Chapter-1

Introduction
1.1. Indian Drug Regulations

Various regulatory aspects related to drug import, manufacture, sale and advertising in India are covered under the Drugs & Cosmetics Act of 1940 and the ensuing Drugs & Cosmetics Rules of 1945, the Pharmacy Act of 1948 and the Drugs & Magic Remedies (Objectionable Advertisements) Act of 1954. Of these, the Drug & Cosmetic Act (Act) of 1940, which led to the Drugs & Cosmetics Rules (Rules) of 1945, is central legislation that regulates India’s drug and cosmetic import, manufacture, distribution and sale. The Act’s main objective is to ensure that available human drugs are safe and efficacious and conform to prescribed quality standards, and marketed cosmetics are safe for use. Over the years, the Act has been amended several times to address public concerns.

The Central Drugs Standard Control Organization (CDSCO), headed by the Drugs Controller General (India) (DCG(I)), discharges the functions allocated to the Central Government (similar to the US Federal Government) under the Act. CDSCO is attached to the office of the Director General of Health Services in the Federal Ministry of Health and Family Welfare. The DCG(I) has statutory authority under the Act with port offices, zonal offices and drug testing laboratories.

The DCG(I)’s office is primarily responsible for:

- Approval of new drugs to be introduced in the country
- Permission to conduct clinical trials
- Registration and control of imported drug quality
- Developing regulatory measures and amendments to acts and rules
- Establishing standards for drugs, cosmetics, diagnostics and devices and updating the Indian Pharmacopoeia
- License approval as Central License Approving Authority for the manufacture of large volume parenterals, vaccines and biotechnology products and operating blood banks and also of such other drugs as may be notified by the federal government from time to time
- Coordinating the activities of the States and advising them on matters relating to uniform administration of the Act and Rules in the country
Despite the Act’s long history, nonuniformity in interpreting and implementing the provisions of laws have made it necessary to update the regulations to bring them up to par with international standards. Historically, regulations have developed mostly de novo, without much interaction with international regulatory agencies. The Act has been reviewed by several government advisory committees since the 1980s, who reached the conclusion that the legislation is reasonably well drafted but its enforcement had been weakened by poor guidelines and variable regulatory personnel interpretation.

After several years of deliberations by various advisory committees, the Indian government plans to strengthen CDSCO and the office of the DCG(I) and considerably expand and reorganize CDSCO along the lines of FDA. DCG(I), along with the Indian Council of Medical Research (ICMR) have adopted international regulatory guidelines and issued Indian versions of the same. ICMR issued the Ethical Guidelines for Biomedical Research on Human Subjects in 2000 and Indian GCP guidelines were released by CDSCO in December 2001. The Drug Technical Advisory Board (DTAB), the highest technical body under the Drugs & Cosmetics Act, has endorsed adoption of GCP guidelines for streamlining clinical studies in India.

1.2 Schedule Y

Schedule-Y defines Requirements and Guidelines for Permission to Import And / Or Manufacture of New Drugs for Sale or To Undertake Clinical Trials in India. (Drugs and Cosmetic Rules 122A, 122B, 122D, 122DA, 122DAA and 122E).

1. Rule 122-A: Application for permission to import new drug

2. Rule 122-B: Application for approval to manufacture new drug

3. Rule 122-D: Permission to import or manufacture FDC


5. Rule122-DAA: Clinical trial Definition

Clinical trial” means a systematic study of new drug(s) in human subject(s) to generate data for discovering and / or verifying the clinical, pharmacological (including pharmacodynamic
Chapter 1

Introduction

and pharmacokinetic) and/or adverse effects with the objective of determining safety and/or
efficacy of the new drug.

6. Rule 122-E: New Drug Definition

- Not been used in the country under labeling conditions

- Approved but now proposed to be marketed with modified or new claims—indications, dosage, dosage form, route of administration

- FDC, individually approved, to be combined for the first time in a fixed ratio or if ratio is changed.

As Per The Schedule-Y of the Drug and Cosmetics Rules, 1945 Requirements And Guidelines For Permission To Manufacture of New Drugs (which is approved outside India but not approved in India) a Phase-III Clinical study and Bioequivalence study is required to carry out in Indian Population.

A Phase-III Clinical Study: For new drugs approved outside India, Phase III studies need to be carried out primarily to generate evidence of efficacy and safety of the drug in Indian patients when used as recommended in the prescribing information. Prior to conduct of Phase III studies in Indian subjects, Licensing Authority may require pharmacokinetic studies to be undertaken to verify that the data generated in Indian population is in conformity with the data already generated abroad.

Bioavailability/Bioequivalence Studies: For drugs approved elsewhere in the world and absorbed systemically, bioequivalence with the reference formulation should be required to carry out.

Clinical trials in India are regulated by Schedule-Y of the Drug and Cosmetics Rules, 1945. The Rules were revised in 2005 and the current rules, the Drugs and Cosmetics (IInd Amendment) Rules, 2005, were released 20 January 2005. Under the updated Rules, the Schedule-Y was extensively revised to bring the Indian regulations up to par with internationally accepted definitions and procedures.

Schedule-Y is the requirements and guidelines for import and/or manufacture of new drugs for sale or for clinical trials. These include details of the application process and components of the application for permission to conduct clinical trials, and the responsibilities of the
sponsor, investigators, and the Independent Ethics Committee (IEC). A clinical trial can only be initiated after obtaining written permission from DCG(I) and an IEC.

1.3 Regulatory bodies of different countries

1. **International**: International Conference on Harmonisation (ICH), World Health Organization (WHO).
2. **Australia**: Australia's Department of Health and Aged Care
3. **Brazil**: National Health Surveillance Agency (Anvisa)
4. **China**: State Food and Drug Administration (SFDA)
5. **Europe**: European Medicines Agency (EMEA)
6. **India**: Central Drug Standard Control Organization (CDSCO)
7. **Japan**: Ministry of Health and Welfare
8. **Malaysia**: National Pharmaceutical Control Bureau
9. **Russia**: Association of International Pharmaceutical Manufacturers
10. **Singapore**: Health Sciences Authority (HSA)
11. **South Africa**: Medicines Control Council (MCC)
12. **Switzerland**: Swiss Agency for Therapeutic Products
13. **UK**: Medicines and Healthcare Products Regulatory Agency (MHRA)
14. **USA**: The Food and Drug Administration (FDA)

In the US, the 1984 Hatch-Waxman Act introduced the Food and Drug Administration's (FDA) Abbreviated New Drug Application (ANDA) scheme to attempt to decrease the time required for approval, thus allowing the savings attributable to generic drugs to commence sooner. Under the ANDA, a generic drug seeking marketing approval must have the same active ingredient(s) as the innovator product; must use the same route of administration; must have a similar rate and extent of absorption of the active ingredient (Bioequivalence with innovator product); and must be produced in facilities that meet good manufacturing process guidelines. Generic manufacturers are not required, however, to include pre-clinical and clinical data to establish safety and effectiveness.

