# DEVELOPMENT OF SOME CHROMATOGRAPHIC METHODS AND THEIR VALIDATION FOR SIMULTANEOUS ESTIMATION OF SOME DRUGS IN BULK AND FORMULATIONS

**A THESIS SUBMITTED TO**
**BHARATI VIDYAPEETH UNIVERSITY, PUNE**
**FOR AWARD OF DEGREE OF**
**DOCTOR OF PHILOSOPHY IN PHARMACEUTICAL CHEMISTRY**
**UNDER THE FACULTY OF PHARMACEUTICAL SCIENCES**

**SUBMITTED BY**
**MR. PRAVIN DEVIDAS PAWAR**

**UNDER THE GUIDANCE OF**
**DR. S. Y. GABHE**

**RESEARCH CENTRE**
**BHARATI VIDYAPEETH DEEMED UNIVERSITY,**
**POONA COLLEGE OF PHARMACY,**
**ERANDWANE, PUNE- 411 038, INDIA**

**- July 2016 -**

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# CERTIFICATE

This is to certify that the work incorporated in the thesis entitled **"Development of some chromatographic methods and their validation for simultaneous estimation of some drugs in bulk and formulations"** for the degree of '**Doctor of Philosophy**' in the subject of **Pharmaceutical chemistry** under the faculty of **Pharmaceutical sciences** has been carried out by **Mr. Pravin Devidas Pawar** in the Department of Pharmaceutical chemistry, Bharati Vidyapeeth Deemed University, Poona College of Pharmacy, Pune during the period from November 2012 to May 2016, under the guidance of **Dr. S. Y. Gabhe**, Professor, Department of Pharmaceutical chemistry, Poona College of Pharmacy, Pune.

Place: Pune

Date:

**Prof. Dr. K. R. Mahadik**
Professor and Principal,
Bharati Vidyapeeth Deemed University,
Poona College of Pharmacy,
Pune - 411038

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Place: Pune

Date:

**Dr. S. Y. Gabhe**
Professor,
Dept. of Pharmaceutical chemistry,
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Pune – 411038.

# DECLARATION BY THE CANDIDATE

I hereby declare that the thesis entitled **"Development of some chromatographic methods and their validation for simultaneous estimation of some drugs in bulk and formulations"** submitted by me to the Bharati Vidyapeeth University, Pune for the degree of Doctor of Philosophy (Ph.D.) in **Pharmaceutical chemistry** under the faculty of "**Pharmaceutical Sciences**" is original piece of work carried out by me under the supervision of **Dr. S. Y. Gabhe**. I further declare that it has not been submitted to this or any other university or institution for the award of any degree or diploma.

I also confirm that all the material which I have borrowed from other sources and incorporated in this thesis is duly acknowledged. If any material is not duly acknowledged and found incorporated in this thesis, it is entirely my responsibility. I am fully aware of the implications of any such act which might have been committed by me advertently or inadvertently.

Place: Pune

Date:

**Mr. Pravin D. Pawar**

Research student

*Dedicated to my*

*Grand Parents*

*&*

*Guide*

## *Acknowledgments*

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*Date:*

*Place: Pune* ***Mr. Pravin Devidas Pawar***

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# ABBREVIATIONS

| | |
|---|---|
| **$\lambda_{max}$** | Wavelength of maximum absorption |
| **ng** | Nanogram |
| **nm** | Nanometer |
| **mm** | Millimeter |
| **cm** | Centimeter |
| **%** | Percentage |
| **$R_t$** | Retention time |
| **$R_f$** | Retardation factor |
| **HPTLC** | High performance thin layer chromatography |
| **HPLC** | High performance liquid chromatography |
| **RP-HPLC** | Reverse phase high performance liquid chromatography |
| **LOD** | Limit of detection |
| **LOQ** | Limit of quantitation |
| **ICH** | International conference on harmonization |
| **SD** | Standard deviation |
| **RSD** | Relative standard deviation |
| **µL** | Microlitre |
| **mL** | Milliliter |
| **µg** | Microgram |
| **mg** | Milligram |
| **r** | Correlation coefficient |
| **$r^2$ or $R^2$** | Square of correlation coefficient |
| **µg $mL^{-1}$** | Microgram per milliliter |
| **ng $band^{-1}$** | Nanogram per band |
| **mL $min^{-1}$** | Milliliter per minute |
| **mM** | Millimolar |
| **ODS** | Octa decyl silane |
| **UV** | Ultraviolet |

| | |
|---|---|
| **h** | Hour |
| **N** | Number of theoretical plates |
| **v** | Volume |
| **°C** | Degree centigrade |
| **µ** | Micron |
| **min** | Minute |
| **Fig** | Figure |
| **AR** | Analytical reagent |
| **i. d.** | Internal diameter |
| $\mathbf{S_{y.x}}$ | Standard deviation of residuals from line |
| **n** | Number of determinations |
| **LSP** | Levosulpiride |
| **ESP** | Esomeprazole |
| **RBP** | Rabeprazole |
| **MTS** | Mometasone furoate |
| **TBF** | Terbinafine hydrochloride |
| **ACN** | Acetonitrile |
| **ICH** | The International Conference on Harmonization |
| **US-FDA/FDA** | The Food and Drug Administration |
| **EI** | Electron ionization |
| **APCI** | Atmospheric pressure chemical ionization |
| **ESI** | Electrospray ionization |
| **APPI** | Atmospheric pressure photoionization |
| **API** | Active pharmaceutical ingredients |
| **DMSO** | Dimethyl sulfoxide |
| **SD** | Standard deviation |
| **RSD** | Relative standard deviation |

# *Introduction*

## 1 Analytical chemistry and its role in pharmaceuticals

Analysis mainly involves separation, identification and determination of the compounds from samples. In simple words in field of chemistry it is explained as "Qualitative and Quantitative analysis". Qualitative mainly involve identification and quantitative involves determination of amount of the compound in the sample. Analytical chemistry is determination of the composition of material in terms of the elements or compound contained; however identification of substance, the elucidation of its structure and quantitative analysis of its composition are the aspects covered by modern analytical chemistry.

The field of chemistry has several techniques to determine quality and quantity of the samples. These techniques can be classified on the basis of principle involved in the determination of the sample.

