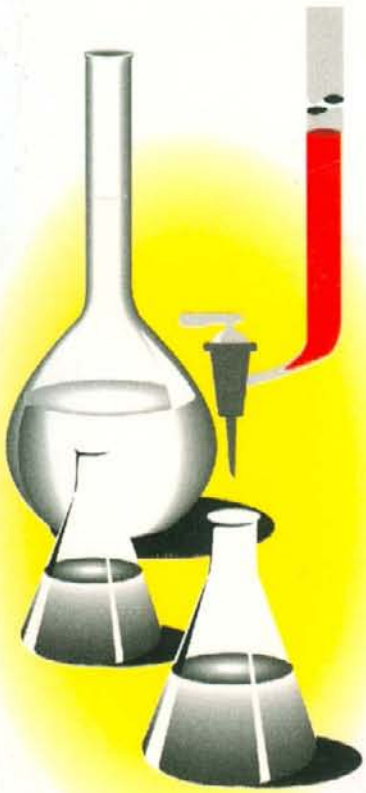
Revised Second Edition

PHARMACEUTICAL DRUG ANALYSIS



Ashutosh Kar

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PHARMACEUTICAL DRUG ANALYSIS

Methodology-Theory-Instrumentation
Pharmaceutical Assays-Cognate Assays

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and
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Preface to the Second Edition

Modern **Pharmaceutical Drug Analysis** essentially involves as a necessary integral component even greater horizons than the actual prevalent critical analysis of not only the active pharmaceutical substances but also the secondary pharmaceutical product(s) *i.e.,* the dosage forms having either single or multi-component formulated product. The fundamental reasons for this sudden legitimate surge in the newer evolving methodologies in the **'analysis of drug substances'** are perhaps due to the tremendous growth in the progress of 'medicinal chemistry' towards achieving one ultimate objective which is to obtain **'better drugs for a better world'.**

With the advent of computer-aided-drug modeling (CADM) the critical, scientific and faster approach to newer drug entities based on the biologically active prototypes, combinatorial chemistry, chiral chemistry and biotechnology has paved the way towards more specific, potent and above all less toxic **'drugs'** to improve the ultimate quality of life in humans.

Keeping in view the above astronomical growth in the design of complicated, specific and highly active drug molecules an equally viable, rigorous, accurate and precise analytical methods have been evolved with the passage of time which have now occupied pivotal and vital positions in most of the **Official Compendia** *viz*., USP, BP, Int.P., Eur. P, IP etc., for the analysis of such compounds both in pure and dosage forms.

The articulated developments in the fields of science and technology being utilized as on date, amalgamated with relatively stringent new regulations, namely: Federal Drug Authority (FDA); International Conference on Harmonization (ICH); Current Good Manufacturing Practices (cGMP); Pre-Approval Inspections (PAIs) and the like are now serving as a **'legal binding'** specifically for the pharmaceutical drug analysis even much more complicated in comparison to the situation prevailing almost two decades ago.

The present revised textbook on **'Pharmaceutical Drug Analysis'** caters for the much needed handbook and reference book, which is absolutely current with regard to the esteemed philosophy of analytical chemistry, an obvious solid support towards drug discovery, development, stability studies, bioavailability and pharmacokinetic studies, and above all the quality assurance of pure drugs together with their respective dosage forms.

The *thirty-two different chapters* meticulously divided into *six parts* invariably covers up analytical techniques being used in most of the **Official Compendia.** Each chapter categorically and explicitly deals with the introduction, theoretical aspect(s), instrumentation, typical examples of pharmaceutical analysis and cognate assays.

The textbook on **'Pharmaceutical Drug Analysis'** would enormously serve the undergraduates, postgraduates, researchers, analytical chemists working in the Quality Assurance Laboratories, new drug development, production and control, teaching, or regulatory authorities.

ASHUTOSH KAR

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Preface to the First Edition

The ever expanding and broad horizon of Pharmaceutical Sciences invariably emphasizes one cardinal aspect that basically they are nothing but 'Applied Sciences.' With the advent of newer drug molecules either partially synthesized, totally synthesized or isolated from naturally occurring microbial and plant products—it has become absolutely necessary to ascertain and examine critically their physical characteristics, chemical equivalence, chemical impurities and their prescribed limits, degradation of products, metabolites and above all their biological features. All these salient features of a '*drug*' help a researcher not only in planning a precise experimental design but also in the interpretation of data in a logical and scientific manner. Pharmaceutical scientists ought to have a good command over the wide-spectrum of chemical analysis so as to achieve completeness in their scientific pursuit of knowledge. Unfortunately, such information is either found scattered in various available literatures or appears as an extremely specific work on a rather scanty and limited subject area.

The main objective of **'Pharmaceutical Drug Analysis'** is to offer not only a ready reference handy textbook but also an intermediate level of coverage for the convenient analysis of pure pharmaceutical substances and their respective dosage forms wherever applicable. The present copious textual compilation of information is solely intended to narrow down the apparently wide gap existing between the available basic texts and the extremely specific research papers from various scientific journals. The contents of this textbook have been meticulously designed to provide fundamentals of various disciplines embodying pharmaceutical drug analysis specifically for the under-graduate students. It will also be useful to the graduate students studying modern methods of pharmaceutical analysis to a great extent. Particular emphasis has been laid on the pharmaceutical substances that are specially found in the *Official Compendia*. It will also cater to scientists and investigators, working in other fields of pharmaceutical sciences who wish to update their personal wealth of knowledge and understanding of the intricacies of modern methods of Pharmaceutical Drug Analysis. Enough literature have been cited at the end of each chapter under 'Recommended Readings' so as to enable the reader to follow up a particular topic with ease.

Part—I has three chapters that exclusively deal with 'General Aspects' of pharmaceutical analysis. Chapter 1 focuses on the pharmaceutical chemicals and their respective purity and management. Critical information with regard to description of the finished product, sampling procedures, bioavailability, identification tests, physical constants and miscellaneous characteristics, such as : ash values, loss on drying, clarity and color of solution, specific tests, limit tests of metallic and non-metallic impurities, limits of moisture content, volatile and non-volatile matter and lastly residue on ignition have also been dealt with. Each section provides adequate procedural details supported by ample typical examples from the *Official Compendia*. Chapter 2 embraces the theory and technique of quantitative analysis with specific emphasis on volumetric analysis, volumetric apparatus, their specifications, standardization and utility. It also includes biomedical analytical chemistry, colorimetric assays, theory and assay of biochemicals, such as : urea, bilirubin, cholesterol; and enzymatic assays, such as alkaline phosphatase, lactate dehydrogenase, salient features of radioimmunoassay and automated methods of chemical analysis. Chapter 3 provides special emphasis on errors in pharmaceutical analysis and their statistical validation. The first aspect is related to errors in pharmaceutical analysis and embodies classification of errors, accuracy, precision and makes

an attempt at minimizing systematic errors. The second aspect is mainly devoted to statistical validation and comprises of statistical treatment of finite samples, distribution of random errors, significant errors, comparison of results, method of least squares and criteria for rejection of an observation.

The various modern techniques involved in Pharmaceutical Drug Analysis mostly covered in the *official compendia* have been adequately dealt with in Part II through Part VI and systematically spread over from Chapter 4 through Chapter 32 in the present textual compilation.

Each chapter has its unique style of presentation that essentially comprises of the following vital features, namely : brief introduction, theory with necessary details and relevant reactions, instrumentation, assay methods—with typical appropriate examples invariably selected from the *Official Compendia* including brief theoretical treatment of individual pharmaceutical substance and dosage form, materials required, procedures, calculations wherever applicable, cognate assays and lastly citation of relevant literature under 'Recommended Readings'.

Part—II contains twelve chapters under the broad category of 'Chemical Methods'. Section—A deals on treatment by 'titrimetric methods' based on acidimetry and alkalimetry. The first arm of this section deliberates on aqueous titrations (Chapter 4), while the second on non-aqueous titrations (Chapter 5). Section—B relates to 'redox methods' with specific reference to permanganate, dichromate and ceric sulphate titration methods (Chapter 6); and also the iodimetric and iodometric titrations (Chapter 7). Section—C concerns with the 'precipitation methods' and focuses on argentometric methods (Chapter 8). Section—D comprises the 'complexometric methods' using organic ligands, such as EDTA. Particular stress has been laid on the effect of pH on complexation, stability of complexes, usage of pM indicators and masking and demasking agents (Chapter 9). Section—E solely embodies the conventional 'gravimetric methods'. The topic has been treated with respect to Law of Mass Action, reversible reactions, principle of solubility of product and common-ion effect. Typical examples have been included of pharmaceutical substances assayed after conversion to free acid, or free base, or free compound and lastly to derivatives or substitution products (Chapter 10). Section—F is entirely devoted to 'thermoanalytical methods' consisting mainly of thermogravimetric analysis (TGA), differential thermal analysis (DTA) and lastly thermometric titrations (TT) (Chapter 11). Section—G particularly embodies the 'miscellaneous methods' which do not fall into the regimen of Section—A through Section—F. It deals with diazotization (Chapter 12), estimation of phenols and related compounds (Chapter 13) using bromine or potassium bromate, potassium iodate solutions; Karl Fischer method for determination of water (Chapter 14); and lastly tetrazolium assay of steroids (Chapter 15).

Part—III exclusively treats 'Electrochemical Methods' invariably and extensively used in the analysis of pharmaceutical substances in the *Official Compendia*. Two important methods, namely; potentiometric methods (Chapter 16) deal with various types of reference electrodes and indicator electrodes, automatic titrator; besides typical examples of nitrazepam, allopurinol and clonidine hydrochloride. Amperometric methods (Chapter 17) comprise of titrations involving dropping-mercury electrode, rotating—platinum electrode and twin-polarized microelectrodes (*i.e*., dead-stop-end-point method).

Part—IV has been entirely devoted to various 'Optical Methods' that find their legitimate recognition in the arsenal of pharmaceutical analytical techniques and have been spread over nine chapters. Refractometry (Chapter 18) deals with refractive index, refractivity, critical micelle concentration (CMC) of various important substances. Polarimetry (Chapter 19) describes optical rotation and specific optical rotation of important pharmaceutical substances. Nephelometry and turbidimetry (Chapter 20) have been treated with sufficient detail with typical examples of chloroetracyclin, sulphate and phosphate ions. Ultraviolet and absorption spectrophotometry (Chapter 21) have been discussed with adequate depth and with regard to various vital theoretical considerations, single-beam and double-beam spectrophotometers; besides typical examples amoxycillin trihydrate, folic acid, glyceryl trinitrate tablets and stilbosterol. Infrared spectrophotometry (IR) (Chapter 22) essentially deals with a brief introduction of group-frequency

region and fingerprint region followed by detailed theoretical aspects covering molecular vibrations and factors influencing vibrational frequencies. Having described the single monochromator infrared spectrophotometers, the applications of IR-spectroscopy have been discussed with respect to pharmaceutical substances and pharmaceutical dosage forms. Analytical aspects of IR-spectroscopy have also been treated adequately for the determination of *cis-trans* isomer ratio in clomiphene citrate, distinction of pri-, sec- and tert-amine salts from one another, studying complex formations and quantitative reaction sequences, identification of functional groups and fingerprinting.

Nuclear resonance spectroscopy (NMR) (Chapter 23) treats the subject with regard to the NMR-phenomenon and proton-NMR. Various theoretical aspects viz., orientations of magnetic nucleus under external-magnetic field (Bo), precessional frequency, saturation of the signal, absorption positions in NMR-spectrum, chemical shift, spin-spin interactions, ^{3}H-NMR, ^{13}C-NMR and 2D-NMR. Special emphasis has been given to the interpretation of a NMR-spectrum, chemical shift, relative peak area, multiplicity of the signal and coupling constant. Instrumentation has been dealt adequately. Applications of NMR-spectroscopy in pharmaceutical analysis, identification testing and assay of drugs have been treated so as to justify their vital importance in modern methods of analysis.

Emission spectroscopy (Chapter 24) provides a brief introduction, theory and instrumentation with regard to its excitation sources, electrodes, sample handling, monochromators, detectors, spectrographs and its applications. Flame spectroscopy (Chapter 25) widely used in the quantitative estimation of various elements *e.g*., Na, K, Ca, Ba has also been included. Both simple flame photometer and internal-standard flame photometer have been discussed in sufficient detail. The assay of Na, K and Ca in blood serum and water; assay of Ba, K and Na in calcium lactate have been described followed by cognate assays. Atomic absorption spectroscopy (AAS) (Chapter 26) treats this versatile aspect of analytical technique at length. It deals with the merits of AAS Vs Flame Emission Spectroscopy (FES) specifically treats both the single-beam and the double-beam AAS followed by the dements of AAS. Instrumentation specifically treats both the single-beam and the double-beam AAS. The various aspects of AAS *e.g*., analytical techniques, detection limit and sensitivity, and interference have been duly covered. A few typical examples of AAS in pharmaceutical analysis *e.g.*, Zn in Insulin-Zinc Suspension, Pd in Carbenicillin Sodium have been described followed by cognate assays.

Part—V is solely confined to the 'Assay Methods based on Separation Techniques' and is spread over five chapters. Liquid-liquid extraction (Chapter 27) mostly treats the subject theoretically and is supported by appropriate examples. Errors due to the volume change and effectiveness of an extraction have been dealt with adequately. Various factors that influence solvent extraction, such as : temperature and inert solutes, pH ion-pair formation and synergistic extraction have been described. A number of typical assay methods, for instance : Cu (I) as the neo-cuproin complex, Fe (III) as the 8-hydroxy quinolate complex, Pb (I) by the dithizone method, Mo (VI) by the thiocyanate method and Ni (II) as dimethylglyoxime complex and by synergistic extraction have been discussed. Thin-layer chromatography (TLC) (Chapter 28) illustrates the versatility of this technique over paper and column chromatography. Various aspects of experimental techniques of TLC viz., preparation of thin layers on plates, choice of adsorbents and solvent systems, activation of adsorbent, purification of adsorbent layers, spotting of components, development of thin layers, special techniques in TLC, chemical reactions on TLC plates, combination of TLC with other techniques and finally detection and evaluation of chromatograms have been expatiated profusely with examples. Applications of TLC in pharmaceutical analysis have been discussed in sufficient length. Gas-liquid chromatography (GLC) (Chapter 29) exclusively treats the subject with regard to various theoretical aspects, namely: plate theory, rate theory, random walk and nonequilibrium theory. Instrumentation comprises mainly different vital components. The working techniques for quantitative analysis *e.g.*, area normalization and internal standard method have been described. Lastly the applications of GLC in pharmaceutical analysis have been described with suitable examples from the *Official Compendia*.

High Performance Liquid Chromatography (HPLC) (Chapter 30) gives an elaborate discussion of theoretical aspects. Instrumentation encompasses the various important components *e.g*., solvent reservoir and degassing system; pressure, flow and temperature; pumps and sample injection system;

columns; detectors; strip-chart recorder; data-handling device and microprocessor control. Another important aspect to facilitate HPLC known as the 'derivatization' has been discussed. The applications of HPLC in the assay of drugs, such as: cephalosporins, frusemide, theophylline, corticosteroids, dichlorphenamide, Human Insulin and lastly cognate assays have been fully elaborated.

Size Exclusion Chromatography (Chapter 31) has also been included as a means of analysis for substances that undergo separation more or less as per their molecular size, viz., insulin and human insulin—for proteins of higher molecular weight; corticotrophin—for impurities of higher molecular weights; and plasma-protein solution—for polymers and aggregates.

Part—VI has been solely devoted to 'Miscellaneous Assay Methods' wherein radioimmunoassay (RIA) (Chapter 32) has been discussed extensively. Various arms of theoretical aspects viz., hapten determinants and purity; importance of antigenic determinants; and analysis of competitive antibody binding of isotopically labeled compounds. The applications of RIA in pharmaceutical analysis, such as : morphine, hydromorphone and hydrocordone in human plasma; clonazepam, flurazepam in human plasma; chlordiazepoxide in plasma; barbiturates, flunisolide in human plasma have been described elaborately. Lastly, the novel applications of RIA-techniques, combined RIA-technique-isotope dilution and stereospecificity have also been included to highlight the importance of RIA in the analytical armamentarium.

It is earnestly believed that **'Pharmaceutical Drug Analysis'** will fulfill the entire requirements of both penultimate and final year students of B. Pharm., for their various courses in analytic chemistry in the universities. It may also help the post-graduate students in their compulsory paper on 'Modern Analytical Techniques' to a great extent.

'Pharmaceutical Drug Analysis' will prove to be a valuable and indispensable guide to those working in Research & Development Laboratories, Quality Assurance Laboratories as well as Drug Testing Laboratories where either new products are being developed or routine analyses are carried out. Academicians and researchers engaged in the evaluation of pharmaceutical drug substances either in pure or dosage forms will also enormously benefit from **'Pharmaceutical Drug Analysis'** by virtue of its ultimate goal of maintaining very high standards of quantitative analysis.

Finally, I wish to record here my special thanks to the numerous colleagues and friends who have not only extended their invaluable help by providing me with relevant sources of material but also by taking an active participation in the discussion of various chapters.

It is hoped that **'Pharmaceutical Drug Analysis'** will soon prove to be an invaluable guide to both undergraduate and postgraduate students and to my esteemed colleagues in the teaching profession. Those working in Research & Development Laboratories, Quality Assurance Laboratories and Drug Testing laboratories will also find the book helpful in solving many of their intricate problems.

ASHUTOSH KAR

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PART I

GENERAL ASPECTS

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1 PHARMACEUTICAL CHEMICALS : PURITY AND MANAGEMENT

CONTAINS :

1.1. INTRODUCTION

Since the Second World War a rapid development of **pharmaceutical chemicals**, and ultimately drugs, has made a quantum progress. Medicinal chemists, pharmacologists, biochemists, analytical chemists and medical professionals have paved the way with their single goal objective to combat the sufferings of human beings. In this integrated effort the role of an analyst *vis-a-vis* the chemical purity of pharmaceutical substances and drugs made therefrom and finally the dosage forms that are usually available for direct patient's usage, has become not only extremely crucial but also equally important and vital. As on date product safety has to be an integral part of all product research in pharmaceutical substances. However, the risk-beneft-ratio has got to be pegged to a bare minimum level. Therefore, it has become absolutely necessary to lay emphasis on product. safety research and development which is very crucial in all the developmental stages of a new secondary pharmaceutical product.

Inspite of all the qualified successes of synthetic drug research achieved in the last four decades to combat infectious diseases of the more than 80,000 different ailments, unfortunately only about one third can be treated with drugs, most of them only symptomatically. The discovery of better, effective and safer drugs is needed to fight the causes of dreadful diseases like cancer, acquired-immuno-deficiency-syndrome (AIDS), arthritis, cardio-vascular diseases, disorders of the central nervous system (CNS), such as : Alzheimer's disease and other vital infectious and metabolic diseases like rheumatoid arthritis.

In order to meet these challenges one needs to adopt novel approaches in pharmaceutical research. Both molecular biology and genetic engineering will be exploited duly in opening up new routes. Genetic

engineering may be explored in the development of new drugs, besides, being used as a research to investigate the molecular causes of severe and dreadful diseases.

It is earnestly believed that towards the beginning of the new century (2001 AD), keeping in view the tremendous global technological competition, one is left with no other choice than to internationalize research and development of pharmaceutical drugs to achieve the common objective '*better drugs for a better world*'.

It is, however, pertinent to mention here that pharmaceutical chemicals must maintain a very high degree of chemical purity. It is quite obvious that a state of absolute purity may not be achievable, but a sincere effort must be exercised to obtain the maximum freedom from foreign substances. Bearing in mind the exorbitant operational costs to attain the 'highest standards' of purity, perhaps some of these processes are not economically viable. Therefore, a compromise has got to be made to strike a balance between the purity of a substance at a reasonably viable cost and at the same time its purity *e.g.*, being fully acceptable for all pharmaceutical usages.

In short, a host of impurities in pharmaceutical chemicals do occur that may be partially responsible for toxicity, chemical interference and general instability.

In this chapter, the purity and management of pharmaceutical chemicals, would be discussed briefly so as to take adequate cognizance of the importance of standardization of these substances, in addition to their management by *Official Methods*.

1.2 PURITY

The standardization of '**pharmaceutical chemicals**' and the dosage forms prepared therefrom plays a vital role so that the patient gets the 'drug' within the permissible limits of potency and tolerance.

The standards for pharmaceutical chemicals and their respective dosage forms, as laid down in, various *Official Compendia* fulfil broadly the following *three* cardinal objectives, namely :

(*a*) Broad-based highest attainable standard,

(*b*) Biological response *versus* chemical purity, and

(*c*) Offical standards *versus* manufacturing standards.

1.2.1. BROAD-BASED HIGHEST ATTAINABLE STANDARD

Keeping in view the various methods of manufacture of a pharmaceutical substance *vis-a-vis* its standards of purity, types of impurity and changing pattern of stability, a broad-based highest attainable standard is always fixed. A few typical examples are stated below :

S.No.	Name of Substance	Standards of Purity* (%)
1.	Aspirin	99.5 - 100.5
2.	Atropine Sulphate	98.5 - 101.5
3.	Bendrofluazide	98.0 - 102.0
4.	Betamethasone	96.0 - 104.0
5.	Busulphan	98.0 - 100.5
6.	Caffeine	98.5 - 101.0
7.	Calcium Levulinate	97.5 - 100.5
8.	Carbamazepine	97.0 - 103.0
9.	Chloramphenicol	98.0 - 102.0
10.	Dexamethasone	96.0 - 104.0
11.	Diethyl toluamide	95.0 - 103.0
12.	Ethacrynic Acid	97.0 - 102.0
13.	Ferrous Sulphate	98.0 - 105.0
14.	Fluphenazine Hydrochloride	98.0 - 101.0
15.	Griseofulvin	97.0 - 102.0

16.	Mecamylamine Hydrochloride	95.0 - 100.5
17.	Nitrofurazone	97.0 - 103.0
18.	Procainamide Hydrochloride	98.0 - 101.0
19.	Salbutamol Sulphate	98.0 - 101.0
20.	Thyroxine Sodium	97.0 - 103.0

* Calculated with respect to the dried substance.

1.2.2. BIOLOGICAL RESPONSE *VS.* CHEMICAL PURITY

Though chemical purity is the topmost priority, yet the biological response of a pharmaceutical substance holds an equal importance. A wide variation of active ingredients ranging between 90% in one sample and 110% (± 10 per cent limit) in another sample could invariably be observed. Therefore, it has become absolutely essential to lay down definite standards so as to ensure that :

- Different laboratories may produce reasonably reproducible products.
- Difference in active ingredients in various lots may be minimised.
- Retention of acceptable level of potency.
- Freedom of toxicity during storage before use.

Examples :

(*i*) Substances to be stored in well-closed, light-resistant containers *e.g.*, isoniazid, nalidixic acid, nandrolone phenylpropionate, nitrofurazone.

(*ii*) Substances to be stored under nitrogen in tightly closed, light-resistant containers at a temperature between 2° and 10°C, *e.g.*, nandrolone decanoate, nystatin, methylergometrine maleate, human normal immunoglobulin.

(*iii*) Substances to be stored in tightly-closed, light-resistant containers in a cool place, *e.g.*, nitrofurantoin, pancreatin, oxyphenonium bromide.

(*iv*) Substances to be stored in tightly-closed, light-resistant containers in a cool place; for parenteral administration, the container should be sterile and sealed so as to exclude micro-organisms. *e.g.*, kanamycin sulphate, novobiocin sodium, benzylpenicillin, lincomycin hydrochloride, chloramphenicol.

(*v*) Substances to be stored in well-closed containers, at a temperature not exceeding 30°C, *e.g.*, procaine penicillin, pepsin, menthol, erythromycin.

1.2.3. OFFICIAL STANDARDS *VIS-A-VIS* MANUFACTURING STANDARDS

The **Offical Standards**, as stipulated in the pharmacopoeias of various countries, *e.g.*, IP BP, Eur. P., Int. P., USSRP, JP etc., of a pharmaceutical substance take cognizance of the purity, nature, methods and hazards of manufacture, precautions of storage and ultimately the conditions under which the product is to be used.

It is a well-known fact that a pharmaceutical substance can be prepared by adopting different routes of synthesis based upon the dynamic ongoing research in the field of organic-reaction-mechanisms. Relentless efforts are exerted vigorously by reputed research laboratories across the world to look for shorter routes of synthesis bearing in mind the cost-effectiveness of the final product. For instance : diclofenac sodium (an NSAID) can be manufactured by two methods, one using a bromo compound as a starting material while the other is based on a non-bromo compound. Nevertheless, the latter product is more in demand because it is completely devoid of bromine residues in the final product.

During the process of manufacture an unavoidable criterion is the loss of active ingredients. Therefore, all *Official Standards* for pharmaceutical chemicals and dosage forms should accomodate such losses caused due to loss in manufacture, unavoidable decomposition and storage under normal conditions for a stipulated period.

It has become an usual practice to include a 'definite overage' in certain dosage forms so as to compensate the noticeable losses caused either due to manufacturing or storage (anticipated decomposition), in order that the finished product may comply with the prescribed offcial standards after the stipulated duration of storage.

Official standards with regard to dosage form and packs, preservation and prevention from contamination in a variety of pharmaceutical products, such as eye-drops, multidose injections and antiseptic creams (external application) that may be prone to spoilage with prolonged repetitive usage should be well defined. The official standards, in general, legislate and control the presence of toxic impurities by prescribed '**limit tests**' and also by more sophisticated analytical techniques using thin-layer chromatography (TLC), high performance thin-layer chromatography (HPTLC), gas-liquid chromatography (GLC) and high-performance liquid chromatography (HPLC).

1.3. MANAGEMENT

Various *Official Compendia* of different countries categorically specify descriptive as well as informative details with regard to the pharmaceutical substances and formulated dosage forms produced therefrom. Hence, all pharmaceutical chemicals and finished products must rigidly conform to the laid-out standards in a particular country and are subjected to various checks at different levels either by Government/State owned drug testing laboratories or by Government/State approved drug testing laboratories.

Official Compendia for pharmaceutical substances usually include the following parameters, namely :

- Description of the Drug or Finished Product
- Identification Tests
- Physical Constants
- Assay of Pharmaceutical Substances
- Assay of Principal Active Ingredients in Formulated Dosage Forms
- Limit Test
- Storage Conditions

1.3.1. DESCRIPTION OF THE DRUG OR FINISHED PRODUCT

The description of a particular drug or finished product may essentially include the following details, namely :

- Brand Name of the Product
- Name of the Active Ingredient
- Strength of Active Igredient in Dosage Form
- Lot/Batch Number
- Date of Manufacture
- Date of Expiry
- Storage Conditions (if any)
- Separate Dosage for Adults and Children

1.3.2. SAMPLING PROCEDURES AND ERRORS

To collect a '*representative sample*' forms a vital aspect of analytical chemistry, because the samples subjected to analysis are assumed to be perfectly homogeneous and truly representative. Thus, sampling may be considered as the most critical aspect of analysis. In other words, the accuracy and significance of measurements may be solely limited by the sampling process. Unless and until the sampling process is performed properly, it may give rise to a possible weak link in the interpretation of the analytical results. For instance, the improper

handling of a blood sample both during and after sampling from a patient prior to an operation may not only pose serious complications but also may prove fatal.

A definite instruction with regard to the sampling of given materials have been duly put forward by a number of professional societies, namely :

- Association of Official Analytical Chemists (AOAC),
- American Society for Testing Materials (ASTM), and
- American Public Health Association (APHA).

However, a good deal of the wisdom of the analyst supported by the application of statisical results and wealth of experience may go a long way in achieving reasonably accurate and reproducible results.

1.3.2.1. Sampling Procedures

Samples may be categorized broadly into *four* heads, namely :

(*a*) **Gross Sample :** A sample that represents the whole lot and may vary from a few grams or less to several pounds based on the nature of the bulk material.

(*b*) **Sample :** A sufficiently small size of the sample exclusively for the purpose of analysis and derived from the representative gross sample.

(*c*) **Analysis Sample :** An aliquot or portion of the '**sample**' being subjected to actual analysis.

(*d*) **Grab Sample :** A single sample usually taken at random and assumed to be representative. It is considered to be the most unreliable way to sample a material.

1.3.2.1.1. *For Solids*

Sampling of solid materials are comparatively more difficult than other materials because of the following *three* reasons, namely :

(*a*) Variation in particle size.

(*b*) Inhomogeniety of the material.

(*c*) Variation within the particle.

Sampling of solids can be best accomplished by adopting the following procedures :

- To take 1/50 to 1/100th of the total bulk for gross samples.
- To take larger gross samples for products having larger particle size.
- To sample large bodies of solid materials while they are in movement to obtain aliquots representing all portion of the bulk.
- To handle tissue samples, several tiny parts of an organ may be taken and combined together.

1.3.2.1.2. *For Liquids*

Sampling of liquids may be carried out by following these procedures :

- Small heterogenous liquid samples are first shaken thoroughly and then followed by immediate sampling.
- Large volumes of liquids are best sampled immediately after a transfer; or if in a pipeline, after passing through a pump where it has undergone the most vigorous mixing.
- Large volumes of stationary liquids are normally sampled with a '*thief sampler*', *i.e.*, a device for collecting aliquots at different levels.
- Samples are best drawn (with a '*thief sampler*') at various depths diagonally instead of vertically down so as to have a better cross-section of the bulk liquid.

- Either separate aliquots of liquid may be analyzed individually and the results combined duly, or the various aliquots may be first combined into one gross sample and replicate analysis carried out. However, the latter method is preferred for obvious reasons since the analysis shall have a better hold on the accuracy and precision of the analysis.
- For sampling of biological fluids the 'time factor' is of utmost importance and hence, should be performed by qualified pathologists attached to clinical laboratories under adequate supervision. A few specific examples are stated below :

  (*a*) A 24 hour urine sample collections are usually more reliable than single specimens.

  (*b*) A sample for blood-sugar analysis is more reliable in a fasting patient.

  (*c*) A sample of cerebro-spinal-fluid (CSF) from the vertebral column by lumber puncture in patients having suspected pyogenic meningitis.

1.3.2.1.3. *For Gases*

A grab-type gas sample is usually satisfactory in certain cases. For example :

(*a*) A breathe sample may be collected by allowing the subject to blow into an evacuated bag. (Persons driving automobile under the '*influence of alcohol*' on high-ways during festive seasons).

(*b*) Auto exhaust may also be collected in large evacuated plastic bag to monitor the pollution by vehicles run by gasoline/diesel/CNG in cities and metropolis.

1.3.2.2. Errors

The famous adage—'*to err is human to forgive divine*'—literally means that it is natural for people to make mistakes. However, errors in analytical chemistry or more precisely in pharmaceutical drug analysis are normally of *three* types, namely :

(*a*) Determinate Errors

(*b*) Instrumental Errors

(*c*) Personal Errors

These above mentioned errors would be discussed briefly here with specific examples. It is pertinent to mention here that errors outside the range of 'permissible errors' in the analyses of pharmaceutical substances may cause serious problems because most of these substances are usually highly toxic, potent and used extensively in life-saving processes across the globe.

1.3.2.2.1. *Determinate Errors*

Errors caused due to either incorrect adoption of an assay method or an incorrect graduation read out by an analyst are termed as **determinate errors.** Such errors, in principle may be determined and corrected. In usual practice the determinate errors are subtle in nature and hence, not easily detected.

A few typical examples of determinate errors are stated below :

(*a*) **Gravimetric Analysis :** Where a compound is precipitated from a solution and the analyst believes that the analyte has been removed from the solution completely. Actually a small portion of the substance under investigation shall remain in solution. This sort of error is normally so insignificant that it is often neglected.

(*b*) **Incomplete Chemical Reaction :** Where a chemical reaction fails to attain the chemical equilibrium, thus virtually invalidating most calculations entirely based on chemical equilibrium characteristics. It may be eliminated by carrying out a detailed study of the reaction kinetics.

(*c*) **Colour-change at Endpoint :** Where a colour change is employed for an endpoint signal in a volumetric analysis. It may require an excess quantity of reagent to affect the colour change which ultimately shows completion of the chemical reaction between reagent and analyte. Hence, it is

absolutely necessary to determine this excess amount of added reagent, otherewise the analytical results may give a positive error. Therefore, in all such analytical procedures a '**blank titration**' is performed simultaneously to determine how much reagent is required to affect the colour change when no analyte is present.

1.3.2.2.2. *Instrumnetal Errors*

The past three decades have witnessed a quantum progress and advancement in the field of analytical chemistry. Nowadays, both **microprocessor based** and **computer-aided analytical instruments** have more or less replaced the manually operated ones in any reasonably good analytical laboratory. One of the most prevalent determinate errors is caused by analytical intruments which are found to be 'out of calibration'. Hence, it is very essential that such instruments need to be calibrated periodically, for instance, a pH meter is calibrated using a buffer solution of known pH, say adjusting the meter to read pH = 7.00 when a buffer of pH 7.00 is measured ; a single-pan electric balance is calibrated by using standard certified weight box; an UV-spectrophotometer is calibrated using standard solutions of known substances.

In a similar manner, the calibration of glassware, such as : volumetric flasks, pipettes, burettes, measuring cylindres are duly carried out by specific methods recommended by Indian Standards Institution (**ISI**), British Standards Institution (**BSI**), National Physical Laboratory (**NPL**), United States Pharmacopoeia (**USP**) at specified temperatures (See Chapter 2).

1.3.2.2.3. *Personal Errors*

In addition to errors caused due to improper assay methods or faulty instruments, it may also be due to the analyst. A few typical examples are cited below :

(*a*) **Physical Impairment :** A person suffering from colour blindness may not be in a position to assess colour-changes precisely ; or if he uses bifocals he may not take the burette readings accurately.

(*b*) **Learning-Curve Syndrome :** An analyst must practise a new assay method employing 'known' samples before making an attempt to tackle an unknown sample, thereby minimising the scope of personal errors.

1.3.3. BIOAVAILABILITY

According to a biopharmaceutic expert, the term bioavailability may be defined as the rate and extent to which the ingredient is absorbed from the drug product into the body or to the site of action. It is measured by blood, serum or plasma levels or from urinary excretion data.

1.3.3.1. Importance

There are *three* major factors that govern the efficacy of a dosage form, namely :

(*a*) Onset of therapeutic activity.

(*b*) Intensity of the therapeutic effect.

(*c*) Duration of the therapeutic effect.

The above three factors are solely responsible for the rate of absorption of the drug, the distribution of the drug throughout the circulatory system and above all the elimination of the active principle from the body.

Official quality control methods adopted, *e.g.*, disintegration time and dissolution rate, do not give ample therapeutic equivalence among drug products belonging to the same class. Moreover, even the products of the same manufacturer may have varying degree of bioavailability in different batches. Therefore, it has become quite necessary to introduce comparative bioavailability studies and skillfully designed fool-proof clinical tests of therapeutic equivalence as an effective true remedial measure of the ultimate performance of drug products.

In 1968, fifty-one patients suffered from an epidemic of anticonvulsant intoxication in Brisbane. A thorough investigation revealed that the intoxication was caused by altering one of the excipients from calcium phosphate to lactose in the drug product Phenytoin Capsule without adequate pre-testing by the manufacturer.

This apparent minor change of excipient was sufficient enough to bring about an appreciable major change in enhancing the bioavailability of the active principles to abnormally high levels in the affected patients.

1.3.3.2. Question of Quality

It has now been established beyond any reasonable doubt that quality of a drug product cannot simply be ensured by inspection or analysis, but a control system has to be built into, from the very beginning of manufacture of a drug. Besides effective quality control measures exercised in every aspects of production including environment, screening of raw materials, process controls, intermediate shelf-life of finished products the most important aspect is to assess the bioavailability of the active principle.

Difference in bioavailability, particularly in drugs with low solubilty, as ascertained by blood level attainment studies, appears to be caused by a number of formulation variables, namely : particlesize, crystalline structure, binding or disintegrating agent, excipient etc., on the release pattern of the drug in its dosage from. For example : the rate of dissolution of the drug in tablet or a capsule in the gastrointestinal fluids.

1.3.3.3. Clinical Efficacy of Drugs

Medical scientists mainly rely on the measurement of bioavailability of a drug as a positive indicator of therapeutic equivalence, because clinical efficacy for orally administered drugs depends on the degree of absorption and the presence of the active ingredient in the blood stream.

Technical information based on *in vivo* standards and specifications are generally incorporated in various official compendia. Hence, in order to record a legitimate assessment of bioavailability, *in vivo* test is an absolute necessity and the relative data obtained therefrom should form an integral part of the standard specifications in the offcial standard.

1.3.3.4. Adverse Drug Reaction

Any dosage-form can produce adverse drug reactions. Hence, a regular feed back of relevant information on such adverse reactions from the medical practitioners to the appropriate regulatory authorities and the concerned manufacturers would not only help to intensify better safety measures but also widen the scope to improve drug-design by meticulous research scientists all over the world.

The following *two* examples convey the implications of adverse-drug reaction. They are :

Example 1 **: Aspirin—**Increased gastric damage and subsequent bleeding caused by some aspirin fomulations have been specifically attributed to the slowly dissolving aspirin particles in the stomach. However, both effervescent and highly buffered dosage forms (antacid-aspirin-tablet), which help in maintaining the aspirin in solution, have been found to minimise gastro-intestinal toxicity.

Example 2 **: Chloramphenicol and Tetracycline—**Sparingly soluble broad-spectrum antibiotics like chloramphenicol and tetracycline found to damage the gastrointestinal epithelium besides changing the normal micro-flora in the GI-tract that are required for normal good health.

1.3.4. IDENTIFICATION TESTS

The true identification of a drug may be accomplished in a number of ways, namely : determination of physical constants, chromatographic tests and finally the chemical tests. The physical constants essentially include the melting point, boiling point, refractive index, weight per millilitre, specific optical rotation, light absorption, viscosity, specific surface area, swelling power, infra-red absorption, and the like. The chromatographic tests include specific spot-tests by thin-layer chromatography (TLC) of pure drug or its presence in a multi-component system. However, the most specific and reliable are the chemical tests which may be categorized separately under tests for inorganic substances and organic substances. The former may be carried out by well defined general quantitative inorganic analysis and the latter by specific reactions of one or more of the functional moieties present in a drug molecule.

1.3.5. PHYSICAL CONSTANTS

A wide range of physical constants, for instance : melting point, boiling point, specific gravity, viscosity, refractive index, solubility, polymorphic forms *vis-a-vis* particle size, in addition to characteristic absorption features and optical rotation play a vital role in characterization of pharmaceutical chemicals and drug substances. These physical constants will be discussed briefly with typical examples as under :

1.3.5.1. Melting Point

It is an important criterion to know the purity of a substance ; however, it has a few limitations. The accuracy and precision of melting point is dependent on a number of factors such as—capillary size, sample size, initial temperature of heating-block and the rate of rise of temperature per unit time (minutes). Keeping in view the different manufacturing processes available for a particular drug the melting point has a definite range usually known as the melting range.

Examples :

S. No.	Pharmaceutical Substance	Melting Point (°C)	
		From	To
l.	Aspirin	141	144
2.	Caffeine	234	239
3.	Metronidazole	159	162
4.	Nicotinic Acid	234	237
5.	Isocarboxazid	105	108
6.	Mestranol	146	154

Thus the melting range takes care of the variance in manufacture together with the storage variance over a stipulated period of time.

1.3.5.2. Boiling Point

It is also an important parameter that establishes the purity of a substance. Depending on the various routes of synthesis available for a substance a boiling point range is usually given in different official compendia.

Examples :

S. No.	Pharmaceutical Substance	Boiling Point (°C)	
		From	To
1.	Chloroform	60	62
2.	Anaesthetic Ether	34	36

1.3.5.3. Refractive Index

It is invariably used as a standard for liquids belonging to the category of fixed oils and synthetic chemicals.

Examples :

S. No.	Pharmaceutical Substance	Refractive Index	
		From	To
1.	Arachis Oil	1.4670	1.4700
2.	Castor Oil	1.4758	1.4798
3.	Shark Liver Oil	1.4590	1.4770
4.	Benzyl Alcohol	1.5360	1.5420
5.	Undecyclenic Acid	1.4470	1.4500

1.3.5.4. Weight Per Millilitre

Weight per millilitre is prevalent in the Pharmacopoeia of India for the control of liquid substances, whereas Relative Density (20°/20°) or Specific Gravity is mostly employed in the European Pharmacopoeia.

Examples :

S. No.	Pharmaceutical Substance	Weight Per Millilitre (g)	
		From	To
l.	Arachis Oil	0.908	0.920
2.	Castor Oil	0.945	0.965
3.	Shark Liver Oil	0.900	0.920
4.	Benzyl Alcohol	1.040	1.050

1.3.5.5. Specific Optical Rotation

As pharmacological activity is intimately related to molecular configuration, hence determination of specific rotation of pharmaceutical substances offer a vital means of ensuring their optical purity.

Examples :

S. No.	Pharmaceutical Substance	Specific Rotation		Remarks
		From	To	
1.	Ergometrine Maleate	+ 50°	+ 56°	For a 1.0% w/v soln. in water
2.	Ethambutol Hydrochloride	+ 6.0°	+ 6.6°	For a 10% w/v soln. in water
3.	Noscapine	– 196°	– 201°	For a 4% w/v soln. in $CHCl_3$ at 20° C
4.	Oestradiol Benzoate	+ 57°	+ 63°	For a 1% w/v soln. in dioxane
5.	Phenylephrine Hydrochloride	– 42°	– 47.5°	For a 2% w/v soln. in water
6.	Morphine Hydrochloride	– 112°	– 115°	Calculated with reference to the dried substance in a 2% w/v soln. in water.

1.3.5.6. Light Absorption

The measurement of light absorption both in the visible and ultraviolet range is employed as an authentic means of identification of offcial pharmaceutical substances.

Examples :

S. No.	Pharmaceutical Substance	Wave Length (nm)	$E_{1\,cm}$	Remarks
1.	Dithranol	354	0.44	0.001% w/v soln. in $CHCI_3$
2.	Ethacrynic Acid	270	0.55-0.60	0.005% w/v soln. in a mixture of 1N. HCl & CH_3OH (1 : 99)
3.	Prednisone Acetate	240	0.365-0.395	0.001% w/v soln. in CH_3OH

1.3.5.7. Viscosity

Viscosity measurements are employed as a method of identifing different grades of liquids.

Examples :

S. No.	Pharmaceutical Substance (Mol. Wt.)	Temp. (°C)	Viscosity (CS*)	
			From	To
1.	Polyethylene Glycol (1500)	100	25	32
2.	Polyethylene Glycol (4000)	100	76	100
3.	Polyethylene Glycol (6000)	100	470	900
4.	Polysorbate – 20	25	240	350
5.	Polysorbate – 80	25	340	450

* Centistokes (Unit of Viscosity)

1.3.5.8. Specific Surface Area

The surface area of powders is determined by subsieve-sizer which is designed for measurement of average particle sizes in the range of 0.2 to 50 microns. The relationship between average particle diameter and specific surface area (SSA) is given by the following expression :

$$\text{SSA} = \frac{6 \times 10^4}{d \times p}$$

where, SSA = Specific surface area in cm^2 per g of material

d = Average diameter in microns

p = True density of material from which the powder was made in g per cm^3

Examples :

S. No.	Pharmaceutical Substance	Specific surface area (cm^2 per g)
1.	Griseofulvin	13,000-17,000
2.	Bephenium Hydroxynaphthoate	7,000

1.3.5.9. Swelling Power

The swelling power of some pharmaceutical products are well defined.

Examples :

(*i*) **Isphagula Husk :** When 1 g, agitated gently and occasionally for four hours in a 25 ml stoppered measuring cylinder filled upto the 20 ml mark with water and allowed to stand for 1 hour, it occupies a volume of not less than 20 ml and sets to a jelly.

(*ii*) **Heavy Kaolin :** When 2 g is titurated with 2 ml of water the mixture does not flow.

1.3.5.10. Infrared Absorption

Measurement and subsequent comparison of the infrared spectrum (between 4000-667 cm^{-1}) of compounds with that of an authentic sample has recently become a versatile method for the identification of drugs having widely varying characteristics.

Examples : Infrared spectroscopy is employed to compare samples of chloramphenicol palmitate (biologically active form) recovered from chloramphenicol palmitate mixture *vis-a-vis* an artificially prepared mixture of authentic sample consisting 10 per cent of the 'inactive polymorph'.

Infrared spectra of known and newly reported compounds are provided in the British Pharmacopoeia (1998) and also in '**Sadtler Standard Spectra**' published by Sadtler Research Laboratories, Philadelphia (USA) is available to check the authenticity of pure drug samples.

1.3.5.11. Miscellaneous Characteristics

A large number of miscellaneous characteristics are usually included in many *official compendia* to ascertain the purity, authenticity and identification of drugs—including : sulphated ash, loss on drying, clarity and colour of solution, presence of heavy metals and specific tests.

1.3.5.11.1. *Sulphated Ash*

Specifically for the synthetic organic compounds, the Pharmacopoeia prescribes values for sulphated ash. The sulphated ash is determined by a double ignition with concentrated sulphuric acid. Metals thus remain as sulphides that are usually stable to heat. The method is one of some precision, and provides results which are rather more reproducible than those obtained by simple ignition.

Examples :

S. No.	Pharmaceutical Substance	Prescribed Limits (%)
1.	Ascorbic Acid	NMT* 0.1
2.	Betamethasone	NMT 0.1
3.	Chlorpheniramine Maleate	NMT 0.15
4.	Carbimazole	NMT 0.1
5.	Digoxin	NMT 0.1
6.	Glibenclamide	NMT 0.2
7.	Mebendazole	NMT 0.1
8.	Methadone Hydrochloride	NMT 0.1
9.	Oxyphenbutazone	NMT 0.1

*NMT = Not More Than

1.3.5.11.2. *Loss on Drying*

Loss on drying reflects the net weight of a pharmaceutical substance being dried at a specified temperature either at an atmospheric or under reduced pressure for a stipulated duration with a specific quantity of the substance.

Examples :

S. No.	Pharmaceutical Substance	Qty. Used (*g*)	Drying Conditions	Duration (Hrs)	Prescribed Limits (%)
l.	Analgin	1.0	105°C	–	NMT* 5.5
2.	Aspirin	1.0	Reduced pressure over Silica Gel	5.0	NMT 0.5
3.	Betamethasone	1.0	In vacuo	–	NMT 0.5
4.	Carbimazole	1.0	In vacuo	24.0	NMT 0.5
5.	Fenfluramine Hydrochloride	1.0	105°C	–	NMT 1.0
6.	Mebendazole	1.0	105°C	–	NMT 0.5

* NMT = Not More Than

1.3.5.11.3. *Clarity and Colour of Solution*

When a pharmaceutical substance is made to dissolve at a known concentration in a specified solvent it gives rise to a clear solution that may be either clear or possess a definite colouration.

Examples :

S. No.	Pharmaceutical Substance	Conc. (% w/v)	Solvent	Remarks
1.	Analgin	5.0	Water	Clear
2.	Digoxin	0.5	Chlorofom : Methanol (1:1)	Clear
3.	Glibenclamide	1.0	Alcohol	Clear and colourless
4.	Methadone Hydrochloride	2.5	Water	Clear and colourless

1.3.5.11.4. *Heavy Metals*

Various tests are prescribed in the offcial compendia to control heavy metal *e.g.*, Ag^{+}, Hg^{2+}, Pb^{2+}, Bi^{2+}, Cu^{2+}, As^{3+}, , Sb^{3+} and Sn^{4+} contamination in organic pharmaceutical substances. Hence, a stringent limit is recommended for the presence of heavy metals in medicinal compounds.

Examples :

S. No.	Pharmaceutical Substance	Prescribed Limits (ppm)
1.	Analgin	NMT* 20
2.	Aspirin	NMT 10
3.	Eucalyptus Oil	NMT 40
4.	Fenfluramine Hydrochloride	NMT 20
5.	Oxyphenbutazone	NMT 10

* NMT = Not More Than

1.3.5.11.5. *Specific Tests*

In fact, certain known impurities are present in a number of pharmaceutical substances. The presence of such impurities may be carried out by performing prescribed specific tests in various *official compendia* in order to ascertain their presence within the stipulated limits.

Examples :

S. No.	Pharmaceutical Substance	Presence of	Test	Prescribed Limit (%)
1.	Aspirin	Salicylic Acid	Dissolve 2.5 g in 25 ml alcohol (TS). To each of two matched cylinders add 48 ml of water and 1 ml of freshly prepared Ferric Ammonium Sulph. Reagent[1]. Into one cylinder pippete 1 ml of stand. salicylic acid soln. (0.01% w/v in water) and into the other pipette 1 ml of TS. Mix the contents of the cylinder, after 30 secs. the colour of the second cylinder is not more intense than the first cylinder.	NMT 0.1
2.	Paracetamol	4-Aminophenol	Dissolve 0.5 g in 10 ml mixture of CH_3OH and H_2O (1:1). Add to it 0.2 ml of alkaline sodium nitroprusside soln. (1.0% w/v in water), mix and allow to stand for 30 mts. The intensity of the colour should not be more than the one produced by using 0.5 g of 4-aminophenol free paracetamol and adding to it 0.5 ml of a 0.005% w/v soln. of 4-aminophenol in the same solvent mixture.	NMT 0.005

3.	Ethambutol Hydro-chloride	(+)-2-Amino-Butan-1-ol	**TLC-Method** : Adsorbent-Silica Gel-G, Mobile Phase-Ethyl acetate : Glacial acetic Acid : HCl : H_2O (11 : 7 : 1 : 1); Apply 2 µl of each of two solns. in MeOH, containing (1) 5% w/v of T.S. & (2)0.050% w/v of (+)-2-aminobutan-1-ol. Remove TLC plates, dry in air, heat at 105°C for 5 mts, cool, spray with cadmium and Ninhydrin soln.[2], & heat to 90°C for 5 mts. The spot obtained with (2) is more intense than with (1).	NMT 1.0
4.	Chlorophensin	Chlorophenol	Dissolve 0.1 g in 5.5 ml of DW, add 3 ml of 4% w/v soln., of sodium hexameta-phosphate, 1.5 ml of lithium and sodium molybdophosphotungstate soln. and 0.4 g of anhydrous Na_2CO_3. Heat on water bath for 5 mts., and cool.	Any blue colour produced is not more intense than that produced when 5.5. ml of 0.001% w/v soln. of 4-chlorophenol is treated likewise.
5.	Adrenaline	Noradrenaline	Dissolve 5.0 mg in 1 ml of a 0.5% w/v soln. of tartaric acid and 4 ml of buffer soln. pH 9.6, mix, add 1 ml freshly prepared 0.5% w/v soln. of sodium 1,2-naphthaquinone-4-sulphonate, mix and allow to stand for 30 mts. Add 0.2 ml of a 1.0% v/v soln. of benzalkoniumchloride soln., mix, add l5 ml of toluene previously washed with buffer soln. pH 9.6 and filtered through a dry filter paper, shake for 30 mts. and allow to separate, centrifuging if necessary.	Any red or purple colour in the toluene layer is not darker than that produced by treating a soln. of 0.40 mg of nor-adrenaline and tartrate and 9 mg of noradrenaline free adrenanline acid tartate in 1 ml of DW in a similar manner.
6.	Digitoxin	Digitonin	Dissolve 10 mg in 2.0 ml of alcohol (96% w/v) in a test tube, the inner walls of which are free from scratches. Add 2.0 ml of a 0.5% w/v soln. of cholesterol in alcohol (96% v/v) and mix by gentle agitation.	No precipitate is formed within 10 minutes.

Reagents :

(1) Dilute 1 ml N. HCl and 2.0 ml ferric ammonium sulphate soln. (10% w/v in H_2O) with suffcient water to produce 100 ml.

(2) Dissolve 50 mg cadmium acetate in a mixture of 5 ml DW and 1 ml glacial acetic acid and dilute with ethyl methyl ketone to 50 ml. Immediately before use add and dissolve suffcient Ninhydrin to produce a soln. containing 0.2% w/v.

(3) Dissolve 10.0 g sodium tungstate and 2.5 g sodium molybdate in 80.0 ml DW in a 250 ml flask; add 5.0 ml phosphoric acid (85-90% w/w) and 10.0 ml HCl (= 11.5 N), connect to a reflux condenser and heat for 10 Hrs. Cool, add 15.0 g lithium sulphate, 5.0 ml DW and 1 drop of bromine and allow to stand for 2 Hrs. Remove the excess bromine by boiling the mixture for 15 mts. without the condenser. Cool, filter and dilute with DW to produce 100 ml.

Caution : (*i*) The prepared soln. should be stored below 4°C, and

(*ii*) The soln. should be used within 4 months after preparation till it retains its original golden yellow colour. It must be rejected if it has a trace of green colour.

1.3.6. LIMIT TESTS *VIS-A-VIS* QUANTITATIVE DETERMINATIONS

In general, limit tests are quantitative or semi-quantitative tests particularly put forward to identify and control invariably small quantities of impurity that are supposed to be present in a pharmaceutical substance. Obviously the amount of any single impurity present in an official substance is usually small, and therefore, the normal visible-reaction-response to any test for that impurity is also quite small. Hence, it is necessary and important to design the individual test in such a manner so as to avoid possible errors in the hands of various analysts. It may be achieved by taking into consideration the following *three* cardinal factors, namely :

(*a*) **Specificity of the Tests :** A test employed as a limit test should imply some sort of selective reaction with the trace impurity. It has been observed that a less specific test which limits a number of possible impurities rather instantly has a positive edge over the highly specific tests.

Exmaple : Contamination of Pb^{2+} and other heavy metal impurities in Alum is precipitated by thioacetamide as their respective sulphides at pH 3.5.

(*b*) **Sensitivity :** The extent of sensitivity stipulated in a limit test varies widely as per the standard laid down by a pharmacopoeia. The sensitivity is governed by a number of variable factors having a common objective to yield reproducible results, for instance :

(*i*) **Gravimetric Analysis :** The precipitation is guided by the concentration of the solute and of the precipitating reagent, reaction time, reaction temperature and the nature and amount of other substance(s) present in solution.

(*ii*) **Colour Tests :** The production of visible and distinct colouration may be achieved by ascertaining the requisite quantities of reagents and reactants, time period and above all the stability of the colour produced.

(*c*) **Personal Errors :** In fact, the personal errors must be avoided as far as possible as explained in Section 1.3.2.2.3 of this chapter.

1.3.7. VARIOUS TYPES OF TESTS FOR QUANTITATIVE DETERMINATIONS

In actual practice, it has been observed that different *official compendia* describe a number of detailed types of tests with a view to obtain a constant and regular check that might be possible to maintain the desired degree of optimum purity both in the pure pharmaceutical substances and the respective dosage-forms made therefrom.

A number of such tests shall be discussed here briefly with specific examples wherever possible and necessary :

1.3.7.1. Limits of Insoluble Matter

The limits of insoluble matter present in pharmaceutical substances and stated in various *official compendia* are given below :

S. No.	Pharmaceutical	Prescribed Test	Inference
1.	Boric Acid	Alcohol insoluble substances	Absence of metallic borates and insoluble impurities
2.	Phenobarbitone Sodium	Dissolve 1 g in a mixture of 2 ml NaOH soln. (20% w/v) and 13 ml DW. Shake with 25 ml of solvent ether for 1-2 mts. Separate the ethereal layer, wash it thrice each with 5 ml DW. Evaporate the ether and dry the residue at 105°C for 1 Hr ; cool and weigh it.	Neutral and basic substances : NMT : 0.3%
3.	Chloramine	Alcohol-insoluble matter : Shake 1 g for 30 mts. with 20 ml of alcohol (96% v/v) and filter on a tared filter. The residue washed with 5 m1 of ethanol (96% v/v) and dried at 100 to 105°C and weighed.	Sodium chloride impurity : NMT 2.0%

4.	Crystal violet	Alcohol-insoluble matter : Weigh 1 g and boil with 50 ml of alcohol (90% v/v) under a reflux condenser for 15 mts. Filter, wash the residue on the filter with hot alcohol (90% v/v) until the washings cease to be coloured violet. Dry to constant weight at 105°C.	Contamination with of Inorganic Salts : NMT : 1.0%

In the same vein, tests for clarity of solution offer another means of limiting insoluble parent drug substances in their correspondingly more highly water-soluble derivatives.

Example :

S. No.	Pharmaceutical Substance	Clarity of Soln. Due To
1.	Phenytoin Sodium	Phenytoin
2.	Pentobarbitone Sodium	Pentobarbitone
3.	Ephedrine Hydrochloride	Ephedrine
4.	Betamethasone Sodium Phosphate	Betamethasone
5.	Amylobarbitone Sodium	Amylobarbitone

1.3.7.2. Limits of Soluble Matter

In order to detect the presence of some very specific impurities normally present in the official substances the limits of soluble impurities have been laid down in different pharmacopoeias. Some typical examples are cited below :

S. No.	Pharmaceutical Substance	Specified Test	Prescribed Limits
1.	Barium Sulphate (radio-opaque medium)	Boil 10 g with a mixture of 10 ml dil. HCl and 90 ml DW for 10 mts. Cool and add DW to restore the original volume and filter through paper, previously washed with a mixture of 10 ml dil. HCl and 90 ml DW to obtain a clear filtrate. Evaporate 50 ml of the filtrate on a waterbath to dryness, and add 2 drops of HCl and 10 ml of hot DW and evaporate the combined filtrate and washings ; dry the residue at 105°C. Digest the residue with 10 ml of water and filter through a paper previously washed with a mixture of 90 ml DW and 8.0 ml dil. HCl. Add 0.5 ml dil. H_2SO_4 to clear the filtrate and set aside for 30 mts.	No turbidity is produced. Water-soluble barium salts are highly toxic.
2.	Light Kaolin	Boil 2 g with 100 ml of 0.2 M HCl under a reflux condenser for 5 mts., cool, filter to dryness. The residue, is obtained after ignition at about 600°C for 30 mts.	NMT : 0.5%
3.	Purified Talc	**Acid-soluble Matter :** **Iron :** Suspend 0.25 g in 40 ml of 1 M H_2SO_4, shake for 15 mts., add 10 ml of a 1% w/v soln. of KCl, dilute to 100 ml with DW and filter quantitatively through filter paper previously washed with HCl and HF (T.S.). Using TS measure transmission by atomic absorption spectrophotometry at 248.3 nm. For the RS add 10 ml of a 1% w/v soln. of KCl and 40 ml of 1 M H_2SO_4 to suitable vols. of iron standard solution (10 ppm Fe) and dilute to 100 ml with water. **Magnesium :** Prepare the sample solution by adding 10 ml of a 1% w/v soln. of KCl to 5 ml of TS and dilute to 100 ml with water. Measure at 285.2 nm. For the RS add 10 ml of a 1% w/v soln. of KCl to suitable vols. of magnesium standard solution (10 ppm Mg) and dilute to 100 ml with DW.	Iron : NMT : 250 ppm Magnesium : NMT : 0.4%

1.3.7.3. Limits of Moisture, Volatile Matter and Residual Solvents

A good number of pharmaceutical substances usually absorb moisture on storage thereby causing deterioration. Such an anomaly can be safely restricted and limited by imposing an essential requirement for the loss in weight (Loss on Drying) when the pharmaceutical chemical is subjected to drying under specified conditions. The quantum of heat that may be applied to the substance varies widely as per the following norms :

(*a*) Nature of the substance

(*b*) Decomposition characterisics of the substance.

Various *official compendia* recommended different temperatures and duration of drying either at atmospheric or reduced pressure (vacuum). A few typical examples are stated below :

S. No.	Pharmaceutical Substance	Drying Conditions (°C)	Drying Time (Hrs.)	Prescribed Limit (%)
l.	Ethynylestradiol	105	3.0	NMT : 1.0
2.	Ethisterone	105	TCW*	NMT : 0.5
3.	Fludrocortisone Acetate	105	TCW	NMT : 1.0
4.	Testosterone	105	TCW	NMT : 0.5
5.	Proxyphylline	100-105	TCW	NMT : 0.5
6.	Quinidine Sulphate	130	TCW	Loses 3.0-5.0
7.	Sodium Chloride	100-105	3.0	NMT : 1.0
8.	Triamcinolone Acetonide	100-105	3.0	NMT : 2.0
9.	Zinc Oxide	500	–	NMT : 1.0
10.	Zinc Undecenoate	100-105	TCW	NMT : 1.5
11.	Chlorotetracycline** Hydrochloride	60	3.0	NMT : 2.0
12.	Drostanolone** Propionate	40	TCW	NMT : 1.0
13.	Fluoxymesterone**	–	24	NMT : 1.0
14.	Streptomycin Sulphate***	60	24	NMT : 7.0
15.	Temazepam**	70	4	NMT : 0.5
16.	Tropicamide**	80	4	NMT : 0.5
17.	Vinblastine Sulphate**	60	16	NMT : 17.0

* To Constant Weight (TCW)

** Dried over P_2O_5 at a pressure not exceeding 0.7 kPa

*** Dried over P_2O_5 at a pressure not exceeding 0.1 kPa

There are *four* types of hydrates which may be observed amongst the pharmaceutical chemicals, namely :

(*a*) Inorganic Salt Hydrates *e.g.*, Magnesium Sulphate ($MgSO_4.7H_2O$) ; Sodium Sulphate ($Na_2SO_4.10\ H_2O$).

(*b*) Salts of Inorganic Cations and Organic Acids *e.g.*, Calcium Lactate, Ferrous Gluconate.

(*c*) Organic Hyrates *e.g.*, Caffeine Hydrate, Theophylline Hydrate.

(*d*) Organic Substances *e.g.*, Acacia, Hydroxymethyl Cellulose.

These substance either lose all or part of their water of crystallization on drying which sometimes attains a considerable value as could be seen in the following data :

S. No. Pharmaceutical Substance	Drying Conditions (°C)	Drying Time (Hrs)	Prescribed Limit (%)
Inorganic Salt Hydrates :			
l. Magnesium Sulphate	110-120 (stage-1)	1	52.0
	400 (stage-2)	TCW*	
2. Sodium Sulphate	30 (stage-1)	1	52.0-57.0
	130 (stage-2)	TCW	
Salts of Inorganic Cations and Organic Acids :			
1. Calcium Lactate Pentahydrate	125	TCW	22.0-27.0
2. Calcium Lactate Trihydrate	125	TCW	15.0-20.0
3. Ferrous Gluconate	100-105	5	7.0-10.5
Organic Hydrates :			
1. Caffeine Hydrate	100-105	1	5.0-9.0
2. Theophylline Hydrate	105	TCW	NMT : 9.5
Organic Substances :			
l. Acacia	100-105	TCW	NMT : 15.0
2. Hydroxymethyl Cellulose	100-105	TCW	NMT : 10.0

* TCW = To Constant Weight

1.3.7.3.1. *Aquametry*

It refers to the determination of water content titrimetrically with **Karl Fischer Reagent (KFR)**. This technique has been used exclusively for the determination of water content in a number of pharmaceutical substances listed below (see Part II G, Chapter 14) :

S.No.	Pharmaceutical Substance	Prescribed Limit (%)
1	Betamethasone Sodium Phosphate	NMT : 8.0
2.	Dexamethasone Sodium Phosphate	NMT : 16.0
3.	Erythromycin	NMT : 6.5
4.	Gentamycin Sulphate	NMT : 15.0
5.	Lymecycline	NMT : 5.0
6.	Lignocaine Hydrochloride	NMT : 5.5-7.0
7.	Procaine Penicillin	NMT : 3.0-4.2

Since the introduction of Gas-Liquid-Chromatography (GLC) (see Part V, Chapter 29) as an essential analytical tool, it has been judiciously exploited as an useful alternative means for not only determining water content in pharmaceutical chemicals but also limiting specific volatile substances present in them. It may be expatiated with the help of the following examples :

Examples : (*i*) **For Determination of Water Content :**

Gonadorelin : (Limit NMT : 7.0 % w/v)

Procedure : Carry out the method for gas chromatography employing the following solutions :

Solution (1) : Dilute 50 µl of anhydrous methanol (internal standard) with sufficient anhydrous propan-2-ol to produce 100 ml.

Solution (2) : Dissolve 4 mg of the sample in 1 ml of anhydrous propan-2-ol.

Solution (3) : Dissolve 4 mg of the sample in 1 ml of solution (1) above.

Solution (4) : Add 10 µl of water to 50 µl of solution (1).

The chromatographic procedure may be carried out by employing :

(*a*) A stainless-steel column (1 m × 2 mm) packed with porous polymer beads *e.g.*, Chromosorb 102 (60 to 80 mesh) and maintained at 120°C.

(*b*) Helium as the carrier gas.

(*c*) A Thermal Conducting Detector (TCD) maintained at 150°C. From the chromatograms obtained and taking into account any water detectable in solution (1), calculate the percentage w/w of water taking 0.9972 as its weight per ml at 20°C.

(*ii*) **For Limiting Specific Volatile Substance :**

Orciprenaline Sulphate : (Limit of Water and Methanol : 6.0% w/w)

Procedure : Perform the method for gas-chromatography using the following three solutions in water containing :

Solution (1) : 0.50% v/v of MeOH and 0.50% v/v of EtOH (96% v/v)—as Internal Standard

Solution (2) : 10% w/v of the sample

Solution (3) : 10% w/v of the sample and 0.50% v/v of the internal standard.

The chromatographic procedure may be performed using a glass column (1.5 × 4 mm) packed with porous polymer beads (80 to 100 mesh) *e.g.*, Porapack-Q and maintained at 140°C.

Calculate the percentage w/v of methanol taking 0.792 as its weight per ml at 20°C.

1.3.7.4. Limits of Non-Volatile Matter

Pharmaceutical chemicals belonging to the domain of inorganic as well as organic substances containing readily volatile matter for which the various *official compendia* prescribe limits of non-volatile matter. It is pertinent to mention here that the Pharmacopoeia usually makes a clear distinction between substances that are readily volatile and substances that are volatile upon strong ignition, for instance :

(*a*) **Readily Volatile :** *e.g.*, **Organic Substances**—alcohol (95% v/v), isopropyl alcohol, chloroform, halothane, anaesthetic ether, chlorocresol and trichloroethylene ; and **Inorganic substances**—ammonia solution, hydrogen peroxide solution, water for injection.

(*b*) **Volatile Upon Strong Ignition :** *e.g.*, hydrous wool fat (lanolin).

1.3.7.5. Limits of Residue on Ignition

In fact, the limits of residue on ignition are basically applicable to the following two categories of pharmaceutical substances, namely :

(*a*) Those which are completely volatile when ignited *e.g.*, Hg.

(*b*) Those which undergo total decomposition thereby leaving a residue with a definite composition *e.g.*, calamine—a basic zinc carbonate that gives rise to ZnO as the residue.

According to BP, 68.0 to 74.0% when ignited at a temperature not lower than 900°C until, after further ignition, two successive weighings do not differ, by more than 0.2% of the weight of the residue.

1.3.7.6. Limits of Loss on Ignition

Official compendia include the limits of 'loss on ignition' which is generally applied to relatively stable pharmaceutical substances that are likely to contain thermolabile impurities. A few typical examples are stated below :

S. No.	Pharmaceutical Substance	Ignition Temp. (°C)	Ignition Time	Prescribed Limits (%)
l.	Dried Calcium Sulphate	Red hot	TCW*	4.5-8.0
2.	Heavy Magnesium Oxide	900	TCW	8.0
3.	Light Kaolin	Red hot	TCW	15.0
4.	Magnesium Trisilicate	900	TCW	17.0-34.0
5.	Magnesium Sulphate	450-500	TCW	31.0-34.0

* TCW= To Constant Weight

1.3.7.7. Limits on Ash Value

The ash values usually represent the inorganic residue present in official herbal drugs and pharmaceutical substances. These values are categorized into *four* heads, namely :

(*a*) Ash Value (Total Ash),

(*b*) Acid-Insoluble Ash,

(*c*) Sulphated Ash, and

(*d*) Water-Soluble Ash.

These values would be explained with the help of some typical examples stated below :

1.3.7.7.1. *Ash Value (Total Ash)*

Ash value normally designates the presence of inorganic salts *e.g.*, calcium oxalate found naturally in the drug, as well as inorganic matter derived from external sources. The official ash values are of prime importance in examination of the purity of powdered drugs as enumerated below :

(*i*) To detect and check adulteration with exhausted drugs *e.g.*, ginger.

(*ii*) To detect and check absence of other parts of the plant *e.g.*, cardamom fruit.

(*iii*) To detect and check adulteration with material containing either starch or stone cells that would modify the ash values.

(*iv*) To ensure the absence of an abnormal proportion of extraneous mineral matter incorporated accidentally or due to follow up treatment or due to *modus operandi* at the time of collection *e.g.*, soil, floor sweepings and sand.

The most common procedure recommended for *crude drugs* is described below :

Procedure : Incinerate 2 to 3 g of the ground drug in a tared platinum or silica dish at a temperature not exceeding 450°C until free from carbon. Cool and weigh. If a carbon-free ash cannot be obtained in this way, exhaust the charred mass with hot water (DW), collect the residue on an ashless filter paper, incinerate the residue and filter paper, add the filtrate, evaporate to dryness and ignite at a temperature not exceeding 450°C. Calculate the percentage of ash with reference to the air-dried drug.

Examples :

S. No.	Pharmaceutical Substance	Prescribed Limit (%)
1.	Aloin	NMT* 0.5
2.	Catechu	NMT 8.0
3.	Sumatra Benzoin	NMT 2.0
4.	Indian Squill	NMT 6.0
5.	Sterculia	NMT 7.0

* NMT = Not More Than

1.3.7.7.2. *Acid-Insoluble Ash*

The method described above for '**total ash**' present in crude drugs containing calcium oxalate has certain serious anomalies, namely :

- Offers variable results upon ashing based on the conditions of ignition.
- Does not detect soil present in the drug efficaciously.
- The limits of excess of soil in the drug are not quite definite.

Hence, the treatment of the '*total ash*' with acid virtually leaves silica exclusively and thus comparatively forms a better test to detect and limit excess of soil in the drug than does the ash.

The common procedure usually adopted for the determination of 'acid insoluble ash' is given below :

Procedure : Place the ash, as described earlier, in a crucible, add 15 ml DW and l0 ml hydrochloric acid (≃ 11.5 N), cover with a watch-glass, boil for 10 minutes and allow to cool. Collect the insoluble matter on an ashless filtre paper, wash with hot DW until the filtrate is neutral, dry, ignite to dull redness, allow to cool in a desiccator and weigh. Repeat until the difference between two successive weighings is not more than l mg. Calculate the percentage of acid-insoluble ash with reference to the air-dried drug.

A few typical examples are listed below :

S. No.	Pharmaceutical Substance	Prescribed Limit (%)
1.	Aniseed	NMT* 2.5
2.	Powdered Belladona Herb	NMT 4.0
3.	Powdered Caraway	NMT 1.5
4.	Cardamom Fruit (of the seeds)	NMT 6.0
5.	Coriander	NMT 1.5
6.	Digitalis Leaf	NMT 5.0
7.	Ipecacuanha	NMT 3.0
8.	Liquorice	NMT 2.0
9.	Lobelia	NMT 5.0
10.	Quillaia	NMT 1.0
11.	Rhubarb	NMT 1.0
12.	Senega Root	NMT 3.0
13.	Alexandrian Senna Fruit	NMT 2.0
14.	Senna leaf	NMT 4.0
15.	Squill	NMT 1.5
16.	Sterculia	NMT 1.0
17.	Stramonium Leaf	NMT 4.0
18.	Valerian	NMT 7.0

* NMT = Not More Than

1.3.7.7.3. *Sulphated Ash*

The estimation of '**sulphated ash**' is broadly employed in the case of :

(*a*) Unorganized drugs *e.g.*, colophony, podophyllum resin, wool alcohols, wool fat and hydrous wool fat.

(*b*) Pharmaceutical substances contained with inorganic impurities *e.g.*,

Natural Origin : Spray-dried acacia, Frangula Bark, Activated Charcoal

Organic Substances : Cephalexin, Lignocaine hydrochloride, Griseofulvin, Diazoxide, Medazapam, Saccharin.

Inorganic Substances : Ammonium chloride, Hydroxy urea.

The general method for the determination of '*sulphated ash*' is enumerated below :

Procedure : Heat a silica or platimum crucible to redness for 30 minutes, allow to cool in a desiccator and weigh. Place a suitable quantity of the substance being examined, accurately weighed in the crucible, add 2 ml of 1 M sulphuric acid and heat, first on a waterbath and then cautiously over a flame to about 600°C. Continue heating until all black particles have disappeared and then allow to cool. Add a few drops of 1 M sulphuric acid, heat to ignition as before and allow to cool. Add a few drops of a 16% solution of ammonium carbonate, evaporate to dryness and cautiously ignite. Cool, weigh, ignite for 15 minutes and repeat the procedure to constant weight.

Following are the examples to depict the '*sulphated ash*' present in various official pharmaceutical chemicals :

S. No.	Pharmaceutical Substance	Prescribed Limit (%)
Substances of Natural Origin :		
1.	Colophony	NMT* : 0.20
2.	Podophyllum Resin	NMT : 1.00
3.	Wool Alcohols	NMT : 0.15
4.	Wool Fat	NMT : 0.15
5.	Hydrous Wool Fat	NMT : 0.10
6.	Spray-dried Acacia	NMT : 5.50
7.	Frangula Bark	TMT : 8.00
8.	Activated Charcoal	NMT : 5.00
Organic Substances :		
1.	Cephalexin	NMT : 0.20
2.	Lignocaine Hydrochloride	NMT : 0.10
3.	Griseofulvin	NMT : 0.10
4.	Diazoxide	NMT : 0.10
5.	Medazepam	NMT : 0.10
6.	Saccharin	NMT : 0.20
Inorganic Substances :		
1.	Ammonium Chloride	NMT : 0.10
2.	Hydroxy urea	NMT : 0.20

* NMT = Not More Than

1.3.7.7.4. *Water-Soluble Ash*

Water-soluble ash is specifically useful in detecting such samples which have been extracted with water.

A detailed procedure as per the official compendium is enumerated below :

Procedure : The ash as described earlier, is boiled for 5 minutes with 25 ml DW, collect the insoluble matter in a sintered-glass crucible or on an ashless filter paper, wash with hot DW and ignite for 15 minutes at a temperature not exceeding 450°. Subtract the weight of the residue thus obtained from the weight of the ash.The difference in weight represents the water-soluble ash. Now, calculate the percentage of water-soluble ash with reference to the air-dried drug.

A typical example of an official drug is that of 'Ginger', the water-soluble ash of which is found to be not more than 6.0%.

1.3.8. LIMIT TESTS FOR METALLIC IMPURITIES

The *official compendia* lay a great deal of emphasis on the control of physiologically dangerous, cumulative poisonous and harmful impurities, such as lead, arsenic and iron present in a host of pharmaceutical chemicals. These impurities very often creep into the final product through a number of means stated below, namely :

(*a*) Through atmospheric pollution.

(*b*) Most frequently derived from the raw materials.

(*c*) From materials used in the process of manufacture.

(*d*) Due to solvent action on the metal of the plant in which the substance is prepared.

In short, all prescribed tests for impurities in the Pharmacopoeia usually fix certain limits of tolerance. For lead, arsenic and iron general quantitative or limit tests are precisely laid down which, with necessary variations and modification are rigidly applicable to pharmaceutical substances.

1.3.8.1. Limit Tests for Lead

Theory : The offcial test is based on the conversion of traces of lead salts present in the pharmaceutical substances to lead sulphide, which is obtained in colloidal form by the addition of sodium sulphide in an alkaline medium achieved by a fairly high concentration of ammonium acetate. The reaction may be expressed as follows :

$$PbCl_2 + Na_2S \longrightarrow PbS \downarrow + 2NaCl$$

The brown colour, caused due to colloidal lead sulphide in the test solution is compared with that produced from a known amount of lead.

Equipment : Nessler Cylinders (or Nessler Glasses) : According to the *British Standard Specification No : 612, 966*—a pair of cylinders made of the same glass and having the same diameter with a graduation mark at the same height from the base in both cylinders (Figure 1).

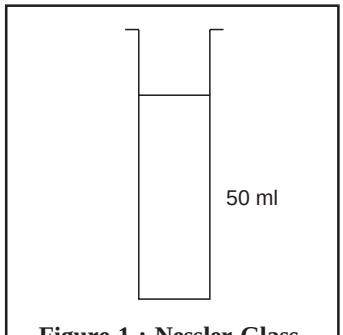

Figure 1 : Nessler Glass.

The final comparison is made by viewing down through the solution against a light background.

Materials Required : (*i*) **Lead Nitrate Stock Solution :** Dissolve 0.1598 g of lead nitrate in 100 ml DW to which has been added 1 ml nitric acid, then dilute with water to 1 Litre.

Note : The solution must be prepared and stored in polyethylene or glass containers free from soluble lead salts.

(*ii*) **Standard Lead Solution :** On the day of use, dilute 10.0 ml of lead nitrate stock solution with DW to 100.0 ml. Each ml of standard lead solution contains the equivalent of 10 microgrammes of lead. A control comparison solution prepared with 2.0 ml of standard lead solution contains, when compared to a solution representing 1.0 g of the substance being tested, the equivalent of 20 parts per million of lead.

(*iii*) **Standard Solution :** Into a 50 ml Nessler Cylinder, pipette 2 ml of standard lead solution and dilute with DW to 25 ml. Adjust with dilute acetic acid Sp. (IP)* or dilute ammonia solution Sp. (IP) to a pH between 3.0 and 4.0, dilute with DW to about 35 ml and mix.

(*iv*) **Test Solution :** Into a 50 ml Nessler Cylinder, place 25 ml of the solution prepared for the test as directed in the individual monograh, dissolve and dilute with DW to 25 ml the specified quantity of the substance being tested. Adjust with dilute acetic acid Sp. (IP) or dilute ammonia solution Sp. to a pH between 3.0 and 4.0, dilute with DW to about 35 ml and mix.

Procedure : To each of the cylinders containing the standard solution and test solution respectively, add l0 ml of freshly prepared hydrogen sulphide solution, mix, dilute with water (DW) to 50 ml, allow to stand for 5 minutes and view downwards over a white surface, the colour produced in the test solution is not darker than that produced in the standard solution.

A few typical examples from the official compendium are given below :

S. No.	Pharmaceutical Substance	Prescribed Limit (ppm)
1.	Benzoic Acid	NMT* : 10
2.	Boric Acid	NMT : 20
3.	Calcium Chloride	NMT : 10
4.	Calcium Gluconate	NMT : 20
5.	Calcium Pantothenate	NMT : 40
6.	Dextrose	NMT : 5
7.	Erythromycin	NMT : 20
8.	Ethylenediamine Hydrate	NMT : 20
9.	Ferrous Gluconate	NMT : 20
10.	Isoprenaline Sulphate	NMT : 10
11.	Heavy Magnesium Oxide	NMT : 20
12.	Nicotinic Acid	NMT : 20
13.	Pentobarbitone Sodium	NMT : 30
14.	Piperazine Hydrate	NMT : 20

* NMT = Not More Than

1.3.8.2. Limit Test for Arsenic

Theory : The official process is a development of the **Gutzeit Test** wherein all arsenic present is duly converted into arsine gas (AsH_3) by subjecting it to reduction with zinc and hydrochloric acid. Further, it depends upon the fact that when arsine comes into contact with dry paper permeated with mercuric (Hg^{2+}) chloride it produces a yellow strain, the intensity of which is directly proportional to the quantity of arsenic present. The various chemical reactions involved may be expressed by the following equations :

$$Zn + 2HC1 \longrightarrow ZnCl_2 + 2(H)$$

$$2As + 6(H) \longrightarrow 2AsH_3 \uparrow$$

$$HgCl_2 + AsH_3 \longrightarrow \underbrace{HgCl_2 \,.\, AsH_3}_{\text{Yellow complex}}$$

The details of experimental procedure described in the Pharmacopoeia are actually based upon a paper by Hill and Collins**, but have been adequately modified from time to time in accordance with the accumulated and acquired experience. Explicitly, the expressions provided in the Pharmacopoeia for limits of arsenic exclusively refer to parts per million, calculated as As.

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* Indian Pharmaeopoeia, 1996

** Chemist and Druggist **67,**548, 1905.

Materials Required : Arsenic limit test apparatus; $HgCl_2$—paper : smooth white filter paper (having thickness in mm of 400 paper = weight in g per Sq. M.), soaked in a saturated solution of $HgCl_2$, pressed to get rid of excess of soln. and dried at about 60°C in the dark ; lead acetate solution 10.0% w/v soln. of $PbAc_2$ in CO_{2-} free water ; KI (AsT), 1.0 g ; Zn (AsT) : l0.0 g ; Dilute Arsenic solution (AST); Standard stains, Test Solutions—are prepared according to the Indian Pharmacopoeia 1996.

Arsenic Limit Test Apparatus (Figure 2)

A wide-mouthed glass bottle capable of holding about 120 ml is fitted with a rubber bung through which passes a glass tube. The latter, made from ordinary glass tubing, has a total length of 200 mm and an internal diameter of exactly 6.5 mm (external diameter about 8 mm). It is drawn out at one end to a diameter of about 1 mm and a hole not less than 2 mm in diameter is blown in the side of the tube, near the constricted part. When the bung is inserted in the bottle containing 70 ml of liquid, the constricted end of the tube is kept above the surface of the liquid, and the hole in the side is below the bottom of the bung. The upper end of the tube is cut off square, and is either slightly rounded or ground smooth.

The rubber bungs (about 25 mm × 25 mm), each with a hole bored centrally and through exactly 6.5 mm in diameter, are fitted with a rubber band or spring clip for holding them tightly in place.

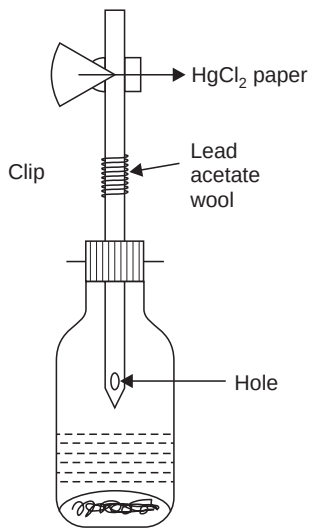


Figure 2 : Aresenic Limit Test Apparatus

Procedure : The glass tube is lightly packed with cotton wool, previously moistened with lead acetate solution and dried, so that the upper surface of the cotton wool is not less than 25 mm below the top of the tube. The upper end of the tube is then inserted into the narrow end of one of the pair of rubber bungs, to a depth of l0 mm (the tube must have a rounded-off end). A piece of mercuric chloride paper is placed flat on the top of the bung and the other bung placed over it and secured by means of the spring clip in such a manner that the holes of the two bungs meet to form a true tube 6.5 mm diameter interrupted by a diaphragm of mercuric chloride paper.

The test solution prepared as specified, is placed in the wide-mouthed bottle, 1 g of KI (AsT) and 10 g of Zn (AsT) are added, and the prepared glass tube is placed quickly in position. The reaction is allowed to proceed for 40 minutes. The yellow stain that is produced on the $HgCl_2$ paper if As is present is compared by daylight with the standard stains obtained by performing in an identical manner with known quantities of dilute arsenic solution (AsT). The comparison of the stains is made immediately at the completion of the test.

By matching the intensity and depth of colour with standard stains, the proportion of arsenic in the substance may be estimated. Thus, a stain equivalent to the 1 ml standard stain obtained by performing on l0 g of a substance implies that the proportion of As is 1 part per million.

Cautions : (*i*) $HgCl_2$ paper should be protected from sunlight during the test to avoid lighter or no stain.

(*ii*) The standard and test stains must be compared immediately as they fade out on retaining.

(*iii*) The reaction may be expedited by the application of heat and 40°C is considered to be the most ideal temperature.

(*iv*) The tube should be washed with HCl (AsT), rinsed with DW, and dried between successive tests.

Special Techniques : The special techniques are usually applicable to a host of pharmaceutical substances before the normal test can be performed. A few typical examples would be discussed briefly here, namely :

(*i*) **Free Acids :** They are first converted to their respective sodium salts with Na_2CO_3 and As^{3+} oxidised to As^{5+} by evaporating the solution with Br_2. The residue is ignited carefully until carbonised to destroy organic matter, while As is kept as non-volatile sodium arsenate. The resulting residue is dissolved in brominated HCl and the test carried out in the normal manner.

Examples : Aspirin, Saccharin, Sodium Salicylate, Sodium Aminosalicylate.

(*ii*) **Substances Reacting Vigorously with HCl :** The As is readily converted to $AsCl_3$ which being volatile in nature is also carried off along with relatively large volumes of CO_2 (generated by the substance and HCl).

***Examples* :** Magnesium Carbonate, Light Magnesium Oxide, Calcium Hydroxide, Chalk, KOH, NaOH.

(*iii*) **Insoluble Substances :** These substances, as those that do not interfere with the solution of As and its subsequent reduction to AsH_3 (arsine). Such substances are suspended in water along with stannated-HCl, and the normal test is performed.

Examples : Magnesium Trisilicate, Bentonite, Barium Sulphate, Light and Heavy Kaolin.

(*iv*) **Metals Interfering with Normal Reaction**

(*a*) **Iron :** It gets deposited on the surface of Zn thereby depressing the intensity of reaction between Zn and HCl to produce H_2.

Remedy : The sample is dissolved in H_2O and stannated HCl to allow conversion of all As to As^{3+} and finally as $AsCl_3$. The latter being volatile in nature can be separated by distillation from remaining metallic salts and the distillate examined in the normal manner.

***Example* :** Ferrous Sulphate.

(*b*) **Antimony :** Sb-compounds are also reduced simultaneously by Zn/HCl to yeild SbH_3 (stilbine) that reacts with $HgCl_2$ paper to give a stain. Therefore, the sample is first distilled with HCl to yield a distillate containing all the As as AsC_3 (volatile), but yields only a fraction of Sb as $SbCl_3$ (non-volatile). A repeated distillation obviously gets rid of even the last traces of Sb.

***Examples* :** Antimony Potassiun Tartrate, Antimony Sodium Tartrate.

A few typical examples are cited below from the *official compendium.*

S. No.	Pharmaceutical Substances	Prescribed Limit (ppm)
1.	Barium Sulphate	NMT* : 1
2.	Benzoic Acid	NMT : 2
3.	Calcium Lactate	NMT : 2
4.	Calcium Hydroxide	NMT : 4
5.	Ferrous Sulphate	NMT : 2
6.	Heavy Kaolin	NMT : 2
7.	Heavy Magnesium Carbonate	NMT : 2
8.	Heavy Magnesium Oxide	NMT : 5
9.	Magnesium Sulphate	NMT : 2
10.	Magnesium Trisilicate	NMT : 4
11.	Potassium Bromide	NMT : 2
12.	Potassium Chloride	NMT : I

* NMT = Not More Than

1.3.8.3. *Limit Test for Iron*

Theory : The limit test for Iron is based on the reaction between iron and thioglycollic acid in a medium buffered with ammonium citrate to give a purple colour, which is subsequently compared with the standard colour obtained with a known amount of iron (0.04 mg of Fe). Ferrous thioglycollate is a **co-ordination compound** that attributes the purple colour ; besides thioglycollic acid converts the entire Fe^{3+} into Fe^{2+}. The reactions involved may be expressed as follows :

$$2Fe^{3+} + 2HS.CH_2.COOH \longrightarrow 2Fe^{2+} + HOOC.CH_2SSCH_2.COOH + 2H^+$$

$$Fe^{2+} + 2HS.CH_2.COOH \longrightarrow \begin{matrix} CH_2S \\ | \\ CO.O \end{matrix} \!\!>\!\! Fe \!\!<\!\! \begin{matrix} O.CO \\ | \\ HS.CH_2 \end{matrix} + 2H^+$$

Ferrous thioglycollate

Materials Required

Nessler cylinder : 1 pair ; Ferric ammonium sulphate : 1.726 g ; Sulphuric acid (0. 1 N) : 10.0 ml ; Iron-free citric acid (20% w/v) : 2.0 ml ; Thioglycollic acid : 0.1 ml; Iron-free ammonia solution : 20 ml.

Standard Iron Solution : Weigh accurately 0.1726 g of ferric ammonium sulphate and dissolve in 10 ml of 0.1 N sulphuric acid and suffcient water to produce 1 Litre. Each ml of this solution contains 0.02 mg of Fe.

Standard Colour : Dilute 2.0 ml of **standard iron solution** with 40 ml DW in a Nessler cylinder. Add 2 ml of a 20% w/v solution of iron-free citric acid and 0.1 ml of thioglycollic acid, mix, make alkaline with iron-free ammonia solution, dilute to 50 ml with DW and allow to stand for 5 minutes.

Procedure : Dissolve the specified quantity of the substance being examined in 40 ml DW, and transfer to a Nessler cylinder. Add to it 2 ml iron-free citric acid solution and 0.1 ml thioglycollic acid, mix, make alkaline with iron-free ammonia solution, dilute to 50 ml with DW and allow to stand for 5 minutes. Any colour produced is not more intense than the standard colour.

Some examples of this test for pharmaceutical substances are listed below :

S. No.	Pharmaceutical Substance	Test	Official Requirements
1.	Calcium Carbonate	Dissolve 0.2 g in 5 ml DW and 0.5 ml HCl, boil and dilute to 40 ml with water.	Complies with the limit test for iron
2.	Calcium Lactate	Dissolve 0.5 g in DW add 2 ml of a 20% w/v soln. of **iron-free-citric acid** and 0.1 ml of thioglycollic acid, mix, make alkaline with **iron-free-ammonia soln.,** dilute to 50 ml with DW and allow to stand for 5 minutes.	Any colour produced is not more intense than that obtained by treating in the same manner 2.0 ml of **iron standard solution** (20 ppm Fe) in place of the soln. being examined.
3.	Tribasic Calcium Phosphate	Dissolve 0.l g in a mixture of 5 ml DW, 0.5 ml HCl and 1 g citric acid. Dilute the solution to 40 ml with DW.	Complies with the limit test for iron.
4.	Heavy Kaolin	Triturate 2 g in a mortar with a 10 ml DW and add 0.5 g sodium salicylate.	Mixture does not acquire more than a slight reddish tint.
5.	Heavy Magnesium Carbonate	Dissolve 0.1 g in 5 ml DW and 0.5 ml HCl.	Complies with the limit test for Iron.
6.	Heavy Magnesium Oxide	Dissolve 40 mg in 5 ml DW and 0.5 ml HCl.	-do-

7.	Magnesium Sulphate	Dissolve 2 g in 20 ml DW.	Complies with the limit test for iron.
8.	Potassium Chloride	20 ml of a 10% w/v solution in **CO_2– free DW** and carry out the test as stated in 2 above.	Complies with limit test for iron.
9.	Salicylic Acid	(I) Boil 12 g with 14 ml of dil. ammonia soln. and 35 ml DW. Cool and adjust the pH to 5.0 to 6.0 by the drop wise addition of dil. NH_4OH/ dil. H_2SO_4. (II) Boil 2 g with 1 ml stand. iron soln., 2 ml of dil. NH_4OH and 45 ml DW, adjust the pH to 5.0 to 6.0 and dilute to 50 ml with DW.	Pink colour produced in (I) should not be deeper than (II).
10.	Sodium Bicarbonate	Dissolve 2.5 g in 20 m1 DW and 4 ml HCl and dilute to 40 ml with DW.	Complies with the limit test of iron.
11.	Sodium Phosphate	20 ml of 10% w/v solution in DW and perform the test as described in 2 above.	Complies with limit test for iron.
12.	Starch	Dissolve the residue obtained, in the test for sulphated ash in 4 ml HCl by heating gently, dilute with DW to 50 ml and mix.	25 ml complies with the limit test for iron.
13.	Zinc Oxide	Dissolve 0.l g in a mixture of 5 ml DW and 0.5 ml HCl, and dilute to 40 ml with water.	Complies with the limit test for iron using 6 drops of thioglycollic acid.
14.	Zinc Sulphate	Dissolve 2.5 g in suffcient CO_2-free DW to produce 50 ml. Dilute 2 ml of this soln. to 10 ml with DW, add 2 m1 of a 20% w/v soln. of **iron-free-Citric acid** and 0.5 ml of thioglycollic acid, mix, make alkaline with **iron-free-ammonia solution, dilute to 50 ml** with DW and allow to stand for 5 minutes.	Complies with the limit test for iron.

1.3.9. LIMIT TEST'S FOR ACID RADICAL IMPURITIES

Acid radical impurities constitute a serious but unavoidable source of impurities in a large number of pharmaceutical chemicals. However, the two most commonly found acid radical impurities are chloride (Cl^-) and sulphate (SO_4^{2-}) that evidently arise from the inevitable use of raw tap-water in various manufacturing operations. As these two acid radical impurities are found in abundance due to contamination, the Pharmacopoeia categorically stipulates limit tests for them which after due minor modifications are applicable to a number of pharmaceutical substances.

In addition to the above two commonly found impurities, there are a number of other acid radical impurities which exist in pharmaceutical substances, namely : arsenate, carbonate, cyanide, nitrate, oxalate, phosphate and silicate.

All these acid radical impurities shall be discussed briefly as under :

1.3.9.1. Limit Test for Chlorides

The limit test for chlorides is based on its precipitation with silver nitrate in the presence of dilute HNO_3, and comparing the opalescence produced due to the formation of AgCl with a standard opalescence achieved with a known quantity of Cl^- ions.

The equation may be expressed as :

$$NaCl + AgNO_3 \longrightarrow AgCl \downarrow + NaNO_3$$

Materials Required : Nessler cylinder 1 pair ; dilute nitric acid (10% w/w of HNO_3) 10.0 ml ; silver nitrate solution (5.0% w/v in DW) 1.0 ml.

Standard Opalescence : Place 1.0 ml of a 0.05845% w/v solution of NaCI in 10 ml of dilute HNO_3 in a Nessler cylinder. Dilute to 50 ml with DW and add 1 ml of $AgNO_3$ solution. Stir immediately with a glass rod and allow to stand for 5 minutes.

Procedure : Dissolve the specified quantity for the substance in DW, or prepare a solution as directed in the text and transfer to a Nessler cylinder. Add 10 ml of dilute nitric acid, except when it is used in the preparation of the solution, dilute to 50 ml with DW, and add 1 ml of $AgNO_3$ solution. Stir immediately with a glass rod and allow to stand for 5 minutes. The opalescence produced is not greater than the standard opalescence, when viewed transversely.

A few typical examples of this test representing a wide spectrum of pharmaceutical substances are enumerated below :

S. No.	Pharmaceutical Substance	Test	Official Requirements
1.	Aluminium Hydroxide Gel	Dissolve 0.5 g in 5 ml dil. HNO_3, boil, cool, dilute to 100 ml with DW and filter 2.5 ml used for the test.	Complies with limit test for chlorides.
2.	Benzocaine	Dissolve 0.2 g in 5 ml alcohol, previously acidified with a few drops of dil. HNO_3 and add a few drops of $AgNO_3$ solution.	No turbidity is produced immediately.
3.	Bephenium Hydroxynapthoate	Boil 2.5 g with 100 ml DW, cool in ice and filter to 20 ml of filtrate, add l0 ml dil. HNO_3 shake and filter. Test with the filtrate.	Complies with limit test for chlorides.
4.	Calcium Aminosalicylate	Dissolve 0.99 g in 10 ml DW and add 3 ml of acetic acid, filter, wash the residue with 5 successive quantities each of 2 ml DW, mix the filtrate and washings and dilute 50 ml with DW. Use 10 ml for the test.	-do-
5.	Calcium Gluconate	Dissolve 1 g in DW, add 10 ml of dilute HNO_3, dilute to 50 ml with DW and add 1 ml of 0.1 M silver nitrate. Stir immediately with a glass rod and allow to stand for 5 minutes protected from light.	When viewed transversely against a black background any opalasecence produced is not more intense then that obtained by treating a mixture of 10.0 ml of chloride standard solution (25 ppm Cl) and 5 ml of DW in the same manner.
6.	Chloramphenicol	Shake 50 mg with 10 ml DW and filter; to the filtrate add a few drops of $AgNO_3$ soln.	No oplescence is produced.
7.	Dextrose	20 ml of a 10% w/v solution in DW is treated as stated in 5 above.	Complies with the limit test for chlorides (125 ppm).
8.	Ephedrine	Dissolve 0.1 g in 1 ml DW and 1 ml dil. HNO_3 and add 0.l ml $AgNO_3$ solution.	No turbidity is produced.
9.	Frusemide	Shake l.0 g with 40 ml DW for 5 minutes and filter. Test with filtrate as stated in 5 above.	Complies with the limit test for chlorides (250 ppm).
10.	Glycerin	20 ml of a 50.0% w/v solution in **CO_2^- free DW** is treated as described in 5 above.	Complies with the limit test for chlorides (25 ppm).

11.	Isocarboxazid	Boil 0.5 g with 5 ml H_2O_2 soln. (30%) and 10 ml NaOH soln. (20% w/v in DW) for 2 minutes. Cool, neutralize to litmus with HNO_3 and add sufficient DW to produce 40 ml. Test with the resulting solution.	Complies with limit test for chlorides (25 ppm).
12.	Heavy Magnesium Oxide	Dissolve 0.4 g in DW by addition of 2 ml HNO_3.	-do-
13.	Phenylbutazone	Boil l.0 g with 30 ml DW for 5 minutes, cool and filter. To 10 ml of the filtrate add 1 ml dil. HNO_3 and 1 ml $AgNO_3$ solution.	No oplascence is produced.
14.	Promethazine Theoclate	Shake 2.0 g with 20 ml DW for 2 minutes and filter. Use 10 ml of filtrate for the test.	Complies with the limit test for chlorides.

1.3.9.2. Limit Test for Sulphates

Theory : The limit test for sulphates is based upon its precipitation as barium sulphate in the presence of barium chloride, hydrochloric acid and traces of barium sulphate. In this combination, hydrochloric acid exerts its common ion effect whereas traces of $BaSO_4$ aids in the rapid and complete precipitation by seeding. Thus, the opalescence caused by the sample is compared immediately with a standard turbidity produced with a known amount of the SO_4^{2-} ion.

The main objective of this test is to provide a rigid control of sulphate as an impurity present primarily in inorganic pharmaceutical substances.

Materials Required : Nessler cylinders 1 pair ; dilute hydrochloric acid (10% w/v of HCl) 2.0 ml.

Barium Sulphate Reagent : Mix 15 ml of 0.5 M barium chloride, 55 ml of DW, and 20 ml of sulphate free alcohol, add 5 ml of a 0.0181% w/v soln. potassium sulphate dilute to 100 ml with DW, and mix. It should always be prepared fresh.

0.5 M Barium Chloride : $BaCl_2$ dissolved in DW to contain in 1 Litre 122.1 g of $BaCl_2$. $2H_2O$.

Standard Turbidity : Place 1.0 ml of a 0.1089% w/v soln. of K_2SO_4 and 2 ml of dilute HCl in a Nessler cylinder, dilute to 45 ml with DW, add 5 ml $BaSO_4$ reagent, stir immediately with a glass rod and allow to stand for 5 minutes.

Procedure : Dissolve the specified quantity of the substance in DW, transfer to a Nessler cylinder, and the preparation of the solution. Dilute to 45 ml with DW, add 5 ml barium sulphate reagent, stir immediately with a glass rod, and allow to stand for 5 minutes. The turbidity is not greater than the standard turbidity, when viewed transversely.

A few examples of this test consisting of a cross-section of pharmaceutical substances are stated below :

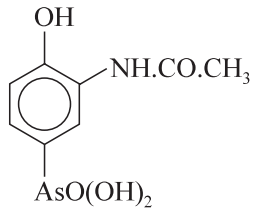
S.No.	Pharmaceutical Substance	Test	Official Requirements
1.	Aluminium Hydroxide Gel	Dissolve 2.5 g in 5 ml of dil. HCl by heating. Cool and dilute to 200 ml with DW. Mix well and filter. To 10 ml of the filtrate add 2 ml of dil. HCl.	Complies with limit test for sulphates.
2.	Ammonium Chloride	To l.0 ml a 25.0% w/v soln. of **barium chloride** in a **Nessler Cylinder** add 1.5 ml of ethanolic sulphate standard solution (10 ppm SO_4), mix and allow to stand for 1 minute. Dissolve l.0 g of NH_4Cl in 15 ml of DW and 0.15 ml of 5 M acetic acid. Add sufficient water to produce 50 ml, stir immediately with a glass rod and allow to stand for 5 minutes.	When viewed transversely against a black background any opalascence produced is not more intense than that obtained by treating in the same manner 15 ml of sulphate standard solution (10 ppm SO_4) in place of the solution being examined (150 ppm).

3.	Calcium Carbonate	Suspend 0.5 g in 5 ml of DW and add dropwise sufficient dil. HCl to effect solution. Add 2 ml dil HCl. Perform the test with the resultant solution.	Complies with the limit test for sulphates.
4.	Calcium Gluconate	Dissolve 1.0 g in 15 ml of DW and carry out the test as described in 2 above.	Complies with limit test for sulphates (150 ppm).
5.	Calcium Lactate	Dissolve 1.0 g in DW by the addition of 2 ml of HCl. Perform the test with the solution.	Complies with the limit test for sulphates.
6.	Dibasic Calcium Phosphate	Dissolve 0.l g in DW by the addition of 1 ml HCl. Perform the test with the solution.	-do-
7.	Dextrose	7.5 ml of a 10% w/v solution of substance diluted to 15 ml with DW and perform the test as stated in 2 above.	Complies with limit test for sulphates (200 ppm)
8.	Ephedrine	Dissolve 0.1 g in 1 ml DW and 1 ml of dil. HCl and add 0.5 ml of $BaCl_2$ solution.	No turbidity is produced within 10 minutes.
9.	Ferrous Gluconate	Dissolve 0.3 g in 5 ml of DW and perform the test as described in 2 above.	Complies with the limit test for sulphates (500 ppm).
10.	Fluorescein Sodium	Dissolve 50 mg in 20 ml DW, and 2.5 ml dil. HCl and filter. Perform the test with the filtrate.	Complies with the limit test for Sulphates.
11.	Lignocaine Hydrochloride	Dissolve 0.2 g in 20 ml DW, add 2 ml 3N HCl, mix, divide into 2 parts. To one part of the soln. add 1 ml $BaCl_2$ soln. (I) and to the other nothing is added (II).	No more turbidity is produced in (I) than in (II).
12.	Mannitol	Dissolve 1.5 g in 10 ml of DW and carry out the test as stated in 2 above.	Complies with the limit test for sulphates (100 ppm).
13.	Phthalylsulphathiazole	Heat 4.0 g with 200 ml DW at 70°C for 5 minutes, cool and filter; Perform test with 50 ml of filtrate.	-do-
14.	Potassium Citrate	Dissolve 0.5 g in DW with addition of 2 ml HCl.	-do-
15.	Quinine Dihydrochloride	Dissolve 0.125 g in. 5 ml of DW and perform the test as described in 2 above.	Complies with the limit test for sulphates (0.12%).
16.	Sodium Bicarbonate	Dissolve 2.0 g in DW with the addition of 2 ml HCl.	-do-
17.	Sodium Hydroxide	Dissolve l.0 g in DW with the addition of 3.5 ml of HCl.	-do-

1.3.9.3. Limit Test for Arsenate

Acetarsol : An organic arsenic compound, being therapeutically active when administered orally, that might be of value in the treatment of *spirochaetal* or *protozoal* diseases, for instance : syphilis, yaws, relapsing fever, sleeping sickness and amoebic dysentry.

It is made from *p*-hydroxyphenylarsonic acid, which may be prepared either by straight forward methods from phenol or from *p*-aminophenylarsonic acid. The resulting compound obtained from either of these reactions is nitrated, reduced and the base is finally acetylated to afford acetarsol.



Acetarsol

Inorganic arsenates are found to be extremely toxic in nature and hence careful control is maintained by the addition of magnesium ammonio-sulphate solution to an aqueous solution of the sample, thereby producing an instant white precipitate.

1.3.9.4. Limit Test for Carbonate

Carbonate impurity in pharmaceutical chemicals usually arise from contamination with atmospheric CO_2.

Examples of a few official compounds subject to this test from the Pharmacopoeia are given below :

S.No.	Pharmaceutical Substance	Test	Official Requirements
1 .	Calcium Hydrogen Phosphate	Dissolve 0.5 g with 5 ml CO_2 free DW and add 1 ml of HCl.	No effervescence is produced.
2.	Calcium Phosphate	5.0 g suspended in 30 ml CO_2 free DW, add 10 ml of HCl.	Dissolves with not more than a slight effervescence.
3.	Sodium Bicarbonate	Measure pH of freshly prepared solution-A (as per BP, 1988).	NMT* 8.6
4.	Purified Talc	When preparing solution A in the test for Calcium, add 1 M. H_2SO_4.	No effervescence produced.

*NMT = Not More Than

1.3.9.5. Limit Test for Cyanide

Cyanide present in Edetate Disodium is assayed by titration with $AgNO_3$ in neutral solution employing dimethylaminobenzylidenerhodamine as an adsorption indicator with a colour change from yellow to orange.

A few typical examples are illustrated below :

A. Edetate Disodium

Materials Required : Edetate disodium 30.0 g ; sodium hydroxide solution (20% w/v in DW) 35.0 ml ; dimethylaminobenzylidenerhodamine solution (0.02% w/v in acetone) 1.0 ml ; 0.01 N $AgNO_3$ solution (1.699 g in 1 litre of DW) 100 ml.

Procedure : Dissolve 30.0 g in a mixture of 100 ml DW and 35 ml NaOH solution, add 1 ml dimethylaminobenzylidenerhodamine and titrate with 0.01N silver nitrate until the colour of the solution changes from yellow to orange. Repeat the operation without the disodium edetate. The difference between the titrations is not more than 1.25 ml.

B. Iodine

Materials Required : Iodine 3.5 g ; zinc powder 10 g ; ferrous sulphate solution (2.0% w/v in boiled and cooled DW) 1.0 ml ; sodium hydroxide solution (20% w/v in DW) 1 ml ; hydrochloric acid ($\simeq$ 11.5 N) 20 ml.

Procedure : Triturate 3.5 g thoroughly with 35 ml DW, filter and decolorise the filtrate by the addition of a little zinc powder. To 5.0 ml of the filtrate add a few drops of ferrous sulphate solution and 1 ml NaOH solution ; warm gently and acidify with HCl, no blue colour or green colour is produced.

C. Potassium Iodide

Materials Required : Potassium iodide 0.5 g ; ferrous sulphate solution (2.0% w/v in boiled and cooled DW) 1 drop ; NaOH solution (20% w/v in DW) 0.5 ml ; HCl 20.0 ml.

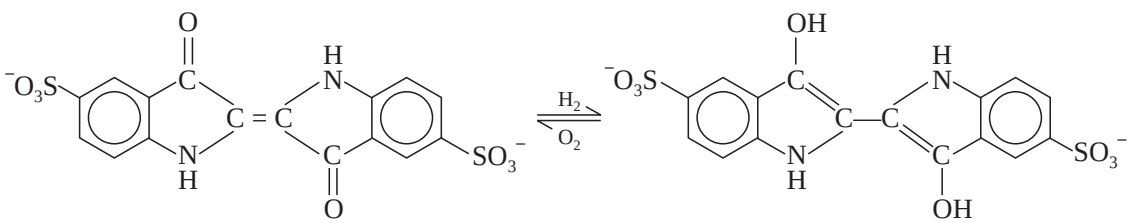
Procedure : Dissolve 0.5 g in 5 ml warm DW, add 1 drop of ferrous sulphate solution and 0.5 ml NaOH solution and acidify with HCl, no blue colour is produced.

1.3.9.6. Limit Test for Nitrate

Basic nitrate is usually found as an impurity in bismuth salts (*e.g.*, bismuth subcarbonate), very often due to the mode of preparation from the metal *via* bismuth nitrate.

BP (1914) first described a limit test, based upon the production of coloured nitro-compounds by the interaction of traces of nitrates with phenol-2, 4-disulphonic acid, and the conversion of these subsequently into dark-yellow ammonium salts. However, this test has a serious disadvantage of correctly matching the yellow colours with great difficulty.

BP (1932) put forward a more reliable test for nitrate based upon the oxidation of indigocarmine to colourless substances by the action of traces of nitrates in presence of hot and fairly concentrated sulphuric acid, and the reaction may be expressed as follows :



The quantities as specifed in the Pharmacopoeia allow an official limit of nitrate equivalent to about 0.29% $BiONO_3$.

A few typical instances of pharmaceutical substances are enumerated below :

S. No.	Pharmaceutical Substance	Test	Official Requirements
l.	Bismuth Subcarbonate	To 0.25 g add 20 ml DW, 0.05 ml indigo-carmine* and then, as a single addition but with caution, 30 ml sulphuric acid. Titrate with indigo-carmine until a stable blue colour is produced.	The volume of indigo carmine required is not more than that equivalent to 1 mg of NO_3 (0.4%).
2.	Calcium Acetate	Dissolve 1.0 g in 10 ml DW, add 5 mg NaCl; 0.05 ml indigo-carmine solution and with stirring 10 ml of N_2-free H_2SO_4.	The blue colour remains for at least 10 minutes.
3.	Magnesium Acetate	-do-	-do-
4.	Pilocarpine Hydrochloride	Dissolve 50 mg in 5 ml DW, and carefully add the soln. to 5 ml of a 0.1% w/v soln. of diphenylamine in H_2SO_4, ensuring that the liquids do not mix.	No blue colour is produced at the liquid interface.
5.	Potassium Acetate	Same as described under (2) above.	Same as stated under (2).
6.	Sulphuric Acid	Carefully add 5.0 ml to a mixture of 5 ml DW and 0.5 ml indigo carmine soln. and allow to stand for 1 minute.	The colour of the solution is discharged.

* **Indigo-Carmine Solution :** To a mixture of 10 ml of HCl and 990 ml of a 20% w/v soln. of N_2-free H_2SO_4 in DW, add sufficient indigo-carmine (about 0.2 g) to produce a solution that complies with the following test :

Add 10 ml to a soln. of l.0 mg of KNO_3 in 10 ml DW, rapidly add 20 ml H_2SO_4 and heat to boiling. The blue colour is just discharged in 1 minute.

1.3.9.7. Limit Test for Oxalate

Oxalate is found to be a frequent impurity in pharmaceutical substances belonging to the category of either organic acids *e.g.*, anhydrous citric acid, tartaric acid; or salts of organic acids *e.g.*, ferrous gluconate, sodium citrate, potassium citrate and sodium cromoglycate. The presence of this impurity is due to the following two prime factors, namely :

(*a*) The use of oxalic acid to get rid of Ca^{2+} during various manufacturing processes.

(*b*) The use of oxalic acid in the isolation and purification of organic bases *e.g.*, ephedrine (thereby resulting into the formation of well defined crystalline oxalates).

A few typical examples are cited below :

S.No.	Pharmaceutical Substance	Test	Official Requirements
1.	Anhydrous Citric Acid	Dissolve 0.8 g in 4 ml DW, add 3 ml HCl and boil for 1 minute with 1 g granulated zinc. Allow to stand for 2 minutes, decant the liquid into a test-tube containing 0.25 ml phenyl-hydrazine HCl* and heat to boiling. Cool rapdily, transfer to a graduated cylinder, add an equal volume of HCl and 0.25 ml potassium hexacyanoferrate** (III), shake and allow to stand for 30 minutes.	Any pink colour produced is not more intense than that produced by treating 4 ml of a 0.01% w/v soln. of oxalic acid at the same time and the same manner (360 ppm, calculated as anhydrous oxalic acid).
2.	Tartaric Acid	-do-	-do-
3.	Sodium Acid Citrate	-do-	Any red colour produced is not more intense than that produced by treating in the same manner 4 ml of a 0.005% sol. of oxalic acid (150 ppm).
4.	Sodium Citrate	-do-	-do-
5.	Ferrous Gluconate	Dissolve 5.0 g in a mixture of 10 ml 1 M H_2SO_4 and 40 ml DW. Shake the soln. with 50 ml ether for 5 minutes. Separate the aqueous layer and shake with a further 20 ml ether for 5 mts. Combine the ethereal layers, evaporate the filtrate to 5 ml and add 1 ml of 2 M acetic acid and l.5 ml solution of $CaCl_2$ ***.	No precipitate is produced within 30 minutes.
6.	Potassium Citrate	Dissolve 1 g in a mixture of 1.5 ml DW and 2.5 ml dilute HCl, add 4 ml alcohol and 4 drops of calcium chloride solution***, and allow to stand for 1 hour.	The mixture remains clear.

* Phenylhydrazine Hydrochloride Solution is 1% w/v in DW.

** Potassium hexacyanoferrate (III) $K_3Fe(CN)_6$ is 5.0% w/v in DW.

*** Calcium Chloride Solution is 7.35% w/v in DW.

1.3.9.8. Limit Test for Phosphate

The limit test for phosphate is based upon the formation of a yellow colour reaction with molybdovanadic reagent (combination of ammonium vanadate and ammonium molybdate) in an acidic medium. However, the exact composition of the molybdovanadophosphoric acid complex is yet to be established.

Three typical examples of pharmaceutical substances are stated below :

S.No.	Pharmaceutical Substance	Test	Official Requirements
l.	Barium Sulphate	To 1.0 g add a mixture of 3 ml 2M HNO_3 and 7 ml DW, heat on a water-bath for 5 minutes, filter, dilute the filtrate to 10 ml with DW, add 5 ml molybdovanadic reagent* and allow to stand for 5 minutes.	Any yellow colour produced is not more intense than that of a standard prepared simultaneously and in the same manner using 10 ml of phosphate standard solution** (= 5 ppm PO_4) (50 ppm).
2.	Sodium Chloride	To 2 ml of a 20% w/v soln. of NaCl in CO_2-free DW, add sufficient DW to make 100 ml. To 100 ml of this soln. add 4 ml of sulphomolybdic solution***, shake, add 0.1 ml of dilute tin(II) chloride solution, allow to stand for 10 minutes and examine 20 ml of the resulting solution.	Any colour produced is not more intense than that produced in 20 ml of a soln. obtained by treating a mixture of 2 ml of phsphate standard solution** (= 5 ppm PO_4) and 98 ml DW in the same manner.
3.	Triclofos Sodium	Dissolve 25 mg in 10 ml of DW, add 4 ml of 1 M H_2S0_4, 1 ml of a 10% w/v soln. of ammonium molybdate and 2 ml of a methylaminophenol-sulphite soln.**** and allow to stand for 15 minutes. Add sufficient DW to produce 25 ml, allow to stand for a further 15 minutes and measure the absorbance of a 4 cm layer of the resulting soln. at 730 nm. Calculate the content of Phosphate from a calibration curve prepared by treating suitable vols. of a 0.00143% w/v soln. of KH_2PO_4 in the same manner.	NMT : 1.0% calculated as PO_4^{3-}.

* **Molybdovanadic Reagent :** Suspend 4.0 g of finely powdered ammonium molybdate and 0.1 g of finely powdered ammonium metavanadate in 70 ml DW and grind until dissolved. Add 20 ml of HNO_3 and dilute to 100 ml with DW.

** **Phosphate Standard Solution** (5 ppm PO_4) : Dilute 0.5 ml of a 0.143% w/v soln. of potassium dihydrogen orthophosphate (KH_2PO_4) to 100 ml with DW.

*** **Sulphomolybdic Solution :** Dissolve with heating, 25 ml ammonium molybdate in 200 ml DW. Separately, with care, add 280 ml H_2SO_4 to 500 ml DW. Cool and mix the two solutions and dilute to 1 Litre with DW.

**** **Methylaminophenol-sulphite Solution :** Dissolve 0.1 g of 4-methylaminophenol sulphate, 20 g sodium metabisulphite and 0.5 g anhydrous sodium sulphite in sufficient DW to produce 100 ml.

1.3.10. LIMIT TESTS FOR NON-METALLIC IMPURITIES

Non-metallic impurities, such as boron, free halogens (I_2, Br_2 and Cl_2) and selenium in pharmaceutical substances usually contribute untoward reactions, skin manifestations and are found to be toxic to healthy tissues.

A few typical examples are described below which essentially contains the above cited nonmetallic impurities :

1.3.10.1. Boron

A. Salbutamol Sulphate : Boron shows its presence in the above compound as a result of the use of sodium borohydride ($NaBH_4$) in the manufacturing process. The estimation depends upon the conversion of boron to borate and the organic matter is subsequently destroyed by ignition with anhydrous sodium carbonate. The quantity of boron is finally determined by colorimetric assay.

Materials Required : Salbutamol sulphate 50 mg ; solution of an equimolar mixture of anhydrous sodium carbonate and potassium carbonate (3% w/v in DW) 5.0 ml ; Solution of curcumin (0.125% w/v in glacial acetic acid) 3.0 ml ; mixture of H_2SO_4 and glacial CH_3COOH (5 ml : 5 ml) 3.0 ml ; ethanol (96%) 100 ml ; solution of boric acid (dissolve 5 g of boric acid in a mixture of 20 ml DW and 20 ml absolute ethanol and dilute to 250 ml with absolute ethanol) : 100 ml.

Procedure : To 50 mg of substance add 5 ml of a 3% w/v solution of an equimolar mixture of anhydrous Na_2CO_3 and K_2CO_3, evaporate to dryness on a water-bath and dry at 120°C. Ignite the residue rapidly until the organic matter has been destroyed, allow to cool and add 0.5 ml DW and 3 ml freshly prepared 0.125% w/v soln. of curcumin in glacial acetic acid. Warm gently to effect solution, allow to cool and add 3 ml of a mixture of H_2SO_4, with stirring, to 5 ml of glacial acetic acid. Mix and allow to stand for 30 minutes. Add sufficient ethanol (96%) to produce 100 ml, filter and measure the absorbance of the filtrate at the maximum of 555 nm. Calculate the content of boron from a reference curve prepared from the absorbance obtained by treating suitable aliquots of a solution of boric acid in the same manner.

Prescribed Limits : Not more than 50 ppm.

1.3.10.2. Free Halogens

A few typical examples of pharmaceutical chemicals in which free halogens like Iodine, Bromine, Fluorine and Chlorine are present as non-metallic impurities are given below.

A. Clioquinol : (Free Iodine)

Materials Required : Clioquinol 1.0 g ; potassium iodide 1.0 g ; H_2SO_4 (1 M) 1.0 ml ; chloroform 2.0 ml ; sodium thiosulphate (0.005 M) 0.1 ml.

Procedure : Shake 1.0 g with a solution of 1 g potassium iodide in 20 ml DW for 30 seconds, allow to stand for 5 minutes and filter. To 10 ml of the filtrate add 1 ml 1 M H_2SO_4 and 2 ml chloroform and shake.

Prescribed Limits : Any colour in the chloroform layer is discharged on the addition of 0.1 ml of 0.005 M sodium thiosulphate.

B. Diethylpropion Hydrochloride : (Free Bromine)

Test : Place 0.05 ml of a 10% w/v solution on starch-iodide paper.

Prescribed Limit : No colour is produced.

C. Doxycycline Hydrochloride : (Free Fluorine)

Materials Required : Doxycyline Hydrochloride : 0.30 g ; oxygen-combustion flask ; 1 L capacity; Nessler cylinder 100 ml ; zirconyl alizarin solution* : 5.0 ml ; fluoride standard solution (10 ppm F) (dilute 5.0 ml of a 0.0442 % w/v soln. of sodium fluoride, previously dried at 300°C for 12 hours, to 100 ml with DW) : 3.0 ml.

Procedure : Burn 0.30 g, in three equal portions, by the method for oxygen-flask combustion (BP), using a 1 Litre flask and a separate 20 ml portion of DW as the absorbing liquid for each combustion, shaking the flask vigorously for about 15 minutes and transferring to the same 100 ml Nessler cylinder. Add 5 ml of acid zirconyl alizarin solution to the combined liquids, adjust the volume to 100 ml with DW and allow to stand for 1 hour.

Prescribed Limit : The colour of the resulting solution is greater than that obtained by repeating the operation with no substance enclosed in the successive portions of filter paper burnt in the method for oxygen flask combustion, but adding 3.0 ml of fluoride standard solution (10 ppm F) to the combined absorption liquids before adding the acid zirconyl alizarin solution.**

---

* **Acid Zirconyl Alizarin Solution :** Dissolve 0.3 g of zirconyl chloride in 50 ml DW, add slowly, while rotating the flask, a solution of 70 mg alizarin red S in 50 ml DW and dilute the clear solution to 1 Litre with a soln. prepared in the following manner. Add 112 ml of HCl in 500 ml of DW ; add 37 ml H_2SO_4 to 400 ml DW, dilute to 500 ml with DW and allow to cool. Mix the two solutions.

** Acid zirconyl alizarin solution should be prepared at least 1 hour before use.

D. Chloroform : (Free Chlorine)

Materials Required : Chloroform 10.0 ml ; cadmium iodide solution (5.0% w/v in DW) 1.0 ml ; starch mucilage 0.1 ml.

Procedure : Shake 10 ml of chloroform with 20 ml of freshly boiled and cooled DW for 3 minutes and allow to separate. To the aqueous layer add 1 ml cadmium iodide soln. and 0.1 ml of 10 ml of starch mucilage.

Prescribed Limit : No blue colour is produced.

E. Tetrachloroethylene (Free Chlorine)

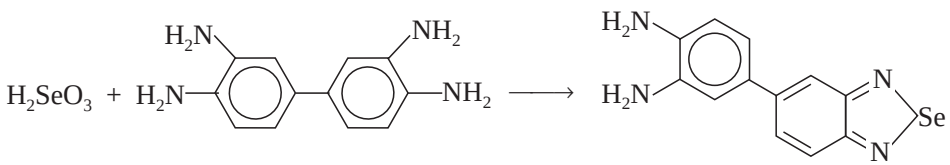
Perform the limit test as stated under chloroform. No blue colour is produced.

1.3.10.3. Selenium

A. Selenium Sulphide

Theory : Selenium is very toxic and its contamination is usually controlled by an absorptiometric method after destruction of the organic compound with fuming nitric acid. The latter converts selenium (Se) as selenous acid (H_2SeO_3), which on subsequent treatment with 3,3′-diaminobenzidine under controlled experimental parameters, results into the formation of a highly coloured compound known as 3,4-diaminophenylpiazselenol. The latter is consequently extracted with toluene after making the aqueous solution alkaline, and the colour compared with a standard prepared likewise from a known amount of selenium. The various reactions involved may be expressed as follows :

$$Se \xrightarrow[(Fuming)]{HNO_3} H_2SeO_3$$



Selenous acid 3, 3′-Diaminobenzidine 3, 4-Diaminophenylpiazselenol

Materials Required : Selenium sulphide : 10.0 g ; formic acid (2.5 M) : 2.0 ml ; 3,3′-diaminobenzedine tetrahydrochloride solution (0.5% w/v in DW) : 2.0 ml ; ammonia (5 M) : 20 ml ; selenium standard solution (1 ppm Se) (Dilute 2.5 ml of a 0.00654% w/v solution of selenous acid to 100 ml with DW) : 5.0 ml.

Procedure : To 10 g of selenium sulphide add 100 ml DW, mix well, allow to stand for 1 hour with frequent shaking and filter. To 10 ml of the filtrate, add 2 ml of 2.5 M formic acid, dilute to 50 ml with DW, adjust the pH to 2.0 to 3.0 with 2.5 M formic acid, add 2.0 ml of a 3,3′-diaminobenzidine tetrahydrochloride in DW, allow to stand for 45 minutes and adjust the pH to 6.0 to 7.0 with 5 M ammonia. Shake the solution for 1 minute with 10 ml of toluene and allow to separate. Measure the absorbance at 420 nm.

Prescribed Limit : The measured absorbance at 420 nm is not greater than that of a solution prepared by treating 5 ml of selenium standard solution (1 ppm Se) in the same manner (5 ppm, calculated as Se).

THEORETICAL AND PRACTICAL EXERCISES

1. What is the importance of 'Purity' in pharmaceutical chemicals for manufacturing drugs ? Discuss with suitable examples.
2. Give a comprehensive account on the following aspects :
   (*a*) Biological response *Vs* chemical purity.
   (*b*) Official standards *Vs* manufacturing standards.
3. Elaborate with specific examples the various sampling procedures and errors commonly encountered in a quality control laboratory.
4. Why do the chemical purity and bioavailability of a **'drug'** equally important to determine the efficiency of a **'dosage form' ?**

5. What are the various **'physical parameters'** that ultimately establish the purity of a drug substance ? Explain with appropriate examples.
6. Discuss the **'miscellaneous characteristic features'** included in **'official compendia'** to establish the purity, authenticity and identification of drugs. Give examples in support of your answer.
7. Give a detailed account on the **'Limit Tests'** *Vs* **'Quantitative Determinations'** by providing suitable examples.
8. Describe the theory, apparatus and procedure involved in the **'limit tests'** for **metallic impurities** *e.g.*, Pb^{2+}, As^{3+} and Fe^{3+}.
9. How will you carry out the **'limit tests'** for **acid radical impurities** *e.g.*, Cl^{-}, SO_4^{2-}, CO_3^{2-}, CN^{-}, NO_3^{-}, Arsenate, Oxalate and Phosphate ?
10. Elaborate the various **'limit tests'** recommended for the **'Non-metallic Impurities'** in **official compendia** *e.g.*, Boron, Halogens, Selenium. Give typical examples to justify your answer.
11. How will you determine the limit test for **'Iron'** in Calcium Lactate and Zinc Oxide ? Explain.

RECOMMENDED READINGS

1. Mandel, J. 'Accuracy and Precision : Evaluation and Interpretation of Analytical Results', In **Treatise on Analytical Chemistry,** ed. by I.M Kolthoff and P.J. Elving, 2nd edn., Vol. 1. New York, Wiley and Sons, Inc., 1978.
2. Pietrzyk, D.J. and C.W. Frank, '**Analytical Chemistry**', 2nd edn., New York, Academic Press, 1979.
3. Diehl, H., D.C. Johnson., R. Markuszewski, and P.M. Moore, 'Colourblindness and Titrations with Visual Indicators', **J. Chem. Edu.62** : 255, 1985.
4. Beckett, A.H. and J.B. Stenlake, '**Practical Pharmaceutical Chemistry**', 3rd ed., Pt. 1, New Delhi, CBS-Publishers & Distributors, 1986.
5. Florene A.T. and Atwood D., '**Physicochemical Principles of Pharmacy**' 2nd Edn., Macmillan Press, London, 1988.

2 THEORY AND TECHNIQUE OF QUANTITATIVE ANALYSIS

CONTAINS :

2.1 INTRODUCTION

The '**technique of quantitative analysis**' is broadly based on the following **three** major heads, namely :

(*a*) Technique of Volumetric Analysis,

(*b*) Technique of Gravimetric Analysis, and

(*c*) Biomedical Analytical Chemistry.

Volumetric analysis essentially comprises of the most precise and accurate measurement of interacting solutions or reagents. It makes use of a number of graduated apparatus, such as : graduated (volumetric) flasks, burettes, pipettes and measuring cylinder of different capacities (volumes).

However, it is pertinent to mention here that quite a few techniques related to measurement of pharmaceutical substances and reagents involved is more or less common to both gravimetric and volumetric analysis. Besides, in gravimetric analysis, some more additional techniques play a vital role, namely : precipitation, filtration, washing of the precipitate and ignition of the precipitate.

Biomedical analytical chemistry happens to be one of the latest disciplines which essentially embraces the principles and techniques of both analytical chemistry and biochemistry. It has often been known as 'clinical chemistry'. This particular aspect of analytical chemistry has gained significant cognizance in the recent past by virtue of certain important techniques being included very much within its scope of analysis, namely : colorimetric assays, enzymic assays, radioimmunoassays and automated methods of clinical analysis.

It is, however, important to mention here that certain other routine procedures also carried out in a clinical laboratory fall beyond the scope of biomedical analytical chemistry, narnely : microbiological assays, heamatological assays, serum analysis, urine analysis and assays of other body fluids.

It will be very much within the scope of this chapter to discuss briefly the various important details, with specific examples wherever necessary, of volumetric analysis, gravimetric analysis and biomedical analytical chemistry.

2.2. VOLUMETRIC ANALYSIS

Volumetric analysis may be broadly defined as those analytical methods whereby the exact volume of a solution of known concentration actually consumed during the course of an analysis is considered as a measure of the amount of active constituent in a given sample under determination (assay).

2.2.1. THEORY

According to the *official method of analysis*, hydrochloric acid can be determined by *first* weighing a given sample accurately, and *secondly*, by adding carefully a solution of known strength of sodium hydroxide in the presence of an appropriate indicator unless and until the exact equivalent amounts of HCl and NaOH have undergone the following chemical reaction :

$$\underset{\text{Analyte}}{HCl} + \underset{\text{Titrant}}{NaOH} \longrightarrow NaCl + H_2O$$

Analyte (or Active Constituent) is the chemical entity under assay *e.g.*, HCl.

Titrant is the solution of known strength (or concentration) employed in the assay *e.g.*, NaOH.

Titration is the process of adding and then actually measuring the volume of titrant consumed in the assay. This volume is usually measured by the help of a calibrated burette.

Indicator is a chemical substance sensitive enough to display an apparent change in colour very close to the point in the ongoing titration process at which equivalent quantities of analyte and titrant have almost virtually reacted with each other.

Equivalence Point (or Stoichiometric Point) is the point at which there appears an abrupt change in certain characteristic of the prevailing reaction mixture—a change that is either ascertained electrometrically or is visibly spotted by the use of indicators.

In usual practice, the volumetric titrations may be accomplished either by direct titration method *e.g.*, assay of HCl employing NaOH as the titrant, or by residual titration method *e.g.*, assay of ZnO in which case a known-excess-measured volume of standardised solution of H_2SO_4, more than the actual amount chemically equivalent to ZnO, is added to the sample ; thereupon, the H_2SO_4 which remain unreacted with ZnO is subsequently titrated (sometimes referred to as **back titration** or **residual titration** in the text) employing standardized NaOH solution.

Thus, we have :

Known amount of H_2SO_4 consumed ≡ Known amount of NaOH + Unknown amount of ZnO

Most *official compendia* usually record the results of drug assays in terms of % w/v, % w/w and % v/v.

2.2.2. DEFINITIONS

In order to have a clear-cut understanding of the various calculations involving volumetric assays throughout this book one needs to gain an in-depth knowledge of the various terms related to '**equivalents**'. They are :

(*a*) **Gram-equivalent Weight (GEW) :** It is the weight in grams that is chemically equivalent to 1 gram-atom of hydrogen (1.0079 g).

It is also sometimes simply referred to as the 'gram-equivalent'. However, GEW has two distinct definitions for neutralization as well as as oxidation-reduction reactions as stated below :

(*i*) **For Neutralization Reactions :** GEW is defined as *that weight of a substance in grams which contains, furnishes, reacts directly or indirectly and replaces 1 gram-atom or ion of hydrogen.*

(*ii*) **For Oxidation—Reduction Reactions**

Explanation : A reaction usually takes place by the combination of oxidizing and reducing agents and this may be considered as the basis for the quantitative measurement of one of the reactants. For instance, $FeSO_4$ can be determined quantitatively by its reaction with ceric sulphate $[Ce(SO_4)_2]$ as expressed by the following equation :

$$Fe^{2+} + Ce^{4+} \longrightarrow Fe^{3+} + Ce^{3+} \qquad ...(a)$$

Equation (*a*) can be split into two half-equations as shown below thereby depicting the loss of electrons by the Fe^{2+} ion [Eq. (*b*)] and the gain of electrons by the Ce^{4+} [Eq. (*c*)] :

$$Fe^{2+} \rightleftharpoons Fe^{3+} + e \qquad ...(b)$$

$$Ce^{4+} + e \rightleftharpoons Ce^{3+} \qquad ...(c)$$

From Eq. (*a*) it is evident that each molecule of $FeSO_4$, upon oxidation, happens to lose one electron. Hence, one mole of $FeSO_4$ loses 6.02×10^{23} electrons which is equivalent to 1 Faraday or 96,500 C. Thus, in electrochemical determination of equivalence point the quantity of electricity is almost identical with that required to reduce 1 mole of $Ce(SO_4)_2$. It follows from here that 1 mole of $FeSO_4$ and 1 mole of $Ce(SO_4)_2$ are chemical equivalents. In other words, 1 g of H, acting as a reducing agent, loses electrons equivalent to 96,500 C.

(*b*) **Equivalent Weight of a Reducing Agent** is that weight which loses electrons equivalent to 96,500 C.

It may be calculated by dividing the gram-molecular weight by the number of electrons lost by each molecule, for instance :

$$Fe^{2+} \longrightarrow Fe^{3+} + e$$

hence, the equivalent weight of $FeSO_4$ oxidizing to $Fe_2(SO_4)_3$ comes out to be 151.919 [$FeSO_4$: molecular weight = 151.91] or 1 gram-molecular weight.

(*c*) **Equivalent Weight of an Oxidizing Agent** is that weight which gains electrons equivalent to 1 Faraday, or to the electrons gained by 1 gram-ion of H^+ ions ($2H^+ + 2e \rightarrow H_2$).

It may be calculated by dividing the gram-molecular weight by the number of electrons gained by each molecule, for example :

(*a*) $Ce^{4+} + e \longrightarrow Ce^{3+}$ (cerous ion)

hence, the equivalent weight of ceric sulphate is 1 gram-molecular weight 332.24 g [$Ce(SO_4)_2$: molecular weight = 332.24]

(*b*) $MnO_4^- + 5e \longrightarrow Mn^{2+}$ (manganous ion)

hence, the equivalent weight of potassium permanganate is 1/5th gram-molecular weight 31.61 g.

($KMnO_4$: $1/5 \times 158.05 = 31.61$)

(c) $Cr_2O_7^{2-} + 6e \longrightarrow 2Cr^{3+}$ (chromous ion)

hence, the equivalent weight of potassium dichromate is l/6 gram-molecular weight 49.03 g.

($K_2Cr_2O_7$: $1/6 \times 294.18 = 49.03$)

(*d*) $I_2 + 2e \longrightarrow 2I^-$ (iodide ion)

hence, the equivalent weight of iodine is 1 gram-molecular weight 126.90 g. (I_2 : Molecular Weight = 126.90)

(*e*) $BrO_3^- + 6e \longrightarrow Br^-$ (bromide ion)

hence, the equivalent weight of potassium bromate is 1/6 gram-molecular weight 27.83 g.

($KBrO_3$: $1/6 \times 167.01 = 27.83$)

(*d*) **Gram-milliequivalent Weight (GmEW)** is nothing but GEW/1000. This term is very much used in all types of volumetric calculations.

(*e*) **Equivalent (equiv)** is the number of gram-equivalents involved in a quantitative method.

(*f*) **Milliequivalent (meq)** is the number of gram-milliequivalents involved in a quantitative method.

However, meq is used more frequently than equiv in quantitative procedures.

(*g*) **Standard Solution** is a solution of known (pre-determined) normality or molarity.

(*h*) **Normality** (expression of concentration) is the number of equivalents of solute per litre (equiv/lire) or milliequivalents per ml. (meg/ml) solution.

(*i*) **Molarity** is the expression of the concentration of a solution in terms of moles per litre.

(*j*) **Standardization** is the actual determination of either the normality or the molarity of a solution.

(*k*) **Primary Standard** is the substance of known purity ('AnalaR'-grade reagents) whose carefully weighed quantity helps in the standardization of an unknown solution (normality or molarity).

(*l*) **Secondary Standard** is another standard solution that is used for standardization of an unknown solution.

Example : An unknown solution of HCl may be standardized volumetrically in *two* ways, namely :

(*i*) by the help of 'AnalaR'-grade Na_2CO_3 *i.e.*, purity is known-'**Primary Standard**', and

(*ii*) by the help of another standard solution of NaOH—'**Secondary Standard**'.

(*m*) **Titer :** is the weight of a substance chemically equivalent to 1 ml of a standard solution.

Example : 1 ml of 1 N HCl contains 0.03646 g (*i.e.*, 0.001 equiv or 1 meq) of HCl and hence is chemically equivalent to 0.04000 g (*i.e.*, 0.001 equiv or 1 meq) of NaOH.

Thus, most calculations in volumetric determinations (titrimetry) are enormously facilitated by using titer values, which may be seen in the following chapters related to various categories of volumetric titrations.

For instance, in the offcial procedure for the assay of tartaric acid, it is stated that 'Each millilitre of 1 N sodium hydroxide is equivalent to 75.04 mg of $C_4H_6O_6$'. The $C_4H_6O_6$ titer of 1 N sodium hydroxide is, therefore, 75.04 mg/ml, a value that may be calculated as follows :

An examination of the equation indicates that 1 mole or 150.09 g of $H_2C_4H_4O_6$

$$\underset{(150.09)}{H_2C_4H_4O_6} + 2NaOH \longrightarrow Na_2C_4H_4O_6 + 2H_2O$$

is 2 equiv, and the equivalent weight of $H_2C_4H_4O_6$ is 75.04 g. Hence, each millilitre of 1 NaOH contains 0.001 equiv of NaOH and is equivalent to 0.001 equiv or $0.001 \times 75.04 = 0.07504$ g or 75.04 mg of $H_6C_4O_6$.

2.2.3. VOLUMETRIC APPARATUS

As we have seen that the volumetric analysis essentially requires the precise and accurate measurement of weights and volumes of interacting solutions. However, the weights are measured upto the fourth place of decimal by using a manually operated good analytical balance or a single-pan electrical balance that need to be calibrated periodically with the help of a standard weight box.

In the broader sense, volumetric apparatus may be divided into *two* categories, namely :

(*a*) To deliver a definite volume of liquid, and

(*b*) To contain a definite volume of liquid.

2.2.3.1. Volumetric Apparatus Meant to Deliver a Definite Volume of Liquid

The two specific volumetric apparatus meant to deliver a defnite volume of liquid are burettes and pipettes which will be discussed very briefly below :

2.2.3.1.1. *Burettes*

Various *official compendia* specifies a standard temperature (°C) for glass volumetric apparatus as mentioned hereunder :

Pharmacopoeia of India (IP) : 27°C ;

United States Pharmacopoeia (USP) and National Formulatory (NF) : 25°C ;

National Bureau of Standards (NBS) : 20°C.

A burette is a graduated glass tube of uniform bore throughout the entire length, used for the accurate delivery and measurement of variable volumes of liquids. Burettes are graduated into millilitres (ml) and 1/10 millilitres (0.1 ml) and are made of varying capacity ranging from 1 ml to 100 ml ; however, the most common size is the 50 ml burette that is used invariably and conveniently for most volumetric titrations. They are usually closed at the bottom either by a Teflon or glass stopcock to monitor and control the outflow of liquid.

Specifications : The design, construction and capacity of volumetric glassware must be in accordance with those laid down by the Indian Standards Institution (ISI). The tolerances on capacity for burettes, as specified in the relevant Indian Standards Institution, specifications are given in Table 2.1.

Table 2.1 : Tolerance on Capacity for Burettes*

Nominal capacity (ml) :	10	25	50	100
Subdivision (ml) :	0.05	0.05	0.1	0.1
Tolerance (±ml) :	0.01	0.03	0.05	0.1

* Burettes : I.S. 1997-1967.

British Standards Institution (B.S. 846 : 1962) has laid down specifications for burettes and these are produced to either Class 'A' or Class 'B' specifications. All Class 'A' and a few of Class 'B' burettes have graduations that extend right round the barrel (or stem) of the burette to minimise errors due to parallax while taking the exact burette reading. It may be noted that Class 'B' burettes are normally graduated on one side only. Permitted tolerances on capacity for burettes used in common practice are stated in Table 2.2.

Table 2.2 : Tolerance on Capacity for Burettes*

Nominal Capacity (ml)	Scale subdivision (ml)	Tolerance on capacity (± ml)	
		Class 'A'	Class 'B'
1	0.01	0.006	0.01
2	0.02	0.01	0.02
5	0.02	0.01	0.02
5	0.05	0.02	0.04
10	0.02	0.01	0.02
10	0.01	0.00	0.05
25	0.05	0.03	0.05
25	0.1	0.05	0.1
50	0.1	0.05	0.1
100	0.2	0.1	0.2

* B.S 846 : 1962

In fact, the tolerance actually represents the maximum error allowed at any point and also the maximum difference allowed between the errors at any two points. For instance, a tolerance of ± 0.05 ml signifies that the burette may have an error at any point by ± 0.05 ml, provided that the difference between the errors at any two given points does not exceed 0.05 ml.

Burettes calibrated at 20°C and 25°C deliver different weights of water for each 10 ml, when weighed with standard brass weights in air at 50% relative humidity (RH) at standard atmospheric pressure, as given below :

At 20°C	9.9718 g*
At 25°C	9.9604 g**

* 1L DW at 20°C weighs 997.177 g

** 1L DW at 25°C weighs 996.04 g

Hence, the true volume for each 10 ml segment of the burette can be calculated from the weights obtained and recorded on a convenient chart.

Leakage : A burette must be tested for any sort of leakage before putting it into operation. Teflon stopcocks are usually adjusted by a knurled nut for perfect use. Glass stopcocks may require a small quantity of a special type of grease or lubricant to allow both ease of operation and to check leakage.

Outlet Tip : From a practical point of view the outlet tip of either types of burette, *i.e.*, having Teflon or glass stopcocks, must be of such diameter and taper as to allow the delivery of a single drop whose volume is significantly less than that which can be held between any two finest graduations of the scale with which the burette is calibrated.

Use of the Burette : The following steps are usually observed while operating a burette, namely :

(*i*) Burette tap is neatly lubricated with a thin-film of grease,

(*ii*) Rinse the burette, before putting it into operation, at least twice with small volumes of the solution (titrant), say about 5.0 ml, carefully draining out the solution between the addition of each portion,

(*iii*) Pour the solution into the burette until the former is little above the zero mark,

(*iv*) Open the burette tap slowly to fill up the tip of the burette and to expel all air bubbles,

(*v*) With the zero at eye-level carefully, drain out the liquid until the lower part of the meniscus is either at level or just below the Zero mark,

(*vi*) Remove the drop on the tip of the burette by just touching rapidly against the inner-neck of a flask or a porcelain tile,

(*vii*) The Class 'B' burettes should be read at level so as to avoid errors due to parallax,

(*viii*) To assist easy and accurate observation of the meniscus (lower for colourless solutions and upper for coloured solutions) it is always advisable to hold a piece of white paper behind the burette at the appropriate level,

(*ix*) Burette readings may be recorded to the nearest 0.02 ml, and

(*x*) Once a titration is completed, 15 seconds duration should be allowed to elapse before the final reading is made, to allow for drainage.

2.2.3.1.2. *Pipettes*

The pipette is the second volumetric apparatus that is meant to deliver a definite volume of liquid. Pipettes are of *two* types, namely :

(*i*) **Transfer Pipettes :** They have only one specific mark engraved on them and are specifically employed to deliver (or transfer) a definite volume of liquid under certain specified conditions, and

(*ii*) **Graduated Pipettes :** They have graduated stems and are used to deliver different small volumes as needed. However, they are not normally used for measuring very exact volumes of liquids.

The tolerances on capacity for pipettes, as specified by the **Indian Standards Institution** (ISI), are stated in Tables 2.3 and 2.4.

Table 2.3 : Tolerance on Capacity for One Mark Pipettes*

Nominal Capacity (ml)	1	2	5	10	20	25	50	1000
Tolerance (± ml)	0.01	0.01	0.02	0.02	0.03	0.03	0.04	0.06

* I.S. 1117-1975

Table 2.4 : Tolerance on Capacity for Graduated Pipettes**

Nominal Capacity (ml)	1	2	5	10	25
Subdivision (ml)	0.01	0.02	0.05	0.10	0.20
Tolerance (± ml)	0.006	0.01	0.03	0.05	0.10

** I.S. 4162-1967

The **British Standards Institution (BSI)** has laid down the permitted tolerances and delivery times for commonly used bulb transfer pipettes as shown in Table 2.5.

Table 2.5 : Tolerances and Delivery Times for One-Mark Pipettes*

Capacity (ml)	Tolerance (± ml)		Delivery Times (secs)		
	Class 'A'	Class 'B'	Min Class 'A'	Min Class 'B'	Max Classes 'A' & 'B'
1	0.007	0.015	7	5	15
2	0.01	0.02	7	5	15
3	0.015	0.03	10	7	20
4	0.015	0.03	10	7	20
5	0.015	0.03	15	10	25
10	0.02	0.04	15	10	25
15	0.025	0.05	20	15	30
20	0.03	0.06	25	20	40
25	0.03	0.06	25	20	40
50	0.04	0.08	30	20	50
100	0.06	0.12	40	30	60
200	0.08	0.16	50	40	70

* B.S 1583 : 1961

The USP specifies the following tolerances accepted by the National Bureau of Standards for transfer pipettes :

Designated Volume (ml) :	1	2	5	10	25	50	100
Limit of Error (ml) :	0.006	0.006	0.01	0.02	0.03	0.05	0.08
Limit of Error (%) :	0.60	0.30	0.20	0.20	0.12	0.10	0.08

The salient features of single-graduation mark transfer pipettes are :

(*a*) Capacity, temperature at which it was graduated (Ex) and reference to delivery time in seconds is stated on the bulb *e.g.*, BOROSIL 1552 25 secs 'A' Ex 20 ml 20°C BS 1583.

(*b*) Class 'A' pipettes do mention the delivery time,

(*c*) Drainage time is specified, though an additional waiting time of 3 seconds after apparent cessation of flow is still important.

Note : The stated times apply only for water and aqueous solutions.

Use of the Transfer Pipette : The following steps mentioned sequentially must be followed while making use of a transfer pipette :

(1) Always rinse the pipette with DW before use and allow it to drain as completely as possible,

(2) Droplets of water remaining in the tip must be removed by touching against filter paper; and at the same time wipe out the outer surface of the pipette to prevent dilution of the solution to be pipetted,

(3) Rinse the pipette with 2 to 3 small portions (5 ml) of the solution and drain out the liquid completely,

(4) Gently suck the liquid up into the pipette a little above the single graduation mark and quickly shut the upper end of the pipette with the tip of the index finger. Now, remove the pipette from the stock solution and carefully wipe out the outer surface of the stem free from any liquid adhering to it. Hold the pipette vertically and keeping the graduation mark at the eye-level, slowly release the pressure on the index finger until the bottom of the meniscus just coincides with the graduation mark. Maintain sufficient pressure on the index-fnger so as to check any escape of liquid from the pipette, and quickly get rid of the drop attached to the tip by gently touching against a porcelain tile. Put the pipette into the receiving container, and permit the liquid to drain out with the tip of the pipette touching the inside of the container at an angle of 60°, taking care that the tip must not be dipping into the delivered liquid. After all the solution has drained out, hold the pipette in this position for at least 3 seconds (waiting time), and then remove the pipette.

Note : (1) The **National Physical Laboratory** (NPL) describes a method of reading meniscus in graduated glassware, *viz*., a dark horizontal line on a white background is placed 1 mm below the meniscus. A slight adjustment of the position of the dark line causes the meniscus to stand out sharply against the white background,

(2) The small drop of liquid that remains in the tip of the emptied pipette is taken into account while doing the calibration, and hence, it must not be added to the delivered liquid by blowing down the pipette.

(3) Liquids having more viscosity and much larger surface tension than water must be provided with adequate draining time *e.g.*, strong solution of iodine.

(4) Presently, many analysts make use of pipette filler for sucking in and draining out of liquids from the transfer pipettes for obvious reasons.

Automatic Pipettes (Transfer Pipettes) : Automatic pipettes are always preferred to ordinary transfer pipettes because of their ability to handle corrosive and toxic liquids in routine analytical laboratories, *e.g.*, determination of Iodine Value in edible oils by iodine-monochloride (ICl) solution.

The automatic pipette (Figure 2.1) dispenses a stated volume of liquid when filled with liquid used in the assay from tip (B) to tip (C) and is allowed to drain out in the normal manner. D is connected to an aspirator which is placed above the pipette so as to enable the solution to flow under gravity.

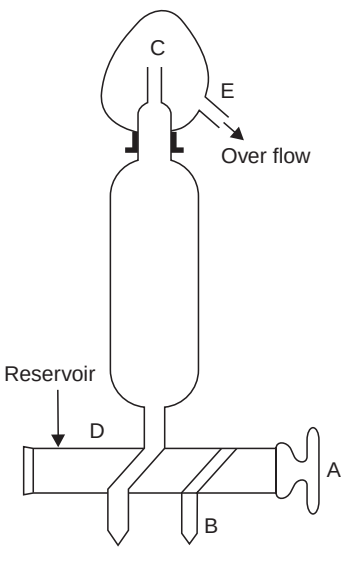


Figure 2.1 : Automatic Pipette.

Operation of the Automatic Pipette : The automatic pipette may be operated by observing the following steps in a sequential manner :

(1) Turn the two-way tap clockwise to open so that the solution starts flowing into the pipette.

(2) After about 5.0 ml has run into the pipette, turn A clockwise through 180°, so that solution now flows from the pipette to fill the delivery tube B.

(3) As soon as B is full upto the tip, again turn A clockwise through 180°, so that the body of the pipette is filled completely to the top-tip.

(4) Close the tap A by turning clockwise through 90° when the solution starts to overflow at C.

(5) The pipette is now full from the lower-tip to the upper-tip and is ready for operation.

(6) Remove the drop of solution from tip B, run out and drain for 15 seconds in the usual way.

2.2.3.2. Volumetric Apparatus Meant to Contain a Definite Volume of Liquid

The two particular volumetric apparatus meant to contain a definite volume of liquid are volumetric flasks (also known as measuring or graduated flasks) and measuring cylinders (also known as graduated cylinders) which will be discussed here briefly :

2.2.3.2.1. *Volumetric Flasks (Syn. Measuring Flasks or Graduated Flasks)*

Volumetric flasks are normally round or pear-shaped, flat-bottomed; having a long-neck, which possesses a single graduation mark round the neck.

Flasks bearing one graduation mark, are meant to contain specified volume of liquid at 20°C, when the lower part of the meniscus coincides with the mark and are known as volumetric flasks.

The long and narrow neck of uniform diameter affords as a measure of accurate adjustment, since the height of the liquid is sensitive enough to small variations of volume.

Units of Capacity

Litre—is defined as '*the volume occupied by one kilogram of water at its temperature of maximum density (4°C) and subjected to normal atmospheric pressure*'. The litre is considered as the standard unit of volume for all volumetric measurements.

The cubic centimetre is the volume occupied by a cube of which each side is 1 cm in length, and thus, 1 litre equals 1000.028 c.c. Therefore, it follows from here that the millilitre and cubic centimetre are not the same, though the difference is quite negligible. Hence, all volumetric apparatus is universally standardized in millilitres.

Standard Calibrations

The **Indian Standard Institution** (ISI) has laid down the tolerances on capacity of volumetric flasks (with different capacities) calibrated at 27°C as stated in Table 2.6.

Table 2.6 : Tolerance on Capacity of Volumetric Flasks*

Nominal capacity (ml) :	5	10	25	50	100	250	500	1000
Tolerance (± ml) :	0.02	0.02	0.03	0.04	0.06	0.1	0.15	0.2

* I.S 915-1975

The **United States Pharmacopoeia** (USP) requirements for volumetric flasks calibrated to contain the indicated volume at 25°C are given in Table 2.7.

Table 2.7 : Designated Volume with Limit of Error for Volumetric Flasks

Designated Volume (ml) :	10	25	50	100	250	500	1000
Limit of Error (ml) :	0.02	0.03	0.05	0.08	0.12	0.15	0.30
Limit of Error (%) :	0.20	0.12	0.10	0.08	0.05	0.03	0.03

The **British Standards Institution** (BSI) and the **National Physical Loaboratory** (NPL) have laid down the tolerances in the capacity of volumetric flasks (*i.e.*, measuring flask) at 20°C by two sets of tolerances *viz*., Grade 'A' and Grade 'B' respectively, evidently to indicate the class of accuracy to which the flask has been subjected to for graduation, followed by the manufacturer's name and finally the BS standard number. However, the permitted tolerances for volumetric flasks commonly used in analytical laboratories are depicted in Table 2.8.

Table 2.8 : Tolerances in the Capacity of Volumetric Flasks*

S.No.	Capacity (ml)	Tolerance Class 'A' (± ml)	Tolerance Class 'B' (± ml)
1	5	0.02	0.04
2	10	0.02	0.04
3	25	0.03	0.06
4	50	0.05	0.10
5	100	0.08	0.15
6	200	0.15	0.30
7	250	0.15	0.30
8	500	0.25	0.50
9	1000	0.40	0.80
10	2000	0.60	1.20

* B.S. 1792

Preparation of Standard Solutions

A reasonably well established analytical laboratory requires a number of standard solutions for its routine as well as specific assays. Therefore, it necessitates to know the intricacies of preparing the standard solutions as detailed in the following steps :

(1) Transfer the requisite quantity of the accurately weighed pharmaceutical substances or solid quantitatively into a beaker and dissolve it in either distilled water (DW) or other specified solvent,

(2) Pour the resulting solution quantitatively, into the funnel placed in the mouth of the volumetric flask with the help of a glass rod and a sharp jet of water from a wash-bottle by holding the beaker with the right hand and the guiding rod with the left hand,

(3) Wash down the contents of the beaker through the funnel by means of the glass rod and the jet of DW. Repeat the process several times till the flask is 2/3rd full,

(4) Remove the funnel, swirl the contents of the volumetric flask and make up the volume upto the mark,

(5) Final adjustment of the volume must be made with the help of a teat pipette by adding DW/solvent dropwise. In doing so, adequate care should be taken to allow suffcient time for water/solvent to drain-down the inside of the neck of the flask, and

(6) Finally shake the contents of the flask thoroughly for 2 to 3 minutes to obtain a perfect homogeneous solution.

Note : (*i*) For precise work, the temperature of the solution must be adjusted to 20°C before making the volume upto the mark,

(*ii*) Standard solutions are usually stored in stock-bottles,

(*iii*) Ensure before any transfer is actually affected that the receiving vessel must be rinsed with at least 2 to 3 successive small quantities of the solution, and

(*iv*) When a standard solution is used a while after preparation, the contents of the stockbottle must be shaken thoroughly before any solution is withdrawn, thereby the condensed droplets of water collected on the inside neck of the container gets mixed with the main bulk of the solution.

2.2.3.2.2. *Graduated Cylinders*

The graduated cylinders are also referred to as the measuring cylinders among volumetric apparatus meant to contain a definite volume of liquid. Measuring cylinders are containers either unstoppered or stoppered having a wide range of capacities varying from 5 ml upto 2000 ml (2 Litres). In usual practice, the smaller cylinders upto 100 ml are normally graduated either in fractions of a millimitre or in millilitres. On the contrary,

the large cylinders are graduated in units of 2, 5, 10, 20, or 50 ml, as per their specific size and volume. However, it is pertinent to mention here that measuring cylinders are used in a broader sense for measuring volumes of solution when only approximate volumes are needed.

2.2.4. GENERAL COSIDERATIONS

Volumetric apparatus invariably used in titrimetric assays, meant either to deliver a definite volume of liquid *viz*., burettes and pipettes, or to contain a definite volume of liquid *viz*., volumetric flasks and measuring cylinders, have essentially the following **three** cardinal general considerations, namely :

(*a*) Cleaning of volumetric apparatus,

(*b*) Calibration of volumetric apparatus, and

(*c*) Effect of temperature on volumetric measurement.

These three aspects will be discussed briefly hereunder :

2.2.4.1. Cleaning of Volumetric Apparatus

New as well as used volumetric apparatus, namely : burettes, pipettes, volumetric flasks and measuring cylinders etc., employed in carrying out most of the pharmacopoeial assays should be extremely clean. It is particularly of great importance where small volumes of liquids are measured.

A positive evidence for a dirty apparatus may be sought by observing the adherence of droplets to the walls of a burette or pipette. However, in a clean volumetric apparatus, the liquid drains down quite uniformly thereby wetting the walls so that no droplets are visible to the naked eye.

A few very effective cleaning fluids that are used in good analytical laboratories are, namely :

(*i*) Chromic Acid Mixture,

(*ii*) Synthetic Detergent Solutions (or Alkaline Cleansing Agents), and

(*iii*) Teepol.

2.2.4.1.1. *Chromic Acid Mixture*

Materials Required : Sodium dichromate : 200 g ; Sulphuric acid : 1500 ml.

Procedure : Weigh 200 g sodium dichromate and transfer to a 2 Litre hard-boroslicate glass beaker. Dissolve it in 100 ml of water and cool in an ice-bath to about 10-15°C. Now, add to it 1500 ml of sulphuric acid (36 N) in small bits at intervals with constant stirring. Chromic acid mixture is extremely corrosive and hygroscopic and must be stored in closed glass-stoppered bottles.

Precautions :

(*i*) Chromate solution should be chilled before addition of H_2SO_4,

(*ii*) Safety goggles should be worn during the addition of the acid,

(*iii*) In case, a green colour develops, discharge the mixture into a sink with continuously flowing water,

(*iv*) Chromic acid must not be used for cleaning calibrated containers employed for optical measurements,

(*v*) Glass apparatus washed with chromic acid mixture must be subjected to adequate prolonged rinsing because glass (silicates and borosilicates) have a tendency to absorb the chromic acid,

(*vi*) Hot solutions should be avoided when cleaning accurately calibrated apparatus, due to the production of a permanent change in volume caused by heat known as *thermal aftereffect*,

(*vii*) All volumetric glasswares must be finally rinsed with purified water (distilled water) before use for analytical purposes.

2.2.4.1.2. *Synthetic Detergent Solutions (or Alkaline Cleansing Agents)*

Detergents are synthetic cleansing agents used with water. The most commonly used anionic surfactants containing carboxylate ions are known as soaps which are generally prepared by the saponification of natural fatty acid glycerides in alkaline solution. Usually a 2 to 5% (w/v) solution of a good detergent powder in water serves as a reasonably effective cleansing agent.

2.2.4.1.3. *Teepol (or Gardinol Type Detergents)*

It is a mixture of the sodium salts of sulphated fatty alcohols made by reducing the mixed fatty acids of coconut oil or cottonseed oil, and fish oils. Sometimes natural waxes such as spermaceti, wool fat and bees wax are sulphated directly.

A 1 to 3% (w/v) solution of Teepol in water may also serve as a good cleansing agent for the removal of stubborn deposits and stains present in glass apparatus.

2.2.5. TECHNIQUE OF VOLUMETRIC ANALYSIS

Following are the various steps that need to be observed carefully so as to achieve reasonably correct and reproducible results in the volumetric titrations :

(1) Conical flasks are considered to be the most suitable vessels meant for volumetric titrations because the mixing can be performed quite rapidly, easily and safely by gently swirling the contents of the flask during the titration,

(2) Beakers are not usually preferred, but in case they are to be used in volumetric analysis, following *two* provisions may have to be made for stirring :

(*a*) use of a magnetic stirrer with a magnetic guide for the solution, and

(*b*) use of a stirring rod,

(3) The titration container or vessel must always be kept polished so as to view the end point vividly,

(4) The solution under titration is normally viewed against a white background *e.g.*, white tile or white paper,

(5) When the end point is being approached it is always advisible to have the drops of titrant split. It can be accomplished by opening the stopcock of the burette in such a manner that only a fraction of a drop flows out and remains adhered to the tip of the burette. Touch of the liquid against the inside of the flask and wash it down into the main bulk of the liquid with a fine jet of DW (from a wash-bottle),

(6) In a situation, where the colour-change at the end-point is rather gradual and not abrupt, it is always useful to have a comparison-solution readily available,

***Example* :** Methyl orange offers a gradual end-point. Hence, two flasks containing the same volume of solution having approximately the same composition as the liquid being titrated may be prepared; first, slightly acidic—Red solution, second, slightly basic—Yellow solution.

In fact, these carefully-prepared comparison solutions would ultimately help in deciding the colour change thereby confirming the actual end-point without any controversy, whatsoever,

(7) All titrations must be carried out in triplicate and the results of two concurrent readings (*i.e.*, whose difference falls within 0.05 ml-based on a 20 ml titration) may be taken into consideration,

(8) Remainder solution in the burette, after titrations have been performed must be rejected and should not be put back to the stock-bottle for obvious reasons of contamination. The burette in operation is then washed thoroughly with DW and allowed to drain by placing it up-side down on a burette stand.

2.3. GRAVIMETRIC ANALYSIS

This topic has been dealt with in sufficient details under 'Chemical Methods' (Part II) of this book.

2.4. BIOMEDICAL ANALYTICAL CHEMISTRY

This particular aspect of analytical chemistry is the outcome of the unique amalgamation of the principles and techniques of analytical chemistry and biochemistry and was initially termed as '*clinical chemistry*' but is more recently and more descriptively known as '**biomedical analytical chemistry**'.

Presently, both serum and urine assays are being used extensively in diagnostic medicine which evidently signifies that the pharmacist of today should be fully conversant with the ever-increasingly important techniques of biomedical analytical chemistry. It is, however, necessary to make a passing reference to microbiological assays and haematological assays, also being carried out in a clinical laboratory, though it should not be treated under this topic since these methods are outside the scope of biomedical analytical chemistry.

Classical example of SGOT-PAS episodes : Patients suffering from tuberculosis (TB) when diagnosed with *para*-aminosalicylic acid (PAS) invariably showed elevated serum levels of the intracellular enzyme serum-glutamic-oxaloacetic-transaminase (SGOT) which was initially considered and treated as a drug-induced hepatic toxicity. Later, an extensive and intensive studies revealed this to be an absolutely false diagnosis. In fact, the apparent enhanced SGOT levels were actually caused on account of the interference of PAS in the SGOT assay.

In the same vein, such analytical and biochemical interferences with respect to drug interference in various biomedical assays are being profusely cited in current scientific and research journals, such as the American Journal of Hospital Pharmacy and Clinical Chemistry.

It has been established beyond any doubt that analytical interferences can only take place when a drug or its resulting metabolite happens to interfere with the analytical method adopted for the assay.

In order to have a comprehensive account on the various aspects of 'Biomedical Analytical. Chemistry', we may have to study the following *four* methods of assay with specific emphasis on their principle and applications, namely :

(*a*) Colorimetric Assays,

(*b*) Enzymatic Assays,

(*c*) Radioimmunoassays, and

(*d*) Automated Methods of Clinical Analysis.

2.4.1. COLORIMETRIC ASSAYS

A. Theory : In fact, *two* fundamental laws actually govern the practice of colorimeteric assays of photometry.

First Law : *Bougner's (1729) or Lambert's (1760) Law defines that—"when a beam of monochromatic light, previously rendered plane-parallel, enters an absorbing medium at right angles to the plane-parallel surfaces of the medium, the rate of decrease in radiant power with the length of light path through the absorbing medium `b' is directly proportional to the radiant power of the beam, i.e., the light will be diminished in geometric (not arithmetic) or exponential progression".*

Alternatively, it may be explained that if a particular thickness absorbs half the light, the thickness which follows the first half and is equal to it will not absorb the entire second half, but instead only half of this half and will consequently reduce it to one-quarter. Thus, we have :

$$-\frac{\partial P}{P} = k\partial b \qquad \ldots (a)$$

Upon integration and changing to logarithms of base 10, and substituting $P = P_0$ when $b = 0$, we may get :

$$2.303 \log (P_0/P) = kb \quad \ldots (b)$$

In other words, the radiant power of the unabsorbed light decreases exponentially as the thickness of the absorbing medium increases arithmetically,

i.e., $$P = P_0 e^{-kb} = P_0 10^{-0.43\,kb} \quad \ldots(c)$$

Second Law : *Bernard's (1852) or Beer's (1852) Law defines that—'the radiant power of a beam of parallel monochromatic radiation decreases in a similar manner as the concentration of the light-absorbing constituent increases".* Thus we have :

$$2.303 \log (P_0/P) = k' C \quad \ldots (d)$$

where, C = concentration of substance, and

k' = constant of proportionality.

Therefore, from Eq. (*b*) and Eq. (*d*), the two Laws may be combined and expressed with a single constant as follows :

$$\log (P_0/P) = abc \quad \ldots(e)$$

or $$P = P_0 10^{-abc} \quad \ldots(f)$$

where, *a* = absorptivity constant*.

[* **and not to be tenned as absorbancy index, extinction coeffcient or specific extinction.**]

In fact, the absorptivity constant '*a*' is dependent upon the wavelength of the radiation as well as the nature of the absorbing material, whose concentration 'C' is usually expressed in grams per litre.

Molar Absorptivity ($\in$) : It is the product of the molecular weight of the substance and its absorptivity and is designated by the symbol $\in$.

Beer's Law (or Beer-Lambert's Law) : The combined law is invariably referred to as '**Beer's Law**', while some texts refer to this as '*Beer-Lambert's Law*'.

Eq. (*f*) is mostly expressed as shown below :

$$A = abc \quad \ldots(g)$$

where, A = absorbance,

a = absorptivity,

b = optical path length, and

c = analyte concentration.

The term $A^{1\%}_{1cm}$ designates the absorbance of a 1 cm layer of solution that essentially contains 1% by weight of absorbing solute.

It is pertinent to mention here that most of the pure pharmaceutical substances (RS) do possess a definite characteristic absorbance (*i.e.*, $A^{1\%}_{1cn}$) that forms the basis of their assay *vis-a-vis* the unknown sample.

Beer's Plot : *It is a plot of the absorbance value (along Y-axis) against a series of unknown solute concentrations in g/litre (along X-axis) thereby yielding a straight line passing through the origin.*

Therefore, the solute-concentration present in an unknown solution can be estimated conveniently from the **Beer's Plot** or sometimes referred to as the **Standard Curve**, merely by measuring the absorbance value of the solution and then finding the concentration value that corresponds to the measured absorbance value as is illustrated in the following Figure 2.2.

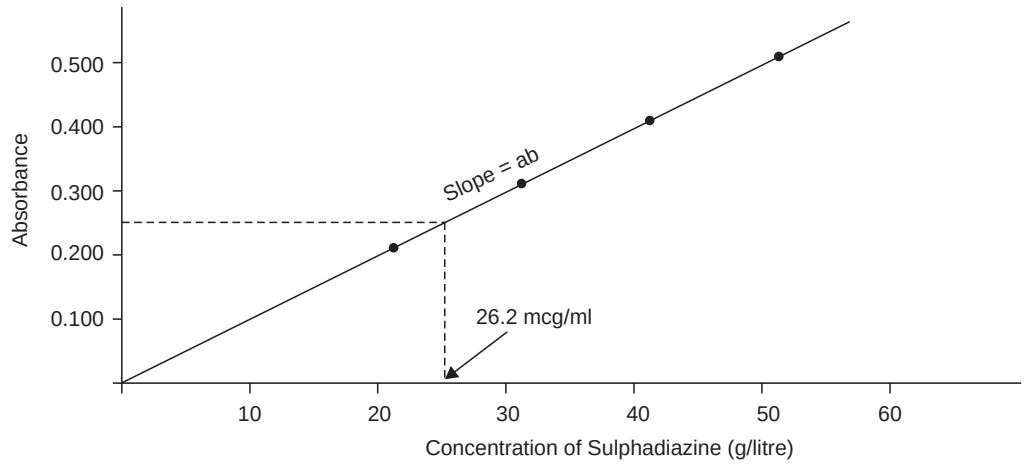
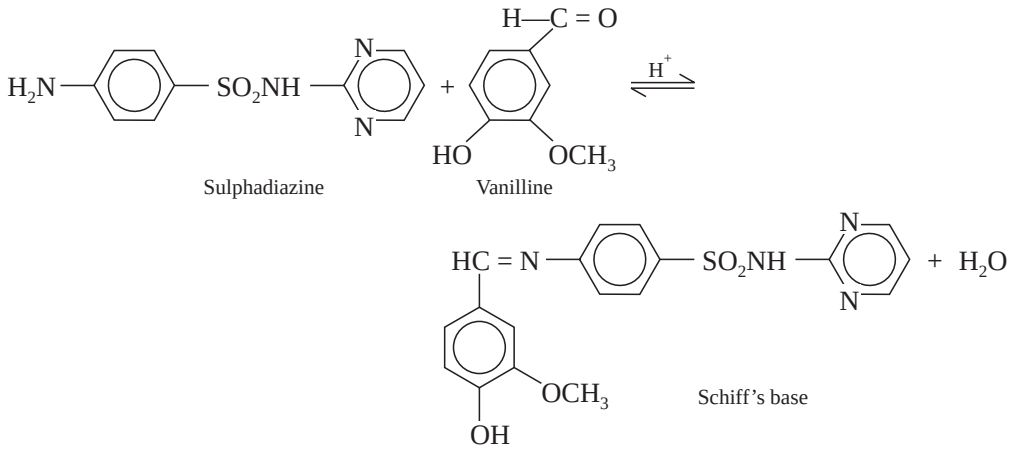


Figure 2.2 : Beer's Plot for Sulphadiazine.

The colorimetric assay of sulphadiazine is based on the **acid-catalysed equilibrium reaction** that occurs between vanillin (an aldehyde) and sulphadiazine (an arylamine). The chemical species that forms as shown below is known as the **Schiff's Base** and is yellow in colour.



Transmittance. The relationship between per cent transmittance and concentration is shown in Figure 2.3.

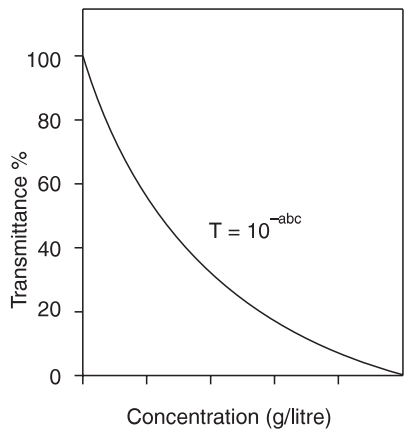


Figure 2.3 : Plot between Transmittance % and Concentration (g/litre or mcg/ml).

From Figure 2.3, it is quite evident that at lower concentrations the per cent trasmission is high and is *vice varsa* at higher concentrations.

However, a direct relationship between per cent transmittance and absorbance is illustrated in Figure 2.4.

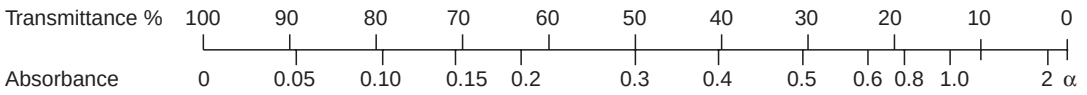


Figure 2.4 : Comparison between scales of Absorbance and Transmittance.

B. Applications in Biomedical Analytical Chemistry Colorimetric assays have a wide spectrum of applications in biomedical analytical chemistry which may be categorized under the following *four* heads, namely :

(*i*) Colorimetric Assays of Biochemicals,

(*ii*) Colorimetric Assays Involving Complexation Reactions,

(*iii*) Colorimetric Assays Involving Redox Reactions, and

(*iv*) Colorimetric Assays of Enzyme Levels.

All these four categories of colorimetric assays shall be discussed briefly with appropriate examples, wherever necessary, to have an indepth knowledge and better understanding of the practical aspects.

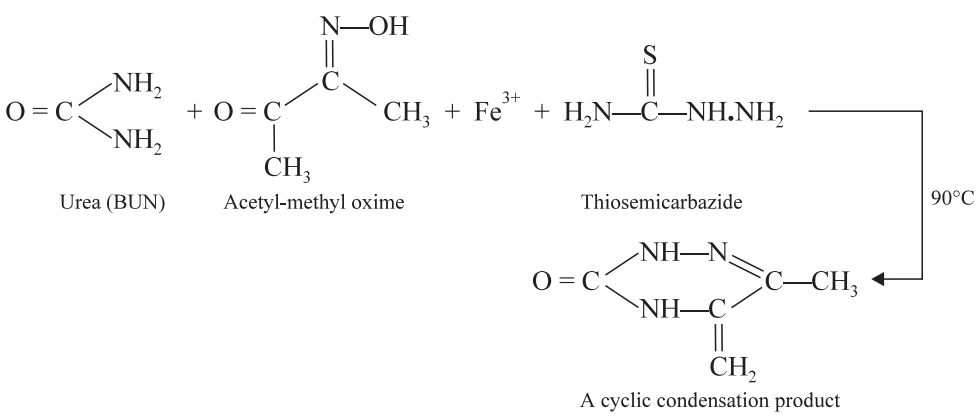
2.4.1.1. Colorimetric Assays of Biochemicals

In this context, the discussion shall be restricted to the colorimetric assays of urea (BUN), bilirubin and cholesterol. However, the clinical significance of these substances and the extent to which they are present in biological fluids; besides the various drugs that usually interfere with their assay are also described adequately in the following pages :

2.4.1.1.1. *Urea (BUN)*

The extent of urea (BUN) present in biological fluids is normally determined in many Auto Analyzers by the following method :

The quantity of substance having an unknown structure is determined at 520 nm spectrophotometrically, while the normal BUN level is determined by averaging the BUN levels of a number of normal subjects.



Profile of BUN-levels

(1) Normal BUN level is 10-15 mg per 100 ml,

(2) Enhanced BUN levels clearly signify a renal dysfunction, for instance urinary tract obstruction and nephritis *i.e.*, inflammation of the kidney.

(3) Increased incidence of BUN is also found in subjects suffering from diabetes, hepatic disorders and gastrointestinal disturbances,

(4) Decreased BUN level is usually indicative of acute hepatic dysfunction and excessive dehydration,

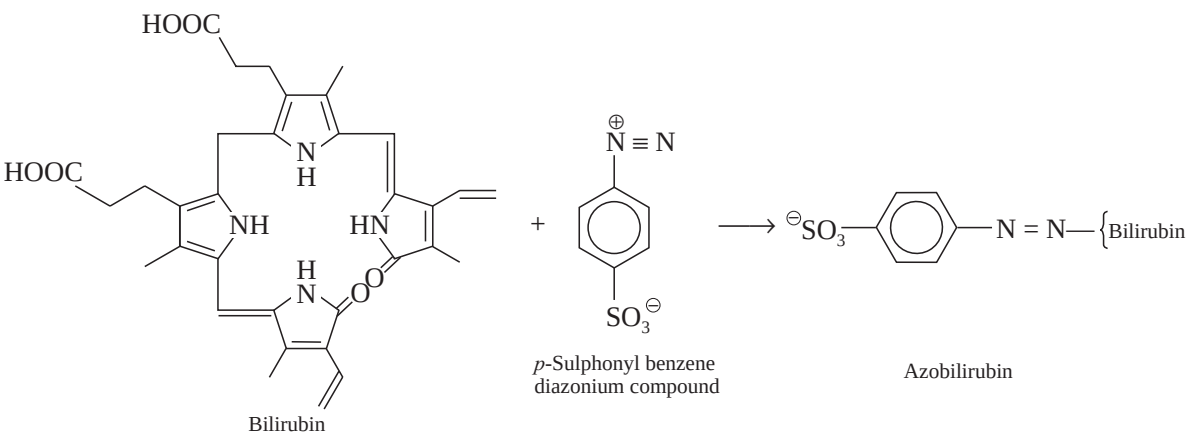
(5) A few important drugs, namely : thiazide diuretics (*e.g.*, chlorothiazide, hydroflumethiazide, bendroflumethiazide, benzthiazide, cyclothiazide etc.), neomycin, tetracyclines, methyldopa etc., help in enhancing the BUN levels perhaps due to interference with normal renal function,

(6) Phenothiazines (*e.g.*, promethazine, chlorpromazine, ethopropazine etc.) on the contrary causes a significant decrease in BUN levels due to lowering of urea production from the liver, and

(7) Substances that are inherently present in the serum and absorb at 520 nm shall interfere with these measurements, and therefore, necessary corrections for these materials have got to be made adequately.

