CHAPTER ONE:

INTRODUCTION TO “RAPID QUANTIFICATION OF PHARMACEUTICALS DRUGS IN BIOLOGICAL FLUID BY CHROMATOGRAPHIC METHODS (LC-MS/MS)”

1.1 THE PERSPECTIVE

Analysis of drugs in biological matrix has witnessed a dramatic surge, both in the development of more selective, effective drugs and in understanding their therapeutic and toxic effects. Knowledge of drug levels in body fluids, such as whole blood, plasma, serum and urine, allows the optimization of pharmacotherapy and provides a basis for studies of patient compliance, bioavailability, pharmacokinetics and the influences of co-medications. The quantitative and qualitative analysis of drugs and their metabolites has been applied extensively in pharmacokinetic studies, since pharmacokinetic variables, such as time to reach maximum plasma concentration, clearance and bioavailability, have to be known for a new drug to be approved. In addition, therapeutic drug monitoring (TDM) is used to improve drug therapy [1] and usually requires specificity to distinguish the drug to be monitored from similar compounds, metabolites or co-administered drugs. In contrast, pharmacokinetic study of a potential drug candidate requires a specific and sensitive method. Thus, development of rapid, selective and sensitive analytical methods for the quantitative evaluation of drugs and their metabolites in biological matrices are critical for the successful conduct of preclinical and/or biopharmaceutics and clinical pharmacology studies.

Several methods have been applied in the analysis of drugs and their metabolites, such as radioimmunoassay (RIA), capillary electrophoresis (CE), gas chromatography (GC), GC-mass spectrometry (GC-MS), high performance liquid chromatography (HPLC) with UV, fluorescence, radioactivity and mass spectrometric detection (MS). Since the introduction of the electrospray ionization (ESI) [2-4] and atmospheric pressure chemical ionization (APCI) [5-7] techniques, LC/MS has become an ideal and widely used method in the analysis of drugs and their metabolites due to its unmatched sensitivity, extraordinary selectivity and rapid rate of analysis [8]. In the pharmaceutical
industry liquid chromatography-tandem mass spectrometry (LC-MS/MS) is already an established method for quality control and quantification of drugs in different matrices. Additionally, this hyphenated analytical tool is becoming more and more important in clinical-chemical analysis (i.e., in therapeutic drug monitoring). Atmospheric pressure ionization (API) techniques are compatible with reversed-phase eluent system, taking into account the use of volatile solvents and additives in chromatographic separation, thus preserving all the advantages of liquid chromatography (LC) [9]. Together, ESI and APCI provide efficient ionization for very different type of molecules including polar, labile and high molecular mass drugs and metabolites. The recently introduced method of atmospheric pressure photoionization (APPI) has expanded the applicability of API techniques towards less polar compounds [10-11].

1.2 THE AIM

i) To develop and validate high throughput and rugged bioanalytical methods for routine sample analyses based on liquid chromatography tandem mass spectrometry (LC-MS/MS) detection.

ii) Efficient and quantitative separation of the drugs from biological matrices like human plasma by employing appropriate choice of extraction methodologies viz. solid phase extraction (SPE), protein precipitation or liquid-liquid extraction techniques.

iii) To apply these validated methods on their formulations in the study of bioequivalency or pharmacokinetics in healthy human male volunteers.

In the present study, the following six important drugs have been studied for their bioanalytical method development, validation and their application to bioequivalence / bioavailability studies in human plasma.

- **Quetiapine** – Antipsychotic
- **Mirtazapine** – Antidepressant
- **Glimepiride**– Blood-glucose-lowering drug
- **Tamsulosin** – Benign prostatic hyperplasia (BPH)
1.3 THE TARGET

The developed methods should have the following merits:

- High sensitivity (lower limit for quantification)
- High selectivity (minimum matrix interference)
- High throughput (shorter analytical run time)
- Minimum sample (biological matrix) volume for processing
- Quantitative and precise recovery of drugs
- Rugged enough for routine sample analysis (human subjects)

1.4 BACKDROP FOR THE TITLE

Chromatography is an analytical tool widely employed for the separation, identification and determination of chemical components in complex mixtures. It is a physical method of separation in which the components/solutes to be separated are distributed between two phases, one of which is stationary (stationary phase) while the other (the mobile phase) moves in a definite direction. The components must interact with the stationary phase to be retained and separated by it. The stationary phase in chromatography can be a solid, liquid coated on solid support or a gel that is fixed in place either in a column or on a planar surface. The mobile phase may be a gas, liquid or a supercritical fluid which moves over or through the stationary phase, carrying the components along with it.

Chromatography can be divided into five categories, based on the mechanism of interaction of the solute with the stationary phase as shown in Figure 1a-e [12].

1.4.1 Adsorption chromatography

It utilizes a solid stationary phase and a liquid or gaseous mobile phase. The solute gets adsorbed onto the surface of this solid stationary phase. Separation in adsorption chromatography results from the interaction of polar functional groups on the solute with
discrete adsorption sites on the adsorbent surface. The selectivity of the separation is dependent on the relative strength of these polar interactions (refer figure 1a).

1.4.2 Partition chromatography
Partition chromatography exploits the fact that a solute in contact with two immiscible liquids (or phases) will distribute itself between them according to its distribution coefficient. This form of chromatography is based on a thin film formed on the surface of a solid support by a liquid stationary phase. The principal intermolecular forces involved are dispersion, induction, orientation and donor-acceptor interactions including hydrogen bonding (refer figure 1b).

1.4.3 Size exclusion chromatography (SEC)
Size exclusion chromatography can be subdivided into “gel filtration” and “gel permeation” chromatography [13]. In gel filtration chromatography, the mobile phase is aqueous, while in gel permeation chromatography, an organic mobile phase is applied. This type of chromatography lacks an attractive interaction between the stationary phase and solute. The liquid or gaseous mobile phase passes through a porous gel which separates the molecules according to its size or in more technical terms, their hydrodynamic volume. The pores are normally small and exclude the larger solute molecules, but allow smaller molecules to enter the gel, causing them to flow through a larger volume. This causes the larger molecules to pass through the column at a faster rate than the smaller ones. The underlying principle of SEC is that particles of different sizes will elute (filter) through a stationary phase at different rates. This result in the separation of a solution of particles based on size (refer figure 1c).

