"DEVELOPMENT AND VALIDATION OF STABILITY INDICATING RP-HPLC METHOD FOR FLUVOXAMINE MALEATE"

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BY

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CERTIFICATE

This is to certify that the dissertation work entitled, "Development and Validation of Stability Indicating RP-HPLC Method for Fluvoxamine Maleate" submitted by ***Ms. SHIKHA GUPTA*** *with reg. no.* ***10MPH308*** *in partial fulfillment for the award of Master of Pharmacy in "Pharmaceutical Analysis" is a bonafide research work carried out by the candidate at ADL, Piramal Pharmaceutical Development Service Ltd., Pharmez under our guidance. This work is original and has not been submitted in part or full for any other degree or diploma to this or any other university or institution.*

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DECLARATION

I declare that the thesis "Development and Validation of Stability Indicating RP-HPLC Method for Fluvoxamine Maleate", has been prepared by me under the guidance of Dr. Priti J. Mehta, Professor & Head, Department of Pharmaceutical Analysis, Institute of Pharmacy, Nirma University and Mr. Anirbanroy Chowdhury, Sr. Group Leader, ADL, Piramal Pharmaceutical Development Service Pvt. Ltd. No part of this thesis has formed the basis for the award of any degree or fellowship previously.

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ABSTRACT

A new, simple, accurate, precise, rapid and selective stability indicating reverse-phase high performance liquid chromatography (RP-HPLC) method has been developed and validated for quantification of Fluvoxamine Maleate from its tablet dosage formulation. The method is based on reverse phase chromatography using Phenomenex Luna C8 (150mm x 46mm, 5µ particle size column. The separation in all the degradation peaks were achieved by using gradient elution of 20 mM KH_2PO_4(pH-3) and Acetonitrile at flow rate 1.0 mL/min and UV detection at 234 nm with the total run time of 40 min. The column is maintained at 30°C throughout the analysis. Stability indicating capability is established by forced degradation experiment of Fluvoxamine Maleate to acid, alkali, neutral, oxidation, thermal and photodegradation conditions according to ICH guidelines. The peaks of degradation samples were found to be very pure with complete resolution. The linear regression analysis data for the calibration plots showed a very good linear relationship with concentration range of 0.05-750µg/mL with the linearity equation of y = 99507x + 64401(r^2 = 0.9999). The method is validated for specificity, accuracy, precision, linearity, and robustness, limit of detection and limit of quantification as per ICH guidelines. The limit of detection and quantitation were found to be 0.015µg/mL and 0.05µg/mLrespectively. Additionally, a mass-compatible method for LC/MS instrument is also developed for the Characterization of different degradation peaks formed during the forced decomposition studies. The degradation behavior was found to be similar in case of Fluvoxamine Maleate API and its marketed dosage form with the % assay value of 98.22%. So, the developed RP-HPLC method can also be applied for the estimation of Fluvoxamine Maleate from its dosage form. As the method could effectively separate the drug from its degradation products, it can be employed as a stability indicating assay method.

CHAPTER-1
INTRODUCTION

1.1 INTRODUCTION TO DISEASE [1-5]

Depression is a state of low mood and aversion to activity that can affect a person's thoughts, behavior, feelings and physical well-being.Depressed people may feel sad, anxious, empty, hopeless, helpless, worthless, guilty, irritable, or restless. They may lose interest in activities that once were pleasurable; experience loss of appetite or overeating, have problems concentrating, remembering details, or making decisions; and may contemplate or attempt suicide. Insomnia, excessive sleeping, fatigue, loss of energy, or aches, pains or digestive problems that are resistant to treatment may be present.

Depressive disorders are mood disorders that come in different forms, just as do other illnesses, such as heart disease and diabetes. Three of the most common types of depressive disorders are discussed below. However, remember that within each of these types, there are variations in the number, timing, severity, and persistence of symptoms. There are also differences in how individuals experience depression based on age.

Major depression

Major depression is characterized by a combination of symptoms that last for at least two weeks in a row, including sad and/or irritable mood (see symptom list), that interfere with the ability to work, sleep, eat, and enjoy once-pleasurable activities. Difficulties in sleeping or eating can take the form of excessive or insufficient of either behavior. Disabling episodes of depression can occur once, twice, or several times in a lifetime.

Dysthymia

Dysthymia is a less severe but usually more long-lasting type of depression compared to major depression. It involves long-term (chronic) symptoms that do not disable but yet prevent the affected person from functioning at "full steam" or from feeling good. Sometimes, people with dysthymia also experience episodes of major depression. This combination of the two types of depression is referred to as double-depression.

Bipolar disorder (manic depression)

Another type of depression is bipolar disorder, which encompasses a group of mood disorders that were formerly called manic-depressive illness or manic depression. These conditions show a particular pattern of inheritance. Not nearly as common as the other types of depressive disorders, bipolar disorders involve cycles of mood that include at least one episode of mania or hypomania and may include episodes of depression as well. Bipolar

disorders are often chronic and recurring. Sometimes, the mood switches are dramatic and rapid, but most often they are gradual.

When in the depressed cycle, the person can experience any or all of the symptoms of a depressive disorder. When in the manic cycle, any or all of the symptoms listed later in this article under mania may be experienced. Mania often affects thinking, judgment, and social behavior in ways that cause serious problems and embarrassment. For example, indiscriminate or otherwise unsafe sexual practices or unwise business or financial decisions may be made when an individual is in a manic phase.

A significant variant of the bipolar disorders is designated as bipolar II disorder. (The usual form of bipolar disorder is referred to as bipolar I disorder.) Bipolar II disorder is a syndrome in which the affected person has repeated depressive episodes punctuated by what is called hypomania (mini-highs). These euphoric states in bipolar II do not fully meet the criteria for the complete manic episodes that occur in bipolar I.

Symptoms of depression and mania

Not everyone who is depressed or manic experiences every symptom. Some people experience a few symptoms and some many symptoms. The severity of symptoms also varies with individuals. Less severe symptoms that precede the more debilitating symptoms are called warning signs.

Depression symptoms of major depression or manic depression [6]

- Persistently sad, anxious, angry, irritable, or "empty" mood
- Feelings of hopelessness or pessimism
- Feelings of worthlessness, helplessness, or excessive guilt
- Loss of interest or pleasure in hobbies and activities that were once enjoyed, including sex
- Social isolation, meaning the sufferer avoids interactions with family or friends
- Insomnia, early-morning awakening, or oversleeping
- Decreased appetite and/or weight loss, or overeating and/or weight gain
- Fatigue, decreased energy, being "slowed down"
- Crying spells
- Thoughts of death or suicide, suicide attempts
- Restlessness, irritability
- Difficulty concentrating, remembering, or making decisions
- Persistent physical symptoms that do not respond to treatment, such asheadaches, digestive disorders, and/or chronic pain

Mania symptoms of manic depression

- Inappropriate elation
- Inappropriate irritability or anger
- Severe insomnia or decreased need to sleep
- Grandiose notions, like having special powers or importance
- Increased talking speed and/or volume
- Disconnected thoughts or speech
- Racing thoughts
- Severely increased sexual desire and/or activity
- Markedly increased energy
- Poor judgment
- Inappropriate social behavior

There are many medications available to treat **depression**.

Different types of **antidepressants** include:

- Selective serotonin reuptake inhibitors (**SSRIs**)
- **Serotonin-norepinephrine reuptake inhibitors** (**SNRIs**)
- Monoamine oxidase inhibitors (MAOIs)
- **Tricyclic antidepressants**
- Miscellaneous other antidepressants.

1.2 INTRODUCTION TO DRUG ANALYSIS [7]

Analytical chemistry is a branch of chemistry that determines the nature and identity of a substance and its composition. In the early twentieth century there were only four accepted branches of chemistry namely, organic chemistry, inorganic chemistry, physical chemistry and biochemistry. Its importance grew, and in the process, absorbed techniques and skills from all other four branches so by the 1950s, analytical chemistry was finally accepted as a branch of chemistry in its own right. The ability to provide timely, accurate, and reliable data is central to the discovery, development, and manufacture of pharmaceuticals. Analytical data are used to screen potential drug candidates, aid in the development of drug synthesis, support formulation studies, monitor the stability of bulk pharmaceuticals and formulated products, and test final products for release. The quality of analytical data is a key factor in the success of a drug development program.

Problems increase as additional people, laboratories and equipments are used to perform the method. When the method is used in the developer‟s laboratory, a small optimization can

usually be made to make the method work, but the flexibility to change it is lost, once the method is transferred to other laboratories or used for official product testing, where methods are submitted to regulatory agencies and changes may require formal approval before they can be implemented for official testing. The best way to minimize method problems is to perform adequate validation experiments during development. The need of the sophisticated analytical instruments and determination using them is almost a routine process for the modern analytical laboratories. It has been vast expanding areas of knowledge as the instrument automation is in ever-increase in power and scope. Further all the manual techniques in the line of analytical studies had steadily been transferred to the instrumental techniques. Analytical methods are generally classified as Physical and Chemical analysis. Physical analysis includes measurement of particle size, dimension, thickens of a solid dosage forms etc. Basically chemical analysis can be divided into three broad categories which are as follow.

1.2.1 TYPES OF ANALYSIS [8]

1.2.1.1 Qualitative Analysis

The Qualitative analysis identifies the nature of substance, and if it is mixture, the nature of the components present.

1.2.1.2 Quantitative Analysis

The Quantitative analysis determines the elemental composition of the substance and the quantitative distribution of each component.

1.2.1.3 Structural Analysis

Analysis which helps in finding the spatial arrangement of atoms in a molecule and the presence or position of certain organic functional group in a given compound. In addition **surface analysis** plays an important role in material studies to obtain surface related physical properties such as topography, depth profiling, orientation of molecule etc. Chemical analysis has some basic steps like, choice of method, sampling, preliminary sample treatment, separations, final measurement and assessment of results. It is with the first step viz. choice of method, care should be exercised to select the proper instrument to carry out fruitful analysis. A wrong selection at this point will leads to a meaningless analysis. Analytical methods are broadly classified as Physical, Chemical and Instrumental analysis.

- Physical observation includes description of the compound, measurements of its dimension (shape, size), colour, odour etc.
- Chemical analysis includes titrimetric analysis of the compound such as Potentiometry, Iodometry, Argentometry, Permagnometry etc.
- Instrumental methods of chemical analysis have become the backbone of the experimental chemistry. The choice of an instrumental method for the determination of a specific element or compound really involves,

1. The instrument to be used

2. The chemical system

The growth of instrumental analysis is related to the developments in the fields of electronic because the generation, transduction, amplification and display of a signal can be done in a convenient manner. Following table (table1.1) lists various instrumental methods along with their major application in pharmaceutical field.

Table 1.1: Various Instrumental Methods of Analysis [8]

Instrumental Method	Property Measured	Application
UV- Visible Spectrophotometry	Absorption of radiation	Identification of the functional group and quantitation of unsaturated compounds.
FTIR Spectroscopy	Absorption of radiation	Quantitative analysis of organic compound at high conc. Level.
Atomic Absorption Spectroscopy	Absorption of radiation	Quantitation of metals or metalloids.
Flame Photometry	Emission of radiation	Quantitation of alkali metals or alkaline earth metals.
X-Ray Diffraction	Diffraction of radiation	Identification of crystal lattice structure.
Magnetic Resonance (NMR)	Nuclear spin energy level of a mol in an applied	Identifies type of hydrogen and carbon in

LC-NMR	magnetic field	organic molecules. Analysis of trace impurity and degradants.
Thermal analysis (DTA \ DSC)	Difference in Temperature \ heat energy	Determination of melting point, Polymorphism, Drug – excipients compatibility
Mass spectrometry LC-MS \ GC-Ms	Mass to charge ratio (m\z)	Mol. Wt. determination Quantification of the analyte in liquid or gas sample. Analysis of the biological sample.

Analytical chemistry deals with methods for determining the chemical composition of samples. A compound can be often measured by several methods. The choice of analytical methodology is based on many considerations, such as chemical properties of the analyte and its concentration, sample matrix, the speed and cost of the analysis, type of measurements i.e., quantitative or qualitative and the number of samples.

- A qualitative method provides numerical information regarding the relative amounts of one or more of the species (the analytes) in the sample.
- Quantitative information is required before a quantitative analysis can be performed.

The presence of unwanted chemicals i.e., impurities even in small amounts may influence the afficacy and safety of the pharmaceutical products. Impurity profiling (i.e. the identity as well as the quantity of impurity in the pharmaceuticals), is now getting important critical attention from regulatory authorities. The different pharmacopoeias, such as the British Pharmacopoeia, United States Pharmacopoeia are slowly incorporating limits to allowable levels of impurities present in the API's or formulation. Hence, there is a need to develop method for the identification of these impurities in the drug substances.

