# CHAPTER ONE

## INTRODUCTION AND OBJECTIVES

**TITLE:** Introduction to the title

**CONTENTS**

- **1.1** THE PERSPECTIVE ........................................... 2
- **1.2** AIM AND OBJECTIVES ...................................... 4
- **1.3** MILIEU FOR THE TITLE ................................... 5
- **1.4** ELEMENTS OF LC/MS APPLICATION ..................... 7
  - 1.4.1 High performance liquid chromatography
  - 1.4.2 Mass spectrometry
  - 1.4.3 Liquid chromatography-mass spectrometry interface
- **1.5** SAMPLE PREPARATION TECHNIQUES ..................... 15
- **1.6** BIOANALYTICAL METHODOLOGY ......................... 16
- **1.7** METHOD VALIDATION ..................................... 18
- **1.8** BIOAVAILABILITY AND BIOEQUIVALENCE .............. 19
- **1.9** PHYSICOCHEMICAL PROPERTIES, PHARMACOLOGY AND MECHANISM OF ACTION OF DRUGS ......................... 23
- **1.10** BRIEF OUTLINE OF THE PROJECT ..................... 30
- **1.11** REFERENCES ................................................. 31
1.1 THE PERSPECTIVE

The field of bioanalysis has matured significantly from early studies in drug metabolism using simple colorimetry, with the proliferation of sophisticated hyphenated techniques linking advanced separations with mass spectrometry and NMR as detection systems, automation and robotics. Reliable data obtained from specific, selective, sensitive and reproducible analysis of a drug and its metabolites in biological samples (plasma, blood, serum, urine, and tissue) is a fundamental and crucial part of every successful drug development program. The information required to determine the metabolic fate of a new chemical entity (NCE) includes detection of metabolites, structure characterization and quantitative analysis. In some cases the concentrations of the metabolites may be extremely low, thus, highly specific and sensitive analytical methods are then required. The study of the metabolic fate of drugs is an essential and important part of the drug development process [1,2]. Pharmacologically active metabolites can contribute significantly to the overall therapeutic and adverse effects of drugs. Therefore, to fully understand the mechanism of action of drugs, it is important to recognize the role of active metabolites. Active metabolites can also be developed as drugs in their own right. Knowledge of drug levels in body fluids 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.

The analysis of drugs and their metabolites is a challenging task and several analytical methods have been applied, such as radioimmunoassay (RIA), gas chromatography/mass spectrometry (GC/MS) and liquid chromatography (LC) with UV, fluorescence, radioactivity and mass spectrometric detection (MS). With the introduction of the electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) techniques, LC/MS has become an ideal and widely used method in the analysis of
metabolites owing to its superior specificity, sensitivity and efficiency. It is now generally accepted as the preferred technique for quantitating small molecule drugs, metabolites, and other xenobiotic biomolecules in biological matrices. Atmospheric pressure ionization (API) techniques are compatible with reversed-phase eluent systems, taking into account the use of volatile solvents and additives in chromatographic separation, thus preserving all the advantages of LC. 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 photo-ionization (APPI) has expanded the applicability of API techniques towards less polar compounds [3,4].

Most of the work in metabolite analysis is carried out by using triple-quadrupole mass spectrometers (QQQ), as their tandem mass spectrometric (MS/MS) scan types are supportive in the identification of metabolites and provide the required specificity and sensitivity. Product ion scans are used for identification and multiple reaction monitoring (MRM) provides the high sensitivity required in quantitative analysis. The unique feature of QQQ is its capability to identify families of metabolites by using neutral loss and precursor ion scans [5,6].

The analytical strategy for metabolite analysis is dependent on the information sought. In the early stages of discovery, metabolic stability, drug–drug interaction and enzyme kinetic studies are based on the quantitative analysis of a parent drug or a few of its metabolites. In these types of analyses, the key issue is high throughput and therefore the analytical method should be as fast as possible. However, the determination of metabolite profiles is usually performed for a limited number of lead molecules in vivo and in vitro, and in these experiments the key issues are high specificity and sensitivity rather than speed.

The identification and quantitation of metabolites requires specific analytical methods and therefore sample preparation is an essential part of the analytical procedure. Interfering matrix compounds, such as proteins, salts and endogenous and background compounds, must be removed in sample pre-treatment, not only to avoid clogging of columns but also to improve the selectivity, sensitivity and reliability of analysis. Common pre-treatment methods for biological samples include protein precipitation (PP) and centrifugation followed by liquid–liquid extraction (LLE) or solid-phase extraction (SPE). Of these, SPE has achieved the widest acceptance owing to the ease of automation and to the availability of a wide variety of commercial sorbent materials. It is worth noting that precipitation of proteins with acids may catalyze the hydrolysis of some conjugates such
as glucuronides and sulphates. This can be avoided by using organic solvents in the precipitation. Also, care must be taken that the highly polar or ionic compounds with low retention factors are not lost in reversed-phase SPE [7].