Most nations require generic drug manufacturers to prove their formulation exhibits bioequivalence to the innovator product. In the U.S., the FDA must approve generic drugs just as innovator drugs must be approved. The FDA requires the bioequivalence of the generic product to be between 80% and 125% of that of the innovator product.
In India, as per the Central Drugs Standard Control Organization (CDSCO) guidelines, to manufacture and market the new drug which is already approved and marketed in other countries but not approved in India, bioequivalence study and Phase III studies need to be carried out locally primarily to generate evidence of efficacy and safety of the drug in Indian patients.

1.4 Bioavailability

In pharmacology, bioavailability (BA) is a subcategory of absorption and is used to describe the fraction of an administered dose of unchanged drug that reaches the systemic circulation, one of the principal pharmacokinetic properties of drugs. By definition, when a medication is administered intravenously, its bioavailability is 100%. However, when a medication is administered via other routes (such as orally), its bioavailability generally decreases (due to incomplete absorption and first-pass metabolism) or may vary from patient to patient.

Bioavailability is one of the essential tools in pharmacokinetics, as bioavailability must be considered when calculating dosages for non-intravenous routes of administration. Bioavailability is defined as the rate and extent to which the active ingredient or active moiety is absorbed from a drug product and becomes available at the site of action. For drug products that are not intended to be absorbed into the bloodstream, bioavailability may be assessed by measurements intended to reflect the rate and extent to which the active ingredient or active moiety becomes available at the site of action. Bioavailability is also defined as the relative amount of drug from an administered dosage form which enters the systemic circulation and the rate at which the drug appears in the systemic circulation.

1.5 Bioequivalence

Bioequivalence is defined as the absence of a significant difference in the rate and extent to which the active ingredient or active moiety in pharmaceutical equivalents or pharmaceutical alternatives becomes available at the site of drug action when administered at the same molar dose under similar conditions in an appropriately designed study.
Chapter 1

Introduction

1.5.1 Pharmacokinetics

The definition of Bioavailability and Bioequivalence emphasize the use of pharmacokinetic measures in an accessible biological matrix such as blood, plasma and/or serum to indicate release of the drug substance from the drug product into the systemic circulation.

1.5.2 Pharmacokinetic terms

1. Peak plasma concentration ($C_{\text{max}}$)- This is the maximum drug concentration achieved in systemic circulation following drug administration.

2. Time of peak concentration ($T_{\text{max}}$)- It is the time required to achieve maximum drug concentration in systemic circulation.

3. Area under the curve (AUC)-
   a) $\text{AUC}_{0-t}$- Area under the plasma concentration-time curve from 0 hr to the last quantifiable concentration to be calculated using trapezoidal rule.
   b) $\text{AUC}_{0-\infty}$- Area under the plasma concentration-time curve from 0 hr to infinity concentration to be calculated as the sum of $\text{AUC}_{0-t}$ plus the ratio of the last measurable concentration to the elimination rate constant.

4. Elimination rate constant ($K_e$)- Apparent first-order terminal elimination rate constant calculated from a semi-log plot of the plasma concentration Vs time curve.

5. Elimination half life ($T_{1/2}$)- Elimination half life of a drug is the time necessary to reduce the drug concentration in the blood, plasma or serum to one-half after equilibrium is reached.

1.6 Bioequivalence Study

Bioequivalence studies are always conducted as per the guidelines published by the regulatory agency of the country where the product is to be register or marketed and ICH GCP guidelines.

1.6.1 Bioequivalence Study design
The bioequivalence study design is depends on the physico-chemical characteristics of the substance, its pharmacokinetic properties and proportionality in composition. The study should be designed in such a way that the formulation effect can be distinguished from other effects.

- **Standard design**
  If two formulations are compared, a randomised, two-period, two-sequence single dose crossover design is recommended. The treatment periods should be separated by a wash out period sufficient to ensure that drug concentrations are below the lower limit of bioanalytical quantification in all subjects at the beginning of the second period. Normally at least 5 elimination half-lives are necessary to achieve this.

- **Alternative designs**
  Under certain circumstances, provided the study design and the statistical analyses are scientifically sound, alternative well-established designs could be considered.
  1. **Parallel design**
     If a drug is known to have very long half life, then parallel design can be used. But it is not widely used for bioequivalence studies because with this design inter subject variability can not be identified or remove because each subject gets only one treatment either test or reference. This design may be considered when inter subject variability is small compared to intra subject variability and the drug is potentially toxic and / or has a very long half life.
  2. **Replicate design**
     This type of study design is used for the drug known for highly variable pharmacokinetic characteristics. Replicate design allows comparison of within-subject variances for the test and reference products. Provides more information about the intrinsic factors underlying formulation. It reduces the number of subjects participating in the bioequivalence study.
  3. **Multiple dose design**
     In the rare situation where problems of sensitivity of the analytical method preclude sufficiently precise plasma concentration measurements after single dose administration and where the concentrations at steady state are sufficiently high to be reliably measured, a multiple dose study may be acceptable as an alternative to the single dose study. Recent development in the bioanalytical methodology, it is unusual that parent drug cannot be measured accurately and precisely hence, use of a multiple dose study instead of a single dose study due to limited sensitivity of the analytical method, is acceptable only in exceptional cases.
Chapter 1

1.6.2 Randomization

Randomization is the process of assigning trial subjects to treatment or control groups using an element of chance to determine the assignment in order to reduce bias\(^9\). Randomization schedule for dosing of investigational products is predetermined by statistician before initiation of study. Randomization depends on the type of study design. Commonly randomization is taken out with help of statistical software.

1.6.3 Study Population\(^10\)

The number of subjects to be included in the study should be based on an appropriate sample size calculation. The number of evaluable subjects in a bioequivalence study should not be less than 12. Bioequivalence studies are generally performed with healthy volunteers. The inclusion/exclusion criteria should be clearly stated in the protocol. Subjects should be 18 years of age or older and preferably have a Body Mass Index between 18.5 and 30 kg/m\(^2\). The subjects should be screened for suitability by means of clinical laboratory tests, a medical history, and a physical examination before conduct of the study.

Subjects could belong to either sex; however, the risk to women of childbearing potential should be considered.

Subjects should preferably be non-smokers and without a history of alcohol or drug abuse.

