1 PHARMACEUTICAL CHEMICALS : PURITY AND MANAGEMENT

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-benefit-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
1.	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
1.	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 $CHCl_3$
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 :

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

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 (%)	
1.	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 tar-trate 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 ml 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 (%)
1.	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 :				
1.	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 :				
1.	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 (%)
1.	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 ($\simeq$ 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 platinum 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 :

$$PbCl_2 + Na_2S \longrightarrow PbS \downarrow + 2NaCl$$
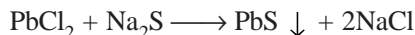
The brown colour, caused due to colloidal lead sulphide in the test solution is compared with that produced from a known amount of lead.

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).

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.

50 ml

Figure 1 : Nessler Glass.

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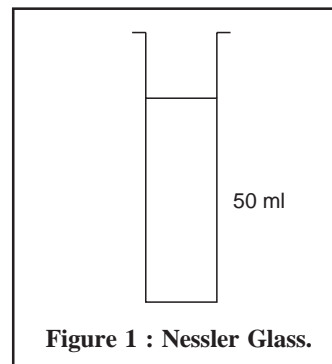
(*iii*) **Standard Solution :** Into a 50 ml Nessler Cylinder, pipette 2 ml of standard lead solution and dilute with DW to 25 ml. Adjust with dilute acetic acid Sp. (IP)* or dilute ammonia solution Sp. (IP) to a pH between 3.0 and 4.0, dilute with DW to about 35 ml and mix.

(*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 10 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.
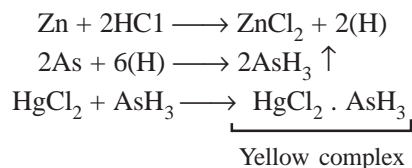
A few typical examples from the official compendium are given below :

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 :

$$Zn + 2HCl \longrightarrow ZnCl_2 + 2(H)$$
$$2As + 6(H) \longrightarrow 2AsH_3 \uparrow$$
$$HgCl_2 + AsH_3 \longrightarrow \underbrace{HgCl_2 . AsH_3}$$
$$\text{Yellow complex}$$

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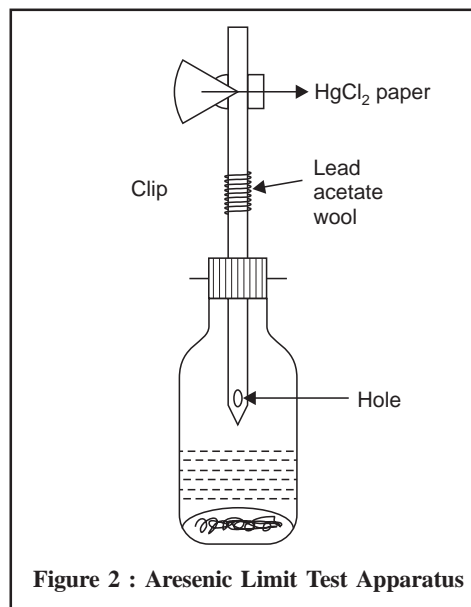
(*i*) **Free Acids :** They are first converted to their respective sodium salts with Na_2CO_3 and As^{3+} oxidised to As^{5+} by evaporating the solution with Br_2. The residue is ignited carefully until carbonised to destroy organic matter, while As is kept as non-volatile sodium arsenate. The resulting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

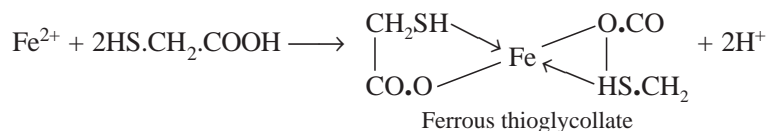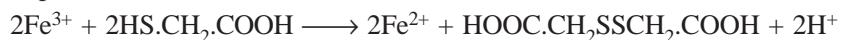*Examples* **:** Antimony Potassiun Tartrate, Antimony Sodium Tartrate.

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 :

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

$$Fe^{2+} + 2HS.CH_2.COOH \longrightarrow \underset{\text{Ferrous thioglycollate}}{\begin{array}{c} CH_2SH \searrow \quad \swarrow O.CO \\ | \qquad Fe \qquad | \\ CO.O \nearrow \quad \nwarrow HS.CH_2 \end{array}} + 2H^+$$

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 0.1 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 :

$$NaCl + AgNO_3 \longrightarrow AgCl \downarrow + NaNO_3$$
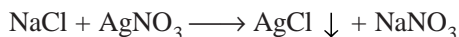
Materials Required : Nessler cylinder 1 pair ; dilute nitric acid (10% w/w of HNO_3) 10.0 ml ; silver nitrate solution (5.0% w/v in DW) 1.0 ml.

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 Hydroxy-napthoate	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 0.1 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
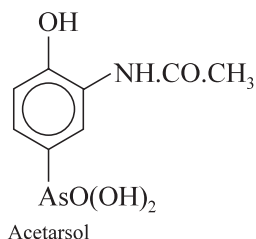
Inorganic arsenates are found to be extremely toxic in nature and hence careful control is maintained by the addition of magnesium ammonio-sulphate solution to an aqueous solution of the sample, thereby producing an instant white precipitate.

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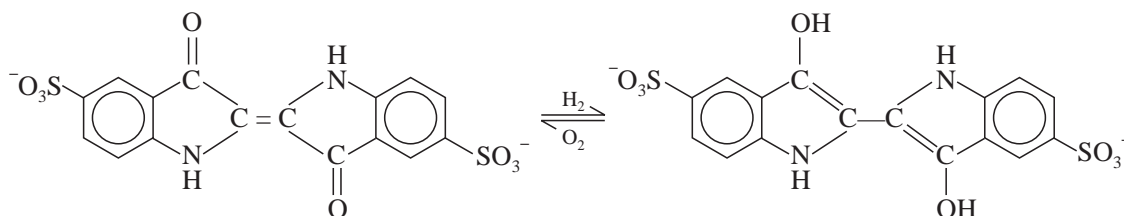
The quantities as specifed in the Pharmacopoeia allow an official limit of nitrate equivalent to about 0.29% $BiONO_3$.

A few typical instances of pharmaceutical substances are enumerated below :

S. No.	Pharmaceutical Substance	Test	Official Requirements
1.	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
1.	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_2SO_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 :

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

Selenous acid   3, 3′-Diaminobenzidine   3, 4-Diaminophenylpiazselenol
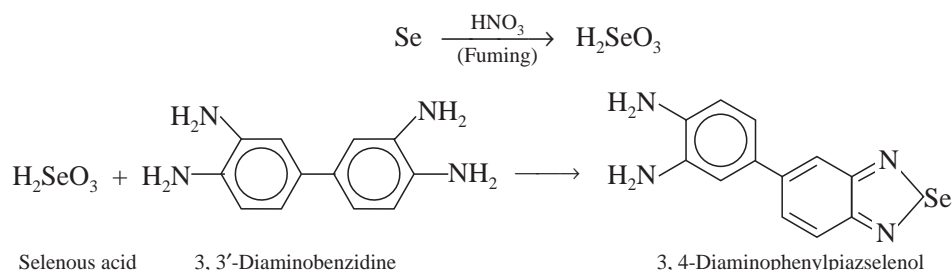
Materials Required : Selenium sulphide : 10.0 g ; formic acid (2.5 M) : 2.0 ml ; 3,3′-diaminobenzedine tetrahydrochloride solution (0.5% w/v in DW) : 2.0 ml ; ammonia (5 M) : 20 ml ; selenium standard solution (1 ppm Se) (Dilute 2.5 ml of a 0.00654% w/v solution of selenous acid to 100 ml with DW) : 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

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 :

$$HCl \quad + \quad NaOH \quad \longrightarrow \quad NaCl \quad + \quad H_2O$$
$$\text{Analyte} \qquad \text{Titrant}$$
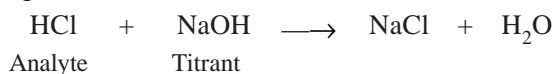
Analyte (or Active Constituent) is the chemical entity under assay *e.g.*, HCl.

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 $\equiv$ 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 through-out 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 :

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

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*)] :

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

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

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 :

$$Fe^{2+} \longrightarrow Fe^{3+} + e$$
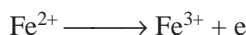
hence, the equivalent weight of $FeSO_4$ oxidizing to $Fe_2(SO_4)_3$ comes out to be 151.919 [$FeSO_4$: molecular weight = 151.91] or 1 gram-molecular weight.

(*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*) $\qquad \qquad Ce^{4+} + e \longrightarrow Ce^{3+} \qquad$ (cerous ion)
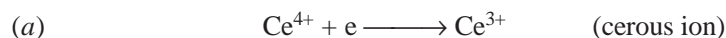
hence, the equivalent weight of ceric sulphate is 1 gram-molecular weight 332.24 g [$Ce(SO_4)_2$: molecular weight = 332.24]

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

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

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

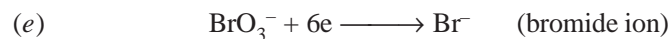(*c*) $\qquad \qquad Cr_2O_7^{2-} + 6e \longrightarrow 2Cr^{3+} \qquad$ (chromous ion)

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

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

(*e*) $\qquad \qquad BrO_3^- + 6e \longrightarrow Br^- \qquad$ (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 $H_2C_4H_4O_6$

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

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.

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.

Figure 2.1 : Automatic Pipette.

(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.

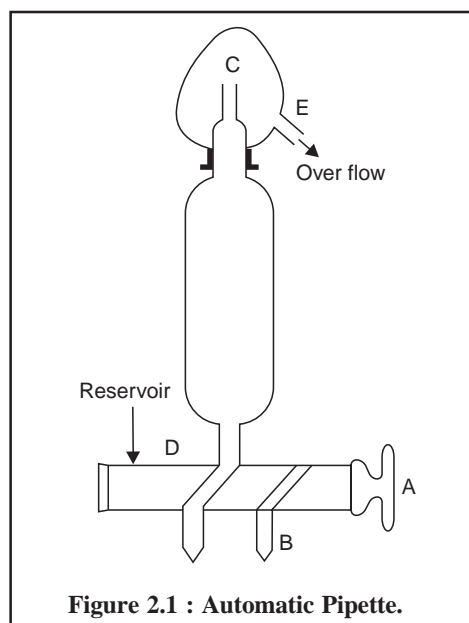(4) Close the tap A by turning clockwise through 90° when the solution starts to overflow at C.

(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 advisable 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 :

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

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

$$2.303 \log (P_0/P) = kb \qquad \qquad ... (b)$$

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

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

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 :

$$2.303 \log (P_0/P) = k'\, C \qquad \qquad ... (d)$$

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 :

$$\log (P_0/P) = abc \qquad \qquad ...(e)$$

or $$P = P_0 10^{-abc} \qquad \qquad ...(f)$$

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 :

$$A = abc \qquad \qquad ...(g)$$

where,   A = absorbance,

   a = absorptivity,

   b = optical path length, and

   c = analyte concentration.