In addition to appropriate sample preparation, good chromatographic performance is often required for sufficient specificity of the LC/MS analysis. The selection of an LC method depends on the complexity of the sample matrix and also on the specificity of the mass spectrometric detection method. In the case of quantitative analysis of a parent drug or a few metabolites in a simple in vitro matrix, the speed of the LC method is a key issue to ensure high sample throughput. Good resolution has clear benefits in drug and metabolite analysis: (i) co-eluting matrix components may decrease the signal several-folds owing to competition in the ionization process, (ii) the metabolism of a drug may lead to the formation of several isobaric compounds that should be separated prior to quantitation and (iii) labile metabolites, such as N-oxide, glucuronides and sulphates, may degrade to give the original drug either by in-source dissociation or by thermal degradation in the heated capillary. In this case, co-elution of the metabolites with the original drug will interfere with quantitation [8]. Reversed-phase LC is most often used in metabolite analysis owing to the universality of the method and its good compatibility with APCI-MS and ESI-MS.

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 in the biological samples. In addition, methods have to be as robust and cost effective as possible, making of particular importance to bioequivalent studies. 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.

1.2 AIM AND OBJECTIVES

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

ii) To realize efficient and selective extraction methodologies for quantitative extraction of selected drugs and their active metabolites from human plasma by
employing suitable extraction technique viz. solid phase extraction (SPE), protein precipitation (PP) or liquid-liquid extraction (LLE).

iii) The developed methods should have the following merits

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

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

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

- Simvastatin and simvastatin acid → Antihyperlipidemic
- Venlafaxine and O-desmethyl venlafaxine → Antidepressant
- Trazodone and m-chlorophenylpiperazine → Antidepressant
- Tramadol and O-desmethyl tramadol → Analgesic
- Sertraline and N-desmethyl sertraline → Antiobsessional/Antidepressant
- Donepezil and 6-O-desmethyl donepezil → Norotropic

1.3 MILIEU FOR THE TITLE

Hyphenated techniques are examples of new tools that pharmaceutical industry adopted for developing fast and cost-effective analytical methods. One of the most prevalent hyphenated techniques, liquid chromatography tandem mass spectrometry (LC-MS/MS), has led to major breakthroughs in the field of quantitative bioanalysis since the 1990s due to its inherent specificity, sensitivity, and speed. This combination of high performance liquid chromatography (HPLC) and mass spectrometry has proven its status as the most powerful analytical tool for screening and identifying drug and their metabolites in modern drug discovery. Bioanalytical functions in the pharmaceutical industry are constantly under pressure to reduce development times. This is often accompanied with an increase in the number of biological samples requiring pharmacokinetic analysis and a decrease in the desired quantitation levels. Thus, it is now generally accepted as the preferred technique for quantitating small molecule drugs, metabolites, and other xenobiotic biomolecules in biological matrices (plasma, blood, serum, urine, and tissue). Continual improvements in
LC/MS interface technologies combined with powerful features for structure analysis, qualitative and quantitative, has resulted in a widened scope of application. The growth in LC/MS applications has been extensive, with retention time and molecular weight emerging as essential analytical features from drug target to product. Liquid chromatography/mass spectrometry (LC/MS)-based techniques provide unique capabilities for pharmaceutical analysis. LC/MS methods are applicable to a wide range of compounds of pharmaceutical interest, and they feature powerful analytical figures of merit (sensitivity, selectivity, speed of analysis, and cost effectiveness). These analytical features have continually improved, resulting in easier-to-use and more reliable instruments. These developments coincided with the pharmaceutical industry's focus on describing the collective properties of novel compounds in a rapid, precise, and quantitative way [9,10].

Four key technical elements have been critical for the acceptance of LC/MS-based techniques in the pharmaceutical industry [11].

1. **The separation science**

The chromatographic method defines the pharmaceutical analysis. Chromatography provides analytical criteria to compare, refine, develop, and control the critical aspects of developing and manufacturing high-quality drug products. Thus, it is common in industry to see LC/MS methods distinguished by the chromatographic technology and features rather than by mass spectrometry performance and capabilities. Indeed, the effective combination of a wide variety of high performance liquid chromatography (HPLC) technologies and formats with mass spectrometry played a vital role in the acceptance of LC/MS. This achievement is significant because HPLC based methods are a universally recognized analysis “currency,” and perhaps, the first to be used throughout every stage of drug development.

2. **The mass spectrometry**

The analytical figures of merit dealing with sensitivity and selectivity provide a powerful platform for analysis. However, it was not until these analytical attributes could be harnessed into a reliable, reproducible, rugged, and high throughput instrument that mass spectrometry techniques could be taken seriously as an integral tool for drug development. The pioneering work performed with LC/MS interfaces has played a significant role in the acceptance of mass spectrometry as a routine tool for pharmaceutical analysis. Furthermore, added dimensions of mass analysis provide enhanced limits of detection for the analysis of complex mixtures and unique capabilities for structure identification.
3. The information
The rate of analysis and subsequent distribution of results has grown tremendously due to the increased use of LC/MS and other information-rich technologies. From strictly an analysis perspective, LC/MS has demonstrated a unique capability for maintaining high quality performance and a rapid turnaround of samples. Yet, it is the accurate and efficient processing of information that has been essential for LC/MS use and acceptance. As a result, LC/MS has developed unique partnerships with tools responsible for sample tracking, interpretation, and data storage. Consequently, LC/MS has become an information-rich, information-dependent technology in the pharmaceutical industry. LC/MS is highly dependent on software to integrate key analysis elements that deal with sample preparation, real-time analysis decisions, and the distribution of results.

