METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS ESTIMATION OF ESOMEPRAZOLE AND LEVOSULPIRIDE BY USING RP-HPLC IN ITS BULK AND PHARMACEUTICAL DOSAGE FORM

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LIST OF ABBREVIATIONS USED

%	:	Percentage
%RSD	:	Percentage Relative Standard Deviation
λ	:	Lambda
α	:	Selectivity factor
°C	:	Degree Celsius
µ	:	Minute
µg/ml	:	Microgram per Millilitre
µl	:	Micro litre
A.R	:	Analytical Reagent
API′S	:	Active Pharmaceutical ingredients
As	:	Peak Asymmetry
AUC	:	Area under curve
B.P	:	British Pharmacopeia
CAS	:	Chemical Abstract service
CFR	:	Code Federal regulations
cm	:	centi meter
DMSO	:	Dimethyl sulfoxide
EC_{50}	:	Half maximal effective concentration
ELSD	:	Evaporate light scattering detector
GERD	:	Gastroesophageal reflux disease
Gms	:	Grams
I.D	:	Internal diameter
IC_{50}	:	Half maximal inhibitory concentration
ICH	:	International conference on Harmonization
IP	:	Indian pharmacopeia
IUPAC	:	International union of pure and applied chemistry
K′	:	Capacity factor
LOD	:	Limit of Detection
LOQ	:	Limit of Quantification
mg/tab	:	Milligram per tablet
min	:	Minute

ml	:	Millilitre
ml/min	:	Millilitre/Minute
mM	:	Milli Mole
MS	:	mass spectroscopy
N	:	column effecient
nm	:	Nanometre
NSAIDs	:	Non steroidal anti- inflammatory drugs
ODS	:	Octadecyl Silica (C_{18} Column)
OPA	:	Ortho Phosphoric Acid
PDA	:	photo diode array
pH	:	Negative Logarithm of Hydrogen Ion
PhoP	:	Transcriptional regulatory protein
Psi	:	pounds for square inch
RI	:	Refractive index
RP-HPLC	:	Reverse Phase-High Performance Liquid Chromatography
Rpm	:	Rotations per Minute
R_s	:	Resolution
RSD	:	Relative Standard Deviation
Rt or t_R	:	Retention time
S.D	:	Standard Deviation
S.E	:	Standard Error
USP	:	United States Pharmacopoeia
UV-VIS	:	Ultraviolet –Visible
v/v	:	Volume/Volume

ABSTRACT

A simple, Accurate, precise method was developed for the simultaneous estimation of the esomeprazole and levosulpiride in Tablet dosage form. Chromatogram was run through ODS (150mm 4.6mm, 5µ). Mobile phase containing Buffer and Acetonitrie in the ratio of 32;68A was pumped through column at a flow rate of 1ml/min. Buffer used in this method was 0.01N KH_2PO_4 pH 5.4 buffer. Temperature was maintained at 30°C. Optimized wavelength for Esomeprazole and Levosulpiride was 290nm. Retention time of Esomeprazole and Levosulpiride were found to be 2.2min and 4.0min. %RSD of the Esomeprazole and Levosulpiride were and found to be 0.97 and 0.50 respectively. %Recover was Obtained as 100.08% and 101.16% for Esomeprazole and Levosulpiride respectively. LOD, LOQ values are obtained from regression equations of Esomeprazole and Levosulpiride were 0.10ppm, 0.34ppm and 1.04ppm, 0.29ppm respectively. Regression equation of Esomeprazole is y = 10568.x + 307.3, and of Levosulpiride is y = 11649.x + 1207.

Key Words: Esomeprazole, Levosulpiride, RP-HPLC.

1. INTRODUCTION

A drug includes all medicines intended for internal or external use for or in the diagnosis, treatment, mitigation or prevention of disease or disorder in human beings or animals, and manufactured exclusively in accordance with the formulae mentioned in authoritative books. [1]

Pharmaceutical analysis is a branch of chemistry involving a process of identification, determination, quantification, purification and separation of components in a mixture or determination of chemical structure of compounds. There are two main types of analysis – Qualitative and Quantitative analysis.

Qualitative analysis is performed to establish composition of a substance. It is done to determine the presence of a compound or substance in a given sample or not. The various qualitative tests are detection of evolved gas, limit tests, color change reactions, determination of melting point and boiling point, mass spectroscopy, determination of nuclear half life etc.

Quantitative analysis techniques are mainly used to determine the amount or concentration of analyte in a sample and expressed as a numerical value in appropriate units. These techniques are based on suitable chemical reaction and either measuring the amount of reagent added to complete the reaction or measuring the amount of reaction product obtained the characteristic movement of a substance through a defined medium under controlled conditions, electrical measurement or measurement of spectroscopic properties of the compound.[2]

1.1 High Performance Liquid Chromatography

Chromatography is the method of separation that finds applications in all branches of science. It was first invented by Russian Botanist Mikhail Twsett. This technique was used to separate various plant pigments like chlorophylls and xanthophylls by passing solutions of these compounds through a glass column packed with finely divided calcium carbonate. The separated species appear as colored bands on the column hence the name of the process (Greek chroma meaning "color" and graphing meaning "writing")[3]

Chromatography is defined as a non- destructive procedure for resolving multi-component mixture of trace, minor, or major constituents into its individual fractions. In chromatography, the sample is dissolved in the mobile phase which may be a gas, liquid, or a supercritical fluid. The principle involved in HPLC is that when a mixture containing different compounds is introduced into the mobile phase and allowed to flow over a stationary phase, the individual compounds travel at different speeds and get separated based on the relative affinities to the stationary phase and the mobile phase. The compounds are separated based on the polarity of the stationary phase and the mobile phase.

Chromatography is of various types based on the physical state of the stationary and the mobile phase:

1. **Solid - Liquid type:** The stationary phase is a solid and the mobile phase is a liquid. Ex: Thin layer chromatography, High performance liquid chromatography (HPLC).
2. **Liquid - Liquid type:** The stationary phase is a liquid and the mobile phase is also a liquid. Ex: Paper Chromatography, HPLC.

3. **Liquid - Gas Type:** The stationary phase is a liquid and the mobile phase is a gas. Ex: Gas chromatography.

High Performance Liquid Chromatography is the most widely used of all the analytical separation techniques. The reasons for its popularity are its sensitivity, ready adaptability to quantitative determination, suitability for non- volatile and thermally fragile species, wide applicability to variety of substances such as amino acids, carbohydrates, nucleic acids, proteins, hydrocarbons, terpenoids, pesticides, steroids, metal-organic species and inorganic species. As high pressures (around 3000 psi) are used for the separation of the analytes down the column, it is often termed as High Pressure Liquid Chromatography.(4

1.2 Types Of HPLC *(5)*

HPLC is classified into various types

a) Based on polarity of stationary and mobile phase

- Normal Phase Chromatography
- Reverse Phase Chromatography

b) Based on the principle of separation

- Adsorption Chromatography
- Partition Chromatography
- Ion Pair Chromatography
- Size Exclusion Chromatography
- Chiral Phase Chromatography

C) **Based on elution technique**

- Isocratic Elution
- Gradient Elution

D) **Based on scale of operation**

- Analytical HPLC
- Preparative HPLC

a) *Based on the polarity of the stationary phase and the mobile phase, it is of two types:*

Normal Phase (NP) HPLC

In this type, the stationary phase is polar and the mobile phase is non-polar, polar compounds are retained for a longer periods because of more affinity towards the stationary phase, hence non-polar compounds travel faster and are eluted first.

Reverse Phase (RP) HPLC

In this type, the stationary phase is non-polar and the mobile phase is polar, non-polar compounds are retained for longer periods as they have more affinity towards the stationary phase. Hence, polar compounds travel faster and are eluted first.

Table 1.1 Types of HPLC Based on Polarity of Stationary Phase and Mobile Phase.

Types	Normal Phase	Reverse Phase
Stationary phase	Polar	Non polar
Mobile phase	Non polar	Polar
Compound eluted first	Non polar	Polar
Compound eluted last	Polar	Non polar

b) *Based on the principle of separation, chromatography is divided into various types,*

Adsorption chromatography

It is one of the oldest types of chromatography. The principle involved is adsorption. Adsorption is a surface phenomenon in which the separation mechanism depends on the difference in the polarity of various drug molecules. The more polar a molecule, the more strongly it will be adsorbed by a polar stationary phase. Similarly the more non-polar a molecule, the more strongly it will be adsorbed by a non-polar stationary phase.(6)

During a surface adsorption chromatography process, a competition for stationary phase adsorption sites exists between the materials to be separated and the mobile phase. In a mixture, molecules of low polarity spend proportionally more time in the mobile phase than those molecules that are highly polar, which are retained for a longer period of time. Therefore the components of a mixture are eluted in order of increasing polarity. (7)

Partition Chromatography

The principle involved is partition in which the solute molecules distribute themselves between the mobile and the stationary phases. In partition chromatography the stationary phase is a non-volatile liquid which is held as a thin layer (or film) on the surface of an inert solid. The mixture to be separated is carried by a gas or a liquid as the mobile phase. With the use of liquid as mobile phases, there is a tendency for the stationary liquid phase to be removed or dissolved. Therefore, the stationary liquid phase has to be chemically bonded to the solid bonding support such as silica gel, cellulose powder, or kieselguhr (hydrated silica). The solute molecules in the mobile phase move through the system at rates determined by their relative solubility's in the stationary and mobile phases.(8)

Ion Exchange Chromatography

Ion Exchange Chromatography is based on the reversible interaction between a charged protein and an oppositely charged chromatography medium. Biomolecules such as proteins, peptides, nucleic acids with even small differences in net surface charge can be separated, and very high resolution is obtained by choosing the optimal ion exchange and separation conditions. The net surface charge of a protein varies according to the surrounding pH. Typically, when the pH is above its isoelectric point, a protein will bind to a positively charged anion exchanger. Below its isoelectric point, a protein will bind to a negatively charged cat ion exchangers.[9]

Anion and cation exchangers are classified as strong or weak, depending on how much the ionization state of the functional groups vary with pH. A strong ion exchange has the same charge density on its surface over a broad pH range, whereas the charge density of a weak ion exchanger changes with pH. The selectivity and the capacity of weak ion exchangers are different at different pH values.[10]

Size Exclusion Chromatography

This process is also known as Gel Permeation Chromatography. In this method the mixture of compounds with various molecular sizes are separated according to size or molecular weight. The stationary phase consists of a porous cross-linked polymeric gel. The pores of the gel normally small and exclude the larger solute molecules, but allows smaller molecules to enter the gel, causing them to flow through a larger volume. This causes the larger molecules to pass through the column at a faster rate than the smaller ones. The components of a mixture therefore elute in order of decreasing size or molecular weight.[11]

Size Exclusion Chromatography is used extensively in the biochemical industry to remove small molecules and inorganic salts from valuable higher molecular weight products such as peptides, proteins and enzymes.

Chiral Phase Chromatography

In this type of chromatography, optical isomers are separated using chiral stationary phases. Two approaches for chiral separation are available, indirect, which utilizes derivatizing agents, and direct, which uses chiral stationary phases or chiral mobile phase additives. In the indirect method, a racemic mixture is made to react with a chiral reagent to form a pair of diastereomers and then chromatographed using a chiral column. Because diastereomers possess different physiochemical properties, they can be separated in a chiral environment.

Direct separation of enantiomers on and a chiral column using a chiral mobile phase additive is applied only in HPLC. In this method, enantiomeric separation is accomplished by the formation of a pair of transient diastereomeric complexes between racemic analyte and the chiral mobile phase additive. Chiral discrimination is due to differences in the stabilities of the diastereomeric complexes, salvation in the mobile phase, and/or binding of the complexes to the solid support.[12]

c) *Based on elution technique, chromatographic methods are of two types,*

Isocratic Elution

In this technique, the composition of the mobile phase is maintained as constant throughout the process of separation. All the compounds in the sample are eluted in a reasonable amount of time, by changing the ratio of polar to non-polar compounds in the mobile phase during the sample run while maintaining peak resolution.

Gradient Elution

A steady change of the mobile phase composition during the chromatographic run is called gradient elution. This is widely used technique when a sample contains components of a wide range of polarities. For a reverse phase gradient, the solvent starts out relatively polar and slowly becomes more non-polar. The gradient elution offers the most complete separation of the peaks, without taking much time. A sample containing compounds of a wide range of polarities can be separated by a gradient elution in a shorter time period without a loss of resolution in the earlier peaks or excessive broadening of later peaks.[13]

Gradient elution also increases quasi-efficiency of the column. In the isocratic elution, the longer a component is retained, the wider its peak. In gradient elution, the tail of the peak is always under the influence of the stronger mobile phase when compared to the peak front. Thus, molecules on the tail of the chromatographic peak will move faster. This will tend to compress zone and narrow the resultant peak. [14]

d) *Based on the scale of operation, chromatography is of two types,*

Analytical HPLC

In Analytical HPLC, quantitative and qualitative determination of a compound is done. It is the most widely used technique. In this method, the sample amount applied to the column is typically in the μg range or lower quantities. The mass ratio of compound to the stationary phase on the column is less than 1: 100000. Under these conditions good separations with sharp and symmetrical peaks are achieved. Recovery of samples is not possible as small quantities of the sample are used. [15]

Preparative HPLC

In Preparative HPLC, isolation and purification of a product is done. It is an expensive technique, when compared to the traditional purification techniques like distillation, crystallization or extraction.

Preparative HPLC is used for the isolation and purification of valuable products in the chemical and pharmaceutical industry as well as in biotechnology and biochemistry. It starts in the µg range for isolation of enzymes in biotechnology (micro purification). For identification and structure elucidation of unknown compounds in synthesis or natural product chemistry it is necessary to obtain pure compounds in amounts ranging from one to a few milligrams. Larger amounts, in gram quantity, are necessary for standards, reference compounds and compounds for toxicological and pharmacological testing. Industrial scale or production scale preparative HPLC, that is, kg quantities of compound, is often done nowadays for valuable pharmaceutical products.

1.3 Instrumentation of HPLC

The main components of HPLC are as given below and are schematicall represented in Fig 1.1

1. Solvent Reservoir
2. Solvent Delivery System (Pump)
3. Injection Port/ Auto sampler
4. Column
5. Detector
6. Data Acquisition system

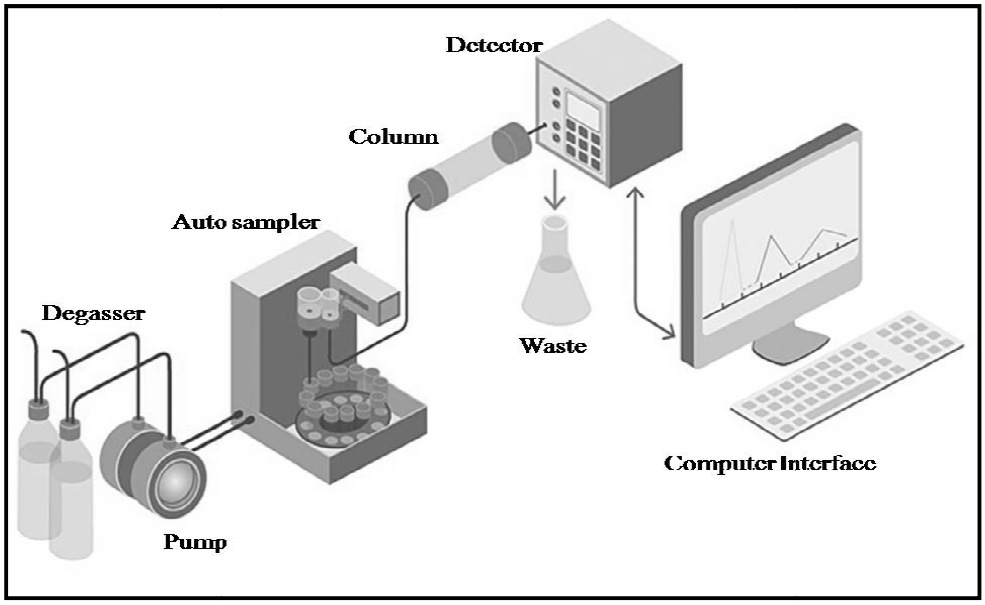

Fig. 1.1 Schematic diagram of instrumentation of HPLC [16]

1.3.1 Solvent Reservoir

Solvent Reservoirs are used to store mobile phase. Scott Duran bottles are commonly used as solvent reservoirs. The solvent reservoir must be made of inert material such as glass and must be smooth so as to avoid growth of microorganisms on its walls. It may be transparent or can be amber colored. A graduated bottle gives a rough estimate of mobile-phase volume in the bottle. Solvent reservoirs are placed above HPLC system (at higher level) in a tray. They should never be kept directly above the system as any spillage of solvent on the system may damage electronic parts of HPLC.

1.3.2 Solvent Delivery System (Pump)

Pumps are an important component of the HPLC system. It delivers a constant flow of mobile phase such that the separation of components of mixture takes place in a reasonable time. There are different types of pumps:

a) Reciprocating Pumps

b) Displacement Pumps

c) Pneumatic Pumps

In reciprocating pumps, a motor driven reciprocating piston controls the flow of mobile phase with the help of two ball check valves that opens and closes with the piston movement. The flow is thus not continuous and as damping of flow is necessary, it is accomplished using pulse dampers which are a long coiled capillary tube. Reciprocating pumps are most widely used in HPLC systems.

Displacement pumps, also known as syringe pump is composed of a one directional motor driven plunger that pushes the mobile phase present in a syringe like chamber. The volume of displacement pumps is limited. A constant flow rate is usually obtained with syringe like pumps.