The term $A_{1cm}^{1\%}$ 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.*, $A_{1cn}^{1\%}$) 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.
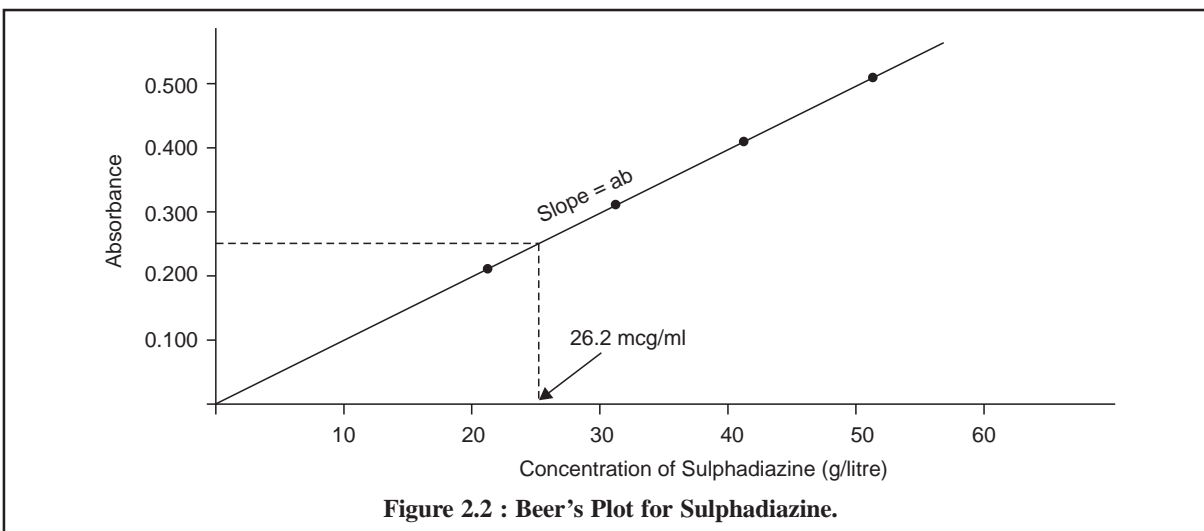
The colorimetric assay of sulphadiazine is based on the **acid-catalysed equilibrium reaction** that occurs between vanillin (an aldehyde) and sulphadiazine (an arylamine). The chemical species that forms as shown below is known as the **Schiff's Base** and is yellow in colour.
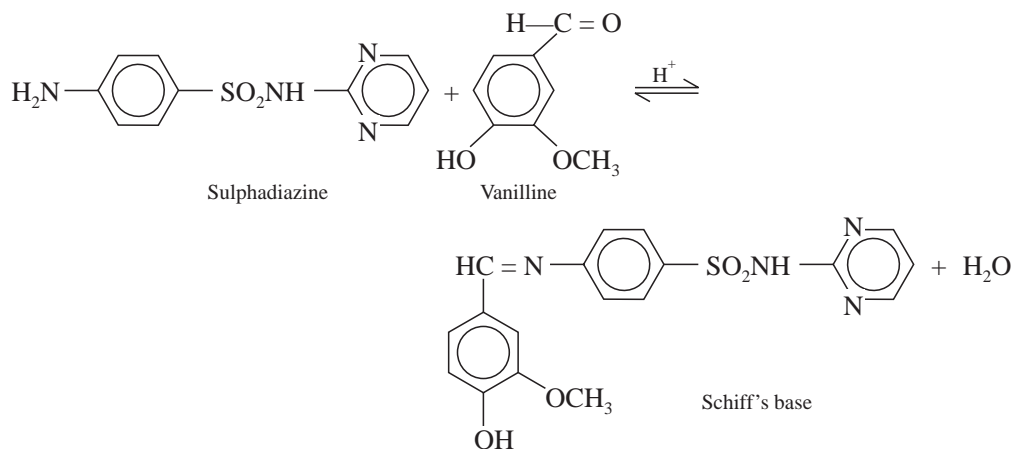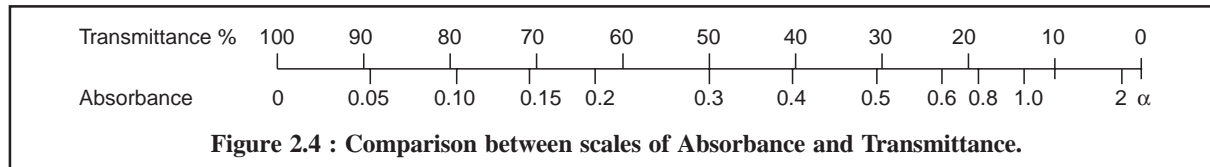

Transmittance. The relationship between per cent transmittance and concentration is shown in Figure 2.3.

Figure 2.3 : Plot between Transmittance % and Concentration (g/litre or mcg/ml).
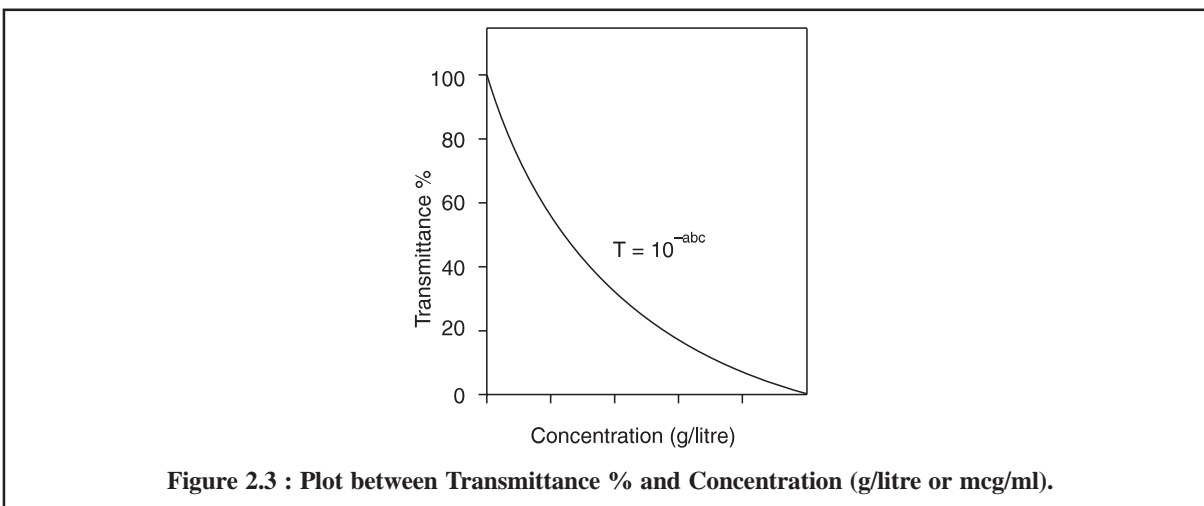
From Figure 2.3, it is quite evident that at lower concentrations the per cent trasmission is high and is *vice varsa* at higher concentrations.

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

Transmittance %	100	90	80	70	60	50	40	30	20	10	0
Absorbance	0	0.05	0.10	0.15 0.2		0.3	0.4	0.5	0.6 0.8 1.0		2 α

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.

Urea (BUN)   Acetyl-methyl oxime   Thiosemicarbazide   90°C

A cyclic condensation product
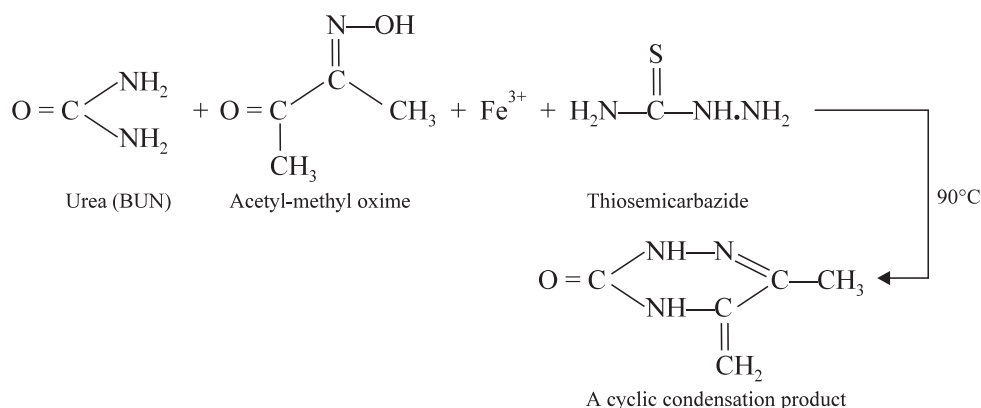
Profile of BUN-levels

(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.


p-Sulphonyl benzene diazonium compound

Azobilirubin
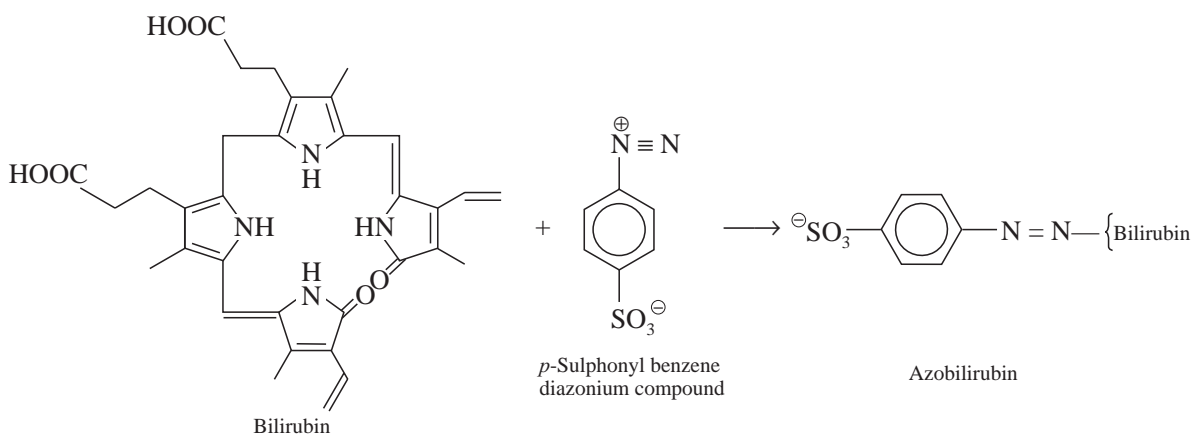
Bilirubin

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 :

$+ CH_3COOH + (CH_3CO)_2 O + H_2SO_4 + $ (2-3 drops)

A blue coloured product
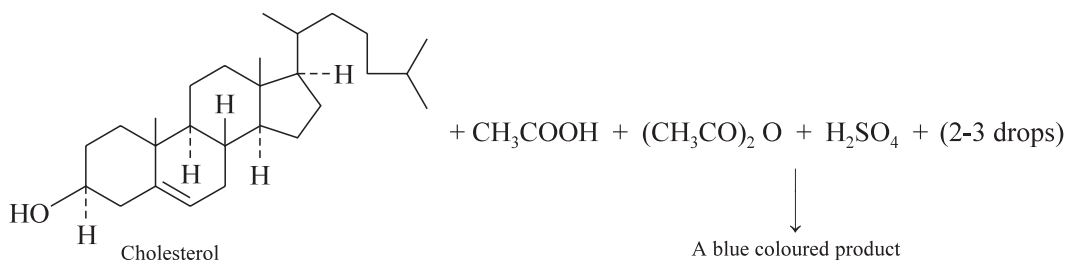
The above reactions is also referred to as the Libermann's Reaction.