4. The widened scope of application
The fact that LC/MS is now routinely used during every stage of drug development is a powerful benchmark for acceptance. The increased performance of applications that incorporate LC/MS have, in turn, stimulated new performance levels for sample preparation, high speed separations, automated analysis, information databases, and software tools, to name a few. Motivated by unmet industry needs, the drive for new applications has stimulated tremendous growth in pharmaceutical analysis marked by invention and creativity.

1.4 ELEMENTS OF LC/MS APPLICATION [11, 12]
From an applications standpoint, the partnership of HPLC and mass spectrometry benefited greatly from the tradition of HPLC within the pharmaceutical industry and from the growing trend to obtain structural and quantitative information during earlier stages of drug development. Ultimately, it has been the power of HPLC to resolve and the ability of mass spectrometry to identify that enabled LC/MS to integrate effectively with drug development and to solve problems. The integrated LC/MS format provides the pharmaceutical industry with a highly efficient platform to conduct a series of online steps to purify the sample and amplify the signal.

1.4.1 High performance liquid chromatography
HPLC-based techniques have been a traditional mainstay of the pharmaceutical industry. It is a powerful technology that allows complex mixtures to be transformed into separated components. It is highly sensitive, reproducible, accessible, and well understood from an operator's standpoint. The output from the HPLC is its unique characteristic that
distinguishes it from all other analytical techniques and the chromatogram, is defined and simple. Each peak is characteristic of a component; each chromatogram is diagnostic of an event or experiment associated with a drug development activity. When combined with the facts that nearly all compounds of pharmaceutical interest are amenable to HPLC methodologies and conditions and that critical information on nearly all events in the drug development cycle can be derived from HPLC chromatograms, it becomes evident why HPLC is a universally accepted analysis tool.

The various forms of chromatography employing a liquid rather than a gas as the moving phase are studied under the head ‘liquid chromatography’. 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. Nowadays, 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 physical 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 from 3-10μm. In general, column of 10 to 300mm length are used with an inner diameter of 1 to 5mm. 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 1 gives a schematic representation of a typical HPLC system.

**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 organoalkoxy-silanes, e.g.

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

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 up to pH-14 (Figure 2). Some of the advantages of bonded-phase materials are relative stability, short equilibration time, and their versatility [13, 14].
Separation process in HPLC: 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. Mixtures of water or aqueous buffers and an organic modifier (methanol, acetonitrile etc.) are used as eluants. 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 [15]. 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. 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 simpler 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 candidates 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 and their active metabolites.

1.4.2 Mass spectrometry [16]
Mass spectrometry has progressed extremely rapidly during the last two decades and this progress has led to the advent of entirely new instruments. Mass spectrometry's characteristics have raised it to an outstanding position among analytical methods: unequalled sensitivity, detection limits, speed and diversity of its applications. In analytical chemistry, the most recent applications are mostly oriented towards biochemical problems, such as proteome, metabolome, high throughput in drug discovery and metabolism, and so on. Other analytical applications are routinely applied in pollution control, food control, forensic science, natural products or process monitoring. Until the widespread commercial introduction of electrospray ionization (ESI)-LC/MS instruments in the early 1990s, mass spectrometry-based techniques had a functional yet limited role in drug development. Primarily geared towards a medicinal chemistry environment, mass spectrometry was a fairly routine tool for molecular weight determination and a speciality tool for complex structure identification problems. Applications and methods were typically defined by the ionization method of choice. Fast atom bombardment (FAB), desorption
chemical ionization (DCI), chemical ionization (CI), and electron ionization (EI) were the predominant choices, with varying degrees of applicability. Once a molecule is ionized, the mass spectrometer provided separation of the resulting molecular ions and dissociation products, according to weight. These masses were assigned to corresponding substructures of the molecule. These approaches were primarily limited to the characterization of low molecular weight compounds (<500Da) with varying degrees of polarity and thermal lability. Detailed analysis was defined by the depth of spectral interpretation for structure identification purposes or by the resolving power of the instrument for exact mass, molecular formula, and purity assessment.

1.4.3 Liquid chromatography-mass spectrometry interface [11, 12, 17-20]

The LC/MS interface provides the connection between the HPLC and the mass spectrometer. It is responsible for the reliable and efficient transfer of analytes from the solution phase to the gas phase. It is also responsible for a critical element of mass spectrometry analysis: the ionization. For most pharmaceutical analyses, the ideal ionization technique would generate a single ion that corresponds to the molecular weight of the drug compound, with little or no fragment ions. The confirmation of structure is facile and quantitation proceeds with a high degree of sensitivity. Elements of selectivity are provided by the HPLC separation (i.e., drug components, biological matrix) and/or MS/MS (i.e., structure elucidation, enhanced quantitation). A block diagram of liquid chromatography-mass spectrometer is depicted in Figure 3.

**Figure 3** — Block diagram of liquid chromatography-mass spectrometer

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 analysed compounds that are separated or filtered on the basis of their mass-to-charge ratio (m/z). This selectivity eases the requirements for the sample preparation or complete chromatographic separation of the components of a mixture. When 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 gas phase ions 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 EI, each method can produce abundant positive or negative ions. The criteria for selection of a particular ionization technique is generally dependent on the physical state of the sample (aqueous solution vs. solid sample), volatility and thermal stability of the sample and the 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 spectrometry (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 (Q₂) 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.