2.4.1.1.2. *Bilirubin*

Bilirubin is diazotized with *para*-sulphonyl benzene diazonium compound and the absorbance of the resulting azobilirubin is measured at 600 nm to determine bilirubin level in the biological fluid *e.g.*, blood serum. In usual practice, a serum blank is run simultaneously by reacting the serum with caffeine, sulphanilic acid and tartaric acid, and the absorbance of the blank is measured at 600 nm which is subsequently subtracted from the azobilirubin absorbance initially obtained before the bilirubin level is finally determined.



Profile of Bilirubin Levels

(1) Normal bilirubin level ranges between 0-1.5 mg per 100 ml,

(2) Enhanced bilirubin level may suggest drug toxicity, bile-tract obstruction, hepatitis and hepatic dysfunction,

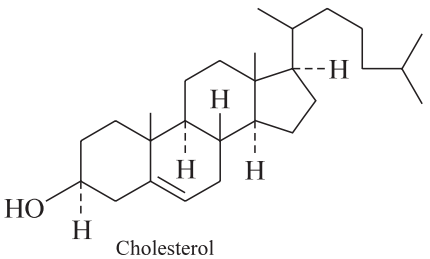
(3) As normal bilirubin level commences from zero, hence conditions responsible for its decrease are practically non-existent,

(4) Increased bilirubin levels are caused due to the intake of large doses of such drugs as : chloroquine, vitamin K, sulpha-drugs, tetracyclines, paracetamol, nicotinic acid and monoamine oxidase inhibitors (*e.g.*, iproniazid RP 1.0 ; nialamide RP 1.8 ; isocarboxazid RP 3.1 ; phenelzine RP 18 ; pheniprazine RP3l ; and tranylcypromine RP 45), where RP designates the 'Relative Potency' based on the tryptamine potentiation test. The elevated levels are due to hepatic injury, and

(5) Drugs that interfere with the assay are, namely : (*a*) phenylazopyridine hydrochloride—a coloured drug, (*b*) azo-compound forming medicinals, and (*c*) degradation product of novobiocin.

2.4.1.1.3. *Cholesterol*

Cholesterol interacts with glacial acetic acid and acetic anhydride to result into the formation of a coloured product whose absorption is measured at 630 nm and this is found to be directly proportional to the level of cholesterol present in the serum. The reaction may be expressed as follows :



Cholesterol

$+ CH_3COOH + (CH_3CO)_2O + H_2SO_4 + (2\text{-}3 \text{ drops})$

↓

A blue coloured product

The above reactions is also referred to as the Libermann's Reaction.

Profile of Cholesterol Levels

(1) Normal total cholesterol level is 200 mg per 100 ml,

(2) Increased cholesterol levels in serum are found in patients suffering from chronic hepatitis, atherosclerosis (deposit of fat in arteries of heart) and hypothyroidism,

(3) Decreased cholesterol levels in serum is indicative of liver ailment and hyperthyroidism,

(4) Corticosteroids (*i.e.*, steroidal compounds) found in urine that possess biological properties resembling those of adrenal cortical extract, either in the increase or decrease of cholesterols levels,

(5) Oestrogens, for instance : estrone, estriol, estradiol etc., are found to lower the cholesterol levels,

(6) The broad-spectrum antibiotic chlorotetracycline and the aminoglycoside antibiotic kanamycin are observed to lower the cholesterol levels by forming salts with bile acids (*e.g.*, cholic acid, deoxycholic acid and chenodeoxycholic acid) in the intestinal canal,

(7) Likewise, the antoconvulsant phenytoin sodium and neomycin—an aminoglycoside antibiotic also decrease the cholesterol levels, and

(8) Interestingly, penicillamine—a degradation product of penicillin and phenothiazines—the histamine H_1—receptor antagonists, such as : promethazine teoclate, methadilazine hydrochloride, trimeprazine tartrate are found to increase the cholesterol levels.

2.4.2. ENZYMATIC ASSAYS

A. Theory : All colorimetric enzymatic assays essentially involve the measurement of the activity of an ezyme under the following *two* circumstances, namely :

(*a*) When substrate is in large excess, and

(*b*) When enzyme concentration is in large excess.

A.1. Substrate Present in Large Excess : In reality, an enzyme reaction is nothing but a special kind of generalized reaction that may best be expressed as follows :

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} P + E \qquad ...(a)$$

where, E = Enzyme,

S = Substrate,

ES = Enzyme-substrate complex, and

P = Product.

From Eq. (*a*), we have,

$$\text{Rate of Product Formation} = V_{max}\,[S]/K_m + [S] \qquad ...(b)$$

where, $K_m = (k_2 + k_3) / k_1$,

V_{max} = Max. rate of reaction

Assuming, [S] to be in large excess [S] >> K_m,

From Eq. (*b*) we have :

$$\text{Rate of Reaction} = V_{max}\,[S]/[S]$$

or $$\text{Rate of Reaction} = V_{max} \qquad ...(c)$$

Example : In order to measure the activity of an enzyme E, such as creatine phosphokinase (CPK), the concentration of the substrate S, for instance creatine, should be in large excesses so that the products measured shall be in the linear portion of the curve (Part 'A') in Figure 2.5.

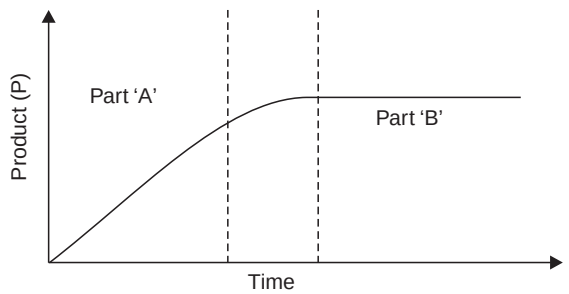


Figure 2.5 : Plot between TIME (P) *Vs* time in Enzymic Reaction.

Therefore, with a view to obtaining the best results, the two experimental parameters, namely : the temperature (constant-temperature-water-bath) and the time (phaser) should always be kept constant in order that the rate of reaction, as determined by the amount of product formed, specially designates the activity of the enzyme under assay, and devoid of the influence of any other variables on the reaction rate.

A.2. Enzyme Concentration in Large Excess

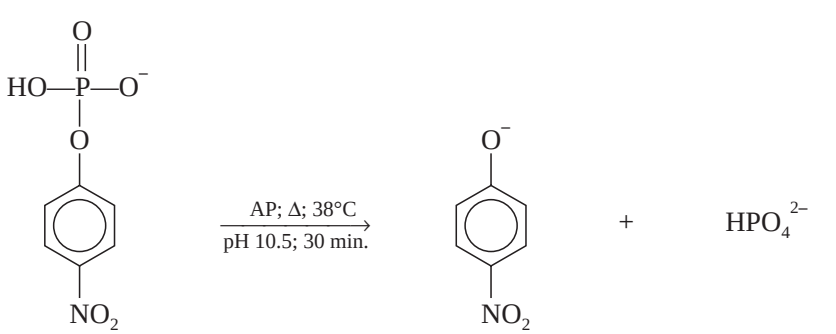
In order to analyze the quantity of substrate (S) present in a biological sample glucose oxidase is added in excess of the actual amount needed for the complete conversion of all the substrate to product ; and to achieve this object the reaction is allowed to run for a fairly long duration (*i.e*., to complete the reaction). It can be seen evidently in Part 'B' of Figure 2.5, wherein the sepecific reaction time the substrate (S) has been consumed completely and consequently, the concentration of the product achieves a maximum value.

2.4.2.1. Assay Methods

A few typical examples of colorimetric assay of enzyme levels will be discussed briefly hereunder :

2.4.2.1.1. *Alkaline Phosphatase (AP)*

Theory : Alkaline phosphatase is responsible for the cleavage of O-P bonds. It is found to be relatively non-specific and this characteristic permits the AP level to be assayed based on the fact that *p*-nitrophenylphosphate ion gets converted to *p*-nitrophenolate anion at pH 10.5; as expressed in the following reaction.



p-Nitrophenylphosphate ion *p*-Nitrophenylphosphate ion Phosphorous acid

In actual practice, *p*-nitrophenylphosphate is present in large excesses, and the reaction is carried out at 38°C for 30 minutes. The resulting amount of *p*-nitrophenolate ion is estimated by the help of an usual standard curve employing known concentrations of *p*-nitrophenolate prepared from *p*-nitrophenol.

Bessey-Lowry Activity : One unit of activity may be defined as the amount of enzyme present in 1 millilitre of serum that liberates 1μ mol of *p*-nitrophenol (0.1391 mg)* per hour at pH 10.5 after 30 minutes at 38°C.

Elimination of Interference due to Coloured Drugs

p-Nitrophenol is colourless, whereas the phenolate ion under basic conditions is yellow in appeanace. Therefore, the elimination of interference due to coloured drugs present in the serum is accomplished effectively by *first*, measuring the absorbance of the serum under basic conditions, and *secondly*, under acidic conditions. Thus we have :

$$A_{p\text{-nitrophenolate}} = A_{basic} - A_{acidic}$$

Profile of AP-levels

(1) Normal AP-levels in adults range between 0.8 to 2.3 Bessey-Lowry units and in children between 2.8 to 6.7,

(2) Increased AP-levels are observed in patients suffering from liver diseases, hyperparathyroidism and in rickets,

(3) Decreased AP-levels could be seen in patients suffering from hypoparathyroidism and pernicious anaemia (*i.e.*, an anaemia tending to be a fatal issue).

Interference due to Bilirubin

Bilirubin is eliminated by dializing the incubated *p*-nitrophenolate ion (at pH 10.5, and maintaining at 38°C for 30 minutes) into 2-amino-2-methyl-1-propanol, without carrying out the blank determination stated earlier.

There are a few medicinals that cause increased bilirubin levels which ultimately enhances AP-levels ; unless and until a corrective measure is taken in the respective procedure one may be left with false AP-level enhancement. Some typical examples are, namely : amitriptyline, chloropropamide, erythromycin, phenylbutazone, sulpha-drugs and tetracyclines.

Materials Required : 0.01 M *p*-Nitrophenol (dissolve 140 mg of *p*-nitrophenol in 100 ml of DW) : 1.0 ml ; 0.02 N NaOH (dissolve 160 mg in 200 ml DW) : 200 ml ; 5 ml of alkaline-buffered substrate (l M *p*-nitrophenylphosphate) (dissolve 7.5 g glycine, 0.095 g anhydrous $MgCl_2$ and 85 ml of 1 N NaOH to 1 litre with DW ; and mixing with an equal volume of a solution prepared by dissolving 0. l0 g of *p*-nitrophenylphosphate in 25 ml of water) ; temperature bath previously set at 38°C ; alkaline phosphatase for unknowns (commercial source) ; working standard [dilute 0.50 ml of a solution of *p*-nitrophenol (10.0 mol/litre, 0.139 g/100 ml) to 100 ml with 0.02 N NaOH].

Procedure :

(1) First of all prepare a standard calibration curve as per Table 2.9.

Table 2.9 : Readings for Standard Calibration Curve

Cuvet	Working Standard (ml)	NaOH 0.02 N (ml)	Alkaline Phosphatase Units/ml	A_{410}
1	1.0	10.1	1.0	-
2	4.0	7.1	4.0	-
3	8.0	3.1	8.0	-
4	10.0	1.1	10.0	-
5	10.5	0.6	10.5	-

*Mol. weight of *p*-Nitrophenol (*i.e.*, $C_6H_5NO_3$) is 139.1.

(2) Plot a graph of absorbance A *Vs* units of alkaline phosphatase per millilitre.

(3) Proceed for the assay of AP in the serum sample sequentially as follows :

(*i*) Pipette 1.0 ml of alkaline—buffered substrate into each of two test tubes and keep in a water-bath preset at 38°C,

(*ii*) When both the test tubes have attained the temperature equlibrium, add 0.10 ml of serum and water to these tubes separately. The one with water serves as a reagent blank and is always needed per set of unknowns. Now, put the two tubes for incubation for exactly 30 minutes period,

(*iii*) Enzyme activity is arrested by adding 10.0 ml of 0.02 N NaOH to each tube. Remove them from the water-bath and mix the contents thoroughly,

(*iv*) Read out the absorbance of the unknown tube at 410 nm against the 'reagent blank' tube,

(*v*) Transfer the contents from the cuvets to the respective test-tubes and add 0.1 ml of HCl ($\simeq$ 11.5 N) to each tube and mix the contents carefully. This operation removes the colour developed due to *p*-nitrophenol,

(*vi*) Again read out the absorbance of the serum sample against the reagent blank tube at 410 nm. This gives the colour due to the serum itself,

(*vii*) Now, the corrected reading is achieved by subtracting the reading obtained in step (*vi*) from the reading in step (*v*). The alkaline-phosphatase activity of the serum as Bessey-Lowery units is obtained from the calibration-curve step (*i*). Under these experimental parameters, we have :

1 Bessey-Lowry Unit = 5×10^{-8} mol of *p*-Nitrophenolate anion.

Thus, one unit of phosphatase activity liberated 1 μ mol of *p*-nitrophenol (l μ mol = 0.1391 mg) per hour per millilitre of serum under specified conditions.

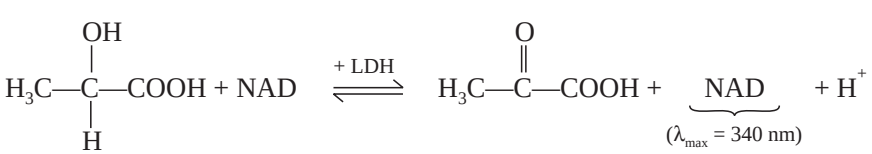
Note : In case, a value more than 10 Bessey-Lowry Units is obtained, it is always advisable to repeat the process either with a smaller volume of serum or a shorter incubation period, and then finally adjust the calculations accordingly.

(4) Report the concentration of AP in units per millilitre.

2.4.2.1.2. *Lactate Dehydrogenase (LDH)*

Theory : The method of LDH assay is based on kinetic analysis. In a kinetic enzymatic assay a unit of enzyme activity is defined as '*the quantity of enzyme that brings about a certain absorbance increase in 30 seconds or 1 minute at a fixed temperature (for instance 25 ± 0.2°C)'*.

The kinetic assay of LDH is based on the conversion of lactic acid to pyruvic acid, in the presence of nicotinamide adenine dinucleotide (NAD), and is closely monitored at intervals of 30 seconds or 1 minute by measuring the increase in absorbance at 340 nm. In this particular instance lactic acid available in an excess to ensure that the increase in pyruvic acid is linear with time, *i.e.*, directly proportional to time. The reaction involved may be expressed as follows :



The liberated nicotinamide-adenine-dinucleotide hydrogenase (NADH) has an absorption maxima at 340 nm, whereas lactic acid. NAD^+ and pyruvic acid do not absorb at all at this wavelenath.

Temperature Correction Factor : The rate of the above reaction is temperature dependent. Hence, if the temperature (experimental) is higher or lower than that used to define a unit of activity, a definite correction factor should be applied as per Table 2.10.

Table 2.10 : Temperature Correction Factor

S. No.	T (°C)	Tf (25°C)	S. No.	T (°C)	Tf (25°C)
1	20	1.45	11	30	0.69
2	21	1.35	12	31	0.64
3	22	1.24	13	32	0.59
4	23	1.15	14	33	0.55
5	24	1.07	15	34	0.51
6	25	1.00	16	35	0.47
7	26	0.92	17	36	0.44
8	27	0.85	18	37	0.41
9	28	0.80	19	38	0.38
10	29	0.74	20	39	0.35
			21	40	0.33

From Table 2.10 it may be observed that :

(*a*) At a temperature beyond 25°C (Tf = 1.0), the absorbance increases at a faster rate than at 25°C due to enhanced rate of reaction and enhanced formation of NADH, thereby lowering the correction factor from 1.0 *e.g.*, 0.80 at 28°C,

(*b*) At a temperature lower than 25°C the rate of reaction is slower than at 25°C, thereby increasing the correction factor from 1.0 *e.g.*, 1.24 at 24°C, and

(*c*) Rule of thumb suggests that for each 10°C rise in temperature the reaction rate is almost doubled and the correction factor is halved, for example : at 35°C the correction factor is 0.47 (or $1.0/2 \simeq 0.47$).

Profile of LDH-levels :

(1) Normal LDH levels are as follows :

Absorbance Units per ml : 42 to 130,

International Units per ml : 0.20 to 0.063

(2) LDH level in serum is found to be increased in 8 to 10 hours after a myocardial infarction (*i.e.*, development or presence of an infarct in the heart) ; obviously the heart muscle is destroyed and consequently the enzymes leak into the serum,

(3) Increased LDH levels are found in patients suffering from diseases related to liver and renal functions, cancer and pulmonary infarction,

(4) Drugs like codeine and morphine help in enhancing LDH levels.

Materials Required : Dermatube LDH provided by Worthington Biochemical, USA.

Procedure : The following steps need to be followed in a sequential manner :

(1) Dissolve the contents of Dermatube LDH (containing NADH and lactic acid) with 2.8 ml of DW,

(2) Put this solution in a cuvette and then insert it in a colorimeter previously warmed up to 25°C. Set the wavelength at 340 nm. Carefully adjust the absorbance of this solution to 0.1 by making use of the proper variable control as explained earlier,

(3) Remove the cuvette and add to it 0.2 ml of serum. Mix the contents of the cuvette and replace it quickly in position. Carefully record the absorbance exactly at intervals of 30 seconds for 2 to 3 minutes. In case, the absorbance happens to rise very rapidly, repeat step 3 by diluting 0.1 ml of the serum to 0.2 ml with DW,

(4) From the foregoing measurement of absorbances calculate an average ΔA/min,

(5) Note the temperature at which the reaction is carried out accurately and then find out Tf from Table 2.10.

(6) Report the LDH concentration as follows :

$$\text{Absorbancy Units of LDH per ml} = \frac{(\Delta A / \text{min}) \times (100) \times \text{Tf}}{\text{ml of serum used}}$$

$$\text{International Units* of LDH per ml} = \frac{(\Delta A / \text{min}) \times (100) \times \text{Tf}}{(6.2 \times 10^3) \times (\text{ml of serum used})}$$

2.4.3. RADIOIMMUNOASSAYS (RIAS)

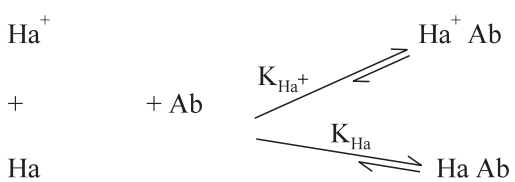
An assay method based on immunological antibody-hapten (Ab-Ha) reaction that makes use of a radioactive tracer is usually known as radioimmunoassay. A hapten (or haptene) is a small molecule that represents the portion of an antigenic molecule or complex which determines its immunologic specificity, for instance : cortisol ; whereas an antibody is a relatively large protein that is specific for certain haptens. An antibody is generated by binding the hapten to a protein, resulting into the formation of an antigen that specifically stimulates the immune system to produce antibodies specific for the hapten.

The assays that utilize protein instead of antibody are normally termed as **competitive protein binding assays**. As an antibody is also a protein, therefore, a radioimmunoassay may be looked upon as a type of competitive protein binding assay.

Theory : Generally, a radioimmunoassay makes use of a radioactive hapten and subsequently the percent of radioactivity bound to the antibody is measured. The radioactivity is determined by the help of a Geiger-Müller Counter or Geiger-Counter or G-M Tube and sometime by a Scintillation Counter.

First of all, a '*Standard Curve*' or a '*Calibration Curve*' is plotted between the reciprocal value (*i.e.*, $1 \times \%^{-1}$ radioactivity bound to the antibody) versus the amount of standard for a series of unknowns. Thus, the amount of hapten present in the unknown sample is measured from the plotted calibration curve conveniently.

The radioimmunoassay is based on the evolved competition between the combination of radioactive (Ha^+) and nonradioactive (Ha) hapten to the antibody as represented below :



Let us assume that the binding constants for Ha^+ and Ha are equal ; now, for a fixed quantity of Ha^+ but an increased concentration of Ha. The ultimate impact would be that lesser Ha^+ shall be bound. In actual practice, however, the use of Tritium (H^3) or Carbon-14 (C^{14}), which helps to render the Ha radioactive, ultimately maintains the equality of these binding constants, namely : $K_{Ha}{}^+$ and K_{Ha} . It also confirms that the

* The amount of enzyme that catalyzes the conversion of l μ mol of lactate per minute.

chemical properties of both radioactive (Ha^+) and nonradioactive (Ha) entities are more or less the same as far as the antibody is concerned.

Salient Features of Radioimmunoassays

(1) They belong to a class of extremely sensitive methods of analysis,

(2) Sample required for assay is usually very small *e.g.*, 1 ml of serum,

(3) Concentrations upto the nanogram range *i.e.*, 10^{-9} g can be measured accurately,

(4) A large number of hormones and drugs which find their abundant usage in a bad way, namely : cortisol (17-hydroxycorticosterone or hydrocortisone), insulin, morphine, barbiturates (sedatives), vitamin B_{12}, digoxin and human growth hormones, such as : somatotropin (elaborated in the placenta),

(5) Incidence of interferences observed in the radioimmunoassays are fairly insignificant by virtue of the highly specific hapten-antibody complexation reaction, and

(6) Exceptions do occur when two 5-substituted barbiturates present together cannot be assayed by this method, obviously due to interference.

2.4.3.1. Cortisol (In Plasma)

Theory : Cortisol (or hydrocortisone) was introduced in the year 1951, for the treatment of rheumatoid arthritis. It has a significant effect on protein metabolism. It also exerts widespread effects on carbohydrates, lipid and protein synthesis (or anabolism). The cardinal side effects such as excessive potassium excretion and sodium retention, enhanced gastric acidity, oedema, psychosis and negative nitogen balance are some of the exaggerated manifestations of the normal metabolite functions of cortisol.

Most importantly, the determination of cortisol levels is considered useful in the diagnosis and treatment of various ailments, namely : Addison's Disease *i.e.*, pernicious anaemia—a condition whereby the maturation of the red cells may not proceed beyond the stage of megaloblasts; Cushing's Syndrome.

Adrenal Tumours : The assay-method is entirely based on the Schwartz-Mann Kit. According to this method, cortisol is first extracted from the plasma using CH_2Cl_2 (methylene chloride). In the actual radioimmunoassay the cortisol present in the extract competes with Cortisol-H^3 (*i.e.*, the radioactive tracer) for the common binding sites on transcortin, which is incidently not an antibody but a cortisol-binding protein. Now, the free cortisol is quantitatively removed by adsorption on dextran-coated charcoal from the one bound to the transcortin. Finally, the bound radioactivity (due to Cortisol-H^3) is measured which is then employed to calculate exactly the amount of cortisol present in the sample by the help of a Standard Curve (or Calibration Curve).

Materials Required : Schwartz-Mann-H^3 Cortisol RIA-Kit ; liquid scintillation counter, centrifuge.

Procedure : The various steps to be followed sequentially for the assay of cortisol in plasma are as follows :

(1) The cortisol is usually extracted from the samples drawn from the patients, as follows :

Place 100 µl of plasma in each of two tubes and add 2.5 ml of methylene chloride. Shake the contents of the tube vigorously for 10 minutes and transfer 0.5 ml of clear extract (*i.e.*, the lower layer) to another tube. Evaporate the methylene chloride either at 35°C in an oven or in a stream of N_2. The extract thus obtained is employed in the following step.

(2) The following steps *viz*., Step 1 to Step 15, related to the procedure for the assay and the calibration curves must be performed simultaneously :

Sequence	Preparation of Calibration Curve	Clinical Determination
Step-1	Consecutively number 18 glass tubes,	Use tubes containing the dried extract,
Step-2	Pipette phosphate buffer (0.04 M having buffer pH 7.4) into tubes as follows :	Pipette 800 µl phosphate buffer into each tube,

Tube	Phosphate Buffer (µl)
l, 2	1300
3, 4	900
5, 6	800
7, g	800
9, 10	700
11, 12	700
13, 14	800
15, 16	700
17, 18	700

Step-3	Add cortisol standard solution (A or B from Schwartz-Mann Kit*) as follows :	Add no standard to patient sample tubes,

Tube	Cortisol Standard	Cortisol ng/Tube
5, 6	NONE	0
7,8	25 µl A	0.5
9, 10	50 µl A	1.0
11,12	100 µl A	2.0
13, 14	25 µl B	4.0
15, 16	50 µl B	8.0
17, 18	100 µl B	16.0

*Manufactured By : Mountain Ave., Orangeburg N.Y.

Step-4	Add 100 µl transcortin solution from SM-Kit to tubes 1, 2 and 5 through 18 and mix gently,	Add 100 µl transcortin solution to each tube,
Step-5	Add 200 µl cortisol (– H^3) to tubes 1 through 18 mix for 3 seconds on a vortex mixer. Set tubes 1 and 2 aside until Step-13,	Add 100 µl cortisol (– H^3) to each tube and mix,
Step-6	From this point onwards the various tubes are treated as follows :	
Step-7	Incubate tubes 3 through 18 and all patient sample tubes in a pre-set constant temperature water-bath at 45°C for exactly 5 minutes,	
Step-8	Immediately after Step-7 incubate tubes 3 through 18 and all patient tubes in an ice-water bath (0 to 4°C) for 30 minutes. Shake the rack several times at short-intervals to ensure that the tubes attain 0-4°C rapidly,	
Step-9	Quickly add 0.5 ml of cold dextran-coated charcoal suspension to tubes 3 through 18 and to all patient sample tubes so as to get rid of free cortisol. Do not add to tubes 1 and 2,	
Step-10	Keep tubes 3 to 18 and all patient sample tubes in an ice-water bath for 10 minutes,	

Step-11	Centrifuge all tubes either at 1240 × g for 10 minutes at 4°C or centrifuge for less time at higher speeds,
Setp-12	Consecutively number a set of scintillation vials,
Step-13	Pipette out 1.0 ml of solution from tubes 1 and 2 into correspondingly numbered scintillation vials. These vials will give the total count per assay. Also pipette 1.0 ml of each clear supernatant into a correspondingly numbered scintillation vial,
Step-14	Add 10.0 ml of UNOGEL to each vial. Shake each vial to solubilize the contents: An emulsion should form, and
Step-15	Count the radioactivity in the vials in sequence for 1 to 10 minutes. The count time should be long enough to accumulate 10,000 to 15,000 counts.

(3) **Results :** Average the counts per minute in vials 3 and 4. This is the blank value. Now, subtract the blank from all other counts per minute to obtain the actual counts per minute and average the counts per minute for vials 1 to 2 to find the total count per minute. The percent bound may be calculated using the following expression :

$$\% \text{ Bound} = \frac{\text{Counts per minute}}{\text{Total counts per minute}} \times 100$$

Finally, plot the percent bound *Vs* nanograms (ng) per tube of cortisol standard either on linear or on semilog paper and make use of this Standard Curve to calculate the amount of cortisol present in the unkown samples.

2.4.4. AUTOMATED METHODS OF CLINICAL ANALYSIS

Theory : An '*Autoanalyzer*' serves as the most versatile and important instrument in a well-equipped '*clinical laboratory*' that caters for the rapid screening of serum levels for upto forty (40) important chemical substances in the field of diagnostic medicine. These autoanalyzers may be either 'Single Channel' *i.e.*, performing only one determination on each sample or Multichannel' *i.e.*, carrying out several different determinations on each sample.

A few important substances that are routinely analyzed in a clinical laboratory with the aid of an 'Autoanalyzer' are, namely : serum-glutamic-oxaloacetic transaminase (SGOT) ; creatine-phophokinase (CPK); alkaline-phosphatase (AP) belonging to the class of enzymes ; and a host of biochemical substances, for instance : bilirubin, serum albumin, blood urea nitrogen (BUN), uric acid, creatinine, total protein, glucose, cholesterol, besides a few common inorganic ions, such as : Cl^-, Ca^{2+}, K^+, Na^+.

The basic principles underlying both automated and unautomated methods of analysis are more or less the same. Out of the broad-spectrum of biological samples blood analysis is the most common one. There exists a number of parameters which may be assayed, and spectrophotometry is ideally suited for nearly all of them, a few typical examples are cited in Table 2.11.

Table 2.11 : Typical Examples of Clinical Analysis Employing Spectrophotometry

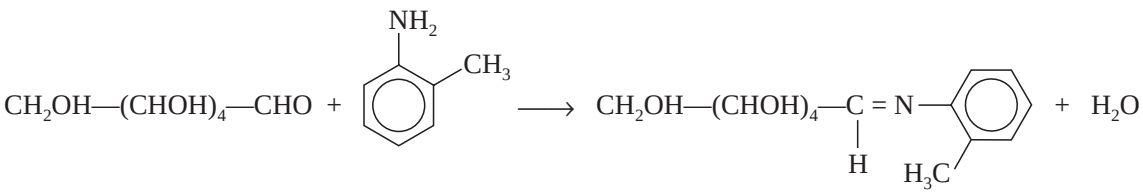
S. No.	Analyte	Reagents(s) and/or Procedure(s)	Wavelength λ (nm)
1.	Bilirubin	Diazotized sulphanilic acid (Ehrlich's Reagent)	540
2.	Cholesterol	Acetic-anhydride (Liebermann-Burchard Reagent)	625
3.	Glucose	Glucose reduces Cu^{2+} to Cu^+ ; & Cu^+ reduces phosphomolybdic acid (Folin-Wu)	420
4.	Glucose	*o*-Toluidine	635
5.	Phosphate (Inorganic)	Na_2MoO_4, *p*-Methylaminophenol sulphate	700
6.	Urea (Nitrogen)	Urease, Na_2WO_4, Nessler's Reagent	490

Explanation : Glucose (having an aldehyde functional moiety) reduces Cu^{2+} to Cu_2O (*i.e.*, Cu^+) as per the following reaction :

$$C_6H_{12}O_6 + 2Cu^{2+} + 6H_2O \rightleftharpoons Cu_2O + C_6H_{12}O_7 + 4H_3O^+$$

As some other sugars are also present in blood sample, and besides the above reaction not being absolutely stoichiometric, it has become necessary in actual practice to establish an emperical calibration curve using known concentrations of glucose. The above reaction is allowed to proceed for exactly 8 minutes at 100°C. To the resulting solution phosphomolybdic acid is added, which is subsequently reduced by Cu_2O to give rise to an intensely coloured 'molybdenum blue' that is measured at 420 nm accurately.

Alternatively, glucose forms a specific complex with *o*-toluidine according to the following reaction that forms the basis of the colorimetric assay :



Glucose *o*-Toluidine Glucose-toluidine complex

The diagnostic green colour is usually developed for exactly 10 minutes at 100°C and measured subsequently at 635 nm.

2.4.4.1. Instrumentation

The schematic diagram of an Auto Analyser is shown in Figure 2.6. The major component parts comprise of the various important sections namely : the preparation section, the reaction section and the analysis section which will be discussed briefly here.

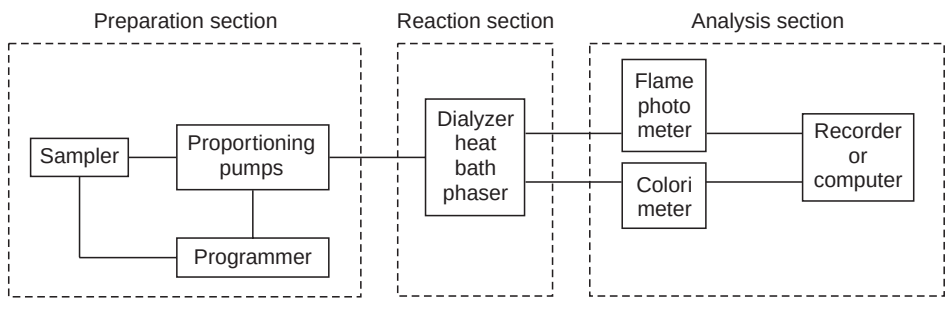


Figure 2.6 : Schematic Diagram of an Auto Analyzer.

2.4.4.1.1. *Preparation Section*

This particular section of the Auto Analyzer consists mainly of the sampler, proportioning pumps, and programmer. First, the sampler introduces a fixed quantity of serum sample into the 'analysis train', which varies from one instrument to another instrument supplied by different manufacturers. For instance, the SMA-12 Survey Auto Analyzer possesses 12 analysis trains or streams as illustrated in Figure 2.7.

The proportioning pump controls the rate of advancement, *viz* 10 inch/minute, of each sample through the analysis stream. Hence, a fixed length of tubing is equivalent to a fixed amount of time. Each analysis stream is made of transparent plastic flexible tubing, and each patient-sample is separated from one another by an air-bubble.

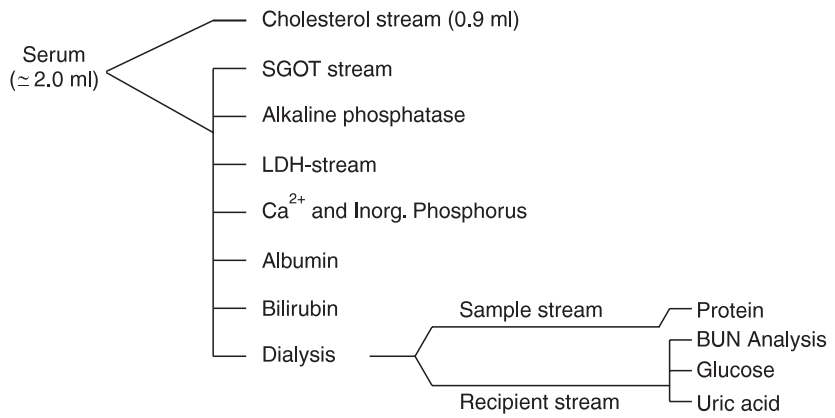


Figure 2.7 : Sample Streams of SMA-12 Survey Auto Analyzer.

2.4.4.1.2. *Reaction Section*

The reaction section essentially comprises of the dialyzer, heat bath and phaser, and obviously the reaction takes place in this zone. Let us consider the following generalized reaction :

$$aA + bB \underset{k_2}{\overset{k_1}{\rightleftharpoons}} cC + dD \qquad ...(a)$$

or

$$K = \frac{[C]^c[D]^d}{[A]^a[B]^b} \qquad ...(b)$$

where, $[C]^c$ = Molar concentration of substance C raised to the *c*th power,

A = Component in serum (*e.g.*, cholesterol), and

B = Reactant that reacts with A to give a coloured product.

Evidently, B is added always in excess to ensure :

(*a*) rapid reaction, and

(*b*) complete reaction by forcing the reaction to the right in accordance to the *Le Chatelier's principle*.

Now, the rate of forward reaction = $k_1 [A]^a [B]^b$

Hence, the rate constant may be expressed as follows :

$$k_1 = Ae^{-Ea/RT} \qquad ...(c)$$

where, R = Gas constant (1.99 cal/K-mol),

T = Temperature, and

E_a = Activation energy of the reaction as depicted in Figure 2.8.

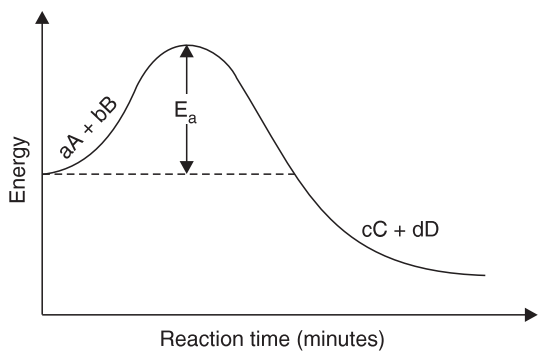


Figure 2.8 : Progress of a Reaction and the Activation Energy Ea.

From Eq. (*c*) it may observed that as the temperature T is enhanced then the rate of reaction also enhances simultaneously because a higher value of T offers a smaller negative exponent of *e* or a larger number. Therefore, in actual experimental operations temperature is increased by the aid of a heat-bath so as to accelerate the reaction which in turn allows the reaction to attain equilibrium state as rapidly as possible.

Naturally at a very high temperature there is every possibility for decomposition of either the products or the reactants.

2.4.4.1.3. *Analysis Section*

The recent advancement in the field of *computer technology* and *anlytical instrumentation* it has become very easy and convenient to have the analyical data from a series of biological samples processed at high speed as digital readouts or on computerized recorders. Many hospitals round the globe make extensive use of advanced computer softwares for data processing as stated beiow :

- Uptodate listing of various laboratory tests,
- Listing of drugs and metabolites that cause interference both biochemically and analytically,
- Storing of levels of biologically important compounds for various disease states, and
- A tentative diagnosis for a patient based on his serum sample under investiation together with the drugs and dosages being administered and the levels of biologically important compounds.

Caution : Nevertheless, the concerned physician or pharmacist must exercise his or her own expertise and knowledge while prescribing drug(s) to a patient along with these computerized data informations.

THEORETICAL AND PRACTICAL EXERCISES

1. Various **'Official Compendia'** *viz.*, IP, BP, USP, NF and NBS (National Bureau of Standards) have laid down **'tolerance on capacity'** for Burettes, Pipettes, Volumetric Flasks. Discuss its importance in volumetric quantitative analysis.
2. (*i*) Discuss briefly the underlying principles of :
   (*a*) Colorimetric assays
   (*b*) Enzymatic assays.
   (*ii*) How would you determine the percentage purity of **'sulphadiazine'** ? Give the theory of the exercise.
3. How would you carry out the assay of **'bilirubin'** or **'cholesterol'** by colorimetric method ? Explain.
4. Describe '*enzymatic assays*' based on colorimetric method of analysis under the following two situations :
   (*i*) When '*substrate*' is in large excess,
   (*ii*) When '*enzyme concentration*' is in large excess.
   Give suitable examples in support of your answer.
5. Give a comprehensive account on the various **'automated methods of clinical analysis'** with an appropriate example.

RECOMMENDED READINGS

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3 ERRORS IN PHARMACEUTICAL ANALYSIS AND STATISTICAL VALIDATION

CONTAINS :

This chapter has been divided into **two** different portions, namely : (*a*) Errors in Pharmaceutical Analysis, and (*b*) Statistical Validation, which will be discussed individually in the following sections :

3A. ERRORS IN PHARMACEUTICAL ANALYSIS

3A.1. INTRODUCTION

The skill, knowledge, expertise and above all the degree of confidence involved in the ultimate result of an analyst is solely governed by the extent of accuracy and precision achieved by the analytical procedure *vis-a-vis* the possible sources of error that may be incorporated inadvertently. In fact, the quantitative pharmaceutical analysis is not merely confined to just taking a random sample, performing a single assay quickly, and finally making a loud claim that the result so obtained cannot be challenged. Truly speaking an ideal analyst must have a total in-depth knowledge of the chemistry involved along with the *pros and cons* of interferences that may be caused due to the host of compounds, elements and ions besides adequate exposure and hands-on experience of the statistical distribution of values.

The terminology 'error' invariably refers to the difference in the numerical values between a measured value and the true value. It has become universally accepted in methods of comparison that the percentage composition of a 'standard sample' provided and certified by the National Institute of Standards and Technology (NIST) or the British Pharmacopoea Chemical Reference Substance (BPCRS) or the European Pharmacopoea

Chemical Reference Substance (EPCRS) must be regarded and treated as absolutely correct, pure and authentic while evaluating a new analytical method. Consequently, the differences thus obtained between the standard values and those by the new analytical methods are then treated as 'errors' in the latest procedure.

3A.2. CLASSIFICATION OF ERRORS

The numerous uncertainties usually encountered in a chemical analysis give rise to a host of 'errors' that may be broadly categorised into *two* heads, namely :

(*i*) Determinate (systematic) Errors, and

(*ii*) Indeterminate (random) Errors.

It is pertinent to mention here that it becomes rather difficult at times to place a particular 'error' into one of the above mentioned categories ; however, the classification may prove to be beneficial with regard to study of the various analytical errors that crop up in the course of routine analysis.

3A.2.1. DETERMINATE (SYSTEMATIC) ERRORS

These are errors that possess a definite value together with a reasonable assignable cause; however, in principle these avoidable errors may be measured and accounted for coveniently. The most important errors belonging to this particular class are :

(*a*) **Personal Errors :** They are exclusively caused due to 'personal equation' of an analyst and have no bearing whatsoever either on the prescribed procedure or methodology involved.

(*b*) **Instrumental Errors :** These are invariably caused due to faulty and uncalibrated instruments, such as : pH meters, single pan electric balances, uv-spectrophotometers, potentiometers etc.

These two errors have been duly discussed under the chapter on 'Pharmaceutical Chemicals : Purity and Management' (Section 1.3.2.2).

(*c*) **Reagent Errors :** The errors that are solely introduced by virtue of the individual reagents, for instance : impurities inherently present in reagents ; high temperature volatalization of platinum (Pt) ; unwanted introduction of 'foreign substances' caused by the action of reagents on either porcelain or glass apparatus.

(*d*) **Constant Errors :** They are observed to be rather independent of the magnitude of the measured amount ; and turn out to be relatively less significant as the magnitude enhances.

Example : Assuming a constant equivalence—point error of 0.10 ml is introduced in a series of titrations, hence for a specific titration needing only 10.0 ml of titrant shall represent a relative error of 1% and only 0.2% for a corresponding 50 ml of titrant consumed.

(*e*) **Proportional Errors :** The absolute value of this kind of error changes with the size of the sample in such a fashion that the relative error remains constant. It is usually incorporated by a material that directly interferes in an analytical procedure.

Example : Estimation of 'chlorate'—an oxidant by iodometric determination. In this particular instance *two* things may happen, namely :

(*i*) Presence of 'Bromate'—another oxidizing agent would give rise to positively higher results, and hence, it must be duly corrected for, and

(*ii*) Absolute error might increase while dealing with large samples, whereas the relative error would remain more or less constant if the sample is perfectly homogenous,

(*f*) **Errors due to Methodology :** Both improper (incorrect) sampling and incompleteness of a reaction often lead to serious errors. A few typical examples invariably encountered in titrimetric and gravimetric analysis are cited below :

S.No.	Titrimetric Analysis	S.No.	Gravimetric Analysis
1.	Failure of reactions to proceed to completion,	1.	Significant solubility of precipitates,
2.	Involvement of either induced or side-reactions,	2.	Co-precipitation and post-precipitation,
3.	Reactions due to subtances other than the one being assayed, and	3.	Decomposition,
4.	A noticeable difference occurring between the stoichiometric equivalence point of a reaction and the observed end-point.	4.	Volatalization of weighing forms on ignition, and
		5.	Precipitation of constituents other than the desired ones.

(*g*) **Additive Errors :** It has been observed that the additive errors are independent of the quantum of the substances actually present in the assay.

Examples : (*i*) Errors caused due to weights, and

(*ii*) Loss in weight of a crucible in which a precipitate is incenerated.

Detection of this error is ascertained by taking samples of different weights.

3A.2.2. INDETERMINATE (RANDOM) ERRORS

As the name suggests, indeterminate errors cannot be pin-pointed to any specific well-defined reasons. They are usually manifested due to the minute variations which take place inadvertently in several successive measurements performed by the same analyst, using utmost care, under almost identical experimental parameters. These errors are mostly random in nature and ultimately give rise to high as well as low results with equal probability. They can neither be corrected nor eliminated, and therefore, form the **'ultimate limitation'** on the specific measurements. It has been observed that by performing repeated measurement of the same variable, the subsequent statistical treatment of the results would have a positive impact of **'reducing their importance'** to a considerable extent.

Example : Figure 3.1, represents the absolute errors in nitrogen analysis by means of micro **Kjeldahl's Method***. Here, each vertical line labelled ($\bar{x}_1 - x_t$) designates the absolute deviation of the mean of the set from the true value. In Figure 3.1, A represents ($\bar{x}_1 - x_t$) the absolute error obtained by **'analyst-1'** for the assay of benzyl-iso-thioureahydrochloride, whereas B represents ($\bar{x}_2 - x_t$) the absolute error obtained by **'analyst-2'** for the assay of the same compound.

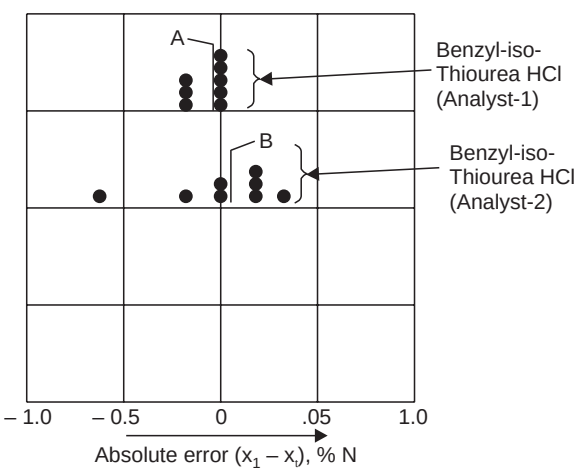


Figure 3.1 : Absolute Errors in N Analysis by a Micro Kjeldahl's Method.

* Willits, C.O. and C.L. Ogg, **J. Assoc. Off. Anal. Chem., 32,** 561, 1949.

Thus, it is evident from Figure 3.1, that the broad spread of individual errors centres around the mean values $(x_n - \bar{x})$ thereby affording a direct indication of indeterminate type uncertainties. Hence, larger indeterminate errors seem to be linked with the performance of **'analyst-2'** than with that of **'analyst-1'**.

Salient Features of Indeterminate Errors

The various salient features of indeterminate errors are enumerated below :

(1) Repeated mesurement of the same variable several times and subsequent refinement to the extent where it is simply a coincidence if the corresponding replicates eventually agree to the last digit,

(2) Both unpredictable and imperceptible factors are unavoidably incorporated in the results what generally appear to be '*random fluctuations*' in the measured quantity,

(3) Recognition of specific definite variables which are beyond anyone's control lying very close to the performance limit of an instrument, such as : temperature variations, noise as well as drift from an electronic circuit, and vibrations caused to a building by heavy vehicular-traffic,

(4) A variation that may be regarded as random by a slipshod analyst may at the same time prove to be quite evident and manageable by a careful observer, and

(5) The average of a number of fine observations having random scatter is definitely more accurate, precise and, hence, more cogent than coarse data that appear to agree perfectly.

3A.2.3. ACCURACY

In connexion with the scientific data the two terminologies **'accuracy'** and **'precision'** are invariably practised synonymously, but there exists a clear distinction between them as discussed below :

In usual practice an accurate result is the one which matches very nearly with true value of a measured amount. The comparison is normally done with regard to the '*error*'; and the accuracy is inversely proportional to it *i.e.*, the greater the accuracy, the smaller is the error. '*Absolute error*' is the difference between the *experimental value* and the *true value*.

Example : An analyst determines a value of 70.55% cineole in a fresh sample of Eucalyptus Oil that actually contains 70.25%, the absolute error is given by :

$$70.55 - 70.25 = 0.30\%$$

The error thus obtained is invariably stated with regard to the actual size of the measured quantity *i.e.*, either in percent (%) or in parts per thousand (ppt). Therefore, the relative error is given by :

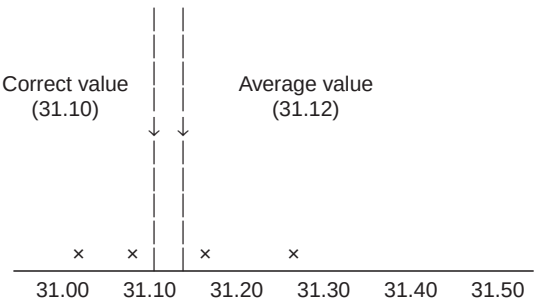
$$\frac{0.30}{70.25} \times 100 = 0.42\% \quad \text{or} \quad \frac{0.30}{70.25} \times 1000 = 4.2 \text{ ppt}$$

3A.2.4. PRECISION

It may be defined as—'*the agreement amongst a cluster of experimental results ; however, it does not imply anything with respect to their relation to the 'true value' '*. Precision designates 'reproducibility' of a measurement, whereas accuracy the correctness of a measurement. Precision invariably forms an integral part of accuracy, but ironically a high degree of precision may not necessarily suggest accuracy.

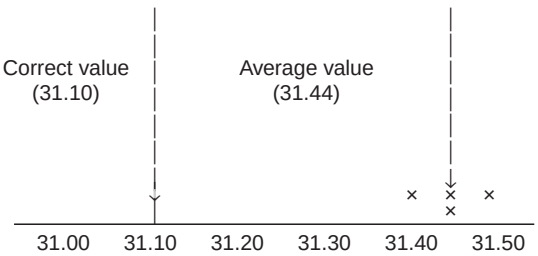
Example : A sample of pure Peppermint Oil is known to contain 30.10 ± 0.03 per cent of Menthone. The results obtained by two Analysts-1 and 2 employing the same sample of peppermint oil and making use of the same analytical reagents and procedure are as stated below :

Analyst 1 : % Menthone : 31.01 ; 31.25 ; 31.08 ; 31.14



The arithmetic mean stands at 31.12% and the results range between 31.01% to 31.25%

Analyst 2 : % Menthone : 31.40 ; 31.46 ; 31.44 ; 31.44



The arithmetic mean is 31.44% and the results vary between 31.40% to 31.46%

The ultimate results of the analysis put forward by the Analysts-1 and 2 may be summarized as under :

(*i*) The results achieved by Analyst-1 are fairly accurate *i.e.*, in close proximity to the correct result ; however, the precision stands at an inferior level to the results obtained by Analyst-2. The results accomplished by Analyst-2 are indeed extremely precise but fail in accuracy,

(*ii*) The results of Analyst-1 lie on either sides of the average value as shown by two 'cross-signs' on each side which might have been caused due to 'random errors' discussed earlier. It is quite evident that there exists a constant (determinate) error in the results obtained by the Analyst-2, and

(*iii*) In case, Analyst-3 had performed the estimations on the very same day in quick succession *i.e.*, one after the other, this type of analysis could be termed as 'repeatable analysis'. If the estimations had been carried out on two separate days altogether, thereby facing different laboratory conditions then the results so obtained would be known as 'reproducible analysis'.

In short, there exists a marked and pronounced distinction between a within-run precision (*i.e.*, **repeatability**) and an in-between-run precision (*i.e.*, **reproducibility**).

3A.2.5. MINIMISING SYSTEMATIC ERRORS

Systematic errors may be reduced substantially and significantly by adopting one of the following procedures rigidly, such as :

(*i*) **Calibration of Instruments, Apparatus and Applying Necessary Corections**

Most of the instruments, commonly used in an analytical laboratory, such as : UV-Spectrophotometer, IR-Spectrophotometer, single—pan electric balance, pH-meter, turbidimeter and nephelometer, polarimeter, refractometer and the like must be calibrated duly, before use so as to eliminate any possible errors. In the same manner all apparatus, namely : pipettes, burettes, volumetric flasks, thermometers, weights etc., must be calibrated duly, and the necessary corrections incorporated to the original measurements.

In some specific instances where an error just cannot be avoided it may be convenient to enforce an appropriate correction for the effect that it ultimately causes ; for instance : the inherent impurity present in a weighed precipitate can be estimated first and then deducted duly from its weight.

(*ii*) **Performing a Parallel Control Determination**

It essentially comprises of performing an altogether separate estimation under almost identical experimental parameters with a quantity of a standard substance that consists of exactly the same weight of the component as is present in the unknown sample. Thus, the weight of the component present in the unknown sample may be calculated with the help of the following expression :

$$\frac{\text{Wt. of component in Standard Substance}}{\text{X}} = \frac{\text{Result obtained for Standard Substance}}{\text{Result obtained for Unknown Sample}}$$

where, X = Weight of the component present in the Unknown Sample.

Note : A good number of Standard Samples, including primary standards, such as : arsenic trioxide, benzoic acid, potassium hydrogen phthalate, sodium oxalate, are available as :

BPCRS = British Pharmacopoeia Chemical Reference Substance,

EPCRS = European Pharmacopoeia Chemical Reference Substance,

CRM = BCS—Certified Reference Materials,

ECRM = EURONORM—Certified Reference Materials.

(*iii*) **Blank Determination :**

In order to ascertain the effect of the impurities present in the reagents employed and reaction vessels used ; besides establishing exactly the extent to which an excess of standard solution required to locate the exact end-point under the prevailing experimental parameters of the unknown sample—a blank determination is an absolute necessity. It may be accomplished by performing a separate parallel estimation, without using the sample at all, and under identical experimental parmeters as employed in the actual analysis of the given sample.

Note : Always avoid using an appreciably large blank correction which gives rise to a vague and uncertain 'exact value' thereby minimising the precision of the analysis.

(*iv*) **Cross-checking Results by Different Methods of Analysis**

In certain specific cases tha accuracy of a result may be cross-checked by performing another analysis of the same substance by an altogether radically different method.

Examples :

(*a*) **HCl-Solution :** It may be assayed either by titration with a standard solution of a strong alkali (NaOH), or by precipitation and weighing as AgCl ; and

(*b*) **Fe^{3+}** : It may be assayed either by gravimetric method as Fe(III) hydroxide after getting rid of the interfering elements and igniting the precipitate to Fe(III) oxide, or by titrimetric method *i.e.*, first reducing to the Fe(II) state and then titrating with a suitable oxidizing agent, for instance Ce(IV) sulphate, $K_2Cr_2O_7$. In short, the results thus obtained by the two fundamentally different techniques must be concordant thereby justifying and ascertaining the fact that the values obtained are fairly small limits of error.

(*v*) **Method of Standard Addition**

Here, a small known quantity of the component under estimation is added to the sample, which is subsequently subjected to analysis for the total amount of component present. The actual difference in the quantity of components present in samples with or without the added component ultimately gives the recovery of the quantum added component. A good satisfactory recovery builds up the confidence in the accuracy of the method of analysis.

Note : The method of 'standard addition' is particularly useful to physicochemical techniques of analysis, for instance : spectrophotometry, turbidimetry.

(*vi*) **Method of Internal Standards**

The specific method is of immense value both in chromatographic as well as spectroscopic determinations. Here, a fixed quantity of a reference substance (*i.e.,* the 'internal standard') is added to a series of known concentrations of the material to be assayed.

A graph is plotted between the concentration values and the ratios obtained from the physical value (*i.e.,* peak area of absorption) of the *'internal standard'* and the series of known concentrations, thereby producing a straight line. Any unknown concentration may be determined effectively by adding the same amount of '*internal standard*' and locating exactly where the ratio obtained falls on the concentration scale.

3B. STATISTICAL VALIDATION

3B.1. INTRODUCTION

After accomplishing the thorough investigation of various aspects of possible 'determinate errors' (Section 3A.2.1) and having applied the relevant corrections, it has been observed that the data thus generated not only show fluctuations but also are found to be random in nature. The powerful and effective technique of statistics may render such results, which scatter in a random manner, into a better form that may be employed intelligently. Besides, the specific statistical treatment of the calibration data, aided by pre-programmable calculators and micro-computers, very often yields a fairly accurate and more presentable determination of the graphs between absorbance and concentration than those produced manually.

3B.2. STATISTICAL VALIDATION

The statistical validation of analytical results will be discussed with regard to the following *six* aspects individually, along with appropriate examples wherever possible, in the sections that follow :

(*i*) Statistical treatment of finite samples,

(*ii*) Distribution of random errors,

(*iii*) Significant errors,

(*iv*) Comparison of results,

(*v*) Method of least squares, and

(*vi*) Criteria for rejection of an observation.

3B.2.1. STATISTICAL TREATMENT OF FINITE SAMPLES

The various techniques by which one may effectively treat the scientific data normally obtained in actual analytical procedures are enumerated below :

3B.2.1.1. Mean

It is the average of a series of results. The mean of a finite number of measurements, x_1, x_2, x_3, x_4, ..., x_n, is commonly represented as $\bar{x}$. It may be calculated by taking the average of individual results as shown below :

$$\bar{x} = \frac{x_1 + x_2 + x_3 + x_4 \ldots\ldots + x_n}{n} = \frac{\sum_{i=1}^{i=n} x_1}{n}$$

It is evident that the mean of *n* results is $\sqrt{n}$ times more reliable than any one of the individual results. Therefore, there exists a diminishing return from accumulating more and more replicate meaurements. In other words, the mean of 9 results is 3 times as reliable as 1 result in measuring central tendency (*i.e.*, the value about which the individual results tend to cluster) ; the mean of 16 results is 4 times as reliable etc.

3B.2.1.2. Median

The median of an even number of results is nothing but the average of the 'two middle values' provided the results are listed in order ; whereas for an odd number of results the median is the 'middle value' itself. However, the '*mean*' and the '*median*' are exactly identical in the case of a truly symmetrical distribution. In short, median is an useful measure specifically when dealing with very small samples.

3B.2.1.3. Average Deviation (or Mean Deviation)

It is the average of the differences between the individual results and the mean. It is regarded as a measure of variability. In the case of a small number of observations the average deviation is found to be not quite significant statistically. The average or mean distribution may be calculated by adopting the following steps, namely :

(*i*) To find the differences between individual results and the mean, without considering the +ve or –ve sign,

(*ii*) To add these individual deviations, and

(*iii*) To divide by the number of results (*i.e.*, *n*).

Hence, an 'average deviation' may be expressed as :

$$\text{Average Deviation} = \bar{d} = \frac{\sum_{i=1}^{i=n}[x_1 - \bar{x}]}{n}$$

3B.2.1.4. Standard Deviation

It is the distance from the mean to the point of inflexion of the normal distribution curve. In comparison to the average deviation the '**standard deviation**' is usually considered to be much more useful and meaningful statistically. For a finite number of values it is normally symbolised as 'S', and may be expressed as follows :

$$S = \sqrt{\frac{\sum_{i=1}^{i=n}[i - \bar{x}]^2}{n-1}}$$

In a situation, where '*n*' is fairly large, say to the extent of 50 or more, it hardly matters whether the denominator in the above expression is either $n - 1$ or n; however, the former (*i.e.*, $n - 1$) is strictly correct.

3B.2.1.5. Coefficient of Variation (ν)

The coefficient of variation (ν) is simply the standard deviation(s) expressed as a percentage of the mean ($\bar{x}$) as stated below :

$$\nu = \frac{s}{\bar{x}} \times 100$$

3B.2.1.6. Variance (s^2)

The variance is the square of the standard deviation(s) *i.e.*, s^2. However, the former is fundamentally more important in statistics than the latter, whereas the latter is employed more frequently in the treatment of chemical data.

3B.2.1.7. Calculations of Fundamental Statistical Parameters

Example : The normality of a solution of sodium hydroxide as determined by an 'analyst' by FOUR different titrations are found to be 0.5038 ; 0.5049 ; 0.5042 ; and 0.5039. Calculate the mean, median, average deviation, standard deviation and coefficient of variation.

Mean : $\bar{x} = \dfrac{0.5038 + 0.5049 + 0.5042 + 0.5039}{4}$

$= \mathbf{0.5042}$

Median : $M = \dfrac{0.5042 + 0.5039}{2}$

$= 0.50405^* = \mathbf{0.5041}$

Average Deviation : $\bar{d} = \dfrac{(0.0004) + (0.0007) + (0.0000) + (0.0003)}{4}$

$= 0.00035^* = \mathbf{0.0004}$

Standard Deviation : $s = \sqrt{\dfrac{(0.0004)^2 + (0.0007)^2 + (0.0000)^2 + (0.0003)^2}{4 - 1}}$

$= 0.00049^* = \mathbf{0.0005}$

Coeffcient of Variation : $= \dfrac{0.0005}{0.5042} \times 100$

$= 0.099^* = \mathbf{0.1\%}$

3B.2.2. DISTRIBUTION OF RANDOM NUMBERS

Results obtained from a given set of measurements that scatter in a random manner are adequately treated by most logical methods of statistics.

In a situation whereby a large number of replicate readings, not less than 50, are observed of a titrimetric equivalence point (continuous variable), the results thus generated shall normally be distributed around the mean in a more or less symmetrical fashion. Thus, the mathematical model which not only fits into but also satisfies such a distribution of random errors is termed as the Normal or Gaussian distribution curve. It is a bell-shaped curve which is noted to be symmetrical about the mean as depicted in Figure 3.2.

The equation of the normal curve may be expressed as given below :

$$y = \frac{1}{\sigma\sqrt{2\pi}}\, e^{-(x-\mu)^2/2\sigma^2}$$

where, y = Relative frequency with which random sampling of the infinite population shall bring forth a specific value x,

σ = Standard deviation, and

μ = Mean.

From the Normal distribution curve (Figure 3.2) it may be observed that 68.26% of results shall fall within one standard deviation on either side of the mean, 95.46% shall fall within two standard deviations, and 99.74% within three standard deviations.

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* Rounding off result to contain only significant numbers. In rounding numbers, drop the last digit if it is less than 5 : *e.g.*, 5.32 becomes 5.3. If the last digit is greater than 5, increase the preceding digit by one : *e.g.*, 5.49 becomes 5.5.

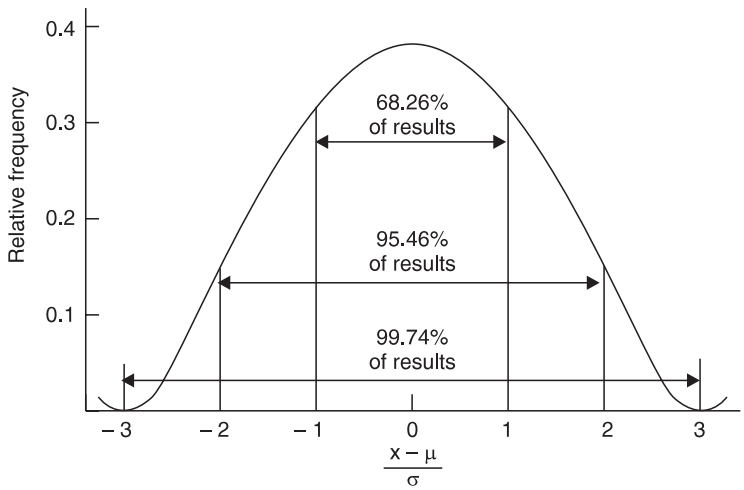

Figure 3.2 : Normal Distribution Curve ; Relative Frequencies of Deviations from the Mean for a Normally Distributed Infinite Population ; Deviations ($x - \mu$) are in Units of σ.

3B.2.3. SIGNIFICANT FIGURES

Generally, significant figures may be defined as—"**All digits* that are certain plus one which contains some uncertainty are said to be significant figures**".

Examples : (*a*) **Burette Reading :** Burettes are mostly graduated with the smallest graduation as 0.1 ml ; hence, while taking the burette reading the figures 6.3 ml can be read off with ample certainty. However, the second place of the decimal is normally estimated by arbitrarily sub-dividing the smallest division into 10 equal parts. Consequently, the final burette reading of 6.32 ml essentially contains three significant figures, of which two are certain, and one with some uncertainty.

(*b*) **Measuring Weights :** In the two measured quantities : 4.7350 g and 4.0082 g the zero is a significant figure ; whereas, in the quantity 0.0065 kg the zeros are not significant figures. Thus, in the latter instance the zeros only serve to locate the decimal point and, therefore, may be eliminated completely by proper choice of units, *e.g.*, 6.5 g. Moreover, the first two numbers do have five significant figures, whilst 0.0065 only has two significant figures.

3B.2.3.1. Computation Rules

The following computation rules are advocated to make sure that a calculated result, arrived at either by addition and subtraction or multiplication and division essentially contains only the number of 'digits' duly justified by the experimental data.

(*a*) **Addition and Subtraction**

In addition and subtraction, retain only as many decimal places as appear in the number that has the fewest decimals.

Example : Add algebraically the numbers given : 16.48 + 9.375 – 3.5450 + 118.9.

Following *three* steps are to be carried out sequentially :

(*i*) All numbers are required to be rounded up preliminarily to two decimal places,

(*ii*) Add the rounded numbers, and

* '**Digit**'—denotes any one of the ten numerals, including the zero.

(*iii*) Final result is then rounded to one decimal place.

+ 16.48	+ 16.48
+ 9.375	+ 9.38
– 3.5450	– 3.55
+ 118.9	+ 118.9
	141.21
Final rounding gives :	**141.2**

Note : This particular method tends to eliminate the accumulation of rounding errors in the final result.

(*b*) **Multiplication and Division**

In multiplication or division, retain in each term one more signifcant figure than is contained in the term with the largest uncertainty. However, the percentage precision of product cannot be greater than the percentage precision of the least precise term entering the calculation. Hence, the multiplication : $2.64 \times 3.126 \times 0.8524 \times 32.9453$ must be accomplished using the values

$$2.64 \times 3.126 \times 0.852 \times 32.95$$

which is equal to 231.6796. Thus, the result obtained may be expressed to five significant figures as 231.68.

(*c*) **Rounding Numbers**

In rounding numbers, always drop the last digit in case it is less than 5, *e.g.*, 8.62 will become 8.6. If the last digit is more than 5, always increase the preceeding digit by one *i.e.*, 9.38 will become 9.4. In case, the digit to be dropped is 5, always round up the preceding digit to the nearest even number *i.e.*, 8.75 will become 8.8 ; and 8.65 will become 8.6. Evidently, this method avoids a tendency to round up numbers in one direction only.

In rounding off quantities to the nearest correct number of significant figures, add one to the last figure retained provided the following figure is either 5 or over. Hence, the average of 0.6526, 0.6521, and 0.6524 is 0.6525 (0.65237).

(*d*) Always retain as many significant figures in a result as will yield only one uncertain figure.

Examples : (*i*) A volume read off from a burette reading that lies between 15.6 ml and 15.8 ml must be recorded as 15.7 ml, but not as 15.70 ml, because the latter would indicate that the reading lies between 15.69 and 15.71 ml.

(*ii*) A weight, to the nearest 0.1 mg, is recorded as 2.4500 g ; and it must not be written as either 2.450 g or 2.45 g, because in the latter instance an accuracy of a centigram is emphasized whereas in the former a milligram.

3B.2.4. COMPARISON OF RESULTS

In a situation where the same sample has been analysed by two separate techniques altogether, each of them repeated several times, and that the mean values obtained are not the same ; statistically it may be possible to ascertain whether the analytical procedure adopted has been either accurate and precise or if it is superior to one of the two methods.

In fact there are two frequently employed methods that may be used to compare the results, namely :

(*a*) Student's *t*-Test, and

(*b*) Variance-Ratio Test (or F-Test).

In order to perform these two tests one should have a clear understanding of the statistical term 'the number of degrees of freedom'.

Degrees of Freedom : It is the number of individual observations which can be allowed to vary under conditions that the mean ($\bar{x}$) and standard deviation(s), once determined, be held constant.

Thus, a sample having **n** values have **n** degrees of freedom, whereas the sum $\Sigma(x - \bar{x})^2$ is considered to have $n - 1$ degrees of freedom, because for any defined value of the mean, $\bar{x}$, only $n - 1$ value can be assigned freely, as the nth is being defined from the other values automatically.

3A.2.4.1. Student's t-Test

It is usually employed for small samples only. It serves *two* main objectives, namely :

(*i*) It is employed to test the difference between the means of two sets of data $\bar{x}_1$ and $\bar{x}_2$, and

(*ii*) It is used to compare the mean obtained from a sample having certain standard value and to express certain degree of confidence in the significance of the comparison.

The value of t may be obtained from the following expression :

$$t = \frac{(\bar{x} - \mu)\sqrt{n}}{s} \qquad ...(i)$$

where, $\bar{x}$ = Mean value,

μ = True value,

s = Standard deviation, and

n = Degree of Freedom.

Example : If $\bar{x}$ the mean of 12 determinations = 9.59, and μ the true value = 9.03, find out whether or not this result is significant provided the standard deviation(s) is 0.16.

From the above Eq. (*i*) we have :

$$t = \frac{(9.59 - 9.03)\sqrt{12}}{0.16}$$

or

$$t = \frac{0.56 \times 3.46}{0.16} = 12.11$$

Now, from the *t*-tables for ($n - 1$ *i.e.*, $12 - 1 =$) 11 degrees of freedom we have :

for	P = 0.10 (10%)	0.05 (5%)	0.01 (1%)
	t = 1.80	2.20	3.11

Evidently, the calculated value for t is 12.11 and the result is highly significant. Besides, the *t*-table also gives the information that the probability of obtaining the difference of 0.56 between the experimental and the true result comes out to be less than 1 in 100, which obviously is indicative of the fact that some kind of bias does exist in the laboratory method adopted.

3B.2.4.2. Variance-Ratio Test (or F-Test)

A test that makes use of the ratio of the variances of two sets of results to determine if the standard deviations (s) are significantly different. Its application may also be extended to compare precisely the results obtained either from two different laboratories or from two different analytical procedures.

It is simply calculated from the following equation :

$$F = s_1^2 / s_2^2 \qquad ...(ii)$$

where, s_1 and s_2 = Standard deviations of two sets of results.

Various steps invoived to determine F-Test are

(*i*) Find the ratio from Eq. (*ii*),

(*ii*) Place the larger s-value in the numerator so that F > 1,

(*iii*) Check for its significance against values in the F-table, and

(*iv*) If the F-value in the table is less than the calculated F-value, then the two standard deviations are significantly different ; otherwise, they are not.

Example : A sample of anhydrous sodium carbonate (Na_2CO_3) is analysed by **two** different methods which give the results for the percentage of Na_2CO_3 as follows :

Method : 'A'	Method : 'B'
$\bar{x}_1 = 24.36$	$\bar{x}_2 = 24.46$
$s_1 = 0.10$	$s_2 = 0.13$
$n = 6$	$n = 5$

Is there any significant difference between the precision of these two sets of results ?

Applying the variance-ratio or F-Test from Eq. (*ii*) we have :

$$F = \frac{(0.13)^2}{(0.10)^2} = \mathbf{1.69}$$

From the standard table having F-values at the 95% probability level, under column $n - 1 = 4$ (since $s_2 > s_1$) and row $n - 1 = 5$, find F = 6.39. Because, 6.39 > 1.69, the standard deviations are not significantly different [see step (*iv*) above].

3B.2.5. METHOD OF LEAST SQUARES

A number of pharmaceutical analytical methods are solely based on instrumental measurements of an absolutely physical nature, such as : measuring peak areas with the help of a gas-chromatograph (GC), and measuring absorbance of a solution using a spectrophotometer (UV). In both these instances, the physical characteristics are directly proportional to the concentration of the analyte under examination. In usual practice, a number of solutions having known concentrations is prepared and the response of the instrument is subsequently measured for each standard solution. Finally, a standard curve or calibration curve is plotted between the observed response *Vs* concentration, which invariably gives rise to straight line. It has been noticed, that the experimental points rarely fall exactly upon a straight line by virtue of the indeterminate errors caused by the instrument readings. At this juncture, an analyst is confronted with the tedious problem to obtain the 'best' straight line for the standard curve based on the observed points so that the error in estimating the concentration of the unknown sample is brought down to the least possible extent. At this stage, instead of deciding to draw the line merely on an analyst's judgement, statistics comes to the rescue by providing a mathematical relationship whereby the analyst not only may calculate the slope objectively but also can obtain the 'best' straight line. The statistical process involved is termed as the method of **least squares.**

Example : The results obtained from the determination of concentration of the standard solutions and measurements of corresponding peak areas with a GC are recorded in Table 3.1 and plotted in Figure 3.3 ; where the former is represented along the *x*-axis and the latter along the *y*-axis. How to draw the' 'best' straight line through all these points ?

Table 3.1 : Method of Least Squares

x	y	xy	x^2	y^2
1.00	2.94	2.94	1.00	8.64
2.00	5.07	10.14	4.00	25.70
3.00	7.05	21.15	9.00	49.70
4.00	8.96	35.84	16.00	80.28
5.00	10.92	54.60	25.00	119.25
$\Sigma x = 15.00$	$\Sigma y = 34.94$	$\Sigma xy = 124.67$	$\Sigma x^2 = 55.00$	$\Sigma y^2 = 283.57$

Considering that the relationship between the concentration and the observed peak areas is a linear one, the equation for a straight line may be expressed as :

$$y = mx + b$$

where, m = Slope of the line, and

b = Intercept on the y-axis.

It may also be assumed that values of x are free of any error.

$$C = \sum x^2 - \frac{(\sum x)^2}{n} = 55.00 - \frac{(15.00)^2}{5} = 10.00$$

$$D = \sum y^2 - \frac{(\sum y)^2}{n} = 283.57 - \frac{(34.94)^2}{5} = 39.41$$

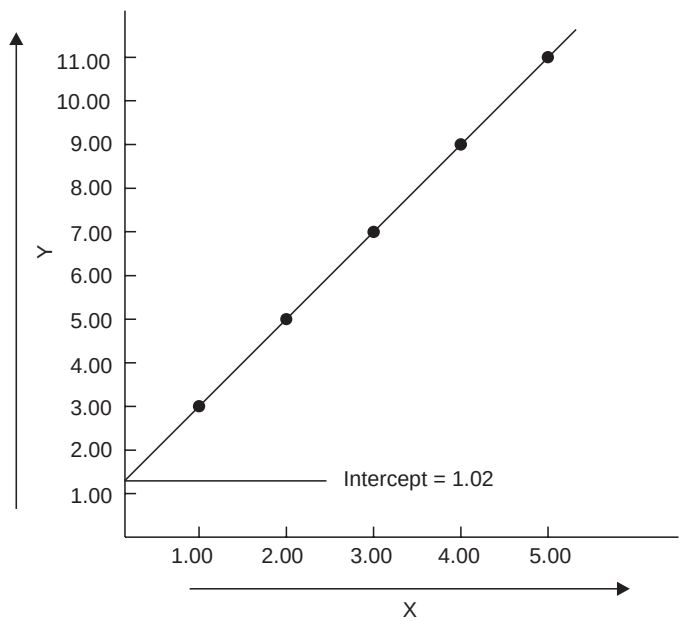

Figure 3.3 : Method of Leat Sqaures.

Presumably, the indeterminate errors caused by the instrument readings, y, are responsible for not allowing the 'data points' to fall exactly on the line. Therefore, the sum of the squares of the deviations obtained from the real instrument readings with respect to the correct values are minimized coinsiderably by adjusting adequately the values of the slope, m, and the intercept, b.

Table 3.1, comprises the values of x and y to enable plot of the graph in Figure 3.3, besides values of x^2, y^2 and xy and also the sums of all these terms.

Statistically, the slope (m) and intercept (b) of the straight line may be obtained by the help of the following equations :

Slope :

$$m = \frac{\sum xy - (\sum x \sum y)/n}{C}$$

$$= \frac{12.67 - (15.00 \times 34.94)/5}{10.00} = \mathbf{1.99}$$

Intercept :

$$b = \frac{\sum y - m\sum x}{n} = \frac{34.94 - 1.00 \times 15.00}{5} = \mathbf{1.02}$$

Therefore, the equation of the line is

$$y = 1.99x + 1.02$$

Thus, the standard deviation of the y values, Sy, is given by :

$$Sy = \sqrt{\frac{(D - m^2C)}{n-2}} = \sqrt{\frac{(39.41) - (1.99)^2\ (10.00)}{5-2}} = \mathbf{0.24}$$

The number of degrees of freedom in the above expression is $n - 2$, because two degrees have already been consumed while calculating the values of m and b earlier.

The standard deviation of the slope, Sm, is given by :

$$Sm = \frac{Sy}{\sqrt{C}} = \frac{0.24}{\sqrt{10.00}} = 0.08$$

At this point, let us suppose that the 'calibration curve' is used to find out the concentration of the 'unknown'. Assuming that three determinations have been carried out separately, thereby giving three y values of 5.85, 5.88, 5.91, or an average value, $\bar{y}_u$, of 5.88. Thus, using the expression : $y = mx + b$, we have :

$$5.88 = 1.99x + 1.02$$

$$\therefore \qquad x = 2.44$$

The standard deviation (Su) in this result is obtained from the expression :

$$Su = \frac{Sy}{m}\sqrt{\frac{1}{n_u} + \frac{1}{n} + \frac{(\bar{y}_u - \bar{y}^2)}{m^2C}}$$

where, n_u = Number of determination of unknown,

n = Number of points in the calibration graph, and

$\bar{y}$ = Average of the y-values in the calibration graph (*i.e.*, 34.94/5 = 6.99)

Therefore,
$$Su = \frac{0.24}{1.99}\sqrt{\frac{1}{3} + \frac{1}{5} + \frac{(5.88 - 6.99)^2}{(1.99)^2(10.00)}}$$

$$Su = \mathbf{0.09}$$

In case, the above statistical analysis has been based on a single determination, for instance : y = 5.88, the value of Su shall come out to be :

$$Su = \frac{0.24}{1.99}\sqrt{\frac{1}{1} + \frac{1}{5} + \frac{(5.88 - 6.99)^2}{(1.99)^2(10.00)}}$$

or
$$Su = \mathbf{0.13}$$

3B.2.6. RECOMMENDATIONS FOR CRITERIA OF REJECTING AN OBSERVATION

An analyst, while carrying out a series of measurements, invariably comes across with ONE specific result in a set of replicates that obviously appears to be quite 'out of place' with the others, and at this juncture he should take an appropriate decision whether to discard (or expunge) this result from any further consideration. Thus, *two* situations often arise, namely :

(*i*) Number of replicates being small, and

(*ii*) Number of replicates being large.

A. Number of Replicates being Small

An analyst in the true sense encounters a serious problem when the number of replicates at his disposal is SMALL. Firstly, the divergent result shows a distinct and significant effect upon the mean value ($\bar{x}$) ; and

secondly, the prevailing scanty available data does not permit getting at the real statistical analysis of the status of the suspected result.

B. Number of Replicates being Large

In this instance, the analyst has the privilege of rejecting one value (*i.e.*, the 'out-of place' value) as it is not an important one by virtue of the following two main reasons :

Firstly, a single value shall exert merely a small effect upon the mean value ($\bar{x}$) ; and secondly, the treatment of data with the real statistical analysis would certainly reveal vividly the probability that the suspected 'out of place' result is a bonafide member of the same population as the others.

Blaedel *et al.** (1951), Wilson** (1952) and Laitinen*** (1960) have put forward more broadly accepted and recommended criteria of rejecting an observation.

3B.2.6.1. Rules Based on the Average Deviation

Both '2.5*d*' and '4*d*' rules are quite familiar to analysts. They may be applied in a sequential manner as follows :

(*i*) Calculate the mean ($\bar{x}$) and average deviation ($\bar{d}$) of the 'good' results,

(*ii*) Determine the deviation of the 'suspected' result from the mean of the 'good' results,

(*iii*) In case, the deviation of the suspected result was found to be either 2.5 times the average deviation of the good results (*i.e.*, '**2.5d' rule**) or 4 times the average deviation of the good results (*i.e.*'**4d' rule**) the suspected result was rejected out right ; otherwise the result was duly retained.

Note : The 'limit for rejection' seems to be too low for both the said rules.

3B.2.6.2. Rules Based on the Range

The Q test, suggested by Dean and Dixon***** (1951) is statistically correct and valid, and it may be applied easily as stated below :

(*i*) Calculate the range of the results,

(*ii*) Determine the difference between the suspected result and its closest neighbour,

(*iii*) Divide the difference obtained in (*ii*) above by the range from (*i*) to arrive at the **rejection Quotient** Q,

(*iv*) Finally, consult a table of Q-values. In case, the computed value of Q is found to be greater than the value given in the table, the result in question can be rejected outright with 90% confidence that it was perhaps subject to some factor or the other which never affected the other results.

Table 3.2, records some of the Q-values as given below :

Table 3.2 : Values of Rejection Quotient, Q

Nos. of Observations	$Q_{0.90}$
3	0.94
4	0.76
5	0.64
6	0.56
7	0.51
8	0.47
9	0.44

* Blaedel, W.J., V.W. Meloche, and J.A. Ramsey., **J. Chem. Ed., 28**, 643, 1951.

** Wilson, E.B. Jr., **An Introduction to Scientific Research,** New York, McGraw Hill Book Company, p-56, 1952.

*** Laitinen, H.A., **Chemical Analysis**, New York, McGraw Hil1 Book Company, p-574, 1960.

***** Dean, R.B. and W.J. Dixon, **Anal. Chem, 23**, 636, 1951.

Example : Five determinations of the ampicillin content in capsules of a marketed product gave the following results : 0.248, 0.245, 0.265, 0.249 and 0.250 mg per capsule. Apply the Q-test to find out if the 0.265 value can be rejected.

The value of Q is :

$$Q = \frac{0.265 - 0.250}{0.265 - 0.245}$$

or $$Q = \mathbf{0.75}$$

The value in Table 3.2, at $n = 5$ is Q = 0.64. Because, the determined value 0.75 > 0.64, according to 'rule based on the range' the result *i.e.*, 0.265 can be rejected.

Note : The Q-test administers excellent justification for the outright rejection of abnormally erroneous values ; however, it fails to eliminate the problem with less deviant suspicious values.

3B.2.7. SAMPLING STATISTICS

The errors that are solely attributed to sampling, specifically in the instance of heterogeneous solids, usually give rise to the most important source of uncertainty in carrying out analysis of pharmaceutical substances.

Thus, the overall standard deviation, s_T, *i.e.*, the total error is given by the following expression :

$$s_T = \sqrt{S_S^2 + S_A^2} \qquad ...(i)$$

where, S_S = Standard deviation of the sampling procedure (*i.e.*, the sampling error), and

S_A = Standard deviation of the analytical procedures (*i.e.*, the analytical error).

The Eq. (*i*) may also be expressed as :

$$S_T = \sqrt{V_S + V_A}$$

where V_S = Variance due to sampling, and

V_A = Variance due to analytical method(s).

The individual determination of V_S and V_A may be accomplished by using the method described under variance (see Section 3B.2.1.6).

Example : If the sampling error is ± 2.8% and the analytical errors by two different analysts come out to be ± 0.9% and ± 0.1% respectively, we may have :

$$S_T = \sqrt{(2.8)^2 + (0.9)^2} = 2.94 \qquad ...(a)$$

$$S_T = \sqrt{(2.8)^2 + (0.1)^2} = 2.80 \qquad ...(b)$$

From Eqs. (*a*) and (*b*) it is quite evident that the actual contribution of the analytical error *viz.*, ± 0.9% and ± 0.1%, to the total error (S_T) is more or less insignificant.

Note : Youden* (1967) suggested that once the analytical error is reduced to 1/3rd of the sampling error, further reduction of the former is not required anymore.

In order to have a meaningful 'sampling plan' the following points should be taken into consideration**, namely :

(1) Number of samples to be taken :

(2) Size of the sample, and

(3) Should separate samples be analysed or should a sample made up of two or more increments (*i.e.*, composite sample) be prepared.

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* Youden, W.J., **J. Assoc. Off Anal. Chem., 50**, 1007, 1967.

** Kratochvil, B., and J.K. Taylor, **Anal. Chem., 53**, 925 A, 1981.

Unknown Bulk Material : A container-load of Paracetamol (10 MT) arrives at a raw-material stores and the composition of the bulk material is unknown, it will be a sensible and logical practice to carry out first and foremost a preliminary investigation by collecting a large number of samples and assaying the analyte of interest.

Thus, the confidence limits are given by the following expression :

$$\mu = \frac{\bar{x} \times tS_S}{\sqrt{n}} \qquad \ldots (c)$$

where, μ = Estimate of the true mean,

$\bar{x}$ = Mean of the analytical results,

t = Parameter depending upon the number of degrees of freedom (ν) and the confidence level required,

S_S = Standard deviation of individual sample, and

n = Number of samples taken.

Example : The estimate in variability of Paracetamol in a consignment of 10 MT, based on 20 determinations, was found to be $\pm$ 1.4%. How many samples must be taken to give (at 95% confidence level) a sampling error of less than 0.5% paracetamol ?

The 0.5% value, in reality, represents the difference between the sample mean $\bar{x}$ and the actual value μ. If this value is designated by E, then Eq. (*c*) may be expressed as :

$$E = \frac{tS_S}{\sqrt{n}} \text{ and hence, } n = \left[\frac{tS_S}{E}\right]^2$$

From the tables [Percentage Points of the *t*-Distribution] the value of t for ($n - 1$), 19 degrees of freedom at 95% confidence level is 2.09.

$$\therefore \qquad n = \left[\frac{2.09 \times 1.4}{0.5}\right]^2 \approx 34$$

Conclusion : From this test it has been established that at least 34 samples are required if the specifications provided in the above cited example are to be fulfilled adequately.

Sample Size : Another major problem associated with the sampling process is that of the sample size. In fact, the sample size withdrawn from a heterogeneous material is solely guided by two factors, namely :

(*a*) Variation in particle size, and

(*b*) Precision required in the results of the analysis.

The sampling variance, V, is inversely proportional to the actual number of sampling increments (n) and may be expressed as :

$$V = \frac{k}{n}$$

where, k = Constant entirely dependent on the size of the increment and variation within the bulk material.

The following points with regard to sampling may be observed carefully :

- A major source of error in sampling may be incorporated from the actual process of taking increments from the bulk material,
- The accuracy of the sample is determined by its total size (based on Random Sampling Theory), and
- The number of increments taken shall directly influence the sampling accuracy provided the bulk material comprises of varying particle sizes.

THEORETICAL AND PRACTICAL EXERCISES

1. What are the two major types of '**errors**' invariably encountered in pharmaceutical analysis ? Explain with suitable examples.

2. How would you differentiate between '**accuracy**' and '**precision**' ? Support your answer with suitable examples.

3. Discuss the various means of minimising '**systemic errors**' with respect to the following aspects :

(*i*) Calibration of instruments and apparatus

(*ii*) Parallel control determination

(*iii*) Blank determination

(*iv*) Verifying results by different methods of analysis

(*v*) Method of standard deviation

(*vi*) Method of internal standards.

4. (*a*) Why is it necessary to apply '**statistical validation**' for analytical results in routine analysis in a Quality Assurance Laboratory (QAL) ?

(*b*) Discuss the following aspects in an elaborated manner :

(*i*) Distribution of random errors.

(*ii*) Method of 'Least Squares'.

5. Elaborate the following statistical methods with suitable examples :

(*i*) Students *t*-test

(*ii*) F-test (Variance-Ratio Test).

6. What are the various recommendations a '**pharmaceutical analyst**' shall propose for rejecting an observation.

7. Describe the various means and ways usually adopted for '**Sampling Statistics**'. Give suitable examples.

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PART II

CHEMICAL METHODS

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A. TITRIMETRIC METHODS : ACIDIMETRY AND ALKALIMETRY

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4 AQUEOUS TITRATIONS

CONTAINS :

4.1. INTRODUCTION

Arrhenius' definition of an *acid* is—'**a substance which yields hydrogen ion (H^+) in an aqueous medium**'; and that of a *base* is—**'a substance which yields hydroxy ions (OH^-) in an aqueous medium'.**

However, these definitions have *two* serious short-comings, they are :

(*a*) they lack explanation of the behaviour of acids and bases in non-aqueous media, and

(*b*) *acidity* is associated with **hydrogen ion**—a relatively simple particle ; whereas, *basicity* is associated with **hydroxyl ion**—a relatively complex entity.

4.1.1. LOWRY AND BRONSTED'S THEORY OF ACIDS AND BASES

Just after the First World War in 1923, Bronsted and Bjerrum in Denmark and Lowry in Great Britain jointly put forward a more acceptable and satisfactory theory of acids and bases which is devoid of objections earlier raised in Arrhenius' definition.

According to Lowry and Bronsted's theory—**'an acid is a substance capable of yielding a proton (hydrogen ion), while a base is a substance capable of accepting a proton'**. Thus, a complementary relationship exists between an acid and a base that may be expressed in a generalized fashion as below :

$$A_{acid} \rightleftharpoons H^+ + B_{base}$$

4.1.1.1. Conjugate Acid-Base Pair

The pair of substances which by virtue of their mutual ability either gain or lose a proton is called a **conjugate acid-base pair**. A few typical examples of such pairs are :

Acid		Base
HNO_3	$\rightleftharpoons$	$H^+ + NO_3^-$
HCl	$\rightleftharpoons$	$H^+ + Cl^-$
CH_3COOH	$\rightleftharpoons$	$H^+ + CH_3COO^-$
NH_4^+	$\rightleftharpoons$	$H^+ + NH_3$
HPO_4^{2-}	$\rightleftharpoons$	$H^+ + PO_4^{3-}$
H_3O^+	$\rightleftharpoons$	$H^+ + H_2O$
HCO_3^-	$\rightleftharpoons$	$H^+ + CO_3^{2-}$
$Al(H_2O)_6^{+++}$	$\rightleftharpoons$	$H^+ + Al(H_2O)_5\ OH^{2+}$

It is quite evident from the above examples that not only molecules but also *anions* and *cations* can act as acids and bases.

In an acid-base titration, the acid will not release a proton unless the base capable of accepting it is simultaneously present ; in other words, in a situation where actual acid-base behaviour exists then an interaction should involve two sets of conjugate acid-base pairs, represented as :

$$\underset{\text{acid}_1}{A_1} + \underset{\text{base}_2}{B_2} \rightleftharpoons \underset{\text{base}_1}{B_1} + \underset{\text{acid}_2}{A_2}$$

$$\underset{\text{acid}_1}{HCl} + \underset{\text{base}_2}{NH_3} \rightleftharpoons \underset{\text{conjugate base}_1}{Cl^-} + \underset{\text{conjugate acid}_2}{NH_4^+}$$

Some other examples include :

$$CH_3COOH + H_2O \rightleftharpoons CH_3COO^- + H_3O^+$$

$$H_2SO_4 + H_2O \rightleftharpoons HSO_4^- + H_3O^+$$

$$H_2O + CN^- \rightleftharpoons OH^- + HCN$$

$$NH_4^+ + S^{2-} \rightleftharpoons NH_3 + HS^-$$

In short, the species which essentially differ from each other by one proton only, are known as **conjugate base and acid** respectively. Sometimes, such a reaction is termed as **protolytic reaction** or **protolysis**, where A_1 and B_1 make the first conjugate acid-base pair and A_2 and B_2 the other pair.

4.1.1.2. Merits of Lowry-Bronsted Theory

It has two points of merit, which are :

(*a*) hydrochloric acid on being dissolved in water undergoes a protolytic reaction, thus :

$$\underset{\text{acid}_1}{HCl} + \underset{\text{base}_2}{H_2O} \rightleftharpoons \underset{\text{base}_1}{Cl^-} + \underset{\text{acid}_2}{H_3O^-}$$

It may be observed that H_3O^+, known as hydronium or oxonium ion is invariably formed when an acid is dissolved in water.

Likewise, ammonia on being dissolved in water is also subjected to protolysis, thus :

$$\underset{\text{base}_1}{NH_3} + \underset{\text{acid}_2}{H_2O} \rightleftharpoons \underset{\text{acid}_1}{NH_4^+} + \underset{\text{base}_2}{OH^-}$$

(*b*) all proton-transfer reactions may be handled, thus :

$$\underset{\text{acid}_1}{NH_4^+} + \underset{\text{base}_2}{S^{2-}} \rightleftharpoons \underset{\text{base}_1}{NH_3} + \underset{\text{acid}_2}{HS^-}$$

4.1.1.3. Demerits of Lowry-Bronsted Theory

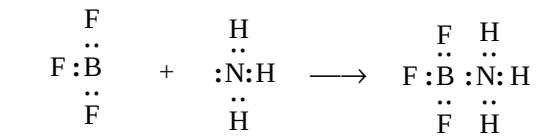
It does not hold good for **nonprotonic** solvents, for instance : BF_3, $POCl_3$ and SO_2.

4.1.2. LEWIS'S THEORY

Lewis (1923) put forward another definition of acids and bases solely dependent on giving or taking of an electron pair. According to Lewis—**'an acid is an electron pair acceptor, whereas a base is an electron pair donor'**. Therefore, it is obvious that whenever any neutralization occurs the formation of an altogether new coordinate covalent bond between the electron pair donor and acceptor atoms take place.

Thus, Lewis's definition is a much broader definition that includes coordination compound formation as acid-base reactions, besides Arrhenius and Lowry-Bronsted acids and bases. Examples :

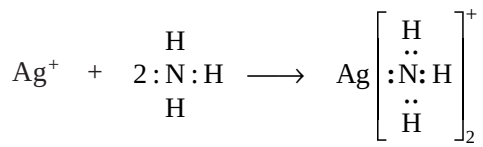
(*i*)



Boron trifluoride (acid) Ammonia (base)

The reaction of borontrifluoride (acid) with ammonia (base) results into a stable octet configuration between mutual sharing of a pair of electrons of latter (donor) and former (acceptor).

(*ii*)



Electron Acceptor (acid) Electron Donor (base)

The reaction of ammonia (base) with Ag^+ (acid) results into a stable configuration due to the mutual sharing of a pair of electrons of latter (donor) and former (acceptor).

4.1.3. USANOVICH THEORY

Usanovich (1934) modified the Lewis concept of acid and base by removing the restriction of either donation or acceptance of the electron pair in a more generalized fashion. According to him :

Acid : *It is a chemical species that reacts with a base thereby giving up cations or accepting anions or electrons.*

Base : *It is a chemical species that reacts with an acid thereby giving up anions or electrons or combines with cations.*

Unlike Arrhenius, Lowry-Bronsted and Lewis acids and bases, the Usanovich's concept in a much broader sense includes all the oxidizing agents as acids and the reducing agents as bases, *e.g.*,

$$\underset{\text{base}}{Fe^{2+}} \rightleftharpoons \underset{\text{acid}}{Fe^{3+}} + e^-$$

In the Iron (II)—Iron (III) system, the ferric ion (III) acts as an oxidizing agent and is an acid ; while the ferrous ion (II) acts as a reducing agent and is a base.

$$\underset{\text{base}}{Ce^{3+}} \rightleftharpoons \underset{\text{acid}}{Ce^{4+}} + e^-$$

Similarly, in the Cerous (III)—Ceric (IV) system, the ceric ion (IV) behaves as an oxidizing agent and acts as an acid ; while the cerous ion (III) behaves as a reducing agents and acts as a base.

4.1.4. LUX-FLOOD CONCEPT

The concept of acid-base reactions with respect to the oxide ion was first introduced by Lux (1929) and supported by Flood (1947). According to the Lux-Flood concept—**'an acid is the oxide-ion acceptor while a base is the oxide donor'**. Examples :

$$MgO + SiO_2 \longrightarrow MgSiO_3$$
$$CaO + SO_3 \longrightarrow CaSO_4$$

In the above reactions both MgO and CaO are the oxide ion donor and hence act as bases, whereas SiO_2 and SO_3 are the oxide-ion acceptor and hence act as acids. Ultimately, the Lux-Flood acid and base react to form magnesium silicate ($MgSiO_3$) and calcium sulphate ($CaSO_4$) salts respectively.

4.2. THEORY OF ACIDIMETRY

Acidimetry, essentially involves the direct or residual titrimetric analysis of alkaline substances (bases) employing an aliquot of acid and is provided usually in the analytical control of a large number of substances included in the various *official compendia*. Examples :

(*a*) **Organic substances :** urea, sodium salicylate, diphenhydramine, emetine hydrochloride, meprobamate, paramethadione, pyrazinamide etc., and

(*b*) **Inorganic substances :** sodium bicarbonate, milk of magnesia, ammonium chloride, calcium hydroxide, lithium carbonate, zinc oxide etc.

The *two* methods, namely : direct titration method and residual titration method are briefly discussed as under :

4.2.1. DIRECT TITRATION METHOD

It is an usual practice that when a solid substance is to be assayed, an aliquot quantity of the same may be weighed accurately and dissolved in sufficient water so that the resulting solution should have more or less the same equivalent concentration as that of the acid used in the titration. Methyl orange (pH range = 3.0 to 4.4) is the indicator of choice for obvious reasons, as phenolphthalein and most other indicators are instantly affected by the carbonic acid (H_2CO_3) generated in the reaction which ultimately cause a change in colour even before the reaction attains completion.

4.2.2. RESIDUAL TITRATION METHOD

Residual titration or back titration is normally employed in the following *two* situations, namely :

Case I : when a chemical reaction proceeds rather slowly or sluggishly, and

Case II : when the substance under determination fails to give a sharp and distinctly visible end-point with an indicator by direct titration.

In usual practice, the residual titration is accomplished by allowing to dissolve the substance under estimation in an accurately measured quantity of a standard solution of known strength present in excess and subsequently titrating the excess of the latter with another previously standardized solution. A good number of examples of this particular method shall be discussed in subsequent exercises.

4.3. ASSAY OF DRUGS

A few typical examples of acidimetric titrations, employing **'direct titration method'** (DTM) and **'residual titration method'** (RTM) from the '*Pharmacopoeia of India*' are described here :

4.3.1. DIRECT TITRATION METHOD

4.3.1.1. Sodium Carbonate (DTM)

Materials Required : 1 g of sodium carbonate ; 0.5 N sulphuric acid.

Procedure : Weigh accurately about 1 g, dissolve in 20 ml of water (DW) and titrate with 0.5 N sulphuric acid, using methyl orange solution as indicator. Each ml of 0.5 N sulphuric acid is equivalent to 0.42 g of $NaHCO_3$.

Equation :

$$\underset{2(84.01)}{2NaHCO_3} + H_2SO_4 \longrightarrow Na_2SO_4 + 2H_2O + 2CO_2\uparrow$$

It is evident from the above equation that 2 ml of $NaHCO_3$ is equivalent to 1 ml of H_2SO_4. Hence, 1 ml of $NaHCO_3$ is 1 equivalent and the equivalent weight is equal to the gram-molecular weight *i.e.*, 84.01/ 2 = 42.0 g. One millilitre of 0.05 N sulphuric acid or 1 milliequivalent is equivalent to 42.0 mg or 1 meq of $NaHCO_3$.

Thus, the purity of the sample assayed may be calculated as follows :

$$\% \ NaHCO_3 = \frac{ml \times N \times 0.8401 \times 100}{\text{wt. of sample}}$$

4.3.1.2. Sodium Hydroxide (DTM)

Materials Required : 1.5 g of sodium hydroxide ; 1 N sulphuric acid.

Procedure : Weigh accurately about 1.5 g of sodium hydroxide and dissolve in about 40 ml of carbon-dioxide free distilled water (*i.e.*, boiled and cooled DW). Cool and titrate with 1 N sulphuric acid using phenolphthalein solution as indicator. When the pink colour of the solution is discharged record the volume of acid solution required.

Equations : Thus, the end-point obtained with phenolphthalein as an indicator designates complete neutralization of all the NaOH as shown by the equation :

$$\underset{2(40.0)}{2NaOH} + H_2SO_4 \longrightarrow Na_2SO_4 + 2H_2O \qquad ...(a)$$

However, in a cold solution, with phenolphthalein as an indicator, the end-point of titration of sodium carbonate with 1 N sulphuric acid is exhibited when the sodium carbonate is fully transformed into sodium carbonate, thus :

$$\underset{2(106)}{2Na_2CO_3} + H_2SO_4 \longrightarrow Na_2SO_4 + 2NaHCO_3 \qquad ...(b)$$

At this juncture, add methyl orange solution and proceed ahead with the titration until a persistant pink colour is produced. Each millilitre of 1 N sulphuric acid is equivalent to 0.040 g (or 40 mg) of total alkali, calculated as NaOH and each millilitre of acid consumed in the titration with methyl orange is equivalent to 0.106 g of Na_2CO_3.

Explanation

1. Titration to a phenolphthalein end-point serves *two* purposes :

   (*a*) Neutralization of sodium hydroxide, and

   (*b*) Conversion of Na_2CO_3 to $NaHCO_3$.

2. When the Na_2CO_3 is converted to $NaHCO_3$ ($CO_3^{2-} + H^+ \rightleftharpoons HCO_3^-$), the H^+ remains low because the CO_3^{2-} is strongly basic, thereby the pH of the resultng mixture ranges between 8 to 9.8. This is when phenolphthalein changes colour till the conversion of Na_2CO_3 to $NaHCO_3$ is complete.

3. The HCO_3^- is weakly basic in nature due to : $HCO_3^- + H^+ \rightleftharpoons H_2CO_3$, and the $NaHCO_3$ thus formed remains unneutralised even though H^+ has been increased to the point where phenolphthalein affords a change in colour.

4. The neutralization of the generated $NaHCO_3$ is complete only when H^+ has been enhanced by further addition of acid, as observed by the change in colour of methyl orange at pH 3.2 to 4.4.

The reaction is represented by the equation :

$$\underset{2(84.01)}{2NaHCO_3} + H_2SO_4 \longrightarrow Na_2SO_4 + 2CO_2 \uparrow + H_2O \qquad ...(c)$$

Calculations : The total volume of 1 N sulphuric acid consumed in the titration was required to neutralize NaOH and Na_2CO_3, thereby converting the latter first to $NaHCO_3$ at the phenolphthalein end-point and then to H_2CO_3 at the methyl orange end-point.

From Eq. (*a*), it may be observed that the equivalent weight of NaOH is 40.00 g. Hence, each millilitre of the total amount of 1 N sulphuric acid consumed is equivalent to 40.00 mg or 1 meq of NaOH. Thus, the total alkalinity calculated as NaOH is therefore :

$$\% \text{ NaOH} = \frac{\text{ml} \times \text{N} \times \text{meq wt} \times 100}{\text{Sample wt}}$$

The volume of 1 N sulphuric acid *i.e.*, the difference between the acid consumed to a methyl orange end-point and the acid consumed to a phenolphthalein end-point, required to neutralize the $NaHCO_3$ as in Eq. (*c*) is equal to the volume needed to generate the $NaHCO_3$ from Na_2CO_3 as in Eq. (*b*). Thus, from Eq. (*b*) it may be calculated that each millilitre of 1 N sulphuric acid is equivalent to 106.0 mg of Na_2CO_3. Hence, the quantity (%) of Na_2CO_3 present in the sample is given by :

$$\% \text{ } Na_2CO_3 = \frac{\text{ml} \times \text{l} \times \text{meq. wt.} \times 100}{\text{wt. of sample}}$$

4.3.1.3. Cognate Assays

Sodium bicarbonate ; sodium salicylate tablets

S. No.	Name of Substance	Quantity Prescribed	Indicator Employed	Calculations
l.	Sodium Bicarbonate	l.0 g	Methyl orange	Each ml of 0.5 N H_2SO_4 ≡ 0.042 g of $NaHCO_3$
2.	Sodium Salicylate Tablets	500 mg	Bromophenol Blue	Each ml of 0.I N HCl ≡ 16.01 mg of $C_7H_5NaO_3$

4.3.2. RESIDUAL TITRATION METHOD

4.3.2.1. Zinc Oxide (RTM)

Materials Required : 1.5 g of zinc oxide ; 1 N sulphuric acid ; 1 N sodium hydroxide ; 2.5 g ammonium chloride.

Procedure : 1.5 g of freshly ignited and cooled zinc oxide is accurately weighed and dissolved with 2.5 g of ammonium chloride in 50 ml of 1 N sulphuric acid with the help of gentle heating. After complete dissolution, add methyl orange and titrate the excess of sulphuric acid with 1 N sodium hydoxide. Each millilitre of 1 N sulphuric acid is equivalent to 40.6 mg of ZnO.

Equation :

$$\underset{(81.38)}{ZnO} + H_2SO_4 \longrightarrow ZnSO_4 + H_2O$$

The requisite quantity of ZnO gets dissolved in the sulphuric acid thereby neutralizing an equivalent amount as shown by the above equation. Thus, the amount of sulphuric acid neutralized by the ZnO is estimated by subtracting, from the total amount of sulphuric acid utilized, the quantity neutralized by the

standard NaOH in the back titration. The equivalent weight of ZnO, as shown in the above equation comes out to be 40.69 g (*i.e.*, 81.38/2 = 40.69). Hence, each millilitre of 1 N sulphuric acid, 1 meq neautralized by the ZnO, is equivalent to 40.68 mg or 1 meq of ZnO.

Thus, the percentage of zinc oxide present in the sample may be calculated as follows :

$$\% \text{ ZnO} = \frac{(\text{ml}_1 \times \text{N}) - (\text{ml}_2 \times \text{N}) \times \text{meq. wt.} \times 100}{\text{wt. of sample}}$$

4.3.2.2. Cognate Assays

Calamine ; Ephedrine ; Lithium carbonate ; Milk of Magnesia ; Magnesium stearate ; Sodium lactate Injection.

S.No.	Name of Substance	Quantity Prescribed	Indicator Employed	Calculations
1.	Calamine	1.5 g	Methyl orange	Each ml of N. H_2S0_4 ≡ 0.04068 g of ZnO
2.	Ephedrine	0.5 g	Methyl Red	Each ml of 0.1 N HCl ≡ 0.01652 g of $C_{10}H_{15}NO$
3.	Lithium Carbonate	1.0 g	Methyl Orange	Each ml of 1 N HCl ≡ 0.03695 g of Li_2CO_3
4.	Milk of Magnesia	5.0 g	Methyl Red	Each ml of 1 N H_2SO_4 ≡ 0.02916 g of $Mg(OH)_2$
5.	Magnesium Stearate	1.0 g	Methyl Orange	Each ml of 0.1 N H_2SO_4 ≡ 0.002015 g of MgO
6.	Sodium Lactate Injection	10.0 ml	Methyl Orange	Each ml 0.1 N H_2SO_4 ≡ 0.01121 g of $C_3H_5NaO_3$

4.4. THEORY OF ALKALIMETRY

Acidic substances are usually determined quantitatively by methods similar to those used for the quantitative determinations of bases. However, *two* methods are generally adopted for the assay of acidic substances, namely :

(*a*) **Direct Titration Methods :** It is accomplished by directly titrating an exact quantity of the acid, acid salt or other acidic substance with standard alkali solutions.

(*b*) **Residual Titration Methods :** It is carried out by the addition of an excess of the standard alkali solution and subsequently determining the amount in excess by residual titration with standard acid solution.

As a general principle, the following guidelines may be observed carefully, namely :

(*i*) the normality of the solution obtained by dissolving the acidic substance must be approximately the same as that of the titrant,

(*ii*) the liquid acidic substance to be titrated must be brought to room temperature (25°C) before titration, because many indicators offer different values at different temperatures, and

(*iii*) the quantity of acid to be taken should be calculated in such a manner that approximately 30 to 40 ml of the previously standardized base shall be utilized for the assay.

Inorganic Acids—for these either methyl red or phenolphthalein may be employed as indicators and the alkali must be standardized with the particular indicator used.

Organic acids—for these phenolphthalein is invariably used, but bromothymol blue, thymol blue and thymolphthalein are also employed as per specific requirements.

Besides, the aforesaid visual methods of assay *i.e.*, observing the change in colour of indicators used, alternative instrumental methods such as : potentiometric, amperometric, polarographic, conductometric methods are also employed in determining the end-point.

4.4.1. DIRECT TITRATION METHOD (DTM)

4.4.1.1. Tartaric Acid

Materials Required : 2 g of Tartaric acid ; 1 N sodium hydroxide.

Procedure : Place 2 g of previously dried and accurately weighed sample of tartaric acid in a conical flask. Dissolve it in 40 ml of DW, add a few drops of phenolphthalein indicator and titrate with standardized 1 N sodium hydroxide. Each millilitre of 1 N sodium hydroxide is equivalent to 75.04 mg of $C_4H_6O_6$.

Equation :

$$\underset{(150.09)}{H_2C_4H_4O_6} + 2NaOH \longrightarrow Na_2C_4H_4O_6 + 2H_2O$$

From the above equation it is evident that two moles of sodium hydroxide is needed to neutralize one mole of tartaric acid, therefore, the equivalent weight of tartaric acid is 75.04 g. Hence, each millilitre of 1 N sodium hydroxide is equivalent to 0.07504 g (*i.e.*, 1 meq) of tartaric acid.

Thus, the percentage of tartaric acid present in the sample is given by :

$$\% \text{ Tartaric Acid} = \frac{ml \times l \times 0.07504 \times 100}{\text{wt. of sample}}$$

4.4.1.2. Busulphan

Materials Required : 0.25 g of Busulphan ; 0.1 N sodium hydroxide.

Procedure : Weigh accurately about 0.25 g of busulphan, add 25 ml of DW and boil gently under a reflux condenser for 30 minutes. Wash the condenser with a small quantity of DW, cool and titrate with 0.1 N sodium hydroxide using phenolphthalein solution as indicator. Each millilitre of 0.1 N sodium hydroxide is equivalent to 0.01232 g of $C_6H_{14}O_6S_2$.

Equation :

$$\underset{(246.29)}{CH_3SO_2.O(CH_2)_4O.SO_2CH_3} + 2H_2O \longrightarrow HO(CH_2)_4OH + \underset{\text{Methane sulphonic acid}}{2CH_3SO_2.OH} \quad ...(a)$$

$$CH_3SO_2OH + NaOH \longrightarrow CH_3SO_2ONa + H_2O \quad ... (b)$$

Busulphan is first hydrolyzed by refluxing it with water and two moles methanesulphonic acid (from one mole of Busulphan) thus generated, titrated with 0.1 N sodium hydroxide employing phenolphthalein as indicator. Hence, the equivalent weight of busulphan is 123.145 g. Therefore, each millilitre of 0.1 N sodium hydroxide is equivalent to 0.01232 g of busulphan.

Thus, the percentage of busulphan present in the sample may be calculated as under :

$$\% \text{ Busulphan} = \frac{ml \times 0.1 \times 0.01232 \times 100}{\text{wt. of sample}}$$

Caution : Busulphan is extremely poisonous. Great care should be taken to avoid inhaling the particles of busulphan or exposing the skin to it.

4.4.1.3. Cognate Assays

Benzoic acid ; cellulose acetate phthalate ; chlorpropamide ; ibuprofen ; indomethacin ; nicotinic acid ; oxyphenbutazone ; phosphoric acid ; phenylbutazone and salicylic acid.

S. No.	Name of Substance	Quantity Prescribed	Indicator Employed	Calculations
1.	Benzoic acid	2.5 g	Phenolphthalein	Each ml of 0.5 N sodium hydroxide ≡ 0.06106 g of $C_7H_6O_2$.
2.	Cellulose Acetate Phthalate (For phthalyl groups)	0.4 g	-do-	Phthalyl groups : $\frac{1.49b}{w} - 1.795$ S% where, b = ml of 0.1 N NaOH used, w = wt. in g of the sample, calcd. with reference to the anhyd. substance, S = %age of Free Acid.
3.	Chlorpropamide	0.5 g	-do-	Each ml of 0.1 N sodium hydroxide ≡ 0.02767 g of $C_{10}H_{13}ClN_2O_3S$
4.	Citric Acid	3.0 g	-do-	Each ml of 1 N NaOH ≡ 0.06404 g of $C_6H_8O_7$
5.	Frusemide	0.5 g	Bromothymol Blue	Each ml of 0.1 N NaOH ≡ 0.03308 g of$C_{12}H_{11}ClN_2O_5S$
6.	Glibenclamide	0.5 g	Phenolphthalein	Each ml of 0.1 N NaOH ≡ 0.0494 g $C_{23}H_{28}ClN_3O_5S$
7.	Ibuprofen	0.5 g	-do-	Each ml of 0.1 N NaOH ≡ 0.02063 g of $C_{13}H_{18}O_2$
8.	Indomethacin	0.45 g	-do-	Each ml of 0.1 N NaOH ≡ 0.03578 g of $C_{19}H_{16}ClNO_4$
9.	Nicotinic Acid	0.3 g	Phenol Red	Each ml of 0.1 N NaOH ≡ 0.01231 g of $C_6H_5NO_2$
10.	Oxyphenbutazone	0.5 g	Bromothymol Blue	Each ml of 0.1 N NaOH ≡ 0.03244 g of $C_{19}H_{20}N_2O_3$
11.	Phosphoric Acid	1.0 g	Phenolphthalein	Each ml of 1 N NaOH ≡ 0.049 g of H_3PO_4
12.	Phenylbutazone*	0.5 g	Bromothymol Blue	Each ml of 0.1 N NaOH ≡ 0.03084 g of $C_{19}H_{20}N_2O_2$
13.	Salicylic Acid	2.0 g	Phenol Red	Each ml of 0.5 N NaOH ≡ 0.06905 g of $C_7H_6O_3$

*It is almost insolubie in water, hence aqueous acetone is employed as the solvent which helps in reducing the apparent pKa of the acid.

4.4.2. RESIDUAL TITRATION METHODS (RTM)

This method is mostly applicable to *official compounds* belonging to the class of esters, acid anhydrides, aldehydes and acid chlorides. In practice this method applies to such substances that normally react too slowly with the titrant because of their poor solubility which may be accomplished either by a heating process or by a precipitation method so as to convert the substance capable for reaction with the standard base.

4.4.2.1. Aspirin Tablets

Materials Required : 20 Aspirin Tablets ; 0.5 N sodium hydroxide ; 0.5 N HCl.

Procedure : Weigh and powder 20 tablets. Accurately weigh a quantity of the powder equivalent to about 0.5 g of aspirin, add 30.0 ml of 0.5 N sodium hydroxide boil gently for 10 minutes and titrate with 0.5 N hydrochloric acid using phenol red solution as an indicator. Repeat the operation without the substance being examined, the difference between the titrations represents the amount of 0.5 N sodium hydroxide required by the aspirin. Each ml of 0.5 N sodium hydroxide is equivalent to 0.04504 g of $C_9H_8O_4$.

Equations : The aspirin is titrated with sodium hydroxide so as to neutralize any free acid formed by hydrolysis of the acetylsalicylic acid as shown by the following equation :

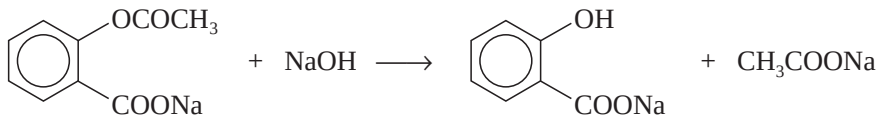
$$C_6H_4OCOCH_3COOH + HOH \longrightarrow C_6H_4OHCOOH + CH_3COOH \quad ...(a)$$

The carbonyl group present in acetylsalicylic acid is subsequently neutralized with NaOH to yield :

$$C_6H_4OCOCH_3COOH + NaOH \longrightarrow C_6H_4OCOCH_3COONa + H_2O \quad ...(b)$$

sodium acetylsalicylate

Further reaction of aspirin with excess of standard NaOH added followed by heating results in the saponification of the sodium acetylsalicylate as shown below :



...(*c*)

From equations (*b*) and (*c*), we have :

$$C_6H_4OCOCH_3COOH \equiv 2NaOH \equiv 2H \equiv 2000 \text{ ml N}$$

or $$0.04504 \text{ g } C_9H_8O_4 \equiv 1 \text{ ml of 0.5 NaOH}$$

4.4.2.2. Cognate Assays

Lactic acid ; Methyl salicylate ; Nicoumalone.

S. No.	Name of Substance	Quantity Prescribed	Indicator Employed	Calculations
1.	Lactic acid	0.2 g	Phenolphthalein	Each ml of 0.1 N NaOH $\equiv$ 0.009008 g of $C_3H_6O_3$
2.	Methyl Salicylate	0.5 g	Phenol Red	Each ml of 0.1 N NaOH $\equiv$ 0.01522 g of $C_8H_8O_3$
3.	Nicoumalone	0.6 g	Bromothymol Blue	Each ml of 0.1 N NaOH $\equiv$ 0.03533 g of $C_{19}H_{15}NO$

THEORETICAL AND PRACTICAL EXERCISES

1. Describe the theory of '**Acids and Bases**' with respect to the following aspects :

(*a*) Lowry-Bronsted's Theory

(*b*) Lewi's Theory

(*c*) Usanovich Theory

(*d*) Lux-Flood Concept.

2. What do you understand by '**direct titration method**' in the context of Aqueous Titrations ? Discuss in details the procedure involved in the assay of :

(*a*) Sodium carbonate

(*b*) Sodium salicylate tablets.

3. "**Residual titration method** is an alternative means of assay of '*drugs*' by "Aqueous Titrations". Justify the statement with the help of assay of the following pharmaceutical substances :

(*a*) Zine Oxide

(*b*) Milk of Magnesia.

4. (*a*) Discuss the various means of assay of '*drug substances*' by DTM (*i.e.,* Direct tiration methods) and RTM (*i.e.,* Residual titration methods).

(*b*) Elaborate the procedures involved in the assay of :

(*i*) Ibuprofen,

(*ii*) Chlorpropamide,

(*iii*) Busulphan.

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5 NON-AQUEOUS TITRATIONS

CONTAINS :

5.1. INTRODUCTION

During the past four decades a plethora of newer complex organic medicinal compounds have taken cognizance in the therapeutic armamentarium. Evidently, these compounds posed *two* vital problems of quality control, both in pure and dosage forms by virtue of their inherent characteristics, namely :

(*a*) poor solubility, and

(*b*) weak reactivity in aqueous medium.

Initially, the above *two* problems were usually circumvented in the following manner :

Example 1 : **Amine salts**—It is first changed to the water-soluble free base, extracted with an appropriate organic solvent and treated with an excess volume of standard acid ; subsequently, the solvent was evaporated, and the remaining acid determined with a standard base.

Example 2 : **Sodium salts**—It is first acidified to release the water-insoluble organic acid, extracted with a suitable organic solvent, the solvent was removed and the residue was subsequently dried and weighed.

Example 3 : **Nitrogen containing compounds**—They are estimated by micro Kjeldahl's Method.

Nevertheless, such specific quantitative methods gave rise to certain serious anomalies and drawbacks.

In order to overcome these shortcomings the non-aqueous titrations were introduced.

Non-aqueous titrations have the following **advantages**, namely :

- Elimination of poor solubility of substances,
- Enhancement of weak reactivity of substances,
- Selective titration by using suitable solvent and titrant of acidic/basic components of physiologically active moiety of a salt,

- Maintenance of speed, precision, accuracy and simplicity at par with classical methods of analysis, and
- Weak bases which have K*b* values less than 10^{-6} can be titrated satisfactorily by non-aqueous titrations. The reason being that in aqueous medium and at higher K*b* values ($> 10^{-6}$) the solvent water competes progressively with the basic species in solution for the proton of the solvent.

5.2. THEORY

The concepts of the Lowry-Bronsted theory may explain the various reactions that take place during many non-aqueous titrations. Thus, an *acid* is a **proton** donor and a *base* is a **proton acceptor**. Therefore, when an acid HA undergoes dissociation it gives rise to a proton and the conjugate base A of the acid :

$$\underset{\text{Acid}}{HA} \rightleftharpoons \underset{\text{Proton}}{H^+} + \underset{\text{Base}}{A^-}$$

In other words, the liberated base A shall unite with a proton to give the corresponding conjugate acid HA of the base A because every base has its conjugate acid and *vice versa.*

Hence, from the above definitions it may be implied that :

(*a*) **an acid :** could be either an electrically neutral molecule *e.g.*, HNO_3 ; or a negatively charged anion *e.g.*, HSO_4^- ; or a positively charged cation *e.g.*, $C_6H_5NH_2^+$, H_3O ;

(*b*) **a base :** could be either an electrically neutral molecule *e.g.*, $C_6H_5NH_2$; or an anion *e.g.*, Cl^-, NO_3^-.

5.2.1. SOLVENTS

These are of *three* types and they will be discussed briefly here :

(*a*) **Protophillic Solvents :** They are essentially basic in nature and normally react with acids to form solvated protons :

Example :

$$\underset{\text{Acid}}{HA} + \underset{\substack{\text{Basic}\\ \text{solvent}}}{\text{Sol.}} \rightleftharpoons \underset{\substack{\text{Solvated}\\ \text{proton}}}{\text{Sol. } H^+} + \underset{\substack{\text{Conjugate}\\ \text{base of acid}}}{A^-}$$

Perchloric acid displays more strongly acidic characteristics than a weak acid, for instance : acetic acid when dissolved in a weakly basic solvent.

(*b*) **Protogenic Solvents :** They are acidic in nature and character *e.g.*, sulphuric acid. They exert a '*levelling effect*' on bases *i.e.*, they become indistinguishable in strength when dissolved in strongly basic solvents due to their enhanced affinity of strong bases for protons.

(*c*) **Amphiprotic Solvents :** They possess both *protophillic* and *protogenic* characteristics.

Examples : Acetic acid, water and alcohols.

They undergo dissociation to a very less extent. Acetic acid is mostly employed as a solvent for the titration of basic substances and its dissociation can be depicted as shown below :

$$CH_3COOH \rightleftharpoons H^+ + CH_3COO^-$$

In the above instance acetic acid is behaving as an acid.

Perchloric Acid : It is a very strong acid and when it is made to dissolve in acetic acid, the latter can behave as a base and forms an '**onium ion**' after combining with protons donated by the perchloric acid. Thus, we have :

$$HClO_4 \rightleftharpoons H^+ + ClO_4^-$$
$$CH_3COOH + H^+ \rightleftharpoons CH_3COOH_2^+$$
Onium ion

As the $CH_3COOH_2^+$ ion can instantly donate its proton to a base, therefore, a solution of perchloric acid in glacial acetic acid, behaves as a strongly acidic solution.

Pyridine, a weak base, when dissolved in acetic acid, the latter exerts its *levelling effect* and subsequently increases the basic characteristics of the pyridine. Therefore, it is practically feasible to titrate a solution of a weak base in acetic acid against a mixture of perchloric acid in acetic acid. Thus, a sharp end point is achieved which otherwise cannot be obtained when the titration is performed in an aqueous medium.

The various reactions with perchloric acid, acetic acid and pyridine are summarized below :

$$HClO_4 + CH_3COOH \rightleftharpoons CH_3COOH_2^+ + ClO_4^-$$
$$C_6H_5N + CH_3COOH \rightleftharpoons C_6H_5NH^+ + CH_3COO^-$$
$$CH_3COOH_2^+ + CH_3COO^- \rightleftharpoons 2CH_3COOH$$

Summing up : $HClO_4 + C_6H_5N \rightleftharpoons C_6H_5NH^+ + ClO_4^-$

Acetonitrile, acetone and dimethylformamide—these non-aqueous solvents exert a greater differential in the protophillic properties of many substances than in the corresponding aqueous solutions, due to the levelling effect of water in the latter solutions. Hence, the most acidic substance in aqueous solutions of a number of acids is the formation of the hydronium ion as shown below :

$$HB + H_2O \longrightarrow H_3O^+ + B^-$$
Acid — Hydronium ion

It is pertinent to observe here that the following inorganic acids almost exhibit equal strength in aqueous solutions, whereas in non-aqueous solvents, their '*acidity*' retards in the following order :

$$HClO_4 > HBr > H_2SO_4 > HCl > HNO_3$$

In glacial acetic acid (an acidic solvent) and in dioxane (a neutral solvent), the perchloric acid ($HClO_4$) behaves as more acidic (*i.e.*, less protophyllic) than HCl; and, therefore, many base-hydrochlorides (*i.e.*, chlorides) may be titrated with standard $HClO_4$, just as carbonates may be titrated in aqueous solution with standard HCl.

In short, it is possible to titrate mixtures of two or three components selectively with a single titration by wisdom of the right choice of solvent for the non-aqueous titrations.

5.3. METHODOLOGY

For non-aqueous titrations, the following *four* steps are usually taken into consideration, namely :

(*i*) Preparation of 0.1 N Perchloric acid,

(*ii*) Standardization of 0.1 N Perchloric Acid,

(*iii*) Choice of Indicators, and

(*iv*) Effect of Temperature on Assays.

5.3.1. PREPARATION OF 0.1 N PERCHLORIC ACID

Materials Required : 8.5 ml of perchloric acid (70.0 to 72.0%) ; 1 Litre of glacial acetic acid ; 30 ml of acetic anhydride.

Procedure : Gradually mix 8.5 ml of perchloric acid to 900 ml of glacial acetic acid with vigorous and continuous stirring. Now add 30 ml acetic anhydride and make up the volume to 1 litre with glacial acetic acid and allow to stand for 24 hours before use.

The acetic anhydride reacts with the water (approx. 30%) in perchloric acid and some traces in glacial acetic acid thereby making the resulting mixture practically anhydrous. Thus, we have :

$$\underset{}{H_2O} + \underset{\text{Acetic anhydride}}{(CH_3CO)_2O} \longrightarrow \underset{\text{Acetic acid}}{2CH_3COOH}$$

Precautions : The following precautions must be observed :

(*a*) Perchloric acid is usually available as a 70 to 72% mixture with water (sp. gr. 1.6). It usually undergoes a spontaneous explosive decomposition and, therefore, it is available always in the form of a solution.

(*b*) Conversion of acetic anhydride to acetic acid requires 40-45 minutes for its completion. It being an exothermic reaction, the solution must be allowed to cool to room temperature before adding glacial acetic acid to volume,

(*c*) Avoid adding an excess of acetic anhydride especially when primary and secondary amines are to be assayed, because these may be converted rapidly to their corresponding acetylated non-basic products :

$$\underset{\text{Primary amine}}{R—NH_2} + (CH_3CO)_2O \longrightarrow \underset{\text{Acetylated product}}{R.NH.(CH_3CO)} + CH_3COOH$$

(*d*) Perchloric acid is not only a powerful oxidising agent but also a strong acid. Hence, it must be handled very carefully.

Perchloric acid has a molecular weight of 100.46 and 1 L of 0.1 N solution shall contain 1 /10th the equivalent weight or 10.046 g. To prepare 1 L of standard perchloric acid solution, it requires 8.5 ml (sp. gr. 1.6) volume and a purity of 72% which will calculate out as 9.792 g of $HClO_4$.

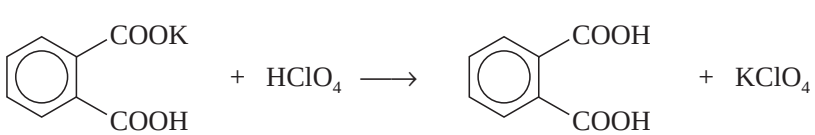
5.3.2. STANDARDIZATION OF 0.1 N PERCHLORIC ACID

Alkaline earth (*e.g.*, Mg, Ca, Ba), and alkali (*e.g.*, Na, K, Rb), salts of organic acids behave as bases in acetic acid solution :

$$R\,COOM \rightleftharpoons R\,COO^- + M^+$$

$$\underset{\text{Onium ion}}{CH_3COOH_2^+} + RCOO^- \rightleftharpoons R\,COOH + \underset{\text{Acetic acid}}{CH_3COOH}$$

In usual practice, potassium hydrogen phthalate (or potassium biphthalate, $KHC_8H_4O_4$) is employed as a standardizing agent for acetous perchloric acid. The reaction may be expressed as follows :



Potassium hydrogen phthalate — Phthalic acid

Therefore, 204.14 g $C_8H_5O_4K \equiv HClO_4 \equiv 1000$ ml N

or 0.02041 g of $C_8H_5O_4K \equiv 1$ ml of 0.1 N $HClO_4$

Procedure : Weigh accurately about 0.5 g of potassium hydrogen phthalate in a 100 ml conical flask. Add 25 ml of glacial acetic acid and attach a reflux condenser fitted with a silica-gel drying tube. Warm until

the salt gets dissolved completely. Cool and titrate with 0.1 N perchloric acid by making use of either of the following *two* indicators :

(*a*) acetous crystal violet-2 drops, end point Blue to Blue-Green (0.5% w/v)

(*b*) acetous oracet blue B-2 drops, end point Blue to Pink.

5.3.3. CHOICE OF INDICATORS

A number of indicators stated below are commonly used in non-aqueous titrations. It is, however, necessary to mention here that the same indicator must be used throughout for carrying out the standardization, titration and neutralization of mercuric acetate solution.

S.No.	Name of Indicator	Colour-change observed		Acidic
		Basic	Neutral	
1.	Crystal violet (0.5% w/v in glacial acetic acid)	Violet	Blue-green	Yellowish green
2.	Oracet Blue B (0.5% in glacial acetic acid)	Blue	Purple	Pink
3.	α-Naphtholbenzein (0.2% in glacial acetic acid)	Blue or blue green	Orange	Dark-green
4.	Quinalidine Red (0.1% in methanol)	Magenta	—	Almost colourless

5.3.4. EFFECT OF TEMPERATURE ON ASSAYS

Generally, most non-aqueous solvents possess greater coeffcients of expansion as compared to water, which is why small differences in temperature may afford significant and appreciable errors that can be eliminated by the application of appropriate correction factors. Hence, it is always advisable to carry out standardization and titration preferably at the same temperature. In a situation where these temperature parameters cannot be achieved, the volume of titrant may be corrected by the application of the following formula :

$$V_c = V\,[1 + 0.001\,(t_1 + t_2)]$$

where, V_c = Corrected volume of titrant,

V = Volume of titrant measured,

t_1 = Temperature at which titrant was standardized, and

t_2 = Temperature at which titration was performed.

5.4. ASSAY BY NON-AQUEOUS TITRATIONS

Assays of various pharmaceutical substances either in pure form or in dosage form may be assayed successfully by non-aqueous titrations. For the sake of convenience these typical titrations can be categorized into *two* broad groups, namely :

(*a*) **Acidimetry in Non-aqueous Titrations—**It can be further sub-divided into *two* heads, namely :

(*i*) Titration of primary, secondary and tertiary amines, and

(*ii*) Titration of halogen acid salts of bases.

(*b*) **Alkalimetry in Non-aqueous Titrations**—*i.e.*, titration of acidic substances.

5.4.1. ACIDIMETRY IN NON-AQUEOUS TITRATIONS

In order to perform feasible titrations of weak bases, the solvent system should be selected specifically in such a fashion so as to eliminate as far as possible the competing reaction of water for the proton besides enhancing the strength of the basic species.

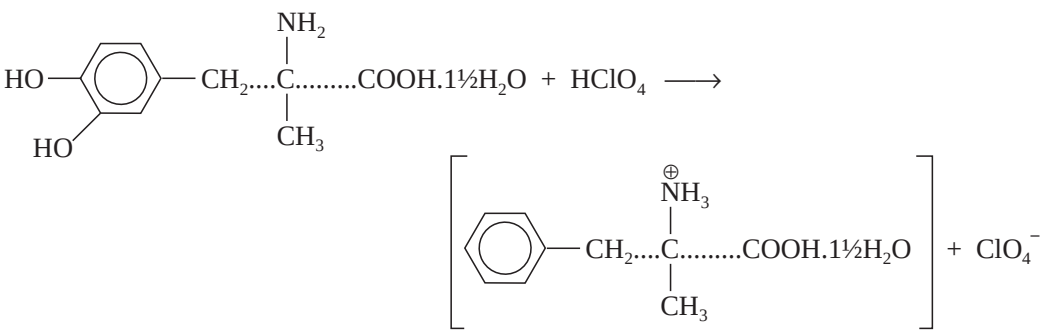
5.4.1.1. Titration of primary, secondary and tertiary amines

5.4.1.1.1. *Methlyldopa*

In general, the reaction taking place between a primary amine and perchloric acid may be expressed as follows :

$$R.NH_2 + HCl_4 \longrightarrow [R.NH_3]^+ + ClO_4^-$$

The specific reaction between methyldopa and perchloric acid is expressed by the following equation :



Hence, 211.24 g of $C_{10}H_{13}NO_4 \equiv HClO_4 \equiv H \equiv 1000$ ml N

or 0.02112 g $C_{10}H_{13}NO_4 \equiv 1$ ml of 0.1 N $HClO_4$

Materials Required : Methyldopa 0.2 g ; anhydrous formic acid : 15 ml ; glacial acetic acid : 30 ml ; dioxane : 30 ml ; 0.1 N perchloric acid and crystal violet solution.

Procedure : Weigh accurately about 0.2 g and dissolve in 15 ml of anhydrous formic acid, 30 ml of glacial acetic acid and 30 ml of dioxane. Add 0.1 ml of crystal violet solution and titrate with 0.1 N perchloric acid. Perform a blank determination and make any necessary correction. Each ml of 0.1 N perchloric acid is equivalent to 0.02112 g of $C_{10}H_{13}NO_4$.

Calculations : The percentage of methyldopa present in the sample is given by :

$$\% \text{ Methyldopa} = \frac{\text{ml} \times 0.1 \times 0.02112 \times 100}{\text{wt. of sample}}$$

5.4.1.1.2. *Methacholine Clloride*

Materials Required : Methacholine chloride : 0.4 g ; glacial acetic acid : 50 ml ; mercuric acetate solution : 10 ml ; 0.1 N perchloric acid and crystal violet solution.

Procedure : Weigh accurately about 0.4 g, previously dried and stored in a vacuum desiccator, and dissolve in 50 ml of glacial acetic acid, add 10 ml of mercuric acetate solution, one drop of crystal violet solution and titrate with 0.1 N perchloric acid to a blue-green end point. Perform a blank determination and make any necessary correction. Each ml of 0.1 N perchloric acid is equivalent to 0.01957 g of $C_8H_{18}ClNO_2$.

Equation :

$$2[CH_3COO\underset{\displaystyle CH_3}{\underset{|}{C}}HCH_2N^+(CH_3)_3]2Cl^- + Hg(OOCCH_3)_2 + 2HClO_4 \longrightarrow$$

$$2[CH_3COO\underset{\displaystyle CH_3}{\underset{|}{C}}HCH_2N^+(CH_3)_3]2ClO_4^- + 2HOOCCH_3 + HgCl_2$$

Mercuric acetate : It is essentially added to prevent the interference of the hydrochloric acid displaced through the formation of the relatively un-ionized $HgCl_2$, thereby making a predominant shift in the equilibrium so that the titrimetric reaction is quantitative.

Blank Titration : It is usually carried out to account for the possible reaction of atmospheric moisture with the titrant perchloric acid and also to check the titrant being employed to bring about the blue-green end-point.

Calculations : The percentage of methacholine chloride in the sample may be calculated by the following expression :

$$\% \text{ Methacholine chloride} = \frac{\text{ml} \times 0.1 \times 0.01957 \times 100}{\text{wt. of sample}}$$

5.4.1.1.3. *Cognate Assays*

Table 5.1, enlists the various cognate determinations using different indicators but employing the same titrant *i.e.*, 0.1 N perchloric acid.

Table 5.1 : Acidimetric Assays : Non-aqueous Titrations with Perchloric Acid using Various Indicators

S.No.	Name of Substance	Qty. Prescribed	Indicator Employed	Calculations
1.	Adrenaline	0.3 g	Crystal violet	Each ml of 0.1 N $HClO_4 \equiv 0.01832$ g of $C_{l9}H_{13}NO_3$
2.	Aminocaproic acid	0.5 g	-do-	Each ml of 0.1 N $HClO_4 \equiv 0.015120$ g of $C_6H_{13}NO_2$
3.	Bephenium hydroxynaphthoate	1.0 g	-do-	Each ml of 0.1 N $HClO_4 \equiv 0.04435$ g of $C_{28}H_{29}NO_4$
4.	Bethanidine sulphate	1.0 g	-do-	Each ml of 0.1 N $HClO_4 \equiv 0.04526$ g of $(C_{l0}H_{15}N_3)_2 \cdot H_2SO_4$
5.	Bisacodyl	0.5 g	I-Naphthol benzein	Each ml of 0.1 N $HClO_4 \equiv 0.03614$ g of $C_{22}H_{19}NO_4$
6.	Chlordiazepoxide	0.8 g	Methyl red	Each ml of 0.1 N $HClO_4 \equiv 0.02998$ g of $C_{16}H_{l4}ClN_3O$
7.	Codeine phosphate	0.4 g	Crystal violet	Each ml of 0.1 N $HClO_4 \equiv 0.03974$ g of $C_{18}H_{21}NO_3$
8.	Ergometrine maleate	0.1 g	-do-	Each ml of 0.1 N $HClO_4 \equiv 0.04415$ g of $C_{19}H_{23}N_3O_2,C_4H_4O_4$
9.	Ethambutal hydrochloride	0.2 g	-do-	Each ml of 0.1 N $HClO_4 \equiv 0.01386$ g of $C_{10}H_{24}N_2O_2 \cdot 2HCl$
10.	Guanethidine sulphate	0.4 g	-do-	Each ml of 0.1 N $HClO_4 \equiv 0.02964$ g of $C_{10}H_{22}N_4.H_2SO_4$
11.	Isoprenaline sulphate	0.4 g	-do-	Each ml of 0.1 N $HClO_4 \equiv 0.05206$ g of $(C_{11}H_{17}NO_3)_2.H_2SO_4$
12.	Levodopa	0.6 g	Oracet Blue-B	Each ml of 0.1 N $HClO_4 \equiv 0.01972$ g of $C_9H_{11}NO_4$
13.	Mepyramine maleate	0.5 g	Crystal violet	Each ml of 0.l N $HClO_4 \equiv 0.02007$ g of $C_{17}H_{23}N_3O.C_4H_4O_4$
14.	Metronidazole	0.45 g	1-Naphthol benzein	Each ml of 0.1 N $HClO_4 \equiv 0.01712$ g of $C_6H_9N_3O_3$

15.	Metronidazole benzoate	0.5 g	Brilliant green	Each ml of 0.1 N $HClO_4$ ≡ 0.02753 g of $C_{13}H_{13}N_3O_4$
16.	Nicotinamide	0.2 g	Crystal violet	Each ml of 0.1 N $HClO_4$ ≡ 0.01221 g of $C_6H_6N_2O$
17.	Nikethamide	0.2 g	-do-	Each ml of 0.1 N $HClO_4$ ≡ 0.01782 g of $C_{10}H_{14}N_2O$
18.	Noscapine	0.5 g	-do-	Each ml of 0.1 N $HClO_4$ ≡ 0.04134 g of $C_{22}H_{23}NO_7$
19.	Phenindamine tartrate	0.8 g	Oracet Blue B	Each ml of 0.1 N $HClO_4$ ≡ 0.04115 g of $C_{19}H_{19}N$, $C_4H_6O_6$
20.	Pholcodine	0.5 g	Crystal violet	Each ml of 0.1 N $HClO_4$ ≡ 0.01993 g of $C_{23}H_{30}N_2O_4$
21.	Piperazine citrate	0.2 g	-do-	Each ml of 0.1 N $HClO_4$ ≡ 0.01071 g of $(C_4H_{l0}N_2)_3$, $2C_6H_8O_7$
22.	Potassium citrate	0.15 g	1-Naphthol benzein	Each ml of 0.1 N $HClO_4$ ≡ 0.01021 g of $C_6H_5K_3O_7$
23.	Prochlorperazine maleate	0.6 g	Crystal violet	Each ml of 0.1 N $HClO_4$ ≡ 0.3030 g of $C_{20}H_{24}ClN_3S$. $2C_4H_4O_4$
24.	Prochlorperazine mesylate	0.8 g	-do-	Each ml of 0.1 N $HClO_4$ ≡ 0.02831 g of $C_{20}H_{24}ClN_3S$, $2CH_3SO_3H$
25.	Promethazine theoclate	1.0 g	Methyl orange	Each ml of 0.1 N $HClO_4$ ≡ 0.0499 g of $C_{17}H_{20}N_2S$
26.	Pyrimethamine	0.5 g	Quinaldine red	Each ml of 0.1 N $HClO_4$ ≡ 0.02487 g of $C_{12}H_{13}ClN_4$
27.	Quinidine sulphate	0.4 g	Crystal violet	Each ml of 0.1 N $HClO_4$ ≡ 0.02490 g of $(C_{20}H_{24}N_2O_2)_2.H_2SO_4$
28.	Quinine bisulphate	0.45 g	-do-	Each ml of 0.1 N $HClO_4$ ≡ 0.05486 g of $C_{20}H_{24}N_2O_2$
29.	Saccharin sodium	0.3 g	-do-	Each ml of 0.1 N $HClO_4$ ≡ 0.02052 g of $C_7H_4NNaO_3S$
30.	Salbutamol sulphate	0.9 g	Oracet Blue-B	Each ml of 0.1 N $HClO_4$ ≡ 0.05767 g of $C_{13}H_{21}NO_3.1/2H_2SO_4$
31.	Sodium acetate	0.25 g	1-Naphthol benzein	Each ml of 0.1 N $HClO_4$ ≡ 0.01361 g of $C_2H_3NaO_2$. $3H_2O$
32.	Sodium benzoate	0.6 g	Crystal violet	Each ml of 0.1 N $HClO_4$ ≡ 0.01441 g of $C_7H_5NaO_2$

5.4.1.1.4. *Potentiometric Titrations*

These non-aqueous titrations may also be carried out with the help of potentiometric titrations which technique shall be discussed at length elsewhere in this book.

It is always preferred to first ascertain the equivalence point of a given neutralization reaction potentiometrically (*i.e.*, an instrumental method of analysis) ; and secondly, by selecting an appropriate indicator that will ensure the sharpest colour change for the least increment of volume of titrant added near the equivalence point.

In actual practice, however, there are quite a number of non-aqueous titrations of pharmaceutical substances either in pure or in dosage forms that can be successfully performed potentiometrically.

Table 5.2, gives the details of such determinations at a glance :

Table 5.2 : Acidimetric Assays : Non-aqueous Titrations with Perchloric Acid using Potentiometry

S.No.	Name of Substance	Qty Prescribed	Calculations
1.	Colchicine	0.05 g	Each ml of 0.02 N $HClO_4$ ≡ 0.007988 g of $C_{22}H_{25}NO_6$
2.	Cyclizine hydrochloride	0.4 g	Each ml of 0.1 N $HClO_4$ ≡ 0.01514 g of $C_{18}H_{22}N_2$, HCl
3.	Diazepam	0.5 g	Each ml of 0.1 N $HClO_4$ ≡ 0.02847 g of $C_{16}H_{13}ClN_2O$
4.	Diphenoxylate hydrochloride	0.6 g	Each ml of 0.1 N $HClO_4$ ≡ 0.0317 g of $C_{19}H_{23}NO$. HCl
5.	Ethionamide Tablets	0.25 g	Each ml of 0.1 N $HClO_4$ ≡ 0.01662 g of $C_8H_{10}N_2S$
6.	Fenfluramine hydrochloride	0.3 g	Each ml of 0.1 N $HClO_4$ ≡ 0.02677 g of $C_{12}H_{16}F_3N$. HCl
7.	Gallamine triethiodide	0.5 g	Each ml of 0.1 N $HClO_4$ ≡ 0.02972 g of $C_{30}H_{60}I_3N_3O_3$
8.	Homatropine hydrochloride	0.3 g	Each ml of 0.1 N $HClO_4$ ≡ 0.03563 g of $C_{16}H_{21}NO_3$. HBr
9.	Hydroxyethyl theophylline	0.3 g	Each ml of 0.1 N $HClO_4$ ≡ 0.02242 g of $C_9H_{12}N_4O_3$
10.	Mebendazole	0.25 g	Each ml of 0.1 N $HClO_4$ ≡ 0.02953 g of $C_{16}H_{13}N_3O_3$
11.	Metformin hydrochloride	0.25 g	Each ml of 0. 1 N $HClO_4$ ≡ 0.008281 g of $C_4H_{11}N_5$. HCl
12.	Phenoformin hydrochloride	0.25 g	Each ml of 0.1 N $HClO_4$ ≡ 0.0120 g of $C_{10}H_{11}N_5$. HCl
13.	Phentolamine hydrochloride	0.5 g	Each ml of 0.1 N $HClO_4$ ≡ 0.03178 g of $C_{17}H_{19}N_3O$. HCl
14.	Physostigmine Injection	30 mg	Each ml of 0.1 N $HClO_4$ ≡ 0.004135 g of $C_{22}H_{27}N_3O_5$
15.	Proguanil hydrochloride	0.3 g	Each ml of 0.1 N $HClO_4$ ≡ 0.01451 g of $C_{11}H_{16}ClN_5$. HCl
16.	Propantheline bromide	0.6 g	Each ml of 0.1 N $HClO_4$ ≡ 0.04484 g of $C_{23}H_{30}BrNO_3$
17.	Scopolamine hydrobromide	0.4 g	Each ml of 0.1 N $HClO_4$ ≡ 0.03843 g of $C_{17}H_{21}NO_4$. HBr
18.	Sodium citrate	0.25 g	Each ml of 0.1 N $HClO_4$ ≡ 0.008602 g of $C_6H_5Na_3O_7$
19.	Triamterene	0.15 g	Each ml of 0.1 N $HClO_4$ ≡ 0.02533 g of $C_{12}H_{11}N_7$
20.	Trimethoprim	0.4 g	Each ml of 0.1 N $HClO_4$ ≡ 0.02903 g of $C_{14}H_{18}N_4O_3$

5.4.1.2. Titration of Halogen Acid Salts of Bases

In general, the halide ions, namely : chloride, bromide and iodide are very weakly basic in character so much so that they cannot react quantitatively with acetous perchloric acid. In order to overcome this problem, mercuric acetate is usually added (it remains undissociated in acetic acid solution) to a halide salt thereby causing the replacement of halide ion by an equivalent amount of acetate ion, which serves as a strong base in acetic acid as shown below :

$$2R.NH_2.HCl \rightleftharpoons 2RNH_3^+ + 2Cl^-$$

$$(CH_3COO)_2Hg + 2Cl^- \longrightarrow \underset{\text{undissociated}}{HgCl_2} + 2CH_3COO^- \quad (\text{undissociated})$$

$$2CH_3COOCH_2^+ + 2CH_3COO^- \rightleftharpoons 4\ CH_3COOH$$

5.4.1.2.A. Amitriptyline Hydrochloride

Materials Required : Amitriptyline hydrochloride : 1.0 g ; mercuric acetate ; crystal violet ; 0.1 N perchloric acid ; glacial acetic acid.

Procedure : Weigh accurately about 1.0 g and dissolve in 50 ml of glacial acetic acid, warming slightly, if necessary, to affect the solution. Cool, add 10 ml of mercuric acetate solution, two drops of crystal violet solution and titrate with 0.1 N perchloric acid to a green end-point. Perform a blank determination and make any necessary correction. Each ml of 0.1 N perchloric acid is equivalent to 0.03139 g of $C_{20}H_{23}N.HCl$.

Equations :

$$2C_{20}H_{23}N.HCl \rightleftharpoons 2C_{20}H_{23}N, H^+ + 2Cl^-$$

$$(CH_3COO)_2\ Hg + 2Cl^- \longrightarrow HgCl_2 + 2CH_3COO^-$$

$$2\ CH_3COOH_2^+ + 2CH_3COO^- \rightleftharpoons 4CH_3COOH$$

Calculations :

$$C_{20}H_{23}N.HCl \equiv Cl^- \equiv CH_3COO^- \equiv HClO_4 \equiv H = 1000 \text{ ml N}$$

or 313.87 g $C_{20}H_{23}N.HCl \equiv 1000$ ml $N.HClO_4$

or 0.03139 g $C_{20}H_{23}N.HCl \equiv 1$ ml of $0.1\ N.HClO_4$

5.4.1.3. Cognate Assays

The following estimations of various pharmaceutical substances can also be carried out by the aforesaid procedure (Table 5.3) :

Table 5.3 : Acidimetric Assays : Non-aqueous Titrations with Perchloric Acid using Mercuric Acetate and different Indicators

S.No.	Name of Substance	Qty. Prescribed	Indicator Employed	Calculations
1.	Amantadine hydrochloride	0.21 g	Crystal violet	Each ml of 0.1 N $HClO_4 \equiv 0.01877$ g of $C_{10}H_{17}N$. HCl
2.	Chlorpromazine hydrochloride	0.6 g	Methyl orange	Each ml of 0.1 N $HClO_4 \equiv 0.3533$ g of $C_{17}H_{19}ClN_2S$. HCl
3.	Clonidine hydrochloride	0.4 g	1-Naphthol benzein	Each ml of 0.5 N $HClO_4 \equiv 0.01333$ g of $C_9H_9Cl_2N_3$. HCl
4.	Cyproheptadiene hydrochloride	0.5 g	Crystal violet	Each ml of 0.1 N $HClO_4 \equiv 0.0323$ g of $C_{21}H_{21}N.HCl$
5.	Dehydroemetine hydrochloride	0.4 g	-do-	Each ml of 0.1 N $HClO_4 \equiv 0.02758$ g of $C_{29}H_{38}N_2O_4$. 2HCl

6.	Dequalinium chloride	0.7 g	-do-	Each ml of 0.1 N $HClO_4$ ≡ 0.02638 g of $C_{30}H_{40}Cl_2N_4$
7.	Diphenhydramine hydrochloride	0.75 g	-do-	Each ml of 0.1 N $HClO_4$ ≡ 0.02918 g of $C_{17}H_{21}NO$. HCl
8.	Ephedrine hydrochloride	0.5 g	-do-	Each ml of 0.1 N $HClO_4$ ≡ 0.02017 g of $C_{10}H_{15}NO$. HCl
9.	Ethylmorphine hydrochloride	0.3 g	-do-	Each ml of 0.1 N $HClO_4$ ≡ 0.03499 g of $C_{19}H_{23}NO_3$. HCl
10.	Fluphenazine hydrochloride	0.6 g	-do-	Each ml of 0.1 N $HClO_4$ ≡ 0.02552 g of $C_{22}H_{26}F_3N_3OS$, 2HCl
11.	Imipramine hydrochloride	0.5 g	-do-	Each ml of 0.1 N $HClO_4$ ≡ 0.03169 g of $C_{19}H_{24}N_2$. HCl
12.	Isoprenaline hydrochloride	0.5 g	Crystal violet	Each ml of 0.1 N $HClO_4$ ≡ 0.02477 g of $C_{11}H_{17}NO_3$. HCl
13.	Lignocaine hydrochloride	0.6 g	-do-	Each ml of 0.1 N $HClO_4$ ≡ 0.02708 g of $C_{14}H_{22}N_2O$. HCl
14.	Meclizine hydrochloride	0.35 g	Quinaldine Red	Each ml of 0.1 N $HClO_4$ ≡ 0.02319 g of $C_{25}H_{27}ClN_2$. 2 HCl
15.	Methadone hydrochloride	0.5 g	Crystal violet	Each ml of 0.1 N $HClO_4$ ≡ 0.03459 g of $C_{21}H_{28}ClNO$
16.	Methylamphetamine hydrochloride	0.4 g	-do-	Each ml of 0.1 N $HClO_4$ ≡ 0.01857 g of $C_{10}H_{15}N$. HCl
17.	Morphine hydrochloride	0.4 g	-do-	Each ml of 0.1 N $HClO_4$ ≡ 0.03218 g of $C_{17}H_{19}NO_3$. HCl
18.	Morphine sulphate	0.5 g	-do-	Each ml of 0.1 N $HClO_4$ ≡ 0.06688 g of $(C_{17}H_{19}NO_3)_2$. H_4SO_4
19.	Neostigmine bromide	0.75 g	-do-	Each ml of 0.1 N $HClO_4$ ≡ 0.0303 g of $C_{12}H_{19}BrN_2O_2$
20.	Oxprenolol hydrochloride	0.4 g	1-Naphthol benzein	Each ml of 0.1 N $HClO_4$ ≡ 0.3018 g of $C_{15}H_{23}NO_3$
21.	Pentazoline hydrochloride	0.65 g	Crystal violet	Each ml of 0.1 N $HClO_4$ ≡ 0.03219 g of $C_{19}H_{27}NO$. HCl
22.	Pethidine hydrochloride	0.5 g	-do-	Each ml of 0.1 N $HClO_4$ ≡ 0.02838 g of $C_{15}H_{21}NO_2$. HCl
23.	Pentobarbitone sodium	0.5 g	1-Naphthol benzein	Each ml of 0.1 N $HClO_4$ ≡ 0.02542 g of $C_{12}H_{11}N_2NaO_3$
24.	Phenylephrine hydrochloride	0.5 g	Crystal violet	Each ml of 0.1 N $HClO_4$ ≡ 0.02037 g of $C_9H_{13}NO_2$. HCl
25.	Phenytoin sodium	0.4 g	1-Naphthol benzein	Each ml of 0.1 N $HClO_4$ ≡ 0.02743 g of $C_{15}H_{11}N_2NaO_2$
26.	Promethazine hydrochloride	1.0 g	Methyl orange	Each ml of 0.1 N $HClO_4$ ≡ 0.03209 g of $C_{17}H_{20}N_2S$. HCl
27.	Propoxyphene hydrochloride	0.6 g	Crystal violet	Each ml of 0.1 N $HClO_4$ ≡ 0.03759 g of $C_{22}H_{29}NO_2$. HCl
28.	Propranolol hydrochloride	0.7 g	1-Naphthol benzein	Each ml of 0.1 N $HClO_4$ ≡ 0.02958 g of $C_{16}H_{21}NO_2$. HCl
29.	Pyridoxine hydrochloride	0.4 g	Crystal violet	Each ml of 0.1 N $HClO_4$ ≡ 0.02056 g of $C_8H_{12}ClNO_3$
30.	Succinylcholine chloride	0.5g	-do-	Each ml of 0.1 N $HClO_4$ ≡ 0.018078 g of $C_{14}H_{30}Cl_2N_2O_4$

31.	Tetramisole hydrochloride	0.5 g	1-Naphthol benzein	Each ml of 0.1 N $HClO_4$ ≡ 0.02408 g of $C_{11}H_{22}N_2S$. HCl
32.	Thiabendazole	0.16 g	Crystal violet	Each ml of 0.1 N $HClO_4$ ≡ 0.02013 g of $C_{10}H_7N_3S$
33.	Verapamil hydrochloride	0.5 g	Crystal violet	Each ml of 0.1 N $HClO_4$ ≡ 0.04911 g of $C_{27}H_{38}N_2O_4$. HCl

5.4.2. ALKALIMETRY IN NON-AQUEOUS TITRATIONS

A plethora of weakly acidic pharmaceutical substances may be titrated effectively by making use of a suitable non-aqueous solvent with a sharp end-point. The wide spectrum of such organic compounds include : anhydrides, acids, amino acids, acid halides, enols (*viz*., barbiturates), xanthines, sulphonamides, phenols, imides and lastly the organic salts of inorganic acids.

However, a weak inorganic acid *e.g.*, boric acid, can be estimated conveniently employing ethylenediamine as the non-aqueous solvent.

5.4.2.1. Preparation of 0.1 N Potassium Methoxide in Toluene-Methanol

Materials Required : Absolute methanol : 40 ml ; dry toluene : 50 ml ; potassium metal : 4 g.

Procedure : Add into a dry flask, a mixture of methanol (40 ml) and dry toluene (50 ml) and cover it loosely. Carefully add freshly cut pieces of potassium metal to the above mixture gradually with constant shaking. After complete dissolution of potassium metal, add enough absolute methanol to yield a clear solution. Toluene 50 ml is added with constant shaking until the mixture turns hazy in appearance. The process is repeated by the alternate addition of methanol and benzene until 1 litre of solution is obtained, taking care to add a minimum volume of methanol to give a visible clear solution.

5.4.2.1.1. *Preparation of 0.1 N Sodiun Methoxide*

It is prepared exactly in a similar manner as for 0.1 N Potassium Methoxide, using 2.3 g of freshly-cut sodium in place of potassium.

5.4.2.1.2. *Preparation of 0.1 N Lithium Methoxide*

It is prepared as for 0.1 N Potassium Methoxide, but using 0.7 g of lithium in place of potassium.

5.4.2.2. Standardization of 0.1 N Methoxide Solution

Materials Required : Dimethylformamide (DMF) : 10 ml ; thymol blue (0.3% in MeOH) ; 0.1 N lithium methoxide in toluene-methanol ; benzoic acid : 0.6 g.

Procedure : The apparatus shown in Figure 5.1, is employed for the standardization of 0.1 N methoxide solution. Transfer 10 ml of DMF in a conical flask and add to it 3 to 4 drops of thymol blue and first neutralize the acidic impurities present in DMF by titrating with 0.1 N lithium methoxide in toluene-methanol. Quickly introduce 0.06 g of benzoic acid and titrate immediately with methoxide in toluene-methanol.

Caution : Care must be taken to avoid contamination of neutralized liquid with atmospheric carbon dioxide.

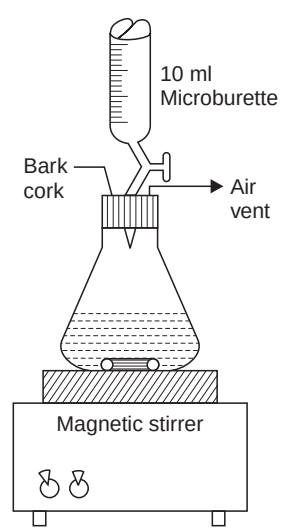


Figure 5.1 : Apparatus for Standadization of Methoxide

Equations : The various equations involved in the above operations are summarized as stated below :

(*i*) $$Na + CH_3OH \longrightarrow CH_3ONa + H\uparrow$$

Interaction between sodium metal and methanol is an exothermic reaction and hence, special care must be taken while adding the metal into the dry solvent in small lots at intervals with adequate cooling so as to keep the reaction well under control.

(*ii*) $$H_2O + CH_3ONa \longrightarrow CH_3OH + NaOH$$

$$H_2CO_3 + 2CH_3ONa \longrightarrow 2CH_3OH + Na_2CO_3$$

The clear solution of sodium methoxide must be kept away from moisture and atmospheric CO_2 as far as possible so as to avoid the above two chemical reactions that might ultimately result into the formation of turbidity.

(*iii*) $$C_6H_5COOH + H{-}CON(CH_3)_2 \underset{\text{DMF}}{\rightleftharpoons} HCON^+H(CH_3)_2 + C_6H_5COO^-$$

$$CH_3ONa \rightleftharpoons CH_3O^- + Na^+$$

$$HCON^+H(CH_3)_2 + CH_3O^- \longrightarrow HCON(CH_3)_2 + CH_3OH$$

Summing up : $C_6H_5COOH + CH_3ONa \longrightarrow C_6H_5COONa + CH_3OH$

Step 1 : It shows the solution of benzoic acid (primary standard) in DMF,

Step 2 : It depicts ionization of sodium methoxide,

Step 3 : It illustrates the interaction between the solvated proton and the methylated ion.

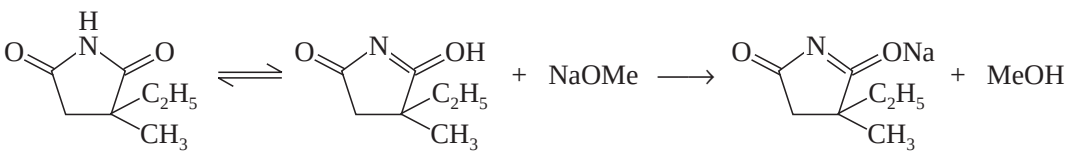
In summing up, the net reaction in the process of standardization has been expressed. The interaction between the water in the solvent (DMF) and the titrant is equivalent to the volume of sodium methoxide consumed by DMF or may be considered as a blank determination.

5.4.2.2.1. *Ethosuximide*

Materials Required : Ethosuximide : 0.2 g ; dimethylformamide : 50 ml ; azo-violet (0.1% w/v in DMF) : 2 drops ; sodium methoxide 0.1 N.

Procedure : Weigh accurately about 0.2 g, dissolve in 50 ml of dimethylformamide, add 2 drops of azo-violet solution and tirate with 0.1 N sodium methoxide to a deep blue end point, taking precautions to prevent absorption of atmospheric carbon dioxide. Perform a blank determination and make any necessary correction. Each ml of 0.1 N sodium methoxide is equivalent to 0.01412 g of $C_7H_{11}NO_2$.

Equations :



Ethosuximide (*keto*-form) | Ethosuximide (*enol*-form) | Sodium salt of Ethosuximide

Calculations :

Therefore, 141.17 g $C_7H_{l1}NO_2 \equiv$ NaOMe $\equiv$ H $\equiv$ 1000 ml N

or 0.01417 g $C_7H_{11}NO_2 \equiv$ 1 ml 0.1 N NaOMe

5.4.2.3. Cognate Assays

The following determinations as stated in Table 5.4 may be carried out effectively by using 0.1 N sodium hydroxide either titrimetrically using an appropriate indicator or potentiometrically :

Table 5.4 : Alkalimetric Assays : Non-Aqueous Titrations using Lithium Methoxide/Sodium Methoxide either Potentiometrically or Titrimetrically

S.No.	Name of Substance	Qty. Prescribed	Indicator Employed	Calculations
l.	Acetazolamide	0.4 g	*	Each ml of 0.1 N $NaOCH_3$ ≡ 0.02222 g of $C_4H_6N_4O_3S_2$
2.	Bendrofluazide	0.2 g	Azo violet	Each ml of 0.1 N $NaOCH_3$ ≡ 0.02107 g of $C_{15}H_{14}F_3N_3O_4S_2$
3.	Allopurinol	0.2 g	Thymol blue	Each ml of 0.1 N $NaOCH_3$ ≡ 0.01361 g of $C_5H_4N_4O$
4.	Mercaptopurine	0.3 g	-do-	Each ml of 0.1 N $NaOCH_3$ ≡ 0.01522 g of $C_5H_4N_4S$
5.	Amylobarbitone	0.5 g	Quinaldine Red	Each ml of 0.1 N $LiOCH_3$ ≡ 0.02263 g of $C_{11}H_{18}N_2O_3$
6.	Nalidixic Acid	0.25 g	Thymolph-thalein	Each ml of 0.1 N $LiOCH_3$ ≡ 0.02322 g of $C_{12}H_{12}N_2O_3$

* Potentiometric determination.

5.4.2.4. Tetrabutylammonium Hydroxide

The alkalimetry in non-aqueous titrations may also be carried out efficiently by using tetrabutylammonium hydroxide along with an appropriate indicatior.

5.4.2.4.1. *Preparation of 0.1 N Tetrabutylammonium Hydroxide in Toluene-Methanol*

Materials Required : Tetrabutylammonium iodide : 40 g ; absolute methanol : 90 ml ; silver oxide : 25 g ; dry toluene : 150 ml.

Procedure : Carefully dissolve 40 g of tetrabutylammonium iodide (Bu_4NI) in 90 ml of absolute methanol, add to it 20 g of finely powdered purified silver oxide and finally shake the mixture thoroughly for 1 hour. Centrifuge about 2-3 ml of the resultant mixture and test for iodide in the supernatant liquid. In case, it gives a positive test, add about 2 g more of silver oxide and shake for an additional period of 30 minutes. The said method may be repeated until the supernatant liquid obtained is completely free from iodide. The mixture thus obtained is filtered through a fine sintered glass filter and finally rinse the container with 3 portions, each of 50 ml of dry toluene. These washings may be added to the filtrate and the final volume is made upto 1 litre with dry toluene. The clear solution may be flushed with CO_2-free nitrogen for at least five minutes and duly protected from both CO_2 and moisture during storage.

Equation :

$$\underset{\text{Tetrabutyl-ammonium bromide}}{2Bu_4NI} + Ag_2O + H_2O \longrightarrow \underset{\text{Tetrabutyl ammonium hydroxide}}{2Bu_4NOH} + 2AgI$$

5.4.2.4.2. Standardization of 0.1 N Tetrabutylammonium Hydroxide

Materials Required : Benzoic acid : 60 mg ; dimethylbromide : 10 ml ; thymol blue solution (0.3% w/v in methanol) ; 0.1 N tetrabutylammonium hydroxide.

Procedure : Accurately weigh about 60 mg of benzoic acid into 10 ml of previously neutralized dimethyl formamide to the blue colour of thymol blue (3 drops) by titration against 0.1 N tetrabutylammonium

hydroxide. Allow the benzoic acid to dissolve gradually and completely and titrate with 0.1 N tetrabutylammonium hydroxide preferably in an atmosphere of CO_2-free nitroaen.

Calculations :

Therefore, $C_6H_5COOH \equiv H \equiv 1000$ ml N

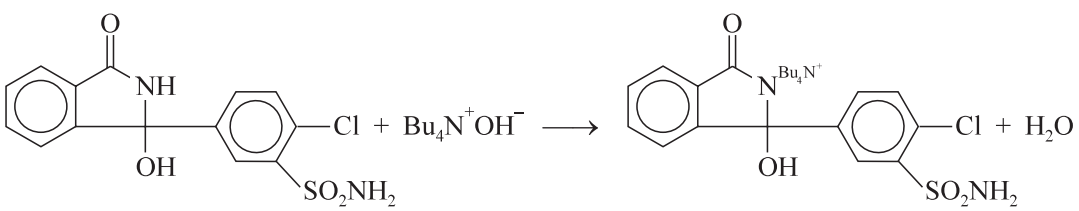
or 0.01221 g $C_7H_6O_2 \equiv 1$ ml of 0.1 N

5.4.2.4.3. *Chlorthalidone*

Materials Required : Chlorthalidone : 0.3 g ; pyridine (dehydrated) : 50 ml ; 0.1 N tetrabutylammonium hydroxide.

Procedure : Weigh accurately about 0.3 g and dissolve in 50 ml of dehydrated pyridine. Titrate with 0.1 N tetrabutylammonium hydroxide, determining the end point potentiometrically and protecting the solution and titrant from atmospheric carbon dioxide throughout the determination. Perform a blank determination and make any necessary correction. Each ml of 0.1 N tetrabutylammonium hydroxide is equivalent to 0.03388 g of $C_{l4}H_{11}ClN_2O_4S$.

Equations :



Calculations :

Therefore, $C_{14}H_{11}ClN_2O_4S \equiv Bu_4N^+OH^- \equiv H \equiv 1000$ ml N

or 338.76 g $C_{14}H_{11}ClN_2O_4S \equiv 1000$ ml N

or 0.0338 g $C_{14}H_{11}ClN_2O_4S \equiv 1$ ml 0.1 N

5.4.2.4.4. *Cognate Assays*

The following pharmaceutical substances may be assayed by employing tetrabutylammonium hydroxide either by using a suitable indicator titrimetrically or potentiometrically as given in Table 5.5.

Table 5.5 : Alkalimetric Assays : Non-Aqueous Titrations using Tetrabutyl-ammonium Hydroxide either Titrimetrically or Potentiometrically

S.No.	Name of Substance	Qty. Prescribed	Indicator Employed	Calculations
1.	Diloxanide Furoate	0.3 g	Potentiometric determination	Each ml of 0.1 N Tetrabutylammonium hydroxide ≡ 0.03282 g of $C_{14}H_{1l}Cl_2NO_4$
2.	Fluorouracil	0.4 g	Thymol blue	Each ml of 0.1 N $Bu_4NOH \equiv 0.01301$ g of $C_4H_3FN_2O_2$
3.	Hydrochlorothiazide	0.3 g	Potentiometric determination	Each ml of 0.1 N $Bu_4NOH \equiv 0.01489$ g of $C_7H_8ClN_3O_4S_2$
4.	Niclosamide	0.3 g	-do-	Each ml of 0.1 N $Bu_4NOH \equiv 0.03271$ g of $C_{l3}H_8Cl_2N_2O_4$

The assay of the aforesaid pharmaceutical substances with tetrabutylammonium hydroxide is on a mole-for-mole basis. As these are monobasic acids in character, therefore, they react quantitatively in a non-aqueous media with the base titrant, employing typical acid-base indicators to detect the end-points.

THEORETICAL AND PRACTICAL EXERCISES

1. (*a*) What is the importance of non-aqueous titrations in pharmaceutical analysis ?

(*b*) How does acetic acid present in acetons-perchloric acid behave as a base ? Give equations to support your answer.

(*c*) What do you understand by '**levelling effect**' ? How does '**pyridine**'–a *weak base* behaves as a *strong base* in acetons-perchloric acid ? Explain the above with the help of chemical reactions involved.

2. (*a*) How would you prepare 1 L of 0.1 M $HClO_4$ solution ? Why is it advised to keep the solution overnight before carrying out the actual assay with it ? Explain.

(*b*) How one would standardised the 0.1 M $HClO_4$? Explain with chemical reactions involved.

(*c*) Name any **three** indicators that are used in non-aqueous titrations with their apparent colour-change at the equivalent point.

(*d*) How does temperature effect the non-aqueous titration ? Explain.

3. Based on '**acidimetry in non-aqueous titrations**', how do we carry out the assay of the following '**drugs**' along with their theory, procedure and calculations :

(*i*) Methyldopa ; (*ii*) Adrenaline

(*iii*) Metronitazole ; (*iv*) Salbutamol sulphate.

4. How would you perform the non-aqueous titrations of pure drugs or their dosage forms petentiometrically ? Explain in details the acidimetric assays of the following '**drugs**' :

(*i*) Diazepam (*ii*) Mebendazole

(*iii*) Physostigmine Injection (*iv*) Trimethoprim.

5. (*a*) Why do we use '**Mercuric Acetate**' in the assay of halogen acid salts of bases ? Explain with suitable examples.

(*b*) Explain in details the assay of the following drugs :

(*i*) Amantadine Hydrochloride (*ii*) Diphenhydramine Hydrochloride

(*iii*) Morphine Sulphate (*iv*) Propranolol Hydrochloride.

6. (*a*) What is the importance of 0.1 M Na-Methoxide, or 0.1 M K-Methoxide, or 0.1 M Li-Methoxide in toluene-methanol particularly for alkalimetry in non-aqueous titrations.

(*b*) Describe the simple apparatus being used for the standardization of 0.1 M sodium-methoxide solution. Explain the various reactions involved using benzoic acid as a primary standard.

(*c*) Discuss the assay of the following drugs either potentiometrically or titrimetrically by non-aqueous titrations using Li-methoxide/Na-methoxide :

(*i*) Ethosuximide (*ii*) Acetazolamide

(*iii*) Allopurinol (*iv*) Nalidixic Acid.

7. How would your assay Niclosamide and Chlorthalidone using tetrabutyl-ammonium hydroxide either potentiometrically or titrimetrically by non-aqueous titrations.

RECOMMENDED READINGS

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B. REDOX METHODS

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6 PERMANGANATE, DICHROMATE AND CERIC SULPHATE TITRATION METHODS

CONTAINS :

6.1. INTRODUCTION

The oxidation and reduction processes essentially take place simultaneously in a reaction, thus one entity gets reduced in the process of oxidizing the second. 'Redox'—is the abbreviated form of reduction—oxidation systems. In the **oxidation—reduction methods of analysis** a change in valence of the reacting products is a must which is contrary to precipitation and neutralization methods of analysis where no change in valence occur. The major oxidizing agents normally employed in volumetric titrations include, potassium permanganate, potassium dichromate, and ceric sulphate.

6.2. THEORY

As a number of elements are capable of exhibiting more than one oxidation state, hence volumetric titration methods based on redox reactions are usually employed widely.

The phenomenon of oxidation may be explained in the following manner :

(*i*) addition of oxygen :

Example : $SO_2 + O \longrightarrow SO_3$

(*ii*) removal of hydrogen :

Example : $H_2S + O \longrightarrow H_2O + S$

(*iii*) enhancement in the ratio of electronegative to the electropositive portion of the molecule :

Examples :

(1) $Sn^{2+} + 2Cl^- + 2HgCl_2 \longrightarrow Sn^{4+} + 4Cl^- + Hg_2Cl_2$

(2) $2Fe^{2+} + 4Cl^- + Cl_2 \longrightarrow 2Fe^{3+}\ 6Cl^-$

In the same vein, the process of reduction may also be explained as stated below :

(*i*) addition of hydrogen :

Example : $C_2H_4 + 2H \longrightarrow C_2H_6$

(*ii*) removal of oxygen :

Example : $CuO + 2H \longrightarrow Cu + H_2O$

(*iii*) enhancement in the ratio of electropositive to electronegative portion of the molecule :

Example : [same as under oxidation (*iii*) above]

It is quite evident from the above cited examples that reduction need not always imply a reaction involving hydrogen, since $HgCl_2$ is reduced to Hg_2Cl_2, and that oxidation may not essentially suggest a reaction involving oxygen, since Fe^{2+} is oxidized by Cl_2 to Fe^{3+}. It is, therefore, pertinent to observe here that whenever one entity undergoes oxidation, definitely some other entity undergoes reduction correspondingly and *vice-versa*. In other words, there always exists a transfer of electrons in oxidation-reduction reactions, because in every such reaction the charge gained or lost by one substance must essentially be lost or gained by another.

A **reducing agent** is the reactant that loses electrons in an oxidation-reduction reaction :

$$Fe^{2+} \longrightarrow Fe^{3+} + e$$

$$Ce^{3+} \longrightarrow Ce^{4+} + e$$

Thus, the reactant containing a constituent atom or atoms are converted to a higher state of oxidation.

An oxidizing agent is the reactant that gains electrons in an oxidation-reduction reaction :

$$Ce^{4+} + e^- \longrightarrow Ce^{3+}$$

$$Fe^{3+} + e^- \longrightarrow Fe^{2+}$$

Thus, the reactant containing a constituent atom or atoms are converted to a lower state of oxidation.

The quantitative measurement of one of the reactants may be accomplished by the reaction derived from the combination of oxidizing and reducing agents, for instance

$$Fe^{2+} + Ce^{4+} \longrightarrow Fe^{3+} + Ce^{3+}$$

and hence, ferrous sulphate can be estimated quantitatively by its reaction with ceric sulphate.

6.3. ASSAY METHODS

The quantitative estimations of a number of pharmaceutical substances may be carried out by using a variety of potential oxidizing agents as stated below :

(*i*) **Permanganate Methods :**

(*a*) Direct Titration Methods,

(*b*) Indirect Titration Methods, and

(*c*) Residual Titration Methods.

(*ii*) **Dichromate Methods :**

Direct titrations with Potassium Dichromate.

(*iii*) **Ceric Sulphate Titration Methods :**

Direct Titrations with Ceric Sulphate

6.3.1. PERMANGANATE METHODS

The vital application of potassium permanganate as a potential oxidizing agent in an acidic medium mainly rests on the reactions designated by the following equations :

Chemically we have :

$$2KMnO_4 + 3H_2SO_4 \longrightarrow K_2SO_4 + 2MnSO_4 + 3H_2O + 5(O)$$

Ionically we have :

$$MnO_4^- + 8H^+ + 5e \longrightarrow Mn^{2+} + 4\ H_2O$$

Therefore, $KMnO_4 \equiv 5e$

or 158.0 g $KMnO_4 \equiv$ 5000 ml N

or 31.60 g $KMnO_4 \equiv$ 1000 ml N

or 3.16 g $KMnO_4 \equiv$ 1000 ml 0.1 N $KMnO_4$

6.3.1.1. Preparation of 0.1 N Potassium Permanganate Solution

Materials Required : Potassium permanganate : 3.5 g.