Pneumatic pumps are the simplest where the mobile phase is pushed out of the mobile phase container by the pressure of a pressurized gas. The flow is dependent on the back pressure of the column and usually the flow is limited to pressures below 2000 psi. [17]

1.3.3 Injection Port or Auto sampler [18]

The Sample Injector enables small liquid samples to be injected into the HPLC system without the sample passing through the pump. The function of the injector is to place an accurate volume of the sample into the high-pressure flow of the mobile phase so that the sample enters the column as a homogeneous, low-volume plug. It is of two types; manual injector or an auto injector. Injection is done through specially designed 6-port rotary injection valve or a Rheodyne valve as shown in Fig

1.2. The sample is introduced at atmospheric pressure by a syringe into a constant volume loop. In the LOAD position the loop is not in the path of the mobile phase. By rotating to the INJECT position the sample in the loop is moved by the mobile phase stream into the column. It is important to allow some sample to flow into waste from loop so as to ensure there are no air bubbles in the loop and previously used sample is completely washed out to prevent previous sample effects. The sample loading can be varied by part filling of the loop or by changing the loop volume.

Automatic injection improves laboratory productivity and eliminates personal errors. Present day advanced HPLC systems are equipped with an auto injector along with an auto sampler. The software programs help filling of the loop and delivery of the sample to the column. The computer controls the sequence of samples for injection from vials kept in numbered positions of the auto sampler. It is important to adopt precautions to ensure consistency of results. [19]

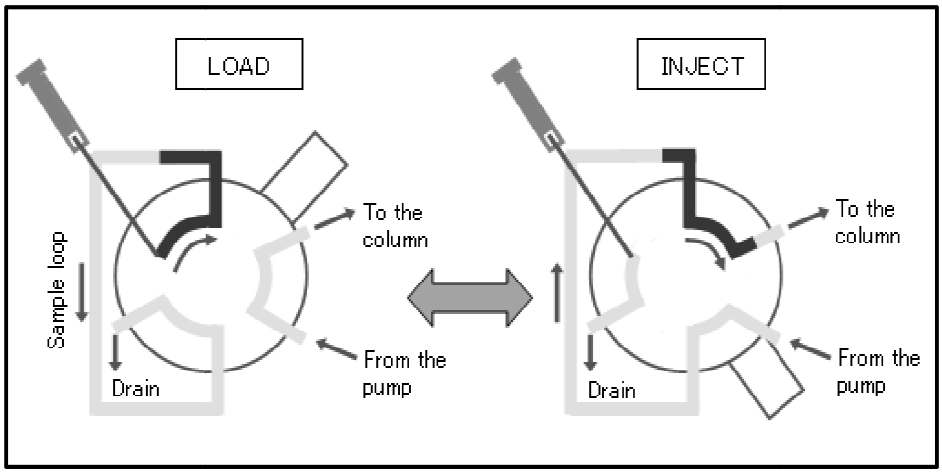

Fig. 1.2 Flow Path of a Manual Injector. [20]

1.3.4 HPLC Column

The HPLC column is referred to as the heart of the process. The stationary phase of the column is used in separating the individual fractions of a sample mixture

by using various physical and chemical parameters. Columns for analytical HPLC are of various sizes ranging from 10 - 25 mm in length and 2.6 – 4.6 mm internal diameter (i.d). Columns for preparative HPLC are in size range of 50 – 250 mm long and i.d greater than 4.6 mm. The columns are generally constructed of stainless steel SS-316 grade to cope with high back pressure (caused by the small particles of the stationary phase) and are glass lined to prevent metal catalysis of solvent- solute reactions at high column pressures (due to force of pumping of the mobile phase through the columns). Columns made of glass are used for the separation of Biomolecules and columns made of PEEK polymer are biocompatible and chemically inert to most solvents. [21]

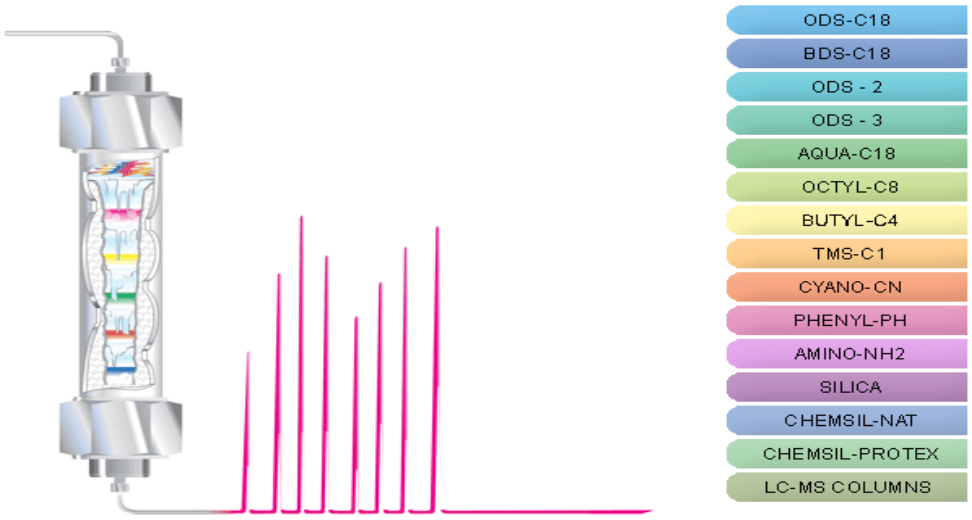

Fig No. 1.3 HPLC Column

Stationary phase

Separation of pharmaceuticals is usually achieved by partition of compounds in the test solution between the mobile and the stationary phases. HPLC systems consisting of polar stationary phases and non-polar mobile phases are described as normal-phase chromatography; those with non-polar stationary phases and polar mobile phases are called reversed-phase chromatography.

There are many types of stationary phases used in HPLC including:

- Unmodified silica, alumina, or porous graphite, used in normal-phase chromatography, where separation is based on differences in adsorption;

- A variety of chemically modified supports prepared from polymers, silica, or porous graphite, used in reverse-phase HPLC, where separation is based principally on partition of the molecules between the mobile phase and the stationary phase;

- Resins or polymers with acid or basic groups, used in ion-exchange chromatography, where separation is based on competition between the ions to be separated and those in the mobile phase;

- Porous silica or polymers, used in size-exclusion chromatography, where separation is based on the relative molecular mass of the molecules.

Most separations are based on partition mechanisms using chemically modified silica as the stationary phase and polar solvents as the mobile phase (reverse-phase HPLC). The surface of the support, e.g. the silanol groups of silica, is reacted with various silane reagents to produce covalently bonded silyl derivatives covering a varying number of active sites on the surface of the support. The nature of the bonded phase is an important parameter for determining the separation properties of the chromatographic system.

For the separation of enantiomers, special chemically modified stationary phases (chiral chromatography) are available, e.g. cyclodextrins, albumins, etc.

Generally, silica-based reverse-phase columns are generally considered to be stable in mobile phases with an apparent pH in the range 2.0 - 8.0, but the column

manufacturer's instructions should be consulted before using the column. Columns containing particles of polymeric materials such as styrene divinyl benzene copolymer are stable over a wider pH range.

For analytical separations the particle size of the most commonly used stationary phases varies between 3 μm and 10 μm. The particles may be spherical or irregular, of different porosities and specific surface area. In the case of reversed-phase, the extent of bonding of the stationary phase is expressed as the carbon-loading. Furthermore, stationary phases may be "end-capped", i.e. the number of residual silanol groups is reduced by methylation. These parameters contribute to the chromatographic behavior of a particular stationary phase. Tailing of peaks, particularly for basic substances, can occur when residual silanol groups are present.

1.3.5 Detector [22]

A detector in the HPLC system measures the compounds after their separation from the column. The detector selected should be capable of responding to changes in the concentration of all the components in the sample with adequate sensitivity even to measure trace amounts. Basically there are two types of detectors: bulk property and solute property detectors. The bulk property detectors, function on some bulk property of the eluent such as refractive index (RI) and is not suitable for gradient elution and are usually less sensitive than solute property detectors. Solute property detectors perform by measuring a physical or chemical property that is specific to the solute. Detectors should have high linear dynamic range and should give faster response. The various detectors used and their applications are given in Table 1.2.

Table 1.2 Commonly used detectors and their application. [23]

Detector	Analyte detected	Solvent requirements	Uses
UV-Visible	Any compounds with chromophore	Non UV absorbing solvents	Wavelength at which maximum absorption occurs can be selected. Has a high degree of selectivity and is useful for many applications.
Photo diode array (PDA) detector	Any compound with chromophore	Non UV absorbing solvents	Detects an entire spectrum simultaneously. Useful for analysis of related substances and impurities.
Fluorescence	Fluorescent compounds	Non UV absorbing solvents	Highly selective and sensitive. Wavelength at which fluorescence occurs is selected. Often used to analyze derivatized samples after treatment with DANSYL chloride to form fluorescent compounds.
Refractive index (RI)	Compound with a different RI to that of the mobile phase	Cannot run mobile phase gradients	Virtually it is a universal detector but has limited sensitivity. Used for the detection of sugars.
Conduct-ivity	Charged or polar compounds	Mobile phase must be conducting	Excellent detector for ion exchange methods.
Electro-chemical	Readily oxidized or reduced compounds	Mobile phase must be conducting	Very sensitive and selective. Used for detection of Biological compounds.
Evaporative light scattering detector (ELSD)	All compounds are detected virtually	Volatile solvents and volatile buffers must be used.	Universal detector, Highly sensitive but not selective. But can be use for gradient analysis. Used for the detection of lipids, sugar and high molecular weight analytes.
Optical rotation detector	Optically active compounds	Mobile phase should be an asymmetric environment	Detects R and L type of isomers

1.3.6 Data Collection devices

Signals from the detector may be collected on chart recorders or electronic integrators that vary in complexity and in their ability to process, store and reprocess chromatographic data. The data storage capacity of these devices is usually limited.

Modern data stations are computer based and have a large storage capacity to collect process and store data for possible subsequent reprocessing. Analytical reports can often be customized to the needs of the analyst.

Integration of peak areas and the setting of threshold levels are not normally problematic in an assay since the peak of the substance to be analyzed should be free of interference. However, in a test for impurities, the selection of the peak area integrator parameters becomes very important, particularly when baseline separations are not always attainable. If baseline separations cannot be obtained, valley-to-valley integration should be employed.

1.4 METHOD DEVELOPMENT AND VALIDATION

Introduction to Method Development

The number of drugs introduced into the market is increasing every year. These drugs may be either new entities or partial structural modification of the existing one. Often a time lag exists from the date of introduction of a drug into the market to the date of its inclusion in pharmacopoeias. This happens because of the possible uncertainties in the continuous and wider usage of these drugs, reports of new toxicities (resulting in their withdrawal from the market), development of patient resistance and introduction of better drugs by competitors. Under these conditions, standards and analytical procedures for these drugs may not be available in the

pharmacopoeias. It becomes necessary, therefore to develop newer analytical methods for such drugs. [24]

Analytical methods should be used within good manufacturing practice (GMP) and good laboratory practice (GLP) environments, and must be developed using the protocols set out in the International Conference on Harmonization guidelines. [25,26]

Method development is a continuous process that progresses in parallel with the evolution of the drug product. The goal and purpose of the method should reflect the phase of drug development. During early drug development, the methods may focus on API behavior. They should be suitable to support preclinical safety evaluations, pre-formulation studies, and prototype product stability studies. As drug development progresses, the analytical methods are refined and expanded, based on increased API and drug product knowledge. The methods should be robust and uncomplicated, while still meeting the appropriate regulatory guidelines. Scouting experiments are frequently performed during method development to establish the performance limits of the method, prior to formal validation experiments. These may include forced degradation studies, which are an integral part of development of a stability-indicating method. API is typically subjected to degradation by acid, base, peroxide, heat, and light. This allows for a determination of the capability of the method to separate and quantify degradation products, while providing insight into the main mechanisms of degradation. Once a stability-indicating method is in place, the formulated drug product can then be subjected to heat and light in order to evaluate potential degradation of the API in the presence of formulation excipients.[27]

Need for the Development of a New Method

Several reasons are available for the development of a new method of analysis.[28]

- There may not be a suitable method for a particular analyte in the specific sample matrix.
- Existing methods may be too erroneous, artefact and/or contamination prone, or they may be unreliable (having poor accuracy or precision).
- Existing methods may be too expensive, time consuming, or energy intensive, or they may not be easily automated.
- Existing methods may not provide adequate sensitivity or analyte selectivity in samples of interest.
- Newer instrumentation and techniques may have evolved that provide opportunities for improved methods, including analyte identification or detection limits, greater accuracy or precision, or better return on investment.
- There may be a need for alternative method to confirm, for legal or scientific reasons, analytical data originally obtained by existing methods.

Method Development Using HPLC

In method development, an attempt to select the best chromatographic conditions like the best column, the best mobile phase, the detection wavelength etc. to be used for routine analysis of any drug is done. For the method development by HPLC method some information about the sample is very essential i.e. number of

components present in the sample, pKa values of different components, UV-Visible Spectra of each analyte, solubility in different solvents, concentration range of each component, nature of sample etc. Prior to method development there must be some technical information i.e. chromatography method selection according to the sample properties, the sample when analyzed with HPLC, the condition where all compounds elute in a reasonable time, optimization of HPLC method with regard to analysis time, resolution, selectivity and sensitivity. [29]

Analyte Standard Characterization

All the information about the analyte is gathered regarding the structure, physical and chemical properties, toxicity, purity, hygroscopicity, solubility and stability. The availability of the 100% pure standard analyte is determined along with its storage and disposal information. If multiple components are to be analyzed in a sample matrix, the number of components to be analyzed is noted and the availability of the standard for each component is checked.[30]

Literature Search and Prior Methodology

The available literature is searched for all types of information related to the analyte. Availability of information regarding the synthesis, physical and chemical properties, solubility or relevant analytical methods is determined. Books, periodicals, regulatory agency compendia, such as IP, USP/NF, and BP etc. should be referred. Chemical Abstracts Service (CAS) automated/ computerized literature searches also should be used. Information pertaining to prior analytical work on the analyte has to be determined within the company and compile the available data, results, reports, memos and publications.

Choosing a Suitable Method

Using the available literature and previous methodology, the methods are adapted and modified. Sample preparation and instrument conditions are adopted to make use of the latest methods and instrumentation.

If no previous methods exist for the analyte in the literature, work from analogy to investigate compounds that are similar in structure and properties. Usually a compound with analytical method exists that is similar to the analyte of interest.

1.5 Optimization

a) Choice of method

The most commonly used chromatographic methods are normal phase chromatography, reverse phase chromatography, reverse phase ion-pair chromatography and ion-exchange chromatography. In the selection of suitable chromatographic method for organic compounds, first reversed phase should be tried, if not successful, normal phase should be tried, then reverse phase ion-pair chromatography should be tried, ion-exchange chromatography at the end.

b) Choice of Mobile Phase

In reversed phase chromatography the selection of mobile phase is very important for the analysis of the drug. We can use Acetonitrile frequently as it is suitable for the entire UV range, methanol and Isopropanol are not suitable below wavelength of 210 nm, acetic acid is suitable above a wavelength of 240 nm, for the preparation of buffers, both K_2HPO_4 and KH_2PO_4 can be used in entire UV range, freshly distilled THF is suitable for HPLC above a wave length of 240 nm, TEA is

suitable above 240 nm, ammonium acetate can be used above 215 nm, EDTA can be used in entire UV range, sodium phosphate is suitable above 210 nm.

The organic phase concentration required for the mobile phase can be estimated by gradient elution method. For aqueous sample mixtures, the best way to start is with gradient reversed phase chromatography. Gradient can be started with 10% organic phase in the mobile phase and organic phase concentration (Methanol & Acetonitrile) can be increased up to 100% within 20 - 60 min. Separation can then be optimized by changing the initial mobile phase composition according to the chromatogram obtained from preliminary run. The initial mobile phase composition can be estimated on the basis of where the compounds of interest were eluted, at what mobile composition.

Changing the polarity of the mobile phase can alter the elution of drug molecules. The elution strength of a mobile phase depends upon its polarity, the stronger the polarity, higher is the elution. Ionic samples (acidic or basic) can be separated, if they are present in un-dissociated form. Dissociation of ionic samples may be suppressed by proper selection of pH.

The pH of the mobile phase has to be selected in such a way that the compounds are not ionized. If the retention times are too short, the decrease of the organic phase concentration in the mobile phase can be in steps of 5%. If the retention times are too long, an increase in 5% steps of the organic phase concentrations is needed. When separating acid or bases, buffered mobile phase is required to maintain consistency in retention time and selectivity. Buffered salts reduce peak tailing for basic compounds by effectively masking silanol groups and also reduce potential ion-exchange interactions with a protonated silanol groups. As potassium is a stronger

counter ion than sodium, it provides improved results compared to sodium (Na^+). Potassium phosphate is used for preparation of buffers of various pH. If band tailing is observed for basic amphoteric compounds few drops of diluted triethylamine or ammonium acetate is added, for acidic or amphoteric compounds, few drops of diluted triethylamine or ammonium acetate is tried. For neutral compounds, the aqueous eluent used in method development is water, for weak to medium acidic compounds, 100 M H_3PO_4 buffer of pH 2.3, for weak to medium basic or acidic compounds in ionized form 100 mM H_3PO_4 buffer of pH 4.0, 50 mM H_3PO_4 buffer of pH 7.5 are used. Unknown sample should be analyzed first with water, then with an acidic and a neutral buffer.

During mixing of the solvents in the preparation of mobile phase, the difference in partial pressure of the individual solvent at a ratio they are to be combined should be considered. The solvent system must be miscible with the previously used mobile phase, if not intermediate solvent may be used, the one that is miscible with previous mobile phase and new mobile phase.

c) Choice of Column

Columns being the heart of HPLC for optimum separation, Stable, high performance column with good selectivity, efficiency is essential requirement for rugged and reproducible method. These characteristics are dependent on the columns manufacturer's production of good quality columns and packing materials.

Column length

- ✓ Longer columns are chosen for increased resolution.

