CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE
INTRODUCTION

To discover and develop a new drug is a very costly process and may run into billions of dollars. Additionally, the time required for this process can run up to approximately 8 to 10 years before the drug can see the light of the day.

In Drug Development process for both new drugs and their generic equivalent drugs, BA and BE studies play a major role. BE studies are generally carried out for generic drugs of innovator drugs to introduce medicine at lower cost into the market as directed by the international regulatory authorities. BE studies are performed not only for the generic products, but also for innovator drugs between early and late clinical trial formulations or if there are changes in excipients of formulation after an innovator drug has been approved. A generic drug does not require to undergo clinical studies if it is bioequivalent to the brand name drug. Because it proves that presence of plasma concentration of active ingredient at the site of action of the generic and innovator medicines are the same; hence the safety and effectiveness will be the same [1]. In the evaluation and interpretation of the bioavailability, bioequivalence and pharmacokinetic data, the bioanalytical methods are employed for estimation of analytes and their metabolites from plasma, urine, saliva and serum [2, 3]. Same applies to methods employed for toxicokinetic studies as well. This data is very critical as it is used to support various regulatory submissions.

Method Validation ascertains the excellence of the analyst’s work. Users of the bioanalytical data thus obtained can be assured of its correctness. Also, validation is mandatory by the regulatory agencies [4, 5]. Regulatory agencies mandate bioavailability and bioequivalence studies to assure them of the therapeutic equivalence between a pharmaceutically equivalent test product and the reference product. These studies give an exact estimate of the release of drug from its intended dosage form and the absorption of same into systemic circulation. Hence, equivalent methods to measure bioavailability should as much as possible be used as in the bioequivalence study [6, 8].

The steps involved in this design process and workflow of BA/BE studies are presented in brief in Figures 1 and 2, respectively.
Figure 1. Process of study design and protocol approval
Figure 2. Work flow of bioavailability/bioequivalence study

This thesis describes developed and validated bioanalytical methods for the simultaneous drug determination in biological matrices. A well-organized development and validation of analytical methods are important components in the development of pharmaceuticals. Hence impetus is given to the fact that the entire procedure right from collection of the sample to final analysis would be as robust, rugged and simple as possible. The intention was to develop methods that can be employed in bioanalytical laboratory.
1. **SAMPLE COLLECTION**

1.1 **Vein puncture technique**

Blood, plasma, serum and urine generally consider as a biological matrix which contain the analyte. By vein puncture technique blood is collected from human volunteer and stored in a container with anticoagulant. After centrifugation at 4000 rpm for 15 min plasma is obtained. About 30 to 50% of the original volume is collected [7].

Nowadays for collection of blood sample dried blood spots (DBS) and dried matrix spots (DMS) are widely used [6-8].

1.2 **Dry blood Spotting**

DBS is latest technique which is useful in the toxicokinetic and pharmacokinetic studies [9]. DBS samplings employ spotting of blood by pricking finger or heal on to a card. Then these cards are kept for air drying and stored in a sealed bag [10].

This technique has several advantages over conventional blood collection techniques. Since the process involves only a single prick for harvesting blood samples, the blood volume required is significantly reduced. This greatly helps in collecting samples from pediatric patients or in animal studies. Serial bleeding of a single animal can thus be conducted instead of using multiple animals. Data quality is also greatly enhanced as DBSs is a friendly technique. Moreover, the cards can be stored at room temperature conditions, thus disposing of the use of low temperature deep freezers. The storage and shipment costs are also reduced [11].

1.2.1 **Dried Blood Spot Sample collection**

DBS analysis can be carried out automatically and semi automatically. Punching of cards and extraction can be carried out with the robotic machine. For sample identification barcode system can be used. In semi-automated cards are fed manually and operator attention required. In automated process the entire process is handled by robotic machine and no operator attention is required. In this, 96 or384 well collection plate is used. The options for automation systems are likely to increase as DBS collection is becoming more widely adopted for bioanalysis [12-15].
1.2.2 Dried Matrix Spot Sample Collection

This is one of the sample collection technique in which DMS card/paper is used for the spotting of the biological matrices collected from the animal and human subjects. The spotted area is punched out and extracted analytes from that spotted area by using suitable technique for analysis. Analytes are extracted in suitable organic solvent and then used for the analysis by LC-MS/MS. Therefore in this technique very less amount of solvent is required as compared to others [16].

![DMS Card](image)

Figure 3. DMS card/paper

DMS technique is cost saving technique due to less sample volume and easy handling DMS technique is most widely used because it is easy for storage and not required deep freezer for storage. Due to less sample size it may occur limitations in sensitivity.

Bond Elute DMS is suitable for automation and standard punching tools a non-cellulose Bond Elute DMS card/paper not soaked into reagents. Non-specific binding of analyte of interest avoids by absence of impregnation. It helps to increase the response and improves signal-to-noise ratio of mass spectrometry. This card required less punching force as compared to others cards. This may leads faster analysis, less error and smoother automation[17].
2. **SAMPLE PREPARATION**

Analytes of environmental or biological origin usually occur in complex matrices. These matrices cannot be injected directly into the instrument without processing.

Due to presence of matrix effect, analysis of drug and its metabolite may get critical. Therefore clean up step in the processing is more important to avoid matrix effect which separates the analyte from endogenous biologic material. This cleanup process is also called as sample preparation. Sample preparation is the major part of the bioanalysis. After processing biological sample interference free homogeneous solution is obtained which is suitable for column and instrument [18].

2.1 **Solid-phase extraction (SPE)**

SPE is a technique used to isolate different analytes from different matrices inclusive of urine, blood, water, soil, animal tissues and several consumer products.

The analytes are transferred to the solid phase (stationary phase) where they are retained for the duration of sampling process, then the analytes are recovered by using reconstitution solution [19]. Solid phase extraction stationary phase includes silica, highly cross linked polystyrene-divinylbenzene copolymer, carbon and other oxide based chemically bonded sorbents of a suitable size.

These SPE cartridges are simple to use and provide ease in sample preparation by combining the right sorbent chemistry with the methodology.

Five Oasis sorbent chemistries designed to meet any sample preparation requirements are available. They are all built upon unique, water-wettable Oasis HLB (Hydrophilic-Lipophilic Balance) copolymer and provide exceptional results. The sulfonic acid MCX - (Mixed-Mode Cation-exchange), and quaternary amine MAX (Mixed-Mode Anion-exchange) cartridges of Oasis provide dual modes of retention (e.g. both reversed- phase and ion exchange retention modes available). This leads to better cleanup and increase in selectivity as well as sensitivity for both acidic and/or basic compounds.
Choosing the Proper Packing Type for Solid Phase Extraction

There are generally three types of sorbents used for solid phase extraction

- **Reversed Phase**: Reversed phase packings such as C_{18}, C_{8} are the most popular and most widely used. In addition to these, C_{4}, C_{2} and phenyl bonded are also available. Reversed phase sorbents generally involves conditioning with an organic solvent (e.g. methanol) followed by an aqueous solvent (e.g. water).

- **Normal Phase**: Normal phase are used to retain polar compounds from non-polar matrices. Silica, Amino and Alumina are commonly used for normal phase. Normal phase packing generally requires conditioning with a non-polar solvent and elution is carried with polar solvents. Compounds which are with basic pH functional groups are retained by silica. However, polar compounds are irreversibly retained on a silica surface and in this case Amino may be used.

- **Ion Exchange**: Ion-exchange retains charged compounds or removes ionic interferences.

**General SPE Extraction Protocols**

Most of SPE protocols consist of the following steps:

- **Conditioning**: Sorbent or the packing bed is usually wetted by methanol followed by water during sample processing which allows efficient solvent compatibility with the incoming sample matrix.

- **Sample introduction or Loading**: Transfer of the sample matrix into the SPE cartridge for extracting out the analyte.