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 :

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

where,   E = Enzyme,

S = Substrate,

ES = Enzyme-substrate complex, and

P = Product.

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

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

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

V_{max} = Max. rate of reaction

Assuming, [S] to be in large excess $[S] \gg K_m$,

From Eq. (*b*) we have :

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

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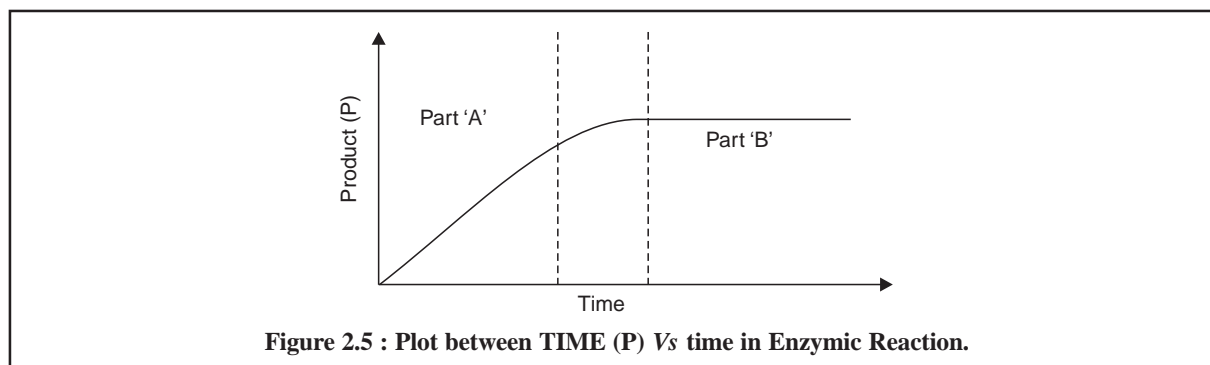

Figure 2.5 : Plot between TIME (P) *Vs* time in Enzymic Reaction.

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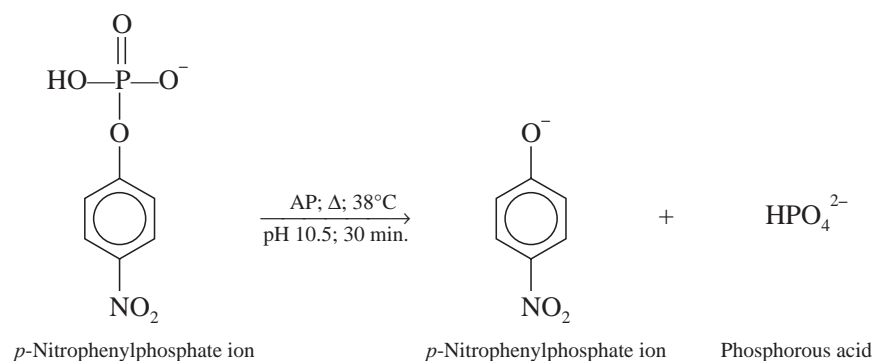
In actual practice, p-nitrophenylphosphate is present in large excesses, and the reaction is carried out at 38°C for 30 minutes. The resulting amount of p-nitrophenolate ion is estimated by the help of an usual standard curve employing known concentrations of p-nitrophenolate prepared from p-nitrophenol.

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-nitro-phenylphosphate 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

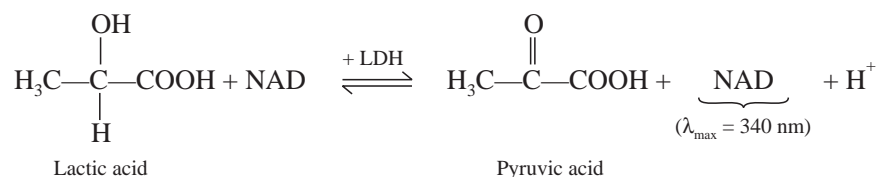| Cuvet	Working Standard (ml)	NaOH 0.02 N (ml)	Alkaline Phosphatase Units/ml	A_{410}
1	1.0	10.1	1.0	-
2	4.0	7.1	4.0	-
3	8.0	3.1	8.0	-
4	10.0	1.1	10.0	-
5	10.5	0.6	10.5	-

*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 (1 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 :

$$
\underset{\text{Lactic acid}}{H_3C-\underset{\underset{H}{|}}{\overset{\overset{OH}{|}}{C}}-COOH} + NAD \quad \underset{}{\overset{+\,LDH}{\rightleftharpoons}} \quad \underset{\text{Pyruvic acid}}{H_3C-\overset{\overset{O}{\|}}{C}-COOH} + \underbrace{NAD}_{(\lambda_{max}\,=\,340\,nm)} + H^+
$$

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 ≃ 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 $\Delta A/min$,

(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 :

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

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

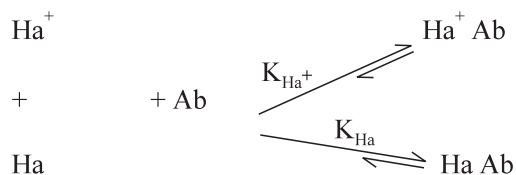## 2.4.3. RADIOIMMUNOASSAYS (RIAS)

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 :

$$
\begin{array}{ccccc}
Ha^+ & & & & Ha^+\ Ab \\
& & & \overset{K_{Ha^+}}{\nearrow} \rightleftharpoons & \\
+ & & +\ Ab & & \\
& & & \underset{K_{Ha}}{\searrow} \rightleftharpoons & \\
Ha & & & & Ha\ Ab
\end{array}
$$

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 (µ*l*)
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

Sequence	Preparation of Calibration Curve	Clinical Determination
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.

Sequence	Preparation of Calibration Curve	Clinical Determination
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 $1240 \times g$ 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 *V*s 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 :

$$C_6H_{12}O_6 + 2Cu^{2+} + 6H_2O \rightleftharpoons Cu_2O + C_6H_{12}O_7 + 4H_3O^+$$
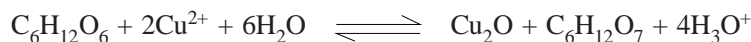
As some other sugars are also present in blood sample, and besides the above reaction not being absolutely stoichiometric, it has become necessary in actual practice to establish an emperical calibration curve using known concentrations of glucose. The above reaction is allowed to proceed for exactly 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
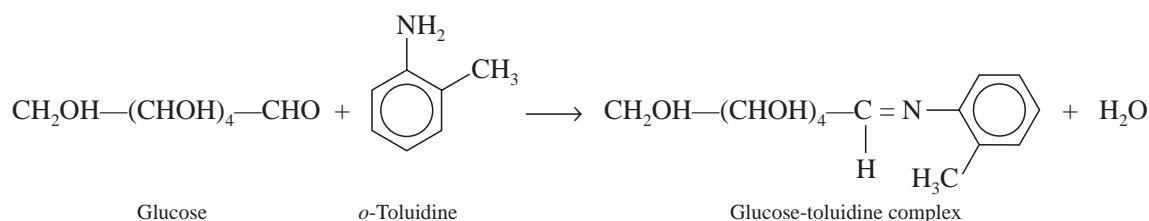
The diagnostic green colour is usually developed for exactly 10 minutes at 100°C and measured subsequently at 635 nm.

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.
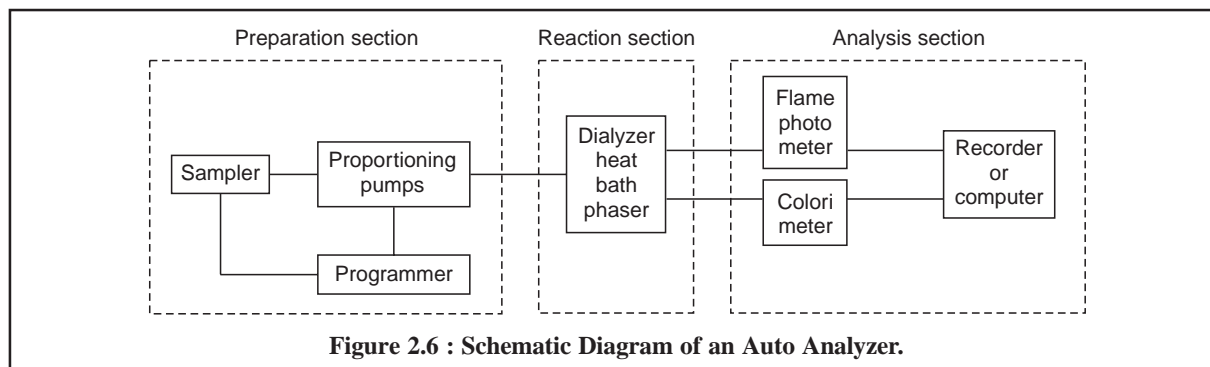

Figure 2.6 : Schematic Diagram of an Auto Analyzer.

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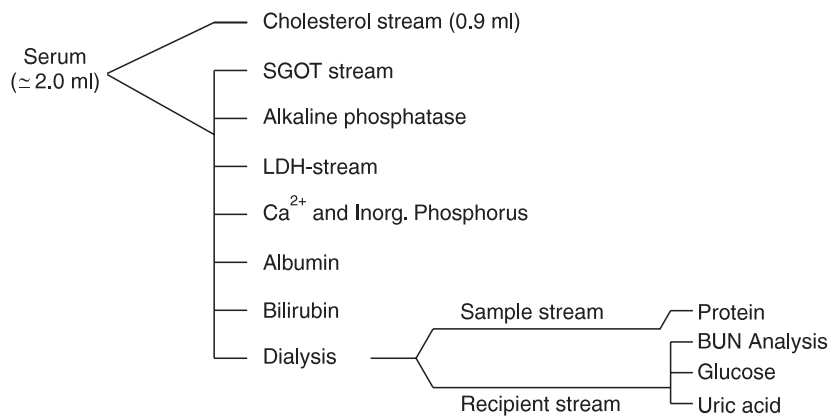
Figure 2.7 : Sample Streams of SMA-12 Survey Auto Analyzer.