Procedure : Weigh accurately about 3.2 g of potassium permanganate on a watch-glass. Transfer the contents to a 250 ml beaker containing cold water and stir vigorously with a glass rod to effect rapid dissolution. Decant the solution through a small plug of glass wool supported by a funnel, into a 1 litre volumetric flask thereby leaving the undissolved residues in the beaker. Add more DW to the beaker and repeat the above process till all the potassium permanganate gets dissolved. Finally make up the volume to the graduated mark and shake well so as to effect uniform mixing.

Note : (*i*) $KMnO_4$ must be weighed on a watch-glass and not on any kind of paper since cellulose fibers are corrosively attacked by it,

(*ii*) Likewise, filtration of $KMnO_4$ solution must be done though cleaned glass wool and not cotton wool, and

(*iii*) Avoid heat in the preparation of $KMnO_4$ solution because traces of grease or other possible contaminants on the glass vessels used can catalyse its decomposition.

6.3.1.2. Standardization of 0.1 N Potassium Permanganate Solution

Materials Required : Oxalic acid : 6.3 g ; sulphuric acid concentrated : 5 ml.

Theory : The standardization of potassium permanganate solution is based upon the following equations :

$$\begin{matrix} COOH \\ | \\ COOH \end{matrix} \longrightarrow 2CO_2 + 2H^+ + 2e$$

Therefore, $H_2C_2O_4.2H_2O \equiv 2e$

or 126.04 g $H_2C_2O_4.2H_2O \equiv$ 2000 ml N

or 63.02 g $H_2C_2H_2.2H_2O \equiv$ 1000 ml N

or 6.302 $H_2C_2O_2.2H_2O \equiv$ 1000 ml 0.1 N $KMnO_4$

Procedure : Weigh accurately about 6.3 g of pure oxalic acid (AnalaR-Grade) into a 1 litre volumetric flask, dissolve in suffcient DW and make up the volume upto the mark. Pipette out 25 ml of this solution, add to it 5 ml of concentrated sulphuric acid along the side of the flask, swirl the contents carefully and warm upto 70°C. Titrate this against the potassium permanganate solution from the burette till the pink colour persists for about 20 seconds.

Precautions :

(*i*) Sufficient acid must be present, otherwise formation of a brown colour during titration may be observed,

(*ii*) Similar brown colouration can also be observed by using too high a temperature or by using a dirty flask, and

(*iii*) To avoid such anomalies always rinse the flask with solution of H_2O_2 and dilute H_2SO_4 before performing the titrations.

6.3.1.3. Direct Titration Methods

Hydrogen peroxide solution and potassium bromide are two pharmaceutical substances that may be estimated by employing 0.1 N potassium permanganate solution and adopting the direct titration method.

6.3.1.3.1. Hydrogen Peroxide Solution

Materials Required : Hydrogen peroxide solution : 10 ml ; 5 N sulphuric acid : 5 ml ; 0.1 N potassium permanganate.

Procedure : Dilute 10 ml of hydrogen peroxide solution to 250 ml with DW in a volumetric flask. To 25.0 ml of this solution add 5 ml of 5 N sulphuric acid and titrate with 0.1 N $KMnO_4$ to a permanent pink endpoint. Each ml of 0.1 N potassium permanganate is equivalent to 0.001701 g of H_2O_2.

Equations :

Chemically, we have :

$$2KMnO_4 + 3H_2SO_4 \longrightarrow K_2SO + 2MnSO_4 + 3H_2O + 5(O)$$

$$5H_2O_2 + 5(O) \longrightarrow 5O_2 + 5H_2O$$

Summing up : $5H_2O_2 + 2KMnO_4 + 3H_2SO_4 \longrightarrow K_2SO_4 + 2MnSO_4 + 8H_2O + 5O_2 \uparrow$

Ionically we have :

$$2MnO^-_4 + 6H^+ + 5H_2O_2 \longrightarrow 2Mn^{2+} + 8H_2O + 5O_2 \uparrow$$

Therefore, $5H_2O_2 \equiv 2MnO_4^- \equiv$ l0e

or 34.02 g $H_2O_2 \equiv 2000$ ml N

or 17.01 g $H_2O_2 \equiv 1000$ ml N

or 0.001701 g $H_2O_2 \equiv 1$ ml 0.1 N $KMnO_4$

Calculations : (For % w/v of H_2O_2)

The 'volume strength' of the hydrogen peroxide solution is the number of ml of oxygen at NTP* which may be produced by the complete thermal decomposition of 1 ml of H_2O_2 solution. Hence, decomposition takes place as designated by the following equation :

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

or 68.04 g $H_2O_2 \equiv 22400$ **ml O_2

or 1 g $H_2O_2 \equiv 329.2$ ml O_2

The IP limit of H_2O_2 solution is 5-7% w/v.

Now, let us consider a sample which contains 6.25 per cent w/v H_2O_2 :

Therefore, 100 ml sample $\equiv 6.25$ g H_2O_2

or 1 ml sample $\equiv 0.0625$ g H_2O_2

$\equiv 0.0625 \times 329.2$ ml O_2

$\equiv 20.58$ ml O_2

Hence, the volume strength of the sample is 20.58.

6.3.1.3.2. *Potassium Bromide*

Materials Required : Potassium bromide : 1.2 g ; sulphuric acid (36 N) ; 10 ml ; 0.1 N $KMnO_4$.

* NTP = Normal temperature and pressure.

** At standard temperature and pressure (STP) 1 mole of $O_2 \equiv 22.4$ L.

Procedure : Weigh accurately about 1.2 g of potassium bromide and dissolve in DW and make up the volume to 1 litre mark with water in a volumetric flask. To 10.0 ml of the solution, add 100 ml of DW and 10 ml of (36 N) sulphuric acid along the side of the flask and a few glass beads (to avoid bumping of solution). Heat to boiling and while the solution is still boiling, titrate with 0.1 N $KMnO_4$ added dropwise until the pink colour just persists. Each ml of 0.1 N $KMnO_4$ is equivalent to 0.01190 g of KBr.

Equations :

The Br^- is oxidised to bromine by acidified $KMnO_4$, thus :

$$2Br^- \longrightarrow Br_2 + 2e$$

or $2KBr \equiv 2e$

or 119 g KBr ≡ 1000 ml N $KMnO_4$

or 0.0119 g KBr ≡ 1 ml 0.1 N $KMnO_4$

6.3.1.4. Indirect Titration Methods

In the indirect method of permanganate oxidation certain compounds are first converted by means of chemical reactions to an equivalent amount of oxalate which is then subsequently oxidized quantitatively by permanganate.

6.3.1.4.1. *Assay of Cherry Juice for Malic Acid*

In this particular assay the malic acid present in the cherry juice is estimated by the following *three* steps sequentially :

Step 1 : Conversion of malic acid to an equivalent amount of calcium salt,

Step 2 : Conversion of calcium salt to corresponding insoluble calcium oxalate, and

Step 3 : Liberation of oxalate and subsequent oxidation with permanganate.

Materials Required : Cherry juice : 10 ml ; calcium carbonate : 1.0 g ; ammonia TS : 1 ml ; ammonium oxalate TS : 15 ml ; diluted ammonia (1 in 49) : 25 ml ; diluted sulphuric acid (1 in 3 ; approximately 9 N) : 30 ml ; potassium permanganate 0.1 N.

Procedure : Place 10 ml of precisely measured cherry juice in a 125 ml flask and add to it 1 g of calcium carbonate. Heat the contents on a water-bath for 15 minutes while swirling periodically and filter. Wash the filter 2 to 3 times with 5 ml portions of DW. Add to the combined filtrate and washings 1 ml of ammonia TS followed by 15 ml of ammonium oxalate TS. Warm the contents on a water-bath for 15 minutes, filter through filter paper and wash the filter with 5 ml portions of a solution previously made by mixing 1 ml of ammonia TS with 49 ml of DW. Perforate the filter paper and wash the precipitate into the same flask with hot DW and followed by 30 ml of diluted sulphuric acid. The resulting solution is heated to 80°C and finally titrated with 0.1 N $KMnO_4$. Each ml of 0.1 N $KMnO_4$ is equivalent to 6.704 g of $C_4H_6O_5$.

Equations : Malic acid first reacts with $CaCO_3$ to yield the soluble calcium malate that goes into the filtrate, whereas the insoluble calcium carbonate is filtered off and rejected. Thus,

$$\underset{\text{Malic acid}}{C_4H_6O_5} + CaCO_3 \longrightarrow \underset{\substack{\text{Calcium malate}\\\text{(soluble)}}}{CaC_4H_4O_5} + CO_2\uparrow + H_2O$$

The interaction between calcium malate and ammonium oxalate results into an equivalent quantity of calcium oxalate by displacement mechanism which is subsequently precipitated :

$$\underset{\text{Calcium malate}}{CaC_4H_4O_5} + \underset{\text{Ammonium oxalate}}{(NH_4)_2C_2O_4} \longrightarrow \underset{\text{Calcium oxalate}}{CaC_2O_4 \cdot H_2O}\downarrow + \underset{\text{Ammonium malate}}{(NH_4)_2C_4H_6O_5}$$

Calculations :

Therefore, $C_4H_6O_5 \equiv CaC_2O_4.H_2O \equiv 2000$ ml N $KMnO_4$

or 134.08 g $C_4H_6O_5 \equiv 2000$ ml N $KMnO_4$

or 67.04 g $C_4H_6O_5 \equiv 1000$ ml N $KMnO_4$

or 0.06704 g $C_4H_6O_5 \equiv 1$ ml 0.1 N $KMnO_4$

6.3.1.5. Residual Titration Methods

The residual titration method for pharmaceutical substances using potassium permanganate solution are mainly of *two* categories, namely :

(*i*) titration wherein an excess of standard oxalic acid is added to the substance and then the excess of oxalic acid is back titrated with $KMnO_4$, and

(*ii*) titration wherein an excess of standard $KMnO_4$ solution is used to oxidize the product, and then the amount in excess is estimated by reduction with either :

(*a*) excess ferrous ammonium sulphate and back titrated with more of standard $KMnO_4$, or

(*b*) excess standard oxalic acid.

6.3.1.5A. *Assay of Sodium Nitrite*

Materials Required : Sodium nitrite : 1.0 g ; 0.1 N potassium permanganate : 50 ml ; sulphuric acid (conc.) : 5 ml ; 0.1 N oxalic acid.

Procedure : Weigh accurately about 1 g of sodium nitrite and dissolve it in DW to make 100 ml in a volumetric flask. Transfer 10 ml of this solution into a mixture of 50 ml of 0.1 N $KMnO_4$, 100 ml of water and add 5 ml of sulphuric acid along the side of the flask. Heat the contents to 40°C, allow it to stand for 5 minutes and add 25 ml of 0.1 N oxalic acid. Warm the resulting mixture to about 80°C on a steam-bath and titrate with 0.1 N $KMnO_4$ solution. Each ml of 0.1 N potassium permanganate is equivalent to 3.450 mg of $NaNO_2$.

Precautions : While adding $NaNO_2$ solution

(*i*) Care should be taken to immerse the tip of the pipette beneath the surface of the permanganate mixture, otherwise the nitrous acid (volatile) generated by $NaNO_2$ and H_2SO_4, would be lost, and

(*ii*) Oxidation of nitrous acid (HNO_2) to nitric acid (HNO_3) takes place sluggishly at ambient temperature and hence, it is necessary to warm it upto 40°C for 5 minutes to expedite completion of reaction.

Equations : Chemically we have :

$$10NaNO_2 + 4\ KMnO_4 + 11H_2SO_4 \longrightarrow 10HNO_3 + 4MnSO_4 + 2K_2SO_4 + 5Na_2SO_4 + 6H_2O$$

(69.0)

Ionically we have :

$$NO_2^- + HO \longrightarrow NO_3^- + 2H^+ + 2e$$

i.e., each molecule of sodium nitrite loses two electrons.

Calculations :

Therefore, $NaNO_2 \equiv NO_2^- \equiv 2e$

or 69.0 g $NaNO_2 \equiv 2000$ ml N

or 0.003450 g $NaNO_2 \equiv 1$ ml of 0.1 N $KMnO_4$

6.3.2. DICHROMATE METHODS

Potassium dischromate ($K_2Cr_2O_7$) is a strong oxidizing agent, quite comparable to $KMnO_4$ that normally shows only one pertinent reduced oxidation state : Thus, chemically we have :

$$K_2Cr_2O_7 + 4H_2SO_4 \longrightarrow K_2SO_4 + Cr_2(SO_4)_3 + 4H_2O + 3(O)$$

Ionically we have :

$$K_2Cr_2O_7 + 14H^+ + 6e^- \longrightarrow 2K^+ + 2Cr^{3+} + 7H_2O$$

Therefore, we have :

$$K_2Cr_2O_7 \equiv 6e$$

or $$294.0 \text{ g } K_2Cr_2O_7 \equiv 6000 \text{ ml N}$$

or $$49.0 \text{ g } K_2Cr_2O_7 \equiv 1000 \text{ ml N}$$

or $$4.90 \text{ g } K_2Cr_2O_7 \equiv 100 \text{ ml of 0.1 N } K_2Cr_2O_7$$

Potassium dichromate exhibits much greater stability in aqueous solution in comparison to potassium permanganate. Potassium dichromate possesses an inherent oranage colour that is not intense enough to serve its own end-point signal, specifically in the presence of the green Cr^{3+} ion, which is supposed to be present at the end-point. Hence, redox indicators are usually employed to locate the exact end-point *e.g.*, barium diphenylamine sulphonate.

6.3.2.1. Preparation of 0.1 N Potassium Dichromate Solution

Materials Required : Potassium dichromate : 4.930 g.

Procedure : Weigh accurately 4.93 g of potassium dichromate previously powdered and dried at 20°C for 4 hours and dissolve in sufficient DW to produce 1 litre in a volumetric flask.

Note : Potassium dichromate can be obtained as a primary standard reagent and hence, standard solutions may be prepared determinately and stored for long periods of time.

Equations : Chemically we have :

$$K_2Cr_2O_7 + 4H_2SO_4 \longrightarrow K_2SO_4 + Cr_2(SO_4)_3 + 4H_2O + 3(O)$$

Ionically we have :

$$K_2Cr_2O_7 + 14H^+ + 6e^- \longrightarrow 2K^+ + 2Cr^{3+} + 7H_2O$$

From this equation it follows that the equivalent weight of potassium dichromate is 1/6th of the molecular weight *i.e.*, 294.22/6 or 49.03 g.

6.3.2.2. Standardization of 0.1 N Potassium Dichromate Solution

It can be achieved by following these steps, namely :

(*a*) **Preparation of Standard Solution of Mohr's Salt $FeSO_4(NH_4)_2.SO_4.6H_2O$:**

Materials Required : Mohr's salt : 4.9 g ; dilute sulphuric acid (1 in 3, approx. 9 N) : 20 ml.

Procedure : Weigh accurately about 4.9 g of pure sample of Mohr's salt and transfer it to a 250 ml volumetric flask. Add 20 ml of dilute sulphuric acid and make up the volume to the mark with DW and finally mix the contents of the flask thoroughly.

Calculations : The quantity of Mohr's salt required for 250 ml of the solution having a normality of 0.05 N can be calculated as follows :

$$\text{Mohr's salt} = \frac{\text{Eq. wt. of Mohr's salt} \times \text{Volume}}{1000} \times 0.05$$

or $$= 4.9 \text{ g}$$

(*b*) **Standardization of 0.1 N $K_2Cr_2O_7$ Solution :**

Materials Required : Standard solution of Mohr's salt (0.05 N) : 250 ml, sulphuric acid (2 N) : 20 ml ; potassium dichromate solution (0.1 N) : 1 litre.

Procedure : Transfer 20 ml of the primary standard solution (Mohr's salt) to the titration flask and add 20 ml of 2 N sulphuric acid. Take the potassium dichromate solution in the burette. Put drops of freshly

prepared potassium ferricyanide, $K_3[Fe(CN)_6]$, solution in the grooves of a porcelain tile. Now, proceed with the titration of Mohr's salt solution against $K_2Cr_2O_7$ solution. Transfer drops of the titrated solution by means of a glass rod and mix with drops of the indicator, already taken in the groove-tile. Alternatively, pre-soaked and dried filter paper with $K_3[Fe(CN)_6]$ solution can also be used in place of the groove-tile method.

In order to arrive at the exact end-point the above titration may be carried out at *three* stages, namely :

Stage 1 : Spot tests are carried out at intervals of 1-2 ml until a blue colour is no longer produced with $K_3[Fe(CN)_6]$, which provides an altogether rough estimate of the $K_2Cr_2O_7$ solution required for the titration,

Stage 2 : Spot tests are only performed near the approach of the end of titration at intervals of 0.1-0.2 ml, and

Stage 3 : Spot tests are finally done only at the end-point.

The above sequential steps give fairly accurate results because the error caused by the removal of part of the solution for the spot tests is made negligibly small. However, the titration is repeated to get a set of concordant readings.

By applying the relationship between N_1V_1 ($K_2Cr_2O_7$) and N_2V_2 (Mohr's salt), the normality of the former may be calculated.

6.3.2.2.1. *Iron Ore*

Materials Required : Iron ore : 0. 1 g ; hydrochloric acid (conc.) : 15 ml ; diphenylamine (1% w/v in conc. H_2SO_4) ; zinc metal (granulated) : 4 g ; ammonium thiocyanate solution (0.1% in water) ; mixture of sulphuric acid and phosphoric acid [dissolve 15 ml of H_2SO_4 (sp. gr. 1.84) in 50 ml of DW, cool and add 15 ml of H_3PO_4 (sp. gr. 1.70) and make the volume to 100 ml with DW] : 25 ml.

Procedure :

(*a*) **Preparation of Standard $K_2Cr_2O_7$ Solution :** Instead of using solutions having definite normality, routine industrial laboratories make use of '*emperical solution*' which is normally expressed in terms of '*titer for the substance determined*'. For this assay, let us prepare an emperical $K_2Cr_2O_7$ solution (250 ml) of such a concentration that 1 ml of the same exactly correspond to 0.0025 g Fe.

Calculations :

1000 ml $K_2Cr_2O_7$ soln. ≡ 0.0025 × 1000 ≡ 2.5 g of Fe

or 250 ml $K_2Cr_2O_7$ soln. ≡ 0.6250 g Fe

By Law of Equivalence, we have :

1 gram-equivalent of $K_2Cr_2O_7$ (49.03 g) ≡ 1 gram-equivalent of Fe (55.85 g)

Hence, $$0.6250 \text{ g Fe} \equiv \frac{0.6250 \times 49.03}{55.85} \equiv 0.5488 \text{ g}$$

Therefore, weigh accurately 0.5488 g of pure $K_2Cr_2O_7$ and transfer it quantitatively into a 250 ml volumetric flask, dissolve in DW, make up the volume and mix thoroughly.

Hence, the '**iron titer**' of this solution is :

$$T\ K_2Cr_2O_7/\ Fe = 0.0025 \text{ g / ml}$$

(*b*) **Preparation of Ore Solution :** Weigh accurately 0.1 g of powdered and dried ore on a clean watch glass and transfer it quantitatively into a 100 ml-volumetric flask. Add 15 ml of concentrated hydrochloric acid, warm the contents of the flask carefully over a sand-bath until most of the dark grains of ore get dissolved completely and only a whitish silica precipitate settles at the bottom of the flask.

(*c*) **Reduction of Fe^{3+} to F^{2+} in the Ore Solution :** Introduce carefully a few pieces of granulated pure zinc metal into the flask, place a funnel in the neck of the flask to avoid splashes and boil the solution gently until the yellow colour has disappeared completely, thereby ascertaining that complete reduction of Fe^{3+} to Fe^{2+} is affected.

Note : It may be further confirmed by doing a spot test with NH_4CNS solution which only shows a blood-red colour with Fe^{3+}.

The contents of the flask is cooled, filtered through cotton wool, washings done with DW and the filtrate diluted to about 350 ml with DW. This dilution is a must so as to avoid any interference caused by its inherent green colour with the estimation of the equivalence point in the titration as per the following chemical reaction :

$$K_2Cr_2O_7 + 6FeCl_2 + 14HCl \longrightarrow 2CrCl_3 + 2KCl + 6FeCl_3 + 7H_2O$$

(*d*) **Final Titration :** The 350 ml solution obtained in (*c*) above is now quantitatively titrated against $K_2Cr_2O_7$ solution employing diphenylamine as an internal indicator. Add 25 ml of a mixture of sulphuric acid and phosphoric acid to the solution along with 2 drops of diphenylamine indicator and titrate the solution with $K_2Cr_2O_7$ solution carefully, by adding small lots at intervals with constant shaking, until a persistant blue-violet colour appears.

Note : (*a*) **The acidity of the solution must be maintained fairly high which can be achieved by adding orthophosphoric acid, H_3PO_4,**

(*b*) **The quantity of diphenylamine must not exceed 2 drops by virtue of the fact that at higher concentration with lower acidity during very slow titration, the indicator undergoes an altogether different type of chemical change that ultimately gives a green colour instead of the desired blue-violet colour.**

(*e*) **Calculations :** Multiply the number of millilitres of $K_2Cr_2O_7$ Solution consumed in the titration by the 'iron titer' and therefrom determine the amount of iron present in the sample. Finally, the percentage of iron present in the ore may be calculated.

6.3.3. CERIC SULPHATE TITRATION METHODS

Ammonium ceric sulphate serves as a powerful oxidizing agent in an acidic medium. The salt has a bright yellow colour and so its solution. On reduction, the resulting cerous salt obtained is colourless in appearance and, therefore, strong solutions may be considered as self-indicating. In general practice, 0.05 N solutions are employed invariably for estimations. As this concentration is very dilute for observation of the respective end-point, hence the inclusion of an appropriate indicator becomes necessary. The oxidation reaction involved may be expressed as follows :

$$Ce^{4+} + e \rightleftharpoons Ce^{3+}$$

It is interesting to observe that the solutions of ammoniurn ceric sulphate possess a number of advantages over permanganate and dichromate methods discussed earlier in this chapter, *viz.*,

(*i*) solutions remain fairly stable even when boiled,

(*ii*) solutions quantitatively react with either arsenite (AsO_3^{3-}) or oxalate $[(COO)_2]^{2-}$ ion, and therefore, either arsenic trioxide or sodium oxalate may be employed as a primary standard,

(*iii*) cerous ion Ce^{3+} is colourless and hence offers no interference with the indicator end-point,

(*iv*) Ce^{3+} always solely results on reduction of Ce^{4+}, whereas permanganate (MaO_4^-) can be reduced to any of several oxidation states,

(*v*) ammonium ceric sulphate unlike potassium permanganate, may be conveniently employed as an oxidizing agent in the presence of high concentrations of HCl, thereby facilitating determinations of Fe^{2+} in the presence of Cl^-, and

(*vi*) ferrous phenanthrolone ion (ferroin) has proved to be a very successful indicator in titrations with ceric salts. Thus, we have :

$$\underset{\substack{\text{Orthophenanthroline}\\\text{(colourless)}\\\text{Base}}}{C_{12}H_8N_2} + Fe^{2+} \longrightarrow \underset{\substack{\text{Ferrous complex}\\\text{(red)}}}{Fe\,(C_{12}H_8N_2)_3^{2+}} \rightleftharpoons \underset{\substack{\text{Ferric complex}\\\text{(blue)}}}{Fe\,(C_{12}H_8N_2)_3^{3+}} + e$$

Orthophenanthroline (base) dissolves rapidly in aqueous solutions of ferrous salts, thereby three moles combine with one Fe^{2+} ion to give a complex termed as '**ferroin**' having an, intense red colour. Now, any strong oxidizing agent converts the ferrous to a corresponding ferric complex having a slight blue colour.

6.3.3.1. Preparation of 0.1 N Ammonium Ceric Sulphate Solution

Materials Required : Ceric ammonium sulphate : 66 g ; sulphuric acid (conc.) : 30 ml.

Procedure : Dissolve 66 g of ceric ammonium sulphate, with the help of gentle heat, in a mixture of 30 ml of sulphuric acid and 500 ml DW. Cool, filter the solution through a fine-porosity sintered-glass crucible, dilute to 1 litre mark in a volumetric flask and mix thoroughly.

Since the oxidation reaction is given by :

$$Ce^{4+} + e \rightleftharpoons Ce^{3+}$$

Therefore, 632.57 g $Ce(SO_4)_2\ 2(NH_4)_2SO_4.2H_2O \equiv$ 1000 ml N

or 63.26 g $Ce(SO_4)_2\ 2(NH_4)_2\ SO_4.2H_2O \equiv$ 1000 ml 0.1 N ammonium ceric sulphate

6.3.3.2. Stadardization of 0.1 N Ammonium Ceric Sulphate Solution

Materials Required : Arsenic trioxide : 0.2 g ; sodium hydroxide solution (8.0% w/v) : 25 ml ; diluted sulphuric acid (10% w/v) : 30 ml ; osmic acid solution (1.0% w/v in water) : 0.15 ml ; ferroin sulphate solution (dissolve 0.7 g of ferrous sulphate in 70 ml of DW and add 1.5 g of 1, 10-phenanthroline and sufficient water to produce 100 ml) : 0.1 ml.

Procedure : Weigh accurately about 0.2 g of arsenic trioxide previously dried at 105°C for 1 hour and transfer to a 500 ml conical flask. Wash down the inner walls of the flask with 25 ml of sodium hydroxide solution, swirl to dissolve, add 100 ml of water and mix. Add 30 ml of diluted sulphuric acid, 0.15 ml of osmic acid solution, 0.1 ml of ferroin sulphate solution and slowly titrate with ceric ammonium sulphate solution until the pink colour is changed to a very pale blue. Each 4.946 mg of arsenic trioxide is equivalent to 1 ml of 0.1 N ammonium ceric sulphate or 0.06326 g of $Ce(SO_4)_2.\ 2(NH_4)_2SO_4\ .\ 2H_2O$.

Equations :

$$As_2O_3 + 6NaOH \longrightarrow 2Na_3AsO_3 + 3H_2O$$

$$2Ce(SO_4)_2 + Na_3AsO_3 + H_2O \longrightarrow Ce_2(SO_4)_3 + Na_3AsO_4 + H_2SO_4$$

It is evident from the above equations that 4 equivalents of ceric sulphate is required to oxidise 1 mole of arsenic trioxide, hence, 1 equivalent weight of arsenic trioxide is 1/4 mole or 197.84/4 or 49.46 g and 1 milliequivalent shall contain 49.46 mg or 0.04946 g.

Calculations : Therefore, the normality of ammonium ceric sulphate solution may be expressed as follows :

$$N = \frac{\text{wt. of arsenic trioxide}}{\text{ml} \times 0.04946}$$

6.3.3.2.1. *Ferrous Fumarate*

Materials Required : Ferrous fumarate : 0.3 g ; diluted H_2SO_4 (10% w/v) : 15 ml ; ferroin sulphate solution ; 0.1 N ammonium ceric sulphate solution.

Procedure : Weigh accurately about 0.3 g of ferrous fumarate and dissolve in 15 ml of dilute sulphuric acid by the help of gentle heating. Cool, add 50 ml of water and titrate immediately with 0.1 N ammonium ceric sulphate, employing ferroin sulphate solution as indicator. Each ml of 0.1 N ammonium ceric sulphate is equivalent to 0.01699 g of $C_4H_2FeO_4$.

Equations and Calculations :

$$C_4H_2FeO_4 \equiv Fe \equiv e$$

Therefore, 169.91 g $C_4H_2FeO_4 \equiv$ 1000 ml N

or 16.99 g $C_4H_2FeO_4 \equiv$ 1000 ml 0.1 N

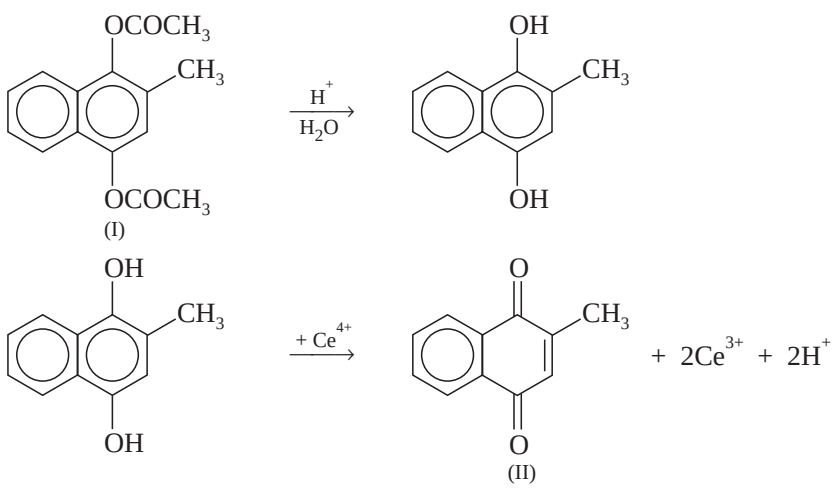
or 0.01699 g $C_4H_2FeO_4 \equiv$ 1 ml 0.1 N Ammonium ceric sulphate

6.3.3.2.2. *Acetomenaphthone*

Materials Required : Acetomenaphthone : 0.2 g ; glacial acetic acid : 15 ml ; dilute hydrochloric acid (10% w/v) : 15 ml ; ammonium ceric sulphate 0.05 N ; ferroin sulphate solution.

Procedure : Weigh accurately about 0.2 g of acetomenaphthone and boil it with 15 ml of glacial acetic acid and 15 ml of dilute hydrochloric acid under a reflux condenser for 15 minutes. Cool the contents carefully and taking adequate precautions to avoid any atmospheric oxidation. Add 0.1 ml of ferroin sulphate solution as indicator and titrate with 0.05 N ammonium ceric sulphate. Repeat the assay without the substance being examined (blank determination) and incorporate the correction, if any. Each ml of 0.05 N ammonium ceric sulphate is equivalent to 0.006457 g of $C_{15}H_{14}O_4$.

Equations :



First, acetamenaphthone (I) undergoes hydrolysis in acidic medium to yield the corresponding phenol and secondly, this phenol is oxidised quantitatively with ammonium ceric sulphate to give the resulting 1, 4-dione derivative (II).

Calculations :

Thus, we have :

$$258.3 \text{ g } C_{15}H_{14}O_4 \equiv 2Ce^{4+} \equiv 2000 \text{ ml N}$$

or 129.15 g $C_{15}H_{14}O_4 \equiv$ 1000 ml N

or 0.12915 g $C_{15}H_{14}O_4 \equiv$ 1 ml N

or 0.012915 g $C_{15}H_{14}O_4 \equiv$ 1 ml 0.1 N

or 0.006457 g $C_{15}H_{14}O_4 \equiv$ 1 ml 0.05 N Ammonium ceric sulphate

6.3.3.2.3. *Cognate Assays*

A number of pharmaceutical substances and dosage forms may be determined by the help of ceric sulphate titration methods as given in Table 6.1.

Table 6.1 : Redox Titrations : Ceric Sulphate Titration Method

S.No.	Name of Substance	Qty. Prescribed	Indicator Employed	Calculations
1.	Ferrous Gluconate	1.5 g	Ferroin sulphate	Each ml of 0.1 N ammonium ceric sulphate = 0.04461 g of $C_{12}H_{22}FeO_{14}$
2.	Ferrous sulphate	1.0 g	-do-	Each ml of 0.1 N ammonium ceric sulphate ≡ 0.0278 g of $FeSO_4 . 7H_2O$
3.	Iron Dextran Injection	2.0 g	-do-	Each ml of 0.1 N ammonium ceric sulphate ≡ 0.005585 g of Fe
4.	Menadione	1.5 g	-do-	Each ml of 0.1 N ammonium ceric sulphate ≡ 0.00861 g of $C_{11}H_8O_2$
5.	Paracetamol	0.3 g	-do-	Each ml of 0.1 N ammonium ceric sulphate ≡ 0.00756 g of $C_8H_9NO_2$
6.	Tocopherol acetate	0.3 g	Diphenyl amine	Each ml of 0.1 N ammonium ceric sulphate ≡ 0.002364 g of $C_{31}H_{52}O_3$

THEORETICAL AND PRACTICAL EXERCISES

l. Discuss the various theoretical aspects involved in the assay of **permanganate, dichromate** and **ceric sulphate** titration methods. Give equations to explain your logical stand.

2. The '**permanganate methods**' essentially consist of *three* ways to assay pharmaceutical substances :

(*i*) Direct titration method, (*ii*) Indirect titration method, and

(*iii*) Residual titration method.

Discuss any *ONE* of these methods explicitely with the help of a typical example.

3. Discuss direct titration method using '**dichromate method**' in the assay of '**Iron Ore**' with reference to the following aspects :

(*i*) Preparation of 0.1 N $K_2Cr_2O_7$ solution 1 L

(*ii*) Standardization of 0.1 N $K_2Cr_2O_7$ solution using Mohr's salt.

4. (*a*) Describe the '**direct titration with Ceric Sulphate**' and enumerate its advantages over '*permanganate*' and '*dichromate*' methods.

(*b*) How would you standardize 0.1 N ammonium-ceric sulphate solution ? Explain.

(*c*) Give the details for the assay of the following drugs by the direct titration with ceric sulphate solution :

(*i*) Ferrous Fumarate (*ii*) Iron-Dextran Injection

(*iii*) Paracetamol (*iv*) Tocophenol acctate.

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7 IODIMETRIC AND IODOMETRIC TITRATIONS

CONTAINS :

7.1. INTRODUCTION

Iodimetric and iodometric titrations constitute another class of oxidation-reduction titrations wherein either iodine solutions are employed directly for the assay or an equivalent amount of iodine is liberated indirectly from the reaction mixture and then assayed.

Iodimetry is a procedure based on the following reversible reaction :

$$2I^- \rightleftharpoons I_2 + 2e$$

Hence, it can be utilized for the quantitative estimation of reducing agents like arsenites (H_3AsO_3) and thiosulphates ($Na_2S_2O_3$) by employing a standard solution of iodine.

Iodometry is an indirect procedure based on the aforesaid reversible reaction whereby the assay of oxidizing agents, for instance : 'available chlorine' in bleaching powder, cupric and ferric salts may be carried out by reducing them with an excess potassium iodide thereby liberating an equivalent quantity of iodine which can be estimated using a standard solution of thiosulphate.

7.2. THEORY

In **iodimetry**, quantitative oxidation of reducing agents, such as arsenious acid (H_2AsO_3) may be carried out by employing standard solutions of iodine as shown under :

$$H_3AsO_3 + H_2O + I_2 \rightleftharpoons H_3AsO_4 + 2H^+ + 2I^-$$

This type of assay is known as **'direct method of iodimetry'.**

In another situation, a known excess quantity of standard iodine solution is added in the substance (a reducing agent) to be assayed and then the excess iodine may be titrated with the help of standard sodium thiosulphate solution, such as : the estimation of sodium bisulphite :

$$NaHSO_3 + I_2 + H_2O \longrightarrow NaHSO_4 + 2HI$$

$$I_2 + \underset{\text{Sodium thiosulphate}}{2Na_2S_2O_3} \longrightarrow 2NaI + \underset{\text{Sodium tetrathionate}}{Na_2S_4O_6}$$

This category of assay is termed as '**residual method of iodimetry**'.

In **iodometry**, an equivalent amount of iodine is liberated when the given sample of an oxidizing agent oxidizes potassium iodide in an acidic medium, for example : the determination of cupric sulphate ($CuSO_4$) :

$$2CuSO_4 + 4\,KI \longrightarrow 2CuI \downarrow + I_2 + 2K_2SO_4$$

Consequently, the equivalent amount of iodine generated by the above reaction may be conveniently assayed by titration against a standard sodium thiosulphate solution. In this context a point of caution must be observed while KI is being oxidized under a strongly acidic medium so as to avoid simultaneous oxidation of the iodide by atmospheric oxygen that may result high erroneous titer values leading to false estimations.

It is, however, pertinent to mention here that iodometric assays are never performed in a strongly basic medium, because of the fact that the reaction between I_2 and OH^- produces hypoiodide and iodate ions respectively as shown below :

$$I_2 + OH^- \longrightarrow HI + IO^-$$
$$3IO^- \longrightarrow IO_3^- + 2I$$

The said two ions partially oxidize thiosulphate to a higher oxidation form, such as sulphate (SO_4^{2-}) thereby the stoichiometry achieved is always false.

7.3. ASSAY METHODS

Assay methods involving iodine can be categorized under the following heads namely :

A. Iodimetric Assays :

(*a*) Direct titration with iodine,

(*b*) Residual titration method : *i.e.*, excess of iodine is titrated with sodium thiosuphate,

B. Iodometric Assays : *i.e.*, release of iodine and subsequent titration with sodium thiosulphate.

7.3.1. IODIMETRIC ASSAYS

In such estimations, the pharmaceutical substances can be measured either directly or back titration of excess iodine with sodium thiosulphate solution.

7.3.1.1. Direct Titration with Iodine

(*a*) **Preparation of 0.1 Iodine Solution**

Theory : Iodine in aqueous solution acts as an oxidizing agent which forms the basis of assay methods involving direct titration with iodine. Thus, we have :

$$I_2 + 2e \longrightarrow 2I^-$$

or $$I_2 \equiv 2e$$

or $$126.9\text{ g } I_2 \equiv 1000\text{ ml N}$$

or $$12.69\text{ g } I_2 \equiv 1000\text{ ml 0.1 N}$$

or $$3.17\text{ g } I_2 \equiv 250\text{ ml 0.1 N}$$

Materials Required : Iodine : 3.2 g ; potassium iodide : 7.5 g.

Procedure : Weigh accurately 3.2 g of crushed iodine crystals on a watch glass and transfer to a beaker containing potassium iodide (7.5 g) and water (10 ml). Dissolve the contents of the beaker with the help of a glass rod and frequent swirling. Transfer the contents of the beaker quantitatively to a 250 ml volumetric flask and make up the volume with DW.

Explanation : Iodine is sparingly soluble in water but undergoes rapid dissolution in the presence of potassium iodide due to the formation of the corresponding triiodide ion :

$$I_2 + I^- \longrightarrow I_3^-$$

Thus, potassium iodide plays dual role, *viz.*, in iodimetry—to solubilize iodine in aqueous KI solution, and in iodometry—as reducing agent, the excess KI helps in retaining liberated I_2 in solution through interaction with KI.

(*b*) **Standardization of 0.1 Iodine Solution with the aid of Arsenic Trioxide (As_2O_3)**

Theory : This particular standardization is solely governed by the following equations, namely :

$$As_2O_3 + 2H_2O \longrightarrow As_2O_5 + 4H^+ + 4e$$

or $$As_2O_3 \equiv 4e$$

or $$197.8 \text{ g } As_2O_3 \equiv 4000 \text{ ml N}$$

or $$49.45 \text{ g } As_2O_3 \equiv 1000 \text{ ml N}$$

or $$0.4945 \text{ g } As_2O_3 \equiv 100 \text{ ml 0.1 N Iodine}$$

Hydroiodic acid (HI) possesses strong reducing characteristics which renders the oxidation with iodine into a reversible reaction as follows :

$$As_2O_3 + 2I_2 + 2H_2O \rightleftharpoons As_2O_5 + 4H^+ + 4I^-$$

In order to shift the equilibrium to the right-hand-side (*i.e.*, towards As_2O_5) in the above reaction, sodium bicarbonate ($NaHCO_3$) is employed to remove the HI generated. It is important to record here that neither sodium hydroxide nor sodium carbonate can be used as both of them produce sodium iodide (NaI) and sodium iodate ($NaIO_3$) as designated below :

$$6NaOH + 3I_2 \longrightarrow 5NaI + NaIO_3 + 3H_2O$$

$$3Na_2CO_3 + I_2 \longrightarrow 5NaI + NaIO_2 + 3CO_2$$

Materials Required : Arsenic trioxide : 0.5 g ; sodium hydroxide solution (20% w/v in water) : 2 ml ; dilute hydrochloric acid (2N) ; sodium bicarbonate : 4 g ; 0.1 N iodine solution.

Procedure : Weigh accurately 0.5 g arsenic trioxide into a beaker, add to it 2 ml of sodium hydroxide solution, and heat to dissolve. Cool and transfer the contents quantitatively to a 100 ml volumetric flask and make up the volume upto the mark with DW. Pipette 20 ml into an iodine-flask, acidify with dilute HCl carefully and confirm it by adding a little $NaHCO_3$ to remove the free excess acid, followed by a further 2 g to get rid of HI formed in the reaction mixture. Now, titrate with 0.1 N iodine solution till the end-point is achieved by the appearance of the first permanent pale straw colour.

(*c*) **Standardization of 0.1 Iodine Solution by the aid of Sodium Thiosulphate**

Theory : Iodine solution may also be standardized by using sodium thiosulphate (AR-Grade) whereby the latter gets oxidized to sodium tetrathionate as expressed below :

$$2S_2O_3^{2-} \longrightarrow S_4O_6^{2-} + 2e$$

or $$I_2 + 2e^- \longrightarrow 2I^-$$

or $$2S_2O_3^{2-} \equiv I_2 \equiv 2e$$

or $$2 \times 248.2 \text{ g } Na_2S_2O_3.5H_2O \equiv 2000 \text{ ml N}$$

or $$248.2 \text{ g } Na_2S_2O_3.5H_2O \equiv 1000 \text{ ml N}$$

or $$24.82 \text{ g } Na_2S_2O_3.5H_2O \equiv 1000 \text{ ml 0.1 N Iodine}$$

Materials Required : Sodium thiosulphate (AR) : 6.025 g ; 0.1 N I_2 solution.

Procedure : Weigh accurately 6.025 g of sodium thiosulphate (AR) to a 250 ml volumetric flask. Dissolve it in DW, shake well and make up the volume to the mark with DW. Pipette 25 ml of 0.1 iodine

solution into an iodine flask and titrate with the standard sodium thiosulphate solution (as primary standard) until the solution becomes almost colourless.

Note : Stock solutions of sodium thiosulphate may be preserved by the addition of a few drops of sodium hydroxide solution (20% w/v) which serves as stabilizer as well as prevents decomposition.

(*d*) **Preparation of Starch Solution**

Material Required : Starch (arrowroot) : 1.0 g.

Procedure : Weigh 1.0 g starch in a glass in a glass pestle-mortar and triturate thoroughly with 10 ml of cold DW. Boil separately 200 ml of DW in a beaker and add the starch paste to it with vigorous stirring. The resulting mixture is boiled gently for a further period of 30 minutes till a transluscent and thin liquid having an uniform consistency is obtained.

Note : (1) The prepared solution of starch undergoes rapid deterioration, hence it is always desired to use freshly prepared solution every day,

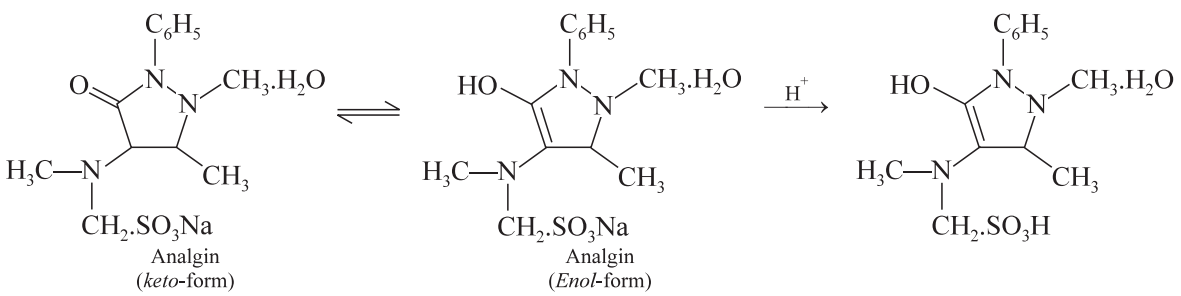
(2) It is now more or less believed that the iodine is held as an 'absorption complex' within the helical chain of the macromolecule β-amylose *i.e.*, a component of most starches. However, another component, α-amylose, is undesirable because it produces a red-colouration with iodine which is not readily reversible, and

(3) 'Soluble Starch' comprises principally of β-amylose, with the α-fraction having been removed. Always, it is a practice to prepare indicator-solutions from this product exclusively.

7.3.1.1.1. *Analgin*

Materials Required : Analgin : 0.4 g ; alcohol (95%) : 40 ml ; 0.01 N hydrochloric acid : 10 ml ; 0.1 N iodine solution.

Theory : The estimation of analgin depends upon the oxidation of the enolic group with iodine. The reaction is not reversible :



Hence, $C_{13}H_{l6}N_3NaO_4S \equiv I_2 \equiv 2e$

or $333.40\ g\ C_{13}H_{16}N_3\ NaO_4S \equiv 2000\ ml\ N$

or $166.70\ g\ C_{13}H_{16}N_3NaO_4S \equiv 1000\ ml\ N$

or $0.01667\ g\ C_{13}H_{16}N_3NaO_4S \equiv 1\ ml\ 0.1\ N\ I_2$

Procedure : Weigh accurately about 0.4 g and dissolve in a mixture of 40 ml of alcohol and 10 ml of 0.01 N hydrochloric acid. Titrate the resulting mixture with 0.1 N iodine solution till a yellow colour that

remains stable for 30 seconds is achieved. Each ml of 0.1 N iodine is equivalent to 0.016670 g of $C_{13}H_{16}N_3NaO_4S$.

7.3.1.1.2. *Acetarsol*

Materials Required : Acetarsol : 0.25 g ; sulphuric acid (conc.) : 7.5 ml ; nitric acid (fuming) : 2.5 ml ; ammonium sulphate : 5 g ; potassium iodide : 1.0 g ; sodium sulphite (0.1 N) : 1.0 ml ; phenolphthalein solution : 2 drops ; NaOH solution (0.1 N) ; dilute sulphuric acid (6 N) ; sodium bicarbonate : 8.0 g ; iodine solution (0.1 N).

Theory : Acetarsol is an organic arsenal, hence arsenic may be estimated by carrying out the oxidation As^{3+} to As^{5+} state with the help of 0.1 N iodine solution.

The organic entity present in acetarsol is destroyed primarily by boiling it with aqua-regia (a mixture of conc. H_2SO_4 and fuming nitric acid). The resulting mixture is heated in the presence of ammonium sulphate to get rid of nitric acid finally in the form of nitrous oxide (N_2O) as follows :

$$2HNO_3 + (NH_4)_2SO_4 \longrightarrow 2NH_4NO_3 + H_2SO_4$$

$$2NH_4NO_3 \xrightarrow{\text{Heat}} 2N_2O \uparrow + 4H_2O$$

Previously added H_2SO_4 maintains an acidic medium which on adding KI liberates HI that reduces the As^{5+} to As^{3+} state. Reduction is completed by boiling the solution which also expels the liberated I_2 as shown below :

$$As_2O_5 + 4HI \longrightarrow As_2O_3 + 2I_2 \uparrow + 2H_2O$$

The resulting mixture is cooled to room temperature and the residual iodine is removed by titration with 0.1 N sodium sulphite solution. Now, the solution is treated with sodium hydroxide solution to make it alkaline and then acidified carefully with dilute H_2SO_4 to remove the free NaOH. Finally, the resulting solution is made alkaline with $NaHCO_3$ so that the equilibrium is shifted to the right (*i.e.*, AS^{3+} gets converted to As^{5+}) quantitatively on carrying out the titration with 0.1 N iodine solution. Thus, we have :

$$As_2O_5 + 2I_2 + 2H_2O \rightleftharpoons As_2O_5 + 4HI$$

or $2C_8H_{10}AsNO_5 \equiv As_2O_5 \equiv As_2O_3 = 2I_2 \equiv 4e$

or 2×275.1 g $C_8H_{10}AsNO_5 \equiv 4000$ ml N

or 550.2 g $C_8H_{10}AsNO_5 \equiv 4000$ ml N

or 137.55 g $C_8H_{10}AsNO_5 \equiv 1000$ ml N

or 0.01375 g $C_8H_{10}AsNO_5 \equiv 1$ ml 0.1 N Iodine

Procedure : Weigh accurately about 0.25 g of acetarsol into a 500 ml iodine flask and add to it sulphuric acid (conc.) 7.5 ml, followed by nitric acid (fuming) 1.5 ml. Boil the contents of the flask gently for 45 minutes preferably in a fume-cupboard. Cool the solution, add 0.5 ml of fuming HNO_3 and boil till brown vapours (N_2O) stop coming. Again cool the contents and add carefully 5 g of ammonium sulphate in small lots at intervals and heat till there is no evolution of N_2O thereby giving rise to a colourless liquid. Bring the solution to room temperature, dilute with 100 ml DW, add 1 g KI and heat gently till the volume becomes 50 ml. Cool and add a few drops of 0.1 N sodium sulphite to effect decolourisation. Add 60 ml DW to dilute the resulting contents and make it just alkaline with NaOH solution by adding phenolphthalein indicator. Finally, acidify with dilute H_2SO_4, neutralize with $NaHCO_3$ and add 4 g of $NaHCO_3$ in excess. Swirl the contents of the flask and titrate with 0.1 N iodine solution. Each ml of 0.1 N iodine solution is equivalent to 0.01375 g of $C_8H_{10}AsNO_5$.

Note : All boiling is to be done in a fume-cupboard.

7.3.1.1.3. *Cognate Assays*

The following pharmaceutical substances can be assayed by direct titration with iodine as stated in Table 7.1.

Table 7.1 : Substances Assayed by Direct Titration with Iodine

S.No.	Name of Substance	Qty. Prescribed	Indicator Employed	Calculations
1.	Ascorbic acid	0.1 g	Starch solution	Each ml of 0.1 N Iodine ≡ 0.008806 g of $C_6H_8O_6$
2.	Sodium ascorbate	0.4 g	-do-	Each ml of 0.1 Iodine ≡ 0.009905 g of $C_6H_7NaO_6$
3.	Sodium thiosulphate	0.8 g	-do-	Each ml of 0.1 N Iodine ≡ 0.02482 g of $Na_2S_2O_3.5H_2O$

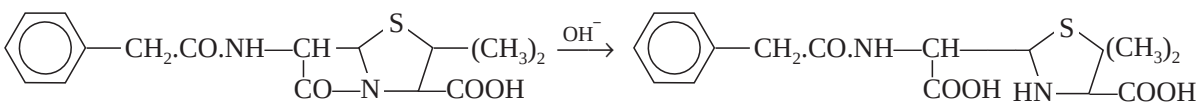
7.3.1.2. Residual Titration Method (*Excess of Iodine Titrated with Sodium Thiosulphate*)

In this titration method an excess of iodine solution is added to the solution of the substance and thus, the latter gets oxidized quantitatively. The excess of iodine is subsequently back titrated with sodium thiosulphate using freshly prepared starch solution as indicator with an end-point from violet to colourless.

7.3.1.2.1. *Benzylpenicillin*

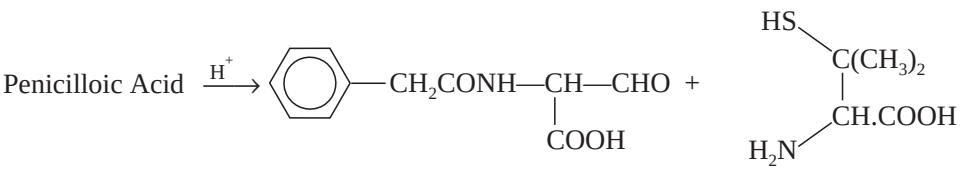
Theory : Benzylpenicillin can be assayed efficiently by adopting the following **three** steps sequentially, namely :

Step 1 : Benzylpenicillin is first converted to the corresponding penicilloic acid (a dicarboxylic acid) by carrying out the hydrolysis with sodium hydroxide solution, as follows :



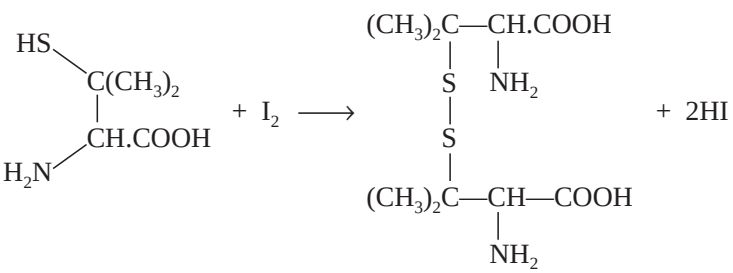
Benzylpenicillin — Penicilloic acid

Step 2 : Penicilloic acid on treatment with acid yields D-penicillamine and benzylpenilic acid, as shown under :



Benzylpenicillic acid — D-penicillamine

Step 3 : D-Penicillamine thus obtained is oxidised quantitatively by iodine to give rise to a disulphide, as expressed in the following equation ; whereas, the excess iodine is back titrated with 0.02 N sodium thiosulphate solution :



From the above reaction, we have :

$$C_{16}H_{17}N_2NaO_4S \equiv I \equiv e$$

In usual practice, however, benzylpenicillin sodium is standardised against a chemical reference substance of pre-determined potency.

Materials Required : Benzylpenicillin : 0.1 g ; (N) sodium hydroxide solution : 5 ml ; buffer solution (5.44% w/v of CH_3COONa and 2.40% w/v of glacial acetic acid) : 20 ml ; (N) hydrochloric acid : 5 ml ; 0.02 N iodine solution : 25 ml ; 0.02 N sodium thiosulphate solution ; starch solution.

Procedure : Weigh accurately about 0.1 g of benzylpenicillin in DW and dilute to 100 ml in a volumetric flask. Transfer 10.0 ml to an iodine flask, add 5 ml of N sodium hydroxide and allow to stand for 20 minutes. Now, add 20 ml of freshly prepared buffer solution, 5 ml of N HCl and 25.0 ml of 0.02 N iodine solution. Close the flask with a wet glass-stopper and allow to stand for 20 minutes in a dark place (*i.e.*, protected from light). Titrate the excess of iodine with 0.02 N sodium thiosulphate, employing freshly prepared starch solution as an indicator added towards the end-point.

To another 10.0 ml of the initial solution add 20 ml of the buffer solution, allow to stand for 20 minutes in the dark and titrate with 0.02 N sodium thiosulphate, using starch solution, added towards the end of the titration as indicator.

The difference between the two titrations represents the volume of 0.02 N iodine equivalent to the total penicillins present in the given sample of benzylpenicillin. An assay may be carried out simultaneously by benzylpenicillin sodium (reference sample) so as to determine the exact equivalent of each ml of 0.02 N iodine.

Calculations : Calculate the potency in Units of penicillin from the declared number of Units of pencillin in benzylpenicillin sodium (reference sample).

7.3.1.2.2. *Sodium Metabisulphite*

Theory : Sodium metabisulphite in acidic medium (HCl) yields SO_2 which reacts with water to produce sulphurous acid. The generated sulphurous acid is quantitatively oxidized by iodine to sulphuric acid, and the excess iodine is subsequently back titrated with sodium thiosulphate. The various reactions can be expressed as shown below :

$$Na_2S_2O_5 + 2HCl \longrightarrow 2NaCl + H_2O + 2SO_2$$

or $$2\,[SO_2 + H_2O \longrightarrow H_2SO_3]$$

or $$2\,[SO_3^{2-} + H_2O \longrightarrow SO_4^{2-}\,2H^+ + 2e]$$

or $$2\,[I_2 + 2e \longrightarrow 2I^-]$$

or $$Na_2S_2O_5 \equiv 2I_2 \equiv 4e$$

or $$190.10 \text{ g } Na_2S_2O_5 \equiv 2I_2 \equiv 4000 \text{ ml N}$$

or $$47.525 \text{ g } Na_2S_2O_5 \equiv 1000 \text{ ml N}$$

or $$0.004753 \text{ g } Na_2S_2O_5 \equiv 1 \text{ ml of 0.1 N Iodine}$$

Materials Required : Sodium metabisulphite : 0.2 g ; 0.1 N Iodine solution ; hydrochloric acid ($\simeq$ 11.5 N) : 1 ml ; 0.1 N sodium thiosulphate ; starch solution.

Procedure : Weigh accurately about 0.2 g of sodium metabisulphite and dissolve in 50.0 ml of 0.1 N iodine solution and add 1 ml hydrochloric acid. Titrate the excess of iodine with 0.1 N sodium thiosulphate employing freshly prepared starch solution, added towards the end of the titration, as indicator. Each ml of 0.1 N iodine is equivalent to 0.0047453 g of $Na_2S_2O_5$.

7.3.1.2.3. *Cognate Assays*

A few other pharmaceutical substances may also be assayed by adopting the residual titration method as shown in Table 7.2.

Table 7.2 : Substances Assayed by Residual Titration of Excess Iodine with Sodium Thiosulphate

S.No.	Name of Substance	Qty. Prescribed	Indicator Employed	Calculations
1.	Polyvinyl pyrrolidone	4.0 g	Starch solution	Each ml of 0.1 N iodine ≡ 0.005557 g of C_6H_9NO
2.	Mechlorethamine hydrochloride	0.10 g	-do-	Each ml of 0.1 N Na_2SO_5 ≡ 9.626 g of $C_5H_{11}Cl_2N$. HCl

7.3.2. IODOMETRIC ASSAYS

In iodometric determinations the pharmaceutical substance oxidizes KI in an acidic medium to produce an equivalent quantity of iodine that may be assayed by titration with a standard solution of sodium thiosulphate.

7.3.2.1. Chlorinated Lime

Chlorinated lime or bleaching powder, $CaOCl_2$, contains about 30% w/w of available chlorine.

Theory : Chlorinated lime reacts with acetic acid to produce a mole each of calcium acetate, hydrochloric acid and hydrochlorous acid. The two acids interact to give water and chlorine, and the latter reacts with HI to liberate iodine that can be estimated by titrating with 0.1 N sodium thiosulphate solution. The various reactions involved may be expressed as given below :

$$CaCl(OCl) + 2CH_3COOH \longrightarrow Ca(CH_3COO)_2 + HCl + HClO$$

$$HCl + HClO \longrightarrow H_2O + Cl_2$$

$$2HI + Cl_2 \longrightarrow 2HCl + I_2$$

or 35.46 g Cl ≡ 1000 ml N

or 0.003546 g ≡ 1 ml of 0.1 N Sodium Thiosulphate

Materials Required : Chlorinated lime : 4 g ; dilute acetic acid : 5 ml ; potassium iodide : 3 g ; acetic acid : 5 ml ; 0.1 N sodium thiosulphate solution.

Procedure : Weigh accurately 4.0 g of chlorinated lime and triturate it in a glass-pestle-mortar with a little DW. Transfer the paste quantitatively into a 1 litre volumetric flask and shake thoroughly. Take a 100 ml volumetric flask, rinse it with a small quantity of the suspension from the 1 litre flask and finally fill it up with the suspension. Rinse out a 250 ml iodine flask containing a little dilute acetic acid and a little of the suspension from the 1-litre flask in order to oxidise any inorganic substance present in the iodine flask. Finally, wash it thoroughly with DW. Now, transfer 100 ml of the suspension completely from the 100 ml volumetric flask to the iodine flask by washing the former repeatedly with DW. Add to it acetic acid 5 ml followed by KI 3.0 g and shake the contents of the flask thoroughly. Titrate the liberated iodine with 0.1 N sodium thiosulphate which is equivalent to 0.003546 g of chlorine.

From this value the percentage of chlorine present in the given sample of chlorinated lime can be calculated.

7.3.2.2. Ferric Ammonium Citrate

Theory : In ferric ammonium citrate it is taken for granted that the entire iron is oxidized to the Fe^{2+} state and practically little Fe^{2+} is present. Thus, the ferric ion present in a known amount of the sample liberates an equivalent amount of iodine from an acidified KI solution. Thus, we have :

$$2[Fe^{3+} + e \longrightarrow Fe^{2+}]$$

or $$2I^- \longrightarrow I_2 + 2e$$

or $2Fe \equiv 2Fe^{3+} \equiv 2e$

or 2×55.85 g Fe $\equiv$ 2000 ml N

or 55.85 g Fe $\equiv$ 1000 ml N

or 0.05585 g Fe $\equiv$ 1 ml of 0.1 N Sodium Thiosulphate

Materials Required : Ferric ammonium citrate : 0.5 g ; sulphuric acid conc. : 1 ml ; 0.1 N $KMnO_4$ solution : 50 ml ; hydrochloric acid : 15 ml ; potassium iodide : 2.0 g ; 0.1 N sodium thiosulphate.

Procedure : Weigh accurately about 0.5 g of ferric ammonium citrate and dissolve the sample in 15 ml DW. Add to it slowly 1 ml of sulphuric acid and warm gently to attain a yellow colouration so as to decompose the iron and ammonium citrate complex completely. Cool and add 0.1 N potassium permanganate solution dropwise from a burette to obtain a pink colour that persists for 5 seconds. To the resulting solution add hydrochloric acid 15 ml and potassium iodide 2.0 g, shake well and set aside for 3 minutes so that iodine may be liberated completely. Now, add 60 ml of water and titrate with 0.1 N sodium thiosulphate solution while shaking the contents continuously till a colourless end-point is achieved.

Precautions :

(*i*) Addition of excess of $KMnO_4$ solution must be avoided, since pink colour developed shall disappear within a short span, which may ultimately give false high results,

(*ii*) Washing down during the course of titration must be checked rigidly in order to maintain the right proportion of various substances in the solution,

(*iii*) End-point is almost colourless, hence starch indicator can be skipped totally, and

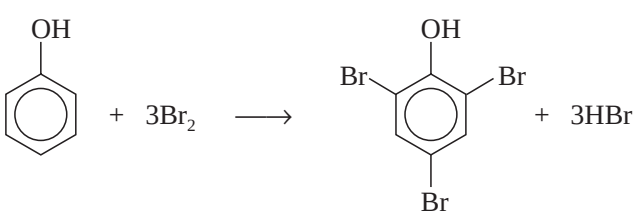
(*iv*) $KMnO_4$ oxidizes the traces of Fe^{2+} to Fe^{3+} in the sample, if any.

7.3.2.3. Thyroid

Thyroxine and diidotyrosine are the two iodine-substituted organic compounds which essentially constitute the active principles present in dried thyroid gland. The latter on being subjected to pyrolysis with anhydrous K_2CO_3, gives rise to an equivalent amount of KI present in the sample. Soon after the completion of carbonization, the crucible is cooled and the residue is extracted with water to dissolve KI, carbonates and other soluble compounds. The resulting solution is filtered and treated with Br_2 in the presence of phosphoric acid (H_3PO_4) so that complete oxidation of iodide to iodate is caused. The following reaction takes place :

$$I^- + 3Br_2 + 3H_2O \longrightarrow IO_3^- + 6HBr$$

The excess of bromine is removed by warming the acidic solution gently till the vapours show a negative test with starch-iodide paper. However, the residual traces of Br_2 are reduced by treatment of the resulting solution with phenol to yield the corresponding 2,4,6-tribromophenol as shown below :



Lastly, iodate (IO_3^-) in a weak acidic medium quantitatively oxidizes KI to an equivalent amount of iodine, as expressed below :

$$IO_3^- + 5I^- + 6H^+ \longrightarrow 3I_2 + 3H_2O$$

It is evident from the above equation that each gram-atomic weight of iodine in thyroid is converted to 1 mol of iodate and finally to 3 mol or 6 equivalent of iodine. Therefore, the equivalent weight of the iodine present in the dried thyroid gland is 21.15 g (*i.e.*, 1/6 × 127 At. wt. of I_2). Hence, each millilitre of 0.01 N sodium thiosulphate is equivalent to 0.0002115 g of iodine (*i.e.*, 0.01 × 0.02115 g).

Materials Required : Thyroid gland dried 1.0 g ; anhydrous potassium carbonate : 17.0 g ; bromine solution (9.6 ml of Br_2 and 30 g of KBr in 100 ml DW) : 7.0 ml ; dilute phosphoric acid (10% w/v) : 42.0 ml ; starch iodide paper ; phenol solution (saturated solution of phenol in water) : 5.0 ml ; potassium iodide solution (10% w/v in water) ; 0.01 N sodium thiosulphate solution ; starch solution.

Procedure : Weigh accurately about 1.0 g of dried thyroid gland in a porcelain crucible, add 7.0 g of anhydrous K_2CO_3, mix thoroughly and overlay with further 10 g more of anhydrous K_2CO_3, finally compact the mixture by tapping gently. Incenerate for 25 minutes at 675°—700°C in a preheated muffle furnace. Cool the contents, add 20 ml of DW, boil gently and decant through a filter paper into a flask. Repeat the extraction by boiling with 20 ml DW, wash the crucible and the residue on the filter with hot water until the filtrate is about 200 ml. To it add 7.0 ml of freshly prepared bromine solution followed by 40 ml of dilute phosphoric acid and continue boiling slowly till starch iodide paper is no longer coloured blue by the vapours. While boiling is in progress top up the volume to 200 ml by adding DW at intervals. Cool and add 5 ml of phenol solution and allow to stand for 5 minutes. Add 2 ml of dilute phosphoric acid and 5 ml of potassium iodide solution and titrate immediately with 0.01 N sodium thiosulphate solution employing starch solution as indicator towards the end-point. A blank estimation is also carried out simultaneously and necessary correction incorporated. Each ml 0.1 N sodium thiosulphate is equivalent to 0.0002115 g of I.

Precautions :

(*i*) Potassium carbonate should be perfectly anhydrous otherwise decrepitation would take place causing loss of material during pyrolysis,

(*ii*) Both the temperature of the muffle furnace and the extent of heating should be monitored closely, because KI is significantly volatile at an elevated temperature and part of it may be lost due to extended heating, and

(*iii*) The solution from which excess Br_2 is removed by heating must be acidic, otherwise a portion of Br_2 shall be fixed in the form of potassium hypobromite (KBrO).

7.3.2.4. Cognate Assays

A few pharmaceutical substances can be assayed by titrating the liberated iodine from potassium iodide with sodium thiosulphate as stated in Table 7.3.

Table 7.3 : Substances Assayed by Titrating the Liberated Iodine from Potassium Iodide with Sodium Thiosulphate

S.No.	Name of Substance	Qty. Prescribed	Indicator Employed	Calculations
1.	Diiodohydroxy quinoline	12 mg	Starch solution	Each ml of 0.02 N $Na_2S_2O_3$ ≡ 0.6616 mg of $C_9H_5I_2NO$
2.	Mannitol	0.4 g	-do-	Each ml of 0.1 N I_2 ≡ 0.001822 g of $C_6H_{14}O_6$
3.	Phenindione	0.3 g	-do-	Each ml of 0.1 N $Na_2S_2O_3$ ≡ 0.01111 g of $C_{15}H_{10}O_2$

THEORETICAL AND PRACTICAL EXERCISES

1. What is the basic difference between '**iodimetric**' and '**iodometric**' titrations ? Explain with the help of equations involved in such typical titrations.

2. Iodimetic titrations may be accomplished by *two* methods :

(*a*) Direct titration with iodine

(*b*) Residual titration method.

Explain the above with the help of preparation of 0.1 N I_2-solution, its standardization and methodologies adopted.

3. How would you assay the following '**drugs**' by iodimetric titrations :
(*i*) Analgin (*ii*) Ascorbic Acid
(*iii*) Benzylpenicillin (*iv*) Mechlorethamine Hydrochloride.

4. Explain the assay of the following '**drug substances**' by using '**direct iodometic method**' :
(*i*) Chlorinated line (*ii*) Ferric Ammonium Citrate
(*iii*) Thyriod (*iv*) Mannitol.

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C. PRECIPITATION METHODS

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8 ARGENTOMETRIC METHODS

CONTAINS :

8.1. INTRODUCTION

In general, titrations governed by precipitation reactions do not really constitute an appreciable number in volumetric determinations in comparison to either redox or acid-base reactions. The interaction between silver-nitrate and sodium chloride in solutions result into the precipitation of silver chloride as shown below :

$$NaCl + AgNO_3 \longrightarrow AgCl \downarrow + NaNO_3$$

In actual practice, however, such titrations are more or less restricted to those involving precipitation of Ag^+ with anions, for instance : halogens (Cl^-, Br^-, I^-) and thiocyanate (SCN^-). Generally, it is quite difficult and tedious to locate the exact point at which further addition of reagent affords no more precipitation. Therefore, the choice and wisdom of a chemical reaction is preferably sought so as to result in either a coloured solution or a coloured precipitate at the end point. A typical instance may be cited by application of potassium chromate (K_2CrO_4) solution in the above case whereby any extra drop of silver nitrate, after all the chloride has been precipitated, immediately causes precipitation of red chromate showing that the end point has been duly achieved.

It is, however, interesting to observe here that such reactions do offer limited usage because of the following *two* facts, namely :

(*i*) Co-precipitation effects do not give a real composition of the precipitate, and

(*ii*) Choice of appropriate indicator is very much limited.

Besides, the foregoing facts another vital aspect to be taken into consideration is the **solubility** product that plays a major role in such titration. Hence, the equilibrium constant of the reaction giving the precipitate of AgCl may be expressed as :

$$AgCl\ (s) \longrightarrow Ag^+\ (aq) + Cl^-\ (aq)$$

or
$$K = \frac{[Ag^+][Cl^-]}{[AgCl]}$$

From the above expression the solubility product constant K*sp* may be designated as :

$$K_{sp} = [Ag^+]\ [Cl^-]$$

assuming the activity of solid AgCl being constant.

Following are the *four* cardinal parameters that may be considered for a feasible argentometric analysis, namely :

(*i*) Precipitate formed must be insoluble,

(*ii*) Precipitation process should be fast and rapid,

(*iii*) Co-precipitation effects must be minimal, and

(*iv*) Detection of equivalence point must be apparently visible.

8.2. THEORY

In the precipitation reaction involving chloride and silver nitrate, the addition of even a small quantity of the latter shall effect precipitation of AgCl provided that K*sp* has been exceeded significantly. At this juncture, the concentrations of both Ag^+ and Cl^- are related by the solubility-product equilibrium constant ; thus, we have :

$$Ag^+ \text{ (titrant)} + Cl^- \text{ (analyte)} \rightleftharpoons AgCl(s)$$

Chromate ion concentration required to initiate the precipitation of Ag_2CrO_4 commences at the equivalence point and may be calculated with the solubility products for AgCl and Ag_2CrO_4 :

$$AgCl : Ksp = 1.8 \times 10^{-10} = [Ag^+]\,(Cl^-)$$

$$Ag_2CrO_4 : Ksp = 1.2 \times 10^{-12} = [Ag^+]^2\,[CrO_4^{2-}]$$

Assuming that at the equivalence point,

$$[Ag^+] = 1.3 \times 10^{-5} \text{ M}$$

the chromate ion concentration must be :

$$[CrO_4^{2-}] = \frac{[AgCrO_4]}{[Ag^+]^2}$$

or

$$= \frac{1.2 \times 10^{-12}}{[1.3 \times 10^{-5}]^2}$$

or

$$= 6.7 \times 10^{-3} \text{ M}$$

In actual practice, the concentration of chromate produces an intense yellow colour to such an extent that the end point is masked. Therefore, normally concentrations of 5×10^{-3} M are employed in analytical procedures. It suggests that $[Ag^+]$ shall be $> 1.3 \times 10^{-5}$ M at the end-point thereby introducing a positive determinate error. However, it has been proved experimentally that even with concentrations as low as 2×10^{-3} M, the extent of error caused is negligibly small.

Adsorption-coprecipitation phenomenon using fluorescein, dichlorofluorescein and tetrabromofluorescein (eosin) essentially impart the fluoresceinate ion that is absorbed on the AgCl particles. At the equivalence point, the AgCl particles change from white to pink due to the coprecipitation of silver fluoresceinate. In short, the adsorption indicator method is quite rapid and capable of providing very accurate results for the estimation of Cl^- with $AgNO_3$.

Furthermore, Br^-, I^- and SCN^- ions can also be titrated with $AgNO_3$ employing eosin as an adsorption indicator.

8.3. ASSAY METHODS

Argentometric titrations may be divided into *two* broad categories, namely :

(*i*) Direct titration with silver-nitrate, and

(*ii*) Ammonium thiocyanate-silver nitrate titrations (Volhard's Method).

8.3.1. DIRECT TITRATION WITH SILVER NITRATE

Pharmaceutical substances essentially containing halides may be estimated by direct titration with silver nitrate solution as a titrant.

8.3.1.1. Preparation of 0.1 N Silver Nitrate Solution

Materials Required : Silver nitrate (AR) : 16.989 g.

Procedure : Weigh accurately 16.989 g of silver nitrate on a watch-glass and transfer quantitatively into a 1 litre volumetric flask. Add freshly prepared DW and make up the volume to 1000 ml. Thus, we have :

$$AgNO_3 + NaCl \longrightarrow AgCl \downarrow + NaNO_3$$

or $AgNO_3 \equiv NaCl \equiv H$

or $169.89 \text{ g } AgNO_3 \equiv 58.45 \text{ g NaCl} \equiv 1000 \text{ ml N}$

or $0.01699 \text{ g } AgNO_3 \equiv 0.005845 \text{ g NaCl} \equiv 1 \text{ ml } 0.1 \text{ N } AgNO_3$

8.3.1.2. Standardization of 0.1 N Silver Nitrate Solution

Materials Required : Sodium chloride : 0.1 g ; acetic acid (33% w/v) : 5 ml ; methyl alcohol (95%) : 50 ml ; eosin solution (0.5% w/v in water) : 5 ml ; 0.1 N silver nitrate solution.

Procedure : Weigh accurately about 0.1 g of sodium chloride, previously dried at 110°C for 2 hours, and dissolve in 5 ml of water. Add 5 ml of acetic acid, 50 ml of methyl alcohol and three drops of eosin solution. Stir thoroughly on a magnetic stirrer and titrate with the silver nitrate solution till the white particles of AgCl change from white to pink. Each 0.005844 g of sodium chloride is equivalent to 1 ml of 0.1 N silver nitrate.

8.3.1.2.1. *Potassium Chlroride*

Materials Required : Potassium chloride : 0.25 g ; potassium chromate solution (5% w/v in water) : 10 ml ; 0.1 N silver nitrate solution.

Procedure : Weigh accurately about 0.25 g of potassium chloride in a conical flask and dissolve it in 50 ml of DW and titrate with 0.1 N silver nitrate solution, using 2-3 drops of potassium chromate solution as indicator till precipitation of red chromate is indicated. Each ml of 0.1 N silver nitrate solution is equivalent to 0.007455 g of KCl.

Equations :

$$AgNO_3 + KCl \longrightarrow AgCl \downarrow + KNO_3$$

or $AgNO_3 \equiv KCl \equiv H$

or $169.89 \text{ g } AgNO_3 \equiv 74.55 \text{ g KCl} \equiv 1000 \text{ ml N}$

or $0.01699 \text{ g } AgNO_3 \equiv 0.007455 \text{ g KCl} \equiv 1 \text{ ml of } 0.1 \text{ N } AgNO_3$

8.3.1.2.2. *Chloral Hydrate*

Materials Required : Chloral hydrate : 4.0 g ; sodium hydroxide (N) : 30 ml ; sulphuric acid (N) ; phenolphthalein solution (1.0% w/v in 50% v/v alcohol) ; 0.1 N silver nitrate solution ; potassium chromate solution (5% w/v in water).

Procedure : Weigh accurately about 4 g of chloral hydrate and dissolve in 10 ml of DW and add 30 ml of N sodium hydroxide solution. Allow the resulting mixture to stand for 2 minutes, and then titrate with N sulphuric acid, employing phenolphthalein solution as indicator till a colour change from pink to colourless is achieved. Titrate the neutralized liquid thus obtained with 0.1 N silver nitrate using potassium chromate solution as indicator till precipitation of red chromate is obtained, Add, now 2/15th of the amount of 0.1 N silver nitrate used to the amount of N sulphuric acid used in the first titration and deduct the figure so obtained

from the amount of N sodium hydroxide added. Each ml of N sodium hydroxide, obtained as difference, is equivalent to 0.1654 g of $C_2H_3Cl_3O_2$.

Explanation :

(*i*) The estimation depends upon the interaction between chloral hydrate and sodium hydroxide as shown by the following equation :

$$C.Cl_3CH(OH)_2 + NaOH \longrightarrow CHCl_3 + HCOONa + H_2O \quad ...(a)$$

$$CCl_2CH(OH)_2 \equiv NaOH \equiv H$$

or $165.40 \text{ g } C_2H_3O_2Cl_3 \equiv 1000 \text{ ml N}$

or $0.1654 \text{ g } C_2H_3O_2Cl_3 \equiv 1 \text{ ml N NaOH}$

(*ii*) As the chloroform generated in Eq. (*a*) undergoes chemical reaction with the alkali to a certain degree; therefore, addition of alkali followed by back titration does not afford the correct assay. Thus, we have :

$$CHCl_3 \xrightarrow{\text{Base}} \underbrace{H.COOH + HCl}_{\downarrow 4NaOH}$$

$$H.COONa + 3NaCl + 4H_2O \quad ... (b)$$

Hence, from Eq. (*b*) we have :

$$CHCl_3 \equiv 4NaOH$$

(*iii*) The ionized chloride generated from the additional side reaction (*b*) may be estimated by titration with 0.1 N silver nitrate solution, and necessarily a correction has got to be made to the alkali-titration reading so as to adequately compensate for this side reaction. Thus, from equation (*b*) we have :

$$CHCl_3 \equiv 4NaOH \equiv 4000 \text{ ml N solution}$$

Also, $3NaCl \equiv 3000 \text{ ml of N } AgNO_3 \text{ solution}$

or $3NaCl \equiv 30{,}000 \text{ ml of 0.1 N } AgNO_3 \text{ solution}$

Therefore, it is evident that 2/15th of the volume of 0.1 N $AgNO_3$ (*i.e.*, 2/15th of 30,000 = 4,000) needed shall give the volume of N NaOH that reacted with chloroform as per Eq. (*b*).

8.3.1.2. Cognate Assays

The pharmaceutical substances in Table 8.1, can be assayed by direct titration with silver nitrate using a suitable indicator.

Table 8.1 : Substances Assayed by Direct Titration with Silver Nitrate

S.No.	Name of Substance	Qty. Prescribed	Indicator Employed	Calculations
1.	Iopanoic acid	0.25 g	Tetrabromo-phenolphthalein ethyl ester	Each ml of 0.05 N $AgNO_3 \equiv 9.516$ mg of $C_{11}H_{12}I_3NO_2$
2.	Benzyltrimethyl ammonium chloride	2.0 ml	Dichloro-fluorescein	Each ml of 0.1 N $AgNO_3 \equiv 18.57$ mg of $C_6H_5CH_2N(CH_3)Cl$
3.	Diatrizoate sodium	0.30 g	Tetrabromo-phenolphthalein ethyl ester	Each ml of 0.05 N $AgNO_3 \equiv 10.60$ mg of $C_{11}H_8I_3N_2NaO_4$

8.3.2. AMMONIUM THIOCYANATE-SILVER NITRATE TITRATIONS (VOLHARD'S METHOD)

Volhard's method is based on *two* major aspects, namely :

(*a*) Complete precipitation of insoluble silver salts from nitric acid solution by adding an excess of silver nitrate solution to a corresponding soluble salt, and

(*b*) Estimation of excess of silver nitrate solution by carrying out residual titration with standard ammonium thiocyanate solution, employing ferric ammonium sulphate as an indicator.

Thus, ammonium thiocyanate reacts with silver nitrate in nitric acid solution as below :

$$NH_4SCN + AgNO_3 \longrightarrow AgSCN \downarrow + NH_4NO_3$$

However, in actual practice the thiocyanate solution is always taken in the burette and is run directly into the silver nitrate solution in the flask that has been duly acidified with nitric acid. Ferric ammonium sulphate is the choicest indicator since the end point is visibly detected by a deep red colour (ferric thiocyanate) due to the interaction of Fe^{2+} ions with a trace of SCN^- ion.

Precautions :

(*i*) Nitric acid must be free from nitrous acid, otherwise thiocyanic acid may give an instant red colouration, and

(*ii*) Temperature of the solution should be maintained below 25°C since at an elevated temperature the red colour of the ferric thiocyanate complex fades away rapidly. Therefore, we have :

$$NH_4SCN \equiv AgNO_3 \equiv H$$

8.3.2.1. Preparation of 0.1 N Ammonium Thiocyanate Solution

Materials Required : Ammonium thiocyanate : 8.0 g.

Procedure : Weigh about 8.0 g of ammonium thiocyanate and transfer it quantitatively in 1 litre volumetric flask. Dissolve it in DW and make up the volume upto the mark.

Equation :

$$NH_4SCN \equiv AgNO_3 \equiv H$$

or $76.12 \text{ g } NH_4SCN \equiv 1000 \text{ ml N}$

or $7.612 \text{ g } NH_4SCN \equiv 1000 \text{ ml } 0.1 \text{ N } AgNO_3$

8.3.2.2. Standardization of 0.1 N Ammonium Thiocyanate Solution

Materials Required : 0.1 N Silver nitrate solution : 25 ml ; nitric acid (16 N) : 2 ml ; ferric ammonium sulphate (10% w/v in water) : 2 ml ; 0.1 N ammonium thiocyanate solution.

Procedure : Pipette 25 ml of a standard 0.1 N $AgNO_3$ solution into a glass-stoppered flask (iodine-flask), dilute with 50 ml of DW, add to it 2 ml of nitric acid and 2 ml of ferric ammonium sulphate solution and titrate with ammonium solution to the first appearance of red-brown colour. Each ml of 0.1 N silver nitrate is equivalent to 0.007612 g of NH_4SCN.

Note : Soon after the addition of ammonium thiocyanate a white precipitate of silver thiocyanate is formed first and then a reddish-brown colour appears that fades out completely upon shaking thereby leaving a white precipitate of silver thiocyanate. The end-point is indicated by the appearance of a permanent faint reddish brown colour that does not vanish upon shaking.

8.3.2.2.1. *Chlorobutol*

Materials Required : Chlorobutol : 0.2 g ; alcohol (95%) : 5 ml ; sodium hydroxide solution (20% w/v in water) : 5 ml ; nitric acid (16 N) : 5 ml ; nitrobenzene : 1 ml ; 0.1 N silver nitrate solution : 50 ml ; ferric ammonium sulphate solution (10% w/v in water) ; 0.1 N ammonium thiocyanate solution.

Procedure : Weigh accurately about 0.2 g of chlorobutol in a flask and dissolve in 5 ml of alcohol. Add to it 5 ml of sodium hydroxide solution, and boil under a reflux condenser for 15 minutes. Cool, dilute with 20 ml of DW, add 5 ml of nitric acid, 1 ml of nitrobenzene and 50 ml of 0.1 N silver nitrate solution. Shake the contents vigorously for 1 minute, add 4 ml of ferric ammonium sulphate solution and titrate the excess of silver nitrate with 0.1 N ammonium thiocyanate solution. Each ml of 0.1 N silver nitrate is equivalent to 0.005917 g of $C_4H_7Cl_3O$.

Explanation : Chlorine combined originally to chlorobutol is being converted by hydrolysis in the presence of sodium hydroxide to ionic chloride that may be estimated quantitatively by Volhard's method in the presence of nitrobenzene.

Thus, we have :

$$CCl_3C(CH_3)_2.OH + 3NaOH \longrightarrow C(OH)_3.C(CH_3)_2.OH + 3NaCl$$

$$\downarrow \text{NaOH}$$

$$(CH_3)_2.C(OH).COONa$$

or $$3NaCl + 3AgNO_3 \longrightarrow 3NaNO + 3AgCl \downarrow$$

or $$C_4H_7OCl_3 \equiv 3NaCl \equiv 3AgNO_3 = 3H \equiv 3000 \text{ ml N}$$

or 186.48 g of $C_4H_7OCl_3 \equiv 3000$ ml N

or 0.006216 g $C_4H_7OCl_3 \equiv 1$ ml of 0.1 N $AgNO_3$

8.3.2.2.2. *Ethionamide*

Theory : Theoretically the cleavage of thioamide link in ethionamide takes place in an acidic medium. Subsequent neutralization with NH_4OH yields ammonium sulphide which on addition of silver nitrate yields a precipitate of Ag_2S. Thus we have :

$$C_8H_{10}N_2S \equiv CS_2$$

or $$CH_2 + NH_4OH \longrightarrow 2(NH_4)_2S + 2H_2O + CO_2$$

or $$(NH_4)_2S + 2AgNO_3 \longrightarrow Ag_2S \downarrow + 2NH_4NO_3$$

Materials Required : Ethionamide : 0.3 g ; dilute sulphuric acid (10% w/w) : 10 ml ; dilute ammonia solution (4.25 ml of strong ammonia solution in 100 ml of water) ; 0.1 N silver nitrate : 50 ml ; dilute nitric acid (10.6 ml of nitric acid to 100 ml of water) : 60 ml ; ferric ammonium sulphate solution (10% w/v in water) : 5 ml ; and 0.1 N ammonium thiocyanate solution.

Procedure : Weigh accurately about 0.3 g of ethionamide in a flask and dissolve in 10 ml of dilute sulphuric acid. Add to it 100 ml of water, 20 ml of dilute ammonia solution and rapidly 50 ml of 0.1 N silver nitrate solution. Allow the resulting mixture to stand for a few minutes, filter and wash the filter paper with three successive quantities, each of 10 ml of DW. To the combined filtrate and washings, add 60 ml of dilute nitric acid, cool and titrate with 0.1 N ammonium thiocyanate employing 5 ml of ferric ammonium sulphate solution as an indicator. Each ml of 0.1 N silver nitrate is equivalent to 0.008312 g of $C_8H_{10}N_2S$.

8.3.2.2.3. *Cognate Assays*

A good number of pharmaceutical substances can be assayed by Volhard's method and are mentioned in Table 8.2.

Table 8.2 : Substances Assayed by Volhard's Method

S.No.	Name of Substance	Qty. Prescribed	Indicator Employed	Calculations
1.	Aminophylline	0.25 g	Ferric ammonium sulphate	Each ml of 0.1 N $AgNO_3 \equiv 0.02102$ g of $C_{16}H_{24}N_{10}O_4$
2.	Chlorophenothane	1.0 g	-do-	Each ml of 0.1 N $AgNO_3 \equiv 0.00709$ g of $C_{14}H_9Cl_5$
3.	Dimenhydrinate (for 8-chloro-theophylline)	0.8 g	-do-	Each ml of 0.1 N $AgNO_3 \equiv 0.02146$ g of $C_7H_7ClN_4O_2$
4.	Gamma Benzene Hexachloride*	0.4 g	-do-	Each ml of 0.1 N $AgNO_3 \equiv 0.009694$ g of $C_6H_6Cl_6$
5.	Oxyphenonium Bromide	0.8 g	-do-	Each ml of 0.1 N $AgNO_3 \equiv 0.04284$ g of $C_{21}H_{34}BrNO_3$
6.	Sodium Chloride	0.1 g	-do-	Each ml of 0.1 N $AgNO_3 \equiv 0.005844$ g of NaCl

* Hydrolysis with ethanolic KOH helps in the conversion of organically combined chlorine to KCl which after due acidification with HNO_3 is assayed by Volhard's Method.

THEORETICAL AND PRACTICAL EXERCISES

1. Explain the following :
(*i*) Precipitation reactions governing '**argentometric methods**'.
(*ii*) Role of '**solubility product**' in precipitation reactions.
(*iii*) Various cardinal parameters required for a feasible argentometric analysis.

2. Discuss the '**theoretical aspect**' of argentometric methods explicitely.

3. Give a comprehensive account of the '**direct titration method**' with silver nitrate with reference to the following :
(*a*) Preparation 0.1 N $AgNO_3$ solution (1 L)
(*b*) Standardization of 0.1 N $AgNO_3$ solution
(*c*) Assay of chloral hydrate.
(*d*) Assay of Benzyltrimethyl ammonium chloride.

4. (*a*) What is Volhard's method ? Explain it with the help of equations and the precautions involved in it.
(*b*) Preparation and standardization of 0.1 N Ammonium Thiocyanate solution.
(*c*) Describe the assay of the following '**drugs**',
(*i*) Chlorobutol (*ii*) Ethionamide
(*iii*) Aminophylline (*iv*) Dimenhydrinate.

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D. COMPLEXOMETRIC METHODS

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9 COMPLEXOMETRIC ANALYSIS

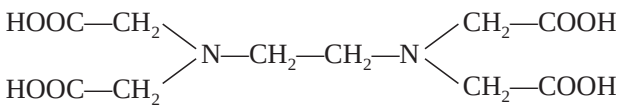
CONTAINS :

9.1. INTRODUCTION

A host of inorganic pharmaceutical substances essentially containing polyvalent and bivalent metal ions, for instance : Al^{3+}, Ca^{2+}, Mg^{2+}, Bi^{2+} and Zn^{2+} were initially analysed quantitatively by the aid of *gravimetric methods*. These historically important procedures have become more or less obsolete since they are extremely time consuming and tedious owing to several steps involved *e.g.*, precipitation, filtration, washing, drying and finally ignition to a constant weight.

Thereafter, analysis was done by faster techniques based on oxalate-permanganate titrations which involved a precipitation procedure followed by quick titration of pre-heated solutions containing oxalate ions.

More recently the introduction of an analytical reagent disodium ethylene-diaminetetraacetate, invariably termed as **EDTA**, an altogether latest titrimetric method has been used exclusively for the estimation of metals using *metal-ion indicators*.



EDTA

9.2. THEORY

Complex is a compound that is formed by the combination of a metal ion with a molecule that is capable of donating electrons, for example :

$[Cu(NH_3)_4]^{2-}$ and $Co[(NH_3)_6]^{3+}$

Cupric ammonium ion Cobaltammine

In the above two examples both Cu^{2+} and Co^{2+} form complexes with lone pair of electrons present in the neutral molecule ammonia *e.g.*, $\ddot{N}H_3$.

Chelate is a complex that is formed by the combination of a polyvalent metal ion with a molecule which essentially contains two or more groups that can donate electrons.

Specifically, disodium ethylenediaminetetraacetate (EDTA) reacts with **polyvalent metal ions** to result in the formation of a fairly stable *water-soluble complex*, or a *chelate compound*.

It is, however, pertinent to mention here that the predominant state of the dissociated forms of EDTA (*viz* . Y^{4-}, HY^{3-} , H_2Y^{2-} and H_3Y^{-}) is solely dependent upon the pH of the medium at which complexation takes place :

where, H_4Y = ethylenediaminetetraacetic acid, and

Y^{4-} = tetracetate ion.

In general, all EDTA complexation reactions essentially have the ratio of EDTA to metal ion as 1 : 1. Thus, we have :

$$Y^{4-} + M^{n+} \rightleftharpoons MY^{n-4}$$

Ligand is a molecule that affords groups for attachment to metal ions such as EDTA.

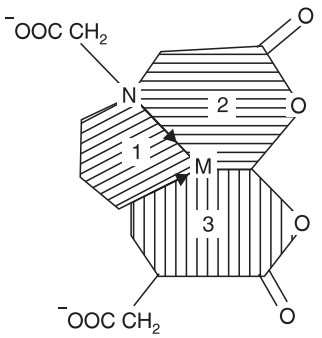
Some examples of polyvalent metal ions are given below :

Bivalent Metal ions : Ca^{2+}, Mg^{2+}, Zn^{2+},

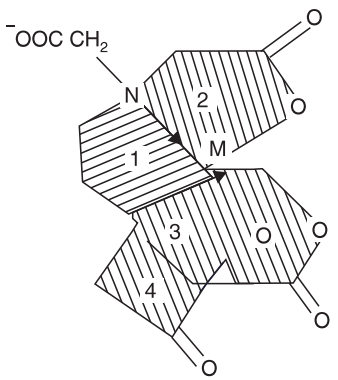
Trivalent Metal ions : Fe^{3+}, Al^{3+}, Cr^{3+},

Tetravalent Metal ions : Sn^{4+}, Ce^{4+}, Cr^{4+}, Pt^{4+}.

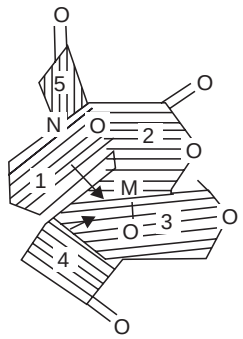
The structures of the complexes formed with *di-*, *tri-* and *tetra*-valent metal ions give rise to *three, four* and *five* rings respectively as depicted below :



M^{2+} [Ca^{2+}, Mg^{2+}, Zn^{2+}]
Nos. of rings formed = 3



M^{3+} [Fe^{3+}, Al^{3+}, Cr^{3+}]
Nos. of rings formed = 4



M^{4+} [Sn^{4+}, Ce^{4+}, Cr^{4+}, Pt^{4+}]
Nos. of rings formed = 5

There are various aspects in complex formation and detection, namely :

(*i*) Effect of pH on complexation,

(*ii*) Stability of complexes,

(*iii*) Colouration of complexes,

(*iv*) Titrability of polyvalent metal ions employing disodium edetate, and

(*v*) Usage of pM indicators in complexometric titrations.

9.2.1. EFFECT OF pH ON COMPLEXATION

Ethylenediamine tetracetic acid (H_4Y) undergoes ionization at *four*, different stages, namely :

$[H^+ + H_3Y^- ; pK_1 = 2.07]$; $[H^+ + H_2Y^{2-} ; pK_2 = 2.75]$;

$[H^+ + HY^{3-} ; pK_3 = 6.24]$; $[H^+ + Y^{4-} ; pK_4 = 10.34]$.

In reality, the actual complexing species is the tetracetate ion *i.e.*, Y^{4-} ; therefore, complexation will take effect more efficiently and be more stable in an aikaline medium. Hence, it is evident that EDTA complexes of many divalent metals are quite stable in ammoniacal solution.

As we have seen earlier that the trivalent metal complexes are normally bound still more firmly due to the formation of four rings (unlike three rings with divalent metal complexes) and stable in strongly acidic solutions, for instance : cobalt (Co^{2+}) EDTA complex is fairly stable in concentrated hydrochloric acid ($\simeq$ 11.5 N).

Though a good number of metal-EDTA complexes are found to be quite stable over a wide-spectrum of pH, yet in actual practice solutions are normally buffered for *two* specific reasons :

(*a*) to stabilize the complex formed, and

(*b*) to achieve the most distinct colour-change of the indicator.

9.2.2. STABILITY OF COMPLEXES

Generally, the formation of a 1 : 1 chelate complex (MX) may be designated by the following equation :

$$M + X \rightleftharpoons MX$$

where, M = Metal ion, and

X = Chelating ion.

Hence, the stability constant, K, may be expressed as :

$$K = \frac{[MX]}{[M][X]}$$

where, items within the 'square brackets' represent activities.

There are *two* cardinal factors which influence the stability constant (K), namely :

(*a*) Elevation in temperature affords a slight enhancement in the ionization of the complex and a slight lowering of K, and

(*b*) Stability constant is decreased on the addition of electrolytes with no common ion ; whereas, ethyl alcohol enhances K, perhaps on account of the suppression of ionization.

Table 9.1, provides the values of the logarithms of stability constants (K) of EDTA-complexes of certain metals normally occurring in pharmaceutical substances :

Table 9.1 : Stability Constants of EDTA-Complexes

S.No.	Cation	Log K	Complexed Metal Ions
l.	Ba^{2+}	7.8	BaY^{2-}
2.	Mg^{2+}	8.7	MgY^{2-}
3.	Ca^{2+}	10.6	CaY^{2-}
4.	Zn^{2+}	16.5	ZnY^{2-}
5.	Cr^{3+}	24.0	CrY^{1-}
6.	Fe^{3+}	25.1	FeY^{1-}
7.	Al^{3+}	15.5	AlY^{1-}

9.2.3. COLOURATION OF COMPLEXES

The formation of EDTA-metal ion complexes invariably attribute a change in the absorption spectrum pattern which ultimately forms the basis of a large number of colorimetric assays.

9.2.4. TITRABILITY OF POLYVALENT METAL IONS EMPLOYING DISODIUM EDETATE

Ethylenediamine tetracetic acid is found to be sparingly soluble in water ($\simeq$ 0.2% w/v) whereas its corresponding disodium salt is almost 50 times more soluble than the parent compound (solubility $\simeq$ 10% w/v). Therefore, it is the disodium salt of EDTA which is normally used in complexometric titrations.

In actual practice, whenever the disodium EDTA solution is added to a solution of a metal ion previously buffered to augment complexation, it has been observed that initially the rate of change of concentration of metal ion is rather slow, but interestingly it picks up quite rapidly as further addition of sodium-EDTA approaches one equivalent.

9.2.5. USAGE OF pM INDICATORS IN COMPLEXOMETRIC TITRATIONS

The equivalence point in complexometric titrations is invariably observed by the help of pM indicators. The relationship amongst pM, concentrations of ligand, chelate complex and stability constant may be established by the following equations :

Assuming K as the stability constant, we have :

$$K = \frac{[MX]}{[M][X]}$$

or $$[M] = \frac{[MX]}{[M][K]}$$

or $$\log [M] = \log \frac{[MX]}{[X]} - \log K$$

or $$p[M] = \log \frac{[X]}{[MX]} - pK \qquad \text{... } (a)$$

Now, considering Eq. (*a*), if a solution is made in such a manner that [X] = [MX], we have :

$$pM = - pK$$

or $$pM = pK' \quad \text{... (}b\text{)}$$

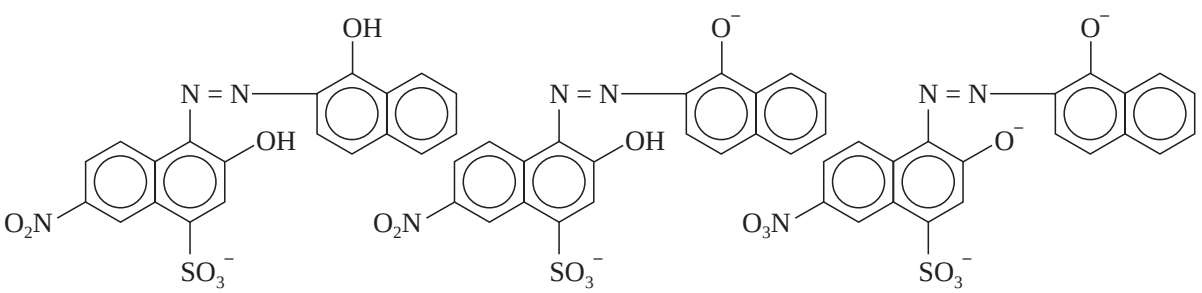
where, K′ is the dissociation constant.

From Eq. (*b*) it may be concluded that a solution having equal activities of free chelating agent and the metal-complex formed, the concentration of metal ions shall remain almost constant and would be buffered exactly in a similar fashion as are H^+ ions in a pH-buffer. As we know that the various chelating agents are mostly basic in character, therefore, the equilibrium attained in a metal-buffer solution is largely influenced by a change in pH. Hence, it may be concluded that the amino acid type chelating agents, such as : ethylenediamine tetracetic acid and ammoniatriacetic acid, when [X] = [MX], pM increases proportionately with pH until it reaches a value pH 10, thereby attaining a constant value. Hence, this particular pH is the '**Ideal pH**' at which complexometric titrations of metals with chelating agents in buffered solution must be performed.

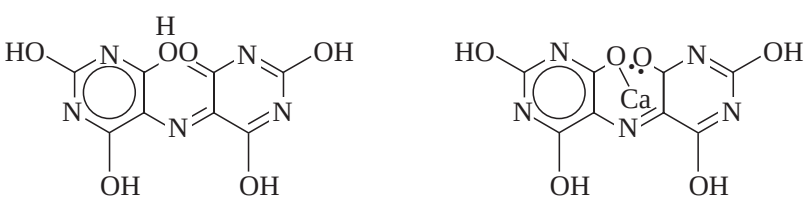
pM Indicator : It is a dye that serves as a chelating agent to yield a *dye-metal complex*, which apparently differs in colour from the original dye, besides possessing a lower stability constant than the corresponding *chelate-metal complex*. Hence, the colour imparted to the solution is mostly attributed due to the dye-complex formed until the end-point, when an equivalent amount of sodium-EDTA has been incorporated. The critical point at which the metal-dye complex decomposes to yield free-dye on addition of the slightest excess of sodium-EDTA, is distinctly shown by a visible change in colour.

Examples :

(*i*) **Mordant Black 2** : (Syn. : Eriochrome Black T ; Solochrome Black T)



(*ii*) **Murexide :** (Syn. : Ammonium Purpurate)



9.3. ASSAY METHODS

The complexometric titrations involving various inorganic pharmaceutical substances may be categorized into *three* broad heads, namely :

(*i*) Direct titration methods,

(*ii*) Masking and demasking agents, and

(*iii*) Residual titration methods.

9.3.1. DIRECT TITRATION METHODS

In direct titration, usually an appropriate buffer solution and a suitable indicator are added to the M^{2+} (metal-ion) solution and subsequently the resulting solution is titrated with previously standardized disodium-EDTA until the indicator just changes colour. Sometimes, a simultaneous blank determination is also recommended to have a check for the presence of traces of metallic impurities in the reagents.

9.3.1.1. Preparation of 0.05 M Disodium Ethylenediamine Tetracetate Solution (Disodium Edetate 0.05 M)

Materials Required : Disodium ethylenediaminetetracetate : 18.6 g.

Procedure : Weigh accurately 18.6 g of disodium ethylenediaminetetracetae, dissolve in sufficient DW in a 1 litre volumetric flask and make up the volume upto the mark.

Calculations :

$$\underset{(372.24)}{C_{l0}H_{14}N_2Na_2O_8,\ 2H_2O} \equiv Ca^{2+} \equiv 1000 \text{ ml M}$$

or 372.24 g $C_{10}H_{14}N_2Na_2O_8, 2H_2O \equiv 1000$ ml M

or 18.612 g $C_{10}H_{14}N_2Na_2O_8, 2H_2O \equiv 1000$ ml 0.05 M

or 0.01861 g $C_{10}H_{14}N_2Na_2O_8, 2H_2O \equiv 1$ ml of 0.05 M Disodium Edetate.

9.3.1.2. Standardization of 0.05 M Disodium Edetate Solution

Materials Required : Granulated zinc : 0.8 g ; dilute HCl (10% w/v of HCl) : 12.0 ml ; bromine water (3 ml Br_2 in 100 ml H_2O) : 5 ml ; sodium hydroxide (2 N) : 20.0 ml ; ammonia buffer (pH 10.0) (dissolve 5.4 g of NH_4Cl in 70 ml of 5 N ammonia and dilute with water to 100 ml) : 100 ml ; Mordant Black II mixture (mixture of 0.2 part Mordant Black II with 100 parts of NaCl) : 50 mg ; disodium edetate : 0.05 M.

Procedure : Weigh accurately about 0.8 g of granulated zinc, dissolve by gentle warming in 12 ml of dilute hydrochloric acid and 5 drops of bromine water. Boil to remove excess bromine, cool and add sufficient DW to produce 200 ml in a volumetric flask. Pipette 20 ml of the resulting solution into a flask and neutralize carefully with 2 N sodium hydroxide. Dilute to about 150 ml with DW, add to it sufficient ammonia buffer (pH 10.0) to dissolve the precipitate and add a further 5 ml quantity in excess. Finally add 50 mg of Mordant Black II mixture and titrate with the disodium edetate solution until the solution turns green. Each 0.003269 g of granulated zinc is equivalent to 1 ml of 0.05 M disodium ethylenediaminetetracetate.

Calculations :

$$ZnCl_2 \equiv Zn^{2+} \equiv 1000 \text{ ml M}$$

or 65.38 g Zn ≡ 1000 ml M

or 3.269 g Zn ≡ 1000 ml 0.05 M

or 0.003269 g Zn ≡ 1 ml of 0.05 M Disodium ethylenediaminetetracetate

9.3.1.3. Calcium Chloride

Materials Required : Calcium chloride dihydrate : 0.15 g ; dilute hydrochloric acid (10% w/w of HCl) : 3.0 ml ; 0.05 M disodium edetate ; sodium hydroxide solution (20% w/v in water) ; calcon mixture (a mixture of 1 part of calcon with 99 parts of freshly ignited anhydrous Na_2SO_4) : 0.1 g.

Equations :

$$Ca^{2+} + [H_2X]^{2-} \longrightarrow [CaX]^{2-} + 2H^+$$

or $CaCl_2.2H_2O \equiv Ca^{2+} \equiv Na_2H_2X, 2H_2O$

or 147.02 g $CaCl_2, 2H_2O \equiv$ 20,000 ml 0.05 M

or 0.007351 g $CaCl_2, 2H_2O \equiv$ 1 ml of 0.05 M Disodium Edetate

Procedure : Weigh accurately about 0.15 g of calcium chloride dihydrate and dissolve it in 50 ml of DW. Titrate with 0.05 M disodium ethylenediamine tetracetate to within a few ml of the expected end point, add 8.0 ml of sodium hydroxide solution and 0.1 g of calcon mixture and continue the titration until the colour of the solution changes from pink to a full blue colour. Each ml of 0.05 M disodium ethylene disodium tetracetate is equivalent to 0.007351 g of $CaCl_2 . 2H_2O$.

9.3.1.4. Magnesium Sulphate

Materials Required : Magnesium sulphate heptahydrate : 0.3 g ; strong ammonia-ammonium chloride solution (6.75 g NH_4Cl in 74.0 ml strong ammonia solution add water q.s. to produce to 100 ml) ; 0.05 M disodium edetate ; Mordant Black II mixture (mixture of 0.2 part mordant black II with 100 parts of NaCl) : 0.1 g.

Equations :

The assay of $MgSO_4.7H_2O$ is based upon the reactions designated by the following equations :

$$Mg^{2+} + [H_2X]^{2-} \longrightarrow [MgX]^{2-} + 2H^+$$

or $MgSO_4.7H_2O \equiv Mg^{2+} \equiv Na_2H_2X, 2H_2O$

or 120.38 g $MgSO_4 \equiv$ 20,000 ml 0.05 M

or 0.00602 g $MgSO_4 \equiv$ 1 ml of 0.05 M Disodium Edetate

Procedure : Weigh accurately about 0.3 g of magnesium sulphate heptahydrate and dissolve in 50 ml of DW. Add to it 10 ml of strong ammonia-ammonium chloride solution, and titrate with 0.05 M disodium ethylenediaminetetracetate employing 0.1 g of mordant black II mixture as indicator, until the pink colour is discharged from the blue. Each ml of 0.05 M disodium ethylenediaminetetracetate is equivalent to 0.00602 g of $MgSO_4$.

9.3.1.5. *Cognate Assays*

A number of pharmaceutical inorganic substances may be assayed by the direct titration method using disodium ethylenediaminetetracetate. A few typical examples are cited in the following Table 9.2.

Table 9.2 : Substances Assayed by Direct Titration with Disodium-EDTA

S.No.	Name of Substance	Qty. Prescribed	Indicator Employed	Calculations
1.	Calcium carbonate	0.1 g	Calcon mixture	Each ml of 0.05 M disodium edetate ≡ 0.005004 g of $CaCO_3$
2.	Dibasic calcium phosphate	0.2 g	Hydroxy naphthol blue	Each ml of 0.05 M disodium edetate ≡ 0.002004 g of Ca
3.	Magnesium chloride	0.5 g	Mordant black II mixture	Each ml of 0.05 M disodium edetate ≡ 0.017017 g of $MgCl_2.6H_2O$
4.	Heavy magnesium oxide	0.1 g	Mordant black II mixture	Each ml of 0.05 M disodium edetate ≡ 0.002015 g of MgO
5.	Magnesium trisilicate (for MaO)	1.0 g	-do-	Each ml of 0.05 M disodium edetate ≡ 0.002015 g of MgO

6.	Zinc chloride	3.0 g	Eriochrome Black-T	Each ml of 0.05 M disodium edetate ≡ 0.006815 g of $ZnCl_2$
7.	Zinc stearate	1 .0 g	-do-	Each ml of 0.05 M disodium edetate ≡ 0.004069 g of ZnO
8.	Zinc sulphate	0.3 g	-do-	Each ml of 0.05 M disodium edetate ≡ 0.01438 g of $ZnSO_4.7H_2O$
9.	Zinc undecylenate	0.5 g	-do-	Each ml of 0.05 M disodium edetate ≡ 0.02160 g of $C_{22}H_{38}O_4Zn$

9.3.2. MASKING AND DEMASKING AGENTS

The disodium ethylenediaminetetracetate usually complexes with a wide spectrum of cations, which ultimately renders the selectivity of the titration procedure adversely, thereby providing enough scope for the accompanying metal impurities to be titrated along with the ion it is aimed at for actual estimation. Therefore, in a situation where one or two ions present in a mixture of cations is specifically required to be determined with a view to eliminate completely the possible effects of unwanted impurities that may enhance the titre value, a third substance is added, which is known as the Masking Agent. These agents must fulfil the following *three* requirements, namely :

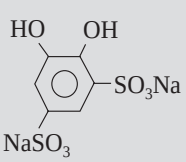
(*a*) should act by precipitation,

(*b*) should form complexes that are definitely more stable than the interfering ion-edetate complex, and

(*c*) colour developed by either precipitates or auxiliary complexes should not obscure the end-point.

A few typical examples are cited below in Table 9.3 where masking has been accomplished by precipitation.

Table 9.3 : Masking Accomplished by Precipitation

S.No.	Interfering Heavy Metal Ions	Complexing Agent	Remarks
1.	Co^{2+}, Cu^{2+}, Pb^{2+}	Na_2S (sodium sulphide), CH_3CSNH_2 (Thioacetamide)	As insoluble sulphides and complexes
2.	Cu^{2+}	$HS.CH_2.CHOH.CH_2OH$ (Thioglycerol)	As insoluble complex
3.	Al^{3+}, Fe^{3+}, Ti^{3+}	NH_4F (Ammonium fluoride)	Complex formation
4.	Hg^{2+}, Cd^{2+}, Zn^{2+}, As^{3+}, Sb^{3+}, SN^{4+}, Pb^{2+}, Bi^{2+}	$HSCH_2.CHSH.CH_2OH$ (Dimercaprol)	Precipitation in weakly acidic medium while soluble in alkaline medium
5.	Hg^{2+}	KI (Potassium iodide)	Masks Hg^{2+} as HgI_4^{4-}
6.	Al^{3+}, Ti^{3+}	 (Disodium catechol-3, 5-disulphonate)	Forms colourless complexes
7.	Al^{3+}, Fe^{3+}, Mn^{3+}	$[N(CH_2CH_2OH)_3]$ (Triethanolamine)	Al-complex : colourless ; Fe-complex : Yellow ; Mn-complex : Green

9.3.3. RESIDUAL TITRATION METHODS

Direct titration method offers a serious limitation for the assay of aluminium and bismuth containing pharmaceutical inorganic substances because of the precipitation of the metal as their corresponding hydroxides in alkaline media thereby introducing undesirable errors.

In actual practice, an excess of the standard solution of disodium edetate is added to the sample, pH is adequately adjusted for the residual titration with a metal-ion solution *e.g.*, $ZnSO_4$ and employing an appropriate indicator which is sensitive enough to the respective titrant. However, the metal ion under estimation remains firmly complexed with the EDTA and offers little interference with the Zn-EDTA complex formed. It has been established experimentally that bismuth readily yields a highly stable complex which may be titrated conveniently between pH 1 and 2. Bismuth forms a stable complex by reacting with EDTA quantitatively at pH 4.0 and, therefore, dithizone is employed as an indicator to detect the end-point for it has a transition state of colour at pH 4.6.

9.3.3.1. Potassium Alum, $KAl(SO_4)_2$, $12H_2O$

Materials Required : Potassium alum : 1.7 g ; 0.05 M disodium edetate : 30.0 ml ; hexamine : 1.0 g ; 0.05 M lead nitrate ; xylenol orange solution (0.1% w/v in water) : 0.4 ml.

Theory : The solution of potassium alum is heated with an excess of disodium edetate to ensure complete formation of aluminium-edetate complex. Hexamine serves as a buffer thereby stabilizing the pH between 5 and 6, the ideal pH for the titration of the disodium edetate not required by the Al with 0.05 M lead nitrate employing xylenol orange as indicator. The various reactions involved may be represented by the following equations :

$$Al^{3+}\ [H_2X]^{2-} \longrightarrow [AlX]^- + 2H^+$$

or $$KAl(SO_4)_2,\ 12H_2O \equiv Al^{3+} \equiv Na_2H_2X,\ 2H_2O$$

or 474.4 g $KAl(SO_4)_2,\ 12H_2O \equiv$ 20,000 ml 0.05 M

or 0.02372 g $KAl(SO_4)_2,\ 12H_2O \equiv$ 1 ml of 0.05 M Disodium Edetate

Procedure : Weigh accurately 1.7 g of potassium alum and dissolve it in suffcient DW in a flask. Heat the contents of flask over a water-bath for 10 minutes to allow completion of complexation and cool to ambient temperature. Now, add 1 g hexamine to act as buffer and titrate with 0.05 M lead nitrate employing 0.4 ml of xylenol orange solution as an indicator. The colour shall change from that of the indicator (yellow at the pH of the titration) to the corresponding reddish purple, the colour of the lead complex of the indicator. Each ml of 0.05 M disodium edetate is equivalent to 0.02372 g of $KAl(SO_4)_2,\ 12H_2O$.

9.3.3.2. Glycobiarsol [Bismethyl-N-glycolyl-arsanilate]

Materials Required : Glycobiarsol : 0.2 g ; 0.05 M disodium edetate : 10.0 ml ; acetic acid-ammonium acetate buffer (mix 13.6 g of sodium acetate and 7.7 g of ammonium acetate in water to make 100 ml. Add 25.0 ml of glacial acetic acid and mix) : 10.0 ml ; alcohol : 25.0 ml : dithizone solution (0.05% w/v in chloroform) : 2.0 ml ; 0.025 M $ZnSO_4$ solution.

Procedure : Weigh accurately 0.20 g of glycobiarsol into a 250-ml conical flask and add 10.0 ml of 0.05 M disodium edetate. Warm the contents of the flask over a water-bath until glycobiarsol gets dissolved completely and then cool the contents to the room temperature (25°C). Add to it 10.0 ml of acetic acid-ammonium acetate buffer, 25.00 ml of alcohol and 2 ml of dithizone solution as an indicator. Titrate the excess of disodium edetate with 0.025 M zinc sulphate until the resulting solution turns rose pink in colour. Each millilitre of 0.05 M disodium edetate consumed is equivalent to 10.45 mg of Bi.

Note : The content of Bi, calculated on dried basis, lies between 38 to 42.5%.

9.3.3.3. Cognate Assays

A number of inorganic pharmaceutical substances may be assayed by adopting the residual titration method as depicted in Table 9.4.

Table 9.4 : Substances Assayed by Residual Titration with EDTA

S.No.	Name of Substance	Qty. Prescribed	Indicator Employed	Calculations
1.	Aluminium glycinate	0.25 g	Methyl Red	Each ml of 0.05 M disodium edetae ≡ 0.002549 g of Al_2O_3
2.	Dried Aluminium Hydroxide	0.8 g	-do-	Each ml of 0.1 M disodium edetate ≡ 0.005098 g of Al_2O_3
3.	Aluminium sulphate	0.5 g	-do-	Each ml of 0.1 M disodium edetate ≡ 0.01711 g of $Al_2(SO_4)_3$
4.	Bismuth subcarbonate	0.5 g	-do-	Each ml of 0.1 M disodium edetate ≡ 0.02090 g of Bi

THEORETICAL AND PRACTICAL EXERCISES

1. What is the underlying principle of '**Complexometric titrations**' ? Give appropriate examples in support to your answer.

2. Discuss the following aspects in an elaborated fashion :

(*i*) Number of '**rings**' formed in the complex with bivalent, trivalent and tetravalent metal ions.

(*ii*) Effect of pH on complexation

(*iii*) Stability of complexes

(*iv*) Usage of pM indicators in complexometric titrations

(*v*) Titrability of polyvalent metal ions employing disodium EDTA.

3. How would your carry out complexometric titrations by the '**direct titration method**' ? Discuss the assay of the following pharmaceutical drugs explicitely :

(*i*) Magnesium sulphate (*ii*) Calcium carbonate

(*iii*) Dibasic calcium phosphate (*iv*) Zine undecylenate.

4. What are 'masking and demasking agents' with reference to complexometric titrations ? Give specific examples to justify your statements.

5. How does '**residual titration method**' help in the complexometric titrations ? Elaborate the assay of the following drugs by this technique :

(*i*) Potassium alum (*ii*) Bismuth subcarbonate

(*iii*) Aluminium glycinate (*iv*) Dried aluminium hydroxide.

RECOMMENDED READINGS

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E. GRAVIMETRIC METHODS

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10 GRAVIMETRIC ANALYSIS

CONTAINS :

10.1. INTRODUCTION

Gravimetric analysis is an unique technique by means of which either an element or a compound is obtained in its purest form through isolation and subsequent weighing. In order to achieve this, the element or compound is first and foremost separated from a specific portion of the pharmaceutical substance being determined and consequently the weight of the constituent in the given sample is calculated on the basis of the weight of the product.

However, in actual gravimetric analysis, the final weight of the product is usually accomplished by adopting anyone of the following standard methods, namely :

(*a*) Solvent extraction,

(*b*) Ignition or volatalization, and

(c) Precipitation from solution.

Gravimetric techniques are broadly based upon the quantitative precipitation of the respective cation or anion from a given solution in *two* different ways :

(*i*) as an insoluble compound that yields a residue having a specific composition after ignition, and

(*ii*) as an insoluble compound having a known composition.

There are *four* vital steps that are essentially required for a successful gravimetric method, namely :

(*a*) Identify an insoluble form with a definite composition,

(*b*) Separate the analyte exclusively from another constituents which may cause interference,

(*c*) Wash the precipitate free of coprecipitants and impurities as far as possible, and

(*d*) Convert the precipitate ultimately to a reasonably measurable form.

10.2. THEORY

The underlying principles and theories of gravimetric analysis are as stated below :

(*i*) Law of mass action and reversible reactions,

(*ii*) Principle of solubility product, and

(*iii*) Common ion effect.

All the above three aspects shall be described briefly *vis-a-vis* their direct impact on the gravimetric analysis.

10.2.1. LAW OF MASS ACTION AND REVERSIBLE REACTIONS

A plethora of chemical reactions that are intimately associated with the quantitative analysis essentially belong to the class of reversible reactions. These reactions under certain prevailing experimental parameters are made to proceed to completion, whereas in certain other conditions they may even attain equilibrium before completion. In the latter instance, erroneous results may creep in with regard to the pharmaceutical substance under estimation. Hence, it has become absolutely necessary first to establish the appropriate conditions whereby the reactions must move forward to attain completion so as to achieve the ultimate objective in all quantitative assays.

In general, there are *three* cardinal experimental parameters that must be observed rigidly in order to check the reversal processes and help the completion of a reaction, namely :

(*a*) formation of very slightly ionized molecules,

(*b*) formation of an insoluble gas, and

(*c*) formation of a sparingly soluble solid.

The '**law of mass action**' advocates that the rate of a reaction is directly proportional to the product of the molecular concentrations of the reacting substances. For example :

$$BaCl_2 + H_2SO \rightleftharpoons BaSO_4 + 2HCl$$

In the above reaction the rate of reaction of barium chloride with sulphuric acid is designated by the following expression :

Forward reaction :

$$\text{Rate} = [BaCl_2] \times [H_2SO_4] \times k \qquad ...(a)$$

where, k = a constant that corrects for all factors which affect the rate other than concentration.

Likewise, in the opposing reaction, we have :

Opposing reaction :

$$\text{Rate} = [BaSO_4] \times [HCl] \times k_1 \qquad ... (b)$$

where, k_1 = another constant.

At equilibrium the rates of the forward reaction (*a*) and opposing reaction (*b*) are equal. Hence, we have :

$$[BaCl_2] \times [H_2SO_4] \times k = [BaSO_4] \times [HCl] \times k_1 \qquad ...(c)$$

Rearranging (*c*) we have :

$$\frac{[BaCl]_2 \times [H_2SO_4]}{[BaSO_4][HCl]} = \frac{k}{k_1} = K \qquad ... (d)$$

As k and k_1 are constants, their quotient K is also a constant known as the equilibrium constant.

From Eq. (*d*), K, the equilibrium constant has a fixed value at a definite temperature, irrespective of concentrations of other components present.

Therefore, if the concentration of sulphuric acid is enhanced, consequently all other concentrations should change accordingly, the concentration of $BaCl_2$ must become less and that of both $BaSO_4$ and HCl be proportionately greater so as to maintain the equilibrium constant, thereby having the net impact of shifting the equilibrium towards the right hand side. Evidently, in most quantitative analysis one entity is added invariably to allow the reaction to proceed as closely to completion as possible.

10.2.2. PRINCIPLE OF SOLUBILITY PRODUCT

The principle of solubility product may be stated as follows :

'*The product of the concentration of the constituent ions in a saturated solution of a difficultly soluble salt for any given temperature is practically a constant, each concentration being raised to a power equal to the relative number of ions supplied by one molecule of the salt upon dissociating*'.

The principle of solubility product is applicable to :

(*i*) difficultly soluble salts in their saturated solutions,

(*ii*) occurrence of precipitation,

(*iii*) prevention of precipitation, and

(*iv*) dissolution of a substance.

For instance, a difficultly soluble salt A*p*B*q* on dissociation provides a relative number of *p* cations and *q* anions. Thus, we have :

$$A_pB_q \rightleftharpoons pA^+ + qB^-$$

Hence, solubility product $A_pB_q = [A^+]^p \times [B^-]^q$

where, [ ] are generally used to express the molar concentrations.

Table 10.1, contains the solubility products of certain difficultly soluble salts generally encountered in pharmaceutical analysis.

Table 10.1 : Solubility Products of Important Inorganic Salts

S.No.	Name of Substance	Temp. °C	Ions Involved	Solubility Product
1.	Aluminium hydroxide	*a*	$Al^{3+} + 3OH^-$	1×10^{-33}
2.	Barium sulphate	*a*	$Ba^{2+} + SO_4^{2-}$	1.1×10^{-10}
3.	Calcium oxalate	*a*	$Ca^{2+} + C_2O_4^{2-}$	2.6×10^{-9}
4.	Lead sulphate	*b*	$Pb^{2+} + SO_4^{2-}$	1.1×10^{-8}
5.	Magnesium oxalate	*b*	$Mg^{2+} + C_2O_4^{2-}$	8.8×10^{-5}
6.	Mercuric sulphide	*a*	$Hg^{2+} + S^{2-}$	1×10^{-50}
7.	Silver chloride	*a*	$Ag^+ + Cl^-$	1.5×10^{-10}
8.	Silver thiocyanate	*a*	$Ag^+ + SCN^-$	1.2×10^{-12}

a = 25°C ; *b* = 18°C.

The interaction of $AgNO_3$ and NaCl results into the formation of AgCl which is slightly soluble in water, the solubility being approximately 0.00001 ml litre^{-1} *i.e.*, 1.5 mg litre^{-1}. On exceeding this concentration, the AgCl gets precipitated which remains in equilibrium with the dissolved AgCl. Therefore, at equilibrium, the clear supernatant liquid is a saturated solution, and at this critical juncture the rate at which the dissolved salt gets precipitated is almost equal to the rate at which the solid undergoes dissolution. This establishes the following equilibria :

$$\underset{\text{Solid precipitate}}{AgCl} \rightleftharpoons \underset{\text{Dissolved unionized}}{AgCl} \rightleftharpoons \underset{\text{Dissolved ionized}}{Ag^+ + Cl^-}$$

Hence, the ionization equilibrium may be expressed as follows :

$$\frac{[Ag^+] \times [Cl^-]\ \text{ionized}}{AgCl\ \text{unionized}} = K$$

where, K = ionization constant.

Considering the following *two* assumptions :

(*i*) solution remains saturated with AgCl at a aiven temperature, and

(*ii*) concentration of unionized AgCl remains constant, it follows, that the product K × [AgCl] too becomes constant.

Therefore, it may be inferred that—'*in a saturated solution of a difficultly soluble salt, the product of the molecular concentration of its ions is constant*'.

For instance :

(*a*) $BaSO_4 \rightleftharpoons Ba^{2+} + SO_4^{2-}$

$[Ba^{2+}] \times [SO_4^{2-}]$ = Solubility product of $BaSO_4$

(*b*) $CaC_2O_4 \rightleftharpoons Ca^{2+} + C_2O_4^{2-}$

$[Ca^{2+}] \times [C_2O_4^{2-}]$ = Solubility product of CaC_2O_4

(*c*) $Al(OH)_3 \rightleftharpoons Al^{3+} + 3OH^-$

$[Al^{3+}] \times [OH^-]\ 3$ = Solubility product of $Al(OH)_3$

It is an usual practice to express the concentration of the solubility product in terms of moles per litre *i.e.*, molar concentrations.

10.2.3. COMNION ION EFFECT

It has been observed that there is no change in the equilibrium constant even if :

(*a*) the concentrations of reacting components may change, and

(*b*) the relative concentration of the reacting substances may change.

When a solution of $BaCl_2$ is added to a solution of sulphuric acid, the sulphate ion for a while is present in a concentration in such a manner that its ionic product with the barium ion exceeds the solubility product of barium sulphate, and the insoluble barium sulphate gets precipitated :

$$Ba^{2+} + SO_4^{2-} \longrightarrow BaSO_4 \downarrow$$

However, at equilibrium the concentration of Ba^{2+} ions shall be exactly equal to the concentration of sulphate ions.

Now, if to the resulting supernatant liquid, which is nothing but a saturated solution of barium sulphate, an additional small quantity of either a soluble barium salt or a soluble sulphate is provided, a slight further precipitation may occur.

Hence, the equilibrium that represents the ionization constant may be expressed as :

$$\frac{[Ba^{2+}] \times [SO_4^{2-}]}{[BaSO_4]} = K \qquad \ldots (a)$$

From Eq. (*a*), it may be derived that if the concentration of Ba^{2+} ion is enhanced by the addition of a soluble barium salt, the concentration of sulphate ion should decrease simultaneously and conversely, that if the concentration of sulphate ion is enhanced by the addition of a soluble sulphate salt, the concentration of Ba^{2+} ion should decrease as their product almost remains constant. Evidently, this decrease in the concentration of the ions in either instance may be achieved by the combination of barium and sulphate ions to give rise to the insoluble barium sulphate thereby forcing the reaction towards completion.

In short, the **common-ion effect** is employed invariably in carrying out the gravimetric analysis of pharmaceutical substances so as to drive reactions toward completion.

Calculations : In gravimetric analysis the percentage of the desired constituent may be achieved by the following expression :

$$\text{Percentage of desired constituent} = \frac{\text{Wt. of precipitate} \times \text{Gravimetric factor}}{\text{Wt. of sample}} \times 100$$

The term '**gravimetric factor**' is generally employed which represents the number of grams of the desired constituent in 1 g of the substance weighed. It can be further expatiated with the help of the following examples :

(*i*) One mole of $BaSO_4$ (233.39 g) contains one mole of SO_4 atoms (96.06 g).

$$\text{Hence, the Gravimetric Factor} = \frac{SO_4}{BaSO_4} = \frac{96.06}{233.39} = \mathbf{0.4116}$$

(*ii*) One mole of AgCl (143.323 g) contains one mole of Cl atoms (35.453 g).

$$\text{Hence, the Gravimetric Factor} = \frac{Cl}{AgCl} = \frac{35.453}{143.323} = \mathbf{0.2474}$$

10.3. ASSAY METHODS

A good number of pharmaceutical substances can be assayed gravimetrically. The gravimetric methods adopted vary according to the nature of the substance under determination. However, most of the substances being estimated gravimetrically fall into one or the other categories stated below, which would be discussed briefly with suitable examples :

(*a*) Substances assayed gravimetrically,

(*b*) Substances assayed after conversion :

(*i*) Substances assayed after conversion to Free Acid,

(*ii*) Substances assayed after conversion to Free Base,

(*iii*) Substances assayed after conversion to Free Compound, and

(*iv*) Substances assayed after conversion to Derivatives or Substitution Products.

10.3.1. SUBSTANCES ASSAYED GRAVIMETRICALLY

A good number of pharmaceutical substances may be determined gravimetrically by obtaining their respective difficultly soluble salts as precipitates, weighing to a constant weight and finding the percentage purity of the substance in question.

A few typical examples are cited below so as to expatiate the procedure as well as the theoretical aspects.

10.3.1.1. Sodium Chloride

Materials Required : Sodium chloride : 0.25 g ; 5% w/v silver nitrate in DW (+ 2-3) drops of conc. HNO_3 ; dilute nitric acid (6 N) ; asbestos fibre.

Theory : The following reaction forms the basis for the calculation of the theoretical amount of silver nitrate solution required as well as the purity of the given sample of NaCl. Thus, we have :

$$\underset{169.87}{AgNO_3} + \underset{58.44}{NaCl} \longrightarrow \underset{143.22}{AgCl} + \underset{84.99}{NaNO_3}$$

Therefore, $NaCl \equiv AgNO_3$

or $$1 \text{ g of NaCl} \equiv \frac{169.87}{58.44} \equiv 2.9067 \text{ g of } AgNO_3$$

As 0.2570 g of NaCl has been used (from experimental data); therefore, the exact amount of $AgNO_3$ required would be :

$$0.2570 \times 2.9067 = 0.7470 \text{ g of } AgNO_3$$

(considering NaCl to be 100% pure)

The $AgNO_3$ solution is 5% w/v :

or $$1 \text{ ml of } 5\% \ AgNO_3 \equiv 0.05 \text{ g of } AgNO_3.$$

Hence, the amount of $AgNO_3$ solution required theoretically would be 0.7470/0.05 = 14.94 ml.

From above, the percentage purity of the given sample of NaCl may be found as shown below :

$$\frac{58.44}{143.22} = 0.4078 \text{ g of NaCl} \equiv 1 \text{ g AgCl}$$

The weight of AgCl is found to be 0.6288 g experimentally, or 0.4078 is the 'gravimetric factor'.

Consequently, the percentage purity of the sample is determined by the formula :

$$\frac{W \times E \times 100}{S} = \%$$

where, W = Wt. of the product of a chemical reaction with the substance under determination,

E = Gravimetric Factor, and

S = Wt. of the sample.

By incorporating the data given above, the amount of sodium chloride present in 100 g of the sample *i.e.*, the percentage purity of NaCl in the given sample may be calculated as follows :

$$\frac{0.6288 \times 0.4078 \times 100}{0.2570} = 99.77\%$$

Procedure : Weigh accurately between 0.20 to 0.30 g of sodium chloride and dissolve in 100 ml of DW. Add to it 1 ml of dilute nitric acid gradually with constant stirring. Check and confirm that the resulting solution is acidic with the help of blue litmus paper. Measure out 5.0 ml in excess of the amount of silver nitrate solution calculated on theoretical basis to precipitate all the available chlorine as silver chloride. The requisite quantity of silver nitrate solution must be added in small lots at intervals with constant stirring with a glass rod. Cover the beaker with a watch-glass and boil the contents very gently with occasional stirring (to avoid bumping of the liquid and loss of volume). Stop heating and digest the mixture for 10 minutes so as to agglomerate the precipitate and enhance settling thereby leaving a clear supernatant liquid. Add 2 drops of silver nitrate solution to the hot supernatant liquid in order to confirm whether precipitation is completed. Keep the beaker away from direct sunlight to allow the precipitate to settle.

Take a properly prepared Gooch crucible, heat to constant weight and fit it into the suction flask. Decant most of the supernatant liquid first into the Gooch crucible by applying gentle suction to hasten filtration. Wash the precipitate on the Gooch crucible at least thrice with 15 ml portions of 0.01 N nitric acid.

Test the above filtrate to be free of $AgNO_3$. Finally wash the precipitate twice with 5 ml portion of DW to get rid of most of the HNO_3 previously retained by the precipitate from the former wash solution. Now, apply vigorous suction to drain out the liquid from the precipitate to the maximum extent. Dry the crucible to a constant weight between 110-120°C in an electric oven until two concurrent weighings are achieved. Thus, the weight of the crucible (tare) must be deducted from the weight of the crucible plus the precipitate to arrive at the weight of silver chloride duly obtained from the sample.

Precautions :

(1) The solution of the substance is usually acidified with HNO_3 to check the precipitation of other substances insoluble in water but soluble in HNO_3 *e.g.*, CO_3^{2-}, O^{2-} and PO_4^{3-}. Besides HNO_3 also helps to coagulate any colloidal AgCl,

(2) The excess of HNO_3 must be avoided to cause solvolysis of silver halides,

(3) Heating should be affected only after the addition of $AgNO_3$, otherwise Cl_2 may be liberated and lost. Thus, we have :

$$NaCl + HNO_3 \longrightarrow HCl + NaNO_3$$

$$6HCl + 2HNO_3 \longrightarrow 3Cl_2 \uparrow + 4H_2O + 2NO$$

(4) The precipitation should preferably be carried out in the absence of strong light because AgCl undergoes decomposition in sunlight with loss of Cl_2,

(5) Washing of the precipitate (AgCl) with 0.01 N HNO_3 is always recommended to prevent loss of AgCl by virtue of its return to colloidal condition (peptization) and to get rid of the soluble salts, namely : AgNO and $NaNO_3$, and

(6) AgCl is significantly volatile on ignition, hence it must always be dried at a comparatively lower temperature.

10.3.1.2. Potassium Alum, $KAl(SO_4)_2$, $12H_2O$

Theory : The percentage of Al in potassium alum can be determined volumetrically by complexometric titration (see Chapter : 9).

However, gravimetric procedure provides a fairly reliable and useful alternative method of analysis for Al which may be accomplished by :

(*a*) precipitation from a solution of the aluminium salt by the addition of NH_4OH in the presence of NH_4Cl, and

(*b*) complexation from a solution of the aluminium salt with 8-hydroxyquinoline (oxine) either from an ammoniacal solution or from acetic acid-acetate buffer.

In the first method, the following reaction takes place :

$$Al^{3+} + 3OH^- \longrightarrow Al(OH)_3 \downarrow$$

The gelatinous white precipitate of $Al(OH)_3$ is duly filtered, washed with dilute NH_4NO_3 solution, transformed to the corresponding oxide and finally weighed as Al_2O_3 .

Disadvantages : There are a number of serious disadvantages of this method, namely :

(*i*) excess of NH_4OH may directly affect the solubility of $Al(OH)_3$,

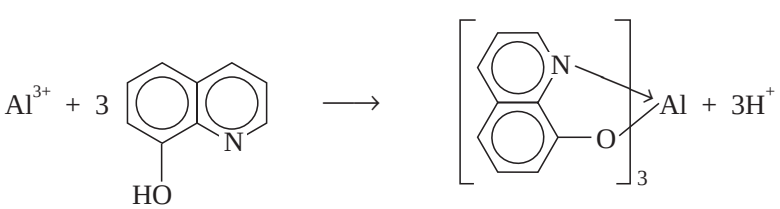
(*ii*) coprecipitation of metal hydroxides that are usually soluble in NH_4OH,

(*iii*) heated oxide (Al_2O_3) is hygroscopic in nature, and

(*iv*) hydroxides may not undergo complete thermal decomposition.

Due to the above short-comings, the second method is usually preferred which shall be discussed below :

Equation :



(Oxine)

Aluminium-oxine complex
(Aluminium-oxinate)

The resulting precipitate of aluminium-oxine complex is crystalline in nature and hence can be filtered conveniently, washed with water and finally dried at 130-150°C to constant weight.

Disadvantages : There are *two* disadvantages of the metal-oxine-complex method, namely :

(*i*) aluminium-oxinate is prone to adsorb oxine, and

(*ii*) lack of selectivity of oxine such that all metals except the alkaline earths (Ba, Mg, Ca, Sr, Be) and alkali (Li, Na, K, Rb, Cs) should be totally absent.

Calculations :

$$KAl(SO_4)_2, 12H_2O \equiv Al(C_9H_6NO)_3$$

or $$26.98 \text{ g Al} \equiv 459.4 \text{ g } Al(C_9H_6NO)_3$$

or $$0.05873 \text{ g Al} \equiv 1 \text{ g of } Al(C_9H_6NO)_3$$

Materials Required : Potassium alum : 0.3 g ; 0.1 N hydrochloric acid : 1.0 ml ; 8-hydroxyquinoline reagent (or oxine-reagent) (25 ml of a 2% w/v solution of oxine in 2 N acetic acid) ; 2 N ammonium acetate (dissolve 15.0 g of ammonium acetate in 20.0 ml of DW, add 0.3 ml of glacial acetic acid and dilute to 100 ml with DW) ; sintered glass crucible No : 3 or 4.

Procedure : Weigh accurately about 0.3 g of potassium alum in a 400-ml beaker, dissolve it in 150 ml of DW containing 1.0 ml of 0.1 N HCl and warm the contents of the beaker to about 60°C. Add the requisite quantity of the oxine reagent and then add a 2 N solution of ammonium acetate gradually from a pipette till precipitation just commences. Add a further portion (50 ml) of ammonium acetate solution with vigorous stirring. Allow the contents of the beaker to stand for 60 minutes with frequent stirring. Filter the precipitate through No : 3 or 4 sintered glass crucible that has been previously dried to a constant weight at 130—150°C. Wash the precipitate throughly with cold DW and dry at 130 to 150°C to constant weight. Each gram of aluminium oxinate is equivalent to 0.05873 g of Al.

10.3.1.3. Cognate Assays

A good deal of pharmaceutical substances are officially assayed gravimetrically as appears in Table 10.2.

Table 10.2 : Substances Assayed Gravimetrically

S.No.	Name of Substance	Qty. Prescribed	Drying Temp. (°C)	Calculations
1.	Barium sulphate	0.60 g	105	Each g of the residue ≡ 0.9213 g of $BaSO_4$
2.	Fluorescein sodium	0.50 g	105	Each g of the residue ≡ 1.132 g of $C_{20}H_{10}Na_2O_5$
3.	Piperazine adipate	0.20 g	105	Each g of the residue ≡ 0.4268 g of $C_4H_{10}N_2$, $C_6H_{10}O_4$
4.	Piperazine hydrate	0.20 g	105	Each g of the residue ≡ 0.3567 g of $C_4H_{10}N_2$, 6 H_2O
5.	Piperazine phosphate	0.20 g	105	Each g of residue ≡ 0.3382 g of $C_4H_{10}N_2$, H_3PO_4
6.	Piperazine phosphate Tabs.	0.15 g	105	Each g of the residue ≡ 0.3714 g of $C_4H_{10}N_2$, H_3PO_4, H_2O
7.	Quinalbarbitone Tablets	0.10 g	105	Each g of the residue ≡ 1.092 g of $C_{12}H_{17}N_2NaO_3$
8.	Quiniodochlor Tablets	0.10 g	105	Each g of the residue ≡ 0.91 g of C_9H_5ClNO
9.	Sodium aurothiomalate (For Na)	0.2 g	600	Each g of the residue ≡ 0.03237 g of Na
10.	Sulphobromophthalein sodium (For Sulphur)	0.2 g	600	Each g of the residue ≡ 0.1374 g of S
11.	Thiocarbazone	0.1 g	105	Each g of the residue ≡ 0.4606 g of $C_{10}H_{12}N_4OS$

10.3.2. SUBSTANCES ASSAYED AFTER CONVERSION

There are certain pharmaceutical substances that can be assayed gravimetrically after their suitable conversion to free acid, or free base, or free compound or corresponding derivatives (or substitution products). All these typical cases shall be discussed briefly with their appropriate examples in the following sections.

10.3.2.1. Substances Assayed after Conversion to Free Acid

A few official pharmaceutical substances may be assayed gravimetrically by affecting separation, purification, and weighing an organic medicinal compound without causing any permanent change in composition. It is an usual practice that before extraction of the organic medicinal compound, the sample of the crushed tablets is carefully washed with petroleum benzene to get rid of undesirable components, for instance : lubricants and binders that would be extracted along with the organic medicinal compound by such solvents as ether or chloroform which is employed subsequently.

In case, the organic medicinal compound is acidic in nature *e.g.*, amobarbital in sodium amobarbital tablets, it is first and foremost extracted with an aqueous solution of an acid or base to cause separation from the neutral substance which might be present. The resulting aqueous solution of the salt of the respective organic medicinal compound is subsequently made acidic and the liberated organic acid (amobarbital) is finally extracted with ether or chloroform.

Interestingly, in a situation where either magnesium stearate or stearic acid forms a component in the formulation, the organic medicinal compound which is acidic (amobarbital) cannot be extracted with NaOH solution for obvious reason that sodium stearate shall also be extracted along with the salt of the organic acid. Therefore, instead a saturated solution of $Ba(OH)_2$ is employed thereby the insoluble precipitate of barium stearate may be discarded by filtration.

10.3.2.1.1. *Phenobarbitone Sodium*

Materials Required : Phenobarbitone sodium : 0.5 g ; hydrochloric acid (2 M) : (dissolve 17.0 ml ($\simeq$ 11.5 N) in 100 ml DW) : 5.0 ml ; ether : 13.5 ml ; absolute ethanol : 2.0 ml.

Procedure : Weigh accurately 0.5 g phenobarbitone sodium and dissolve in 15 ml of DW. Add to it 5 ml of 2 M hydrochloric acid and extract with 50 ml of ether and then with successive 25 ml quantities of ether until complete extraction is affected. Wash the combined extracts with two 5 ml quantities of DW and wash the combined aqueous extracts with 10 ml quantities of ether. Add the ether to the main ethereal extract, evaporate to low bulk, add 2 ml of absolute ethanol, evaporate to dryness and dry the residue to constant weight at 105°C. Each g of residue is equivalent to $C_{12}H_{11}N_2NaO_3$.

Calculations :

$$C_{12}H_{11}N_2NaO_3 \equiv C_{l2}H_{l2}N_2O_3$$

or $$254.2 \text{ g } C_{12}H_{11}N_2NaO_3 \equiv 232.2 \text{ g } C_{12}H_{12}N_2O_3$$

or $$1.095 \text{ g } C_{l2}H_{11}N_2NaO_3 \equiv 1 \text{ g of } C_{l2}H_{12}N_2O_3$$

10.3.2.1.2. *Cognate Assays*

There are certain pharmaceutical substances that may be assayed after their conversion to the respective free acids as shown in Table 10.3.

Table 10.3 : Substances Assayed Gravimetrically by Conversion to Free Acid

S.No.	Name of Substance	Qty. Prescribed	Drying Temp. (°C)	Calculations
l.	Amobarbital sodium	0.5 g	l05	Each g of residue ≡ 1.097 g of $C_{11}H_{17}N_2NaO_3$
2.	Pentobarbital sodium Tablets	0.3 g	105	Each g of residue ≡ 0.1097 g of $C_{11}H_{17}N_2NaO_3$
3.	Phenytoin sodium	0.3 g	105	Each g of residue ≡ 1.087 g of $C_{l5}H_{11}N_2NaO_2$
4.	Secobarbital sodium	0.5 g	100	Each g of the residue ≡ 1.092 g of $C_{12}H_{17}N_2NaO_3$

10.3.2.2. Substances Assayed after Conversion to Free Base

In a specific instance where the organic medicinal substance is basic in nature *e.g.*, papaverine in papaverine hydrochloride, it is primarily treated with an aqueous solution of a base and subsequently the liberated organic base is extracted with either chloroform or ether.

A typical example is described below :

10.3.2.2.1. *Papaverine Hydrochloride Tablets*

Materials Required : Sodium hydroxide (2 M) (dissolve 8.0 g of NaOH pellets in 100 ml of CO_2 free DW : 50 ml ; chloroform : 100 ml ; absolute ethanol : 5 ml.

Calculations :

$$C_{20}H_{21}NO_4 \,.\, HCl \equiv C_{20}H_{21}NO_4$$

or $$374.45 \text{ g } C_{20}H_{21}NO_4 \,.\, HCl \equiv 339.0 \text{ g } C_{20}H_{21}NO_4$$

or $$1.105 \text{ g } C_{20}H_{21}NO_4 \,.\, HCl \equiv 1 \text{ g of } C_{20}H_{21}NO_4$$

Procedure : Weigh 20 tablets and crush them in a pestle mortar and find out the average weight of a single tablet. Accurately weigh 0.5 g equivalent of papaverine hydrochloride and dissolve in 15 ml of DW. Add to it 15 ml of 2 M sodium hydroxide and extract with 50 ml of chloroform and then with successive 25 ml quantities of chloroform until complete extraction is affected. Wash the combined extracts with two 5 ml quantities of DW and wash the combined aqueous extract with two 10 ml quantities of chloroform. Add the chloroform to the main chloroform extract, evaporate to a small volume, add 2 ml of absolute ethanol, evaporate to dryness and dry the residue to constant weight at 105°C.

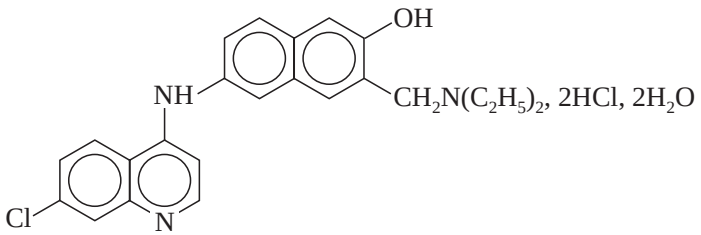
Each g of the residue is equivalent to 1.105 g of $C_{20}H_{21}NO_4$. HCl.

10.3.2.2.2. *Amodiaquine Hydrochloride*

Materials Required : Amodiaquine hydrochloride : 0.3 g ; dilute ammonia solution (42.5 ml of strong ammonia solution to 100 ml in water) ; NO. 4 sintered glass crucible.

Theory : Amodiaquine hydrochloride possesses two moles of inherent water of crystallization, and hence the precentage base is calculated with reference to the substance dried over P_2O_5 at a pressure not exceeding 5 mm of Hg. Usually, the assay is performed on one portion of the sample and the drying on a separate portion altogether.

The underlying principle of the method is based upon the precipitation of amodiaquine base that is generated as a precipitate when the salt is decomposed in aqueous medium with dilute ammonia.



$$C_{20}H_{22}ON_3Cl,\ 2HCl,\ 2H_2O + 2NH_3 \longrightarrow \underset{\text{Amodiaquine base}}{C_{20}H_{22}ON_3}\downarrow + 2NH_4Cl + 2H_2O$$

or 464.35 g $C_{20}H_{22}ON_3Cl$, 2HCl, $2H_2O \equiv$ 355.4 g $C_{20}H_{22}ON_3$

or 1.306 g $C_{20}H_{22}ON_3Cl$, 2HCl, $2H_2O \equiv$ 1 g of $C_{20}H_{22}ON_3$

Procedure : Weigh accurately 0.3 g of previously dried amodiaquine hydrochloride into a 100 ml beaker provided with a stirring rod and watch glass cover. Dissolve it in 50 ml of DW and dilute ammonia solution with constant gentle stirring until the solution is just alkaline (to litmus paper). Allow the contents of the flask to stand for 30 minutes and then quantitatively filter through a NO. 4 sintered glass-crucible previously dried to a constant weight at 105°C. Wash the precipitate several times with DW, until the washings do not give a positive test for chloride (test with standard $AgNO_3$ Solution). Dry the residue to a constant weight at 105°C. Each gram of residue is equivalent to 1.306 g of $C_{20}H_{22}ON_3Cl$, 2HCl, $2H_2O$.

10.3.2.2.2. *Cognate Assays*

A few other pharmaceutical substances are also determined after conversion to free bases as recorded in Table : 10.4.

Table 10.4 : Substances Determined Gravimetrically by Conversion to Free Base

S.No.	Name of Substance	Qty. Prescribed	Drying Temp. (°C)	Calculations
1.	Phenacaine hydrochloride	0.5 g	105	Each g of residue ≡ 1.122 g of $C_{18}H_{22}N_2O_2$. HCl

10.3.2.3. Substances Assayed After Conversion to Free Compound

In certain specific cases either the pure pharmaceutical substance or dosage forms are quantitatively converted to free compound. This conversion to free compound is quantitative and hence forms the basis of gravimetric analysis. A few typical examples belonging to this category are, namely : progesterone suspension sterile, progesterone tablets, sodium lauryl sulphate, mephobarbital tablets and sorbitan monooleate.

10.3.2.3.1. *Mephobarbital Tablets*

Materials Required : Mephobarbital : 300 mg ; hexane : 100 ml ; chloroform : 150 ml ; alcohol (95% v/v) : l0 ml.

Procedure : Weigh and finely powder not less than 20 mephobarbital tablets. Transfer an accurately weighed portion of the powder equivalent to about 300 mg of mephobarbital to an extraction thimble. Extract with 15 ml of solvent hexane, allow the thimble to drain, transfer to a continuous extraction apparatus provided with a tared flask, and extract the mephobarbital with chloroform for 2 hours. Evaporate the chloroform on a steam bath with the aid of a current of air, cool, dissolve the residue in about 10 ml of alcohol, evaporate, dry the residue at 105°C for 1 hour, cool and weigh.

The weight of the residue represents the weight $C_{l3}H_{14}N_2O_3$ in the portion of the tablets taken.

10.3.2.4. Substances Assayed after Conversion to Derivatives or Substitution Products

In pharmaceutical drug analysis a host of organic pharmaceutical substances are invariably converted quantitatively to their corresponding derivatives by virtue of interactions with certain functional entities, namely : aldehyde, ketone, amino, carboxyl, phenolic, hydroxyl etc. However, in some cases it may be feasible to obtain uniform substitution products of organic pharmaceutical substances quantitatively, for instance : tetraido derivative of phenolphthalein is obtained from the phenolphthalein tablets. It is important to mention here that the number of organic pharmaceutical substances which may be analysed by this method is limited because of *two* vital reasons, they are :

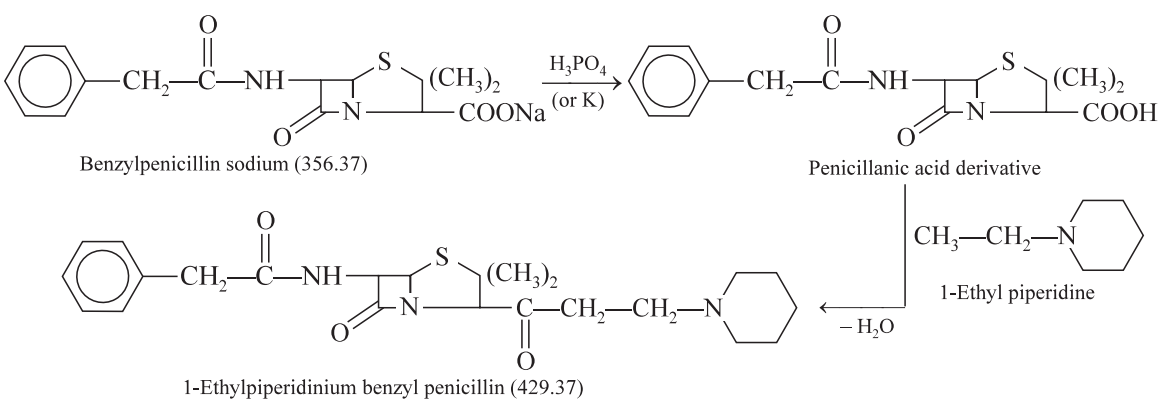
(*a*) the reversible nature of reactions, and

(*b*) the formation of products of side reactions simultaneously.

10.3.2.4.1. *Benzylpenicillin(Syn : Benzylpenicillin Sodium or Potassium Salt)*

Materials Required : Benzylpenicillin sodium (say) : 0.12 g ; amyl acetate (previously saturated with 1-ethylpiperidinium benzylpencillin at room temperature, cooled in ice and filtered) : 5.0 ml ; phosphoric acid (20% v/v) : 0.5 ml ; anhydrous sodium sulphate (freshly ignited and powdered) : 0.5 g ; dry acetone (previously saturated with 1-ethylpiperidinium benzylpenicillin at room temperature cooled in ice and filtered) : 3.0 ml ; 1-ethylpiperidine amyl acetate solution (prepared from l-ethyl piperidine, 1 .0 ml, and amyl acetate, 8.0 ml, saturated at room temperature with 1-ethylpiperidinium benzylpenicillin, cooled in ice and filtered) : 1.5 ml ; dry acetone in amyl acetate (1 : 1) previously saturated with 1-ethylpiperidinium benzylpenicillin : 2.0 ml ; solvent ether : 4.0 ml.

Theory : Benzylpenicillin (sodium or potassium salt) may be assayed gravimetrically by quantitative conversion to the 1-ethylpiperidinium benzylpenicillin derivative. The ultimate precipitation is caused by l-ethyl piperidine after the respective sodium or potassium salt of benzylpencillin has been duly converted with phosphoric acid to the corresponding penicillanic acid (*i.e.* parent acid) and the latter finally extracted with amyl alcohol. The reactions may be expressed as follows :



Therefore, we have :

$$C_{16}H_{17}N_2NaO_4S \equiv C_{23}H_{31}N_3O_3S$$

or $\quad 356.37 \text{ g } C_{16}H_{17}N_2NaO_4S \equiv 429.37 \text{ g } C_{23}H_{31}N_3O_3S$

or $\quad 0.8300 \text{ g } C_{16}H_{17}N_2NaO_4S \equiv 1 \text{ g of } C_{23}H_{31}N_3O_3S$

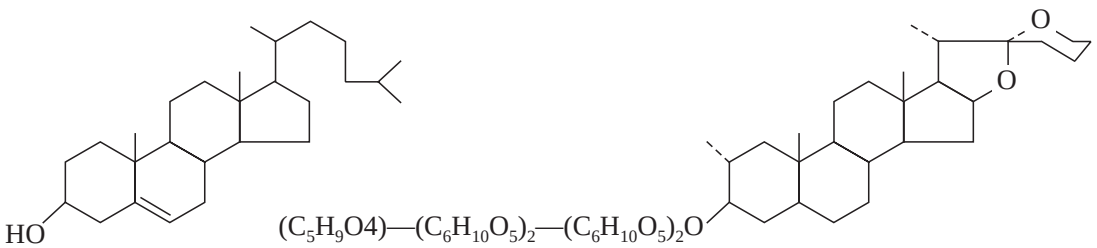
Procedure : Weigh accurately 0.12 g of benzyl penicillin sodium, dissolve in 5 ml of ice-cold DW in a flask and cool in an ice-bath. Add to it 5.0 ml of amyl acetate followed by 0.5 ml of ice-cold H_3PO_4, stopper, shake the contents immediately for 15 seconds, and centrifuge for 30 seconds. Remove the aqueous layer as completely as possible with the help of a pipette. Add 0.5 g anhydrous Na_2SO_4, stir the contents vigorously and cool in an ice-bath for 5 minutes. Centrifuge for about 30 seconds and again cool in ice-bath for 5 minutes. Pipette 3.0 ml of the supernatant liquid into a tared centrifuge tube. Add to it

3.0 ml of ice-cold acetone and 1.5 ml of 1-ethylpiperidine amyl acetate solution, stir, stopper the tube and cool in ice-bath for 2 hours. Now, centrifuge for 1 minute, break the surface with the help of a pointed glass rod, so that all crystalline particles are covered by liquid, and again centrifuge for 1 minute. Decant off the supernatant liquid, wash the precipitate with 2 ml of ice-cold dry acetone in amyl acetate (1 : 1) and again centrifuge for 1.5 minutes. Decant the supernatant liquid, wash twice with 2.0 ml portion of solvent ether, centrifuging for 1.5 minutes and decanting each time. Dry to constant weight under vacuum at room temperature. Each gram of residue is equivalent to 0.8300 g of $C_{16}H_{17}N_2NaO_4S$.

10.3.2.4.2. *Cholesterol*

Materials Required : Cholesterol : 0.1 g ; ethanol (90% v/v) : 12.0 ml ; digitonin solution (0.5% w/v in 90% v/v ethanol) : 40.0 ml ; ethanol (90% v/v) : 100 ml ; acetone ; carbon tetrachloride.

Theory : The assay of cholesterol is solely based on the fact that practically all 3 β-hydroxysterols *e.g.*, cholesterol, readily produces an insoluble molecular addition complex with pure digitonin (1 : 1)—a steroidal saponin isolated from either *Digitalis purpurea* or *Digitalis lanata.* The complex thus obtained is crystalline in nature, fairly stable and possesses very low solubilities.



The complexation of cholesterol and digitonin may be expressed as follows :

$$\underset{\text{Cholesterol}}{C_{27}H_{46}O} + \underset{\text{Digitonin}}{C_{56}H_{92}O_{29}} \longrightarrow \underset{\text{Digitonide Complex}}{C_{83}H_{138}O_{30}}$$

Therefore, 386.3 g $C_{27}H_{46}O \equiv 1616$ g $C_{83}H_{138}O_{30}$

or 0.2390 g $C_{27}H_{46}O \equiv 1$ g of $C_{83}H_{138}O_{30}$

Procedure : Weigh accurately about 0.1 g of cholesterol into a 100 ml flask and dissolve it in 12.0 ml ethanol. Insert the stopper and allow to stand at room temperature (25 ± 2°C) for 12 hours, filter through a Gooch crucible, and wash with 5.0 ml of ethanol. Mix the washings to the filtrate and add to it 40.0 ml solution of digitonin and make it warm to 60°C to ensure that the complexation is almost complete. Filter the precipitate of the resulting complex through a prepared Gooch crucible, previously dried to constant weight at 105°C. Wash the precipitate with ethanol followed by acetone and carbon tetrachloride, allow to drain as completely as possible, and dry to a constant weight at 105°C. Each g of the residue is equivalent to 0.2390 g of cholesterol.

Note : All solutions must be ice-cold.

10.3.2.4.3. *Thiamine Hydrochloride*

Materials Required : Thiamine hydrochloride : 0.5 g ; hydrochloric acid (≃ 11.5 N) : 2.0 ml ; silicotungstic acid solution (10% w/v in water) : 4.0 ml ; NO : 4-sintered glass-crucible ; dilute hydrochloric acid (1 part HCl + 19 parts H_2O) : 50 ml.

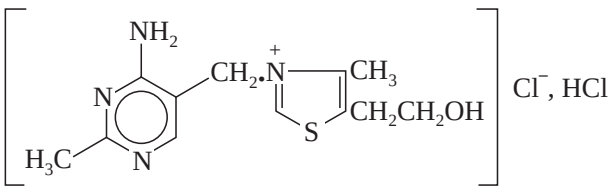
Theory : The gravimetric assay of thiamine hydrochloride is based upon the precipitation of it as thiamine silicotungstate with silicotungstic acid in a slightly acidic medium. It has been observed that the precipitating reagent is a complex silicate SiO_2, 12 WO_2, *n* H_2O having somewhat variable composition with regard to the degree of hydration. For a reasonably precise and accurate determination the precipitating reagent must contain ≮ 1.85% SiO_2 and ≮ 85% WO_3. Interestingly, the thiamine silicotungstate complex possesses more or less a constant composition.

The precipitation of insoluble thiamine silicotungstate may be designated by the following reaction :

$$2C_{12}H_{17}ON_4SCl, HCl + [SiO_2, 12WO_3] + 6H_2O \longrightarrow (C_{12}H_{17}ON_4SCl)_2, [SiO_2(OH)_2, 12WO_3], 4H_2O$$

or 674.6 $C_{12}H_{17}ON_4SCl, HCl \equiv$ 3480 g of Thiamine Silicotungstate

or 0.1958 g $C_{12}H_{17}ON_4SCl, HCl \equiv$ 1 g of Thiamine Silicotungstate



Thiamine Silicotungstate

Procedure : Weigh accurately 0.05 g of thiamine hydrochloride, previously dried at 105°C, and dissolve it in 50 ml DW in a 250 ml beaker having a stirring rod and watch glass cover. Add to it 2.0 ml of hydrochloric acid, heat to boiling and then add 4.0 ml of silicotungstic acid solution as rapidly as possible. Now, boil the solution gently for 2 minutes and quickly filter through a NO. 4 sintered-glass crucible, previously dried to a constant weight at 105°C. Wash the residue with a boiling mixture of HCl and H_2O (1 : 19) about 40 ml, then with DW 10.0 ml and ultimately with two portions of 5 ml each of acetone. Finally dry the residue to constant weight at 105°C. Each g of thiamine silicotungstate residue is equivalent to 0.1938 g of $C_{12}H_{17}ON_4SCl, HCl$.

Precautions :

(*a*) An excess of HCl is a must so as to produce a readily filterable precipitate,

(*b*) In case the sample is pure enough, the rate of addition of silicotungstic acid has little influence on the result, but on the contrary if the sample has significant impurity it may afford poor results,

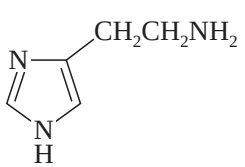
(*c*) To achieve complete complexation boiling must be done for more than 2 minutes, otherwise it would yield low results, and

(*d*) A 50-ml wash-liquid is quite ideal, further washings (volume) may offer poor results.

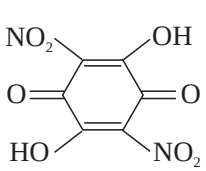
10.3.2.4.4. *Histamine Acid Phosphate ($C_5H_9N_3$, $2H_3PO_4$)*

Materials Required : Histamine : 0.15 g ; nitranilic acid solution (3.5% w/v in 95% ethanol) : 10.0 ml ; ethanol (95%) : 30.0 ml ; sintered-glass crucible (NO : 3) ; ether : 10.0 ml.

Theory : The gravimetric assay of histamine acid phosphate is based upon the formation of insoluble histamine-nitranilic acid complex as depicted in the following equation :



Histamine



Nitranilic acid

Therefore, we have :

$$\underset{\text{Histamine acid phosphate}}{C_5H_9N_3, 2H_3PO_4} + C_6H_2N_2O_8 \longrightarrow \underset{\text{Histamine-nitranilic acid complex}}{C_5H_9N_3.C_6H_2N_2O_8}\downarrow + 2H_3PO_4$$

or 307.1 g $C_5H_9N_3, 2H_3PO_4 \equiv$ 341.3 g $C_5H_9N_3.C_6H_2N_2O_8$

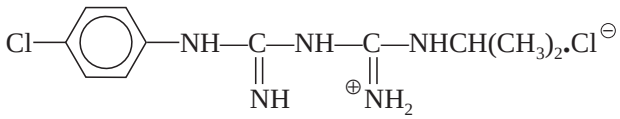
or 0.8998 g $C_5H_9N_3, 2H_3PO_4 \equiv$ 1 g of $C_5H_9N_3.C_6H_2N_2O_8$

Procedure : Weigh accurately about 0.15 g of histamine acid phosphate into a 250 ml beaker provided with a stirring rod and watch glass cover. Add to it 10.0 ml of DW to dissolve the sample. Now, add 10.0 ml of nitranilic acid solution, stir and allow to stand for 15 ininutes. Pour in 10.0 ml of ethanol, keep it in an ice-bath for 3 hours and filter through a No. 3 sintered-glass crucible, previously dried to a constant weight at 130°C. Transfer the precipitate quantitatively and wash it thoroughly with four quantities each of 5.0 ml of ethanol and ultimately with 10.0 ml of ether. Dry to constant weight at 130°C. Simultaneously, determine the loss in weight on drying a separate portion of the sample at 105°C. Each gram of the histamine-nitranilic acid complex is equivalent to 0.8998 g of $C_5H_9N_3$, 2 H_3PO_4.

10.3.2.4.5. *Proguanil Hydrochloride*

Materials Required : Proguanil hydrochloride : 0.6 g ; ammoniacal cupric chloride solution (dissolve 22.5 g of copper (II) chloride in 200 ml of DW and mix with 100 ml of 13.5 M ammonia) ; NO. 4 sintered-glass crucible ; mixture of dilute solution of ammonia and DW (1 : 5).

Theory : Gravimetric analysis of proguanil hydrochloride involves the precipitation of the proguanil-cupric complex that results on the addition of ammoniacal cupric chloride solution to a solution of proguanil hydrochloride. The reaction can be expressed by the following equation :



Proguanil hydrochloride

$$2C_{11}H_{l6}N_5Cl,\ HCl\ +\ CuCl_2 \xrightarrow{NH_3} (C_{11}H_{15}N_5Cl)_2Cu\ +\ 4\ HCl$$

Proguanil cupric complex

or 580.2 g $C_{11}H_{l6}N_5Cl$, HCl ≡ 568.9 g $(C_{11}H_{15}N_5Cl)_2Cu$

or 1.0199 g $C_{11}H_{l6}N_5Cl$, HCl ≡ 1 g of $(C_{11}H_{15}N_5Cl)_2Cu$

Procedure : Weigh accurately 0.6 g of proguanil hydrochloride into a 250 ml beaker fitted with a stirring rod and watch-glass cover. Add to it 50.0 ml of DW and heat gently to dissolve the sample. Chill the solution below 10°C in an ice-bath and then add ammoniacal-cupric-chloride solution with continuous stirring till the resulting solution attains a permanent deep-colour. Allow the solution to stand for 90 minutes to complete the complexation and then filter through a No. 4 sintered glass crucible previously dried to constant weight at 130°C. Transfer the precipitate quantitatively into the crucible, wash first with a mixture of dilute solution of ammonia and DW (1 : 5) adequately followed by cold water until the washings are practically colourless thereby showing the complete absence of soluble copper salts. Dry the precipitate to a constant weight at 130°C. Simultaneously, find out the loss in weight on drying with a separate portion of the sample at 105°C and incorporate this in the calculation. Each gram of proguanil-cupric-complex is equivalent to 1.0199 g of $C_{11}H_{l6}N_5Cl$, HCl.

10.3.2.4.6. *Benzethonium Chloride*

Theory : In general, quaternary nitrogen containing compounds like—choline chloride, acetylpyridinium chloride, benzethonium chloride, and bethanechol chloride readily form insoluble salts quantitatively with tetraphenyl boron and this puts forward the basis for the gravimetric assay of the above cited pharmaceutical substances.

The various reactions involved may be summarized and expressed as follows :

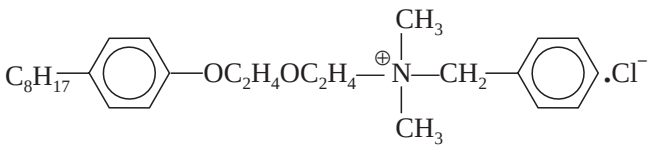
$$R_4N.Cl\ +\ Na(C_6H_5)_4B \longrightarrow R_4N(C_6H_5)_4B\downarrow\ +\ NaCl \qquad ...(a)$$

$$R_4N.Cl\ +\ Ind^- \longrightarrow R_4N.Ind\ +\ Cl^- \qquad ...(b)$$

$$R_4N.Ind\ +\ Na(C_6H_5)B \longrightarrow R_4N.(C_6H_5)_4B\ +\ Na^+\ +\ Ind^- \qquad ...(c)$$

Eq. (*a*) shows that the quaternary salt gets quantitatively precipitated by sodium tetraphenyl boron as the complexing agent. Eq. (*b*) depicts that quaternary compounds shall readily react with certain anionic dye, such as bromophenol blue, to yield a blue, chloroform-soluble complex.

Eq. (*c*) finally illustrates that the blue-coloured complex shall react quantitatively with sodium tetraphenyl boron to give an insoluble compound.



Bezethonium chloride

Therefore, we have :

$$C_{27}H_{41}O_2NCl + Na(C_6H_5)_4B \longrightarrow C_{27}H_{41}O_2N.(C_6H_5)_4B \downarrow + NaCl$$

or $C_{27}H_{41}O_2NCl \equiv C_{27}H_{41}O_2N.(C_6H_5)_4B$

or 446.45 g $C_{27}H_{41}O_2NCl \equiv$ 729.82 g $C_{27}H_{41}O_2N \cdot (C_6H_5)_4B$

or 0.6117 g $C_{27}H_{41}O_2NCl \equiv$ 1 g of $C_{27}H_{41}O_2N \cdot (C_6H_5)_4B$

Materials Required : Benzethonium chloride : 0.15 g ; Chloroform : 50 ml ; bromophenol blue solution (Dissolve with heating 0.2 g of bromophenol blue in 3 ml of 0.1 M NaOH and 10 ml of ethanol (96%). Allow to cool and dilute to 100 ml with ethanol 96%] : 50 ml ; sodium tetraphenyl borate solution (1% w/v in chloroform) : 50 ml ; sintered-glass crucible No : 4.

Procedure : Weigh accurately about 0.15 g of benzethonium chloride sample into a 250-ml beaker placed on a magnetic-stirrer and watch-glass cover. Add to it 25 ml of chloroform and warm gently to dissolve. Cool to ambient temperature and add suffcient bromophenol blue solution gradually till the solution yields a blue Chloroform-soluble complex. Now, add sodium tetraphenyl borate solution in small lots at intervals with constant stirring until the complete precipitation of insoluble benzethonium tetraphenyl borate complex takes place. Allow the solution to stand for 60 minutes to complete the complexation and subsequently filter through a No. 4 sintered-glass crucible previously dried to constant weight at 130°C. Transfer the precipitate quantitatively into the crucible and wash the precipitate with cold chloroform. Dry the precipitate to a constant weight at 110°C. Each gram of benzethonium tetraphenyl borate complex is equivalent to 0.6117 g of $C_{27}H_{41}O_2NCl$.

10.3.2.4.7. *Cognate Assays*

Quite a few official pharmaceutical substances and their respective dosage forms can be assayed gravimetrically after conversion to their corresponding derivatives or substitution products. Table 10.5 records some examples from *official compendia*.

Table 10.5 : Substances Assayed Gravimetrically by Conversion to Derivatives or Substitution Products

S.No.	Name of Substance	Qty. Prescribed	Drying Temp. (°C)	Calculations
1.	Piperazine citrate Tabs.	0.2 (≡ Piperazine Hydrate)	105	Each g of dipicrate residue ≡ 0.3568 g of $(C_4H_{10}N_2)_3$, $2C_6H_8O_7$
2.	Iodochlorhydroxyquin Tabs.	0.1	105	Each g of copper complex residue ≡ 0.9750 g of C_9H_5NOCl
3.	Phentolamine hydrochloride	0.5	105	Each g of trichloroacetate residue ≡ 0.7448 g of $C_{17}H_{19}N_3O$. HCl

THEORETICAL AND PRACTICAL EXERCISES

1. What are the advantages of '**gravimetric analysis**' over '**titrimetric analysis**' ? Give suitable examples to expatiate your answer.

2. How does the '**Law of Mass Action and Reversible Reactions**' help in accomplishing the gravimetric analysis ? Explain.

3. '**The principle of solubility product**' is the major factor in governing the gravimetric analysis. Justify the statement adequately with appropriate examples.

4. What is the critical role played by '**common ion effect**' in gravimetric analysis ? Explain the theoretical aspect and calculations involved in such an analysis.

5. How would you assay the following '**drugs**' gravimetrically :

(*i*) Sodium chloride (*ii*) Potassium alum

(*iii*) Barium sulphate (*iv*) Piperazine phosphate.

6. Gravimetric analysis may be accomplished by one of the following means and ways :

(*a*) Substances assayed after conversion to Free Acid,

(*b*) Substances assayed after conversion to Free Base,

(*c*) Substances assayed after conversion to Free Compound, and

(*d*) Substances assayed after conversion to Derivatives.

Give at least **ONE** suitable example from each method in support of your answer.

RECOMMENDED READINGS

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F. THERMOANALYTICAL METHODS

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11 THERMOANALYTICAL ANALYSIS

CONTAINS :

11.1. INTRODUCTION

Thermoanalytical methods essentially encompass such techniques that are based entirely on the concept of heating a sample followed by well-defned modified procedures, such as : gravimetric analysis, differential analysis and titrimetric analysis. In usual practice, data are generated as a result of continuously recorded curves that may be considered as 'thermal spectra'. These thermal spectra also termed as **'thermograms**, often characterize a single or multicomponent system in terms of :

(*a*) temperature dependencies of its thermodynamic properties, and

(*b*) physicochemical reaction kinetics.

Broadly speaking the thermoanalytical methods are normally classified into the following three categories, namely :

(*i*) Thermogravimetric Analysis (TGA),

(*ii*) Differential Thermal Analysis (DTA), and

(*iii*) Thermometric Titrations.

All the above mentioned techniques shall be discussed briefly with specific reference to their theory, instrumentation, methodology and applications wherever necessary.

11.2. THERMOGRAVIMETRIC ANALYSIS (TGA)

11.2.1. THEORY

A large number of chemical substances invariably decompose upon heating, and this idea of heating a sample to observe weight changes is the underlying principle of thermogravimetric analysis (TGA). However, TGA may be sub-divided into *two* heads, namely :

(*a*) Static (or Isothermal) Thermogravimetric Analysis, and

(*b*) Dynamic Thermogravimetric Analysis.

11.2.1.1. Static Thermogravimetric Analysis

In this particular instance the sample under analysis is maintained at a constant temperature for a period of time during which any changes in weight are observed carefully.

11.2.1.2. Dynamic Thermogravimetric Analysis

In dynamic thermogravimetric analysis a sample is subjected to conditions of predetermined, carefully controlled continuous increase in temperature that is invariably found to be linear with time.

11.2.2. INSTRUMENTATION

The essential requirements for an instrument (Figure 11.1) meant for thermogravimetric analysis are, namely :

(*a*) A high-precision balance,

(*b*) A furnace adequately programmed for a linear rise of temperature with time, and

(*c*) A sensitive recorder.

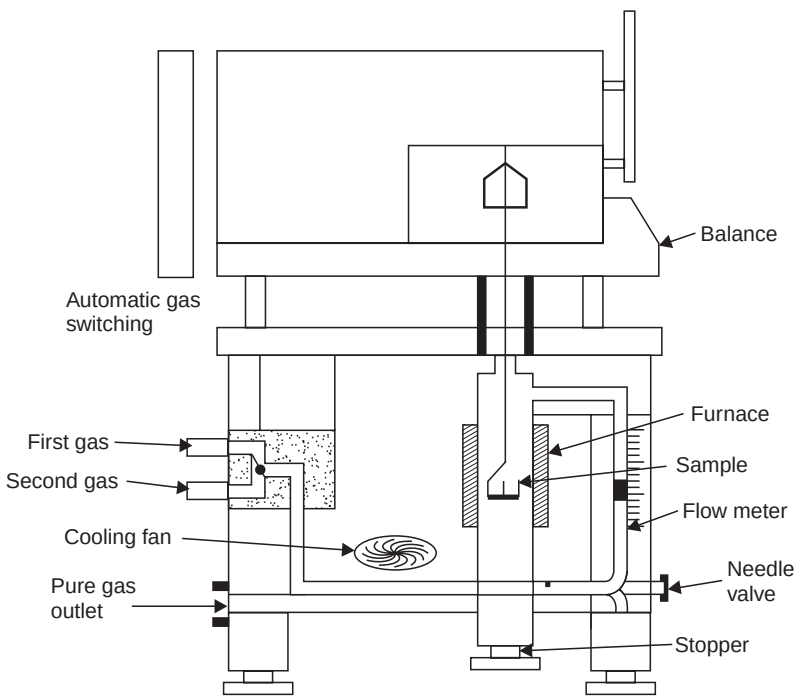

Figure 11.1 : Thermobalance for Thermogravimetric Analysis in a Controlled Atmosphere upto 1000°C.