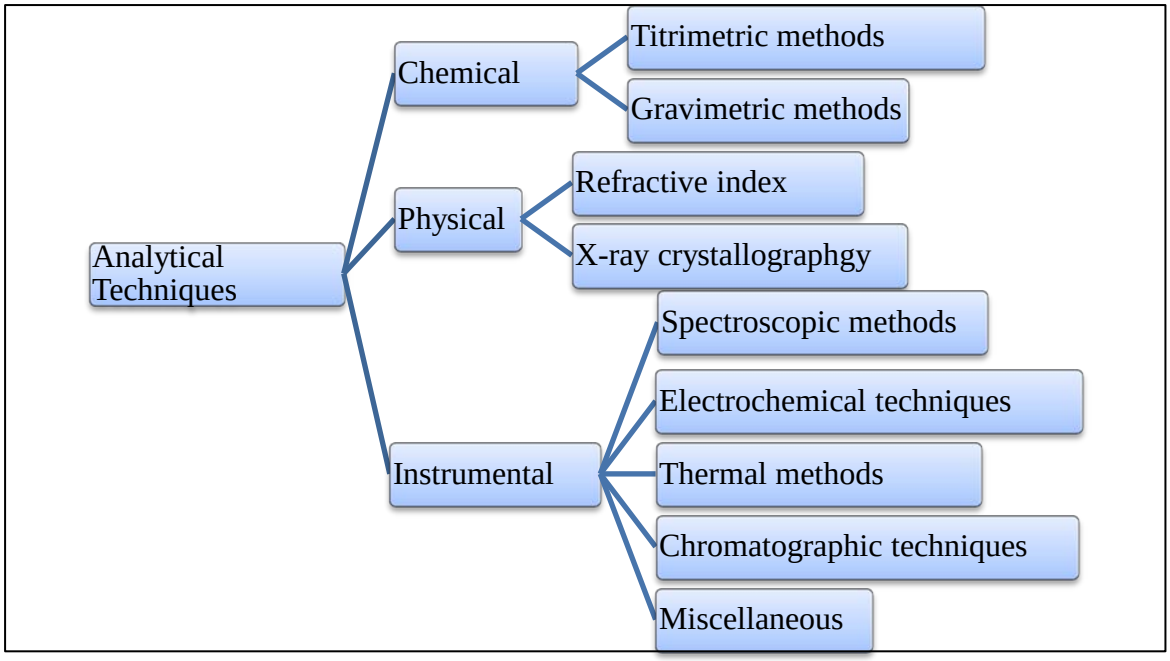


**Figure 1: Classification of analytical techniques**

Chemical entities; may be synthetically obtained or from natural sources, are included in pharmaceuticals when they show desired pharmacological or biological activity. Potency of drug is a fundamental factor in deciding their dose as well as designing their formulation. Analytical chemist with known knowledge and methodology

always tries to maintain quality and quantity of drug in formulations and bulk. It starts with development of the method for analysis of drugs and its validation[1-4].

## 2 Significance of analytical method development

Analytical methods are needed to analyze bulk samples of drugs and excipients as well as formulations. Nowadays newer drugs and their combination with other drugs are coming in the market. It is the core responsibility of the manufacturer to follow stringently the regulatory guidelines to provide safe and effective medicines. Thus the quality assurance and quality control departments are the most essential sections of any manufacturing unit. The qualitative and quantitative analysis of the active pharmaceutical ingredients in bulk and in formulations is carried out by different methods like physical and chemical testing and analytical methods like volumetric, spectroscopic or chromatographic methods. These analytical procedures could be assay methods or stability indicating methods.

Increased count of active pharmaceutical ingredients, their formulations, newer drugs and newer type of formulations have increased widespread distribution of standard and counterfeit drugs in their formulations. Assurance for quality and quantity of the pharmaceutical chemicals and formulations are essential for the general public health. Pharmaceutical analysis plays a key role in the assurance of quality of the formulations. Pharmaceutical analysis is indispensable in the process of quality control for statutory certification of drugs and their formulation either by the industry or by the regulatory authorities. Thus constant development of new and improved analytical methods for accurate determination of drugs in raw materials and in pharmaceutical dosage forms is essential for quality control studies.

New analytical method development is based on following basic criteria:

- The combination of drugs or drugs may not be approved in any official book.
- The procedure for analysis for the combination or active pharmaceutical ingredient may not be available because of patent regulation.
- Excipient interference may create problems in the application of the available method for the drug in the formulation or combination.

- Method for quantitative analysis of the active pharmaceutical ingredients in the biological samples may not be available.
- Method for quantitative estimation of drugs in presence of other active pharmaceutical ingredients may not be available.

## 3 Multicomponent formulation or fixed-dose combinations (FDCs)

Greater patient suitability, manifold actions, less side effects, improved potency and quicker relief drives fixed dose combinations or multicomponent formulations towards important formulation type from the public health prospective. This ancient phenomenon gives relief from symptoms and simultaneously it can combat with the disease cause. Hence the role of analytical chemist to admit the challenge to develop a steadfast method for quantitative estimation of drugs in multicomponent pharmaceutical preparations is more important.

Analyses of multicomponent systems utilize simultaneous analysis procedures which avoid tedious and expensive procedures like solvent extraction and separation. The volumetric and spectroscopic methods are bit time consuming and laborious as it involves individual assessments and mathematical or statistical treatment of the data. These shortfalls are cleverly overcome by chromatography methods. Chromatographic methods concurrently separate and quantify all components of multicomponent systems. The chromatographic methods mainly include gas chromatography (GC), high performance liquid chromatography (HPLC), and high performance thin layer chromatography (HPTLC). They have wide utilization in establishment of purity, uniformity, and stability of pharmaceuticals and biological samples analysis. It is done by measuring specific and non-specific property of the substance as detector response with respect to its concentration in the mixture, formulation or biological sample[5-6].

## 4 Different techniques used for multi-component analysis

### 4.1 Spectrophotometric multi-component analysis

The ability of an electron to absorb electromagnetic radiation and go to excited state is the principle behind absorption spectroscopy. There is a quantitative relationship between the concentration of analyte and the light absorbed. It is the simplest tool that

exists for quantitation. In case of multi-component analysis spectroscopic methods uses several methods like simultaneous equation method, absorption ratio method, single standard and double standard methods. This method is simple, rapid, precise, highly accurate, and less time consuming.

### 4.2 High performance liquid chromatography (HPLC)

This is a higher end instrumental technique available as a substitute for classical column chromatography. It works on same principle that when a compound travels through a column it distributes itself based on its affinity towards the stationary and mobile phase. The principle helps to resolve mixture based on difference in affinity of components towards the stationary and mobile phase. This technique is generally adsorption, partition, ion exchange and gel permeation. Sensitivity of the method is the key factor due to which it is extensively used among all chromatographic separation methods. It is also suitable for separation of nonvolatile and thermally unstable compounds. The technique has wide applications in quantitative determinations.