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 :

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

or

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

where, $[C]^c$ = Molar concentration of substance C raised to the cth 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 :

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

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

T = Temperature, and

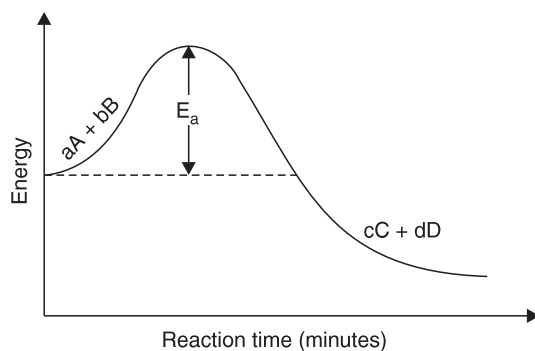E_a = Activation energy of the reaction as depicted in Figure 2.8.

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.

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7.   Thomas LC and GJ Chamberlain, **Colorimetric Chemical Analytical Methods**, 9th ed., Wiley-Interscience, New York, 1980.

8.   Sawicki, E, **Photometric Organic Analysis**, Part I, Wiley-Interscience, New York, 1970.

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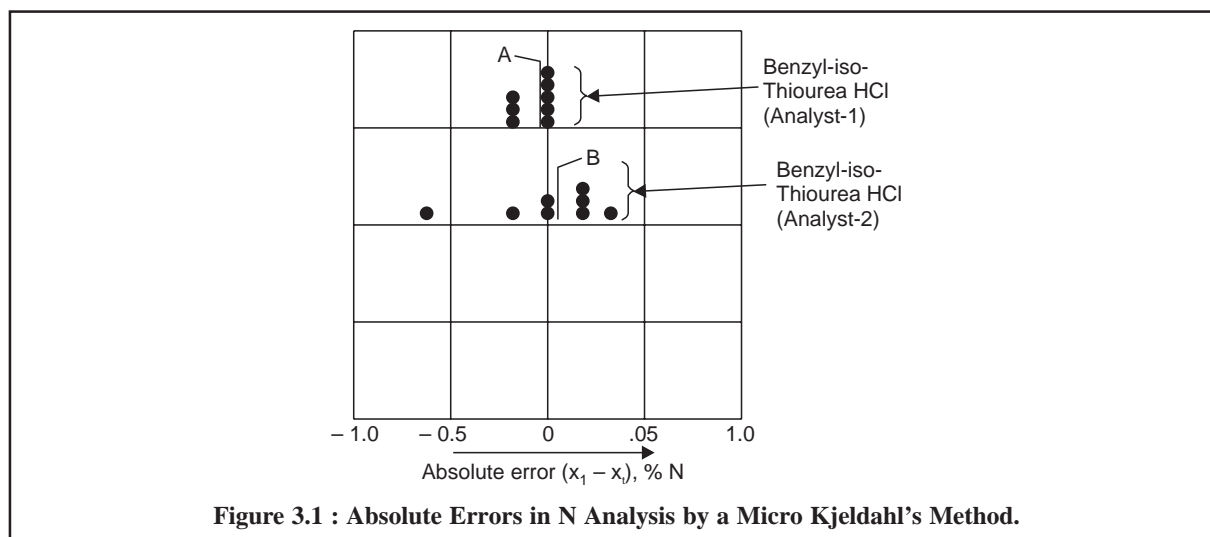
* Willits, C.O. and C.L. Ogg, **J. Assoc. Off. Anal. Chem., 32,** 561, 1949.

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
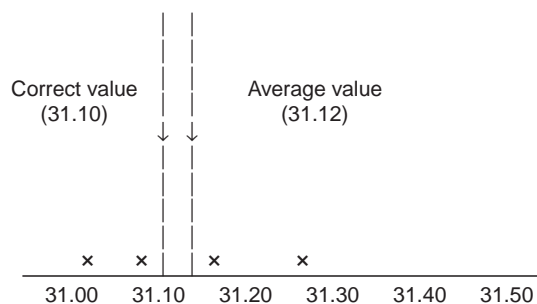
The arithmetic mean stands at 31.12% and the results range between 31.01% to 31.25%

Analyst 2 : % Menthone : 31.40 ; 31.46 ; 31.44 ; 31.44
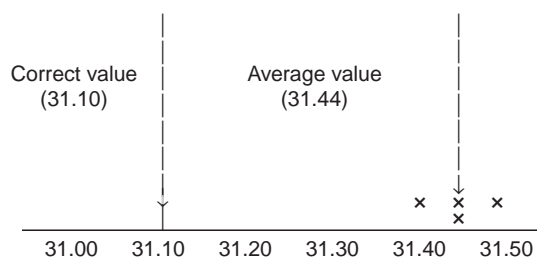
The arithmetic mean is 31.44% and the results vary between 31.40% to 31.46%

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 :

$$\frac{\text{Wt. of component in Standard Substance}}{X} = \frac{\text{Result obtained for Standard Substance}}{\text{Result obtained for Unknown Sample}}$$

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*) **Fe³⁺** : 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 :

$$\bar{x} = \frac{x_1 + x_2 + x_3 + x_4 ...... + x_n}{n} = \frac{\sum\limits_{i=1}^{i=n} x_1}{n}$$

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 :

$$\text{Average Deviation} \quad = \bar{d} = \frac{\sum\limits_{i=1}^{i=n}[x_1 - \bar{x}]}{n}$$

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 :

$$S = \sqrt{\frac{\sum\limits_{i=1}^{i=n}[i - \bar{x}]^2}{n-1}}$$

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 (s²)

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 :
$$\bar{x} = \frac{0.5038 + 0.5049 + 0.5042 + 0.5039}{4}$$

$$= \mathbf{0.5042}$$

Median :
$$M = \frac{0.5042 + 0.5039}{2}$$

$$= 0.50405^* = \mathbf{0.5041}$$

Average Deviation :
$$\bar{d} = \frac{(0.0004) + (0.0007) + (0.0000) + (0.0003)}{4}$$

$$= 0.00035^* = \mathbf{0.0004}$$

Standard Deviation :
$$s = \sqrt{\frac{(0.0004)^2 + (0.0007)^2 + (0.0000)^2 + (0.0003)^2}{4-1}}$$

$$= 0.00049^* = \mathbf{0.0005}$$

Coeffcient of Variation :
$$= \frac{0.0005}{0.5042} \times 100$$

$$= 0.099^* = \mathbf{0.1\%}$$

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.

---

* 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.
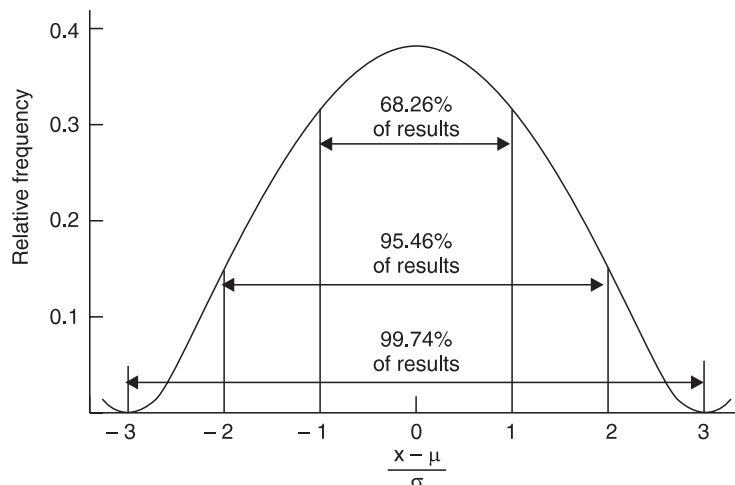
**Figure 3.2 : Normal Distribution Curve ; Relative Frequencies of Deviations from the Mean
for a Normally Distributed Infinite Population ; Deviations ($x - \mu$) are in Units of σ.**

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 :

<div align="center">

Method : 'A'	Method : 'B'
$\bar{x}_1 = 24.36$	$\bar{x}_2 = 24.46$
$s_1 = 0.10$	$s_2 = 0.13$
$n = 6$	$n = 5$

</div>

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 ?

<div align="center">

Table 3.1 : Method of Least Squares

</div>

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.
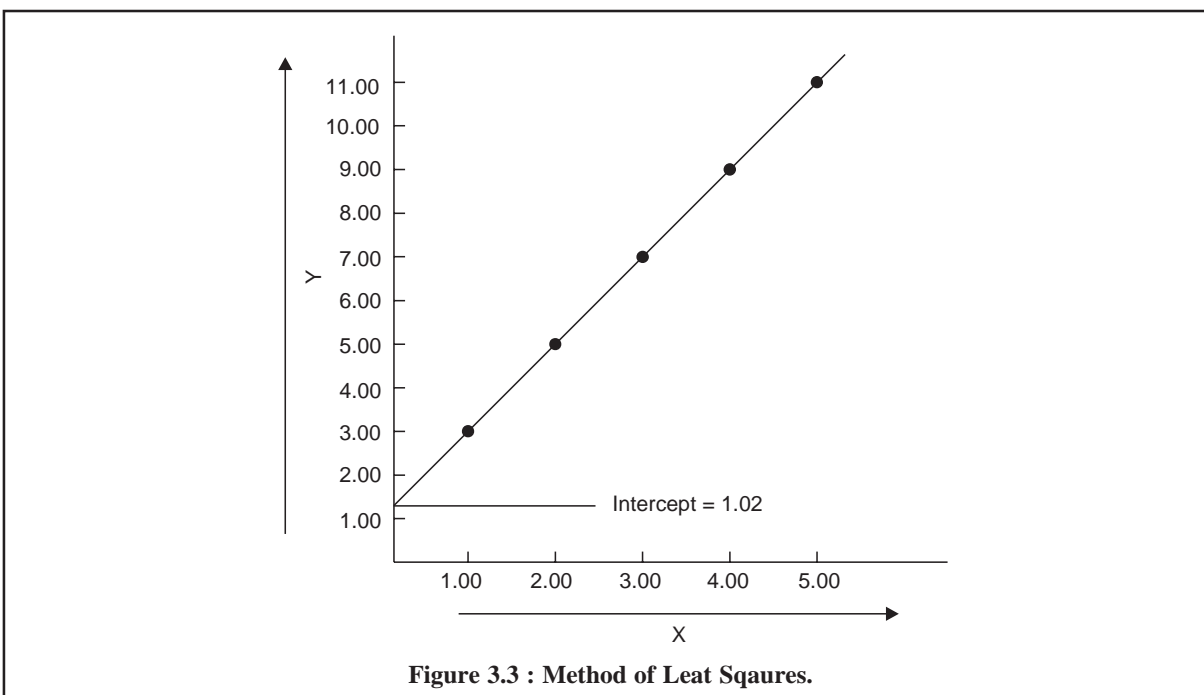
Presumably, the indeterminate errors caused by the instrument readings, y, are responsible for not allowing the 'data points' to fall exactly on the line. Therefore, the sum of the squares of the deviations obtained from the real instrument readings with respect to the correct values are minimized coinsiderably 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 :

$$5.88 = 1.99x + 1.02$$

$$\therefore \qquad\qquad x = 2.44$$

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 $\qquad\qquad\qquad$ Q = **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 :

$$s_T = \sqrt{S_S{}^2 + S_A{}^2} \qquad\qquad\qquad ...(i)$$

where, $\quad S_S$ = Standard deviation of the sampling procedure (*i.e.*, the sampling error), and

$\quad S_A$ = 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 $\quad V_S$ = Variance due to sampling, and

$\quad V_A$ = 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 $\pm 2.8\%$ and the analytical errors by two different analysts come out to be $\pm 0.9\%$ and $\pm 0.1\%$ respectively, we may have :

$$S_T = \sqrt{(2.8)^2 + (0.9)^2} = 2.94 \qquad\qquad\qquad ...(a)$$

$$S_T = \sqrt{(2.8)^2 + (0.1)^2} = 2.80 \qquad\qquad\qquad ...(b)$$

From Eqs. (*a*) and (*b*) it is quite evident that the actual contribution of the analytical error *viz.*, $\pm 0.9\%$ and $\pm 0.1\%$, to the total error (S_T) is more or less insignificant.