- **Washing**: In washing step, use of weak solvent is mainly done to clean the sample matrix of impurities so that the analyte is retained on the sorbent or packing bed.

- **Elution**: In elution step the desired analyte is removed from the sorbent or packing bed by use of elution solvent. Methanol and Acetonitrile are commonly used for elution of analytes.

**Benefits of Using SPE**

- SPE gives greater reproducibility as compared to other techniques.
- Cleaner extracts are obtained using Oasis SPE products.
- High recovery of the analyte.
- No emulsion is formed during sample preparation by using SPE.
• SPE gives increased productivity as it is easy to use.
• Sample processing of about 40-50 matrix samples in a batch can be processed with an inexpensive vacuum manifold.

2.2 Liquid-Liquid Extraction (LLE)

LLE is oldest and most widely used sample preparation technique. In LLE aqueous solvent is mixed with organic solvent which is immiscible with each other to extract drugs from organic part. This organic part is evaporated till dryness and reconstituted in suitable solvent for analysis.

Compared to other sample preparation techniques, LLE has advantages such as large sample capacity and direct analysis after concentration of the clean organic extract. Disadvantages of the classical LLE approach are that it is labor intensive, difficult to automate and uses large volume of expensive/ and environmentally harmful organic solvents. To phase out these disadvantages, some modern approaches to classical LLE have appeared during the past 10 years. Examples of modern approaches to LLE are single drop-liquid phase microextraction [21], liquid phase microextraction (LPME) [22] and supported membrane extraction (SME) [21]. In single drop -LPME organic solvent drop is collected from the syringe needle.

As the organic droplet is placed in the aqueous sample, based on passive diffusion, the analytes are extracted into the droplet. The droplet is withdrawn after extraction procedure for analysis.

The use of porous hollow fibers made of polypropylene, which contain the extraction phase. Further, the pores of the hollow fiber are filled with an immobilized organic liquid. As the fiber assembly is placed in a sample vial, the analytes are extracted through the organic liquid immobilized within the pores of the hollow fiber before they are trapped in the acceptor phase. To speed up the extraction time, the samples may be stirred or vibrated. Although, this procedure is much more robust than single drop- LPME, it suffers from disadvantages such as difficulty to automate and long extraction times (up to 60 minutes per sample). However, by utilizing an electrical potential difference across the membrane the researchers could speed up analysis time considerably (5 minutes per sample). This procedure was named electro membrane isolation (EMI) [16].
2.3 Protein Precipitation

It is a very simple extraction technique as compared to LLE or SPE. It can be done by using any suitable organic solvent in which the analyte has better solubility and protein precipitating properties. Acetonitrile is the first choice of solvent for protein precipitation. In this sample preparation approach mainly removal of protein by denaturation and precipitation is carried out. The supernatant solution obtained post protein precipitation can be directly injected into the HPLC system or it can be evaporated and reconstituted to concentrate the sample for better clean up. This process can be carried out in a micro centrifuge at accelerated speed [2, 3].

2.4 Recent Trends in Sample Preparation

Nowadays in extraction techniques very less amount of solvents or solvent free extraction preferred.

There are several new methods for better sample preparation, such as use of conventional methods with automation for SPE, protein precipitation, 96-well plates and liquid liquid extraction. Nowadays micro extraction techniques are preferred like liquid liquid micro extraction (LLME), solid phase micro extraction (SPME) and micro extraction using packed sorbent (MEPS), etc.

Although there are several advantages to these new techniques, each one of these has its own set of drawbacks thus necessitating the use of the conventional off line sample preparation techniques in laboratories.

2.2.1 Solid Phase Microextraction

Initially this technique was used for the environmental samples analysis. Now a day this technique is also used for the analysis of lipophilic analytes from biological matrices. In solid phase microextraction, the probe which is consisted of silica fibre and coated with polymer film is inserted into solution containing analyte for extraction. Analyte gets adsorbed on the fibre [24]. The extracted analytes are then thermally desorbed for GC/MS and for HPLC injected via sample loop [26-28]. In this techniques, very less solvent or no solvent is used.
2.2.2 Solid-phase dynamic extraction

Solid-phase dynamic extraction (SPDE) is a technique which is utilized for sampling wall coated needles [29]. The inner part of needle wall is prepared with a 7 µm thick film of polydimethylsiloxane (PDMS) and activated carbon as stationary phase. The sampling is carried out by pulling and pushing a fixed volume of the sample through the needle. The adsorbed analytes are then recovered using carrier gas or indoor air into a GC injector. The technique can be used for vapour and liquid samples.

2.2.3 Stir Bar Sorptive Extraction

Stir Bar Sorptive Extraction (SBSE) is a very handy way of preparing samples for analysis by LC-MS. With SBSE, the extraction medium takes the form of a magnetic stir bar coated with a thick, polydimethyl siloxane film. To extract an aqueous sample, the stir bar is whizzed up in the sample for an hour or two, and all but the most polar compounds partition into the PDMS film. For volatile samples, rinse the stir bar with distilled water, blot it dry and thermally desorb the stir bar into a GC-MS. However, SBSE extracts organics of all kinds, even those that run on LC and the stir bars can be conveniently back-extracted into an organic solvent, ready for injection into an LC (19).

2.2.4 Microextraction In Packed Syringe

MEPS is a latest technique of SPE miniaturization which involves less solvent consumption. In this technique syringe with needle is used as an extraction device. The actual sample preparation is done on a packed bed to facilitate selective sampling. In MEPS needle consisted of sorbent. This is used as a cartridge which takes near about 100 injections depending upon sample matrix being analysed [28]. MEPS can also take up sample volumes from upto 10 µL and up to 1000 µL. MEPS can be either manual or fully automated. Before sample loading biological sample is diluted. For plasma 5 dilution factor and for blood 25 are consider. Then sample loaded and step of loading can be repeated in order to get a better recovery. Washing of the sorbent is carried out in next step.
This is done in order to erase proteins or other endogenous substances. The proceeding step is elution which can be done using organic solvent directly to the LC or GC injector[31].

**Figure 4.** MEPS BIN in the syringe needle.

### 2.2.5 Dispersive solid-phase extraction (DSPE)
This technique is cheap, easy and focused on selectivity enhancement. DSPE is used for the estimation of pesticides in fruits and vegetables. In this technique, extraction of analytes is carried out with acetonitrile and partitioning is carried out in presence of salt like anhydrous sodium sulfate and sodium chloride. Small amount of dispersive sorbent (in the milligram range) is added after centrifugation. Those sorbent are selected which are able to interact with endogenous components of matrix but not with the analyte. After this step, clean organic extract is obtained which contains analyte of interest. Organic extract can be used directly for instrumental analysis [32].
2.2.6 Disposable Pipette Extraction (DPX)

In this technique minimum amount of solvent is used and extraction carried out from liquid solution which gives high recovery. The sorbent is packed inside the pipette tip. When the sample is taken into the pipette tip, the analyte which is present in the sample interact with the solid phase. Mixing of the phases is carried out by use of drawn air from the tip. This gives high productivity, throughput and fast extraction. [33]. In this technique, equilibrium time is essential because recovery of analyte is depends on it and it is easy to control. Conditioning and washing steps are not required here. Hence minimum solvents are used for the elution which minimizes evaporation and concentration steps.

![Disposable Pipette Extraction process](image)

Figure 5. Disposable Pipette Extraction process
3. ANALYSIS

One of the most fundamental separation techniques used in the industry is Liquid chromatography. For nonvolatile and thermally fragile molecules gas chromatography is not suitable. Large numbers of organic compounds are separated by liquid chromatography. Liquid chromatography-mass spectrometry (LC-MS/MS) is a method that uses liquid chromatography (or HPLC) with mass spectrometric detection. It is commonly used in laboratories for the qualitative and quantitative analysis of drug products and/or biological samples.

