts such a sodium chloride are often included in freeze-dried formulations to provide an isotonic reconstituted solution. Their use should be approached with some caution, however, for several reasons. First, when used in combination with amorphous excipients, added salt tends to decrease the collapse temperature (discussed later), making the process less efficient and, in some cases, increasing the risk of not being able to make a pharmaceutically acceptable product (7). Added salt may also inhibit crystallization of components of the formulation for which crystallization is needed. When added salt is needed in a formulation, it is particularly important to systematically vary the amount of salt and study the effect of salt concentration on the freeze-drying characteristics of the formulation.

THE FREEZING PROCESS

Freezing is a critical step in the freeze-drying process, since the physical state of the frozen system influences quality attributes of the final product as well as the process efficiency. Characterization of freezing behavior is an important step in the development of a freeze-dried product for several reasons. First, the driving force for freeze-drying is the vapor pressure of ice, and the vapor pressure of ice is very temperature dependent. A goal of process optimization is to carry out freeze-drying at the highest rate possible without causing damage to the product. Every frozen formulation has an upper temperature limit (more about this below) during the primary drying process, and it is important to know this upper temperature limit and use it in process development so that the product temperature remains safely below this limit during primary drying, but not so far below the limit as to make the process unnecessarily time consuming. Second, process validation involves assuring that the cycle conditions are appropriate for the formulation. To be able to validate a process, "benchmark" data must be available to assess the adequacy of the process conditions, and these data are provided by characterization of the formulation. Finally, there is ongoing regulatory scrutiny of the pharmaceutical development process as well as manufacturing operations. Regulatory authorities expect a scientific rationale for freeze dry cycle conditions, with appropriate documentation.

Types of Freezing Behavior

An overview of the events that can take place during freezing is shown by the diagram in Figure 4. For simplicity sake, it is assumed that the starting solution is a simple aqueous system. It is important to recognize that even pure water does not freeze at 0°C, but instead

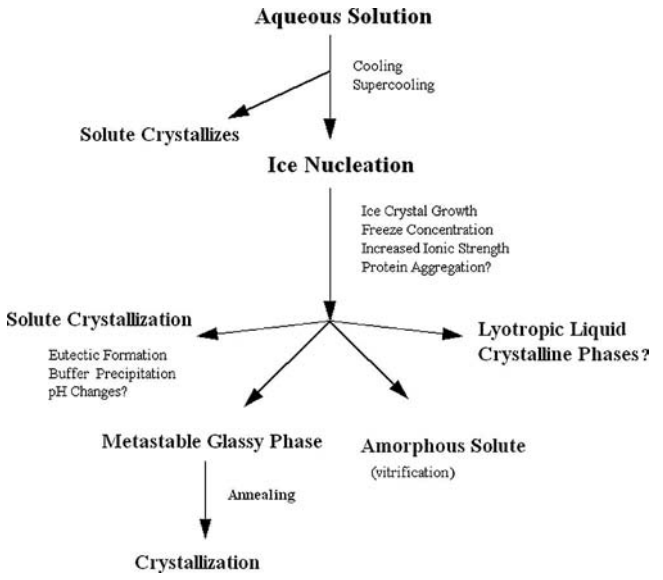

Figure 4 Schematic diagram of events taking place during freezing.

undergoes *supercooling*, where the water remains liquid well below the melting point of ice. Supercooling is important because the actual *freezing rate* of a solution is determined by the time elapsed between nucleation of the first ice crystals and complete solidification of the system. This is sometimes confused with the *cooling rate*, which is the rate at which the temperature of the shelves is decreased during freezing.

Ice nucleation, like any crystallization process, can be either *homogeneous*, where water molecules spontaneously order themselves into nuclei, or *heterogeneous*, where nucleation is triggered by a surface or by extraneous particulate matter. In practice, nucleation is always heterogeneous. The solutions we deal with in freeze-drying have been sterile filtered, and the containers (usually glass vials) have been cleaned and sterilized, so there is relatively little in the way of extraneous particulate matter to trigger ice nucleation. Nevertheless, there are still irregularities in the microstructure of the glass that can serve as nucleation sites, but aqueous solutions intended to be freeze-dried can supercool by as much as 12 to 15°C before ice crystals nucleate. High degrees of supercooling result in rapid freezing, which in turn influences ice crystal morphology and the amount of water in the system that remains unfrozen.

It is important to first consider ice morphology before considering the behavior of components of a formulation during freezing. Different ice morphology, including regular and irregular dendrites, as well as spherulitic systems (thin fibers if ice radiating outward from the nucleation site), forms during freezing, depending on the freezing rate and the type and concentration of solutes present. Ice crystal morphology and size distribution have been shown to influence the rates of primary (8) and secondary drying, as well as protein aggregation in freeze-dried protein formulations. Searles et al. (8) describe three stages of the freezing process. The first is the heterogeneous nucleation of ice or primary nucleation. This is followed by secondary nucleation, during which a visible front propagates through some portion of the sample at a rate on the order of several millimeters per second. This process stops as the temperature of the system approaches the equilibrium freezing temperature. Secondary nucleation is followed by solidification, which takes place at a slower rate as the heat released by ice crystallization is conducted out of the sample and ultimately to the heat transfer fluid. These investigators further describe two different freezing mechanisms. In the first, termed *global supercooling*, the entire liquid volume reaches the same degree of supercooling and the secondary nucleation zone includes the entire solution volume. In *directional solidification*, a portion of the liquid volume is cooled to the point of primary and secondary nucleation, and the nucleation and solidification fronts move together into the previously unnucleated portion of the solution. Both mechanisms can apply to the type of

freezing that takes place in pharmaceutical freeze-drying; that is, vials filled with liquid that are frozen on the freeze dryer shelf. However, directional solidification usually requires some type of ice nucleating agent. The freezing mechanism was demonstrated to be reflected in the morphology of the freeze-dried cake.

Most people do not think of freezing as a dehydration process, but it certainly is in the sense that, when the water freezes, it becomes a separate phase, and the material in the interstitial space between ice crystals becomes much more concentrated. If a solution of normal saline is frozen for example, the sodium chloride concentration in the initial solution is 0.15 N. When this solution is frozen, the sodium chloride concentration in the interstitial space between ice crystals reaches nearly 4 N before sodium chloride precipitates from the freeze concentrate. In the case of formulations containing sodium chloride or other salts, this high ionic strength environment can be damaging, particularly to biological materials such as proteins and cells.

A main point of Figure 4 is that both freeze-drying behavior and quality attributes of the product are determined by the physical state of the solute, or solutes, in the frozen system. For the sake of simplicity, we will assume a single solute dissolved in water. After ice crystal growth has essentially completed, and the solute has been concentrated as much as possible in the interstitial space between ice crystals, what happens next depends on whether the solute crystallizes from this freeze concentrate.

Solute Crystallizes from the Freeze Concentrate

When the solute crystallizes from the freeze-concentrated solution, the phase behavior is represented by a temperature-composition phase diagram as shown in Figure 5. In a simple solution, 100% A represents pure water and 100% B represents pure solute. We will use sodium chloride as B in this example. The curve AC represents the melting point of ice as a function of sodium chloride concentration, and the curve BC represents the solubility of sodium chloride in water as a function of temperature. If the system is cooled to a temperature below point C, the solute is no longer soluble, and it crystallizes and precipitates. This is the *eutectic* (from Greek, meaning "easily melted") *composition*, and the temperature corresponding to C is the *eutectic melting* temperature.

What the phase diagram tells us about freeze-drying of a solution of sodium chloride in water is the following. Starting with a solution of sodium chloride in water, say at point x in Figure 5, and cooling this solution to perhaps −40°C, we follow a vertical line on the phase diagram. Below the freezing point curve, we have a two-phase system—ice and a freeze-concentrated solution of sodium chloride. In the two-phase region, as the temperature is

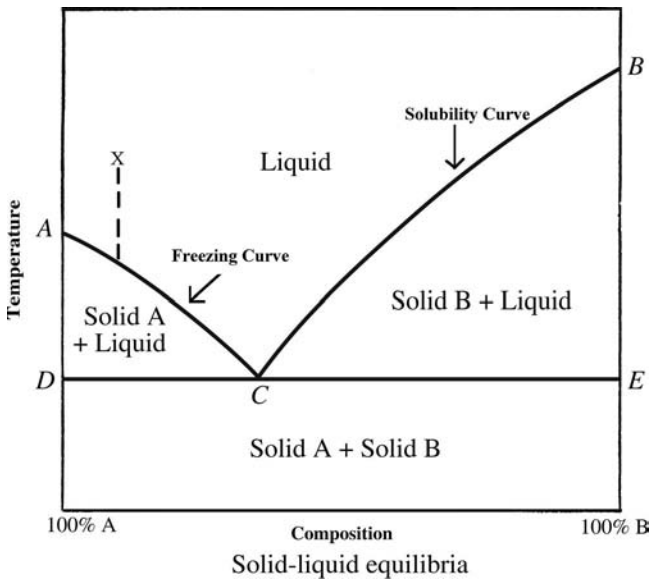

Figure 5 Temperature-composition phase diagram of a binary system.

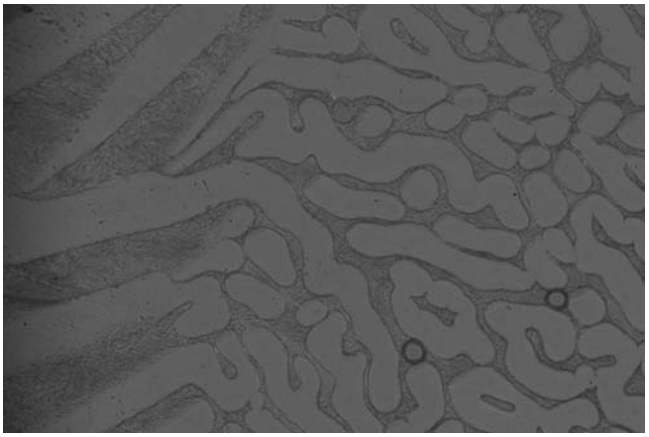

Figure 6 (*See color insert*) Photomicrograph of a frozen solution of sodium chloride in water.

decreased, the ice crystals grow and the freeze concentrate becomes more concentrated. At any temperature in this region, the composition of the system is given by a horizontal line (called a "tie line") through this temperature. Thus, the composition of the freeze concentrate is given by the line AC. When this concentration reaches point C, a eutectic mixture of sodium chloride and ice precipitates form the freeze concentrate. It is only at this point that the system is completely solidified. A photomicrograph of a frozen sodium chloride solution is shown in Figure 6, where the dark material in the interstitial space between ice crystals is the eutectic mixture. This mixture consists of ice and crystalline sodium chloride. The reason that these crystals cannot be seen in the photograph is that the crystallite size is too small to be resolved by an optical microscope.

In reality, the crystallization of solute is just as unpredictable as the crystallization of water. While it cannot be shown on an equilibrium phase diagram, supercooling in these systems occurs twice—once prior to crystallization of ice, and again prior to crystallization of the eutectic mixture. Both events require nucleation, which is a stochastic process.

Eutectic mixtures melt at a sharply defined temperature, as if they were a single, pure compound. The significance of the eutectic melting temperature to freeze-drying is that it represents the maximum allowable product temperature during primary drying. Exceeding this temperature during the process would result in puffing, foaming, perhaps expulsion of solids from a vial, and loss of pharmaceutical acceptability. A list of eutectic melting temperatures of some pharmaceutically relevant materials is shown in Table 2. However, despite the attention that eutectic solidification has attracted in the literature and the considerations given to it in this text, it is not seen with most formulations. The most common behavior is formation of a glassy mixture, discussed in the following text.

Eutectic crystallization is the underlying cause of a phenomenon in freezing of formulations intended for freeze-drying that is worthy of mention. Solutions of sodium phosphate, the most common buffer in freeze-dried formulations, are known to undergo a pH shift accompanying freezing, such that the effective pH in the freeze concentrate formed

Table 2 Eutectic Melting Temperatures of Representative Materials

Material	Eutectic melting temperature (°C)
Mannitol	About −1
Glycine	−3.5
Sodium phosphate dibasic	−0.5
Polyethylene glycols	−14 to −16
Sodium chloride	−21.5
Citric acid	−12
Potassium chloride	−10.7
Sodium acetate	−18

during freezing can be significantly lower than that of the starting solution. This happens because the dibasic buffer salt is less soluble at low temperature than the monobasic salt. Crystallization of the dibasic sodium phosphate causes the equilibrium to shift according to LeChatlier's Principle, resulting in decreased pH. Gomez and Rodriguez-Hornedo (9) used a special pH electrode designed to withstand freezing to study the influences of initial buffer solution pH and concentration on subsequent pH changes during freezing, as well as the influence of other species in solution on buffer salt crystallization. These investigators reported that the pH changes associated with crystallization of a sodium phosphate buffer solution initially at pH 7.4 are directly related to the initial concentration of buffer in the range of 8 to 100 mM. Further, the lower the initial pH of the buffer, the higher the observed pH at -10°C. Addition of NaCl increases the ion product of dibasic sodium phosphate, thereby leading to larger pH changes. Solutes such as sucrose and mannitol inhibited crystallization of buffer species, resulting in smaller pH shifts upon freezing. The presence of sucrose and mannitol at concentrations above 3 moles per mole of dibasic sodium phosphate completely prevented buffer salt crystallization. In this case, the pH change upon freezing was only 0.5 units, which was attributed to the effect of freeze concentration. It is worth emphasizing that pH shifts only occur when the dibasic sodium phosphate salt crystallizes, so just because a formulation contains sodium phosphate does not mean that the pH will shift during freezing. Other components of formulation, particularly those that remain amorphous during and after freezing, as well as rapid freezing rates, tend to inhibit this crystallization.

Other pharmaceutically relevant buffer systems have not been as well characterized as phosphate with respect to pH changes accompanying freezing. Larsen (10) reported that acetate, citrate, glycine, and Tris show only small pH shifts upon freezing.

Many drugs crystallize readily from freezing aqueous solutions, including sodium ethacrynate, pentamidine isethionate, nicotinamide, ribavirin. Common excipients that readily crystallize, in addition to sodium chloride and sodium phosphate dibasic, are mannitol, glycine, and polyethylene glycols.

Solute Remains Amorphous After Freeze Concentration

Again, to simplify the discussion, we are considering aqueous systems containing only one solute. Consider sucrose, a common excipient in freeze-dried protein formulations. In this case, the first part of the process is the same; that is, ice crystals nucleate after considerable supercooling and grow. The freeze concentrate becomes more concentrated in sucrose and more viscous. However, unlike sodium chloride, sucrose does not crystallize from the freeze concentrate regardless of the freezing temperature used, and its behavior cannot be described by an equilibrium phase diagram. The glassy mixture resulting from freeze concentration does undergo a *glass transition* as the temperature decreases, where the viscosity of the mixture may increase by orders of magnitude over a temperature range of a few degrees. This glass transition is a reversible change of state (it is not a phase change) between a viscous liquid above the glass transition to a solid below the glass transition. The glass transition temperature of the maximally freeze-concentrated solute, known as T_g', is the physical chemical basis for *collapse* in freeze-drying. If the temperature of the product is held below T_g', the glassy mixture of solute and unfrozen water is rigid enough to support its own weight as the supporting structure of ice crystals is sublimed away. This results in retention of the microstructure that was established by the freezing process. If, however, the temperature of the system is increased above T_g' during primary drying, the glassy mixture of solute and water can undergo viscous flow under the force of gravity when ice is sublimed, a phenomenon known as collapse.

A pharmaceutically acceptable freeze-dried solid generally has the same size and shape as the liquid that was originally filled into the vials, and has a uniform color and texture. These qualities are generally lost as a result of collapse. In addition, collapse results in a decrease in the specific surface area of the freeze-dried solids, and this can result in longer reconstitution time relative to a system that retains the microstructure established by freezing. Perhaps more importantly, collapsed systems tend to have higher levels of residual moisture, perhaps because of decreased surface area available for evaporation of the water that was part of the glassy mixture. This, in turn, can adversely influence stability of the freeze-dried solid. A

Figure 7 The vial on the left shows complete collapse and the second vial from the left is an example of partial collapse. The two vials on the right are pharmaceutically acceptable.

photograph of a vial exhibiting collapse is shown in Figure 7, and a list of T_g' temperatures of representative materials is given in Table 3. It is important to recognize that both T_g' and collapse temperatures are more subjective measurements than, for example, melting temperatures. This is discussed further in the section later on dealing with characterization of frozen systems.

Figure 8 is a cartoon intended to illustrate the concepts discussed above. There are important differences in freeze-drying behavior between systems where the solute crystallizes and those where it remains amorphous. First, when the solute crystallizes, nearly all of the water in the system is frozen, either as pre-eutectic ice or ice that is part of the eutectic mixture. This means that there is very little secondary drying required. Amorphous systems, on the other hand, contain a significant amount of unfrozen water. Maximally freeze-concentrated sucrose, for example, contains about 20% unfrozen water, which requires removal during secondary drying. Second, eutectic melting temperatures of most pharmaceutically relevant materials tend to be fairly high—in the range of $-1^{\circ}C$ to about $-15^{\circ}C$. Glass transition temperatures, on the other hand, vary over a much wider range (Table 3), and can be so low that the system cannot be completely solidified in a freeze dryer, where the lowest temperature achieved on the shelf is seldom below about $-45^{\circ}C$.

In addition to different behavior during freeze-drying, the physical state of the drug can dramatically influence the stability of the freeze-dried solid. Amorphous drugs can undergo solid-state degradation at substantially higher rates than the same drug as a crystalline solid (11).

Of course, actual formulations usually consist of several components, so it is not uncommon to see both types of behavior within the same formulation, where there is a crystalline component and an amorphous component. In this case, the freeze dry cycle conditions must be based on the lowest of either the eutectic melting temperature or the collapse temperature. This is usually the collapse temperature.

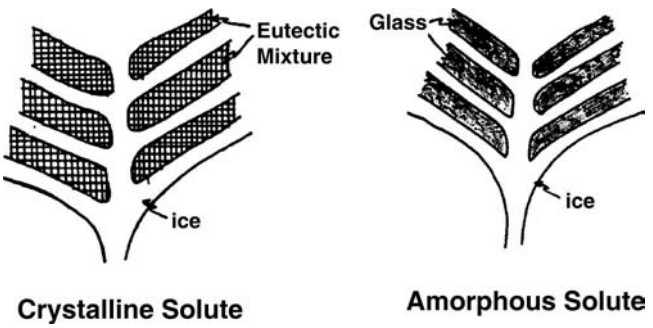

Figure 8 Cartoon showing the microstructure of a frozen systems in which the solute is crystalline and amorphous.

Table 3 T_g' Temperatures of Representative Materials

Material	T_g'
Dextran	−9°
Fructose	−48°
Gelatin	−8 to −10°C
Sucrose	−32° to −34°
Lactose	−32°
Maltose	−32°C
Trehalose	−30°
Albumin	−10°
Glycine (amorphous)	<−60°

Solute Forms a Metastable Glass

Sometimes, perhaps because of a high degree of supercooling and a subsequent rapid freezing rate, a compound will first form a glassy mixture in the interstitial space between ice crystals; however, with some subsequent heating, the solute will crystallize from this glassy mixture. Mannitol is the most common example of metastable glass formation. For this reason, *annealing* is sometimes used in a freeze-drying process. Annealing is simply heating the "frozen" system after the initial freezing process—not enough to melt the product, but enough to promote crystallization of components of the formulation that have initially formed glassy mixtures. Typically, an annealing step would consist of heating the frozen system to a temperature higher than T_g' but lower than the onset of melting, and holding for two to three hours. Gatlin and DeLuca (12) investigated three cephalosporins that all form glassy mixtures upon initial freezing and, unless annealed, remained in the less desirable amorphous form after freeze-drying.

Solute Forms a Lyotropic Liquid Crystal

States of matter which have degrees of order intermediate between amorphous and crystalline are called liquid crystals. Liquid crystals are broadly categorized as *thermotropic*, which are formed by heating, and *lyotropic*, which are formed by addition of solvent to a solid. Compounds that form liquid crystals are generally surface active, and the liquid crystal represents a more ordered structure than a micelle. These higher-ordered structures are a result of freeze concentration and may be either lamellar or rod shaped. There have been few reports of lyotropic liquid crystal formation in aqueous solutions of drugs, and even fewer that are relevant to freeze-drying. Powell and co-workers (13) reported peptide liquid crystal formation by the luteinizing hormone releasing hormone deterelix and the effect of added salts on thermodynamic stability of the liquid crystal phase. Vadas et al. (14) reported that a leukotriene D_4 receptor antagonist forms lyotropic liquid crystalline phases when lyophilized from aqueous solution. Bogardus (15) studied the phase equilibria of nafcillin sodium-water and reported a lamellar mesophase in aqueous solutions containing more than 55% nafcillin sodium. Milton and Nail (16) extended this work by characterizing the low-temperature differential scanning calorimetry (DSC) thermogram of frozen aqueous solutions of nafcillin as well as the freeze-dried solid. Freeze-drying of frozen systems containing lyotropic mesophases appears to result in a unique x-ray diffractogram consisting of a single sharp peak at low angle (less than about 5° 2θ) in addition to the "halo" that is characteristic of amorphous solids. Herman et al. (17) reported a similar x-ray powder diffraction pattern in methylprednisolone sodium succinate. The influence of liquid crystal formation during freezing on critical quality attributes of freeze-dried products is a subject that remains largely unexplored.

Characterization of Freezing Behavior

The purpose of characterizing the freezing behavior of a formulation intended for freeze-drying is primarily to determine the maximum allowable product temperature during the primary drying phase as well as to gain insight into the physical state of the material during

and after freeze-drying. While a variety of methods have been reported in the literature, we will briefly describe the two most common characterization methods—low-temperature thermal analysis and freeze dry microscopy.

Thermal Analysis

Thermal analysis of frozen systems intended for freeze-drying has become a standard tool for formulation and process development (18–20). Physical or chemical changes in a material occurring with changes in temperature are accompanied by the absorption or release of energy in the form of heat. Thermal analysis measures the temperatures at which these transitions occur, as well as the energy associated with the transitions and whether they are endothermic (absorption of energy) or exothermic (release of energy). The types of transitions observed in frozen formulations are illustrated in Figure 9. These are only three. The glass transition of the maximally freeze-concentrated solute is observed as a shift in the baseline toward higher heat capacity. Eutectic melting is an endothermic peak, and crystallization of a formulation component during the time course of the DSC experiment is observed as an exotherm. Thus, interpretation of DSC thermograms of frozen systems is simple in principle. However, several factors contribute to uncertainty in interpretation of the DSC data. First, eutectic melting may take place at a temperature very near the melting endotherm of ice; for example, eutectic mixtures of mannitol/ice and dibasic sodium phosphate/ice undergo eutectic melting at about -1 and -0.5°C, respectively. These endotherms are not resolved from the melting of ice. Therefore, resolution of eutectic melting from ice melting can be a significant source of uncertainty. Regarding the glass transition, in some cases the heat capacity change is too small to be detected by DSC with certainty, so sensitivity can become an issue. It should also be noted that glasses are broadly defined as *strong* or *fragile*. This does not refer to mechanical properties directly, but rather to the temperature dependence of molecular mobility in the region of the glass transition. Fragile glasses have relatively narrow glass transition regions and relatively high heat capacity change associated with the glass transition. Strong glasses are the opposite—they have broad glass transition regions and small heat capacity change associated with this transition. Therefore, the glass transition can be difficult to detect for strong glasses. Fortunately, most pharmaceutically relevant amorphous materials (with the exception of proteins) are fragile glasses. Interpretation of low-temperature DSC thermograms can also be more uncertain in formulations containing many components, probably because of interactions between components. Finally, the glass transition region, particularly for systems containing more than about 10% of an amorphous solute, may be observed as more than a

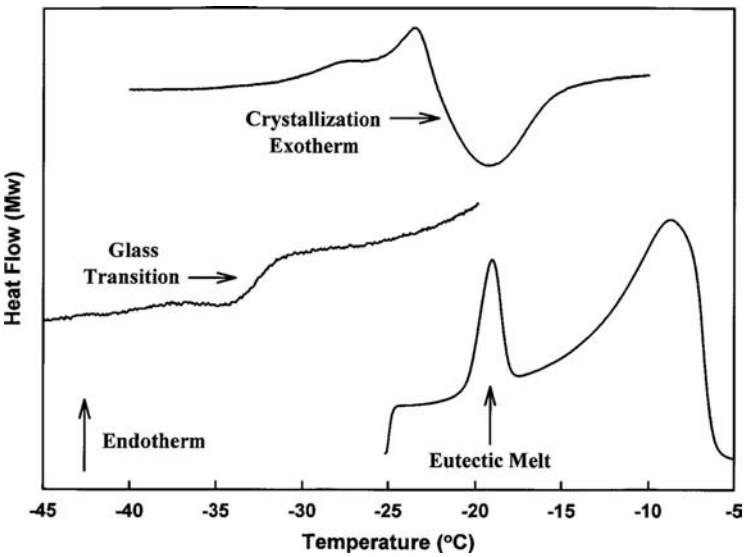

Figure 9 Thermal transitions occurring in frozen systems.

single transition. Sacha and co-workers (21) have shown that disaccharides share this "double transition" feature, and that the higher-temperature transition is the transition that is most predictive of collapse in freeze-drying.

In the past 15 years or so, *modulated* DSC has become a common tool for characterization of frozen systems. In this method, the temperature is changed linearly with superposed sinusoidal temperature modulation, and the sample thermal response is observed in comparison with that of the thermally inert reference material. The sample thermal response is separated by Fourier transformation into a response in-phase with the temperature modulation and a response that is out of phase with the modulation. The response that is in-phase is recorded as the reversing component of the thermogram, and the out-of-phase response is recorded as the nonreversing component. Modulated DSC helps to resolve different thermal events occurring in the same temperature range; for example, a crystallization exotherm could tend to obscure a glass transition, and modulated DSC separates these events into the two components of the thermogram. Modulated DSC is useful both in characterization of frozen systems and in characterization of freeze-dried solids—particularly amorphous solids. A detailed discussion of modulated DSC is beyond the scope of this chapter. The reader is referred to reviews by Coleman and Craig (22), Schawe (23), and Ozawa (24).

Freeze Dry Microscopy

Freeze dry microscopy refers to observation of freezing and freeze-drying behavior using a freeze-drying stage mounted on an optical microscope. Such stages were, in earlier days, homemade devices but are now available commercially. The stage shown in Figure 10 consists of a metal block with a hole to allow the sample to be illuminated with transmitted light. The temperature of this block is controlled by a combination of an electrical heater embedded in the block and the circulation of the nitrogen that boils off a Dewar containing liquid nitrogen. A sample volume of no more than about 5 μL is placed on a microscope cover slip, which is placed on the block, and another cover slip is placed on top. It is good practice to use a small drop of a coupling fluid such as silicone oil to assure good thermal contact between the metal block and the bottom cover slip. There is a removable lid on the stage with a window for viewing. The stage is connected to a vacuum pump and to a pressure gauge. The experiment then consists of freezing the sample, evacuating the stage, and carrying out primary drying. Sample temperatures and ramp rates can generally be programmed.

It is helpful to use a microscopy with polarizing capability to gain information about the physical state of the sample. Crystalline materials, generally having more than one refractive index, give rise to retardation colors, whereas amorphous materials are dark under normal conditions of illumination. The working distance of the microscope—the distance between the

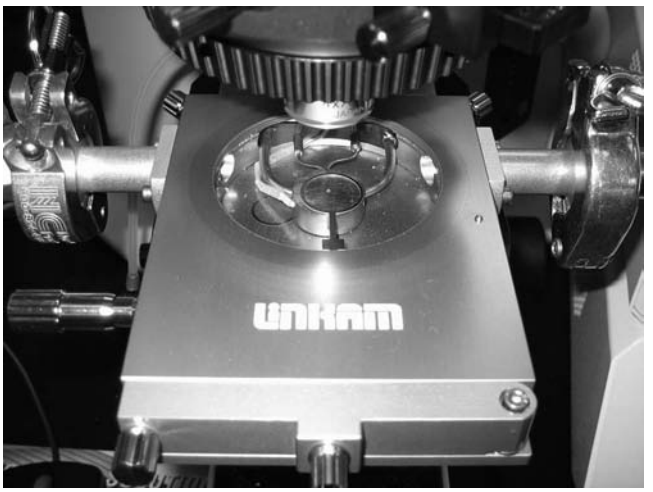

Figure 10 Stage for freeze dry microscopy.

sample and the tip of the ocular—should be about 1 cm to allow space for the lid on the top of the stage.

During primary drying, a distinct sublimation front can be observed moving through the frozen material. At the onset of collapse for amorphous systems, viscous flow of the freeze-concentrated material can be observed as the supporting structure of ice crystals sublimes away. This is illustrated by the photomicrograph in Figure 11. While the underlying event behind collapse of the sample is the glass transition of the freeze-concentrated material, collapse of the sample is not always observed at the same temperature as T_g'. The reason for this is that even though primary drying and secondary drying are different phases of the drying process, once the sublimation front passes through a given volume element of a sample, secondary drying of the partially dried material in that volume element begins, even though there is still ice in other regions of the sample. As secondary drying proceeds, unfrozen water is removed, which deplasticizes the material, causing the glass transition temperature of the partially dried material to increase. For this reason, collapse is generally observed at a somewhat higher temperature than T_g'. How much higher depends on both the rate of primary drying and the rate of secondary drying, but about 3°C is representative.

Collapse is caused by viscous flow of the partially dried material when ice is removed, so it is observed in the dried layer, usually immediately adjacent to the sublimation front (because it is in this region that the level of unfrozen water is highest). Eutectic melting, on the other hand, is observed in the frozen layer, and is usually accompanied by bubbling of the sample as air bubbles formed during freezing expand into the vacuum.

Freeze dry microscopy is also useful for observing annealing effects in freeze-drying. As the frozen material is annealed, the morphology of the ice is likely to change as ice crystals grow. Annealing also may promote crystallization of materials that remain amorphous initially after freezing. Figure 12 illustrates the crystallization of nafcillin during annealing of the frozen solution. The circles represent areas where crystallization has taken place.

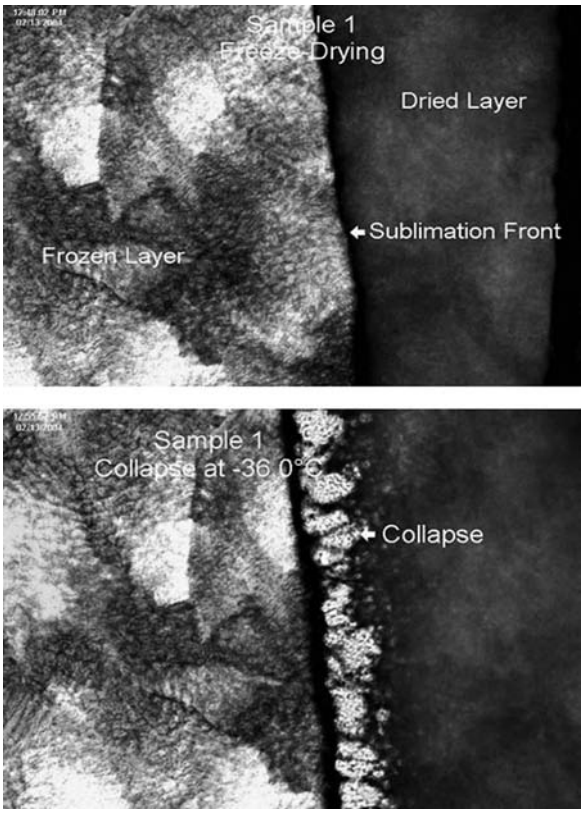

Figure 11 (*See color insert*) Photomicrographs taken during freeze-drying showing retention of structure (*top*) and collapse (adjacent to sublimation front).

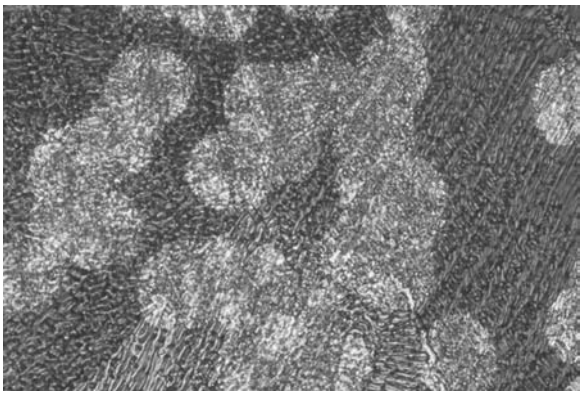

Figure 12 (*See color insert*) Photomicrograph showing crystallization of a solute from a frozen system during annealing.

Perhaps the most important source of uncertainty in the use of a freeze-drying microscope is the uncertainty around determination of a collapse temperature. Some formulations undergo collapse rather abruptly as the temperature of the system is increased. Others, however, collapse very gradually—perhaps over a range of 10°C or more. The most relevant information is the level of collapse that can be detected visually in the freeze-dried solids. However, the level of microscopic collapse that would correspond to visually observable collapse is not obvious.

HEAT AND MASS TRANSFER OPERATIONS IN FREEZE-DRYING

Sublimation of ice is not inherently slow. The maximum rate of evaporation (or sublimation) is a function of the vapor pressure of the substance at a given temperature, as well as the molecular weight of the material. Using appropriate values for water, and assuming 2 mL of water in a vial, leads to the conclusion that if the ice were allowed to sublime at its maximum rate, the ice would be removed in about one minute. Why, then, does it take two days or more to freeze dry many actual formulations? The answer is because of limitations in the rate at which the heat of sublimation can be provided where it is needed, and the rate at which water vapor can be transported from the sublimation front and deposited on the condenser.

Heat and mass transfer in freeze-drying are related through the heat of sublimation of ice, ΔH_s, which is about 2828 J/g. Heat and mass transfer, like all transfer operations, follow the general form of

$$\text{Flux (or flow rate)} = \text{Driving force} \times \text{Conductance}$$

Alternatively, the flow term can be expressed as a driving force divided by a resistance. For heat transfer, the flow rate term refers to the rate of heat transfer, the driving force is a temperature difference, and the conductance term might be the thermal conductivity of a material, such as ice. For mass transfer, the flow rate term is the sublimation rate, the driving force is a pressure difference, and conductance term is generally expressed as a resistance to mass transfer. Complications arise, though, because freeze-drying takes place in a system at low pressure, and the transport properties of the vapor are a function of the system pressure. Before proceeding further, it is helpful to briefly discuss the transport properties of gases at low pressure, since this is important to understanding both heat transfer and mass transfer in freeze-drying.

Transport Properties of Gases at Low Pressure

A fundamental property of gases at low pressure as regards transport properties of gases is the *Knudsen number*, or *Kn*, which is the ratio of the *molecular mean free path*, *L*, to a characteristic dimension of the system, *a*. For water vapor, the mean free path is roughly

$$L(\text{cm}) = \frac{3}{P_\mu}$$

where the pressure, *P*, is expressed in microns of mercury (μHg) or millitorr (mT). For example, at 50 μHg, which is representative of pressures used in freeze-drying, the mean free

path of water vapor is about 0.06 cm. The characteristic dimension of the system depends on what aspect of freeze-drying we are addressing. For example, if the issue is flow of vapor through the pores of the partially dried solids, a is the average diameter of the pores in the cake. For heat transfer from the shelf to the vial, we must take into account the fact that the bottom of the vial is not flat, and is therefore not in intimate contact with the shelf. In this case, the value of a is the average thickness of the "gap" between the bottom of the vial and the shelf. If the issue is flow of vapor through the duct connecting the chamber of the freeze dryer with the condenser, a would be the diameter of the duct.

When the mean free path is small compared to the characteristic dimension a, collisions between gas molecules are much more frequent than collisions with the boundaries of the system under consideration. Since collisions between molecules determine the viscosity of the gas, this flow regime is called *viscous flow*. If L is large compared to a, then collisions of molecules with the boundaries of the system dominate, and the flow is termed *free molecular*, or *Knudsen*, flow. A third flow regime is *transition* flow, which lies between viscous and Knudsen flow. Approximate values of Kn delineating the different flow regimes are:

$$\begin{aligned} Kn &< 0.01 \quad \text{viscous flow} \\ 0.01 < Kn &< 1 \quad \text{transition flow} \\ 1 &< Kn \quad \text{free molecular flow} \end{aligned}$$

Flow regime has a dramatic effect on transport properties. This will be discussed separately for heat transfer and mass transfer.

Heat Transfer at Low Pressures

Under viscous flow conditions, the conductance of heat through a gas is independent of pressure, and Fourier's law applies:

$$\frac{dQ}{dt} = kA \times \frac{dT}{dx}$$

where dQ/dt represents the rate of heat transfer; k is the *thermal conductivity* of a material; A, the area at right angles to the direction of heat flow; and dT/dx is the temperature gradient (the driving force). For water vapor, the thermal conductivity is about 0.64 J/hr cm °K at 273°K.

Heat transfer under free molecular flow conditions is more complicated. Knudsen developed the theory of thermal conductance of gases in this flow regime based on collisions of individual molecules with a surface. As discussed earlier, the gas behavior in this range is determined by collisions of molecules with the boundaries of a system, not on collisions between gas molecules. When a molecule at a temperature T_i strikes a surface at a higher temperature T_s, the incident molecule picks up thermal energy from the collision. The extent to which the energy is increased by the collision is expressed by a term known as the *accommodation coefficient*, α, where

$$\alpha = \frac{T_r - T_i}{T_s - T_i}$$

and T_r is the temperature of the gas molecule rebounding from the collision with the warmer surface. If $\alpha = 1$, then the exchange of energy is complete, and the molecule acquires the temperature of the warmer surface after one collision. Using the kinetic theory of gases, it can be shown that the rate of energy transfer from a hot surface to a cold surface per unit area is

$$E_0 = \alpha \Lambda_0 P (273/T_i)^{1/2} (T_s - T_i)$$

Note that, in the free molecular flow regime, the rate of energy transfer is directly related to the system pressure. The quantity Λ_0 is the *free molecular heat conductivity* at 0°C. Table 4 gives some values of Λ_0 for representative gases. Note that the conductance of water vapor of water vapor is significantly higher than that of air. Note also that the rate of energy transfer is *independent* of the distance separating the bodies exchanging heat. This makes sense in light of

Table 4 Values of Free Molecular Heat Conductivity for Representative Gases

Gas	Free molecular heat conductivity (J/cm^2 hr °Kμ)
Hydrogen	21.8×10^{-2}
Helium	10.5×10^{-2}
Water vapor	9.54×10^{-2}
Nitrogen	5.76×10^{-2}
Oxygen	5.58×10^{-2}

Source: From Ref. 25.

the fact that the behavior of the gas is dependent on collisions of gas molecules with the surfaces, not with other gas molecules.

Consider, as an example, steady-state heat transfer between two parallel plates separated by a distance x of 0.1 cm, with one plate at a temperature of 0°C and the other at −20°C. One millimeter would be roughly the effective separation distance resulting from the bottom of glass vials not being flat. First consider a system under vacuum containing only water vapor at a pressure of 1000 mT, where the molecular mean free path is 0.003 cm, and viscous flow conditions apply. Fourier's law applies and, assuming that k does not change much with temperature and the areas are constant,

$$\begin{aligned}\frac{Q}{At} &= \frac{k\Delta T}{x} \\ &= (0.64\ \text{J/cm hr}\ ^\circ\text{K})(20^\circ\text{K})/0.1\ \text{cm} \\ &= 128\ \text{J/cm}^2\ \text{hr}\end{aligned}$$

Now consider the same system, but at a pressure of 1 μHg (1 mT), where the mean free path is about 3 cm and free molecular flow conditions apply. Assuming an energy accommodation coefficient value of 0.9 and a water vapor temperature of 0°C,

$$\begin{aligned}E_0 &= (0.9)(9.54 \times 10^{-2}\ \text{J/hr cm}^2\ ^\circ\text{K mT})(1\ \text{mT})(20^\circ\text{C}) \\ &= 1.71\ \text{J/hr cm}^2\end{aligned}$$

Thus, for the same driving force (the temperature difference between the two surfaces), evacuating the system to a pressure in the free molecular flow regime decreases the rate of heat transfer by about a factor of roughly 75. Of course, actual freeze-drying takes place at pressures intermediate between these pressures, so the conductive heat transfer consists of components of both viscous flow and free molecular flow, but the viscous flow component would tend to dominate over the free molecular component.

Mass Transfer at Low Pressures

Consider the flow of gas through a tube for both viscous flow and free molecular flow. Viscous flow is described by the Poiseuille equation, where the flow of gas, Q, through a straight tube of constant circular cross section is

$$Q = \frac{\pi r^4 P_a (P_2 - P_1)}{8\eta l}$$

where r is the tube radius; l, the tube length; and η, is the viscosity of the gas. P_a is the average of the upstream and downstream pressures, P_2 and P_1, respectively. The conductance F of this tube is:

$$F = \frac{Q}{P_2 - P_1} = \frac{\pi r^4 P_a}{8\eta l}$$

Note that the conductance increases with the fourth power of the radius, is directly proportional to the average pressure in the tube, and is inversely proportional to the viscosity of the vapor and the length of the tube.

For *molecular flow* through a similar tube of constant cross-sectional area A, perimeter H, and length l, the flow rate is:

$$Q = \frac{(4/3)v_a A^2 (P_2 - P_1)}{Hl}$$

where v_a is the mean molecular speed and is given by

$$v_a = \left(\frac{8R_0T}{\pi M}\right)^{1/2}$$

where R_0 is the gas constant (8.31×10^7 ergs/°K g mole) and M is the molecular weight of the gas. The conductance is then

$$F = \left(\frac{4}{3}\right)\left(\frac{A^2}{Hl}\right)\left(\frac{8R_0T}{\pi M}\right)^{1/2}$$

Note that, for molecular flow, the conductance of the tube is independent of pressure. This makes sense, since the flow properties are determined by collisions of gas molecules with the boundaries of the system, and not by collisions between gas molecules. For a given gas at a constant temperature, the conductance depends only on the geometry of the tube. Conductance increases with the square of the area and is inversely proportional to the length of the tube.

To illustrate the influence of flow regime on conductance of a tube, consider a cylindrical tube with a radius of 1 cm and a length of 100 cm. For water vapor at an average pressure of 1000 mT and a temperature of −20°C, the mean free path, L, is 0.003 cm, so viscous flow conditions apply. The conductance of this tube is

$$F = \left(\frac{\pi r^4}{8\eta l}\right)P_a$$

The viscosity of water vapor at −20°C is 1.55×10^{-4} poise. Therefore,

$$F = \frac{\pi(1\,\text{cm})^4(1000\,\text{mT})}{8(1.55 \times 10^{-4}\,\text{g/cm sec})(1\,\text{cm})} = 101.3\,\text{L/sec}$$

Now consider the same tube at a pressure of 1 μHg, where the mean free path is about 3 cm and molecular flow conditions apply.

$$\begin{aligned} F &= (4/3)(\text{A}^2/\text{HI})(8R_0T/\pi M)^{1/2} \\ &= (4/3)[9.68\,\text{cm}^4/(6.28\,\text{cm})(100\,\text{cm})][8(8.31 \times 10^7\,\text{ergs/°K g mole})(253°\text{K})/\pi(18\,\text{g})]^{1/2} \\ &= 1.14\,\text{L/ sec} \end{aligned}$$

Note the two order of magnitude difference in conductance between viscous flow conditions and free molecular flow conditions for the same tube. These are approximations only. There are several assumptions in the use of the Poiseuille equation to describe conductance. The reader is referred to Dushman and Lafferty for a more detailed discussion (25).

Heat Transfer in Freeze-Drying

There are three basic mechanisms for heat transfer—conduction, convection, and thermal radiation. Conduction is the transfer of heat by molecular motion between one volume element of a material and the next. Convection is the transfer of heat by flow of a fluid—either a liquid or a gas. Convection can be either *natural* convection, where the flow arises from density changes with temperature, or *forced* convection, where an external force is applied. Thermal radiation is electromagnetic radiation arising from thermal excitation of materials. It is generally accepted that, because freeze-drying takes place at a fairly low pressure, on the order

of 0.1 mmHg, convection plays little, if any, role in freeze-drying, and it will not be discussed further here.

As discussed earlier, heat transfer by conduction is governed by Fourier's law:

$$Q = k \cdot \Delta T / \Delta x$$

where Q is the heat flux, or the rate of heat flow per unit area per unit time; k, the thermal conductivity of a given material; ΔT is the temperature difference between the two bodies exchanging heat, and Δx is the thickness of the material. Conductive heat transfer often takes place through a series of different materials; for example, heat transfer from the freeze dryer shelf to a vial undergoing sublimation requires conductance from the glass vial, the frozen formulation, and perhaps a tray between the vials and the shelf. In this case, a resistance term is defined as

$$R_i = \frac{x_i}{k_i}$$

where x_i and k_i are the thickness and thermal conductivity, respectively, of a given material. Thermal conductivities of representative materials are given in Table 5. In heat transfer through a series of resistances, there is usually one resistance that dominates the others, called the *limiting resistance*. In the case of freeze-drying formulations in vials, the limiting resistance arises from the fact that the bottom of a vial is not flat, and not in intimate contact with the heat source. The heat transfer rate is thus governed by the gas phase between the shelf and the vial as discussed earlier. Before proceeding further, it is important to discuss another mechanism of heat transfer—*thermal radiation*.

Heat Transfer by Thermal Radiation

Heat transfer by thermal radiation is fundamentally different from heat transfer by conduction or convection, since some form of matter between the heat source and the heat sink is required for convection or conduction, whereas any matter between heat source and heat sink only impedes heat transfer by radiation. When thermal radiation strikes a surface, it may be absorbed, reflected, or transmitted. For most solids, the transmissivity is essentially zero, since they are opaque to thermal radiation. A hypothetical material, called a *black body*, has an absorptivity value of 1 and neither transmits nor reflects thermal radiation. Instead, all incident energy is absorbed and re-radiated. Real materials do not absorb all incident radiation and are termed *gray*. The *emissivity*, ε, is defined as the ratio of the total emissive power of a surface to the total emissive power of an ideally radiating surface, or black body, at the same temperature. At thermal equilibrium, the absorptivity and emissivity of a material are equal. Emissivity values for materials common to freeze-drying are listed in Table 6. It is important to note that emissivity of a given material is determined not only by the nature of the material,

Table 5 Thermal Conductivities of Representative Materials

Material	Thermal conductivity (J/cm hr°K)
Borosilicate glass	39.3
Aluminum	1.08×10^4
Stainless steel, type 304	618.6
Ice	78.2
Air (atmospheric pressure)	0.87

Table 6 Thermal Emissivity of Representative Materials

Material	Emissivity
Stainless steel, smooth	0.64
Glass, smooth	0.94
Aluminum, polished	0.04

but also by the surface finish. In general, the more "shiny" a surface, the lower the thermal emissivity.

The rate of heat transfer by thermal radiation is given by the Stefan–Boltzmann law:

$$Q = \sigma \varepsilon T^4$$

where σ, the Stefan–Boltzmann constant, has a value of 2.04×10^{-8} J/cm^2 hr °K^4 and T is the absolute temperature. The quantity of heat transferred by a black body at temperature T_1 to a black body at a lower temperature T_2 is given by

$$\frac{Q_{12}}{A} = \sigma F_{12}(T_1^4 - T_2^4)$$

where F_{12} is the "view factor", which represent the fraction of total radiation leaving body 1 that strikes body 2. For gray body radiation, the view factor takes into account the emissivities of the two bodies in addition to the system geometry:

$$\mathcal{F}_{12} = [(1/F_{12}) + (1/\varepsilon_1 - 1) + (A_1/A_2)(1/\varepsilon_2 - 1)]^{-1}$$

These relationships can be used to estimate the contribution of thermal radiation to freeze-drying. Consider radiative heat transfer between two parallel plates of equal area, one representing a stainless steel freeze dryer shelf at 0°C and the other a glass plate representing an array of vials at −20°C. The thermal emissivities of the stainless steel and glass are assumed to be 0.64 and 0.94, respectively. Further, we assume that all of the thermal radiation from the stainless steel plate strikes the glass surface. The view factor is then

$$F_{12} = [1 + (1/0.64 - 1) + (1)(1/0.94 - 1)]^{-1}$$
$$F_{12} = 0.61$$

And

$$Q/A = q = (2.04 \times 10^{-8}\text{J/cm}^2\,\text{hr}\,^\circ\text{K}^4)(0.61)[(273)^4 - (253)^4]$$
$$= 18.1\ \text{J/cm}^2\,\text{hr}$$

Note that this value is independent of the spacing between the plates and independent of pressure. We previously estimated the conductive contributions at 1000 mT (viscous) and 1 mT (molecular) as 128 and 1.7 J/cm^2 hr, respectively. The actual heat transfer by thermal conduction will be somewhere in between these values, so we can conclude that the contribution of thermal radiation is less than the conductive component, but should not be ignored. Given that thermal radiation increases with the fourth power of temperature, it will become relatively more important at higher shelf temperatures.

Thermal radiation becomes more significant in light of warm surfaces in proximity to the product, such as the chamber walls and, particularly, the door of the freeze dryer. Thermal radiation has been shown to be an important contributor to the "edge effect" in freeze-drying, where the vials at the edge of an array of vials dry at a significantly higher rate than vials away from the edge (Fig. 13). Rambhatla and co-workers (26) studied this by sputter coating vials with gold to substantially decrease the thermal emissivity of the glass (note that glass has an unusually high thermal emissivity). Sublimation rate was measured gravimetrically for gold-coated versus uncoated vials both at the edge of the array and at the middle of the array. Three different shelf temperatures were used. In each case, sublimation rate was fastest in uncoated vials at the front (close to the Plexiglass door) of the array. Coated vials at the front of the array underwent sublimation at a rate of about half that of uncoated vials. Differences between coated and uncoated vials were much smaller for vials placed somewhere in the middle of the array, which supports the conclusion that thermal radiation is a major contributor to the edge effect. The data also supports the idea that the edge effect is much more pronounced when freeze-drying at low shelf temperature. This makes sense, since the thermal radiation is coming from the environment outside the freeze dryer, and there is a greater driving force for thermal radiation when the shelf temperature is controlled at a low level.

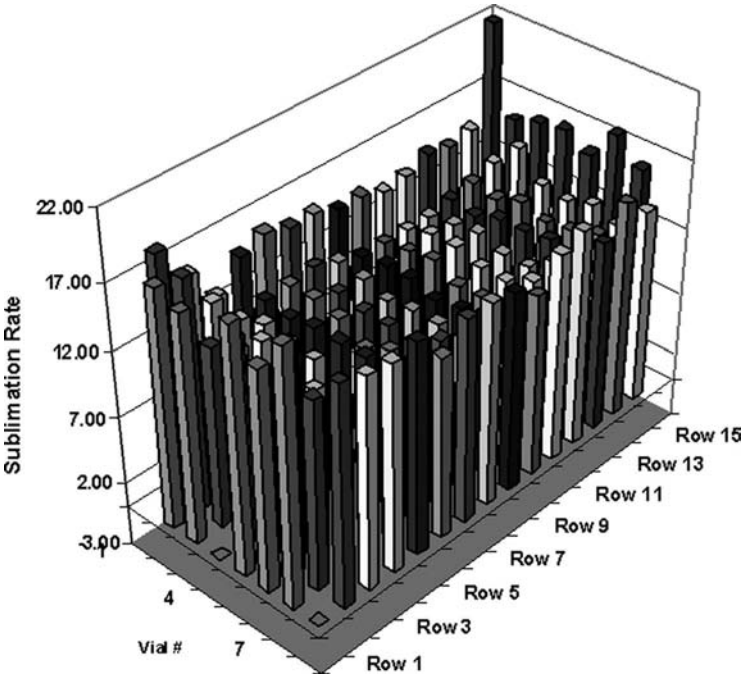

Figure 13 (*See color insert*) Distribution of sublimation rates for a laboratory scale freeze dryer showing the relative magnitude of "edge effects."

The Vial Heat Transfer Coefficient

The vial heat transfer coefficient is typically measured by filling vials with a representative volume of water and carrying out an abbreviated freeze-drying cycle for sufficient time to sublime perhaps half of the vial contents. A number of vials that were filled, marked for identification, and weighed initially are weighed again after the abbreviated cycle. The heat transfer coefficient is determined using the following equation (27):

$$k_v = q(T_s - T_b)$$

where T_b is the temperature at the bottom center of the vial and T_s is the shelf surface temperature. The rate of heat transfer per unit area per unit time, q, is then

$$q = \frac{\Delta H_s \Delta w}{\Delta t}$$

where Δw represents the weight loss of a given vial and Δt is the sublimation time. The vial heat transfer coefficient actually includes three individual terms that reflect underlying heat transfer mechanisms:

$$k_v = k_r + k_c + k_g$$

where k_r is the component due to radiative heat transfer; k_c, the component arising from direct contact between the vial and the shelf; and k_g, the component attributable to conduction through the gas phase resulting from lack of direct contact between vial and shelf. The later term is generally the rate-limiting conductance and is expressed as

$$k_g = \frac{\alpha \Lambda_0 P}{1 + 1(\alpha \Lambda_0 / \lambda_0) P}$$

where λ_0 represents the thermal conductivity of water vapor, l represents the average separation distance between the bottom of the vial and the shelf, and the other terms are defined earlier. This expression takes into account heat transfer arising from both flow regimes—viscous and molecular flow—as a function of the average separation distance. When

the term $l(\alpha\Lambda_0/\lambda_0)P$ is much larger than unity, then viscous flow conditions apply and the gas conduction term reduces to

$$k_g = \frac{\lambda_0}{l}$$

That is, the conduction term is directly related to the thermal conductivity of water vapor, inversely related to the separation distance, and is independent of pressure. At very small separation distances, where $l(\alpha\Lambda_0/\lambda_0)P \ll 1$, then molecular flow conditions apply and

$$k_g = \alpha\Lambda_0 P$$

That is, the conduction term is independent of separation distance and is directly dependent on pressure.

Measurement of the vial heat transfer coefficient is a useful way to evaluate the effect of changing vial specifications on the suitability of a freeze dry cycle for a given formulation. If the vial heat transfer coefficients are not significantly different, then there should be no effect. A significant difference would require re-examination of the freeze dry cycle.

Mass Transfer in Freeze-Drying

In the same way that there is a series of resistances to heat transfer from the shelf to the sublimation front during primary drying, there is also a series of resistances to mass transfer from the sublimation front to condensation of water vapor on the condenser. These resistances are typically the partially dried product layer, the headspace of the vial including the slot, or slots, in the partially seated stopper, and the resistance associated with the flow of water vapor in the chamber, the duct connecting the chamber with the condenser, and the condenser itself. Not surprisingly, the limiting resistance is almost always the porous bed of partially dried solids. The resistance associated with the vial headspace/stopper is generally quite low, assuming that the stopper is appropriately positioned. The resistance of the chamber/condenser can, under very aggressive drying conditions, become a controlling resistance because of choked flow, discussed in the following text.

The sublimation rate again takes the form of a flow term equaling a driving force divided by a resistance:

$$\text{Sublimation rate} = \frac{P_i - P_c}{R_p}$$

Where P_i is the vapor pressure of ice at the sublimation front; P_c, the partial pressure of water vapor in the chamber; and R_p, the resistance of the partially dried layer of solids. Since the sublimation front moves from the top of the vial to the bottom during primary drying, the depth of the partially dried layer increases and the resistance increases. This causes the sublimation rate to decrease and, since the rate of heat flow from the shelf remains approximately constant, the product temperature increases. Of course, this also increases P_i and increases the driving force for sublimation. The increased driving force does not completely offset the increased resistance, however, which explains why the product temperature tends to increase gradually during primary drying. Thus, under the same set of primary drying conditions, the product temperature profile can vary widely depending on the resistance characteristics of the formulation. This is illustrated in Figure 14. As resistance to mass transfer increases, control of product temperature becomes more uncertain. For formulations that have a relatively high resistance to flow of water vapor, it is important to limit the depth of the fill. It is generally good practice to limit the fill volume to no more than about one third of the capacity of the vial.

Searles et al. reported that primary drying rate is affected by the nucleation temperature of ice, where high degrees of supercooling result in more rapid freezing once ice crystals nucleate. Fast freezing results in small ice crystals that, in turn, have a relatively high resistance to mass transfer. Conversely, low degrees of supercooling result in larger ice crystals, relatively low resistance to vapor flow, and higher sublimation rate. One benefit of annealing, as reported by Searles et al. (8), is to allow Ostwalt ripening of smaller ice crystals such that a more

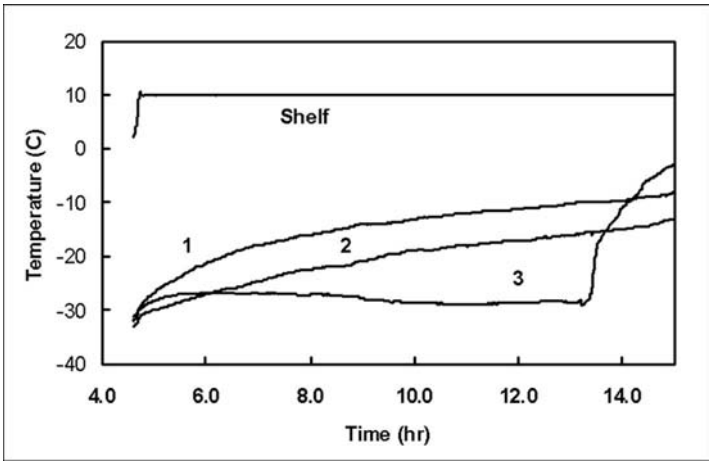

Figure 14 Product temperature versus time during primary drying for (1) 8% mannitol, (2) 4% mannitol, and (3) 5% lactose.

uniform distribution of ice crystal sizes results, with faster average sublimation rate and better vial-to-vial uniformity of sublimation.

There is a need in freeze-drying technology development for better control of the freezing step. This would not only make the freezing step more efficient, but would also improve consistency of drying. Approaches to improved control of freezing include the use of ultrasound (28), an electric field (29), and freezing under a slight vacuum (30). The practical application of any of these techniques has yet to be established however. As of this writing, Praxair, Inc., has reported a technique for controlled nucleation of ice that could be readily scalable, but there appear to be no publications as yet describing the details of the technique.

Change in morphology of the partial dried cake during primary drying can result in a change in resistance. "Microcollapse" of lactose during freeze-drying was reported by Milton and Nail (31), where scanning electron microscopy was used to study the microstructure of the solids. Microcollapse results in holes appearing in plates of amorphous substance, with an accompanying decrease in resistance of the dried layer. This would be expected to result in an increased sublimation rate and a decrease in product temperature during primary drying.

Measurement of Sublimation Rate

The sublimation rate can be measured in several ways. If a sample thief is available to remove samples from the freeze-dryer during the process, then several vials can be pre-weighed and identified. The thief is then used to remove these vials at various times during the primary drying process, reweighed, and a weight loss versus time curve is constructed. If no thief is available, the same approach can be used except that the cycle is terminated before primary drying is completed. Of course, this is a destructive test, and only one drying time point is possible, but the vials can be reweighed and an average sublimation rate over the time interval can be calculated. The most sophisticated method of measuring the sublimation rate is to use tunable diode laser absorption spectroscopy (TDLAS), which is discussed below.

Mass Transfer During Secondary Drying

Secondary drying refers to removal of water that did not freeze during the freezing process. The amount of this unfrozen water depends largely on the nature of the formulation. In formulations with a relatively high content of amorphous solid, the unfrozen water level is relatively high. Since ice is no longer present during secondary drying, higher shelf temperatures are generally used as compared with primary drying. However, for amorphous formulations in particular, collapse can take place during secondary drying if the shelf temperature is increased too rapidly, or to a temperature that is too high.

There is not a large body of published information on secondary drying. Pikal and coworkers (32) studied the rate of secondary drying as a function of shelf temperature and

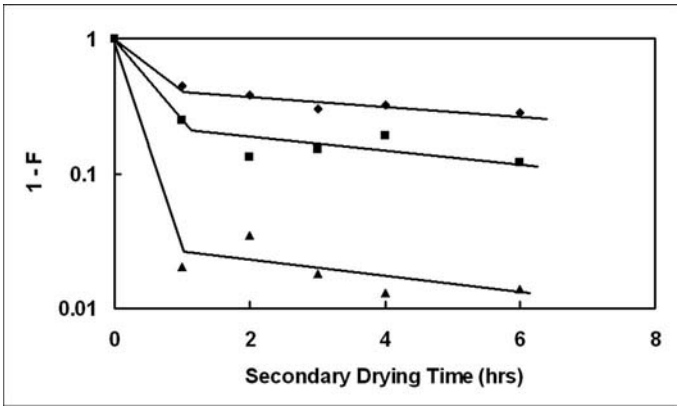

Figure 15 Relative rates of secondary drying for different materials.

chamber pressure for various formulations, and representative results are shown in Figure 15. The rate of water removal during secondary drying is determined by, not surprisingly, the formulation, as well as the shelf temperature. For the formulations examined, secondary drying seems to take place in two stages—an early "fast" phase, followed by a "slow" phase, where a plateau is reached in the residual moisture as a function of drying time. This plateau level is determined largely by the shelf temperature during secondary drying. Rate of secondary drying was shown to be, at least for the model systems studied, independent of the chamber pressure. This is counter to the common point of view that the chamber pressure should be reduced to the lowest practical attainable level during secondary drying, and supports the idea that the rate-limiting step in secondary drying is either diffusion of water through the glassy matrix or evaporation of water at the surface of the solid—most likely the former.

Choked Flow in Freeze-Drying

Normally, the resistance of the dried product layer is the controlling resistance to mass transfer in freeze-drying. However, under aggressive drying conditions, another resistance has been shown to be significant, this one arising from the duct connecting the chamber with the condenser in freeze dryers with external condensers (33). Water vapor flows through this cylindrical duct because the upstream pressure P_u is higher than the downstream pressure, P_d. As water vapor flows through this duct, the pressure decreases and, since the mass flow rate is constant for any axial position along the duct, the velocity of the vapor increases. The kinetic theory of gases shows, however, that there is a limit to the vapor velocity, which is the speed of sound in water vapor, about 360 m/sec or Mach 1. As the speed of sound is approached, further reduction of the pressure on the condenser side of the duct will cause no change in the mass flow rate through the duct. In this case, flow through the duct is said to be *choked*. This represents the maximum sublimation rate that the freeze dryer will support at any given chamber pressure. Attempting to operate at a higher sublimation rate would result in the inability to control chamber pressure.

The choke point is a function of chamber pressure—the higher the chamber pressure, the higher the choke point. The choke point can be determined by testing the system using ice slabs, where tray rings are lined with plastic and filled with perhaps 1 to 2 cm of water. All of the shelves are utilized for this. The water is frozen and the system is evacuated. Starting at the low end of the operating pressure range for the freeze dryer, say 50 mT, the pressure is allowed to stabilize, then the shelf temperature is increased, either by ramping the temperature or by making stepwise increases in the shelf temperature set point. A temperature will be reached where the chamber pressure will drift above the set point. This is the choke point for that pressure. A new set pressure set point is then established, and the process is repeated until the operating pressure range has been covered. The actual sublimation rate at each point would need to be determined gravimetrically; that is, by carrying out a brief sublimation cycle and determining the weight of ice sublimed, then converting to an averaged sublimation rate.

PROCESS MONITORING

The traditional method of monitoring the status of the product is to place a thermocouple, or another temperature measuring device such as a resistance temperature detector (RTD), in several vials of product. This technique provides important information on product temperature during primary drying, when primary drying is complete, as well as an indication of the end point of secondary drying (Fig. 3). While this is necessary when developing freeze dry cycles, it has significant drawbacks as a monitoring method in a manufacturing setting. First, monitored vials are not truly representative of nonmonitored vials, since the temperature measuring device promotes heterogeneous nucleation of ice. This results in lower degrees of supercooling, larger ice crystals, and faster drying rates. In general, monitored vials undergo sublimation at a rate roughly 10% faster than nonmonitored vials. Second, placing thermocouple probes in individual vials is a manual process that inevitably compromises sterility assurance. Some manufacturers try to avoid this by placing monitored vials in the front row of vials, closest to the chamber door. However, as discussed earlier, this position is the most subject to the "edge effect", making data from monitored vials even more nonrepresentative. Finally, advancing technology in parenteral manufacturing has made automated loading/unloading systems common in freeze-drying. Such systems are not compatible with placing temperature measuring devices in individual vials.

There is a continuing need in the industry for better process monitoring, and the past several years have seen considerable activity in process monitoring tools. Below is a brief survey of methods intended to monitor the status of the entire batch.

Comparative Pressure Measurement

Comparative pressure measurement is based on the use of two types of pressure sensors—a capacitance manometer and a thermal conductivity-type gauge (a thermocouple gauge or, more commonly, a Pirani gauge) (34). The capacitance manometer is based on capacitance changes associated with a flexible metal diaphragm between a sealed reference cell and the process gases. Thus, it measures force per unit area independently of gas phase composition. The thermocouple-type gauge, on the other hand, is preferentially sensitive to water vapor because of the higher thermal conductivity of water vapor relative to nitrogen or oxygen. In comparative pressure measurement, chamber pressure is both monitored and controlled with the capacitance manometer while it is also monitored with the thermal conductivity-type gauge. A representative graph of a cycle monitored with comparative pressure measurement is shown in Figure 16. During primary drying, the apparent pressure as measured by a Pirani gauge is nearly constant, and is considerably higher than the "true" pressure as measured by capacitance manometer, since the composition of the vapor in the chamber is nearly all water vapor. As primary drying ends and the partial pressure of water vapor decreases, the Pirani reading decreases. As the shelf temperature is increased during secondary drying, the Pirani pressure increases again as unfrozen water is released from the partially dried solids. The

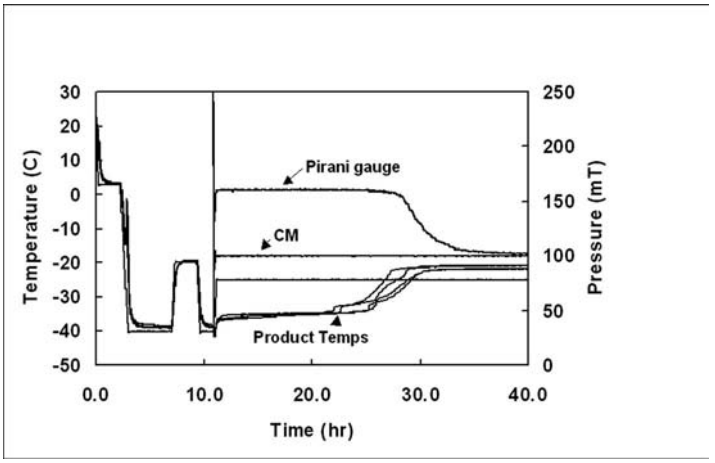

Figure 16 Process variables illustrating monitoring by comparative pressure measurement.

pressure decreases again and approaches a steady-state value near the capacitance manometer reading as the product approaches dryness.

Comparative pressure measurement has proven to be a robust method of monitoring the status of the entire batch. It is independent of scale of operations and is inexpensive. Despite these advantages, it has been rather slow to be adopted by the industry.

Electronic Hygrometer

Electronic hygrometers measure the dew point, or frost point, of a process gas, and are based on either an optical measurement or a capacitance measurement (35). While not commonly used in freeze-drying, the instrument demonstrated to be successful for monitoring freeze-drying is based on capacitance changes due to sorption of water vapor. The process data using the electronic hygrometer is qualitatively very similar to the data shown in Figure 16.

Pressure Rise

The pressure rise technique consists simply of closing the valve between the chamber and condenser for a brief interval as the end of drying is approached. As the rate of water vapor evolution from the product decreases, the amount of pressure rise approaches the background leak rate of the chamber/condenser. The method is simple and robust, but must be applied with some caution. If the sequence of opening the valve periodically begins during primary drying, it is important to assure that the valve does not stay closed so long that the high chamber pressure causes damage to the product.

Manometric Temperature Measurement

Milton and co-workers (31) described a method based on a pressure rise measurement, except that the valve between the chamber and condenser is closed for a brief interval during primary drying. The transient pressure response is measured, and this response is fit to an equation based on fundamental heat and mass transfer consisting of three components: the continued sublimation of ice during the time course of the measurement, continued heat transfer to the vial from the shelf during the measurement, and dissipation of the temperature gradient across the frozen layer during the measurement. The composite equation contains three unknowns: the vapor pressure of ice (thus the temperature at the sublimation front), the resistance of the product to mass transfer, and the vial heat transfer coefficient. A nonlinear least squares algorithm is then used to obtain values of these variables that provide the best fit of the equation to the actual transient pressure response. Using the manometric temperature measurement (MTM) method, reasonable agreement has been observed between product temperatures measured by MTM and those measured by traditional methods like the thermocouple, particularly given that they do not measure the temperature in the same location. Thermocouple measurements typically measure the temperature at the bottom center of the vial, whereas MTM calculates the temperature at the sublimation front, and there is a temperature gradient across the frozen layer of 1 to 2°C. Likewise, reasonable values are obtained for the resistance of the dried product layer to flow of water vapor, and this has led to a better understanding of the role of dried layer morphology on resistance of the solid layer to mass transfer, as discussed earlier. This technique has been advanced further as commercially available software, the SMART® Freeze Dryer, to control the product temperature at the desired value, thus decreasing the amount of trial and error experiments needed in cycle development (36).

While manometric temperature measurement is a very useful laboratory tool, it requires a quick-acting valve between the chamber and condenser to record the transient pressure response appropriately. Since the main valves on production scale freeze dryers have relatively slow-acting valves, the method has not yet been applied to freeze-drying on a commercial scale.

Tunable Diode Laser Absorption Spectroscopy

Tunable diode laser absorption spectroscopy, or TDLAS, is a new and still developing technology that shows significant promise as a process analytical technology in freeze-drying. TDLAS is a near-infrared method that provides real-time measurement of the mass flow rate of water vapor flowing from the chamber to the condenser during freeze-drying. The hardware is

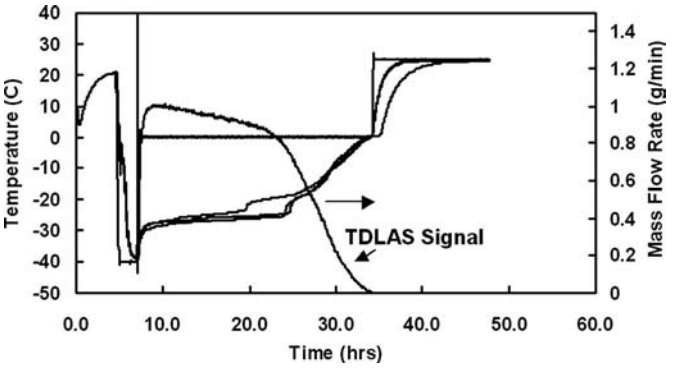

Figure 17 Representative graph of freeze drying process variables showing mass flow rate as measured by tunable diode laser absorption spectroscopy (TDLAS).

mounted on the duct connecting the chamber to the condenser and consists of a fiber-optic laser source aligned at about a 45° angle to a detector on the opposite side of the duct. Water vapor concentration is measured by traditional absorption spectroscopy. The velocity measurement is based on the fact that moving water vapor has a frequency of maximum absorption that is shifted relative to stationary water vapor by an amount that is proportional to the speed of the vapor. The λ_{max} of the moving vapor is compared with that of stationary water vapor sealed in a reference cell. The calculation of average velocity is based on a computational fluid dynamic model of vapor flow in the duct. The velocity measurement, along with the concentration of water vapor, is used to calculate the instantaneous mass flow rate. The instantaneous flow rate data is integrated over the time course of the freeze dry cycle to give the cumulative amount of water removed.

TDLAS has been shown to be a useful tool in cycle development (37). For example, the influence of pressure on sublimation rate can be quantitated simply by changing the set point pressure and observing the resulting sublimation rate. Freeze dryer capability can be readily measured by determining the maximum sublimation rate supported. Measuring capability of both laboratory and production freeze dryers facilitates scale-up by preventing development of aggressive cycles on laboratory equipment that cannot be supported by production scale equipment. Finally, accurate cycle end points can be determined by observing the time at which the flow rate approaches zero (Fig. 17).

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Index

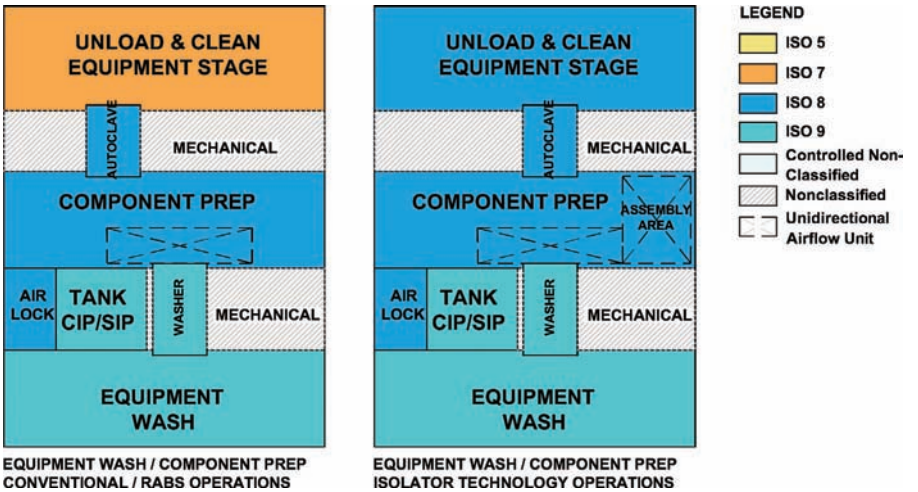

Figure 1.1 Weigh and dispense (*see page 5*).

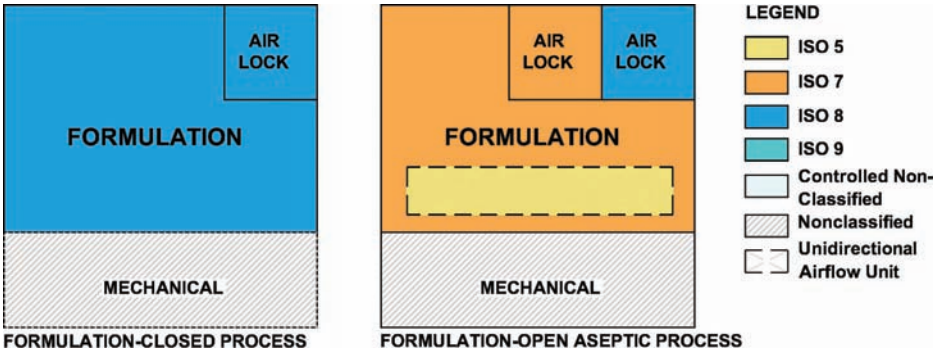

Figure 1.2 Formulation (*see page 7*).

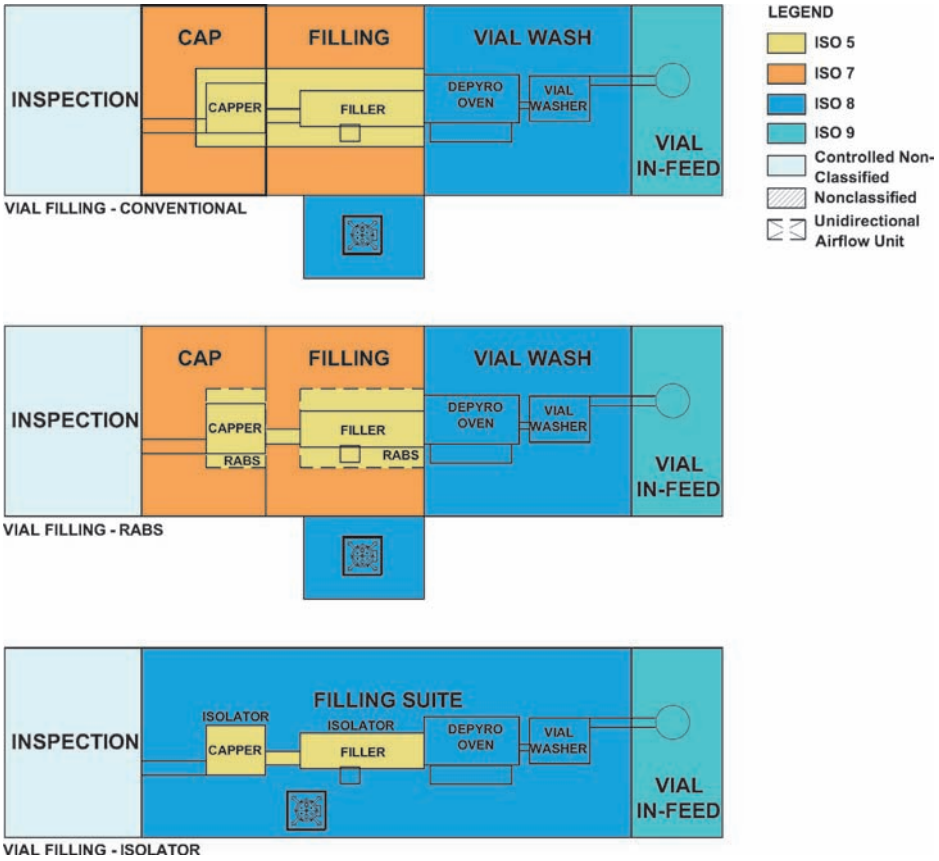

Figure 1.4 Vial filling (*see page 10*).

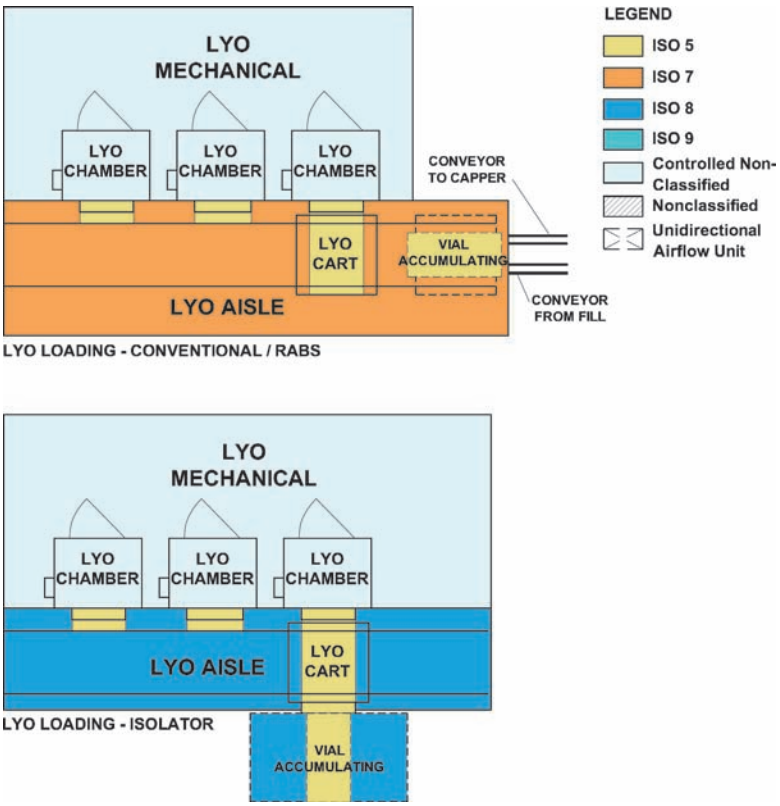

Figure 1.10 LYO load/unload (*see page 18*).

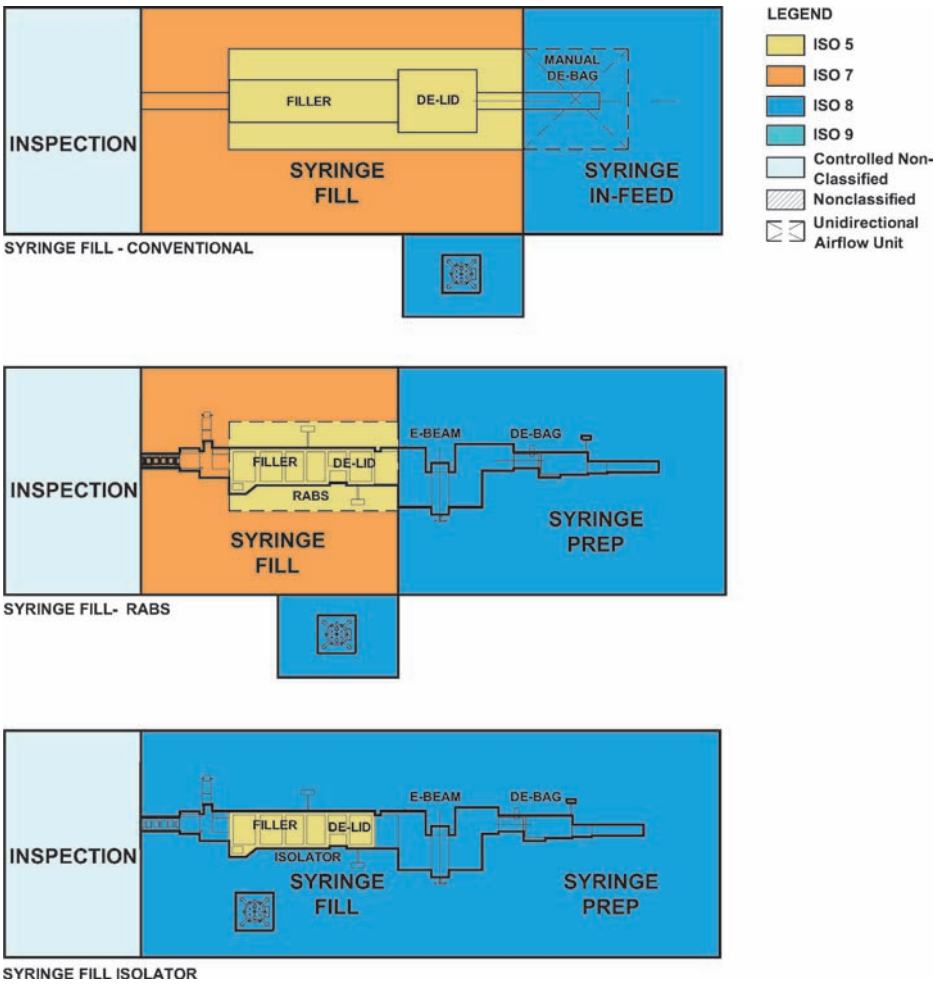

Figure 1.15 Syringe fill (*see page 22*).

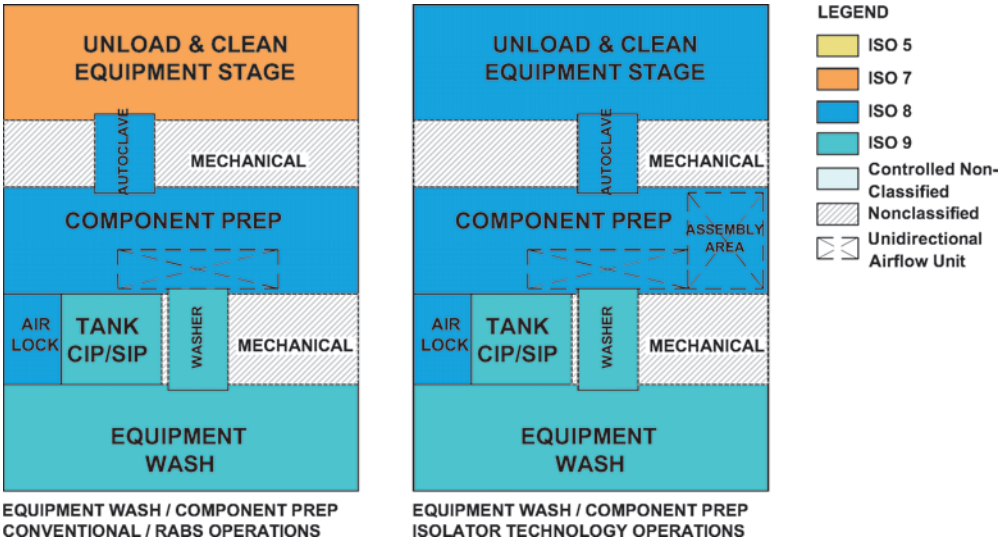

Figure 1.23 Equipment wash/component prep (*see page 30*).

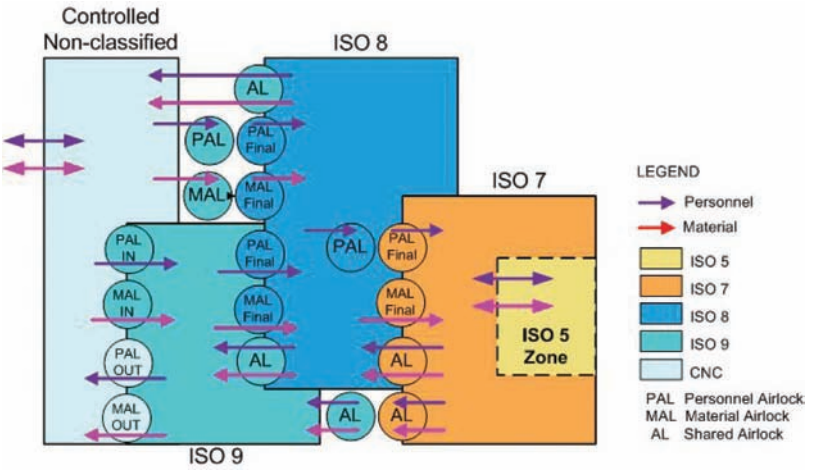

Figure 1.26 General airlock concept by classification (*see page 40*).

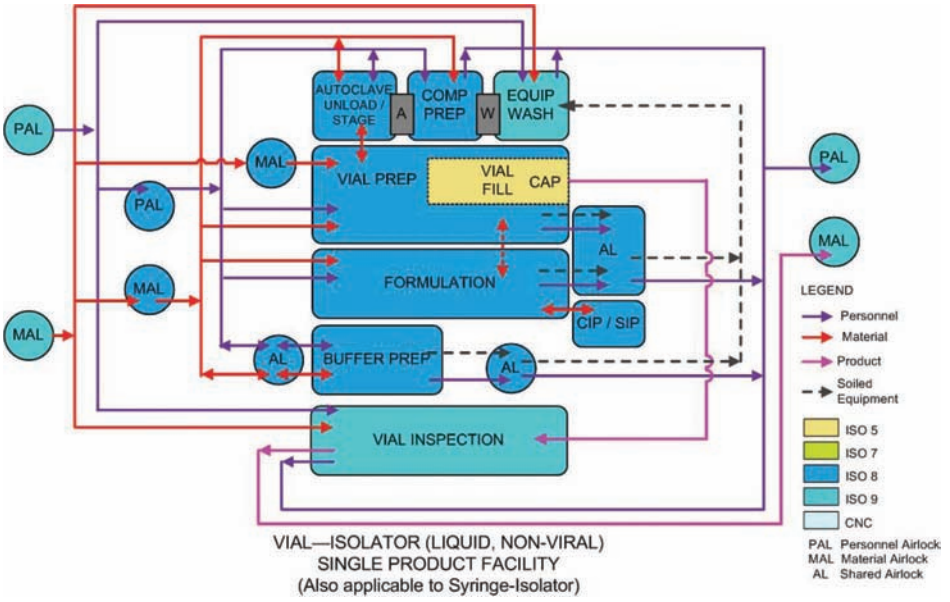

Figure 1.27 Single product—single-suite module (*see page 41*).

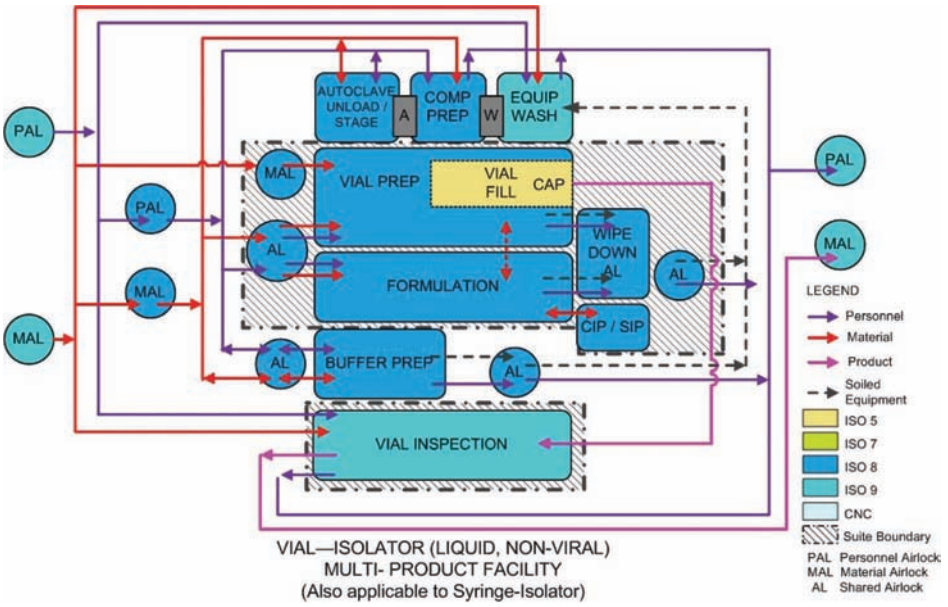

Figure 1.28 Multiproduct—multi-suite module (*see page 42*).

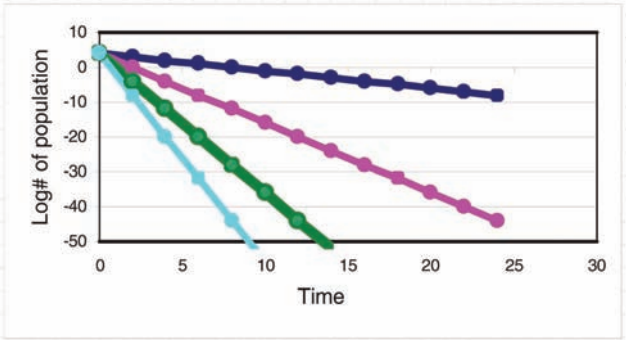

Figure 9.15 Overkill approach (*see page 231*).

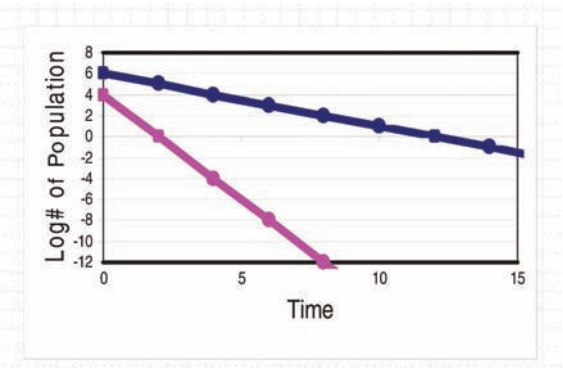

Figure 9.16 BB/BI method with survival count (*see page 232*).

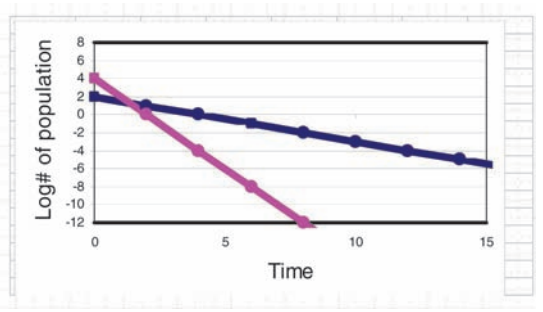

Figure 9.17 BB/BI method with total BI kill (*see page 232*).

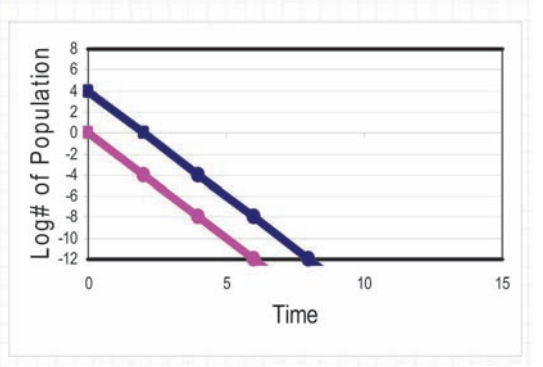

Figure 9.18 Bioburden approach (*see page 233*).

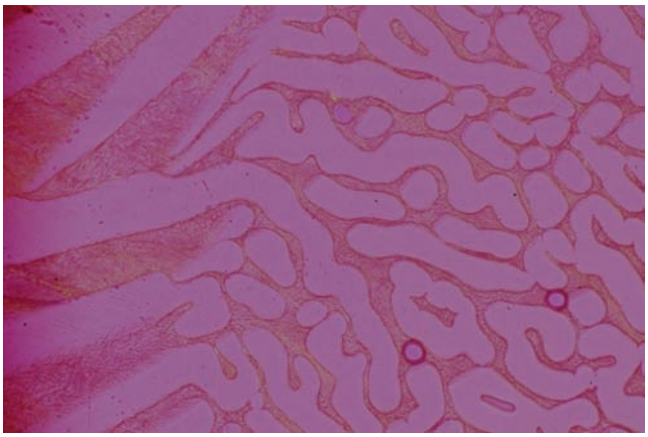

Figure 15.6 Photomicrograph of a frozen solution of sodium chloride in water (*see page 361*).

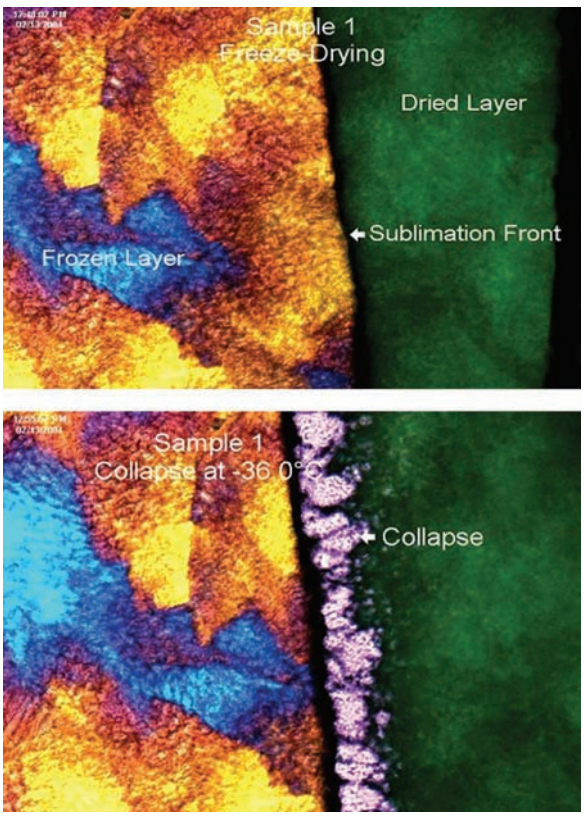

Figure 15.11 Photomicrographs taken during freeze-drying showing retention of structure (*top*) and collapse (adjacent to sublimation front) (*see page 367*).

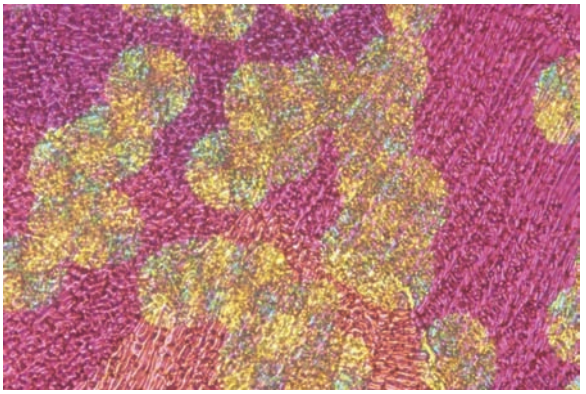

Figure 15.12 Photomicrograph showing crystallization of a solute from a frozen system during annealing (*see page 368*).

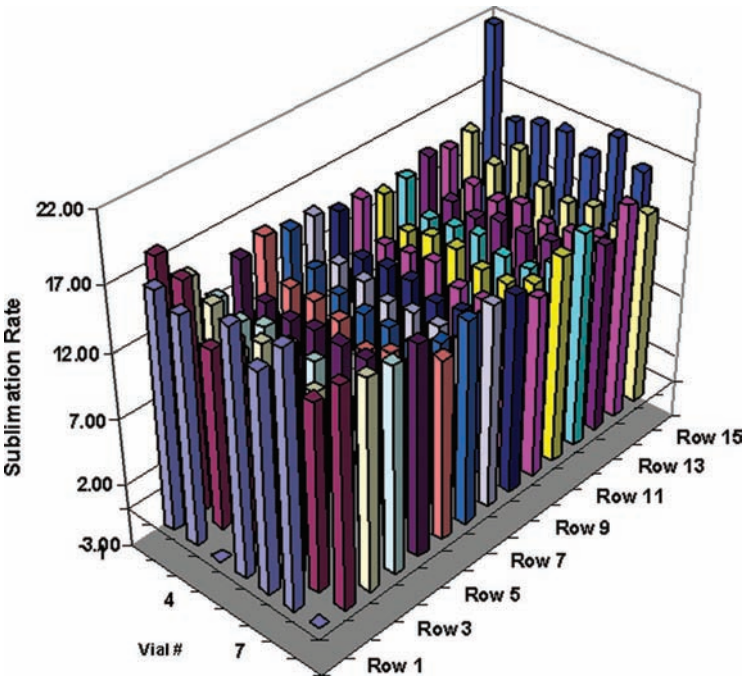

Figure 15.13 Distribution of sublimation rates for a laboratory scale freeze dryer showing the relative magnitude of "edge effects" (*see page 374*).

Pharmaceutical Dosage Forms

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Pharmaceutical Dosage Forms

Parenteral Medications
Third Edition

Volume 3
Regulations, Validation and the Future

Edited by

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We dedicate this work to those who have inspired us.
To my parents Walter and Ruth Ludwig and my wife Sue Ludwig
To my parents Hari and Pratibha Nema and my wife Tina Busch-Nema

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Foreword

I was a faculty member at the University of Tennessee and a colleague of Dr. Kenneth Avis when he conceived, organized, and edited (along with H.A. Lieberman and L. Lachman) the first edition of this book series that was published in 1984. It was so well received by the pharmaceutical science community that an expanded three-volume second edition was published in 1992. Dr. Avis did not survive long enough to oversee a third edition, and it was questionable whether a third edition would ever be published until two of his graduate students, Drs. Nema and Ludwig, took it upon themselves to carry on Dr. Avis' tradition.

Their oversight of this third edition is work that their mentor would be highly pleased and proud of. From 29 chapters in the second edition to 43 chapters in this new edition, this three-volume series comprehensively covers both the traditional subjects in parenteral science and technology as well as new and expanded subjects. For example, separate chapter topics in this edition not found in previous editions include solubility and solubilization, depot delivery systems, biophysical and biochemical characterization of peptides and proteins, container-closure integrity testing, water systems, endotoxin testing, focused chapters on different sterilization methods, risk assessment in aseptic processing, visual inspection, advances in injection devices, RNAi delivery, regulatory considerations for excipients, techniques to evaluate pain on injection, product specifications, extractables and leachables, process analytical technology, and quality by design.

The editors have done an outstanding job of convincing so many top experts in their fields to author these 43 chapters. The excellent reputations of the authors and editors of this book will guarantee superb content of each chapter. There is no other book in the world that covers the breadth and depth of parenteral science and technology better than this one. In my opinion, the editors have achieved their primary objectives—publishing a book that contains current and emerging sterile product development and manufacturing information, and maintaining the high standard of quality that readers would expect.

Michael J. Akers
Baxter BioPharma Solutions
Bloomington, Indiana, U.S.A.

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Preface

Pharmaceutical Dosage Forms: Parenteral Medications was originally published in 1984 and immediately accepted as a definitive reference in academic institutions and the pharmaceutical industry. The second edition was published in 1993. The ensuing years have produced incredible technological advancement. Classic small-molecule drugs are now complemented by complex molecules such as monoclonal antibodies, antibody fragments, aptamers, antisense, RNAi therapeutics, and DNA vaccines. There have been significant innovations in delivery devices, analytical techniques, in-silico modeling, and manufacturing and control technologies. In addition, the global regulatory environment has shifted toward greater emphasis on science-based risk assessment as evidenced by the evolving cGMPs, quality by design (QbD), process analytical technology (PAT), continuous processing, real time release, and other initiatives. The rapidly changing landscape in the parenteral field was the primary reason we undertook the challenging task of updating the three volumes. Our objectives were to (*i*) revise the text with current and emerging sterile product development and manufacturing science and (*ii*) maintain the high standard of quality the readers expect.

The third edition not only reflects enhanced content in all the chapters, but also more than half of the chapters are new underscoring the rapidly advancing technology. We have divided the volumes into logical subunits—volume 1 addresses formulation and packaging aspects; volume 2, facility design, sterilization and processing; and volume 3, regulations, validation and future directions. The authors invited to contribute chapters are established leaders with proven track records in their specialty areas. Hence, the textbook is authoritative and contains much of the collective experience gained in the (bio)pharmaceutical industry over the last two decades. *We are deeply grateful to all the authors who made this work possible.*

Volume 1 begins with a historical perspective of injectable drug therapy and common routes of administration. Formulation of small molecules and large molecules is presented in depth, including ophthalmic dosage forms. Parenteral packaging options are discussed relative to glass and plastic containers, as well as elastomeric closures. A definitive chapter is provided on container closure integrity.

Volume 2 presents chapters on facility design, cleanroom operations, and control of the environment. A chapter discussing pharmaceutical water systems is included. Key quality attributes of sterile dosage forms are discussed, including particulate matter, endotoxin, and sterility testing. The most widely used sterilization techniques as well as processing technologies are presented. Volume 2 concludes with an in-depth chapter on lyophilization.

Volume 3 focuses on regulatory requirements, risk-based process design, specifications, QbD, and extractables/leachables. In addition, we have included chapters on parenteral administration devices, siRNA delivery systems, injection site pain assessment, and control, PAT, and rapid microbiology test methods. Volume 3 concludes with a forward-looking chapter discussing the future of parenteral product manufacturing.

These three volumes differ from other textbooks in that they provide a learned review on developing parenteral dosage forms for *both* small molecules and biologics. Practical guidance is provided, in addition to theoretical aspects, for how to bring a drug candidate forward from discovery, through preclinical and clinical development, manufacturing, validation, and eventual registration.

The editors wish to thank Judy Clarkston and Lynn O'Toole-Bird (Pfizer, Inc.) for their invaluable assistance and organizational support during this project, and Sherri Niziolek and Bianca Turnbull (Informa Healthcare) for patiently leading us through the publishing process.

We also acknowledge the assistance of Pfizer, Inc. colleagues Lin Chen and Min Huang for reviewing several of the chapters.

We would like to express special gratitude to the late Kenneth E. Avis (University of Tennessee College of Pharmacy) for his dedication to teaching and sharing practical knowledge in the area of parenteral medications to so many students over the years, including us. Finally, we acknowledge the contributions of Dr Avis, Leon Lachman, and Herbert A. Lieberman who edited the earlier editions of this book series.

Sandeep Nema
John D. Ludwig

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1 cGMP regulations of parenteral drugs

Terry E. Munson

INTRODUCTION

This chapter presents an overview of the current Good Manufacturing Practice (cGMP) regulations for parenteral drugs. Since most of the major world regulatory authorities follow either the U.S. or the European Union (EU) model for their GMP regulations, this chapter will focus only on these two regulations.

U.S. REGULATIONS

Food, Drug, and Cosmetic Act

In the United States, the law that is violated when a parenteral drug product is not manufactured according to cGMPs is the Food, Drug, and Cosmetic Act (Act). Failure to follow GMPs is covered under Section 501, Adulterated Drugs and Devices section of the Act. Section 501(a)(2)(B) states:

> A drug or device shall be deemed to be adulterated— . . . if it is a drug and the methods used in, or the facilities or controls used for its manufacture, processing, packing, or holding do not conform to or are not operated or administered in conformity with current good manufacturing practice to assure that such drug meets the requirements of this Act as to safety and has the identity and strength, and meets the quality and purity characteristics, which it purports or is represented to posses. . . .

This is the section of the law that requires manufacturers to produce drug in conformity with GMP practices. Adverse observations from manufacturing site inspections typically fall under this section of the Act.

Two other sections of the action that should be noted, although they do not directly apply to the GMP regulations, are as follows:

Section 501(b): "A drug or device shall be deemed to be adulterated— . . . If it purports to be or is represented as a drug the name of which is recognized in an official compendium, and its strength, quality, or purity falls below, the standards set forth in such compendium."

Section 501(c): "A drug or device shall be deemed to be adulterated— . . . If it is not subject to the provisions of paragraph (b) of this section and its strength differs from, or its purity or quality falls below, that which it purports or is represented to posses."

Sections 501(b) and (c) citations result from the analysis of drug samples picked up either during inspections or at end-user sites in one of the Food and Drug Administration (FDA) laboratories.

To fail the requirements in 501(b), the product must be tested exactly by the methods in the compendial monograph. One key element of this section is that the product only needs to be represented in the compendia to fall under the jurisdiction of this section, irrespective of whether or not it purports to be United States Pharmacopeia (USP)/National Formulary (NF). For example, Sodium Chloride Injection USP, Bacteriostatic Sodium Chloride Injection, and allergenic extract diluent (0.9% sodium chloride in water) are all represented in the compendia and thus subject to 501(b) of the Act.

Section 501(c) is used for drugs not meeting the requirements that are in the drug application or in-house specifications. This could also apply to specifications that are in addition to the requirements in a compendial monograph.

cGMP Regulations

To implement the provisions of the Federal Food, Drug, and Cosmetic Act, Congress delegated to the FDA, through the Secretary of Health, Education, and Welfare, broad authority to promulgate regulations for the efficient enforcement of the Act under Section 701(a). The

exceptions to this authority are those provisions of the Act that are cited in Section 701(e). These include several drug provisions relating to certain types of adulteration and misbranding. Regulations issued under Section 701(e) require an opportunity for a public hearing under formal rule-making procedures, referred to as an evidentiary public hearing. Regulations promulgated under Section 701(a) on the contrary are subject only to notice and comment or informal rule-making under the provisions of the Administrative Procedures Act (1).

To implement Section 501(a)(2)(B) of the Act, the FDA issued regulations, in accordance with Section 701(a) of the Act, defining what it considered "current good manufacturing practice." The latest revisions of the cGMP regulations for human and veterinary drugs were published in the Federal Register of September 29, 1978, and became effective on March 28, 1979 (2). Because these regulations provide legal standards for controlling the quality of drugs, they should be of interest to all health professionals. They can also provide an insight into standard operating procedures that may serve those who are called upon to set up a quality *control program on the handling and administration of* parenterals in health care facilities. Unlike other regulations, regulatory controls are based primarily on inspections of establishments manufacturing, processing, packing, or holding human and veterinary drugs.

The cGMP regulations are contained in Title 21 Code of Federal Regulations parts 210 and 211.

Part 210—Current Good Manufacturing Practice in Manufacturing, Processing, Packing, or Holding of Drugs; General

Part 210 gives the status of the cGMP regulations. It indicates that failure to follow the regulations in parts 211 through 226 would render drug adulterated under Section 501(a)(2)(B) of the act. It also states that the person who is responsible for the failure to comply shall be subject to regulatory action. In the United States, typically the president or chief executive officer of the company is held responsible. In some cases the top manager of quality has also been cited in regulatory actions.

Paragraph 210.2 describes the applicability of cGMP regulations. It states that parts 210 through 226 pertain to drugs, parts 600 through 680 pertain to biological products, and part 1271 pertains to human cell, tissue, or cellular or tissue-based products subject to Section 505 of the act or Section 351 of the Public Health Service Act, shall be considered to supplement, not supersede, each other, unless the regulation explicitly provide otherwise. This means that the cGMP regulation in parts 210 and 211 also apply to biological products and biotechnology products. It also states that investigational drugs used in phase 1, 2 or 3 clinical studies must comply with the regulations in part 211. The FDA has further clarified that all cGMP provisions except labeling and process validation requirements are to be applied to clinical products.

The last section of part 210 lists definitions for some of the terms used in part 211. On September 8, 2008, the FDA published a revision to the non-fiber-releasing filter definition in part 210.3 (3). The reference to asbestos filters as fiber-releasing filters was deleted.

Part 211—Current Good Manufacturing Practice for Finished Pharmaceuticals

Subpart A: general provisions, including scope and definitions. This subpart is a repeat of the sections in 210.1 and 210.3. There is a statement that cGMPs in part 211 do not apply to Over-The-Counter (OTC) drug products that are ordinarily marketed and consumed as human foods. This is the case for vitamins and herbal products.

Subpart B: organization and personnel.

1. Responsibilities and authority of a quality control unit are to be spelled out in writing.
2. Personnel qualification for assigned functions and training in CGMP shall be conducted on a continuing basis.
3. Only authorized personnel shall enter those areas of the buildings and facilities designated as limited-access areas.
4. Consultants advising on CGMP shall be qualified, and records shall be maintained on their employment and qualifications.

Subpart C: buildings and facilities.

1. Buildings—their size, construction, and operational areas—are to be designed so that they are suited to the types of products produced or held therein to prevent contamination or mix-ups.
2. Special operations require more detailed criteria as to the adequacy of the building and facilities. Thus, the requirements for aseptic processing must include floors, walls, and ceilings of smooth, hard surfaces that are easily cleanable.

   Temperature and humidity controls.

   An air supply filtered through high-efficiency particulate filters under positive pressure, regardless of whether the flow is laminar or nonlaminar.

   A system of monitoring environmental conditions.

   A system for cleaning and disinfecting the room and equipment to produce aseptic conditions.
3. Equipment for adequate[a] control over air pressure, microorganisms, dust, humidity, and temperature shall be provided when appropriate[a] for the manufacture, processing, packing, or holding of a drug product.
4. Sanitation shall be assured by requiring written procedures for cleaning and assigning responsibility of seeing that they are followed. Rodenticides, insecticides, or fumigating agents shall not be used unless registered and used in accordance with the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA). Sanitation procedures shall apply to work performed by contractors.

Subpart D: equipment.

1. Adequacy of equipment design, size, and location should be validated.
2. Equipment cleaning and maintenance record keeping is essential.

Subpart E: control of components and drug product containers and closures.

1. Take appropriate measures to establish suitable specifications, and assure conformance with the specifications by proper records.[a]
2. Retest components, drug product containers, and closures as necessary due to conditions or passage of time that might adversely affect them.
3. Assure that drug product containers and closures are not reactive, additive, or absorptive, so as to alter the strength, quality, or purity of the drug beyond the applicable specifications.
4. Enforce standards and specifications to ensure that such hazards as pyrogens are eliminated from containers and closures for parenterals.

Subpart F: production and process controls.

1. Provide written procedures and change control with approval by the quality control unit.
2. Validate each process to demonstrate that it will consistently do what it purports to do.

[a]Such words as *adequate* and *appropriate* are used frequently in this and other sections of the CGMP. This puts the burden on the manufacturer of showing through data and performance records that the selections are "adequate" and "appropriate." Such flexibility is viewed by industry as a desirable attribute in the cGMP.

3. Control against microbiological contamination, including validation of the sterilization process.
4. Reprocessing must be based on procedures that *will ensure* that reprocessed batches will conform to *all* established standards, specifications, and product characteristics.

Subpart G: packaging and labeling control.

1. Provide written procedures and documentation to assure that every stage, from the design, receipt, identification, storage, and handling of labeling and packaging to their application to the drug product, is adequately controlled.
2. Use of gang-printed labeling for different drug products, or different strengths or net contents of the same drug product, is prohibited unless the labeling from gang-printing sheets is adequately differentiated by size, shape, or color.
3. Labeling reconciliation is waved for cut or roll labeling if a 100% examination for correct labeling is performed.
4. All prescription drug products and most OTC drug products shall have expiration dates on their labeling on the basis of adequate stability studies. However, the commissioner proposed in a separate *Federal Register* document published at the same time as the cGMP final rule that certain OTC drug products be exempted from expiration dates. These included those OTC drug products used without dosage limitation provided that it could be shown that they are stable for at least three years. Drug products to be reconstituted at time of dispensing shall bear expiration information for both the reconstituted and unreconstituted products.

Subpart H: holding and distribution.

1. There shall be written procedures to describe the warehousing. Where necessary to produce product, there shall be appropriate environmental controls.
2. There shall be written distribution procedures so that any recalls, if required, can be handled expeditiously.

Subpart I: laboratory controls.

1. Any specifications, standards, sampling plans, test procedures, or other laboratory controls, such as stability testing, are to be approved by the quality control unit.
2. The laboratory controls required are specified.
3. Testing and release procedures are specified for the usual drug products and exceptions in the case of short-lived radiopharmaceutical parenterals where batches may be released prior to completion of sterility and/or pyrogen testing. Appropriate laboratory testing is provided for, as necessary, of each batch of drug product required to be free of objectionable microorganisms.
4. A written stability program on the basis of studies conducted in the same container-closure system in which it will be marketed is required.
5. Products purporting to be sterile and/or pyrogen-free must be batch tested prior to release.
6. Reserve samples must have at least twice the quantity necessary for all tests except for sterility and pyrogen testing.
7. Animals used in testing components, in-process materials, or drug products for compliance with established specifications shall be maintained and controlled in a manner that assures their suitability for their intended use.
8. If a reasonable possibility exists that a non-penicillin drug product has been exposed to cross-contamination with penicillin, the non-penicillin drug product shall be tested for the presence of penicillin.

Subpart J: records and reports.

1. Documentation through written procedures and records is now required for practically all operations. The items to be reported in a laboratory assay report are spelled out for the first time in the cGMP.
2. Complaints must be documented, and procedures must be followed in the investigation of complaints by quality control.
3. The quality control unit is responsible for review of all production and control records.

Guidelines

Guidelines are a tool used by the FDA to explain its interpretation of what is required to meet the regulations. Whenever the FDA perceives that the pharmaceutical industry does not understand the regulations or their intent, a group of in-house experts is assembled to generate the proposed guideline. Once the proposed document is submitted for public review and comment, it is finalized. Below is a list of guidelines that have been written and can be applied to parenteral drug products.

GMP Guidelines

Guideline on General Principles of Process Validation, May, 1987, and November 2008 Draft

Guideline on Sterile Drug Products Produced by Aseptic Processing, September, 2004

Guideline on Validation of the Limulus Amebocyte Lysate Test as an End-Product Endotoxin Test for Human and Animal Parenteral Drugs, Biological Products, and Medical Devices, December, 1987

Guideline on the Preparation of Investigational New Drug Products, February, 1990

Guideline on Submission of Documentation in Applications for Parametric Release of Human and Veterinary Drug Products Terminally Sterilized by Moist Heat Processing, August 2008, Draft

The most significant guideline for parenteral drug manufacture is the guideline for aseptic processing.

The first aseptic guideline was issued in 1987. In an aseptic process, the drug product, container, and closure are first subjected to sterilization methods separately, as appropriate, and then brought together. It described the facilities, equipment, environmental conditions, and process validation requirements for products produced using aseptic processing. In addition, it described environmental monitoring and laboratory testing requirements. The only specific acceptance criteria given was nonviable particulate limits for clean rooms and adjacent areas and the acceptance criteria for the media fills used to validate the aseptic processes. Particulate limits are based on Federal Standard 209 (withdrawn). Media fill acceptance criteria was listed at 1 positive unit per 1000 units filled with media. This represents a contamination rate of 0.1%. A minimum of 3000 units was required to be able to detect the contamination rate with 95% confidence.

In 2004, the FDA issued a revised guideline (4). The revision was necessary to incorporate improvement made by the pharmaceutical industry and new concepts being promoted by the FDA. During the development of the guideline, a great deal of effort was made to try to harmonize as much as possible with the EU requirements. In addition, the FDA assembled an expert panel made up of industry experts to assist in answering specific questions. This is the first time the industry has had any influence on guidelines during the development phase. Most of the recommendations made by the expert panel were adopted by the FDA.

The most significant changes in the 2004 guideline are as follows:

- Microbial limits were added to guideline.
- The nonviable and viable airborne particulate limits that are stated in the EU guide were adopted. The major exception is that settling plates are not required or expected by the FDA. The FDA also adopted the International Organization for Standardization (ISO) designations for clean room classification instead of the A to D designations used by the EU.

Clean area classification (0.5 μm particles/ft^3)	ISO designation	>0.5 μm particles/m^3	Microbiological active air action levels (cfu/m^3)	Microbiological settling plates action levels (diam. 90 mm; cfu/4 hr)
100	5	3,520	1	1
1,000	6	35,200	7	3
10,000	7	352,000	10	5
100,000	8	3,520,000	100	50

- Differential pressure is now expressed in pascals and as a range instead of a single value, that is, 10 to 15 Pa versus 0.05 in. of water. This range is the same as that in the EU.
- The requirement that the velocity in a unidirectional flow area should be 90 ± 20 ft/min has been deleted. Instead each site must justify and validate that the velocities used are appropriate. Typically, airflow studies are used to demonstrate that at the measured velocity the airflow sweeps particles away from the product without generating turbulence. It also prevents extrinsic particulate matter from getting into the product.
- The FDA expects environmental monitoring data to be trended and analyzed for any trends that could indicate a potential risk to the products.
- A recommendation that the quality unit view all process simulations (media fill) was added. While this is a recommendation, it appears that the FDA investigators expect to see evidence that QA does observe and take notes on activities occurring during the process simulations.
- The FDA recommends that the media fill program address applicable issues such as
  - factors associated with the longest permitted run on the processing line that can pose contamination risk (e.g., operator fatigue);
  - representative number, type, and complexity of normal interventions that occur with each run, as well as nonroutine interventions and events (e.g., maintenance, stoppages, equipment adjustments);
  - lyophilization, when applicable;
  - aseptic assembly of equipment (e.g., at start-up, during processing);
  - number of personnel and their activities;
  - representative number of aseptic additions (e.g., charging containers and closures as well as sterile ingredients) or transfers;
  - shift changes, breaks, and gown changes (when applicable);
  - type of aseptic equipment disconnections/connections;
  - aseptic sample collections;
  - line speed and configuration;
  - weight checks;
  - container closure systems (e.g., sizes, type, compatibility with equipment); and
  - specific provisions in written procedures relating to aseptic processing (e.g., conditions permitted before line clearance is mandated).

  This is the first time that the FDA has given such details concerning what should be covered by the process simulation procedure/protocols.

- The process simulation acceptance criteria was changed as follows:
  When filling fewer than 5000 units, no contaminated units should be detected.
  - One contaminated unit is considered cause for revalidation, following an investigation.

  When filling from 5000 to 10,000 units
  - One contaminated unit should result in an investigation, including consideration of a repeat media fill.
  - Two contaminated units are considered cause for revalidation, following investigation.

  When filling more than 10,000 units
  - One contaminated unit should result in an investigation.
  - Two contaminated units are considered cause for revalidation, following investigation.

It should be noted that it does not matter if 10,000 units or 150,000 units are filled, the acceptance criteria is the same. This is a significant change from the previous contamination rate concept where the more units filled with media, the more positive units allowed. Both the pharmaceutical industry and the FDA agreed that this approach was inappropriate. The goal of aseptic process is to produce a sterile product, that is, zero units contaminated. On the basis of industry input it was determined that the above criteria represented the current industry capabilities.

Three new sections were added to the aseptic guideline to address the following:

- **Aseptic processing isolators**
  The main concerns raised were as follows:
  - Glove integrity.
  - Proper isolator design.
  - Pressure differentials—promotes the need for ISO 5 protection at opening in the isolator.
  - Isolators must be in classified rooms. ISO 8 is recommended. The isolator is prohibited from being in an unclassified room.
  - There is an extensive discussion of decontamination of isolators. For example, biological indicators must be used, if decontamination is used for product contact parts, a six-logarithm reduction must be proven and the frequency of decontamination must be justified and have supporting data.
- **Blow/fill/seal technology**
  Blow/fill/seal (BFS) machines must be designed to prevent extraneous contamination. The room environment can be ISO 8. Another major concern is container/closure integrity. The FDA requires that reliable and sensitive inspection processes must be established to make sure every unit is intact.
- **Processing prior to filling and sealing operations**
  Process simulations must cover all aseptic manipulations that occur to the product prior to the manufacturing process, including the holding times for sterile bulks.

Compliance Policy Guides

Compliance policy guides (CPGs) were developed as a mechanism of disseminating the FDA policy to the district offices. CPGs were developed by centers or other headquarters units to explain how the FDA will enforce various aspects of the regulations or various situations that the field investigator may find. They are usually developed in response to questions on how to interpret a specific regulation or what is the agency policy concerning a specific subject. CPGs can be obtained through the National Technical Information Service or at http://www.fda.gov.

One CPG that is particularly applicable to parenteral drugs is guide 7132a.13, "Parametric Release—Terminally Heat Sterilized Drug Products." In this guide the FDA defines parametric release as "a sterility release procedure based upon effective control,

monitoring, and documentation of a validated sterilization process cycle in lieu of release based upon end-product sterility testing." The FDA will only accept parametric release for terminally heat sterilized parenteral drug products. Parametric release of drug products sterilized by filtration or ethylene oxide will not be allowed. For those products that are the subject of a new drug application, the manufacturer must submit a supplement and obtain approval prior to initiation of parametric release. Parametric release of drug products that do not require new drug applications cannot be used until the above requirements have been met. The firm should have the data to support parametric release at the manufacturing site. Firms planning on parametric release of non-New Drug Application (NDA) drug products should contact the local FDA district office prior to initiation so that the FDA can determine that they have met all the required criteria. There are four requirements listed in the guide that must be met before parametric release can be considered by the FDA.

1. The sterilization process cycle has been validated to achieve microbial bioburden reduction to 10° with a minimum safety factor of an additional six-logarithm reduction. All cycle parameters must be identified by the manufacturer as critical, for example, time, temperature, and pressure, or noncritical, for example, cooling time and heat-up time. Failure of one of the critical parameters must result in automatic rejection of the sterilizer load. Biological indicators can be used to evaluate cycle lethality where equipment malfunction prevents measurement of one critical cycle parameter. If more than one critical parameter is not met, the batch is considered nonsterile despite biological indicator sterility.
2. Integrity for each container/closure system has been validated to prevent in-process and postprocess contamination over the product's intended shelf life.
3. Bioburden testing, covering total aerobic and spore counts, is conducted on each batch of presterilized drug product. Resistance of any spore-forming organism found must be compared to that of the organism used to validate the sterilization cycle.
4. Chemical or biological indicators are included in each truck, tray, or pallet of each sterilizer load. Both chemical and biological indicators must be fully characterized and documented. Chemical indicators cannot be used to evaluate cycle lethality due to lack of time/temperature accuracy.