> **Note :** $\quad$ 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.

[*] $\quad$ 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 :

$$\mu = \frac{\bar{x} \times tS_S}{\sqrt{n}} \qquad\qquad ...\ (c)$$

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.

$$\therefore \qquad\qquad n = \left[\frac{2.09 \times 1.4}{0.5}\right]^2 \approx 34$$

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

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 :

$$A_{acid} \rightleftharpoons H^+ + B_{base}$$
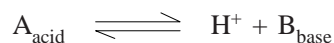
4.1.1.1. Conjugate Acid-Base Pair

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 :

$$\text{Acid} \qquad\qquad \text{Base}$$

$$HNO_3 \rightleftharpoons H^+ + NO_3^-$$

$$HCl \rightleftharpoons H^+ + Cl^-$$

$$CH_3COOH \rightleftharpoons H^+ + CH_3COO^-$$

$$NH_4^+ \rightleftharpoons H^+ + NH_3$$

$$HPO_4^{2-} \rightleftharpoons H^+ + PO_4^{3-}$$

$$H_3O^+ \rightleftharpoons H^+ + H_2O$$

$$HCO_3^- \rightleftharpoons H^+ + CO_3^{2-}$$

$$Al(H_2O)_6^{+++} \rightleftharpoons H^+ + Al(H_2O)_5\,OH^{2+}$$
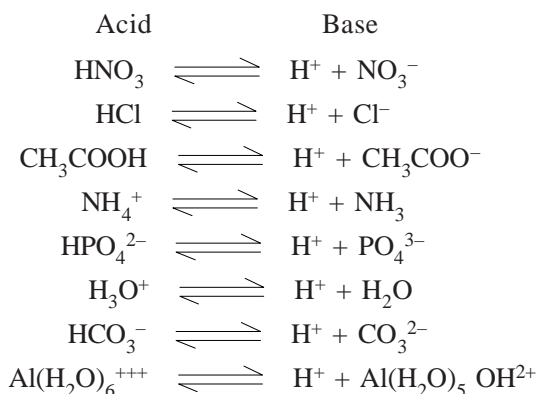
It is quite evident from the above examples that not only molecules but also *anions* and *cations* can act as acids and bases.

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 :

$$A_1 \ + \ B_2 \rightleftharpoons B_1 \ + \ A_2$$
$$\text{acid}_1 \qquad \text{base}_2 \qquad\quad \text{base}_1 \qquad \text{acid}_2$$

$$HCl \ + \ NH_3 \rightleftharpoons Cl^- \ + \ NH_4^+$$
$$\text{acid}_1 \qquad \text{base}_2 \qquad\quad \text{conjugate} \ \ \text{conjugate}$$
$$\text{base}_1 \qquad \text{acid}_2$$

Some other examples include :

$$CH_3COOH + H_2O \rightleftharpoons CH_3COO^- + H_3O^+$$

$$H_2SO_4 + H_2O \rightleftharpoons HSO_4^- \ + H_3O^+$$

$$H_2O + CN^- \rightleftharpoons OH^- + HCN$$

$$NH_4^+ + S^{2-} \rightleftharpoons NH_3 + HS^-$$
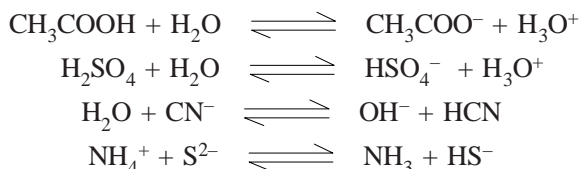
In short, the species which essentially differ from each other by one proton only, are known as **conjugate base and acid** respectively. Sometimes, such a reaction is termed as **protolytic reaction** or **protolysis**, where A_1 and B_1 make the first conjugate acid-base pair and A_2 and B_2 the other pair.
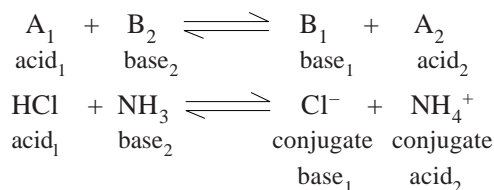
4.1.1.2. Merits of Lowry-Bronsted Theory

It has two points of merit, which are :

(*a*) hydrochloric acid on being dissolved in water undergoes a protolytic reaction, thus :

$$HCl \ + \ H_2O \rightleftharpoons Cl^- \ + \ H_3O^-$$
$$\text{acid}_1 \qquad \text{base}_2 \qquad\quad \text{base}_1 \qquad \text{acid}_2$$
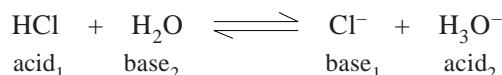
It may be observed that H_3O^+, known as hydronium or oxonium ion is invariably formed when an acid is dissolved in water.

Likewise, ammonia on being dissolved in water is also subjected to protolysis, thus :

$$NH_3 \ + \ H_2O \rightleftharpoons NH_4^+ \ + \ OH^-$$
$$\text{base}_1 \qquad \text{acid}_2 \qquad\quad \text{acid}_1 \qquad\quad \text{base}_2$$
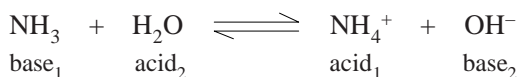
(*b*) all proton-transfer reactions may be handled, thus :

$$NH_4^+ \ + \ S^{2-} \rightleftharpoons NH_3 + \ HS^-$$
$$\text{acid}_1 \qquad\ \text{base}_2 \qquad\quad \text{base}_1 \qquad \text{acid}_2$$
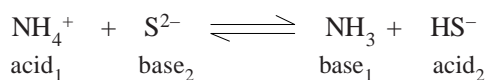
4.1.1.3. Demerits of Lowry-Bronsted Theory

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 :

$$
(i) \quad
\begin{matrix}
F \\
\ddot{} \\
F : B \\
\ddot{} \\
F
\end{matrix}
\quad + \quad
\begin{matrix}
H \\
\ddot{} \\
: N : H \\
\ddot{} \\
H
\end{matrix}
\quad \longrightarrow \quad
\begin{matrix}
F \quad H \\
\ddot{} \quad \ddot{} \\
F : B : N : H \\
\ddot{} \quad \ddot{} \\
F \quad H
\end{matrix}
$$

<div align="center">

Boron trifluoride   Ammonia
(acid)   (base)

</div>

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).

$$
(ii) \quad Ag^+ \quad + \quad 2 : N : H \quad \longrightarrow \quad Ag \left[\begin{matrix} H \\ \ddot{} \\ : N : H \\ \ddot{} \\ H \end{matrix} \right]_2^+
$$

<div align="center">

Electron   Electron
Acceptor   Donor
(acid)   (base)

</div>

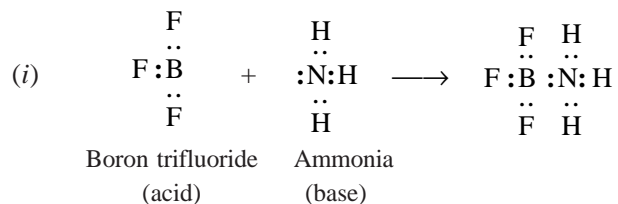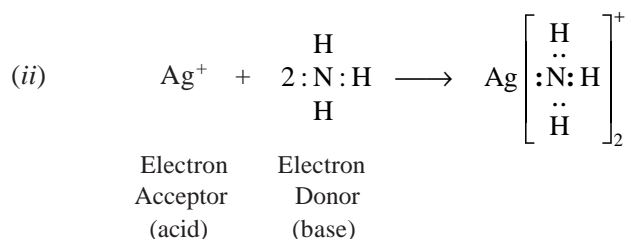The reaction of ammonia (base) with Ag^+ (acid) results into a stable configuration due to the mutual sharing of a pair of electrons of latter (donor) and former (acceptor).

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.*,

$$
\underset{base}{Fe^{2+}} \quad \rightleftharpoons \quad \underset{acid}{Fe^{3+}} \quad + \quad e^-
$$
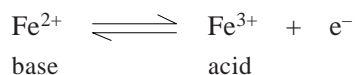
In the Iron (II)—Iron (III) system, the ferric ion (III) acts as an oxidizing agent and is an acid ; while the ferrous ion (II) acts as a reducing agent and is a base.

$$
\underset{base}{Ce^{3+}} \quad \rightleftharpoons \quad \underset{acid}{Ce^{4+}} \quad + \quad e^-
$$
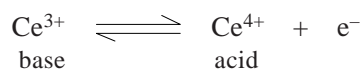
Similarly, in the Cerous (III)—Ceric (IV) system, the ceric ion (IV) behaves as an oxidizing agent and acts as an acid ; while the cerous ion (III) behaves as a reducing agents and acts as a base.

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 :

$$MgO + SiO_2 \longrightarrow MgSiO_3$$
$$CaO + SO_3 \longrightarrow CaSO_4$$
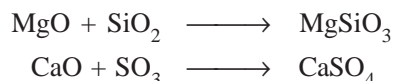
In the above reactions both MgO and CaO are the oxide ion donor and hence act as bases, whereas SiO_2 and SO_3 are the oxide-ion acceptor and hence act as acids. Ultimately, the Lux-Flood acid and base react to form magnesium silicate ($MgSiO_3$) and calcium sulphate ($CaSO_4$) salts respectively.