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. Very often there is a time occupied 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. The different activity of R&D includes drug development, synthesis, manufacturing, formulation, clinical trials, evaluation and finally launching i.e. finished products. Regulatory and quality assurance functions are closely associated with these processes[6]. Before submitting the drug product for approval to the regulatory authorities, it is necessary to assure that all batches of drug products comply with specific standards, utilization of approved ingredients and production methods. It becomes the responsibility of pharmaceutical analysts in quality control (QC), quality assurance (QA) department. The methods are generally developed in an analytical R&D and transferred to QC department, or other departments. Quality assurance and quality control play a central role in estimating the safety and efficacy of medicines. A highly specific and sensitive analytical technique holds the key to design, development, standardization and quality control of medicinal products. They are equally important in pharmacokinetics and in drug metabolism studies, both of which are fundamental to the assessment of bioavailability and the duration of clinical response[6]. The pharmaceutical analysts play a major role in assuring the identity, safety, efficacy and quality of the drug product. Safety and efficacy studies require that drug substance and drug product meet two critical requirements are:

- Established identity and purity
- Established bioavailability and dissolution

In brief, the reasons for the development of newer methods of drug analysis are [9]:

- The drug or drug combination may not be official in any pharmacopoeias.
- A proper analytical procedure for the drug may not be available in the literature due to patent regulations.
- Analytical methods may not be available for the drug in the form of a formulation due to the interference caused by the formulation excipients.
- Analytical methods for the quantitation of the drug in biological fluids may not be available.
- Analytical methods for a drug in combination with other drugs may not be available.
- The existing analytical procedures may require expensive reagents and solvents.

- Analytical techniques that are generally used for drug analysis are biological and microbiological methods, radioactive methods, physical methods and miscellaneous techniques like conventional titrimetric, gravimetric and polarimetric methods.

1.3 INTRODUCTION TO HPLC

HPLC originally referred to the fact that high pressure was needed to generate the flow required for liquid chromatography in packed columns. In the beginning, instrument components only had the capability of generating pressures of 500psi (35 bars). This was called High Pressure Liquid Chromatography (HPLC). The early 1970's saw a tremendous leap in technology. These new "HPLC" instruments could develop up to 6,000psi (400 bars) of pressure, and included improved detectors and columns. HPLC really began to take hold in the mid to late 1970's. With continued advances in performance, the name was changed to High Performance Liquid Chromatography (HPLC). High Performance Liquid Chromatography (HPLC) is now one of the most powerful tools in analytical chemistry, with the ability to separate, identify and quantitate the compounds that are present in any sample that can be dissolved in a liquid. Today, trace concentrations of compounds, as low as "parts per trillion" (ppt), are easily obtained. HPLC can be applied to just about any sample, such as pharmaceuticals, food, nutraceuticals, cosmetics, environmental matrices, forensic samples, and industrial chemicals.

High Performance Liquid Chromatography is the most widely used analytical separation technique. HPLC has been rapidly developed with the introduction of new pumping methods, more reliable columns and a variety of detectors. Most of the drugs in multicomponent dosage forms can be analyzed by HPLC methods because of the several advantages like rapidity, specificity, accuracy, precision and ease of automation in this method. HPLC method eliminates tedious extraction and isolation procedures.

Some of the advantages are: **[10]**

- speed (analysis can be accomplished in 20 minutes or less)
- greater sensitivity (various detectors can be employed)
- precise and reproducible
- calculations are done by integrator itself
- improved resolution (wide variety of stationary phases)
- reusable columns (expensive columns but can be used for many analysis)

- ideal for the substances of low volatility
- easy sample recovery, handling and maintenance
- instrumentation tends itself to automation and quantitation (less time and less labor)
- suitable for preparative liquid chromatography on a much larger scale

In HPLC, the analyst has a wide choice of chromatographic separation methodologies from normal to reverse phase and a whole range of mobile phases using isocratic (or) gradient elution techniques.

1.3.1 DIFFERENT MODES OF SEPARATION [11]

1.3.1.1 Normal Phase Chromatography:

In the normal phase mode, the stationary phase is polar and the mobile phase is nonpolar in nature. In this technique, nonpolar compounds travel faster and are eluted first. This is because of the lower affinity between the nonpolar compounds and the stationary phase. Polar compounds are retained for longer time because of their higher affinity with the stationary phase. These compounds, therefore take more time to elute. Therefore Normal phase mode of separation is not generally used for pharmaceutical applications because most of the drug molecules are polar in nature and hence take longer time to elute.

1.3.1.2 Reverse Phase Chromatography:

Reversed phase mode is the most popular mode for analytical and preparative separation of compounds of interest in chemical, biological, pharmaceutical, food and biomedical sciences. In this mode, the stationary phase is nonpolar hydrophobic packing with octyl or octa decyl functional group bonded to silica gel and the mobile phase is polar solvent. An aqueous mobile phase allows the use of secondary solute chemical equilibrium (such as ionization control, ion suppression, ion pairing and complexation) to control retention and selectivity. The polar compounds get eluted first in this mode and nonpolar compounds are retained for longer time. As most of the drugs and pharmaceuticals are polar in nature, they are not retained for longer time and hence elute faster. The different columns used are octa decyl silane (ODS) or C-18, C-8, C-4, etc., in the order of increasing polarity of the stationary phase.

1.3.1.3 Ion-Exchange Chromatography:

In Ion-exchange chromatography, a charged stationary phase is used containing oppositely charged counter-ions having property to exchange with solute ions of the same charge in the mobile phase. The technique is known as cation-exchange or anion-exchange

chromatography, depending on whether the solutes to be exchanged are positively or negatively charged.

1.3.1.4 Size Exclusion Chromatography:

Size exclusion chromatography is suitable for solutes with molecular weight of 2000 Daltons or more and it separates molecules according to their molecular mass. Largest molecules are eluted first and the smallest molecules last. It is also useful for preliminary investigation of unknown samples.

1.3.1.5 Affinity Chromatography:

Affinity chromatography uses highly specific biochemical interactions for separation. The stationary phase contains specific groups of molecules which can adsorb the sample if certain stearic and charge related conditions are satisfied. This technique can be used to isolate proteins, enzymes as well as antibodies from complex mixtures.

1.3.1.6 Adsorption Chromatography:

Here the analyte adsorbed onto the surface of a polar packing. The nature of the adsorption involves the interaction of polar molecules with a very polar solid stationary phase.

1.3.1.7 Partition Chromatography:

It is the most widely used liquid chromatographic procedure to separate most kinds of organic molecules. Here the components present in the analyte mixture distribute themselves between the mobile phase and stationary phase as the mobile phase moves through the column. The stationary phase actually consists of a thin liquid film either adsorbed or chemically bonded to the surface of finely divided solid particles.

1.3.2 INSTRUMENTATION [10]

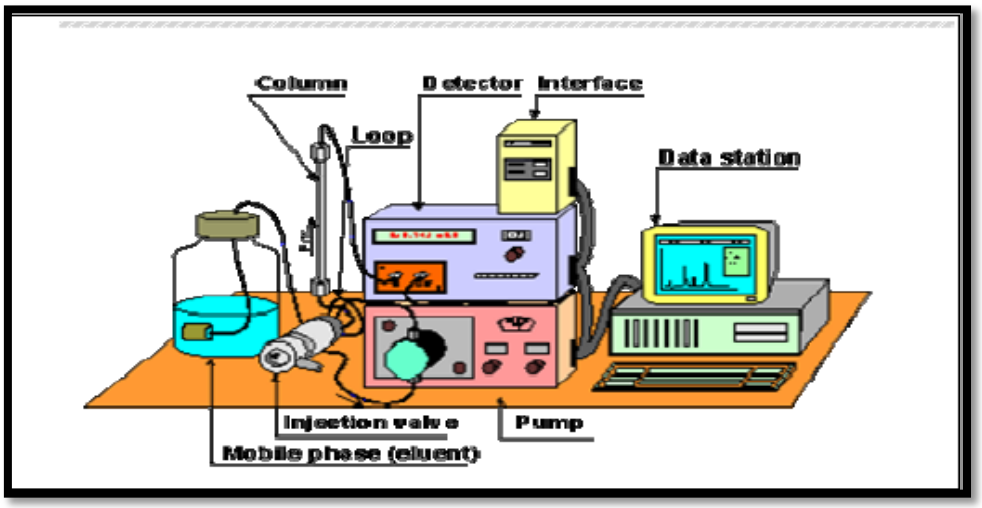


Figure 1.1: Schematic Diagram of HPLC Instrument

1.3.2.1 Various Components of HPLC [12]

1.3.2.1.1 Solvent Delivery System Including Pump

A mobile phase is pumped under pressure from one or several reservoir and flows through the column at a constant rate. Eluting power increases with increasing polarity of the solvent but decreases with increasing polarity. A degasser is needed to remove dissolved air and other gases from the solvent. Special grades of solvents are available for HPLC and these have been purified carefully in order to remove absorbing impurities and particulate matter to prevent them from damaging injection system or clogging the column.

Pumps

The pump is one of the most important components of HPLC, since its performance directly affects retention time, reproducibility and detector sensitivity. Three main types of pumps used in HPLC are:

i) Displacement pump

It produces a flow that tends to independent of viscosity and back pressure. But it possesses limited capacity (250 ml) though having pulse free output.

ii) Reciprocating pump

It has small internal volume (35 to 400 μl), their high output pressure (upto 10,000 psi) and their constant flow rates. But it produces a pulsed flow.

iii) Pneumatic or constant pressure pump

They are pulse free; suffer from limited capacity as well as a dependence of flow rate on solvent viscosity and column back pressure. They are limited to pressure less than 2000 psi.

1.3.2.1.2 Sample Injection System

The peak broadening attributable to this step is negligible because of insertion of the sample into the pressurized column must be as a narrow plug so that. The injection system itself should have no dead (void) volume. There are three important ways of introducing the sample into injection port:

i) Loop injection A fixed amount of volume is introduced by making use of fixed volume loop injector.

ii) Valve injection A variable volume is introduced by making use of an injection valve.

iii) On column injection A variable volume is introduced by means of a syringe through a septum.

1.3.2.1.3 Chromatographic Column

The column is usually made up of heavy glass or stainless steel tubing to withstand high pressure. The columns are usually 10-30 cm long and 4-10 mm inside diameter containing stationary phase at particle diameter of 25 μ or less. Columns with an internal diameter of 5 mm give good results because of compromise between efficiency, sample capacity, and the amount of packing and solvent required.

Column Packing

The packing used in modern HPLC consist of small, rigid particles having a narrow particle size distribution. There are three main types of column packing in HPLC:

i) Porous, polymeric beds

These are based on styrene divinyl benzene co-polymers used in ion exchange and size exclusion chromatography. For analytical purpose these have now been replaced by silica based, packing which are more efficient and more stable.

ii) Porous layer beds

Consisting of a thin shell (1-3 μ) of silica or modified silica on a spherical inert core (e.g. Glass). After the development of totally porous micro particulate packing, these have not been used in HPLC.

iii) Totally Porous silica particles (diameter < 10 μ)

These packing have widely been used for analytical HPLC in recent years. Particles of diameter >20 μ are dry packed. While particles of diameter <20 μ are slurry packed in which particles are suspended in a suitable solvent and slurry so they can driven into the columns.

1.3.2.1.4 Detector

The function of the detector in HPLC is to monitor the mobile phase as it merges from the column. Detectors are usually of two types:

i) Bulk property detectors

It compares overall changes in a physical property of the mobile phase with and without an eluting solute. E.g. refractive index, dielectric constant or density.

ii) Solute property detectors

It works on the basis of a physical property of the solute which is not exhibited by the pure mobile phase. These detectors are about 1000 times more sensitive giving a detectable signal for a few nano grams of sample. E.g. UV absorbance, fluorescence or diffusion current.

1.3.3 QUANTITATION IN HPLC [13]

Peak height or peak area measurements only provide a response in terms of detector signal. This response must be related to the concentration or mass of the compound of interest. To accomplish this, some type of calibration must be performed.

The three primary techniques for quantitation are:

- External standard method
- Internal standard method
- Method of standard addition

1.3.3.1 External Standard Method

For good quantitation using external standards, the chromatographic conditions must remain constant during the separation of all standards and samples. The external standard method involves the use of a single standard or up to three solutions. The peak area or the height of the sample and the standard used are compared directly. One can also use the slope of the calibration curve based on standard that contain known concentration of the compound of interest.

1.3.3.2 Internal Standard Method

A most widely used technique involves the addition of an internal standard to compensate various analytical errors. In this approach, a known compound of a fixed concentration is added to the known amount of samples to give separate peaks in the chromatograms to compensate for the losses of the compounds of interest during sample pretreatment steps. Any loss of the component of interest will be accompanied by the loss of an equivalent fraction of the internal standard. The accuracy of this method depends on the structural equivalency of the compounds of interest and the internal standard. The requirements for an internal standard are:

- It must be completely resolved without any interference.
- It must elute close to the compound of interest.
- It must not be present in the original sample.
- It must be stable, unreactive with sample components, column packing and the mobile phase.
- It must behave equivalent to the compound of interest for analysis like pretreatments, derivative formations, etc.
- It must be added at a concentration that will produce a peak area or peak height ratio of about unity with the compound.