1.4.4 Ion Exchange Chromatography
Ion-exchange entails a reversible, stoichiometric exchange between sample ions in the mobile phase and ions of like charge associated with the ion-exchange surface [14]. The stationary phase is a rigid matrix, the surface of which carries fixed positively or negatively charged functional groups (A). Counter ions (Y) of opposite charge are associated with each site in the matrix and these can exchange with similarly charged
ions in the mobile phase. If the matrix contains negatively charged acidic functional 
groups then it is capable of exchanging cations and is called a cation-exchanger; if it 
bears positively charged basic groups it is an anion-exchanger capable of exchanging 
anions (refer figure 1d). If the sample ions are depicted as $M^+$ or $X^-$ the process can be 
represented as:

cation exchange

\[ \text{Matrix} - A^- Y^+ + M^+ \rightarrow \text{Matrix} - A^- M^+ + Y^+ \]

anion exchange

\[ \text{Matrix} - A^+ Y^- + X^- \rightarrow \text{Matrix} - A^+ X^- + Y^- \]

1.4.5 Affinity Chromatography

Affinity chromatography is a highly specific separation method based on biochemical 
interactions such as between antigen and antibody, enzyme and substrate, or receptor and 
ligand [15]. The specificity of the interaction is due to both spatial and electrostatic 
effects. The stationary phase consists of a bioactive ligand chemically bonded to a solid 
support through a spacer. Only components that match the ligand properties are adsorbed. 
Elution is performed by the use of a mobile phase containing a component with a larger 
affinity to the ligand than the analyte or by changes of pH or ionic strength of the mobile 
phase (refer figure 1e).

1.4.6 Liquid chromatography (LC)

The various forms of chromatography employing a liquid rather than a gas as the moving 
phase are studied under the head liquid chromatography [16]. A useful classification of 
different LC techniques is based on the type of distribution mechanism applied in the 
separation. The four most widely studied LC methodologies include, partition (liquid-
liquid), adsorption (solid-liquid), ion-exchange and size exclusion chromatography. Now 
a days, high performance liquid chromatography (HPLC) is the most widely used 
analytical technique for the qualitative and quantitative analysis of pharmaceuticals,
biomolecules, polymers, and other organic compounds. HPLC is a separation technique conducted in the liquid phase in which a sample is separated into its constituent components (or analytes) by distributing between the mobile phase (a continuous flowing liquid) and a stationary phase (sorbents packed inside a column). Modern HPLC uses high pressure to force solvent through closed columns containing very fine particles that give high-resolution separations. The column is the heart of the system. The efficiency of a packed column increases as the size of the stationary phase particles decreases, where the particle size range is from 3-10 µm. In general, column of 10 to 300 mm length are used with an inner diameter of 1 to 5 mm. A typical modular/integrated HPLC system consists of a multi-solvent delivery pump, an on-line degasser, an autosampler, a high pressure column, a column oven, a detector and a data-handling workstation. Figure 2 gives a schematic representation of HPLC system with different modules.

Normal phase adsorption HPLC utilizes a polar stationary phase and less polar mobile phase. It is mainly used for the analysis of relatively nonpolar compounds. The retention of the components in a mixture increases with increasing polarity of the analytes. Silica gel is used as adsorbent in most applications, although alumina and chemically bonded stationary phases (with diol groups) are also used as well. The specific adsorption used in normal phase is the result of electrostatic forces between the permanent dipole of the silanol groups on the silica gel surface and the permanent or induced dipoles on the analyte molecules.

Reversed-phase system are used for most of the present LC applications, especially because the chemically bonded non-polar packing materials (stationary phase) are more easy to use. It is ideally suited for the analysis of polar and ionogenic analytes. In the reversed phase systems, the stationary phase is non polar or weakly polar and the mobile phase is more polar. Silica gel can be easily modified at the surface by chemical reactions with organochloro- or organoalkoxysilanes, e.g.

\[
\begin{align*}
\text{Si} - \text{OH} + \text{Cl} - \text{Si} - (\text{CH}_3)_2 - R & \rightarrow \text{Si} - \text{O} - \text{Si} - (\text{CH}_3)_2 - R + \text{HCl}
\end{align*}
\]

The siloxane bond is stable in organic and aqueous solvents in the pH range 2.5 – 8.0. Important R-groups that can be introduced in this way, are octyl (C8), octadecyl
(C18), phenyl, n-propylamine, alkylol, alkyl-N+(CH3)3 and phenylsulfonate (C6H4-SO3-). Non-polar, chemically modified silica gel or other non-polar packing materials, such as styrene-divinylbenzene copolymers (XAD, PRP) are used as stationary phases for aqueous-organic solvent mixtures upto pH-14 (Figure 3). Some of the advantages of bonded-phase materials are relative stability, short equilibration time and their versatility [13].

1.4.7 Separation process in HPLC- An overview

The sample is injected by means of an injection port into the mobile phase stream delivered by the high-pressure pump and transported through the column where the separation takes place and the analytes are monitored with a flow-through detector like UV, fluorescence, photo diode array, refractive index, electrochemical or evaporative light-scattering.

The fundamental important steps in creating highly selective phase systems include rigorous control of surface chemistry and adjustment of the final stationary phase properties by appropriate mobile phase selection, leading to specific solute-surface interaction and suppressing undesired side interactions.