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 :

$$2NaHCO_3 \ + \ H_2SO_4 \ \longrightarrow \ Na_2SO_4 \ + \ 2H_2O \ + \ 2CO_2\uparrow$$
$$2(84.01)$$
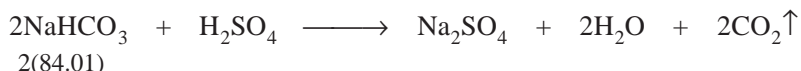
It is evident from the above equation that 2 ml of $NaHCO_3$ is equivalent to 1 ml of H_2SO_4. Hence, 1 ml of $NaHCO_3$ is 1 equivalent and the equivalent weight is equal to the gram-molecular weight *i.e.*, 84.01/ 2 = 42.0 g. One millilitre of 0.05 N sulphuric acid or 1 milliequivalent is equivalent to 42.0 mg or 1 meq of $NaHCO_3$.

Thus, the purity of the sample assayed may be calculated as follows :

$$\% \ NaHCO_3 = \frac{ml \times N \times 0.8401 \times 100}{wt.\,of\,sample}$$

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 :

$$2NaOH \ + \ H_2SO_4 \ \longrightarrow \ Na_2SO_4 \ + \ 2H_2O \qquad\qquad ...(a)$$
$$2(40.0)$$

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 :

$$2Na_2CO_3 \ + \ H_2SO_4 \ \longrightarrow \ Na_2SO_4 \ + \ 2NaHCO_3 \qquad\qquad ...(b)$$
$$2(106)$$

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 :

$$2NaHCO_3 \ + \ H_2SO_4 \ \longrightarrow \ Na_2SO_4 \ + \ 2CO_2 \uparrow \ + \ H_2O \qquad \qquad ...(c)$$
$$2(84.01)$$
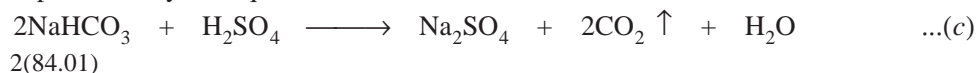
Calculations : The total volume of 1 N sulphuric acid consumed in the titration was required to neutralize NaOH and Na_2CO_3, thereby converting the latter first to $NaHCO_3$ at the phenolphthalein end-point and then to H_2CO_3 at the methyl orange end-point.

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 :

$$\% \ NaOH = \frac{ml \times N \times meq \ wt \times 100}{Sample \ wt}$$

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 :

$$\% \ Na_2CO_3 = \frac{ml \times 1 \times meq. \ wt. \times 100}{wt. \ of \ sample}$$

4.3.1.3. Cognate Assays

Sodium bicarbonate ; sodium salicylate tablets

S. No.	Name of Substance	Quantity Prescribed	Indicator Employed	Calculations
1.	Sodium Bicarbonate	1.0 g	Methyl orange	Each ml of 0.5 N H_2SO_4 $\equiv$ 0.042 g of $NaHCO_3$
2.	Sodium Salicylate Tablets	500 mg	Bromophenol Blue	Each ml of 0.I N HCl $\equiv$ 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 :

$$ZnO \ + \ H_2SO_4 \ \longrightarrow \ ZnSO_4 \ + \ H_2O$$
$$(81.38)$$
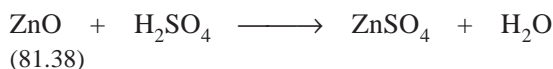
The requisite quantity of ZnO gets dissolved in the sulphuric acid thereby neutralizing an equivalent amount as shown by the above equation. Thus, the amount of sulphuric acid neutralized by the ZnO is estimated by subtracting, from the total amount of sulphuric acid utilized, the quantity neutralized 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 :

$$\% \ ZnO = \frac{(ml_1 \times N) - (ml_2 \times N) \times meq. \ wt. \times 100}{wt. \ of \ sample}$$

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_2SO_4 $\equiv 0.04068$ g of ZnO
2.	Ephedrine	0.5 g	Methyl Red	Each ml of 0.1 N HCl $\equiv 0.01652$ g of $C_{10}H_{15}NO$
3.	Lithium Carbonate	1.0 g	Methyl Orange	Each ml of 1 N HCl $\equiv 0.03695$ g of Li_2CO_3
4.	Milk of Magnesia	5.0 g	Methyl Red	Each ml of 1 N H_2SO_4 $\equiv 0.02916$ g of $Mg(OH)_2$
5.	Magnesium Stearate	1.0 g	Methyl Orange	Each ml of 0.1 N H_2SO_4 $\equiv 0.002015$ g of MgO
6.	Sodium Lactate Injection	10.0 ml	Methyl Orange	Each ml 0.1 N H_2SO_4 $\equiv 0.01121$ 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, conducto-metric 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 :

$$H_2C_4H_4O_6 \; + \; 2NaOH \; \longrightarrow \; Na_2C_4H_4O_6 \; + \; 2H_2O$$
$$(150.09)$$

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 :

$$\% \text{ Tartaric Acid} = \frac{ml \times 1 \times 0.07504 \times 100}{wt. \text{ of sample}}$$

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 :

$$CH_3SO_2.O(CH_2)_4O.SO_2CH_3 \; + \; 2H_2O \longrightarrow HO(CH_2)_4OH \; + \; 2CH_3SO_2.OH \qquad ...(a)$$
$$(246.29) \hspace{11cm} \text{Methane sulphonic acid}$$

$$CH_3SO_2OH \; + \; NaOH \longrightarrow CH_3SO_2ONa \; + \; H_2O \qquad\qquad ... (b)$$

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 :

$$\% \text{ Busulphan} = \frac{ml \times 0.1 \times 0.01232 \times 100}{wt. \text{ of sample}}$$

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 $\equiv 0.06106$ g of $C_7H_6O_2$.
2.	Cellulose Acetate Phthalate (For phthalyl groups)	0.4 g	-do-	Phthalyl groups : $\dfrac{1.49b}{w} - 1.795$ 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 $\equiv 0.02767$ g of $C_{10}H_{13}ClN_2O_3S$
4.	Citric Acid	3.0 g	-do-	Each ml of 1 N NaOH $\equiv 0.06404$ g of $C_6H_8O_7$
5.	Frusemide	0.5 g	Bromothymol Blue	Each ml of 0.1 N NaOH $\equiv 0.03308$ g of $C_{12}H_{11}ClN_2O_5S$
6.	Glibenclamide	0.5 g	Phenolphthalein	Each ml of 0.1 N NaOH $\equiv 0.0494$ g $C_{23}H_{28}ClN_3O_5S$
7.	Ibuprofen	0.5 g	-do-	Each ml of 0.1 N NaOH $\equiv 0.02063$ g of $C_{13}H_{18}O_2$
8.	Indomethacin	0.45 g	-do-	Each ml of 0.1 N NaOH $\equiv 0.03578$ g of $C_{19}H_{16}ClNO_4$
9.	Nicotinic Acid	0.3 g	Phenol Red	Each ml of 0.1 N NaOH $\equiv 0.01231$ g of $C_6H_5NO_2$
10.	Oxyphenbutazone	0.5 g	Bromothymol Blue	Each ml of 0.1 N NaOH $\equiv 0.03244$ g of $C_{19}H_{20}N_2O_3$
11.	Phosphoric Acid	1.0 g	Phenolphthalein	Each ml of 1 N NaOH $\equiv 0.049$ g of H_3PO_4
12.	Phenylbutazone*	0.5 g	Bromothymol Blue	Each ml of 0.1 N NaOH $\equiv 0.03084$ g of $C_{19}H_{20}N_2O_2$
13.	Salicylic Acid	2.0 g	Phenol Red	Each ml of 0.5 N NaOH $\equiv 0.06905$ 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 :

$$C_6H_4OCOCH_3COOH + HOH \longrightarrow C_6H_4OHCOOH + CH_3COOH \qquad ...(a)$$

The carbonyl group present in acetylsalicylic acid is subsequently neutralized with NaOH to yield :

$$C_6H_4OCOCH_3COOH + NaOH \longrightarrow C_6H_4OCOCH_3COONa + H_2O \qquad ...(b)$$
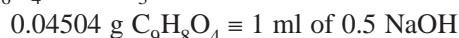sodium acetylsalicylate
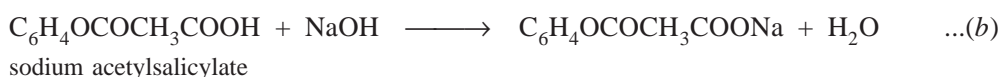
Further reaction of aspirin with excess of standard NaOH added followed by heating results in the saponification of the sodium acetylsalicylate as shown below :

$$...(c)$$
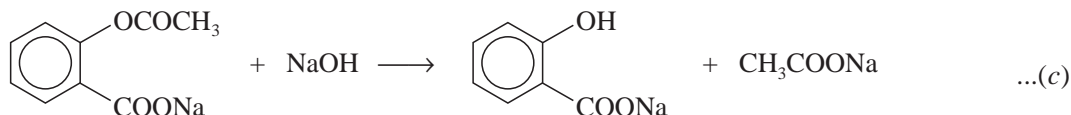
From equations (b) and (c), we have :

$$C_6H_4OCOCH_3COOH \equiv 2NaOH \equiv 2H \equiv 2000 \text{ ml N}$$

or $\qquad 0.04504 \text{ g } C_9H_8O_4 \equiv 1 \text{ ml of } 0.5 \text{ NaOH}$

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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7. Freiser, H and Q. Fernado : **Ionic Equilibria in Analytical Chemistry**, John Wiley, New York, 1963.

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5 NON-AQUEOUS TITRATIONS

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 Kb values less than 10^{-6} can be titrated satisfactorily by non-aqueous titrations. The reason being that in aqueous medium and at higher Kb 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 :

$$HA \rightleftharpoons H^+ + A^-$$

Acid   Proton   Base
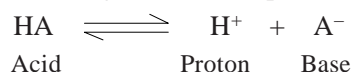
In other words, the liberated base A shall unite with a proton to give the corresponding conjugate acid HA of the base A because every base has its conjugate acid and *vice versa.*

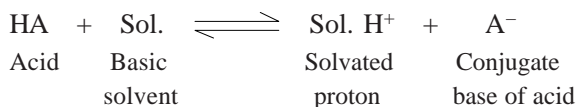Hence, from the above definitions it may be implied that :

(*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 :

$$HA + Sol. \rightleftharpoons Sol. H^+ + A^-$$

Acid   Basic   Solvated   Conjugate
       solvent   proton   base of acid

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 :

$$CH_3COOH \rightleftharpoons H^+ + CH_3COO^-$$
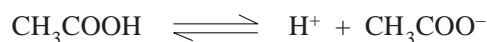
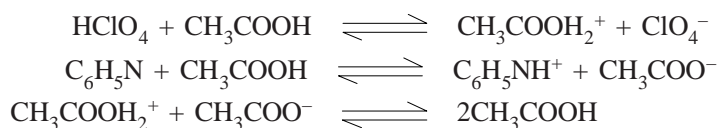In the above instance acetic acid is behaving as an acid.

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
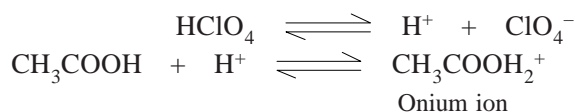
As the $CH_3COOH_2^+$ ion can instantly donate its proton to a base, therefore, a solution of perchloric acid in glacial acetic acid, behaves as a strongly acidic solution.