The FDA issued a draft guideline on the documentation that must be submitted in applications to support parametric release of human, biological, and veterinary drugs (4). The definition of parametric release has been revised to conform to the new focus of FDA. It is defined as "a sterility assurance release program where demonstrated control of the sterilization process enables a firm to use defined critical process controls, in lieu of the sterility test." The release program should be based on the results of a risk assessment of the terminal sterilization cycle, demonstration of process understanding, and prior knowledge of the production and sterilization process.

EU REGULATIONS

EU Directives

Directives in the EU are the same laws as in the United States. The directive that requires the EU member states to ensure that pharmaceutical manufacturers comply with GMP is in Chapter IV of Directive 75/319/EEC for human products and Chapter V of Directive 81/851/EEC for veterinary products. Another Directive, 92/25/EEC, requires all wholesale distributors to be authorized and comply with guidelines on Good Distribution Practice (GDP).

The principles and guidelines of GMP are stated in two directives. Directive 91/356/EEC and 2003/94/2003 are for human medicinal products and 91/412/EEC is for veterinary products (5). It must be noted that while these are termed guidelines they should be treated the same as the GMP regulations in the United States. They are enforceable under the member state laws.

GMP Regulations

The GMP regulations are organized into nine general chapters.

Chapter 1 Quality Management
This chapter covers the requirements for quality assurance and quality control.

The system of quality assurance appropriate for the manufacture of medicinal products should ensure that

1. medicinal products are designed and developed in a way that takes account of the requirements of GMP and Good Laboratory Practice;
2. production and control operations are clearly specified and GMP adopted;
3. managerial responsibilities are clearly specified;
4. arrangements are made for the manufacture, supply, and use of the correct starting and packaging materials;
5. all necessary controls on intermediate products and any other in-process controls and validations are carried out;
6. the finished product is correctly processed and checked, according to the defined procedures;
7. medicinal products are not sold or supplied before a qualified person has certified that each production batch has been produced and controlled in accordance with the requirements of the marketing authorization and any other regulations relevant to the production, control, and release of medicinal products;
8. satisfactory arrangements exist to ensure, as far as possible, that the medicinal products are stored, distributed, and subsequently handled so that quality is maintained throughout their shelf life; and
9. there is a procedure for self-inspection and/or quality audit that regularly appraises the effectiveness and applicability of the quality assurance system.

It should be noted that the legal responsibility for the quality of products rests with the qualified person. This is very different from the United States, where the CEO/president is ultimately held responsible for the quality of the products manufactured by the company. A qualified person must be noted in each Market Authorization Application.

The basic requirements of quality control are that

1. adequate facilities, trained personnel, and approved procedures are available for sampling, inspecting and testing starting materials, packaging materials, intermediate, bulk, and finished products, and, where appropriate, for monitoring environmental conditions for GMP purposes;
2. samples of starting materials, packaging materials, intermediate products, bulk products, and finished products are taken by personnel and by methods approved by quality control;
3. test methods are validated;
4. records are made, manually and/or by recording instruments, which demonstrate that all the required sampling, inspecting, and testing procedures were actually carried out. Any deviations are fully recorded and investigated;
5. the finished products contain active ingredients complying with the qualitative and quantitative composition of the marketing authorization, are of the purity required, and are enclosed within their proper containers and correctly labeled;
6. records are made of the results of inspection and that testing of materials, intermediate, bulk, and finished products is formally assessed against specification. Product assessment includes a review and evaluation of relevant production documentation and an assessment of deviations from specified procedures;
7. no batch of product is released for sale or supply prior to certification by a qualified person that it is in accordance with the requirements of the marketing authorization; and
8. sufficient reference samples of starting materials and products are retained to permit future examination of the product if necessary and that the product is retained in its final pack unless exceptionally large packs are produced.

In addition to describing the function of the quality assurance and control, this chapter also describes the elements of the annual review of medicinal products. They are as follows:

1. A review of raw materials used in the product, especially those from new sources
2. A review of critical in-process controls and finished product results
3. A review of all batches that failed to meet established specification(s)
4. A review of all critical deviations or nonconformances and related investigations
5. A review of all changes carried out to the processes or analytical methods
6. A review of marketing authorization variations submitted/granted/refused, including those for third country dossiers
7. A review of the results of the stability monitoring program
8. A review of all quality-related returns, complaints and recalls, including export only medicinal products
9. A review of adequacy of previous corrective actions
10. For new marketing authorizations, a review of postmarketing commitments
11. A list of validated procedures and their revalidation dates
12. A list of qualified equipment and their requalification dates

Chapter 2 Personnel

This chapter describes the duties of key personnel, including the qualified person. Since the qualified person is unique to the EU regulatory system, we will look at the duties of the qualified person. The duties are described in Article 51 of Directive 2001/83/EC and are summarized below.

1. For medicinal products manufactured within the European Community, a qualified person must ensure that each batch has been produced and tested/checked in accordance with the directives and the marketing authorization.
2. For medicinal products manufactured outside the European Community, a qualified person must ensure that each imported batch has undergone, in the importing country, the testing specified in paragraph 1 (b) of Article 51.
3. A qualified person must certify in a register or equivalent document, as operations are carried out and before any release, that each production batch satisfies the provisions of Article 51.

As can be seen, the qualified person is responsible for ensuring the quality of all drug products introduced into the EU market. The person must obtain training and pass a test to become a qualified person. This training and test is to ensure that the person understands the requirements of the job. In some of the member states there are other basic requirements, such as the person must be a pharmacist or have a minimum number of years of experience. If the qualified person releases a product that lacks the required quality, they can be fined or sent to jail for exposing the public to a potential risk of injury.

Chapter 3 Premises and Equipment

The basic requirement is that premises and equipment must be located, designed, constructed, adapted, and maintained to suit the operations to be carried out.

There is a very specific indication that separate facilities are required for highly sensitizing materials or biological preparations. In addition, certain antibiotics, certain hormones, certain cytotoxics, and highly active drugs should not be produced in the same facilities. The quality control laboratory should be separated from production areas, especially microbiology laboratories. U.S. regulations only mention penicillin as requiring separate facilities.

Basically the requirements for the EU and the United States are essentially the same. The main difference is that the EU regulations give more detail as to what is expected.

Chapter 4 Documentation
This chapter describes the types of documents that are required. Documents required are as follows:

- Specifications for starting and packaging materials, intermediate and bulk products, and finished products
- Manufacturing formula and processing instructions
- Packaging instructions
- Batch processing records
- Batch packaging records
- Procedures
  - Receipt of materials
  - Sampling
  - Testing
  - Release and rejection
- Other documents
  - Validation protocol and reports
  - Equipment assembly and calibration
  - Maintenance, cleaning, and sanitation
  - Personnel training, clothing, and hygiene
  - Environmental monitoring
  - Pest control
  - Complaints
  - Recalls
  - Returns

As can be seen there is no difference between the type of procedures that are required by both the EU and the FDA. Again the most striking difference is that the EU guide gives more details.

Chapter 5 Production
The basic requirements are as follows:

- Production operations must follow clearly defined procedure
- Production operations must prevent cross-contamination
- Production rooms and equipment should be identified with the product or material being processed
- All drug production processes and equipment should be validated and revalidated after significant changes to the equipment or process
- Starting materials must come from approved vendors and should be sampled and tested for compliance with applicable specifications
- Printed packaging materials should be controlled and issued by authorized personnel
- There should be physical separation between packaging operations so that mix-ups are prevented. In addition there should be procedure to inspect packaging area prior to use to ensure that all previous product and labels have been cleared from the area
- After packaging, all containers should be inspected to ensure that packaging is complete and contains all required information, especially lot identification and expiration date
- Rejected materials must be stored in separate restricted areas
- Reprocessing of rejected products should be a rare occurrence
- Returned product should be destroyed, but could be used in subsequent batches after testing by the quality control department

You will note that the above requirements are not that different from the U.S. GMP requirements.

Chapter 6 Quality Control
This chapter discusses the elements of Good Quality Control Laboratory Practice. Some of these elements are as follows:

- Documentation—specifications, test procedures, sampling procedures, validation records, and out-of-specification/out-of-trend investigation procedures.
- Sampling—methods, sampling equipment, storage conditions, etc.
- Testing—method validation, analytical results review process, training of analysts, reagent preparation and documentation, glassware cleaning and use, and reference standard handling.
- Special attention is given to on-going stability testing programs. The program should be applied to both bulk active pharmaceutical ingredients and finished dosage forms. Protocols should be developed that describe all testing that is to be performed on products. All on-going stability testing should extend to the end of the shelf life of the product. The results of the stability testing should be made available to key personnel and the qualified person(s).

Chapter 7 Contract Manufacture and Analysis
This chapter outlines the responsibilities of the company that hold the marketing authorization when they contract out the manufacturing and/or analysis of the product. It states that there should be a contract between the parties so that there is a clear understanding of the responsibilities of all the parties involved. The contract must clearly state how the qualified person releasing each batch will exercise their legal responsibilities.

The U.S. regulations are silent concerning this topic. While the FDA encourages quality agreements that state who is responsible for which aspects of the GMP regulations and drug application commitments, there is no regulation that requires the agreement.

Chapter 8 Complaints and Product Recall
All complaints and other information concerning potentially defective products must be reviewed carefully according to written procedures. During review, if a product defect is discovered or suspected in a batch, consideration should be given to check other batches. There should be a periodic review of complaints for trends.

There should be established written procedure to organize any recall activities. Recall operations should be capable of being initiated promptly and at any time. If recall is initiated, all regulatory authorities in all countries where the product was distributed must be notified. All recalled material must be properly identified and stored separately in a secure area. The effectiveness of the recall should be assessed during the recall.

Chapter 9 Self-Inspection
This chapter covers the requirements that manufacturers should conduct self-audits of their operations. Audits can be conducted by independent personnel within the company or by outside experts. All audits must be documented and corrective actions developed for any adverse observations during the audit. While the FDA does not have an equivalent section in their GMP regulations, they do consider it a duty of the quality organization to perform self-audits.

In addition to the above general chapters, there are 20 annexes that give more detailed requirements for specific product types or dosage forms. The annexes are as follows:

Annex 1	Manufacture of sterile medicinal products (revision November 2008). The revised annex should be implemented by March 1, 2009, except for the provisions on capping of vials, which should be implemented by March 1, 2010.
Annex 2	Manufacture of biological medicinal products for human use
Annex 3	Manufacture of radiopharmaceuticals

Annex 4 Manufacture of veterinary medicinal products other than immunological veterinary medicinal products
Annex 5 Manufacture of immunological veterinary medicinal products
Annex 6 Manufacture of medicinal gases
Annex 7 Manufacture of herbal medicinal products
Annex 8 Sampling of starting and packaging materials
Annex 9 Manufacture of liquids, creams, and ointments
Annex 10 Manufacture of pressurized metered dose aerosol preparations for inhalation
Annex 11 Computerized systems
Annex 12 Use of ionizing radiation in the manufacture of medicinal products
Annex 13 Manufacture of investigational medicinal products
Annex 14 Manufacture of products derived from human blood or human plasma
Annex 15 Qualification and validation (July 2001)
Annex 16 Certification by a qualified person and batch release (July 2001)
Annex 17 Parametric release (July 2001)
Annex 18 Good manufacturing practice for active pharmaceutical ingredients requirements for active substances used as starting materials from October 2005 covered under part II
Annex 19 Reference and retention samples (December 2005)
Annex 20 Quality risk management (February 2008)

Annex 1—Manufacturer of Sterile Medicinal Products

Of the annexes, the one that has received the most attention is Annex 1 concerning the manufacture of sterile medicinal products. This annex gives very specific guidance as to what is required to produce sterile drug products.

While the FDA guidelines, in general, do not give specific values, the FDA does require manufacturers to have documentation to justify that what they are doing is appropriate. In Annex 1, the EU gives very specific requirements and limits. The current version became official on March 1, 2009, except for the provision on capping of freeze-dried vials, which becomes official on March 1, 2010.

Probably the most confusing requirement in the annex is the testing of nonviable airborne particulates in the "at-rest" and "in-operation" states. Limits are given for both states. These non-viable particulate limits are now closer to the limits in ISO14644 than in the previous version of annex 1. Except for grade A there is a different limit for the two states. Normally the at-rest state is only tested during commissioning of a new clean room or after significant changes are made to an existing clean room. But it appears that the EU investigators are requesting that at-rest testing be performed periodically on a routine basis. Currently, the FDA only considers the in-operation state for all of its recommendations. They are concerned with the condition of the environment when product is being exposed to it.

The following table gives the particulate limits for the four clean room grades.

	Maximum permitted number of particles per m^3 equal to or greater than the tabulated size			
	At rest		In operation	
Grade	0.5 μm	5.0 μm	0.5 μm	5.0 μm
A	3,520	20	3,520	20
B	3,520	29	352,000	2,900
C	352,000	2,900	3,520,000	29,000
D	3,520,000	29,000	Not defined	Not defined

As can be seen, the other major difference between the EU and the FDA is the requirement to measure the particles at 5.0 μm. This has been a very controversial difference. The industry has argued that the 5 μm particles do not have to be measured. If you are going to market a product in the EU, you will have to monitor for both 0.5 and 5.0 μm particles.

In addition, there is a requirement that after operations have been completed, the at-rest limits should be attained after a 15 to 20 minutes "clean-up" period. During the at-rest stage no activities or personnel are present.

The annex also gives limits for microbiological monitoring of clean room, as shown in the following table.

	Recommended limits for microbial contamination			
Grade	Air sample cfu/m^3	Settle plates (diameter 90 mm) cfu/4 hr	Contact plates (diameter 55 mm) cfu/plate	Glove print 5 fingers cfu/glove
A	<1	<1	<1	<1
B	10	5	5	5
C	100	50	25	–
D	200	100	50	–

It should be noted that all of the values in the above table are average values. There is no explanation of what is to be averaged. From discussions with EU inspectors, it appears that all of the plates in a grade A zone can be averaged and then compared to the given limits. This is a dangerous concept since a problem area could be averaged out by the other areas in the same zone. For example, there could be a high count at the area where sterile stopper are placed in a hopper but very low values at other locations. The average would indicate no problem but the individual value would indicate a potential contamination risk at the stopper hopper area.

The annex gives grades under which operations should be performed. For instance filling of high-risk terminally sterilized products should be done in a grade A area with a grade C background. Formulation is to be performed in a grade C area. For aseptically filled products, filling must be conducted in grade A with a grade B background. For isolators, the EU allows the background to be grade D at rest, while the FDA requires grade C in operation. But for a BFS machine, the background must be grade C with personnel in grade A/B clothing.

One other area of difference between the EU and the FDA is the issue of capping of vials. The EU requires that vials with missing or displaced stoppers be rejected prior to capping and that the capper be either in the clean room or in a restricted access barrier system (RABS) supplied with grade A air. The major problem with this concept is that caps are very dirty from a particulate matter viewpoint and should never be in a clean room. In addition, if the capper is in the clean room then all of the caps have to be sterilized. Since the caps take up a lot of space, this could have a significant impact on the sterilization capacity of the facility. While the FDA requires that the vials be protected with grade A air prior to capping, they have not required that the enclosure be a RABS design. Capping machines typically take a lot of adjustments in the beginning and sometimes during production. Making the adjustments through glove ports would be a problem, not only from an equipment design viewpoint but also from a personnel safety viewpoint. Currently when one of the doors is opened, the capper automatically shuts off. With glove ports, a new detector system would have to be developed and installed that would shut off the capper when someone inserts their hand into a glove. The requirements in this section of the EU guidance will have very little impact on the sterility assurance of parenteral products. Missing stoppers are already detected during the 100% inspection that takes place after capping. Slightly raised stoppers are only a concern for lyophilized products where the stopper has slits in the sides. Even here the stopper would have to be significantly displace to present a high risk and should be able to be removed during lyophilizer unloading operations.

The other section of the EU GMP guide gives the same information as required by the other regulatory authorities and has caused little discussion over the years.

This chapter would be remiss if it did not discuss one of the guidelines in volume 3 of "The rules governing medicinal products in the European Union." The EU has published a series of scientific guidelines to harmonize the manner in which the EU member states and the EMEA interpret and apply the detailed requirements for the demonstration of quality,

safety, and efficacy container in the Community Directives. In addition, these guidelines are also intended to help companies make sure that their marketing authorization applications will be recognized as valid by the EMEA. This means the EMEA investigators will audit against these guidelines.

In guideline CPMP/QWP/486/95 concerning the manufacture of the finished dosage form, there is a statement that

> "For sterilisation by filtration the maximum acceptable bioburden prior to the filtration must be stated in the application (6). In most situations NMT 10 CFU's/100 mL will be acceptable, depending on the volume to be filtered in relation to the diameter of the filter. If this requirement is not met, it is necessary to use a pre-filtration through a bacteria-retaining filter to obtain a sufficiently low bioburden."

Over the years this has become a mandatory expectation by the EMEA and has forced manufacturers to dual filter parenteral preparations that are filter sterilized. This author has never heard any justification for the 10 cfu/100 mL limit. EU's requirement for dual filtration has caused a lot of discussions and misinterpretations. Many companies have interpreted this to mean that to sterilize a product you need two 0.2 μm filters, and both must be integrity tested and pass to declare the sterilization process successful. But the statement indicates that the first filter is a bioburden reduction filter that will reduce the bioburden to NMT 10 cfu/100 mL. The second filter is the sterilizing filter. So, only the final filter should be integrity tested. If the second filter fails and the first filter passes, you still have a sterility failure from a regulatory viewpoint because the sterilizing filter did not pass integrity. This is another example where the EU has enforceable guidelines, whereas the FDA guidelines are not enforceable.

In this chapter we have looked at the GMP regulations in the United States and Europe. These regulations form the basis for most of the regulations in the other markets of the world. The good news is that the regulatory authorities have been working hard to harmonize their requirements to lessen the burden on pharmaceutical companies, while at the same time protecting the users of the medicinal products from harm.

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2 Risk assessment and mitigation in aseptic processing

James Agalloco and James Akers

INTRODUCTION

Sterile products are frequently administered to patients through the dermal layer to attain rapid therapeutic response and accurate dosing. Delivery in this manner intentionally circumvents the body's protective mechanisms, and mandates that the product be largely free of infectious microorganisms and endotoxin. These concerns are heightened when the drug is delivered to patients whose health is already compromised as is common in clinical settings. Awareness of the patient has prompted regulatory preference for the use of terminal sterilization (1,2). While the use of lethal processes on finished formulations in their final container is favored because of their lethality, material considerations have limited their application such that an estimated 85% of all sterile products are manufactured by aseptic processing that are less abusive of essential material and container properties.[a]

Aseptic processing customarily use a variety of sterilization procedures for the individual components of the formulated product, container, and product contact parts, enabling the sterilizing process to be chosen for preservation of the key quality attributes of the materials. The core aseptic process assembles the sterilized items into the final dosage form in an environment specifically designed for that purpose. Because product containers are closed after the individual sterilization processes are carried out, the potential for contamination ingress is ever present during aseptic processing. In the belief that knowledge of the conditions under which the aseptic process is carried out would be valuable in determining the acceptability of the resulting product, environmental monitoring has historically been considered essential. Microbial sampling of air and surfaces as well as personnel gloves and gown within the aseptic environment were instituted as a means of environmental monitoring, which ultimately evolved into a program thought to provide critical information regarding sterility assurance. When monitoring was first instituted, the environmental conditions and gowning systems were markedly less capable than those presently in use. As a consequence, performance expectations and demonstrated performance were understood to be nonabsolute. Nevertheless, it was certainly understood that improvement in contamination control performance was both desirable and attainable. The gradual refinements in aseptic processing technology and performance expectations took place over a period of some 50 years.

THE MYTH OF STERILITY

The manufacture of sterile products is closely associated with expectations for sterility of the finished dosage form. This is customarily defined by the probability of a nonsterile unit (PNSU) or sterility assurance level (SAL).[b] The minimum expectation for PNSU in terminally sterilized products is that it be no greater than 1 nonsterile unit in one million units or 1×10^{-6}. The origins of this target lie in the food industry as it was initially developed for canned foods where the concern was the avoidance of *Clostridium botulinum*, an anaerobic spore former. The goal was not sterility of the canned foods but an acceptable level of safety for the consumer. In actuality, it defines a maximum level of risk that a consumer might be exposed to in the consumption of the sterilized material. This approach is essentially the same as that employed for the terminal sterilization of pharmaceutical products, which while stated as a PNSU, it is really a statement of the level of material safety (or risk minimization). Aseptic processing relies on the component and material sterilization methods for success, but differs in that

[a]This is estimated as a percentage of products and not as a percentage of the number of containers.
[b]The current preference is for the use of PNSU rather that SAL, because PNSU is a far easier concept for the novice to interpret.

calculation of a PNSU (or SAL) is impossible there being no directly lethal element of the aseptic manufacturing process. Aseptic processing performance is evaluated using process simulation studies in which the maximum contamination rate in the exercise demonstrates the capability of the overall aseptic process during that exercise and that exercise alone. Suggestions that the success in a process simulation defines the sterility assurance capability of an aseptic process are entirely fallacious. The process simulation is a singular event comprised of a number of individual sterilization, manual decontamination, and manipulations that cannot support the ability of those practices under different circumstance. The simulation demonstrates potential capability in a limited manner, but there are no means to extend the results to the same aseptic procedures in a separate event.

There is a common belief that the environmental monitoring performed in conjunction with every aseptic process provides a means for extension of the simulation performance to production operations. The limitations of microbial recovery from environmental samples in present-day manned clean rooms are such that these claims are certainly spurious. Extension of this thinking to advanced aseptic processing technologies is similarly inappropriate. What has been demonstrated for every aseptic processing is that it can be most realistically described as safety. Aseptic processes are essentially considered safe because the patient outcomes are successful and contamination in aseptically filled products has only infrequently been linked to known product contamination derived from the aseptic process. Our industry's ability to detect contamination in aseptic processes through any form of environment monitoring is extremely limited both in terms of analytical limit of detection and sampling statistics. The monitoring sample sizes are too small to afford any meaningful evaluation of the conditions, and the cultural methods employed do not have a limit of detection approaching zero. The sterility test is of such limited value in assessing process efficacy that it could be more aptly termed "the test for gross microbial contamination." The FDA's recalls of aseptically produced products are rarely the result of demonstrated contamination in the finished product, but rather an absence of appropriate conditions or inadequate documentation during the production operations. What has been attained with aseptic processing is more properly described as "safety." Sterility of aseptically filled products is completely unprovable, as it would require the evaluation of an infinite sample size with analytical method capable of detecting *any* contamination present. This is simply impossible, so realistically proving sterility in aseptic processing is not simply a matter of being willing to make a greater effort in terms of sampling intensity.

The improvements in aseptic processing were instituted to effect greater control over the environment, as influenced by its basic design, decontamination method, and operator involvement with a singular goal of reducing the contamination potential. The true objective has always been reduction of risk to the patient receiving the aseptically produced product. Aseptic processing systems in their most evolved forms have reached the point where the means to establish their acceptability are no longer adequate to provide any meaningful indication of performance.

As the processing capability evolved, closely followed (or at times preceded) by regulatory expectations, a critical component of the monitoring system remained unchanged. With each refinement of aseptic processing technology, the microbial sampling methods were increasingly taxed. In today's advanced aseptic processing systems, the environmental monitoring is being asked to prove an absolute negative—that no microorganisms are present anywhere in either the processing environment or the product. While particularly true of isolators, Restricted Access Barrier Systems (RABS) and many newer conventional aseptic facilities suffer the same limitations. This presents industry with a substantial conundrum of some magnitude with respect to the evaluation, selection, and ongoing control of aseptic processing technologies.

RISK ASSESSMENT

We first noted the limitations of monitoring programs nearly 20 years ago when new facilities began to exhibit environmental control capabilities that challenged the sensitivity and resolution of monitoring methods then available. When contamination was detected in these environments, it was increasingly associated with personnel. This was consistent with the long-standing understanding across the industry and regulators that personnel are responsible for the majority of contamination in an aseptic process (3,4). Deceased former FDA inspector

Hank Avallone had expressed this exact belief in direct manner during the 1980s, "It is useful to assume that the operator is always contaminated while operating in the aseptic area. If the procedures are viewed from this perspective, those practices which are exposing the product to contamination are more easily identified" (5). Actually, from experience and published research, we need not merely assume that an operator is a source of contamination, rather we can take it as an absolute certainty that clean room personnel function as mobile contamination generators. The idea that it is possible to have "sterile" clean rooms or sterile gowned operators has in fact been completely debunked.

In late 2004, when we began the development of our risk analysis method, we drew heavily on the concept that the release of contamination by operators was not merely possible but rather inevitable. With this simple truth in mind we focused our method on the human actions that are central to any aseptic process (6,7). The Agalloco-Akers (A-A) methods attribute risk almost exclusively to human activity within the aseptic process. The closer, longer, and more invasive the personnel intervention, the greater the risk is for contamination introduction. In taking this tact we discounted the more traditional approaches to risk assessment that endeavored to associate risk with contamination transfer to open containers from the air or surfaces (8,9). While we agree with the basic premises of these methods, the calculations required to calculate a risk value include values for which there is no reliable input data. It is our belief that because these methods utilize microbial recovery determination as a fundamental factor in the determination of the contamination ingress potential (and thus risk), they are inherently limited where the background microbial levels are largely devoid of recoverable microorganisms. Also, it is clear, given the variability of microbial analysis and the extremely limited sample size, that it is not possible for monitoring to give us much insight regarding patient risk. It therefore follows that it is not possible to determine through monitoring that an appropriate level of "sterility assurance" has been attained, or to assess anything but truly gross changes in environmental control.

Since the publication of the A-A method, it has been successfully utilized by several firms to evaluate and improve their aseptic processing operations.[c] Katayama and his colleagues compared its application to other risk methods and concluded that the A-A method offered the closest correlation to the historical performance at several aseptic sites when compared with other aseptic risk methodologies (10). A more general means for risk assessment related to sterile products has been developed by Parenteral Drug Association (PDA) (11). Regardless of the risk assessment methodology employed, it is essential that firms consider how their designs, practices, and expectations impact the contamination potential. Assumptions about outcomes must be evaluated in a rigorous manner to provide the greater confidence in the eventual design. Not only is this scientifically sound but is also expected by regulators (12,13).

Discussion of aseptic processing risk, or truly any risk assessment, should not end with completion of the assessment. It must be acknowledged that while risk assessment is an important task, it is not an end onto itself. It must be followed by a far more important activity, which is risk mitigation. Consider the driver of an automobile who notices that it is beginning to rain. This is the risk assessment, and although necessary it does not effect any improvement in the driver's safety. Until the driver mitigates the risk in an effective manner, there is no real benefit. Unless the driver adjusts the speed and turns on the wipers and the lights, there is no reduction in its risk potential. The assessment of risk is only the first step that must be accomplished, and aseptic processing is no different.

RISK MITIGATION

Risk assessment alone however is not enough; if the fundamental concepts adopted are inadequate, the resulting risk might be lowest for a specific design, but not the lowest possible. It would be far preferable to define and utilize design principles that ultimately result in the best aseptic processing design for a specific application. In considering what criteria to utilize, we believe adherence to the core principles of advanced aseptic processing is most appropriate: "An advanced aseptic process is one in which direct intervention with open product containers or exposed product contact surfaces by operators wearing conventional cleanroom garments is not required and never permitted" (14).

[c]Akers J and Agalloco J, personal communications, 2005–2009.

Full consideration of this expectation can be utilized to define the elements of facility design, equipment selection, container/closure selection, product delivery, personnel, procedure definition, and environmental monitoring.

Facilities

The selection of an appropriate advanced aseptic technology is central to nearly all of the subsequent design choices. The choice is often between closed RABS and isolators; however, other designs and technologies should be given due consideration. Once that basic choice has been made, there are options within those alternatives that should be considered as well to further define the technology to be implemented. The design process for an aseptic facility is a lengthy process: proceeding from conceptualization to preliminary and detailed design with a myriad of choices and decisions to be made throughout. Considering the core objective, the following preferences can be defined:

- Design for ease of execution through the choice of construction materials, design for ease of access, and detail elements that facilitate both cleaning and decontamination of the core environment.
- The material, personnel, and equipment flows should be defined to minimize mix-ups and contamination potential.
- The heating, ventilating, and air conditioning (HVAC) system should provide adequate air quality and pressurization to prevent the ingress of contamination.
- Air flow should be sufficient to provide high dilution rates, particularly within the most risk intensive locations within the environment.[d]
- Air systems should be supplied with high efficiency particle air (HEPA) filters that are periodically integrity tested.
- Differential pressures for the system should be controlled, monitored, and alarmed to support continuous integrity of the critical core area.
- Temperature and humidity should be controlled to maximize personnel comfort during operations consistent with product stability/safety requirements.
- Materials and personnel airlocks should be utilized to increase separation between environments of different classification
- Facility and enclosure surfaces must be resistant to the potential corrosive action of sanitizing and decontamination agents, especially sporicidal agents because of their generally greater chemical activity.
- The core aseptic environment should be maintained in an "aseptic" condition when in an operational state and periodically sanitized or decontaminated. Isolators and closed RABS should be decontaminated with sporicidal agents on a periodic basis.
- Only a minimum of materials should be retained in the aseptic portion of the facility through the utilization of just-in-time delivery to the aseptic area.
- Subjective regulatory tenants of aseptic processing such as smoke studies, air velocity measurements, unidirectional air flow, absence of eddy's should be considered but not overly weighted in the definition of HVAC design details. The absence of turbulence in any aseptic production environment is not physically achievable, and there are no objective metrics to define acceptable or unacceptable conditions.
- The completion of operation of RABS should be possible in a "closed" mode. Open door interventions during aseptic processing are never acceptable

Equipment/Utensils

In aseptic processing, the processing equipment located within the enclosure is always critical to success. The reliability of the equipment and the sophistication of its design are paramount in minimizing the need for interventions within the enclosure.

[d]The use of high air dilution rates in isolators has not been demonstrated to be of any meaningful benefit as it is with other aseptic processing designs.

- All product contact surfaces should be sterilized using validated methods (vibratory feed systems may be exempted from this requirement) provided they are high-level decontaminated with a sporicidal agent in situ. Their installation following sterilization often entails substantial and lengthy interventions that can result in contamination risk. Even in separative technologies, the need to curtail interventions persists.
- Sterilization-in-place and clean-in-place should be utilized wherever possible for product and gas delivery lines and filters. At the current state of technology, sterilization-in-place is possible for all types of aseptic filling processes including powder fill.
- Equipment and utensils should be sterilized in hermetically sealed containers/wrapping. The container design should be supported by scientific proof of their integrity. In separative technologies decontaminating utensils in situ may be the best alternative.
- Equipment and utensils should remain within sterile containers/wraps until entry into the critical zone just prior to use to avoid contamination that would occur if they were exposed in the adjacent less-clean environment.
- Equipment and utensils should be sterilized/depyrogenated using a just-in-time approach.
- Processing equipment should be selected for high reliability, ease of changeover, and remote adjustment. Wherever possible they should be self-clearing to eliminate the need for personnel intervention in the event of a miss-feed, jam, or other fault.
- Equipment change over from one format to another should be possible with a minimum of manual intervention.
- The process equipment should use Process Analytical Technologies (PAT) and other feedback systems for ease of control, operation, and documentation. This can result in fewer interventions in both the critical and background environments.
- Non–product contact portions of the equipment should be easily decontaminated and noninvasive of the critical zone.
- Equipment and enclosure surfaces should be resistant to the potential corrosive action of sanitizing and decontamination agents.
- Equipment surfaces within closed RABS should be easily accessible for high-level decontamination, automatic decontamination systems in RABS should be favored over manual decontamination activities as they are inherently lower risk since they can be accomplished with the system in a fully closed configuration and without human contamination.

Containers/Closures/Components

The containers and closures necessary are perhaps the most important items in an aseptic process. The ease of their introduction, transfer, movement, placement, and closure must all be successfully accomplished by the equipment with a minimum of human intervention. It should be immediately evident that they need to consistently process throughout the system, and thus high-quality components with extremely tight dimensional tolerances may be a required when compared to what might be customary in a less advanced (and thus markedly less capable) processing system part. As more complex and multifaceted combination products and medical devices are aseptically produced, the ability to sterilize, introduce, and feed components with complex shapes and special fitments have become an absolute requirement. Robotics, which can now withstand frequent exposure to agents such as vapor phase hydrogen peroxide (VPHP), can often handle complex parts and by utilizing vision systems and laser guidance achieve levels of flexibility and precision that would be impossible by more conventional means.

- Containers/closures/components must be prepared and sterilized/depyrogenated using validated processes.
- Containers/closures/components should be introduced in a manner that retains at least one layer of sterilized container or hermetic wrapping until entry into the critical zone. It is important to remember that in advanced aseptic processing systems such as

isolators, the entire enclosure must be considered the critical zone. The container should have a defined level of integrity. An important rule in isolator systems or closed RABS is that nothing should ever be transferred into the enclosure that is not equal to or lower than that environment. This necessitates the use of VPHP pass boxes, E-beam tunnels, or pass through systems that can be validated using biological indicators or in the case of radiation dosimetry. With proper design, execution, and procedures, Rapid Transfer Ports (RTPs) can also serve as transfer devices that ensure that the objective of taking in objects of equal or better contamination control quality than the environment is met.

- Containers/closures/components should be selected for reliability of handling in the processing equipment to avoid the need for corrective interventions. Higher Acceptable Quality Levels (AQLs) for defects can result in a reduction in the need for interventions.
- Containers/closures/components should be sterilized/depyrogenated using a just-in-time approach. Inventories of materials within the aseptic environment (especially the critical environment) should be minimized. In a typical separative technology–based aseptic processing space does not allow for substantial accumulation of parts and they are therefore typically transferred in on an as needed basis. However, if the criterion of transferring only materials of equal or better contamination controls quality than the enclosure environment, there is no reason to be concerned that such objects might become contaminated within the validated use or campaign period of an enclosure. Materials do not become less microbiologically clean over time in a well-controlled, separative, and unmanned environment.

Product

Delivery of sterile product to the critical zone is easily accomplished with minimal risk using either directly piped connections or RTP connection systems.

- Production materials must be prepared and sterilized using validated methods.
- Liquid product delivery piping should be cleaned and sterilized in place. Gas delivery piping should be sterilized in place.
- Any product delivery and other aseptic connections (e.g., inert gas) should be made within the enclosure.
- Where product is supplied to the critical zone in sterile container (e.g., sterile powders), it should be introduced in a manner that retains at least one layer of sterilized protective covering or wrap until entry into the critical zone.

Personnel

The operating personnel must be diligent in the operation of the equipment and adherence to the core principles of aseptic processing technique at all times. The permanent use of thicker gloves on an enclosure must not be misinterpreted as permission to operate in violation of defined aseptic procedures.

- Personnel must receive initial and periodic formal training in current Good Manufacturing Practices, aseptic processing, microbiology, aseptic gowning, and job specific tasks they must perform.
- Where appropriate personnel should be initially and periodically thereafter assessed for their proficiency in aseptic gowning; of course in many advanced aseptic processing systems gowning is limited and nonaseptic and will require no real training since it is not a critical risk mitigation factor in isolators and potentially in closed RABS as well.
- Personnel should be initially and periodically thereafter assessed for their proficiency in aseptic technique. Specific training should also be provided for those individuals performing the initial set-up of the equipment prior to the aseptic process. Obviously, in highly automated systems that do not rely in personnel or gowned operators, conventional clean room practices and traditions are not necessary.

- Personnel shall conform to the highest standards of aseptic technique at all times even when working with a closed RABS or isolator.
- Personnel should be periodically monitoring when exiting from the aseptic core. Isolator or closed RABS gloves, however, need only be tested at the end of a production run or campaign. It is not desirable to leave media residues on gloves and sleeve assemblies. Also, in separative technologies, glove integrity is the key to risk mitigation. That which does not leak cannot pass microorganisms, therefore physical testing is generally a better solution.
- Gown materials should be cleaned and sterilized using validated methods. It is not necessary to use sterile gowns in rooms surrounding isolators. Also, it is worth remembering that sterility is always a trade-off between microbial "kill" and damage to materials. Thus, extreme overkill is unwarranted where gowning materials are concerned; damage to the gown's integrity is a far greater concern than achieving extreme sterilization lethality levels that are meaningless anyway.
- Gloves must never contact product contact surfaces within an enclosure. Also, the gloves when used to make adjustments must never be put at risk from punctures, tears, or pinching. The operator should also avoid stretching glove/sleeve assemblies in an attempt to reach something within an enclosure. Stretching beyond the initial point of resistance can lead to wear at the glove/sleeve junction and perhaps even a full-blown separation. We cannot overemphasize the need for careful ergonomic design, and should flaws in ergonomics be found in operations they should be corrected immediately. It is possible in many enclosures to relocate gloves to better access positions.

Procedures

Interventions always increase the risk of contamination in an aseptic process even those using advanced technologies (however, the superior environmental control inherent in advanced aseptic technologies makes personal risk a far lower risk factor than in conventional clean rooms). The design of the facility, equipment, component, and product supply should serve to reduce the complexity, duration, and number of interventions. The "perfect" intervention is the one that is not necessary (15).

- Procedures should be critically reviewed to eliminate and/or simplify interventions throughout the aseptic processes.
- All interventions should be designed for minimal risk of contaminating sterile materials.
- Interventions performed during aseptic processing must be recognized as increasing the risk of contamination dissemination.
- All interventions should be performed using sterilized tools whenever possible.
- Defined procedures should be established in detail for all inherent interventions and more broadly for expected corrective interventions (where some flexibility in execution is necessary due to their greater diversity).

Monitoring

The monitoring of an advanced aseptic processing system plays a substantially less important role than it does in ordinary manned aseptic clean rooms, and it is important to recognize that even in standard clean rooms monitoring has a point of diminished return. Its eventual elimination as an anachronism in these extremely clean environments can be expected at some future time. In the interim, any monitoring performed must be accomplished in as minimally invasive and disruptive manner as possible.

- Monitoring of any type must not subject the product to increased risk of contamination. No monitoring is preferable to monitoring that increases the risk of contamination for sterile materials.

- Environmental monitoring activities must be recognized as interventional activities and subject to the similar constraints and expectations (including detailed procedures) as any other intervention.
- Monitoring must be recognized as subject to adventitious contamination pre- and postsampling that is unrelated to the environment, material, or surface being sampled. Methods to minimize that potential beyond what is incorporated into monitoring of conventional manned clean rooms may be necessary.
- Viable monitoring should not be considered an "in-process sterility test" regardless of whether the sample is taken in the enclosure or of a so-called "critical" product contact surface.
- Environmental monitoring results should not be considered as "proof" of either sterility or nonsterility.
- It must be recognized that microbial monitoring can never recover all microorganisms present in an environment nor on a surface.
- The absence or presence of microorganisms in an environmental sample is not confirmation of asepsis nor is it uniformly indicative of process inadequacy.
- Significant excursions from the routine microbial prolife within the enclosure and background environments should be investigated.
- Detection of low numbers of microorganisms in manned clean rooms should be considered a rare, but not unusual event.
- Investigations into recoveries of low numbers of microorganisms in manned clean rooms should be recognized as predominantly make work exercises.
- Process simulations are indicators of process capability but cannot definitely establish the sterility of material produced at another time.
- Process simulations in excess of 5 to 10,000 units are of relatively limited value; their greatest utility is in the evaluation of aseptic set-up practices.

CONCLUSION

What has been presented above represents a major departure from the established doctrine for aseptic processing control. The ever increasing capabilities of aseptic processing technologies have dramatically reduced the utility of the classical monitoring tools that this industry has used for decades. If some future technology enables effective monitoring at the extremely sensitive levels that advanced aseptic processing systems presently provide, there may be justification in their use. We might postulate that by the time those systems become available, future aseptic processing technologies demonstrating superior capabilities to those presently available might make those new monitoring tools moot as well. In the interim, it is clearly time to shift the paradigm for advanced aseptic processing systems away from monitoring and toward their design. Where monitoring is used, total particulate monitoring has significant advantages over microbiological monitoring. Total particulate monitoring provides a real-time indication of a major change in the physical performance of HEPA filters or a significant increase in particles produced by processing equipment, something that conventional microbiological monitoring cannot do.

Our industry has always sought to improve the sterility of aseptically produced products. For many years this was accomplished through measures that were more or less instinctive rather than reflective of real science and engineering. The adoption of risk-based approaches is a relatively new concept, but it is essential that the practitioner take the next step. Mitigation is of far greater importance in the overall effort and provides a greater measure of safety to aseptic operations.

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3 Development challenges and validation of fill and finish processes for biotherapeutics

Karoline Bechtold-Peters

INTRODUCTION

The therapeutic antibodies presently sold on the international markets are all administered via parenteral route, that is, by subcutaneous or intramuscular injection or intravenous infusion, with one product injected into the vitreous humor of the eye (Table 1). Approximately, two-thirds of these preparations are liquid-stable preparations with 1 to 100 mL nominal volume; one-third of the preparations are marketed as lyophilized powder for reconstitution. With this selection of routes of application and dosage forms, it becomes quickly clear that the manufacturing procedure of choice for therapeutic antibody products is a liquid filling process under aseptic conditions, optionally with an additional freeze-drying process directly in the vial. Sterilization in the final container is practically excluded because of the thermolability of proteins.

Monoclonal antibodies, as well as other therapeutic proteins, are sensitive toward various stresses such as heat, shear, interfaces, and foaming (Fig. 1), and since aseptic processes in general are high-level risk processes, suitable attention must be dedicated to the development of the manufacturing procedure, process parameters, as well as their validation.

In the first section of this chapter, manufacturing steps up to the final product will be described, including challenges in the development of the fill and finish process based on various case studies. The second part will go into details as regards to the validation of the pharmaceutical manufacturing process taking into account current authority views like the "risk-based approach" of the FDA.

COMPONENTS AND PROCESS STEPS IN FILL AND FINISH

Typically the process chain for the finished drug product starts with the final formulation step. Often times this is the last step in the downstream drug substance process (Fig. 2). The concentrated bulk is diluted, if necessary, after thawing, preferentially under controlled conditions with a formulation buffer containing functional excipients to obtain the required protein concentration. The bulk solution is generally isotonic at a physiologically acceptable pH range and contains various stabilizers, for example, nonreducing sugars, amino acids, complexing agents, antioxidants, or cake formers.

The tanks containing the formulated solution are placed in proximity to the grade A area. For aseptic processes in isolators, the tank resides in grade D (or better), while for classical clean room operation the tank resides in grade C area. One or two sterile filters in series are placed as a part of transfer assembly to sterile filter the bulk solution into the grade A room for filling. The sterilizing grade filters are checked for integrity immediately before the sterile filtration and are again checked for membrane integrity following completion of the filtration step. The sterile filtration can occur off-line into a second hold container (Fig. 3). After finalization of filtration this hold container is connected to the filling machine and the filling starts. For additional sterility assurance, an additional sterile filter may be placed very close to the filling needle (EU GMP Guide, Annex 1, 2008).

If large volume batches are to be filled, an in-line filtration (Fig. 3) directly from the bulk tank, via one or two sterile filters, into a smaller intermediate container (surge vessel) does make sense. In this case, the bulk tank can be placed outside of grade A area avoiding extra manipulations and equipment in the clean area. Reduction in the process time occurs as the sterile filtration and filling proceed in parallel and there is no need to clean and sterilize at least one or more stainless steel tanks. Before the start of the sterile filtration, a sample for bioburden is collected for every batch of drug product (EU Guidelines to GMP, Annex 1, 2008). To avoid false positive results for the bioburden, closed, presterilized disposable systems are useful (1).

Table 1 Therapeutic[a] Monoclonal Antibodies on the Market (December 2009)

INN name	Trade name	Type of antibody	Dosage form	Application route
Oncology				
Alemtuzumab	MabCampath®	Humanized	Liquid, vial	IV infusion
Bevacizumab	Avastin®	Humanized	Liquid, vial	IV infusion
Cetuximab	Erbitux®	Chimeric	Liquid, vial	IV infusion
Panitumumab	Vectibix®	Human	Liquid, vial	IV infusion
Rituximab	MabThera®, Rituxan®	Chimeric	Liquid, vial	IV infusion
Gemtuzumab	Mylotarg®	Humanized, conjugated to calicheamicin	Lyophilized powder, amber vial	IV infusion
Ibritumomab	Zevalin®	Murine, ^{90}Y conjugated	Liquid, vial, radiolabeled	IV infusion
Tositumomab	Bexxar®	Murine, ^{131}I-conjugated	Liquid, vial, radiolabeled	IV infusion
Trastuzumab	Herceptin®	Humanized	Lyophilized powder, vial	IV infusion
Ofatumumab	Arzerra	Human	Liquid, vial	IV infusion
Catumaxomab	Removab	Rat-murine	Liquid, prefilled syringe	IV infusion
Autoimmune diseases, transplantation therapy				
Adalimumab	Humira®	Human	Liquid, prefilled syringe	SC injection
Basiliximab	Simulect®	Chimeric	Lyophilized powder, vial	IV infusion or injection (bolus)
Daclizumab	Zenapax®	Humanized	Liquid, vial	IV infusion
Infliximab	Remicade®	Chimeric	Lyophilized powder, vial	IV infusion
Muromonab	Orthoclon OKT3®	Murine	Liquid, ampoule	IV injection (bolus)
Natalizumab	Tysabri®	Humanized	Liquid, vial	IV infusion
Efalizumab	Raptiva®	Humanized	Lyophilized powder, vial	SC Injection
Certolizumab pegol	Cimzia®	Humanized	Lyophilized powder, vial AND Liquid, prefilled syringe	SC Injection
Golimumab	Simponi®	Human	Prefilled syringe with needle guard or in autoinjector	SC injection
Tocilizumab	Actemra®, RoActemra®	Humanized	Liquid, vial	IV infusion
Canakinumab	Ilaris®	Human	Lyophilized powder, vial	SC injection
Other indications				
Abciximab	ReoPro®	Chimeric (Fab_2 fragment)	Liquid, vial	IV injection followed by infusion
Palivizumab	Synagis®	Humanized	Liquid, vial (new dosage form)	IM Injection
Ranibizumab	Lucentis®	Humanized (Fab fragment)	Liquid, vial	Intravitreal injection
Omalizumab	Xolair®	Humanized	Lyophilized powder, vial	SC injection
Eculizumab	Soliris™	Humanized	Liquid, vial	IV infusion

[a]In addition, monoclonal antibody-based imaging agents are available; e.g., CEA-Scan (Arcitumomab Tc-99), LeukoScan (Sulesomab Tc-99), ProstaScint (Capromab pendetide Indium-111), and Verluma (Nofetumomab merpentan Tc-99).

Dispensing into the vials, cartridges, or syringes occurs by pumps, that is, peristaltic pumps, piston pumps, or rolling diaphragm pumps, or by time-pressure or mass flow filling systems. Formulation solution is placed into sterile containers via 1 to 16 needles. The containers are closed immediately by sterilized stoppers or sealing discs (with cap). Filling and stoppering process steps are highly critical as they are ran while the containers are open. Particles and microbes can reach the open product. With therapeutic proteins such as monoclonal antibodies, a heat sterilization in the final container is not feasible. The foreign particulate matter specification is particularly stringent for large-volume solutions for infusion to avoid embolism in the patient (Table 2).

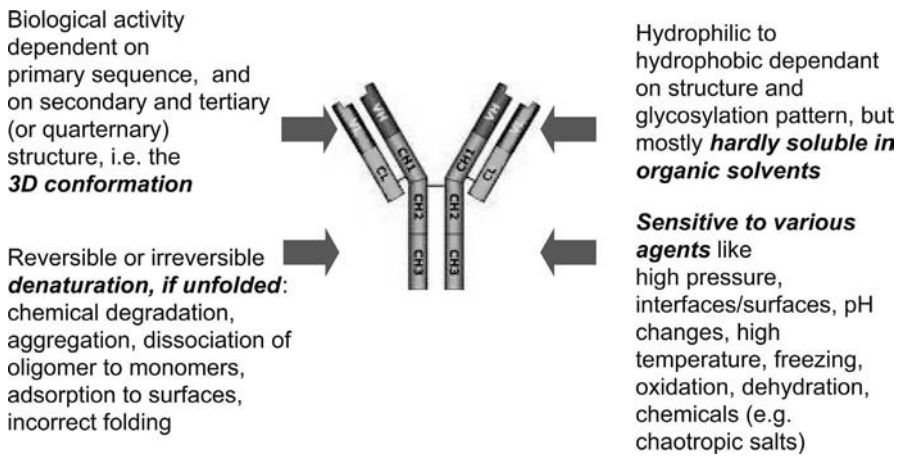


Figure 1 Special properties of biologics as drugs.

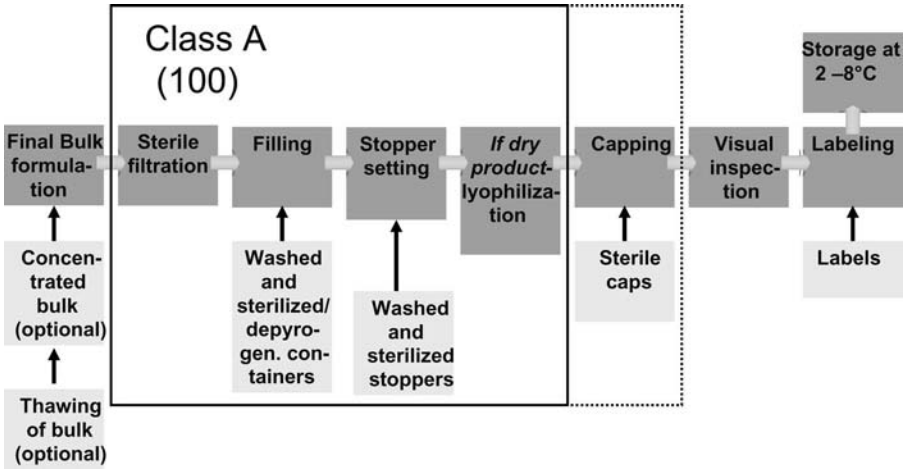


Figure 2 Typical fill and finish process into vials.

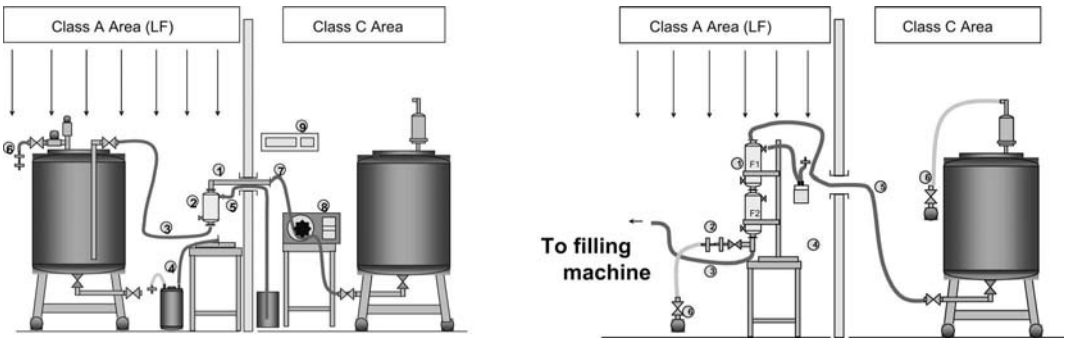


Figure 3 Examples for off-line and in-line sterile filtration setups.

Table 2 Pharmacopoeial Requirements as Regards to Particles in Injectables

USP 30-NF25 <788>	Ph Eur. 6.8	JP XIV (foreign insoluble matter test and insoluble particulate matter test)
Subvisible particles		
Limits for Light Obscuration Test Particle Count[a] *Small volume injections (≤100 mL volume)* ≥10 μm: 6000 particles/container ≥25 μm: 600 particles/container *Large volume injections (>100 mL volume)* ≥10 μm: 25 particles/mL ≥25 μm: 3 particles/mL	Limits for Light Obscuration Test Particle Count[b] *≤100 mL volume* ≥10 μm: 6000 particles/container ≥25 μm: 600 particles/container *>100 mL volume:* ≥10 μm: 25 particles/mL ≥25 μm: 3 particles/mL	Criteria ≥10 μm: 25 particles/mL ≥25 μm: 3 particles/mL
Limits for Microscopic Method Particle Count[a] *Small volume injections* ≥10 μm: 3000 particles/container ≥25 μm: 300 particles/container *Large volume injections* ≥10 μm: 12 particles/mL ≥25 μm: 2 particles/mL	Limits for Microscopic Method Particle Count[b] *≤100 mL volume* ≥10 μm: 3000 particles/container ≥25 μm: 300 particles/container *>100 mL volume:* ≥10 μm: 12 particles/mL ≥25 μm: 2 particles/mL	
Visible particles		
Injectable solutions, including solutions constituted from sterile solids intended for parenteral use, are essentially free from particulate matter that can be observed on visual inspection.	*Parenteral preparations:* solutions for injection/infusion examined under suitable conditions of visibility (2,000–3,750 lux) are clear and practically free from particles *Monoclonal antibodies for human use:* liquid or reconstituted freeze-dried preparations are clear or slightly opalescent and colourless or slightly yellow, without visible particles	Injections must be clear and free from readily detectable foreign insoluble matter (8,000–10,000 lux)

[a]Unless solely intended for IM and SC administration or the label states that the product is to be used with a final filter are exempt from these requirements.
[b]In the case of preparations for SC or IM injection, higher limits may be appropriate; radiopharmaceuticals and preparations for which the label states that the product is to be used with a final filter are exempt from these requirements.

In the last step of the manufacturing process, the stopper is fixed, in case of vials, by a cap. Because the vial is sealed using a suitable stopper/vial combination and the fact that the stopper is pushed-in (2), the vial capping theoretically can be performed in grade C area—although under laminar air flow. However, due to current EU regulation (EU GMP Guide, Annex 1), many manufacturers have upgraded the capping process to occur under grade A conditions.

The manufactured vials, syringes, or cartridges are 100% visually inspected by trained examiners for various defects, either manually or using automatic machines. The quality of 100% visual inspection is verified by taking random product samples according to Acceptable Quality Level (AQL) tables that are based on Military Standard 105D.

In the case of lyophilizates, the freeze-drying stopper is pressed only partially into the container and the half-closed containers are then loaded into the freeze-dryer. At the end of the lyophilization process, while the vials are still in the freeze-dryer, the stoppers are pushed into the containers as the shelves are pressed together. Following unloading, the capping is performed in grade A or C areas under laminar flow according to the procedure specified by the company.

The filling process is preceded by the preparation of the packaging materials that are washed and if necessary siliconized and sterilized after unpacking. However, the use of RTS (ready-to-sterilize) or RTF (ready-to-fill) quality stoppers or caps are widespread throughout the industry. A special "Closed-Vial" technology (3) is based on prewashed, irradiated, already stoppered cyclic olefin copolymer (COC) vials that are filled through the stopper and then resealed by laser light and equipped with a flip-off cap. For lyophilization process, closed stopper systems that allow moisture to escape but prevent bacteria or particulates from entering are offered by at least two companies.

SELECTED CASE STUDIES EXEMPLIFYING DEVELOPMENT CHALLENGES DURING FILL AND FINISH

Problems can occur with each of the mentioned process steps under production conditions, if the procedures, process parameters, and materials in contact with the product were not examined carefully for possible influence on the product (Table 3). The whole manufacturing process must be broken down, during development, into unit operation steps and the criticality of every step be examined.

Detailed descriptions of this process are exemplified in the following case studies.

Filtration

Because of the thermolability of the biologically active substance, the sterile filtration constitutes the only possible sterilization procedure for the protein or antibody-containing formulated bulk. The aspects that are to be followed and examined for definition of the sterile

Table 3 Instability of Therapeutic Proteins and Stress Factors Occurring During Final Drug Product Manufacture

Protein instability		
Type of instability	Stress factors	Time point of occurrence during manufacture of final drug product
Physical Denaturation Aggregation (reversible, irreversible) Precipitation Adsorption Chemical Hydrolysis/deamidation/fragmentation Oxidation Isomerization Disulfide exchange	pH Ionic strength Denaturants Metal ions Oxygen Light Temperature Shear Interfaces, surfaces (air/liquid, liquid/glass, or metal or plastic)	Mixing Transport/transfer Filtration Filling Freezing Drying Visual inspection

Table 4 Considerations for Sterile Filter Selection

Nature and type of membrane material
- Cellulose esters
- Polyvinylidenfluoride
- Polyethersulfone
- Nylon
- Polycarbonate

As capillary (with cylindrical straight-through pores) or noncapillary filters (8)

Membrane and solution factors	Filtration parameter
• Adsorptives behavior • Expected bioburden/particle burden • Total filtration volume • Logistics/supplier relation	• Product contact time, filtration time • Filtration pressure • Flow rates per unit area • Filtration temperature • Area-specific bioload per unit area (and specific bioburden microorganisms if relevant)

Types of filter studies
- Adsorption of formulation components
- Flow rate over time/after interruption
- Membrane integrity (bubble point, diffusive flow, forward flow), with WFI/purified water and product
- Bacterial challenge/bacterial retention performance including viability of test bacteria in formulation
- Correlation between bacterial retention and integrity test method
- Extractables (chemicals, particles)
- Sterilizability (sterility with/without manifolds attached, effect of sterilization on filter integrity)
- Chemical compatibility

filter are summarized in Table 4. The PDA Technical Report No. 26 (4) gives a good overview of the key considerations during sterile filtration of solutions.

As a rule, 0.2- or 0.22-μm filters are used for the sterile filtration. This filter rating refers to the smallest size of microorganisms and particles that are removed rather than to the actual size or form of the filter pores (5). Usually pressure applied during sterile-filtration process is $\leq$2 bars. Because the composition of the bulk formulation can influence the ability of the filter to hold back bacterial loads, bacteria-retaining filters must be validated specifically for a product. Bracketing can be considered with very similar products (6). During the bacterial retention study, the filter is challenged with $>10^7$ cfu of *Brevundimonas diminuta* (ATCC 19146) per cm^2 under worst-case process conditions expected during filtration of the drug product, and the ability to retain challenge microorganism (and hence ability to achieve a sterile product) is examined (4,7). Unfortunately, the bulk volumes required for these studies are substantial (several liters), even after usual scale-down to 47-mm diameter disks, which renders the study an expensive investigation, especially for highly concentrated formulations. Although complete filter validation package is only available at the time of process validation, it is recommended that at least a risk assessment or an abbreviated bacterial retention should be performed during clinical development for novel formulations, because filtration is the only sterilization step for the heat-unstable protein preparations.

Coarser, foreign particles and large aggregates are removed during sterile filtration using a 0.2-μm filter. Consideration must be given to the desired removal of contaminants like bioburden and the undesirable removal of active substance or excipients by adsorption to the large surface of the filter membranes. The typical area of a 0.22-μm filter is >100 m^2/g (8). Furthermore, the sterile filter must be compatible with the bulk protein solution under filtration conditions. For most protein formulations, this is uncritical given the aqueous nature, low-processing temperature between 0 and 30°C, and a pH range between 5 and 8. However, during the process development stage, studies must be conducted to confirm the compatibility. Studies by Pitt (9) show that different filter materials bind protein weakly or very strongly depending on the polymer material. Pitt found that mixed cellulose ester and nylon have stronger affinity to proteins than poly(vinylidene fluoride), or PVDF, and polysulfone.

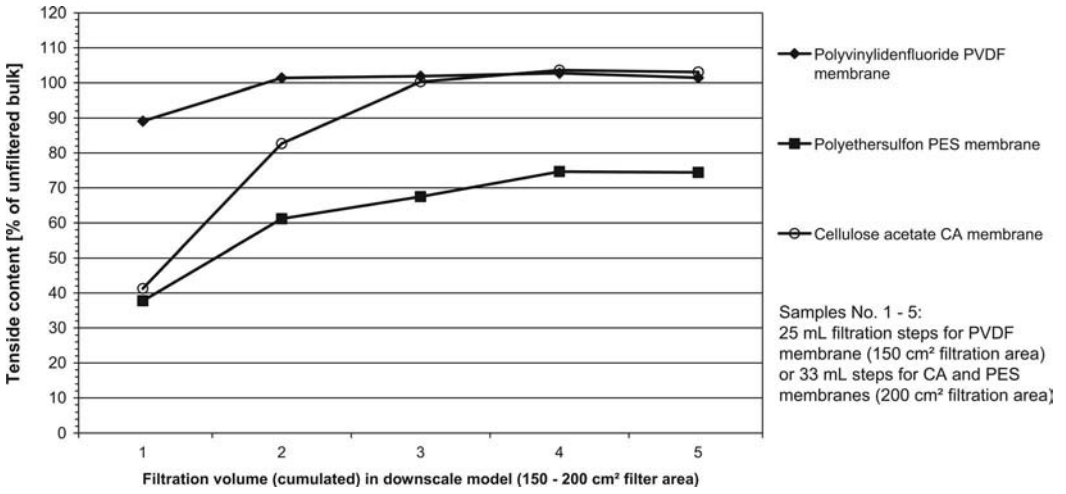


Figure 4 Adsorption of surfactant (Tenside) from a monoclonal antibody containing formulated bulk (0.01% of polysorbate 80, pH 5.5) during filtration through three filter membranes, filter area 150 to 200 cm^2.

Excipients can also bind to filters with varying avidity. Surfactants, for example, are preferentially distributed at surfaces because of their interfacial properties, their amounts can be reduced significantly if the formulation has low concentration of surfactant and the process involves multiple filtrations. In an unpublished study with antibody-containing bulk (0.01% polysorbate 80 and 5 mg/mL of an antibody), three sterile filters, composed of PVDF, polyether sulfone (PES), and cellulose acetate (CA), were examined (Fig. 4). The following order of loss of polysorbate 80 was observed: PVDF < CA <<PES.

Similar investigations must also be undertaken for preservatives added to multiple-dose preparations, for example, growth hormone or insulin, as they can also be removed in significant amounts by filtration.

In the case of undesirable adsorption of formulation components or protein, saturating the membrane by prerinsing with formulation buffer can be a mitigation strategy. In addition, choosing a suitable, small surface area with low absorption filter material will be important. With in-line filtration, filtrate might be discarded before the beginning of the main filling process to guarantee the homogeneity of the drug product over the whole batch.

During process development, migration of potential extractables from filter membrane, filter housing, supporting fabrics, sealing disks, O-rings, and tubing into the formulation should be examined. These extractable studies are conducted following the sterilization of the filters by steam or by irradiation. Potential extractable sources from sterilizing filters may include surfactants and wetting agents, additives used in the plastic component manufacture, manufacturing debris, monomers of materials of construction, etc. Unfortunately, neither the pharmacopoeias nor official guidances give concrete information regarding the acceptable amount of extractable substance from the filters. Therefore, the toxicological assessment and check of compatibility is left eventually with the user. Current filter manufacturers offer services where they can conduct studies on extractables or provide information to the user on the basis of previous experience.

As a part of filter extractable study, filters from several lots are eluted following worst-case pretreatment (several sterilization cycles, higher sterilization temperature, and sterilization time) and under more aggressive model conditions (as regards to time, temperature, solvent/power of elution, pH). The eluates are analyzed gravimetrically (NVR, nonvolatile residues), by TOC (total organic carbon), liquid chromatography (RP-HPLC), capillary electrophoresis (HPCE), gas chromatography (GC and GC-MS), or Fourier transform infrared (FTIR) spectroscopy. Because protein and formulation components interfere with the analyses, water-based solvent system is mostly used as representative extraction media. The found extractables must be classified afterward as toxic or nontoxic. Additional testing like biological

reactivity in vitro and in vivo according to USP <87> and <88> may have to be performed to provide added safety assurance. In the end, a recommendation for a preflush volume (e.g., 2 L for a 4 in. capsule) is made, so that possible extractables are already removed to a great extent before the main filtration commences.

With protein preparations, the testing for membrane integrity is not trivial. The filter must be rinsed with large amounts of water to remove surface active materials, often at raised temperature, before the established value for bubble point, forward flow/diffusive flow, or pressure decay is reached. For the postuse membrane integrity determination, a product-specific bubble-point value is recommended.

In the end, the filter dimension must be chosen so that the filtration rate fits with the filling process, in particular for the on-line/in-line filtration. A decrease of the filtration rate over time or after an interruption with downtime due to increasing filter blockage resulting in protein fouling can be mostly avoided by determining the adequate size of the filter membrane surface. Nevertheless, an oversizing should also not occur because of the already described adsorption losses and large dead volumes in the filter of expensive drug product.

Maa and Hsu (8) compared the fouling behavior of different proteins [recombinant human growth hormone (rhGH), recombinant human deoxyribonuclease (rhDNAse), recombinant tissue plasminogen activator (rt-PA), an anti–immunoglobulin E (anti-IgE) antibody] during sterile filtration and examined some possible fouling mechanisms: pore narrowing, adsorption because of nonspecific binding between membrane and protein, shear-induced adsorption and aggregation, and adsorption due to hydrophobic surfaces. The filtration flow over the time of 0.09 molar solutions was noted. All solutions, including anti-IgE solutions, showed a decline of filtration flux with increasing filtration volume due to membrane fouling, although this was relatively slightly pronounced for the anti-IgE solution (slope, -0.004). A clear correlation between initial flow and molecular weight was noted with the monoclonal antibody solution filtering being very slow due to the high molecular weight (~ 2.7 mL/min/cm^2 at about 13 mg/mL concentration, Millipore filter from mixed cellulose ester, 9.4 psi filtration pressure). Fouling at filtration membranes, tubing, or other surfaces can be affected by the respective protein formulation (pH, ionic strength) and is often reduced by the addition of surfactants such as polysorbates (8–10).

Filling

Protein-containing solutions tend to foam so in some cases they not only compromise filling process but also cause a reduction of the protein integrity. In downscale models, which shake the formulated bulk or press it with increasing velocity through narrow cannulas, the foam behavior can be explored a priori as a part of the formulation development. Because excessive foaming leads to inaccuracy in dosing, the filling speed must be lowered drastically or filling of larger volumes performed in two steps by means of two filling stations in series. In the end, the filling mode also plays an important role. Most filling machines at production scale are equipped with a movable filling needle that follows during the filling process the upward moving liquid meniscus. Hereby, the filling needle can be led below the meniscus, at the meniscus, or above the meniscus.

In a filling test with an antibody-containing bulk solution (protein concentration 5 mg/mL), the filling "under the meniscus" turned out to be advantageous compared with the filling "above the meniscus" (unpublished results) (Fig. 5). For a further model describing foaming behavior see Maa and Hsu (11).

A decisive influence on the filling process is the choice of filling or dosing system (principle). The following systems are available:

- Peristaltic pumps
- Piston pumps (180° or 360°)
- Rolling diaphragm pumps
- Time-pressure filling systems
- Mass flow filling systems

These dosing systems differ both in dosing precision and in shear stress applied to the protein solution. While peristaltic pumps enable very smooth filling, piston pumps may be

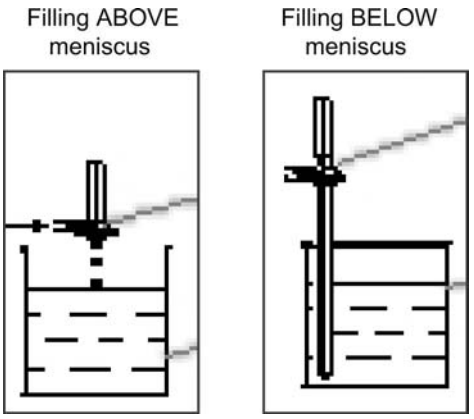


Figure 5 Filling modes into vials, cartridges, and syringes.

Table 5 The Excess Volumes Recommended by USP Usually Sufficient to Permit Withdrawal and Administration of the Labeled Volumes (USP <1151>)

	Recommended excess volume	
Labeled size	For mobile liquids	For viscous liquids
0.5 mL	0.10 mL	0.12 mL
1.0 mL	0.10 mL	0.15 mL
2.0 mL	0.15 mL	0.25 mL
5.0 mL	0.30 mL	0.50 mL
10.0 mL	0.50 mL	0.70 mL
20.0 mL	0.60 mL	0.90 mL
30.0 mL	0.80 mL	1.20 mL
50.0 mL or more	2%	3%

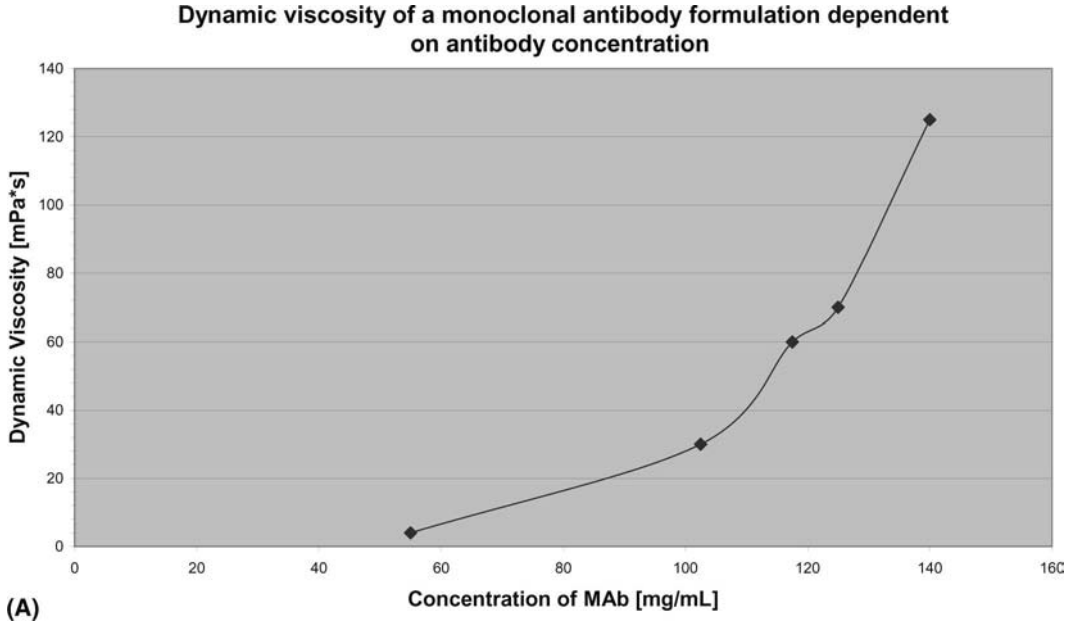
more accurate dosing devices. If feasible, at least in development, various dosing systems should be tested to meet the needs of the product.

At the beginning of the filling process, a target in-process specification with alert and action limits is defined by determination of the minimal fill volume, which will assure that specified extractable volume can be withdrawn. USP Chapter <1151> provides recommendation for injectable excess fill volumes for low- and high-viscosity products (Table 5). Proteinaceous solutions are typically between the two classes. Because of the high molecular weight of proteins and antibodies (5–150 kDa), the colloidal solutions formed may be rather viscous at high concentrations. To cope with the volume restrictions as regards subcutaneous application (max. 2–3 mL volume), concentrations of 100 to 200 mg/mL may be the goal with viscosities far beyond water-like liquids (Fig. 6A). Also, since viscosity varies with temperature as depicted in Figure 6B, a dosing system such as the time-pressure filler is specifically challenged and requires an experimentally obtained temperature compensation algorithm into the process control system.

Effect of Contact Surfaces

During final formulation, storage of bulk and filling of the antibody preparations glass, steel, and plastics are typical materials in contact with product (e.g., silicone tubes, polypropylene filter housings, polyvinylidene difluoride filter membranes).

Although the pharmaceutical industry uses high-quality stainless steels, 1.4404/1.4435 (316L) and 1.4539 (904L), the potential to release small quantities of metal ions must not be neglected. Lam et al. (13) described considerable reduction in the oxidation during the storage, particularly at Met255, by replacing the stainless steel filler by a filler from an alternative material (52% oxidized Fc compared with 18% after 2 weeks at 40°C). This oxidation was ascribed to the corrosion of steel by chloride ions after contact with NaCl containing formulation buffer at low pH (pH 5.0) and extraction of iron ions. Metal ions such as iron ions can react with peroxide



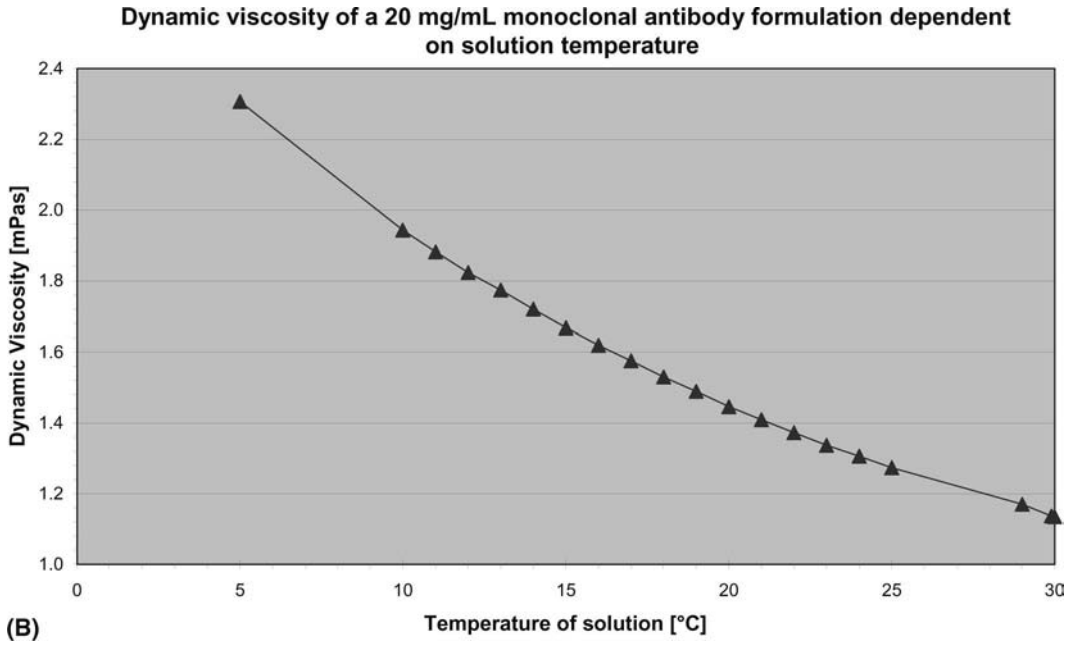


Figure 6 (**A**,**B**) Dynamic viscosities of monoclonal antibody containing bulks. Influence of temperature and antibody concentration on viscosity. *Source*: From Ref. 12.

impurities (e.g., from polysorbates) or the protein itself in the formulation to form free radicals that initiate oxidative degradation. In the same study, Lam et. al. could generate 26% increase of the anti-HER2 oxidation after storage only by manufacture of the NaCl containing diluent buffer in a steel tank. After production of the buffer in a glass container no oxidation occurred (13). Furthermore after three months of storage of the NaCl containing anti-HER2 formulation in a 30 mL stainless steel container, up to 3 ppm of extracted iron ions were determined. However, such data can be transferred only to a limited extent to tanks in the pilot and production scale because of the unfavorable surfaces to volume ratio in such minitanks.

Metallurgically it is important to distinguish reactions that lead to either rouging or leaching of metal ions from steel surfaces. The latter is favored in acidic solution and in the presence of chloride ions. The rouging progress on the other hand represents an inversion of the formerly chromoxide-rich passive layer to an iron oxide–dominated porous surface layer due to the environmental influences (medium, temperature). These environmental factors are responsible for disturbing the dynamic balance of the former passive layer (14). Rouging on stainless-steel surface is often detected during preventive maintenance or by presence of reddish to brownish coloring, for example, in water for injection conduit systems, reddish iron oxide particles are then found on particle filters and sieve inserts. By chemical cleansing cycles, the corrosion layer can be stripped off (e.g., citric acid, EDTA, H_3PO_4, HF + HNO_3) and the surface repassivated (oxidizing materials such as HNO_3), so that the corrosion resistance of the stainless steel is restored (15). The cleanliness and absence of particles from stainless steel components is necessary for the production of parenterals. Substantially higher quality stainless steel can be obtained after electropolishing than after mechanical polishing procedures (removal of Beilby layer) (16).

Air-Water Interface

Antibodies and other therapeutic proteins can be damaged by shear or stressing at interfaces in various steps of the process. These stresses occur not only in upstream and downstream processes such as during aeration and agitation, recirculation, centrifugation and filtering for cell separation, purification, buffer exchange, and concentration, but also during production of the pharmaceutical final product. Interface stress occurs during final mixing of drug product, sterile filtration, filling, conveying, transporting, and shaking during visual control. A summary of stress factors during production of the final product is represented in Table 3.

Proteins diffuse to and subsequently orient to interfaces formed. This process may be followed by unfolding of globular configurations to the denatured state. Ultimately aggregation and precipitation may occur dependent on the conformational stability of flexible segments of the protein molecule. During many of the processes mentioned in the previous paragraph, the interfacial film is continually renewed with progressively more protein exposed to the interface resulting in further loss in activity (17). Harrison et al. examined the effect of high shearing (20,000/sec) on scFv fragments in an underfilled cylindrical minireactor with impeller. In the absence of protective additives, 80% irreversible loss of activity was measured compared with the unsheared scFv fragment (17). Other reports suggest that shear, without generation of new interface, has minimal effect on protein activity.

Listed in Table 6 are the shear rates during pharmaceutical process steps under usual conditions. For a noncompressible Newtonian liquid, the shear rate occurring at the capillary walls γ_{wall} in sec^{-1} is calculated according to equation (1) (18,19):

$$\dot{\gamma}_{wall} = \frac{4Q}{\pi R^3} = \frac{8V}{D} = \frac{4V}{R} = \frac{32Q}{\pi D^3} \quad (1)$$

where Q is the volumetric flow rate in cm^3/sec; V, the average flow velocity in cm/sec; D, the inner diameter of the capillary in cm; and R, the inner radius of the capillary in cm.

Table 6 Estimates of Shear Stress Applied During Fill and Finish of Antibody Solutions (20 mL Fill Volume, 10 mg/mL Antibody Concentration, Newtonian Behavior, One Filling Station)

Process step	Conditions and assumptions for calculation	Shear rate per orifice (sec^{-1})
0.2 μm filtration	130 L filtered within 30 min (off-line) or 6 hr (on-line), filter with 10^8 pores/cm^2 at a diameter of 0.2 μm/pore, 1900 cm^2 filtration area (Opticap XL4)	4.8×10^5 (off-line) 4.0×10^4 (on-line)
Rotary pump	130 L filled within 6 hr, 25% of time flow through outlet, ID outlet 5 mm	2.0×10^3
Time-pressure filler or peristaltic filler	130 L filled within 6 hr, 80% of time valves opened, ID 7 mm	2.2×10^2
Needle size A	130 L filled within 6 hr, 50% of time flow though needle, ID 7 mm	3.6×10^2
Needle size B	130 L filled within 6 hr, 50% of time flow though needle, ID 5 mm	9.8×10^2

Abbreviations: ID, inner diameter.

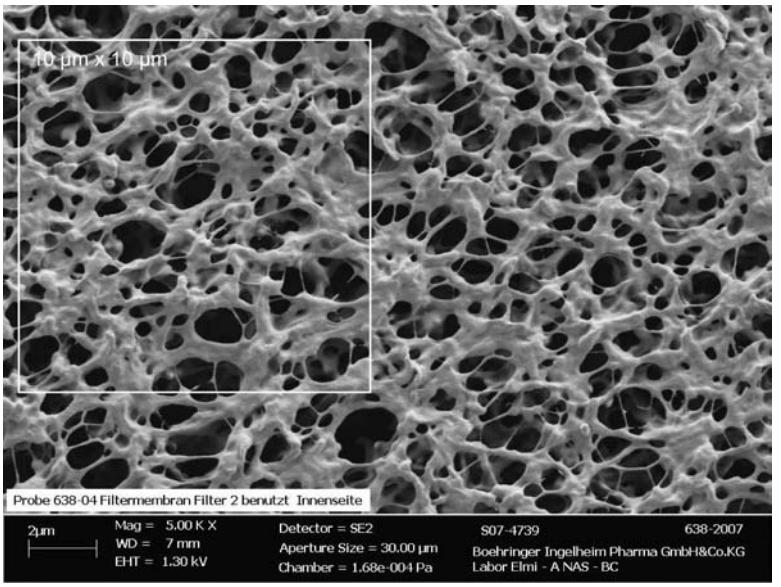


Figure 7 SEM photograph of sterile filter membrane (Durapore) 0.2 μm pore structure after use.

It becomes obvious that filtration through narrow pores under pressure (Fig. 7) imposes the comparatively highest stress effects on the antibody, with the filter structure and the pore size having a considerable influence. Shear stress in piston pumps or time-pressure pumps as well as during dosing by needles is considerably lower. Nevertheless, the calculations show that with the reduction of the inner diameter of the filling needle the shear rate increases and that peristaltic pumps as well as time-pressure pumps are markedly more gently dosing than piston pumps.

For the development of protein products stress studies during the process development are important. The following systems may be used as downscale models: vial and bottle shakers, pumps, impeller systems, Couette flow systems, ultrafiltration modules, capillaries, static and dynamic concentric cylinder systems, and rotating disk reactors. These differ clearly in the applied shear rates in the range of a few 100/sec up to 26,000/sec (20). Examples of such shear stress test systems are represented in Figure 8.

Light, Oxygen, and Temperature

The influence of light must be considered on the stability of protein. Under the influence of light (2 weeks, 20,000 lux, 27°C), the recombinant antibody anti-HER2 in liquid formulation showed oxidation at Met255 in the heavy chain of the Fc region. The light-induced oxidation of recombinant monoclonal antibody anti-HER2 occurs through the singlet oxygen pathway and could be controlled effectively by antioxidants like 3.5 mM methionine or 6.3 mM sodium thiosulfate (13). With very photosensitive proteins, precautionary controls to reduce photo-degradation during manufacturing should be in place, for example, use of stainless steel tanks, in place of glass vessels, since they are impervious to light, use of opaque tubings, colored plastic carton boxes to minimize light exposition during intermediate storage and visual inspection, etc. If necessary manufacturing and filling under red or yellow light is an option for the parenteral product based on the knowledge of the molecule sensitivity to the various wavelengths of light. In general, the room light mapping is also performed, and work processes are designed that avoid unnecessary light exposure and define the maximum total light exposure to the product over the entire manufacturing, packaging, and labeling operations.

The effect of molecular oxygen on oxidation of recombinant monoclonal antibody anti-HER2 was examined by Lam et al. by replacing headspace oxygen with nitrogen before stress

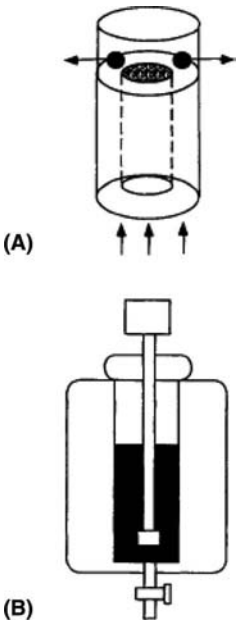


Figure 8 Schematic representation of a rotor/stator assembly (**A**) and of a homogenizer system (**B**) used by Maa and Hsu in shear stress studies for proteins. *Source*: From Ref. 20.

storage at 40°C for two weeks (13). Whereas the control sample developed 52% oxidized Fc after two weeks at 40°C, the removal of oxygen in the sample vials after repeated pulling of vacuum and replacement by nitrogen was as effective as the addition of antioxidants.

Packaging System and Its Preparation

For the parenteral products containing proteinaceous solutions vials, prefilled syringes and cartridges are the most relevant packaging systems. The vial, syringe, and cartridge bodies are mostly made of type I tubing glass. Plastic bags or vials (e.g., blow-fill-seal bottles) play a minor role except for plasma products and the storage of intermediate or final bulk. Rubber is used as sealing discs and stoppers.

The selected packaging system has to be checked for the following main attributes:

- Container closure integrity
- Potential interactions between protein solution and packaging material

Examples are given below to illustrate that packaging selection is a vital task during drug product development of protein products.

Package Integrity

The primary sealing zones of the vial and stopper combinations are located at the flange and neck of the vial; hence, appropriate stoppers should preferably be uncoated in this area.

If packaging manufacturers provide material with too broad dimension tolerances, the stoppered vials may not maintain integrity prior to capping (Fig. 9). Adequate investigations of stopper sealing performance, supplier and incoming controls of the packaging materials, for example, conformance to predefined dimensional tolerances, are mandatory.

Protein—Packaging Material Interaction

The interaction can have two directions: components may leach from the packaging material into the proteinaceous solution, but also components from the proteinaceous formulation may adsorb to the packaging material.

In regards to the former phenomenon, essentially silicone detaching from syringe or cartridge bodies or siliconized stoppers are of concern because silicone can cause aggregation

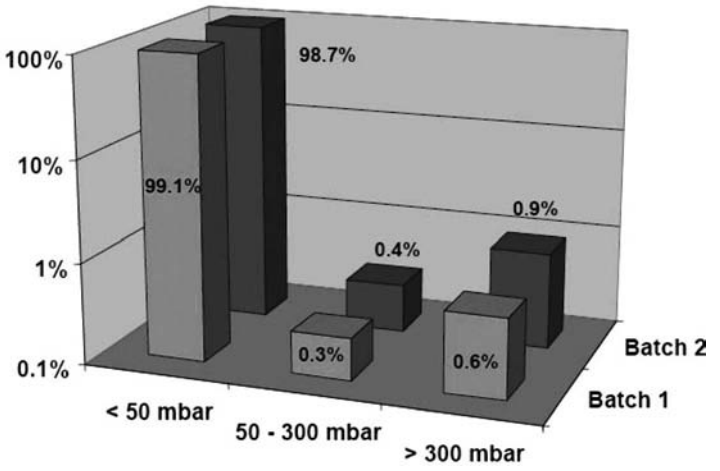


Figure 9 Incidence of lyophilized vials with reduced or lost vacuum after final sealing, 6 R vials with blow back ring and 20-mm uncoated, siliconized stopper. *Source*: From Ref. 21.

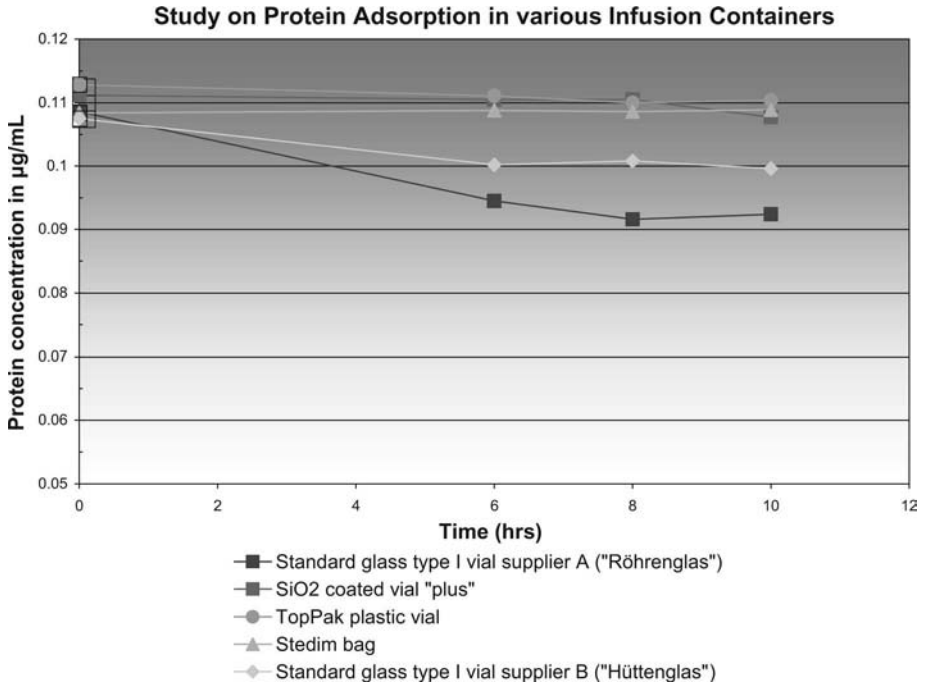


Figure 10 Comparison of various container materials as regards to adsorption of protein from a low concentration protein solution. *Source*: From Ref. 22.

of sensitive proteins. Tungsten, in trace amounts, from the syringe forming process has been described to cause aggregation of therapeutic proteins; however, now tungsten-free syringes are on the market.

Concerning adsorption of the protein to the packaging walls, less thoughts must be spent in case of high-concentration liquid antibody formulations than in case of low-protein concentrations. A study could nicely show that for a small chemokine protein, at a concentration of as low as 0.1 µg/mL, the packaging material indeed mattered. Glass revealed massive adsorption of the protein as opposed to EVAM bags, COC vials, and SiO_2 plasma-coated glass vials (Fig. 10).

Lyophilization

In case of problems with the stability during storage of liquid protein preparations, which cannot be repaired by formulation measures such as pH optimization, buffer exchange, or addition of surfactants, the product will be mostly freeze-dried. Other measures such as freezing are usually not an attractive option for commercial preparations, except for intermediate products before conjugation. Lyophilization belongs to a category of rather difficult pharmaceutical processes, since many parameters must be adjusted. Although smart freeze-drier technology and predictive modeling have advanced significantly, often experiments conducted at small scale are of limited value.

After filling the product into vials or other containers (e.g., double chamber cartridges), special lyo-stoppers are at first only partially pushed into the containers and the lot is loaded manually using a loading cart or automatically onto the lyophilizer shelves that may be precooled. The subsequent freezing process may be in some instances interrupted by annealing to drive partially amorphous sugars to completely crystallize (predominantly mannitol containing formulations). Following primary drying process, the shelf temperature is typically increased stepwise, for example, from -50 to 0°C. The solvent is collected by the condenser. At a vacuum of typically $\leq$100 mbar energy must be introduced into the frozen product via the shelves to sublimate the water, since vacuum is a bad heat conductor. Care must be taken not to exceed the glass transition temperature T_g', because this would lead to a collapse of the cake and hence to unacceptable appearance of the lyophilizate, and in some cases even lead to damage of the protein. In other cases, to the contrary, collapse of the lyo cake had no negative effect on protein activity, monomer content, or even on the secondary structure (infra-red spectra) of an IgG and of lactate dehydrogenase (LDH) initially and following storage (23,24). As ice sublimes during primary drying, collapse is prevented by maintaining the structural integrity of the maximally freeze-concentrated amorphous phase that surrounds the ice crystals. Below its glass transitions temperature T_g', this amorphous phase exists as a "glass," which is hard and brittle and has negligible mobility. The final residual moisture, which is also very much dependent on the formulation (25), is reached during secondary drying by the removal of adsorbed water. Here the shelf temperature is again raised, for example, to $+25^{\circ}$C. A low residual moisture is prerequisite for storage stability since residual water lowers the glass transition temperature T_g of the lyophilizate. According to a general rule, T_g should be at least 10°C (preferably 50°C) above the targeted storage temperature. Under vacuum or after partial or complete break of vacuum with sterile air or nitrogen, the partially pushed-in stoppers are moved to their final, closed, position by collapsing the shelves. After unloading of the freeze-dryer, the caps or seal caps are fixed to the container by crimping. There exist a large number of books that deal exclusively with drying and lyophilization (26–30). These books along with a chapter on freeze-drying in this book series cover this topic and hence the rest of the chapter will not focus on lyophilization process.

Residual Moisture of the Stopper

The moisture content of lyophilized product, during storage, is influenced by insufficiently dry stoppers that may lead to instabilities. The ratio of mass of stopper to mass of lyophilizate is decisive: the smaller the mass of lyophilizate, the more significant the effect (31). In Figure 11, the storage-related moisture contents of lyophilizates upon usage of stoppers with a residual moisture of 0.3% and 0.05% are compared. The permeation of water through the stopper, to the contrary, is of subordinate relevance.

Change of Native Structure of the Monoclonal Antibody by Lyophilization

In an aqueous environment, the driving forces for the protein conformation are the hydrophobic effect. Nonpolar amino acid residues are pushed into the inner of the protein core and thus removed from the solvent; hydrogen bonds form between the polar amino acid residues at the protein surface and the surrounding water. For this reason, the removal of tightly bound water can change the protein structure and lead to insoluble or soluble protein aggregates. Described in various publications, the partial exchange of the binding partner water by water substitutes like sugars can physically stabilize the protein. Andya et al.

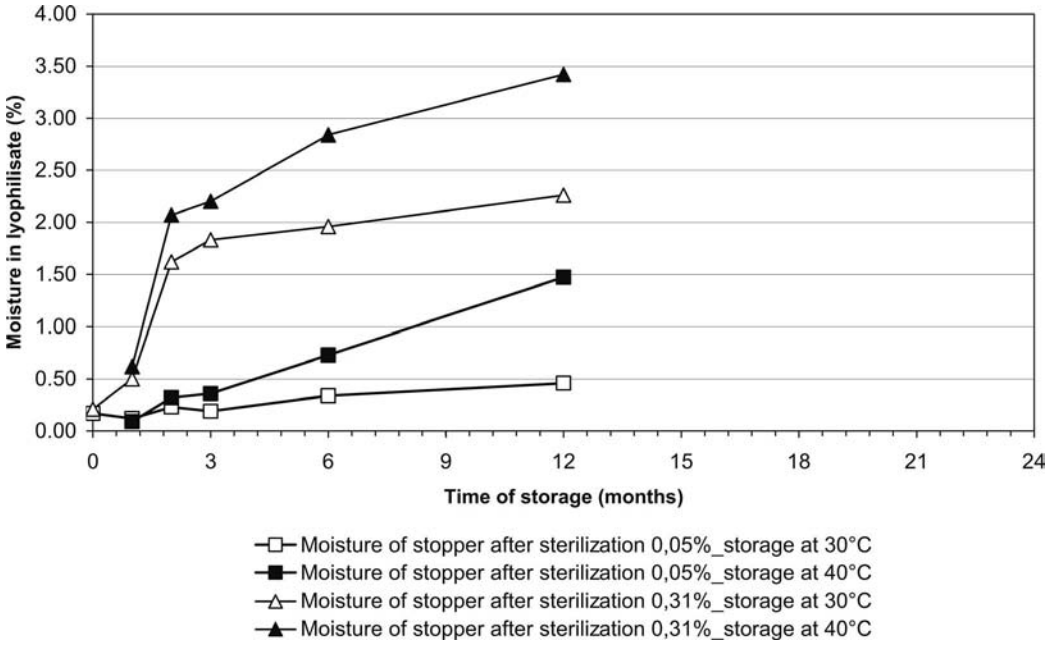


Figure 11 Dependence of moisture content of lyophilized cake during storage on stopper moisture after stopper sterilization.

compared, by FTIR spectrometry and circular dichroism (CD), the secondary structure of a purified recombinant human monoclonal antibody after lyophilization without excipients and together with sucrose or trehalose in ratios of 260:1 up to 2000:1 (25). Indeed in the presence of sugars, a structure very similar to the native one was found even in the solid state. After one year of storage at 30°C, covalent aggregates through free thiol groups had formed without the sugars. A comparison of the measured and calculated water monolayer by use of the Brunauer–Emmett–Teller equation found that the residual moisture values of the excipient-free lyophilizate showed approximately 25% less water was present in the cake than needed for the complete saturation of all surface-accessible hydrophilic groups (25). The authors attributed the observed differences in the solid state and the storage-related instabilities of the sugar-free preparation to the uncovered charged sites due to dehydration.

QUALITY PROCESSES BY DESIGN

(Refer to chap. 13, "Application of Quality by Design in CMC development.")

Philosophy Changes Due to Risk-Based Approach and QbD

In the context of the Quality-by-Design (QbD) philosophy, as outlined in the ICH Guideline Q8 Pharmaceutical Development (2006, followed by revisions), during development profound process understanding must be *systematically* attained and be developed further in the product life cycle. By definition, a well defined design space and convincing justification in the regulatory submission documents should allow future flexibility as far as the proposed change is within the established design space. The prerequisites for allowing regulatory flexibility are good design of product and process, good risk management strategies (ICH Q9), and good quality systems (ICH Q10).

The introduction of new technologies and continuous process improvements have been purposely omitted so far by (bio)pharmaceutical manufacturers, since regulatory requirements made implementation of optimized production almost impossible. Very detailed batch records, which defined numerous non-key parameters, led to deviations and laborious investigations. Primarily, compliance led to rising costs, reduced yields with minimal to no

improvement in the product quality. The FDA and EMEA have acknowledged these problems and embraced the Process Analytical Technology (PAT) and developed the 21st century Good Manufacturing Practice (GMP) initiative. The goals are to allow industry to implement the latest technology to produce high-quality products with optimized processes. Assurance of regulatory relief, if a QbD concept was implemented, is also a potential benefit.

However, this benefit for the submitter is yet to be shown in practice broadly. Large skepticism exists around the concept of allowing more freedom during change control, if additional information in the submission documents and the development studies are provided to establish design space. Little concrete, executable instructions are available from the regulatory authorities in how to request and implement flexibility.

A multivariate experimental study should be conducted to define the design space. Traditionally, proven acceptable ranges (PARs) were defined for each parameter on the basis of development studies that were often univariate in design. Useful, but not a must, is to know the edge of failure, the range in which the process no longer works as desired. For validation and the dossier, the question arises whether a target range is sufficient, or whether the PARs are to be indicated. The concept of PAR and NOR (normal operating range) may be in conflict with the design space concept, since they are typically derived from one-factor-at-a-time (OFAT) experimentation or univariate analysis where the interaction terms are not taken into account (32).

QbD—Systemic Approach and Use of Risk Analysis

Key to QbD is based on good science and performing risk assessment. Critical step for assuring the product quality is the identification and control of parameters within the manufacturing process, active substance, excipient, components, and packaging materials.

Practice of performing a *systematic risk analyses* has proven to be a very helpful tool for the process development, process verification, and preparation of the process validation. Different systems for the risk analysis are shown in Figures 12 to 14.

- Failure mode and effect analysis (FMEA)
- Ishikawa's cause-and-effect diagram (commonly referred to as fishbone diagram)
- Tree analysis

The result of the risk analyses can serve as the basis for a systematic and detailed understanding, for example, by design of experiment (DoE). According to ICH Q8, it is essential to establish to what extent the variation of the process parameter settings affect product quality. It is also important to know where the variability of the process parameters and the product quality attributes stem from. Fluctuations in the measuring systems should be accounted for prior to drawing any conclusions. Different experimental designs are available including full factorial design, fractional factorial design, central composite design, or Box-Behnken design. These designs can be provided by off the shelf DoE software such as JMP (SAS), Statistica (StatSoft), Modde (Umetrics), etc.

A systematic representation of the approach is provided in Figure 15. As an example parameter, the pressure during sterile filtration of the proteinaceous bulk was selected. Depending on sensitivity of the protein, filtration pressure can cause damage to the active substance (input = parameter filtration pressure, output = critical quality attribute, for example, aggregate content).

PROCESS VALIDATION

Legal Basis for Process Validation

United States

U.S. guidances that give details on how to validate (aseptic) pharmaceutical processes include the following:

- 21 CFR 211.100 and 110
- Guidance for Industry: Process Validation: General Principles and Practices (Draft published Nov 2008 replacing the Guidance in place since 1987)

	FMEA - Risk analysis for product XXX mg Purpose of this FMEA: process robustness testing in Pilot Plant and transfer to Production Plant									
Criticality	**Characteristic (main branch)**	**Potential failures**	**Potential effects**	**Potential causes**	**O**	**S**	**D**	**RPN**	**Action required**	**To be actioned by**	**Review / Documentation**
	Bulk										
1	Bulk container (type - stainless steel)	Vessel leaks	High bioburden, endotoxins in finished product	Defective valve / flange / seal	3	8	1	24			
4	Bulk container (type - plastic bag)	Bag leaks	High bioburden endotoxins in finished product	Material defects excessive mechanical stress, embrittlement of material	8	10	1	80			
5		Leachables	Product quality inadequate (foreign substances)	Bag material not suitable for formulation	3	5	10	150			
6		Gas permeation during long/short term storage	Product quality inadequate (oxidatation of drug or excipients, loss of solvent)	Bag material does not inhibit gas permeation such as oxygen uptake and water vapour permeation	8	10	8	640			
7	Holding of bulk after thawing	Hold time/temp too long/high	Reduction in product quality (OOS result)	Wrong storage temp. Wrong storage time	1	10	1	10			
	Packaging material Rubber stoppers										
12	Machinability (in Pilot Plant and in Production Plant)	Poor machinability	Increased aseptic risk, increased process time	Stopper dimensions Incorrect degree of siliconisation Incorrect sterilisation / drying process Rubber formulation (stoppers too tacky) Change parts unsuitable or unavailable	3	8	1	24			
15	Sterilisation (in Pilot Plant and in Production Plant)	Incorrect sterilisation process	Non-sterile product	Sterilisation process not valid	1	10	8	80			
17	Quality	Stoppers contain endotoxins and/or particles, degree of siliconisation not as per order, non-compliance with dimensional tolerances, stopper defects	OOS result for stoppers	Manufacturing / cleaning process at stopper manufacturer's not valid	3	5	1	15			
18	Delivery capability Delivery lead times	Stoppers cannot be delivered on time	Filling of batches delayed	Order placed too late Supply problems on manufacturer's part	3	5	1	15			
19	Sensitivity of product to silicone oil (from stoppers)	Sensitivity to silicone oil	Reduction in product quality (OOS result)	Siliconisation of stoppers	5	10	5	250			

Figure 12 Example of an FMEA (failure mode and effect analysis).

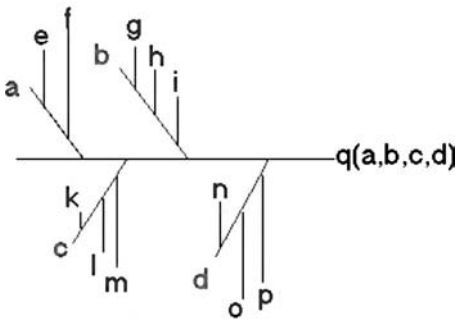


Figure 13 Ishikawa's cause-and-effect diagram ("fishbone").

- Compliance Policy Guide (CPG), Process Validation Requirements for Drug Products and Active Pharmaceutical Ingredients Subject to Pre-Market Approval, rev. Dec. 2004
- FDA Guidance for Industry: Sterile Drug Products Produced by Aseptic Processing—Current Good Manufacturing Practice, Sep 2004
- Guidance for Industry for the Submission of Documentation for Sterilization Process Validation in Applications for Human and Veterinary Drug Products, Nov. 1994

	CTQ_1	CTQ_2	CTQ_3	CTQ_4	CTQ_5	CTQ_6	CTQ_7	CTQ_8	CTQ_9
Processstep 1	○	○	●	X	○	○	○	○	○
Processstep 2	○	XX	○	○	●	○		○	○
Processstep 3	○	X	○	○	○	●	○	○	X
Processstep 4	X	●	●	○	XX		○	●	X

○ No influence
● Small influence
X Big influence

CTQ = critical to quality attribute

Figure 14 Fault tree analysis.

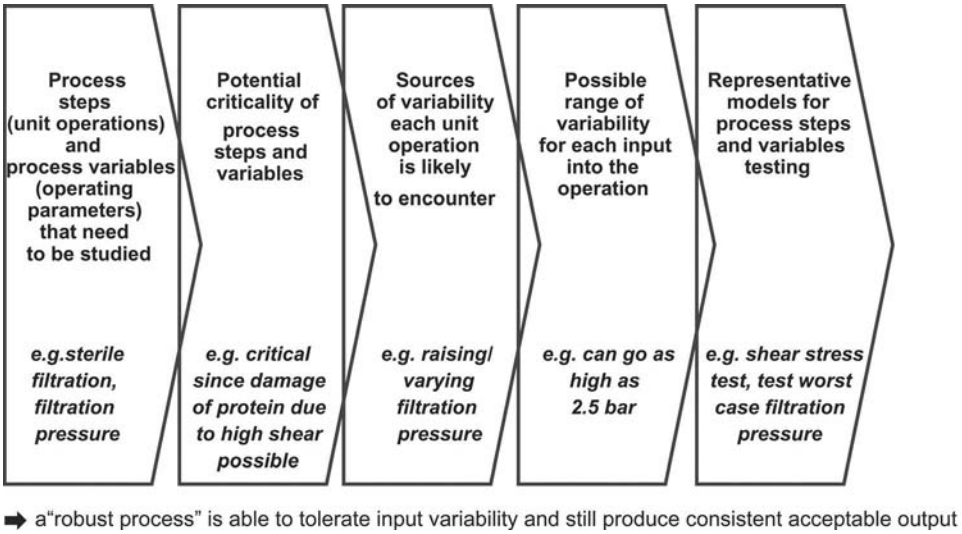


Figure 15 Systematic analysis of critical process variables and example from aseptic fill and finish manufacture of a protein product.

- FDA Guidance for Industry, PAT—A Framework for Innovative Pharmaceutical Development, Manufacturing, and Quality Assurance, Sep 2004
- Pharmaceutical cGMPs for the 21st Century—A Risk-Based Approach, FDA Final Report, Sep 2004

In the guidance "Process Validation Requirements for Drug Products and Active Pharmaceutical Ingredients Subject to Pre-Market Approval" of 2004 (which however is actually not directly applicable to recombinant proteins) it is stated that—"the proof of validation is obtained through rational experimental design and the evaluation of data, preferably beginning from the process development phase and continuing through the commercial production phase."

The process validation guideline from 1987 has been revised by the FDA and a draft has been published for public comment in November 2008. The draft revision picks up the ideas of the CPG and addresses the relationship between modern quality systems and manufacturing science advances to the conduct of process validation. The revision focuses on a process validation life-cycle approach including four key phases—design, confirm, verify (three of four listed) (Fig. 16). The current focus on the commercial process "validation" will be shifted toward the design phase in agreement with the new FDA science-based approach, the application of QbD by gathering complete product/process knowledge, a "continuous quality verification system," and an effective monitoring/assessment program to address effective

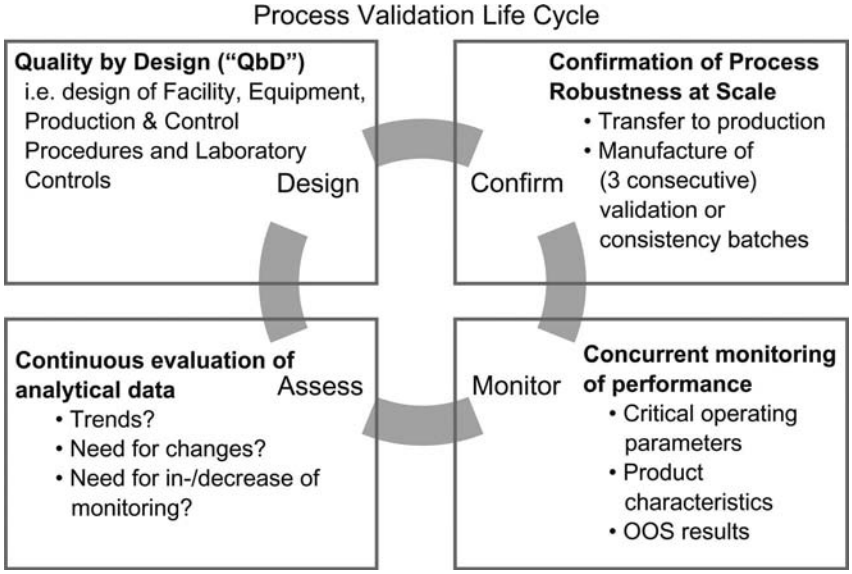


Figure 16 Validation concept according to three-stage concept. *Source*: Adapted from Ref. 33.

process control and continuous improvement as the key factors for reducing the risk to the product quality. This new perspective is different from the current process validation approach. Process validation for the purposes of the new draft directive does not limit itself to the pure process qualification. The linguistic usage for "process validation" and "process qualification" is somewhat different between the European Union and U.S. Guidelines (see EU GMP Guide, Annex 15).

Other important basic ideas and elements of the validation in the new guideline are as follows:

- Integrated team approach to process validation that embraces expertise from a variety of disciplines including process engineering and statistics.
- Project plans, along with the full support of senior management "... all studies should be planned and conducted according to sound scientific principles, appropriately documented, and should be approved in accordance with the established procedure appropriate for the stage of the lifecycle ...."
- Modeling of procedures, ideally at small scale, and transfer by appropriate simulations or virtual programs from the pilot to production scale; at least the process understanding can be improved significantly by modeling.
- Importance of process controls that can reduce input variation, adjust for input variation during manufacturing, or combine both approaches. PAT for in-process controls is essential in those cases where quality is not readily measurable later in the product, for example, microbial contamination.
- Statistician or person with adequate training in statistical process control techniques to develop the data collection plan and statistical methods and procedures used in measuring and evaluating process stability and process capability.
- Representation of the processes by process flow diagrams.

The terms conformance batches and validation batches are equivalent. The approval of the product can take place still before the successful manufacture of the conformance batches (for small molecule parenterals). However, the validation should be completed prior to the start of marketing. For biologics, validation batches need to be manufactured prior to filing the

BLA. For biotherapeutics, typically drug product process validation is already described in the dossier and can thus be reviewed during preapproval inspection (PAI).

Exemptions to process validation requirements are made for orphan drugs for health-economic reasons, drugs with very limited shelf life, and drugs with limited use like radiopharmaceuticals. In these cases, release for the market and validation can take place in parallel (concurrent validation).

EU and International

EU and international guidance that address validation of pharmaceutical processes include the following:

- CPMP Note for Guidance on Process Validation + Annex I Process Validation Scheme, Sep 2001
- CPMP Note for Guidance on Development Pharmaceutics, Jan 1998
- Annex II, Note for Guidance on Process Validation—Non Standard Processes, Jan 2005
- EU Guide to GMP, Annex 15, Qualification and Validation, Sep 2001
- ICH Q8 and ICH Q9

In the *Note for Guidance (NfG) on Process Validation* with its Annex I, validation is described as the verification of the process at scale. Usually on the basis of three full-scale batches, and development data, small to pilot scale that is 10% of production scale, proof of process validation is established (*critical steps, critical parameters*). It is expected that in the process validation, additional tests are accomplished beyond the spectrum of the release tests.

Also in the EU, revalidation is understood as periodic continuum. As already mentioned in the previous section, definitions of PV (process validation), new processes/products and PQ (process qualification) validation using product or simulating product, are somewhat different from the linguistic usage in the United States, in particular from the FDA draft on process validation.

An accomplished process validation is not necessary at the time of the submission, but the protocol of the planned studies for the production batches is part of the dossier and/or the Pharmaceutical Expert Report. This is not valid for nonstandard methods of production (*Annex II to NfG on Process Validation*), nonstandard sterilization procedures, aseptic manufacturing processes, certain lyophilization procedures, microencapsulation procedures, and sustained release products. In these cases, before approval, three consecutive batches at production scale are demanded. Comparable to the FDA regulations, the *EU GMP Guide, Annex 15*, defines that even though process validation should normally be completed prior to the distribution and sale of the medicinal product (prospective validation), "in exceptional circumstances, where this is not possible, it may be necessary to validate processes during routine production (concurrent validation)." There is also a retrospective validation, which is applicable only to well-established processes without changes.

Significant changes in the manufacturing process can initiate variations in the market authorization that need approval by the authorities prior to implementation (type II variations). In case of products with biological active substances, even small changes of process are nearly always type II variations in accordance with *guideline on the details of the various categories of variations to the terms of marketing authorizations for medicinal products for human use and veterinary medicinal products*. Very detailed descriptions of process and equipment design are expressly not demanded for the dossier in the *NfG on Process Validation*.

In the EU, the basic ideas of parametric release have been introduced. It however covers only terminally sterilized products (*NfG on Parametric Release, 2001; Annex 17 to the EU Guide to GMP*).

In the *Annex 13 of the EU GMP Guide*, it is stated that manufacturing processes for clinical supplies do not have to be validated, with the exception of buildings and equipment. For products labeled as sterile, sterilization processes must be validated according to the same standard as market products. The same holds true for virus inactivation or removal processes. Aseptic processes must be validated at this stage, whereby the smaller batch sizes and the semimanual steps during the production are valid.

Cleaning validation for similar products and processes can be accomplished with a representative product and on the basis of one worst-case consideration.

Validation of an Aseptic Fill and Finish Process for a Monoclonal Antibody or Therapeutic Protein Product

Before the product-specific validation can be started, it is understood that any process validation facilities and equipment must be qualified for the purpose by DQ/IQ/OQ. The focus of the drug product validation runs should be to monitor and control critical operational parameters (COPs).

The validation of the pharmaceutical process will be described referencing the example of a classical monoclonal antibody product. The following steps must be considered in the context of the validation, and the list is not meant to be exhaustive:

- Thawing and pooling of the frozen bulk, potential additions and dilutions
- Cleaning and sterilization of multiprocess equipment [e.g., needles, pumps, tanks, pipes, disposable tubings, and bottles (unless provided in ready-to-use quality); also the hold time between end of use and cleaning as well as between cleaning and reuse are part of the cleaning validation]
- Bioburden reduction filtration and sterile filtration
- Cleaning and sterilization of packaging materials (e.g., vials, stoppers, and crimp caps)
- Filling and sealing procedures, to include freeze-drying if necessary
- Aseptic procedures, interventions, and the facility/personnel involved (media fills)
- Visual inspection
- Transportation of the filled and controlled vials (e.g., to the final packer, warehouse, etc.)
- Hold times of the bulk after thawing, the final product after the filling at various temperatures, and the hold times during process stops
- Decontamination of equipment in vaporized hydrogen peroxide (VHP) material locks

Naturally the analytical and in-process control procedures must be validated. Starting from phase 2 and 3, methods complying to cGMP are expected in accordance with the Draft FDA Guidance on Process Validation.

Because of fact that aseptic processes for therapeutic protein preparations belong to high-risk processes, some process steps are already validated during the clinical phase. For aseptic manipulations, sterilization and decontamination procedures guarantee the absence of contamination. Process steps, that determine other quality parameters, are usually validated prospectively and fully only with the conformance batches and are covered during the clinical phase by concurrent validation.

Design of Process

As shown in sections "Selected Case Studies Exemplifying Development Challenges During Fill and Finish" and "Quality Processes by Design," prior to validation, the process must be developed and investigated. Hereby "design" means to identify critical operation parameters and acceptable operating ranges by development studies such as

- Design of experiments
- Laboratory or scale-up experimental batches to gain process understanding

Furthermore mechanisms to limit or control variability, based on experimental data, must be established. A "robust process" is able to tolerate input variability and still produce consistent, acceptable output.

What exactly are COPs? The definitions given in the PDA Technical Report No. 42 (1998) are very useful even though the report does not explicitly target the aseptic processes:

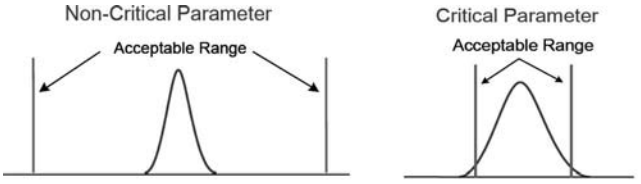
- Operational parameter = *input variable* or condition of manufacturing process that can be directly controlled in the process.
- Critical operational parameter (COP): input process parameter that should be controlled within a *narrow* operating range to ensure *quality* attributes meet specifications.

- Non–critical operational parameter (Non-COP): input process parameters that fall outside definition for COPs. Divided into:
  - Key operational parameters (KOP): input parameter that should be carefully controlled within a *narrow* range and is essential for *process performance* (does *not* affect quality).
  - Non–key operational parameters (Non-KOP): input parameter that has been demonstrated to be easily controlled and has a *wide acceptable limit* (quality or process performance impacted if acceptable limits exceeded).

Other sources differentiate between COP or non-COP and KOP and non-KOP depending on whether (critical) *quality* attributes (CQA) or *performance* attributes (= *output variables*) of the product or the process are affected. Non-COP or non-KOP are parameters that have a wide tolerance and need not be narrowly controlled. This is depicted in Figure 17. Table 7 lists some of the operational parameters during drug product manufacturing and their hypothetical classification. The table should be considered an example since the classification of parameters as COP or KOP will change depending on the product and process used. In ICH guideline, the terminology used is "process" rather than "operational," for example, instead of COP or KOP, the terms CPP and KPP are used.

Conformance Batches

The conformance batches, that are exactly consistent with the classical validation batches, are manufactured after the transfer of the process into the production facility. The EU GMP Guide,



Critical process Parameter: a process parameter that must be controlled within predetermined criteria to ensure the bulk drug substance or drug product meets its specification/quality attributes. A process parameter is critical if the target range (TR) is near the acceptable range (AR) or as determined by a technical expert

Figure 17 Definition of operational parameters.

Table 7 Example for a List of Operational Parameters and Their Classification

Controllable operational parameter	Classification[a]	Acceptable range (as of development)	Normal range (conformance batches)
Hold time of bulk outside cold room (hours)	COP	3 mo at 25°C within specification	Up to 2 wk at room temperature
Filtration pressure or rate (bar or mL/min)	Non-KOP	Up to 2 bar	1.0–1.5 bar
Fill volume and precision (mL)	COP	At 10.5 mL fill with 10.1 mL extractable volume	10.7 mL ± 0.2 mL
Bulk temperature (°C)	Non-KOP	No density change between 0 and 30°C	5–25°C
Fill rate (vials/min/needle)	Non-KOP	Up to 30 vials/needle	25 vials/needle
Hold time of product outside cold room (hours)	COP	3 mo at 25°C within specs (drug product or drug substance)	Up to 2 wk at room temperature
Hold time of product exposed to light (hours)	Non-KOP	No impact after 1.2 Million lux hours (ICH)	Up to 24 hr exposed to room light
Capping force or conditions (N or mm)	COP	± ... mm capping height	± ... mm capping height

[a]This classification is hypothetical and is for illustration purpose only.
Abbreviations: COP, critical operational parameter; KOP, key operational parameter.

Annex 15 states that "It is generally considered acceptable that *three* consecutive batches/runs within the finally agreed parameters, would constitute a validation of the process." According to the current Compliance Policy Guides Manual of the FDA, however, no concrete number of conformance batches is required (CPG 7132c.08 Sec. 490.100).

Hereby, "transfer" means the transfer of developmental knowledge to production (technology transfer), that is, after transfer:

- Batch records and standard operating procedures (SOPs) are written and equipment and facilities equivalency is established.
- All raw materials and the suppliers are qualified.
- Measurement systems are qualified to include QC lab as well as production floor test instrumentation.
- Personnel training is completed.

Conformance stands for the following:

- Execution of conformance batches (usually 3 per dose strength) with appropriate sampling points and sampling level, that is,
  - Evidence that process can function at commercial scale by production personnel.
  - Demonstrate reproducibility.
  - New process and packaging components are considered in the media fill concept (validation of aseptic procedures).
- Full sample and data analysis of the consistency batches
  - Data may confirm process as is, point to major process design change(s) or suggest process improvement(s).
  - Changes are implemented via change control procedures
    - Assess need for additional conformance batch(es) or limited testing. Amount/degree of additional work commensurate with the significance of the change and its impact on product quality.

All activities and the underlying validation policy must be established prospectively in a Validation Master Plan (VMP) as well as in individual validation protocols (EU GMP Guide, Annex 15, 2001). The review process must be adhered to. The validation protocols define sampling, analysis, and acceptance criteria for judging whether validation can be classified as successful or not. Furthermore, operating parameters, processing limits, and component (raw material) inputs should be described in the validation protocol. It is very helpful to have tested these acceptance criteria and also the statistical approach during process transfer. The acceptance criteria, however, should have been derived from development studies and clinical material manufactured previously, except for those tests that are predefined by pharmacopoeias or other guidelines.

In our example of a monoclonal antibody product, it would be reasonable to fix acceptance criteria during validation of the fill and finish process for the following parameters (as appropriate):

- Homogeneity and quality of the bulk after thawing and pooling, for example, determined by protein concentration, aggregates, monomers, particle number, and turbidity.
- Bioburden of the bulk prior to sterile filtration.
- Homogeneity and quality of the bulk after sterile filtration, for example, surfactant concentration, protein concentration, aggregates, monomers, number of particles, turbidity, further biophysical or chemical properties depending on the product.
- Quality of the final product at the beginning, middle, and end (where appropriate). This is important for suspension products.
- Sterility of the bulk formulation.
- Additional IPC (in-process control) specifications, for example, fill volume, stopper seating, quality of capping.

- Final product properties, for example, extractable volume, residual moisture, and aggregate or monomer content.
- Yield after filling and capping.
- Yield after visual inspection
- Maximal filtration time, filling time, and capping time.
- Maximal hold time of bulk and final product at room temperature or at other relevant temperatures.

After performance and analysis, validation or conformance drug product manufacturing report is written and must be reviewed against the protocol. Deviations from the protocol must be explained and their impact (if any) on the validation should be evaluated.

Validation During Product Life Cycle
After successful manufacture of the conformance batches, PAI and market authorization, validation is to be continued, according to the current FDA philosophy, during the entire product lifetime. This means:

- Monitor
  - Routine commercial manufacturing
    - Monitor critical operating and performance parameters
      - Utilize appropriate tools, for example, Statistical Process Control (SPC)
      - Monitor product characteristics, for example, stability, product specifications.
    - Monitor state of personnel training and material, facility/equipment, and SOP changes.
    - Investigate out of specifications (OOS) for root cause and implement corrective and preventive actions (CAPA).
- Assess
  - Analyze monitoring data
    - Trend data upon regular review.
    - Evaluate need to increase/decrease level of monitoring/sampling on the basis of accumulated data.
  - Periodic evaluation
    - To determine the need for changes, for example, manufacturing procedures, control procedures, drug product specifications.
  - Study OOS and OOT (out-of-trend) data.
  - Assess impact of process and product changes made over time.
  - Feedback into design stage for significant process shifts or changes.

The CFR [section 211.180(e)] requires "that information and data about product performance and manufacturing experience be periodically reviewed to determine whether any changes to the established process are warranted. Ongoing feedback about product performance is an essential feature of process maintenance." EU GMP Guide, Annex 15, also demands periodic examination whether the process is still in the validated condition or whether changes make revalidation necessary. It is not explicit whether or not changes originate from the feedback of the product performance.