1.6.4 Sampling schedule

A sufficient number of samples should be collected to adequately describe the plasma concentration-time profile. The sampling schedule should include frequent sampling around predicted \(T_{\text{max}}\) to provide a reliable estimate of peak exposure. In particular, the sampling schedule should be planned to avoid \(C_{\text{max}}\) being the first point of a concentration time curve. The sampling schedule should also cover the plasma concentration time curve long enough to provide a reliable estimate of the extent of exposure which is achieved if \(\text{AUC}_{0-t}\) covers at least 80% of \(\text{AUC}_{0-\infty}\).\(^{10}\)

The sampling can continue for atleast three or more terminal half lives of the drug. Wash out period should be adequate atleast 5 half lives of the drug to be measured.\(^8\)

After collection and segregation of study samples, samples are stored below \(-20^\circ\text{C}\) in deep freezers till the completion of analysis.
1.6.5 Independent Ethics Committee (IEC)

An independent body constituted of medical professionals and non-medical members, whose responsibility it is to ensure the protection of the rights, safety and well-being of human subjects involved in a trial and to provide public assurance of that protection, by, among other things, reviewing and approving / providing favorable opinion on, the trial protocol, the suitability of the investigator(s), facilities, and the methods and material to be used in obtaining and documenting informed consent of the trial subjects. IEC should consist of at least 5 members out of which at least one member whose primary area of interest is in nonscientific area and at least one member who is independent of the institution / trial site.

1.6.6 Informed Consent

The subjects can get enroll in the study only after signing informed consent form. Informed Consent is the process by which a subject voluntarily confirms his or her willingness to participate in a particular trial, after having been informed of all aspects of the trial that are relevant to the subject's decision to participate.

1.6.7 Statistical Interpretation of Data

After the collection of the data, statistical methods must be applied to determine the level of significance of any observed difference in the rate and / or extent of absorption in order to establish bioequivalence between two or more drug products. Analysis of Variance (ANOVA) method should be applied to determine statistical differences between test and reference products. The 90 % confidence interval (C.I.) for the test to reference ratio at the 5% significance level for the parameter under consideration should be considered for testing bioequivalence. To meet the assumptions of normality of the data underlying the statistical analysis, the logarithmic transformation should be carried out for the pharmacokinetic parameters C\text{max} and AUC before performing statistical analysis.

To establish the bioequivalence, the calculated 90 % CI for C\text{max} and AUC should fall within the bioequivalence range, usually 80 % to 125 %.

1.7 Method development

Bioanalysis is a term generally used to describe the quantitative measurement of a compound in biological fluids primarily blood, plasma, serum, urine or tissue extracts. A sensitive,
accurate, precise and specific bioanalytical method is required to monitor drug levels in plasma and urine in clinical studies.\(^\text{15}\).

A bioanalytical method consists of following steps:

- Selection of Analytical techniques
- Optimization Chromatographic conditions
- Optimization of sample purification technique

### 1.7.1 Selection of Analytical Technique

Method development starts with the choice of the techniques e.g. chromatography or spectrophotometric technique. Scientific data and results have to be accurate, precise and reliable and are subject to ever increasing scrutiny by regulators in industry, the environment and medicine, in validation and also in research and development. Therefore, the choice of instrument to be used in particular circumstances is an important decision.\(^\text{16}\). Since many years chromatographic estimation play a very important role in field of analysis. HPLC instrument was method of choice in many areas. Analytical methods and techniques are constantly undergoing changes and improvements in many instances. In the scientific area small molecule bioanalysis has been revolutionized by LC-MS/MS over last two decades. Tremendous progress has been made towards achieving greater sensitivity, specificity and speed.\(^\text{17}\).

Our choice of technique was Liquid chromatography. HPLC with UV detector and Mass Spectrometer were techniques of choice for the method development.

### 1.7.2 Optimization of chromatographic conditions

It is further divided into three parts

1. Optimization of mobile phase
2. Column Selection
3. Optimization of other chromatographic parameters

**(A)Optimization of Mobile Phase**\(^\text{18}\)

**Composition of mobile phase**: usually water is used as a base solvent to which varying concentrations of miscible organics are added. Solvent strength is usually adjusted by varying the composition of the solvent mixture. Methanol is the most commonly used organic solvent. Acetonitrile and tetrahydrofuran are next most commonly used solvents in that order.
pH of mobile phase: Changes in pH can change the separation selectivity for ionised or ionisable solutes, since charged molecules are distributed preferentially into the aqueous or more polar phase. Variation in pH normally are not effective in obtaining desired changes in selectivity with solute that do not ionize. Acidic drug shows decrease in retention time if pH of the mobile increases, till pH exceeds pKa. In contrast to these acids, the retention time of basic drugs increases as pH increases. A neutral compound shows little change in retention with variation in pH, because it does not contain ionisable groups. Occasionally various salts are added to the aqueous mobile phase in reverse phase to vary solute retention or selectivity.

(B) Column Selection
The column is the heart of HPLC separation process. The availability of a stable, high performance column is essential in developing a rugged reproducible method. Different columns can vary in plate number, band symmetry, retention, band spacing and life time. Traditionally, HPLC columns were polar, e.g. silica, and the mobile phases used were relatively nonpolar in nature. This mode of HPLC was called normal phase chromatography. More common today is ‘reversed’ phase HPLC where the columns are hydrophobic, e.g. C-8, C-18 bonded phase. Solvents used in reversed phase HPLC include any miscible combination of water and various organic modifiers (the most common are methanol or acetonitrile). A change in column length, particle size or flow rate can sometimes be used to achieve an acceptable final separation, especially when only a minor improvement in resolution is required. A smaller particle size is capable of providing better resolution with no increase in run time, or faster separations with no loss in resolution. Column packed with porous polymeric particles can also be useful for developing HPLC methods. The main advantage of porous polymers is that are applicable in the pH range 1 to 13. These columns can be used for separating highly basic solutes at high pH. The use of porous polymers at high pH often produces good peak shape with highly basic compounds in non ionized state.

(C) Optimization of other chromatographic conditions
Optimization of column temperature, flow rate, injection volume and autosampler temperature, help in improving peak shape, increasing the response of analyte or increasing the stability of compound.
(D) Optimization of sample purification technique

Sample preparation, also known as sample treatment/sample clean-up/sample extraction, is an integral part of bioanalytical method. Biological matrix has a complex biochemical nature and comprises numerous components, viz. salts, acids, bases, proteins, cells, exogenous/endogenous small organic molecules like lipids and lipoproteins. Sample preparation is a process which aims at selective isolation of the analyte of interest from the matrix, minimization/elimination of matrix components in the processed sample and, if required, concentration of the analyte of interest. Sample preparation is an extremely important part of an analysis programme. It is very important that the matrices are removed by proper sample clean up procedure before the sample is introduced into a chromatographic system.