- It is desirable that this compound is commercially available in high purity.

The internal standard should be added to the sample prior to sample preparation procedure and homogenized with it. To be able to recalculate the concentration of a sample component in the original sample, we have to demonstrate first the response factor. The response factor (RF) is the ratio of peak areas of sample component (Ax) and the internal standard (AISTD) obtained by injecting the same quantity. It can be calculated by using the formula,

$$RF = Ax / AISTD$$

On the basis of the response factor and strength of the internal standard (NISTD), the amount of the analyte in the original sample can be calculated using the formula,

$$X = AS / RF * AISTD \times NISTD$$

The calculations described above can be used after proving the linearity of the calibration curve for the internal standard and the analytical reference standard of the compound of interest. When more than one component is to be analyzed from the sample, the response factor of each component should be determined in the calculations using similar formula.

1.3.3.3 Standard Addition Method

In the standard addition method, a known quantity of the standard compound added to the sample solution is to be estimated. This method is suitable when sufficient amount of the sample is available and allows calibration in the presence of excipients or other components. It is also necessary to keep the flow rate and the mobile phase composition constant. The sample should be dissolved in the mobile phase. The solvent used in sample solution and the mobile phase are identical otherwise analysis can become less accurate.

1.4 INTRODUCTION TO STABILITY INDICATING ASSAY METHODS (SIAMs)

1.4.1 STABILITY

Stability of a pharmaceutical preparation can be defined as: The capability of particular formulation in a specific container/closure system to remain within its physical, chemical, microbiological, therapeutic and toxicological specifications throughout its shelf life. [14]

Nowadays, stability testing has become an integral part of formulation development. It is also a part of dossier submission to regulatory agencies for lincensing approval. The purpose of stability testing is to provide evidence on how the quality of a drug substance or drug product varies with time under the influence of a variety of environmental factors such as temperature, humidity and light, and to establish retest period for the drug substance or a shelf life for the drug product and recommended storage conditions. [15] To known what might happen to drug product when exposed to stressed and a normal conditions it is equally

important to know first what happens to the drug substance when exposed alone to such conditions, which gives an idea of the intrinsic behavior of the substance and help in predicting how it will behave when formulated into a new internal environment under the influence of external environment.[16] According to the, ICH and FDA guidance documents, stress testing is conducted to fulfill three main purposes[17]:

1. To provide a stability assessment of the drug substance or the drug product.
2. To elucidate the possible degradation pathways of the drug substance or the active pharmaceutical ingredient in the drug product.
3. To investigate the stability-indicating power of the analytical procedures applied for the drug substance and the drug product.

1.4.2 SIAMs

The stability-indicating assay is a method that is employed for the analysis of stability samples in pharmaceutical industry. According International Conference on Harmonization (ICH) guidelines, the requirement of establishment of explicitly require conduct of forced decomposition studies under a variety of conditions, like pH, light, oxidation, dry heat, etc. and separation of drug from degradation products. The method is expected to allow analysis of individual degradation products. [18]

This definition in the draft guideline of 1998 reads as: Validated quantitative analytical methods that can detect the changes with time in the chemical, physical, or microbiological properties of the drug substance and drug product, and that are specific so that the contents of active ingredient, degradation products, and other components of interest can be accurately measured without interference.

1.4.2.1 Types of SIAMs [19]

1.4.2.1.1 Specific SIAMs

It can be defined as: A method that is able to measure unequivocally the drug(s) in the presence of all degradation products, in the presence of excipients and additives, expected to be present in the formulation. e.g. Titrimetric and UV methods that seperates drug but not all components.

1.4.2.1.2 Selective SIAMs

Whereas it can be defined as: A method that is able to measure unequivocally the drug(s) and all degradation products in the presence of excipients and additives, expected to be present in

the formulation. e.g. Chromatographic methods that seperates drug and all degradation products and more relevant for new drugs.

1.4.2.2 Techniques Employed in SIAM Development[19]

1.4.2.2.1 Titrimetric and Spectrophotometric Methods

In these methods, usually the objective is the analysis of the drug of interest alone in the matrix of excipients, additives, degradation products, impurities, etc. and also other drugs in case of the combination products. Their advantage is the precise, low cost and simplicity, though sometimes they are not sensitive. Due to limitation of specificity, there are hardly many reports these days on their use for the assay of stability samples. However, a few reports involving derivative spectroscopy have been published lately.

1.4.2.2.2 Chromatographic Methods

Various chromatographic methods that have been used are thin-layer chromatography (TLC), high-performance thin-layer chromatography (HPTLC), gas chromatography (GC), High Performance Liquid Chromatography (HPLC) and newer technique like capillary electrophoresis (CE). TLC is a simple technique that has been used in the past for developing SIAMs. Its disadvantages, such as variability and non-quantitative nature, limit its use as a basic technique for SIAM development.

1.4.2.2.3 Miscellaneous Methods

A few studies have also reported the use of proton nuclear magnetic resonance (NMR) spectroscopy for the development of SIAMs. CE is the latest entry to the techniques for the development of SIAMs. It has the advantage of high sensitivity, resolution and high efficiencies with minimal peak dispersion. The other techniques are GC-MS, LC-MS, LCMS-MS, LC-NMR and CE-MS.

1.5 INTRODUCTION TO METHOD DEVELOPMENT & VALIDATION

Method development and optimization in liquid chromatography is an attractive field of research for academia and industry. Complex mixtures or samples required systematic method development involving accurate modeling of the retention behavior of the analyte [20].

Information on a sample, define separation goals

↓

Need for special HPLC procedure, sample pretreatment, etc.

↓

Choose detector and detector settings

↓

Choose LC method; preliminary run; estimate best separation conditions

↓

Optimize separation conditions

↓

Check for problems or requirement for special procedure

↓

Recover purified material, Quantitative calibration, Qualitative method

↓

Validate method for release to routine laboratory

Table 1.2: Preferred experimental conditions for the initial HPLC run [20]

Separation variable	**Initial condition**
Column Dimensions (length, diameter) Particle size Stationary phase	150x4.6mm 5µm(3.5µm alternatively) C8 or C18
Mobile phase Solvent A and B % of strong solvent Buffer (compound, pH, and conc.) pH range Additives (e.g. Ion pair reagent, amine modifier)	 Buffer- Acetonitrile 80-100% 25mM Potassium phosphate 2.0 < 3.0 Do not use initially
Flow rate	1.5-2.0 ml/min.
Temperature	350-450˚C.
Sample size Volume Weight	 < 25µl < 100µg

1.5.2 METHOD DEVELOPMENT AND DESIGN OF SEPERATION IN HPLC [21-22]

The knowledge about the nature of the sample, namely its molecular weight, polarity, ionic character and the solubility parameter is required for development of methods to analyze drugs in multicomponent dosage forms. An exact recipe for HPLC, however, cannot be

provided because method development involves considerable trial and error procedures. The most difficult problem is where to start, what type of column is worth trying with what kind of mobile phase. In general one begins with reversed phase chromatography, when the compounds are hydrophilic in nature with many polar groups are generally water soluble. 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 5-10% organic phase in the mobile phase and the organic phase concentration (methanol or acetonitrile) can be increased up to 100% within 30-45 min. Separation can then be optimized by changing the initial mobile phase composition and the slope of the gradient according to the chromatogram obtained from the preliminary run. The initial mobile phase composition can be estimated on the basis of where the compounds of interest were eluted, mainly at what mobile phase composition. Changing the polarity of mobile phase can alter 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 including acidic or basic can be separated, if they are present in un-dissociated form. Dissociation of ionic samples may be suppressed by the proper selection of pH.

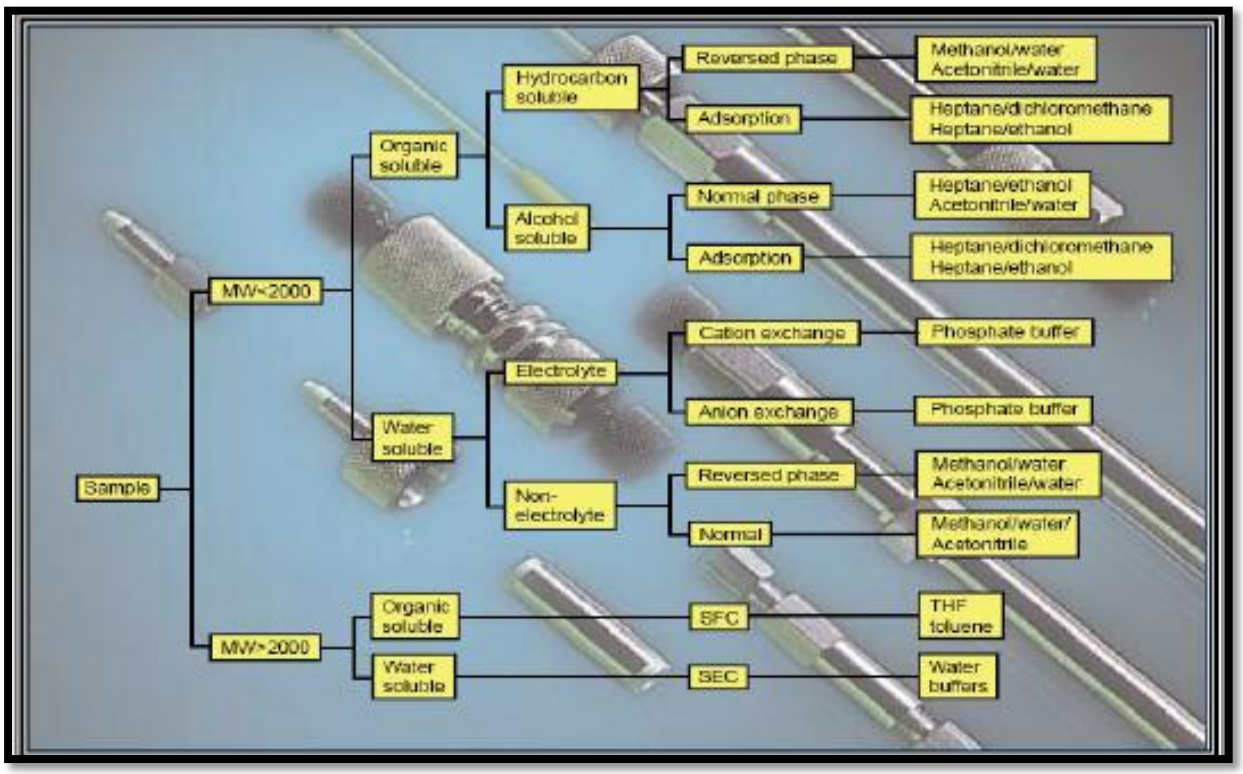


Figure 1.2 Phase Selection Process

The pH of the mobile phase has to be selected in such a way that the compounds are not ionized. If the retention time is too short, the decrease of the organic phase concentration in the mobile phase can be in steps of 5% is needed. If the retention time is too long, an increase of the organic phase concentration is needed.

In UV detection, good analytical results are obtained only when the wavelength is selected carefully. This requires knowledge of the UV spectra of the individual components present in the sample. If analyte standards are available, their UV spectra can be measured prior to HPLC method development. The molar absorbance at the detection wavelength is also an important parameter. When peaks are not detected in the chromatograms, it is possible that the sample quantity is not enough for the detection. An injection of volume of 20 µl from a solution of 1 mg/ml concentration normally provides good signals for UV active compounds around 220 nm. Even if the compounds exhibit higher ƛmax, they absorb strongly at lower wavelength. It is not always necessary to detect compounds at their maximum absorbance. It is, however, advantageous to avoid the detection at the sloppy part of the UV spectrum for precise quantitation. When acceptable peaks are detected on the chromatogram, the investigation of the peak shapes can help for further method development. The addition of peak modifiers to the mobile phase can affect the separation of ionic samples. For examples, the retention of the basic compounds can be influenced by the addition of small amounts of triethylamine, a peak modifier to the mobile phase. Similarly for acidic compounds small amounts of acids such as Acetic acid can be used. This can lead to useful changes in selectivity. When tailing or fronting is observed, it means that the mobile phase is not totally compatible with the solutes. In most case, the pH is not properly selected and hence partial dissociation or protonation takes place. When the peak shape does not improve by lower (1-2) or higher (8-9) pH, then ion-pair chromatography can be used. For acidic compounds, cationic ion pair molecules at higher pH and for basic compounds, anionic ion-pair molecules at lower pH can be used. For amphoteric solutes or a mixture of acidic and basic compounds, ion-pair chromatography is the method of choice. The low solubility of the sample in the mobile phase can also cause bad peak shapes. It is always advisable to use the same solvents for the preparation of sample solution as the mobile phase to avoid precipitation of the compounds in the column or injector.