SPECIFICATIONS AND CONTROL LIMITS

The definition of specifications and control ranges, both during the development phases and prospective validation are complex and not clearly agreed upon across the (bio)pharmaceutical industry or regulated at this time.

According to the QbD concept, there should be differentiation between critical quality attributes (CQAs) and parameters used to monitor process consistency. "Specifications and the corresponding limits as applied to CQAs serve to ensure that the product is fit for use, whereas control limits are a manufacturer's tool to monitor shifts and trends in the manufacturing

process. In the current paradigm, inappropriate use of specifications creates a disincentive for continuous process understanding ..." (34). The vision of QbD and design space cannot be achieved when control limits are used as specifications. The design space may be viewed as the region of process settings that yields acceptable product (*i.e., product that meets specifications*). When control limits are used as specifications, the design space reverts to the control space for the process, leaving no opportunity for process improvement (35).

This risk is likewise borne in validation studies. Samples are taken from multiple locations of the production process, or at multiple levels of a process parameter, and subject to specifications. As with stability testing, in which multiple samples are taken over time, validation samples are subject to excess risk of OOS due to multiplicity. This acts as a disincentive to collecting data for better process understanding (35). It is likely that as industry and regulators gain experience, some of these difficulties could be overcome so that process can be improved over time without undue regulatory burden.

SUMMARY

In this chapter, key factors in developing and validating aseptic drug product process for biologics has been covered. Readers are advised that a sound scientific practice should always be used in conjunction with a knowledge of current regulatory environment. Although, this chapter provides a high-level overview of QbD as relates to drug product validation, a more thorough discussion is included in chapter "Application of Quality by Design in CMC Development" in this volume.

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4 Visual inspection

Maria Toler and Sandeep Nema

OVERVIEW

The inspection of parenteral products is driven by the need to minimize the introduction of unintended particulate matter to patients during the administration of injectable medications. Visual inspection also allows for the opportunity to detect and reject other categories of nonconforming units, such as those with cracks and or incomplete seals, which can affect the integrity and sterility of the product. In most cases, these defects will occur randomly and at low frequency. This has led to the current expectation that each finished unit will be individually inspected 100%.

Particulate matter is defined by the United States Pharmacopeia (USP) as "mobile undissolved particles, other than gas bubbles, unintentionally present in the solutions." It is the expectation of the USP that "each final container of injection be subjected individually to a physical inspection, whenever the nature of the container permits, and that every container whose contents show evidence of contamination with visible foreign material be rejected." Visible particulate matter can be defined by the size of the particles. It is generally accepted that the human eye can detect particles once the size approaches 50 μm. The detection of a particle is based on the probability of being able to see it within a container, with the probability increasing with increasing particle size. Analysis of visual inspection results from several studies involving different groups of inspectors showed that the probability of detecting a single 50-μm particle, in a clear solution within a 10-mL vial with diffuse illumination between 2000 and 3000 lux, is just slightly above 0%. However, this probability increases to approximately 40% for a 100-μm particle and becomes >95% for particles ≥200 μm (1). This and similar studies show the dependence of visual detection and particle size. Other factors, such as the refractive index and luster of the particle will also affect the ability to detect.

Why inspect for visible particulates? There is no clear consensus on the safety of having a small number of visible particles in an injectable drug product. The primary evidence for safety can be found in the literature on drug abuse. There is some evidence that addicts who injected drugs had manifestations of pulmonary foreign body emboli and granulomas, along with abnormal pulmonary function (2–4). It has been observed that granulomas are generally associated with fibers and silicosis with glass particulates, while fungal particles have been associated with pyretic issues (5). Protein particles, both subvisible and visible are being investigated for their effect on immune responses. There is a lack of controlled studies in humans to better understand the effect of small amounts of visible particles. Rather, it is generally accepted that injectables should be clear and essentially free of particles that can be seen by the unaided eye. This primarily applies to drugs being infused via the peripheral veins (IV). The presence of particulate matter in intramuscular or subcutaneous injections is not of great concern, especially since small volumes are usually injected and tissue phagocytosis as well as local immobilization of the particles would make them almost harmless.

The major effects and pathological conditions that have been linked in the literature to the injection of particulate matter include the following (6):

- Direct blockage of a blood vessel by foreign particulate matter
- Platelet agglutination, leading to the formation of emboli
- Local inflammatory reactions caused by the impaction of particles in the tissues
- Antigenic reactions with subsequent allergenic consequences
- The distribution of injected particles will depend on size and to lesser extent on particle composition
  - Large particles (≥50 μm) on the basis of circulation (venous infusion → right heart → lung) will be retained in the lung
  - Particles that are ≥10 μm pass the pulmonary vasculature slowly

- Particles <10 μm in size are retained in the liver and spleen for long periods
- Particles <10 μm in size are significantly cleared by phagocytosis by cells of the reticuloendothelial system

In addition to safety issues, the presence of particulate matter in the product can be an indication of formulation unsuitability, improper container closure system, degradation or lack of process cleanliness. Because it can be considered a product quality attribute, particulate matter should be controlled in intramuscular and subcutaneous dosage forms as well as intravenously injected products.

REGULATORY ASPECTS

The purpose of a visual inspection is to satisfy the regulatory agencies and ensure the safety and quality of the drug product. A survey was presented at a Parenteral Drug Association (PDA) forum in 2008 describing regulatory observations that had been reported over the last 12 years (7). Fifty percent of the firms surveyed were challenged on their inspection programs. Having an appropriate inspection program can aid an organization in avoiding a Form 483 or Warning Letter from the U.S. Food and Drug Administration (FDA). The Form 483 is referred to as a "Notice of Inspectional Observations." It is issued by an FDA field investigator after an on-site inspection and will list areas of noncompliance with current Good Manufacturing Practices (cGMPs) or other deficiencies in the quality system. The organization must respond to the Form 483 and identify a course of action to correct the findings, along with a timeframe that issues will be addressed.

Some findings from Form 483s issued over the last 10 years include the areas of documentation, quality limits, and process. Documentation findings included lack of training procedures, standard operating procedures (SOPs) for visual inspection, and inspector retesting schedules. An example quoted from a Form 483 was "Observation #3 from the FDA-483 states that there was no documentation that your firm performed a visual, unit-by-unit examination of containers, vials, and ampoules for defects. You also did not visually inspect each component, diluent, or product for visible contamination." Another Form 483 example, relating to inspector training stated that:

> "There is no written procedure to describe the training required for employees performing visual inspection of containers from either media fill operations or of final products. Additionally, the control standards used to train individuals who perform visual inspections are incomplete in that there are no standards that describe the criteria for sizing and characterizing particulate matter, examples of over or under filled containers or bottles containing glass, metal or rubber contaminants."

Poor documentation can indicate a lack of proper control over the inspection program. Findings related to quality limits include no definition of critical, major, and minor defects that would trigger an investigation. Defining the acceptance criteria and level of defect is a good manufacturing practice and allows for an unbiased approach to rejection of defective product. Examples quoted from Form 483s include "SOP BV1019, entitled 'Visual Inspection of BoTox Product,' does not specify limits for critical, major and minor defects which, when exceeded, would trigger an investigation, and does not instruct operators to place rejected vials in the specific bins"; "there is no specified action level or limit for the filled product container visual inspection performed by the filling department."

Findings related to process include the lack of a separate labeled container/area for rejected samples and a failure of a machine inspection station to completely clear a lot of product before running a new lot of product. There is a greater chance of mixing lots or introducing rejects into an acceptable lot of product, as described in this Form 483:

> A Seidenader™ inspection machine was being used for visual inspections of a parenteral. The machine had an exit arm in which several vials would remain in the machine and were not pushed out. These vials could not be clearly seen without bending down to look at the exit arm. As a result of this, an incident occurred where the line was not cleared of these vials and they became mixed with the beginning of the next lot of product. The next lot of product looked the same as the previous product and as a result mislabeled product was distributed.

VISIBLE PARTICULATE MATTER INSPECTION REQUIREMENTS

In the early part of the 1900s, the USP recognized the need for injectable compounds to be true solutions. In 1936, a requirement for the "clarity" of injectable solutions was specified.

Currently the USP states that all "inspection processes shall be designed and qualified to ensure that every lot of all parenteral preparations is essentially free from visible particulate" with no inspection method specified. The pharmacopeias from other countries that participate in the International Conference on Harmonization (ICH) are similar in requirements, having some differences in the amount of detail provided in the description of the inspection methods.

The USP (Chapter <1> Injections) states that all

> articles intended for parenteral administration shall be prepared in a manner designed to exclude particulate matter as defined in Particulate Matter in Injections <788> and other foreign matter ... The inspection process shall be designed and qualified to ensure that every lot of parenteral preparations is essentially free from visible particulates. Qualification of the inspection process shall be performed with reference to particulates in the visible range of a type that might emanate from the manufacturing or filling process. Every container that shows evidence of visible particulates shall be rejected.

The Japanese Pharmacopoeia (JP) states that unless otherwise stated, injections should meet the requirements of the Foreign Insoluble Matter Test for Injections <6.06>. There are two inspection methods described.

Method 1 "is applied to injections either in solutions or in solution constituted from sterile drug solids" and uses the following procedure: "Clean the exterior of containers, and inspect with the unaided eyes at a position of light intensity at approximately 8000 to 10,000 lux, with an incandescent lamp at appropriate distances above and below the container."

Method 2 "is applied to injections with constituted solution" and uses the following procedure: "Clean the exterior of the containers, and dissolve the contents with constituted solution or with water for injection carefully, avoiding any contamination with extraneous foreign substances. The solution thus constituted must be clear and free from foreign insoluble matter that is clearly detectable when inspected with the unaided eyes at a position of light intensity of approximately 1000 lux, right under an incandescent lamp."

The requirements for the freedom of parenteral solutions from the presence of particulate matter are very strict in Japan. The inspection process for individual containers is more rigorous than the USP (8).

The European Pharmacopoeia (EP) states that "Solutions for injection, examined under suitable conditions of visibility, are clear and practically free from particles." The inspection procedure states that "Gently swirl or invert the container and observe for about 5 seconds in front of the white panel. Repeat the procedure in front of the black panel. Record the presence of any particles." The method includes a description of the viewing station and lighting requirements (2000–3750 lux at the viewing point). The presence of particles is recorded but no sample quantity or acceptance criteria are provided.

The various compendia have similar statements for the requirements for visible particulate matter in injectables:

USP: "essentially free from particles that can be observed on visual inspection"
EP: "clear and practically free from particles"
JP: "clear and free from readily detectable foreign insoluble matter"

There is currently a proposal to the USP to revise the General Chapter—Injections <1> sampling requirements (9). The proposal is based on the General Inspection Level II sampling plan as described in ANSI/ASQ Z1.4 with an Acceptable Quality Limit (AQL) of 0.65%. This AQL was chosen based on the median value obtained from a recent benchmarking survey of industry practice that was conducted by the PDA (7). This inspection procedure would apply to retesting of product in distribution (having undergone 100% inspection) or when a limited subset of the batch is available for inspection (e.g., from retained or returned samples). A sampling of 60 units would be inspected. The batch would be considered to meet the requirement "essentially free" when no more than one (1) unit with one or more particles is

observed. This sampling plan has an AQL of 0.60%, which is acceptably close to the ANSI/ASQ Z1.4 AQL of 0.65%.

It does, however, need to be noted that several parenteral products have recently been approved that contain visible particulate matter. Vectibix (Amgen, Thousand Oaks, California, U.S.) is a marketed product that specifies in the product insert that visible particles may be observed. Upon examination, it is observed that this product contains a great deal of visible particles in solution. It is also specified that the drug is filtered right before administration. Stelara (ustekinumab, Centocor, Radnor, Pennsylvania, U.S.) is described as a colorless to light yellow product that may contain a few small translucent or white particles. Arzerra (GlaxoSmithKline, Brentford, Middlesex, U.K.) is a product stating that it is a colorless solution that may contain a small amount of visible translucent-to-white amorphous drug product particles. The product is supplied with an in-line filter. Similarly, Erbitux (Imclone/Bristol-Myers Squibb, New York, New York, U.S.) is a product that is also instructed to be used with an in-line filter due to the presence of protein particles. All four cases (Vectibix, Stelara, Arzerra, and Erbitux) are a result of the inherent nature of protein formulations, which can result in the formation of intrinsic protein particles. These visible, translucent, white particles are removed via in-line filtration during IV administration without noticeable loss of assay/potency since only a small fraction of protein is in the form of visible particulates. It could be debated if a better formulation could be developed to eliminate such protein particles is or is not possible. However, these types of products present a significant challenge during visual inspection. A reference to an acceptable lot must be made and the inspection assay should be validated to ensure that the product meets this reference. It would be a good practice to provide a quantitative or semiquantitative determination of these visible particles. In addition, some attempt should be made to understand the composition of the visible particles and to ensure that it remains consistent during storage under preferred conditions.

The Inspection Process

The levels of subvisible and visible particulates in a sample are a useful measure of the product quality. Monitoring of visible particles is an important product attribute and a regulatory requirement. There are two very different approaches to detection of visible particulate matter in parenterals. One method utilizes people and the other utilizes machines for detection.

Human Visual Inspection

The inspection apparatus that is normally used is comprised of an inspection station containing a lamp at a specified intensity. The EP provides a figure of the type of apparatus to be used in visual inspections. The lighting may be fluorescent, incandescent, spot and/or polarized, with fluorescent being the most common. The light source may be positioned above, below, or behind the units being inspected, with a range of intensity from 100- to 350-ft candles (note that the JP requires 740- to 930-ft candle light intensity for inspection of plastic containers) (19). The inspection station has both white and black backgrounds. Some inspection stations also include a magnification lens. The variables that are of concern during human inspection, such as fatigue and visual acuity, are addressed when appropriate training procedures are in place. The parameters that should be adhered to during visual inspection are referred to in the compendia. Figure 1 shows a manual inspection room. The inspectors in this

Figure 1 A manual inspection room. Inspectors are situated at inspection stations.

facility sit comfortably at stations with a gray/black background (black and white backgrounds are most commonly used). In this example, they are inspecting prefilled syringe products. The inspectors will view the syringes at several positions (Figs. 2–4), they are not only looking for particulate matter within the syringe but also looking at the condition of the container, inspecting all dimensions of the units for any defects. Figure 5 shows an inspector looking at vials. Vials are placed into a clear holder to allow for more than one unit to be inspected at a time for defects.

Some of the factors that are of concern during human visual inspection are listed below. The factors are categorized as either inspection process variables (these variables are controllable) or product characteristics (may not be controllable).

Inspection process variables:

Visual acuity (close vision capability)
Proper motion of the container to suspend the particles

Figure 2 An inspector begins by observing the prefilled syringes in their packaging trays.

Figure 3 Prefilled syringes are viewed at the light source.

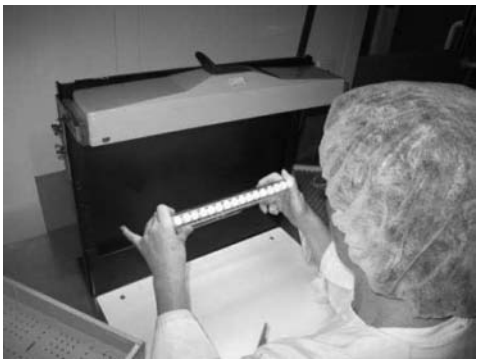

Figure 4 Prefilled syringes are inspected, ensuring that all dimensions of the product are monitored. Here the inspector is viewing the top of the syringe for any defects.

Figure 5 An inspector is monitoring vials. Here vials are placed in a clear holder so more than one unit can be inspected at a time.

Manual dexterity of the inspector
Type and intensity of lighting
Background to particle contrast
Time of inspection
Total background illumination
Accurate illumination at the point of inspection
Use of magnification
Initial position of the particle at the commencement of inspection
The presence of interfering signals such as cavitation bubbles or glare
Inspector fatigue

Product characteristics:

Total volume in container
Container clarity
Particle density and optical properties of particles and solution
Optical defects in the wall of the container and markings on container
Foaming properties and viscosity of the product

In general, a manual inspection procedure should include the following steps:

1. Any labels, if present, must be removed from the container. Container should be cleaned using low particle shedding wipes.
2. Hold the container by the neck and swirl container to set particles in motion. Care should be taken to avoid creating air bubbles. Air bubbles will rise to the surface, which will help differentiate them from particulate matter.
3. The container is inspected while being held at a 45° to 60° angle from the vertical, about 10 in. below the light source [some sources state 4 in. (8)]. The container should be inspected in front of both a black and white background. Light should be directed away from the inspector's eyes and the container should not be placed directly under lighting to avoid glare.
4. If no particles are seen so far, the container can be slowly inverted and inspected for any heavy particles that may not have been suspended during swirling.
5. Containers with visible particles should be set aside for further investigation/rejection.
6. Small volume vials and ampoules (<50 mL) should be viewed for approximately 5 seconds (10 seconds per the JP). Containers that are 50 to 100 mL should be viewed for 10 to 20 seconds. Containers that are 250 to 1000 mL should be viewed for 30 to 60 seconds.

The manual inspection is considered the benchmark for all other particle inspection methods and devices, therefore no validation studies are required. However, it is important to

note that human inspectors must undergo appropriate training and testing to become qualified for inspection. The qualification is then maintained through good supervision, proper procedures, and continued retraining. It has been shown that none of the commercially available inspection systems can, with a single reading, show performance that is equivalent to a single manual inspection (10). According to Knapp, even two serial machine inspections cannot achieve the security and discrimination of a trained inspector. With the advances in automated inspection machines, this statement may not be as accurate today, but it does show the power of the visual inspection procedure. However, it is imperative that the operators be properly trained for performing visual inspections. The accept/reject decision by the inspector has been shown to be a probabilistic determination, with the probability of being able to detect a particle increasing with increasing particle size (11).

The initial position of the particle at the commencement of inspection is an interesting aspect of a good inspection and should be further considered. Particle movement during the inspection is necessary to differentiate a contaminating particle from the container markings or other optical distortions. The inspector should be trained to provide maximum velocity to the particle while avoiding an excess of energy so that bubbles are not formed. If a formulation composition affects the formation of bubbles, the inspection procedure must be modified accordingly. An important factor in the velocity of a particle is the initial position on the container bottom. Those particles that are closer to the axis of rotation require greater rotational energy to achieve adequate movement during inspection. Care should be taken to avoid impact or excessive transport of the container to avoid cavitation bubbles from forming. It can be very difficult to differentiate between bubbles and solid particulates in solution. Also, it should be noted that the viscosity and container volume will affect the velocity of the particles in solution.

Inspector Training/Calibration

As stated previously, proper inspector training is critical to a properly designed visual inspection program. The handling of the sample as well as the "calibration" of the inspector is important to obtaining accurate and repeatable results. The concept of creating a calibration curve during training has been explored (11). The idea is similar to using a calibration curve for a particle counter. The rejection probability calibration curve is generated using a test set of containers, each with a single, durable, and accurately measured visible particle in a suspending fluid. Knapp has described the use of glass and stainless steel particles for the test set. Ideally, the test set should include samples that are representative of the entire particle contamination spectrum from clean to must-reject contamination. This test set can be applied to multiple inspectors at multiple sites, defining the test environment. Results should be obtained until there is sufficient data to support an analysis at the 0.05 significance level. This result set can be used to obtain a standard "visibility" reference curve. This reference curve can then be used to assure the competency of the inspectors and, ultimately, the quality of the production batch. Once the visibility reference curve is established, this information can be used to qualify new inspectors or machine inspection. A good practice would be to demonstrate that the inspection security is achieved to at least the same level as the qualified manual inspection (12).

Another aspect of training that can be easily overlooked is the requirement of an appropriate vision test. A study was performed to determine the efficiency of a group of inspectors (12). It was found that the group fell into three categories, low, middle, and high false reject rates. The high false reject rate occurrence in the high group was traced to the fact that only the standard distance eye test was specified for the inspectors. Following this observation, a close focus eye test was required to resolve the problem. It is critical to add appropriate visual acuity testing to the qualification of the inspectors.

Machine Inspection

There are numerous machines that have been developed to aid in the visual inspection process. The throughput is much higher when utilizing these systems versus manual human inspection. Machines for semiautomated inspection can perform most of the mechanical manipulations normally done by the human inspector. These manipulations include swirling

the vials, inverting samples, stopping the container, and the ability to remove units flagged as defective. These semiautomated systems can provide additional lighting, such as Tyndall or polarized light filters and adjustable container holders to change the angle of inspection or container rotation rate. The visualization process can be performed via an imaging system, which reduces eye strain to the operator. There are also completely automated systems available. Eisai, for example, has automated inspection instruments capable of inspecting vials, ampoules, and syringes (13). The AIM (Automatic Inspection Machine) is fully automatic and is capable of detecting particulate matter as well as cosmetic defects at up to 24,000 vials or ampoules per hour. Various areas of the container are inspected including the body, heel, neck, and crimp/cap area. There is also a system for the inspection of syringes. The EIS inspection system is fully automated and used primarily for prefilled syringe inspection at up to 36,000 units per hour.

These Eisai automated systems are based on transmitted light (static division) technology. Static division refers to the ability of the machine to differentiate between moving and static objects, for instance a moving particle versus a scratch on the container surface. Particles in solution will block a portion of the transmitted light passing through the container. The particle will block a portion of the light causing a shadow that is detected by an array of small diodes. Since the instrument is looking at the blockage of light, the color and reflectivity of the particle is not a factor in detection. In addition, the change in light intensity is monitored so only the signals from moving objects are recorded. During operation, the container is spun at high speeds (1000–5000 rpm) just before reaching the inspection station. At that point a brake is applied and the liquid inside the container will continue to rotate due to inertia. Any insoluble particle matter will be suspended and float past the detection system. A prespin step can be used to dislodge and remove any bubbles in the container, reducing the incidence of false rejects. Since the system utilizes transmitted light, it can be set up in any work area regardless of the external lighting. These machines are designed to perform two inspections per container. This improves both sensitivity and reproducibility. Human inspection capabilities are the benchmark used for determining the sensitivity of machine inspection. These machines claim to have a sensitivity better or equal to the human eye. Figure 6 shows an Eisai system, with vials being fed into the machine for inspection. A closer view, with a vial being illuminated, is shown in Figure 7.

Another inspection machine is made by Seidenader. The machine is used for inspection of clear liquids, suspensions, lyophilized products, all in containers that range from ampoules, cartridges, vials, or bottles up to 100 mL. Common particulates that can be detected include foreign material, floating particles, fibers, and glass shards. For lyophilized materials, inspection criteria include melt back, shrunken cake, discoloration, particulates on the cake, unlyophilized product, and fill level. The machine can also be used, with optional features, for inspecting vial and ampoule defects such as position and color of cap, missing stoppers, seal

Figure 6 A photo showing the Eisai system showing vials being fed to the instrument.

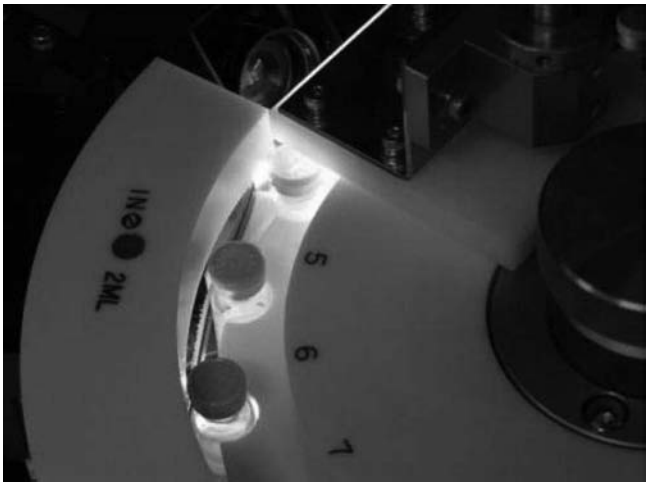

Figure 7 Vials moving into the inspection window, with a vial being illuminated.

crimp defects, scratches, dirt, cracks in sidewall or bottom, deformed ampoule tip, burn marks, and color ring code. The machines are made to analyze a wide range of products from 1 to 100 mL. The inspection mechanism is different for this machine. Each container is inspected in up to three stations. An image subtraction method is used for the detection of particulate matter. The inspection process has the following steps:

1. Container is rotated at high speed.
2. Rotation is stopped and the liquid continues to move, and particles within the liquid move.
3. A central inspection mirror moves with the transport of the container.
4. A camera acquires a sequence of images.
5. The images are sent to a processor and compared with each other via an overlay image (pixel by pixel).
6. Objects that change position between consecutive images are identified as free moving particles. Defects like scratches or glare will not change position between images.

Seidenader claims that this process of detection requires less agitation than other systems and is therefore more applicable to delicate samples such as biopharmaceuticals and viscous products. Production speeds to 36,000 vials/hr are possible. All images are stored and can be printed for further examination. The machine has some desirable options such as integration of NIR technology for product identification and residual moisture testing. An optional head space analyzer is available as well. Figures 8 and 9 show two views of a Seidenader inspection machine set up.

Important Considerations in Visual Inspection

The visual inspection process is performed not only to detect particulates within the product but also to monitor for any container and/or product defects. A useful approach to visual inspection is to create a list of criteria, categorized as critical, major, and minor defects. A typical list of inspection criteria used by the pharmaceutical and biopharmaceutical industry is shown in Table 1. Once a list of criteria has been determined, an AQL can be applied to each category of defect. The AQLs are based on historical information collected during development and manufacturing and represent the highest percentage of defective units unacceptable for releasing the batch. Having acceptance limits is a good manufacturing practice, these limits are used to trigger when an investigation should ensue. Examples of various container defects are shown in Figures 10 to 15. Most of these defects would be considered critical and easily observed by a trained inspector.

Figure 8 The Seidenader inspection machine.

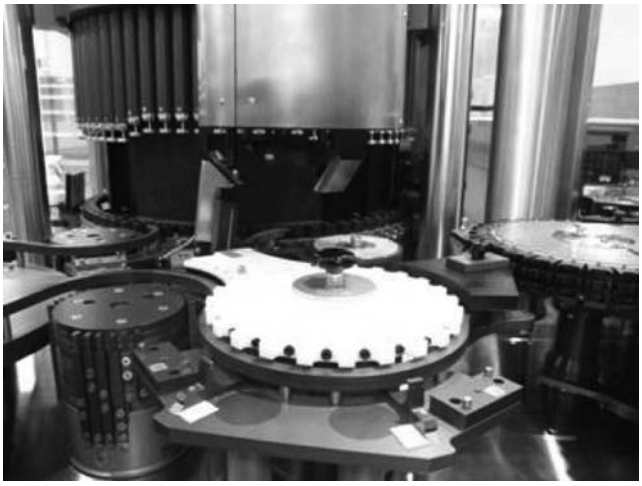

Figure 9 The Seidenader inspection machine, a closer view showing the feedback module.

Table 1 Inspection Criteria, Categorized by Defect Impact

Critical defects (AQL 0.1–0.5%)	Major defects (AQL 1.0%)	Minor defects (AQL 2.5–5.0%)
Incorrect product	Extraneous color of cake	Bubbles in the glass
Melt back (lyophilized cake)	Presence of foreign material	Rough seam wave wrinkle in glass
Overfill of lyophilized product (results in superpotent dose on reconstitution)	Chipped vial	Poor appearance: dirt or specks imbedded in glass or minor scratches
Dried product on vial neck	Scratch in vial (double deep)	Crimp with poor appearance
Cracks in glass	Color variation in stopper	Presence of product on outside of container
Broken vial	Stones in glass (outside)	Broken lyophilized cake[a]
Stones in glass (inside)	Empty container	Uneven cake-cake surface on incline (lyophilized cake)
Incorrect stopper	Plastic cap from flip-off seal missing	Gross excess of product on inside shoulder
Missing/misaligned stopper	Substances on stopper	
Inadequate crimp (not tight)	Flanging incorrect	
Incorrect color of cap or seal	Amount of fill incorrect	

Example AQL limits are shown in parenthesis.
[a]For some products like antibiotics broken cake is normal and is not classified as a defect.

Figure 10 During a manual inspection, a crack along the bottom and side of a vial containing lyophilized product was detected.

Figure 11 During a manual inspection a broken vial neck was observed.

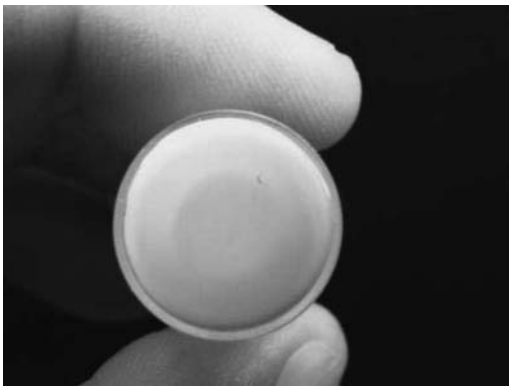

Figure 12 During manual inspection, a dark particle on a lyophilized cake is observed through the bottom of the vial.

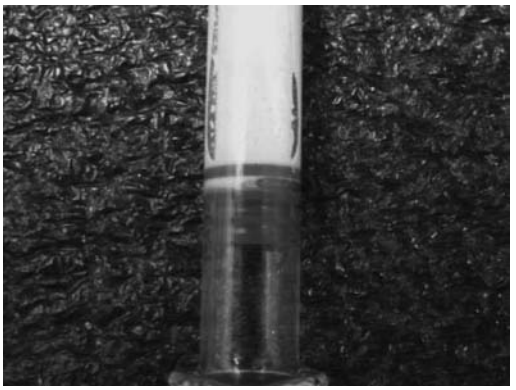

Figure 13 Liquid product is seen below the stopper in a prefilled syringe product. This was observed during a manual inspection.

Figure 14 Photo showing vials that failed due to crimp defects, on the vial farthest right, no crimp/overseal was applied.

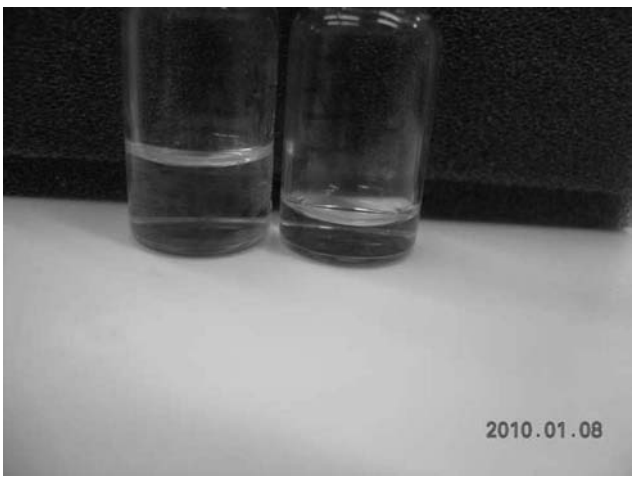

Figure 15 The vial on the right failed inspection due to less than acceptable fill volume (properly filled vial shown on the left).

The International Organization for Standardization (ISO) provides extensive guidelines on establishing sampling plans and determining AQLs. The international standard ISO 2859 *Sampling procedures for inspection by attributes* describes attribute sampling schemes and plans. The guidance document is divided into six parts, with part 1 being of most relevance for visual lot inspections. ISO 2859 Part 1—*Sampling schemes indexed by acceptance quality limits (AQL) for lot-by-lot inspection* provides sampling schemes indexed by AQLs. The quality measure used is percent nonconforming (or parts per 100). It was developed primarily for the inspection of a continuing series of lots that originate from the same production process. The remaining parts of the ISO guidance describe a general introduction to the series as well as sampling plans and procedures for specialized cases.

Facility Inspections/Requirements

In many cases, processing of parenteral products may be done at an external facility or contract organization. The opportunity to audit the facility is also an aspect of good process control. Some criteria that should be included in the quality audit of a contract manufacturing organization are as follows:

- Are product contact surfaces maintained under aseptic conditions?
- Are periodic evaluations of the facility performed and documentation available?

- How are out of specification results dealt with?
- How is the customer notified?
- What is the overall appearance of the facility?
- Do SOPs exist for each operation?
- Are training records current?
- Are operators trained in detecting particulate matter (visual acuity etc.)?
- Where are particulate matter investigations performed?
- Are they utilizing current/appropriate technology?
- Are they utilizing current compendial methods?

PARTICULATE MATTER CHARACTERIZATION

Particulate matter in a sample can originate from a variety of sources. Particulates can be intrinsic, coming from the product itself (as in the case of protein aggregates in a biological product, crystallization of API/excipients, precipitation of insoluble impurities or degradation products etc.) or extrinsic (sourced from a process or the environment the sample is in contact with). Any successful attempts to reduce the amount of particulate matter should include an attempt to identify particles. This will help in understanding the source of the particulate matter. Once samples have been identified as having visible particles, selecting a representative sample set and isolation of the particulate matter are the first steps in the identification process. As much as possible, the first examination of the particulate matter should be done without disrupting the integrity of the sample container, for instance, viewing the particulates in the container using a stereomicroscope. This is an opportunity to examine the population within the container—a look at the material in situ. The optical properties and morphological features of the particles should be recorded at this time.

Next, the particles should be isolated and examined by light microscopy. The use of polarized light can be very helpful in the identification of many types of foreign matter including glass, cellulosic fibers, and inorganic metallic particles. The morphological and optical properties of the particle should be recorded. If the identification is not complete, additional analysis can be performed on the isolated particles. These include spectroscopic techniques such as FTIR-microscopy and elemental techniques such as scanning electron microscopy–energy dispersive X ray (SEM-EDX).

Particle Isolation

There are several approaches that can be used to isolate the particulate matter. Filtration is a common approach, where a portion of the sample containing particulate matter is isolated on a filter membrane. The filter material should be appropriate for the type of particles being collected. For most foreign material, a Nucleopore® membrane filter, where particles are captured on the flat surface, works well. An advantage to the filtration approach is that the contents of the entire container can be captured for analysis. The process is more challenging for soft or gelatinous materials such as protein particles. These particles tend to form a film that is very difficult to recover from the filter membrane. For more challenging materials, other approaches may prove successful. Another common isolation approach involves the drawing up of particles via a microcapillary pipette. The particles are deposited onto a glass microscope slide. They can be washed, stained, or otherwise treated for further analysis. A third approach is to use centrifugation to sediment the particulate matter. This technique is useful for isolating very small particles that may be difficult to collect by other means. For larger particles, filtration or pipetting works very well. It is important to reduce the chances of introducing contamination (additional particulate matter) during the isolation process. The use of clean areas such as laminar flow hoods is encouraged. In addition, analyzing blank samples (such as neat filter membranes) can help identify any artifacts emanating from the procedure itself.

Microscopy

Light microscopy should be the first step in the identification process. A sensible approach is to first view the container in room light, then in an inspection light box. Next, the particles should be examined by low magnification using a stereomicroscope or similar instrument, recording

the characteristics of the material in situ. An example of a white particle in a vial observed first by visual examination under room lighting, then inspected using a stereomicroscope is shown in Figure 16. The particle is easily detected and some additional characteristics such as the morphology can be recorded before opening the container. There are several references in the literature describing important characteristics of particulates, a comprehensive list of which is shown in Table 2 (14). Microscopy provides excellent sensitivity and can provide useful information from subnanogram amounts of material. The minimum particle length that can be resolved by microscopy varies with instruments and optical properties of the material, but is generally accepted to be 1 μm (15). The analysis can be done quickly and with a high degree of accuracy when performed by an experienced microscopist. In addition to the visual observations, microscopy can be used to aid in microchemical testing of the particles. Solubility can be an important indicator in the composition of the material, providing information on functional groups or elemental composition. Proteinaceous materials can be identified by the use of specific stains applied to the particles. Also, the use of fluorescence staining and microscopy can be advantageous for certain types of particulate matter.

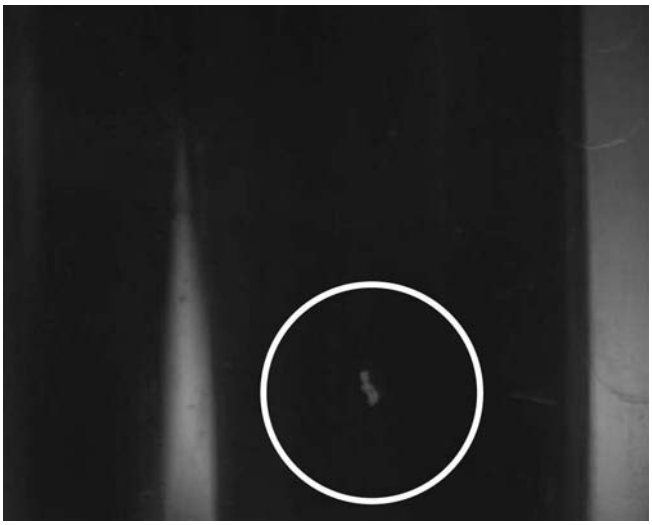

Figure 16 White particle observed in a sealed vial (*circled*).

Table 2 Particle Characteristics Obtainable by Optical Microscopy with Polarized Light

Particle characteristic	Properties
Morphology	Particle shape
Size	Linear dimensions and thickness
Surface texture	Is it smooth, rough, scaly? Is there evidence of tool marks?
Hardness	Does it deform, is it brittle?
Reflectivity	Is it dull, semi-dull, or very reflective?
Transparency	Is it transparent, translucent, or opaque?
Color	Color using transmitted and/or reflected light
Magnetism	Is it magnetic?
Refractive indices	Can determine using refractive index oils? Can be very useful in identification?
Melting point	Does the particle melt when heated, and at what temperature? This requires the use of a hot stage
Chemical composition	What elements or functional groups might be present? This requires the use of microchemical or spectroscopic tests

The associated properties for each characteristic are listed in the right column.
Source: Adapted from Ref. 14.

It should be noted that there can be heterogeneity within an individual particle as well as within the particle population. It is critical that good sampling plans are utilized, and analysis of an appropriate number of particles is employed. Evaluating the sample first by optical microscopy provides the opportunity to identify heterogeneity and avoid misinterpretation of the results.

Another type of microscopy widely used in particle identification is SEM, especially when coupled with EDX analysis for elemental analysis. In SEM, a beam of high-energy electrons is rastered over a sample and an image is produced by means of low-energy secondary electrons and backscattered electrons (16). The SEM will give a topographical picture of the material, including size, shape, and detailed information on the texture. The addition of EDX provides elemental information by measuring the energy of X rays emitted from the sample when it is exposed to an electron beam. Sample preparation is minimal, usually just requiring a thin coating of gold, carbon, or other conductive material to prevent the charging by the electron beam. Instruments are available that can analyze samples under various environments and require no coating. The spatial resolution for SEM is around 0.1 μm or slightly less, making it a good technique for looking at particle homogeneity. The combination of high-resolution images and elemental composition can be a powerful characterization tool.

A fairly new microscopic technique gaining popularity is the use of flow microscopy. This technique provides particle counts as well as a digital image of each particle analyzed, using small sample amounts (as small as 500 μL) and detecting particles as small as 1 μm. Two examples of this technology are the FlowCAM and the Brightwell flow microscopes. The technique is powerful, allowing the user to view the morphology of the particles. In some cases, the morphology alone can provide critical information in identifying the type of particulate matter. Spherical particulate matter such as oil droplets and bubbles can be easily differentiated from more irregular shaped particles by evaluating shape factors associated with the particles analyzed. There has been some recent work on showing the differentiation of silicone oil droplets in the presence of protein particulates using the Brightwell flow microscope (17). A sample containing silicone oil droplets and protein particles was analyzed. Particles $\geq$5 μm were shown to be easily resolved by the flow microscope software. Because spherical particles such as oil droplets have very high aspect ratios, a filter was used in the data analysis to separate the particle population with a size $\geq$5 μm and an aspect ratio $\geq$0.85. This population was verified to be primarily comprised of silicone oil droplets. The technique is gaining popularity in the area of subvisible particle analysis since the technology claims to be more sensitive than the traditional light obscuration methodology. It is also being investigated for analysis of protein particulates since the technology also claims to be more sensitive to detecting near-transparent particles (18,19).

Within the category of dynamic imaging, another instrument, the Eyetech (Ankersmid Ltd., Antwerpen, Belgium) has been used for visualization of particulate matter. The Eyetech uses a rotating laser beam to scan individual particles within a sample zone. The instrument offers a variety of accessories for imaging wet, dry, or airborne particles. Focusing on the wet sample analysis, the instrument can provide similar information to the flow microscope. Here the detection system is based on the rotating laser beam obscuration time and signal interaction as detected by a photodiode. The duration of the laser obscuration is used in determining the particle size. Images of individual particles are recorded and can be recalled for later reprocessing. One area of concern for all of the dynamic imaging systems is the ability to only analyze particles that are in focus. The Eyetech addresses this issue by using a sophisticated algorithm that measures the angle of the laser path and particle boundary. If that angle is significantly $<90^{\circ}$, it will be due to off center or out-of-focus particles. This will result in signals that have a wider pulse transition with small amplitude. These data points are then discarded, ensuring the user is not analyzing out-of-focus particles. Another feature of the Eyetech is that there are no assumptions of particle sphericity. The size measurements are solely based on the length of the cord crossed by the laser, providing an accurate length measurement for each particle. These imaging systems all provide information on particle size and shape, with some differences in how the data is presented. It is critical that the user understand the mechanism of detection and what information is being produced. This data can then be used to better understand the physical properties of the particles being analyzed.

Spectroscopic Techniques

The most useful molecular techniques include FTIR and Raman spectroscopy for particle identification. An FTIR microscope is a useful tool in particle identification. The sample can be viewed directly and in many instruments, a photomicrograph of the sample being analyzed can be obtained for reference. Variable masking options allow the analyst to measure the spectra of small areas within the field of view. There is minimal sample preparation, and many particles can be directly mounted onto potassium bromide (KBr) plates for analysis in transmission mode. Particle thickness and shape can have an effect on the spectra. Flattening the sample onto the KBr plate or using a diamond compression cell can help reduce these effects.

Raman spectroscopy can also be used for particle identification. For example, the RapID Single Particle Explorer (SPE) is an automated Raman system that allows for analysis of particles isolated on a gold filter membrane. The particles are filtered onto the membrane and it is placed into the RapID system. Run parameters are specified by the operator, including the area of the filter to be scanned. The instrument is capable of analyzing count, size, and shape of particles from 0.5 to 5000 μm. The filter is scanned and images of the scanned areas are recorded, along with the associated Raman spectra for the particles detected. The instrument compares the spectra obtained to defined libraries and reports particle composition and ID based on the libraries used. A ranking of how well spectra match library spectra is reported. An advantage of this type of system is that it can provide analysis of a great deal of particles in one run. The RapID system is also 21 CFR Part 11 compliant, making it a possible technique in a GMP setting. As with any analytical technique, care should be taken in the interpretation of the results and the analyst should use this information in conjunction with other information gathered during the identification process (e.g., optical microscopy).

In general, spectroscopy is sensitive and requires minimal sample preparation. The spectra can provide valuable information on functional groups related to the analyte. Most systems are equipped with comprehensive libraries, but care should be used when interpreting the results from automated identification software.

SOURCES AND PREVENTION

The ultimate goal of any particulate investigation is to determine the source of the particulate matter and reduce or prevent its recurrence. The procedures described thus far allow for the detection and identification of the particulate matter. This information can lead to an understanding of the source and can provide insight into methods for elimination. By understanding sources of contamination, controls can be implemented for prevention in future processes.

For biological compounds, air-water interface stress should be considered when agitating product for inspection. Such stress can create or increase particulates in these products, sometimes even with moderate agitation. Agitation can also cause the loss of visible particulates in some products (redissolution of precipitates). Visible particles that were loosely bound aggregates have been observed to break into smaller subvisible particles.

Particulate matter can come from a wide variety of sources including packaging, facilities/environment, or the formulation itself. There are numerous examples where packaging was the source of particulate matter. Glass vials have been known to delaminate, causing a haze-like contamination within liquid formulations. In addition, there are many reports of lubricants (such as silicone oil) leaching into liquid formulations, causing haze or opalescence within the sample. A list of some contaminants reported in IV solutions along with their sizes is shown in Table 3 (6). As shown, there are a large number of particles that are below visible detection (<50 μm). These particles, for the most part, will be detected during sub-visible particle analysis. However, the particle size listed is a primary particle size, and one must remember that many of these particles can be found in aggregated form putting their size in the visible region.

Environmental contamination can be the result of poor air quality and improper operator techniques during processing. It is recommended that processing and filling procedures are performed in laminar air flow devices and class-100 environments. Even in a controlled environment, particles can originate from air handling and filtration systems, room construction

Table 3 List of Contaminants Observed in IV Solutions, Along with Associated Size Range

Contaminant	Size range (μm)
Insect parts	20–1000
Glass fragments	1–1000
Glass delamination	1–100 (extremely thin particles)
Rubber fragments	1–500
Metal particles	1–100
Cellulose fibers	1–100
Machine oil droplets	1–100
Plastic fragments	1–100
Starch	5–50
Calcium carbonate	1–10
Plasticizer droplets	1–10
Silicone oil droplets	0.01–10
Carbon black, clay, diatomaceous earth	1–5
Bacterial fragments	0.1–5
Viruses	0.05–0.1

Source: Adapted from Ref. 6.

Table 4 Theoretical Distribution of Particulate Matter Sources Under Controlled Environmental Conditions

		%Contribution	
Area	Factor	$\geq$10 μm	$\geq$50 μm
Clean lab	Work area/equipment/materials	30	55
	Process flow/adjacent areas	1	5
	Personnel (worker and activity)	70	>30
Aseptic filling	Work area/equipment/materials	15	33
	Process flow/adjacent areas	<5	10
	Personnel (worker and activity)	85	40
Device production	Work area/equipment/materials	55	55
	Process flow/adjacent areas	5	5
	Personnel (worker and activity)	25	10

Source: Adapted from Ref. 6.

materials, personnel, equipment (primarily due to wear or malfunction), and instrumentation in the area. Barber, on the basis of his research, developed a theoretical distribution of particulate matter contamination (at 10 μm and 50 μm sizes) and their sources as shown in Table 4. As shown, the contribution from personnel is significant. Overall, the particle burden can be reduced by following good aseptic procedures including good personnel training, use of appropriate low shedding garb, room access control, and environmental particle monitoring.

The formulation itself may be the source of particulate matter. Excipients can be the source of particles, even if filtered before use. Degradation or interactions with materials used in processing/packaging can lead to particle formation. Storage conditions or sample-handling procedures can also induce particulate formation.

One source of particulate matter that is sometimes overlooked is filtration units. There are two types of filters commonly used, depth filters and screen filters. The choice of filter will depend on the intended application, flow rate, viscosity of the solution, and compatibility with the solution to be filtered. It has been observed that depth filters can contribute cellulose fibers, mineral fibers, animal fibers, glass fibers, or sintered steel particles into the filtered solution (5).

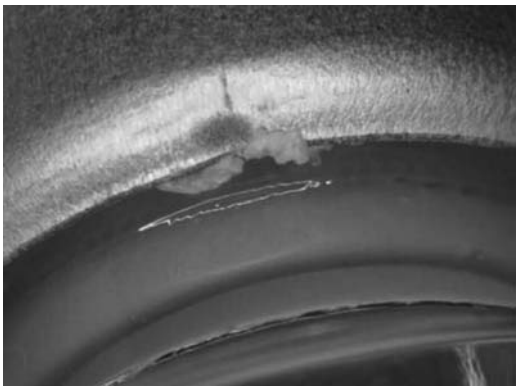

Figure 17 Photomicrograph showing the outside of a filled vial, with crystalline material at the vial crimp.

Screen filters generally contribute fewer particles to the solution. Screen filters are made of cellulose acetate or mixed cellulose esters with or without regenerated cellulose.

Case Study

There is considerable interest in monitoring both subvisible and visible particle load for the purpose of process and formulation evaluation. An increase in particle load during a stability study, for instance, can be an indication that the process or formulation is not well controlled. Further determination of the particle type can provide rich insight into the processing, providing information to obtain a rugged formulation or process leading to a high-quality product. For instance, during a development program, a formulation group noticed particulate matter on the outside of filled liquid product in vials as shown in Figure 17. The material was inspected by light microscopy and isolated for further analysis. The composition was found to be primarily crystalline mannitol, a main component in the drug product. The container integrity was tested on several vials and found to be acceptable. With further investigation it was determined that drug product solution was being deposited on the exterior of the vial during filling. The filling needle was not properly aligned and would drip between vial fills. This was easily corrected and this rapid investigation led to a more robust procedure for filling.

SUMMARY

An important part of particulate matter control is prevention early in the development process. The earlier controls are put in place, the less crisis situations and regulatory concerns that will have to be dealt with later. Personnel working in the processing areas should be effectively trained with regard to particle sources and particulate matter control. Since it is more difficult to control larger areas, it is advisable to create minienvironments and use isolators for various applications. Process design is critical to the control of particulate matter contamination. If particulate matter is found, techniques used in the inspection and identification of visible particulate matter have advanced to the point where investigations can be performed in a timely fashion. With a properly equipped laboratory, the detection and composition of particles can be determined, allowing for identification of the source and, ultimately, control over processes and formulations to greatly reduce or eliminate their recurrence. Many inspectors have used the visual inspection data during preapproval inspections and annual GMP inspections to identify weak links in the manufacturing process or controls. High reject rates during inspection can point to specific problems, for example,

high/low fill volumes—filling not controlled, lot checks and
lyophilized cake appearance/meltback—lyo controls, nonrobust lyo cycle.

It is important that the (bio) pharmaceutical industry utilizes this information to proactively address these potential issues. This will lead to robust and well-controlled processes for the development and manufacture of injectable drug products.

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5 Advances in parenteral injection devices and aids

Donna L. French and James J. Collins

BENEFITS OF INJECTION DELIVERY SYSTEMS

The growth of the injectable market in the last decade has led to the development of numerous injection device technologies for product preparation and administration. Injection device technologies facilitate injection preparation, ease administration, improve dose accuracy, and ensure safety, all of which contribute to improved user acceptance and compliance. Device technologies covered in this chapter include tools for injection preparation, needlestick prevention devices, and delivery devices used to administer injectable drugs.

FACTORS INFLUENCING SELECTION OF A DEVICE TECHNOLOGY

The selection of an injection device technology depends on the attributes of the patient population and drug product. Users of device technologies can be patients or health care professionals. For patient administration at home, the patient's experience with injectables, mental acuity, physical dexterity, and product storage conditions need to be considered. For the health care professional, prevention of accidental needlesticks and clinic operating procedures are the primary considerations. As for the drug product attributes, formulation factors such as preservatives, liquid versus lyophilized, and viscosity are critical. In addition, dosing factors such as the route of administration, frequency of administration, deliverable volume, and fixed versus variable dosing can have an impact on device selection. Although the selection of a device has historically been constrained by such factors, many current devices have new capabilities that overcome previous limitations and accommodate a wider range of product attributes.

Customer-Based Selection Factors

Route of Administration

The route of drug delivery—intravenous (IV), subcutaneous (SC), or intramuscular (IM)—affects the selection and design of the injection device. Devices used for IV administration must be universally compatible with clinical procedures and equipment, and most of these devices are tools that provide minor improvements to existing IV administration procedures. Injection devices are primarily designed for SC or IM use by a patient or health care provider. Key considerations in the design of devices for SC and IM use include injection depth and volume. The needles for these routes of administration vary in length and needle gauge. An injection device must be designed to ensure that the drug is injected in the appropriate SC or IM space. Incorrect administration could result in discomfort and/or alter the pharmacokinetics, efficacy, and safety of the drug.

Frequency of Administration

The frequency of drug administration impacts the selection of the type of device (e.g., cartridge pen or prefilled syringe with an autoinjector) as well as the decision to use a reusable or disposable design. Drugs may be administered frequently, such as daily or more often, or infrequently, such as once weekly or less often. For frequently administered products, devices that are portable and contain multiple doses in a compact design, such as multidose pens, are least disruptive to a patient's lifestyle and most cost effective. Infrequently administered products are likely to be supplied as a disposable single-dose device. A simple, intuitive, ready-to-use system is particularly appropriate in this setting, so that patients do not have to familiarize themselves with the instructions each time they use the device or perform a complex procedure with a product that is used only occasionally. The best example of this scenario is the use of emergency antidote devices, which may only be used once in a person's

lifetime for a medical emergency. An easy, quick, and intuitive injection procedure is essential to prevent the occurrence of a serious medical condition.

The choice of a reusable or disposable device is a balance between convenience and cost. Although reusable devices require more manipulation by the end user, they are more cost effective. If a drug is frequently administered, the unit cost of disposable devices may be cost prohibitive because of the waste associated with frequent disposal.

Acute Vs. Chronic Therapies

In acute therapies, a drug is used only for a discrete period of time, whereas with chronic therapies, the drug is used for extended periods of time, and in many instances, for the duration of the patient's life. For acute therapies, ease of use is essential so that the patient does not have to be trained on a complex injection procedure when the product will be used only for a limited time. For chronic therapies, easy-to-use devices are preferred but the extended use also allows additional features to be considered. Chronic therapies may require long-term monitoring of the disease, which may require that the patient use electronic features on the injection device to record injection times, dosing, and other information useful for monitoring the compliance or other disease status indicators. Although such features are inherently more complex, they can provide significant value and are not an issue after the patient becomes accustomed to using them.

Considerations for Self-Injecting Populations

The selection and design of an injection device requires careful consideration of the needs of the end-user population—children, elderly, and physically challenged. In self-injecting populations, physical impairments, cognitive challenges, the user's degree of experience with injections, and patient age (e.g., pediatric, elderly) are important considerations in device design. For example, rheumatoid arthritis patients will require a device that is easy to grip and activate, and therefore careful study of the physical challenges is critical to ensuring the device can be used by the patient population. Elderly patients may have difficulty reading instructions or dosing information on the device. Aside from special considerations for specific end-user populations, human factors must be a key consideration in all delivery device designs. Not only are human factors a good design practice, human factors studies are required as part of the regulatory expectations for medical devices. Never assume you understand how a user will use the designed device. Experienced users will provide different feedback than inexperienced users, and the exact same device may have different challenges for each therapeutic for which it is applied. In all cases, device designs that are intuitive are less likely to have issues with end-user training and compliance.

Considerations for Health Care Professional Users

A key consideration for health care professionals is safety—prevention of cross-contamination between patients or between the patient and the health care provider. Prevention of patient to patient contamination is easily achieved with single-use disposable devices. For multiuse devices, explicit instructions not to share or reuse devices with other patients should be provided. Currently the only type of device used for multiple patients are reusable needle-free devices that have been used for mass immunization worldwide. Such devices are uncommon in other clinical settings, where single-use disposable syringes and other devices are typically used. These devices are equipped with needlestick prevention mechanisms to protect the health care provider.

Needlestick prevention devices that safeguard health care providers from contracting serious or fatal diseases from accidental sharps injuries have become a key focus area for the pharmaceutical and medical device industries within the last decade. Injuries from needles or other sharps contaminated with bloodborne pathogens such as human immunodeficiency virus, hepatitis B virus, and hepatitis C virus have been a serious problem. The Center for Disease Control and Prevention estimated that 600,000 to 800,000 needlestick and other percutaneous injuries occurred among health care workers annually, and that 62% to 88% of sharps injuries could be prevented by the use of safer medical devices (1). In 2000, the United States enacted the Needlestick Safety and Prevention Act requiring that hospitals and clinics

take appropriate measures to prevent needlestick injuries to reduce the risk of transferring bloodborne pathogens to the health care provider (1). A revised Occupational Safety and Health Administration (OSHA) Bloodborne Pathogens Standard became effective in 2001. New engineering controls in this standard included the use of sharps with engineered sharps injury protections (ESIP) and needleless systems. Products that are administered by health care providers in hospital clinical settings must have a needlestick prevention mechanism, and only some device types are amenable to this design feature. Devices with engineered sharps protection are discussed in more detail in sect. "Needlestick Prevention Devices."

Product Property–Based Selection Factors

Liquid Vs. Lyophilized Products

Injectable products are supplied as liquids or lyophilized powders. A lyophilized formulation is used when the drug product is not sufficiently stable during its shelf life storage as a liquid. A disadvantage of lyophilized products is that they require reconstitution prior to injection, which is an additional step that must be performed compared with a liquid product. Some device technologies are designed solely to ease the inconvenience of the reconstitution step, and then the drug is injected with a conventional needle and syringe. When a lyophilized product is used with an injection device, the design must have a mechanism for reconstitution prior to injection. While reconstitution can be designed to be easier, a liquid formulation will always be an advantage. The more inexperienced the user, the more physically or mentally challenged the user, or the more frequent the need for reconstitution, the greater the value of a ready-to-use liquid formulation.

Viscosity

Even slightly viscous products (~5 cp) impact the delivery of a product with a device, and higher viscosity products (15–30 cp) may require significant adaptation of the drug delivery system for administration. The viscosity of the product affects the needle gauge that can be used with the injection device technology and the speed of injection. Designing a device for a viscous product will require trade-offs between the needle size, injection time, and the force applied to administer the product. To mimic a typical injection speed (less than ~10 seconds), the size of the needle must be increased or the force that the patient or device must apply to expel the product through the needle must increase. Increasing the size of the needle is potentially more painful for the patient, and the force that can manually be applied to a syringe or device to expel the product is limited. High forces can be applied using automated types of injection devices, but a specialized design may be required.

Preserved Vs. Nonpreserved Formulations

Whether the product can be stabilized with preservatives will also be a key factor in the delivery technology selection. Multiuse products such as pen and cartridges systems require the pharmaceutical product to be preserved. If the drug product is not stable with preservative, then a single-use system will be required.

Fixed Vs. Variable Dosing

Some products are dosed according to the weight or specific therapeutic needs of the patient (variable dosing). Other products are the same dose despite differences between patients (fixed or flat dosing). This is an important development parameter as some device designs are more amenable to variable dosing.

Volume of Administration

The injection volume to be administered is a key factor in device design and selection as it affects dose accuracy, ease of administration, and injection site tolerability. The administered volume is dependent primarily on the potency, physicochemical properties, and stability of the drug as a function of concentration. The administered volume of most SC products is between 0.1 and 1.0 mL, and most injection devices are designed to deliver volumes in this range.

Because of the large doses required for efficacy of some drugs and limitations in drug concentration for some products because of their stability and physicochemical properties (e.g., viscosity), an increasing number of injectable products may require administration of volumes more than 1 mL. A general clinical rule of thumb is that SC injectable volumes should be less than 1 mL to avoid injection site discomfort. However, the tolerability of higher injection volumes is not well understood, and the impact of the formulation (excipients, pH, drug properties) and the needle type (e.g., size, needle point geometry) add additional complexity to understanding the injection experience. From a technology perspective, current bolus injection devices are typically designed to deliver 1 mL or less, although technologies can be adapted to deliver larger volumes that are clinically tolerated. Infusion pumps can deliver larger volumes subcutaneously over a longer period of time (minutes to hours) than a typical bolus injection ($\leq$10 seconds). From a clinical perspective, other options to administer the product can be considered, such as IM or IV administration. Many of the options to deliver large doses are also less convenient for the patient.

Injection of small volumes poses different challenges. Because of limitations in glass-forming and filling technology, prefilled syringes and autoinjectors are not recommended for volumes below 100 μL. If a prefilled syringe is going to be utilized, the formulation should target a volume above 100 μL. If a pen cartridge device is going to be utilized, the International Standards Organization regulatory expectation (ISO 11608) for dose accuracy is an absolute value of ±10 μL up to a volume of administration of 200 μL, and then ±5% for volumes greater than 200 μL. Given this requirement, the system dose accuracy will be impacted by the dose volume chosen. At a dose volume of 10 μL, the dose accuracy would be ±100% and the dose accuracy as a percentage would improve as the dose volume increases.

THE PRIMARY CONTAINER

The drug product is contained in a container closure system, or primary container, to prevent microbial contamination, solvent loss, or exposure to gases or water vapor. Type I borosilicate glass is the most common construction material due its excellent barrier properties and inertness. Current devices are designed to be used with three types of primary containers: vials, cartridges, and prefilled syringes.

Vials

Glass vials are the most prevalent primary container for injectable drugs. Plastic resins may be more prevalent materials for this container closure system in the future. Vials are cylindrical containers with a stopper and seal, which is crimped onto the top of the vial to maintain container closure (Fig. 1). Vials can contain either liquids or lyophilized powders. Most injection devices used with vials are tools to facilitate reconstitution (if the product is lyophilized), transfer product between vials and other containers (e.g., IV bags, syringes, or other vials), or ease removal of the product from the container.

Cartridges

Glass cartridges are used as a primary container for injection pens (described in sect. "Injection Pens" in more detail). For preserved multiuse formulations, glass cartridges are the commonly used primary container. As with other vials, plastic resins may be a more prevalent material for this primary container system in the future. A cartridge has a tubular barrel that is sealed on each end with a rubber or elastomeric closure (Fig. 1). The drug product is prefilled into the container and retained by a stopper to which a needle assembly can be attached on one end and a plunger on the other end. Dual-chamber cartridges are used for lyophilized drug products in which the dried product is contained in one chamber and the diluent in the other. A channel between the chambers allows mixing and reconstitution of the product at the time of use.

Prefilled Syringes

Prefilled syringes are syringes supplied to the patient or health care provider that already contain the drug. These systems offer a more convenient alternative to standard drug vials—the user does not need to perform the steps to prepare and administer the product from vials, such as air pressure adjustments, aspirating the drug from the vial, changing needles, and

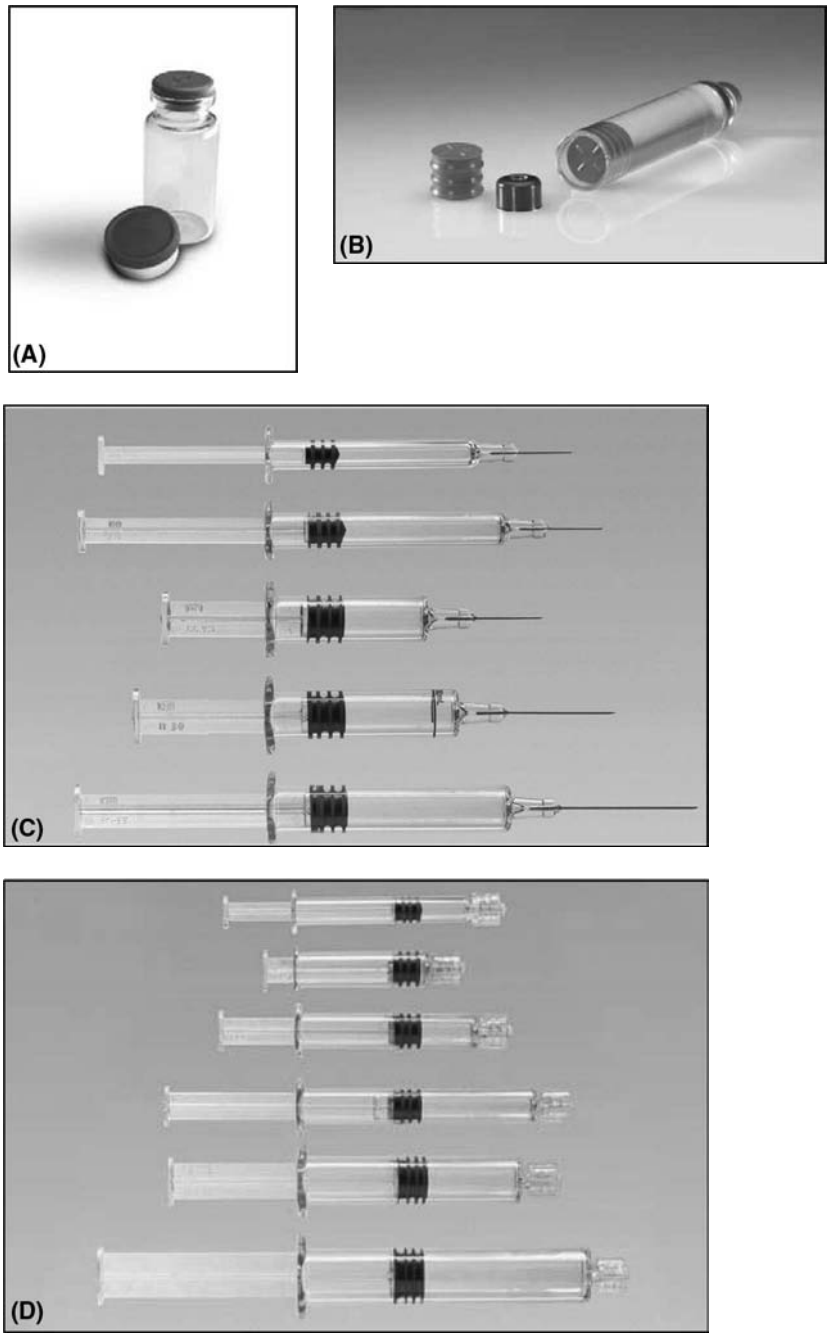

Figure 1 Primary containers: (**A**) vial, (**B**) cartridge, (**C**) staked needle prefilled syringe, and (**D**) luer-lock prefilled syringe. *Source:* Parts A and B courtesy of West Pharmaceutical Services and parts C and D courtesy of Becton, Dickinson and Company.

reconstitution and handling of diluent if the product is lyophilized. Prefilled syringes reduce or eliminate these preparation steps, improve dosing accuracy, and reduce the risk of contamination because of fewer transfers of the product prior to injection. Prefilled syringes can be used as the injection device or can be used with an autoinjector (described in sect. "Autoinjectors") to automate the injection.

Prefilled syringes consist of a cylindrical glass or plastic barrel; by far, the most common prefilled syringes are made of type I borosilicate glass. Plastic prefilled syringes are used commercially to a limited extent but may become a more prevalent technology in the future. Other components include the plunger (or stopper), plunger rod, and syringe tip, which may be a luer tip or staked needle (Fig. 1). Luer-tipped syringes have a tip cap for product containment and maintaining sterility. Prior to injection, the user removes the tip cap and attaches a needle to the syringe. Staked needle systems have the needle permanently affixed to the tip of the syringe barrel, and the needle tip is embedded into an elastomeric or rubber needle shield to maintain product containment and sterility. The preattached needle offers greater convenience because the user does not need to perform the steps to attach the needle. However, the user does not have the ability to choose the needle, which can be a disadvantage. Because of manufacturing and container closure design requirements for these systems, staked needles have different injection and glide force properties, and the injection experience may be different than that of nonstaked needles. Prefilled syringes with staked needles are intended for SC or IM administration, rather than IV use through ports. Depending on the application or preference of the user, a luer-tip system with the needle of choice may be preferable. The decision to use a staked needle or luer-tip syringe depends on various factors for which needle selection is important, such as the route of administration, product viscosity, compatibility with injection devices or aids, and market preferences for convenience versus flexibility in needle choice.

Prefilled syringes are available for lyophilized drugs. These syringes consist of a glass dual chamber container in which one chamber contains the diluent and the other contains the drug product. At the time of injection, the drug is reconstituted by moving the diluent from its chamber into the drug product chamber by pushing the plunger rod. After the reconstitution step, the product is directly injected into the skin as with any syringe. These systems are available commercially with a luer-tip system.

Drug Product and Container Compatibility

Assessment of the compatibility between the drug product, primary container, and device as a system is an essential element of product development and an area of increased scrutiny by regulatory agencies. The stability of biopharmaceutical products is impacted by the physical, chemical, and mechanical properties of the primary container and device system. Factors that must be considered in assessing compatibility include materials of construction, surface preparation or treatment, and any added excipients. Glass, plastic, elastomeric, and rubber components have inherent chemical and physical properties that can impact product stability. The integrity of the product upon actuation of the device must be also evaluated to ensure that the device is not detrimental to the product. The shear forces applied to the product by some devices have the potential to cause instability for biopharmaceuticals. The impact of manufacturing and component preparation processes including washing, sterilizing, and storage conditions must be evaluated, and understanding how these components are tested for product quality is essential. Lot-to-lot and vendor-to-vendor variability can pose unforeseen issues. In addition to drug stability issues, the functionality of the device can also be impacted by these same factors and may also vary with the drug product formulation. The compatibility and functionality of the drug-container-device system needs to be assessed under both normal and stressed conditions, and it must be understood not only initially but also as the components age. The stability of the drug and functionality of the device must be retained over the shelf life of the product.

Leachables and extractables in primary containers commonly pose chemical and physical stability challenges for injectable drug products. Primary container components can contain trace metals, plasticizers, antioxidants, accelerators, silicone, vulcanizing agents, and other chemicals that can impact product stability. For example, silicone is in most primary containers to provide lubricity for proper functionality. Silicone enables the plunger to glide smoothly through the syringe or cartridge for drug administration. However, many pharmaceutical proteins are incompatible with silicone and the product can become unstable and form particulates upon storage or shipping. Another example is tungsten, a heavy metal residual from the prefilled syringe manufacturing process, which can lead to serious stability issues for

some biopharmaceuticals. Other protein compatibility issues include adherence of the drug to the glass walls of the container and delamination of glass surfaces.

Some technologies have been developed to avoid compatibility problems with primary container components. These include fluoropolymer coatings on rubber components as well as thermoplastic elastomers and fluoropolymers as materials of construction for plungers and stoppers. Improvements in silicone chemistry and application have been developed that increase adherence of the silicone to the containers and reduce the amount of free silicone available. Plastic prefilled syringes, which are composed of cyclic olefin polymer or copolymer, offer potential advantages over glass with respect to the leachable/extractable profile. In addition, plastic syringes offer the advantages of being lightweight and less prone to breakage. One type of plastic syringe is silicone-free, which is an advantage for silicone-sensitive biopharmaceuticals. These syringes are more permeable to gases than glass, which is a factor that must be considered in development of these prefilled syringe products, particularly with respect to stability of the biopharmaceuticals.

INJECTION DEVICES AND USER AIDS

Autoinjectors

Autoinjectors are spring-based systems that automatically inject drug from a prefilled syringe into the skin (Figs. 2 and 3). Insertion of the needle into the skin and delivery of the drug occurs automatically upon activation. These devices can be used for SC or IM injection. All commercial autoinjectors are fixed single-dose systems, although injectors with variable dose capabilities could be developed. Reusable, semidisposable, and disposable systems are used commercially.

Reusable systems are cost-effective options for frequently administered products in self-administering populations. However, these delivery systems require a significant amount of end-user manipulation to perform an injection, and the complexity of use has potentially limited their popularity. Numerous steps are required to operate reusable systems because the user must assemble the device for use and disassemble it after use. Typically, a user will have to separate the device into two parts, set the activation mechanism, insert the prefilled syringe, assemble the device, unlock the actuator, and activate the device for injection. After injection,

Figure 2 Single-use disposable autoinjector. *Source:* Courtesy of Scandinavian Health Limited Medical.

Figure 3 Reusable autoinjector designed for Rheumatoid Arthritis patients. *Source:* Courtesy of Owen Mumford Ltd.

the device is disassembled and the syringe is removed and discarded. Most systems are designed for staked needle prefilled syringes but some are designed to be used with a cartridge, luer-tip prefilled syringe, or a syringe that has been loaded with drug from a vial by the end user. Currently, reusable devices do not have needlestick prevention features; such a feature would require a permanent locking mechanism over the needle tip that would prevent reuse.

Single-use disposable autoinjectors were historically used for emergency medicine but are now used with pharmaceuticals. These systems are supplied preassembled with a prefilled syringe containing the drug. These systems have integrated needlestick protection mechanisms, which makes them suitable for clinical or home administration. These systems are easier to use than their reusable counterparts as they are supplied preassembled and ready to activate. The user simply removes the needle cover, unlocks the actuation mechanism, activates the device to inject, and then discards the entire device after injection. These systems are good choices for infrequently administered products or acute application because of their ease of use; disposable systems are more cost effective with short-term or infrequent use.

With semidisposable systems, the component containing the drug product is discarded after each use but the activation mechanism component is reused. All commercial disposable and semidisposable autoinjectors are used only with liquid products, although systems for use with lyophilized systems are under development.

Injection Pens

Pen injectors (Fig. 4) were first used for frequent self-administration of preserved multidose drug formulations requiring weight-based dosing. However, fixed-dose and single-use pens are now in use. Pen injectors are the most widely used injection devices and have been used in the diabetes management and human growth hormone (hGH) market for the last 20 years. Pen injectors are portable and provide greater ease of use and convenience compared with traditional vials and syringes. Cartridges are the primary drug containers used with pens. Small gauge needles designed for SC injections are used with these devices. The user is required to place a needle onto the tip of the device, set the dose, manually insert the needle into the skin, and push a button to inject the drug. Prior to the next injection, the system may need to be reset, and the needle must be replaced. The original pen injectors were reusable devices, which require the end user to insert a prefilled cartridge into the pen and to periodically replace the cartridge when it is empty. More recently, disposable pen injectors have been introduced in which a prefilled cartridge is preassembled into the pen. The entire device is discarded when the cartridge is empty. An advantage of pen injectors is that they can easily be used with liquid or lyophilized formulations. For lyophilized formulations, either the cartridge is a dual chamber system (as described in sect. "Cartridges") or an adapter is provided to reconstitute the powder in the cartridge. After the initial reconstitution, injections are identical to that of a liquid formulation. Hence, the inconvenience of reconstitution is minimized with these systems because it is performed only when a new cartridge is used, and the reconstitution process is easier with the pen than with conventional vials and syringes. More recent developments in pen devices include the use of needle safety devices, automated needle insertion and injection, smaller dosing capabilities, and electronics. Electronic capable systems allow the patient to record and review their dosing information.

Figure 4 Injection pen. *Source:* Courtesy of Y posomed.

Figure 5 Needle-free injector. *Source:* Courtesy of Antares Pharma Inc.

Needle-Free Injectors

Needle-free injection systems enable the injection of drug products without the use of a needle (Fig. 5). A high-pressure gas or spring drives the drug product through a small orifice in the device with sufficient force to create a hole in the skin and inject the drug. Needle-free injections are not pain-free but have obvious attractions for needle-phobic patients. These technologies also best meet the requirements of the U.S. Needlestick Safety and Prevention Act. Reusable needle-free devices are commercially marketed for hGH and insulin, and have historically been used for mass immunizations. These systems typically require that the drug be transferred from a vial into a cartridge that is used in the device. Either a liquid drug product or a reconstituted lyophilized drug product can be transferred into the device for injection, and then the end-user sets the dose and injects. Reusable systems offer variable dosing and formulation flexibility, but as is typical of all reusable devices, they are more difficult to use than disposable versions. Disposable needle-free devices are in development by a number of companies. As with other prefilled disposable devices, fewer steps are required for injection. Disposable devices are designed to be filled from a vial or prefilled to give a single fixed dose. Historically, disposable needle-free technologies have been limited to liquid formulations and low administrable volumes, but recent developments include the ability to dose a lyophilized formulation using a dual chamber cartridge and larger volumes.

Reconstitution Aids

Reconstitution of a lyophilized product prior to injection is an inconvenience that can be offset by the use of dual chamber syringe/cartridge systems or adaptors that enable needle-less reconstitution in vials. Dual chamber systems contain the diluent and powdered drug in the same primary container and enable mixing via a channel that connects the drug and diluent. These are available as prefilled syringes (as described in sect. "Prefilled Syringes") or as prefilled cartridges for use with an injection device.

In addition to dual chamber syringes, a variety of reconstitution aids are available to facilitate the preparation of lyophilized drug products (Fig. 6). Vial adapters provide an interface between the product and diluent that enables the reconstitution of the product by a luer or other connection rather than a needle. Some adapters connect product and diluent-filled vials with a syringe. Others connect the vial with a syringe, in which case a prefilled diluent syringe provides additional convenience. Some adapters are preattached to the product vial and diluent syringe or have preattached needles. Vial adapters for multidose formulations are also available. These systems have a mechanism in the adapter to maintain container closure between injections.

Tools to assist the patient in handling components such as removing caps on vials and syringe caps and/or handling of needles have also been marketed. Some injection device companies have developed devices that enable reconstitution and automate injection, but none have been commercialized to date.

Pumps

Pumps (Fig. 7) have been utilized for the clinical delivery of many IV products and for the SC delivery of insulin by diabetics for over 20 years. The growth of biotechnology products is increasing the opportunities for pumps, and pump technology choices for clinically and patient-administered products are likely to grow. There are three broad categories of pumps

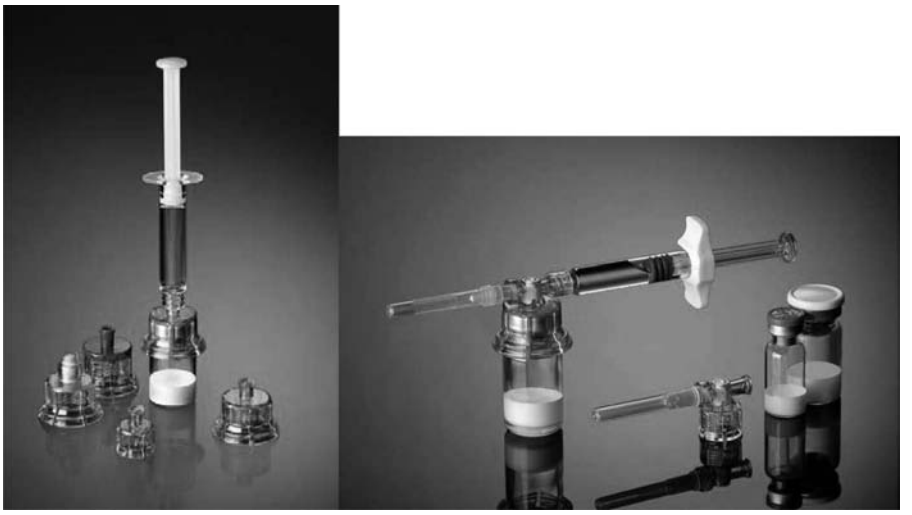

Figure 6 Reconstitution aids. *Source:* Courtesy of West Pharmaceutical Services.

Figure 7 External injection pump. *Source:* Courtesy of Insulet Corporation.

that can be considered: IV pumps, patient-administered external pumps, and implantable pumps.

While not an option for all products, clinical IV administration with a pump is a viable option for infrequently delivered products or for very serious diseases in which IV administration provides a clinical advantage. An IV formulation provides very good systemic distribution and bioavailability, and the vial container closure system utilized to store the pharmaceutical product prior to pump administration is well established. The challenge with formulations administered by an IV pump is the end-user inconvenience.

With regard to patient-administered external pumps, there are three different types of external pumps being developed that balance ease of use with additional features and cost. These three external pump types can be categorized as disposable patch pumps, semi-disposable electromechanical pumps, and reusable electromechanical pumps. Disposable patch pumps are simple needle-based systems with an adhesive patch attached to the skin. These disposable patches are primarily designed to deliver a fixed basal rate and are utilized for one to three days. Semidisposable electromechanical pumps utilize a disposable drive system and incorporate a reusable electronic module that can provide a more advanced feature set such as a variable basal rate or bolus dosing. In some cases, the pump worn by the patient can be smaller as the electronic module that controls the pump can be carried separately. External reusable electromechanical pumps are full-featured pumps that have variable basal

rates, bolus dosing, data connectivity, and dose memory options. These reusable pumps can provide very accurate dosing and can incorporate a number of features required by the therapeutic. Currently, patient-administered external pumps utilize a cartridge container that is filled by the patient from a vial. Patient-administered pumps are complex and costly, and they need to provide a clinical benefit over other options to gain customer acceptance.

The last pump category is internal implantable pumps. These pumps provide a unique benefit of being able to deliver drug product directly to specific areas such as the central nervous system (CNS) to provide a unique clinical benefit. Implantable pumps are primarily being applied in diabetes, pain, and CNS applications.

Needlestick Prevention Devices

The U.S. Federal Needlestick Prevention Act of 2001 (see sect. "Considerations for Heath Care Professional Users") has driven the commercialization ESIP systems for their injection devices, prefilled syringes, and needles. These devices are supplied with needle products designed for withdrawing body fluids, accessing veins or arteries, and administering medications in a clinical setting. Needles intended to be attached to a syringe are available with a needle cover mechanism that the user slides over the tip of the needle after use (Fig. 8). For drug products in prefilled syringes with staked needles, needlestick prevention devices can be provided preassembled with the syringe (Fig. 8). These systems typically consist of a main body attached over the syringe body with a component that slides over the tip of the syringe and needle after use. Manual, active, and passive needlestick prevention devices are commercially available. However, manual systems are becoming obsolete with the introduction of newer technologies. With manual systems, the health care provider manually slides the protective guard over the

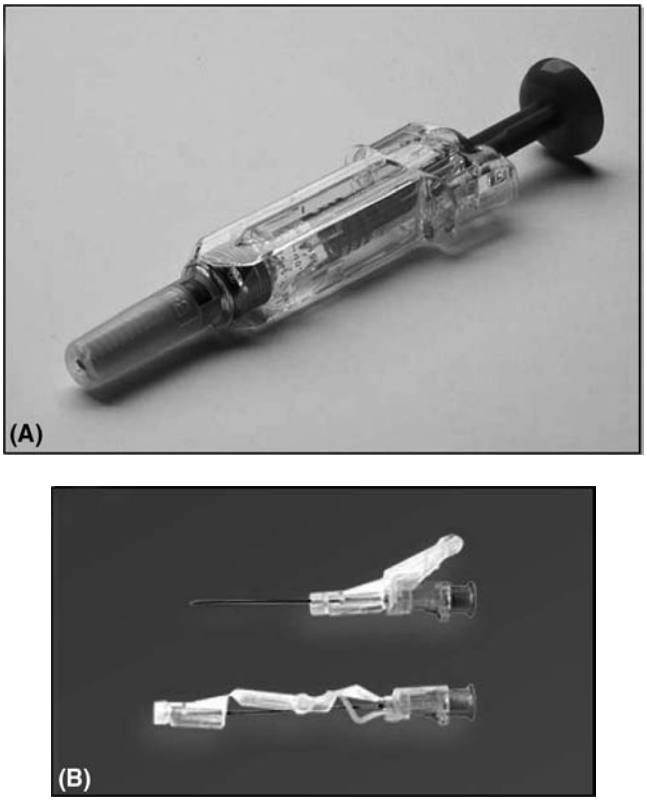

Figure 8 (**A**) Automatic needlestick prevention device with staked needle prefilled syringe and (**B**) needle-based needlestick prevention device. *Source:* Part A courtesy of Safety Syringes, Inc. and part B courtesy of Becton, Dickinson, and Company.

needle at the end of the injection. The manual design is suboptimal as users must physically pass their hand over the needle to cover it; mistakes or incorrect use of the device have the potential to cause a needlestick. With active systems, an actuation step is required to automatically activate/slide the needle protection guard, but it does not require the user to pass their hand over the needle. Passive devices are automated and safest; these systems activate the needle protection guard at the end of the injection without any additional action by the user.

DELIVERY DEVICE DEVELOPMENT QUALITY SYSTEM REQUIREMENTS

Incorporating a device into the dosage form requires the development scientist or engineer to understand the medical device regulations as well as the pharmaceutical regulations. The expectation is that a medical device incorporated into a pharmaceutical delivery system will meet the key elements of the device regulations. The two primary global governing regulations for these combination products are the ISO 13485 and the Food and Drug Administration's (FDA) Title 21 Code of Federal Regulations (CFR) Part 820. These regulations share many common requirements. In addition, the emerging Quality-by-Design initiative at the FDA contains a number of elements that are consistent with the device requirements such as working to establish patient requirements early and understanding the key patient risk in the product design.

Device Quality Management for Parenteral Delivery Device Forms

International Standards Organization—ISO 13485

This international standard outlines the requirements of a quality system for an organization to provide a delivery device that will consistently meet the customer requirements and regulatory requirements applicable to medical devices. The primary objective of this international standard is to assist in harmonizing global medical device requirements and quality systems. For this reason, ISO 13485 has many elements that are common to the FDA's Quality System Requirement (QSR) and Japan's Ministry of Health Labor and Welfare (MHLW) requirements for medical devices. In comparing the ISO 13485 to the FDA's QSR, the ISO guidance is not as prescriptive with regard to device history records, complaint handling, and device master records, but is more detailed with regard to understanding customer requirements and planning for demand realization. The ISO 13485 standard specifically focuses on medical devices, and therefore some of the requirements for ISO 9001 (Quality Management System—Requirements) have been excluded. An organization that meets the requirements of ISO 13485 will need to add the requirements for ISO 9001 if they desire to also claim conformance with that standard.

Quality System Requirements (21 CFR Part 820)

The QSR represents the FDA's requirements for medical device design, development, manufacturing, and postmarket surveillance as defined in Part 820 of the Food, Drug, and Cosmetic Act. The FDA does not expect companies that are developing combination products (containing a medical device constituent part) to operate under two separate quality systems, but they do expect that the device constituents will meet the primary device regulations and the drug components will meet the primary drug regulations. The FDA provides companies two options with regard to compliance for a combination product. A company can demonstrate that each constituent part meets the applicable regulations or demonstrate compliance with either the drug current Good Manufacturing Practices (cGMPs) or device Quality System regulations while also meeting certain conditions of the other quality system. If operating under a pharmaceutical quality system defined by Part 210/211, the FDA requires that the organization also demonstrate compliance with six specific provisions of the device regulations: Management responsibility 820.20, Design controls 820.30, Purchasing controls 820.50, Corrective and preventative actions (CAPA) 820.100, Installation 820.170, and Servicing 820.200. The use of CAPA systems is becoming more prevalent within pharmaceutical companies, but it is important to understand that a CAPA system is a critical element of the QSR and will nearly always be inspected as part of a device regulatory visit. If a company is

operating under a quality system defined primarily by the device QSR contained in Part 820 and developing a product that includes a parenteral dosage form, the following elements of the Part 210/211 regulations should be included: Testing and approval or rejection of components, drug product containers, and closures 211.84; Calculation of yield 211.103; Tamper-evident packaging for over-the-counter (OTC) human drug products 211.132; Expiration dating 211.137; Testing and release for distribution 211.165; Stability testing 211.166; Special testing requirements 211.167; and Reserve samples 211.168 (2).

DESIGN AND DEVELOPMENT PLANNING

Design and development planning is a critical requirement for medical delivery devices. It includes requirements to understand the key elements of the design. The FDA requires that each company establish and maintain plans that describe the design and development activities and define responsibility for implementation. The plan needs to identify and describe the interfaces with different groups that provide input to the design and development process. The plans need to be reviewed, updated, and approved as design and development evolves (3).

Design Inputs, Design Outputs, Design Review, Design Verification, Design Transfer, and Risk Management

As devices are incorporated into a pharmaceutical dosage form, specific requirements with regard to medical device design inputs, design outputs, design reviews, design verification, design transfer, and device risk management should be included.

Design inputs are the physical and performance requirements of a device that are used as a basis for device design. The manufacturer must establish and maintain procedures to ensure that the design requirement relating to a device are appropriate and address the intended use of the device, including the needs of the user and patient. This includes a mechanism for addressing incomplete, ambiguous, or conflicting requirements. It is important to understand that market research concepts are not design inputs. The expectation is that some development will be required to transform the initial market research concepts into a more comprehensive set of documents that define the design inputs as per the QSR (3).

Design outputs are the results of a design effort at each design phase and at the end of the total design effort. The manufacturer must have procedures for defining and documenting design output in terms that allow an adequate evaluation of conformance to design input requirements. Design output procedures shall contain or make reference to acceptance criteria and shall ensure that those design outputs that are essential for the proper functioning of the device are identified. The finished delivery device design output must be the basis for the device master record. The total finished delivery device design output consists of the device, its packaging and labeling, and the device master record. Design output must include production specifications (assembly drawings, component and material specifications, production and process specifications, software machine code, work instructions, quality assurance specifications and procedures, installation and servicing procedures, packaging and labeling specifications, including methods and processes used) as well as descriptive materials that define and characterize the design (3).

Design reviews are a formal, documented, comprehensive, systematic examination of a design to evaluate the adequacy of the design requirements, to evaluate the capability of the design to meet these requirements, and to identify problems. Establishment of a formal process of reviewing the delivery system design at each stage of development and documentation of the development of the design in the design history file is required. The formal design review must have at least one qualified independent reviewer as part of the process as well as representatives of all functions concerned with the design stage being reviewed (3).

Design verification requires that an organization establish and maintain procedures for verifying the device design with objective evidence that the specified requirements of the drug delivery device have been met. Design verification shall ensure that the design outputs meet the required design inputs. These activities precede design validation, which measures whether the completed drug delivery system meets the user requirements (3).

Design technical transfer requires that each manufacturer establish procedures to ensure that the delivery device design is correctly translated into production specifications. This is not a unique requirement to delivery device technology as all pharmaceutical products must have the core elements of drug's development translated into a production specification. However, in some cases, the delivery device may be developed by a third party incorporating a unique technology platform, and in these cases a specific focus should be placed on the technical transfer of knowledge for these systems.

Design changes are common during the development of the device. Each manufacturer should have procedures for the identification, documentation, validation, or where appropriate, verification, review, and approval of design changes before their implementation (3).

Risk management is a key expectation as part of the design control process. The expectation is that an organization designing a delivery device will identify, analyze, control, and monitor the risks associated with bringing the delivery system to market. Risk to the user may be inherent in the design of the product, part of the production process, or created by the patient's use of the product. Performance of risk management identification and analysis early and throughout the design process is critical and should be part of the definition of design inputs. Risks identified late in the design process are often more challenging to mitigate and will often cause a delay in the launch of a new delivery system.

Design Validation, User Studies, and Clinical Testing

Design validation requires that an organization ensure the delivery device specifications meet the user's needs and intended use. Objective evidence that the delivery device meets the intended use of the product will typically include the design verification activities as well as evaluation of the device with the end user. The user evaluations can be completed by user studies simulating actual use or clinical testing. Design validation must be completed with delivery devices that are representative of the final product and manufactured using the same methods that will be used in final production. Labeling, packaging, and user instructions are considered part of the product, and these elements must be part of the design validation activities (3).

DESIGN HISTORY FILE

A design history file is a record of the development history of the delivery device. Unlike pharmaceutical products that have development history reports, medical devices primarily utilize a design history file to document the design and development history. The design history file is specified by the FDA QSR. A design history file is not specifically designated by ISO 13485:2003, but there is a requirement for the creation of documentation and records for design control. The primary elements of a design history file that meets the FDA requirements also meet the basic intent of the ISO 13485, so creation of a design history file or technical file is strongly recommended (3).

DELIVERY DEVICE MANUFACTURING REQUIREMENTS

The basic GMP requirements apply to delivery device manufacturing. Both the FDA and ISO requirements call for production and process controls as well as monitoring of customer feedback. An assembled mechanical medical device can have unique characteristics depending on the combination of parts used. It is very important to have well-defined component specifications and a robust test plan that will enable the manufacturer to fully characterize the production lot and ensure that any major production anomalies are detected. When a dosage form incorporates a delivery device, the need to monitor patient feedback is critical given the potential questions that may arise with regard to how the product should be used, the potential that misuse might lead to complaints, or even the possibility that an issue might cause an adverse event. Robust surveillance systems must be in place prior to launch.

PARENTERAL DEVICE REGULATORY SUBMISSIONS

510k or New Drug Application (NDA) Submission as a Combination Product

Primary oversight of device regulatory submissions is provided by the Center for Device and Radiological Health (CDRH). Drug delivery devices integrate device technology with a pharmaceutical product, and this integration of device technology is supported by the FDA's

Office of Combination Products. The Office of Combination Products helps to determine which of the FDA's centers (CDRH, CDER, CBER) will have primary jurisdiction for review of the submission. Primary oversight of a combination device regulatory submission is determined by whether the device or the pharmaceutical product is the primary mode of action. If the device is integral to the dosage form, such as a prefilled pen with a drug cartridge or a disposable autoinjector with a prefilled syringe, then the submission will likely require an NDA submission because of the primary mode of action rule. Given that the drug will most likely be the primary mode of action, the submission review will likely be led by either CDER or CBER, depending on the type of drug, with CDRH providing consultation.

European Union Regulations and CE Mark Requirements

The primary guidance with regard to medical devices in Europe is the European Union (EU) Medical Devices Directive. A conformity assessment by a notified body that will lead to a CE mark is a requirement for medical devices that are developed in compliance with the EU Medical Device Directive. Therefore, reusable delivery devices such as pen injectors, needle-free devices, or pumps would require a CE mark in Europe. Drug delivery devices that integrate device technology with a pharmaceutical product in a single prefilled unit are regulated by the EU Medicinal Products Directive. The EU Medicinal Products Directive does not require CE marking, but it is expected that the device component of the delivery system will meet the essential requirements of the Medical Device Directive.

Japan Requirements for the MHLW

The Japanese regulatory process with requirements for delivery device submissions is similar for both reusable and prefilled delivery technologies. For either a reusable or prefilled device, a separate submission is provided for review. The submission typically would include a medical device description, materials of construction, device specifications typically as defined by applicable ISO guidance, list of countries launched, address of the manufacturer, risk management process, any malfunctions reported, and a photo of the delivery device. After the medical device technology for a prefilled system is approved, it can be cross-referenced for use with other pharmaceutical products.

CONCLUDING REMARKS

Injection devices and tools are a rapidly growing and important segment of injectable products markets in the pharmaceutical and biopharmaceutical industry. The integration of a device, primary container, and drug product poses complexity for development and commercialization that are unlike the simpler presentations of the past. The rapidly evolving and unique regulatory requirements for combination products also pose additional new challenges. In the end, the most essential element for success is ensuring the combination product meets the needs of the patient in a way that provides convenience, eases discomfort or apprehension associated with injection, and enables better quality of life.

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6 siRNA targeting using injectable nano-based delivery systems

Lan Feng and Russell J. Mumper

Abstract: The 2006 Nobel Prize in physiology or medicine was awarded to Andrew Fire and Craig Mello who demonstrated a fundamental control of gene expression called RNA interference (RNAi). Since the first time small interfering RNA (siRNA) was shown to knock down the expression of a target protein in mammal cells in 2001, a significant surge of interest has been focused on this promising area. This chapter will provide an overview of RNAi, siRNA, and siRNA-based therapeutics, as well as review the current state of the art of injectable siRNA nanodelivery systems and targeting strategies. The review will also discuss the chemical, physical, and biological barriers, as well as ideal criteria for effective siRNA nano-based therapeutics.

OVERVIEW

RNAi Mechanisms and siRNA

Antisense is a ubiquitous and conserved phenomenon in cells. Antisense nucleotides suppress the gene expression through several distinct mechanisms, such as RNaseH-induced degradation of complimentary mRNA through antisense oligonucleotides hybridizing to their target mRNA; sterical inhibition of mRNA translation or pretranslational splicing; cleavage of target mRNA by some ribozymes or deoxyribozymes because of their intrinsic catalytic activity; RNA-induced silencing complex (RISC)-mediated degradation of target mRNA by double-stranded RNA (dsRNA) (1–3).

RNA interference (RNAi) is the antisense effect caused by RNA (Fig. 1). dsRNAs are important regulators of gene expression in eukaryotic cells. Interfering dsRNAs cleave mRNA through several steps. First, the "DICER" enzyme and its cofactors cleave dsRNA to 21 to 23 base-pair (bp) segments, which are called small interfering RNAs (siRNAs) and assist their loading onto the RISC. RISC removes the sense strand, uses the antisense strand as a guide to seek the complimentary region in the mRNA, and pairs the antisense strand to its target. RISC contains an important protein Argonaute 2 (Ago 2) that has an RNaseH-like domain carrying the activity of RNA cleavage. After cleavage, the resulting 5′ and 3′ fragments are subsequently subjected to full degradation by other nucleases (2–4). Interfering dsRNA can be either endogenously produced or exogenously provided. However, exogenous dsRNAs longer than 30 bp cause severe toxic responses in mammals, which limit their applications (5). In 2001, Elbashir and colleagues published a paper in *Nature*, reporting the use of synthetic 19 bp duplexes siRNAs with 2-nucleotide (nt) 3′ overhangs to mediate RNAi in mammalian cell culture systems (6). Later, researchers extended this to recombinant DNA expressing similar short interfering RNA to have longer effect in cells. siRNA has quickly become one of the most powerful and indispensable tools in molecular biology.

Therapeutic Target and Applications

Since siRNA is a highly specific tool for target gene knockdown, it has been used in the field of molecular biology to understand gene function, as well as to identify and validate genes (7–11). On the basis of knowledge of gene function, siRNA designed to target gene encoding disease-associated protein is currently under intensive investigation as a potent and specific therapeutic agent.

RNAi was found as an anti-viral defense in plants (12). Thus, siRNA as a treatment of human virus diseases may hold the greatest promise in the clinic. Recently, several groups have explored the therapeutic effects of RNAi on hepatitis B virus (HBV) (13), hepatitis C virus (14), human immunodeficiency virus type-1 (15–17), herpes simplex virus 2 (18), respiratory syncytial virus (19,20), human papillomavirus (21,22), as well as others through inhibiting viral

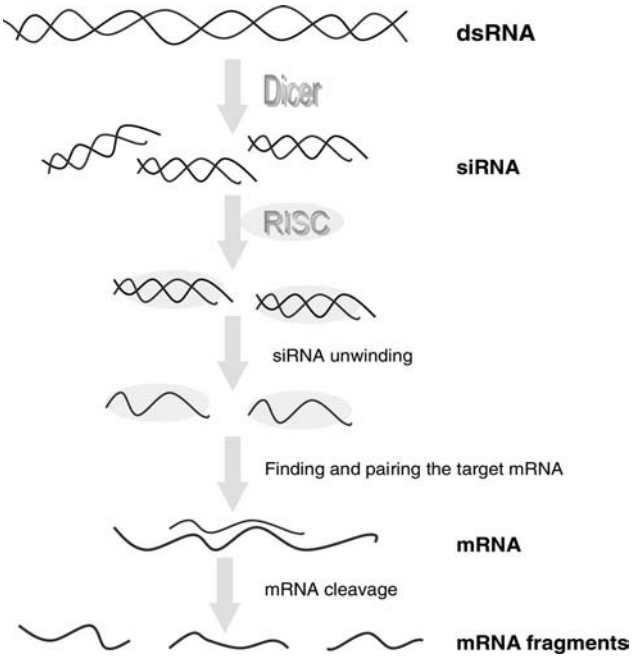

Figure 1 (*See color insert*) Mechanism of RNA interference.

replication and production mechanisms. All the studies have yielded encouraging results. Another strategy is to inhibit the host proteins for pathogen invasion or signaling pathways that initiate the inflammatory response such as cell death receptor Fas (23–25) and caspase-8 (26,27).

A second therapeutic application for RNAi is the treatment of dominant genetic diseases. Autosomal dominant diseases caused by mutant gene encoding essential proteins can be treated by siRNA targeting the mutated alleles. Studies have demonstrated that many familial neurodegenerative diseases, such as Huntington's disease, spinobulbar muscular atrophy, and slow channel congenital myasthenic syndrome (SCCMS) caused by the overexpression of mutated genes or CAG-repeat expansions that encode polyglutamine in the disease protein might be treated by siRNAs (28,29). Another example is the Cu,Zn superoxide dismutase (SOD1) gene in amyotrophic lateral sclerosis (ALS). Schwarz et al. reported that siRNA was specific enough to discriminate single-nucleotide polymorphism. Many SOD1 mutations are single-nucleotide mutations that make siRNA a promising potential therapeutic strategy for the treatment of ALS (30).

Along with the intensive research in molecular biology on cancer, the involvement of more and more signaling pathways and oncogenic genes has been demonstrated, which in turn makes RNAi anticancer therapy possible. Oncogenic genes are often important for cell survival and growth when normally expressed and strictly regulated. In addition, inhibitors of oncogenic proteins are not specific and often cause severe side effects. The high specificity of siRNA allows the selective knockdown of mutated oncogenes without influencing normal cells. Mutations of Ras are present in many cancers such as pancreatic cancers, colon cancers, leukemia, as well as others. In oncogenic K-RasV12, a point mutation results in a valine instead of a glycine in wild-type K-Ras. A viral siRNA transfection targeting this region strongly inhibited the expression of K-RasV12 and tumor formation in nude mice (31,32). Besides targeting oncogenes like Bcr-Abl (33), Bcl-2 (34), Survivin (35), some alternative strategies have also been investigated and have obtained success to some extent. Suppression of tumor angiogenesis by effectively silencing epidermal growth factor receptor (EGFR) gene and vascular endothelial growth factor (VEGF) receptors inhibited the in vivo growth of non–small lung cancer (36) and PC-3 prostate cancer cells (37), respectively. An RNAi approach also enhanced the effects of chemotherapy in resistant breast cancer cells because of the suppression of MDR1 (38,39).

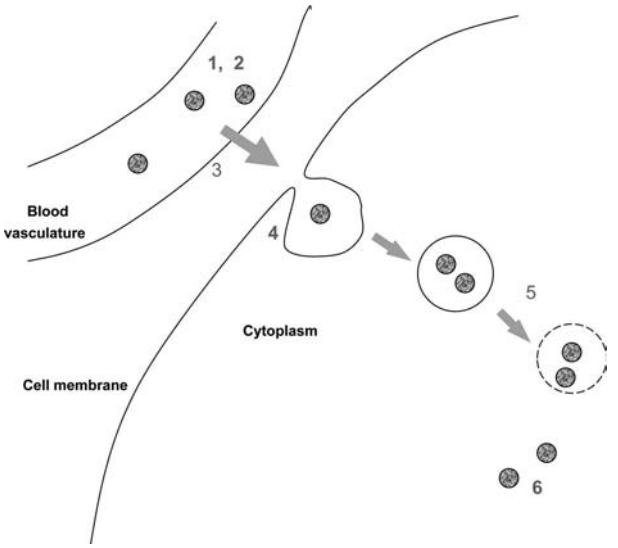

Figure 2 Challenges for siRNA delivery. The barriers include (1) susceptibility in the blood circulation and tissues after injection, (2) rapid clearance by renal excretion and RES uptake, (3) extravasation across the endothelium and to the target tissue, (4) penetration through the cell membrane, (5) endosomal escape, and (6) transient persistence in cells. *Abbreviations*: siRNA, small interfering RNA; RES, reticuloendothelial system.

Delivery Barriers and Challenges

As a potential therapeutics to treat human disease, siRNA needs to be efficiently delivered in vivo. Before designing an effective delivery system for siRNA, it is crucial to understand the six main challenges and barriers of siRNA delivery (Fig. 2).

First, siRNAs are vulnerable to nucleases in serum and tissues. Second, siRNA would be rapidly cleared from the circulation by renal excretion and the reticuloendothelial system (RES) uptake, especially delivered in a nanoparticulate formulation that was prone to RES uptake and elimination. Third, extravasation of siRNA across the endothelium and access to the target tissue is difficult because of its size and negative charge. Fourth, as hydrophilic, negatively charged macromolecules, siRNAs may have poor plasma membrane penetrating properties. Furthermore, if the siRNAs enter cells through an endocytosis mechanism, another important barrier is endosomal escape. Eventually siRNA would end up in late endosome or lysosome and be digested if they could not be released to cytoplasm where its effect takes place. Finally, the persistence of siRNA effect is not permanent because of its inability to reproduce itself.

Available Delivery Approaches

Both noncarrier and carrier strategies are available for in vivo siRNA delivery. Aimed at overcoming individual delivery barriers, various noncarrier systems or methods have been developed. Chemical modifications have been applied to improve the nuclease stability of siRNA, for example, sulfur substitution for a nonbridging oxygen in the phosphodiester linkages (40). Simple conjugation of siRNAs with ligands represents a large portion in this category. Cholesterol siRNA conjugation reduces renal excretion and increases circulation half-life by binding to plasma albumin. Long-chain fatty acid conjugation of siRNA may facilitate the cellular uptake of siRNA by receptor-mediated endocytosis (41). A considerable effort has been devoted to cell-penetrating peptide (CPP) conjugate investigation. These small polycationic peptides rich in arginine and lysine promote the cell penetration of the coupled cargo, which could be siRNA or siRNA-containing complexes. However, the mechanism of uptake and the delivery efficiency is still controversial. An intravenous injection of naked siRNA in massive volume through mouse tail vein has been performed to increase the transport of siRNA through capillary endothelial cells. This method is termed hydrodynamic injection and induces hepatic gene silencing (42). Other noncarrier methods include topical application and the gene gun, among others. Generally, these methods are less efficient and/or practical than carrier strategies.

As for carrier strategies, these can be further divided into viral and nonviral carriers. To this point, viruses are still the most efficient vehicles for gene delivery. Because of their

intrinsic nature and function, they can easily penetrate capillary membranes, cell membrane, and even nuclear membranes to reach their destination. When the siRNA containing nucleic acid is inserted to the genetic DNA, it enables long-term expression, and therefore has the ability to chronically suppress gene expression. However, the disadvantages are obvious and inevitable. For example, viral carriers have the difficulties of preparation and storage, immunogenicity, and potential carcinogenicity if they either suppress tumor suppressor genes or activate oncogenes. Hence, extensive attention has been attracted to the design and study of nonviral nanoscale siRNA delivery systems. Although this is a relatively novel area, a growing number of achievements have been made in the recent years as will be discussed in detail.

Differences Between siRNA and pDNA Delivery

As double-stranded nucleic acids, siRNA and double-stranded DNA (dsDNA) share many common properties. They have similar backbone structure with the same negative charge to nucleotide ratio. They both can interact electrostatically with positively charged agents so that many delivery systems are designed on the basis of this principle. Plasmid DNA (pDNA) has been investigated and delivered for at least two decades. Considering the similarity between siRNA and pDNA, applying the knowledge from pDNA delivery systems can facilitate rationale approaches to the delivery of siRNA. However, understanding the key differences between pDNA and siRNA is critical for designing the most efficient and safe siRNA delivery systems.

First, RNA is more sensitive to enzymatic degradation than DNA. The 5′-carbon sugar in RNA nucleotides is ribose instead of deoxyribose in DNA. This structure makes the RNA backbone more susceptible to spontaneous breakdown and hydrolysis by nucleases. Moreover, DNase and RNase are present in various environments both in vitro and in vivo. To avoid unexpected degradation during the handling and preparation process, creating a DNase/RNase-free environment is of great importance. However, DNase inhibition can be easily achieved while RNase inhibition is much more difficult. In particular, RNase A is extremely stable in an aqueous environment (43). Chemical modifications have been performed to increase the stability of dsRNA, for example, 2′-*O*-methyl modification, incorporation of locked nucleic acids (LNAs), phosphorothioate, etc. (40). The greater susceptibility of RNA highlights the critical need for a protective carrier to effectively deliver siRNA.

Second, the delivery destination or intracellular location needed for pDNA and siRNA action is quite different. pDNA requires delivery into the nucleus of the host cell where it can use the transcriptional machinery of the host cell to carry out its therapeutic effect. Unlike pDNA exerting its effect in the nucleus, the target of siRNA is its complementary mRNAs that have already been released from the nucleus after transcription. Therefore, siRNA only needs to be delivered to the cytoplasm. For this reason, pDNA delivery often requires a nuclear localization mechanism such as involving the inclusion of a nuclear localization sequence or carriers that can transport their cargo to the nucleus.

Third, depending on their different action mechanisms, the duration of siRNA and pDNA effects differs as well. Naked siRNAs, unlike pDNA-expressed siRNAs, are not regenerated in cells. Thus, in rapidly dividing cells, the typical gene-silencing duration is three to seven days because of the dilution of siRNAs below a certain level. In contrast, in slowly or nondividing cells, the gene knockdown effect can last as long as three weeks depending on the stability and half-life of the suppressed protein (44). The therapeutic effects with pDNAs not only depend on their own stability but also on the strength of their promoters if they are nonintegrative. In comparison, it is well known that the therapeutic effects of integrated DNA vectors could be long term or even permanent. Hence, the contrast between the pDNA and siRNA requirements above highlight the fact that successful siRNA therapy will necessitate repeated treatment, which makes selection of the carrier with low cytotoxicity and immunogenicity even more important for siRNA.

Another obvious difference between pDNA and siRNA is the molecular weight and size of the molecules. The pDNAs used in gene therapy are usually several kilobase pairs while siRNAs are only 21 to 23 bp. In pDNA delivery, it is often complexed and condensed to nanometric-sized particles directly with cationic agents. However, it is well known that many types of cationic condensing agents (polymers, lipids, etc.) often lead to aggregation of the condensed particles. Because of its smaller size, siRNA is perhaps easier to complex with

cationic condensing agents. However, these complexes with siRNA are often unstable and decomplex since the smaller siRNA is not condensed and the ionic interaction is much easier to compete off with counterions. RNA is somewhat stiffer than DNA. The persistence length, which is a basic mechanical property quantifying the stiffness of a long-chain molecule, of dsDNA is 450 to 500 Å and that of dsRNA is approximately 700 Å (45). At 2.7 Å/bp, the persistence length for RNA is 260 bp. Therefore, 21- to 23-bp siRNA behaves as a rod and is not likely to be further condensed. Thus, electrostatic interaction between siRNA and cationic agents could lead to a relatively uncontrolled interaction and form complexes of large sizes and poor stability, with the consequence of incomplete encapsulation (46).

Considering the differences discussed above, the strategy for the delivery of pDNA and siRNA should be interrogated carefully. One should not assume that a delivery system that works for pDNA could be simply transferred to an siRNA delivery system before a more thorough investigation is performed.

IDEAL INJECTABLE NANO-BASED SYSTEMS FOR siRNA DELIVERY

For an ideal injectable nano-based delivery system to efficiently deliver siRNA, regardless if it is for topical or systemic administration, certain criteria must be met. For systemic injection of siRNA, additional criteria must be considered.

Generally speaking, at the cellular level, a successful delivery vehicle must be formulated to have the following characteristics: (*i*) provide protection to siRNA against degradation in extracellular fluids, (*ii*) facilitate efficient cellular uptake, (*iii*) facilitate endosomal escape before the early endosome becomes late endosome or lysosomes in which the siRNA will be destroyed, (*iv*) be able to readily release siRNA upon arrival at the cytosol where the RNAi effect takes place, (*v*) be nontoxic to the cells, (*vi*) be stable during storage and in the vehicle for administration solution, that is, chemically and physically stable.

For a systemically administered siRNA nanocarrier, there are some additional concerns: (*i*) provide protection to siRNA against degradation not only in the extracellular fluids but also in the systemic circulation, (*ii*) be stable in the systemic circulation with limited breakdown and/or aggregation before it arrives at the target site, (*iii*) be able to extravasate blood vessels and penetrate tissues to gain access to the target site, (*iv*) maintain proper particle size and surface properties to avoid clearance and/or elimination via the kidneys and RES.

To further increase the efficiency of in vivo siRNA delivery, target strategies are widely applied. As for the targeted systemic nanocarrier, choosing a suitable targeting ligand is critical as well. First of all, the targeting should be specific enough, that is, the expression of the receptor on the target cells should be highly specific, highly expressed, and not shed, among others. Second, the targeting ligand should have high affinity with the target receptor to ensure sufficient retention time as well as trigger cellular uptake via receptor-mediated endocytosis instead of remaining bound to the receptor. Last but not least, the targeting ligand should be amenable to the required chemistries needed to attach the ligand to the nanocarrier, as well as have low or no immunogenicity.

NANO-BASED DELIVERY SYSTEMS

Complexes

RNA is a molecule consisting of a chain of nucleotide units. Each nucleotide is composed of a nitrogenous base, a ribose sugar, and a phosphate. RNA is a negatively charged molecule because of the negative charge on phosphate groups at physiological pH. siRNA molecules are dsRNA with 19 to 21 bp. Calculating the charge density gives about three negative charges per kilodalton (kDa) molecular weight of siRNA.

To date, complexes of siRNA with various positively charged materials by electrostatic interaction represent the largest portion of active research. In this category, there are two major subgroups and some others.

Lipoplex

The most often referenced formulation in this group is cationic liposomes. When cationic liposomes are mixed with negatively charged siRNA, the organized bilayer structure of the

liposome is altered by electrostatic interaction so that they are no longer referred to as liposomes but have a new name of "lipoplexes."

The DOTAP Liposomal Transfection Reagent is a commercially available liposome formulation of the monocationic lipid 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) that can be used for the transfection of nucleic acids. Mixing the DOTAP reagent with the negatively charged siRNA results in a spontaneously formed stable complex that can be directly added to the tissue culture medium with or without serum. Commercially available DOTAP is not only used as an instrumental tool for in vitro siRNA delivery to investigate gene functions in molecular biology, it has been used to deliver siRNA in mice to prove the concept and feasibility of certain therapeutic ideas (47–50). Dioleoyl-phosphatidylethanolamine (DOPE) is a neutral helper lipid usually used with DOTAP to formulate transfection reagent. It is generally believed that DOPE enhances transfection because of its tendency to form hexagonal phase structures at temperatures above 10°C, which facilitates siRNA endosomal escape (51).

On the basis of this classical liposome formulation, targeting ligands have been included to deliver siRNA to specific tissues. For example, Chang and coworkers developed a tumor targeting immunoliposome that takes advantage of elevated transferring receptor (TfR) levels on tumor cells to deliver pDNA, antisense oligonucleotides, imaging agent, or siRNA (52–54). The anti-transferrin receptor single-chain antibody fragment was incorporated into the liposomes and formed immunoliposomes. This intravenously administered immunoliposome delivered its cargo (which could be pDNA, antisense oligonucleotides, imaging agent or siRNA) specifically and efficiently to primary/metastatic tumors. In addition, a pH-sensitive histidine-lysine peptide (HoKC) was included in the complex to further increase the endosomal escape. In a recent report, the results showed increased potency of the liposome-HoKC complex and their ability to carry anti-HER2 siRNA to target and sensitize tumor cells, silencing the target gene, and inhibiting tumor growth in vivo (55). Cardoso et al. associated transferrin instead of the TfR antibody to DOTAP/cholesterol liposome, another conventional cationic liposome, to target TfR expressing cells (56). In vitro experiments by the group showed enhanced gene knockdown activity of transferrin-associated liposome compared with the conventional liposomes by anti-GFP (anti–green fluorescent protein) siRNA. Besides tumor targeting, siRNA liposomes are targeted to other tissues and organs such as the liver. Kim and his colleagues formulated anti-HBV siRNA into a complex of DOTAP/cholesterol liposome and apolipoprotein A-I (apo A-I) (57). Apolipoprotein is recognized by class B, type-1 scavenger receptor (SR-BI) that is predominantly expressed in the liver. When the liver-targeted formulation was injected intravenously into a HBV carrying mouse model, the viral protein expression was reduced to about 30% and its effect lasted up to eight days upon a single treatment.

In addition to the commercially available lipids, some cationic lipids have also been designed and synthesized to improve the transfection efficiency and reduce the cytotoxicity. It has been reported that an ether linkage containing cationic lipid, such as 1,2-dioleyloxypropyl-3-trimethylammonium chloride (DOTMA), has higher in vivo transfection efficiency than the corresponding ester analogue DOTAP (58). On the basis of the structure-activity information, Chien et al. synthesized ether-linked cationic cardiolipin analogue (CCLA) where the phosphate groups of cardiolipin were replaced with quaternary ammonium groups as shown in Figure 3A (59).

Their report showed that the transfection efficiency of the luciferase reporter gene in mice was sevenfold higher than the commercially available DOTAP-based liposome, and the CCLA-based liposome had lower toxicity than DOTAP transfection reagent. When the CCLA-based liposome was used to deliver the c-raf siRNA in mice bearing human breast cancer (MDA-MB-231) xenografts, the tumor growth was inhibited 73% as compared with free siRNA treatment. For the same reason, many groups synthesized other cationic lipids to meet the needs of in vitro and in vivo delivery such as cationic cholesterol–based polyamine lipid *N*′-cholesteryloxycarbonyl-3,7-diazanonane-1,9-diamine (CDAN) (46), 2-(3-[bis-(3-amino-propyl)-amino]-propylamino)-*N*-ditetradecylcarbamoylmethyl-acetamide (RPR209120) (60), and multivalent lipid 5 or pentavalent lipid (MVL5) (61). Their structures are shown in Figure 3B–D. Positive charges could also be incorporated by adding aminoglycoside to the lipid. Desigaux et al. synthesized a series of cationic lipids (DOST, DOSK, DOSP, DOSN)

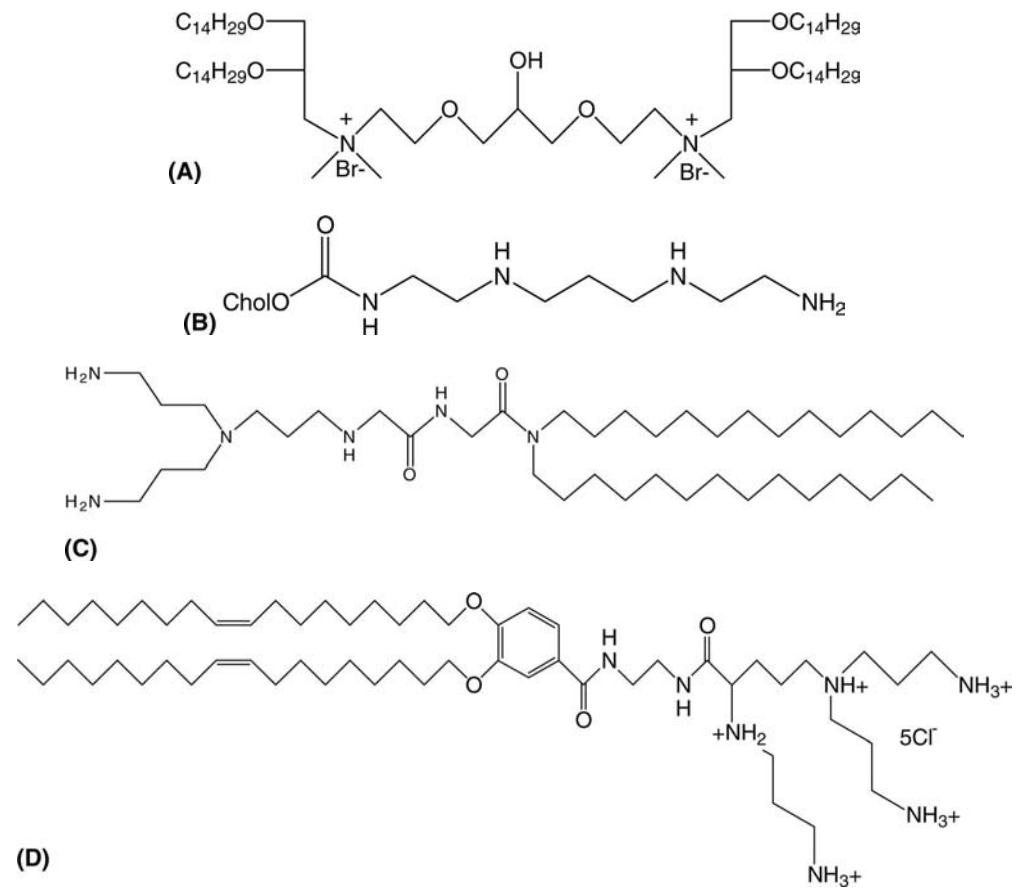

Figure 3 Structures of (**A**) CCLA, (**B**) CDAN, (**C**) RPR209120, and (**D**) MVL5. *Abbreviations*: CCLA, cationic cardiolipin analogue; CDAN, cationic cholesterol–based polyamine lipid *N′*-cholesteryloxycarbonyl-3,7-diazanonane-1, 9-diamine; RPR209120, 2-(3-[bis-(3-amino-propyl)-amino]-propylamino)-*N*-ditetradecylcarbamoylmethyl-acetamide; MVL5, multivalent lipid 5 or pentavalent lipid.

bearing various aminoglycosides (tobramycin, kanamycinA, paromomycin, and ethylthioneomycin B, respectively) linked to two dioleyl chains by a succinyl spacer for specific interaction with siRNA (62).

Besides lipid-aided cellular delivery, some positively charged CPPs have been incorporated into conventional liposomes. In a study by Mudhakir et al., liposomes composed of egg phosphatidylcholine (EPC) and cholesterol were modified by direct conjugation of a novel peptide IRQRRRR (IRQ) to the surface of liposomes (63). IRQ is a peptide ligand that targets skeletal muscle found by in vivo phage display. Since the novel peptide IRQ is rich in arginine, it not only serves as a tissue-target moiety but also triggers the cellular uptake via caveolar endocytosis.

An interesting concept called site-specific release has been applied to liposomal siRNA targeting delivery as well. It is well known that under pathological conditions the expression of many proteins are altered including intracellular receptors and enzymes, as well as others. Most of the recent studies have focused on targeting modified receptors using either an antibody or a small molecular receptor substrate. However, altered expression of enzymes in the pathological tissue could also serve as a novel target by triggering site-specific release of a therapeutic agent. For example, $sPLA_2$ is an enzyme upregulated in cancer and inflammatory tissues, but it is present at low levels in the blood circulation. Foged et al. formulated a liposome including lipid dipalmitoylphosphatidylglycerol (DPPG), which is favored by human group IIA $sPLA_2$. They hypothesized that the liposome could site-specifically release siRNA in inflammatory tissue but not in the systemic circulation or other tissues (64).

Moreover, the hydrolysis products were thought to disturb the cellular membrane and facilitate the uptake of siRNA. Although their data showed that the $sPLA_2$ degradable liposomes did not silence enhanced green fluorescent protein (EGFP) expression in HeLa cells, they did show that the siRNA from the liposomal formulation was taken up by HeLa cells and that uptake was augmented by the addition of $sPLA_2$. The concept of site-specific release with no active targeting moieties opens an alternative avenue and deserves more attention.

Polyplex

Polymers, either natural or synthetic, represent another major group of complexing agents for siRNA delivery. The formulation of nucleic acids complexed with polymers is generally called "polyplex" in this chapter even though in various literatures they are sometimes referred to as nanoparticles or micelles.

Cationic polymers, for example, polyethylenimine (PEI), polypropylenimine (PPI), poly-L-lysine (PLL), polyallylamine (PAA), cationic dextran, and chitosan are the most commonly used materials for siRNA complexation. Among them, PEI has been the most widely used polymer for complexing with siRNA.