### 4.3 High performance thin layer chromatography (HPTLC)

It is also called as planar chromatography or open column chromatography. The mobile phase normally moves on thin layer of stationary phase, which is most of the time silica, by capillary action. This method utilizes more than one development step to analyze sample. After chromatographic development quantitation is carried out by absorbance-reflectance mode or visualization regents by derivatization.

### 4.4 Gas chromatography (GC)

Gas chromatography is a separation technique in which sample molecules get separated from the mixture when it travels with gaseous mobile phase through solid or liquid stationary phase. The molecule should possess some degree of thermal stability to be in gaseous state.

### 4.5 Hyphenated techniques

An interface is generally used to combine two different analytical techniques. When two or more analytical techniques are combined they are referred to as hyphenated techniques. One technique will separate the components of the mixture and another will detect the analyte in the mixture. Most often the other technique is some form of

mass spectrometry.

Examples of hyphenated and tandem techniques:

- LC-MS or HPLC-MS : Liquid Chromatography or High performance Liquid Chromatography-Mass Spectrometry
- TLC-MS: Thin layer chromatography- Mass spectrometry
- GC-MS: Gas Chromatography- Mass Spectrometry
- CE-MS: Capillary Electrophoresis- Mass Spectrometry
- CE-UV: Capillary Electrophoresis- Ultraviolet-Visible Spectroscopy
- LC-MS/MS: Liquid Chromatography- Mass Spectrometry/ Mass Spectrometry
- GC-MS/MS: Gas Chromatography- Mass Spectrometry/ Mass Spectrometry

## 5 TLC-MS

Planer chromatography is identified as a rapid tool for separation of components in mixture. The advantages of HPTLC over HPLC or any other chromatographic methods are well established and accepted. The low cost fast separation obtained from HPTLC with straight visual analysis has increased and maintained demand of this simple method. The hyphenated system in which mass spectrometry is coupled with TLC where the ratio m/z can be easily determined for the separated compounds. The two systems are connected with the help of TLC-MS interface.

**Figure 2: TLC-MS interface (Source- CAMAG)**

The thin layer chromatography- mass spectrometry interface is the most adaptable instrument for quick and contamination free elution of TLC/ HPTLC bands directly from the stationary phase of layer and subsequent analysis by online handover into the mass spectrometer. Mass spectrometry is the ultimate tool for the confirmation of the unknown compound. The mass spectrometric identification of substances separated by TLC/HPTLC has been known for a long time. Though, in the earlier methods the

target zones or bands had to be scraped off or removed from the plate, extracted into a tube with suitable solvent and transferred offline into the MS system. Hyphenation of HPLC with different MS systems is also well known but not all analytes may be separated by HPLC due to limitations like absence of chromophore, heavy matrix load or compatibility issue with mobile phases and MS solvents. To overcome these limitations HPTLC may be a more effective separation technique. TLC-MS Interface extracts the bands of interest and directly transfers them into MS techniques (APCI-MS, ESI-MS or APPI-MS). It never encounters with the mobile phase of the TLC system used to achieve the separation. The great advantage of the instrument is that the entire bands are transferred into the MS for evaluation and that within less than one minute sensitive mass spectrometric information becomes available.

The TLC-MS interface extracts bands or zones from a TLC / HPTLC plate. For the extraction, ACN, methanol or any other suitable solvent can be used at the ideal flow rate of the LC-MS system (e.g. 0.1 mL / min). The elution head is situated with the integrated laser crosshairs or based on the coordinates determined by the TLC Scanner or TLC Visualizer. After abstraction the eluate is either transferred into the MS or collected in a sample vial for further offline analysis. TLC-MS pairing is the noteworthy key to the hyphenation of thin-layer chromatography and mass spectrometry (MS) and thus unlocks novel capacities for both techniques. Hence it's very useful in determining the degradation products in stability indicating assay.[7-12]

## 6 Method validation

Validation of developed method is a step by step procedure to ensure the quality of it. It mainly involves various parameters to check whether it meets the requirements for intended laboratory purpose[13]. These developed methods need to be validated or revalidated before their use in analytical laboratories. ICH[14, 15] has given guidelines for validation of such analytical procedures for technical requirements for the registration of pharmaceuticals for human use. 'The guidelines proposed by USFDA mainly include samples and analytical data for method validation. The guidelines for method validation for compound evaluation are proposed by USP. The guidelines mainly include definition for eight parameters necessary to be studied. An extension with more detailed methodology is in preparation and nearly completed[16]. The United States Environmental Protection Agency (US EPA) has given guidelines for the

analytical method development and validation for the Resource Conservation and Recovery Act (RCRA)[17]. The most complete published document was titled as 'Conference Report of the Washington Conference on Analytical Methods Validation: Bioavailability, Bioequivalence and Pharmacokinetic Studies' held in 1990 (sponsored by the American Association of Pharmaceutical Scientists, the AOAC and the US FDA, among others)[18-22].

According to different guidelines, validation is defined as follows:

FDA-guidelines:

Validation is establishing documented evidence which provides a high degree of assurance that a specific process will consistently produce a product meeting its pre-determined specifications and quality attributes.

EU-guidelines

Action of proving, in accordance with GMP-principles that any procedure, process, equipment, material, activity or system actually leads to the expected results.

ICH-guidelines

Methods validation is the process of demonstrating that analytical procedures are suitable for their intended use[14, 15]

### 6.1 Need of Method Validation Studies[13, 23, 24]

- To measure specific system performance
- To identify and quantify potential for error
- To recognize differences between each method
- To assure regulatory guidelines

### 6.2 Categories of Analytical Procedures to be validated

The four most common types of analytical methods for validation are,

- Identification tests.
- Quantitative tests for impurities content.
- Limit tests for the control of impurities.

- Quantity determination tests for the active pharmaceutical ingredients in samples or in formulation or other selected components in the formulation[17].

## 6.3 Parameters for Method Validation[25-32]

The various parameters given by different authorities, organization and ICH mainly include specificity, selectivity, precision, repeatability, intermediate precision, reproducibility, accurate, range, LOD, LOQ, robustness and ruggedness.

### 6.3.1 Specificity

Specificity should be checked during determination of impurities and the assay and validation of identification tests. The procedures used to prove specificity will depend on the proposed objective of the analytical procedure. Perhaps analytical procedure for the specific analyte cannot be always demonstrated. In such a condition more than one analytical procedure is suggested to accomplish the needed level of judgment.