Optimization can be started only after a reasonable chromatogram has been obtained. A reasonable chromatogram means that more or less symmetrical peaks present on the chromatogram can detect all the compounds. By slight change of the mobile phase

composition, the position of the peaks can be predicted within the range of investigated changes. An optimized chromatogram is the one in which all the peaks are symmetrical and are well separated within less run time. The peak resolution can be increased by using a more efficient column with higher theoretical plate number (N), which can be achieved by using a column of smaller particle size, or a longer column. These factors, however, will increase the analysis time. Flow rate does not influence resolution, but it has a strong effect on the analysis time.

1.5.3 STRATERGY FOR DEVELOPMENT OF VALIDATED SIAMs

Step I: Critical Study of the Drug Structure to Assess the Likely Decomposition Route(s)

This should be the first element whenever one takes up the project on establishment of a SIAMs. Much information can simply be gained from the structure, by study of the functional groups and other key components[25].

Step II: Collection of Information on Physicochemical Parameters.

It is generally important to know various physicochemical parameters like pKa, log P, solubility, absorptivity and wavelength maximum of the drug in question before the method development in question, before method development is taken up.The knowledge of log P for the drug and the identified degradation products provides good insight into the separation behavior likely to be obtained on a particular stationary phase. The knowledge of pKa is important as most of the pH- related changes in retention occur at pH of the buffer to be used in the mobile phase. The availability of the solubility data in aqueous, organic and commonly used HPLC solvents and their combinations can thus prove to be very useful in the selection of the sample solvents and the mobile phase. For the analysis of the drug or degradation products, it requires that they are soluble in HPLC compatible solvents in the first place.

Step III: Stress (Forced Decomposition) Study [23-24]

The third step in the development of SIAM is the conduct of stress studies to generate degradation products of the drug. The ICH guideline Q1A suggests the following conditions to be employed: (i) 10 0C increments above the accelerated temperatures, E.g. 50 0C, 60 0C etc., (ii) humidity where appropriate, E.g. 75 % or greater, (iii) hydrolysis across a wide range of pH values, (iv) oxidation and (v) photolysis. However, the guideline provides no details on

how hydrolytic, photolytic and oxidative studies have to be actually performed. Depending upon the results, Decision is taken on whether to increase the strength of the reaction conditions.

Step IV: Preliminary Separation Studies on Stressed Samples [25]

The stress samples are subjected to preliminary analysis to study the number and types of degradation products formed under various conditions. The simplest way is to start analysis with a RP C-18 column, preferably optimized chromatographic conditions. Well-separated and good quality peaks at the outset provide better resolution.

Step V: Final Method Development and Optimization [26]

The method is optimized, by changing the mobile phase ratio, pH, gradient, flow rate, temperature, solvent type, and the column and its type in such a way that no merging between close or co-eluting peaks.

Step VI: Identification and Characterization of Degradation Products and Preparation of Standards

Before moving to the validation of a SIAM, it necessary to identify the degradation products of drug and arrange for their standards. These are required to establish specificity/selectivity of the method. The work on this aspect can even be initiated once an idea on the nature and number of degradation products formed under different degradation conditions is obtained from preliminary separation studies. Peak purity of the active substance is checked by photo-diode array detector to verify that the method is selective, and a single component peak is quantified.

Step VII: Validation of Stability Indicating Assay Methods

Validation of analytical methods, in general, has been extensively covered in the ICH guidelines Q2A and Q2B, in the FDA guidance and by USP. Here validation has been carried out as per ICH guidelines Q2A and Q2B. Overall, there are two stages in the validation of SIAM. First stage is early in the development cycle when drug substance is subjected to forced decomposition study and the SIAM is established based on the knowledge of drug degradation behavior. Main focus of validation is on establishment of specificity/selectivity followed by other validation parameters like accuracy, precision, linearity, range, robustness, etc. In the second stage, when the SIAM so developed is extended to formulations or other

matrices, the emphasis gets limited to just prove the pertinence of the established validation parameters in the presence of excipients or other formulation constituents.

1.5.6 METHODOLOGY OF ANALYTICAL METHOD VALIDATION

1.5.6.1 Definition

Validation is a process of establishing documented evidence, which provides a high degree of assurance that a specific activity will consistently produce a desired result or product meeting its predetermined specifications and quality characteristics. It is an act of proving that any procedure, process, equipment, material, activity and system performs as expected under given set of conditions and also give the required accuracy, precision, sensitivity, ruggedness, etc26. When extended to an analytical procedure, depending upon the application, it means that a method works reproducibly, when carried out by same or different persons, in same or different laboratories, using different reagents, different equipments, etc.

1.5.6.2 Benefits of Analytical Method Validation [27]

- The biggest advantage of method validation is that it builds a high degree of confidence, not only for the developer but also to the user.
- Although the validation exercise may appear costly and time consuming, it results inexpensive, eliminates frustrating repetitions and leads to better time management in the end.

Minor changes in the conditions such as reagent supplier or grade, analytical setup are unavoidable due to obvious reasons but the method validation absorbs the shock of such conditions and pays for more than invested on the process. For analytical method validation of pharmaceutical guidelines from the International Conference on Harmonisation (ICH), United States Food and Drug Administration (USFDA), American Association of official Analytical Chemists (AOAC) and United States of Pharmacopoeia (USP) provide a framework for validation in a more efficient and productive manner.

1.5.6.3 Analytical Method Validation: The Regulatory Perspective [28]

1.5.6.3.1 USFDA:

According to this, Validation is the process of establishing documented evidence which provides a high degree of assurance that a specific process will consistently produce a product meeting its predetermined specifications and quality attributes.

***1.5.6.3.2 WHO*:**

Defines Validation as an action of providing any procedure, process, equipment, material, activity or system actually leads to the expected results.

***1.5.6.3.3 EUROPEAN COMMITTEE*:**

Defines Validation as an action of providing in accordance with the principles of GMP that any procedure, process, material, activity or system actually lead to expected results. The WHO published guidelines under the title, "Validation of analytical procedures used in the examination of pharmaceutical materials". It appeared in the 32nd report of the WHO expert committee on "specifications for pharmaceutical preparations" which was published in 1992. The international Conference on Harmonization (ICH), which has been on the forefront of developing the harmonized tripartite guidelines for adoption in the US, Japan and EC, has issued two guidelines under the titles "Text on validation of Analytical procedures Q2 (R1)" and "validation of Analytical procedure Methodology Q2 (R1)."[29]

1.5.6.4 Data Elements Required for Assay Validation

There are various analytical methods used for the examination of pharmaceutical materials. Analytical methods may be broadly classified as Per WHO as follows:

Class A: Tests designed to establish identity, whether of bulk drug substances or of a particular ingredient in a finished dosage form.

Class B: Methods designed to detect and quantitative impurities in a bulk drug substance or finished dosage form.

Class C: Methods used to determine quantitatively the concentration of a bulk drug substance or of a major ingredient in a finished dosage form.

Class D: Methods used to assess the characteristic of finished dosage forms, such as dissolution profiles and content uniformity.

Table 1.3: Characteristics that should be considered for Different Types of Analytical Procedure (As per WHO guidelines)

Sr. No	Parameter	Class A	Class B		Class C	Class D
			Quntitative test	Limit Test		
1.	Accuracy	--	X	--	X	X
2.	Precision	--	X	--	X	X
3.	Robustness	X	X	X	X	X
4.	Linearity and Range	--	X	--	X	X
5.	Selectivity	X	Yes	X	X	X
6.	Limit of Detection	X	--	X	--	--

Where, X indicates the tests should be performed.

1.5.6.5 Analytical Method Validation Parameters [30-31]

Before performing validation of analytiacal method it is necessary to understand the validation parameters. The various Performance parameters, which are addressed in a validation exercise, are grouped as follows.

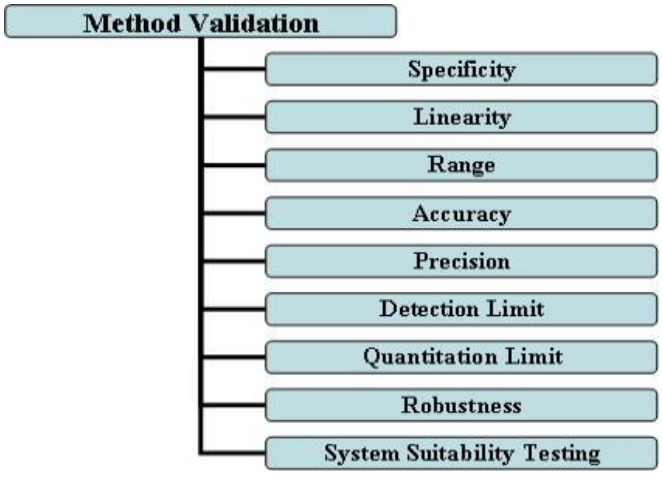


Figure 1.3: Method Validation Parameters as per USP and ICH

1.5.6.5.1 Selectivity and Specificity

The selectivity of an analytical method is its ability to measure accurately and specifically the analyte of interest in the presence of components that may be expected to be present in the sample matrix. If an analytical procedure is able to separate and resolve the various

components of a mixture and detect the analyte qualitatively the method is called selective. On the other hand, if the method determines or measures quantitatively the component of interest in the sample matrix without separation, it is said to be specific. Hence one basic difference in the selectivity and specificity is that, while the former is restricted to qualitative detection of the components of a sample, the latter means quantitative measurement of one or more analytes.

Selectivity may be expressed in terms of the bias of the assay results obtained when the procedure is applied to the analyte in the presence of expected levels of other components, compared to the results obtained on the same analyte without a added substances. When the other components are all known and available, selectivity may be determined by comparing the test results obtained on the analyte with and without the addition of the potentially interfering materials. When such components are either unidentified or unavailable, a measure of selectivity can often be obtained by determining the recovery of a standard addition of pure analyte to a material containing a constant level of the other components.

1.5.6.5.2 Linearity and Range

The linearity of an analytical method is its ability to elicit test results that are directly (or by a well defined mathematical transformation) proportional to the analyte concentration in samples within a given range. Linearity usually expressed in terms of the variance around the slope of regression line calculated according to an established mathematical relationship from test results obtained by the analysis of samples with varying concentrations of analyte. The linear range of detectability that obeys Beer‟s law is dependent on the compound analyzed and the detector used. The working sample concentration and samples tested for accuracy should be in the linear range. The claim that the method is linear is to be justified with additional mention of zero intercept by processing data by linear least square regression. Data is processed by linear least square regression declaring the regression co-efficient and b of the linear equation y= ax + b together with the correlation coefficient of determination r^2. For the method to be linear, the r2 value should be close to1. The range of an analytical method is the interval between the upper and lower levels of the analyte (including these levels) that have been demonstrated to be determined with precision, accuracy and linearity using the method as written.

1.5.6.5.3 Accuracy

The accuracy of an analytical method may be defined as the closeness of the test results obtained by the method to the true value. It is the measure of the exactness of the analytical method developed. Accuracy may often express as percent recovery by the assay of a known amount of analyte added. Accuracy may be determined by applying the method to samples or mixtures of excipients to which known amount of analyte have been added, both above and below the normal levels expected in the samples. Accuracy is then calculated from the test results as the percentage of the analyte recovered by the assay. The ICH documents recommend that accuracy should be assessed using a minimum of nine determinations over a minimum of three concentration levels, covering the specified range (i.e. three concentrations and three replicated of each concentration).

1.5.6.5.4 Precision

The precision of an analytical method is the degree of agreement among individual test results when the method is applied repeatedly to multiple samplings of homogenous samples. This is usually expressed as the standard deviation or the relative standard deviation (coefficient of variation). Precision is a measure of the degree of reproducibility or of the repeatability of the analytical method under normal operating circumstances. Repeatability involves analysis of replicates by the analyst using the same equipment, method and conducting the precision study over short period of time while reproducibility involves precision study at

- Different Laboratories
- Different Batch of Reagent
- Different Analysts
- Different Equipments

i) Determination of Repeatability

Repeatability can be defined as the precision of the procedure when repeated by same analyst under the same operating conditions like same reagents, equipments, settings and laboratory over a short interval of time. It is normally expected that at least six replicates should be carried out and a table showing each individual result provided from which the mean, standard deviation and co-efficient of variation should be calculated for set of n value. The

RSD values are important for showing degree of variation expected when the analytical procedure is repeated several time in a standard situation. It should be below 2% for bulk drugs and below 2% for assay in finished product.

The ICH documents recommend that repeatability should be assessed using a minimum of nine determinations covering the specified range for the procedure (i.e. three concentrations and three replicates of each concentration or using a minimum of six determinations at 100% of the test concentration).

ii) Determination of Reproducibility

Reproducibility means the precision of the procedure when it is carried out under different conditions, usually in different laboratories on separate, putatively identical samples taken from the same homogeneous batch of material. Comparisons of results obtained by different analysts, by the use of different equipments or by carrying out the analysis at different times can also provide valuable information.