Figure 4 - Major components of API 4000 mass spectrometer with different pumps
1.5 SAMPLE PREPARATION TECHNIQUES [21]

Adequate sample preparation is a key aspect of quantitative bioanalysis and can often be the cause of bottlenecks during high-throughput analysis. Sample preparation is a process required for the transformation of a sample to make it amenable for chemical analysis or to improve the analysis. This is necessary when a given sample cannot be directly analyzed or when direct analysis generates poor results. Typical problems with analyses are interferences and low sensitivity. Sample preparation is usually needed to eliminate interferences and to increase sensitivity. Samples are made from two distinct parts, the analytes and the matrix. The analytes are the compounds of interest that must be analyzed, while matrix is the remainder of the sample, which does not require 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 [22]. 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 [23]. 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 [24]. 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 [25]. One phase in LLE is usually the aqueous, and the second phase will be an organic solvent. More hydrophilic compounds prefer the polar aqueous phase, whereas more hydrophobic compounds will be found mainly in the organic solvent. Analytes extracted into the organic phase are easily recovered by evaporation of the solvent; analytes extracted into the aqueous phase often can be injected directly onto a reversed phase column column. It takes advantage of the relative solubilities 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) → extraction → pH adjustment (base or acid) → back extraction" offers an effective generic approach. Liquid-liquid extraction is labour intensive as it involves drying followed by reconstitution to meet the desired quantitation limits.

iii) **Solid phase extraction (SPE)** is routinely used in the sample preparation for quantifying analytes in biological fluids such as plasma and urine [26]. SPE uses a 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. It plays a significant role in the evaluation and interpretation of bioavailability, bioequivalence and pharmacokinetic data [27]. 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 [28]. 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 [29]. Method development involves evaluation and optimization of the various stages of sample preparation, chromatographic separation, detection and
quantitation. 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 in gas chromatography (GC).

ii) Choice of internal standard, 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 [30,31].

Matrix effect, often described as matrix ionization effect or ion suppression effect, is a phenomenon observed when the signal of analyte can be either suppressed or enhanced due to the co-eluting components that originate from the sample matrix [9]. When a rather long isocratic or gradient chromatographic program is used in the quantitative assay, matrix effect may be not present at the retention time for an analyte. However, in the case of high-throughput LC-MS/MS analysis, matrix effect is one of the major issues to be addressed in method development and validation, especially when analyte is not well separated from the LC-front. One problem brought by matrix suppression effect is reduced sensitivity when analyte signal is suppressed. Detailed studies on matrix effects revealed that the ion suppression or enhancement is frequently accompanied by significant deterioration of the precision of the analytical method as demonstrated by Matuszewski et al. [31]. The authors studied the precision (%CV) upon repetitive injection of post-extraction spiked plasma samples as a function of the analyte.
concentration for a single lot and for five different lots of plasma. While for the single plasma lot the precision is acceptable, it may not be when different plasma lots are taken into account. Generally, matrix effect impacts more on the low end of calibration curve than the mid range or highly end. When discussing matrix effects, it is useful to discriminate between ion suppression (or enhancement) by the matrix at one hand, and different matrix effects exerted by different sample lots at the other hand. The difference in response between a neat solution sample and the post-extraction spiked sample is called the absolute matrix effect, while the difference in response between various lots of post-extraction spiked samples is called the relative matrix effect. If no counteraction is taken, an absolute matrix effect will primarily affect the accuracy of the method, while a relative matrix effect will primarily affect the precision of the method.

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 [30]. 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 [32]. Additionally, 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 [33].

1.7 METHOD VALIDATION

The search for the reliable range of a method and continuous application of this knowledge is called validation [33]. It can also be defined as the process of documenting that the method under consideration is suitable for its intended purpose [34]. 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 [28]. 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 [35]. 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 [32].

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 some differences exist between their documents [34].

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) [36]. According to them the revised version of terminology to be included are bias (accuracy), precision, specificity, limit of detection, limit of quantitation, 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 (Societe Francaise des Sciences et Techniques Pharmaceutiques) [34].

On the other hand the guideline for industry by FDA [37] 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) sensitivity, (2) selectivity, (3) accuracy and precision, (4) carryover, (5) calibration curve, (5) recovery, (6) matrix effect, (7) dilution integrity and (8) 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 QC samples.

1.8 BIOAVAILABILITY AND BIOEQUIVALENCE [38,39]

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) Pharmaceutical 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.

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.

**Objective of bioavailability studies**

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 \( (C_{\text{max}}) \):** 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).

**Figure 5** - A typical plasma concentration-time profile showing pharmacokinetic-pharmacodynamic parameters.

**Time of peak concentration (T<sub>max</sub>):** 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 T<sub>max</sub>. 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 μg/mL x h. 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.

Recently, the ‘incurred’ or study sample reanalysis (ISR) has become mandatory for bioanalytical methods used to support the drug development process. Viswanathan et al. [40] have suggested that an evaluation of the reproducibility in the analysis of incurred
samples be performed on each species used for Good Laboratory Practices (GLP) toxicology assessments, as well as an appropriate evaluation of incurred sample reproducibility from clinical studies. Incurred or study samples can vary in their composition when compared with the standards and quality control samples used to validate the method and analyze these samples. During the 3rd American Association of Pharmaceutical Scientists (AAPS)/Food and Drug Administration (FDA) Bioanalytical Workshop, it was suggested that the reproducibility in the analysis of incurred samples be evaluated in addition to the usual prestudy validation activities performed. Although every attempt is made to formulate standards and QCs to be as similar to the study samples being analyzed as possible, "incurred" or study samples can differ in a variety of ways. These differences are dependent in part on whether the analyte(s) in question are small molecules or macromolecules. Moreover, it becomes even more important when metabolites are measured, as they may convert in vitro to their parent drug molecule [41].