Still improved techniques such as ultra-performance liquid chromatography (UPLC) with small particles (2mm) provide better efficiency when compared to other chromatographic techniques. In liquid chromatography, selection of the mobile phase is major criteria because chromatographic separation of analyte is dependent on interaction of analyte with mobile phase and the stationary phase. In liquid chromatography the choice and variation of the mobile phase is of prime importance in order to achieve the best results.

A full-scan LC/MS examination can suggest probable oxidative and/or conjugative metabolic conversion by considering ionic species observed.

Tandem mass spectrometry (MS/MS) involves the practice of carrying out one mass selective operation after another. The purpose of first ionization is to separate an ion species called as parent ion or molecular ion, while that of latter ionization is to find out the mass to charge ratio (m/z) of the fragment or daughter ion, produced due to collision induced dissociation (CID) of the parent ions. The instrument can be operated either using a full scan mode or in the more sensitive selected-ion monitoring mode (SIM) detecting positive or negative ions.

3.1 Ion Sources

There has been a significant advancement over the past decade in LC-MS instrumentation especially in ion sources and different techniques which ionize analytes and separate the ions thus formed from the liquid phase. Ion source is only the device where ionization of the analyte takes place.
The principles of ionizing neutral molecules are based on protonation, electron ejection, electron capture, cationization or deprotonation. Alternatively, they work by transferring of a charged moiety from the condensed phase to a gaseous phase [35].

The different ionization techniques are as follows; CI, ESI, EI, APCI, fast atom/ion bombardment (FAB), thermospray ionization (TSP), field desorption/field ionization (FD/FI), MALDI. In most of these methods, positive or negatively charged sample ions are created.

The most widely used ionization sources used in mass spectrometry are ESI and MALDI as they offer the best mass range and excellent sensitivity.

With the advent of API (Atmospheric Pressure ionization) methods, there has been a wide increase in the number of compounds that can be quantified using LC-MS. In this technique, the molecules are ionized using atmospheric pressure first. They are then electrostatically and mechanically separated from the other neutral molecules present.

The API techniques are as follows

- Electrospray ionization (ESI),
- Atmospheric pressure chemical ionization (APCI)
- Atmospheric pressure photoionization (APPI).

### 3.1.1 Electrospray ionization

This technique is based partly on the generation of ions in solution prior to reach the mass spectrometer. In the presence of a strong electrostatic field and heated drying gas eluent nebulized by LC using atmospheric pressure. In ESI, the gaseous phase ion formation is significantly varied as compared to Chemical Ionization, Electron Impact and Fast Atom Bombardment. This technique employs a needle maintained at a potential (around 3.5 kV) through which the sample solution is aspirated.

This potential causes the spray to become charged during the nebulisation. In a chamber charged droplets are vaporized in presence of vacum till the charged ions left behind. In an ESI spectrum the resultant ions often carry multiple charges, which reduces their mass-to charge ratio as compared to a singly charged species. These feature help to obtain large molecules mass spectra.
Thus ESI is a very sensitive method, well suited for quantitation of large molecules, small molecules, labile molecules, etc. These include all kinds of organometallic compounds, peptides, proteins, polymers, etc.

![Electrospray ionization](image)

**Figure 6. Electrospray ionization**

**3.1.2 Atmospheric pressure chemical ionization**

In this technique, the eluent from the LC is typically sprayed through a heated capillary at atmospheric pressure. This heat is responsible for vaporizing the eluent. The gaseous phase molecules thus formed and from corona needle get ionized by electron discharged. Through chemical ionization, the solvent ions transfer their charge to the analyte molecules. These then pass into mass analyzer through a capillary sampling orifice. For polar and nonpolar compounds APCI is preferred. It is used molecules less than 1,500 u without multiple charging. APCI also required high temperature due to this for large biomolecule analysis which are thermally unstable it is less suitable.

APCI generally used for nonpolar compounds with normal phase chromatography more often than electrospray.
3.1.3 Atmospheric pressure photoionization

Atmospheric pressure photoionization (APPI) is newer as compared to other techniques. The APPI technique is similar to APCI in the sense that a vaporizer converts the LC eluent into gaseous phase. Photons in a narrow range of ionization energies are then generated via a discharge lamp. The range of energies is optimized so as to ionize most of the analyte molecules and keep the ionization of solvent molecules to a bare minimum. The resulting ions then pass through the capillary sampling orifice and finally into the mass analyzer. APPI is applicable to many of the same compounds that are typically analyzed by APCI. It shows particular promise in two applications, highly nonpolar compounds and low flow rates (<100 ml/min), where APCI sensitivity is sometimes reduced.
3.2 Mass Analyzers

There are four types of analysers used in mass spectrometry. They are listed as Quadrupole, Time-of-flight Ion trap, Orbitrap, Fourier transform-ion cyclotron resonance (FT-ICR or FT-MS). Each has its own advantages and disadvantages and varies as per the analytical requirement.

3.2.1 Quadrupole

A quadrupole mass analyzer has square shaped four parallel rods. The charged ions are sent down to the center of this square. Electromagnetic fields are generated through voltages applied across these rods. It is these fields that allow ions of only a particular m/z ratio to pass at a given time. Linear quadrupoles are the easiest to operate and least costly. They can be run either in scanning mode or selected ion monitoring (SIM) mode [33].

In the Scan mode, one can work in a range of mass-to-charge ratios. Whereas in SIM mode only designated m/z ratios are monitored. It is a more sensitive technique than the Scanning mode but it provides information about only a few ions. Hence for qualitative purposes, scan mode is preferred. SIM mode is used for quantifying and monitoring specific target molecules [34-37].

Oscillating electrical fields are used by quadrupole mass analyzers. At a time only ions of a particular m/z ratio are allowed to pass through. The path changes in accordance with the potential applied on the magnetic lenses. This allows for a wide range of m/z values to be swept rapidly. This can be either in succession of well-spaced hops or in a continuous motion.

A quadrupole mass spectrometer acts as a mass-selective filter. It is closely similar to an ion trap model, especially like a quadrupole ion trap. It is hence named as a transmission quadrupole. For tandem MS analysis using a quadrupole instrument, three quadrupoles are placed in series. Function of each quadrupole is different; the first quadrupole (Q1) is act like mass filter it scans only specific m/z value. In second quadrupole (Q2) fragmentation of ion takes place by collision gas (argon or helium) so it also known as the collision cell. The third Quadrupole (Q3) gives final mass spectrum [38-42].
3.2.2 Time-of-flight
TOF-MS a measure of the time that it takes for an ion to reach from ion source to a detector. Generally lighter ion reaches to the detector first because it travels fast that’s why m/z calculated by its arrival times. In this a uniform electromagnetic force is applied to all ions at the same time, causing them to accelerate down a flight tube. Time-off light mass analyzers have a wide mass range and can be very accurate in their mass measurements.

3.2.3 Ion trap
Ion trap mass analyzers have a chamber which consists of a circular ring electrode plus two end caps. In chamber ions are “trapped” by electromagnetic fields. Another field can be applied to selectively eject ions from the trap. Advantage of ion traps is to perform multiple stages of mass spectrometry without additional mass analyzers.

3.2.4 Orbitrap
Latest development in trapping devices is Orbitrap mass spectrometer used as an m/z analyzer which has high mass accuracy, dynamic range and sensitivity[46]. This mass analyser i.e orbitrap consists of axial electrode and coaxial electrode which produces an electrostatic field with quadro-logarithmic potential distribution [43-45]. Orbitraps have a high mass accuracy (1–2 ppm), a high resolving power (up to 200,000) and a high dynamic range (around 5000) [45].