11.2.2.1. Balances

They are usually of *two* types :

(*a*) **Null-point Type :** It makes use of an appropriate sensing-element which aptly detects any slightest deviation of the balance beam and provides the application of a restoring force, directly proportional to the change in weight, thereby returning the beam to its original null-point. The restoring-force is subsequently recorded either directly or with the aid of a transducer.

(*b*) **Deflection Type :** It is essentially based on either a conventional analytical balance consisting of helical spring, cantilever beam and strain gauze or a torsion analytical balance involving the conversion of deviations directly into a record of the weight change.

11.2.2.2. Furnace

The furnace must be designed in such a fashion so as to incorporate an appropriate smooth input thereby maintaining either a fixed temperature or a predetermined linear-heating programme (*e.g.*, 10°C-600°C per hour).

The temperature control of the furnace is satisfactorily achieved via a thermocouple mounted very close to the furnace-winding. The maximum operational temperature may be obtained by using different thermocouples as indicated below :

S.No.	Specifications	Max. Temp. (°C)
1.	Nickel-Chrome (Nichrome)	1100
2.	Platinum-Rhodium	1450
3.	Graphite-Tube Furnace*	> 1500

*Control and measurement of temperatures are critical and problematic.

11.2.2.3. Recorder

The recording device must be such so as to :

(*i*) record both temperature and weight continuously, and

(*ii*) make a definite periodic record of the time.

11.2.3. METHODOLOGY

The '*thermogram*' for calcium oxalate monohydrate ($CaC_2O_4.H_2O$) is presented in Figure 11.2. The successive plateaus correspond to the anhydrous oxalate (100-250°C), calcium carbonate (400-500°C), and finally calcium oxide (700-850°C). In other words, these plateaus on the decomposition curve designate *two* vital aspects, namely :

(*a*) clear indication of constant weight, and

(*b*) stable phases within a specified temperature interval. The chemical reactions involved may be summarized as follows :

$$CaC_2O_4.H_2O \xrightleftharpoons{100\text{-}250^\circ C} CaC_2O_4 \xrightleftharpoons{400\text{-}500^\circ C} CaCO_3 \xrightleftharpoons{700\text{-}850^\circ C} CaO$$

[Stage 1] [$+H_2O\uparrow$] [Stage 2] [$+CO\uparrow$] [Stage 3] [$+CO_2\uparrow$]

11.2.3.1. Interpretation of Thermogram

In the thermogram (Figure 11.2), which vividly illustrates the thermogravimetric evaluation of $CaC_2O_4.H_2O$, it is ensured that the weight of this product decreases in several stages, namely :

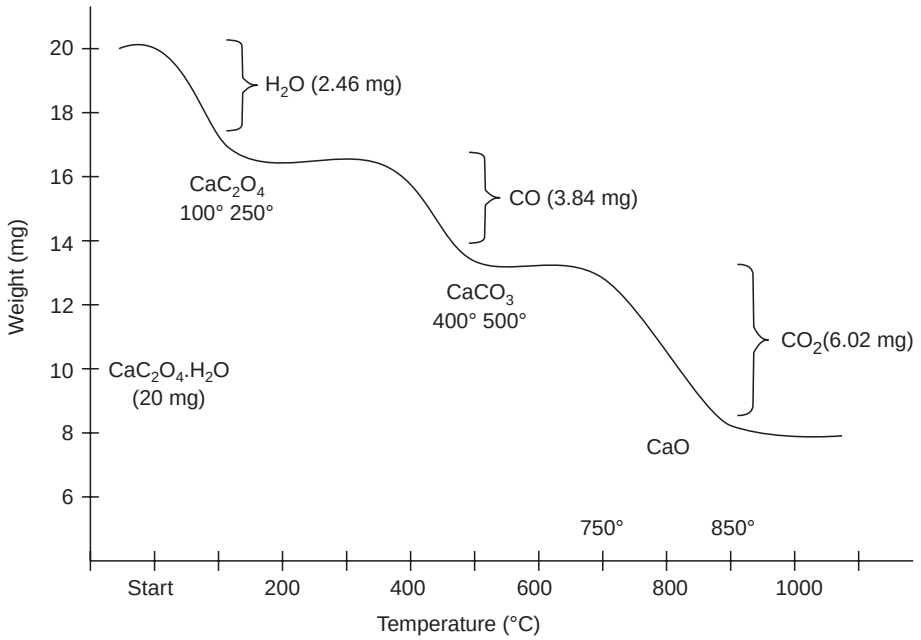

Figure 11.2 : Thermogravimetric Evaluation of Calcium Oxalate Monohydrate heated at the rate of 6°C min^{-1}.

Stage 1 : The water of hydration (or crystallization) from calcium oxalate monohydrate is lost which corresponds to 2.46 mg (12.3%) equivalent to 1 mole of H_2O in the temperature range 100-250°C.

Actually, the 12.3% weight loss that took place between 100-250°C should correspond to 12.3% of the original formula weight for $CaCO_3\ H_2O$ (FW = 146). Hence, the product being lost has a formula weight of $0.123 \times 146 = 17.958$ ($\simeq$ 18.0), and it corresponds to H_2O.

Stage 2 : One mole of carbon monoxide is evolved subsequently from calcium oxalate, corresponding to 3.84 mg (19.2%) in the temperature range 400-500°C.

The 19.2% weight loss that occurred between 400-500°C should correspond to 19.2% of the original formula weight of 146. Therefore, the product being given out has a formula weight of $1.92 \times 146 = 28.0$, that corresponds to CO.

Stage 3 : Finally, a mole of CO_2 is evolved from calcium carbonate that corresponds to 6.02 mg (3.01%) in the temperature range 700-850°C.

The weight loss amounting to 3.01% which took place in the range 700-850°C must, in fact, corresponds to 3.01% of the original formula weight of 146. Therefore, the product being evolved has a formula weight of $0.301 \times 146 = 43.946$ ($\simeq$ 44), and it corresponds to CO_2.

It is quite evident that in a multicomponent system wherein more than one component exhibits weight variations and that too at different temperature regions, the composition of the original compound may be estimated as depicted in Figure 11.2.

In a situation whereby an inert material is present along with a pure substance, from the generated thermogram the composition of the mixture may be derived from the percentage weight variation which takes place relative to the percentage weight variation observed with the pure compound (A), by employing the following expression :

$$\text{Component A (wt \%)} = \frac{\text{\% wt. change for mixture}}{\text{\% wt. change for pure compound A}} \times 100\ (\%)$$

11.2.4. APPLICATIONS

The most broadly based application of the thermogravimetric analysis (TGA) has been visualized and exploited in the investigation of analytical methods, such as :

(*i*) Determining appropriate forms for many elements,

(*ii*) Screening and testing of substances which may be used as potential analytical standards (primary standard), and

(*iii*) Direct application of the technique in analytical assays.

A few typical applications of TGA are, namely :

(*a*) Plateaus for hydrates are sometimes based on the initial water content (*i.e.*, water of crystallization). It has been observed that in humidified air at low heating rates, hydrates usually give rise to good plateaus.

Example : Dehydration of sodium tungstate 28-hydrate [Na_2WO_4.28 H_2O (5 : 12)]

Experimental parameters* :

A. Humidified air, 300°C/hour,

B. Humidified air, 150°C/hour,

C. Humidified air 10°C/hour,

D. Room air, 10°C/hour,

Sample weight : 0.5000 g ;

n = Moles water per $5Na_2O$, 12 WO_3

(*b*) Analysis of flue-gas scrubber system in environmental analysis.

The flue-gas emitted from a coal-fired-power-plant is subjected to scrubbing by the aid of wet limestone to get rid of sulphur dioxide (SO_2) as completely as possible. TGA helps in monitoring the system by carrying out the analysis of the products resulting from the scrubbing process, that mainly consist of (*i*) $CaCO_3$; (*ii*) $CaSO_3$. $CaSO_3$. 1/4 H_2O, and (*iii*) $CaSO_4$. $2H_2O$.

The thermogram obtained from TGA provides the following valuable informations which suggests the decomposition occurring at three distinct stages

S.No.	Conversion		Wt. Loss Region (°C)	Wt. Loss (%)	Due To
	From	To			
1.	$CaSO_4.2H_2O$	$CaSO_4$	100-200	66	$2H_2O$
2.	$CaSO_3.CaSO_4.1/2\ H_2O$	$CaSO_3.CaSO_4$	420	31	$1/2H_2O$
3.	$CaCO_3$	CaO	630-800	03	CO_2

thereby causing the loss due to two moles of water, half-a-mole of water and one mole of CO_2.

(c) The stepwise degradation of organic polymers has received adequate attention which has broadened the in-depth knowledge of polymer chemistry. In this specific instance the sample is either heated under vacuum or in an inert atmosphere (of N_2).

(*d*) The thermogravimetric data may be employed to evaluate the kinetic parameters of weight variations in reactions.

* After, Newkirk A.E., **Anal. Chem., 32** 1560 (1960).

11.3. DIFFERENTIAL THERMAL ANALYSIS (DTA)

11.3.1. THEORY

The difference of temperature between the sample under estimation and a thermally-inert reference material is continuously recorded as a function of furnace temperature in differential thermal analysis (DTA). In actual practice both TGA and DTA are regarded as complementary techniques whereby information gathered by the usage of one approach is invariably supplemented and enhanced by the application of the other method. The range of phenomena measurable during a DTA-run is found to be much larger than in a TGA-run.

It is pertinent to mention here that in the course of TGA many vital processes, for instance : crystallization, crystalline transitions, pure fusion reactions, glass transitions, and solid-state reactions devoid of volatile components might not be indicated as they happen to cause little change in weight of the sample. TGA invariably describes with ample precision the stoichiometry related to chemical changes that are indicated during DTA by an endothermal or exothermal duration from the base-line.

11.3.2. INSTRUMENTATION

A differential thermal analyzer is composed of *five* basic components, namely :

(*i*) Sample holder with built-in thermocouple assembly,

(*ii*) Flow-control system,

(*iii*) Furnace assembly,

(*iv*) Preamplifier and Recorder, and

(*v*) Furnace Power Programmer and Controller.

A typical commercial differential thermal analyzer is schematically illustrated in Figure 11.3.

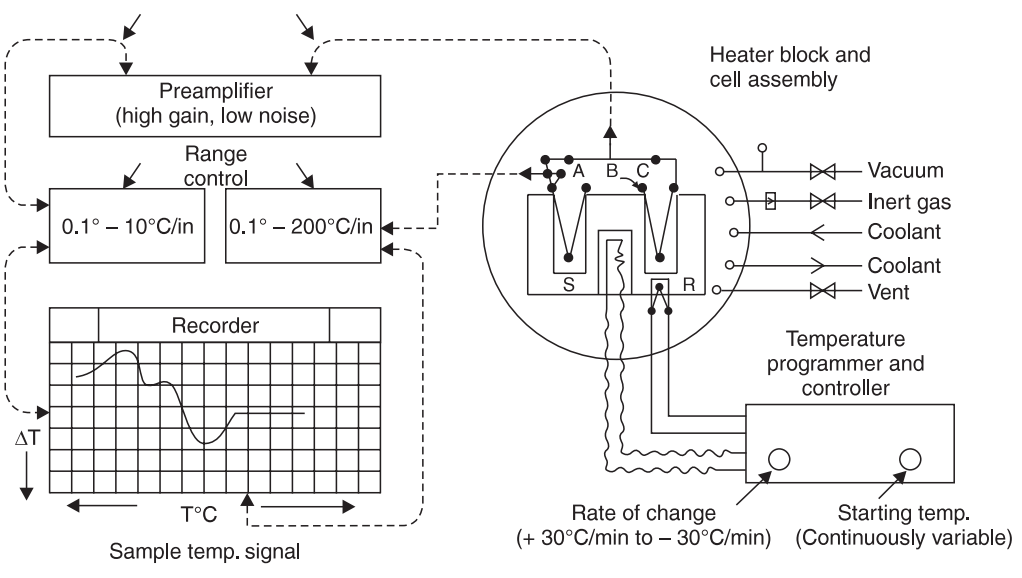

Figure 11.3 : Schematic Diagram of a Differential Thermal Analyzer.
(*Source :* E.I. Du Pont De Nemours, Inc, USA)

(*a*) Thermocouples employed are normally unsheathed Platinum *Vs* Platinum and Sodium *Vs* 10% Rhodium. The said two thermocouples help in measuring the difference in temperature between a sample S and an absolutely inert reference substance R, as both are subjected to heating in a ceramic or metal block inside a furnace being operated by a temperature programmer and controller.

(*b*) The output of the differential thermocouple is amplified adequately through a high gain, low-noise preamplifier and subsequently hooked to the recorder, one axis of which is driven by the block temperature signal and is measured by a third thermocouple.

(*c*) **Heating/Cooling Device :** A sufficient versatility is achieved by the aid of a pressure-vacuum, high-temperature electric furnace. An almost constant heating rate is usually achieved by using a motor-driven variable auto transformer.

Both heating rates and cooling rates may be conveniently adjusted continuously :

(*i*) From 0°-30°C/minute by some instruments, and

(*ii*) From a choice of several commonly employed heating rates *viz*., 2°, 4°, 8° and 16°C/minute.

Usual workable sample temperatures are upto : 500°C. Exceptional maximum temperatures are upto : 1000°C.

(*d*) Relatively small sample volumes help in *two* ways : *first*, they make evacuation easy ; and *secondly*, they minimize thermal gradients.

11.3.3. METHODOLOGY

(*i*) Insert a very thin thermocouple into a disposable sample tube 2 mm in diameter and containing 0.1-10 mg of sample,

(*ii*) Another identical tube is either kept empty or filled with a reference substance, such as quartz, sand, alumina or alundum powder,

(*iii*) The two tubes are simultaneously inserted into the sample block and subsequently heated (or cooled) at a uniform predetermined programmed rate, and

(*iv*) DTA—being a dynamic process, it is extremely important that all aspects of the technique must be thoroughly standardised so as to obtain reproducible results. A few of these aspects vital aspects are :

- Pretreatment of the specimen,
- Particle size and packing of the specimen,
- Dilution of the specimen,
- Nature of the inert diluent,
- Crystalline substances must be powdered, and sieved through 100-mesh sieve,
- For colloidal particles (*e.g.*, clays), micelle-size is very critical, and
- Either to supress an unwanted reaction (*e.g.*, oxidation), or to explore the study of a reaction (*e.g.*, gaseous reaction product)—the atmosphere should be controlled adequately.

Figure 11.4, depicts the differential thermal analysis investigation of calcium acetate monohydrate at a uniform programmed heating rate of 12°C/minute.

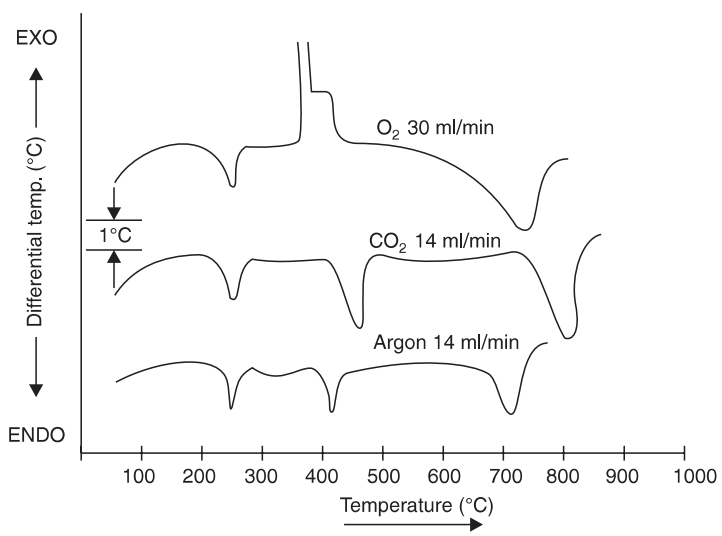

Figure 11.4 : DTA-Investigation of Calcium Acetate Monohydrate, 12°C. min^{-1} (After-American Inst. Co. Inc., USA, Bulletin).

The chemical reactions involved in the differentiated thermal analysis of calcium acetate monohydrate may be expressed as follows :

$$Ca(CH_3COO)_2.H_2O \underset{\text{(Stage-I)}}{\overset{200\text{-}250^\circ C}{\rightleftharpoons}} Ca(CH_3COO)_2 \underset{\text{(Stage-II)}}{\overset{350\text{-}400^\circ C}{\rightleftharpoons}} CaCO_3 \underset{\text{(Stage-III)}}{\overset{800\text{-}950^\circ C}{\rightleftharpoons}} CaO$$

Stage I : The endothermal dehydration of calcium acetate monohydrate occurs giving rise to the anhydrous salt. It is easily noticed by an endothermal band on DTA curve between 200°C and 250°C.

Stage II : The anhydrous salt undergoes endothermal decomposition reaction at 350-400°C resulting into the formation of $CaCO_3$. It has been observed that this decomposition reaction seems to be almost alike in the presence of either CO_2 or Ar.

Stage III : The decomposition of calcium carbonate to calcium oxide, which is a function of the partial pressure of the CO_2 in contact with the sample. The endothermal band for the carbonate decomposition is sharply peaked spread over a relatively narrower temperature range in an atmosphere of CO_2.

11.3.4. APPLICATIONS

The various important applications of DTA are :

(*i*) Rapid identification of the compositions of mixed clays,

(*ii*) Studying the thermal stabilities of inorganic compounds,

(*iii*) Critically examining in a specific reaction whether a new compound is actually formed or the product is nothing but an unreacted original substance, and

(*iv*) DTA offers a wide spectrum of useful investigations related to reaction kinetics, polymerization, solvent retention, phase-transformations, solid-phase reactions and curing or drying properties of a product.

11.4. THERMOMETRIC TITRATIONS (T T)

11.4.1. THEORY

The thermometric titrations (TT) make use of '**heats of reaction**' to obtain titration curves. In usual practice, the temperature of solution is plotted against the volume of titrant. TT is performed by allowing the titrant to flow from a thermostated-burette directly into a solution contained in a thermally-insulated vessel, and subsequently the observed change in temperature of the solution is recorded precisely either during continuous addition of titrant or after every successive incremental addition. The end-point is aptly indicated by a sharp break in the curve.

As the dielectric constant of a solvent exerts little effect on the thermometric titrations, the latter may be employed effectively in most non-aqueous media.

Hence, in a broader-sense TT may be utilized in a number of reactions with greater efficacy, for instance : complexation, precipitation, redox, neutralization. Further, TT can be used to titrate gases against other gases devoid of a liquid-phase ; and to titrate liquid solutions with gaseous reagents.

11.4.2. INSTRUMENTATION

A standard thermometric titration assembly essentially consists of the following *four* vital components, namely :

(*i*) Motor-driven Burette,

(*ii*) Adiabatic Titration Chamber

(*iii*) Thermister Bridge Assembly, and

(*iv*) Recorder.

Fiaure 11.5, represents the schematic thermometric titration assembly complete with a bridge-circuit. To minimise heat transfer losses from the solution by its immediate surroundings, the thermometric titrations are usually carried out in an isolated-beaker tightly closed with a stopper having provision for a burette-tip, a motorized-glass stirrer, and a temperature-monitoring arrangement.

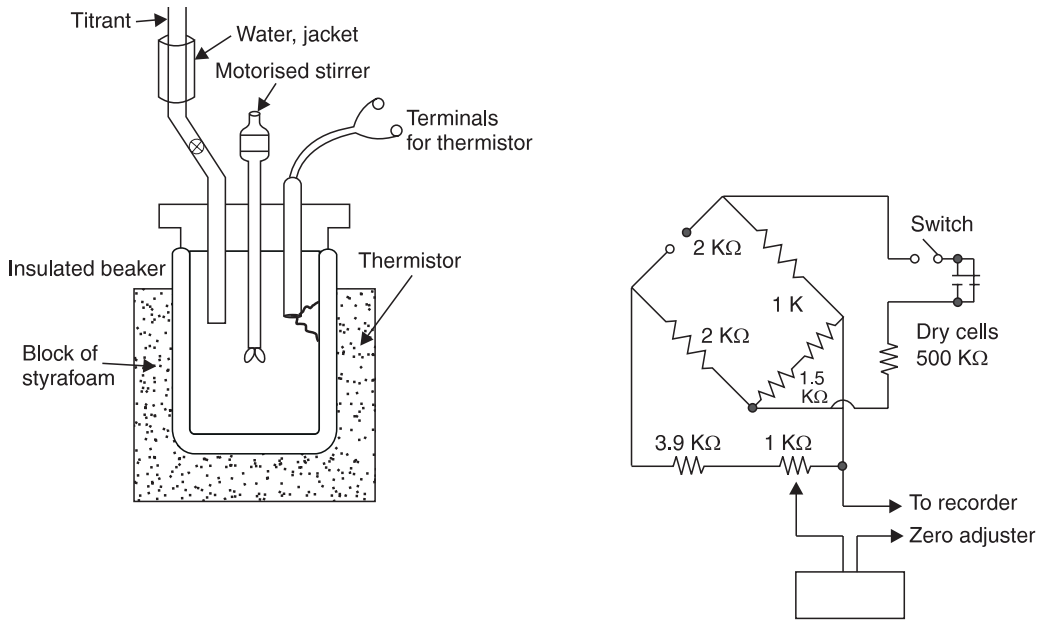

Figure 11.5 : Schematic Thermometric Titration Assembly Complete with a Bridge-Circuit (After Linde *et al.*, Anal Chem. 25, 494, 1953).

Procedure :

(*a*) Introduce the titrant from a burette that is duly mounted in a thermostated-water-jacket to maintain the temperature of the titrant within ± 0.05°C,

(*b*) Experimental parameters are predetermined in such a fashion such that the volume of titrant needed for each titration must lie between 1-3 ml,

(*c*) Automated device delivering reagent at a steady and constant rate of 600 µl per minute usually permits recording,

(*d*) Constant-speed motorized stirrer at 600 rpm is employed to effect uniform mixing of solution,

(*e*) Variations in temperature are measured with the help of a sensitive thermister-sensing-element with fast response, that is sealed completely in glass and immersed in solution,

(*f*) In the course of a thermometric titration, the thermister attached to the insulated-beaker is connected to one arm of the Wheatstone Bridge as displayed in Figure 11.5. The values of the circuit component listed are for a thermister having an approximate resistance of 2 KΩ and a sensitivity of $^{-}$0.04 Ω/Ω/°C in the 25°C temperature range. Hence, an observed change of 1°C ≡ an unbalanced potential of 15.7 mV, and

(*g*) The heat of reaction is either absorbed or generated upon addition of the titrant to the beaker, thereby unbalancing the Wheatstone Bridge caused by simultaneous variations in the resistance (temperature) in the insulated-beaker thermister. Thus, the bridge unbalance potential is promptly plotted by the recorder.

Note : (*i*) **To minimise the temperature variations between the titrant and the solution and also to obviate volume corrections, the concentration of the titrant is invariably maintained 10–100 times higher than that of the reactant, and**

(*ii*) **To obtain optimum results, temperatures of the titrant and the solution must be always within 0.2°C of each other before a titration is commenced.**

11.4.3. METHODOLOGY

Thermometric titration curves usually represent both the entropy and the free energy involved. The titrant is added to the solution at a constant rate in order that the voltage output of the thermister-temperature-transducer changes linearly with time upto the equivalence point.

TT-method affords exact end-point due to :

(*a*) Coloured solutions, and

(*b*) Poisoning of Electrodes.

In usual practice it has been observed that thermometric titrations are mostly feasible with such systems that provide rates of temperature change more than 0.01°C/second.

A few typical examples are cited below :

S.No.	Titrant (M)	Solution (M)	Temp. Change (°C)
1.	NaOH (1 M ; 1 ml)	HCl (0.33 M ; 30 ml)	+ 0.4°C
2.	Na_2-EDTA (1 M)	$MgCl_2$ (0.033 M)	– 0.08°C

Precautions :

(*i*) Lower limit of concentrations which can be titrated effectively is 0.002 M,

(*ii*) No transfer of heat between the titration vessel and its immediate surroundings is allowed, and

(*iii*) During titration temperature fluctuation must not exceed 0.001°C.

11.4.4. APPLICATIONS

Various important applications of thermometric titrations are enumerated below :

(*i*) **Precipitation Reactions :** *e.g.*, Chloride ions (Cl^-) with Ag^+ ions. Besides, phase relations have been studied extensively in precipitation reactions.

(*ii*) **Ion-combination Reactions :** *e.g.*, divalent cations like Ca^{2+}, Mg^{2+} with EDTA (complexometric estimation),

(*iii*) **Conversion of Amides to Amines :** *e.g.*,

An aromatic sulphonic acid amide $\xrightarrow[\substack{\text{Sodium Hypochlorite}\\(\text{Neutral/Alk. Medium})}]{NaClO_3}$ A Monochloramine

(*iv*) **Estimation of H_2O and $(CH_3CO)_2O$ concentrations in a mixture :** The concentration of either of these reactions in the presence of the other may be determined successfully by measuring the rise in temperature taking place during the exothermic reactions of water and acetic anhydride in glacial acid solution along with a trace of perchloric acid ($HClO_4$) acting as a catalyst, and

(*v*) **Benzene in Cyclohexane :** Benzene may be estimated rapidly with fairly good accuracy in cyclohexane by measuring the heat of nitration, whereby a previously prepared standard nitrating acid mixture (benzene and cyclohexane) and the subsequent temperature rise is noted which is a direct function of the quantity of benzene present.

Details involving various experimental parameters for the above estimation are enumerated below :

11.4.4.1. Estimation of Benzene in Cyclohexane

Materials Required : Thermometric titration assembly as per Figure 11.5, minus the burette; a stop-watch or timer ; standard nitrating acid mixture [mix 2 volumes of 70% HNO_3 (d = 1.41) with 1 volume of 95% H_2SO_4 (d = 1.82)] ; Bakelite screw-cap bottle (4 oz. capacity) : 2.

Procedure :

(1) Weigh 50 g of sample in a Bakelite screw-cap bottle and in a similar bottle put the standard nitrating mixture. Place these two bottles in a thermostat maintained at 20°C until the contents have attained an equilibrium temperature,

(2) Transfer 50 ml of the standard nitrating-acid to the insulated vessel and insert the motorised stirrer. Just wait for about 3-5 minutes and then start the motorized stirrer. After exactly 1 minute record the initial temperature,

(3) Stop the motor. Insert the sample into the reaction vessel and start the stirrer. Now, start taking readings of the rise in temperature after each interval 1, 2, 3 and 5 minutes respectively, and

(4) Plot a 'calibration curve' between the observed temperature-rise in a 3 minute interval *Vs* percent benzene present in cyclohexane. Run pure cyclohexane and standards containing 0.5-5.0 percent benzene by weight.

THEORETICAL AND PRACTICAL EXERCISES

1. How does '**thermoanalytical analysis**' give rise to various types of '**thermograms**' that help in characterizing either a single or multicomponent system ?

2. Discuss, the fundamental theory of '**thermogravimetric analysis**', and its instrumentation aspects in an elaborated manner.

3. What are the pharmaceutical applications of TGA ? Explain the interpretation of thermogram obtained by TGA of calcium oxalate monohydrate being heated at the rate of 6°C per minute.

4. Attempt the following aspects of **'differential thermal analysis' :**

(*a*) Theory (*b*) Instrumentation

(*c*) Methodology (*d*) Applications.

5. How would your differentiate '**thermometric titrations**' from TGA and DTA ? Explain.

6. Describe a '**standard thermometric titration**' assembly comprising of the following **FOUR** important components and explain its working :

(*i*) Motor-driven burette (*ii*) Adiabatic titration chamber

(*iii*) Thermister bridge assembly (*iv*) Recorder.

How does it help in **'complexometric titrations'** ? Explain.

RECOMMENDED READINGS

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6. Atkins, P.W., **Physical Chemistry**, 5th ed., Freeman, San Fransisco, 1994.

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G. MISCELLANEOUS METHODS

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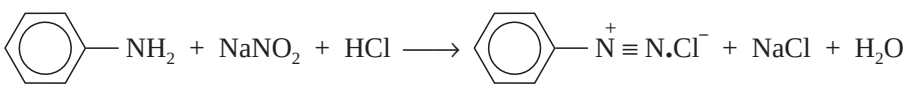
12 DIAZOTIZATION (SODIUM NITRITE TITRATION)

CONTAINS :

12.1. INTRODUCTION

In general, aromatic primary amino moiety (*i.e.*, Ar-NH_2), as present in a host of sulphadrugs *viz.*, succinyl sulphathiazole, sulphamethoxazole, sulphaphenazole and other potent pharmaceutical substances, for instance sodium or calcium aminosalicylate, isocarboxazid, primaquine phosphate, procainamide hydrochloride, procaine hydrochloride and dapsone react with sodium nitrite in an acidic medium to yield the corresponding diazonium salts as expressed below :



Aniline — Phenyl diazonium chloride

It is interesting to observe here that the above reaction is absolutely quantitative under experimental parameters. Therefore, it forms the basis for the estimation of pharmaceutical substances essentially containing a free primary amino function as already illustrated earlier.

12.2. THEORY

Nitrous acid is formed by the interaction of sodium nitrite and hydrochloric acid as follows :

$$NaNO_2 + HCl \longrightarrow NaCl + HNO_2$$

The end-point in the sodium nitrite titration is determined by the liberation of iodine from iodide which may be expressed by the following equations :

$$KI + HCl \longrightarrow HI + KCl$$

$$2HI + 2HNO_2 \longrightarrow I_2 + 2NO + 2H_2O$$

In other words, the small excess of HNO_2 present at the end-point can be detected visually by employing either starch-iodide paper or paste as an external indicator. Thus, the liberated iodine reacts with starch to form a blue green colour which is a very sensitive reaction. Besides, the end-point may also be accomplished electrometrically by adopting the dead-stop end-point technique, using a pair of platinum electrodes immersed in the titration liquid.

12.3. ASSAY METHODS

A number of pharmaceutical substances can be assayed by official methods employing sodium nitrite titrations. A few typical examples are described below to get an indepth knowledge about sodium nitrite titrations.

12.3.1. PREPARATION OF 0.1 M SODIUM NITRITE SOLUTION

Materials Required : Sodium nitrite : 7.5 g.

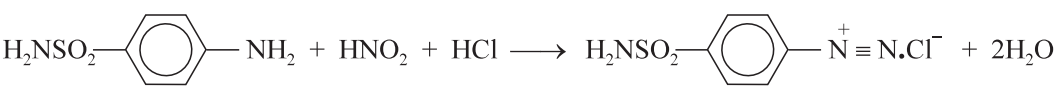
Procedure : Weigh accurately 7.5 g of sodium nitrite and add sufficient DW to produce 1 litre in a 1000 ml volumetric flask.

12.3.2. STANDARDIZATION OF 0.1 M SODIUM NITRITE SOLUTIOIN WITH SULPHANILAMIDE

Materials Required : Sulphanilamide (previously dried at 105°C for 3 hours) : 0.5 g ; hydrochloric acid ($\simeq$ 11.5 N) : 20 ml ; 0.1 M sodium nitrite.

Theory : The nitrous acid, generated on the introduction of sodium nitrite solution into the acidic reaction mixture, reacts with the primary amino group of sulphanilamide quantitatively, resulting into the formation of an unstable nitrite that decomposes ultimately with the formation of a diazonium salt. The diazonium salt thus produced is also unstable, and if the reaction mixture is not maintained between 5-10°C, it shall undergo decomposition thereby forming phenol products which may react further with nitrous acid. The reactions involving the formation of the diazonium salt may be expressed in the following manner :

$$NaNO_2 + HCl \longrightarrow HNO_2 + NaCl$$



Sulphanilamide (172.2)

Diazonium salt

$$C_6H_8N_2O_2S \equiv NaNO_2$$

or $$172.2 \text{ g } C_6H_8N_2O_2S \equiv 1000 \text{ ml M}$$

or $$17.22 \text{ g } C_6H_8N_2O_2S \equiv 1000 \text{ ml } 0.1 \text{ M}$$

or $$0.01722 \text{ g } C_6H_8N_2O_2S \equiv 1 \text{ ml of } 0.1 \text{ M } NaNO_2$$

At the equivalence point a slight excess of HNO_2 is present which must persist for at least 1 minute. This excess HNO_2 may be detected by employing either starch iodide strip or paste and designated by the following equation :

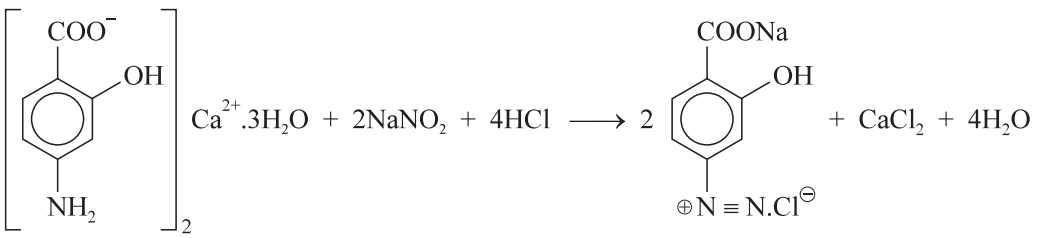
$$2I^- + 2HNO_2 + 2H^+ \longrightarrow I_2 + 2NO + 2H_2O$$

Procedure : Weigh accurately 0.5 g of suphanilamide and transfer to a beaker. Add to it 20 ml of hydrochloric acid and 50 ml of DW, stir until dissolved and cool to 15°C in an ice-bath. Add to it 25 g of crushed ice, and titrate slowly with sodium nitrite solution, stirring vigorously, until the tip of the glass rod dipped into the titrated solution immediately produces a distinct blue ring on being touched to starch-iodide paper. The titration is supposed to be complete when the end-point is deducible after the resulting mixture has been allowed to stand for 1 minute. Each 0.01722 g of sulphanilamide is equivalent to 1 ml of 0.1 N sodium nitrite.

12.3.3. CALCIUM AMINOSALICYLATE

Materials Required : Calcium aminosalicylate : 0.5 g ; hydrochloric acid (≃ 11.5 N) : 10.0 ml ; potassium bromide : 1.0 g ; 0.1 M sodium nitrite ; starch-iodide paper.

Theory : The assay of calcium aminosalicylate is based upon the reaction designated by the following equation :



344.38

Therefore, 344.38 g $C_{14}H_{l2}CaN_2O_6 \equiv 2NaNO_2 \equiv 2000$ ml M

or 172.1 g $C_{14}H_{l2}CaN_2O_6 \equiv 1000$ ml M

or 17.21 g $C_{14}H_{l2}CaN_2O_6 \equiv 1000$ ml 0.1 M

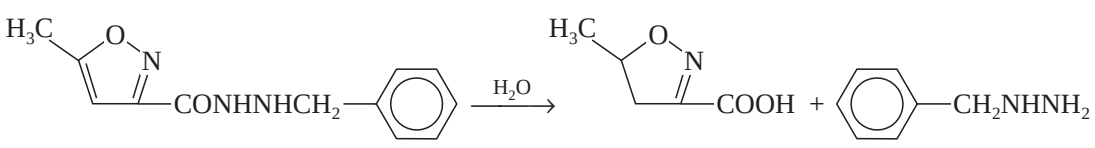
or 0.1722 g $C_{14}H_{l2}CaN_2O_6 \equiv 1$ ml of 0.1 M $NaNO_2$

Procedure : Weigh accurately about 0.5 g of calcium aminosalicylate, into a funnel placed in the mouth of a 250 ml volumetric flask. Wash through with 10 ml of hydrochloric acid and enough DW to dissolve, add 1.0 g potassium bromide and make up the volume upto 250 ml mark. Pipette 50 ml into a conical flask, cool to below 15°C (in ice-bath) and titrate gradually with 0.1 M sodium nitrite solution while shaking the contents of the flask vigorously and continuously until a distinct blue colour is achieved when a drop of the titrated solution is placed on a starch-iodide paper 5 minutes after the last addition of the 0.1 M $NaNO_2$ solution. Care must be taken to add $NaNO_2$ solution at the rate of 0.1 ml near the end of the titration. Each ml of 0.1 M sodium nitrite is equivalent to 0.01722 g of $C_{14}H_{l2}CaN_2O_6$.

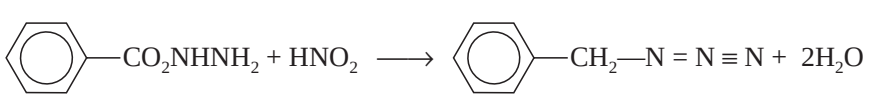
12.3.4. ISOCARBOXAZID

Materials Required : Isocarboxazid : 0.5 g ; glacial acetic acid (99% w/w or 17.5 N) : 20.0 ml ; hydrochloric acid (≃ 11.5 N) : 20.0 ml ; 0.1 M sodium nitrite ; starch-iodide paper.

Theory : The estimation is based on the fact that isocarboxazid undergoes rapid cleavage in acidic medium to produce benzylhydrazine. The latter reacts quantitatively with nitrous acid ($NaNO_2$ and HCl) to give rise to benzylazide. The various reactions involved are expressed as follows :



(231.25) Benzylhydrazine



Benzylazide

Therefore, $C_{12}H_{13}N_3O_2 \equiv NaNO_2 \equiv 1000$ ml M

or 231.25 g $C_{12}H_{13}N_3O_2 \equiv 1000$ ml M

or 23.125 g $C_{12}H_{13}N_3O_2 \equiv 1000$ ml 0.1 M

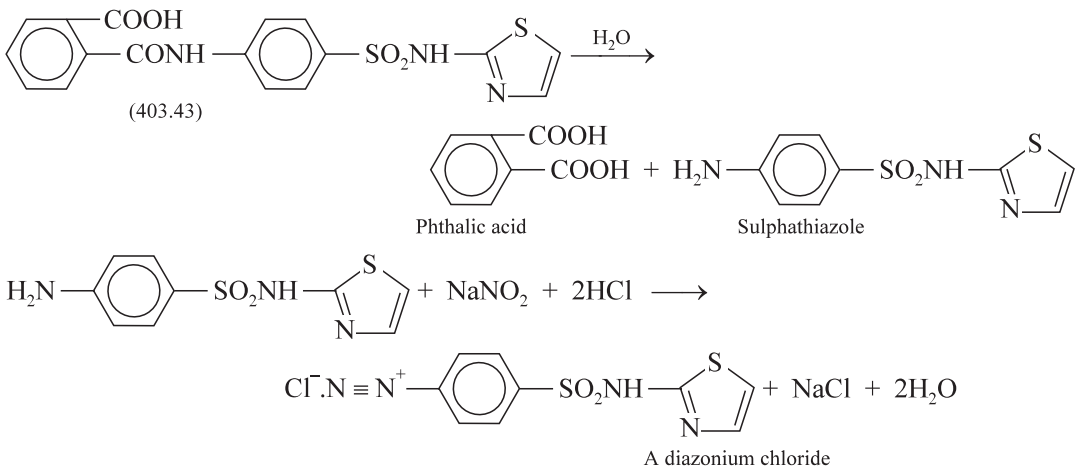
or 0.02313 g $C_{12}H_{13}N_3O_2 \equiv 1$ ml 0.1 M $NaNO_2$

Procedure : Weigh accurately about 0.5 g of isocarboxazid and dissolve it in 20 ml of glacial acetic acid. Add to it 20 ml of hydrochloric acid and 50 ml of DW. Cool to about 15°C in an ice-bath and titrate slowly with 0.1 M $NaNO_2$ while shaking vigorously and continuously until a distinct blue colour is obtained on a starch-iodide paper that lasts for 5 minutes after the final addition of the 0.1 M $NaNO_2$ solution to the titrated solution. Add $NaNO_2$ solution very carefully at the rate of 0.1 ml at a time as the end-point is approached. Each mole of 0.1 M sodium nitrite is equivalent to 0.02313 g of $C_{12}H_{13}N_3O_2$.

12.3.5. PHTHALYLSULPHATHIAZOLE

Materials Required : Phthalylsulphathiazole : 0.5 g ; sodium hydroxide solution (20% w/v in water) : 10.0 ml ; hydrochloric acid (≃ 11.5 N) : 20.0 ml ; 0.1 M sodium nitrite ; starch-iodide paper.

Theory : The assay is based upon the reactions designated by the following equations :



Therefore, $C_{17}H_{13}N_3O_5S_2 \equiv NaNO_2 \equiv 1000$ ml M

or 403.43 g $C_{17}H_{13}N_3O_5S_2 \equiv 1000$ ml M

or 40.343 g $C_{17}H_{13}N_3O_5S_2 \equiv 1000$ ml 0.1 M

or 0.04034 g $C_{17}H_{13}N_3O_5S_2 \equiv 1$ ml 0.1 M $NaNO_2$

Phthalylsulphathiazole undergoes hydrolysis to give phthalic acid and sulphathizole. The latter reacts with nitrous acid to yield the corresponding diazonium salt quantitatively.

Procedure : Weigh accurately about 0.5 g of phthalylsulphathiazole and heat on a water-bath for 2 hours after the addition of 10.0 ml of sodium hydroxide solution. Cool the contents of the flask to 15°C in an ice-bath, add to it 10.0 ml of water and 20.0 ml of hydrochloric acid and carry out the titration slowly with 0.1 M sodium nitrite solution. The contents of the flask are shaken thoroughly and continuously until a distinctly visible blue colour is obtained when a drop of the titrated solution is placed on a starch-iodide paper 5 minutes after the last addition of the 0.1 M $NaNO_2$ solution. Towards the approach of the end-point the addition of $NaNO_2$ solution must be at the rate of 0.1 ml. Each ml of 0.1 M sodium nitrite is equivalent to 0.04034 g of $C_{17}H_{13}N_3O_5S_2$.

12.3.6. COGNATE ASSAYS

A plethora of pharmaceutical substances that can be assayed by the help of sodium nitrite titrations are mentioned in Table 12.1.

Table 12.1 : Substance Assayed by Direct Titrations with Sodium Nitrite

S.No.	Name of Substance	Qty. Prescribed	Indicator Employed	Calculations
1.	Dapsone	0.3 g	Starch-iodide paper/paste	Each ml of 0.1 M $NaNO_2$ ≡ 0.01242 g of $C_{12}H_{12}N_2O_2S$
2.	Primaquine phosphate	1.0 g	-do-	Each ml of 0.1 M $NaNO_2$ ≡ 0.04553 g of $C_{15}H_{21}N_3O$, $2H_3PO_4$
3.	Procainamide hydrochloride	0.5 g	-do-	Each ml of 0.1 M $NaNO_2$ ≡ 0.02718 g of $C_{l3}H_{21}N_3O$, HCl
4.	Procaine hydrochloride	1.0 g	-do-	Each ml of 0.1 M $NaNO_2$ ≡ 0.02728 g of $C_{13}H_{20}N_2O_2$, HCl
5.	Sodium amino-salicylate	2.5 g	- do-	Each ml of 0.1 M $NaNO_2$ ≡ 0.01752 g of $C_7H_6NNaO_3$
6.	Succinylsulpha-thiazole	0.5 g	-do-	Each ml of 0.1 M $NaNO_2$ ≡ 0.03554 g of $C_{13}H_{13}N_3O_5S_2$
7.	Sulphacetamide sodium	0.5 g	-do-	Each ml of 0.1 M $NaNO_2$ ≡ 0.02362 g of $C_8H_9N_2NaO_3S$
8.	Sulphadiazine	0.5 g	-do-	Each ml of 0.1 M $NaNO_2$ ≡ 0.02503 g of $C_{10}H_{10}N_4O_2S$
9.	Sulphadimethoxine	0.5 g	-do-	Each ml of 0.1 M $NaNO_2$ ≡ 0.0313 g of $C_{12}H_{14}N_4O_4S$
10.	Sulphadimidine	0.5 g	-do-	Each ml of 0.1 M $NaNO_2$ ≡ 0.02783 g of $C_{12}H_{14}N_4O_4S$
11.	Sulphadimidine Sodium	0.5 g	-do-	Each ml of 0.1 M $NaNO_2$ ≡ 0.03003 g of $C_{l2}H_{13}N_4NaO_2S$
12.	Sulphalene	0.5 g	-do-	Each ml of 0.1 M $NaNO_2$ ≡ 0.02803 g of $C_{11}H_{12}N_4O_3S$
13.	Sulphamethizole	0.5 g	-do-	Each ml of 0.1 M $NaNO_2$ ≡ 0.02703 g of $C_9H_{10}N_4O_2S_2$
14.	Sulphamethoxazole	0.5 g	-do-	Each ml of 0.1 M $NaNO_2$ ≡ 0.02533 g of $C_{l0}H_{11}N_3O_3S$
15.	Sulphaphenazole	0.5 g	-do-	Each ml of 0.1 M $NaNO_2$ ≡ 0.03144 g of $C_{15}H_{l4}N_4O_2S$

THEORETICAL AND PRACTICAL EXERCISES

1. What is the '**diazotization**' reaction ? How does it help in the assay of drugs ? Explain.

2. Why is it necessary to perform '**sodium nitrite titrations**' invariably in an acidic medium ? Provide a plausible explanation based on chemical reactions.

3. (*a*) How would you prepare 1 L of 0.1 M $NaNO_2$ solution ? How can we standardize the above solution using pure sulphanilamide ?

Explain with various reactions involved, along with the procedural details.

(*b*) Discuss the assay of calcium aminosalicylate.

4. Based on the '**diazotization reaction**' how would you carry out the assay of the following '**drug substances**' :

(*i*) Isocarboxazid (*ii*) Phthalylsulphathiazole

(*iii*) Sulphamelthoxazole (*iv*) Primaquine phosphate.

RECOMMENDED READINGS

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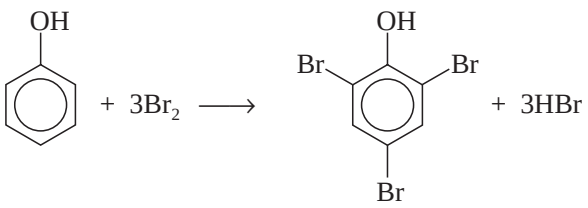
13 ESTIMATION OF PHENOLS AND RELATED COMPOUNDS

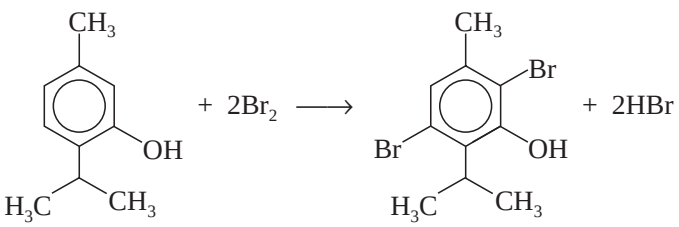
CONTAINS :

13.1. INTRODUCTION

In oxidation-reduction methods bromine is employed as an oxidizing agent in place of iodine, because it is reduced quantitatively be the readily oxidized pharmaceutical organic substances in a reaction which results in either water-insoluble bromine substitution products, for instance :

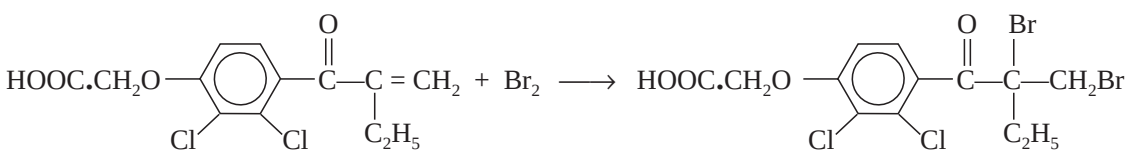


Phenol — 2, 4, 6-Tribromophenol

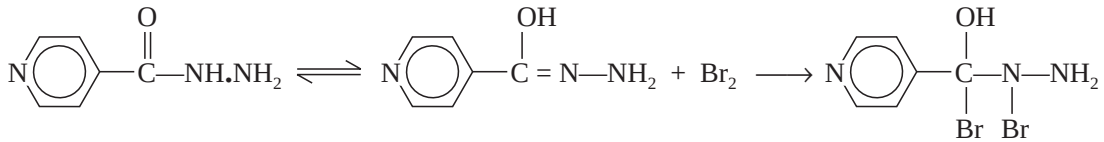


Thymol

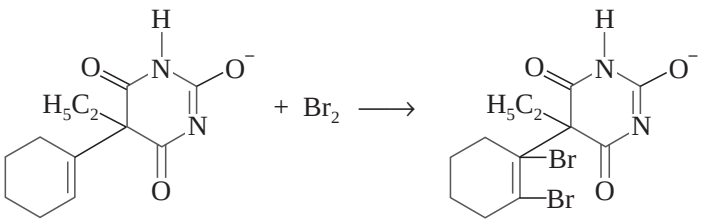
or corresponding water-insoluble bromine-addition products, such as :



Ethacrynic acid — Dibromo derivative



Isoniazid (‘*keto*’-form) Isoniazid (‘*enol*’-form) Dibromo derivative



Cyclobarbitone Dibromo derivative

However, the standard solution used does not have bromine (Br_2) as such but it does contain an equivalent amount of potassium bromate and an excess of potassium bromide and the resulting mixture on subsequent acidification liberates bromine. The reaction may be expressed as follows :

$$5KBr + KBrO_3 + 6HCl \longrightarrow 6KCl + 3Br_2 + 3H_2O$$

The liberated bromine helps in oxidizing iodide to an equivalent amount of iodine as shown below :

$$2KI + Br_2 \longrightarrow 2KBr + I_2$$

The free iodine thus produced is titrated with previously standardized sodium thiosulphate solution as depicted below :

$$I_2 + \underset{\text{Sodium thiosulphate}}{2Na_2S_2O_3} \longrightarrow 2NaI + \underset{\text{Sodium tetrathionate}}{Na_2S_4O_6}$$

13.2. THEORY

In oxidation-reduction assays the use of bromine is judiciously carried out as an oxidizing agent effectively for such specific compounds which ultimately results into the formation of both bromine substitution and bromine additive compounds. These products of reaction are produced quantitatively and are mostly water-insoluble in characteristics ; and more interestingly they take place in an acidic medium.

As it has been discussed earlier, iodine cannot be used directly as an oxidizing agent in such type of assays, whereas the liberated iodine quantitatively produced by the oxidation of iodide with bromine (excess) may be assayed by titrating against sodium thiosulphate solution.

13.3. ASSAY METHODS

Assay methods based on bromine may be classified under the following *three* heads, namely :

(*i*) Titrations with 0.1 N Bromine,

(*ii*) Titrations with Potassium Bromate, and

(*iii*) Titrations with Potassium Iodate.

13.3.1. TITRATIONS WITH 0.1 N BROMINE

This involves the preparation of 0.1 N bromine solution and subsequent standardization with 0.1 N sodium thiosulphate solution. Bromine solution is also known as Koppeschaar’s Solution in some literature.

13.3.1.1. Preparation of 0.1 N Bromine Solution

Materials Required : Potassium bromate : 3.0 g ; potassium bromide : 15 g.

Procedure : Weigh 3 g of potassium bromate and 15 g of potassium bromide in a beaker and dissolve with water. Transfer it quantitatively into a 1 litre volumetric flask and make up the volume with DW.

13.3.1.2. Standardization of 0.1 N Bromine with 0.1 N Sodium Thiosulphate Solution

Materials Required : 0.1 N Bromine solution ; hydrochloric acid ($\simeq$ 11.5 N) : 5 ml ; potassium iodide solution (10% w/v in water) : 5.0 ml ; 0.1 N sodium thiosulphate ; starch solution.

Procedure : Transfer 25 ml of 0.1 N bromine solution with the help of a pipette into a 500 ml iodine flask and dilute it with 120 ml of DW. Add to it 5 ml of hydrochloric acid, moisten the glass-stopper with water and insert the stopper in the flask. Shake the contents gently. Now, add 5 ml of potassium iodide solution, again lace the stopper and allow the resulting mixture to stand for 5 minutes in the dark. Titrate the liberated iodine with previously standardized 0.1 N sodium thiosulphate solution, adding 3 ml of freshly prepared starch solution towards the end-point. Each ml of 0.1 N sodium thiosulphate is equivalent to 0.01598 g of Br_2.

13.3.1.3. Thymol

Materials Required : Thymol : 0.1 g ; N sodium hydroxide : 25.0 ml ; dilute hydrochloric acid (10% v/v of HCl) : 20.0 ml ; 0.1 N bromine ; methyl orange solution (0.1% w/v soln. in 20% alcohol).

Procedure : Weigh accurately about 0.1 g of thymol, transfer to a 250-ml iodine flask and dissolve in 25.0 ml of N sodium hydroxide. Add to it 20.0 ml of dilute hydrochloric acid and immediatelv titrate with 0.1 N bromine to within 1 to 2 ml of the calculated end-point. Warm the solution to about 75°C, add 2 drops of methyl orange solution and continue the titration gradually while swirling the contents of the flask thoroughly after each addition. When the colour of the methyl orange is discharged, add 2 drops of 0.1 N bromine, shake well, add 1 drop of methyl orange solution and shake vigorously. If the colour of the solution is still red, continue the titration dropwise and with constant stirring until the red colour of the indicator is discharged completely. Repeat the alternate addition of 0.1 N bromine and methyl orange solution until the red colour is discharged after the addition of the methyl orange solution. Each ml of 0.1 N bromine is equivalent to 0.003755 g of $C_{10}H_{14}O$.

Calculations :

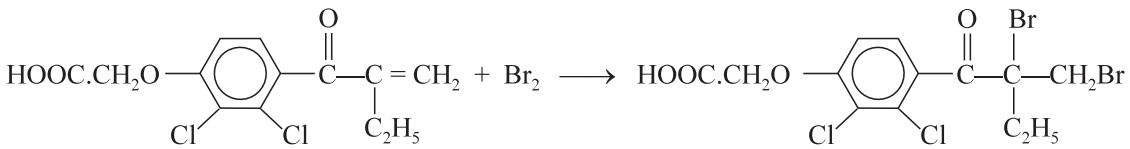
$$\underset{150.22}{C_{10}H_{14}O} + 2Br_2 \longrightarrow C_{10}H_{l2}Br_2O + 2HBr$$

Since, 1 mole of thymol reacts with 2 mol, 4 equivalent of bromine under the conditions of the assay, the equivalent weight of thymol is 37.55 g, 1/4 gramme molecular weight (*i.e.*, 150.22/4 = 37.55). Therefore, each milliliter of 0.1 N bromine consumed in the reaction with thymol is equivalent to 0.1×0.03755 = 0.003755 g or 0.1 meq. of thymol ($C_{10}H_{14}O$).

13.3.1.4. Ethacrynic Acid

Theory : Active bromine is liberated from the standard solution of bromine in an acidic medium (HCl) that subsequently attacks the double bond present in the side chain of the ethacrynic acid molecule thereby resulting into the formation of the corresponding dibromo derivative. This particular reaction takes place quantitatively. Hence, the reactions involved in this assay may be expressed as follows :

$$KBrO_3 + 5KBr + 6HCl \longrightarrow 6Br^{\ominus} + 6KCl + 3H_2O$$



Ethacrynic acid — Dibromo derivative

A blank determination is always performed simultaneously to account for the losses caused by the bromine as well as iodine vapours due to the interaction of excess bromine on potassium iodide.

Materials Required : Ethacrynic acid : 0.2 g ; glacial acetic acid : 40.0 ml ; 0.1 N bromine : 20.0 ml ; hydrochloric acid (≃ 11.5 N) : 3.0 ml ; potassium iodide solution ; (10% w/v in water) : 20 ml ; 0.1 N sodium thiosulphate ; starch solution.

Procedure : Weigh accurately about 0.2 g of ethacrynic acid, dissolve in 40 ml of glacial acetic acid in a 250 ml iodine flask. Add to it 20 ml of 0.1 N bromine and 30.0 ml of hydrochloric acid, immediately place in position the moistened stopper to the ffask, mix the contents vigorously and allow it to stand in a dark place for 60 minutes (to complete the reaction with bromine). Add to it 100 ml of water and 20 ml of KI Solution and titrate immediately with 0.1 sodium thiosulphate, employing freshly prepared starch solution as an indicator towards the end of the titration. Repeat an operation without the pharmaceutical substance (blank titration) ; thus the difference between the titrations represents the amount of bromine required by the ethacrynic acid. Each ml of 0.1 N bromine is equivalent to 0.01516 g of $C_{13}H_{12}Cl_2O_4$.

Calculations : From the above equations, we have :

$$C_{13}H_{12}Cl_2O_4 \equiv Br_2 \equiv 2e$$

or $303.14 \text{ g } C_{13}H_{12}Cl_2O_4 \equiv 2000 \text{ ml N}$

or $151.57 \text{ g } C_{13}H_{12}Cl_2O_4 \equiv 1000 \text{ ml N}$

or $0.01516 \text{ g } C_{13}H_{12}Cl_2O_4 \equiv 1 \text{ ml of 0.1 N Bromine}$

Alternatively, we have :

$$C_{13}H_{12}Cl_2O_4\ \% = \frac{\text{ml difference} \times \text{N} \times (303.14/2000) \times 100}{\text{wt. of sample}}$$

13.3.1.5. Cognate Assays

A number of pharmaceutical substances may be determined quantitatively by titration with bromine as given in Table 13.1.

Table 13.1 : Substances Assayed by Direct Titration with Bromine

S.No.	Name of Substance	Qty. Prescribed	Indicator Employed	Calculations
1.	Compound Benzoic acid Ointment (For Salicylic acid)	2.5 g	Starch solution	Each ml of 0.1 N bromine ≡ 0.002302 g of $C_7H_6O_3$
2.	Cyclobarbitone Tablets	0.5 g	-do-	Each ml of 0.1 N bromine ≡ 0.01277 g of $C_{24}H_{30}CaN_4O_6$
3.	Isoniazid	0.4 g	-do-	Each ml of 0.1 N bromine ≡ 0.003429 g of $C_6H_7N_3O$
4.	Methylparaben	0.1 g	-do-	Each ml of 0.1 N bromine ≡ 0.002536 g of $C_8H_8O_3$
5.	Phenylephrine hydrochloride	0.1 g	-do -	Each ml of 0.1 N bromine ≡ 3.395 g of $C_9H_{13}NO_2$. HCl

13.3.2. TITRATIONS WITH POTASSIUM BROMATE

Potassium bromate can also be employed as an oxidizing agent in the assay of a number of pharmaceutical substances, namely : mephenesin, phenol, and sodium salicylate. This particular method solely depends upon the formation of iodine monobromide (IBr) in relatively higher concentration of hydrochloric acid solution.

13.3.2.1. Preparation of 0.1 N Potassium Bromate

Theory : Potassium bromate can be estimated by the addition of potassium iodide and dilute hydrochloric acid. Thus, we have :

$$KBrO_3 + HI \longrightarrow HIO_3 + KBr$$

$$IO_3^- + 5I^- + 6H^+ \longrightarrow 3I_2 + 3H_2O$$

or $KBrO_3 \equiv IO_3^- \equiv 3I_2 \equiv 6e$

or 167.02 g $KBrO_3 \equiv$ 6000 ml N

or 27.84 g $KBrO_3 \equiv$ 1000 ml N

or 0.002784 g $KBrO_3 \equiv$ 1 ml of 0.1 N Sodium thiosulphate

Materials Required : Potassium bromate : 2.784 g.

Procedure : Weigh accurately 2.784 g of potassium iodide into a beaker and dissolve it in suffcient DW. Transfer the solution quantitatively into a 1 litre volumetric flask and make up the volume to the mark.

13.3.2.2. Standardization of 0.1 N Potassium Bromate Solution with the help of 0.1 N Sodium Thiosulphate

Materials Required : 0.1 N Potassium bromate ; potassium iodide : 3.0 g ; hydrochloric acid ($\simeq$ 11.5 N) : 3.0 ml ; 0.1 N sodium thiosulphate ; starch solution : 3.0 ml.

Procedure : Transfer an accurately measured volume of about 30.0 ml of 0.1 N potassium bromate solution into a 250 ml iodine flask. Add to it 3.0 g potassium iodide, followed by 3.0 ml of potassium iodide, followed by 3.0 ml of hydrochloric acid. Mix the contents thoroughly and allow it to stand for 5 minutes with its stopper in position. Titrate the liberated iodine with previously standardized 0.1 N sodium thiosulphate, using 3.0 ml of freshly prepared starch solution as an indicator at the end-point. Carry out a blank run using the same quantities of the reagents and incorporate the necessary corrections, if any. Each ml of 0.1 N sodium thiosulphate is equivalent to 0.002784 g of $KBrO_3$.

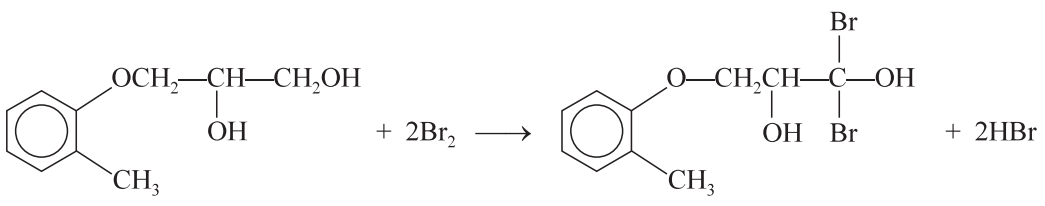
13.3.2.3. Mephenesin

Theory : Mephenesin undergoes oxidation with bromine to yield a dibromo derivative as expressed in the following equation :

$$BrO_3^- + 6e + 6H^+ \longrightarrow Br^- + 3H_2O \quad ...(a)$$

$$2BrO_3^- + 10e + 12H^+ \longrightarrow Br_2 + 6H_2O \quad ...(b)$$

In this instance an excess of potassium bromate is employed. Therefore, any bromide formed [Eq. (*a*)] is oxidized to bromine, and the excess bromate and the bromine are assayed bromometrically. The reduction of bromate to bromine may be designated as in [Eq. (*b*)].



Mephenesin Dibromo derivative

Hence, we have :

$$C_{10}H_{l4}O_3 \equiv Br_2 \equiv 2e$$

or $182.22 \text{ g } C_{10}H_{l4}O_3 \equiv 2000 \text{ ml N}$

or $91.11 \text{ g } C_{10}H_{l4}O_3 \equiv 1000 \text{ ml N}$

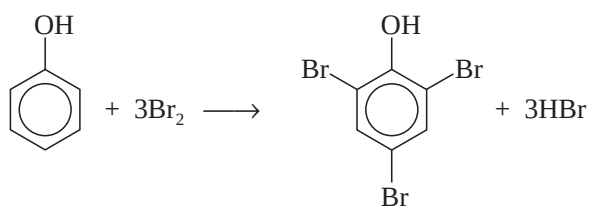
or $0.00911 \text{ g } C_{10}H_{l4}O_3 \equiv 1 \text{ ml of } 0.1 \text{ N } KBrO_3$

Materials Required : Mephenesin : 0.15 g ; 0.1 N potassium bromate : 25.0 ml ; potassium bromide powder : 10.0 g ; hydrochloric acid (25% w/v) : 10.0 ml ; potassium iodide solution (10% w/v in water) : 10.0 ml ; 0.1 N sodium thiosulphate solution ; starch solution.

Procedure : Weigh accurately 0.15 g of mephenesin and dissolve in 50 ml of DW into a 250 ml iodine-flask. Add to it 25.0 ml of 0.1 N potassium bromate solution and 10.0 g of powdered potassium bromide. After the dissolution of KBr, add 10 ml of hydrochloric acid, insert the moistened stopper, and after 10 seconds add 10 ml of potassium iodide solution. Titrate with 0.1 N sodium thiosulphate using starch solution as indicator. Each ml of 0.1 N potassium bromate is equivalent to 0.00911 g of $C_{10}H_{l4}O_3$.

13.3.2.4. Phenol

Theory : Phenol interacts with bromine whereby the former undergoes bromination to yield a water-insoluble 2, 4, 6-tribromophenol. This reaction takes place quantitatively as shown below :



Phenol — 2, 4, 6-Tribromophenol

Thus, we have :

$$C_6H_5\text{—}OH \equiv 3Br_2 \equiv 6e$$

or $94.11 \text{ g } C_6H_5\text{—}OH \equiv 6000 \text{ ml N}$

or $15.685 \text{ g } C_6H_5\text{— } OH \equiv 1000 \text{ ml N}$

or $0.001569 \text{ g } C_6H_5\text{— } OH \equiv 1 \text{ ml } 0.1 \text{ N Potassium Bromate}$

Materials Required : Phenol : 0.5 g ; 0.1 N potassium bromate : 25.0 ml ; potassium iodide (powdered) : 1.0 g ; dilute hydrochloric acid (10% w/w of HCl) : 10.0 ml ; potassium iodide (10% w/v in water) : 10 ml ; chloroform : 10.0 ml ; 0.1 N sodium thiosulphate ; starch solution.

Procedure : Weigh accurately 0.5 g of phenol and dissolve in sufficient water to produce 500 ml in a volumetric flask. Mix 25.0 ml of this solution with 25.0 ml of 0.1 N potassium bromate in a 250 ml iodine flask and add to it 1 g of powdered KI and 10.0 ml of dilute hydrochloric acid. Moisten the glass stopper with a few drops of KI solution and place it in position. Set it aside in a dark place for 20 minutes while shaking the contents frequently in between. Add to it 10 ml of KI solution, shake the contents thoroughly and allow it to stand in the dark for a further duration of 5 minutes. Wash the stopper and neck of the flask carefully with DW, add 10 ml chloroform and titrate with the liberated iodine with 0.1 N sodium thiosulphate using freshly prepared starch as an indicator. Carry out a blank titration simultaneously and incorporate any necessary correction, if required. Each ml of 0.1 N potassium bromate is equivalent to 0.001569 g of C_6H_6O.

13.3.2.5. Cognate Assays

A few other pharmaceutical substances may also be assayed by titrating with 0.1 N potassium bromate as indicated in Table 13.2.

Table 13.2. Substances Assayed by 0.1 N Potassium Bromate

S.No.	Name of Substance	Qty. Prescribed	Indicator Employed	Calculations
1.	Sodium Salicylate	0.1 g	—	Each ml of 0.2 N $KBrO_3 \equiv 0.005336$ g of $C_7H_5NaO_3$
2.	Chlorocresol	0.07 g	Starch solution	Each ml of 0.0167 M $KBrO_3 \equiv 0.003565$ g of C_7H_7ClO

13.3.3. TITRATIONS WITH POTASSIUM IODATE

Potassium iodate is a fairly strong oxidizing agent that may be used in the assay of a number of pharmaceutical substances, for instance : benzalkonium chloride, cetrimide, hydralazine hydrochloride, potassium iodide, phenylhydrazine hydrochloride, semicarbazide hydrochloride and the like. Under appropriate experimental parameters the iodate reacts quantitatively with both iodides and iodine. It is, however, interesting to observe here that the iodate titrations may be carried out effectively in the presence of saturated organic acids, alcohol and a host of other organic substances.

The oxidation-reduction methods with potassium iodate invariably based on the formation of iodine monochloride (ICl) in a medium of strong hydrochloric acid solution.

13.3.3.1. Preparation of 0.05 M Potassium Iodate

Theory : First of all the potassium iodate is dried to a constant weight at 110°C to make it completely free from moisture and then brought to room temperature in a desiccator. It is pertinent to mention here that KIO_3 is a very stable salt and may be obtained in a very pure form. Therefore, it is possible to prepare the standard solutions of KIO_3 by dissolving the calculated weight of the salt in water and diluting the same to an approximate volume.

Since, the normality of iodate solution varies significantly depending on the nature of the reaction, therefore, in usual practice standard iodate solutions of known molarity are used.

The reduction of potassium iodate to iodide is usually not feasible in a direct titrimetric method (unlike the reduction of potassium bromate to bromide) and hence, has no viable application in the official procedures :

$$IO_3^- + 6e + 6H^+ \longrightarrow I^- + 3H_2O \qquad ...(a)$$

In this type of reaction, 1 mol of KIO_3 is 6 equivalent and a 0.05 M solution would be 0.3 N.

In a situation, whereby excess of potassium iodate is employed, any I^- formed [Eq. (*a*)] is readily oxidized to iodine, and subsequently the excess iodate and the iodine are estimated by the iodometric procedure. Thus, the reduction of the iodate to iodine may be expressed as shown below :

$$2IO_3^- + 10e + 12H^+ \longrightarrow I_2 + 6H_2O \qquad ...(b)$$

In such a reaction, 1 mol of iodate is 5 equivalent and a 0.05 M solution would be 0.25 N. This reaction of iodate is never used in the offcial assay methods.

Interestingly, at higher concentrations of hydrochloric acid, both the iodide and iodine obtained as reduction products of iodate [Eqs. (*a*) and (*b*)] are quantitatively converted to I^+. It forms the basis of official procedures for iodate titrations.

The iodine produced intially by the reduction of iodate [Eq. (*b*)] undergoes solvolysis in a polar solvent as expressed in the following reaction :

$$I_2 \rightleftharpoons I^+ + I^-$$

The iodine cation forms iodine monochloride (ICl) in a medium having sufficiently high concentration of HCl and the latter is subsequently stabilized by complex ion formation. Thus, we have :

$$I^+ + HCl \rightleftharpoons ICl + H^+ \quad ...(c)$$

$$ICl + HCl \rightleftharpoons ICl_2^- + H^+ \quad ...(d)$$

Adding Equations (*c*) and (*d*), we may have :

$$I^+ + 2HCl \rightleftharpoons ICl_2^- + 2H^+$$

In actual practice, either carbon tetrachloride or chloroform is usually added so as to make the end-point distinctly visible. Iodine is liberated at the initial stages of the titration which renders the chloroform layer coloured. At that material point when all the reducing agent under estimation has been duly oxidized, the iodate completes the oxidation of iodine and iodide to I^+, and hence the colour from the chloroform layer disappears.

In official methods of analysis *i.e.*, the iodine monochloride method, the reduction of KIO_3 can be expressed as follows :

$$IO_3^- + 4e + 6H^+ \longrightarrow I^+ + 3H_2O \quad ...(e)$$

In Eq. (*e*), 1 mol of KIO_3 is 4 equivalent, and a 0.05 solution would be 0.2 N.

Materials Required : Potassium iodate : 10.7 g.

Procedure : Weigh accurately 10.7 g of pure potassium iodate, previously dried at 110°C to constant weight, in sufficient DW to produce 1 litre in a volumetric flask.

13.3.3.2. Benzalkonium Chloride

Materials Required : Benzalkonium chloride : 4.0 g ; chloroform : 60.0 ml ; 0.1 N sodium hydroxide : 10.0 ml ; potassium iodide (5% w/v in water) : 10.0 ml ; hydrochloric acid ($\simeq$ 11.5 N) : 40.0 ml ; 0.05 M potassium iodate.

Procedure : Weigh accurately benzalkonium chloride 4.0 g and dissolve it in sufficient DW to make 100 ml. Pipette 25.0 ml into a separating funnel, add 25 ml of chloroform, 10 ml of 0.1 N NaOH and 10 ml of potassium iodide solution. Shake the contents thoroughly, allow to separate and collect the chloroform layer in another separating funnel. Treat the aqueous layer with 3 further quantities each of 10 ml of chloroform and discard the chloroform layer. To the aqueous layer add 40 ml of hydrochloric acid, cool and titrate with 0.05 M potassium iodate till the solution becomes pale brown in colour. Add 2 ml of chloroform and continue the titration until the chlorofonn layer becomes colourless. Titrate a mixture of 29 ml of water, 10 ml of KI solution and 40 ml of hydrochloric acid with 0.05 M potassium iodate under identical conditions (Blank Titration). The differences between the titrations represent the amount of 0.05 M potassium iodate required. Each ml of 0.05 M potassium iodate is equivalent to 0.0354 g of $C_{22}H_{40}ClN$.

Calculations :

$$2C_{22}H_{40}ClN \equiv 2KI \equiv KIO_3$$

or $$354 \text{ g } C_{22}H_{40}ClN \equiv 1000 \text{ ml } 0.5 \text{ M } KIO_3$$

or $$35.4 \text{ g } C_{22}H_{40}ClN \equiv 1000 \text{ ml } 0.05 \text{ M } KIO_3$$

or $$0.0354 \text{ g } C_{22}H_{40}ClN \equiv 1 \text{ ml of } 0.05 \text{ M } KIO_3$$

13.3.3.3. Potassium Iodide

Theory : The iodine monochloride method described earlier employing standard potassium iodate is the basis for the official assay of potassium iodide. Vigorous shaking is a prime requirement, as the end-point is approached in this assay, because of the fact that both iodine and iodate in different phases attribute a heterogeneous medium. However, the reaction involving the oxidation of KI by iodate may be designated as shown below :

$$\underset{2(166.0)}{2KI} + KIO_3 + 6HCl \longrightarrow 3ICl + KCI + 3H_2O$$

The reduction of KIO_3 may be expressed as :

$$IO_3^+ + 4e + 6H^+ \longrightarrow I^+ + 3H_2O$$

Hence, from the above equation we have, 1 mol of KIO_3 is 4 equivalent and a 0.05 M solution would be 0.2 N.

Thus, we have :

$$2KI \equiv IO_3^- \equiv 4e$$

or $$166 \text{ g KI} \equiv 2e \equiv 2000 \text{ ml N}$$

or $$83 \text{ g KI} \equiv 1000 \text{ ml N}$$

or $$16.60 \text{ g KI} \equiv 1000 \text{ ml } 0.2 \text{ N} \equiv 1000 \text{ ml } 0.05 \text{ M}$$

or $$0.01660 \text{ g KI} \equiv 1 \text{ ml } 0.05 \text{ M } KIO_3$$

Materials Required : Potassium iodide : 0.5 g ; hydrochloric acid ($\simeq$ 11.5 N) : 35 ml ; chloroform : 5 ml ; 0.05 M potassium iodate.

Procedure : Weigh accurately 0.5 g of potassium iodide and dissolve it in about 10 ml of DW. Add to it 35 ml of hydrochloric acid and 5 ml of chloroform. Titrate with 0.05 M potassium iodate till the purple colour of iodine disappears from the chloroform layer. Add the last portion of the iodate solution carefully and dropwise while shaking the contents of the flask vigorously and continuously. Allow to stand for 5 minutes. In case any colour still develops in the chloroform layer continue the titration. Each ml of 0.05 M potassium iodate is equivalent to 0.0166 g of potassium iodide.

13.3.3.4. Cognate Assays

A host of other pharmaceutical substances, namely : cetrimide, hydralazine hydrochloride, phenylhydrazine hydrochloride may be assayed by titration with potassium iodate as mentioned in Table : 13.3.

Table 13.3 : Substances Assayed by Potassium Iodate

S.No.	Name of Substance	Qty. Prescribed	Calculations
1.	Cetrimide	2.0 g	Each ml of 0.05 M $KIO_3 \equiv$ 0.03364 g of $C_{17}H_{38}BrN$
2.	Hydralazine hydrochloride	0.15 g	Each ml of 0.02 M $KIO_3 \equiv$ 0.03933 g of $C_8H_8N_4$. HCl
3.	Phenylhydrazine hydrochloride	0.2 g	Each ml of 0.05 M $KIO_3 \equiv$ 0.007231 g of $C_6H_5NHNH_2$. HCl

THEORETICAL AND PRACTICAL EXERCISES

1. Why is '**bromine**' preferred to '**iodine**' in redox methods for the assay of pharmaceutical organic substances ? Explain with suitable examples.

2. (*a*) How would your prepare 1 L of 0.1 N Bromine solution and standardize it with $Na_2S_2O_3$ solution ? Explain.

(*b*) Using 0.1 N Br_2 solution how would you carry out the assay of the following '**drugs**' :

(*i*) Thymol (*ii*) Ethacrynic acid

(*iii*) Isoniazid (*iv*) Methylparaben.

3. (*a*) Explain the procedural details of preparing 1 L of 0.1 N $KBrO_3$ solution and its subsequent standardization with 0.1 N $Na_2S_2O_3$ solution.

(*b*) Discuss the assay of the following medicinal compounds :

(*i*) Phenol (*ii*) Mephenesin

(*iii*) Chlorocresol (*iv*) Sodium salicylate.

4. (*a*) Give the sequential procedure which one may adopt to prepare 1 L of 0.05 M KIO_3 solution, and standardize it.

(*b*) How would you carry out the assay of the following '**drugs**' :

(*i*) Cetrimide (*ii*) Hydralazine Hydrochloride

(*iii*) Phenylhydrazine Hydrochloride.

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14 KARL FISCHER METHOD FOR DETERMINATION OF WATER

CONTAINS :

14.1. INTRODUCTION

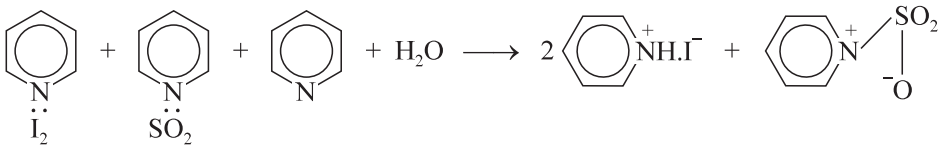
A plethora of chemical compounds for the determination of small amounts of water present in organic solids, pharmaceutical substances and organic solvents have been devised over a length of time. But unquestionably the most important of these is the one proposed by Karl Fischer (1935), which is considered to be relatively specific for water*. It essentially makes use of the Karl Fischer reagent which is composed of iodine, sulphur dioxide, pyridine and methanol.

Note : Both pyridine and methanol should be anhydrous.

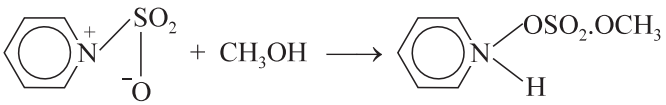
14.2. THEORY

Water present in the analyte reacts with the Karl Fischer reagent in a two-stage process as shown below :

Stage 1 :



Stage 2 :



...(*b*)

From Eq. (*a*) step l, it is obvious that the oxidation of sulphur dioxide takes place by iodine to yield sulphur trioxide and hydrogen iodide thereby consuming one mole of water. In other words, each one molecule

* Mitchell, J., **Anal. Chem 23,** 1069 (1951).

* Mitchell, J., and D.M. Smith, '**Aquametry**', 2nd ed., New York, Interscience, (I977).

of iodine disappears against each molecule of water present in the given sample. It is pertinent to mention here that in the presence of a large excess of pyridine (C_5H_5N), all reactants as well as the resulting products of reaction mostly exist as complexes as evident from Eqs. (*a*) and (*b*).

Stability of the Reagent : The stability of the original Karl Fischer reagent initially prepared with an excess of methanol was found to be fairly poor and hence, evidently needed frequent standardization. However, it was estabtished subsequently that the stability could be improved significantly by replacing the methanol by 2-methoxyethanol.

It has been observed that the titer of the Karl Fischer reagent, which stands at 3.5 mg of water per milliliter of reagent, falls rapidly upon standing with the passage of time. Hence, the following precautions must be observed rigidly using the Karl Fischer reagent, namely :

(*a*) Always prepare the reagent a day or two before it is to be used,

(*b*) Great care must be taken to prevent and check any possible contamination either of the reagent or the sample by atmospheric moisture,

(*c*) All glassware(s) must be thoroughly dried before use,

(*d*) Standard solution should be stored out of contact with air, and

(*e*) Essential to minimise contact between the atmosphere and the solution during the course of titration.

End-point Detection : The end-point of the Karl Fischer titration may be determined quite easily by adopting the electrometric technique employing the dead-stop end-point method. When a small quantum of e.m.f. is applied across two platinum electrodes immersed in the reaction mixture, a current shall tend to flow till free iodine exists, to remove hydrogen and ultimately depolarize the cathode. A situation will soon arise when practically all the traces of iodine have reacted completely thereby setting the current to almost zero or very close to zero or attain the end-point.

Limitations of Karl Fischer Titration : The Karl Fischer titration has a number of serious limitations due to possible interferences tantamount to erroneous results, namely :

(*i*) **Oxidizing agents**, for instance : chromates, Cu(II), Fe(III), $Cr_2O_7^{2-}$, peroxides, salts, higher oxides,

Example :

$$MnO_2 + 4C_5H_5NH^+ + 2I^- \longrightarrow Mn^{2+} + 4C_5H_5N + I_2 + H_2O$$

(*ii*) **Reducing agents**, such as : Sn(II) salts, sulphides, and $S_2O_3^{2-}$, and

(*iii*) Compounds that have a tendency to form water with the ingredients of the Karl Fischer reagent, for instance :

(*a*) **basic oxides :** *e.g.*, ZnO ;

Example : $ZnO + 2C_5H_5NH^+ \longrightarrow Zn^{2+} + C_5H_5N + H_2O$

(*b*) **salts of weak oxy-acids** *e.g.*, $NaHCO_3$;

Example : $NaHCO_3 + C_5H_5NH^+ \longrightarrow Na^+ + H_2O + CO_2 + C_5H_5N$

Note : As H_2CO_3, carbonic acid, is very unstable ; hence it splits up to yield a mole each of water and CO_2.

14.3. INSTRUMENTATION

Figure 14.1 illustrates a simple dead-stop end-point assembly or a Karl Fischer titration apparatus. The titration vessel is fitted with a pair of identical platinum electrodes, a mechanical stirrer with adjustable speed, and a burette. It will be observed that absolutely little or no current may flow unless and until the solution is totally free from any polarizing substances ; this could perhaps be due to the absorbed layers of oxygen and hydrogen on the anode and cathode respectively. However, the current shall flow only when the two electrodes

get depolarized. The Karl Fischer reagent is pumped into the burette by means of hand bellows, the eccess of moisture is usually prevented by employing an appropriate arrangement of desiccant tubes. Alternatively, the stirring may also be accomplished either by using a magnetic stirrer or by means of a suitably dried nitrogen passed gently through the solution during the course of titration.

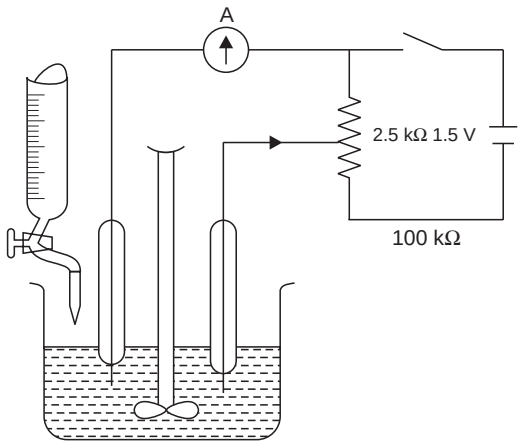


Figure 14.1 : Dead-Stop End-point Assembly.

The end-point is achieved by employing an eiectrical circuit comprising of a microammeter (A), platinum electrodes, together with a 1.5 V to 2.0 V battery connected across a variable resistance of about 2.5 kΩ. First of all the resistance is adjusted in such a manner that an initial current passes through the platinum electrodes in series with a microammeter (A). After each addition of reagent, the pointer of the microammeter gets deflected but quickly returns to its original position. At the end of the reaction a deflection is obtained which persists for 10-15 seconds.

14.3.1. AUTOMATED ELECTROCHEMICAL KARL FISCHER ANALYSIS

Commercially available Modern KF-Titrators are usually equipped with specifically designed titration vessels that are exclusively meant to check and prevent the contact with atmospheric moisture. Quite a few such devices are armed with microprocessors that will perform the requisite operations sequentially in a programmed manner automatically ; and may also dish out a print-out of the desired results including the percentage moisture content. In fact, these Modern KF-Titrators not only afford greater accuracy and precision in results but also offer much ease and convenience in routine analysis as compared to the classical techniques based on either caulometry or controlled current potentiometry using two indicator electrodes.

In this procedure the iodide needed for the reaction with water is normally generated within the titration vessel *caulometrically* as shown below :

$$H_2O + I_2 + SO_2 + 3C_5H_5N + CH_3OH \longrightarrow 2C_5H_5N.HI + C_5H_5NH.SO_4.CH_3$$

Thus, the basis of the analysis rests upon the quantitative relationship existing between charge passed and iodine produced by the reagent according to the above reaction. Therefore, the generation of iodine is automatically stopped when an excess of it is detected by the indicator electrode. It essentially consists of two platinum electrodes across which an AC is applied and subsequently a marked drop in voltage between the electrodes takes place as soon as an excess of iodine is present. Normally such automated instruments make use of *proprietory reagents exclusively*.

The major advantage of this approach to KF-analysis being that no calibration is required as the method is absolute and is entirely based on the stoichiometry of the aforesaid equation. It is noteworthy that one may determine the amounts of water ranging between 10 mcg and 10 mg in solid as well as liquid samples.

14.4. APPLICATIONS OF KARL FISCHER METHOD FOR DETERMINATION OF WATER IN PHARMACEUTICAL ANALYSIS

The Karl Fischer method for the determination of water is used for prednisolone sodium phosphate as described below.

14.4.1. PREDNISOLONE SODIUM PHOSPHATE

Materials Required : Karl Fischer Reagent* : 100 ml ; prednisolone sodium phosphate : 0.2 g ; anhydrous methanol : 20.0 ml.

Procedure : Add about 20 ml of anhydrous methanol to the titration vessel and titrate to the amperometric end-point with the Karl Fischer reagent. Quickly add 0.2 g of prednisolone sodium phosphate sample, stir for 1 minute and again titrate to the amperometric end-point with the Karl Fischer reagent. The difference between the two titrations gives the volume (v) of Karl Fischer reagent consumed by the sample.

The minimum water equivalent is 3.5 mg of water per ml of Karl Fischer reagent. Hence, the percentage of water w/w in the given sample may be calculated by the following expression :

$$\text{Water \% (w/w)} = \frac{v \times 3.5}{\text{wt. of sample (mg)}} \times 100$$

Precautions :

(1) The reagents and solutions used must be kept anhydrous and necessary care should be taken throughout to prevent exposure to atmospheric moisture,

(2) The Karl Fischer reagent should be protected from light and preferably stored in a bottle fitted with an automatic burette, and

(3) The water equivalent of Karl Fischer reagent should always be determined before use.

14.4.2. COGNATE ASSAYS

A number of other *official pharmaceutical substances* may be assayed for their water content by the Karl Fischer method as summarized in the following Table 14.1.

Table 14.1 : Cognate Assays of Pharmaceutical Substances by Karl Fischer Method

S.No.	Name of Substance	Qty. Prescribed	Prescribed Limit of Water % (w/w)
1.	Rifamycin Sodium	0.2 g	12.0-17.0
2.	Sodium Methyl Hydroxybenzoate	1.0 g	NMT** 5.0
3.	Triamcinolone Acetonide	0.2 g	NMT 2.0

* The use of commercially available Karl Fischer Reagent must be validated in order to verify in each case the stoichiometry and the absence of incompatibility between the substance being examined and the reagent.

** NMT = Not More Than.

THEORETICAL AND PRACTICAL EXERCISES

1. How would you explain the presence of water in an '**anlyte**' usually reacts with Karl Fischer reagent in a *two-stage process* ? Give the chemical reactions involved in the above procedure.

2. Give a brief account on the following :

(*a*) Stability of the KF-reagent

(*b*) End-point detection

(*c*) Limitations of Karl Fischer Titration.

3. (*a*) With the help of a neat-labeled-diagramatic sketch explain the working of a '**Dead-Stop End-point**' assembly.

(*b*) Describe a Modern KF-Titrator using a chemical reaction associated with it. Why is it advantageous in comparison to the dead-stop end-point apparatus.

4. How would you assay the following medicinal compounds :

(*i*) Prednisolone sodium phosphate (*ii*) Rifamycin sodium

(*iii*) Sodium methyl hydroxybenzoate (*iv*) Triamcinolone acetonide.

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15 TETRAZOLIUM ASSAY OF STEROIDS

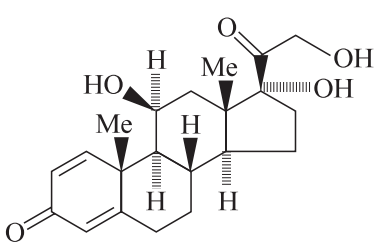
CONTAINS :

15.1. INTRODUCTION

A number of steroids essentially having a α-ketol (21-hydroxy-20 keto) side-chain group, for instance : hydrocortisone, hvdrocortisone acetate, prednisolone, methylprednisolone, methylprednisolone acetate, flucocinolone acetonide, triamcionolone acetonide and the like—are quantitatively reduced by tetrazolium salts to their respective coloured formazan derivatives. Thus, it is possible to carry out the assay of a number of formulations that contain corticosteroids by using triphenyltetrazolium chloride. The said reaction is usually performed in an alkaline medium (tetramethylammonium hydroxide) between a temperature ranging between 30° to 35°C for a duration of 1 to 2 hours. The absorbance of the resulting *formazan derivative* producing a **red product** is usually measured around 484 nm.

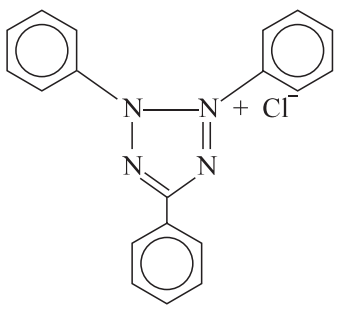
15.2. THEORY

The oxidation of the α-ketol moiety present in the steroid under examination and the subsequent reduction of triphenyltetrazolium chloride to the corresponding triphenylformazan are depicted in the following reaction :

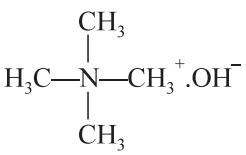


Prednisolone
(C-21 : —CH_2OH)

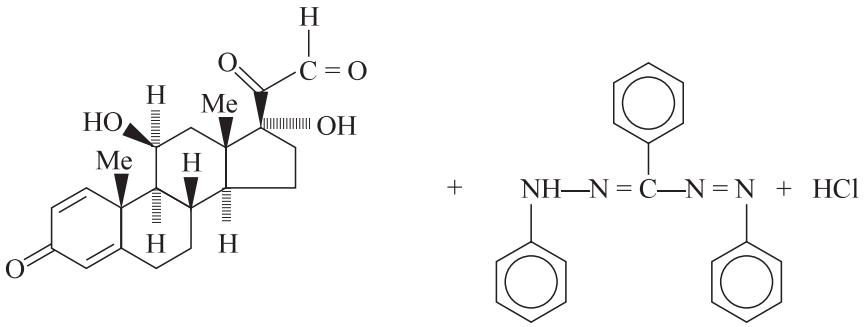
+



Triphenyltetrazolium chloride



Tetramethyl ammonium hydroxide
(30–35° ; 1–2 Hours)



Prednisolone
(C-21 : —CHO)

Triphenylformazan
(Red product)

The triphenyltetrazolium chloride ring undergoes cleavage, as shown by the dotted line, and 2H-atoms are given out by the steroid prednisolone in being converted from C-21, —CH_2OH to C-21, —CHO function ; one of the H-atoms from above is utilized in the formation of the open-chain compound *i.e.*, triphenylformazan derivative ; whereas, the second H-atom abstracts the Cl^- ion as a mole of HCl. The above interaction is of a quantitative nature.

However, it is pertinent to mention here that certain steroids esterified at C-21 position, such as : hydrocortisone acetate, methylprednisolone acetate are duly hydrolyzed in the alkaline medium to give rise to the corresponding free C-21 hydroxy steroids and hence, may also be assayed by adopting the same procedure.

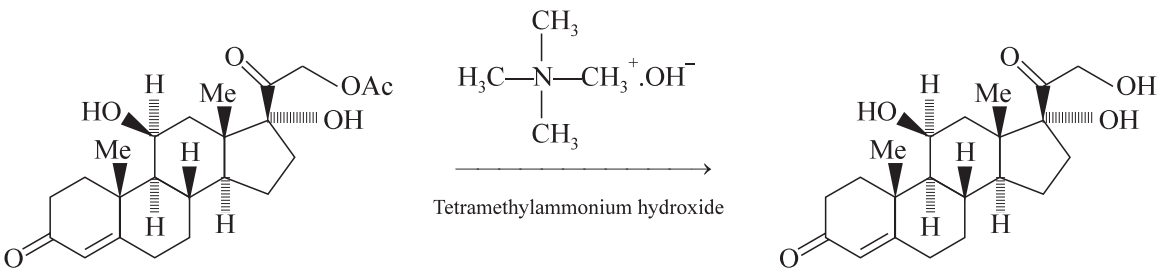
Precautions : All these assays are to be carried out strictly in the absence of light and atmospheric oxygen to get optimum results.

15.3. ASSAY OF PHARMACEUTICAL SUBSTANCES

A number of steroidal pharmaceutical substances listed in the *official compendia* may be assayed by the tetrazolium method of analysis. A few typical examples are described below :

15.3.1. HYDROCORTISONE ACETATE

Theory : Hydrocortisone acetate is first hydrolysed by strong trimethylammonium hydroxide solution to yield the free 21-hydroxysteroid *i.e.*, hydrocortisone as shown below :



Hydrocortisone acetate

Tetramethylammonium hydroxide

Hydrocortosone

The resulting hydrolysed product is then treated with triphenyltetrazolium chloride and the coloured triphenylformazan is measured at 525 nm.

Materials Required : Hydrocortisone acetate : 0.350 g ; aldehyde-free absolute ethanol : 100 ml ; triphenyltetrazolium chloride solution [a 0.5% w/v solution of 2,3,5,-triphenyltetrazolium chloride in aldehyde-free ethanol (96%)] : 10 ml ; dilute tetramethylammonium hydroxide solution [Dilute 10 ml of tetramethylammonium hydroxide solution (10%) to 100 ml with aldehyde-free ethanol (96%). It contains about 1% w/v of $C_4H_{13}NO$. To be prepared immediately before use] : 10 ml.

Procedure : The following steps are to be followed sequentially strictly protected from light :

(1) Dissolve accurately weighed hydrocortisone acetate 300 to 350 mg in 10 ml aldehyde-free absolute ethanol,

(2) Transfer 10 ml to a 25 ml graduated flask, add 2 ml of triphenyltetrazolium chloride solution, displace the air in the flask with oxygen-free nitrogen,

(3) Immediately add 2 ml of dilute tetramethylammonium hydroxide solution and again displace the air with oxygen-free nitrogen,

(4) Stopper the flask, mix the contents by gently swirling and allow to stand in a water-bath maintained at 30°C for 1 hour,

(5) Cool rapidly, add sufficient aldehyde-free absolute ethanol to produce 25 ml,

(6) Mix well and immediately determine the absorbance of the resulting solution in a stoppered cell at the maximum at 485 nm, using in the reference cell a solution prepared at the same time and in the same manner using 10 ml of aidehyde-free absolute ethanol, and

(7) Repeat the operation using the hydrocortisone acetate EPCRS* in place of the substance being examined under the same experimental parameters.

15.3.2. COGNATE ASSAYS

The following pharmaceutical substances may also be assayed by the above method, namely :

(*a*) Methylprednisolone,

(*b*) Hydrocortisone,

(*c*) Prednisolone, and

(*d*) Prednisone.

THEORETICAL AND PRACTICAL EXERCISES

1. What is the underlying principle of '**tetrazolium assay of steroids**' ? Explain with necessary equations involved in it.

2. Describe the assay of the following steroidal drugs :

(*i*) Hydrocortisone acetate (*ii*) Hydrocortisone

(*iii*) Prednisolone (*iv*) Prednisone.

RECOMMENDED READINGS

1. Beckett, A.H., and J.B. Stenlake, '**Practical Pharmaceutical Chemistry**', 4th ed., London, The Athlone Press, 1988.

2. **British Pharmacopoeia,** International Edition, Vols. I & II, HMSO, 1993.

* European Pharmacopoeia Chemical Reference Substances.

PART III

ELECTROCHEMICAL METHODS

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16 POTENTIOMETRIC METHODS

CONTAINS :

16.1. INTRODUCTION

Generally speaking the actual concentration of a broad spectrum of solutes may be measured conveniently by forming an appropriate electrochemical cell. Thus, most electrochemical cells invariably comprise of *two* electrodes, namely : (*a*) *an Indicator Electrode*—the voltage of which solely depends on the thermodynamic activity (*i.e.*, concentration) of one specific component in the solution ; and (*b*) *a Reference Electrode*—the voltage of which must be absolutely independent of the nature and composition of the solutions wherein it is immersed. Placing together of these two electrodes in a solution obviously gives rise to an electrochemical cell ; and consequently the voltage thus generated across the electrodes may be determined by connecting it either to a potentiometer or a millivoltmeter that has a sensitivity to measure ± 0.2 mV, besides possessing a high impedence-input of minimum 10^{12} ohms (Ω).

Under these experimental parameters when an extremely feeble current, of the order of less than 5 pA, is drawn from the electrodes, the e.m.f. of the cell may be expressed as below :

$$E_{cell} = E_{+} - E_{-} + E_{j} \qquad ...(a)$$

where, E_j = e.m.f. at the liquid junction.

In Eq. (*a*), E_j may be eliminated completely by emp1oying a saltbridge integral with the reference electrode. In usual practice, the loss of electrons or reduction occurs from the prevailing chemical system at the cathode ; whereas the gain of electrons or oxidation takes place at the anode.

16.2. THEORY

In a situation, where a metal M is placed in a solution containing its own ions M^{n+}, an electrode potential is established across the two electrodes, whose actual value is provided by the Nernst equation as shown below :

$$E = E^{-} + (RT/nF)\ {}^{1}n\ a\ M^{n+} \qquad ...\ (b)$$

From Eq. (*b*) the relationship to a cationic electrode, *i.e.*, sensitive only to a cation concentration, may be expressed as :

$$E = E^{-} Y^{n+}, Y + (RT/nF)\ {}^{1}n\ aY^{n+} \qquad ...(c)$$

to an anionic electrode :

$$E = E^{-} X^{n-}, X - (RT/nF)\ {}^{1}n\ a\ X^{n-} \qquad ...(d)$$

or to a redox electrode :

$$E = E^{-}_{ox,\ red} + (RT/nF)\ {}^{1}n\ \frac{a_{ox}}{a_{red}} \qquad ...(e)$$

where, E^{-} = Standard electrode potential (SEP) (or reduction potential of the half-cell involved),

a = Thermodynamic activity of the ion to which the electrode is sensitive,

R = Gas constant (8.314 $JK^{-1}\ mol^{-1}$),

T = Absolute temperature (K),

F = Faraday (96500 C/mole of electrons), and

n = Number of electrons involved in the electrode reaction.

Direct Potentiometry : The procedure adopted of employing a single measurement of electrode potential to determine the concentration of an ionic species in a solution is usually termed as **direct potentiometry**.

Disadvantages : Direct potentiometry has the following *two* serious disadvantages namely :

(*a*) **From the Nernst Eq. (b) :** Considering n = 1, temperature 25°C, RT/nF being a constant, and introducing the factor for the conversion of natural logarithms to logarithms to base 10, the term RT/nF shows a value of 0.0591 V. Therefore, for an ion M^{+} (monovalent) a ten-time change in the electrode potential E by approximately 60 millivolts (mV) ; whereas for an ion M^{2+} (bivalent) a change in identical magnitude of activity shall bring forth alternation of E by about 30 mV. Hence, it is evident that to attain a desired accuracy and precision to the extent of 1% in the estimated value for the direct concentration using the technique of direct potentiometry, for M^{+} ion—the E should be measurable correctly within 0.26 mV ; and for M^{2+} ion-within 0.1 mV.

(*b*) **Uncertainty due to liquid-junction potential (E_j) :** It has been observed that the liquid-junction potential (E_j) occurring between the two solutions, one related to the reference-electrode and the other to the indicator-electrode gives rise to a certain quantum of uncertainty with regard to e.m.f. measurement.

Remedial Measures : There are *two* ways to eliminate the above anomaly, namely :

(*i*) to replace the reference electrode with a concentration-cell *i.e.*, with an electrode comprised of a rod of the same metal as that employed in the indicator electrode plus a solution having the same cation as present in the test-solution, but with a known concentration. Thus, the ionic activity of the metal ion present in the test-solution may be represented by the following expression :

$$E_{cell} = (RT/nF)\ {}^{1}n\ \frac{(activity)_{known}}{(activity)_{unknown}} \qquad ...(f)$$

(*ii*) by using one solution which contains a high concentration of KCl or NH_4NO_3 *i.e.*, such electrolytes that offer almost identical values for ionic conductivities for both cation as well as anion.

Keeping in view the above serious anomalies commonly encountered with direct potentiometry, such as : an element of uncertainty triggered by liquid junction potential (E_j) and high degree of sensitivity required to measure electrode potential (E), it promptly gave birth to the phenomenon of potentiometric titrations,

which subsequently received a high level of sophistication and ultimately turned into a versatile analytical method. As the name suggests, it is indeed a titrimetric method whereby a series of potentiometric measurements are recorded so as to locate the end-point as correctly as possible. In this procedure, it is particularly of more interest to know the exact changes in the observed electrode potential after each addition of the titrant, rather than a precise and accurate electrode potential often brought about by a given solution. Thus, in a way the impact due to liquid-junction-potential (E_j) has been eliminated completely. It is pertinent to mention here that in a potentiometric titration procedure the apparent change in cell e.m.f. takes place not only most rapidly but also most distinctly in the vicinity of the end-point.

16.2.1. GENERAL CONSIDERATIONS

The potentiometric titrations invariably cover a broad-spectrum of chemical reactions that may be classified as follows :

(*i*) Neutralization reactions,

(*ii*) Redox reactions,

(*iii*) Precipitation reactions,

(*iv*) Complexation reactions, and

(*v*) Potentiometric titrations in non-aqueous solvents.

The general principles which govern the above different types of reactions will be discussed briefly in the sections that follow :

16.2.1.1. Neutralization Reactions

The accuracy and precision with which the end-point can be determined potentiometrically solely depends upon the quantum of change in the observed e.m.f. in the vicinity of the equivalence point, which in turn entirely depends upon the strength and the concentration of acid and base employed.

Merits of the Method : It is found to be useful to titrate a mixture of acids having a significant difference in their strengths, for instance : HCl and CH_3COOH (alcoholic). In this case, the first-break in the titration curve signifies that the stronger of the two acids *i.e.*, HCl, gets neutralized ; whereas, the second-break represents the entire completion (*i.e.*, HCl + CH_3COOH).

In order to get fruitful and reproducible results it is quite necessary that the strengths between either the two acids or bases in question must vary by at least 10^5 to 1.

Demerits of the Method : The neutralization reactions often found to be giving unsatisfactory results in the following *two* instances. They are :

(*a*) when both the acid and the base are appreciably weak, and

(*b*) when either the acid or the base is very weak (*i.e.*, $K < 10^{-8}$) and also the prevailing solutions are dilute.

Note : In (*a*) above, an accuracy upto 1% is achievable in 0.1 M solution.

Choice of Electrodes :

Indicator Electrodes : Hydrogen, Glass or Antimony electrodes ;

Reference Electrode : Calomel electrode.

16.2.1.2. Redox Reactions

In this particular case the ratio of the concentrations of the oxidized and reduced forms of ionic species establishes the determining factor. Considering the following reaction,

$$\text{Oxidised form} + \underline{n}\ \text{electrons} \rightleftharpoons \text{Reduced form}$$

The electrode potential E is given by the following expression :

$$E = E^{-} + \frac{0.0591}{n} \log \frac{[Ox]}{[Red]} \qquad \ldots (g)$$

where, E^{-} = Standard potential of the system.

In other words, the potential of the immersed indicator electrode is solely controlled and monitored by the ratio of the ionic concentrations in Eq. (*g*). Furthermore, in the course of either reduction of an oxidizing agent or *vice-versa i.e.* the said ratio, and hence the observed potential, undergoes an instant rapid change in the proximity of the end-point of the redox reaction.

Example : A typical example is that of titrations of Fe^{2+} with potassium permanganate or potassium dichromate or cerium (IV) sulphate.

Choice of Electrode : Indicator Electrode : Pt wire or foil.

The oxidizing agent is usually taken in the burette.

16.2.1.3. Precipitation Reactions

In this the determining factor mainly rests on the solubility product of the resulting nearly insoluble material generated in the course of a precipitation reaction and its ionic concentration at the equivalence point. It is, however, pertinent to mention here that the indicator electrode must readily come into equilibrium with one of the ions.

Example : Titration of Ag^{+} with a halide (Cl^{-}, Br^{-} or I^{-}) or with SCN^{-} (thiocyanate ion).

Choice of Electrodes :

Reference Electrodes : Saturated Calomel Electrode (SCE) :

Silver-silver chloride Electrode ;

Indicator Electrodes : Silver wire or Platinum wire or gauze plated with silver and sealed into a glass-tube.

(It should readily come into equilibrium with one of the ions of the precipitate).

Salt-Bridge : For the determination of a halide the salt-bridge should be a saturated solution of potassium nitrate.

Note : Ion-selective electrode can also be employed.

16.2.1.4. Complexation Reaction

Complexation invariably occurs by the interaction of a sparingly soluble precipitate with an excess amount of the reagent, for instance : the classical example of titration between KCN and $AgNO_3$ as expressed by the following reactions :

$$KCN + AgNO_3 \longrightarrow AgCN + KNO_3 \qquad \ldots(h)$$

$$AgCN + KCN \longrightarrow \underset{\text{(Complex Ion)}}{K[Ag(CN)_2]} \qquad \ldots(i)$$

In Eq. (*h*) the precipitate of AgCN is produced at first instance ; consequently, the precipitate of AgCN initially produced gets dissolved by further addition of KCN to afford the complex ion $[Ag(CN)_2]^{-}$ Eq. (*i*) and only a negligible quantum of Ag^{+} ions remain in the solution. Thus, the entire process from *ab initio* to the final stage of titration may be divided into *three* distinct portions, namely :

(*i*) **Upto end-point :** Here, all the available CN^{-} ion has been virtually converted to the complex ion. At this stage the ever increasing concentration reflects a gradually increasing concentration of Ag^{+} ions, thereby slowly enhancing the potential of the Ag-electrode dipping in the solution,

(*ii*) **At the end-point :** It is usually visualized by a distinct and marked rise in potential, and

(*iii*) **Beyond end-point :** Further addition of $AgNO_3$ brings about only a gradual change in e.m.f. and AgCN gets precipitated. Ultimately, a second sudden change in potential may be visualized at this juncture when practically most of the CN^- ion gets precipitated as AgCN.

Choice of Electrodes :

Indicator Electrode : Silver electrode ;

Reference Electrodes : Colomel electrode ; Mercury-mercury (I) sulphate electrode.

Salt-Bridge : A saturated solution of KNO_3 or K_2SO_4 isolated from the reference electrode.

16.2.1.5. Potentiometric Titration in Non-Aqueous Solvents

The potentiometric technique has proved to be of great significance and utility for determining end-points of titrations in a non-aqueous media. The mV scale rather than the pH scale of the potentiometer must be used for obvious reasons, namely :

(*i*) pH scale based upon buffers has no logical significance in a non-aqueous media, and

(*ii*) the potentials in non-aqueous media may exceed the pH scale.

The resulting titration curves are more or less emperical and afford a reasonably dependable and reproducible means of end-point detection.

Choice of Electrodes :

Indicator Electrodes : Glass electrode ;

Reference Electrode : Calomel electrode ;

Salt-Bridge : A saturated solution of KCl.

16.2.2. END-POINT DETERMINATION

In fact, there are several acceptable means to graph the potentiometric titration data generated from an actual titration (Section : 16.2.1.1 to 16.2.1.5) in order to locate the exact (or nearest) end-point. These may be illustrated exclusively by employing the titration data provided in Table 16.1, between 25 ml of 0.01 M NaF and 0.01 M La $(NO_3)_3$.

Table 16.1 : Data of Potentiometric Titration of 25.0 ml 0.01 M NaF Against 0.01 M La $(NO_3)_3$

Volume of La $(NO_3)_3$ (ml)	E (mV)	$\Delta E/\Delta V$ (mV ml^{-1})	$\Delta^2E/\Delta V^2$ (mV ml^{-2})
2.0	– 250	0.5	0.25
4.0	– 249	1	0
5.0	– 248	1	1
6.0	– 247	2	4
6.5	– 246	4	32
7.0	– 244	20	0
7.2	– 240	20	25
7.4	– 236	– 25	50
7.6	– 231	35	75
7.8	– 224	50	200
8.0	– 214	90	600
8.1	– 205	150	900
8.2	– 190	260	– 700
8.3	– 164	190	– 800

8.4	– 145	110	– 300
8.5	– 134	80	– 225
8.6	– 126	45	– 75
8.8	– 117	30	– 50
9.0	– 111	20	– 35
9.2	– 107	13	– 23
9.5	– 103	6	– 6
10.0	– 100	3	– 1
11.0	– 97	2	– 1
12.0	– 95	0.5	– 0.75

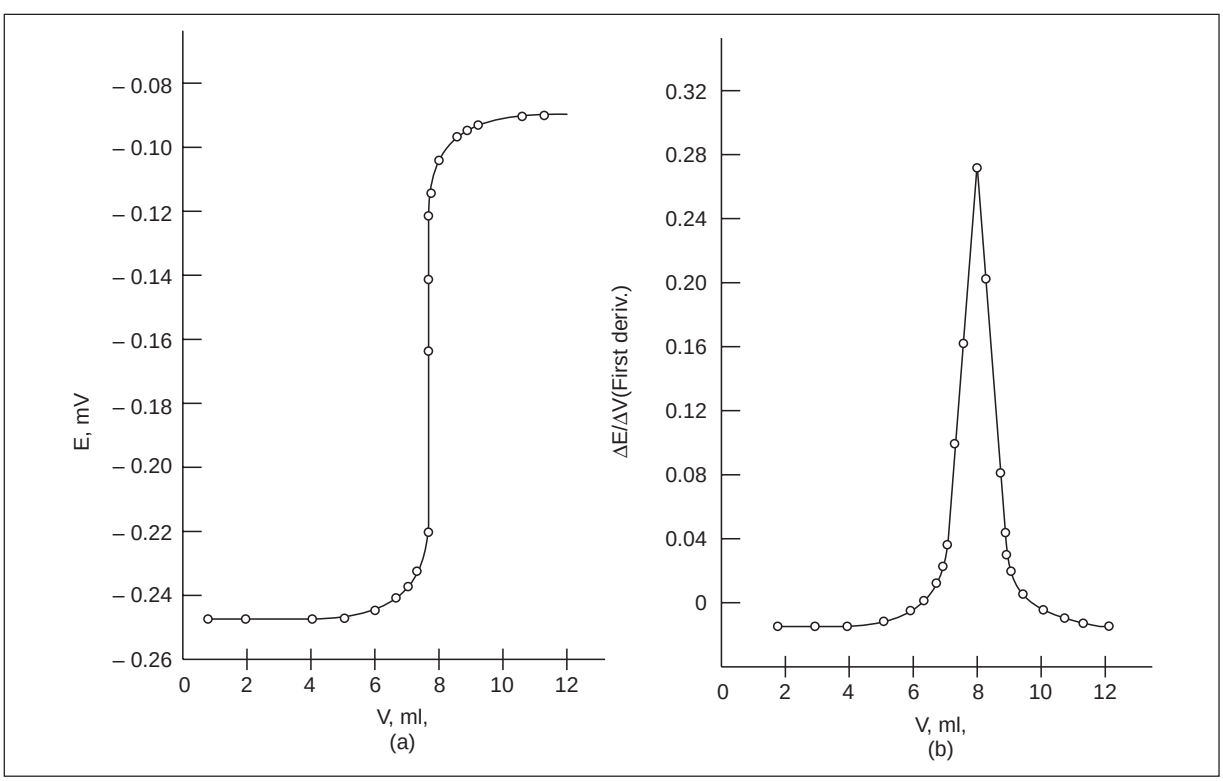
The simplest and the most commonly used method is to plot the cell voltage E, millivolts (mV), versus the volume (ml) of titrant added. Ultimately, the end-point is determined from the point of maximum slope of the curve *i.e.*, the point of inflexion, as depicted in Figure 16.1 (*a*). However, the degree of accuracy and precision with which this point of inflexion can be located from the plotted graph largely depends on the individual number of data points observed in the close proximities of the end-point.

Figure 16.1 (*a*) gives rise to a **sigmoid-curve** (or **S-shaped curve**) obtained either by using an appropriate equipment (automatic titrators) that plots the graph automatically* as the titration proceeds, or manually by plotting the raw experimental data. The central portion of the sigmoid curve, in fact is the critical zone where the point of inflexion resides and this may be located by adopting any one of the following *three* procedures, namely :

(*i*) Method of parallel tangents,

(*ii*) Method of bisection, and

(*iii*) Method of circle fitting.



* Automatic titrators do not necessarily produce results that are more accurate than those obtained manually, but are much faster and capable of handling large samples.

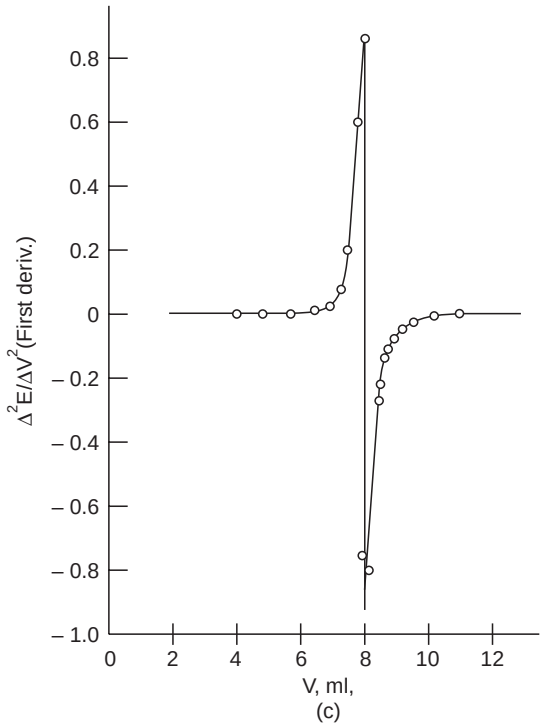


Figure 16.1 : Potentiometric Titration Curves of 25 ml of 0.01 M Sodium Fluoride *Vs* 0.01 M Lanthanum Nitrate ; (*a*) Sigmoid (Regular) Curve ; (*b*) First Derivative Curve ; (*c*) Second Derivative Curve.

Figure 16.1 (*b*) is obtained by plotting $\Delta E/\Delta V$ against V which is termed as the **first derivative curve**. It gives a maximum at the point of inflexion of the titration curve *i.e.*, at the end-point.

Figure 16.1 (*c*) is achieved by plotting the slope of the frst derivative curve against the volume of titrant added *i.e.*, by plotting $\Delta^2E/\Delta V^2$ Vs V and is known as the **second derivative curve**. Thus, the second derivative becomes zero at the point of inflexion and hence, affords a more exact measurement of the equivalence point.

The titration error (*i.e.*, difference between end-point and equivalence point) is found to be small when the potential change at the equivalence point is large. Invariably, in most of the reactions employed in potentiometric analysis, the titration error is normally quite small and hence may be neglected.

16.3. INSTRUMENTATION

Figure 16.2 illustrates a typical assembly for carrying out a potentiometric titration. Broadly speaking, the titration essentially comprises of measuring and subsequently recording a cell potential in terms of either mV or pH, after each sequentially known addition of reagents.

In usual practice, the titrant (*e.g.*, Lanthanum Nitrate) is added in large amounts at the initial stage ; as the end-point is approached, which is marked by distinct larger potential changes per addition, the subsequent increments are made smaller to the tune of 0.1 ml for each addition.

It is always advisable to allow sufficient time lapse after each addition of titrant so as to attain equilibrium. A gentle and uniform stirring by means of a magnetic stirrer also helps in hastening the ultimate achievement of equilibrium :

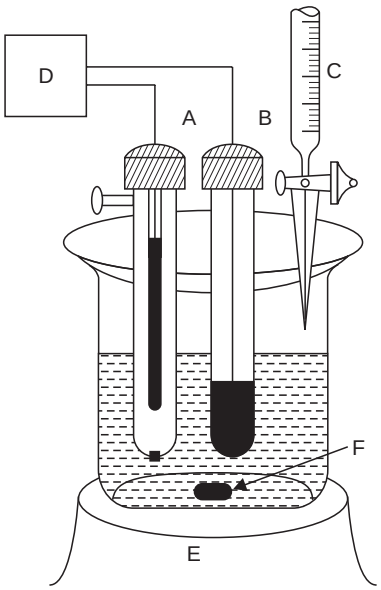


Figure 16.2 : A Potentiometric Titration Assembly.

The various components shown in Figure 16.2, are as follows :

A = Saturated Calomel Electrode (SCE),

B = Indicator Electrode,

C = Burette to discharge titrant in the reacting vessel,

D = pH Meter with a mV scale,

E = Magnetic stirrer with variable speed, and

F = Magnetic Guide.

16.3.1. ELECTRODES

The accurate, precise and effective potentiometric measurements are evidently made with the aid of the following *two* types of electrodes namely :

(*i*) **Reference Electrodes,** such as :

(*a*) Standard Hydrogen Electrode,

(*b*) Saturated Calomel Electrode, and

(*c*) Silver-silver Chloride Electrode.

(*ii*) **Indicator Electrodes**, such as :

(*a*) Metal Indicator Electrode, and

(*b*) Membrane Indicator Electrode.

These various kinds of electrodes will be discussed briefly, along with a diagrammatic representation wherever possible, in the sections that follow :

16.3.1.1. Reference Electrodes

In general, reference electrodes exhibit a potential which is absolutely independent of the solution wherein it is used. Besides, it must not display any significant change even when a small quantum of current is passed through it.

16.3.1.1.1. *Standard Hydrogen Electrode (SHE)*

The standard hydrogen electrode (SHE), as shown in Figure 16.3, is considered to be the universally accepted reference electrode. The metal electrode comprises of a small piece of platinum foil with a finely divided platinum, usually termed as **platinum black** because of its dark look. The coated foil is immersed in an acidic medium having a hydrogen ion activity of 0.1, and through which H_2 gas is bubbled at a partial pressure of 1.0 atm (unit activity). The Pt-black-foil possesses a relatively large-surface-area thereby enabling it to absorb an appreciable amount of H_2 gas, ultimately bringing it into direct contact with the surrounding H^+ ions at the electrode surface. Consequently, the Pt-electrode attains a potential which is finally estimated by the relative tendencies of H^+ ions to undergo reduction and H_2 (*g*) to undergo oxidation simultaneously. It is an usual convention to assign the potential of SHE a value exactly equal to zero at all temperatures.

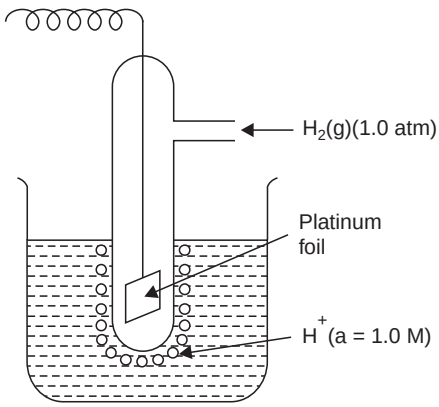


Figure 16.3 : A Standard Hydrogen Electrode.

16.3.1.1.2. *Saturated Calomel Electrode*

The schematic diagram of a commercial saturated calomel electrode (SCE) is depicted in Figure 16.4. It essentially consists of a platinum wire immersed in a slurry made up of pure mercury, solid mercurous chloride Hg_2Cl_2 (commonly known as **calomel**), and aqueous saturated solution of KCl, packed in the inner-tube (*c*) having a small hole (B). The outer-tube contains a saturated solution of KCl (D) having a porous ceramic fiber (A) at its lower end. It serves as a salt-bridge which allows the entire set-up immersed directly into the solution to be measured. The porous ceramic fiber permits establishment of electrical contact between one side of the salt-bridge and the solution under the examination and serves as a barrier between the said two solutions. The small opening at the top end of the salt-bridge tube serves as a fill-hole (E) through which either KCl solution may be filled or replaced as and when required. The different parts of the saturated calomel electrode are as follows :

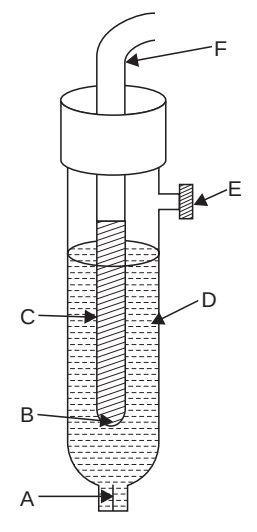


Figure 16.4 : A Saturated Calomel Electrode.

A = Porous ceramic fiber,

B = Small-hole,

C = Slurry of Hg, Hg_2Cl_2 and saturated KCl,

D = Saturated KCl solution,

E = Fill-hole, and

F = Electrical lead.

The half-cell of SCE may be expressed as :

$$Hg \mid Hg_2Cl_2 \text{ [sat'd], KCl [sat'd]} \parallel$$

for which the half-reaction is :

$$Hg_2Cl_2(s) + 2e^- \rightleftharpoons 2Hg\ (l) + 2Cl^-$$

According to the Nernst equation, the potential of the electrode is represented by :

$$E = E^{\circ}_{Hg_2Cl_2} / Hg - \frac{0.0592}{2} \log \frac{(1)[Cl^-]^2}{1}$$

assuming the activities of Hg and Hg_2Cl_2 solid are both unity.

Advantages : The *two* major advantages of SCE are, namely :

(*a*) Concentration of Cl^- does not alter appreciably even if some of the solvent gets evaporated, and

(*b*) Generates a comparatively small junction potential (E_j) at the two salt-bridge solution interfaces.

16.3.1.1.3. *Silver-silver Chloride Electrode*

Figure 16.5 shows a silver-silver chloride electrode which comprises of a silver wire coated with silver chloride (B) and is duly placed in a 1 M KCl solution saturated with AgCl (C).

The half-cell of silver-silver chloride electrode may be represented as :

$$Ag(s) \mid AgCl\ [sat'd]\ ,\ KCl\ [xM] \parallel$$

for which half-reaction would be :

$$AgCl(s) + e^- \rightleftharpoons Ag(s) + Cl^-$$

According to the Nernst equation, the potential of the electrode is expressed as :

$$E = E^{\circ}_{AgCl/Ag^+} - \frac{0.0592}{1} \log [Cl^-]$$

considering that the potential of the electrode is solely dependent on the concentration of Cl^-.

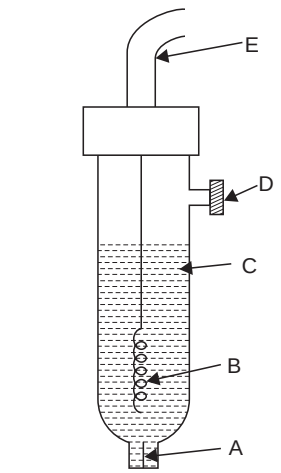


Figure 16.5 : A Silver-silver Chloride Electrode.

The various components of a silver-silver chloride electrode are, namely :

A = Porous ceramic fiber,

B = Ag wire coated with AgCl,

C = 1 M KCl saturated with AgCl,

D = Fill-hole, and

E = Electrical lead.

16.3.1.2. Indicator Electrodes

An indicator electrode is invariably used exclusively in conjunction with a reference electrode the response of which solely depends upon the concentration of the analyte.

16.3.1.2.1. *Metal Indicator Electrode*

Metal indicator electrodes develop a potential which is usually determined by the equilibrium position of a redox half-reaction at the electrode surface. These are further classified into the following *three* types, namely :

(*i*) First order electrodes,

(*ii*) Second order electrodes, and

(*iii*) Inert electrodes.

which shall be discussed briefly below.

16.3.1.2.1.1. *First-order electrodes*

They are essentially comprised of a metal placed in a solution of its respective ions, for instance : a silver wire immersed into a $AgNO_3$ solution. Hence, the reversible half reaction may be represented as :

$$Ag^+ + e^- \rightleftharpoons Ag(s) \quad E° = 0.800 \text{ V}$$

and the corresponding Nernst equation would be as follows :

$$E = 0.800 - \frac{0.0592}{1} \log \frac{1}{Ag^+}$$

The metals that display reversible half reactions with their respective ions and are found to be suitable for employing as first-order electrodes are Ag, Hg, Cu, Cd, Zn, Bi, Pb and Sn. However, several other metals like : Fe, Co, Cr and W are not useful due to the following reasons :

(*i*) Non-reproducible potentials largely influenced by impurities,

(*ii*) Irregular crystal structures in the solid-state, and

(*iii*) Formation of oxide layers on their surfaces.

16.3.1.2.1.2. *Second-order electrodes*

Sometimes a metal electrode may be directly responsible to the concentration of an anion which either gives rise to a complex or a precipitate with the respective cations of the metal. Therefore, they are termed as **second-order electrodes** as they respond to an ion not directly involved in the electron transfer process. The silver-silver chloride electrode, as already described in Section 16.3.1.1.3, is a typical example of a second-order electrode. In this particular instance, the coated Ag wire when dipped in a solution, sufficient AgCl dissolves to saturate the layer of solution just in contact with the respective electrode surface. Thus, the Ag^+ ion concentration in the said layer of solution may be determined by the status of the solubility product (K_{sp}) equilibrium :

$$AgCl(s) \rightleftharpoons Ag^+ + Cl^-$$

or $$K_{sp} = [Ag^+]\,[Cl^-]$$

Disadvantages : The *four* serious disadvantages are, namely :

(*a*) May be used effectively over a certain range of anion concentration only so that the solution must remain saturated with the substance coating the metal,

(*b*) In the case of Ag-AgCl electrode, a very low Cl^- ion concentration would dissolve the AgCl-coating to a great extent,

(*c*) Likewise, a very high concentration of Cl^- ion would result into the formation of soluble complex ions as shown below :

$$AgCl(s) + Cl^- \rightleftharpoons AgCl_2^-$$
$$AgCl_2^- + Cl^- \rightleftharpoons AgCl_3^{2-}$$

(*d*) Ions like Br^-, I^- SCN^-, CN^- and S^{2-} cause interference while using a Ag-AgCl electrode to estimate Cl^- ion concentrations because of the facts that these ions usually form salts with Ag^+ ion which are significantly less soluble than AgCl.

16.3.1.2.1.3. *Inert electrodes*

Inert electrodes comprise of chemically inert conductors, for instance : Au, Pt and C which do not necessarily take part either directly or indirectly in the various redox processes. However, the potential developed at an inert electrode solely depends upon both the nature as well as the prevailing concentration of the different redox-reagents present in the solution.

Example : A Pt-electrode placed in a solution consisting of both Fe^{3+} and Fe^{2+} ions develops a potential which is duly represented by the Nernst equation for ions as given below :

$$E = E^\circ_{Fe^{3+}/Fe^{2+}} - \frac{0.0592}{1} \log \frac{[Fe^{2+}]}{[Fe^{3+}]}$$

Advantages : The *two* main advantages of inert-electrodes are, namely :

(*a*) Exhibit no chemical selectivity, and

(*b*) Respond to any reversible redox-system.

16.3.1.2.2. *Membrane Indicator Electrodes (or Ion-Selective Electrodes)*

The underlying principle of this type of electrode is that the potential developed due to an unequal charge generated at the opposing surfaces of a 'special' membrane. The resulting charge at each surface of the membrane is exclusively controlled and monitored by the exact position of an equilibrium involving analyte ions, which in turn, solely depends upon the concentration of those ions present in the solution. Ion-selective electrodes occupy a very important place in the analytical chemistry by virtue of the fact that one may use the acquired skill, expertise and wisdom to design and commercially prepare membranes that are practically selective towards a specific ion besides producing potentials according to the Nernst-type equation. These are classified further into the following *four* kinds, namely :

(*i*) Glass membrane electrodes,

(*ii*) Polymer (liquid) membrane electrodes,

(*iii*) Crystalline membrane electrodes, and

(*iv*) Gas-sensing electrodes,

which will be described below briefly :

16.3.1.2.2.1. *Glass Membrane Electrodes*

The diagram of a typical glass-membrane electrode is depicted in Figure 16.6. The internal element essentially comprises of a Ag-AgCl electrode (B) dipped in a pH 7 buffer saturated with AgCl (A). The thin, ion-selective glass membrane (I) is carefully fused to the bottom of a high resistance non-responsive glass tube (H) so that the entire membrane may be immersed while taking measurements.

The half-cell of glass-membrane electrode may be expressed as :

$$Ag\ (s) \mid AgCl\ [saturated],\ Cl^-\ (inside),\ H^+\ (inside) \mid glass\ membrane \mid H^+\ (outside)$$

According to the Nernst equation, the potential of the electrode is represented by :

$$E = E^\circ_{AgCl/Ag} - \frac{0.0592}{1} \log [Cl^-] + \frac{0.0592}{1} \log \frac{[H^+]_{outside}}{[H^+]_{inside}} \qquad ...(i)$$

Now, separating the ratio of H^+ ion concentrations into two log terms we may have :

$$E = E^\circ_{AgCl/Ag} - 0.0592 \log [Cl^-] + 0.0592 \log \frac{1}{[H^+]_{inside}} + 0.0592 \log [H^+]_{outside} \qquad ...(ii)$$

As the activities (*i.e.*, concentrations) of H^+ and Cl^- in the internal electrolyte solution are constant, the first three components on the right hand side of Eq. (*ii*) may be confined into a single constant, K, and the equation could be rewritten as :

$$E = K + 0.0592 \log [H^+]_{outside} \qquad ...(iii)$$

The various components of Figure 16.6 are as follows :

A = pH 7.0 buffer solution saturated with AgCl,

B = Ag-AgCl internal reference electrode,

C = Mercury connection,

D = Connecting wire,

E = Shield,

F = Cap,

G = Shielded and insulated connecting wire,

H = High resistance non-responsive glass, and

I = H^+–selective glass membrane.

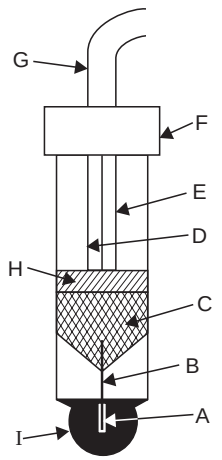


Figure 16.6 : A Glass-Membrane pH Electrode

16.3.1.2.2.2. *Polymer (Liquid) Membrane Electrode*

Figure 16.7. illustrates a calcium-ion polymer (liquid) membrane electrode. It has a close similarity to the glass pH electrode, and it essentially comprises of an internal Ag-AgCl electrode (B) and an internal reference solution having a fixed composition *e.g.*, aqueous $CaCl_2$ saturated with AgCl (C). The liquid calcium di (*n*-decyl) phosphate, $[\{CH_3(CH_2)_8CH_2O\}_2PO_2]_2$ Ca serves as the membrane, positioned at the lower end of the electrode, and strategically immobilized by a thin disk of PVC (polyvinyl chloride) (A) which is not penetrable with water.

Thus, the calcium di (*n*-decyl) phosphate forms an equilibrium with its ions at every membrane surface :

$$\underset{\text{(membrane)}}{[(RO)_2PO_2]_2\ Ca} \rightleftharpoons \underset{\text{(non-aqueous liquid membrane)}}{2(RO)_2PO_2^-} + \underset{\text{(aqueous)}}{Ca^{2+}}$$

where, R = $CH_3(CH_2)_8$ –CH_2– *i.e.*, *n*-decyl hydrocarbon chain.

Interestingly, the didecylphosphate anion represents a fixed component of the non-aqueous liquid membrane. As the concentration of Ca^+ ions present in the solutions on either side of the membrane varies ; hence, the concentration of didecylphosphate anion at every membrane surface would also vary accordingly, thereby causing a potential that may be expressed by the following equation :

$$E = E^\circ_{AgCl/Ag^+} - \frac{0.0592}{1}\log\,[Cl^-] + \frac{0.0592}{2}\log\frac{[Ca^{2+}]_{outside}}{[Ca^{2+}]_{inside}} \qquad ...(i)$$

Again, separating the ratio of Ca^{2+} ion concentration into two log terms we have :

$$E = E^\circ_{AgCl/Ag^+} - \frac{0.0592}{1}\log\,[Cl^-] + \frac{0.0592}{2}\log\frac{1}{[Ca^{2+}]_{inside}} + \frac{0.0592}{2}\log[Ca^{2+}]_{outside} \qquad ...(ii)$$

Since the activities of Ca^{2+} and Cl^- in the internal electrolyte solution are more or less constant, the first three terms on the right hand side of Eq. (*ii*) may be combined to a single constant, K, and the same equation may be rewritten as follows :

$$E = K + \frac{0.0592}{2}\log[Ca^{2+}]_{outside} \qquad ...(iii)$$

The different essential components of Figure 16.7 are as stated below :

A = Calcium di (*n*-decyl)-phosphate immobilizes in PVC,

B = Silver-silver chloride electrode, and

C = Aqueous $CaCl_2$ saturated with AgCl.

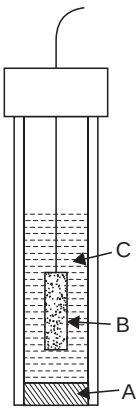


Figure 16.7 : A Calcium-ion Polymer (Liquid) Membrane Electrode.

16.3.1.2.2.3. *Crystalline Membrane Electrodes*

The crystalline membrane electrodes have a very close similarity to those of glass-membrane electrodes (see Section 16.3.1.2.2.1) except that glass has been replaced with crystalline membrane. In fact, these electrodes offer a means to devise responsive to anions by making use of a membrane containing specific anionic sites.

Example : Fluoride-ion Electrode : In this particular instance the membrane essentially comprises of a single crystal of lanthanum fluoride (LaF_3), usually doped with a slight trace of europium (II), Eu^{2+}, so as to initiate the crystal defects required for establishing its electrical conductivity. Therefore, the potential developed at each surface of the membrane is finally determined by the exact status of the equilibrium :

$$\underset{\text{(membrane)}}{LaF_3} \rightleftharpoons \underset{\text{(membrane)}}{La^{3+}} + \underset{\text{(aqueous)}}{3F^-}$$

and is represented by the following equation :

$$E = K + \frac{0.0592}{1} \log [F^-]_{outside} = K - 0.0592 \log [F^-]_{outside}$$

Salient features of Fluoride-Ion Electrode are, namely :

(*a*) At low pH, F^- ion gets readily converted to the weak acid HF (pKa = 3.17) thereby rendering the electrode insensitive,

(*b*) It is almost 10^3 times more specific and selective for F^- ion as compared to other common anions, of course with the exception of OH^- ion, and

(*c*) This electrode can tolerate conveniently the maximum concentration of OH^- ion to the extent of $\frac{1}{10}$th as compared to the F^- ion concentration.

Table 16.2 records the characteristics of certain selected crystalline-membrane electrodes.

Table 16.2 : Characteristics of Certain Selected Crystalline Membrane Electrodes

S.No.	Analyte Ion	Membrane Composition	Conc. Range (M)	Recommended pH Range	Selectivity Coefficients
1.	Br^-	$AgBr/Ag_2S$	$10° - 10^{-5}$	2 – 12	$Cl^- = 0.003$; $OH^- = 3 \times 10^{-5}$; $I^- = 5000$; $CN^- >> 1$
2.	CN^-	$AgCN/Ag_2S$	$10° - 10^{-6}$	11 – 13	$Cl^- = 1 \times 10^{-6}$; $Br = 2 \times 10^{-4}$; $I = 1.5$
3.	Ag^+	Ag_2S	$10° - 10^{-7}$	2 – 9	$Hg^{2+} >> 1$
4.	Pb^{2+}	PbS/Ag_2S	$10^{-1} - 10^{-6}$	3 – 7	$Cd^{2+} = 0.3$; $Zn^{2+} = 2 \times 10^{-4}$; $Fe^{2+} = 0.05$

16.3.1.2.2.4. *Gas-Sensing Electrode*

The schematic diagram of a gas-sensing electrode is illustrated in Figure 16.8, that comprises of essentially a reference electrode (E), a specific-ion electrode (B), and an internal electrolyte solution (F) contained in a cylindrical plastic tube (G). One end of the plastic tubing is provided with a thin, replaceable, gas-permeable membrane that separates the internal electrolyte solution from the external solution containing gaseous analyte. However, the exact composition and specifications of this gas-permeable membrane is usually described by its respective manufacturers. It is normally made up of a thin microporous film fabricated from a hydrophobic plastic material.

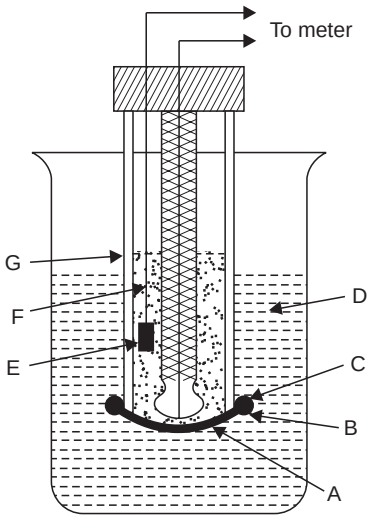


Figure 16.8 : A Schematic Diagram of Gas-Sensing Electrode.

The various components of Figure 16.8 are as follows :

A = Gas permeable membrane,

B = Specific ion electrode (a glass electrode),

C = 'O'-Ring to hold the membrane,

D = External solution containing dissolved gaseous analyte,

E = Reference electrode (a Ag/AgCl electrode),

F = Internal electrolyte solution, and

G = Plastic tube.

In general, it must fulfil the following requirements, namely :

(*a*) It should act as a 100% barrier for both water and electrolytes *i.e.*, they must not pass through this membrane,

(*b*) Pores of the film contain exclusively air or other gases to which it is exposed, and

(*c*) A solution containing a particular gaseous-analyte, for instance CO_2, when comes in contact with the membrane the former migrates swiftly into the pores of the latter, as expressed by the following reaction :

$$\underset{\text{(aqueous)}}{CO_2} \rightleftharpoons \underset{\text{(gaseous)}}{CO_2} \qquad ...(a)$$

External solution — Membrane pores

As the number of pores in the gas-permeable membrane are plenty, therefore, an equilibrium is established. Evidently, the carbon-dioxide present in the pores is in direct contact with the internal-electrolyte solution (F), thereby giving rise to a second equilibrium reaction that may be represented as follows :

$$\underset{\text{(gaseous)}}{CO_2} \rightleftharpoons \underset{\text{(aqueous)}}{CO_2} \qquad ...(b)$$

Membrane Pores — Internal Solution

As a result of the above two reactions, Eq. (*a*) and Eq. (*b*), the external solution containing dissolved gaseous analyte (D) immediately attains an equilibrium with the film of internal electrolyte solution (F) present very close to the gas-permeable membrane (A). Thus, another equilibrium gets established that affords the pH of the internal-surface film to alter according to the following expression :

$$\underset{\text{(aqueous)}}{CO_2 + 2H_2O} \rightleftharpoons HCO_3^- + H_3O^+ \qquad \text{... } (c)$$

Internal Solution — Internal Solution

The above change in pH is instantly detected by means of a Ag/AgCl reference electrode pair (E) dipped in the film of internal solution as shown in Figure 16.8.

Therefore, the net overall reaction caused by the entire aforesaid process may be achieved by simply summing up the *three* chemical reactions (*a*), (*b*), and (*c*) to give :

$$\underset{\text{(aqueous)}}{CO_2 + 2H_2O} \rightleftharpoons H_3O^+ + HCO_3^- \qquad \text{... } (d)$$

External Solution — Internal Solution

The equilibrium constant, K, for Eq. (*d*) may be represented by :

$$K = \frac{[H_3O^+][HCO_3^-]}{[CO_2\ (\text{aqueous})]_{\text{external}}} \qquad ...(e)$$

Assuming that the concentration of HCO_3^-present in the internal-electrolyte solution (F) is made comparatively high such that its concentrations do not undergo any appreciable change due to the migrating CO_2, we may have :

$$K_g = \frac{[H_3O^+][HCO_3^-]}{[CO_2\ (\text{aqueous})]_{\text{external}}} = \frac{K}{[HCO_3]} \qquad ...(f)$$

Thus, Eq. (*f*) may be rewritten as follows :

$$a_1 = [H_3O^+] = K_g\ [CO_2\ (\text{aqueous})]\ \text{external} \qquad ...(g)$$

where, a_1 = Internal hydrogen ion activity

It is given that :

$$E_{cell} = L + 0.0592 \log a_1 \qquad ...(h)$$

Consequently, the potential of the electrode system present in the internal-electrolyte solution (F) is solely dependent on a_1 according to Eq. (*h*). Hence, substituting Eq. (*g*) into Eq. (*h*), we may have :

$$E_{cell} = L + 0.0592 \log K_g\ [CO_2\ (\text{aqueous})]_{\text{external}}$$

or

$$E_{cell} = L' + 0.0592\ \text{Log}\ [CO_2\ (\text{aqueous})]_{\text{external}}$$

where, $L' = L + 0.0592 \log K_g$

In short, therefore, the potential of the cell comprising of the Ag/AgCI reference electrode (E) *i.e.*, the internal reference and the specific ion electrode (B) *i.e.*, the indicator electrode is normally determined by the CO_2 concentration of the external solution containing dissolved gaseous analyte.

Notes : (*i*) **None of the electrodes (reference & indicator) ever gets in contact directly with the analyte solution, and**

(*ii*) **The only substances which may cause interference with the measurement of potential are dissolved gases which may have a free-access through the membrane, and in turn may affect the pH of the internal solution accordingly.**

Selectivity of Gas-sensing Electrode : The selectivity of the gas-sensing electrode may be enhanced by making use of such an internal electrode which is particularly sensitive enough to certain species other than the H^+ ion.

Example : Nitrate-sensing electrode is employed to cater for a cell which will be sensitive exclusively to nitrogen dioxide (NO_2). The equilibrium of such a reaction may be represented as follows :

$$\underset{\text{(aqueous)}}{2\,NO_2} + H_2O \rightleftharpoons \underset{\text{Internal}}{NO_2^-} + NO_3^- + 2\,H^+$$

External Solution — Internal Solution

The nitrate-sensing electrode allows the determination of NO_2 in the presence of certain specific gases only, for instance, NH_3, SO_2 and CO_2, that will also affect the change in pH of the internal electrolyte solution significantly.

16.3.2. AUTOMATIC TITRATOR (PRESET END-POINT TITRATOR)

The schematic diagram of an automatic titrator* is shown in Figure 16.9.

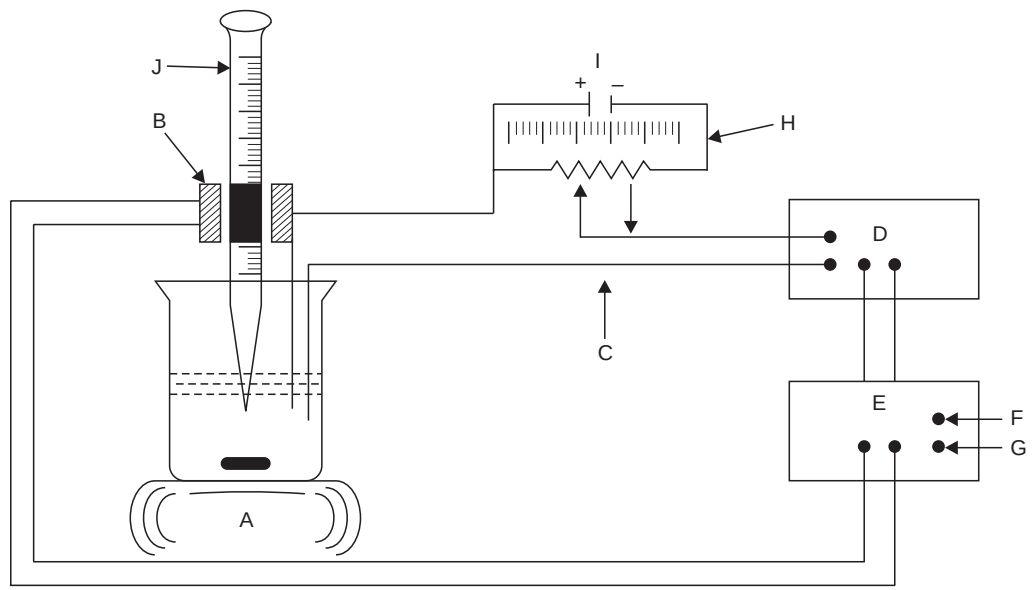


Figure 16.9 : Diagram of An Automatic Titrator.

The various components of Figure 16.9 are, namely :

A = Magnetic stirrer with a Regulator,

B = Solenoid valve, and

C = Error Signal,

D = Amplifier,

E = Electronic Switch,

F and G = AC-Source,

H = End-point Potential,

I = Calibrated Potentiometer, and

J = Accurately calibrated Burette.

In this case a preset equivalence point potentiometer is applied at the two electrodes with the aid of a calibrated potentiometer (I). It will give rise to an "error" signal (C) provided a difference is caused between this potential and that of the electrodes. The feeble signal thus generated is duly amplified (D) and closes an electronic switch (E) which allows the electricity to flow through the solenoid operated value (B) of the burette (J). As the titration proceeds, the error signal (C) starts approaching a zero value, subsequently the

*Svehla, G., **Potentiometric Titrations**, New York, Pergamon Press, 1978.

current to the solenoid valve (B) is instantly switched off, and finally the flow of titrant from the burette (J) comes to a halt. The solution of the sample is constantly and uniformly stirred with the help of a magnetic stirrer (A).

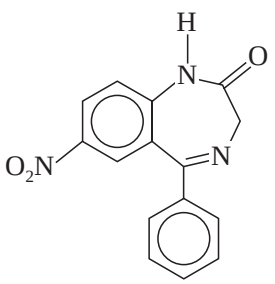
16.4. APPLICATIONS OF POTENTIOMETRIC TITRATIONS IN PHARMACEUTICAL ANALYSIS

Potentiometric titrations have been used extensively for assay of a number of official compounds. A few typical examples would be described here, namely : Nitrazepam ; Allopurinol ; and Chloridine hydrochloride.

A. Assay of Nitrazepam :

Materials Required : Nitrazepam : 0.25 g ; acetic anhydride : 25.0 ml ; perchloric acid (0.1 M) : 250 ml ; a Potentiometer ; a Magnetic Stirrer ; Burette (50 ml) ;

Theory : Nitrazepam is a weakly basic compound and hence, it may be titrated conveniently by means of a non-aqueous titration technique and determining the end-point potentiometrically.



Nitrazepam

Procedure : Weigh accurately 0.25 g of nitrazepam and dissolve in 25.0 ml of acetic anhydride. Titrate with 0.1 M perchloric acid placed in a burette and adding it carefully into the beaker kept on a magnetic stirrer potentiometrically. Each ml of 0.1 M perchloric acid is equivalent to 28.13 mg of $C_{15}H_{11}N_3O_3$.

B. Assay of Allopurinol :

Materials Required : Allopurinol : 0.12 g ; dimethylformamide : 100.0 ml ; tetrabutylammonium hydroxide (0.1 M) : 1 L ;

Preparation of 0.1 M Tetrabutylammonium hydroxide (1 Litre) : Dissolve 40 g of tetrabutylammonium iodide in 90 ml of anhydrous methanol, add 20 g of finely powdered silver oxide and shake vigorously for 1 hour. Centrifuge a few ml of the mixture and test the supernatant liquid for iodides. If a positive reaction is obtained add a further 2 g of silver oxide and shake for 30 minutes. Repeat this procedure until the mixture is free from iodides, filter through a fine sintered-glass filter and wash the reaction vessel and filter with three 50-ml quantities of toluene. Add the washings to the filtrate and add sufficient toluene to produce 1000 ml. Pass dry carbon-dioxide free N_2 through the solution for 5 minutes.

Standardization of 0.1 M Tetrabutylammonium Hydroxide : To 10 ml of dimethylformamide add 0.05 ml of a 0.3 % w/v solution of thymol blue in methanol and titrate with the tetrabutylammonium hydroxide solution until a pure blue colour is produced. Immediately add 0.2 g of benzoic acid, stir to effect solution and titrate with the tetrabutylammonium hydroxide solution until the pure blue colour is restored. Protect the solution from atmospheric CO_2 throughout the titration. The volume of titrant used in the second titration represents the amount of tetrabutylammonium hydroxide required. Each ml of 0.1 M tetrabutylammonium hydroxide Vs is equivalent to 12.21 mg of $C_7H_6O_2$.

Procedure : Dissolve 0.12 g of accurately weighed allopurinol in 50 ml of dimethylformamide, with gentle heating, if necessary. Titrate to the colour change of the indicator that corresponds to the maximum absolute value of dE/dV in a potentiometric titration (where E is the electromnotive force and V is the

volume of the titrant). Each ml of 0.1 M tetrabutylammonium hydroxide Vs is equivalent to 13.61 mg of $C_5H_4N_4O$.

C. Clonidine Hydrochloride :

Materials Required : Clonidine hydrochloride : 0.2 g ; ethanol (96%) : 100 ml ; 0.1 M ethanolic sodium hydroxide Vs : 1 L (Add 3.3 g of 10 M sodium hydroxide solution to 250 ml of absolute ethanol).

Standardization of 0.1 M Ethanolic Sodium Hydroxide Solution Vs : Dissolve 0.2 g of benzoic acid in a mixture of 10 ml of ethanol (96%) and 2 ml of water and titrate with the ethanolic sodium hydroxide solution using 0.2 ml of thymolphthalein solution (a 0.1 % w/v solution of thymolphthalein in ethanol (96%) as indicator. Each ml of 0.1 M ethanolic sodium hydroxide Vs is equivalent to 12.21 mg of $C_7H_6O_2$.

Procedure : Dissolve 0.2 g of clonidine hydrochloride in 70 ml of ethanol (96%) and titrate with 0.1M ethanolic sodium hydroxide Vs determining the end-point potentiometrically. Each ml of 0.1 M ethanolic sodium hydroxide Vs is equivalent to 26.66 mg of $C_9H_9Cl_2,N_3$, HCl.

16.4.1. COGNATE ASSAYS

A plethora of official drugs are assayed by the potentiometric method in various *official compendia*, and a few selected examples are given in Table 16.3, which may be assayed potentiometrically :

Table 16.3 : Cognate Assays of Official Compounds

S.No.	Name of Substance	Qty. Prescribed	Titrant/ Indicator	Calculations
l.	Apomorphine Hydrochloride	0.25 g	Perchloric Acid (0.1M)	Each ml of 0.1 M $HClO_4$ ≡ 30.38 mg of $C_{17}H_{17}NO_2$. HCl
2.	Azathioprine	0.25 g	Tetrabutyl-ammonium hydroxide (0.1 M)	Each ml of 0.1 M Tetrabutylammonium hydroxide ≡ 27.73 mg of $C_9H_7N_7O_2S$
3.	Bendrofluazide	0.2 g	-do-	Each ml of 0.1 M Tetrabutylammonium hydroxide = 21.07 mg of $C_{15}H_{14}F_3N_3O_4S_2$
4.	Bisacodyl	0.3 g	Perchloric Acid (0.1 M)	Each ml of 0.1 M $HClO_4$ ≡ 36.14 mg of $C_{22}H_{19}NO_4$
5.	Carbidopa	0.15 g	-do-	Each ml of 0.1 M $HClO_4$ = 22.62 mg of $C_{10}H_{14}N_2O_4$
6.	Cimetidine	0.2 g	-do-	Each ml of 0.1 M $HClO_4$ ≡ 25.23 mg of $C_{10}H_{16}N_6S$
7.	Disulfiram	0.45 g	Silver Nitrate (0.1 M)	Each ml of 0.1 M $AgNO_3$ ≡ 59.30 mg of $C_{10}H_{20}N_2S_4O$
8.	Ethinyloestra-diol	0.2 g	Sodium Hydroxide (0.1 M)	Each ml of 0.1 M NaOH ≡ 29.64 mg of $C_{20}H_{24}O_2$
9.	Etofylline	0.2 g	Perchloric Acid (0.1 M)	Each ml of 0.1 M $HClO_4$ ≡ 22.42 mg of $C_9H_{12}N_4O_3$
10.	Flunitrazepam	0.25 g	-do-	Each ml of 0.1 M $HClO_4$ ≡ 31.33 mg of $C_{16}H_{12}FN_3O_3$
11.	Glutethimide	0.15 g	Ethanolic NaOH (0.1M)	Each ml of 0.1 M NaOH ≡ 21.73 mg of $C_{13}H_{15}NO_2$
12.	Lomustine	0.2 g	Silver Nitrate (0.05 M)	Each ml of 0.05 M $AgNO_3$ ≡ 11.68 mg of $C_9H_{16}ClN_3O_2$

THEORETICAL AND PRACTICAL EXERCISES

1. 'The actual strength of a broad spectrum of '**solutes**' can be determined quantitatively by forming an appropriate electrochemical cell' Justify the above statement with the help of Nernst Equation.

2. (*a*) What are the two major disadvantages of '**Direct Potentiometry**' ? Explain.

(*b*) How can one implement '**remedial measures**' to make potentiometric titrations into an efficacious method of quantitative analytical technique ? Explain.

3. Discuss in an elaborated manner the various means of '**potentiometric titrations**' in the following reaction variants :

(*a*) Neutralization reactions, (*b*) Redox reactions,

(*c*) Precipitation reactions, (*d*) Complexation reactions, and

(*e*) Potentiometric titrations in non-aqueous solvents.

4. Potentiometric titration curves between 25 ml of 0.01 M NaF and 0.01 M La $(NO_3)_3$ may be obtained as the following **three** predominant variants, namely :

(*a*) Sigmoid (Regular) Curve, (*b*) First Derivative Curve, and

(*c*) Second Derivative Curve.

With the help of a diagramatic neat-sketch of each curve explain and affirm which one gives the most reliable '**equivalence point**' and why.

5. Describe a potentiometric titration assembly with a well-labelled diagram. Briefly enumerate its working systematically.

6. What are the tow major types of 'Electrodes' one may come across in potentiometric method of analysis ? Discuss the working of at least one electrode from each category along with its diagramatic description, working and advantages.

7. Do you think an '**Automatic Titrator**' (Preset End-Point Titrator) is a technological advancement in potentiometric titration ?

Expatiate its efficacy and advantages in a busy '**quality assurance laboratory**' with a neat-labelled diagram and its *modus operandi.*

8. How would you carry out the assay of the following '**drugs**' ?

(*i*) Nitrazepam, (*ii*) Allopurinol,

(*iii*) Bendrofluazide, (*iv*) Cimetidine,

(*v*) Lomustine, and (*vi*) Ethinyloestradiol.

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17 AMPEROMETRIC METHODS

CONTAINS :

17.1. INTRODUCTION

An **amperometric method** or **amperometry** is concerned with the measurement of current under a constant applied voltage ; and under such experimental parameters the concentration of the **'analyte'** exclusively determines the quantum and magnitude of the current. Hence, these measurements may be employed effectively to record the alteration in concentration of an ion in question in the course of a titration, and ultimately the end-point is established. This specific process is commonly referred to as *amperometric method* or *amperometry.*

In this particular case, the total current flowing shall remain almost equal to the current carried by the ions that undergoes equal electrolytic migration together with the current caused on account of the diffusion of the ions. Thus, we have :

$$I = Id + Im$$

where I = Total current,

Id = Diffusion current, and

Im = Migration current.

An awkward situation arises when dealing with a dilute solution where it has been observed that the depletion of the electrode layer ultimately leads to an enhancement of the resistance of the solution and thereby affecting subsequently an alteration in the Ohm's Law potential drop (I × R) in the cell. This ultimately gives rise to a doubtful observed potential operative at the electrode. In order to overcome this serious anomaly, it is a normal practice to add an excess of an indifferent electrolyte to the system, such as : 0.1 M KCl, which renders the solution to remain stable at a low and constant resistance, whereas the migration current (Im) of the species under examination almost vanishes *i.e.*, I = Id.

The ion under investigation, whose rate of diffusion at the electrode surface is governed by **Fick's Law** represented as under :

$$\frac{\partial c}{\partial t} = \frac{D\partial^2 c}{\partial x^2}$$

where, D = Diffusion coefficient,

C = Concentration,

t = Time, and

x = Distance from the electrode surface.

Thus, the potential of the electrode is controlled and monitored by the **Nernst Equation** as shown below :

$$E = E^\circ + \frac{RT}{nF} \ln \frac{{}^{a}ox}{{}^{a}red}$$

Salient Features of Amperometric Methods : The various salient features of amperometric titrations are enumerated below :

(*a*) It is less dependent upon the characteristics of the electrode,

(*b*) It is quite independent of the nature and type of the supporting electrolyte,

(*c*) It does not require a constant temperature in the course of a titration but it should not necessarily be fixed accurately,

(*d*) The substance under investigation may not essentially be reactive at the electrode ; whereas either a reactive reagent or a product is just sufficient for a successful amperometric titration, and

(*e*) The amperometric method is inherently more accurate and precise, and therefore, has an edge as compared to the polarographic method.

17.2. THEORY

Assuming that the migration current (Im) is virtually eliminated by the addition of a reasonably enough supporting electrolyte then the only cardinal factor which would affect the limiting current would be the rate of diffusion of the electro-active substance from the main body of the solution to the surface of the electrode.

Thus, we may have :

Diffusion current = Limiting current – Residual current

It follows from above that the diffusion current is directly proportional to the concentration of the electro-active substance present in the solution. Now, if a situation is created whereby a portion of the electro-active substance is eliminated by interaction with a specific reagent, the diffusion current shall decrease significantly. It represents the fundamental underlying principle of amperometric method or amperometry. Hence, at an appropriate applied voltage the apparent diffusion current is measured as a function of the volume of the titrating solution added. Now, if a graph is plotted between the *'current'* against the *'volume of reagent added'*, the end-point will be represented by the point of intersection of two lines indicating the change of current both before and after the equivalence is achieved.

17.2.1 TITRATION CURVES

The most commonly obtained various kinds of curves encountered in amperometric methods are illustrated in Fig. 17.1 (*a*) through (*d*) ; and each of them shall be discussed briefly as follows :

Fig. 17.1 (a) : It represents a titration wherein the analyte reacts at the electrode whereas the reagent does not. In other words, only the substance under titration gives rise to a diffusion current ; whereby the electro-active substance is removed from the solution by means of precipitation with an inactive substance.

Example : The titration of Pb^{2+} with SO_4^{2-} or $C_2O_4^{2-}$ ions. An appreciably high potential is usually applied to yield a diffusion current for lead. From Fig. : 1(A), one may evidently observe a linear decrease in current because Pb^{2+} ions are removed from the solution by precipitation. The small curvature just prior to the end-point (or equivalence point) shows the incompleteness of the analytical reaction in this particular region. However, the end-point may be achieved by extrapolation of the linear portions, as shown in the said figure.

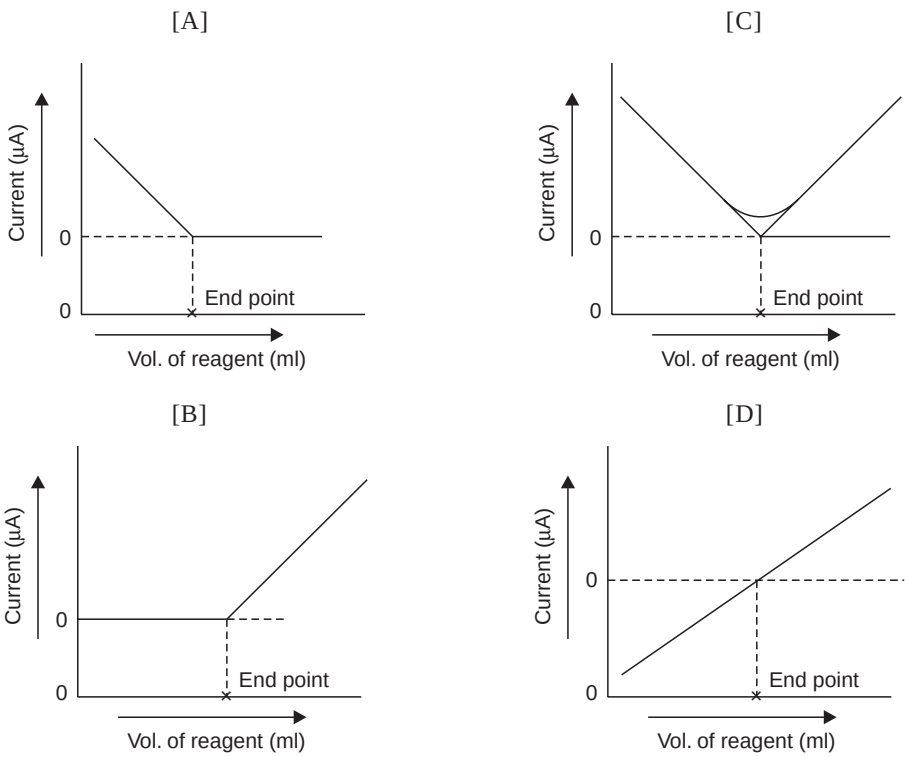

Figure 17.1 : Typical Amperometric Titration Curves; (a) 'A' is Reduced 'R' is not ; (b) 'R' is Reduced 'A' is not; (c) Both 'R' & 'A' are Reduced (d) End-point Indicated by Zero-current 'A' = Analyte ; 'R' = Reagent.

Figure 17.1 (b) : It designates typical of an amperometric titration curve wherein the reagent exclusively reacts at the microelectrode surface and the analyte does not. In other words, the reagent gives rise to a diffusion current, whereas the solute does not ; it means an electro-active precipitating reagent is being added to an inactive substance.

Examples : (a) Titration of Mg^{2+} with 8-hydroxyquinoline. In this particular instance, a diffusion current for 8-hydroxyquinoline is normally achieved at – 1.6 V *Vs* Standard Calomel Electrode (SCE), whereas Mg^{2+} ion is more or less inert at this potential.

(*b*) Titration of Ba^{2+} or Pb^{2+} ions with SO^{4-} ions.

Figure 17.1 (c) : It represents an amperometric method wherein the solute as well as the titrating reagent afford diffusion currents ; and give rise to a sharp V-shaped curve. The end-point may be obtained by extrapolation of the lower-end of the V-shaped portion of the curve as depicted in the above Figure.

Examples : (a) Titration of Ph^{2+} ion with $Cr_2O_7^{2-}$ ion. The Figure : 17.1 (*c*) corresponds to the amperometric titrations of Pb^{2+} and $Cr_2O_7^{2-}$ ion at an applied potential more than – 1.0 V ; when both these ions afford diffusion currents at this very potential and the end-point is duly signalled corresponding to a minimum in the curve.

(*b*) Titration of Ni^{2+} ion with dimethylglyoxime ion, $\begin{bmatrix} CH_3—C=NO \\ | \\ CH_3—C=NO \end{bmatrix}^{2-}$, and

(*c*) Titration of Cu^{2+} ion with benzoin α-oxime ion, $\begin{bmatrix} C_6H_5—CH—O \\ | \\ C_6H_5—C=NO \end{bmatrix}^{2-}$

Figure 17.1 (d) : In this particular instance the current undergoes a change from cathodic to anodic or *vice-versa.* Thus, the final end-point of the potentiometric titration is indicated by a zero-current as depicted in Figure 17.1 (*d*). Since the resulting diffusion coefficient of the reagent is found to be slightly different from the corresponding substance under titration, therefore, the slope of the line just before the end-point actually differs very slightly from that after the end-point. However, in actual practice it is rather convenient to add the reagent unless and until the current attains a zero value.

Examples: (*a*) Titration of I^- ion with Hg^{2+} ion (as nitrate),

(*b*) Titration of Ti^{3+} ion in an acidified tartaric acid,

$[CH(OH)COOH]_2$, medium with Fe^{3+} ion.

In addition to the above *four* types of amperometric methods cited, there also exist a plethora of titrations involving neutralization and complex ion formation that have been accomplished successfully, for instance :

(*i*) Amperometric method for the study of precipitation reactions, *e.g.*, salicylaldoxime (or salicylaldehyde oxime), dimethylglyoxime, have been used for such type of studies.

(*ii*) Halides, such as : I^-, Br^- and Cl^- have been titrated at a less negative potential by virtue of the fact that in these titrations the main indicator reaction is the deposition of silver from aquo-silver ions.

(*iii*) Micromolecular solutions of Cd^{2+} ions against ethylene diaminetetra-acetic acid (EDTA) have been carried out amperometrically.

17.2.2. CORRECTIONS FOR THE VOLUME CHANGE

The corrections for the volume change may be affected by adopting either of the *two* methods described below namely :

Method I : In order to obtain plots between current (μA) and volume of reagent (ml) specifically with linear regions both before and after the end-point (or equivalence point), it is absolutely necessary to apply the corrections for the volume change which results from the added titrant. This correction is applied by multiplying the measured corresponding diffusion current (Id) by the following factor :

$$\frac{V + v}{V}$$

where, V = Initial volume of the solution, and

v = Volume of the titrating reagent added.

Method II : The above correction caused due to the volume change may be eliminated to a great extent by making use of the reagent at a concentration of 10 to 20 times higher than that of the corresponding solute, and subsequently adding the same from a semimicro-burette very carefully. The use of concentrated reagents have the following advantages, namely :

(*a*) Relatively very small amount of dissolved O_2 is incorporated into the system, which eliminates completely the prolonged bubbling of inert gas (*e.g.*, N_2) through the medium after each addition of the reagent, and

(*b*) Elimination of '*migration current*' by simple addition of enough supporting electrolyte. If need be, an appropriate maximum suppressor can also be incorporated judiciously.

17.2.3. ADVANTAGES OF AMPEROMETRIC TITRATIONS

A few cardinal advantages of amperometric titrations are described below, namely :

1. The amperometric titration may normally be performed very quickly, because the equivalence point (or end-point) is determined graphically. A series of measurements at constant applied voltage just prior and latter to the end-point are more than enough.
2. The titrations can be carried out both satisfactorily and effectively in such situations where the solubility relations offer erroneous and unsatisfactory results given by visual indicator and potentiometric methods. For instance :

(*a*) A reaction product which is hydrolysed significantly *e.g.*, acid base titrations, and

(*b*) A reaction product that is appreciably insoluble *e.g.*, precipitation reaction.

It is quite evident that the readings in the vicinity of end-point offer practically no specific value and importance in amperometric titrations. Because the readings are mostly taken in particular zones where there exists either an excess of reagent or of titrant, and which specific points the hydrolysis or solubility is entirely suppressed by the effect of Mass Action. The point of intersection of these lines ultimately gives rise to the desired end-point.

3. A good number of amperometric titrations may be performed on considerably dilute solutions (say, 10^{-4} M) at which neither potentiometric nor visual indicator methods ever can give precise and accurate results, and
4. In order to eliminate the migration current (Im) completely either the '*foreign salts*' already present cause little interference or invariably added so as to serve as the '*supporting electrolyte*'.

17.3. INSTRUMENTATION

The amperometric titrations can be accomplished by any one of the *three* methods, namely :

(*i*) Amperometric titrations with the dropping mercury electrode,

(*ii*) Amperometric titrations with a rotating platinum microelectrode, and

(*iii*) Amperometric titrations with twin-polarized microelectrodes (or **Biamperometric Titrations** or **Dead-stop-end-point method**).

These *three* techniques will be discussed in the sections that follow.

17.3.1. AMPEROMETRIC TITRATIONS WITH THE DROPPING MERCURY ELECTRODE

Figure 17.2 (*a*) and (*b*) illustrates the schematic diagram of amperometric titrations with the dropping mercury electrode having a titration-cell and an electric circuit respectively.

The titration-cell Figure 17.2 (*a*) essentially comprises of a pyrex 100-ml, four-necked, flat-bottomed flask. A semimicro burette (B) (graduated in 0.01 ml), a 2-way gas-inlet tube (A) to enable N_2 to pass either through the solution or simply over its surface, a dropping mercury electrode (C) and an agar-potassium salt-bridge* are duly fitted into the four necks with the help of air-tight rubber stoppers.

The electrical circuit, Figure 17.2 (*b*), consists of two 1.5 V dry cells that provides a voltage applied to the above titration cell. It is duly controlled and monitored by the potential divider (R) and is conveniently measured with the help of a digital voltmeter (V). Finally, the current flowing through the circuit may be read out on the micro-ammeter (M) installed.

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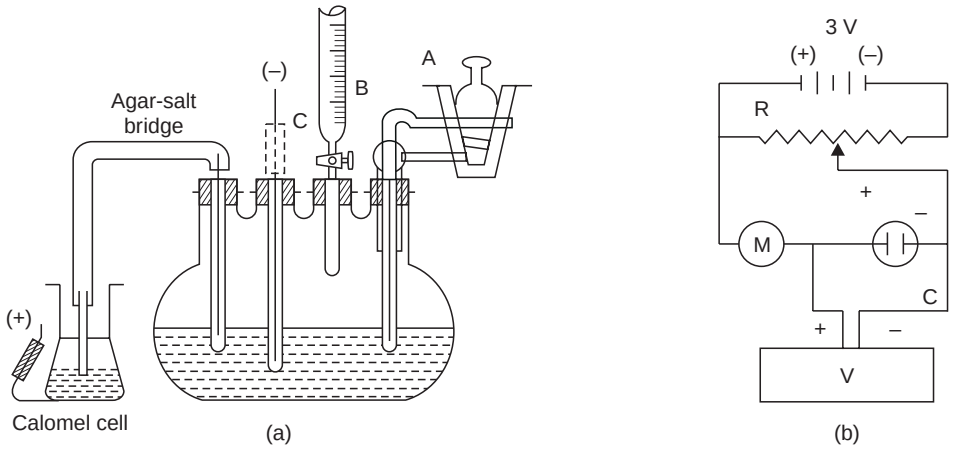
*The agar-salt bridge is usually made from a gel which is 3% agar and contains enough KCl to saturate the solution at room temperature. An agar-KNO_3 bridge is used when Cl^- ion interferes.



(*a*) Titration-Cell ; (*b*) Electrical Circuit

Figure 17.2 : A Schematic Diagram of Amperometric Titrations with Dropping Mercury Electrode.

For Figure : 17.2 [a]

A = 2-Way gas-inlet tube,

B = Semimicro burette (graduated in 0.01 ml), and

C = Dropping mercury electrode.

For Figure : 17.2 [b]

R = Potential divider (a 50 – 100Ω variable resistance),

C = Cell,

M = Micro-ammeter (μA), and

V = Digital voltmeter.

The following steps may be carried out in a sequential manner for an amperometric titration, namely :

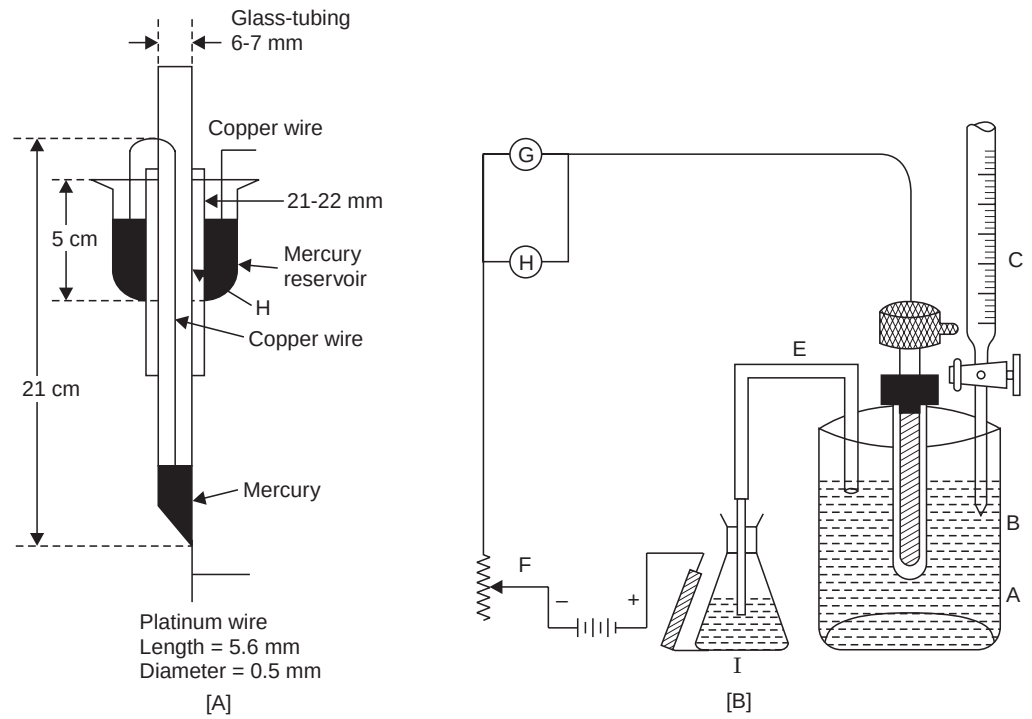
1. A known volume of the solution under investigation is introduced in the titration cell,
2. The apparatus is assembled and electrical connections are duly completed with dropping mercury electrode (C) as cathode and saturated calomel half-cell as anode,
3. A slow stream of pure analytical grade N_2 – gas is bubbled through the solution for 15 minutes to get rid of dissolved O_2 completely,
4. Applied voltage is adjusted to the desired value, and the initial diffusion current (Id) is noted carefully,
5. A known volume of the reagent is introduced from the semimicro burette (B), while N_2 is again bubbled through the solution for about 2 minutes to ensure thorough mixing as well as complete elimination of traces of O_2 from the added liquid,
6. The flow of N_2 gas through the solution is stopped, but is continued to be passed over the surface of the solution gently so as to maintain an O_2 free inert atmosphere in the reaction vessel,
7. The current (μA) and microburette readings are recorded simultaneously, and
8. Finally, the said procedure is repeated until sufficient readings have been obtained to allow the equivalence point to be determined as the intersection of the two linear portions of the graph thus achieved.

17.3.2. AMPEROMETRIC TITRATIONS WITH A ROTATING PLATINUM MICROELECTRODE

The rotating platinum microelectrode was first introduced by Laitinen and Kolthoff in 1941. Figure 17.3 (*a*) depicts a simple rotating platinum microelectrode which is made out from an usual standard 'mercury seal'. A platinum wire (length : 5.0 mm ; diameter : 0.5 mm) protrudes from the lower end wall of a 21

cm long 6 mm glass tubing, which is bent at an angle of 90°. There are holes (H) in the stem of the mercury reservoir for making electrical contact with it. The mercury reservoir is provided with a flange fitted inward to prevent Hg from being thrown out.