#### 6.3.1.1 Identification

The compounds which are closely or structurally related to analyte; are likely to be present and should be differentiated by suitable identification tests. The test/s which are employed should be able to differentiate the presence of compound/s (of interest or not) by positive and negative results of same compound. The positive results of samples should be compared with the reference material and the negative results should be carried out for the samples which do not contain the compound. In addition to this the procedure should be applied and confirmed to the compound which are structurally similar and or very closely matches with analyte but producing negative results. The selection of such compounds which may interfere should be based on scientific principles with a deliberation that interference could occur.

#### 6.3.1.2 Assay and Impurity Test(s)

The chromatograms should be used to prove the specificity in case of chromatographic procedures. The individual components should be properly labeled in the chromatograms. The other separation techniques should follow the same consideration. In case of critical separation, specificity can be proved by separating and resolving two different components which elute with a very small difference. In non-specific cases supporting procedure should be used to prove overall specificity.

This could be understood by example where a titrimetric method is accepted to assay an API for release; it could be combined with the suitable test for impurities. The method is same for assay and impurity tests.

#### *6.3.1.2.1 Estimation of analytes when impurities are available*

In case of assay where impurities are available specificity can be proved by adding pure analyte (API or product) with correct levels of impurities or excipients. The results obtained by spiking analyte with the impurities and/or excipients should be unaffected due to presence of these materials. The results should be compared with the results of unspiked samples. In case of impurity tests the differentiation could be established by spiking API or product with correct level of impurities and results should demonstrate separation of impurities from other components of the matrix.

#### *6.3.1.2.2 Estimation of the analyte when impurities are not available*

If impurity or degradation product standards are unavailable, specificity may be demonstrated by comparing the test results of samples containing impurities or degradation products to a second well-characterized procedure e.g. pharmacopoeial method or other validated analytical procedure (independent procedure). As appropriate, this should include samples stored under relevant stress conditions: light, heat, humidity, acid/base hydrolysis and oxidation (forced degradation studies, discussed later in detail).

- For the assay, the two results should be compared
- For the impurity tests, the impurity profiles should be compared

In case of chromatographic study, purity of the peak may be useful to demonstrate that the peak due to analyte is not due to more than one component.
(e.g. diode array, mass spectrometry).

### 6.3.2 Linearity

The range of analytical procedure should be evaluated for linearity. It should be proved directly on active pharmaceutical ingredient by preparation of stock solution and or by weighing separately the synthetic mixture of components of product by using suggested procedure.

Graphical evaluation of signals as a function of analyte concentration should be used for linearity evaluation. In case of linear relationship of the plot signals and

concentration; data should be further evaluated by appropriate statistical methods. These statistical methods used are like regression line by using least squares method. The deviation of data points from mean can also be helpful to evaluate the linearity of the data. Linearity establishment should be done with minimum of 5 concentrations.

### 6.3.3 Range

The range for the analytical procedure is usually derived from the linearity study results. The range mainly rests on the intention and use fullness of the procedure. It is recognized by approving that the analytical method offers an acceptable validation parameters like linearity, accuracy and precision. It is established when applied to samples containing analyte amount within or at extreme specified range of the analytical procedure.

The following points should be considered for minimum specified ranges:

- In case of assay of an active pharmaceutical ingredient or a finished product 80 to 120% of the test concentration.
- In case of impurity determination; from the reporting level of an impurity 1 to 120%. In case of potent impurities or toxic or impurities showing unexpected pharmacological effects, LOD and LOQ should be equal to the level at which the impurity must be controlled. In case of validation during development, it is essential to study the range around the proposed limits.
- When assay and purity are studied together as one test and when 100% standard is used, linearity should be analyzed over the range from reporting levels of the impurities 1 to 120% to the assay specification.

### 6.3.4 Accuracy

Accuracy should be studied and recognized over the identified linearity range of analytical procedure.

#### 6.3.4.1 Assay

#### 6.3.4.2 Active Substance

Accuracy should be established by considering following points

- An analytical procedure should be applied to analyte having known purity. (Reference material)
- The results of the proposed analytical methods should be compared with second

most well established procedure, whose accuracy is defined.

- After establishment of linearity, specificity and precision; accuracy may be concluded.

#### 6.3.4.3 Medicinal Product

Numerous approaches for determination of accuracy are available:

- A known quantity of the substance to be analyzed has been added to the synthetic mixture of the product component and apply analytical procedure.
- If samples of all product components are not available then it can be accepted either to add recognized amounts of the analyte to the product or to compare the results from second well established procedure whose accuracy is defined.
- After establishment of linearity, specificity and precision; accuracy may be concluded

### 6.3.5 Impurities (Quantitation)

Accuracy of the samples (API or product) spiked with known amounts of impurities should be evaluated. If samples with certain impurities and/or degradation products are not available then results should be compared with independent procedures and considered to be accepted.

### 6.3.6 Recommended Data

Calculation of accuracy should be done by using minimum of 3 concentration levels covering the range of analytical procedure and using minimum of 9 determinations. (3 concentrations and 3 replicates in the specified range of total analytical procedure). Accuracy should be stated as percentage recovery.

### 6.3.7 Precision

Validation of tests for assay and for quantitative determination of impurities includes an investigation of precision.

#### 6.3.7.1 Repeatability

Repeatability should be evaluated using:

- A minimum of 9 replicates covering the range for the proposed analytical procedure. (3 replicates of 3 concentrations each)
- A minimum of 6 replicates at 100 % of the test concentration

#### 6.3.7.2 Intermediate Precision

The level to which intermediate precision should be evaluated rests on the conditions under which the procedure is intended to be used. The analyst should establish and study the influence of random events on the precision of the proposed analytical procedure. The random event to be studied includes analysts, different days, different equipment etc. It is not considered essential to study these effects independently.

#### 6.3.7.3 Reproducibility

Reproducibility of proposed analytical method is evaluated by means of an inter-laboratory trial. This parameter of validation is mainly carried out for those analytical procedures which are intended to record in the official books, elsewhere the reproducibility need not be carried out for the market oriented products.

#### 6.3.7.4 Recommended data

To establish the precision of proposed method SD, RSD (CV) and confidence interval should be reported.

### 6.3.8 Detection limit

Depending on whether the proposed procedure is a non-instrumental or instrumental; several approaches for determination of detection limit are established. Methods enumerated below may be acceptable.

