BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF ESOMEPRAZOLE IN HUMAN PLASMA BY LC-MS/MS

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In
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Submitted by

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Under the Guidance of

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MARCH - 2010.

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CERTIFICATE

This is to certify that the works embodied in this dissertation entitled **"BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF ESOMEPRAZOLE IN HUMAN PLASMA BY LC-MS/MS"** submitted to The Tamil Nadu Dr. M.G.R. Medical University, Chennai, was carried out by **M.SATHIYARAJ (Reg.No.26084829)**, for the partial fulfillment for the degree of **MASTER OF PHARMACY** in Pharmaceutical Analysis under the guidance of **Mr.R.VIJAY AMIRTHARAJ, M.Pharm., Ph.D.,** Head of the Department of Pharmaceutical Analysis, J.K.K.Munirajah Medical Research Foundation College of Pharmacy, Komarapalyam, during the academic year 2009-2010.

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DECLARATION

The work presented in this dissertation entitled **"BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF ESOMEPRAZOLE IN HUMAN PLASMA BY LC-MS/MS"** was carried out by me, under the guidance of, **Mr. R. VIJAY AMIRTHARAJ M.Pharm., Ph.D.,** Head of Department of Pharmaceutical Analysis, J.K.K.Munirajah Medical Research Foundation College of Pharmacy, Komarapalayam.

This work is original and has not been submitted in part or full for the award of any other degree or diploma of any other university.

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TO WHOMSOEVER IT MAY CONCERN

This is to certify that **Mr.M.SATHIYARAJ,** M. Pharm (Pharmaceutical Analysis) , student of Dr.MGR Medical university, Tamilnadu has done his project work on "BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF ESOMEPRAZOLE IN HUMAN PLASMA BY LCMS/MS" from June,2009 to December, 2009 in our organisation.

We found him sincere and honest in his work.

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INTRODUCTION

This thesis deals with the studies carried out by the writer in this laboratory for the past one year on the development and validation method used for the Esomeprazole in biological fluids. Before discussing the experimental results, a brief introduction to biopharmaceutical analysis, analysis of drugs and metabolites in biological media, preliminary treatment of biological samples, extraction procedure for drugs and metabolites from biological sample, estimation of drugs in biological sample by LC-MS , quantitative techniques in LC-MS.[1,2,3,4,5,6,7]

1.1 BIO PHARMACEUTICAL ANALYSIS:

Need for bio pharmaceutical analysis:

Methods of measuring drugs in biological media are increasingly important problems related to following studies are highly dependent on bio pharmaceutical analytical methodology.

- Bio availability and Bio equivalence studies,
- New drug development,
- Clinical pharmacokinetics,
- Research in basic bio medical and Pharmaceutical sciences.

1.2 ASSAY OF DRUGS AND THEIR METABOLITES:

A number of allusions have been made to analytical methods that distinguish drugs from their metabolites. Drug metabolism reaction can be divided into phase I and phase II categories. Phase I typically involves oxidation, reduction and hydrolysis reaction. In contrast phase II transformation entail couplins or condensation of drugs or their phase I metabolites with common body constituents (e.g. sulfate, glucuronic acid). Except for reduction process, most phase I and phase II reactions yield metabolites that are more polar and hence more water soluble than the parent drug. Assay must distinguish between drug and its metabolites. If this fact is ignored, erroneous data may be generated.

1.3 ANALYSIS OF DRUGS IN VARIOUS BIOLOGICAL MEDIA:

The most common samples obtained for bio pharmaceutical analysis are blood and urine. Feces are also utilized, especially in the drug or metabolite is poorly absorbed or extensively excreted in the bile. Other media that can be utilized include saliva, breath and tissue.

The choice of sampling media is determined largely for the nature of the drug study. For example drug levels in clinical pharmacokinetic study demand the use of blood, urine, the possibly saliva. A bioavailability study may require drug level data in blood and or urine where is a drug identification of drug abuse problem may be solved with only one type of biological sample.

Detection of a drug or its metabolites in biological media is usually complicated by the matrix. Because of this, various types of clean up procedures involving techniques such as solvent extraction and chromatography are employed to effectively separate drug components from endogenous biological material. The ultimate sensitivity and selectivity of the assay method may be limited by the efficiency of the clean up methodology.

Blood :

Whole blood is collected from venipuncture with either a hypodermic syringe or vaccutainers apparatus. The volume of blood collected at any one sampling time is usually limited to 5 to 15ml (depending on the assay sensitivity and the total number of samples taken for a given study). If the blood is allowed to clot and is then centrifuged about 30 to 50% of the original volume is collected as serum (upper level). Thus plasma generally is preferred because of its greater yield from blood. The greater the yield the greater the amount of drug and the fewer the problems with sensitivity. Blood, serum, or plasma samples can be utilized for drug studies and may require protein denaturation steps before further manipulation.

If plasma or serum is used for the analytical procedure the fresh whole blood should be centrifuged immediately at 4000rpm for approximately 5 to 15mins. and the supernatant should be transferred by means of a suitable device such as Pasteur pipettes. To clean a container of appropriate size for storage. The remaining blood cells can then be discarded or stored for further studies such as drug binding.

Urine:

Urine is the easiest to obtained from the patient and also permits collection of large and frequently more concentrated samples. The lack of protein in a healthy individuals urine obviates the need for denaturation steps. Because urine samples are readily obtained and often provide the greatest source of metabolites. They are frequently analyzed in drug metabolism studies.

Feces:

With human feces are collected in an aluminum foil pan placed under a toilet seat. Once collected the foil is folded around the material and the sample lyophilized. Fecal specimens contain high protein content and difficulties arises is their handling and analysis (even after lyophilization) because of the large ratio of solid mass to drug. Denaturation of protein is usually required before further analytical manipulations are begun.

Saliva:

Saliva is obtained from humans via non invasive sampling techniques. Saliva is advantageous in drug studies done with children. Certain drugs exists a constant ratio between plasma and salivary levels. Although the concentration of drug in saliva are more equal to those in plasma, a constant ratio (over on effective therapeutic range) permits calculation of plasma levels based on salivary analysis. The amount of drug excreted in saliva is related to the degree of ionization at physiologic pH 7.4 and the extent of protein binding. That is un-ionized drugs, which are not highly protein bound occur at highest concentrations in saliva. Theophylline can be analyzed in saliva because the plasma/ saliva ratio is 2:1.

Breath:

The analysis of drugs in breath is traditionally reserved for low molecular weight compounds that posses relatively low boiling points. The extent of eliminating of drugs via the lungs is also related to the solubility of a drug in the blood and to extent of metabolism of the drug in the body. Blood levels of drugs can be reduced through Ostwald solubility coefficients (osc), and drug level can be determined in alveolar (“deep-lung”) breath. The determination blood alcohol levels through breath

analyses are commonly employed in many states to determine whether individuals are legally intoxicated.

1.4 STORAGE REQUIREMENT OF BIOLOGICAL SAMPLE:

In order to avoid decomposition or other potential chemical changes in the drugs to be analyzed. Biological samples should be frozen immediately upon collection and thawed before analysis. When drugs are susceptible to plasma esterase, the addition of esterase inhibitors such as sodium fluoride to blood samples immediately, after collection helps to prevent drug decomposition.

When collecting and storing biological samples, the analyst should be wary of artifacts from tubing or storage vessels that can be contaminate the sample. For example plastic ware frequently contains the high boiling liquid bis (2 ethythexyl) phthalate, similarly the plunger plugs of vaccutainers are known to contain tri-butoxyethyl phosphate , which can be interfere in certain drug analysis.

In the case of feces, lyophillization of the sample before storage is highly desirable unless prior investigations have revealed little or no reactivity of the drug components with the endogenous intestinal micro-organisms.

1.5 PRELIMINARY TREATMENT OF BIOLOGICAL SAMPLES:

In most cases, preliminary treatment of a sample is needed before the analyst can be proceeding to the measurement step. Analysis is required for drug in sample as diverse as plasma, urine, feces, saliva, bile, sweat and seminal fluid. Each of these samples has its own set of factors that must be considered before an appropriate pretreatment method can be selected. Such factors are texture and chemical composition of the sample, degree of drug protein binding. Chemical stability of the drug and types of interferences can affect the final measurement step.

1.5.1 Protein precipitation or Denaturation:

Biological materials such as plasma, feces and saliva contain significant quantities of protein which can bind a drug. The drug may have to be freed from protein before further manipulation. Protein denaturation is important because the presence of proteins, lipids, salts and other endogenous material in the sample can cause rapid determination of LC-MS method and interfere the assay.

The protein denaturation procedure includes the use of tungstic acid, ammonium sulfate, heat, alcohol, trichloro acetic acid, methanol, Acetonitrile, and perchloric acid or ultra filtration and dialysis. These methods may reduce rather than eliminate the protein problem. After addition of reagent to a sample, a milky white precipitate is obtained. This is separated by centrifugation at 3000 rpm for 10 to 20 mins.

Ultra filtration and dialysis procedures they have been used to separate protein from Biological sample. Ultra filtration and dialysis procedure are not widely used because they are slow.

1.5.2 Hydrolysis of conjugates:

The conjugate (glucuronides and sulfates) of the drug or metabolite is usually hydrophilic and or ionized at physiologic pH. Thus, conjugates are not amendable to classic solvent extraction techniques. To overcome these problems, samples are usually hydrolyzed using enzymes (Glucurase or snail 8-glucuranidase /aryl sulfatase) or acid. The resulting unconjugated drug or metabolite is less hydrophilic and can be extracted from the biological matrix.

A nonspecific acid hydrolysis can be accomplished by heating a biologic sample for 30 mins at 90 to 100˚C in 2 to 5N hydrochloric acid. Upon cooling the pH of the sample can be adjusted to the desired level and the drug or metabolite may be isolated by solvent extraction.

1.5.3 Homogenization:

For sample containing insoluble protein such as muscle or other related tissues, a homogenization or solubilising step using 1N hydrochloric acid may be required before treating the sample further. For gelatinous samples such as seminal fluid or septum liquefaction is achieved via sonication. A solid sample such as feces can be homogenized with a minimum amount of methanol. Homogenization is usually performed with a blade.

1.6 EXTRACTION PROCEDURE FOR DRUG AND METABOLITE FROM BIOLOGICAL SAMPLES:

The biological samples are treated with drugs and metabolites. After that it can be extracted with extracted solvent by using suitable extraction procedure. The extraction procedure may classified into following types,

- Liquid-Solid Extraction
- Liquid-Liquid Extraction

1.6.1 Liquid-Solid Extraction:

Liquid-Solid extractions occur between a solid phase and a liquid phase. Among the solids that have been used successfully in the extraction (usually via adsorption) of drugs from liquid samples are charcoal, alumina, silica gel and aluminium silicate. Liquid-Solid extraction is often particularly suitable for polar compounds that would otherwise tend to remain in the aqueous phase. The method could also be useful for amphoteric compounds that cannot be extracted easily from water. Solid can adsorb organic compounds by hydrophobic interactions, vander waals forces, hydrophobic bonding and dipole-dipole interaction.

A factor governing the adsorption includes,

- Solvent polarity
- Flow rate of the solvent
- Degree of contact the solvent has with the adsorbent

In the adsorption process, the hydrophobic portion of the solute that has little affinity for the water phase is preferentially adsorbed on the adsorbent surface while the hydrophilic portion of the solute remains in the aqueous phase.

Biological sample can be prepared for cleanup by passing the sample through the resin bed where drug (Metabolite) components are adsorbed and finally eluted with an appropriate solvent.

The liquid-solid extraction method provides a convenient extraction procedure for blood samples. Thus avoiding solvent extraction process, precipitation, drug losses

and emulsion formulation. It is possible forever that strong drug protein binding could prevent sufficient adsorption of the drug to resin.

1.6.2 Dehydration method:

An aqueous biological sample is treated with a sufficient quantity of anhydrous salt (sodium or magnesium sulfate) to create a dried mix. This mix is then extracted with a suitable organic solvent to remove the desired drug or metabolite.

1.6.3 Liquid-Liquid Extraction:

Liquid-Liquid Extraction is probably the most widely used techniques because,

- The analyst can remove the drug or metabolite from larger concentration of endogenous materials that might interfere with the final analytical concentration.
- The technique is simple, rabid and has a relatively small cast factor per sample.
- The extract containing the drug can be evaporated to dryness and the residue can be redissolved in a smaller volume of a more compatible with a particular Analyte methodology in the measurement step, such as a mobile phase in LC-MS determination.
- The extracted material can be redissolved in smaller volume (e.g. 100 to 500µl of solvent thereby extending the sensitivity limits of an assay.
- It is possible to extract more than one sample concurrently.
- Near quantitative recoveries (90% or better) of most drugs can be obtained through multiple or continuous extraction.

Partitioning or distribution of a drug between two possible liquid phases can be expressed in terms of a partition or distribution coefficient usually called P. A partition coefficient is constant only for a particular solute temperature and pair of solvent used. By knowing the P value for the extracted drug and the absolute volume of the two phases to be utilized, the quantity of drug extracted after a single extraction

can be obtained. In multiple extraction methodology, the original biological sample is extracted several times with fresh volumes of organic solvent used as much drug as possible is obtained. Because the combined extracts contain the total extracted drug. It is desirable to calculate the number of extraction necessary to achieve maximum extraction.

1.7 FACTORS AFFECTING PARTITION COEFFICIENT:

Factor that influence partition coefficient and hence recovery of drugs in Liquid-Liquid Extraction are,

- Choice of organic solvent
- Effect of pH
- Ionic strength of the aqueous phase

1.7.1 Choice of organic solvent:

- The solvent should be immiscible with an aqueous phase,
- It should have less polarity than water and should solubilize the desired extractable compound to a large extent.
- It should also have a relatively low boiling point so that it can be easily evaporated.
- Other considerations are cost, toxicity, flammability and nature of the solvent.
- It is generally accepted that diethyl ether and chloroform are the solvents of choice for acidic and basic drugs respectively.
- When the identity of a drug is known the extraction solvent is chosen with more case because a P value either is known or can be approximated based on existing solubility data.
- Control of pH is another important factor is successful solvent extraction procedures. Because most drugs are classified chemically as weak acids or bases, they can be easily converted into their respective salts by treatment with a strong inorganic acid or base. These salts are charged species and therefore

have appreciable solubility in polar solvent such as water but have little, if any solubility in polar solvents such as diethyl ether.

1.7.2 Effect of pH:

- Proper pH adjustment of a biological sample permits conversion of an ionized drug to an un-ionized species, which is more soluble in a nonpolar solvent and therefore, extractable from an aqueous environment.
- The proper pH for extraction can be calculated from the Henderson Hasselberch equation using the pka of the compound. If the species to be analyzed is unknown, the pka must be approximated based on the chemical nature of the suspected agent.
- A general rule of thumb for basic drug is to extract the drug from the sample at a pH 2 to 3 unit above the pka value for the drug for acidic drugs, a pH value 2 to 3 unit below the pka is indicated. This ensures that at least 99.9% of the unionized form of the drug is available for extraction.

1.7.3 Ionic strength of the aqueous phase:

- Addition of highly water soluble ionized salts, such as sodium chloride, to an aqueous phase creates a high degree of interaction between the water molecules and the inorganic ions in solution. Fewer water molecules are free to interact with an unionized drug.
- Therefore the solubility of the drug in the aqueous phase decreases, thereby increasing the partitioning or distributing in favor of the nonpolar or organic phase.
- The technique of adding inorganic salts to an aqueous phase in an extraction procedure is commonly called “salting out”. Addition of inorganic salts (Ammonium carbonate) to a biologic sample also lessens emulsion formation and facilitates extraction of a drug into an organic solvent.