Figure 17.3 (*b*) illustrates the electric circuit. The electrical connection is duly done to the electrode by means of a strong amalgamated Cu-wire passing through the glass tubing to the lower end of the Hg covering the sealed-in platinum wire ; the upper end of which passes through a small hole made in the stem of the stirrer and dips well into the Hg present in the Hg seal. Subsequently, a wire from the Hg seal is connected to the source of applied voltage. The glass tubing serves as the stem of the electrode that is rotated at a constant speed of 600 rpm.



(*a*) Rotating Platinum Microelectrode (*b*) Electrical-Circuit

Figure 17.3 : A Schematic Diagram of Amperometric Titrations with The Rotating Platinum Microelectrode.

For Figure 17.3 (*a*)

H = Hole in the stem of glass-tubing for making electrical contact with the Mercury Reservoir ;

F = Flange fitted inward of the Hg-Reservoir to prevent Hg from being thrown out.

For Figure 17.3 (*b*)

A = Platinum wire,

B = Hg filled glass tubing,

C = Semimicro burette (graduated in 0.01 ml),

D = Rotating platinum microelectrode,

E = Salt bridge,

F = Potential divider,

G = Galvanometer,

H = Sensitivity shunt, and

I = Calomel cell.

17.3.3. AMPEROMETRIC TITRATIONS WITH TWIN-POLARIZED MICROELECTRODES (BIAMPEROMETRIC TITRATIONS OR DEAD-STOP-END-POINT METHOD)

Dead-stop-end-point method was first introduced by Foulk and Bawden* in 1926. Evidently, this particular technique is a modification of the classical amperometric titration. This technique is specifically applicable to only such systems where the phenomenon of oxidation-reduction exists both before as well as after the equivalence point has been duly accomplished.

It essentially makes use of two identical, stationary microelectrodes immersed in a well stirred solution of the sample. A small potential ranging between these electrodes ; and the resulting current is measured subsequently as a function of the volume of reagent added. The end-point is distinctly characterized by a sudden current rise from zero or a decrease in the current to zero or a minimum at zero in a V-shaped curve.

Though this technique was first used in 1926, but it received its due recognition only around 1950**.

Figure 17.4 represents a simple diagram of an amperometric titration assembly with twin-polarized microelectrodes.

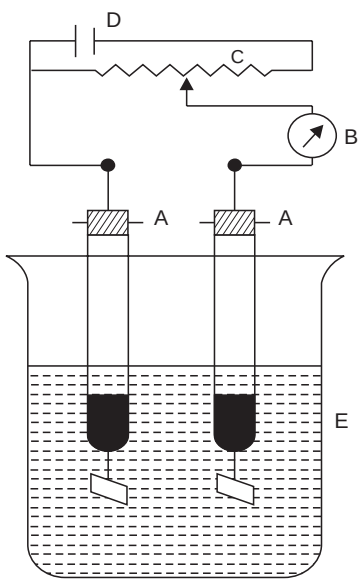


Figure 17.4 : Diagram of Amperometric Titrations With Twin-Polarized Microelectrodes.

The various components are as follows :

A, A = Twin-polarized Platinum microelectrodes,

B = Micro-ammeter (μA),

C = 500 Ω, 0.5 watt potentiometer,

D = 3-Volt dry torch cell or a 2-volt accumulator

E = Reaction vessel.

The potentiometer is adjusted in such a fashion that there is a distinct potential drop of about 80 to 100 millivolts between the two platinum electrodes.

* Foulk, C.W., and A.T. Bawden, **J. Amer. Chem. Soc., 48,** 2025, 1926

** Lingane, J.J. **Electroanalytical Chemistry,** 2nd. ed., New York, Interscience, 1958.

17.4. APPLICATIONS OF AMPEROMETRIC TITRATIONS IN PHARMACEUTICAL SUBSTANCES

Some pharmaceutical substances are assayed by amperometric titrations, namely : procainamide hydrochloride ;

17.4.1. PROCAINAMIDE HYDROCHLORIDE

Materials Required : Procainamide hydrochloride : 0.25 g ; 2M hydrochloric acid : 100 ml ; potassium bromide : 3 g ; 0.1 M sodium nitrite Vs (dissolve sodium nitrite in sufficient water to produce 1000 ml) ; Standardization of 0.1 M Sodium Nitrite Vs : Dissolve 0.3 g of sulphanillic acid in 50 ml of 2M hydrochloric acid, add 3 g of KBr, cool in ice and titrate with 0.1 M sodium nitrite Vs determining the end-point amperometrically. Each ml of 0.1 N sodium nitrite Vs is equivalent to 17.32 mg of $C_6H_7NO_3S$.

Procedure : Dissolve 0.25 g of procainamide hydrochloride in 50 ml of 2 M hydrochloric acid, add 3 g of potassium bromide, cool in ice and titrate slowly with 0.1 M sodium nitrite Vs, stirring constantly and determining the end-point amperometrically. Each ml of 0.1 M sodium nitrite Vs is equivalent of 27.18 mg of $C_{13}H_{21}N_3O$. HCl.

17.4.2. COGNATE ASSAY

Procaine hydrochloride can be assayed exactly in a similar manner by using 0.4 g of the substance. Each ml of 0.1 M sodium nitrite Vs is equivalent to 27.28 mg of $C_{13}H_{20}N_2O_2$, HCl.

17.4.3. ASSAY OF NICKEL WITH DIMETHYLGLYOXIME

Materials Required : 0.001 M Nickel solution ; supporting electrolyte [a mixture of NH_4OH (1.0 M) and NH_4Cl (0.2 M)] ; gelatin solution (0.2%) : 2 ml ;

Procedure : The following steps may be followed in a sequential manner :

1. Weigh accurately a sample of Ni-salt to yield a 0.001 M Ni-solution. To 25 ml of this solution placed in a titration cell add an equal volume (25.0 ml) of a supporting electrolyte and 2 ml of gelatin solution,
2. The solution must be deoxygenated. Set the applied e.m.f. to 01.85 V Vs SCE (standard-calomel electrode),
3. The diffusion current is measured, and
4. Finally, titrate with dimethylglyoxime solution (0.02 M) using the standard general method and obtain a V-shaped graph.

Each ml of dimethylglyoxime solution is equivalent to 0.5869 mg of Nickel.

17.4.4. ASSAY OF LEAD WITH POTASSIUM DICHROMATE SOLUTION

Materials Required :

(*i*) **Buffered supporting electrolyte :** Dissolve 10 g of KNO_3 and 8.2 g of sodium acetate in 500 ml of DW. Add glacial acetic acid carefully until a pH of 4.2 is achieved (pH Meter) (approximately 10 ml of the acid will be required),

(*ii*) **Standard 0.01 M $K_2Cr_2O_7$ Solution :** Weigh accurately 'ANALAR'-grade 1.47 g $K_2Cr_2O_7$ into a 500-ml volumetric flask. Dissolve in DW and make up the volume upto the mark, and

(*iii*) **0.1% w/v Gelatin Solution :** Dissolve 0.1 g gelatin in 100 ml of boiling DW.

Procedure : The amperometric titration may be carried out in a 100 ml beaker. A saturated KNO_3 salt bridge is employed to provide contact between the saturated calomel electrode and the analyte solution. The various steps involved are as follows :

1. Weigh accurately a sample of Pb-salt to give a 0.01 to 0.02 M lead solution,

2. Transfer 10.0 ml aliquot to the titration vessel,
3. Add to it 25 ml of the buffered supporting electrolyte, and 5 ml of the gelatin solution,
4. Determine the current at zero applied potential,
5. Add $K_2Cr_2O_7$ (0.01 M) solution, in 1 ml increments, and measuring the resulting current after each addition,
6. Continue the addition to at least 5.0 ml beyond the equivalence point,
7. Correct the currents for the volume change, and plot the graph. Determine the end-point and calculate the number of milligrams of Pb in the given sample, and
8. Repeat the titrations at – 1.0 V. It is essential to bubble N_2 through the solution for 10–15 minutes before the titration and while addition of reagents are made. However, the flow of N_2 must be stopped at the time of measuring the current. Again, correct the currents for dilution, plot the graph, determine the end-point, and report the number of milligrams of Pb present in the given sample.

THEORETICAL AND PRACTICAL EXERCISES

1. (*a*) Give a plausible explanation of the theoretical aspects of **'amperometric method'** of analysis with specific reference to both Fick's Law and Nernst Equation.

(*b*) Give a brief account of the various salient features of **'amperometry'.**

2. Discuss the four typical amperometric titration curves obtained in amperometric method of analysis and examine them critically with appropriate examples.

3. Attempt the following with regard to **'amperometry'** :

(*a*) Corrections for the volume change, and

(*b*) Advantages.

4. What are the **three** methods to accomplish amperometric titrations effectively. Discribe any ONE method exhaustively with its diagram, components and working. Enumerate briefly the advantages of one such method over the other.

5. How would you assay the following medicinal compounds amperometrically :

(*i*) Procaine hydrochloride, (*ii*) Procainamide hydrochloride,

(*iii*) Presence of Ni with dimethylglyoxime, and (*iv*) Presence of Pb with $K_2Cr_2O_7$ solution.

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PART IV

OPTICAL METHODS

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18 REFRACTOMETRY

CONTAINS :

18.1. INTRODUCTION

Light passes more rapidly through a vacuum than through a substance (medium). It has been observed that when a ray of light happens to pass from one medium (*a*) into another medium (*b*) it is subjected to refraction (Figure 18.1). In other words, the ray travels at a lower velocity in the relatively more optically dense medium (*b*) than in medium (*a*) which is less optically dense. It is a common practice to compare the refractive indices of liquids to that of air.

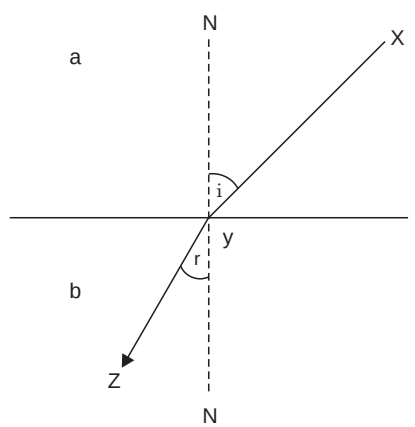


Figure 18.1 : The Path of Light between Two Media 'a' and 'b'.

According to Snell's Law we have :

$$a^n b = \frac{\sin i}{\sin r} \qquad ...(1)$$

where, i = Angle of incidence,

r = Angle of refraction, and

n = Refractive index of medium (*b*) relative to medium (*a*)

Critical Angle *vis-a-vis* Refractive Index

Figure 18.2, represents the critical angle which is used invariably in refractometry. Considering a narrow band of rays, *x-y*, held near to the boundary between the two media '*a*' and '*b*' (Figure 18.2), and

viewed at Z, one may observe a band of light. This particular band has a sharp edge at *y*, where the actual ray (*y*-*y*) may be seen. However, no rays are to be seen in the *y*-*y*′ region. Therefore, we have :

$$a^n b = \frac{\sin i}{\sin r} = \frac{\sin 90°}{\sin \theta} = \frac{1}{\sin \theta} \qquad ...(2)$$

Thus, a measurement of the critical angle θ may ultimately offer the exact refractive index of medium (*b*).

It is pertinent to mention here that the refractive index of a substance is not a static (constant) property of the substance but it alters with (*a*) wavelength and (*b*) temperature.

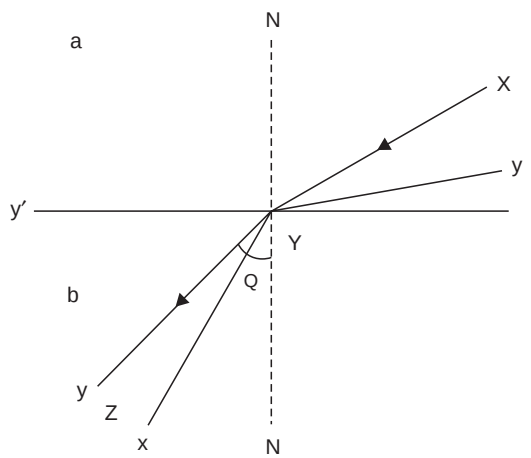


Figure 18.2 : Critical Angle.

Therefore, conventionally the temperature at which the refractive index is measured is usually designated as a superscript numerical on *n* ; whereas the wave-length of light employed as a subscript capital. Thus, we have : n_D^{20}, where 20 specifies the temperature expressed in (°C) at which RI has been measured and D represents the sodium D-light (λ = 589.3 nm).

18.2. THEORY

Lorentz and Lorentz in 1880, introduced the terminology **specific refraction** or **refractivity** which may be expressed as :

$$[n] = \frac{n^2 - 1}{n^2 + 2} \cdot \frac{1}{p} \qquad ...(3)$$

where, n = Refractive index,

p = Density of the substance*

Hence, the specific refraction (Eq. 3) is considered to be a more useful property and is characteristic of the substance, being absolutely independent of temperature.

Molar Refractivity : Later on, a still more useful property termed as the molar refraction (or refractivity) was introduced which could be expressed as follows :

$$R = \left(\frac{n^2 - 1}{n^2 + 2} \right) \frac{M}{p} \qquad ...(4)$$

*Density measured at the same temperature as the refractive index RI

where, R = Molar refraction,

P = Density of the substance, and

M = Molecular weight.

Interestingly, both specific refraction [*n*] and molar refraction (R), being temperature independent, should have the same values for a given substance either in the solid, liquid or gaseous state, provided the molecular structure is unchanged.

Unit of Molar Refraction : As the refractive index is a dimensionless quantity, the units of molar refraction are simply those of molar volume, M/*p i.e.*, $cm^3 . mol^{-1}$.

The molar refractivity is more or less an additive property.

Atomic Refractivities : Atomic refractivities may be attributed by virtue of :

(*a*) Structural features *e.g.*, double bond, triple bond or nature of ring structure (3-member/4-member rings), and

(*b*) Individual atoms, *e.g.*, H, C, Cl, Br, I and O. However, 'O' contributes different values for different groups, for instance : hydroxyl (—OH), carbonyl (—CO—) and ethereal (—O—) moieties.

A few representative atomic refractivities and bond contributions are given in Table 18.1 below :

Table 18.1 : Atomic Refractivities for Na D-Light (λ = 589.3 nm)

Atom	R ($cm^3\ mol^{-1}$)
H	1.100
C	2.418
C (C = C)	1.733
C (C ≡ C)	2.398
O (—OH)	1.525
O (—CO—)	2.11
O (—O—)	1.643
Cl	5.967
Br	8.748
I	13.900
3-Member Ring	0.71
4-Member Ring	0.48

Based on the atomic refractivities given in Table 18.1, it may be possible to calculate the molar refractivities of various pharmaceutical substances theoretically and compare the same with values found experimentally. A few typical examples are cited below :

(*a*) **Acetone, CH_3COCH_3 [or C_3H_6O] :**

$$R_{C_3H_6O} = 3\ R_C + 6R_H + R_O$$

$$= 3 \times 2.418 + 6 \times 1.100 + 2.211$$

$$= \mathbf{7.254 + 6.600 + 2.11 = 15.964}$$

Calculated : $R_{C_3H_6O} = 15.964\ cm^3\ mol^{-1}$

Experimental : $R_{C_3H_6O} = 15.985\ cm^3\ mol^{-1}$

(*b*) **Methyl Alcohol, CH_3OH [or CH_4O] :**

$$R_{CH_3OH} = R_C + 4R_H + R_O$$
$$= 2.418 + 4 \times 1.100 + 1.525$$
$$= 2.418 + 4.400 + 1.525$$
$$= \mathbf{8.343}$$

Calculated : $R_{CH_4O} = 8.343\ cm^3\ mol^{-1}$

Experimental : $R_{CH_4O} = 8.296\ cm^3\ mol^{-1}$

(*c*) **Chloroform $CHCl_3$:**

$$R_{CHCl_3} = R_C + R_H + 3R_{Cl}$$
$$= 2.418 + 1.100 + 3 \times 5.967$$
$$= 2.418 + 1.100 + 17.901$$
$$= \mathbf{21.419}$$

Calculated : $R_{CHCl_3} = 21.419\ cm^3\ mol^{-1}$

Experimental : $R_{CHCl_3} = 21.393\ cm^3\ mol^{-1}$.

18.3. INSTRUMENTATION

In Figure 18.3, the optical system of **Abbe' Refractometer** has been shown based on the critical angle principle.

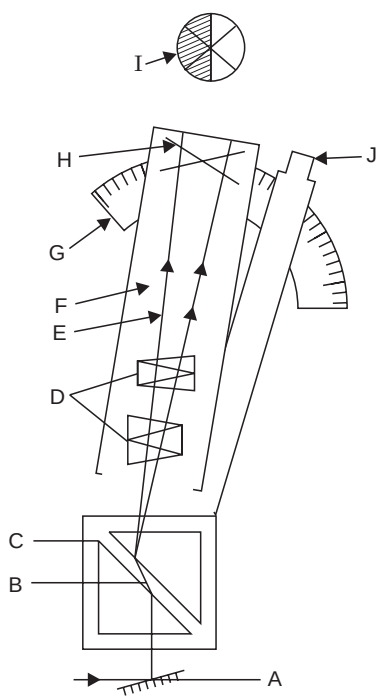


Figure 18.3. Abbe' Refractometer : Optical System.

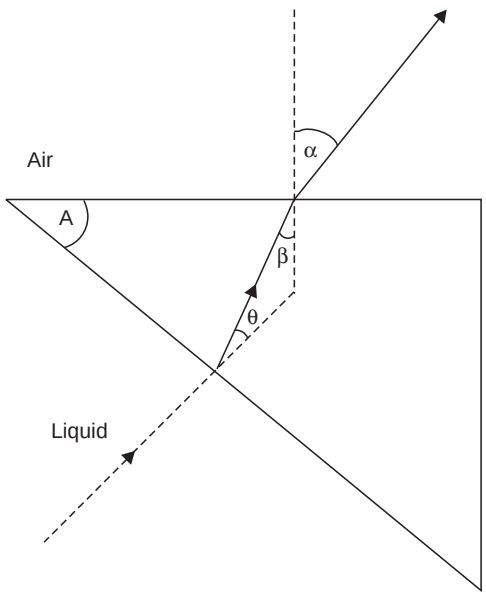


Figure 18.4. Abbe' Refractometer : Optical Path for Upper Prism.

The various parts in Figure 18.3, are stated as below :

A = Mirror ; F = Telescope ;

B = Liquid (Sample) ; G = Scale ;

C = Prism box ; H = Cross hair ;

D = Amici Prisms ; I = Field of view ;

E = Critical ray ; J = Eye-piece for reading scale ;

Procedure : The liquid whose RI is to be determined is placed between the two prisms (B). The upper face of the lower prism has a ground surface so as to diffuse the light rays in every possible direction. The rays passing from the liquid to the upper prism undergoes refraction in the normal manner, thereby providing a bright field in the eye-piece. The critical ray is originated by virtue of the rays that strike the liquid glass interface at the grazing incidence. As an outcome of these combined effects the '*field of view*' is represented as a distinct dark and light area having a sharp dividing line.

Fig. 18.4, designates the optical path for the upper prism in Abbe' Refractometer. When a ray of light passes from the liquid medium and enters the upper prism, it gets refracted by an angle θ between the lower face of the prism and the normal, an angle β between the emerging refracted ray at the upper face and the normal, and finally an angle α between the reflected ray at the upper face and the normal. Thus, we have :

$$N = \frac{\sin \alpha}{\sin \beta} \qquad ...(a)$$

$$\sin \theta = \text{glass } {}^{n} \text{ liquid} = \frac{n}{N} \qquad ...(b)$$

where, N = Refractive index of the prism compared to air, and

n = Refractive index of the liquid (air to liquid)

and $A = \beta + \theta$

or $\theta = A - \beta \qquad ...(c)$

From Eq. (*b*) we have :

$$n = N \sin \theta \qquad ...(d)$$

Putting the value of θ from Eq. (*c*) in Eq. (*d*), we have :

$$n = N \sin (A - \beta)$$

or $$n = N \sin A \cos \beta - N \cos A \sin \beta \qquad ...(e)$$

As we know $\sin^2 \beta + \cos^2 \beta = 1 \qquad ...(f)$

From Eq. (*a*) we have :

$$N^2 = \frac{\sin^2 \alpha}{\sin^2 \beta}$$

or $$\sin^2 \beta = \frac{\sin^2 \alpha}{N^2} \qquad ...(g)$$

From Eqs. (*f*) and (*g*) we may have :

$$\frac{\sin^2 \alpha}{N^2} + \cos^2 \beta = 1$$

or $$\cos \beta = \sqrt{\left\{1 - \frac{\sin^2 \alpha}{N^2}\right\}} \qquad ...(h)$$

Substituting the value of cos β from Eq. (*h*) in Eq. (*e*) and also sin β = sin α/N from Eq. (*a*) we have :

$$n = N \sin A \sqrt{\left\{1 - \frac{\sin^2 \alpha}{N^2}\right\}} - \cos A \sin \alpha$$

or

$$n = \left\{\sin A \sqrt{(N^2 - \sin^2 \alpha)}\right\} - \sin \alpha \cos A \qquad ...(i)$$

Now, based on the two constants, *viz.*, A and N, for a specific prism and a measurable angle α it is convenient to determine the refractive index of the liquid *n* relative to air from Eq. (*i*). With the help of the Abbe' refractometer the angle α lying between the normal and the critical ray emerging from the upper surface of the prism may be measured. By the aid of the two constants A and N (for a particular prism) the angle α has been converted into the refractive index directly and the scale of the instrument has been duly calibrated and printed accordingly.

The telescope (F) of the Abbe' refractometer is fixed (Figure 18.3) and the prism box (C) is directly attached to the scale. When C is made to rotate gradually the critical ray (E) falls on the cross hair (H) of the telescope (F). At this juncture the value of the refractive index of the liquid (*n*) can be measured directly from the scale (G).

It is, however, important to mention here that the calibration of Abbe's refractometer may be checked periodically by making use of standard liquids whose refractive index are stated in the *European Pharmacopoea* (as Reference Liquids).

For instance

S.No.	Standard Liquid	Refractive Index*
1.	Carbon Tetrachloride	1.4603
2.	α-Methylnaphthylamine	1.6176
3.	Toluene	1.4969

18.4. DETERMINATION OF REFRACTIVE INDEX OF PHARMACEUTICAL SUBSTANCES

A large number of pharmaceutical substances such as volatile oils, namely : peppermint oil, lemon oil, aniseed oil have a definite range of refractive index. Based on this physical characteristic it is possible to ascertain the purity of this volatile oil precisely and accurately.

Materials Required : Abbe' refractometer, volatile oil, xylene, capillary tubes ;

Procedure : In order to obtain precise and accurate measurements the prism case of Abbe' refractometer is attached to a thermostat bath whose temperature is previously maintained at 25°C. Open the prism box gently and place a few drops of pure volatile oil on the lower prism with the help of a capillary tube and finally close the box. The mirrors are duly adjusted so as to obtain a bright illumination of the field of view. The knurled knob is turned gradually until the field of view displays a dark and light zone. In case, a coloured-fringe is observed between the two zones it becomes necessary to adjust the Amici prisms carefully to achieve a sharp and black boundary. It is important to adjust this on the cross hair and finally the reading of refractive index is noted. After use, the prism box is opened and cleaned thoroughly with a lens cleansing tissue moistened with xylene/acetone. Thus, the refractive index, n_D^{25} for certain volatile oils as per BP (1993) are as follows :

*European Pharmacopoea Reference Liquids

Peppermint oil	:	1.460—1.467
Lemon oil	:	1.474—1.476
Aniseed oil	:	1.553—1.560
Clove oil	:	1.528—1.537
Dill oil	:	1.481—1492
Eucalyptus oil	:	1.458—1.470

18.5. APPLICATIONS OF REFRACTIVITY

The various applications of refractivity are enumerated below :

(*a*) It is feasible to determine the molar refractivities of different substances experimentally and subsequently comparing their values with theoretical ones as discussed in section 18.2,

(*b*) Based on the fact that molar refractivity is an additive property, it may be utilized to determine the refractivities of homogeneous mixtures (as solutions).

Thus, the molar refraction of a solution having two components (*viz.*, the solute and the solvent) is given by the expression :

$$R_{1,2} = N_1R_1 + N_2R_2 \qquad ...(i)$$

where, N_1 = Mole fraction of the solute,

N_2 = Mole fraction of the solvent,

R_1 = Molar refractivity of the solute, and

R_2 = Molar refractivity of the solvent.

Evidently, from Eq. (*i*), it is quite possible to determine the molar refractivity of an unknown solute R_1 provided we know the mole fraction N_1 and N_2 and the refractivities of the solute R_2 and the homogeneous solution $R_{1,2}$.

Besides, the concentration of the solute in the solution may be determined by employing the following expression, provided the refractivities of the solute, the solvent and the solution are known :

$$R_{1,2} = \frac{(n^2 - 1)}{(n^2 + 2)}\left[\frac{N_1M_1 + N_2M_2}{p}\right] \qquad ...(ii)$$

(*c*) **Determination of Critical Micelle Concentration (CMC)**

In general, substances that form micelles in water offer two distinct regions in their molecules : first, the hydrophobic entity (caused due to the hydrocarbon chain), and secondly, the hydrophilic entity (caused due to the polar group). It has been observed that a number of monomers usually hold all the hydrocarbon chains together specifically in the centre of the micelle which are ultimately responsible for minimising the free energy of the system. Thus, the particular concentration at which the micelles are first observed is termed as the **critical micelle concentration (CMC)**. Interestingly, the physical characteristics of the substances forming micelles afford sharp changes at the CMC. Therefore, a plot of refractive index (RI) Vs concentration (g/L) must depict a visible change in slope at the CMC.

Example :

Determination of critical micelle concentration (CMC) of Butyric Acid by refractometry :

Butryic acid ($CH_3CH_2CH_2COOH$) is found to form micelles in an aqueous medium in a concentration range fairly suitable for measurements with the Abbe' refractometer.

Materials Required : Butyric acid solution (25%, w/v in DW) : 200 ml ; volumetric flasks (50 ml) : 6 ;

Procedure : Prepare precisely 2.5, 5.0, 7.5, 10.5, 15.0 and 20.0% solutions of butyric acid in water by measuring suitable volumes (from a stock solution of 25% w/v) with the help of a burette into six 50 ml volumetric flasks, and finally making up the volume with DW. Using Abbe' refractometer measure the refractive indices of all the above six solutions besides the stock solution (25%) at 25°C. Measure also the refractive index of DW.

Results : Plot a graph having the various concentrations of butyric acid along the abscissa and the refractive indices along the ordinate, whereby two straight lines are obtained intersecting at the CMC as shown in Figure 18.5 below :

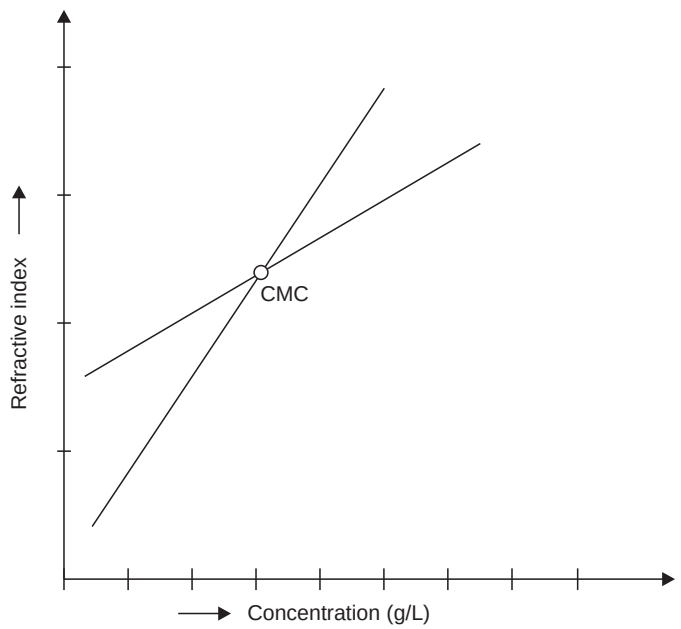


Figure 18.5 : Plot of RI Vs Conc. In Determining CMC of Butyric Acid-Water Mixture.

THEORETICAL AND PRACTICAL EXERCISES

1. Explain the following with reference to '**Refractometry**' :

(*a*) Snell's Law,

(*b*) Critical angle *vis-a-vis* Refractive index,

(*c*) Molar refractivity, and

(*d*) Atomic refractivities.

2. Describe the optical system of Abbe's Refractometer, its optical path for upper prism and its operational procedure.

3. How would you derive the mathematical expression '*n*' *i.e.*, the refractive index ?

4. How would you determine the 'refractive index' of pharmaceutical substances ? Give suitable examples.

5. Discuss the applications of refractivity with special reference to Critical Micelle Concentration (CMC).

RECOMMENDED READINGS

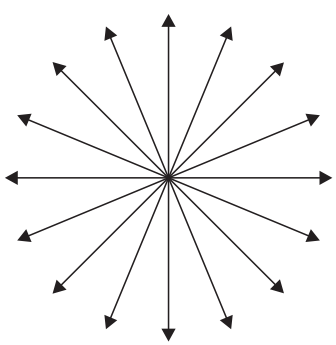
1. Glasstone, S., **'Textbook of Physical Chemistry',** New York, D. Van Nostrand, 1960.
2. Barrow, G.M., **'Physical Chemistry',** New York, McGraw Hill, 1961.
3. Nachod, F.C. and J.J. Zuckerman, Eds., **'Determination of Organic Structures by Physical Methods',** New York, Academic Press, 1971.
4. Weissberger, A and B.W. Rossiter, **'Techniques of Chemistry', Vol. I : Physical Methods of Chemistry,** New York, John Wiley & Sons Inc., 1972.

19 POLARIMETRY

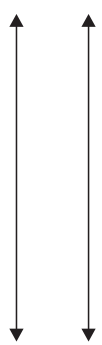
19.1. INTRODUCTION

The classical electromagnetic theory of light put forward by Maxwell advocates that the electric and magnetic fields associated with a beam of monochromatic light vibrate in all directions perpendicular to the direction of propagation of light. In fact, there exists an indefinite number of planes that pass through the line of propagation, and an ordinary light usually vibrates in all the planes. This is also referred to as **unpolarized light**. Under certain specific circumstances, the vibrations may all be restricted to one direction only, in the perpendicular plane and this is termed as **plane-polarized light**.

Figures 19.1 (*a*) and (*b*) depict the ordinary or unpolarized light and plane-polarized light respectively.



(a) Ordinary Light



(b) Plane-polarized Light

Figure 19.1

A few crystalline substances, for instance : Iceland spar, Calcite (a form of $CaCO_3$) or Polaroid, possess different refractive indices for light whose field oscillates either perpendicular or parallel to the principal plane of the crystal. Thus, an ordinary light (unpolarized light) gets converted into a plane-polarized light by simply passing it through a lens made of the above cited materials and traditionally called a **Nicol prism** (after **William Nicol-the inventor**).

Therefore, an optically active substance is one that rotates the plane of polarized light. In other words, certain specific substances by virtue of their internal structure may be able to transmit only such vibrations that are oriented along certain directions and entirely block vibrations in other directions.

Figure 19.2 evidently shows the electric field of a plane-polarized light which consists of two components of fixed magnitude rotating in opposite directions to one another ; the right circularly polarized light ; and the

left circularly polarized light. However, it is worth mentioning that the plane-polarized beam is the vector-sum of these two components.

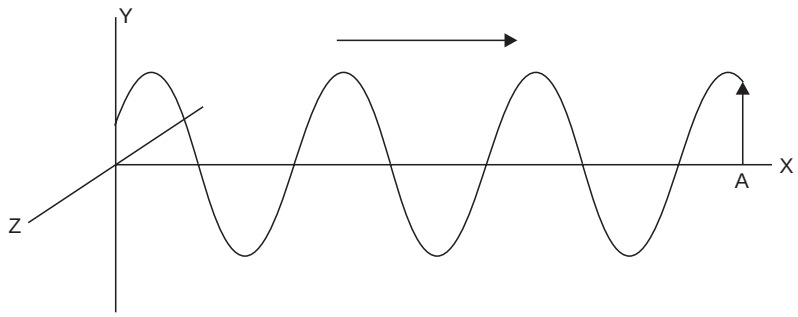


Fig. 2 (a)

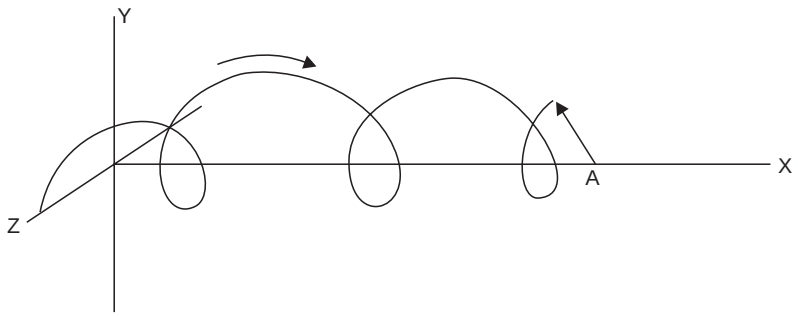


Fig. 2 (b)

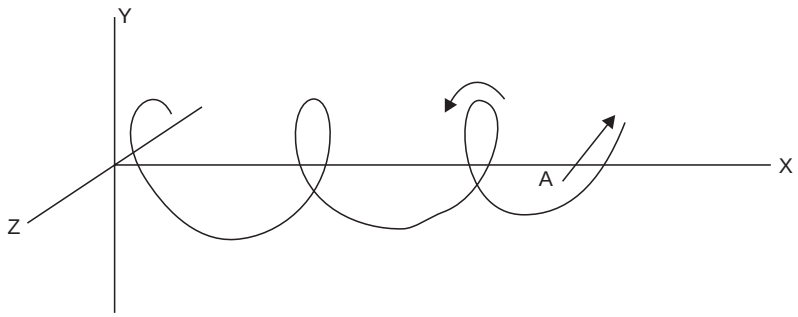


Fig. 2 (c)

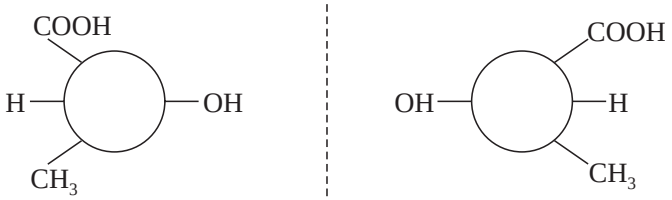
Figure 19.2 : (a) Plane polarized ; (b) Right circularly polarized ; (c) Left circularly polarized.

19.2. THEORY

An **optically active substance is one that rotates the plane of polarized light**. In other words, when a polarized light, oscillating in a specific plane, is made to pass through an optically active substance, it happens to emerge oscillating in an altogether different plane.

In general, organic molecules having a central carbon atom to which are attached four altogether different moieties, as C (WXYZ) thereby rendering the molecule asymmetric, are all optically active. Such types of molecules usually exist in two stereoisomeric forms as mirror images of each other. For example :

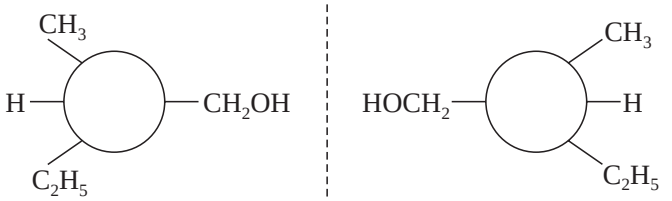
(*i*) Lactic Acid



d-Lactic acid
i(*a*)

l-Lactic acid
i(*b*)

(*ii*) 2-Methyl-1-butanol



(d) 2-Methyl-1-butanol
ii(*a*)

(I) 2-Methyl-1-butanol
ii(*b*)

In the above cited example [*i* (*a*)] the rotation of the plane of polarization is to the right (clockwise), the lactic acid is dextrorotatory (Latin : *dexter* = right) designated by '*d*' ; if the rotation is to the left (counterclockwise), the lactic acid [*i* (*b*)] is levorotatory (Latin : *laevus* = left) designated by '*1*'. In the same vein, the example [*ii* (*b*)] represents 1-2 methy1-1-butanol ; a product derived from fusel oil.

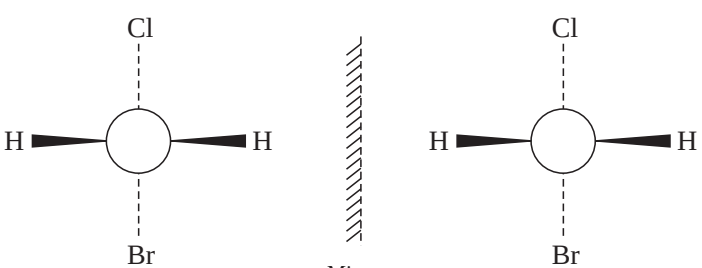
Non-superimposability and Optical Activity : Interestingly, in these two specific examples of lactic acid (*d*- ; and *1*-isomers) and 2-methy-1-butanol (*d*- ; and *1*-isomers) one criterion is common *i.e.,* the two mirror images are not superimposable. In other words, such compounds whose mirror images display non-superimposability exhibit optical activity. Furthermore, in the particular instance of C (WXYZ) it may be observed that the molecule whose mirror-image is not just another identical molecule but gives rise to a molecule of an altogether different isomeric compound. Thus, a pure sample of a single enantiomer must fulfil the following *three* important characteristic features, namely :

(*a*) No molecule can serve as the mirror image of another molecule,

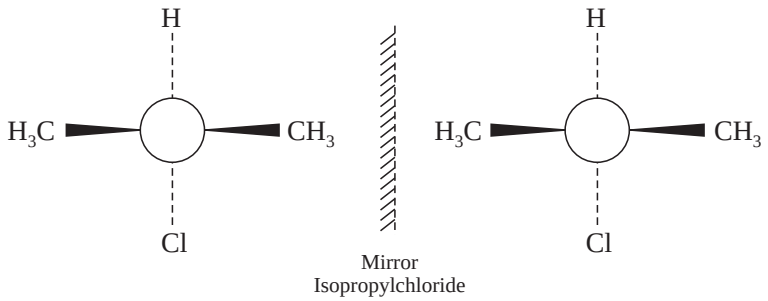
(*b*) Exact cancelling out of rotations (of plane of polarized light) do not occur, and

(*d*) Net result is offered in terms of the '*optical activity*'.

Superimposability and Loss of Optical Activity : In a situation where molecules exist as C (W_2XY), that is when two of the four groups become identical, as may be observed in bromochloromethane and isopropylchloride as shown below :



Mirror
Bromochloromethane



Isopropylchloride

It may be observed clearly that the two mirror images are superimposable and hence they do not exhibit any optical activity.

19.3. INSTRUMENTATION

The rotation of the plane of polarized light and hence the optical activity may be detected and measured accurately by an instrument known as the polarimeter.

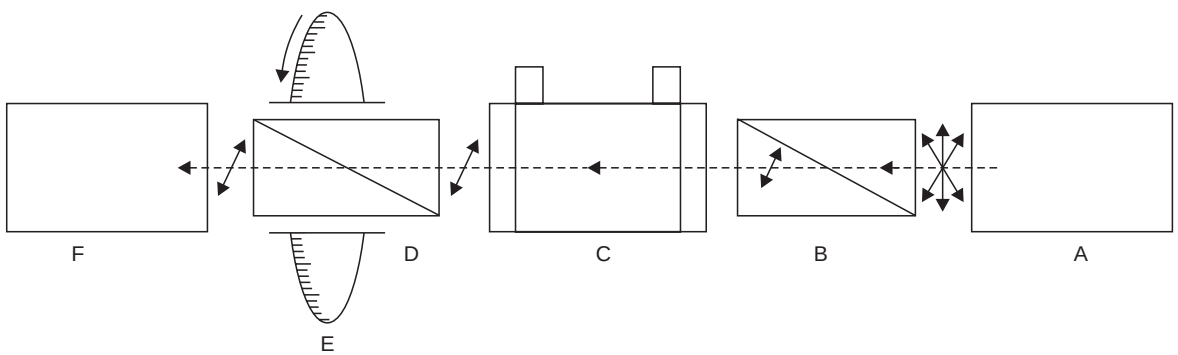


Figure 19.3 : Optical System of A Polarimeter.

Figure 19.3, represents the optical system of a polarimeter,

A = Collimated monochromatic light source,

B = Polarizing prism (Nicol),

C = Polarimeter glass tube (20 cm) with glass windows,

D = Analyzing rotator prism (Nicol),

E = Circular scale with vernier,

F = Null detector (Eye or Photoelectric Cell).

Principle : The underlying principle of a polarimeter is that light from the source, usually a sodium vapour lamp, first gets collimated at A, and subsequently falls upon polarizer B (a **calcite prism**). The polarizer permits only the light polarized in a particular direction to pass it. The emergent polarized ray now passes through the sample under investigation, kept in the polarimeter glass tube C to the analyzer D, which happens to be another polarizing prism. The analyzing rotator prism D (**Nicol**) is fixed in such a manner that it can be rotated easily about the axis of the incident light ray. Two situations arise when the analyzing rotator prism (D) is put into action, *firstly*, the prism being parallel to the plane of polarization of the incident light—the net result is that the intensity of light reaching the Null detector F is maximum ; and *secondly*, the prism being perpendicular to the plane of the polarized light—the net result is observed by the intensity of light reaching the detector as minimum. Hence, the overall difference in the position of the analyzer, as noted from the circular

scale E, that provides minimum light intensity with and without the sample in the cell is the observed 'rotation' of the sample in question.

Specific Rotation : A polarized light when passed through an optically active substance, each molecule of it encountered by the light beam rotates the plane of polarization by a constant amount characteristic of the substance. Consequently, a measure of the rotary power of the individual molecule, irrespective of the two parameters, namely : the path length and the concentration, is achieved by converting the measured rotation into a specific rotation by the help of the following expressions :

$$[\alpha]_{\lambda}^{T} = \frac{100\,\alpha_{\lambda}^{T}}{LC} \qquad ...(a)$$

or

$$= \frac{\alpha_{\lambda}^{T}}{L\rho} \qquad ...(b)$$

where, T = Temperature (°C),

λ = Wavelength (= D is used to denote the sodium D line, which is a doublet at 5890° A),

$[\alpha]_{\lambda}^{T}$ = Specific rotation at temperature T and wavelength λ,

L = Length of the path of the light through the sample in decimeters,

C = Concentration of the optically active substance (in grams per 100 ml of solution),

α_{λ}^{T} = Observed angle of rotation, and

ρ = Density of the substance.

19.4. DETERMINATION OF OPTICAL ACTIVITY OF PHARMACEUTICAL SUBSTANCES

The two characteristic parameters related to optical activity of the pharmaceutical substances, namely : (*a*) optical rotation, and (*b*) specific optical rotation, can be measured satisfactorily by the help of a Polarimeter as stated below :

19.4.1. DETERMINATION OF OPTICAL ROTATION OF PHARMACEUTICAL SUBSTANCES

The optical rotation of a number of pure pharmaceutical substances may be measured accurately by noting the angle through which the plane of polarization is rotated when polarized light passes through the substance, if liquid or through a solution of the substance, if solid.

A few typical examples of ibuprofen and levodopa are discussed below :

19.4.1.1. Ibuprofen

Materials Required : 2.5% (w/v) solution of ibuprofen and a polarimeter ;

Procedure : First and foremost it is absolutely necessary to check the linearity of the scale of a polarimeter either using **certified quartz plates** or using **known solution of sucrose**.

The sample tube of the polarimeter is rinsed with the drug solution (2.5% w/v) and filled up with the same solution. The end glass-windows are closed properly. The angle of rotation of ibuprofen is now measured at 19.5° to 20.5°, using the D-line of polarized sodium light. Take at least five measurements and determine the mean value.

19.4.1.2. Levodopa

Theory : It has been observed that the specific rotation of levodopa in the visible region is rather on the lower side *i.e.*, ($[\alpha]_{D}^{20}$ = – 12° in 1 M hydrochloric acid). Therefore, it is necessary to enhance the optical

rotation to a reasonable extent by some suitable means. It is, however, achieved by the formation of a complex with hexamine.

Materials Required : Dried levodopa : 0.2 *g* ; hexamine : 5.0 *g* ; hydrochloric acid (1 M) : 50 ml (dissolve 85 ml of HCl in 1 L of DW) ;

Procedure : Dissolve a quantity equivalent of 0.2 *g* of the dried substance and 5 *g* of hexamine in 10 ml of 1 M hydrochloric acid, add sufficient 1 M HCl to produce 25 ml and allow to stand for 3 hours, protected from light. The optical rotation is measured by a previously calibrated polarimeter.

Optical Rotation : – 1.27° to – 1.34°

19.4.1.3 Cognate Assays

The optical rotation of a number of substances official in the pharmacopoeia may be determined conveniently as stated in Table 19.1.

Table 19.1 : Optical Rotation of Some Official* Compounds

S.No.	Substance	Conc. Used (% w/v)	Length of Tube (dm)	Optical Rotation	Remarks
1.	Atropine Methobromide	10	2	– 0.25° to + 0.05°	—
2.	Atropine Methonitrate	10	2	-do-	—
3.	Atropine Sulphate	10	2	– 0.50° to + 0.05°	—
4.	Racemic Camphor	10 (in 96 %) EtOH)	—	– 0.15° to + 0.15°	—
5.	Dihydrotachysterol	—	—	+ 70° to + 80°	—
6.	Lemon Oil	—	—	+ 57° to + 70°	—
7.	Dementholised	—	—	– 22° to – 29°	Brazilian Oil,
	Mint Oil			– 17° to – 24°	Chinese Oil
8.	Orange Oil	—	—	+ 94° to + 99°	—
9.	Spearmint Oil	—	—	– 45° to – 60°	—

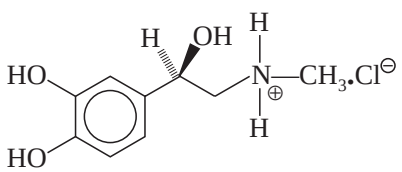
19.4.2 DETERMINATION OF SPECIFIC OPTICAL ROTATION OF PHARMACEUTICAL SUBSTANCES

The **specific optical rotation** of a solid substance is usually determined by measuring the angle of rotation at the wavelength of the sodium D-line at a temperature of 20°C, and calculating the result with reference to a layer 1 dm thick of a solution containing 1 *g* of the substance per ml. It is pertinent to mention here that the specific optical rotation of a solid is always expressed to a given solvent and concentration.

Example : Adrenaline ;

19.4.2.1. Adrenaline

Theory : As pure adrenaline is sparingly soluble in distilled water, therefore, its solution is made in 1 M hydrochloric acid whereby the N-atom gets protonated and results into the formation of a quaternary ammonium compounds as shown in under :



*British Pharmacopoeia, 1993.

Materials Required : 4% *w/v* solution of adrenaline in 1 M HCl (dissolve 85.0 ml of HCl in 1 L of DW) ;

Procedure : Determine the angle of rotation of the freshly prepared 4% w/v solution of adrenaline in 1 M hydrochloric acid with the help of a previously checked polarimeter. The mean value of at least five similar determinations is employed in the calculation of the specific optical rotation.

Calculations : Calculate the specific optical rotation using the following expression, namely :

For Liquids, $[\alpha]_D^{20} = \frac{\alpha}{1d}$

For solids, $[\alpha]_D^{20} = \frac{100\alpha}{1c}$

where, l = the length in dm of the polarimeter tube,

d = the relative density of the substance, and

c = the concentration of the substance expressed as a percentage w/v.

19.4.2.2. Cognate Assays

The specific optical rotation of a large number of potent pharmaceutical substances may be determined by the above mentioned procedure but specific concentrations and method of preparation of solutions is according to the *official compendium* as stated in Table 19.2 below :

Table 19.2 : Specific Optical Rotation of Some Important Official *Compounds

S.No.	Substance	Concentration/Preparation of Solution	Specific Optical Rotation $[\alpha]_D^{20}$
1.	Acetylcysteine	Dissolve 1.25 g in a mixture of 1 ml of a 0.1% w/v soln. of disodium edetate, 7.5 ml of 1 M NaOH and sufficient mixed phosphate buffer (pH 7.0) to produce 25 ml.	+ 21° to + 27°
2.	Alanine	Dissolve 2.5 g in sufficient 7 M HCl to produce 25 ml.	+ 13.5° to + 15.5°
3.	Amoxycillin Sodium	A 0.25% w/v soln. in a 0.4 w/v soln. of potassium hydrogen phthalate	+ 240° to + 290°
4.	Ampicillin	0.25 w/v solution.	+280° to + 305°
5.	Ampicillin Sodium	0.25% soln. in a 0.4% soln. of potassium hydrogen phthalate.	+258° to + 287°
6.	Apomorphine Hydrochloride	1% w/v soln. in 0.02 M HCl.	– 48° to – 52°
7.	Ascorbic Acid	10% w/v solution.	+ 20.5° to + 21.5°
8.	Beclomethasone Dipropionate	1% w/v solution in 1, 4-dioxan	+ 88° to + 94°
9.	Benethamine Penicillin	1% w/v in chloroform	+ 120° to + 125°
10.	Benzylpenicillin Potassium	2% w/v in CO_2-free water	+ 270° to + 300°
11.	Betamethasone	0.5% w/v in 1, 4-dioxan	+ 114° to + 122°
12.	Calcium Pantothenate	5.0% w/v in CO_2 free water	+ 25.5° to + 27.5°
13.	Carbidopa	Dissolve 0.25 g in sufficient $AlCl_3$ soln. (BP, 1993) to produce 25 ml with the aid of ultra sound.	– 22.5° to – 26.5°

*British Pharmacopoeia, 1993.

14.	Cephalexin	0.5% w/v in phthalate buffer (pH 4.4)	+ 149° to + 158°
15.	Chloramphenicol	6% w/v in absolute ethanol	+ 18.5° to + 20.5°
16.	Cindamycin Hydrochloride	4% w/v solution	+ 135° to + 150°
17.	Cocaine	Dissolve 0.6 g in 2.5 ml of 1 M HCl and add sufficient DW to produce 25 ml	– 79° to – 81°
18.	Colchicine	50 mg in ethanol (96%) to produce 10 ml	– 235° to – 250°
19.	Cytarabine	0.25 g in DW to produce 25 ml	+ 154° to + 160°
20.	Deslanoside	2% w/v soln. in anhydrous pyridine	+ 6.5° to + 8.5°
21.	Dexamethasone	1% w/v soln. in 1, 4-dioxan	+ 75° to + 80°
22.	Dicloxacillin Sodium	1% w/v soln.	+ 128° to + 143°
23.	Digitoxin	2.5% w/v soln. in chloroform	+ 16.0° to + 18.5°
24.	Emetine Hydrochloride	1.25 g of the dried substance in sufficient DW to produce 25 ml	+ 16° to + 19°
25.	Ephedrine	Dissolve 2.25 g in 15 ml of 2 M HCl and dilute to 50 ml with DW	– 41° to – 43°
26.	Ergocalciferol	Dissolve 0.2 g rapidly without heating, in aldehyde-free ethanol (96%) to produce 25 ml	+ 103° to + 107°
27.	Erythromycin	2% w/v soln. in absolute ethanol	– 71° to – 78°
28.	Ethinyloestradiol	5% w/v in pyridine	– 27° to – 30°
29.	Framycetin Sulphate	10% w/v soln.	+ 52.5° to + 55.5°
30.	Gentamycin Sulphate	10% w/v soln.	+ 107° to + 121°
31.	Griseofulvin	1% w/v soln. in dimethylformamide	+ 354° to + 464°
32.	Hydrocortisone Acetate	1% w/v soln. in 1, 4-dioxan	+ 158° to + 167°
33.	Hyoscyamine Sulphate	5% w/v soln.	– 24° to – 29°
34.	Naproxen	2% w/v soln. in chloroform	+ 63° to + 68°
35.	Sucrose	20% w/v soln.	+ 66.8°
36.	Testosterone	1% w/v soln. in absolute ethanol	+ 83° to + 90°

THEORETICAL AND PRACTICAL EXERCISES

1. What is the fundamental theory of 'polarimetry' ? How would you depict the plane polarized light, right circularly polarized light and left circularly polarized light diagramatically ?

2. (*a*) Describe the optical system of a polarimeter with labelled diagram.

(*b*) Explain the following explicitely :

(*i*) Non-superimposability and optical activity,

(*ii*) Superimposability and loss of optical activity, and

(*iii*) Specific optical rotation.

3. How would you determine the optical rotation of the following pharmaceutical substances ?

(*i*) Ibuprofen, (*ii*) Levodopa,

(*iii*) Atropine sulphate, and (*iv*) Spearmint oil.

4. How would you carry out the determination of specific optical rotation of the following official compounds ?

(*i*) Adrenaline, (*ii*) Betamethasone,

(*iii*) Chloramphenicol, (*iv*) Dicloxacillin sodium,

(*v*) Ergocalciferol, and (*vi*) Griseofulvin.

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20 NEPHELOMETRY AND TURBIDIMETRY

CONTAINS :

20.1. INTRODUCTION

When light is passed through moderately stable suspensions, a portion of the incident radiant energy is dissipated by virtue of the absorption, refraction, and reflection, whereas the remaining portion gets transmitted. It is quite evident that the optical characteristics of each suspension shall alter according to the concentration of the dispersed phase. In fact, the measurement of the intensity of the transmitted light through such suspensions *vis-a-vis* the concentration of the dispersed phase serves as the basis of *turbidimetric analysis*.

In another situation when the aforesaid suspension is viewed at 90° (*i.e.*, right angles) to the direction of the incident light (Figure 20.1) the system appears opalescent on account of the reflection of light from the particle of the suspension. This scattering of light is termed as the **Tyndall effect.** The observed opalescence or cloudiness is the net result caused by irregularly and diffusely reflected light from the suspension. Consequently, the ultimate measurement of the intensity of the scattered light as a true representation of the actual concentration of the dispersed phase forms the basis of nephelometric analysis (derived from Greek : **nephele**-means cloud). It is found to be most sensitive and effective specially in the case of very dilute suspensions having a concentration not greater than 100 mg L^{-1}. However, it is interesting to observe that the technique of turbidimetric analysis resembles that of flame photometry ; and nephelometric analysis to that of fluorimetry.

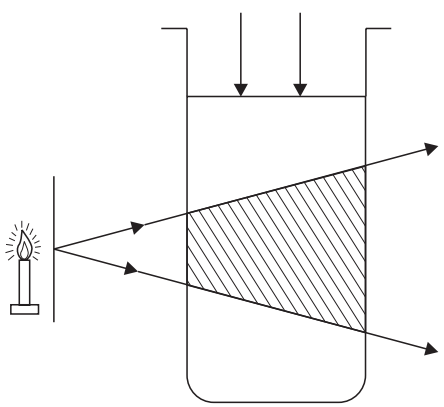


Figure 20.1 : Tyndall Effect.

20.2. THEORY

In short, **turbidimetry** is the measurement of the degree of attenuation of a radiant beam incident on particles suspended in a medium, the measurement being made in the directly transmitted beam. Thus, turbidity (T) may be expressed as :

$$T = \frac{1}{l} . \ln . \frac{I_o}{I_t} \qquad ...(a)$$

where, T = Turbidity,

l = Length of dispersion through which the light passes,

I_o = Intensity of incident light,

I_t = Intensity of transmitted light, and

n = Refractive index of the dispersion medium.

The **International Pharmacopoeia** describes **Turbidance (S)** as—'*a measure of the light-scattering effect of suspended particles*' ; and **Turbidity** (*r*) as—'*a measure of the decrease in incident beam intensity per unit length of a given suspension*'.

Nephelometry exclusively refers to the *measurement of the light scattered by suspended particles at right angles (perpendicular) to the incident beam.*

Turbidimetry or nephelometry may be employed judiciously for the measurement of precipitates produced by the interaction of very dilute solutions of reagents, or other particular matter, for instance : concentration of colloidal dispersion of organic and inorganic compounds and suspensions of bacterials cells (microbial assays).

It is, however, pertinent to mention here that in order to achieve the prime objective of obtaining fairly reproducible analytical results and absolutely consistent results the following experimental parameters may be observed strictly with regard to the production of suspensions of reasonably uniform characteristic features, namely :

(*i*) the extremely dilute suspensions of bacterial cells may be employed to encounter the problems caused due to birefringence,

(*ii*) the concentrations of the two ions that combine to yield the respective precipitate, besides the ratio of the concentrations in the solutions that are mixed,

(*iii*) the procedural details including the order and the rate of mixing,

(*iv*) the amounts of other salts and substances present *e.g.*, the protective colloids such as : dextrin, gelatin, gum arabic ; and

(*v*) the temperature.

20.3. INSTRUMENTS FOR NEPHELOMETRY AND TURBIDIMETRY

Nephelometric and turbidimetric measurements may be made with a fairly reasonable accuracy and precision by using either standard instruments available commercially or by improvising other similar devices. A brief description of such available means shall be discussed below :

20.3.1. INSTRUMENTS FOR NEPHELOMETRY

In general, nephelometric measurements essentially require an instrument with a photocell placed in position so that it may receive selectively the scattered light rather than the transmitted light. As this principle and geometry also hold good specifically to fluorimeters; and, therefore, these can be employed as nephelometers by selecting proper filters.

The following instruments are used invariably for nephelometric measurements, namely :

20.3.1.1. Duboscq Colorimeter

In actual practice, the so called '*visual*' **nephelometer** (comparator type) have been more or less superseded by the photoelectric instruments Nevertheless, a **Duboscq Colorimeter** with a slight modification may be used conveniently for nephelometric analysis, for instance :

(*a*) the path-of-light should be arranged in such a fashion that the light enters the side of the cups at right angles to the plungers rather than through the bottoms,

(*b*) clear-glass-tube with opaque bottoms are to be used instead of the normal cups,

(*c*) the glass-plungers are precisely fitted with opaque sleeves, and

(*d*) the light that enters at right angles to the clear-glass-tubes should be monitored carefully so as to achieve an equal-illumination on either sides.

Now, a standard suspension is placed in one clear-glass-tube, and the unknown solution is treated exactly in an identical fashion and placed in the other clear-glass-tube. Finally, the dividing line existing between the two fields in the eye-piece (Figure 20.2) must be distinctly thin and sharp, and it must disappear when the two fields are matched properly.

The Duboscq Colorimeter should always be maintained meticulously neat and clean. The clear-glass-tubes and the plungers are either rinsed with distilled water or with the solution to be measured.

First of all, it is necessary to ensure that the readings are zero when the plungers just touch the bottoms of the clear-glass-tubes. Now, the standard solution is placed in one clear-glass-tube, whereas an equal volume of the solution in question (unknown) in the other ; bearing in mind the fact that the clear-glass-tubes should never be filled above their respective shoulders.

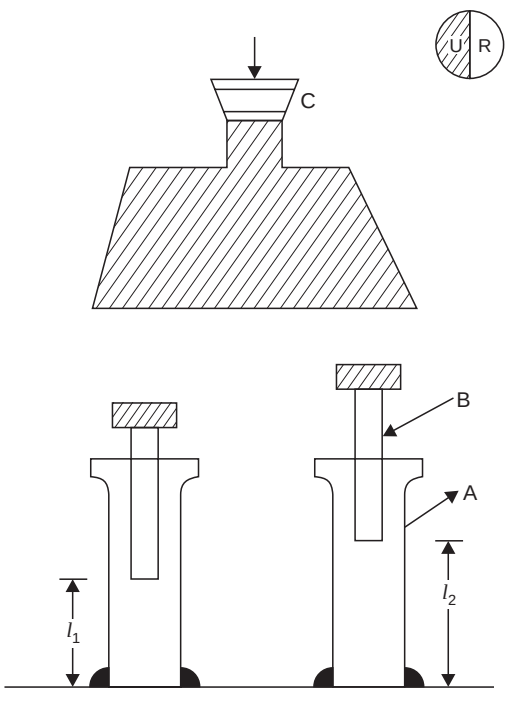


Figure 20.2 : DUBOSCQ COLORIMETER.

The various components of a Duboscq Colorimeter are as follows :

A = Clear glass tube with opaque bottom,

B = Glass plungers fitted with opaque sleeves, and

C = Eye piece.

Subsequently, set the unknown solution at a scale reading of 10.0 mm and simultaneously adjust the standard until the fields are matched equally. Perform at least five similar adjustments with the clear-glass-tube (A) containing the standard solution, and calculate the mean value. Care should always be taken that the plungers (B) always remain below the surface of the liquid. However, it is advised to visualize the match-point from above and below :

Assuming **Beer's Law** holds good the concentration of the solution in question (unknown) may be determined by the help of the following expression :

$$\underset{\text{(known)}}{c_1 l_1} = \underset{\text{(unknown)}}{c_2 l_2}$$

or
$$c_2 = \frac{c_1 l_1}{l_2} \qquad ...(b)$$

where, l_1 = Average readings for the clear-glass-tube having the solutions of known concentration,

l_2 = Average reading for the clear-glass-tube having the solution of the unknown concentration,

c_1 = Concentration of the known solution, and

c_2 = Concentration of the unknown solution.

It may, however, be observed that if $l_2 = 10.0$, the standard scale when multiplied by 10 shall give the percentage concentration of the sample in terms of the standard.

20.3.1.2. Nephelometer

The most important characteristic feature of a **nephelometer** is the '*reflector*' that has been specifically designed so as to collect the light which has undergone scattering by the particles present in a turbid or cloudy solution. A typical nephelometer is illustrated in Figure : 20.3, below :

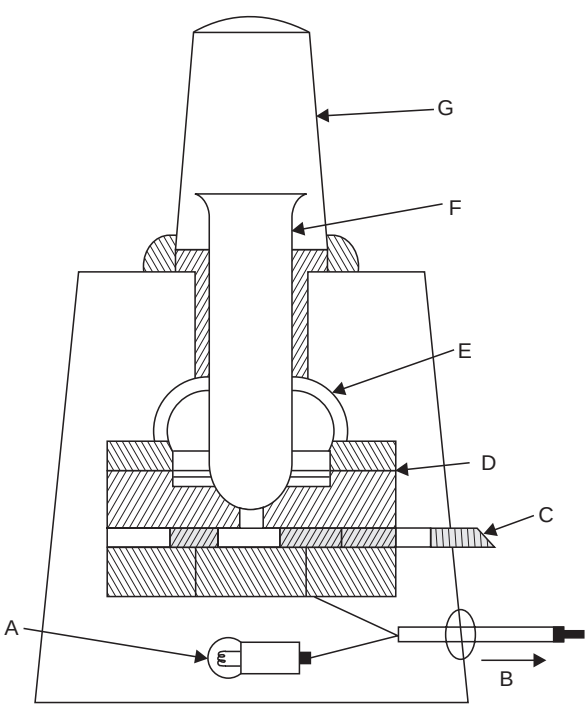


Figure 20.3 : A Nephelometer.

Following are the different parts of a nephelometer :

A = A light source,

B = A sensitive micro-ammeter,

C = Filter wheel with a series of colour filters,

D = An annular photocell,

E = A reflector to collect the scattered light,

F = A test tube, and

G = A metal test tube cover to exclude extraneous light.

The test solution (sample) is placed in a test tube (F) that has been duly rested on a light source (A) as exhibited in Figure 20.3. The scattered light caused by the particles in a turbid or cloudy solution is immediately directed by the reflector (E) on to an annular photocell (D). A series of standard colour filters are usually provided in the form of a filter-wheel (C) so as to facilitate analysis of coloured solutions ; taking care that the filter chosen must be similar to colour to that of the solution. The current generated after passing through the photocell (*i.e.*, light energy is being converted to electrical energy) is recorded by a sensitive micro-ammeter (B). The test tube is provided with a metallic cover (G) to get rid of any extraneous light. Usually a nephelometer is provided with zero-setting controls, sensitivity adjusting device and a set of previously matched test tubes.

20.3.2. INSTRUMENTS FOR TURBIDIMETRY

In fact, either visual or photoelectric colorimeters may be satisfactorily employed as turbidimeters. However, the use of the blue filter normally enhances the sensitivity appreciably. It has been observed that the light transmitted by a turbid solution does not normally obey the *Beer-Lambert Law* accurately and precisely. Therefore, as an usual practice it is advisable to construct a **'calibration curve'** by employing several standard solutions. The concentration of the unknown solution may be read off directly from the above calibration curve as is done in the case of colorimetric assays.

20.4. ASSAY OF PHARMACEUTICAL SUBSTANCES

A number of pharmaceutical substances are assayed either turbidimetrically or nephelometrically. The assay methods of these *two* techniques shall be discussed briefly below with the help of appropriate examples :

20.4.1. TURBIDIMETRIC ASSAY

A large number of antibiotics, namely : chlortetracycline, doxycyline, gentamicin, neomycin, streptomycin, tobramycin and the like may be assayed tubidimetrically with fairly good accuracy.

20.4.1.1. Assay of Chloretracycline

Theory : Inoculate a medium consisting of : peptone : 6 g, beef extract : 1.5 g, yeast extract : 3 g, sodium chloride : 3.5 g, D-glucose monohydrate : 1.0 g, dipotassium hydrogen orthophosphate : 3.68 g, potassium hydrogen orthophosphate : 1.32 g and dissolve in sufficient water to produce 1 L with a known quantity of a suspension of *Staphylococcus aureus* (NCTC 6571*) so as to obtain a readily measured opacity after an incubation of about 4 hours. The micro-organisms must exhibit a sensitivity to the antibiotic under investigation to such an extent that a sufficiently large inhibition of growth takes place in the prevailing conditions of the test.

In actual practice, it is always advisable that the inoculated medium should be used immediately after its preparation. Using a phosphate buffer of pH 4.5 (dissolve 13.61 g of $KH_2 PO_4$ in about 750 ml of water, adjusting the pH to 4.5 with 0.1 M NaOH and diluting to 1 L with water), prepare solutions of the Standard Preparation and the substance under investigation at concentrations presumed to be equal.

* NCTC = National Collection of Type Culture

To enable the validity of the assay to be examined, it is desirable to use at least three doses of the Standard Preparation and of the substance being examined. It is also advisable to use doses in logarithmic progression in a parallel line assay.

Materials Required : Standard chlortertracyline ; sterilized media (as described above) : 1 L ; authentic and pure strain of microorganism *Staphylococcus aureus* (NCTC 6571) ; formaldehyde solution (34-37% w/v) 10 ml ; matched identical test tubes : 20 ;

Procedure : Distribute into identical test-tubes an equal volume of standard tetracycline solution and the sample to be examined (having presumed equal concentrations) and add to each tube an equal volume of inoculated nutrient medium (for instance 1 ml of the solution and 9 ml of the medium). Prepare at the same time two control tubes without the chlortetracycline, one containing the inoculated medium and the other identical with it but treated immediately with 0.5 ml of formaldehyde solution. These tubes are used to set the optical apparatus employed to measure the growth.

Place all the tubes, randomly distributed, in a water-bath or other suitable means of bringing all the tubes rapidly to 35-37 °C *i.e.*, the incubation temperature and maintain them at that temperature for 3 to 4 hours, taking due precautions to ensure uniformity of temperatures and identical incubation times. After incubation, stop the growth of the microorganisms by adding 0.5 ml of formaldehyde solution, each tube and subsequently measure the opacity to at least three significant figures using a suitable optical apparatus. From the results calculate the potency of the substance being examined *i.e.*, chlortetracycline by standard statistical methods.

Note : (a) Rectilinearity* of the dose-response relationship, transformed or untransformed, is often obtained only over a very limited range. It is this range that must be used in calculating the activity and it must include at least three consecutive doses in order to permit rectilinearity to be verified,

(*b*) Use in each assay the number of replications per dose sufficient to ensure the required precision. The assay may be repeated and the results combined statistically to obtain the required precision and to ascertain whether the potency of the antibiotic being examined is not less than the minimum required.

20.4.1.2 Cognate Assays

A few other official antibiotics in BP (1993) may also be assayed by adopting the method stated above, but using specific micro-organism, definite final pH of the medium, pH of the phosphate buffer, potency of solution (U per ml) and the incubation temperature. A few typical examples are given in Table 20.1 below :

Table 20.1 Assay of Antibiotics Turbidimetrically

S. No.	Antibiotic	Micro-organism	Medium Final pH	Phosphate Buffer pH	Potency of Solution U per ml	Incubation Temperature (°C)
1.	Doxycycline	**Staphylococcus aureus** (NCTC 7447)**	7.0	4.5	0.003 to 0.010	35 to 37
2.	Gentamycin	-do-	7.0	8.0	0.6 to 1.25	35 to 37
3.	Neomycin	**Klebsiella pneumoniae** (NCIMB 9111)***	7.6	8.0	1.5 to 4	35 to 37
4.	Streptomycin	-do-	7.0	8.0	2.4 to 3.8	35 to 37
5.	Tobramycin	**Staphylococcus aureus** (NCTC 7447)	7.0	7.0	0.75 to 1.875	35 to 37

* In order to obtain the required rectilinearity it may be necessary to select from a large number three consecutive doses, using corresponding doses of the standard preparation and of the substance being examined. (BP, 1993, Appendix XIV A, p, 167 and 168).

**NCTC : National Collection of Type Cultures

*** NCIMB : National Collection Industrial and Marine Bacteria

20.4.2. NEPHELOMETRIC ASSAY

Nephelometric assay may be employed for the determination of sulphate (SO_4^{2-}) and phosphate (PO_4^{3-}) ions quite efficiently. These two estimations shall be discussed below in an elaborated manner :

20.4.2.1. Assay of Sulphate Ion (SO_4^{2-})

Theory : From actual experience it has been observed that it is always difficult to reproduce the turbidity of a dilute barium-sulphate-suspension. Hence, it is very important to adopt the underlying experimental procedure very closely and rigidly so as to obtain reasonably good results, namely :

(*i*) The rate of formation (velocity) of the precipitation along with the concentration of the reactants should be monitored and controlled by the addition of pure solid barium chloride having a definite grain size,

(*ii*) The rate at which barium chloride undergoes dissolution controls the velocity of the reaction,

(*iii*) Both NaCl and HCl (reagent) are added before the commencement of the precipitation so as to check the growth of microcrystals of $BaSO_4$,

(*iv*) An optimum pH must be maintained that essentially decreases the effect of variable quantities of the other electrolytes, possibly present in the sample, upon the size of the suspended $BaSO_4$ particles,

(*v*) Most importantly the presence of glycerol-ethanol solution helps to stabilize the turbidity,

(*vi*) Each reaction-vessel must be shaken gently both at the same rate and the same number of times so as to obtain a uniform particle size ($BaSO_4$),

(*vii*) The unknown sample should be treated exactly in an identical manner (as the standard solution), and

(*viii*) The time-gap between the time of precipitation and the time of measurement (of turbidity) should always be kept constant.

Materials Required

(*i*) **Standard Sulphate Solution :** 1.814 g of K_2SO_4 (dry) is dissolved in DW and diluted to 1 L in a graduated flask :

96.08 g of SO_4^{2-} present in 174.26 g of K_2SO_4, therefore,

1.000 g of SO_4^{2-} ion is present in $\frac{174.26}{96.08}$ = 1.8136 g of K_2SO_4 in 1 L

= 1.814 g of K_2SO_4 in 1 L

or 1.000 mg of SO_4^{2-} ion present in 1 ml

i.e., the solution contains 1.000 mg of SO_4^{2-} per ml

(*ii*) **Sodium Chloride-Hydrochloric Acid Reagent :** 60 g of NaCl is dissolved in 200-ml of DW, add to it 5 ml of concentrated HCl (AR) and dilute to 250-ml with DW,

(*iii*) **Barium Chloride :** The $BaCl_2$ crystals that pass through the 20 mesh sieve and retained by the 39 mesh sieve are only used,

(*iv*) **Glycerol-Ethanol-Solution :** Prepared by dissolving pure glycerol in absolute ethanol (1 : 2).

Procedure

1. Transfer 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 ml of the standard potassium sulphate solution from a burette into each separate 100-ml volumetric flask and number them from 1 to 8,
2. To each flask (1 to 8) pipette out 10 ml of the NaCl-HCl reagent and 20 ml of the glycerol-ethanol solution, and dilute to 100 ml mark with DW,
3. Weigh and add 0.3 g of sieved $BaCl_2$ to each flask (1 to 8) stopper them, and shake for exactly one minute by inverting flask once in one second (All $BaCl_2$ must dissolve),

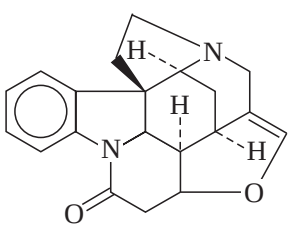
4. Permit each flask to stand for 2-3 minutes and read out the turbidity in the nephelometer,

   Caution : Avoid any tiny air-bubbles sticking to the inner walls of the matched test-tubes.

5. By employing the most-concentrated, K_2SO_4 solution, as standard, and by the help of the sensitivity control, adjust the micro-ammeter reading to 100-divisions,
6. A '*Blank*' solution is prepared by adopting the above operations sequentially, but without the addition of the K_2SO_4 solution,
7. Insert the Blank solution in the nephelometer and adjust to zero reading of the scale by the aid of zero-control-knob,
8. Check the reading of the most-turbid-solution, and adjust any deviation from 100 by means of the sensitivity control,
9. Repeat the measurements with the remaining standard sulphate solution and plot the nephelometer reading V_S the SO_4^{2-} ion content per ml,
10. 'Unknown Solution'—Determine the SO_4^{2-} ion content of an unknown solution, for instance : 0.4 mg per ml, by means of the standard-calibration-curve.

20.4.2.2. Assay of Phosphate Ion (PO_4^{3-})

Theory : The underlying principle for the assay of PO_4^{3-} ion by nephelometry is the formation of strychnine-molybdophosphate complex (I).



$24MoO_3 \cdot P_2O_5 \cdot xH_2O$

(I)

The turbidity thus obtained is white in appearance and consists of very fine particles of the above complex. Extra care must be taken for **not** agitating the precipitate so as to avoid agglomeration of the same quickly. Likewise, temperature variation should also be avoided as far as possible because the precipitate is somewhat sensitive.

Materials Required

1. **Standard Phosphate Solution :** 1.721 g of KH_2PO_4, previously dried at 110 °C, is dissolved in 1 L of DW in a 1000-ml volumetric flask and make up the volume with DW upto the mark.

The resulting diluted solution contains 0.01 mg P_2O_5 ml^{-1}.

2. **Molybdate-Strychnine Reagent**

Solution 'A' : (Acid Molybdate Solution) : Weigh 30 g of molbdenum trioxide (Mo_2O_3) in a 500-ml conical flask, add to it 10 g of Na_2CO_3 and 200 ml of DW. Boil the contents of the flask until a clear solution is achieved. Filter the hot solution, add 200 ml of 5 M. H_2SO_4, allow to cool and dilute to 500 ml with DW.

Solution 'B' (Strychnine-Sulphate Solution) : Weigh 1.6 g strychnine sulphate in 100 ml of DW. Warm it gently, cool and dilute to 500 ml with DW.

Molybdate-strychnine reagent is prepared by dissolving solution-B shaking the resulting mixture vigorously. The bluish-white precipitate thus obtained is filtered through What man No : 42 filter paper and the resulting clear solution may be used within 20 hours.

Note : (*i*) Strychnine must be handled with gloves on as it is a very toxic alkaloloidal substance and under no condition it should be ingested,

(*ii*) Molybdate-strychnine reagent is always prepared afresh by mixing solution-B to solution-A, because the addition of the acid-molybdate solution to the strychnine-sulphate solution gives a precipitate after 24 hours, and

(*iii*) Solutions A and B can be stored indefinitely.

3. **Saturated Sodium Sulphate Solution :** A saturated aqueous solution of sodium sulphate is prepared at 50 °C, cooled to room temperature and filtered before use.
4. **Sulphuric Acid (1 M) :** 27.0 ml of concentrated H_2SO_4 is diluted to 500 ml in a graduated flask.

Procedure

1. Transfer accurately 1.0, 2.0, 4.0, 6.0, 8.0, and 10.0 ml of the standard phosphate solution with a burette into each 100 ml volumetric flasks.
2. 18 ml of 1 M . H_2SO_4 is added to each flask, followed by 16 ml of saturated sodium sulphate solution, and diluted to 95 ml with DW.
3. Add 2.0 ml of the molybdate-strychnine reagent to the resulting solution and make up the volume to 100 ml.
4. The contents of the flask is mixed by gently inverting it a number of times, but without shaking vigorously.
5. Keep the flasks aside for at least 20 minutes so as to allow the turbidities to develop before making the measurements.
6. A 'blank' solution is prepared by performing the above operations sequentially, but without the addition of the phosphate solution.
7. By employing the most concentrated solution as the initial standard, adjust the microammeter reading to 100 divisions.
8. Place the 'blank' solution into the matched test-tube of the nephelometer and adjust the reading to zero.
9. Check the reading of the most turbid solution, and adjust any deviation from 100 by the help of the sensitivity control.
10. Repeat the measurements with the remaining standard phosphate solution and plot the nephelometer reading V_S the mg P_2O_5 per ml.
11. **Unknown Solution :** Determine the phosphate content of an unknown solution, for example : containing 0.005 mg P_2O_5 per ml by the help of the standard-calibration graph.

THEORETICAL AND PRACTICAL EXERCISES

1. What is Tyndall Effect ? How does it affect 'nephelometry' and 'turbidimetry ' ? Explain.

2. (*a*) Define 'turbindance' and 'turbidity' as per the International Pharmacopoeia.

(*b*) Discuss the 'theoretical aspect' and 'experimental parameters' for turbidimetry.

3. Describe the under mentional analytical instruments with the help of a neat diagram and working modalities :

(*a*) Duboscq colorimeter,

(*b*) Nephelometer, and

(*c*) Photoelectric colorimeter.

4. How would you accomplish the '**turbidimetric assay**' of the following medicinal compounds :

(*i*) Chlortetracycline, (*ii*) Doxycycline,

(*ii*) Gentamycin, and (*iv*) Tobramycin.

5. Desocibe in details the assay of the following '**drug substances**' by using a '**nephelometer**' :

(*i*) SO_4^{2-} ion, (*ii*) PO_4^{3-} ion.

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3. Svehla, G, Ed., **'Comprehensive Analytical Chemistry',** Vol. XIX (1986) ; Vol. XX (1985) ; Vol. XXII (1986), Amsterdam, Elsevier Publishers, 1986.

4. Jeffrey, G.H. J. Bassett., J. Mendham and R.C. Denney, **'Vogel's Textbook of Quantitative Chemical Analysis*,'** 5th ed., New York, Longman Scientific and Technical, 1989.

5. Schirmer, R.E., **'Modern Methods of Pharmaceutical Analysis,** 2nd. Vol. 1, Boston, CRC Press 1991.

21 ULTRAVIOLET AND ABSORPTION METHODS

CONTAINS :

21.1. INTRODUCTION

In the earlier sections of this part, the various analytical methods based upon the measurements of mass and volume have been described at sufficient length with their typical applications in the analysis of pharmaceutical substances. Comparatively older methods of analysis, such as **colorimetry** is entirely based upon the interaction of specifically visible light with a sample. In this particular instance, just the visible portion of the electromagnetic radiation spectrum within the range of 400 and 700 nanometers (nm) to which a human eye is sensitive, has been employed. In a situation whereby the sample is made to interact with a wide spectrum of wavelengths in a given zone of electromagnetic radiation, consequently giving rise to a collection of measurement signals as a function of wavelength is termed as a **spectrum**, ultimately putting forward the most common terminology **spectrochemical analysis** or **spectroscopy.**

21.2. THEORY

21.2.1. ELECTROMAGNETIC SPECTRUM

It has been established beyond any reasonable doubt that the absorption and the emission of energy in the **electromagnetic spectrum** take place in distinct separate pockets or photons. The relationship

existing between the energy of a photon and the frequency matching its propagation may be expressed as follows :

$$E = h\nu \qquad ...(a)$$

where, E = Energy (in ergs),

ν = Frequency (in cycles sec^{-1}), and

h = Universal constant termed as Planck's constant (6.6256×10^{-27} erg sec).

However, the relationship between wavelength and frequency may be expressed as follows :

$$\nu = c/\lambda \qquad ...(b)$$

where, λ = Wavelength (in cms),

c = Velocity of propagation of radiant energy in vacuum (which is nothing but the speed of light in vacuum ; and is equivalent to 2.9979×10^{10} cm sec^{-1}).

The radiant power of a beam is designated by its intensity of radiation, which in turn is directly proportional to the number of photons per second that are propagated in the beam.

Monochromatic Beam : A beam that carries radiation of only one distinctly separate wave length is known as **monochromatic**.

Polychromatic or Heterochromatic : A beam that carries radiation of several wavelengths is termed as **polychromatic or heterochromatic**.

21.2.2. SCHEMATIC REPRESENTATION OF ELECTROMAGNETIC SPECTRUM

Figure 21.1, provides a schematic representation of electromagnetic spectrum, whereby the beam of a white light from an incandescent solid (*e.g.*, the filament of an electric bulb consisting of numerous separate waves of different wavelengths) is passed through a prism thereby giving rise to a continuous spectrum wherein each colour corresponds to waves of a particular individual wavelength.

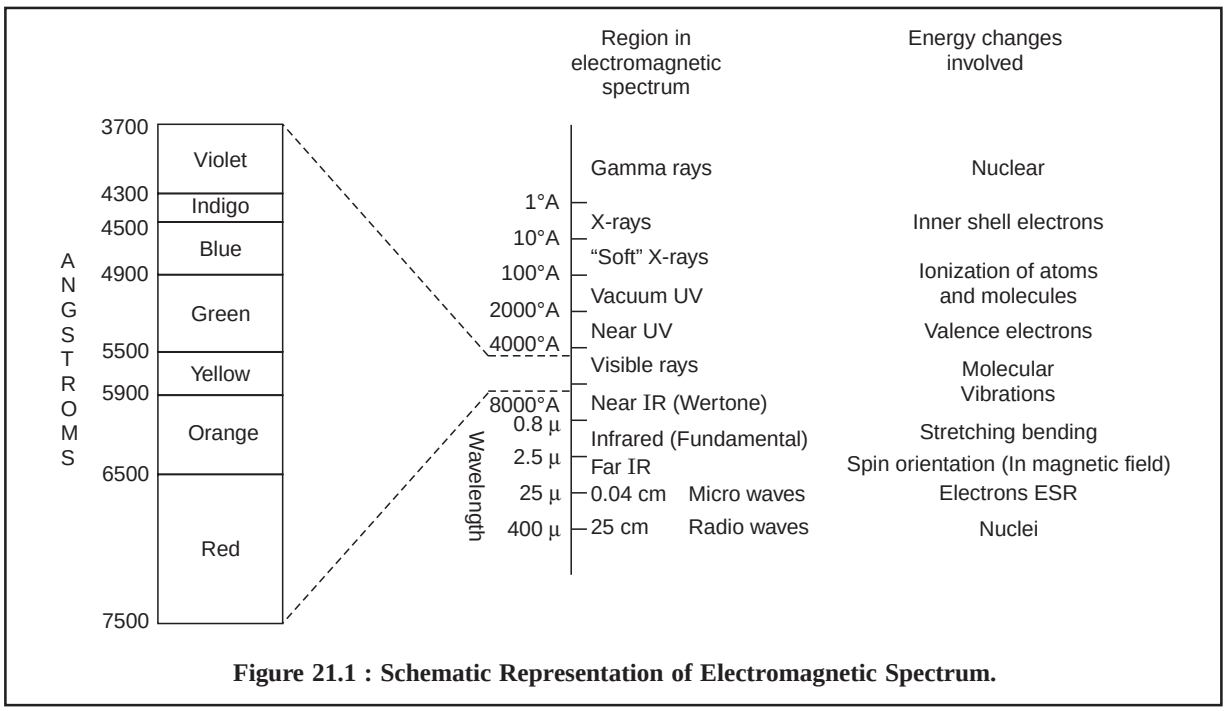


Figure 21.1 : Schematic Representation of Electromagnetic Spectrum.

A few salient points from Figure 21.1 are enumerated below :

(*a*) The visible spectrum constitutes a small portion of the complete electromagnetic radiation spectrum that extends from the ultra-short wave gamma rays at one end to that of the radio-waves at the other (400-700 nm),

(*b*) The wave length scale is nonlinear,

(*c*) **γ-Rays Region :** *Mossbauer Spectroscopy* (due to absorption) and *γ-Ray Spectroscopy* (due to emission) are used as analytical means.

(*d*) **Inner-shell Electrons :** *X-Ray absorption spectroscopy* (due to absorption) and *X-Ray Fluorescence spectroscopy* (XRF) (due to emission) are employed as analytical means.

(*e*) **From Vacuum-UV to Infra-Red Region :** *UV-VIS, IR-spectroscopy, spectrophotometry, atomic absorption spectroscopy* (AAS) (due to absorption) and *atomic emission spectroscopy* (AES, ESS, ICP) ; *atomic fluorescence spectroscopy* (AFS) (due to emission) are used as analytical techniques.

(*f*) **Microwave Region :** *Microwave spectroscopy* and *electron spin resonance* (ESR) (due to absorption) are employed as analytical methods.

(*g*) **Radiowave Region :** *Nuclear Magnetic Resonance* (NMR) (due to absorption) is used as analytical method.

21.2.3. MOLAR ABSORPTIVITY

Usually, a molecule exists in the state of *lowest energy* the **ground state**. However, absorption of light of the right frequency (in the UV-region) raises a molecule to an **excited state** *i.e.,* a state of *higher energy*. Considering the example for ethylene *two* situations arise, namely :

(*a*) **Ground State :** Here, both π electrons are in the π orbital. This configuration is designated as π^2, where the superscript represents the number of electrons in that orbital.

(*b*) **Excited State :** Here, an electron is in the π orbital while the other in the π* orbital (having an opposite spin). Thus, the resulting configuration ππ* is obviously less stable due to the fact that :

(*i*) only one electron helps to hold the atom together, and

(*ii*) the other electron tends to force them apart.

The **molar absorptivity** is mostly controlled by *two* vital factors, namely :

(*i*) polarity of the excited state, and (*ii*) probability of the electronic transition. So as to materialize an interaction, a photon should evidently strike a molecule very closely within the space of the molecular dimensions. The probability of the electronic transition, designated as '*g*', shall be responsible for the target hits that may ultimately lead to absorption. However, the molar absorptivity may be expressed as follows :

$$\frac{-\partial P}{P} = \frac{1}{3}\, g\, C\, N_A\, A(\partial b/1000) \qquad ...(c)$$

where, N_A = Avogadro Number,

A = Cross-sectional target area*

$\frac{1}{3}$ = Statistical factor (to permit random orientation),

g = Probability of the electronic transition

By inserting numerical constants and integration Eq. (*c*) we have :

$$\log (Po/P)\, bC = \in = (0.87 \times 10^{20})\, g\, A \qquad ...(d)$$

where, $\in$ = Molar absorptivity

Absorption with $\in > 10^4$ is considered high-intensity absorption.

* May be obtained from X-Ray Diffraction Data.

21.2.4. LAWS OF PHOTOMETRY

The '*Laws of Protometry*' has been discussed in Chapter-1 of this text under section 4.1.

21.2.5. SPECTRAL PRESENTATION

Absorption spectra may be presented in a number of fashions as depicted in Figure 21.2, namely :

(*a*) Wavelength *Vs* Absorbance,

(*b*) Wavelength *Vs* Molar Absorptivity, and

(*c*) Wavelength *Vs* Transmittance.

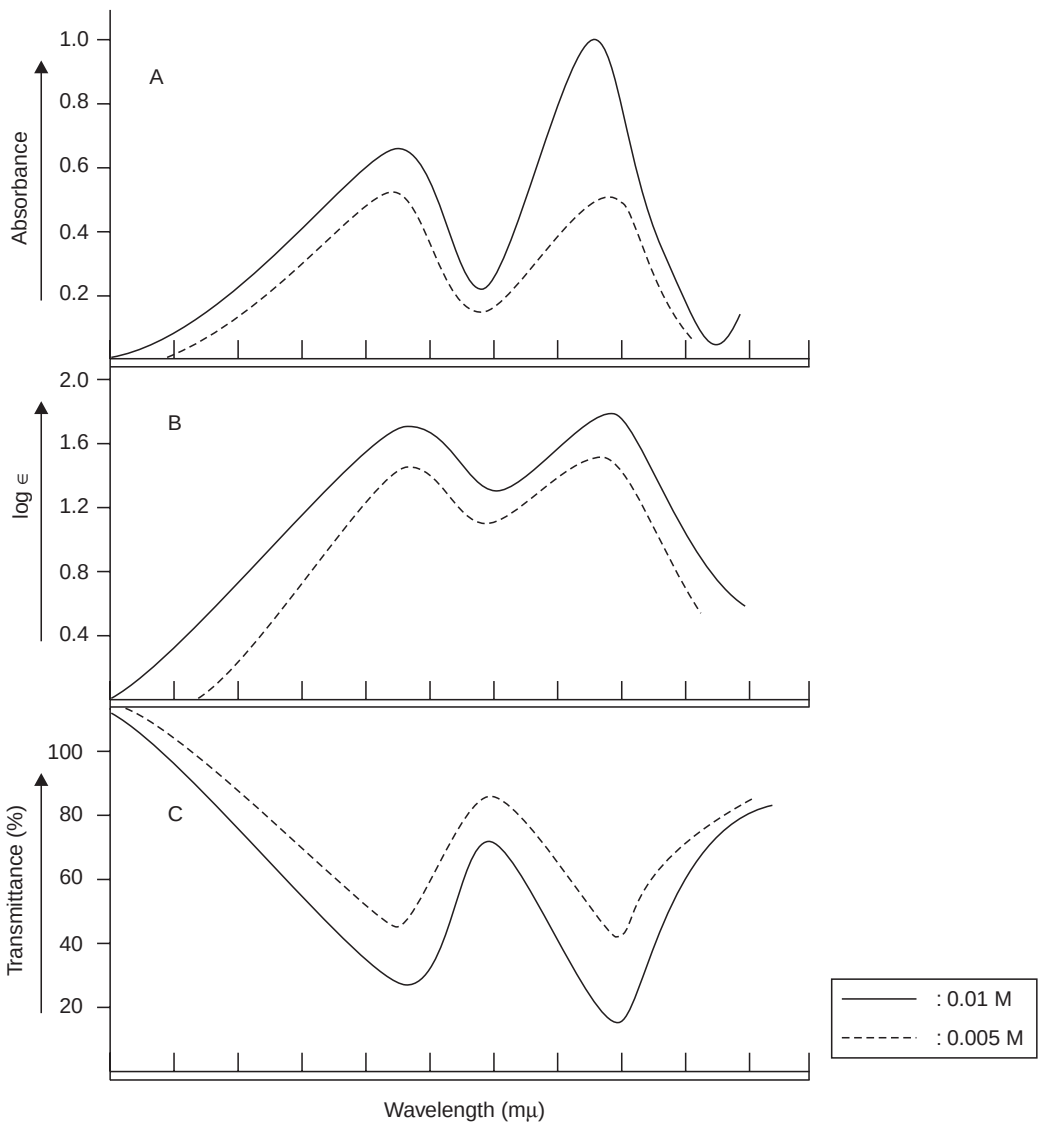


Figure 21.2 : Profile of Spectral Absorption Curves.

A few important features related to spectral presentation are enumerated below :

(*a*) In order to simplify the conversion of spectra in qualitative identification the spectral data should be plotted either as log A or as log ∈ *Vs* wavelength, thereby giving rise to the following expression :

$$\log A = \log \in + \log b + \log c \qquad ...(e)$$

where, b = Cell-length, and

c = Sample concentration.

From Eq. (*e*), one may observe that the resulting curve is independent of both cell-length and sample concentration,

(*b*) The identity and nonconformity of sample may be ascertained by simply carrying out the comparison of spectral presentation both up or down the ordinate scale,

(*c*) In order to obtain both reproducible and fairly consistent accurate plot the ordinate in absorbance values must be plotted on graph paper having 1 mm equivalent to 0.005 absorbance,

(*d*) Most importantly all relevant informations pertaining to : solvent employed, concentrations used, the band pass and ultimately the Model/Make of the Spectrophotometer,

(*e*) **Choice of Solvents :** For instance :

Water-common solvent for a number of inorganic substances,

Ethanol (96% w/v)-good choice as fairly polar solvent,

Cyclohexane-common solvent for a number of aromatic compounds.

21.2.6. STRUCTURAL FEATURES

While discussing the structural features special emphasis shall be laid only to those molecules that are capable of absorption within the wavelength region from 185 to 800 mμ.

A few *salient structural features* are enumerated below :

(*i*) Compounds having single bonds involving σ-valency electrons usually display absorption spectra below 150 mμ. Such spectra will be observed only in interaction with other types.

(*ii*) Excitation help in promoting a *p*-orbital electron into an antibonding σ orbit thereby giving rise to an $n \rightarrow \sigma^*$ transition, for example : ethers, sulphides, amines, and alkyl halides.

(*iii*) Unshared *p*-electrons exist besides σ-electrons in saturated compounds having covalent bonds and heteroatoms, for instance : N, S, O, Cl, Br, I,

(*iv*) Unsaturated compounds give rise to the absorption spectra by the displacement of π-electrons.

(*v*) Molecules that have single chromophores (*i.e.,* absorbing groups)-normally undergo transitions almost very close to their respective wavelengths,

(*vi*) Interestingly, a molecule containing only a single chromophore of a particular species shall absorb light of approximately the same wavelength as that of a molecule having two or more insulated chromophores, however, the intensity of the absorption shall be directly proportional to the number of the latter type of chromophore present in the compound.

Examples : (*a*) *meta*-orientation about an aromatic ring, and

(*b*) interposition of a single methylene (= CH_2) moiety.

The above two instances are sufficient to insulate chromophores from each other totally,

(*vii*) **Hyperconjugation—**is usually observed when slight interaction takes place with alkyl radicals attached to chromophores.

(*viii*) In fact, *four* different types of absorption bands have so far gained cognizance in the spectra of organic compounds, which are namely : *K-bands* ; *R-bands* ; *B-bands* ; and *E-bands*.

These bands will be discussed briefly here with regard to the structural features.

(*a*) **K-bands :** They normally arise from π-π structures and result from $\pi \rightarrow \pi^*$ transitions. These are invariably characterized by high molar absorptivity.

Examples :

(*i*) A diene : C = C—C = C to C^+—C = C—C^- ; where K-band is due to the resonance transition,

(*ii*) Vinyl benzene or acetophenone : *i.e.,* aromatic compounds having chromophoric substitution.

(*b*) **R-bands :** They usually arise from $n \rightarrow \pi^*$ transitions. They seldom display very noticeable results in aliphatic compounds, but marked and pronounced bathochromic shifts (*i.e.,* shifting of absorption towards longer wavelengths—as in extended open-chain-conjugated systems) do take place when—SH, —OH and —NH_2 replace hydrogen atom in unsaturated groups. Thus, R-bands help in the confirmation of a particular structure whereby additional bands are obtained by appropriate modifications in the electronic-structure of the parent compound.

(*c*) **B-bands :** These are rather weak-type of absorption bands. They are characteristic of both heteroatomic and aromatic molecules and may also consist of fine vibrational sub-bands.

(*d*) **E-bands :** They usually result from oscillations of electrons in aromatic-ring systems,

(*ix*) **Conjugated Systems :**

It is quite evident that the conjugated systems might fail to display the expected conjugated bands due to the following *two* reasons, namely :

(*a*) Orbitals of adjacent multiple bonds are at right angles instead of being parallel, and

(*b*) Resonating dipolar structures cannot be envisaged.

The resulting spectrum may seem to appear as a mere superimposition of the spectra of the individual chromophoric groups.

Examples : Allene and ketene systems

Polyphenyls (*e.g., m*-terphenyl)

(*x*) **Steric Hindrance :** The attachment of bulky functional entities to ring systems offering steric-hindrance may ultimately prevent the coplanarity of two resonating structures either completely or partially.

However, partial hindrance specifically leads to such characteristic bands pertaining to those parts of conjugated system.

21.2.7. ABSORPTION OF RADIANT ENERGY BY MOLECULES

In reality, the molecules are as energetic as the modern teenagers. They invariably rock, roll, twist, jerk, and bend, and if the music is of the right rhythm, choice, and frequency, the electrons within the molecule shall move from the '*ground state'* to the '*excited state'*.

Explicitly, the total energy in a molecule is the sum of the energies associated with the translational, rotational, vibrational and electronic motions of the molecule/or electrons/or nuclei in the molecule. These *four* motion-related-energies are briefly explained below :

(*a*) **Transational Energy :** It is associated with the motion (velocity) of the molecule as a whole.

(*b*) **Rotational Energy :** It is associated with the overall rotation of the molecule.

(*c*) **Vibrational Energy :** It is associated with the motion of atoms within the molecule.

(*d*) **Electronic Energy :** It is associated with the motion of electrons arounds the nuclei.

Electrons generally found in the conjugated double bonds invariably give rise to spectra in the UV and visible regions of the electromagnetic spectrum.

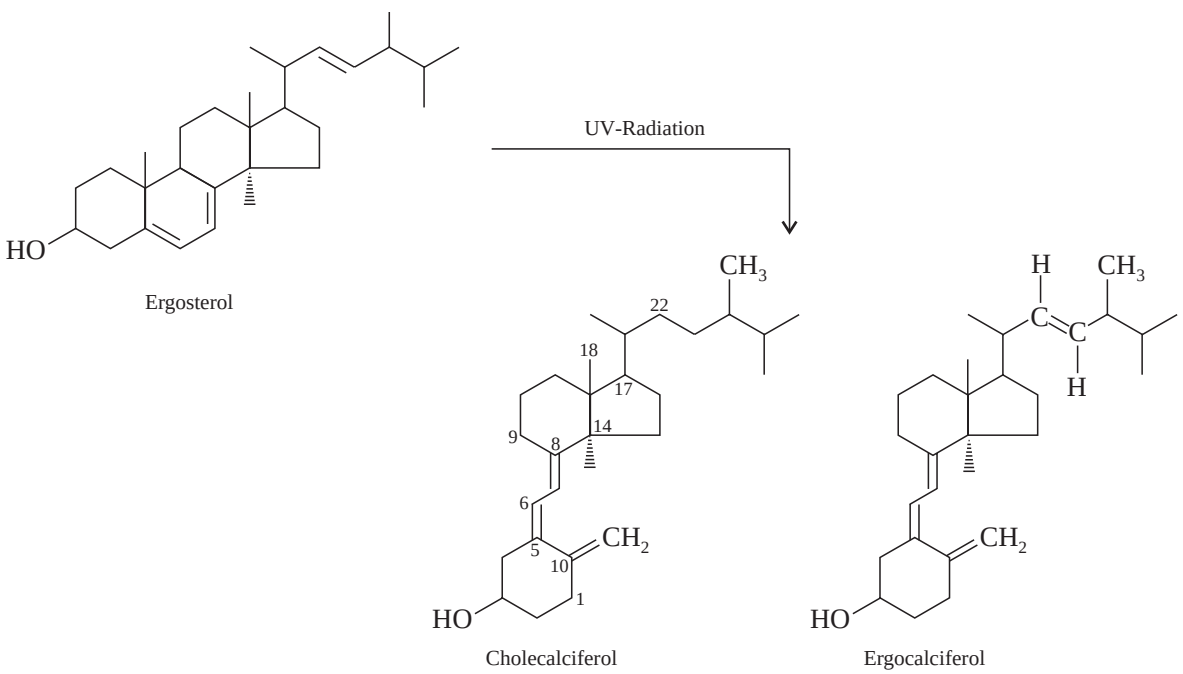
It is pertinent to mention here that an excited electron normally returns to the ground state in about 10^{-9} to 10^{-8} seconds. Consequently, energy must now be released to compensate for the energy absorbed by the system. In actual practice however, the following *three* situations arise, namely :

Firstly, if the electron returns directly to the ground state, the net effect would be evolution of heat.

Secondly, if the electron returns to the ground state by passing through a second excited state, the net outcome would be release of energy in the form of heat and light.

Thirdly, if a large amount of energy is absorbed by certain substances, bonds may be ruptured and thereby giving rise to altogether new compounds.

For instance : ergosterol on being subjected to UV radiation yields cholecalciferols which are, in fact, altogether new substances.



Ergosterol

Cholecalciferol

Ergocalciferol

In general, the changes incurred are usually minimal and for this very reason the UV-spectrophotometry is considered to be a non-destructive method of analysis.

However, the relative energies due to electrons (*d*), vibration (*c*), and rotation (*b*) are more or less in the order of 10,000 : 100 : 1 ; and the total energy for any one state at any material time may be depicted by the following expression :

$$E_{Total} = E_{Electronic} + E_{Vibrational} + E_{Rotational}$$

The diagrammatic representation of the potential energy of a diatomic molecule showing :

(*i*) Potential energy-nuclear separation curves, and

(*ii*) Relationship between electronic transitions and absorption curves ; is illustrated in Figure 21.3.

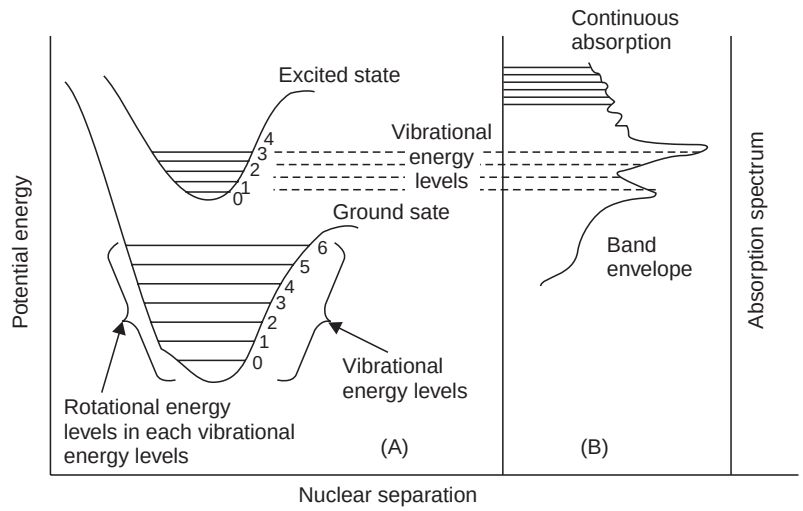


Figure 21.3 : Diagrammatic representation of Potential Energy of a diatomic molecule showing : (A) Potential Energy-Nuclear Separation Curves ; (B) Relationship between Electronic Transitions and Absorption Curves.

Explanation of various features in Figures 22.3 :

(*i*) The mutual forces are also zero when the nuclei are at infinity ; but as the latter come closer to one another, forces of attraction start operating and the potential energy decreases,

(*ii*) The potential energy records an increase when the nuclei get very close to one another thereby causing repulsion,

(*iii*) The atoms, therefore, can vibrate about the minimum position RC at the vibrational level 0,

(*iv*) The electronic configuration of the molecule gives rise to different quantum of energy associated with it which may be indicated and represented by the horizontal lines in Figure 21.3 ($0 \rightarrow 6$),

(*v*) At ambient temperature, the molecule is in the lowest ebb of the vibrational level of the ground state,

(*vi*) The corresponding electronic transition from the ground state to an excited state, is represented by the upper curve in Figure 21.3,

(*vii*) Rotational energy variations usually accompany electronic variations, however, they are comparatively smaller in size and often yield a fine structure superimposed on the electronic-vibrational change,

(*viii*) The frequency of the absorption bands associated with the transition is put forward by the following expression :

$$h\nu = E_{\text{Excited state}} - E_{\text{Ground state}} \qquad ...(e)$$

where, h = *Planck's Constant*,

ν = Frequency, and

E = Energy level.

In reality, their appearance as a pattern comes into being chiefly from transitions to the various vibrational levels of the excited state as shown in Figure 21.3.

21.2.8. FACTORS INFLUENCING ABSORPTION OF RADIANT ENERGY

There are various cardinal factors that govern measurement of absorption of radiant energy, namely :

(*a*) Absorbing groups (or Chromophores),

(*b*) Solvent effects,

(*c*) Effect of temperature, and

(*d*) Inorganic ions.

These vital factors would be discussed briefly with specific examples hereunder :

21.2.8.1. Absorbing Groups (or Chromophores)

A **'chromophore'** is a group which when attached to a saturated hydrocarbon produces a molecule that absorbs a maximum of visible of UV energy at some specific wavelength.

A few typical examples having electronic absorption bands for various representive chromophores are provided in the following Table : 21 : 1 :

Table 21.1 : Absorption Bands for Representative Chromophores with Examples :

S.No.	Chromophore	System	λ_{max}	ϵ_{max}	λ_{max}	Examples
1.	Acetylide	–C ≡ C–	175-180	6000	—	Acetylene
2.	Azo	–N = N–	285-400	3-25	—	Azomethane
3.	Aldehyde	–CHO	210	strong	280-300	Acetaldehyde
4.	Carboxyl	–COOH	200-210	50-70	—	Acetic acid
5.	Nitrile	–C ≡ N	160	—	—	Acetonitrile
6.	Nitro	$-NO_2$	210	strong	—	Nitromethane
7.	Thioketone	C = S	205	strong	—	Thiobenzophenone
8.	Esters	–COOR	205	50	—	Ethyl acetate
9.	Ether	–O–	185	1000	—	Diethyl ether
10.	Amine	$-NH_2$	195	2800	—	Methyl amine
11.	Thiol	–SH	195	1400	—	Thiophenol
12.	Iodide	–I	260	400	—	Methyl iodide
13.	Bromide	–Br	208	300	—	Ethyl bromide
14.	Sulphone	$-SO_2-$	180	—	—	Dapsone
15.	Nitroso	–N = O	302	100	—	*p*-Nitroso phenol

21.2.8.2. Solvent Effects

The absorption spectrum of a pharmaceutical substance depends partially upon the solvent that has been employed to solubilize the substance. A drug may absorb a miximum of radiant energy at a particular wavelength in one solvent but shall absorb practically little at the same wavelength in another solvent. These apparent changes in spectrum are exclusively due to various characteristic features, namely :

(*a*) Nature of the solvent,

(*b*) Nature of the absorption band, and

(*c*) Nature of the solute.

Some salient features of '*Solvent Effects*' are enumerated below :

(*i*) Absorption bands of many substances are relatively sharper and may also exhibit fine structure when measured in solvents of low dipole moment,

(*ii*) Interactions of solvent-solute are found to be much stronger in such substances where strong dipole forces are involved,

(*iii*) Solvent effects do help in reorganizing electronic transitions of the type n—π^* that essentially involve the nonbonding electrons of nitrogen and oxygen,

(*iv*) The nonbonding electrons of nitrogen and oxygen usually interact with polar solvents that ultimately give rise to a characteristic shift to shorter wavelengths.

Example : The spectrum of Iodine in a nonpolar solvent like $CHCl_3$ is found to be distinctly different (purple to the naked eye) when the same is compared in a polar solvent such as C_2H_5OH (brownish to the naked eye) in Figure 21.4.

(*v*) A spectrum normally shows appreciable changes with varying pH when an ionizable moiety is present in the molecule and thereby constitutes part of the chromophore structure.

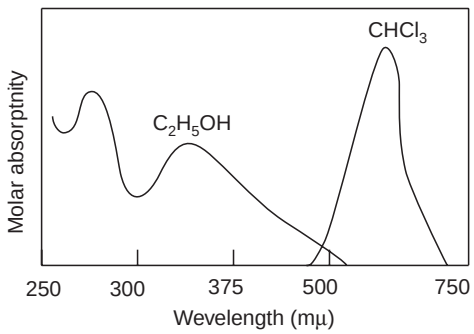


Figure 21.4 : Absorption spectrum of Iodine in a nonpolar and polar solvent.

21.2.8.3 Effect of Temperature

- Low temperature offfer sharper absorption bands of many pharmaceutical substances than at room temperature,
- Vibrational resolutions are definitely well-defined at low temperatures because of the following two reasons, namely :

  (*a*) Fewer vibrational levels are occupied, and

  (*d*) Degree of solute-solvent interaction is minimised,
- Samples in highly rigid or viscous media (*e.g.*, glass) is examined frequently in phosphorescence methods and also in some fluorescence methods.

21.2.8.4 Inorganic Ions

The 'chromophoric entities' present in the inorganic compounds are of two types, namely :

(*a*) **Involving several atoms :** such as : permanganate (MnO_4^-) and dichromate ($Cr_2O_7^-$) moieties, and

(*b*) **Involving single atoms :** Those having incomplete outer *d*-electron shells where closely spaced, unoccupied energy levels are available in abundance for instance : coordination compounds with Rare Earths : *e.g.*, Be, Sr, Ra, and Transition Elements : Cr, Mn, Ni, Pt, Ag, Pd, Cd, Hg, Au,

It is worth while to note that the absorption spectra for these elements are caused due to a charge-transfer-process whereby an electron gets transferred form one part of the ion to another.

Interestingly, inclusion of readily polarizable atoms do exert an effect likewise to lengthening a conjugated chain. Examples :

Inorganic Ions	Colour	Molar Absorptivity
$FeCl_3$	Yellow	Lower
$FeBr_3$	Orange	Higher

21.3. INSTRUMENTATION

A spectrophotometer is an instrument which is capable of isolating **'monochromatic'** radiation ; or that which specifically contains a dispersing element : a prism or a grating.

It is pertinent to mention here that there are a plethora of commercially available spectrophotometers of varying design *i.e.,* single-beam (simple), double-beam (more precise and accurate) and microcomputer controlled built-in-recorder with separate printer ; and obviously having a wide-price-range from Rs 3.0 Lacs to Rs 17.5 Lacs. Evidently, it is practically impossible to describe either all or even a major fraction of, the various spectrophotometers available.

Therefore, in this particular section the following *two* types of spectrophotometers shall be discussed briefly :

(*a*) Single-beam Spectrophotometer, and

(*b*) Double-beam Spectrophotometer.

21.3.1. SINGLE BEAM SPECTROPHOTOMETER

The desired wavelength is isolated by using a prism or grating and auxiliary mirrors and slits that collectively from a microchromator of the instrument. The wavelength dial on a spectrophotometer is adjusted to a specific value, but the radiation leaving the exit-slit is found to be rarely monochromatic. The schematic diagram of the Beckman Model DU-Spectrophotometer is illustrated in Figure 21.5.

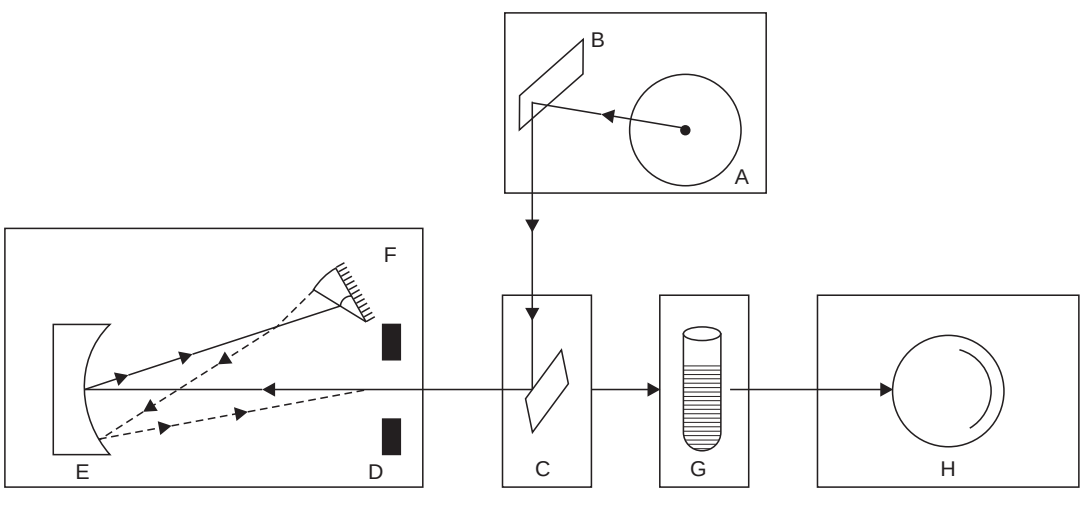


Figure 21.5 : Schematic Diagram of A Beckman Model DU-Spectrophotometer.

The various components of Figure 21.5 are given below :

A = Source of light ;
B = Condensing mirror ;
C = Slit-entrance mirror ;
D = Adjustable slit ;
E = Collimator mirror ;
F = Prism (Reflecting) ;
G = Cuvette containing sample ;
H = Phototube ;

Light from the source (A) is focussed on the condensing mirror (B) and directed in a beam to the 45° slit-entrance mirror (C). The slit-entrance mirror subsequently deflects the beam through the adjustable slit (D) and into the monochromator to the collimator mirror (E). As a result the light falling on the collimator mirror is rendered parallel and reflected to the prism (F), where it undergoes refraction. The back surface of the prism is aluminized, so that the light refracted at the first surface is reflected back through the prism, undergoing further refraction as it emerges. The desired wavelength of light is selected by rotating the wavelength selector fixed on top of the monochromator case. This control, in fact, adjusts the position of the

prism. The spectrum from the prism is directed back to the collimating mirror which centres the chosen wavelength of light on the slit and the sample (G). Light passing through the sample strikes the phototube (H), causing a voltage to appear across a load-resistor. The voltage is duly amplified and registered on either the strip-chart recorder or the null-meter.

The **Milton Roy Spectronic$^{(R)}$-20** definitely provides a low-cost and easy to operate instrument, that is still capable of achieving absorbance readings accurate to ± 1 or 2%.

Beckman Instruments, one of the pioneers in Analytical Instruments and dominating this field since 50 years, has come up with their latest **Beckman DU Series 60 Spectrophotometer,** which essentially makes use of *two* different sources of light, namely :

(*a*) H_2 or D_2 Lamp-for measurement in UV-region, and

(*b*) Tungsten Lamp-for measurement in visible region,

thereby permitting measurements from 190-1000 nm. A computer system has also been provided to enable automatic spectrochemical measurements and perform calculations simultaneously.

21.3.2. DOUBLE BEAM SPECTROPHOTOMETER

The quantum leap amalgamated with qualified success in the advancement of Analytical Instruments necessitated for more rapid and precise and accurate measurements in UV and visible spectroscopy. It could be accomplished by the help of the following *two* cardinal modifications, namely :

(*a*) Need for a continuous change in wavelength so that light through the blank and through the sample may be monitored continuously, and

(*b*) Measurements done with a recording spectrophotometer.

The above two modifications have been duly incorporated in a double-beam spectrophotometer. Figure 21.6, depicts the schematic diagram of the optical system involved in a Lambda-2 microcomputer-controlled UV-VIS Spectrophotometer (Perkin-Elmer).

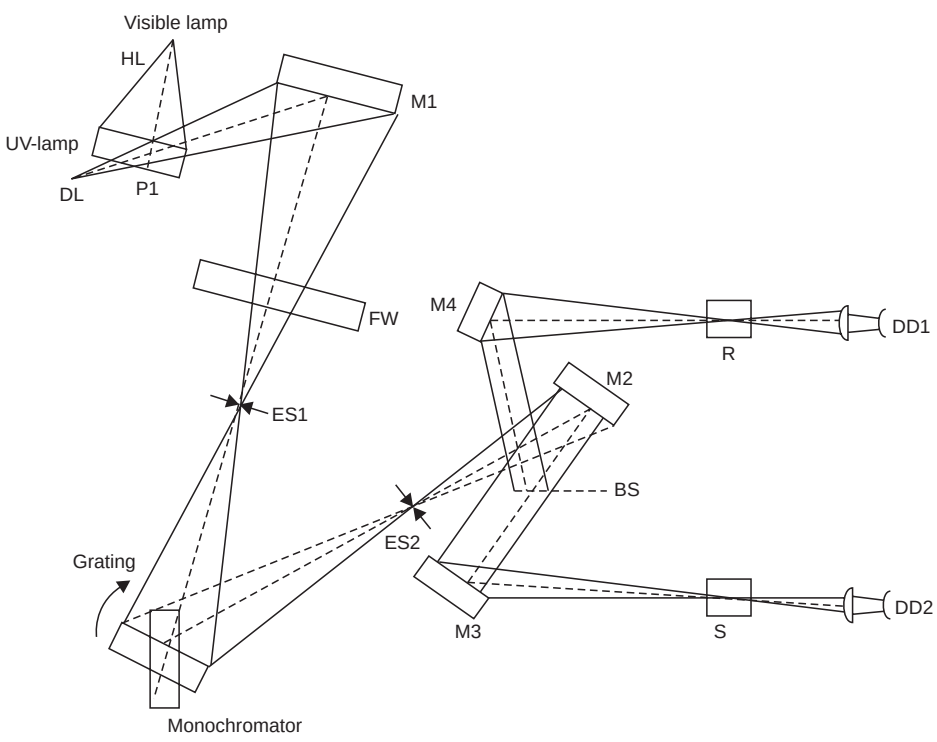


Figure 21.6 : Schematic Diagram of the Optical System in a Double-Beam Spectrophotometer Model : Lambda-2 Micrometer controlled UV-VIS Spectrophotometer (Perkin-Elmer).

The various components of Figure 21.6 are stated below :

VIS-LAMP = Tungstem Lamp.

UV-LAMP = Hydrogen Lamp (HL),

= Deuterium Lamp (DL),

P_1 = Movable source-selection mirror,

M_1, M_2, M_3, M_4 = Mirrors,

FW = Filter wheel,

ES 1 = Entrance slit,

ES 2 = Exit slit,

BS = Beam Splitter,

R = Reference Sample Holder,

S = Sample holder (Test), and

DD 1, DD 2 = Diode detectors.

In fact, the source beam is usually split in two different manners, namely :

(*a*) **Separated in Space :** In this instance, the source beam is split between the sample cell-path and the reference cell-path, and finally detected by two diode detectors. Here, the two detectors should be adequately matched so that no changes occur relative to each other during the measurements,

(*b*) **Separated in Time :** In this case, the source beam is split with the help of an optical chopper which permits the source beam to alternate between the sample cell-path and the reference cell-path. Here, the source should be stable enough so that no changes take place in the radiant energy during the chopping time.

Keeping in view, this specific, rigid and stringent requirement, the separation-in-space method is found to be normally of lower precision and accuracy than the separation-in time-method.

Evidently, the optical choppers are quite expensive, and therefore, the instrument manufacturers very often utilize the separation-in-space method for the routine measurement spectrophotometers.

However, the most sophisticated double-beam spectrophotometer is usually pretty expensive by virtue of the following facts, namely :

(*i*) Greater operating stability,

(*ii*) Rapid speed compared to single-beam instruments,

(*iii*) Complicated optical system involved, and

(*iv*) Recording device for recording absorbance Vs wavelength.

The source beam after passing through the movable source selection mirror (M1), gets reflected and subsequently makes an entry through the filter wheel (FW) and the entrance-slit (ES 1) to the monochromator. The grating is adjusted duly to allow the beam to pass through the exit slit (ES 2) and fall upon the mirror (M 2). At this juncture the beam splitter (B S) splits the reflected beam from mirror (M 2) into two halves : one gets reflected through the mirror (M 4), and passes through the reference sample holder (R) to the diode-detector (DD 1) ; whereas the second one is reflected through the mirror (M 3), passes through the sample holder (S) to the diode detector (DD 2). In fact, Figure 21.6, represents the double-beam operation of a beam separated-in-space.

Double beam spectrophotometers are being manufactured by various well-known manufacturers across the world, such as : SUMADZU ; VARIAN ; CECIL ; BECKMAN ; PERKIN ELMER ; etc., to name a few. These instruments are mostly based on microcomputer-controlled devices with built-in recorder to accomplish faster speed and greater operating stability.

21.4. ASSAY METHODS

21.4.1. METHODOLOGY

In general, when a radiation is made to pass through a layer of a solution containing an absorbing pharmaceutical substance, a portion of the radiation is absorbed by it, whereas the intensity of the radiation emerging from the solution is always found to be less than the intensity of the radiation entering it, Therefore, the quantum of the absorption is designated in terms of the extinction E, that is represented by the following expression :

$$E = \log 10 (I_o/I)$$

where, I_o = Intensity of radiation passing into the absorbing layer, and

I = Intensity of radiation passing out of the absorbing layer.

Extinction is solely dependent upon the following *two* factors, namely :

(*a*) Concentration of the absorbing substance present in the solution, and

(*b*) Thickness of the absorbing layer taken for measurement.

Bearing in mind the ease in calculations and also the convenience of reference, the extinction of a 1-cm layer of a 1% w/v solution is usually recommended in most of the official compendia (*i.e.,* USP ; BP ; EP : IP :) for many pharmaceutical substances and is evaluated by the following expression :

$$E (1\% ; 1\text{-cm}) = E/cl$$

where, c = Concentration of the absorbing substance represented as a percentage (w/v) ; and

l = Thickness of the absorbing layer (cm).

It is however, pertinent to mention here that most pure pharmaceutical substances possess a characteristic value of E (1% ; 1-cm) at a specific wavelength in a given spectroscopic-grade solvent (UVASOL$^{(R)}$-Merck). This particular property is the basis for most assay methods included in pharmacopoeia that are absolutely free from interfering materials, besides being utilized for identifying substances.

In all other instances, the recommended tests specified in pharmacopoeia and prescribed assay methods normally call for comparison against Reference Substances (RS) to ensure measurements under conditions identical for the substance under examination and the reference substance.

In actual practice, where a test or an assay recommends the usage of a Reference Substance, the spectrophotometric measurements are always performed first with the solution prepared from the Reference Substance by the directions provided in the specific monograph and then with the corresponding solution prepared from the substance under examination. Nevertheless, the second measurement must be done immediately after the first, by employing the same cell and the same instrumental parameters.

21.4.2. SPECTROPHOTOMETERS

Any appropriate spectrophotometer capable for measuring both in the ultra-violet (UV) and visible range of the spectrum must essentially consist of an optical system that should produce monochromatic light in the range 190-780 nm and a suitable device for measuring the extinction (E) precisely and accurately.

Besides, the two empty cuvettes (or cells) normally employed for the solution under examination and the reference substance (RS) should have exactly the same spectral features and characteristics. Importantly, when a double bond recording instrument is being employed the solvent cell is always placed in the reference beam.

21.4.3. PREPARATION OF SAMPLE

The pharmaceutical substance under examination is usually dissolved in a spectroscopic grade UVASOL$^{(R)}$ SOLVENT. Particular care must be taken to employ solvents free from contaminants absorbing in the specific spectral region being used. In measuring the extinction of a solution at a given wavelength, the extinction of the solvent cell and its contents must not exceed 0.4 and should be preferably less than 0.2 when measured with reference to air at the same wavelength. Particularly, the solvent in the solvent cell should always be of the same purity, grade and batch as that employed to prepare the respective solution and above all it must be free from fluorescence at the wavelength of measurement.

Ethyl alcohol, methyl alcohol and cyclohexane (UVASOL$^{(R)}$-Grade) employed as solvents shall have an extinction, measured in a 1 cm cell at 240 nm with reference to water (spectroscopic grade), not exceeding 0.10.

21.4.4. MEASUREMENT OF EXTINCTION (E)

(*a*) Unless otherwise prescribed, measure the extinction (E) or the absorbance (A), at the prescribed wavelength using a path-length of 1 cm at 25 ± 1°C (IP) and at 20 ± 1°C (BP). All the measurements are normally performed with reference to the solvent used to prepare the solution being examined, unless otherwise indicated in the individual monograph.

(*b*) In the case of an assay or a limit test where the extinction forms the basis for a quantitative determination, a manually scanning instrument is employed invariably. In tests for identification, a recording instrument is always preferred ; besides, the concentration of the solution and the path-length are specifically monitored. In case, the laid down conditions are not suitable for a particular instrument, the thickness of the solution (*i.e.*, path-length) may be varied without altering the concentration of the solution,

(*c*) Each assay of a pharmaceutical substance by UV-method specifies a wavelength at which maximum absorption takes place which implies the maximum occurring either precisely at or in the vicinity of the given wave length,

(*d*) Pharmaceutical assays (*i.e.,* quantitative determinations) are normally performed at wavelength above 235 nm,

(*e*) In case, the measurements are specifically to be carried out at a wavelength between the range 190-210 nm, the following extra and special precautions must be adhered to rigidly, namely :

(*i*) Purging the cell compartments with N_2,

(*ii*) Making use of only spectroscopic grade solvents *e.g.,* UVASOL$^{(R)}$ (Merck), and

(*iii*) Making use of cells that are absolutely transparent in the region 190-210 nm.

(*f*) The requirements for light absorption in the *official compendia* invariably apply to the dried, anhydrous, or solvent free material in all such monographs in which standards for loss on drying, water or solvent content are provided.

21.4.5. EXAMPLES

A few typical examples for the assay of pharmaceutical substances by UV-spectrophotometric method are described below :

A. Amoxycillin Trihydrate

Materials Required : Amoxycillin trihydrate : 0.17 *g* ; 100-ml volumetric flask ; 2 ; buffer solution pH 9.0 (Solution : I : Boric acid and Potassium Chloride (0.2 M)-Dissolve 12.366 *g* of Boric acid and 14.911 g of KCl in DW and dilute with water to 1000 ml ; Solution : II NaOH (0.2 N) : Dissolve 8.0 g of NaOH in CO_2-free DW to produce 1000 ml ; Now, transfer 50 ml of solution I into a 200-ml volumetric flask

and add to it 20.8 ml of solution II, then add sufficient DW to make up the volume to 200 ml) : 10 ml ; acetic anhydride-dioxan solution (add 1 ml of acetic anhydride to 50-ml of dioxan) : 1.0 ml ; imidazole-mercury reagent (dissolve 8.25 g of recrystallized imidazole in 60 ml of DW and add 10 ml of 5N HCl. Stir the solution magnetically and, add dropwise, 10 ml of a 0.27% w/v solution of Hg_2Cl_2. Adjust the pH to 6.8 ± 0.05 with 5 N HCl (about 4.0 ml is needed) and add sufficient DW to produce 100 ml) : 10.0 ml ;

Procedure : Weigh accurately about 0.17 g of amoxycillin trihydrate and dissolve in sufficient DW to produce 500 ml. Now, transfer 10 ml of this solution into a 100 ml volumetric flask, add 10 ml of buffer solution pH 9.0 followed by 1 ml of acetic anhydride-dioxan solution, allow to stand for 5 minutes, and add sufficient water to produce 100 ml. Pipette 2 ml of the resulting solution into each of the two stoppered tubes. To tube 1 add 10 ml of imidazole-mercury reagent, mix, stopper the tube and immerse it in a water-bath previously maintained at 60 °C for exactly 25 minutes, with occasional swirling. Remove the tube from the water-bath and cool rapidly to 20 °C (Solution-1). To tube 2 add 10 ml of DW and mix thoroughly (Solution-2). Immediately, measure the extinctions of Solutions 1 and 2 at the maximum at about 325 nm, as detailed above, employing as the blank a mixture of 2 ml of DW and 10 ml of imidazole-mercury reagent for Solution-1 and simply DW for Solution-2.

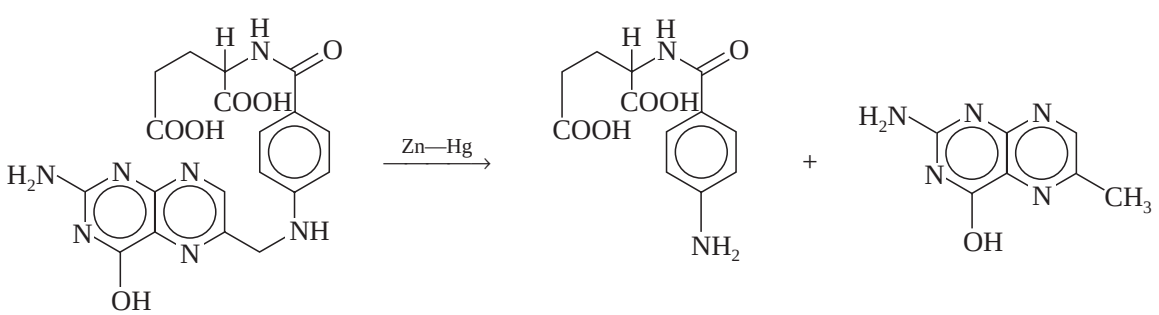
Calculations : The content of $C_{16}H_{19}N_3O_5S$ may be calculated from the difference between the extinctions of Solution-1 and that of Solution-2 and from the difference obtained by repeating the operation using 0.17 *g* of amoxycillin trihydrate (RS), instead of the sample being examined and the declared content of $C_{16}H_{19}N_3O_5S$ in the amoxycillin trihydrate (RS).

Cognate Assays : Ampicillin can also be assayed by employing the above method using 0.15 g of the sample.

B. Folic Acid

Theory : Folic acid (I) undergoes cleavage by reduction with Zn-Hg in acidic medium to yield *p*-aminobenzoylglutamic acid (II). The primary aromatic amino group present in the latter is subsequently diazotized in the usual manner and coupled in acidic solution with N-(1-naphthyl)-ethylenediamine hydrochloride in the absence of light (caution). The colour thus produced has a maximum absorption at 550 nm and the extinction (E) is consequently compared with a calibration curve obtained from *p*-aminobenzoic acid (PABA) that has been duly diazotized and coupled exactly in the same fashion as the *p*-aminobenzoylglutamic acid.

The reaction involved is expressed by the following equation :



(I) (II)

Note : In order to ensure that the extinctions recorded exclusively refer to folic acid (I), and also that they do not necessarily include a contribution from a free-primary-amino-aromatic-moiety obtained from a decomposition product, a blank estimation is always performed with the unreduced solution and an appropriate correction is applied. The colour thus corresponds to a definite quantity of $C_{16}H_{19}O_6N_7$. Thus, we have :

$$C_7H_7O_2N = C_{19}H_{19}O_6N_7$$

or 137 g of $C_7H_7O_2N$ = 447 g of $C_{19}H_{19}O_6N_7$

or 1 g of $C_7H_7O_2N$ = 3.22 g of $C_{19}H_{19}O_6N_7$

Materials Required : Folic acid : 0.05 g : 0.1 N NaOH : 100 ml ; 2 N HCl : 30 ml ; Zn-powder : 0.5 g ; sodium nitrite solution (0.1% w/v in DW) : 5 ml ; ammonium sulphamate (0.5% w/v in DW : 5 ml ; N-(1-naphthyl) ethylene-diamine hydrochloride solution (0.1 % w/v in DW) : 5 ml ;

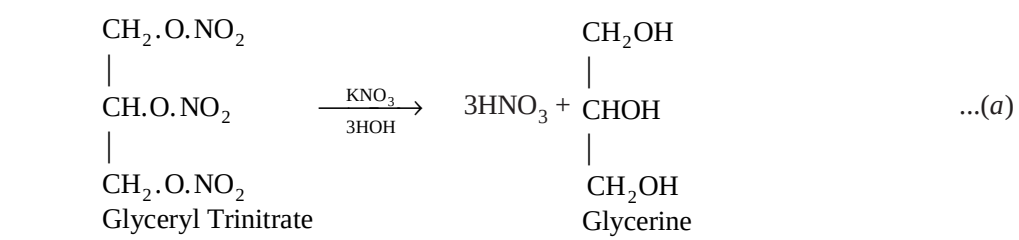
Procedure : Accurately weigh about 0.5 g, dissolve in 50 ml of 0.1 N NaOH and add sufficient 0.1 N NaOH to produce 100 ml (Solution-1). To 3 ml add 20 ml of 2 N HCl and dilute to 100 ml with DW. To 50 ml of this solution, add 0.5 g of zinc powder, allow to stand in a dark place for 20 minutes with intermittent shaking and filter, Dilute 10 ml of the filtrate to 25 ml with DW, add 5 ml of 2N HCl and 5 ml of a 0.1% solution of sodium nitrite, mix and allow to stand for 2 minutes. Add 5 ml of a 0.5% w/v solution of ammonium sulphamate, mix and allow to stand for 2 minutes. Now, add carefully 5 ml of a 0.1% solution of N-(1-naphthyl) ethylene diamine hydrochloride, mix thoroughly and allow to stand for 10 minutes. Add sufficient DW to produce 50 ml and measure the extinction of the resulting solution at about 550 nm, as discussed earlier, using as blank a solution prepared exactly in a similar manner but employing 25 ml of DW and beginning the procedure at ''add 5 ml of 2 N HCl...''

To a further portion of 30 ml of solution-1, add 20 ml of 2N HCl and sufficient DW to produce 100 ml. Mix 10 ml of this solution with 15 ml of DW and repeat the operations stated above beginning the procedure at ''add 5 ml of 2 N HCl ...''

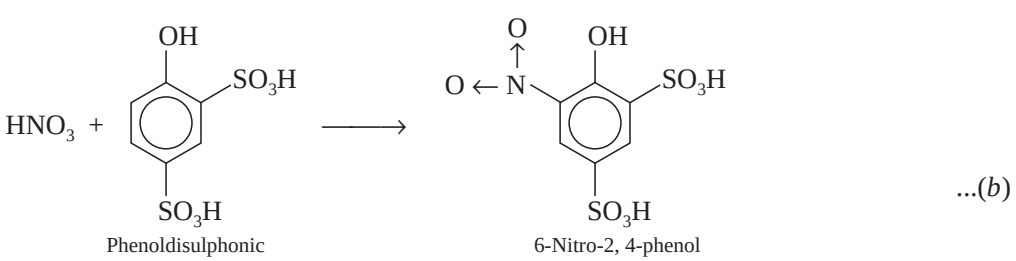
Finally, substract 1/10th of the extinction of the unreduced solution from that of the reduced solution and from the result thus obtained calculate the amount of $C_{19}H_{19}O_6N_7$, using the result obtained by repeating the operation using folic acid (RS) instead of the substance being examined and the declared content of $C_{19}H_{19}O_6N_7$ in folic acid (RS).

C. Glyceryl Trinitrate Tablets

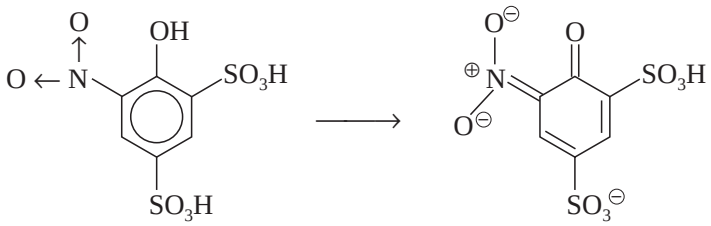
Theory : First and foremost the active ingredient *i.e.,* glyceryl trinitrate is extracted completely from the tables by shaking with glacial acetic acid. To an aliquot of the resulting acetic acid solution an excess of phenoldisulphonic acid is added to produce a yellow colour which is subsequently intensified by adding an excess of ammonia. The following reactions take place :



...(*a*)



...(*b*)



...(*c*)

The standard substance in this assay is KNO_3, which conforms to the nitric acid released by acidolysis in the test solution.

Materials Required : Glyceryl trinitrate tablets : 20 ; glacial acetic acid (90% v/v) : 5 ml ; phenoldisulphonic acid solution (heat 3 g of phenol with 20 ml of sulphuric acid on a water-bath for 6 hours, and transfer the resulting liquid to a stoppered vessel) : 2 ml ; strong ammonia solution ; 20 ml ; potassium nitrate (previously dried at 105 °C) : 1 g ;

Procedure : Weigh and powder 20 tablets. Now, weigh accurately a quantity of the powder equivalent to 0.5 mg of glyceryl trinitrate, add 5 ml of glacial acetic acid, shake thoroughly for 1 hour and then centrifuge. To 2 ml of the supernatant liquid add 2 ml of phenoldisulphonic acid solution and allow to stand for 15 minutes. Add 8 ml of DW, make alkaline with strong ammonia solution, cool to about 20 °C, dilute to 20 ml with DW and filter. Finally, measure the extinction of a 1-cm layer of the filtrate at 405 nm, as described earlier, employing as blank 2 ml of glacial acetic acid, treated exactly in a similar fashion, beginning at ''add 2 ml of phenoldisulphonic acid solution .....''.

Dissolve 133.5 mg of potassium nitrate, in sufficient DW to produce 100 ml ; to 10 ml add sufficient glacial acetic acid to produce 100 ml. Taking 2 ml of this solution, just repeat the assay beginning the procedure at ''add 2 ml of phenoldisulphonic acid solution.....''.

Calculations : We have : $KNO_3 \equiv C_3H_5N_3O_9$

or $101 \text{ g } KNO_3 \equiv 227 \text{ g of } C_3H_5N_3O_9$

or $101 \text{ g of } KNO_3 \equiv 227/3 \text{ g } C_3H_5N_3O_9$*

or $\equiv 75.66 \text{ g of } C_3H_5N_3O_9$

or $1 \text{ ml } (\equiv 1.335 \text{ mg}) \text{ of } KNO_3 \equiv 0.1 \text{ mg of } C_3H_5N_3O_9$

The content of $C_3H_5N_3O_9$ may be calculated from the values of the extinctions thus obtained. Each ml of the potassium nitrate solution is equivalent to 0.1 mg of $C_3H_5N_3O_9$.

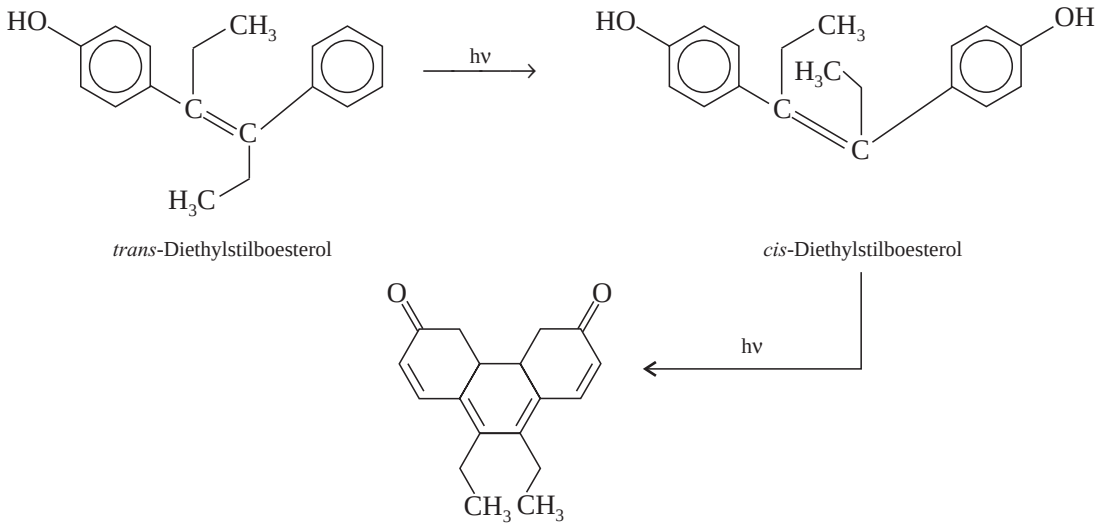
Cognate Assays : The following two pharmaceutical products, namely : Pentaerythritol tetranitrate Tablets and Diluted Isosorbide dinitrate are assayed by using a solution of phenoldisulphonic acid as detailed below :

S.No.	Name of Substance	Qty. Prescribed	Extinction (∈)	Calculation
1.	Pentaerythyritol tetranitrate Tablets	50 mg	405 nm	Each ml of KNO_3 soln. ≡ 0.5 mg of $C_5H_8N_4O_{12}$
2.	Diluted Isosorbide dinitrate	25 mg	405 nm	Each ml of KNO_3 soln. ≡ 0.934 mg of $C_6H_8N_2O_8$

D. Stilboesterol

Theory : The assay of stilbonesterol is exclusively based upon photochemical reactions whereby the *trans*-isomer firstly gets converted into its corresponding *cis*-isomer (*Geometrical Isomerism*) and then followed by intramolecular rearrangement therby causing ring closure as expressed in the (see next page) equations :

* Because glycerine has thre replaceable-OH groups.



trans-Diethylstilboesterol

cis-Diethylstilboesterol

A Diketo-system
(Intramolecular Rearrangement)

The highly conjugated diketo system obtained as a result of irradiation of the stilbosterol solution placed in a closed spectrophotometer cell for a duration of 10 minutes and exposed to a 15-watt short-wave ultraviolet lamp. Ultimately the extinction is duly measured at 418 nm and compared with stilboesterol (RS) treated exactly in the same manner.

Materials Required : Stilbosterol : 20 mg ; ethyl alcohol (absolute) : 250 ml ; dipotassium hydrogen phosphate solution (dissolve 1 *g* in 55 ml of DW) : 25 ml ;

Procedure : Weigh accurately about 20 mg of stilbosterol in sufficient ethyl alcohol to produce 100 ml ; and dilute 10 ml of this solution to 100 ml with ethyl alcohol. To 25 ml of the resulting solution add 25 ml of dispotassium hydrogen phosphate solution, transfer a portion of the mixture to a 1-cm closed quartz cell, place the cell 10 cm from a 15 watt short-wave UV-lamp, and subject it to irradiation for 10 minutes. Now, measure the extinction of the irradiated solution at the maximum at about 418 nm as described earlier.

Calculations : Calculate the content of $C_{18}H_{20}O_2$ from the extinction obtained by repeating the operation with stilbosterol (RS).

21.4.6. UV-ABSORPTION CHARACTERISTICS OF SOME OFFICIAL PHARMACEUTICAL SUBSTANCES

The ultra-violet absorption characteristics of a number of official pharmaceutical substances have been duly provided in Table 21.2.

Table 21.2 : UV-Absorption Characteristics of Pharmaceutical Substances

S.No.	Name of substance	Qty. Prescribed	Solvent Used	(nm)	E (1% ; 1-cm ;)
1.	Amodiaquine hydrochloride	0.3 g	0.1 N HCl	343	—
2.	Ampicillin	0.15 g	Water	325	—
3.	Betamethasone sodium phosphate	0.2 g	-do-	241	391 (as Betamethasone)
4.	Carbamazepine	0.1 g	Alcohol (95%)	285	490
5.	Carbimazole	50 mg	Water/0.1 N HCl	291	557
6.	Chloramphenicol	0.125 g	Water	278	288
7.	Chloramphenicol Palmitate	60 mg	Ethyl Alcohol	271	178
8.	Cyanocobalamine	25 mg	Water	361	207

9.	Deslanoside	30 mg	Methanol	490	—
10.	Dexamethasone Sodium Phosphate	0.2 g	Water	241	297
11.	Digitoxin	40 mg	Ethanol	495	—
12.	Dithranol	20 mg	Glacial Acetic Acid	450	550
13.	Ergotamine Tartrate	10 mg	Tartaric Acid (1% w/v)	578	—
14.	Ethinylestradiol	150 mcg	Methanol	538	—
15.	Ethipropazine HCl Tablets	50 mg	Ethanol (95%)	252	845
16.	Griseofulvin	80 mg	Ethanol	291	686
17.	Imipramine HCl Tablets	75 mg	0.1 N HCl	250	264
18.	Indomethacin Capsules	50 mg	Methanol	318	193
19.	Isoprenaline HCl Injection	5 mg	Water	540	—
20.	Isoprenaline Tablets	0.1 g	Water	540	—
21.	Isoxsuprine HCl	50 mg	Water	269	71.5
22.	Lanatoside-C	0.03 g	Methanol	490	—
23.	Megestrol Acetate	10 mg	Ethanol	287	630
24.	Methandienone	50 mg	-do-	245	516
25.	Methadilazine HCl	0.1 g	Water	275	—
26.	Methylergometrine Maleate	20 mg	Water	550	—
27.	Nalidixic Acid Tablets	0.1 g	N . NaOH	258	1120
28.	Nalorphine HCl	25 mg	Water	285	—
29.	Nandrolone Decanoate	10 mg	Ethanol	239	407
30.	Nandrolone Phenylpropionate	10 mg	Ethanol	240	430
31.	Nicoumalone Tablet	1 mg	Methanol	306	521
32.	Nitrofurantoin	0.12 g	DMF/Acetate Buffer	367	765
33.	Nitrofurazone	0.1 *g*	DMF/Water	375	822
34.	Oestradiol Benzoate	10 mg	Ethanol	231	490
35.	Oestradiol Dipropionate	40 mg	Methanol	520	—
36.	Oxprenolol Tablets	20 mg	Water/Methylene Chloride	273	74.5
37.	Oxyphenonium Bromide Tablets	20 mg	Water	620	—
38.	Phenylephrine HCl Injection	50 mg	NH_2SO_4	273	95
39.	Psoralen	0.1 g	Methanol	247	—
40.	Riboflavine Phosphate Sodium	0.1 g	Water/Acetate Buffer	444	323
41.	Rifampicin	0.1 g	Methanol	475	187
42.	Sodium Cromoglycate	0.1 g	Buffer Soln. (pH 7.4)	326	164
43.	Spironolactone	10 mg	Methanol	238	470
44.	Stilboesterol Diphosphate	0.1 g	Ethanol/Water	241	—
45.	Testosterone Propionate	10 mg	Ethanol	241	490
46.	Triamcinolone Acetonide	25 mg	-do-	240	354
47.	Tubocurarine Chloride	25 mg	Water	280	105

THEORETICAL AND PRACTICAL EXERCISES

1. Give a brief and comprehensive account of the following terminologies :

(*a*) Electromagnetic spectrum, (*b*) Molar absorptivity,

(*c*) Absorption spectra, (*d*) Structural features, and

(*e*) Absorption bands.

2. (*a*) What are the **four** motion-related energies exhibited by a '**drug molecule**' ? Explain.

(*b*) Enumerate the **three** distinct situations that may arise in the transformation of an '**excited electron**' to its '**ground state**'. Give suitable examples, wherever necessary, to support your answer.

3. (*a*) Discuss the various salient features of '**potential energy**' of a *diatomic molecule* diagrammatically.

(*b*) What are the various factors that essentially influence the absorption of radiant energy ? Explain with typical examples.

4. (*a*) Why a '**double-beam**' **spectrophotometer** gives more precise, reliable and reproducible results in comparison to a '**single-beam**' **spectrophotometer** ? Explain.

(*b*) Describe an UV-Spectrophotometer with a neat-labelled block diagram and explain its operational mode.

5. Discuss the theory, procedure and calculations for the assay of the following medicinal compounds :

(*i*) Folic acid,

(*ii*) Glyceryl trinitrate tablets, and

(*iii*) *Trans*-Diethylstilbesterol.

6. UV-Spectrophotometric method employed for the assay of the following '**drug substances**' :

(*i*) Ampicillin, (*ii*) Ergotamine tartrate,

(*iii*) Nalorphine hydrochloride, (*iv*) Nitrofurazone,

(*v*) Rifampicin, and (*vi*) Spironolactone.

RECOMMENDED READINGS

1. Jaffe, H.H. and M. Orchin., '**Theory and Applications of Ultraviolet Spectroscopy',** New York, Wiley, 1962.

2. IUPACC Commission on Spectrochemical and other Optical Procedures for Analysis **Spectrophotometric Data for Colorimetric Analysis,** London, Butterworth Publishers Ltd., 1963.

3. West, W., (Ed.), '**Techniques of Organic Chemistry',** Vol. IX., **In Chemical Applications of Spectroscopy,** 2nd ed., New York, Interscience, 1968.

4. Rao, C.N.R., '**Ultraviolet and Visible Spectroscopy',** London, Butterworths, 1975.

5. Knowles, A. and C. Burgess, Eds., '**Practical Absorption Spectrometry',** New York, Chapman and Hall, 1984.

6. Alremore, I.R., '**Evaluation of Instrumentation for UV-Visible Spectrophotometry',** *J. Chem. Educ.*, **63 :** A 216, 1986.

7. Day, R.A. Jr. and A.L. Underwood., '**Quantitative analysis',** 6th ed,. New Delhi, Prentice-Hall of India Pvt. Ltd., 1993.

22 INFRARED SPECTROPHOTOMETRY

CONTAINS :

22.1. INTRODUCTION

The **infrared spectrum** provides the largest number of characteristic properties of a compound. It also serves as a powerful '*analytical tool*' for the extensive and intensive study of molecular structure.

In fact, **infrared absorption spectra** are due to changes in vibrational energy accompanied by changes in rotational energy. Broadly speaking, the range in the electromagnetic spectrum that extends from 0.8 to 200 μ is referred to as the infrared region. In usual practice, however, either the wavelength (μ) or the wave number (cm^{-1}) is employed to measure the position of a given infrared absorption. More precisely, the infrared regions may be categorized into three distinct zones based on their respective wave numbers and wavelengths as stated below :

S. No.	Region	Wave Number (cm^{-1})	Wavelength (μ)
1.	Ordinary Infrared	4000-667	2.5-15
2.	Near Infrared	12,500-4,000	0.8-2.5
3.	Far Infrared	667-50	15-200

Besides, the infrared region is found to be normally rich in peaks by virtue of the fact that there exist a number of vibrational modes (3*n*-6, where, *n* = number of atoms for any nonlinear molecule).

Another school of thought advocates that there are two general regions in the infrared spectrum, namely : (*a*) **Group frequency region :** having a wavelength ranging from 2.5 to 8.0 μ and a wave number from 4000-1300 cm^{-1} ; (*b*) **Fingerprint region :** having a wavelength ranging from 8.0-2.5 μ and a wave number from 1300-400 cm^{-1}.

22.1.1. GROUP FREQUENCY REGION

Here, the stretching and bending vibrational bands associated with specific structural or functional groups are observed frequently.

Example : The C = O stretching frequency is about 1700 cm^{-1} ; whereas the C—H stretching frequency is about 3000 cm^{-1} and both of them are almost independent of the rest of the molecule as depicted in Table 22.1.

Table 22.1 : Stretching Frequencies found in Group Frequency Region

C—H Stretch			C = O Stretch		
S. No.	**Molecule**	**Frequency (cm^{-1})**	**S. No.**	**Molecule**	**Frequency (cm^{-1})**
1.	$CHCl_3$	3019	1.	CH_3COCH_3	1715
2.	$C_2H_2Cl_2$	3089	2.	CH_3CHO	1729
3.	$CH_2 = CH_2$	3105, 2990	3.	$H_5C_2COC_2H_5$	1720
4.	C_6H_6	3099	4.	HCOOH	1729
5.	CH_3OH	2977	5.	CH_3COOH	1718
6.	$CH \equiv CH$	3287	6.	CF_3COOH	1776

22.1.2. FINGERPRINT REGION

Here, the vibrational modes depend solely and strongly on the rest of the molecule.

Example : The C—C stretching frequency depends largely on what else is bonded to the carbon atoms.

It is interesting to observe here that this particular region of the spectrum is densely populated with bands. As we know that no two **'fingerprints'** could be identical in human beings, exactly in a similar manner no two compounds may have the same 'fingerprint region'. Thus, each and every molecule essentially gives rise to a unique spectrum which offers a characteristic feature of the same.

22.2. THEORY

The underlying principle of infrared spectroscopy is based upon the molecular vibrations which is further composed of the stretching and the bending vibrations of a molecule.

Therefore, it would be necessary to have a clear concept of various modes of vibrations often encountered in different molecules having a variety of functional moieties, laws governing them and the mathematical derivations related to them.

22.2.1. MOLECULAR VIBRATIONS

A molecule may not be looked upon as a rigid assemblage of atoms. Rather it may be regarded as a sort of flexible system comprising of balls of varying masses representing the atoms of a molecule and springs of varying strengths representing the chemical bonds of a molecule.

The vibrations for molecules are of *two* types, namely :

(*a*) Stretching, and

(*b*) Bending (or deformation).

22.2.1.1. Stretching

Vibration causes stretching whereby the distance between the two atoms increases or decreases, but the atoms remain in the same bond axis.

22.2.1.2. Bending (or Deformation)

Vibration causes bending whereby the position of the atom changes relative to the original bond axis.

Therefore, the various stretching and bending vibrations of a bond usually take place at particular quantized frequencies. Thus, in a situation where upon the infrared light having the same frequency is incident on the molecule, energy is absorbed, and the net effect could be observed by an increase in the amplitude of that vibration. In another situation, whereby the molecule reverts from the excited state to the ground state, the absorbed energy is released in the form of heat.

The various stretching and bending vibrations that can exist within a molecule may be represented schematically as shown below in Figure 22.1 :

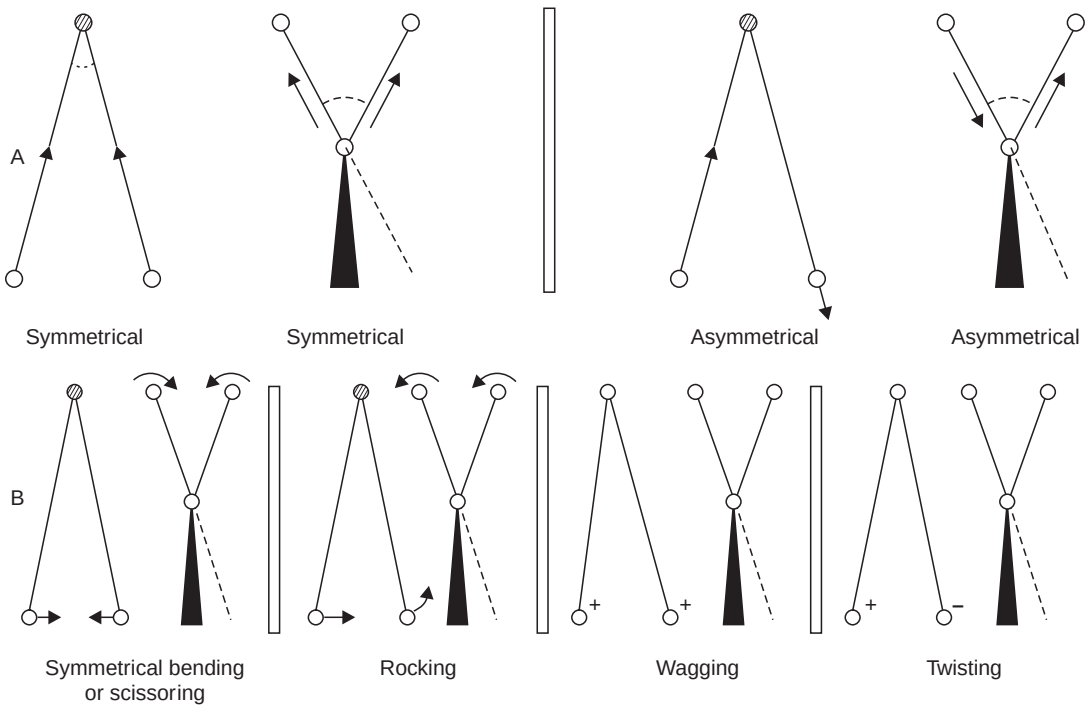


Figure 22.1 : Stretching Modes (A) and Deformation Modes (B)

There are *two* types of Bending (or deformation) Modes, namely :

(*i*) Below the plane of paper and perpendicular to it designated by (+) sign, and

(*ii*) Above the plane of paper and perpendicular to it represented by (–) sign.

22.2.1.3. Stretching Vibrations

In this particular instance, the atoms move invariably along the bond that joins them *e.g.*, C—H ; C = O ; O—H ; N—H.

The stretching vibrations may be further sub-divided into *two* categories, namely :

(*a*) **Symmetrical Stretching :** In this case, the two hydrogen atoms either move towards or away from the central carbon atom in unison, thereby either altering the interatomic distance or causing no change in valence angle (Figure 22.1).

(*b*) **Asymmetrical Stretching :** In this instance, one hydrogen atom approaches the carbon atom while the other moves away from the carbon atom (Figure 22.1).

22.2.1.4. Bending (or Deformation) Vibrations

In the event when a three-atom system forms part of a larger molecule, it is quite possible to have bending (or deformation) vibrations which essentially involve oscillation of the atoms, or group as a whole and is perpendicular to its chemical bond (Figure 22.1).

Such bending vibrations can take place either in-plane or out-of-plane.

22.2.1.4.1. *In-Plane Bending Vibrations*

These are *two* types :

(*a*) **Scissoring or Symmetrical Bending :** In this case, the two atoms connected to a central atom either move toward or away from each other with certain deformation of the valence angle.

(*b*) **Rocking :** In this case, the structural unit swings back and forth in the plane of the molecule.

22.2.1.4.2. *Out-of Plane Bending Vibrations*

These are also of *two* kinds, namely :

(*a*) **Wagging :** In this case the structural unit swings back and forth out of the plane of the molecule.

(*b*) **Twisting :** In this case the structural unit rotates about the bond that joins it to the rest of the molecule.

22.2.1.4.3. *Explanations of Bending and Stretching Vibrations*

The bending (or deformation) vibrations generally require less energy and take place at longer-wavelength than the corresponding stretching vibrations.

In contrast, the stretching vibrations are observed to occur with respect to their corresponding bond-strengths.

Examples : The typical examples of triple-bond, double-bond and single-bond are given below :

S. No.	Type of Bond	Examples	Force Constants (dynes/cm)	Absorption At	
				Wavelength (μ)	Frequency (cm^{-1})
1.	Triple Bond	C ≡ C ; C ≡ N ;	15×10^5	4.4-5.0	2300-2000
2.	Double Bond	C = O ; C = C ; C = NH ;	10×10^5	5.3-6.7	1900-1500
3.	Single Bond	C—C ; C—OH ; C—N ;	5×10^5	7.7-12.5	1300-800

Whenever a very small proton like : C—H ; O—H ; or N—H is involved in a single bond, the stretching vibrations normally take place at much higher frequency *i.e.*, 3700-2630 cm^{-1} (or 2.7-3.8 μ). It is, however, interesting to note that O—H bond absorbs at 2.8 μ (or 3570 cm^{-1}), whereas O—D bond absorbs at 3.8 μ (or 2630 cm^{-1}). In this specific case, the strengths of the two bonds are more or less the same, but the mass of one atom is almost doubled.

22.2.1.4.4. *Calculation of Vibrational Frequencies*

The vibrational frequency may be calculated with fairly remarkable accuracy by the help of Hooke's Law and is expressed as :

$$\nu = \frac{1}{2\pi}\left(\frac{k}{m_1 m_2/(m_1 + m_2)}\right)^{\frac{1}{2}} \qquad ...(a)$$

where, ν = Frequency,

k = Force constant of the bond, and

m_1 and m_2 = Masses of two atoms.

The quantity $m_1m_2/(m_1 + m_2)$ is often expressed as μ, the reduced mass of the system.

Example : Calculate the approximate frequency of the C—H stretching vibration from the following data :

$k = 500\ \text{Nm}^{-1} = 5.0 \times 10^5\ \text{gs}^{-2}$ (since 1 newton = 10^3 gm s^{-2}) ;

m_c = mass of the carbon atom = 20×10^{-24} g ;

m_H = mass of the hydrogen atom = 1.6×10^{-24} g ;

Solution : By putting the values of k, m_1 and m_2 in Eq. (*a*) we have :

$$\nu = \frac{7}{2 \times 22}\left(\frac{5.0 \times 10^5\,\text{gs}^{-2}}{(20 \times 10^{-24}\ \text{g})(1.6 \times 10^{-24}\ \text{g})(20 + 1.6)10^{-24}\ \text{g}}\right)^{\frac{1}{2}}$$

or $\nu = 9.3 \times 10^{13}\ \text{s}^{-1}$

Using the relationship between frequency and wave number, we have :

$$\bar{\nu} = \nu/c$$

where, $\bar{\nu}$ = Wave number,

ν = Frequency, and

c = Velocity of light ($\equiv 2.998 \times 10^8\ \text{ms}^{-1}$)

Therefore, we have :

$$\bar{\nu} = \frac{\nu}{c} = \frac{9.3 \times 10^{13}\ \text{s}^{-1}}{3.0 \times 10^8\ \text{ms}^{-1}}$$

or $= 3.1 \times 10^5\ \text{cm}^{-1}$

or $= 3100\ \text{cm}^{-1}$

However, it is pertinent to restate the underlying principles embodied in these calculations, that is **"the vibrational frequency of a bond is expected to increase when the bond strength increases, and also when the reduced mass of the system decreases".**

22.2.1.5. Salient Features of IR-Spectroscopy

There are, in fact, *three* vital points that may be noted with regard to IR-spectroscopy, namely :

(*a*) Most intense peaks in the IR-spectrum are solely due to absorption peaks caused by the stretching vibrations,

(*b*) Molar extinction coefficient in IR-spectroscopy varies from 0-2000 cm^{-1}, and

(*c*) Molar extinction coefficient is directly proportional to the square of the change in the dipole moment* of the molecule that the particular vibration affords.

22.2.2. FACTORS INFLUENCING VIBRATIONAL FREQUENCIES

There are a number of factors that influence the precise frequency of a molecular vibration, namely :

(*a*) Vibrational coupling,

(*b*) Hydrogen bonding,

---

* Dipole Moment = Electric charge × Distance between the charges

or $\mu = e \times d = 10^{-10}$ e.s.u. $\times\ 10^{-8}$ cm = 10^{-18} esu cm (or D)

The unit of Dipole Moment is DEBYE (D) after the name of the researcher.

(*c*) Electronic Effects, and

(*d*) Field Effects.

All the above factors shall be discussed briefly with appropriate examples and explanations, wherever necessary, below :

22.2.2.1. Vibrational Coupling

The following four vibrations may be observed in the high-resolution spectra of compounds containing both —CH_2 and —CH_3 groups.

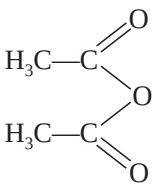
C—H : One stretching frequency,

H—C(H) : Two coupled vibrations having different frequencies *i.e.*, ν_{anti} and ν_{symn} (for asymmetric and symmetric), and

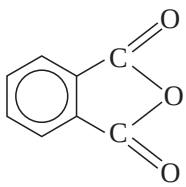
H—C(H)(H) : Equivalent coupled vibration.

Examples : The *three* typical examples are described below, namely :

(*a*) **Carboxylic Acid Anhydrides :** For instance :



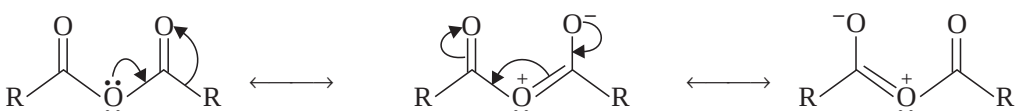
Acetic anhydride
(Aliphatic anhydride)



Phthalic anhydride
(Aromatic anhydride)

Following are the salient features :

- It affords two C = O stretching absorptions *viz.*, ν_{anti} and ν_{symm} between frequency 1800-1900 cm^{-1} having a separation of about 65 cm^{-1} ;
- The coupling solely occurs between the two carbonyl groups, that are indirectly linked through —O— ;
- **Resonance :** The interaction is probably encouraged due to the single as well as double-bond character prevailing in the carbonyl-oxygen bonds brought about by resonance as shown below :



Anhydrides : Various resonance forms

(*b*) **Amides (—C(=O)—NH_2) :** The functional moiety 'amide' shows two distinct bands around 1600-1700 cm^{-1} caused due to C = O str. and N—H def. These bands are also known as Amide-I and Amide-II bands.

Amide-I : is formed due to C = O str. and amounts to a level as high as 80% ;

Amide-II : is a strongly coupled interaction between N—H def. and C—N str. and accounts for the remaining 20%.

(*c*) **Aldehydes (—C(=O)—H) :** The functional group 'aldehyde' offers C—H str. absorption band which appears as a doublet because of interaction between the two components, namely : C—H str. fundamental and C—H def. overtone.

22.2.2.2. Hydrogen Bonding

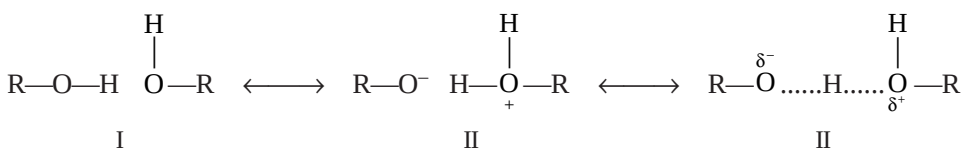
In general, the hydrogen bonding present in O—H and N—H compounds give rise to a number of effects in the IR-spectra. However, the carbonyl groups or aromatic rings present in the same molecule as the O—H or N—H group may cause similar shifts by intramolecular action.

A few typical examples involving hydrogen bonding in a wide-range of organic compounds are discussed below :

(*a*) **Alcohols and Phenols :** Interestingly, alcohols and phenols, in condensed phases (KBr-Disc/ liquid film), are strongly hydrogen-bonded thereby forming dimers, trimers and tetramers that ultimately leads to a wide envelope of absorptions thus causing broadening of the absorption band.

In diluted solution and in an inert solvent (*e.g.*, 1-butanol 1% in CCl_4) the proportion of free molecules enhances that gives rise to the 3650 cm^{-1} band.

Another school of thought suggests that the hydrogen-bond may be regarded as a resonance hybrid I and II (approximating overall to III)-thereby the original O—H bond undergoes a lengthening as depicted below :

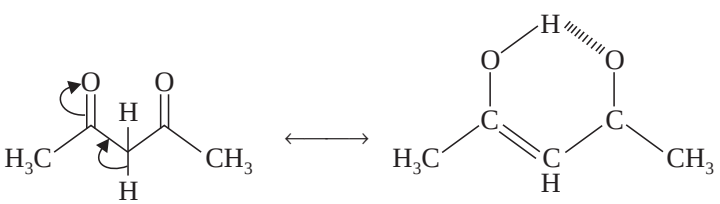


I II II

(*b*) **Enols and Chelates :** The hydrogen-bonding existing in enols and chelates is found to be specifically strong and the observed O—H str. frequencies may be very low (2800 cm^{-1}). In this particular instance, the dilution by an inert solvent (*e.g.*, 1-butanol 1% in CCl_4) cannot even break these bonds, therefore, free O—H str. may not be observed at low concentrations.

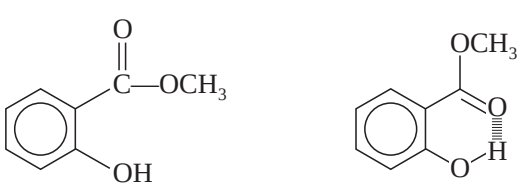
The above observations may be explained by citing the example of acetyl dimethyl ketone (for enols) and methyl salicylate (for chelates) as shown below :

Enols :



Acetyl dimethyl ketone (*keto*-form) Acetyl dimethyl ketone (*enol*-form)

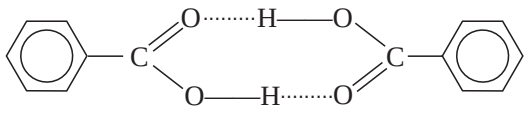
Chelates :



Methyl salicylate Methyl salicylate (Chelate)

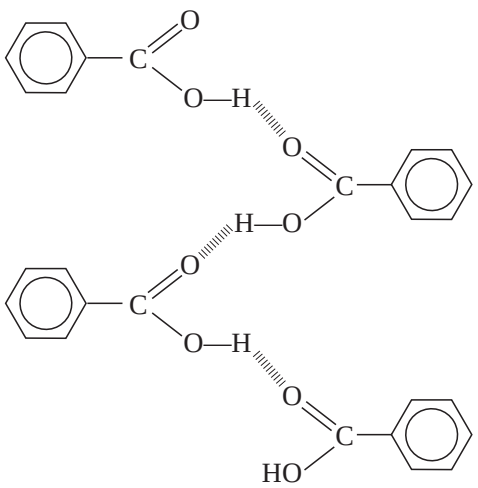
(*c*) **Carboxylic Acids :** Let us consider the example of benzoic acid IR-spectrum in KBr-disc wherein we may observe a relatively broad band caused due to hydrogen-bonded O—H str. between 2500-3500 cm^{-1}. These broad bands are formed due to the dimeric and polymeric associations of benzoic acid as shown below :

Dimeric Association :



A dimer of benzoic acid

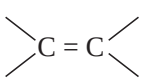
Polymeric Association :



A polymer of benzoic acid

(*d*) **π-Cloud Interactions :** Since alkene and aromatic π-bonds behave as Lewis bases, they may form hydrogen bonds to acidic hydrogens *e.g.*,

π-Bonds :



Ethylene

Benzene

Example : The frequency of O—H str. in phenols is lowered by 40-100 cm^{-1} when the IR-spectrum is recorded in a benzene solution as compared to a carbon tetrachloride solution.

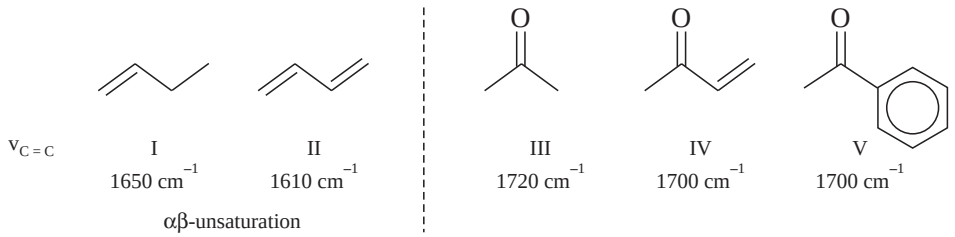
(*e*) **Amines :** Amines usually show two distinct bands due to N—H stretching in different environments. In condensed-phase spectra, amines show **bonded N—H** str. around 3300 cm^{-1} and in dilute solution a new band near 3600 cm^{-1} corresponding to free N—H str. It may be attributed due to the fact that the electronegativity of nitrogen is less than that of oxygen and hence the hydrogen bonds in amines are weaker than in alcohols.

22.2.2.3. Electronic Effects

On the basis of theoretical principles one may explain the frequency shifts that normally take place in molecular vibrations when the substituents are altered :

A few such classical examples are enumerated with appropriate explanations.

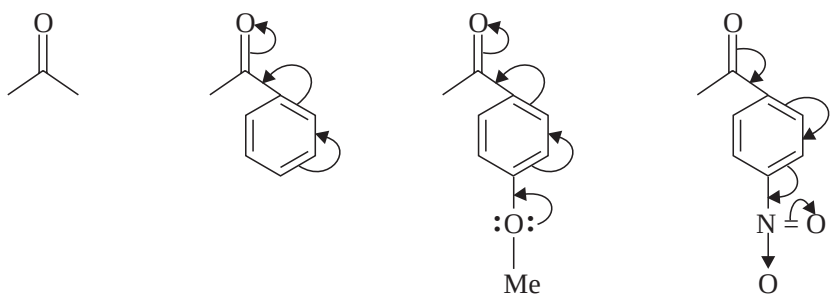
(*a*) **Conjugation Effect :** It is observed to lower the frequency of both C = C str. and C = O str., irrespective of the fact it is brought about by either αβ-unsaturation or by an aromatic ring as shown below :



Explanation : In II, lowering of band order in the C = C bond is observed due to conjugation effect whereby the stretching vibration frequency is decreased by 40-50 cm^{-1} (compare I and II above). In a similar manner, in V delocalization of π-electrons between C = O and the benzene ring enhances the double-bond character of the bond joining the C = O to the ring. It ultimately leads to a lower band order in the C = O bond thereby decreasing the stretching vibration frequency by 20-30 cm^{-1} (compare III and V above).

(*b*) **Mesomeric (or Resonance) Effect :** Whenever a molecule can be represented by two or more structures that differ only in the arrangement of electrons—that is, by structures that have the same arrangement of atomic nuclei-there is resonance.

It may be further expatiated with the help of the following typical examples :



—OMe : Electron Releasing Moiety

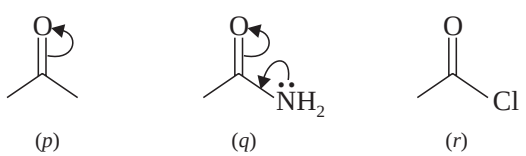
—NO_2 : Electron Withdrawing Moiety

In (*b*) above, the presence of a phenyl ring increases the *mesomeric shift* thereby lowering C = O str. frequency.

+ M Group : *viz*., *p*-OMe (an electron releasing function)—its presence as depicted in (*c*) above will further lower frequencies due to *enhanced mesomeric effect*.

– M Group : *viz*., *p*-NO_2 (an electron withdrawing function)—its presence as shown in (*d*) above will further increase frequencies due to *decreased mesomeric effect*.

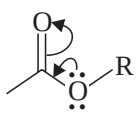
(*c*) **Inductive Effects :** The inductive effects solely depends upon the 'intrinsic' tendency of a substituent to either release or withdraw electrons-by definition, its electronegativity acting either through the molecular chain or through space. This effect usually weakens steadily with increasing distance from the substituent.



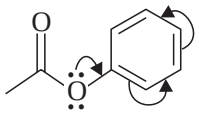
The 'inductive effects' shall now be discussed specifically with regard to the various functional moieties such as : amides, acyl chlorides, alkyl esters and aryl esters :

(*i*) **Amides :** (*q*), The + M effect causes weakening of the C = O bond, leading to the corresponding ketone (*p*). In this particular instance, the – I effect of nitrogen is being dominated by + M effect.

(*ii*) **Acyl Chlorides :** (*r*), the – I effect of Cl is more effective than + M effect, thereby causing an opposite shift (to higher frequency).



(*x*)



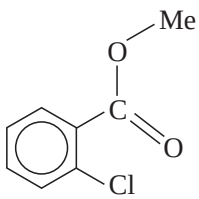
(*y*)

(*iii*) **Alkyl Esters :** (*x*), it has been observed that a conflict between I and M effects invariably takes place in the case of esters. Here, the non-bonding electrons residing on oxygen enhance the + M conjugation thereby decreasing the C = O frequency.

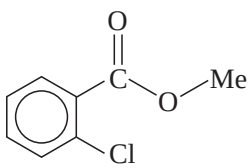
(*iv*) **Aryl Esters :** (*y*), here the non-bonding electrons located on oxygen are partially drawn into the benzene ring and thereafter their conjugation with C = O is minimised. The net effect would be that – I effect of oxygen becomes dominant and consequently C = O moves to a higher frequency.

(*d*) **Field Effects :** It has been observed that two functional groups often influence each other's vibrational frequencies by a through-space interaction that may be either steric and/or electrostatic in nature.

A typical example of *ortho*-chlorobenzoic acid esters is shown below :



(*k*)



(*l*)

In the above instance, the field effect shifts the C = O frequency in the rotational isomer (*k*) and not in the isomer (1). As both isomers are usually found to be present together, therefore, two C = O str. absorptions are observed in the spectrum of this compound.

The various aspects discussed above in Sections 22.2.1 to 22.2.2.3, give a sufficient in-depth knowledge of theoretical considerations related to the better understanding of infrared spectroscopy.