Unlike in adsorption chromatography, the specific analyte-solvent interactions e.g. solubility effects are most important in reversed-phase HPLC, since the interaction of the analyte and the bonded-phase material are weak nonspecific Van der Waals interaction. The retention decreases with increasing polarity of the analyte. A buffer is frequently used in reversed-phase systems as it reduces the protolysis of ionogenic analytes, which in ionic form show little retention. Phosphate buffers are widely applied for that purpose, since they span a wide pH range and show good buffer capacity. The use of buffer is obligatory in real world applications e.g. bioanalysis, where many of the matrix components are ionogenic.

In the analysis of acidic or basic compounds, adjustment of the pH is not always successful. The addition of an organic lipophilic ionic compound as a counter-ion for the ionic analytes results in the formation of ion-pairs that are well retained on the reversed-phase material. Mixtures of water or aqueous buffers and an organic modifier (methanol, acetonitrile etc.) are used as eluents. The percentage and type of organic modifier is the
most important parameter in adjusting the retention of non-ionic analytes. Considerable attention is given to automated optimization of reversed-phase LC separations [17-18]. Traditionally, most pharmaceutical assays are isocratic analysis employing the same phase throughout the elution of the sample. Isocratic analyses are particularly common in quality control applications since they use simple HPLC equipment and premixed mobile phase. In contrast, gradient analysis is suited for complex samples and those containing analytes of wide polarities in which the strength of the mobile phase (organic modifier content) is increased with time during sample elution. Hence the separation can be achieved in shorter time and with better efficiency.

Analysis by HPLC-UV generally lacks sensitivity and specificity, especially in analysis of drug in biological matrices. Thus, a need for more sensitive and selective detectors is paramount in such cases, where the drug is extensively metabolized and yet the retention time and UV spectral character remains the same as that of parent compound. Mass Spectrometry detection and characterization is now a vital new tool in bioanalytical method development and pharmacokinetic analysis of drugs.

1.4.8 Liquid chromatography-Mass spectrometry (LC-MS)
Liquid chromatography coupled with MS is widely used in drug discovery and development. Innovative and successful research efforts in the past decades on the design of an effective interface connection between LC (operated under atmospheric pressure) and MS (operated under a high vacuum) have made LC congenial with MS. LC-MS is an integrated hybrid analytical system, where LC plays a role in separation and introduction of sample components to the mass spectrometer. The mass spectrometer acts as a highly selective detector for the analyte of interest and gathers both qualitative and quantitative information. The principle of MS is the production of ions from separated compounds on the basis of their mass-to-charge ratio \( (m/z) \). This selectivity eases the requirements for complete chromatographic separation of the components of a mixture. When it operated in a mode that is highly selective for a particular analyte, the mass spectrometer increases the signal-to-noise ratio in quantitative analysis and lowers the detection limit for the analyte. The use of a mass detector for peak detection in a chromatographic process offers some special advantages.
Various procedures are used to form ions in gas phase from molecules, depending on the physical state of the analyte. Choices are available as to the types of ions produced (positively and negatively charged, radical cations, protonated molecules, etc.) and the degree to which these ions are internally excited. Internally excited molecular ions dissociate to produce fragment ions, which may reveal details of molecular structure. On the other hand, an intact molecular ion provides information on molecular weights. The major ionization methods used for organic or biological compounds can be grouped into four categories: electron ionization (EI), chemical ionization (CI), desorption ionization (DI) including matrix-assisted laser desorption ionization (MALDI) and spray ionization (SI) like thermospray (TS), electrospray (ES) and atmospheric pressure chemical ionization (APCI) techniques. Except for electron ionization, each method can produce abundant positive or negative ions. The criteria for selection of a particular ionization technique is generally dependent on, physical state of the sample (aqueous solution vs. solid sample) volatility and thermal stability of the sample.

Type of information sought (molecular structure vs. sequence analysis)

Ions can be separated on the basis of their mass-to-charge ratios using electric or magnetic fields arranged so as to spread them in time or space. Among many others, five distinct mass analyzers are: sector magnetic fields, time-of-flight analyzers, quadrupole mass filters, quadrupole ion traps and ion cyclotron resonance. In some of these analyzers, physical separation of ions in space is achieved, in others the mass dependent frequency of ion motion is examined and in still others ion velocity is measured using timing circuitry. Most applications for quantitative bioanalysis use tandem mass spectrometers (MS/MS) to characterize individual compounds in a complex mixture or to identify a compound’s structure. These goals are achieved by separating the ionization step from the fragmentation process and thus controlling the degree of fragmentation. Mass analysis is performed twice in a tandem instrument to identify both the parent and product ion. This can be done in two distinct ways (i) by separating the mass analysis operations in space or (ii) by separating them in time. Separation in space can be achieved by coupling two mass analyzers. For example, a sector magnet can be coupled to a quadrupole mass filter. Parent ions are selected by the sector magnet, and so separated from all other ions generated from the sample. These selected ions are activated.
by a collision process, and the resulting set of product ions is subjected to mass analysis with the quadrupole mass filter. Fragmentation is achieved by raising the internal energy of the ions by collision-induced dissociation (CID). This process involves passing the energetic beam of parent ions through a cell containing a collision gas, such as He, N2 or Ar. The most important separation-in-space tandem mass spectrometer is the triple quadrupole as shown in Figure 4. In this tandem analyzer instrument, an intermediate quadrupole (Q1) is used to confine ions to the axis in the presence of a collision gas. This quadrupole is not operated in a mass analysis mode but is set to transmit all ions. The products of CID are passed into the third quadrupole (the second mass analyzer) for mass analysis. The second major type of tandem mass spectrometer uses a single mass analyzer to perform two steps of mass analysis, which are separated from each other in time. Both the quadrupole ion trap and ion cyclotron resonance instrument can be used in this way. The ion of interest is first isolated in the trap, activated by collision, and finally its dissociation products are mass analyzed [19].