1.8 CHROMATOGRAPHIC METHODS:

The presence of metabolites or of more than one drug in a biologic sample usually demands a more sophisticated separation for their measurement especially, when two or more drugs are of similar physical and chemical nature. Chromatography is a separation technique that is based on differing affinities of a mixture of solutes between at least two phases. The result is a physical separation of the mixture into various components. The affinities or interaction can be classified in terms of a solute adhering to the surface of a polar solid (adsorption), a solute dissolving in a liquid (partition), and a solute passing through or impeded by a porous substances based on its molecular size (exclusion).

Individual chromatographic techniques relation to their usefulness as separation tools for drugs or metabolites in biological samples are

- ✓ HPTLC
- ✓ GC
- ✓ HPLC
- ✓ LC-MS

1.8.1 High performance thin layer chromatography:

Thin layer chromatography usually is performed by a solute mixture is placed near the bottom of a plate (a technique is called as spotting) at a definite location (called the migin), and the mobile phase ascends the plate by capillary action (a process called development or elution). The separation process is carried out in a closed chamber in which the atmosphere is allowed to become saturated with the vapors of the mobile phase before elution.

Depending on the chromatographic behavior of a particular solute, a separation results and, later substances is observed at a definite distance from the origin. After the development process visualization of the metabolite in each zone on a TLC plate can be achieved by destructive and nondestructive techniques. Many of the techniques are based on functional group chemistry and thus the use of reagents offers specially and increased sensitivity for the analyzed drug or metabolite.

1.8.2 Gas chromatography:

Gas chromatography (GC) is one of the most extensively used tools for quantitative analysis of drugs in biologic samples. Gas chromatography offers the advantages of speed sensitivity, resolution and simplicity for both quantitative and qualitative drug analysis. In gas liquid chromatography the stationary phase is a liquid that is coated onto an inert solid support. The process is a form of partition chromatography, where the components of a drug mixture are separated based on the solutes vapor pressure (or B.P), solubility in the stationary phase (partition coefficient) and to some degree molecular weight.

Various liquid phases are chosen depending on the chemical nature of the drug to be separated. The most common liquid phases used for biologic analyzes are substituted siloxanes (e.g. OV-1, OV-17) and polyethylene glycol. A GC column contains the stationary phase, which is usually a liquid that is coated (usually 1 to 5% w/w) onto a solid support, such as a diatomaceous earth (80 to 120 mesh size) or onto the walls of a column (e.g. open tubular capillary columns).

The stationary phase is nonvolatile at the column temperatures employed and must possess suitable selectivity for the drug mixture that is to be separated. The packed column is usually 1 to 2 m length and 2 to 4 mm in diameter. Recently, a trend, toward using capillary columns has emerged. The advantage of these columns is their high efficiencies (10,000 to 100,000 theoretical plates). They are usually 0.25 to 1.25 mm in diameter and approximately 20 m in length.

Detector like flame ionization detector (FID), electron capture detector (ECD) and nitrogen phosphorous (N-P) detector are employed for the drug analysis in biological samples because of their uniqueness and high sensitivity. The combination of GC-MS is currently a powerful tool in drug and metabolite identification in biological samples. Sensitivity of a mass spectrometer is in the nano gram to high fenito gram range.

1.8.3 High performance liquid chromatography:

Most of the drugs in biological samples can be analyzed by HPLC method because of several advantages like rapidity, specificity, accuracy, precision, ease of automation and eliminates tedious extraction and isolation procedure. HPLC is directly derived from classic column chromatography in that a liquid mobile phase is pumped under pressure rather than by gravity flow through a column filled with a stationary phase. This has resulted in a sharp reduction in separation time, narrow peak zones, and improved resolution. This mobile phase is placed in a solvent reservoir for pumping into the system. In case of liquid-solid HPLC, solvent are chosen from the elutropic series. A solvent system is usually degassed by vacuum treatment or sonication before use.

There are different modes of separation in HPLC. They are normal phase mode reversed phase mode, reversed phase ion pair chromatography, ion exchange chromatography, affinity chromatography and size exclusion chromatography (gel permeation and gel filtration chromatography). The different types of detection used in HPLC methods based on ultraviolet (UV), fluorescence, refractive index, mass Spectrophotometric and electrochemical. In most cases, method development in HPLC is carried out with UV detection using a variable wave length Spectrophotometric detector or a diode array detector (DAD).

Chemical derivatization procedures for HPLC are performed in order to improve detectability to improve selectivity (or specificity) to modify chromatographic properties and in some cases to provide favorable mass spectral fragmentation patterns for structure elucidation when a mass spectrophotometer is used either as an on-line or off-line detector.

When a drug or metabolite is difficult to derivatize but possesses reasonable Lewis acid or base properties, an ion pair reagent is added to the mobile phase to form an ion-pair with the compound. Thereby enhancing detection and chromatographic properties ion pair techniques such as this can be approached with both pre and post column methodology. Both chromogenic and fluorescing counter ions can be employed depending on the sensitivity requirements of the assay.

1.8.4 LC-MS:

Introduction:

Chromatography is an essential separation technique in life sciences and related chemistry fields. Traditional detectors such as ultraviolet-visible, electrochemical, refractive index, flame ionization, thermal conductivity, etc are widely used to quantify compounds as bidimensional data are produced (response x time).

The mass detectors are characterized by generating tridimensional data (response x time x ionic specimen), that is, mass spectrum that can provide very important information on the sample molecular weight, its molecular structure, identity, quantity and purity. Data from the mass spectra add specificity to both quantitative and qualitative analysis.

For most of the compounds, the mass detectors are more sensitive and much more specific than traditional detectors. They can analyze several compounds and identify components in non-separated chromatograms, thus reducing the need of a perfect chromatography. The mass spectral data may supplement data from other detectors. Although two compounds can have similar UV or Mass spectra, such as in LC-MS, this phenomenon is hardly simultaneous; therefore, both types of data together can be used to identify, confirm and quantify compounds with highly correct results.

Some mass spectrometers have the characteristic of performing multiple mass spectrometry steps in a single sample. They can generate a mass spectrum, select a specific ion and then generate a new spectrum. Some of them are able to repeat this cycle several times until the structure is determined (MS/MS or MS^n).

Technique principle:

The mass spectrometer basically operates through the ionization and fragmentation of molecules. Afterwards, the resulting ions are identified according to their mass/charge ratio. The three key components of the process include: ions source, analyzer and detector. The purpose of the ions source is to generate ions. There are several kinds of ions source in the chromatography connected to mass detectors. Each

type is specific for a given class of compounds. There are also several kinds of analyzers for the separation of ions and detectors for the generation of measurable signals.

Each one has advantages and drawbacks depending of the type of information searched. Details concerning the most common sources, analyzers and detectors will be discussed next:

Ionization source:

The molecules ionization and fragmentation will take place in the source. Several ionization techniques are available, including: Atom bombing - FAB, Laser Desorption - LD, Thermospray - TS, Particles beam, etc. However, the most commonly used sources are:

Electrons impact (EI):

The Electrons Impact (EI) source uses a filament in charge of emitting electrons with a defined energy of 70 eV. Once the electrons beam energy is much higher than the first ionization potential in most of the compounds of the sample, this energy is ionized and then fragmented. This kind of ionization is related to the GC.

Chemical ionization (CI):

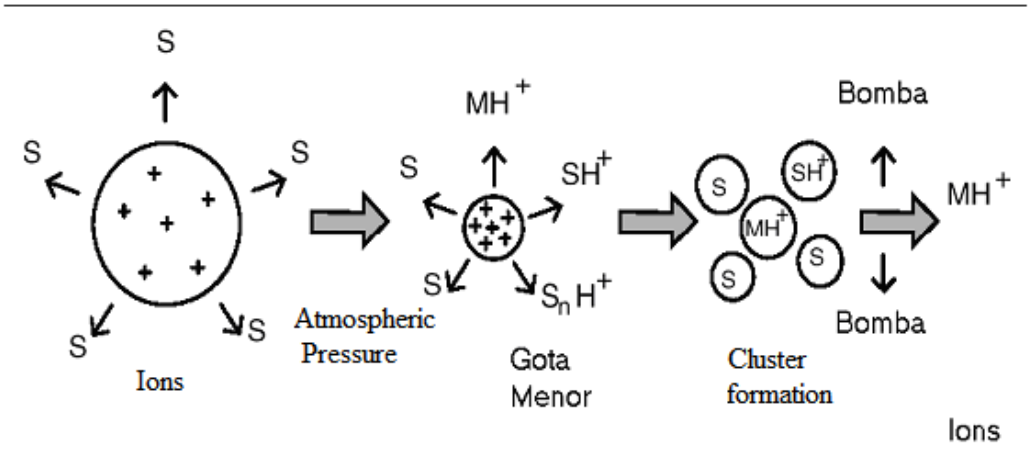
The Chemical Ionization (CI) source uses liquid or gas agents to react with molecules. Ionization usually takes place by means of the transference of a proton to the molecule, thus forming specimens called molecular pseudo-ions. As this ionization is much “smoother” than the electrons impact, the spectrum produced contains a few fragments and almost exclusively the molecular pseudo-ion; therefore, it is employed in the determination of the molecular weight and/or quantitative analyses. This kind of ionization is related to GC.

Electro spray Ionization (ESI):

The electro spray ionization has a great impact on the use of mass spectrometry applied to biological researches in the last years. It was the first method to expand the instruments useful mass range to above 50,000 Da. However introduced in its present model in 1984, the technique returns to the investigations of electrically

assisted liquid dispersion in the beginning of this century. In fact, the main discovery was almost accidental in 1968 when Malcolm Dole and cooperators were able to bring macromolecules to the gas phase at atmospheric pressure. It was possible by spraying a sample solution to a small tube with a strong electric field in the presence of a warm nitrogen flow to help in the dissolvation and then measuring the ions formed. Later, innovative experiences in this field led to the introduction of an ES ionization source. Since then, a wide range of biomolecules was investigated by ES. The sample is usually dissolved in a mixture of water and organic solvent, typically methanol, isopropanol and acetonitrile: it can be directly infused or injected in continuous flow that is, contained in the eluant of a HPLC column and CE capillary column.

The ES source is simples, forming a spray occurring in a high voltage field as showed in Figure. In a proposed mechanism, it is believed that the ion formation is the result of an ionic evaporation process, first proposed in 1976. A droplets spray is generated by the electrostatic dispersion of the liquid applied by the capillary end. Favored by a heated gas (usually nitrogen), the droplets are disaggregated, lose solvent molecules in the process and occasionally produce individual ions. In another proposed mechanism, the droplets dissolvation leads to an increasing charge density on the droplet surface that will cause a coulomb explosion eventually producing individual ions.



Regardless the proposed mechanism, the ions are formed at atmospheric pressure and enter into a bore located in the vortex of a cone acting as the first barrier to the vacuum phase. A skimmer collects the ions and guides them to the mass spectrometer.

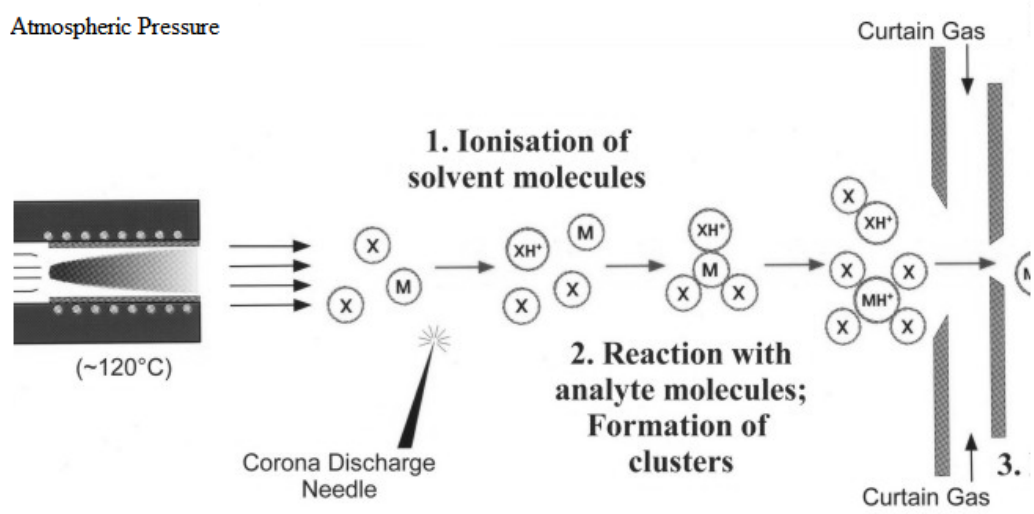
The spray formation is the most import part of the ES technique. It is usually advised to filter all solvents; high electrolyte concentrations should be avoided as they can cause ionization suppressions and unstable operating conditions. High flows compatible to those used in HPLC, can be now used through a heated nebulizing gas to help in producing the spray.

For macromolecules, each ion that usually enters the mass spectrometer has a high charge number. As the mass spectrometers measure the mass/charge ratio instead of the mass, it is possible that high molecular mass molecules have enough charge to fall within the *m/z* range of a linear quadrupole, typically *m/z* 20-4000.

Atmospheric Pressure Chemical Ionization:

In APCI, the LC eluent is sprayed through a heated (typically 250°C – 400°C) vaporizer at atmospheric pressure. The heat vaporizes the liquid. The resulting gas-phase solvent molecules are ionized by electrons discharged from a corona needle. The solvent ions then transfer charge to the analyte molecules through chemical reactions (chemical ionization).The analyte ions pass through a capillary sampling orifice into the mass analyzer.

APCI is applicable to a wide range of polar and nonpolar molecules. It rarely results in multiple charging so it is typically used for molecules less than 1,500 μ. Due to this, and because it involves high temperatures, APCI is less well-suited than electro spray for analysis of large biomolecules that may be thermally unstable. APCI is used with normal-phase chromatography more often than electro spray is because the analytes are usually nonpolar

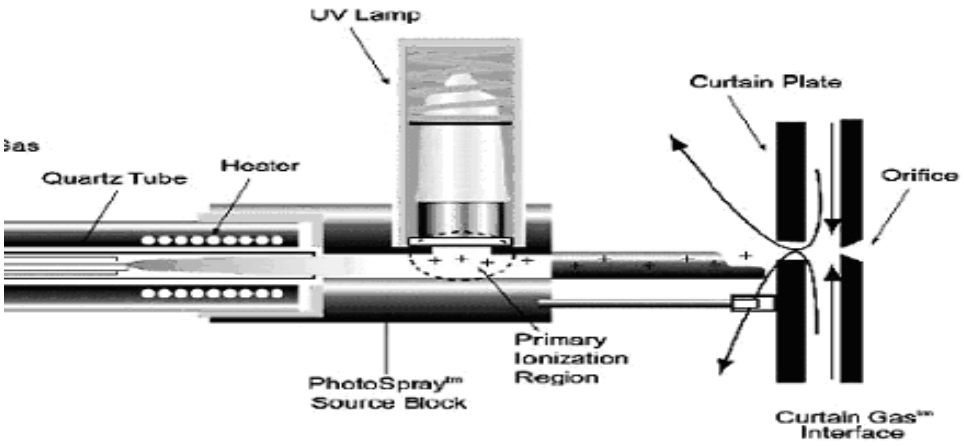


Atmospheric Pressure Photo Ionization:

Atmospheric pressure photo ionization (APPI) for LC/MS is a relatively new technique. As in APCI, a vaporizer converts the LC eluent to the gas phase. A discharge lamp generates photons in a narrow range of ionization energies. The range of energies is carefully chosen to ionize as many analyte molecules as possible while minimizing the ionization of solvent molecules. The resulting ions pass through a capillary sampling orifice 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 non polar compounds and low flow rates (<100 µl/min), where APCI sensitivity is sometimes reduced.