1.5.6.5.5 Limit of Detection and Limit of Quantitation

i) Limit of Detection

The limit of detection is the parameter of limit tests. It is the lowest level of analyte that can be detected, but not necessarily determined in a quantitative fashion, using a specific method under the required experimental conditions. The limit test thus merely substantiates that the analyte concentration is above or below a certain level. The determination of the limit of detection of instrumental procedures is carried out by determining the signal-to-noise ratio by comparing test results from the samples with known concentration of analyte with those of blank samples and establishing the minimum level at which the analyte can be reliably detected. A signal-to-noise ratio of 2:1 or 3:1 is generally accepted. The signal-to-noise ratio is determined by dividing the base peak by the standard deviation of all data points below a set threshold. Limit of detection is calculated by taking the concentration of the peak of interest divided by three times the signal-to-noise ratio. For spectroscopic techniques or other methods that rely upon a calibration curve for quantitative measurements, the IUPAC approach employs the standard deviation of the intercept (Sa) which may be related to LOD and the slope of the calibration curve, b, by:

LOD = 3.3 Sa / b.

ii) Limit of Quantitation

Limit of quantitation is a parameter of quantitative assays for low levels of compounds in sample matrices such as impurities in bulk drugs and degradation products in finished pharmaceuticals. The limit of quantitation is the lowest concentration of analyte in a sample that may be determined with acceptable accuracy and precision when the required procedure is applied. It is measured by analyzing samples containing known quantities of the analyte and determining the lowest level at which acceptable degrees of accuracy and precision are attainable. Where the final assessment is based on an instrumental reading, the magnitude of background response by analyzing a number of blank samples and calculating the standard deviation of this response. The standard deviation multiplied by a factor (usually 10) provides an estimate of the limit of quantitation. In many cases, the LOQ is approximately twice the limit of detection. Sa is the standard deviation of the intercept which may be related to LOQ and the slope of the calibration curve, b, by: LOQ = 10 Sa / b

1.5.6.5.6 Robustness and Ruggedness

i) Robustness

The robustness of an analytical method is a measure of its capacity to remain unaffected by small but deliberate variation in method parameters and provides an indication of its reliability during normal usage. The determination of robustness requires that methods characteristic are assessed when one or more operating parameter varied.

ii) Ruggedness

The ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of normal test conditions such as different laboratories, different analysts, using operational and environmental conditions that may differ but are still within the specified parameters of the assay. The testing of ruggedness is normally suggested when the method is to be used in more than one laboratory. Ruggedness is normally expressed as the lack of the influence on the test results of operational and environmental variables of the analytical method.

1.5.6.5.7 Stability of Analytical Solution Stability of the sample, standard and reagents is required for a reasonable time to generate reproducible and reliable results. For example, 24 hour stability is desired for solutions and reagents that need to be prepared for each analysis. System suitability test provide the added assurance that on a specific occasion the method is giving, accurate and precise results. System suitability test are run every time a method is used either before or during analysis.

1.5.6.5.8 System Suitability

System suitability is the checking of a system to ensure system performance before or during the analysis of unknowns. Parameters such as plate count, tailing factors, resolution and reproducibility (% RSD, retention time and area for six repetitions) are determined and compared against the specifications set for the method. These parameters are measured during the analysis. Similar to the analytical method development, the system suitability test strategy should be revised as the analysts develop more experience with the assay. List of the terms to be measured and their recommended limits of the system suitability samples are given in Table 1.4

Table 1.4: System Suitability Parameters and Recommendations

Parameters	**Recommendation**
Capacity Factor (k)	The peak should be well-resolved from other peaks and the void volume, generally K > 2
Repeatability	RSD ≤ 1% N ≥ 5 is desirable
Relative retention	Not essential as the resolution stated
Resolution (Rs)	Rs of > 2 between the peak of interest and the closest eluting potential interferent (impurity, excipients, degradation products, internal standard, etc.)
Tailing factor (T)	T ≤ 2
Theoretical plates (N)	In general should be > 2000

Table 1.5: Characteristics to be validated in HPLC

Characteristics	**Acceptance Criteria**
Accuarcy / trueness	Recovery 98-102% (individual)
Precision	RSD < 2%
Repeatability	RSD < 2%
Intermediate Precision	RSD < 2%
Specificity/ Selectivity	No interference
Detection Limit	S/N > 2 or 3
Quantitation Limit	S/N > 10
Linearity	Correlation coefficient r2 > 0.999
Range	80-120%
Stability	>24 h or > 12 h

CHAPTER-2
DRUG PROFILE

2. DRUG PROFILE OF FLUVOXAMINE MALEATE [32-34]:

Table 2.1: Chemical Data of Fluvoxamine Maleate

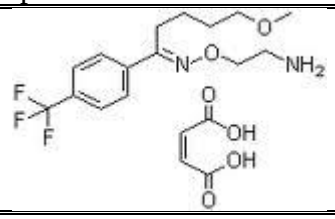
Drug Name	Fluvoxamine Maleate
Brand Name	Luvox
Drug Class	Anti-depressant
Chemical Structure	
Chemical IUPAC Name	5-methoxy-4'-(trifluoromethyl) valero phenone-(E)-O-(2-aminoethyl)oxime maleate
Chemical Formula	$C_{15}H_{21}O_2N_2F_3 \bullet C_4H_4O_4$
Molecular Weight	434.4
CAS No.	54739-18-3
Melting Point	120-122.5
Dissociation constant (pka)	8.7
Partition coefficient (Log P)	0.4 (n-heptane/water)
Solubility	Sparingly soluble in water, freely soluble in ethanol and chloroform and practically insoluble in diethyl ether.
State	A white or off white, odorless, crystalline powder

Table 2.2: Pharmacokinetic Data of Fluvoxamine Maleate

Oral absorption	The oral bioavailability of fluvoxamine is 53%.
Plasma protein binding	The plasma protein binding is about 80%.
Metabolism	Fluvoxamine is strongly metabolized in the liver, mostly by the processes of oxidative demethylation (producing fluvoxamine acid and its N-acetyl analog) and deamination (producing fluvoxethanol). Only fluvoxamine acid has been shown to have SERT inhibitor activity, roughly 1-2 orders of magnitude less potent than the parent compound. Radio-labeled administration of a dose of fluvoxamine produced nine identifiable metabolites, constituting 85% of the absorbed dosage (thus 15% of the fluvoxamine remained unchanged). This isolate of metabolites was empirically proven to contain 60% fluvoxamine acid and its N-acetyl analog, and 10% fluvoxethanol, with the other six metabolites making up 30%.

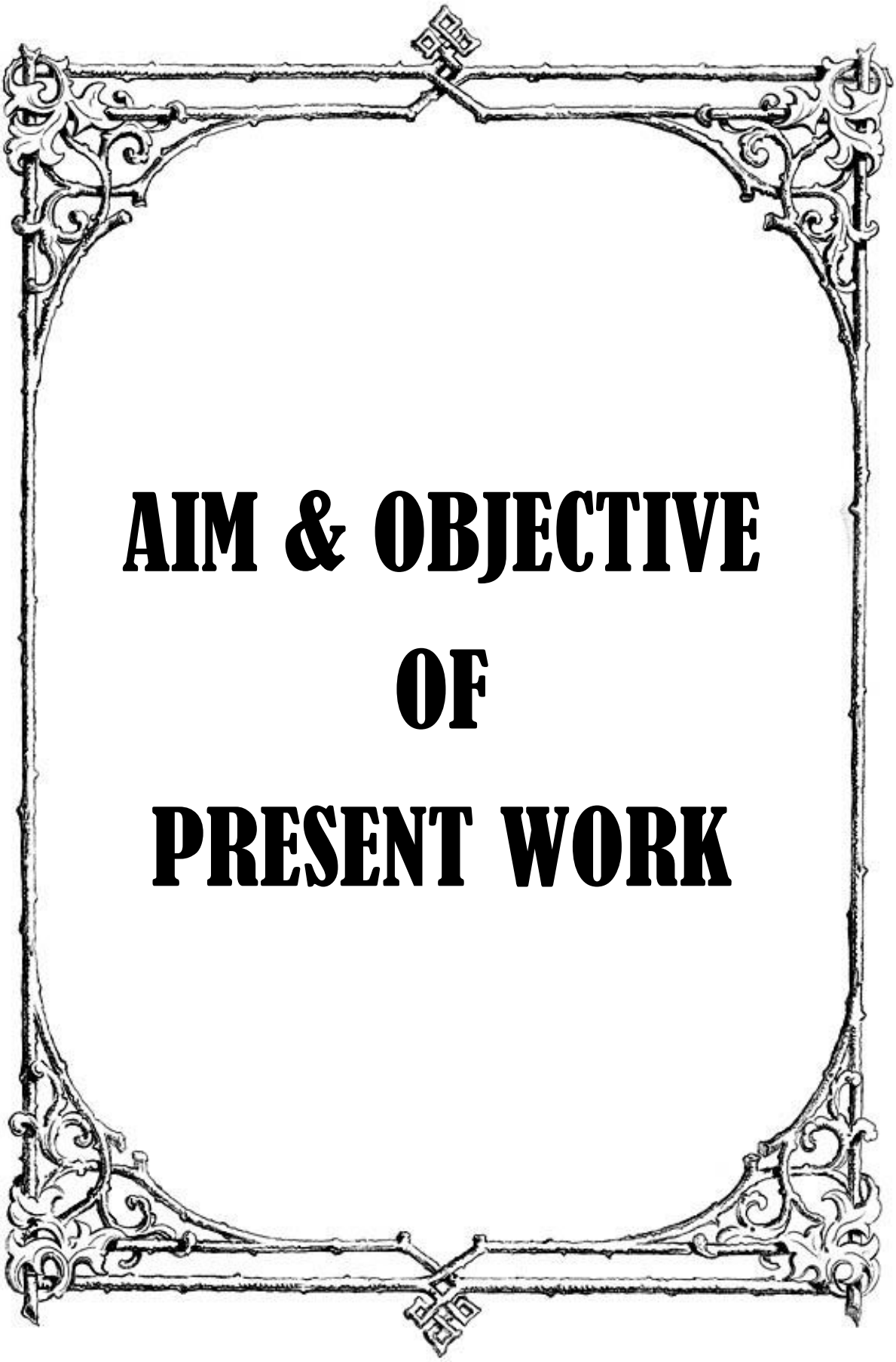
Excretion	Fluvoxamine has the shortest serum half-life of all SSRIs, with a mean of 15.6 hours.
Plasma half life	15.6 hours

Table 2.3: Therapeutic Consideration of Fluvoxamine Maleate

Drug Category	selective serotonin reuptake inhibitor (SSRI)
Dosage and Administration	Several weeks (up to 8 weeks) may pass before you feel the full effect of this medicine. Follow the directions for using this medicine provided by your doctor. Store this medicine at room temperature, away from heat and light. Continue to take this medicine even if you feel better. If you miss a dose of this medicine and you are taking 1 dose daily, take the missed dose if you remember the same day. Skip the missed dose if you do not remember until the next day. If you miss a dose of this medicine and you are taking more than 1 dose a day, skip the missed dose and go back to your regular dosing schedule. Do NOT take 2 doses at once.
Phamacology	It is a potent and selective serotonin reuptake inhibitor with approximately 100-fold affinity for the serotonin transporter over the norepinephrine transporter. It has negligible affinity for the dopamine transporter or any other receptor, with the sole exception of the $\sigma_1$receptor. It behaves as a potent agonist at this receptor and has the highest affinity of any SSRI for doing so.
Mechanism of Action	The mechanism of action of fluvoxamine maleate in Obsessive Compulsive Disorder is presumed to be linked to its specific serotonin reuptake inhibition in brain neurons. In preclinical studies, it was found that fluvoxamine inhibited neuronal uptake of serotonin.

Contraindications	Co-administration of terfenadine (Seldane), astemizole (Hismanal), or cisapride with fluvoxamine maleate is contraindicated. Fluvoxamine maleate is contraindicated in patients with a history of hypersensitivity to fluvoxamine maleate.
Drug Interactions	Combined use of fluvoxamine and MAO inhibitors is contraindicated. DO NOT TAKE THIS MEDICINE with terfenadine (Seldane), astemizole (Hismanal), or cisapride.
Precautions	During premarketing studies, seizures were reported in 0.2% of fluvoxamine-treated patients. Fluvoxamine maleate should be used cautiously in patients with a history of seizures. It should be discontinued in any patient who develops seizures. Suicide: The possibility of a suicide attempt is inherent in patients with depressive symptoms, whether these occur in primary depression or in association with another primary disorder such as OCD. Close supervision of high risk patients should accompany initial drug therapy. Prescriptions for fluvoxamine maleate should be written for the smallest quantity of tablets consistent with good patient management in order to reduce the risk of overdose.
Adverse effects	Most commonly observed with fluvoxamine include nausea, vomiting, drowsiness, insomnia, dizziness, nervousness, feeling anxious, dry mouth, abdominal pain, constipation, diarrhea, heart burn, loss of appetite, muscle weakness, pins and needles, abnormal taste, headache, faster heart beat, sweating, weight gain, weight loss or unusual bruising. Other side effects which are observed more frequently in children include abnormal thoughts or behaviour, cough, increased period pain, nose bleeds, increased

	restlessness, infection and sinusitis. Sexual side effects with fluvoxamine are less pronounced than with other SSRIs.
Storage	**Immediate-release tablets** Store at 68° to 77°F. Protect from high humidity. Dispense in a tightly closed, light-resistant container. **Extended-release capsules** Store at 59° to 86°F. Protect from high humidity. Avoid exposure to temperatures above 86°F.