1.5 SAMPLE PREPARATION TECHNIQUES

Sample preparation is a series of steps required to transform a sample so that it is suitable for analysis. Sample preparation could include dissolving the sample, extracting analyte from a complex matrix, concentrating a dilute analyte to a level that can be measured, chemically converting analyte to a detectable form and removing or masking interfering species [12]. The commonly used extraction techniques for sample preparation in bioanalysis include protein precipitation, liquid-liquid extraction and solid phase extraction.

i) Protein precipitation (PP) is a technique used to separate the drug from the biological matrices by means of precipitating the proteins using organic solvents such as acetonitrile, methanol or acidified solutions such as trifluoroacetic acid and perchloric acid [20]. The mixtures are centrifuged or filtered to afford a clear supernatant or filtrate solution, which can be directly injected into HPLC and LC-MS system. Protein precipitation does not require very extensive method development and can be implemented as a simple generic method to prepare samples from discovery
pharmacokinetic studies [21-23]. Protein precipitation is a fast method to produce analyzable samples, however in term of cleanliness, the sample is considered crude.

**ii** Liquid-Liquid extraction (LLE) with two immiscible liquids is useful for sample cleanup of complex biological samples such as blood, plasma, urine and tissues. It offers cleanup of matrices and concentration enhancement in preparing samples for HPLC and LC-MS analysis [20, 24-26]. It takes advantage of the relative solubility's of the solutes in immiscible solvents as they concentrate in the solvent with the highest solubility, governed by their partition or distribution coefficients. For acidic or basic analytes, a sequence of “sample pH adjustment (acid or base) $\rightarrow$ extraction $\rightarrow$ pH adjustment (base or acid) $\rightarrow$ back extraction” offers an effective generic approach. Liquid-liquid extraction is labor intensive as it involves drying followed by reconstitution to meet the desired quantification limits.

**iii** Solid Phase extraction (SPE) is routinely used in the sample preparation for quantifying analytes in biological fluids such as plasma and urine [27-28]. In a solid phase extraction of use of small volume of a chromatographic stationary phase to isolate desired analytes from a sample. This technique allows both removal of interfering biological matrix components and enhancing the concentrations of analytes in HPLC and LC/MS samples. SPE can also be performed on a liquid handling system using a 96 well plate format, which are pre-packed SPE blocks with different sorbents. In general, the biological samples are loaded onto SPE cartridges and the biological matrices are then washed out of the cartridges. The retained analytes are often eluted using small aliquots of an organic solvent so that the desired analytes are concentrated enough for analysis without further evaporation and concentration procedures. Other solid-liquid extraction techniques include solid phase micro extraction, accelerated fluid extraction, supercritical fluid extraction and microwave-assisted extraction.

1.6 BIOANALYTICAL METHODOLOGY

Bioanalytical chemistry is the qualitative and quantitative analysis of drug substances in biological fluids such as blood, plasma, serum, urine and tissues [29]. It plays a significant role in the evaluation and interpretation of bioavailability, bioequivalence and pharmacokinetic data [30]. The process by which a specific bioanalytical method is
developed, validated, and used in routine sample analysis can be divided into (1) reference standard preparation, (2) bioanalytical method development and establishment of assay procedure (Full Method Validation) and (3) application of validated bioanalytical method to routine drug analysis and acceptance criteria for the analytical run and/or batch. The main analytical segments that comprise bioanalytical methodology are method development, method validation and application in routine sample analysis.

A bioanalytical method is a set of all of the procedures involved in the collection, processing, storing and analysis of a biological matrix for an analyte [31]. Analytical methods employed for quantitative determination of drugs and their metabolites in biological fluids are the key determinants in generating reproducible and reliable data for sample analysis [32]. Method development involves evaluation and optimization of the various stages of sample preparation, chromatographic separation, detection and quantification. Initially, an extensive literature survey on the same or similar analyte is done followed by summarizing the main features of the work, which is of primary importance. Based on this information, the following selections could be made:

i) The choice of instrument that is suitable for the analysis of the analyte of interest. This includes the choice of the column associated with the instrument, the detector, the mobile phase in the high performance liquid chromatography (HPLC) and the choice of carrier gas in gas chromatography (GC).

ii) Choice of internal standard (IS), which is best for the study. It must have similar chromatographic and ionization properties compared to the analyte.

iii) The choice of extraction procedure, which is quick and efficient, gives the highest possible recovery without interference at the elution time of the analyte of interest and has acceptable accuracy and precision which meets the intended study requirement.

Method performance is determined primarily by the quality of the procedure itself. The two factors that are most important in determining the quality of the method are selective recovery and standardization. Analytical recovery of a method refers to whether the analytical method in question provides response for the entire amount of analyte that is contained in a sample. Recovery is usually defined as the percentage of the reference material that is measured, to that which is added to a blank. This should not be confused with the test of matrix effect in which recovery is defined as the response,
measured from the matrix (e.g. plasma) as a percentage of that measured from the pure solvent (e.g. water). Results of the experiment that compare matrix to pure solvent is referred to as relative recovery and true test of recovery is referred to as absolute recovery [33-34].

Another important issue in method development stage is the choice of internal versus external standardization. Internal standardization is common in bioanalytical methods especially with chromatographic procedures. The assumption for the use of internal standard is that the partition coefficient of the analyte and the internal standard are very similar [33]. For internal standardization, a structural or isotopic analogue of the analyte is added to the sample prior to sample pre-treatment and the ratio of the response of the analyte to that of the internal standard is plotted against the concentration [35].

Another important point is that the tests performed at the stage of method development should be done with the same equipment that will actually be used for subsequent routine analysis. The differences found between individual instruments representing similar models from the same manufacturer is not surprising and should be accounted for [36].