3.2.5 Fourier transform ion cyclotron resonance
The principle of Fourier transform mass spectrometry (FT-MS) is to produce ions to measure the mass spectra in presence of magnetic field. In magnetic ion trap, injected ions form a part of circuit which is determined by its m/z ratio, this can be deconvoluted by performing a Fourier transform on the signal. The advantage of this analyser is that it gives high sensitivity and high resolution and thus precision.
4. DETECTORS

The detector is used to record the signal obtained by an ions. Due to electron multiplier ions gets separated according to its m/z ratio by mass analyzer and detected through the signal. An electron multiplier made up of dynodes at high potential. An ion which arrives at dynode produces secondary electrons. In the electric field it gets accelerated and strikes a second dynode to produce more electrons. These dynodes amplify the current and produces high amplification. [47].

![Continuous Dynode Electron Multiplier](image)

**Figure 9. Continuous Dynode Electron Multiplier**

Electron multiplier and detectors like Faraday cups and ion-to-photon are used. These detectors are used because the mass analyzer ions are small and significant amplification is often necessary to get a signal. Micro channel Plate Detectors are commonly used in modern commercial instruments[48]. In the mass analyzer/ion trap region, the detectors of FTMS and orbitraps made up of metal surface which the ions only pass near as they oscillate[49].
CHAPTER 1

5. **RECENT DEVELOPMENTS IN HIGH-THROUGHPUT QUANTITATIVE BIOANALYSIS**

LC-MS/MS is very essential for the development of new methods for antibodies and routine analysis [50]. Today in pharmaceutical industry and laboratory LC-MSMS widely used. It is used for toxicology, endocrinology and metabolic TDM analysis. There is enormous improvement in this technology.

Nowadays LC-MS/MS, technology is used for the xenobiotic biomolecules, metabolites and small compounds from biological matrices [51]. The analyte which is present in matrices cannot be injected directly to the LC-MS/MS for analysis, therefore sample preparation technique is the most important part of the analysis. Manual sample preparation techniques are very time consuming and laborious. Recent years sample processing generated a lot of interest due to short time for analysis by using direct injection of plasma using an online extraction method and SPE, LLE in 96 well plates.

To reduce run time gradient techniques, use of small columns is generally preferred. Removal of matrix effect in bioanalytical methods is essential for separation of endogenous biological components from analytes and for better chromatography [52]. Therefore new techniques have been developed such as ultra-performance LC with sub-2 mm particles and monolithic chromatography which show higher speed, better resolution and sensitivity for high-throughput analysis while minimizing matrix effects. Automation in LC-MS/MS is great advancement in instrumentation field for high-throughput analysis. Sample preparation is bottle neck of high-throughput analysis which carried out using latest technology i.e. 96 well plates for quantitative bioanalysis.

In LC sample extraction carried out using 4 principles:

Protein precipitation carried out by addition of inorganic acids, organic solvents and chaotropic salts, protein filtration, LLE, and SPE. The increasing demand for high throughput causes a unique situation of balancing cost versus analysis speed as each sample preparation technique offers unique advantage. Protein precipitation techniques widely used in bioanalysis because it is fast, cost effective and easily automated. Major disadvantage of PPT is higher variation from sample to sample due to endogenous material which causes ion suppression. SPE and LLE give very clean
extracts. Therefore ion-suppression effects due to residual matrix components are reduced with solvent extraction and SPE compared to mere PPT [53-57]. A novel 96-well SPE plate has been designed to minimize the elution volume required for quantitative elution of analytes and the plate is packed with 2mg of a high-capacity SPE sorbent that allows loading of up to 750 µL of plasma. The novel design permits elution with as little as 25 µL solvent [56].

The on-line SPE technique is fully automated and offers speed, high sensitivity by the pre-concentration factor, and low extraction cost per sample [58-61]. On-line introduction of internal standard (IS) and generic method for the fast determination of a wide range of drugs in serum or plasma for quantitative analysis has been presented for the Spark Holland system [62-64]. These methods are very useful because minimum or no sample pre-treatment is required and significant sample preparation time is saved [65-68]. SPE materials can be packed in permanently used syringes of autosamplers as well and also referred to as solid phase micro extraction (SPME) or microextraction by packed sorbents (MEPS) [71-73].
6. **STEPS AND CONSIDERATIONS FOR METHOD DEVELOPMENT**

The development of a sound bioanalytical method is of paramount importance during the process of drug discovery and development culminating in marketing approval. It is a set of various procedures, mainly used for the determination of drugs and their metabolites in biological matrices such as urine, plasma, and serum.

6.1 **Information about the sample**

The knowledge about the sample can provide valuable clues for the best choice of initial conditions for a chromatographic separation. Depending on the use made of this sample information, different approaches to LCMS method development are possible. The necessary information required about an analyte before beginning the method development process.

- Chemical structure (functionality) of compounds
- Molecular weight
- pKa value
- Sample Solubility and stability
- Toxicity, purity
- Concentration range of compounds in samples of interest
- Metabolites

6.2 **Chromatographic Approaches**

Chromatographic optimisation is the process of finding a set of conditions that adequately separate and enable the quantification of the analyte of the interestin the presence of an endogeneous material with acceptable accuracy, precision, sensitivity, specificity, cost, ease and speed.

Main aim of the chromatographic optimisation is to achieve the resolution between the analyte and the endogenous material generating a response at a detector.
6.3 Sample Preparation Approaches
Sample preparation is an essential part of high importance when an LC-MS/MS method is developed for the bioanalysis. Because of the large amount of proteins in plasma samples, conventional HPLC column will not tolerate the direct introduction of plasma, therefore most bioanalytical processes have a sample preparation stage. The main aim of sample preparation step is to provide a reproducible and homogenous solution suitable for injection into column. The main characteristics of the sample which are used after sample preparation step for injection into the column are:
1. It should be free from possible interferences.
2. It should not damage the column.
3. Compatible with LCMS method.
4. Solvents used for preparation of the samples should be compatible with mobile phase and should not significantly affect the retention and resolution of the analyte.
Other important reason to have a sample preparation step includes reducing the matrix components and also elimination of ion suppression. Solid phase extraction, Liquid liquid extraction and protein precipitation are mainly used for bioanalytical method development process.

6.4 Parameters Influencing LC-MS/MS Method Development
Once the extraction method for each of the analytes under study is optimized, other parameters involved in Liquid Chromatography are also studied. All the instruments used during the development and validation stages are checked for calibration as per the respective schedules. Only when calibration was confirmed did the analysis part should be resumed.
The literature survey for each individual analyte under study provided the base for initial selection of the chromatographic conditions. The conditions were then suitably modified in our laboratory to obtain satisfactory results.
Detailed explanations for choice of particular parameters for the finalized chromatographic conditions are discussed in individual chapters and the parameters are-
CHAPTER 1

• Selection of Internal Standard
• Selection of Mobile phase
• Selection of Column
• Role of pH
• Role of Buffers
• Role of flow rate
• Role of Temperature

6.4.1 Selection of Internal Standard
In LC-MS/MS based assays, use of an internal standard is always advisable to account for the general variability seen during analysis. There are so many conditions which could account for variability in such assays, such as laboratory room temperature, variability in extraction, improper sample addition or processing errors, etc.
An ideal Internal Standard should be able to compensate for these small variations during analysis so that the actual analysis is not compromised in any way. Also, in biological assays the sample quantity that the analyst has to work with is very low as they are all precious plasma samples.
A best internal standard for an LC-MS/MS based assay would be a stable labeled compound of the analyte under study. This would very much help with the assay, as the internal standard in this case would behave almost in an identical manner as the analyte. However, the high cost factor and the minimal availability of these stable labeled compounds is the biggest drawback for a small scale laboratory. In absence of a stable labeled compound, the most logical choice for an internal standard should be a compound belonging to the same therapeutic category as the analyte being monitored.
In most of the methods discussed in this thesis, structurally similar compounds or compounds from the same therapeutic category as the analyte under study were selected as internal standards.
The choice and suitability of these internal standards in the discussed methods were justified on application of these methods, after complete validation, to actual study samples.
6.4.2 Selection of Mobile Phase

One of the most important parameters is the selection of a proper mobile phase. In LC-MS/MS based assays, it is preferred to use simple volatile buffers like Formic acid solution, acetic acid solution, etc. of very mild concentrations. Other buffers generally used are weak solutions of ammonium acetate or ammonium formate. Strong buffers containing ionic salts and phosphates are not very well tolerated by the mass spectrometer and hence their use is very minimal to negligible.