1.9 PHYSICOCHEMICAL PROPERTIES, PHARMACOLOGY AND MECHANISM OF ACTION OF DRUGS [42]

1. Simvastatin

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IUPAC Name</td>
<td>(1S,3R,7S,8S,8aR)-8-{2-{[(2R,4R)-4-hydroxy-6-oxooxan-2-yl]ethyl}-3,7-dimethyl-1,2,3,7,8,8a-hexahydonaphthalen-1-yl} 2,2-dimethylbutanoate</td>
</tr>
<tr>
<td>Molecular Formula</td>
<td>C_{25}H_{35}O_{5}</td>
</tr>
<tr>
<td>Molecular Weight</td>
<td>418.56g/mol</td>
</tr>
<tr>
<td>Percentage Composition</td>
<td>C 71.74%, H 9.15%, O 19.11%</td>
</tr>
<tr>
<td>Monograph Number</td>
<td>8613</td>
</tr>
<tr>
<td>CAS Registry Number</td>
<td>79902-63-9</td>
</tr>
<tr>
<td>H₂O-Solubility</td>
<td>0.76mg/L</td>
</tr>
<tr>
<td>LogP / Hydrophobicity</td>
<td>4.7</td>
</tr>
<tr>
<td>Protein Binding</td>
<td>95% (to human plasma proteins)</td>
</tr>
<tr>
<td>pKa</td>
<td>-</td>
</tr>
<tr>
<td>Melting point</td>
<td>135-138°C</td>
</tr>
<tr>
<td>Half Life</td>
<td>3h</td>
</tr>
<tr>
<td>Category</td>
<td>Cardiovascular agent</td>
</tr>
<tr>
<td>BCS Class (cLogP)</td>
<td>Class II</td>
</tr>
</tbody>
</table>
**Indication:** For the treatment of hypercholesterolemia

**Pharmacology:** Simvastatin, the methylated form of lovastatin, is an oral antilipemic agent which inhibits 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase. Simvastatin is used in the treatment of primary hypercholesterolemia and is effective in reducing total and LDL-cholesterol as well as plasma triglycerides and apolipoprotein B. Simvastatin is a powerful lipid-lowering drug that can decrease low density lipoprotein (LDL) levels by up to 50%. It is used in doses of 5mg to 80mg. Higher doses (160mg) have been found to be too toxic, while giving only minimal benefit in terms of lipid lowering. There is no real effect on HDL and triglyceride levels. From recent research it has become apparent that simvastatin and other statins inhibit the progression of atherosclerosis beyond their effects on LDL. Many explanations have been proposed, for example its inhibitory effect on macrophages in the atherosclerotic plaque lesions. All statins act by inhibiting HMG-CoA reductase, the rate-limiting enzyme of the HMG-CoA reductase pathway, the metabolic pathway responsible for the endogenous production of cholesterol. Statins are more effective than other lipid-regulating drugs at lowering LDL-cholesterol concentration but they are less effective than the fibrates in reducing triglyceride concentration. However, statins reduce cardiovascular disease events and total mortality irrespective of the initial cholesterol concentration.

**Mechanism of action:** The 6-membered lactone ring of simvastatin is hydrolyzed in vivo to generate mevinolinic acid, an active metabolite structurally similar to HMG-CoA. Once hydrolyzed, simvastatin competes with HMG-CoA for HMG-CoA reductase, a hepatic microsomal enzyme. Interference with the activity of this enzyme reduces the quantity of mevalonic acid, a precursor of cholesterol. Simvastatin is in the form of an inactive lactone that is readily hydrolyzed in vivo to the corresponding β-hydroxyacid, a potent inhibitor of HMG-CoA reductase. Inhibition of HMG-CoA reductase is the basis for an assay in pharmacokinetic studies of the β-hydroxyacid metabolites (active inhibitors) and following base hydrolysis, active plus latent inhibitors (total inhibitors) in plasma following administration of simvastatin.

**Absorption:** Absorption of simvastatin, estimated relative to an intravenous reference dose, in each of two animal species tested, averaged about 85% of an oral dose. In animal studies, after oral dosing, simvastatin achieved substantially higher concentrations in the liver than in non-target tissue.

**Toxicity:** It is used in doses of 5mg to 80mg. Higher doses (160mg) have been found to be too toxic, while giving only minimal benefit in terms of lipid lowering.
2. Venlafaxine

<table>
<thead>
<tr>
<th>IUPAC Name</th>
<th>(RS)-1-[2-dimethylamino-1-(4-methoxyphenyl)-ethyl]cyclohexanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular Formula</td>
<td>C_{17}H_{23}NO_{2}</td>
</tr>
<tr>
<td>Molecular Weight</td>
<td>277.40g/mol</td>
</tr>
<tr>
<td>Percentage Composition</td>
<td>C 73.61%, H 9.81%, N 5.05%, O 11.54%</td>
</tr>
<tr>
<td>Monograph Number</td>
<td>10008</td>
</tr>
<tr>
<td>CAS Registry Number</td>
<td>93413-69-5</td>
</tr>
<tr>
<td>H_{2}O-Solubility</td>
<td>512mg/mL</td>
</tr>
<tr>
<td>LogP / Hydrophobicity</td>
<td>2.8</td>
</tr>
<tr>
<td>Protein Binding</td>
<td>27% (to human plasma proteins)</td>
</tr>
<tr>
<td>pKa</td>
<td>9.4</td>
</tr>
<tr>
<td>Melting point</td>
<td>215-217°C</td>
</tr>
<tr>
<td>Half Life</td>
<td>5h</td>
</tr>
<tr>
<td>Category</td>
<td>Central Nervous System Agent</td>
</tr>
<tr>
<td>BCS Class (cLogP)</td>
<td>Class I</td>
</tr>
</tbody>
</table>