#### 6.3.8.1 Based on Visual Evaluation

Non-instrumental and sometimes instrumental methods may be evaluated visually. The limit of detection of the analyte is determined by analysis of samples of known concentrations of analyte at minimum level at which it can be reliably detected.

#### 6.3.8.2 Based on Signal-to-Noise ratio

The analytical methods which show baseline noise can be validated by determination of detection limits. Signal to noise ratio is determined by performing comparison of signals from samples of analyte of known low concentration with blank samples and establishing the lowest concentration at which analyte can be reliably detected. A ratio of 3 or 2:1 is adequate for estimation of limit of detection.

#### 6.3.8.3 Based on the Standard Deviation of the Response and the Slope

The detection limit (DL) may be expressed as:

$DL = 3.3/\sigma*S$

where $\sigma$ = the standard deviation of the response

S = the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte. The estimate of $\sigma$ may be carried out in a variety of ways, for example:

#### *6.3.8.3.1 Based on the standard deviation of the blank*

Suitable numbers of blank samples are scanned and extent of their background response is measured. Finally SD of these responses is calculated.

#### *6.3.8.3.2 Based on the calibration curve*

A calibration curve is plotted in the specific range of detection limit by using samples containing analyte. The residual SD of a regression line or the SD of y-intercept of regression line may be used as the SD.

### 6.3.8.4 Recommended Data

The limit of detection and method used for determination of limit of detection should be presented. If limit of detection determination is based on visual assessment or signal to noise ratio then the relevant chromatogram presentation is acceptable. If it is determined by calculation or statistical method, then this may be consequently validated by the independent analysis of appropriate number of samples near to be the limit of detection.

## 6.3.9 Quantitation Limit

Limit of quantitation is determined by several methods, depending on instrumental or non-instrumental procedures. Method other than listed below may be acceptable.

### 6.3.9.1 Based on Visual Evaluation

Non instrumental and sometimes instrumental methods may be evaluated by visual evaluation. The limit of quantitation is generally determined by analyzing samples with known lowest concentration of analyte with acceptable accuracy and precision.

### 6.3.9.2 Based on Signal-to-Noise ratio approach

Analytical procedures showing baseline noise can only be applied with this approach. Signal to noise ratio is determined by comparing measured signals with known lowest

concentrations of analyte and samples of blank. Minimum concentration is established at which the analyte can be consistently quantified. A characteristic signal to noise ratio is 10:1.

#### 6.3.9.3 Based on the Standard Deviation of the Response and the Slope

The quantitation limit (QL) may be expressed as:

$QL = 10/ \sigma * S$

where $\sigma$ = the standard deviation of the response, S = the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte. The estimate of $\sigma$ may be carried out in a variety of ways including:

##### *6.3.9.3.1 Based on Standard Deviation of the Blank*

Suitable numbers of blank samples are scanned and extent of their background response is measured. Finally, SD of these responses is calculated.

##### *6.3.9.3.2 Based on the Calibration Curve*

A calibration curve is plotted in the specific range of quantitation limit by using samples containing analyte. The residual SD of a regression line or the SD of y-intercept of regression line may be used as the SD.

#### 6.3.9.4 Recommended Data

The limit of quantitation and the method used for determination of limit of quantitation should be given. The method may be consequently validated by appropriate number of samples near to the limit of detection.

### 6.3.10 Robustness

The evaluation of robustness should be considered during the development phase and depends on the type of procedure under study. It should show the reliability of an analysis with respect to deliberate variations in method parameters. If measurements are susceptible to variations in analytical conditions, then analytical conditions should be suitably controlled or a precautionary statement should be included in the procedure. One consequence of the evaluation of robustness should be that a series of

system suitability parameters (e.g., resolution test) is established to ensure that the validity of the analytical procedure is maintained whenever used.

Typical variations which are studied are stability of analytical solutions and extraction time. In the case of HPLC, examples of typical variations are mainly variations in pH along with that composition of mobile phase, columns of different lots and make, temperature, flow rate of the mobile phase. In case of GC, typically variations studied are columns of different lots and make, temperature and flow rate.

### 6.3.11 System Suitability Testing

System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such. System suitability test parameters to be established for a particular procedure depend on the type of procedure being validated. Following table gives information about validation characteristics regarded as the most important for the validation of different types of analytical procedures.

**Table No. 1: Validation parameters to be included for different analytical procedures**

| **Type of Analytical Procedure** | **Identi--fication** | **Testing for impurities** | | **Assay -dissolution -content/potency** |
|---|---|---|---|---|
| **Characteristics** | | **Quantitative** | **Limit Test** | |
| **Accuracy** | - | + | - | + |
| **Precision** | | | | |
| **Repeatability** | - | + | - | + |
| **Intermediate precision** | - | +(1) | - | +(1) |
| **Specificity(2)** | + | + | + | + |
| **Detection limit** | - | -(3) | + | - |
| **Quantitation limit** | - | + | - | - |
| **Linearity** | - | + | - | + |
| **Range** | - | + | - | + |

- signifies that this characteristic is not normally evaluated

+ signifies that this characteristic is normally evaluated

(1) In cases where reproducibility has been performed, intermediate precision is not needed

(2) Lack of specificity of one analytical procedure could be compensated by other supporting analytical procedure(s)

(3) May be needed in some cases

## 7 Stability Indicating Method [33-38]

Stability Indicating Method (SIM) is one of the extended part of validation and assay procedure. Though it is the application of the developed and validated method, it is very essential criteria according to the different regulatory bodies which govern the pharmaceutical field. Stability of drug substance and drug product is of concern to maintain its efficacy and safety. The FDA and ICH guidelines state the importance and regulations in terms of maintaining, testing and documenting the stability profile of drug substance and products.

To register a new drug it is mandatory to develop its stability and safety profile by stability studies. Two main studies which are compulsorily included are long term studies of one year (12 months) and accelerated stability studies of half a year (6 months). Intermediate stability studies can also be performed with milder conditions than of accelerated studies with same duration. All these studies include separation of degradants from the stable product, identification and quantitation of the degradants. Stability indicating methods (SIM) (forced degradation studies) as compared to above mentioned require less time and favors getting the degradation products in very short span of time (few weeks). The data generated from SIM can be applied for the analysis of accelerated and long term study samples, doing this will definitely reduce the time required to produce final stability conditions of concerned drug substance or product.