The native branched PEI (25 kDa) is a prototype polymeric transfection agent that has gained widespread use. Branched PEI contains primary, secondary, and tertiary amines in the molar ratio of 1:2:1. The primary amines are mainly responsible for nucleic acid condensation while the secondary and tertiary amines provide buffering capacity and therefore facilitate endosomal escape via the so-called "proton-sponge" effect. The transfection efficiency of PEI, along with its cytotoxicity, strongly depends on its molecular weight. Usually, high molecular weight PEI has higher transfection efficiency but with higher toxicity as well, while low molecular weight PEI has lower cytotoxicity with reduced transfection efficiency. To enhance the gene delivery efficiency and minimize cytotoxicity of PEI, there has been a great deal of effort focused on structurally modifying PEI. For example, Dong et al. cross-linked low molecular weight PEI 800 Da with short diacrylate linkages to form higher molecular weight PEI structures (65). The modification combines the favorable low toxicity of low molecular weight PEI with the higher transfection efficiency of high molecular weight PEI. The biodegradable ester bonds are hydrolyzed under physiological conditions within the cell after delivery and convert the cross-linked high molecular weight PEI into low toxic low molecular weight PEI. In a study of pDNA transfection, an optimal cross-linked PEI, EGDMA-PEI 800-4h (the product of conjugation of amino groups of PEI 800 Da to EGDMA for four hours), resulted in a 9-fold increase in gene delivery efficiency in B16F10 cells and a 16-fold increase in 293T cells compared to with commercially available PEI 25 kDa control. Later the modified PEI was used to deliver plasmid-encoded focal adhesion kinase-1 (FAK1) siRNA in vivo and prolonged the survival of the tumor-bearing mice (66). To address the associated cytotoxicity with the use of PEI for siRNA delivery, Swami et al. cross-linked PEI with 1,4-butanediol diglycidyl ether (bisepoxide) (67). The modification converted primary amines, which are believed to be the main source of cytotoxicity, to secondary and secondary to tertiary amines. The system was found to deliver siRNA more efficiently into HEK cells as compared with native PEI 25 kDa with significantly reduced cytotoxicity.

Jere et al. conjugated low molecular weight PEI and polyethylene glycol (PEG) with biodegradable poly(β-amino ester) (PAE) (68). The high repetitive PEI units are thought to result in high delivery efficiency while PEG units and the ester linkage facilitate more rapid intracellular siRNA release and lead to enhanced polymer degradation resulting in lower cytotoxicity. As a result, PAE as a carrier was found to be less toxic and 1.5-fold more effective than standard PEI 25 kDa. Several other PEI modifications have been investigated by Zintchenko et al. (69). The group performed a number of modifications including ethyl acrylate, acetylation of primary amines, or introduction of negatively charged propionic acid or succinic acid groups to the PEI structure. All the conjugates led to reduced toxicity in comparison to the unmodified PEI. In particular, succinylation of PEI resulted in up to 10-fold lower toxicity in Neuro2A cells.

In order to facilitate release of siRNA in the cell, branches of PEI have been derivatized with ketal linkages (70). Ketal linkages are acid-degradable under mild acidic pHs (e.g., pH

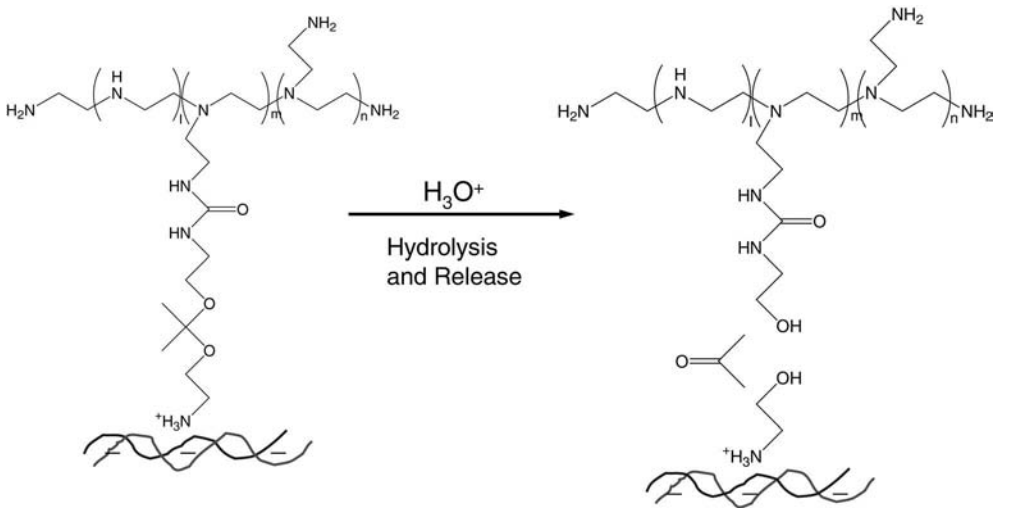

Figure 4 Dissociation of nucleic acids from ketalized PEI upon hydrolysis. *Abbreviation*: PEI, polyethylenimine. *Source*: Adapted from Ref. 70.

5.0) and facilitate the release of siRNA in the endosomal environment as shown in Figure 4. The ketalized PEI complexed with siRNA into siRNA/PEI polyplexes with a particle size range of 80 to 200 nm showed enhanced delivery efficiency with reduced cytotoxicity.

One of the primary disadvantages of the use of positively charged complexing agents is that they are prone to aggregation or disassociation in the blood when complexed to siRNA. Moreover, positively charged complexing agents tend to interact with the negatively charged proteins in the systemic circulation and are taken up by the RES. To address this potential problem, PEG has been utilized to shield the surface of the complex which serves to provide enhanced stability. PEG has either been conjugated directly to siRNA or to the cationic polymer. For example, Kim et al. conjugated PEG to siRNA via a disulfide linkage that could then be cleaved in the reductive environment in endosomes and cytoplasm. The PEG-siRNA conjugate was then complexed with PEI to form a nanoparticle (71). The resulting nanoparticle has an inner core composed of siRNA/PEI surrounded by a hydrophilic PEG shell. This kind of structure is similar to amphiphilic lipidic micelle and could be spontaneously formed, so it is called a self-assembled micelle even though the particle size is often not in the traditional micellar range. In vivo imaging results from Kim et al. showed enhanced accumulation of micelles in the tumor region following intravenous injection. Pegylated siRNA has also been complexed with other cationic polymers such as PLL (72).

PEG has also been conjugated to the cationic polymer. For example, PEG derivatized diblock or triblock copolymers have been designed and synthesized by many groups. A recent publication reported the synthesis of a triblock polymer consisting of monomethoxy PEG, poly (3-caprolactone) (PCL), and poly(2-aminoethyl ethylene phosphate) (PPEEA) (73). The polymers in an aqueous solution spontaneously formed positively charged micelles surrounded by PEG corona. siRNA was postloaded into the formed micelles resulting in complexes with an average particle size from 98 to 125 nm depending on the nitrogen to phosphate (N/P) ratio (Fig. 5).

Besides the linear copolymers, cationic graft comb-like copolymers were synthesized and used to deliver siRNA. Sato et al. prepared and evaluated a series of cationic comb-type copolymers (CCCs) consisting of a PLL backbone and PEG or dextran side chains (74). The water soluble dextran side chains of the copolymer are in abundance (>70 wt.%) and the highly dense PEG brush reinforced the electronic interaction between copolymers and siRNA instead of hindering it. The most remarkable property of the CCC with higher side-chain content (10 wt.% PLL and 90% wt.% PEG) is that it increased circulation time of siRNA in mouse bloodstream by 100-fold (74). Interestingly, even when the CCC was injected into

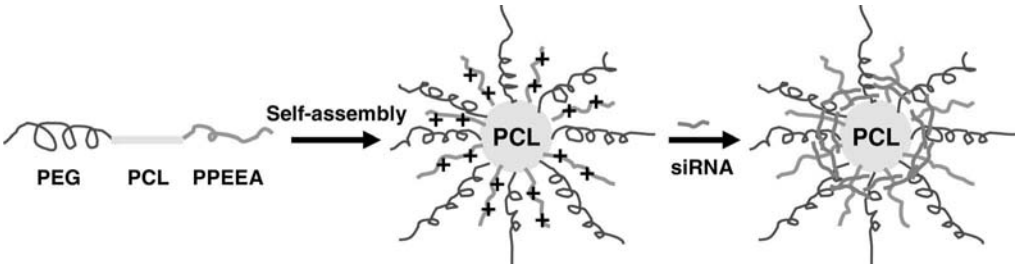

Figure 5 (*See color insert*) Self-assembling of cationic micellar nanoparticles and loading of siRNA. *Abbreviation*: siRNA, small interfering RNA. *Source*: Adapted from Ref. 73.

mouse intravenously 20 minutes prior to the injection of siRNA, the CCC still increased the half-life of the postinjected siRNA by more than 60-fold suggesting that the CCC prefers interaction with siRNA to other anions existing in blood.

While some investigators increase the stability and systemic half-life of siRNA polyplexes by incorporating PEG, others provide protection to the polycation/siRNA complex with another layer of lipid coating. Kim et al. synthesized a water soluble lipopolymer (WSLP) by conjugating cholesteryl chloroformate to PEI 1.8 kDa through a hydrophobic lipid anchor (75). The lipopolymer combined the advantages of both liposomes and cationic PEI. While the positively charged headgroup PEI complexed with siRNA and enhanced endosomal escape, the lipid coating on the complex further protected the complex from aggregation and RES clearance and increased the cell membrane permeability. The in vivo data showed that WSLP/VEGF siRNA complexes reduced tumor volume by 55% at 21 days and by 65% at 28 days relative to control tumors.

While most of the approaches discussed so far increase the transfection efficiency of PEI by reducing its cytotoxicity or provide protection against systemic clearance to some extent, a novel approach is to directly attach PEI with a membrane-active peptide. Melittin (Mel) is the major bioactive component of bee venom. Mel has been conjugated to pegylated PEI or PLL (76). To avoid its extracellular lytic activity, the amines of Mel were modified with dimethylmaleic anhydride that was cleaved under acidic pH in the endosome and enhanced the endosomolytic activity of Mel. PEG-PEI-Mel and PEG-PLL-Mel showed 70% and more than 90% in vitro luciferase gene knockdown, respectively.

To achieve targeted delivery, targeting moieties have been attached to PEI. PEI is usually pegylated with ligands conjugated to the distal end of the PEG, while direct attachment of ligands to PEI is performed as well. Schiffelers et al. targeted tumor neovasculature expressing integrins by conjugating an Arg-Gly-Asp (RGD) peptide to PEI 25 kDa (77). siRNA specific to vascular endothelial growth factor receptor-2 (VEGFR2) was complexed with the modified PEI at a N/P ratio of 2 to 6, resulting in the formation of polyplexes with average particle size of about 100 nm. The intravenous administration of these polyplexes to nude mice showed tissue-specific accumulation of PEI-PEG-RDG/siRNA. Kim et al. utilized a similar approach to complex siRNA with PEI-PEG-folate (78). Interestingly, their results showed that the delivery of siRNA led to the most pronounced gene-silencing effect compared with the delivery of antisense oligodeoxynucleotide (AS-ODN) or siRNA-expressing plasmid DNA. Another recently published paper reported on the use of hyaluronic acid (HA) as a ligand to target lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1) (79). In vitro data showed increased siRNA uptake in HA receptor expressing cells but not in nonexpressing cells, and that the gene-silencing effect was inhibited by free HA in a concentration-dependent manner.

Compared with the relative extensive investigation of PEIs and PLLs for siRNA delivery to date, studies on the use of other polymers is limited. Chitosan is one polymer being investigated for siRNA delivery. Although chitosan has been studied for more than a decade as a delivery system for pDNA, there are only few studies using it as a carrier of siRNA. Chitosan is a copolymer of *N*-acetyl-D-glucosamine (GlcNAc) and D-glucosamine (GlcN) produced by the alkaline deacetylation of chitin. As a natural polymer, chitosan is considered

to be biocompatible and nontoxic, although this depends on various physical-chemical properties such as purity, % deacetylation, and molecular weight, among others. The primary amines in the chitosan backbone become positively charged at the pH levels below the pK_a of the primary amine (pK_a 6.5) so that chitosan forms a complex with siRNA with electrostatic interaction. Several studies of chitosan/siRNA complex have shown that the ability of the chitosan to deliver siRNA to cells is dependent on the weight ratio, molecular weight of chitosan, and the degree of deacetylation (80–83). Similar to other complexes, chitosan/siRNA complexes can be formed by a simple mixing and stirring process. Different from other synthetic polymers, the N/P ratios to prepare chitosan/siRNA are relatively much higher. For example, Howard et al. used N/P ratio as high as 285; however, these high ratios reduced cell viability (80). The in vitro data showed that chitosan/siRNA complexes formed using high molecular weight (114 and 170 kDa) and deacetylation degree (84%) at N/P 150 were most stable with particle size about 200 nm (81). The group showed that 80% EGFP gene–silencing efficiency was obtained after 24 hours in H1299 green cells in vitro. Effective in vivo gene silencing was achieved in mice bronchiole epithelial cells (37% and 43% reduction of EGFP positive cells compared with scramble siRNA and untreated control, respectively) after nasal administration. However, the ability of the complexes to deliver siRNA systemically requires further investigation.

Thiamine pyrophosphate (TPP) has been used to form salts with chitosan to improve chitosan water solubility (83). Chitosan is a weak base with a pK_a value of 6.2 to 7.0, and thus has poor solubility at neutral to alkaline pH. TPP is a zwitterionic compound, which can increase the water solubility of chitosan due to the phosphate groups. However, the amine groups of TPP together with chitosan bind to negatively charged siRNAs to form complexes. The maximal EGFP gene–silencing effect mediated by chitosan-TPP/siRNA was 70% to 73%. Another study by Katas and Alpar used sodium tripolyphosphate to ionically cross-link chitosan to form nanoparticles (82). siRNA was either mixed with sodium tripolyphosphate and then dripped into a chitosan salt solution, or adsorbed to preformed chitosan/tripolyphosphate particles. The particle size of chitosan/tripolyphosphate was 510 $\pm$ 22.9 nm and 276 $\pm$ 17.9 nm formed using chitosan glutamate 470 kDa and 160 kDa, respectively. The particle size of chitosan/tripolyphosphate was 709 $\pm$ 50.3 nm and 415 $\pm$ 44.6 nm formed using chitosan hydrochloride 270 kDa and 110 kDa, respectively.

Leng et al. synthesized several branched peptide polymers composed of histidine and lysine (HK polymer) (84). Figure 6 shows the structure of a branched HK polymer with eight terminal branches and histidine-rich domains (H^3K8b). An integrin-binding ligand RGD was further added to increase the delivery efficiency of siRNA. Although the sizes of HK polymer/siRNA polyplexes were over 400 nm, the in vitro delivery efficiency was significant. The complex of H^3K8b and anti-β-galactosidase (β-gal) siRNA inhibited β-gal expression by more than 80% after 48 hours in SVR-bag4 cells that stably expressed β-gal. The H^3K8b/anti-luciferase siRNA complex inhibited more than 90% luciferase activity in MDA-MB-435 cells, which were cotransfected with a luciferase expression plasmid.

Others

In addition to the two larger families of cationic complexing reagents, lipids and polymers, there are several other molecules that have been proposed to make nano-based siRNA delivery systems.

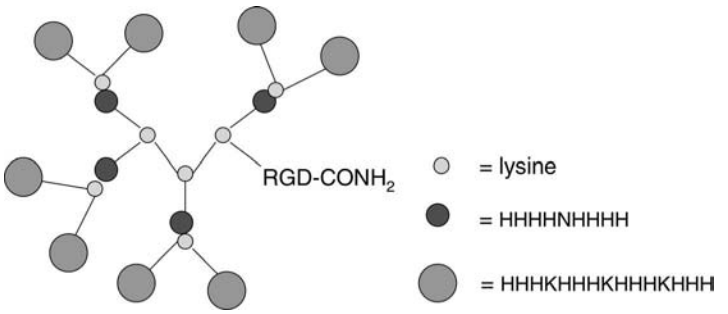

Figure 6 (*See color insert*) Schematic structure of H^3K8b polymer.

Positively charged natural proteins are a pool of convenient reagents in terms of their potential to complex and deliver siRNA. In a broad sense, proteins are also a group of polymers. To date, atelocollagen is the only protein used alone to deliver siRNA both in vitro and in vivo (85). Atelocollagen is a highly purified decomposition product of type I collagen derived from the dermis of cattle with a molecular weight of 300 kDa. The amino acid sequence at both N- and C-terminal of a collagen called telopeptide is the main source of the immunogenicity. Therefore, since atelocollagen obtained by pepsin treatment of collagen lacks immunogenic telopeptides, atelocollagen itself has low relative immunogenicity. It is a rod-like molecule with a length of 300 nm and a diameter of 1.5 nm. Atelocollagen, which is positively charged, interacts with the negatively charged siRNA to form an atelocollagen/siRNA complex with a diameter of 100 to 300 nm. An interesting property of atelocollagen is that it is soluble at a lower temperature but solidifies at a temperature over 30°C. Therefore, the atelocollagen/siRNA complexes were prepared and stored at 4°C. Once introduced into animals, the complex becomes solidified and releases siRNA in a controlled manner for a period of time due to the biodegradable nature of atelocollagen. Direct intratumoral injection of human HST-1/FGF-4 (fibroblast growth factor) siRNA complexed with atelocollagen resulting in about 12-fold and 8-fold tumor growth inhibition compared with atelocollagen alone and control siRNA, respectively, in an orthotopic xenograft of a human non-seminomatous germ cell tumor at 21 days after treatment.

On the basis of the barriers that must be overcome to deliver siRNA, some innovative carriers have been synthesized to fulfill multiple functions in one system. 1,4,7-Triazanonylimino-bis [*N*-(oleicyl-cysteinyl-histinyl)-1-aminoethyl) propionamide] (THCO) (Fig. 7) and (1-aminoethyl)imino-bis [*N*-(oleicyl-cysteinyl-histinyl-1-aminoethyl)propionamide] (EHCO) are two molecules containing a protonatable amine head group of different pK_as, two cysteine residues and two 8-heptadecenyl tails (86,87). They form stable complexes with siRNA through charge and hydrophobic interaction. The protonatable amino head group consists of primary, secondary, and tertiary amines having different pK_as (the pK_a values of primary, secondary, and tertiary amines are approximately 6.5, 7.0, and 6.0, respectively), which is similar to branched PEI. Thus, these molecules not only complex siRNA but also facilitate endosomal escape. The dithiol groups in the molecules can be polymerized by forming disulfide bonds to further provide stability to the formed siRNA complex. The disulfide bonds may be reduced in the endosome and cytoplasm resulting in the dissociation and release of siRNA. The multifunctional compounds mediated 40% to 88% silencing of luciferase expression with 100 nM siRNA in U87-luc cells.

Additionally, there are some interesting carriers that are quite unique in terms of geometry and other physical-chemical properties. For example, a cone-shaped macrocyclic

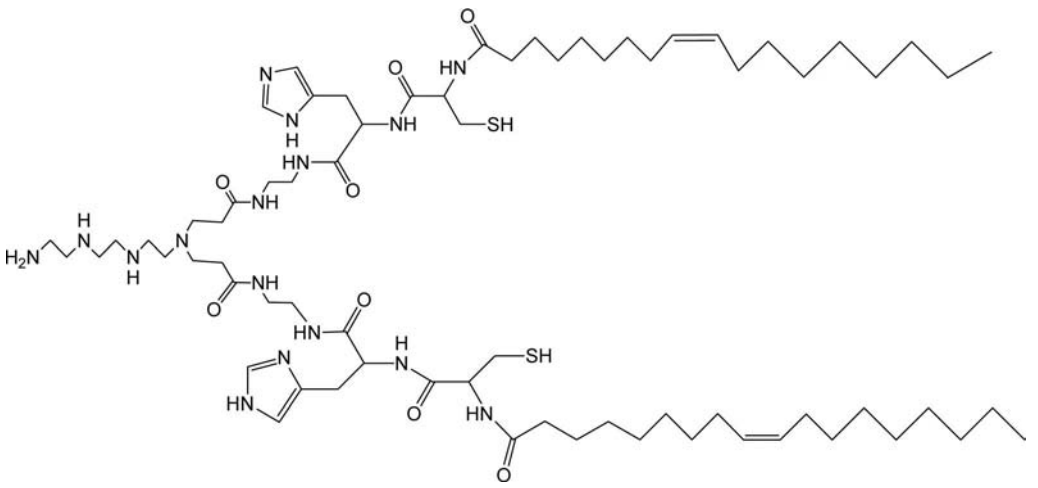

Figure 7 Structure of THCO. *Abbreviation*: THCO, 1,4,7-triazanonylimino-bis [*N*-(oleicyl-cysteinyl-histinyl)-1-aminoethyl) propionamide].

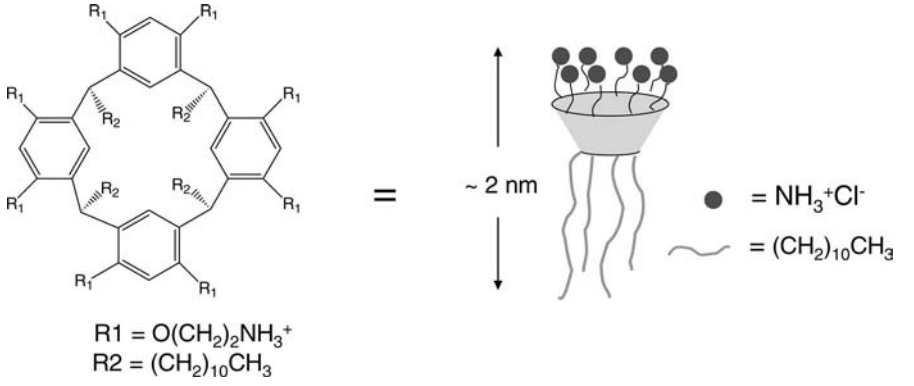

Figure 8 (*See color insert*) Cone-shaped structure of macrocyclic octaamine. *Source*: Adapted from Ref. 88.

octaamine as shown in Figure 8 has been proposed by Matsui et al. (88). The novel carrier has four long alkyl chains and eight amino groups on the opposite side of the calix[4]resorcarene macrocycle. What makes the macrocyclic octaamine different from other cationic lipids or polymers is that being a small and single molecule (molecular weight 1740), the compound unimolecularly presents a positive charge cluster motif with a well-defined geometry. Like amphiphilic micelle-forming polymers, the macrocyclic octaamine itself may form small micelle-like particles, with hydrophilic amino groups outside and lipophilic chain inside as illustrated in Figure 8. As a result, the cone-shaped macrocyclic octaamine formed complexes with siRNA in a compact size of approximately 10 nm. Although the in vitro delivery of macrocyclic octaamine/siRNA complex occurred with 90% to 95% knockdown of luciferase expression in HeLa, HepG2, and HEK293 cells at 48 hours, its in vivo performance remains to be investigated.

The KALA peptide (WEAK LAKA LAKA LAKH LAKA LAKA LKAC EA) is a well-known cationic, amphiphilic, and fusogenic peptide, which has been popularly studied as an endosomal escaping peptide complexing with various nucleic acids. However, it was reported that KALA/siRNA complexes did not show sufficient gene-silencing effect in the presence of serum proteins. In a recent study, two cysteine residuals were added to both terminals of KALA (89). The cysteine-KALA-cysteine peptide (CWEAK LAKA LAKA LAKH LAKA LAKA LKAC) self-cross-linked through reducible disulfide linkage. The cross-linked KALA (cl-KALA) formed more stable and compact complexes with siRNA. To further improve the colloidal stability, siRNA was modified with PEG. According to a previous report of the same group, direct PEG conjugation to siRNA could form more stable complexes than those by PEG-modified cationic polymers (71). Although cl-KALA/siRNA and cl-KALA/siRNA-PEG only showed 23.6% and 47% knockdown of GFP expression in MDA-MB-435-GFP cells at the N/P ratio of 64, the data showed their potential as a nano-based delivery system for siRNA.

Nanoparticles

In a broad sense, all particles in the nanoscale range are called nanoparticles. However, in this chapter, nanoparticles are differentiated from nanocomplexes by their more organized structures, that is, well-defined shell and core structures.

Huang et al. has developed a targeted nanoparticle formulation for siRNA systemic delivery to metastatic tumors overexpressing the sigma receptor (90,91). The core of the nanoparticle is a complex of siRNA, calf thymus DNA, and protamine, a highly positively charged peptide. The shell of the nanoparticle is a reorganized liposome structure consisting of DOTAP and cholesterol (1:1 molar ratio). Thus, the nanoparticle is referred to as "LPD," or liposome-polycation-DNA (Fig. 9). The nanoparticles are formed spontaneously by mixing the core complexes with preformed cationic liposomes. To create a sterically stabilized particle and for subsequent targeting, DSPE-PEG_{2000} (1,2-distearoyl-sn-glycerol-3-phosphoethanolamine-*N*-[methoxy(polyethylene $glycol)_{2000}$]) or DSPE-PEG-anisamide was postinserted into the preformed LPD. The in vitro results showed that the delivery efficiency of the targeted

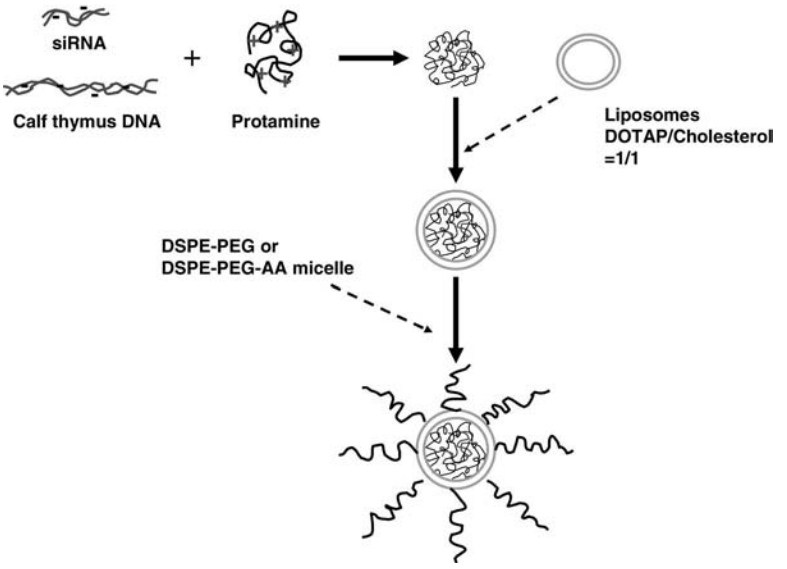

Figure 9 (*See color insert*) Preparation of PEGylated LPD. *Abbreviation*: LPD, liposome-polycation-DNA. *Source*: Adapted from Ref. 90.

nanoparticles was four- to seven-fold higher than the nontargeted nanoparticles. The in vivo tissue distribution results suggested that LPD surface-modified by PEG delivered a therapeutic dose to the tumor and avoided substantial accumulation in the liver with either targeted or untargeted LPD. These results suggest that the tumor accumulation of LPD with particle size around 100 nm is primarily due to the EPR (enhanced permeability and retention) effect as compared with targeting. After a single IV injection of 150 μg/kg anti-luciferase siRNA, 70% to 80% luciferase activity was silenced in a metastatic mouse tumor model. To avoid potential immunogenicity and inflammatory responses with calf thymus DNA, the calf thymus DNA has also been replaced with HA to produce LPH (liposome-polycation-hyaluronic acid) nanoparticles (92). The results showed that while the gene-silencing effect of LPH nanoparticles is comparable to LPD nanoparticles, the immunotoxicity of LPH is much lower.

A similar structure to LPH has also been reported by Peer et al. to develop leukocyte-directed nanoparticles to deliver anti-cyclin D1 siRNA (93). The preformed liposome is composed of phosphatidylcholine (PC), dipalmitoylphosphatidylethanolamine (DPPE), and cholesterol (Chol). High molecular weight hyaluronan (850 kDa), was attached to the outer surface of the liposomes by covalent linkage to DPPE to provide steric stabilization. The resulting nanoparticles were equipped with targeting function by covalently conjugating to the hyaluronan, a monoclonal antibody FIB504 against β7 integrins, which are highly expressed in gut mononuclear leukocytes. Anti-cyclin D1 siRNA loaded nanoparticles were formed by rehydrating lyophilized liposomes with water containing protamine-condensed siRNA. In an experimentally induced colitis mouse model, the β7 integrin–targeted nanoparticles knocked down the cyclin D1 expression to the normal level and ameliorated the colitis score.

An organic-inorganic hybrid nanoparticle was developed by Kakizawa et al. (94,95). The organic-inorganic shell-core structure is a core composed of nanocrystals of siRNA/CaP (calcium phosphate) complexes surrounded by a hydrophilic shell of a PEG-PAA block copolymer (polyethylene glycol-aspartic acid). Because of its potential biocompatibility, CaP is widely applied in various biomedical applications. Its binding affinity to a variety of molecules including proteins, nucleic acid, and small-molecule drugs makes it a potential controlled release material. However, one of the difficulties in using CaP to form nanoparticles is the relatively rapid crystallization rate of CaP. In the absence of other materials, the growth of siRNA/CaP complex crystals is rapid and precipitates are formed within minutes after mixing siRNA and CaP solutions. However, in the presence of a PEG-polycarboxylate block

copolymer, such as PEG-PAA, the rapid crystal growth is controlled or even prevented through the absorption of the PAA segment of PEG-PAA on the formed crystal surface. The resulting complex nanoparticles have diameters ranging from 100 to 300 nm depending on the PEG-PAA and CaP concentrations. Moreover, the CaP core dissociates in the intracellular environment with lower calcium concentration compared with the extracellular fluids, allowing the controlled release of siRNA from the core matrix. However, since the complex nanoparticles lack the ability to escape the endosomes, in vitro gene knockdown experiments are performed by pretreatment of the cells with chloroquine, a well-known adjuvant to provide endosomal escape. Although the in vitro luciferase expression was silenced by the siRNA/CaP/PEG-PAA nanoparticles to about 40% in 293 cells, the requirement of chloroquine makes this formulation less practical for siRNA delivery. To facilitate endosomal escape provided by the nanoparticle itself, PAA was replaced with polymethacrylic acid, another polyanion that undergoes a conformational change at pH 4 to 6 to expose a more hydrophobic structure which is able to interact with the endosomal membrane and disturb its structure. As a result, the luciferase activity was inhibited to 20% in 293 cells using as low of a siRNA concentration of 25 nM without the use of chloroquin.

Bartlett and Davis designed a modular-delivery vehicle that utilized an inclusion complex for targeted delivery of siRNA (96). The inclusion complex was comprised of siRNA and a synthesized cyclodextrin-containing polycation (CDP) that provided two functions. First, the polycation contains 2 mol of positive charge per CDP monomer, which complexes with negatively charged siRNA and self-assembles to nanoparticles. Second, the cyclodextrin motifs on the surface of the nanoparticle serve as a "loading dock" to incorporate PEGs and target ligands. PEG molecules containing adamantane (AD) on the proximal end and either methoxy (AD-PEG) or a targeting ligand such as transferrin (AD-PEG-Tf) on the distal end was mixed with CDP at a 1:1 AD-PEG/β-CD (mol/mol) ratio in water. AD-PEG or AD-PEG-Tf was attached to the polymer via inclusion complex formation between AD and the β-CD motifs on the polycation backbone. A calculation of the stoichiometry of each particle estimated that a 70-nm particle contained about 2000 siRNA molecules and around 100 AD-PEG-Tf molecules. Thus, each CDP nanoparticle could theoretically deliver a large payload of siRNA with a large ratio of siRNA to targeting ligand (20:1). The functional efficiency of CDP nanoparticles was demonstrated through knockdown of luciferase reporter protein expression. HeLa cells treated with CDP nanoparticles containing both pGL-3 plasmid DNA expressing firefly luciferase and siRNA against luciferase showed 50% lower expression of luciferase than cells that received either the plasmid alone or the plasmid plus control siRNA.

While most of the nanoparticle designs tend to entrap or hide siRNA in the nanoparticle core, thus providing siRNA protection against degradation, a few groups have attempted to adsorb siRNA on the surface of solid nanoparticles. For example, Kim et al. developed cationic solid lipid nanoparticles consisting of natural components of protein-free low-density lipoprotein (LDL) to deliver siRNA (97). LDLs are natural nanocarriers abundant in the bloodstream, transporting lipids, cholesterol, proteins, and hydrophobic drugs throughout systemic circulation. Solid lipid nanoparticles, mimicking natural LDL, have been shown to be very stable and behave similarly to native LDL when injected into the bloodstream. The solid lipid nanoparticles were composed of 45% (wt/wt) cholesteryl ester, 3% (wt/wt) triglyceride, 10% (wt/wt) cholesterol, 14% (wt/wt) DOPE, and 28% (wt/wt) 3β-[*N*-(*N*′, *N*′-dimethylamino-ethane)-carbamoyl]-cholesterol (DC-chol). The function of the cationic DC-chol was to make the surface of the nanoparticles positively charged with a zeta potential of about +40 mV. siRNA was conjugated to PEG via a disulfide linkage and anchored onto the surface of cationic solid lipid nanoparticles through charge interaction. Under an optimal weight ratio of DC-chol and siRNA-PEG conjugate, the LDL-like nanoparticles silenced the expression of GFP and VEGF to 40% and showed much less cytotoxicity than PEI 25 kDa in MDA-MB-435 cells. Although work with the LDP-like particles has only progressed to in vitro studies, it is expected that the LDL-like nanoparticle may be useful for in vivo tumor-targeting delivery of siRNA since elevated levels of low-density lipoprotein receptor (LDLR) are reported in various cancer cells such as myeloid leukemic cells, colon, kidney, and brain tumor cells.

Finally, like DOTAP liposomes, nanoparticles for nucleic acid delivery including siRNA are also patented and commercially available for the purpose of scientific research. Bioalliance

(Paris, France) patented a chitosan-coated polyisohexylcyanoacrylate (PIHCA) nanoparticle in 2004. The nanoparticle was directly utilized by Pille et al. to deliver anti-RhoA (Ras homologous A) siRNA in mice and to prove the therapeutic potential of the strategy to treat aggressive breast cancers (98).

Nanocapsules

Nanocapsules are functionally similar to nanoparticles except for having a liquid-filled core instead of a solid core. To date, there are just a few publications on the use of nanocapsules as siRNA delivery carriers. The following will discuss two such nanocapsules that have novel properties as potential siRNA delivery systems.

Ideally, to entrap siRNA in the internal core of a nanocapsule, the core should be aqueous to accommodate the hydrophilic siRNA. The preparation of nanocapsules usually involves the preparation of an emulsion. An oil-in-water emulsion is unable to encapsulate the hydrophilic siRNA alone. In addition, a water-in-oil emulsion leads to nanocapsules suspended in an oil phase, which may not be desirable for intravenous administration or would have to be removed prior to injection. To facilitate the formulation of siRNA in a nanocapsule, Toub et al. developed a nanocapsule with an aqueous core that also could suspended in an aqueous vehicle (99). A water-in-oil nanoemulsion was first prepared by adding an aqueous phase containing siRNA to an oil phase composed of Miglyol and Span 80. Then, isobutylcyanoacrylate (IBCA) monomer was added to the nanoemulsion under mechanical stirring. When IBCA polymerized, it formed a shell structure surrounding the aqueous core containing entrapped siRNA. Later, the oil phase and surfactant were removed by ultracentrifugation. The resulting pellet was resuspended in water to produce a nanosuspension with a particle size of 350 ± 100 nm. In vitro studies in NIH/3T3 cells stably transfected with human EWS-Fli1 gene showed that siRNA against EWS-Fli1 oncogene delivered in the nanocapsules inhibited the EWS-Fli1 mRNA level to 40%. When tested in vivo in xenograft mice bearing EWS-Fli1-expressing tumors, the nanocapsules were found to inhibit 80% of the tumor growth after intratumoral injection when compared with the saline treated control mice. This was the first study reporting on the use of aqueous core nanocapsules for the delivery of siRNA with resulting efficacy in vivo.

To facilitate endosomal escape and release siRNA to the cytosol where RNAi events take place, various endosomal escaping agents have been utilized, such as fusogenic lipids and peptides, polymers exerting proton-sponge effect, etc. A novel endosomal breaking formulation called thermosensitive hydrogel nanocapsules were developed by Lee et al. (100). The thermosensitive Pluronic F-127/PEI 2 kDa nanocapsules were synthesized by interfacial cross-linking reaction between preactivated Pluronic F-127 and low molecular weight PEI 2 kDa at the oil-in-water interface. The resulting Pluronic/PEI 2 kDa nanocapsules had an interior structure filled with aqueous fluid surrounded by a cross-linked Pluronic/PEI 2 kDa shell. Most pluronic copolymers have the critical micelle temperature (CMT) ranging from 25°C to 40°C. Above the CMT, the pluronic copolymers self-assemble to form a spherical micellar structure by dehydration of the poly-(propylene oxide) (PPO) moieties within the structure. The average particle size of Pluronic/PEI 2 kDa nanocapsules was 118.9 ± 15.3 nm at 37°C and 412.3 ± 83.2 at 15°C, respectively. According to the temperature-dependent property of pluronic, the collapse of the nanocapsules with increasing temperature is primarily caused by enhanced hydrophobic interactions between the PPO blocks in the Pluronic F-127 copolymers. PEG-conjugated siRNA was anchored to the surface of Pluronic/PEI 2 kDa nanocapsules through charge interaction. During in vitro cell transfection experiments, three hours after the cells treated with the nanoparticles at 37°C, 15 minutes of 15°C cold shock was given to the cells. The increased particle size under 15°C caused a 41.7-fold volume change, which disrupts the endosomal membrane by physical strength. With cold shock treatment, the expression of GFP in HeLa cells and VEGF in PC-3 cells was reduced to 37.3% and 3.2%, respectively.

Dendrimers

Polycationic dendrimers such as poly-(amidoamine) (PAMAM) dendrimers have long been used to deliver DNA. Recent studies have shown that PAMAM may also serve as siRNA delivery carriers (101). PAMAM dendrimers contain primary amine groups on the surface and

tertiary amine groups in the internal architecture. The primary amines bind siRNA, whereas the tertiary amines act as a proton-sponge and facilitate the endosomal release of siRNA into the cytoplasm. The siRNA-PAMAM complexes are very stable, which could only have been dissociated under very strong ionic strength conditions. PAMAM dendrimers are termed as G*n* with *n* denoting dendrimer generation number. As the generation number increases, the number of terminal amines increases. Thus, similar to DNA-PAMAM affinities, an increase in PAMAM generation leads to stronger interactions between the dendrimer and the siRNA. Zhou et al. showed that GL3Luc siRNA-G_7 complex reduced the expression of luciferase to 20% in A549Luc cells in vitro (102). To lower the cytotoxicity of G_7 PAMAM dendrimers while maintaining the siRNA binding affinity, surface PAMAM-NH_2 was acetylated with acetic anhydride and internal PAMAM-OH was quaternized with methyl iodide (103). Both modifications generate neutral outer surface with internal positive charges. It was found that the modifications did not interfere with the binding ability but significantly decrease the cytotoxicity of G_7 PAMAM dendrimers. An effort was also made to further increase the cellular uptake of siRNA-PAMAM complex by conjugating the cell-penetrating peptide, Tat; however, the conjugation of Tat did not improve the efficiency of the dendrimer (104).

The terminal groups of G_3 PAMAM dendrimer have been partially conjugated with α-cyclodextrin (α-CDE) to deliver siRNA (105). CDE, at high concentration, disturbs the cellular membrane components such as phospholipids and cholesterol, leading to increased membrane permeability. Moreover, the α-CDE has low cytotoxicity even at high charge ratio of α-CDE/nucleic acid. Thus, the G_3 PAMAM dendrimer/α-CDE conjugate was developed to reduce the cytotoxicity and increase the delivery efficiency for nucleic acids. A pilot study showed that siRNA against pGL3 luciferase delivered by G_3 PAMAM dendrimer/α-CDE conjugate suppressed the luciferase gene expression level in vitro by about 50% in NIH3T3-luc cells.

Dendritic poly(L-lysine) generation 6 (KG_6) was used to deliver several siRNAs by Inoue et al. (106). KG_6 was used in combination with the amphiphilic weak-base peptide Endo-Porter (EP), which is a commercially available cellular delivery reagent available from Gene Tools. Neither KG_6 nor EP could efficiently deliver glyceraldehyde 3-phosphate dehydrogenase (GAPDH) siRNA when KG_6 or EP was used alone. However, when KG_6 and EP were used together, GADPH was efficiently knocked down both protein levels and mRNA levels in H4IIEC3 cells.

Other Novel Carriers

In addition to the traditional or conventional siRNA delivery carriers discussed above, there are several highly innovative new strategies that are being developed and tested as potential delivery systems for siRNA.

Quantum dots (QDs) are nanoscaled semiconductor inorganic materials that have provided greatly enhanced capabilities for medical imaging and diagnostics. Yezhelyev et al. developed a class of dual-functional nanoparticle for both siRNA delivery and imaging based on the use of QDs (107). Highly luminescent QDs were first synthesized and encapsulated in the poly-(maleic anhydride-alt-1-tetradecene) bearing surface carboxylic acid groups. The carboxylic acid groups were then partially converted to tertiary amines (Fig. 10). It was found that by balancing the ratio of the carboxylic acid and tertiary amine moieties, the proton-sponge effect could be precisely controlled. The resulting polymer-coated QDs were suitable

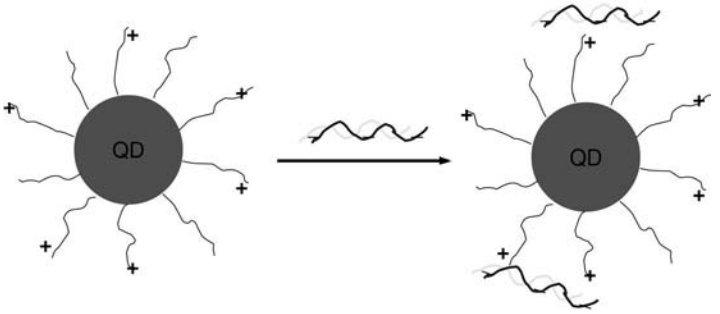

Figure 10 (*See color insert*) Adsorption of siRNA onto surface-modified QDs. *Abbreviations*: siRNA, small interfering RNA; QDs, quantum dots.

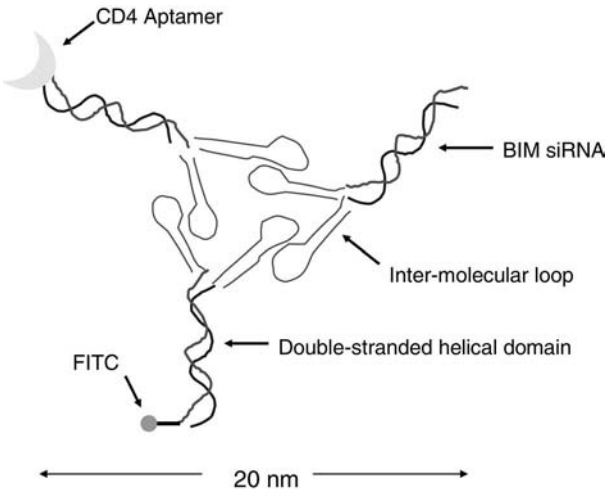

Figure 11 (*See color insert*) Schematic structure of engineering pRNA nanoparticle containing siRNA, aptamer, and fluorescent label. *Abbreviations*: pRNA, packing RNA; siRNA, small interfering RNA.

for siRNA binding, penetrating the cell, and for providing a mechanism for endosomal escape. In comparison to cationic lipids and polymer-based siRNA delivery systems, the QD-based nanoparticles have much smaller size and more uniform size distribution. A QD core size of 6 nm yielded polymer-coated dots with sizes of 13 and 17 nm before and after siRNA binding, respectively. The QD nanoparticles efficiently delivered siRNA against cyclophilin B in a human breast cancer cell line and led to nearly complete suppression of cyclophilin B expression, which was superior to three most commonly used transfection reagents (Lipofectamine^{TM}, TransIT-TKO^{TM}, and JetPEI^{TM}). Another advantage of the QD-siRNA particles is that they afford simultaneous delivery with imaging allowing for real-time tracking and intracellular localization of QDs during delivery.

Khaled et al. have also engineered protein- and lipid-free multifunctional RNA nanoparticles to deliver siRNA and combine targeted therapy and imaging in a natural modality, pRNA (packing RNA), by utilizing RNA nanotechnology (108). pRNA is a vital component of molecular motor, which uses ATP as energy to package DNA into the procapsid during the replication of linear dsDNA viruses. The 117-nucleotide pRNA monomer contains two functional domains: the intermolecular-interacting domain and the double-stranded helical DNA packaging domain. The intermolecular-interacting domain contains left and right loops like two arms that interlock with other pRNA monomers via base-pairing to form dimer, trimer, or hexamers of size 10 to 30 nm. Figure 11 shows the structure of a pRNA trimer. According to their study, the replacement of pRNA helical region with siRNA, or connection of the RNA aptamer, or connection of other chemical components did not interfere either with the folding and trimering of the pRNA, as long as the two strands were paired, or with the function of siRNA and other connected moieties. Therefore, they tried to replace the helical region with small RNA fragments and connect RNA aptamer or other chemical components to this region to engineer a variety of chimeric pRNAs. The pRNA trimers with the size of about 20 nm are extremely compact and versatile nanoparticles with lots of advantages. For example, as shown in Figure 11, a trimeric complex composed of pRNA/aptamer (CD4), pRNA/siRNA (BIM), and pRNA/fluorescein isothiocyanate (FITC) could target CD4 positive cells and simultaneously deliver siRNA against proapoptosis factor Bcl-2 interacting mediator of cell death (BIM) and imaging molecule FITC to these cells. In addition, more than one siRNA could be constructed to the pRNA nanoparticles to inhibit the expression of multiple oncogenes. RNA aptamers, compared with antibodies and phage-displaying peptides, have very low immunogenicity. Furthermore, the size, shape, stoichiometry, and the functions of the final product are highly controllable.

FUTURE PERSPECTIVE

The promise of siRNA applications as a powerful therapeutic agent relies on a successful delivery vehicle. In this chapter, a series of criteria of an "ideal" nano-based siRNA delivery system were addressed and can be summarized as efficient, specific, and safe. It is obvious that

a great deal of effort has been devoted to pursuing the ideal nano-based systems for siRNA delivery, and the field is developing rapidly. However, all current-reported formulations have recognizable gaps.

Delivery efficiency depends on many factors. First, the structures of carrier materials are critical. Currently, although there are some general rules to design and synthesize siRNA complexing agents (e.g., the presence of positive charges), the investigation of structure-efficiency relationship is still under a trial-and-error mode. In the future, when a large amount of compounds have been studied, databases could be built, and thus computer simulation and modeling would be performed to rationally design the delivery agent and to predict the binding and assembly with siRNA.

Particle size is another factor controlling the in vivo efficiency of siRNA nanoparticles. Nanoparticles with a broad range of particle size (from 20 nm up to about 800 nm) have been reported in the literature to deliver siRNA in vitro. However, since most of the studies have stopped at in vitro experiments, the in vivo efficacy of the siRNA nanoparticles remains a question. What is not fully understood yet is how the particle size and surface properties minimize systemic clearance and optimize target tissue penetration, and cellular uptake.

In addition to particle size and size distribution, other properties such as shape, mechanical properties, and surface texture and morphology are also important factors affecting siRNA delivery efficiency of nanoparticles both in vitro and in vivo. While chemical modifications of carrier materials are the major strategy to increase the efficiency of siRNA delivery nowadays, the influence of physical properties of the nanoformulation has been underestimated. Together with particle size, these physical properties and their influence on the nanoparticle behavior in circulation, tissue distribution, cell penetration, and cellular trafficking require more attention.

The specificity of siRNA delivery primarily depends on the selection of a target and ligand, both of which would benefit from progress and advances in other fields. The advances in molecular biology would help find more specifically expressed targets such as receptors, integrins, or enzymes in pathological tissues as well as more specific and high-affinity ligand via, for example, in vivo phage display.

Years ago, the incorporation of PEG in various nanoformulations dramatically decreased their nonspecific RES clearance and increased their circulation half-life. As the nonspecific RES clearance decreases, the accumulation of nanoparticles in target organ or tissue increases. Hence, to increase the delivery specificity, active targeting using a targeting ligand is preferred; however, improved delivery by passive targeting may also have therapeutic potential and utility.

In terms of the safety for nano-based siRNA delivery systems, on one hand, efforts need to be made to further decrease the cytotoxicity of carrier materials and to look for less immunogenic targeting ligands. On the other hand, the toxicity of different formulations is mostly identified and/or estimated by in vitro experiments. However, cytotoxicity is often cell-type dependent. Thus, the field also is in need of improved, predictive, in vitro models to more accurately reflect the in vivo environment.

There is no doubt that delivering siRNA safely and efficiently is a challenging task. The field is in need of a breakthrough.

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7 Excipients for parenteral dosage forms: regulatory considerations and controls

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INTRODUCTION

A survey of commercial parenteral products confirms an interesting observation—the active drug molecule typically comprises only a small percentage of the drug product formulation whereas the excipients make up the primary components. Excipients provide the enhanced vehicle for the active pharmaceutical ingredient (API) and are typically referred to as inactive or inert ingredients, where "inactive" or "inert" indicates the compound does not directly contribute to the intended therapeutic or diagnostic activity of the drug product. Pharmaceutical excipients or additives are compounds added to the finished drug product with a specific functional role [other than that defined for API or in case of biologics, drug substance (DS)]. These functions include increasing the bulk to aid in manufacturing, stabilizing of the active drug, improving delivery and targeting, and modifying the safety or pharmacokinetic profile of the active drug. Compounds considered excipients also encompass ingredients that are used for the production of dosage forms, but may or may not be present in the finished dosage forms. Examples are water for lyophilized product and inert gases in the head space of container (1). It is clear that excipients have a "functionality" in the dosage form synonymous to the pharmacological activity of API/DS, which is currently being acknowledged by various pharmacopoeias (2). It is noted, however, that many of the existing excipient monographs do not address this functionality aspect and its control.

With the current Quality-by-Design (QbD) initiative, pharmaceutical companies are achieving a better understanding of how the functionality and performance of excipients can influence the drug product (3). Concurrently, steps are being initiated to rectify the failure of existing pharmacopoeial excipient monographs to directly address excipient functionality. These activities include the proposal of an USP (United States Pharmacopoeia) general chapter on functionality of excipients and Ph. Eur. (European Pharmacopoeia) nonmandatory Functionality-Related Characteristics section in the monographs of some excipients. The QbD approach will also provide better insight into the potential impact of excipient variability on product quality. Design space studies of excipient variability and functional performance will provide a higher level of assurance that excipient standards accurately reflect excipient quality (4).

While the functional role of the inert or inactive excipient does not include the therapeutic or diagnostic activity of the active drug ingredient, it may have some level of pharmacological activity. Therefore, restrictions have been placed on the type or amount of excipient that can be included in the formulation of parenteral drug products because of safety issues. For example, Japan, the United States, and the European Union (EU) prohibit or discourage the use of amino mercuric chloride or thimerosal, yet these excipients are still widely used in several products in rest of the world.

A typical definition of parenteral is *not oral or not through the alimentary canal*. As defined in the Ph. Eur. and the British Pharmacopoeia (BP), "Parenteral preparations are sterile preparations intended for administration by injection, infusion, or implantation into the human or animal body"; however, for the purposes of this chapter, only sterile preparations for administration by injection or infusion into the human body will be surveyed (5,6). Injectable formulations are subject to a strict set of requirements. The formulated product has to be sterile, pyrogen-free, and in the case of solution, free of particulate matter. Coloring agents added solely for the purpose of coloring the parenteral preparation are not allowed. An isotonic formulation is preferred, and depending on the route of administration, some excipients may be prohibited. Certain drugs administered by injection, rather than orally, may pose a higher risk for an adverse event or the drug's effect may be especially difficult to reverse

because the injected drug bypasses natural defense barriers and is quickly distributed throughout the body. The excipient must be able to withstand the rigors of the sterilization process such as the very high temperatures required for terminal steam sterilization, or filtration and lyophilization in aseptic processing. All of the above factors can limit the choice of excipients available to the formulator.

When choosing acceptable excipients for a parenteral formulation, a formulator should first look for those excipients already used in approved parenteral drug products and/or designated for parenteral use in regulatory listings such as the FDA's inactive ingredient database. Using these excipients provides increased assurance to the formulator that they will probably be safe for their new drug product. However, it should be understood that as excipients are combined with other additives and/or with a new drug molecule, unforeseen potentiation or synergistic toxic effects could result. Utilizing excipients previously approved in an injectable product will often ease the regulatory scrutiny and may require less safety data. In contrast, a new additive will certainly require additional studies adding to the cost, time, and risk to product development. It is important to note that inclusion of an excipient in the GRAS (Generally Recognized as Safe) list or pharmacopoeia such as the USP-NF (United States Pharmacopoeia–National Formulary) does not mean that the excipient has been deemed safe by the regulatory agencies for use in parenteral products.

In Japan, if the drug product contains an excipient with no precedence of use in Japan, then the quality and safety attributes of the excipient must be evaluated by the Subcommittee on Pharmaceutical Excipients of the Central Pharmaceutical Affairs Council concurrently with the evaluation of the drug product application (7). Precedence of use means that the excipient has been used in a drug product in Japan, which is administered via the same route and in a dose level equal to or greater than the excipient in question in the new application.

This chapter offers a comprehensive review of excipients that have been included in injectable products marketed in the United States, Europe, and Japan. A review of the literature indicates that only limited articles have been published which specifically deal with the selection of parenteral excipients (8–17). However, excipients included in other sterile dosage forms not administered parenterally, such as solution for irrigation, ophthalmic or otic drops, and ointments, are not covered in this chapter.

Several sources of information were used to summarize the information in this chapter (9–22). The tables are categorized on the basis of the excipient's primary function in the formulation. For example, ascorbates are categorized as antioxidants, although they can also serve as buffers. This classification system minimizes redundancy and results in a simplified format. Excipient concentration is expressed as percent weight by volume (wt/vol) or volume by volume (vol/vol). For lyophilized or powder products, the percentages were calculated on the basis of the most commonly used reconstitution volume.

TYPES OF EXCIPIENTS

Solvents and Cosolvents

Table 1 list solvents and cosolvents used in parenteral products. Water for injection (WFI) is the most common solvent that can be produced by a variety of technologies. The Ph. Eur. recognizes WFI produced by the distillation process only, even though WFI produced by reverse osmosis will meet all the specifications (23,24). WFI may be combined or substituted with a cosolvent to improve the solubility or stability of drugs (25,26). The dielectric constant and solubility parameters are among the most common polarity indices used for solvent blending (27,28). For more than 50% of parenteral cosolvent systems, ethanol and propylene glycol are used either alone or in combination with other solvents. Interestingly, propylene glycol is used more often than polyethylene glycols (PEGs) in spite of its higher myotoxicity and hemolyzing effects (29–32). A review of toxicity for commonly used parenteral cosolvents is summarized in an article by Mottu et al. (33). The hemolytic potential of cosolvents is as follows (31):

Dimethyl acetamide < PEG 400 < ethanol < propylene glycol < dimethylsulfoxide

Degradation of the drug in the cosolvent system may result due to the possible presence of residual peroxide from the bleaching of PEG or the generation of peroxides in PEG. Hence, it is important to use unbleached and/or low-peroxide PEGs in the formulation.

Table 1 Solvents and Cosolvents

Excipient	Frequency	Range	Example
Benzyl benzoate	3	20–44.7% wt/vol	Delestrogen® 40 mg/mL (Bristol Myers) 44.7% wt/vol
Castor oil	2	11.50%	Delestrogen 40 mg/mL (Bristol Myers)
Cottonseed oil	1	73.6–87.4% wt/vol	Depo® Testosterone (Pfizer) 73.6% wt/vol
N,N dimethylacetamide	2	6–33% wt/vol	Busulfex® (Orphan Medical) 33%
Ethanol/ethanol dehydrated	34	0.6–100%	Prograf® (Fujisawa) 80% vol/vol, Alprostadil (Bedford Lab) 100%
Glycerin (glycerol)	17	1.6–70% wt/vol	Multitest CMI® (Pasteur Merieux) 70% wt/vol
N-methyl-2-pyrrolidone	1	[a]	Eligrad 7.5 mg (Sanofi)
Peanut oil	1	[a]	Bal in Oil® (Becton Dickinson)
Polyethylene glycol			
PEG[b]	5	0.15–50%	Secobarbital sodium (Wyeth-Ayerst) 50%
PEG 300	4	50–65%	VePesid® (Bristol Myers) 65% wt/vol
PEG 400	4	11.2–67% vol/vol	Busulfex® (Orphan Medical) 67%
PEG 600	1	5% wt/vol	Persantine® (Dupont-Merck)
PEG 3350	4	0.3–3%	Depo-Medrol® (Upjohn) 2.95% wt/vol
PEG 4000	1	0.3–3%	Invega Sustenna® (Janssen)
Poppyseed oil	1	[a]	Ethiodol® (Savage)
Propylene glycol	32	0.0025–80%	Ativan® (Wyeth-Ayerst) 80%
Safflower oil	2	5–10%	Liposyn II® (Abbott) 10%
Sesame oil	7	100%	Solganal Inj.® (Schering)
Soybean oil	1	10% wt/vol	Diprivan Inj. (Zeneca)
Vegetable oil	2	[a]	Virilon IM Inj.® (Star Pharmaceuticals)

[a]Not applicable or no data available.
[b]PEG molecular weight not specified.

Oils such as cottonseed, castor, safflower, and soybean have additional specifications if they are used in parenterals. These specifications include saponification value, iodine number, test for unsaponifiable matter, test for free fatty acid, solid paraffin test at 10°C, and acid value. Oils are used to dissolve drugs with low aqueous solubility and provide a mechanism to slowly release drug over a long period of time. In total parenteral nutrition (TPN) products, oils serve as a fat source and as carriers for fat-soluble vitamins. Two important concerns when using fixed oils in injectable products are (*i*) oil degradation, which leads to rancidity and production of free fatty acids, and (*ii*) presence of mineral oil or paraffin that the body cannot metabolize.

Polymeric and Surface Active Compounds

Table 2 includes a broad category of excipients whose functions include the following:

1. Viscosity enhancing and suspending agents such as carboxymethylcellulose, sodium carboxymethylcellulose, acacia, Povidone, hydrolyzed gelatin, sorbitol
2. Solubilizing, wetting, or emulsifying agents such as Cremophore EL, sodium desoxycholate, Polysorbate 20 or 80, PEG 40 castor oil, PEG 60 castor oil, sodium dodecyl sulfate, lecithin, or egg yolk phospholipid
3. Gelling agent such as aluminum monostearate that is added to fixed oil to form a viscous or gel-like suspension medium.
4. Complexing agents such as cyclodextrins

Polysorbate 80 is the most common and versatile solubilizing, wetting, and emulsifying agent. Again, the level of residual peroxides present in polysorbates and protecting them from light and air to prevent further oxidation is an important concern (34**)**. Polysorbate 80 is a polyoxyethylene sorbitan ester of oleic acid (unsaturated fatty acid) while Polysorbate 20 is a polyoxyethylene sorbitan ester of lauric acid (saturated fatty acid). Thus, stability differences in the drug product formulated with Polysorbate 80 versus Polysorbate 20 may occur in some

Table 2 Solubilizing, Wetting, Suspending, Emulsifying, or Thickening Agents

Excipient	Frequency	Range	Example
Acacia	2	7%	Tuberculin Old Test® (Lederle) 7%
Aluminum monostearate	1	2%	Solganal Inj.® (Schering) 2%
Carboxymethylcellulose	4	0.50–0.55%	Bicillin® (Wyeth-Ayerst) 0.55%
Carboxymethylcellulose, sodium (Croscarmellose sodium)	21	0.15–3.0%	Nutropin Depot® (Genentech) 3%
Cremophor EL[a]	3	50–65% wt/vol	Sandimmune® (Sandoz) 65% wt/vol
Cyclodextrin-γ	1	5.0%	Cardiotec (BMS)
Cyclodextrin-α	1	0.14%	Edex (Schwartz)
Hydroxypropyl-β-cyclodextrin	2	16–40%	Sporanox (Janssen)
Sulfobutylether cyclodextrin sodium	3	15–29.4%	Geodon (Pfizer)
Desoxycholate sodium	1	0.4% wt/vol	Fungizone® (Bristol Myers) 0.41% wt/vol
Egg yolk phospholipid	3	1.2%	Cleviprex® (The Medicines Co.)
Gelatin, hydrolzyed	1	16% wt/vol	Cortone® (Merck) 16% wt/vol
Lecithin	8	0.4–1.2% wt/vol	Diprivan® (Zeneca) 1.2% wt/vol
Polyoxyethylated fatty acid	1	7% wt/vol	AquaMephyton® (Merck) 7% wt/vol
Polysorbate 80 (Tween 80)	72	0.001–100%	Taxotere® (Aventis) 100%
Polysorbate 20 (Tween 20)	22	0.001–0.4%	Calcijex® (Abbott) 0.4% wt/vol
PEG 40 castor oil[b]	1	11.5% vol/vol	Monistat® (Janssen) 11.5% vol/vol
PEG 60 castor oil[c]	1	20% wt/vol	Prograf® (Fujisawa) 20% wt/vol
Poloxamer-188 (Pluronic F68)	5	0.005–0.3%	Norditropin (NovoNordisk) 0.3%
Povidone (Polyvinyl pyrrolidone, Crospovidone)	7	0.5–0.6% wt/vol	Bicillin® (Wyeth-Ayerst) 0.6% wt/vol
Sodium dodecyl sulfate (sodium lauryl sulfate)	1	0.018% wt/vol	Proleukin® (Cetus) 0.018% wt/vol
Sorbitol	3	25–50%	Aristrospan® (Fujisawa) 50% vol/vol
Triton X-100 (Octoxynol-9)	1	0.0085% wt/vol	Fluarix® (GSK)

[a]Cremophor EL, Etocas 35, polyethoxylated castor oil, polyoxyethylene 35 castor oil.
[b]PEG 40 castor oil, polyoxyl 40 castor oil, castor oil POE-40, Croduret 40, polyoxyethylene 40 castor oil, Protachem CA-40.
[c]PEG 60 hydrogenated castor oil, Cremophor RH 60, hydrogenated castor oil POE-60, Protachem CAH-60.

cases. This has been noted with Neupogen®, which when exposed to a high concentration of Polysorbate 20 exhibited substantially less oxidation than when exposed to similar concentration of Polysorbate 80 (35). In many other formulation studies with proteins no such advantage of polysorbate could be confirmed as the stability is molecule dependent.

Several new excipients, such as cyclodextrins, are being evaluated to increase the solubility or improve the stability of parenteral drugs. Currently, there are two FDA-approved parenteral products that utilize α- and γ-cyclodextrins. β-Cyclodextrin is unsuitable for parenteral administration because it causes necrosis of the proximal kidney tubules upon IV and SC administration (36). Hydroxypropyl-β-cyclodextrin (HPβCD) and sulfobutylether-β-cyclodextrin (SBE-7-β-CD) have shown the most promise. Captisol™ is the trade name of SBE-7-β-CD and is anionic. Currently, three Captisol-based drug formulations have been approved in the United States. One parenteral formulation is in phase II/III clinical trial that utilizes HPβCD (Cavitron®) and another (Sporanox) has been approved by the FDA. Manufacturers of HPβCD and SBE-7-β-CD have established a Drug Master File (DMF) with the FDA. A detailed review of cyclodextrins has been recently published (37,38). A caution when using cyclodextrin is it can accelerate drug product degradation (39) and can sequester preservatives rendering them ineffective (40).

Chelating Agents

Table 3 lists the relatively few chelating agents that are used in parenteral products. These agents complex heavy metals allowing for improved efficacy of antioxidants or preservatives. Citric acid, tartaric acid, and some amino acids can also act as chelating agents. There has been some misunderstanding that ethylenediaminetetraacetic acid (EDTA) (as calcium or sodium salt) has

Table 3 Chelating Agents

Excipient	Frequency	Range	Example
Calcium disodium EDTA	11	0.01–0.1%	Wydase® (Wyeth-Ayerst) 0.1% wt/vol
Disodium EDTA	48	0.01–0.11%	Calcijex® (Abbott) 0.11% wt/vol
Sodium EDTA	1	0.20%	Folvite® (Lederle) 0.20%
Calcium versetamide sodium	1	2.84% wt/vol	OptiMARK® (Mallinckrodt)
Calteridol	1	0.023% wt/vol	Prohance® (Bracco Diagnostics, Inc.)
DTPA	3	0.04–1.2%	Omniscan™ (GE Healthcare) 1.2%

Abbreviations: EDTA, ethylenediaminetetraacetic acid; DTPA, diethylenetriaminepentaacetic acid, pentetic acid.

not been used in an approved injectable product in Japan. There are some drug products that contain calcium disodium EDTA on market currently in Japan, and this excipient is also listed as an official excipient in Japan (refer to sect. "Special Additives" for details). One possible advantage calcium EDTA has over the tetrasodium salt is that it does not contribute sodium and does not chelate calcium from the blood.

Complexing agents should not be used in metalloprotein formulations, where the protein subunits are held by the metal (41). EDTA, in rare instances, can increase the oxidation rate due to binding of EDTA-metal complex to protein, resulting in site-specific generation of radicals (42).

Antioxidants

Antioxidants are used to prevent the oxidation of active substances and excipients in the finished product and may be categorized into three groups:

1. True antioxidants: They react with free radicals via a chain termination mechanism for example, butylated hydroxytoluene.
2. Reducing agents: They have a lower redox potential than the drug and get preferentially oxidized, for example, ascorbic acid. Thus, they can be consumed during the shelf life of the product.
3. Antioxidant synergists: These enhance the effect of antioxidants, for example, EDTA.

Table 4 summarizes the antioxidants, their frequency of use, concentration range, and examples of product containing them. Sulfite, bisulfite, and metabisulfite constitute the majority of antioxidants used in parenteral products despite several reports of incompatibility and toxicity (43,44). Butylated hydroxy anisole, butylated hydroxy toluene, α-tocopherol, and propyl gallate are primarily used in semi-/nonaqueous vehicles because of their low aqueous solubility (45). Ascorbic acid/sodium ascorbate may serve as an antioxidant, a buffer, and a chelating agent in the same formulation. Some amino acids such as methionine and cysteine also function as effective antioxidants.

The Committee for Proprietary Medicinal Products (CPMP) guideline calls for a full explanation and justification for including antioxidants in the formulation (46,47). Specific evidence must be provided that their use cannot be avoided and its concentration must be justified in terms of efficacy and safety. Thus, it is imperative to first try an inert gas such as nitrogen or argon in the head space to prevent oxidation. Antioxidants such as sulfites and metabisulfites are especially undesirable.

Some antioxidants such as propyl gallate and butylated hydroxy anisole possess antimicrobial properties. Compatibility of antioxidants with the drug, packaging system, and the body should be studied carefully. For example, tocopherols may be absorbed on to plastics; ascorbic acid is incompatible with alkalis and oxidizing materials such as phenylephrine; propyl gallate forms complexes with metal ions such as sodium, potassium, and iron.

Preservatives

Benzyl alcohol is the most common antimicrobial preservative present in parenteral formulations (Table 5). This observation is consistent with other surveys (12,48). Parabens are the second most common preservatives. Surprisingly, thimerosal is also common,

Table 4 Antioxidants and Reducing Agents

Excipient	Frequency	Range	Example
Acetone sodium bisulfite	4	0.2–0.4% wt/vol	Novocaine® (Sanofi-Winthrop) 0.4% wt/vol
Argon	–	100%	Used to fill headspace of lyophilized or liquid products. TechneScan MAG3® (Covidien)
Ascorbyl palmitate	1		Visudyne® (QLT)
Ascorbate (sodium/acid)	10	0.1–4.8% wt/vol	Vibramycin® (Pfizer) 4.8% wt/vol
Bisulfite sodium	31	0.02–0.66% wt/vol	Amikin® (Bristol Myers) 0.66% wt/vol
Butylated hydroxy anisole (BHA)	3	0.00028–0.03% wt/vol	Aquasol A® (Astra) 0.03% wt/vol
Butylated hydroxy toluene (BHT)	4	0.00116–0.03% wt/vol	Aquasol A (Astra) 0.03% wt/vol
Cystein/Cysteinate HCl	4	0.07–1.3% wt/vol	Acthrel® (Ferring) 1.3% wt/vol
Dithionite sodium (sodium hydrosulfite, sodium sulfoxylate)	1	0.10%	Numorphan® (Endo Lab) 0.10%
Gentisic acid	1	0.02% wt/vol	OctreoScan® (Mallinckrodt) 0.02% wt/vol
Gentisic acid ethanolamine	1	2%	M.V.I. 12® (Astra) 2%
Glutamate monosodium	1	0.1% wt/vol	Varivax® (Merck) 0.1% wt/vol
Glutathione	1	0.01% wt/vol	Advate® (Baxter) 0.01% wt/vol
Formaldehyde sulfoxylate sodium	10	0.075–0.5% wt/vol	Terramycin solution (Pfizer) 0.5% wt/vol
Metabisulfite potassium	1	0.10%	Vasoxyl® (Glaxo-Wellcome) 0.10%
Metabisulfite sodium	33	0.02–1% wt/vol	Intropin® (DuPont) 1% wt/vol
Methionine	5	0.01–0.15%	Depo-subQ provera 104 (Upjohn)
Monothioglycerol (thioglycerol)	8	0.1–1%	Terramycin solution (Pfizer) 1%
Nitrogen	–	100%	Used to fill headspace of lyophilized or liquid products
Propyl gallate	3	0.02%	Navane® (Pfizer) 0.02%
Sulfite sodium	8	0.05–0.2% wt/vol	Enlon® (Ohmeda) 0.2% wt/vol
α-Tocopherol	2	0.005–0.075%	Torisel (Wyetth) 0.075%
α-Tocopherol hydrogen succinate	1	0.02% wt/vol	Fluarix® (GSK) 0.02% wt/vol
Thioglycolate sodium	1	0.66% wt/vol	Sus-Phrine® (Forest) 0.66% wt/vol

Table 5 Antimicrobial Preservatives

Excipient	Frequency	Range	Example
Benzalkonium chloride	1	0.02% wt/vol	Celestone Soluspan® (Schering) 0.02% wt/vol
Benzethonium chloride	4	0.01%	Benadryl® (Parke-Davis) 0.01% wt/vol
Benzyl alcohol	90	0.75–5%	Dimenhydrinate Inj., USP (APP Pharmaceuticals) 5%
Chlorbutanol	19	0.25–0.5%	Codine phosphate (Wyeth-Ayerst) 0.5%
m-Cresol	13	0.1–0.315%	Humalog® (Lilly) 0.315%
Myristyl γ-picolinium chloride	2	0.0195–0.169% wt/vol	Depo-Provera® (Pharmacia-Upjohn) 0.169% wt/vol
Paraben methyl	55	0.05–0.18%	Inapsine® (Janssen) 0.18% wt/vol
Paraben propyl	45	0.005–0.1%	Xylocaine w/epinephrine (Astra) 0.1% wt/vol
Phenol	55	0.15–0.5%	Calcimar® (Rhone-Poulanc) 0.5% wt/vol
2-Phenoxyethanol	4	0.50%	Havrix® (SmithKline Beecham) 0.50% wt/vol
Phenyl mercuric nitrate	3	0.001%	Antivenin® (Wyeth-Ayerst) 0.001%
Thimerosal	50	0.003–0.012%	Atgam® (Pharmacia-Upjohn) 0.01%

especially in vaccines, even though some individuals are sensitive to mercurics. Several preservatives can volatilize easily (e.g., benzyl alcohol and phenol) and therefore should not be used in a lyophilized dosage form. Chlorocresol is purported to be a good preservative for parenterals, but our survey did not find any examples of commercial products containing chlorocresol. The British Pharmaceutical Codex and Martindale list chlorocresol to be used as a

Table 6 Maximum Permissible Amount of Preservatives and Antioxidants

Excipient	Maximum limit in USP (%)
Mercurial compounds	0.01
Cationic surfactants	0.01
Chlorobutanol	0.50
Cresol	0.50
Phenol	0.50
Sulfur dioxide or an equivalent amount of the sulfite, bisulfite, or metabisulfite of potassium or sodium	0.20

preservative in multidose aqueous injections at concentration of 0.1% but no examples of injectable products have been provided (49,50).

Antimicrobial preservatives are allowed in multidose injections to prevent growth of microorganisms that may accidentally enter the container during withdrawal of the dose. In the United States, preservatives are discouraged from being used in single-dose injections, while Ph. Eur. and BP allow aqueous preparations that are manufactured using aseptic techniques to contain suitable preservatives. However, Ph. Eur. and BP prohibit antimicrobials from single-dose injections where the dose volume is greater than 15 mL or if the drug product is to be injected via intracisternal or any route (e.g., retro-ocular) that gives access to the cerebrospinal fluid (CSF). It is imperative that preservatives should never be used as a substitute for inadequate cGMP (current Good Manufacturing Practices). The primary reason for minimizing the use of antimicrobial preservatives is toxicity. For example, many individuals are allergic to mercury preservatives, and benzyl alcohol is contraindicated in children under the age of two years. The USP has also placed some restrictions on the maximum concentration of preservatives allowed in the formulation to address toxicity and allergic reactions (Table 6). The World Health Organization has set an estimated total acceptable daily intake for sorbate (as acid, calcium, potassium, and sodium salts) as not more than 25 mg/kg body weight. Recently, concerns have been raised on the safety of parabens in pediatric formulations based primarily on reports by one Japanese laboratory between 2001 and 2004, which indicated effects on reproductive apparatus of juvenile male rats given propyl (51) or butyl paraben (52), but lack of effects for methyl and ethyl parabens (53). However, toxicological data suggests otherwise (54). Until a comprehensive assessment is done, formulators should take into account current view of the regulatory agencies (e.g., Agence Francaise de Securite Sanitaire des Produits de Sante (AFSSAPS), Scandinavian, etc.) and may opt for other preservative options for pediatric products.

Preservative efficacy is assessed during product development using Antimicrobial Preservative Effectiveness Testing, PET (55–57). Satisfactory PET results on finished aqueous-preserved parenteral product in the commercial package can be used up to a maximum of 28 days after the container has been opened (58). Unpreserved product should preferably be used immediately following opening, reconstitution, or dilution.

Similar to antioxidants, addition of preservatives in medicinal products requires justification. Wherever possible, their use should be avoided, particularly for pediatric products, but if required, minimal concentrations should be determined (47).

Buffers

Buffers are added to a formulation to adjust the pH to optimize solubility and stability. For parenteral preparations, it is desirable to target the pH of the product to physiological pH. Consideration of the buffer concentration (ionic strength) and the buffer species is important. For example, citrate buffers in the range of 5 to 15 mM are used in the formulations, but increasing the buffer concentration to >50 mM will result in excessive pain on subcutaneous injection and toxic effects because of the chelation of calcium in the blood if large volumes of product are injected.

Buffers have maximum buffer capacities near their pK_a. It is important to select buffers with a small $\Delta pK_a/^{\circ}C$ for products that may be subjected to excessive temperature fluctuations during processing such as steam sterilization, thermal cycling, or lyophilization. Tris, whose

$\Delta pK_a/°C$ is large (−0.028/°C), the pH of buffer, made at 25°C will change from 7.1 to 5.0 at 100°C, which could dramatically alter the stability or solubility of the drug. Similarly, the preferred buffers for a lyophilized product may be those that show the least pH change upon cooling, do not crystallize out, and will remain in the amorphous state protecting the drug. For example, replacing succinate with glycolate buffer improves the stability of lyophilized interferon-γ (59). During the lyophilization of mannitol containing succinate buffer at pH 5, monosodium succinate crystallizes reducing the pH and resulting in the unfolding of interferon-γ. This pH shift is not seen with glycolate buffer.

Table 7 lists buffers and chemicals used to adjust the pH of parenteral formulations and maintain the product pH range. The most common buffers used are phosphate, citrate, and acetate. Mono- and di-ethanolamines are added to adjust pH and form corresponding salts. Hydrogen bromide, sulfuric acid, benzene sulfonic acid, and methane sulfonic acids are added to drugs that are salts of bromide (Scopolamine HBr, Hyoscine HBr), sulfate (Nebcin, Tobramycin Sulfate), besylate (Tracrium Injection, Atracurium besylate), or mesylate (DHE 45 Injection, dihydroergotamine mesylate). Glucono-δ-lactone is used to adjust the pH of Quinidine Gluconate. Benzoate buffer, at a concentration of 5%, is used in Valium Injection. Citrates are a common buffer that can serve a dual role as chelating agents. Amino acids, histidine, arginine, aspartic, and glycine, function as buffers and stabilize proteins and peptide formulations. These amino acids are also used as lyo-additives and may prevent cold denaturation. Lactate and tartrate are occasionally used as buffer systems. Acetates are good buffers at low pH, but they are not generally used for lyophilization because of the potential sublimation of acetates.

Bulking Agents, Protectants, and Tonicity Adjusters

Table 8 lists additives that are osmolality adjusters and bulking or lyo-/cryoprotective agents. The most common tonicity adjusters are dextrose and sodium chloride. Additives that serve as lyophilization bulking agents and also as stabilizers and/or as buffers include some amino acids such as glycine, alanine, histidine, imidazole, arginine, asparagine, and aspartic acid. Other commonly used lyo-additives are monosaccharides (dextrose, glucose, maltose, lactose), disaccharides (sucrose, trehalose), polyhydric alcohols (inositol, mannitol, sorbitol), glycols (PEG 3350), Povidone (polyvinylpyrrolidone, PVP), and proteins (albumin, gelatin). Hydroxyethyl starch (hetastarch) and pentastarch, which are currently used as plasma expanders in commercial injectable products such as Hespan and Pentaspan, are also being evaluated as protectants during freeze-drying of proteins.

PVP has been used in injectable products (except in Japan) as a solubilizing agent, a protectant, and as a bulking agent. Only pyrogen-free grade, with low molecular weight (K-value less than 18), should be used in parenteral products to allow for rapid renal elimination. PVP not only solubilizes drugs such as rifampicin, but it may also reduce the local toxicity as seen in case of Oxytetracycline Injection.

Protein stabilization in the lyophilized state can be achieved if the stabilizer and protein do not phase separate during freezing or the stabilizer does not crystallize out. For Neupogen (GCSF), mannitol was replaced with sorbitol in the formulation to prevent the loss of activity of the liquid formulation upon accidental freezing (35). If the solution freezes, mannitol crystallizes while sorbitol will remain in an amorphous state protecting GCSF. However, a recent report suggests that sorbitol can also crystallize under certain conditions (60). Similarly, it is preferred that the drug remains dispersed in the stabilizer upon freezing of the solution to maximize protection. For example, cefoxitin, a cephalosporin, is more stable when freeze-dried with sucrose than with trehalose. Although the glass transition temperature and structural relaxation time is much greater for trehalose than sucrose (61), FTIR data indicates that the trehalose-cefoxitin system phase separates into two nearly pure components resulting in no protection (stability). Similarly, sucrose was found to be a better cryoprotectant than dextran for protein because dextran and protein underwent phase segregation as the solution started to freeze. The mechanism of cryoprotection in the solution has been explained by the preferential exclusion hypothesis (62).

Trehalose is a nonreducing disaccharide composed of two D-glucose monomers. It is found in some plants and animals that can withstand dehydration (anhydrobiosis) and

Table 7 Buffers and pH Adjusting Agents

Excipient	pH range	Example
Acetate		
Sodium	2.5–7.0	Syntocinon® (Novartis)
Acetic acid	2.5–7.2	Syntocinon (Novartis)
Glacial acetic acid	3.5–7.0	Brevibloc® (Ohmeda)
Ammonium	6.8–7.8	Bumex Injection® (Roche)
Ammonium sulfate	–	Innovar® (Astra)
Ammonium hydroxide	–	Triostat® (Jones Medical)
Arginine	7.0–7.4	Retavase® (Boehringer)
Aspartic acid	5.0–5.6	Pepcid® (Merck)
Benzene sulfonic acid	3.25–3.65	Nimbex® (Glaxo Wellcome)
Benzoate sodium/acid	3.5–6.9	Valium® (Roche)
Bicarbonate, sodium	5.5–11.0	Cenolate® (Abbott)
Boric acid/sodium		Comvax® (Merck)
Carbonate, sodium	4.0–11.0	Hyperab® (Bayer)
Carbon dioxide	–	Serentil® (Boehringer) Used to fill headspace
Citrate		
Acid	2.5–9.0	DTIC-Dome® (Bayer)
Sodium	3.0–8.5	Amikin® (Bristol Myers)
Disodium	6.1	Cerezyme® (Genzyme)
Trisodium	6.1	Cerezyme® (Genzyme)
Diethanolamine	9.5–10.5	Bactim IV® (Roche)
Glucono δ-lactone	5.5–7.0	Quinidine Gluconate (Lilly)
Glycine/Glycine HCl	2.5–10.8	Hep-B Gammagee® (Merck)
Histidine/Histidine HCl	5.0–6.5	Doxil® (Sequus)
Hydrochloric acid	Broad range	Amicar® (Immunex)
Hydrobromic acid	3.5–6.5	Scopolamine (UDL)
Lactate sodium/acid	2.7–5.8	Innovar® (Janssen)
Lysine (L)	–	Eminase® (Roberts)
Maleic acid	3.0–5.0	Librium® (Roche)
Meglumine	6.5–11.0	Magnevist® (Berlex)
Methanesulfonic acid	3.2–4.0	DHE-45® (Novartis)
Monoethanolamine	8.0–9.0	Terramycin (Pfizer)
Phosphate		
Acid	6.5–8.5	Saizen® (Serono Labs)
Monobasic potassium	6.7–7.3	Zantac® (Glaxo-Wellcome)
Dibasic potassium	6.7–7.3	Aminosyn® (Hospira)
Monobasic sodium[a]	2.5–8.0	Pregnyl® (Organon)
Dibasic sodium[b]	2.5–8.3	Zantac® (Glaxo-Wellcome)
Tribasic sodium	–	Synthroid® (Knoll)
Sodium hydroxide	Broad range	Optiray® (Mallinckrodt)
Succinate sodium/disodium	5.0–6.0	AmBisome® (Fujisawa)
Sulfuric acid	3.0–7.0	Nebcin® (Lilly)
Tartrate sodium/acid	2.5–6.2	Methergine® (Novartis)
Tromethamine (Tris)	6.5–9.0	Optiray® (Mallinckrodt)

[a]Sodium biphosphate, sodium dihydrogen phosphate, or sodium dihydrogen orthophosphate.
[b]Sodium phosphate, disodium hydrogen phosphate.

therefore had been suggested to stabilize drugs that undergo denaturation during spray or freeze-drying (63). Herceptin® (trastuzumab) is a recombinant DNA-derived monoclonal antibody (MAb) used for treating metastatic breast cancer. The MAb is stabilized in the lyophilized formulation using α,α-trehalose dihydrate. Trehalose is also used as a cryoprotectant to prevent liposomal aggregation and leakage. In the dried state, carbohydrates such as trehalose and inositol exert their protective effect by acting as a water substitute (64).

Formulations may require additives for specific gravity adjustments, especially for drugs which, upon administration, may come in contact with CSF. CSF has a specific gravity of 1.0059 at 37°C. Solutions with the same specific gravity as that of CSF are termed isobaric while those with a specific gravity greater than that of CSF are called hyperbaric. Upon

Table 8 Bulking Agents, Protectants, and Tonicity Adjusters

Excipient	Example
Alanine	Thrombate III® (Bayer)
Albumin	Bioclate® (Arco)
Albumin (human)	Botox® (Allergan)
Amino acids	Havrix® (Smith Kline Beecham)
Arginine (L)	Activase® (Genentech)
Aspargine	Tice BCG® (Organon)
Aspartic acid (L)	Pepcid® (Merck)
Calcium chloride	Xyntha® (Wyeth)
Cyclodextrin-α	Edex® (Schwartz)
Cyclodextrin-γ	Cardiotec® (Squibb)
Dextran 40	Etopophos® (Bristol Myers)
Dextrose	Betaseron® (Berlex)
Gelatin (cross-linked)	Kabikinase® (Pharmacia-Upjohn)
Gelatin (hydrolyzed)	Acthar® (Rhone-Poulanc Rorer)
Lactic and glycolic acid copolymers	Lupron Depot® (TAP)
Glucose	Iveegam® (Immuno-US)
Glycerine	Tice BCG® (Organon)
Glycine/glycine hydrochloride	Atgam® (Pharmacia-Upjohn)
Histidine	Antihemophilic Factor, human (American Red Cross)
Imidazole	Helixate® (Armour)
Inositol	OctreoScan® (Mallinckrodt)
Lactose	Caverject® (Pharmacia-Upjohn)
Magnesium chloride	Terramycin Solution (Pfizer)
Magnesium sulfate	Tice BCG® (Organon)
Maltose	Gamimune N® (Bayer)
Mannitol	Elspar® (Merck)
Polyethylene glycol 3350	Bioclate® (Arco)
Polylactic acid	Lupron Depot® (TAP)
Potassium chloride	Varivax® (Merck)
Povidone	Alkeran® (Glaxo-Wellcome)
Sodium chloride	WinRho SD® (Univax)
Sodium cholesteryl sulfate	Amphotec® (Sequus)
Sodium succinate	Actimmune® (Genentech)
Sodium sulfate	Depo-Provera® (Pharmacia-Upjohn)
Sorbitol	Panhematin® (Abbott)
Sucrose	Prolastin® (Bayer)
L-Threonine	Temodar® (Schering)
Trehalose (α, α)	Herceptin® (Genentech)

administration of a hyperbaric solution in the spinal cord, the injected solution will settle and affect spinal nerves at the end of the spinal cord. For example, dibucaine hydrochloride solution (Nupercaine® 1:200) is isobaric, while Nupercaine 1:500 is hypobaric (specific gravity of 1.0036 at 37°C). Nupercaine heavy solution is made hyperbaric by addition of 5% dextrose solution. This solution will block (anesthetize) the lower spinal nerves as it settles down in the spinal cord.

Special Additives

Table 9 lists special additives that have been included in pharmaceutical formulations to serve specific functions. Some of the special additives are summarized in the following along with their intended use:

1. Calcium D-saccharate tetrahydrate 0.46% wt/vol is used in Calcium Gluconate Injection, a saturated solution of 10% wt/vol, to prevent crystallization during temperature fluctuations.

Table 9 Special Additives

Excipient	Example
Acetyl tryptophanate	Human Albumin (American Red Cross)
Aluminum hydroxide	Recombivax HB® (Merck)
Aluminum phosphate	Tetanus Toxoid Adsorbed (Wyeth)
Aluminum potassium sulfate	TD Adsorbed Adult (Pasteur Merieux)
Amino acids [leucine, isoleucine, lysine (as acetate or HCl salt), valine, phenylalanine, threonine, tryptophan, alanine, aspartic acid, glutamic acid, proline, serine, tyrosine, taurine]	Travasol 10% Injection (Baxter) Aminosyn-PF10% (Hospira)
ε-Aminocaproic acid	Eminase® (Roberts)
Calcium D-saccharate	Calcium Gluconate (American Regent)
Caprylate sodium	Human Albumin (American Red Cross)
8-Chlorotheophylline	Dimenhydrinate® (Steris)
Creatine	Dalalone DP® (Forest)
Creatinine	Decadron® (Merck)
Cholesterol	Doxil® (Sequus)
Cholesteryl sulfate sodium	Amphotec® (Sequus)
Cyclohexanedione dioxime	Cardiotec® (BMS)
Diethanolamine	Bactrim® IV Infusion (Roche)
Distearyl phosphatidylcholine	DaunoXome® (Nexstar)
Distearyl phosphatidylglycerol	MiKasome® (NeXstar)
L-α-Dimyristoylphosphatidylcholine	Abelcet® (The Liposome Co.)
L-α-Dimyristoylphosphatidylglycerol	Abelcet (The Liposome Co.)
Dioleoylphosphatidylcholine (DOPC)	DepoCyt® (Chiron)
Dipalmitoylphosphatidylglycerol (DPPG)	DepoCyt (Chiron)
(*R*)-hexadecanoic acid, 1-[(phosphonoxy)methyl]-1,2-ethanediyl ester, monosodium salt (DPPA)	Definity® (Lantheus Medical Imaging)
(*R*)-4-hydroxy-*N*,*N*,*N*-trimethyl-10-oxo-7-[(1oxohexadecyl) oxy]-3,4,9-trioxa-4-phosphapentacosan-1-aminium, 4-oxide, inner salt (DPPC)	Definity (Lantheus Medical Imaging)
(*R*)-[6-hydroxy-6-oxido-9-[(1-oxohexadecyl)oxy]-5,7,11-trioxa-2aza-6-phosphahexacos-1-yl]-ω-methoxypoly(ox-1, 2-ethanediyl), monosodium salt (MPEG5000 DPPE)	Definity (Lantheus Medical Imaging)
MPEG-distearoyl phosphoethanolamine	Doxil® (Sequus)
Ethyl lactate	Ergotrate maleate (Lilly)
Ethylenediamine	Aminophylline (Abbott)
L-Glutamate sodium	Kabikinase® (Pharmacia-Upjohn)
Hyaluronate sodium	Trivaris® (Allergan)
Hydrogenated soy phosphatidylcholine	Doxil® (Sequus)
Iron ammonium citrate	Tice BCG® (Organon)
Lactic acid	Cipro IV® (Bayer)
D,L-Lactic and glycolic acid copolymer	Zoladex® (Zeneca)
Meglumine	Magnevist® (Berlex)
Methyl boronic acid	Cardiotec® (BMS)
Niacinamide	Estradurin® (Wyeth-Ayerst)
Paraben methyl	Adriamycin RDF® (Pharmacia-Upjohn)
Phosphatidylglycerol, egg (EPG)	Visudyne® (QLT)
Potassium sodium tartrate	CEA-Scan® (Immunomedics)
Protamine (as sulfate)	Insulatard NPH® (Novo Nordisk)
Simethicone	Premarin Injection® (Wyeth-Ayerst)
Saccharin sodium	Compazine Injection® (Smith Kline Beecham)
Sodium D-gluconate	Myoview® (Amersham)
Sodium hypochlorite	Ultratag™ RBC (Covidien)
Sodium sulfate	Depo-Provera® (Pfizer)
Stannous chloride	Myoview® (Amersham)
Sulfosalicylate disodium	Myoview® (Amersham)
Tin chloride (stannous and stannic)	Ultratag™ RBC (Covidien)
Tri-*n*-butyl phosphate	Venoglobulin® (Alpha Therapeutic)
Tricaprylin	DepoDur® (SkyePharma)
Triolein	DepoCyt® (Chiron)
von Willebrand factor	Bioclate® (Arco)
Zinc	Lente Insulin® (Novo Nordisk)
Zinc acetate	Nutropin Depot® (Genentech)
Zinc carbonate	Nutropin Depot® (Genentech)
Zinc oxide	Humalog® (Lilly)

2. Lactic acid is used in Cipro IV® as a solubilizing agent for the antibiotic.
3. Simethicone is used in the lyophilized product Premarin Injection® to prevent the formation of foam during reconstitution.
4. Creatine or creatinine are used in the dexamethasone formulations Dexamethasone acetate and Dexamethasone sodium phosphate, which are available as a suspension or a solution.
5. Methyl paraben (0.2 mg/mL) is used in Adriamycin RDF® to increase dissolution (65).
6. 0.1% Ethyl lactate is used in Ergotrate maleate as a solubilizing agent.
7. Niacinamide (12.5 mg/mL) is used in Estradurin Injection® as a solubilizing agent. Hydeltrasol® also contains niacinamide. The concept of hydrotropic agents to increase water solubility has been tried on several compounds including proteins (66,67).
8. Aluminum, in the form of aluminum hydroxide, aluminum phosphate, or aluminum potassium sulfate, is used as adjuvant in various vaccine formulations to elicit an increased immunogenic response.
9. Lupron Depot® Injection is lyophilized microspheres of gelatin and glycolic-lactic acid for intramuscular injection. Nutropin Depot® consists of polylactate-glycolate microspheres.
10. Sodium caprylate (sodium octoate) has antifungal properties, but it is also used to improve the stability of albumin solution against the effects of heat. Albumin solution can be heat pasteurized by heating at 60°C for 10 hours in the presence of sodium caprylate. Acetyl tryptophanate sodium is also added to albumin formulations.
11. Meglumine (*N*-methylglucamine) is used to form in situ salt. For example, diatrizoic acid, an X-ray contrast agent, is more stable when autoclaved as meglumine salt than as sodium salt (68). Meglumine is also added to Magnevist®, a magnetic resonance contrast agent.
12. Tri-*n*-butyl phosphate is present as an excipient in human immune globulin solution (Venoglobulin®). Its exact function in the formulation is not known, but it may serve as a scavenging agent.
13. von Willebrand factor is used to stabilize recombinant antihemophilic factor (Bioclate®).
14. Epsilon amino caproic acid (6-amino hexanoic acid) is used as a stabilizer in anistreplase (Eminase Injection®).
15. Zinc, zinc acetate, zinc carbonate, and protamine have been added to growth hormone and insulin to form complexes and control the duration of action.
16. Lipids (natural or modified) and cholesterols are used in liposomes and lipid complexes (e.g., PC, DMPC, DMPG, DOPC, DPPG, DSPC, DSPG, DPPA, DPPC, MPEG5000DPPE).
17. Several amino acids are used as either stabilizers or as part of amino acid solution for parenteral nutrition.

The FDA has published the "Inactive Ingredient Guide," which lists excipients in alphabetical order (20). The Inactive Ingredient database is reasonably comprehensive and is updated on a quarterly basis, but it does not include several excipients used in recently approved drug products. Each listed ingredient is followed by the route of administration, the CAS #, the UNII #, and in some cases, the range of concentration used in the approved drug product. However, this list does not provide the name of commercial product(s) corresponding to each excipient. Table 10 lists excipients that are included in the FDA database but were not found in our survey.

Similarly, in Japan the "Japanese Pharmaceutical Excipients Directory (JPED)" is published by the Japanese Pharmaceutical Excipients Council, with the cooperation and

Table 10 Excipients Listed in the FDA Inactive Ingredient Guide but Not Found in Our Survey

Excipient	Route/dosage form	Conc. (%)
Acetic anhydride	IV; injection	
Acetylated monoglycerides	IV; injection	
Activated charcoal	IM; injectable	0.3
Adipic acid	IM; injection	1
Alcohol, denatured	IV; injection	
Benzyl chloride	IV; injection	1.00E-03
Bibapcitide	IV; injection	0.01
Brocrinat	IV(infusion); injection	
Calcium gluceptate	IV; injection	5
Calcium hydroxide	IV; injection	0.37
Caldiamide sodium	IV; solution, injection	1.2
Calteridol calcium	IV; injection	0.02
Cellulose, microcrystalline	IV; injection	14.9
Corn oil	IM; injection	
Deoxycholic acid	IV(infusion); powder, for injection solution	
Diatrizoic acid	IM; injection	59.7
Dimethyl sulfoxide	IV(infusion); powder, for injection suspension, lyophilized	
Dimyristoyl lecithin	IV; powder, for injection solution, lyophilized	7.05
Disofenin	IV(infusion); injection	2
Docusate sodium	IM; injection	0.01
Edetic acid	Submucosal; solution, injection	0.05
Ethanolamine hydrochloride	IV; injection	0.15
Ethyl acetate	IM; injection	
Exametazime	IV; injection	
Fampridine	IV; injection	
Ferric chloride	IV; injection	6.05
Fructose	IV(infusion); powder, for injection solution, lyophilized	5
Gadolinium oxide	IV; injection	
Gluceptate sodium	IV; powder, for injection solution	20
Gluceptate sodium dihydrate	IV; injection	7.5
Glucuronic acid	IV; injection	
Glycocholic acid	IV; powder, for injection solution, lyophilized	14
Guanidine hydrochloride	IV; injection	0.25
Hydroxyethylpiperazine ethane sulfonic acid	IV; injection	
Insulin beef	SC; injection	0.1
Insulin pork	SC; injection	0.1
Iodine	IV; injection	
Iodoxamic acid	IV; injection	31
Iofetamine hydrochloride	IV; injection	
Isopropyl alcohol	IV; injection	
Lactobionic acid	IV(infusion); powder, for injection solution	
Lecithin, egg	IV; injectable	1.2
Lidofenin	IV; injection	
Magnesium stearate	Implantation; injection	1.50E-03
Mebrofenin	IV; injection	
Medronate disodium	IV; injection	1
Medronic acid	IV; injection	2.5
Metaphosphoric acid	IV(infusion); injection	0.13
Methylcellulose	Intra-articular; injection	0.1
Methylene blue	IV; injection	1
N-(carbamoyl-methoxy PEG-40)-1,2-distearoyl-cephalin sodium	IV; injection, suspension, liposomal	0.31
Nioxime	IV; injection	0.2
Octanoic acid	IV; injection	0.01
Oxidronate sodium	IV; injection	0.2
Oxyquinoline	IV; injection	5.00E-03
PEG sorbitan isostearate	IM; injection	
PEG vegetable oil	IM, SC; injection	7

(*continued*)

Table 10 Excipients Listed in the FDA Inactive Ingredient Guide but Not Found in Our Survey (*Continued*)

Excipient	Route/dosage form	Conc. (%)
Pentetate calcium trisodium	Intrathecal; injection	
Pentetate pentasodium	IV; injection	0.5
Perflutren	IV; injection	
Polysiloxane	IV; injectable	
Polysorbate 40	IM, IV; injection	
Polyvinyl alcohol	IM; injection, microspheres	
Potassium bisulfite	IV; injection	
Potassium hydroxide	IV; injection	
Silicone	IM, IV; injection	
Sodium bisulfate	IM, IV; injection	0.32
Sodium chlorate	IV(infusion); injection	15.4
Sodium cysteinate hydrochloride	Intradiscal; powder, for injection solution	
Sodium iodide	IV; powder, for injection solution	5
Sodium pyrophosphate	IV; injection	1.2
Sodium thiomalate	IM, IV; injection	
Sodium thiosulfate	IV; solution	
Sodium thiosulfate anhydrous	IV; solution	0.19
Sodium trimetaphosphate	IV; powder, for injection solution	
Sorbitan monopalmitate	IM; injection	
Stannous fluoride	IV; injection	0.07
Stannous tartrate	IV; injection	8.00E-03
Starch	IM; injection	0.6
Succimer	IV; injection	
Succinic acid	IM, IV; injection	
Sulfur dioxide	IV(infusion); solution, injection	0.15
Sulfurous acid	IM; injection	
Tetrakis(1-isocyano-2-methoxy-2-methyl-propane)-copper(i) tetrafluoroborate	IV; injection	0.1
Tetrofosmin	IV(infusion); powder, for injection solution, lyophilized	0.02
Theophylline	IV(infusion); injection	
Trifluoroacetic acid	IV(infusion); powder, for injection solution, lyophilized	
Urea	IM; injection	
Zinc chloride	Intradermal; injection	0.04

guidance of the Ministry of Health, Labour and Welfare (69). This directory divides the excipients into the following two categories:

1. Official excipients—those that have been recognized in the Japanese Pharmacopoeia (JP), Japanese Pharmaceutical Codex, and Japanese Pharmaceutical Excipients (JPE) and for which testing methods and standards have been determined.
2. Nonofficial excipients—those that have been used in pharmaceutical products sold in Japan and are planned to be included in the official book or in supplemental editions.

JPED lists the excipients, the route of administration, and the maximum amount or concentration that has been approved. An excipient used within the listed concentration limits is considered "precedented" and no additional data is needed. If the excipient concentration is outside of the limits, additional safety info may be needed (experimental or published literature). Unprecedented or novel excipients may have to be placed on stability and quality standards developed. If the excipient is listed in JP, JPE, or JPED, it must meet the specifications listed in the monograph; however, if the excipient is not listed in any of the above three books then USP, Ph. Eur., or other pharmacopoeial standards should be used. For excipients not listed in pharmacopoeias, in-house specifications are used.

REGULATORY PERSPECTIVE

The International Pharmaceutical Excipients Council (IPEC) has classified excipients into four classes on the basis of the safety testing information available (70).

1. New chemical excipients: require a full safety evaluation program. EU directive 75/318/EEC states that new chemical excipients will be treated in the same way as new actives. Safety studies for a new chemical excipient have been estimated to cost about $3.5 million over four to five years. In the United States, relevant information from these safety studies will need to be filed with the FDA in a DMF, and in Europe a dossier needs to be established. The IPEC Europe has issued a guideline (Compilation of Excipient Masterfiles Guidelines) that provides guidance to excipient producers on how to construct a dossier that will support MAA (Marketing Authorization Application) while maintaining the confidentiality of the data.
2. Existing chemical excipient—first use in man: implies that animal safety data exist since it may have been used in some other application. Additional safety information may have to be gathered to justify its use in humans.
3. Existing chemical excipient: indicates that it has been used in humans but change in route of administration (say from oral to parenteral), new dosage form, higher dose, etc. may require additional safety information.
4. New modifications or combinations of existing excipients: a physical interaction NOT a chemical reaction. No safety evaluation is necessary in this case.

Formulators should understand that just because an excipient is listed as GRAS does not mean that it can be used in a parenteral dosage form. The GRAS list includes materials that have been demonstrated as safe for food (oral administration) but have not necessarily been deemed safe for use in an injectable product. Therefore, additives included in this list are of very limited value for selecting excipients for parenteral formulations.

The USP, JP, Ph. Eur., BP, and other pharmacopoeias may have monographs for identical excipients, which differ considerably with regards to specifications, test criteria, and analytical methods. This presents a significant testing burden on a pharmaceutical manufacturer intending to supply a global product because they will have to perform testing on the same excipient numerous times to meet the various compendial specifications. Under the auspices of the Pharmacopoeial Discussion Group (PDG), there is ongoing harmonization of excipient monographs. PDG has been working on several commonly used excipients to achieve a single monograph for each excipient. Presently, 26 General Chapters and 40 excipient monographs have been harmonized (stage 6 of the process). For example, benzyl alcohol undergoes degradation by a free radical mechanism to form benzaldehyde and hydrogen peroxide. The degradation products are much more toxic than the parent molecule. The USP, JP, and Ph. Eur. require three different chromatographic systems to test for organic impurity (mainly benzaldehyde). The harmonized monograph of benzyl alcohol has eliminated unnecessary repetition, which does not contribute to the overall quality of the product (71).

In addition to testing and specifications, regulatory bodies are also focusing their attention on excipient manufacturing processes. There have been major initiatives on the part of IPEC to improve the quality of additives, which has resulted in a publication titled "Good Manufacturing Practices Guide for Bulk Pharmaceutical Excipients" (72). The excipients may be manufactured for food, cosmetic, chemical, agriculture, or pharmaceutical industries, and the requirement for each industry is different. The purpose of this guide is to develop a quality system framework that may be used for excipient suppliers, which will be acceptable to the pharmaceutical industry, and to harmonize the requirements in the United States, Europe, and Japan.

The United States and Europe require all excipients to be declared, along with their quantity, on the label if the product is an injectable preparation. In Japan, only the names of excipients are required in the labeling (information that is included with the product like the package insert); however, information of the quantity of each excipient is not required on the label. EU Article 54(c) requires that all excipients need to be declared on the labeling if the medicinal product is an injectable or a topical or an eye preparation. The European guide for

the label and package leaflet also lists excipients, which have special issues, and are addressed in an Annex (73). Table 11 is a summary of some of these ingredients that are commonly used as parenteral excipients and the corresponding safety information that should be included in the leaflet. The package leaflet must include a list of information on those excipients, knowledge of which is important for the safe and effective use of the medicinal product.

Table 11 Excipients for Label and Corresponding Information for Leaflet

Name	Threshold level	Information for the package leaflet
Arachis oil	Zero	Whenever arachis oil appears, peanut oil should appear besides it. If you are allergic to peanut or soya, do not use this medicinal product
Benzoic acid and benzoates	Zero	It may increase the risk of jaundice in newborn babies
Benzyl alcohol	Exposures less than 90 mg/kg/day	Must not be given to premature babies or neonates. May cause toxic reactions and allergic reactions in infants and children up to 3 yr old.
	90 mg/kg/day	Must not be given to premature babies or neonates. Due to the risk of fatal toxic reactions arising from exposure to benzyl alcohol in excess of 90 mg/kg/day, this product should not be used in infants and children up to 3 yr old.
Castor oil polyoxyl and castor oil polyoxyl hydrogenated	Zero	May cause severe allergic reactions
Chlorocresol	Zero	May cause allergic reactions
Ethanol	<100 mg/dose	This medicinal product contains small amounts of ethanol (alcohol), <100 mg/dose.
	100 mg–3 g/dose	This medicinal product contains . . . vol % ethanol (alcohol), i.e., up to . . . mg/dose, equivalent to . . . mL beer, . . . mL wine per dose. Harmful for those suffering from alcoholism. To be taken into account in pregnant or breast-feeding women, children and high-risk groups such as patients with liver disease or epilepsy.
	3 g/dose	This medicinal product contains . . . vol % ethanol (alcohol), i.e., up to . . . mg/dose, equivalent to . . . mL beer, . . . mL wine per dose. Harmful for those suffering from alcoholism. To be taken into account in pregnant or breast-feeding women, children and high-risk groups such as patients with liver disease or epilepsy. The amount of alcohol in this medicinal product may alter the effects of other medicines. The amount of alcohol in this medicinal product may impair your ability to drive or use machines.
Fructose	Zero	If you have been told by your doctor that you have intolerance to some sugars, contact your doctor before taking this medicinal product. Patients with rare hereditary problems of fructose intolerance should not take this medicine.
	5 g	Contains *x* g fructose per dose. This should be taken into account in patients with diabetes mellitus.
Galactose	Zero	If you have been told by your doctor that you have intolerance to some sugars, contact your doctor before taking this medicinal product. SPC proposal: patients with rare hereditary problems of galactose intolerance, e.g., galactosemia should not take this medicine.
	5 g	Contains *x* g galactose per dose. This should be taken into account in patients with diabetes mellitus

Table 11 (*Continued*)

Name	Threshold level	Information for the package leaflet
Glucose	5 g	Contains *x* g glucose per dose. This should be taken into account in patients with diabetes mellitus.
Heparin (as an excipient)	Zero	May cause allergic reactions and reduced blood cell counts that may affect the blood clotting system. Patients with a history of heparin-induced allergic reactions should avoid the use of heparin-containing medicines.
Organic mercury compounds (like thiomerosal phenylmercuric nitrate, acetate, borate)	Zero	This medicinal product contains (thiomerosal) as a preservative and it is possible that you/your child may experience an allergic reaction. Tell your doctor if you/your child have/has any known allergies. Tell your doctor if you/your child have/has experienced any health problems after previous administration of a vaccine.
Parahydroxybenzoates and their esters	Zero	May cause allergic reactions (possibly delayed), and exceptionally, bronchospasm.
Phenylalanine	Zero	This medicine contains phenylalanine. May be harmful for people with phenylketonuria.
Potassium	<1 mmol/dose	This medicine contains potassium, <1 mmol (39 mg) per dose, i.e., essentially "potassium-free."
	1 mmol/dose	This medicine contains *x* mmol (or *y* mg) potassium per dose. To be taken into consideration by patients with reduced kidney function or patients on a controlled potassium diet.
	30 mmol/L	May cause pain at the site of injection.
Propylene glycol and esters	400 mg/kg adults 200 mg/kg children	May cause alcohol-like symptoms.
Sesame oil	Zero	May rarely cause severe allergic reactions.
Sodium	<1 mmol/dose	This medicinal product contains <1 mmol sodium (23 mg) per dose, i.e., essentially "sodium-free."
	1 mmol/dose	This medicinal product contains *x* mmol (or *y* mg) sodium per dose. To be taken into consideration by patients on a controlled sodium diet.
Sorbitol	Zero	If you have been told by your doctor that you have intolerance to some sugars, contact your doctor before taking this medicinal product. SPC proposal: Patients with rare hereditary problems of fructose intolerance should not take this medicine.
Soya oil (and hydrogenated soya oil)	Zero	Medicinal product contains soya oil. If you are allergic to peanut or soya, do not use this medicinal product.

Abbreviation: SPC, Summary of Product Characteristics.
Source: From Ref. 73.

Similarly, 21 CFR 201.22 requires prescription drugs containing sulfites to be labeled with a warning statement about possible hypersensitivity. An informational chapter in USP <1091> Labeling of Inactive Ingredients provides guidelines for labeling of inactive ingredients present in dosage forms.

According to the Notes for Guidance on Pharmaceutical Development (CHMP/ICH/167068/04), the choice of excipients, their grade, compatibility, concentration, and function should be described in the P2 section of the Common Technical Document. It is necessary to justify inclusion of all the ingredients in the drug product and describe their intended function. Generally, a specification of ±10% at the end of shelf life is acceptable except for antioxidant and preservatives where performance data from PET or stability data may justify broader limits.

The bioburden and endotoxin limits of excipients used in the manufacture of sterile medical products shall be stated. However, this individual testing of excipients may be omitted if bioburden and endotoxin testing of the solution is checked prior to sterilization.

If an excipient is present in Ph. Eur. or other major pharmacopoeias, the monograph specifications are generally acceptable in the registration file. However, excipients that are not described in any pharmacopoeia, specifications should include physical characterization, identification tests, purity test, assay, and impurity tests. A certification is also included to confirm that excipients are of non-animal (specifically non-ruminant) origin. If this is not the case, a regulatory agency will require documentation to demonstrate freedom from viral and transmissible spongiform encephalopathies (TSE) risks (74).

CRITERIA FOR THE SELECTION OF EXCIPIENT AND SUPPLIER

Excipient selection during formulation development of parenteral dosage forms is focused on providing a safe, stable, efficacious, and functional product. The choice and the characteristics of excipients should be appropriate for the intended purpose.

> An explanation should be provided with regard to the function of all constituents in the formulation, with justification for their inclusion. In some cases, experimental data may be necessary to justify such inclusion e.g. preservatives. The choice of the quality of the excipient should be guided by its role in the formulation and by the proposed manufacturing process. In some cases it may be necessary to address and justify the quality of certain excipients in the formulation (75).

Normally a pharmaceutical development report is written in the United States, which should be available at the time of preapproval inspection (PAI). The development report captures the choice of excipients, their purpose and level in the drug product, their compatibility with other excipients, drug or package system, and how they may influence the stability and efficacy of the finished product. This information is similar to that included in the P2 section of the Common Technical Document submission.

The following key points should be considered in selecting an excipient and its supplier for parenteral products:

1. Influence of the excipient on the overall quality, stability, and effectiveness of drug product.
2. Physical, chemical, and biological compatibility of the excipient with drug and the packaging system (76).
3. Compatibility of the excipient with the manufacturing process; for example, preservatives may be adsorbed by rubber tubes or filters, acetate buffers will be lost during lyophilization process, etc.
4. The amount or percentage of excipients that can be added to the drug product. Table 6 summarizes the maximum amount of preservatives and antioxidants allowed by various pharmacopoeias.
5. Route of administration. The USP, Ph. Eur., and BP do not allow preservatives to be present in injections intended to come in contact with brain tissues or CSF. Thus, intracisternal, epidural, and intradural injections should be preservative-free. Also, it is preferred for a drug product to be administered via IV route to be free of particulate matter. However, if the size of the particle is well controlled, as in fat emulsion or colloidal albumin or amphotericin B dispersion, they can be administered by IV infusion.
6. Dose volume. All large-volume parenterals (LVPs) and those small-volume parenterals (SVPs) where the single-dose injection volume can be greater than 15 mL are required by the Ph. Eur./BP to be preservative-free (unless justified). The USP recommends that special care be observed in the choice and the use of added substances in preparations for injections that are administered in volumes exceeding 5 mL (77).
7. Whether the product is intended for single or multiple-dose use. According to USP, single-dose injections should be preservative-free. The FDA takes the position that even though a single-dose injection may have to be aseptically processed, the manufacturer should not use a preservative to prevent microbial growth. European agencies have taken a more lenient attitude on this subject.

8. The length or duration of time that the drug product will be used once the multidose injection is opened.
9. How safe is the excipient? Does it cause tissue irritation, hemolysis, or other toxic effects on cells, tissues, or organs?
10. Does the parenteral excipient contain very low levels of lead, aluminum, or other heavy metals?
11. Does a dossier or DMF exist for the excipient?
12. Has the excipient been used in humans? Has it been used via a parenteral route and in the amount and concentration that is being planned?
13. Has the drug product containing this excipient been approved throughout the world?
14. What is the cost of the excipient and is it readily available?
15. Is the excipient vendor following the GMP guide? Is the vendor ISO 9000 certified?
16. Will the excipient supplier certify the material to meet USP, BP, Ph. Eur., JP, and other pharmacopoeias?
17. Has the supplier been audited by the FDA or the company's audit group? How did they fare?

Presence of impurities in excipients can have a dramatic influence on the safety, efficacy, or stability of the drug product. Monomers or metal catalysts used during a polymerization process are toxic and can also destabilize the drug product if present in trace amounts. Because of safety concerns, the limit of vinyl chloride (monomer) in polyvinyl pyrrolidone is $\leq$10 ppm and for hydrazine (a side product of polymerization reaction) is $\leq$1 ppm. Monomeric ethylene oxide is highly toxic and can be present in ethoxylated excipients such as PEGs, ethoxylated fatty acids, etc.

An FDA guidance suggests that the animal-derived materials (e.g., egg yolk lecithin, egg phospholipid) used in drug product, originating from Belgium, France, and Netherland, between January to June, 1999, should be investigated for the presence of dioxin and polychlorinated biphenyls. Contaminated animal feed is the likely source of contamination in the animal-derived product.

Excipients such as dextrose, citric acid, mannitol, and trehalose are manufactured by fermentation processes and should be specially controlled for endotoxin levels. Mycotoxin (highly toxic metabolic products of certain fungi species) contamination of an excipient derived from natural material has not been specifically addressed by regulatory authorities. The German health authority has issued a draft guideline in 1997 where a limit has been specified for aflotoxins M_1, B_1, and the sum of B_1, B_2, G_1, and G_2 in the starting material for pharmaceutical products.

Heavy metal contamination of an excipient is a concern, especially for sugars, phosphate, and citrate. Several rules have been proposed or established. For example, Ph. Eur. sets a limit of $\leq$1 ppm of nickel in polyols. California Proposition 65 specifies a limit of $\leq$0.5 μg of lead per day per product (78). Similarly, the USP and FDA have issued guidelines that limit the aluminum content for all LVPs used in TPN therapy to 25 μg/L (79). Further, it requires that the maximum level of aluminum in SVPs intended to be added to LVPs and pharmacy bulk packages, at expiration date, be stated on the immediate container label.

An excipient's physical and chemical stability will determine the frequency for retesting. Because of the relatively small amount of active ingredient compared with the amount of excipients in most parenteral formulations, the degradation of even a small percentage of excipient can lead to levels of impurities sufficient to react or degrade a large percentage of active material. For example, in the presence of light and oxygen, benzyl alcohol decomposes via a free radical mechanism to form benzaldehyde (x% of benzaldehyde is approximately equivalent to 1/3 x% of hydrogen peroxide). Hydrogen peroxide can rapidly oxidize sulfhydryl groups of amino acids such as cysteine present in peptides or proteins.

Thorough due diligence and risk analysis should be conducted in the selection of a pharmaceutical excipient supplier. Because excipients are often commodity (low value–high volume) products, suppliers focus on improving manufacturing efficiency to reduce cost, which frequently results in manufacturing process changes that potentially could impact the

quality or characteristic profile of the excipient. Generally, the pharmaceutical industry is a relatively small customer (in terms of volume of material purchased) of these suppliers and has limited business leverage. For example, the pharmaceutical industry uses approximately 20% of gelatin produced. Of this 20%, most is for production of oral dosage forms. The parenteral portion is approximately 5% of this 20%. Therefore, it is imperative that the drug manufacturer negotiates a detailed contract with the excipient supplier, which strictly prohibits the supplier from making any changes in the process or quality of the material without informing the customer well in advance. Also, the pharmaceutical manufacturer should investigate, and even consider qualifying, alternate suppliers who could be used in case of an emergency. A change in the supplier should not be made without consulting the pertinent regulatory bodies, since such an event may require prior regulatory approval.

The pharmaceutical manufacturer should have an active Vendor Certification Program and assure that the vendor is ISO 9000 certified. An audit of the excipient manufacturer is essential since the pharmaceutical industry is ultimately responsible for the quality of the drug product that includes the excipient(s) as one of the components. A useful audit tool is the IPEC GMP guide that is written in the format of ISO 9000 using identical nomenclature and paragraph numbering. The audit should determine and ensure that the quality is being built into the excipient, which may be difficult to measure by incoming quality control assessment of the material. This is especially true for parenteral excipients where not only chemical, but also microbiological, attributes are critical. Bioburden and endotoxin limits may be needed for each of the excipients, and several guidelines are available to establish the specifications (80,81).

There are no legal requirements for excipient GMPs in Europe (82). The Qualified Person (QP) is responsible to assure that the quality of excipients is appropriate on the basis of pharmacopoeial specifications or a company's quality systems.

Unfortunate events in Haiti highlight the importance of assuring the quality of excipients to the same degree that one normally does for active ingredients. From November 1995 through June 1996, acute anuric renal failure was diagnosed in 86 children. This was associated with the use of diethylene glycol contaminated glycerin used to manufacture acetaminophen syrup (83). The FDA is advising pharmaceutical companies to test for melamine down to a 2.5 ppm level in certain nitrogen-rich drug ingredients (raw materials that contain more than 2.5% nitrogen and those for which purity or strength is determined on the basis of nitrogen content) (84). The list of excipients includes albumin, amino acids derived from casein protein hydrolysates, ammonium salts, protamine sulfate, povidone, lactose, gelatin, etc. This guidance is in response to the incidents of pet food and Chinese milk doped with melamine.

The FDA recognizes the importance of excipients in the product for performance and safety. An injectable generic product should have identical nonexceptional excipients (qualitatively and quantitatively) as that of reference listed drug if the generic drug product is to follow the simplest path of registration, otherwise additional data must be submitted to demonstrate that the differences do not affect the safety or performance (85). For parenteral products, nonexceptional excipients are ingredients other than preservatives, pH adjuster, antioxidant, and buffers.

SAFETY ISSUES

Clinical experience with many of the excipients has resulted in some safety watch outs. For example, sensitization reactions have been reported for the parabens, thimerosal, and propyl gallate. Sorbitol is metabolized to fructose and can be dangerous when administered to fructose intolerant patients. Table 11 lists safety concerns that need to be included on the labeling.

Progress in drug delivery systems and new proteins/peptides being developed for parenteral administration has created a need to expand the list of excipients that can be safely used. The informational chapter in the USP presents a scientifically based approach for a safety assessment of new pharmaceutical excipients (86). A new or novel excipient is defined as one that has not been previously used in a pharmaceutical preparation or has not been fully qualified by existing safety data with respect to the proposed level of exposure, duration of exposure, or route of administration (87). Besides the baseline toxicity data (either through literature or experimentation), if the drug (excipient) will be administered short term

(<14 days of consecutive days per treatment episode), intermediate term (14–90 days), or long term (>3 months), additional safety information on the excipient is needed (87).

Currently, there are concerns regarding TSE via animal-derived excipients such as gelatin (88). TSE are caused by prions that are extremely resistant to heat and normal sterilization processes. Hence, a risk assessment is done at early stage of product development to make sure that the excipients do not contribute to this risk.

Several guidelines are available that address the issue of animal-derived excipients and scientific principles to minimize the possible transmission of TSE via medicinal products (89,90). The current situation indicates that there are negligible concerns for lactose, glycerol, fatty acids, and their esters, but the situation is less clear for gelatin. Gelatin is still a necessary ingredient for some medicinal products, and the European Medicines Agency (EMEA) has updated its guidance to allow gelatin from category I and II countries if gelatin is produced by the acid process and category I, II, and III countries if produced by the alkali process (91). Additional information on risk assessment of ruminant materials originating from United States, Canada, and other countries can be found in Refs. 90, 92, and 93.

In the current regulatory environment, if feasible, it may be beneficial to select non-animal-derived excipient. There have always been concerns in using bovine serum albumin (BSA) or human serum albumin (HSA) because they may have been possibly derived from virus contaminated blood. Recombinant human erythropoietin and darbepoetin alfa formulations were changed to replace albumin with Polysorbate 80. Currently, recombinant HSA is available from several companies, which reduces probability of TSE (94).

European Commission directive EMEA/410/01/rev. 2 requires manufacturers to provide a "Certificate of Suitability" or the underlying "scientific information" to attest that their pharmaceuticals are free of TSEs.

Vegetable origin polysorbate should be used. If older products contain animal sourced polysorbate then a switch should be made. The EMEA has stated that such a change of a Polysorbate 80 source will not result in reperforming viral inactivation studies (95). It is also important to know the vegetable source (e.g., is trehalose being made from corn or tapioca) and if during the manufacturing of the excipient any processing aids (e.g., enzymes during production of lactose) are being used that are derived from an animal source.

FUTURE DIRECTION

Biodegradable polymeric materials such as polylactic acid, polyglycolic acid, and other poly-α-hydroxy acids have been used as medical devices and also as biodegradable sutures since the 1960s (96). Currently, the FDA has approved for marketing only devices made from homopolymers or copolymers of glycolide, lactide, caprolactone, *p*-dioxanone, and trimethylene carbonate (97). Such biopolymers are finding increased application as a matrix to deliver parenteral drugs for prolonged delivery (98). At least four drug products—Lupron Depot®, Decapeptyl®, Nutropin Depot®, and Zoladex®—have been approved. All four drug products are microspheres in polyglycolic acid(PLG), polylactic acid(PLA), or the co-polylactic-glycolic acid(PLGA) matrix. Decapeptyl is approved in France and is a microsphere for IM administration. It contains drug in a matrix of PLGA and carboxymethylcellulose with mannitol and Polysorbate 80.

Several phospholipid-based excipients are finding increased application as solubilizing agents, emulsifying agents, or as components of a liposomal formulation. The phospholipids occur naturally and are biocompatible and biodegradable, for example, egg phosphatidylcholine, soybean phosphatidylcholine, hydrogenated soybean phosphatidylcholine (HSPC), DMPC, DSPC, DOPC, DSPE, DMPG, DPPG, and DSPG. Spartaject™ technology uses a mixture of phospholipids, to encapsulate poorly water soluble drug, to form a microsuspension that can be injected intravenously. Busulfan drug product uses this technology and is currently undergoing phase I clinical trials. Many liposomal and liposomal-like formulations (DepoFoam®) are either approved (DepoCyt®) or are undergoing clinical trials to reduce drug toxicity, improve drug stability, prolong the duration of action, or to deliver drug to the central nervous system (99). Two amphotericin formulations have been approved in the United States, which are a liposomal or lipid complex between the antifungal drug and the positively

charged lipid. Amphotec® is a 1:1 molar ratio complex of amphotericin B and cholesteryl sulfate, while Abelcet® is a 1:1 molar complex of amphotericin B with phospholipids (7 parts of L-α-dimyristoylphosphatidylcholine and L-α-dimyristoylphosphatidyl glycerol).