It is also equally important to use more part of the organic modifier in the mobile phase like methanol or acetonitrile to facilitate quick and effective evaporation in the heated capillary of the source.

The ratio of the aqueous to organic part needs to be optimized so that there is sufficient retention of the analytes of interest without compromising on the run time of the assay or the peak shapes of the analytes under study.

In all the methods discussed in this thesis, different combinations of mobile phase ratios were run either isocratically or using gradient elution to confirm the best possible chromatographic conditions. In all the four methods, isocratic elution was seen to best suit the purpose due to the ease of preparing the mobile phase.

In addition to this, the flow rate for each method was also optimized to get the best possible results.

6.4.3 Selection of Column

Always referred to as the heart of the chromatographic instrument, the selection of the column is the most important part of method development. For LC-MS/MS based assays, it is generally preferred to use columns of small lengths to facilitate faster elution to reduce the time of analysis. This is because, an analytical chemist will have to analyse almost 100 to 200 samples a day during the course of a Bioequivalence or Bioavailability study. With a smaller run time, it would facilitate faster analysis wherein all samples from a particular subject could be processed and analysed under one acquisition batch. This does not however mean that one cannot use a column of longer length and dimensions, if absolutely necessary.
Columns from different makes were checked during development like Thermo Hypurity C$_{8}$ and C$_{18}$, Symmetry Shield RP$_{18}$ and RP$_{8}$, Zorbax Eclipse C$_{18}$, Thermo Hypurity Advance, Kromasil C$_{8}$ and C$_{18}$, etc. of different lengths and dimensions. Columns with different chemistries as stationary phases were also employed to check for suitability. In two out of four methods, C$_{18}$ columns with dimensions of 50 cm X 4.6 mm, 5 micron gave the best response and peak shapes. For one of the methods, a monolithic column, with dimensions of 50 cm X 4.6 mm was selected whereas for the remaining one method, the best results were seen on a C$_{8}$ column of dimensions 150 cm X 4.0 mm, 5 microns.

6.4.4 Role of pH

Buffer pH selection causes separation of ionisable compounds by Reversed-Phase HPLC. Hence for good peak shape with nice chromatography proper selection of buffer pH is carried out. Due to this tailing, fronting and splitting problem of peak does not occur. Peak tailing is minimized at low pH. For anlyte retention and selectivity intermediate pH is preferred. Due to this method ruggedness is maximized.

6.4.5 Role of Buffer

Buffers are needed if sample solution is outside the pH range. Generally ammonium formate; ammonium acetate and formic acid were used as a buffer solution.

6.4.6 Role of Flow rate

Flow rate, can be used to increase the resolution. The lower flow rate can give lower column back pressure but increase in the run time. Flow rate can be adjusted according to chromatography Method development involves considerable trial and error procedures.

6.4.7 Role of temperature

Temperature is the main criterion which is taken for the consideration. If temperature increases retention time of peak decreases for the normal compound.
7. **STEPS AND CONSIDERATIONS FOR METHOD VALIDATION**

7.1 **Benefits of Bioanalytical Method Validation**

Highest degree of method assurance, quality of product is provided by validation process to meet quality specification.

With this note, method validation is also beneficial in many ways and it:

- Decreases the risk of preventing problems and thus assures the smooth running of the process.
- Decreases the running cost.
- Decreases the risk of regulatory non-compliance and
- Fully validated process may require less in-process controls and end product testing.

7.2 **Fundamental Elements of Method Validation**

Some elements or points on which any method validation process stands are:

- Validation plan and standard operating procedures (SOP).
- Establishment of acceptance criteria (i.e., testing parameters, limits of acceptability of the components).
- Demonstration of proper equipment system calibration and operation.
- Demonstration of meeting of process with an established range of operation for multiple runs.
- Demonstration of accuracy and precision for any analytical test methods.

7.3 **Validation parameters [74-78]**

After development of the method validation, the method is carried out for routine analysis. For validation of the method guidelines from the Food and Drug Administration (FDA) and the International Conference on Harmonization (ICH) are followed [74-78].

Following parameters are considered for the validation:

- **Accuracy and precision**

Accuracy is a parameter that defines the closeness between the measured value and a reference or true value and is determined by replicate analysis of samples of a known concentration. Precision describes the distribution or agreement between multiple analysis of a single homogenous sample.
• **Recovery**
Recovery experiments performed to determine the amount of the analyte Peak area obtained after spiking the specified amount spiked in the biological samples. Recovery of the analyte(s) and internal standard should be close to 100% and consistent for each three levels of the quality control samples.
Extracted samples Peak areas of the analyte at three different concentrations were compared to the aqueous unextracted samples. Recovery of an analytes should be consistent throughout the concentration levels, drug and IS recovery should be close.

• **Specificity/Selectivity**
Selectivity experiment is the capability of an analytical method to separate and identify analyte peak in the presence of other components from matrix sample.
Bioanalytical method selectivity exercise performed using at least 6 lot/sources of matrix. Each blank sample should be tested for interference from endogenous matrix components, decomposition products, metabolites etc.

• **Sensitivity**
Sensitivity is measured in terms of LLOQ (lower limit of quantification).
Prepare six spiked LLOQ from the same stock solution used to prepare the LLOQ standard of the calibration curve.

• **MatrixEffect**
In different matrix lot ion enhancement or suppression observed because of co-eluting matrix component at the retention time and MRM of the analyte(s) and internal standard. Hence matrix effect carried out to ensure that selectivity and precision are not compromised with the ion suppression.
Matrix effect is well known in LC/MS/MS analysis it is related to method of sample extraction and the chromatographic separation of analytes on HPLC column [44].
It is performed by Post extraction spiking of analytes in six lots of plasma at LQC and HQC concentration levels and demine the consistency of area in different Lots.
Exercise passed if % CV of chromatographic peak area ratio found between ± 15% at each concentrations level for analyte and internal standard.
Stability

Stability of the analyte during sample analysis is a requirement for reliable quantification. The stability of the analyte in the biological matrix at intended storage temperatures for the various stages of analysis including storage prior to analysis should be established. The different types of stability studies performed in validation are listed below.

- **Long-term stability**
  Long-term stability is carried out to assess the stability of analyte(s) in biological fluids during its storage in deep freezer at analyte(s) specific storage temperature for a period of time that equals or exceeds the period of time between the date of first sample collection from the subject in first period and the date of last sample analysis. Generate long-term stability data on three different occasions.

- **Short-term stability (Bench Top Stability)**
  Bench top stability is carried to assess the stability of the analyte(s) in biological fluids over a period of time during which the samples are expected to be kept on the bench while processing.

- **Post-preparative stability (Auto sampler Stability)**
  Autosampler stability is carried out to assess the stability of the processed samples placed in the autosampler at specific temperature for the period of time depending on the anticipated run time or the complete analysis of bioanalytical batch.

- **Freeze/Thaw stability**
  Freeze-thaw stability is carried out to assess the stability of the analyte (s) in biological fluids during repeated freezing and thawing cycles. Establish the influence of minimum three freeze thaw cycles. Whenever there is simultaneous estimation of more than one analyte with different methods, a similar number of freeze thaw cycles should be established.

- **Linearity and range**
  The linearity range gives relationship between the response function y and the concentration x.