- ✓ Shorter columns are chosen for shorter analysis time, lower back pressure, fast equilibration and less solvent consumption. Column internal diameter

- ✓ Wider diameter columns are chosen for greater sample loading.

- ✓ Narrow columns are chosen for more sensitivity and reduced mobile phase consumption.

Particle shape

- ✓ Columns with spherical particle shapes are preferred when lower back pressure column stability and greater efficiency is required.

- ✓ Columns with irregular particle shapes are preferred when large surface area and high capacity is required.

Particle size

- ✓ Columns with small particle size of 3 - 4 µg are preferred for complex mixtures with similar components. Combination of a short column (10 - 50 mm) with small particle size is used for fast, high resolution separations.

- ✓ Columns with larger particle size of 5 – 10 µg are preferred for structurally different compounds.

- ✓ Columns with large particle of 15 – 20 µg are used for preparative separations.

Surface area

- ✓ Columns with high surface area packing are selected for more capacity, greater resolution and longer retention.

- ✓ Columns with low surface area packing are selected for quicker equilibration time.

Carbon load

- ✓ Columns with high carbon load are chosen for greater column capacities and resolution.

- ✓ Columns with low carbon load for faster analysis time.

End capping

- ✓ Columns with end capped packing are selected to eliminate unpredictable secondary interactions with base material

- ✓ Columns with non-end capped packing are selected for selectivity differences for polar compounds by controlling secondary interactions.

a) Choice of Detector

Detectors are eyes of the liquid chromatography system and measure the compounds after their separation on the column. Selected detector should be capable of responding to change in concentrations of all the components in the sample with adequate sensitivity even to measure trace substances. The detectors must have certain characteristics i.e. high sensitivity, higher linear dynamic range, application to most of the solutes, does not contribute to band broadening, non-destructive, faster response.

Further Optimization

After the selection of a suitable method, mobile phase, column and detector, further optimization can be done to obtain a well developed method.

For shorter analysis time

- Change to isocratic method. The suitable mobile phase composition is estimated from the gradient run.

- Use of shorter column, if proper resolution is obtained.

For better resolution

- Use of longer column.
- Use of stationary phase with smaller particles (3 – 4 µg).

For better selectivity and sensitivity

- Other stationary phases e.g. phenyl, CN etc.
- pH control with ion-forming compounds
- Use of methanol or THF instead of Acetonitrile.
- Detection at the absorption maximum of the substance
- All factors which leads to narrower and higher peaks as gradient elution, smaller particle, and micro bore columns. [31]

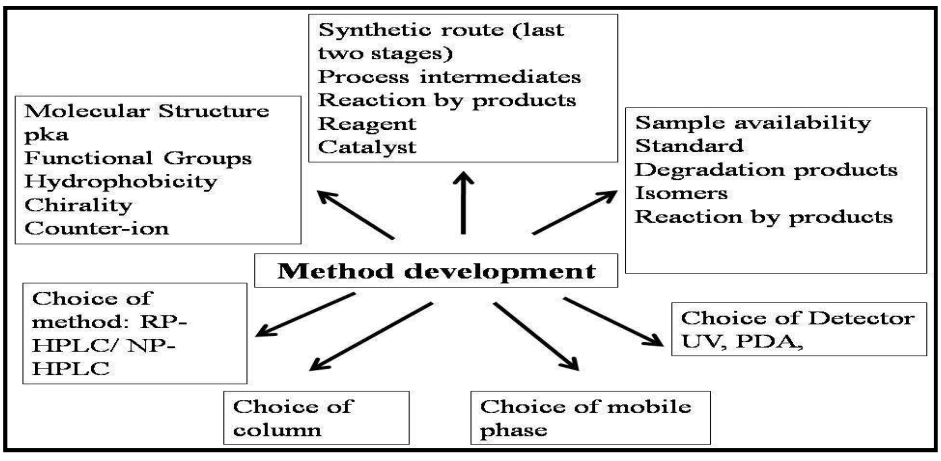

Fig 1.4 Outline of the process involved in method development

1.6 Parameters Affecting Changes in Chromatograph

The various parameters affecting the changes in chromatographic conditions are

1. Flow rate
2. Temperature
3. pH
4. Ion pair reagent
5. Column efficiency
6. Capacity factor
7. Resolution
8. Retention time
9. Peak asymmetry

1. Effect of Flow Rate

The efficiency of a HPLC column varies with flow rate. It is sometimes useful and readily utilized to increase the resolution. A faster flow rate of the eluent minimizes the time required to run a column and thereby minimizes diffusion, resulting in a better separation (less band broadening). However, the maximum flow rate is limited because a finite time is required for analyte to equilibrate between stationary phase and mobile phase. A slower flow rate will decrease the column back pressure and a corresponding increase in the run time is observed.[32]

Table 1.3 Flow rates for column with different internal diameter.

Internal diameter of column (mm)	Standard flow rate (µl / min)
4.6	1000
2.1	200
1.0	50
0.30	4

2. Effect of Temperature

Elevated temperatures decrease viscosity and increase solubility and diffusivity. Retention, peak shape, column efficiency, and total analysis time are affected by temperature because both the thermodynamics and kinetics of adsorption processes are functions of temperature. Additionally, temperature control results in improved reproducibility. In liquid chromatography temperature is used in the optimization of a separation. At higher temperature, peaks will be sharper and elute earlier. System pressure is affected by temperature. The viscosity of the mobile phase decreases with increasing temperature. For example if the HPLC system pressure is too high for a given solvent system temperature of the column may be raised to 40 ^{0}C or even 60 ^{0}C. Higher temperature will lead to a shorter column lifetime and some columns may not be able to tolerate 60 ^{0}C. The combination of smaller diameter packing with shorter column lengths at elevated temperatures facilitates efficient and fast separations, meeting the high throughput performance requirements of the pharmaceutical industry. [33]

3. Role of pH

For some preparations the effect of changing pH is minimal. However for acids and bases, a small change in pH is significant. Changing the pH changes the

degree of ionization of molecules in solution, affecting polarity of the solution thereby changing the retention times in an HPLC separation. In a sample mixture, the retention time of the components of the mixture are also changed to different extents. Hence it affects the degree of selectivity, where the peaks become further apart or at a particular pH they may co-elute and then the peak elution order will change. Selection of a proper buffer pH is necessary to reproducibly separate ionizable compounds by RP-HPLC. Selection of an improper pH for ionizable analytes leads to asymmetric peaks that are broad, tall or split. [34]

During the selection of a buffer, pKa of the analyte should be considered. A buffer with 2 pH units above or below pKa of the analyte is recommended for a good peak shape. From Henderson- Hassel back equation, [35]

$$pH = pKa + \log ([A^-]/ [HA])$$

It can be determined that 99% of the analyte is in a single form, Good peak shape is possible only when an analyte is in a single form. [36]

4. Role of Ion-Pair Reagent

Most of these compounds are ionic or polar; hence the use of reversed phase-high performance liquid chromatography (RP-HPLC) is somewhat restricted. Initially when deciding to select RP-HPLC or RP-HPLC with ion-pairing, the nature of the analyte of interest is considered. If the sample is neutral, RP-HPLC is used first; and if the sample is ionic, RP-HPLC with ion pairing is used. Thus RP-HPLC and RP-HPLC with ion pairing are similar except that the latter consists of an ion-pair reagent in the mobile phase to improve the selectively of ionic samples. The ionic pair

reagents are large ionic molecules having a charge opposite to the analyte of interest, as well as a hydrophobic region to interact with the stationary phase. The counter-ion combines with the ions of the eluent, becoming ion pairs in the stationary phase. This results in different retention, thus facilitating separation of analytes.

The use of an ion-pair reagent is suggested only when separation is not adequate with reversed-phase HPLC. This is because using an ion-pair reagent introduces additional experimental parameters that need to be controlled, such as selection of a suitable ion-pair reagent to use and its concentration. Because of this added variable, reversed-phase HPLC should be utilized on any ionic analyte first before trying ion-pair reversed–phase HPLC. The approach used in RP-HPLC to separate charged analytes is ionic suppression. This technique is based on the pH adjustment of the mobile phase to result in a non-ionized analyte. However, this requires extensive method development and is only suitable for single compounds or simple mixtures where the pKa's of the analytes lie close together. [37]

5. Column Efficiency (N)

The efficiency of a chromatographic column is given terms of number of theoretical plates (plate number), N

$$N = 16\left(\frac{t_r}{w}\right)^2$$

Where, t_r- retention time measured from the time of injection, w- peak width peak width obtained by drawing tangents to the sides of the Gaussian curve at the inflection points and extrapolating the tangents to intercept the baseline as in fig 1.6

Efficiency of the column is also expressed as height equivalent to theoretical plate, (or plate height) HETP (or h)

Where**,** L- length of the column,

$$h = \frac{L}{N}$$

N- Number of theoretical plates. *(38)*

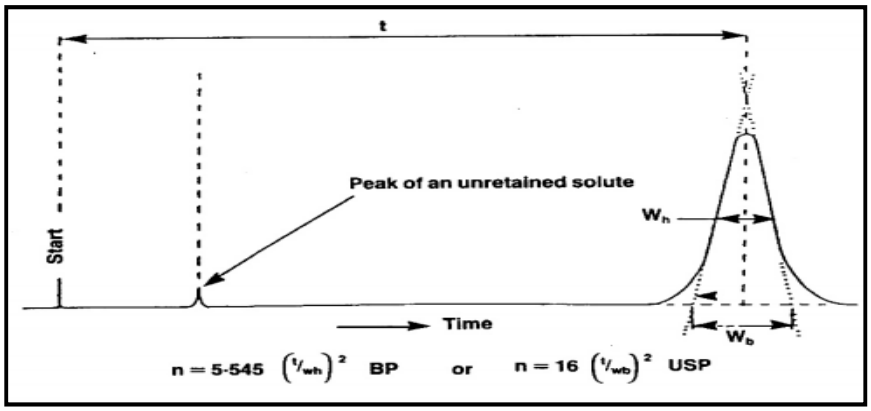

Fig. 1.5 Pictorial Representation of number of theoretical plates.

6. Capacity Factor (k')

It is measure of the position of a sample peak in the chromatogram, being specific for a given compound, a parameter which specifies the extent of delay of substance to be separated.

$$k' = \frac{t_r - t_m}{t_m}$$

Where, t_r– retention time of the solute, t_m – retention time of the unretained compound by the column packing. Fig 1.7 shows capacity factor of a solute.

k' depends at stationary phase, mobile phase, temperature and quality of column packing. For good chromatographic performance with isocratic separation, k' value should be in the range of 1-10. If k' < 1.0, the bands are inadequately separated from excessively unretained material, if k' > 10 separation takes too long and bands broadened, if k' > 30, satisfactory isocratic separation using present column and mobile phase is not obtained and gradient elution should be tried.

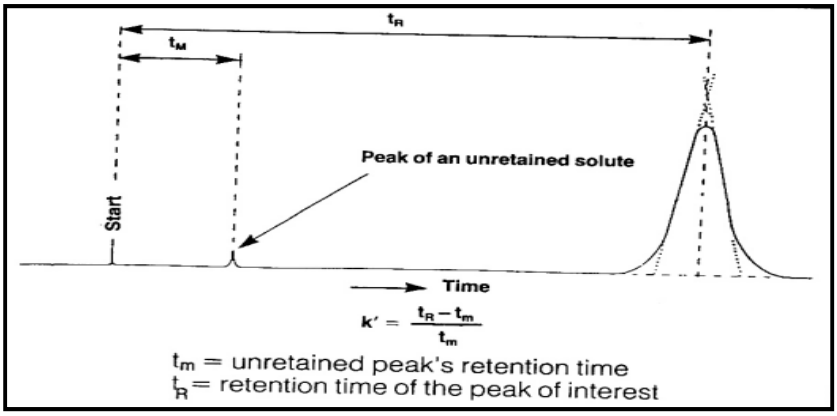

Fig.1.6 Pictorial representation of capacity factor.

7. Resolution (R_s)

The ability of the column to separate two solutes. In a chromatogram it is the distance of separation of two peaks.

$$R_s = \frac{t_{r2} - t_{r1}}{0.5(w_1 - w_2)}$$

Where,t_{r1} ,t_{r2} – retention time of two immediately adjacent peaks,

w_1, w_2 – peak widths of two immediately adjacent peaks as shown in fig 1.8.

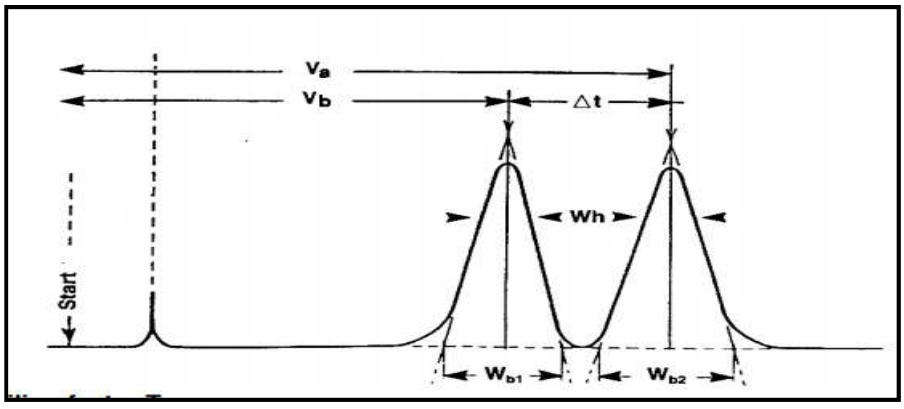

Fig.1.7 Pictorial Representation of Resolution.

1. Selectivity factor (α) / Relative Retention

This describes the relative position of two adjacent peaks. Ideally, it is calculated using the capacity factor because the peaks separation depends on the components interaction with the stationary phase.

Where,

$$\alpha = \frac{k'_b}{k'_a}$$

k'_a, k'_b– capacity factors of peak a and peak b respectively.

The value of the separation factor is always greater than unity. The separation factor is also identical to the ratio of the corresponding distribution constants. If the capacity factor is used, the separation factor should be consistent for a given column, mobile phase composition and specified temperature, regardless of the instrument used.

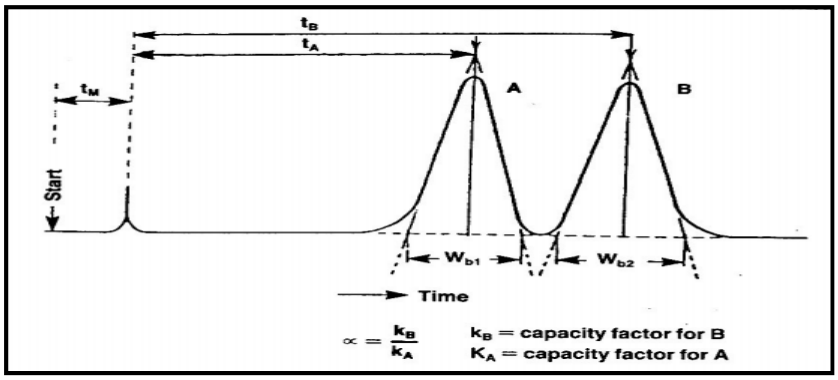

Fig.1.8 Pictorial representation of selectivity factor.

9. Peak Asymmetry (A_s)

It is also known as tailing factor. The asymmetry factor for a peak can be calculated using the following formula.

$$A_s = \frac{W_{0.05}}{2f}$$

Where, $W_{0.05}$– peak width at 5% height from the base line,

f – distance between maximum and leading edge of the peak as in Fig 1.9

It is also calculated from $$A_s = \frac{b}{a}$$

Where, b - distance from the point at peak maxima to the trailing edge,

a – distance from the leading edge of the peak to the peak maxima (both measured at 10% height of the baseline) as shown in Fig 2.0.

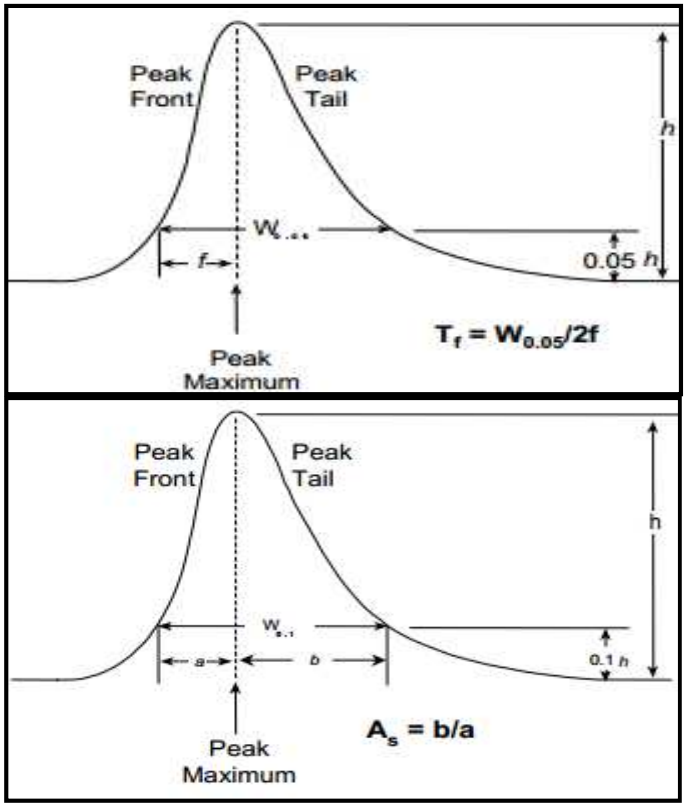

Fig. 1.9 Pictorial representation of peak asymmetry.

Introduction to Method Validation

Validation is an integral part of quality assurance; it involves the systematic study of systems, facilities and processes aimed at determining whether they perform their intended functions adequately and consistently as specified. Validation in itself

does not improve processes but confirms that the processes have been properly developed and are under control.