The benefits of suitable sample preparation are listed below:

1. Samples are free of interferences,
2. The column is not damaged
3. Sample becomes compatible with the intended HPLC method that is the sample solvent will dissolve in the mobile phase without affecting sample retention or resolution.
4. It may also possible to concentrate the analyte and/or derivatize them for improved detection or better separation.
5. Method precision and accuracy is determined by the sample pretreatment procedure.
6. It provides quantitative recovery of analyte
7. Involves a minimum number of steps and be easily automated.

Sample pretreatment techniques mainly comprises of Protein Precipitation, Liquid-Liquid extraction and Solid Phase Extraction.

1.7.3 Protein precipitation

This technique mainly depends on precipitation of matrix proteins. The acids like trichloroacetic acid and perchloric acid or solvents like methanol and acetonitrile are used in various proportions to precipitate the proteins.

Advantages:

1. It is very easy and rapid sample processing technique
2. The cost of the sample processing technique is very less
3. The solvents and acids required for this technique are easily available
4. This technique is very useful when analyte is not extractable
Chapter 1

Introduction

Disadvantages

1. Chance of incomplete precipitation of proteins
2. It decreases the life of HPLC column
3. It decreases the sensitivity of the method by dilution effect
4. Sometimes selectivity may not be obtained

Advances in Protein Precipitation technique

Protein precipitation plates

Protein Precipitation plates have been developed so that, after precipitation of protein, filtration can be carried out in the same well without centrifugation and supernatant transfer steps. Most commonly, PP plates are available in 96-well format, which enables both manual and robotic automation for the PP process. Structurally, these plates contain tubes with membrane/depth filter and are attachable to vacuum filtration. The filtration collection plates are made to be compatible with most of the modern autosamplers for easy automation of the whole process.

1.7.4 Liquid-Liquid Extraction

Liquid-Liquid extraction (LLE) involves partitioning of the sample between two immiscible phases and is used for separating analytes from interfering matrix. Aqueous phase is biological matrix and the analyte is extracted into the organic phase. Analyte are easily recovered by evaporation of the solvent. Chemical equilibria involving changes in pH, ion pairing, complexation and so on can be used to enhance analyte recovery and / or the elimination of interferences.

Advantages:

1. This method is more selective and sensitive
2. Acceptable percentage recovery achievable
3. This technique is also not too costly

Disadvantages:

1. Time consuming process
2. Chances of formation of emulsion
3. Chances of error are more and precautions are required
4. Chances of environmental pollution is more
5. It requires special equipments like shakers, evaporators etc
6. Some times low / variable recoveries
(A) Advances in LLE

1. Salting out assisted LLE
When an inorganic salt is added to mixture of water and a water-miscible organic solvent, it leads to the separation of the solvent from the mixture and forms a biphasic system, a phenomenon commonly termed as ‘salt-induced phase separation’. The effectiveness of the salting-out depends on the physicochemical properties of the analyte and the type of salt used. Sodium chloride, ammonium sulphate, magnesium sulphate, calcium chloride, potassium carbonate, calcium sulphate can be used for this purpose.29

2. Flash Freezing
Use of dry ice in liquid-liquid extraction of plasma, serum or blood helps in further solidification of precipitated impurities. After extraction with organic liquid test tubes racks are placed in dry ice all the matrix components solidifies and removal of upper organic layer becomes easy. This technique can also be used in combination with protein precipitation technique.

1.7.5 Solid Phase Extraction19, 21
Solid phase extraction (SPE) is the most important technique used in sample pretreatment for chromatography.

Advantages:
1. SPE is a very fast and easy so more efficient process than LLE.
2. It is easier to obtain better recovery of the analyte.
3. With SPE it is possible to obtain a more complete removal of interferences from the analyte fraction.
4. Reverse-phase SPE (RP-SPE) techniques are most popular, as only small amounts of organic solvent are required for elution.

Disadvantages:
1. It is very expensive technique as the cost of SPE cartridges is very high.
2. It requires sophisticated instrumentation, which is also very expensive.

The application of SPE generally involves four steps
1. Conditioning of packing
2. Sample application
3. Washing the packing (removal of interferences)
4. Recovery of the sample
Step 1: carried out prior to addition of sample, the packing is conditioned by the passage of a few bed volumes of solvents, typically methanol or acetonitrile through the cartridge. The role of the conditioning step is (1) removes any impurities that may have collected while the cartridge was exposed to laboratory environment and (2) allows the sorbent to be solvated. Salvation is important because reverse-phase packing that have been allowed to dry out often exhibit decreased sample retention.

Step 2: in the SPE experiment involves sample application where the sample is added to the cartridge. The sample for SPE can be applied with pipette or syringe or pumped into the cartridge.

Step 3: Provides for the removal of interferences by washing the cartridge with solvents. Interferences that are weaklier retained than the analyte are washed from the cartridge, but no loss of analyte occurs. Water, buffer or small amount of organic solvent can be used to wash solvent in RP-SPE.

Step 4: Provides for elution and collection of the analyte fraction. If detection sensitivity is a major goal, the analyte should be collected in as small a volume or can evaporate to dryness is often required in any event to concentrate the sample.

(A) Advances of SPE technique

1. Molecularly Imprinted Polymer SPE

Molecularly imprinted polymers are highly cross-linked polymers which have artificially generated recognition sites that are intentionally engineered and specific to the chemistry of a target analyte or class of analytes to provide high selectivity. It offers three mechanisms for the selective analyte retention viz. covalent, non-covalent and semi-covalent bonding. As a result selectivity is significantly improved, the interfering background can be reduced and much lower detection limits can be achieved.

2. On-line SPE

On-line solid phase extraction (OLSPE) methods are easier and faster prior to transfer to an analytical column. Two types of online SPE columns are commercially available: the RAM column turbulent flow chromatography (TFC) column

RAMs have been appearing in the scientific literature in the past two decades. RAMs are used mainly for the analysis of substances with low molecular mass (e.g. drugs, endogenous substances and xenobiotics) in complex matrices containing high molecular substances (most frequently proteins). RAM HPLC columns eliminate the sample clean-up and can be used as a
pre-column for the direct injection of biological samples such as serum and plasma. In most cases, they function as a pre-column, in combination with an analytical column and column switching, to remove proteinaceous materials prior to chromatographic separation. RAM columns are characterized by hydrophilic/hydrophobic, ion-exchange or size exclusion mechanisms. The hydrophilic phase covers the outer surface of a sorbent and large biomolecules such as proteins are restricted from the adsorpive surfaces inside silica particles. The hydrophobic or ion-exchange phases cover the internal surface of sorbent pores where small analyte molecules are able to penetrate into pores and interact with a stationary phase bonded on the inner surface. As a result, protein molecules pass through the column rapidly and analytes of interest are retained on the adsorptive sites. Depending on the application, the analyte molecules are directed to MS detection or transferred onto an analytical column for separation prior to MS detection.