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.
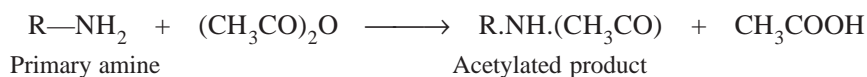
Procedure : Gradually mix 8.5 ml of perchloric acid to 900 ml of glacial acetic acid with vigorous and continuous stirring. Now add 30 ml acetic anhydride and make up the volume to 1 litre with glacial acetic acid and allow to stand for 24 hours before use.

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 :

$$H_2O \ + \ (CH_3CO)_2O \longrightarrow 2CH_3COOH$$
$$\text{Acetic anhydride} \qquad\qquad \text{Acetic acid}$$
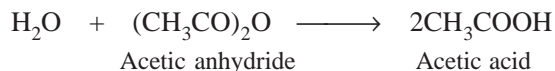
Precautions : The following precautions must be observed :

(*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 :
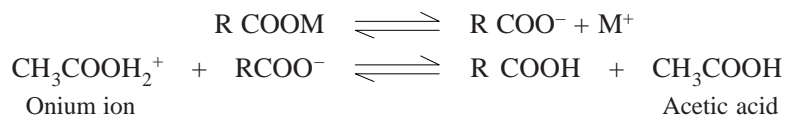
$$R—NH_2 \ + \ (CH_3CO)_2O \longrightarrow R.NH.(CH_3CO) \ + \ CH_3COOH$$
$$\text{Primary amine} \qquad\qquad\qquad \text{Acetylated product}$$

(*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 :

$$R\ COOM \ \rightleftharpoons \ R\ COO^- + M^+$$
$$CH_3COOH_2^+ \ + \ RCOO^- \ \rightleftharpoons \ R\ COOH \ + \ CH_3COOH$$
$$\text{Onium ion} \qquad\qquad\qquad\qquad \text{Acetic acid}$$

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
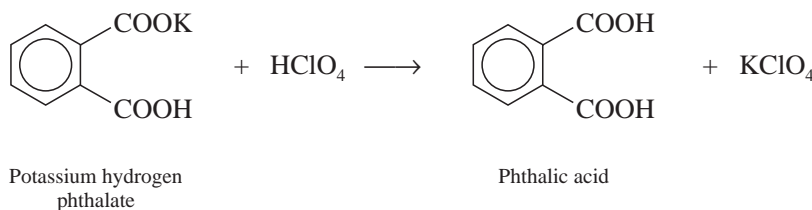
Therefore, $\qquad$ 204.14 g $C_8H_5O_4K \equiv HClO_4 \equiv 1000$ ml N

or $\qquad$ 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.
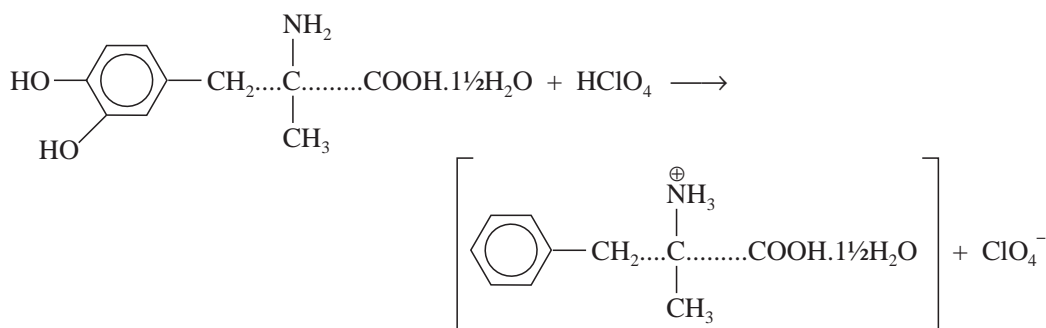
5.4.1.1. Titration of primary, secondary and tertiary amines

5.4.1.1.1. *Methlyldopa*

In general, the reaction taking place between a primary amine and perchloric acid may be expressed as follows :

$$R.NH_2 \ + \ HCl_4 \ \longrightarrow \ [R.NH_3]^+ \ + \ ClO_4^-$$
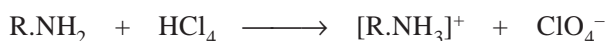
The specific reaction between methyldopa and perchloric acid is expressed by the following equation :

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 $C_{10}H_{13}NO_4$.

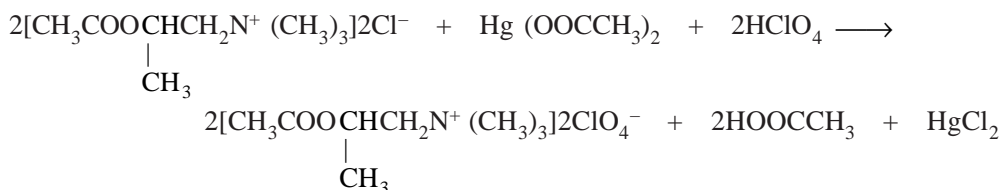**Calculations :** The percentage of methyldopa present in the sample is given by :

$$\% \ \text{Methyldopa} = \frac{ml \times 0.1 \times 0.02112 \times 100}{wt. \ of \ sample}$$

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 :

$$2[CH_3COOCHCH_2N^+ (CH_3)_3]2Cl^- \ + \ Hg \ (OOCCH_3)_2 \ + \ 2HClO_4 \ \longrightarrow$$
             |
             CH_3

$$2[CH_3COOCHCH_2N^+ (CH_3)_3]2ClO_4^- \ + \ 2HOOCCH_3 \ + \ HgCl_2$$
                      |
                      CH_3

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 :

$$\% \text{ Methacholine chloride} = \frac{ml \times 0.1 \times 0.01957 \times 100}{\text{wt. of sample}}$$

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 $C_{19}H_{13}NO_3$
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 $(C_{10}H_{15}N_3)_2 . H_2SO_4$
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 $C_{16}H_{14}ClN_3O$
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 $C_{10}H_{24}N_2O_2 . 2HCl$
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 0.1 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 $HClO_4 \equiv 0.02753$ g of $C_{13}H_{13}N_3O_4$
16.	Nicotinamide	0.2 g	Crystal violet	Each ml of 0.1 N $HClO_4 \equiv 0.01221$ g of $C_6H_6N_2O$
17.	Nikethamide	0.2 g	-do-	Each ml of 0.1 N $HClO_4 \equiv 0.01782$ g of $C_{10}H_{14}N_2O$
18.	Noscapine	0.5 g	-do-	Each ml of 0.1 N $HClO_4 \equiv 0.04134$ g of $C_{22}H_{23}NO_7$
19.	Phenindamine tartrate	0.8 g	Oracet Blue B	Each ml of 0.1 N $HClO_4 \equiv 0.04115$ g of $C_{19}H_{19}N, C_4H_6O_6$
20.	Pholcodine	0.5 g	Crystal violet	Each ml of 0.1 N $HClO_4 \equiv 0.01993$ g of $C_{23}H_{30}N_2O_4$
21.	Piperazine citrate	0.2 g	-do-	Each ml of 0.1 N $HClO_4 \equiv 0.01071$ g of $(C_4H_{10}N_2)_3, 2C_6H_8O_7$
22.	Potassium citrate	0.15 g	1-Naphthol benzein	Each ml of 0.1 N $HClO_4 \equiv 0.01021$ g of $C_6H_5K_3O_7$
23.	Prochlorperazine maleate	0.6 g	Crystal violet	Each ml of 0.1 N $HClO_4 \equiv 0.3030$ 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 $HClO_4 \equiv 0.02831$ 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 $HClO_4 \equiv 0.0499$ g of $C_{17}H_{20}N_2S$
26.	Pyrimethamine	0.5 g	Quinaldine red	Each ml of 0.1 N $HClO_4 \equiv 0.02487$ g of $C_{12}H_{13}ClN_4$
27.	Quinidine sulphate	0.4 g	Crystal violet	Each ml of 0.1 N $HClO_4 \equiv 0.02490$ 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 $HClO_4 \equiv 0.05486$ g of $C_{20}H_{24}N_2O_2$
29.	Saccharin sodium	0.3 g	-do-	Each ml of 0.1 N $HClO_4 \equiv 0.02052$ g of $C_7H_4NNaO_3S$
30.	Salbutamol sulphate	0.9 g	Oracet Blue-B	Each ml of 0.1 N $HClO_4 \equiv 0.05767$ 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 $HClO_4 \equiv 0.01361$ g of $C_2H_3NaO_2 . 3H_2O$
32.	Sodium benzoate	0.6 g	Crystal violet	Each ml of 0.1 N $HClO_4 \equiv 0.01441$ 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.
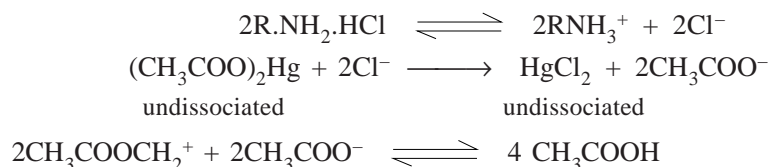
Table 5.2, gives the details of such determinations at a glance :

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 :
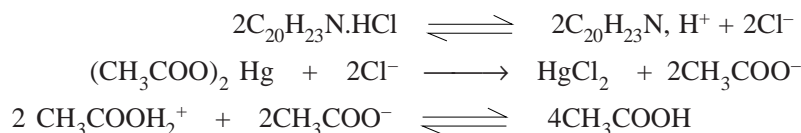
$$2R.NH_2.HCl \rightleftharpoons 2RNH_3^+ + 2Cl^-$$

$$(CH_3COO)_2Hg + 2Cl^- \longrightarrow HgCl_2 + 2CH_3COO^-$$
$$\text{undissociated} \qquad\qquad \text{undissociated}$$

$$2CH_3COOCH_2^+ + 2CH_3COO^- \rightleftharpoons 4\ CH_3COOH$$

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 :

$$C_{20}H_{23}N.HCl \equiv Cl^- \equiv CH_3COO^- \equiv HClO_4 \equiv H = 1000\ ml\ N$$