Method validation is defined as the process of proving (through scientific studies) that an analytical method is acceptable for its intended use. To ensure compliance with quality and safety standards, the United States, Europe, Japan, and other countries have published compendia, or pharmacopeias, that describe official test methods for many marketed drug products. For example, analytical methods found in United States Pharmacopeia (USP) are legally recognized analytical procedures under section 501 (b) of the Federal Food, Drug, and Cosmetic Act. For these compendia methods, USP provides regulatory guidance for method validation. In addition, validation of analytical methods is covered by the United States Code of Federal Regulations (CFR). A great deal of effort has been devoted to the harmonization of pharmaceutical regulatory requirements in the United States, Europe, and Japan. As part of this initiative, the International Conference on Harmonization (ICH) has issued guidelines for analytical method validation. The recent FDA methods validation draft guidance documents as well as U.S. both refer to ICH guidelines. [39]

The required validation parameters, also termed analytical performance characteristics or analytical figs of merit. Methods should be validated or revalidated

- Before their introduction and routine use.
- Whenever the conditions change for which the method has been validated, e.g., instrument with different characteristics.
- Wherever the method is changed and the change is outside the original scope of the method.

The validation of analytical procedures is directed to the four most common types of analytical procedures: Identification tests; Quantitative tests for impurities' content; Limit tests for the control of impurities; Quantitative tests of the active moiety in samples of drug substance or drug product or other selected component(s) in the drug product.

Method Validation (ICH Guidelines)

1. Accuracy,

2. Precision,

- Repeatability,
- Intermediate precision.

3. Specificity / Selectivity,

4. Limit of Detection,

5. Limit of Quantitation,

6. Linearity,

7. Range,

8. Robustness,

9. System Suitability.

The validation terminology in ICH guidelines differ from the validation given in USP with two exceptions. Ruggedness is not included in ICH guideline and treats system suitability as a part of method validation, whereas the USP considers it in a separate chapter.

Accuracy

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 should be established across the specified range of the analytical procedure.

Accuracy is measured as the percentage of the analyte recovered by assay, spiking samples in a blind study.

Accuracy studies for drug substance and drug product are recommended to be performed at the 80, 100 and 120% levels of label claim as stated in the Guidelines for Submitting Samples and Analytical Data for Methods Validation

Accuracy should be assessed using a minimum of 9 determinations over a minimum of 3 concentration levels covering the specified range (e.g.3 concentrations /3 replicates each of the total analytical procedure). Accuracy should be reported as percent recovery by the assay of known added amount of analyte in the sample or as the difference between the mean and the accepted true value together with the confidence intervals.[40]

The acceptance criterion for accuracy is the Relative Standard Deviation (RSD) for all the recovery values should not be more than 2.0%.

Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision

may be considered at three levels: repeatability, intermediate precision and reproducibility.

The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements.

The relative standard deviation (RSD) for the assay of six sample preparations should not be more than 2.0%.

Specificity

Specificity is the ability to assess accurately the analyte in the presence of components which may be expected to be present in the sample matrix. Typically these might include impurities, degradants, matrix, etc. it is a measure of the degree of interference from such other things such as other active ingredients, excipients, impurities, and degradation products, ensuring that a peak response is due to a single component only.

Specificity is divided into two separate categories: identification and assay/ impurity tests. For identification purpose, specificity is demonstrated by the ability to discriminate between compounds of closely related structures or comparison to a known reference standard. For assay/ impurity tests, specificity is demonstrated by the resolution of the two closely eluting compounds. These compounds are usually the major component or the active ingredient and an impurity.

Limit of Detection (LOD)

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an

exact value. It is a limit test that specifies whether or not an analyte is above or below a certain value.

It is expressed as a concentration at a specified signal to noise ratio usually a 2 or 3-to-1 ratio. Two other method can also be used to determine LOD: Visual non-instrumental methods and a means of calculation: Visual non-instrumental methods may include techniques such as thin-layer chromatography (TLC) or titrations. LODs may also be calculated based on the standard deviation (SD) of the response and the slope (S) of the calibration curve at levels approaching the LOD according to the formula:

Where,

$$LOD = 3.3\left(\frac{SD}{S}\right)$$

SD- standard deviation

S- Slope

The standard deviation of the response can be determined based on the standard deviation of the blank, on the residual standard deviation of the regression line, or the standard deviation of y-intercepts of regression lines. The method used to determine LOD should be documented and supported, and an appropriate number of samples should be analyzed at the limit to validate the level.

Limit of Quantitation (LOQ)

The limit of quantitation (LOQ) is defined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated operational conditions of the method.

The ICH has recognized the 10-to-1 signal –to-noise ratio as typical, and as for LOD, lists the same two additional options that can be used to determine LOQ: visual non-instrumental methods and a means of calculation. The calculation method is again based on the standard deviation (SD) of the response and the slope (S) of the calibration curve according to the formula,

Where,

$$LOQ = 10\left(\frac{SD}{S}\right)$$

SD- standard deviation

S- Slope

Again, the standard deviation of the response can be determined based on the standard deviation of the blank, on the residual standard deviation of the regression line, or the standard deviation of y-intercepts of regression lines. As with LOD, the method used to determine LOQ should be documented and supported, and an appropriate number of samples should be analyzed at the limit to validate the level.

Linearity and Range

Linearity is the ability of the method to elicit test results that are directly proportional to analyte concentration within a given range. Linearity is generally reported as the variance of the slope of the regression line. Range is the (inclusive) interval between the upper and lower levels of analyte that have been demonstrated to be determined with precision, accuracy, and linearity using the method. The range is normally expressed in the same units as the test results obtained by the method.

A minimum of five concentration levels, along with certain minimum specified ranges are to be determined. For assay tests, the minimum specified range is

80-120% of the target concentration. For impurity tests, the minimum range is from the reporting level of each impurity to 120% of the specification.

The relationship between the concentration (in %) of drug in sample and area of should be linear in the specified range and the correlation should not be less than 0.9.

Robustness

Robustness is the capacity of a method to remain unaffected by small deliberate variations in method parameters. The robustness of a method is evaluated varying method parameters such as percent organic solvent, pH, ionic strength, or temperature and determining the effect (if any) on the results of the method.

The RSD for the assay of drugs in a sample under deliberately modified chromatographic conditions should not be more than 2.0%.

System Suitability

System suitability tests are an integral part of gas and liquid chromatographic methods. They are used to verify that the resolution and reproducibility of the chromatographic system are adequate for the analysis to be done. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as a whole. *(41)*

System suitability is the checking of a system to ensure system performance before or drying the analysis unknowns. Parameters such as plate count, tailing factor, resolution and reproducibility (% RSD retention time and area for repetitive. injections) are determined and compared against the specifications set for the method.

These parameters are measured during the analysis of a system suitability sample that is a mixture of main components and expected by-products.

Table 1.4 Acceptance Limits for System Suitability Test.

Parameter	Limit
Capacity Factor	k' > 2
Injection precision	RSD < 1% for n ≥ 5
Resolution	R_s > 2
Tailing factor	$A_s \leq$ 2
Theoretical plates	N > 2000

Definitions and Formulas Used in Method Validation

Mean/ Average (x_i)

The average result (ā) is calculated by summing the individual results and dividing the sum by the number (n) of individual values.

$$x_i = \frac{x_1 + x_2 + x_3 \ldots}{n}$$

Where, $x_1, x_2, x_3..$ = Values of individual results

n = Number of individual results

Standard Deviation (SD)

It is the root mean square deviation of values from their average.

$$SD = \sqrt{\frac{\sum(x - x_i)}{n - 1}}$$

Where Σ = Sum of observations

x_i = Mean or arithmetic average ($\Sigma x / n$)

x = Individual observed value

$x - x_i$ = Deviation of a value from the mean

n = Number of observations

Relative Standard Deviation (RSD)

It is defined as standard deviation expressed as the percentage of mean.

$$RSD = \frac{SD}{x_i} \times 100$$

Where SD = Standard deviation

x_i = Mean or arithmetic average ($\Sigma x / n$

Correlation Co-Efficient (R)

The correlation coefficient is used to indicate the relationship of two random variables. It provides a measure of the strength and direction of the correlation varying from -1 to +1. Positive values indicate that the two variables are positively correlated, meaning the two variables vary in the same direction. Negative values indicate that the two variables are negatively correlated, meaning the two variables vary in the contrary direction. Values close to +1 or -1 reveal the two variables are highly related.

$$R = \frac{n(\sum xy) - (\sum x)(\sum y)}{\sqrt{[\sum x^2 - (\sum x)^2]\,[[\sum y^2 - (\sum y)^2]}}$$

Where n = number of observations

x = first value

y = second value

Σxy = sum of products of first and second value

Σx = sum of first values

Σy = sum of second values

Σx^2 = sum of squares of first value

Σy^2 = sum of squares of second value

Linear Regression

A regression is a statistical analysis assessing the association between two variables. It is used to find the relationship between two variables.

The equation of straight line is

$$y = a + bx$$

Where

B = slope

a = intercept

$$Slope\ (b) = [n(\Sigma xy) - (\Sigma x)\ (\Sigma y)] / [n(\Sigma x^2) - (\Sigma x)^2)$$

$$Intercept\ (a) = [(\Sigma y) - b(\Sigma x)] / n$$

Where

n = number of observations

x = first value

y = second value

Σxy = sum of products of first and second value

Σx = sum of first values

Σy = sum of second values

Σx^2 = sum of squares of first value

Σy^2 = sum of squares of second value

2. DRUG PROFILE

ESOMEPRAZOLE:

Brand Name : Nexium

Application : A gastric proton pump inhibitor

CAS Number : 217087-09-7

Purity : ≥98%

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

Molecular Mass : 345.417g/mols

Description

Esomeprazole Magnesium is an enantiomer of omeprazole which acts as a gastric proton pump inhibitor.

Structure

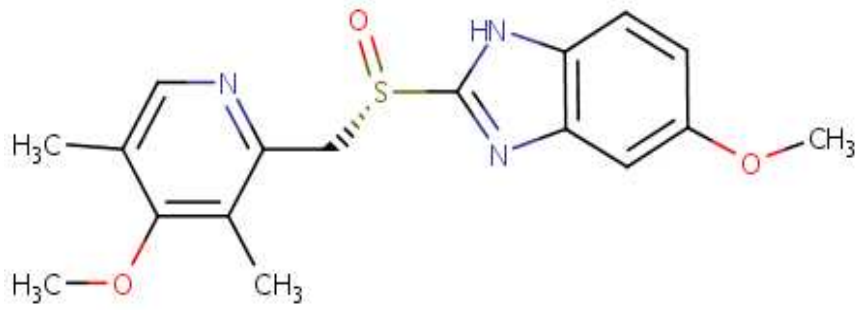

Fig.2.1 Structure of Esomeprazole

Technical Information

Appearance : Powder

Physical State : Solid

Solubility

Soluble in methanol, DMSO (143 mg/ml at 25 °C), ethanol (143 mg/ml at 25 °C), and water (<1 mg/ml at 25 °C).

Storage **:** Store at -20° C

Melting Point **:** 171-174 °C (dec.)

Refractive Index **:** $n^{20}D$ ~1.67 (Predicted)

Optical Activity **:** α20/D -139.5º±2.5º, c = 1 in methanol

IC50

The PhoP regulon in Salmonella Typhimurium: $EC_{50}50$ >50 µM;

the PhoP regulon in Salmonella Typhimurium: IC_{50} >75 µM

Indication

For the treatment of acid-reflux disorders (GERD), peptic ulcer disease, H. pylori eradication, and prevention of gastroinetestinal bleeds with NSAID use.

Pharmacodynamics

Esomeprazole is a compound that inhibits gastric acid secretion and is indicated in the treatment of gastroesophageal reflux disease (GERD), the healing of erosive esophagitis, and H. pylori eradication to reduce the risk of duodenal ulcer recurrence. Esomeprazole belongs to a new class of antisecretory compounds, the substituted benzimidazoles, that do not exhibit anticholinergic or H2 histamine antagonistic properties, but that suppress gastric acid secretion by specific inhibition of the H^+/K^+ATPase at the secretory surface of the gastric parietal cell. By doing so, it

inhibits acid secretion into the gsatric lumen. This effect is dose-related and leads to inhibition of both basal and stimulated acid secretion irrespective of the stimulus.

Mechanism of Action

Esomeprazole is a proton pump inhibitor that suppresses gastric acid secretion by specific inhibition of the H^+/K^+ATPase in the gastric parietal cell. By acting specifically on the proton pump, Esomeprazole blocks the final step in acid production, thus reducing gastric acidity.

Absorption **:** 90%

Volume of distribution **:** 16 L [healthy volunteers]

Protein binding **:** 97%

Half life **:** 1-1.5 hours

Metabolism

Mainly hepatic. Esomeprazole is completely metabolized by the cytochrome P450 system via CYP2C19 and CYP3A4. Metabolism produces inactive 56ydroxyl and desmethyl metabolites, which have no effect on gastric acid secretion. Less than 1% of the parent drug is excreted in urine.

Route of Elimination

Approximately 80% of the administered dose of esomeprazole is excreted as metabolites in urine and the remaining 20% is excreted in feces.

LEVOSULPIRIDE

Synonym : Levosulpiride

Application : A D2DR inhibitor

CAS Number : 23672-07-3

Purity : ≥99%

Molecular Weight : 341.43

Molecular Formula : $C_{15}H_{23}N_3O_4S$

Structure

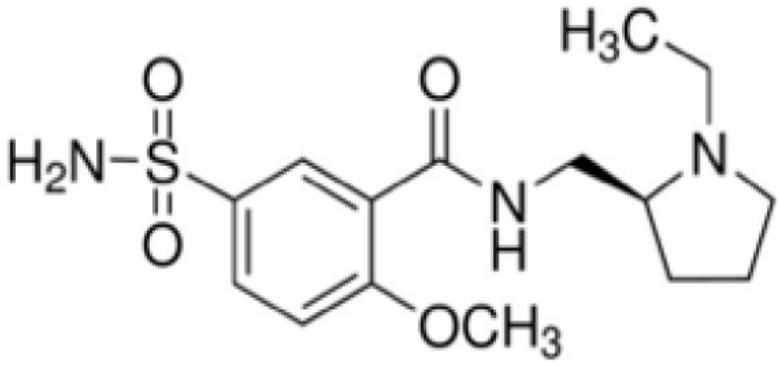

Fig.2.2 Structure of Levosulpiride

Description

(S)-(-)-Sulpiride is a D2DR(D2 dopamine receptor) and D3DR(D3 dopamine receptor) inhibitor.

Technical Information

Appearance : Crystalline powder

Physical State : Solid

Solubility

Soluble in water (partly), DMF, DMSO (69mg/ml at 25 °C), water (<1 mg/ml at 25 °C), and ethanol (10 mM).

Storage : Store at 4° C

Melting Point : 183-186 °C (lit.)

Boiling Point : 529.85 °C (Predicted)

Density : 1.24 g/cm3 (Predicted)

Refractive Index : $n^{20}D$ 1.55 (Predicted)

Optical Activity : α25/D -67.5º±1.5º, c = 2 in DMF

IC50

D3DR: IC_{50} = 123 nM (human); D2DR: IC_{50} = 128 nM (human); Dopamine D2 receptor: IC_{50} = 210 nM (rat)

pK Values : pKa: 9.98 (Predicted), pKb: 8.97 (Predicted)

Indication : Sulpiride is indicated for the treatment of schizophrenia.

Side Effects

- **Genitourinary** - Absence of menstrual period, breast enlargement in male, spontaneous milk secretion, and changes in libido.
- **Potentially Fatal** - Neuroleptic malignant syndrome.

Storage : store at room temperature 25°C

Mechanism of Action

In contrast to most other neuroleptics which block both dopamine D_1 and D_2 receptors, sulpiride is more selective and acts primarily as a dopamine D_2 antagonist. Sulpiride appears to lack effects on norepinephrine, acetylcholine, serotonin, histamine, or gamma-aminobutyric acid (GABA) receptors.

Pharmacodynamics

Sulpiride is a substituted benzamide derivative and a selective dopamine D2 antagonist with antipsychotic and antidepressant activity. Other benzamide derivatives include metoclopramide, tiapride, and sultopride.

Absorption

Sulpiride is absorbed slowly from the gastrointestinal tract. Its oral bioavailability is only 25 to 35% with marked interindividual differences.

Half life : 6 – 8 hour

LITERATURE REVIEW

- **Deepak Kumar Jain, Nitesh Jain,1 Rita Charde,1 and Nilesh Jain2. et al** [42] reported as A simple, precise, reliable, rapid, sensitive and validated RP-HPLC method has been developed to determine esomeprazole magnesium trihydrate (ESO) and naproxen (NAP) in synthetic mixture form. Chromatographic separation achieved isocratically on Phenomenex, Luna C18 column (5 µm, 150mm × 4.60mm) and acetonitrile: phosphate buffer (pH 7.0) in the ratio of 50:50 (v/v) as the mobile phase, at a flow rate of 0.5 ml/min. Detection was carried out at 300 nm. The retention times for NAP and ESO was found to be 2.67 ±0.014 and 5.65 ±0.09 min respectively. Parameters such as linearity, precision, accuracy, recovery, specificity and ruggedness are studied as reported in the ICH guidelines. The method was linear in the concentration range of 50-250 µg/ml for NAP and 2-10 µg/ml for ESO with correlation coefficient of 0.999 and 0.998 respectively. The mean recoveries obtained for NAP and ESO were 100.01% and 97.76 % respectively and RSD was less than 2. The correlation coefficients for all components are close to 1. Developed method was found to be accurate, precise, selective and rapid for simultaneous estimation of NAP and ESO.