1.7 METHOD VALIDATION
The search for the reliable range of a method and continuous application of this knowledge is called validation [36]. It can also be defined as the process of documenting that the method under consideration is suitable for its intended purpose [37-38]. Method validation involves all the procedures required to demonstrate that a particular method for quantitative determination of an analyte(s) in a particular biological matrix is reliable for the intended application [31]. Validation is also a proof of the repeatability, specificity and suitability of the method. Bioanalytical methods must be validated if the results are used to support the registration of a new drug or a new formulation of an existing one. Validation is required to demonstrate the performance of the method and reliability of analytical results [37]. If a bioanalytical method is claimed to be for quantitative biomedical application, then it is important to ensure that a minimum package of validation experiments has been conducted and yields satisfactory results [35].
Before discussing how to carry out the validation experiment, it is important to stress that validation in bioanalysis should not be considered as an isolated field. A consensus on common terminology for all analytical fields is therefore required. For the moment it is not yet possible to propose a validation terminology that is also in agreement with the recommendations of important international organizations such as the ISO (International Standard Organization), IUPAC (International Union of Pure and Applied Chemistry) and AOAC (Association of Official Analytical Chemists), since differences exist between their documents [37].

On the other hand the guideline for industry by FDA [39] states that the fundamental parameters of validation parameters for a bioanalytical method validation are accuracy, precision, selectivity, sensitivity, reproducibility and stability. Typical method development and establishment for bioanalytical method includes determination of (1) selectivity, (2) accuracy, (3) precision, (4) recovery, (5) calibration curve and (6) stability. For a bioanalytical method to be considered valid, specific acceptance criteria should be set in advance and achieved for accuracy and precision for the validation of the Quality Control samples. Validations can be subdivided into the following three categories:

**Full validation**

This is the validation performed when developing and implementing a bioanalytical method for the first time. Full validation should be performed to support pharmacokinetic, bioavailability, bioequivalence and drug interaction studies in a new drug application (NDA) [32].

**Partial validation**

Partial validations are performed when modifications of already validated bioanalytical methods are made. Partial validation can range from as little as one intra-assay and precision determination to a nearly full validation. Some of the typical bioanalytical method changes that fall into this category include bioanalytical method transfer between laboratories or analyst, change in analytical methodology, change of matrix within species, change of species within matrix [32, 39]. The decision of which parameters to be revalidated depend on the logical consideration of the specific validation parameters likely to be affected by the change made to the bioanalytical method.
**Cross validation**

Cross validation is a comparison of validation parameters when two or more bioanalytical methods are used to generate data within the same study or across different studies. An example of cross validation would be a situation when the original validated bioanalytical method serves as the reference and the revised bioanalytical method is the comparator [32, 39].

For the validation of pharmaceutical drug formulations the discussion on a consensus terminology is relatively advanced. It is suggested to follow in general the proposal elaborated for the validation of drug formulation by the joint initiative of the pharmaceutical industry and the regulatory agencies of the three major regulatory authorities (the European Union, the USA and Japan), the International Conference on Harmonization (ICH) [40]. According to them the revised version of terminology to be included are bias (accuracy), precision, specificity, limit of detection, limit of quantification, linearity, range and stability. The term stability is also specifically considered in the validation strategy for bioanalytical methods, which is prepared by the French group SFSTP (Societies Francaise des Sciences et Techniques Pharmaceutiques) [37].

**1.8 BIOAVAILABILITY AND BIOEQUIVALENCE**

The therapeutic effectiveness of a drug depends upon the ability of the dosage form to deliver the medicament to its site of action at a rate and amount sufficient to elicit the desired pharmacologic response. This attribute of the dosage form is referred to as physiologic availability, biologic availability or simply bioavailability. For most drugs, the pharmacologic response can be related directly to the plasma levels. Thus, the term bioavailability is defined as the rate and extent (amount) of absorption of unchanged drug from its dosage form. Absolute bioavailability is the fraction of drug effectively absorbed after extravascular administration, when compared to the administration of the same drug intravenously. Relative bioavailability or bioequivalence between drugs, administered by the same extra vascular route, may be evaluated by comparing pharmacokinetic parameters related to bioavailability, i.e., to the quantity absorbed and to the rate of the absorption process. Bioequivalent drugs are pharmaceutical equivalents (same
pharmaceutical formulation and quantity of the same active ingredient) that, when given in the same molar dose, in the same condition, does not present significant statistical differences regarding bioavailability. The rate or rapidity with which a drug is absorbed is an important consideration when a rapid onset of action is desired as in the treatment of acute conditions such as asthma attack, pain, etc. A slower absorption rate is however desired when the aim is to prolong the duration of action or to avoid the adverse effects. On the other hand, extent of absorption is of special significance in the treatment of chronic conditions like hyper-tension, epilepsy, etc.

If the size of the dose to be administered is same, then bioavailability of a drug from its dosage form depends upon 3 major factors:

i) Pharmaceutic factors related to physicochemical properties of the drug and characteristics of the dosage form

ii) Patient related factors

iii) Route of administration

The influence of route of administration on drug’s bioavailability is generally in the following order: parenteral > oral > rectal > topical with few exceptions. Within the parenteral route, intravenous injection of a drug results in 100% bioavailability as the absorption process is bypassed. However, for reasons of stability and convenience, most drugs are administered orally. In such cases, the dose available to the patient, called as the bioavailable dose, is often less than the administered dose. The amount of drug that reaches the systemic circulation (i.e. extent of absorption) is called as systemic availability or simply availability. The term bioavailability fraction F (Bioavailable dose/Administered dose) refers to the fraction of administered dose that enters the systemic circulation [41].

In the relationship between dose and effectiveness or dose response, not only the amount of drug administered and the pharmacological effect of the drug are of importance but many other factors are responsible for the entrance of a drug into the body. These factors are based on the physical and chemical properties of the drug substance and of the drug product. What happens to the active ingredient in the body after administration of a drug product in its various dosage forms? This entire cycle of processes is termed fate of drugs. Whether a blood level curve will reach its peak rapidly
or slowly depends on the route of administration, the dosage form, the liberation rate of the drug from the dosage form, diffusion, penetration and permeation of the drug, its distribution within the body fluids and tissues, the type, amount and rate of biotransformation, recycling processes and elimination. In addition to these factors there are also others, depending on the individual disposition, diseases, etc.