Poloxamer or Pluronic are block copolymers composed of polyoxyethylene and polyoxypropylene segments. They exhibit reverse thermal gelation and are being tried as solubilizing, emulsifying, and stabilizing agents. Thus, a depot drug delivery system can be created using Pluronics whereby the product is a viscous injection that gels upon intramuscular injection (100). Pluronics can prevent protein aggregation or ad/absorption and can help in the reconstitution of lyophilized products. Pluronic F68 (Polaxamer-188), F38 (Poloxamer-108), and F127 (Poloxamer-407) are the most commonly used Pluronics. For example, a liquid formulation of human growth hormone and Factor VIII can be stabilized using Pluronics. Fluosol® is a complex mixture of perfluorocarbons, with a high oxygen-carrying capacity, emulsified with Pluronic F68 and various lipids. It was recently approved by the FDA for adjuvant therapy to reduce myocardial ischemia during coronary angioplasty. A highly purified form of Poloxamer-188 (Flocor™), intended for IV administration, is undergoing phase III clinical trials for various cardiovascular diseases. Purification of Poloxamer-188 has been shown to reduce nephrotoxicity. Another nonionic surfactant, Solutol HS 15 (Macrogol-15-Hydroxystearate), has been approved by the Health Protection Branch (Canada) in vitamin K1 formulation for human application.

Polymeric materials such as Poloxamer and albumin may coat micro- or nanoparticles, alter their surface characteristics, and reduce their phagocytosis and opsonization by reticuloendothelial system following IV injection. Such surface modifications often result in prolongation in the circulation time of intravenously injected colloidal dispersions (101). Poloxamers have also been used to stabilize suspensions such as NanoCrystal™ (102).

Fluosol-DA®, manufactured by Green Cross Corporation in Japan, was the first successfully developed injectable perfluorocarbons-based commercial product. It is a dilute (20% wt/vol) emulsion based on perfluorodecalin and perfluorotripropylamine emulsified with potassium oleate, Pluronic F68, and egg yolk lecithin. These perfluorocarbons are inert and can also be used to formulate nonaqueous preparations of insoluble proteins and small molecules (103). Perfluorocarbons have also been approved by the FDA in one ultrasound contrast agent, Optison®, which is administered via the IV route. Optison is a suspension of microspheres of HSA with octafluoropropane. Heat treatment and sonication of appropriately diluted human albumin, in the presence of octafluoropropane gas, is used to manufacture microspheres in Optison injection. The protein in the microsphere shell makes up approximately 5% to 7% (wt/wt) of the total protein in the liquid. The microspheres have a mean diameter range of 2.0 to 4.5 μm with 93% of the microspheres being less than 10 μm.

Sucrose acetate isobutyrate (SAIB) is a high-viscosity liquid system, which converts into free flowing liquid when mixed with 10% to 15% ethanol (104). Upon SC or IM injection, the matrix rapidly converts to water insoluble semisolid, which is capable of delivering proteins and small molecules for a prolonged period. SAIB is biocompatible and biodegrades to natural metabolites.

Several other biodegradable, biocompatible, injectable polymers being investigated for drug delivery systems include polyvinyl alcohol, block copolymer of PLA-PEG, polycyanoacrylate, polyanhydrides, cellulose, chitosan, alginate, collagen, modified HSA, albumin, starches, dextrans, hyaluronic acid and its derivatives, and hydroxyapatite (105). It is impossible to cover all the aspects in the field of excipient development, control, and usage in a single chapter, but it is clear that many of the new drug modalities like delivery of genes, immunomodulators, RNAi, anti-sense, aptamers, and other novel therapeutic agents will invariably require new excipients to be successful (106).

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8 Techniques to evaluate damage and pain on injection

Gayle A. Brazeau, Jessica Klapa, and Pramod Gupta

BACKGROUND AND OPTIMIZING PARENTERAL FORMULATIONS

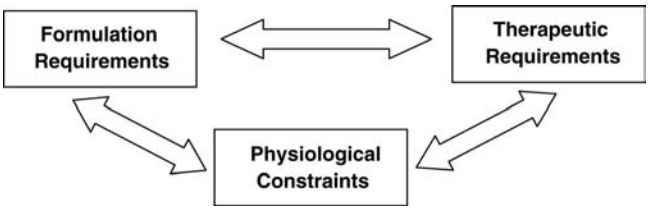
Injectable products have and will continue to be an important aspect in the medication management of patients for cancer, acute cardiovascular disease, infection, central nervous system disorders, and traumatic injuries. It is often also important for the development of parenteral product formulations for existing oral drugs for use in institutional, long-term, and home health care settings, given many of the chronic conditions may necessitate treatment in these types of facilities. The formulation of injectables can be a challenging project, given the complexity of the formulations from the perspective of optimizing the formulation requirements for the product, the physiological constraints associated with administration of the product, and the therapeutic characteristics of the drug (Fig. 1).

With respect to a given formulation, pharmaceutical scientists must consider the specific therapeutic requirements such as the indication or use of the drug, the optimal route of drug administration for the treated condition or disease, the targeted patient population(s) for the condition or disease treatment, the type of product (viz., immediate vs. sustained release), and the pharmacokinetic and pharmacodynamic profile of the drug. These therapeutic considerations must be balanced with the formulation requirements in optimizing the type of dosage form (e.g., solution, suspension, emulsion, or the newer and innovative drug delivery systems), solubility, stability, compatibility, injection volume, and viscosity. Finally, the formulation and therapeutic requirements must be optimized in considering the physiological constraints associated with parenteral administration, such as the route and site of injection, specifically the injection volume, injection speed, frequency of injections, and the local site reactions, namely the tissue damage on injection and pain on injection.

While the tools and methodological approaches are readily available for optimizing and understanding the elements with respect to the formulation requirements and characterizing the therapeutic requirements, one specific area that is often difficult to characterize during formulation development is the evaluation of the potential for causing tissue damage and/or pain on injection. The goal of this chapter is to provide pharmaceutical scientists with a general overview of available in vitro and in vivo methods in animals to screen drugs, excipients, and formulations for their potential to cause tissue damage and pain. While this chapter will provide a general discussion and summary of these topics, readers are encouraged to review the specific references for additional details. Furthermore, the characterization and determination of the extent of tissue damage and/or pain associated with a parenteral formulation is an ideal example of the need for professional collaboration between pharmaceutical scientists, pharmacologists, toxicologists, and neuroscientists, given the complexity of the physiological, biological, and biochemical interactions between the formulation and the site of injection.

Definitions and Relationship Between Tissue Damage and Pain on Injection

It is critical to understand the key definitions with respect to tissue damage and/or pain associated with injectables. Tissue damage can be defined as a *formulation*-induced reversible or irreversible change in the anatomy, biochemistry, or physiology at the injection site. *Formulation* in this specific definition can range from a single drug to one or more excipient(s) to final product composed of the drug and other excipients or a delivery system. The specific type of tissue damage includes hemolysis or phlebitis associated with intravenous administration and myotoxicity associated with intramuscular administration. For subcutaneous injections, the damage could be associated with those structures associated with this injection space such as the skin or skeletal muscle. The evaluation of the extent of tissue damage on intramuscular or intravenous injection is relatively easy to evaluate, given the availability of a



Formulations requirements	Physiological constraints	Therapeutic requirements

• Dosage form type
• Solubility
• Stability and compatibility
• Injection volume
• Viscosity
	• Route/site injection
	  ◦ Injection volume
	  ◦ Injection speed
	  ◦ Frequency of injection
	• Local site reactions
	  ◦ Tissue damage
	  ◦ Pain
		• Therapeutic indication and use
		  ◦ Administration route
		  ◦ Patient population
		• Formulation release profile
		• Pharmacokinetic and pharmacodynamic profile

Figure 1 Optimization considerations in the formulation of injectable products.

Table 1 In Vitro and In Vivo Markers to Evaluate Tissue Damage

In vitro markers	In vivo tissue markers

• Hemoglobin—erythrocytes
• Cytosolic cellular components
  ◦ Creatine kinase
  ◦ Lactate dehydrogenase
  ◦ Potassium
• Histological evaluation
  ◦ Extracellular membrane disruptions
  ◦ Intracellular membrane disruptions
  ◦ Changes in intracellular organelles
	• Release of proteins/cytosolic components
	  ◦ Creatine kinase—specifically MM isozyme
	  ◦ Lactate dehydrogenase
	  ◦ Myoglobin
	  ◦ Aldolase
	  ◦ Carbonic anhydrase III
	  ◦ Myloperoxidase—indicative of neutrophils
	  ◦ *N*-acetyl-β-glucosaminidase—indicative of monocytes
	  ◦ Potassium
	• Blinded histological examination
	  ◦ Lesion size
	  ◦ Severity
	  ◦ Presence of necrosis/degeneration
	  ◦ Presence of inflammatory cells
	  ◦ Edema
	  ◦ Hemorrhage

wide array of biochemical and histological markers associated with these specific sites. A listing of the various available in vitro and in vivo markers for evaluating tissue damage is provided in Table 1.

Pain on injection is an unpleasant sensation associated with the injection of a *formulation* (as defined in the above paragraph). Pain on injection is often acute in nature as it is limited to the normal time for healing or the time necessary for neutralization of the initiating or causative factors. Evaluating the potential of a *formulation* to cause pain has been found to be more difficult to quantify experimentally as this process is associated with the activation of pain receptors, nociceptors, at the injection site. The sensation of pain is mediated in the periphery by multiple sets of specialized afferents called nociceptors. A brief overview is discussed in the following text as an introduction to this topic, but for additional and more specific information about acute versus chronic pain, the reviews by Brazeau, Schmelz, Dussor, and Mense are useful in this regard (1–4).

There are three different relationships linking tissue damage with pain on injection. The most likely relationship is the *formulation* causes tissue damage, and this damage results in the release of intracellular molecules that activate nociceptors, resulting in pain as suggested by outward behavioral indicators such as licking the injection site or guarding/minimizing the use of the limb. Alternatively, a formulation could result in the direct activation of nociceptors and produce pain without any specific tissue damage. A third potential relationship is tissue damage associated with the *formulation*, but the *formulation* itself may inhibit the nociceptive pathways. This later relationship may be the hardest to screen *formulations* unless specific markers of tissue damage and approaches are included in the evaluation. An easy way to consider the relationships and considerations between tissue damage in muscle with pain following intramuscular injection is provided in Figure 2.

Why the Importance of In Vitro and In Vivo Animal Studies to Evaluate Tissue Damage and/or Pain on Injection?

It might be questioned why it is necessary for the utilization of in vitro and in vivo animal methods to evaluate and screen *formulations* for tissue damage and/or pain on injection. Ideally and initially, it is advantageous and cost effective to identify any potential tissue damage and/or pain on injection of a given formulation prior to the clinical trials. However, in vitro methods can provide formulators with the opportunity to screen various excipients, evaluate different formulation compositions and delivery systems, as well as evaluate the mechanisms of acute tissue damage to optimize the initial selection of a formulation. In vivo studies not only provide the opportunity to further confirm the in vitro results but can also allow investigators to look at the effect of blood flow, the immune system, and the intact pain system as shown in Figure 3. As such, formulators are encouraged to consider both in vitro and in vivo *studies* to thoroughly optimize injectable formulations prior to commencing any clinical studies.

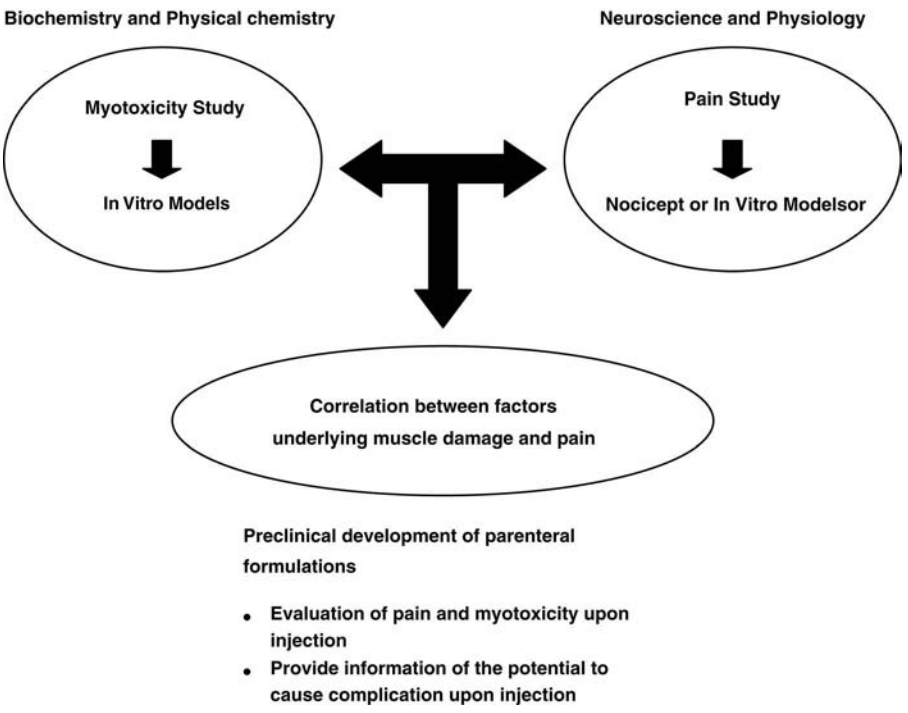


Figure 2 Link between myotoxicity and pain on intramuscular injection.

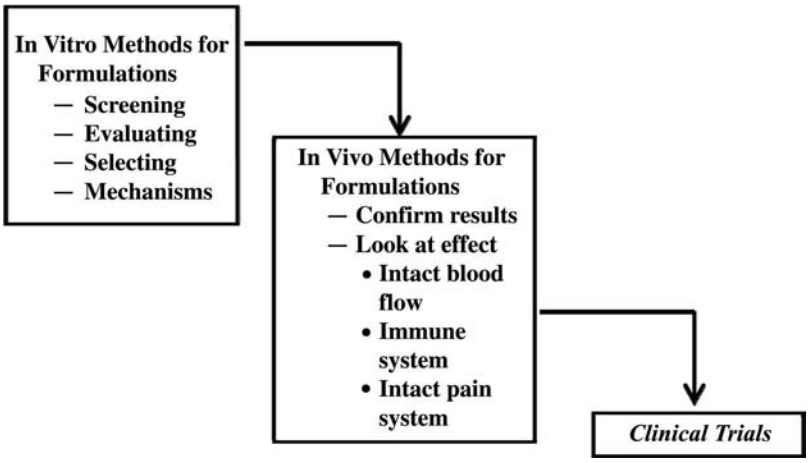


Figure 3 Importance of in vitro and in vivo methods in optimizing formulations.

General Overview on the Mechanisms of Tissue Damage

It is important to define key terms when considering tissue damage or pain on injection. An irritant is the molecule that can be linked to the source of irritation, either pain or tissue damage. Alternatively, a vesicant is a highly reactive molecule that combines with DNA, proteins, or other cell components resulting in cellular alterations that can be reversible or irreversible. It is essential to know and characterize the chemistry of molecules in a parenteral formulation as this can provide insight as to whether the structural elements may be likely to react with cellular components at the injection site. The knowledge of the structural elements provides a key as to whether the excipients or the therapeutic agent in the parenteral formulation has the potential to be an irritant or a vesicant. This highlights the importance of systematically screening all the components in a formulation or to avoid the use of specific agent if there is a potential for tissue damage/pain based on the chemical structure, the literature, or previous experimental findings. One needs to consider all parenteral formulations from the pathological perspective, specifically whether a given injectable component can result in inflammation, soreness, or irritability of a cell, tissue, or organ system, and from the physiological perspective, whether this compound results in an elicitation of an activity or response in an organ or tissue that could result in a pathological alteration.

It becomes critical for investigators to become familiar with the various types of mechanisms that could result in damage to the tissues at the site of injection. Consultation with toxicologists can provide important insight into identifying the potential mechanisms responsible for tissue damage at the injection site. For example, in skeletal muscle, there are several mechanisms that can be initially considered when evaluating formulations for their potential to cause tissue damage. These potential mechanisms by which a molecule could cause muscle damage include (*i*) a disruption of the sarcolemma (the muscle membrane), which could disrupt intracellular homeostasis; (*ii*) a disruption or alteration in the mechanisms responsible for maintaining intracellular calcium homeostasis as this is essential to muscle functioning, and increased cytosolic calcium is associated with tissue damage; (*iii*) an interference in mitochondrial functioning thus disrupting homeostatic processes; (*iv*) an increased oxidative stress leading to formulation of reactive molecules, thus disrupting cellular functioning; and (*v*) dramatic changes in intracellular or extracellular pH or tonicity, which can result in cellular distress (5–13).

General Overview on the Mechanisms for Pain on Injection

Pain on injection involves the activation of nociceptors at the injection sites (1). Three types of nociceptors seem to be involved primarily with pain on injection and involve chemical,

thermal, or mechanical sensitivity. This includes the acid-sensing ion channels that are activated by protons and have a preference for sodium, the heat-gated vanilloid receptors (VR-1 capsaicin) that are activated by heat ($> 45^{\circ}$) and capsaicin and nonselective to cations, and the mechanosensitive or stretch-activated channels that are responsive to membrane stress and mechanical forces when cells are exposed to either hypo-osmotic or hyperosmotic fluids.

CONSIDERATIONS IN MODEL SELECTION FOR TISSUE DAMAGE

The selection of the in vitro or in vivo model for evaluating the potential of a drug, excipient, or formulation to cause tissue damage on injection requires the investigator to be knowledgeable of the particular aspects of these particular methodologies. These aspects include the advantages and disadvantages of the model; the parameters utilized to evaluate the tissue damage; the key experimental assumptions; important experimental cautions, limitation, and the requirements or approaches for data analysis. An investigator who neglects to take these aspects into consideration in utilizing these approaches may end up with experimental results that may not be that useful for screening, evaluation, and selection of parenteral formulations that are not associated with tissue damage on injection.

IN VITRO METHODS FOR EVALUATING TISSUE DAMAGE

In vitro methods can play a critical role in the selection of excipients or the development and comparison of various parenteral formulations. These methods, in general, can be easily developed and implemented in any laboratory setting and can provide an approach for the establishment of a database related to specific excipients and formulations useful for future studies, given the experimental assumptions and limitations are taken into consideration.

Red Blood Cell Hemolysis Methods

The utilization of red blood cell hemolysis with the release of hemoglobin as a marker for evaluating formulation-induced irritation continues to be an important approach in developing and optimizing injectables, particularly those intended for intravenous injection. Two types of experimental systems have been implemented, and involved either a static evaluation or flow through dynamic evaluation of the acute interaction between the test formulation and red blood cells as reported by Yalkowsky and coworkers (14–21) and Obeng and Cadwallader (22). Yalkowsky and his team have contributed significantly to the use of red blood cell hemolysis as an indicator of tissue damage (14,21). In a static evaluation of the interaction of a formulation with red blood cells, there are several key issues to be addressed. This includes limiting the sources of the red blood cells and ensuring adequate and consistent time for the interaction of the formulation with the red blood cells as this will minimize the variability. Furthermore, it is critical to keep the ratio of the test vehicle to the red blood cells constant, to incorporate in the study design the appropriate negative or positive controls, and to incorporate during the hemoglobin quantification an extraction method that avoids possible changes in hemoglobin absorption maxima by the test solution through the use of a standard matrix for the spectrophotometric analysis. Additional considerations for the dynamic flow through system include ensuring there is a consistent flow through the system to allow adequate mixing and interaction with between the test solution and the red blood cells (22). One advantage of the dynamic flow through system is it enables the investigator to vary the injection speed to look at dilutional effects and the impact on this interaction between the formulation and red blood cells.

Cell Culture Methods

The use of muscle cell cultures can be an important tool for evaluating tissue damage on injection. Two muscle cell lines have been found to be particularly useful in looking at parenteral induced tissue damage, specifically for intramuscular injectables. These cell lines are the rat L6 myoblasts and mouse C2C12 myoblasts, and both are available commercially (23–29). Cell culture methods can be easily adopted in the laboratory and are advantageous, given this is a relatively rapid approach to evaluate the acute effect of the test compound. Cell culture methods to evaluate tissue damage can employ the release of intracellular components (often cytosolic enzymes such as creatine kinase or lactate dehydrogenase) into the medium,

the concentration of intracellular components remaining in the cells after removal of the medium or an assessment of cell viability or cell death caused by the treatment. If the measure of tissue damage is the release of cytosolic enzymes into the medium, the investigator must also conduct the requisite preliminary studies showing the presence of the treatment does not interfere with the activity of the specific enzyme. It is critical to evaluate the extent to which a treatment formulation may reduce the number of cells as they may be lifted from the plate during the experiment, particularly if one is analyzing the release or retention of intracellular components. As such, it becomes critical for the investigators to always normalize their experimental findings for cell number, protein, DNA, or other markers useful to characterize the cell population. Furthermore, it is critical to include in the experimental design the appropriate negative and positive control treatments as the benchmark for evaluating the extent of tissue damage.

A limitation of any cell culture approach is that the investigators must be cognizant of the specific passage number for the cell line. Secondly, experimental results can be confounded by complications associated with formulations that are not isotonic, as this could result in cell swelling and lysis associated with hypotonic solutions and cell shrinkage associated with hypertonic solutions. Cell passage number and tonicity can impact upon the concentrations of intracellular components often utilized as parameters for evaluating tissue damage, thus confounding experimental results.

Another key issue associated with muscle cell culture methods is whether to utilize either myoblast (immature muscle cells) or to differentiate the cells into mature muscle cells (myotubules) as this can impact on the concentration of intracellular components used as markers in the screening process. Both the L6 and C2C12 cell lines can be differentiated into myotubules as judged by increases in cytosolic enzymes and morphological changes. Figure 4 shows the difference in L6 and C2C12 in growth medium (2% fetal bovine serum in Dulbecco's

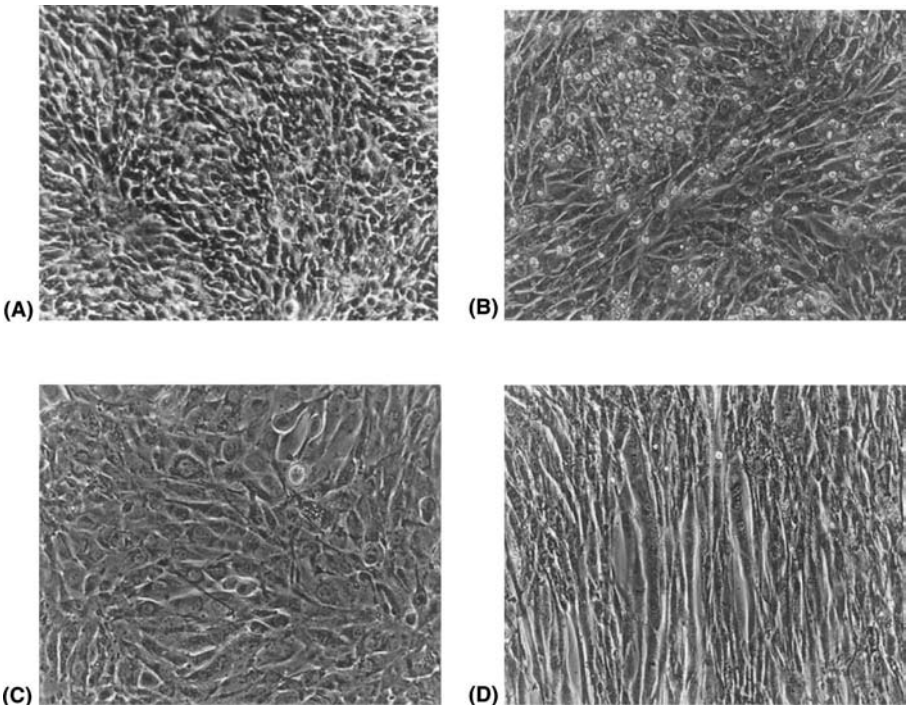


Figure 4 (*See color insert*) (**A**) Four-day-old L6 myoblasts in GM. (**B**) L6 myotubules at day 6 in DM (2% FBS in DMEM) during fusing process. (**C**) Four-day-old C2C12 in GM. (**D**) C2C12 myotubules at day 6 in DM (10% HS in DMEM). *Abbreviations*: GM, growth medium; DM, differentiation medium; FBS, fetal bovine serum; HS, horse serum.

Table 2 Creatine Kinase Activity During Differentiation in L6 and C2C12 Cell Lines

	Creatine kinase activity (U/L)[a]	
Cell line	Myoblasts	Myotubules
L6	132.2 ± 19.8	73.6 ± 11.6[b]
C2C12	2905 ± 46	3599 ± 308[c]

[a]Means $\pm$ SEM. Data was obtained from the appropriate initial cell density of 6×10^6 cells per sample.
[b]Significantly lower than that of myoblasts ($p < 0.05$).
[c]Significantly higher than that of myoblasts ($p < 0.05$).

Modified Essential Medium (DMEM)) compared with these cell lines in differentiation medium (10% horse serum in DMEM). There is a pronounced morphological alteration during differentiation process as shown by the change formation of myotubules in panels B and D. Futhermore, there can be a pronounced difference between the two cell lines in the creatine kinase activity in myoblasts versus myotubules as shown in Table 2.

Tissue Reactivity Model

Silva and colleagues reported a tissue reactivity model that can be useful to look at biocompatibility or toxicity of biomaterials, parenteral formulations, or delivery systems (30). In this experimental system, L-929 cells are grown to near confluent monolayers followed by removal of the culture medium that is replaced with agar-containing medium and neutral red vital stain (marker of cell viability). Following solidification of the agar, the treatment is placed on the cells with control treatments (on filter paper), and the cells are then incubated for 24 hours at 37°C. The culture can be evaluated microscopically around the treatments, and toxicity is measured by the loss of the vital stain. The investigator is able to evaluate the biological reactivity (cellular degeneration, lysis, malformation, and sloughing) by calculating a zone index (ZI) with a range of reactivity of the treatment ranging from 0 with no detectable zone around the sample to 5, which involves the entire dish (as the numerator) and a lysis index ranging from none to severe (80% of the zone affected) as the denominator for the controls and treatments. It is critical to include the appropriate positive and negative control treatments in this system (30).

Isolated Skeletal Muscle Systems

Rodent isolated muscles can also be useful in screening *formulations* for their potential to cause tissue damage for both intramuscular and subcutaneous injectables (31,32). This method involves direct administration of small volume (15 μL) of the treatment into either the extensor digitorum longus (EDL) muscle or the soleus (SOL) muscle. These two muscles are utilized because (*i*) they can be easily isolated and removed via the tendon connections from the rear legs without directly touching or damaging the respective muscle, (*ii*) the treatments can be injected easily into the body of the muscle belly using a small gas chromatographic syringe, and (*iii*) the muscles can be saved at the end of the experiment for possible histological evaluation. It is recommended to utilize both muscles in these studies as the EDL and SOL muscles can provide an indication of potential tissue damage to fast twitch glycolytic muscles or slow twitch oxidative muscles, respectively, and most human skeletal muscle is primarily composed of mixtures of these two muscle fiber types. The experimental design can involve using the two EDL and SOL muscles for one specific treatment, thus enabling duplicates for each animal for both muscles. Alternatively, the experimental design can utilize one EDL or SOL as the treated muscle, while the contralateral muscles could be used as the control (no treatment or solvent control).

In general, this experimental protocol involves male Sprague Dawley or one consistent strain of rats, six weeks old, 150 to 200 g, that are humanely sacrificed using cervical

dislocation after being anesthetized. After carefully isolating and dissecting out the EDL or SOL muscles (their weight is ~200 mg for an adult rat), the treatment being investigated is injected lengthwise into the belly of the muscle. An optimal injection volume is 15 μL as this causes a small welt on the muscle and the investigator can visualize whether the treatment has leaked out of the muscle. Larger volumes (25 μL) are associated with more difficulties in the treatment leaking out of the muscle, while smaller volumes (5 μL) may not be sufficient to elucidate a response in the skeletal muscle. Once the muscle has been injected, it is suspended in the incubation vessel via placing the muscle into a small basket (a long narrow circumference teflon tube with holes to prevent the muscle from floating or being disrupted by the aeration process) and placed into 8 to 10 mL of balanced salt solution through which is being bubbled a carbogen (95% O_2–5% CO_2) at 37°C. One such balanced salt solution that has been utilized is composed of 116 mM sodium chloride, 5.4 mM potassium chloride, 5.6 mM dextrose, and 262 mM sodium bicarbonate adjusted to pH 7.4. This solution does not contain calcium, which has been shown to exacerbate skeletal muscle damage (33).

The extent of tissue damage can be measured by the release of cytosolic enzymes into the incubation medium over a specific period of time. The most useful markers of tissue damage are the release of enzymes such as creatine kinase or lactate dehydrogenase that can be easily quantified using available spectrophotometric kinetic assays. The most useful approach has been to measure the activity of the released enzymes at 30-minute intervals from the time the muscle was injected with the formulation. This is easily facilitated by draining the incubation medium at 30-minute intervals and replacing the incubation vessel with fresh balanced salt solution. Experiences with these isolated muscles indicate that tissue viability is maintained for 90 to 120 minutes as noted by a dramatic increase in enzyme release after 90 or 120 minutes. As such, tissue damage is quantified by the cumulative release of the enzyme (as measured by activity) over the experimental period. One caution, though it may be minor, given the injection volume of the test formulation (15 μL), the muscle size (~200 mg), and the incubation medium (8–10 mL) in this study design, is to always consider whether the treatment has the potential to interfere with enzyme activity or with the measurement of this enzyme activity. This issue can easily be addressed through simple preliminary studies looking at enzyme activity in the absence or presence of the treatment.

One overall advantage of this experimental system is it involves direct injection into the muscle tissue like an intramuscular administration. In addition, it can also provide a basis for evaluating subcutaneous injectables for their potential to cause tissue damage since this injection site is often adjacent to muscle tissue that may become damaged. Additional advantages of this type of experimental system for screening *formulations* for their potential to cause tissue damage is that the process is relatively rapid, uses a minimal amount of the test formulation, can easily be learned by new investigators, and is reproducible over time and location with minimum variability as measured by coefficient of variation in the experimental results of 10% to 20%. This system has also been shown to correlate well with in vivo results in animals and clinical trials (32,34,35).

A limitation in this experimental system is that it measures only the acute toxicity to the muscle tissue caused by either a direct effect on the muscle membrane (sarcolemma) or rapid biochemical changes as a result of the injected formulation. It is also critical to include the appropriate negative and positive control formulations in the study design as a basis for evaluating the magnitude of the tissue damage caused by a given formulation. Useful negative controls (those formulations that do not cause tissue damage) can include an uninjected muscle, a needle puncture alone with no vehicle, normal saline, and 5% dextrose, while positive controls (those formulations that have been shown to cause tissue damage) can include directly slicing or damaging the muscle, slicing the muscle in half, or other formulations such as surfactants at higher than normally used concentrations, solvents such as propylene glycol at 40% vol/vol or higher or available parenteral formulations that have been shown to cause muscle damage (32). Cautions, as stated earlier, in utilizing this experimental system or in selecting the appropriate positive or negative controls must take into account the viability of the isolated muscle and ensuring there is no interference in the measurement of the released enzyme or other cytosolic component being evaluated as the marker for tissue damage (36).

IN VIVO METHODS FOR EVALUATING TISSUE DAMAGE

Infusion-Related Thrombosis

Several studies have reported infusion-related thrombosis; however, this seems to be attributed most frequently to device used in the drug administration. Examples include continuous infusion of interleukin-2 and total parenteral nutrition (37,38).

Rabbit Model

The rabbit lesion model is a generally accepted method for the prediction of muscle damage following intramuscular administration of drugs (39,40). This is because the damaged area is readily visible and can be quantified using histological approaches. If the damaged area is sufficiently large, it is generally considered a lesion. This model has been extensively referenced since 1949 (41) and remains the "gold standard" for predicting formulation tolerability in humans (42). Being more sensitive to intramuscular inflammation than humans, rabbits serve as a good and gentle animal model to screen formulations that might be intolerable in humans.

The typical method of rabbit lesion assessment involves injecting groups of animals with 1 mL of test and reference articles, approximately 0.6 cm deep into the sacrospinalis muscle using 23-guage sterile needles. The animals are euthanized over period of time postdosing, for example, 1, 2, 3, 6, 12 through 24 days, and lesions monitored for hemorrhage, lesion volume, and histology. Protocols that benefit with creatine kinase measurements in blood involve sample and testing blood samples through 72 hours postinjection. The results are typically converted into area under the creatine kinase curve and used to compare formulations versus control treatments or other treatments.

Given inherent variability with animals, typically a group of six animals are advised per treatment, which allows good differentiation between different formulations and drug concentration effects (43). Key advantages of the method include its broad acceptability, opportunity for testing multiple treatments per animal to permit crossover comparison, correlation of test data with historical or published data, and ability to monitor the lesion size. The disadvantages of the method are somewhat inherent to animal models, like relatively more expensive over the in vitro methods and need for training in handling of animals, including dosing of test articles and blood sampling from the ear vein for creatine kinase measurements.

Rodent Model

While the rabbit model has certainly been useful in evaluating tissue damage associated with parenteral injections, the cost, time, and difficulties associated with the use of this animal model may limit the enthusiasm of such an approach. Alternatively, a rat model can be useful for evaluating injectable formulations, given the reduced costs, easier experimental design, and time considerations (35,41,43). The rodent model has been shown to be useful and complements the findings observed with the isolated muscle model (35,44–46).

In this specific experimental design, the rodent is canulated via the jugular vein and allowed to recover for at least 12 hours prior to initiating the study. In previous studies, this 12-hour period is sufficient to allow the serum creatine kinase levels (the marker of tissue damage) to return to baseline following the surgery for the placement of the jugular cannula. The treatment (200–500 μL) can then be injected either into the gastrocnenius muscle (one of the two main muscles of the calf with the SOL adjacent to the gastrocnemius) or the gluteus medius muscle (in the pelvic area on the dorsal side). The advantage of using a rat versus the rabbit is the duration of the experiment one needs to utilize in characterizing the serum creatine kinase levels. Experiences with measuring serum creatine kinase levels at specific times as a marker of tissue damage may only require up to 72 hours and in most cases will only require 24 hours (as compared to an average 7–10 days in the rabbit). This would enable the investigator to easily design crossover studies as based on the patency of the jugular cannula. An additional advantage of the rodent system in studies have shown that peak creatine kinase levels occur at two hours after injection and is independent of the magnitude of the tissue damage caused by the formulation (in rabbit studies peak creatine kinase levels varied as to the severity of the tissue damage, with the most damaging formulations peaking at an earlier time compared with less damaging formulations) (35,39). This would enable investigators to

evaluate serum creatine kinase levels as a second measure of tissue damage. Additional advantages are the ease of working with rodents versus rabbits, given their size differences and costs in housing and caring between the two species. A limitation of using rats is the volume of and number of blood samples that can be taken daily or during the course of the study.

IN VIVO METHODS FOR EVALUATING PAIN ON INJECTION

Pain on injection can occur following local administration of drug (e.g., subcutaneous and intramuscular) as well as on infusion, like in the case of intravenous or intra-arterial injection. Often model selection for assessing pain on injection is dependent on the route, frequency, and duration of injection. Three animal models have been successfully applied for assessing pain on injection for both local as well as infused drug administrations.

Rabbit and Rat Vein Models

In these models, the respective animal is used for infusing test and comparator samples intravenously, and often the results are assessed visually in terms of local site reaction and the associated changes. Implicitly, an article with least local visual change on infusion is considered the least painful to the animal.

Rabbit ear vein has been used to assess pain on injection. In this model, groups of three to five animals receive a fixed drug concentration (e.g., 1–10 mg/mL) and a set total dose (e.g., 1–10 mg) over a predetermined infusion rate through their marginal ear vein (e.g., 1 mL/min). Use of set dosing parameters allows comparison of results among different formulation groups as well as with negative control like saline or dextrose. Following dosing, each animal is examined carefully at the site of injection, for up to 24 or 48 hours, for swelling, bruises, and/or discoloration of the injection site and surrounding tissue. Generally, no change on injection is indication of a relatively well-tolerated formulation. This model has been used for assessment of numerous drugs known to be painful on injection such as clarithromycin (47). The model assumes that pain on injection will translate into visual change at and around the site of injection.

The rabbit vein irritation test has been shown to be effective in that a comparison of a lactobionate solution of macrolide antibiotic clarithromycin with its emulsion formulation and a dextrose control demonstrated the negative control (dextrose) to cause no local changes. The solution formulation of drug caused flushing of blood at the site of injection in all three animals in this test group, with bruises lasting through 24 hours after dosing. However, an emulsion formulation intended to reduce pain on injection caused no local change in two of the three animals in this test group immediately on injection, one of which demonstrated no change through 24 hours after dosing and the other showed limited bruising during this period. The third animal in this group exhibited some bruises immediately on injection that lasted through the 24 hours observation period. The emulsion formulation was deemed to be more tolerable than the solution formula based on correlation of the results from this animal model with those from other models (48).

The rat tail vein test complements and provides data comparable with the rabbit ear vein model. Because of smaller size, typically six animals are used per test group in this model. The infusion rate is kept low, for example, 0.3 mL/min, and the results can be compared against controls after one or multiple dosing. Again, in a study comparing an emulsion formulation of macrolide clarithromycin against its solution form, the negative control dextrose demonstrated purple, pink, and red spots near the area of injection in five out of the six animals in this group. Upon administration of the drug lactobionate solution, all six animals in this group demonstrated pink, red, and purple area covering large portion of the tail around the injection site. As a comparison, the emulsion formulation indicated limited spots near the site of injection in fewer animals (48). The similarity in results in these two animal models is generally believed to be a good predictor of similar manifestations in human clinical trials.

Conscious Rat Model

Subjectivity and lack of good correlation in assessment of pain on injection based on visual scores and patient response led Marcek et al. (49) to investigate the response to the intravenous

injection of test articles in rats restrained in a tube that, in turn, is connected with a data acquisition system. The model is based on the premise that the rapid onset of vocalization and struggle in the restraint tube following injection, and the disappearance of these signs on completion of injection, is indicative of pain caused by the product injected to the animal. The authors have validated this model using isotonic and hypertonic formulations, increasing concentrations of pain-inducing chemicals that demonstrated good correlation with the results, as well as with range of marketed products known to cause pain on injection. Although the model has been shown to discern formulations with differing pain-inducing abilities on injection, it is somewhat complex to step up specifically for rapid screening in preclinical drug development.

Rat Paw-Lick Model

This model is based on the theory that if a substance is injected in the paw of a rat, the frequency of paw licks by the animal are proportional to the pain at the site of injection. Implicitly, a formulation that does not cause pain on injection, for example, saline or dextrose, would not stimulate the animal for paw licks; however, a more painful chemical injected in the same area would trigger the animal to lick its paw. The model was initially developed for testing local pain on injection, for example, subcutaneous injection (50). The authors demonstrated good correlation between concentrations of pain-inducing drugs cefoxitin and cefazolin and paw licks over a 12-minute interval after injection. In addition, the authors were able to prove local anesthetic effect, and hence reduction in paw licks, after injection of these drugs that also contained lidocaine. The model also seems to correlate reasonably well with creatine kinase levels on injection, a marker of pain/irritation following injection (51).

A good correlation has been noted between normal and extreme pH of injectable samples with rat paw licks, as well as between cosolvent concentration in treatment and rat paw licks (52). Finally, a good correlation has been demonstrated between pain-causing formulation of macrolide clarithromycin and its less-painful emulsion formula with paw licks, and the results corroborate well with other models like rabbit ear vein and rat tail vein results.

A major limitation of this model is local drug administration and small injection volume. Although these limitations may not play a role in testing samples intended for local injection, the model may identify pain through paw licks for formulations that may not cause the same physiological response on intravenous injection due to dilution.

Now and the Future Use of Molecular Genetic Methods in Evaluating Tissue Damage and Pain on Injection

With continued advancements in the area of molecular and genomic technologies, parenteral formulators will have the opportunity to employ screening techniques to identify specific biomarkers for tissue damage or pain on injection and whether these biomarkers are upregulated or downregulated with given excipients, drugs, or formulations. The availability of specific quantitative polymerase chain reaction (QPCR) gene-array systems to evaluate specific biochemical pathways will enable investigators to employ experimental systems ranging from cells culture methods and animal studies to even simple initial clinical trials to rapidly screen formulations for their potential to activate cellular entities thought to be associated with tissue damage and/or pain on injection. In clinical studies, this could be crucial if there is concern that repeated injections may result in the development of tissue damage. Furthermore, these experimental methods would enable investigators to identify and quantify specific biomarkers as a measure for tissue damage or pain for a given formulation or classes of compounds.

CONCLUSIONS

While not all injectables may be associated with damage and/or pain on injection during preclinical and clinical trials, when this occurs in an injectable product it can be a challenge to the subsequent optimization of the final formulation and to the acceptance by clinician and patients. It becomes necessary, therefore, for pharmaceutical scientists to be aware of the available experimental approaches, both in vitro and in vivo, to screen and evaluate excipients, drugs, or various formulations for their potential to cause tissue damage and/or pain early on in the development of injectables. The available literature can provide important insight into

the types of excipients, drugs, or formulations, which have been associated with these adverse effects during injection. Careful design of the parenteral formulation based on early screening and evaluation studies for any potential tissue damage and/or pain on injection can result in the savings of time and financial resources during subsequent studies in the development and approval processes. Furthermore, the development of an in-house database related to the potential for chemicals to cause damage and/or pain using existing experimental methods will enable the rational design of future formulations intended for parenteral administration.

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9 Parenteral product specifications and stability

Michael Bergren

INTRODUCTION

Specifications and stability of parenteral products are set in the broader context of drug product development of small molecules, biologics, and devices. The specifications for a finished pharmaceutical product are an accepted list of requirements that a product must meet before it is released into distribution. Typically, these requirements are laboratory tests and associated acceptance criteria. International Conference on Harmonisation (ICH) Q6A (1) provides a suitable working definition of specifications. For purposes of this chapter, the definition should be broadened to extend beyond "new" drug substances and products.

> A specification is defined as a list of tests, references to analytical procedures, and appropriate acceptance criteria, which are numerical limits, ranges, or other criteria for the tests described. It establishes the set of criteria to which a new drug substance or new drug product should conform to be considered acceptable for its intended use. "Conformance to specifications" means that the drug substance and/or drug product, when tested according to the listed analytical procedures, will meet the listed acceptance criteria. Specifications are critical quality standards that are proposed and justified by the manufacturer and approved by regulatory authorities as conditions of approval.

The concept of specifications is central to globally accepted principles of Good Manufacturing Practice (GMP). Specifications are product-specific and must ensure, in the words of U.S. GMP regulations, that "drug products conform to appropriate standards of identity, strength, quality, and purity" (2). Further, existing U.S. GMP regulations state "For each batch of drug product, there shall be appropriate laboratory determination of satisfactory conformance to final specifications for the drug product." Specifications must also be developed for investigational products used in human clinical trials. In contrast to marketed products, specifications for clinical trial materials generally reflect the more limited understanding of the product and assume greater commensurate restrictions on its use in a carefully monitored clinical setting.

The topic of specifications for the broad category of parenteral medications shares elements common to all drug products, but it includes many additional complexities unique to parenterals, particularly within the broad scope of modern parenterals.

1. Because parenteral administration is fundamentally invasive, specifications always include requirements for sterility, limits on byproducts of microbes (e.g., endotoxin), and limits on particulates, particularly in the case of intravenous administration.
2. A large number of parenteral products are not sufficiently stable to be marketed as solutions. These products must be reconstituted prior to injection, in many cases from a lyophilized solid or sterile powder. Specifications on these products must incorporate an understanding of factors that influence the solid-state stability of lyophiles, frequently incorporating partially or completely amorphous drugs.
3. Biopharmaceuticals are a large and rapidly growing category of complex substances that are delivered almost exclusively via the parenteral route. Specifications for biopharmaceuticals generally reflect a qualitative difference in both our capacity for analytical characterization of these molecules and the robustness of these products.
4. Increasingly, parenteral medications are being developed to achieve either sustained or targeted delivery. Many of these products require specifications based on chemical or functional tests to help ensure consistent drug release or targeting. While there is some overlap with considerations applied to other categories of modified release drugs, the physiology, chemistry, and requirements are usually quite different.

5. Parenterals are commonly in intimate contact with product packaging and may be delivered via admixtures or through a variety of devices. Specifications may need to include formulation quality attributes affected by packaging or administration sets.

The topic of biopharmaceuticals will benefit from a brief explanation of terminology in this introductory section. For purposes of this chapter, drug substances will be classified in one of two broad categories: synthetic/semisynthetic drug substances or biopharmaceutical drug substances. This is a somewhat arbitrary division that is largely drawn to recognize two quite different situations from a regulatory and specifications perspective (3). While these categories appear to relate to the origin of the drug substance, they are equally associated with our capability to achieve complete molecular characterization of the drug using modern analytical methods. A prototypical synthetic drug substance is a single chemical entity produced largely or entirely by organic chemical synthesis, with an impurity profile and a degradation profile that can be almost completely known and measured with available analytical methods. For such a substance, a chemical assay, or a collection of chemical assays, can be used with confidence to assure both biological potency and drug safety. Indeed, in many cases the terms "assay" and "potency" are used interchangeably. From a product and process design perspective, as well as a regulatory and specifications perspective, there are clear benefits to achieving this detailed level of understanding. In contrast, a prototypical biopharmaceutical drug substance is the isolated high molecular weight product of a biological organism. It is less well understood on a molecular level, sometimes much less well understood. Existing analytical tools fail to completely measure the molecular attributes that contribute to the biological activities—desired or undesired—of the prototypical biopharmaceutical drug substance, and an array of physicochemical analyses need to be supplemented by biological or biochemical assays to assess both potency and safety with confidence. The prototypical biopharmaceutical drug substance is more susceptible to changes resulting from stresses encountered in processing, formulation, or storage. Clearly, these prototypical examples are constructs that represent two extremes, but the categories they represent require significant differences in approach to specifications development. These differences are reflected in the regulatory guidances covering specifications and stability, and they will be emphasized periodically throughout this chapter.

Specifications are a broad topic that can be discussed in different contexts, but the focus of this chapter will be specifications in the context of parenteral product stability. Consequently, the final product specifications will be discussed with an emphasis on the relationship between specifications and product shelf life. Other aspects of specifications—including specifications on components, drug substance, raw materials, or in-process specifications—will not be specifically addressed. Even with this restriction, it is not possible to cover all areas in depth in a single chapter, in particular for areas where approaches to setting specifications are still evolving, such as biopharmaceuticals and controlled release parenterals.

DEVELOPMENT OF SPECIFICATIONS

Specifications gain acceptance as an outcome of the regulatory process associated with product registration. This application process is a formal dialog between the product manufacturer, or applicant, and the appropriate regulatory authority. Acceptability of final product specifications is predicated on the following elements of the application.

- Product definition
  The rationale for specifications is predicated on a well-defined product—including formulation, process, and packaging—manufactured according to current GMP standards. Although specifications on components will not be discussed in this chapter (note—other chapters address this topic), it should be noted that there are significant and stringent global requirements for specifications on raw materials, especially water, and packaging used in parenteral products.
- Product performance
  The product development process is expected to result in clear expectations for product performance and a corresponding understanding of attributes critical to

product performance. Specifications must be designed to ensure that marketed product performs safely and effectively, consistent with the performance in clinical studies that provided basis for approval.

- Regulatory expectations
  Proposed specifications are considered within the context of current regulatory expectations for the category and region in which the product will be marketed. Because of the intrinsically invasive nature of parenteral therapy, a number of specific requirements are integral to specifications for parenteral dosage forms.
- Stability
  Stability of the product must be understood and supported by confirmatory data. Storage conditions should be established that ensure the product continues to meet specifications throughout its shelf life or expiration dating period. Product attributes, and related test results, are expected to change over time, but specifications should be designed to ensure that products meet requirements throughout shelf life.
- Test procedures
  Test procedures and sampling procedures are integral to product specifications. Test procedures must be validated, and validation is specific to the formulation and process. Likewise, acceptance criteria are specific to the test procedure; changes in procedures may require revalidation of the method and the associated acceptance criteria may need to be revised, even if the product is unchanged.

From a manufacturing perspective, the central outcomes of drug product development are the final product specifications and the manufacturing process. Specifications created for products in early stages of clinical investigation are designed to ensure the safety of the product in the clinical setting, and to provide reasonable assurance of the integrity of conclusions derived from the outcome of the study. (In these early stages, risks are concomitantly reduced because clinical exposure is also limited, and there is significantly greater control over clinical setting.) Because knowledge is limited, specifications with quantitative acceptance criteria are commonly fewer in number and acceptance criteria may be less restrictive, with the exception of specifications for impurities. As formulation, and clinical, and process experience with the drug develops, specific product attributes may be identified that are critical to product quality (4). Identification of these attributes provides a framework for defining the experimental studies and data required to establish manufacturing process and specifications.

As a useful example, consider the case where release rate of drug from the formulation is critical for achievement of effective blood levels, and one of the important determinants of release rate is suspected to be particle size. Specific developmental studies—including combinations of clinical, nonclinical, and in vitro studies—may be designed to assess the dependence of blood levels on particle size, including interactions with other formulation factors. In addition, data collected from processing studies provide an assessment of the capability of the process to manufacture drug within a targeted range of particle size. In the end, the limits on particle size (i.e., acceptance criteria for the specification) may be established on the basis of requirements imposed by drug release, which has an established relationship to safe and therapeutic blood levels. However, if the manufacturing process is typically capable of producing particles in a much tighter range, the limits may instead be chosen to reflect the process capabilities. A third outcome is that developmental studies could be used to justify the absence of a specification for particle size, in the event that the process is shown to be consistently capable of producing material in a range where no meaningful variation in drug release could be demonstrated. The rational development of specifications based on an understanding of critical quality attributes is fundamental to concepts of Quality by Design, which are elaborated in a subsequent chapter.

REGULATORY EXPECTATIONS

Many long-standing requirements for parenterals have tests and acceptance criteria that are thoroughly vetted and well documented in regulations and compendia. Although requirements have long been similar across market regions, they were sufficiently distinct to create

great complexity in specifications for a "global" pharmaceutical product. Multiple tests were occasionally required for the same attribute to ensure regional regulatory approvals.

During the last two decades however, members of the Pharmacopoeial Discussion Group (PDG) have harmonized several important tests in the regional compendia in traditional major pharmaceutical markets—the United States, Europe, and Japan. This progress is reflected in significantly improved consistency among the compendia in these three regions, including *The United State Pharmacopoeia* (USP) (5), the *European Pharmacopoeia* (Ph. Eur.) (6), and the *Japanese Pharmacopoeia* (JP) (7). (For clarity and brevity, in subsequent text, references will be made to titles of specific compendial chapters without additional parenthetic references to the bibliography. All such references should be understood to refer to the editions, through indicated supplements.) All three compendia have specific umbrella discussions of requirements for parenterals: USP *<1> Injections*, Ph. Eur. *Parenteral Preparations*, and JP *General Rules for Preparations 11. Injections*, and many of the test requirements in these chapters provide references to chapters that have been partially or entirely harmonized.

In a parallel manner, regulatory expectations have been increasingly harmonized through the initiatives of regulatory and industry groups in these three major market regions. Their collaboration across a broad range of topics, under the banner of the *International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use*, has resulted in agreements commonly referred to as ICH guidelines (8). As an integral part of the ICH process, the ICH guidelines are adopted by regulators in participating regions. In some cases these guidelines are new, but commonly they displace prior guidance documents that were not harmonized. A parallel process for harmonization efforts for veterinary drugs have occurred under the auspices of VICH (9), which in many cases has resulted in quality guidelines analogous to those from ICH. The ICH harmonization process is woven together with the compendial harmonization efforts through a process described in ICH Q4B: Evaluation and Recommendation of Pharmacopoeial Texts for Use in the ICH Regions (10). Through this process, the ICH Expert Working Group recommends harmonized compendial text for adoption by regulators as interchangeable across ICH regions. The harmonized tests, along with considerations for implementation in each region, are published in annexes to the ICH Q4B guidance.

Although the ICH process centered on regulatory process in regions associated with major pharmaceutical research, from the beginning it involved observers from other bodies representing non-ICH regions, particularly the World Health Organization (WHO). As a consequence, ICH guidelines have had significant influence on regulatory processes and expectations well beyond Europe, the United States, and Japan. Guidelines have provided reasonable frameworks for regional harmonization initiatives, such as ASEAN Harmonization effort (11). Not infrequently however, regional initiatives have made changes to the guidelines to meet regional requirements. As a consequence, for example, the initial ICH guideline (12) on stability in Climate Zones III and IV was withdrawn because of concerns in Zone IV countries that stability conditions were insufficiently stressful to reflect climatic conditions prevalent in some locations. WHO and the regional initiatives are filling the gap through introduction of regionally harmonized guidelines (13). Hence, despite the value of global efforts toward regulatory harmonization, it is still important to understand regional regulatory expectations. Subsequent discussion will draw largely on the ICH guidelines, which adequately capture central global themes for parenterals specifications and stability, but regional details may differ, particularly outside the ICH regions.

Shelf Life Specifications and Release Specifications

Specifications are universally understood to be requirements that the product is expected to meet throughout its shelf life, when it is stored and dispensed according to the instructions provided in its labeling. Few products are timeless however. When products age, results of some tests will change over time. Stability studies are conducted to confirm that these changes occur in a reproducible manner that is characteristic of the product design. For those tests that change with time, test results at the time the product is released into distribution must be sufficiently within the acceptance criteria to ensure the product remains within the acceptance criteria throughout its shelf life. In effect, the product must be released according to a narrower

set of acceptance criteria. In some regulatory regions, the European Union in particular, "release specifications" must be submitted for regulatory approval in addition to "shelf life specifications." Whether release specifications are submitted for approval or not, Quality Assurance groups in most manufacturing organizations necessarily decide on product release by utilizing a set of criteria narrower than the shelf life specification. These criteria are normally designed to ensure the product meets specifications when tested throughout shelf life, accounting for changes that result from both product stability as well as measurement variability. Generally, and throughout the remainder of this chapter, "specifications" will refer to shelf life specifications. Where requirements refer to release specifications, they will specifically be noted as such.

Individual Specification Requirements

This section provides brief introduction to elements of common parenterals specifications as presented in ICH Q6A guideline (1) and major regional compendia. ICH recognized the distinctive challenges posed by biopharmaceutical products and consequently issued ICH Q6B (14), the scope of which covers "proteins and polypeptides, their derivatives, and products of which they are components (e.g., conjugates). These proteins and polypeptides are produced from recombinant or nonrecombinant cell-culture expression systems." ICH Q6B provides additional guidance for this category of biopharmaceutical products but refers to compendial testing requirements for many standard parenteral tests.

Volume of Injection

For liquid parenteral products, fill volume must be sufficient for withdrawal of the specified volume of injection from the container using the recommended configuration of needle and syringe for injection. Some overfill is generally required, the magnitude of which depends on both container volume and product viscosity. Specific requirements for volume of injection, both for single-dose and multiple-dose products, are found in USP *<1> Injections*, which is harmonized with JP *6.05 Test for Extractable Volume of Parenteral Preparations* and Ph. Eur. *2.9.17. Test for Extractable Volume of Parenteral Preparations*. The methods are considered interchangeable within the current compendia, as indicated in ICH Q4B Annex2 (15). The volume of injection may be determined as part of in-process testing, but the limit should be justified on the basis of the actual volume requirement for administration. Normally, volume of injection would not be evaluated as a stability-related attribute unless the product was packaged in a semipermeable container.

Description or Appearance

Description is a standard requirement that provides valuable qualitative information on the visual appearance of a product relative to a standard description. It typically includes an assessment of color, clarity of solution, physical integrity of lyophilized cake, homogeneity of dispersion, or visual indication of presence of foreign matter. Attributes such as physical separation of emulsions or dispersions or cake collapse, may be readily detected by appearance testing. As a supplement to the qualitative appearance test, specific quantitative compendial tests allow measurement of color or turbidity relative to a set of standards (e.g., Ph. Eur. *2.2.2. Degree of coloration of liquids*; *2.2.1. Clarity and degree of opalescence of liquids*; USP *<851> Spectrophotometry and light-scattering*). These tests may provide particularly appropriate tools for establishing thresholds of acceptability for appearance, or for allowing more quantitative trending of color and clarity on stability. Appearance is a vital requirement for stability testing.

Visible Particles

Solutions for parenteral administration are expected to meet compendial requirements. USP31 *<1> Injections* states that solutions should be "essentially free from visible particulates." Ph. Eur. 6 *Parenterals* requires that solutions for injection "are clear and practically free from particles," and JP XV requires that "Injections must be clear and free from readily detectable

foreign insoluble matter." Products are expected to meet these criteria at the time of release on the basis of established and qualified visual or machine-based inspection methods of each unit. Extensive history and many of the details associated with the requirement for visual inspection are provided in references (16). The limit on visible particles applies to all small volume parenterals, infusions, and reconstituted solutions. Where the character of the product does not allow for 100% inspection—either because of packaging or because the product must be reconstituted—the product must be suitably sampled for visual inspection. In some cases, the product may need to be transferred to an alternate vessel for inspection.

Parenteral products should typically be examined for visible particles as part of developmental and registration stability program for parenterals to ensure that instability related to decomposition or incompatibility does not give rise to the appearance and growth of visible particles. Although the product will typically be examined for subvisible particulates, some common mechanisms for particle nucleation and growth may result in visible particles without exceeding the limits for subvisible particulate. In general, visible particles should not appear on stability, but because of the probabilistic nature of the inspection process, visible particles may occasionally be evident in product on stability, even though product met inspection criteria at time of release and even in the absence of stability-related particle formation. Therefore, when particles are observed, it is particularly helpful to identify and characterize the particle to assess whether it is a foreign contaminant from the manufacturing process (extrinsic) or whether it is formulation-related (intrinsic). Trends in the occurrence of intrinsic particles may signify important stability-related changes in the product.

Subvisible Particulate

The requirement for subvisible particulates in parenteral solutions is harmonized among the three major compendia (USP *<788> Particulate Matter in Injections*; Ph. Eur. *2.9.19. Particulate Contamination: Sub-Visible Particles*; and JP *6.07 Insoluble Particulate Matter Test for Injections*). This requirement is uniformly applicable to both large- and small-volume injectables. However, the limits may be relaxed for injectables that are strictly administered via the subcutaneous or intramuscular route or for powders that are reconstituted prior to injection. (There is, however, a possibility that this exclusion may be dropped.) The test currently can be conducted via light obscuration (LO) or membrane microscopy (MM) methods. Although LO is frequently preferred, the microscopic method has advantages for formulations where optical or flow characteristics preclude the use of LO method. Additionally, if the results of the LO test exceed the limits, the procedure prescribes a second stage of testing using the MM method to assess whether the sample meets the requirement. Second stage testing may be required, for example, in the testing of prefilled syringes, where silicone oil droplets may contribute to high LO counts, but the oil droplets are filtered during the MM procedure. Applicable current limits for compendial articles are listed in Table 1.

Until demonstrated otherwise, the quantity of subvisible particulate should be considered a stability-related attribute. During product development, the MM procedure may be particularly useful for tracking and trending types of particles on stability, allowing the user to

Table 1 Subvisible Particulate Matter Acceptance Criteria for Parenterals

Nominal volume of container	Test attribute (μm)	Acceptance criteria	
		Light obscuration	Membrane microscopy
>100 mL[a]	Count ≥10	NMT 25/mL	NMT 12/mL
	Count ≥ 25	NMT 3/mL	NMT 2/mL
≤100 mL[a]	Count ≥10	NMT 6000/container	NMT 3000/container
	Count ≥ 25	NMT 600/container	NMT 300/container

[a]In the JP, containers with a nominal volume of 100 mL have the same requirement as containers with nominal volume of >100 mL.

Abbreviations: JP, Japanese Pharmacopoeia; NMT, not more than.

identify particles and the possible root cause associated with specific stability-related trends. Subvisible particulates, in the range of 1 to 10 μm, are increasingly of interest for therapeutic protein products because particulates containing aggregated proteins may have greater potential for stimulating undesired immune responses (17).

Sterility/CCI

Sterility is an absolute, universal requirement, specifically required by statute for essentially all types of parenteral products—human and veterinary drugs, biologics, and devices. Although sterility testing is commonly required, it is widely acknowledged that sterility testing of the final packaged product is a relatively weak tool for demonstrating acceptable levels of sterility assurance, which depends on establishing, maintaining, and monitoring appropriate process and environmental controls throughout the manufacturing process. Sterility testing only provides a final confirmatory evaluation of sterility for a validated sterile manufacturing process. Sterility test methods are in final stages of harmonization among three major compendia (USP <71> *Sterility Tests*; Ph. Eur. *2.6.1 Sterility*; JP *4.06 Sterility Test*). The harmonized texts are referenced in ICH Q4B Annex 8 (18), which provides references to the compendial editions/supplements containing versions of the test method that will be considered interchangeable by regulators across ICH regions. The methods are written to apply to a broad variety of parenteral products, and they include requirements for assessing the test suitability. Under some conditions, a terminally sterilized product may qualify for parametric release (19), in which case sterility testing is not part of the product release specification.

Sterility must be maintained throughout product shelf life, and therefore sterility testing is an essential requirement of specifications for stability testing, even if sterility testing is not required for product release in a parametric release environment. The critical factor in maintaining the sterility of the product is usually the integrity of container/closure system. A recent guidance from the FDA (20) allows for container/closure integrity (CCI) testing in lieu of sterility testing in stability protocols. The guidance does not specify or recommend a specific method for CCI testing, but requires that the validated method, and its relationship to microbial integrity, be discussed in the application or supplement. A growing variety of approaches to CCI testing (16) may offer useful alternatives to sterility testing on stability protocols. In contrast to sterility testing, many of these methods have the advantage of being nondestructive.

Endotoxin or Pyrogen Testing

Limits on bacterial endotoxin or pyrogen are a standard requirement for parenterals, and "endotoxins/pyrogen" is listed as a recommended test for parenterals in ICH Q6A. Bacterial endotoxin is an impurity introduced during product manufacture. The sterility of parenteral products ensures that bacterial endotoxin content will not increase over time, therefore endotoxin is not a common test requirement for stability.

Measurements of endotoxin are based on response to test reagents prepared from *Limulus* amoebocyte lysate (LAL). The response of the reagent is standardized relative to Reference Standard Endotoxin, the strength of which is expressed in endotoxin units, or EU (1 EU = 1 IU or international unit). Compendial methods for endotoxin are largely harmonized (Ph. Eur. *2.6.14 Bacterial Endotoxins*; USP <85> *Bacterial Endotoxins Test*; JP *4.01 Bacterial Endotoxins Test*). Results for endotoxin, as measured by these procedures, are expressed in EU/unit of dose—typically EU/mg or EU/mL. The general acceptance criteria for compendial tests are based on calculation of K/M, where K is a threshold dose for pyrogen response in EU/kg body weight, and M is the maximum recommended dose per kg body weight in a one-hour period. The threshold pyrogen dose is 5 EU/kg for most parenterals, but 0.2 EU/kg for parenterals administered intrathecally. Specific compendial monographs may contain alternative acceptance criteria, and alternative criteria for new drugs may be proposed on the basis of development experience and route of administration. Endotoxin specifications are also commonly required for medical devices, where the endotoxin limit is commonly expressed as 0.5 EU/mL of extract solution obtained by rinsing the device.

If the endotoxin method cannot be suitably validated, animal-based testing for pyrogens may provide an acceptable alternative. Each of the three compendia include pyrogen tests, but the tests have not been harmonized and therefore differ somewhat with respect to factors such as number of animals tested and acceptance criteria for temperature deviations (Ph. Eur. *2.6.8 Pyrogens*; USP *<151> Pyrogen Test*; and JP *4.04 Pyrogen Test*).

The displacement of the animal-based pyrogen test with the LAL test for bacterial endotoxin is an interesting example of widespread industry and regulatory adoption of, and transition to, improved methodology. Two recent books (21,22) review test methods, history, and test applications.

Uniformity of Dosage Units

Individual dosage units are required to be uniform with respect to drug content (ICH Q6A, ICH Q6B) based on standards established in compendia. The test and acceptance criteria for dose uniformity are being harmonized among compendia in the ICH regions (Ph. Eur. 2.9.4 *Uniformity of Dosage Units*; USP *<905> Uniformity of Dosage Units*; JP 6.02 *Uniformity of Dosage Units*). Specific regional considerations for acceptability and publication dates for harmonized texts are given in Annex 6 of ICH Q4B (23). As written, the dose uniformity test applies to a broad variety of dosage forms, including many single-dose parenterals. Dose uniformity can always be tested by measuring content uniformity, using an assay method for the active ingredient, or it may be assessed by the simpler alternative of mass variation in special cases. With one exception, content uniformity approach is required for single-dose injectable suspensions, emulsions, gels, and many solids containing additional ingredients. Mass variation can be employed for solid powder fills where the active is the sole ingredient, and for lyophilized products where the product was filled as a true solution and subsequently lyophilized. Content uniformity testing is always required for single-dose products containing multiple active substances, and the limits apply to each active. Dose uniformity is not a requirement for single-dose solutions because these meet requirements for both assay and extractable volume.

Identification

Identification is a requirement common to specifications for all drug products. As stated in ICH Q6A, the identification test should be able to establish the identity of the drug substance in the drug product and should discriminate among drugs closely related in structure. Multiple test methods and acceptance criteria may be applied in the event that a single method fails to show "sufficient" specificity. High-performance liquid chromatography (HPLC) retention times alone are regarded insufficient to establish identity, but the combination of retention time with distinctive UV spectrum or mass spectrum is usually considered adequate. An infrared spectral match of the extracted active ingredient is broadly regarded as a good example of a single identification method that is sufficient for lower molecular weight drugs.

ICH Q6B also requires identification testing in product specifications, although it is referred to as an "Identity" test in the Q6B guideline. Identity tests for biopharmaceuticals may be significantly more challenging because the molecular weight and molecular diversity of the drug may be greater, and it may be necessary to differentiate between materials that are more similar in structure. Alternative biological, immunochemical, or biochemical test methods may be utilized.

Identification tests are qualitative in nature, and method validation requirements (24) only include demonstration of specificity. Identity is not, therefore, a stability-indicating test, and the identity of the product is not typically reconfirmed as part of stability-testing protocols. Clearly, however, the identification test must retain suitable specificity to ensure that it can be used to identify the product throughout its shelf life.

Assay or Potency

On the basis of ICH Q6A, ICH Q6B, and broadly by statute in most regions, drug products require testing to assess content of the active substance (i.e., "strength" of the drug product as

required by regulation). For many drugs, and particularly for synthetic drug substances, drug content is commonly and readily measured by precise analytical assay of quantity per dose, or of drug concentration per unit volume administered. Assays are expected to be specific—meaning they can differentiate the active drug from closely related substances including components of the formulation matrix and potential drug impurities. In addition, assays normally should be stability-indicating, meaning they can analyze the drug accurately in the presence of its degradation products. HPLC, which has been developed in a wide variety of formats, is the methodology most commonly utilized for assay. Less specific methods, such as titrations, may still be utilized, if justified, but under these circumstances, supplemental analysis of degradation products on stability is required. Low-specificity methods are not commonly utilized for synthetic drugs in new drug applications, but they are common to many pharmacopoeial monographs.

Establishing an appropriate acceptance range for assay in the case of small-molecule pharmaceuticals requires an understanding of manufacturing variation, analytical method variation, and product stability. While there are no rigorous requirements for limits on assay, a range of 90% to 110% of labeled content or concentration is typical for small-molecule pharmaceuticals. Values outside of this range generally require some justification. A maximum range of 95% to 105% of label claim at time of release is required by European Union guidance on release specifications (25), unless special circumstances justify a broader range. This release specification is consistent with common manufacturing and analytical variation, as well as the broader acceptance range of the shelf life specification. Frequently, these ranges can be reduced on the basis of manufacturing experience. If analytical methods employed for content uniformity determinations are adequate, the assay value can typically be taken as an average of the content uniformity measurements.

For biopharmaceuticals, ICH Q6B recognizes significant challenges that complicate this assay of the active ingredient. Biopharmaceuticals—by virtue of their size, complexity, and sometimes molecular diversity—may be very difficult to analyze in the presence of substances that are chemically similar but not pharmaceutically active. Further, biopharmaceuticals typically have complex higher-order structures that depend on noncovalent interactions to achieve and retain molecular conformations, or shapes, associated with activity. Analyzing the population of active molecular species in properly folded conformations is challenging, and in many cases is only achieved by a combination of tools, including physicochemical assays, immunochemical assays, and biological assays. Physicochemical alterations are frequently monitored by a combination of methods, including chromatography, electrophoresis, and a variety of spectroscopic and spectrometric methods. Biological potency assays, either in vitro or in vivo, are typically required to ensure therapeutic efficacy throughout shelf life, and to provide some ability to select and interpret the physicochemical measurements. Specific acceptance criteria are unique to each product, but the same general principles apply: quantitative ranges must account for variation in assay, manufacture, and the maximum extent of change anticipated on stability. In addition, biopharmaceuticals typically require a specification for quantity, or total concentration, in the final product. The method may be nonspecific, such as an assay for total protein concentration.

Degradation Products

Impurities appearing in the product as a result of chemical changes in the drug over time are classified as "degradation products." These substances may result from a variety of reaction pathways: unimolecular decomposition, interaction with light or radiation, or from specific chemical reactions with components of the formulation or packaging system. They are distinct from "process impurities," which are substances produced as byproducts of synthesis of the drug substance, although some degradation products (DgPs) may also be process impurities. ICH Q3B (26) establishes expectations for specifications on DgPs in new drug products for small-molecule pharmaceuticals. The principles and language of this guidance have broader application, so the guidance is worth summarizing here because it provides a sound set of principles for the case where modern analytical technology is brought to bear on specifications development process for the category of drugs that are presently capable of relatively thorough

chemical characterization with these tools. Biopharmaceutical molecules, however, may degrade in ways that cannot be adequately or comprehensively characterized by current analytical methods.

On the basis of ICH Q3B, specifications for substances related to the drug in the drug product are required for DgPs only. Process impurities in a new drug product are assumed to be limited by specifications on the drug substance using principles of ICH Q3A (27), and the process impurities need not be monitored in the drug product unless they are also DgPs. To establish suitable specifications, a chemical understanding of degradation pathways (i.e., a degradation profile) must be acquired from scientific stability studies conducted with highly specific analytical methods during drug development. Final analytical methods should be capable of selectively quantifying individual DgPs and should exclude process impurities from the analysis. The exclusion of a related substance from the list of DgPs requires justification—on the basis of their chemical structure and established chemistry and/or data from scientific stability studies, including studies commonly referred to as "stress-stability studies" (28). Because DgPs are usually close structural relatives of the drug, the quantity of a DgP is expressed as a weight percentage relative to the drug at its label content.

With respect to specifications, DgPs are classified in three ways in the guidance.

- Identified vs. unidentified
  Identified DgPs have known molecular structure. Unidentified DgPs are specific substances with undetermined molecular structure. They are recognized by some characteristic behavior in analytical systems, such as chromatographic retention time.
- Qualified vs. unqualified
  Qualified DgPs have established biological safety profiles that support their safe administration under a dosing protocol when levels are below an established "qualification level." Unqualified DgPs are those for which a qualification level has not been established on the basis of safety data.
- Specified vs. unspecified
  If an individual specification (i.e., procedure and acceptance criterion) is associated with the DgP, it is considered a "specified" DgP. An "unspecified" DgP is subject to a general acceptance limit, which is applied to all individual DgPs that are not specified DgPs.

In addition to the requirement for individual degradation products, a specification must be established for total degradation products, which is defined as the sum of all reported DgPs.

ICH Q3B established clear guidelines for specific levels above which DgPs should be (*i*) detected and reported, (*ii*) identified, and (*iii*) qualified. These levels are derived in a manner that is dose-dependent, because exposure to impurities decreases with decreasing dose. The guidelines therefore provide a framework for establishing acceptance criteria during drug product development based on stability studies, analytical method characteristics, manufacturing experience, and safety studies. For some reconstitutable products, use-period stability studies may reveal increases in DgPs that need to be factored in to provide acceptable ranges of DgPs throughout product shelf life.

The fundamental concepts for biopharmaceutical products—as discussed in ICH Q6B—are similar, although the terms are further refined to capture distinctions less commonly encountered for small molecules. Further, precise guidance on thresholds for identification and qualification is not provided. Impurities in molecules derived from biotechnological synthetic pathways are classified in ICH Q6B as "process-related impurities" and "product-related impurities." *Process-related impurities* typically cover a broad range of cell-derived and process-derived constituents that must be evaluated and monitored, typically in the drug substance. Process-related impurities are structurally unrelated to *product-related impurities*, which are regarded as "molecular variants of the desired product which do not have properties comparable to those of the desired product with respect to activity, efficacy, and safety." The guidance further differentiates "product-related substances" as molecular variants that are comparable to the desired product in terms of activity, efficacy, and safety, and therefore are not considered impurities. ICH Q6B requires that specific tests and acceptance criteria

be developed to monitor product-related impurities when they increase during manufacturing or storage of the drug product, but there is no need to establish specifications for the product when evidence indicates that these impurities do not change during manufacture or storage. Clearly, a great deal of effort may be required during development to develop methods for product-related impurities (e.g., truncated forms, isomers, posttranslational modifications, deamidated forms) and to assess whether they can be appropriately classified as product-related substances.

Residual Solvents

ICH Q3C (29) set guidelines for a broad variety of residual solvents in drug products based on safety considerations for permissible daily exposure. Solvents are divided into three classes on the basis of risk:

Class 1: Solvents to be avoided
Class 2: Solvents to be limited
Class 3: Solvents with low toxic potential

Limits are provided and monitoring for specific solvent is strongly recommended for class 1 and class 2 solvents. The guideline recommends a general limit of 50 mg/day for class 3 solvents, and nonspecific test methods, such as loss-on-drying, are considered suitable for monitoring. The residual solvent requirement applies to drug products, but it is commonly met by limiting the concentration of residual solvents in all drug substances and excipients. To simplify, a broadly acceptable set of concentration limits are provided in ICH Q3C on the basis of a total daily product dose of 10 g/day. Alternatively, limits for individual substances can be calculated on the basis of their specific contributions to the daily intake (option 2).

ICH Q3C requires validation of analytical methods for residual solvents, but does not provide specific methods. Broadly applicable general test methods are provided in compendia (USP *<467> Residual Solvents*; Ph. Eur. *2.4.24 Identification and Control of Residual Solvents*) on the basis of methods originating in Ph. Eur. Both USP and Ph. Eur. adopt the ICH Q3C limits for compendial articles, unless otherwise stated in specific monographs, and the limits have thus become legal requirements for drug products covered by compendial monographs in these regions. Residual solvents are not commonly considered a stability-related attribute.

Leachables/Extractables

Leachables are a category of impurities that originate in packaging and migrate into a pharmaceutical product under the normal range of storage conditions. Leachables are specifically excluded from consideration in the ICH Q3B guidance, and there is currently relatively little prescriptive policy guidance that lists specific regulatory expectations for monitoring of leachables in injectable products. To a large extent, many concerns associated with leachables are addressed by development studies that demonstrate safety of packaging extractables—substances extracted from packaging using forcing conditions, typically combinations of solvents and temperature. Both the existing FDA packaging guidance (30) and ICH Q6A raise concern for the influence of package chemistry on safety and compatibility of parenterals. These guidances focus on demonstrating acceptable levels of packaging extractables in product through development studies, including stability studies, if needed. An EMEA guidance addresses specific requirements for studies on extractables in plastic materials (31), including a requirement for migration studies or stability studies on leachables to confirm the extractables from packaging do not migrate into product to significant extent.

Leachables in general are an active topic for guidance development. Detailed guidances are available for inhalers and nasal products, and regulatory expectations for leachables in ophthalmics are also under discussion. Parenterals are typically packaged in complex materials, including glass, polymers, and elastomers (32). They have a documented history of product/package interactions, including various examples where small amounts of leached substances—including metal ions, silicones, benzothiazoles, formaldehyde, and a variety of

organics—have altered product appearance, safety, or stability (33,34). Large volume parenterals represent a particular case where low concentrations may still give rise to a large total exposure because of the volume administered, and analytical methods with very low quantitation limits may be needed. During development studies, leachables should be considered as a candidate for potential specification development unless levels are low enough to justify these substances are not a concern. The rationale needs to be revisited as a consequence of packaging changes. Their exclusion from the specifications should be based on sound rationale developed from packaging extractable studies and confirmed through migration studies or through leachable analysis conducted during the formal stability program.

Water Content

ICH Q6A recommends a specification on water content for sterile solids for reconstitution as well as nonaqueous parenteral products. Water may be assessed by a variety of methods, the most common being loss-on-drying or Karl Fischer titration (Ph. Eur. *2.05.12 Water Semi-micro Determination, 2.05.32 Water Micro-determination*; USP *<921>Water Determination*; JP *2.41 Loss on Drying Test, 2.48 Water Determination Karl Fischer Method*), but a variety of other methods exist, including near-infrared spectroscopy, which can be utilized as a nondestructive probe of water content. The potential influence of water on solid-state chemical and physical stability of solids, particularly amorphous solids and lyophilized powders, has been extensively documented (35–37). Likewise, water may play a critical role in stability of nonaqueous parenterals, including physical stability of some nonaqueous suspensions (38). Water content may change significantly on stability, depending on permeability of packaging and water sorption characteristics of components, such as stoppers.

pH

ICH Q6A recommends a specification on pH for parenteral products where applicable. Changes in pH may alter product characteristics including pain on injection or product stability. pH is commonly measured potentiometrically with glass electrodes using compendial methods (Ph. Eur. *2.02.03 Potentiometric Determination of pH*; USP *<791> pH*; JP *2.54 pH Determination*). Acceptance criteria for pH should be developed on the basis of batch data and product development studies that address the influence of pH on key quality attributes. Stability attributes, such as rates of appearance of degradation products, may be strongly sensitive to pH (39). Developmental kinetic studies with supporting data may be needed to ensure the acceptance range for pH is consistent with the acceptance range for the degradation product throughout shelf life. Changes in pH should be monitored through the stability program.

Preservative Effectiveness and Antimicrobial Preservative Content

With few exceptions, multidose parenterals incorporate a chemical preservative (40), and suitable levels of the preservative must be maintained throughout shelf life. Minimum effective levels of preservative are typically established during product development based on results of pharmacopoeial preservative effectiveness testing (Ph. Eur. *5.1.3 Efficacy of Antimicrobial Preservation*; USP *<51> Antimicrobial Effectiveness Testing*) across a range of preservative concentrations. Although preservative effectiveness testing directly assesses the resistance of the formulation to microbial growth, it has some distinct disadvantages. Results of the test are difficult to trend quantitatively, and the test requires extended times, 14 to 28 days, for culturing of microbes. Therefore, in the registered product specifications, antimicrobial preservatives are commonly assayed using a stability-indicating method such as HPLC, which provides a suitable measure of preservative effectiveness as long as the formulation parameters (e.g., pH) critical to preservative action are properly controlled and understood. The rationale for acceptance of preservative content is captured in ICH Q6A guidance, which also recommends testing of preservative effectiveness through development and scale-up, including stability studies.

Acceptance criteria for preservative levels throughout shelf life should be clearly supported by the relationship established between preservative levels and preservative effectiveness during product development and confirmed in the new drug product stability studies. In many cases, for preservatives in common use, there may be considerable decrease in preservative levels throughout shelf life or usage duration of the product, in part because preservatives are frequently sorbed into plastics or elastomers. The European Union guidance (25) on product release specifications stipulates that preservative content should be 90% to 110% at time of release, unless suitable justification is provided for a broader specification. Since levels of preservatives also need to be justified (41), the stability of the preservative system needs to be understood well enough to allow reasonable initial preservative levels to be established.

Antioxidant/Chelating Agent

Antioxidants and/or chelating agents are sometimes utilized in parenteral formulations to enhance product stability, and development of specifications for these components is aligned with recommendations of ICH Q6A. Specific identification and test procedures are required for release specifications and stability specifications (41). Depending on the stability of the antioxidant and its specific role in the formulation, stability testing may not be required, but this should be justified.

Functionality Testing

ICH Q6A recommends that parenteral formulations packaged with delivery devices, for example, prefilled syringes, include testing to ensure specific functional characteristics of the delivery system (e.g., syringability, extrusion force, glide force, or break force), have test procedures and acceptance criteria. Depending on requirements for the device in question, functionality may be required for the drug/device combination on stability to ensure that delivery characteristics are consistent through shelf life. Syringability should also be properly regarded as a formulation attribute of injectable products that reflects the ease with which the product flows through the needle, including the force required to deliver it. This characteristic is largely related to rheology of the formulation, but it may be more easily measured by practical subjective assessment of ejection force through an appropriate gauge needle. In some cases, it may be appropriate to include a test for syringability, or a rheological test, in the product specification, particularly during development and primary stability studies.

Osmolarity or Osmolality

Osmolarity or osmolality specifications are expected when the tonicity of a parenteral product is declared in its labeling. Osmolarity is commonly a consistent function of composition and is not likely to change on stability unless there is significant breakdown of product or loss of water. Osmolarity is typically determined based on standard compendial methods (Ph. Eur. *2.2.35 Osmolality*; USP *<785> Osmolality and Osmolarity*; JP *2.47 Osmolarity Determination*). Data from development and validations studies may allow reduction of testing for osmolarity in the marketed product.

Particle Size Distribution

Key performance attributes, including drug release rate and rheology, may depend on the particle size distribution of injectable dispersions, such as suspensions, emulsions, and liposomes. ICH Q6A recommends consideration of testing for particles size distributions with quantitative acceptance criteria. If release rate is a primary concern, developmental data should be considered when determining the need for either a dissolution procedure or a particle size distribution procedure. A variety of methods exist for measuring particle size distribution of suspended particles. The most commonly used methods are laser light diffraction, electrozone particle counting, and optical microscopy (42,43). For nanoparticle distributions, more suitable tools include dynamic light scattering, field-flow fractionation, and analytical ultracentrifugation. None of these methods are chemically specific, so particle size

distribution measurements on products are difficult at best in cases where multiple species are present that must be chemically differentiated.

Particle size distribution specifications commonly include assessments of various percentage points on a cumulative distribution curve representing the fraction of particles below a given size range. ICH Q6A recommends specifications based on some measure of the mean of the distribution, as well as an upper and/or lower percentage point of the distribution. It would be common to utilize the median, or 50th percentile (a.k.a. D_{50}), as well as the 90th percentile D_{90} to measure the large particle tail of the distribution and the 10th percentile to measure the small particle tail of the distribution. The volume-weighted mean diameter would also be a common measure of mean particle size and a suitable replacement for D_{50}. There are numerous complexities associated with particle size measurements, including selection of methods, sampling, and sample preparation. It is important to recognize that instrumental methods for particle size differ fundamentally in their response to particles of differing sizes and shapes. Hence, acceptance criteria developed for the method and suspension have little meaning when the method is changed or if the process is changed in ways that alter particle shapes. Validation of particle size methods includes assessment of precision and robustness, but not accuracy.

ICHQ6A recommends "acceptance criteria should be set based on the observed ranges of variation, and should take into account the dissolution profiles of the batches that showed acceptable performance in vivo and the intended use of the product." While it would be uncommon to include acceptance criteria for particle size in specifications for early clinical batches, the measurement of particle sizes at this stage would be provide important data for subsequent justification of acceptance criteria. In some cases, specific clinical studies may need to be designed to establish the limits of an acceptable range of particle sizes. Particle size is a stability-related attribute, and changes in particle size on stability should be assessed. The particle size distribution may change through dissolution and regrowth, or through aggregation/agglomeration.

Redispersibility

ICH Q6A recommends a test for resuspendability of injectable suspensions that settle on storage (produce sediment). Shaking is considered an appropriate procedure, if properly controlled, and the time required to achieve resuspension provides a measurement of redispersibility. If adequate redispersibility is demonstrated on the basis of product development and stability studies, elimination of this attribute from the specifications may be proposed. Redispersibility is a stability-related attribute: significant changes in redispersibility occasionally occur because of caking of suspension solids, which may only become evident over time.

Reconstitution Time

For parenteral products that require reconstitution, acceptance criteria for reconstitution time should be provided on the basis of ICH Q6A guidance. The choice of diluent should be justified. Data generated during product development and process validation may be sufficient to justify skip lot testing or elimination of this attribute from the specification for rapidly dissolving products. Reconstitution time can be affected by physical changes in powders or solid cakes, so reconstitution time should probably be assessed on stability unless there is evidence that the solids dissolve sufficiently rapidly that such changes are not likely to affect reconstitution time.

Other Specifications

A wide variety of other specifications may be appropriate or expected depending on product attributes or specific product type. Examples include testing unique to liposomal formulations, lipid emulsions, or microparticulate controlled release formulations. The interested reader should consult current literature and guidance sources, including compendia.

Biopharmaceuticals include numerous requirements for process-related impurities like residual DNA, residual host cell proteins, Protein A, mycoplasma, antibiotics, and viruses.

Similar to residual solvent testing, where specifications for the drug substance eliminate the need to test for solvents in the drug product, testing for these process-related impurities is rarely done as part of drug product specifications but is commonly managed at the drug substance stage.

One major topic—in vitro release testing for parenteral suspensions or parenteral controlled release dispersions—represents a persistent area of interest, which is not discussed extensively in current guidance or compendial monograph testing (44). (Interestingly, ICH Q6A indicates that under some circumstances particle size testing may be performed in lieu of dissolution testing, but it does not mention dissolution testing as a requirement.) A variety of approaches have been developed for in vitro release testing of parenteral dispersions, partially in an effort to develop a test procedure suited to specifications and capable of measuring an attribute correlated to in vivo release rate. The monograph on injectable dispersed systems by Burgess is recommended (45). Some guidelines regarding preferred test systems and methods for setting acceptance criteria have been published by professional associations (46).

DRUG PRODUCT STABILITY

The investigation of stability for a new parenteral product progresses through the same stages as all new drug products that ultimately gain approval on the basis of successful regulatory applications. In the end, wherever appropriate, these applications follow established guidelines for submission and approval of data to support the marketed product shelf life. Most of the remainder of this chapter will summarize the content of these guidelines, but first it must be stressed that registration stability studies are only intended to confirm an understanding of product stability. The fundamental stability characteristics of the drug must be elucidated during the formulation design stage. Formulation choices at the design stage provide the opportunity to eliminate, or at least minimize, formulation factors that lead to poor stability and to optimize formulation properties that protect the drug from stress factors in the surrounding environment, sometimes including the environment in vivo. In a limited sense, "poor stability" may be interpreted as a shelf life shorter than desired. In the broadest sense, poor stability should equally be understood to include inconsistency. Consistent stability performance requires an adequate level of control over factors that affect stability, and the importance of achieving an understanding of the comprehensive array of factors that yield consistent batch-to-batch stability cannot be overemphasized.

Stability and Formulation Development

Successful formulation development requires expertise in analytical, bioanalytical, and physical organic chemistry to design studies that elucidate the major chemical degradation pathways. For biopharmaceuticals, an array of tools may be needed to explore the consequences of stresses imposed by time and temperature as well as processing and packaging. Assessments for biopharmaceuticals may be chemical, physical, and biological in an effort to not only identify changes but to correlate changes in physical and chemical measurements with changes in activity and overall biological response. From a stability perspective, package-related liabilities should also be identified early, because parenterals belong to a relatively limited category of pharmaceutical products that are typically in intimate contact with packaging. These packaging studies include some characterization of packaging extractables and the potential effect they may have on stability. In addition to chemistry, physical attributes of the parenteral product may become stability-limiting, particularly in dispersed systems, and it is not uncommon to find that chemical stability is predicated on physical stability or physical consistency. Finally, largely because of considerations related to stability, successful formulation development goes hand-in-hand with successful analytical method development. There is extensive literature on chemical and physical stability of drugs, which should be consulted for design of studies as well as specific reaction chemistries (see Refs. 29 and 40 for good introductions).

Registration stability studies or formal stability studies are lengthy and relatively expensive, and are usually undertaken immediately prior to registration. To reduce the risk of failure, the fundamental science needed to support the understanding of the product should be acquired before initiating formal stability studies for product registration. Ideally, at the

initiation of formal stability, there should be a thorough knowledge of product attributes (specifications) that are most likely to restrict shelf life, and their kinetic behavior. In most cases, this level of stability understanding develops concurrently with clinical studies and nonclinical safety studies that provide some of the basis for setting acceptance limits used in product specification development.

Stability Requirements for Product Registration

Currently, regulatory expectations for stability studies of new parenteral products are best reflected in ICH guidelines. Stability requirements for product registration were an important topic in ICH guidelines related to quality, because disparities in regional requirements frequently resulted in excessive stability testing. The resulting guidelines reduced the number of conditions, test points, and approaches to data evaluation that were needed to establish shelf lives for a global product, and distilled most of the sound principles developed across multiple regions into a common and rational framework.

ICH Q1A:	Stability Testing of New Drug Substance and Products (47)
ICH Q1B:	Photostability Testing of New Drug Substances and Products (48)
ICH Q1C:	Stability Testing of New Dosage Forms (49)
ICH Q1D:	Bracketing and Matrixing Designs for Stability Testing of New Drug Substances and Products (50)
ICH Q1E:	Evaluation of Stability Data (51)
ICH Q1F:	Stability Data Package for Registration Applications in Climatic Zones III and IV (withdrawn) (12)
ICH Q5C:	Stability Testing of Biotechnological/Biological Products (52)

ICH Q1A is the parent guideline that establishes a number of key definitions. Most of the terms are in common use, but they have less precise meanings. The precision of some of the ICH definitions is sufficiently important for the current discussion to repeat the definitions here. In a few cases the definitions have been abbreviated but not otherwise altered.

Formal Stability Studies and Shelf Life

Formal stability studies are "Long term and accelerated (and intermediate) studies undertaken on primary and/or commitment batches according to a prescribed stability protocol to establish or confirm the retest period of a drug substance or the shelf life of a drug product." The results of these studies should provide sufficient confirmatory data to allow the marketing authority to approve the product shelf life. The studies are conducted based on written protocols, with samples stored in chambers under controlled conditions, and pulled for testing at preselected times. The testing is regulated under GMPs. Test methods must be validated and associated equipment and laboratories must be qualified to do the testing. Laboratory records must be maintained for inspection, and in many cases these will be reviewed prior to product approval. The *shelf life*, or *expiration dating period*, is "the time period during which a drug product is expected to remain within the approved shelf life specification, provided that it is stored under the conditions defined on the container label."

Batch Requirements for Formal Stability

On the basis of ICH Q1A, formal stability studies require a minimum of three *primary batches* of drug product. Two of the three primary batches should be at least pilot scale batches, and the third may be smaller, although laboratory batches are not acceptable. A *pilot scale batch* is "a batch of a drug substance or drug product manufactured by a procedure fully representative of and simulating that to be applied to a full production scale batch," and typical pilot scale batches are at least 1/10 of production scale. *Commitment batches* are "Production batches of a drug substance or drug product for which the stability studies are initiated or completed post approval through a commitment made in the registration application." ICH Q1A requires the evaluation of stability from the first three production batches on the basis of the approved protocol and specifications as a postapproval commitment.

Multiple batches are utilized in formal stability to provide some understanding of consistency in stability performance across factors that vary between batches. Therefore, different batches of drug substance should be utilized where possible. Preferably, although not specifically stated in ICH Q1A, different batches of key excipients should be used as well. The formulation of primary batches must be the same as requested for production batches for the product, and the process must at least simulate the process used for production batches. Samples for the formal stability study must be packaged in the same package (i.e., primary container/closure and secondary packaging) that will be used for marketed product. Primary batches should meet the same specifications requested for the marketed product, and they should be manufactured to provide the labeled amount of drug.

One factor that is not well addressed in current guidance is container orientation on stability. The only ICH guidance that addresses container orientation is ICH Q5C for biopharmaceutical products, which recommends "stability studies should include samples maintained in the inverted or horizontal position (i.e., in contact with the closure), as well as in the upright position, to determine the effects of the closure on product quality." The withdrawn 1998 FDA draft stability guidance (53) was more specific on the issue of orientation, requiring that solutions, dispersed systems, and semisolids be stored on stability in both horizontal/inverted and upright orientations until the effect of storage orientations could be assessed and stability could be restricted to the most stressful orientation. The draft guidance for metered dose inhaler products takes a similar approach (54). It is reasonable to anticipate the need for some data to demonstrate the influence of container orientation on storage stability of the product, in accord with ICH Q5C.

Stability Storage Conditions and Test Intervals

In considering the appropriate storage conditions for stability studies, ICH Q1A exploits concept of climate zones, which provides different conditions to account for regional variations in two major stability factors: temperature and relative humidity. (The influence of remaining environmental factors is accounted for in special studies, such as photostability testing as discussed in ICH Q1B.) Pharmaceutical markets are divided among four major climate zones, which differ with respect to ambient annual averages in these two variables, and therefore require long-term stability under conditions that are sufficiently suitable to reflect the upper extremes of average ambient storage in each area. Zahn provides a recent review (55), including data that cover most global regions. The ICH regions are largely part of Climate Zones I and II, corresponding to temperate and subtropical or Mediterranean climates.

ICH Q1A recommendations appropriate for Zones I and II are summarized in Table 2, on the basis of the storage condition specified for the product. There are two cases where

Table 2 ICH Stability Storage Conditions for Zones I and II

Case;	Temperature/relative humidity[a] and duration of study		
Recommended storage conditions	Long term	Intermediate	Accelerated
General case; Room temperature storage	25°C/60% RH through shelf life	30°/65% RH 12 mo	40°/75% RH 6 mo
Aqueous product/semipermeable package; Room temperature storage	25°/40% RH through shelf life	30°/65% RH 12 mo	40°/ NMT 25% RH 6 mo
General case; Refrigerator storage	5°C ± 3°C through shelf life	None	25°/40% RH 6 mo
General case; Freezer storage	−20°C ± 5°C through shelf life	None	None

[a]Where tolerances are not specifically indicated in the table, they are ±2°C for temperature and ±5% RH for relative humidity.

Abbreviation: NMT, not more than.

packaging plays a role. For products in impermeable packaging (e.g., sealed glass ampoules), relative humidity is not significant and storage conditions do not need to provide for controlled relative humidity. For aqueous products in semipermeable packaging (e.g., large-volume parenterals in low-density polyethylene bags, ophthalmic solutions in flexible polyolefin containers) intended to be stored under ambient conditions, water loss can be a significant problem. Clearly for these products, high water activity inside of the package is not a stressful condition, but water loss in a dry environment may be a significant limitation for stability. Storage at alternate lower relative humidities is required.

Product testing is recommended at minimum test intervals of three months through the first year, six months through the second year, and annually thereafter. This recommendation is clearly a general minimum requirement. Where specific kinetic information is available to guide selection of test points, points may be added at suitable locations to improve the analysis. Not all testing is required at all intervals or for all batches, as noted in the discussions on specifications, particularly for the microbiological tests. In general, testing at the intermediate condition is not needed if the product does not undergo "significant change" at the accelerated condition, as discussed in the data evaluation section of ICH Q1A and ICH Q1E, and in the subsequent section on Stability Data Evaluation. Recommendations for stability data at time of regulatory submission are a minimum of 12 months long-term data and 6 months of accelerated or intermediate data.

Climate Zones III and IV cover hotter regions of the globe. Zone III climates are drier, and Zone IV climates are more humid. Temperature requirements for room temperature storage in these zones are $30^{\circ}C \pm 2^{\circ}C$. Relative humidity requirements for testing in Zone IV are split between 65% and 75% for the general case, because some Zone IV countries (specifically those that declare themselves to be Zone IVb) have conditions where products are consistently subjected to higher relative humidities. Where global stability products are considered, this leaves manufacturers with a range of options, depending on specific regions and willingness to consider different expiration periods for the various regions.

Label storage statements differ by region. Examples of regional label storage statement recommendations are provided in USP 31, an EMEA guidance (56), and a WHO guideline (57).

In-Use Stability

Parenterals include groups of reconstitutable or multidose products with specific requirements for in-use stability testing. ICH Q1A requires in-use stability testing for products through the label use-period after reconstitution or dilution. This in-use stability testing should be conducted as part of the formal stability protocol at both initial and final (or end of shelf life) time points for long-term stability of primary batches. In addition, data should be collected at 12 months or the intermediate time point immediately prior to submission. The type of testing that might be conducted through the use-period on a reconstituted product was probably best covered in the now-withdrawn 1998 FDA draft stability guidance (53). It included appearance, clarity, color, pH, assay, preservative (if present), degradation products/aggregates, sterility, pyrogenicity, and particulate matter.

An EMEA guidance (58) recommends testing to be completed prior to submission to ensure that multidose products retain quality throughout their label use period, when product is taken from the multidose container on the basis of label instructions under normal environmental conditions. At least one of these protocols should be conducted on a batch near the end of shelf life.

Analysis of Stability Data

Basic principles of data analysis for formal stability studies are presented in the ICH Q1A parent guidance. These principles are further elaborated in the ICH Q1E guidance, particularly for instances where the requested shelf life at time of submission for a new drug product requires extrapolation beyond existing long-term data. Data for submission are expected to be properly tabulated and reviewed, with results and trends for each attribute individually discussed, as appropriate. The shelf life for the product should not exceed the shelf life predicted for any individual attribute.

The combination of accelerated, intermediate, and long-term stability data are first evaluated for significant change. The following definition of significant change is adapted directly from ICH Q1A:

- A 5% change in assay from its initial value, or failure to meet the acceptance criteria for potency when using biological or immunological procedures.
- Any degradation product's exceeding its acceptance criterion.
- Failure to meet the acceptance criteria for appearance, physical attributes, and functionality test (e.g., color, phase separation, resuspendability, caking, hardness, dose delivery per actuation); however, some changes in physical attributes (e.g., softening of suppositories, melting of creams) may be expected under accelerated conditions.
- Failure to meet the acceptance criterion for pH.
- Failure to meet the acceptance criteria for dissolution for 12 dosage units.
- Loss of water from semipermeable containers greater than 3% after three months at 40°C/NMT 25%RH, unless justified for small containers (1 mL or less).

ICH Q1A provides a decision tree to help evaluate the data and assess whether, and to what extent, extrapolation can be used to justify a shelf life request. As an example, consider a product to be stored at ambient conditions. The outcomes of the ICH decisions tree are tabulated in Table 3, which shows the greater the extent of change with time and temperature, the less extrapolation can be used to justify shelf life beyond that allowed by existing long-term data. It also shows the advantage of analyzing data statistically, where possible, to provide justification for extrapolation beyond that which could be justified in the absence of statistical analysis. A similar table can readily be constructed for refrigerated storage, where in general the extent of allowed extrapolation is shorter.

For biologics, generally extrapolation of not more than six months beyond the real-time stability data is granted due to potential concerns of non-Arrhenius stability behavior of drug product.

Statistical Analysis of Stability Data

The ICH Q1E guidance provides a suitable framework for statistical trend analysis of quantitative data, where the data can be modeled using linear regression. Simpler data, such as discrete pass/fail responses, or more complex data, such as dissolution profile data, can also be trended statistically (59), but these are beyond the scope of current guidance. Because the extent of degradation of the drug in most drug products is relatively small throughout shelf life, most stability data can be trended accurately with simple zero-order kinetics, even if the fundamental model kinetics are first order or more complex orders. (In this case, the models are commonly referred to as pseudo-zero-order.) Carstensen (60) has shown the relative difference between first-order and zero-order expressions is quite small as long as the extent of

Table 3 Summary of Allowed Extrapolation Based on (no) Changes in Quality Attributes

Significant change at 6 mo?	Little change over time or little variability	Statistical analysis	Allowed extrapolation[a]
No—Accel.	Yes—both accelerated and long term	Not required	2X but NMT +12 mo
	No—accelerated or long term	Yes	2X but NMT +12 mo
		No	1.5X but NMT + 6 mo
Yes—Accel.	Not relevant	Yes	1.5X but NMT + 6 mo
No—Inter.		No	NMT + 3 mo
Yes—Accel. Yes—Inter.	Not relevant	Not relevant	None

[a]Times and multipliers are referenced to the time established by current long-term data. Extrapolations must be supported by statistical analysis or supportive data.

change is below about 15%. The appearance of primary degradation products also commonly follows pseudo-zero-order kinetics, unless the products themselves degrade, in which case more complex models may need to be considered (39). Changes in other components, such as antioxidants or preservatives, may cover much more significant fractions of the initial content, so alternate kinetic models may also be required in these cases. For simplicity, the overview of statistical treatment presented here will focus on zero-order kinetic models, but many of the concepts can be extended to alternate kinetic models via variable transformations or nonlinear regression modeling.

Linear regression, as utilized for analysis of zero-order trends in stability data for a single batch, is a straightforward application of linear regression as described in multiple introductory texts on applied statistics (61,62). To briefly summarize, consider the assay values as the dependent variable, y_i, which is modeled as a simple linear function of time, t_i, for stability time points $i = 1, \ldots n$. The stability data at each time point are limited in precision by variability in measurements and sampling. Although they follow a general zero-order trend, deviations of individual points about the line are common. The linear model is given in equation (1), where the least squares slope β and intercept α are the values that minimize the sum-of-square errors $\sum \varepsilon_j^2$ in the model expression over all time points j. The errors, ε, are assumed to be normally distributed, with mean of zero, and standard deviation of σ.

$$y_j = \alpha + \beta t_j + \varepsilon_j \qquad \text{(Model 1)} \tag{1}$$

To exemplify, consider the stability data for assay collected for a one of three primary stability batches (Batch A). Assay values collected at 0, 3, 6, 9, 12, and 18 months are plotted in Figure 1. In Figure 1, a horizontal line is drawn through the lower specification limit (LSL) of 90% of label. The solid trend line obtained by standard linear regression computer program is shown in Figure 1. Where the trend line crosses the LSL just below 30 months, it provides a "median" estimate of the shelf life for this batch. However, because there is error in the fitted data, there is inferential uncertainty in the shelf life. The standard error of the data about the line in this example is 0.78%, a value that is very consistent with the precision of analytical methods for assay. The "true" shelf life for the batch has equal likelihood of being longer or shorter than 30 months, because the variability in the data limits the degree of certainty in the estimation of the shelf life. The greater the variation of data about the line, the greater the degree of uncertainty. Clearly, since products are expected to meet specifications throughout

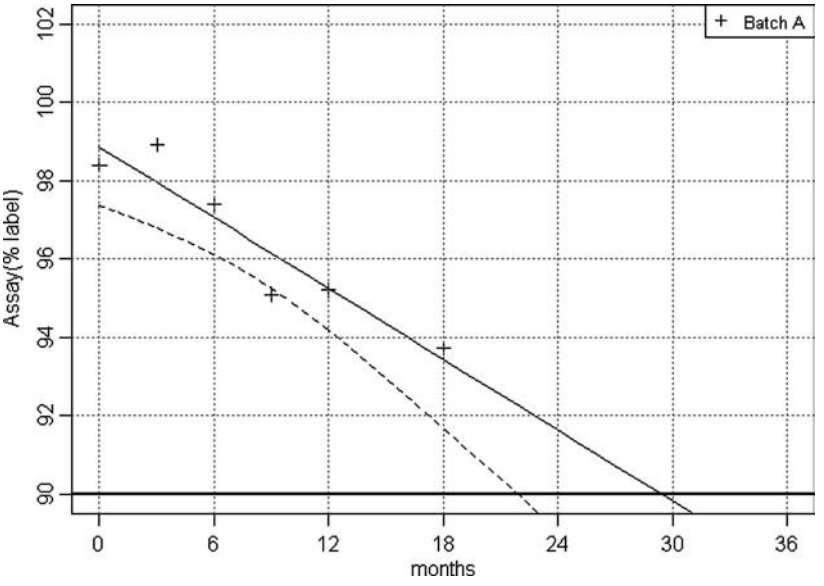

Figure 1 Linear regression analysis of assay results for 18 months of stability from a single batch. The dashed line is the lower 95% two-sided confidence interval for the regression line.

shelf life, the regression line estimate of shelf-life is a poor choice because it is very likely (50% probability) to overestimate the true shelf life.

The accepted resolution to this dilemma, as recommended in ICH Q1E, is to calculate a confidence limit about the regression line. Assuming the errors are normally distributed, confidence limits can be calculated on the basis of standard formulas. These limits estimate the region within which the true trend line for the batch is expected to lie at a chosen level of confidence. The dashed line in Figure 1 represents the lower 95% two-sided confidence limit for the regression line. In this case, the true trend line has a 97.5% likelihood of being above the region shown by this curve. If the tentative shelf life is conservatively assigned at not-more-than (NMT) 22 months, the point where the 95% confidence limit intersects the LSL, there is only a small likelihood that the shelf life will be an overestimate and the batch is likely to remain within specifications through shelf life. Note, however, that there is considerable difference between the regression line estimate of NMT 29 months and this accepted shelf life estimate of NMT 22 months. This difference is the penalty of uncertainty. The ability of the statistical tools to account for uncertainty in conservatively estimating shelf lives is the major reason why ICH Q1E allows more extended extrapolation in cases where the data are analyzed statistically.

Typically, the formal stability studies will include results from three batches. ICH Q1E requires that the shelf life of the product cannot be longer than the shortest estimated shelf life of these batches, using the intersection point of the 95% confidence interval with the lower or upper specification limits. With appropriate statistical software, data from multiple batches can be analyzed together using the model given by equation (2).

$$y_{ij} = \alpha_i + \beta_i t_{ij} + \varepsilon_{ij} \qquad \text{(Model 2)} \tag{2}$$

In this model, the "i" subscript denotes the batch. Each batch has an independent slope and intercept. Although equation (2) may seem equivalent to three instances of equation (1), the regression using equation (2) assumes a single pooled estimate of the residual standard error. In some cases, this pooled estimate may extend the shelf life estimate somewhat by reducing the estimated standard deviation associated with the batch that has the shortest estimated crossing point. As an example, consider Figure 2, where data from two additional batches have been added to the data from Batch A. Regression lines, and the lower 95%

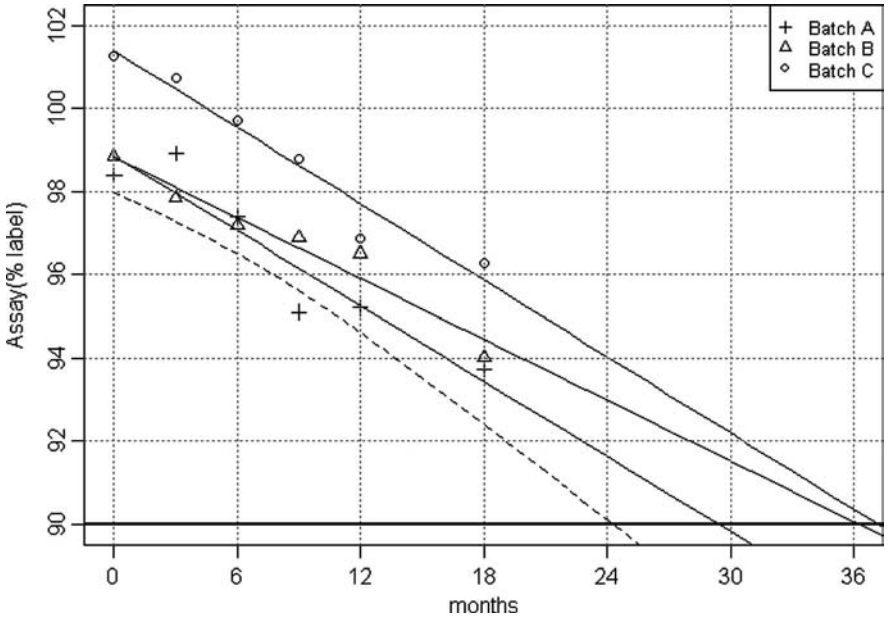

Figure 2 Linear regression of assay results for 18 months of stability from three batches. The model was pooled for residuals. The dashed line is the lower 95% two-sided confidence interval for the regression line of Batch A.

confidence interval for Batch A have been plotted based on analysis of Model 2. Batch A is still the batch with the shortest estimated shelf life. The regression line associated with Batch A has not changed, but the confidence limit has moved closer to the line, and it crosses the lower specification at a point just beyond 24 months, an increase of greater than two months beyond the confidence limit in Figure 1. The additional data included in the model of equation (2) resulted in an improved estimate of the residual standard error (0.59%), an increase in the residual degrees of freedom, and a shrinkage of the confidence limit about the line. An increase in the estimated shelf life is common, but by no means universal. The residual standard error may increase when the estimate is improved, but if it results in a more accurate assessment of actual measurement precision, it should also result in a better conservative estimate of shelf life.

Additional improvements in shelf life estimation can be achieved through batch pooling, as discussed in ICH Q1E. Where batch data pooling can be justified, based on a set of appropriate decision criteria, more extended shelf lives can frequently be justified, and in most cases more realistic shelf life estimates can be obtained. The decision criteria are based on ANCOVA (analysis of covariance) comparison of regression models (60), with time as a covariate and batches (or strengths, package, orientation, etc.) as a factor. As a typical example of how this procedure is applied to batch pooling, consider the first regression model to be that given by equation (2), where slope and intercept are allowed to vary by batch. The second regression model is the pooled slope model, given by equation (3), where intercepts are allowed to vary by batch but the slope is equal for all batches.

$$y_{ij} = \alpha_i + \beta t_{ij} + \varepsilon_{ij} \qquad \text{(Model 3)} \tag{3}$$

Regression analysis of the same data shown in Figure 2, with the model equation given in equation (3), provided the results shown in Figure 3. In this case, equality of slopes has decreased the slope of the regression line for Batch A, so it intersects with the LSL at a point

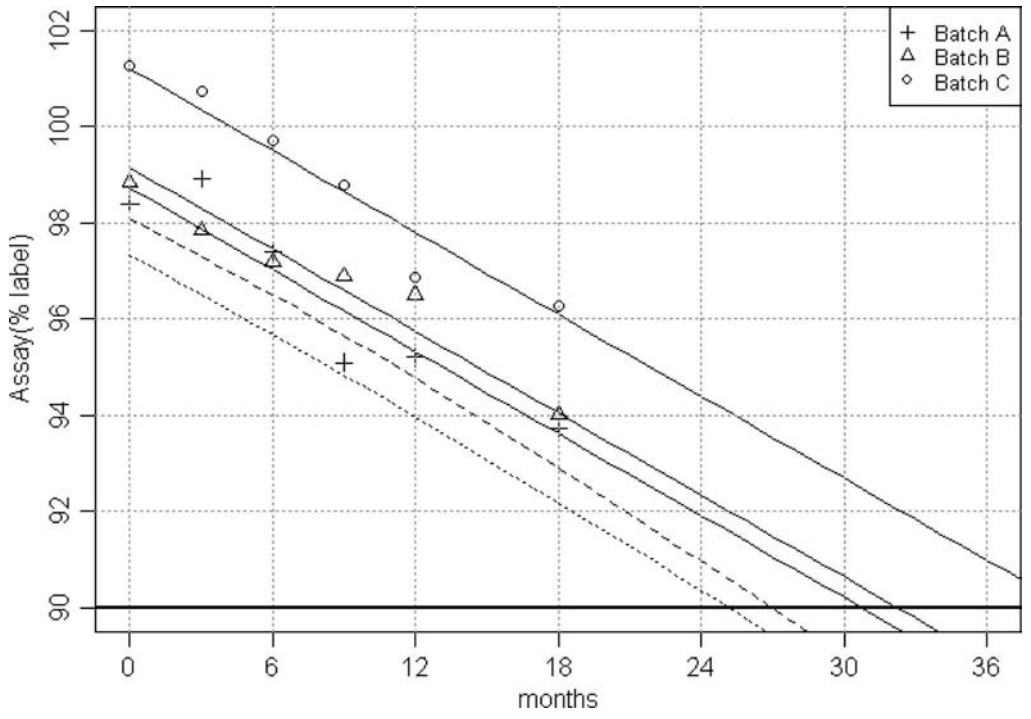

Figure 3 Linear regression of assay results for 18 months of stability from three batches. The model allowed pooling for estimation of a common slope. The dashed line is the lower 95% two-sided confidence interval for the regression line of Batch A. The dotted line is the lower 95% two-sided prediction interval for the regression line of Batch A.

just beyond 30 months. The 95% confidence limit for Batch A, given by the dashed line, now intersects the LSL at 27 months. (The additional dotted line will be discussed further below.) For this example, pooling has increased the estimated shelf life from 22 to 27 months, a substantial improvement if allowable.

The question arises, "How can we justify pooling of slopes within the guidelines of ICH Q1E?" Intuitively, if the slopes visibly differ to a greater extent than the distribution of points about each line, the difference in slopes would seem to be meaningful, and pooling of the slopes would be expected to obscure real differences between batches. However, if differences in the slopes over the length of the line segment appear small relative to the distribution of points about the line, it would seem unlikely that the differences would contribute much to the overall fit of the model. In this case, the slopes could be pooled without loss of valuable information regarding batch-to-batch differences in stability behavior. This intuitive assessment can be readily translated into a test statistic when the data are distributed normally about the regression lines. Let $SSE_x = \Sigma\varepsilon^2$ for model x. Also, let the residual degrees of freedom for model x be given by DF_x, which is equal to the number of data points minus the number of fitted parameters (independent slopes and intercepts). The test statistic for comparison of model 2 and model 3 is given by the expression.

$$F = \frac{(SSE_3 - SSE_2)/(DF_3 - DF_2)}{SSE_2/DF_2}$$

Both the numerator and denominator are mean square errors, which are random variables with chi-square distributions because the data are normally distributed. Their ratio, F, is therefore distributed as the F-distribution with $DF_3 - DF_2$ and DF_2 degrees of freedom, and values of the ratio can be compared with percentage points of standard F-distribution to assess the statistical significance of model improvements as additional terms are added. In the case of the examples given above, $DF_3 = 14$, $DF_2 = 12$, and the value of F computed from the sum of square error terms is $F = 0.7332$. This value corresponds to a probability point on the $F_{2,14}$ distribution of $p = 0.50$, which suggests that any difference between the two models is not statistically significant. Under these circumstances, pooling is accepted. ICH Q1E recommends batches not be pooled when $p < 0.25$, which is a relatively conservative choice that helps ensure any true batch-to-batch differences are retained in the analysis. A similar analysis can be completed for pooling of intercepts. In this case, for the data shown, $p < 0.0001$, and the intercepts were not poolable. Where factors do not involve cross-batch comparisons (e.g., package sizes, orientations), ICH Q1E recommends that batches pooling be disallowed only when the significance level is $p < 0.05$.

Within the limitations of the linear regression models, the ANCOVA analysis is versatile enough to account for multiple factors and sequential comparisons of models. This type of evaluation can readily be conducted with a number of standard statistical packages, including SAS (63) and R, an open-source software package (64).

Two caveats should to be kept in mind. (*i*) Future batches may show variation that is not evident in the primary batches. For example, if a batch similar to those in Figure 3 had an assay intercept of 96%, it likely would not meet acceptance criteria through a 24-month shelf life. Possible variations in future batches need to be considered when release criteria are established, and the relationship between expiration period, shelf life specifications, and release specifications must be understood. (*ii*) For batches that will be tested on stability as part of a commitment, or as part of an ongoing annual stability program, the risk of out-of-specification (OOS) test events may need to be assessed and managed. The 95% confidence interval reflects confidence in the estimation of linear model parameters, and this confidence interval shrinks as the number of points included in the analysis increases. However, when the distribution of future individual test measurements is considered, it is affected by both the inferential uncertainty associated with the model parameters and the measurement uncertainty associated with the precision of individual measurements. This contribution does not diminish. The 95% prediction interval represents both of these contributions and delineates the range within which 95% of future measurements are predicted to lie. An example of the lower two-sided 95% prediction interval is shown as the dotted line in Figure 3. When expiration

periods are set, it may be useful to consider whether the prediction intervals show significant likelihood of OOS prior to the end of shelf life. Approaches may need to be developed to manage this risk.

Matrixing and Bracketing

Products that are available in multiple container volumes or strengths may benefit from either bracketing or matrixing (ICH Q1D). These practices allow for some overall reduction in the stability testing workload. Bracketing is the practice of testing stability for products at the extremes of strength or container size, and using the results to support the expiration period for the product at intermediate strength or container size. For example, results from a solution formulated at 0.5% and 2% wt/vol concentrations could be used to support a 1.0% concentration as long as the formulations did not differ significantly with respect to type and concentrations of excipients. If the high and low strengths differ in stability, the expiration period of the intermediate strength is constrained to the shorter of the two expiration periods. Some care is obviously required for multiphase products like suspensions, where changes in strength may be associated with change in the distribution of drug among phases that differ in stability.

Matrixing is the practice of testing multiple combinations of factors in a design that allows for reduction of testing at some time points. In statistics, matrix designs are commonly referred to as fractional factorial designs. Matrix designs commonly include combinations of factors such as strength, container size, container type, and even minor changes in formulation. Where a full factorial design would require testing of all combinations at all time points, a matrixed design allows for a subset of the samples to be tested at many of the time points. While the stability workload can be reduced, there is a risk that the expiration period that can be supported in a matrixed design will be shorter than that given by the full design, particularly in cases where measurement variability is large. Depending on the product and testing schedule, stability testing may be reduced by as much as 30% to 50% or more, a substantial decrease for designs that include multiple factors. Matrixing may be applied to bracketed designs. For additional discussion, see Chow (59) and references therein.

Special Considerations in Stability of Biopharmaceutical Products

Recommendations for stability of new biopharmaceutical products are captured in ICH Q5C. Basic principles are similar to those for ICH Q1A, but specific recommendations differ from those of ICH Q1A, reflecting both increased challenges of biopharmaceuticals, as well as the more limited capacity to capture comprehensive product quality attributes through a collection of precise analytical measurements. Briefly, the major points are as follows:

- Data from three primary batches on formal stability studies of at least six months duration are expected at time of filing. There are no prescribed stability storage conditions. Each product is considered unique with respect to definition of appropriate storage conditions.
- Lots may be pilot scale, but process and final package should be the same as batches to be manufactured.
- The shelf life request is granted on the basis of real time data (no extrapolations) at the label storage condition. Accelerated stability studies are recommended to help characterize the degradation profile and support excursions.
- In all cases, some measurement of biological potency of stability samples is required.

Photochemical Stability

ICH Q1B guidance describes a standard confirmatory photostability stress testing scheme for assessing susceptibility of both drug substance and a new drug product to light. The light sources have well-defined visible and near UV spectral characteristics typical of filtered daylight. The product is exposed to a minimum of 1.2 million lux hours and an integrated near ultraviolet energy of not less than 200 watt hours/m^2. It is subsequently analyzed for changes in appearance, color, clarity, assay, and degradants. Products should be exposed in a

transparent container, in the immediate package, and in the market pack to assess whether the product is photolabile, and whether the packaging is sufficiently protective to prevent the product from degradation that could affect its shelf life. If the product is not sufficiently stable after exposure to light, changes in packaging or special labeling may be required.

Stability Studies to Support Excursions

Although pharmaceutical products may generally be stored in controlled environments prior to dispensing, they may be shipped under conditions where they are subjected to short-term temperature excursions or rapid temperature changes. The increasing proportion of biopharmaceutical products, which are usually more sensitive to these excursions, has drawn considerable attention to shipping and distribution practices and the types of stability information required to support them. ICH Q1A accelerated stability studies provide useful information regarding exposure to higher temperatures, but at least two types of additional short-term studies are commonly run. Temperature excursion studies expose samples to high- or low-temperature excursions (including $-20^{\circ}C$ exposure), typically for intervals of two days. Temperature cycling studies cycle the product through drastic changes in temperature over short intervals. For instance, where drug product labeled for room temperature storage might be exposed to subfreezing temperatures, thermal cycling would expose the product to three successive cycles of $-20^{\circ}C$ for two days, followed by $40^{\circ}C$ for two days. At the end of the cycle, the product would be examined for appearance, assay, and degradation products, and physical attributes including precipitation, aggregation, or phase segregation. Designs for cycling studies are somewhat specific to both the product and the stresses present in the intended distribution network. Specific designs are not currently outlined in regulatory guidances, except for the Metered Dose Inhaler guidance and the withdrawn 1998 stability guidance, but they are discussed in the Parenteral Drug Association Technical Report 39 (65).

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10 The management of extractables and leachables in pharmaceutical products

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INTRODUCTION: ORIGIN AND IMPORTANCE OF EXTRACTABLES AND LEACHABLES

In this chapter the important elements of extractables and leachables, with which those in the pharmaceutical industry should be familiar, are discussed. On completion of this chapter, the reader should be able to

- Understand the extractables/leachables expectations in guidelines, guidances, United States Pharmacopoeia (USP), International Conference on Harmonization (ICH), U.S. Code of Federal Regulations (CFR), and other regulatory documents
  What is expected or required?
- Identify sources of extractables from packaging and process materials such as plastic, glass, and rubber
  Where do E&L (extractables and leachables) originate?
- Know what specific extractables/leachables information must be present in the chemistry, manufacturing, and controls documentation (CMC) sections of applications for the various types of drug products (injectables, oral, etc.)
  What information must be contained in new drug applications?
- Design and execute an extractables/leachables study
  What are the elements of an E&L study?

Packaging has allowed the widespread distribution of drugs; without it there would be no pharmaceutical industry, and the quality and quantity of life we have come to expect would not exist. Try to imagine a product that you use that comes to you without the use of packaging, perhaps a home-grown tomato from your garden! Packaging serves many functions (1,2) such as

- Protection and containment—packaging is expected to maintain the quality and quantity of a drug product until expiration from the filling line to the patient. Packaging must not interact with nor alter the efficacy of the drug, and leachables levels must not present a toxicity risk to patients. In addition, packaging must meet all pharmacopoeial requirements where marketed.
- Transportation and storage—drug products are transported and stored in packaging containers until used. Stability studies provide information on the finite "shelf life" of a drug product in a specific container.
- Identification—printed components provide the product name, strength, expiry date, dose, precautions and contraindications, and other information to healthcare professionals and patients.
- Compliance—necessary dose quantities can be conveniently packaged to facilitate the delivery of specific quantities of drug per day.
- Delivery—some packaging components, such as the prefilled syringe, provide the additional function of being delivery or administration devices.