or $\qquad\qquad$ 313.87 g $\quad C_{20}H_{23}N.HCl \equiv 1000\ ml\ N.HClO_4$

or $\qquad\qquad$ 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 $HClO_4 \equiv 0.02638$ g of $C_{30}H_{40}Cl_2N_4$
7.	Diphenhydramine hydrochloride	0.75 g	-do-	Each ml of 0.1 N $HClO_4 \equiv 0.02918$ g of $C_{17}H_{21}NO . HCl$
8.	Ephedrine hydrochloride	0.5 g	-do-	Each ml of 0.1 N $HClO_4 \equiv 0.02017$ g of $C_{10}H_{15}NO. HCl$
9.	Ethylmorphine hydrochloride	0.3 g	-do-	Each ml of 0.1 N $HClO_4 \equiv 0.03499$ g of $C_{19}H_{23}NO_3 . HCl$
10.	Fluphenazine hydrochloride	0.6 g	-do-	Each ml of 0.1 N $HClO_4 \equiv 0.02552$ g of $C_{22}H_{26}F_3N_3OS, 2HCl$
11.	Imipramine hydrochloride	0.5 g	-do-	Each ml of 0.1 N $HClO_4 \equiv 0.03169$ g of $C_{19}H_{24}N_2 . HCl$
12.	Isoprenaline hydrochloride	0.5 g	Crystal violet	Each ml of 0.1 N $HClO_4 \equiv 0.02477$ g of $C_{11}H_{17}NO_3 . HCl$
13.	Lignocaine hydrochloride	0.6 g	-do-	Each ml of 0.1 N $HClO_4 \equiv 0.02708$ g of $C_{14}H_{22}N_2O . HCl$
14.	Meclizine hydrochloride	0.35 g	Quinaldine Red	Each ml of 0.1 N $HClO_4 \equiv 0.02319$ g of $C_{25}H_{27}ClN_2 . 2 HCl$
15.	Methadone hydrochloride	0.5 g	Crystal violet	Each ml of 0.1 N $HClO_4 \equiv 0.03459$ g of $C_{21}H_{28}ClNO$
16.	Methylamphetamine hydrochloride	0.4 g	-do-	Each ml of 0.1 N $HClO_4 \equiv 0.01857$ g of $C_{10}H_{15}N . HCl$
17.	Morphine hydrochloride	0.4 g	-do-	Each ml of 0.1 N $HClO_4 \equiv 0.03218$ g of $C_{17}H_{19}NO_3 . HCl$
18.	Morphine sulphate	0.5 g	-do-	Each ml of 0.1 N $HClO_4 \equiv 0.06688$ 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 $HClO_4 \equiv 0.0303$ g of $C_{12}H_{19}BrN_2O_2$
20.	Oxprenolol hydrochloride	0.4 g	1-Naphthol benzein	Each ml of 0.1 N $HClO_4 \equiv 0.3018$ g of $C_{15}H_{23}NO_3$
21.	Pentazoline hydrochloride	0.65 g	Crystal violet	Each ml of 0.1 N $HClO_4 \equiv 0.03219$ g of $C_{19}H_{27}NO. HCl$
22.	Pethidine hydrochloride	0.5 g	-do-	Each ml of 0.1 N $HClO_4 \equiv 0.02838$ g of $C_{15}H_{21}NO_2 . HCl$
23.	Pentobarbitone sodium	0.5 g	1-Naphthol benzein	Each ml of 0.1 N $HClO_4 \equiv 0.02542$ g of $C_{12}H_{11}N_2NaO_3$
24.	Phenylephrine hydrochloride	0.5 g	Crystal violet	Each ml of 0.1 N $HClO_4 \equiv 0.02037$ g of $C_9H_{13}NO_2 . HCl$
25.	Phenytoin sodium	0.4 g	1-Naphthol benzein	Each ml of 0.1 N $HClO_4 \equiv 0.02743$ g of $C_{15}H_{11}N_2NaO_2$
26.	Promethazine hydrochloride	1.0 g	Methyl orange	Each ml of 0.1 N $HClO_4 \equiv 0.03209$ g of $C_{17}H_{20}N_2S . HCl$
27.	Propoxyphene hydrochloride	0.6 g	Crystal violet	Each ml of 0.1 N $HClO_4 \equiv 0.03759$ g of $C_{22}H_{29}NO_2 . HCl$
28.	Propranolol hydrochloride	0.7 g	1-Naphthol benzein	Each ml of 0.1 N $HClO_4 \equiv 0.02958$ g of $C_{16}H_{21}NO_2 . HCl$
29.	Pyridoxine hydrochloride	0.4 g	Crystal violet	Each ml of 0.1 N $HClO_4 \equiv 0.02056$ g of $C_8H_{12}ClNO_3$
30.	Succinylcholine chloride	0.5g	-do-	Each ml of 0.1 N $HClO_4 \equiv 0.018078$ 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.
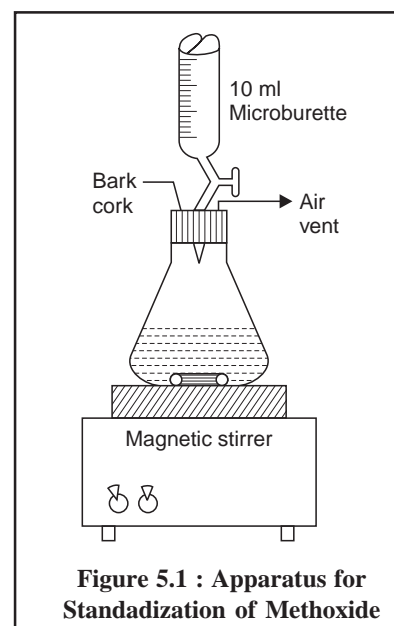

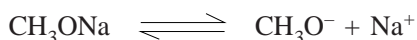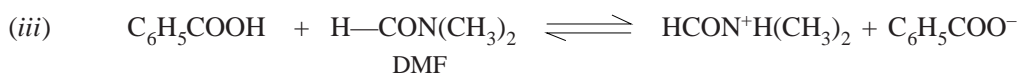**Figure 5.1 : Apparatus for Standadization of Methoxide**

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*) $$H_2O + CH_3ONa \longrightarrow CH_3OH + NaOH$$
$$H_2CO_3 + 2CH_3ONa \longrightarrow 2CH_3OH + Na_2CO_3$$
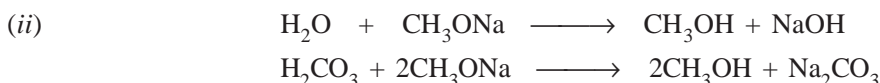
The clear solution of sodium methoxide must be kept away from moisture and atmospheric CO_2 as far as possible so as to avoid the above two chemical reactions that might ultimately result into the formation of turbidity.

(*iii*) $$C_6H_5COOH + H\text{—}CON(CH_3)_2 \rightleftharpoons HCON^+H(CH_3)_2 + C_6H_5COO^-$$
$$\text{DMF}$$

$$CH_3ONa \rightleftharpoons CH_3O^- + Na^+$$
$$HCON^+H(CH_3)_2 + CH_3O^- \longrightarrow HCON(CH_3)_2 + CH_3OH$$

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 |
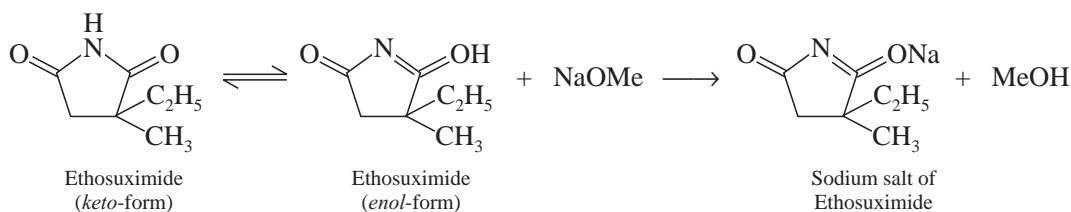
Calculations :

Therefore,   141.17 g $C_7H_{11}NO_2 \equiv$ 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
1.	Acetazolamide	0.4 g	*	Each ml of 0.1 N $NaOCH_3 \equiv 0.02222$ g of $C_4H_6N_4O_3S_2$
2.	Bendrofluazide	0.2 g	Azo violet	Each ml of 0.1 N $NaOCH_3 \equiv 0.02107$ g of $C_{15}H_{14}F_3N_3O_4S_2$
3.	Allopurinol	0.2 g	Thymol blue	Each ml of 0.1 N $NaOCH_3 \equiv 0.01361$ g of $C_5H_4N_4O$
4.	Mercaptopurine	0.3 g	-do-	Each ml of 0.1 N $NaOCH_3 \equiv 0.01522$ g of $C_5H_4N_4S$
5.	Amylobarbitone	0.5 g	Quinaldine Red	Each ml of 0.1 N $LiOCH_3 \equiv 0.02263$ g of $C_{11}H_{18}N_2O_3$
6.	Nalidixic Acid	0.25 g	Thymolph-thalein	Each ml of 0.1 N $LiOCH_3 \equiv 0.02322$ g of $C_{12}H_{12}N_2O_3$

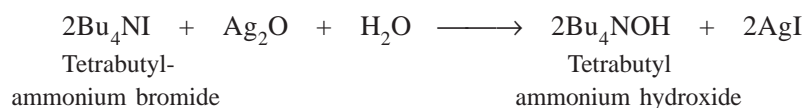* Potentiometric determination.

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 :

$$2Bu_4NI \ + \ Ag_2O \ + \ H_2O \ \longrightarrow \ 2Bu_4NOH \ + \ 2AgI$$

Tetrabutyl-
ammonium bromide

Tetrabutyl
ammonium hydroxide

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.
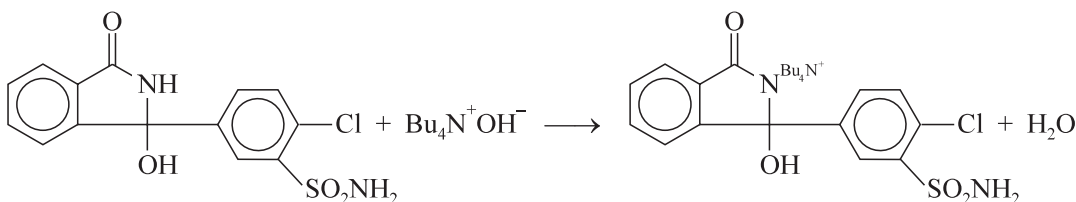
Calculations :

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 $C_{14}H_{11}ClN_2O_4S$.

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 $\equiv 0.03282$ g of $C_{14}H_{11}Cl_2NO_4$
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 $C_{13}H_8Cl_2N_2O_4$

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

1. Kucharsky, J., and L. Safarik, '**Titrations in Non-Aqueous Solvents**', New York, Elsevier North-Holland, 1965.

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3. Pietrzyk, DJ, and CW Frank, '**Analytical Chemistry**', London, Academic Press, 2nd ed., 1979.

4. Beckett, AH, and JB Stenlake, '**Practical Pharmaceutical Chemistry**', London, Athlone Press, 4th ed., Part-I, 1988.

5. Kolthoff IM and S Bruckenstein, Chap. 13 in **Treatise on Analytical Chemistry**, Part-I, Vol. I : IM Kolthoff and PJ Elving (eds.). Interscience, New York, 1959.

6. Bruckenstein S and IM Kolhoff, Chap. XIII in **Pharmaceutical Analysis**, T. Higuchi and E. Brochmann–Hanssen (eds.), Interscience, New York, 1961.