In all cases, the nature of the analyte(s) and the separation conditions has a strong influence on which ionization technique: electro spray, APCI, or APPI, will generate the best results. The most effective technique is not always easy to predict.



Mass analyzers:

After the admittance of the molecules into the ions source and subsequent ionization, it is necessary to determine the corresponding masses of ions formed so as to obtain the mass spectrum. The function of the mass analyzer is to separate ions according to their mass/charge ratios and transmit them to the detector. There are several kinds of mass analyzers. The most common and widely used are the "Quadrupole" and "Ion Traps" analyzers.

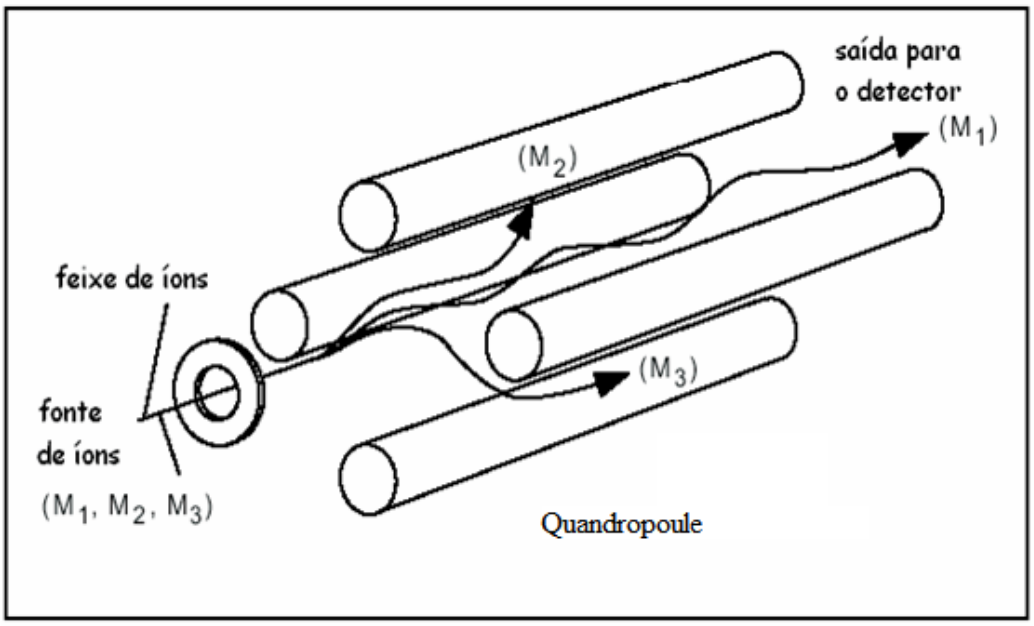
The quadrupole are scanning analyzers, that is, after the admittance of a mixture of ions with different mass/charge ratios (m/z) and different abundances, electric fields are applied; at a given time, only ions with a specific mass can leave intact. By varying the electric field applied, one can select and record different ions. The "Ion Trap" analyzers, however, are not regarded as pure scanning devices, as the ions are stored before the scanning itself.

Quadrupole mass analyzers:

The instrument is based on four parallel bars in a quadrangular area where the ions beam is focused on the central axis of these bars, a fixed electric potential (DC) and a radio frequency potential (RF) are applied to these diagonal and opposite bars. For a given RF and DC combination, ions of a specific mass range m/z have their path changed from the central axis. The mass spectrum is obtained from the DC voltage and RF components in a synchronized fashion that is, keeping the RF/DC ratio constant. The potential applied to the opposite bar pairs are determined as follows:

$$\pm\phi_\circ = \mathbf{U + V \cos\phi_t}$$

Where in U is a DC voltage and V cos ϕ_t the time-dependent voltage in which V the RF amplitude and ϕ the RF frequency.



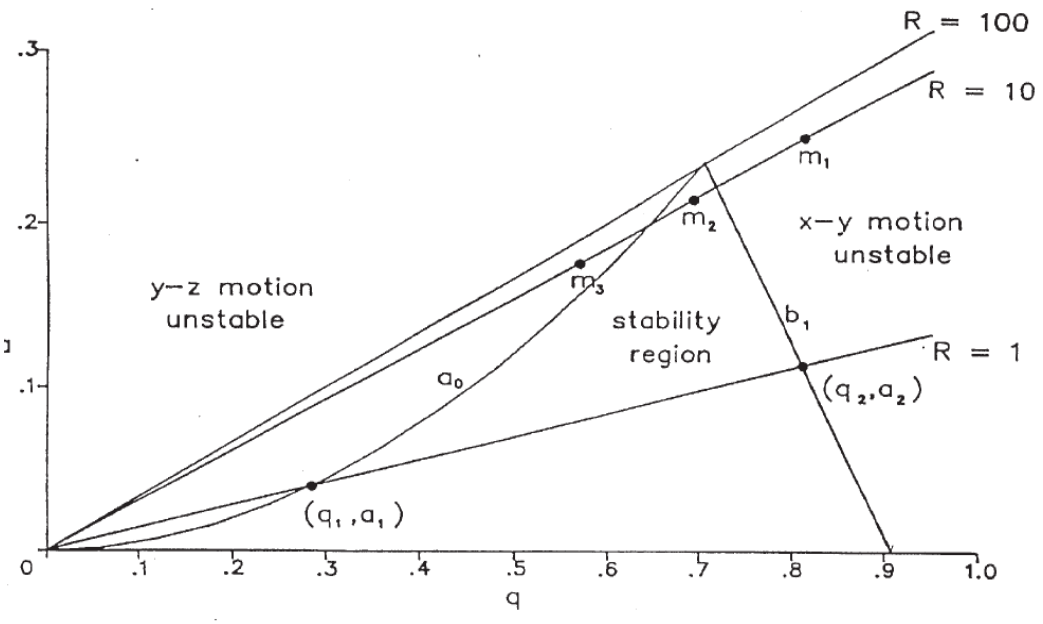
The quadrupole operation (as well as the ion trap to be discussed later) can be qualitatively discussed through the stability diagram corresponding the DC potential amplitude, the RF potential amplitude with the path of a stable ion (that is, an ion that can remain intact after passing the quadrupole). This will be represented in the equations and in graph.

The motion equation of a charged particle can be expressed as a Mathieu equation in which the a_u and q_u parameters can be defined.

$$a_u = a_x = -a_y = 4zU / m\phi^2 r_o^2$$

$$q_u = q_x = -q_y = 2zV / m\phi^2 r_o^2$$

wherein m/z is the ion mass/charge ration, and ro is half of the distance between the two opposite bars. There is no parameter for z, since the RF field acts on the x/y plane (z is the main axis of the linear quadrupole).



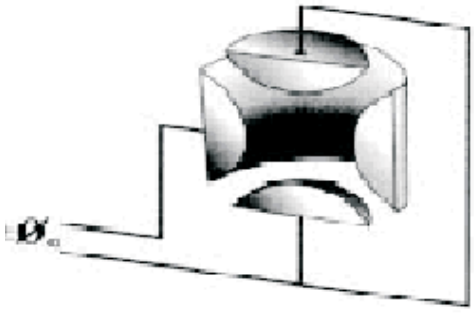
Stability diagram in the analyzer

In this case, the ion m_2 is the only ion that remains stable (observe it is within the stability region of the graph), while m_3 and m_1 cannot reach the detector. The R=100 2e R=10 straight lines represent two distinct combinations of the DC/RF ratio; by changing these ratios, it is possible to change the mass filter resolution, that is, the capacity to differentiate or filter masses very close to each other. The “a” and “q” parameters are respectively proportional to the DC and RF values.

“Ion trap” quadrupole mass analyzers:

This mass analyzer has the same mathematic operation principles of the conventional quadrupole, that is, ions stability within a specific path. However, the major difference is that in the ion trap the ions do not follow a single path towards the detector, but are “trapped” in orbits within the trap structure, thus giving raise to the name “ion trap”. While in the quadrupole the ions are formed in the ions source and then expelled towards the mass analyzer, in the ion trap the ions are formed ions and the mass analyzed in the same space region. This space region where the “trapped” ions are found corresponds to approximately the volume of 1 cm-side cube.

The figure below shows a schematic representation of the ion trap analyzer.



Schematic representation of the "ion trap" analyzer

As the quadrupole analyzers, the ion traps analyzers also use electric fields; these electric fields are intended to keep the ions confined and separate the masses (mass analysis). In this kind of mass analyzer, the electric field used is purely RF (radio frequency consisting in a sine wave with a frequency of about 1 Mhz) applied directly on the central annular electrode. Thus, depending on the RF amplitude applied, the ions can remain stable inside the trap. By increasing this amplitude, the ions with greater masses are "ejected" from the confinement region and then reach the detector.

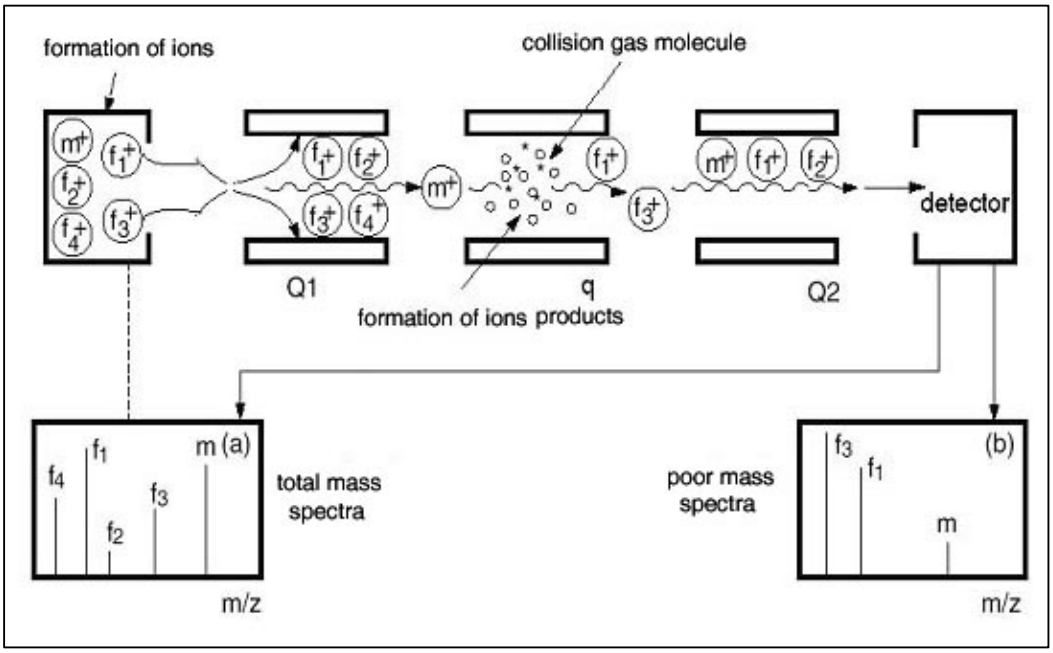
Another peculiarity of the ion trap analyzers is the need to control the number of ions within the structure, aiming to avoid reactions of these ions with molecules still present. These interactions can lead to a slight change in the final spectrum of some compounds, thus making interpretation/ identification difficult. This problem is eliminated in the quadrupole analyzers, as the ions are almost immediately "thrown" from the ions source after the formation and do not have the chance to interact with the molecules present.

On the other hand, the final sensitivity in the full scanning mode is greater in trap analyzers since all ions formed are necessarily detected once they are confined. This is not the situation for the quadrupole analyzers. That is, while the quadrupole is scanning the mass range aiming to produce a spectrum, the unstable ions get "lost" and, therefore, do not generate a detectable signal. It is possible, however, to significantly increase the quadrupole sensitivity by defining a fixed mass (or a few masses) of interest; this technique is called SIM (Single Ion Monitoring).

Tandem mass spectrometry:

The tandem mass spectrometry, MS/MS or MSn wherein n=2, 3..., uses two or more mass analysis steps, one to preselect an ion and the others to analyze the induced fragments, for example, by collision (CID) with an inert gas, such as argon or helium. This can be a tandem-in-space or tandemin- time analysis.

Tandem-in-space means several mass analyzers in series. Various combinations are possible, the most common include: triple quadrupole (Q1qQ2), four sectors and hybrid instruments. Q represents a quadrupole mass filter and q a RF quadrupole only (collision chamber). In the case of the triple quadrupole, an ion of interest generated in the ionization source is selected with the first quadrupole Q1, dissociated in the collision chamber q with energies up to 300 eV; the fragmentation products are analyzed with the second quadrupole Q2.



Thus, it is possible to obtain information on the sequence of a peptide by selecting the ion corresponding to the protoned peptide (called precursor ion) and analyzing the fragments of its structure using Q_2. This process is called ions-product scanning. Several other types of scanning or analytical experiments can be performed. For example, the search of all precursors of a given fragment is called ions-precursors scanning. This can be reached by keeping Q_2 steady at the mass/ charge ratio of the

ion concerned and scanning all ions present at Q_1, while the collision dissociations at q take place. In another scanning mode, ions that lose a specific fragment can be identified by scanning Q_1 and Q_2 simultaneously, keeping the mass difference between the quadrupole analyzers equal to the mass of the neutral lost fragment.

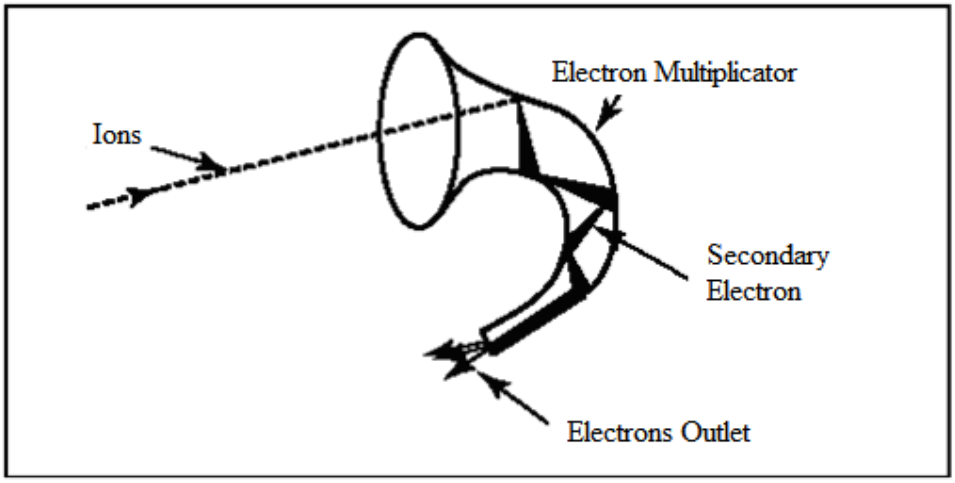
Tandem-in-time can be obtained using “ion trap” devices and ICR mass spectrometers (also called FTMS). In fact, these devices are not limited to MS/MS experiments, but can reach multiple stages (MS/MS/MS…/MS or MSn). In such devices, the ions are selected by applying specific voltage pulses and dissociations normally occur by collisions with other gases.