Recently, TFC, a high flow chromatographic technique, has been reported for the automated on-line clean-up of biological fluids. TFC methods are based on the direct injection of biological samples without extraction or treatment onto a column with small internal diameter (1mm or 0.18µm) and packed with large particles (20–60µm), which imparts an additional level of selectivity via the stationary phase chemistry. TFC offers the advantage of unique flow dynamics occurring inside the column with high flow rates in the range of 1.5–5.0 mL/min. When the mobile phase flows through a TFC, higher linear velocities 100 times greater than those typically seen in HPLC columns are generated. The large interstitial spaces between column particles and high linear mobile phase velocity create turbulence within the column. As small molecular weight molecules diffuse faster than large molecular weight molecules, the small compounds diffuse into the particle pores and bind to the internal surfaces of the stationary phase particles. The turbulent flow of the mobile phase quickly flushes the large molecular weight compounds such as proteins through the column to waste before they have an opportunity to diffuse into the particle pores. TFC columns with a variety of chemistries to accommodate different analyte types are commercially available.

1.7.6 Selection of Internal standards

During the maturation of modern bioanalysis the use of internal standards has become an integral part of bioanalytical methods. The main purpose of utilizing internal standards is to correct any variation other than that related to the amount of analyte present in a sample, such as the variability in dilutions, evaporation, degradation, recovery, adsorption, derivatisation, injection and detection. Hence internal standard should be added in the sample processing as
early as possible, usually added immediately after the aliquoting of samples\textsuperscript{22}. Internal standard should have the same or similar physicochemical properties as its analyte, which means that they should usually have similar molecular weights and synthesizing routes for their reference standards\textsuperscript{23}.

The selection of internal standard is based on two different approaches one a structural analogue related to analyte and other a labeled internal standard with stable isotopes like (2H or D, 13C etc). The labeling sites in a molecule should be carefully chosen because stable and sensitive ion is required for quantitation during LCMS analysis.

Stable isotope is preferred in LC-MS/MS as it shows similar properties as that of analyte also it shows same retention time. Any variation during processing or injection like voltage / gas fluctuation can be encountered by using stable isotope as an internal standard.

The structural analogues are generally useful in HPLC analysis as in HPLC analysis adequate resolution between analytical peak and internal standard peak is required.

### 1.8 Method Validation

The main objective of validation of the method is to demonstrate the reliability of a particular method for the quantitative determination of an analyte’s concentration in a specific matrix. Reliability of the analytical results depends upon\textsuperscript{20}: (i) Stability of the analytes in the biological matrix under processing conditions and during the entire period of storage (ii) Specificity (iii) Accuracy (iv) Sensitivity (v) Precision (vi) Response function.

Validation of all the developed methods was performed as per the international guideline for method validation\textsuperscript{24-27}. The procedure followed for estimation of all the method validation parameters, is as follows.

#### 1.8.1 Carry over effect

Carry over effect is performed to study the rinsing cycles or wash program of auto injector required to wash the injection needle properly so as not to get any interference from the previous injection.

Prepare sufficient number of recovery comparison sample of 1.5 to 1.8 times of non-extracted upper limit of quantitation containing internal standard(IS), extracted sample containing 1.5 to 1.8 times concentration of highest calibration standard with IS, Lower Limit of Quantiataion (LLOQ) sample in duplicate with IS from biological matrix and extracted blank samples from the same matrix lot. Injection program of high concentration sample followed by blank or
mobile phase should be used to study the carry over effect. Check for the interference in mobile phase.

Acceptance criteria:
- Percentage of carry over observed at the retention time of analyte or IS in mobile phase and blank plasma samples must be \( \leq 20\% \) of the mean area response of analyte obtained in extracted LLOQ samples and \( \leq 5\% \) of the mean area response of IS obtained in extracted LLOQ samples.

### 1.8.2 Selectivity

Selectivity exercise is performed to access the ability of the bioanalytical method to differentiate and quantify the analyte(s) in presence of other components in the sample. In case of plasma and serum as biological matrix, procure at least six individual batches of plain plasma/ serum and plasma /serum separated from hemolysed blood Process and inject all the blank matrix lots along with LLOQ spiked samples.

The run time of blank samples should be at least four times the retention time of the last eluting peak of interest to check for late eluting peaks in case of HPLC -UV.

Evaluate the interference in terms of the response of the interfering peak at the retention time of analyte and internal standard in blank sample compared against the mean response obtained in the processed sample of LLOQ for analyte and internal standard, respectively.

Acceptance criteria:
- The response of the interfering peak at the retention time of analyte(s) should be \( \leq 20\% \) of the mean response of the processed LLOQ.
- The response of the interfering peak at the retention time of internal standard should be \( \leq 5\% \) of the mean response of the internal standard in processed LLOQ samples.
- At least 80% of all the processed blank matrix lots should meet the above mentioned acceptance criteria.

### 1.8.3 Matrix effect (only for mass spectrometric methods)

Matrix effect is investigated to ensure that selectivity and precision are not compromised within the matrix screened. Matrix effect may arise due to co-elution of some unintended components present in biological samples or which are added as part of analysis. These components result in ion suppression / enhancement, decrease / increase in sensitivity of
analytes over a period of time, increase in baseline, imprecision of data, drift in retention time and distortion or tailing of chromatographic peak. Thus assessment of matrix effect constitutes an important and integral part of validation for quantitative LC-MS/MS method. Extract 3 blank samples from each of at least six batches of matrix under screening. Prepare Low Quality Control (LQC), Medium Quality Control (MQC) and High Quality Control (HQC) spiking dilutions and internal standard dilution. Spike LQC, MQC and HQC spiking dilutions and IS dilution into the above extracted blank samples. Prepare recovery comparison sample at LQC, MQC and HQC concentration level along with IS. Inject the spiked samples and 6 replicates of the recovery comparison samples. Calculate the mean and precision (% C.V.) for area response obtained for both Analyte and internal standard in spiked samples as well as in recovery comparison samples. 

Acceptance criteria
- Precision of IS normalized Matrix factor at LQC, MQC and HQC level should be ≤ 15%.

1.8.4 Sensitivity
Sensitivity is measured in terms of LLOQ (lower limit of quantification). Process and inject the six LLOQ samples along with calibration curve standards and calculate the concentration of the LLOQ samples.

Acceptance criteria:
- The accuracy of the mean concentration of LLOQ samples must be within 80% to 120% of the nominal concentration. The precision around the mean concentration should be ≤ 20%.
- At least 75% of the LLOQ samples should meet the acceptance criteria for accuracy.