**Indication:** For the treatment of severe depression

**Pharmacology:** Venlafaxine, an antidepressant agent structurally unrelated to other antidepressants, is used to treat melancholia, generalized anxiety disorder (GAD), panic disorder, post-traumatic stress disorder, and hot flashes in breast cancer survivors.

**Mechanism of action:** Venlafaxine and its active metabolite, O-desmethyl venlafaxine (ODV), inhibit the reuptake of both serotonin and norepinephrine (NE) with a potency greater for the 5-hydroxytryptamine (5-HT) than for the NE reuptake process. Both venlafaxine and the ODV metabolite have weak inhibitory effects on the reuptake of dopamine but, unlike the tricyclics and similar to selective serotonin reuptake inhibitors (SSRI), they are not active at histaminergic, muscarinic, or alpha (1)-adrenergic receptors.

**Absorption:** Bioavailability is 45% following oral administration.

**Toxicity:** Most patients overdosing with venlafaxine develop only mild symptoms. However, severe toxicity is reported with the most common symptoms being CNS depression, serotonin toxicity, seizure or cardiac conduction abnormalities. Venlafaxine's toxicity appears to be higher than other SSRIIs, with a fatal toxic dose closer to that of the tricyclic antidepressants than the SSRIIs. Doses of 900mg or more are likely to cause moderate toxicity. Deaths have been reported following large doses.
3. Trazodone

<table>
<thead>
<tr>
<th>IUPAC Name</th>
<th>2-(3-[4-(3-chlorophenyl)piperazin-1-yl]propyl)-[1,2,4]triazolo[4,3-a]pyridin-3(2H)-one</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular Formula</td>
<td>C_{19}H_{22}ClN_{2}O</td>
</tr>
<tr>
<td>Molecular Weight</td>
<td>371.86g/mol</td>
</tr>
<tr>
<td>Percentage Composition</td>
<td>C 61.37%, H 5.96%, Cl 9.53%, N 18.83%, O 4.30%</td>
</tr>
<tr>
<td>Monograph Number</td>
<td>9654</td>
</tr>
<tr>
<td>CAS Registry Number</td>
<td>19794-93-5</td>
</tr>
<tr>
<td>H_{2}O-Solubility</td>
<td>Sparingly soluble</td>
</tr>
<tr>
<td>LogP / Hydrophobicity</td>
<td>2.553</td>
</tr>
<tr>
<td>Protein Binding</td>
<td>89-95% (to human plasma proteins)</td>
</tr>
<tr>
<td>pKa</td>
<td>6.14 (50% ethanol)</td>
</tr>
<tr>
<td>Melting point</td>
<td>86-87°C</td>
</tr>
<tr>
<td>Half Life</td>
<td>3-6h</td>
</tr>
<tr>
<td>Category</td>
<td>Second Generation Antidepressants</td>
</tr>
<tr>
<td>BCS Class (cLogP)</td>
<td>-</td>
</tr>
</tbody>
</table>

**Indication:** For the treatment of depression

**Pharmacology:** Trazodone is an antidepressant and hypnotic chemically unrelated to tricyclic, tetracyclic or other known antidepressant agents. The mechanism of trazodone’s antidepressant action in human is not fully understood. In animals, trazodone selectively inhibits serotonin uptake by brain synaptosomes and potentiates the behavioral changes induced by the serotonin precursor, 5-hydroxytryptophan (5-HTP). Cardiac conduction effects of trazodone in the anesthetized dog are qualitatively dissimilar and quantitatively less pronounced than those seen with tricyclic antidepressants. Trazodone is not a monoamine oxidase inhibitor and, unlike amphetamine-type drugs, does not stimulate the central nervous system. In human, trazodone is well absorbed after oral administration without selective localization in any tissue. Since the clearance of trazodone from the body is sufficiently variable, in some patients trazodone may accumulate in the plasma.

**Mechanism of action:** Trazodone binds at 5-HT2 receptor; it acts as a serotonin agonist at high doses and a serotonin antagonist at low doses. Like fluoxetine, trazodone’s antidepressant activity likely results from blockage of serotonin reuptake by inhibiting serotonin reuptake pump at the presynaptic neuronal membrane. If used for long time periods, postsynaptic neuronal receptor binding sites may also be affected. The sedative
The effect of trazodone is likely the result of alpha-adrenergic blocking action and modest histamine blockade at H1 receptor. It weakly blocks presynaptic alpha2-adrenergic receptors and strongly inhibits postsynaptic alpha1 receptors. Trazodone does not affect the reuptake of norepinephrine or dopamine within the CNS.