- **Dipali Patel, Nishitkumar Patel, Reeta Vaishy, Viral Patel, Chiragsinh Solanki, and Mitul Patel et al** [43] reported as A simple, specific, precise, and accurate reversed-phase HPLC method was developed and validated for simultaneous estimation of aspirin and esomeprazole magnesium in tablet dosage forms. The separation was achieved by HyperChrom ODS-BP C18 column (200□mm × 4.6□mm; 5.0□µm) using acetonitrile: methanol: 0.05□M phosphate buffer at pH 3 adjusted with orthophosphoric acid (25□:□25□:□50, v/v) as

eluent, at a flow rate of 1 mL/min. Detection was carried out at wavelength 230 nm. The retention times of aspirin and esomeprazole magnesium were 4.29 min and 6.09 min, respectively. The linearity was established over the concentration ranges of 10–70 µg/mL and 10–30 µg/mL with correlation coefficients () 0.9986 and 0.9973 for aspirin and esomeprazole magnesium, respectively. The mean recoveries were found to be in the ranges of 99.80–100.57% and 99.70–100.83% for aspirin and esomeprazole magnesium, respectively. The proposed method has been validated as per ICH guidelines and successfully applied to the estimation of aspirin and esomeprazole magnesium in their combined tablet dosage form.

- **Dilip G Maheshwari a and Priti D Trivedi b,* et al** [(44)] reported as A simple, accurate, reliable and reproducible HPLC method was developed for the simultaneous determination of esomeprazole and domperidone in combined dosage forms. The method employed C18 phenomenex column, acetate buffer: acetonitrile: methanol (55:35:10) as mobile phase and detection was made at 290nm. The retention times were found to be 6.76 and 4.42 min for ESO and DOMPE respectively. The method was validated as per ICH guidelines. The method shows good linearity, accuracy, and precision, limit of detection and limit of quantification. The linearity range was found between 4-19 µg/mL for both ESO and DOMPE with relative standard deviation of 0.022 and 0.076 respectively. The value for LOD was found to be 0.3 µg/mL and 0.4 µg/mL and LOQ was found to be 1.5 µg/mL and 2.5 µg/mL for ESO and DOMPE respectively. The main recovery was found to be 99.81 ± 1.27 and 100.43 ± 1.15 for ESO and DOMPE respectively. The method was suitable for routine analysis of ESO and DOMPE both individually and in combined dosage forms.

- **ArmAĞAn ÖnAl* And Aysel ÖZtunÇ et al** [45] reported as A simple, selective and accurate high performance liquid chromatographic (HPLC) method was developed and validated for theanalysis of esomeprazole magnesium trihydrate (ES) in tablets. Chromatographic separation was achieved isocratically on a C18column utilizing a mobile phase of acetonitrile/phosphate buffer (60:40, v/v, pH 7) at a flow rate of 1.0 mL/min with UV detection at205 nm. Lansoprazole was used as an internal standard (IS). The calibration curve of ES was linear in the range of 100~1000 ng/mL(r = 0.9992, n = 4). The RSD values for intra- and inter-day precision were 0.66~0.86% and 0.84~1.11%, respectively. The proposed method was successfully applied to the determination of ES in tablets. The mean recovery for ES from the tablets ranged between97.82~98.22%. ES was subjected to neutral, acid and alkali hydrolysis as well as oxidation, dry heat treatment and photo degradation. Being simple, accurate and selective, the method can be used for routine quality control analysis.

- **Ia Hultman, Helene Stenhoff, Mathias Liljeblad et al** [46] reported as 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 less than 9.5% with accuracy between 97.7% and 100.1% for all analytes.

- **Patil Shamkant S1,*, Dhabale Pandurang N1, Kuchekar Bhanudas S2 et al** [47] reported as 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 294310nm. 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.

- **Santaji Uttam Nalwadea, b, et al** [48] reported as A novel gradient reversed-phase ultra performance liquid chromatographic method has been developed for quantitative determination of Esomeprazole magnesium and its seven impurities in pharmaceutical dosage forms. Chromatographic separation has been achieved on an Acquity BEH C18, 50 mm × 2.1 mm, 1.7 µm with buffered mobile phase consisting solvent A (0.04 molar (M) glycine (pH 9.0) buffer) and solvent B (mixture of acetonitrile and Milli-Q water in the ratio 90: 10 (v/v); respectively) delivered at flow rate of 0.21 mL min−1 and the detection wavelength 305 nm. Resolution of Esomeprazole magnesium and all the seven potential impurities has been achieved greater than 2.0 for all pairs of compounds. The drug was subjected to the stress conditions of oxidative, acid, base, hydrolytic, thermal and photolytic degradation. Esomeprazole magnesium was found to degrade significantly in oxidative and acid hydrolysis stress conditions and stable in base, hydrolytic and

photolytic degradation conditions. The degradation products were well resolved from main peak and its impurities, thus proved the stability indicating power of the method. The stress samples were assayed against a reference standard and the mass balance was found to be close to 99.1%. So this method was also suitable for Assay determination of Esomeprazole magnesium in pharmaceutical dosage forms. The developed method was validated as per ICH guidelines with respect to specificity, linearity, limit of detection, limit of quantification, accuracy, precision and robustness.

- **Jin SE1, Ban E, Kim YB, Kim CK. et al** [49] reported as A rapid and simple high performance liquid chromatography (HPLC) method was developed and validated for determination of levosulpiride in human plasma. After extraction with ethylacetate/methylene chloride (5:1, v/v), analysis of levosulpiride in plasma samples was carried out using a reverse phase C18 column with fluorescence detector (maximum excitation at 300 nm and maximum emission at 365 nm) for separation and quantification. A mixture of methanol-20 mM phosphate buffer (pH 3.5, 16:84, v/v) was used as a mobile phase. The method was specific and sensitive with a limit of quantification of 5 ng/ml. This HPLC method was validated by examining the precision and accuracy for inter- and intra-day analysis in the concentration range of 5-150 ng/ml. The relative standard deviation (R.S.D.) in inter- and intra-day validation were 8.16-19.75 and 3.90-11.69%, respectively. In stability tests, levosulpiride in human plasma was stable during the storage and assay procedure. The method was applied to the bioequivalence study of two levosulpiride tablet formulations (25 mg) after a single oral administration.

- **A. Sirisha*, A. Ravi Kumar et al** [50] reported as A new simple, precise, accurate and selective RP- HPLC method has been developed and validated for estimation of Levosulpiride and Rabeprazole in pharmaceutical formulation. The detection was carried out at 216nm for both drugs. The retention time for LEVO and RAB were found to be 4.918 min. and 5.873 min. respectively. The method was validated for precision, recovery, robustness, specificity, and detection and quantification limits, in accordance with International Conference on Harmonization guidelines. Linearity was observed in the concentration range from 50% to 150% of nominal concentration of Rabeprazole and Levosulpiride Correlation coefficient was 0.999 for both the drugs .The limit of detection and quantification of LEVO were 0.021 mg/ml and 0.0731 mg/ml respectively. While for RAB it was 0.06 mg/ml and 0.20 mg/ml, respectively. The % recovery was found to be within the limits of the acceptance criteria with average recovery of 101.3% for LEVO and 99.3% for RAB. The % RSD below 2.0 shows the high precision of proposed method.

- **Huang MC1, Ho HO, Yeh GC, Ke WT, Lin LC et al** [51] reported as An improved HPLC method using a silica gel column with fluorescence detection (excitation at 300 nm and emission at 365 nm) was developed for the determination of sulpiride concentrations in plasma. Analysis of sulpiride in plasma samples was simplified by a one-step liquid-liquid extraction after alkaline treatment of only 1 ml of plasma. The low limit of quantitation was 20 ng/ml with a coefficient of variation of less than 20%. A linear range was found from 20 to 1500 ng/ml. This HPLC method was validated with the precision for inter-day and intra-day runs being 0.36-8.01% and 0.29-5.25%, respectively, and the accuracy (standard deviation of mean, SD) for inter-day and intra-day runs being -1.58 to

5.02% and -2.14 to 5.21%, respectively. Bioequivalence of the two products was evaluated in 12 normal healthy male volunteers in a single-dose, two-period, two-sequence, two-treatment cross-over study. Sulpiride plasma concentrations were analyzed with this validated HPLC method. Results demonstrated that the two tablet formulations of sulpiride appear to be bioequivalent.

- **Su-Eon Jin, Eunmi Ban, Yang-Bae Kim, Chong-Kook Kim et al** [(52)] reported as A rapid and simple high performance liquid chromatography (HPLC) method was developed and validated for determination of levosulpiride in human plasma. After extraction with ethylacetate/methylene chloride (5:1, v/v), analysis of levosulpiride in plasma samples was carried out using a reverse phase C18 column with fluorescence detector (maximum excitation at 300 nm and maximum emission at 365 nm) for separation and quantification. A mixture of methanol—20 mM phosphate buffer (pH 3.5, 16:84, v/v) was used as a mobile phase. The method was specific and sensitive with a limit of quantification of 5 ng/ml. This HPLC method was validated by examining the precision and accuracy for inter- and intra-day analysis in the concentration range of 5–150 ng/ml. The relative standard deviation (R.S.D.) in inter- and intra-day validation were 8.16–19.75 and 3.90–11.69%, respectively. In stability tests, levosulpiride in human plasma was stable during the storage and assay procedure. The method was applied to the bioequivalence study of two levosulpiride tablet formulations (25 mg) after a singleoraladministration

OBJECTIVE AND PLAN OF STUDY

- Based on literature review, it is found that a number of studies involving method development for estimation of Esomeprazole and Levosulpiride have been carried out in formulations/plasma (Human). Thus, a number of analytical methods including LC-MS, LC with UV detection in biological specimen, spectrophotometry, HPTLC have been developed, but review of literature reveals that no method has been developed which can be stability indicating for the estimation of Esomeprazole and Levosulpiride in the bulk and capsule dosage form by RP- HPLC.

- No chromatographic methods were reported for estimation of both these drugs from their pharmaceutical formulation. Therefore, the objective is to develop simple, precise, accurate RP-HPLC method for estimation of Esomeprazole and Levosulpiride in its pharmaceutical formulation.

- Hence the goals of the present work are,

1. Development and validation of RP-HPLC method for estimation of Esomeprazole and Levosulpiride in bulk and pharmaceutical formulation.

- Selection of solvent
- Optimization of mobile phase
- Preparation of calibration curve
- Validation of the proposed method
- Application of the proposed method for estimation of drugs in their dosage form.

MATERIALS AND METHODS

MATERIALS

Table 5.1 List of Equipment/Instruments

S. No.	Name	Make/ Model
1	Analytical balance	Aicoset
2	HPLC instrument Series Software	A HPLC system (WATERS) Alliance e2695 EMPOWER- 2
3	Columns	INERTSIL ODS3(250mm,4.6mm,5µ) SUNFIRE C_{18}(250mm,4.6mm,5µ) HYPERSIL BDS C_{18} (100,4.6 mm,5µ)
4	Detector	UV-Visible detector
5	Sonicator	SONICA 2200MH
6	pH meter	Metler Toledo
7	Vacuum filter	Model XI 5522050 of Millipore

Table 5.2 List of Chemicals and Reagents

REAGENT	SPECIFICATIONS
Potassium dihydrogen orthophosphate (Merck – HPLC grade)	Mol. Formula – KH_2PO_4 Mol. Weight – 136.09
Orthophosphoric acid (Merck – HPLC grade)	Mol. Formula – H_3PO_4 Mol. Weight – 98
Ammonium acetate (Merck-GR)	Mol. Formula-CH_3COONH_4 Mol. Weight-77.0825
Methanol (Merck hplc grade)	Mol. Formula – CH_3OH Mol. Weight – 32.04
Acetonitrile (Merck – HPLC grade)	Mol. Formula – CH_3CN Mol. Weight – 41.05
WATER	Milli Q grade

METHODS

Preparation of buffer (0.01N KH_2Po_4)

Accurately weighed 1.36gm of potassium dihydrogen Ortho phosphate in a 1000ml of Volumetric flask add about 900ml of milli-Q water and degas in a sonicator and finally make up the volume with water and pH adjusted to 5.4 with dil. OPA

Standard Preparation:

Accurately Weighed and transferred 15mg of levosulpiride and 8mg of Esomeprazole working Standards into a 10ml clean dry volumetric flask, add 3/4th volume of diluent, sonicated for 5 minutes and make up to the final volume with diluents. 1ml from the above two stock solutions was taken into a 10ml volumetric flask and made up to 10ml.

Sample Preparation:

5 tablets were weighed and powdered and transferred into a 50mL volumetric flask, 35mL of diluent added and sonicated for 25 min, further the volume made up with diluent and filtered. From the filtered solution 0.2 ml was pipeted out into a 10 ml volumetric flask and made upto 10ml with diluent.

Linearity:

Linearity solutions are prepared such that 0.25ml, 0.5ml, 0.75ml, 1ml, 1.25ml, 1.5ml from the Stock solutions of Esomeprazole and Levosulpiride are taken in to 6 different volumetric flasks and diluted to 10ml with diluents to get 20ppm, 40ppm,

60ppm, 80ppm, 100ppm, 120ppm of Esomeprazole and 37.5ppm, 75ppm, 112.5ppm 150ppm, 187.5ppm, 225ppm of Levosulpiride.

Standard Preparation:

Accurately Weighed and transferred 15mg of levosulpiride and 8mg of Esomeprazole working Standards into a 10ml clean dry volumetric flask, add 3/4th volume of diluent, sonicated for 5 minutes and make up to the final volume with diluents. 1ml from the above two stock solutions was taken into a 10ml volumetric flask and made up to 10ml.

Sample Preparation:

5 tablets were weighed and powdered and transferred into a 50mL volumetric flask, 35mL of diluent added and sonicated for 25 min, further the volume made up with diluent and filtered. From the filtered solution 0.2 ml was pipeted out into a 10 ml volumetric flask and made upto 10ml with diluent.

Accuracy

Standard Preparation:

Accurately Weighed and transferred 15mg of levosulpiride and 8mg of Esomeprazole working Standards into a 10ml clean dry volumetric flask, add 3/4th volume of diluent, sonicated for 5 minutes and make up to the final volume with diluents. 1ml from the above two stock solutions was taken into a 10ml volumetric flask and made up to 10ml.

Sample preparation

50%: 5 tablets were weighed and calculate the average weight of each tablet then 750mg tablet powder was transferred into a 50mL volumetric flask, 30mL of

diluent added and sonicated for 25 min, further the volume made up with diluent and filtered. From the filtered solution 0.2ml was pipeted out into a 10 ml volumetric flask and made up to 10ml with diluent.

100%: 5 tablets were weighed and calculate the average weight of each tablet then 1500mg tablet powder was transferred into a 50mL volumetric flask, 30mL of diluent added and sonicated for 25 min, further the volume made up with diluent and filtered. From the filtered solution 0.2ml was pipeted out into a 10 ml volumetric flask and made up to 10ml with diluent.

150%: 5 tablets were weighed and calculate the average weight of each tablet then 2250mg tablet powder was transferred into a 50mL volumetric flask, 30mL of diluent added and sonicated for 25 min, further the volume made up with diluent and filtered. From the filtered solution 0.2ml was pipeted out into a 10 ml volumetric flask and made up to 10ml with diluent.

Method Development

Many trials were done by changing columns and Mobile phases and were reported below.

Trial 1

This trial was run through ods 250 column with mobile phase composition of 55:45 Buffer and Acetonitrile, Flow rate set at 1ml/min.

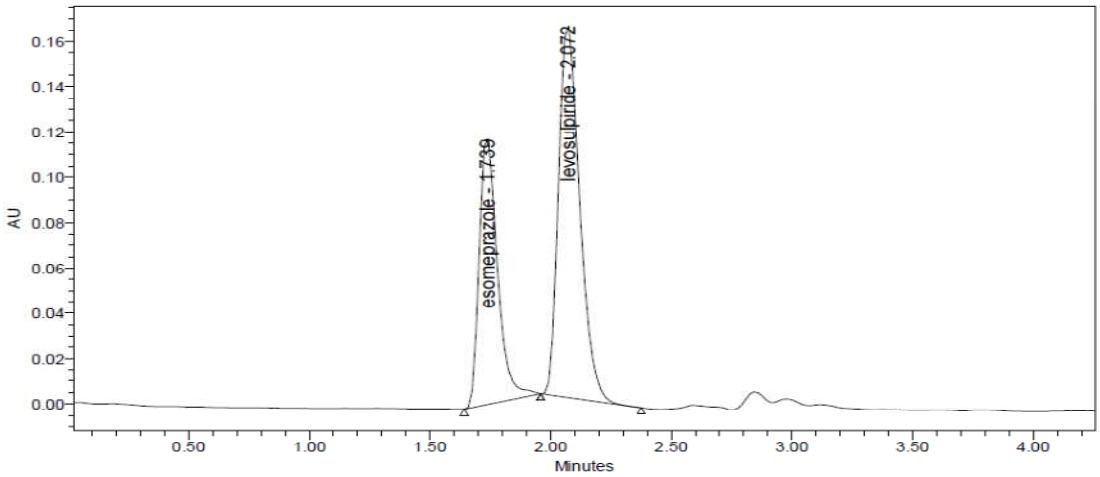

Fig 5.1 Trial chromatogram 1

Observation: resolution is not good

Trial 2

This trial was run through ODS 250mm column with mobile phase composition of 50:50 A water and Acetonitrile, Flow rate set at 1ml/min.