22.3. INSTRUMENTATION

The infrared spectrophotometers are based on either single monochromation or double monochromation :

(*a*) Single-Monochromator Infrared Spectrophotometer, and

(*b*) Double-Monochromator Infrared Sepctrophotometer.

The optical diagrams, components used and their modes of operation shall be discussed briefly in this context under different heads.

22.3.1. SINGLE MONOCHROMATOR INFRARED SPECTROPHOTOMETERS

The important features of an infrared spectrophotometer are as follows :

(*i*) Infrared sources,

(*ii*) Monochromators,

(*iii*) Detectors, and

(*iv*) Mode of Operation.

22.3.1.1. Infrared Sources

The most common infrared sources are electrically heated rods of the following types :

(*a*) Sintered mixtures of the oxides of Zirconium (Zr), Yttrium (Y), Erbium (Er) etc., also known as **'Nernst Glower',**

(*b*) Silicon Carbide **'Globar'**, and

(*c*) Various ceramic (clay) materials.

It is quite evident that the infrared output from all these different sources invariably varies in intensity over a definite frequency range, therefore, a compensating variable slit is usually programmed to operate in unison with the scanning over the individual frequencies.

22.3.1.2. Monochromators

Three types of substances are normally employed as monochromators, namely :

(*i*) **Metal Halide Prisms :** Various metal halide prisms, such as : KBr (12-25 μm), LiF (0.2-6 μm) and CeBr (15-38 μm) have been used earlier, but they have become more or less obsolescent nowadays.

(*ii*) **NaCl Prism (2-15 μm) :** Sodium chloride prism are of use for the whole of the region from 4000-650 cm^{-1}. First, it offers low resolution at 4000-2500 cm^{-1}, and secondly, because of its hygroscopic nature the optics have got to be protected at 20 °C above the ambient temperature.

(*iii*) **Gratings :** In general, gratings are commonly employed in the design of the instruments and offer better resolution at higher frequency than the prisms. They offer much better resolution at low frequency, *viz.*, typical rulings are 240 lines per nm for the 4000-1500 cm^{-1} region and 120 lines per nm for the 1500-650 cm^{-1} region.

22.3.1.3. Detectors

There are ion all *three* different types of detectors that are used in the infrared region :

(*a*) **Thermocouples (or Thermopiles) :** The underlying principle of a thermocouple is that if two dissimilar metal wires are joined head to tail, then a difference in temperature between head and tail causes a current to flow in the wires. In the infrared spectrophotometer this current shall be directly proportional to the intensity of radiation falling on the thermocouple. Hence, the thermocouples are invariably employed in the infrared region, and to help in the complete absorption of 'available energy' the 'hot' junction or receiver is normally blackened.

(*b*) **Golay Detector :** In this specific instance the absorption of infrared radiation affords expansion of an inert gas in a cell-chamber. One wall of the cell-chamber is provided with a flexible mirror and the resulting distortion alters the intensity of illumination falling on a photocell from a reflected beam of light. Thus, the current from the photocell is directly proportional to the incident radiation.

(*c*) **Bolometers :** These are based on the principle that make use of the increase in resistance of a metal with increase in temperature. For instance, when the two platinum foils are appropriately incorporated into a Wheatstone bridge, and radiation is allowed to fall on the foil, a change in the resistance is observed ultimately. This causes an out-of-balance current that is directly proportional to the incidental radiation. Just like the thermocouples, they are used in the infrared region.

22.3.1.4. Mode of Operation

The schematic layout of a single-monochromator infrared spectrophotometer has been duly depicted in Figure 22.2.

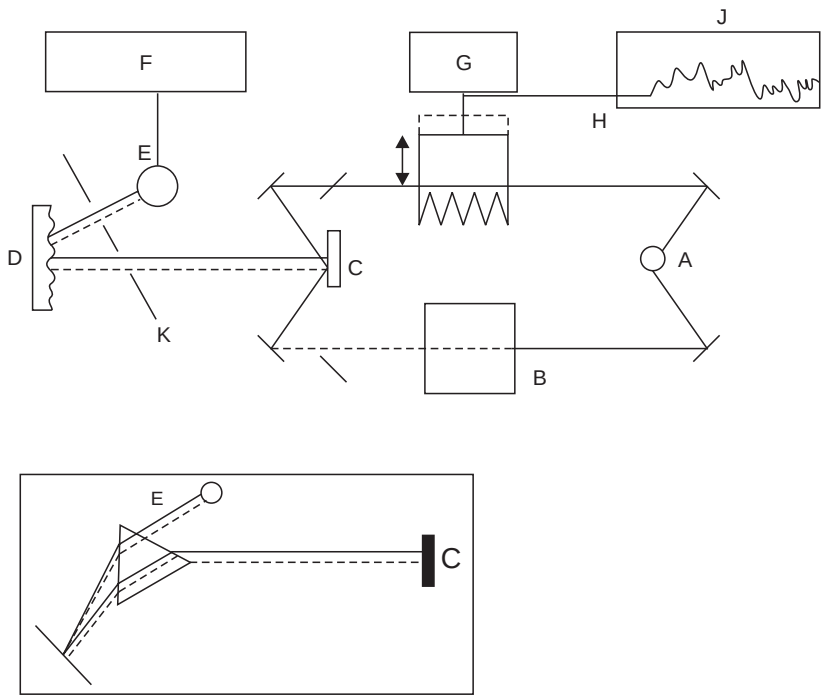


Figure 22.2. Optical Diagram of a Single-Monochromator Infrared Spectrophotometer.

The various vital components of Figure 22.2 are as follows :

A = Infrared source,

B = Sample beam,

C = Chopper—a rotating segmented mirror,

D = Monochromator grating,

E = Detector thermopile,

F = Amplifier,

G = Servo-motor,

H = An optical Wedge,

I = Prism,

J = Ink-pen recorder, and

K = Slits.

The sequential steps observed in the mode of operation are as stated below :

(*i*) The light from infrared source A is split equally into two beams ; one of which B is made to pass through the sample *i.e.*, the sample beam while the other serves as reference beam.

The main objective of such a double beam operation is to measure the difference in intensities between the two beams at each wave length.

(*ii*) The two beams are subsequently reflected on a rotating segmented mirror called chopper C. The chopper rotating ≈ 10 times per second helps the sample beam and the reference beam to be reflected alternatively to the monochromator grating D.

(*iii*) Thus, the grating rotates slowly and transmits individual frequencies to the detector thermopile (E), that consequently converts the infrared (thermal) energy to the corresponding electrical energy.

(*iv*) When a sample has absorbed a certain quantum of light of specific frequency the detector shall be receiving alternatively from the chopper an intense beam (due to reference beam) and a relatively weak beam (due to sample beam). It will generate a pulsating or alternating current (AC) flowing from the detector to the amplifier F.

(*v*) This out-of-balance signal received by the amplifier, is coupled to a small servo-motor G, that drives an optical wedge (H) into the reference beam until the detector receives light of equal intensity from sample and reference beams.

(*vi*) The slightest movement of the wedge (or attenuator) is further coupled to a ink-pen recorder J, so that movement of the former, both 'in' and 'out' of the reference beam, is adequately recorded on the printed chart at various absorption bands.

This specific type of the **'double-beam optical-null recording spectrophotometer'** is termed so because it critically balances out by the help of optical means the differential between the two beams.

The 'inset diagram' in Figure 22.2 shows the use of a **'prism'** in place of the **'grating'**. However, underlying principle being identical, a rotating mirror affords the scanning of individual frequencies.

22.3.2. DOUBLE-MONOCHROMATOR INFRARED SPECTROPHOTOMETER

The schematic optical diagram of a double-beam infrared spectrophotometer has been shown in Figure 22.3 as per Beckman Model IR-9.

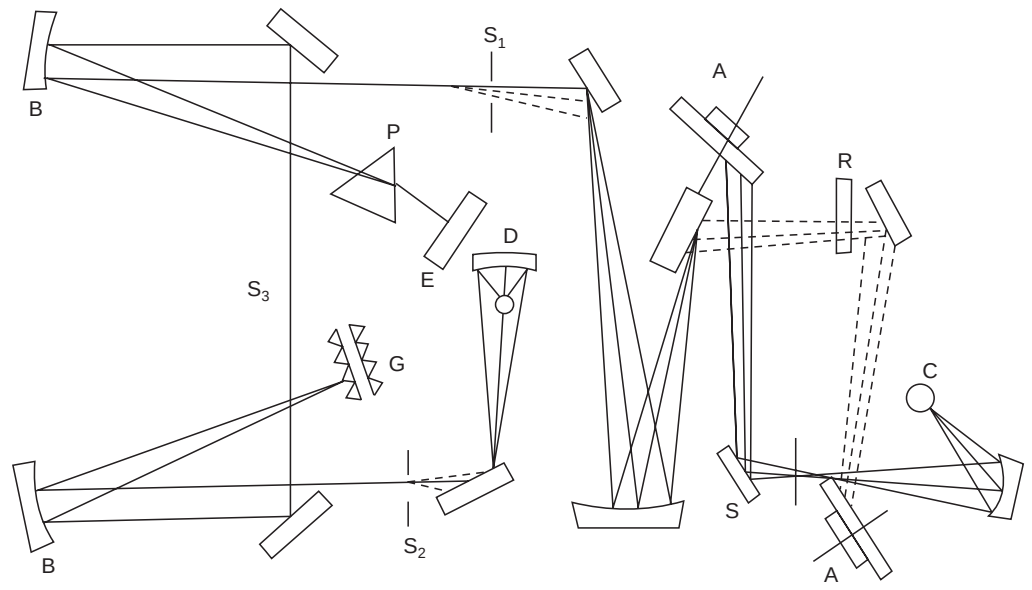


Figure 22.3 : Optical Diagram of a Double-Monochromator Infrared Spectrophotometer (Beckman Model IR-9)

The various components of a double-monochromator infrared spectrophotometer shown in Figure 22.3 are as follows below :

A = Rotating mirror,

B = Collimating mirror,

C = Infrared source,

S = Sample beam,

R = Reference beam,

D = Detector,

S_1 = Entrance, slit,

S_2 = Exit slit,

S_3 = Intermediate slit,

E = Littrow mirror,

G = Monochromator Gratings, and

P = Prism.

The various steps that may be followed sequentially to operate a double-monochromator infrared spectrophotometer are described below :

(*i*) The light from the infrared source C is made to split into two beams one of which passes through the sample (*i.e.*, the sample beam) while the other caters as the reference beam. This sort of double-beam arrangement facilitates in measuring difference in intensities between the *two* beams at each wavelength,

(*ii*) In this instance two monochromators have been employed in series with an intermediate slit (S_3) as shown in Figure 22.3,

(*iii*) The optical train affords as much as twice the dispersion and the ultimate resolution is fairly comparable to any single-monochromator instrument (Figure 22.2),

(*iv*) All stray radiant energy is virtually eliminated,

(*v*) In Figure 22.3, (Beckman Model IR-9) one of the two prism monochromators has been replaced with a dual grating, and

(*vi*) Finally, the detector picks up light of equal intensity from sample and reference beams.

22.3.1.5. Experimental Profile of Infrared Spectroscopy : Quantitative Analysis

In usual practice, there are *two* methods that are frequently employed for the determination of the transmittance ratio in quantitative analysis namely :

(*a*) Emperical ratio method, and

(*b*) Base-line method.

The above *two* methods shall be discussed briefly with the help of certain typical examples as detailed below :

22.3.1.5.1. *Emperical Ratio Method*

This particular method is often employed in a situation where the absorption bands of the analyte are found to be very close to those of the main constituent or the internal standard.

The quantitative analysis of pharmaceutical substances may be achieved by emperical-ratio method either by plotting percentage transmittance against wavelength or by plotting the log T^1o/T^1 against concentration as illustrated in Figure 22.4.

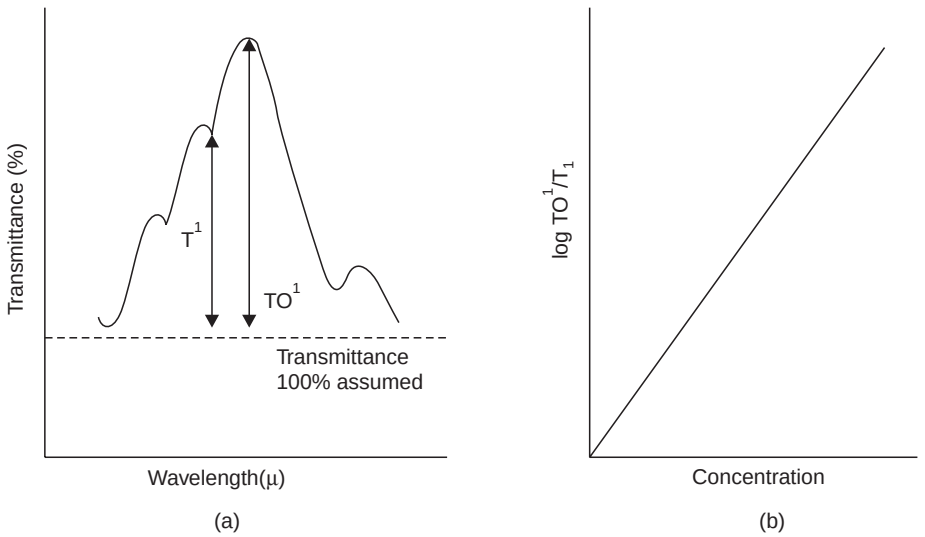


Figure 22.4 : (*a*) Plot of Transmittance Vs Wavelength (*b*) Plot of log T^1o/T^1Vs Concentration.

22.3.1.5.2. *Base-Line Method*

It essentially involves the selection of an absorption band of an analyte which does not remain very close to the bands of other constituents present in the matrix.

Figure 22.5 depicts the absorption bands of the sodium salt of Penicillin G at 703 cm^{-1}.

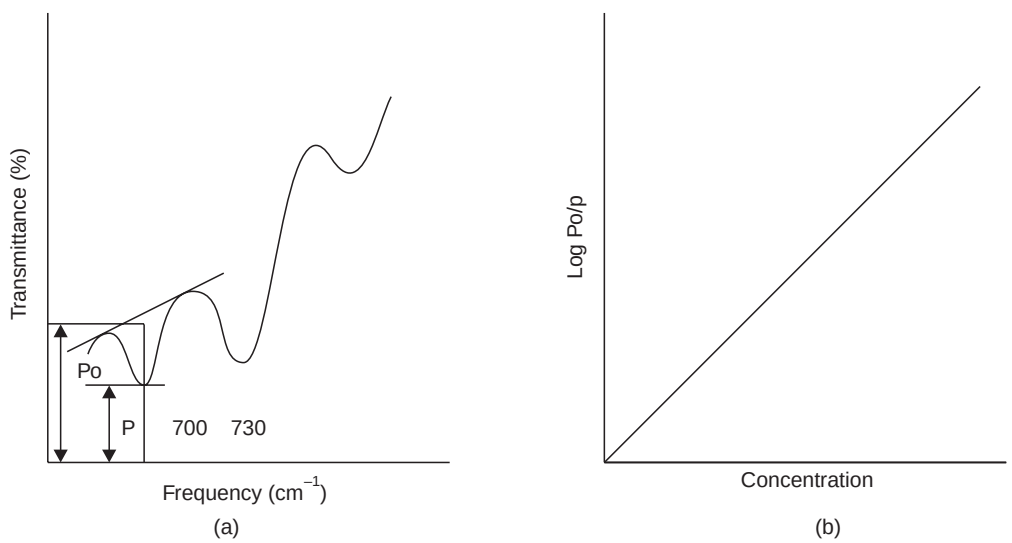


Figure 22.5 : (*a*) Plot of Transmittance Vs Frequency ; (*b*) Plot of log Po/P Vs Concentration.

The value of the incident radiant energy Po may be achieved by drawing a straight line tangent to the spectral absorption curve at the position of the analyte's absorption band. Consequently the transmittance P is usually measured at the point of maximum absorption. Finally, the value of log Po/P is plotted against the concentration as shown in Figure 22.5.

It is, however, pertinent to mention here that the application of both emperical ratio method and base-line method help in eliminating to a great extent the errors caused due to changes in source intensity and adjustment of the optical system.

22.3.1.6. Determination of the Absorption Spectrum of a Solid Compound (or a Pharmaceutical Substance)

The determination of the absorption spectrum of a solid pharmaceutical substance is invariably accomplished by any one of the *two* following techniques namely :

(*a*) Mull Technique, and

(*b*) Potassium Bromide Disc Technique.

These *two* different techniques shall be described below :

22.3.1.6.1. *Mull Technique*

Procedure : Take about 15-20 mg of sample in a previously cleaned small agate mortar and powder it thoroughly (about 200 mesh). Add to it 2 drops of purified paraffin (commonly known as Nujol) or any other suitable mulling liquid and continue the trituration until a very smooth paste of uniform consistency is achieved. Now, transfer the slurry to a sodium chloride window, placing it carefully into the cavity made by the spacer. Consequently, place the other window on top and thus assemble the cell. With the help of a clean piece of tissue-paper wipe out the excess paste that has squeezed out from the cell windows. Finally, introduce the cell in the respective cell-compartment.

Salient Features : The salient features of Mull Technique are as follows :

(*i*) Particle size of the sample has got to be reduced below 200 mesh or 3 μm so as to avoid scattering of radiation thereby causing poor absorption spectrum.

(*ii*) Hydrogen bonding and crystal forces usually influence the trace obtained.

(*iii*) Paraffin itself gives rise to strong band either at 1460-1380 cm^{-1} or at 2820-2850 cm^{-1}.

22.3.1.6.2. *Potassium Bromide Disc Technique*

Procedure : For a window of diameter 1.3 cm, take 100 mg of spectroscopic grade KBr in a previously cleaned agate pestle and mortar and grind it thoroughly with 0.05-0.5 mg of the sample. Now, carefully place the sample mixture into the pressing chamber of the mould in such a manner that it is held between the polished surfaces of the bottom and top pressing dies. Subsequently, attach the chamber to the vacuum line and switch-on the vacuum pump ; initially applying a slight negative pressure so as to compact the powder and then gradually increasing it to ≤ 15 mm Hg for 30 seconds. Finally, enhance the pressing force to 100,000 lb/in^2 or 10-12 $tons/in^2$ for a period of 1-2 minutes. Carefully, release the pressure and dismantle the dies. Now, remove the window from the mould and keep it in position onto the sample holder.

Salient Features : The salient features of KBr-disc technique are stated below :

(*i*) There exists a possibility of interaction between vibrations of the sample and the potassium halide lattice,

(*ii*) It is considered to be the most suitable method for other screening of very minute quantities of substances being eluted from the columns in Gas Liquid Chromatography (GLC). In actual practice, about 300 mg of the spectroscopic grade KBr is placed in a short column immediately after the detector. Consequently, the solid is powdered, pressed into a disc in the normal procedure and ultimately the absorption spectrum of the trapped substance is studied,

(*iii*) It enjoys the advantage of producing spectra absolutely free from any solvent peaks (unlike Mull Technique) and hence it is employed extensively in routine analysis.

Internal Standard for KBr-Disc Technique : In quantitative analysis it is essential to examine absolutely uniform discs of identical weights. To achieve this, known weights of both KBr and analyte are required in the preparation of the KBr-disc and finally from the absorption data a calibration-curve may be obtained. In this process, it is a must to weigh the discs and also to measure their thickness at different points

on their surface with the help of a dual micrometer. In order to overcome this tedious process of measuring disc thickness carefully the use of an internal standard has been introduced.

Potassium thiocyanate (KSCN) is considered to be the **choicest internal standard**. In usual practice, it must be preground, dried and subsequently reground, and used at a concentration of 0.2% (w/w) along with the dried spectroscopic grade KBr. The mixture of KBr-KSCN is stored over P_2O_5.

Procedure : A standard calibration curve is plotted by thoroughly mixing together about 10% (w/w) of the analyte with the KBr-KSCN mixture and then grinding the same intimately. Now, the ratio of the thiocyanate absorption at 2125 cm^{-1} to a selected band absorption of the analyte is plotted against the percent concentration of the sample. Likewise, an identical disc is prepared with the unknown sample and the same KBr-KSCN mixture. Finally, its absorbance ratio is determined and the concentration (of unknown sample) is read off directly from the standard calibration curve.

22.3.1.7. Calibration of Infrared Spectrophotometers

The wavelength (or wave number) scale calibration of infrared spectrophotometers is usually carried out with the aid of a strip of polystyrene film fixed on a frame. It consists of several sharp absorption bands, the wavelengths of which are known accurately and precisely. Basically, all IR-spectrophotometers need to be calibrated periodically as per the specific instructions so as to ascertain their accuracy and precision.

22.4. APPLICATIONS OF IR-SPECTROSCOPY IN PHARMACEUTICAL ASSAYS

22.4.1. APPLICATIONS OF IR-SPECTROSCOPY IN THE ANALYSIS OF PHARMACEUTICAL SUBSTANCES

A host of pharmaceutical substances can be identified and critically examined with the help of infrared spectroscopy. Hence, the latest versions of British Pharmacopoeia (BP) and United States Pharmacopoeia (USP) contain the complete IR-spectrum of such pure pharmaceutical substances that are essentially included in the respective *official compendium*. These authentic IR-spectra are profusely used in many well-equipped Quality Assurance Laboratories in checking the purity of commercially available drugs before employing them in various formulations.

Following is the detailed procedure laid out in the Pharmacopoeia of India (IP) for the preparation of KBr-disc or KCl-disc :

Procedure : Triturate about 1 mg of the pharmaceutical substance with approximately 300 mg of dry, finely powdered KBr or KCl of spectroscopic grade, as directed. Grind the mixture thoroughly, spread it uniformly in a suitable die and compress under vacuum at a pressure of about 10 t in^{-2}. Mount the resultant disc in a suitable holder in the spectrophotometer and obtain the IR-spectrum.

Precautions : The following precautions may be observed carefully :

(*i*) Several factors *e.g.* excessive or insufficient grinding, absorption of moisture or other impurities in the halide carrier, may ultimately result in the formation of unsatisfactory discs,

(*ii*) Unless its preparation presents certain specific difficulties a disc should be rejected if visual inspection shows lack of uniformity, or if the transmittance at about 2000 cm^{-1} (5 μm) in the absence of a specific absorption band is less than 75% without compensation, and

(*iii*) If the other ingredients of tablets, injections or other dosage forms are not completely removed from the substance being examined, they may contribute to the spectrum.

Example : The infrared absorption spectrum of the following pharmaceutical substances do exhibit maxima which are only at the same wavelengths as, and have similar relative intensities to those in the spectrum of the corresponding reference samples, namely :

Ampicillin sodium ; Amylobarbitone ; Betamethasone ; Betamethasone valerate ; Carbenicillin disodium ; Chloroquine phosphate ; Chloroquine sulphate ; Cemetidine ; Clofazimine ; Clofibrate ; Clonidine

hydrochloride ; Cloxacilline sodium ; Colchicine Cyclophosphamide ; Cyproheptadine hydrochloride ; Dexamethasone ; Activated dimethicone ; Diphenylpyraline hydrochloride ; Erythromycin estolate ; Ethambutol hydrochloride ; Ethirylestradiol ; Ethiosuximide ; Fludrocortisone acetate ; Fluphenazine hydrochloride ; Iburprofen ; Diluted isosorbide dinitrate ; Lincomycin hydrochloride ; Mebendazole ; Metoformin hydrochloride ; Methdilazine hydrochloride ; Methotrexate ; Nalidixic acid ; Nandrolone decanoate ; Nandrolone phenylpropionate ; Niclosamide ; Nitrofurantoin ; Nitrofurazone ; Norethisterone ; Oxprenolol hydrochloride ; Pentazocine hydrochloride ; Pentolamine hydrochloride ; Phentolamine mesylate ; Primidone ; Prochlorperazine mesylate ; Proguanil hydrochlorde ; Pyrazinamide ; Pyrimethamine ; Rifampicin ; Spironolactone ; Stilbosterol diphosphate ; Sulphadimethoxine ; Sulphalene ; Sulphamethizole ; Testosterone propionate ; Thiabendazole ; Trifluoperazine hydrochloride ; Triflupromazine hydrochloride.

22.4.2. APPLICATIONS OF IR-SPECTROSCOPY IN THE ANALYSIS OF PHARMACEUTICAL DOSAGE FORMS

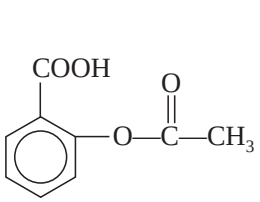
A number of pharmaceutical dosage forms can be assayed conveniently with the help of IR-spectroscopy. A few typical examples are enumerated below for ready reference, namely :

22.4.2.1. Determination of Aspirin, Phenacetin and Caffeine in Tablets

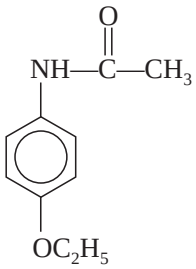
Theory : The quantitation is solely based on the intensities of the carbonyl bands at 1764, 1511 and 1665 cm^{-1} for aspirin, phenacetin and caffeine respectively.

Materials Required : APC-Tablets ; Chloroform ;

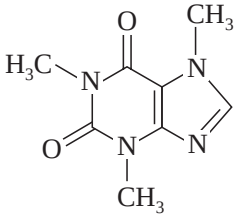
Procedure : The drug contents of an appropriate number of tablets are directly extracted into chloroform, filtered if necessary so as to remove the insoluble tablet components, and the final concentration of chloroform solution is made in such a way so that it should contain : 90 mg ml^{-1} of aspirin ; 64 mg ml^{-1} of phenacetin, and 134 mg ml^{-1} of caffeine. The IR-spectrum is now recorded in a 0.1 mm NaCl-cell between 1400-2000 cm^{-1}.



Aspirin



Phenacetin



Caffeine

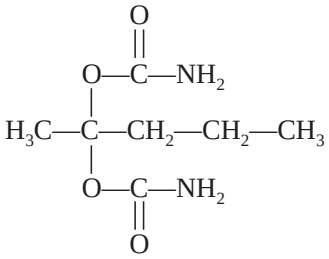
22.4.2.2. Determination of Codeine Phosphate in Tablets

Codeine phosphate was duly extracted into CS_2 and quantitatively determined by measuring its absorption at 942 cm^{-1}.

22.4.2.3. Determination of Meprobamate in Tablets

Maynard (1960) carried out the analysis of meprobamate by dissolving it in chloroform (spectroscopic grade) and subsequently determining the intensity of the amide carbonyl band ($—\overset{\overset{O}{\|}}{C}—$) at 1582 cm^{-1}. Later Shearken (1968) adopted a modified method of assay by using chloroform as an extracting medium, but instead of the carbonyl band measured the N—H stretching band at 3436 cm^{-1}. However, this particular method essentially requires the complete removal of both water and ethanol ; the latter is present in $CHCl_3$ as a stabilizer which is required to be eliminated completely to avoid interference from O—H stretching bands. To achieve this objective activated alumina columns have been used extensively. However, Zappala and Post

(1977) got rid of the interferences occurring at lower frequencies by measuring meprobamates by employing the primary amine combination band at 5107 cm^{-1} in the near IR region.

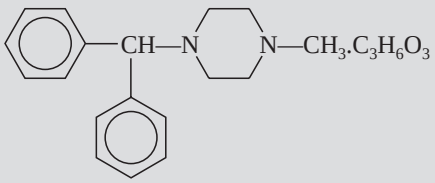
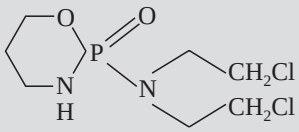
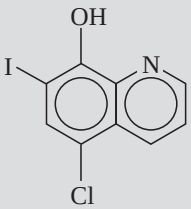
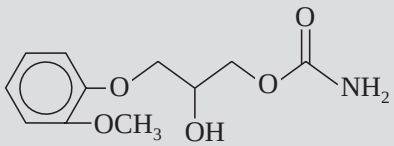
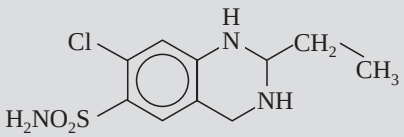


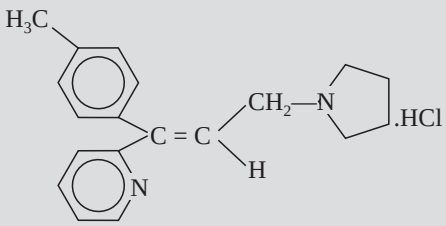
Meprobamate

22.4.2.4. *Cognate Assays*

The USP (XIX) and NF (XIV) have described the assays of various pharmaceutical dosage forms in appropriate solvent at different frequencies (cm^{-1}). A few typical examples are given in Table 22.2.

Table 22.2 : Quantitative IR Analysis Profile of some Dosage Forms as per USP* and NF**

S. No.	Pharmaceutical Substance/Preparation	Chemical Structure	Solvent used	Frequency (cm^{-1})
1.	Cyclizine Lactate Inj.		Cyclohexane	704
2.	Cyclophosphamide Injection/Tablets		KBr Pellet with Fe $(SCN)_3$ Internal Standard	1053
3.	Iodochlorhydroxyquin Cream/Ointment		Carbon Sulphide	694
4.	Methocarbamol Inj./ Tablets		Chloroform	1730
5.	Quinethazone Tablets		Cyclohexane	1342

6.	Simethicone Tablets/ Suspension	$(CH_3)_3SiO[(CH_2)_2SiO]_n^- Si(CH_3)_3$	Carbon Tetrachloride	1269
7.	Triprolidine Hydrochloride		Cyclohexane	824

*USP (XIX) ; ** N.F. (XIV) ;

22.5. APPLICATIONS OF IR-SPECTROSCOPY IN ANALYTICAL CHEMISTRY

The technique of infrared spectroscopy has been adequately exploited in the domain of analytical chemistry. This aspect is duly expatiated with the aid of the following typical examples, namely :

22.5.1. DETERMINATION OF *CIS-TRANS* ISOMER RATIO IN CLOMIPHENE CITRATE

It is a gonad stimulating principle.

Theory : It is an established fact that *cis*- and *trans*-substituted double bonds have slightly different absorption bands in the region of 13 μm. This specific feature forms the basis of the present determination.

Besides, the pharmacological actions of many compounds are invariably dependent on the shape of molecules and hence, usually play a very significant role. Therefore, if both *cis*- and *trans*-isomers are produced in the course of a particular synthesis it may be absolutely necessary to incorporate in the product profile a specific test for the relative proportions of one to the other. This type of 'control measure' strictly conforms the uniformity of composition in the bulk-drug industry and ensures a check on the batch-to-batch variation.

Procedure : Dissolve accurately 22.5 mg of *trans*-clomiphene citrate and 52.5 mg of cis-clomiphene citrate (approx. 1 : 2.3) into 10 ml of DW in a clean 50 ml separating funnel. Add to it 1 ml solution of sodium hydroxide (5% w/v in DW). In the alkaline medium the base is liberated which is extracted successively with 3 portions of solvent ether (10 ml each). The combined ethereal layer is washed with two portions of DW (10 ml each). The resulting ethereal fraction is dried over anhydrous sodium sulphate, filter, evaporate to dryness carefully over an electric water-bath and dissolve the residue in 1 ml of CS_2. Now, record the absorption curve in a 0.2 mm cell over the range 12.50 to 14.00 μm. Calculate the absorbance for the peaks at 13.16 and 13.51 μm respectively by employing the base-line method (see section 3.1.B in this chapter) between the minima at 12.66 and 13.89 μm.

Finally, repeat the assay with a 1 : 1 mixture (75 mg) of *cis* and *trans*-clomiphene citrates and also with clomiphene citrate (75 mg) as such. Thus, calculate the ratio as follows :

$$\frac{\text{Absorbance at 13.16 μm}}{\text{Absorbance at 13.51 μm}}$$

with regard to each assay and therefrom confirm at the ratios of the sample falls very much within the ratios for the standards thereby indicating that the sample contains 50-70% *cis*-clomiphene citrate.

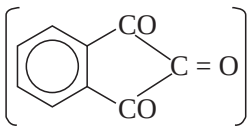
22.5.2. TO DISTINGUISH AND CHARACTERIZE THE PRI-, SEC-AND TERT-AMINE SALTS FROM ONE ANOTHER

Example : (+) Amphetamine Sulphate-a pri-amine salt, χ-Ephedrine Sulphate-a sec-amine salt, and Quinine Hydrochloride-a tert-amine salt.

22.5.3. IR-SPECTROSCOPY IN THE STUDY OF COMPLEX FORMATIONS

The IR-spectroscopy has been judiciously used for the study of complex formations.

Examples :

(*a*) **Ninhydrin :**  *i.e.*, 1, 2, 3-Triketone derived from indane reacts with amino acids under appropriate conditions to result in the formation of a *deep blue complex*. This reaction is so sensitive that it forms the basis of quantitative complex formation studies by IR-spectroscopy.

(*b*) **1 : 10-Phenanthrolin :** reacts with Fe^{2+} ion quantitatively to give rise to a deep red complex due to formation of phenanthroline-ferrous complex, which being extremely sensitive in nature is usually exploited as the basis of quantitative complex formation studies by IR-spectroscopy.

22.5.4. IR-SPECTROSCOPY IN QUANTITATIVE REACTION SEQUENCE STUDY

IR-spectroscopy technique has been used meaningfully in the qualitative reaction sequence studies with regard to various organic synthesis, namely : reduction of —NO_2 group to —NH_2 ; reduction of $(—\overset{\overset{O}{\|}}{C}—)$ carbonyl group to —CH (OH) ; oxidation of methyl-group to —COOH ; etc.

22.5.5. IR-SPECTROSCOPY IN THE IDENTIFICATION OF FUNCTIONAL GROUPS

A few salient features in this context are, namely :

(*a*) The absence of a specific characteristic absorption may be more informative than its presence, *e.g.*, the presence *vis-a-vis* absence of a C = O str. absorption.

(*b*) Multifunctional compounds invariably exhibit altogether separate absorption peaks due to the presence of individual functional groups. In a situation where these functional groups interact with each other either absorption peaks merge with one another or they shift from their original positions, for instance :

Glycine : $H_2N—CH_2—COOH$ (α-amino acetic acid *i.e.*, and aliphatic amino acid) ;

Pentane-2, 4-dione, acetylacetone : $CH_3CO\ CH_2\ COCH_3$-$\alpha\beta$-diketone ;

***para*-Hydroxybenzoic acid :** $HO—C_6H_4—COOH$-$\alpha\gamma$-hydroxy acid (aromatic) ;

(*c*) Graphically presented correlation tables, as cited in specialist texts of Cross, Bellamy and Van der Mass, are found to be fairly precise and accurate for the critical identification of functional groups. It is, however, pertinent to mention here that the degree of accuracy lies between $\pm$ 5 cm^{-1} for ordinary routine IR-spectrophotometers having lesser observed accuracy at higher frequencies.

(*d*) Keeping in view the vast wealth of expertise and experience, it may be inferred that the maximum weightage can be solely rested on the absorptions either below 900 cm^{-1}, or above 1400 cm^{-1}, for obvious reasons as the 'fingerprint region' 900-1400 cm^{-1} mainly contains a plethora of unassigned absorptions.

(*e*) Group frequencies are invariably more readily accountable and hence valuable in comparison to the corresponding single absorption bands. It may be further expatiated due to the fact that a functional group which often results in many specific and characteristic absorption bands can be identified more precisely and definitely than a function which produces only one characteristic absorption band. For instance :

(*i*) **Esters (—C(=O)—OR) :** It affords group frequencies due to C = O str., and C—O str. and hence more readily identified than the ketones (C = O str.)

(*ii*) **Amides (—C(=O)—NH.H) :** It gives rise to group frequencies on account of (C = O str., N—H str., N—H def.) and, therefore, more easily identified than the corresponding amides.

22.5.6. IR-SPECTROSCOPY : IDENTIFICATION BY FINGERPRINTING

The 'fingerprint region' lies between 1300-400 cm^{-1} which is considered to be the most valuable component of the spectra and mainly comprises of a specifically large number of unassigned vibrations. Therefore, IR-spectroscopy aids in the identification of unknown compound by comparing its spectrum with a standard spectra recorded under exactly similar experimental parameters. Thus, pharmaceutical substances that exhibit the same infrared spectra may be inferred as identical.

Precisely in the domain of analysis by physico chemical property IR-spectroscopy offers a far more characteristic, valid and qualified '**proof of identity'** than the comparison of any other physical property.

Precautions : Certain precautions may be observed readily so as to obtain really identical spectra, namely :

(*a*) Sampling to be done under identical conditions,

(*b*) Same IR-spectrophotometer may be used for obtaining the various spectra,

(*c*) Experimental parameters like : slit-width, scan-speed etc., must be identical,

(*d*) An attempt should be made to obtain the maximum number of peaks in the '*fingerprint region*' thereby ascertaining the **proof of identity** more confidently.

Computer Aided Analysis : With the advent of spectacular and quantum jump in the field of instrument technology over the past two decades a good number of world-renowned manufacturers, such as : Beckman, Bio-Rad, Brüker, Cecil, Hitachi, Nicolet, Perkin-Elmer, Schumadzu have introduced various sophisticated fully computerized FT-IR spectrophotometers. These instruments have the advantage of storing in their computer-memory-banks of sizable number of digitalized information obtained from the infrared spectra of standard compounds. Now, with the flick of a keyboard button the spectrum of an unknown compound, previously fed to the same digital storage bank, may be conveniently compared with the standards and finally to get at the identical infrared absorptions to the unknown.

22.5.7. INTERPRETATION OF AN IR-SPECTRUM

There exist no hard and fast rules with regard to the interpretation of an IR-spectrum, but based on the vast wealth of experience and wisdom of the analyst amalgamated with a storehouse of general observations go a long way towards the exact interpretation of the same. However, following different aspects must be taken into consideration while interpreting the spectrum :

(*a*) In usual practice, the absence of a strong group absorption definitely indicates the absence of that group in the molecule, based on the assumption that no other factors are influencing which might shift the absorption band to the other regions *e.g.*, hydrogen bonding. In other words, intramolecular or intermolecular changes caused due to the hydrogen bonding help in shifting the expected absorption band either to the higher region or to the lower region. For instance : the clear absence of a sharp and strong absorption band in the region 1850-1640 cm^{-1} (or 5.40-650 μ) completely excludes the possibility of carbonyl groups from the molecular structure under investigation.

(*b*) It is quite important to carry out all the preliminary examination of the IR-spectrum of an unknown compound exclusively and definitely on the regions above 900-650 cm^{-1} (11.1-15.4 μ) and above 1350 cm^{-1} (below 7.40 μ). For example :

Free NH_2 and free NH	:	3300-3500 cm^{-1}
Bonded NH	:	3100-3400 cm^{-1}
= NH	:	3300-3400 cm^{-1}
Free OH	:	3550-3650 cm^{-1}
Intermolecular Bonded OH	:	Dimeric — 3450-3550 cm^{-1}
		Polymeric — 3200-3400 cm^{-1}
Intramolecular Bonded OH	:	3420-3600 cm^{-1}

(*c*) **'Fingerprint Region'** *i.e.*, the intervening region 1300-400 cm^{-1} essentially provides very useful information, specifically when examined with reference to bands in the lower and higher regions. It frequently consists of a relatively large number of bands the origin of which is neither located nor determined so easily. Broadly speaking, the 'fingerprint region' helps in the identification of unknown pharmaceutical substances with the aid of reference samples and comparing the two spectra by superimposing them on one another. For this reason many *official compendia* like BP, USP provide the spectra of many pure and authentic pharmaceutical substances that may be compared with the ones under investigation.

(*d*) **Assignment of Bands to Specific Groups by Employing Isotopes :** Deuterium exchange is specifically beneficial for assignment to A-H vibrations in a situation where the hydrogen is exchangeable.

For a simple diatomic molecule X-Y the sole vibration which may take place in a periodic stretching along the X-Y band. Thus, the stretching vibrations may be visualized as the oscillations of two entities connected by a spring and the same mathematical treatment, known as **Hooke's Law,** holds good to a first approximation. Hence, for stretching of the band X-Y, the vibrational frequency (cm^{-1}) may be expressed by the following equation :

$$\bar{\nu} = 1302 \sqrt{k/\mu} \qquad ...(a)$$

where, k = Force constant, and

μ = Reduced mass of the two atoms.

Therefore, for bands having the same force constant k :

$$\bar{\nu} \propto \mu^{-1/2} \qquad ...(b)$$

Thus, it may be shown that the absorption frequencies for a bond involving deuterium are, to a rough approximation $1/\sqrt{2}$ times the frequencies of the corresponding bonds involving hydrogen.

Examples :

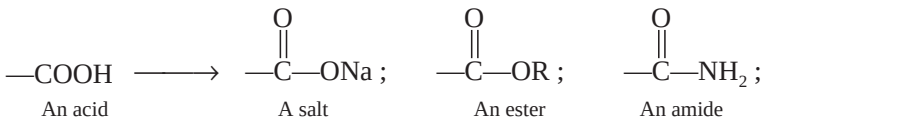
(*i*) Free OH shows absorption at 3550-3650 cm^{-1} ; whereas OD shows absorption at 2400-2800 cm^{-1} ;

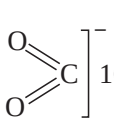
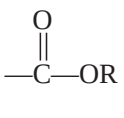
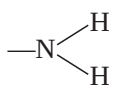
(*ii*) Free NH shows absorption at 3300-3500 cm^{-1} ; whereas free ND shows absorption at 2400-2600 cm^{-1}.

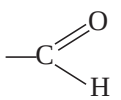
In addition to the above cited typical instances the hydrogen bonding can also be studied at length by subsequent replacement of proton by deuterium.

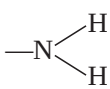
(*e*) **Assignment of Bands to Specific Groups by Affecting Chemical Changes :** Various chemical changes brought about in the organic compounds may be assigned different absorption peaks on the specific modified chemical entities. This can be explained with the help of the (see next page) examples, namely :

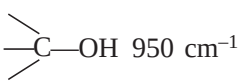
(*i*) Conversion of an acid to its corresponding salt, or an ester or a primary amide :



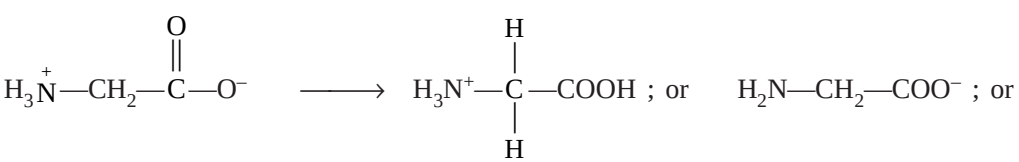
Absorption : O—H 2900 cm^{-1}  1600 cm^{-1};   3300 cm^{-1}

Bands :  1650 cm^{-1} 1900-2000 cm^{-1} C = O 1600 cm^{-1}

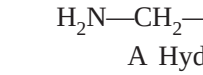
—C = O 1200 cm^{-1}  1580 cm^{-1}

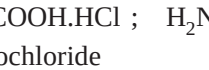


(*ii*) Conversion of an Amino Acid to its corresponding hydrochloride or salt :



NH_3^+ 3050-3150 cm^{-1} (*m*) ; NH_3^+ 2550-2750 cm^{-1} (*w*) ;





THEORETICAL AND PRACTICAL EXERCISES

1. Explain the following **'terminologies'** explicitely in IR-Spectrophotometry :

(*i*) Group frequency region, (*ii*) Fingerprint region,

(*iii*) Molecular vibrations, (*iv*) Vibrational coupling,

(*v*) Hydrogen Bonding (in IR), (*vi*) Electronic effects, and

(*vii*) Field effects.

2. IR-Spectrophotometer variants are of two types :

(*a*) Single monochromation, and

(*b*) Double monochromation.

Describe any ONE of them with a neat-labeled optical diagram and its *modus operandi.*

3. Discuss the experimental profile of IR-Spectroscopy with regard to :

(*a*) Emperical ratio method, and

(*b*) Base-line method.

4. What are the two commonly used techniques invariably employed for the determination of '**absorption spectrum**' of a solid '*drug*'. Explain.

5. How do we assay the following dosage forms by IR-spectroscopy ? Explain.

(*i*) Meprobamate Tablets, (*ii*) Cyclizine lactate Injection,

(*iii*) Cyclophosphamide Tablets, (*iv*) Methocarbamol Injection,

(*v*) Simethicone Tablets, and (*vi*) Tripolidiue hydrochloride.

6. Explain IR-spectroscopy in '**analytical chemistry**' for the determination of :

(*a*) *cis-trans* Isomer ratio in clomiphene citrate.

(*b*) Differentiation of *pri-sec*-and *tert*-Amine salts.

(*c*) Quantitation reaction sequence studies.

(*d*) Complex formations.

(*e*) Identification of functional groups.

7. Give a comprehensive account on the interpretation of an IR-Spectrum. Explain.

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23 NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

CONTAINS :

23.1. INTRODUCTION

Nuclear Magnetic Resonance (NMR) spectroscopy just like IR and UV is regarded as a process whereby energy from an external source is absorbed and brings about a change or resonance to an 'excited' or high energy state. The energy required for NMR lies in the low energy or long wavelength radio-frequency end of the electromagnetic spectrum.

Consequent to the magnetic properties of nuclei arising from the axial spin, the emerging radio-frequency gets absorbed in a magnetic field. Therefore, for a particular nucleus an NMR absorption spectrum invariably comprises one to several groups of absorption lines in the ratio-frequency portion of the electromagnetic spectrum. Evidently, the location of peaks indicate the chemical nature of the nucleus, whereas the multiplets provide information regarding the spatial positions of the neighbouring nuclei. Hence, NMR is also known as **Nuclear Spin Resonance (NSR)** spectroscopy.

NMR has accomplished a growth in a geometrical progression since the early sixties and virtually developed into extremely potential analytical tools not only useful for elucidation of complex structural determinations but also equally beneficial in the assay of pharmaceutical substances.

In reality, NMR spectroscopy has broadened the scope and absolute possibility for performing more extensive as well as intensive studies with regard to recording the spectrum of isolated and synthesized organic molecules in addition to their mechanistic and stereochemical details hitherto inaccessible. Therefore, NMR spectroscopy finds its applications for compound identification, by means of a **'fingerprint technique'** very much identical to that used in *IR-spectroscopy*. Besides, it is invariably utilized as a specific method of assay for the individual constituents of a mixture. A few typical examples of drug assays will be dealt separately at the end of this chapter to justify its efficacy and usefulness.

23.1.1. THE NMR PHENOMENON

Following are the *five* different aspects that essentially govern the NMR phenomenon, namely :

A. The Spinning Nucleus : The nucleus of the hydrogen atom, *i.e.*, the proton, just behaves as if it is a small spinning bar magnet. It does so because it evidently possesses an electrical charge as well as a mechanical spin. Consequently, a spinning charged body will generate a magnetic field, and hence the nucleus of hydrogen atom is not an exception.

B. The effect of an External Magnetic Field : As a **'compass needle'** possesses an inherent tendency to align itself with the earth's magnetic field, the proton not only responds to the influence of an external magnetic field but also tends to align itself with that field. However, because of restrictions as applicable to nuclei (not to compass needles) the proton can only adopt the following two orientations with regard to an external magnetic field. At this juncture *two* situations normally arise, namely :

(*a*) when proton is aligned with the field (*i.e.*, at lower energy state), and

(*b*) when proton is opposed to the field (*i.e.*, at higher energy state).

Some NMR-analysts describe these proton orientations as **'parallel'** with or **'antiparallel'** with the applied field.

C. The Precessional Motion : The proton appears to be behaving as **'spinning magnet'** and therefore, not only can it align itself with or oppose an external field, but also may move in a characteristic manner under the influence of the external magnet.

Figure 23.1, represents the precessing nuclei *vis-a-vis* the transition energy (ΔE) of reorientation of magnetic dipole between the aligned and opposed conditions when subjected to an external magnetic field (Bo). It is absolutely clear from this Figure that the proton gets aligned with the external magnetic field only at a lower energy states, while it becomes opposed to the field at higher energy states.

However, the energy of the reorientation of magnetic dipole, ΔE, may be expressed as follows :

$$\Delta E = h\nu$$

where, h = Planck's constant, and

ν = Frequency of radiation.

In order to understand the precessional motion more vividly, let us take the example of a spinning 'top' and its spinning motion. The top will (unless absolutely vertical) also perform a comparatively slower *waltz-like motion* whereby the spinning axis of the top moves slowly around the vertical. This particular phenomenon is known as the **precessional motion** and hence, the 'top' is generally said to be precessing around the vertical axis of the earth's gravitational field. In other words, the precession comes into effect due to the interaction of spin (*i.e., gyroscopic motion*) with the earth's gravity vertically downwards. Therefore, a spinning top will precess, whereas a static top will fall over (not precess).

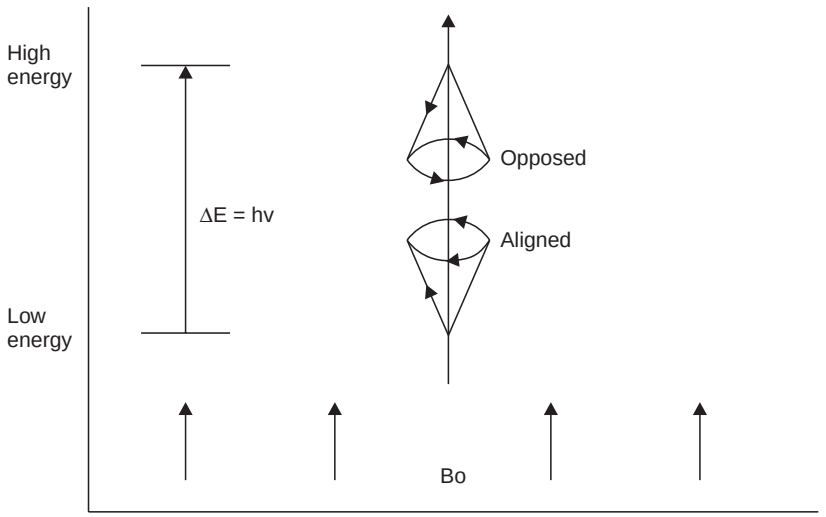


Figure 23.1 : The Precessing Nuclei *vis-a-vis* The Transition Between Aligned and Opposed Conditions.

D. The Precessional Frequency : The spinning frequency of the nucleus does not change at all, whereas the speed of precession does. Therefore, $\nu \propto$ Bo, *i.e.*, the precessional frequency is directly proportional to the strength of the external field Bo.

It designates one of the most important relationships in NMR-spectroscopy.

Example : A proton expressed to an external magnetic force of 1.4 T ($\equiv$ 14, 000 gauss) will precess $\approx$ 60 million times per second so that the precessional frequency ν = 60 MHz ; and for an external field of 2.3 T, $\nu \approx$ 100 MHz, and at 5.1 T, $\nu \approx$ 200 MHz.

E. The Energy Transitions : Whenever a proton is precessing in the aligned orientation (low energy) it can absorb energy and pass into the orientation (high energy) ; and subsequently it can lose this extra energy and relax back into the aligned state.

Interestingly, the precessing proton can only absorb energy from the radio frequency source if the precessing frequency is exactly the same as that of the radio frequency beam ; and when this particular situation arises, the nucleus and the radio frequency beam are said to be in resonance, thereby justifying the term 'nuclear magnetic resonance'.

In NMR spectroscopy, the precessing protons of an organic molecule, after being duly exposed to a powerful external magnetic field (ranging between 60-400 MHz), are irradiated with radio frequency energy of the appropriate frequencies, thereby promoting protons from the low-energy (aligned state) to the high-energy (opposed state). The absorption of energy is ultimately recorded in the form of NMR spectrum as shown in Figure 23.2.

23.1.2. INFORMATIONS PROVIDED BY ^{1}H-NMR (PROTON-NMR)

^{1}H-NMR provides a number of valuable informations stated below, which are employed for the structural elucidation as well as assay of important pharmaceutical substances, namely :

(*i*) To record differences in the magnetic properties of the various nuclei present,

(*ii*) To deduce in large measure the exact locations of these nuclei within the molecule,

(*iii*) To deduce how many different types of hydrogen environments are present in the molecule,

(*iv*) To deduce which hydrogen atoms are present on neighbouring carbon atoms, and

(*v*) To measure exactly how many H-atoms are actually present in each of these environments.

Example : Figure 23.2 depicts the NMR-spectrum of toluene (C_6H_5—CH_3), which essentially possesses *two* different species of H-atoms, for instance :

(*a*) methyl hydrogen atoms (—CH_3), and

(*b*) aromatic ring hydrogen atoms (—C_6H_5).

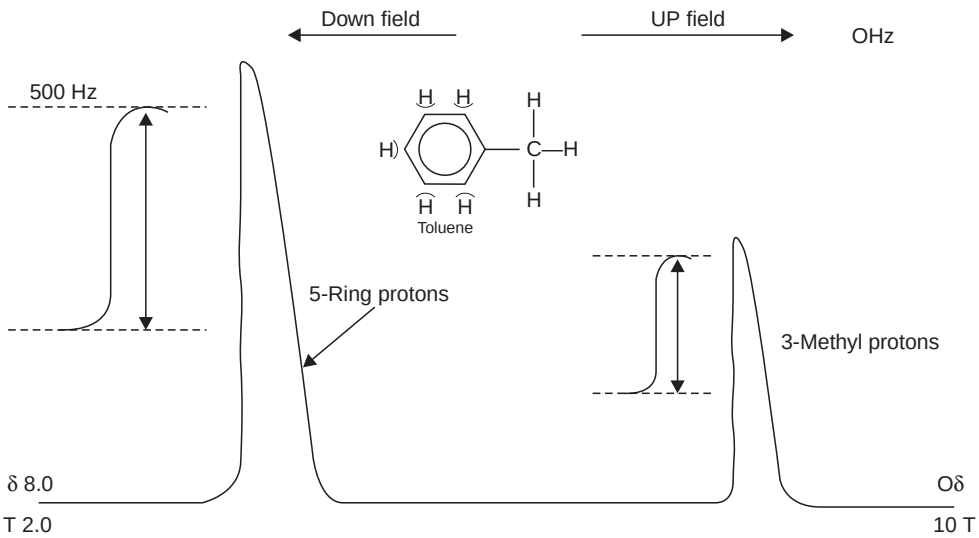


Figure 23.2 : NMR-Spectrum of Toluene Showing Two Signals in the Intensity Ratio [5 : 3].

Hence, two signals will show-up in the NMR-spectrum corresponding to these two different chemical and magnetic environments. Furthermore, the areas under each signal are in the ratio of the number of protons in each part of the molecule, and thus actual measurement will reveal that the ratio of these areas is 5 : 3.

23.2. THEORY

Almost fifty per cent of the nuclei known so far behave as if they were spinning as a whole about an axis just like a minute bar magnet, the axis of which happens to be coincident with the axis of spin. The angular momentum of the charge created by the spinning electrons may be expressed in terms of spin quantum number designated as 'I' (in units of $h/2\pi$ were h is **Planck's constant**). Therefore, for a nuclei to exhibit NMR phenomenon the spin quantum number I is always greater than 0. The spin quantum number I is directly associated with the mass number and the atomic number of the nuclei. Pope *et al*. (1959) has put forward a detailed list of spin quantum values *vis-a-vis* mass number and atomic number so as to facilitate in establishing the value of I empirically as shown below :

Mass Number	Atomic Number	Spin Quantum Number
Odd	Odd or Even	$I = \frac{1}{2}$,
Even	Even	I* = 0,
Even	Odd	I = 1, 2, 3, 4 .....

*(*i*) The nuclei of such type of isotopes possess essentially a spherically symmetrical charge distribution, and

(*ii*) They do not have angular momentum, and hence do not give nuclear magnetic resonance spectra.

* Pope, J.A., Schneider, W.G. and Bernstein, H.J., **High Resolution Nuclear Magnetic Resonance,** London, McGraw-Hill, 1959.

From the above useful data provided one may draw an inference that spin numbers have either 'integral' or 'half-integral' values ranging from $\frac{1}{2}$ to at least 9/2 for different nuclei. The spin number is obtained by the addition of individual protons and neutron spin numbers of $\frac{1}{2}$ each, with the restriction that neutrons can cancel only neutrons and protons can cancel only protons.

Precisely *three* classes of nuclei may be neatly distinguished, namely :

(*a*) **Zero-spin (I = O) :** Those where both the number of protons and neutrons are even, for instance : ^{12}C, ^{16}O, and ^{32}S. Nuclei in this category do not interfere with a NMR-signal from other nuclei. In case, ^{12}C and ^{16}O has also been magnetic per chance, the NMR spectra of organic molecules certainly would have been much more difficult and complex.

(*b*) **Half-Integral Spin** $\left(\mathbf{I = \frac{1}{2}}\right)$ **:** Those where either the number of protons or the number of neutrons is odd. This constitutes the most important group of nuclei for their immense applications and utility to a medicinal chemist and an organic chemist.

Examples :* They are ^{1}H ; ^{3}H ;^{13}C ; ^{19}F ; ^{31}P ; ^{15}N ; ^{29}S ;

(*c*) **Integral Spin (I = 1) :** Those where both the number of protons and the number of neutrons is odd.

Examples : Where 1 = 1, are : ^{2}H (Deuterium) and ^{14}N ; and where I > 1 are : ^{10}B ; ^{11}B ; ^{35}Cl ; ^{17}O ; ^{27}Al ;

In other words, isotopes having a spin value equal to, or greater than one exhibit an ellipsoidal charge distribution and have spin. They invariably possess a nuclear electric quadrupole moment, designated as 'Q'.

23.2.1. ORIENTATIONS OF MAGNETIC NUCLEUS UNDER Bo

Under the influence of external magnetic field, Bo, a magnetic nucleus may take up different orientations with regard to that field, for instance :

(*i*) **Two orientations :** The number of possible orientation is given by (2I + 1), so that for nuclei with spin $\frac{1}{2}$ *e.g.*, ^{1}H, ^{13}C, ^{19}F only two orientations are allowed, and

(*ii*) **Three orientations :** Both ^{2}H (Deuterium) and ^{14}N have I = 1 and, therefore, can take up three orientations. These nuclei essentially possess both electric quadrupoles and magnetic dipoles.

23.2.2. PRECESSIONAL FREQUENCY (ν)

In any magnetic field, magnetic nuclei like the proton precess at a frequency ν, which is proportional to the strength of the applied field. The exact frequency is expressed by :

$$\nu = \frac{\mu \beta_N Bo}{hI}$$

where, Bo = Strength of the external field experienced by the proton,

I = Spin quantum number,

h = Planck's constant (6.626×10^{-34} Js),

μ = Magnetic moment of the particular nucleus, and

β_N = Nuclear magnet on constant.

* Harris, R.K. and B.E. Mann., **'NMR and the Periodic Table',** New York Academic Press, 1978.

Table 23.1 records some typical approximate values of 'ν' for (*a*) selected values of field strength Bo, and (*b*) common magnetic nuclei.

Table 23.1 : Precessional Frequencies* as a Function of Increasing Field Strength

Bo/tesla (Nucleus)	External Magnetic Force					
	1.4	**2.1**	**2.3**	**5.1**	**5.8**	**7.1**
^{1}H	60	90	100	220	250	300
^{2}H	9.2	13.8	15.3	33.7	38.4	46.0
^{13}C	15.1	22.6	25.2	55.0	62.9	75.5
^{14}N	4.3	6.5	7.2	15.8	17.9	21.5
^{19}F	56.5	84.7	93.0	206.5	233.4	282.0
^{31}P	24.3	36.4	40.5	89.2	101.5	121.5

(Free Electron) 3.9×10^4

The data from Table 23.1, reveals that :

(*a*) magnetic moments of ^{1}H and ^{19}F are relatively large and, therefore, detection NMR-signals with these nuclei are fairly sensitive,

(*b*) Magnetic moment of ^{13}C is almost 1/4 that of ^{1}H *i.e.*, the former is less sensitively detected in NMR as compared to the latter,

(*c*) Similarly, magnetic moment of ^{2}H (Deuterium) is approximately 1/6th that of ^{1}H (less sensitively detected in NMR), and

(*d*) Even with very large magnetic fields, upto 7.1 T, the energy difference ($\Delta = h\nu$) is very small, upto 300 MHz ; because the difference is so small ($\simeq 10^{-4}$ kJ mol^{-1}) the number of protons in the two energy states are almost equal.

23.2.3. SATURATION OF THE SIGNAL

It has been observed that nuclei in the lower energy state undergo transitions to the higher energy state. The populations of the two states may approach equality, and if this situation arises no further net absorption of energy take place, and the observed NMR resonance signal will fade out. This particular situation is termed as **saturation of the signal**.

It is pertinent to mention here that in a small NMR spectrum the populations in the two spin states never become equal, by virtue of the fact that higher energy nuclei are constantly returning to the lower energy spin state.

23.2.4. ABSORPTION POSITIONS IN NMR-SPECTRUM

For different types of organic compounds and pharmaceutical substances the resonance positions for protons lie usually within a narrow range (~ 600 Hz). As the differences in the signal's positions are small in comparison to the applied frequency (60×10^6 Hz or 60 MHz), therefore, the absolute measurements of absorption positions cannot be made with the required degree of accuracy (0.1 Hz 60×10^6 Hz, *i.e.*, 1 part in 10^8). However, it is quite possible to measure the differences in frequency relative to a standard substance with the required degree of accuracy and precision.

23.2.5. CHEMICAL SHIFT

The chemical shift (δ) is defined as the difference between the resonance position of a nucleus and that of a standard reference compound. It is normally expressed in terms independent of Ho (or the related applied resonance frequency ν) :

* Precessional Frequencies in MHz

$$\text{Chemical shift } (\delta) = \frac{\Delta\nu\,(\text{Hz})}{\text{Applied resonance frequency} \times 10^6\,(\text{Hz})} \times 10^6 \text{ ppm}$$

where, $\Delta\nu$ = Difference in frequency (Hz) between the observed signal and that of the standard (reference compound).

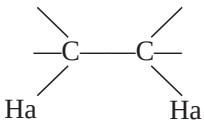
Reference Compound : For ^{1}H NMR *i.e.*, Proton-NMR, tetramethyl silane (TMS), $(CH_3)_4Si$, is employed mostly as the reference compound, because of the fact that its protons resonate at higher field strength than most other protons.

Convention for δ : TMS assigned ($\delta = 0$), values for other protons are measured positively downfield. In other words, increasing δ corresponds to increasing de-shielding of the nucleus.

23.2.6. SPIN-SPIN INTERACTIONS

High resolution NMR spectra very often exhibit signals as multiplets, invariably showing a more or less symmetrical appearance.

Multiplicity is brought about due to the splitting of the signal of one set of equivalent nuclei by the magnetic fields of adjacent sets of nuclei *i.e.*, **spin-spin interactions.** The distance between the peaks of a regular multiplet is termed as the coupling constant, designated as J, and measured in Hz.



The protons Ha and Hb are totally in different chemical environments. There is a significant difference in their chemical shifts because of the variance in the resonance positions of their nuclei. Thus, Ha experiences a total magnetic field comprising of : external field (Ho) and local field due to Hb as shown in Figure 23.3.

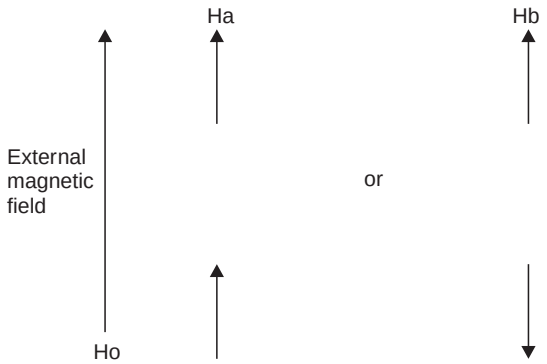
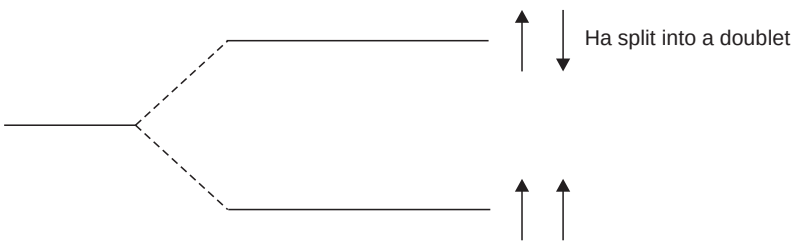


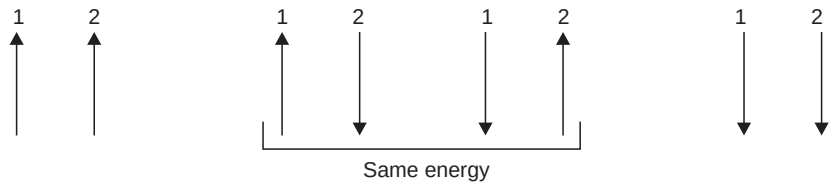
Figure 23.3 : Ha and Hb Exposed to External Magnetic Field.

From Figure 23.3, it is evident that Ha, at a given constant, experiences the local field of Hb that may be either aligned with or against that of Ha.

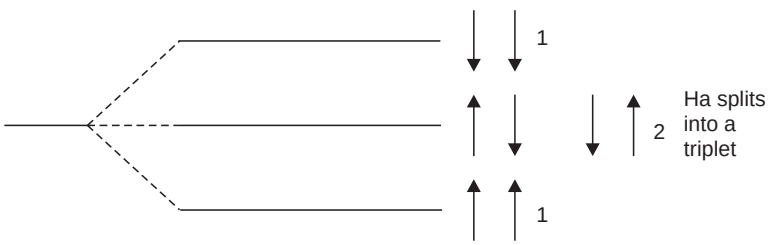
The Ha signal is split into a doublet and the peaks of this doublet will be equal in height, because each alignment of spins has equal probability.



Based on the same reasonings, the signal emerged from Hb is split into a doublet as shown below : The spin-alignments of Hb_1 and Hb_2 may be designated as :



For Ha the corresponding energy levels would be as depicted below :



The probability of each of the above cited four spin arrangements is equal, two having the same energy ; thereby giving rise to a triplet for the signal of Ha, having peak heights in the ratio 1 : 2 : 1.

Therefore, generalizing the spin-spin interactions cause a signal to be split into (n + 1) peaks, where 'n' is the number of interacting nuclei on the adjacent carbon atom.

Hence, *two* important observations are usually made, namely :

(*a*) Coupling constant, J, is independent of Ho (contrast with δ), and

(*b*) Regular multiplets are produced when the difference in chemical shifts (in Hz) between nuclei A and X (*i.e.*, $\Delta\nu_{AX}$) is large relative to the coupling constant J_{AX}, *i.e.*, when $\Delta\nu A_X/JAX \geq \sim 10$.

The spectra obtained in this manner designated as First Order, and these may be analysed with the help of the following FOUR general RULES, namely :

RULE : 1 : Multiplets caused by mutual interaction of nuclei A and nuclei X have identical J values,

RULE : 2 : Interaction of nucleus A with a group of n magnetically equivalent nuclei X (of spin IX), produces a multiplet of ($2n_x$, Ix + 1) peaks,

RULE : 3 : Intensities of the multiplet are asymmetrical about the mid-point of the signal, that corresponds to the origin of the multiplet and is equal to the chemical shift.

Pascal's Triangle : The nuclei having spin quantum number I = 1/2, relative intensities of the multiplet's peak are given by the coefficients of the binomial expansion, $(1 + x)^n$, where n = number of nuclei interacting with the specific nucleus emitting the signal ; or by **Pascal's Triangle** as given below :

n = 0					1					singlet
= 1				1		1				doublet
= 2			1		2		1			triplet
= 3		1		3		3		1		quartet
= 4	1		4		6		4		1	quintet

RULE : 4 : Interaction is normally observable between close groups of magnetically non-equivalent nuclei.

This chapter exclusively deals with nuclei of spin 1/2 and, therefore, the examples and applications shall be given from ^{1}H *i.e.*, **proton magnetic resonance (PMR) spectroscopy.**

However, a brief description of the following *three* types of NMR-spectroscopy will be made here so as to apprise the readers about their principles and main usages only, such as :

(*i*) ^{3}H-NMR (Tritium NMR Spectrocopy),

(*ii*) ^{13}C-NMR Spectroscopy, and

(*iii*) 2D-NMR (Two Dimensional Correlation Spectrocopy ; or Two Dimensional COSY Spectrum).

23.2.7. ^{3}H-NMR (TRITIUM NMR-SPECTROSCOPY)

The ease with which **'tritium'** could be employed for labelling organic compounds, having fairly high molar specific activity, has turned it into a very useful and versatile β-emitting radionuclide for chemical and life sciences research. The unique novel characteristic feature of tritium tracers being that it may be used as a tracer for carbon as well as hydrogen structures. A non-destructive method of analysis was initiated in Great Britain* employed elaborated sophistically designed instrumentations** armed with **'supercon'** magnets and latest computer technology.

The comprehensive dedicated research ultimately made it possible to decode the patterns of labelling in almost any type of tritium labelled compound at low isotopic abundance (*e.g.*, 3×10^{-4} to 3×10^{-2} per cent. ^{3}H per site) with the aid of ^{3}H-NMR directly, rapidly, reliably and non-destructive analytical means. Since, 1971, the ^{3}H-NMR spectroscopy, utilizing only millicurie (mCi) quantities of radioactivity, emerged as a most useful analytical tool for the study of tritium labelled compounds.

The *two* major advantages of ^{3}H-NMR spectroscopy based on the characteristic magnetic features of the tritium nucleus are, namely :

(*a*) High receptivity of the tritium nucleus, *i.e.*, only small amounts of radioactivity (0.1 to 10 mCi per site, 3.7 to 370 MBq) are needed to bring about a well-defined spectrum, and

(*b*) Similarity of the chemical shifts of ^{3}H nuclei and those of ^{1}H nuclei (protons) *i.e.*, the copious volume of available data on 'proton-chemical shifts' may be applied directly for the interpretation of ^{3}H-NMR spectra. In other words no new correlations need to be determined, as in the case for ^{13}C-NMR-sepctroscopy.

The various advantages of ^{3}H-NMR spectroscopy are, namely :

- a rapid direct and non-destructive method,
- provides direct information on regiospecificity,
- gives quantitative distribution of the label,
- caters for accurate and precise information on the stereochemistry of the label, and
- requires only millicuries (mCi) rather than microcuries or lesser amounts of radioactivity.

Table 23.2, records the nuclear properties of ^{1}H, ^{3}H (T) and ^{13}C isotopes being employed particularly in various arms of **'Life Sciences' :**

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* Amersham International plc & Dept. of Chemistry, University of Surrey, (UK)—a collaboration project (1968) ;

** A solid-state 90 MHz Fourier Transform Instrument with a 96 MHz channel for ^{3}H observation, proton-spin decoupling and a ^{2}H field-frequency lock with computer control and hard-disc storage of acquired data.

Table 23.2 : Nuclear Characteristics* of ^{1}H, ^{3}H (T) and ^{13}C Isotopes Employed Specifically in 'Life Sciences'

S. No.	Nuclear Characteristics	^{1}H	^{3}H(T)	^{13}C
1.	Natural Abundance (%)	99.984	$< 10^{-16}$	1.11
2.	Nuclear spin	$\frac{1}{2}$	$\frac{1}{2}$	$\frac{1}{2}$
3.	Magnetic momentum ($\mu/\mu N$)	4.8371	5.1594	1.2162
4.	Magnetogyric ratio ($\gamma/10^7$ radians $T^{-1}s^{-1}$)	26.7519	28.5336	6.7263
5.	Resonance frequency (MHz at 2.114 T)	90.0	96.0	22.6
6.	Relative sensitivity for equal numbers of nuclei at constant field	1.0	1.21	1.59×10^{-2}
7.	Half-life	—	12.43 *y*	—
8.	Radiation	Stable	—	Stable
9.	Maximum (MeV)	—	0.018	—

23.2.8. ^{13}C-NMR-SPECTROSCOPY

The 'carbon-skeleton' has been viewed directly with the help of Carbon-13 NMR spectroscopy on a particle basis since early 1970's ; whereas ^{1}H-NMR spectrometry started in late 1950's. The valuable contribution made by various researchers**, between 1976 and 1980, has virtually placed ^{13}C-NMR to a strategically much advanced stage where it gives a clear edge over ^{1}H-NMR in terms of not only its versatility but also its wide application in analysis.

^{13}C-NMR refers to recording another NMR-spectrum but of the C-13 atoms rather than the hydrogen atoms. In actual practice, however, -'these spectra are recorded in such a manner that each chemically distinct carbon gives rise to single peak, without any coupling or fine structure'.

Hence, simply a count of the peaks can be used to see how many carbons are actually present in the molecule. But this particular technique is not reliable for a molecule that exhibits symmetry, because this would ultimately reduce the number of peaks.

It is interesting to note that ^{12}C nucleus is not magnetically **'active'** (spin quantum number I = 0), whereas the ^{13}C nucleus, like the ^{1}H nucleus, has a spin number I = $\frac{1}{2}$. Keeping in view the nuclear characteristic features one may observe that the natural abundance of ^{13}C is equal to 1.1% that of ^{12}C and also the sensitivity of ^{13}C is equal to 1.6% that of ^{1}H. Therefore, the overall sensitivity of ^{13}C compound with ^{1}H stands at 1/5700.

There are *three* short-comings of ^{13}C-NMR spectra, namely :

(1) Only 1% of the carbon in the molecule is carbon-13,

(2) Sensitivity is consequently low, and

(3) Recording the NMR-spectra is a tedious and time consuming process. However, with the advent of recent developments in NMR-spectroscopy it is quite possible to eliminate some of these short comings adequately. They are :

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* Harris, RK, NMR and the Periodic Table, **Chem. Soc. Rev.,** 5, 1, 1976

Weast, RC, **Handbook of Chemistry and Physics,** 62nd, ed., Cleveland, CRC-Press, 1981-1982.

** Wehrli, FW, and T. Wirthin., **'Interpretation of Carbon-13 NMR spectra'**, London, Heyden, 1976

Abraham, RJ, and P. Loftus., **'Proton and Carbon-13 NMR spectroscopy',** London, Heyden, 1978

Levy, GC, RL Lichter, and GL Nelson, **'Carbon-13 Nuclear Magnetic Resonance,** 2nd, ed., New York, Wiley-Interscience, 1980.

(*a*) Development of powerful magnets (**'supercon' magnets**) has ultimately resulted in relatively stronger NMR-signals from the same number of atoms,

(*b*) Improved hardware in NMR-spectroscopy has gainfully accomplished higher sensitivity, and

(*c*) Development of more sensitive strategies has made it possible to record these C—H correlation spectra in a much easier manner.

Therefore, it is now possible either to record the ^{13}C-NMR signal and place the hydrogens in the undetected **'second dimension'** or to record the signal from the hydrogens and place the ^{13}C resonances in the **'indirect dimension'**.

In actual practice, the latter mode is technically more demanding and affords results that are much higher in sensitivity. Recent developments in NMR-spectrometer hardware and technique have made this more-sensitive-mode of operation, termed as **'inverse-detection',** rather readily applicable to modern analysis.

23.2.9. 2D-NMR (TWO DIMENSIONAL CORRELATION SPECTROSCOPY OR TWO DIMENSIONAL COSY SPECTRUM)

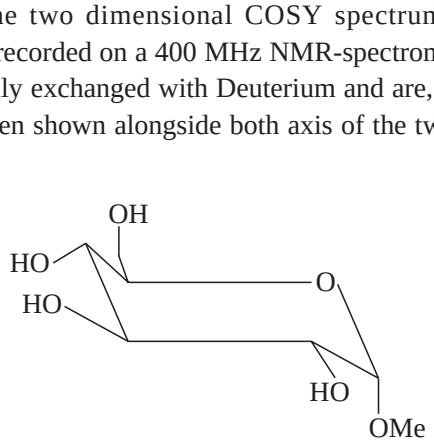
The interaction between different hydrogens in a molecule, known as **'scaler'** or **'spin-spin coupling',** transmitted invariably through chemical bonds, usually cover 2 or 3 at the most. Therefore, when a hydrogen with a chemical shift 'A' is coupled to a hydrogen with chemical shift 'B', one would immediately make out that the hydrogens must be only 2 or 3 bonds away from one another. To know exactly with particular hydrogens are coupled to one another it is necessary to record a two-dimensional **'Correlation Spectroscopy'** (COSY) spectrum.

Generally, a normal NMR-spectrum has amplitude plotted *Vs* just one frequency-dimension (the ppm scale). In 2D-NMR, the amplitude is plotted **Vs** two frequency-dimensions (two ppm scales), normally in the form of a counter plot, just like a topographic map.

The most important aspect about these 2D-NMR spectra is that they show the relation between the peaks in an NMR-spectrum.

Example : A peak at ordinate A ppm in one dimension and B ppm in the other simply indicates that a hydrogen with shift A is duly coupled to a hydrogen with shift B. In short, this is all the information which one needs to interpret in a COSY-spectrum. Thus, the resulting chemical shifts of coupled protons may be simply read off the spectrum.

Figure 23.4, illustrates the two dimensional COSY spectrum of a sugar : 1-0-methyl α-D-glucopyranoside (1) that has been recorded on a 400 MHz NMR-spectrometer ; the sample was dissolved in D_2O so that the OH protons get duly exchanged with Deuterium and are, therefore, not seen at all. Besides, the ^{1}H-NMR-spectrum has also been shown alongside both axis of the two dimensional spectrum in Figure 23.4.



1-o-Methyl α-D-glucopyranoside

Salient features of ^{1}H-NMR and 2D-NMR spectra are, namely :

1. At 3.25 ppm a sharp intense peak, labelled B, is characteristic of an —OMe moiety,

2. At 4.6 ppm there appears another strong peak which is due to the presence of some residual HDO in the D_2O that was initially employed to dissolve the sample,
3. At 4.65 ppm, the two peaks or multiplets are due to H which are distinctly separated from the rest of the spectrum. These are known to belong to the aromatic proton H_1 which is present adjacent to two oxygen atoms, and
4. The COSY-spectrum (Figure 23.4) depicts a so called cross-peak CH at F_1 = 4.65 ppm ; F_2 = 3.4 ppm which means that the proton is coupled to another proton whose shift is 3.4 ppm.

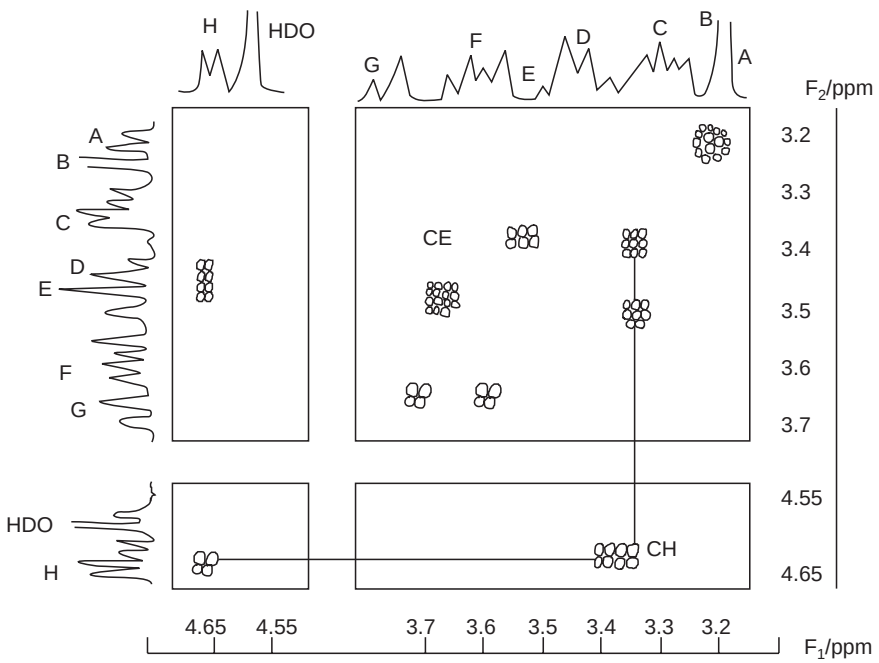


Figure 23.4 : 2D-NMR COSY Spectrum of 1-0-Methyl α-D-Glucopyranoside.

Thus, looking at the structure of compound (1), the resonance C at 3.4 ppm may be due to H_2 by connecting it to H_1.

Following along it may be observed that from multiplet C there exists another cross-peak, CE at F_1 = 3.5 ppm, F_2 = 3.4 ppm, suggesting thereby that the proton resonating at 3.4 ppm is duly coupled to one resonating at 3.5 ppm ; this identifies multiplet E as H_3. In fact, from this single COSY-spectrum (Figure 23.4) one may identify the complete chain of coupled protons as it goes round the pyranose ring.

However, in actual practice with a little skill and expertise one may :

(*i*) Read off the bonding network from the spectrum,

(*ii*) Interpret a COSY spectra easily, because without it finding the coupled pairs of hydrogens is mostly not only ambiguous but also time-consuming, and

(*iii*) Reveal a few chains of coupled resonances.

23.3. INTERPRETATION OF A NMR-SPECTRUM

The interpretation of a NMR-spectrum can be accomplished by determining the following parameters for each signal methodically as described below :

23.3.1. CHEMICAL SHIFT (δ) (RELATIVE TO REFERENCE COMPOUND, USUALLY Me_4Si)

The chemical shift indicates the environment of the proton. One may refer to the tables and charts in various reference books* for approximate ranges of δ for ^1H in different environments.

23.3.2. RELATIVE PEAK AREA

This is equal to the height of step of the integration trace.

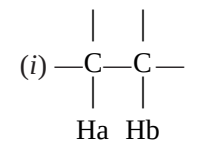
In fact, peak area is proportional to the number of protons causing the signal. Always look for simple ratios *e.g.*, 3 : 1, rather than (say) 14 : 4. A strong singlet (or upfield triplet) may indicate CH_3 ; the corresponding integration steps provide a good starting point for ascertaining the relative number of protons present in the molecule under investigation.

23.3.3. MULTIPLICITY OF THE SIGNAL

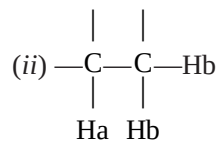
The number of peaks in a regularly split signal (*e.g.*, a regularly spaced triplet, quartet etc.,) or other recognisable splittings (*e.g.*, doublet of doublets etc.,), should be noted carefully.

Therefore, multiplicity and the relative peak heights in a multiplet provide an useful additional check on the relative number of protons obtained from the integration of peak areas.

Thus, coupling ^1Ha to another ^1Hb may give rise to a doublet or a triplet or a doublet-of-doublet as shown below :

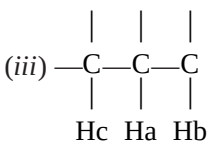


Multiplet
1 : 1 Doublet



Triplet
1 : 2 : 1
$(n + 1)$-plet

n identical ^1H



1 : 1 Doublet of 1 : 1 Doublet

[Hb ≠ Hc]

n protons different from $(n + 1)$-plet of $(m + 1)$-plets where *m* other protons.

23.3.4. COUPLING CONSTANT (J)

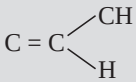
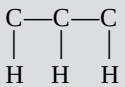
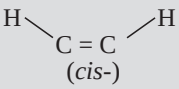
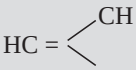
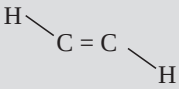
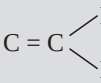
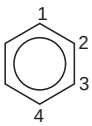
It represents regular multiplets. Actually, J is the separation (in Hertz ; Hz = sec^{-1}) between the peaks of regular multiplets.

The coupling constants help in the identification of the coupled nuclei because Jab = Jba : and are therefore, useful in characterizing the relative orientations of interacting protons.

---

* Silverstein, R.M. and G.C. Basler, **Spectrometric Identification of Organic Compounds,** New York Wiley, 1968. Jackman, L.M. and S. Sternhell, **Applications of NMR Spectroscopy in Organic Chemistry,** London, Pergamon Press, 1969.

A few examples of ^1H coupling constants, J (Hz) are given below :

C—C Linkages	Value of J	C—C Linkage	Value of J
	7		4—10
	O	 (*cis*-)	6—14
	0.5—2	 (*trans*-)	11—20
	0—3		
	1, 2 ~ 8 1, 3 ~ 2 1, 4 ~ 0 } Value of J		

23.4. INSTRUMENTATION

The NMR-spectrum can be scanned either by changing the frequency of the radio-frequency oscillator or by changing the spacing of the energy levels while making a small change in the applied magnetic field.

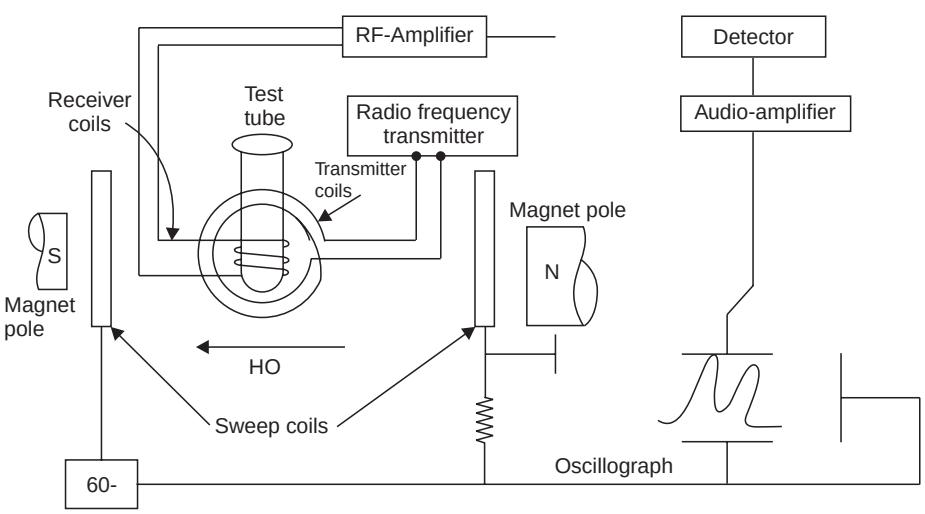


Figure 23.5 : Schematic Block Diagram of an NMR-Spectrometer.

The sample is introduced in a test-tube between the pole faces of a DC-electromagnet whose gap field can be varied from zero upto 14,092 gauss and even scaled upto 23,000 gauss in sophisticated versions of the instrument. The pole pieces are nearly 12 inches in diameter and are spaced approximately 1.75 inches apart. In order to flip the rotating nuclear axis with regard to the magnetic field an oscillating radio-frequency field, supplied by low power, crystal-controlled oscillator is strategically placed at right angles that would be perpendicular to the plane of the paper. The coil that transmits the radio-frequency field is made into two-halves to allow insertion of the sample holder, and the two halves are placed in the gap of the magnetic poles.

Coils located within the pole gap allow a sweep to be made through the applied magnetic field that produces resonance in the range of precession frequencies.

A few turns of wire wound tightly around the sample tube forms a separate radio-frequency coil which picks up the resonant signals emitted from the sample. The receiver coil is perpendicular to both the stationary field and the radio-frequency transmitter coil so as to minimise pick-up from these fields. Thus, energy is absorbed from these receiver coils when nuclear transitions are induced. Absorption of energy causes the radio-frequency voltage across the receiver coil to drop. This voltage change is amplified and detected by a high-gain-radio frequency amplifier and a diode-detector which is tuned to the same frequency as the ratio frequency transmitter.

The resulting DC-voltage is placed on the vertical plates of an oscilloscope to produce an intensity as a function of frequency which is nothing but the desired NMR-spectrum.

23.5. APPLICATIONS OF NMR-SPECTROSCOPY IN PHARMACEUTICAL ANALYSIS

NMR-spectroscopy has been extensively employed for the identification testing as well as quantitative analysis of pharmaceutical substances. These *two* aspects shall be discussed in the sections that follow :

23.5.1. IDENTIFICATION TESTING

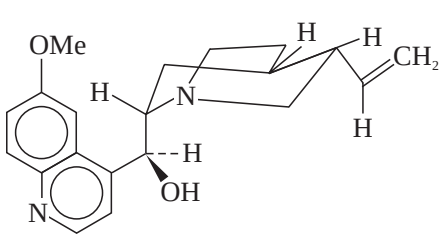
The versatility and ability of NMR to distinctly differentiate nuclei in various intramolecular environments has placed it as the most reliable and dependable technique for carrying out the identification testing of a host of pure drugs. Hence, any apparent deviations of the spectrum of a sample under investigation *vis-a-vis* the spectrum of the pure and the authentic pharmaceutical substance usually give rise to an enormous information not only confined to the true identity of the substance but also the probable nature of the impurities it possesses.

The survey of literature provides ample evidence of the NMR spectra of a good number of medicinal compounds belonging to various categories, namely : sulphonamides ; barbiturates* ; amphetamines** ; steroids ; antihistamines*** ; penicillins and cephalosporins**** to name a few.

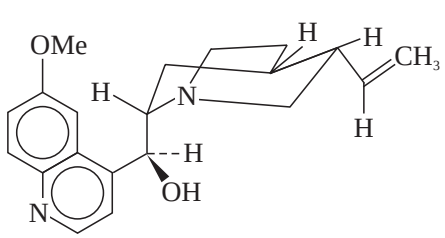
23.5.2. ASSAY OF DRUGS

A plethora of pure drugs, their respective combinations and their dosage forms have been assayed by NMR-spectroscopy quantitatively by various researchers and the result(s) thus obtained were duly verified and compared with the standard methods prescribed in various *official compendia*. A few typical examples of such drugs shall be described briefly here :

A. Quinidine in Mixtures and Hydroquinidine***



I



II

* Fratiello, A., M. Mardirossian and E. Chavez., **J. Magn. Reson., 12**, 221, 1993.

** Warren, RJ, PP Bagosh and J.E. Zarembo, **J. Assoc. Offic. Anal. Chem., 54,** 1179, 1971.

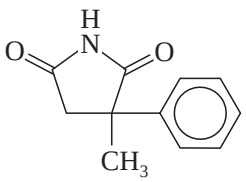
*** Chang, CJ and CE Peck, **J. Pharm. Sci., 65,** 1019, 1976.

**** Wilson, WL, HW Avodich and D.W. Hughes, **J. Assoc. Offic. Anal. Chem., 57,** 1300, 1974.

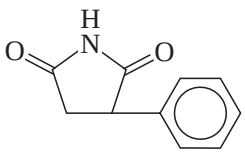
***** Huynh-Ngoc, T. and G. Sirois, **J Pharm. Sci., 62,** 1334, 1973.

A given sample containing a mixture of quinidine (I) and hydroquinidine (II) is dissolved in requisite quantity of deutrochloroform ($CDCl_3$) along with 2, 3, 5-triboromothiophene as the **internal standard**. The quantitative determination is carried out by comparing the peak area attributed by ethylene of (I) at 5.16 ppm to the internal standard peak at 6.93 ppm. The coefficient of variation was found to be 1%.

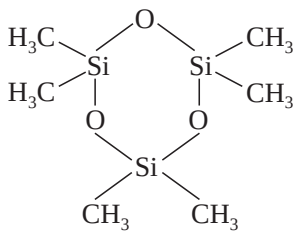
B. Assay Methsuximide and Phensuximide Capsules*



(I)



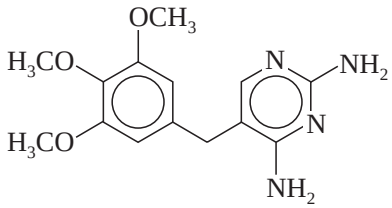
(II)



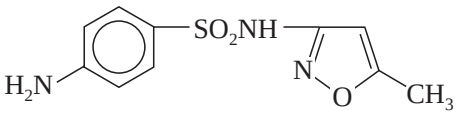
(III)

The analysis of methsuximide (I) is performed in carbon tetrachloride and of phensuximide (II) in 10% v/v dichloromethane in carbon tetrachloride. In this particular analysis hexamethylcyclotrisiloxane (III) is employed as an **internal standard** for (I) and (II) ; whereas the frequencies are referenced to usual tetramethylsilane (TNS).

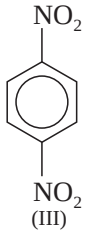
C. Assay of Trimethoprim and Sulphamethoxazole in Tablets and Powders**



(I)



(II)

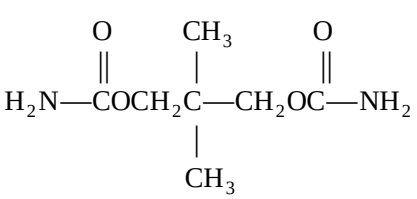


(III)

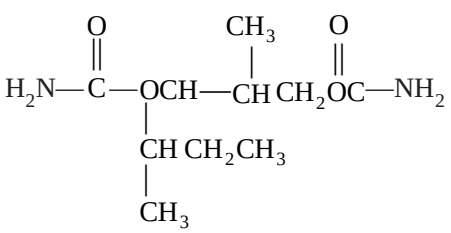
The simultaneous assay of trimethoprim (I) and sulfamethoxazole (II) present either in tablets or powder may be done effectively by NMR method.

Here, a powdered sample comprising 1 mg of (1), 50 mg of (II), and 30 mg of pure 1,4-dinitrobenzene (III) as **internal standard** is carefully dissolved by heating in 1 ml of dimethylsulphoxide-d_6 and subsequently centrifuged to eliminate solid residues, if any. For trimethoprim (I) : the assay is solely based on the singlets at 3.40 and 3.55 ppm on account of the aromatic and methoxy protons of (I) respectively. For sulfamethoxazole (II) the singlet at 2.3 ppm is particularly due to the methyl group of (II) ; and the singlet at 8 ppm is due to (III). It is, however, pertinent to mention here that the assay results were fairly in agreement with British Pharmacopoeial method of analysis. Finally, the NMR-spectroscopic method coefficient of variation was found to be only 0.9%.

D. Assay of Meprobamate and Mebutamate



(I)



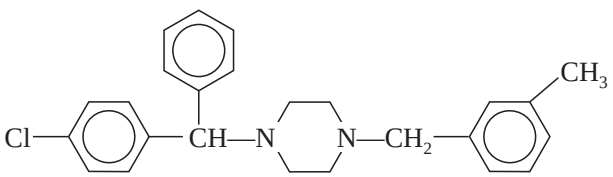
(II)

* Turczan, JW and BA Coldwitz, **J. Pharm. Sci., 62,** 1705, 1973.

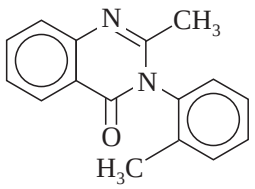
** Rodriguez, MR, MT Pizzorna and S.M. Albonico, **J. Pharm. Sci., 66** 121, 1977.

The assay of meprobamate (1) and mebutamate (II) have been accomplished* by using malonic acid as the **internal standard** and acetone as the solvent. The results obtained were fairly comparable to the lengthy official procedures.

E. Assay of Meclizine and Methaqualone



(I)



(II)

NMR-assay of meclizine (I) and methaqualone (II), besides a number of other potent hypnotics and their corresponding mixtures have been successfully carried out using an external standardization procedure reported**. It is, however, interesting to observe that additional sources of variability are usually incorporated into an assay employing external standardization, and the same has been duly shown in the results thus obtained *i.e.*, a large coefficient of variation to the extent of 4% achieved.

F. Assay of Iodine Values of Natural Oils

Natural oils like : olive, peanut, sunflower seed contain mostly the triglycerides, which usually give rise to *four* characteristic sets of signals in their corresponding PMR-spectra due to the resonance of alkenyl protons, namely :

(*i*) 4, C-1 glyceride methylene protons,

(*ii*) 1, C-2 glyceride methylene proton,

(*iii*) Methylene protons directly linked to a double-bond, and

(*iv*) Remaining protons on saturated carbon atoms.

Hence, it is possible to measure accurately the integration curve given out by the combined C-1, and C-2 glyceride methylene protons that occurs almost separately at 4. Now, employing these as an internal calibration one may determine conveniently the following *two* vital informations, such as :

(*a*) the total number of alkenyl protons, which is a measure of degree of unsaturation, and,

(*b*) the total number of protons, which is a measure of the average molecular weight.

Thus, the iodine values assayed (calculated) from the alkenyl proton integration*** and the corresponding molecular weight match quite favourably with the results obtained by Wijs Method as shown below :

Natural Oils	NMR-Method	Wijs Method
Olive Oil	80.8 ± 0.9	83.0-85.3
Peanut Oil	94.5 ± 0.6	95.0-97.2
Sunflower Seed Oil	135.0 ± 0.9	135.0-137.7

In addition to the above cited typical examples there are a quite a few other drug substances which have been duly assayed by NMR-spectroscopy, thus suggesting the versatility of this technique as an important analytical tool.

---

* Turezan, JW and TC Kram, **J. Pharm. Sci. 56,** 1643, 1967.

** Rucker G. and PN Natrajan, **Arch Pharm., 300,** 276, 1967.

*** Johnson, LF and JN Shoolery, **Analyt. Chem., 34,** 1136, 1962.

THEORETICAL AND PRACTICAL EXERCISES

1. What do you understand by NMR-Spectroscopy ? Discuss the NMR-phenomenon including the following vital aspects :

(*i*) Spinning Nucleus, (*ii*) Effect of an External Magnetic Field,

(*iii*) Precessional Motion, (*iv*) Precessional Frequency, and

(*v*) Energy Transitions.

2. Explain the following terminologies usually encountered in NMR-Spectroscopy :

(*a*) Spin quantum number, (*b*) Chemical shift,

(*c*) Spin-spin interactions, and (*d*) Pascal's triangle.

3. Discuss expatiately the underlying variants with respect to NMR-Spectroscopy :

(*a*) ^{3}H-NMR, (*b*) ^{13}C-NMR,

(*c*) 2D-COSY SPECTRUM.

Give suitable examples whereve necessary to justify your plausible explanation.

4. What essential steps you would consider in order to interpret a NMR-spectrum ? Explain.

5. Explain with the help of a neat, labeled, schematic block diagram of an NMR-Spectrometer ; and describe its operational modalities briefly.

6. Can one make use of NMR-Spectroscopy as an '**identification testing**' method for pharmaceutical substances ? Explain with typical examples.

7. How would you carry out the '**assay**' of the following pharmaceutical products :

(*a*) Quinidine in mixtures of hydroquinidine,

(*b*) Methsuximide and Phensuximide Capsules,

(*c*) Trimethoprim and Sulfamethoxazole Tablets,

(*d*) Meprobamate and Mebutamate, and

(*e*) Meclizine and Methaqualone.

Explain with procedural details sequentially.

8. Is it possible to exploit NMR-spectroscopy in the '**assay**' of **iodine values** of Natural Oils ? Explain with theoretical aspects and typical examples.

RECOMMENDED READINGS

1. Bhacca, NS and DH Williams, **Applications of NMR-Sectroscopy in Organic Chemistry, Illustrated from the Steroid Field,** San Fransisco, Prentice-Hall, 1964.

2. Jackman, LM and S. Sternhell, **Application of Nuclear Magnetic Resonance in Organic Chemistry,** 2nd., ed., New York, Oxford, 1969.

3. Flynn, EH, **Cephalosporins and Penicillins,** Yew York, Academic Press, 1972.

4. Heftmann, E., Ed., **Modern Methods of Steroid Analysis,** New York, Academic Press, 1973.

5. Kasler, F., **Quantitative Analysis by NMR-Spectroscopy,** London, Academic Press, 1973.

6. Chamberian, NF, **The Practice of NMR-Spectroscopy,** New York, Plenum Press, 1974.

7. Evans, EA, DC Warrell, JA Elvidge and JR Jones, **Handbook of Tritium NMR-Spectroscopy and Applications,** New York, John Wiley and Sons, 1985.

24 EMISSION SPECTROSCOPY

CONTAINS :

24.1. INTRODUCTION

Emission spectroscopy is exclusively related to atoms whereas a number of other spectroscopic techniques deal with molecules. The fundamental fact of emission spectroscopy is very simple, wherein the atoms present in a sample undergo excitation due to the absorption of either electrical or thermal energy. Subsequently, the radiation emitted by atoms in an excited sample is studied in an elaborated manner both qualitatively and quantitatively. Therefore, emission spectroscopy is considered to be an useful analytical tool for the analysis of :

(*i*) elemental analysis of metals,

(*ii*) identification and quantitative determination of metallic elements,

(*iii*) estimation of metalloids *e.g.*, arsenic, silicon, selenium, present is extremely low concentrations, and

(*iv*) analysis of solids, liquids or gases as follows :

solids-as such or evaporated solutions,

liquids-atomized spray, analyzed occasionally, and

gases-analyzed rarely.

In short, emission spectroscopy is considered to be the most accurate, precise and reliable means of quantitative analysis of elements as on date. If proper skill, precautions and wisdom are applied together this method may be adopted safely and conveniently to analyze approximately seventy elements from the **'periodic table'** at a concentration as low as 1 ppm.

24.2. THEORY

The theoretical aspects of emission spectroscopy may be categorized into the following *four* heads, namely :

(*a*) **Spectra :** A beam of light on being passed either through a Nicol's prism or a grating, is split-up right into its constituent array of colours frequently termed as **spectrum**. However, the complete

spectrum has a wide range that may be further divided into various regions based on their respective wavelengths (0 to 35,000° A) :

(*i*) **Ultraviolet Region :** It embraces radiations of wavelengths between 0 to 4000° A,

(*ii*) **Visible Region :** It includes radiations of wavelengths between 4000 to 7300° A, and

(*iii*) **Infrared Region :** It has radiations of wavelengths between 7300 and 35,000° A.

(*b*) **Classes of Spectra :** There exist, in fact, two major types of spectra commonly termed as *emission spectra* and the *absorption spectra* which shall be discussed briefly as follows below :

(*i*) **Emission Spectra :** An element on being heated to a very high temperature either by electrical method or a thermal method-usually emits light. This particular light after passing through either a prism or a grating when studied directly with the help of a spectroscope, gives rise to a spectrum, that is termed as **emission spectrum.**

(*ii*) **Absorption Spectra :** A source of light emits a continuous spectrum when first made to pass through an absorbing substance and subsequently through a spectroscope. It has been noticed that a few lines are missing in the observed spectrum thereby leaving either dark bands or lines at their respective places. Because the light of wavelength exactly corresponding to these dark bands (or lines) is found to be absorbed by the substance through which light is passed, the resulting spectrum is called as an **absorption spectrum.**

(*c*) **Classification of Emission Spectra :** The emission spectra may be classified into the following *three* types, namely :

(*i*) **Band Spectra (or Molecular Spectrum) :** Each molecule upon excitation gives out a band spectrum (or bands) that are characteristics of the molecule. In fact, a band spectrum comprises of groups of lines so near to one another that under normal circumstances they more or less seem to appear as continuous bands.

However, in emission spectroscopy the band spectra provided by molecules may be eliminated completely by giving energy to the corresponding molecules so that they may be split-up into separate atoms.

(*ii*) **Continuous Spectra :** A continuous emission spectrum is obtained when solids are heated to incandescence. The thermal radiation of this nature is termed as black-body radiation, which has the following *three* characteristic features, namely :

(*a*) Dependent more on the temperature of the emitting surface than the material of which the surface is made of,

(*b*) Caused by the innumerable atomic and molecular oscillations excited in the condensed solid by the thermal energy, and

(*c*) Independent of the chemical composition of the substance.

Example : Incandescent solids, *e.g.*, carbon and iron give rise to continuous emission spectra when they are heated until they glow.

Hence, it is pertinent to mention here that the continuous spectrum cannot be employed effectively for spectrochemical analysis and these spectra may be eliminated completely by volatalizing the material (sample) before excitation.

(*iii*) **Line Spectra :** Line spectra are usually encountered when the light emitting substance *i.e.*, the radiating species are separate atomic entities (particles) which are distinctly separated from one another, as in gas. Therefore, it is invariably known as 'atomic spectrum'. As the line spectrum depends solely upon the type of an atom, hence it enjoys the status of a predominant type of emission spectroscopy.

Bohr's theory rightly explains the fundamental origin of **'line spectrum'** according to which :

- An atom in the ground state has its electrons present in the lowest permitted energy-levels,
- An excited atom (by thermal or electrical means) has its electrons migrate from inner orbitals (specifically valence electrons) to outer orbitals,
- The excited electrons quickly give a photon of energy of immediately take the position in an orbital having the lowest energy (or ground state), and
- The emission of radiation from the excited atoms give rise to distinct spectral lines thereby forming the basis of emission spectroscopy.

Figure 24.1, depicts the energy-level diagrams both for an atom and a simple molecule illustrating the source of a line-spectrum and a band-spectrum as discussed above in (*iii*) and (*i*).

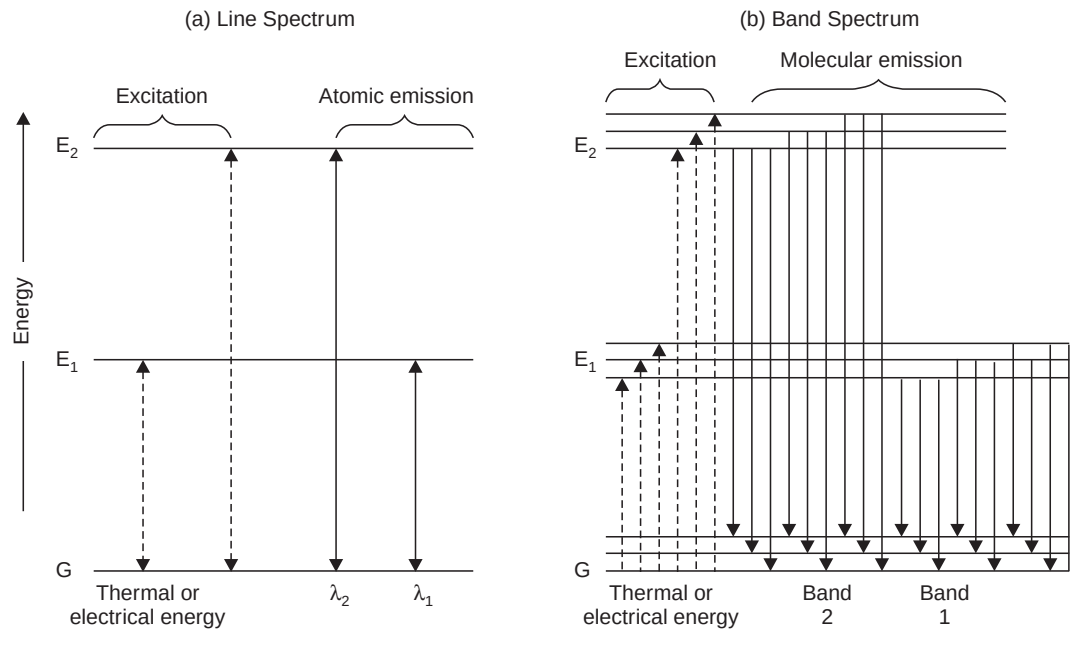


Figure 24.1 : Energy level Diagrams for an Atom and a Molecule Depicting the source of (a) a Line Spectrum and (b) a Band Spectrum.

Figure 24.1 (*a*), designates the energy level diagram displaying the source of the lines in a typical spectrum of an element, where :

G = The horizontal line represents the ground state energy or the lowest energy of an atom (say Na atom), and

E_1 and E_2 = Represent the two higher energy electronic levels of the atom (say Na atom).

For a Na atom the single-outer-electron in the lowest ground state G is situated in the 3*s* orbital. Consequently, the energy level E_1 might designate the energy of the atom when this 'single electron' has been duly raised to the 3*p* state by virtue of its absorption of thermal, electrical or radiant energy. This phenomenon has been clearly shown with the help of the dotted-line in Figure : 24.1 (*a*). However, the atom ultimately gets back to its ground state, may be after 10^{-8} s, thereby emitting radiation whose frequency is given by the following expression :

$$\upsilon_1 = (E_1 - G)h$$

or

$$\lambda_1 = hc/(E_1 - G)$$

This particular phenomenon is depicted by the solid-line in Figure 24.1 (*a*). In the case of Na atom E_2 designates the highly energetic 4*p* state and the radiation λ_2 obtained therefrom will appear at a relatively shorter wavelength.

Figure 24.1 (*b*), represents the energy level diagram of a molecule where the energy differences among the various quantized vibrational and rotational states are comparatively much smaller as compared to the electronic states. The horizontal lines are due to the many excited vibrational states whereas the energy differences due to rotational states have not been shown in the said Figure. Thus, the multitude of various energy states is clearly shown by the solid lines in Figure 24.1 (*b*), whereby two distinct bands of radiation are obtained, each of which consists of a huge number of closely spaced lines.

(*d*) **Effect of Concentration on Line and Band Spectra :** The radiant power by virtue of the radiant energy, of a line or band exclusively depends directly on the total number of excited atoms or molecules present, which is subsequently proportional to the total concentration of the species present in the source. Therefore, we may have the following expression :

$$P = kC$$

where, P = Radiant power,

C = Total concentration of the species, and

k = Constant of proportionality

The aforesaid relationship forms the basis of quantitative emission spectroscopy.

(*e*) **Excitation-Energy Requirements :** A single spectral-line is emitted from an element only when the energy equivalent to the excitation potential of the element is usually absorbed. This particular requirement is very critical and important. Exactly in a similar manner, the full-fledged complete spectrum is obtained possibly only when the energy equivalent to the ionization potential is absorbed by a molecule.

(*f*) **Limitations of Emission Spectroscopy :** The emission spectroscopy has a number of limitations that are enumerated below briefly :

(1) Perhaps all the elements present in the periodic table might be excited to yield respective emission spectra by employing a huge energetic source. However, it has a serious drawback because most of the spectral lines invariably fall within the vacuum-ultraviolet region thereby rendering their critical studies rather difficult. Hence, the emission spectroscopy is exclusively limited to metals and metalloids. The non-metals, for instance : Phosphorus, Sulphur, Carbon etc. are not limited to these studies.

(2) **Emission spectroscopy of sodium *vis-a-vis* uranium :** Emission spectroscopy is mainly based on sensitivity which is inversely proportional to the complexity of the atomic spectra. In actual practice, it has been observed that the spectra of alkali-metals, like : K, Na, Li, Rb appear to be very simple and hence they may be studied conveniently without any difficulty. It is also pertinent to mention here that these spectra usually comprise of 13 to 14 adequately spaced lines having reasonably good sensitivity and possessing wavelengths.

In the specific case of sodium the resulting emission spectrum shall exhibit characteristic yellow lines. The spectrum is so highly sensitive that even the traces of Na show yellow lines distinctly.

In the case of other elements, for instance : Uranium, the emission spectrum normally displays thousands of narrowly spaced lines. However, the emission source possesses a fixed amount of energy which shall be spread up eventually amongst the thousands of lines thereby minimizing the sensitivity of each line. Hence, it is rather difficult to examine the less sensitive complex spectra of elements such as uranium.

24.3. INSTRUMENTATION

The various essential components of a reasonably good emission spectrograph are as follows, namely :

(*i*) Excitation sources,

(*ii*) Electrodes,

(*iii*) Sample Handling,

(*iv*) Monochromators,

(*v*) Detectors, and

(*vi*) Spectrographs.

24.3.1. EXCITATION SOURCES

The excitation sources may be sub divided into the following *two* heads, namely :

(*a*) **Salient Features of Excitation Sources :** These should fulfil the following procedural requirements :

- Sample should be changed into its vaporised form,
- Vaporised form of sample must be dissociated into atoms,
- Electrons present in the atoms should be excited from the ground state to higher-energy-levels,
- Capable of exciting atoms of most of the elements of interest (in the *Periodic Table*),
- To produce sufficient line-intensity in order to detect these lines within the scope of the '*detection limit*', and
- Must essentially achieve reproducible excitation conditions of various samples.

(*b*) **Types of Excitation Sources :** The various types of excitation sources are as follows :

(*i*) **Flames :** A flame is generally employed for such molecules that do not need either very high temperatures for excitation or dissociation into atoms. Flames are comparatively inexpensive and cater for both stable and reproducible sources of excitation that can effectively handle a wide-range of typical analytical problems. However, the temperature of the flame is guided by a number of vital factors, such as :

- Types of Fuel and Oxidant,
- Fuel to Oxidant Ratio,
- Type of Burner Employed, and
- Zone (or region) in flame which is focussed into the entrance-slit of spectral-isolation-unit.

Table 24.1, records the temperatures of commonly used fuels and oxidants in flames in emission spectroscopy.

Table 24.1 : Temperatures of Commonly Used Fuels and Oxidants in Flames

S. No.	Type of		Temperature (°C)
	Fuel	Oxidant	
1.	Natural Gas	Oxygen	2700
2.	Natural Gas	Air	1700
3.	Acetylene	Oxygen	3200
4.	Acetylene	Air	2200
5.	Hydrogen	Oxygen	2800
6.	Acetylene	Nitrous Oxide	3400

Note : (1) The temperature of the flame and the composition of the flame afford a direct influence on interferences which may give rise to erroneous results,

(2) The dissociation of molecules and excitation of atoms usually occur at a specific temperature.

(*ii*) **Direct Current Arc :** It is considered to be one of the most versatile excitation modes used extensively for quantitative spectrochemical emission analysis. Figure 24.2 represents the different essential components of the circuit for a direct current are

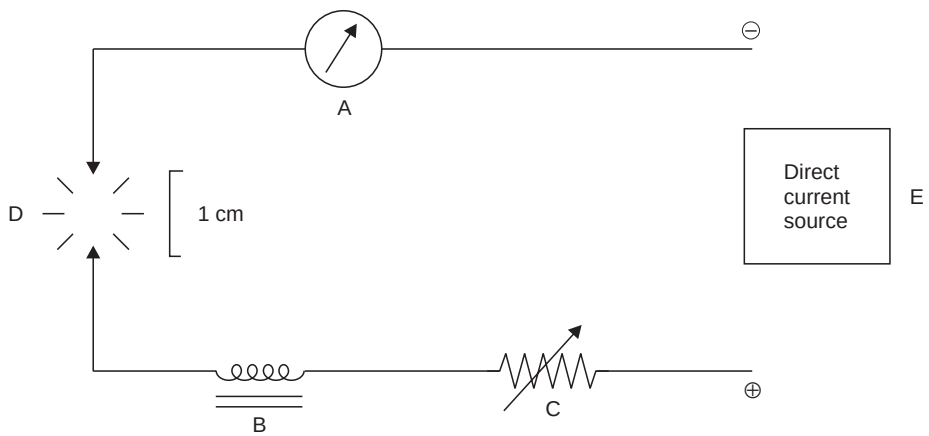


Figure 24.2. Circuit Diagram for a Direct Current Arc.

A = An Ammeter (Range 3 to 30 A),

B = Inductance Coil,

C = Variable Resistance (Range 10 to 40 Ω)

D = Arc Gap (Range from 20 mm to 1 cm), and

E = Direct Current Source (Range 110 to 220 V at 3 to 30 A).

Procedure : The various procedural steps are as follows :

- Current is passed across the arc-gap in series with the help of a variable resistor C (10 – 40 Ω) and an inductance coil B.
- Initial resistance caused due to air-gap is very high to allow conduction of current. Hence, the arc is first initiated by narrowing its gap momentarily while 110-220 V DC is applied. Once the current picks up flow, the temperature across the arc-gap shoots up promptly. The electrodes are pulled apart leaving a gap of 20 mm to 1 cm, thereby establishing the electric arc whose temperature varies from 4000 to 8000° K.
- Sample (solid or liquid) is usually introduced upon the lower electrode between the arc-gap, and
- Variable resistance (C) adjusts the intensity of current, whereas inductance coil (B) stabilizes its flow.

Merits of Direct-Current-Arc : They are as follows :

- Provides a very sensitive excitation source :
- Excitation energy is solely thermal and not electrical which is more than enough for exciting all the metal elements, and
- DC-arc gives rise to emission species that are exclusively neutral atoms rather than ions.

(*iii*) **Alternating Current Arc :** Figure 24.3 depicts the various essential components of the circuit diagram for an alternating current arc :

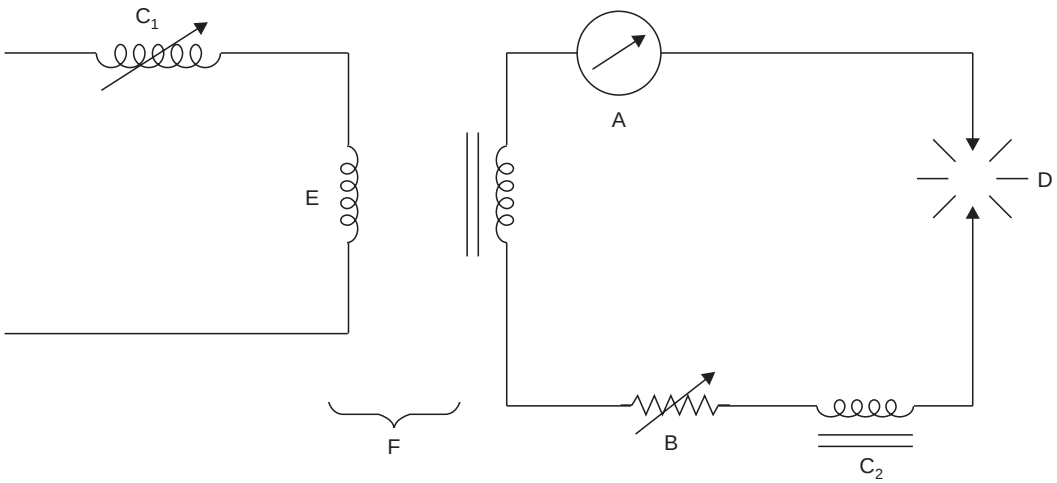


Figure 24.3 : Circuit Diagram for an Alternating Current Arc.

Where, A = Ammeter (Range 3 to 30 A)

B = Variable Resistance,

C_1 = Variable Inductance in the Primary Circuit,

C_2 = Inductance Coil in the Main Circuit,

D = Arc Gap (Range from 20 mm to 1 cm),

E = Primary Circuit, and

F = Step-up Transformer (Range 2000 to 5000 V).

Procedure : The procedural details are stated below :

- Step-up transformer (F) maintains a high voltage of 2000 to 5000 V, which helps the arc to jump the gap,
- Variable inductance (C_1) is adjusted duly to maintain a current of 1 to 5 A in the primary circuit,
- Current in the main circuit is alternating at a frequency of 60 Hz thereby extinguishing the arc 120 times in one second, and
- After each cycle the arc picks out a new surface area whereby the entire surface of the sample under examination, is exhaustively arced and subsequently excited.

It is worthwhile noting that the arc-gap temperature in this case is considerably lower than the direct-current arc, due to the stop-and start nature of the source, which ultimately offers a much lower sensitivity.

24.3.2. ELECTRODES

The electrodes normally employed in emission spectroscopy are of *two* types, namely :

(*a*) **Self Electrodes :** If the material (sample) under probe is itself not only a good conductor but also can tolerate very high temperatures (in the *arc-gap*), the material may be used as the electrode ; and such electrodes are termed as **self-electrodes.**

Examples : Pure metal powders may be compressed into solid discs or cylinders which can be used as electrodes. Likewise, the analyzing alloys can also be used.

(*b*) **Graphite Electrodes :** If the material (sample) under study is neither a good conductor nor can afford to tolerate high temperatures, it is usually kept in a small cavity of the lower graphite electrode whereas the upper electrode (graphite) is given a pointed sharp-shape. These electrodes have centre posts which minimises *wandering-of-the-arc source* thereby improving the reproducibility ; and their narrow neck improves the sensitivity appreciably.

24.3.3. SAMPLE HANDLING

Two types of samples are usually examined by emission spectroscopy, namely :

(*a*) **Solids :** Solid samples can also be sub-divided into two categories, such as : (*i*) Those possessing good conductance characteristics and can withstand high temperatures : it can be achieved by making electrodes with the material directly to be used for the electrical discharge ; (*ii*) Those having poor conductance and cannot withstand high temperatures : it can be powdered mixed with the powdered graphite (known as buffer) and placed in the depression of the lower graphite electrode. On passing the electrical discharge the material (sample) is first vaporised into the body of the discharge and subsequently the spectrographic emission occurs.

(*b*) **Liquids :** Liquid samples may be dispensed conveniently with the aid of two types of small-holders, namely : *firstly*, wherein the porous base of the cup gradually releases the sample into the discharge from the top ; and *secondly*, wherein the rotating-disc carriers take up the sample into the discharge from the bottom steadily.

Note : (1) Both types are found to be suitable for either aqueous or non-aqueous solvents, and

(2) Samples dissolved in organic solvents usually ignite in the discharge which may produce erratic emission. It is more prominent in the rotating-disc type sample carriers.

24.3.4. MONOCHROMATORS

Monochromators help to isolate and separate the various lines of the sample's emission spectrum. Two types are generally used in the emission spectroscopy, namely :

(*a*) **Prism Monochromators :** In usual practice, the materials of construction of prisms are either quartz or silica (fused) because of their absolute transparency to UV-radiation. Prism monochromators normally bring forth *two serious shortcomings* which are discussed briefly here, namely :

First, when light from a single emission-line (of one particular wavelength) is made to pass through a quartz (or glass) prism, it emerges from the other side of the prism as two different lines as shown in Figure 24.4 (*a*). This splitting-up of one line into two separate lines affords not only the loss of the emerged light's intensity but also complicates the interpretation of the spectrum ; thereby rendering its use both in qualitative and quantitative analysis rather difficult. Cornu Type Prisms eliminate this lacuna completely. In this case, two-half prisms are joined together : the first half-prism splits the incident emission line into two separate beams, whereas the second-half prism recombines them into a single emergent beam as shown in Figure 24.4 (*b*).

Secondly, the dispersion of a prism is never constant over a wide range of wavelength, whereby the identification of either the emission lines or the unknown wavelengths is rather difficult on the basis of simply measuring their dispersions.

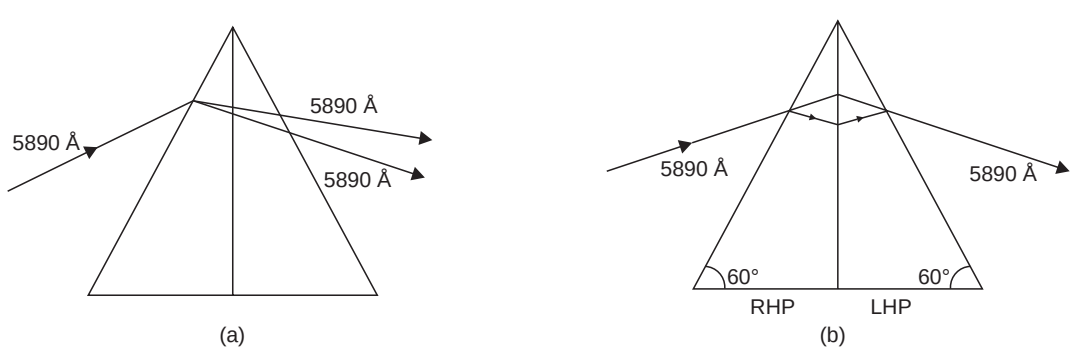


Figure 24.4. (*a*) Emergence of two lines from a single Quartz Prism ; (*b*) Cornu Type Prism-with two half-prisms-emergence of a single line. (RHP = Right Hand Polarization and LHP = Left Hand Polarization).

(*b*) **Grating Monochromators :** The various advantages of grating monochromators are as follows :

- **Much better resolution achieved :** thereby resulting in the development of many sophisticated equipments,
- **Offers absolute linear dispersion :** thereby replacing prisms completely as the dispersing element inspite of its high-cost, and
- **Resolution is constant and independent of wavelength :** thereby the identification of the wavelength of emission lines on a photographic plate is simplified *i.e.*, once a known reference line is identified, other lines may be known very conveniently.

Disadvantage : The major disadvantage of grating monochromators is that its higher-order-wavelengths overlap which may be eliminated completely either by using filters or by employing detectors that are not sensitive to the higher-orders.

24.3.5. DETECTORS

There are *two* types of detectors that are used most frequently in emission spectroscopy, namely :

(*a*) **Photographic Detectors-**used for qualitative analysis, and

(*b*) **Photomultiplier Detectors-**used for quantitative analysis.

The *two* detectors shall be discussed here briefly.

24.3.5.1. Photographic Detectors

Many spectrographs record the intensity of spectral lines on a photographic emulsion directly, which is subsequently developed by an appropriate **'developer'** in the prescribed duration at a specific recommended temperature.

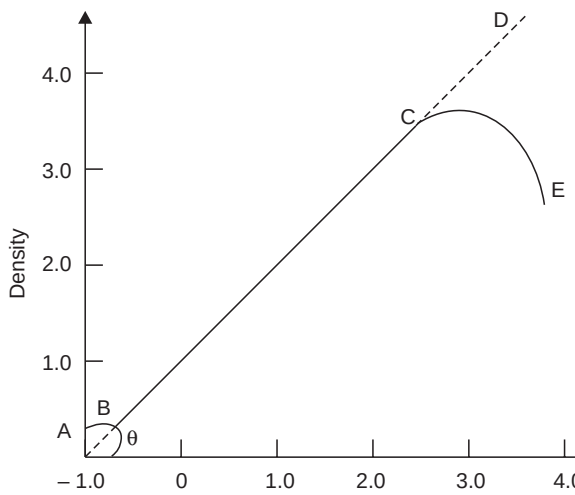


Figure 24.5 : Photographic Detection Curve Density Vs Log Exposure.

Procedure : The various steps involved are as follows :

1. A beam of light is passed through a clear zone of the film and subsequently the intensity of the transmitted beam is measured by means of a phototube fitted in the densitometer,
2. A beam of light is then passed through the darkened zone of the film and the intensity is measured as stated above,
3. The logarithm of the ratio of the intensity of the light transmitted through the clear zone and the darkened zone is computed ; and is plotted against the logarithm of the exposure as shown in Figure 24.5.

4. The region BC in Figure 24.5 clearly shows that the density is directly proportional to the logarithm of the intensity of the curve and represents the most useful zone of the curve, and
5. The slope of region BC is usually called as the 'gamma' (γ) of the emulsion of photographic plate and is expressed as :

$$\gamma = \tan \theta$$

Consequently, it may be inferred that when the value of γ is high, it is indicative of the fact that high-degree of contrast is expected ; and if γ has a low value, naturally low-degree of contrast is deemed for.

(*b*) **Photomultiplier Detectors :** Spectrographs that record the direct-reading emissions exclusively essentially make use of photomultiplier detectors instead of a photographic plate. It requires a large number of photomultiplier tubes for carrying out the detection of different emission lines simultaneously and that is way the direct-reading devices are relatively much costlier. By virtue of its convenience, fast and more accurate and precise results, this type of detectors is always preferred.

However, it is worthwhile to have a comparison of the merits and demerits of photographic and photomultiplier detectors side-by-side as follows :

Photographic Detector	Photomultiplier Detector
Merits :	
1. Large number of spectral lines may be recorded at the same time.	It cannot be achieved.
2. Provides a permanent record of the spectrum that may be stored.	It is less versatile.
3. Emission intensity may be integrated by a photographic emulsion over a period of time.	It cannot be obtained.
4. Photographic emulsions have a very high degree of sensitivity throughout the visible and UV-regions.	It is not so sensitive.
5. Cost-effective detector.	Very costly detector.
Demerits :	
1. Requires controlled photographic development that involves a lot of time and enhances the risk of errors.	Does not require either controlled photographic development or have risk of error.
2. Does not display quick response to spectral lines.	It shows immediate response to spectral lines.
3. Interpretation of spectral lines not so necessary.	Interpretation is easier and hence makes it the most desirable detectors.

24.3.6. SPECTROGRAPHS

The resulting **'emission spectra'** from the detector may be thoroughly studied with the aid of an effective optical arrangement which will critically identify the frequencies and their respective intensities. The optical arrangement varies from one instrument to another based on the device used, and hence the nomenclature also varies, namely :

S. No.	Nomenclature	Device Used	Measurement Performed
1.	Spectroscope	Visual	Frequencies
2.	Spectrograph	Photographic	Wavelengths (intensities)
3.	Spectrometer	Scanning a spectrum	Wavelengths (intensities)

However, the various commercially available spectrographs may be differentiated solely by the fact whether they make use of either a **'prism'** or a **'grating'** as the vital dispensing medium. A good 'spectrograph' using either a prism or a grating shall be discussed briefly here.

(*a*) **Littrow Type Spectrograph (*i.e.*, a Prism Instrument)**

Figure 24.6, shows the schematic diagram of a **Littrow Type spectrograph** which essentially has the following components, namely :

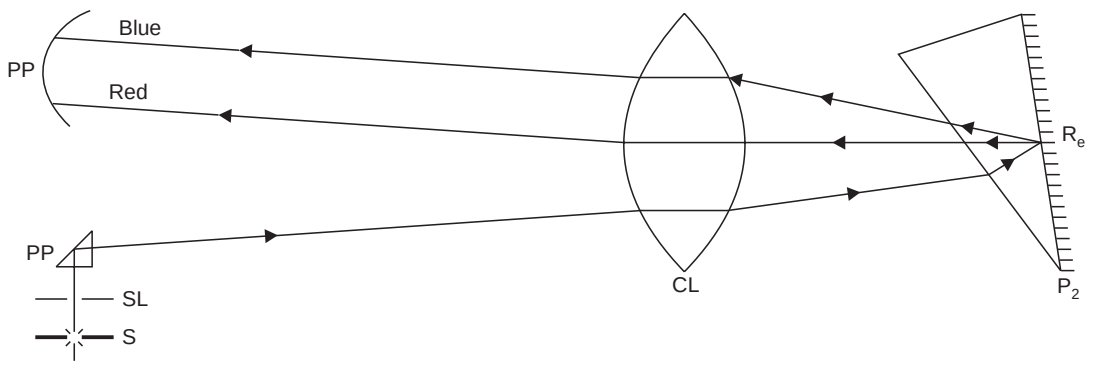


Figure 24.6 : A Schematic Optical Diagram of a Littrow-Type Spectrograph.

S = Excitation source,

SL = Slit,

P_1 = A reflecting prism,

CL = A collimating lens,

P_2 = Littrow prism,

RC = Reflective coating (mirrored surface), and

PP = Photographic plate.

A **Littrow type spectrograph** makes use of a Littrow-type prism exclusively which is made from a single piece of Quartz with its rear-surface mirrored or metallized (with Silver). This sort of prism completely eliminates the polarization effects as the beam of light moves back and forth through the body of the same prism. Thus, a beam of light from the source of light (S) passes through the slit (SL), gets reflected through the reflecting prism (P_1), penetrates through the collimating lens (CL), enters the Littrow prism (P_2), again gets reflected by its reflective coating (RC), enters the collimating lens (CL) and finally comes out as a spectrum that is recorded on the photographic plate (PP).

It is interesting to observe that a typical large Littrow Spectrograph having a single Quartz prism covers a wavelength range from 2000 to 80000 Å.

(*b*) **Ebert-Mounting Spectrograph (*i.e.*, a Grating Instrument)**

An **Ebert-mounting spectrograph** exclusively makes use of a plane-grating rather than a concave-grating as employed either in *Rowland mounting* or in *Eagle arrangement*. It enormously helps as the ruling of the grating is a lot easier and less complicated. In this particular optical device a concave mirror (CM) is used to render the radiation striking the grating (G) parallel and also to focus the dispersed (-o-o-o-) radiation on the photographic plate of the camera.

Figure 24.7, depicts the schematic diagram of a **Ebert-Mounting Spectrograph** with the following vital components.

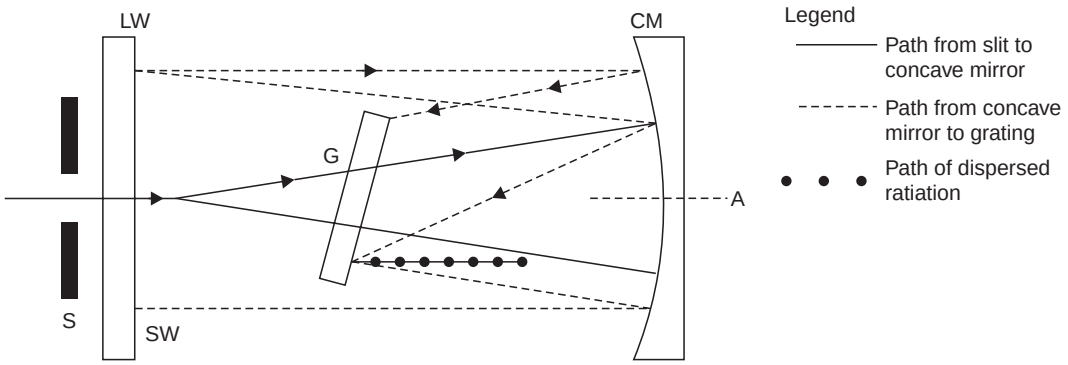


Figure 24.7 : A Schematic Diagram of a Ebert-Mounting Spectrograph.

S = Slit,

G = Grating,

CM = Concave Mirror,

LW = Longer wavelength,

SW = Shorter wavelength, and

A = Axis.

Salient Features of Ebert-Mounting Spectrographs : The various salient features are, namely :

- Gratings normally have 600 to 120 lines per mm,
- Covers a wavelength range from 1800-30,000 Å,
- Possess the highest wavelength range, and
- Possible to observe high-order visible and UV-spectra.

24.4. APPLICATIONS OF EMISSION SPECTROSCOPY

In general, prepare not fewer than three reference solutions of the element to be determined covering the concentration range recommended by the manufacturers for the element and instrument used. Any reagent used in preparing the solution of the substance being examined must be added to the reference solution in the same concentration. Besides, where solids are present in solutions they may give rise to interferences and for that reason the solid content of the solutions must be below 2% wherever possible.

1. Emission spectroscopy has been employed for the analysis of various alloys, namely : aluminium, copper, magnesium, zinc, lead, and tin.
2. It has been used for the analysis of a number of elements, for instance : Na, K, Zn, Cu, Ca, Mg, Ni and Fe present in various tissues of human beings. Changes in trace-metal concentrations have been studied at length with regard to the ageing process.
3. Trace amounts of Ca, Cu, and Zn have been examined in blood samples.
4. Presence of Zn has been examined in the pancreas tissue.
5. To determine the extent of elements present in **'crude oil'** by virtue of the fact that some of these may poison the catalysts used in the cracking-process *e.g.*, V, Cu, Ni, and Fe.

THEORETICAL AND PRACTICAL EXERCISES

1. Discuss the fundamental theory of **'Emission Spectroscopy'**. Substantiate your explanation based on the energy-level diagrams for an 'atom' and a 'molecule'.

2. How would you explain the following cardinal aspects in Emission spectroscopy ?

(*i*) Effect of concentration on **'Line'** and **'Band'** spectra.

(*ii*) Limitations of Emission spectroscopy.

3. With the help of a neat-labeled circuit diagram explain the following :

(*a*) Direct Current Arc

(*b*) Alternating Current Arc

Discuss their procedural steps, merits/demerits explicitely.

4. Describe the two 'common detectors' invariably used in emission spectroscopy. Differentiate the plus and negative aspects encountered in : (*a*) Photographic Detector ; and (*b*) Photomultiplier Detector, briefly.

5. How would you identify the **'frequencies'** and the **'intensities'** of emission spectra by the help of :

(*a*) Littrow type spectrograph, (*b*) Ebert-mounting spectrograph.

Explain the working with a schematic diagram.

6. Enumerate the various applications of **'Emission Spectroscopy'** with respect to the following entities :

(*i*) analysis of alloy,

(*ii*) analysis of elements in tissues,

(*iii*) analysis of elements in blood samples,

(*iv*) analysis of Zn in pancreas tissue, and

(*v*) elements present in 'crude oil sample'.

RECOMMENDED READINGS

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3. **'The International Pharmacopoeia',** Vol. I., **General Methods of Analysis,** Geneva, World Health Organization, 3rd., ed. 1979.
4. Hargis, H.G., **'Analytical chemistry',** New Jersy, Prentice Hall, 1988.

25 FLAME SPECTROSCOPY

CONTAINS :

25.1. INTRODUCTION

Metallic salts (or metallic compounds) after dissolution in appropriate solvents when introduced into a flame (for instance : acetylene burning in oxygen at 3200°C), turns into its vapours that essentially contain mostly the atoms of the metal. Quite a few such gaseous metal atoms are usually raised to a particular high energy level that enables them to allow the emission of radiation characteristics features of the metal : for example-the characteristic flame colourations of metals frequently encountered in simple organic compounds such as : Na-yellow, Ca-brick-red ; Ba-apple-green. This forms the fundamental basis of initially called **Flame Photometry,** but more recently known as **Flame Emission Spectroscopy (FES).**

It is quite evident that a relatively large proportion of the gaseous metal atoms shall remain in the ground state *i.e.,* in an unexcited form. It has been observed that such ground-state atoms shall absorb radiant energy pertaining to their own particular resource wavelength. Therefore, when a light having the same resonance wavelength is made to pass through a flame consisting of such atoms, a portion of the light shall be absorbed accordingly. Furthermore, the extent or degree of absorption would be directly proportional to the total number of ground-state present in the flame. And this is the basis of **Atomic Absorption Spectroscopy** (AAS).

The emission spectrum thus obtained is made up of a number of lines that actually originate from the resulting excited atoms or ions ; and these steps may be shown diagrammatically as represented in Figure 25.1.

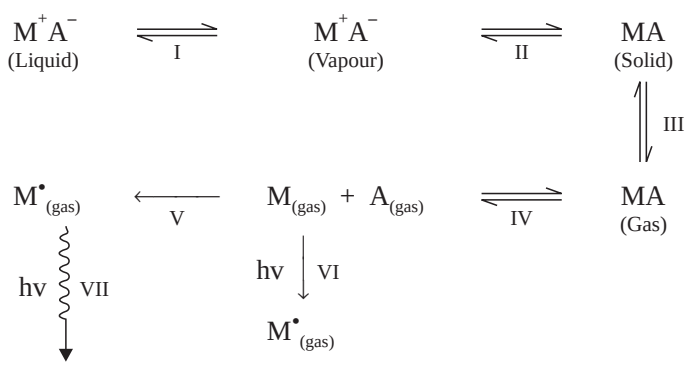

Figure 25.1 : Diagrammatic Representation of Emission Spectrum from excited Atoms or Ions.

The various steps (I to VII) in Figure 25.1, above are explained as under :

Step-I : The liquid sample containing a suitable compound of the metal ($M^+ A^-$) is aspirated into a flame, thereby converting it into its vapours or liquid droplets,

Step-II : The evaporation of vapours (or droplets) give rise to the corresponding solid residue,

Step-III : The vapourization of the solid residue into its gaseous state occurs,

Step-IV : The dissociation of the gaseous state into its constituent atoms, namely : $M_{(gas)} + A_{(gas)}$ take place, that initially, is in ground state,

Step-V : The thermal excitation of some atoms into their respective higher energy levels will lead ultimately to a condition whereby they radiate energy (flame emission) measured by Flame Emission Spectroscopy (FES), and

Step-VI : The absorption of radiant energy by some atoms into their higher energy levels enable them to radiate energy (atomic absorption) measured by Atomic Absorption Spectroscopy (AAS).

25.2. THEORY

The underlying principle of **Flame Emission Spectroscopy (FES)** may be explained when a liquid sample containing a metallic salt solution under investigation is introduced into a flame, the following steps normally take place in quick succession, namely :

(*i*) the solvent gets evaporated leaving behind the corresponding solid salt,

(*ii*) the solid salt undergoes vaporization and gets converted into its respective gaseous state, and

(*iii*) the progressive dissociation of either a portion or all of the gaseous molecules gives rise to free neutral atoms or radicals.

The resulting neutral atoms are excited by the thermal energy of the flame which are fairly unstable, and hence instantly emit photons and eventually return to the ground state (*i.e.,* the lower energy state). The resulting emission spectrum caused by the emitted photons and its subsequent measurement forms the fundamental basis of FES.

Bohr's Equation : If we consider two quantized energy levels *e.g.,* higher as E_2 and lower as E_1, the radiation given out during the transition from E_2 to E_1 may be expressed by the following equation :

$$E_2 - E_1 = h\,\nu \qquad ...(a)$$

where, h = Planck's constant, and

ν = Frequency of emitted light,

now, the frequency ν may be defined as follows :

$$\nu = c/\lambda \qquad ...(b)$$

where, c = Velocity of light, and

λ = Wavelength of the absorbed radiation.

Combining equations (*a*) and (*b*) we have :

$$E_2 - E_1 = hc/\lambda$$

$$\lambda = hc/E_2 - E_1 \qquad ...(c)$$

The expression (*c*) is the **Bohr's equation** which enables us to calculate :

- Wavelength of the emitted radiation which is characteristic of the atoms of the particular element from which it was initially emitted,

- Wavelength of radiation given out from a flame is indicative of the element(s) that might be present in that flame, and
- Intensity of radiation may quantify the exact amount of the elements present.

Boltzmann Equation : The fraction of free atoms which are excited thermally, or in other words, the relationship between the ground-state and the excited-state quantum is exclusively represented by the Boltzmann equation given below :

$$N_1/N_0 = (g_1/g_0)\, e^{-\Delta E/kT} \qquad .....(d)$$

where, N_1 = Number of atoms in the excited state (high energy level),

N_0 = Number of ground state atoms,

g_1/g_0 = Ratio of statistical weights for ground and excited states,

E = Energy of excitation (= $h\upsilon$),

k = The Boltzmann's constant, and

T = Temperature (in Kelvin).

Form equation (*d*) it may be observed that :

- Fraction of atoms excited (N_1) solely depends upon the temperature of the flame (T), and
- Ratio N_1/N_0 is dependent upon the excitation energy (ΔE).

Therefore, the fraction of atoms excited critically depends on the temperature of the flame thereby emphasizing the vital importance of controlling the temperature in **Flame Emission Spectroscopy (FES).**

25.3. INSTRUMENTATION

There are *two* types of **Flame Photometers** that are used invariably in *Flame Emission Spectroscopy* (FES), namely :

(*a*) Simple Flame Photometer, and

(*b*) Internal Standard Flame Photometer.

These *two* typical instruments shall be discussed briefly here highlighting their various components and procedural details.

25.3.1. SIMPLE FLAME PHOTOMETER

The line-sketch of a simple flame photometer is shown in Figure 25.2.

In general, Flame Photometers are designed and intended mainly for carrying out the assay of elements like : Sodium, Potassium, Calcium, and Lithium that possess the ability to give out an easily excited flame spectrum having sufficient intensity for rapid detection by a photocell.

Procedure : The compressed and filtered air (A) is first introduced into a Nebulizer (E) which creates a negative pressure (suction) enabling the liquid sample (C) to gain entry into the atomizer (E). Thus, it mixes with the stream of air as a fine droplet (mist) which goes into the burner (G). The fuel gas (D) introduced into the mixing chambers (F) at a given pressure gets in touch with the air and the mixture is ignited. Consequently, the radiation from the resulting flame (H) is made to pass through a convex lens (I) and ultimately through an optical filter (J) that allows specifically the radiation characteristic of the element under examination to pass through the photocell (K). Finally, the output from the photocell is adequately amplified (L) and subsequently measured on an appropriate sensitive digital-read-out device.

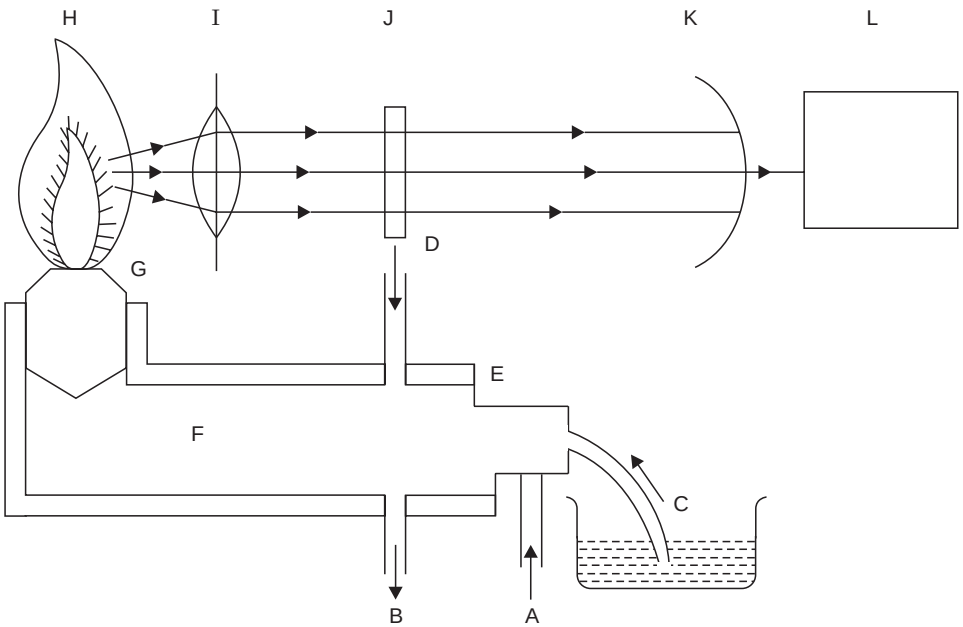

Figure 25.2 : Layout of a simple Flame Photometer
[Corning model 410 Flame Photometer is based on this pattern].

A = Inlet for compressed Air,

B = Drain outlet (to maintain constant pressure head in the mixing Chamber),

C = Liquid sample (sucked into the Nebulizer),

D = Inlet for Fuel-Gas to the Laminar-Flow-Burner,

E = Nebulizer to atomize the liquid sample,

F = Mixing Chamber for Fuel Gas, Compressed Air, and Atomized Liquid Sample,

G = Burner,

H = Flame,

I = Convex lens,

K = Optical filter to transmit only a strong-line of the element, and

L = Amplifier to amplify the feeble electrical impulse and a built-in direct read-out device.

25.3.2. INTERNAL STANDARD FLAME PHOTOMETER

The layout of an internal standard flame photometer is illustrated in Figure 25.3.

A = Inlet for compressed air,

B = Inlet for Acetylene (Fuel-Gas),

C = Liquid sample sucked in by an atomizer,

D = Flame,

E = Mirror,

F = An optical filter to allow the transmission of only one strong-line of the element,

G = Amplifier to amplify the weak electrical current,

H = A Null detector to record the intensity of the element under study and the internal-standard (Lithium),

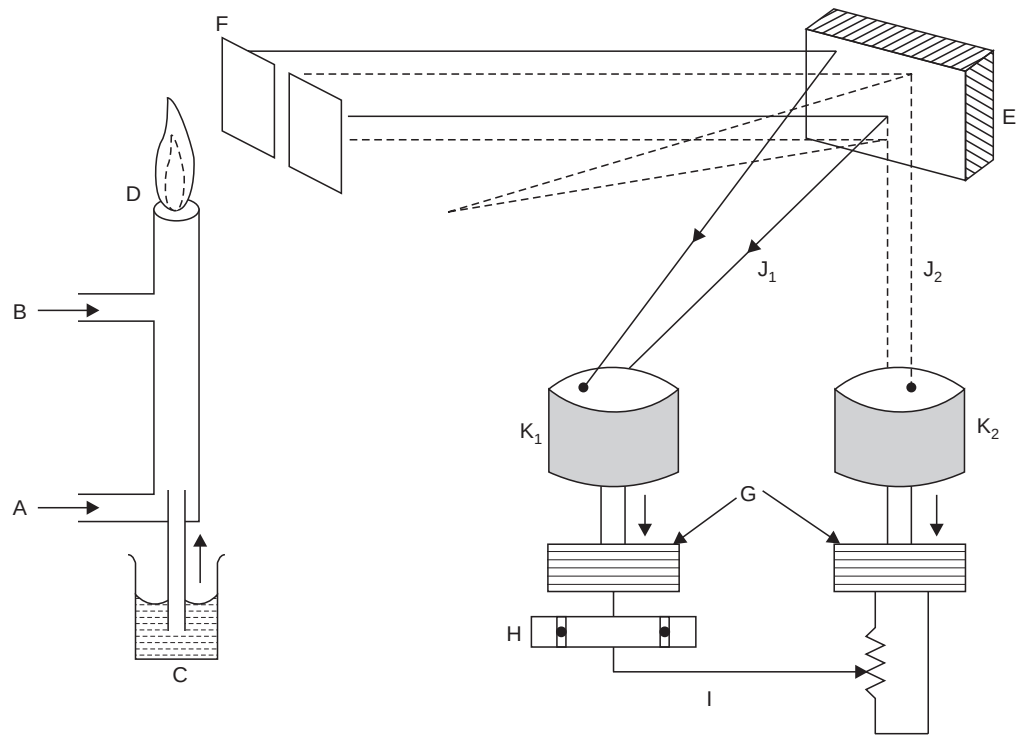

Figure 25.3 : Layout of an Internal Standard Flame Photometer.

I = A calibrated potentiometer,

J_1 = Lines due to the 'sample'

J_2 = Lines due to the Internal Standard 'Lithium', and

K_1 & K_2 = Photocells to convert light-energy to electrical impulse.

The use of an internal standard flame photometer not only eliminates the visible effects of momentary fluctuations in the flame characteristics produced by variations in either the oxidant or under full pressures, but also the errors caused due to differences in surface tension and in viscosity are minimised to a great extent.

Procedure : In this particular instance 'Lithium' is employed as an internal standard and an equal concentration is added simultaneously to the sample and the standard solutions. The sample (C) solution having the internal standard (Lithium) is sucked in by an atomizer and a fine spray is thereby introduced into the flame (D). The radiation thus emitted is subsequently passed through a filter (F) and then collected by a mirror (E). The emitted radiation reflected from the mirror is split up into two parts : the first part is caused due to the internal standard (Lithium), whereas the second part arises due to the element under examination. Both these lines J_1 and J_2 are passed through the respective photocells K_1 and K_2 whereby the light energy is transformed into the electrical impulses. These electrical impulses are usually very weak and feeble and hence, they are duly amplified by a suitable amplifier (G) individually and are subsequently introduced into the common detecting device (H) *i.e.,* a **'Null detector'**-so as to enable it to record the intensity of the element under investigation and also the internal standard (Lithium) accurately using a calibrated potentiometer (I).

In short, an **internal-standard flame photometer** provides a direct and simultaneous result with respect to the ratio of intensities.

25.4. APPLICATIONS OF FLAME EMISSION SPECTROSCOPY IN PHARMACEUTICAL ANALYSIS

A few typical example of **'flame-emission spectroscopy'** are given below.

25.4.1. ASSAY OF SODIUM, POTASSIUM AND CALCIUM IN BLOOD SERUM AND WATER

(*i*) **Standard potassium and sodium solutions, approximately 500 ppm :** Weigh accurately 0.95 *g* of dried KCl and 1.25 *g* of dried NaCl into separate 1-litre volumetric flasks. Dissolve in water and dilute to the mark.

(*ii*) **Standard calcium solution, approximately 500 ppm :** Weigh accurately 1.25 g of $CaCO_3$, which has been dried at 110°C, into a 500-ml breaker. Add about 200 ml of DW and 10 ml of conc. HCl. Cover the breaker with a watch-glass during addition of acid to prevent loss of solution as CO_2 is evolved. After the solution is complete, transfer it quantitatively into a 1-litre volumetric flask and dilute to the mark with DW.

(*iii*) **Radiation buffer* for sodium determination :** Prepare a saturated solution with reagent-grade $CaCl_2$, KCl, $MgCl_2$, in that order.

(*iv*) **Radiation buffer for potassium determination :** Prepare a saturated solution with reagent-grade NaCl, $CaCl_2$ and $MgCl_2$, in that order.

(*v*) **Radiation buffer for calcium determination :** Prepare a saturated solution with reagent-grade NaCl, KCl, $MgCl_2$ in that order.

Procedure

(*a*) **Preparation of working curves :** Transfer 5 ml of the appropriate radiation buffer to each series of 100-ml volumetric flasks. Add a volume of the standard solution which will cover a concentration ranging between 0 to 100 ppm. Dilute to 100 ml with DW and mix well. Measure the emission intensity of these samples by taking at least three readings for each. Between each set of measurements, aspirate DW through the burner. Correct the average values for background luminosity, and prepare a working curve from these data.

(*b*) **Analysis of blood serum/water sample :** Prepare aliquot portions of the sample as described in the above paragraph (*a*). If necessary, use a standard to calibrate the response of the spectrometer to the working curve. Then measure the emission intensity for the unknown. After correcting the data for background, determine the concentration by comparison with the working curve.

25.4.2. ASSAY OF BARIUM, POTASSIUM AND SODIUM IN CALCIUM ACETATE

The technique of flame emission spectroscopy is used to determine the concentration of Ba, K, and Na ions by measuring the intensity of emission at a specific wavelength by the atomic vapour of the element generated from calcium acetate *i.e.,* by introducing its solution into a flame.

25.4.2.1. For Emission Measurements

Introduce water into the atomic vapour generator, adjust the instrument reading to zero, introduce the most concentrated solution into the generator and adjust the sensitivity to give a suitable reading ; again introduce water or the prescribed solution into the generator and when the reading is constant readjust, if necessary, to zero.

25.4.2.2. Method of Standard Addition

The various steps are as follows :

(1) Place in each of not fewer than three similar graduated flasks equal volumes of the solutions of the substance being examined, prepared as follows :

* Radiation buffers are used to minimise the effect of each ion upon the emission intensity of the others.

(*a*) Prepare a 5.0% w/v solution and use barium solution ASp*, suitably diluted with water to prepare the standard solution.

(*b*) Prepare a 1.25% w/v solution and use potassium solution ASp**, suitably diluted with mater, to prepare the standard solution.

(*c*) Prepare a 1.0% w/v solution and use sodium solution ASp***, suitably diluted with water, to prepare the standard solution.

(2) Add to all but one these flasks a measured quantity of the specified standard solution (marked *; **; ***; above) to produce a series of solution containing increasing amounts of the element being determined.

(3) Dilute the contents of each flask to the required volume with water.

(4) After having calibrated the instrument as directed above, introduce each solution into the generator three times and record the steady reading. If the generator is a flame, wash the apparatus thoroughly with water ; if a furnace is used fire it after each introduction.

(5) Plot the mean of the readings against concentration on a graph the axes of which intersect at zero added element and zero reading.

(6) Extrapolate the straight line joining the points until it meets the extrapolated concentration axis. The distance between this point and the intersection of the axes represents the concentration of the element (*e.g.*, Mg, K, Na) being determined in the solution of the substance being examined.

25.4.2.3. Limits of Elements present in Calcium Acetate Sample

Mg : Not more than 500 ppm of Mg ;

K : Not more than 0.1% of K, and

Na : Not more than 0.5% of Na.

25.4.3. COGNATE ASSAYS

The following substance, namely : magnesium acetate ; potassium citrate ; potassium hydroxide ; potassium nitrate and sodium chloride can also be assayed for their respective elements as shown in Table 25.1 below. However, all the respective solutions of the said pharmaceutical substance and their standard solutions must be prepared as prescribed in BP (1993) strictly to obtain the best results.

Table 25.1 : Assay of Pharmaceutical Substances by Flame Emission Spectrophotometry (or FES):

S.No.	Name of Substance	Elements Present	Qty. Prescribed (% *w/v*)	Limits Prescribed***
1.	Magnesium Acetate	K ; Na ;	For K = 0.50 ; For Na = 1.00 ;	NMT 0.1% of K ; NMT 0.5% of Na ;
2.	Potassium Citrate	Na ;	For Na = 1 mg ; of Na per ml in DW ;	NMT 0.3% of Na ;

* **Barium Solution ASp :** Dissolve 1.778 g of $BaCl_2$ in sufficient water to produce 1000 ml. Dilute it with water so that 1 ml contains 1 mg of Ba.

** **Potassium Solution ASp :** Dissolve 1.144 g of KCl, previously dried at 100° to 105° for 3 hours, in sufficient water to produce 1000 ml. (It contains 600 meg of K in 1 ml).

*** **Sodium Solution ASp :** Dissolve 0.5084 g of NaCl, previously dried at 100° to 105° for 3 hours, in sufficient water to produce 1000 ml. (It contains 200 mcg of Na in 1 ml).

3.	Potassium Hydroxide	Na ;	For Na = 1.0 g in 50 ml DW + 5 ml of 5M H_2SO_4. Dilute to 100 ml with DW. Dilute 1 ml to 10 ml in DW.	NMT 1.0% of Na ;
4.	Potassium Nitrate	Na ;	For Na = 1.0 and measuring at 589 nm	NMT 0.1% of Na ;
5.	Sodium Chloride	K ;	For K = 1.0 ;	NMT 500 ppm

*British Phrmacopeia, Vol. I and II, 1993.

THEORETICAL AND PRACTICAL EXERCISES

1. What is **'Flame Photometry'** ? Explain.

2. Discuss the following theoretical aspects of flame spectroscopy :

(*a*) Bohr's Equation, and (*b*) Boltzmann Equation.

3. What are the two types of Flame Photometers commonly used in **Flame Emission Spectroscopy** (FES) ? Describe them individually with a neat layout and explain their *modus operandi.*

4. How would you assay sodium, potassium and calcium in blood serum and water ? Explain.

5. Explain the assay of Ba, K and Na in calcium acetate using the **'method of standard addition'.**

6. Discuss the assay of the following pharmaceutical substances by **FES-method** :

(*i*) Magnesium acetate, (*ii*) Potassium nitrate,

(*iii*) Potassium nitrate, and (*iv*) Sodium chloride.

RECOMMENDED READINGS

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2. Kolthoff, I.M. and P.J. Elving., **'Treatise in Analytical Chemistry',** Part I, New York, Interscience, 1965.

3. Dean, J.A. and T.C. Bains, Ed., **'Flame Emission Atomic Absorption Spectroscopy',** Vol. I, **Theory,** New York, Marcel Dekker, Inc., 1974.

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6. Beckett, A.H. and J.B. Stenlake, Eds., **'Practical Pharmaceutical Chemistry',** Part II, 4 th ed., London, The Athlone Press, 1988.

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26 ATOMIC ABSORPTION SPECTROSCOPY

CONTAINS :

26.1. INTRODUCTION

Allan Walsh, in 1955*, was the pioneer for the introduction of **atomic absorption spectroscopy** (AAS), which eventually proved to be one of the best-known-instrumental-techniques in the analytical armamentarium, that has since been exploited both intensively and extensively in carrying out the quantitative determination of trace metals in liquids of completely diversified nature, for instance : blood serum-for Ca^{2+}, Mg^{2+}, Na^{+} and K^{+} ; edible oils-Ni^{2+} ; beer samples-Cu^{+} ; gasoline (petrol)-Pb^{2+} ; urine-Se^{4+} ; tap-water-Mg^{2+} ; Ca^{2+} ; lubricating oil-Vanadium (V).

The atomic absorption spectroscopy (AAS) may be accomplished either by using a **flame**-whereby the sample solution is aspirated directly into a flame or by using an **electrothermal** device-whereby the sample solution is first evaporated and then ignited on a hot surface. It has been noticed that gaseous metal atoms in an unexcited form *i.e.,* ground state atoms, will absorb radiant energy related to their own specific resonance wavelength. Hence, when a light with the same resonance wavelength is passed through a flame comprising of such atoms, a part of the light will be absorbed accordingly. Besides, the degree of absorption would be directly proportional to the total number of ground-state atoms present in the flame, which ultimately forms the basis of **Atomic Absorption Spectroscopy (AAS)**.

AAS facilitates the estimation of a particular element in the presence of many other elements efficaciously. In other words, there is absolutely no necessity to separate the '*test element*' from the rest thereby not only saving a great deal of time but also eliminating the possibility of various sources of error incurred by these processes. In addition AAS may be used for the estimation of both aqueous and non-aqueous solutions.

*Walsh, A., **Spectrochim Acta., 7,** 108, 1955.

Because of the fact that AAS is free from any cumbersome-sample-preparation, it has proved to be an ideal, dependent and versatile analytical tool for the non-chemists specifically, for instance : biologists, clinicians and the engineers, whose interest lies only in the significance of the results.

26.2. THEORY

The underlying principle of atomic absorption spectroscopy (AAS) is the absorption of energy exclusively by ground state atoms while they are in the gaseous form.

It may be further expatiated as follows below :

A solution consisting of certain metallic species when aspirated into a flame, it will give rise to the corresponding vapours of metallic species. As it has already been discussed under flame emission spectroscopy (FES) [Chapter-25] : Some metal atoms would be raised directly to an energy level to such an extent as to emit the particular radiation of the metal. At this critical point, a sufficiently large quantum of the metal atoms of a particular element would still remain in the non-emitting ground-state, which in turn shall be receptive of light radiation having their own specific wavelength. Consequently, when a light of this wavelength is passed through a flame ; along the atoms of the metallic species, a portion of the same would be absorbed ; and the resulting absorption has been found to be directly proportional to the density of the atoms present in the flame at that material time. In AAS, one logically determines the amount of light absorbed. In other words, the concentration of the metallic element may be determined directly from the value of absorption.

The total amount of light absorbed may be provided by the following mathematical expression :

$$\text{Total amount of light absorbed (at } \upsilon) = \frac{\pi e^2}{mc} \text{N}f \qquad ...(a)$$

where υ = Frequency of the light path,

e = Charge on the electron,

m = Mass of the electron,

c = Speed of light,

N = Total number of atoms which can absorb at υ, and

f = Ability for each atom to absorb at υ (oscillator strength).

The components in Eq. (a), namely : π, e, m and c are constants, therefore, it can be further written in a simplified form as below :

$$\text{Total amount of light absorbed} = \text{K} \times \text{N} \times f \qquad ...(b)$$

Hence, from Eq. (b) it may be inferred that :

(a) it is independent of the wavelength, and

(b) it is independent of temperature,

More explicitly, the absorption by atom is independent of both the wavelength of absorption and the temperature of the atoms. And these two specific characteristic features give AAS a clear distinct and positive edge over FES.

26.2.1. MERITS OF AAS OVER FES

The various points of merit of atomic absorption spectroscopy over flame spectroscopy are enumerated below :

S. No.	AAS		FES
1.	This technique is superior and specific because of the fact that only the atoms of a particular element can absorb radiation of their own characteristic wavelength.	**1.**	Spectral interferences usually take place in this technique.
2.	A relatively large number of metal atoms produce an atomic absorption signal whereby the effect of flame-temperature variation is negligible in AAS *i.e.,* independent of flame-temperature.	**2.**	A much smaller number of metal atoms do produce an emission signal in FES, showing that this technique is not independent of flame, temperature.
3.	The detection limits of sensitivity of the following elements are more by AAS technique, such as : Ag, As, Au, B, Bi, Cd, Co, and Fe.	**3.**	The detection limits (sensitivity) of the under mentioned elements are higher by FES technique, for instance : Al, Ba, Ca, Eu, Ho, In, K and La.

Note : The detection limits of Cr, Cu, Mn, Mo, Pd, Rh, Ni, and V are almost equal by AAS and FES techniques.

26.2.2. DEMERITS OF AAS

The various points of demerit of atomic absorption spectroscopy are as follows :

(*i*) It essentially requires a separate lamp for each element to be determined ; and this serious lacuna is usually overcome either by using a line-source with the introduction of flame or by using a continuous source with the introduction of a very high resolution monochromator,

(*ii*) AAS cannot be employed very effectively for such elements that produce their corresponding oxides when exposed in the flame, for example : Al, Mo, Si, Ti, W, V. Nevertheless, these estimations may be performed under suitably modified experimental parameters, and

(*iii*) When the solutions of metal salts are made in an aqueous medium the predominant anion present affects the resulting signal to a negotiable extent.

26.3. INSTRUMENTATION

The atomic absorption spectrophotometers are essentially of *two* types, namely :

(*a*) Single-beam Atomic Absorption Spectrophotometer, and

(*b*) Double-beam Atomic Absorption Spectrophotometer.

These two instruments shall be discussed briefly here along with their vital components.

26.3.1. SINGLE-BEAM ATOMIC ABSORPTION SPECTROPHOTOMETER

The schematic diagram of a single-beam atomic spectrophotometer in illustrated in Figure 26.1.

A = Anode (Tungsten),

B = Glass-shield,

C = Neon or Argon at 1-5 torr,

D = Quartz or Pyrex Window,

E = Inlet for Acetylene,

F = Inlet for air,

G = Liquid sample sucked in by an atomizer,

HCL = Hollow cathode Lamp,

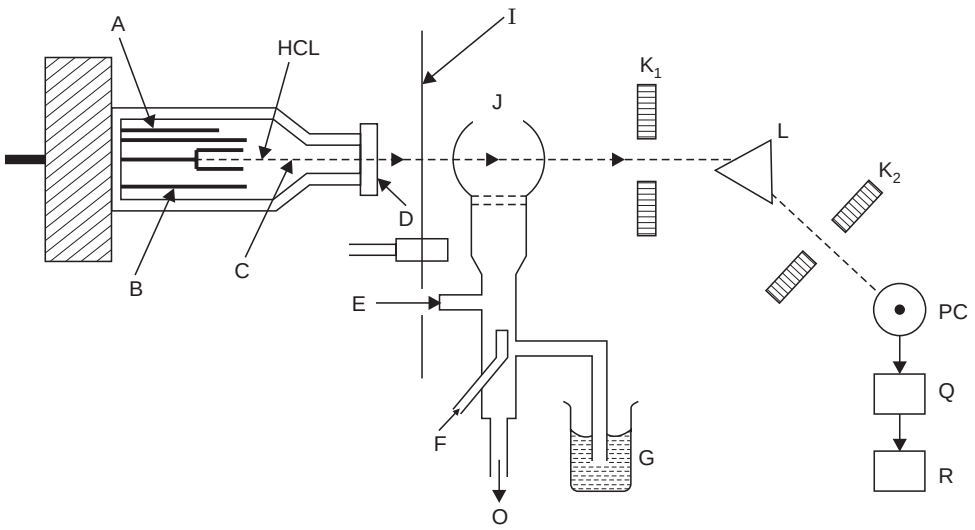

Figure 26.1 : Schematic Diagram of a Single-Beam Atomic Absorption Spectrophotometer.

I = Chopper (a rotating shutter),

J = Flame,

K_1 and K_2 = Slits,

L = Prism or Grating,

M = Photocell,

O = Drain outlet to maintain a constant pressure head in the mixing chamber,

PC = Photocell,

Q = Photodetector, and

R = Amplifier and Recorder.

The most common source for atomic absorption measurements in the 'hollow-cathode-lamp', which essentially consists of a Tungsten anode (A) and a cylindrical cathode (HCL) sealed in a glass tube (B) that is duly filled with neon or Argon (C) at a pressure of 1 to 5 torr. It is a practice to have the cathode constructed of the metal whose spectrum is desired or serves to support a layer of that particular metal. The chopper* (I) is interposed between the hollow-cathode-lamp (HCL) and the flame (J). Subsequently, the liquid sample (G) is sucked in by an atomizer into the flame (J). Just prior to its entry to the flame, the sample solution first gets dispersed into a mist of very small droplets that evaporates in the flame to yield initially the dry salt, and subsequently the vapour of the salt. At this particular stage a portion of this vapour will be dissociated into atoms of the element required to be measured. In this manner, the flame possesses free ground state (*i.e.*, unexcited) atoms that are worthy of absorbing radiations, from an external source when the radiation eventually matches exactly to the energy needed for a transition element from the lower ground-state-level to the upper excited-state-level. The resulting unabsorbed radiation from the flame (J), firstly passes through the slit (K_1) and then through the monochromator *i.e.,* the prism or grating (L) that exclusively isolates the exciting spectral lines of the light source ; secondly, through the slit (K_2) into the photocell (PC), thirdly, into a photodetector (Q) and fourthly, its output is adequately amplified and registered on a recorder (R). It is worthwhile to mention here that the final absorption is measured by the difference in the transmitted signal both in the absence and presence of the element under investigation.

*Chopper is a rotating wheel whose function is to break the steady stream of light from the hollow-cathode-lamp into an intermittent light that gives rise to a **Pulsating current** into the photocell ; this current is duly amplified and recorded.

26.3.2. DOUBLE-BEAM ATOMIC ABSORPTION SPECTROPHOTOMETER

The major disadvantage of a single-beam atomic absorption spectrophotometer (Figure 26.1) lies in its very low stability. The introduction of a **double-beam atomic absorption spectrophotometer** (Figure 26.2) has completely eliminated the above main lacuna and provides much enhanced stability. In this particular instance the chopped beam of light from the hollow-cathode-lamp is split into two parts. The first portion, passes through the flame, while the second portion is made to bypass the flame completely. However, the two separate beams of light are recombined meticulously by an unique optically-designed assembly, passes through a monochromator to a strategically placed detector and ultimately to a sensitive read-out device.

It is pertinent to mention here that a double-beam atomic absorption spectrophotometer is absolutely independent of (*a*) lamp drift, (*b*) sensitivity of detector with time.

The optical path of a double-beam atomic absorption spectrophotometer is depicted in Figure 26.2. The various essential components comprising the optical arrangement in Figure 26.2 are enumerated after the figure.

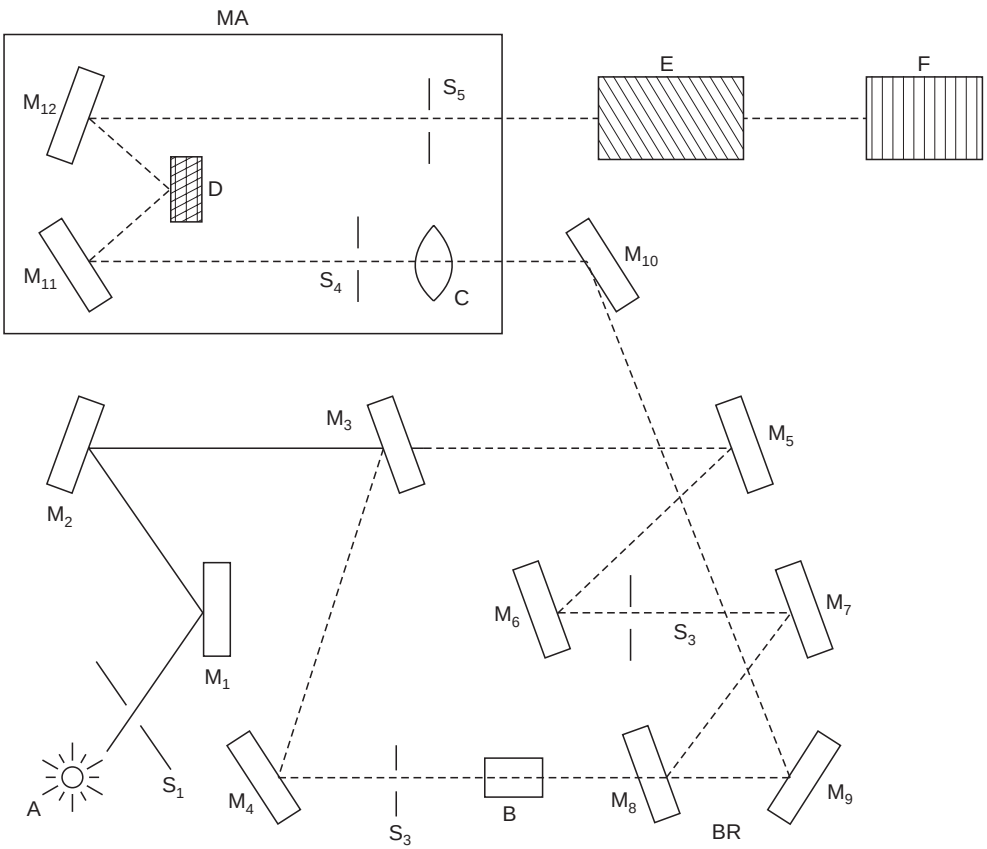

Figure 26.2 : Optical path of a Double-beam Atomic Absorption Spectrophotometer.

A = Source of light (Hollow-Cathode-Lamp),

B = Flame,

C = Field lens,

D = Grating,

E = Detector,

F = Read-out device,

BR = Beam recombination zone,

MA = Monochromator assembly,

S_1 to S_4 = Slits,

S_5 = Exit slit, and

M_1 to M_{12} = Mirrors

The light hollow-cathode-lamp source (A) passes through the slit S_1 and strikes at mirrors M_1 and M_2. The Mirrors M_3 splits chopped beam from the source into two parts ; one passes through the mirror M_4-slit S_2-flame (B)-mirror M_8 and strikes at mirror M_9 to reach mirror M_{10}, and the second strikes at mirror M_6-slit S_3-mirror M_7, M_8 and M_9 respectively to reach the mirror M_{10}. The mirror M_8 and M_9 serve as a **beam recombination zone** (BR). The recombined beam gets reflected by mirrors M_{10} passes through the field lens (C), slit S_4, strikes at M_{11}, passes through the grating (D), to the mirror M_{12} and ultimately passes out through the exit (S_5) and the monochromator assembly (MA) into the detector (E) and finally to the read-out device (F).

26.4. IMPORTANT ASPECTS OF ATOMIC ABSORPTION SPECTROSCOPY

The following *three* important aspects of atomic absorption spectroscopy shall be discussed here briefly, namely :

(*i*) Analytical Techniques,

(*ii*) Detection Limit and Sensitivity, and

(*iii*) Interferences.

26.4.1. ANALYTICAL TECHNIQUES

In atomic absorption spectroscopy (AAS) the technique using *calibration curves* and the *standard addition method* are both equally suitable for the quantitative determinations of elements.

26.4.1.1. Calibration Curves

Theoretically, the absorbance must be proportional to concentrations, however, deviations from linearity usually take place. Therefore, it is necessary to prepare an **empirical calibration curve** (ECC). For this, the standard solutions of the element(s) to be determined are employed to plot the ECC from which the contents in the '**test solutions'** may be measured conveniently.

Figure 26.3, represents the typical calibration curves of copper at 3428 Å, where :

X = Sample absorption reading,

Y = Sample concentration reading, and

Z = Calibration curve.

It is quite evident from the calibration curve (Z) in Figure 26.3, that the linearity between the concentration of Cu (in ppm) and absorbance prevails over the range 2.0 to 10.0 ppm specifically, whereas at higher concentrations the said relationship does not hold good anymore. Hence, it is pertinent to mention here that whenever the quantitative analysis of an element is to be carried out, the absorbance is preferably measured almost under the same experimental parameters whereby the calibration curve was initially constructed.