1.8.5 Hemolysis effect
Hemolysis effect is investigated to ensure that precision and accuracy are not compromised for plasma/ serum separated from hemolysed blood. Prepare six replicates of LQC, MQC and HQC using plasma / serum separated from hemolysed blood. Process and analyse all above Quality Ccontrol (QC) sample sets along with calibration curve (CC) standards prepared in plain plasma.

Acceptance criteria:
- The accuracy of the mean value of QCs should be within 85% to 115% of the nominal value.
- The precision around the mean value should be ≤ 15%.
Section 1.8.6 Linearity
A calibration (standard) curve is the relationship between instrument response and known concentrations of the analyte. Use a regression equation with an appropriate weighing factor to determine the detector response/concentration. 
Acceptance criteria:
- Coefficient of correlation \(r^2\) should be \(\geq 0.9800\)
- Accuracy at LLOQ should be within 80% to 120% of the nominal concentration.
- Accuracy at concentrations other than LLOQ should be within 85% to 115% of the nominal concentration.
- At least 75% or at least six of the non-zero calibration curve standards (whichever is greater) including the LLOQ and highest calibration curve standard should meet the above criteria.
- Interference if any observed in blank/blank + IS at retention times of analyte(s) and internal standard(s) should be \(\leq 20\%\) of LLOQ and \(\leq 5\%\) of mean IS response of CC respectively.

Section 1.8.7 Precision and accuracy
The accuracy of an analytical method describes the closeness of mean test results obtained by the method to the true value (concentration) of the analyte. Accuracy is determined by replicate analysis of samples containing known amounts of the analyte. The precision of an analytical method describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of biological matrix.
Calculate between and within batch precision and accuracy by analyzing a minimum of three precision and accuracy batches.
A precision and accuracy batch should contain the following samples with internal standard.
- Blank sample (matrix sample processed without internal standard)
- Blank + IS (matrix sample processed with internal standard)
- Six to eight non-zero concentration spiked calibration curve standards covering the expected range, including LLOQ and the highest standard of the calibration curve
Chapter 1

Introduction

Six LLOQ QC samples, Six LQC samples, Six MQC samples, Six HQC samples

Calculate QC sample concentration from the respective calibration curve.

Calculations:

Calculate the mean concentration, standard deviation (STDEV), coefficient of variance (% C.V.) and accuracy (% Nominal) for all CC standards and at each QC concentration level.

Acceptance criteria:

- Mean % Nominal concentration at each QC sample level other than LLOQ QC must be between 85% and 115% and the precision (% C.V) should be ≤ 15%.
- Mean % Nominal concentration at LLOQ QC must be between 80% and 120% and the precision (% C.V) should be ≤ 20%.
- At least 67% (four out of six) of total QC samples and at least 50% QC samples at each level should meet the above acceptance criteria.

1.8.8 Stability Studies

Drug stability in a biological fluid is a function of the storage conditions, the chemical properties of the drug, the matrix, and the container system. Stability procedures should evaluate the stability of the analytes during sample collection and handling, after long-term (frozen at the intended storage temperature) and short-term (bench top, room temperature) storage, and after going through freeze and thaw cycles and the analytical process. Conditions used in stability experiments should reflect situations likely to be encountered during actual sample handling and analysis.

(A) Standard stock solution stability

1. Short term bench top stock solution stability

Short-term bench top stock solution stability is performed to assess stability of solution left on the bench while preparing the stock dilutions.

Prepare stability stock dilution mixture of analyte(s) and IS of suitable concentration from the stock kept on bench for at least six hours. Also prepare a comparison stock dilution of equivalent concentration as that of stability stock dilution from the freshly prepared stock (s). Inject six replicates of stability stock dilution and comparison stock (fresh) dilution [mixture of analyte(s) and IS]. Calculate the mean area response of fresh stock and stability stock.

Acceptance criteria:

- Percentage of Comparison response should be between 90% and 110%.
Chapter 1

Introduction

2. Refrigerated stock solution stability

Refrigerated stock solution stability is performed to assess the stability of stored stock over a period of time in refrigerator, during which it can be used. Prepare fresh stock solution of analyte(s) and internal standard. Prepare suitable stock dilutions of both, the refrigerated stock (stability sample) and fresh stock (comparison sample).

Inject six replicates of stability stock dilution and comparison stock dilution [mixture of analyte(s) and internal standard]. Calculate % comparison response.

Acceptance Criteria:

- Percentage of Comparison response should be between 90% and 110%.

(B) Freeze-thaw (FT) stability

FT stability is performed to assess the stability of the analyte(s) in biological fluids during repeated freezing and thawing cycles. Establish the influence of minimum three FT cycles. Withdraw at least six replicates of LQC and HQC from deep freezer after at least 24 hours of continuous freezing and thawed unassisted at bench top (FT Cycle -1). Refreeze the samples after complete thawing. Withdraw these samples after a period of minimum of 12 hours and thaw (FT Cycle-2) Refreeze the samples after complete thawing. Again withdraw these samples after a period of minimum of 12 hours and after thawing (FT Cycle-3) analyse the samples. Process and analyse the above QC samples, along with freshly prepared calibration curve standards.

(C) Bench top stability

Bench top temperature stability is performed to assess the stability of the analyte (s) in biological fluids over a period of time during which the samples are expected to be kept on the bench while processing. Withdraw six replicates of LQC and HQC from deep freezer and leave them at room temperature for atleast four hours (stability samples). Process and analyse the stability samples along with freshly prepared calibration curve standards.

(D) Auto-sampler Stability

Autosampler stability is performed to assess the stability of the processed samples placed in the autosampler at specific temperature for the period of time depending on the anticipated run time or the complete analysis of bioanalytical batch. Place the processed samples in the autosampler at decided temperature. Record the time of placing autosampler stability QC
samples in the autosampler, Store QC samples for desired stability period in autosampler. Analyse the stability samples along with freshly prepared calibration curve standards.

(E) **Long-term stability (Stability Below – 50°C)**

Long-term stability is performed to assess the stability of analyte(s) in biological fluids during its storage in deep freezer below - 50°C. Withdraw six sets of LQC, MQC and HQC stored below – 50°C from deep freezer. Analyse the stability samples along with freshly prepared calibration curve standards. Compare the concentrations of the stability QC samples with concentration for the QC samples on the first day of analysis (considered as nominal concentration).

(F) **Post preparative stability**

Post preparative stability exercise is conducted to assess the stability of the processed samples in laboratory conditions for temporary storage.