AIM & OBJECTIVE OF PRESENT WORK

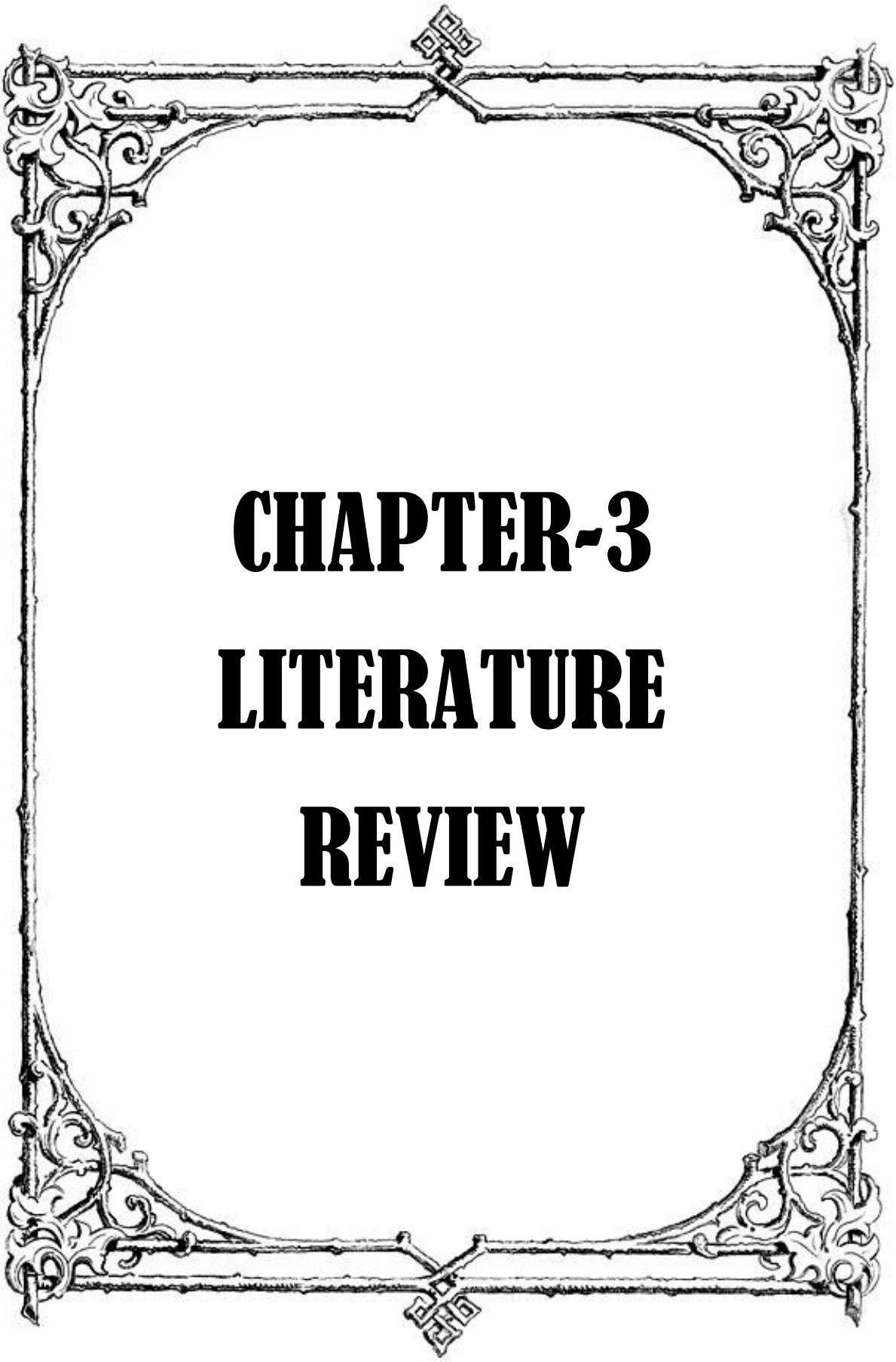
The purpose of the stability testing is to provide evidence on how quality of a drug substance or drug product varies with time under the influence of variety of environmental factors such as temperature, humidity and light. Stability testing permits the establishment of recommended storage conditions, retest periods and shelf-lives.

Many pharmaceutical substances are known to deteriorate during distribution and storage particularly in hot, humid climates. Nonetheless, little precise information is available on the degradation characteristics of many long-established substances. More is known of the stability of recently introduced substances since relevant data must now be generated as a condition of registration to support proposed expiry dates. The results, however, are rarely published. So, in order to attain the detailed behavior of the drug in presence of different stress conditions, the stability indicating assay method can be developed.

From the literature survey, it can be accomplished that the several methods are reported for the estimation of Fluvoxamine Maleatealone and its combination with other drugs but no single stability indicating method has been reported for the drug in bulk and in its dosage form. Hence, it was endeavored to develop an accurate, precise and sensitive stability indicating method for the drug in bulk and in its dosage form.

OBJECTIVES OF THE PRESENT WORK

1. To develop a suitable stability indicating RP-HPLC method for the Fluvoxamine Maleate in bulk and in its dosage form.
2. To validate the developed method in terms of Linearity, Range, Accuracy, Precision, Limit of Detection, Limit of Quantification, Robustness, and Repeatability.
3.To develop the mass compatible method for the characterization of the different degradation peaks for Fluvoxamine Maleate by LC-MS.



CHAPTER-3
LITERATURE REVIEW

3. Review of Literature

Fluvoxamine Maleate is official in US Pharmacopoeia.

Table-3.1 Official method in U.S.P

Sr No.	Matrix	Column	Mobile Phase	Conditions	Ref No.
1	Tablet	4.6 mm x 250mm column that contains packing L7	Buffer solution : Acetonitrile (62:38)	**Flow rate** : 1.7ml/min **Column temperature** :40˚C **λmax** : 234 nm **Working concentration** : 50 µg/ml **Injection volume**: 10 µl	35

Table 3.2 Reported Methods for Estimation of Fluvoxamine Maleate

Sr No.	Type of Reaction	Reagent Used	λ_{max} nm	Molar Absorptivity L mole^{-1} cm^{-1}	Beer's Limits µg /mL	Ref No.
1	Condensation	Nin or NH-AA	560	1.542×10^3	20 -120	36
2	Complex formation	CTC	620	8.732×10^3	5 - 30	
3	Inner complex formation	SNP-HA	440	1.324×10^4	4 - 24	
4	Oxidative coupling Reaction	Brucine-NaIO4	520	8.862×10^2	25 - 150	
5	Complex formation & Redox reaction	Chloranilic acid	540	3.063 x 103	10 - 60	

Table 2.3 Reported HPLC Method

Sr No.	Matrix	Column	Mobile Phase	Conditions	Ref No.
1	In Human plasma and urine using 1,2-naphtho quinone-4-sulphonic acid sodium salt	Phenomenex C_{18} (250 mm x 4.6 mm ID, 5µm)	Acetonitrile:Water (80:20 v/v)	Flow rate: 1 ml/min. Internal standard: Tryptamine	37
2	In Human Plasma	C-8 column	Acetonitrile + 0.4 ml diethylamine : water (37:63)	λ_{max}: 254 nm LOD: 10 ng/ml	38

3	In Plasma by Column-Switching High-Performance Liquid Chromatography	250 mm x 4.6 mm C-8 column	Acetonitrile: Methanol: 0.01M phosphate buffer eluent (188:578:235 by volume)	Flow rate: 1.5 ml/min Detection: 214nm. Linearity: 0 to 1000 pg/L.	39

Table-2.4 Reported method in Dossage form

Sr No.	Matrix	Method	Condition	Remarks	Ref No.
1	Tablet	UV spectrophotometric method using Urea as Hydrotropic agent	λ_{max} :271 nm	Linear range: 5 - 25 µg/ml, r^2 = 0.991	40
2	Tablet & Plasma	Spectrofluorimetric Determination via Derivatization with 4-Chloro-7-Nitrobenzo-2-Oxa-1,3-Diazole.	Excitation λ_{max} : 470nm Emission λ_{max} : 535nm	Linear range: 65 - 800 ng/ml, r^2 = 0.9995	41
3	Tablet & Plasma	Spectrofluorimetric Determination via Derivatization with fluorescamine	Excitation λ_{max} : 383nm Emission λ_{max} : 481nm	Linear range: 0.1-1.1 µg/ml , r^2 = 0.9995	

CHAPTER-4
IDENTIFICATION TESTS

4.1 Instruments used for the identification tests:

4.1.1 Melting Point Apparatus: Melting point apparatus with model no MR08211005, LabIndia Pvt. Ltd. was used.

4.1.2 UV-Visible Spectrophotometer: UV-Visible spectrophotometer with model UV-1800 of SHIMADZU Corporation, Japan was used.

4.1.3 FT-IR: FT-IR spectrophotometer with model IR-Affinity, range of 7800-350cm-^{1}, of SHIMADZU Corporation, Japan was used.

4.2 Melting point Determination

Melting point of Fluvoxamine Maleate has been determined using melting point apparatus. The melting point of the compounds was taken by open capillary method.

Table-4.1: Melting Point of Fluvoxamine Maleate

Drug	Reported Melting Point (˚C)[35]	Observed Melting Point (˚C)
Fluvoxamine Maleate	121-123˚C	118-123˚C

4.3 UV Spectra of drug

Table-4.2: UV Spectra of Fluvoxamine Maleate (In Acetonitrile : Water, 1:1, %v/v)

Drug	Reported Peaks (nm) [42]	Observed Peaks (nm)
Fluvoxamine Maleate	251nm	251.3nm

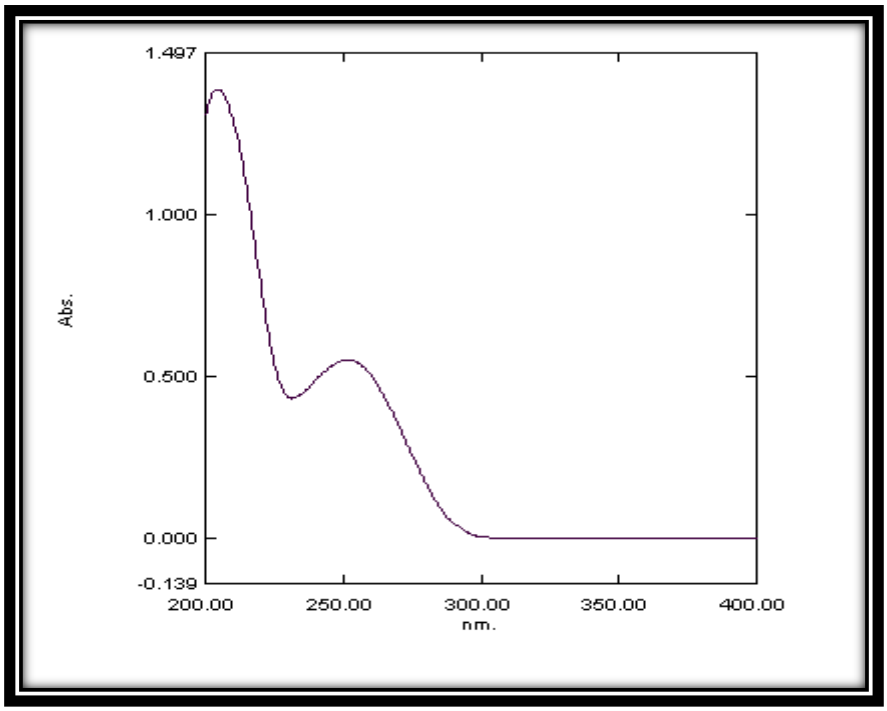


Figure-4.1: UV Spectra of Fluvoxamine Maleate- 50µg/ml (In Acetonitrile : Water, 1:1, V/V)