  Regression equation with an appropriate weighting factor is used to determine the detector response / concentration.
CHAPTER 1

8. REVIEW OF LITERATURE OF SELECTED MOLECULES

8.1 Piroxicam and Paracetamol

Piroxicam (C$_{15}$H$_{13}$N$_3$O$_4$S) [4-hydroxy-2-methyl-N-(2-pyridyl)-2H-1,2-benzothiazine-3-carboxamide-1, 1-dioxide] is a non-steroidal anti-inflammatory drug (NSAIDs) with analgesic and anti-pyretic activities[79,80]. It exhibits a rapid and effective response in the treatment of many diseases such as ankylosing, spondylitis, gout juvenile rheumatoid arthritis, musculoskeletal disorders, postpartum pain and sport injuries [81]. It acts by reducing hormones which induces inflammation and pain in the body caused by osteoarthritis and rheumatoid arthritis [82]. Due to reversible inhibition of cyclooxygenase, piroxicam shows anti-inflammatory effect. It causes the peripheral inhibition of prostaglandin synthesis [83, 84]. Cox-1 enzyme produces the prostaglandins [85]. Piroxicam stops the formation of thromboxane A$_2$, an aggregating agent, by the platelets and inhibits production of prostaglandins by blocking the Cox-1 enzyme and migration of leucocytes in to sites of inflammation[86, 87].

Paracetamol (C$_8$H$_9$NO$_2$) is chemically N-(4-hydroxyphenyl)acetamide. Paracetamol is used as an antipyretic and analgesic [88, 89]. Acetaminophen is a weak inhibitor of the synthesis of prostaglandins (PGs) [89]. It decreases PG concentrations but does not suppress the inflammation of rheumatoid arthritis like the selective COX-2 inhibitors [90-92].

NSAIDs were clearly more effective in post-operative pain, the addition of a piroxicam to paracetamol may confer additional analgesic efficacy compared to paracetamol alone, especially because of the superior onset of action and the low incidence of adverse effects, and should be the preferred choice in high risk patients like diabetics and renal failures patients [93].

In previous literature numerous analytical methods have been reported for the determination of piroxicam and paracetamol in single or in combination with other drugs in pharmaceuticals or in biological fluids, including spectroscopic [85-89, 94, 95, 96], chromatographic methods [97, 98] and mass fragmentography. So far, to our present knowledge, no UFLC-MS/MS method has been reported for simultaneous
estimation of piroxicam and paracetamol in human plasma. These reported methods required more run time, maximum sample volume, time consuming extraction procedures which were not useful for bioavailability, pharmacokinetic and bioequivalence studies. This developed method is highly sensitive and specific, which can be used for routine analysis of paracetamol and piroxicam as the active ingredients with short preparation and analysis time. This method has a short runtime of 3 minutes which allows more sample analysis per day.

### 8.2 Metformin and Letrozole

Metformin hydrochloride (C₄H₁₁N₅•HCl) [N,N-dimethylimidodicarbonimidicdiamide hydrochloride] is an antidiabetic agent [99]. Metformin is antihyperglycemic agent [100]. Its use in type 2 diabetes mellitus to reduce hyperglycemia is similar to that of sulfonylureas, thiazolidinediones, and insulin [113]. Metformin is used for the treatment of polycystic ovary syndrome (PCOS) in reproductive medicine and also reduce inflammation and has an effect on steroidogenesis inovarian granulosa cells and thecal cells [101]. Metformin acts in PCOS patients by improving pregnancy rate and the metabolic situation and by decreasing the complications of pregnancy such as gestational diabetes [102].

Letrozole(C₁₇H₁₁N₅)[4,4'-(1H-1,2,4-triazol-1-yl)methylene]dibenzonitrile] is a potent and selective non-steroidal aromatase inhibitor [103]. It is approved for use in post-menopausal women who have breast cancer that has progressed after antiestrogen therapy [104].

Polycystic ovary disease (PCOD) is the major cause of anovulation, the incidence of which has been reported to reach 6% in infertile females [105]. Letrozole is a newly designed selective aromatase inhibitor, which can be used to induce ovulation in infertile women with polycystic ovary syndrome (PCOS) [106].

A variety of methods have been employed for the detection of the individual component or combination in plasma, including HPLC, HPLC-UV, LC–MS or LC–MS/MS. [107-115]. However, these reported methods required more time, laborious extraction procedures, relatively large sample volume, long chromatographic analysis time and also showed low sensitivity which were not adequate for pharmacokinetic and bioequivalence studies. Recently, a LC–MS/MS method was reported for the
determination of letrozole in human plasma by protein precipitation extraction with a lower limit of quantitation (LLOQ) of 0.40 and 50.0 ng/mL. The method achieved sufficient sensitivity in long run time, which is not useful for high throughput analysis. No previous methods with a simultaneous determination of the letrozole and metformin had been described. Therefore, a highly sensitive and simple LC–MS/MS method was developed and validated for the simultaneous determination of metformin and letrozole in human plasma. This developed method offered higher sensitivity, more simple procedure, smaller sample volume and shorter run time; resulting in high-throughput analysis of letrozole and metformin using single IS in a single experiment for bioequivalence studies, with a chromatographic run time of 2.5 min only.

8.3 Salbutamol and Beclomethasone

Salbutamol is selective beta2-adrenergic receptor agonist. In the treatment of asthma and COPD salbutamol used [116-118]. It is selective for β2 adrenergic receptors than beta1 receptors which are present in bronchial smooth muscle of the lung. It relaxes muscles to keep the airways open, making it easier to breathe Salbutamol is well absorbed but due to presystemic metabolism in the GI tract and liver its systemic bioavailability is only 50% [119].This drug is fast acting by the inhaled route due to its direct action on bronchial smooth muscle and also be given orally or intravenously. Beclomethasone is generally used in the treatment of severe asthma and lung diseases. Due to asthma and lung disorder coughing, chest tightness, wheezing, and shortness of breath, caused. Beclomethasone reduces swelling in the airways.

salbutamol and beclomethasone dipropionate combination generally used to treat asthma. There are many methods for the determination of these drugs alone or in combination by spectroscopic [128] and chromatographic methods [117-120,123-127, 129] but no LCMSMS method reported. Hence developed LCMSMS method with less run time and plasma volume.
8.4 Ramipril, Ramiprilate and Felodipine

Ramipril is a member of the second generation of angiotensin-converting enzyme (ACE) inhibitors [130,131,132]. The pro-drug is hydrolysed in vivo releasing the active metabolite ramiprilate [4,5]. The metabolite has a long elimination half-life (≈100 h) permitting once-a-day administration [130, 131]. Ramiprilate binds to ACE with very high affinity, and is active at concentrations similar to those of the enzyme, establishing the equilibrium slowly [135].

Felodipine is a long-acting calcium channel blocker and used to control high blood pressure. It acts by inhibiting movement of calcium ions across cell membrane in systemic and coronary vascular smooth muscle. Systemic bioavailability of felodipine is 20% and $T_{\text{max}}$ is 2.5 to 5 h. Combination of ramipril and felodipine work in different ways to lower blood pressure. Clinical trials show that using Ramipril & felodipine combination is more effective than using either ramipril or felodipine alone.

Felodipine prevents calcium from being released within muscle cells of the small arteries and thereby causes the muscle to relax and the arteries to dilate or expand [136-139]. In the quantitative determination of felodipine in body fluids and pharmaceutical dosage forms, the previous studies were realized by titrimetry [140], spectrophotometry [141], HPLC [142-146] and gas-liquid chromatography [147].
9. DRUG PROFILE OF SELECTED MOLECULES[148-150].