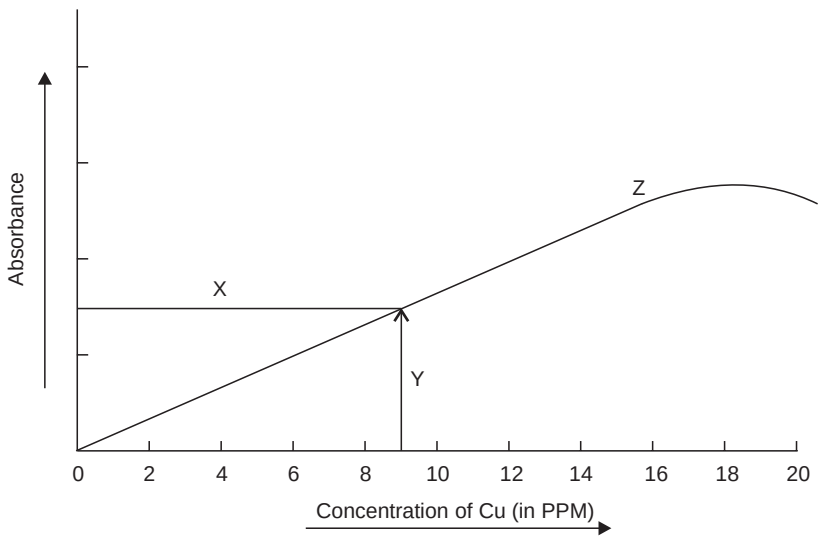

Figure 26.3 : Calibration Curve of Copper at 3428 Å.

26.4.1.2. Standard Addition Method

The **standard addition method** is widely employed in AAS. In this case, two more aliquots of the sample are transferred to volumetric flasks. The first, is diluted to volume, and the absorbance of the solution is measured. The second, receives a known quantity of analyte, whose absorbance is also measured after dilution to the same volume. Likewise, data for other standard additions may also be obtained.

If a plot between absorbance and concentration reveals a linear relationship, which may be accomplished by several stepwise standard additions, the following expressions hold good, namely :

$$A_X = k\, C_X \qquad ...(a)$$

$$A_T = k\, (C_S + C_X) \qquad ...(b)$$

where, C_X = Analyte concentration in the diluted sample,

C_S = Contribution of the added standard to the concentration, and

A_X and A_T = Measured absorbances of C_X and C_S.

Combining Eqs. (*a*) and (*b*) we have :

$$C_X = C_S \frac{A_X}{A_T - A_X} \qquad ...(c)$$

When a number of stepwise additions are performed, A_T can be plotted against C_X. Thus, the resulting straight line may be extrapolated to $A_T = 0$. By substituting this value in Eq. (*c*) we may have at the intercept :

$$C_X = - C_S \qquad ...(d)$$

Advantage : The major plus points of the standard addition method is that it tends to compensate for variations caused by physical and chemical interferences in the sample solution.

26.4.2. DETECTION LIMIT AND SENSITIVITY

Detection Limit : It may be defined as the concentration (meg ml^{-1}) of an element that gives rise in the shifting of absorbance signal to an amount which equals to the peak-to-peak noise of the base-line.

Sensitivity : It may be defined as the concentration of element present in the sample solution that produces 1% absorption.

From the above definition it is quite evident that the sensitivity takes no cognizance of the noise-level of the base-line, therefore, it is more or less of no use as a definite guide to the least quantity of an element which may be estimated. However, the sensitivity of a 1% absorption-is a pure theoretical number only that would undergo a change solely depending on the efficiency of the lamp (hollow-cathode-lamp), atomizer, flame-system employed, monochromator (prism, grating used), and finally the photomultiplier used.

The sensitivity for 1% absorbance is determined by the help of the expression given below :

$$C_1\% = \frac{C_{0.1} \times 0.0044}{0.1}$$

where, $C_1\%$ = Concentration that yields 1% absorption, and

$C_{0.1}$ = Concentration that yields an absorption of 0.1

Sensitivity is usually expressed in terms of mcg ml^{-1} for 1% absorbance.

It is an usual practice to perform an actual-test-run over a sufficiently large range by employing the necessary prevailing expansion facility so as to ascertain fully whether or not the atomic absorption technique is reasonably applicable to a specific low-level estimation. Such a data may ultimately reveal the exact and true detection limit which is normally equals to twice the noise level.

26.4.3. INTERFERENCES

In general, atomic absorption methods are subject to *three* types of interferences, namely :

(*i*) Spectral Interferences,

(*ii*) Chemical Interferences, and

(*iii*) Ionisation Interferences.

The different interferences shall be discussed briefly below :

26.4.3.1 Spectral Interferences

This type of interference normally takes place when the absorption of an interfering species either overlaps or lies very near to the analyte absorption, with the result that resolution by the monochromator almost becomes impossible, Hollow-cathode-source invariably give rise to extremely narrow emission-lines, hence interference caused due to overlap of atomic spectral lines is rather rare.

A few typical examples of spectral interferences are given below :

(*a*) Spectral interferences caused either by the combustion products which show broad-band absorption or the particulate products which scatter radiation. In fact, both these products distinctly lower the power of the transmitted beam of light and ultimately give rise to positive analytical errors.

Remedy

(*a*) When the source of the combustion or particulate products is the full and oxidant mixture alone, then a blank is aspirated into the flame and the necessary corrections are effected from the observed absorbances.

(*b*) Spectral interferences may be produced due to an emission line of another element, radical or molecule and also by unresolved band spectra. Here, the lines are read together proportionately to the extent of overlap if the spectral band after passing through the monochromator allows the undersired radiation to reach the photoreceptor finally.

For instance : Manganese triplet (at 4031°, 4033° and 4035° A) : potassium doublet (at 4044° and 4047° A) and the gallium line (at 4033° A).

Remedy : The overlapping of this nature may be eliminated either by prior chemical separation or by selection other spectral lines.

(*c*) **Sample Matrix :** A relatively more complex and troublesome problem is usually faced when the source of scattering originates right in the sample matrix itself. In such a situation, it has been noticed that the power of the transmitted beam-designated as P, is reduced by the nonanalyte components, whereas the incident beam power-designated as Po, is not ; thereby resulting in a positive error in absorbance and hence in concentration.

Example : Determination of Barium in alkaline-earth mixtures affords a potential matrix interference due to absorption. It has been observed that an intense and useful absorption line for barium atoms, occurring at 553.6 nm, lies in the centre of a broad absorption band for Ca $(OH)_2$, that extends from 540 to 560 nm.

Remedy : (1) The effect due to sample matrix is quickly and effectively eliminated by replacing nitrous oxide for air as the oxidant for the acetylene, whereby the higher temperature completely decomposes the Ca $(OH)_2$ and eliminates the absorption band.

(2) If the source of interference is known, an excess of the interfering substance may be added to the sample as well as the standards ; provided the 'excess' is sufficient enough with respect to the concentration from the sample matrix, the concentration of the latter will thus become insignificant. Such an added substance is sometimes referred to as a radiation buffer.

26.4.3.2. CHEMICAL INTERFERENCES

In usual practice, the chemical interferences are found to be more common than the spectral interference. However, their effects may very often be minimized by appropriate choice of experimental parameters.

Examples : (*i*) **Chemical Interferences due to Anion (PO_4^{3-}) :** Phosphate ions have been found to interfere with determination of Mg and Ca by AAS. The absorption due to Mg and Ca are appreciably weaker in the presence of PO_4^{3-} ions than in their absence. This is evidently on account of the formation of fairly stable phosphates of Mg and Ca which do not readily split-up into the respective atoms in the mantle of a flame.

Remedy : The addition of an excess of strontium (Sr), or lanthanum (La), or thorium (Th) ion remarkably minimizes the interference of PO_4^{3-} ion in the determination of Mg, and Ca by replacing the analyte in the analyte in the compound formed with the respective interfering species. In short, these ions do combine preferentially with PO_4^{3-} ions.

(*ii*) **Chemical Interference due to Cations :** In certain specific cases cations also interfere in atomic absorption measurements, for instance : Boron interferes with Mg and Ca ; whereas aluminium interferes with alkaline earth elements.

Protective Agents : These agents are found to inhibit the interferences by virture of their ability to form relatively stable but volatile species with the respective analyte. There are *three* reagents that are employed commonly for this purpose, namely :

(*a*) Ethylenediaminetetra-acetic acid (EDTA).

(*b*) 8-Hydroxyquinoline, and

(*c*) Ammonium salt of 1-pyrrolidinecarbodithioic acid (APDC). EDTA helps to eliminate the interferences of Al^3, Si^{4+}, PO_4^{3-} and SO_4^{2-} in the determination of Ca.

26.4.3.3. Ionization Interferences

It has been observed that the ionization of atoms or molecules is comparatively very small in magnitude in combustion mixtures which essentially involve air as the oxidant and, therefore, may be ignored and neglected. Consequently, the substitution of air with either oxygen or nitrous oxide, however, gives rise to

temperatures which are high enough to cause appreciable ionization., Hence, as a consequence of the attained equilibrium-a fairly significant concentration of electron exists as shown below :

$$M \rightleftharpoons M^+ + e^-$$

where, M = Neutral atom or molecule,

M^+ = Its corresponding ion, and

e^- = An electron

Hence, if the medium has the species B in addition to species M, and if the former ionizes according to the following equation :

$$B \rightleftharpoons B^+ + e^-$$

then the extent of ionization of the latter will be minimized substantially be the *Law of Mass Action* of the electrons originated from the former species (*i.e.,* B).

Example : The intensity of atomic absorption lines for the alkali metals, such as : potassium (K) ; rubidium (Rb) ; and caesium (Cs), is found to be affected by temperature in a complex way. Under certain experimental parameters a noticeable decrease in absorption may be observed in hotter flames. Hence, lower excitation temperatures are invariably recommended for the analysis of alkali metals.

Remedy : The resulting effects of shifts in ionization equilibrium may be eliminated effectively by the addition of an ionization suppressor, that promptly gives a comparatively high concentration of electrons to the flame. This ultimately results in the suppression of ionization by the respective analyte.

26.5. APPLICATION OF ATOMIC ABSORPTION SPECTROSCOPY IN PHARMACEUTICAL ANALYSIS

The elements present in a host of pharmaceutical substances are determined quantitatively by atomic absorption spectroscopy, for example : Pd in carbenicillin sodium ; Cu, Pb and Zn in activated charcoal ; Fe in ascorbic acid ; Ag in cisplatin ; Ph and Zn in copper sulphate ; Zn in glucogen ; Zn in insulin ; Pb in oxprenolol hydrochloride ; Ni in prazosin hydrochloride ; Zn in sodium sulphite heptahydrate, and Cd and Pb in zinc oxide.

26.5.1. ASSAY OF TOTAL ZINC IN INSULIN ZINC SUSPENSION

Theory : Insulin zinc suspension is nothing but a neutral suspension of insulin in the form of water insoluble complex with $ZnCl_2$. Determination of both total zinc and zinc in solution is performed on a sample of the supernatant liquid obtained by centrifuging the suspension. The percentage of total zinc and of zinc in solution varies according to the strength of the preparation *viz*., 40, 80 or 100 units ml^{-1}.

Materials Required : Stock solution of Zn (5000 mcg ml^{-1}) : Dissolve Zn metal (Anala-R-Grade) 2.5 g in 5 M HCl (20 ml) and dilute to 500 ml with DW ; HCl (0.1 M) : 10 ml ;

Procedure : To the 2 ml of well-shaken suspension add HCl (0.1 M ; 1 ml) and dilute with water to 200 ml. Spray the solution by adopting the standard procedure and read off the concentration of zinc from a calibration curve prepared with solution containing 0.5, 1, 2, and 3 mcg ml^{-1} of Zn.

26.5.2. ASSAY OF PALLADIUM IN CARBENICILLIN SODIUM

Materials Required : Carbenicillin sodium : 1.0 g ; sulphuric acid (36 N or 18 M) : 2.0 ml ; mixture of nitric acid (70% w/v) and hydrochloric acid (35% w/w or 11.5 M) [3 : 4] : 5.0 ml ; hydrochloric acid (11.5 M) : 3.0 ml ; palladiun solution (standard) [Dissolve 1.670 g of Palladium (II) chloride in 200 ml of a 50% v/v solution of hydrochloric acid (11.5 M) with the aid of heat, cool and add sufficient water to produce 1 litre] : This standard palladium solution contains 1 mg of Pd in 1 ml ;

Procedure* : Moisten 1 g of carbenicillin sodium in a silica crucible with 2 ml of sulphuric acid. Heat, gently at first, then more strongly until all carbon is removed and a white ash is obtained. Allow to cool and add 5 ml of a mixture of nitric acid and hydrochloric acid and evaporate to dryness on a water-bath. Add 3 ml of hydrochloric acid, warm to dissolve and add sufficient water to produce 25 ml.

Place in each of three similar graduated flasks equal volumes of the solution of the substance prepared as above. Add to all but one of these flasks a measured quantity of the specified standard solution of palladium to produce a series of solutions containing increases amounts of Pd. Dilute the contents of each flask to the required volume with DW.

After calibrating the instrument as stated above, introduce each solution into the generator 3 times and record the steady reading at 248 nm. If the generator is a flame, wash the apparatus thoroughly with DW after each introduction ; if a furnace is used, fire it after each introduction. Plot the mean of the readings against concentration on a graph the axes of which intersect at zero added Pd and zero reading. Extrapolate the straight line joining the points until it meets the extrapolated concentration axis. The distance between this point and the intersection of the axes represents the concentration of Pd present in the prepared solution of carbenicillin sodium.

26.5.3. COGNATE ASSAYS

A number of pharmaceutical substances official in BP (1993) can be assayed by adopting the above procedures of AAS as detailed in the following Table 26.1.

Table 26.1 : Assay of Pharmaceutical Substances by Atomic Absorption Spectroscopy :

S. No.	Name of Substance	Elements Assayed	Qty. Prescribed	Comp. of Flame	Measured at(nm)	Limits Prescribed
1.	Activated Charcoal	Cu ; Pb ; Zn ;	2.0 g	Air-Acetylene	325.0	Cu = NMT** 25 ppm ; Pb = NMT 10 ppm ; Zn = NMT 25 ppm ;
2.	Ascorbic Acid	Fe ;	20% w/v soln. in 0.1 M HNO_3	—	248.3	Fe = NMT 2 ppm ;
3.	Cisplatin	Ag ;	0.1 g	Lean air-Acetylene	328 (Silver-hollow-cathode lamp)	Ag = NMT 250 ppm ;
4.	Copper Sulphate	Pb ; Zn ;	1.25 g in 10 ml HCl	—	Pb : 283.3 ; Zn : 213.9 ;	Pb = NT 75 ppm ; Zn = NMT 500 ppm ;
5.	Glucagon	Zn ;	0.2% w/v Air-in 0.01 M HCl	Acetylene (11.2) Vol	213.9 (Zn hollow-cathode-lamp)	Zn = NMT 0.15% of Zn ;
6.	Oxprenolol Hydrochloride	Pb ;	4% w/v	—	217.0 (Pb-hollow-cathode-lamp)	Pb = NMT 5 ppm ;

*British Pharmacopoeia, Vol. I & II, 1993.

7.	Prazosin Hydrochloride	Ni ;	0.1 g	—	232	Ni = NMT 50 ppm ;
8.	Sodium Sulphite Heptahydrate	Zn ;	20.0 g	Air-Acetylene	213.9 (Zn-hollow-cathode-lamp)	Zn = NMT 12 ppm ;
9.	Zinc oxide	Cd ; Pb ;	2.0 g	Air-Acetylene	Cd : 228.8 ; Pb : 217. 0;	Cd = NMT 10 ppm ; Pb = NMT 50 ppm ;

*NMT = Not More Than ;

THEORETICAL AND PRACTICAL EXERCISES

1. Explain the following statements with respect to atomic absorption spectroscopy (AAS) :

(*i*) One of the best known analytical methods for quantitative estimation of trace metals.

(*ii*) AAS may be accomplished either by electrothermal device or by flame.

(*iii*) AAS facilitates estimation of a specific element in the presence of other elements accurately and precisely.

(*iv*) Merits of AAS over FES.

(*v*) Demerits of AAS.

2. Discuss the underlying principle of AAS. How would you explain the mathematical expression derived from the total amount of light absorbed ?

3. Describe the various components that are essentially involved in any one of the following two types AAS :

(*a*) Single-beam AAS, (*b*) Double-beam AAS

with the help of a schematic diagram.

4. Give a comprehensive account on the following *two* vital aspects of AAS, namely :

(*a*) Analytical techniques, (*b*) Detection limit and sensitivity.

5. How do the following *three* types of interferences affect the atomic absorption spectroscopic methods :

(*a*) Spectral interferences, (*b*) Chemical interferences, and

(*c*) Ionisation interferences.

Support your answer with appropriate examples.

6. Discuss the assay of the following medicinal compounds in an elaborated manner :

(*i*) Total Zn in insulin Zn suspension, (*ii*) Pd in carbenicillin sodium,

(*iii*) Ag in cisplatin, (*iv*) Fe in Ascorbic acid,

(*v*) Pb in Oxprenolol hydrochloride, and (*vi*) Ni in Prazosin hydrochloride.

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PART V

ASSAY METHODS BASED ON SEPARATION TECHNIQUES

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27 LIQUID-LIQUID EXTRACTION

CONTAINS :

27.1. INTRODUCTION

Liquid-Liquid extraction is a versatile and dependable separation technique wherein an aqueous solution is usually brought into contact with another organic solvent, exclusively immiscible with the former, so as to affect a legitimate and actual transfer of either one or more solutes into the latter. The normal-feasible separations which can thus be achieved are found to be rather easy, fast, convenient and effective resonably. Invariably such separations may be performed by shaking the two liquids in a separatory funnel for a few minutes ; and may be extended either to large quantities of pharmaceutical substances or trace levels.

In the case of pharmaceutical chemicals that are mostly **'organic solutes',** the liquid-liquid extraction system may very often make use of two immiscible organic solvents (*e.g.*, alcohol and ether) instead of the aqueous-organic type of extraction. On the contrary, the **'inorganic solutes'** normally encountered are invariably in aqueous solutions ; therefore, it has become absolutely necessary to produce such neutral substances out of them, for instance *ion-association complexes* and *metal-chelates* (using organic-ligands) that may be extracted into an appropriate organic solvent.

In short, **liquid-liquid extraction** has been employed predominantly and effectively not only for the pre-concentration and isolation of a 'single' chemical entity just before its actual estimation, but also for the extraction of classes of organic compounds or groups of metals, just prior to their usual estimation either by *chromatographic techniques* or by *atomic-absorption methods*.

27.2. THEORY

The behavioural pattern of two immiscible solvents, say '*a*' and '*b*', is essentially nonideal with respect to one another. Now, if a third substance is made to dissolve in a two-phase mixture of the solvents (*i.e.,* '*a*' and '*b*'), it may behave ideally in either phases provided its concentration in each individual phase is approximately small. Therefore, under these prevailing experimental parameters the ratio of the mole fractions of the solute in the two respective immiscible phases ('*a*' and '*b*') is found to be a constant which is absolutely independent of the quantity of solute present. It is termed as the **Nernst Distribution Law** or the **Partition Law** and may be expressed as follows :

$$K_p = \frac{[A]_a}{[A]_b} = \frac{\text{Concentration of solute in solvent '}a\text{'}}{\text{Concentration of solute in solvent '}b\text{'}} \qquad ...(a)$$

where, $[A]_a$ = Mole fraction of solute A in Phase '*a*'

$[B]_b$ = Mole fraction of solute B in Phase '*b*', and

K_p = A constant.

The constant (K_p) is also known as the **distribution coefficient** or the **partition coefficient**. Interestingly, this particular relation [Eq. (*a*)] was originally derived for ideal solutions only, but it caters for a fairly good description of the behavioural pattern of a number of real-extraction-systems encountered in the analysis of pharmaceutical substances. However, the **Partition Law** offers the following *two* limitations, namely :

(*a*) It is not thermodynamically rigorous *i.e.,* it takes no cognizance of the activities of the different species. In other words, it is solely applicable to very dilute solutions in which case the ratio of the activities almost approaches unity, and

(*b*) It does not hold good when the distributing substances encounters association or distribution in either phases (*i.e.,* '*a*' and '*b*').

Consequently, a more rigorous treatment particularly specifies K_p as the ratio of the activities of the substance (A) in the two solvents instead of their concentrations. Hence, for dilute solutions, at a specified constant pressure and temperature, the mole fraction of a solute is directly proportional to its concentration in molarity or mass per unit volume ; which implies that these may be employed instead of mole-fraction in Eq. (*a*).

Thus, the **Partition Coefficient** K_p is also given by the following expression :

$$K_p = \frac{S_1}{S_2} \qquad ...(b)$$

where, S_1 = Solubility of substance A in solvent '*a*', and

S_2 = Solubility of substance A in solvent '*b*'.

Adequate precaution and care must be exercised in determining partition coefficients based on the solubility data as S_1 is not the solubility of substance 'A' in pure Solvent '*a*', but rather the solubility in Solvent '*a*' saturated with Solvent '*b*'.

***Example* :** In order to determine the exact partition coefficient of substance 'A' between water and ethyl acetate, the appropriate solubilities would be those of the substance 'A' in 3.3% ethyl acetate in water (composition of the 'aqueous' layer) and 8.7% in water in ethyl acetate (composition of the **'ester'** layer).

Likewise, the following Table 27.1, records the mutual solubilities of a few typical solvent pairs that are used frequently for **liquid-liquid extraction** procedures.

Table 27.1 : Physical Data on Binary Solvent Systems*

S.No.	Solvent Pair	Percentage Composition Phases		Composition of Azeotrope (%)	Boiling Pt. of Azeotrope (°C)	Boiling Pt. of Solvents (°C)
		Upper	Lower			
1.	1-Butanol-	79.9	7.7	55.5	93.0	117.7
	Water	20.1	92.3	44.5		100.0
2.	*n*-Butyl ether-	99.97	0.19	66.6	94.1	142.0
	Water	0.03	99.81	33.4		100.0
3.	Carbon tetrachloride-	0.03	99.97	95.9	66.8	76.8
	Water	99.97	0.03	4.1		100.0
4.	Chloroform-	0.8	99.8	97.0	56.3	61.2
	Water	99.2	0.2	3.0		100.0
5.	Dichloromethane-	2.0	99.9	99.0	38.8	40.0
	Water	98.0	0.1	1.0		100.0
6.	Ethyl ether-	98.53	6.04	98.2	34.2	34.6
	Water	1.47	93.96	1.2		100.0
7.	Hexane-	85.0	42.0	73.1	69.0	50.0
	Methanol	15.0	58.0	26.9	64.7	
8.	1-Octanol-	—	0.1	10.0	99.4	195.0
	Water	—	99.9	90.0		100.0
9.	Toluene-	99.95	0.06	79.8	85.0	110.6
	Water	0.05	99.94	20.2		100.0
10.	*m*-Xylene-	99.95	0.05	60.0	94.5	139.1
	Water	0.05	99.95	40.0		100.0

In liquid-liquid extractions the following *two* aspects are very crucial and important, namely :

(*a*) Error due to the Volume Change, and

(*b*) Effectiveness of an Extraction.

These *two* aspects shall be discussed briefly at this juncture.

27.2.1. ERROR DUE TO THE VOLUME CHANGE

In a situation wherein two immiscible solvents are employed in an extraction, the volumes of the two individual phases after attainment of equilibrium may be appreciably different in comparison to the initial volumes of the solvents used. Therefore, a number of procedures have been adopted to avoid **'error due to the volume change'** incurred thereby, namely :

(*i*) Measure the volume of the phase employed for the analysis and incorporate this volume in the calculations,

(*ii*) Separate the phase quantitatively and subsequently dilute to a known volume,

(*iii*) Separate the phase quantitatively and make use of the entire volume in the remaining steps of the ongoing analysis, and

(*iv*) Carry a marker substance through the extraction to automatically compensate for volume changes.

However, the latter procedure finds its abundant use in chromatographic methods of analysis.

* Leo, A., Hansch, C. and Elkins, D., **Chem Rev., 71** 525, 1971.

27.2.2. EFFECTIVENESS OF AN EXTRACTION

Based on the appropriate partition coefficient of an immiscible solvent pair it is possible to calculate the 'effectiveness of an extraction'.

Let us assume that '*x*' moles of solute present initially in a volume V_2 of Solvent '*b*'. Now, this particular sample undergoes extraction with a volume V_1 of Solvent '*a*' and subsequently '*y*' moles of compound are left in V_2 at equilibrium.

Substituting these values in Eq. (*a*) and using molarity instead of mole fraction, we have :

$$K_p = \frac{M_1}{M_2} = \left(\frac{x-y}{V_1}\right) \Big/ \left(\frac{y}{V_2}\right) \qquad ...(c)$$

after simplifying and rearranging :

$$K_p = \left(\frac{x}{V_1} - \frac{y}{V_1}\right)\frac{V_2}{y}$$

or $$= \frac{x}{y} \cdot \frac{V_2}{V_1} - \frac{V_2}{V_1}$$

or $$= \frac{V_2}{V_1}\left(\frac{x}{y} - 1\right)$$

or $$K_p \cdot \frac{V_1}{V_2} = \frac{x}{y} - 1$$

or $$K_p \cdot \frac{V_1}{V_2} + 1 = x/y$$

or $$y/x = \left(\frac{V_1}{V_2} K_p + 1\right)^{-1} = f \qquad ...(d)$$

where, f = Fraction not extracted.

Figure 27.1, represents the nomogram from which the unextracted fraction for various values of V_2/V_1 and K may be obtained.

From Eqs. (*d*) it is quite evident that the fraction extracted is absolutely independent of the initial solute concentration. Hence, the fraction left unextracted after '*n*' extraction may be given by the following expression :

$$fn = \left(\frac{V_1}{V_2} K_p + 1\right)^{-n} \qquad .....(e)$$

assuming that the same volumes of solvents have been used for each extraction.

Form Eq. (*e*) it is distinctly obvious that a series of several extractions would definitely prove to be more efficacious than one single extraction using the same total volume of solvent.

From figure 27.1, the following steps may be adopted in order to determine the percentage of the analyte left in the sample after a single extraction :

(*i*) Hold a straight edge in such a manner that it is made to pass though the point on the 'left scale' which exactly corresponds to that ratio of extracting solvent to sample solvent volume and through the point representing the partition coefficient (K_p) on the **'middle scale'**, and

(*ii*) The percentage of the '**analyte**', left behind in the sample solvent after a single extraction is given by the intersection of the straight-edge with the **'right scale'**.

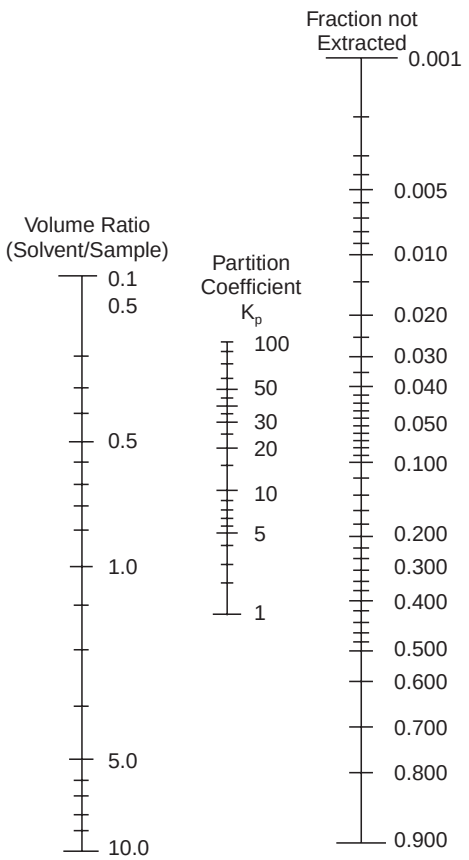


Figure 27.1 : A nomogram to calculate the 'fraction not extracted' after a single step liquid-liquid extraction process.

27.3. FACTORS INFLUENCING SOLVENT EXTRACTION

A number of cardinal factors exert a positive influence on the phenomenon of solvent extraction, namely :

(*a*) Effect of temperature and inert solutes,

(*b*) Effect of pH on extraction,

(*c*) Effect of ion-pair formation, and

(*d*) Effect of synergistic extraction.

These factors shall be discussed briefly below :

27.3.1. EFFECT OF TEMPERATURE AND INERT SOLUTES

The physical as well as chemical interactions of a solute is capable of changing its apparent partition coefficient between a pair of solvents. Therefore, it is absolutely necessary to take this into consideration while selecting an appropriate extraction-system. Craig and Craig* have advocated that the partition coefficients

* Craig, L.C. and Craig, D., Laboratory Extraction and Countercurrent Distribution, in, **Techniques of Organic Chemistry,** Vol III, I, Separation and Purification, 2nd ed., Weissberger, A., Ed., New York, Interscience 171, 1956.

are normally not sensitive to temperature when the two solvents in question are more or less immiscible and also the concentrations are fairly low in both the phases. Thus, the effect of temperature on the partition coefficient may be estimated conveniently from its effect on the solubilities of the substance in the two respective solvents. By substituting the solubilities (*e.g.*, S_1 and S_2) in Eq. (*b*) it is possible to estimate K.

The effect of inert solutes, such as : calcium chloride, magnesium chloride and sucrose, can also be employed judiciously and efficaciously in the development of solutions to difficult extraction problems by allowing efficient extractions from the water into such solvents as acetone, ethanol and methanol that are found to be completely miscible with water in the absence of salt. Matkovitch and Cristian* found the above three inert solutes to be the best agents for salting acetone out of water. It has been observed that the acetone layer that separated from a saturated aqueous solution of $CaCl_2$ exclusively contained 0.32 $\pm$ 0.01% water (v/v) and 212 ppm salt (w/w) at equilibrium.

27.3.2. EFFECT OF pH ON EXTRACTION

Generally, it has been found that the organic acids and bases do exist in aqueous solution as equilibrium mixtures of their respective neutral as well as ionic forms. Thus, these neutral and ionic forms may not have the same identical partition coefficients in a second solvent ; therefore, the quantity of a substance being extracted solely depends upon the position of the acid-base equilibrium and ultimately upon the pH of the resulting solution. Hence, extraction coefficient (E) may be defined as the ratio of the concentrations of the substance in all its forms in the two respective phases in the presence of equilibria ; and it can be expressed as follows :

$$E = \text{Extraction Coefficient} = \frac{\Sigma[Si]_2}{\Sigma[Si]_1} \qquad ...(i)$$

where, $\Sigma[Si]_2$ = The sum total of all forms of the compound in Phase-'2', and

$\Sigma[Si]_1$ = The sum total of all forms of the compound in Phase '1'.

In fact, the actual effect of the equilibrium on the extraction may be shown by determining the extraction coefficient for the system :

$$A + H \rightleftharpoons AH \quad \text{or} \quad K = \frac{[AH]_1}{[A]_1\,[H]_1} \qquad ...(ii)$$

where, A = Extract with partition coefficient K_p, A and

AH = Extract with partition coefficient K*p*, AH

Hence, $$K_p, A = \frac{[A]_2}{[A]_1} \text{ and } K_p, AH = \frac{[AH]_2}{[AH]_1} \qquad ...(iii)$$

Therefore, for this particular system the efficiency coefficient E may be expressed as follows :

$$E = \frac{[A]_2 + [AH]_2}{[A]_1 + [AH]_1} \qquad ...(iv)$$

Now, substituting Eq. (*ii*) and Eq. (*iii*) into Eq. (*iv*) and subsequently simplifying, we shall get :

$$E = \frac{K_p, A[A]_1\, K_p, AH[AH]_1}{[A]_1 + [AH]_1} \quad \text{From Eq. } (iii)$$

or $$= \frac{[A]_1\,\{K_p, A + K_p, AH[AH]_1 / [A]_1\}}{[A]_1\,\{1 + [AH]_1 / [A]_1\}}$$

or $$E = \frac{K_p, A + K_p, AH.\, K[H]_1}{1 + K[H]_1} \quad \text{From Eq. } (ii) \qquad ...(v)$$

* Matkovitch, C.E., and Cristian, G.D., **Anal. Chem., 45,** 1951, 1973

From Eq. (*v*) it is quite evident that E approaches K_p, A as $K[H]_1$ becomes small and K_p, AH as $K[H]_1$ becomes large.

Now, assuming that only A extracts (*i.e.,* A being a neutral organic base and AH the conjugate acid), Eq. (*v*) may be expressed as :

$$E = K_p, A \frac{1}{1 + K[H]_1} = K_p, A \frac{[A]}{[A] + [AH]} \quad ...(vi)$$

The following inferences may be arrived at on the basis of Eq. (*vi*), namely :

(*a*) Extraction coefficient (E) is just the partition coefficient times the fraction of the analyte which is present in the extractable form,

(*b*) Under a given set of experimental parameters the ultimate effect of the 'equilibrium' shall be to reduce the amount extracted, and

(*c*) Forcibly shifting the 'equilibrium' toward the extractable species by adjusting the pH helps to minimise the effect of the equilibrium thereby rendering E almost equal to K_p, A.

In conclusion, it may be observed that the pH for an **'extraction system'** must be selected in such a fashion so that the maximum quantum of the analyte is present in the extractable form, that obviously suggests that the analyte should always be in the form of either a free base or a free acid. From the actual practical experience it has been noticed that a good-working range lies between 95 to 97% present in the extractable form.

27.3.3. EFFECT OF ION-PAIR FORMATION

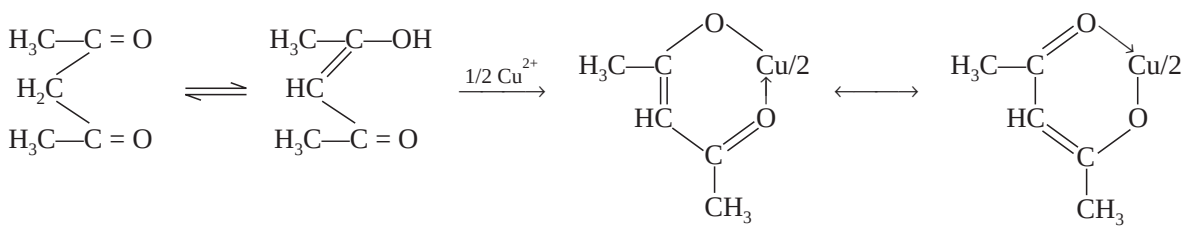
Ion-pair formation needs its due recognition because it very often gives rise to unexpected extractions. In true sense, ion-pair may be regarded as a close association of an anion and cation, and therefore, it usually takes place either in a polar or a non-polar solvent. In reality, the ion-pairs are invariably formed by virtue of the union between comparatively large organic anions and (much smaller) cations. Interestingly, the resulting ion-pairs have been found to show their appreciable solubility in polar solvents ; and hence, these species may be extracted conveniently under such experimental parameters where neither individual component ion could.

A few vital criteria towards the formation of an improved aqueous extractable ionic species are, namely :

- Formation of a neutral metal-chelate complex or by ion association, and
- Creation of larger and more hydrophobic molecular species.

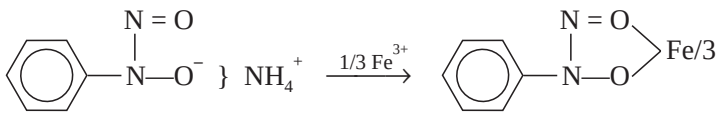
A few typical examples shall be discussed here to explain the chelate-formation :

Example 1 **: Cu^{2+} with 'acetylacetonate'** forms a fairly stable ring compound :



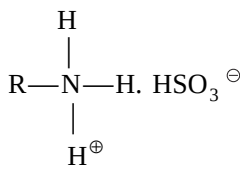
Acetylacetonate (*keto*-form) | Acetylacetonate (*enol*-form) | Copper(II)-acetylacetonate complex (Resonating ring compound)

Example 2 **: Iron (III) 'cupferrate'** gives rise to a stable ring compounds as shown below :



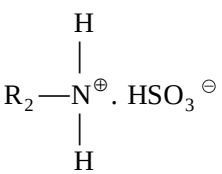
Example 3 **: Sulphonic acids** rapidly pair with a plethora of **'protonated amines'** to form an easily extractable complex

Primary amine (1°) :



A complex

Secondary amine (2°) :



A complex

Example 4 **: Cl^- ion** serves as an 'appropriate anion' that favourably combines with many aromatic amines and alkaloids which may ultimately be extracted from the corresponding aqueous solutions into chloroform as their respective chlorides*.

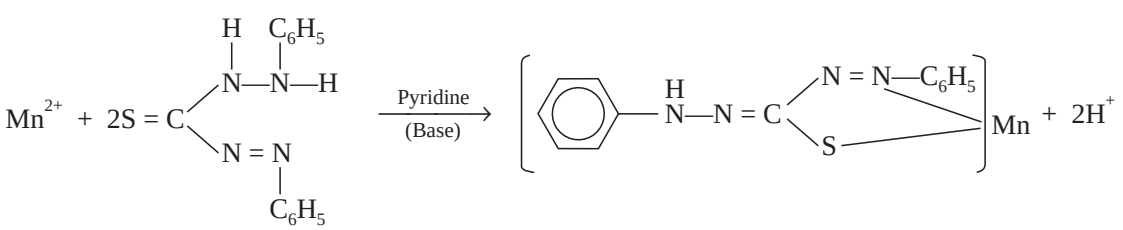
27.3.4. EFFECT OF SYNERGISTIC EXTRACTION

Synergism : It may be defined as **'the process whereby two different reagents when employed together are capable of extracting a metal ion with a distinct and marked efficiency, in comparison to a condition when the same two reagents are used individually'.**

Example **: (*i*) : Complexation of Mn^{2+} with dithizone and pyridine :**

It has been observed that the complex formed by Mn^{2+} with dithizone alone is of no practical analytical utility because of the fact that it undergoes decomposition very quickly. However, the addition of a base, such as : pyridine into the Mn^{2+} plus dithizone complex yields a red-complex, which is fairly stable to oxidation and light; and, therefore, forms the basis for a very sensitive **photometric method** employed in estimating trace amounts of Mn^{2+}.

Equation : Following is the chemical reaction of the above complex formation :



Dithizone
(or Diphenylthiocarbazone)

Manganese-dithizone
(red-complex)

(*ii*) Complexation of Ni^{2+} with dithizone and 1, 10-phenanthrolone :

Noticeably, the reaction of Ni^{2+} with dithizone is quite slow and sluggish. Nevertheless, this slow reaction is significantly accelerated by the addition of nitrogen-containing bases like 1, 10-phenanthroline. The resulting complex may be represented by the following equation :

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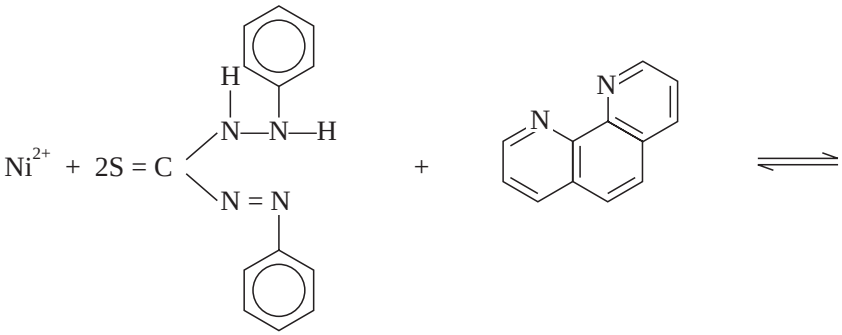
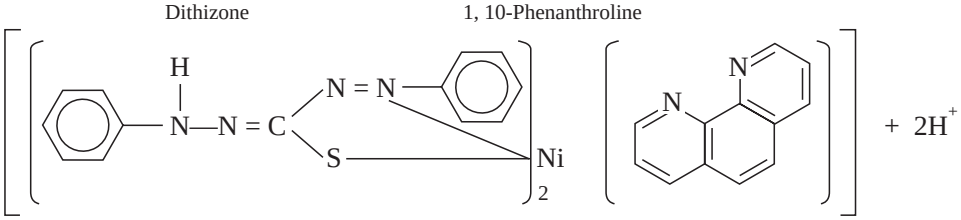
* Manske, R.H.F., **Can. J. Chem., B14**, 347, 1936.

Equation :



Dithizone 1, 10-Phenanthroline



Nickel-dithizone-phenanthroline complex

It is the basis of a very sensitive synergistic extraction photometric procedure for trace amounts of Ni^{2+}.

27.4. EMULSION PROBLEM ENCOUNTERED IN EXTRACTIONS

Emulsion : It may be defined as-**'a dispersed system containing at least two immiscible liquid phases'.**

The effective and meaningful extraction of an analyte is rendered almost impossible when one encounters an emulsion formation during an extraction process thereby the separation of the two phases becomes difficult. Actually, it offers a frequent and serious problem when dealing with the extraction of drugs from biological as well as pharmaceutical formulations.

Emulsion formation enhances the area of the interface between the two immiscible solvents and as a result also enhances the **'free energy'** of the system, which may be designated by the following expression :

$$\text{Free energy} = \gamma \times \Delta A$$

where γ = Interfacial tension, and

A = change in surface area resulting from emulsification.

Obviously the **'lowest free energy'** is given by the most stable state for a system at constant pressure and, therefore, in due course an emulsion shall **'break'** spontaneously to the two-layered system. However, the breaking of an emulsion could be relatively a rather slow phenomenon. There are a number of factors which may be responsible for the slow-coalescence of an emulsion, namely :

(*a*) Finely divided powders, albumin, gelatin and natural gums have a tendency to coat the droplets formed in an emulsion which ultimately prevent them from coalescing,

(*b*) Usually surfactants decrease the interfacial tension between the two immiscible liquids which help in stabilizing an emulsion, and

(*c*) Ionic species may get absorbed at the interface of two immiscible layers resulting in the formation of a net charge on the droplets. Because all droplets shall essentially bear the similar charge, naturally they will repel one another thereby preventing coalescence.

In fact, there are many natural and synthetic products that are profusely incorporated in the 'formulation of drugs' which are found to stabilize emulsions either by coating the droplets or by minimizing the interfacial tension, namely :

(*i*) **Coating the droplets :** *e.g.,* starch acacia, silica, gelatin finely divided talc, and

(*ii*) **Minimizing the interfacial tension :** *e.g.,* mono-and di-glycerides ; stearates and sorbitan monoleate.

It has been observed that once an emulsion is formed it is rather difficult to break it. Therefore, it is absolutely necessary to adhere to the following guidelines, as far as possible, in order to avoid forming emulsions in the course of an extraction process :

(1) Always affect very cautious and gentle agitation besides employing a sufficiently large liquid-liquid interface to obtain a reasonably good extraction. Especially when the two-liquid layers have a large contact surface in an extraction process, vigorous or thorough shaking of the two phases is not required at all,

(2) The removal of any finely divided insoluble material(s) in a liquid phase must be done by filtration before carrying out the extraction process,

(3) Always prefer and use such solvent pairs that have a large density difference and a high interfacial tension, for instance : water and hexane, as they are less prone to emulsion problems. In contrast, such solvent pairs as water and benzene should not be used in the extraction process,

(4) When performing extraction from water always ensure not to work at pH extremes and particularly at high pH ranges to avoid emulsification, and

(5) In cases, of acute emulsion-problems substances like-anion exchangers alumina or silicagel are used specifically to resolve the problem by adsorption of the emulsifying agents. In fact, it would be advisable to employ the technique of column chromatography for the effective separation of the analyte as compared to an extraction process.

Breaking of an Emulsion (*i.e.,* Coalescence) : Following are the various techniques invariably used so as to break an emulsion or to achieve coalescence, namely :

(1) **Mechanical Means :** Coalescence may be achieved by mechanically creating turbulence on the surfaces of the droplets either by passing the emulsion through a bed of glass-wool or by stirring with the help of a glass-rod simply,

(2) **Centrifugation :** In cases where the densities of the two liquids are appreciably different coalescence may be afforded by centrifugation-a physical means,

(3) **Addition of Monovalent and Divalent Ions :** Relatively simple emulsions are broken by adding monovalent salts like sodium chloride ; whereas charge-stabilized emulsions are specifically sensitive to the divalent ions, such as : $CaCl_2$; $MgCl_2$ etc.

(4) **Ethanol or Higher Alcohol :** Addition of small quantities of either ethanol or sometimes a higher homologous alcohol shall aid in coalescing an emulsion,

(5) **Sudden Cooling of Emulsion (Thermal Shock) :** Sudden temperature drop or freezing (*i.e.,* giving a thermal shock) of an emulsion mostly enhances the interfacial tension between the two immiscible phases thereby causing coalescence.

(6) **Altering the Ratio of Solvents :** Coalescence of an emulsion may also be achieved either by altering the ratio of the prevailing dispersed phase or even by partial evaporation of the solvent,

(7) **Silicone Defoaming Agent :** A few drops of the silicone-defoaming agent sometimes help in breaking an emulsion, and

(8) **Thin-Bed of an Adsorbent :** Sometimes simply passing an emulsion through a thin-bed of an adsorbent remarkably helps in achieving coalescence taking note of the fact that the analyte will not be absorbed from either solvent.

27.5. ASSAY METHODS BASED ON LIQUID-LIQUID EXTRACTION

A number of specific elements may be determined quantitatively based on liquid-liquid extraction method or **'solvent-extraction'** technique, namely :

(*a*) Determination of copper (I) as the neo-cuproin complex,

(*b*) Determination of Iron (III) as the 8-hydroxyquinoline complex or Iron (III) oxinate,

(*c*) Determination of lead (I) by the dithizone method,

(*d*) Determination of molybdenum (VI) by the thiocyanate method,

(*e*) Determination of Ni (II) :

(*i*) as dimethylglyoxime complex, and

(*ii*) by synergistic extraction.

All these assay methods shall be discussed in the following sections :

27.5.1. DETERMINATION OF COPPER (I) AS THE NEO-CUPROIN COMPLEX

Theory

'Neo-cuproin' (*i.e.*, 2, 9-dimethyl-1 : 10-phenathroline) under specific experimental parameters almost behaves as a critical reagent for copper (I). The resulting complex is freely soluble in chloroform and absorbs at 457 nm.

Materials Required : hydroxyammonium chloride solution (10% w/v) : 25 ml ; sodium citrate solution (30% w/v) : 50 ml ; ammonia solution ; **'neo-cuproin'** solution (0.1% w/v in absolute ethanol) : 50 ml ; chloroform ;

Procedure : The following steps may be adopted :

(1) Transfer 10.0 ml of the sample solution (containing upto 200 mcg of copper) in a separatory funnel, add 5 ml of hydroxyammonium chloride solution to affect the reduction of Cu (II) to Cu (I),

(2) To the resulting solution add 10 ml of solution citrate solution to enable complexation of any other metals that may be present,

(3) Add ammonia solution gradually until the pH is about 4.0 (use Congo Red) followed by 10 ml **'neo-cuproin'** solution,

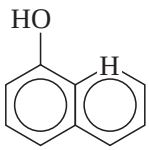
(4) Shake for about 30 seconds with 10 ml of chloroform and allow the layers to separate,

(5) Repeat the extraction with a further 5 ml of chloroform, and

(6) Finally, measure the absorbance at 457 nm against a blank on the reagents that have been used identically to the sample.

27.5.2. DETERMINATION OF IRON (III) AS THE 8-HYDROXY QUINOLATE COMPLEX [IRON (III) OXINATE]

Theory : Iron (III) upto an extent of 50-200 mcg can be extracted effectively from an aqueous solution with a 1% solution of 8-hydroxyquinoline (symbolized as HQ) in chloroform by carrying out a double extraction when the pH of the resulting aqueous solution ranges between 2 and 10. Evidently, between pH 2.0 to 2.5 metals like Ni, Co, Ce (III) and Al do not interfere at all. However, iron (III) oxinate is dark-coloured in chloroform and absorbs at 470 nm.



8-Hydroxyquinoline (oxime)

The reaction may be expressed as follows :

$$3\,(HQ)_{org} + (Fe^{3+})_{aq} \rightleftharpoons (FeO_3)_{org} + (H^+)_{aq}$$

Materials Required : Hydrated ammonium iron (III) sulphate : 0.0266 g ; oxine solution ('AnalaR'-Grade, 1% w/v in chloroform) : 50 ml ; chloroform ; 100 ml ;

Procedure : The following steps may be followed :

(1) Weigh accurately 0.0226 g of hydrated ammonium iron (III) sulphate and dissolve it in 1 litre of DW in a volumetric flask ; 50 ml of this solution contains 100 mcg of iron,

(2) Place 50 ml of the solution (≡ 100 mcg of Fe) in a 100-ml separatory funnel, and add to it 10 ml of 1% oxine solution, and shake for 1 minute,

(3) Separate the chloroform layer,

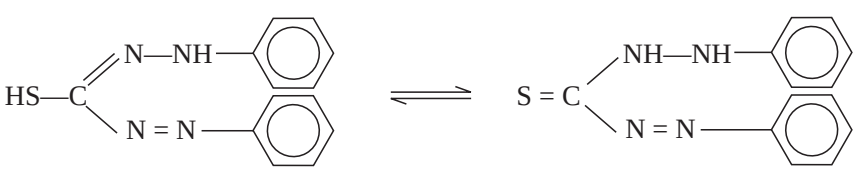
(4) Transfer a portion of the chloroform layer to a 1 cm absorption cell and determine the absorbance at 470 nm in a UV-spectrophotometer, employing the solvent (chloroform) as a blank or reference, and

(5) Repeat the extraction with a further 10 ml quantity of 1% oxine solution, and measure the absorbance again so as to confirm whether all the iron was extracted or not. Usually three extractions suffice the complete extraction of Fe (III).

Note : From a glimpse of typical analytical results it may be seen that absorbance after first extraction 0.0592 ; after second extraction 0.0050 ; after third extraction 0.0010 ;

27.5.3. DETERMINATION OF LEAD (I) BY THE DITHIZONE METHOD

Theory : In solution, dithizone (diphenylthiocarbazone) exhibits tautomerism as shown below :



Dithizone (*enol*-form) I — Dithizone (*keto*-form) II

The enol-form of Dithizone (I) behaves as monoprotic acid having a dissociation constant *pKa* = 4.7 upto a pH range of about 12 : obviously, the acid proton is inherited due to the thiol moiety in (I). In reality, two kinds of **'metal dithizonates'** are invariably formed, namely :

(*a*) **'Primary' metal dithizonates :** These are produced as per the following reaction :

$$Mn^+ + n\,H_2\,Dz \rightleftharpoons M\,(HDz)_n + n\,H^+$$

They are of greater analytical value because of their high stability and greater solubility in organic solvents.

(*b*) **'Secondary' metal dithizonates :** These are specifically formed by some metals, such as : Cu, Ag, Au, Hg, Bi and Pd. The second complex are produced under the following *two* conditions, namely :

(*i*) deficiency of the reagent, and

(*ii*) higher pH range,

and may be expressed as follows :

$$2\,M\,(HDz)_n \rightleftharpoons M_2\,Dz_n + n\,H_2\,Dz$$

The poor stability and miserable solubility in organic solvents render these products of insignificant analytical importance.

It is, however, pertinent to mention here that dithizone* is an extremely sensitive reagent and, therefore, helps in the determination of lead either from a neutral or faintly alkaline medium to the extent of a few micrograms.

Materials Required : Pure lead nitrate : 0.0079 g ; ammonia-cyanide-sulphite mixture (dilute 35 ml of conc. ammonia solution having sp. gr. 0.88 and 3 ml of 10% w/v solution potassium cyanide **(Caution : deadly poisonous, use protective gloves while handling)** to 100 ml, and then dissolving 0.15 g of sodium sulphite in this solution) : 75 ml ; dithizone (pure) solution (0.005% w/v in chloroform)** : 7.5 ml ; chloroform : 17.5 ml ;

Procedure : Dissolve 0.0079 g of pure lead nitrate in 1 litre of DW in a volumetric flask. To 10 ml of this solution (equivalent to about 50 mcg of Pb) contained in a 250-ml separatory funnel, add 775 ml of ammonia-cyanide-mixture, and adjust the pH of the resulting solution to pH 9.5 by the careful addition of HCl. Now, add 7.5 ml of dithizone solution and 17.5 ml of chloroform rapidly. Shake the contents of the separatory funnel thoroughly for 1 minute, and allow the phases to separate. Determine the absorbance at 510 nm *vis-a-vis* a blank solution in a 1.0 cm absorption cell. However, a further extraction of the same solution yields zero absorption thereby indicating that complete extraction of lead has taken place.

27.5.4. DETERMINATION OF MOLYBDENUM (VI) BY THE THIOCYANATE METHOD

Theory : Molybdenum (VI) is mostly converted to molybdenum (V) when an acidic solution of the former is treated with tin (II) chloride preferably in the presence a little Fe^{2+} ion. The resulting molybdenum (V) form a red complex with thiocyanate ion as follows :

$$Mo^{6+} \xrightarrow[SnCl_2\ ;\ Fe^{2+}\ (traces)]{H^+\ ;} Mo^{5+}$$

$$Mo^{5+} + 5\ SCN^- \longrightarrow \underset{\text{Red-complex}}{Mo\ (SCN)_5}$$

Consequently, the red-complex is extracted with either solvents possessing donor oxygen atoms, such as : 3-methyl butanol. However, Mo (VI) may also be extracted with diethyl ether-an oxygenated solvent, because it yields the maximum percentage extractive with 7.0 M NH_4 SCN as could be seen from the following Table 27.2.

Table 27.2 : Diethyl Ether Extraction of Mo (VI) with Various Anions

Metal	HCl[a] (6.0 M)	HBr[b] (6.0 M)	HI[c] (6.9M)	NH_4SCN[b] (7.0M)	HNO_3[b] (8.0 M)
			PERCENTAGE EXTRACTIVES		
Mo (VI)	85	54	6.5	97	0.6

a-3 : 2 volume ratio of organic to aqueous phase

b-1 : 1 volume ratio of organic to aqueous phase

c-4 : 1 volume ratio of organic to aqueous phase

The molybdenum complex exhibits maximum absorption at 465 nm.

Materials Required :

(*i*) **Standard Molybdenum Solution :** Dissolve 0.184 g of ammonium molybdate $(NH_4)_6\ [Mo_7O_{24}]\ 4H_2O$ in 1 litre of distilled water in a volumetric flask : this yields a 0.01% solution which can be

* Dithizone of the **'purest quality'** must be used for the assay, since it readily oxidizes to form diphenylthiocarbadiazoles $[S = C\ (N = N - C_6H_5)_2]$ which is non-reactive to metals.

** 1 ml of dithizone solution ≡ 20 mcg of Pb ;

diluted to 0.001% with 0.1 M HCl, thereby giving a Mo solution containing 100 mcg ml^{-1},

(*ii*) **Ammonium Iron (II) Sulphate Solution :** Dissolve 10 g of the salt in 100 ml of very dilute sulphuric acid,

(*iii*) **Tin (II) Chloride Solution :** Dissolve 10 g of Tin (II) chloride dihydrate in 100 ml of 1 M HCl, and

(*iv*) **Potassium Thiocyanate Solution :** Prepare a 10% w/v aqueous solution from the pure salt ('AnalaR'-Grade).

Procedure : The various steps involved are as follows :

(1) First of all construct a calibration curve by transferring accurately 1.0, 2.0, 3.0, 4.0 and 5.0 ml of the 0.001% Mo solution (*i.e.*, containing 10, 20, 30, 40, and 50 mcg Mo respectively) in individual 50-ml separatory funnels and diluting each of them with an equal volume of water.

(2) Add to each funnel 2 ml of conc. HCl, 1 ml of ammonia iron (II) sulphate solution, and 3 ml of the potassium thiocyanate solution,

(3) Shake gently and then induce 3ml of the tin (II) chloride solution,

(4) Add water to bring the total volume in each separatory funnel to 25 ml and mix thoroughly,

(5) Introduce exactly 10 ml of redistilled 3-methyl butanol into each funnel and shake them separately for 30 seconds,

(6) Allow the two phases to separate completely and carefully drain out the lower aqueous layer,

(7) Remove the glass-stopper and pour the alcoholic extract through a small plug of purified glass wool in a small funnel and transfer the organic extract to a 1 cm absorption cell,

(8) Measure the absorbance at 465 nm in a UV spectrophotometer against a 3-methyl butanol blank,

(9) Plot the graph by taking absorbance against concentration of Mo in Mcg, thereby obtaining a straight line spreading over a range 0-50 mcg of Mo (obeying **Beer's Law**), and

(10) Finally, determine the concentration of Mo in unknown samples provided and containing less than 50 mcg Mo per 10 ml ; make use of the calibration curve, and subject the unknown samples to the same treatment as the standard solutions.

27.5.5. DETERMINATIONS OF NICKEL (II)

A. As Dimethylglyoxime Complex

Theory : In ammoniacal solution, Ni (II) forms an insoluble red coordination compound with dimethylglyoxime ($C_4H_8O_2N_3$). Nickel dimethylglyoximate is only sparingly soluble in chloroform (35-50 mcg Ni ml^{-1}). It is, however, necessary to know the approximate amount of Ni present in the sample, so as to avoid adding a large excess of dimethylglyoxime, which is not very soluble in water and may precipitate easily along with the nickel-complex. The optimum pH range at which the extraction of this complex should be carried out ranges between 7-12 in the presence of citrate. It has been observed that the nickel-complex is quite bulky in nature when first precipitated and hence, shows a tendency to move up along the walls of the container. Therefore, care should be taken that the sample must not contain more than 50 mg of Ni. Lastly, the nickel complex absorbs at 366 nm and also at 465-470 nm.

The formation of nickel dimethylglyoximate complex may be expressed as follows :

$$Ni^{2+} + 2\ C_4H_8O_2N_2 \xrightarrow{NH_3} Ni\ (C_4H_7O_2N_2) + 2\ H^+$$

Materials Required : Ammonium nickel sulphate (pure) : 0.135 g ; citric acid : 5.0 g ; dilute ammonia solution ; dimethylglyoxime solution (dissolve 0.50 g of dimethyl-glyoxime in 250 ml of ammonia solution and diluting to 500 ml with water) : 20 ml ; chloroform : 50 ml ;

Procedure

(1) Weigh accurately 0.135 g of pure ammonium nickel sulphate ($NiSO_4$, $(NH_4)_2$ SO_4, $6H_2O$) and dissolve in 1 litre of distilled water in a volumetric flask,

(2) Transfer 10 ml of the resulting solution (Ni $\simeq$ 100 mcg) into a breaker containing 90 ml of water,

(3) Add to it 5 g of citric acid, and then dilute ammonia solution carefully until the pH is 7.5,

(4) Cool and transfer to a separatory funnel, add 20 ml of dimethylglyoxime solution and, after standing for a minute 12 ml of chloroform,

(5) Shake the contents of the funnel for 1 minute, permit the two phases to separate out completely,

(6) Collect the lower red chloroform layer and determine the absorbance at 366 nm in a 1 cm absorption cell against a blank, and

(7) Extract once again with a 12 ml portion of chloroform and measure its absorbance at 366 nm ; usually very negligible Ni (II) may be found.

B. Synergistic Extraction

Theory : Dithizone and 1, 10-phenanthroline (see Section 27.3.3) help in the synergistic extraction of Ni (II) both quantitatively and rapidly over a wide range of pH between 5.5 to 11.0 to give rise to a dark coloured mixed-ligand complex that absorbs at 520 nm. The resulting complex bears the following vital characteristic features, namely :

(*i*) It is fairly stable to allow the complete removal of excess dithizone by back-titration with 0.1 M NaOH, so as to make a 'monocolour' method feasible,

(*ii*) The molar absorptivity of the complex stands at 4.91×10^4 mol^{-1} L cm^{-1}, and

(*iii*) The synergistic method is predominantly much more sensitive as compared to any other method for the determination of Ni (II).

Materials Required : Ammonium nickel sulphate* (pure) : 0.0135 g ; phthalate or acetate (ethanoate) buffer (pH 6.0) : 5 ml ; dilute ammonia solution ; chloroform : 15 ml ; sodium hydroxide (0.1 M) : 10.0 ml ;

Procedure

(1) To 5 ml of a solution containing from 1 to 10 mcg of Nickel (II) 5 ml of a phthalate or acetate buffer,

(2) In case, the sample is acidic, adjust the pH to 6.0 with dilute ammonia solution carefully,

(3) To the resulting solution add 15 ml of chloroform solution of dithizone and 1, 10-phenanthroline,

(4) Moderately shake the two phases for 5 minutes in a separatory funnel, allow them to separate distinctly into aqueous and chloroform (lower) layers,

(5) Excess dithizone may be removed from the chloroform layer by back-extraction with 10 ml of 0.1 M NaOH, (a through shaking for 60 seconds will suffice this extraction),

(6) Once again separate the chloroform layer (lower) and measure its absorbance in a 1 cm absorption cell at 520 nm *Vs* an identically treated blank, and

(7) Finally, draw a calibration curve using standard Ni (II) solution containing 2, 4, 6, 8, and 10 mcg in 10 ml (obeying **Beer's Law**).

Caution : All glassware must be rinsed with dilute acid and then thoroughly with distilled water.

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* Dissolve 0.0135g of ammonium nickel sulphate in 1 litre of DW ; dilute 10 ml of this solution to 100 ml with DW (1 ml of this solution ≡ 2 mcg of Ni).

Note : The reagent must be prepared afresh using 'AnalaR-Grade' dithizone and 1, 10-phenanthroline, preferably taken from a new or recently opened reagent bottle.

THEORETICAL AND PRACTICAL EXERCISES

1. What is the importance of '*liquid-liquid extraction*' in the domain of actual estimation ? Explain.

2. Discuss the **Nernst Distribution Law** or **Partition Law** with reference to the theoretical aspects of liquid-liquid extraction support your answer with suitable examples.

3. Expatiate the *two* following vital aspects of liquid-liquid extraction :

(*a*) Error due to the volume change, (*b*) Effectiveness of an '*extraction*'.

4. Enumerate the following **four** cardinal factors which influence the solvent extraction mostly :

(*i*) Effect of temperature and inert solutes, (*ii*) Effect of pH on extraction,

(*iii*) Effect of ion-pair formation, and (*iv*) Effect of synergistic extraction

Provide suitable examples wherever possible to make your explanation more plausible and understandable.

5. What do you understand by the term '*free energy*' of the system between two immiscible solvents ? How would you encounter the emulsion problem in liquid-liquid extractions ? Explain.

6. Discuss the assay of Ni(II) :

(*a*) As dimethylglyoxime complex, and (*b*) By synergistic extraction.

7. Describe the theory and methodology for the assay of Cu(I) as its neo-cuprin complex.

8. Discuss the theoretical and procedural aspects for the assay of Fe(III) as its Fe(III) oxinate complex.

9. How would you determine Pb(I) by the dithizone method ? Explain.

10. Give the theory and procedure for the determination of Mo(VI) by the thiocyanate method.

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4. Alders, L., **'Liquid-Liquid Extraction : Theory and Laboratory Practice',** 2nd ed., Amsterdam, Elsevier, 1959.

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6. Karger, B.L., L.R. Snyder and C. Horvath., **'An Introduction to Separation Science',** New York, John Wiley & Sons, Inc., 1975.

7. Miller, J.M., **'Separation Methods in Chemical Analysis',** New York, John Wiley & Sons, Inc., 1975.

8. Jeffrey, G.H., J. Basett, J. Mendham and R.C. Denney, **'Vogel's Textbook of Quantitative Chemical Analysis',** 5th ed., ew York, Longman Scientific & Technical, 1989.

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28 THIN LAYER CHROMATOGRAPHY (TLC)

CONTAINS :

The following *four* aspects of chromatography shall be discussed in Part V of this book in addition to applications of these chromatographic techniques exclusively to quantitative analyses of pharmaceutical substances :

- Thin-Layer Chromatography (TLC),
- Gas-Liquid Chromatography (GLC),
- High Performance Liquid Chromatography (HPLC), and
- Size Exclusion Chromatography (SEC).

28.1. INTRODUCTION

Thin-layer chromatography (TLC) is an altogether new, versatile and specialized laboratory technique that was evolved in early Fifties, and since then has become an indispensable means of separation for analysts and researchers round the globe. It can be employed conveniently both for organic and inorganic substances,

either derived from natural sources or synthesized in the laboratories, on quantities ranging from the nanogram to microgram levels.

Kirchner in 1950 was the first who used adsorption chromatography on impregnated glass-plate coated with silicic acid or alumina. It may be emphasized, however, that **Egon Stahl's fundamental work** stands as a landmark in the world-wide acceptance of this new technique in the laboratory. Later on, Stahl in 1958, introduced a standard equipment for preparing uniform thin-layers of known thickness, which eventually led to the ultimate acceptance of this new technique as an additional modern tool for analytical chemistry.

This is invariably referred to in various literature as : **'open-column chromatography'; 'drop chromatography' ; 'strip-chromatography' ; 'spread-layer chromatography' ; 'surface chromatography'.**

TLC in addition to combining the meritorious plus points of column and paper chromatography, also considered to be extraordinarily superior to either of the two in certain aspects.

28.2. THEORY

The adsorbent used in TLC is a thin, uniform layer (normally 0.24 mm thick) of a dry, finely powered material applied to an appropriate support, such as a glass plate or an aluminium sheet or a plastic foil. Subsequently, the mobile phase is permitted to move across the surface of the plate (usually by capillary action) and the chromatographic phenomenon may solely depend upon adsorption, partition, or a combination of both, depending on the adsorbent, its treatment, and the nature of the solvents employed. During the chromatographic separation procedure the TLC-plate is placed in a chromatographic chamber, mostly made up of glass to enable clear observation of the movement of the mobile phase up the plate, that is pre-saturated with the solvent vapour. The inert solid supports invariably employed are, namely : alumina, silica gel, kieselguhr and cellulose, to these may be added appropriate substances, for instance : calcium sulphate (gypsum) so as to provide adequate adhesion to the solid support, example : silica gel-G (G-stands for gypsum).

The prepared layer may be impregnated with suitable materials to achieve specific purpose, namely :

(*i*) **Buffering materials :** To afford acidic, basic or neutral layers,

(*ii*) **Silver nitrate :** To modify its characteristics *e.g.*, for separation of methyl esters of fatty acids, and

(*iii*) **Ion-exchange materials :** To modify its properties, *e.g.*, admixture of cellulose with ion-exchange resins used for the separation of nucleic acids and their respective derivatives.

Therefore, the application of skill and wisdom may give rise to a fairly wide spectrum of possible layers, employed in conjunction with a vast combination of solvent systems permits and affords an almost infinite variation of separating power that really makes TLC such a versatile and useful technique in the domain of pharmaceutical analysis.

28.2.1. VERSATILITY OF TLC OVER PAPER AND COLUMN CHROMATOGRAPHY

In general, TLC essentially not only amalgamates the meritorious plus points of both paper and column chromatography but also it is distinctly superior and more versatile to either of the two methods. However, the versatility of TLC over paper and column chromatography are quite evident from the following points, namely :

(*i*) **Simple equipments :** TLC mostly requires very simple equipments, such as : micro-slides ; specimen jars with lid ; glass-sprayers ; strips of glass sheet ; small chromatank etc.

(*ii*) **Short development time :** In TLC, the separation is very rapid *i.e.*, the development time is of short duration (say 1 hour) for reasonably good separation on inorganic adsorbent layers. Hence, it has a positive edge over paper and column chromatography which normally takes several hours or days.

(*iii*) **Wide choice of stationary phase :** TLC may be used for adsorption, partition (including reversed phase) or ion-exchange chromatography,

(*iv*) **Quick recovery of separated constituents :** TLC permits the possibility of removal of the adsorbent coating on the plates by scraping with a spatula. In other words, a spot or a zone can be removed

quantitatively, and the separated constituent dissolved in an appropriate solvent is estimated either by suitable spectrophotometric or colorimetric analysis.

(*v*) **Separation effects :** The separation effects obtained by TLC are more distinctive and superior than those of paper chromatography,

(*vi*) **Easy visualization of separated components :** Detection of fluorescence components when exposed to UV light is much easier than on paper by virture of the fact that inorganic material (*i.e.*, adsorbent) has intrinsic fluorescence,

(*vii*) **Detection Limit :** TLC affords extremely sharp delineated spots and offer lower detection limit *i.e.*, one decimal power less than that in paper chromatography,

(*viii*) **Variable thickness of think layers :** The method employed in TLC may be further extended to preparative separations by using thicker layers and also to meet separations by column chromatography,

(*ix*) **Chemically inert stationary phase :** Use of inorganic adsorbents *e.g.*, alumina and silica, in TLC allows the application of corrosive sprays to detect fractionated substances, for instance : carbohydrates by 70% conc. H_2SO_4, and

(*x*) **Trace analysis :** TLC method is suitable as micromethod in trace analysis.

28.3. EXPERIMENTAL TECHNIQUES OF TLC

The various techniques with regard to thin layer chromatography (TLC) are as stated below, namely :

28.3.1. PREPARATION OF THIN LAYERS ON PLATES

The paramount importance with regard to the preparation of thin layer is that it must be uniform and consistent throughout. Various means have been put forward to apply thin layers of powdered or their suspensions or their slurries to the carrier plates with a view to achieve an uniform layer throughout the length of the plates. These are namely :

(*a*) **Pouring of Layers :** In order to obtain layers of equal thickness, a measured amount of the suspension or slurry is placed on a given-size plate that is rested on an absolutely labelled surface. The plate is subsequently tipped backward and forward to permit the slurry (or suspension) to spread uniformly on the surface of the plate.

(*b*) **Dipping :** Peifer* in 1962, was pioneer in introducing this technique, whereby two plates at a time back-to-back are dipped together in a slurry of the adsorbent in either chloroform or chloroform-methanol. However, this particular methods is not much in use now-a-days.

(*c*) **Spraying :** Reitsema** was first to develop this method by making use of a small paint-sprayer for the distribution of the suspension or slurry onto the surface of the glass-plate.

Disadvantages : There are mainly *two* major disadvantages of this technique, namely :

(*i*) Non-uniformity of layers on a single-plate, and

(*ii*) Variation observed from one plate to the other was significant.

Belgian Patent No : 625012 : It essentially consists of spraying either molten or partially molten absorbent onto a glass plate, for instance : an alumina film prepared by melting and aluminium rod with an oxyacetylene flame and subsequently spraying the molten adsorbent onto a glass plate.

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* Peifer, J.J., **Mikrochim Acta,** 529, 1962.

** Reitsema, R.H., **Anal. Chem., 26,** 960, 1954.

(*d*) **Spreading :** In this particular case, the suspension or slurry is put in an 'applicator', which is subsequently moved either over the stationary glass-plate or *vice-versa i.e.*, it is held stationary while the glass plate is pulled or pushed through. This technique termed as 'spreading' usually yields uniform thin layers on the glass plates.

Kirchner's* technique essentially consists of :

- selecting uniform surfaced glass plates,
- placing them between glass or metal guides which are thicker than glass plates by the amount that is desired for the adsorbent layer, and
- spreading the slurry on the glass plate with the help of a glass rod.

Egon Stahl's apparatus exclusively designed for the application of thin-adsorbent layers which broadly comprises of *two* major parts, namely :

(*i*) **Aligning Tray :** It is a tray on which the glass plates are placed in a series or in-a-line, and

(*ii*) **Spreader :** It holds the spreading mixture (as a slurry or suspension) and applies it uniformly in a thin-layer.

Figure 28.1 (*a*) shows the operation of thin-layer spreader ; while Figure 28.1 (*b*) depicts the aligning tray with glass-slide partially coated.

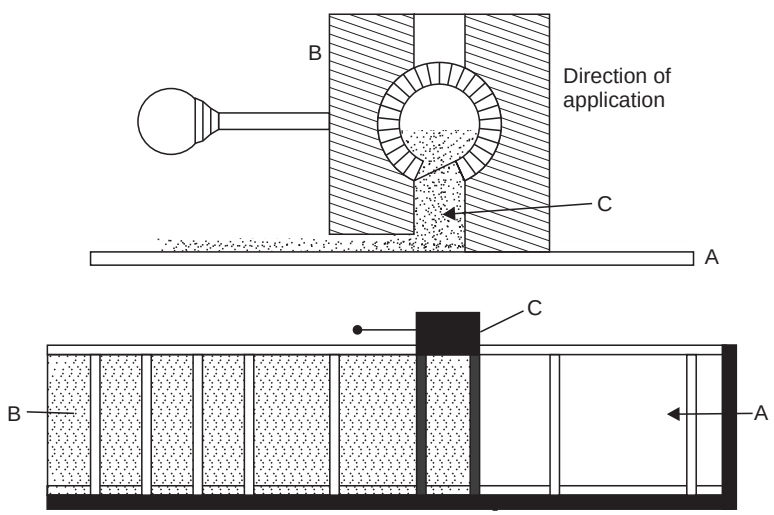

Figure 28.1 (*a*) Operation of thin layer spreader
(*b*) Aligning Tray with Glass-slide Partially Coated.

In Figure 28.1 (*a*) A = Glass plate,
B = Spreader, and
C = Slurry of the adsorbent.

Here, the slurry (C) is put in the spreader (B) and then moved along the direction of application onto the surface of the glass plate (A) to obtain an uniform layer,

In Figure 28.1 (*b*) A = Glass plate,
B = Layer of adsorbent, and
C = Aligning tray

* Kirchner, J.G., Miller J.M. and Keller, G.J., **Anal, Chem., 23, 420, 1951.**

The slurry of the adsorbent is introduced into the aligning tray (C) which is then moved onto the glass plate (A) to obtain an uniform layer of adsorbent (B).

In 1962, a well known West German firm DESAGA introduced a much improved and simplified version of a TLC-spreader that could conveniently give uniform layer thickness raging from 0.00 to 2,0 mm.

(*e*) **'TLC-Plates ready-for Use' (or Pre-coated Plates)**

E. Merck AG (Darmstadt, West Germany) was pioneer in introducing *two* types of ready-for-use TLC plates either on glass or polysheets (ethyleneterephthalate), namely :

(*i*) Ready-for-use TLC Plates with Cellulose-F, and

(*ii*) Ready-for-use TLC Plates with Silica Gel F-254.

Interestingly, these precoated TLC-plates essentially have : first, a special abrasive-resistant layer containing no gypsum ; and secondly, the layer contains a reliable fluorescent indicator that is excited to emit a fluorescence under either a short-wave or a long-wave UV light.

Advantages : These '*ready-for-use*' TLC-plates have the following advantages namely :

- It may be safely activated at 110-120° C, before, use,
- The properties of the layer minimise spot-diffusion that helps both more strong concentration of spots and more distinctive separations with higher sensitivity,
- It may accept more corrosive spray-reagents, for example : conc. sulphuric acid, phosphoric acid, phosphomolybdic acid, perchloric acid on antimony trichloride ; and the sprayed plates could be heated upto 110-120 °C without any darkening whatsoever,
- The migration rate is slightly enhanced when compared to hand coated plates, and
- The TLC plates may be cut into strips by the aid of a glass cutter applied on the reverse side.

28.3.2 CHOICE OF ADSORBENTS

The choice of proper adsorbent in TLC plays a vital role in the separation of components either belonging to natural origin or to purely synthetic origin. It is chiefly based on certain crucial informations like :

(*i*) Solubility of the substance *e.g.*, hydrophilic and lipophilic,

(*ii*) Nature of the compound *i.e.*, whether it is acidic/basic/neutral/amphoteric

(*iii*) Reactivity of compound with either the solvent or the adsorbent, and

(*iv*) Chemical reactivity of compounds with the binders.

In actual practice, the adsorbents are of two types : firstly the *inorganic*, and secondly, the *organic* adsorbents. A host of substances from each type are used in TLC and these shall be discussed briefly as below :

28.3.2.1. Inorganic adsorbents

These are namely :

(*i*) **Aluminium oxide- (Al_2O_3) :** The alkali (Na_2CO_3 ; $NaHCO_3$) present in alumina very often gives rise to secondary reactions that may be eliminated by washing with dilute mineral acid or with water, followed finally by methanol and ultimately by heating at 200 °C.

Note : Justisaz and Teichner* in 1947 suggested that 1 g of alumina for TLC has 90 sq. M surface area and the one having less than 6 sq. M is useless. Alumina is usually available in three grades :

* Jutisaz, M. and S. Teichner, **Bull. Soc. Chim.** France, 389, 1947.

(*a*) acidic (pH $\simeq$ 4.0) ; (*b*) basic (pH $\simeq$ 9.0) ; and (*c*) neutral (pH $\simeq$ 7.5).

(*ii*) **Aluminium Silicate :** It permits the adsorption of sterols and sterol glycosides from oils without the use of solvent.

(*iii*) **Bauxite (aluminium oxide ore) :** Zechmeister used bauxite for the separation of enzymic hydrolysates of chitin (a nitrogen-containing polysaccharide found in certain fungi *e.g.*, ergot) ; whereas La Lande employed it for the refining of sugar.

(*iv*) **Bentonites :** It is used mostly for the separation of Vitamin D from vitamin A and sterols and 2,4-dinitrophenyl hydrazones of aldehydes and ketones.

(*v*) **Calcium Carbonate :** It is used as such for the separation of xanthophylls and napthaquinones or other pigments and elution is done with dilute acid to isolate the various components present.

Note : Vaterite-the unstable crystalline modification of calcium carbonate has much greater adsorbent capacity than aronite or calcite.

(*vi*) **Calcium Hydroxide :** It is used as an adsorbent for the separation of carotenoids.

(*vii*) **Calcium Oxalate :** It is used for the separation of anthraquinones and related hypericins (*i.e.*, a dianthrone pigment found in the leaves and petals of *Hypericum perforatum*, Family ; *Guttiferae*).

(*viii*) **Calcium Silicate :** It is employed frequently for the separation of carbohydrates and the corresponding phenylosazones.

(*ix*) **Calcium Sulphate :** It is found to be suitable for the separation of steroids and lipids.

(*x*) **Dicalcium Phosphate :** It is used for the purification of carotene-the natural red pigment.

(*xi*) **Fuller's Earth :** It is hydrous magnesium aluminosilicate which is employed extensively in the petroleum industry for the decolaration of oils. It is also employed for the separation of amino acids and pteridines.

(*xii*) **Hydoxyl-Apatite :** It is a complex calcium phosphate hydroxide which is used for the separation of proteins and glycerides. In may be used with/without binder.

(*xiii*) **Kieselguhr (Diatomaceous Earth) :** (pH 7.0) : It is available both with and without a binder. Its capacity of resolving constituents is less than either silica gel or alumina.

(*xiv*) **Magnesium Silicate (Magnesol : MgO 2.5 SiO_2.H_2O) :** It is usually employed for the separation of sugar acetates ; whereas, magnesium trisilicate is used for the separation of steroids, acetylate gycosides, esters, glycerides, lactones etc.

(*xv*) **Silica Gel :** (pH 6.0) : It is used extensively for the separation of sterols, fatty acids, glycerides, azoated carbohydrates, sugar acetates, amino acids.

(*xvi*) **Tri-calcium Phosphate :** It is mostly used for the separation of enzymes.

(*xvii*) **Water-soluble salts :** A number of water-soluble salts are used in TLC for affecting separation of constituents, namely :

$CuSO_4$ (anhydrous) : for azobenzene derivatives,

$CuSO_4.5H_2O$: found to be better than alumina,

$ZnSO_4$; $MnSO_4$; $Al_2(SO_4)_3$ and $MgSO_4$: anhydrous salts good for azobenzene derivatives,

$Al_2(SO_4)_3$: for hydroxyl anthraquinones, and

Na_2CO_3 : for Vitamin A

(*xviii*) **Zinc Carbonate :** It is used for the separation of carotenoids and coloured derivatives of amino acids.

28.3.2.2. Organic Adsorbents

The organic adsorbents are known for their relatively milder action for the separation of good number of components, namely :

(*i*) **Cellulose and Acetylated Cellulose :** These adsorbents are commercially available in various forms *e.g.*, particle size, degree of acetylation, with or without binders like starch or Plaster of Paris.

(*ii*) **Charcoal and Activated Carbon :** Tiselius used charcoal for the frontal analysis of sugars, amino acids and other substances. Charcoal absorbs strongly aromatic substances, such as : amino acids, which may be explained by virtue of the fact that the carbon-carbon spacings in graphite are almost of the same order as those present in benzene. Charcoal is also employed for the adsorption of fatty acids.

Weiss* used impregnated activated carbon with fatty acid or non-electrolyte thereby modifying and attributing special and improved adsorption characteristics.

(*iii*) **Dextran Gels :** Proteins and nucleotides can be separated by using cross-linked dextran gels available in various types and particle sizes. The molecular weight of dextran-gels vary considerably depending upon the extent of cross-linked nature.

(*iv*) **Cellulose Ion-Exchange Powder :** Interestingly, the cellulose powder have been modified by state-of-the-art technique that they invariably mimic as real ion-exchangers, namely :

DEAE-Cellulose = Diethaminoethyl cellulose,

ECTEOLA-Cellulose = Epichlorhydrin linked triethanolamine cellulose, and

PEI-Cellulose = Polyethylenimine cellulose.

Note : These absorbents may be used both with or without binders, such as : colloidion.

(*v*) **Ion-Exchange Resins :** Nucleic acids and their respective derivatives may be separated either by using ion-exchange resins alone or in conjunction with cellulose powder.

(*vi*) **Polyamide :** Flavanoids-the phenolic substances may be separated effectively using polyamide as such or with a binder, for instance : plaster of Paris or starch.

(*vii*) **Polyethylene Powder :** Fatty acids and their corresponding esters are separated by using polyethylene powder.

(*viii*) **Sucrose :** Both xanthophylls and chlorophylls (*i.e.*, chlorophyll-*a* and -*b*) are separated by using sucrose powder effectively.

28.3.3. CHOICE OF SOLVENT SYSTEM IN TLC

The choice of solvent or a mixture of solvents used in TLC is solely guided by two important factors : (*a*) the nature of the constituent to be separated *i.e.*, whether it is polar or non-polar ; and (*b*) the nature of the process involved *i.e.*, whether it is a case of 'adsorption' or 'partition chromatography'. It has been observed that the rate of migration of a substance on a given adsorbent depends upon the solvent used ; therefore, the latter may be arranged in order of the elutive power, usually termed as the **elutropic series** as shown in the following Table 28.1.

* Weiss, D.E., **Nature, 162,** 372, (1948).

Table 28.1 : Eluotropic Series of Solvents for Adsorbents Containing Oxygen

Series-'A'*		Series-'B'**
Petroleum Ether	↓	*n*-Pentane
Carbon Tetrachloride	I	Petroleum Ether (bp 30-50 °C)
Trichloro Ethylene	N	Petroleum Ether (bp 80-100 °C)
Benzene	C	*n*-Hexane
Dichloromethane	R	*n*-Heptane
Chloroform	E	Cyclohexane
Diethyl Ether	A	Carbon Tetrachloride
Dimethyl Formamide	S	Trichloroethylene
Ethyl Acetate	I	Benzene
Pyridine	N	Dichloromethane
Acetone	G	Chloroform (Alcohol-Free)
n-Propanol	E	Diethyl Ether (Absolute)
Ethyl Alcohol	L	Ethyl Acetate
Methyl Alcohol	U	Pyridine
Formamide	T	Acetone
Water	I	*n*-Propanol
Glycol	V	Ethyl Alcohol
Glycerine	E	Methyl Alcohol ; Water
	P	
	O	
	W	
	E	
	R	
	↓	

Note : (*i*) These series are not always valid in precisely the same order for all substances,

(*ii*) These series may be regarded as good guides for selecting a specific solvent only, and

(*iii*) These series are good for hydrophyllic adsorbents and not for hydrophobic ones *e.g.*, charcoals.

From actual experimental results it has been established beyond any reasonable doubt that the mixtures of two or three solvents of different polarity mostly offer distinct and much improved separation as compared to chemically homogeneous solvents. Table 28.2 records the elutropic series of one and two component solvents.

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* Hesse, G.**J. Anal. Chem., 181,** 274, 1961.

** Wohleben, G., Cited in **Handbuch der Lebensmitted Chemic,** Band II, Tell I, (Springechu Verlag, Berlin), 584, 1965.

Table 28.2 : Eluotropic Series of 1 and 2-Component Solvents

Benzene	↓	Benzene : Ether (1 : 9)
Benzene : Chloroform (1 : 1)	I	Ether : Methanol (9.9 : 0.1)
Chloroform	N	Ether
Cyclohexane : Ethyl Acetate (8 : 2)	C	Ether : DMF (9.9 : 0.1)
Chloroform : Acetone (95 : 5)	R	Ethyl Acetate
Benzene : Acetone (9 : 1)	E	Ethyl acetate : Methanol (9.9 : 0.1)
Benzene : Ethyl Acetate (8 : 2)	A	Benzene : Acetone (1 : 1)
Chloroform Ether (9 : 1)	S	Chloroform : Methanol (9 : 1)
Benzene : Methanol (95 : 5)	I	Dioxane
Benzene : Ether (6 : 4)	N	Acetone
Cyclohexane : Ethyl Acetate (1 : 1)	G	Methanol
Chloroform : Ether (8 : 2)	E	Dioxane : Water (9 : 1)
Benzen : Acetone (8 : 2)	L	
Chloroform : Methanol (99 : 1)	U	
Benzene : Methanol (9 : 1)	T	
Chloroform : Acetone (8.5 : 1.5)	I	
Benzene : Ether (4 : 6)	V	
Benzene : Ethyl Acetate (1 : 1)	E	
Chloroform : Ether (6 : 4)	P	
Cyclohexane : Ethyl Acetate (2 : 8)	O	
Butyl Acetate	W	
Chloroform : Acetone (7 : 3)	E	
Benzene : Ethyl Acetate (3 : 7)	R	
Butyl Acetate : Methanol (9.9 : 0.1)	↓	

Note : The numbers refer to proportionate volumes.

28.3.4. ACTIVATION OF ADSORBENT

In fact, it is extremely important to eliminate as completely as possible the solvent imbedded into the thin layer of coated adsorbent. It is achieved conveniently first by air-drying the TLC plates for a duration of 30 minutes and then in a hot-air oven maintained at 110 °C for another 30 minutes and subsequently cooling them in a dessicator. This drying process helps a great extent in rendering the adsorbent layer active. In order to achieve very active layers, silica gel and alumina coated plates may be heated upto 150 °C for a duration of 4 hours and colling them in a dessicator.

28.3.5. PURIFICATION OF SILICA GEL-G LAYERS

The iron present as an impurity in silica gel-G affords an appreciable distortion of the ‘chromatogram’. Hence, it has become almost a necessary step to purify the adsorbent. The ‘iron-free’ layers may be achieved by providing the pre-coated and air-dried plates a preliminary development with a mixture of methanol and

concentrated hydrochloric acid* (9 : 1). By this process the entire iron gets migrated with the solvent front to the upper boundary of the TLC plate. Consequently, the purified plates are again dried and activated at 110°C.

The cleaning process usually washes out the $CaSO_4$ originally present as binder. Therefore, the silica gel thus obtained by purification may be reused to prepare TLC-plates with other appropriate binders like gypsum, starch etc.

28.3.6. SPOTTING OF THE COMPONENTS

The following points may be strictly adhered to while spotting the component or mixture of components on a TLC plate, namely :

(*i*) The sample is normally applied as a solution in a '*non-polar solvent*' as far as possible, since the use of a polar solvent may cause:

(*a*) spreading out of the starting spot, and

(*b*) affect directly the Rf value of components,

(*ii*) The solvent employed for dissolving the sample must be easily volatile-in-nature so that it should be removed from the TLC plate before development commences,

(*iii*) The 'area of application' should be smallest as far as possible so as to achieve a sharper resolution,

(*iv*) To maintain the size of the spot 'small' repeated applications is made by allowing the solvent to evaporate after each application. It can be easily achieved by :

(*a*) Pre-warming the TLC-plate, and

(*b*) Passing a stream of hot-air right below the sample spot (from a hair-drier).

(*v*) For exclusively 'preparative work' the sample is applied in a narrow-band the width of which must be kept as narrow as possible, and

(*vi*) Use of 'spotting templates', available commercially** may provide

(*a*) Device for making the 'starting-line'

(*b*) Device for making the 'finishing-line', and

(*c*) Means for uniformly spacing the spots on the starting-line.

28.3.7. DEVELOPMENT OF THIN LAYERS

The spotted TLC plates, after evaporation of the sample solvent, is placed in a closed chamber saturated with vapours of the developing solvent(s). One end of the plate is then wetted with the developer by means of either 'ascending-technique or the 'descending-technique' as shown in Figure 28.2 (*a*), (*b*). After the developer has traversed one-half to two-thirds the total length of the TLC plate, the latter is removed from the chamber, air-dried and the positions of the components are located by any of several methods.

There are *three* major factors which essentially govern the 'development of thin-layers', namely :

(*i*) Equilibration of the chamber (or chamber-saturation),

(*ii*) Protection against oxidation (temperature and light), and

(*iii*) Visualization.

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* Both Methanol and conc. HCl must be of 'AnalaR'-Grade Reagent.

** Manufactured by M/S DESAGA ; CAMAG ; CAMDEN ; & CHEMETRON ;

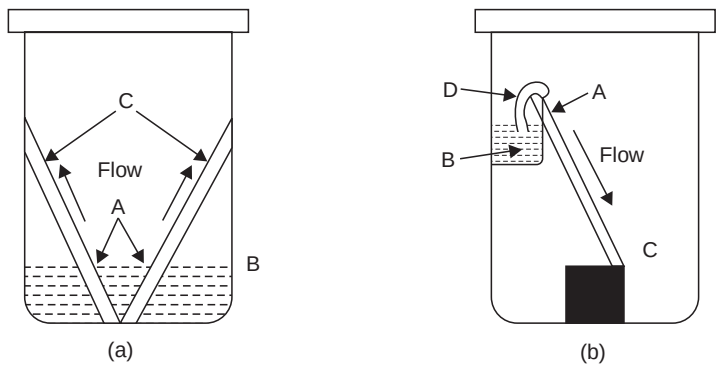


Figure 28.2 (*a*) : Ascending Flow A = Starting position of sample(s) ; B = Solvent System ; C = Chromatographic Surface ;
Figure 28.2 (*b*) : Descending Flow A = Starting position of sample(s) ; B = Developer ; C = Chromatographic surface ; D = Cotton wick ;

28.3.7.1. Equilibration of the Chamber

The equilibration of the chamber or chamber-saturation is a vital factor to obtain reproducible Rf values. It may be achieved by allowing the solvent system to remain in the chamber for at least 1 to 2 hours so that the vapours of the solvent(s) would pre-saturate the latter adequately. This is done to obtain distinct separation of constituents, uniform solvent from and prevent evaporation of the solvent on TLC-plates.

28.3.7.2. Protection against Oxidation

Both temperature and light augments oxidation and, therefore, ideally the following experimental parameters must be observed to obtain the best development of thin-layers, *viz*.,

Temperature : 18-23°C, and

Light : Diffused daylight both natural and artificial,

However, direct sunlight (UV) or drought may give rise to 'oblique formation' of the solvent front.

28.3.7.3. Visualization

As a result of both intensive as well as extensive research a number of organic and inorganic substances have been identified that positively demonstrate an '*improved visualization*'. Such substances are termed collectively as '**fluorescent indicators**'.

Examples : Barium diphenylamine sulphonate ; 2,7-dichlorofluorescein ; Fluorescein (0.2% w/v in Ethanol) ; Morin (0.1% w/v in Ethanol) ; Sodium fluorescinate (0.4% w/v in water) ; Rhodamine B ; Zinc Silicate ; Calcium silicate ; Methylumbelliferone (or 7-hydroxy-4-methyl coumarin).

28.3.8. SPECIAL TECHNIQUES IN TLC

The various special techniques applicable in TLC are enumerated below briefly with specific examples wherever possible, namely :

(*i*) **Horizontal TLC :** Mistryukov in 1961 introduced this technique whereby the horizontal development of loose-layer TLC plates were made by using a shallow dish having a ground glass cover. The TLC plate was carefully rested on a T-shaped glass piece and the starting end was pressed duly against a filter paper held by another glass strip, which allowed the solvent to move to the thin-layer-film from the bottom of the dish by capillary action.

Example : Methyl esters of mixed fatty acid may be separated on loose-layer of alumina using suitable solvent-system.

(2) **Continuous TLC :** It is good for the separation of such components having small as well as very close Rf values and may be achieved by using :

(*a*) Rectangular horizontal plates where the solvent is allowed to move over them and subsequently evaporated after it has almost reached the end of the run, and

(*b*) Triangular glass-plates-where the mixture to be separated is spotted near the apex on a thin-layer and two different solvent mixtures are fed from two sides to the thin-layer and fractions subsequently collected at the base (Reisert,* 1963 ; Turins** ; 1965 ;).

(3) **Preparative TLC :** TLC may be skillfully extended to cater for extremely useful method for preparative separations. To maintain uniformity, as a rule, plates of 20 cm height and 20-100 cm length with layers between 0.5 and 0.2 mm thickness are normally employed. It essentially has three cardinal features, namely :

(*a*) Component mixtures is always obtained either in streaks or bands,

(*b*) Separation is invariably accomplished by multiple development, and

(*c*) Localization of separated components is only done under UV-light.

Note : Amount of constituents ranging between 0.1 and 100 g can be separated without any loss easily.

(4) **Multiple Dimensional TLC :** It can be regarded as a variant of multiple development chromatography. It could be expatiated with the help of the following typical example, namely :

Example : Separation of mixture of fatty acids, cholesterols and their esters ; lecithins and polar lipids*** :

S. No.	Solvent System	Position of Plate	Separation Caused
1.	Propanol : Ammonia (2 : 1)	Up-right	(*i*) Resolution of polar lipids and lecithins ; (*ii*) Carried fatty acids, cholesterol and its esters to the solvent front
2.	Chloroform : Benzene (3 : 1)	-do-	(*i*) Separated fatty acids and free cholesterols (*ii*) Carried esters to the solvent front.
3.	Carbontetrachloride	Plate turned to 180°	Separation of only cholesterol esters.

(5) **Two-Dimensional Chromatography :** It is also termed as **two-dimensional planar chromatography.** Here, the sample is spotted in one corner of a square TLC plate (size : 20 cm × 20 cm) as shown in Figure 28.3. The development is first carried out in the ascending direction using solvent-1 (see legend of Figure 28.3). The solvent is then eliminated by evaporation and the plate is rotated through 90°, following which ascending with the second solvent is accomplished. After

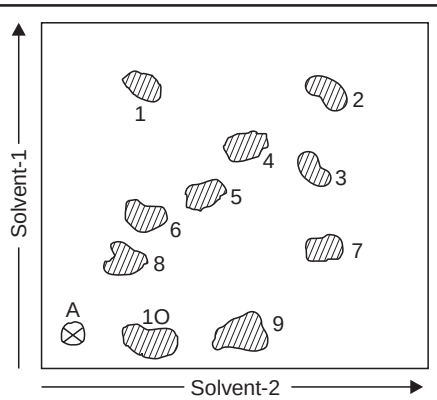


Figure 28.3 : Two-Dimensional Thin-Layer Chromatogram of some Amino-Acids : A = Sample spot *Solvent-1* : Toluene : 2-Chloroethanol : Pyridine; *Solvent - 2* : Chloroform : Benzylalc : Acetic Acid

* Reisert, P.M. and D. Schumacher., **Experientia, 19** (84), 1963.

** Turins, S. and V. Marianovio-Krojowan, V. Obradovic and M. Obradovic., **Anal. chem., 46,** 1905, 1965.

*** Weicker, H., **Klin Wache, 37,** 763, 1959.

removal of the solvent the spots of separated constituents are located by spraying with specific reagents.

Example : Mixture of amino acids obtained from protein hydrolysates are separated by this method* and spots located by using Ninhydrin Reagent that forms a pink to purple product with amino acids.

(6) **Centrifugal Chromatography :** It essentially makes use of the 'centrifugal force' so as to accelerate the flow of solvent through the thin-layer of the chromatogram. Korzum and Brody** in 1964, first applied this method to TLC, whereby the layers of plaster-of-paris bound alumina or silica gel were directly applied to either circular glass or aluminium plates with a hole in the centre to enable it to fit into the centrifuge. As usual, the sample mixture is applied 2.5 cm from the centre hole and the solvent system is set to allow a constant flow, with the centrifuge rotating at 500-700 RPM. In this manner, the usual developing time of 35 minutes is drastically reduced to mere 10 minutes by acceleration.

(7) **Wedged-Tip Chromatography :** Reindel *et al**** (1953) and Mathias***** (1954) introduced the wedged-tip technique in TLC which essentially exhibit the following *two* plus points, namely :

(*a*) Improved separation, and

(*b*) Constituents forced to assume an almost band-like path.

Figure 28.4, depicts the TLC-plate with wedged-tip divisions. The following steps are to be adopted sequentially, namely :

(*i*) Draw dividing lines 0.5 to 1.0 mm broad on the surface of the layer with a narrow-metal spatula,

(*ii*) Pentagons are facilitated by the help of a stencil made of transparent plastic material, and

(*iii*) Sample mixture are applied to the narrow portion of the wedge to get the best results.

Figure 28.5, illustrates TLC of an urine sample by the normal TLC-technique *vis-a-vis* the wedged-tip technique (Figure 28.5(*b*)). One may clearly visualize the beautiful separated bands in the latter as compared to the several odd and irregular-shaped spots in the former. Both the clarity of separation and the reproducibility of the results are predominant in the latter technique. Figure 28.5 (*a*) and (*b*) represent the typical analysis of a urine sample containing glucose, arabinose, lactose and fructose respectively.

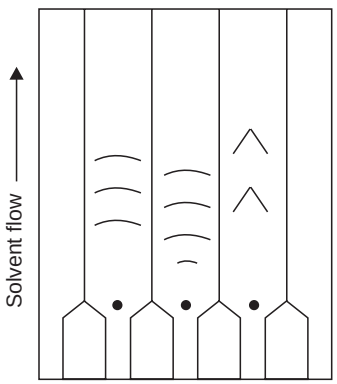


Figure 28.4 : TLC with Wedged-Tip Divisions
A = It occurs only with large quantities

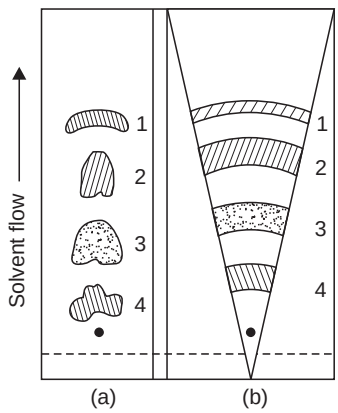


Figure 28.5 (a) TLC-Normal technique 28.5
(b) TLC-Wedged-Tip Technique
1 = Glucose (Blue-Violet) ; 2 = Arabinose (Blue)
3 = Lactose (violet) ; 4 = Fructose (Red) ;

* Von Arx. E., and R, Ann Nehr., **J. Chromatog, 12,** 329, 1963.

** Korzum, B.P., and Brody, S., **J. Pharm Sci., 53,** 454, 1964.

*** Reindel, F., and W. Hoppe., **Naturwissenschaften, 40,** 245, 1953.

**** Mathias, W., **Naturwissenschaften, 41,** 18, 1954.

28.3.9. CHEMICAL REACTIONS ON TLC PLATES

Glass being an inert material used for TLC-plates renders it ideal for utilization with strong corrosive reagents.

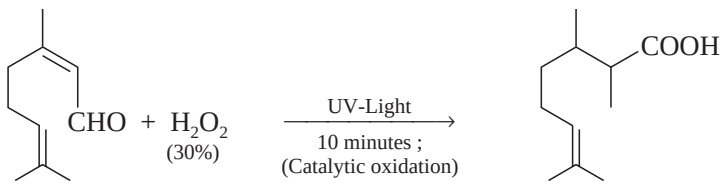
Miller and Kirchner* in 1953, were the pioneer in originating and developing the novel ideal of performing chemical unit-process reactions directly on TLC-plates. The *two* major steps involved in achieving this objective are, namely :

(*a*) Sample is spotted on a TLC plate in the usual manner and subsequently covered with a specific reagent, and

(*b*) Soonafter the reaction is completed, the TLC plate is developed using an appropriate solvent thereby separating the products of the reaction.

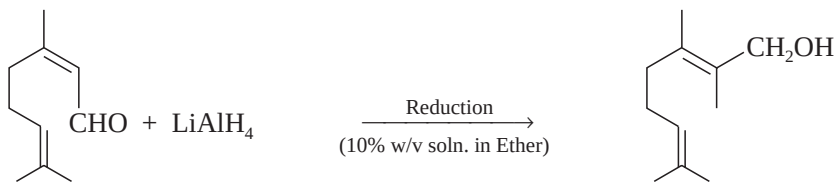
In actual practice, the resulting Rf value of the original compound together with the chromatographic results of the reaction are usually good enough to identify a compound accurately and precisely.

Example : (*i*) Citral reacts with 30% H_2O_2 in the presence of UV-light for a duration 10 minutes and undergoes catalytic oxidation to yield geranic acid as shown below :



Citral — Geranic acid

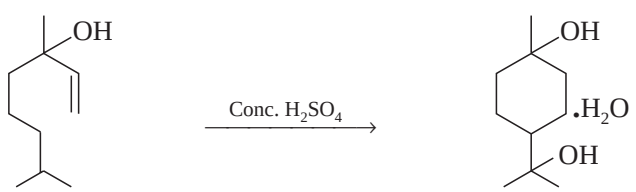
(*ii*) Citral undergoes reduction in the presence of 10% w/v solution of $LiAlH_4$ in ether to produce geraniol as represented in the following reaction :



Citral — Geraniol

Exactly in the same manner, a number of other chemical unit-process reactions may be accomplished on TLC plates as stated here briefly :

(*a*) **Dehydration :** Sample spot of terpene alcohols *e.g.*, linalol, be converted to hydrocarbons by adding a drop of conc. H_2SO_4 as shown below :



Linalol — Terpin hydrate

Consequently, the TLC plate is developed with hexane and since oxygenated compounds, do not move in hexane (*i.e.*, stay-back), only the hydrocarbons thus generated move away from the specific-reaction zone.

* Miller, J.M., and J.G. Kirchner, **Anal. Chem., 25,** 1107, 1953.

(*b*) **Bromination :** Cargill* in 1962, separated cholestanol from cholesterol by TLC. The mixture is spotted on a TLC plate and reacted with a soln. of Br_2 (0.1% w/v in $CHCl_3$), taking care that its quantity must be 2 to 3 times the weight of the sample mixture. Development in a solvent system consisting of benzene and ethyl acetate (2 : 1) would result in a clear distinction of cholestanol and reaction products of cholesterol with Br_2.

(*c*) **Enzymatic Reaction :** Randerath and Randerath** in 1964, demonstrated an enzymatic reaction directly on an anion-exchange layer of cellulose impregnated with polyethylene imine. A buffered solution of phosphodiesterase is applied to the sample spot of cytidine dipohosphate glucose, which is subsequently covered with paraffin and allowed to stand for 45-60 minutes at 23°C. Chromatography of the resulting degradation products gives rise to cytidine 5-monophosphate and glucose 1-phosphate.

(*d*) **Esterification :** Benneth and Heftmann*** in 1962, showed that it was feasible to esterify the C-3, hydroxy steroids directly on TLC plates by means of tri-fluoroacetic anhydride. After treating the compounds with the anhydride, it is absolutely necessary to dry the pate in the hood for several minutes so as to get rid of the trifluoroacetic acid that is produced as a by-product.

28.3.10. COMBINATION OF TLC WITH OTHER TECHNIQUES

TLC may be combined with *column chromatography* and *vapour-phase chromatography* as discussed here briefly :

(*a*) **Column Chromatography :** TLC helps in selecting the best combination of solvent and adsorbent for a given column separation. Miller and Kirchner**** (1952) developed this combination thoroughly and employed it extensively for the separation of a large number of difficult types of compounds.

(*b*) **Vapour-phase Chromatography :** The various spots obtained from TLC may be eluted, concentrated and then subjected to vapour-phase chromatogrphy studies. Ikeda *et al****** (1961, 1962) exploited this combination for the analysis of a variety of naturally occurring constituents, namely :

(*i*) Citrus oils and other essential oils,

(*ii*) Oestrogens in urine sample,

(*iii*) Testosterone in urine sample, and

(*iv*) Progesterone in plasma.

28.3.11. DETECTION OF COMPONENTS

After development of TLC plates, the next important step is to detect the separated components so as to determine their respective Rf values.

Example : (*i*) **Coloured Substances :** *e.g.*, Xanthophylls, Chlorophylls, Carotenes, etc., may be located visually.

(*ii*) **Colourless Substances :** *e.g.*, alkaloids, steroids, amino acids and the like may be detected under short-wave UV-light or a long-wave UV-light. These substances may also be detected as brown/dark brown spots when exposed to I_2-vapours in a closed dessicator.

(*iii*) **Specific Detecting Reagents :** A few specific detecting reagents are normally used for a particular class of compounds *e.g.*,

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* Cargill, D.I., **analyst, 87,** 865, 1962.

** Randerath, K., and E. Randerath., **Angew Chem Intern,** Ed, 3, 442, 1964.

*** Benneth, R.D., and E. Heftmann., **J. Chromatog., 9,** 353, 1962.

**** Miller, J.M. and J.G. Kirchner., **Anal Chem., 24,** 1480, 1952.

***** Ikeda *et al.*, **Food Technol, 15,** 379, 1961 ; Ikeda *et al*, **Food Science, 27**, 454, 1962.

Aniline-phthalate reagent : for carbohydrates ;

Ninhydrin reagent : for amino-acids, and

Dragendorff's reagent : for alkaloids

(*iv*) **Chromic acid/conc. H_2SO_4 :** These corrosive reagents usually char the organic material on TLC plates and may be seen as dark brown spots.

28.3.12. EVALUATION OF THE CHROMATOGRAM

After completing the detection procedure the various separated solutes on the TLC plate are marked with the help of a sharp needle (*e.g.*, pithing needle) ; subsequently, their evaluation may be carried out either qualitatively or quantitatively, as stated below :

28.3.12.1. Qualitative Evaluation

The Rf value (Retention Factor) various separated solutes is determined accurately. The Rf value represents the differences in rate of movement of the components duly caused by their various partition coefficients *i.e.*, their different solubility in the mobile and stationary phases. In order words, the Rf value (relate to front) is-'the ratio between the distance starting point-centre of spot and distance starting point-solvent front', thus it may be expressed as :

$$\text{Rf} = \frac{\text{Distance of centre of spot from starting point}}{\text{Distance of solvent front from starting point}}.$$

Important Points : (*i*) Due to the always longer path of the solvent front, the Rf value is invariably lesser than 1.

(*ii*) Rf value is always constant for each component only under identical experimental parameters, and

(*iii*) Rf value depends upon a number of governing factors, such as : quality of the layer material ; activation grade of the layer ; thickness of layer ; quality of solvent ; equilibration of chamber ; chromatographic technique employed (*e.g.*, ascending, descending) ; presence of impurities ; and conc. of simple applied ; and

(*iv*) All possible anomalies in (*iii*) above may be eliminated by performing a co-chromatogram of a standard substance along with that of a sample. Thus, the distance traversed by a substance is compared with that of the standard (or reference). This 'new' relation is usually designated as Rst-value. Therefore, in short, it is expressed as follows :

$$\text{Rst} = \frac{\text{Rf of the substance}}{\text{Rf of the standard}}$$

Unlike the Rf value, the Rst value may be more than 1.00 because here the substance under investigation (*i.e.*, sample) usually travels further than the standard.

In TLC, the qualitative evaluation is solely based on the determination of Rf values of unknown spots *vis-a-vis* Rf values of standard substances preferably on the same TLC plate so as to avoid any possible error whatsoever.

28.3.12.2. Quantitative Analysis

The quantitative analysis of chromatographically separated constituents may be carried out with high degree of accuracy and precision in *two* manners, namely :

(*i*) **Direct Method :** *i.e.*, the quantitative determinations is performed directly on the adsorbent layer, and

(*ii*) **Indirect Method :** *i.e.*, the separated constituents are quantitatively removed from, the adsorbent and subsequently estimated after elution.

28.3.12.2.1. *Direct Methods*

The various methods under this category are, namely :

(*i*) **Measurement of Spot-areas :** This method is solely based on a mathematical relationship existing between the prevailing spot area and the amount of component present. It is not quite accurate due to high random errors.

(*ii*) **Densitometry :** The intensity of the colour of a component is measured on the chromatogram using a densitometer.

(*iii*) **Spectrophotometry :** Characterization of the separated spots by reading the absorption or fluorescence curves directly from TLC plates is carried out with the help of Chromatogram Spectrophotometer devised by Zeiss, Stahl and Jork.

Besides, IR-spectroscopy, reflectance spectroscopy, spark chamber method etc., may also be employed for the direct evaluation of chromatograms.

28.3.12.2.2. *Indirect Methods*

These methods are based on elution techniques, followed by micro-analysis of the resultant eluate by adopting one or more of the undermentioned known methods, namely :

Colorimetry ; Fluorimetry ; Radiometry ; Flame-photometry ; UV-Spectrophotometry ; Gravimetry ; Polarography ; Vapourphase Chromatography ;

28.4. APPLICATIONS OF TLC IN PHARMACEUTICAL ANALYSIS

The technique of thin-layer chromatography (TLC) has been used extensively in the domain of pharmaceutical analysis for a variety of specific and useful applications, for example :

(*i*) To identify the presence of undesirable specific organic compounds present as impurities in a number of pharmaceutical substances, namely : morphine in apomorphine hydrochloride ; hydrazine in carbidopa ; 3-aminopropanol in dexampanthenol ; etc.,

(*ii*) Related substances present in official drugs, namely : related substances present in a wide number of potent pharmaceutical substances *e.g.*, aminophylline ; baclofen ; chloramphenicol ; carbamazepine etc.,

(*iii*) Foreign alkaloids present in alkaloidal drugs, for instance : atropine sulphate ; codeine ;

(*iv*) Foreign steroids present in steroidal drugs, for example : betamethasone valerate ;

(*v*) Ninhydrin positive substances in official amino acids *e.g.*, glutamic acid ; leucine ;

The various applications of TLC as cited above would be discussed in the sections that follow :

28.4.1. PRESENCE OF SPECIFIC SUBSTANCES AS IMPURITIES IN DRUG SUBSTANCES

Examples : (1) **Morphine in Apomorphine Hydrochloride**

Materials Required : Silica gel-G ; Mixture of Acetonitrile : Dichloromethane : Ethyl acetate ; Anhydrous formic acid : Water (30 : 30 : 30 : 5 : 5) ; solution (1) : 0.20% w/v of apomorphine in methanol ; solution (2) : 0.004% w/v of apomorphine HCl in methanol ; (3) Morphine : 2% w/v in methanol ; sodium nitrite solution (3% w/v in DW) ;

Procedure : Prepare the chromatogrphic tank by lining the walls with sheets of filter paper ; pour the mobile-phase into the tank, saturating the filter paper in the process, to a depth of 5 to 10 mm, close the tank and allow it to stand at 20° to 25 °C for 1 hour for equilibration of the mobile-phase in the chromatank. Apply separately to the TLC plate 5 μ*l* of each of two solutions (1) and (2) of apomorphine hydrochloride and (3) of morphine in the form of circular spots about 2 to 6 mm in diameter, and 15 to 20 mm from one end of the plate and not nearer than 10 mm to the sides ; the two spots must be at least 10 mm apart. Mark the sides of the plate 15 cm from the line of application. Allow the solvent to evaporate and place in the chromatank,

ensuring that it is nearly vertical as possible and that the spots are above the level of the mobile-phase. Close the tank and allow to stand at 20° to 25°, unless the mobile-phase has ascended to the marked lines. Remove the plate and dry it in a current of cold air until all traces of solvent has disappeared and spray with a solution of sodium nitrite. Expose the plate to ammonia vapour for a few minutes and allow to stand in daylight for about 1 hour.

Observations : In the chromatogram obtained with solution (1), there is no reddish orange spot with an Rf value of 0.3 to .5 relative to the principal spot (about 2% of morphine). The test in not valid unless there is a clearly visible spot in the chromatogram obtained with solution (2).

28.4.1.1. Cognate Assays

A number of other typical examples of pharmaceutical substances containing specific organic compounds, that may be identified by adopting the similar TLC technique are stated in Table 28.3 :

Table 28.3 : Cognate Assays of Specific Organic Compounds Present in Pharmaceutical Substances

S. No.	Name of Substance	Name of Impurities	Adsorbent	Mobile Phase	Solutions	Detection	Observations
1.	Amino benzoic acid	4-Nitro-benzoic acid	Cellulose-F254 (Merck)	2-Mthyl propan-1-ol : water : 5M ammonia (700 : 30 : 15)	(1) 10% w/v of amino-benzoic acid ; (2) 0.020% w/v of 4-nitro benzoic acid ; [in EtOH (96%)]	UV-Light (254 nm)	Spot obtained with soln. (1) for impurity should not be more than the spot with soln. (2)
2.	Benztropine Mesylate	Tropine	Silica gel-G	Ethanol (96%) : Ammonia (13.5 M) (75 : 15)	(1) 4.0% w/v of sample ; (2) 0.20% w/v of atropine ; [in acetone]	(1) Sod* iodobis-muthate soln. (2) 0.4% w/v soln. of H_2SO_4	Spot obtained with soln. (1) for impurity should not be more than the spot with soln (2).
3.	Cyproheptadiene Hydrochloride	Dibenzo-cycloheptatriene	Silica gel-60 ; F 254 (Merck)	Chlorofom : Methanol (90 : 10)	(1) 0 1.% w/v of sample ; (2) 0.0020% w/v dibenzo-cycloheptatriene ; [in chloroform]	Spray with ethanolic H_2SO_4 (10%) heat at 110 °C and examine under UV-light 365 nm ;	

* **Sodium iodobismuthate solution :** Boil for a few minutes a mixture of 2.6 g of bismuth oxycarbonate, 7.0 g of sodium iodide and 25 ml of glacial acetic acid. Allow to stand for 12 hours and filter through sintered glass. To 20 mm of the filtrate add 8 ml of ethyl acetate (solution A). Immediately before use, mix 2 ml of solution A, 20 ml of glacial acetic acid and 40 ml of ethyl acetate.

Note : (*i*) For TLC, the sensitivity may be enhanced by spraying first with the above solution and then with sulphuric acid (0.2%), and

(*ii*) Solution A must be kept in a well-closed container.

28.4.2. RELATED SUBSTANCES PRESENT IN OFFICIAL DRUGS

(1) **Aminophylline : Presence of Related Substances**

Materials Required : Silica gel-G F254 ; Mobile-phase (butan-1-ol : acetone : chloroform : 13.5 ammonia : : 40 : 30 : 30 : 10) : 100 ml ; Solution-1 : dissolve 0.2 g of sample in 2 ml of DW, warm and dilute to 10 ml with methanol ; Solution (2) : dilute 1 vol. of soln. 1 to 200 vols. with methanol ;

Procedure : Apply separately to the coated plate of silica get GF254 10 µl each of solution (1) and (2). Follow the procedure as detailed in Part II section 4.1, using the above mobile phase. After removal of the plate, allow it to dry in air and examine under UV-light (254 nm).

Observations : Any secondary spot in the chromatogram obtained with solution (1) is not more intense than the spot in the chromatogram obtained with solution (2).

28.4.2.1. Cognate Assays

A good number of pharmaceutical substances do contain 'related substances' which can be identified by TLC methods as summarized in Table 28.4 below :

Table 28.4 : Cognate Assays of Related Substances Present in Pharmacopoeial Drugs :

S. No.	Name of Substance	Adsorbent	Mobile Phase	Solutions	Detection	Observations
1.	Bisacodyl	Silica gel GF254 (Merck)	Butane-2-one : Xylene (1 : 1) ; Solvent front to run only 10 cm from line of application	(1), (2), (3) and (4) contain 2.0% w/v ; 0.20% w/v ; and 0.020% w/v ; and 0.010% w/v of sample in acetone ; (5) contains 0.20% w/v Bisacodyl EPCRS in acetone ;	Dried plate examined under UV-light (254 nm)	Any secondary spot obtained with soln. (1) is not more intense than spot with soln. (3) and not more than one spot is more intense than spot with sol. (4)
2.	Chloram-phenicol	Silica gel GF254 Apply separately to TLC plate 1 µ*l* and 20 µl of sol (1) ; 1 µl of sol (2) and 200 µ*l* of sol. (3)	Butane-2-one : Xylene (1 : 1) ; Solvent front to run only 10 cm from line of application	(1), (2), (3) and (4) contain 2.0% w/v ; 0.02% w/v ; and 0.01% w/v of sample in acetone ; (5) contains 0.2% w/v Bisacodyl EPCRS in acetone ;	Dried plate examined under UV-light (254 nm)	Any secondary spot obtained with 20 µl of soln. (1) is less intense than the spot in the chromatogram obtained with sol. (3)
3.	Chloram-bucil	Silica gel GF254 Apply separately to TLC plate 5 µl of sol. (1), (2) and (3)	Chloroform : Methanol : Water (90 : 10 : 1)	(1) 1% w/v of sample in acetone ; (2) 1% w/v of chloramphenicol EPCRS in acetone ; and (3) dilute 0.5 ml of (2) to 100 ml with acetone	Under UV-light (254 nm)	Any secondary spot obtained with soln. (1) is less intense than spot with soln. (2), and not more than one such spot is more intense than spot with soln. (3)

28.4.3. FOREIGN ALKALOIDS PRESENT IN ALKALOIDAL DRUGS

Examples :

(1) **Atropine Sulphate : Foreign Alkaloids and Development Products :**

Materials Required : Silica gel G ; mobile-phase (acetone : water : 13, 5 M ammonia : : 90 : 7 : 3) : 100 ml ; solution (1, 2% w/v of sample in methanol ; solution (2) : 0.02% w/v of sample in methanol ; solution (3 : 0.01% w/v of sample in methanol ; dilute potassium iodobismuthate solution (dissolve 100 g of (+) – tartaric acid in 500 ml of water and add 50 ml of potassium iodobismuthate solution RI*) : 100 ml ;

Procedure : Apply separately to the coated TLC plate 1 µ*l* of each of three solutions (1), (2) and (3). Develop the plate in the above mobile-phase such that the solvent front is allowed to ascend only 10 cm above the line of application. After removal of the plate, dry it at 100 °C to 105 °C for 15 minutes, allow to cool and spray with dilute potassium iodobismuthate solution until spots appear.

Observations : Any secondary spot in the chromatogram obtained with solution (1) is not more intense than the spot obtained with solution (2), and not more than one such spot is more intense than the spot obtained with solution (3).

28.4.3.1. Cognate Assay

The presence of **'foreign alkaloids'** in Codeine (BP)** may be determined by more or less an identical method as already discussed in section 28.4.3 earlier.

28.4.4. FOREIGN STEROIDS PRESENT IN STEROIDAL DRUGS

Example :

(1) **Betamethasone Valerate : Related Foreign Steroids :**

Materials Required : Silica gel G ; mobile-phase : (1, 2-dichloroethane : methanol : water : : 95 : 5 : 0.2) : 100 ml ; mixture of chloroform and methanol (9 : 1) : 50 ml ; solution (1) : betamethasone valerate sample : 1. 5% w/v ; solution (2) : betamethasone valerate BPCRS/EPCRS*** : 1.5% w/v ; solution (3) : a solution containing 0.030% w/v each of betamethasone EPCRS and betamethasone 21-valerate BPCRS ; alkaline tetrazolium blue solution**** q.s. ;

Procedure : Apply separately to the coated TLC plate 1 µl of each of three solutions (1), (2) and (3) prepared in a mixture of chloroform/methanol stated above. After removal of the plate, allow it to cool dry in air until the solvents have evaporated, heat at 105 °C for 10 minutes, cool and spray with alkaline tetrazolium blue solution.

Observations : (1) The principal spot in the chromatogram obtained with soln. (1) corresponds in position, colour and intensity to that obtained with soln. (2),

(2) Any secondary spot in the chromatogram obtained with soln. (1) is not more intense than the proximate spot in the chromatogram, with soln. (3).

---

* Dissolve 100 g of (+)-tartaric acid in 400 ml of water and add 8.5 g of bismuth oxynitrate. Shake for 1 hour, add 200 ml of a 40% w/v solution of KI and shake well Allow to stand for 24 hours and filter. Store and protect from light.

** BP (1993)

*** British Pharmacopoeia Chemical Reference Substance ; European Pharmacopoeia Chemical Reference Substance ;

**** Immediately before use mix 1 volume of a 0.2% w/v soln. of tetrazolium blue in methanol with 3 volumes of 12% w/v soln. of sodium hydroxide in methanol ;

28.4.5. NINHYDRIN POSITIVE SUBSTANCES PRESENT IN OFFICIAL AMINO ACIDS

Example :

1. **Glutamic Acid**

Materials Required : Silica gel-G ; mobile-phase (glacial acetic acid : water : butan-1-ol : : 20 : 20 ; 20 ; 60) : 100 ml ; solution (1) : dissolve 0.1 g of sample in 5 ml of 2 M ammonia* ; solution (2) : dilute 1 ml of soln. (1) to 50 ml with water ; solution (3) : dilute 5 ml of solution (2) to 20 ml with water ; Solution (4) : dissolve 10 mg of glutamic acid EPCRS in sufficient water to produce 50 ml ; solution (5) dissolve 10 mg of glutamic acid EPCRS and 10 mg of aspartic acid EPCRS in sufficient water to produce 25 ml ; ninhydrin solution (0.2% w/v solution of ninhydrin in a mixture of 95 vols. of butan-1-ol and 5 vols of 2 M acetic acid**) : 50 ml ;

Procedure : Apply separately to the silica gel G coated plates 5 µl of each of sols (1), (2), (3), (4) and (5) and dry the TLC plates in a current of air for 15 minutes before commencing development. Carry out the development using the above mentioned mobile-phase as usual. After removal of the plate, allow it to dry in air, spray with ninhydrin solution and heat at 100° to 105 °C for 15 minutes.

Observations

(1) Any secondary spot in the chromatogram obtained with soln. (1) is less intense than the spot obtained with soln. (3).

(2) The test is not valid unless the chromatogram obtained with soln. (5) show two distinctly separated spots.

28.4.5.1. Congnate Assays

The assay of leucine-an amino acid official in BP (1993) may also be carried out by adopting a similar procedure using the same adsorbent and mobile-phase but different solution from (1) to (5).

THEORETICAL AND PRACTICAL EXERCISES

1. Attempt the following aspect of '*Thin-layer Chromatography*' (TLC) :

(*a*) Importance of TLC,

(*b*) Theory of TLC, and

(*c*) Versatility of TLC over paper and column chromatography.

2. Discuss comprehensively the various *experimental techniques* of TLC :

(*i*) Preparation of TLC plates, (*ii*) Choice of 'adsorbents',

(*iii*) Choice of 'solvent system in TLC', and (*iv*) Activation of 'adsorbent'.

3. How would you accomplish the following requirements in TLC ?

(*a*) Purification of silica gel-G layers, (*b*) Spolting of components (analytes),

(*c*) Development of thin layers.

Explain with appropriate examples.

4. Explain the following '*Special Techniques*' in TLC with suitable examples :

(*i*) Multi dimensional TLC, (*ii*) 2D-Chromatography,

(*iii*) Centrifugal chromatography, and (*iv*) Wedged-Tip Chromatography.

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* Dissolve 15 ml of 13.5 M ammonia (25% w/w of NH_3) to 100 ml in water.

** Dilute 11.4 ml of 17.5 M glacial acetic acid to 100 ml with water.

5. Enumerate the various '*chemical reactions*' that can be carried out on TLC plates. Support your answer with typical examples, reactions and procedure involved.

6. Elaborate these aspects of TLC explicitely :

(*a*) Combination of TLC with other techniques *e.g*., column chromatography and vapour-phase chromatography,

(*b*) Detection of separated components,

(*c*) Qualitative evaluation of the '*chromatogram*', and

(*d*) Quantitative analysis of the '*chromatogram*'.

7. What are the various aspects of TLC in pharmaceutical analysis with regard to :

(*a*) Presence of specific substances as impurities in **'drugs'.**

(*b*) Presence of related substances n **'official drugs'.**

(*c*) Presence of foreign alkaloids in **'alkaloidal drugs'.**

(*d*) Presence of steroids present in **'steroidal drugs'.**

(*e*) Ninhydrin positive substances present in **'official amino acids'.**

Give at least **ONE** typical example to support your answer.

RECOMMENDED READINGS

1. Marini-Bettolo, G.B., **Thin Layer Chromatography,** Amsterdam, Elsevier, 1964.
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3. Stahl, E. and H. Jork, **Thin Layer Chromatography : A Laboratory Handbook',** New York, Springer Verlag, 1969.
4. Touchstone, J.C. Ed., **'Quantitative Thin Layer Chromatography',** New York, Wiley 1973.
5. Stock, R. and C.B.F. Rice, **Chromatographic Methods,** 3rd ed., London, Chapman and Hall, 1974.
6. Heftmann, E., **Chromatography,** 3rd ed., New York, Van Nostrand Reinhold Co., 1975.
7. Touchstone, J.C. and M. F. Dobbins, **Practice of Thin Layer Chromatography,** New York, John Wiley 1978.
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29 GAS LIQUID CHROMATOGRAPHY (GLC)

CONTAINS :

29.1. INTRODUCTION

One of the most difficult and frustrating problems ever encountered in the domain of pharmaceutical analysis is that of the simultaneous separation, identification and above all the quantitation of more than one compound from a complex mixture in a pharmaceutical product.

A good of sophisticated chromatographic techniques of separation have been put forward since early fifties that may be categorized into the following *four* groups, namely :

(*a*) Gas-Solid adsorption Chromatography (GSC),

(*b*) Gas-Liquid partition Chromatography, (GLC),

(*c*) Liquid-Solid adsorption Chromatography (LSC), and

(*d*) Liquid-Liquid partition Chromatography (LLC).

The first two groups have been collectively termed as **'Gas Chromatography'.** Its phenomenal growth at almost logarithmic pace may be attributed to its unparalleled potential in resolving components of a complex mixture. **Gas chromatography** fundamentally is a separation technique that not only essentially provides *prima facie* indentification of a compound but also caters for quantitative estimation after due calibration.

Gas chromatography makes use, as the stationary phase, a glass or metal column filled either with a powdered adsorbent or a non-volatile liquid coated on a non-adsorbent powder. The mobile-phase consists of an inert-gas loaded with the vapourised mixture of solutes flowing through the stationary phase at a suitable temperature. In the course of the passage of the vapour of the sample through the column, separation of the components of the sample occurs in two ways, namely :

(*a*) **due to adsorption effects-***i.e.*, when the prepared column consists of particles of adsorbent only, and

(*b*) **due to partition effects-***i.e.*, when the particles of adsorbent are coated with a liquid that forms a stationary phase.

Martin and Synge in 1952, became the Nobel Laureates for their excellent, innovative research work on the development of **partition chromatography**.

It is, however, pertinent to mention here that GLC has a much greater application in the field of pharmaceutical analysis which extends over to most organic constituents that have a measurable vapour present at the temperature employed.

The principal advantages of GC are enumerated below, namely :

- It has high frequency of separation and even complex mixtures may be adequately resolved into constituents,
- It has a very high degree of sensitivity in detection of components *i.e.*, only a few mg of sample is enough for complete analysis,
- Speed of analysis is quite rapid,
- Gives reasonably good accuracy and precision,
- The technique is fairly suitable for routine analysis because its operation and related calculations do not require highly skilled personnel, and

The overall cost of equipment is comparatively low and its life is generally long.

29.2. THEORY

There are, in fact, *three* theories that have gained virtually wide recognition and acceptance in describing a gas chromatographic separation, namely :

(*a*) Plate theory,

(*b*) Rate theory, and

(*c*) Random walk and nonequilibrium theory.

These different theories will be discussed briefly in the sections that follows :

29.2.1. PLATE THEORY

Martin and Synge* first proposed the '**plate theory**' in 1941, whereby they merely compared the GC separation to fractional distillation. Thus, the 'theoretical' plate is the portion of the column wherein the solute is in complete equilibrium with the mobile and the stationary phase.

* Martin, AP, and RLM Synge, **Biochem. J., 35,** 1358, 1941.

This equilibrium is represented by the following expression :

$$K_D = \frac{\text{Conc. of solute in Stationary Phase}}{\text{Conc. of solute in Mobile Phase}}$$

where, K_D = Distribution coefficient.

Thus, the distribution of a solute after '*n*' equilibrium (plates) may be defined by the expansion of the binomial in Eq. (*a*) below :

$$(a + b)^{n-1} \qquad ...(a)$$

where, $(n - 1)$ = Number of transfers between the plates,

$a = 1/(K_D + 1)$, and

$b = K_D/(K_D + 1)$.

29.2.2. RATE THEORY

As the '*Plate Theory*' has two serious limitation, *viz*., first : it does not speak of the separating power of a definite length of column, and second : it does not suggest means of improving the performance of the column ; the '**Rate Theory**' has been introduced which endeavours to include the vital fact that-'**the mobile-phase flows continuously, besides the solute molecules are constantly being transported and partitioned in a gas chromatographic column**'. It is usually expressed by the following expression :

$$h = 2\lambda d_p + \frac{2\gamma D_G}{u} + \frac{8k' d_f^{\,2}}{\pi^2 (1+k')^2 D_L}\, u \qquad ...(b)$$

where, u = Average linear gas velocity,

λ = Measure of the packing irregularities

d_p = Particle diameter,

γ = Tortuosity factor,

D_G = Coefficient of gaseous diffusion of the solute in the carrier gas,

k' = Ratio of the amount of solute in the stationary phase to that in the gas phase,

d_f = Film thickness (usually in μm), and

D_L = Diffusion constant of solute in liquid phase.

Eq. (*b*) was first advocated by Van Deemter* in 1956, and may be rewritten as given below [Eq. (*c*)] wherein all terms except '*u*' are constant :

$$h = A + \frac{B}{u} + Cu \qquad ...(c)$$

29.2.3 RANDOM WALK AND NONEQUILIBRIUM THEORY

Giddings** in 1958, first proposed the captioned theory wherein he suggested a chromatographic separation in terms of a random walk. Based on a statistical concept the virtual spreading of a '**solute band**' may be considered by virtue of **molecular diffusion, mass transfer**, and **Eddy diffusion** (*i.e.*, *flow pattern effects*) were equated to standard deviation. In fact, this particular approach correlates the spreading of a chromatographic peak to various parameters, for instance : mass transfer, diffusion coefficient D_G, particle diameter, velocity of mobile-phase, and finally the length of column. Thus, coefficient D_G, particle diameter, velocity of mobile-phase, and finally the length of column. Thus, the plate height '*h*' employing the random walk approach may be expressed as in Eq. (*d*) in the next page :

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* Van Deemter, FJ Zuiderweg and A. Klinkengerg, **Chem., Eng., Sci., 5,** 271, 1956.

** Giddings JC, **J. Chem Ed., 35,** 588 1958.

$$h = \frac{B}{u} + Cu + \sum \frac{1}{1/A + (1/Cm)u} \quad ...(d)$$

where, C*m* = Resistance to mass transfer in gas phase should be treated independently,

A = Eddy diffusion,

B = Longitudinal molecular diffusion in both mobile and stationary phases, and

C = Kinetic or mass transfer term originating in the stationary phase.

In actual practice, there are *two* basic considerations that prevail upon in gas chromatography, namely :

(*a*) **Retention :** The phenomena affecting retention or hold up on the column, sometimes referred to as the thermodynamic effect, and

(*b*) **Column Efficiency :** The phenomena affecting column efficiency or the kinetic aspect that governs the tendency for a particular solute band to '*broaden*' as it traverses through the column.

However, the resolution or extent of separation of any two peaks from a column is solely dependent upon both *retention* and *column efficiency*.

Although separations may be caused by elution, frontal and displacement analyses, yet the elution technique is the most common. This method makes use of a stream of carrier-gas flowing through the column. Precisely, a sample is injected into the carrier-gas as a '**plug**' of vapour that is swept into the head of the packed chromatographic column. Separation of components that comprise the sample results from a difference in the multiple forces by which the column materials tend to retain each of the components.

Irrespective of the nature of the retention that is due to adsorption, solubility, chemical binding, polarity or molecular filtration, the column does retain some components longer than others. When in the gas phase the components are moved toward the column outlet, they are selectively retarded by the stationary phase. Consequently, all components pass through the column at **varying speeds** and **emerge in the inverse order of their retention by the column materials**. The aforesaid process may be outlined schematically as shown in Figure 29.1.

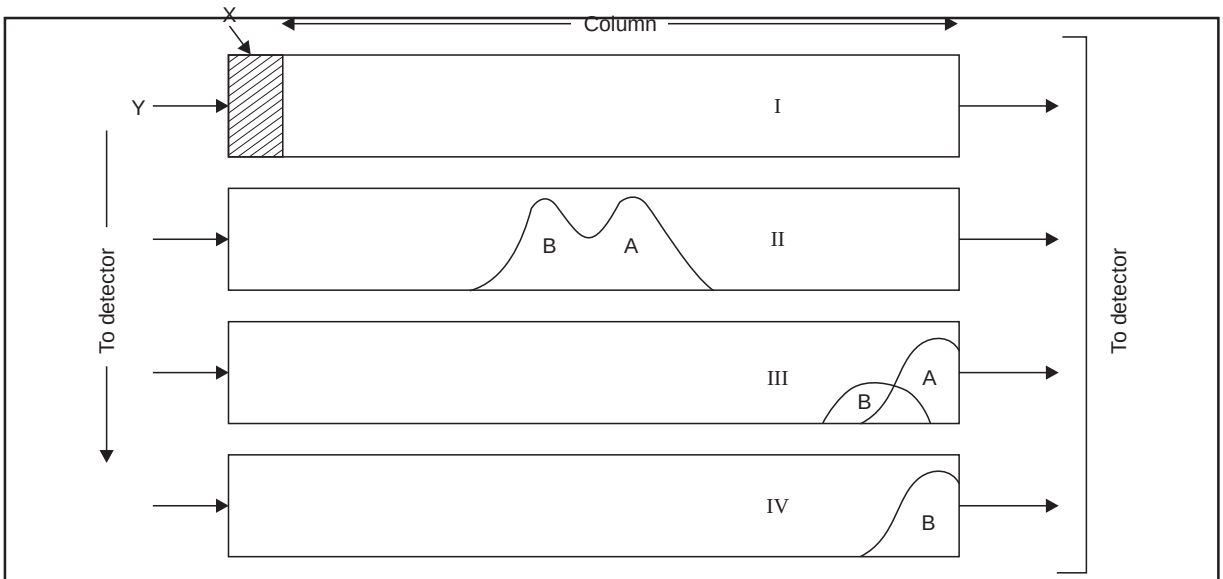

Figure 29.1 : Schematic Elution of Components A and B From A Column Vs Time
[X = 'Plug' of A and B ; Y = Carrier gas inlet]

Upon emerging from the column, the gaseous phase immediately enters a 'detector' attached to the column. Here, the individual components register a series of signals that appear as a succession of peaks above a base line on the chromatogram.

From Figure 29.1, it is evident that 'plug' of mixture A and B just enters the column 1 at time T_1, moves to the middle of the column I at time T_2, part of A has passed through the column III at time T_3 and finally A has passed completely and part of B passed through the column IV at time T_4.

29.3. INSTRUMENTATION

A gas chromatograph essentially comprises of *six* vital components, namely :

(*a*) Carrier Gas Regulator and Flow Meter,

(*b*) Sample Injection System,

(*c*) Separation Column,

(*d*) Thermal Compartment,

(*e*) Detectors,

(*f*) Recording of Signal Current, and

(*g*) Integrator.

These components shall be discussed briefly in the sections that follow : Figure 29.2, gives the schematic diagram of a standard GLC equipment showing the various parts :

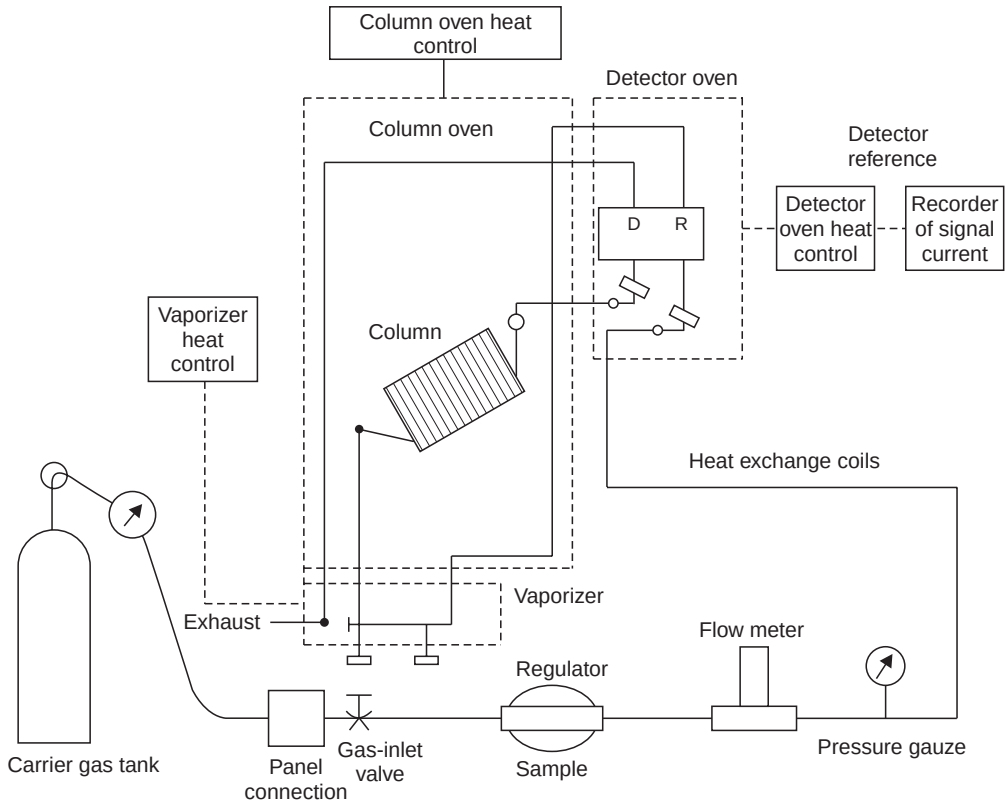

Figure 29.2 : Schematic Diagram of A Standard GLC equipment.

The sample is introduced into the vaporizer and enters the column along with the carrier gas at a constant flow through the detector oven. The reference sample also passes through the detector oven into the column which is maintained by column-oven heat control device. The detector picks up the signals of the sample as well as the reference substance one after the other which is duly amplified and the signal current recorded on a strip-chart recording device or other suitable means. After passing through the detector oven the vapours of the sample plus the carrier gas leaves the equipment through an exhaust pipe.

Note : Ultrapure N_2 for use in flame-ionization devices may be generated by the Serfass Apparatus available commercially.

29.3.1. CARRIER GAS PRESSURE REGULATOR AND FLOW METER

The various carrier gas used in GC along with their characteristic features are stated below :

H_2 : It has a distinctly better thermal conductivity and lower density. Demerits are its reactivity with unsaturated compounds and hazardous explosive nature,

He : It has an excellent thermal conductivity, low density, inertness and it permits greater flow rates. It is highly expensive,

N_2 : It offers reduced sensitivity and is inexpensive, and

Air : It is employed only when the atmospheric O_2 is beneficial to the detector separation.

Importantly, the operating efficiency of a chromatograph is directly dependent on the maintenance of a highly constant carrier gas-flow-rate. Carrier gas passes from the tank through a toggle value, a flow meter, a few feet of metal capillary restrictors, and a 0-4 m pressure gauze. The flow rate could be adjusted by means of a needle value mounted on the base of the flow meter and is controlled by the capillary restrictors. On the downstream side of the pressure regulator, a tee (T) may split the flow and direct it to the sample and the reference side of the detector.

29.3.2. SAMPLE INJECTION SYSTEM

The sample injection system is very important and critical because GC makes use of very small amounts of the samples. A good and ideal sample injection system should be the one where the sample must not—

(*i*) be decomposed at the point of injection,

(*ii*) create pressure surges, and

(*iii*) undergo fractionation, condensation or adsorption of components during the course of transfer to the column.

There are different modes of handling liquid, solid and gaseous samples in a GC which will be discussed briefly here :

(*a*) **Liquid Samples :** They are usually injected by hypodermic syringes through a self-sealing silicon-rubber septum into a preheated-metal-block flash evaporator. The sample is vapourized as a 'plug' and carried right into the column by the respective carrier gas. Sample size ranges between 1–10 μ*l*.

(*b*) **Solid Samples :** These are either dissolved in volatile liquids (solvents) or temporarily liquefied by exposure to infra-red heat.

(*c*) **Gas Samples :** They are best handled and injected by gas-light syrings or a gas-sampling valve, usually termed as a *stream-splitter*. In the simplest form this is merely a glass-system (Figure 29.2A) made up of three stop-cocks, between two of which there is a standard

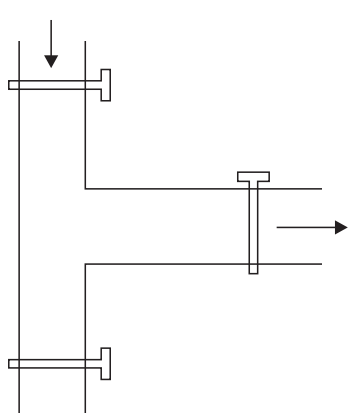

Figure 29.2A : A Stream-Splitter.

volume wherein the 'gas' is trapped. Gas from this bypass-capillary-loop is introduced right into the column by sliding or rotating a valve to connect the loop with the stream of carrier gas.

29.3.3. SEPARATION COLUMN

It is also known as the '**chromatographic column**'. In reality the heart of a GC is the column duly packed or capillary in which the separation of constituents is materialized. The packed-column is usually a tubing having an internal diameter of 4.0 mm and made up of stainless-steel, copper, cupronickel or glass either bent in U-shape or coiled. Its length varies from 120 cm to 150 M.

The general requirements of a liquid phas are :

- Differential partitioning of sample components,
- Reasonably good solvent properties for components,
- High thermal stability, and
- A lower vapour pressure at the column temperature.

Table 29.1, illustrates the characteristic features of some typical liquid-phases used in GC :

Table 29.1 : Typical Liquid Phases

S.No.	Solvent	Suitable for Solute Type	Upper Temp. Limit (°C)
1.	Paraffin Oil (Nujol)	Paraffin, Olefin, Halide,	150
2.	Silicone Oil	Paraffin, Olefin, Ester, Ether,	200
3.	Polyglycols (Carbowaxes)	Amine, Nitrile, Ether, Ketone, Ester, Alcohol, Aromatics,	100-200
4.	Apiezon L-Grease	General for polar types	300

29.3.4. THERMAL COMPARTMENT

A precise control of the column temperature is not only a must but also a requisite, whether it is intended to maintain an invariant-temperature or to provide a programmed-temperature. Importantly, the temperature of the column oven must be controlled by a system that is sensitive enough to changes of 0.01°C and that maintains an accurate control to 0.1°C. In normal practice, an air-bath chamber surrounds the column and air is circulated by a blower through the thermal compartment. However, separate temperature controls are very much desirable for the vaporizer block as well as the detector-oven.

More recently, programmes are also available that features both in linear and non-linear temperature programming as sample and reference columns. The compartment temperature can also be raised at various rates upto a maximum of 60 °C min^{-1} in the lower-temperature ranges and about 35 °C min^{-1} at higher temperatures.

29.3.5. DETECTORS

There are in all *six* different kinds of detectors used in 'Gas Chromatography', namely :

(*i*) Thermal conductivity detector (TCD),

(*ii*) Flame ionization detector (FID),

(*iii*) Electron capture detector (ECD),

(*iv*) Thermionic detector (NP–FID)

(*v*) Flame photometric detector (FPD), and

(*vi*) Photoionization detector (PID).

The *first three* detectors are invariably used in GLC and shall be discussed in details below ; whereas a passing reference shall be made with respect to the *second three* detectors.

29.3.5.1. Thermal Conductivity Detector (TCD)

The thermal conductivity detector, or **katharometer**, was the first ever detector employed for GLC; and is still being used today be virtue of its *versatility*, *stability*, *simplicity* and above all the *low-cost*.

Principle : The underlying principle of TCD is that the ability of a gas to dissipate heat, *i.e.*, its thermal conductivity, from a heated body shall change with the composition of the gas. It may be further explained by the fact that each specific carrier gas shall have a characteristic thermal conductivity that is picked up first-and-foremost by the equilibrium temperature of the detecting element to afford a baseline signal. Evidently, the thermal conductivity of the mixture of carrier gas plus sample must be altogether different from that of pure carrier gas ; and while the mixture takes its course through the detector, an obvious change in the temperature of the detecting element is duly recorded as a signal.

Figure 29.3 shows a simple diagram of a thermal conductivity detector. It essentially consists of two cells of small volumes, made within a metal block, termed as **reference cell** and **sample cell**. Each cell has a resistance wire or thermister or filament that possesses a high temperature coefficient or resistance *i.e.*, the resistance varies appreciably with slight variation in temperature. These two resistances, namely : reference cell (R) and sample cell (S) are included in two arms of a Wheatstone Bridge. Now, the carrier gas is passed into both the cells, but the column-effluents are allowed to enter only the sample cell. Thus, the temperature of the sample cell changes due to widely different thermal conductivity of the sample component than that of the carrier gas, thereby causing a change in resistance of (S) and the Wheatstone Bridge gets unbalanced. The off-balance current is transmitted to the recorder that finally draws the elution-curve for the sample(s) undergoing chromatographic separations.

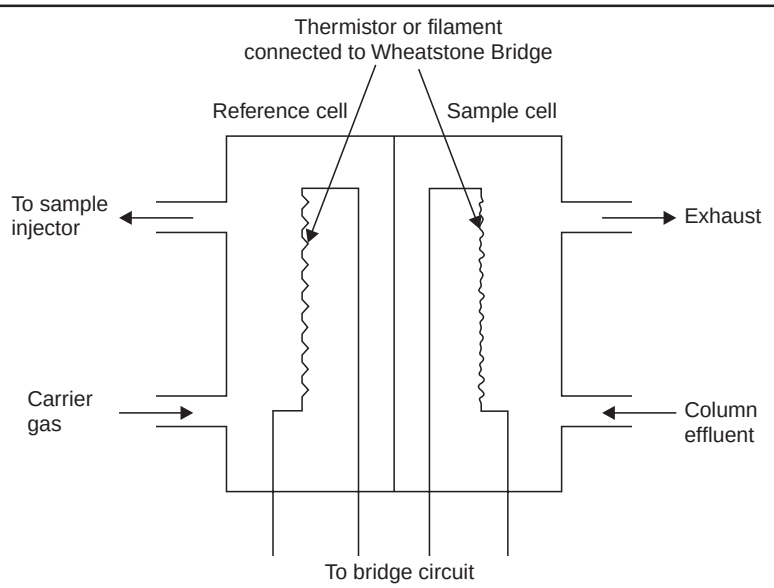

Figure 29.3 : Diagram of A Thermal Conductivity Detector [TCD].

Cautions

(*i*) First turn the carrier gas on and then switch on filament-current/detector block heater, and

(*ii*) Do not off the carrier gas before switching off the detector current or before the detector block has attained ambient temperature. This saves the filament from being damaged and enhances its life-span considerably.

29.3.5.2. Flame Ionization Detector (FID)

The general class of '**ionization detectors**' comprise of the following important detectors, namely :

- Flame ionization detector,
- Electron capture detector,

- Thermionic detector, and
- Photoionization detector.

No other detector till date has surpassed the flame ionization detector (FID) as a universal gas chromatographic detector. It hardly meets, all the characteristic features of TCD in terms of simplicity, stability, and versatility besides having *two* distinctly positive plus points :

(*i*) Its linearity over a wider concentration range, and

(*ii*) It being more sensitive with less flow and temperature dependency.

Principle : First, the principles of operation for all ionization detectors shall be discussed briefly and then the actual principles with specific details would be described under each particular detector.

Generally, the fundamental physical process underlying the operation of an ionization detector is the conduction of electricity by gases. At normal temperatures and pressures a gas essentially behaves as a perfect electrical insulator. However, if electrically charged particles (ions and electrons) are produced in a gas, it becomes a conductor. In other words, their free motion in the direction of the electrical field renders the gas conducting. Assuming a situation, when a vapour is held between two electrodes to which a voltage is applied, practically and absolutely no current shall flow at all in the electrical circuit until and unless charged particles are introduced. The quantum of electric current thus generated would become the signal of the ionization detector. On applying adequate voltage to the electrodes, all of the ions would be collected, and hence the ion-current shall be directly proportional to the number of ions between the electrodes. As the presence of only a few ions are capable of exhibiting the conductivity of the gas; therefore, ionization detectors are usually very sensitive.

Principle of FID : The underlying principle of FID is that invariably a mixture of hydrogen-oxygen or hydrogen-air flame burns with the generation of comparatively fewer ions, but when an organic compound *viz*., most pharmaceutical substances is ignited in such a flame, ion production gets enhanced dramatically. Therefore, when such a flame is held between two electrodes to which a voltage ranging between 100-300 V is applied, it would instantly give rise to an ion current on burning an organic compound in the flame.

Figure 29.4, illustrates a schematic diagram of a **flame-ionization detector**. It comprises of a positively charged ring (also referred to as cylindrical collector electrode), whereas the flame jet serves as the negative electrode. The flame jet has two inlets ; from the bottom of the column effluent is introduced and from the side H_2 to form the fuel, whereas air is let in uniformly around the base of the jet.

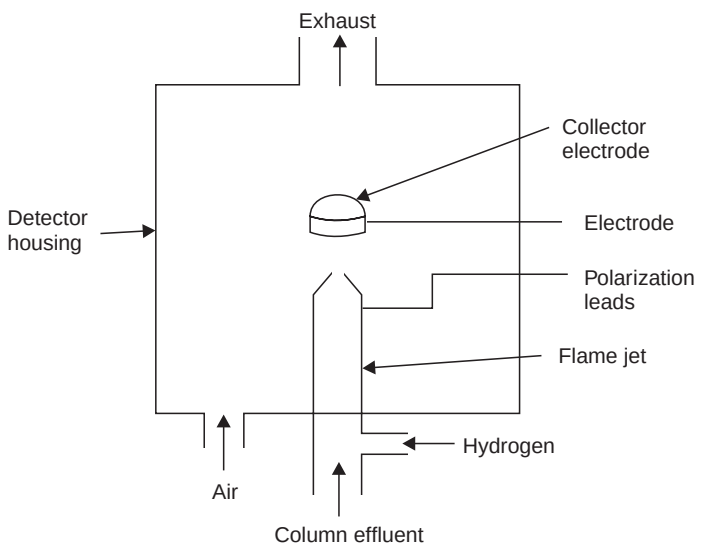

Figure 29.4 : Schematic Diagram of A Flame Ionization Detector [FID].

29.3.5.3. Electron Capture Detector (ECD)

In the domain of gas chromatography the **electron capture detector** (ECD) enjoys the reputation of being one of the *most sensitive* as well as *selective detectors*. However, this valuable detector needs to be handled with a lot of skill and expertise so as to achieve wonderful and dependable results.

Principle : ECD belongs to the general class of ionization detectors, the underlying principles of which have already been discussed. In ECD specifically a β-emitter serves as a source of radiation to generate the ions that helps in ionizing the carrier gas molecules to form positive ions and free electrons as expressed in the following Equation (*e*) :

$$C + \text{radiation} \rightarrow C^+ + e^- \qquad ...(e)$$

In a situation when the said phenomenon is conducted between a pair of charged electrodes, the mobility of the lighter negative ions *i.e.,* the electrons, would be much higher in comparison to the heavier positive ions *i.e.,* the charged carrier-gas-molecules, thereby ruling out the possibility of their 'recombination'. Thus mostly the cations and electrons will be collected, while generating a standing current that forms the baseline-signal of the ECD detector. At this stage, if an organic molecule, (*i.e.,* a pharmaceutical substance) possessing a comparatively high electron affinity is introduced, a portion of the electrons shall be captured to produce negatively charged ions. These heavy-negative-ions will have less mobility as compared to the electrons ; therefore, they will have no other coice than to unite with positive ions. Thus, the net result would be fewer ions and electrons available to migrate to the electrodes, thereby causing a marked and pronounced reduction in the standing current of the detector. Ultimately, this observed current decrease represent as the 'signal' of the electron capture detector.

Figure 29.5, depicts the diagram of an electron capture detector. The metal block of the detector housing itself serves as a cathode, whereas an electrode polarizing lead suitably positioned in the centre of the detector housing caters for a collector electrode (anode). The radioactive source from a beta-emitter is introduced from either sides of the detector housing below the electrode polarizing lead.

The column-effluent is passed into the detector from the bottom whereas its exhaust goes out from the top.

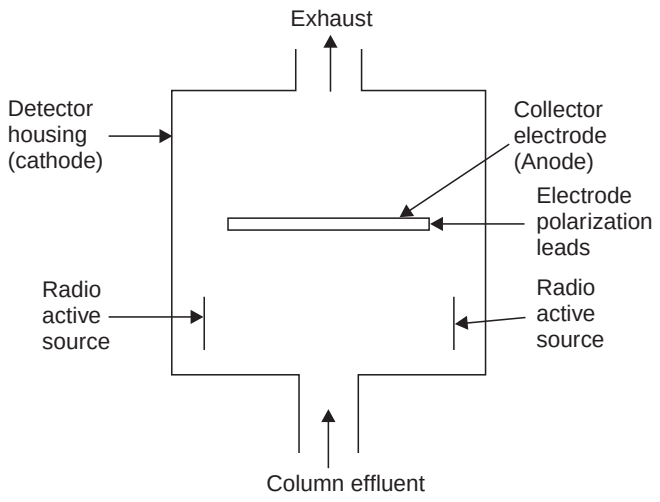

Figure 29.5 : Diagram of an Electron Capture Detector [FCD]

29.3.5.4. Thermionic Detector (NP-FID)

The very name suggests, the thermionic detector functions on the principle of ion-current generated by the thermal production of ions. It may also be invariably termed as a **nitrogen detector**, a **sulphur detector**, a **phophorus detector**, and a **halogen detector** by virtue of the fact that its specificity in detecting organic compounds essentially containing these elements. Furthermore, it is also widely known as NP-FID because it is invariably employed for carrying out the *analysis of N- or P-containing organic compounds*.

29.3.5.5. Flame Photometric Detector (FPD)

Brody and Chaney* in 1966, were the first and foremost to describe the **flame photometric detector** (FPD) which unfortunately could not get enough recognition in the field of gas chromatographic analysis due to the following reasons, namely :

(*i*) Its selectivity, and

(*ii*) Its poor commercial availability.

It solely operates on the principle of photon emission. If P- or S-containing hydrocarbons are ignited in a hydrogen-rich flame, it gives rise to *chemiluminescent species spontaneously* which may subsequently be detected by a suitably photomultiplier device. Hence, FPD is regarded as a specific detector for P- or S-containing compounds.

29.3.5.6. Photoionization Detector (PID)

Lovelock** in 1960, first introduced the photoionization detector but unfortunately its reported usages have been more or less scarce.

PID belongs to the generic class of ionization detectors whose principles have already been discussed earlier. As the very name signifies the PID induces ionization *via* photons emitted by an UV-lamp. A PID detector makes use of a photon energy of 10.2 electron volts (eV) emitted as a **Lyman alpha line***. Only such compounds having ionization potentials less than 10.2 eV shall absorb the UV-radiation and be subsequently converted to positive ions. Two-charged electrodes serve as an electric field in the detector, the cathode becoming the collector electrode for the ions. The ion-current thus generated, that will be directly proportional to the ion concentration, then becomes the signal of the detector.

29.3.6. RECORDING OF SIGNAL CURRENT

In general, the signal from a gas chromatograph is recorded continuously as a function of time by means of a potentiometric device. Most frequently, a recorder of 1-10 mV full-scale deflection ($\simeq$ 10 inches) and having a response time 1 second or less is quite adequate.

Variable chart speeds between the range of 5-50 mm. min^{-1} are most preferable in GC.

Essentially in a potentiometric recorder, the input signal is balanced continuously by a feedback signal making use of a servomechanism ; whereby a pen strategically connected to this system moves proportionally along the width of the chart paper, thus recording the signal, whereas simultaneously the chart paper keeps moving at a constant speed along its length.

The following important points should be noted before operating a recorder, namely :

(*i*) Its 'zero' must be adjusted (or synchronized) with the 'input zero' otherwise the baseline might shift with alterations in attenuation of the signal,

(*ii*) The amplifier gain must also be adjusted duly so as to avoid completely the dead-base and oscillation,

(*iii*) A recorder with inadequate shielding from AC circuits would display shifting of its zero point, and

(*iv*) A reasonably good recorder having quality performance must be employed so as to achieve correct recording of analog-signal, a topmost priority towards quantitative accuracy and precision.

29.3.7. INTEGRATOR

An '**intergrator**' may be regarded as a device that essentially facilitates simultaneous measurement of areas under the chromatographic peaks in the chromatogram either by *mechanical* or *electronic* means. It is, however, pertinent to mention here that '**manual techniques**' for determining peak areas are known, such as :

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* Brody, SS and JE Chaney., **J. Gas Chromatography, 4,** 42, 1966.

** Lovelock, JE, **Nature, 188,** 401, 1960.

*** Driscoll, JN, **Amer. Lab., 8,** 71, 1976.

'triangulation', **cutting and weighting of peaks**, planimetry, but all these methods are quite time consuming, tedious and not accurate. Hence, based on the actual need, incorporation of an appropriate integrator in a reasonably good GC-set up is an absolute necessity.

There are *two* types of integrators generally employed in GC, namely :

(*a*) **Ball and Disk Integrator :** This is nothing but a purely mechanical device and installed at one end of the very strip-chart recorder itself. It carries a pen that writes along a span of about one inch, reserved for integrator on the recorder chart paper at the end. The zero line of the integrator moves almost parallel to the base line of the chromatogram and as soon as a peak appears on the recorder, the integrator-pen starts moving from right to left the *vice-versa* within its one-inch strip. Each one-inch traverse (counted along projection parallel to signal axis) is usually assigned a value of 100 counts ; the total number of counts corresponding to a peak are directly proportional to the area of the peak.

The type of mechanical integrator* affords fairly good accuracy and precision ; and above all it is quite cheap.

(*b*) **Electronic Integrator :** An '**electronic integrator**' is definitely a much superior, accurate and dependable device wherein the GC-signal is converted to a frequency pulse that are accumulated corresponding to a peak and later on digitally printed out as a measure of the peak area. The main advantages of an electronic integrator are, namely :

(*i*) Provides a much wider linear range,

(*ii*) Changing the 'attenuation' is not required, and

(*iii*) Offers highest precision in peak-area measurement.

Of course, the electronic integrators are quite expensive

Precision of the TWO methods : The '**electronic integrator**' is almost 3 times** more accurate and precise than the '**ball and disc integrator**' :

Method	Standard Deviation (%)
Ball and Disc Integrator	1.30
Electronic Integrator	0.40

GC-Computer System : Nowadays, a large number of data-processing-computer-aided instruments for the automatic calculation of various peak parameters, for instance : relative retention, composition, peak areas etc., can be conveniently coupled with GC-systems. A commercially available*** fairly sophisticated computer system of such type are available abundantly that may be capable of undertaking load upto 100 gas-chromatographs with ample data-storage facilities. In fact, the installation such **as** '**multi GC-systems**' in the routine analysis in oil-refineries and bulk pharmaceutical industries, and chemical based industries have tremendously cut-down their operating cost of analysis to a bare minimum.

29.4. WORKING TECHNIQUES FOR QUANTITATIVE ANALYSIS

In actual practice, the following *three* working techniques are not only widely popular but also provide optimum accuracy and precision for the quantitative analysis of pharmaceutical substances, namely :

(*i*) Area Normalization,

(*ii*) Internal Standard Method, and

(*iii*) Comparison Method.

* Manufactured by : Disc Instrument Company, USA.

** Source : M/s Varian Aerograph, USA.

*** Sumadzu, Japan ; Varian Aerograph, USA ; Perkin-Elmer, USA.

There three techniques will be discussed briefly in the sections that follow :

29.4.1. AREA NORMALIZATION

Assuming that the chromatogram is as represented in Figure 29.6, the formula employed is :

$$\% \text{ X} = \frac{\text{A}x \,.\, fx}{\text{A}x \,.\, fx \;+\; \text{A}y \,.\, fy \;+\; \text{A}z \,.\, fz} \times 100$$

$$= \frac{\text{A}x \,.\, fx}{\Sigma \text{A}f} \times 100 \qquad ...(f)$$

where, A = Peak area, and

f = Response factor.

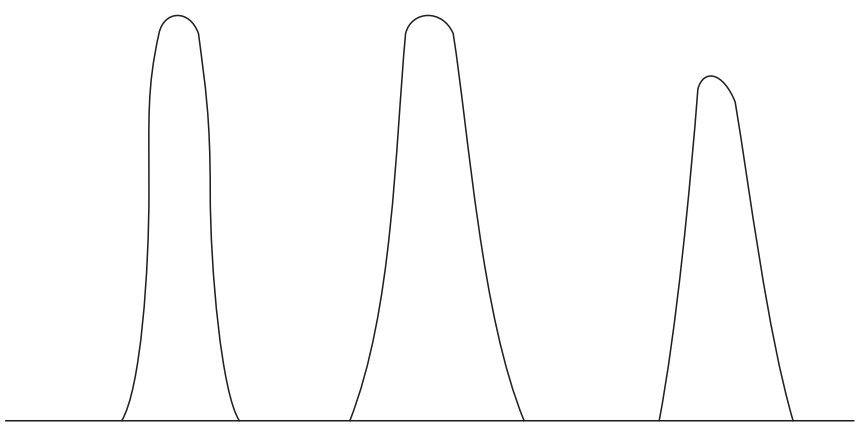

Figure 29.6 : Area Normalization Method.

Generally, different components possess different response factors, application of which not only compensates for different detector response for different components but also take into consideration the other factors inherent with the procedure. However, these factors may be calculated by preparing a synthetic mixture absolutely identical to what is expected in the sample, and subsequently carrying out the gas-chromatography of this mixture exactly under idential experimental parameters as described in the method of analysis. Thus, we have :

$$fx = \frac{\text{W}x \,/\, \text{A}x}{\text{W}r \,/\, \text{A}r} \qquad ...(g)$$

where, W = Weight or conc. of component in the mixture, and

r = A reference component present in the mixture which is assumed to have response factor of unity.

In certain instances, like petroleum fractions, where it may be possible to assume that most of the components possess almost equal response factors, the area normalization formula in Eq. (*f*) may be further simplified to :

$$\% \text{ X} = \frac{\text{A}x}{\text{A}} \times 100 \qquad ...(h)$$

Salient features of Area Normalization Method are as follows :

(*i*) Very suitable for routine type of samples where the variations in composition are only marginal *i.e.*, in such cases where the response factors need to be checked periodically only when necessary, and

(*ii*) An obligatory condition of this method being that all the components of the sample should elute and be recorded.

29.4.2. INTERNAL STANDARD METHOD

In this particular method it is necessary to select a reference compound (known as-**internal standard**) that should meet the following requirements rigidly :

(*i*) It is not a component of the sample but as far as possible, is chemically identical,

(*ii*) It is resolved from various components of the sample, and

(*iii*) It elutes near the components of interest.

The internal standard (IS) is usually added to the sample in such a concentration that matches favourably with that of components to be evaluated. Now, the respective chromatogram is obtained by the GC-method. The percentage of the sample is obtained by the following expression :

$$\%X = \frac{Ax \,.\, fx}{A_{IS} \,.\, f_{IS}} \cdot \frac{w}{W} \times 100 \qquad ...(i)$$

where, W = Weight of the sample,

w = Weight of the internal standard,

A_{IS} = Peak area of internal standard, and

f_{IS} = Response factor of the internal standard.

Graphical Approach : Many a times a '**graphical approach**' as illustrated in Figure 29.7 is also applied for the quantitative determination of components in the sample.

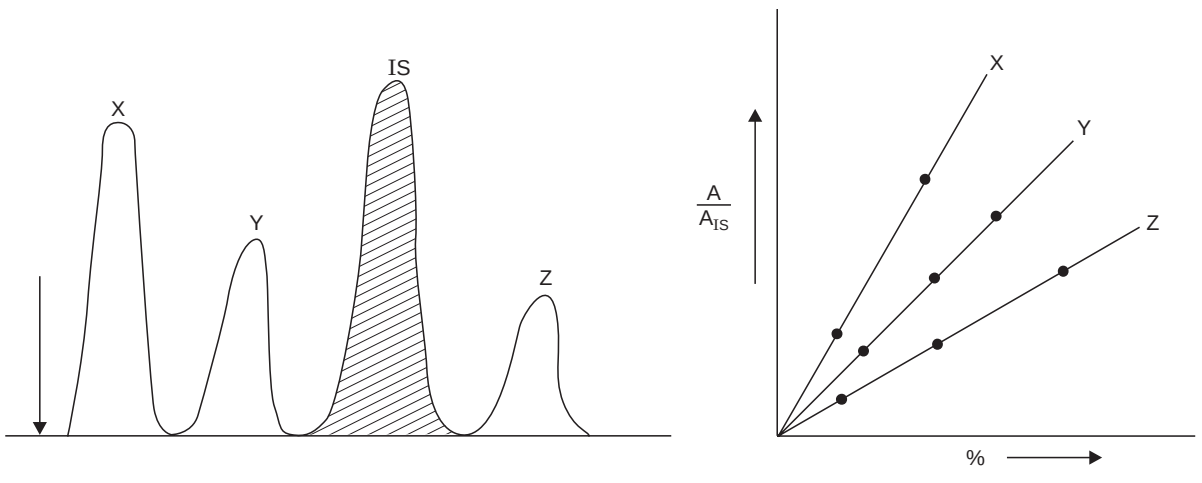

Figure 29.7 : Graphical Approach : Internal Standard Method.

First and foremost, the calibration curves are plotted for each component by GC-method using synthetic blends (containing varying concentrations of the component and fixed known concentration of IS) and also plotting A/A_{IS} Vs %-concentration. Then running separately the sample (plus IS) in a similar manner and determining A/A_{IS} value, %-concentration of the component may be observed from the calibration curve.

Salient features of **Internal Standard Method**-are as follows :

(*i*) It gives very accurate and precise results,

(*ii*) It completely eliminates possibility of error caused due to loss of some part of the sample (other than the determined components) during the initial preparation stage,

(*iii*) It eliminates error due to incomplete elution of all the sample components, and

(*iv*) It eliminates error caused due to inaccurate measurement of sample size before injection.

29.4.3. COMPARISON METHOD

The **'comparison method'** makes use of a purely synthetic blend containing the component to be determined in the same order of concentration as expected in the sample. In fact, the very purpose of this synthetic-blends is only to simulate a typical sample. Now, exactly equal (or known) amounts of both, the '**synthetic blend**' and the '**sample**'are separately injected and chromatograms obtained. Thus, by actually comparing the areas of the desired component in both the chromatograms, the 'unknown concentration' may be determined by the following expression :

$$\%X = \frac{A}{A'_X} \text{ (\% X in synthetic blend)}$$

where, A'_X = Peak area of component X in the chromatogram of 'synthetic blend'.

However, this method is less accurate in comparison to the first two methods described earlier for quantitative analysis. It should be used judiciously if only a few components present in small concentration (*i.e.*, < 5%) in the sample are required to be estimated *e.g.*, in trace-analysis.

Precautions : Following are certain precautions that must be observed in the quantitative analysis, namely :

(*i*) Detector response should always be linear in the concentration range covered in the analysis,

(*ii*) Distortion of the peak caused due to detector and recorder performance must be as negligible as possible,

(*iii*) Both sample decomposition and adsorption in any portion of the GC-assembly must be avoided, and

(*iv*) Adequate and precise sampling technique must be followed to permit injection of representative sample only. Obviously, this part of analyis is as vital and critical as the gas chromatorgraphic part of analysis.

29.5. APPLICATIONS OF GLC IN PHARMACEUTICAL ANALYSIS

Gas liquid chromatography (GLC) or **gas chromatography** (GC) finds its abundant applications in the accurate and precise analysis of plethora of official pharmaceutical substances covering a wide range as enumerated below :

(*i*) Assay of Drugs,

(*ii*) Determination of specific organic compounds as impurities in official pharmaceutical substance,

(*iii*) Determination of related substances in official drugs,

(*iv*) Determination of water in drug, and

(*v*) Determination of chloroform with head-space chromatography.

29.5.1. ASSAY OF DRUGS

Assay of Cetostearyl Alcohol

Materials Required : Solution (1) (1% w/v of cetostearyl alcohol sample in 96% ethanol) ; solution (2) (0.6% w/v of cetyl alcohol EPCRS in 96% ethanol) ; (3) (0.4% w/v of stearyl alcohol EPCRS in 96% ethanol ; solution (4) [mix 1 ml of solution (2) and 1 ml of solution (3) and add sufficient 96% ethanol to produce 10 ml] ;

Chromatographic Parameters-are as follows :

(*i*) **Column :** Made of glass or stainless steel ; size : (3 M × 4 mm) ; adsorbent : diatomaceous support (125 to 180 mesh) impregnated with 10% w/w of polydimethylsiloxane and maintained at 200 °C,

(*ii*) **Inlet-port and Detector :** are maintained at 250 °C,

(*iii*) **Flow rate of Carrier Gas** (N_2) : 30 ml minute^{-1}, and

(*iv*) **Resolution Factor :** between the two principle peaks in the chromatogram obtained with solution (1) must not be less than 1.25 (it may be achieved by adjusting the flow rate), and

(*v*) **Detector :** Flame Ionization Detector (FID).

Procedure : After having maintained the above mentioned experimental conditions for gas chromatography inject 2µ*l* of solutions (1) through (4) sequentially.

Observations : The assay is not valid unless the chromatogram obtained with solution (4) shows two principal peaks with a signal-to-noise ratio of at least 5.

Calculations : Calculate the content of cetylalcohol and of stearyl alcohol from the chromatogram thus obtained with solution (1) by normalization. Identify the peaks by visual comparison with the chromatograms obtained with solutions (2) and (3) respectively.

29.5.1.1. Cognate Assays

A few other drugs can also be assayed by the same procedure and are stated below in Table 29.2 :

Table 29.2 : Cognate Assay of Drugs by GLC-Method

S.No.	Name of Substance	Column Parameters	Solutions	Calculations
1.	Ethyloestrenol	Glass column (1.0 M × 4 mm) packed with acid-washed silanised diatomaceous support (80 to 100 mesh) coated with 3% w/w of phenyl methyl silicone fluid (50% phenyl) maintained at 200 °C. OV-17 is also suitable.	(1) 0.2 w/v of ethyloestrenol BPCRS and 0.1% w/v of arachidic alcohol (internal standard) ; (2) 0.2% w/v of sample ; (3) 0.2% of sample plus 0.1% w/v of the internal standard.	Calculate the content of $C_{20}H_{32}O$ using the declared content of $C_{20}H_{32}O$ ethyloestrenol BPCRS.
2.	Lincomycin Hydrochloride	Glass column (1.5 M × 3 mm) packed with acid-washed silanised diatomaceous support impregnated with 3% w/w of phenyl methyl silicone fluid (50% of phenyl) (OV-17 is also suitable) and maintained at 260 °C ; Inlet-port and detector maintained at 260-290 °C ; Carrier gas : Helium-with a flow rate of about 45 ml minute^{-1}.	Solutions (1), (2) and (3)* ;	Calculate the content of $C_{18}H_{34}N_2O_6S$, HCl, H_2O in lincomycin hydrochloride BPCRS

* BP (1993) Vol. I, p-384.

29.5.2. DETERMINATION OF SPECIFIC ORGANIC COMPOUNDS AS IMPURITIES IN OFFICIAL PHARMACEUTICAL SUBSTANCES

A number of organic compounds, such as : N, N-dimethylaniline-present in amoxycillin trihydrate ; cephalexin ; cloxacillin sodium ; dicloxacillin sodium ; 2-ethylhexanoic acid-in amoxycillin sodium ; 4-chlorophenol-in clofibrate ; acetone and butanol-in daunorubacin hydrochloride ; cineole : limonene ratio-in dementholised mint oil etc ;

A. Determination of N, N-dimethylaniline in Cephalexin

Materials Required : Cephalexin sample : 1.0 g ; Solution A [0.005% w/v soln. of naphthalene (internal standard) in cyclohexane] : 20 ml ; Solution B (mix 50 mg of N, N-dimethylaniline with 2 ml HCl and 20 ml of water, shake to dissolve, add enough DW to produce 50 ml ; and dilute 5 ml of the resulting solution to 250 ml with DW) : 20 ml ; solution (1) (add 5 ml of 1 M NaOH and 1 ml of solution A to 1 ml of solution B, shake vigorously for 1 minute, centrifuge and use the upper layer) ; solution (2) (dissolve 1 g of cephalexin sample in 5 ml of 1 M NaOH, add 1 ml of solution A, shake vigorously for 1 minute, centrifuge and use the upper layer) ;

Chromatographic Conditions : are as stated under :

Column* : Glass column ; size (2 M × 2 mm) ; adsorbent : packed with acid-washed, silanized diatomaceous support (80 to 100 mesh) impregnated with 3% w/w of phenyl methyl silicone fluid (50% phenyl, and maintained at 120 °C,

Detector : Flame Ionization Detector (FID),

Inlet-port and Detector : maintained at 150 °C, and

Flow Rate : 30 ml minute^{-1} for N_2 as the carrier gas.

Procedure : After having set the above experimental conditions for gas chromatography, inject 1 µ*l* of the solutions (1) and (2) sequentially into the column. Repeat the determinations so as the ensure a consistent response. Determine the peak areas**.

Calculations : From the value obtained calculate the content of N, N-dimethylaniline present in the given sample of cephalexin. However, according to BP(1993) it should not be more than 20 ppm.