Detectors:

After selection by the analyzer, the ions are guided towards the detector where they will be converted into a measurable signal. Detectors can be divided in three groups. Photosensitive plates and Faraday cages are included in the first group and directly correlate the measured signal to the amount of the analyzed ion. The second group includes the electrons multipliers, photomultipliers and microchannel plates that amplify the intensity of the received signal. These are the most commonly used detectors and will be discussed herein. The third group is used in ICR devices (FTMS) and consist in a radio frequency detector applied to the trapped ions.

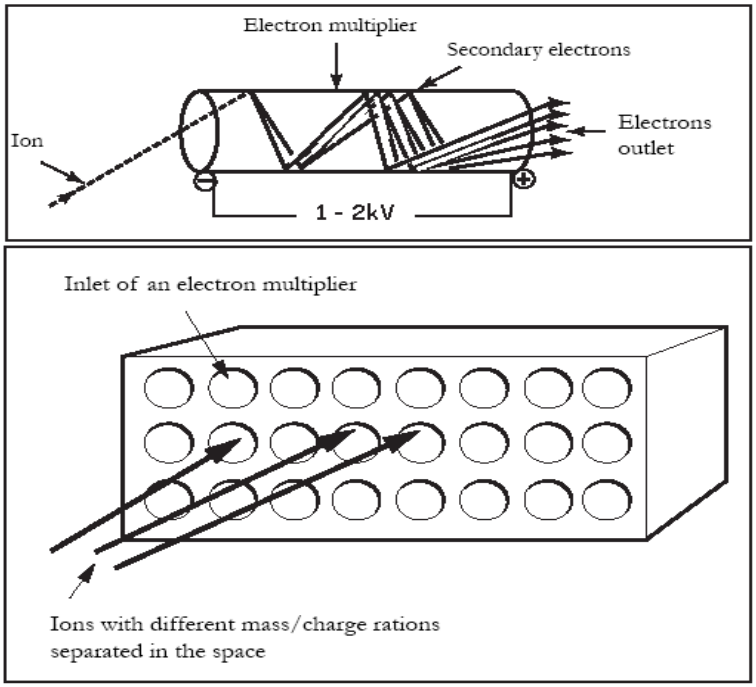
Electrons multipliers:

When reaching the conversion dinode where a negative potential is applied, positive or negative ions emit several secondary electrons. These electrons are accelerated towards an electrons multiplier and hit the walls with sufficient energy to remove some electrons. These electrons will hit the other side of the wall, thus releasing more electrons. This cascade effect continues until a measurable current is finally created at the end of the electrons multiplier.



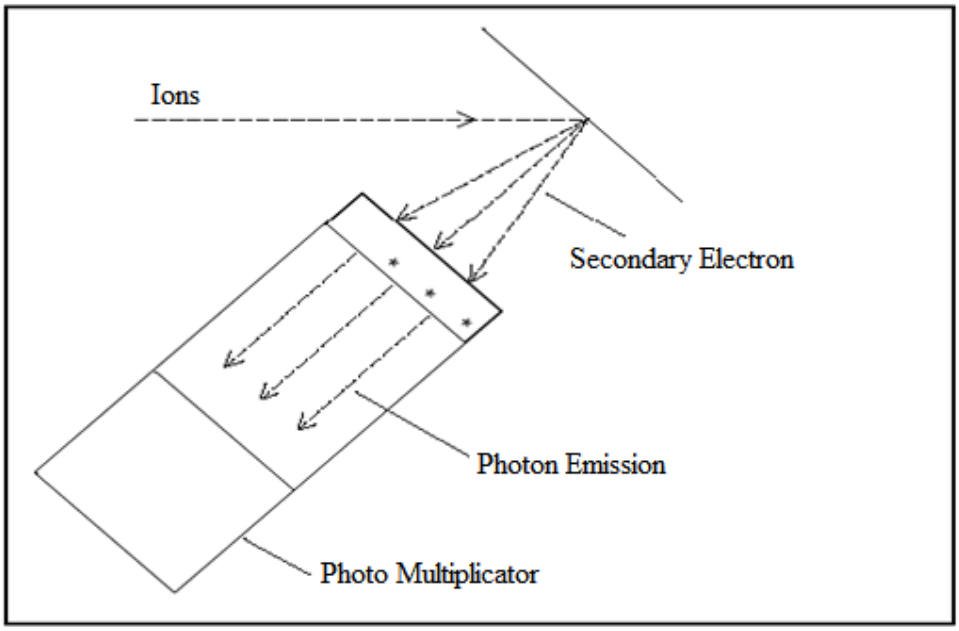
Microchannel plates:

The microchannel plates consist in a plate containing parallel cylindrical microchannels. The inlet side of these microchannels is kept with a negative potential of approximately 1kV when compared to the outlet side. The electrons multiplication, started with the collision of an ion in these channels, occurs through a semiconductive substance that coats each channel and generates secondary electrons. Curved channels prevent the acceleration of positive ions towards the inlet. The cascade effect inside the channels can multiply the number of electrons in the order of 105 and the use of several coupled plates allow an amplification that can reach 108. At the outlet of each channel, a metal anode collects the electrons current and the signal is transmitted to the processor. Another characteristic of these microchannel detectors is an extremely low signals multiplication time, making them inadequate for detection in devices such as time-of-flight analyzer.



Photomultiplier:

This kind of detector comprises two conversion dinodes; a phosphorescent screen and a photomultiplier. This detector, as the electrons multiplier and the microchannel plates, allows the detection of positive and negative ions. Upon detection, the ions are accelerated towards the dinode having a reverse inverse polarity of that of the ion; the electrons are then released and accelerated towards the phosphorescent screen where they are converted into photons. The photons are then detected by the photomultiplier. The phosphorescent screen surface is coated with a fine conductive aluminum layer so as to avoid the formation of charges that could refrain new electrons from reaching it. The amplification reaches values from 104 to 105.



Data acquisition and processing:

Specific computer programs integrally perform data acquisition and processing. These programs are in charge of several tasks, ranging from the control of the monitoring time of an ion to the construction of calibration curves where the areas (or heights) of unknown sample peaks are interpolated, thus producing the desired quantitative datum. Each manufacturer has a data acquisition and treatment program with different resources and limitations. Therefore, the comprehension of these programs and the future preparation of standard operating procedures (SOP) are crucial for conducting any study.

Application of LC-MS:

- Peptide mapping.
- Selective detection of compound in a complex mixture.
- Efficient analysis of biological sample.
- To identify degradation procedures in stability studies
- Identification of metabolites.
- Quantification of compounds in biological matrix.

1.9 ESTIMATION OF DRUGS IN BIOLOGICAL SAMPLE BY LC-MS:

MS has emerged as an ideal technique for the identification of such structurally diverse metabolites. When coupled with online HPLC the technique is extremely robust, rapid, sensitive, and easily automated. Not surprisingly, LC-MS/MS have become the methods of choice for pharmacokinetic studies, yielding concentration versus time data for drug compounds from in vivo samples such as plasma.

LC-MS instrument consist of three major components

- LC (to resolve a complex mixture of components)
- An interface (to transport the analyte in to the ion source) of a mass spectrometer
- Mass spectrometer (to ionize and mass analyze the individually resolved components)

Reverse phase (RP) HPLC is a widely pretended mode of chromatography and is a major contributing factor to advances made in several areas of pharmaceutical science. Mobile phase composition is a very critical in achieving selectivity in RP-HPLC separation. Although a large number of buffer system have been used in conventional RP-HPLC, only the volatile ion paring reagent can be used in LC-MS analysis.

Most of the Drugs in Biological sample can be analyzed by LC-MS method. Because of several advantages like rapidity, specificity, accuracy, precision, ease of automation and eliminates tedious extraction and isolation procedures some of the advantages are,

- Speed (analysis can be accomplished in 20 minutes or less),
- Greater sensitivity (Various detector can be employed),
- Improved resolution (wide variety of stationary phases),
- Reusable column (Expensive columns but can be used for many samples),
- Ideal for the substances of low volatility,
- Easy samples recovery, handling and maintenance,

- Instrumentation lends itself to automation and quantitation (less time and less labour),
- Precise and reproducible,
- Calculation are done by integrator itself and
- Suitable for preparative chromatography on a much large scale.

1.10 QUANTITATIVE ANALYSIS IN LC-MS

These methods are generally used for quantitative analysis. They are the external standard method, the internal standard method and the standard addition method.

1.10.1 External Standard method:

The external standard method involves the use of a single standard or up to three standard solutions. The peak area or the height of the samples and the standard used are compared directly or the slope of the calibration curve based on standards that contain known concentration of the compounds of interest.

1.10.2 Internal Standard Method:

A widely used technique of quantitation involves the addition of an internal standard to compensate for various analytical errors. In this approach, a known compound of a fixed concentration is added to the known amount of samples to give separate peaks in the chromatograms. To compensate for the losses of the components of the interest will be in companied by the loss of equivalent fraction of internal standard. The accuracy of this approach obviously dependents on the structural equivalence of the compounds of interest and the internal standard.

- The requirement for an internal standard must be,
- Give a completely resolved peak with no interference
- Elute close to the compound of interest
- Behave equivalent to the compounds of interest for analysis like pretreatment, derivative formation, etc.
- Be added at a concentration that will produce a peak area or peak height ratio of about unity with the compounds of interest.
- Not be present in the original sample,

- ❖ Be stable, uncreative with sample components, column packing and the mobile phase.
- ❖ Be commercially available in high purity.

The internal standard should be added to the sample prior to sample preparation procedure and homogenized with it. Response factor is used to determine the concentration of a sample component in the original sample. The response factor (RF) is the ratio of peak areas of sample component (A_s) and the internal standard (A_{ISTD}) obtained by injecting the same quantity.

1.11 METHOD VALIDATION

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

Method validation involves all the procedures required to demonstrate that a particular method for quantitative determination of the concentration of an analyte (or a series of analyses) in a particular biological matrix is reliable for the intended application. 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. 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.

The guideline for industry by FDA states that the fundamental parameters of validation parameters for a bioanalytical method validation are accuracy, precision, selectivity, sensitivity, reproducibility and stability. Typical method development and establishment for bioanalytical method includes determination of (1) selectivity, (2) accuracy, (3) precision, (4) recovery, (5) calibration curve, and (6) stability.

For a bioanalytical method to be considered valid, specific acceptance criteria should be set in advance and achieved for accuracy and precision for the validation of the QC samples.

Validations are subdivided into the following three categories

Full validation:

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

Partial validation:

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

Cross validation:

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

1.11.1 Selectivity:

A method is said to be specific if it produces a response for only a single analyte. Method selectivity is the ability of a method to produce a response for the target analyte distinguishing it from all other interferences. Interferences in biological samples arise from a number of endogenous (analyte metabolite, degradation products, co-administered drugs and chemicals normally accruing in biological fluids) and exogenous sources (impurities in reagents and dirty lab-ware). Zero level interference of the analyte is desired, but it is hardly ever the case. The main thing one must take care of is that, the response of the LLOQ (Lower Limit of Quantification) standards should be greater than the response from the blank biological matrix by a defined factor. If all the efforts to get rid of interferences in the chromatographic process fail, changing to a more selective detector such as Mass Spectrometry (MS) or MS-MS may give a better result.

The following practical approach may be used during method development to investigate the selectivity of an analytical method.

Processing blank samples from different sources will help to demonstrate lack of interference from substances native to the biological sample but not from the analyte metabolite. Processing of reagent blank in the absence of biological matrix is normally adequate to demonstrate selectivity with regard to exogenous interferences mentioned above.

Although it would be preferable that all tested blanks, if obtained under controlled conditions, be free from interferences, factors like food and beverage intake and cigarette smoking can affect selectivity. Evaluation of a minimum of six matrix sources to approve the selectivity of the method.

1.11.2 Precision:

The precision of analytical procedure expresses the closeness of agreement between a series of measurements from multiple sampling of the same homogenous sample under prescribed condition. It is expressed as the percentage coefficient of variance (% CV) or relative standard deviation (R.S.D.) of the replicate measurements.

$$\% CV = \frac{\text{Mean}}{\text{Standard deviation}} \times 100$$

Precision may be considered at three levels,

- Repeatability
- Intermediate Precision
- Reproducibility

Repeatability:

Repeatability expresses the precision under the same operating conditions over a short interval of time.

Intermediate precision:

Intermediate precision expresses within the laboratory in different days, different analyst, different equipment, etc.

Reproducibility:

Reproducibility expresses the precision between the laboratories. It also known as inter assay precision.

The reproducibility of a method is of prime interest to the analyst since this will give a better representation of the precision during routine use as it includes the variability from a greater number of sources.

A minimum of three concentrations in the range of expected concentrations is recommended.

The %CV determined at each concentration level, should not exceed 15 % except for the LLOQ, where it should not exceed 20%.

1.11.3 Accuracy*:*

The accuracy of a bioanalytical method is a measure of the systematic error or bias. The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found.

Accuracy is best reported as percentage bias that is calculated from the formula:

$$\% \text{ bias} = \frac{\text{Measured value} - \text{True value}}{\text{True value}} \text{ X } 100$$

Some of the possible error sources causing biased measurement are: error in sampling, sample preparation, preparation of calibration line and sample analysis. The method accuracy can be studied by comparing the results of a method with results obtained, by analysis of certified reference material (CRM) or standard reference material (SRM).

Accuracy should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected concentrations is recommended.The mean value should be within 15 % of the actual value except at LLOQ, where it should not deviate by more than 20 % .

1.11.4 Recovery:

The recovery of an analyte in an assay is the response of the detector to a quantity of analyte added and/or separated from a biological matrix. The recovery is associated with the efficacy of the analytical separation method, within variability levels. The recovery of an analyte does not need to be of 100%, however, the quantity of analyte recovered and of the internal standard must be consistent, accurate and reproducible. Experiments for recovery should be made comparing analytical results of samples with three concentrations (low, medium, high) with standard solutions at the same concentrations, representing 100% recovery.

$$\text{Absolute Recovery} = \frac{\text{Response of spiked plasma (processed)}}{\text{Response of standard solution (unprocessed)}} \text{ X } 100$$

Good precision and accuracy can be obtained from methods with moderate recoveries, provided they have adequate sensitivity. Indeed it may be desirable to intentionally sacrifice high recovery in order to achieve better selectivity with some sample extraction procedure.

Solvents such as ethyl acetate normally give rise to high recovery of analyte; however these solvents simultaneously extract many interfering compounds. Therefore, provided that an adequate sensitive detection limit is attained with good precision and accuracy, the extent of recovery should not be considered an issue in bioanalytical method development and validation.

1.11.4 Matrix effect:

Biological matrix can play a role in affecting the selectivity, sensitivity and precision. This happens due to direct or indirect alteration of the response of analyte from the unintended interferences present in biological matrix. The quantitation measure of matrix effect is the matrix factor and was calculated by using following equation.

$$\text{Matrix effect} = \frac{\text{Response in post extracted spiked sample}}{\text{Response in standard solution}} \text{X}100 - 100$$

1.11.6 Stability:

The stability of the analyte is often critical in biological samples even over a short period of time. Degradation is not unusual even when all precautions are taken to avoid specifically known stability problems of the analyte (e.g. light sensitivity). It is therefore important to verify that there is not sample degradation between the time of collection of the sample and their analysis that would compromise the result of the study.

Stability evaluation is done to show that the concentration of analyte at the time of analysis corresponds to the concentration of the analyte at the time of sampling.

An essential aspect of method validation is to demonstrate that analyte(s) is (are) stable in the biological matrix and in all solvents encountered during the sample work-up process, under the conditions to which study samples will be subjected.