1. **Bench top post preparative stability**

Process six sets of LQC and HQC as per method of extraction and keep on bench at room temperature for sufficient time period e.g. 12 hours, 24 hours etc. After storage period inject the samples along with freshly prepared calibration curve standards.

2. **Dry extract refrigerator stability**

This is performed when procedure of evaporation is involved in method of extraction. Process six sets of LQC and HQC as per method of extraction only up to stage evaporation to dryness. Store these samples without reconstitution for the period for which stability testing is carried out. After storage period inject the samples along with freshly prepared calibration curve standards.

**Acceptance criteria for all the stabilities**

- Percentage of nominal of QC samples should be between 85% to 115% and precision of QC samples should be within ± 15% CV.
- At least 67% (4 out of 6) of total QC samples and 50% of the QC samples at each level must meet the above acceptance criteria of % nominal.
- Stability / % Change must be within ± 15 % for the stability samples from the nominal concentration.
1.8.9 Reinjection reproducibility

Reinjection reproducibility is performed to assess the reproducibility of the back-calculated concentration of the reinjected samples. Process calibration curve standards and six replicates of LQC and HQC. Store the QC samples in the autosampler. Inject these QC samples along with processed calibration curve standards. Reinject the above QC samples stored in autosampler and back calculate the QC concentrations using initial calibration curve standards.

Acceptance criteria:
- % Nominal should be within 85% to 115%.
- At least 67% (4 out of 6) of the total reinjected QC samples and at least 50% of reinjected QC samples at each level should meet above acceptance criteria.

1.8.10 Recovery

The recovery of an analyte in an assay is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the true concentration of the pure authentic standard. Recovery pertains to the extraction efficiency of an analytical method within the limits of variability. Recovery of the analyte need not be 100%, but the extent of recovery of an analyte and of the internal standard should be consistent, precise, and reproducible.

Process and inject QC samples (extracted samples). Prepare solution mixture of drug(s) and/or metabolite(s) if any, including IS representing 100% extraction at LQC, MQC and HQC concentration along with the IS (non-extracted sample). Inject six replicates each of LQC, MQC and HQC along with IS (non-extracted samples). Calculate recovery by comparing extracted and non-extracted area response of analyte and IS.

Acceptance criteria:
- The % C.V. of the mean analyte(s) and IS recoveries must be ≤ 15% at LQC, MQC and HQC concentration level.

1.8.11 Dilution integrity

This exercise is performed to access the ability of the method to quantify the analyte and yield accurate and precise results after the sample dilution. Prepare QC samples spiked with about 1.5 – 1.8 times the concentration of the highest calibration curve standard. Process six sets of above QC samples by diluting them twice and four times prior to extraction by addition of
screened interference free blank matrix. Inject these QC samples along with calibration curve standards processed without dilution and calculate the QC concentrations using dilution factor as 2 (for two times diluted samples) and 4 (for four times diluted samples).

Acceptance criteria:
- Within batch precision of QC sample having same dilution factors should be ≤ 15%.
- Within batch accuracy of QC sample having same dilution factors should be ± 15% of the nominal value.
- At least 67% (four out of six) of total QC samples having same dilution factor.

1.8.12 Partial volume test

This exercise is performed to access the ability of the method to quantify the analyte using partial processing volume of the matrix, keeping the volume of all the other reagents and solvents same.

Process six sets each of MQC and HQC using both 50% and 25% of processing volume.

Inject these quality control samples along with calibration curve standards processed using full volume and calculate the concentrations using multiplication factor as 2 (for 50% processing volume) and 4 (for 25% processing volume).

Acceptance criteria:
- Within batch precision of QC samples having same multiplication factor should be ≤ 15%.
- Within batch accuracy of QC samples having same multiplication factor should be ± 15% of the nominal value.

Note: At least 67% (four out of six) of total QC samples and at least 50% QC samples at each level having same multiplication factor should meet the above acceptance criteria.

1.8.13 Ruggedness

One precision and accuracy batch should be processed using different sets of reagents and analysed using different column (same type, same dimension) on a different instrument.

Note: The entire ruggedness precision and accuracy batch has to be processed by one analyst.

Acceptance criteria:
- The batch should pass the linearity and within batch precision and accuracy criteria.
1.9 Application of Validated Method to Routine Drug Analysis

Each bio-analytical batch consists of calibration curve standards, quality control samples and unknown samples of one or more volunteers of a bioequivalence study.

Calibration curve and quality control samples are prepared by spiking known concentrations. The volume of spiking solution should not exceed 2% of the total matrix volume to avoid change in matrix properties. CC and QC samples are bulk spiked together and required volume of aliquot is taken out in small vials. These tubes caped tightly and stored below – 50°C in deep freezer until analysis.

(A) Calibration Curve

A calibration curve should be generated for each analyte and should be used to calculate the concentration of the analyte in the unknown samples in the run. A calibration curve is the relationship between instrument response and known concentrations of the analyte. The range of calibration curve should be established to allow adequate description of the pharmacokinetics of the analyte of interest. A calibration curve should consist of a blank sample (matrix sample processed without internal standard), a zero sample (matrix sample processed with internal standard), and six to eight non-zero samples covering the expected range, including LLOQ and Upper Limit of Quantitation (ULOQ). LLOQ concentration should be selected at least 5% of the reported C_max value and ULOQ should be selected at least double the reported C_max concentration to cover subject to subject variation in analyte concentrations.

Acceptance criteria for calibration curve-

- Coefficient of correlation($r^2$) should be ≥ 0.9800
- Accuracy at LLOQ should be within 80% to 120% of the nominal concentration.
- Accuracy at concentrations other than LLOQ should be within 85% to 115% of the nominal concentration.
- At least 75% or at least six of the non-zero calibration curve standards (whichever is greater) including the LLOQ and highest calibration curve standard should meet the above criteria.
- Interference if any observed in blank / blank + IS at retention times of analyte(s) and internal standard(s) should be ≤ 20% of LLOQ and ≤ 5% of mean IS response of CC respectively.
(B) Quality Control samples

The minimum number of Quality Control samples (in multiples of three) should be at least 5% of the number of unknown samples or six total QCs, whichever is greater. Placement of QC samples within a run should be designed to detect assay drift over the run.

Quality Control Samples should consist of minimum of three concentrations, one within 3 times of the LLOQ, one in the midrange and one approaching the high end of the range should be incorporated into each run.

Acceptance criteria for quality control samples-
At least 67% (four out of six) of the QC samples and 50% of QC samples at each level should be within ±15% of their respective nominal (theoretical) values.

(C) Subject samples

Samples of all the periods of one subject should be processed and run in one analytical batch. Interference observed at retention time of analyte in Predose sample should be less than 5% of the C_{max} value of the respective period. Predose sample should also be processed with internal standard for this calculation.