In essence, good packaging must be "suitable for use," that is, it must provide the necessary protection, compatibility, safety, and performance. E&L studies measure two of the four suitability requirements: compatibility and safety.

Despite the necessity and positive functions of packaging, there is one significant disadvantage that must be evaluated for every pharmaceutical product. That is, *packaging materials interact with drug products*. In fact an article in Chemical & Engineering News (3) stated

that "It is not a question of whether packaging components will leach into a product, it's a question of how much." A statement in the U.S. current Good Manufacturing Practices (cGMPs) acknowledges and reflects this concern (4).

> § 211.94 (a) Drug product containers and closures shall not be reactive, additive, or absorptive so as to alter the safety, identity, strength, quality, or purity of the drug beyond the official or established requirements.

Drug product-packaging interactions are commonly classified into four types:

- Adsorption—some part of a drug product is sorbed or concentrated onto the contact surface of a packing component.
- Absorption—following adsorption, the substance may penetrate the surface of the packaging material and migrate into the material.
- Permeation—further migration may lead to migration of a substance through the packaging to the noncontact surface and beyond.
- Leaching—substances may migrate from a packaging component into the drug product.

All four or any combination of the interactions may and probably do take place in any given packaging situation; however, as long as the interactions do not "alter the safety, identity, strength, quality, or purity of the drug beyond the official or established requirements" during the shelf life of drug product, the packaging is deemed acceptable for use. Although all four interactions are important, only leaching or migration of packaging substances into drug products or related materials will be discussed in this chapter.

Thus far, the two terms, extractables and leachables, have been mentioned but not defined. They are often used interchangeably but they have distinctly different meanings.

- Extractables are chemical substances that are removed from a material by the exertion of an artificial or exaggerated force. That force may be a strong solvent, a high temperature, a long extraction time, or a combination of the three. An extractables test is a *packaging test* performed on a packaging component or material.
- Leachables are chemical components that migrate from a contact material into drug products during storage at "normal" conditions. A leachables test is a *drug test* performed on a drug product or related material to identify and quantify substances that have migrated into it from a packaging component or other related component.

Extractables tests may be performed on a component or material without specific knowledge of what drug may be ultimately in contact with it. Lists of extractables may be generated by manufacturers or suppliers of packaging materials and components using solvent systems that have come to be standardized as common industry practice. A list of extractables represents a list of *potential leachables* that may be targeted for identification and quantification is a leachables study. Leachables are usually a subset of extractables—and not all extractables are leachables. Exceptions will be discussed later in this chapter.

E&L studies have different goals.

- Controlled extractables studies—to identify as many chemical compounds as possible that have the potential to become leachables.
- Leachables stability studies—to identify, quantify, and qualify as many compounds as possible that migrated from packaging materials into a drug product. Qualification is the process of acquiring and evaluating data that establishes the biological safety of an individual impurity or a given impurity profile at the level(s) specified. It may not be necessary to identify, quantify, and qualify a leachable if the amount present in the drug does not present a safety concern. This will be discussed later in the chapter when the work of the Product Quality Research Institute (PQRI) is discussed (5).

Extractables studies should always precede leachables studies since the target compounds for the leachables studies are identified in the extractables studies.

Why have leachables become an issue in the world of pharmaceuticals? The obvious reason is because substances that have migrated into a drug product may "alter the safety, identity, strength, quality, or purity of the drug" (4). Some commonly listed reasons are that leachables

- May interfere with drug product assays (e.g. have the same retention time as the drug substance in a high performance liquid chromatography (HPLC) assay)
- May interfere with medical diagnostic tests
- May cause appearance change in drug product (e.g., color change)
- May increase the impurity level of drug product to an unacceptable level
- May react with one or more of drug product components [e.g., may cause precipitate, pH change, or degradation of the active ingredient; Zn/epinephrine reaction in dental anesthetics (6)]
- May increase toxicity of drug product (e.g., may require identification, risk assessment, qualification, and quantification)

Taking into account all the reasons listed above, the goal of any leachables study is to reduce risk to patients who receive the drug products. A recent example of product that caused adverse affects due to the lack of leachables data was EPREX®, a product of Janssen-Cilag (7). The history of events surrounding EPREX is listed below.

- EPREX in vials contained human serum albumin (HSA), 1994.
- EPREX with HSA was offered in a prefilled syringe containing an uncoated rubber plunger, 1994.
- HSA-free EPREX was introduced in 1998; HSA was replaced by Polysorbate 80 as the protein stabilizer.
- Polysorbate 80 increased the extraction of adjuvant-like leachables from the rubber plunger causing an unwanted side effect called PRCA (pure red-cell aplasia) in EPREX patients.
- Recall occurred and a coated plunger, which reduced the amount of leachables, was substituted for the uncoated closure.

The authors of the paper who describe the EPREX problem recommend that "... an active program of monitoring products for the presence of extraneous molecules is prudent. The necessity of using multiple techniques for the detection, identification, and quantitation of the leachables in this study indicates that no single method is sufficient and suggests that multiple, orthogonal techniques be routinely employed." In this particular case, even if a thorough E&L study was performed, the connection of the rubber leachable with PRCA probably could not have been predicted.

An older study of a dental anesthetic presents a simpler situation (6). In 1981, Astra Pharmaceutical Products, now Astra Zeneca, produced and marketed a dental anesthetic in a syringe cartridge. The cartridge had two rubber components: a thin rubber seal on the needle end, which is pierced by a double-ended needle in the syringe body to allow expulsion of the drug, and a chlorobutyl rubber plunger that seals the opposite end of the cartridge and is pushed forward by a plunger rod to deliver the anesthetic to the patient. The drug product contained a vasoconstrictor, epinephrine, to localize the anesthetic in the area of the dental procedure. Reports from dentists that the anesthetic was less effective than usual in several cases led Astra to the search for the cause. Dentists reported that the anesthetic effect was no longer localized, leading to an investigation of the level of epinephrine. It was known that oxygen reacts with epinephrine and that oxygen could be permeating through or around the thin rubber seal or around the thicker plunger. Neither was occurring; the oxygen level in the cartridges was not out of acceptable limits. Further investigation led to a correlation of high soluble aluminum with low levels of epinephrine. The source of the aluminum was calcined clay, which was a reinforcing agent in the rubber plunger. A change in the calcining temperature of the clay caused an increase in the solubility of the aluminum from 5000 to 25,000 ppm in the dental cartridges. It was determined in subsequent studies (8,9) that aluminum catalyzes the degradation of epinephrine.

In the 1980s, there were no leachables studies as we would do them today, and extractables testing on rubber components was confined to compendial tests such as the USP and the European Pharmacopoeia (Ph. Eur.). In this particular case, there were no material or process changes made by Astra or their rubber supplier, but changes were made in the process of manufacturing the clay by the rubber manufacturer's clay supplier. Extractables tests and leachables tests would have identified aluminum as a significant leachable and alerted Astra to perhaps monitor its concentration in incoming rubber. And certainly quality agreements, which included change controls, between Astra and the rubber supplier, and the rubber supplier and its clay supplier, would have given all parties the opportunity to evaluate the impact of the proposed change in calcining temperature.

The key lessons learned from the Astra studies are

- E&L studies, done prior to marketing, are necessary to identify possible harmful leachables in a drug product.
- E&L studies are not a "one time and over" study; periodic monitoring of packaging components and drug products is necessary.
- Quality agreements, emphasizing change control, are necessary to reduce the chance of process or material changes.
- Information-sharing relationships between user and supplier are key to maintaining quality, not only knowing what is needed from each other but why it is essential.

Leachables from both food and drug packaging was the lead article in an issue of the widely distributed Chemical & Engineering News (3). In it the author highlighted two cases of interaction of protein-based drugs with components of prefilled syringes. In one case, a syringe manufacturer was using an epoxy adhesive to attach the metal needle to the syringe barrel. Unfortunately, a solvent from the partially dried epoxy leached into the liquid drug product, oxidized the protein, and caused it to aggregate. In the second case, a tungsten wire used to make the hole in the tip of the syringe to hold the needle left a tungsten oxide residue that later migrated into the drug product and also caused protein aggregation.

Leachables in a drug product have many sources. Since leachables have several sources, E&L studies must not focus only on the primary drug package. Both drug contact and noncontact materials may be sources. The chief concern of any health authority is safety—how do leachables affect the drug and what direct affect do leachables have on the body (toxic effects)? Figure 1 illustrates the many routes though which leachables may enter the body. These routes are primarily through the drug, but leachables, if that is what we wish to call them, may also enter the body by direct contact with packaging materials such as with a drug patch, catheter, or implanted drug delivery device. In Figure 1, this is represented by the arrow from the material to the body. The other routes are

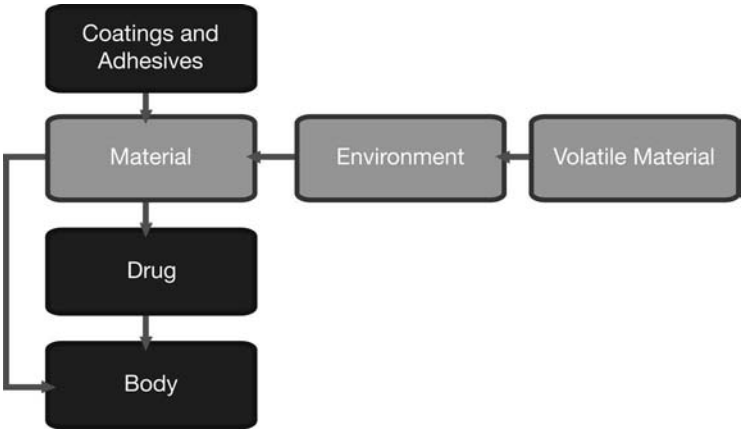

Figure 1 Routes of leachables.

- From material (packaging) into a drug, then into the body
- From coatings and adhesives into packaging materials, then into a drug, then into the body. This occurs most often in flexible and rigid plastic containers with labels where adhesives and ink components can migrate through the plastic container walls into the drug. This does not occur with glass containers.
- From the environment—volatile compounds in the environment can be adsorbed and absorbed by packaging materials and then move into a drug and ultimately the body.
- Volatile materials—volatile materials that are in close proximity to packaging materials may get into the environment and eventually into the body using the route described above. For example, wooden pallets are often fumigated with methyl bromide to rid them of insects. Plastic and rubber packaging materials shipped and stored on wooden pallets could absorb the fumigant if temperature is high and the space is confined.

Consider the case of a glass prefilled syringe with a staked needle (10). The drug is in contact with the following components/materials:

- Glass barrel—glass, although quite nonreactive, is not inert. Metal ions from the glass and pH shifts are the chief concerns. Tungsten, a residue from the manufacturing process, is also a concern. Tungsten wires are used to make the orifice in the tip of the glass barrel where the stainless steel needle will be placed.
- Rubber plunger—rubber is a source of both organic and inorganic extractables. Extractables from both glass and rubber will be discussed in detail later in the chapter.
- Rubber needle shield—the tip of the needle is imbedded in rubber to seal the syringe. Although the area of contact is very small, the needle shield must be considered in an E&L study.
- Stainless steel needle—iron and nickel are chief metal ions extracted.
- Adhesive—an adhesive is used to bind the steel needle to the glass barrel. Contact area is small, but organics may be extracted into the drug product.
- Silicone oil—although not thought of as a packaging component, silicone oil is placed on the inside surface of the glass to reduce the break loose and gliding forces of the rubber plunger. Silicone is often a concern with biotechnology-derived products.

In designing an E&L study there are many choices to be made and each has associated risks. Some of these choices are

- Level of study for a drug product. The U.S. Food and Drug Administration's (FDA) guidance on packaging (11) contains graduated levels of E&L studies for different routes of administration.
- Packaging materials/components to study. It is obvious that a packaging material that will be in direct contact with the drug (primary packaging materials) must always be considered. But, how about labels, or over raps that are not in contact with the drug?
- Extracting solvent systems. How many? Which ones?
- Extraction conditions. How long? What temperature? What quantity/surface area per volume of solvent?
- Minimum quantity to identify, quantify, and qualify.
- Analytical methods to use and validate.
- Participating laboratories. What skill sets? In-house or contract?
- Frequency of leachables measurements over shelf life.

Even after considerable study of the E&L requirements and best practices, those considering an E&L study have the same common questions. These questions are not easily answered and there is not only one correct study protocol. The correctness of a protocol depends on the drug (e.g., route of administration, frequency of use, patient population), the packaging materials (type and degree of potential interaction with the drug), division of FDA

or regulatory authority that regulates the drug product, drug company making the submission, and many other variables. The common questions are as follows:

- What do the regulations say? There are several guidances, guidelines, ICH (www.ich.org), and CFR (www.gpoaccess.gov/cfr/retrieve.html) documents to consider.
- How far do we go? Scientists ponder how much effort is expected to identify, or quantify, or qualify a chemical compound that is found in an E&L study. Many extractable compounds may not have published reference spectra or chromatographic retention time data or toxicity data, nor is authentic reference material available, and the quantity extracted may be at the limit of detection. Regulations (11) often contain terms such as "appropriate solvent," "significantly exceed," and "may be advisable," which make definitive interpretation difficult.
- How is a practical science-based E&L study performed?

These and other questions will be answered in this chapter giving the reader enough information to design a "correct" protocol and to avoid inappropriate, non-science-based methods and procedures.

COMMON TERMS DEFINED

The language of E&L studies is still evolving and many common terms have specific meanings when used in the context of E&L. Even the terms "extractable" and "leachable", though very distinct, are often used interchangeably in both written and oral presentations. Common terms are defined here, rather than in an appendix, so that the reader is familiar with the language of E&L before moving into more detailed sections of this chapter. The most important two terms, extractables and leachables, have been defined previously so the reader is referred back to section "Introduction: Origin and Importance of Extractables and Leachables."

Packaging

- Container closure system: Refers to the sum of packaging components that together contain and protect the dosage form. This includes primary packaging components and secondary packaging components.
- Packaging component: Any single part of a container closure system.
- Packaging materials: May refer to packaging components or to materials of construction.
- Primary packaging component: A packaging component that is or may be in direct contact with the dosage form. Examples of primary components are

☐ Ampoules	☐ Bottles [glass, high-density polyethylene (HDPE)]
☐ Flexible bags	☐ Closure liners
☐ Tube with/without liner	☐ Desiccant container
☐ Pouch	☐ Filler (cotton, rayon)
☐ Cap inner seal	☐ Rubber vial stopper
☐ Blister packaging	☐ Glass, plastic vial
☐ Bulk containers	☐ Prefilled syringe

  Primary packaging components *are the major source of leachables* because they are in intimate contact with drug products.
- Secondary packaging component: A packaging component that is not and will not be in direct contact with the dosage form. Some examples are as follows:

☐ Overwraps	☐ Cartons
☐ Inks	☐ Labels
☐ Plastic plunger rods	☐ IV tubing clamp
☐ Aluminum and aluminum/plastic crimp seals for vials	

  Secondary components *may be a source of leachables*. Adhesive from labels on plastic containers must be considered as potential leachable but not when on glass

containers. Volatile substances from secondary components may also migrate into primary components and become leachables.

- Associated component: Component intended to deliver the dosage form to the patient but not stored in contact with the dosage form. Examples are

☐ Dosing cups ☐ Calibrated spoons
☐ Sterile empty syringe ☐ Medicine dropper

Since these components are only intended for short-term contact (minutes) with drug products, *leachables are unlikely*; however, if it is possible and likely that drug products could be stored in these components for a significant time (hours or days), such as in a sterile empty syringe or even in a dosing cup, then these components should be evaluated for leachables.

Drug

- Drug: A therapeutic agent or any substance other than food used in the prevention, diagnosis, alleviation, treatment, or cure of diseases.
- Drug product: The dosage form in the final immediate packaging intended for marketing. Leachables studies are performed on the drug product for a time equal to the shelf life.
- Drug substance or active pharmaceutical ingredient (API): An active ingredient that is intended to furnish pharmacological activity or other effect in the prevention, diagnosis, alleviation, treatment, or cure of diseases. The unformulated drug substance that may be subsequently combined or formulated with excipients to produce the drug product.
- Drug product vehicle: The entity (or mixture of entities) that delivers the drug to the site of application. For a liquid dosage form, the drug product vehicle is every part (or component) of the liquid preparation except the drug substance or API. In certain circumstances, such as when the API would interfere with the analysis of extractable substances in the drug product, leachables testing may be performed with the drug product vehicle and not the drug product. To justify this, the analyst would need to demonstrate that the API does not alter the extraction properties of the drug product.
- Solvent: An organic or inorganic liquid used as a vehicle for the preparation of solutions or suspensions in the synthesis or manufacture of a new drug substance. Also, an organic or inorganic liquid used in extraction studies that will extract chemical components that are potential leachables but will not dissolve the material or component being studied.
- Simulated solvent: Solvents commonly used to mimic the extraction properties of foods and beverages to be used for extractables testing prescribed in the food additive regulations. The food simulating solvents are generally water, heptane, and 8% and 50% alcohol. Extraction conditions are based on conditions of use and type of food. In the leachables testing of drugs, the "drug product vehicle," may be used as a "simulating solvent" when the API interferes with analytical testing.
- Degradation product: An impurity resulting from a chemical change in the drug substance brought about during manufacture and/or storage of the new drug product by the effect of, for example, light, temperature, pH, water, or by reaction with an excipient and/or the immediate container closure system (12).
- Identified degradation product: A degradation product for which a structural characterization has been achieved (12).
- Unidentified degradation product: A degradation product for which a structural characterization has not been achieved and that is defined solely by qualitative analytical properties (e.g., chromatographic retention time) (12).
- Specified degradation product: A degradation product that is individually listed and limited with a specific acceptance criterion in the new drug product specification. A specified degradation product can be either identified or unidentified (12).
- Impurity: Any component of the new drug product that is not the drug substance or an excipient in the drug product (12).

- Impurity profile: A description of the identified and unidentified impurities present in a drug product (12).
- Identified impurity: An impurity for which a structural characterization has been achieved (13).
- Potential impurity: An impurity that theoretically can arise during manufacture or storage. It may or may not actually appear in the new drug substance (13).
- Specified impurity: An impurity that is individually listed and limited with a specific acceptance criterion in the new drug substance specification. A specified impurity can be either identified or unidentified (13).
- Unidentified impurity: An impurity for which a structural characterization has not been achieved and that is defined solely by qualitative analytical properties (e.g., chromatographic retention time) (13).
- Unspecified impurity: An impurity that is limited by a general acceptance criterion, but not individually listed with its own specific acceptance criterion, in the new drug substance specification (13).

Extractions and Leachables Studies

- Extraction profile: Analysis (usually by chromatographic means) of extracts from a container-closure system, usually qualitative. A profile is usually presented as a chromatogram or as a table showing the identity, relative peak height, and retention time or as a table.
- Quantitative extraction profile: An extraction profile in which the amount of each substance is determined.
- Qualification: The process of acquiring and evaluating data that establishes the biological safety of an individual impurity or a given impurity profile at the level(s) specified.
- Thresholds [from ICH Q3B(R2): Impurities in New Drug Products] (12):
  - ☐ Reporting threshold: A limit above which an impurity needs to be reported.
  - ☐ Identification threshold: A limit above which an impurity needs to be identified.
  - ☐ Qualification threshold: A limit above which an impurity needs to be qualified.

- PQRI: Product Quality Research Institute—a nonprofit consortium of organizations working together to generate and share timely, relevant, and impactful information that advances drug product quality and development (14). The PQRI has completed and published one E&L study of inhalation products (OINDP) (5) and is currently completing another study of parenteral and ophthalmic products (PODP). (See definitions of OINDP and PODP below). An important objective of these studies is to define toxicological and analytical limits for E&L studies.
- OINDP: PQRI study of Orally Inhaled and Nasal Drug Products such as
  - ☐ Metered dose inhalers
  - ☐ Dry powder inhalers
  - ☐ Inhalation solutions
  - ☐ Inhalation suspensions
  - ☐ Spray products
  - ☐ Nasal sprays

- PODP: PQRI study of Parenteral and Ophthalmic Drug Products such as injectable SVPs (small volume parenterals), injectable LVPs (large volume parenterals), and all ophthalmic products—injectable and noninjectable.
- SCT: PQRI term for Safety Concern Threshold, which is the threshold below which a leachable would have a dose so low as to preset negligible safety concerns from carcinogenic and noncarcinogenic toxic effects. For OINDP products, this threshold was concluded to be 0.15 μg/day (*n*). For PODP products the threshold may differ.
- QT: PQRI term for Qualification Threshold, which is the threshold below which a given leachable is not considered for safety qualification (toxicological assessment) unless the leachable presents structure-activity relationships (SAR) concerns (5). For OINDP products this threshold was concluded to be 5.0 μg/day. For PODP products the QT threshold may differ also.

REGULATORY REQUIREMENTS

Drug products and medical devices are regulated worldwide for the purpose of protecting all consumers; although guiding principles are intended to achieve the same end result, the legislation is not international. Drug products, medical devices, and their raw materials are obtained globally, in part, or finished product, from both established and emerging economies. Regulatory bodies in each country will have their own set of expectations for the safety, quality, effectiveness, and performance of drugs and medical devices. For instance, in Europe, licensing can be granted at a national and/or European Union level and a number of different regulatory agencies may be involved. Through the ICH process, considerable harmonization has been achieved among the three regions (Japan, Europe, and the United States) in the technical requirements for the registration of pharmaceuticals for human use. These products are licensed through a market application and approval process and the FDA, European Union Medicines and Healthcare Products Regulatory Agency (MHRA), and Japanese Ministry of Health, Labor and Welfare (MHLW) are among the regulatory bodies driving the standards for governance of drugs and medical devices. The regulations for pharmaceuticals, biologics, and medical devices are not totally harmonized but the expectations and process have much in common. In this chapter, the U.S. legislation, FDA guidance documents, and recognized standards will be discussed in relation to qualification of container closure systems for drug products with consideration given to the medical device regulation.

In 1906, the original U.S. Food and Drugs Act was passed by Congress to prohibit interstate commerce of misbranded and adulterated foods, drinks, and drugs. This was later revised in 1938 to the Federal Food, Drug, and Cosmetic (FDC) Act to contain new provisions to extend the control to cosmetics and therapeutic devices. New drugs were also required to be shown safe before marketing. Amendments were made in 1976 and 1990 for medical devices so that all devices are to be divided into classes with varying amounts of control required and indication of safety. The sections of the United States Code (USC) Sections 501, 502, and 505 are associated with container closure systems for drug products; the following transcriptions are noted:

- "a drug is deemed adulterated if its container is composed in whole or part of a poisonous or deleterious substance that may render the contents injurious to health . . ."
- "an application shall include a full description of the methods used in the manufacturing, process, and packaging of such a drug. This includes facilities and controls used in the packaging and drug product."

The rulings for drugs were codified in 1978 under the CFR Title 21 parts 210 and 211, more commonly known as current Good Manufacturing Practices; devices were regulated under 21 CFR 820 Quality System Regulation, sections specific to these products are described as follows (15,16):

21 CFR 211.160 General Requirements

Laboratory Controls shall include the establishment of scientifically sound and applicable written specifications, standards, sampling plans, and test procedures including resampling, retesting, and data interpretation procedures designed to ensure that components, drug product containers, closures, in-process materials, labeling, and drug products conform to appropriate standards of identity, strength, quality, and purity.

21CFR 211.94 Drug Product Containers and Closures

Device containers should not be reactive, additive, or absorptive as to alter the safety, identify, strength, quality, or purity of the drug beyond the official or established requirements of drug product.

Standards or specifications, methods of testing, and, where indicated, methods of cleaning, sterilizing, and processing to remove pyrogenic properties shall be written and followed for drug product container and closures.

21 CFR 820

cGMP requirements are set forth in this quality system regulation. The requirements in this part govern the methods used in, and the facilities and controls used

> for, the design, manufacture, packaging, labeling, storage, installation, and servicing of all finished devices intended for human use. The requirements in this part are intended to ensure that finished devices will be safe and effective and otherwise in compliance with the FDC Act.

Applications for drug products and devices are submitted to one of the three FDA centers: Center for Biologics Evaluation and Research (CBER), Center for Devices and Radiological Health (CDRH), and Center for Drug Evaluation and Research (CDER). Each center governs specific to the nature of the product and its intended use. This means the information required for a drug or biological application is similar but may not be necessary for that of a device, and the information for a device may not pertain to that of a drug product. Over time as drug products and administration forms have evolved, combination products have entered the market. A combination product is defined under the FDC Act as

1. A product comprising two or more regulated components, i.e., drug/device, biologic/device, drug/biologic, or drug/device/biologic, that are physically, chemically, or otherwise combined or mixed and produced as a single entity.
2. Two or more separate products packaged together in a single package or as a unit and comprised of drug and device products, device and biological products, or biological and drug products.
3. A drug, device, or biological product packaged separately that according to its investigational plan or proposed labeling is intended for use only with an approved individually specified drug, device, or biological product where both are required to achieve the intended use, indication, or effect and where upon approval of the proposed product the labeling of the approved product would need to be changed, e.g., to reflect a change in intended use, dosage form, strength, route of administration, or significant change in dose.
4. Any investigational drug, device, or biological product packaged separately that according to its proposed labeling is for use only with another individually specified investigational drug, device, or biological product where both are required to achieve the intended use, indication, or effect.

Combination products raise a variety of regulatory and review challenges since the products share many of the same basic features, they are also each somewhat unique.

Drugs, devices, and biological products each have their own types of marketing applications, GMP regulations, and adverse event reporting requirements. When drugs and devices, drugs and biologics, or devices and biologics are combined to create a new product, questions are sometimes raised about how the combination product as a whole will be regulated as there is no special type of marketing application for combination products. Under Section 503 of the FDC Act, a combination product is assigned to a center with primary/lead jurisdiction based on a determination of the primary mode of action (PMOA) of the combination product. A combination product is assigned through the FDA Office of Combination Products (OCP) (17).

The type and amount of container closure information required in a given application can vary and the interpretation of the legislation can be dependent on different factors. Requirements mandated by the FDA are found in applicable monographs (those with numbers under 1000) of the USP/National Formulary (18). Beyond the container closure information specified in the monographs, the FDA recommends additional information to be provided on the basis of guidance documents. The FDA's Guidance for Industry does not suggest a comprehensive list of tests, specific test methods, or acceptance criteria. Batch-to-batch consistency of packaging components and acceptance criteria should be based on good scientific principles for each specific system and product (11). The guidance documents concerning cGMP and the container closure guidance will be discussed here. A list of other related guidance documents will be included in "References".

In 2006, the modernization of the cGMPs was initiated to bridge the 1978 regulation with current understanding of quality systems, harmonize with other widely used quality systems,

and establish a framework for a more systematic risk-based approach to manufacturing of pharmaceuticals. The FDA issued a report in 2004 titled, "21st Century" Initiative on the Regulation of Pharmaceutical Manufacturing, which described plans for forthcoming guidance on the new quality system. The Guidance for Industry *Quality Systems Approach to Pharmaceutical CGMP Regulations* was published in 2006 by CDER, CBER, Center for Veterinary Medicines (CVM), and Office of Regulatory Affairs (ORA), providing the framework to instill the philosophy that "Quality should be built into the product, and testing alone cannot be relied on to ensure product quality" (19). The concept was subsequently more fully detailed in the following Guidance for Industry documents developed by CDER/CBER within the expert working group of ICH (20–22):

– 2009 Q8(R2) Pharmaceutical Development
– 2006 Q9 Quality Risk Management
– 2009 Q10 Pharmaceutical Quality System

The concept of design space and building quality through process development and improvements are presented in Q8. The choice and rationale for the container closure system should be consistent with the Common Technical Document (CTD) format. Information on both leachables and extractables should be included in Module 3 (Quality) Manufacturing Process Development section under Container Closure System (3.2.P.2.4.). When warranted, E&L related impurities, the correlations and specifications should be included, if leachables are confirmed through shelf life. Q8 cites "The degree of regulatory flexibility is predicated on the level of relevant scientific knowledge provided." Relevant scientific knowledge is grounded in the principles of risk management, which is described in Q9 and illustrated as shown in Figure 2.

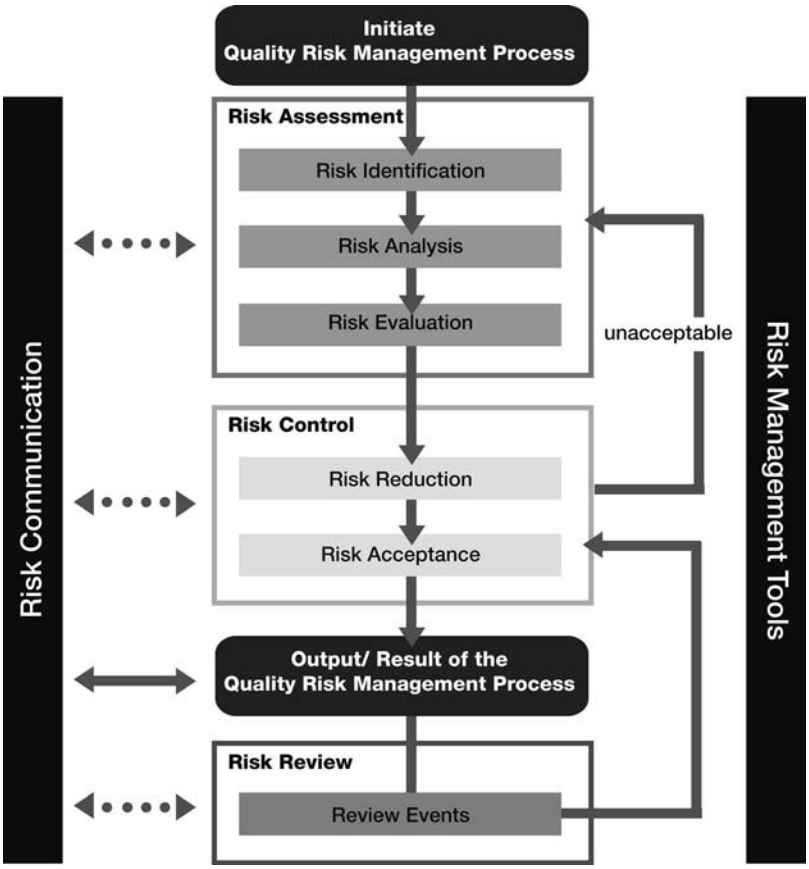

Figure 2 Quality risk management process.

Examples of risk tools such as Failure Mode Effects Analysis (FMEA), Fault Tree Analysis (FTA), Preliminary Hazard Assessments (PHA), and Hazard Analysis and Critical Control Points (HACCP) are referenced. Quantifying the probability of any particular extractable migrating into the drug product and the severity of the impact is the goal of a leachable study.

Three main objectives of Q10 are to (*i*) achieve product realization, (*ii*) establish and maintain a state of control, and (*iii*) facilitate continual improvement.

The ICH Q10 model outlines the pharmaceutical quality system on the basis of the International Organization for Standardization (ISO) quality concepts. Quality systems to support the technical activities for pharmaceutical development, technology transfer, commercial manufacturing, and product discontinuation are explained. ICH Q10 augments the cGMPs by providing details on specific quality elements such as process performance monitoring systems, corrective action/preventative action, change management, and management responsibilities. Implementation of Q10 throughout the product lifecycle should facilitate innovation and continual improvement and strengthen the link between pharmaceutical development and manufacturing activities. Leachables and extractables, although not specifically mentioned, is key in achieving the Q10 objectives. Container closure systems must be manufactured under GMP conditions and satisfy the same quality elements.

The Guidance for Industry: Container Closure Systems for Packaging Drugs and Biologics was published by CDER/CBER in 1999 and provides recommendations for the information to be provided in an application for any drug product. Since the publication of this guidance there have been additional recommendations on inhalation products as well as those of the PQRI. These recommendations will be cited in the "References" section but are too specific to include in this section (5,23,24).

The market package for a drug product includes the primary packaging components, secondary package, external packaging, and associated components. The FDA recommends the packaging to be suitable based on assessments in four main categories:

Protection
: Ensure the container closure system shields the product from light, solvent loss, reactive gases, moisture, microbial contamination and filth.

Compatibility
: The container closure system must safeguard against loss of potency, degradation of drug substance, reduced concentration of an excipient, changes in drug product pH, discoloration of either the dosage form or the packaging component or increase in brittleness of the packaging.

Safety
: The container closure system will not leach harmful or undesirable amounts of substances to which a patient will be exposed when being treated.

Performance
: The container closure system will function and deliver in the manner for which it was designed.

Each suitability category is associated with a level of testing in which level 1 indicates the greatest degree of evaluation required. The safety category provides guidance for E&L studies to determine what chemical species may migrate into the dosage form and the toxicological evaluation of those migrated substances. The concern for package component–product interaction is ranked according to the physical state of the product (liquid vs. solid) and type of liquid (organic, organic-aqueous, and aqueous). For example, inhalation aerosol products that contain highly extracting organic solvents are ranked HIGH while solid oral tablets are ranked LOW. The type of drug product, according to route of administration and concern for interaction, is evaluated in the Guidance Document Matrix as shown in Figure 3. The recommended level (1S–5S) of safety testing is noted with respect to the different types of drug products. The highest level of testing is 1S and the testing recommendations are gradually reduced as 5S is approached.

In general, recommended testing may include any combination of the USP monographs <661> for plastic containers, <381> for elastomeric closures, <660> for glass containers,

Degree of Concern Associated with Route of Administration	Likelihood of Packaging Component-Dosage Form Interaction		
	High	**Medium**	**Low**
Highest	• Inhalation Aerosols and Solutions **(1S)** • Injections and Injectables Suspensions **(2S)**	• Sterile Powders and Powders for Injection **(2S)** • Inhalation Powders **(5S)**	
High	• Ophthalmic Solutions and Suspensions **(2S)** • Transdermal Ointments and Patches **(3S)** • Nasal Aerosols and Sprays **(1S)**		
Low	• Topical Solutions and Suspensions **(3S)** • Topical and Lingual Aerosols **(3S)** • Oral Solutions and Suspensions **(3S)**	• Topical Powders; Oral Powders **(4S)**	• Oral Tablets • Oral Capsules **(4S)**

Figure 3 Guidance Document Matrix.

and <87> <88> for biologic reactivity tests, plus generation of qualitative extraction profiles, quantitative extraction profiles, and reference to 21 CFR Indirect Food Additive Regulation (25).

The ICH recommends identification and acceptance criteria for leachables in ICH Q3B (R), Impurities in New Drug Products, which applies only to the reaction products of the drug substance with the immediate container/closure system in amounts $\geq$0.1% (12). Also, ICH Q6A, Specifications: Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products, has provisions for extractables specifications when data demonstrates the need that acceptance criteria for extractables from container/closure components are appropriate (26).

The ISO published ISO 10993 Biological Evaluation of Medical Devices, a multiple part standard in which chemical characterization is required to evaluate potential leachable chemicals and their bioavailability (27). This standard is intended for medical devices and has limited utility for container closure systems, although the chemical characterization and specification parts have common elements. The applicant has the overall responsibility to ensure the suitability of the container closure/device system throughout the shelf life of the product. A program to evaluate for leachables during stability studies will indicate which controls will be needed to show that the product is consistent with respect to container closure/device system interaction. In all cases, the guidance documents are not prescriptive and there are other approaches that can be taken to indicate container closure/ device system safety. The information provided in an application should have a science base rational that is data supported following cGMP.

E&L FROM RUBBER COMPONENTS

Rubber has been in commercial use as material for packaging components, especially parenteral packaging, since the early part of the 20th century. It possesses unique physical properties that are important to the functions of the total parenteral packaging system. Even before that, soon after the discovery of the vulcanization process by Charles Goodyear in 1839, the use of rubber in medical applications such as bandages, gloves, tubing, hot water bottles, and syringes was described (28,29).

In this section, rubber as a material for packaging components will be reviewed. The discussion will be divided into three parts:

- Composition of rubber components
- Sources of extractables/leachables
- Reduction of leachables

Composition of Rubber Components

Rubber is a unique material. It can be molded into an almost limitless variety of shapes and forms; it is flexible and conformable; and, when penetrated, it is resealable (28). Rubber is commonly formed into packaging components such as syringe plungers, needle shields, dropper bulbs, and vial stoppers. Syringe plungers conform tightly to the cylindrical syringe barrel and effectively seal the drug product in the syringe without leakage. Vial stoppers are forced and held against the top finish of glass vials by aluminum seals, effectively sealing any irregularities in the glass-rubber interface. When penetrated by needle cannula, rubber reseals the opening once the needle is withdrawn. No other materials, such as glass, thermoform plastic, and metal possess these abilities.

Rubber can be divided into natural and synthetic. Natural rubber was largely used pre-1940 for pharmaceutical packaging components (30). In 1940, butyl rubber was invented. Butyl has better gas barrier properties and more thermal oxidation resistance than natural rubber and therefore was quickly adopted for use in rubber stoppers. In the 1960s, first chlorobutyl and then bromobutyl rubber were introduced, and today most stoppers are made from these polymers. It is not only the barrier and oxidation resistance properties of the halogenated butyls (chloro- and bromobutyls) that have made them the predominate choice for stoppers over both natural and butyl rubber; the additional advantage is that halobutyls can be cured using low levels of "clean" curing agents, including sulfur- and zinc-free ones. This is a great advantage when E&L are a concern. So halobutyls have both physical (moisture and oxygen barrier, resistance to oxidation) and chemical (lower levels of less toxic curing agents) advantages over natural rubber. Today, it is estimated that 80% to 90% of all injection and infusion stoppers are based on halobutyl rubber. A new polymer, brominated isobutylene paramethylstyrene terpolymer (BIMSM), was recently introduced, which is said to yield very low levels of extractables that heretofore have only been possible with polymer-coated stoppers (30). More on this subject later in the chapter.

Rubber is composed of several materials, each of which is necessary for a particular physical or chemical property. Typical key materials are listed below.

Pharmaceutical Rubber Formulation Materials

Material	Function
• Elastomer	• Base elastomer or polymer
• Curing agent	• Forms cross-links between chains, also known as a vulcanizing agent
• Accelerator	• Affects the type and rate of cross-linking
• Activator	• Modifies the efficiency of curing agents
• Antioxidant	• Reduces oxidation of polymer
• Plasticizer	• Acts as processing aid
• Filler	• Modifies physical properties such as hardness
• Pigment	• Coloring agent

A rubber formulation may contain more than one elastomer; blends of natural and chlorobutyl are common. More than one pigment may be used; mixtures of carbon black and titanium dioxide are used to produce gray-colored rubber components. Similarly, multiple types of other materials may be used also; therefore, a rubber formulation may contain many

ingredients, each of which may contribute to leachables in drug products. A typical chlorobutyl pharmaceutical formulation, materials and percentage, is listed below.

Typical Chlorobutyl Rubber	Formulation Used for Pharmaceutical Packaging
Material	Percentage by weight
Chlorobutyl rubber (elastomer)	53.1
Calcined clay (filler)	39.8
Paraffin oil (plasticizer)	4.2
Titanium oxide (pigment)	1.1
Carbon black (pigment)	0.13
Thiuram (curing agent)	0.14
Zinc oxide (activator)	1.0
Butylated hydroxytoluene, BHT (anti-oxidant)	0.53

Sources of Extractables and Leachables

Each of the ingredients in a rubber formulation can be a source of leachables. Examples are as follows:

- Elastomer—monomers; oligomers (short chains of monomer units both cyclic and noncyclic); halogenated oligomers from chloro- and bromobutyl rubber; polymer additives and by-products such as BHT, antioxidants, calcium stearate, and epoxidized soybean oil (ESBO). Some of the monomers and oligomers are volatile and can migrate from rubber stoppers into dry lyophilized drug products during storage (30).
- Fillers—metal ions such as Ca, Al, Mg, Mn, Si.
- Plasticizers—volatile oligomers.
- Pigments—metals ions such as Ti, Fe, and Ca; and polynuclear aromatics from carbon black.
- Curing/vulcanizing agents and accelerators—original chemical compounds and reaction products.
- Activators—metal ions (e.g., Zn) and stearates.
- Antioxidants—original chemical compounds (e.g., amines and phenols) and degradation products.

Many factors affect both the number of chemical compounds and their amounts extracted from rubber into a drug product. These factors are as follows:

- Type of rubber formulation—bromobutyl rubbers are generally "cleaner" (have lower extractables) than other rubber types in the order:
  bromobutyls > chlorobutyls > butyls > natural.
- Number and type of chemical compounds in the rubber formulation—modern rubber formulations may have only 6 to 8 ingredients, while older natural rubber formulations have 10 to 15 ingredients.
- Drug product vehicle—aqueous (pH, ionic strength); organic. Vehicles with higher percentages of organic solvents have more leachables. For example, metered dose inhalers (MDI) utilize chloroflorocarbons (CFC) and hydrofloroalkanes (HFA) for both the vehicle and propellant in OINDP. These organic solvents can extract larger amounts of and greater numbers of organic compounds from rubber packaging components than aqueous solutions commonly utilized for injectable or ophthalmic drugs.
- Rubber surface area to drug product vehicle volume ratio—the larger the surface area of the rubber component exposed to the drug product, the greater the opportunity of extraction. Using the smallest rubber components possible or using film-coated components [e.g., West's Flurotec® (31) or Helvoet's Omniflex® (32) stoppers] are viable strategies to minimize drug-rubber contact and therefore leachables.

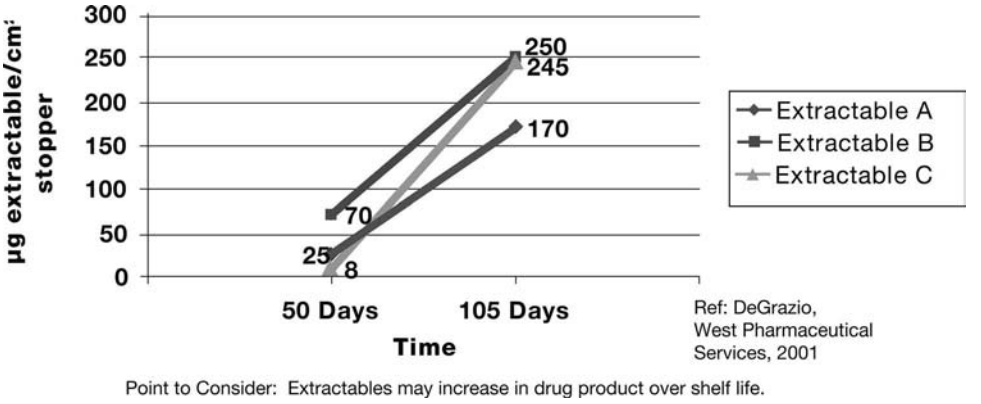

Figure 4 Extractables over time in aqueous solution.

- Temperature—temperature during terminal sterilization, transport, and storage. The rate of migration of chemical compounds to the surface of a packaging component and the solubility of the compounds in the drug product vehicle increase with temperature. Products that are terminally sterilized by heat are especially vulnerable to higher leachables since the drug product is in direct contact with packaging components at 121°C or higher for 30 or more minutes. Refrigerated (2–8°C) and frozen (−25 to −10°C) products mitigate the rate of migration of packaging extractables. Transport and storage at temperatures higher than that recommended on the label not only affects the stability of the product but also may increase leachables (33).
- Time—the longer the shelf life of a product, the greater the opportunity for increased leachables. This is illustrated in the data presented by DeGrazio in Figure 4.

The analysis of E&L from rubber is particularly challenging for the following reasons:

- The composition of rubber is usually proprietary; therefore, getting information about potential extractables from suppliers is unnecessarily difficult. Drug manufacturers generally must perform extractables studies prior to leachables studies to qualify rubber components. It is recommended that the first step in any E&L study is contact with the supplier to get as much rubber composition information as possible. Having information about what is and what is not in a rubber formulation *before* extractables studies are initiated will save time and money.
- There are many raw materials in a rubber formulation. Refer to section "Composition of Rubber Components" discussed earlier.
- The raw materials are not pure. They contain many impurities that may or may not be known to the rubber manufacturer. Also, these impurities are not commonly listed in the ingredient list found in a Drug Master File. Typical impurities found in the vulcanizing agent, *N*-*t*-butyl-2-benzothiazyl-sulfenamide (TBBS), are listed below.
  - $C_{(7–9)}$ alkyl benzyl phthalate
  - Benzothiazyl disulfide
  - *t*-Butylamine
  - 2-Mercaptobenzothiazole
  - Benzothiazole
  - Mineral oil (*anti-dust agent*)

  Inorganic materials, such as fillers, are also complex materials. The composition of a typical kaolin clay is shown below.
  - Al_2O_3 44.48%
  - SiO_2 52.41%
  - Water 5,000 ppm, maximum
  - TiO_2 17,900 ppm, typical

- Na_2O 2,800 ppm
- CaO 300 ppm
- Fe_2O_3 5,800 ppm
- Co 200 ppm
- K_2O 1,500 ppm

- Impurities may be present in very small quantities.
- Many compounds, including impurities, are changed chemically during and after the manufacturing process. During the vulcanization or cross-linking process reactive curing agents, accelerators, and antioxidants are chemically changed; antioxidants continue to change postmanufacturing as they react with oxygen to protect the polymer.
- The reaction products of vulcanization are often not known, are present in very small quantities, and pure standards are not available.

These factors make identification, quantification, and qualification very challenging.

The type of rubber or elastomer that was discussed thus far is *thermoset* rubber. Thermosets are polymers that have been chemically cross-linked to form the final structure of the material. Cross-linking and forming of the rubber into a functionally shaped packaging component take place in a mold in the presence of heat and pressure. Once formed, thermosets do not melt and cannot be easily reformed due to the permanence of the chemical cross-links (29). The necessity of a curing system (curing agent, accelerator, activator) in thermosets increases the number of chemical compounds in the rubber and thus the opportunity for them and their reaction products to become leachables.

Thermoplastic rubber, called TPE or thermoplastic elastomer, is another type of rubber. These materials have functional properties similar to thermoset rubber but can be melted and reformed into a different shape if desired like common plastics such as polyethylene. Thermoplastic rubbers are not chemically permanently cross-linked like thermosets. The cross-link in thermoset polymers is a covalent bond created during the curing process. However, the cross-link in thermoplastic elastomer polymers is a weaker dipole or hydrogen bond that can be broken when sufficient heat is applied and reformed when cooled. Because of the absences of a chemical curing system, thermoplastics have simpler chemistries than thermosets and potentially lower levels of leachables. Unfortunately, thermoplastics have found limited use to date as pharmaceutical packaging components because of their tendency to deform during terminal sterilization.

Reduction of Leachables

Leachables are an inevitable companion of packaging, but one can take steps to reduce them to levels that are safe for the purpose intended.

- Choose the "most compatible" rubber formulation. Perform accelerated extractables screening studies with the drug product. There are several approaches to this prescreening.
  - Information due diligence—compare available rubber formulation information with known drug product chemistry. Discuss any likely incompatibilities with the rubber supplier. Also, review the information with toxicologists for any likely concerns.
  - Drug product spiking—prepare a concentrated extract of the rubber formulation by extracting the rubber with the drug product vehicle or a solvent system that mimics the drug product vehicle. Perform the extract at high temperature (e.g., reflux or autoclave) using rubber with a large surface area (e.g., cutting the rubber component into small pieces). Mix a portion of the extract with drug product and observe/analyze product for interactions.
  - Accelerated stability testing—store drug product in contact with rubber component at the highest temperature that the product will tolerate for two to four weeks. Observe/analyze product for interactions.
- Use the smallest possible packaging component to minimize drug-rubber contact. A typical 20-mm vial stopper has more than twice the drug product contact surface area than a 13-mm stopper (20-mm S-127 stopper = 3.65 cm^2 vs. 13-mm V-35 stopper = 1.65 cm^2) (34).

- Limit contact time between drug product and rubber component by limiting the shelf life.
- Pre-extract rubber components before use to reduce the amount of substances available for migration into drug products. This method was very common for natural rubber vial stoppers and syringes when older sulfur-containing cure systems were used. Rubber components were typically autoclaved in the drug product vehicle before use. The introduction of synthetic halobutyl stoppers with cleaner curing systems has become much less common. However, for rubber components used in contact organic solvents, such as valves and o-rings in inhalation drug containers, pre-extraction of components is still commonly used (3).
- Use the best possible contact conditions. Freeze drying, refrigerating, and freezing the drug product will reduce the rate of extraction of impurities from rubber. Terminal sterilization, in which the drug product and rubber packaging component are in contact at high temperatures for a short time (~121°C for 30 minutes), can produce large amounts of extractables compared with normal storage conditions (~RT for 3 years). When undesirable drug product-packaging interactions are anticipated, avoidance of terminal sterilization in favor of aseptic processing is recommended.
- Use a coated stopper. Stoppers coated with "a more inert than rubber" coating can reduce leachables. Refer to the EPREX example discussed earlier.

  Coated stoppers are commercially available in two types: partially coated and totally coated. In the partially coated type, a thin film of inert polymer is laminated onto the surface of the rubber closure in the molding process. The West Flurotec (31) stopper, which is laminated with a copolymer of ethylene and tetrafluroethylene (ETFE), is an example of a partially coated stopper. The ETFE laminate can be applied to either the bottom plug or product contact area of the stopper or to both the top and bottom of the stopper. The function of the laminate on the top of the stopper is not to reduce extractables but rather to provide a nonstick surface on lyophilization stoppers so that they do not stick to the lyophilizer shelves during stoppering. Illustrations of these stoppers are shown in Figures 5 and 6. The black area represents the rubber and the gray represents the coating.

  The Helvoet Omniflex3G® (32) product is an example of a totally coated stopper. In the Helvoet process, stoppers are coated on all surfaces with a

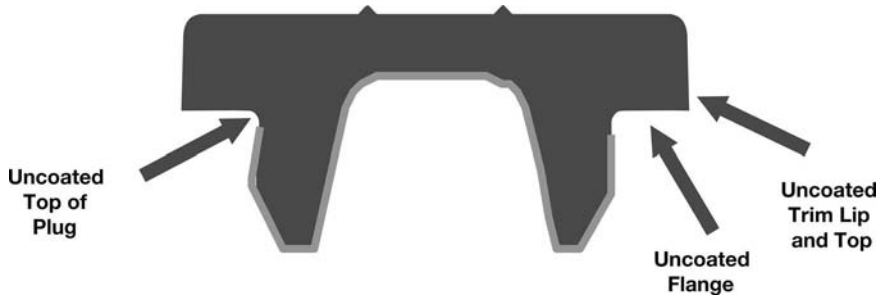

Figure 5 Plug coated stopper.

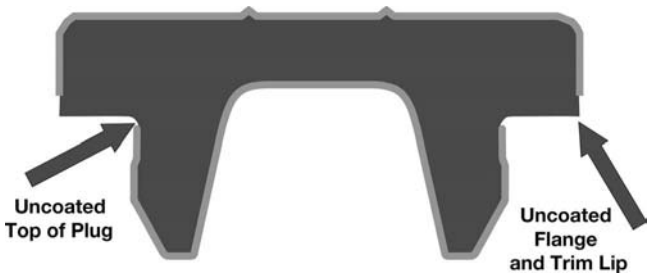

Figure 6 Plug and top coated stopper.

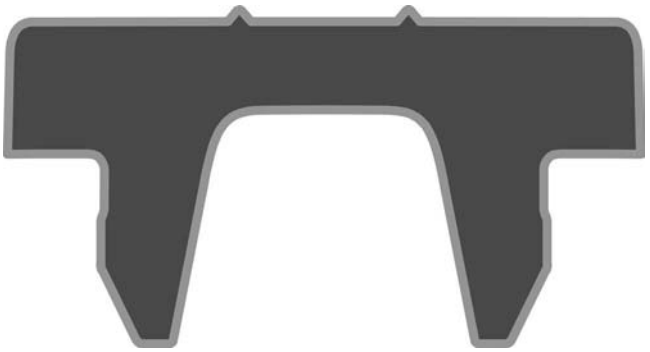

Figure 7 Totally coated stopper.

fluoropolymer material after the stopper is molded. An illustration of this stopper is shown in Figure 7.

The West Flurotec film is available on syringe plungers also.
In managing leachables from rubber components it is important to note the following:

- Extractables can migrate into both liquid and solid products (powder, lyophilized).
- Rubber components are composed of several raw materials, each of which has the potential to migrate into drug products and become a leachable.
- Raw materials used in pharmaceutical and medicinal rubber components are not pure and may be composed of several chemical compounds and contain impurities.
- Chemical compounds are changed during and after the manufacturing process. Therefore, leachables found may differ in identity from those used in the rubber formulation recipe.
- The number and quantity of leachables found will depend on the composition of the elastomeric formulation, rubber processing and sterilization cycles, time, and the unique characteristics of the drug product.
- Identity and quantification of extractables from rubber is a complex process requiring expertise in both chemical analysis and rubber chemistry.

E&L FROM GLASS COMPONENTS

Although glass, in most applications, is less reactive with drug products than rubber or plastics, it is not inert. In dealing with E&L there are two major differences between glass and rubber. The first is that glass compositions are more uniform from supplier to supplier. The percentage of each raw material may differ slightly but the materials themselves are quite uniform. The second difference is that glass compositions are usually not proprietary and are readily shared by the supplier with the drug packager. These differences make the identity and quantification of glass E&L much easier than in either rubber or plastics.

The history of glass as a packaging material is a long one; glass containers existed in Egypt around 1500 BC (1). Glass has been used as a pharmaceutical packaging component for several hundred years particularly during the 20th century during the tremendous growth of pharmaceutical industry. Even though many predicted doom for the glass industry when modern plastics became available some 50 years ago, the use of glass for pharmaceutical containers has endured. There are several unique advantages of glass that account for this endurance.

- Excellent chemical resistance, but NOT INERT
- Impermeable to gases
- Easily cleaned, sterilized, and depyrogenated
- Transparent
- Rigid, strong, and dimensionally stable

Glass packaging components are made by either forming them in a rigid mold from molten glass (molded glass) or by forming them from heated extruded glass tubing (tubing

glass). Size determines the optimum manufacturing method. Small components are made easily from available small diameter tubing while large components must be made by molding because large diameter glass tubing is not available. The transition point is about 100-mL capacity. Common glass components and their method of manufacture are listed below.

- Ampoules (tubing glass)
  - Inert, low cost, high particulate from opening, not user-friendly
- Vials (tubing or molded)
  - Ease of filling, multiple use, user-friendly
- Bottles (>100 mL, molded)
  - Used for LVPs but being replaced by plastic
  - Smaller bottles used for solid dosage forms

In this section, glass as a material for packaging components will be reviewed. The discussion will be divided into three parts:

- Composition of glass components
- Sources of extractables/leachables
- Reduction of leachables

Composition of Glass Components

Commercial glass is an inorganic product of fusion that has cooled to a rigid state without crystallization (35,36). It is essentially a rigid liquid. Glass may be thought of as a thermoplastic—it is softened by heat, capable of being formed into a wide spectrum of shapes, and can be reheated and remolded into new shapes without degradation of the material properties. The essential difference between glass and common thermoplastic materials, such as polyethylene and polypropylene, is that glass is an inorganic material while the cited plastics are organic materials.

Glass is composed of the following materials:

- Matrix material—SiO_2 or sand
- Fluxing agents that lower the melting point
  - Na_2CO_3 soda ash that converts to Na_2O
  - K_2CO_3 potash that converts to K_2O
- Stabilizers
  - $CaCO_3$ (lime) converted to CaO for hardness and chemical resistance
  - Al_2O_3 (aluminum oxide) for chemical resistance
  - B_2O_3 (boron trioxide) lowers melting point
  - Cerium oxide increases resistance to discoloration by γ-radiation
- Coloring agents
  - Fe_2O_3 (iron oxide) and TiO_2 (titanium oxide) for amber glass
  - Cobalt and copper oxides for blue glass
  - Iron, manganese, and chromium oxides for green glass

Pharmaceutical glasses fall into two types that differ in their essential compositions. These are as follows:

- Soda-lime glass, which is composed of the following:
  - 71% to 75% SiO_2
  - 12% to 15% Na_2O (soda ash)
  - 10% to 15% CaO (lime)

The name is derived from the two compounds that predominate—*soda* ash and *lime*.

- Borosilicate glass, which contains the following:
  - 70% to 80% SiO_2 (silicate or sand)
  - 7% to 13% B_2O_3 (boron trioxide)

- 4% to 6% Na_2O and K_2O
- 2% to 4% Al_2O_3

The name again comes from the two predominate components—*boron* trioxide and *silica* dioxide.

A survey of the compositions of commercial borosilicate glasses (37) demonstrates the subtle differences in properties (Table 1). Flint glass is colorless while amber is yellow-brown in color; amber glass is used for drug products that are sensitive to light.

Glass can be divided into types in many ways—by composition (soda lime/borosilicate), color (flint/amber), forming method (molded/tubing), coefficient of expansion (33/50/90)—but in the world of E&L, the most meaningful method is by chemical resistance. The USP classifies glass (38) into three types according to chemical resistance requirements as show in Table 2.

The glass with the highest resistance is borosilicate glass or type I. It is differentiated from soda-lime glass (type III) by a Powdered Glass (PG) test in which the alkalinity of the glass or its capacity to exchange sodium ions (Na^+) in the glass for hydrogen ions (H^+) in solution is measured by a titration with dilute sulfuric acid. The lower the amount of acid consumed at the endpoint, the higher the resistance of the glass. The USP specification for type I glass is 1.0 mL or less; for type II glass the limit is 8.5 mL or less. Since ion exchange (extraction of sodium and other ions in the glass by replacement with hydrogen ions from the solution) is the principal reaction between glass and aqueous solutions, this test is a measure of the extractability of the glass.

There is a third type of USP glass—type II treated soda-lime glass. Type II glass is created by chemically pre-extracting or "treating" containers made from type III glass. The previously discussed tests for type I and III glass are performed on powdered glass made by crushing glass containers (vials, ampoules, bottles) with a steel mortar and pestle prior to testing. But the Water Attack (WA) test for type II glass is performed on intact containers because only the inside surface is treated to lower the extractability. The limits for type II glass containers are 0.7 mL per 100 mL of test solution for those with a capacity of 100 mL or less, and 0.2 mL per 100 mL for those over 100 mL capacity.

Table 1 Compositions of Commercial Borosilicate Glasses

	Glass "A", flint	Glass "B", flint	Glass "C", amber
Composition %			
Silicon dioxide (SiO_2)	81.0	75.0	70.0
Boron trioxide (B_2O_3)	13.0	10.5	7.0
Aluminum oxide (Al_2O_3)	2.0	5.0	6.0
Sodium oxide (Na_2O)	4.0	7.0	7.0
Potassium oxide (K_2O)	–	–	1.0
Calcium oxide (CaO)	–	1.5	<1.0
Titanium dioxide (TiO_2)	–	–	5.0
Physical properties			
Thermal expansion ($\times 10^{-7}$ in./in./°C)	33	49	54
Annealing point °C	560	565	560
Chemical resistance			
USP Powdered Glass Test (max. limit = 1 mL)	0.26	0.30	0.35

Table 2 USP <660> Types of Glass

Type	Composition	Test	Size, mL	Max. mL of 0.02 N Acid
I	Highly resistant, borosilicate	PG	All	1.0
II	Treated soda lime	WA	100 or less	0.7
			Over 100	0.2
III	Soda lime	PG	All	8.5

USP 31 <660>
Abbreviations: PG, Powdered Glass; WA, Water Attack.

Type I glass is the predominate choice for injectable drug products. Type II is not used as much as previously because of the variability in treatment effectiveness and the environmental impact of the treatment processes. Type III glass is not used for parenterals. This subject will be discussed in the section "Reduction of Extractables."

Sources of Extractables/Leachables

Several factors affect the number and amount of leachables from glass containers into drug products.

- Drug composition
  - Type and concentration of ions
  - pH
- Method of glass container fabrication
  - Molded, tubing, fabrication temperature, glass treatments
- Methods of container processing
  - Terminal sterilization, aseptic processing, depyrogenation
- Container size and shape relative to the drug product volume
- Storage conditions of filled container
  - Time, temperature, orientation of container
- Glass composition
  - Borosilicate, soda lime

Glass–drug product interactions may be divided into several types.

- Ion exchange: It is the predominate method of interaction. Na^+, K^+, Ba^{2+}, and Ca^{2+} are the major extractables from glass via ion exchange.
- Glass dissolution: Phosphates, oxalates, citrates, and tartrates can accelerate the dissolution of glass. Silicates and Al^{3+} are released by the dissolution.
- Pitting: EDTA can form complexes with many divalent and trivalent ions and accelerate dissolution of glass resulting in pitting of the glass surface.
- Adsorption: Proteins such as insulin and albumin are known to adsorb on glass surfaces.

In a comprehensive study (39), Borchert et al. investigated the extractables from borosilicate glass with an accelerated procedure using unbuffered aqueous solutions at pH = 4, 6.5, 8, and 10.4 and buffered solutions at pH = 8 and 10. Accelerated extraction was performed at 121°C for one hour. The authors concluded the following:

- Low levels of extractables were leached from glass with solutions of pH 4 to 8. Significantly higher levels were observed when the glass was exposed to alkaline media (pH > 9).
- Silicon was the major extractable.
- Sodium was another major extractable. In acidic solutions, Na is extracted from the glass by ion exchange (H^+ into glass, Na^+ out of glass); in basic solutions, Na^+ is released during dissolution of the glass.
- Minor extractables observed were K, Al, Ba, and Ca—Al by dissolution and K, Ba, and Ca via ion exchange.
- Other elements extracted were Mg, Fe, and Zn but at the level of detection, that is, <0.1 ppm.
- A positive shift in the pH of the unbuffered extracts was observed when the initial pH of medium was <7. Conversely, a negative shift was observed with medium of pH > 8. The pH of acidic solutions is raised by ion exchange—H^+ ions leave the aqueous medium and become part of the glass. The pH of basic medium is lowered by the consumption of OH^- ion during glass dissolution.
- Treated glass had less extractables than untreated glass.

Borchert's observations are important for anyone using glass containers for aqueous drug products. It is important to note that

- Unbuffered solutions with initial pH values <7 or >8 will shift in pH. Drug products with narrow acceptable pH ranges over shelf life may require a buffer. The reactions of glass in acidic and basic medium are illustrated below.

  Acidic/Neutral (ion exchange)

  $$Na^+(glass) + H_3O^+(sol) \rightarrow Na^+(sol) + H_3O^+(glass)$$

  Basic (dissolution)

  $$2OH^-(sol) + (SiO_2)_x \rightarrow SiO_3^{2-} + H_2O$$

- Glass, although less extractable than rubber and some plastics, is not inert. pH shifts and metal ion extracts are probable.
- With glass containers, it is not typically necessary to precede a leachables study with an extractables study since the metals extracted from glass are very consistent from glass to glass and are well known. Since the purpose of an extractables study is to identify potential leachables, this is not necessary. Also methods of metal ion identification and measurement, such as inductively coupled plasma/mass spectrometry (ICP/MS) and inductively coupled plasma/atomic emission spectroscopy (ICP/AES), are able to analyze many elements in one test.

  With rubber, extracts are not consistent from one rubber to another, so an extractables test is a necessary step prior to a leachables study.

Reduction of Leachables from Glass

There are three primary sources of glass that will reduce leachables—treated glass, glass manufactured using special methods, and coated glass. Combinations of these methods such as chemical treatment of containers made from special glasses are common.

Glass Treatments

Glass treatments are physical or chemical processes used to modify the physical or chemical durability of glass.

- Physical: The most common methods of improving the physical durability of glass are fire polishing and annealing. Fire polishing is a method of smoothing the surface or edges of glass by exposing it to a flame or heat. By melting the surface of the material, surface tension smoothes the surface. This process removes cracks or scratches that make glass containers more susceptible to breakage (1). Annealing is the process of reheating then cooling glass containers at a controlled rate to relieve stresses that are imparted during the forming process. The annealing process takes place in an oven called a lehr (35). The annealing temperature of borosilicate glass is 580°C and 560°C for soda-lime glass (1).
- Chemical: Chemical treatments reduce extractable substances, mainly sodium, from the surface of glass containers. Several types of chemical treatments can be used (see following text), but ammonium sulfate is the most widely used for containers for drug products.

  Sulfur treatments

  $$SO_2, SO_3, (NH_4)_2SO_4 \rightarrow Na_2(SO_4)$$

  Chlorides salts and chlorine gas

  $$NaCl, (NH_4)Cl, Cl_2 \rightarrow NaCl$$

  Fluorine compounds

  $$C_4F_8, CClF_3, C_2F_4 \rightarrow NaF$$

With sulfur treatments, sodium sulfate [$Na_2(SO_4)$] is formed on the surface of the glass container; it is then easily washed away during the container washing process. With chlorine/chloride treatment, sodium chloride (NaCl) is the resulting salt deposited. When fluorides are used, sodium fluoride (NaF) results. All three treatments remove sodium from the surface and convert it to a soluble salt that is easily removed. The chemistry of the ammonium sulfate treatment is shown below. In this treatment, a small amount of an aqueous solution of ammonium sulfate is added to each container (e.g., vial, ampoule). The containers are then conveyed into an oven at approximately 550°C where the ammonium sulfate is converted to ammonium bisulfate and ammonia gas. The ammonia bisulfate reacts with the sodium in the surface of the glass, and in an ion exchange reaction, the sodium in the glass is replaced with hydrogen ions. Sodium sulfate and ammonia gas are by-products of the treatment.

$$(NH_4)_2SO_4 \rightarrow (NH_4)HSO_4 + NH_3$$
$$2Na^+(\text{glass}) + (NH_4)HSO_4 \rightarrow Na_2SO_4 + NH_3 + 2H^+(\text{glass})$$

Treatment of glass with ammonium sulfate is most commonly used to convert type III glass to type II ("treated glass") or to improve the chemical properties of type I glass.

$$Na^+(\text{glass}) \rightarrow H^+(\text{glass})$$
$$\text{Type III} \rightarrow \text{Type II}$$
or
$$\text{Type I} \rightarrow \text{Type I (improved chemical resistance)}$$

Treatment decreases both the amount of metal ions extracted from glass container surfaces and the pH shift of solutions in contact with glass. Data from a presentation by Aldrich (40) demonstrated the improvement of glass from treatments.

Treated Vs. Untreated Glass
Type I glass, 10 mL vials, extraction @ 121°C for 1 hr

Component	Untreated	Treated
SiO_2	20.7 ppm	0.6 ppm
Ba	0.7	<0.1
Al	1.3	<0.1
Na	3.1	0.3

Borchert (41) and Aldrich (40) also showed that treatments only affect the surface of glass containers. In the untreated containers, the percentage of sodium in the matrix of the glass is 15% but higher near the surface (down to 6000 Å). This increase in surface sodium results from migration of sodium to the surface during the formation process, especially with tubing glass. Areas that are heated frequently, such as the bottom and top (shoulder, neck, and finish) of the vial, have higher percentages of surface sodium than the sides of the vial. Treatment reduces the sodium to very low levels near the surface but does not affect the depth below approximately 8000 Å. Data from Aldrich is shown in the following table.

Percentage Na Vs. Depth

Depth, *A*	Untreated (%)	Treated (%)
1300	25–41	<1
4150	16–19	
6000	17–18	↓
>8000	15	15

Since the bottom and top areas of tubing glass containers contain more surface sodium than the walls, it is important to minimize contact of drug with these high sodium areas. This is done by both using the appropriately sized container for the volume to be packaged (small

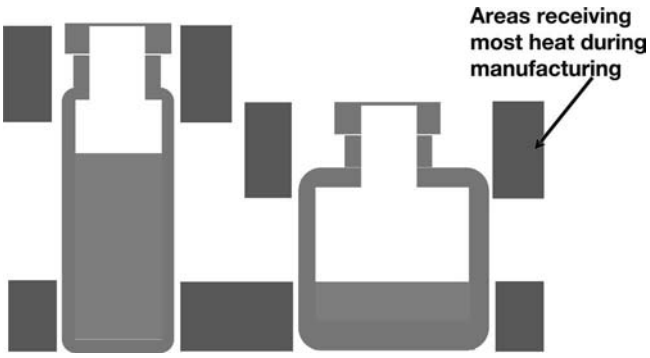

Figure 8 Effects of high sodium zones on the interior surface and filled volume.

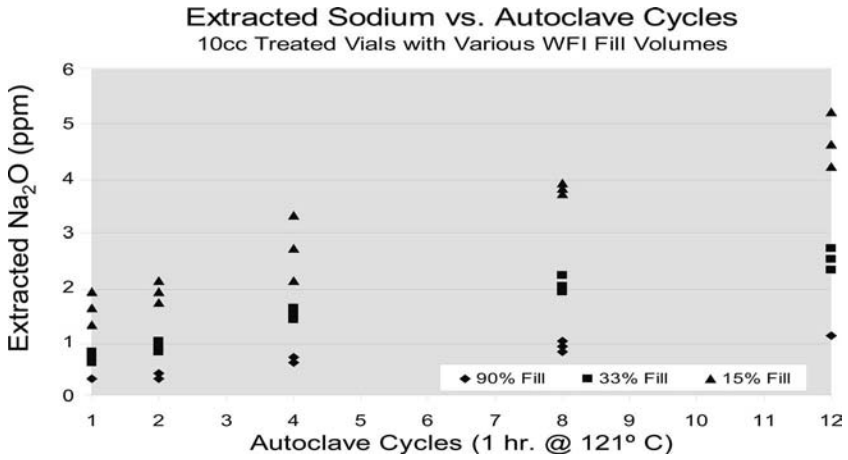

Figure 9 Extracted sodium versus fill volume and time.

volumes in small containers) and optimum-shaped containers (height and diameter). As illustrated in Figure 8, tall narrow vials minimize drug contact with the bottom and maximize contact with the low-sodium vial sides (42). However, vials that are too tall and narrow are not stable so the objective is to balance height and diameter.

The effect of fill volume and exposure time on the amount of extracted sodium was investigated by Swift (37). Figure 9 demonstrates the following:

- Extractable sodium increases with time of solution-container contact. In this case, time is measured in autoclave cycles.
- As the fill volume decreases from 90% to 33% to 15% of capacity, the amount of sodium extracted increases. At 15% of fill capacity, a proportionally larger amount of the solution is in contact with the bottom compared with the sides.

Sulfur treatment of glass, despite its disadvantages, is still a widely used method of producing type II glass and of improving the chemical resistance (reducing extractables) of type I glass. Some disadvantages are as follows:

- Can add cost
- Damages production equipment
- Adds stress to washing system
- Contaminates the washing system
- Causes an environmental issue because of disposal of salts
- Treatment can be inconsistent. Excessive treatment causes pitting of glass surfaces.

- Introduces risk of sulfate residues
- No standards or tests for the amount/concentration of ammonium sulfate used
- Restricts user's ability to test "as formed" surface quality

The chief advantages are as follows:

- Significant reduction in surface alkalinity
- Reduction in other metal ion extractables

Special Glass Manufacturing Methods
A method that produces glass with lower extractables is preferred over the posttreatment glass produced with higher extractables. The amount of sodium on the surface of the glass is dependent on the glass-forming temperatures; the higher the temperatures, the more the migration and volatilization of sodium. Modern manufacturing methods are characterized by the following:

- Moderate and controlled forming temperatures
- Process monitoring
- Process control
- Prevention of the recondensation of volatilized sodium and other constituents

A comparison of the hydrolytic resistance of untreated, treated, and control-manufactured glass is shown on Figure 10 (42). The control-manufactured glass is much better than the untreated and uncontrolled process glass and about the same as the treated and uncontrolled glass, but the controlled process glass is much more consistent in hydrolytic resistance.

Special glasses are being developed to meet specific needs such as low aluminum type I glass for injectable nutritional and blood-derived products. An example is SGD's Asolvex® type 1 glass, which SGD claims reduces the aluminum content by a third (43).

Even with properly treated and/or control manufactured glass, the proper-sized container will significantly reduce the amount of extractable substances.

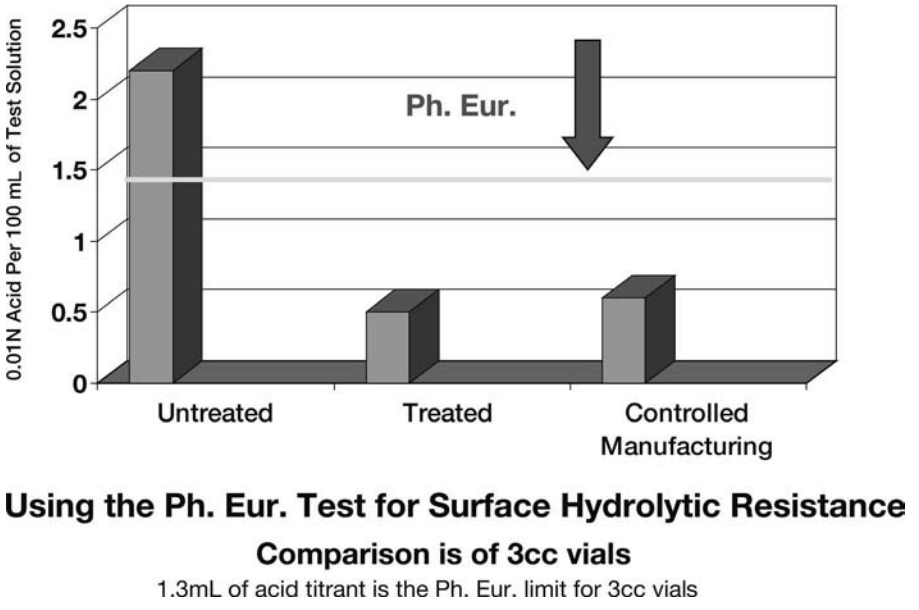

Figure 10 Comparison of the hydrolytic resistance of glasses.

Coatings for Glass

As with FluroTec-coated rubber (*v*) to reduce extractables, glass coatings have been developed to minimize glass-solution interactions. Schott has developed a coated vial marketed as Type I plus® (44). Schott Type I plus containers are made of pharmaceutical type I glass with a chemically bonded, ultrathin (~150 nm) layer of pure SiO_2 on their inner surface. Since the SiO_2 layer contains no sodium or other metal ions such as K, Al, Ba, Ca, Mg, Fe, or Zn, pH shifts and extractables are significantly reduced as shown in the data from Schott.

Comparison of Extractables from Type I and Type I plus® Glass

Metal ion	Type I	Schott Type I plus	Reduction factor
Na	3.5 ppm	<0.01 ppm	>350
Ca	1.1	<0.05	>22
B	3.5	<0.01	>35
Si	5.0	<0.3	>15
Al	2.3	<0.05	>45

Extraction conditions—autoclaving for 6 hr @ 121°C with WFI.

Glass containers remain a viable option to the pharmaceutical packager, but it is important to note that

- Glass is NOT inert
- Extractables and leachables are
  - Si, Na—major
  - K, B, Ca, Al—minor
  - Mg, Fe, Zn—trace
- All type I glasses are NOT equivalent
- Methods are available to reduce extractables and leachables.

E&L FROM PLASTIC COMPONENTS

Plastics as pharmaceutical packaging materials are the "new kids on the block." It was not until the early 1950s, with the full commercialization of polyethylene, that plastic emerged as a packaging material (1). The development of polymer technology has made plastics the material of choice over glass bottles for LVPs. Plastics are also becoming an alternative for SVPs. Plastic containers have the following advantages over glass:

- Less fragile
- Lighter in weight
- More easily fabricated into complex shapes
- Less expensive in some cases

There are also some disadvantages compared with glass:

- Clarity can be an issue with many plastics
- More permeable to gases, water vapor, and secondary packaging chemicals from inks and label adhesives
- Less stable during handling because of lighter weight
- More complex extractable substances
- Not as easily sterilized and depyrogenated
- Can cost more than glass

Today there are more than 50 different plastic materials used in various pharmaceutical and medical applications. As with rubber and glass, extractable materials from plastics are a concern for the pharmaceutical packager.

In this section, plastic as a material for packaging components will be reviewed. The discussion will be divided into three parts:

- Composition of plastic components
- Sources of extractables/leachables
- Factors that affect migration: thermodynamics and kinetics

Composition of Plastic Components

As with rubber and glass, modern plastics are formulated with several types of chemical substances and additives, each of which imparts specific chemical and/or physical properties to the plastic component. The most important of these substances are the following (1,45,46):

- Polymers—impart basic desired properties to component. Classes of plastics used in pharmaceutical applications will be discussed in a later section.
- Fillers—reduce degradation, reduce cost, affect moisture absorption and shrinkage. For example, carbon black, calcium carbonate, talc, clays, silica, and magnesium carbonate.
- Lubricants—ease the movement of the melted polymer against itself during processing and may enhance end-use lubricity. For example, zinc stearate, PE waxes, fatty acids, amides, and polydimethyl siloxane.
- Antioxidants—reduce the degradation of polymers exposed to heat, light, ozone, oxygen, radiation, or mechanical stress. For example, hindered phenols and cresols, secondary amines, phosphites, thioesters, BHT, and butylated hydroxyanisole (BHA).
- Heat stabilizers—protect polymers from the effects of heat, pressure, and shear during the polymerization process or secondary processes. For example, octyl thiotin complexes, calcium-zinc salts, epoxidized materials, and estertins.
- Plasticizers—impart flexibility, resilience, or softness. Plasticizers are used mainly in polyvinylchloride and may be used in polyvinyl alcohol/acetate copolymers, polyvinyl acetate and polyvinyl alcohol formulations, polymethyl methacrylate, and nylon. For example, dimethyl phthalate (DMP), diethyl phthalate (DEP), dibutyl phthalate (DBP), and di(2-ethylhexyl) phthalate (DEHP or DOP).
- Pigments/colorants—used to color, tint, or hide the color of the base polymer; may affect the physical, chemical, or mechanical properties of the plastic. For example, carbon black and inorganic oxides such as TiO_2, ZnO, Fe_2O_3; fillers can also function as pigments; organic dyes and organometallic complexes such as Phthalocyanine Green G (Pigment Green 7), which is a complex between copper (II) and chlorinated phthalocyanine and are not good choices for pharmaceutical applications where extractables are an important factor.
- Other additives, some of which are not commonly used in pharmaceutical applications, are antistatic agents, catalysts, bactericides, impact modifiers, release agents, brighteners, flame retardants, ultraviolet (UV) absorbers, inhibitors, nucleating agents, and blowing agents.

Polymers

A wide variety of plastics are used by the pharmaceutical industry but fewer are used in packaging and process materials where extractables are a concern. The reader is directed to other texts for broader information on the manufacturing and properties of polymers used in the plastics industry (1,2,47). The following is a selected list of plastics divided by application.

- Vials: Polypropylene, polyethylene terephthalate, polyethylene glycol terephthalate, high-density polyethylene, polycarbonate, cyclic olefin polymer
- Bottles: Polycarbonate, high-density polyethylene, polypropylene, polyethylene terephthalate, polyethylene glycol terephthalate, polycarbonate, cyclic olefin polymer
- Syringe barrels: Polypropylene, high-density polyethylene, polycarbonate, cyclic olefin polymer

- Form-fill-seal vials: Low-density polyethylene
- SVP and LVP flexible bags: Ethylene vinyl alcohol copolymer, ionomers [Surlyn® (48)], polyamides (nylons), low-density polyethylene, linear low-density polyethylene, polyvinylchloride, polyethylene terephthalate
- Single-use process bags (1–100 L): Low-density polyethylene, ethylene vinyl alcohol copolymer, ethylene vinyl acetate, polyamides (nylons), very low density polyethylene, ultralow density polyethylene
- Process tubing: Polytetrafluoroethylene and fluorinated ethylene propylene–lined rubber, silicone, polyvinylchloride [Tygon® (49)], ethylene-vinyl acetate
- Filters: Cellulose acetate, polyethersulfone, cellulose nitrate, polyvinylidine chloride, polyamide, polycarbonate, polytetrafluoroethylene, polypropylene
- Inhalation containers and devices: Polypropylene, polyvinylchloride
- Thermoforms: Polypropylene, polyethylene glycol terephthalate, polymonochlorotrifluoroethylene [Aclar® (50)], polyethylene terephthalate, polyvinylchloride, polystyrene
- Films: Polytetrafluoroethylene, fluorinated ethylene propylene, ethylene tetrafluoroethylene, poly(para-xylene) (Parylene)
- Adhesives: Polyvinyl acetate, ethylene-vinyl acetate

Sources of Extractables/Leachables

Because of the complexity of formulations, the leachability of plastics is virtually impossible to predict a priori. Some common plastics and extractables found are listed in the following table (40,51).

Acetal polyoxymethylene (POM) (Delrin®) (52)	Phenolic antioxidant, nitrogen stabilizer, residual formaldehyde, formic acid, trioxane, calcium, magnesium, silicon, zinc, sodium, phosphorus, oligomers
Polypropylene (PP)	Calcium stearate, butylated hydroxytoluene (BHT), dilaurylthiodipropionate, titanium dioxide, aluminum, titanium, chlorine, potassium, sodium, C_6–C_{16} oligomers
Polyethylene terephthalate (PET)	Cyclic dimers and trimers, terephthalic acid, diethylene and ethylene glycol, acetaldehyde, fatty acids, aluminum, calcium, cobalt, tin, zinc
Polyethylene (PE)	Phenolic antioxidant, phosphite antioxidant, amide lubricant, aluminum, chloride, titanium, zinc, calcium, sulfur, phosphorus, *t*-butyl alcohol, oligomers, glycerol monostearate
Polyvinylchloride (PVC)	Phthalate esters, sulfur stabilizer, fatty acid, benzene, chlorine, silicon, magnesium, zinc, calcium, aluminum, residual vinyl chloride, HCl, cartenoids, diphenyl polyenes, oligomers, calcium stearate
Polystyrene (PS)	Residual monomer, ethyl benzene, phenolic antioxidant, glycol esters, oligomers

Factors That Affect Migration: Thermodynamics and Kinetics

An article by Jenke (53) on the leaching of substances from plastics identifies both thermodynamic and kinetic factors that impact migration. Thermodynamics defines the absolute maximum equilibrium interaction between a material and solution (amount extracted) and kinetics defines the rate at which that equilibrium is achieved (speed of extraction).

Thermodynamics

Estimates of how much substance (e.g., an antioxidant such as Irganox 1076) will migrate from a plastic (e.g., polyethylene) into an aqueous solution can be calculated from the partition coefficient, E_b (i.e., the ratio of the equilibrium concentrations of the substance in the plastic and the concentration in the aqueous solution). However, these partitions coefficients between plastic and water are rarely available in the literature. They can be calculated, however, from other more readily obtained partition coefficients, $P_{o/w}$, specifically between octanol and water. $P_{o/w}$ values can be obtained from the literature or by experimentation. Once relationship between E_b and $P_{o/w}$ is established for a particular plastic, E_b can be calculated from $P_{o/w}$.

Many factors affect the partitioning process. If the extractable substance is ionizable, its dissociation constant (p*K*) and pH of the aqueous solution play a role. Concentrations of other ionic and nonionic substances in the aqueous phase (drug product) also affect partitioning. These substances may affect the apparent "polarity" of the drug product; this may affect the level of leachables. The effect of added Polysorbate 80 (Tween 80) on the extraction of antioxidant from a rubber plunger into the Eprex drug product was discussed earlier and is a good example of the effect of polarity on partitioning (7). The utilization of octanol/water partition coefficients to model solution-plastic interactions has been described in detail for several plastic materials by Jenke et al. (54).

Kinetics

Kinetic factors determine the level of migration if equilibrium has not been reached. In long-term contact situations, such as a drug filled into an LVP container, terminally sterilized, and stored before use, equilibrium may be reached. In the case of short contact situations, such as a drug being filled into a sterile empty syringe for immediate injection, it is the kinetics of migration which determines the amount of migration that has taken place.

E&L from plastics is a growing area of research for several reasons:

1. Compared with glass
   - The number of different substances that can migrate is very large since the compositions of plastics are more diverse than the compositions of glass.
   - The migrating substances may be both organic and inorganic.
2. Compared with rubber
   - The drug product contact surface area of plastics (containers and closures) is usually much larger than rubber (closures), increasing the potential for leachables.
3. Growth of plastic applications
   - Flexible plastic applications such as SVP, LVP, and large volume disposable applications (2–1000 L single-use 2D and 3D bags).
   - Blister packaging for tablets and capsules.
   - Vial materials with low extractables and high clarity [e.g., Cyclic olefin polymers (COP/COC) such as Ticona's (Celanese Corp, Florence, KY, USA) Topas and Nippon Zeon's (Zeon Chemicals L.P., Louisville, KY, USA) Zeonex resins].

E&L FROM INKS, ADHESIVES, AND COATINGS

Label components such as inks, adhesives, and coatings are not primary packaging materials since they are not in direct contact with drug products or substances. However, substances from the materials may migrate through the walls of plastic containers and appear as leachables. Examples of unexpected migrations of materials abound. One such report described the migration of a UV protective coating from a label through a hard plastic bottle into the drug product (55); another described the migration of two photoinitiators, 1-benzoylcyclohexanol and 2-hydroxy-2-methylpropiophenone, from ink on labels through HDPE bottles into a solid dosage form product (56).

Inks

Inks may be composed of the following types of substances:

- Oligomers—polyesters, epoxy compounds
- Photoinitiators—α-hydroxyketones, α-aminoketones, phenylglyoxylates
- Monomers—acrylates
- Stabilizers—4-methoxyphenol
- Pigments—carbon black, talc, organic pigments

The composition of inks for food products is regulated by the FDA in several parts of 21 CFR (57). 21 CFR 178.3297 concerns colorants for polymers. Other components of inks may be the subject of separate regulations such as 21 CFR 177.1520 for olefins in inks.

Adhesives

Even more so than inks on labels, label adhesives are in direct contact with the drug product container. And like inks, adhesive components can migrate through plastics. Pressure-sensitive label adhesives are typically formulated from acrylics or modified acrylics, wetting agents, and biocides. There are also adhesives based on rubber/resin blends. Each of these materials may be composed of several chemical substances that make the formulation quite complex, and, as a rule, it is very difficult to obtain a comprehensive list of adhesive ingredients from label suppliers making the extractables/leachables process difficult.

Adhesives for food applications is regulated by the FDA under 21 CFR 175.105 (Adhesives) (58). Section 175.105(a)(2) states that manufacturers of finished food packaging must ensure that the adhesive is either separated from the food by a functional barrier or is subject to limits of GMP practices. Of course, there are no CFR limits for drugs.

Identification of Extractables from Labels

Because of the difficult in obtaining credible and comprehensive information about the extractable substances from labels suppliers, pharmaceutical companies must perform a controlled extraction study to identify extractable substances. An example of extractables protocol for labels is described below.

Label Extractables Protocol

Before beginning any study, the label supplier should be requested to supply as much information on the label composition as is possible. Discussion with the suppliers/manufacturers of the ink, adhesive, and paper portions of the label are also desirable. Review of suppliers/manufacturers literature (paper and web based) will prepare the user with the proper questions for suppliers. Any upfront knowledge of the label composition will provide dividends in time and money saved during the extraction study.

A controlled extraction study of a packaging label involves exposing a sample to an appropriate solvent system at elevated temperatures to accelerate the extraction process followed by chemical analysis. The label, which is not intended in ordinary applications to be ever exposed to solvents, will not only be subject to extraction but likely will partially dissolve in some solvents. At least two solvents are recommended—water or an aqueous system that mimics the drug product (e.g., buffer at drug pH) and 2-propanol. The extraction is performed by soaking the label in a solvent for a fixed time at a controlled temperature or by refluxing the solvent over the label. Extraction profiling of the label is divided into three parts: nonvolatiles, volatiles, and semivolatiles.

Nonvolatile Profile

Label solvent extracts are analyzed for residual substances. An UV spectrum of the extract will provide identity information on the possible unsaturated extractables. A portion of the solvent extract is evaporated to dryness and prepared for analysis. Nonvolatile residue (NVR) is calculated on the basis of label weight. This provides generalized information on possible extractables when comparing labels. Infrared (IR) spectroscopy or Fourier Transform IR (FTIR) spectroscopy of the residue provides general identification of the significant functional groups of the extracts. Qualitative trace elements in the residue are then analyzed by scanning electron microscopy and energy dispersive X ray (EDX). Residual extracts are then analyzed by ICP/MS.

Semivolatile Profile

After extraction, the extract is separated by chromatographic methods and identified by retention information or by mass spectrometry (MS). Typical methods used may be (51):

- Gas chromatography/mass spectrometry (GC/MS)
- Liquid chromatography/mass spectrometry (LC/MS)
- Liquid chromatography/diode array detection (LC/DAD)
- Gas chromatography/flame ionization detection (GC/FID)

- Liquid chromatography/ultraviolet detection (LC/UV)
- Inductively coupled plasma/mass spectroscopy (ICP/MS)
- Inductively coupled plasma/optical emission spectroscopy (ICP/OES)

Volatiles Profile

Samples of the labels are profiled without solvent extraction by head space GC/MS. Labels are cut into small pieces and placed into headspace vials along with a small amount of WFI. The headspace vials are heated for an appropriate amount of time and temperature to generate any volatile substances and then analyzed by chromatography with an MS detector.

Identification of extractable and volatile substances is made by comparison to standard libraries and comparison to reference standards when available. Results should be shared with the label supplier to validate, where possible, the origin of the substance is identified. Protocols for leachables studies will be discussed later in the chapter.

PACKAGING STANDARDS AND COMPENDIA TESTS

In this section, the compendia tests on rubber, glass, and plastic from the USP, Ph. Eur., and, to a lesser extent, the Japanese Pharmacopoeia (JP) will be reviewed.

Compendial test procedures are specific for certain types of materials, yet the data does not typically provide compound specific information. While this appears to be an enigma, compendial methods denote that a standardized approach can be used to provide general information to identify certain characteristics of the materials and the physicochemical nature of the materials. There are specifications associated with many of the monographs and those specified materials must be compliant to indicate suitability for use with a pharmaceutical product. Standardized tests indicating biological responses to the component materials as well as certain functional tests are also included in the compendia. Data from compendia tests are limited and considered as first-line information to be acquired when qualifying a container closure system. Complete stability, compatibility, and safety assessment of the container closure system are necessary to ensure it is appropriate for the intended use. An example of container closure systems materials types covered in the USP and Ph. Eur. monographs are shown in Table 3 (59).

Materials that are in contact with pharmaceutical container closure systems must comply with the appropriate pharmacopoeia monographs as required by regulatory agencies around the world. Unfortunately, there are no global specifications for container closure systems and requirements are enforced according to the terms of each country. Even though the materials of construction may have general characteristics that are standard, these attributes are not necessarily consistent on a global level. A single container closure system may be composed of several different materials types, and these materials types can be intended for different uses. The monographs in the Ph. Eur. are based on aspects associated with specific materials together with the applications so that rubber closures, ophthalmic containers, single-use syringes, lubricants, containers for parenteral, intravenous infusions, and parenteral nutritionals as well as systems used with blood products are to be tested according to the specified monograph. The extraction procedures, analysis, and specifications vary according to each

Table 3 USP/Ph. Eur. Material Types

Material	USP	Ph. Eur.
Elastomers	X	X
Glass	X	X
Polypropylene	X	X
Polyethylene	X	X
Polyvinyl chloride	–	X
Polyethylene-vinyl acetate	–	X
Polyethylene terephthalate	X	X
Silicone oil/elastomers	–	X

monograph within Ph. Eur. and even more so compared with USP monographs. The specifications in the USP monographs have been standardized on the basis of the materials used in containers in general and elastomeric closures for injection. The USP monographs have a broader application, but as mentioned previously, compendia testing is first-line assessment and the data provided is not definitive enough to qualify a container closure system for its intended use. The scope of compendia test standards are related to the chemical and biological characteristics for individual material formulations with functional tests associated with performance aspects of a particular system. There are efforts to harmonize compendia but the impact of changing test criteria and specifications for marketed container closure systems are far reaching and consensus is challenging so the process for harmonization is slow moving.

Rubber

The ISO publishes a set of procedures for elastomers, which encompass the majority of the USP, Ph. Eur., and JP test methods. ISO standards are voluntary and provide baseline information but again limited with respect to a complete assessment for any particular material and use. A listing of ISO standards for elastomers is below (60):

ISO 8871 Elastomeric parts for parenterals and for devices for pharmaceutical use

Part 1: Extractables in aqueous autoclavates
Part 2: Tests for identification and evaluation
Part 3: Determination of particles
Part 4: Biological requirements and test methods
Part 5: Functional requirements and testing

The multitude of standardized testing for the chemical and biological attributes of elastomeric closures currently required by the USP, Ph. Eur., and JP (61) is summarized in Tables 4 to 6. The ISO tests that correlate to the compendia tests are also noted. Although the tests between the three pharmacopoeias and ISO may have some commonalities, the extraction

Table 4 Compendia Extraction Tests

Test	USP	Ph. Eur.	JP	ISO
Appearance	X	X	X	X
Absorbance	X	X	X	X
Acidity/alkalinity	X	X	X	X
Ammonium	X	X	–	X
Reducing substances	X	X	X	X
Extractable zinc	X	X	X	X
Residue on evaporation	–	X	X	X
Volume sulfides	X	X	–	X
Heavy metals	X	X	–	X
Turbidity/color	–	–	–	X
Conductivity	–	–	–	X
Foam	–	–	X	–

Table 5 Compendia Nonextraction Tests

Test	USP	Ph. Eur.	JP	ISO
Total Cd and Pb	–	–	X	–
IR pyrolyzate	–	X	–	X
Resistance to steam	–	–	–	X
Ash	–	X	–	X
Density	–	–	–	X
Hardness	–	–	–	X
Elasticity	–	X	–	X

Table 6 Biological Tests

Test	USP	Ph. Eur.	JP	ISO
In vitro cell culture	X	–	–	X
In vivo systemic inj.	X	–	X	X
In vivo intracutaneous	X	–	–	X
Pyrogen	–	–	X	X
Hemolysis	–	–	X	–
Bioburden	–	–	–	X

methods and procedures can be unique, thereby making it a challenge to show compliance with all three regulatory bodies. So the question lingers, what data is meaningful, useful, and scientifically sound for the initial testing phase?

The compendia summary tables indicate ISO has the most comprehensive set of tests, but these methods are unique and are not mandated as those of the pharmacopoeias. The ISO tests are parallel to those of the pharmacopeias such that mainly initial information on the chemistry and biological response is provided and a full study is still needed to show suitability of the container closure for its intended purpose, and it is conceivable that some of these tests may not be relevant and/or redundant. The specificity of standardized tests presents a challenge when qualifying materials on a global basis because it is difficult to standardize acceptance criteria when test procedures are not consistent. The sampling, extraction conditions (solvents and exposure), and analysis conditions (techniques and conditions) vary, and results are specific to those conditions. The sampling in some cases is intended to evaluate the container closure material's formulation and others, the actual container closure item or configuration (component or system). The USP and Ph. Eur. chemical and biological tests for elastomeric closures can be done on each formulation and the functional tests on each product (item-formulation combination). The JP chemical and biological tests for elastomeric closures must be done on each product due to the weight/weight ratio used in preparation of extracts. The JP method favors large items; large items are more likely to meet JP specifications than smaller items.

Requirements for functionality and cleanliness must also be considered when assessing container closure suitability, and standards have been developed for the functional evaluation of elastomeric closures that include specifications for penetrability, fragmentation, self-sealing capacity, and container closure integrity. Standards for visible (>25 μm) and subvisible (>2 μm and <25 μm) have also been developed.

The pharmacopoeia monographs provide a wide-range of test procedures and intended for materials used within a certain context. As a result of some of the generalities, often these tests can be applied to materials that may not fall into the intent of the specification. There are also new or combinations of materials being used in container closures system that may not have existed when specifications were set. As old materials maybe discontinued or new materials enter the market, updates of the compendia may not keep pace, and standardization will become more challenging. The USP monograph <381> Elastomeric Closures was only recently updated to include provisions for the required physicochemical, functionality, and biological testing relative to the types of coating on closures as well as responsibilities of suppliers and end users. This section is also more similar to Ph. Eur. and states test limits for type I (aqueous preparations) and type II (typically nonaqueous preparations).

Classification schemes have been established, which integrate the significance of the data and guide in the initial selection of materials. Categories for plastic, elastomers, and glass materials have been developed to differentiate suitability for a particular application.

Glass

There are three classifications for glass; the USP and Ph. Eur. classify glass into type I, II, and III. Type I is highly resistant borosilicate glass used for parenteral preparations of all pH

values. Type II is treated soda-lime glass and is also used for parenteral application of all pH values where stability studies have demonstrated suitability. Type III is a soda-lime glass much less durable and only allowed for parenteral use if stability studies were found to be acceptable. In addition to the type I, II, and III designations, ISO has distinguished glass in the same manner as Class HC 1, 2, and 3. The classification for glass is based on the characteristic of the solubility of glass in water when autoclaved. The USP test for solubility is referred to as Chemical Resistance and Water Attack; in Ph. Eur., Hydrolytic Resistance; and in JP, Soluble Alkali. The procedures between pharmacopoeias are different, but the solubility for all is measured on the basis of titration of water, after exposure to glass, with a weak acid to detect the amount of alkali (base) present. There are other pertinent tests and specifications for glass in each of the pharmacopoeias, but these are not factored in to the classification scheme.

Plastics

Pharmaceutical products and container closure systems continue to evolve to meet the needs of patients and caregivers. Packaging is no longer limited to the protection and storage of a drug product, with the rising demand for innovative delivery and administration devices, the boundaries for regulation between container closure systems and devices have blurred. The FDA, European Union and Health Canada have designation for classes of medical devices, but the USP classes are intended to qualify the materials. The USP addresses materials requirements for container closure systems and devices as well as ISO; the Ph. Eur. and JP do not deal directly with medical devices.

Regulatory controls for device materials are grounded in biological reactivity tests, and the degree of testing is linked to the material classifications. Plastics are assigned the USP class designation of I to VI on the basis of results of the biological reactivity data.

Injection tests are used to assign the class designations I, II, III, V to plastics; implantation tests must be used to assign USP Classes IV and VI. The numerical class increases relative to the duration (risk) of contact between the body and device. In the category of implantable devices, exclusive use of Class VI is mandated.

The USP chapters over 1000 are not mandated but recommended, and USP<1031> has established a set of recommendations for "Biocompatibility of Materials Used in Drug Containers, Medical Devices, and Implants" that describes tests and classes required for medical devices and implants based on the following:

- Similarity and uniqueness of product relative to a previously marketed (predicate) product
- Extent contact between product and patient, etc.
- Duration of contact
- Material composition of product

Plastics must meet the requirements of the USP <87> Biological Reactivity Tests in vitro test (cell culture) to be suitable for a drug container. No further testing is necessary for containers to establish biocompatibility. The USP <1031> tests are designed to detect the nonspecific, biologically reactive, physical, or chemical characteristics of medical products or the materials used in their construction. ISO has available a more comprehensive set of test procedures, ISO 10993 for "Biological Evaluation of Medical Devices."

The requirements for bacterial endotoxins must be met for medical devices listed in USP Chapter <161>, Transfusion and Infusion Assemblies and Similar Medical Devices. The requirements apply to sterile and nonpyrogenic assemblies and devices in contact directly or indirectly with the cardiovascular and lymphatic systems and cerebrospinal fluid such as but not limited to solution administration sets, extension sets, transfer sets, blood administration sets, intravenous catheter, implants, dialyzers and dialysis tubing and accessories, heart valves, vascular grafts, intramuscular drug delivery catheters, and transfusion and infusion assemblies.

In summary, portions of devices made of plastics or other polymers meet the requirement of Biological tests—Plastics and Other Polymers under USP Containers <661>. Portions of devices made of elastomeric materials should meet the requirements of Elastomeric

Closures for Injection <381>. If a class designation is needed for plastics the requirements under <88> Biological Reactivity Tests, in vivo, apply. Compliance with compendial standards is necessary for regulatory approval but not sufficient to indicate the suitability of a container closure or device. Additional compatibility, functionality, and performance tests are necessary to prove suitability with a specific drug product and application.

PROCESS WORK FLOWS FOR MANAGING PACKAGING EXTRACTABLES AND LEACHABLES

There is a logical progression to the framework for identification and control of leachables involving a set of steps to guide the degree of qualification relative to the phase of development. The qualification of leachables is associated with not only the discovery and amount of leachables but also the toxicological impact to the patient. There are various routes that can be taken to qualify a suitable container closure system and the drug product type, route of administration, duration of exposure, and patient population are among the variables to be considered. The qualification process is detailed, spanning a long period of time, and a team of analytical chemists, toxicologists, quality/regulatory professionals, engineers, and procurement specialists would facilitate development of a process map. The first step and most vital step of the process is to identify the primary container closure components and drug product contact materials and other critical components to be evaluated for extractables. These materials would then be assessed for potential extractables, starting by obtaining supplier information and results of compendia tests for materials that have an applicable monograph. The compendia test will not provide adequate information to correlate to patient safety, so the next step would entail developing a study design to obtain a chemical profile of the potential extractables for all the critical materials.

The protocol for an extractable study should employ multiple solvents having varying propensity to extract constituents from the container closure materials using aggressive conditions. The conditions for extractions should be adequate to provide a chemical profile but not so extreme as to create anomalous results. The extracts should then be analyzed using multiple techniques to detect organic and inorganic constituents of the container closure system. After a chemical profile is obtained, the data can be compared with the supplier information and evaluated for any compounds of concern. In the initial stage of the evaluations, much of the data will be qualitative or at best semiquantitative having only tentative identifications. It is not always evident if there is a toxicological concern until positive identification is made and the compounds are measured. Once extractable compounds are identified and measured, an assessment for toxicological impact can be attained and alternative materials may be considered if necessary. The measurement of extractables in container closure systems should be made using well-characterized methods to have a level of confidence to guide in the decision-making process. Measurements should be direct, provide high assurance of reproducibility, have purpose, and be sufficient and timely to provide a meaningful evaluation of quality (62). The methods should be fully validated if they are to be used to control the container closure materials.

Selection of the extractable compounds to be evaluated in a leachable study will need to have careful consideration; an extractable will not necessarily become a leachable. It is also conceivable that an extractable may form an interaction product or degradation product once in contact with the drug product. Migration of extractables or interaction products may occur under certain conditions relative to the drug product that may take place over a period of time. It is necessary that the leachable methods have the appropriate sensitivity and specificity as well as be free of interferences from the drug product. These methods must be validated under the guidelines provided by the regulatory agencies. The drug products can then be set-up on stability and evaluated at the specified time points. Assessments of the toxicological impact should be made throughout the studies and a correlation between E&L should be made to enable control of leachable compounds. A summary of these steps is illustrated in Figure 11. The process flow diagram describes a 14-step process, each step being a building block for the next (51). Another approach for a process map is shown in Figure 12 (51).

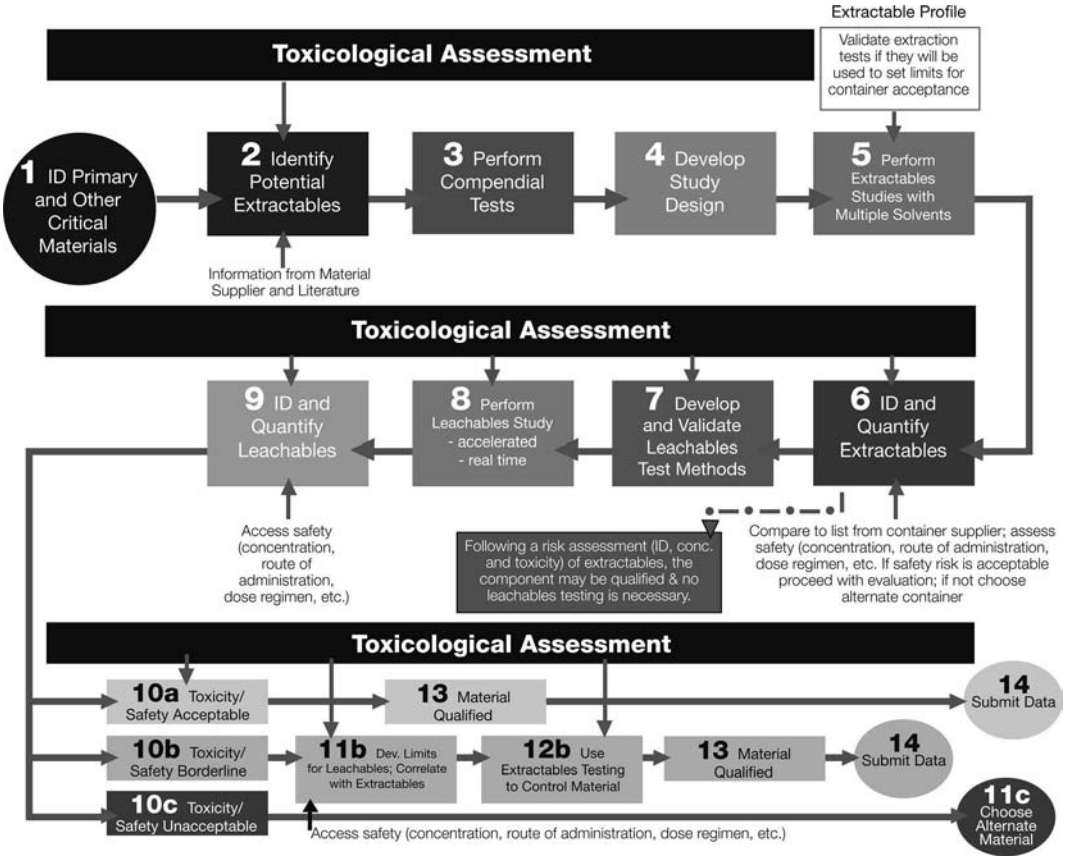

Figure 11 Extractables and leachables management.

MANAGING E&L FROM SINGLE USE AND PROCESS COMPONENTS

There is a potential for pharmaceutical products to be contaminated from contact materials during any phase of production or storage. While it is true that the guidance documents use the term container closure systems, evaluation of these systems are not limited to only the primary containers; secondary and ancillary materials can also contribute to leachables as well as any materials that may be in intermediate contact with the pharmaceutical product during manufacture. According to GMPs, 21CFR 211.65, packaging and the *equipment shall be constructed so that surfaces that contact components, in-process materials, or drug products shall not be reactive, additive, or absorptive so as to alter the safety, identity, strength, quality, or purity of the drug product beyond the official or other established requirements*.

The process materials and equipment used in manufacturing biopharmaceutical products fall into this category and can introduce leachables, albeit the intermediate or upstream nature of the processing materials. Certain bioprocess conditions may serve to filter or concentrate a given extractable compound introducing a leachable into the drug product with potential to cause harm to a patient. This presents the challenge to the manufacturer of biopharmaceutical products such that the critical materials to be evaluated for extractables must be understood early in the pharmaceutical development process so that this can be incorporated into leachable studies. Extractables, therefore, potential leachables from components of manufacturing systems and process materials may include filters, capsules, tubing, pumps, films connectors, and fittings for bioprocess containers (BPCs), single-use bags,

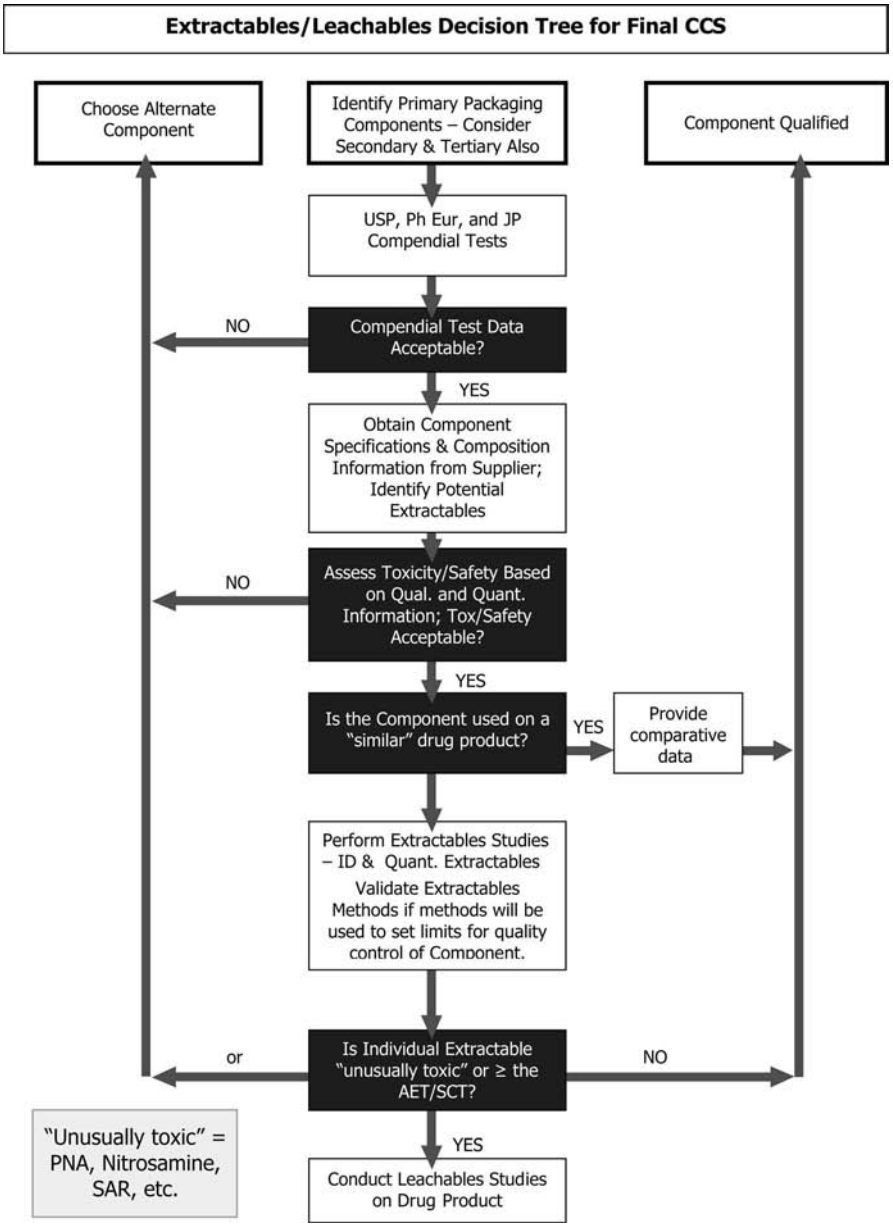

Figure 12 Extractables leachables decision tree. *Abbreviations*: PNA, polynuclear aromatics; SAR, structure-activity relationships; SCT, Safety Concern Threshold; AET, analytical evaluation threshold; CCS, container closure system.

and other product-contact materials (PCMs). Typical conditions of drug product exposure to a final container closure system compared with that of a disposable or process component is shown in Table 7.

The likelihood of interaction of single use or process components with a biopharmaceutical product will depend on four major conditions: (*i*) the direct contact to the actual drug product, (*ii*) indirect contact of solutions or materials that are precursors to the drug product, (*iii*) immediate contact of the drug product or precursor that is not processed further after contact, and (*iv*) remote contact of the drug product or precursor that is processed further after contact (63). The degree of safety qualification for the components used in the processing and

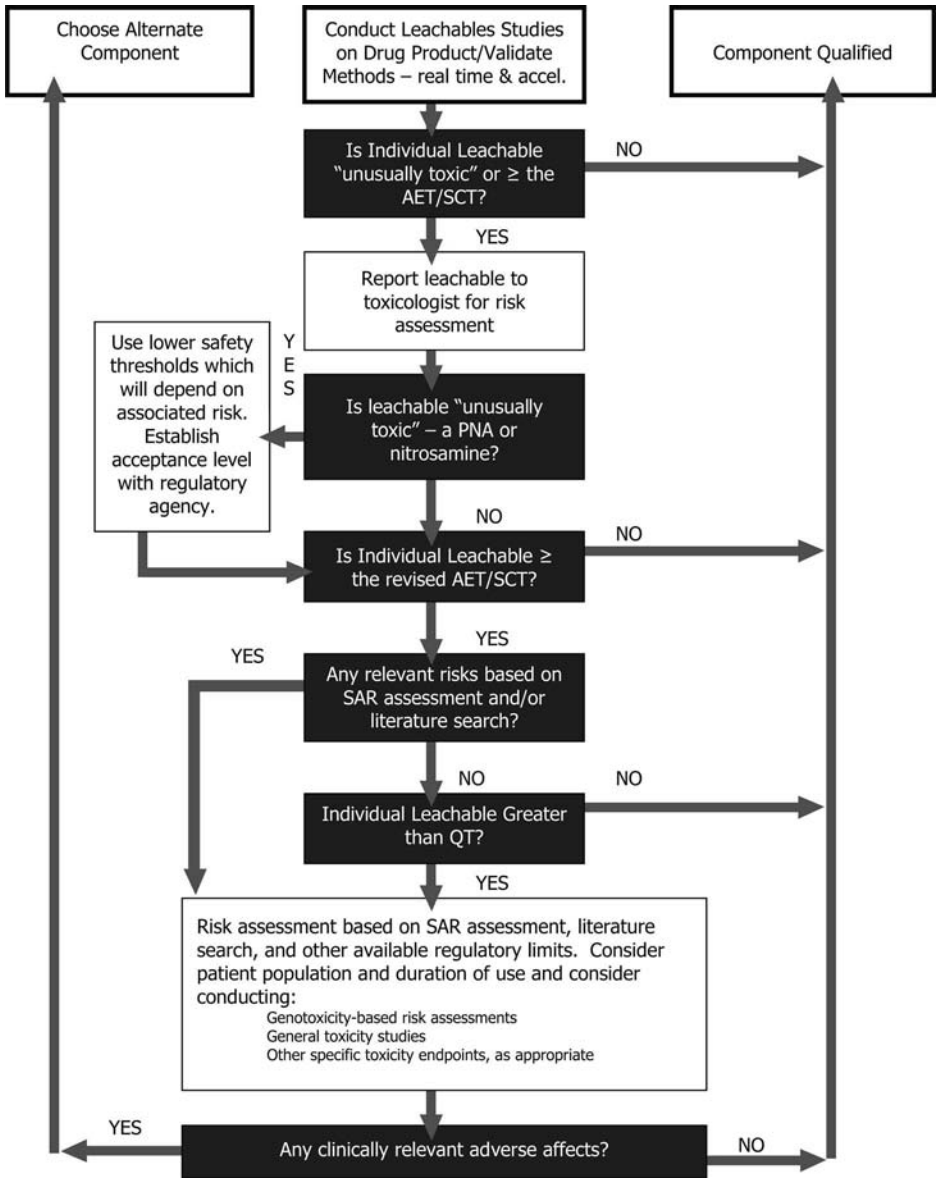

Figure 12 Continued

Table 7 Drug Product Exposure to Process Components

Final container closure	Disposable and process components
Contact time 18 mo. to 4 yr	Contact time seconds to years
Only contacts drug product	May contact drug product and precursors
Surface area to volume rations are in a relatively narrow range	Surface area (SA) to volume (V) rations are in a relatively wide range. (As size increases SA/V decreases)
Temp. are limited to freezing to autoclave, approx. −20 to +121°C; storage −20 to +25°C	Temp. may be from −80 to +121°C
Narrow range of materials—glass, plastic, rubber	Wide range of materials—metals, glass, more types of plastics and rubbers (cellulose, neoprene, silicones, nylons)

manufacture of biopharmaceuticals can be best managed by first considering which components have the highest risk for interaction with the pharmaceutical product. Contamination of a biopharmaceutical product can pose a safety risk to a patient causing a toxic or allergic effect; contamination can also change the properties of the drug product having a negative impact to the product and putting the patient at risk by not receiving intended therapy. The risk of constituents migrating from the contact material into the drug product or the precursors must take into consideration the compatibility of the component materials, proximity of the component to the final product, the product or precursor composition, the surface area of the contact material, the contact time and temperature. Other issues of concern are possible pretreatments and intended use such as exposure of the components to steam sterilization or γ radiation and if the components will be rinsed and reused. All of these factors combined create a dilemma when designing a process for qualification and validation of surface contact and in-process materials.

To select the appropriate materials for evaluation, the risk assessment tools, described in ICH Q9, can be incorporated to judge the critical disposable and bioprocess components of interest. Risk scores can be assigned based on conditions such as those listed in Table 8. A risk score can be developed on the basis of the probability of extractables occurring relative to the severity of harm caused from contamination of the biopharmaceutical.

Risk scores are generally based on predictive models developed for particular materials in a particular system and would need to be developed for each specific application. Although there will always be a degree of uncertainty in the risk values, an informed decision can be made by considering each material and potential for migration.

Once the surface contact materials from processing equipment and components are evaluated for the potential to contaminate the biopharmaceutical product, a decision can be made as to whether an alternative material should be considered or to proceed with an extraction study. According to the 1999 FDA Guidance for Industry: Container Closure Systems for Packaging Drugs and Biologics, an extraction study would employ at least three extraction solutions followed by the analysis of extracts using multiple analytical techniques. Table 9 shows solutions that may be considered for an extraction study of a bioprocess container. The use of multiple solvents should include clean solvents to provide a chemical profile as well as solution simulating the drug product. Conditions of exposure should be exaggerated to indicate worst case and provide data for the chemical profile. On the basis of the extractables data constituents would be evaluated for potential impact to patient safety as

Table 8 Considerations for Risk Evaluation

Proximity to API	Area of exposure
Type of solvents(s)	Known extractables
Length of contact	Known toxicity
Temperature of exposure	Resistance to extraction

Abbreviation: API, active pharmaceutical ingredient.

Table 9 Solutions for Extracting Bioprocess Containers

High pH solutions
3M NaOH, 6M urea, 2M Na bicarbonate, 4M NaCl, 1M Tris Base
Mid pH solutions
WFI, 1M Na phosphate buffer @ pH = 7, 1M succinate, 10M ethylene glycol
Low pH solutions
2M Na carbonate, 4M guanidine, 2M HCl
Solvents
50% EtOH/WFI, 10% DMSO
Solutions specific to company drug products
4M acetic acid, NaCl/Na phosphate/DMSO 0.5M/0.2M/3%, 10 mM histidine/0.50M arginine

well as to the drug product, at this point another decision can be made to seek alternative material or to proceed to a leachable assessment (51).

The target analytes for leachable studies, derived from the extraction of the surface contact materials from processing equipment and components, can be combined with those from the primary container closure system to understand the required sensitivity, those that are in common or may interfere with the drug product or other target analytes before developing the leachable study plan. Several methods may need to be developed and validated to encompass all of the constituents of interest. Leachable measurements must be accurate and precise having potential to indicate the presence of interaction products associated with the drug product formulation. Like the final container closure system, disposables and process components also require extractables assessment and leachables control. Manufactures of processing materials and equipment may provide baseline information on extractables but the materials suitability for one process may not be valid for a different biopharmaceutical product. The ultimate proof of suitability relies on user-specific studies. The degree of scrutiny for single use and processing components depends on several factors, and a risk assessment is commonly used to identify and prioritize studies to qualify and control the materials and components.

EXTRACTABLE STUDIES: MATERIAL AND TESTING CONSIDERATIONS

Extraction studies are conducted to achieve a greater understanding of the materials that are in contact and critical to a pharmaceutical product in an effort to protect the product and patient from adverse effects. There are different study approaches for conducting extraction studies depending on the intended outcome of the study. The choice of extraction conditions and analysis techniques can be relative to the goal of obtaining qualitative profiles, quantitative profiles, predictive modeling, and/or actual conditions of exposure. A comprehensive extractable study can entail a combination of all the above objectives. The FDA guidance on container closure systems classifies the degrees of concern for likelihood of interaction between the container closure system and drug product from high to low depending on the route of administration. Inhalation and injectable dosage forms are among the highest level of concern. The PQRI Leachables and Extractables Working Group has published guidelines for inhalation products titled *Safety Thresholds and Best Practices for Extractables and Leachables in Orally Inhaled and Nasal Drug Product*. This recommendation document, available at www.pqri.org, details a systematic comprehensive approach for investigation and control of leachables by employing controlled extraction studies followed by correlation to leachables and control. These recommendations incorporated threshold values to answer the question of "how low to go," but these thresholds currently apply only to inhalation products. Investigations for extractables in container closure systems would be more rigorous as concern for interaction increases and future recommendations for parenterals and ophthalmics are planned (64).

The solvents and exposure conditions used for extractions studies considers not only the nature and use of the drug product, but the physical and chemical nature of the material under investigation. Typical materials used in the container closure systems for different dosage forms are shown in Table 10.

The critical components for evaluation is the first and most important step since qualifying container closure systems is a long process, and taking steps backward to include an overlooked critical component will cause a disappointing delay.

There are certain factors that are relevant to selecting critical components for evaluations and include patient contact, product contact, device performance, type of secondary packaging, and if there are ancillary components. With respect to other components of concerns, the intermediate package, bulk containers, and process materials may also be germane. The FDA recommends that a stronger extracting solvent than the drug product would be used to obtain a qualitative extraction profile.

The composition of the critical components along with information on the drug product matrix will drive the type of solvents to employ and most suitable exposure conditions. Information from the supplier and downstream suppliers will aid in developing an extraction protocol. An understanding of the base material, additives and processing aids, polymerization and fabrication processes, as well as a type of cleaning, pretreatment, storage and shipping

Table 10 Example Container Closure Components

Dosage form	Components	Example material
Inhalation	MDI/DPI components, canisters, valves, gaskets, blister packs, bottles, actuators, mouthpiece, pumps, closures, liners, label/inks	Polyolefins, SBR and EDPM rubber, thermoplastic elastomers, polyacetal, polyesters, polyamides, acrylics, epoxies, paper/paperboard, metals, glass
Injectable	SVP $<$ 100 mL/LVP $>$ 100 mL cartridge, syringe, vial, ampoules, flexible bag, closures/plungers, injection ports, needles, adhesives, inks, overwraps	Polyolefins, butyl rubber, EPDM rubber, polyvinyl chloride, polyurethanes, polycarbonate, acrylics, polyamides, polystyrene, thermoplastic elastomers, silicones polyesters, epoxides, cellophane, fluoropolymers, styrenics, paper/paperboard, metals, glass
Ophthalmic	Bottles, droppers, screw caps, liners, tips, tubes/liners, labels/ink	Polyolefins, acrylics, vinyls, epoxies, polyamides, thermoplastic elastomers, polyesters, cellophane, glass, paper/paperboard, metals
Transdermal	Adhesives, membranes, barrier films, reservoir, coatings, blister packs, preformed trays, overwraps, substrates, topical aerosol components	Polyolefins, acrylics, vinyls, polyamides, polyesters, styrenics, rubber material, thermoplastics, metal
Associated components	Nebulizers, dosing spoons, dropper, dosing cups	Polyolefins, glass, rubber, thermoplastic polyesters

Abbreviations: MDI, metered dose inhalers; DPI, dry powder inhalers; SVP, small volume parenterals; LVP, large volume parenterals.

will all factor into the decision for not only extraction but also the appropriate analytical techniques.