According to the recommendations on the Washington conference report by Shah et al. (1992), the stability of the analyte in matrix at ambient temperature should be evaluated over a time that encompasses the duration of typical sample preparation,

sample handling and analytical run time. Similarly Dagar & Brunett (1995) gave the following details to be investigated

Long term stability:

This is done to assess whether the analyte is stable in the plasma matrix under the sample storage conditions for the time period required for the samples generated in a clinical study to be analyzed.

Standard stock solution stability:

The stability test for the standard stock solution must be done at the same temperature, container and solvent as that to be used for the study. The time period should be at least six hours.

Short term matrix stability:

This must be evaluated following the storage under laboratory conditions used for sample work-up for a period of e.g. 6 h to 24 h, and compared with data from the same samples prepared and analyzed without delay.

On-instrument sample stability:

This should be evaluated over the maximum time from completion of sample work-up to completion of data collection, with allowance for potential delay in analysis due to equipment failure. This stability study is conducted at the temperature at which processed study samples will be held prior to data collection.

Freeze -thaw stability:

This stability test is done to ensure that the sample remains stable after it is subjected to multiple freeze-thaw cycles in the process of the study. This can be done by thawing samples at high, medium and low concentrations unassisted and allowing them to freeze again for at least 12-24 hrs. The cycle is repeated twice and the sample is processed at the end of the third cycle and its result is compared with freshly prepared sample. If the analyte is not stable after three cycles, measures must be taken to store adequate amounts of aliquots to permit repeats, without having to freeze and thaw the sample more than once.

Acceptable stability is 2 % change in standard solution or sample solution response relative to freshly prepared standard. Acceptable stability at the LLOQ for standard solution and sample solution is 20 % change in response relative to a freshly prepared sample.

1.11.7 Sensitivity:

According to IUPAC as cited in Roger Causon, a method is said to be sensitive if small changes in concentration cause large changes in the response function.

Sensitivity can be expressed as the slope of the linear regression calibration curve, and it is measured at the same time as the linearity tests. The sensitivity attainable with an analytical method depends on the nature of the analyte and the detection technique employed. The sensitivity required for a specific response depends on the concentrations to be measured in the biological specimens generated in the specific study.

LITERATURE REVIEW

♣ ***Udupa N. et al***[8] developed a simple sensitive and specific method for the determination of Esomeprazole in Capsule and Human plasma. Lansoprazole was used as an internal standard. Separation of Esomeprazole and Internal standard was carried out an Reverse phase C18 Column (250 x 4.6 mm, 5µ) using Mobile phase acetonitrile and 20mM Phosphate buffer pH 3.2 (30:70 V/V) and UV-Visible detection at 300 nm without interference from endogenous materials. The Limit of Detection and Quantification of Esomeprazole in human plasma was 10 ng/ ml and 20 ng /ml respectively.

♣ ***Mathias Liljeblad et al***[9] reported a LC-MS/MS method was developed for quantitative determination of Esomeprazole, and its two main metabolites 5-hydroxyesomeprazole and omeprazole sulphone in 25 µl human, rat or dog plasma. The analytes and their internal standards were extracted from plasma into methyl tert-butyl ether-dichloromethane (3:2 v/v). After evaporation and reconstitution of the organic extract the analytes were separated on a reversed-phase LC column and measured by atmospheric pressure positive ionisation MS. The linearity range was 20 -20,000 nmol/L for Esomeprazole and omeprazole sulphone, and 20-4000 nmol/l for 5-hydroxyesomeprazole. The extraction recoveries ranged between 80 and 105%. The intra and inter-day imprecision were has lessthan 9.5% with accuracy between 97.7% and 100.1% for all analytes.

♣ ***Veleri A.Frerichs et al***[10]**.,** reported a method has been developed and validation for the quantitation of midazolam, alphahydroxy-midazolam, omeprazole and hydroxyl omeprazole from one 250µl sample of human plasma using high performance liquid chromatography coupled to tandem mass spectrometry. The method was validated for a daily working range of 0.400-100 ng/ml, with limits of detection between 2 and 15 pg/ml. The inter-assay variation was less than 15% for all analysis condition and 24 h in the post-preparative analysis matrix. This method was used to analyze samples in support of clinical studies probing the activity of the cytochrome P-450 enzyme system.

♣ ***Patel B.H. et al[11]*** a simple, sensitive, and precise high performance liquid chromatographic method for the analysis of Pantoprazole, Rebeprazole, Esomeprazole, domperidone and itopride, with ultraviolet detection at 210 nm, has been developed, validated, and used for the determination of compounds in commercial pharmaceutical products. The compounds were well separated on a Hypersil BDS C_{18} reversed-phase column by use of a mobile phase consisting of 0.05 M, 4.70 pH, potassium dihydrogen phosphate buffer - Acetonitrile (720:280 v/v) at a flow rate of 1.0 mL min^{-1}. The linearity ranges were 400–4,000 ng mL^{-1} for Pantoprazole, 200–2,000 ng mL^{-1} for Rebeprazole, 400–4,000 ng mL^{-1} for Esomeprazole, 300–3,000 ng mL^{-1} for domperidone and 500–5,000 ng mL^{-1} for itopride. Limits of detection (LOD) obtained were: Pantoprazole 147.51 ng mL^{-1}, Rebeprazole 65.65 ng mL^{-1}, Esomeprazole 131.27 ng mL^{-1}, domperidone 98.33 ng mL^{-1} and itopride 162.35 ng mL^{-1} . The study showed that reversed-phase liquid chromatography is sensitive and selective for the determination of Pantoprazole, Rebeprazole, Esomeprazole, domperidone and itopride using single mobile phase.

♣ ***Patil Shamkant S. et al[12]*** three simple, precise and economical UV methods have been developed for the estimation of Esomeprazole in bulk and pharmaceutical formulations. Esomeprazole has the absorbance maxima at 303nm (Method A), and in the first order derivative spectra, showed zero crossing at 303nm, with a sharp peak at 292nm when n=1 (Method B), Method C applied was Area under Curve (AUC) for analysis of Esomeprazole in the wavelength range of 294- 310nm. Drug followed the Beer's Lamberts range of 5-40 µg /ml for the Method A, B C. Results of analysis were validated statistically and by recovery studies and were found to be satisfactory.

♣ ***Nafisur Rahman et al[13]*** two simple, sensitive and economical spectrophotometric methods have been developed for the determination of Esomeprazole magnesium in commercial dosage forms. Method A is based on the reaction of Esomeprazole magnesium with 5-sulfosalicylic acid in methanol to form a yellow product, which absorbs maximally at 365 nm. Method B utilizes the reaction of Esomeprazole magnesium with N-bromosuccinimide in acetone-chloroform medium to form α -bromo derivative of the drug peaking at 380 nm.

Under the optimized experimental conditions, Beer's law is obeyed in the concentration ranges of 2-48 and 10-100 μgmL^{-1}withmolar absorptivity of 2.11 X 10^4 and 4.57 X 10^4Lmol^{-1} cm^{-1} for methods A and B, respectively. The limits of detection for methods A and B are 0.35 and 0.46 μg mL^{-1}, respectively. No interference was observed from excipients commonly present in tablet formulations. Methods A and B are successfully applied to the commercial tablets for the estimation of Esomeprazole magnesium with good accuracy and precision. The results compare favorably with the reference spectrophotometric method indicating no significant difference between the methods compared

♣ ***Mehta R.J et al[14]*** developed a simple, precise, rapid and economical HPLC method for the estimation Rabeprazole sodium in bulk and in tablets using mobile phase containing Methanol and Water in volume of ratio of (90:10 V/V) at flow rate of 1.0 ml/min. A ODS C18 Reverse phase column (250 x 4.6 mm, 5 μ) was used as a stationary phase. Quantification was performed using diode array detector at 282 nm and the runtime observed was 10 mins. The retention time of the drug was found to be 3.49 mins. The linearity was observed in the range of 8-16 μg/ml with correlation coefficient 0.9958. The percentage assay of Rabeprazole Sodium was 101.2 %.

♣ ***Prasanna Reddy Attu et al[15]*** a simple, selective, accurate High Performance Liquid Chromatographic (HPLC) method was developed and validated for the analysis of Rebeprazole sodium. Chromatographic separation achieved isocratically on a C18 column [Use Inertsil C18, 5μ , 150 mm x 4.6 mm] utilizing a mobile phase of Acetonitrile/phosphate buffer (70:30, v/v, pH 7.0) at a flow rate of 0.8 ml/min with UV detection at 228 nm. The retention time of Rebeprazole sodium was 2.44 min. The method is accurate (99.15-101.85%), precise (intra-day variation 0.13-1.56% and inter-day variation 0.30-1.60%) and linear within range 0.1-30μg/ml (R^2=0.999) concentration and was successfully used in monitoring left over drug. The detection limit of Rebeprazole sodium at a signal-to noise ratio of 3 was 1.80ng/ml in human plasma while quantification limit in human serum was 5.60 ng/ml. The proposed method is applicable to routine analysis of Rebeprazole sodium in pharmaceutical formulations as well as in human plasma samples.

♣ ***Lakshmi S. et al***[16]a simple, fast and precise multi component mode analysis UV Spectrophotometric method has been developed for the simultaneous estimation of Omeprazole and Domepridone in combined Capsule dosage form. Shimadzu UV-1601 instrument was used and λ max of Omeprazole and Domepridone was found to be 272 nm and 286 nm using methanol as solvent and linearity lies between 10-60 µg/ml for Omeprazole and 5-30 µg/ml for Domepridone at their respective wavelengths.

♣ ***Zarna Dedania et al***[17] a RP-HPLC method is developed for simultaneous estimation of Omeprazole and ondansetron in combined tablet dosage form. Omeprazole is a proton pump inhibitor and in the treatment of gastro-oesophageal reflux disease (GERD), peptic ulcer and Zollinger-Ellison syndrome. Ondansetron is used as selective 5-HT3 receptor antagonist and used in the management of nausea and vomiting induced by cytotoxic chemotherapy and radio therapy and also post operative nausea and vomiting. The mobile phase used was a combination of Methanol: Acetonitrile (90:10). The detection of the combined dosage form was carried out at 218 nm and a flow rate employed was 0.5 ml/min. The retention time for Omeprazole and ondansetron was found to be 5.39 and 11.08 min respectively. Linearity was obtained in the concentration range of 4 to 20 µg/ml of Omeprazole and 4 to20 µg/ml of ondansetron with a correlation coefficient of 0.997 and 0.9967. Detector consists of photodiode array detector; the reversed phase column used was RP-C18 (2.27µm size, 250 mm X 4.6 mm i.d.) at ambient temperature. The developed method was validated according to ICH guidelines and values of accuracy, precision and other statistical analysis were found to be in good accordance with the prescribed values. Thus the proposed method was successfully applied for simultaneous determination of ondansetron and Omeprazole in routine analysis.

♣ ***Ozaltn N***[18]**.** Two different UV Method were developed for determination of Lansoprazole in pharmaceutical dosage forms. The solution of the standard and the sample prepared in 0.1M Sodium hydroxide and Phosphate buffer pH 6.6. Both UV Spectrophotometric techniques were applied. Second order derivative spectra were between 200 nm and 400 nm at N=9. The linear range for the UV Spectrophotometric method was 3.0-25.0 µg/ml. The developed methods

were applied to three different pharmaceutical preparations. The percentage recovery was 100.2%.

♣ ***Yeniceli D. et al***[19] the direct determination of Lansoprazole by using a Flow Injection Analysis (FIA) with detection and its application to the pharmaceutical capsule is described. In this study carrier solvent was found to be 0.01M Sodium Hydroxide and it was determined at optimum condition as flow rate of 1 ml/min and wavelength of 292 nm. Examining the repeatability of that was found to be 1.72% for intraday and 2.13% for inter day precision.

♣ ***Bhavna Patel et al***[20] *a* RP-HPLC method has developed for simultaneous estimation of Lansoprazole and domperidone in combined capsule dosage form. Lansoprazole is a proton pump inhibitor and used in the treatment of duodenal and gastric ulcers and gastro esophageal reflux disease (GERD), a condition in which the acid in the stomach washes back up into the esophagus. Sometimes Lansoprazole is used in combination with antibiotics to treat ulcers associated with infection caused by the *H. pylori* bacteria, gastro-oesophageal reflux disease (GERD), peptic ulcer and Zollinger- Ellison syndrome. Domperidone is indicated for treating symptoms associated with upper gastrointestinal motility disorders caused by chronic and sub acute gastritis. It is a gastrointestinal emptying (delayed) adjunct, a peristaltic stimulant and exhibit antiemetic properties.

♣ ***Jain N.K et al***[21] A novel, simple, sensitive, rabid and Spectrophotometric method has been developed for simultaneous estimation of amoxicillin tri hydrate and Rabeprazole Sodium. The method involves solving of simultaneous evaluations based on measurement of absorbance at two wavelengths 247nm and 292nm. Both the drugs obey Beer's law in the concentration range of 0-50 µg/ml and 0-20 µg/ml. Results of the method were validated statistically and by recovery studies.

♣ ***Venisee N***[22] a Sensitive, Specific HPLC assay was developed to quantitated Omeprazole in plasma and gastric fluid. The HPLC system consist of a multi-phase column combining anion exchange and reverse phase separation(Omnipac pax-500, Dionx) and variable wavelength UV detector set at

254nm. The mobile phase was a mixture of Sodium phosphate buffer: Methanol: Acetonitrile (60:20:20) with final pH adjusted to 7.0 Metronidazole and Omeprazole were extracted by adsorption on to a C”2 bonded silica gel SPE column, and eluted with methanol. The dried reconstituted in a solution of Acetyl Salicylic Acid (ASA) and then injected into the HPLC system. Under these conditions Metronidazole, Omeprazole and ASA were well separated and recoveries in plasma were greater than 80% Omeprazole was fastly measured in gastric fluid because of rabid decomposition.

AIM AND PLAN OF WORK

Studies to measure Bioavailability and/or establish Bioequivalence of a product are important elements in support of orally administered drug products in investigational new drug applications (INDs), new drug applications (NDAs), abbreviated new drug applications (ANDAs), and their supplements. The systemic exposure profile determined during clinical trials in the IND period can serve as a benchmark for subsequent BE studies.

Until recently, bioavailability (rate and extent of absorption of medicaments from drug delivery systems) of drugs was not emphasized. It was more or less assumed that if the physical and chemical integrities of a drug product were assured pharmacologic performance would be observed. It is now recognized that formulation factors can influence the biologic availability of a medicament from a dosage unit in mammalian systems. Consequently, it has become common practice to establish bioavailability by measurement of blood levels of drugs following administration of dosage forms.

However, it should be noted that either bioavailability or bioequivalence data could be generated without analytical methodology to accurately measure drugs in biological fluids.

Currently there is a need in the pharmaceutical environment to develop analytical methods for the determination of Esomeprazole in human plasma. The developed method could then be applied to clinical trials to obtain accurate pharmacokinetic parameters in human plasma.

HPLC-UV and LC-MS/MS methods have been reported. Some of these methods use complicated extraction equipments, long and tedious extraction procedures, and large amounts of solvents or biological fluids for extraction while other methods have a long turn around time during analysis.

In this project work the aim is to develop a suitable, highly specific, and sensitive analytical method for the quantitation of Esomeprazole in the low picogram range in human plasma.

The main objective of this work is to develop rapid, selective and sensitive LC-MS/MS method that have short and simple extraction procedures, consume small amounts of solvent and biological fluid for extraction and a short turn-around time.