If interference in predose sample is more than 5% of the Cmax value then the subject is not considered for the statistical analysis. Interference at retention time of internal standard in predose sample should be less than 5% of mean internal standard response observed in the batch.

(D) Statistical analysis

To establish the bioequivalence, the calculated 90% CI for Cmax and AUC should fall within the bioequivalence range, usually 80-125%^{28}.

Statistical analysis is carried out with the help of statistical software. In this research work SAS software version 9.1.3 is used for the statistical calculations of ANOVA. The In-transformed least square mean and 90% confidence interval (CI) based on least square mean is calculated from ANOVA and ratio of geometric means for the pharmacokinetic parameters C_{max}, AUC_{0-t} and AUC_{0-\infty} for was calculated for each analyte.

1.10 Clinical trials

Clinical trials are a set of procedures in medical research and drug development that are conducted to allow safety (or more specifically, information about adverse drug reactions and
adverse effects of other treatments) and efficacy data to be collected for health interventions (e.g., drugs, diagnostics, devices, therapy protocols). These trials can take place only after satisfactory information has been gathered on the quality of the non-clinical safety, and Health Authority/Ethics Committee approval is granted in the country where the trial is taking place.\(^{30}\)

Clinical trials should be designed, conducted and analysed according to sound scientific principles to achieve their objectives; and should be reported appropriately.

### 1.10.1 Phases of clinical trials\(^{31}\):

#### (A) Phase-I (Most typical kind of study: Human Pharmacology):

Phase-I starts with the initial administration of an investigational new drug into humans. Although human pharmacology studies are typically identified with Phase I, they may also be indicated at other points in the development sequence. Studies in this phase of development usually have non-therapeutic objectives and may be conducted in healthy volunteer subjects or certain types of patients, e.g. patients with mild hypertension. Drugs with significant potential toxicity, e.g. cytotoxic drugs, are usually studied in patients. Studies in this phase can be open, baseline controlled or may use randomisation and blinding, to improve the validity of observations.

Studies conducted in Phase I typically involve one or a combination of the following aspects:

1. **Estimation of Initial Safety and Tolerability**
   
   The initial and subsequent administration of an investigational new drug into humans is usually intended to determine the tolerability of the dose range expected to be needed for later clinical studies and to determine the nature of adverse reactions that can be expected. These studies typically include both single and multiple dose administration.

2. **Pharmacokinetics**
   
   Characterisation of a drug's absorption, distribution, metabolism, and excretion continues throughout the development plan. Their preliminary characterisation is an important goal of Phase I. Pharmacokinetics may be assessed via separate studies or as a part of efficacy, safety and tolerance studies. Pharmacokinetic studies are particularly important to assess the clearance of the drug and to anticipate possible accumulation of parent drug or metabolites and potential drug-drug interactions. Some pharmacokinetic studies are commonly conducted in later phases to answer more specialised questions. For many orally administered drugs,
especially modified release products, the study of food effects on bioavailability is important. Obtaining pharmacokinetic information in sub-populations such as patients with impaired elimination (renal or hepatic failure), the elderly, children, women and ethnic subgroups should be considered. Drug-drug interaction studies are important for many drugs; these are generally performed in phases beyond Phase I but studies in animals and in vitro studies of metabolism and potential interactions may lead to doing such studies earlier.

3. Assessment of Pharmacodynamics
Depending on the drug and the endpoint studied, pharmacodynamic studies and studies relating drug blood levels to response (PK/PD studies) may be conducted in healthy volunteer subjects or in patients with the target disease. In patients, if there is an appropriate measure, pharmacodynamic data can provide early estimates of activity and potential efficacy and may guide the dosage and dose regimen in later studies.

4. Early Measurement of Drug Activity
Preliminary studies of activity or potential therapeutic benefit may be conducted in Phase I as a secondary objective. Such studies are generally performed in later phases but may be appropriate when drug activity is readily measurable with a short duration of drug exposure in patients at this early stage.

(B) Phase-II (Most typical kind of study: Therapeutic Exploratory)
Phase-II is usually considered to start with the initiation of studies in which the primary objective is to explore therapeutic efficacy in patients.
Initial therapeutic exploratory studies may use a variety of study designs, including concurrent controls and comparisons with baseline status. Subsequent trials are usually randomised and concurrently controlled to evaluate the efficacy of the drug and its safety for a particular therapeutic indication. Studies in Phase-II are typically conducted in a group of patients who are selected by relatively narrow criteria, leading to a relatively homogeneous population and are closely monitored.
An important goal for this phase is to determine the dose(s) and regimen for Phase-III trials. Early studies in this phase often utilise dose escalation designs to give an early estimate of dose response and later studies may confirm the dose response relationship for the indication in question by using recognised parallel dose-response designs (could also be deferred to phase-III). Confirmatory dose response studies may be conducted in Phase-II or left for
Phase-III. Doses used in Phase II are usually but not always less than the highest doses used in Phase-I.

Additional objectives of clinical trials conducted in Phase-II may include evaluation of potential study endpoints, therapeutic regimens (including concomitant medications) and target populations (e.g. mild versus severe disease) for further study in Phase-II or III. These objectives may be served by exploratory analyses, examining subsets of data and by including multiple endpoints in trials.

(C) Phase-III (Most typical kind of study: Therapeutic Confirmatory)

Phase-III usually is considered to begin with the initiation of studies in which the primary objective is to demonstrate, or confirm therapeutic benefit. Studies in Phase-III are designed to confirm the preliminary evidence accumulated in Phase-II that a drug is safe and effective for use in the intended indication and recipient population. These studies are intended to provide an adequate basis for marketing approval. Studies in Phase-III may also further explore the dose-response relationship, or explore the drug’s use in wider populations, in different stages of disease, or in combination with another drug. For drugs intended to be administered for long periods, trials involving extended exposure to the drug are ordinarily conducted in Phase-III, although they may be started in Phase-II. ICH E1 and ICH E7 describe the overall clinical safety database considerations for chronically administered drugs and drugs used in the elderly. These studies carried out in Phase-III complete the information needed to support adequate instructions for use of the drug (official product information).

(D) Phase-IV (Variety of Studies: - Therapeutic Use)

Phase-IV begins after drug approval. Therapeutic use studies go beyond the prior demonstration of the drug’s safety, efficacy and dose definition. Studies in Phase-IV are all studies (other than routine surveillance) performed after drug approval and related to the approved indication. They are studies that were not considered necessary for approval but are often important for optimising the drug’s use. They may be of any type but should have valid scientific objectives. Commonly conducted studies include additional drug-drug interaction, dose-response or safety studies and studies designed to support use under the approved indication, e.g. mortality/morbidity studies, epidemiological studies.