9.1 DRUG PROFILE OF PIROXICAM

<table>
<thead>
<tr>
<th>Name</th>
<th>PIROXICAM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Structure</td>
<td><img src="image" alt="Structure of PIROXICAM" /></td>
</tr>
<tr>
<td>Chemical Formula:</td>
<td>$\text{C}<em>{15}\text{H}</em>{13}\text{N}<em>{3}\text{O}</em>{4}\text{S}$</td>
</tr>
<tr>
<td>IUPAC Name</td>
<td>4-hydroxy-2-methyl-1,1-dioxo-N-(pyridin-2-yl)-2H-1{6},2-benzothiazine-3-carboxamide</td>
</tr>
<tr>
<td>Molecular weight:</td>
<td>331.35</td>
</tr>
<tr>
<td>Appearance and color:</td>
<td>White to off-white crystalline powder</td>
</tr>
<tr>
<td>Solubility:</td>
<td>It is sparingly soluble in water and most organic solvents.</td>
</tr>
<tr>
<td>Dosage And Administration</td>
<td>10 mg or 20 mg Piroxicam, Oral administration</td>
</tr>
<tr>
<td>Category</td>
<td>non-steroidal anti-inflammatory drug (NSAID)</td>
</tr>
<tr>
<td>Half life</td>
<td>Up to 50h</td>
</tr>
<tr>
<td>Plasma protein binding</td>
<td>99%</td>
</tr>
</tbody>
</table>
## 9.2 DRUG PROFILE OF PARACETAMOL

<table>
<thead>
<tr>
<th>Name</th>
<th>PARACETAMOL (ACETAMINOPHEN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Structure</td>
<td>![Structure Diagram]</td>
</tr>
<tr>
<td>Chemical Formula</td>
<td>C₈H₉NO₂</td>
</tr>
<tr>
<td>IUPAC Name</td>
<td>N-(4-hydroxyphenyl)acetamide</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>151.17</td>
</tr>
<tr>
<td>Appearance and color</td>
<td>It is a white crystalline powder.</td>
</tr>
<tr>
<td>Solubility</td>
<td>It is insoluble in water; very soluble in ethanol.</td>
</tr>
<tr>
<td>Dosage And Administration</td>
<td>500 mg per dose and 750 mg per day.</td>
</tr>
<tr>
<td>Category</td>
<td>Analgesic, Antipyretic</td>
</tr>
<tr>
<td>Half life</td>
<td>2 to 3 h</td>
</tr>
<tr>
<td>Plasma protein binding</td>
<td>25%</td>
</tr>
</tbody>
</table>
### 9.3 DRUG PROFILE OF LETROZOLE

<table>
<thead>
<tr>
<th>Name</th>
<th>LETROZOLE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Structure</strong></td>
<td><img src="image" alt="Structure Image" /></td>
</tr>
<tr>
<td><strong>Chemical Formula</strong></td>
<td>C\textsubscript{17}H\textsubscript{11}N\textsubscript{5}</td>
</tr>
<tr>
<td><strong>IUPAC Name</strong></td>
<td>4{(<a href="1H1,2,4triazol1yl">cyanophenyl</a>-methyl]-benzonitrile</td>
</tr>
<tr>
<td><strong>Molecular weight</strong></td>
<td>285.31</td>
</tr>
<tr>
<td><strong>Appearance and color</strong></td>
<td>white to yellowish crystalline powder, practically odorless</td>
</tr>
<tr>
<td><strong>Solubility</strong></td>
<td>Dichloromethane, ethanol</td>
</tr>
<tr>
<td><strong>Dosage And Administration</strong></td>
<td>2.5 mg tablets for oral administration.</td>
</tr>
<tr>
<td><strong>Category</strong></td>
<td>non-steroidal aromatase inhibitor</td>
</tr>
<tr>
<td><strong>Half life</strong></td>
<td>about 2 days</td>
</tr>
<tr>
<td><strong>Plasma protein binding</strong></td>
<td>Weakly protein bound</td>
</tr>
</tbody>
</table>
### 9.4 DRUG PROFILE OF METFORMIN

<table>
<thead>
<tr>
<th>Name</th>
<th>METFORMIN</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Structure</strong></td>
<td>![Structure Diagram]</td>
</tr>
<tr>
<td><strong>Chemical Formula:</strong></td>
<td>C₄H₁₁N₅•HCl</td>
</tr>
<tr>
<td><strong>IUPAC Name</strong></td>
<td>N,N- dimethylimidodicarbonimidic diamide hydrochloride</td>
</tr>
<tr>
<td><strong>Molecular weight:</strong></td>
<td>165.63</td>
</tr>
<tr>
<td><strong>Appearance and color:</strong></td>
<td>White to off-white crystalline compound.</td>
</tr>
<tr>
<td><strong>Solubility:</strong></td>
<td>freely soluble in water</td>
</tr>
<tr>
<td><strong>Dosage And Administration</strong></td>
<td>500 mg and 1000 mg, Extended Release Tablets</td>
</tr>
<tr>
<td><strong>Category</strong></td>
<td>antidiabetic</td>
</tr>
<tr>
<td><strong>T max and C max of tablet</strong></td>
<td>7.8 h and 1.3±0.33µg/ml</td>
</tr>
</tbody>
</table>
### 9.5 DRUG PROFILE OF SALBUTAMOL

<table>
<thead>
<tr>
<th>Name</th>
<th>SALBUTAMOL</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Structure</strong></td>
<td><img src="image" alt="Structure Diagram" /></td>
</tr>
<tr>
<td><strong>Chemical Formula:</strong></td>
<td>C\textsubscript{13}H\textsubscript{21}NO\textsubscript{3}</td>
</tr>
<tr>
<td><strong>IUPAC Name</strong></td>
<td>(RS)-4-[2-(\textit{tert}-butylamino)-1-hydroxyethyl]2(hydroxymethyl)phenol</td>
</tr>
<tr>
<td><strong>Molecular weight:</strong></td>
<td>239.311</td>
</tr>
<tr>
<td><strong>Appearance and color:</strong></td>
<td>White crystalline powder; odorless and almost tasteless.</td>
</tr>
<tr>
<td><strong>Solubility:</strong></td>
<td>Methanol</td>
</tr>
<tr>
<td><strong>Dosage And Administration</strong></td>
<td>As aerosol: 100 or 200 mcg ((12\text{ puffs})3\text{times daily})</td>
</tr>
</tbody>
</table>
| **Category**          | • Bronchodilator Agents  
                          • Adrenergic beta-Agonists  
                          • Tocolytic Agents |
| **Half life**         | 4.6 to 6 h          |
| **T max and C max of tablet** | 2 h and 18 ng/mL respectively |
9.6 DRUG PROFILE OF BECLOMETHASONE

<table>
<thead>
<tr>
<th>Name</th>
<th>BECLOMETHASONE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Structure</td>
<td>![Structure Diagram]</td>
</tr>
<tr>
<td>Chemical Formula</td>
<td>C_{28}H_{37}ClO_{7}H_{2}O</td>
</tr>
<tr>
<td>IUPAC Name</td>
<td>9-chloro-11β, 17, 21-trihydroxy-16β-methylpregna-1, 4-diene-3, 20-dione 17, 21 dipropionate, monohydrate</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>539.07</td>
</tr>
<tr>
<td>Appearance and color</td>
<td>It is a white to creamy-white, odorless powder</td>
</tr>
<tr>
<td>Solubility</td>
<td>Chloroform, acetone, ethanol</td>
</tr>
<tr>
<td>Dosage And Administration</td>
<td>1 or 2 nasal inhalations (42 to 84 mcg) in each nostril twice a day</td>
</tr>
<tr>
<td>Category</td>
<td>Anti-Asthmatic Agents</td>
</tr>
<tr>
<td>Half life</td>
<td>0.5 and 2.7 h</td>
</tr>
</tbody>
</table>
## 9.7 DRUG PROFILE OF RAMIPRIL