Plan of present study:

The plan of present study is as follows,

1. **Optimization of Chromatographic condition** were proposed to be developed and optimized,
    - Selection of Mass Condition,
    - Selection of initial separation condition,
    - Nature of stationary phase
    - Nature of mobile phase (pH, peak modifier, solvent strength, ratio and flow rate),
    - Sensitivity and
    - Selection of Internal standard.
2. The developed method were also proposed to be **Validated** using the various validation parameter such as,
    - Accuracy,
    - Precision,
    - Linearity and Range,
    - Selectivity / Specificity,
    - Matrix Effect,
    - Recovery,
    - Robustness / Ruggedness,
    - Stability

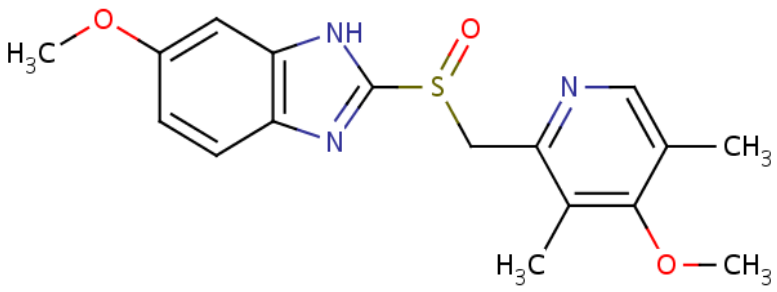
DRUGPROFILE

Analyte Name: **ESOMEPRAZOLE**[23,24]

Chemical Name: 6-methoxy-2-[(4-methoxy-3,5-dimethylpyridin-2-yl)methylsulfinyl]-1H-benzimidazole

Molecular Formula: $C_{17}H_{19}N_3O_3S$

Molecular Structure:



Molecular Weight: 345.417 g/mol

Solubility:

- Easily soluble in methanol.
- Partially soluble in acetone.
- Very slightly soluble in cold water, hot water.

CLINICAL PHARMACOLOGY: [25,26]

Description:

Esomeprazole belongs to a class of medications known as proton pump inhibitors (PPIs). PPIs, via the inhibition of H^+/K^+ ATPase enzyme pumps located in the gastric lumen, decrease the amount of gastric acid produced. Esomeprazole consists only of the active isomer (S-isomer), where as its counterpart, omeprazole, contains both active and inactive isomers (R- and S-isomers).

Studies have shown that a decrease in the amount of gastric acid produced was beneficial for patients with erosive esophagitis, symptomatic gastroesophageal reflux disease (GERD), NSAID-associated gastric ulcers, *Helicobacter pylori* (*H. pylori*) infections, and Zollinger-Ellison Syndrome. In patients with GERD, PPIs have been shown to relieve patients of GERD symptoms. In other cases, such as patients with *H. pylori* infections or the development of ulcers with the use of NSAIDs, PPIs have again proven to be efficacious in symptomatic and gastric damage relief.

Mechanism of Action:

Esomeprazole, like other PPIs, is a prodrug that is activated in an acidic environment to its active form (sulfenamide). The active form of the prodrug creates a covalent bond to H^+/K^+ ATPase pumps located on parietal cells in the gastric lumen. H^+/K^+ ATPase pumps are involved in the final step of the acid secretion pathway. This bond irreversibly inhibits the subsequent release of hydrogen ions. Inhibition of acid production is maintained until new H^+/K^+ ATPase pumps are regenerated (~18 hours).

Pharmacokinetics: [27]

Absorption:

Esomeprazole, intended for the oral route of administration, is formulated with an enteric coating to prevent rapid dissolution in the acidic environment of the gastric cavity. The different strengths of oral Esomeprazole (20 mg and 40 mg) create the ranges seen in C_{max}, T_{max} and AUC. A single 40 mg oral dose, taken without food, reaches its peak of 4.7 µmol/L (C_{max}) within 1.5 hours (T_{max}) with a bioavailability of 64%. The plasma concentration-time curve (AUC) for this single dose is

4.32 µmol*h/L. After multiple doses in fasting conditions, bioavailability increases to ~90% and the AUC increases to 11.2 µmol*h/L. The AUC decreases by 43-53% when a single dose is taken with food. Due to the differences in isomeric composition, the AUC of Esomeprazole is 80% higher than that of Omeprazole. This is due to a decrease in the clearance and first-pass metabolism of the S-isomer.

Intravenous forms of Esomeprazole have a slightly different pharmacokinetic profile than the oral forms. The different strengths of IV Esomeprazole (20 mg and 40 mg) create the ranges seen in C_{max} and AUC. After the IV administration of a 40 mg dose, given once daily for a total of 5 days, the AUC is 16.2 µmol*h/L with a peak concentration (C_{max}) of 7.51 µmol/L.

Distribution:

Esomeprazole has an apparent volume of distribution of 16 L. Additionally, esomeprazole is approximately 97% protein bound. This applies to both the oral and intravenous forms.

Metabolism:

Esomeprazole metabolism is mediated via the cytochrome P450 enzyme system (CYP450). The hydroxyl and desmethyl metabolites are formed by the CYP2C19 isoenzyme, while the sulphone metabolite is formed via the CYP3A4 enzyme. All metabolites formed are inactive.

Excretion:

The inactive metabolites of esomeprazole are mainly renally excreted (80%) with some fecal elimination (20%). A minimal amount of the active parent drug is excreted in the urine (1%). The elimination half-life for both the oral and intravenous forms ranges from 1-1.5 hours, with a slightly longer half-life with the oral forms of esomeprazole. Elimination is complete with no accumulation of drug.

Indications and Dosages:

FDA Approved Indications:

Gastroesophegeal reflux disease (GERD) with or without erosive esophagitis (oral, IV):

- **Healing/symptomatic relief:**
  - ✓ 20-40 mg QD for 4 to 8 weeks
  - ✓ Additional 4 to 8 weeks of therapy is indicated if initial therapy is ineffective.
- **Maintenance dose:**
  - ✓ 20-40 mg QD for up to 6 months.
  - ✓ Studies have not been carried out past 6 months.
- **Intravenous dose:**
  - ✓ 20-40 mg QD infusions given over 3-30 minutes for up to 10 days.
  - ✓ Convert patient to oral dosage form if possible after 10 days.

NSAID-associated gastric ulcer prophylaxis (oral):

- **Maintenance dose:**
  - ✓ 20-40 mg QD.
  - ✓ Studies have been done for up to 26 weeks of use.

Helicobacter pylori (H. pylori)-associated duodenal ulcer (oral):

- **Maintenance dose:**
  - ✓ 40 mg QD for 4 weeks.
  - ✓ Eradication of *H.pylori* is seen when esomeprazole is used in combination with amoxicillin and clarithromycin.

Zollinger-Ellison Syndrome (oral):

- **Maintenance dose:**
  - ✓ Typical dose is 40 mg BID.
  - ✓ Patients have been shown to see therapeutic results with daily dosages ranging from 80-240 mg.

Non-FDA Approved Indications:

- *H. pylori*-associated gastric ulcer
- nephropathic cystinosis
- pyrosis (heartburn)

Dosage Adjustment:

Hepatic insufficiency: Do not exceed 20 mg/day in patients with severe hepatic insufficiency (Child Pugh Class C).

Dosage Limits:

- **Adults (18 years and up):** 40 mg/day (for Zollinger-Ellison Syndrome, 240 mg/day)
- **Elderly:** 40 mg/day (for Zollinger-Ellison Syndrome, 240 mg/day)
- **Adolescents and children ages 12-17 years:** 40 mg/day PO
- **Children ages <12 years:** Safe and effective use has not been established.

Drug interaction:

Drug Name		Interaction
Atazanavir	-	This gastric pH modifier decreases the levels/effects of atazanavir.
Enoxacin	-	The agent decreases the absorption of enoxacin.
Indinavir	-	Omeprazole decreases the absorption of indinavir.
Itraconazole	-	The proton pump inhibitor decreases the absorption of imidazole.
Ketoconazole	-	The proton pump inhibitor decreases the absorption of imidazole.

Side Effects:

Common side effects include headache, diarrhea, nausea, gas, decreased appetite, constipation, dry mouth, and abdominal pain. More severe side effects are severe allergic reactions, chest pain, dark urine, fast heartbeat, fever, persistent sore throat, severe stomach pain, unusual bruising or bleeding, unusual tiredness, and yellowing of the eyes or skin.

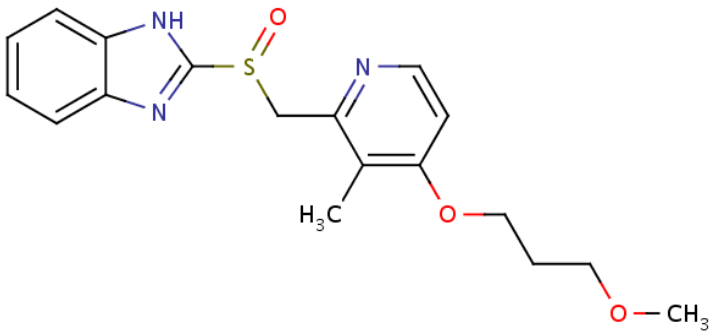
Proton pump inhibitors may be associated with a greater risk of hip fractures, clostridium difficile-associated diarrhea. Patients are frequently administered the drugs in intensive care as a protective measure against ulcers, but this use is also associated with a 30% increase in occurrence of pneumonia.

IS Name: **RABEPRAZOLE**[28,29]

Chemical Name: 2- [[4- (3-methoxypropoxy) -3- methylpyridin-2-yl] methyl sulfinyl]-1H-benzimidazole

Molecular Formula: $C_{18}H_{21}N_3O_3S$

Molecular Structure:



Molecular Weight: 359.444 g/mol

Solubility:

- Very soluble in methanol and Water.
- Freely soluble in Ethanol, Chloroform, Ethyl acetate.
- In soluble in Ether and n-Hexane.

CLINICAL PHARMACOLOGY: [30, 31]

Rabeprazole prevents the production of acid in the stomach. It reduces symptoms and prevents injury to the esophagus or stomach in patients with gastroesophageal reflux disease (GERD) or ulcers. Rabeprazole is also useful in conditions that produce too much stomach acid such as Zollinger-Ellison syndrome. Rabeprazole may also be used with antibiotics to get rid of bacteria that are associated with some ulcers. Rabeprazole is a selective and irreversible proton pump inhibitor, suppresses gastric acid secretion by specific inhibition of the H^+, K^+ -ATPase enzyme system which is found at the secretory surface of parietal cells. It inhibits the final transport of hydrogen ions (via exchange with potassium ions) into the gastric lumen.

Mechanism of Action:

Rabeprazole belongs to a class of antisecretory compounds (substituted benzimidazole proton-pump inhibitors) that do not exhibit anticholinergic or

histamine H_2-receptor antagonist properties, but suppress gastric acid secretion by inhibiting the gastric H^+/K^+ATPase (hydrogen-potassium adenosine triphosphatase) at the secretory surface of the gastric parietal cell. Because this enzyme is regarded as the acid (proton) pump within the parietal cell, rabeprazole has been characterized as a gastric proton-pump inhibitor. Rabeprazole blocks the final step of gastric acid secretion. In gastric parietal cells, rabeprazole is protonated, accumulates, and is transformed to an active sulfenamide. When studied in vitro, rabeprazole is chemically activated at pH 1.2 with a half-life of 78 seconds.

Pharmacokinetics:

Absorption:

Absolute bioavailability for a 20 mg oral tablet of rabeprazole (compared to intravenous administration) is approximately 52%. When rabeprazole is administered with a high fat meal, its T_{max} is variable and may delay its absorption up to 4 hours or longer, however, the C_{max} and the extent of rabeprazole absorption (AUC) are not significantly altered. Thus rabeprazole may be taken without regard to timing of meals.

Distribution:

Rabeprazole is 96.3% bound to human plasma proteins.

Metabolism:

Rabeprazole is extensively metabolized. The thioether and sulphone are the primary metabolites measured in human plasma. These metabolites were not observed to have significant antisecretory activity. In vitro studies have demonstrated that rabeprazole is metabolized in the liver primarily by cytochromes P450 3A (CYP3A) to a sulphone metabolite and cytochrome P450 2C19 (CYP2C19) to desmethyl rabeprazole. The thioether metabolite is formed non-enzymatically by reduction of rabeprazole. CYP2C19 exhibits a known genetic polymorphism due to its deficiency in some sub-populations (e.g. 3 to 5% of Caucasians and 17 to 20% of Asians). Rabeprazole metabolism is slow in these sub-populations, therefore, they are referred to as poor metabolizers of the drug.

Elimination:

Following a single 20 mg oral dose of 14 C-labeled rabeprazole, approximately 90% of the drug was eliminated in the urine, primarily as thioether carboxylic acid; its glucuronide, and mercapturic acid metabolites. The remainder of the dose was recovered in the feces. Total recovery of radioactivity was 99.8%. No unchanged rabeprazole was recovered in the urine or feces.

Indication and Dosages:

Short-term treatment in healing and symptomatic relief of duodenal ulcers and erosive or ulcerative gastroesophageal reflux disease (GERD); maintaining healing and reducing relapse rates of heartburn symptoms in patients with GERD; treatment of daytime and nighttime heartburn and other symptoms associated with GERD; long-term treatment of pathological hypersecretory conditions, including Zollinger-Ellison syndrome and in combination with amoxicillin and clarithromycin to eradicate *Helicobacter pylori*.

- Gastric ulcer
- Peptic ulcer disease (PUD)
- Maintenance of healing of erosive or ulcerative GERD
- Healing of erosive and ulcerative GERD
- Healing of duodenal ulcers.
- Treatment of symptomatic GERD
- Treatment of pathological hypersecretory conditions (Zollinger-Ellison syndrome)
- *Helicobacter pylori* eradication to reduce risk of duodenal ulcer recurrence

Side Effects:

All medicines may cause side effects, but many people have no, or minor, side effects. Check with your doctor if any of these most common side effects persist or become bothersome when using Rabeprazole:

- Diarrhea
- Head ache.

Seek medical attention right away if any of these severe side effects occur when using Rabeprazole:

Severe allergic reactions (rash; hives; itching; difficulty breathing; tightness in the chest; swelling of the mouth, face, lips, or tongue; unusual hoarseness); chest pain; fast or irregular heartbeat; fever, chills, or sore throat; red, swollen, blistered, or peeling skin; severe or persistent stomach pain; unusual bruising or bleeding; unusual tiredness; vision changes.

Drug Interaction:

DRUG		**INTERACTION**
Atazanavir	-	This gastric pH modifier decrease the levels/ effects of atazanavir
Dasatinib	-	Possible decreased levels of dasatinib
Digoxin	-	Rabeprazole increases the effect of digoxin
Enoxacin	-	The agent decreases the absorbtion of enoxacin
Indinavir	-	Omeprazole decreases the absorptionof indinavir
Itraconazole	-	The proton pump inhibitor decreases the absorptionof imidazole.
Ketaconazole	-	The proton pump inhibitor decreases the absorptionof imidazole.

LIST OF AVAILABLE FORMULATION FOR ESOMEPRAZOLE

The pharmaceutical dosage forms of Esomeprazole are available as tablets in Indian market. The list of manufactures is given below.