US-FDA in 1987, stated in their guidelines that SIM for analytical methods for quantitative purpose which are based on biological, structural and chemical properties of each API of the product will be able to measure the content accurately and differentiate each API from its degradation product. International Conference on Harmonization (ICH) emphasizes the stability –indicating assay methods (SIAM) to be conducted through the forced degradation studies under various stressed conditions like extreme pH, light exposure, oxidation effect, exposure to dry and wet heat etc. It also included the separation and identification of the degraded products.

## 8 Analytical Methods to Perform SIAMs

Titrimetric, spectrophotometric and chromatographic techniques are generally employed for the analysis of the samples obtained from the stressed studies.

Nowadays the combination of chromatography and spectrophotometry is mostly used for separation and identification of degradants. The hyphenated technique (LC-MS, LC-NMR, TLC-MS) lowers the total study period and provides more accurate and elaborated data of qualitative as well as quantitative analysis of the degradation study. It is well understood that the chromatographic techniques are very versatile in separation of the components. Its adaptability of different stationary phases, mobile phases and detection methods allow performing various kinds of analysis. This makes chromatography a super powerful method to be employed in SIM development.

Among chromatography, HPLC and HPTLC are most widely used. HPLC has very high resolution capacity, high sensitivity and specificity. It works for versatile compounds like non-volatile, polar/nonpolar, and thermo-liable compounds. Thus maximum SIAMs have been developed using HPLC.

HPTLC is preferred because of its reliability, accuracy, less cost (low amount of mobile phase) and mainly it gives simultaneous estimation which makes the method rapid for quantitative analysis. TLC- MS is recently added technique to the hyphenated list, which has made HPTLC more valuable and useful in terms of qualitative analysis. Stability indicating methods are carried out by forced degradation studies. The qualitative, quantitative studies and identification of impurities and active drug are very important for the following reasons:

1. To determine the conditions that causes degradation through the degradation pathways.
2. To identify the impurities and structure elucidation of degradants.
3. To enlist the assured shelf life conditions.
4. To distinguish clearly the degradants from the drugs and excipients in the formulation.
5. To determine the best physical state (solution, solid, semi-solid etc) for the dosage form of the drug.

## 9 Forced Degradation Studies [33, 37, 39]

### 9.1 Introduction

Forced degradation studies are premeditated to generate chemical entities which are related to the parent product and analyzing the same by developed and validated analytical method. The different conditions to be studies are various levels of pH solutions, presence of oxygen and light, elevated temperatures and humidity levels. The range of degradants is between 5-20 % for the degradation studies carried out by chromatography. It is not mandatory that every force degradation study will give the positive results. The drug/product is declared to be stable and the test is terminated, when no results are obtained under the studied conditions which are equal to those mentioned in accelerated study.

Over stressing a molecule can lead to degradation profiles that are not representative of real storage conditions and perhaps not relevant to method development. Therefore, stress testing conditions should be realistic and not excessive. In this regard, it is the amount of stress that is important and not necessarily the extent of degradation. Indeed, some compounds may not degrade significantly after considerable exposure to stress conditions.

The ICH guidance recognizes that, it is impossible to provide strict degradation guidelines and allows certain freedom in selecting stress conditions for biologicals. The choice of forced degradation conditions should be based on data from accelerated pharmaceutical studies and sound scientific understanding of the products decomposition mechanism under typical use conditions. A minimal condition to be considered for forced degradation studies are

- Hydrolytic
- Oxidative
- Photolytic
- Neutral
- Thermal (dry heat and wet heat)

Regulatory guidance does not specify pH, temperature ranges, specific oxidizing agents, or conditions to use, the number of freeze thaw cycles, or specific wavelengths and light intensities. The design of photolysis studies is left to the applicant's discretion although Q1B recommends that the light source should produce combined visible and ultraviolet (UV, 320-400 nm) outputs. The exposure level should be justified. The advice of appropriate regulatory authorities should be considered on a case-by-case basis to determine guidance for light-induced stress.

Based on the extensive studies by Saranjit Singh and Monika Bakshi[33, 37], they have classified the stability of drugs into specific categories and they have defined stress conditions for each of them. The following six classes can be identified:

- Class I: Extremely labile
- Class II: Very labile
- Class III: Labile
- Class IV: Stable

- Class V: Very stable
- Class VI: Practically stable

### 9.2 Hydrolytic Conditions

The hydrolytic degradation of a new drug in acidic and alkaline condition can be studied by refluxing the drug in 0.1 N HCl or 0.1 N NaOH. If reasonable degradation is seen, then testing can be stopped at this point. However, in case no degradation is seen under these conditions, the drug should be refluxed in acid/alkali of higher strength and for longer duration of time. Alternatively if total degradation is seen after subjecting the drugs to initial condition, then acid/alkali strength can be decreased along with decrease in reaction temperature.