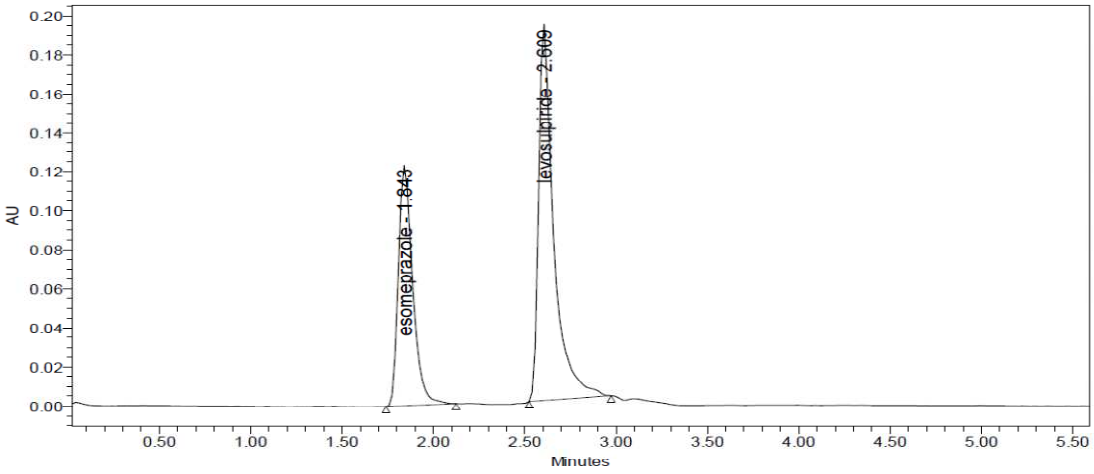

Fig 5.2 Trial chromatogram 2

Observation: Esomeprazole eluted in void range.

Trial 3

This trial was run through ODS 250mm column with mobile phase composition of 40:60 A water and Acetonitrile, Flow rate set at 1ml/min.

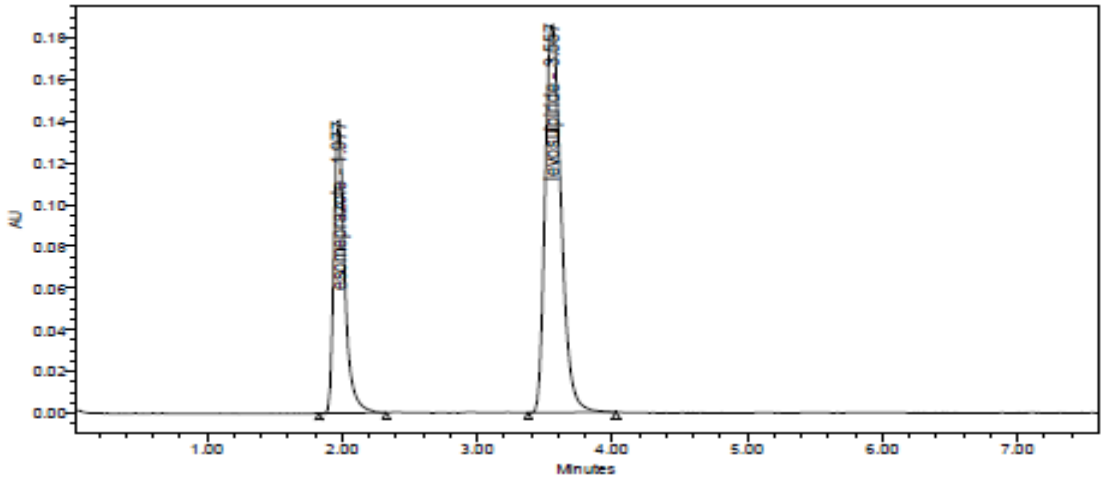

Fig 5.2 Trial chromatogram 3

Observation: Esomeprazole eluted in void range.

Optimized Method: Drugs were eluted with good retention time, resolution; all the system suitable parameters like Plate count and Tailing factor were within the limits.

Mobile phase:

Buffer and Acetonitrile taken in the ratio 32B:68A

Chromatographic conditions:

Flow rate	**:**	**1**ml/min
Column	**:**	ODS 150mm x 4.6 mm, 5µ.
Detector wave length	**:**	290nm
Column temperature	**:**	30°C
Injection volume	**:**	10µL
Run time	**:**	7min
Diluent	**:**	water:methanol 50:50

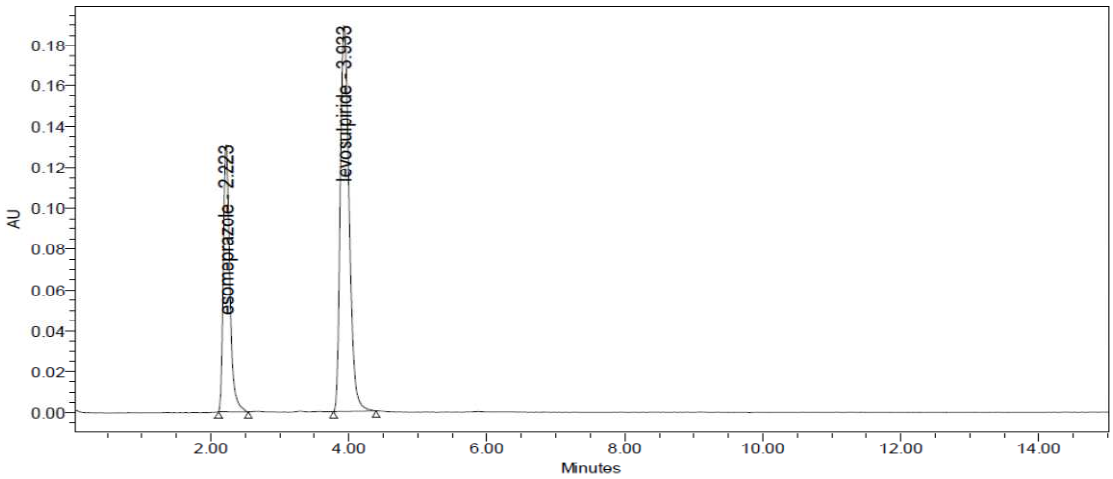

Fig 5.4 Optimized chromatogram of Esomeprazole and Levosulpiride

RESULTS AND DISCUSSIONS

1. System suitability

All the system suitability parameters are within range and satisfactory as per ICH guidelines.

Table: 6.1 System suitability studies of Esomeprazole and Levosulpiride method

Property	Esomeprazole	Levosulpiride
Retention time (t_R)	2.21± 0.3 min	4.01±0.3min
Theoretical plates (N)	2641 ± 163.48	4414± 163.48
Tailing factor (T)	1.45 ± 0.117	1.36± 0.117

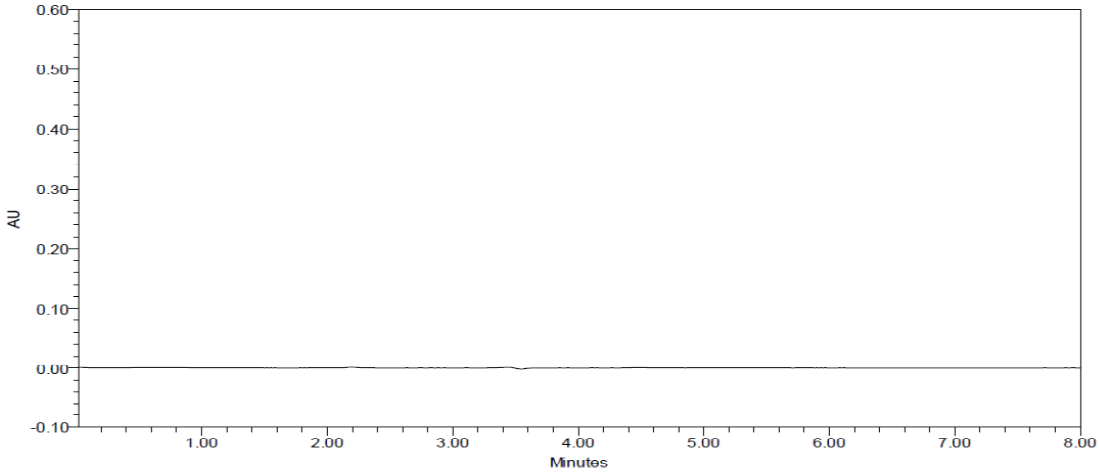

Fig : 6.1 Chromatogram of blank

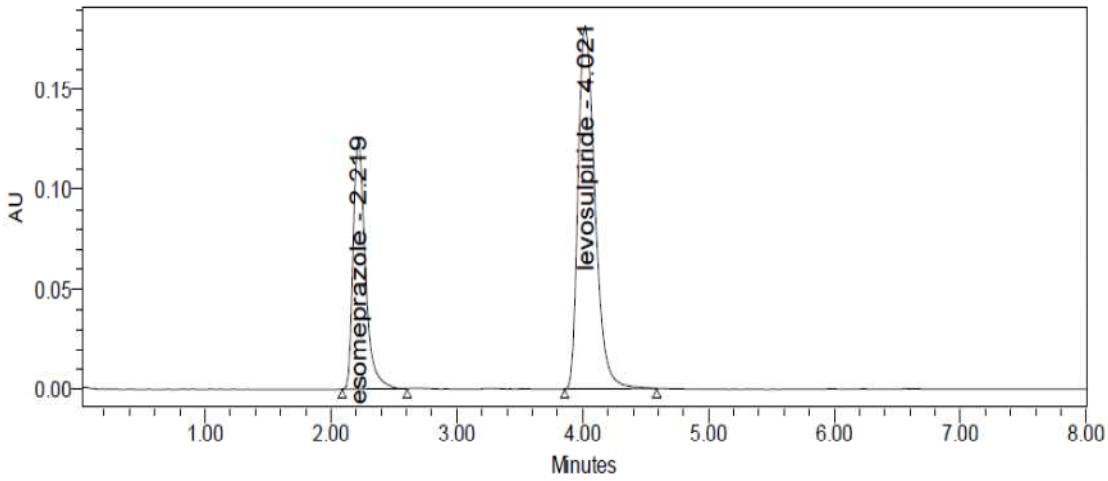

Fig: 6.2 Typical chromatogram of Esomeprazole and Levosulpiride.

2. Linearity

Six Linear concentrations of Esomeprazole(20-120ppm) and Levosulpiride (37.5ppm to 225ppm) are prepared and Injected. Regression equation of the the Esomeprazole and Levosulpiride are found to be, y = 10568x + 307.3, y = 11649.x + 120.7 And regression co-efficient was 0.999.

Table: 6.2 Calibration data of Esomeprazole and Levosulpiride method

S. No.	Concentration Esomeprazole (µg/ml)	Response	Concentration Levosulpiride (µg/ml)	Response
1	0	0	0	0
2	20	219743	37.5	436194
3	40	420301	75	891483
4	60	632028	112.5	1323196
5	80	839036	150	1697713
6	100	1049902	187.5	2202932
7	120	1279655	225	2630631

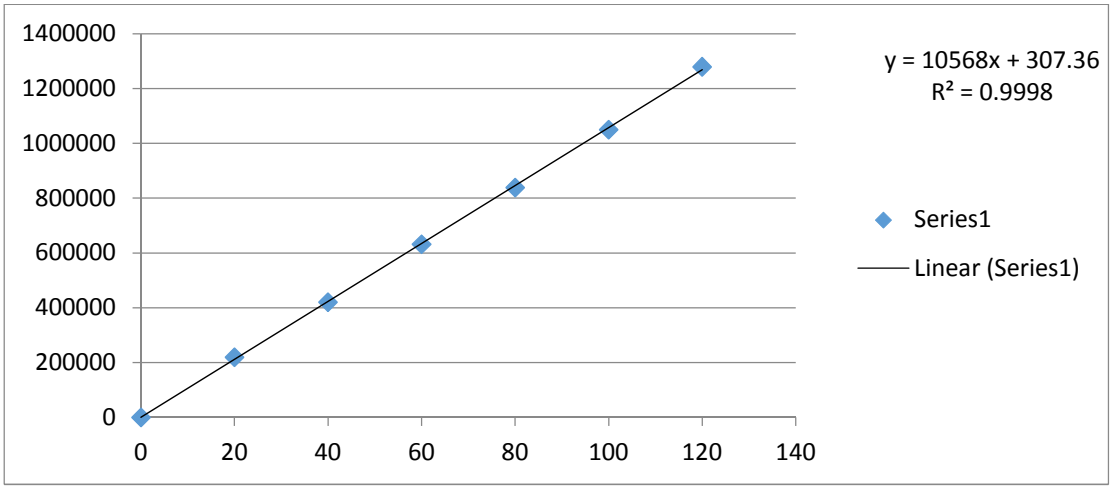

Fig: 6.3 Calibration curve of Esomeprazole

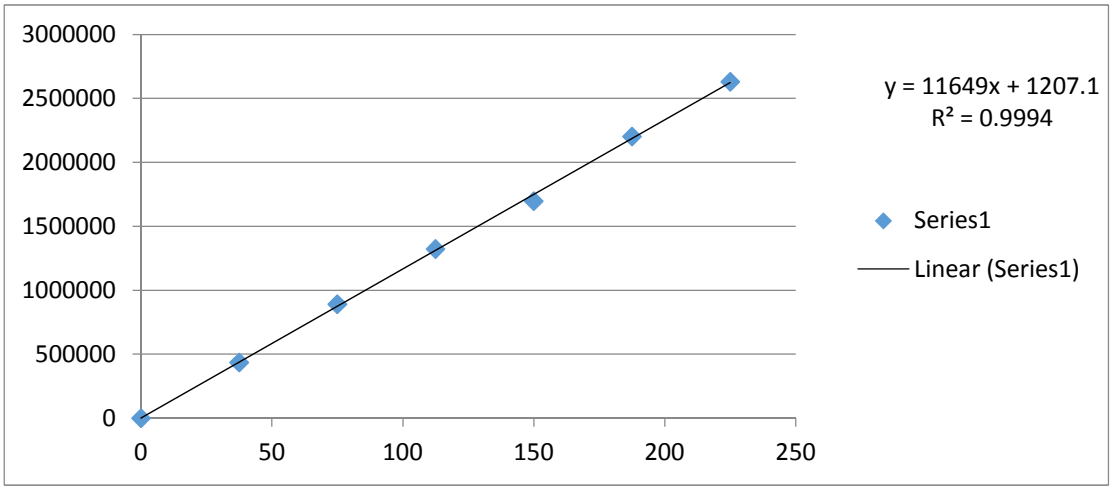

Fig: 6.4 Calibration curve of Levosulpiride

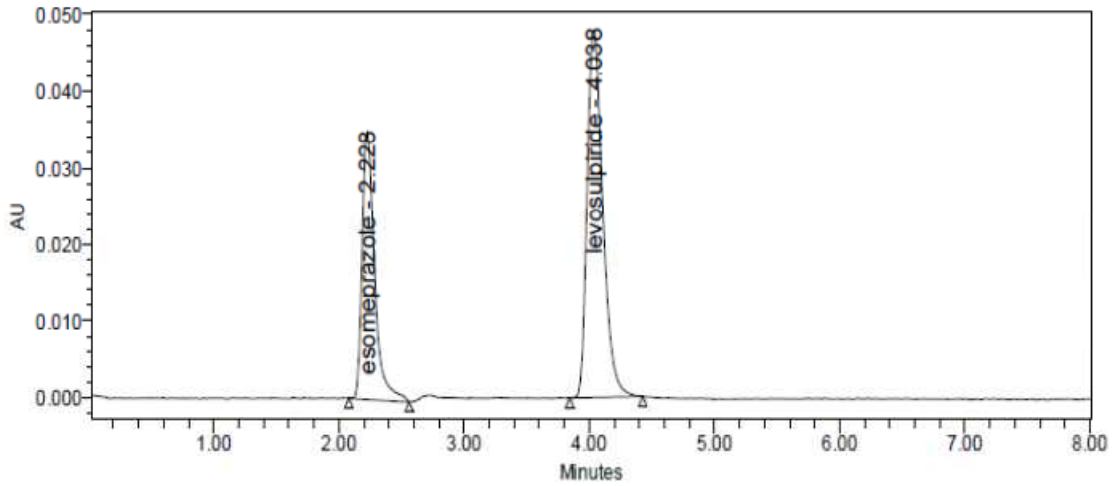

Fig: 6.5 Linearity 25% Chromatogram of Esomeprazole and Levosulpiride method.

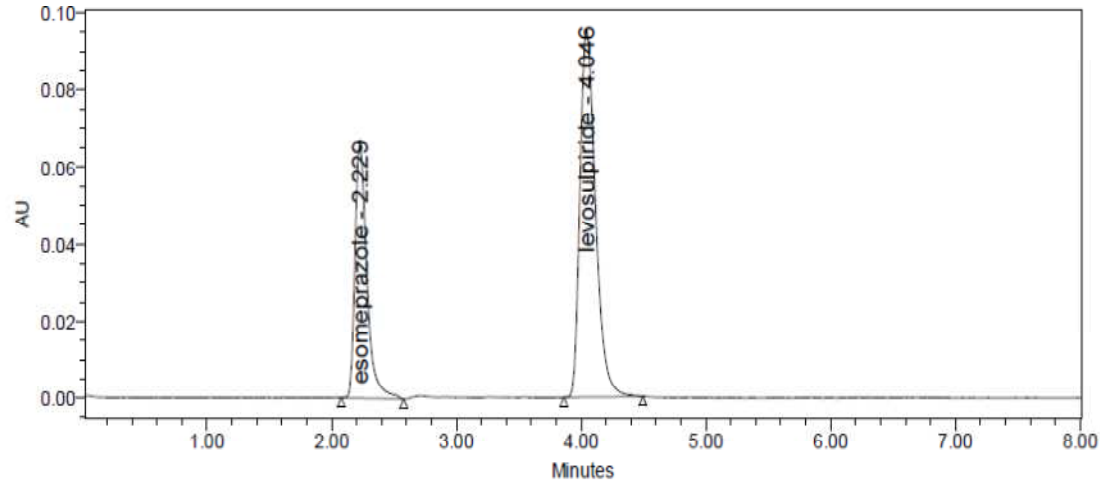

Fig: 6.6 Linearity 50% Chromatogram of Esomeprazole and Levosulpiride method.