**Absorption:** Well absorbed following oral administration

**Toxicity:** LD_{50}=96mg/kg (i.v. in mice)

### 4. Tramadol

<table>
<thead>
<tr>
<th>IUPAC Name</th>
<th>(1R,2R)-rel-2-[(dimethylamino)methyl]-1-(3-methoxyphenyl)cyclohexanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular Formula</td>
<td>C_{16}H_{23}NO_{2}</td>
</tr>
<tr>
<td>Molecular Weight</td>
<td>263.37g/mol</td>
</tr>
<tr>
<td>Percentage Composition</td>
<td>C 72.97%, H 9.57%, N 5.32%, O 12.15%</td>
</tr>
<tr>
<td>Monograph Number</td>
<td>9642</td>
</tr>
<tr>
<td>CAS Registry Number</td>
<td>27203-92-5</td>
</tr>
<tr>
<td>H₂O-Solubility</td>
<td>Soluble in water, 0.75mg/mL</td>
</tr>
<tr>
<td>LogP / Hydrophobicity</td>
<td>2.4</td>
</tr>
<tr>
<td>Protein Binding</td>
<td>20% (to human plasma proteins)</td>
</tr>
<tr>
<td>pKa</td>
<td>9.41</td>
</tr>
<tr>
<td>Melting point</td>
<td>180-181°C</td>
</tr>
<tr>
<td>Half Life</td>
<td>6.3h</td>
</tr>
<tr>
<td>Category</td>
<td>Central Nervous System Agents</td>
</tr>
<tr>
<td>BCS Class (cLogP)</td>
<td>Class I</td>
</tr>
</tbody>
</table>

**Indication:** Indicated in the treatment of moderate to severe pain

**Pharmacology:** Tramadol, a centrally-acting analgesic, exists as a racemic mixture of the trans-isomer, with important differences in binding, activity, and metabolism associated with the two enantiomers. Although tramadol is a synthetic analog of codeine, it has a significantly lower affinity for opioid receptors than codeine. Tramadol is used to treat postoperative, dental, cancer and acute musculoskeletal pain and as an adjuvant to nonsteroidal anti-inflammatory drug (NSAID) therapy in patients with osteoarthritis.

**Mechanism of action:** Tramadol and its O-desmethyl metabolite (M1) are selective, weak μ-opioid (OP3) receptor agonists. Opiate receptors are coupled with G-protein receptors and function as both positive and negative regulators of synaptic transmission via G-proteins that activate effector proteins. As the effector system is adenylate cyclase and cAMP located at the inner surface of the plasma membrane, opioids decrease
intracellular cAMP by inhibiting adenylate cyclase. Subsequently, the release of nociceptive neurotransmitters such as substance P, γ-aminobutyric acid (GABA), dopamine, acetylcholine and noradrenaline is inhibited. The analgesic properties of tramadol can be attributed to norepinephrine and serotonin reuptake blockade in the central nervous system (CNS), which inhibits pain transmission in the spinal cord. The (+) enantiomer has higher affinity for the OP3 receptor and preferentially inhibits serotonin uptake and enhances serotonin release. The (-) enantiomer preferentially inhibits norepinephrine reuptake by stimulating alpha (2)-adrenergic receptors.

**Absorption:** Recemic tramadol is rapidly and almost completely absorbed after oral administration. The mean absolute bioavailability of a 100mg oral dose is approximately 75%. The mean peak plasma concentration of racemic tramadol and M1 occurs at two and three hours, respectively, after administration in healthy adults.

**Toxicity:** LD₉₀=350mg/kg (orally in mice)

---

**5. Sertraline**

<table>
<thead>
<tr>
<th>IUPAC Name</th>
<th>(1S,4S)-4-(3,4-dichlorophenyl)-N-methyl-1,2,3,4-tetrahydronaphthalen-1-amine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular Formula</td>
<td>C₁₇H₁₁Cl₂N</td>
</tr>
<tr>
<td>Molecular Weight</td>
<td>306.23g/mol</td>
</tr>
<tr>
<td>Percentage Composition</td>
<td>C 66.68%, H 5.60%, Cl 23.15%, N 4.57%</td>
</tr>
<tr>
<td>Monograph Number</td>
<td>8541</td>
</tr>
<tr>
<td>CAS Registry Number</td>
<td>79617-96-2</td>
</tr>
<tr>
<td>H₂O-Solubility</td>
<td>3.5mg/L</td>
</tr>
<tr>
<td>LogP / Hydrophobicity</td>
<td>5.567</td>
</tr>
<tr>
<td>Protein Binding</td>
<td>98%</td>
</tr>
<tr>
<td>pKa</td>
<td>9.48 (water)</td>
</tr>
<tr>
<td>Melting point</td>
<td>243-245°C</td>
</tr>
<tr>
<td>Half Life</td>
<td>26h</td>
</tr>
<tr>
<td>Category</td>
<td>Central Nervous System Agents</td>
</tr>
<tr>
<td>BCS Class (cLogP)</td>
<td>Class I</td>
</tr>
</tbody>
</table>

**Indication:** For the treatment of post-traumatic stress disorder, depression, obsessive-compulsive disorder and panic disorder.

**Pharmacology:** Sertraline, an antidepressant drug similar to citalopram, fluoxetine and paroxetine, is of the SSRI type. Sertraline has one active metabolite and is used to treat depression, obsessive-compulsive disorder (OCD), panic disorder and post-traumatic stress disorder.
Mechanism of action: It is believed that sertraline inhibits reuptake of serotonin at the neuronal membrane. SSRIs have less sedative, anticholinergic and cardiovascular effects than the tricyclic antidepressant drugs because of decreased binding to histamine, acetylcholine and norepinephrine receptors.