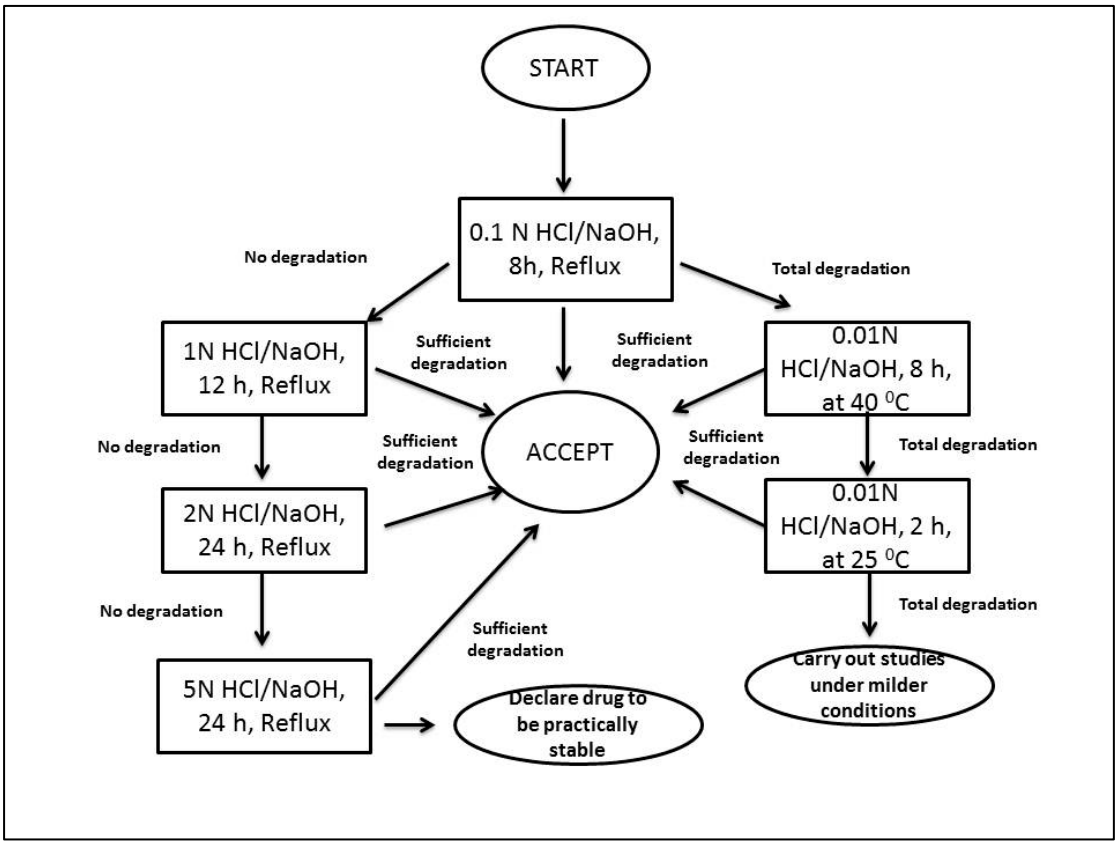


**Figure 3: Flow chart for performing stress studies for hydrolytic degradation under acid and alkali conditions**

**Table No. 2. Classification system for acidic and alkaline hydrolysis**

| Category of drug | Strength of acid/alkali | Time of exposure | Temperature | Extent of Decomposition |
|---|---|---|---|---|
| **Practically stable** | 5 N | 2 day | Refluxing | None |
| **Very stable** | 2 N | 1 day | Refluxing | Sufficient |
| **Stable** | 1 N | 12 h | Refluxing | Sufficient |
| **Labile** | 0.1 N | 8 h | Refluxing | Sufficient |
| **Very labile** | 0.01 N | 8 h | 40 °C | Sufficient |
| **Extremely labile** | 0.01 N | 2 h | 25 °C | Sufficient |

### 9.3 Oxidation Conditions

To test for oxidation, it is suggested to use hydrogen peroxide in the concentration range of 3 to 50 %. In some drugs extensive degradation is seen when exposed to 3% hydrogen peroxide for very short time period at room temperature. In other cases exposure to high concentration of hydrogen peroxide, even under extreme condition does not cause any significant degradation. The behavior is on expected lines, as some drugs are oxidized fast, while others are not.

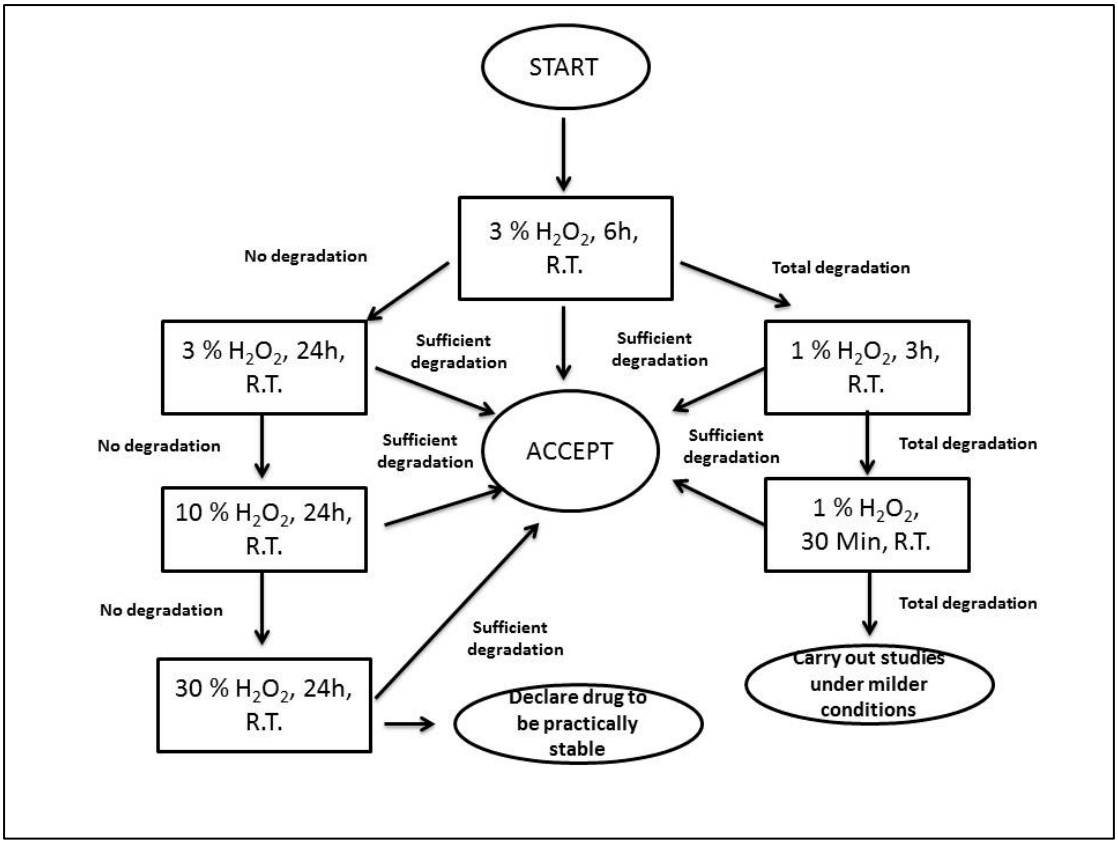


**Figure 4: Flow chart for performing stress studies for degradation under oxidative conditions**

**Table No. 3. Classification system for oxidative degradation**

| **Category of drug** | **Strength of hydrogen peroxide** | **Time of exposure** | **Temperature** | **Extent of Decomposition** |
|---|---|---|---|---|
| **Practically stable** | 30% | 48 h | R.T. | None |
| **Very stable** | 10% | 24 h | R.T. | Sufficient |
| **Stable** | 3% | 24 h | R.T. | Sufficient |
| **Labile** | 3% | 6 h | R.T. | Sufficient |
| **Very labile** | 1% | 3 h | R.T. | Sufficient |
| **Extremely labile** | 1% | 30 min | R.T. | Sufficient |

### 9.4 Photolytic Conditions

UV light: The photolytic studies should be carried out by exposure to light using a combination of cool white and UV fluorescent lamp. Exposure energy should be minimum of 1.2 million lux hrs fluorescent light and if decomposition is not seen then intensity should be increased by 5 times. In case still no decomposition takes place, the drug can be declared photo stable.