<table>
<thead>
<tr>
<th>Name</th>
<th>RAMIPRIL</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Structure</strong></td>
<td><img src="image" alt="Ramipril Structure" /></td>
</tr>
<tr>
<td>Chemical Formula:</td>
<td>C(<em>{23})H(</em>{32})N(<em>{2})O(</em>{5})</td>
</tr>
<tr>
<td>IUPAC Name</td>
<td>((2S,3aS,6aS)-1(((S)-N-((S)-1-Carboxy-3-phenylpropyl) alanyl) octahydrocyclopenta [b]pyrrole-2-carboxylic acid, 1-ethyl ester)</td>
</tr>
<tr>
<td>Molecular weight:</td>
<td>416.5</td>
</tr>
<tr>
<td>Appearance and color:</td>
<td>Ramipril is a white, crystalline substance</td>
</tr>
<tr>
<td>Solubility:</td>
<td>Soluble in polar organic solvents</td>
</tr>
<tr>
<td>Dosage And Administration</td>
<td>Capsule: 1.25 mg, 2.5 mg, 5 mg, 10 mg</td>
</tr>
<tr>
<td>Category</td>
<td>Antihypertensive Angiotensin-converting enzyme (ACE) inhibitor</td>
</tr>
<tr>
<td>Serum protein binding</td>
<td>73%</td>
</tr>
<tr>
<td>T max and C max of tablet</td>
<td>1 h and 19 ng/mL respectively.</td>
</tr>
</tbody>
</table>
### 9.8 DRUG PROFILE OF RAMIPRILAT

<table>
<thead>
<tr>
<th>Name</th>
<th>RAMIPRILAT</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Structure</strong></td>
<td>![Structure Image]</td>
</tr>
<tr>
<td>Chemical Formula:</td>
<td>$C_{21}H_{28}N_2O_5$</td>
</tr>
<tr>
<td>IUPAC Name</td>
<td>$(2S,3aS,6aS)-1-((S)-N-((S)-1-Carboxy-3-phenylpropyl)alanyl)tetrahydrocyclopenta[b]pyrrole-2-carboxylic acid.$</td>
</tr>
<tr>
<td>Molecular weight:</td>
<td>388.46</td>
</tr>
<tr>
<td>Appearance and color:</td>
<td>White to Off-White Solid</td>
</tr>
<tr>
<td>Solubility:</td>
<td>Methanol</td>
</tr>
<tr>
<td>Category</td>
<td>Antihypertensive Angiotensin-converting enzyme (ACE) inhibitor</td>
</tr>
<tr>
<td>Serum protein binding</td>
<td>56%</td>
</tr>
<tr>
<td>$T_{max}$ and $C_{max}$ of tablet</td>
<td>2–4 hours and 5.12 ng/mL respectively</td>
</tr>
</tbody>
</table>
### 9.9 DRUG PROFILE OF FELODIPINE

<table>
<thead>
<tr>
<th>Name</th>
<th>FELODIPINE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Structure</strong></td>
<td><img src="image" alt="Chemical Structure" /></td>
</tr>
<tr>
<td><strong>Chemical Formula:</strong></td>
<td>C(<em>{18})H(</em>{19})Cl(_2)NO(_4)</td>
</tr>
<tr>
<td><strong>IUPAC Name</strong></td>
<td>Ethyl methyl 4-(2,3-dichlorophenyl)1,4-dihydro-2,6-dimethyl-3,5 pyridine dicarboxylate</td>
</tr>
<tr>
<td><strong>Molecular weight:</strong></td>
<td>384.26</td>
</tr>
<tr>
<td><strong>Appearance and color:</strong></td>
<td>slightly yellowish, crystalline powder</td>
</tr>
<tr>
<td><strong>Solubility:</strong></td>
<td>freely soluble in dichloromethane and ethanol</td>
</tr>
<tr>
<td><strong>Dosage And Administration</strong></td>
<td>2.5mg and 10 mg oral</td>
</tr>
<tr>
<td><strong>Category</strong></td>
<td>Antihypertensive</td>
</tr>
<tr>
<td><strong>Half life</strong></td>
<td>8.5-19.7 h in healthy volunteers</td>
</tr>
<tr>
<td><strong>Plasma protein binding</strong></td>
<td>99%</td>
</tr>
<tr>
<td><strong>T max and C max of tablet</strong></td>
<td>1 h and 8 ng/mL respectively</td>
</tr>
</tbody>
</table>
10. **AIM AND OBJECTIVE OF THE PRESENT RESEARCH WORK**

The aim of the present research work was to develop simple, accurate, precise and fast methods for quantification of the different categories of drugs and/or their metabolites from human plasma by using the best chromatographic device available in the market today, which is LC-MS/MS.

Literature reported methods are lengthy, tedious, long chromatographic analysis time and required higher sample volume by using HPLC, LC–MS, GC-MS and HPLC-UV etc which were not adequate for high throughput analysis for bioequivalence and pharmacokinetic studies.

The objectives of the present research work are:

I] To develop sensitive, rapid, accurate and precise methods of different categories of drugs from a combination formulation by using LC MS/MS in human plasma, which have higher sensitivity, more simple procedure, smaller sample volume, consume less solvents and shorter run time; resulting in high-throughput analysis.

II] To perform validation of developed methods according to international regulatory guidelines, to provide greater assurance that the method will work well in other laboratories, where different operators, instruments, and reagents are involved and where it will be used over much longer period of time.

III] To employ methods for estimation of analytes and its metabolites from human plasma in the evaluation and interpretation of the bioavailability, bioequivalence and PK data. Same applies to methods employed for toxicokinetic studies as well.
CHAPTER 1

11. PLAN OF WORK

11.1 Literature Survey

- **Drug profile** - Chemical structure, molecular weight, molecular formula, pKa, pH and solubility.
- **Pharmacokinetic parameters** - dose, formulation, Cmax, Tmax, t₁/₂, AUC and protein binding etc.
- **Published articles** on selected molecules.

11.2 Experimental Work

- **Materials and Solvents**
  Reference standards of analyte and internal standard, HPLC grade solvents like methanol, acetonitrile, n-Hexane, ethanol, TBME etc, GR grade buffers like ammonium acetate, ammonium formate, ammonia, acetic acid and formic acid etc. Human plasma, microtips, hand gloves, mask, ria vials, HPLC vials etc.

- **Instruments and Equipments**
  Mass Spectrometer (Applied Biosyste Mds Sciex, API4000) Analytical balance (Sartorius, CPA26 P), SPE processor (Orochem Technologies), vortex mixer (Spinix), Reciprocating Shaker (GFL 3018), Hot Plate Magnetic Stirrer (Spinot), Milli Q Water System (Millipore), Weight Box (Sartorius, 1mg to 5 g), Deep Freezer -80°C (Sanyo, Mdf-U53v), Freezer -20°C (Sanyo, Mdf-U537), Auto Pipette (Eppendorf), Multipette (Eppendorf), Ultra Sonicator (Life Care Pvt Ltd), pH Meter (Thermoscientific, Mgl-16831), Refrigerated Centrifuge (Hittich, Rotanta 460 R), Nitrogen Evaporator (Takashche Analytical)
CHAPTER 1

- **Bioanalytical Method Development**-
  1. Mass parameter optimization
  2. Chromatographic approaches
  3. Selection of Internal Standard, Mobile phase, Column
  4. Sample preparation techniques

- **Bioanalytical Method Validation**-
  After development of the method validation of the method is carried out for routine analysis. For validation of the method guidelines from the Food and Drug Administration (FDA) and the International Conference on Harmonization (ICH) are followed

  1. Accuracy and precision
  2. Specificity/Selectivity
  3. Sensitivity
  4. Recovery
  5. Matrix Effect
  6. Stability
  7. Long-term stability
  8. Bench Top Stability
  9. Auto sampler Stability
  10. Freeze/Thaw stability

11.3 **Application of the Method**

  Bioequivalence study