The fate of drugs is described in the leading literature on biopharmaceutics and pharmacokinetics by the LADME-system showing that liberation, absorption, distribution, metabolism and excretion are involved [42].

The LADME-system is the key to the following tasks:

- Development of new active compounds
- Development of dosage forms with desired release characteristics
- Determination of pharmacokinetic parameters and pharmacokinetic drug product profiles
- Determination and evaluation of bioavailability
- Selection of the most appropriate route of administration
- Determination of effective dose sizes

Adjustment of dosage regimen for different body weight and required minimum effective concentration of drug in the body in patients without or with renal failure.

The importance of bioavailability studies are due to

i) Primary stages of development of a suitable dosage form for a new drug entity.

ii) Determination of influence of excipients, patient related factors and possible interaction with other drugs on the efficiency of absorption.

iii) Development of new formulations of the existing drugs.

iv) Control of quality of a drug product during the early stages of marketing in order to determine the influence of processing factors, storage and stability on drug absorption.

**Plasma Drug Concentration-Time Profile**

A direct relationship exists between the concentration of drug at the biophase (site of action) and the concentration of drug in plasma. A typical plasma drug concentration-
time curve obtained after a single oral dose of a drug and showing various pharmacokinetic and pharmacodynamic parameters is depicted in Figure 5. Such a profile can be obtained by measuring the concentration of drug in plasma samples taken at various intervals of time after administration of a dosage form and plotting the concentration of drug in plasma (Y-axis) versus the corresponding time at which the plasma sample was collected (X-axis).

The three important pharmacokinetic parameters that describe the plasma level-time curve and useful in assessing the bioavailabilities of a drug from its formulation are:

- **Peak Plasma concentration (Cmax):** The point of maximum concentration of drug in plasma is called as the peak and the concentration of drug at peak is known as peak plasma concentration. It is also called as peak height concentration and maximum drug concentration. The peak level depends upon the administered dose and rate of absorption and elimination. The peak represents the point of time when absorption rate equals elimination rate of drug. The portion of curve to the left of peak represents absorption phase i.e. when the rate of absorption is greater than the rate of elimination. The section of curve to the right of peak generally represents elimination phase i.e. when the rate of elimination exceeds rate of absorption. Peak concentration is often related to the intensity of pharmacologic response and should ideally be above minimum effective concentration (MEC) but less than the maximum safe concentration (MSC).

- **Time of peak concentration (tmax):** The time for drug to reach peak concentration in plasma (after extravascular administration) is called as the time of peak concentration. It is expressed in hours and is useful in estimating the rate of absorption. Onset time and onset of action are dependent upon tmax. The parameter is of particular importance in assessing the efficacy of drugs used to treat acute conditions like pain and insomnia which can be treated by a single dose.

- **Area under the Curve (AUC):** It represents the total integrated area under the plasma level-time profile and expresses the total amount of drug that comes into the systemic circulation after its administration. AUC is expressed in mcg/mL x hours. It is the most important parameter in evaluating the bioavailability of a drug from its dosage form as it represents the extent of absorption. AUC is also important for drugs that are administered repetitively for the treatment of chronic conditions like asthma or epilepsy.
Pharmacokinetic and bio-equivalency studies require very precise and accurate assay methods that are well validated to quantify drugs in biological samples. The assay methods have to be sensitive enough to determine the biological sample concentration of the drug and/or its metabolite(s) for a period of about five elimination half-lives after dosage of the drug. The assay methods also have to be very selective to ensure reliable data, free from interference of endogenous compounds and possible metabolites in the biological samples. In addition, methods have to be as robust and cost effective as possible, which is extremely vital for bioequivalent studies but definitely not at the cost of quality of the data generated. Above all, the assay methods must be able to withstand the scrutiny of national drug registration authorities who judge them on the basis of criteria established by international consensus.
1.9 PHYSICOCHEMICAL PROPERTIES, PHARMACOLOGY AND MECHANISM OF ACTION OF DRUGS STUDIED

**Quetiapine**

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IUPAC Name</td>
<td>2-[2-(4-benzo[b][1,5]benzothiazepin-6-ylpiperazin-1-yl) ethoxy]ethanol</td>
</tr>
<tr>
<td>Molecular Formula</td>
<td>C21H25N3O2S</td>
</tr>
<tr>
<td>Mol wt</td>
<td>383.5099 g/mol</td>
</tr>
<tr>
<td>CAS Registry No</td>
<td>111974-69-7</td>
</tr>
<tr>
<td>H,2O-Solubility</td>
<td>moderate</td>
</tr>
<tr>
<td>LogP / Hydrophobicity</td>
<td>2.8</td>
</tr>
<tr>
<td>Protein Binding</td>
<td>83% (to human plasma proteins)</td>
</tr>
<tr>
<td>pK&lt;sub&gt;a&lt;/sub&gt;</td>
<td>~6.8 and 3.3</td>
</tr>
<tr>
<td>Melting point</td>
<td>-</td>
</tr>
</tbody>
</table>

Pharmacology: Quetiapine is a psychotropic agent belonging to the chemical class of benzisoxazole derivatives and is indicated for the treatment of schizophrenia. Quetiapine is a selective monoaminergic antagonist with high affinity for the serotonin Type 2 (5HT2) and dopamine type 2 (D2) receptors. Quetiapine is an antagonist at serotonin 5-HT1A and 5HT2, dopamine D1 and D2, histamine H1, and adrenergic alpha 1 and alpha 2 receptors. Quetiapine has no significant affinity for cholinergic muscarinic or benzodiazepine receptors. Drowsiness and orthostatic hypotension associated with use of quetiapine may be explained by its antagonism of histamine H1 and adrenergic alpha 1 receptors, respectively. Quetiapine's antagonism of adrenergic a1 receptors may explain the orthostatic hypotension observed with this drug.