A container closure will be suitable if it protects the drug product, functions properly, and found to be compatible with the dosage in addition to ensuring harmful chemicals will not leach into the product. The compatibility of a container closure has many variables; the complexity of the process for assessing risk is represented in the Ishikawa diagram Figure 13 (65).

Results from extractable studies should be representative of appropriate sampling. All lots are not created equal and the variability must be realized to set specifications and acceptance criteria. The sample to surface ratio should be adequate so that required sensitivity can be achieved and is consistent with the sample preparation techniques. A container closure system may be multicomponent, multilayered, coated, or have surface treatments or pretreatments that would also have a bearing on the sampling plan.

The conditions of extractions may serve different purposes and there are three general types: (*i*) accelerated extraction that is intended to reduce experimental time to reflect actual use; (*ii*) exaggerated or aggressive extractions, which are conditions that are intended to maximize the amount of extractables; and (*iii*) simulated extractions, which are conditions intended to mimic actual use such as those used in the CFR for indirect food additives (66).

As a rule of thumb, suitable extraction solvents would have the following properties (67):

- Range of boiling points
- One of similar extracting properties to drug product vehicle
- High purity and relatively nonreactive
- Easily and safely handled and readily available

The probability that constituents will migrate from the container closure materials into the drug product are related to diffusion of the entity from the polymer and solubility in the drug product. The purpose for the extraction may be intended to provide a qualitative profile or materials control methods, in this case exaggerated or aggressive extraction conditions would be optimal. If the intent is to provide a predictive model, accelerated or simulated extraction

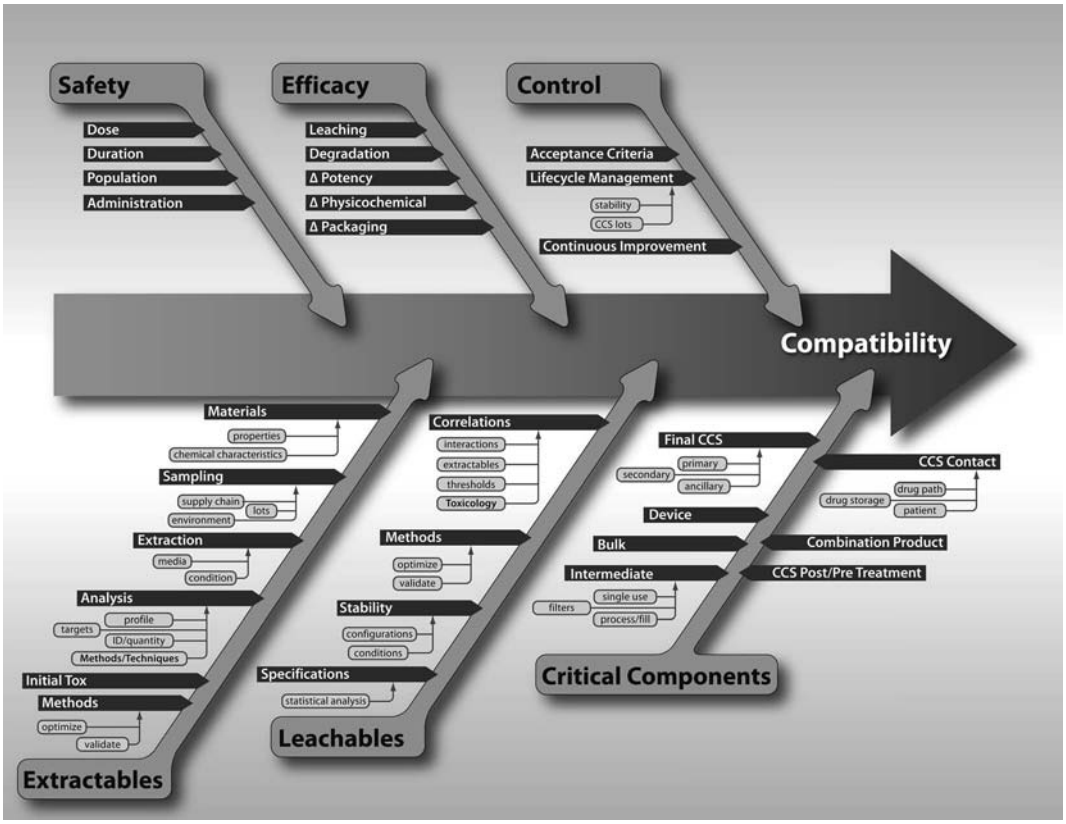

Figure 13 Assessing risk for container closure system.

techniques would be more practical. Examples of data representing exaggerated extractions under reflux conditions, accelerated extractions at 50 and 70°C, and simulated extractions for sterilization at 50°C are shown in Figure 14.

Aggressive and exaggerated extraction techniques would include reflux, Soxhlet, autoclave, microwave, accelerated extractors, and sonication. The solvents would be harsh such as hexane, ethanol, isopropanol, or chlorinated solvents. Whereas simulated extraction may only have water or diluted alcohol. The nonvolatile residue of the extracts, infrared spectroscopy, total organic carbon, or other broad-based information can be acquired to aid in developing conditions for more sensitive and selective methodology. Identification of the extractables is usually acquired by mass spectrometry; gas and liquid chromatography are typically employed for trace organic compounds. Inductively coupled plasma spectroscopy and ion chromatography are commonly used techniques for detection of inorganic species. Multiple analytical techniques should be employed to allow the most comprehensive profile. Complementary techniques and authentic standards can confirm species identifications. Once identifications are confirmed, methods can be optimized and validated for measuring the species of interest.

As it can be seen from the GC/MS profile chromatograms, run under identical conditions, a simulated extraction would not provide any information on a chemical profile of a container closure component; the aggressive and exaggerated conditions provide a wealth of information, but there is danger in creating anomalies using harsh conditions.

Not all of the data generated during the exploratory phase of an extraction will be useful to correlate to leachables, but it is prudent to have too much data rather than not enough. Interaction, hydrolysis, and degradation products may also occur and would not be evident in

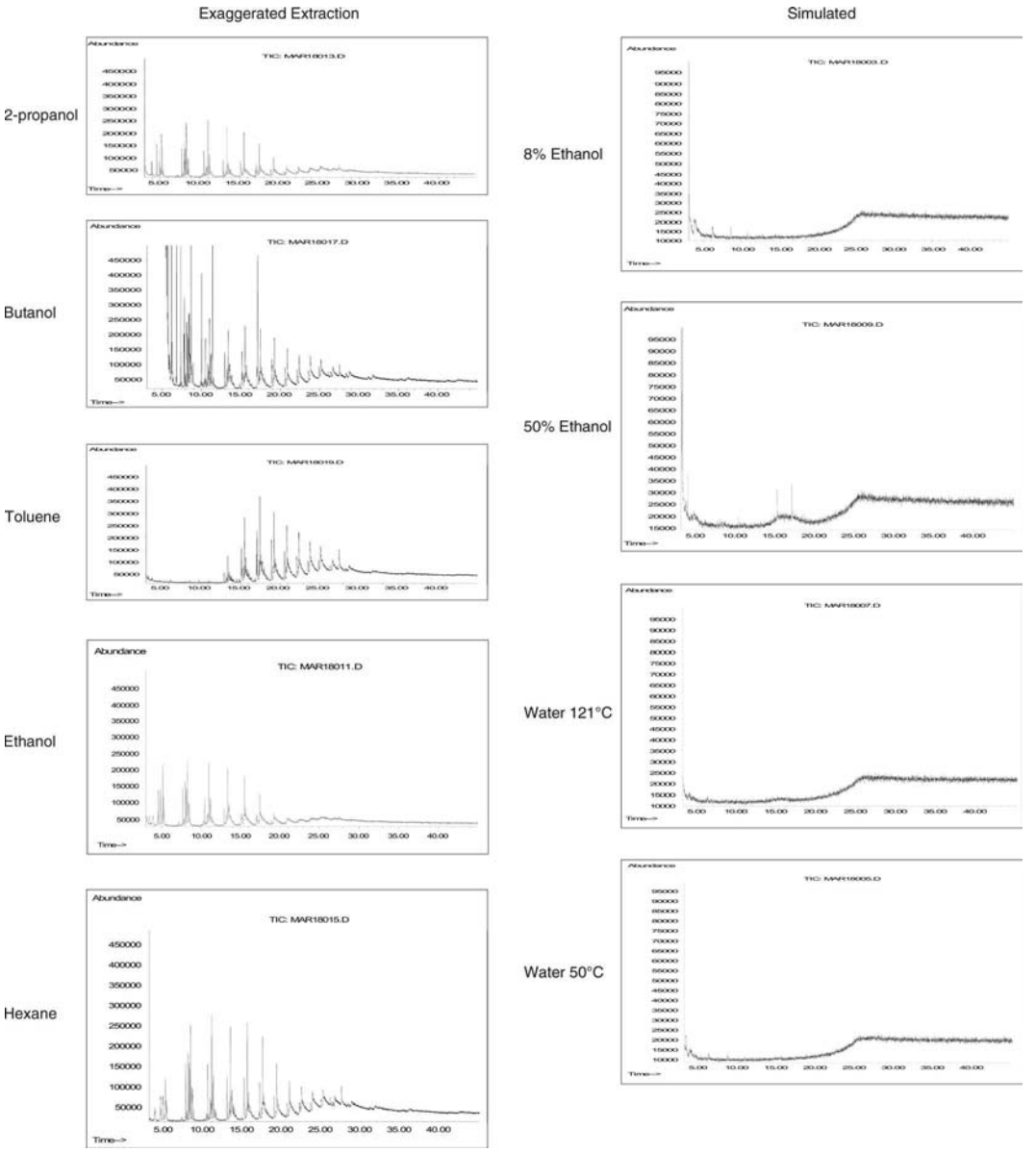

Figure 14 GC/MS qualitative profiles.

the extractables data. For this reason, the leachable methods would need to be adequate to detect unspecified species at the stability time points. In any event, the information required in drug product application is regulatory policy and the expectation is that it can change on a case-by-case basis.

LEACHABLE STUDIES: DRUG PRODUCT AND TESTING CONSIDERATIONS

Potential leachables are indicated from the component parts evaluated in the extraction studies. A comprehensive analysis of appropriately prepared extracts should detect, when present, residual starting materials from the polymerization process, primary or secondary additives, extractable contaminates from known or unknown sources, processing aids, and

additive impurities, oxidation or breakdown products. Leachables can also arise from a reaction of an extractable with drug product or secondary components that may have been overlooked when selecting components for evaluation.

The data and analysis conditions from the extraction studies can be used to develop leachable methods and include development of optimal conditions for the analytical techniques to measure target potential leachables at required sensitivity. Sample preparation trials can be carried out on the drug product control to optimize the leachable methods. The method should be evaluated to indicate suitability for validation by performing spiking, recovery, repeatability, and linearity studies. Assessment of the proposed method can then be accomplished by analysis of the initial drug product samples in contact with packaging materials and accelerated and shelf life stability samples stored in the final package. The leachable methods should be validated according to regulatory guidelines before routine and stability testing are performed.

Several lots of drug product, stored at different orientations, should be evaluated to realize variability and provide adequate information that can be used to: (*i*) determine maximum leachable levels and establish acceptance criteria if necessary, (*ii*) perform a risk assessment of leachable species on the basis of actual stability time points, and (*iii*) provide the ability to correlate leachable data to extractables to determine packaging specifications if appropriate. An extraction study should indicate greater concentrations of extractables compared with leachables. This means that methods to measure extractables should be valid and reliable. A correlation can be established if the leachables detected can be quantitatively linked, directly or indirectly to an extractable. The maximum leachable levels can be predicted based on achieving asymptotic levels of extractables. It is conceivable that routine analysis and control of the packaging components could ensure acceptable levels of leachables over the shelf life of the product. In the end, the container closure system suitable for one drug product may or may not be suitable for another drug product.

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11 Process analytical technology and rapid microbiological methods

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INTRODUCTION

In September 2004, the Food and Drug Administration (FDA) Guidance for Industry PAT—A Framework for Innovative Pharmaceutical Development, Manufacturing, and Quality Assurance was issued to encourage pharmaceutical manufacturers to develop and implement effective and efficient innovative approaches in providing quality pharmaceuticals to the public (1). The linkage of rapid microbiological methods (RMMs) to Process Analytical Technology (PAT) is largely based on real-time release, which is the ability to evaluate and ensure the acceptable quality of in-process and/or final product on the basis of the collection and analysis of in-process data. As stated in the FDA guide, the PAT component of real-time release typically includes a valid combination of assessed material attributes and process controls. Material attributes such as bioburden, endotoxin content, and sterility could be assessed using direct and/or indirect process analytical methods. The combined process measurements and other test data gathered during the manufacturing process could serve as the basis for real-time release of the final product and would demonstrate that each batch conforms to established regulatory quality attributes. The FDA considers real-time release to be comparable with alternative analytical procedures to the compendial microbiological tests for final product release. It is notable that the guidance document stated that real-time release as defined in this guidance builds on parametric release of terminally heat sterilized drug products, a practice in the U.S. large-volume parenteral industry since 1985. In real-time release, material attributes such as formulation, bioburden, container size, and load pattern, as well as process parameters such as sterilization parameters, are measured and controlled.

In this chapter, the authors will attempt to define the role of RMM in PAT and discuss the application of RMM to aseptic filling, biopharmaceutical upstream and downstream processing, environmental monitoring and control in clean rooms; the selection, development, validation, and implementation of RMM for PAT applications; industry, regulatory, and compendial guidelines for RMM; regulatory approval of RMM and the future of RMM in parenteral medication manufacturing.

TRADITIONAL MICROBIAL TEST METHODS

Unexpectedly to some, the standard-setting organization for drug products marketed in the United States is not the U.S. Federal Food and Drug Administration but the U.S. Pharmacopoeial Convention Inc., an independent standards organization, empowered by the U.S. Federal Food, Drug, and Cosmetic Act as the official drug standard-setting organization in the United States for drug products. The U.S. Pharmacopoeial Convention publishes and maintains the U.S. Pharmacopoeia (USP), National Formulary (NF), and USP Reference Standards and sets the official tests and quality standards for both drug products and pharmaceutical ingredients. In Europe and Japan, the compendia, that is, European Pharmacopoeia (Ph. Eur.) and Japanese Pharmacopoeia (JP) are government-controlled organizations that play a similar role to the USP.

Traditional USP microbial testing methods, as referee tests, rely on the growth of microorganisms in culture media for detection, enumeration, and selective isolation. These traditional methods continue to be used because of their long history of use, simplicity, effectiveness, low cost, and suitability for use in all microbiological testing laboratories. However, serious questions can be raised if the continued use of these traditional methods is the right strategy to improve quality and efficiency in the pharmaceutical industry. Those traditional methods were originally designed for the detection of human pathogens and not for the microbiological quality control of pharmaceutical processes and products. The drivers of

the microbial testing should be the critical microbiological quality attributes associated with a specific drug product and the risk assessment of the potential for microbial contamination of that drug product and resulting patient infection. The next few paragraphs will discuss the industry experience with compendial microbial testing.

Bioburden Testing

Nonsterile drug substances, pharmaceutical excipients, and drug products are evaluated for bioburden using microbial limit or microbiological examination tests. On November 8, 2005, at the Pharmacopoeial Discussion Group meeting in Chicago, Illinois, representatives of the three major compendia, that is, JP, Ph. Eur., and USP signed off on the harmonized microbiological examination tests. The USP published the General Test Chapters <61> *Microbiological Examination of Non-sterile Products: Microbial Enumerations Tests* and <62> *Microbiological Examination of Non-sterile Products: Tests for Specified Microorganisms*, and the General Informational Chapter <1111> *Microbiological Quality of Non-sterile Pharmaceutical Products* in the Second Supplement to USP 29/NF 24 in June 2006 with an official date of August 1, 2007. On November 14, 2006, the USP announced on their website a postponement of the official implementation date to May 1, 2009, to allow companies more time for method qualification, change control, and regulatory. The companion Ph. Eur. chapters are 2.6.12 *Microbiological Examination of Non-sterile Products (Total Viable Aerobic Count)*, Ph. Eur. 2.6.13 *Microbiological Examination of Non-sterile Products (Test for Specified Micro-organisms)*, and Ph. Eur. 5.1.4 *Microbiological Quality of Pharmaceutical Preparations.* These referee tests are clearly unsuitable for PAT applications due to their extended incubation times, relative insensitivity, and low precision, and even have limitation as release test methods as they may not detect all objectionable microorganisms that could be present in a nonsterile drug product.

Sterility Testing

Sterility testing was traditionally been conducted by inoculating a microbiological broth with an aliquot of the test material and scoring growth by the detection of turbidity. The compendial sterility tests have been harmonized in terms of media, growth-promotion requirements, suitability tests, incubation conditions, number of containers and amounts of material tested, and observation and interpretation of the results. Limited local requirements from the different pharmacopoeias were included in the compendial tests and these will be removed in May 2009. The membrane filtration test is the preferred test over the direct inoculation test as it has the capacity to test the entire contents of a product container and inhibitory substances may be rinsed from the membrane. The details of the tests may be found in USP Chapter <71> *Sterility Tests* and Ph. Eur. 2.6.1 *Sterility*. The incubation period for the test is at least 14 days, making it clearly unsuitable for a PAT application.

Bacterial Endotoxin Testing

Bacterial endotoxins are pyrogenic materials, for example, lipopolysaccharide, present in the cell wall of gram-negative bacteria. Bacterial endotoxins, if present in injectable products, can lead to dose-related adverse reactions in patients receiving injections ranging from chills to fever to death. A threshold pyrogenic dose is 5 EU per kg of body weight for a parenteral administration. In terms of weight and not potency, this is about 1 ng per kg of body weight for *Escherichia coli* and 50 to 70 ng/kg for *Pseudomonas aeruginosa* in both rabbits and humans. For *E. coli*, this represents some 10,000 whole cells per kg. The in vivo rabbit pyrogen test was replaced by the in vitro *Limulus* amebocyte lysate (LAL) endotoxin test in the mid-1970s making the test suitable for both in-process and finished product testing. This test is largely responsible for the elimination of pyrogens from parenteral drug products. As different sources of endotoxin have differing potency, the standard was assigned potency in endotoxin units (EU).

The compendial bacterial endotoxins assays and reference standards have been harmonized in terms of test methods, that is, gel-clot, turbidimetric (end-point and kinetic) and chromogenic (end-point and kinetic) assays, reagents, reference standard, calculation of endotoxin limits for drug products, suitability testing, and assay validation. It should be noted that the gel-clot method is semiquantitative in that it determines the lowest two-fold dilution

where clot formation occurs. Despite this limitation, in the event of a dispute as to the endotoxin content of a product, the referee test is the longer established gel-clot method. The details of the tests may be found in USP <85> *Bacterial Endotoxins Assay* and Ph. Eur. 2.6.14 *Bacterial Endotoxins*. Also, the 1987 FDA *Guideline on Validation of the LAL Test as an End-Product Endotoxin Test for Human and Animal Parenteral Drugs, Biological Products, and Medical Devices* contains details on the assay validation requirements. In general, a kinetic turbidimetric or chromogenic method would be preferred to the gel-clot method to fully quantify the bacterial endotoxin and remove the subjectivity of the gel-clot method using dilution one-half, one-quarter, or one-tenth of the maximum valid dilution. As the incubation period is one hour or possibly less, endotoxin screening has a high potential as a PAT application.

Other Testing

Other tests conducted during parenteral manufacturing may not be compendial. A bioburden evaluation of a drug substance, excipient, in-process material, presterile bulk solutions, packaging component, or nonsterile drug product is a noncompendial procedure to evaluate the number and type of microorganisms per unit weight, item, or unit surface area of the material. Typically in product development, a bioburden evaluation is a non–Good Manufacturing Practice (non-GMP) screening test that may not be fully validated or have regulatory status undertaken as part of a risk assessment during formulation and manufacturing process development. For example, as part of sterilization process development, the numbers, cellular morphology, cell size, staining reactions, and spore-forming capabilities of the predominant microbial population associated with the material would be determined to establish the appropriate sterilization parameters for sterile filtration, steam sterilization, or dry heat sterilization. When bioburden testing is used in routine production, it would be considered a GMP test and would be fully validated and included in regulatory filings.

For aseptically filled injectable products, emphasis would be given to the numbers and size of the microorganisms in a presterile bulk solution and the size retention, bulk solution volume, and filtration area subject to sterile filtration. With moist or dry heat sterilization, the numbers of spores and their relative resistance, that is, D-value, to the sterilization process would be considered.

With presterile bulk solutions, the bioburden requirements would be more conditional on the bulk volume, nominal pore size, and the filter size than the nature of the product. The 2004 FDA Aseptic Processing Guidance document overemphasizes toxicogenic materials, especially bacterial endotoxins, derived from the presterile filtration bioburden. The rating of a sterilizing filter is the retention of 10^7 colony-forming units of the challenge organism *Brevundimonas diminuta* per square centimeter of filter surface. It should be noted that the current EU guidelines for presterile bulk solutions are 10 cfu/100 mL, and tandem sterilizing filters are typically employed (2).

WHAT ARE RAPID MICROBIOLOGICAL METHODS?

A RMM is an alternate microbiological test that is completed in shorter time than the classical tests that depend on incubation for microbial growth to detect microorganisms as either colonies on a plate or turbidity in a broth. It may involve reducing the incubation time for plate count by at least half, processing a sample to obtain a result in two to three hours or a direct analytical method. The latter two approaches are typically not growth-based, hence move toward real-time analysis.

As pharmaceutical microbiologists, our primary objectives are to determine which microorganisms, if any, are in our pharmaceutical ingredients, intermediates, plant environment, or drug products; if present, how many microorganisms and what microorganisms they are and their potential impact, to help the quality unit make decisions to proceed with manufacturing and release product to the market. The test methods are classified as detection, screening, enumeration, and identification (3). Examples from the compendial microbial tests are sterility testing (detection/qualitative), absence of specified microorganisms (screening/qualitative), and microbial count (enumeration/quantitative). In addition, there is the noncompendial microbial identification (identification/qualitative).

The classification systems for rapid methods proposed in the 2002 PDA (Parenteral Drug Association) Technical Report No. 33 are based on how the technology works, for example, growth of microorganisms, viability of microorganisms, presence/absence of cellular components or artifacts, nucleic acid methods, traditional methods combined with computer-aided imaging, and combination methods (4,5). Similar, but slightly different, classifications may be found in compendial chapters discussing the validation of alternative microbiological test methods (6,7).

Growth-Based Technologies

These methods are based on measurement of biochemical or physiological parameters other than turbidity or colony formation, used in classical methods that reflect the growth of the microorganisms. Examples include ATP bioluminescence, colorimetric detection of carbon dioxide production and measurement of change in head-space pressure, impedance, advanced imaging, and biochemical assays.

Viability-Based Technologies

These types of technologies do not require growth of microorganisms for detection. Differing methods, including vital staining and fluorogenic substrates, are used to determine if the cell is viable or nonviable, and, if viable cells are detected, they can be enumerated. Examples of this technology include solid-phase cytometry and flow fluorescence cytometry.

Cellular Component or Artifact-Based Technologies

These technologies look for a specific cellular component or artifact within the cell for detection and/or microbial identification. Examples include fatty acid profiles, matrix-assisted desorption ionized—time of flight (MALDI-TOF) mass spectrometry, enzyme-linked immunosorbent assay (ELISA), fluorescent probe detection and bacterial endotoxin LAL test.

Nucleic Acid–Based Technologies

These technologies use nucleic acid methods as the basis of operation for detection, enumeration, and/or identification. Examples include DNA probes, ribotyping polymerase chain reaction (PCR), and ribosomal DNA-based sequencing.

A SURVEY OF RAPID MICROBIOLOGICAL METHODS

In most cases, RMMs may be divided into classes on the basis of their principle of detection. In this survey of RMMs, a ranking is made on the basis of successful implementation in the pharmaceutical industry (Tables 1 and 2). Note: This is provided as useful information to the reader and is not intended to be an endorsement from the authors of this chapter. Other systems may be available or become available that are not included in the table.

Table 1 Some Representative RMM Frequently Implemented in the Pharmaceutical Industry

System	Supplier	Technology	Major application
ChemScan RDI	Chemunex, Princeton, New Jersey, U.S.	Solid-phase LASER fluorescence scanning microscopy	AVC
MicroPRO (RBD 3000)	AATI, Ames, Iowa, U.S.	Fluorescence flow cytometry	AVC, P/A
RapiScreen/AkuScreen	Celsis, Chicago, Illinois, U.S.	ATP bioluminescence	P/A
BacT/ALERT	bioMerieux, Durham, North Carolina, U.S.	CO_2 colorimetric detection	P/A
Pallchek	Pall Corp. East Hills, New York, U.S.	Membrane filtration ATP bioluminescence	P/A
BACTEC 9000	BD, Corkyville, Delaware, U.S.	CO_2 detection fluorescence	P/A
Endosafe PTS	Charles River Laboratories, Wilmington, Massachusetts, U.S.	Handheld chromogenic LAL endotoxin assay	Bacterial endotoxin assay

Abbreviations: AVC, aerobic viable count; P/A, presence/absence; LAL, *Limulus* amebocyte lysate.

Table 2 Some Representative RMM with the Potential to Be Implemented in the Pharmaceutical Industry

System	Supplier	Technology	Major application
Q-PCR: Micro Compass	Lonza, Basel, Switzerland	RT-PCR	AVC
BacTrac 4300	Sy-Lab, Vienna, Austria	Impedance	AVC,P/A
Soleris Pathogen Detection Systems	Biosys (Centrus), Kingsport, Tennessee, U.S.	Optical biochemical	AVC
RABIT	Don Whitley Scientific, Shipley, England	Impedance	AVC
PyroSense	Lonza, Basel, Switzerland	Chromogenic LAL, recombinant Factor C based	On-line endotoxin detection
Biovigilant Air Monitoring System	Biovigilant, Tucson, Arizona, U.S.	Direct cell detection	On-line air monitoring
Growth Direct	Rapid Microbial System, Bedford, Massachusetts, U.S.	Autofluorescence Advanced imaging	AVC
Kikkoman ATP Swabs for Hygiene Testing	Kikkoman, Tokyo, Japan	ATP detection	Surface monitoring

Abbreviations: AVC, aerobic viable count; P/A, presence/absence.

It can be concluded that some of the most successful RMMs are the ChemScan, AkuScreen, and BacT/ALERT systems. More companies use these RMMs for in-process controls than product release. The latter is often product dictated. Rate of success of implementation is determined by the ability to focus and reserve manpower on the qualification and validation work.

WHAT IS A REAL-TIME MICROBIOLOGICAL METHOD?

In general, decision makers (i.e., physicians, production managers, and quality units) claim that the microbiological testing laboratories in the hospitals, food production sector, and pharmaceutical industry are the rate-limiting steps for patient treatment and product release. As microbiologists, we recognized the truth in their criticisms that microbial tests are imprecise with long incubation times. In Table 3, typical incubation times are shown for a range of microbial tests.

Microbiology laboratories count the time in days or even weeks to obtain a result. Furthermore, the results may need to be interpreted, reviewed, and approved before they can be reported. And that is not all! The time to ship the samples to the laboratory must be considered. It is a simple addition calculation: *Time to report = Time to ship the sample to the laboratory + administrative time + analysis time + incubation time + verify time + approval time + time to report the result.* Product release cycle times are protracted and are the sum of all these sequential activities. That means seven (7) items to work on to speed up the overall testing process. With RMM, in most cases, only the analysis time and incubation time is considered.

Table 3 The Incubation Requirement for Microbial Tests Used in Drug Manufacturing

Test	Incubation time
Total aerobic microbial count	3–5 day
Total yeast and mold count	5–7 day
Sterility tests	14–18 day
Absence of specified microorganisms tests	18–72 hr
Limulus amebocyte lysate endotoxin tests	1 hr
Microbial identification, phenotypic	3–5 day
Microbial identification, genotypic	1 day
Preservative efficacy tests	7–28 day
Mycoplasma test	28 day

It is important not to forget the other time-consuming factors in the analytical process when considering RMM implementation.

Other important differences that we recognize is that among in-process RMM testing for production process control, RMM testing for troubleshooting, and RMM testing for product release. All three may have different goals. Some definitions are in order before discussing the goals. The normal way we perform microbial analysis (when the sample is taken to the microbiology laboratory) is called *off-line testing*, if an analysis takes place near the production line but the sample is taken out of the production process, it is called *at-line testing*, and the last one is *in-line testing*, where there is a continuous analysis ongoing in the production process.

Conventional microbial testing, in most cases, is *off-line testing* with a few cases *at-line testing* (depending on the manufacturing infrastructure). If we examine RMMs, they also belong to these two categories with some exceptions that have the potential to be used *in line* (see Table 2 for an overview of different RMMs).

What determines now whether a RMM can be used *off line*, *at line*, *or in line*? In most cases, it is the underlying principle of the technique. For this discussion, RMMs can be subdivided into different categories on the basis of their detection principle: (*i*) detection of early growth, (*ii*) viability-based testing, and (*iii*) detection of microbial cell components. RMMs based on the early detection of growth principle are the slowest; the other two will be faster depending on the kind of application. Some examples: Detection of CO_2 production is a growth-depended technique that may be used for sterility testing. This application is unlikely to be an *in-line* application because of the aseptic handling that is inherent to the sterility test. In best case, it could be an *at-line* application. Detection via flow cytometry has a viability-based detection principle. Although it is not on the market, we can imagine that an *in-line* application could be possible to detect and count microorganisms via a laser detection principle. In fact, there are some techniques available that are potential *in-line* detection systems based on viability cell detection (Biovigilant, Tucson, Arizona, U.S.). The last category: Detection of cell component has many applications: detection of DNA, fatty acids, ATP, etc. In most cases, it requires a sample preparation that automatically converts it to an *off-line* application. There are several examples of *at-line* detection of bacterial endotoxin that may be used in parenteral manufacturing. They are the Endosafe PTS (*at line*), Charles River Laboratories, Wilmington, Massachusetts, U.S., and the PyroSense System (*on line*), Lonza, Basel, Switzerland.

Is the conclusion that the only real-time RMM is a system that is based on viability cell detection in an *in-line* PAT application? (time to report = real-time result) In principle, the answer is yes. However, in most cases, it is not possible to use the viability cell detection principle *in line*. What is the best possible option for the production departments and microbiology laboratories that serve them? The most practical option would be *at-line* testing with a viability-based cell detection principle (time to report < 30 minutes). However, because the viability-based cell detection systems have the technical limitations of a lack of sensitivity (limit of detection/quantification) and specificity (differentiating between cells and particulates), we end up with an *at-line* testing option of detection of early growth/cell component to eliminate ambiguity (time to report 24–48 hours).

It must be emphasized that with RMMs, the objective of the testing determines what kind of system is needed. For RMM testing for product release for the market, an *off-line* testing system is the right choice because there is no need for testing at the production floor. RMM testing for troubleshooting, in contrast to product release, can be both *at-line* testing and *off-line* testing. RMM testing for in-process testing would be preferably done *at line*. With the latter, the difficulty and workability of a test method determines the *at-line* or *off-line* application of a test.

THE APPLICATION OF RMM TO ASEPTIC PROCESSING

Aseptic processing may be divided into: (*i*) aseptic bulk processing most often employed with biologics and (*ii*) aseptic filling and lyophilization with both biologics and small molecules. On the basis of a risk assessment, critical control points can be established and, if necessary, monitored to minimize the risk of microbial contamination and loss of environmental control (8). This monitoring would be more effective if conducted in real time to provide the opportunity to take corrective action to reduce the possibility of contamination.

The following microbial tests may be used during in-process monitoring:

- Microbial limits and bacterial endotoxin testing of incoming pharmaceutical ingredients and packaging components
- Microbial counts and bacterial endotoxin testing of water for pharmaceutical use, buffers, and other intermediates
- Presterile filtration bioburden monitoring
- Biological indicator monitoring
- Sterility testing of sterile bulk drug substances
- Microbial monitoring of air, surfaces, and personnel in clean rooms

Bacterial Endotoxin Testing

As pointed out earlier, with endotoxin monitoring, two major innovations are notable. They are handheld bacterial endotoxin monitoring units (Endosafe) that are used by manufacturing personnel to test water for injection points of use immediately prior to delivering ingredient water and at-line monitoring systems (PyroSense) that continuously monitor endotoxin levels in a water-for-injection loop at preset time intervals. These instruments can mitigate risk of using endotoxin-contaminated water.

Water Testing

Microbial counts are used to monitor pharmaceutical water systems for alert and action levels to identify possible out-of-trend conditions that require corrective action. The monitoring can identify potential point-of-use, loop, or entire water system problems. As the European requirements specify the use of membrane filtration with R2A agar incubated at 30 to 35°C for at least five days, excursions are identified long after the ingredient water has been used. RMMs that have been used for monitoring water systems include the Milliflex Rapid System (Millipore Corp, Bedford, Massachusetts, U.S.) based on membrane filtration, ATP bioluminescence and advanced imaging, the Scan RDI system (AES-Chemunex, Princeton, New Jersey, U.S.) based on membrane filtration, a fluorogenic substrate and solid phase LASER scanning microscopy, and the MicroPro System (AATI, Ames, Iowa, U.S.) based on vital stain flow cytometry. These systems may be used to obtain microbial counts within the order of 18 hours, 3 hours, and 30 minutes, respectively. Of these technologies, only the flow cytometry system meets the definition of real-time, at-line testing suitable for a PAT application, although the method may be too insensitive (level of quantification on the order of 100 bacterial cells per mL) for many applications that depend on enumeration and not just screening for gross contamination.

Bioburden and Sterility Testing

For aseptically filled injectable products, emphasis would be given to the numbers and size of the microorganisms in a presterile bulk solution, the volume of bulk solution to be filtered, and the size retention and filtration area of the sterilizing filter. The rating of a sterilizing filter is the retention of 10^7 colony-forming units of the challenge organism *B. diminuta* per square centimeter of filter surface. As noted earlier, the current EU guidelines for presterile bulk solutions are 10 cfu/100 mL, and tandem sterilizing filters are typically employed in Europe. With tandem sterilizing filters, monitoring the bioburden of the bulk solution challenging the second filter may be eliminated. To demonstrate that the bulk solution meets this requirement, a 100-mL sample would be tested using a membrane filtration method. Given the stringent requirement, RMMs must have a limit of detection and quantification commensurate with the 10 cfu/100 mL limit as well as a rapid turnaround time. This severely limits the options available for bioburden monitoring.

A possible option is to use a RMM as a presence/absence test for water for injection, low-pyrogen purified water, and in-process material to screen out samples that contain no microorganisms where processing would continue and concentrate on additional enumeration of those sample that contain microorganisms.

Sterility testing of sterile bulk drug substances and sterile bulk solution prior to aseptic filling is typically conducted using a 10-mL sample inoculated into broth and incubated for at least 14 days. With sterile drug substances that are being stored for future use, there is no time constraint for sterility testing unless there is a need to reprocess the drug substance, to prevent

product loss, if it is found to be not sterile. Sterile bulk sterility tests are legal requirements for biologics marketed in the United States, using the tests according to 21 CFR 610.13.

The MicroCompass™ Detection system (Lonza) based on detection of universal sequences of RNA using a one-step real-time reverse transcriptase PCR assay and MGB™ Eclipse probe technology is a promising new technology. Universal sequences detected are based on ribosomal 16S rRNA (bacteria) and 18S rRNA (yeast and molds). The sensitivity is 50 fg of RNA or as little as 100 cfu. This technology has a detection limit that has sensitivity on the edge of bioburden limit.

Environmental Monitoring

Microbial monitoring of air, surfaces, and personnel in clean rooms is conducted during each manufacturing shift. The results are delayed for five to seven days due to the incubation of the microbiological culture media. As environmental monitoring is by far the largest microbial testing in an aseptic filling facility, the automation of the sampling, incubation, and reading of plates would increase the efficiency and timeliness of the monitoring. A technology that will achieve this goal is the Growth Direct System (Rapid Microbial Systems, Bedford, Massachusetts, U.S.) based on the early detection of microcolonies on plates using advanced imaging.

A technology that will achieve real-time environmental monitoring is the Biovigilant Air Monitoring System that is capable of counting both viable and nonviable particles in a clean room setting. This may be used as a PAT application detecting high-efficiency particular air (HEPA) filter failures, isolator system leaks, human interventions generating airborne microorganisms, and the ingress of microorganisms from supporting areas that would enable immediate corrective action such as line clearance, changes in clean room behavior, and even aborting aseptic filling operations.

THE APPLICATION OF RMM TO BIOPHARMACEUTICAL UPSTREAM AND DOWNSTREAM PROCESSING

In the bioprocessing, microbiological control plays an important role. The definition of the bioprocessing is important. Bioprocessing is the manufacture of therapeutic proteins using mammalian, bacterial, yeast, or other living (plants, insects) cells. This process can be divided into two parts: (*i*) upstream processing, in which the cell culture step takes place, and (*ii*) downstream processing, where the protein is recovered and purified using a range of biochemical purification techniques, especially large-scale column chromatography.

The scale of the bioprocess has increased in the last 10 years. It started with small-scale culture $<$10 L but increased to larger volumes of the order of 15,000 L. The challenge to prevent contamination of those giant fermentors is huge. Financial risks are high (50 euro/L medium, which means that only the costs of one contaminated fermentor can be of the order of 750K euro).

Looking at the downstream processing, we see the same kind of evolution in scale. It started with small columns and currently large columns, and their associated resins are used that are expensive to maintain and difficult to replace once contaminated.

The golden rule in bioprocess industry is the following:

1. Prevent contamination from input materials and equipment.
2. Detect a contamination as fast as possible.
3. Monitor your process on critical control points.
4. Take corrective action as soon as possible to isolate the incidence and find the root cause analysis.

Sterile media and equipment is achieved using validated sterilization processes and released by the use of a validated rapid microbial method. To be useful, RMMs must generate real-time results within the processing area and not a microbiology laboratory.

If the decision is taken to implement RMM in bioprocessing operations, a series of steps have to be taken to prove the PAT concept. Most important is the first step: The selection of "the most valuable sample," or in risk analysis terminology, the critical control point. These are the key samples that mark a critical step in the process. For example, before the inocula are transferred from a smaller to the next larger fermentor, it is wise to take a sample before the processing reaches the scale of 15,000 L. It goes without saying that all the input materials

(media, buffers, cells, compressed air, etc.) are critical samples. If a contamination occurs, RMMs are very useful instruments to troubleshoot the process. The first 24 hours after a contamination occurs is vital. The longer it takes to collect and analyze data, the more difficult it will be actually to find the root cause of the contamination and take corrective action. An important tool for root cause analysis is also a rapid identification technology. The identity of a microbial contaminant can help to find the root cause. Rapid identification, that is, within one day can be very useful. Automatically, a genotyping based technology will be the method of choice, for example, 16s rRNA sequencing, due to its rapidity and accuracy.

THE ROLE OF RMM IN ENVIRONMENTAL MONITORING AND CONTROL

What is the role of environmental microbiological monitoring? In general, monitoring is performed to get insight into the microbiological quality of the manufacturing environment. Depending on the classification of the production environment, critical locations are selected and are sampled by contact plates, settle plates, or active air monitoring. Monitoring can be divided into monitoring of surfaces, air, and personnel. The specifications of the monitored places depend also on the classification of the area and the criticality of the operation. Strict limits are used in a grade A area (ISO 5) (<1 cfu/settle plate), whereas grade B (ISO 6–8) or lower classified areas have less stringent limits. As incubation times are long for monitoring media (3–5 days), the results represent the past history of that sampled area and not the current status. That is widely recognized in the industry; hence, we follow the trend of the microbiological cleanness with respect to sampling times. As soon as an adverse trend is detected in the microbiological quality of a sampling location, corrective action is taken such as additional disinfection, retraining of the personnel, or screening for changes in the environment. Immediate action to an out of limit in monitoring in general is difficult because of the time lag in the actual monitoring action and the time the result is known.

The results that are obtained with the current monitoring techniques give, as expected, a relative value. Monitoring efficiency depends on the type of surface, the contact time, the type of media, and incubation time. This also adds up to the relative value of environmental monitoring, and stresses the importance of performing trend surveys to assure control of the microbiological quality of the environment. What is then the role of RMMs in environmental monitoring? The conventional methods give a good insight into the microbiological quality of the environment; however, they have the disadvantage that manufacturing errors, for example, a wrong disinfection procedure, are detected at a later point or not even detected at all. That could be the benefit of RMM in environmental monitoring. A timely corrective action can be performed and the risk of production in a dirty environment is diminished. RMMs contribute to the validated state of the production process. The link to the actual batch of product that is being produced is difficult to make with environmental monitoring. If production takes place in a microbiologically dirty environment, the chance of getting a contaminated product is higher. If RMM is used, it may be easier to link the actual microbiological measurement to the microbiological quality of the product. Parametric release could be easier using these RMM technologies.

At this moment, there is no definitive RMM for environmental monitoring available which gives results the same day. Direct cell detection by ChemScan/ScanRDI technology was tested by some companies for air monitoring but is not a widespread application because of the low throughput and cost in testing with this technology. ATP measurement could be the method of choice, as instrumentation is available that can process many samples and the technology has been successfully used for hygiene monitoring in the food industry. However, the sensitivity is insufficient to measure low microbial counts on the very clean surfaces that are common in pharmaceutical production. The ultimate RMM for environmental monitoring should give results within 30 minutes and is quantitative and very easy to operate in a clean room environment.

INDUSTRY, REGULATORY, AND COMPENDIAL GUIDELINES FOR RMM

Since May 2000, when the PDA Technical Report No. 33 was published, a number of regulatory and compendial documents have been issued that were strongly influenced by the technical report to address the selection, purchase, implementation, and regulatory submission of alternate microbiological methods including RMMs. They include the following.

PDA Technical Report No. 33

The PDA was the first organization to develop guidance for the evaluation, implementation, and validation of RMMs (4). Guidance information was published as Technical Report No. 33. This document was developed by a committee of individuals from industry, regulatory agencies, compendial groups, and instrument vendors and chaired by one of the authors of this chapter. This guidance provided definitions in microbiological terms for validation criteria similar to the information in USP <1225> for chemistry methods.

USP Informational Chapter <1223> on Validation of Alternative Microbiological Methods

The USP Information Chapter <1223> defined the validation criteria to be used for RMMs, along with definitions of these criteria in terms of microbiology, in contrast to chemistry as found in USP <1225> (6). The proposal also identifies how to determine which criteria are applicable to different technologies, on the basis of the type of testing being performed.

GMPs for the 21st Century

The FDA initiated a program to modernize requirements for pharmaceutical manufacturing and quality. This modernization included encouraging early adoption of new technologies, facilitation of industry application of modern quality management technologies, encouraging implementation of risk-based approaches in critical areas, ensuring that policies for review of a submission, compliance, and facility inspection are based on state-of-the-art technologies, and enhancing the consistency and coordination of FDA regulatory programs. This resulted in an initiative titled "Pharmaceutical cGMPs for the 21st Century—A Risk-Based Approach" in 2004 (9).

FDA Guidance on Aseptic Processing 2004

In 2004, FDA published an updated guidance document on aseptic processing of pharmaceutical products. It includes a provision for the use of alternative microbiological test methods. This guideline was titled "Guidance for Industry Sterile Drug Products Produced by Aseptic Processing—Current Good Manufacturing Practice" (10).

Ph. Eur. Chapter on RMM

In 2006, the Ph. Eur. published 5.1.6. *Alternative Methods for Control of Microbiological Quality* (7). This chapter provided an overview of some RMMs available and potentially applicable to pharmaceutical processes, and how they may be used for microbiological control of products and processes. It also provides guidance on how to choose and validate an appropriate method using the ATP bioluminescence technology as an example.

THE SELECTION, DEVELOPMENT, VALIDATION, AND IMPLEMENTATION OF AN RMM FOR PROCESS ANALYTICAL TECHNOLOGY APPLICATIONS

The implementation of an RMM in the production area is a considerable challenge, although it is becoming easier compared with the situations five years ago. This process can be divided in different steps to be taken, which are important to follow to assure a successful implementation. The goal of implementation of each rapid method can be different (like earlier mentioned). A reduction of cycle time is a common goal. In this case, the testing will be conducted on the end product of the production process. Another goal is risk mitigation for microbial contamination in the production process (preventative) and troubleshooting failures to determine the root cause (reactive). In this case, the RMM is assurance against microbial contamination and will safeguard the production process. The following steps should be taken:

1. Discuss in detail with the manufacturing the details of the production process and select the most valuable sample or critical control points.
2. Select the most suitable detection method (growth based, direct cell detection, or detection of cell components) that is compatible with the nature of the sample, the expected contamination, and the sensitivity to be achieved.
3. Select the instrumentation that fits the best for the sample and the technology.
4. Select an equipment supplier.
5. Perform pilot or proof-of-concept testing to prove that the instrumentation fits the specific application. Perform method suitability testing for a range of test materials.

6. Purchase the instrumentation and perform the equipment validation, that is, Installation Qualification (IQ), Operational Qualification (OQ), and Performance Qualification (PQ) using vendor supplied document whenever possible.
7. Perform method suitability testing at least on three independent batches.
8. Assemble all the GMP documentation (Standard Operating Procedures, calibration programs, regulatory submissions, and change controls).
9. Implement in routine testing.

REGULATORY APPROVAL OF RMM

With the FDA, three avenues are possible for the approval of RMMs. A New Drug Application (NDA) submission for an RMM may be used with a new product and an NDA supplement for existing product, filing a comparability protocol, or using the PAT initiative pathway. The FDA prefers the comparability protocol approach (11) as it accommodates the fact that the FDA approvals are typically drug product specific, and a comparability protocol gives the FDA the opportunity to review your method validation plan prior to executing it for a range of drug products. In general, it is advisable to discuss the application and validation strategy with the regulatory agency in advance.

The most important RMM validation issue is equivalence to the current method. Other standard validation issues include accuracy, sensitivity, precision, and linearity of response. Microbiologist should use supplier-generated validation protocols whenever possible. IQ is best timed with the delivery of the equipment to your laboratory. OQ will demonstrate the functionality of the equipment while PQ will be directly related to your application and products. Remember it is acceptable to include supplier-generated reports and publication from peer-reviewed journals within your validation report so you may avoid repeating the generation of preexisting data. Validation protocols and reports must include the validation rationale, acceptance criteria, and deviations from protocol or acceptance criteria, and the documents must be reviewed and approved by the quality unit.

THE FUTURE OF RMM IN PARENTERAL MEDICATION MANUFACTURING

What is the future of RMMs in parenteral drug manufacturing? The major trends are (*i*) the move away from traditional growth-based methods to RMMs on the basis of vital cell staining, ATP, or nucleic acid concentration, (*ii*) the move from the microbiology laboratory to the production floor as the site of the microbial testing, and (*iii*) the use of RMM to PAT applications by the real-time testing in-process samples.

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12 Quality assurance

Michael Gorman

INTRODUCTION

Quality assurance is particularly important in aseptic manufacturing. This type of manufacturing must strictly follow carefully established and validated methods of preparation and procedures. Quality needs to be built into the operations and process and not be placed on just the end-product testing. Quality must be applied to facilities, preparation of materials, and to all aspects of processing.

Key quality systems and key aspects of those quality systems as they apply to aseptic processing are discussed in this chapter. In general, all operating conditions and treatment of materials should be such as to prevent microbial contamination and follow a proven control strategy (1). The output from the control systems of the operating conditions and treatment of materials should then be assessed as part of the lot disposition process (Fig. 1).

To maintain the sterility of the components and the product during aseptic processing, the control strategy needs to include the following: environment, personnel, critical surfaces, container/closure sterilization and transfer procedures, maximum holding period of the product before filling into the final container, and sterilizing processes (1).

In general, two basic areas can be defined—physical assets and process systems. These two basic areas need systems to collect and track information to ensure sterility assurance. The physical assets include facilities, equipment, and utilities (e.g., air handling systems, compressed air, nitrogen, steam generator, and water). The process systems include key points to the quality systems like training, material management, calibrations, validations, processes, batch records, investigations, quality control laboratory, environmental monitoring, cleaning equipment/facilities, and quality information management. The role of quality assurance in product development for an aspect process will be briefly presented.

The output from the control systems for routine monitoring of the physical assets (not in use and during production), coupled with the output from the process systems associated with the production batch, should be included in the quality information management system for assessing status of each lot produced by aseptic processing.

PHYSICAL ASSETS

The physical assets should be designed to support the specific type of production and to reduce the chance for contamination of the product. Many aspects of the physical assets have been discussed in detail throughout other chapters. This section will focus on quality aspects of physical assets related to design preferences and control. Quality plays a key role with physical assets by aligning with operations and provides guidance for design, systems for monitoring, change control, and qualifying. Also, operations and quality need to work closely together to resolve investigations related to the physical assets.

Facilities

For aseptic processing, the facility layout should control the flow of materials and personnel with respect to environment quality needed for the stage of processing. For example, the facility should have a cascade of room classifications from less to more as the process flows toward aseptic requirements. Air locks should be used to separately transfer materials and the flow of personnel into the critical aseptic processing areas to prevent the chance of contamination (Fig. 2).

The construction materials for the production areas should be chosen for durability to allow for frequent cleaning/sanitizing. In clean areas, all exposed surfaces should be smooth, impervious, and unbroken to minimize the shedding or accumulation of particles or microorganisms (2). The wall and room designs should not have areas to collect dust or cause difficulties for cleaning. Examples of adequate design features include seamless and

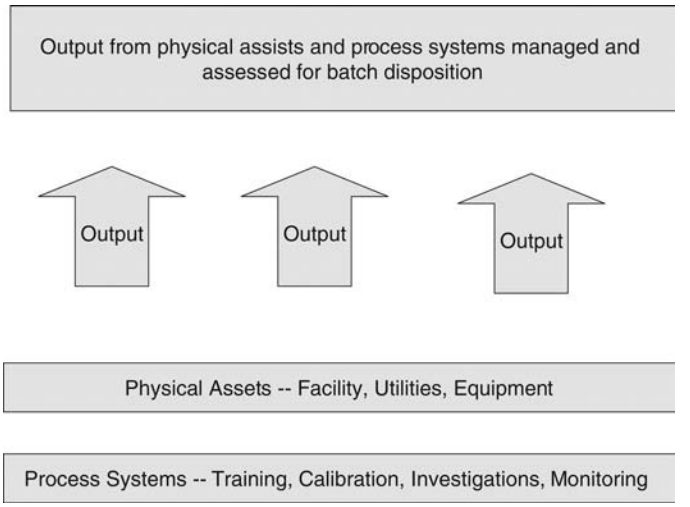


Figure 1 Overview of aseptic control.

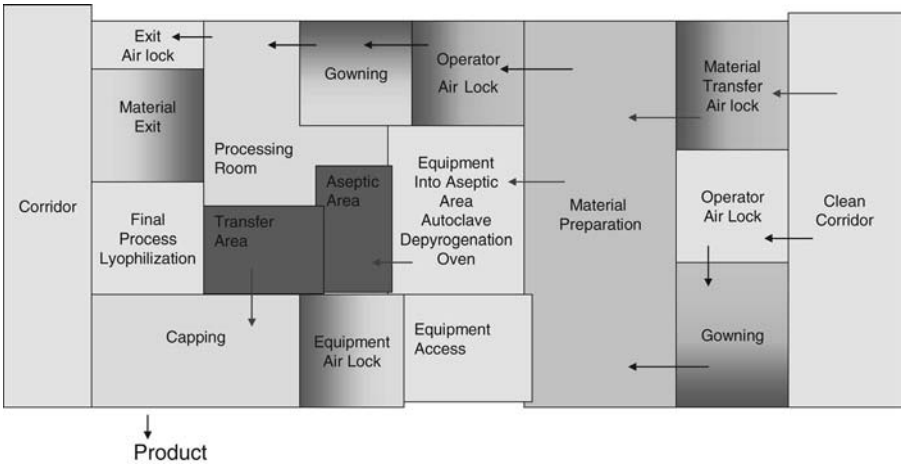


Figure 2 Basic facility diagram.

rounded floor to wall junctions as well as readily accessible corners. Ceilings and associated HEPA filter banks should be designed to protect sterile materials from contamination (3). False ceilings should be sealed to prevent contamination from the space above them. Sinks and drains should not be located in areas used for aseptic manufacture.

In quality control microbiology, sterility testing area should have the same or better quality environment as the aseptic processing area. This is done to minimize the potential of false positives during testing.

The facility should be routinely inspected for the need of wall, floor, and ceiling repairs. These inspections should be documented and repairs preformed promptly to keep the facilities in a good state of control to prevent the chance for product contamination. In general, the facility should be inspected before each batch and thoroughly inspected and repaired at defined frequencies (e.g., every 6 months). These inspections and repairs proactively keep the facilities in good working order to prevent contamination of the product.

Clean area control parameters should be supported by microbiological and particle data obtained during qualification studies. Initial clean room qualification should include an assessment of air quality under as-built, static conditions and dynamic conditions. It is

Table 1 Air Classifications

Clean area classification (0.5 μm particles/ft^3)	ISO designation[a]	> 0.5 μm particles/m^3	Microbiological active air action levels[b] (cfu/m^3)	Microbiological settling plates action levels[b,c] (diam. 90 mm; cfu/4 hr)
100	5	3,520	1[d]	1[d]
1,000	6	35,200	7	3
10,000	7	352,000	10	5
100,000	8	3,520,000	100	50

All classifications based on data measured in the vicinity of exposed materials/articles during periods of activity.
[a]ISO 14644-1 designations provide uniform particle concentration values for clean rooms in multiple industries. An ISO 5 particle concentration is equal to Class 100 and approximately equals EU grade A.
[b]Values represent recommended levels of environmental quality. You may find it appropriate to establish alternate microbiological action levels because of the nature of the operation or method of analysis.
[c]The additional use of settling plates is optional.
[d]Samples from Class 100 (ISO 5) environments should normally yield no microbiological contaminants.

important for area qualification and classification to place most emphasis on data generated under dynamic conditions (i.e., with personnel present, equipment in place, and operations ongoing). Table 1 summarizes clean area air classifications and recommended action levels of microbiological quality (4).

The facility should be designed to meet room classifications appropriate for each stage of manufacturing. The facility is key for maintaining appropriate environmental conditions to protect the product from contamination for routine aseptic manufacturing. The facility needs to be properly maintained, monitored, and used for intended purpose.

Equipment

Aseptic processing equipment should be appropriately designed to facilitate ease of sterilization (5). Equipment should be designed to be easily assembled and disassembled, cleaned, sanitized, and/or sterilized. Fixed equipment (e.g., large mixing tanks) should be properly designed with attention to features such as accessibility to sterilizing agent, piping slope, and proper condensate removal. Additionally, the effect of equipment design on the clean room environment should be addressed. For example, horizontal surfaces or ledges that accumulate particles should be avoided. Equipment should not obstruct airflow and, in critical areas, its design should not disturb unidirectional airflow (3).

In the aseptic processing area, smoke studies should be used to verify unidirectional airflow. Videotaping smoke studies provide thorough evidence showing air flow patterns. If changes to equipment or facilities are needed, air flow patterns need to be carefully assessed and recorded.

Equipment shall be constructed so that surfaces that contact components, in-process materials, or drug products shall not be reactive, additive, or absorptive so as to alter the safety, identity, strength, quality, or purity of the drug product. Ideally, product contact surfaces should be disposable or made of materials that are product dedicated (e.g., 316 stainless steel). In multiproduct facility, key product contact surfaces should be dedicated to a product. For instance, filling needles should either be disposable or dedicated to a product. This is done to prevent the chance of cross-contamination.

Adequate cleaning, drying, and storage of equipment will aid in controlling bioburden and prevent contribution of endotoxin load. If adequate procedures are not used, endotoxins can be introduced into the process by the equipment (3). Records should be kept showing cleaning schedules and the performance of the cleaning procedures.

Equipment surfaces that contact sterilized drug product, or its sterilized containers or closures, must be sterile so as not to alter purity of the drug (5). Where reasonable contamination potential exists, surfaces that are in the vicinity of the sterile product should also be clean and free of microorganisms. The validation of cleaning procedures is important to show removal of microorganisms, processing materials, and cleaning agents.

Monitoring devices should be used whenever feasible. Equipment monitoring provides proof that the equipment functioned properly during use. The output from the monitoring devices should be recorded to provide assurance for the proper equipment performance during manufacturing.

Records need to be kept for equipment showing routine and nonroutine maintenance, usage, and calibration of monitoring devices. If equipment does not operate within intended limits, an investigation should be preformed.

Utilities

Utilities for an aseptic processing facility should be designed to prevent contamination. Utilities actually bring processing materials into contact with the product. These materials should be sterilized. For example, the compressed air system may introduce air into a lyophilizer before the product is stoppered. Thus, the air becomes the headspace of the product vial. Quality aspects will be illustrated for the following utilities: air systems (HVAC), compressed air system, nitrogen gas supply, water, and steam generator.

HVAC

The main purpose for the HVAC system is to provide clean air into the processing areas. The HVAC system needs to be designed to deliver particulate- and microbial-free air. Most systems contain prefilters with >95% efficiency filters with terminal or final filters >99.9% efficiency (HEPA). In the aseptic areas, HVAC systems should deliver single-pass air. Therefore, the system should not recirculate air and the air supply should consist of 100% fresh makeup air. This is done to prevent cross-contamination.

The HVAC system should be capable of keeping the processing areas very cool for operator comfort. Typically the environment should be around <65°F and <60% RH. The main reason for this type of temperature and humidity control is to keep the operators, who are generally double gowned, comfortable and free from perspiration to decrease shedding.

Monitoring systems should target continuous monitoring for temperature, humidity and pressure differentials across filters and pressure differentials between rooms. The continuous monitoring should have appropriate ranges. If conditions fall outside of set ranges, an investigation should be triggered with an assessment to the impact on the product.

Compressed Air System

Like the HVAC system, the compressed air system should be designed to provide essentially a source for sterile air. The air system should be monitored at frequencies to show that air is delivered free from contaminates like microorganisms and hydrocarbons. At use-points that come into contact with the product terminal, sterile filters should be used. These filters should be tested for integrity. Records should be maintained for the proper routine performance and lot performance of the air system.

Nitrogen Gas

Nitrogen gas is often used during the production process to control equipment and sometimes used to produce an environment free from oxygen. The nitrogen gas supply should be tested for identity and moisture. Often a plant may use a bulk liquid nitrogen tank coupled with evaporators to supply nitrogen gas. In these systems, each charge of the bulk nitrogen tank should be tested at a minimum for identity. If by accident the wrong liquid was loaded into the bulk tank, this could cause major damage to the nitrogen system and contaminate the production facility. The nitrogen system needs to be routinely monitored for performance. Routine and nonroutine maintenance need to be documented. Additionally, on key locations throughout the nitrogen system, point-of-use sterilizing filters should be used and integrity tested.

Water

Other chapters outline water systems in detail for aseptic processing. From the quality perspective, the water system should be monitored before use to ensure that the appropriate

quality is used during processing. Ideally, the water system should be continuously monitored for key parameters like pressure, temperature, conductivity, and total organic carbon. Additionally, the water system should be sampled throughout key points in the system and points of use. Records need to be kept for routine and nonroutine maintenance. To ensure the proper control of the water system, the monitoring data should be analyzed by trending, and reviewed routinely. During the course of monitoring a water system, alert and action limits need to be established. If a limit is exceeded, an appropriate action/investigation should be preformed.

Steam Generator

Steam systems should be supplied with clean water that is free from hydrocarbons, salts, and microorganisms, ideally, water-for-injection quality. The steam quality needs to be routinely tested throughout the distribution system and at key points of use. Like the other utility systems, records should be kept for the maintenance and performance of the steam generator.

QUALITY SYSTEM

Quality assurance needs to remain proactive in aseptic processing by providing guidance to operations for developing systems. A proactive quality system for aseptic processing has a rigorous monitoring, evaluation, and response/corrective action component. Proactive quality needs the right systems in place to react before major problems happen. The monitoring aspects of the quality system should be evaluated for trends and reviewed frequently by quality and operations management. Quality should evaluate and assess the output from the physical assets and quality systems for each batch manufactured. Components of the process system that are discussed in this chapter are the following: training, material management, calibration, validation, process, batch records, investigations, quality control laboratories, environmental monitoring, cleaning equipment/facility, and quality information management.

Training

Each employee has a responsibility to the company to ensure records and training activities are current. All regulations have requirements for training and qualifications of personnel. For example, 21 CFR 211.25(a) states that "Each person engaged in the manufacture, processing, packing, or holding of a drug product shall have education, training, and experience, or any combination thereof, to enable that person to perform the assigned functions." (5) Training shall be in the particular operations that the employee performs and in current good manufacturing practice on an ongoing basis.

Another point about training that extends to each employee is contained in 21 CFR 211.28(a), stating that "Personnel engaged in the manufacture, processing, packing, or holding of a drug product shall wear clean clothing appropriate for the duties they perform. Protective apparel, such as head, face, hand, and arm coverings, shall be worn as necessary to protect drug products from contamination." (5) Additionally, 21 CFR 211.28(b) states that "Personnel shall practice good sanitation and health habits." (5) These types of regulations are particularly important for aseptic manufacturing to protect the product from contamination from the employees.

A well-designed, maintained, and operated aseptic process minimizes personnel intervention (e.g., isolator or barrier use). As operator activities increase in an aseptic processing operation, the risk to finished product sterility also increases. To ensure product sterility, it is critical for operators involved in aseptic activities to use aseptic technique at all times.

Appropriate training should be conducted before an individual is permitted to enter the aseptic manufacturing area. Fundamental training topics should include aseptic technique, clean room behavior, microbiology, hygiene, gowning, patient safety hazards posed by a nonsterile drug product, and the specific written procedures covering aseptic manufacturing area operations.

After initial training, personnel should participate regularly in an ongoing training program. Supervisory personnel should routinely evaluate each operator's conformance to written procedures during actual operations. Similarly, the quality control unit should provide regular oversight of adherence to established, written procedures and aseptic technique during

manufacturing operations. Some of the techniques aimed at maintaining appropriate levels of sterility assurance include the following:

- Contact sterile materials only with sterile instruments
- Move slowly and deliberately
- Keep the entire body out of the path of unidirectional airflow
- Approach a necessary manipulation in a manner that does not compromise sterility of the product
- Maintain proper gown control

Written procedures should adequately address circumstances under which personnel should be retrained, requalified, or reassigned to other areas. Training activities should be clearly documented in records for each employee.

Material Management

Material management needs attention with respect to aseptic processing. The main focus for material management needs to always insure that the integrity of the material delivered to the aseptic process has not been comprised. When materials are received they should be carefully inspected for the condition of the containers for damage and any possible breech of container integrity. The materials should be placed into a state of quarantine until released by quality according to specifications/procedures.

Samples for release testing need to be carefully removed under aseptic conditions to prevent any possible chance of contamination of the material during the sampling procedure. The sampling needs to be performed in an environment of the same or better classification to which the material will be charged into the process. The material needs to be delivered to the production areas in a controlled manner to prevent any possible chance of mix-up or contamination. Records need to show complete accountability, traceability, and handling of the material.

Calibrations

Calibrations should focus on monitoring devices for equipment and facilities. As previously discussed, the calibration devices should be routinely reviewed and the information recorded. Monitoring devices are integral for documenting the performance of the process in relation to sterility assurance.

Monitoring devices need to be calibrated to tolerances that allow for reliable accuracy over the monitoring range of measurement. For example, a thermocouple should not be calibrated with a tolerance of $\pm 2^{\circ}C$ if the accuracy of the measurement needs to be $\pm 0.1^{\circ}C$. Also, the calibration should span the range of measurement that the monitoring device will routinely record.

Records need to be kept for monitoring devices. The records need to clearly show calibration results as well as any adjustments and maintenance made to the device. Monitoring devices should be routinely verified before use in manufacturing. For example, a balance should be checked for accuracy by weighing a check weight and recording the results. If a device is found out of tolerance, corrective actions should be taken. Also, an assessment should be documented for what the impact of the out-of-tolerance device had on the facility and processes.

Validations

Other chapters have described key technical aspects about validations. From a quality perspective, validations should be done on facilities, utilities, and equipment. For aseptic manufacturing, validations need to clearly show that the item will routinely perform in a way needed to assure product integrity.

As mentioned previously, the facilities should be proven to provide an environment suitable for the specific type of manufacturing. Typical parameters for validation of facilities are temperature, relative humidity, pressure differentials, and particulate matter (viable and nonviable).

Equipment validation should thoroughly confirm that the performance is appropriate for the process/product. Standard equipment should follow the traditional validation plan of supplier information, installation qualification, operational qualification, and performance qualification. The qualification process should prove that the monitoring and control aspects of the equipment are suitable and in a state of control for the process.

Custom designed equipment should consider following a validation plan that ensures equipment is designed correctly for intended use. An approach for customized equipment is design qualification, factory acceptance testing, installation qualification, operational qualification, and performance qualification. Equipment should be routinely requalified on a routine basis defined by procedures or when significant changes are made.

Sterilizing equipment cycles should be validated to the specific load or cycle to support the process. Additionally, sterilizing cycles need to be routinely revalidated, or if a change occurs to the equipment or the utilities, revalidation should be considered.

Process

Process validation in aseptic manufacturing has two key aspects—can the process reliably manufacture product and maintain sterility. Validation should prove that following the parameters outlined in a control strategy, the process can manufacture product that has the safety, identity, strength, quality, and purity required. The reliability of the manufacturing process traditionally is shown from three validation lots.

To ensure the sterility of products sterilization, aseptic filling and closing operations must be adequately validated (5). The goal of even the most effective sterilization processes can be defeated if the sterilized elements of a product (the drug formulation, the container, and the closure) are brought together under conditions that contaminate any of those elements.

An aseptic processing operation should be validated using a microbiological growth medium in place of the product, media fill. Normally a media fill includes exposing microbiological growth medium to product contact surfaces of equipment, container closure systems, critical environments, and process manipulations to closely simulate the same exposure that the product itself will undergo during the process. The sealed containers filled with the medium are then incubated to detect microbial contamination. Results are then assessed for the potential of a unit of drug product to become contaminated during actual operations (e.g., start-up, sterile ingredient additions, aseptic connections, filling, and closing). Environmental monitoring data from the process simulation can also provide useful information for the processing line evaluation.

A media fill program should incorporate the contamination risk factors that occur on a production line and accurately assesses the state of process control. Media fill studies should closely simulate aseptic manufacturing operations incorporating, as appropriate, worst-case activities and conditions that provide a challenge to aseptic operations. Media fill programs should address applicable issues such as

- Run time
- Representative interventions, routine and nonroutine
- Lyophilization, when applicable
- Aseptic assembly of equipment
- Number of personnel and their activities
- Representative number of aseptic additions or transfers
- Shift changes, breaks, and gown changes (when applicable)
- Type of aseptic equipment disconnections/connections
- Aseptic sample collections
- Line speed and configuration
- Weight checks
- Typical environmental conditions
- Run size
- Container closure systems

A batch record should be followed for media fill studies. Additionally, documentation should be created that notes production conditions, operations, and simulated activities. A video recording can be very useful during media fills. The recording can be used as a record of the event and referred to during training exercises.

In general, a microbiological growth medium, such as soybean casein digest medium, should be used. Use of anaerobic growth media (e.g., fluid thioglycollate medium) should be

considered in special circumstances when a nitrogen environment is required for the process. The media selected should be demonstrated to promote growth of gram-positive and gram-negative bacteria, yeast, and mold. The QC laboratory should determine if indicator organisms sufficiently represent production-related isolates. Environmental monitoring and sterility test isolates can be substituted (as appropriate) or added to the growth promotion challenge.

The records from the media fill study should be carefully reviewed in the same way a production batch record would be reviewed. If any aberrant result is observed, an investigation should be initiated.

Batch Records

Batch records are the basic production record. Batch records should provide clear directions to execute the process as well as be the collection point for appropriate information throughout the process. The batch record should have adequate information and verification of collected information to reliably produce the desired product. During aseptic manufacturing, output from environment, facility, equipment, and personnel should be collected. This output should be assessed and compared to proven limits.

During the production run, if any value is collected and is outside of set ranges, this aberrant value should be investigated. The investigation needs to be referenced in the batch record. Any aspect of aseptic manufacturing should be investigated and assessed for impact to product before the lot disposition decision is made.

Following the execution of the batch record, typically the record is peer reviewed by a lead operator. Once the peer review is completed, the manufacturing authority needs to review the record for completeness and accuracy. Any questions or comments should be resolved by the operators. Following the manufacturing review, quality should review the record and verify that all collected data meets the control strategy requirements.

Investigations

Quality assurance needs to approach investigations from a science and risk-management prospective. Investigations tend to be a huge learning opportunity for most operations. The focus of an investigation should be on science and risk to generate an understanding of root cause and formulate a corrective and preventive action. Basically, when an aberrant result/trend is observed or a nonroutine event occurs, an investigation should take place to understand, learn, and make corrections.

In aseptic manufacturing, an investigation should occur when any aberrant result is obtained or unexpected event takes place, from physical assets and/or from process systems. The initial part of the investigation should assess what lots are impacted by the aberrant result and hold all lots in question until the investigation is fully understood and appropriate corrective actions are taken.

In general, investigations usually take the following steps:

- Discovery of an investigational situation
- Confirmation of the need for an investigation
- Notification of the investigation—hold product and operations
- Clearly record the cause/reason for the investigation
- Information collection
- Formation of hypothesis for why the aberrant result was obtained
- Conformational testing of hypothesis
- Validate hypothesis
- Assess impact to product
- Formulate corrective action
- Test corrective action
- Implement corrective action

The basic concept of investigation process is to follow the scientific model and learn more about the process/facility capabilities and to formulate a decision point on the initial aberrant result or unexpected event.

Quality Control Laboratories

Regulations generally state that the quality unit has the authority to approve or reject all components and materials used in processing and products produced. 21 CFR 211.22(a) states that (5)

> There shall be a quality control unit that shall have the responsibility and authority to approve or reject all components, drug product containers, closures, in-process materials, packaging material, labeling, and drug products, and the authority to review production records to assure that no errors have occurred or, if errors have occurred, that they have been fully investigated. The quality control unit shall be responsible for approving or rejecting drug products manufactured, processed, packed, or held under contract by another company.

The quality control laboratory needs to have the same level of control as the manufacturing operations. The laboratory should be able to perform testing and provide very accurate results. The laboratory systems should be able to collect, store, and handle samples without compromising the integrity of the sample or having any mix-ups. Production operational points should be applied to the operations of the quality control laboratory (Table 2).

As shown in Table 2, many of the operational concepts about manufacturing apply to the quality control laboratory. The focus may be slightly different in that operations focus is on product while the laboratory focus is on the test result. But the concepts are comparable and when working together allow for the production of a quality product.

Environmental Monitoring

In aseptic processing, one of the most important laboratory controls is the environmental monitoring program. This program provides key information on the state of control of the aseptic processing environment during operations as well as routine steady state. Environmental monitoring may be able to identify potential routes of contamination, allowing for implementation of corrections before product contamination occurs.

Evaluating the quality of air and surfaces in the clean room environment should start with a well-defined written program and scientifically sound methods. All environmental monitoring locations should be described in procedures with sufficient detail to allow for reproducible sampling of a given location surveyed. Procedures should also address elements like the following:

Table 2 Comparison of Operations with Quality Control Laboratory

	Operations	Quality control laboratory
Training	Employees need appropriate training and experience to perform assigned responsibilities	Same
Material management	Materials are handled to insure appropriate integrity is maintained and to prevent mix-ups	Samples need to ensure integrity, storage, and traceability in laboratory systems are maintained
Calibration/validation	Production equipment and monitoring devices need to show appropriate level of control	Laboratory equipment need to be treated in a way to insure reliability of results
Process	The operations to produce a sterile drug product	The activities to produce reliable test results
Records	Batch records provide directions and a collection point for all process information	Test records are kept to provide accuracy for testing
Investigations	Focus areas are performing the process and product	Focus areas are method performance and test result
Environment	Production environment needs to be clean, monitored, and kept in a way so as not to contaminate the product	The laboratory environment needs to have appropriate conditions to ensure samples can be handled without causing contamination
Equipment	Clean, maintained, calibrated/qualified	Same

- Frequency of sampling
- When the samples are taken (i.e., during or at the conclusion of operations)
- Duration of sampling
- Sample size (e.g., surface area, air volume)
- Specific sampling equipment and techniques
- Alert and action levels
- Appropriate response to deviations from alert or action levels.

The monitoring program should cover all production shifts and include air, floors, walls, and equipment surfaces, including the critical surfaces that come in contact with the product, container, and closures. Locations that present the most microbiological risk to the product need to be a key part of the program. Data needs to be collected to ensure that the microbiological quality of the critical areas shows whether or not aseptic conditions are maintained during filling and closing activities.

Environmental monitoring data needs to be analyzed looking for trends. From a practical point of view, if the data has all 0 values then a review of sampling and testing needs to occur. If the data shows more positive values in an area, a review of the cleaning procedures needs to occur. In a robust sampling and environmental monitoring program the data will show positives in a more random fashion. But, in the aseptic areas (ISO 5), the data should confirm the required conditions.

The collective output from the environmental monitoring program needs to be carefully evaluated on a routine frequency. Additionally, environmental monitoring data needs to be assessed during the routine manufacturing of one batch.

Cleaning Equipment/Facilities

Cleaning and sterilizing are important activities for aseptic manufacturing. Equipment cleaning procedures should be validated and routinely verified. Critical product contact surfaces need to be sterilized before using in the manufacturing process. Some keep points to consider from a quality perspective of an aseptic cleaning validation program are the following:

- Training of operators
- Sampling methods to account for process materials and microorganisms
- Sanitizing agent contact times need to qualify for effectiveness (e.g., do a small study on coupons of process surfaces spiked with known levels of microorganisms and hold agent for contact time to verify the absence of microorganisms)
- Equipment/material hold times before use
- Transfer and setup of equipment

Ideally, whenever possible in aseptic processing, disposable or single-use critical product contact items should be used. If disposable items cannot be used, then dedicated equipment should be used to protect the product from cross-contamination. If dedicated equipment cannot be used, then the importance of a rigorous cleaning validation and verification plan is extremely critical.

The equipment and facilities should be verified that they have been cleaned and are within the allowable hold times before use. This information should be recorded in the batch records.

Quality Information Management

The principle philosophy of quality information management begins early in product development. The combination of ICH Q8, Q9, and Q10 has provided a road map of key features of information management and how the organization should use that information (6–9). Early in product development, the design space for processing parameters needs to start being developed. As the procedure goes through the development process, refinements are made and knowledge is gained. This information needs to be captured and used to develop the design space and process control strategy.

For aseptic processing, key elements of the control strategy and process knowledge are the following:

- Process hold times
- Product contact surfaces
- Container closure assurance
- Confirmation of material handling
- Sterilization/sanitization procedures of equipment
- Equipment hold times
- Sterilization cycles
- Equipment normal operating parameters
- Confirmation of sterility assurance for the process
- Product interactions with filters and process surfaces

Once the control strategy is set, information should be collected for each batch. The information should be compared with the historical information collected. If any parameter is outside of the normal operating ranges for the process, an investigation should occur to understand why the aberrant result was obtained.

On a set frequency, the information collected according to the control strategy should be reviewed. This information needs to be evaluated for trends over a number of batches. Ranges should be assessed for applicability to quality aspects of the process. Related or repeated events should be assessed and corrective and preventive actions should be done to minimize reoccurrence.

Quality information management systems may include the following:

- Building Management System
- Laboratory Information Management Systems
- Document Information Management Systems
- Equipment Information Management Systems for calibration and validation
- Batch Records
- Deviations and Investigation
- Material Management Systems

A key aspect of the information management systems is change control. The systems need to evolve as process knowledge and systems gain more information. During the change control process, a key point is what impact will the change have to the process, as well as aseptic processing impact. Any impact to aseptic processing needs to be carefully assessed and tested to ensure the appropriate sterility assurance levels are maintained during the process.

The fundamental important point of quality information management is that information is to be collected and this information assessed for the state of control of the entire process. This is the fundamental philosophy behind quality assurance science. Each batch should be assessed against the entire information set collected, information from the physical assets and process systems outputs.

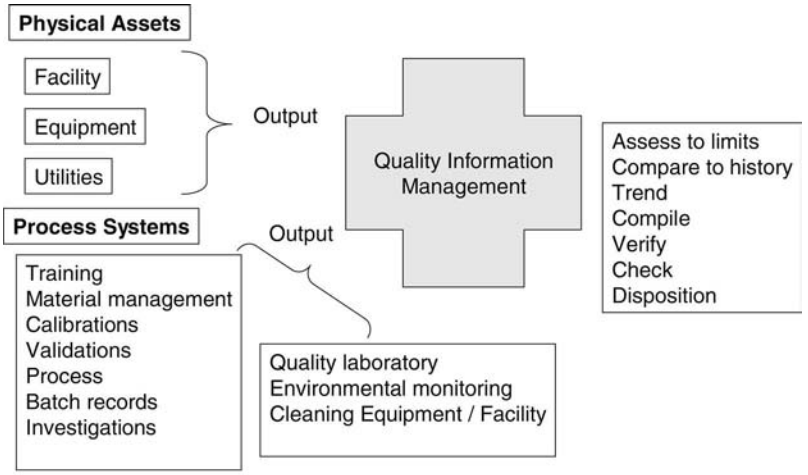
QUALITY ASSURANCE ROLE IN PRODUCT DEVELOPMENT

Quality assurance has an important role in product development. Quality needs to be able to make lot decisions on the basis of the information available about the process. In early development, with only having manufactured the development drug once or twice, a lot may not be known about the process. For aseptic processing, facility and process controls also apply and must be in place so that batch results and process observations represent the specific product/process for which little is known at the outset.

Guidance documents from health authorities have been developed helping refine approach to clinical manufacturing (Fig. 3) (10,11). Quality needs to draw upon all experience and provide input into development team about paths forward when issues occur during manufacturing. As the process is developed, quality can play a key role to the development

ICH Q8	ICH Q9	ICH Q10	Annex 13 (EU GMPs)	FDA Guidance
Pharmaceutical Development	**Quality Risk Management**	**Pharmaceutical Quality System**	**Manufacture of Investigational Medicinal Products**	**cGMP for Phase 1 Investigational Drugs**
• Parameters for Manufacturing Control (i.e., Design Space) • Enhanced knowledge of product performance	• Facilitates decision making • Proactive means to identify and control quality issues	• Facilitates continual improvement over product life cycle • Maintains a state of control	• Procedures need to be flexible as process is developed. • Protects trial subjects • Maintain batch consistency	• Facilitates the initiation of the human clinical studies • Protects trial subjects

Figure 3 Fit-for-purpose regulatory guidance documents.



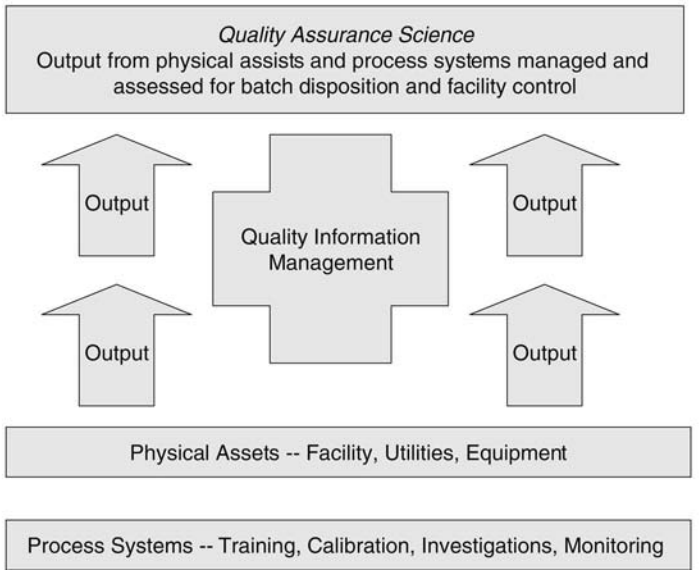


Figure 4 Information flow diagram of aseptic control strategy.

team by helping managing the information collected. This information can be used to help define the design space for the process. Once the process is close to becoming commercial, a control strategy should be prepared. The control strategy should define all monitoring and control parameters for the process.

An important aspect in product development when the product requires to be manufactured by aseptic techniques sterility assurance aspects should be the same across all phases of development. The difficulty of a development drug is the lack of process experience. If the development drug has only been made once or twice, the development team needs to use experience and education to make decisions.

CONCLUSIONS

Every person involved with parental drug manufacturing has the responsibility to assure the quality of the product produced. As stated previously, many of the technical aspects are described in detail in other chapters. The aim of this chapter was to illustrate quality aspects throughout the process. Additionally, within aseptic manufacturing, certain monitoring and information need to be collected on a routine basis to continually assess the state of control of the complete operation. The basis for assessing the state of control is to have rigorous and defined information flow processes (Fig. 4). Once the information is collected, quality assurance should have the ability to assess, evaluate, and make appropriate decisions to ensure the product has the required safety, identity, strength, quality, and purity.

Quality Assurance Science is the process of bringing all of the information together, evaluating the information, making decisions, refining systems, and applying process knowledge. This process begins in the early stages of drug development when not a lot of specific process information is known, but it is important to allow for development to progress, building the process knowledge. However, even in early development, process sterility assurance requirements should be largely the same at all stages of development and routine commercial manufacturing.

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13 Application of Quality by Design in CMC[a] development

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Abstract: This chapter will summarize conceptual development of the Quality-by-Design (QbD) approach from the platform principles outlined in the ICH, Q8R, Q9, and Q10 guidelines. The chapter will characterize contemporary definitions of the important elements of QbD and provide examples of the application of QbD in the technical development and management of pharmaceutical products throughout their life cycle in alignment with regulatory expectations. The application of QbD affords the opportunity to capitalize on experience and knowledge using a systematic scientific and risk-based approach to understand the *variability* of quality and material attributes and process parameters with the purpose of improving quality assurance in the safety and efficacy of the product for the patient.

INTRODUCTION

In August 2002, the FDA announced an initiative, *Pharmaceutical Current Good Manufacturing Practices (CGMPs) for the 21st Century* (1,2). The intent of this initiative was to modernize FDA's regulation of the quality of pharmaceutical products by implementing science-based policies and standards. Companies have also been encouraged to use risk-based assessments, in particular when identifying product quality attributes, and adopt integrated quality systems throughout the life cycle of a product. A number of guidance documents have been published related to this initiative (3–8).

The movement toward science-based regulations has not been limited to the United States. The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) issued two draft guidelines, further focusing on how to incorporate Quality by Design (QbD) into the preparation of Common Technical Documents (CTD). ICH Q8R addresses Section 3.2.P.2 Pharmaceutical Development (9) and ICH Q9 discusses the use of risk assessment (10). ICH Q10 was subsequently issued to address pharmaceutical quality systems (PQS) (11). Together, the development and adoption of these guidelines stimulated several companies to formally embrace the concepts and apply them to develop their products. While many elements associated with QbD, that is, risk assessments, design of experiments (DoE), operational control strategies, etc., have been employed well before the adoption of the ICH guidelines, application was frequently not systematic, concerted, or prospective, but rather retrospective in response to issues or problems encountered during development or after commercial launch. In addition, provisions in traditional regulatory guidelines did not offer regulatory incentive to pursue or provide additional scientific details describing the breadth of process understanding and product knowledge beyond empirical results from direct manufacturing experience. Consequently, companies were reluctant to pursue a QbD approach or introduce supplemental studies on process capability for fear of unnecessarily increasing regulatory "burden" and potentially delaying regulatory approvals.

In 2005, the FDA launched a pilot program (12) that encouraged pharmaceutical companies to submit science-based New Drug Applications that contained elements of FDA's vision for CGMPs for the 21st century that aligned with the recently issued ICH Q8R and Q9 guidelines. The pilot was largely successful in that it engaged regulators, inspectors, and industry scientists in a meaningful exchange of how to prosecute QbD concepts with real projects and products. In addition, the industry response to the pilot program transformed a largely theoretical opportunity into actual regulatory applications describing the use of concepts,

[a]Chemistry, Manufacturing, and Controls

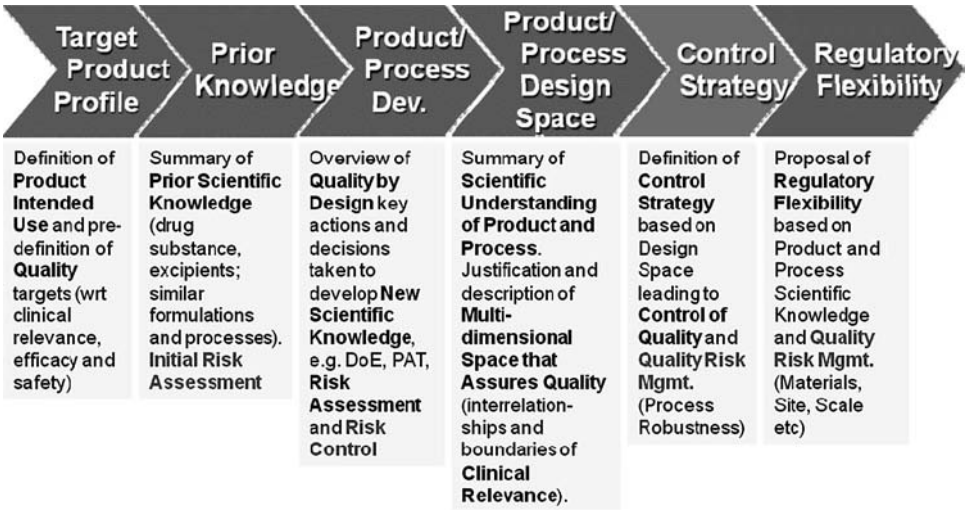


Figure 1 Outline of approach to application of Quality by Design.

which generated a variety of concrete approaches (13–23). The differences in those approaches highlighted the need for clarity and further conceptual refinements of the concepts and their application, and raised several questions:

- How to adequately and appropriately characterize commitments versus data in a regulatory application?
- How is prior knowledge substantiated?
- What level of detail is required to justify risk assessments?
- How should design space be presented and conveyed to demonstrate quality assurance?
- How can modeling be used to justify commercial manufacturing process changes?
- How should control strategy connect drug product quality attributes to process parameters and material attributes?
- Is there an attenuation of regulatory latitude for postapproval optimization and continual improvement?
- What roles do regulatory assessors and inspectors have respectively in assessing QbD relative to the pharmaceutical quality system?

Far from suppressing progress, these, among many other questions, stimulated regulatory authorities and industry to pursue clarification. A fair measure of subsequent progress has improved the consistent application and value of these concepts. The QbD approach, which is outlined in Figure 1 (24), is sequentially consistent with, and intrinsically similar to, a traditional development paradigm. However, risk- and science-based development of product and process design criteria are addressed in a systematic and prospective manner. The objective is to achieve product knowledge and process understanding.

Perhaps, most importantly, the application of a QbD approach and investment in robust pharmaceutical quality systems are expected to reduce *unexpected variability* in manufacturing processes and unanticipated failures in product quality, thereby improving quality assurance of products.

RELEVANCE OF QUALITY BY DESIGN

Value of QbD

During the last several years, the words "Quality by Design" have become synonymous with pharmaceutical process improvement. QbD has seemingly assumed pervasive and even mystical proportions in the media as it has evolved from a conceptual initiative to a "panacea"

for improving pharmaceutical development and manufacturing. However, while many large innovator pharmaceutical companies have embraced QbD principles and conceptual approaches to product development and process understanding, much of the rest of the pharmaceutical industry, including companies that manufacture generic medicines and excipient and raw material suppliers, have remained tentative (25,26). And so, comprehensive adoption of the concepts may still be an aspiration within the industry even where modest incremental efforts to introduce elements of the principles during development or retrospectively to improve manufacturing processes for approved commercial products have increased.

With respect to traditional pharmaceutical development paradigms, contemporary and compelling benefits to legitimately account for the immediate and short-term costs of investing in process understanding are, admittedly, conspicuous in their absence. The resistance to embrace the paradigm shift and adopt the QbD approach has been perceived as operationally cost prohibitive. To date, the return on investment for QbD is largely anecdotal, circumstantial, or academic (27). Accordingly, examples of meaningful return on investment for adopting and implementing QbD principles have been limited and modest, lending credence to the contention that there is no financial incentive to adopt QbD or, alternatively, QbD may be selectively useful only for certain products (28). Certainly, the paradigm shift toward introducing systematic risk assessments that leverage prior knowledge and stimulate studies to understand the dynamic nature of manufacturing processes requires change from a minimalist, empirical, and reactive orientation to a holistic, scientifically designed, and prospective approach.

The intrinsic advantages of investing in process understanding increases confidence and assurance of product quality. Tangible benefits, reductions in manufacturing costs associated with improved efficiencies and expeditious innovations, reduction in manufacturing recalls, failures or extraneous investigations attributed to uncertainty are largely realized over the long term as the life cycle of a product matures. Table 1 provides a list of expected benefits, most of which have been realized by application of QbD principles by companies who participated in the FDA pilot program (15,16,21–23). The proliferation of studies reflecting the application of QbD is a credible testament to its inherent value.

From a business perspective, the logic for adopting a QbD approach is not obvious, especially when current regulatory requirements neither prescribe QbD nor provide immediate incentive for investment. The relatively high probability of product attrition during early clinical development does not justify ancillary investments in establishing design space when adherence to current statutory expectations is satisfactory for achieving regulatory approval. After all, the adoption of QbD is optional (9). However, the principles promulgated in ICH Q8R, Q9, and Q10 are not new. Pharmaceutical companies have applied the elements of QbD during development and postregulatory approval to develop robust manufacturing processes and/or assess process consistency for several years before ICH Q8R was conceived. What is relatively novel is the systematic and mechanistic approach using prior knowledge to assess

Table 1 Anticipated Benefits of Quality by Design

Reduce	Improve
Uncertainty and risk	Process understanding
Recalls, technical anomalies, quality investigations, manufacturing failures	Innovation and process improvement
Manufacturing costs	Quality assurance
Need for repetitious process validation exercises	Regulatory flexibility
Quantity of postapproval regulatory submissions and/or regulatory expectations	Application of technology, e.g., PAT, modeling, scale-down models
Nonscientific regulatory and/or compliance exercises	Regulatory review criteria
	Scientific/technical literacy
	Capitalization experience
	Development efficiency
	Global harmonization

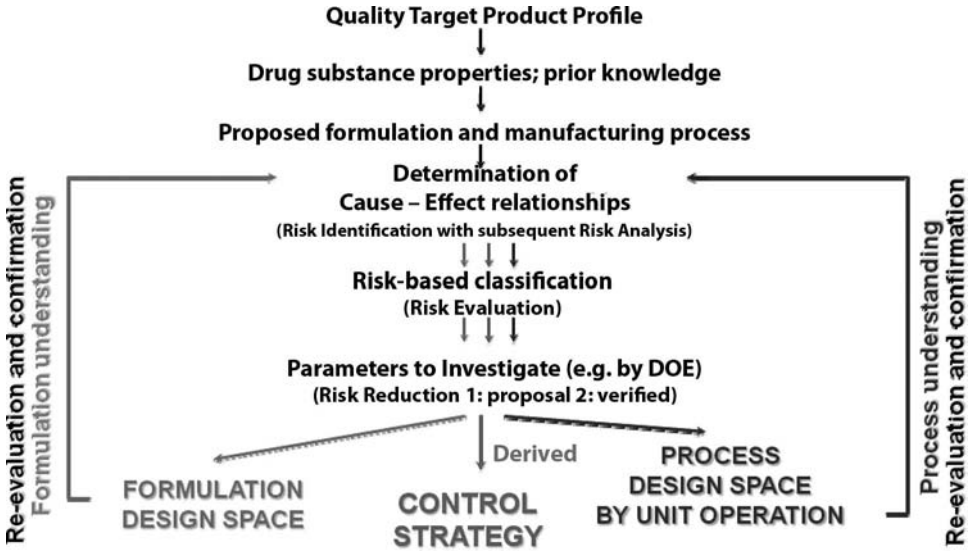


Figure 2 (*See color insert*) Process flow diagram describing the approach to developing process understanding and building quality into formulation and manufacturing process design.

risk, establishing process boundaries to understand and reduce variability and developing holistic control strategies in a prospective focus toward continual improvement.

WHAT DOES QUALITY BY DESIGN MEAN?

The paradigm shift or change in orientation toward adoption and application of QbD principles has been acknowledged by several companies who have incorporated the approach as an integral part of their business. For example, once clinical proof of concept has been demonstrated for a product, development project teams embark on systematic and scientific/risk-based design of the commercial product formulation and process, where elements of QbD are integral to development as illustrated in Figure 2 (29).

From company to company, the sequence may vary in detail and formality, but the elements of the approach are essentially the same. The therapeutic profile of a medicinal candidate and its preliminary quality criteria provide the definition of the product and its intended use. The properties of the drug substance are confirmed and analyzed and, in conjunction with "prior knowledge," form the basis for understanding material attributes that lead to formulation design. An assessment of the formulation in consideration of prior relevant knowledge and experience may reveal functional relationships between material properties and quality attributes that may warrant experiments to establish important properties and characteristics of the formulated product and their influence on quality. Likewise, design of the manufacturing process with subsequent assessment in conjunction with prior relevant knowledge and experience may reveal functional relationships between process parameters and quality attributes. These functional relationships may lead to experiments that establish design space, whose boundaries can contribute to the understanding and development of control strategy. The process is iterative and can provide a useful understanding of the product and manufacturing process from which subsequent improvements can be planned and executed.

PHARMACEUTICAL QUALITY SYSTEM

Investing in efforts to increase product knowledge and process understanding through continual improvement across the product life cycle is paramount to pharmaceutical innovation. Adopting a QbD approach serves the patients by improving confidence in the assurance and consistency of product quality; reorients regulatory scrutiny on scientifically

and technologically relevant commitments and data and balances regulatory expectations for appropriate, continual life cycle improvement; and benefits industry by promoting and progressing scientific and technological innovation. Of course, the application of QbD concepts is predicated on a robust pharmaceutical quality system as described in ICH Q10. In fact, the connectivity between scientific and technological development and routine manufacture is imperative.

The competent quality management system ensures "effective monitoring and control systems for process performance and product quality, thereby providing assurance of continued suitability and capability of processes" (11). This includes knowledge management and quality risk management "by providing the means for science and risk based decisions related to product quality" (11). In the absence of these systems, the application of QbD is effectively rendered impotent. For instance, an API (active pharmaceutical ingredient) manufacturer may have developed an acute understanding of how perturbations in the parameters of the process affect a critical quality attribute (CQA) such as genotoxic impurity levels. However, if the system monitoring levels of those impurities during the process is not robust, the control of those impurities may be suspect. In addition, any subsequent improvement or optimization of the process would be vulnerable to failure.

ICH Q10 reinforces adherence to regional GMP requirements, ISO standards, and the ICH Q7 guideline. In principle, the quality standards described in ICH Q10 are the foundation on which science- and risk-based QbD approaches are "enabled and qualified" (11). In particular, continual improvement relies entirely on robust technology transfer, change management, and knowledge management systems to ensure appropriate continuity. A design space defined by the combination of parameter boundaries that governs roller compaction, lyophilization, hydrogenation reactions, performance of a dry powder inhaler, or a packaging operation is only as good as the systems used to assure consistency of any of those processes. Where the pharmaceutical quality system aligns most directly with the application of QbD is in the establishment of the control strategy. A comprehensive control strategy is generally not confined to those measureable attributes in a specification, but can include the relevant systems governed by PQS. In fact, control strategy is defined in ICH Q10 (11):

> A planned set of controls, derived from current product and process understanding that assures process performance and product quality. The controls can include parameters and attributes related to the drug substance and drug product materials and components, facility and equipment operating conditions, in-process controls, finished product specifications, and the associated methods and frequency of monitoring and control.

The aspects of a PQS that should be addressed in a regulatory submission have not been definitively established. In general, and from experience to date, regulatory authorities expect to understand the criteria used to assess risk, both for parameters and attributes that are important or critical and worthy of study and/or control, as well as justification for noncritical attributes and parameters (13,14). In addition, an understanding of how the manufacturing process and design space are monitored and the approach to change management are useful for regulatory assessors to confirm that systematic and robust processes maintain control.

QUALITY TARGET PRODUCT PROFILE

The importance of understanding the therapeutic expectations for the product cannot be underestimated. The connection between the quality of the product and its impact on safety and efficacy for the patient is paramount. Certainly, physical and chemical properties of the product that influence quality attributes required to ensure safety and efficacy are important. However, other relationships between product quality and patient need should also be considered. For example, patient compliance, which is necessary to ensure efficacy, may be linked to a CQA like the integrity of the product package or ease of patient use. A portion of the intended patient population may have tolerability or allergic responses to specific components typically used in a formulation, that is, lactose intolerance, which may influence formulation design or warrant the use of less desirable substitutes.

Fundamentally, while product quality is inherently assured by how robust a company's pharmaceutical quality systems are, that is, preventing contamination, maintaining

manufacturing consistency, training of personnel, etc., science- and risk-based approaches to developing product knowledge and process understanding ultimately assure product quality is aligned with safety and efficacy for the patient. The quality target product profile (QTPP) is therefore a direct reflection of product attributes that warrant attention and may be critical to assuring appropriate product quality. As presented in ICH Q8R (9) "the quality target product profile forms the basis of design for the development of the product" and could include:

- Intended use in clinical setting, route of administration, dosage form, delivery systems
- Dosage strength(s)
- Container closure system
- Therapeutic moiety release or delivery and attributes affecting pharmacokinetic characteristics
- Drug product quality criteria (e.g., sterility, purity, stability and drug release) appropriate for the intended marketed product

The schematic in Figure 3 shows how the elements of the QTPP may be translated to the CQAs of the product. While not all potential attributes are critical for every product, a formal and concerted risk assessment can determine which are important to evaluate to demonstrate their relative influence on safety and efficacy for the patient. Ultimately, the QTPP may serve as the basis for deriving the product specification.

What is not present in Figure 3 schematic are the business attributes for which considerations must also be addressed in the QTPP. Decisions regarding commercialization of a product must be balanced with technical and regulatory expectations. The QTPP should address the following criteria as well:

- Patient population differentiation, that is, pediatric versus geriatric, which often determines preferential dosage forms.
- Ethnic and religious proclivities, that is, use of components derived from bovine or porcine sources.
- Specific market needs, for example, preferential formulations to accommodate local pharmacy practices, for example, sachets, crushed tablets, or for example, multiple packaging configurations/quantities to accommodate bulk distribution versus individual administration.

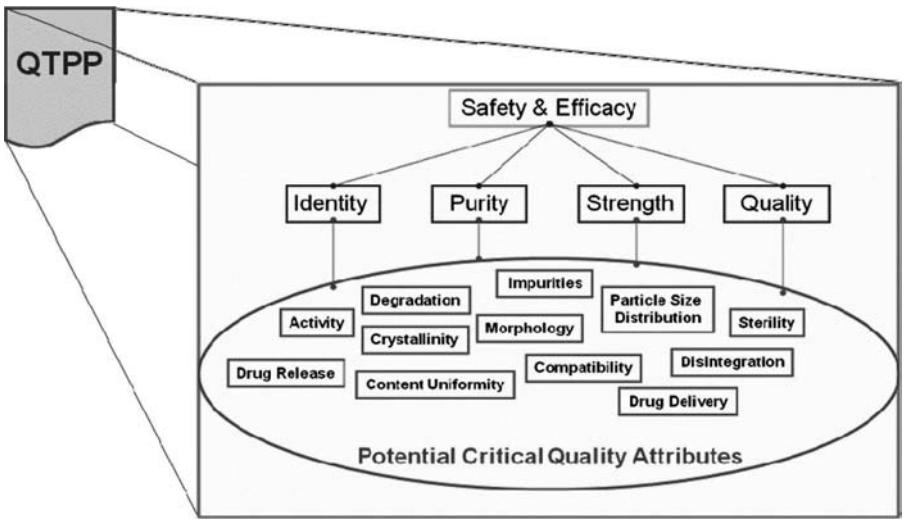


Figure 3 Derivation of critical quality attributes from the quality target product profile.

Finally, cost is, and should be, a consideration. While quality may be the primary driver for determining CQAs of the product, the cost of manufacturing will have an impact on the formulation options and manufacturing process selected to meet the commercial criteria in the QTPP.

QUALITY RISK MANAGEMENT

Quality risk management is seminal to QbD. While a robust pharmaceutical quality system serves as the foundation on which design space and control strategy are developed and managed, quality risk management provides the scaffold for creating meaningful design space and establishing an effective and robust control strategy. The inherent complexity of developing a drug product that behaves identically for each individual is a daunting challenge. As noted in ICH Q9, "the manufacture and use of the drug product necessarily entail some degree of risk" (10). Understanding risk and assessing which risks are important are at the core of QbD. In fact, it is the process of assessing, controlling, and reviewing risks throughout a product life cycle that instigates a systematic approach to developing process understanding and generates the development of design space and results in the establishment of a robust control strategy.

During the evolution of QbD, and particularly during the FDA pilot program and subsequent EMEA/EfPIA PAT Team sponsored workshops (30), the emphasis on quality risk management has engendered tremendous interest and engagement among industry and regulatory assessors and inspectors. Much of the focus has been on the quality of risk assessment justifications that lead to delineation of critical versus non-CQAs and process parameters. A robust quality risk management process typically requires the collaboration of a cross-functional team of experts from a variety of pharmaceutical science disciplines. Evaluating risk based on scientific knowledge that may reflect their collective prior experience or theoretical or conceptual analysis is extremely important to adequately address all of the potential sources of variability in a manufacturing process. Understanding what is known and recognizing and acknowledging uncertainty about what is not known is the beginning of the risk management process and can only be adequately addressed by adhering to the primary principles of quality risk management:

- The evaluation of the risk to quality should be based on scientific knowledge and ultimately link to the protection of the patient
- The level of effort, formality, and documentation of the quality risk management process should be commensurate with the level of risk (10)

Certainly an increase in the level of risk warrants concomitant and proportionate diligence in characterizing and evaluating and managing risk. In addition, transparency in describing and conveying the judgment basis of risk assessments, regardless of the level of risk, is useful for anticipating and potentially preventing failure.

Risk Assessments

Variables evaluated in a risk assessment should be judged relative to the following questions (10,31):

- What might go wrong?
- What is the likelihood it will go wrong?
- If it does go wrong, what is the impact?
- If it does go wrong, will the failure be detected?

Answers to these questions provide the relevant criteria by which risk is judged, namely the severity, uncertainty, and probability a risk may pose and whether or not a risk can be detected. Severity is defined as the measure of possible consequences of a hazard (10). Many companies in the industry employ a scale that differentiates catastrophic from negligible impact (32). Uncertainty is the unknown level of understanding for which the variability of a process parameter or quality attribute influences the severity and/or probability of risk to the

safety, efficacy, and quality of the product. In many instances, experiments or studies can reduce the level of uncertainty. Probability is the likely occurrence of impact on the safety, efficacy, and quality of a product. Probability is generally characterized by an estimate of the degree of variability of a parameter or attribute to impact quality and may consider the combination of operational controls in place that reduces the level of occurrence. Detectability is the ability to discover or determine the existence, presence, or fact of a hazard (10). The ability to detect variability of a parameter or attribute and the relative sensitivity to variability can provide appropriate mitigation for a risk. The combination of these criteria are used together to assess the risk parameters and attributes may pose on the quality of the product.

Perhaps most significantly, quality risk management provides the basis for creating design space and establishing a robust control strategy as illustrated in Figure 4.

The QTPP yields a number of CQAs that for all intents and purposes represent the drug product specification. Process parameters functionally related to CQAs are evaluated to determine their relative risk to those CQAs. Risk assessments are judgments. They may rely on a combination of prior knowledge, experience, and/or experimentation. Figure 5 is illustrative

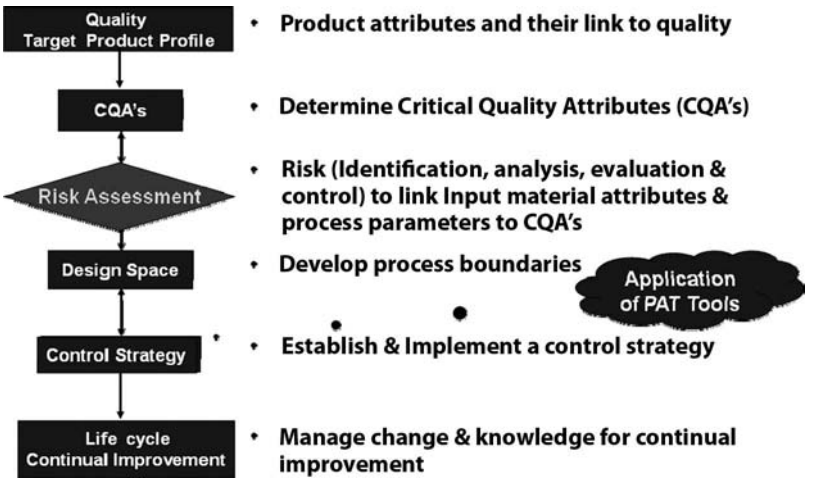


Figure 4 Sequence of elements of Quality by Design. *Source*: From Ref. 15.

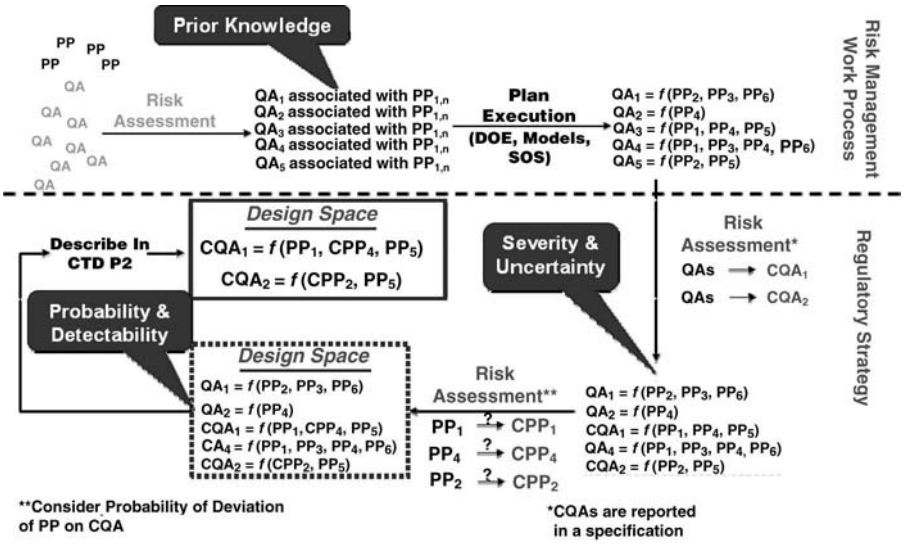
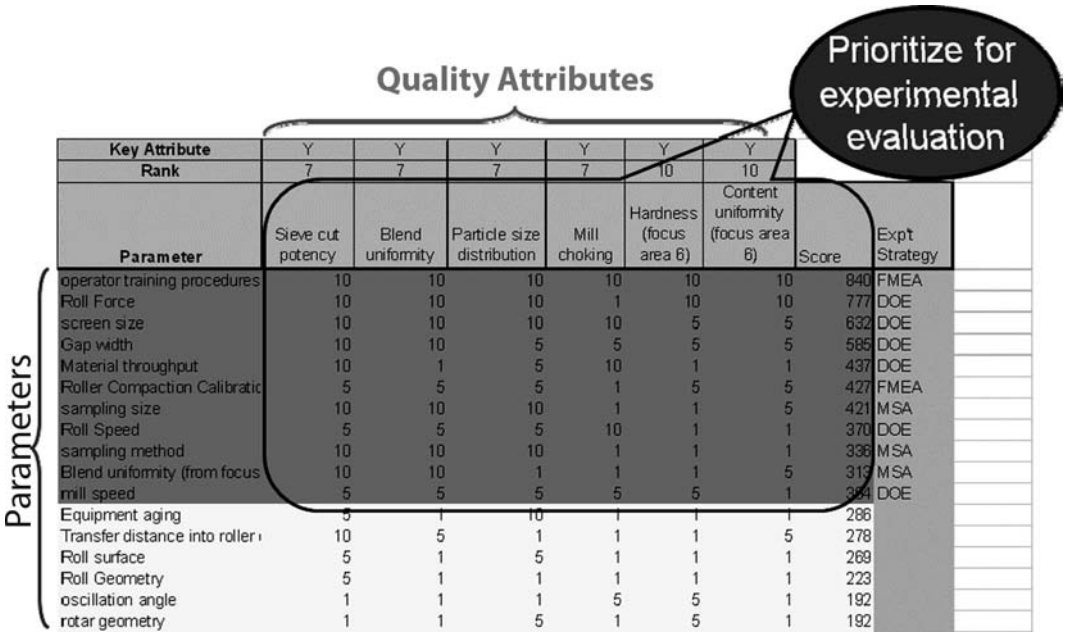


Figure 5 (*See color insert*) Schematic example of the quality risk management process. *Abbreviations*: PP, process parameters; CQA, critical quality attribute; CTD, Common Technical Document. *Source*: From Refs. 22,23,26.



Key Attribute	Y	Y	Y	Y	Y	Y		
Rank	7	7	7	7	10	10		
Parameter	Sieve cut potency	Blend uniformity	Particle size distribution	Mill choking	Hardness (focus area 6)	Content uniformity (focus area 6)	Score	Expt Strategy
operator training procedures	10	10	10	10	10	10	840	FMEA
Roll Force	10	10	10	1	10	10	777	DOE
screen size	10	10	10	10	5	5	632	DOE
Gap width	10	10	5	5	5	5	585	DOE
Material throughput	10	1	5	10	1	1	437	DOE
Roller Compaction Calibratic	5	5	5	1	5	5	427	FMEA
sampling size	10	10	10	1	1	5	421	MSA
Roll Speed	5	5	5	10	1	1	370	DOE
sampling method	10	10	10	1	1	1	336	MSA
Blend uniformity (from focus	10	10	1	1	1	5	313	MSA
mill speed	5	5	5	5	5	1	304	DOE
Equipment aging	5	1	10	1	1	1	286	
Transfer distance into roller	10	5	1	1	1	5	278	
Roll surface	5	1	5	1	1	1	269	
Roll Geometry	5	1	1	1	1	1	223	
oscillation angle	1	1	1	5	5	1	192	
rotar geometry	1	1	5	1	5	1	192	

Figure 6 (*See color insert*) Cause-and-effect matrix for distinguishing important quality attributes and process parameters for subsequent evaluation.

of the approach that may be used to discriminate what is known from what is unknown about a product and the process to manufacture it.

A vast variable space exists—often referred to as "knowledge space" —that contains inputs and outputs that can be described and labeled as process parameters (PP) and quality attributes (QA), respectively. Through a risk assessment, what is not known is identified from what is known or judged to be understood. In this step of the process, the question of what can go wrong is addressed and a list of potential hazards is catalogued.

Risk Identification and Analysis

An estimation of the risk may be qualitative or quantitative and may be the result of ranking risk in a cause-and-effect matrix associating process parameters with their potential impact on quality attributes as shown in Figure 6.

The analysis of functional relationships can distinguish the level of risk and serves to prioritize relevant studies or experiments required to evaluate the risk. Another way to identify and analyze risks and organize them in an orderly fashion is to use an Ishikawa diagram. An example of a template for an Ishikawa diagram organizes potential causes into four categories as shown in Figure 7. Frequently, Ishikawa diagrams are used to identify potential causes of a specific problem. If the problem is *why isn't the telephone being answered on time*, the potential causes can be traced to specific sources. In much the same way, the risk of having production defects in a tablet can be traced to the potential sources of variability that create that risk. The Ishikawa diagram provides an alternative tool for identifying risks.

Other options for identifying and analyzing risk include, but are not limited to, the following tools:

- Quality function deployment—a qualitative and structured analysis that translates "customer" requirements into technical options.
- Influence matrix—quantitative measure of the effect a specific parameter has on a measurable product characteristic or attribute.

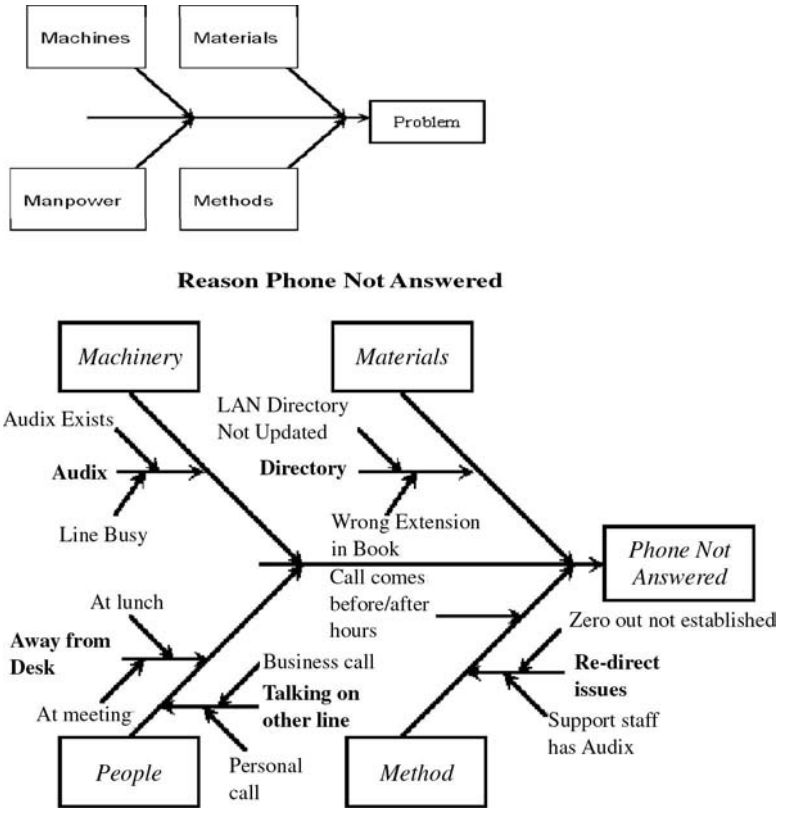


Figure 7 Example of Ishikawa diagrams. *Source*: From Ref. 33.

- Failure mode effects analysis (FMEA)—structured approach to identify, estimate, prioritize, and evaluate risk that aims at failure prevention and is primarily used to limit risk in change management.

Each of these tools alone or in combination with one another can provide a preliminary and systematic assessment of risk. However, it is subsequent evaluation of risk, where scientific experiments, models, and simulations can increase understanding of the risk and lead to design space to describe the area within which risk can be controlled. Subsequent assessments may distinguish acceptable risks from risks that require controls and/or methods for measuring control. The threshold of acceptable risk may ultimately be described in a design space and is fundamentally based on an evaluation of severity of impact, relative uncertainty, probability of occurrence, and an ability to detect variability.

Risk Evaluation

Identifying the sources of variability among process parameters that may pose risk to quality attributes allows for an analysis of the impact and probability of that risk causing harm. The importance or magnitude a risk poses often leads to the development of an experimental strategy to evaluate the level of risk. The functional relationships between process parameters and quality attributes within the focus areas of a manufacturing process provide the opportunity to evaluate the risk quantitatively and characterize boundaries of that risk through experimentation. Figure 8 provides an example of how a DoE may be derived from a set of focus areas containing several unit operations.

Design of Experiment (33–39) is a structured and statistical approach to evaluating the interactions of process parameters and their impact on quality attributes. Multiple parameters are studied simultaneously that allows estimation of interactions between factors. The designs

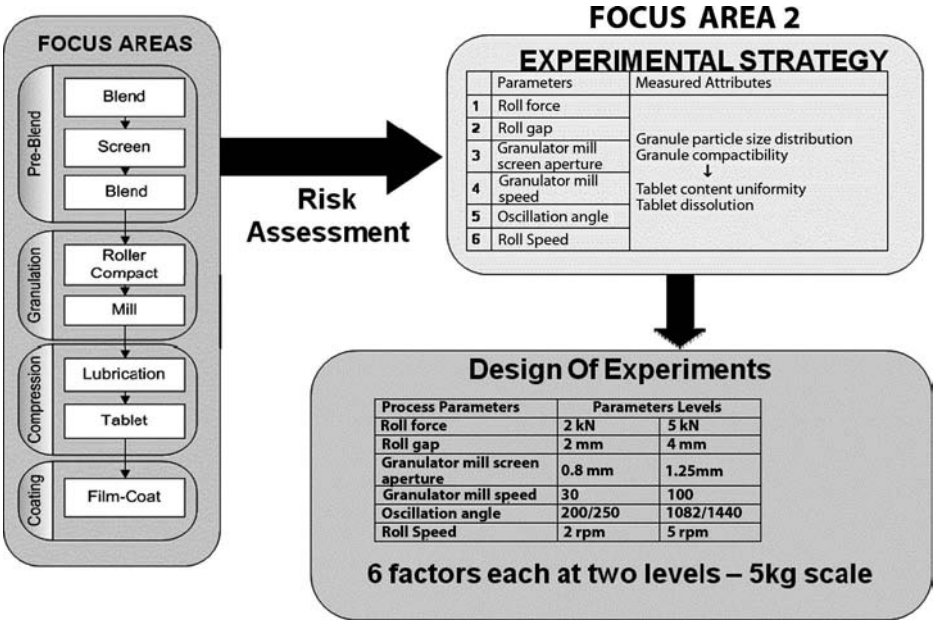


Figure 8 Translation of focus area variables into experimental strategy and plan.

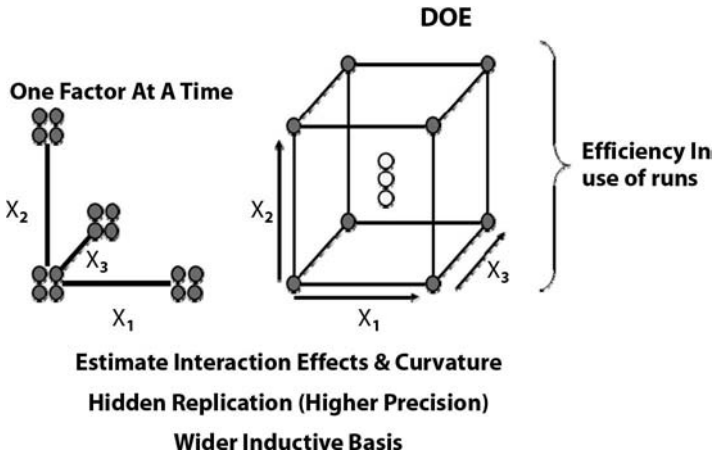


Figure 9 Generic example of design of experiment scheme.

can be structured to specific objectives, that is, factor screening or response surface exploration as well as identifying resource constraints. The use of DoE offers efficiency for estimating parameter effects and control over precision of response prediction in the case of response surface designs. They are comprehensive in nature, eliminate subjective assessments, and provide data with a wide inductive basis. Model building can be used to condense the raw data into systems of equations that describe relationships and thereby facilitate interpretation. Sequential experimentation provides for incremental understanding of the relationship between parameters and quality attributes by converging to conditions that produce the desired product.

Consider the case of three parameters evaluated over two levels. In Figure 9, the left-hand scheme represents the one-factor-at-a-time approach (OFAT). Four separate trials are run at 4 different points—16 total runs. In each instance, one factor is varied while the other two remain at the baseline level. This design allows estimation of each parameter effect (main effect) given that the other two parameters are at their baseline level.

The design scheme on the right requires only 11 runs and estimates the parameter effects with the same precision. In addition, interaction effects can be estimated. The replicated center points allow an estimate system curvature and pure experimental error. If any parameter does not alter the response, the design projects into a replicated design in the other parameters. Estimation of all effects, including interactions, provides a wider inductive basis for the experiment.

The statistical approach to DoE is useful in quantitatively characterizing the level of risk that any given parameter or attribute may pose in a multivariate expression. In addition, there are a variety of statistical designs, such as factorial, resolution of factorial, irregular fraction, D-optimal, Plackett–Burman, central composite, and mixture designs, etc., that may be employed to investigate specific effects or the extent to which a given parameter or attribute will impact quality attributes of a product. The variety of statistical approaches generate data that can be used to optimize the understanding of the boundaries of parameters and attributes in a design space and thereby improve the understanding their relative risk may have on the quality of the product.

Scientific- and risk-based assessments meet several fundamental objectives of QbD:

- Risk assessments are useful for characterizing and *ranking* attributes process parameters semiquantitatively relative to their impact on safety and efficacy.
- The risk assessment approach may be applied to drug substance synthesis and drug product manufacture.
- Use of formal risk assessment criteria to identify and differentiate critical from noncritical sources of variability and determine which variables are important to study and control.
- Design and performance of multivariate experiments to understand the interaction of variables with one another and their relative impact on quality attributes that affect patient safety and efficacy.
- Development of a well-characterized design space "or multidimensional combination and interaction of variables that demonstrates assurance of quality."
- Establishment of a coherent and concerted control strategy that may include the adoption of innovative technology, that is, PAT, to monitor or measure process variables directly.
- Sequential and iterative risk assessments → experimental plan → design space → control strategy.

The risk assessment process is iterative. As the life cycle of a product evolves from pharmaceutical development through technology transfer, during commercial manufacture and with the introduction of product enhancements and alternative formulations, the functional relationships between parameters and attributes and quality attributes of the product may change. Reassessing functional relationships, adjusting design space boundaries to accommodate changes in the manufacturing process, and establishing new design space increase process understanding and product knowledge and provide improved quality assurance of the product.