4.4 FT-IR Spectra of Fluvoxamine Maleate

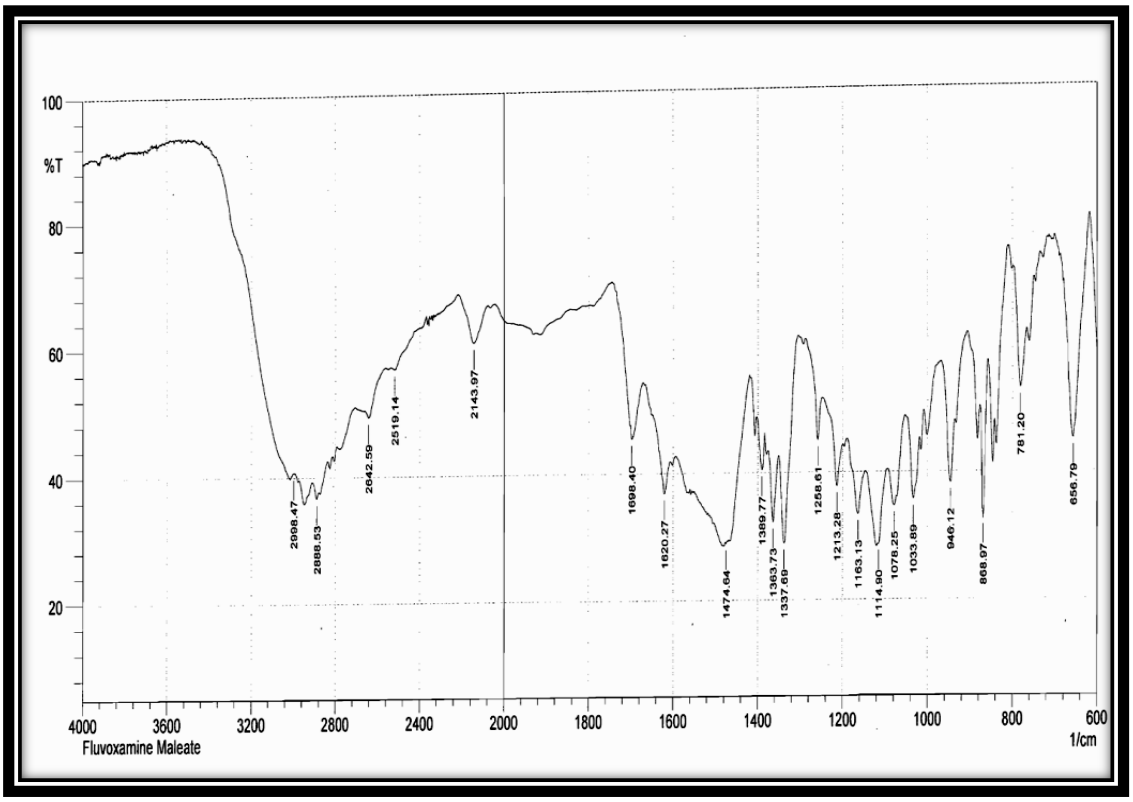


Figure-4.2: FT-IR Spectra of Fluvoxamine Maleate

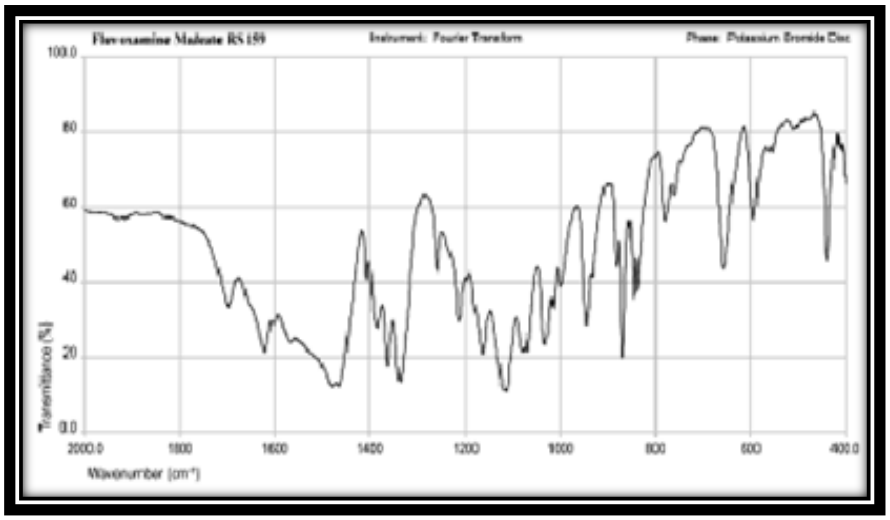


Figure-4.3: Reported FT-IR Spectra of Fluvoxamine Maleate [42]

Table-4.3: Interpretation of IR Spectra

Sr No.	Reported frequency[43] (cm^{-1})	Obtained frequency (cm^{-1})	Functional Group
1	2885-2865	2888.53	Methylene C-H stretch
2	1695-1690	1698.40	Lower C=O vibration
3	1650-1590	1620.27	Primary amine strong assymetry
4	1420-1300	1389.77	Symmetric stretch of carboxylic acid
5	1365-1320	1363.73	Symmetric Nitro stretch
6	1510-1450	1474.64	Aromatic Ring Stretch
7	1150-1000	1033.89	C-F strong stretching

CHAPTER-5
EXPERIMENTAL WORK

5.1 INSTRUMENTS

5.1.1 HPLC: HPLC with 2695 Pump, 2489 UV Detection, 2998 PDA Detection of Waters Alliance, Waters India Pvt. Ltd. was used for the HPLC analysis.

5.1.2 pH meter: pH meter of PICO+ pH meter, Labindia Pvt. Ltd. was used to measure the accurate pH of the different aqueous solutions.

5.1.3 Ultra sonicator: Power Sonicator 420 was used for the sonication of the different solutions.

5.1.4 Vaccum oven: Vaccum oven of Thermolab Pvt. Ltd. was used for giving the heat to the different samples of analysis.

5.1.5 Analytical Balance: Analytical balances with different weighing capacity of 2 mg – 200 mg, 20 mg – 100 mg, 200 mg- 500 g of Meter Toledo Pvt Ltd. were used for accurate weighing of the samples.

5.1.6 Vaccum Pump: Vaccum pump of Millipore Pvt. Ltd was used to filter the different solvents for analysis.

5.2 REAGENTS AND MATERIALS

5.2.1 API: The gift sample of active pharmaceutical ingredient, Fluvoxamine Maleate was gifted from the Torrent Research Centre,Gujarat, INDIA.

5.2.2 Tablets: The tablets containing the drug Fluvoxamine Maleate (Fluvoxin 50) was procured form local market manufactured by the Sun Pharmaceuticals Pvt. Ltd.

5.2.3 Reagents:

Liquid reagents:

Acetonitrile - HPLC Grade, Hydrochloric Acid, Hydrogen peroxide from Merck Specialties Pvt. Ltd, Worli, Mumbai

Water (TKA): Genpure, Pacific water system

Solid reagents:

Potassium hydrogen phosphate, Sodium Hydroxide pellets, Ammonium formate, AR Grade

5.3 SELECTION OF ANALYTICAL PARAMETERS

5.3.1 Selection of the detection wavelength

Absorption maxima (λmax) is the point at which a drug shows its maximum absorbance. In the quantification of drug, it is very necessary to have the peak height (response) with sufficient absorbance. So, in order to achieve proper peak response, the UV Spectra of the drug containing 50µg/mL concentration was prepared in the diluent (Acetonitrile : Water, 1: 1, v/v) and the spectrum was scanned in the range of 190-400 nm to find the maximum

absorbance of the drug. The absorption maxima was found to be at 251.00 nm but when trial of degradation study was performed on this wavelength it was found that the formed degradation products have very less response at this wavelength so by scanning the spectrum of trial of degradation in range of 190 – 400nm it was found that at 234nm the API as well as formed degradation products have good response so it was selected as the analytical wavelength.

5.3.2 Selection of column [44]

The column is the heart of the system. Based on the bonding chemistry of the column, the elution order affects the method development process. Depending on the nature of analyte and matrix column selection was made for the method development.

C18-PFP phase utilises a specially developed ligand combining a C18 chain with integral PFP functionality, resulting in a phase that maintains the hydrophobic, stability and low bleed characteristics of leading C18 phase, yet provides the multiple retention mechanisms including hydrophobic, Л-Л interaction and dipole-dipole hydrogen bonding of a PFP phase that are responsible for the unique selectivity of C18-PFP.

Due to its integral penta fluoro phenyl functionality C18-PFP is recommended for seperations that involve halogenated aromatic compounds and it improve retention of polar basic compounds for better seperations.

As the Fluvoxamine Maleate is a polar basic compound containing fluorine atoms Thermosil PFP C18 column was selected to initiate the method development. But with this column observed peak shape of FXA was not proper so it was thought to use the column with low carbon loading to reduce tailing of main peak.

Additionally, the C-8 columns are considered for its better separation and less tailing with low carbon loading. So, it was thought to go ahead for the method development with this column.

Phenomenex Luna is One of the World's Leading HPLC Columns. It is dependable, ultra-pure silica-based HPLC columns that offer an extensive variety of selectivities which are scalable from micro-bore to preparative and purification scale solutions.

- Extensive line of rugged USP phases
- Wide pH stability for long column lifetime and method flexibility
- Easy method scalability from Fast LC to Preparative and Bulk Purification

It is available in Si, C5, C8, C8(2), C18, C18(2), Phenyl-Hexyl, CN, NH2 phases specifically to provide excellent, highly reproducible chromatography well suited for pharmaceutical and other biologically important compounds.other biologically important compounds.

Table 5.1 Technical Specifications for seperation of compound

Phase	Particle Sizes (µm)	Pore Size (Å)	Surface Area (sq. m/g)	Carbon Load (%)	pH Range	Recommended Use
C18	5, 3, 10	100	440	19	1.5-10.0	Separation of hydrophobic compounds
C18(2)	3, 5, 2.5, 10, 15	100	400	N/A	1.5-10.0	Separation of hydrophobic compounds
C8	5, 10	100	440	14.75	1.5-10.0	Separation of very hydrophobic compounds
C8(2)	3, 5, 10, 15	100	400	13.5	1.5-10.0	Separation of very hydrophobic compounds
CN	3, 5, 10	100	400	7	1.5-7.0	Separation of polar compounds
HILIC	3, 5	200	200*	5.7	2.0-7.5	Separation of polar compounds
NH2	3, 5, 10	100	400	9.5	1.5-11.0	Separation of polar compounds
PFP(2)	3, 5	100	400	11.5	1.5-8.0	Separation of aromatic and halogenated compounds
Phenyl-Hexyl	3, 5, 10, 15	100	400	17.5	1.5-10.0	Separation of aromatic compounds
SCX	5, 10	100	400	0.55 % Sulfur Load	2.0-7.0	Separation of positively charged compounds
Silica (2)	3, 5, 10, 15	100	400	N/A	2.0-7.5	Separation of polar compounds

5.4 PREPARATION OF THE STANDARD STOCK SOLUTION OF FLUVOXAMINE MALEATE (FXA)

A 50 mg of standard FXA was weighed and transferred to 100 ml of volumetric flask and dissolved in the diluents, Acetonitrile : Water, 1:1, v/v. The flask was shaken and volume was made up to the mark with the diluent to get the solution containing 500 µg/ml of FXA. This solution was used further for all the trials related to the optimization of the mobile phase.

5.5 OPTIMIZED MOBILE PHASE AND CHROMATOGRAPHIC CONDITION

Mobile phase optimization was performed with many trials starting with the phosphate buffer and acetonitrile in the gradient elution flow. The main purpose of the optimization was to achieve proper peak shape with sufficient height, theoretical plates, resolution and purity.

- **Mobile phase A:** 20 mM KH_2PO_4 (pH: 3.0)
- **Mobile phase B:** Acetonitrile
- **Flow Rate:** 1.0 mL/min
- **Injection Volume:** 20 µL
- **Column Temperature:** 30°C (± 5°C)
- **Sample Temperature:** Ambient
- **Needle Wash:** Extended
- **Wavelength:** 234 nm
- **Sampling rate:** 10 points/sec

Time (Min)	% A	% B
0	75	25
2	75	25
30	25	75
35	25	75
35.1	75	25
40	75	25

5.6 ANALYSIS OF TABLET SAMPLES

Total 20 tablets (Fluvoxin 50) were weighed accurately and powdered. An amount equivalent to 50 mg of Fluvoxamine Maleate was taken and dissolved in 40-50 ml diluent (Acetonitrile:Water) in 100 ml volumetric flask. The solution was sonicated for 5 minutes and then diluted with diluent up to the mark. The solution was filtered by using 0.45 µ PVDF filter. The filtered solution was injected and the chromatogram was recorded.

5.7 PREPARATION OF THE SOLUTIONS & OPTIMIZED CONDITIONS FOR THE FORCED DEGRADATION STUDIES

A 50 mg of standard FXA was weighed and transferred to 100 ml of volumetric flask and dissolved in the diluent (Acetonitrile: Water, 1:1, v/v). The flask was shaken and volume was

made up to the mark with the diluents to get the solution containing 500 µg/ml of FXA. The solution was degraded according to the instructions below.