FORMULATION FOR ESOMEPRAZOLE

S.No	Brand Name	Generic Name	Dose	Formulation	Company	Country
01	ESOMAC	Esomeprazole	20/40mg	Tablets	Cipla	India
02	ESOREST	Esomeprazole	20/40mg	Tablets	Centaur	India
03	ESTAB	Esomeprazole	20/40mg	Tablets	Indoco	India
04	ESOFAG	Esomeprazole	20/40mg	Tablets	Micro Labs	India
05	ESCZ	Esomeprazole	20/40mg	Tablets	Glen Mark	India
06	IZRA	Esomeprazole	20/40mg	Tablets	Unichem	India
07	NEXIUM	Esomeprazole	20/40mg	Tablets	Ranbaxy	India
08	NEXPRO	Esomeprazole	20/40mg	Tablets	Torrent	India
09	SOMPRAZ	Esomeprazole	20/40mg	Tablets	Solares	India

MATERIALS AND METHODS

5.1 Materials used:

Drugs Used

S.NO	Standards used	Manufacturer
1.	Esomeprazole Magnesium Trihydrate (working standard)	Smilax laborateries ltd, Hyderabad
2.	Rabeprazole Sodium (Internal standard)	Nifty Labs, Hyderabad

Chemicals and reagents used

Chemicals	Manufacturers	Grade
Acetonitrile	Merck	HPLC
Methanol	Lobachemie	HPLC
Sodium Bicarbonate	Qualigens	Excelar
Ethyl acetate	Qualigens	HPLC
Diethyl amine	Qualigens	HPLC
Ammonia	Qualigens	SQ

- Water of HPLC grade from Milli-Q RO system was used.
- Blank plasma (K_2 EDTA) procured from AZIDUS Laboratories.

5.2 Instruments used:

Equipment	Configuration/Model	Make
MS/MS	Micromass Quattro Micro^{TM} API with Masslynx V 4.1	Waters
HPLC	2695- Separation Module 2487- Dual λ Absorbance Detector	Waters
Centrifuge	5810 R	Eppendrof
Column	Gemini C18 / 50 X 4.6 mm I.D 5µ	Phenomex
Microbalance	MX5	Mettler Toledo
Vortex Mixer	Heidolph	Vibramax110
Water purification system	Milli Q	Millipore
pH meter	Picco	Labindia
Micro pipettes	variable	Eppendrof
Deep freezers	-86°C Deep Freezer	Sanyo
Low Volume Evaporator	Turbovap® LV	Caliper life sciences

5.3 Method development

5.3.1 Optimization of the LC-MS/MS system:

MS/MS is a much more specific and selective method of detection than UV. Interference by co-eluting components is not considered as significant problem as with UV detection system although the so-called "matrix effect" needs to be tested, for this reason the whole method development process was focused on mobile phase and extraction process optimization.

The LC- MS/MS instrument was calibrated with polypropylene glycol (PPG) standard in positive and negative ionisation mode. Infusion was done using 500ng/ml of Esomeprazole and Omeprazole separately in Methanol: Water (50:50). Using the spectra of the infused solutions, mass spectrometer parameters were optimized.

Preferred MS/MS detection conditions:

LC-MS/MS detector is at unit resolution in the multiple reaction monitoring (MRM) mode. The transition of the protonated molecular ions m/z 346.31 and m/z 360.21 to the product ions m/z 198.22 and m/z 242.3 for the analyte and internal standard respectively. Electron Spray ionisation (ESI) was used for ion production.

Compound parameters:

The compound parameters namely Declustering potential (DP), Entrance potential (EP), Collision energy (CE), Collision cell entrance potential (CEP), Collision cell exit potential (CXP) were optimized separately for Esomeprazole and Rabeprazole.

Declustering Potential (DP):

The DP parameter controls the potential difference between ground (usually the skimmer) and the orifice plate. It is used to minimize solvent cluster ions, which may attach to sample. The higher the voltage, the greater the amount of fragmentation. If the declustering potential is too high, the sample ion itself may fragment.

Entrance Potential (EP):

The EP parameter controls the potential difference between the voltage on Q_0 and ground. The entrance potential guides and focuses the ions through the high-pressure and Q_0 region.

Collision Energy (CE):

The CE parameter controls the potential difference between $Q_0$and Q_2 (collision cell). It is used only in MS/MS-type scan. This is the amount of energy that the precursor ions receive as they are accelerated into the collision cell, where they collide with gas molecules and fragment. In Q_1 and Q_3 scans the voltage applied to the collision cell is RO_2 (collision cell rod offset)

Collision Cell Entrance Potential (CEP):

The CEP parameter controls the collision cell entrance potential, which is the potential difference between Q_0 and IQ_2. It focuses ions into Q_2 (collision cell). CEP is used in Q and MS/MS-type scans. Note that for Q_3 scans, this voltage is called IQ_2 and by default is in fixed mode.

Collision Cell Exit Potential (CXP):

The CXP parameter controls the potential difference between RO_2 and IQ_3. It is only used in Q_3 and MS/MS-type scans, where it transmits the ions into Q_3. In Q_1 scans, the voltage applied to IQ_3 is accessed through the IQ_3 parameter.

Source parameter

The source parameters namely Curtain gas (CUR), collision activated dissociation (CAD), Ion spray voltage, the heated nebulizer temperature, GS_1, GS_2 were optimized using flow injection analysis (FIA) mode.

CUR (Curtain Gas™):

The CUR parameter controls the gas flow of the curtain Gas interface. The curtain Gas interface is located between the curtain plate and the orifice. It prevents solvent droplets from entering and contaminating the ion optics. The gas flow should be maintained as high as possible without losing sensitivity.

CAD (CAD Gas):

The CAD parameter controls the pressure of collision gas in the collision cell during Q3 and MS/MS-type scans. For Q3 scans, the collision gas helps to focus the ions as they pass through the collision cell; by default the CAD parameter is in fixed mode. For MS/MS-type scans, the collision gas acts as a target to fragment the precursor ions. When the parent ions collide with the collision gas, they can dissociate to fragment ions.

Ion Spray voltage:

The IS parameter controls the voltage applied to the needle that ionizes the sample in the ion source. It depends on the polarity and it affects the spray stability and the sensitivity.

Ion Transfer Voltage:

For the Photo Spray source, the IS parameter controls the voltage that transfers the ions from the primary ionization region towards the curtain plate orifice.

Temperature (TEM):

The TEM parameter controls the temperature of the turbo gas in the Electron Spray® probe or the temperature of the probe in the heated nebulizer (or APCI). It is used to help evaporate the solvent to produce gas phase sample ions.

GS1 (Gas 1):

The GSI parameter controls the nebulizer gas. The nebulizer gas helps generate small droplets of sample flow and affects spray stability and sensitivity.

GS2 (GAS 2):

The GS2 parameter controls the auxiliary, or turbo gas. It is used to help evaporate the spray droplets and prevent solvent from entering the instrument.

5.3.2 Column Selection:

Different reverse phase HPLC column tested for the analysis of Esomeprazole were C-8 (150x4.6, 5µ), C-18 (150x4.6, 5µ), (50x4.6, 5µ) of different makes and of different lots. The different makes are Hypersil column, Symmetry Column, Phenomenox column etc. Out off these columns Phenomex, Gemini C-18 (50x4.6, 5u) was selected because of its better resolution and reproducibility. The retention of the analyte (Esomeprazole) was 0.66 minutes and the retention time of the internal standard (Rabeprazole) was found to be 0.66 minutes.

5.3.3 Mobile phase optimization:

For LC-MS/MS system, a volatile mobile phase is required, hence a buffer of 5mM ammonium acetate was prepared and acetic acid was added until the pH of the solution was adjusted to 5.

Since electro spray ionisation is more efficient the higher the concentration of the organic modifier in the mobile phase, so methanol is used as organic modifier with ammonium acetate and found low sensitivity due to less ionization or suppression.

To enhance the sensitivity, 0.1 % formic acid was used as mobile phase with methanol when using the above mobile phase sensitivity was good but the peak shape was not satisfactory. To get a symmetric peak the concentration of formic acid was 0.1%.

A second mobile phase was developed using Acetonitrile as organic solvent. Since methanol is more polar with better eluting power than Acetonitrile in reversed phase chromatography, But the amount of Acetonitrile in the mobile phase needs to be more to get better retention times. Since the objective of this study was to develop a simple reliable method that would facilitate analysis of in human plasma in a large number of samples over a relatively short period of time (Bioequivalence Studies), in a cost effective manner, the suitable mobile phase is 80 % Acetonitrile as organic modifier with 20% of Aqueous. Aqueous pH maintained at pH-7.0.

5.3.4 Effect of flow rate:

To obtain some information about effect of flow rate the analysis was done at different flow rates with the optimized mobile phase. With an isocratic run using 80% acetonitrile and 20% of milliQ water(pH-7.0) at a flow rate of 0.6ml per minute Esomeprazole eluted at 0.53 min with void volume and this retention time increased to 0.66 minutes when the flow rate was set at 1.0 ml per minute. The flow rate was optimized as 1.0 ml per minute to avoid interference at low concentrations.

5.3.5 Effect of injection volume:

The process of quantification at very low concentrations is a competition between signal and noise. In order to keep the instrument clean the amount of sample reaching into the mass spectrometer should be kept as low as possible. This means that, only very pure solvents should be used for the mobile phase and the amount of extract injected should be as small as possible, i.e. a small injection volume is preferable. A comparison was made between injecting 20 µL and 10 µL of a reconstituted extract of Esomeprazole from a 40 pg/ml plasma sample.

Since in both cases the Esomeprazole peak had a similar signal to noise ratio (S/N = 15) it was decided that the injection volume of extracts should be fixed at 10 µL.

5.3.6 Selection of internal standard:

Different compounds such as Omeprazole, Pantaprazole, and Lansaprazole were tried for internal standard and there was no effective response and finally Rabeprazole was tried as internal standard which showed effective response with good elution and recovery.

5.3.7 Extraction of analytes from plasma:

Since the objective of this study was to develop a simple reliable method that would facilitate analysis of Esomeprazole in human plasma in a large number of samples over a relatively short period of time (bioequivalence studies), in a cost effective manner, it was decided to investigate a number of different extraction procedures.

It was therefore decided to investigate this procedure as a first approximation. To do this, a pool of blank human plasma was obtained and spiked with relevant concentrations of Esomeprazole before extraction.

The ***liquid liquid extraction*** procedure was attempted with some variations.

Aliquots of 80 µl of spiked plasma in a glass tube was added with 50 µl of 1000 ng/ml Rabeprazole and tried with different extraction solvents. The ratio of the solvents and % recovery obtained is shown in table

Solvents	**Volume**	**% recovery of analyte**	
		Analyte	**IS**
n Hexane : n Butanol (80:20)	2ml	35%	75%
Diethyl ether : Dichloromethane(70:30)	2ml	30%	62%
TBME : Ethyl acetate (50:50)	2ml	42%	78%
TBME	2ml	40%	65%
Ethyl acetate	3ml	75%	80%

The ***Solid phase extraction*** procedure was also carried using solid phase extraction cartridges of Strata-X 33µm polymeric reversed phase (30mg/1ml) from phenomenex.

Solid phase extraction involves the following steps:

- The cartridges were first conditioned using 1 mL of 100% Methanol.
- Then Equilibrated with 100% Water
- Aliquot of 80 µl of spiked plasma with 50 µl of 1000 ng/ml Rabeprazole (Internal standard) was added.
- Then washed with 1 mL of 5% Methanol / 5%HCl.

- And finally eluted with 100% Methanol.
- Evaporate it in Low volume evaporator at 40ºC under nitrogen.
- Reconstitute the residue with 250 µL of Acetonitrile: water (50:50)

The Solid phase extraction method is costly and time consuming process, However since the selectivity of MS/MS detection is same as SPE when sample is extracted using LLE method since the objective of this study was to develop a simple reliable method that would facilitate analysis of Esomeprazole in human body fluids in a cost effective manner. So the LLE method using Ethyl acetate was selected because of its greater recovery.

The final optimized extraction procedure was as follows

- Transfer 80 µL of sample into a polypropylene RIA vial.
- Add 50 µL of 1000 ng/mL internal standard solution.
- Vortex for about 60 sec.
- Add 200 µL of 100mM Sodium bicarbonate in Water.
- And add 2 ml of Ethyl acetate
- Vortex for 15 minutes in Vibromax shaker at 2500rpm.
- Centrifuge the tubes at 4000 rpm at 4 ºC for 15 minutes.
- Collect the supernatant liquid in another polypropylene RIA vial.
- Evaporate it in Low volume evaporator at 40ºC under nitrogen.
- Reconstitute the samples with 200 µL of (Water: 0.02 % Diethyl amine (DEA) in Methanol / 3:7 / v: v).
- Vortex it for 1 min.

5.4 Optimized chromatographic conditions:

5.4.1 HPLC Condition:

Waters 2695 Separation Module consisting of,

- HPLC Pump,
- Auto Sampler and
- Degasser.

S.No.	Parameters	Condition
1	Column	Gemini C18 / 50 X 4.6 mm 5µ
2	Mobile phase	Acetonitrile : Milli Q Water (pH 7.0 with Ammonia) / 80:20 / v:v
3	Flow rate	1.0 mL/minute with 1:1 split
4	Auto Sampler Temperature	4˚C
5	Injection volume	10 µL
6	Run Time	2.2min

5.4.2 Mass Spectrometric Conditions:

Quattro Micro™ API (LC-MS/MS)

Sl.No.	Parameters	
1	Mode/Polarity	ESI +ve
2	Capillary (KV)	4
3	Extractor (V)	2
4	Multiplier (V)	650
5	Gas cell pirani (mbar)	$3.66 * 10^{-3}$
6	RF Lens (V)	0.3
7	Desolvation Temperature (°C)	350
8	Source Temperature(°C)	120
9	Desolvation Gas (L/hr)	750
10	Cone Gas	50
11	Calibration Static	2
12	LM 1 Resolution	15.0
13	HM 1 Resolution	15.0
14	IE 1	0.5
15	Entrance	1
16	Collision	20
17	Exit	1
18	LM 2 Resolution	15.0
19	HM 2 Resolution	15.0
20	IE 2	3

Sl.No.	Parameter	Esomeprazole	Rabeprazole (IS)
1	**Parent Ion**	346.31	360.21
2	**Daughter ion**	198.22	242.3
3	**Cone Voltage**	23.0	19.0
4	**Collision Energy**	11.0	10.0

5.5 METHODS:

5.5.1 Preparation of Reagents and Solutions:

Preparation of water pH 7.0:

Transfer 1000 mL of Milli Q Water into a 1000 mL reagent bottle. Adjust the pH to 7.00 ± 0.02 with ammonia solution and filter through 0.2 µm membrane filter, sonicate for 10 minutes.

Preparation of Diluent (0.02 % DEA in Methanol):

Transfer 500 mL of Methanol and 0.1 mL of DEA was added into 500 mL reagent bottle, mix well and sonicate for 10 minutes.

Preparation of Diluent [Water: 0.02 % DEA in Methanol / 30:70 (v/v)]

Transfer 70 mL of 0.02 % DEA in Methanol and 30 mL of Milli Q Water into 100 mL reagent bottle, mix well and sonicate for 10 minutes.

Preparation of Mobile phase [Acetonitrile: Water pH 7.0 / 80:20 (v/v)]

Transfer 300 mL of Type I milliQ Water pH 7.0 into a clean 1000 mL reagent bottle. Add 700 mL of Acetonitile, mix well and sonicate for 10 minutes.

5.5.2 Stock Solution Preparation and Dilution:

Stock Solution Preparation for Esomeprazole:

Weigh approximately 10 mg of Esomeprazole and transfer into a 10mL volumetric flask. Dissolve it in Methanol and make up the volume with the same to produce a solution of 1 mg/mL strength of Esomeprazole.