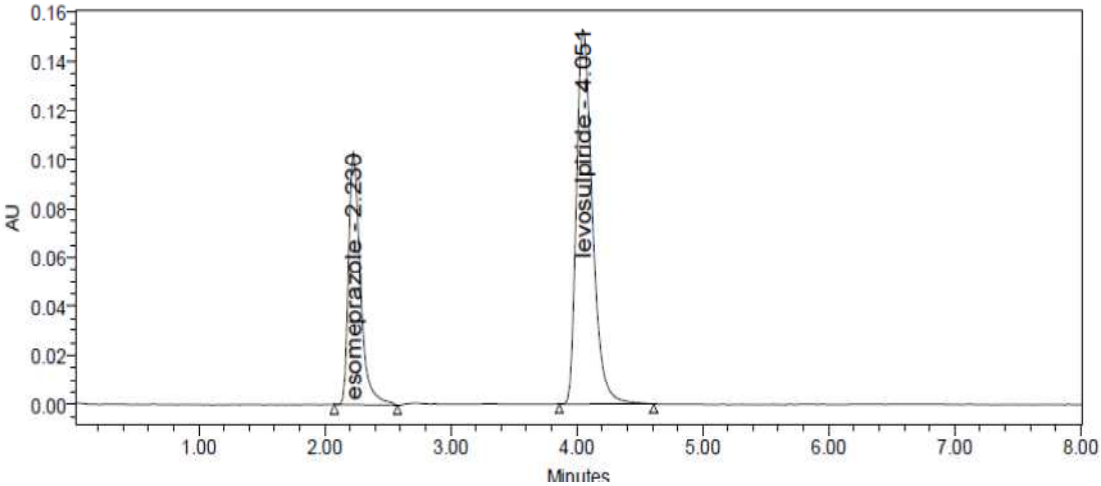

Fig: 6.7 Linearity 75% Chromatogram of Esomeprazole and Levosulpiride method.

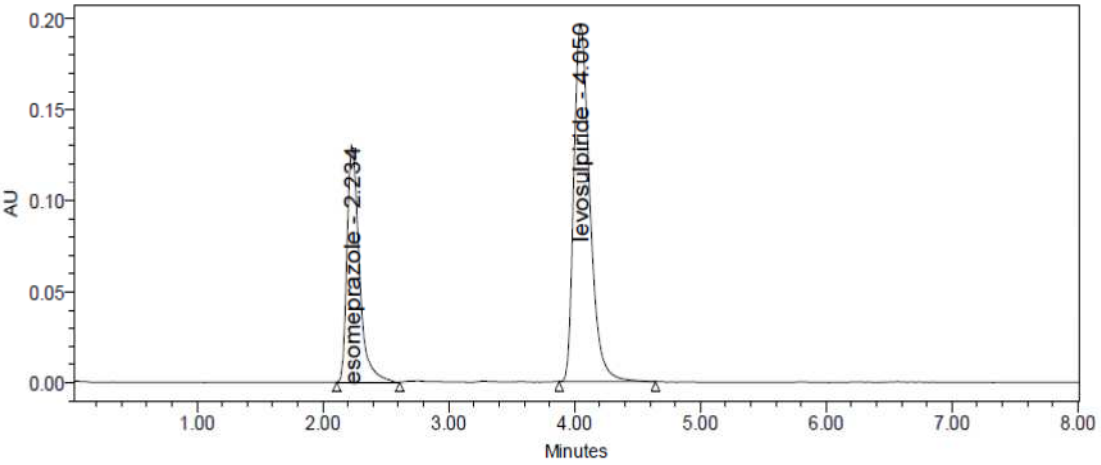

Fig: 6.8 Linearity 100% Chromatogram of Esomeprazole and Levosulpiride method.

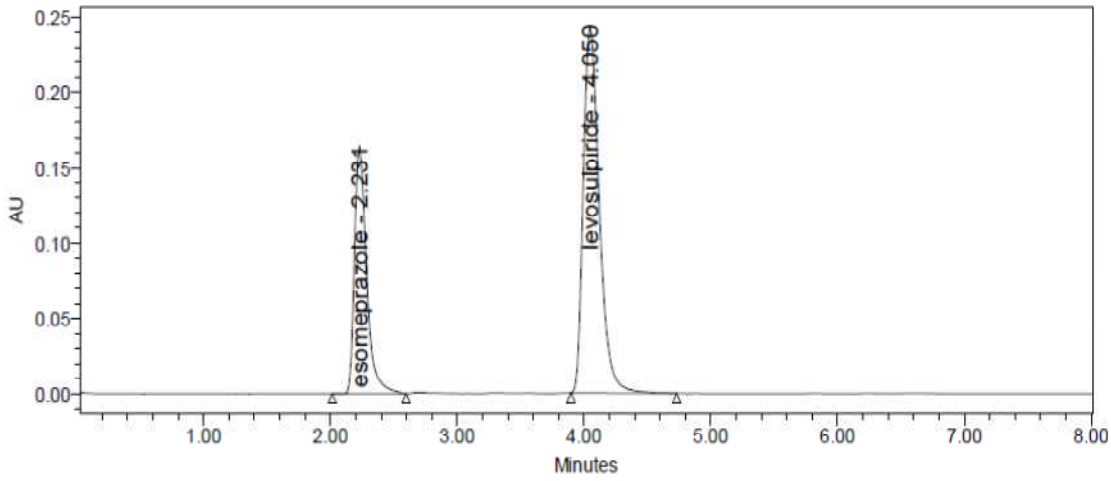

Fig: 6.9 Linearity 125% Chromatogram of Esomeprazole and Levosulpiride method.

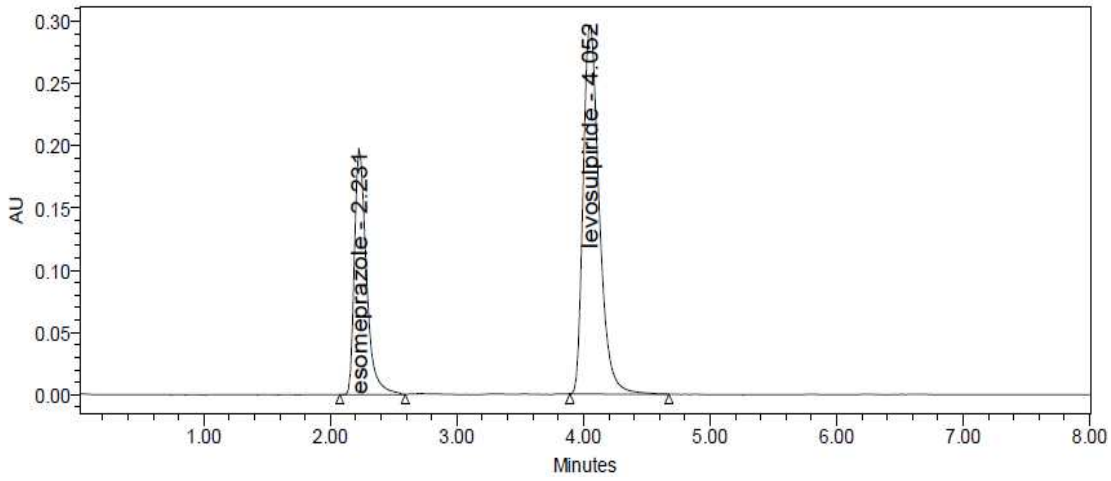

Fig: 6.10 Linearity 150% Chromatogram of Esomeprazole and Levosulpiride method.

3. Precision:

Intraday precision (Repeatability)

Intraday Precision was performed and % RSD for Esomeprazole and Levosulpiride were found to be 1.3% and 0.1% respectively.

Table: 6.3 Repeatability results for Esomeprazole and Levosulpiride .

Sr. No.	Esomeprazole	Levosulpiride
1	814986	1754081
2	825795	1754437
3	840851	1758333
4	819155	1752184
5	822084	1752359
6	839797	1754366
Mean	827111	1754293
Std. Dev.	10835.5	2217.2
%RSD	1.3	0.1

*Average of six determinations

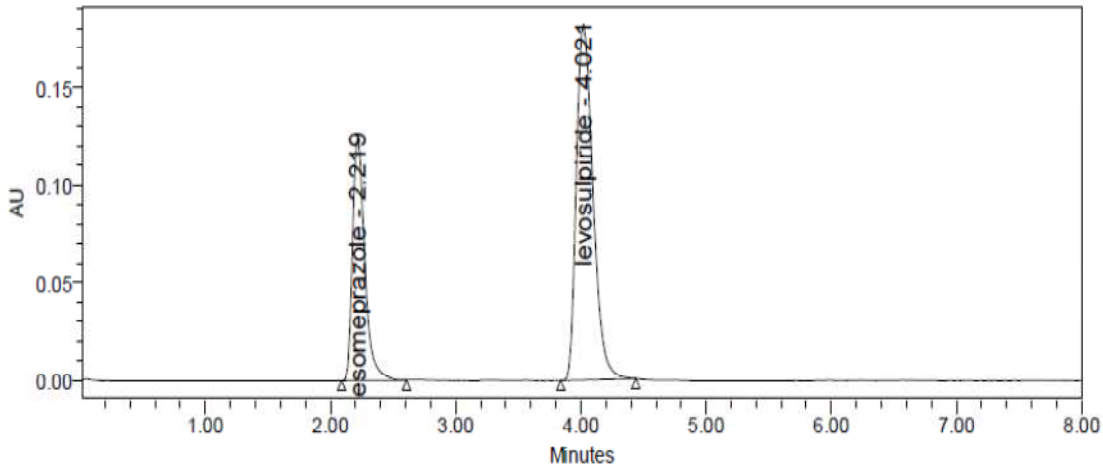

Fig: 6.11 Repeatability Chromatogram of Esomeprazole and Levosulpiride method.

Inter day precision

Inter day precision was performed with 24 hrs time lag and the %RSD Obtained for Esomeprazole and Levosulpiride were 1.0% and 0.5%.

Table 6.4 Inter day precision results for Esomeprazole and Levosulpiride

Sr. No.	Esomeprazole	Levosulpiride
1	834986	1743927
2	843663	1732187
3	830708	1728243
4	848290	1726706
5	832084	1720428
6	848665	1739622
Mean	839732.7	1731852
Std. Dev.	8135.6	8675.3
%RSD	1.0	0.5

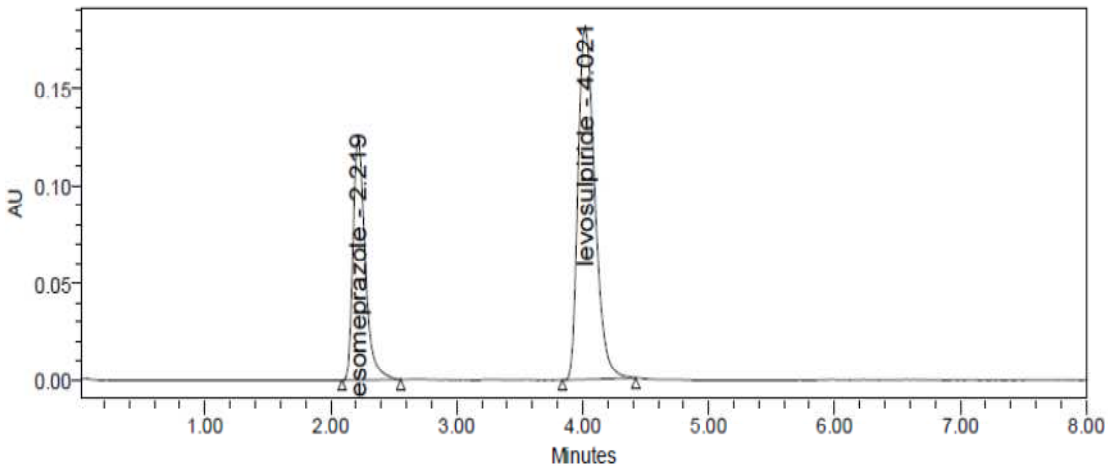

Fig: 6.12 Inter Day precision Chromatogram of Esomeprazole and Levosulpiride method

4. Accuracy

Three concentrations 50%, 100%, 150%, were injected in a triplicate manner and amount Recovered and % Recovery were displayed in Table 6.5.

Table: 6.5 Accuracy results of Esomeprazole and Levosulpiride

Sample	Amount added (µg/ml)	Amount Recovered (µg/ml)	Recovery (%)	% RSD
Esomeprazole	40	39.04	99.97	0.2
	80	79.82	99.99	0.48
	120	120.99	100.32	0.71
Levosulpiride	75	75.72	100.250	0.79
	150	150.15	101.58	0.33
	225	225.55	101.48	0.18

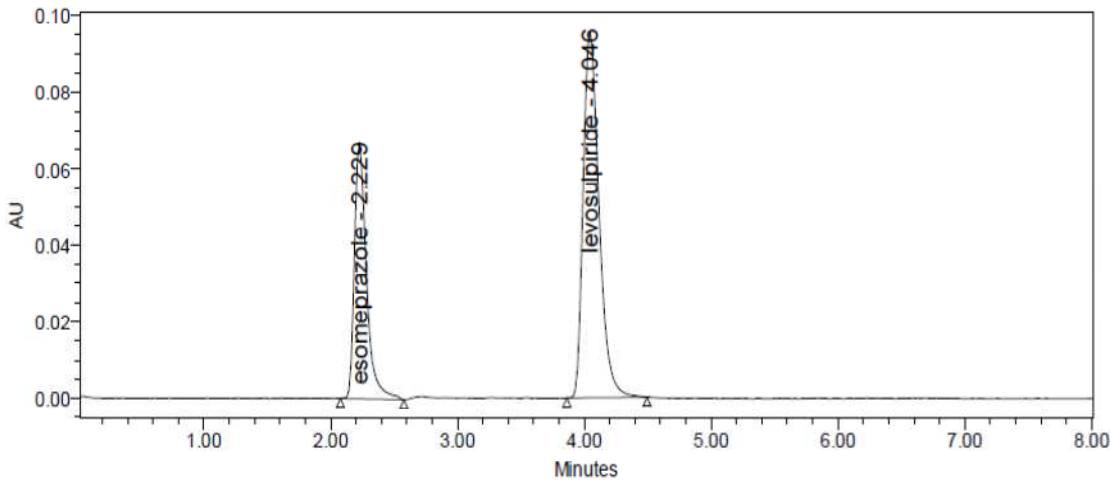

Fig: 6.13 Accuracy 50% Chromatogram of Esomeprazole and Levosulpiride method.

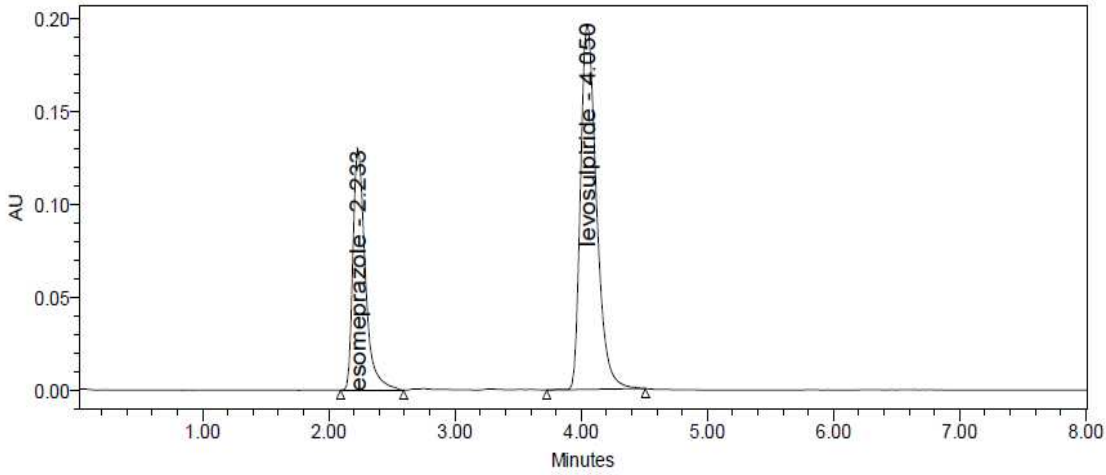

Fig: 6.14 Accuracy 100% Chromatogram of Esomeprazole and Levosulpiride method.

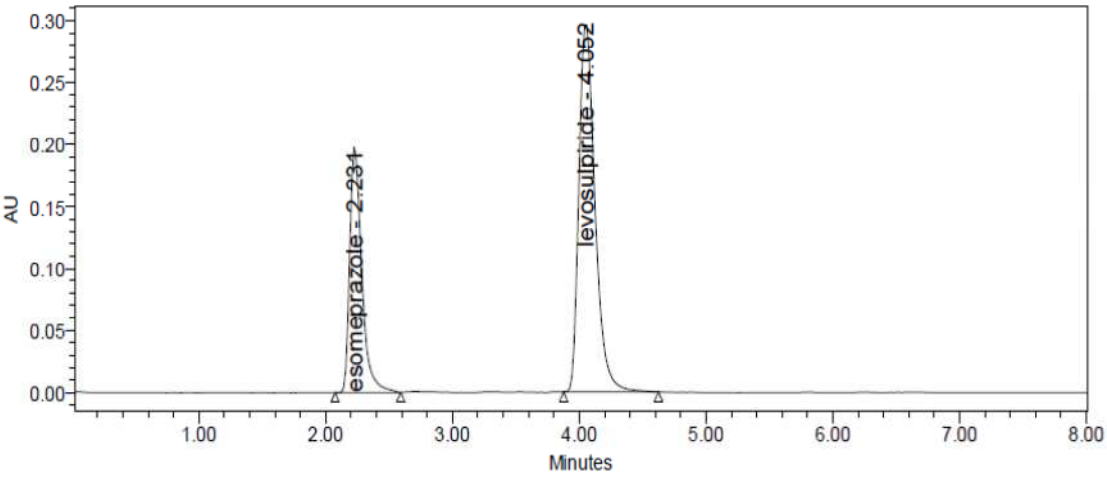

Fig: 6.15 Accuracy 150% Chromatogram of Esomeprazole and Levosulpiride method.