Absorption: The effects of food on the bioavailability of the sertraline tablet and oral concentrate were studied in subjects administered a single dose with and without food. For the tablet, AUC was slightly increased when drug was administered with food but the $C_{\text{max}}$ was 25% greater, while the time to reach peak plasma concentration ($T_{\text{max}}$) decreased from 8h post-dosing to 5.5h. For the oral concentrate, $T_{\text{max}}$ was slightly prolonged from 5.9 to 7.0h with food.

Toxicity: Symptoms of toxicity include alopecia, decreased libido, diarrhea, ejaculation disorder, fatigue, insomnia, somnolence and serotonin syndrome.

6. Donepezil

<table>
<thead>
<tr>
<th>IUPAC Name</th>
<th>(RS)-2-[(1-benzyl-4-piperidyl)methyl]-5,6-dimethoxy-2,3-dihydroinden-1-one</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular Formula</td>
<td>C$<em>2$H$</em>{29}$NO$_3$</td>
</tr>
<tr>
<td>Molecular Weight</td>
<td>379.49g/mol</td>
</tr>
<tr>
<td>Percentage Composition</td>
<td>C 75.96%, H 7.70%, N 3.69%, O 12.65%</td>
</tr>
<tr>
<td>Monograph Number</td>
<td>3453</td>
</tr>
<tr>
<td>CAS Registry Number</td>
<td>120014-06-4</td>
</tr>
<tr>
<td>H$_2$O-Solubility</td>
<td>2.931mg/L</td>
</tr>
<tr>
<td>LogP / Hydrophobicity</td>
<td>3.6</td>
</tr>
<tr>
<td>Protein Binding</td>
<td>96%</td>
</tr>
<tr>
<td>pKa</td>
<td>-</td>
</tr>
<tr>
<td>Melting point</td>
<td>211-212°C</td>
</tr>
<tr>
<td>Half Life</td>
<td>70h</td>
</tr>
<tr>
<td>Category</td>
<td>Central Nervous System Agents</td>
</tr>
<tr>
<td>BCS Class (cLogP)</td>
<td>Class I</td>
</tr>
</tbody>
</table>

Indication: For management of symptoms associated with Alzheimer’s disease.

Pharmacology: Donepezil is a centrally acting reversible acetyl cholinesterase inhibitor. Its main therapeutic use is in the treatment of Alzheimer’s disease where it is used to increase cortical acetylcholine. It is well absorbed in the gut with an oral bioavailability of 100% and easily crosses the blood-brain barrier. Because it has a half life of about 70 h, it can be taken once a day. Initial dose is 5mg per day, which can be increased to 10mg per day after an adjustment period of at least 4 weeks. Donepezil is a parasympathomimetic,
specifically, a reversible cholinesterase inhibitor. Donepezil is postulated to exert its therapeutic effect by enhancing cholinergic function. This is accomplished by increasing the concentration of acetylcholine through reversible inhibition of its hydrolysis by acetylcholinesterase, if this proposed mechanism of action is correct, donepezil's effect may lessen as the disease process advances and fewer cholinergic neurons remain functionally intact. There is no evidence that donepezil alters the course of the underlying dementing process.

**Mechanism of action:** Donepezil's proposed mechanism of action involves the increase in the concentration of acetylcholine through reversible inhibition of its hydrolysis by acetylcholinesterase.

**Absorption:** Donepezil is well absorbed with a relative oral bioavailability of 100% and reaches peak plasma concentrations in 3 to 4h.

**Toxicity:** Symptoms of overdose include severe nausea, vomiting, salivation, sweating, bradycardia, hypotension, respiratory depression, collapse and convulsions. Increasing muscle weakness is a possibility and may result in death if respiratory muscles are involved.

**1.10 BRIEF OUTLINE OF THE PROJECT**

The development and validation of bioanalytical assay methods suitable for quantitation of the selected drugs and their active metabolites (simvastatin and simvastatin acid, venlafaxine and O-desmethyl venlafaxine, trazodone and m-chlorophenylpiperazine, tramadol and O-desmethyl tramadol, sertraline and N-desmethyl sertraline & donepezil and 6-O-desmethyl donepezil) 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. Further, literature search is done to collect information on assay methods reported for the selected drugs (discussed in respective chapters). The different aspects of these assay methods viz. extraction, instrumentation, total turn-around time and sensitivity, selectivity etc. were assessed. 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 rapid compared to previously established methods. Systematic validation as per USFDA guidelines is done for all the methods. The parameters investigated include selectivity, sensitivity, cross specificity, carry over effect,
linearity, accuracy and precision, absolute and relative recovery, absolute and relative matrix effect, stability in plasma and dilution integrity. The application of these methods for bioequivalence study is conducted with test and reference formulation of the selected drugs on healthy human subjects. The pharmacokinetic parameters investigated include \( C_{\text{max}} \), \( T_{\text{max}} \), \( t_{1/2} \), AUC and \( K_e \). The study is conducted strictly in accordance with guidelines laid down by International Conference on Harmonization and USFDA.

### 1.11 REFERENCES


   iii. PharmGKB: http://www.pharmgkb.org