Mechanism of action: The mechanism of action of quetiapine, as with other drugs used to treat schizophrenia, is unknown. However, it is thought that the drug's therapeutic activity in schizophrenia is mediated through a combination of dopamine type 2 (D2) and serotonin type 2 (5HT2) receptor antagonism. Although quetiapine is known to bind other receptors with similar affinity, only the dopamine D2 and serotonin 5HT2 receptor binding is responsible for quetiapine's therapeutic activity in schizophrenia.
### Mirtazapine

<table>
<thead>
<tr>
<th>IUPAC Name</th>
<th>1,2,3,4,10,14b-hexahydro-2-methylpyrazino[2,1-a]pyrido[2,3-c][2]benzazepine</th>
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<tbody>
<tr>
<td>Molecular Formula</td>
<td>C17H19N3</td>
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<tr>
<td>Mol wt</td>
<td>265.1579 g/mol</td>
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<tr>
<td>CAS Registry No</td>
<td>61337-67-5</td>
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<tr>
<td>H₂O-Solubility</td>
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<td>LogP / Hydrophobicity</td>
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<td>Protein Binding</td>
<td>85%</td>
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<tr>
<td>pka</td>
<td>-</td>
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<td>Melting point</td>
<td>114-116°C</td>
</tr>
</tbody>
</table>

**Pharmacology:** Mirtazapine, an antidepressant of the piperazinoazepine class, is a tetracyclic compound with an anxiolytic effect. Mirtazapine has fewer ADRs than tricyclic antidepressants and is better tolerated. Selective blockade of specific serotonin receptors by mirtazapine likely minimizes side effects typical of other antidepressants.

**Mechanism of action:** Mirtazapine acts as an antagonist at central pre-synaptic alpha (2)-receptors, inhibiting negative feedback to the presynaptic nerve and causing an increase in NE release. Blockade of heteroreceptors, alpha (2)-receptors contained in serotenergic neurons, enhances the release of 5-HT, increasing the interactions between 5-HT and 5-HT1 receptors and contributing to the anxiolytic effects of mirtazapine. Mirtazapine also acts as a weak antagonist at 5-HT1 receptors and as a potent antagonist at 5-HT2 (particularly subtypes 2A and 2C) and 5-HT3 receptors. Blockade of these receptors may explain the lower incidence of adverse effects such as anxiety, insomnia and nausea. Mirtazapine also exhibits significant antagonism at H1-receptors, resulting in sedation. Mirtazapine has no effects on the reuptake of either NE or 5-HT and has only minimal activity dopaminergic and muscarinic receptors.

### Glimepiride
Pharmacology: Glimepiride, like glyburide and glipizide, is a "second-generation" sulfonylurea agents. Glimepiride is used with diet to lower blood glucose by increasing the secretion of insulin from pancreas and increasing the sensitivity of peripheral tissues to insulin.

Mechanism of action: The mechanism of action of glimepiride in lowering blood glucose appears to be dependent on stimulating the release of insulin from functioning pancreatic beta cells and increasing sensitivity of peripheral tissues to insulin. Glimepiride likely binds to ATP-sensitive potassium channel receptors on the pancreatic cell surface, reducing potassium conductance and causing depolarization of the membrane. Membrane depolarization stimulates calcium ion influx through voltage sensitive calcium channels. This increase in intracellular calcium ion concentration induces the secretion of insulin.

Tamosulosin
<table>
<thead>
<tr>
<th>IUPAC Name</th>
<th>(-)-(R)-5-[2-[[2-(o-Ethoxyphenoxy) ethyl]amino]propyl]-2-methoxybenzenesulfonamide, monohydrochloride.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular Formula</td>
<td>$C_{20}H_{28}N_2O_5S$</td>
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<td>Mol wt</td>
<td>408.51 g/mol</td>
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<tr>
<td>CAS Registry No</td>
<td>106133-20-4</td>
</tr>
<tr>
<td>H$_2$O-Solubility</td>
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</tr>
<tr>
<td>LogP / Hydrophobicity</td>
<td>2.3</td>
</tr>
<tr>
<td>Protein Binding</td>
<td>94-99%</td>
</tr>
<tr>
<td>pka</td>
<td>-</td>
</tr>
<tr>
<td>Melting point</td>
<td>226-228 °C</td>
</tr>
</tbody>
</table>

Pharmacology: The symptoms associated with benign prostatic hyperplasia (BPH) are related to bladder outlet obstruction, which is comprised of two underlying components: static and dynamic. The static component is related to an increase in prostate size caused, in part, by a proliferation of smooth muscle cells in the prostatic stroma. However, the severity of BPH symptoms and the degree of urethral obstruction do not correlate well with the size of the prostate. The dynamic component is a function of an increase in smooth muscle tone in the prostate and bladder neck leading to constriction of the bladder outlet. Smooth muscle tone is mediated by the sympathetic nervous stimulation of alpha1 adrenoceptors, which are abundant in the prostate, prostatic capsule, prostatic urethra and bladder neck. Blockade of these adrenoceptors can cause smooth muscles in the bladder neck and prostate to relax, resulting in an improvement in urine flow rate and a reduction in symptoms of BPH. Tamsulosin, an alpha1 adrenoceptor blocking agent, exhibits selectivity for alpha1 receptors in the human prostate. At least three discrete alpha1-adrenoceptor subtypes have been identified: alpha1A, alpha1B and alpha1D; their distribution differs between human organs and tissue. Approximately 70% of the alpha1-receptors in human prostate are of the alpha1A subtype.