29.5.2.1. Cognate Assay

A few cognate determinations are listed in Table 29.3 :

Table 29.3 : Cognate Determination of N, N, Dimethylaniline in Official Pharmaceutical Substances

S.No.	Name of Substance	Chromatographic Conditions	Sample Injection only	Solns for GLC***	Pharmacopoeal Requirement
1.	Amoxicillin Sodium	Same as for Cephalexin	1 µ*l*	Solution A ; Soln. (1) and (2) ;	NMT 20 ppm ;
2.	Cloxacillin Sodium	-do-	1 µ*l*	Solution A ; Soln. (1) and (2) ;	NMT 20 ppm ;
3.	Dicloxacillin Sodium	-do-	1 µ*l*	Solution A ; Solution B ; Solutions (1), (2) and (3) ;	NMT 20 ppm ;

* OV-17 is also suitable ;

** BP (1993) Vol I ;

*** Applications where temperature programming is required in GLC, peak-areas determinations are to be used only.

29.5.3. DETERMINATION OF RELATED SUBSTANCES IN OFFICIAL DRUGS

Related substance present in good number of official drugs may be determined by GLC method, for instance : Bromopheneramine maleate ; Bronopol ; Cepahloridine ; Chlorocresol ; Chloroform ; Chloroxylenol., Cindamycin hydrochloride ; Griseofulvin ; Isometheptene mucate ; Levomenthol ;

A. Related Substances in Bromopheneramine Maleate

Materials Required : Solution (1) dilute 1 volume of solution (3) to 200 volumes with a 0.005% w/v solution of N-phenylcarbazole (internal standard) in toluence : 10 ml ; Solution (2) : (add 5 ml of DW to 0.1 g of bromopheneramine maleate sample, make the resulting solution alkaline with 13.5 M ammonia, add 2.5 ml of toluene, shake for 5 minutes, centrifuge and use the upper layer) ; solution (3) : (prepare it exactly in the same manner as solution-'2' but using the internal standard solution in place of toluene) ; Solution (4) : (dissolve 10 mg of bromopheneramine maleate BPCRS in 5 ml of DW, make alkaline with 13.5 M ammonia, add 2.5 ml of toluene, shake for 5 minutes, centrifuge and dilute 1 volume of the upper layer to 20 volumes with toluene) ;

Chromatographic Parameters : These are as mentioned below :

Column : Glass column ; size : (1.5 m × 4 mm) ; adsorbent : packed with acid-washed, silanized diatomaceous support (80 to 100 mesh) impregnated with 3% of phenyl methyl silicone fluid (50% phenyl) and maintained at 220 °C.

Flow rate of Carrier Gas : 30 l minute^{-1} ;

Carrier Gas : Nitrogen ;

Detector : Flame Ionization Detector (FID) ;

Procedure : After having maintained the aforesaid experimental parameter for gas chromatography, inject 1 μ*l* each of solutions (1), (2), (3) and (4) in a sequential manner.

Calculations

(*i*) Calculate the ratio (γ) of the area of the peak due to bromopheneramine to that of the peak due to the internal standard in the chromatogram obtained with solution (1) ; and

(*ii*) In the chromatogram obtained with solution (3) the ratio of the sum of the areas of any secondary peaks to the area of the peak due to the internal standard is not greater than γ and the ratio of the area of any secondary peak to the area of the peak due to the internal standard is not greater than 0.4γ.

29.5.3.1. Cognate Determinations

A few cognate assays are summarized in Table 29.4 ; below :

Table 29.4 : Cognate Determinations of Related Substances in Official Drugs*

S.No.	Name Substance	Chromatographic Conditions	Sample Injection Qty.	Solutions for GLC*	Calculations/ Observations
1.	Bronopol	Same as under Bromopheneramine Maleate	1 μ*l*	Solution A ; Solution B ; Solutions (1), (2) and (3)	Calculate ratio for sample and internal stand. from soln. (1) ; for soln. (3) ratio of area of any secondary peak and is not > γ.

* BP (1993) Vol I & II.

2.	Clindamycin Hydrochloride	Same as above except adsorbent (60 to 80 mesh) ; Column Temp. 170 °C. Inlet-port and Detector Temp. 200° to 210 °C. Record the chromatogram for soln. (1) twice the retention time of cindamycin.	1 µ*l*	Solution (1) ; Solution (2) ;	(1) Test is not valid unless the height of the principal peak obted. with soln. (1) measured from base-line is at least 70% of full-scale deflection. (2) For soln. (1) the sum of the areas of any second ary peak is not > 3% and the area of any one such peak is not 2% by normalization.

29.5.4. Determination of Water in a Drug

Assay of Water Present in Mentrophin

Materials Required : Solution (1) [dilute 15 µ*l* of anhydrous methanol (internal standard) with sufficient anhydrous propan-2-ol to produce 100 ml] ; Solution (2) (dissolve 4 mg of mentrophin sample in 0.5 ml of anhydrous propan-2-ol) ; Solution (3) [dissolve 4 mg of mentrophin sample in 0.5 ml of solution (1)] ; Solution (4) and 10 µ*l* of water to 50 ml of solution (1) ;

Chromatographic Conditions

Column : Stainless steel ; size : (1 M × 2 mm) ; adsorbent : packed with porous polymer beads (60 to 80 mesh) and maintained at 120 °C.

[**Note :** Chromosorb 102 is also suitable.]

Carrier Gas : Helium ;

Detector : Thermal Conductivity Detector (TCD)-maintained at 150 °C.

Procedure : After having maintained the various experimental parameters stated above for gas chromatography and using throughout absolutely dry glassware which may be siliconized, inject 1 µ*l* of solution (1) through solution 4 sequentially and obtain the chromatogram.

Calculations : From the chromatograms obtained and taking into account any water detectable in solution (1), calculate the percentage of water taking 0.9972 g as its weight per ml at 20 °C.

29.5.5. DETERMINATION OF CHLOROFORM IN COLCHICINE BY HEAD-SPACE GAS CHROMATOGRAPHY

Head-space gas chromatography is an analytical device specifically suitable for the separation and simultaneous determination of volatile constituents present in solid or in liquid samples.

Principle : The underlying principle of **head space gas chromatography** is the analysis of the vapour phase in equilibrium with the solid or liquid phase.

Apparatus : The introduction of sample(s) may be accomplished by using airtight syringes and a simple conventional gas chromatograph. Nevertheless, the equilibrium has got to be carried out in a separate chamber and the vapour phase is subsequently conveyed to the column taking necessary and every possible precautions so as to avoid any minute changes in the equilibrium.

Materials Required : Solution (1) dissolve 0.4 g of colchicine sample in sufficient water to produce 10 ml and place 1 ml of the solution in each of three identical stoppered vials ; solution (2) dilute 5 µ*l* of chloroform to 10 ml with water and place 10 µ*l* of the resulting solution and 1 ml of solution (1) in each of three identical stoppered vials ;

Chromatographic Conditions

Column : Fused-silica capillary column ; size : (50 M × 0.32 mm) coated with a 5-μm film of chemically-bonded polymethyl siloxane ;

Detector : Flame Ionization Detector (FID) maintained at 250 °C.

Carrier Gas : Nitrogen

Flow Rate : 4 ml minute^{-1} for the carrier gas ;

Procedure : First of all maintain the above experimental parameters of the gas chromatograph and then maintain the six solutions at 90 °C for 20 minutes, pressurise for a duration of 30 seconds only and transfer subsequently to the column at a temperature of 120 °C. Repeat the operation using a vial containing 1 ml of water. Perform each measurement at least three times.

Calculations : Calculate the percentage w/w of chloroform, taking into consideration 1.48 as the weight per ml at 20 °C.

THEORETICAL AND PRACTICAL EXERCISES

1. 'Nobel laureates Martin and Synge's innovative work on the development of '*partition chromatography*' introduced Gas Liquid Chromatography (GLC) as a versatile analytical tool'. Justify the above statement with plausible explanation.

2. Enumerate the following aspects of GLC :

(*i*) separation occurs due to adsorption effects,

(*ii*) separation occurs due to partition effects, and

(*iii*) advantages of GLC over TLC, column chromatography.

3. Give a comprehensive account on the theoretical aspect of GLC with regard to :

(*a*) Plate theory, (*b*) Rate theory, and

(*c*) Random walk and nonequilibrium theory.

4. Discuss the working of various components required for an efficient GLC equipment with the help of a neat diagramatic sketch.

5. What are the various **'detectors'** used in GLC equipment ? Describe the following two commonly used detectors in an elaborated manner :

(*a*) Thermal Conductivity Detector (TCD) (*b*) Flame Ionization Detector (FID).

6. **'Chromatographic Peaks'** in GLC may be measured accurately either by

(*a*) Ball and Disc Integrator ; (*b*) Electronic Integrator.

Discuss the two aforesaid integrators and also affirm which one gives better results.

7. What are the *three* widely popular working techniques for **'quantitative analysis'** by GLC ? Expatiate each method along with graphic presentation wherever necessary.

8. How would you carry out the assay of the following drugs :

(*i*) Cetostearyl alcohol, (*ii*) Ethyloestrenol, and

(*iii*) Lincomycin hydrochloride.

9. How GLC helps in the determination of specific organic compounds in **'official drug substances' :**

(*i*) N, N-Dimethylaniline in CEPHALEXINE, and

(*ii*) N, N-Dimenthylaniline in CLOXACILLIN SODIUM.

10. How would assay the *related substances* in **'Official Drugs'** ?

(*i*) Bromopheneramine moleate, (*ii*) Bronopol, and

(*iii*) Clindamycin hydrochloride.

11. Give details for the assay of :

(*a*) Water present in mentrophin, and (*b*) Chloroform present in colchicine.

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30 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

CONTAINS :

30.1. INTRODUCTION

The excellent and the most wonderful technique of **'high performance liquid chromatography'** (HPLC) is nothing but an outcome of the various theories and instrumentation that were originally advocated for liquid chromatography (LC) and gas chromatography (GC). By the late 1960's, analysts invariably used to have the bliss of excellent experience of achieving the goal of superb separations of complex mixtures in seconds rather than in minutes, with the aid of electronic integrators to get an exact access of areas under elution bands, and above all the **'computer-printouts'** of the complete analysis with the flick of a finger. In many favourable instances the smallest possible quantities ranging from nanogram to picogram* could be detected with utmost ease and convenience.

* nanogram = 10^{-9} g ; 1 picogram = 100^{-12} g ;

In reality, some of the serious limitations too often encountered in GC ultimately brought about the development of HPLC, for instance :

(*i*) In GC the mixture of components are usually screened in the vapour phase. Hence, either a stable vapour from the mixture is obtained directly or indirectly converting the substance in it to such derivatives that are thermally stable. One 20% of chemical compounds usually come across in analysis are suitable for GC directly *i.e.*, without making their corresponding appropriate derivatives,

(*ii*) The remainder 80% of the chemical compounds are either thermally unstable or involatile in nature, and

(*iii*) Compounds essentially having highly polar or ionizable function groups are very prone to **'tailing'** by GC-analysis.

Therefore, HPLC has been evolved as a dire confluence of need, technological supremacy, the emergence of newer theoretical concepts and ideas towards development along rational lines, and above all-**'the human desire to minimise work'. HPLC offers numerous advantages as stated below :**

- Capable of handling **'macromolecules'**,
- Suitable for **pharmaceutical compounds,**
- Efficient analysis of **'labile natural products',**
- Reliable handling of **inorganic or other ionic species,** and
- Dependable analysis of **biochemicals**.

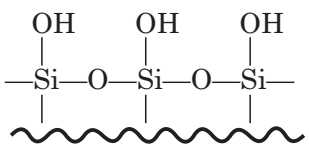
Interestingly, in HPLC the stationary phase and the mobile-phase is able to interact with the sample selectively. Besides, such interactions as hydrogen bonding or complexation which are absolutely not possible in the GC-mobile phase may be accomplished with much ease in the HPLC-mobile phase. Furthermore, the spectrum of these selective interactions may also be enhanced by an appropriate chemical modification of the silica surface *i.e.*, the stationary phase. Therefore, HPLC is regarded as a more versatile technique than GC and capable of achieving more difficult separations.

30.2. THEORY

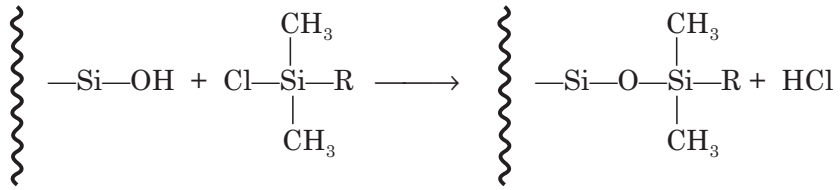
The particle size of the stationary phase material plays a very vital and crucial role in HPLC. In fact, high-efficiency-stationary-phase materials have been researched and developed exclusively for HPLC having progressively smaller particle size termed as microparticulate column packings. These silica particles are mostly uniform, porous, with spherical or irregular shape, and having diameter ranging from 3.5 to 10 μm.

Bonded-Phase Supports : The bonded-phase supports usually overcome plethora of the nagging problems which is mostly encountered with adsorbed-liquid phases. Here the molecules, comprising the stationary phase, *i.e.*, the surfaces of the silica particles, are covalently bonded to a silica-based support particle.

However, the most popular bonded-phase, **siloxanes**, are formed by heating the silica particles-in dilute acid for a day or two so as to generate the reactive silonal group :



which is subsequently treated with an organochlorosilane :



These bonded phases are found to be fairly stable between the pH range 2 to 9 and upto temperatures of about 80 °C. The nature of the R group of the silane solely determines the surface polarity of the bonded phase. A fairly common bonded phase is made with a linear C_{18} hydrocarbon, also known as ODS (octadecyl silane) bonded phases, wherein the groups appear to be protruding out from the silica particle surface just as the bristles on a toothbrush. It takes care of almost 75% of the samples in HPLC.

Note : The exact mechanism by which the respective bonded phases actually alter the nature of the sorption mechanism is still not yet clear.

When such microparticulate-bonded-phases are packed compactly into a column by means of a suitable device, the small size of these particles offers a significant resistance to solvent flow ; therefore, the mobile phase has to be pumped through the column under a high positive pressure. For an analytical HPLC, the mobile-phase is pumped through the column at a flow rate of 1-5 cm^3. min^{-1}.

At this juncture usually two varying situations arise. These are, *firstly*, isocratic elution - *i.e.*, when the composition of the mobile-phase is constant, and

Secondly, gradient elution-*i.e.*, when the composition of the mobile phase can be made to change in a predetermined fashion during the course of separation.

Note : Here, the gradient elution may be simply compared to the temperature programming in GC.

In-line Detector : It broadly helps to sense the separated solutes, after they exit through the column. Invariably the detector is an electrical signal whose variation is displayed on a potentiometer recorder or a computing integrator or a video-screen. Modern HPLC units are provided with detectors having selective-devices thereby categorically restricting the response to all the solutes present in a mixture.

Note : However, no universal detector has so far been discovered for HPLC to cater for a wide-spectrum of components ; as the Flame-Ionization-Detector (FID) used for GC in Chapter 29.

Post-Column Derivatisation : There are certain stubborn and fairly difficult components that are not easily detectable in HPLC. Therefore, such component(s) have to be appropriately converted into their corresponding detectable form once they emerge from the column.

Table 30.1 records the comparison of HPLC and GLC specifically with respect to their advantages.

Table 30.1 : Comparison of HPLC and GLC

S. No.	HPLC	GLC
1.	Can accommodate non-volatile and thermally unstable samples	Not applicable.
2.	Applicable to inorganic ions	Not applicable.
3.	Complicated and expensive equipment	Simple and inexpensive equipment.
4.	Overall analysis is not that fast	Quite rapid
5.	No universal detector available	FID-as Universal Detector

Both HPLC and GLC are :

- Efficient, highly selective and widely applicable
- Only small quantum of sample required

- Ordinarily non-destructive of sample
- Readily adaptable to *'Quantitative Analyses'*
- Provide dependable, accurate and precise and reproducible results.

30.3. INSTRUMENTATION

Modern HPLC essentially comprises of the following main components namely :

(*i*) Solvent reservoir and degassing system,

(*ii*) Pressure, flow and temperature,

(*iii*) Pumps and sample injection system,

(*iv*) Columns,

(*v*) Detectors,

(*vi*) Strip-chart recorder, and

(*vii*) Data handling device and microprocessor control.

All these vital components will be discussed with adequate details, wherever necessary, in the various sections that follow :

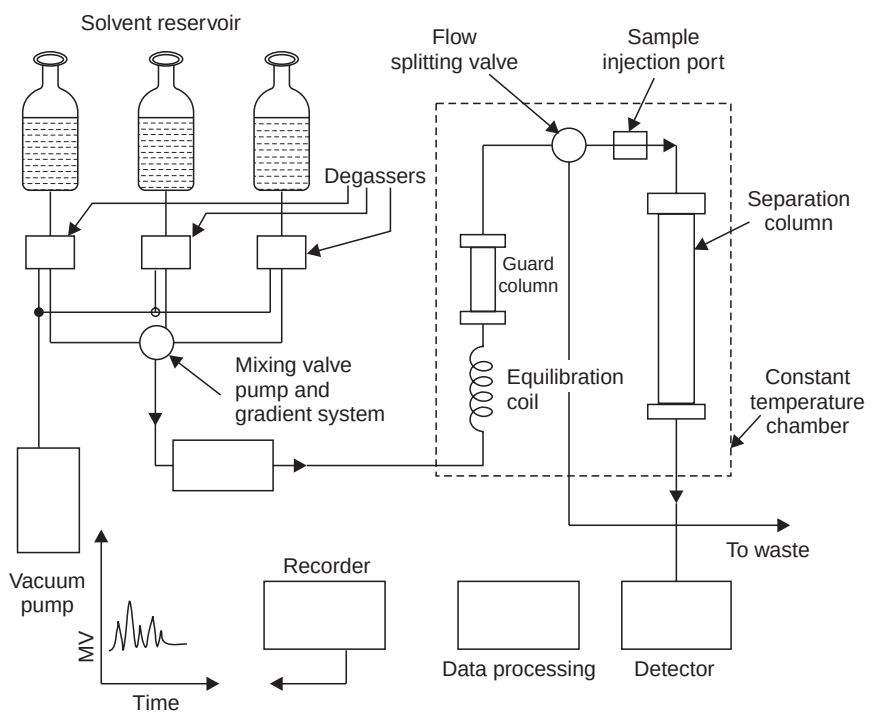


Figure 30.1 : Flow Diagram of a High-Performance Liquid Chromatograph (HPLC).

The Figure 30.1, illustrates the flow diagram of a high performance liquid chromatograph, wherein all the vital components have been duly represented.

The mobile phase, that may be either a single liquid or a mixture of two or more liquids, is pumped at high pressure into a temperature controlled oven, where it first, gains its entry into an equilibration coil to bring it to the operating temperature, and secondly, through a 'guarded column' specially designed and strategically positioned to protect the analytical column from impurities and ultimately extend its lifetime. In case a differential

type of detector is employed, the flow may be split at this juncture, with a portion going directly to the reference side of the detector and a portion to the analytical column housed in a constant temperature chamber. Just like GC, the sample is introduced to the mobile phase prior to the column. Finally, the column-effluent is made to pass through the sample side of all the detector, followed by a data-processing unit and then to the recorder.

30.3.1. SOLVENT RESERVOIR AND DEGASSING SYSTEM

Mobile-phase consisting of a mixture of organic solvents or an aqueous-organic mixture or a buffer solution may be employed depending upon the chromatographic method *vis-a-vis* the detector to be used. Special grades of solvents are commercially available for HPLC that have been adequately refined to eliminate completely the UV-absorbing impurities and any particulate matter., In case, other grades of solvents are employed, purification may have to be done at all cost because impurities present would, if strongly UV-absorbing, affect the detector or, if of higher polarity (*e.g.*, traces of H_2O or EtOH, commonly included as a stabilizer, in $CHCl_3$), influence the separation.

Solvent-reservoir comprises of a 1 dm^3 glass bottle having a lid and a 1/8 inch diameter ptfe-tube to convey the mobile phase from the reservoir to the degassers and then to the pump. As described above, any liquid entering the pump should be free from dust and particulate matter, otherwise these foreign substances may invariably give rise to irregular pumping action, damage seals and valves, irregular behaviour of column owing to its contamination, and ultimate blockade of column. Sometimes a stainless steel filter element (of filter size 2 μm) that could be conveniently positioned either in the ptfe-tube in the reservoir or an in-line-filter may be employed.

Degassing : Many liquids dissolve appreciable amounts of atmospheric gases *e.g.*, air or suspended air-bubbles that may be a major cause of practical problems in HPLC, specifically affecting the operation of the pump and the detector. However, all such problems may be avoided by degassing the mobile-phase by subjecting the mobile-phase under vacuum, distillation, spurging with a fine spray of an inert gas of low solubility such as Argon or Helium or by heating and ultrasonic stirring*.

30.3.2. PRESSURE, FLOW AND TEMPERATURE

Pressure : HPLC columns are packed usually upto 700 times atmospheric pressure and, therefore, the operating inlet-column-pressure in HPLC may be to a maximum of 200 times atmospheric pressure.

Hence, 1 N atmospheric pressure = 10^5 Pa (Pascal)**

or $1 \text{ Pa} = 1 \text{ Nm}^{-2}$

or $1 \text{ Bar} = 10^5 \text{ Pa}$

Pressures may also be expressed as psi (*i.e.*, pounds per square inch) or in kg cm^{-2}.

Conversion between bar and psi : It is known that—

1 pound = 0.4536 kg ; 1 inch = 2.54 cm ; and $g = 9.81 \text{ ms}^{-2}$; Therefore, 1 psi = a force of 0.4536×9.81 N acting over an area of 0.0254^2 m^2

or $1 \text{ psi} = .4536 \times 9.81/(.0254)^2 = 6897 \text{ Pa}$

or $1 \text{ bar} = 10^5/6897 = 14.5 \text{ psi}$

the conversion for kg cm^{-2} are :

$$1 \text{ kg cm}^{-2} = 0.981 \text{ bar} = 14.2 \text{ psi}$$

However, it is pertinent to mention here that most of the analytical HPLC is performed using pressures between 25 to 100 bar only.

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* Scott, RPW, **Contemporary Liquid Chromatography,** New York, Wiley, 1976.

** Standard International Unit of Pressure.

There are, in fact, several factors that are solely responsible for the '*pressure*' developed in a column, namely :

(*a*) the length of the column,

(*b*) particle size of the stationary phase,

(*c*) viscosity of the mobile-phase, and

(*d*) flow-rate of the mobile-phase.

The pressures mentioned above correspond to mobile-phase flow rates of approximately 1-5 $cm^3\ min^{-1}$ through the column.

Flow : The flow can be measured periodically at the column outlet by collecting the liquid for a known period, and thereafter, either measuring the volume or weighing it physically.

Temperature : In reality, the maintenance of strict 'temperature control' plays a vital role in measuring the retention-data correctly and precisely. It makes use of the refractometer detectors specifically. In HPLC, difficult separations may be achieved by increasing the temperature carefully, but this must be done initially on a hit and trial basis.

30.3.3 PUMPS AND SAMPLE INJECTION SYSTEM

Pumps : The *two* major functions of the pump in a modern HPLC are, namely :

(*i*) To pass the mobile-phase through the column at a high pressure, and

(*ii*) At a constant a controlled flow rate.

HPLC makes use of two types of pumps. They are :

(*a*) **Constant Pressure Pump :** A constant-pressure pump acts by applying a constant pressure to the mobile-phase. The flow rate through the column is determined by the flow resistance of the column.

(*b*) **Constant Flow Pump :** A constant-flow pump affords and maintains a given flow of liquid. The pressure developed entirely depends upon the flow resistance.

Importantly, in a constant-pressure pump the flow rate will change if the flow resistance changes. Whereas in the constant flow pumps the changes in flow resistance are compensated duly by a change of pressure. Therefore, it is always advisable to use constant flow pump in HPLC determinations.

Salient features of HPLC pump are as follows :

(*i*) Interior of the pump must not be corroded by any solvent to be used in the system,

(*ii*) Variable-flow-rate device must be available to monitor flow rate,

(*iii*) Solvent flow must be non-pulsing,

(*iv*) Changing from one mobile-phase to another must be convenient, and

(*v*) It should be easy to dismantle and repair.

The pump is a very delicate and sensitive part of HPLC unit ; therefore, all buffer solutions should be removed carefully after use either by pumping water (HPLC-grade) or an appropriate solvent (HPLC-grade) for several minutes.

Reciprocating Pump : Figure 30.2 represents the schematic diagram of a typical reciprocating pump along with its various essential components. The piston is moved in and out of a solvent chamber by an eccentric cam or gear. The forward-stroke closes the inlet-check value while the outlet valve opens and the respective mobile phase is duly pumped into the column. Consequently, the return-stroke-closes the outlet valve and it refills the chamber.

Advantages : It has the following advantages, namely :

(*i*) It has an unlimited capacity,

(*ii*) The internal-volume can be made very small from 10-100 μ*l*,

(*iii*) The flow-rate can be monitored either by changing the length of the piston or by varying the speed of the motor, and

(*iv*) It has an easy access to the valves and seals.

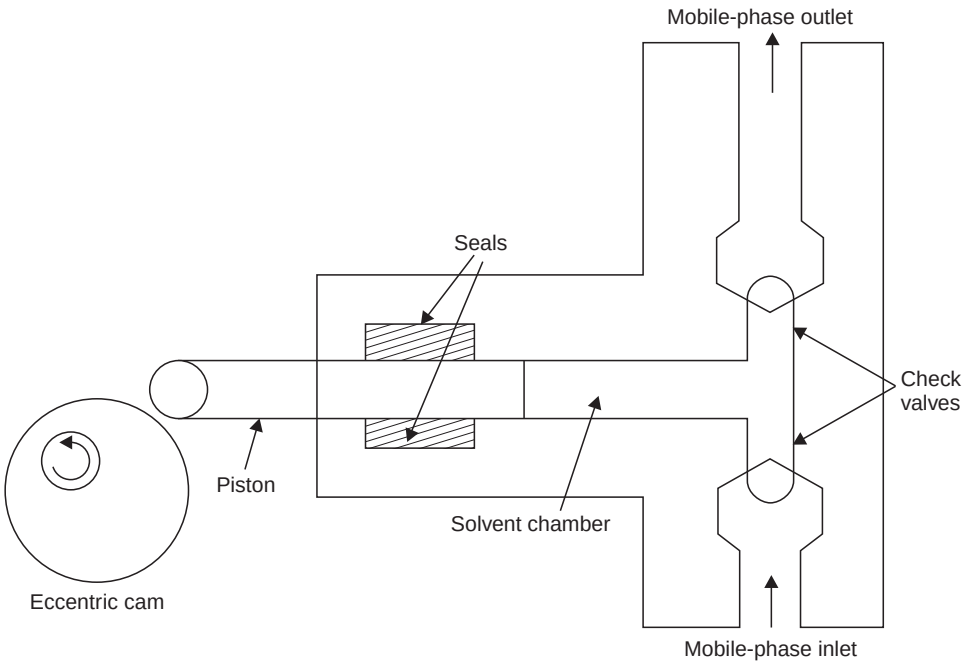

Figure 30.2 : Schematic Diagram of a Reciprocating Pump.

The use of twin-head reciprocating pump (*i.e.*, having the two heads operated 180° out of phase) functions in such a manner that while one head is pumping, the other is refilling as could be seen in Figure 30.3.

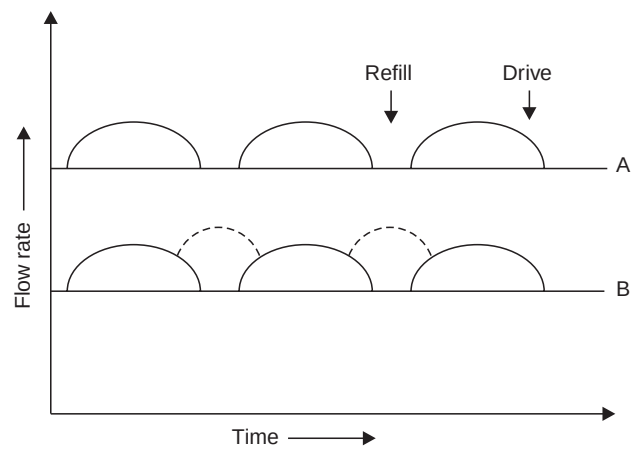

Figure 30.3 : Working of A Twin-Head Reciprocating Pump.

In Figure 30.3, the flow rate of a twin-head reciprocal pump has been plotted against time. The stage-A depicts the drive while the refill zone is vacant ; while the stage-B evidently shows the two-heads functioning simultaneously thereby the drive and the refill both zones could be visualized.

Sample Injection System : There are in all *three* different modes of sample injection system that are used in HPLC, namely :

(*a*) **Septum Injectors :** They usually permit the introduction of the sample by a high pressure syringe through a self-sealing elastometer septum. The major drawback associated with this type of injectors is **'leaching effect'** of the mobile-phase just in contact with the septum, thereby resulting in the formation of **'ghost peaks'** or **'pseudo peaks'.** In short, in HPLC the mode of syringe injection brings about more problems than in GC.

(*b*) **Stop-flow Septumless Injection :** Here, most of the problems associated with septum-injectors have been duly eliminated. The flow of the mobile-phase through the column is stopped for a while, and when the column reaches an ambient pressure the top of the column is opened and the sample introduced at the top of the packing.

The first two methods are relatively very cheap.

(*c*) **Microvolume Sampling Valves :** Highly sophisticated modern HPLC frequently make use of microvolume sampling valves for injection which not only give fairly good precision, but also are adaptable for automatic injection. These valves enable samples to be introduced reproducibly into pressurized columns without causing the least interruption of the mobile-phase flow.

Figure 30.4, displays the operation of a sample loop in two different modes *i.e.*, (*a*) sampling mode and (*b*) injection mode. Here, the sample is loaded at atmospheric pressure into an external loop in the microvolume-sampling valve, and subsequently injected into the mobile-phase by a suitable rotation to the valve. However, the volume of sample introduced usually ranges between 2 μl to over 100 μl ; but can be varied either by altering the volume of the sample loop or by employing specific variable-volume sample valves.

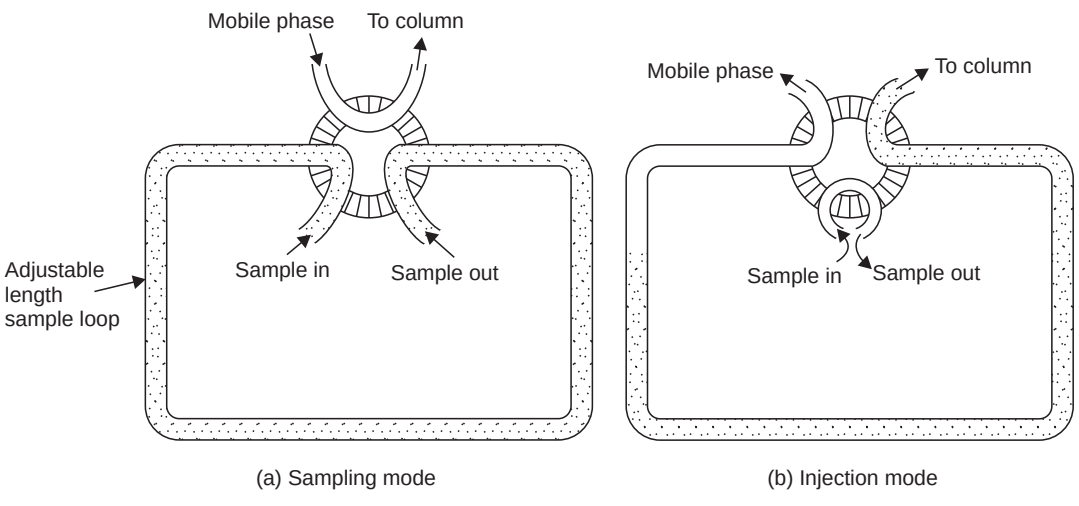

Figure 30.4 : Microvolume Sampling Valve : Operation of a Sample Loop.

Therefore, it is always preferred for most quantitative work by virtue of its very high degree of precision and accuracy.

30.3.4. COLUMNS

(*a*) **Dimensions and Fillings :** Following are the various dimensions of HPLC columns :

Material	: Stainless-steel (highly polished surface)
External Diameter	: 6.35 mm (or ≡ 0.25 inch),
Internal Diameter	: 4-5 mm (usual : 4.6 mm), and
Length	: 10-3 cm (usual : 25 cm).

(*b*) **Fittings :** Each end of the column is adequately fitted with a stainless-steel gauze or frit with a mesh of 2 μm or less so as to retain the packing material (usually having a particle diameter 10, 5 and 3 μm).

A stainless-steel-reducing union for a column of ID 4.6 mm type makes use of a 1/4-1/6 inch union with a short length of 0.25 mm (or 0.01 inch) ID ptfe tube so as to connect the column to the detector.

In actual practice, three conventional reducing unions available are employed, namely :

(*i*) Large Dead Volume (LDV)) Union : Loss of efficiency,

(*ii*) Zero Dead Volume (ZDV) Union : Loss of efficiency, and

(*iii*) Low Dead Volume (LDV) Union : Most efficient, most expensive, and dead-volume 0.1 μ*l*.

Figure 3.5 depicts the diagram of a typical LDV-Union having a SS-frit of 2 μm and a ptfe tubing of 1/6 inch.

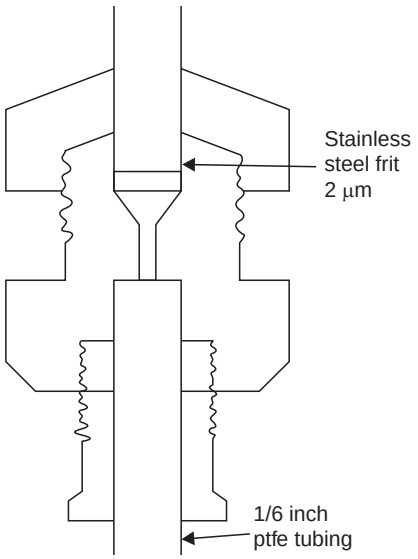

Figure 30.5 : LDV-Union.

(*ii*) **Performance :** Inside a column the concentration of a band of solute decreases as it moves through the system. The column performance or the efficiency of a column entirely depends on the amount of spreading that takes place. The measurement is represented in Figure 30.6, below :

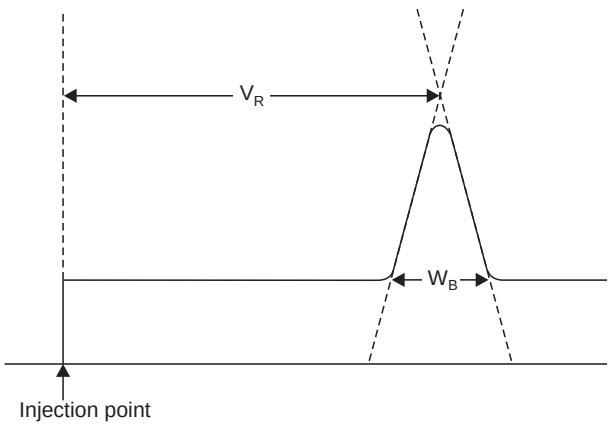

Figure 30.6 : Measurement of Column Performance.

The efficiency or performance of a column may be measured by the following expression :

$$N = 16(V_R/W_B)^2 \quad ...(a)$$

or $$H = L/N \quad ...(b)$$

V_R = Retention volume of a solute,

W_R = Volume occupied by a solute (or **'Peak-Width'**). Evidently, for a more efficient column, W_B will be smaller at a given value of V_R,

N = Plate number of the column (dimensionless),

H = Plate height of the column (mm × μm), and

L = Length of the column (cm).

Based on Eq. (*b*) one may clearly observe that for a more efficient column 'N' gets larger and correspondingly 'H' gets smaller.

(*iii*) **Types of Packing :** Modern HPLC makes use of packing which essentially consist of small and rigid particles with a very narrow particle size distribution. Broadly speaking three types of packing are invariably used in HPLC column, namely :

(*a*) **Styrene-divinylbenzene copolymers** based porous polymeric beads have been employed exclusively for size-exclusion and ion-exchange chromatography, but now mostly been replaced by silica-based packings that proved to be more efficient and mechanically stable.

(*b*) **Porous-layer beads** with a diameter ranging between 30-35 μm comprising of a thin shell (1-3 μm) of silicon or modified silica, on an inert spherical core material, such as : glass beads are still being employed for certain ion-exchange procedures ; but of late their usage as such in HPLC have been superseded by 100% porous microparticulate packings, and

(*c*) **Porous-silica particles** (100%) with a diameter less than 1 μm and narrow-particle size range, nowadays, form the basis of most abundantly available important column packings used in analytical HPLC. In comparison to the porous-layer beads, as detailed in (*b*) above, the porous-silica particles yield significant improvements not only in column efficiency but also in sample capacity and speed of analysis.

30.3.5. DETECTORS

The main function of the detector in HPLC is to monitor the mobile-phase coming out of the column, which in turn emits electrical signals that are directly proportional to the characteristics either of the solute or the mobile-phase.

The various detectors often used in HPLC may be categorized into *three* major heads, namely :

(*i*) **Bulk-property detectors :** They specifically measure the difference in some physical property of the solute present in the mobile-phase in comparison to the individual mobile-phase, for instance :

(*a*) Refractive-index detectors, and

(*b*) Conductivity detectors.

(*ii*) **Solute-property detectors.** They critically respond to a particular physical or chemical characteristic of the solute (in question), which should be ideally and absolutely independent of the mobile-phase being used. But complete independence of the mobile-phase is hardly to be seen, however, signal discrimination is good enough to enable distinctly measurable experimental procedures with solvent changes, such as : gradient-elution.

The solute-property detectors include :

(*a*) UV-detectors, and

(*b*) Fluorescence Detectors.

(*iii*) **Multipurpose detectors :** Besides, providing a high degree of sensitivity* together with a broad-linear-response-attainable range, invariably a particular situation critically demands detectors of more selective nature in the domain of 'analytical chemistry' *vis-a-vis* 'Pharmaceutical Analysis' that could be accomplished by using 'multipurpose detectors', such as : **"Perkin-Elmer '3D' System"** that combines UV absorption, fluorescence and conductometric detection.

(*iv*) **Electrochemical detectors : 'Electrochemical detector'** in HPLC usually refers to either amperometric or coulometric detectors, that specifically measure the current associated with the reduction or oxidation of solutes. As only a narrow spectrum of compounds undergo electrochemical oxidation, such detectors are quite selective ; and this selectivity may be further enhanced by monitoring the potential applied to the detector so as to differentiate between various electroactive species. Naturally, electrochemical detection essentially makes use of conducting mobile phases, for instance : inorganic salts or mixtures of water with water-miscible organic solvents.

The **five important types of detectors** shall be discussed along with their simple diagrammatic sketches, in the sections that follow :

30.3.5.1. UV-Detectors

Principle : An UV-detector is based on the principle of absorption of UV visible light from the effluent emerging out of the column and passed though a photocell placed in the radiation beam.

Figure 30.7 represents the schematic diagram of a double-beam UV detector used in HPLC system. Initially, dual-wavelength instruments having 254 and/or 280 nm were introduced which is presently being replaced by more sophisticated and up-dated variable wavelength detectors spread over wide range between 210-800 nm capable of performing more selective detection possible.

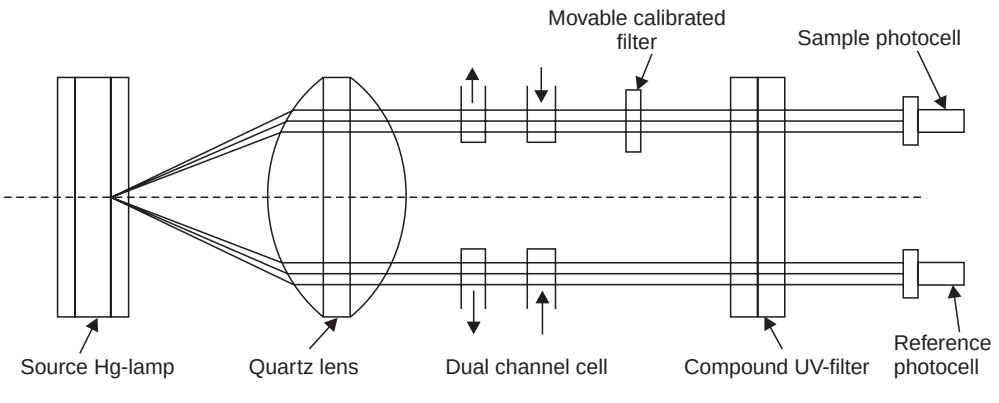

Figure 30.7 : Schematic Diagram of a Double-Beam UV Detector.

Diode Array Detector (or **Multichannel Detector**) is also a UV detector wherein a polychromatic light is made to pass through the flow cell. A strategically placed grating diffracts the outcoming radiation and subsequently meets an array of photodiodes whereby each photodiode receives a different narrow wavelength band. Here, a microprocessor scans the array of diodes several times in one second and the resulting spectrum is visualized on the screen of a VDU or subsequently stored in the instrument for a printout as and when required. Another extremely important and useful characteristic feature of a diode-array detector is that it may be 'programmed' so as to affect changes in the detection wavelength at particular points in the chromatogram. This versatile criterion is used to **'clean up'** a chromatogram *i.e.,* to discard all interfering peaks caused due to components irrelevantly present in the sample.

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* High Sensitivity of about 1 in 10^9 being attainable using UV and fluorescence detectors.

Advantages : Various advantages are, namely :

(*a*) A very selective detector which will detect only such solutes that specifically absorb UV/visible radiation *e.g.*, alkenes, aromatics and compounds having multiple bonds between C, O, N and S.

(*b*) The mobile-phase* employed ideally must not absorb any radiation.

30.3.5.2. Fluorescence Detector

A plethora of compounds (solutes) present in the mobile-phase on being passed as column effluent through a cell irradiated with Xenon or Deuterium source first absorb UV radiation and subsequently emit-radiation of a longer wavelength in two different manners, namely :

(*a*) Instantly-termed as **'Fluorescence',** and

(*b*) After a time-gap-known as **'Phosphorescence'**.

Fluorescent compounds : A relatively small proportion of inorganic and organic compounds exhibit natural fluorescence, whereas a larger number of pharmaceutical substances and environmental contaminants [*e.g.*, polycyclic aromatic hydrocarbons (PAH)] having a conjugated-cyclic system are fluorescent. Such compounds having absorbed energy being re-emitted from 0.1-1.0 can be detected by a fluorescence detector. However, non-fluorescent compounds can be converted to fluorescent derivatives by treatment with appropriate solvents.

Figure 30.8, illustrates the diagram of a fluorescence detector :

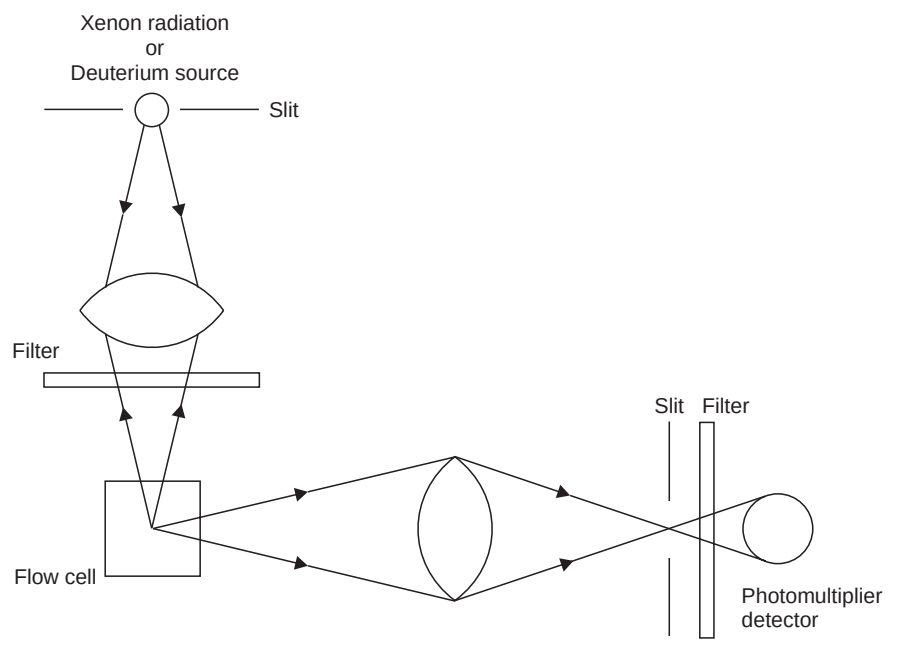

Figure 30.8 : Diagram of a Fluorescence Detector.

Radiation from a Xenon-radiation or a Deuterium-source is focussed on the flow cell through a filter. The fluorescent radiation emitted by the sample is usually measured at 90° to the incident beam. The second filter picks up a suitable wavelength and avoids all scattered light to reach ultimately the photomultiplier detector.

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* Always use only HPLC-Grade solvents for better precision and reproducibility of results.

30.3.5.3. Refractive Index Detector

It is also known as **'RI-Detector'** and **'Refractmeter'.** Figure 30.9, represents the block-diagram of a refractive-index detector.

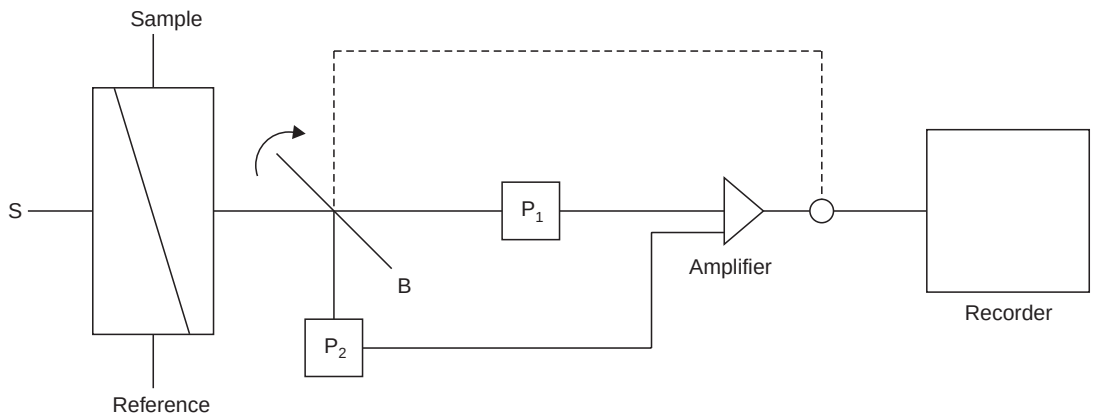


Figure 30.9 : Block Diagram of a Refractive Index Detector.

Light from the source(s) is focused into the cell, that consists of sample and reference sample ; and the two chambers are separated by a diagonal sheet of glass. After passing through the cell, the light is diverted by a beam-splitter (B) to two photocells (P_1 and P_2 respectively. A change in the observed refractive index (RI) of the sample stream causes a difference in their relative output, which is adequately amplified and recorded duly.

The RI of a few commonly used mobile-phase is stated below :

Mobile-Phase	Refractive-Index
Benzene	1.501
Decane	1.410
Hexane	1.375
Octane	1.397
Tetrayhydrofuran	1.405

Any solute can be detected as long as there exists a measurable difference in refractive index between the solute and the mobile-phase.

30.3.5.4. Multipurpose Detector

A multipurpose detector essentially comprises of three detectors combined and housed together in a single unit. A typical example of such a detector is the one developed by Perkin-Elmer known as **"Perkin-Elmer '3D' System"** which is depicted in Figure 30.10.

The functions of the three different detectors used in Figure 30.10 are enumerated as under :

(*i*) **Fluorescence Function :** It can monitor emission above 280 nm, based on excitation at 254 nm,

(*ii*) **UV-Function :** It is fixed wavelength 254 nm detector, and

(*iii*) **Conductance-Function :** The metal inlet and outlet tubes serve as electrodes to measure the conductance of the ions.

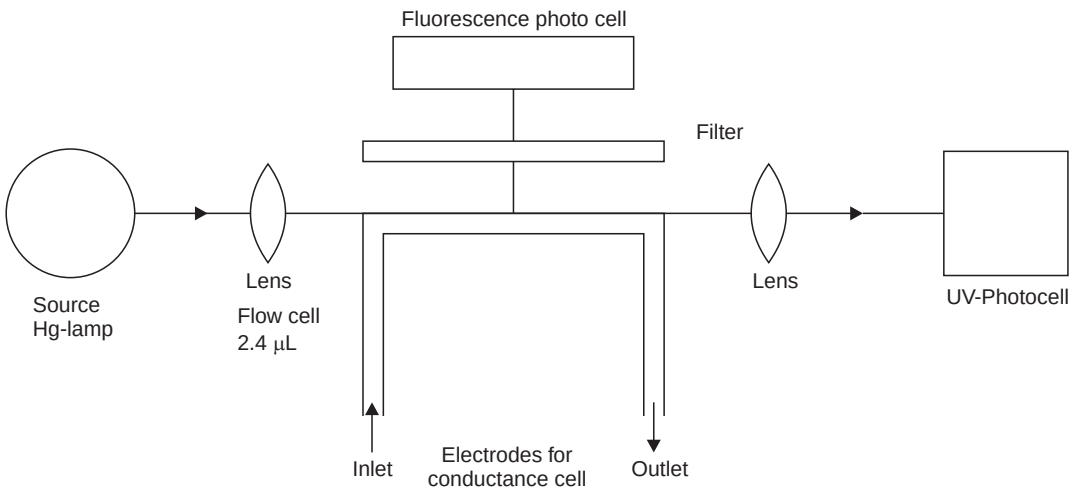


Figure 30.10 : Block Diagram of a Multipurpose Detector.

30.3.5.5. Electrochemical Detectors

In actual practice, however, it is rather difficult to utilize the functions of electrochemical reduction as a means of detection of HPLC by virtue of the fact that the serious interference (*i.e.*, large background current) generated by reduction of oxygen in the mobile phase. As complete removal of oxygen is almost difficult, therefore, electrochemical detection is normally based upon the oxidation of the solute.

Examples : The various compounds that may be detected conveniently are, namely : aromatic amines, phenols, ketones, and aldehydes and heterocyclic nitrogen compounds.

In short, the **amperometric detector** is presently considered to be the best electrochemical detector having the following distinct advantages, such as :

(*i*) very small internal cell-volume,

(*ii*) high degree of sensitivity,

(*iii*) more limited range of applications, and

(*iv*) excellent for trace analyses as UV-detector lacks adequate sensitivity.

Table 30.8, provides a comprehensive comparison of various typical detector characteristics invariably used in HPLC, such as : response, concentration expressed in g ml^{-1} and the linear range. However, the linear range usually refers to the range over which the response is essentially linear. It is mostly expressed as the factor by which the lowest factor (*i.e.*, Cn) should be multiplied in order to obtain the highest concentration.

Table 30.2 : Pattern of Typical Detector Characteristics in HPLC

S. No.	Type	Response	Cn (g ml^{-1})	Linear Range
1.	Amperometric	Selective	10^{-10}	10^4-10^5
2.	Conductometric	-do-	10^{-7}	10^3-10^4
3.	Fluorescence	-do-	10^{-12}	10^3-10^4
4.	UV/Visible Absorption	-do-	10^{-8}	10^4-10^5
5.	Refractive Index	Universal	10^{-6}	10^3-10^4

30.3.6. STRIP CHART RECORDER

The signal emerging from the detector of a HPLC is recorded continuously as function of time most commonly with the help of a potentiometric recorder. Invariably, a recorder of 1 to 10 mV full-scale deflec-

tion over a stretch of approximately ten inches and having a response-time of one second or even less is regarded as most appropriate. Strip-chart recorder with variable chart speeds ranging between 5 to 5 mm min^{-1} are usually preferred.

The input signal of a potentiometric-recorder is balanced continuously with the help of a feedback signal arrangement (device) using a *servomechanism*. A pen attached to this device moves proportionately, with preadjusted attenuation, along the width of the chart-paper thereby recording the signal accurately, while the chart-paper moves at a fixed speed along the length.

It is pertinent to mention here that before commencing the operation of a recorder, its zero point must be adjusted with the input zero, otherwise the baseline will also shift with slight changes in the attenuation of the signal.

Besides, it is also equally important to adjust properly the amplifier gain so as to eliminate completely the dead-band and the oscillations. A recorder having inadequate shielding from the AC circuits may display shifting of its zero point.

30.3.7. DATA HANDLING DEVICE AND MICROPROCESSOR CONTROL

Modern HPLC is adequately provided with complete data handling devices. Thousands of samples routinely analysed in Quality Assurance Laboratories in Pharmaceutical Industries/Bulk Drug Industries etc. are duly processed and the data stored in the computerised data-handling devices. Each stored data may be retrieved from the memory of the computerised device with the flick of a finger, as and when needed, in the form of print-out.

Microprocessor based analytical equipments is no longer an uncommon phenomenon towards the modernization, automation, and above all the ease of function and handling of sophisticated devices, for instance : a microprocessor scans the array of diodes many times a second in a **'diode array detector'** ; a microprocessor does the temperature programming of a constant temperature chamber of HPLC unit.

30.4. DERIVATIZATION

The main purpose of derivatization in HPLC is to improve detection specifically when determining traces of solutes in complex matrices, for example :

(*i*) Pharmaceutical substances lacking an UV-chromophore in the 254 nm region but possessing a reactive functional group,

(*ii*) Biological fluids *e.g.*, blood, serum, urine ; cerebrospinal fluid (CSF); and

(*iii*) Environmental samples.

Derivatization may be accomplished by *two* means, namely :

(*a*) Pre-column off-line derivatization.

(*b*) Post-column on-line derivatization.

These two methods shall be discussed briefly at this juncture :

30.4.1. PRE-COLUMN OFF-LINE DERIVATIZATION

Merits : This technique has the following merits :

(*a*) Requires no modification to the instrument *i.e.*, a plus point when compared to the post-column methods, and

(*b*) Imposes fewer limitations with regard to reaction-time and conditions.

Demerits : The demerits include :

(*a*) Formation of a stable and well-defined product is an absolute necessity,

(*b*) Presence of excess reagent or by products may invariably interfere with separation, and

(*c*) Very often derivatization may altogether change the chromatographic properties of the sample which facilitated separation.

30.4.2. POST-COLUMN ON-LINE DERIVATIZATION

The following experimental parameters should be maintained, namely :

(*a*) Derivatization performed in a special-reactor strategically positioned between the column and the detector,

(*b*) Reaction must be completed rapidly at moderate temperatures,

(*c*) Derivatization reaction need not even go to completion provided it can be made reproducible,

(*d*) No detector-response should exist due to any excess reagent present, and

(*e*) Reaction must be carried out in a medium other than the mobile-phase.

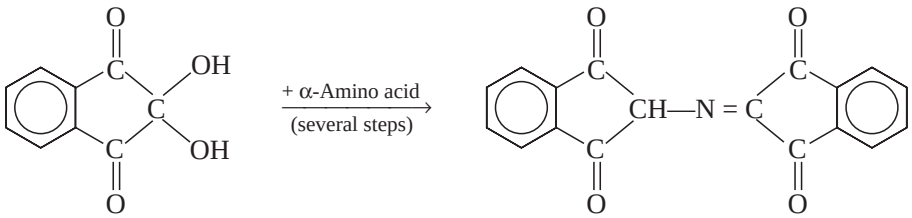
Merit : The main merit of post-column-on-line derivatization is that ideally the separation and detection processes can be optimized individually.

30.4.3. REAGENTS FOR DERIVATIZATION

There are potentially viable reagents available that may be employed for the derivatization of compounds either for enhancing UV/visible radiation (called **chromatags**) or for reaction of non-fluorescent reagent molecules (called **fluorotags**) with solutes to yield fluorescent derivatives.

Examples : (*i*) **Derivatization for UV-Detectors :**

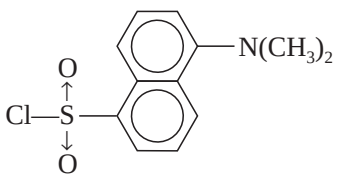
Ninhydrin (a **chromatag** is commonly employed to yield corresponding derivatives of amino acids that show absorption specifically at about 570 nm as shown in the following reaction :



Ninhydrin ‘Blue-coloured complex’

(*ii*) **Derivatization for Fluorescence Detectors :**

Dansyl Chloride (a **fluorotag**) is invariably used to obtain fluorescent derivatives of proteins, amines and phenolic compounds, the excitation and emission wavelengths being 335 to 365 nm and 520 nm respectively.



Densyl chloride

Some other important **‘Fluorotags’** are stated below :

S. No.	Reagent	Reacting Functional Groups
1	Ce^4-salts + H_2SO_4	Dicarboxylic acids
2	Fluorescamine [Fluram$^{(R)}$]	1°-Amines
3	*o*-Phthalaldehyde [Fluoropa$^{(R)}$]	1°-Amines

30.5. APPLICATIONS OF HPLC IN PHARMACEUTICAL ANALYSIS

Modern HPLC finds its abundant applications not only confined to isolation of natural pharmaceutically active compounds, control of microbiological processes but also assay of pure drugs and their dosage forms. A few typical examples will be discussed below :

30.5.1. ISOLATION OF NATURAL PHARMACEUTICALLY ACTIVE COMPOUNDS

Some plant alkaloids and glycosides can be isolated as stated below :

Category of Natural Products	Constituents	Used as
Alkaloids	Morphine; Codeine	Analgesic, Antitussive
Glycoside	Digitalis glycosides Sennosides	Cardiovascular diseases, Laxatives

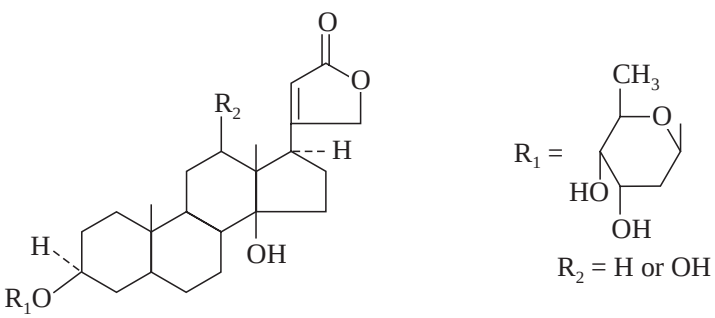
Chromatographic Conditions :

Column : Size-25 cm × 4.6 mm ID ;

Adsorbent : Lichrosorb RP-8 ;

Mobile-phase : Water/Acetonitrile-Gradient Elution ;

Detector : UV 254 nm



Digoxin : R_2 = OH ; R_1 = as above ;

Digitoxin : R_2 = H ; R_1 = as above ;

30.5.2. CONTROL OF MICROBIOLOGICAL PROCESSES

Various microbiological processes are used in the production of a number of antibiotics, for instance : **penicillins**, **tetracyclines**, **chloramphenicol** and **streptomycins**. The major areas of such operations being :

- kinetics of the microbiological process,
- monitoring of the on-going process,
- isolation and purification of active ingredients,
- purity control of active constituents, and
- monitoring derivatization reactions of these compounds.

HPLC-controlled analysis of a microbiological process during Penicillin Production : Chromatographic conditions are as follows :

Column : Size-25 cm × 4.6 mm ID ;

Adsorbent : Lichrosorb-$NH_2^{(R)}$ (10 μm) ;

Mobile-phase : 0.005 M H_2SO_4 buffer (pH 4.4))/acetonitrile (50 : 50) ; Flow rate : 3 ml min^{-1} ;

Detector : UV-220 nm ;

Microbial cleavage of Penicillin-G into 6-AMP and phenylacetate is as shown below :

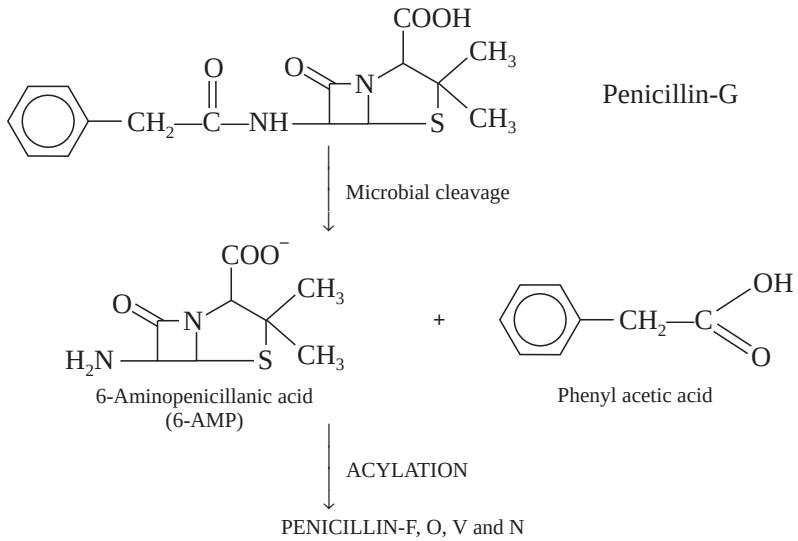


Figure : 11 (*a*) and (*b*) shows the HPLC chromatograms of penicillins in **'production sample'** and **'standard solution'** respectively :

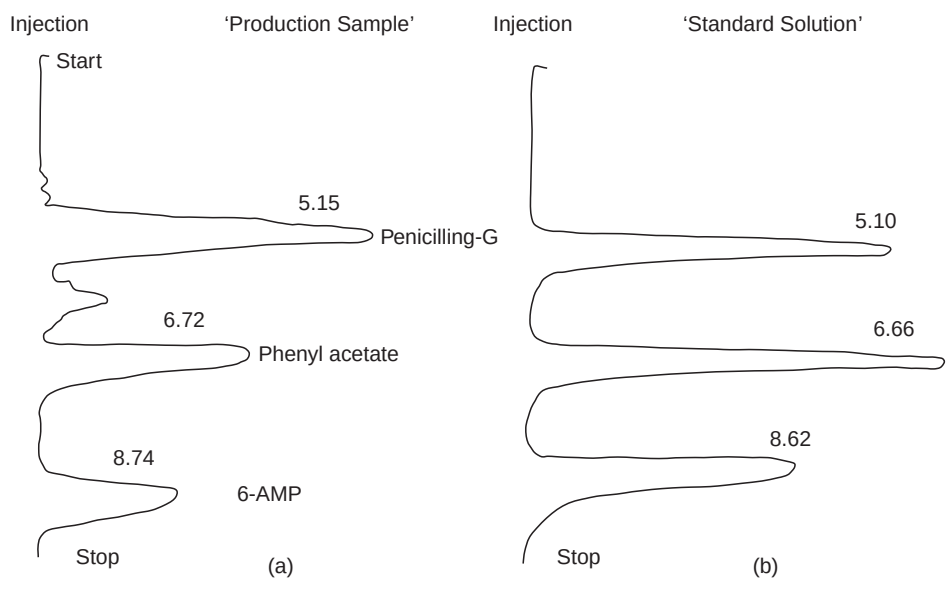


Figure 30.11 (*a*) and (*b*) : HPLC Chromatograms of Penicillins In 'Production Sample' and 'Standard Solution'.

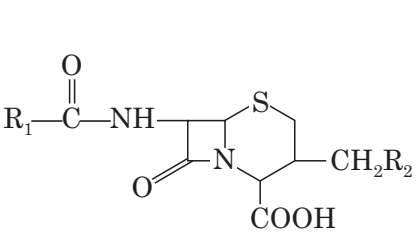
30.5.3. ASSAY OF CEPHALOSPORINS

Several commercially available cephalosporin antibiotics have been adequately separated by HPLC methods under the following experimental parameters

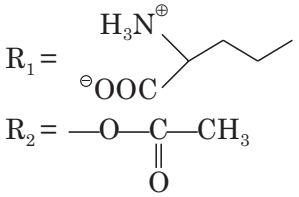
Column : ODS-SIL-X-II,

Mobile-phase : 0.95 M Ammonium Carbonate/Methanol (95 : 5) ;

Detector : UV-220 nm ;



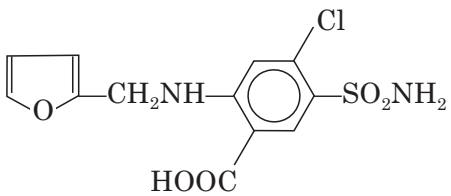
Cephalosporin C :



The loss of absorption with cleavage of the β-lactam was used by Marrelli* to analyze the concentrations of cephalosporin C in the presence of other UV absorbing species.

30.5.4. ASSAY OF FRUSEMIDE**

Theory : HPLC analysis of frusemide and its decomposition products is carried out by using simultaneous fluorescence and UV detection.



4-Chloro-N-furfuryl-5-sulphamoylanthranilic acid

It is noteworthy that fluorescence detection is a very specific technique, especially when excitation and emission wavelengths can be selected. In addition to this, sensitivity for compounds with photoluminescence properties can be higher by factors of 100 to 1000 when compared with that of other detectors.

The chromatographic conditions for Frusemide determination are as stated below :

Column : Size : 250 × 4.6 mm ID ;

Adsorbent : Lichrosorb$^{(R)}$ RP-8, 10 μm ;

Mobile-phase : Gradient elution-2 minutes, from 20% B to 37% B in 15 minutes, where, A = Water (pH 2.7) and B = Acetonitrile ;

Detector : (*i*) Fluorescence : Excitation : 275 nm ; Emission : above 405 nm ;

Figure : 12 (*a*) and (*b*) depict the HPLC chromatograms of frusemide by fluorescence and UV detectors respectively.

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* Flynn, EH, Ed., **'Cephalosporins and Penicillins',** Academic Press, New York, 1972.

** The name of this diuretic in the **Eur. Ph.** is **'furosemide'.**

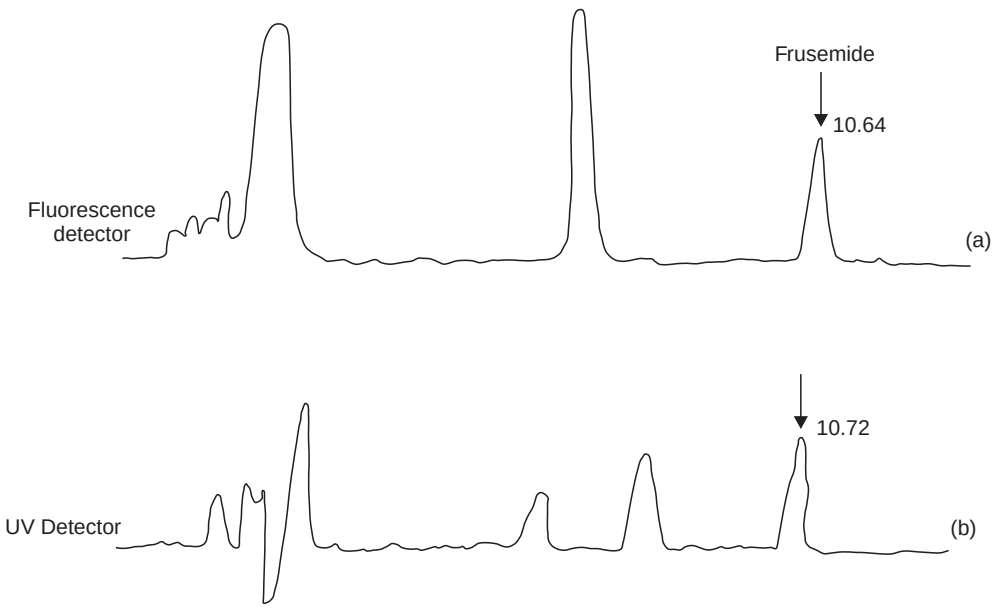
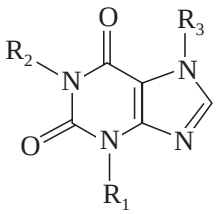


Figure 30.12 : HPLC-Chromatogram of Frusemide (a) Fluorescence Detector ; (b) UV-Detector.

30.5.5. ASSAY OF THEOPHYLLINE

Theophylline invariably contains other related substances as impurities, namely : theobromine, caffeine and β-hydroxypropyltheophylline.



Caffeine : $R_1 = R_2 = R_3 = —CH_3$;

Theophylline : $R_1 = R_2 = —CH_3$; $R_3 = —H$;

Theobromine : $R_2 = —H$; $R_1 = R_2 = —CH_3$;

β-Hydroxypropyltheophylline : $R_1 = R_2 = —CH_3$; and

$R_3 = —CH_2—\underset{\displaystyle OH}{\underset{|}{CH}}—CH_3$;

The chromatographic conditions for HPLC are as stated below :

Sample size : 10 μL ;

Column : size – 250 × 4.6 mm ID ;

Adsorbent : Lichrosorb (R) RP-8, 10 μm ;

Mobile-phase : 0.02 M KH_2PO_4 Buffer (pH 3.5)/Acetonitrile (95 : 5) ;

Detector : UV-254 nm ;

Figure 30.13, illustrates the HPLC chromatogram of **theophylline** assay with *four* distinct peaks.

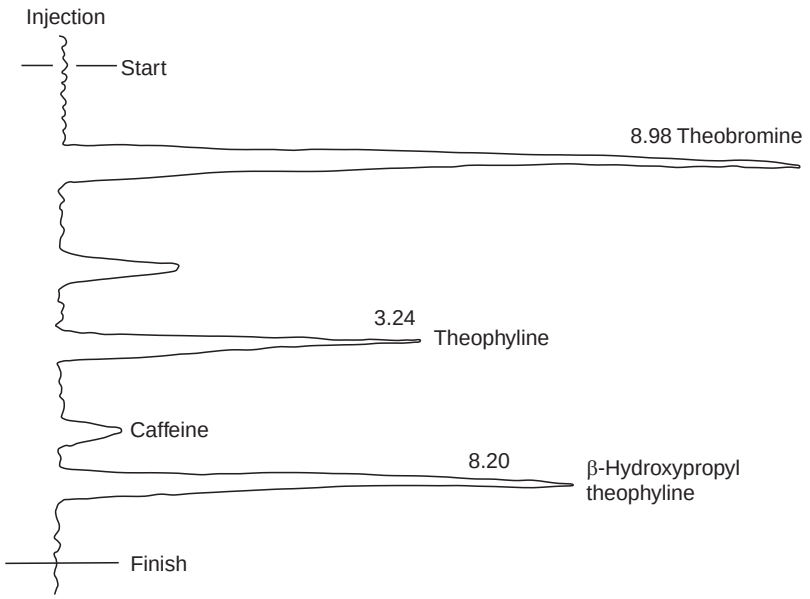


Figure 30.13 : HPLC Chromatogram of Theophylline.

30.5.6. ASSAY OF CORTICOSTEROIDS

A mixture of **six corticosteroids**, namely : *deoxycortisone*, *hydrocortisone acetate*, *cortisone*, *prednisone*, *hydrocortisone* and *prednisolone* can be assayed by HPLC method. The chromatogrpahic parameters for the assay are as follows :

Sample size : 10 μL
Column : size-250 × 4.6 mm ID ;
Adsorbent : Lichrosorb$^{(R)}$ DIOL : 10 μm ;
Mobile-phase : Gradient elution of A (*n*-Hexane) and B (Isopropanol) ;
Detector : UV-254 nm ;

Figure 30.14, clearly shows the six well-elaborated and distinct peaks of all the constituents stated earlier under the above HPLC parameters.

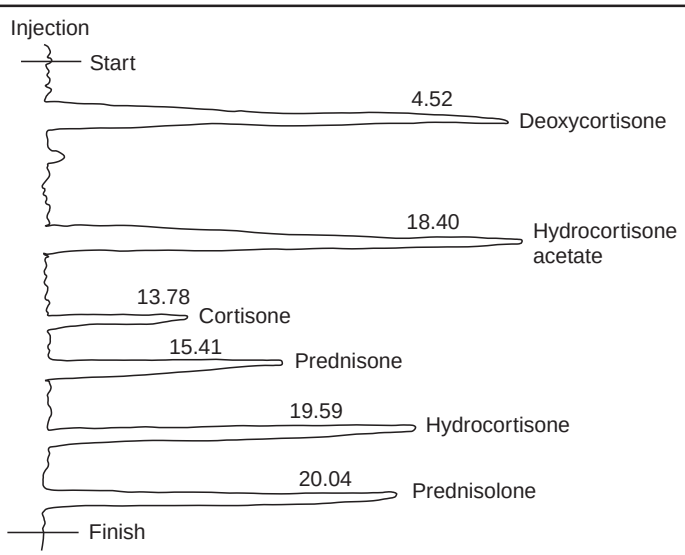


Figure 30.14 : HPLC Chromatogram of Corticosteroids.

30.5.7. ASSAY OF DICHLORPHENAMIDE*

Materials Required : Dichlorphenamide sample : 100 mg ; dichlorphenamide RS : 100 mg ; Mobile-phase [solution containing 0.02 M NaH_2PO_4 and 0.2 M Na_2HPO_4 in a mixture of equal volumes of acetonitrile and water] : 50 ml ;

Procedure : The chromatographic procedure may be performed using µ **Bondapack C18 column** as the stationary phase and the above mentioned mobile-phase with a flow rate of 1.0 ml per minute and a detection wavelength of 280 nm. Carry out the HPLC analysis using solutions in the mobile-phase containing (1) 0.05% w/v of dichlorphenamide RS and (2) 0.05% w/v of dichlorphenamide sample.

Calculations : Calculate the content of $C_6H_6Cl_2N_2O_4S_2$ using the declared content of the same in dichlorphenamide RS.

30.5.8. ASSAY OF HUMAN INSULIN*

Materials Required : Solution (1) : dissolve 40 mg of Human Insulin in sufficient of 0.01 M HCl (0.3648 g of HCl in one litre DW) to produce 10 ml ; solution (2) : Mix thoroughly 900 µL of 0.01 M HCl to 100 µL of solution (1) ; Solution (3) : dissolve 40 mg of Human Insulin EPCRS in sufficient 0.01 M HCl to produce 10 ml ; Solution (4) : mix 1 ml of solution (3) with 1 ml of a solution containing 4 mg of porcine insulin RS : Mobile-phase 'A' : Dissolve 28.4 g of anhydrous Na_2SO_4 in sufficient water to produce 1000 ml, add 2.7 ml of orthophosphoric acid, adjust of pH 2.3, if necessary, with ethanolamine, filter and degas by passing He through the solution ; Mobile-phase 'B' : Mix 500 ml of mobile-phase 'A' with 500 ml of acetonitrile, filter and degas by passing He through the solution.

Procedure : The HPLC is carried out using (*a*) a **Vydac C18 column**, for proteins and peptides, maintained at 40 °C, (*b*) as the mobile phase at a flow rate of 1 ml per minute, a mixture of 48 volumes of mobile phase 'A' and 52 volumes of mobile phase 'B' prepared and maintained at a temperature of not less than 20 °C, and (*c*) a detection wavelength of 214 nm.

Adopt the following steps sequentially :

Step	Inject	Test is not valid unless
I	10 µL of soln. (4)	The resolution between the peaks corresponding to human and porcine insulin is at least 1.3. (If necessary, adjust the conc. of acetonitrile in the mobile-phase by slight decrease or increase until the required resolution is obtained.
II	Soln. (3) six times	The relative standard deviation of the area of the principal peak is at most 2%.
III	10 µL of each of solns. (1), (2) and (3)	The area of the principal peak in the chromatogram with soln. (1) is 10 ± 0.5 times the area of the principal peak in the chromatogram obtained with soln. (2).

Calculations : Calculate the content of human insulin, $C_{257}H_{383}N_{65}O_{77}S_6$, from the peak areas and using the declared content of $C_{257}H_{383}N_{65}O_{77}S_6$ in human insulin EPCRS.

30.5.9. COGNATE ASSAYS

A number of pharmaceutical substances can be assayed by HPLC method as detailed in Table 30.1 (see next page).

* BP (1993)

Table 30.1 : Cognate Assays by HPLC Method

S. No.	Name of Substance	Stationary Phase	Mobile Phase/ Flow Rate	Solutions	Detection	Calculations
1.	Betamethasone Valerate	Spherisorb CDS 1	Abs. Ethanol and Water (42 : 58)/2 ml per minute	(1), (2) and (3)*	UV-Detector 238 nm	Based on the declared content of betamethasone valerate BPCRS ($C_{27}H_{37}FO_6$)
2.	Fluocinolone Acetonide	μ Bondapack C18	Methanol : Water (62 : 38)/2ml min^{-1} ;	(1), (2) and (3)	UV-Detector 254 nm	Based on the declared content of $C_{24}H_{29}Cl_2FO_5$ in BPCRS.
3.	Methotrexate	Nucleosil C18 in Acetonitrile	Phosphate Buffer (pH 6.0) and Acetonitrile (92 : 8)/1.4 ml min^{-1}.	(1), (2) and (3)	UV-Detector 303 nm	Based on the declared content of $C_{20}H_{22}N_8O_5$ in EPCRS
4.	Vinblastine Sulphate	Zorbax C8	Methanol : 1.5% w/v soln. of diethylamine adjusted to pH 7.5 with *o*-phosphoric acid : Acetonitrile (50 : 38 : 12)/1.0 ml min^{-1}	(1) to (5) kept in ice before use	UV-detector 262 nm	Based on the declared content of $C_{46}H_{58}N_4O_9.H_2SO_4$ in EPCRS.

* BP (1993) Vol. 1 ;

Along similar lines, the presence of related substances found in pharmaceutical drugs may be estimated by using HPLC method, for examples : atenolol, buclizine hydrochloride, ibuprofen, and the like.

THEORETICAL AND PRACTICAL EXERCISES

1. What are the major advantages of High Performance Liquid Chromatography (HPLC) over GLC ? Explain with typical examples.

2. Enumerate the vital theoretical aspects of HPLC giving suitable examples.

3. Describe the working of a HPLC-equipment highlighting the various important components with a labelled diagramatic presentation.

4. Elaborate on the following aspects of HPLC :

(*a*) Reciprocatng pump,

(*b*) Working of a twin-head reciprocating pump,

(*c*) Microvolume sampling valve (*i*) Sampling mode ; and (*ii*) Injection mode, and

(*d*) Measurement of column performance.

5. Discuss briefly the following detectors used in HPLC alongwith a diagramatic sketch :

(*i*) Double-beam uv-detector, (*ii*) Fluorescence detector,

(*iii*) Refractive index detector, and (*iv*) Multipurpose detector.

6. What do you understand by '*pre-column off-line derivatization*' and '*post-column on-line derivatization*' ? Explain.

7. Explain how one may use HPLC to accomplish the following :

(*a*) Isolation of alkaloid and glycoside, (*b*) Control of microbiological processes.

Give suitable examples in support of your answer.

8. How would you assay the following **'medicinal compounds'** enlisted in *Official Compendia* :

(*i*) Cephalosporins, (*ii*) Frusemide,

(*iii*) Theophylline, (*iv*) Corticosteroids,

(*v*) Dichlorphenamide, and (*vi*) Human Insulin.

RECOMMENDED READINGS

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2. Johnson, EL and R. Stevenson, **'Basic Liquid Chromatography',** Palo Alto, Varian Associates Inc., 1978.

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4. Engelhardt, H., **'High Performance Liquid Chromatography : Chemical Laboratory Practice',** New York, Springer-Verlag, 1979.

5. Snyder, LR and JJ Kirkland, **'Introduction to Modern Liquid Chromatography',** 2nd ed., New York, Wiley, 1979.

6. Hamilton, RJ and PA Sewell **'Introduction to High Performance Liquid Chromatography',** London, Chapman and Hall, 1982.

7. Knox, JH, Ed., **'High Performance Liquid Chromatography',** Edinburgh, Edinburgh University Press, 1982.

8. Simpson, CF, Ed., **'Techniques in Liquid Chromatography',** New York, John Wiley, 1982.

31 SIZE EXCLUSION CHROMATOGRAPHY

CONTAINS :

31.1. INTRODUCTION

The **size-exclusion chromatography** (or **gel-chromatography**) is a means of separation which is exclusively dependent on the exchange of solute molecules between the solvent of the mobile-phase and the same solvent within the pores of the column-packing material. In reality, it is the pore-size-range of the packing material that solely determines the molecular-size-range within which a particular separation can take place effectively.

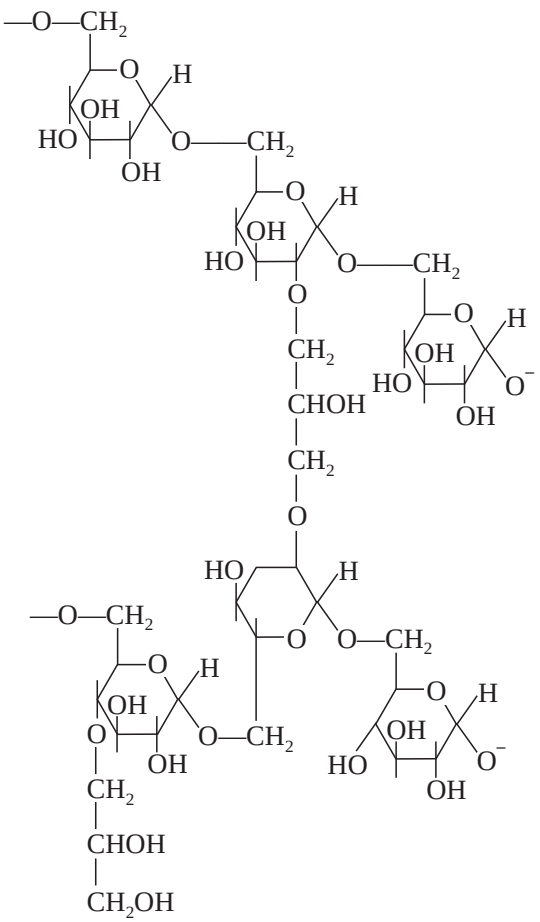
The timely adoption of the *cross-linked dextran gels* (*i.e.*, **Sephadex**) in late-fifties as a packing material for column chromatography opened an altogether new horizon of chromatographic separation whereby substances, in general, undergo separation more or less as per their molecular size.

In actual practice, the inert gels of dextran (I)-a polyglucose or other types of polymers, for instance : agarose and polyacrylamides, wherein the macromolecules invariably are cross-linked to afford a reasonably porous 3D-structure*, served as the stationary phases in size-exclusion chromatography.

The salient features of 'gels' are enumerated below :

(*i*) The extent or degree of cross-linking and obviously the sizes of the pores within the body of the gels are rigidly monitored and controlled during the course of manufacture,

* Three-dimensional (3D) structure.



(I)

(*ii*) Mostly the gels are hydrophilic in nature and evidently they swell-up in contact with water,

(*iii*) Gels having a large degree of cross-linking and a relatively large pore size usually need a larger volume of water in order to fill up the pores available within the gel-structure in comparison to the tightly linked gels, as could be seen in Table 31.1.

Table 31.1 : Characteristics of Dextran Polymer Gels

Sephadex Grade	Water Regain (g/g)	Mol.Wt.	Bed* Vol. (ml g^{-1})	Swelling Time (*h*)
Sephadex-15	1.5	1,500	2.5-3.6	3
Sephadex-75	7.5	50,000	13	24
Sephadex-200	20	200,000	30	72

(*iv*) Buffered aqueous solutions normally serve as mobile phases in size-exclusion chromatography. However, highly modified gel polymers are also available commercially (*e.g.,* Sephadex-LX) that exclusively make use of organic solvents.

31.2. THEORY

The efficiency and ability of a gel to slow down the movement of various substances downwards in a packed column with the respective gel entirely depends on the molecular size of the substance *vis-a-vis* to the

* Dry polymer basis.

pore sizes prevailing within the gel matrix. Evidently, a substance with high molecular weight is unable to diffuse into the pores of the gel and thereby moves down the column more rapidly through the channels between the grains of the gel. On the contrary, a substance having molecular size distinctly smaller than the largest pores of the gel shall naturally penetrate the pores and move with a slower pace down the column. In this manner the substances having molecular size greater than the pores shall undergo exclusion thereby affecting their elution from the column into the space immediately ahead of the relatively small molecular weight components. In other words, the substances are found to be eluted from the column strictly in order of the decreasing molecular size.

The liquid phase which is absorbed by the synthetic polymer granules (*e.g.*, Sephadex) is mostly available in a wide range as solvent for solute molecules in contact with the gel. It has been observed that the actual distribution of the solute in between the inside and outside of the respective gel granules is nothing but a criterion of the available space. However, the underlying distribution coefficient occurring between the granular and interstitial aqueous phases is found to be independent of *three* major factors, namely :

(*a*) pH

(*b*) Ionic strength, and

(*c*) Concentration of the solvent.

31.2.1. DISTRIBUTION COEFFICIENT (K_D)

The distribution coefficient is defined by the underlying expression :

$$K_D = \frac{V_R - V_O}{V_T - V_O}$$

where, V_R = Retention volume for the component of interest,

V_O = Retention volume for a non-retained component (or exclusion volume), and

V_T = Retention volume for a component that has full access to all the pores of the support (or total permeation volume).

31.2.2. PERFORMANCE

The column performance may be determined from the number of theoretical plates per metre (*n*), calculated by the help of the following expression :

$$n = \frac{5.54 V_R^2}{L W_h^2}$$

where, V_R = Retention volume* for the component of interest,

W_h = Width of the peak of interest at half peak height, measured in the same units as V_R.

31.2.3. MATERIALS

There are usually three types of materials that are employed in the size-exclusion chromatography for pharmaceutical substances which have been discussed briefly as under :

(*a*) **Agarose FC**

Presentation : Swollen beads 60 to 140 μm in diameter, available as a 4% suspension in water,

Applications : (1) It is used for the separation of proteins having molecular weights ranging from 6×10^4 to 2×10^7, and

(2) It is employed for the separation of polysaccharides having molecular weights varying from 3×10^3 to 5×10^6.

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* Retention Volume-is the distance along the baseline between the point of injection and a perpendicular dropped from the maximum of the peak of interest.

(*b*) **Agarose FC, Cross-linked**

Presentation : Prepared from agarose by reaction with 2, 3-dibromopropan-1-ol in strongly alkaline enviroment. It occurs as swollen beads 60 to 140 μm in diameter and is available as a 4% suspension in water.

Applications : (1) It is employed for the separation of proteins having molecular weights ranging between 6×10^4 to 20×10^6, and

(2) It is used for the separation of polysaccharides having molecular weights varying between 3×10^3 to 5×10^6.

(*c*) **Silica Gel FC**

Presentation : It is very finely divided power having an average particle size 10 μm with a very hydrophilic surface. It has an average pore diameter of about 30 nm. It is fairly compatible with aqueous solutions of pH 2 to 8 and also with various organic solvents.

Applications : It is employed for the separation of proteins having molecular weights ranging from 1×10^3 to 3×10^5.

31.3. APPARATUS

The apparatus for '**size-exclusion chromatography**' essentially comprises of a chromatographic column generally made up of glass having a diameter to height ratio of between 1 : 10 and 1 : 20, packed with an appropriate separation material (*e.g.,* different grades of **Sephadex**) which is capable of fractionation in the suitable range of molecular size and may be adequately temperature controlled. It is an usual practice to allow the mobile phase to pass through the column at a constant rate either by the aid of a suitably pump or simply by gravity.

31.3.1. APPLICATION OF SAMPLE

The sample is normally applied to the column by adopting one of the *five* following methods, namely :

(*i*) Directly to the drained-bed-surface with permitting the bed to dry,

(*ii*) Layered beneath the mobile-phase, provided the sample is denser than the mobile-phase,

(*iii*) Using a flow adaptor,

(*iv*) Using a syringe through a septum, and

(*v*) Using an injection valve.

31.3.2. DETECTION AND RECORDING

The outlet from the column is connected to a '**detector**' usually fitted with an **'automatic recorder'** that permits exclusively the monitoring of the relative concentrations of the various constituents present in the sample. However, one may also make use of an **automatic fraction collector** duly attached to the outlet from the column, if required. The various experimental parameters stated below are normally given in the *official monograph*, namely :

(*a*) Temperature of the column, if other than ambient,

(*b*) Nature of the packing material*,

(*c*) Composition of the mobile-phase,

(*d*) Flow rate of the mobile phase,

(*e*) Means of detection** of the sample components.

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* It must be treated, and the column packed, as per the manufacturer's instructions.

** UV-Specrophotometry.

31.4. APPLICATIONS OF SIZE EXCLUSION CHROMATOGRAPHY IN PHARMACEUTICAL ANALYSIS

The size-exclusion-chromatography may be used for *two* specific purposes in the analysis of pharmaceutical substances, such as :

(*i*) Determination of relative component composition, and

(*ii*) Determination of molecular weight.

31.4.1. DETERMINATION OF RELATIVE COMPONENT COMPOSITION

The assay method along with specific experimental parameters are duly stated in the *official monograph*. Here, *two* situations arise, namely :

(*a*) **Equivalent Responses :** In case, all of the components of the sample exhibit equivalent responses to the detector, then the relative quantity of each component may be determined conveniently by dividing each peak area by the sum of the peak areas of the components of interest, and

(*b*) **Non-equivalent Responses :** In case, the responses achieved are not equivalent, calculate the relative component composition either from the calibration curve obtained with the calibration standards specified in the official monograph or by any other method stated in the official monograph.

31.4.2. DETERMINATION OF MOLECULAR WEIGHT

The following steps may be followed in a sequential manner to determine the molecular weight of a pharmaceutical substance :

(*i*) Follow the method on the sample by employing the specified procedure laid down in the official monograph,

(*ii*) Plot a graph of the retention volume of the calibration standards as a function of the logarithm of the molecular weight,

(*iii*) The curve, thus obtained, normally approximates to a straight line within the exclusion and total permeation limits,

(*iv*) The molecular weight of the component of interest may be determined from the calibration curve, and

(*vi*) The calibration is valid only for the particular system employed under the specified experimental parameters.

The techniques of **size-exclusion chromatography** has been used effectively in checking the purity of the following pharmaceutical substances for their respective impurities, such as :

(*i*) Corticotrophin : For impurities of higher molecular weight,

(*ii*) Insulin : For proteins of higher molecular weight,

(*iii*) Human Insulin : For proteins of higher molecular weight, and

(*iv*) Plasma Protein Solution : For polymers and aggregates.

31.4.3. CORTICOTROPHIN : FOR IMPURITIES OF HIGHER MOLECULAR WEIGHTS

Materials Required : Corticotrophin : 1 mg ; acetic acid (1 M) [prepared by dissolving 57 ml of glacial acetic acid in 1000 ml of DW] : 100 ml ; sodium dodecyl sulphate (1% w/v) : 10 ml ;

Procedure : Dissolve accurately weighed 1 mg of corticotophin in 1 ml of 1 M acetic acid containing 1% w/v of sodium dodecyl sulphate. Heat the solution at 100 °C for 10 minutes and allow to cool.

The chromatographic procedure may be performed using (*a*) a column (about 85 cm × 10 mm) packed with polyacrylamide or cross-linked dextran for chromatography having a fractionation range for peptides with

relative molecular weights of approximately 1000 to 10,000 ; (*b*) 1 M acetic acid as the mobile phase with a flow rate of 7 ml per hour, and (*c*) a detection wavelength of 276 nm. Now, connect the detector, fitted with a flow-cell suitable for liquid chromatography having a volume of not more than 1 ml, to a strip-chart recorder. Set the detector and chart recorder at a full-scale sensitivity of 0.5 absorbance unit.

Equilibrate the column with 1 M acetic acid. Apply the cold solution to the top of the column using 0.4 ml per cm^2 of column cross-sectional area. The sum of the areas of any peaks eluted before the principal peak is not greater than 5.0% of the sum of the areas of all the peaks in the chromatogram.

31.4.4. INSULIN : FOR PROTEINS OF HIGHER MOLECULAR WEIGHT

Materials Required : Solution (1) : Dissolve 10 mg of insulin in 1 ml of the mobile phase ; Solution (2) Dilute 100 µ*l* of solution (1) to 10 ml with the mobile phase ; and Solution : (3) Dissolve 10 mg of procine insulin EPCRS* of bovine insulin EPCRS, as appropriate, in 1 ml of the mobile phase.

Procedure : The chromatographic procedure may be carried out using (*a*) a column (60 cm × not less than 7.5 mm) packed with silica gel for chromatography (10 µm ; pore size about 13 nm) Water 1-125 ; Toyo Soda TSK 2000 SW ; and Zorbax GF 250 are suitable ; (*b*) as the mobile phase with a flow rate of 0.5 ml per minute a filtered and degassed solution prepared by mixing 20 volumes of glacial acetic acid and 50 volumes of water, adjusting the pH to 3.0 by the addition of a 25% v/v solution of ammonia, adding 30 volumes of acetonitrile and mixing 1 and (*c*) a detection wavelength of 276 nm.

Inject 50 µl of each solution. Adjust the sensitivity of the detector so that the height of the principal peak in the chromatogram obtained with solution (2) is 50-70% of full-scale deflection. In the chromatogram obtained with solution (1) the sum of the area of any peak eluting before the principal peak is not greater than the area of the principal peak in the chromatogram obtained with solution (2) (1.0%).

31.4.5. HUMAN INSULIN : FOR PROTEINS OF HIGHER MOLECULAR WEIGHT

Materials Required : Solution (1) : Dissolve 10 mg of human insulin in 1 ml of the mobile phase, Solution (2) : Dilute 100 µL of Solution (1) to 10 ml with the mobile-phase, and Solution (3) : Dissolve 10 mg of human insulin EPCRS in 1 ml of the mobile-phase.

Procedure : The chromatographic procedure may be performed using (*a*) a column (60 cm × not less than 7.5 mm) packed with silica gel for chromatography (10 µm ; pore size about 13 nm) Water 1-125 ; Toyo Soda TSK 2000 SW and Zorbax GF 250 are suitable, (*b*) as the mobile phase with a flow rate of 0.5 ml per minutes of a filtered and degassed solution prepared by mixing 20 volumes of glacial acetic acid and 50 volumes of water, adjusting the pH to 3.0 by the addition of a 25% v/v solution of ammonia, adding 30 volumes of acetonitrile and mixing, and (*c*) a detection wavelength of 276 nm.

Inject 50 µL of each solution. Adjust the sensitivity of the detector so that the height of the principal peak in the chromatogram obtained with solution (2) is 50 to 70% of full-scale deflection. In the chromatogram obtained with solution (1) the sum of the areas of any peaks eluting before the principal peak is not greater than the area of the principal peak in the chromatogram obtained with solution (2) (1.0%).

31.4.6. PLASMA PROTEIN SOLUTION : FOR POLYMERS AND AGGREGATES

Materials Required : Plasma protein solution : 2.0 ml ; mixed phosphate buffer pH 7.0 with azide [To 1000 ml of a solution containing 1.8% w/v of disodium hydrogen orthophosphate and 2.3% w/v of sodium chloride and sufficient of a solution containing 0.78% w/v of sodium dihydrogen orthophosphate and 2.3% w/v of sodium chloride (about 280 ml) to produce a pH of 7.0 Dissolve sufficient sodium azide in the resulting solution to give a 0.02% w/v solution] : 1000 ml ;

Procedure : The chromatographic procedure may be carried out at room temperature using (*a*) a column (1 M × 25 mm) packed with a cross-linked dextran suitable for fractionation of globular proteins in the range of molecular weights from 5,000 to 350,000 (Sephadex G-150 is suitable), (*b*) mixed phosphate buffer pH 7.0 with azide as the mobile-phase with a flow rate of about 20 ml (4 ml per square centimetre) of column cross-sectional area) per hour, and (*c*) a detection wavelength of 280 nm.

Collect the eluate in fractions of about 4 ml and combine the fractions corresponding to each peak. For each combined fraction carry out the determination of nitrogen as per BP (1993). Not more than 10% of the total nitrogen is present in the combined fraction associated with non-retained proteins.

THEORETICAL AND PRACTICAL EXERCISES

1. What is the importance of **'Size-exclusion chromotography'** (SEC) in the assay of '*drugs*' ? Explain.

2. Expatiate the theoretical aspects of SEC with specific reference to the following :

(*a*) Distribution coefficient, (*b*) Performance, and

(*c*) Materials.

3. How would you accomplish the following **'assays'** with SEC :

(*i*) Determination of relative component composition,

(*ii*) Determination of molecular weight,

(*iii*) Impurities of high-molecular weight in **'Corticotrophin'**,

(*iv*) Proteins of high molecular weight in **'Insulin'/'Human Insulin'**,

(*v*) Polymers and aggregates in **'Plasma Protein Solution'.**

RECOMMENDED READINGS

1. Beckett, AH and JB Stenlake, **'Practical Pharmaceutical Chemistry',** Part II, 4th ed., London, The Athlone Press, 1988.

2. Jeffery, GH, J. Bassett, J. Mendham and RC Denney, **'Vogel's Textbook of Chemical Analysis,** 5th ed., New York, Longman Scientific & Technical, 1989.

3. **British Pharmacopoeia,** Vol. I and II, London, HMSO, 1993.

PART VI

MISCELLANEOUS ASSAY METHODS

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32 RADIOIMMUNOASSAY

CONTAINS :

32.1. INTRODUCTION

The introduction of **radioimmunoassay** (RIA) and its subsequent development as a possible versatile tool in wide spheres of science, occurred empirically to the initiators. Radioimmunoassay was primarily developed by Berson and Yalow* (1959) for the quantitative measurement of insulin in human plasma, which eventually not only revolutionized endocrinilogy as such but also paved the way for the clinical chemistry laboratory practice in general. As on date RIA principles have found wide application in the field of drug analysis, pharmacokinetic studies, drug-therapy monitoring and above all the immunodiagnosis in medicine to mention but a few. Specifically RIA measures the actual effect of changing concentrations of a particular substance present in a biological fluid (*e.g.*, blood, plasma, urine) based on an *in vitro* system consisting of radioactive standards of the same substance and a specific antibody. In a true sense, RIA is nothing but an indirect method of analysis because it does not make use of either the radioactive standard or the antibody present in the original sample.

Before the emergence of radioimmunoassay as an acceptable analytical technique, a number of other methods were employed for the analysis of 'drugs' in the plasma. Prominent among these methods were thin-

* Berson, SA and RS Yalow, **J. Clin. Invest., 38,** 1996, 1959.

layer chromatography (TLC), **gas-liquid chromatography** (GLC), **spectrofluometry** (SPF) and ordinary **radiolabelling assay**. The above methods, undoubtedly, have certain advantages to their credit ; however, the disadvantages outnumbered the advantages, as stated below :

Disadvantages

(1) Non-specificity of the technique,

(2) Non-sensitivity of the method,

(3) Involvement of the processes of extraction, purification and concentration of the specimen under investigation,

(4) Heat treatment of the specimen resulted invariably in degradation and destruction of the substances, and

(5) Many processes involved ultimately make the analysis rigorous and unnecessarily sluggish.

On the contrary, RIA provided a specific, sensitive, rapid, convenient, reliable, reproducible and less expensive assay methods for biological fluids.

The introduction of enzyme immunoassay (EIA) and similar allied immunoassay techniques in early eighties showed, in fact, a brighter path towards quantitative analysis.

RIA technique has splendidly made available to the drug analyst, endocrinologist, physiologist, pharmacologist, clinical chemist and biochemist a very sensitive, specific and comparatively easier method for the quantitative measurement of serum or plasma drug, hormones, enzyme concentrations, besides, drug concentrations in biological fluids. It has also proved to be equally important in pharmacokinetic studies and in acute monitoring of patient drug therapy according to Mule *et al** (1974).

Skelley *et al*** (1973) listed a number of substances that may be determined quantitatively by the help of the RIA method, namely : **nucleic acids, proteins, enzymes, prostaglandins, steroidal hormones, antibodies, cancer and viral antigens, vitamins, and drugs together with their respective metabolites.**

Importantly, the pioneer work or Oliver and coworkers*** (1968) and followed by valuable and meaningful contributions by Landon and Moffat**** (1976) proved beyond any reasonable doubt the efficacy of RIA in the quantification of a host of **pharmaceutical substances**.

32.2. THEORY

The basic underlying principle of **radioimmunoassay** utilizes the reaction between an antigen• (hapten)•• and its specific antibody. Small molecules (micromolecular) for instance : drugs that may serve as haptens and can normally be made antigenic by coupling them chemically to a macromolecular substance, such as : *protein polysaccharide, carbohydrate* etc. The hapten is obtained from a non-antigenic compound (micromolecule) *e.g., morphine, cartelol etc.*, which is ultimately conjugated••• convalently to a carrier•••• macromolecule to render it antigenic.

Animals normally develop antibodies••••• to the injected immunogenic substance as part of their natural immune response. The serum derived from these animals is used as the antibody source and tested with reference

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* Mule, SJ, ML Bastos and D. Jukofsky, **Clin. Chem., 20,** 243, 1974.

** Skelley, DS, LP Brown and P.K. Besch, **Clin, Chem., 19,** 146, 1973.

*** Oliver, GC et al, **J. Clin. Invest., 47,** 1035, 1968.

**** Landon, J., and AC Moffat, **Analyst, 101,** 225, 1976.

• **Antigen :** The substance against which antibody formation was intended (in this case, the **drug**) ;

•• **Hapten : (Ligand) :** A small, molecule, such as a drug, that must be coupled to a carrier molecule to induce an immune response, but capable of combining with specific antibodies. ;

••• **Conjugate :** The combined hapten and carrier ;

•••• **Carrier :** A protein, polypeptide, or inert matrix that is coupled to the hapten to form an immunogen;

••••• **Antibody :** An immunoglobulin that will bind with an antigen (ligand or hapten) or immunogen ;

to their specificity, sensitivity or affinity at their **titer level**. By specificity, is meant the lowest concentration of a compound which can be detected in undiluted body fluid. Generally, it is referred to as the ''**detection limit**'' or the ''**cut off level**''.

Sensitivity defines the degree to which an assay can distinguish one compound from another of the same nature and an **immunoassay** is a function of the particular antibody molecules contained in the antiserum. Specificity of the antiserum is a function of the particular antigen used to immunize the animal. Affinity usually measures how strongly bound is the antigen to the antibody. **Titer** refers to the concentration level of, in the context of the usage, antibody contained in the obtained serum.

Immunological reactions by virtue of their specificity allow the discrete identification of single molecular entities in the presence of many-fold higher concentrations of either multiple or chemically identical molecular entities. However, it is pertinent to be noted here that both **immunological** and **immunochemical techniques** are capable of providing the much sought after assay systems for *pharmaceutical substances* present in complex mixtures without the necessity of undergoing through the tedious and cumbersome process of prior extraction and purification required frequently for their respective biological and chemical tests. Interestingly, the **radioimmunochemical methods** possess the additional advantages of offering exquisite sensitivity as well as enhanced specificity*.

32.2.1. HAPTEN DETERMINANTS AND PURITY : THE KEY TO IMMUNOLOGICAL SPECIFICITY

It has since been recognized as a well established phenomenon that is possible to hook-up a micromolecule (drug) to a macromolecule (protein, polypeptide, polysaccharide) to render it antigenic, inject the resulting conjugate into an immunologically competent animal and subsequently harvest antibodies which includes those bound to the hapten moiety. Nevertheless, the animal should be genetically a responder with regard to the specific macromolecule carrier and even so to the micromolecule moiety of the immunogenic conjugate. Apparently, it may appear as the most efficient and easiest means to hook-up the micromolecule being made haptenic by any of its available chemically reactive functional groups to the selected carrier molecule.

But unfortunately, no matter how many competent animals are immunized with such an immunogenic conjugate, the antisera thus generated cannot contain a population in the total antibody immunoglobulin (IgG) pool that will recognize the chemically reactive group used for coupling to the carrier portion of the conjugate moiety. In case, only a small quantum of antigenic determinants** exist in the hapten before conjugation to macromolecule the loss of even one functional group can turn out to be critical.

32.2.2. IMPORTANCE OF ANTIGENIC DETERMINANTS

These are, namely :

(*i*) The functional groups of the hapten should remain unblocked in the conjugate molecule,

(*ii*) These chemical functions are primarily responsible for metabolic activity ; besides, all active functions of a small hapten should remain accessible in the hapten carrier conjugate to obtain the most exquisitely specific antibody immunoglobulin (IgG) population of which the immune system is capable,

(*iii*) The fewer the active functions are available to serve as haptenic determination, the lesser will be the specificity of the reaction in radioimmunoassay ; in other words, the greater the number of antigenic determinants in a hapten molecule the more specific shall be its reaction with its antibody.

Example : Blockade of a single hydroxyl group of morphine in the preparation of morphine immunogen results in an antiserum that is entirely unable to distinguish **homologous morphine forms** from its **corresponding surrogates with unavailable hydroxyl(s)*****. Further, the antiserum produced by immunization with such a morphonyl immunogen reacts with codeine either equally or better than morphine.

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* Pincus, G., KV Thimann, and E.B. Astwood, **The Hormones,** New York, Academic Press, 557, 1964.

** Functional groups that determine antigenic properties.

*** Spector, S. and CW Parker, **Science, 168,** 1347, 1970.

(*iv*) All chemically reactive functions of a pure derivative, not particularly those which coincide with physiological activity, must remain undistorted and accessible to avail themselves as immunological determinants.

32.2.3. ANALYSIS BY COMPETITIVE ANTIBODY BINDING OR ISOTOPICALLY LABELLED COMPOUNDS

Radioimmunoassay is nothing but a competitive binding assay employing the principle of reversible binding of a labelled antigen to its specific antibody ; and the ability of unlabelled antigen not only to compete in the reaction but also to displace labelled antigen from antibody. Nevertheless, the antibody and labelled antigen are always present as limiting factors and the concentration of unlabelled antigen (present either as standard solution or as sample under examination) is increased continually. It has been observed that the percentage of antibody-bound labelled antigen declines progressively as a consequence of saturation of the combining sites on the antibody molecule.

The principle governing radioimmunoassay has been duly illustrated in Figure 32.1.

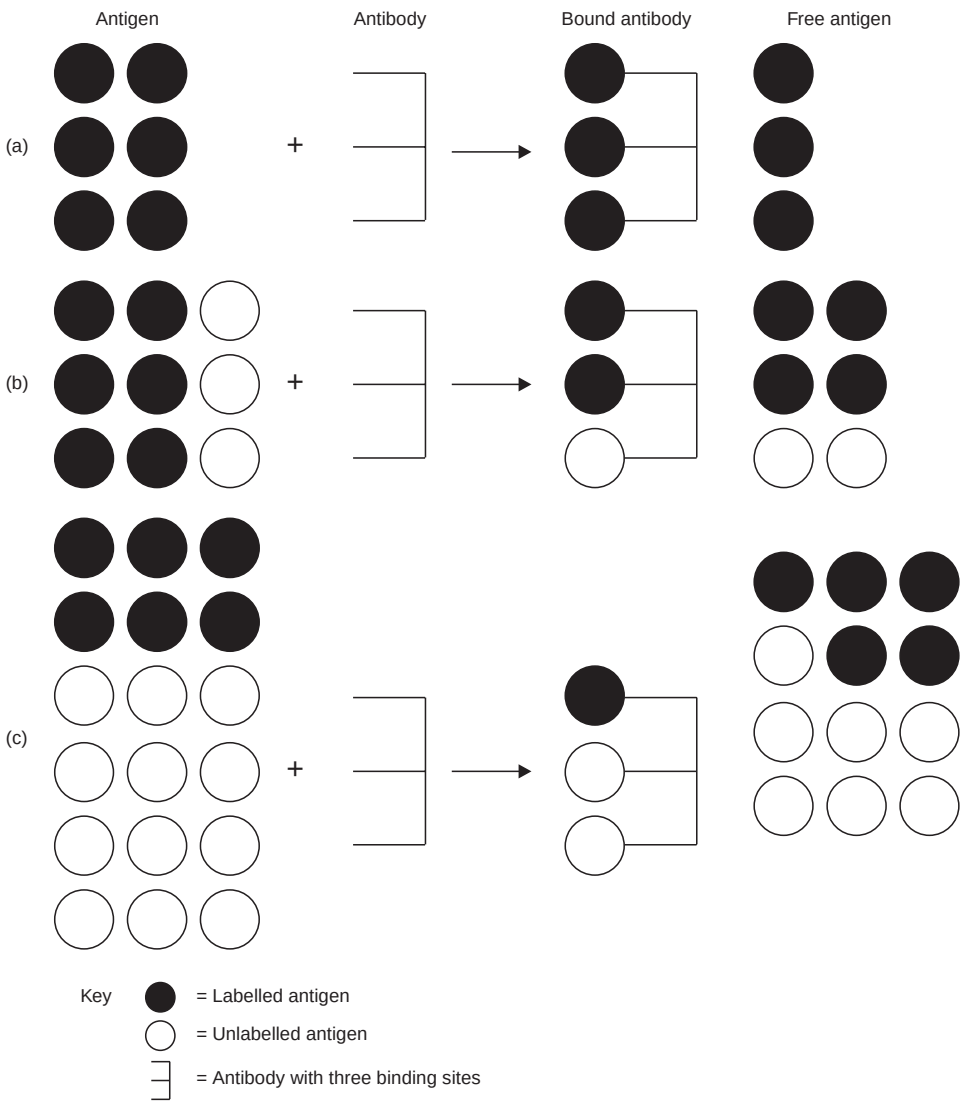


Figure 32.1 : Schematic Illustration of Competitive Binding in Ideal Radioimmunoassay.

An ideal behaviour has been assumed in Figure 32.1, whereby most radioimmunoassay very closely approach this condition. In order to fulfill the requirements of an ideal behaviour the following criteria must be accomplished, namely :

(*i*) The non-radioactive antigen (A) and radioactive antigen (A*) are indistinguishable chemically *i.e.*, both of them are identical chemically,

(*ii*) The two reactions ultimately go to completion *i.e.*, the equilibrium constants of the binding of labelled and unlabelled antigen to antibody are not only equal but also are so huge in number that they may be regarded as infinite,

(*iii*) The antigen and antibody usually react in the ratio 1 : 1, and

(*iv*) There are no cross reactions observed in the medium *i.e.*, the antibody being specific only for the single antigen indicated in the reaction or being determined.

The main objective of RIA is to determine the concentration 'C' of a non-radioactive antigen (unlabelled). Hence, in order to conduct RIA-a standard curve first to be made where 'C', concentration of non-labelled antigen in standard solution, is plotted as a function of radioactivity. It is usually accomplished by saturating the antibody binding sites with radioactive or labelled antigen, adding known concentration of the non-radioactive (hapten) antigen, in standard solution, to the reaction mixture for the unlabelled antigen from its binding site on the antibody. It is a normal practice, to measure radioactivity with each known unlabelled antigen added (concentration) which is plotted along the X-axis against the radioactivity Y-axis. This is also known as the **'close-response curve'**.

If a radioactive-labelled form of a substrate (A*) is added to a plasma containing unlabelled-substrate (A) and a limited amount of its specific binding antibody (P), then assuming a dynamic equilibrium exists between (A) and (P), (A*) shall distribute itself evenly among the unlabelled substrate (A). If the binding affinity between (A) and (P) is very high, virtually all the (A*) added will be found until (P) is saturated and at equilibrium. Thus, we have :

$$\frac{(A - P + A^* - P)}{\text{Total } (A + A^*)} \text{ or } \frac{A^* - P}{\text{Total } A^*} \text{ and } \frac{A - P}{\text{Total } A}$$

where, (A* – P) = Antibody labelled antigen-complex, and

= (A – P) = antibody unlabelled antigen-complex.

At this juncture, if further (A) is added, it will also compete for the same binding site so that (A* – P) shall be reduced. Still further additions of (A) will cause the (A* – P) concentration to be reduced further.

Under these prevailing circumstances the reduction in (A* – P) complex concentration taking place may be predicted as follows :

Assuming that P (antibody) has 200 binding sites available and at the initial stage only 20 molecules of (A) is present, sufficient (A*) is added so as to saturate P *i.e.*, 180 molecules of (A*). Therefore, virtually all are bound so that :

$$\frac{(A - P + A^* - P)}{\text{Total } (A + A^*)} \times \frac{100}{1} = 99 \text{ to } 100\%$$

If, then 100 molecules of A are added, there is a total of 300 molecules of (A* + A) competing for 200 binding sites on the antibody (P). Now, when an equilibrium is established, the percentage bound is given by the expression, :

$$\frac{(A - P + A^* - P)}{\text{Total } (A + A^*)} \times \frac{100}{1} = \frac{200}{300} \times \frac{100}{1} = \frac{A^* - P}{\text{Total } A^*} \times \frac{100}{1}$$

or

$$= \frac{120}{80} \times \frac{100}{1} = 66.6\%$$

If a further 100 molecules of A are added at this stage, the percentage bound shall become :

$$\frac{200}{400} \times \frac{100}{1} = \frac{90}{180} \times \frac{100}{1} = 50\%$$

Thus, continuing with further additions of (A), each of 100 molecules at a time will ultimately give rise to two typical RIA-Standard Curves as depicted in Figure 32.2 and Figure 32.3 respectively.

Form Figure 32.2, it is quite evident that the percentage of radioactive compound bound A* decreases with the continual addition of unlabelled compound A.

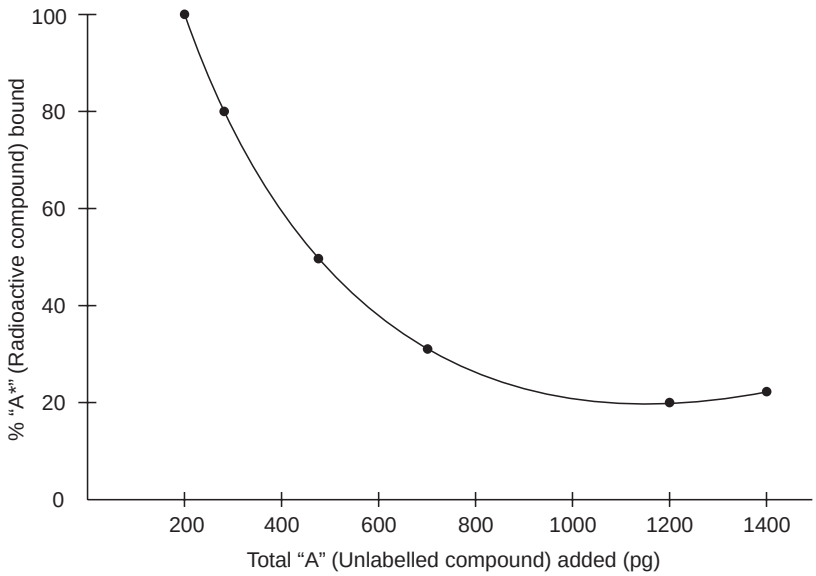


Figure 32.2 : A Typical RIA Standard Curve.

Figure 32.3, depicts the plotting of the percentage inhibition of labelled compound binding A* against the continual addition of unlabelled compound A thereby giving rise to a straight line.

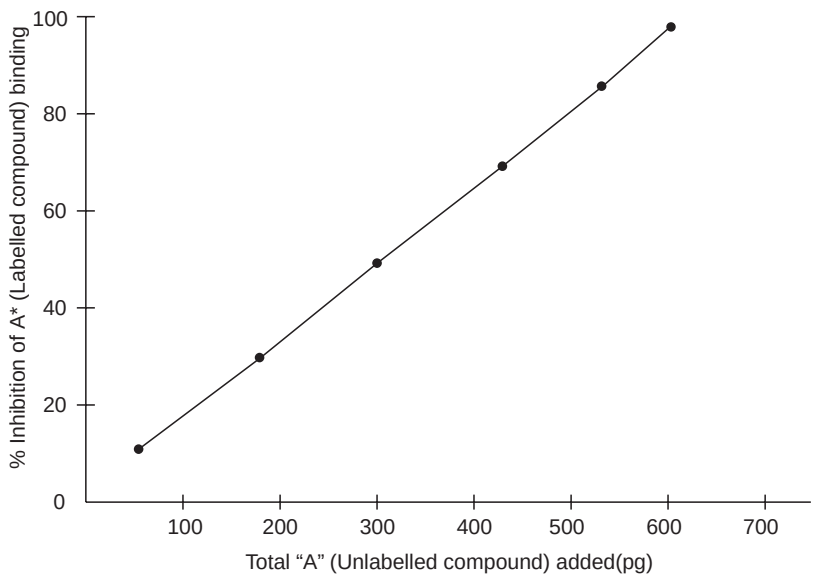


Figure 32.3 : A Typical RIA Standard Curve.

The following important points may be observed :

(*a*) In place of pure unlabelled A, a sample of plasma from which all the antibody P has been removed duly, and which contains an unknown amount of A, is added to the same system, it may be quantitated as per the respective observed fall in A* – P complex concentration that it causes ultimately,

(*b*) It is pertinent to mention here that the validity of radioimmunoassay procedure solely depends upon the identical behaviour of standards as well as unknowns (*i.e.,* unlabelled antigenic substance in unknown sample being assayed). However, this particular condition may be tested and verified by making multiple dilutions of an unknown sample and subsequently determining whether the curve of competitive inhibition of binding is superimposable on the standard curve employed for the respective assay. Failure to fulfill this condition precludes a truly quantitative estimation[+], and

(*c*) A crude hormone preparation is found to be satisfactory enough both for immunization and for use as a standard, but for the purpose of comparison of values collected from various laboratories, a generally available reference preparation must be used as a standard solution.

32.3. INSTRUMENTATION

The *two* most vital equipments essentially required for radioimmunoassay (RIA) are, namely :

(*i*) Centrifuge, and

(*ii*) Radioactive Counters.

These two equipments shall now be discussed briefly as follows :

32.3.1. CENTRIFUGE

A centrifuge which is capable of generating 1200-2500 rpm using swing-bucket-rotor or 3500 to 4000 rpm using a fixed-angle-head rotor can be employed effectively. However, the former type is preferred because of the fact that here the pellet is formed at the bottom of the test tube and the supernatant layer is more easily removed in comparison to the latter type where the pellet is formed at an angle. In case, a centrifuge having relatively less gravitational force is employed then it is absolutely necessary to enhance the centrifugation time until suitable pellets are formed duly.

32.3.2. RADIOACTIVE COUNTERS

In usual practice, *two* types of radioactive counters are mainly employed depending on the type of radioactive substance used, namely :

(*a*) Gamma Counters, and

(*b*) Scintillation Counters.

32.3.2.1. Gamma Counters

These are used invariably for the **gamma-energy emitting isotopes**, for instance : ^{125}I-the more common iodine-isotope.

32.3.2.2. Scintillation Counters

These are mostly used for counting **beta-energy-emitting isotopes**, such as : tritium ^{3}H and ^{14}C-(Carbon-14) isotopes.

First and foremost, radioimmunoassays were universally based on the ^{3}H or ^{14}C isotope labelling technique, but this has the main disadvantage of using liquid-scintillation counting. Therefore, the comparatively much simpler technique of gamma-ray counting by labelling compounds with ^{124}I, ^{125}I, or ^{131}I is now being increasingly utilized wherever such labelling is practically feasible.

+ Yellow, RS, SM Glick, J. Roth and SA Barson, Radioimmunoassay of human plasma ACTH., **J. Clin, Endocrinol., 24,** 1219, 1974.

32.4. METHODOLOGY OF THE ASSAY

The methodology of the radioimmunoassay have been studied extensively and outlined in a sequential manner as follows :

(1) Mix a fixed volume (fixed concentration) of antiserum containing the specific antibody with a constant amount of radiolabelled antigen,

(2) Incubate it for come specified duration at an appropriate temperature, usually + 4 °C,

(3) A definite volume of the sample containing the hapten to be measured is added to the reaction test-tube,

(4) The antibody reacts with both the radioactive and unlabelled hapten forming an antibody-radiolabelled antigen and antibody-unlabelled antigen complexes,

(5) Since, both the radioactive and non-radioactive antigens (haptens) are more or less chemically and immunochemically the same, they will eventually compete for the limited number of antibody sites available ; thus, the amount of radioactivity that ultimately combines with the antibody will be an inverse function of the amount of unlabelled hapten competing for these sites,

(6) The radioactivity falls because the unlabelled antigen dilutes it *i.e.*, reducing the number of labelled hapten combining with the antibody,

(7) The counts obtained from the radioactivity are used to determine the hapten concentration in the sample, the interpretation being done on the standard curve, and

(8) RIA is an exquisitely sensitive assay method that is capable of measuring with great accuracy (hapten) concentrations in nanograms and picograms utilizing very small volumes of the sample.

Note :

(*i*) *In order to measure the radioactivity in the labelled hapten-antibody complex of the free hapten* (*labelled*) *a convenient means of separating these fractions is usually adopted,*

(*ii*) *The method of assaying the radioactivity of the bound and/or unbound fraction following separation, solely depends on the nature of the isotope and on the method utilized for the separation of the bound and unbound fractions,*

(*iii*) *Thus, one may actually determine either the antibody bound fraction or the unbound fraction routinely, but in the preliminary experiments it is always necessary to determine both these fractions, and compare them with a standard containing the total number of counts added in order to make sure that there are no losses unaccounted for,*

(*iv*) *The validity of RIA entirely depends upon the identical behaviour of standard and labelled substance unknown, and not on the identity of the labelled tracer and the unknown. Hence, the experimental conditions of incubation of standards and unknowns must be identical for any factors that might affect the extent of the immunochemical reaction, pH, ionic composition, protein content or any other substances of interest. However, these conditions may be tested conveniently and can be controlled effectively by preparing standards in hormone free plasma at the same dilution at which unknowns are assayed.*

32.5. APPLICATIONS OF RADIOIMMUNOASSAY (RIA) IN PHARMACEUTICAL ANALYSIS

The scope of applicability of radioimmunoassay is rapidly expanding with the dawn of each day as RIA is being developed for newer pharmaceutical substances. It has attained wide recognition and application both *in vitro* and *in vivo* measurements of compounds of interest like insulin, gastrin, glucagon, and growth hormones on one hand ; whereas drugs like :

Morphine	—	Narcotic analgesic,
Hydromorphone and Hydrocodone	—	Narcotic analgesic, antitussive and antipyretic,

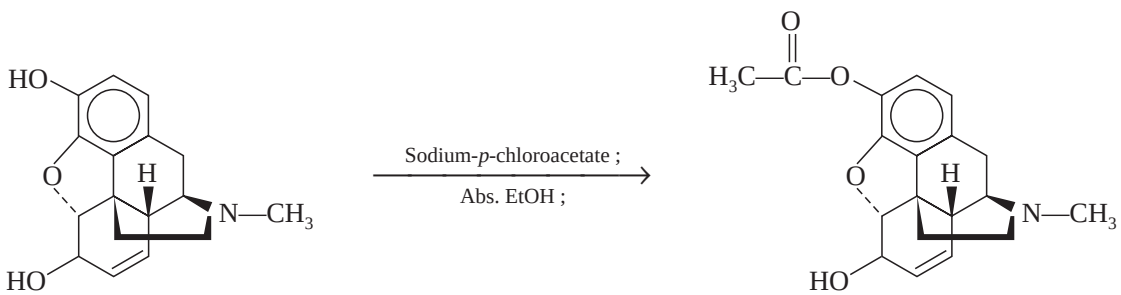
on the other hand.

Clonazepam	—	Sedative and anticonvulsant,
Flurazepam	—	Hypnotic and anticonvulsant,
Chlordiazepoxide	—	Sedative
Barbiturates	—	Hypnotic and anticonvulsant,
Flunisolide	—	A steroid having marked anti-inflammatory activity,
Neobentine	—	A novel antidysrhythmic and antifibrillatory agent,
Carteolol	—	B_1-Adrenoreceptor blocker used in hypertension and arrthmias,

RIA of some of these drugs will be discussed in the sections that follows :

32.5.1. RADIOIMMUNOASSAY OF MORPHINE

Synthesis of Immunogen : Morphine is first converted to 3-*o*-carboxymethyl-morphine by reacting the free base with sodium-*p*-chloroacetate in absolute ethanol :



Morphine
(I)

3-*o*-Carboxymethyl-morphine
(m.p. 292-293°C)
(II)

The product (II) is coupled to bovine-serum albumin by dissolving the former in distilled water containing the latter, maintaining the pH of the resulting mixture to 5.5 and 1-ethyl-3-(3-dimethyl-aminopropyl) carbidiimide was added. The mixture is incubated overnight at room temperature and then dialyzed against distilled water to cause purification. The resulting purified product carboxy-methyl-bovine-serum conjugate is then labelled with tritium.

Antiserum Production : The immunogen, carboxymethylmorphine-bovine-serum-albumin, is emulsified with equal volume of complete Freund's adjuvant*. Initial immunization doses are injected into the New Zealand albino rabbits and later on this followed up with booster injections after a period of 6 weeks. The antiserum titer is determined with each booster dose injection and is duly harvested when the titre value is maximum. This is diluted suitably and employed in the radioimmunoassay**.

RIA-Procedure : The various steps followed are as stated below, namely :

(1) Various dilutions of antiserums are incubated in the presence of fixed concentration of (^{3}H)-dihydromorphine, and after incubation, a neutral saturated ammonium sulphate solution is added to all the tubes,

(2) The complete precipitate, sedimented by centrifugation at 5000 rpm is washed twice with 50% ammonium sulphate solution,

(3) The washed-precipitate, containing antibody-bound morphine, is dissolved in NCS-solubilizer, and the radioactivity is counted with the help of a Packard-Iri-card Liquid Scintillation Spectrometer,

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* **Adjuvant—**An oily substance that will form an emulsion aqueous solutions/suspensions of immunogen to enhance the antibody response. Complete adjuvant also contains *Tubercele bacilli.*

** Spector, S. and CW Parker, **Science, 168,** 1347, 1970.

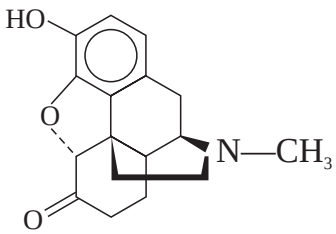
(4) The tube which contained radioactive dihydromorphine and antiserum but no unlabelled morphine, served as a measure of maximum antibody-bound radioactivity,

(5) The addition of increasing amount of unlabelled morphine to a fixed amount of (^{3}H) dihydromorphine and antiserum results a competitive inhibition of the labelled dihydromorphine for the formation of the antibody-hapten complex, and

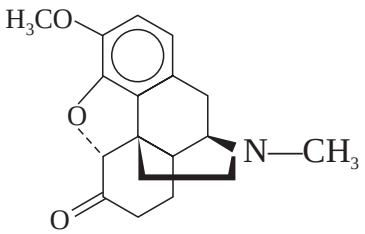
(6) The assay sensitivity limit is found to be 100 pg of unlabelled-morhine per tube that caused 20% binding inhibition of labelled-dihydromorphine, (see Figure 32.3).

32.5.2. RADIOIMMUNOASSAY OF HYDROMORPHONE AND HYDROCODONE IN HUMAN PLASMA

Hydromorphone (I) and **hydrocodone** (II) belong to the **morphine group of drugs** and are used invariably in combination with other ingredients in a number of proprietory antitussive and analgesic antipyretic mixtures. However, interest in the pharmacokinetics of hydromorphone and hydrocodone in human subjects required an adequate assay for drug levels in plasma.



Hydromorphone
(I)



Hydrocodone
(II)

RIA for hydromorphone*, ** and hydrocodone*** are fairly sensitive in the nanogram per millilitre range but essentially require the preparation of a specific antibody. The laid-out RIA method is quite capable of estimating the above drugs within a range of 2.5-20 ng ml^{-1} using standard 100 µl plasma sample only.

RAI is carried out using morphine-6-antiserum and tritiated dihydromorphine (commercially available). The free-drug is separated from bound drug using dextran coated charcoal and an aliquot of the supenate containing the antiserum-bound-drug is subsequently counted for radioactivity. However, the radioactivity measurements are normally ascertained in a Liquid Scintillation Counter provided with 20-ml glass scintillation vials.

Materials Required

(*i*) **Lyophilized morphine-6-antiserum :** It is diluted 1 : 20 with phosphate buffer prior to use,

(*ii*) **^{3}H-Dihydromorphine Solution :** It is prepared by diluting 2 µl of the radiolabelled compound in ethanol to 10 ml with phosphate buffer so that each 0.1 ml of solution contained 83 pg (0.5 mole),

(*iii*) **Dextran-coated chrocoal suspension :** It is prepared by mixing 2.5 g of charcoal in 100 ml of distilled water with 2.5 g of dextran in 100 ml of distilled water, and eliminating the fine particles by centrifugation, and

(*iv*) **Preparation of Saturated Solutions :** Individual stock solutions containing the equivalent of 200 µg of I or II base line are prepared using weighed quantities of the respective powders dissolved in distilled water. Dilutions of the drugs are made in individual 10 ml volumetric flasks to yield drug concentrations of 2.5, 5.0, 10.0 and 20 ng ml^{-1} for I and 10.0, 20.0, 40.0 and 80.0 ng ml^{-1} for II. The dilutions are made using blank plasma and phosphate buffer solutions.

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* Wainer, BH, EW Fitch, J. Friend and R.M. Rothberg, **Clin. Immunol. Immunopathol, 3,** 155, 1974.

** Findlay, WJA, EC Jones and RM Welch, **Drug Metab, Dispos., 7,** 310, 1979

*** Findlay, WJA, JT Warren, JA Hill and R.M. Welch, **J. Pharma Sci 70,** 642, 1981

RIA-Procedure : The different steps to be followed are stated below, namely :

(1) Various dilutions of unknown plasma, morphine-6-antiserum, ^{3}H-dihydromorphone are prepared afresh,

(2) The unknown plasma (0.1 ml) is incubated directly with morphine-6-antiserum (0.1 ml) and buffer (0.3 ml) for a duration of 50 minutes at room temperature (20 ± 2 °C) and immediately followed by 10 minutes at 4°C,

(3) The ice-cold dextran-coated-charcoal suspension (0.1 ml) is added to all the above tubes, followed by immediate mixing and incubation for 10 minutes at 4°C,

(4) All the tubes are then centrifuged for a period of 15 minutes at 3000 rpm,

(5) A small portion (0.2 ml) of the supernate is removed and placed in a scintillation vial containing 0.5 ml of distilled water and 5 ml of scintilation fluid,

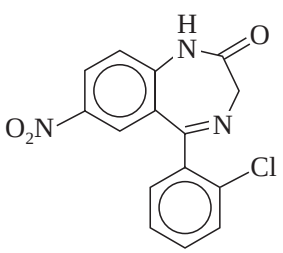
(6) The contents of the scintillation vial are mixed thoroughly, and the radioactivity is measured in a Liquid Scintillation Counter for 10 minutes,

(7) Duplicate hydromorphone 2.5, 5.0, 10.0 and 20.0 ng ml^{-1} or hydrocodone 10.0, 20.0, 40.0, and 80.0 μg ml^{-1} standards are accurately assayed concurrently and the data is plotted in a graph, and

(8) The regression equation, calculated from the standard solutions in each collection, is employed to determine quantitatively the drug concentration present in individual plasma samples.

32.5.3. RADIOIMMUNOASSAY OF CLONAZEPAM

Colonazepam belongs to the class of **1, 4-benzodiazepines** that has been found to be therapeutically effective in controlling minor motor seizures *i.e.,* petitmal epilepsy in humans*



Clonazepam

Synthesis of Immunogen : The 3-hemisuccinyloxy derivative of clonazepam is covalently coupled to bovine serum albumin employing the mixed-anhydride method suggested by Erlanger and coworkers** (1959). After successive dialysis against dioxane-water borate buffer and water, the immunogen *i.e.,* clonazepam-bovine-serum-albumin conjugate is isolated by lyophilization.

Preparation of ^{3}H-Labelled Clonazepam : ^{3}H-Clonazepam is prepared by tritium exchange employing dimethyl formamide-titrated water having a specific activity*** of 100 ci g^{-1}. The resulting product is subsequently purified by silica-gel-column-chromatography, thereby yielding a material which has a specific activity of 4.32 mci mg^{-1}. This specific method of introducing ^{3}H (tritium) probably provided exchange chiefly at C-3 position****.

Antibody Production : A thick emulsion of the immunogen (clonazepam-bovine-serum-albumin-conjugate) is prepared employing complete Freund's adjuvant and two New Zealand white female rabbits are immunized intradermally at multiple sites with the immunogen emulsion. The animals are then administered

* Kruse, R., **Epilepsia, 12,** 287, 1971

** Erlanger, BF, R. Borker, S.M. Beiser, and S. Liebermann, **J. Boil. Chem, 234,** 1090, 1959.

*** **Specific activity**-is a unit that expresses the amount of radioactivity as a function of the mass of the material.

**** Dixan, W.R., K.E. Fahrenholtz, W. Burger and C. Perry, **Res. Commun. Chem. Pathol. Pharmacol. 16,** 121, 1977.

with booster doses intravenously with immunogen emulsion at monthly intervals, and serum is harvested 10-14 days after each administration. Both rabbits produced satisfactory titers of antibodies to clonazepam within a period of three months following the initial immunization. The resulting serum is pooled, diluted suitably and employed in the radioimmunoassay.

RIA-Procedure : The various steps involved in the RIA-procedure for clonazepam are enumerated below, namely :

(1) A constant small (volume) portion of the control plasma is added to constant small (volume) portion of standard clonazepam in small test-tubes to generate a calibration (standard) curve,

(2) Appropriate controls are included by adding the control plasma to small portion of buffer solutions,

(3) Each unknown plasma sample is added to tubes containing buffer solution then the titrated (^{3}H)-colnazepam solution followed by diluted antiserum is added,

(4) The contents of each tube are mixed thoroughly and allowed to stand at room temperature for sometime,

(5) Saturated ammonium sulphate solution is added to precipitate the globulin-bound-^{3}H- clonazepam and after mixing, the tubes are allowed to stand for 15 minutes at 0 °C,

(6) The tubes are subsequently centrifuged at 3000 rpm,

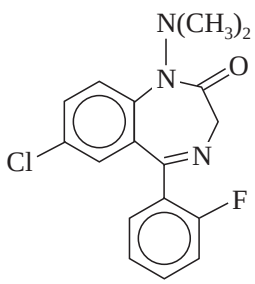
(7) Each supernate, containing the unbound ^{3}H-clonazepam, is decanted into a scintillation vial and toluene is added,

(8) The samples are assayed for ^{3}H-activity in a liquid scintillation counter, and

(9) All samples including the standards, unknowns and controls are assayed in duplicate and the average of the ^{3}H-counts is employed for the percentage of binding.

32.5.4. RADIOIMMUNOASSAY OF FLURAZEPAM IN HUMAN PLASMA

Flurazepam belongs to the class of **hypnotic agent** used for the treatment of insomnia.



Flurazepam

Synthesis of Immunogen (Hapten) : 3-Hydroxy flurazepam is refluxed with succinic anhydride in dichloromethane containing triethylamine to produce the desired hapten, 3-hemisuccinyloxy-flurazepam. It is coupled covalently to bovine-serum-albumin by the mixed-anhydride procedure developed by Erlanger *et al* (1959). The resulting conjugate is purified by dialysis against sodium bicarbonate solution followed by dialysis against distilled water and finally isolated by lyophilization.

Immunization and Antibody Production : The immunogen 3-hemisuccinyloxyflurazepam, is emulsified with complete Freund's adjuvant. It is injected intradermally into two female New Zealand albino (white) rabbits. Repeated doses are administered twice at interval of two weeks. Subsequently, booster injections of the thick-immunogen-emulsion-paste are administered after a span of 6-weeks. The antibody is harvested when its titer level is high enough, diluted to the suitable-level and employed in the RIA.

RIA-Procedure : The different steps followed in the RIA-procedure are as given below :

(1) A calibration curve is generated by adding ^{3}H-Flurazepam in 0.1 ml of buffer containing 0.03-0.2 ng range of flurazepam in buffer,

(2) Following preparation of the standards, duplicate portions of the reconstituted unknown flurazepam fractions are added to tubes containing ^{3}H-Flurazepam,

(3) Diluted antiserum is added to all tubes except the non-specific-binding control specimen to which buffer is added,

(4) The contents of each tube is mixed gently on a Vortex Mixer and allowed to stand at room temperature,

(5) Following incubation, the antibody-bound radio ligand is separated from the unbound fraction by precipitation with saturated ammonium sulphate,

(6) After the pellet is dissolved in water add 3 ml of scintillation fluid to produce a clear solution, and

(7) The radioactivity in each tube is quantified in a modified scintillation liquid counter.

RIA-Specificity* : The specificity of the antiserum initially is evaluated by cross-reactivity** studies involving all the **flurazepam metabolites** known to be present in plasma. The mono-as well as di-desethylmetabolites exhibited a cross-reactivity of 17 and 3.7% respectively, while other possible competitors cross-reacted less than 1% as shown in Table 32.1.

Table 32.1 : Flurazepam and Hapten 3-Hemisiccinyloxyl-flurazepam *vis-a-vis* the Cross-Reactivity of Metabolites Present in Plasma :

S.No.	Compound	Cross-Reactivity (%)
1.	Flurazepam	100
2.	3-Hemisuccinyloxyflurazepam	—
3.	3-Hydroxyflurazepam	—
4.	Monodesethylflurazepam	17.0
5.	Didesethylflurazepam	3.5
6.	N-1-Hydroxyethylflurazepam	1.0
7.	N-1-Desalkylflurazepam	1.0

Evidently, due to the cross-reactivity of both mono- and di-desethyl metabolites, a specific assay of flurazepam could not be developed successfully without first separating it from its metabolites effectively by the help of column chromatography.

32.5.5. RADIOIMMUNOASSAY OF CHLORDIAZEPOXIDE IN PLASMA

Chlordiazepoxide is the pioneer member of the **1, 4-benzodiazepines** to be employed clinically as an antianxiety agent in humans***. A number of methods based on extraction processes are available for the assay of this drug, namely : *spectrofluorometry*, *polarography* and *electron-capture GC-technique* ; but RIA measures it directly in the blood without involving extraction and possesses very low sensitivity.

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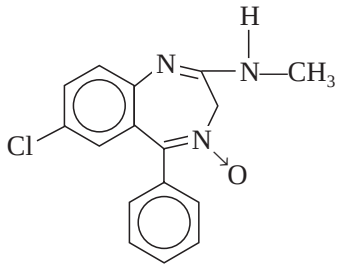
* **Specificity :** The degree of freedom from interference by substances other than the antigen.

** **Cross-reactivity :** The amount of a similar substance that will cause the same displacement of labelled antigen from the antibody as an arbitrary amount of the antigen. The usual definition is ID_{50}, which is the concentration of cross-reacting material required to displace 50% of labelled hapten from the antibody.

*** Harris, TH, **J. Amer. Med. Asso., 172,** 1162, 1960.



Chlordiazepoxide

Synthesis of Immunogen : Chlordiazepoxide as suspension in N-methylformamide is treated with HCl in dioxane to yield a pale-yellow solution. The resulting mixture is cooled to -30 °C and isoamyl nitrite in dioxane is added. The solution is stirred at – 30° to – 40 °C and aqueous ammonium sulfamate is added with continuous stirring.

The chilled azide solution is added slowly, dropwise with constant vigorous stirring into a solution of bovine-serum albumin. The pH is maintained at 8.0 to 8.7 by the careful addition of NaOH solution. The resulting pale-yellow solution is kept at 4°C for a duration of 36 hours and then dialysed against trimethamine buffer. After further dialysis for two days against distilled water, the immunogen is isolated by lyophilization.

Immunization and Antibody Production : The lypphilized immunogen obtained above is dissolved in normal saline and emulsified with equal volumes of complete Freund's adjuvant into a thick paste. Three New Zealand albino rabbits are immunized with the immunogen-paste through intradermal injections. The process is repeated twice at 2-weeks intervals followed by booster doses at monthly intervals. The antiserum is harvested when the plasma titer value is attained maximum.

RIA-Procedure : The various steps involved in the RIA procedure are enumerated below :

(1) A constant volume of control human plasma is added to a constant volume of each standard of chlordiazepoxide to produce a calibration curve of 2 to 100 ng per tube,

(2) The same volume of the unknown plasma samples is added to tubes containing constant volume of the solution of the labelled chlordiazepoxide and constant volume of the antiserum solution is now added to all the tubes,

(3) The volumes in all the tubes are made upto 1 ml with buffer solution, mixed thoroughly on a Vortex Mixer, and each tube is immersed in an ice-water bath,

(4) An equal volume of saturated ammonium sulphate solution is added to enable complete precipitation of globulin-bound chlordiazepoxide ^{14}C,

(5) After mixing the contents of the tubes thoroughly on a Vortex Mixer and allowing them to stand for a while at 4°C, the tubes are centrifuged at 3000 rpm,

(6) The supernate thus obtained containing unbound chlordiazepoxide-^{14}C is decanted into a counting vial and toluene is added, and

(7) The radioactivity in the supernate and that in the precipitate are separately counted in a scintillation counter.

Specificity of Antibody binding of Chlordiazepoxide : A good number of benzodiazepines are tested for their ability to complete with labelled chlordiazepoxide for the respective antibody binding site. The various competitors are adequately tested at a concentration of 200 ng *i.e..*,10-times the concentration of chlordiazepoxide required to produce a 50% inhibition of binding as shown in Table 32.2.

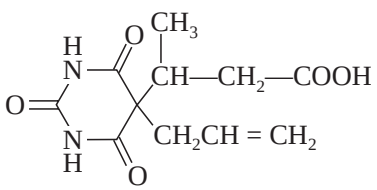
Table 32.2 : Specificity of Antiserum for Chlordiazepoxide*

S.No.	Compound	Cross-Reactivity (%)
1.	Chlordiazepoxide	100
2.	N-Desmethylchloriazepoxide	5
3.	Demoxepam	<1
4.	N-Desmethyldiazepam	<1
5.	Diazepam	<1
6.	Clonazepam	<1

From table 32.2 it is evident that the highest cross-reaction is 5% with N-desmethylchlordiazepoxide while demoxepam, N-desmethyldiazepam, diazepam and clonazepam displayed less than 1% inhibition. However, the RIA method appears to be reliable over a range of 2-100 ng per tube of chlordiazepoxide and, therefore, the sensitivity limit stands at 20 ng ml^{-1} using a 1.0 ml sample of plasma.

32.5.6. RADIOIMMUNOASSAY OF BARBITURATES

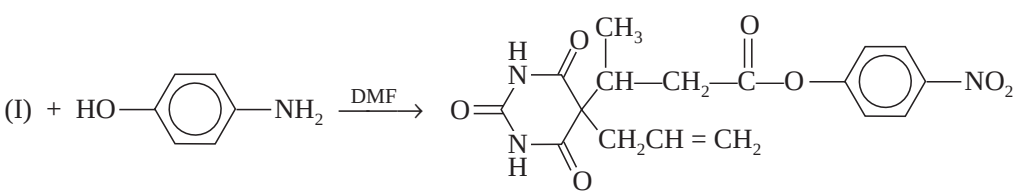
Barbiturates represent a class of **sedative and hypnotic drugs** employed extensively in medicine. RIA provides a rapid, sensitive specific and reliable means for their determination in plasma levels upto 5 ng without indulging in any type of extraction, filtration or evaporation as required for other conventional analytical methods**.



(I)

5-Allyl-5-(1-carboxyisopropyl) barbituric acid.

Synthesis of Immunogen (Hapten) : The barbiturate, 5-allyl-5-(1-carboxyisopropyl) barbituric acid (I) is first converted to 5-allyl-5-(1-*p*-nitrophenyloxycarbonylisopropyl) barbituric acid (II) by the interaction of the base with *p*-nitrophenol in N, N-dimethylformamide (DMF) as shown below :



p-Nitrophenol (II)

The resulting product (II) is subsequently coupled to bovine-serum-albumin in a glycerol-water mixture in the presence of dicyclohexylcarbodiimide. The mixture is incubated overnight at 4°C, and the protein-hapten complex is dialysed against distilled water thereby causing its purification. Conjugation of the respective barbiturate to the protein carrier, comparison of the barbiturate BGG-conjugate to control BGG-solution and preparation of ^{14}C-pentobarbital sodium are carried out respectively.

Preparation of Antiserum : The barbiturate-bovine-serum-albumin conjugate is duly emulsified with an equal volume of complete Freund's adjuvant and New Zealand albino rabbits are subsequently im-

* Dixon, WR, J. Earley and E. Postma, **J. Pharm. Sci., 64,** 937, 1975.

** Utiger, RD, **J. Clin. Invest., 44,** 1277, 1965.

munized with this particular emulsion. Six weeks after the initial does, booster doses are administered to the animals in each of their foot pads. Blood samples are collected 5-7 days after the booster injections and the serum is examined for antibodies to barbiturates. The antiserum is harvested when the serum antibody titer has attained its maximum level.

It has been observed that while normal, rabbit serum failed to bind labelled phenobarbital, the serum from immunized rabbits bound 75 to 80% of the added pentobarbital and there exists a linear relationship between ^{14}C-phenobarbital and the concentration of added antibody. Besides, when variable quantities of ^{14}C-pentobarbital are added to a constant quantity of antibody, there exists a linear relationship between added and bound ^{14}C-phenobarbital as depicted in Figure 32.4.

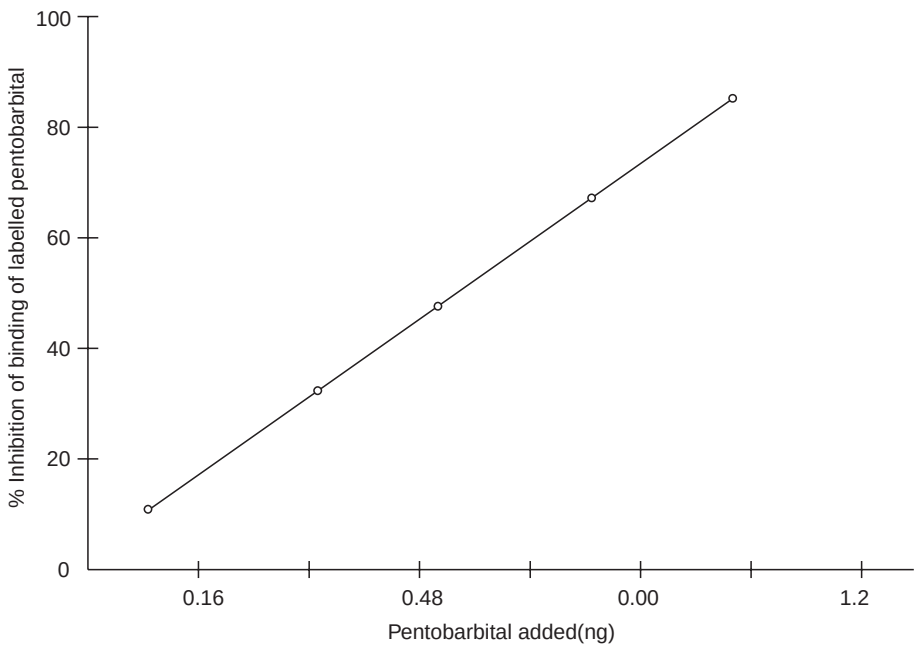
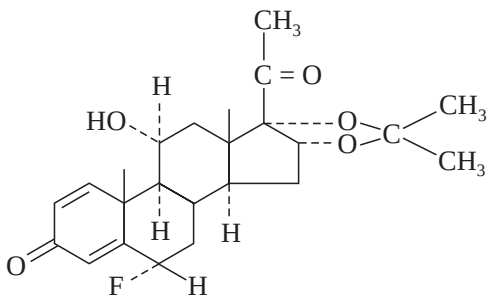


Figure 32.4 : Percentage Inhibition of Labelled Phenobarbital Binding by Added Unlabelled phenobarbital.

32.5.7. RADIOIMMUNOASSAY OF FLUNISOLIDE IN HUMAN PLASMA

Flunisolide is a fast-acting corticoid designed for the treatment of allergic rhinitis, asthma, and other allied respiratory disorders in humans*. As the quantum of drug delivered by inhalation (*i.e.*, the usual route of administration of the drug), is invariably small, the plasma-levels attained can also be fairly small. Hence, there is a dire need for a sensitive method of plasma concentration evaluation which is satisfied by radioimmunoassay.



Flunisolide

* Turkeltaub, PC, PS Norman, and S. Crepea, **J. Allergy Clin. Immunol., 55,** 120, 1975
Lowel, FC, JL Ohman. and M. Williams, **J. Allergy Clin. Immunol., 57,** 257, 1876.

Synthesis of Hapten Immunogen and Antiserum Production : The hapten, flunisolide-bovine-serum-albumin conjugate is prepared by coupling the 21-hemisuccinate of flunisolide to bovine-serum-albumin with a water-soluble carbodiimide coupling reagent*. The reaction mixture is dialysed exhaustively against normal saline to cause purification and the extent of conjugation is estimated by measuring the protein concentration**. However, the flunisolide residues are determined by UV-absorption method.

An emulsion of the hapten (*i.e.*, conjugate) in normal saline is prepared by mixing with an equal volume of Freund's complete adjuvant. The prepared emulsion is injected subcutaneously into four different sites in New Zealand albino rabbits. Six weeks after the initial injection, all the animals are placed on a regimen of weekly booster shots. After a period of six months, antiserum from these animals are harvested and dilutions of 1 : 10,000 to 1 : 30,000 produced 50% binding or more and is employed in the RIA.

RIA-Procedure : The following steps are to be adopted in a sequential manner, namely :

(1) Flunisolide standards required for the preparation of the standard curve are obtained by dilution of a stock solution of 10 mg of it in 10 ml of ethanol,

(2) A series of standard solution *viz*., 20, 50, 100, 200, 300, 500 and 600 pg per 0.1 ml in tris-(hydroxymethyl)-aminomethane/hydrochloric acid buffer and stored duly at 0 °C temperature,

(3) An ethanolic solution of ^{3}H-Flunisolide is diluted with tris-(hydroxymethyl)-aminomethane/hydrochloric acid buffer and 0.1% gelatin such that 0.1 ml portion contains 8,000 to 10,000 cmp activity,

(4) The antiserums are diluted in the said buffer with 0.1% gelatin to give rise to a total binding of between 35-50%,

(5) The charcoal stock solution is diluted as and when required with the aforementioned buffer immediately before, use,

(6) RIA is conducted by mixing together various dilutions of antiserum, buffer solution, ^{3}H-Flunisolide and various dilutions of flunisolide standard solutions in a set of test tubes,

(7) A second set of test tubes containing various dilutions of antiserum, buffer solution, ^{3}H-Flunisolide and various dilutions of the plasma being analysed of flunisolide content are prepared separately,

(8) The two sets of test tubes are incubated at temperature of 0 °C after adding constant volume of charcoal suspension to each of the tubes and mixing them thoroughly on a Vortex Mixer,

(9) The incubation is done overnight,

(10) The tubes are then centrifuged at 2500 rpm for 4 minutes and immediately 0.5 ml of the supernate is transferred into scintillation vials, and

(11) The scintillation fluid is added and the solutions are counted for 10 minutes in Scintillation Counter***.

The percentage inhibition is calculated and the values obtained from the first set of tubes is used to plot a standard curve. The concentrations of flunisolide from the standard curve values from their calulated percentage inhibition value as depicted in figure 32.5 below :

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* Sheenhan, JC and JJ Hlarka, **J. Org. Chem., 21,** 439, 1956.

** Lowry, OH, JJ Rosenbrough, AL Parr, and R. Randall., **J. Biol. Chem., 193,** 265, 1951.

*** Nerenburg, C., and SB Matin., **J. Pharm. Sci., 70,** 900, 1981.

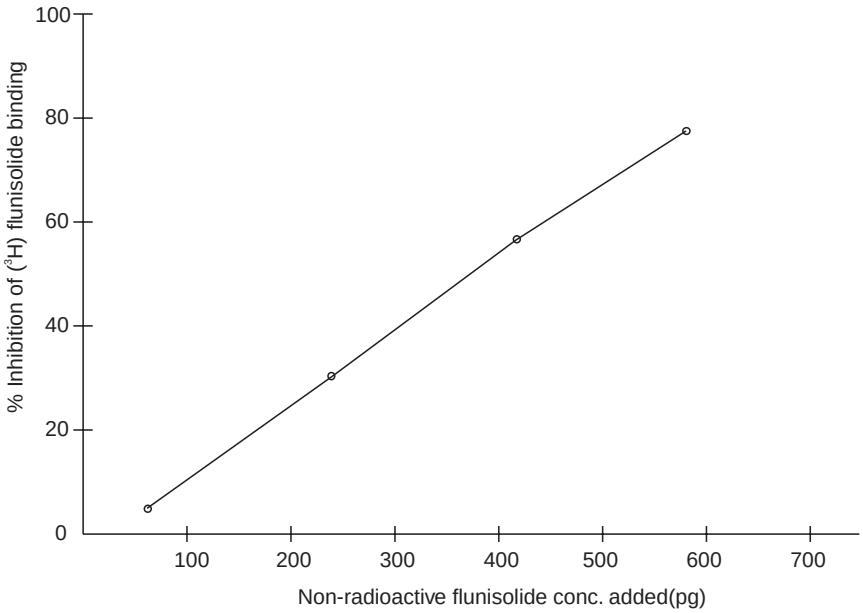


Figure 32.5 : Standard Curve (Displacement Curve) of Tritiated Flunisolide.

32.6. NOVEL APPLICATIONS OF RIA-TECHNIQUES

The radioimmunoassay technique has been gainfully exploited in a variety of novel applications of which only the *two* important aspects stated below will be discussed briefly, namely :

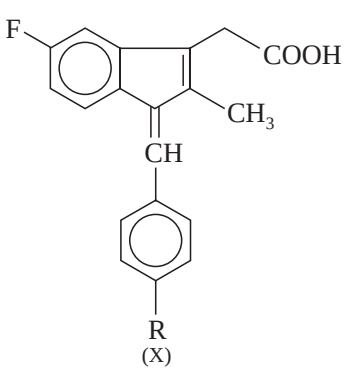
(*i*) Combined RIA Technique-Isotope Dilution, and

(*ii*) Stereospecificity.

32.6.1. COMBINED RIA-TECHNIQUES ISOTOPE DILUTION

In normal RIA-procedures the labelled drug or metabolite not only serves as the tracer for recovery but also for RIA quantification. However, the isotope dilution method categorically makes a clear separation of the drug and its metabolites. Consequently, a non-specific antiserum is employed to actually quantify the total amount of both unlabelled and labelled substance present.

The combined RIA-technique and isotope dilution has been successfully developed to estimate **SULINDAC*** along with its two prominent metabolites, namely : its sulphone and its sulphide present in the plasma-level as shown in the following chemical structures X and Y.

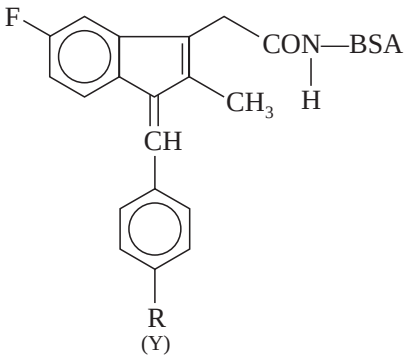


(X)

R	COMPOUND
$SOCH_3$	Sulindac
SCH_3	Sulindac Sulphide
SO_2CH_3	Sulindac Sulphone

[Sulindac and its metabolites]

* Hare, LE, CA Ditzler, M. Hichens, A. Rosegay and DE Duggan., **J. Pharma. Sci., 66,** 414, 1977.



R	COMPOUND
$SOCH_3$	Sulindac
SCH_3	Sulindac Sulphide
SO_2CH_3	Sulindac Sulphone

[Sulindac-Bovine-Serum-Albumin (BSA)]

CONJUGATE STRUCTURES

After due corrections have been incorporated with regard to recovery, it is possible to quantify the amount of standard sulindac or unknown ; besides standard metabolite or unknown.

Advantages : The various advantages are as follows :

(*i*) The methodology is not only very specific but also fairly sensitive, and

(*ii*) It serves as a substitute to simple RIA-procedure when specific antisera are not readily accessible.

Disadvantages : It has *two* main disadvantages :

(*i*) The method is time-consuming, and

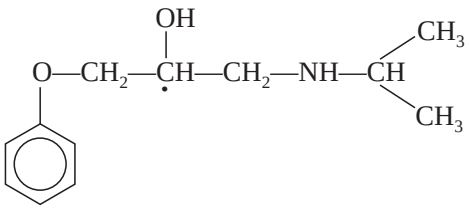
(*ii*) It involves tiresome and meticulous process of isotope dilution.

32.6.2. STEREOSPECIFICITY

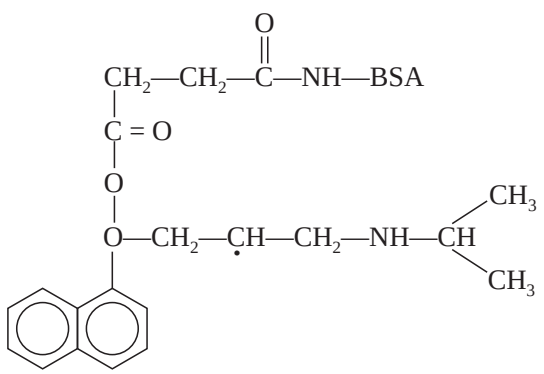
The **stereospecificity** of antigen-antibody reactions has gained its due recognition more than half-a-century ago*. However, an intensive and extensive stereospecificity radioimmunoassay procedures have been adequately applied to a number of pharmaceutical substances since mid-seventies, for instance : *atropine, propranolol, methadone*-to name a few.

Propranolol** which represents a comparatively better conceived example shall be discussed briefly as under with regard to its stereospecificity :

Propranolol is a recemic mixture *i.e.*, it contains an equimolecular portion of *d*- and *l*- isomers as given below :



Propranolol



Propranolol-Hemisuccinate-Bovine-Serum-Albumin (BSA) Conjugate

[• It shows the assymmetric carbon atom.]

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* Landsteiner, K., **'The Specificity of Serological Reactions',** Combridge, Harvard University Press, p. 168, 174, 1945.

** Kawashima, K., A. Levy and S. Spector., **J. Pharmaeol. Exp. Ther., 196,** 517, 1976.

Interestingly, only the *l*-propranolol exhibit β-blocking activity. In actual practice, two antisersa have been developed experimentally, namely :

(*a*) Antisera against the *dl*-racemic mixture, and

(*b*) Antisera against the *l*-isomer (active form only).

The *dl*-propranolol antiserum exhibits an almost equal affinity for both *d*- and *l*-isomers ; whilst the *l*-propranolol shows exclusively a marked and pronounced affinity for the *l*-isomer, By the application of these two RIA-techniques, it was practically feasible to quantify the plasma and heart concentrations of *dl*- and *l*-propranolol individually. Thus, the concentrations of *d*-propranolol could be arrived at by subtracting the concentration of *l*-isomer from the *dl*-mixture. It has been clearly demonstrated by Kawashima and coworkwers* that the *d*-propranolol undergoes distribution *in vivo* very sluggishly besides being metabolized more rapidly whereas the *l*-isomer gets distributed rather quickly to various tissues including the heart.

The RIA-procedure for propranolol is solely based on antisera derived from conjugates through the asymmetric carbon (*i.e.*, the optical carbon) as shown in the above chemical structures. Perhaps it could be possible that the stereospecificity of propranolol is caused due to the conformation of the drug-hapten in relation to the carrier protein to a great extent, through this hypothesis remains to be ascertained scientifically.

Consequently, the stabilization of the optical carbon by virtue of the conjugation to respective protein might improve upon the status of the specificity to a considerable extent. In order to prove the validity of this phenomenon one may carry out a definitive methodology whereby a closely monitored and controlled study of the antisera obtained by conjugates specifically prepared at the 'asymmetric-carbon' and at 'another-site' are both compared simultaneously under identical experimental parameters.

THEORETICAL AND PRACTICAL EXERCISES

1. What is Radioimmunoassay (RIA) ? Discuss its '*merits*' and '*demerits*' articulately.

2. Elaborate the theoretical aspects of RIA with suitable examples/explanations.

3. Discuss the various steps that are sequentially adopted in the **'Methodology of RIA'.**

4. How would you perform the assay of **'drugs'** by RIA as listed below ? Give structures, equations wherever applicable to strengthen your answer :

(*a*) Morphine,

(*b*) Hydromorphons and hydrocordone in Human Plasma,

(*c*) Clonazepam,

(*d*) Flurazepam in Human Plasma,

(*e*) Chlordiazepoxide in Plasma,

(*f*) Barbiturates, and

(*g*) Flunisolide in Human Plasma.

5. Give a brief account on the **'Novel Applications of RIA-Techniques'.** Support your answer with appropriate examples.

RECOMMENDED READINGS

1. Hayes, R., F. Goswitz and B. Murphy, Eds., '**Radioisotopes in Medicine : *In Vitro* Studies',** 1968.

2. Odell, WD and WH Daughaday, Eds., '**Principle of Competitive Proteinbinding Assays',** Philadelphia, Lippincott, 1971.

* Kawashima, K., A. Levy and S. Spector., **J. Pharmaeol. Exp. Ther., 196,** 517, 1976.

3. Davies, DS and BNC Prichard, Eds., '**Biological Effects of Drugs in Relation to their Plasma Concentration',** New York, McMillan, 1973.

4. '**Radioimmunoassay and Related Procedures in Medicine'** Vols 1 and 2., Vienna, International Atomic Energy Agency, 1974.

5. Croll, MN, LW Brady, T. Honda and RJ, Waliner., Eds., '**New Techniques in Tumour Localization and Radioimmunoassay',** New York, Wiley 1974.

6. Mule, SI, I. Sunshine, M. Braude and RE Willeta, Eds' '**Immunoassays for Drugs Subject to Abuse',** Cleveland Inc., Ohio CRC-Press, 1974.

7. Gennaro AR (ed.) : **Remington : The Science and Practice of Pharmacy**, 20th edn. Vol. I & II, Lippincott Williams & Wilkins, New York, 2004.

ATOMIC WEIGHTS

Element	Symbol	Atomic Number	Atomic Weight
Actinium	Ac	89	227.028
Aluminium	Al	13	26.98154
Americium	Am	95	(243)
Antimony	Sb	51	121.75_3
Argon	Ar	18	39.948
Arsenic	As	33	74.9216
Astatine	At	85	(210)
Barium	Ba	56	137.33
Berkelium	Bk	97	(247)
Beryllium	Be	4	9.01218
Bismuth	Bi	83	208.9804
Boron	B	5	10.811_5
Bromine	Br	35	79.904
Cadmium	Cd	48	112.41
Calcium	Ca	20	40.078_4
Californium	Cf	98	(251)
Carbon	C	6	12.011
Cerium	Ce	58	140.12
Cesium	Cs	55	132.9054
Chlorine	Cl	17	35.453
Chromium	Cr	24	51.9961_6
Cobalt	Co	27	58.9332
Copper	Cu	29	63.546_3
Curium	Cm	96	(247)
Dysprosium	Dy	66	162.50_3
Einsteinium	Es	99	(252)
Erbium	Er	68	167.26_3
Europium	Eu	63	151.96
Fermium	Fm	100	(257)
Fluorine	F	9	18.998403
Francium	Fr	87	(223)
Gadolinium	Gd	64	157.25_3
Gallium	Ga	31	69.723_4
Germanium	Ge	32	72.59_3
Gold	Au	79	196.9665
Hafnium	Hf	72	178.49
Helium	He	2	4.002602
Holmium	Ho	67	164.9304
Hydrogen	H	1	1.00794_7
Indium	-In	49	114.82
Iodine	I	53	126.9045
Iridium	Ir	77	192.22_3
Iron	Fe	26	55.847_3
Krypton	Kr	36	83.80
Lanthanum	La	57	138.9055_3
Lawrencium	Lr	103	(260)
Lead	Pb	82	207.2
Lithium	Li	3	6.941_2
Lutetium	Lu	71	174.967
Magnesium	Mg	12	24.305
Manganese	Mn	25	54.9380
Mendelevium	Md	101	(258)
Mercury	Hg	80	200.59_3
Molybdenum	Mo	42	95.94
Neodymium	Nd	60	144.24_3
Neon	Ne	10	20.179
Neptunium	Np	93	237.048
Nickel	Ni	28	58.69
Niobium	Nb	41	92.9064
Nitrogen	N	7	14.0067
Nobelium	No	102	(259)
Osmium	Os	76	190.2
Oxygen	O	8	15.9994_3
Palladium	Pd	46	106.42
Phosphorus	P	15	30.97376
Platinum	Pt	78	195.08_3
Plutonium	Pu	94	(244)
Polonium	Po	84	(209)
Potassium	K	19	39.0983
Praseodymium	Pr	59	140.9077
Promethium	Pm	61	(145)
Protactinium	Pa	91	231.036
Radium	Ra	88	226.025
Radon	Rn	86	(222)
Rhenium	Re	75	186.207
Rhodium	Rh	45	102.9055
Rubidium	Rb	37	85.4678_3
Ruthenium	Ru	44	101.07_2
Samarium	Sm	62	150.36_3
Scandium	Sc	21	44.95591
Selenium	Se	34	78.96_3
Silicon	Si	14	28.0855_3
Silver	Ag	47	107.8682_3
Sodium	Na	11	22.98977
Strontium	Sr	38	87.62
Sulphur	S	16	32.066_6
Tantalum	Ta	73	180.9479
Technetium	Tc	43	(98)
Tellurium	Te	52	127.60_3
Terbium	Tb	65	158.9254
Thallium	Tl	81	204.38_3
Thorium	Th	90	232.0381
Thulium	Tm	69	168.9342
Tin	Sn	50	118.710_7
Titanium	Ti	22	47.88_3
Tungsten	W	74	183.85_3
Uranium	U	92	238.0289
Vanadium	V	23	50.9415
Xenon	Xe	54	131.29_3
Ytterbium	Yb	70	173.04_3
Yttrium	Y	39	88.9059
Zinc	Zn	30	65.39_2
Zirconium	Zr	40	91.224_2

Subscripted digit represents the uncertainty in the preceeding digit. The absence of a subscripted digit means the uncertainty in the last digit M. For the most stable isotopes the mass number is given in parentheses.

FORMULA WEIGHTS

$AgBr$	187.77
$AgCl$	143.32
AgI	234.77
$AgNO_3$	169.87
$AgSCN$	165.95
$Al(C_9H_6NO)_3$ (Al quinolinate)	459.44
$AlCl_3$	133.34
$Al(NO_3)_3$	213.00
$Al(OH)_3$	78.00
Al_2O_3	101.97
As_2O_3	197.84
$BaCl_2$	208.24
$BaCl_2.2H_2O$	244.27
$BaCrO_4$	253.32
BaS	169.40
$BaSO_4$	233.39
$Ba_3(PO_4)_2$	601.93
C_2H_5OH	46.07
$C_9H_{12}O_6$ (glucose)	180.16
$CO(NH_2)_2$ (urea)	60.06
CO_2	44.01
$CaCO_3$	100.09
CaC_2O_4	128.10
$CaC_2O_4.H_2O$	146.11
$CaCl_2$	110.98
CaO	56.08
$CaSO_4$	136.14
$CuCl_2$	134.45
CuO	79.55
$CuSO_4$	159.61
$Fe(NH_4)_2(SO_4)_2.6H_2O$	392.14
FeO	71.85
$FeSO_4$	151.91
Fe_2O_3	159.69
$Fe_2(SO_4)_3$	399.88
Fe_3O_4	231.54
HCl	36.46
$HClO_4$	100.46
HNO_3	63.01
$(HOCH_2)_3CNH_2$ (THAM)	121.14
$HONH_3Cl$	69.49
$(HO_2C)_2$ (oxalic acid)	90.04
$(HO_2C)_2.2H_2O$ (oxalic acid)	126.07
HO_2CH (formic acid)	46.03
HO_2CCH_3 (acetic aid)	60.05
HO_2CCHCl_2 (dichloroacetic acid)	128.94
$(HO_2CCH_2)_2$ C(OH) CO_2H (citric acid)	192.13
HO_3SNH_2 (sulphamic acid)	97.09
H_2O	18.02
H_2O_2	34.01
H_2SO_4	98.08
H_3BO_3	61.83
H_3PO_4	98.00
$HgCl_2$	271.50
Hg_2Cl_2	472.09
KBr	119.00
KCN	65.12
KCl	74.55
$KHC_8H_4O_4$ (KHPhthalate)	204.22
$KH(IO_3)_2$	389.91
KH_2PO_4	136.09
KI	166.00
KIO_3	214.00
$KMnO_4$	158.03
KNO_3	101.10
KOH	56.11
$KSCN$	97.18
$K_2Cr_2O_7$	294.18
K_2HPO_4	174.18
Li_2SO_4	109.95
$MgCO_3$	84.31
$Mg(C_9H_6NO)_3$ (Mg quinolinate)	456.76
$MgCl_2$	95.21
$MgSO_4$	120.37
$Mg_2P_2O_2$	222.55
MnO_2	86.94
NH_3	17.03
NH_4HF_2	57.04
NH_4Cl	53.49
NH_4NO_3	80.04
$(NH_4)_2C_2O_4.2H_2O$	160.13
$(NH_4)_2Ce(NO_3)_6$	548.23
$(NH_4)_2SO_4$	132.14
N_2H_2	32.05
$NaBr$	102.89
$NaCN$	49.01
$NaCl$	58.44
NaF	41.99
$NaHCO_3$	84.01
NaH_2PO_4	119.98
$NaNO_2$	69.00
$NaOCl$	74.44
$NaOH$	40.00
NaO_2CCH_3 (sodium acetate)	82.03
$Na_2B_4O_7$	201.22
$Na_2B_4O_7.10H_2O$	381.37
Na_2CO_3	105.99
$Na_2C_2O_4$	134.00
Na_2HPO_4	141.96
$Na_2H_2Y.2H_2O$ (Y = EDTA)	372.24
Na_2SO_3	126.04
Na_2SO_4	142.04
$Na_2S_2O_4$	158.11
$Na_2S_2O_3.5H_2O$	248.19
Na_3PO_4	163.94
$Pb(NO_3)_2$	331.21
$PbSO_4$	303.26
SO_2	64.06
SO_3	80.06
Sb_2S_3	339.70
$SnCl_2$	189.62
SnO_2	150.71
$Th(IO_3)_4$	931.65
ThO_2	264.04
TiO_2	79.88
Tl_2CrO_4	524.76
U_3O_8	842.08
V_2O_5	181.88
$ZnCO_3$	125.40
$Zn_2P_2O_7$	304.72
$Zr(HPO_4)_2$	283.18
ZrP_2O_7	265.17

COMPOUNDS FOR PREPARING STANDARD SOLUTIONS

Element	Compound	Formula weight (g)	1000 ppm* (g/L)	Sol. Net	Comments
Aluminium	Al metal	26.982	1.0000	Hot, dil. HCl	*b*
Antimony	$KSbOC_4H_4O_6.H_2O$	333.92	2.7427	Water	*e*
Arsenic	As_2O_3	197.84	2.6406	Dil. HCl	*a, i*
Barium	$BaCO_3$	197.35	1.4369	Dil. HCl	
Bismuth	Bi_2O_3	465.96	1.1148	HNO_3	
Boron	H_3BO_3	61.833	5.7200	Water	*f*
Bromine	KBr	119.00	1.4894	Water	*b*
Cadmium	CdO	128.40	1.1423	HNO_3	
Calcium	$CaCO_3$	100.09	2.4972	Dil. HCl	*a*
Cerium	$(NH_4)_2Ce(NO_3)_6$	548.23	3.9126	Water	
Chromium	$K_2Cr_2O_7$	294.18	2.8290	Water	*a*
Cobalt	Co metal	58.933	1.0000	HNO_3	*b*
Copper	Cu metal	63.546	1.0000	Dil. HNO_3	*b*
	CuO	79.545	1.2517	Hot HCl	*b*
Fluorine	NaF	41.988	2.2101	Water	*c*
Germanium	GeO_2	104.60	1.4410	Hot 1*M* NaOH	
Gold	Au metal	196.97	1.0000	Hot Aqua Regia	*b*
Iodine	KIO_3	214.00	1.6863	Water	*a*
Iron	Fe metal	55.847	1.0000	Hot HCl	*b*
Lanthanum	La_2O_3	325.82	1.1728	Hot HCl	
Lead	$Pb(NO_3)_2$	331.21	1.5985	Water	*b*
Lithium	Li_2CO_3	73.890	5.3243	HCl	*b*
Magnesium	MgO	40.304	1.6583	HCl	
Manganese	$MnSO_4H_2O$	169.01	3.0764	Water	*g*
Mercury	$HgCl_2$	271.50	1.3535	Water	
Molybdenum	MoO_3	143.94	1.5003	1 *M* NaOH	
Nickel	Ni metal	58.69	1.0000	Hot HNO_3	*b*
Palladium	Pd metal	106.42	1.0000	Hot HNO_3	
Phosphorus	KH_2PO_4	136.09	4.3937	Water	
Platinum	K_2PrCl_4	415.12	2.1278	Water	
Potassium	KCl	74.551	1.9065	Water	*b*
	$KHC_8H_4O_4$	204.22	5.2228	Water	*a. i*
	$K_2Cr_2O_7$	294.18	3.7618	Water	*a. i*
Scandium	Sc_2O_3	137.91	1.5339	Hot HCl	
Selenium	Se metal	78.96	1.0000	Hot HNO_3	
Silicon	Si metal	28.086	1.0000	Conc. NaOH	
	SiO_2	60.085	2.1391	HF	
Silver	$AgNO_3$	169.87	1.5748	Water	*b d*
Sodium	NaCl	58.442	2.5428	Water	*a*
	$Na_2C_2O_4$	134.00	2.9146	Water	*a. i*

* Weight of substance per liter to give an element concentration of 1000 ppm.

Strontium	$SrCO_3$	147.63	1.6849	HCl	*b*
Sulphur	K_2SO_4	174.27	5.4351	Water	*b*
Thallium	Ti_2CO_3	468.75	1.1468	Water	
Tin	Sn metal	118.71	1.0000	HCl	
	SnO	134.71	1.1348	HCl	
Titanium	Ti metal	47.88	1.0000	9M H_2SO_4	*b*
Tungsten	$Na_2WO_42H_2O$	329.86	1.7942	Water	*h*
Uranium	UO_2	270.03	1.1344	HNO_3	
	U_3O_6	842.08	1.1792	HNO_3	*a. i*
Vanadium	V_2O_5	181.88	1.7852	Hot HCl	
Zinc	ZnO	81.39	1.2448	HCl	*b*

a : Primary standard.

b : These compounds conform very well to the criteria and approach primary standard quality.

c : Sodium fluoride solutions will etch glass and should be freshly prepared.

d : When kept dry. silver nitrate crystals are not affected by light Solutions of silver nitrate should be stored in brown bottles ?

e : Antimony potassium tartrate loses the H_2O with drying at 110 °C. After drying f.e.w. 324.92, 1000 ppm-2. 6687. The water is not rapidly regained, but the compound should be kept in a desicator after drying and should be weighed quickly once it is removed. The dried compound is water soluble.

f : Boric acid may be weighed accurately directly from the bottle. It will lose one H_2O_2 molecule at 100 °C and a second H_2O_2 molecule at approximately 130-140 °C and is difficult to dry to a constant weight.

g : $MnSO_4.H_2O$ may be dried at 110 °C without losing the water of hydration.

h : Sodium tungstate loses both water molecules at 110°C. After drying *f.w*. = 293.83 1000 ppm = 1.5982g. The wate is not rapidly regained but the compound should be kept in a desicator after drying and should be weighed quickly once it is removed.

i : These compounds are sold as primary standards by the National Bureau of Standards Office of Standards Reformer Materials.

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Symbols

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