Stock Dilution for Esomeprazole:

From Esomeprazole Stock Solution prepare Stock Dilutions ranging from 100.0 ng/mL – 20000.0 ng/mL with Methanol containing 0.02 % DEA, as provided in the Table No. 1

Table No. 1 - Stock Dilution for Esomeprazole Calibration Standards

Sl.No.	Initial Solution ID	Initial Solution Conc. (ng/mL)	Volume of Spiking Solution (mL)	Volume of Diluent (mL)	Total Volume of Final Solution (mL)	Final Solution Conc. (ng/mL)	Final Solution I.D.
1	STOCKA	1000000.000	0.080	3.920	4.000	20000.000	STD 8 SS
2	STOCKA	1000000.000	0.068	3.932	4.000	17000.000	STD 7 SS
3	STOCKA	1000000.000	0.040	3.960	4.000	10000.000	STD 6 SS
4	STD 8 SS	20000.000	0.800	3.200	4.000	4000.000	STD 5 SS
5	STD 8 SS	20000.000	0.320	3.680	4.000	1600.000	STD 4 SS
6	STD 8 SS	20000.000	0.100	3.900	4.000	500.000	STD 3 SS
7	STD 5 SS	4000.000	0.200	3.800	4.000	200.000	STD 2 SS
8	STD 5 SS	4000.000	0.100	3.900	4.000	100.000	STD 1 SS

Stock Dilution for Esomeprazole QC samples:

From Esomeprazole Stock Solution prepare Stock Dilutions ranging from 100.0 ng/mL – 16000.0ng/mL with Methanol containing 0.02 % DEA, as provided in the Table No.2

Table No.2 - Stock Dilution for Esomeprazole Quality Control Samples

Sl.No.	Initial Solution ID	Initial Solution Conc. (ng/mL)	Volume of Spiking Solution (mL)	Volume of Diluent (mL)	Total Volume of Final Solution (mL)	Final Solution Conc. (ng/mL)	Final Solution I.D.
1	STOCK B	1000000.000	0.064	3.936	4.000	16000.000	HQC SS
2	STD 8 SS	20000.000	0.440	3.560	4.000	2200.000	MQC SS
3	STD 8 SS	20000.000	0.060	3.940	4.000	300.000	LQC SS
4	STD 8 SS	20000.000	0.020	3.980	4.000	100.000	LLOQ SS

Stock Solution Preparation for Rabeprazole:

Weigh approximately 10 mg of Rabeprazole and transfer into a 10 mL volumetric flask. Dissolve it in Methanol and make up the volume with the same to produce a solution of 1 mg/mL strength of Rabeprazole.

Stock Dilution for Rabeprazole:

From Rabeprazole Stock Solution prepare Working Concentration of the Rabeprazole Solution (1000 ng/mL) as provided in the Table No.3

Table No. 3 – Stock Dilution for Rabeprazole

Sl.No.	Initial Solution ID	Initial Solution Conc. (ng/mL)	Volume of Spiking Solution (mL)	Volume of Diluent (mL)	Total Volume of Final Solution (mL)	Final Solution Conc. (ng/mL)	Final Solution I.D.
1	STOCKA	1000000	0.200	9.800	10.000	20000.000	STOCK B
2	STOCKB	20000	2.500	47.500	50.000	1000.000	STOCK C

5.5.3 Esomeprazole Calibration Standards in Human Plasma:

From Esomeprazole Stock Dilutions prepare Calibration Standards in Human plasma ranging from 5 ng/mL – 1000 ng/mL, as provided in the Table No.4

Table No.4 - Esomeprazole Calibration Standards in Human plasma

Sl.No.	Initial Solution ID	Initial Solution Conc. (ng/mL)	Volume of Spiking Solution (mL)	Volume of Diluent (mL)	Total Volume of Final Solution (mL)	Final Solution Conc. (ng/mL)	Final Solution I.D.
1	STD 8 SS	20000.000	0.200	3.800	4.000	1000.000	STD 8
2	STD 7 SS	17000.000	0.200	3.800	4.000	850.000	STD 7
3	STD 6 SS	10000.000	0.200	3.800	4.000	500.000	STD 6
4	STD 5 SS	4000.000	0.200	3.800	4.000	200.000	STD 5
5	STD 4 SS	1600.000	0.200	3.800	4.000	80.000	STD 4
6	STD 3 SS	500.000	0.200	3.800	4.000	25.000	STD 3
7	STD 2 SS	200.000	0.200	3.800	4.000	10.000	STD 2
8	STD 1 SS	100.000	0.200	3.800	4.000	5.000	STD 1

Esomeprazole Quality Control Samples in Human Plasma:

From Esomeprazole Stock Dilutions prepare Quality Control samples in Human Plasma ranging from 5.0 ng/mL – 800.0 ng/mL, as provided in the Table No.5

Table No.5- Esomeprazole Quality Control samples in Human Plasma

Sl.No.	Initial Solution ID	Initial Solution Conc. (ng/mL)	Volume of Spiking Solution (mL)	Volume of Diluent (mL)	Total Volume of Final Solution (mL)	Final Solution Conc. (ng/mL)	Final Solution I.D.
1	HQC SS	16000.000	0.200	3.800	4.000	800.000	HQC
2	MQC SS	2200.000	0.200	3.800	4.000	110.000	MQC
3	LQC SS	300.000	0.200	3.800	4.000	15.000	LQC
4	LLOQ SS	100.000	0.200	3.800	4.000	5.000	LLOQ

5.6 Extraction Procedure:

Type: Liquid- Liquid Extraction

- Transfer 80 µL of sample into a polypropylene RIA vial.
- Add 50 µL of 1000 ng/mL internal standard solution.
- Vortex for about 60 sec.
- Add 200 µL of 100 mM Sodium bicarbonate in Water.
- And add 2 ml of Ethyl acetate
- Vortex for 15 minutes in Vibromax shaker at 2500rpm.
- Centrifuge the tubes at 4000 rpm at 4 ºC for 15 minutes.
- Collect the supernatant liquid in another polypropylene RIA vial.
- Evaporate it in Low volume evaporator at 40ºC under nitrogen.
- Reconstitute the samples with 200 µL of (Water: 0.02 % Diethyl amine (DEA) in Methanol / 3:7 / v: v).
- Vortex it for 1 min.

Data Processing:

The concentration of unknown is calculated from the following equation by utilizing linear regression with $1/x^2$ as weighting factor

$$Y = mX+b$$

Where, X = Concentration of Esomeprazole

m = Slope of the Calibration Curve

Y = Peak area ratio of Esomeprazole

b=Intercept of the Calibration Curve

RESULT AND DISCUSSION

6.1 Selectivity:

Six lots of Blank Human plasma were chosen to evaluate the specificity of the method. Two replicates from each of those 6 lots of Esomeprazole / Rabeprazole free blank human plasma samples spiked with Esomeprazole at LLOQ concentration and Rabeprazole were then extracted along with two replicates from each of those 6 lots of Esomeprazole / Rabeprazole free blank human plasma samples without spiking Esomeprazole / Rabeprazole.

Average interference response obtained at Esomeprazole Retention Time from two replicates of each lot of Esomeprazole / Rabeprazole free blank human plasma is ≤ 20% of the average Esomeprazole response obtained from the corresponding lot of human plasma containing Esomeprazole at LLOQ concentration along with Rabeprazole. **(Table No: 6)**

Average interference response obtained at Rabeprazole Retention Time from two replicates of each lot of Esomeprazole / IS free blank human plasma is ≤ 10% of the average Rabeprazole response obtained from the corresponding lot of human plasma containing Esomeprazole at LLOQ concentration along with IS. **(Table No: 6)**

6.2 Precision and Accuracy:

The method has been found to be reproducible by performing three Precision and Accuracy (P&A) batches consisting of one intra day batch and two inter day batches. Each analytical run in P&A consists of two replicates of Standards at LLOQ and ULOQ, and one replicate at other levels along with 6 replicates of QC at all levels.

Intra day run is evaluated from the Precision and Accuracy of 6 replicates of QC samples at LLOQ, LQC, MQC and HQC levels from the first three accepted analytical runs individually.

Precision and Accuracy across all QC levels range from 2.0 % to 12.9 % and -19.5 % to 13.1 % in the Intraday batch. **(Table No: 8)**

Inter day run is evaluated from the Precision and Accuracy of 18 replicates of QC samples at LLOQ, LQC, MQC and HQC levels obtained from the first three accepted analytical runs.

Precision and Accuracy across all QC levels range from 5.0 % to 13.9 %, -5.5 % to10.9 % in the Inter day batches. **(Table No: 9)**

Precision and Accuracy for STD at LLOQ and ULOQ across three core P&A batches are 16.6% and -5.8% for LLOQ and 3.2 % and 3.2 % for ULOQ respectively. **(Table No: 7)**

6.3 Linearity and Lower Limit of Quantitation:

In order to establish the linearity of the method, a series of calibration standards ranging from 5.0 ng/ml to 1000.0 ng/ml were prepared. Three linearity curves containing eight non-zero concentrations were analyzed. Ratio of detector response for Esomeprazole to internal standard was used for regression analysis. Each calibration curves was analyzed individually by using least square weighted ($1/X^2$) linear regression. All the curves were forced through zero. Back calculation were made from the calibration curves to determine the concentration of Esomeprazole in each calibration standards.

Calibration curves were found to be linear over the range of 5.0 – 1000.0 ng/ml with the lower limit of quantitation of 5.0 ng/ml. The co-efficient of correlation were found to be better than 0.99 for the all calibration curves analyzed. **(Table No: 7).**

6.4 Ruggedness:

Ruggedness is evaluated from the Precision and Accuracy batch (Analytical Run # 3) processed by different days consisting of six replicates of QC samples at LLOQ, LQC, MQC and HQC levels.

Precision and Accuracy across all QC levels range from 2.2 % to 4.6 % and 3.0 % to 13.1 % in the Ruggedness Batch. **(Table No: 8,9)**

6.5 Freeze Thaw Stability:

Stability of Esomeprazole has been evaluated after three Freeze/Thaw Cycles.

Accuracy of Esomeprazole QC samples at LQC and HQC levels determined from six replicates is found to be ranging from 4.3 % to 12.3 % respectively by comparing against freshly spiked Calibration Standards. **(Table No: 10)**

Esomeprazole is found to be stable in Human plasma after being frozen -20°C or lower and thawed at ambient temperature three times (i.e., undergoing three Freeze Thaw cycles).

6.6 Bench Top Stability:

Stability of Esomeprazole has been evaluated in Bench Top conditions over a period of six hr at ambient temperature.

Accuracy of Esomeprazole QC samples at LQC and HQC levels determined from six replicates is found to be ranging from 3.3 % to 9.1 % respectively by comparing against freshly prepared Calibration Standards. **(Table No: 11).**

Esomeprazole is found to be stable in Human plasma after six hr exposure at ambient temperature at the Bench Top.

6.7 Dilution Integrity:

Integrity of Esomeprazole after dilution has been evaluated by diluting the Dilution QC (twice the concentration of ULOQ) with a Dilution Factor (DF) of 5.

Accuracy of Esomeprazole QC samples determined from six replicates of Dilution QC diluted with a DF of five is found to be 9.8 %.**(Table No: 12)**

Upon diluting Esomeprazole QC samples with DF 5 dilution integrity is observed to be preserved.

6.8 Recovery:

Recovery of Esomeprazole / Rabeprazole has been evaluated in human plasma by comparing six replicates of extracted QC samples at LQC, MQC and HQC levels against unextracted samples containing equivalent concentrations at the end of extraction.

Mean Recovery is found to be 73.4 % for Esomeprazole and 76.2 % for Rabeprazole respectively **(Table No: 13 and 14)**

Extraction efficiency calculated in terms of amount of Esomeprazole and Rabeprazole recovered from human plasma is found to be consistent.

6.9 Matrix Effect:

Matrix effect of Esomeprazole / Rabeprazole has been evaluated in human plasma by comparing six replicates of unextracted QC samples at LQC, MQC and HQC levels against neat solutions containing equivalent concentrations at the end of extraction.

% Mean Matrix effect is found to be -0.5 % for Esomeprazole and -1.7 % for Rabeprazole respectively. **(Table No: 15 and 16).**

6.10 Reinjection Stability:

Reinjection Stability of the samples stored in the auto sampler over a period of 68 hours are evaluated by reinjecting two replicates of extracted STD and QC samples at all levels (except LLOQ).

Accuracy of Esomeprazole QC samples at LQC, MQC and HQC levels determined from two replicates are found to be ranging from -1.2 % to 11.8 % **(Table No: 17)**

Esomeprazole is found to be stable in the Autosampler temperature for 68 hr.

6.11 Post Preparative Stability:

Post preparative Stability of the samples is evaluated by comparing two replicates of extracted STD and QC samples at all levels (Except LLOQ) stored in the autosampler against freshly extracted STD.

Accuracy of Esomeprazole QC samples at LQC,MQC and HQC levels determined from two replicates are found to be ranging from 12.0 % to -10.3 % **(Table No: 18)**.

Esomeprazole is found to be stable in the Autosampler temperature for 20 hr.

6.12 Short Term Stock Solution Stability:

Stability of Esomeprazole has been evaluated in Methanol after exposing the stock solution (Methanol) for six hr at room temperature.

Response ratio obtained from six replicates of aqueous solution at Mid QC levels prepared from Stock Solution of Esomeprazole exposed at room temperature for six hr is compared against the response obtained from six replicates of aqueous solution at Mid QC levels prepared from Stock Solution stored at -20 °C for the same duration.

Response ratio of two stock solutions is within 3.9 % each other. **(Table No: 19)**

Esomeprazole is found to be stable in Methanol over a period of 6 hr at room temperature.

6.13 Long Term Stock Solution Stability:

Stability of Esomeprazole has been evaluated in methanol after storing the stock solution for 23 days at -20 °C.

Response Ratio obtained from six replicates of aqueous solution at Mid QC levels prepared from Stock Solution of Esomeprazole stored for 23 days is compared against the response obtained from six replicates of aqueous solution at Mid QC levels prepared from freshly made Stock Solution of Esomeprazole.

Response Ratio of two stock solutions is within -0.2 % each other. **(Table No: 20).**

Esomeprazole is found to be stable in solvent over a period of 23 days at -20 °C.

6.14 Long Term Frozen Stability:

Stability of Esomeprazole has been evaluated after storing QC samples -70°C for 29 days.

Accuracy of Esomeprazole QC samples at LQC and HQC levels determined from six replicates is found to be ranging from 0.7 % to 3.6 % respectively by comparing against freshly prepared Calibration Standards. **(Table No: 21)**

Esomeprazole is found to be stable in Human plasma over a period of 29 days at -70 °C.

SUMMARY AND CONCLUSION

Esomeprazole is a proton pump inhibitor which reduces gastric acid secretion through inhibition of H^+/K^+-ATPase in gastric parietal cells. By inhibiting the functioning of this enzyme, the drug prevents formation of gastric acid.

The bioanalytical methodology described in this manuscript was specific, sensitive, accurate and precise. The method employed HPLC coupled with electrospray ionization mass spectrometric detection (LC-ESI-MS). The method involved a simple sample preparation by liquid- liquid extraction followed by isocratic chromatographic separations.

A sensitive method that is precise and accurate over a linear assay range of 5.000 – 1000.000 ng/mL has been validated for the determination of Esomeprazole in Human plasma using LC-MS/MS Method.

The LC-ESI-MS method was capable of estimating 5 ng/ml of Esomeprazole accurately in human plasma with high degree of reproducibility.The method can be useful for further BA/BE studies Pharmacokinetic studies.

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