Risk Control

Decisions on what level of risk is acceptable have frequently centered on which parameters and attributes are "critical." Designating a level of criticality for attributes and parameters, that is, continuum of criticality, can be useful in delineating risk acceptance from risk reduction. Certainly, unacceptable risk requires mitigation or avoidance. However, there are risks where the severity of impact may be high, such as safety and efficacy to the patient, but the uncertainty and probability very low, for example, process parameters and attributes demonstrate that the risk is mitigated because the functional relationships are well understood and controlled within specified boundaries. For example, genotoxic impurities can be purged and controlled in the third step of a six-step synthesis of a drug substance. Stoichiometry, temperature, and pH of the reaction have been demonstrated to impact control of genotoxic

impurities. Therefore, control of genotoxic impurities in the drug substance (a CQA of the product) is functionally related to the combination of these parameters.

$$\mathrm{CQA} = f(\mathrm{PP}_{\mathrm{stoichiometry}}, \mathrm{PP}_{\mathrm{temperature}}, \mathrm{PP}_{\mathrm{pH}})$$

The design space described by the multivariate interactions of these parameters defines the boundaries within which control for this CQA is demonstrated. Subsequent demonstration that the process consistently operates within the design space reduces the risk.

Not all risk can be eliminated. In many instances, an appropriate risk management strategy will reduce the risk to an acceptable level where severity and probability may be mitigated by adherence to parameter and attribute boundaries, that is, control may be demonstrated by direct or indirect measurements of specific quality attributes. The acceptability of risk is often a decision that balances the presumed impact of the risk relative to appropriate controls to mitigate that impact. For example, the presence of genotoxic impurities produced during manufacture of a drug substance at residual levels that exceed the Threshold of Toxicological Concern (TTC) poses a safety risk to the patient. However, if the drug itself is mutagenic and is indicated for first-line therapy for breast cancer, the presence of these impurities should be balanced with the benefit of the drug and its duration of use. If reduction or elimination of genotoxic impurities is cost prohibitive or results in other quality issues, then acceptance of limits for these impurities that exceed the standard regulatory expectation may be justified.

Risk Communication and Risk Review

The other elements of quality risk management that support the scientific approach to decision-making are communication and review. Risks should be characterized by their respective and relevant relationship to quality attributes and process parameters and documented in a logical manner that shows the relationships between product quality and the attributes and parameters that influence quality. A general summary of the risk assessment approach and justifications for decisions regarding the attributes and parameters that warrant concern is helpful to regulatory authorities and should be transparent and reproducible.

In a regulatory submission, a description of the process used to evaluate and characterize risks should be provided. Regulators are keen to understand how a company distinguishes which attributes and parameters to study from those parameters and attributes that are noncritical (13,14,18–20). Summary examples of the evaluation tools and their respective results, for example, cause-and-effect matrix (Fig. 6), are useful ways to convey the outcomes from these risk assessments. In addition, descriptions of functional relationships between CQAs and the attributes and process parameters that may influence those CQAs provide context for describing the multivariate results from experiments and the design space created from those variable interactions.

DESIGN SPACE FOR DRUG SUBSTANCE

Design space can, and often is, the outcome of a robust quality risk management process. Table 2 and Figure 10 are a tabular summary and schematic representation of the outcomes from a comprehensive quality risk management process (40). Table 2 provides a summary and culmination of the results from risk assessment and evaluation including experimental results to establish design space for an example of drug substance manufacturing. Figure 10 is an example of the depiction of the design space created from these results. The drug substance manufacturing process can be separated into focus areas, that is, steps 1 and 2, steps 3 and 4, steps 5 and 6, etc., as shown in Figure 10, which may include one or more steps in the chemical synthesis. Once potential CQAs are defined, experiments can be performed to determine those process parameters that impact them, and subsequently identify acceptable operating ranges for which acceptable product is made. In some instances, edges of failure are identified for process parameters that lead to the production of an intermediate or drug substance that is not in compliance with the acceptance criteria for a CQA. However, most often edges of failure are not identified for the operating ranges investigated. The investigated range may be very large for the parameter being controlled, thereby providing more than enough "space" within which the

Table 2 Tabular Summary of Design Space Criteria

Quality attribute	Type	Process parameter or attributes impacting critical quality attribute	Type	Normal operating range	Design space range	Control strategy
Drug substance particle size	CQA	Temperature of API addition to acid	KPP	<60°C	Up to 60°C	Batch record, API specification
		Milling of API	KPP	Screen: 12–20 mesh Impeller speed: 1500 ± 100 RPM	Screen: 12–20 mesh Impeller speed: 1000–2000 RPM	
Drug substance purity	CQA					Intermediate and API specifications
Quality of intermediate "B"	CQA	Catalyst filtration	KPP	<20 ppm	<40 ppm	Batch record, intermediate specifications
		Quantity of reagant D	KPP	1.4–1.6 Meq	1.0–1.9 Meq	
		Reagant "D addition rate	KPP	60–90 min	45–120 min	
		pH of crystallization	KPP	5.0–6.0	3.0–8.0	
Quality of intermediate "A"	CQA	Quantity of acid #1	KPP	2–3 Meq	1–4 Meq	Bach record, intermediate specifications
		Quantity of acid #2	KPP	2–3 Meq	1–4 Meq	

Abbreviations: API, active pharmaceutical ingredient; CQA, critical quality attribute; KPP, key process parameter.

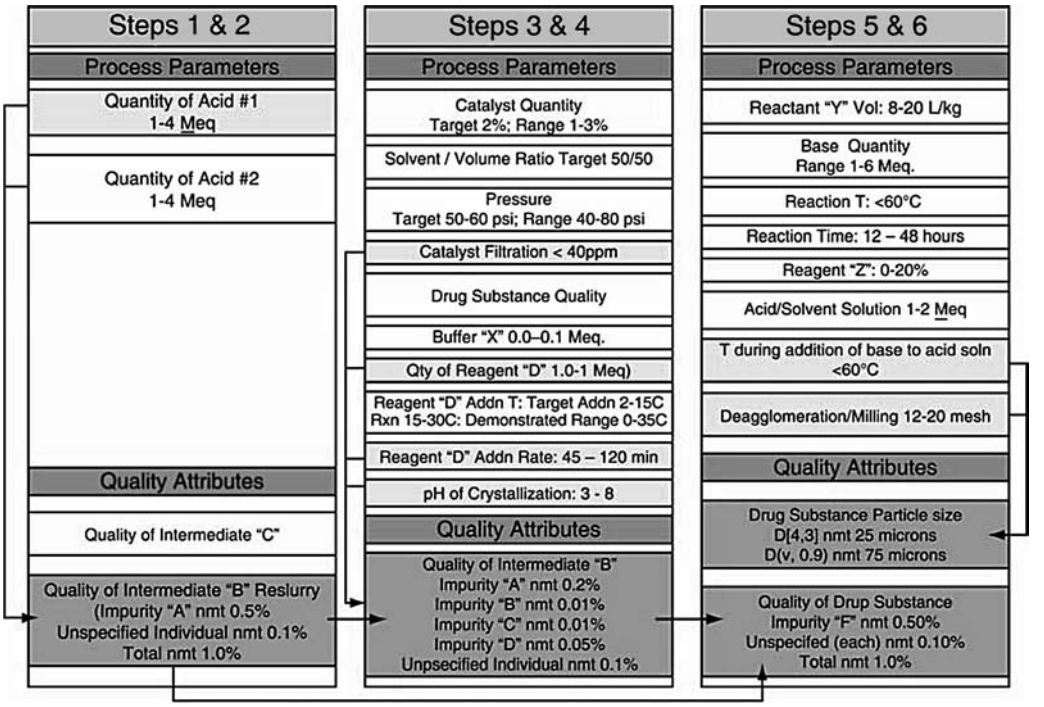


Figure 10 (*See color insert*) Schematic description of design space criteria.

process can effectively operate. In these cases, unless changes to the manufacturing process are anticipated, there is little business incentive to expand the investigated design space region any further. In other instances, attempting to expand the operating boundary ranges may exceed the capability of the equipment.

The pictorial representation of design space (Fig. 10) contains a series of columns, one for each focus area investigated. Each column is built from information contained in the summary table that summarized the knowledge obtained for each individual focus area. Cells that are shaded in pink highlight CQAs or process parameters, while those shaded in yellow contain key process parameters, those parameters that may influence CQAs, where risk assessment suggests probability and detectability warrant monitoring or further evaluation. Unshaded cells denote non-CQAs or process parameters. The focus areas are tied together by functional relationships that link the quality attributes and process parameters both within and across columns to other factors that they impact.

Biologicals

QbD principles are applicable to both small molecule drugs and large molecular biologics. However, the challenges of executing risk assessments are greater for a biological because the large size molecule is vastly more complex and the impact of attributes and process parameters on product quality attributes is generally more uncertain than for small molecules. In addition, the complicated nature of a biologics molecular generation from living organisms can lead to significant product heterogeneity. The inherent complexity of biological molecules can render the link between product attributes and clinical performance highly equivocal. The inability to associate quality attributes to safety and efficacy increases the level of uncertainty in assessing risk. Furthermore, the inherent difficulty to precisely characterize many biological molecules reduces the opportunities to develop concrete process understanding. However, examples and case studies describing the application of QbD principles and, in particular, quality risk management approaches have demonstrated limited success (41–45). In a similar fashion to Center for Drug Evaluation and Research (CDER), FDA's Center for Biologics Evaluation and Research (CBER) has initiated a pilot program for biological molecules that have been developed using QbD principles.

DESIGN SPACE FOR DRUG PRODUCT

Table 3 and Figure 11 illustrate the outcomes from the execution of a quality risk management process for a drug product (40). Focus areas that include one or more unit operations can be used to separate the drug product manufacturing process into manageable pieces. Statistically designed experiments and modeling can be used to identify CQAs and the process parameters that impact them. The use of univariate approaches to define the process may provide useful knowledge, but does not constitute a design space. Multivariate approaches (such as the use of factorial or central composite experimental designs or latent variable modeling) are encouraged as they detect interactions between multiple variables, which would otherwise likely go undetected. Following the completion of the development work for a given focus area, a table is prepared that summarizes the parameters investigated and the quality attributes that they impact. The table should also include columns describing the design space, the category that the quality attribute or process parameter falls into, and the control mechanism. Table 3 contains an example of a summary table of "knowledge space" i.e., all the formulation and process knowledge generated during product development for a dry granulation operation.

Table 3 Summary of Knowledge Space for a Dry Granulation Focus Area

	Boundary results			
Process parameters	Roller compactor "A"	Roller compactor "B"	Control	CPP/KPP
Roll force	X–Y kN	Y–Z kN	Batch record	KPP
Roll speed	X–Y RPM	Y–Z RPM	Batch record	No
Roll type	Pocketed Knurled	Serrated	Batch record	No
Gap width	X–Y	Y–Z mm	Batch record	KPP
Granulator screen size	X–Y mm	Y–Z mm	Batch record	KPP
Granulator speed	X RPM	Y–Z RPM	Batch record	No

Abbreviation: KPP, key process parameter.

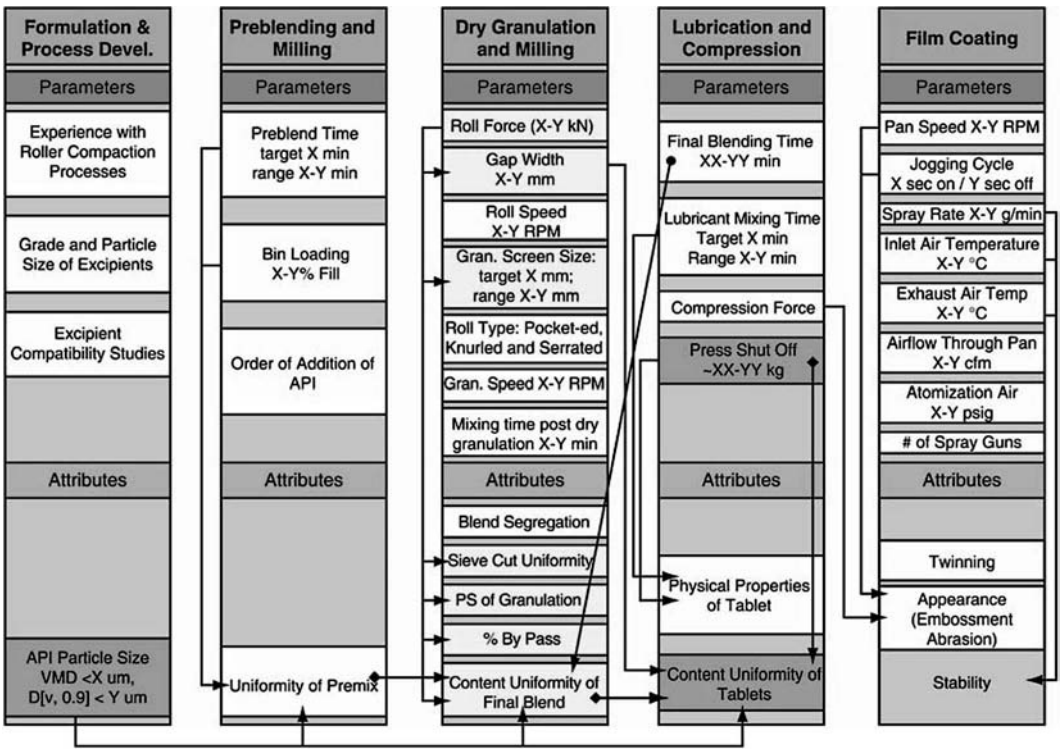


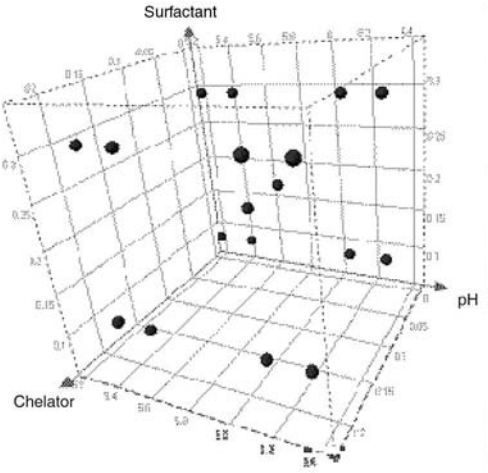
Figure 11 (*See color insert*) Design space for a drug product manufacturing process.

Figure 11 provides an example of the design space for the drug product. Much like the drug substance design space diagram, cells that are shaded in pink highlight CQAs or process parameters, while those shaded in yellow contain key process parameters, those parameters that may influence CQAs, where risk assessment suggests probability and detectability warrant monitoring or further evaluation. Unshaded cells denote non-CQAs or process parameters. Furthermore, links between drug substance and drug product attributes demonstrate the potential impact that drug substance quality attributes have on drug product quality attributes. For example, particle size distribution may be a CQA of the drug substance because it can influence drug product manufacturability. Drug substance particle size distribution translates to an important process parameter, or input, to the drug product CQA for content uniformity of the granulation blend or dosage form. Arrows show how the parameters and attributes are linked to each other, prospectively (feed-forward) and retrospectively (feed-backward), in the manufacturing process.

In addition to highlighting the parameters and attributes that define the design space (yellow and pink boxes), Figures 10 and 11 provide useful context to compare the relevant design space to noncritical parameters and attributes (white boxes) that constitute the knowledge space. Of course, these types of representations also reflect the outcomes from risk-based evaluation of the manufacturing process relative to CQAs of the drug product.

For a biological drug product, the formation of oxidative species and aggregation monomers can be reduced by introducing specific components in the formulation design to retard degradation. The effect of certain excipients, that is, surfactant and chelators, and pH on these CQAs can be built into the design of the formulation. On the basis of a risk assessment focused on limiting oxidation, a multifactorial DOE can be developed as described in the example presented in Figure 12.

On the basis of results from the DOE, a design space was created that demonstrates the optimal concentration of formulation process parameters, that is, excipient, chelators, and pH that will reduce generation of aggregate monomer and oxidative species and produce a stable product. Figure 13 illustrates the simple design space boundaries for this example. These simple diagrams describe (*i*) control of aggregation/monomer generation and (*ii*) control of oxidative species based on results from the multifactorial design described in Figure 12.



6	5.3	0.3	0.02	84	20
7	5.3	0.3	0.2	84	20
8	5.3	0.1	0.2	84	20
11	5.3	0.1	0.02	84	20
1	5.5	0.3	0.2	84	20
15	5.5	0.3	0.02	84	20
17	5.5	0.1	0.2	84	20
18	5.5	0.1	0.02	84	20
19	5.8	0.2	0.05	84	20
12	5.8	0.2	0.11	84	20
13	5.8	0.2	0.11	84	20
2	6.1	0.3	0.2	84	20
5	6.1	0.1	0.02	84	20
9	6.1	0.3	0.02	84	20
16	6.1	0.1	0.2	84	20
3	6.3	0.3	0.02	84	20
4	6.3	0.3	0.2	84	20
10	6.3	0.1	0.02	84	20
14	6.3	0.1	0.2	84	20

Figure 12 (*See color insert*) Example of multifactorial design to determine optimum concentrations of formulation parameters for a biologic.

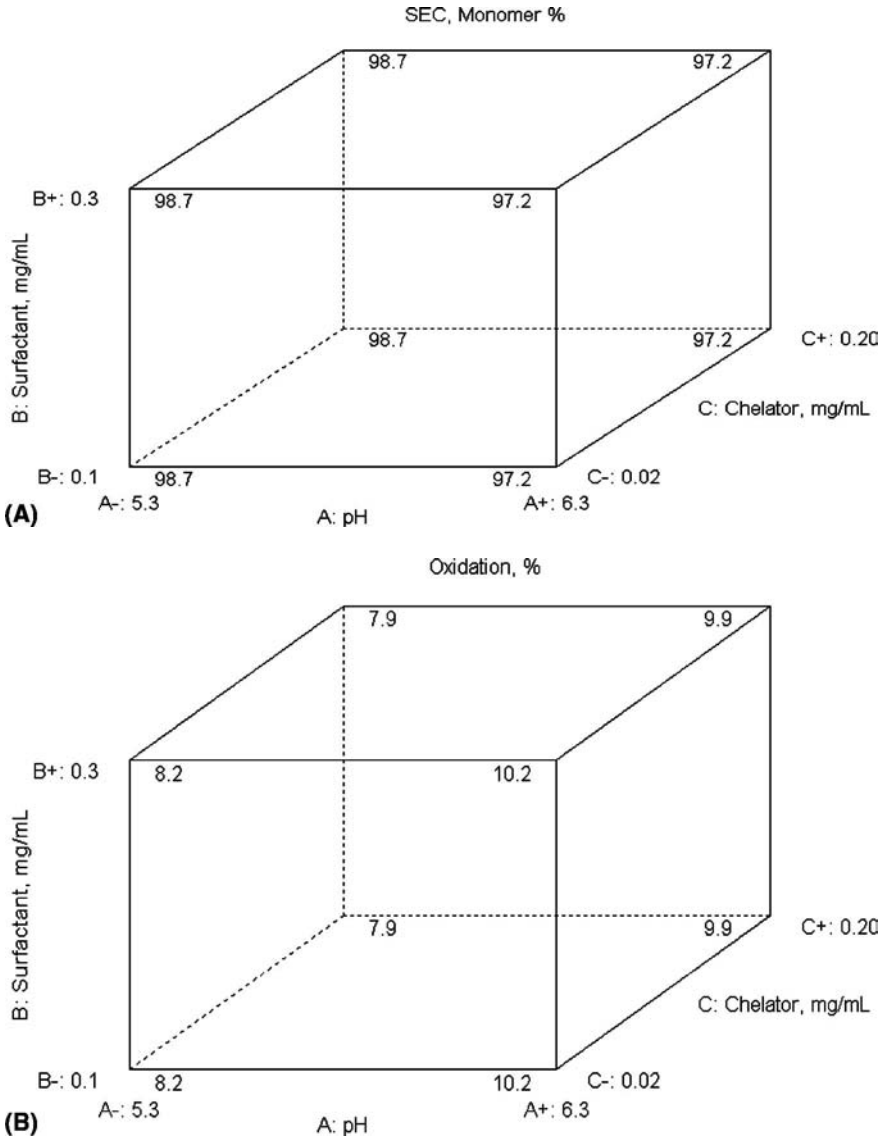


Figure 13 Design spaces for surfactant and chelator levels relative to pH that produce a stable product with respect to (**A**) aggregation/monomer content and (**B**) oxidation.

Finally, there are several ways to convey data from experiments where the multivariate interactions of attributes and parameters have been evaluated. Overlay or contour plots are frequently used to present design space and are useful for "seeing" where multivariate design space resides in contrast to areas where the risk of failure increases. Figure 14 is an example of a contour plot of design space for drug substance crystallization yield.

The area in the center of the plot (blue) defines the space within which the desired crystallized form of the drug substance is produced as a function of total solution volume, percentage of ethanol and yield. The area toward the edges reflects space that may lead to product with undesirable physical form. This contour plot shows the balance of process parameter boundaries required to deliver desirable physical form of the drug product.

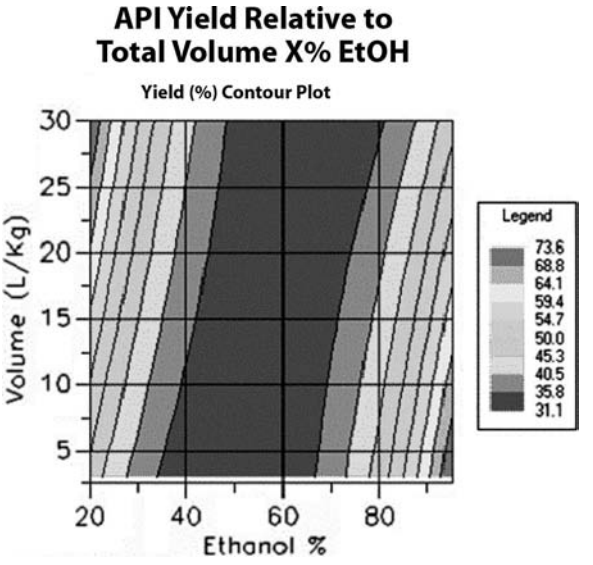


Figure 14 (*See color insert*) Example of a contour plot of design space for drug substance crystallization yield.

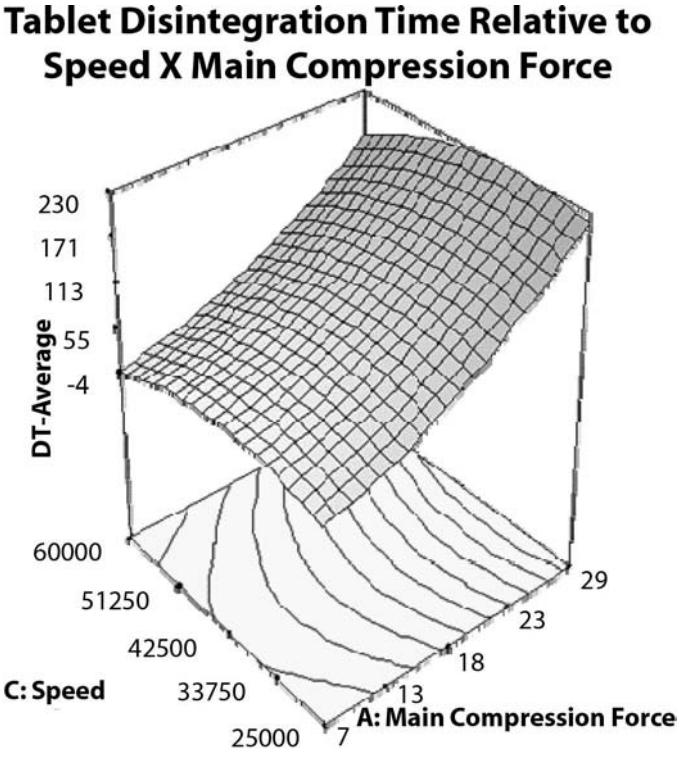


Figure 15 (*See color insert*) Contour plot describing design space for tablet disintegration time.

Similarly, a contour plot (Figure 15) describing the design space for tablet disintegration time relative to speed and compression force of the tablet press provides a profile for disintegration time that may influence tablet dissolution. The expression of data from experiments to describe a drug product attribute as a function of process parameters provides a reflection of how multiple variables interact to influence a drug product attribute.

CONTROL STRATEGY

The control strategy for a product is a comprehensive set of *planned* controls that reflect existing product knowledge and process understanding. From a holistic perspective, a control strategy includes reference to and demonstration of a robust pharmaceutical quality system and consists of appropriate qualitative confirmation and quantitative measurements that demonstrate risks to CQAs of the drug product are eliminated, reduced, or otherwise mitigated by a measure of control. A robust pharmaceutical quality system that effectively manages regulatory commitments adheres to and reinforces thorough and robust product release and is compliant with pharmaceutical quality standards is unequivocal. In particular, an effective and contemporary change management system and knowledge management process ensures continuity and consistency in the quality control of the product.

In adopting a QbD approach and applying the science and risk-based principles to assess quality attributes and process parameters, design space can be created to describe the boundaries within which unit operations of a manufacturing process may operate. In essence, design space can demonstrate control of variables that may impact a CQA, and a control strategy can be established to accommodate design space. In fact, a combination of well-defined design space boundaries and real-time release testing can effectively demonstrate and confirm control and serve as the basis for release of the product without the need for specific end-product testing. In fact, where the risk is understood and the severity and probability of impact are controllable, the demonstration of process control through the creation of design space could conceivably reduce the need to perform in-process testing as well. Continuous formal verification to demonstrate process capability in accordance with well-grounded design space criteria could serve as the basis for product release to a specification derived largely from CQAs.

CONTINUOUS IMPROVEMENT

QbD is by definition a mechanism to develop and improve process understanding and product knowledge. The approach and principles therefore are intended to be iterative.

The nature of quality risk management is and should be inherently iterative in that the development of product knowledge and process understanding stimulates regular reassessment to improve mechanistic understanding and potential control of variability. The characterization of the severity, uncertainty, probability, and detectability of risk through the life cycle also allows for accommodation of optimizations to support business objectives. The investment in QbD should therefore be construed as the appropriate cost of doing business, prospectively moving toward a paradigm of continual improvement rather than retrospectively reacting to unanticipated variability in the manufacture of products. Specific evaluations and studies are usually inserted/included into the development timeline or life cycle plan as a complement to or in concerted alignment with other business critical investments, such as, standard and in-use stability studies, impurity purge studies, formulation compatibility, packaging moisture vapor transmission, sterility evaluations, etc. However, QbD can, and perhaps should, be more than a collection of scientific exercises that incrementally improve understanding and may increase opportunities to improve a manufacturing process and reduce costs. In fact, several proponents of QbD have argued that the intrinsic value of QbD is the "full understanding of how product attributes and process variables relate to or influence product performance" (46).

The principles embodied in QbD provide valuable opportunities to increase understanding of how the quality of a pharmaceutical product contributes to patient safety and efficacy. Understanding properties and characteristics of raw materials and components; their relative combination and compatibility with one another; the influence of basic conditions of temperature, pressure, and time; and the operational criteria of manufacturing processes can collectively improve assurance of quality regardless of the product or process to which it is applied. While not all development timelines will permit a comprehensive execution of certain elements of QbD, that is, evaluation of all critical and important variables via complicated experiments, performing a preliminary risk assessment as part of process development is useful in delineating what may be important to control in a process and can provide the basis for subsequent systematic, mechanistic, and science-based studies retrospectively. The

adoption and implementation of principles of QbD is a responsible and advantageous approach to managing the life cycle of pharmaceutical products.

ACKNOWLEDGMENTS

The authors recognize that the concept of Quality by Design can effectively be applied using several approaches. In fact, common conceptual threads that reflect how the principles of QbD have been prosecuted have progressed incrementally. The variety of tactics demonstrated through the application of actual practice has been useful substrate from which to draw general and definitive positions. In addition, frequent and exhaustive deliberations among many individuals within the pharmaceutical industry at multiple forums and conferences and among individual exchanges have yielded a tremendous wealth of meaningful scientific and regulatory experience and relevant opportunities to improve collective technical understanding of the merits of these concepts. We appreciate the contributions from many Pfizer colleagues, including Leslie Bloom, Tim Watson, John Groskoph, Graham Cook, Holly Bonsignore, T.G. Venkateshwaran, Ron Ogilvie, Zena Smith, and Alton Johnson. We also appreciate contributions from Chris Potter, John Berridge at ISPE, Chris Sinko (BMS), John Donaubauer (Abbott), Joanne Barrick (Lilly), Wim Oostra (Merck), Mette Kraemer-Hansen (Novonordisk), Tom Schultz (J & J), Chris Brook (GSK), and Steve Tyler (Abbott) for their valuable insights and experience. We are grateful for dynamic engagement with Moheb Nasr, FDA ONDQA, Joe Famulare and Rick Friedman, FDA OC, Yukio Hayama, MHLW Division of Drugs @ NIHS, and Jean-Louis Robert, Laboratoire National de Santé (LNS), Luxembourg for their concerted discourse and erudite perspectives. We welcome the collective enthusiasm to challenge prevailing dogma and traditional paradigms and encourage the pursuit of scientific- and risk-based approaches to pharmaceutical development and continuous improvement.

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14 Future of parenteral manufacturing

James Agalloco, James Akers, and Russell Madsen

We began the development of this chapter with an open mandate from the editors. We were asked to consider what parenteral manufacturing might be like in the future. As we had all separately and sometimes collectively developed papers and presentations on various elements of this subject, we believed we were up to the task (1,2). Our opening discussions revealed we had a substantial amount of work to do. We considered many more subjects for inclusion; the one's we have included met two important criteria. First, we were unanimous in our belief that they would be relevant in the next 10 to 15 years; and second, one or more of us felt sufficiently well versed in the technical area to make a meaningful effort as to what the future circumstance might be. The result is what you see here, a collection of brief essay's outlining what might be the state of the art in the not too distant future. We had one consolation in developing this, unlike a technical paper or even a commentary, references to what we are predicting would be beneficial, but their absence is a reflection of the future tense of this entire chapter. Undoubtedly, we will be wrong with respect to some portion of the following, but since we have made a rather substantial number of predictions, some of them might be completely accurate. Your difficulty as a reader will be to decide which is which, an effort that will be increasingly easier as the future becomes the present.

OVERALL CHANGES

Outsourcing

The pharmaceutical has expanded its use of contract manufacturing organization (CMO) substantially in recent years, and this is a trend likely to continue in the future. There had always been a segment of the parenteral industry that has utilized contract services. The predominant usage of CMOs for many years was largely in four areas:

- Filling of sterile penicillin and cephalosporin formulations
- Filling of prefilled syringes
- Filling of lyophilized formulation
- Filling of clinical trial materials

The motivation for the use of a contractor was customarily to avoid the added expense of separate or additional facilities for these unique formulations and presentations. These were perhaps the predominant use of contract manufacturing services until the advent of the biotechnology industry.

The first products developed by the biotechnology industry were large molecular weight proteins, many of which were lyophilized or required cold chain distribution. The simplest and most direct means for evaluation of these materials in a clinical setting required a parenteral dosage form, and in the majority of cases a freeze dried formulation was most appropriate. This led to an increase in the use of outside formulation and filling services. Initially, much of this outsourced production was accomplished by traditional parenteral manufacturers, although from the beginning CMOs sought to fill this increased demand. The biotechnology pioneers quite properly focused their attention to the unique parts of their processes, which was the fermentation and biochemical purification of these macromolecular entities. The seemingly scientifically simpler parenteral manufacturing steps that followed were important, but would be outsourced so that the many start-up biotech firms could rightly focus their technical and financial resources on the unique aspects of their technology.

The initial explosion in CMO usage came in the late 1980s and early 1990s as a result of two parallel but largely unrelated events. First, the initial success of biotechnology fostered a substantial increase in clinical (and later commercial) scale parenteral manufacture. By the

early 1990s, the explosion in biotechnology product development resulted in approximately half of all Investigational New Drug (IND) applications being for these "large-molecule" products. Second, the restructuring of the world's major pharmaceutical firms in which mergers and a desire to "right size" led to a realization that there was substantial excess capacity in all areas of the industry. The result of this effort was a transfer of facilities to entrepreneurs, sometimes consisting of the prior employees of the site, and initial contracts for the production of prior products by the new CMO. In some instances, traditional pharmaceutical firms have supplemented their own operations at production sites by offering their services as CMOs to leverage their facility utilization.

These trends have continued and perhaps increased. Recent years have witnessed a new driver for CMO usage, the virtual pharmaceutical company mimicking a trend that originated in the consumer products and microelectronics industries. In its most extreme mode, the virtual company outsources all aspects of product life cycle from development, production, and marketing. The virtual firm by virtue of its small size can operate profitably at very modest volumes due to the absence of internal infrastructure. Slightly larger virtual firms operate similarly, through purchase of New Drug Applications (NDAs) and Abbreviated New Drug Applications (ANDAs) of older and lower volume products from larger firms, securing CROs (contract research organizations)/CMOs for production and using others for distribution and marketing.

Outsourcing in parenteral manufacturing has expanded in ways that have never been considered previously. These applications are less comprehensive than those of the fully functional CMO, but are nevertheless represent a clear trend across the industry.

- Suppliers of packaging components, primarily rubber closures, but occasionally of other items, have vertically integrated their offerings. They are offering for sale, at a premium of course, ready-to-sterilize or ready-to-use components. Many companies large and small find it economically attractive to buy components ready to use to avoid operating costs, capital equipment costs (and maintenance), as well as improving inventory turns on components.
- Contract laboratories offering a variety of chemical and microbiological testing to virtual firms or to CMOs that lack extensive internal laboratory capabilities.
- Contract development/research firms that assist with product formulation and process development activities.
- Contract service providers offer virtually every conceivable form of assistance necessary to firms large and small including consultation, regulatory submission, training services, and audit execution.

Some recent statements by executives at major pharmaceutical firms suggest that even greater usage of CMOs might be forthcoming. There was some quick retraction of that perspective, perhaps because of the potential backlash at internal production sites. That a large pharmaceutical firm might consider outsourcing all of its production related activities is certainly significant. There is a clear belief that manufacturing of pharmaceuticals, and perhaps most importantly, is a costly activity with substantial infrastructure and overhead that could perhaps be eliminated. Could a multinational pharmaceutical giant go "virtual"? The notion itself, suggests that the economics of virtual operation might be substantially better than those of more fully integrated firms. If that is the case, then the future will certainly see outsourcing play an increasingly important role in pharmaceutical manufacturing, and certainly in parenterals.

Harmonization of Regulations and Inspections

The pharmaceutical industry is highly regulated, primarily by agencies such as FDA (United States), EMA (Europe), and MHLW (Japan)[a]. These agencies seek to ensure the safety and efficacy of pharmaceuticals by means of registration requirements and through inspections

[a]Food and Drug Administration (FDA), European Medicines Agency, Ministry of Health, Labour and Welfare (MHLW).

designed to ensure that the manufactured products comply with the standards contained in their approved applications and that appropriate manufacturing controls required by the various Good Manufacturing Practice (GMP) regulations are in place. Organizations such as PIC/S also influence the direction of manufacturing practices for sterile products. The industry must also concern itself with pharmacopoeial standards contained in the USP, JP, and Ph. Eur.[b] Also, compliance with other regulations such as environmental and employee safety and health is a necessity in the industry.

As expected, there are differences in regulations in various regions and in the interpretation of those regulations by the agencies charged with ensuring compliance with them. This leads to variation in quality systems and manufacturing controls employed by multinational pharmaceutical companies, and it increases cost.

International Conference on Harmonization

The International Council on Harmonization (ICH), which was formed in 1990, has been effective in producing a series of guidance documents designed to provide a common framework for pharmaceutical regulatory compliance. ICH develops guidance through a Steering Committee that meets twice a year. Members include, EFPIA, MHLW, JPMA, FDA, and PhRMA[c], representing regulatory bodies and research-based pharmaceutical companies in Europe, Japan, and the United States. Nonvoting observers (WHO, EFTA[d], and Health Canada) provide a link between the ICH and non-ICH countries and regions.

These meetings have resulted in guidance documents that provide a framework of concepts and practices acceptable in the three regions. Examples include Q7 "Good Manufacturing Practice Guide for Active Pharmaceutical Ingredients," Q8 "Pharmaceutical Development," Q9 "Quality Risk Management," and Q10 "Pharmaceutical Quality System." Other important guidance documents have been produced covering the topics of Stability (Q1A–F), Analytical Validation (Q2), Impurities (Q3A–C), Pharmacopoeias (Q4, Q4A and B, with Appendices), Quality of Biotechnological Products (Q5A–E), and Specifications (Q6A–B) (3). Under ICH auspices, a Common Technical Document has been developed to facilitate a harmonized approach and format for regulatory filings relating to new drugs.

Pharmaceutical Inspection Convention/Scheme

Harmonization of regulatory inspections in terms of scope, focus, and interpretation of the applicable regulations and guidance documents is an important issue from the viewpoint of the regulated industry as well as the involved regulatory agencies. Consistency of inspectional approach leads to improved regulatory compliance since expectations are clearly defined and production and quality systems can be presented to the inspectors in ways that are understandable to all concerned parties.

PIC/S develops harmonized GMP standards and guidance documents, trains inspectors, assesses inspectorates, and promotes cooperation for competent authorities and international organizations. The participating authorities represent most of the countries of the European Union, as well as Australia, Canada, Singapore, and South Africa, to name a few. Partners and observers include the European Directorate for the Quality of Medicines and HealthCare (EDQM), and WHO.

The FDA is not, as of this writing, a member of PIC/S, but the agency has made application for membership. Inclusion of the FDA could result in progress toward the harmonization of international inspectional practices. As experience is gained, and with increased cooperation and interaction among inspectorates, harmonized inspections should become a reality. The hoped for outcome of harmonization at the inspection and enforcement

[b]United States Pharmacopeial Convention (USP), Japanese Pharmacopoeia (JP), and European Pharmacopoeia (Ph. Eur.).

[c]European Federation of Pharmaceutical Industries and Associations (EFPIA), Japan Pharmaceutical Manufacturers Association (JPMA), and Pharmaceutical Research and Manufacturers of America (PhRMA).

[d]European Free Trade Association (EFTA).

level would be the mutual recognition of inspections. This would obviate the need for the same firm operating under the jurisdiction of a competent authority that participated in the harmonization scheme from undergoing numerous inspections from other competent authorities. It is not uncommon for multinational firms to undergo 10 or more inspections per year, which typically cover the same technical and compliance subject matter. This duplication of effort is neither useful to the firms subjected to these redundant inspections nor does the end user accrue any benefit. Mutual recognition of inspections could significantly reduce costs for industry and regulatory agencies while providing necessary oversight to protect the end user.

Pharmacopoeias

Pharmacopoeias have been established in many countries, regions, and internationally in an effort to standardize the testing of active pharmaceutical ingredients, excipients, containers and closures, and pharmaceutical products and to ensure the efficacy of the medicines delivered to the consumer. There are many minor differences in the monographs and chapters of the various pharmacopoeias. These differences result in extra testing in the event pharmaceutical products are marketed in these countries and regions. Pharmacopoeial differences can be particularly troublesome for active pharmaceutical ingredients and excipients since these materials are often widely distributed. Pharmacopoeial harmonization can, therefore, result in better product uniformity, reduced testing, cost savings, and reduced regulatory burden. The Pharmacopoeial Discussion Group (PDG) was created to foster pharmacopoeial harmonization.

The PDG consists of members representing the EDQM, the USP, and the JP. The PDG usually meets in conjunction with ICH and provides the ICH Steering Committee with updates on pharmacopoeial harmonization issues. The PDG works to harmonize general chapters and excipient monographs in the three pharmacopoeias. A chapter or monograph is harmonized when "a substance or preparation tested by the harmonized procedure yields the same results and the same accept/reject decision is reached" according to the PDG. Full harmonization is not achieved until the text becomes official in all three pharmacopoeias.

In conclusion, there appears to be consensus among all parties involved, that is, regulators, the regulated industry, the pharmacopoeias, and organizations such as ICH and WHO to harmonize inspectional practices and regulations, resulting in improved compliance levels, patient safety, and decreased cost.

Globalization of Manufacturing

The majority of the world's production of pharmaceutical products was for many years the near exclusive province of multinational firms located in Japan, Western Europe, and the United States. While there certainly were plants located in other areas of the globe, distribution from those facilities was predominantly local. This began to change with the opening of mainland China to outside investment. The availability of extremely low cost labor for facility construction and operation along with financial support from government sources and less restrictive environmental regulation led to an influx of pharmaceutical manufacturing. India and Brazil offer similar opportunities. These countries offer added possibilities in the domestic and nearby markets.

The availability of low cost labor is perhaps the greatest single motivator in the placement of pharmaceutical facilities in these environments. Labor rates in these locales are a fraction of that paid in the traditional pharmaceutical manufacturing locations, and is clearly a major driver in the global transition to them. Parenteral manufacturing in these environments raises some concerns in the minds of many. Sterile products, especially those made using aseptic processing requires a proficient work force with unique skills. Training of operators to work in aseptic manufacturing is no mean task. In transitioning to these new production centers, firms must be prepared to make a substantial training commitment to bring their new workforce up to an appropriate level of competence. This is presumed easier in India, where the English language competency makes the task somewhat less daunting.

Recent experience in China as a production site has raised concerns relative to the integrity of its supply chain. The difficulties encountered in 2008 with contamination of heparin, baby food, and other materials have made it a difficult choice. These difficulties may be in the past; however, the negative experience is perhaps too recent for continued expansion to continue at the same pace. A commonly applied strategy has been to maintain tighter control over new high-value and high-profit products by continuing to manufacture them in the United States, Japan, or Europe while moving products to lower cost areas only near the end of their patent life. This enables firms to move older less profitable products out and newer and perhaps more technically challenging products in to take their place. This is also consistent with the philosophy of developing manufacturing sites within an organization that specialize in a particular technology. This core-competency approach has proven popular in an era of consolidation and rationalization of capacity, and in a world where redundancy of supply can be achieved easily through outsourcing rather than maintaining "back-up capacity" within the organization.

The drive toward globalized manufacturing is largely driven by economics, with the cost of labor in the developed world being one of the major factors. If the technology advances described elsewhere in this chapter reduce the labor content associated with parenteral manufacturing, then the labor cost driver is somewhat or even fully mitigated. A parallel element of this shift would be the need for those workers who do remain to be substantially better trained to enable them to operate and maintain the more sophisticated equipment that will be utilized. Also, if global environmental policies become more harmonized another major cost driver in moving manufacturing to a developing nation may no longer exist.

DEVELOPMENT

Elimination of Conventional Manual Filling

The production of sterile injectable products began with gowned personnel manually assembling containers. We could also envision that shortly after these first fills were performed that the innate hazards associated with intimate contact between personnel and sterile materials were recognized. Many years have passed since the origins of sterile product preparation by manned personnel, and with that passage our industry has witnessed substantial improvements in processing technology. The 1950s saw the introduction of HEPA filters and machine filling. The 1960s witnessed wholesale changes in sterilizing filtration with the adoption of the 0.2-μm filter. Validation as a requirement for sterilization and other processes came about during the 1970s. The next decade brought forth automated systems, parametric release, and isolation technology. The 1990s saw the first Restricted Access Barrier Systems (RABS) utilized for aseptic processing. In the 21st century, we have seen Process Analytical Technology (PAT), Quality by Design (QbD), and improved analytical technologies. Clearly, a lot has changed over the last 60 years, yet one surprising constant remains—hand filling of sterile products is still a common practice.

The extensive number of improvements adopted for parenteral manufacturing on a larger scale has undoubtedly improved the quality and safety of sterile products (4). That manual filling persists is likely due to the fact that it is somewhat hidden. Hand fills are the province of clinical supply production, orphan drugs, and very low volume operations, the very sort of operation unlikely to distinguish the firm using it. Would anyone tout the performance of their hand fill operations? It may be performed in a better environment, with superior gowning materials and even a modicum of automation, but in many ways it is little different from the practices of a long ago era. Gowned personnel still serve as the means for transfer of components from filler to stoppering and then to crimping. The operator's role in hand filling is largely unchanged from what it originally was, and that strikes us as an unacceptable compromise in today's far more capable and demanding environment.

The difficulty with manual filling lies in the required intimate involvement of personnel with sterile materials. Perhaps the simplest way to understand the increased contamination risk associated with hand fills is through the Agalloco-Akers Aseptic Risk Evaluation Methodology (5,6) One of the elements of this method is the Intervention Risk, which is the number of operator touches per container produced. In a manual filling process, this number

cannot be less than one, and could approach three or four. When determined for a machine fill, the Intervention Risk can be anywhere from 100- to 10,000-fold lower. The continued use of an aseptic process that the "best case" is one hundred times more risky than the poorest machine supported process unnecessarily exposes the patient to potential microbial contamination, which could easily be avoided using other technologies.

It is clearly an anachronism, a relic of an earlier time that belongs in our past, not in our future. Closed isolators are a near ideal substitute for the production of small batches using largely the same practices used in hand filling in clean rooms. The isolator provides

- The ability to effectively decontaminate the processing environment in a more effective manner
- A near perfect separation of the operator from the sterile materials, approaching the goal of the "sterile field"
- Means for the safety introduction/removal of items without compromising the integrity of the isolator through the use of rapid transfer ports

There are isolators-based systems being developed that will take the entire hand fill process to an entirely new level. Vision equipped robots will be used to prepare sterile materials without operator involvement during the entire process. These offer opportunities for potent and hazardous materials where even the use of an isolator means that some operator risks are unavoidable. These systems also eliminate the fatigue factor common in a heavily manual process of any kind.

Given the availability of technologies that can dramatically enhance the certainty of the process, and thus raise the capability of hand filling to levels commensurate to commercial scale production (where isolators and RABS are becoming more and more common), the elimination of manual aseptic filling in clean rooms appears near certain. The elimination of manual filling could be hastened if inspections really did focus on end-user risk. There have been examples of inspections that focused on the enforcement of arcane local requirements for certain practices while overlooking hand-filling or manual aseptic interventions that would easily be either automated or separated by RABS or Isolator technology.

QbD/Design Controls

Ensuring the quality of pharmaceutical products and medical devices is a primary concern of manufacturers, regulatory agencies, and patients globally. QbD and associated design controls have been used for many years in the aerospace, automobile, and the electronics and computer-related industries to ensure the quality and reliability of manufactured products. The pharmaceutical industry has relied on pharmaceutical development, clinical studies, and conformance to the manufacturing processes and controls contained in the approved marketing applications to ensure product quality.

The FDA's GMP for the 21st century initiative effectively cleared the way for a different approach to drug development, licensing, and manufacturing (7). The initiative allowed manufacturers to continuously evaluate drug product quality during the development and marketing phases and make changes to improve those processes without undue regulatory burden. The ICH Q8 and Q9 guidance documents, mentioned in the previous section, provided manufacturers with a road map to improve the development and evaluate and manage risk.

QbD incorporates comprehensive prior knowledge about the product and process, some of which is derived from similar products and processes, scientific studies such as design of experiments, and quality risk management throughout the product's life cycle. This leads to improved understanding of the product and its manufacturing process, forming a basis for a more flexible regulatory approach. Regulatory flexibility is directly related to the level of scientific knowledge contained in the registration application. The application should contain information substantiating the scientific relevancy of the data submitted.

Important elements of a QbD-based pharmaceutical development program include accurately defining the product as it relates to dosage form, bioavailability, safety, efficacy, and

stability; identifying critical quality attributes of the drug product so that they may be appropriately controlled; evaluating characteristics of the active ingredient(s) and excipients to ensure they possess the requisite attributes; selecting and defining the manufacturing process; identifying and implementing an appropriate control strategy; and providing a system of continuous improvement capable of integrating information and knowledge gained over the product's life cycle, which can be the basis for refining the development and manufacturing process.

QbD allows the use of a manufacturing process that is adjustable within the design space as contrasted with the one that is fixed. Instead of validation based on a few initial full-scale production batches, validation is carried out over the life of the product through continuous process verification and statistical process control. In such a system, process operations are tracked to support continuous improvements and postapproval. Instead of product specifications based on data available at the time of product registration, specifications become part of the control strategy based on product performance and process capability. In such a system, evaluation and control of product quality shifts further and further upstream (through "retroactive" development, API and excipient characteristics, process control) thereby eliminating problems before they occur. This allows for a risk-based control strategy rather than quality control, primarily through intermediate and finished product testing.

As QbD evolves, it is conceivable that the principles can be applied to custom products formulated for specific patients. These products are produced in small quantities, typically to meet a specific diagnostic or therapeutic requirement of a single patient. In therapeutic applications, the promise of these products includes improving safety with reduced risk from adverse reactions. Advanced QbD, design control strategies, and product development processes will provide the tools to ensure patient safety in this environment.

COMPOUNDING

Single-Use Technologies ("Disposables")

The production of parenteral formulations has typically involved the preparation of solutions and suspensions using vessels equipped with a means for agitation and a method for heating/cooling of the contents. The scale of this activity varied from the laboratory bench to the large commercial scale equipment, but the principles were essentially identical. Cleaning of the equipment was always required between products, and customarily between lots of the same product; but it was not until the early 1990s that cleaning became a concern of substantially greater magnitude (8). Cleaning validation was certainly something that had been considered necessary for years by those responsible for validation at parenteral firms, but the paramount difficulty facing all was the selection of an appropriate (read that as acceptable to a regulatory agency) criteria for allowable residual. This was the inevitable stumbling block that no firm wanted to topple alone. The interest in cleaning validation was such that the PDA (Parenteral Drug Association) formed a task group that developed an industry guidance document that defined several means for the establishment of a suitable acceptance criterion (9). The logjam with respect to acceptance criterion removed, cleaning validation began in earnest across the industry. However, it soon became evident that the effort required in cleaning validation and subsequent in-process cleaning and testing was substantial and were not going to be reduced substantially by the availability of a limit for residues. Uncertainties with respect to selection of appropriate sampling locations, rinse versus swab testing, residual recovery uncertainty and confirmation of process parameters, and other issues have hampered progress on cleaning validation. With the growing frustration regarding the scope and difficulties associated, a Gordian knot–like solution was identified.

Disposables are perhaps the solution to cleaning validation by eliminating the issue at the very core. If an equipment item was not reused, it would not have to be cleaned. If it was not cleaned, there is no need for cleaning validation. In one simple stroke, the issues with cleaning validation could be swept away: both cleaning and cleaning validation could be entirely eliminated where a disposable item could replace a reusable one.

The earliest applications of disposables were rather surprising, it was not a simple hose connection or a modest size bag, but rather large containers utilized for buffer and media

preparation in biologic manufacturing. The largest initial concern with respect to these applications was not their preparation or in their cleaning (they were all relatively simple solutions), but rather the desire to minimize initial capital expenditure. Also, disposables had the secondary positive effect of eliminating the need to validate production vessels including, in some cases, fermentors. This brought about additional cost savings and reduced facility construction and validation timelines. Using a single-use plastic container for the buffer made the overall process substantially easier and cheaper, at least initially. Extension of this disposable concept to single-use bioreactors was the next logical step. These vessels were somewhat more complex and the residues more diverse, so while the design of the disposable unit is more complex (and expensive) this is more than offset by the elimination of the difficult and lengthy cleaning process that must follow, as well as eliminating cleaning validation for this equipment.

Potential applications for disposable systems in parenteral manufacturing and filling are limited only by the imagination of the end user. Small-scale filling sets, process filtration assemblies, aseptic sampling systems, and other items are all available for use as disposable assemblies, which can be either a "standard" configuration or customized for a specific application. The increased use of disposable systems can be certain, given the operational advantages to their use, because the benefits extend beyond cleaning validation. The smaller disposable systems and assemblies offer substantial advantages, including increased certainty of assembly; reduced labor costs for system preparation, cleaning, and sterilization; reduced needs for internal sterilization time; enhanced confidence in sterilization; and greater system integrity.

The use of disposables does have some negatives to be considered. The polymeric materials utilized must not interact with the materials being processed in any way. The potential for extractables and leachables materials from the polymers to enter the product must be evaluated to assure that no adverse effects are present. Similarly, the adsorption/adherence of formulation components by the polymeric materials must also be considered. The issues here are comparable to those associated with the use of membrane filters; however, the exposure periods are likely to be longer and the conditions of use more variable. These issues can be overcome by careful selection and evaluation of the materials to be used. Environmental issues are of growing concern. Throwing away expensive and very large polymer containers and tubing after a single use runs counter to the current green ethos taking shape around the globe. It seems logical that sooner rather than later single-use technology vendors will consider means by which their products can be recovered and recycled. The use of disposable fluid systems offers enough advantages that increased use in the future is assured.

Filter Pore Size

Filtration has been used to remove microorganisms from parenteral and ophthalmic solutions for many years. During the first half of the last century, many sterilizing filters were made of asbestos or unglazed porcelain materials and achieved bacterial retention mainly through depth filtration and absorption. Microporous membrane filters came into widespread use for sterilization of pharmaceutical solutions during the 1950s. Use of these filters facilitated integrity tests, such as the bubble point, which could be directly correlated with the size of the largest pore(s) in the membrane, paving the way for validation studies that could be used to demonstrate the ability of a membrane to remove specific microorganisms from a solution.

The first microporous membranes used for sterilizing filtration had a pore size designation of 0.45 μm (10). When these filters were initially introduced it was thought that they retained particles and microorganisms exclusively through sieving. It was soon discovered, however, that other retention mechanisms were at work and microorganisms larger than the filters' pore-size rating were sometimes not retained. Dr Frances Bowman, an FDA scientist, discovered in 1960 that small numbers of certain microorganisms could penetrate 0.45 μm membrane filters and that this penetration was linked to the challenge level of those microorganisms, that is, penetration occurred when the challenge level was between 10^4 and 10^6 microorganisms per cm^2 of effective filtration area (11).

Further study revealed that this microorganism (at the time known as *Pseudomonas diminuta*) was retained by membrane filters rated at 0.2 μm. This led to the use of this microorganism to differentiate between 0.45- and 0.2-μm-rated filters. Another interesting fact emerged: *P. diminuta* could penetrate even 0.2-μm-rated filters if the challenge level was high enough (12). The concept of the absolute sterilizing-grade filter was abandoned.

The discovery that small bacteria could exist in pharmaceutical process streams under conditions where nutrient levels were extremely low led to the search for membrane filters of still smaller pore size. Currently, 0.1-μm-rated filters are used for process streams and products where these microorganisms may be present. If mycoplasma may be present, 0.1-μm-rated filters are generally employed. Use of these filters can restrict throughput and result in relatively long filtration times, and the filters tend to cost more than filters with larger pore-size ratings.

Membrane filters are classified according to pore size. Microfilters have nominal pore size ratings ranging from 1.2 μm to 0.04 μm. Ultrafilters are classified in kilodaltons, with pore-size ratings of about 0.05 μm to 0.0005 μm. Ultrafilters retain large molecules such as gamma globulin and serum albumin. Nanofilters have pore-size ratings of between about 0.005 μm and 0.0002 μm. Nanofilters are typically used for virus removal in the pharmaceutical and biopharmaceutical industries. Reverse osmosis membranes are rated with pore sizes between 0.005 μm and 0.00005 μm, and they are the tightest of the porous membranes. They find their use in the pharmaceutical industry primarily in water purification (13).

For all but the most unusual situations, 0.2- and 0.1-μm-rated membrane filters will continue to be used in pharmaceutical and biopharmaceutical sterilizing filtration applications, assuming viral removal is not an issue. These filters can be integrity tested and validated, and they have combinations of flow-rate and throughput that do not unduly impact their utility as process filters. It is unlikely that filters with smaller pore-size ratings will be used unless new technologies are discovered to increase throughput and to reduce expense.

FILLING

Plastic Containers

Glass vials, ampoules, and bottles have been used extensively to package parenteral products. Glass containers are clear, offer high chemical resistance, and can be securely closed either by fusion or with elastomeric closures, thereby preserving product sterility. These containers also withstand autoclaving and have low vapor and gas transmission characteristics.

Glass containers must be processed before they can be used. Washing and depyrogenation steps consume relatively large amounts of purified water and water for injection and electrical energy. Validation of washing and depyrogenation steps must be performed. Stoppers must also be washed to ensure cleanliness and freedom from endotoxins unless they are provided ready to use by the manufacturer. Failure to adequately prepare glass container closure systems can result in undesirable particulate matter in the finished product. Finally, glass containers are fragile. Breakage can be especially problematic if the pharmaceutical products are highly potent, toxic, or carcinogenic. Plastic containers solve many of these problems while offering some new ones of their own.

The first widespread use of plastic containers for injectable products was for large-volume parenterals (14). These infusion bags were easier and safer to handle in clinical practice than glass and did not require washing and depyrogenation. However, special autoclave cycles employing air overpressure had to be developed to prevent damage to the containers during postfilling terminal sterilization. Also, it was more difficult to inspect the finished product for the presence of particulate matter since the containers were not as clear as glass, although modern inspectional systems along with statistical sampling have provided means to ensure that appropriate control of foreign matter in infusion bags and other opaque or semiopaque products is achieved. Additionally, filters at the point of use can be employed with all products including infusion bags. Also, there are now infusion bags in which the drug and diluent are aseptically filled in advanced technologies such as automation and isolator technology.

Blow-fill-seal (BFS) and form-fill-seal technologies using both low- and high-density polyethylene and other polymers have been used for ophthalmic and for parenteral products (15). In this process, the container is formed from melted polymer pellets, filled, and sealed sequentially in a controlled environment designed to preserve the sterility of the components and the finished product. Container washing and depyrogenation are not required. Like infusion bags, the containers are translucent, which hampers visual inspection of the contents.

Plastic containers, however, have several disadvantages compared with glass. Leachable compounds from the polymers themselves, from plasticizers, and from inks and label adhesives can potentially find their way into the finished product. Careful formulation development and choice of polymers can effectively mitigate this problem. Plastic containers are in some cases relatively more permeable to atmospheric gases and water vapor than glass. Oxygen-sensitive will require careful consideration gas permeation properties in the development process; however, they can provide a vapor barrier that compares well with glass. Again, careful formulation development and packaging configurations utilizing vapor-resistant barrier overwraps can mitigate vapor and gas transfer issues. It is apparent that the percentage of plastic containers used for pharmaceutical packaging has been increasing. It is likely that the percentage will continue to increase, especially with the discovery of new polymers and packaging technologies that will reduce vapor transmission and leachables, improve container clarity, and provide increased safety for highly potent products. Industry has seen remarkable development in the properties of plastic containers over the last 15 to 20 years and we have reason to suspect that there will be an even more rapid rate of delivery system and container development over the next decade.

Closed Vial Technology

Glass vials and elastomeric closures have been used for decades to package parenteral products. Generally, the vials and closures must be washed and depyrogenated before use and in the case of aseptically filled products rendered sterile. These processes consume relatively large quantities of purified water and water for injection and the WFI production and glass depyrogenation processes are energy intensive. After filling, the closures are inserted and aluminum seals applied. Filling, stoppering, and sealing are performed in controlled environment areas, ISO Class 5 for aseptically filled products. The controlled environment must be monitored to ensure it remains within its designed operating parameters. Product contact surfaces of the vials and closures must remain sterile. This means that stopper hoppers, tracks, and vibratory bowls must be sterilized using validated processes and assembled so that they remain sterile throughout the filling and stoppering processes (16).

Closed vial filling offers an attractive alternative to conventional container closure systems. It eliminates the need for container and closure washing, depyrogenation, and sterilization, and it eliminates the need for the attendant systems such as purified water, water for injection, vial and stopper washers, depyrogenation/sterilization tunnels, and highly controlled environmental conditions related to container-closure preparation. Closed vial filling significantly decreases energy consumption, eliminates the costs of installation and operational qualification for these unneeded systems, and significantly reduces validation costs, especially those associated with environmental monitoring and media fills.

In the closed vial filling system, product is aseptically filled into presterilized closed containers inside the filling machine that maintains an ISO Class 5 environment, resulting in a high level of sterility assurance. Vials and stoppers are manufactured and assembled robotically in an ISO 5 clean room, resulting in very low levels of subvisible and no visible particulates. The assembled container-closure system is subsequently exposed to a γ-irradiation sterilization process at 25 kGy (minimum). The closed vial filling system incorporates e-beam irradiation for surface sterilization of the closure immediately prior to filling through the vial closure (stopper), laser resealing of the closure puncture, and application of the flip-away cap.

Other advantages include high levels of safety for operators, supply chain, and medical personnel when handling potent or cytotoxic products since the process design uses polymeric vials that eliminate breakage.

Separative Technologies

Separative technologies are defined as environmental control systems that fully separate the human operator from the aseptic production environment. Separative technologies by preventing direct human intervention proactively minimize the risk associated with human-borne and released contamination into the aseptic manufacturing environment. It has long been recognized that humans are the only significant source of contamination and hence risk in aseptic processing. Therefore, the implementation of separative technologies marks a very significant step forward in the effort to reduce microbiological risk to the end user. Two general categories of separative technologies are used in the aseptic processing industries: isolator technology and RABS.

Isolation Technology

Isolation technology can be expected to become the dominant environmental control system for the filling of aseptically filled products. The number of conventional human occupied clean rooms being constructed in our industry has been diminished in number over the last 10 to 15 years. Newly constructed facilities are most commonly isolator or RABS-based designs. The reasons for the introduction of these technologies are obvious; they offer an enhanced means to separate personnel from sterile materials. One of us was bold enough to predict that isolators would become the dominant means of production for aseptic production (17). It now seems certain that isolators will ultimately become the technology of choice for aseptic filling. RABS will play a role in the future of our industry, but it seems to us that that RABS cannot realize the performance and return on investment available with isolation technology and thus will play only a secondary role (see following text).

Isolation technology provides undeniable advantages over other environmental control systems for aseptic processing:

- Has the ability to decontaminate reproducibly and automatically
- Provides the best possible separation between operators and sterile materials
- Eliminates aseptic gowning for personnel
- Reduces external environmental conditions relative to other technologies
- Reduces operating costs
- Provides superior environmental conditions

For these reasons and others the trend toward increased use of isolators is likely to continue (18). We predict they will become the technology of choice for virtually all newly constructed aseptic processing facilities.

Although isolators have noteworthy advantages, they may not be the best choice for all product types or for all drug delivery system/drug product combinations. In 2010, when we think of isolator technology we immediately think of vapor-phase hydrogen peroxide (VPHP) decontamination of the system as well. However, protein and peptide products can, in some circumstances, be exquisitely sensitive to H_2O_2 residues. There have been residue targets as low as 10 to 50 ppb established, and this can be a practical difficulty depending on the need to decontaminate product contact materials in situ and also depending on the complexity of the processing equipment. It will be critical for research and development into alternative decontamination/sterilization methods to continue apace as we move forward, and it may also be necessary for industry and regulators to consider that decontamination as currently practiced may not be required in all cases.

Restricted Access Barrier Systems

The production of sterile products has been dominated by the conventional manned clean room since the 1960s. Around 1990, after they had been implemented for sterility testing at a number of firms, isolator-based filling systems were first introduced into the sterile products sector. Hailed as extraordinary breakthrough, the initial interest in their capabilities was overwhelmingly favorable. Regrettably, some of the initial enthusiasm was tempered by firms that had difficulty with their implementation. Whether this was the fault of the technology in

asking too much too soon or the result of overstated expectations on the part of some firms is unclear. What is certain was that there was a desire for a system with the performance capabilities of an isolator with the simplicity of a manned clean room. Thus, the RABS was born, as a less complex alternative to isolation technology. As this is written, the use of RABS is expanding, but evidence is starting to appear that the rate of growth may not match that of isolators. After something of a plateau in implementation rate in the late 1990s and early part of the current decade, it seems clear that isolator implementation is accelerating. This may be largely due to the fact that industry and the regulatory community have learned that many of the concerns associated with mouse holes, rapid transport systems, enclosure leakage, decontamination efficacy, mouse holes, and gloves were significantly exaggerated. Actually, none of these issues have proven to be significant contamination risks. Plus, the general understanding of these issues has improved and effective countermeasures have been implemented to mitigate the perceived risk associated with these devises.

The glove system is a wonderful example of industry responding in a logical commonsense manner to reports of higher than expected glove leak rates in the mid-1990s. A wholesale move to Hypalon gloves, which have proven more reliable than the Neoprene gloves originally used, and the implementation of effective glove leak testing, inspection, and management programs have resulted in a manifold reduction in risk. Similar commonsense approaches have reduced concern regarding the so-called RTP ring of uncertainty as well.

Perhaps most importantly though, the isolator has effectively matched the contamination control benefits advocates of this technology foresaw over two decades ago. The best argument in favor of isolators is that they have, when well designed and implemented, proven to be exceedingly successful. Isolator technology has further benefited from the redesign of processing equipment to make it more isolator friendly. Manufacturers have learned to design and engineer equipment that functions much better in the isolator environment and which require far fewer interventions using gloves. So effectively has equipment been designed to work in isolators that gloveless isolators which seemed a pipe dream in the near past are now quite possible.

It is evident to all that isolators have superior capabilities to RABS in some areas. There is a belief that the simpler RABS designs may offer advantages and indeed where VPHP decontamination is a persistent problem to the product, closed RABS systems would be a viable solution worth considering.

The long-term prognosis for aseptic processing technology is thus somewhat clouded. RABS appear to offer capability without the validation headaches sometimes associated with VPHP and thus have considerable appeal. It is certain that as a retrofit, or replacement of an existing filler system within an operating facility, RABS can perform better than the human scale clean room. Converting an existing parenteral operation over to isolation technology seems a near impossible task; the facility alterations are so significant that such a project seems fraught with pitfalls. RABS can be utilized as a suite-by-suite approach to upgrading an operating facility without the difficulties a conversion to isolation technology might entail. This is perhaps the greatest opportunity for their application, and given the longevity of parenteral facilities, it is in this mode that we might see the greatest application of RABS in the future.

The isolator by virtue of its superior decontamination, operator protection, and reduced operational cost will be the preferred approach for new construction. This has been the conclusion reached by many firms across the industry, and is likely to continue in the foreseeable future. Thus, some 20 years from now, we will likely see both RABS and isolators in everyday use across the industry.

Robotics

In 2004, the first robot designed to operate in a VPHP decontaminated environment was installed and validated in an isolator technology filling line in Japan. In this installation the robots were used for unloading stopper containers from the autoclave and also were used to charge the stopper bowl. Robots have also been used successfully in aseptic cell culture isolators and in radiopharmaceuticals applications. There are now at least two firms offering robots designed to operate in an isolator environment and therefore capable of routine, frequent exposure to VPHP.

If we consider that the real benefit of separative technology is the elimination of the direct human intervention, it becomes clear that the robot can be equally effective at mitigating contamination risk. Clearly, eliminating the human with automation can work just as effectively at controlling contamination as separating the human from the aseptic environment. Recently, at least one vendor has begun marketing robots that are both resistant to VPHP and capable of remote operation using hand controls. These hand controls can also be used to train the robot increasing flexibility and reducing the need for complicated programming when process change is required.

The reluctance toward the use of robotics in the pharmaceutical and biopharmaceutical industries is puzzling given the widespread implementation of robotics in nearly all manufacturing industries. Robots have proven to be economically advantageous not only in heavy industries but also in light manufacturing including electronic component assembly. As this chapter is written our industry continues to lag behind other manufacturing industries in robotic applications and in some other forms of machine automation as well. This is very likely to change over the next decade as our industry learns the myriad advantages of robots, and success breaks down the prevailing reluctance to use them.

Robots and automation will also reduce interest in finding lower cost manufacturing venues. In fact, logic tells us that as they continue to develop, countries like India, China, and others will see their standards of living and regulatory climates change such that they will not be able to compete on price advantage alone. Once, not so long ago, Japan was considered a relatively low-priced site of manufacture, but this is certainly no longer the case. There is no reason to believe that nations now moving up the development trajectory will not follow a similar course. Thus, at some point, shipping and short supply lines may be of more interest in low costs of labor, low taxation, and lax environmental compliance requirements, all of which are likely to go away as a country develops. Robots could make possible economic and low cost manufacturing closer to the target populations for a given group of patients.

Robots are also likely to play a greater role as more customized healthcare products are developed. These products will not require high-throughput operations but rather flexible manufacturing in ultralow risk aseptic environments, since in effect most of these products will be released aseptically and process validation in the manner we understand it now may not be fully possible. Gene therapy and regenerative medicine products promise to be game changers in the therapeutic world and they need to be made close to the point of use. Manual assembly or fill as already covered is not desirable. Therefore, the only logical solution will be robotics and quite often robotics in conjunction with separative technology of one kind or another.

BFS

BFS systems are quite simple in concept but extremely complex in terms of engineering, manufacture, and operation. Earlier generations of BFS systems required not infrequent interventions to clear solidified plastic from fill nozzles and other interior parts. These systems did not qualify as advanced aseptic processing systems as they did not effectively eliminate direct operator interventions.

Fortunately, in the current generation of BFS systems, intervention-free operation is not only possible, it is generally achieved. As previously stated, plastic resin is the starting material for the BFS container. These resins are fed from large holding containers to hoppers on the machine; the plastic resin generally in the form of beads is melted and blown into molds under relatively high temperature and sufficient pressure to form the container. Filling systems, generally of the piston-pump variety, dose product into the container, which is then heat sealed. All filing and sealing is accomplished under an air shower that provides unidirectional Class 5 HEPA filtered air to the aseptic critical zone of the equipment. The containers, which may range from single-dose ampoules of 1 mL volume or less to up to a liter or more, are effectively sterilized by the heat and pressure of molding. The filling systems are sterilized and in most cases cleaned in place, thus no aseptic connections are required. The result is a very low contamination risk aseptic production system.

There are only two risk modalities associated with the current generation of BFS equipment. The first is the maintenance of a sterile supply of drug to the filling system.

BFS lends itself to quite long campaigns that may reach or exceed seven days. Therefore, the ability of the compounding and filtration system to deliver sterile product over an extended period of time is vital. This requires very careful design and engineering to ensure that bioburden can be very well controlled through the campaign duration. Obviously, the higher the probability of the formulation supporting microbial growth and therefore being prone to the amplification of contaminants, the greater the inherent risk and the most careful the design, engineering, installation, validation, and process control requirements. The second contamination route that has been observed in BFS originates from the cooling system. Generally the molds are cooled by water, and the water circulation system is not sterile in any current design. Given the long running times these cooling water systems can be a source of contamination, should leaks occur. Antimicrobial agents could be used in the cooling water system, but this raises a risk of chemical contamination, should aerosols occur. Fortunately, advancements in the design and therefore safety of cooling water systems are evolving rapidly.

Blow molding in-line of bottles made of (polyethylene terephthalate) PET or other plastics is possible, and aseptic filling systems using such bottles are in use and validated. In most cases the bottles are subjected to a sterilization using a chemical or more recently e-beam sterilization. Prefilled syringes can also be blow-molded inline and sterilized en route to the filling process generally using e-beam.

In situ or in-line blow molding of plastic bottles is a technology that will continue to evolve and which can be applied to even rather complex dosing systems in the future. It seems logical that the closures compatible with hypodermic syringes can certainly be developed and implemented in the coming decade. In-line or in situ blow molding with or without instantaneous heat sealing is likely to be with us for many decades to come. Also, as is the case with robotics we may see blow molding in use with isolators or RABS systems. In fact, hybridization of different manufacturing, environmental control, and automation/robots systems in a single-production operation seems increasingly likely.

Aseptic Filling Systems

Dramatic changes in filling technology, particularly for aseptically processed containers are anticipated. The equipment would be highly automated and specifically designed to operate without human access and would include operating capabilities and features such as

- Provision for all routine interventions
- Elimination of corrective interventions
- Clean-in-place or sterilize-in-place capabilities for all product contact surfaces
- Weight verification or adjustment on all containers
- Container integrity control and confirmation on all containers
- Continuous monitoring of critical process variables
- The use of PAT where appropriate
- Automated in-feed and discharge of components without human intervention
- Automated environmental monitoring of isolator internal air and surfaces
- Automated setup and transition from clean-in-place or sterilize-in-place to aseptic filling
- Self-clearing filling systems (for jam-free operation)
- No-container, no-fill to eliminate spillage

The reader is encouraged to seek out the many detailed reference texts that cover the technologies introduced in this section in far more technical detail than this brief chapter allows. Suffice it to say that the variety of aseptic manufacturing systems in the near future is limited only by the imagination. The authors are convinced though that each of these systems will have one critical thing in common, which is that they will all fully eliminate the direct human intervention. The future of aseptic processing will not be just one technology for manufacturing or one technology for environmental control, rather many possibilities will exist. However, they will all completely eliminate human contamination risk and thus result in aseptically produced products that are so safe that whole new regulatory approaches will have to be required. A blind adherence to clean room–derived regulatory policies will slow development and implementation, and therefore result in both safety and economic harm to the end user.

STERILIZATION

ClO_2, H_2O_2 and O_3 Sterilization/Decontamination

Sterilization of materials is at the core of almost every parenteral manufacturing process. The processes utilized are dominated by heating processes, with steam and dry heat among the most common. Solutions of course have been sterilized by membrane filtration for many years, a situation that is unlikely to change given the unique nature of fluids. For materials that are susceptible to heat, radiation and ethylene oxide (ETO) are the predominant alternatives. Radiation sterilization is seeing increased use (see following text), but is not compatible with some materials. ETO sterilization is widely used in the sterilization of medical devices, as well as many plastic items utilized in the pharmaceutical industry including filters, wipes, and containers. Despite its sterilization efficacy and wide use, ETO is hardly a method of choice because of the extreme environmental, toxicity, and safety issues associated with its use. Much of the pharmaceutical industry moved away from in-house sterilization using ETO to avoid the extensive measures required to handle it safely. As a consequence, contract sterilization firms now provide the bulk of ETO sterilization capacity worldwide. Movement away from ETO mixtures with CFC-12 (dichlorodifluoromethane) has accelerated because of potential ozone layer depletion as a result of CFC emissions (19). As a consequence, the global healthcare industry has sought alternatives to ETO that could provide comparable sterilization effectiveness for heat-sensitive materials without its substantial negative consequences. Contract sterilization sites no longer utilize ETO/CFC mixtures, and many have converted to 100% ETO systems increasing the explosion hazards and worker safety concerns accordingly.

Chlorine dioxide, hydrogen peroxide and ozone have demonstrated a broad range of antimicrobial activity against both vegetative cells and spore-forming microorganisms (20,21). The broader application of these agents for sterilization has been in part limited by the extensive experience and installed base of ETO sterilization units. As long as ETO use is considered acceptable and contract sterilization is available, there is minimal incentive to pursue alternatives. Nevertheless, development of ClO_2, H_2O_2 and O_3 has been pursued by firms seeking a safer and environmentally friendly alternative.

Ozone decontamination of classified rooms was incorporated into the initial design of an aseptic processing facility for Novartis in Stein, Switzerland.[e] This facility also utilized tightly sealed double door airlocks with ozone for the sanitization of items being introduced into the aseptic core. Both installations have been proven effective for microbial control in the facility. TSO_3 of Quebec City, Canada, obtained 510K approval of an ozone sterilizer by the FDA in 2003.

The detection of *Bacillus anthracis* in the U.S. post office and government buildings required a means for removal of spores of this toxic microbe. Chlorine dioxide was one of the agents utilized for this treatment, and it demonstrated excellent efficacy with minimal complications. This experience has led to additional applications on facilities dealing with mold and other forms of microbial contamination. Chlorine dioxide also has been successfully utilized for decontamination of isolator environments (22).

These agents have both demonstrated excellent lethality against spore-forming microorganisms, and expanded usage of each agent can be expected in the future. The only drawback to their application is perhaps the substantial installed capacity of contract ETO units across the globe. Given the explosion hazard and worker safety issues associated with ETO, it can be anticipated that a slow shift to ClO_2, H_2O_2 and O_3 is possible. For firms seeking in-house gas sterilization capability, these agents may become more common as knowledge of their efficacy grows. They each offer the industry a sterilization alternative to current processes.

Sterilization by Radiation

Radiation is widely utilized in the medical device industry for the sterilization of a wide variety of items including bandages, implants, latex gloves, wipes, and countless other items using either γ rays or electron beams. The extensive use of radiation for medical devices has been supported through the development of consensus documents that aid the practitioner in defining, validating, and maintaining a consistent process (23,24). These documents formed

[e]J Agalloco, personal communication, 1995.

the basis for global standards for radiation sterilization with the device industry, but were poorly suited for applications in pharmaceuticals. The lethality assumptions inherent in the older radiation sterilization standards are based on bioburden assumptions from common materials utilized in medical devices. The methods relied heavily on microbial testing of large numbers of fractionally sterilized units, with the results utilized to establish the minimum amount of radiation required. The application of these methods for sterilizing pharmaceutical products was considered quite difficult, and as a consequence only a handful of pharmaceuticals were ever successfully sterilized using radiation.

The success with radiation sterilization processes within medical device industry led to increasing consideration for application in pharmaceutical processing. Perhaps the single greatest factor in the expanded use of radiation sterilization has been the emergence of the VD_{MAX} methods for establishing an effective radiation dose for sterilization of materials (25). In contrast to the other dose-setting methods utilized that are heavily device oriented, the VD_{MAX} method utilizes substantially smaller samples of materials to establish a sterilization process. This seemingly modest change is better suited to pharmaceutical development, where limited material availability and high cost make use of the other dose-setting methods impractical. Several finished dosage forms have been introduced using radiation sterilization in a terminal process, something that would have proved impractical and prohibitively expensive previously. Applications for postaseptic fill lethal treatment using adaptations of the VD_{MAX} dose-setting method are also possible (see following section).[f] The continued use of radiation within the medical device industry has aided other applications. Not only is there a growing body of knowledge regarding radiation sterilization, makers of plastic containers, elastomeric closures, and other plastic materials have developed formulations and polymers that are less susceptible to the damaging effect of radiation, expanding the possibilities for application in many settings. Another factor influencing radiation usage is the increase in isolator installations, where electron beam systems are well suited for use for continuous material in-feed of heat-sensitive materials.

Several new radiation sterilization technologies are in active development, with a range of applications as diverse as full pallet e-beam and X-ray systems, and small-scale systems that can be inserted into individual containers. As with any technology, once the initial resistance has been overcome, increasing usage follows. It seems clear that radiation sterilization will play an increasingly prominent role in future production methods for parenteral dosage forms, expanding upon the modest but very promising use now being experienced.

Postaseptic Fill Lethal Treatments

The preparation of sterile products has been dominated by two distinct approaches for many years. Products are either manufactured using aseptic processing or terminal sterilization. The distinction between the processes has always been rather sharp, the processes were considered distinct and separate. Although preference was always given to the use of a terminal approach because of its increased reliability and certainty, aseptic processing was utilized in the majority of formulation because the adverse impact of the expected terminal processes in use proved destructive of product properties in many cases.

Some years back, there was an exchange of missives among industry regarding the potential for a postaseptic treatment to provide a higher level of sterility assurance to the end product (26–29). The discussion went on for some time and while perhaps educational, none of those who participated seized on what was perhaps the salient point that lay just beneath the surface of that discussion on sterility assurance level (SAL). While we quibbled on terminology, we all glossed over what should have been the focus of our interaction—would a postaseptic fill treatment of some kind provide a safer product for the end user? We never quite reached that issue in our discussion. Reflecting back on that dialog, I suppose we all knew that it would, and yet somehow we never broached that question or its answer directly. Hindsight is of course 20-20, and the answer to that unasked question has to be a resounding—yes, of course it most certainly would.

[f]JB Kowalski and JP Agalloco, personal communication, 2008.

In recent years, the climate for regulation has changed dramatically, with FDAs proposal for Risk-Based Compliance dramatically influencing industry thinking (30). It seems obvious that, if a postaseptic lethal treatment is provided to what is already accepted as a sterile product then the likelihood of an isolated contaminant surviving in the container would be reduced. The FDA made the point during the revised Aseptic Processing guidance, that aseptically filled products were the cause of the vast majority of recalls for lack of sterility assurance (31). In discussions held with the FDA to review the first draft of that document, a brief discussion was held regarding the potential desirability of a supplemental treatment following aseptic processing, but as the Product Quality Research Institute (PQRI) group was pressed for time, we tabled the subject, and regrettably never returned to it subsequently (32). The European Community regulatory community approached the subject of process selection rather differently and unfortunately in an extremely rigorous manner (33). The decision tree sets forth strict requirements for minimal treatments that would be accepted as terminal sterilization. Any lethal process not attaining a minimum F_0 of eight minutes shall be produced by aseptic processing. Some years earlier, the FDA endeavored to mandate an explicit preference for terminal sterilization through a modification of the 211 regulations (34). There was substantial discussion of this proposal, and it was eventually tabled as something not easily implemented. One of the many discussion items at that time was a request by industry for a defined minimum F_0 threshold that FDA would accept (35). This would have resulted in a situation much like that resulting from the PIC/S decision tree, albeit somewhat earlier. The absence of a single minimum value has not hampered firms seeking to assure greater safety for their products, and we are aware of firms that have utilized a number of very flexible approaches and minimum F_0 targets well below eight minutes. Nevertheless, the conjunction "or" is far more commonly used than "and" when consideration of processes for sterile products involves both aseptic processing and terminal sterilization.

Given the desire to mitigate patient risk, and in full consideration of all that has occurred in the past, it seems obvious that subjecting an aseptically filled (supported by a process simulation program) to some form of lethal treatment afterward will become increasingly common in the not too distant future. Possible moist heat processes that could be utilized include an A_0 process in the range of 70 to 90°C (A_0 is a process for microbial control of vegetative cells in hospitals); intermediate temperature ($\sim$100°C) for destruction of non-thermophilic spore formers; or low F_0 processes ($<$8 minutes). Adaptations of the VD_{MAX} radiation process can be utilized for a comparable objective where modest radiation doses, below those considered minimal for sterilization, could provide comparable improvements in microbial control following an aseptic fill.

TESTING AND INSPECTION

Elimination of Sterility Testing for Terminally Sterilized Products

Sterility testing has been an accepted practice for the acceptance of sterile products since their inception. It has been a mandatory requirement since 1932 and first appeared in USP 11 and was official in 1936 (36). The test provides a laboratory test component that endeavors to establish that sterile products are not microbially contaminated. When first introduced into the pharmacopeia, the precepts of validation for sterilization and process simulation for aseptic processing were many years in the future. Under those circumstances, it was wholly appropriate to require that a test able to detect microbial contamination be added to monographs for sterile products. Batch sizes at the time were relatively small and production methods for sterile products were primitive by today's standards. Contamination levels in sterile products at that time are largely unknown, but presumably substantially higher than at present. Under those circumstances, testing of a sample for sterility might be a reasonable expectation. The first mention media fills defined a contamination rate for aseptic process in the 1970s of approximately 0.3%, a number that is at least an order of magnitude higher that what is considered attainable in the industry today (37).

With the passage of years since it became a compendial requirement, the sterility test monograph has gone through considerable change with respect to the methods, media, sample selection, response to positive results, and a number of other changes. These changes have adapted how the test is performed and the results interpreted, but throughout the years the

basic objective of the test has remained unaltered. The sterility test has remained a mandatory part of sterile product release systems. Produced lots, whether manufactured by terminal sterilization or aseptic processing, must be subject to the requirements of the sterility test unless regulatory approval has been granted for parametric release.

The first efforts toward parametric release of terminally sterilized products by moist heat were developed within Baxter Healthcare, which received FDA approval for its submission in 1985 after a 4-year review period. After that approval, the FDA outlined its expectations, perhaps drawn from the Baxter submission in a 1987 FDA Compliance Policy Guide CPG 460.800 (38). That document outlined the regulatory expectations for submission to use parametric release in lieu of sterility testing. The next submission for pharmaceuticals was approved in the mid-1990s, and several additional filings have been submitted and approved subsequently. Parametric release submissions and approvals have also been granted for medical devices using ETO and radiation sterilization.

The PDA provided an initial consensus industry perspective on parametric release in which it endeavored to define expectations for application of parametric release (39). The document outlines the components of a validation effort that could support parametric release with the goal of defining practices prior to regulatory initiatives. An updated version of the document is currently in development dealing with technology changes and more recent regulatory pronouncements.

The European Medicines Agency for Evaluation of Medicinal Products (EMA) CPMP committee issued a parametric release position statement in 2001, which updated thinking and provided a broader regulatory perspective (40). This was followed closely by an Annex to the EU CGMPs on the same subject (41). These efforts addressed parametric release in the broader context of operations with pharmaceuticals; nevertheless they did specifically address application for sterile products. The perspectives voiced in these documents brought parametric release out of a guidance setting and into formal regulation. The essence of these documents relative to sterility testing can be summarized in a single sentence, "Elimination of the sterility test is only valid on the basis of successful demonstration that predetermined, validated sterilizing conditions have been achieved." The focus of parametric release clearly resides in the sterilization validation effort. The documents correctly speak to the severe statistical limitations inherent in the sterility test.

The FDA has provided long anticipated parametric release guidance in a 2008 document that included the following definition (42):

> *Parametric release* is defined as a sterility assurance release program where demonstrated control of the sterilization process enables a firm to use defined critical process controls, in lieu of the sterility test, to fulfill the intent of 21 CFR 211.165(a), and 211.167(a).

The basic principle behind the use of parametric release in lieu of sterility testing is a heavy reliance on rigorous production controls largely defined from sterilization validation. A firm using parametric release must establish criteria for evaluation of each sterilization cycle that are used to establish conformance to the validated sterilization process. To what extent does the validation effort required for parametric release differ from that for sterilization processes used for nonparametric application? The answer to this question is that all modern sterilization processes are validated in an identical manner. Sterilization processes for the preparation of components, filling parts, and product formulations where the end product is sterile but where the treatment is in-process are validated in an identical manner, but there are almost no in-process sterility tests performed on these materials. Thus, parametric release is widespread, but somewhat "sub rosa" in that these processes are not considered within the same context because they occur in-process rather than as a terminal treatment. For aseptically filled products, which comprise an estimated 85% of all sterile products, consideration is expected in release for a review of all records impacting the sterility of the finished product. Thus parametric release is in daily usage worldwide for virtually every in-process sterilization performed. Given that these processes are so widespread, without scrutiny, and followed by a far less certain aseptic process does not alter the inherent risk associated with this common practice.

Why then should terminal sterilization processes where there are no subsequent contamination introduction steps be held to a more rigorous standard of requiring formal

approval prior to adoption of parametric release? In Annex 17, EMEA cautions that parametric release should not be considered for initial use, "It is unlikely that a completely new product would be considered as suitable for Parametric Release because a period of satisfactory sterility test results will form part of the acceptance criteria. There may be cases when a new product is only a minor variation, from the sterility assurance point of view, and existing sterility test data from other products could be considered as relevant." The FDA's more recent effort maintains consideration of prior history with sterility testing, "Experience with the proposed or similar product (and container closure system), the overall risks to sterility, and the steps you have taken to assess and control these risks." These cautions seem misplaced, historical performance with a statistically invalid test is really of no relevance. The proper perspective with respect to initiation of parametric release has perhaps been enunciated by Dr T. Sasaki of the Japanese Ministry of Health, "Everybody knows that sterility testing is meaningless for terminally sterilized products, but sterility testing is still carried out on terminally sterilized pharmaceutical products in many countries. In Japan, we have investigated the introduction of PR for terminally sterilized pharmaceutical products from a scientific viewpoint for the past two years " (43). The conclusion to that investigation is certainly somewhat different from what might be expected from a major regulatory body; the JP has included the following in its general notices (44).

> When a high level of sterility assurance is maintained consistently, based on the records derived from validation studies of the manufacturing process and the in-process controls, the sterility test usually required for the release of the products may be omitted.

Dr Sasaki indicates in his article that the JP did not restrict parametric release merely to terminally sterilized products, and that with appropriate controls it could be considered for aseptically produced materials as well.[g] The JP has provided a three-tiered approach to terminal sterilization process validation including a process in which an aseptically filled product is acceptable for release if its subsequent heat treatment it receives an $F_0 > 2$ minutes as a minimum.

The JP initiative addresses the subject of parametric release from a purely scientific perspective, and takes the discussion in an entirely new direction level. Objective science would certainly preclude the imposition of the sterility test for terminally sterilized products, where it quite literally serves no beneficial purpose. The statistical limitations of the test are such that if it were submitted to the global compendia as a proposed new general test, it would likely be rejected as lacking any real utility. It is our considered opinion that the sterility test will be eliminated for any material subjected to a validated terminal sterilization process. Sterility testing will be acknowledged for the anachronism that it is for terminal processes; a means for release of sterile products that was conceived in an era of substantially less capability that has been largely transcended by present day validation capabilities. Its continuance in the global compendia is not justifiable considering its statistical limitations that could have been overlooked at the time of its adoption, but given the present state of the sterilization proficiency is a useless and arbitrary constraint on operations. We can also envision a time in the future, where this same statement could be made with respect to some future aseptic processing technology.

Visual and Automated Parenteral Inspection

Visual inspection of parenteral products is driven by the need to minimize the introduction of unintended particulate matter to patients during the delivery of injectable medications. Such inspection also offers the opportunity to reject nonintegral units, such as those with cracks or incomplete seals, which pose a risk to the sterility of the product. The desire to detect these defects at a very low frequency and the randomness of their occurrence has resulted in the current expectation for inspection of each finished unit (100% inspection).

[g]Dr Sasaki explained that in the case of aseptic processing, there is no means to demonstrate a SAL of $<10^{-6}$ in terms of aseptic processing parameters. So at this time, it is impossible to permit parametric release for aseptically filled products. In the future, it may be possible to accept parametric release for aseptic processing from some parameter(s) as yet undefined or currently undeterminable.

Human-based visual inspection historically has been done against a white and a black background using 100-W incandescent lighting. Such inspection is labor intensive and the results are subjective, depending on the skill and training of the inspectors. The results can be influenced by the inspection technique vis-à-vis manipulation of the inspected containers to optimize the location of any particulate matter that may be present and interpretation of what constitutes a defect, for example, bubble in the wall of the container, tightness of aluminum seal, etc.

Automated inspection equipment has been designed to improve the efficiency and accuracy of the inspection process, eliminating the subjectivity of human inspection. Automated inspection equipment is designed to detect particles in the product being inspected and to detect container and closure defects that may compromise the product, that is, cracks, pinholes, leaks, and loose seals. While human inspectors can inspect for both types of defects, machine systems are usually designed to inspect for one or the other, although some automated inspection systems perform both tasks. Automated inspection equipment utilizes light extinction, light scattering, lasers, light-emitting diodes, high-intensity lighting, video cameras, polarization, moving and stationary inspection technologies coupled with microprocessors to detect and categorize the various types of defects.

Automated inspection should be validated to ensure the results are at least as good as those obtained by a visual inspection performed by a well trained and qualified human inspector performing the inspection according to pharmacopoeial standards.

New technologies will certainly be developed to improve the speed and accuracy of automated inspection. One important factor will be manipulation of the container to focus the location of visible particulate matter to improve its detection capability.

PAT for On-line Release of Lots

PAT was introduced to the pharmaceutical industry by FDA's Office of Pharmaceutical Science, a branch of CDER, in 1996 as the Process Analytical Technology Initiative. Part of the initiative was a document titled "Guidance for Industry PAT—A Framework for Innovative Pharmaceutical Development, Manufacturing, and Quality Assurance." According to the guidance, an important FDA goal was "to tailor the Agency's usual regulatory scrutiny to meet the needs of PAT-based innovations that (1) improve the scientific basis for establishing regulatory specifications, (2) promote continuous improvement, and (3) improve manufacturing while maintaining or improving the current level of product quality" (45). More than 10 years later, pharmaceutical companies are still wrestling with ways to implement PAT principles and technologies in their manufacturing operations.

Historically, and as required in the GMP regulations, manufacturers had produced pharmaceutical products and tested them at various intervals in the production process to ascertain whether they meet predetermined quality standards. The manufacturing process and testing requirements were predicated on the contents of the approved application, which could in general only be changed through resubmission and approval of a supplement. The FDA initiative titled "Pharmaceutical CGMPs for the 21st Century—A Risk-Based Approach," introduced in 2002, effectively paved the way for implementation of PAT. The initiative provided for the following:

- Encourage the early adoption of new technological advances by the pharmaceutical industry.
- Facilitate industry application of modern quality management techniques, including implementation of quality systems approaches, to all aspects of pharmaceutical production and quality assurance.
- Encourage implementation of risk-based approaches that focus both industry and agency attention on critical areas.
- Ensure that regulatory review, compliance, and inspection policies are based on state-of-the-art pharmaceutical science.
- Enhance the consistency and coordination of FDA's drug quality regulatory programs, in part, by further integrating enhanced quality systems approaches into the agency's business processes and regulatory policies concerning review and inspection activities.

PAT, coupled with robust and effective product development, risk analysis, and risk management practices, can improve manufacturing efficiencies and the quality of products reaching the consumer. Minor and unavoidable differences in active ingredients, excipients, and packaging components can be accommodated through the use of on-line, at-line sampling and testing coupled with automated process control. As sensor technology improves and automated control systems are implemented, PAT will replace conventional manufacture, hold, sample, test, and release quality control practices.

A PAT system used for tablet production provides an example of the efficiencies and process control advantages of such a system. The process starts with the automated addition and in-line blending of the active ingredient and excipients, which are discharged into a process hopper. Rates of addition and mixing are automatically controlled. Sensors in the hopper, coupled with mass-balance sensors in the feed system, ensure the correct concentration of the active ingredient and monitor blend uniformity.

The hopper discharges into a series of high-speed tablet presses equipped with hardness monitoring and weight control sensors. The produced tablets are also examined on-line with optical sensors to detect physical defects that may be present. Automated filling machines package and label the tablets as they are discharged from the compression step. Cap torque and correct labeling, control number, and expiration date are automatically verified. The packaged tablets are packed into shippers, the labeling of which is verified on-line, and the shippers are transferred by an automated conveyor system to a quarantine area in the warehouse, awaiting final batch record review and release. The batch records are generated automatically with input from the on-line process control systems and are reviewed by the quality control unit. Once production is complete, the process train, with the exception of the tablet presses, is cleaned in place. PAT systems are used in clean-in-place systems, monitoring and controlling those processes to ensure systems are clean and free of objectionable levels of residues. The process is estimated to reduce time between the start of production and batch release by 80% compared with the conventional production system it replaced.

PAT is likely in the next 10 years to become the norm for many types of products, stimulated by improved levels of process understanding and control and production efficiencies. The PAT and GMP for the 21st century initiatives, coupled with ICH Q8, 9, and 10, will make this a reality.

CONCLUSION

The materials included in this chapter likely represent only a portion of those likely to impact the manufacture of sterile products. Those who might read this chapter 20 or more years in the future will be either surprised by our insight, or humored by our misconceptions. In either case, we have little doubt, that by that time the preparation of sterile products will have been altered by one or more of the potential influences described above. More than 2000 years ago, the Greek historian, opined that "the only constant is change" (46). We wholeheartedly agree.

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Index

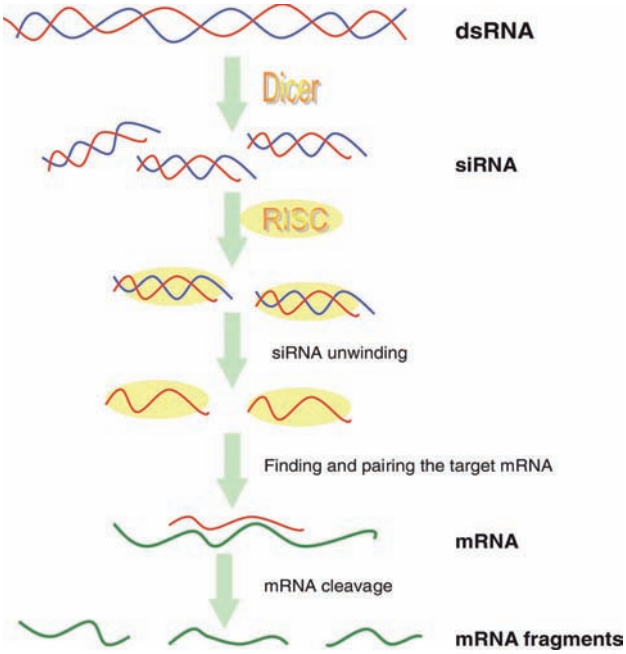


Figure 6.1 Mechanism of RNA interference (*see page 87*).

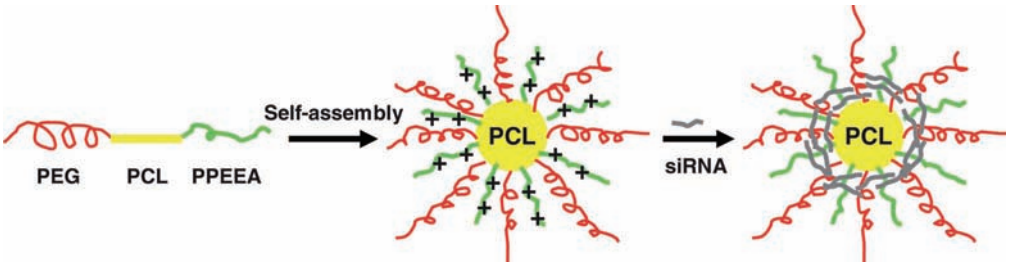


Figure 6.5 Self-assembling of cationic micellar nanoparticles and loading of siRNA. *Abbreviation*: siRNA, small interfering RNA. *Source*: Adapted from Ref. 73 (*see page 95*).

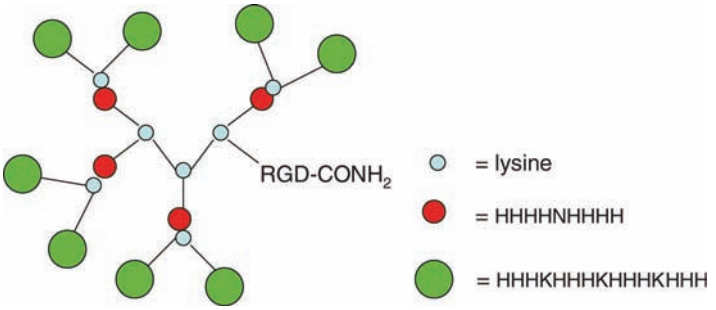


Figure 6.6 Schematic structure of H^3K8b polymer (*see page 96*).

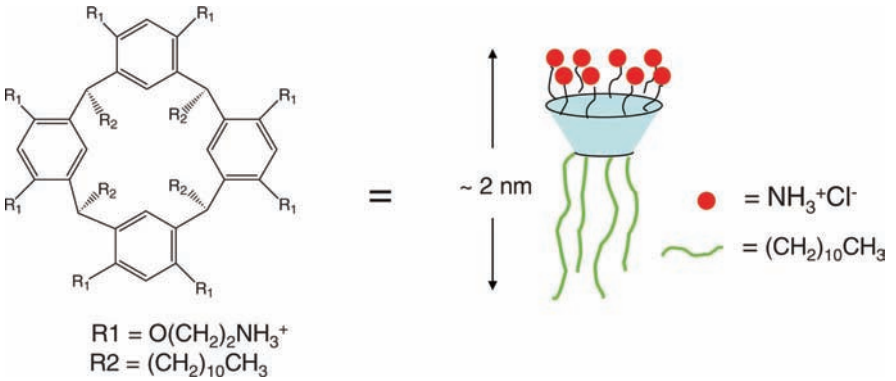


Figure 6.8 Cone-shaped structure of macrocyclic octaamine. *Source*: Adapted from Ref. 88 (*see page 98*).

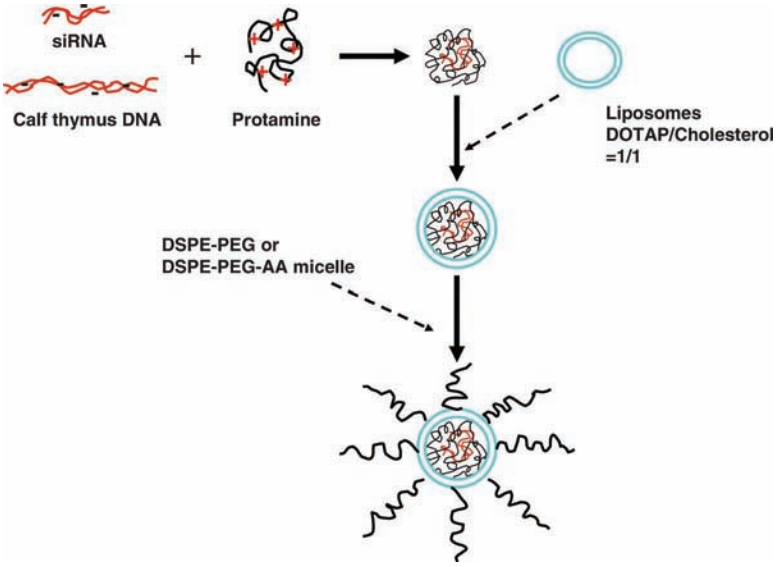


Figure 6.9 Preparation of PEGylated LPD. *Abbreviation*: LPD, liposome-polycation-DNA. *Source*: Adapted from Ref. 90 (*see page 99*).

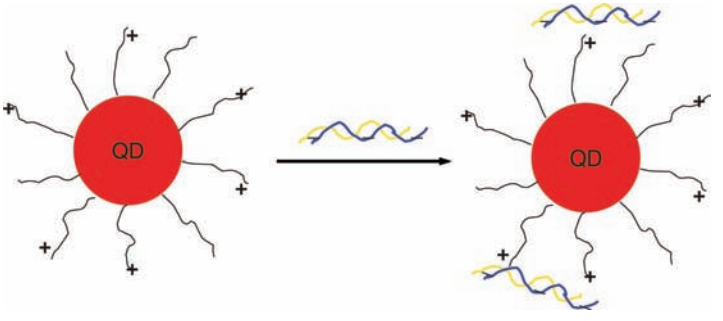


Figure 6.10 Adsorption of siRNA onto surface-modified QDs. *Abbreviations*: siRNA, small interfering RNA; QDs, quantum dots (*see page 102*).

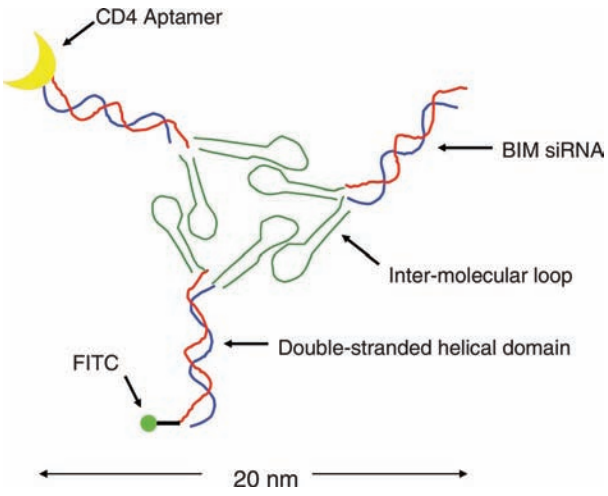


Figure 6.11 Schematic structure of engineering pRNA nanoparticle containing siRNA, aptamer, and fluorescent label. *Abbreviations*: pRNA, packing RNA; siRNA, small interfering RNA (*see page 103*).

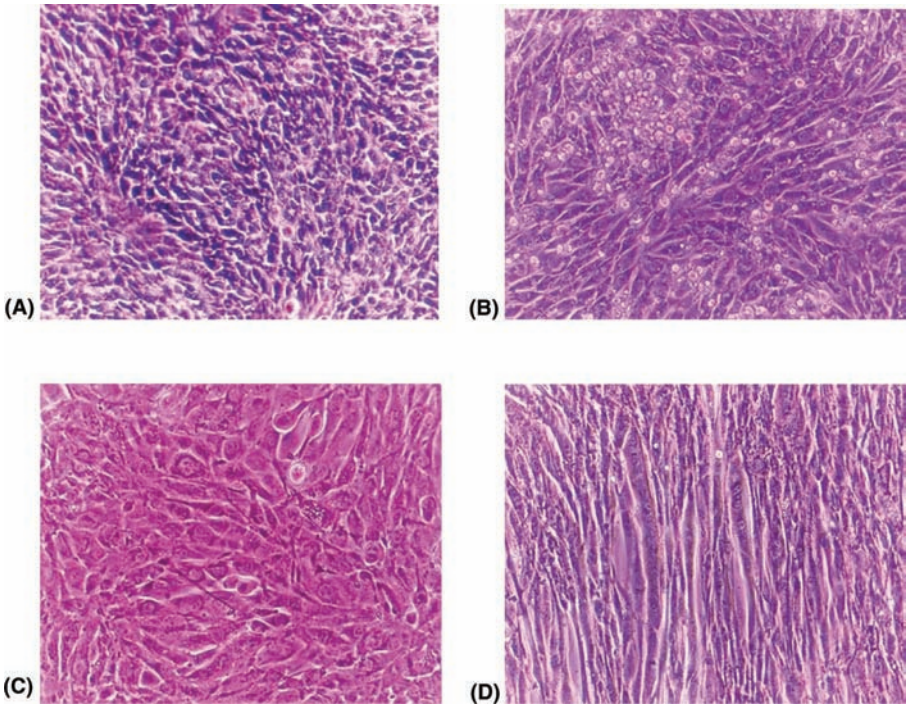


Figure 8.4 (**A**) Four-day-old L6 myoblasts in GM. (**B**) L6 myotubules at day 6 in DM (2% FBS in DMEM) during fusing process. (**C**) Four-day-old C2C12 in GM. (**D**) C2C12 myotubules at day 6 in DM (10% HS in DMEM). *Abbreviations*: GM, growth medium; DM, differentiation medium; FBS, fetal bovine serum; HS, horse serum. (*see page 140*).

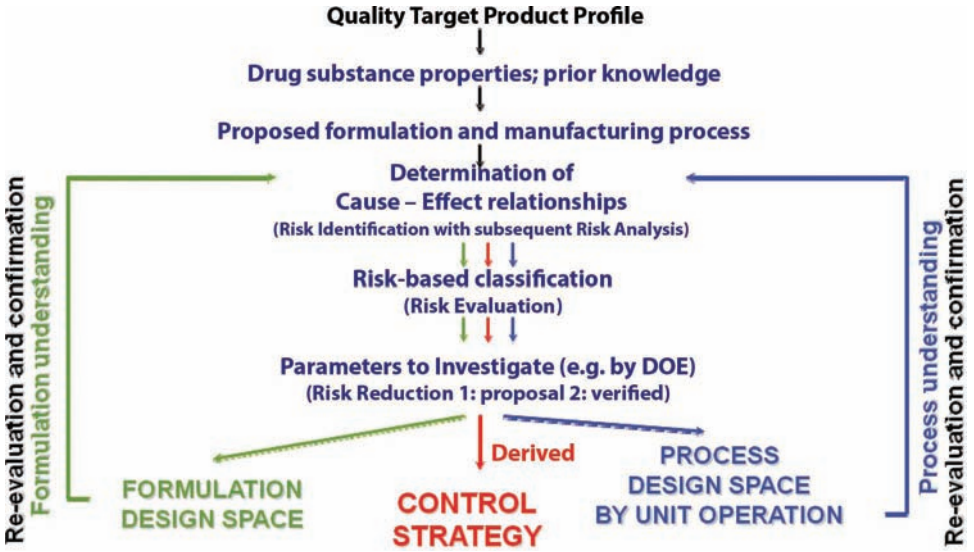


Figure 13.2 Process flow diagram describing the approach to developing process understanding and building quality into formulation and manufacturing process design (*see page 249*).

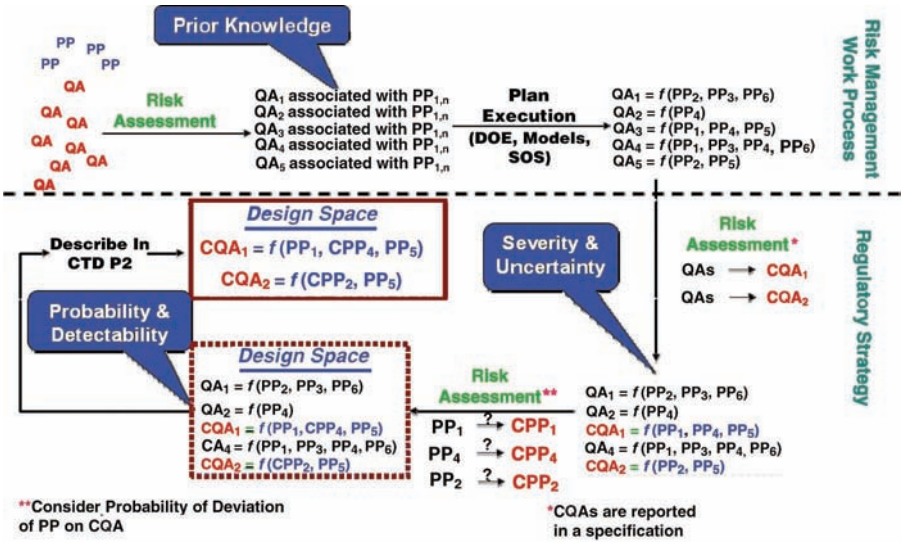
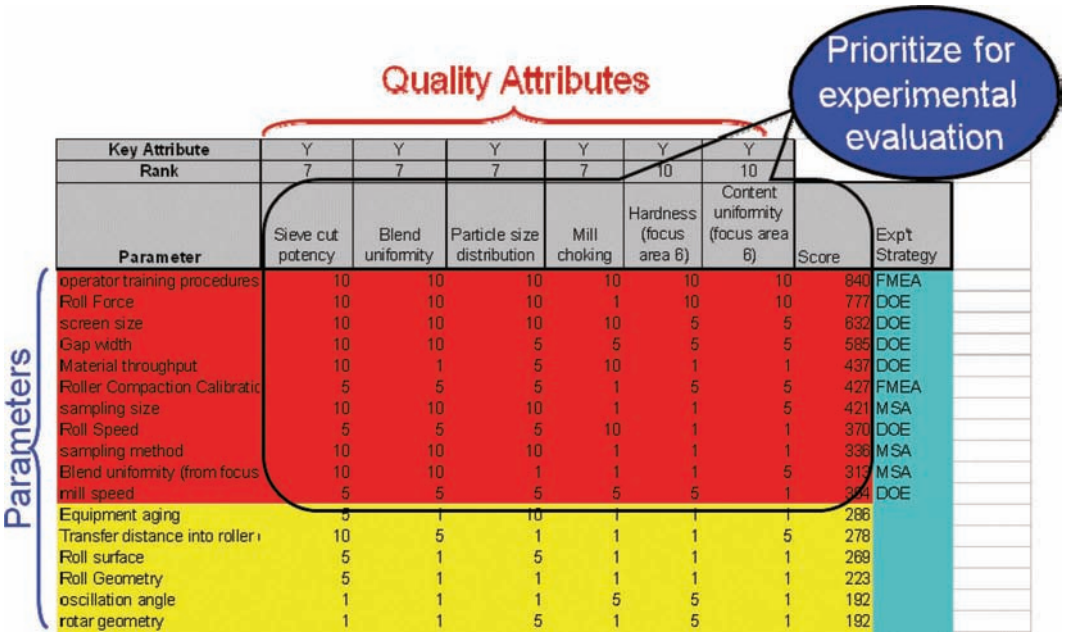


Figure 13.5 Schematic example of the quality risk management process. *Abbreviations*: PP, process parameters; CQA, critical quality attribute; CTD, Common Technical Document. *Source*: From Refs. 22,23,26 (*see page 253*).



Key Attribute	Y	Y	Y	Y	Y	Y		
Rank	7	7	7	7	10	10		
Parameter	Sieve cut potency	Blend uniformity	Particle size distribution	Mill choking	Hardness (focus area 6)	Content uniformity (focus area 6)	Score	Expt Strategy
operator training procedures	10	10	10	10	10	10	840	FMEA
Roll Force	10	10	10	1	10	10	777	DOE
screen size	10	10	10	10	5	5	632	DOE
Gap width	10	10	5	5	5	5	585	DOE
Material throughput	10	1	5	10	1	1	437	DOE
Roller Compaction Calibratic	5	5	5	1	5	5	427	FMEA
sampling size	10	10	10	1	1	5	421	MSA
Roll Speed	5	5	5	10	1	1	370	DOE
sampling method	10	10	10	1	1	1	336	MSA
Blend uniformity (from focus	10	10	1	1	1	5	313	MSA
mill speed	5	5	5	5	5	1	304	DOE
Equipment aging	5	1	10	1	1	1	286	
Transfer distance into roller	10	5	1	1	1	5	278	
Roll surface	5	1	5	1	1	1	269	
Roll Geometry	5	1	1	1	1	1	223	
oscillation angle	1	1	1	5	5	1	192	
rotar geometry	1	1	5	1	5	1	192	

Figure 13.6 Cause-and-effect matrix for distinguishing important quality attributes and process parameters for subsequent evaluation (*see page 254*).

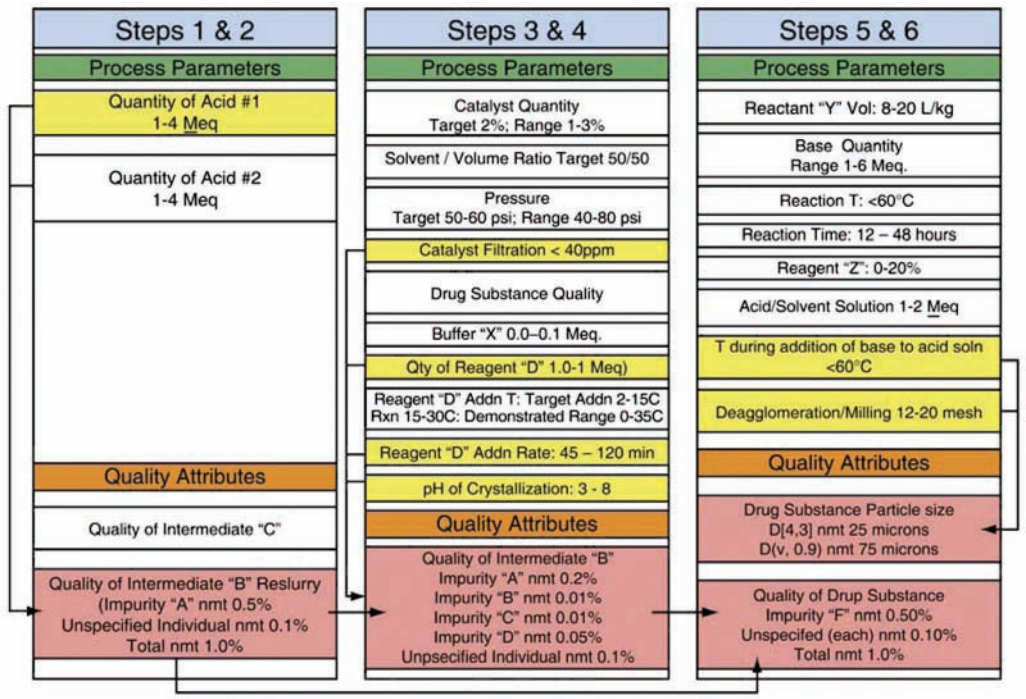


Figure 13.10 Schematic description of design space criteria (*see page 260*).

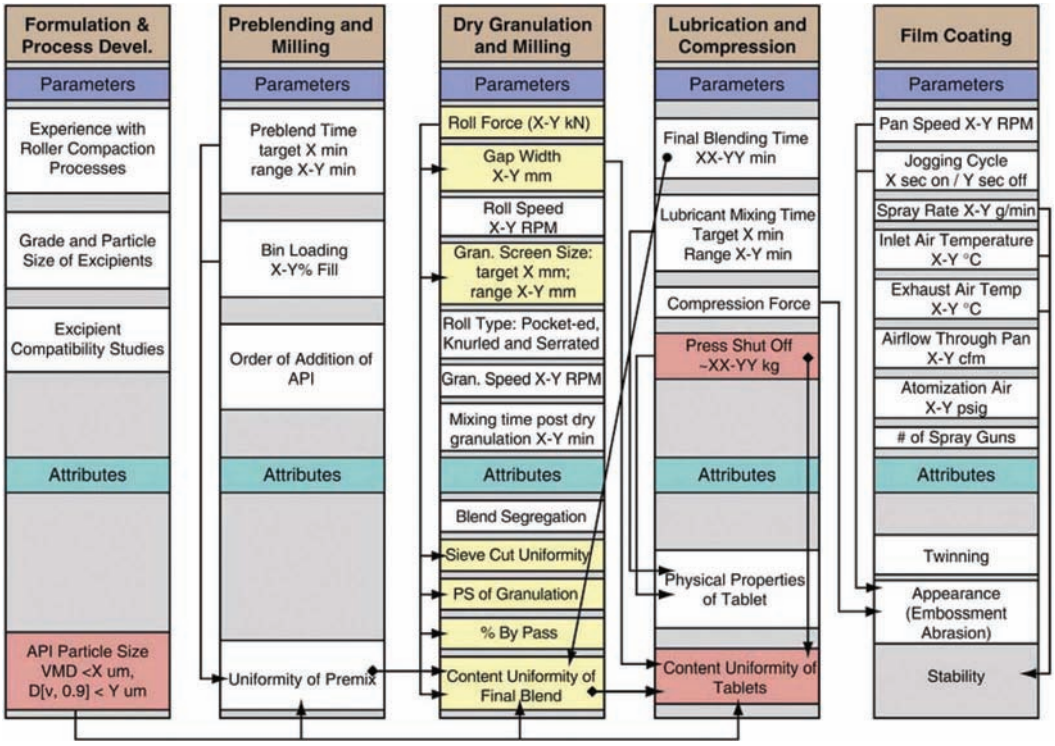
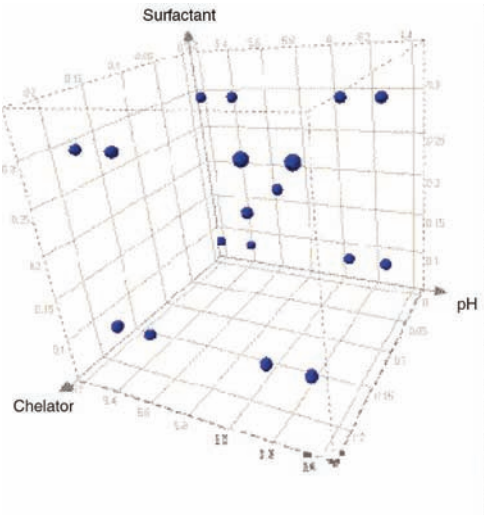


Figure 13.11 Design space for a drug product manufacturing process (*see page 261*).



6	5.3	0.3	0.02	84	20
7	5.3	0.3	0?	84	20
8	5.3	0.1	0.2	84	20
11	5.3	0.1	0.02	84	20
1	5.5	0.3	0.2	84	20
15	5.5	0.3	0.02	84	20
17	5.5	0.1	0.2	84	20
18	5.5	0.1	0.02	84	20
19	5.8	0.2	0.05	84	20
12	5.8	0.2	0.11	84	20
13	5.9	0.2	0.11	04	20
2	6.1	0.3	0.2	84	20
5	6.1	0.1	0.02	84	20
9	6.1	0.3	0.02	84	20
16	6.1	0.1	0.2	84	20
3	6.3	0.3	0.02	84	20
4	6.3	0.3	0.2	84	20
10	6.3	0.1	0.02	04	20
14	6.3	0.1	0.2	84	20

Figure 13.12 Example of multifactorial design to determine optimum concentrations of formulation parameters for a biologic (*see page 262*).

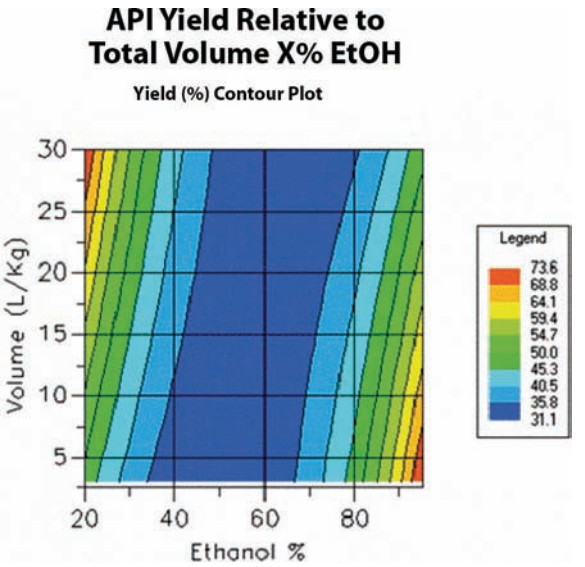


Figure 13.14 Example of a contour plot of design space for drug substance crystallization yield (*see page 264*).

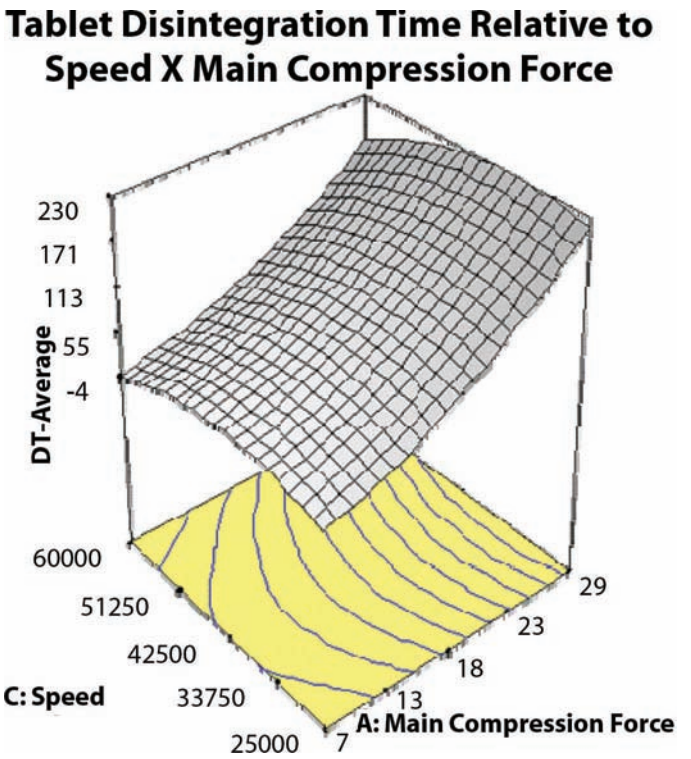


Figure 13.15 Contour plot describing design space for tablet disintegration time (*see page 264*).

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