Mechanism of action: Tamsulosin is a selective antagonist at alpha1A and alpha1B adrenoceptors in the prostate, prostatic capsule, prostatic urethra and bladder neck. At least three discrete alpha1 adrenoceptor subtypes have been identified: alpha1A, alpha1B and alpha1D; their distribution differs between human organs and tissue. Approximately 70% of the alpha1 receptors in human prostate are of the alpha1A subtype. Blockage of these receptors causes relaxation of smooth muscles in the bladder neck and prostate.
Topiramate

<table>
<thead>
<tr>
<th>IUPAC Name</th>
<th>2,3:4,5-Bis-O-(1-methylethylidene)-beta-D-fructopyranose sulfamate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular Formula</td>
<td>C_{12}H_{21}NO_{8}S</td>
</tr>
<tr>
<td>Mol wt</td>
<td>339.3620 g/mol</td>
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<td>CAS Registry No</td>
<td>97240-79-4</td>
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<tr>
<td>H_{2}O-Solubility</td>
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<td>LogP / Hydrophobicity</td>
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<td>Protein Binding</td>
<td>15-41%</td>
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<td>pka</td>
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</tr>
<tr>
<td>Melting point</td>
<td>-</td>
</tr>
</tbody>
</table>

Pharmacology: Topiramate is an anticonvulsant indicated in the treatment of epilepsy and migraine. Topiramate enhances GABA-activated chloride channels. In addition, topiramate inhibits excitatory neurotransmission, through actions on kainate and AMPA receptors. There is evidence that topiramate has a specific effect on GluR5 kainate receptors. It is also an inhibitor of carbonic anhydrase, particular subtypes II and IV, but this action is weak and unlikely to be related to its anticonvulsant actions, but may account for the bad taste and the development of renal stones seen during treatment. Its possible effect as a mood stabilizer seems to occur before anticonvulsant qualities at lower dosages. Topiramate inhibits maximal electroshock and pentylenetetrazol-induced seizures as well as partial and secondarily generalized tonic-clonic seizures in the kindling model, findings predecdive of a broad spectrum of antiseizure activities clinically.

Mechanism of action: The precise mechanism of action of topiramate is not known. However, studies have shown that topiramate blocks the action potentials elicited repetitively by a sustained depolarization of the neurons in a time-dependent manner, suggesting a state-dependent sodium channel blocking action. Topiramate also augments the activity of the neurotransmitter gamma-aminobutyrate (GABA) at some subtypes of the GABAA receptor (controls an integral chloride channel), indicating a possible mechanism through potentiation of the activity of GABA. Topiramate also demonstrates
antagonism of the AMPA/kainate subtype of the glutamate excitatory amino acid receptor. It also inhibits carbonic anhydrase (particularly isozymes II and IV), but this action is weak and unlikely to be related to its anticonvulsant actions.

### Olanzapine

<table>
<thead>
<tr>
<th>IUPAC Name</th>
<th>5-methyl-8-(4-methylpiperazin-yl)-4thia-2,9diazatricyclo[8.4.0.0^{3,7}]tetradeca-1(10),2,5,7,11,13-hexane</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular Formula</td>
<td>C_{17}H_{20}N_{4}S</td>
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<tr>
<td>Mol wt</td>
<td>312.432 g/mol</td>
</tr>
<tr>
<td>CAS Registry No</td>
<td>132539-06-1</td>
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<td>LogP / Hydrophobicity</td>
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<tr>
<td>Protein Binding</td>
<td>93%</td>
</tr>
<tr>
<td>pka</td>
<td>-</td>
</tr>
<tr>
<td>Melting point</td>
<td>-</td>
</tr>
</tbody>
</table>

Pharmacology: For the acute and maintenance treatment of schizophrenia and related psychotic disorders, as well as acute treatment of manic or mixed episodes of bipolar I disorder. Intramuscular olanzapine is indicated for the rapid control of agitated patients. Olanzapine, an atypical antipsychotic agent, used to treat both negative and positive symptoms of schizophrenia, acute mania with bipolar disorder, agitation and psychotic symptoms in dementia.

Mechanism of action: Olanzapine’s antipsychotic activity is likely due to a combination of antagonism at D2 receptors in the metabolism pathways and 5HT2A receptors in the frontal cortex. Antagonism at D2 receptors relieves receptor relieves positive symptoms while antagonism at 5HT2A receptors relieves negative symptoms of schizophrenia.

### 1.10 BRIEF OUTLINE ABOUT THE WORK UNDERTAKEN

The development and validation of bioanalytical assay methods suitable for quantification of the selected drugs (quetiapine, mirtazapine, glimepiride, tamsulosin, topiramate and olanzapine) in biological matrices is discussed in this work. Relevant literature sources were consulted to understand the different parameters that must be
included in method development and validation, to identify what constitutes a good assay method and to know the international regulations pertaining to bioanalytical methodology that determine whether a developed assay method is acceptable or not. The information on assay methods reported for the selected drugs (discussed in respective chapters) has been carried out. The different aspects of these assay methods viz. extraction, instrumentation and total turn-around time were assessed. Some of the methods used were long and tedious extraction procedures and large amounts of solvents or biological fluids for extraction while other methods have a long analysis time. Thus, an objective was set to develop selective, sensitive and rapid LC-MS/MS assay methods that have short and simple extraction procedures, consume small amounts of solvent and biological fluid for extraction and a short turn-around time compared to previously established methods. The developed methods were suitable for bioavailability / bioequivalence studies to obtain accurate pharmacokinetic parameters in human plasma.
Figure 1a: Adsorption chromatography

Figure 1b: Partition chromatography
Figure 1c: Size exclusion chromatography

Figure 1d: Ion-exchange chromatography
Figure 1e: Affinity chromatography

One kind of molecule in complex mixture becomes attached to molecule that is covalently bound to stationary phase

All other molecules simply wash through
Figure 2: Schematic diagram of modern HPLC
Figure 3: The retention capacity of acids and bases for the total pH range
Figure 4: Major components of TSQ Quantum mass spectrometer
Figure 5: A typical plasma concentration-time profile showing pharmacokinetic-pharmacodynamic parameters, obtained after oral administration of single dose of a drug.
REFERENCES