Table 5.2 Preparation & Optimized Degradation Condition

Degradation condition	Preparation & Optimized Degradation Condition
Control	Standard solution
Thermal	Heat approximate 100mg of API at 60˚C for 72 hours.
Photo	Transfer 10ml solution into a 10 ml flask. Expose the solution in photo stability chamber 1.2 million lux hours (UV) and 200 Whr/m^2 (visible).
Acidic	Add 8 ml standard solution to a 10 ml flask. Add 1ml 1N HCl. Heat 60˚C for 8 hours.
Basic	Add 8 ml standard solution to a 10 ml flask. Add 1ml 5N NaOH. Heat 80˚C for 24 hours.
Oxidation	Add 9ml standard solution to a 10ml flask. Add 1ml 3%H_2O_2 & Store at room temperature for 8 hours.
Neutral	Add 50mg of API to a 10 ml flask. Dissolve it in 5ml of water. Heat 80˚C for 24 hours.

The calculation of the % Degradation and % Assay were done according to following formula.

$$\% \text{ Degradation} = \frac{\text{Area dropped of sample}}{\text{Area of standard}} \times \frac{\text{Standard weight}}{\text{Standard Dilution factor}} \times \frac{\text{Sample Dilution Factor}}{\text{Sample weight}} \times \text{Potency}$$

Potency of the drug = 99.90 %

5.8 PREPARATION OF SOLUTIONS FOR METHOD VALIDATION

5.8.1 Linearity Curve

A 100 mg of standard FXA was weighed and transferred to 100 ml of volumetric flask and dissolved in the diluent, Acetonitrile: Water, 1:1, v/v. The flask was shaken and volume was made up to the mark with the diluent to get solution containing 1000 µg/ml of FXA.

From this, further the dilutions were prepared as follow to plot Linearity curve.

Table 5.3 Dilution Pattern of the solutions

Level (% of Target Concentration)	Volume standard stock solution (S) (mL)	Final Volume (mL)	Further Dilution (mL to mL)	Target Concentration (ppm)
0.01%	1	100	0.5ml to 100ml	0.05
0.02%	1	100	1ml to 100ml	0.1
0.05%	1	100	2.5ml to 100ml	0.25
0.1	1	100	5ml to 10 ml	0.5
1	1	10	5ml to 100 ml	5
50	2.5	10	NA	250
80	4.0	10	NA	400
100	5.0	10	NA	500
120	6.0	10	NA	600
150	7.5	10	NA	750

For estimation of Fluvoxamine Maleate, calibration curve was plotted in the range of 0.05-750µg/ml.

5.8.2 Precision

The intraday precision was carried out by taking the chromatogram of working concentration (500 µg/ml) with the repetition of 6 times in a day and interday precision was carried out by taking the chromatogram of working concentration (500 µg/ml) with the repetition of 6 times on a three different days.

5.8.3 LOD and LOQ

For LOD, the solutions were prepared in the lower concentration range starting from the 0.01 µg/ml (diluted 1 ml from 1 µg/ml of FXA solution to 100 ml with diluent) and the signal to noise ratio was measured. The LOD was considered at a concentration at which S/N ratio was 3. For LOQ, the solutions were prepared in the lower concentration range starting from the

0.05 µg/ml (diluted 5 ml from 1 µg/ml of FXA solution to 100 ml with diluent) and the signal to noise ratio was measured. The LOQ was considered at a concentration at which S/N ratio was 10.

5.8.4 Accuracy

The accuracy of the method was determined by performing the recovery studies from tablet powder by standard addition method at three different levels, 80%, 100% and 120%.

5.8.5 Robustness

The robustness studies were performed on the Fluvoxamine API with the deliberate changes in the flow rate, wavelength and change in column temperature.

CHAPTER-7
SUMMARY

The optimization of the mobile phase was done with the different trial of the gradient flow with phosphate buffer, acetate buffer and acetonitrile. The method is based on reverse phase chromatography usingPhenomenex Luna C-8 (150×4.6 mm) 5µ particle size column. The separation in all the degradation peaks was achieved by using gradient elution of 20 mM potassium phosphate buffer and Acetonitrile at flow rate 1.0 mL/min and UV detection at 234.00 nm. The column was maintained at 30°C through out the analysis. The total run time is 40 min. Forced degradation studies were carried out according to ICH Guidelines. The objective of the study was to find out the likely degradation products which in turn help degradation pathway and intrinsic stability of the molecule. Stability indicating capability is established by forced degradation experiment of Fluvoxamine Maleate to acid, alkali, neutral, oxidation, thermal and photo degradation conditions. The peaks of degradation samples were pure and well resolved with the resolution of more than 1.5. The labelling of the peak is according to the similar UV spectra of the degradation peaks observed from the PDA detector.

Table 7.1: Summary of validation parameters

Stastical Parameter	**Fluvoxamine Maleate**
Linear Range	0.05-750µg/ml
Regression Equation	y = 99507x + 64401
R2 value	0.999
Accuaracy (Recovery Studies)	100.67 ± 0.376
Intraday Precision (% RSD)	0.090% -0.241%
LOD	0.015 µg/ml
LOQ	0.05 µg/ml

Table 7.2: Summary of percentage degradation of Fluvoxamine Maleate

No.	**Degradation Condition**	**Optimized Degradation Condition**	**% Degradation**
1	Acid	1N HCl, 8 Hour, Reflux/Heat, 60°C	18.48%

2	Base	5N NaOH, 24 Hour, Reflux/Heat, 80°C	<1%
3	Peroxide	0.3% H2O2, 8 Hour, RT	5.4%
4	Neutral	water, 24 Hour, Reflux/Heat, 80°C	7.67%
5	Thermal	80°C, 72 Hour	3.77%
6	Photo	1.2 million lux hours	<1%

7.2 CONCLUSION

The RP-HPLC method developed for the quantification of Fluvoxamine Maleate is fully validated as per the ICH Guidelines, thus indicating general applicability of the method for routine analysis of formulation those marketed in regulated countries. The proposed method is simple, accurate, precise and specific and has the ability to separate the drug Fluvoxamine Maleate, its degradation products from each other in the tablet dosage form. Sample solution stability was established by determination of assay over the period of 72 hours. The simplicity of the method allows the method for the application in the laboratory for routine quality check as well as for the stability studies for the formulated product. Overall, method provides high throughput solution for the determination of Fluvoxamine Maleate in the tablet with excellent selectivity, precision and accuracy.

CHAPTER-8
REFERENCES

1. http://en.wikipedia.org/wiki/Depression (mood) access on 23 April 2012.
2. Sandra Salmans (1997). *Depression: questions you have - answers you need*. People's Medical Society.
3. Depression, National Institute of Mental Health, 2009-09-23, Retrieved 2010-05-22, access on 23 April 2012.
4. Schmidt, Peter, Mood, depression, and reproductive hormones in the menopausal transition, The American Journal of Medicine, 2005.
5. Wright SL, Persad C. Distinguishing between depression and dementia in older persons: Neuropsychological and neuropathological correlates. *Journal of geriatric psychiatry and neurology*. 2007, 20(4), pp. 189–98.
6. http://www.medicinenet.com/depression/article.htm access on 23 April 2012.
7. Swarbrick J, Boyal JC, Encyclopedia of Pharmaceutical Technology, Vol. I. New York: Marcel Dekker Inc., 1998, pp. 217-24.
8. Mendham J, Denney RC, Baraes JD, Vogel's Textbook of Quantitative chemical Analysis 5th Edn, Singapore: Pears Edjucation, 1991, pp. 5- 11.
9. Sharma PP, How to Practice GMPs, Good Manufacturing Practices. 3rd Edn, New Delhi: Vandana Publication Pvt Ltd; 2001, pp. 214.
10. Available online at: http://en.wikipedia.org/wiki/High_Performance_liquid chromatography access on 23 April 2012.
11. Meyer VR, Practical High Performance Liquid Chromatography. 2nd Edn, London: John Wiley and Sons; 1993, pp. 26-7, 40, 222, 246 and 256.
12. Available online at: http://www.chem.new.edu/ courses/U331/CHMI222HPLC ppt access on 23 April 2012.
13. Ahuja S, Modern Pharmaceutical Analysis: An Overview. In: Ahuja S, Scypinski S, editors. Handbook of Modern Phamaceutical Analysis. Vol. III. United States of America: Academic Press, 2001, pp. 349.
14. Mendham J, Denney RC, Baraes JD, Thomas MJK, Vogel's Textbook of Quantitative Chemical Analysis. 5th Edn, 1991, pp. 217-35.
15. Szepei Gabor, HPLC in Pharmaceutical Analysis. Vol. I, 1990, pp. 101-73.
16. Kulkarni GT, Stability Testing of Pharmaceutical Products: An Overview, Indian J Pharm Edu. 2004, pp. 38, 194.
17. Achrya MM, Pharmaceuticals Stability Testing and Studies: An Overview, The Eastern Pharmacist.1999, pp. 31-32.

18. Klick S, Toward a Generic Approach for Stress Testing of Drug Substances and Drug Products, Pharm Tech. 2005, 2, pp. 48-66.
19. ICH QIA (R2). Stability Testing of New Drug Substances and Products, 2003.
20. Billet and Ripper, In; Brown R, Phyllis E. Advance in chromatography: selectivity optimization in HPLC. Vol. 39, 1998, pp. 264-265.
21. Singh SS, Bakshi M, Development of Validated Stability Indicating Assay Methods: Critical Review, J Pharm Bio Anal. 2002, 28, pp. 1011–1040.
22. Snyder LR, Kirkland JJ, Glajch JI, Practical HPLC Method Development. 2nd Edn, 1997, pp. 2-21.
23. Available online at: http://www.mac_mod.com access on 23 April 2012.
24. Available online at: http://www.sge.com access on 23 April 2012.
25. Singh SS, Bakshi M, Guidance on Conduct of Stress Test to Determine Inherent Stability of Drugs, Pharm Tech. 2000, 4, pp. 1-14.
26. International Conference on Harmonization of Technical Requirement for Registration of Pharmaceutical for Human use. Stability Testing of New Drug Substance and Products ICH Q1A (R2), 2003.
27. Validation of Analytical Procedure: Methodology, ICH Harmonised Tripartite Guidelines. 1996, pp. 1-8.
28. Green JM, A Guide to Analytical Method Validation, J Amer Chem. 1996, 68, 305A-9A.
29. Chandran S, Singh RSP, Comparision of various international guidelines for analytical method validation, Pharmazie. 2007, 62, pp. 4-14.
30. Quality Assurance of Pharmaceuticals, A compendium of guidelines and related materials, WHO Geneva. Vol. I, 1997, pp. 119-24.
31. International Conference on Harmonization of Technical Requirement for Registration of Pharmaceuticals for Human use. Validation of Analytical Procedure: Text and Methodology ICH Q2 (R1), 2005.
32. http://www.drugs.com/search.php?searchterm=fluvoxamine access on 23 April 2012.
33. Available Online at: http://en.Wikipedia.org/ wiki/ Fluvoxamine access on 23 April 2012.
34. Harry G. Brittain, Analytical Profiles of Drug Substances and Excipients, Volume 24, pp. 175.

35. U.S Pharmacopoeia NF-31, The United States Pharmacopoieal Convention Inc., 12601, Twinbrook Parkway, MD20852.
36. V. Annapurna, G. Jyothi, V. Nagalakshmi and B. Sailaja, Spectrophotometric Methods for the Assay of Fluvoxamine Using Chromogenic Reagents, E-Journal of Chemistry, 2010, 7(4), pp. 1539-1545.
37. V. Van-der-Meersch, Mougeot, Sensitive one step extraction procedure for column chromatographic determination of Fluvoxamine Maleate in human plasma J. Chromatogr.biomed. appl.105, pp. 441-449.
38. J.P. Foglia, L.A.Birder, J.M. Perel determination of Fluvoxamine in human plasma by High Performance Liquid Chromatography, J. Chromatogr.Biomed. Appl.105, pp. 441-449.
39. R.H. Pullen, A.A. Fatmi, determination of Fluvoxamine by High Performance Liquid Chromatography with fluoroscence detector, J. Chromatogr.biomed. appl.112, pp. 101-107.
40. R K Jat, R. C. Chhipa, S Sharma, Spectrophotometric Estimation of Fluvoxamine Maleate in Tablets Using Hydrotropic Agent, International Journal of Pharmaceutical Quality Assurance, 2(4), pp. 73-75.
41. El-Didamony A., Gouda A, A novel spectrofluorimetric method for the assay of pseudoephedrine hydrochloride in pharmaceutical formulations via derivatization with 4-chloro-7-nitrobenzofurazan, Journal of Biological and Chemical Luminescence,Volume 26, Issue 6, p.p 510–517.
42. Clark J., Analytical Profile of drug substance and excipients, Vol-12, pp. 1060.
43. Coates, J., Interpretation of Infrared Spectra, a Practical Approach in Encyclopedia of Analytical Chemistry R.A. Meyers (Ed.), pp. 10815–10837.
44. http://www.phenomenex.com/Products/HPLCDetail/Luna access on 23 April 2012.