5. LOD

The parameter LOD was determined by analysis of sample with known concentration of analyte and by establishing the minimum level at which the analyte can be reliably detected. LOD for Esomeprazole and Levosulpiride were found to be 0.10 and 1.04respectively.

Fig : 6.16 LOD Chromatogram of Esomeprazole and Levosulpiride method.

6. LOQ

The parameter LOD was determined by analysis of sample with known concentration of analyte and by establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision. LOQ for Esomeprazole and Levosulpiride were found to be 0.29 and 1.04 respectively.

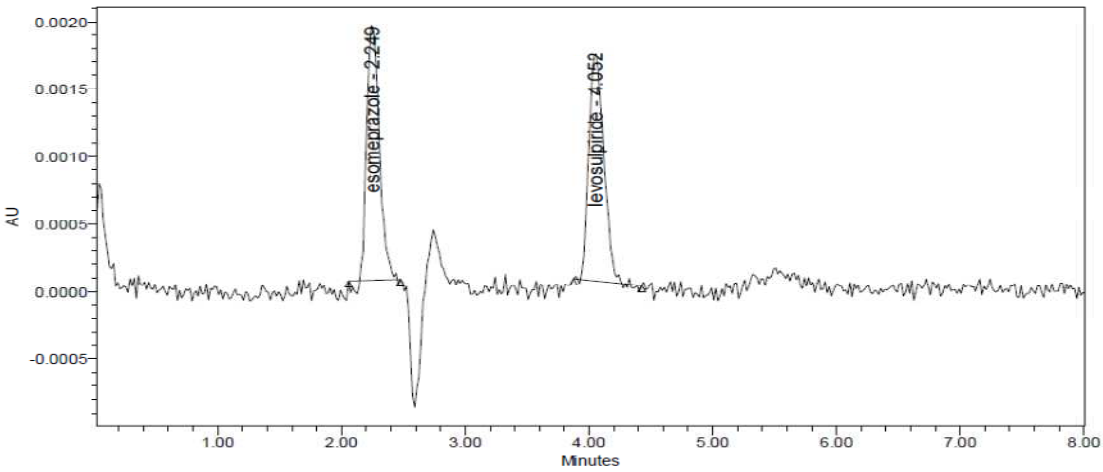

Fig : 6.17 LOQ Chromatogram of of Esomeprazole and Levosulpiride method.

7. **Robustness**

Small deliberate changes in method like Flow rate, mobile phase ratio, and temperature are made but there were no recognized change in the result and are within range as per ICH Guide lines.

Table 6.6 Robustness data of Esomeprazole and Levosulpiride method.

S.NO	Robustness condition	Esomeprazole %RSD	Levosulpiride %RSD
1	Flow minus	0.4	1.0
2	Flow Plus	0.4	0.4
3	Mobile phase minus	0.1	0.0
4	Mobile phase Plus	0.1	0.1
5	Temperature minus	0.2	0.5
6	Temperature Plus	0.3	0.5

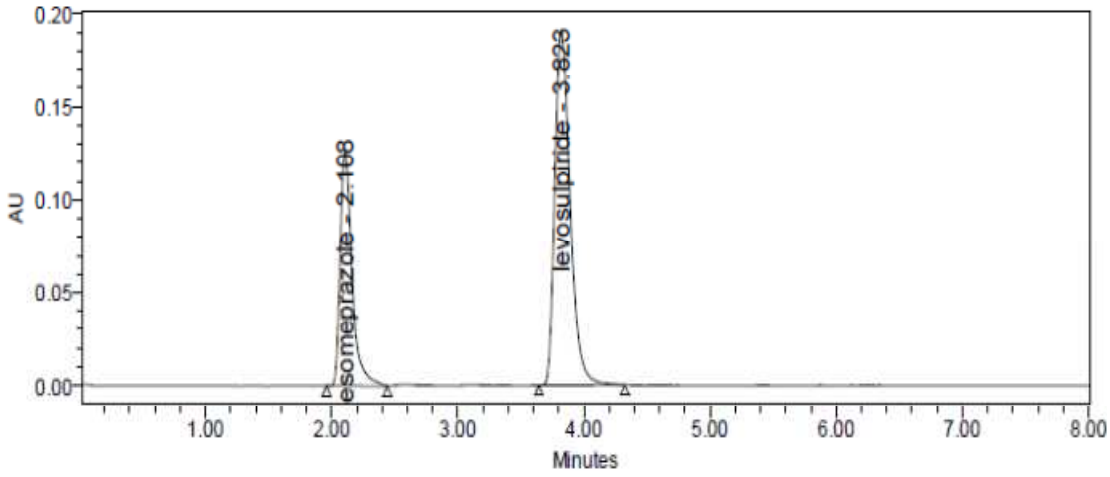

Fig: 6.18 Flow minus Chromatogram of Esomeprazole and Levosulpiride method.

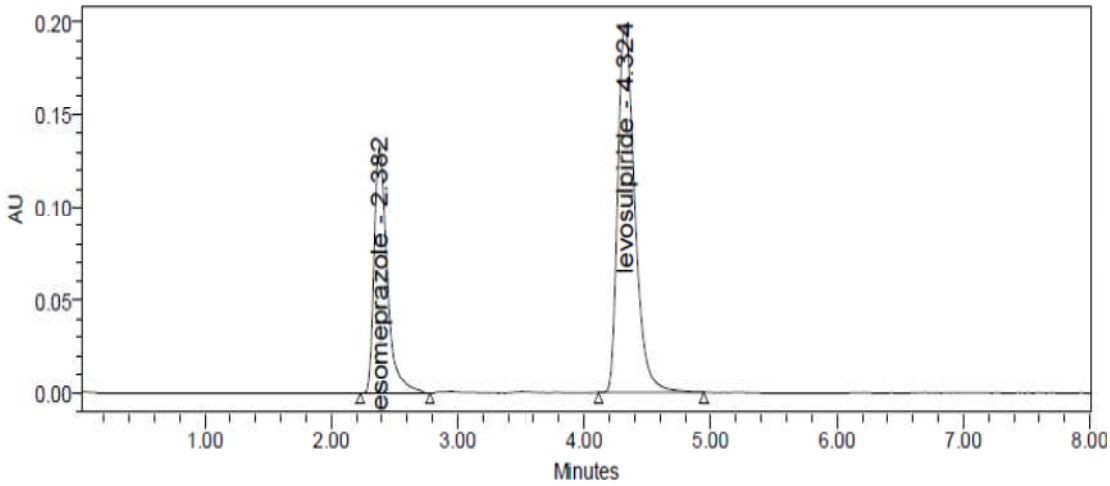

Fig: 6.19 Flow plus Chromatogram of Esomeprazole and Levosulpiride method.

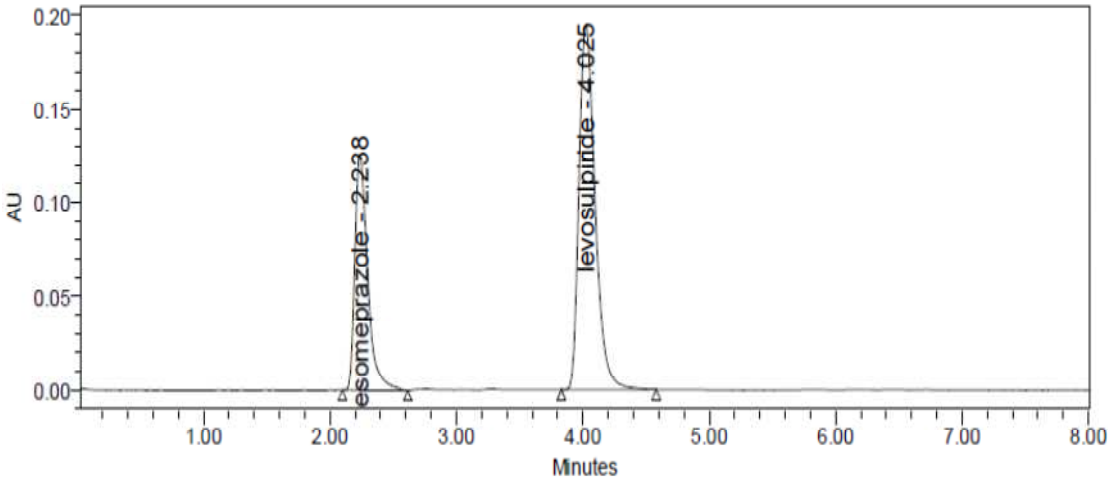

Fig: 6.20 Mobile phase minus Chromatogram of Esomeprazole and Levosulpiride method.

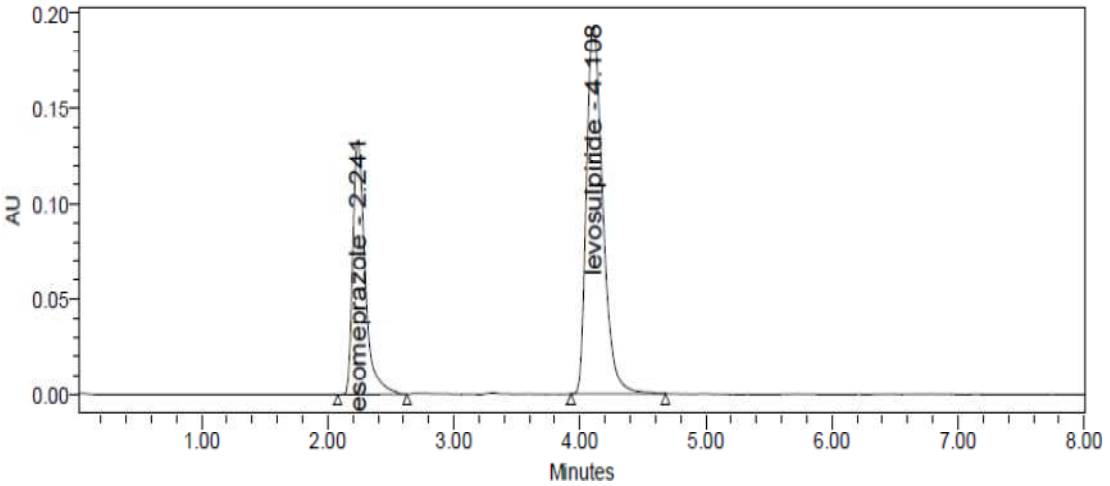

Fig: 6.21 Mobile phase Plus Chromatogram of Esomeprazole and Levosulpiride method.

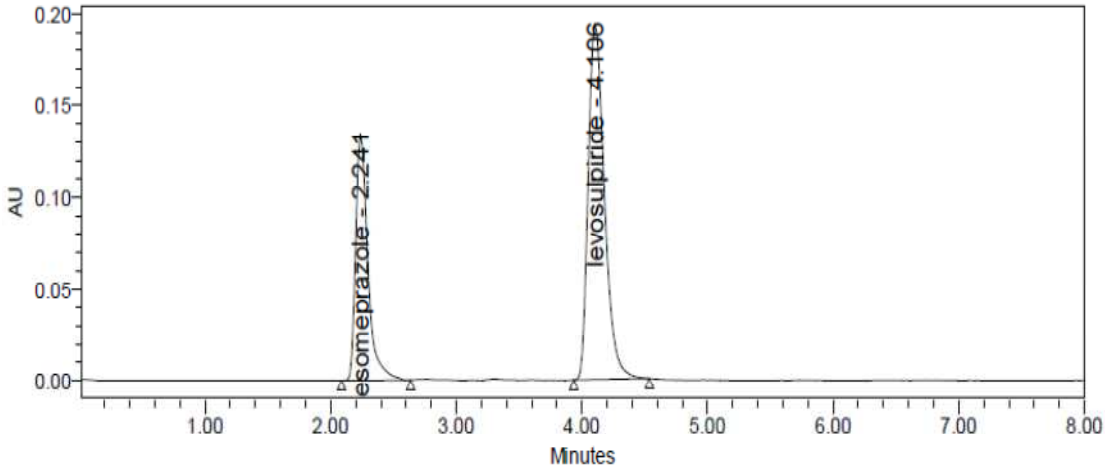

Fig: 6.22 Temperature minus Chromatogram of Esomeprazole and Levosulpiride method.

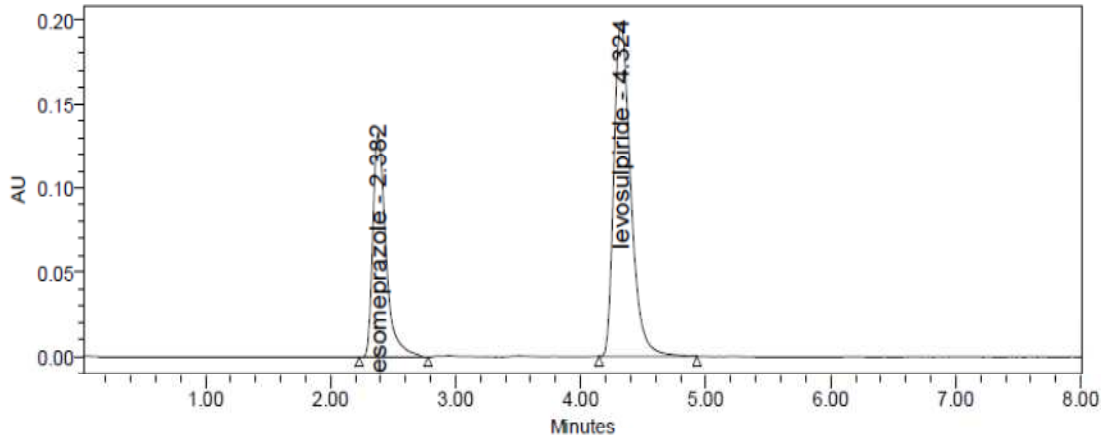

Fig: 6.23 Temperature Plus Chromatogram of Esomeprazole and Levosulpiride method.

Assay

Standard preparations are made from the API and Sample Preparations are from Formulation. Both sample and standards are injected six homogeneous samples. Drug in the formulation was estimated by taking the standard as the reference. The Average %Assay was calculated and found to be 100.08% and 101.16% for Esomeprazole and Levosulpiride respectively.

Table 6.7 Assay of Tablet

S. No.	Esomeprazole %Assay	Levosulpiride %Assay
1	99.51463	101.865
2	100.5488	101.1793
3	99.00477	100.9489
4	101.1002	100.8591
5	99.16876	100.4924
6	101.1449	101.6136
AVG	100.08	101.16
STDEV	0.97	0.51
%RSD	0.97	0.50

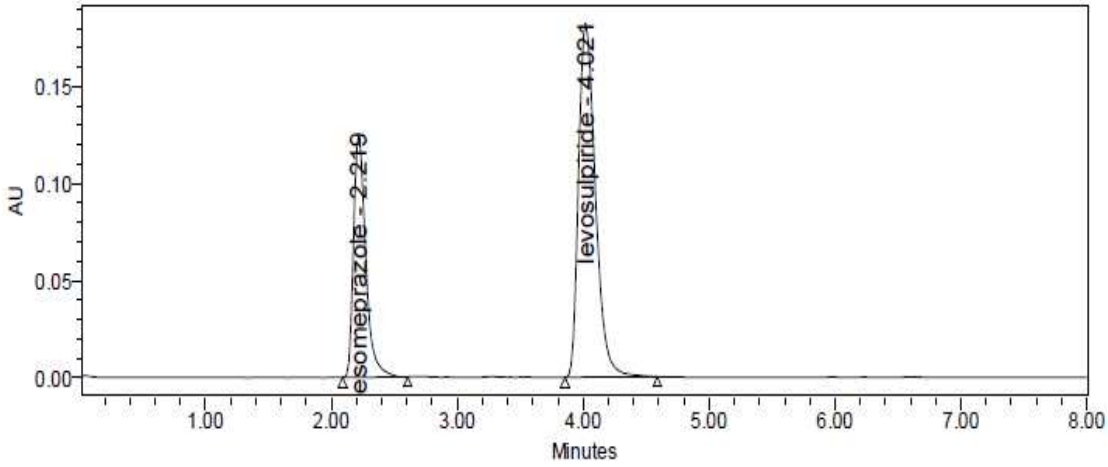

Fig: 6.24 Assay of Tablet

SUMMARY AND CONCLUSION

7.1 Summary Table

Parameters	Esomeprazole	Levosulpiride
Calibration range (mcg / ml)	20-120ppm	37.5-225ppm
Optimized wavelength	290nm	290nm
Retention time	2.2min	4.0min
Regression equation (Y*)	y = 10568.x + 307.3	y = 11649.x + 1207
Correlation coefficient(r^2)	0.999	0.999
Precision (% RSD*)	0.97	0.50
% Recovery	100.08%	101.16%
Limit of Detection (mcg / ml)	0.10ppm	0.34ppm
Limit of Quantitation (mcg / ml)	0.29ppm	1.04ppm

Conclusion

A simple, Accurate, precise method was developed for the simultaneous estimation of the Esomeprazole and Levosulpiride in Tablet dosage form. Retention time of Esomeprazole and Levosulpiride were found to be 2.2min and 4.0min. %RSD of the Esomeprazole and Levosulpiride were and found to be 0.97 and 0.50 respectively. %Recover was Obtained as 100.08% and 101.16% for Esomeprazole and Levosulpiride respectively. LOD, LOQ values are obtained from regression equations of Esomeprazole and Levosulpiride were 0.10ppm, 0.34ppm and 0.29ppm, 1.04ppm respectively. Regression equation of Esomeprazole is y = 10568x + 307.3, and of Levosulpiride is y = 11649.x + 1207. Retention times are decreased and that run time was decreased so the method developed was simple and economical that can be adopted in regular Quality control test in Industries.

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