Sunlight: The photolytic studies should cover the exposure of drug solution to sunlight. The exposure period ranges from few hours to several months. The photolytic studies are carried out at room temperature.

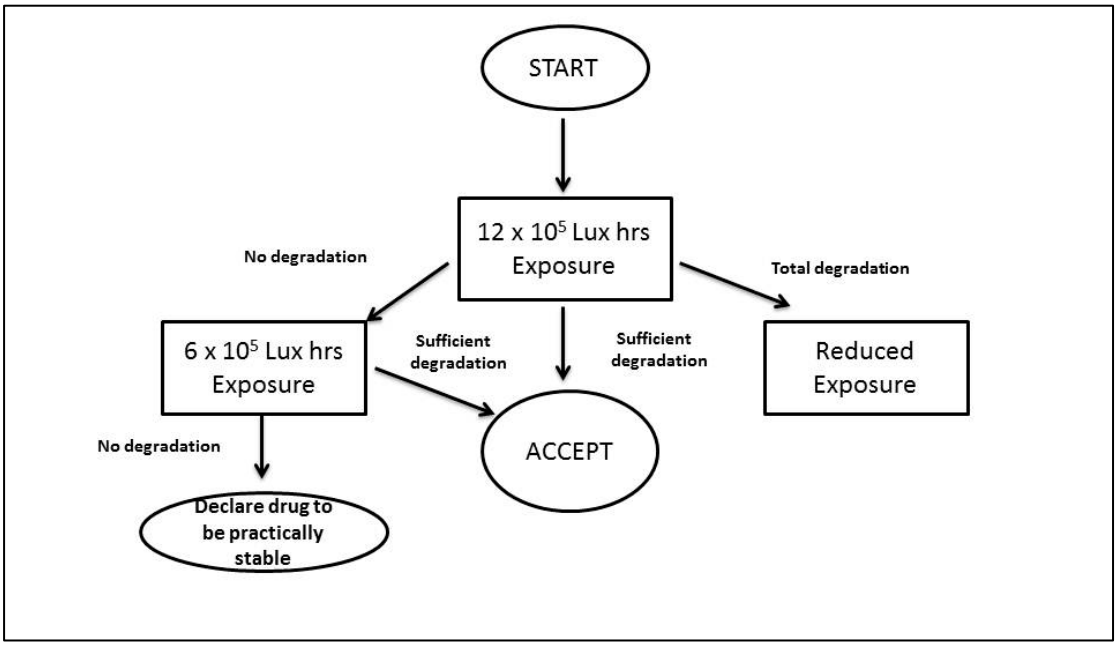


**Figure 5: Flow chart of photo-degradation**

**Table No. 4. Classification system for photolytic degradation**

| **Category of drug** | **Total exposure** | **Temperature** | **Extent of Decomposition** |
|---|---|---|---|
| **Photo labile** | 1.2 X 106 lux h | R.T. | Sufficient or total |
| **Photo stable** | 6 X 106 lux h | R.T. | None |

### 9.5 Neutral Conditions

Stress testing under neutral condition can be carried out by refluxing the drug in water for 12 hrs. Refluxing time should be increased or decreased to obtain 10 to 20 % degradation of drug in 12 hrs.

**Table No. 5. Classification system for hydrolysis under neutral conditions**

| **Category of drug** | **Time of exposure** | **Temperature** | **Extent of Decomposition** |
|---|---|---|---|
| **Practically stable** | 5 days | Refluxing | None |
| **Very stable** | 2 days | Refluxing | Sufficient |
| **Stable** | 1 day | Refluxing | Sufficient |
| **Labile** | 12 h | Refluxing | Sufficient |
| **Very labile** | 8 h | 40 °C | Sufficient |
| **Extremely labile** | 1 h | 25 °C | Sufficient |

### 9.6 Thermal Conditions

#### 9.6.1 Dry Heat

Heating the drug powder at higher temperature in oven can be followed to carry out stress testing for dry heat degradation.

#### 9.6.2 Wet Heat

Wet heat degradation can be studied by keeping the drug solution at 50°C, 75% relative humidity for 3 months in humidity chamber.

## 10 Levosulpiride

Levosulpiride is a substituted benzamide derivative. Dopamine is a neurotransmitter involved in regulation of behavior and mood. Disturbance in neurotransmitter leads to psychotic illness like schizophrenia. It is reported as selective antagonist of dopamine D2 receptors. As an antipsychotic it is mainly indicated in psychotic illness like schizophrenia, depression. It is also indicated in somatoform disorders, emesis and dyspepsia. It is a levo entiomer of sulpiride. The levo enantiomer shows better pharmacological actions and lower incidence of toxic effects than both dextro as well as the racemic forms of the drug.

### 10.1 Mechanism of action

Levosulpiride is an atypical antipsychotic agent that blocks the presynaptic dopaminergic D2 receptors. Like its parent compound, levosulpiride shows antagonism at D3 and D2 receptors present pre-synaptically as well as post-synaptically in the rat striatum or nucleus accumbens. The preferential binding of the presynaptic dopamine receptors decreases the synthesis and release of dopamine at low doses whereas it causes postsynaptic D2 receptor antagonism at higher dose. This receptor profile of the drug along with its limbic selectivity explains its effectiveness in the management of both positive and negative symptoms of schizophrenia. [40-43]

It is prescribed in combination with proton pump inhibitors. The available marketed formulations mainly include combination of levosulpiride with pantoprazole, esomeprazole and rabeprazole.

## 10.2 Drug profile:

**Levosulpiride**

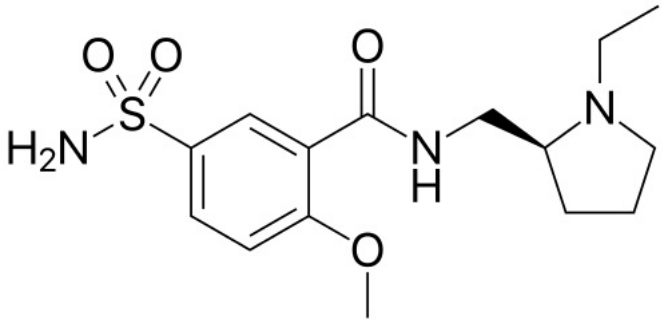


**Figure 6: Chemical structure of Levosulpiride**

| | |
|---|---|
| **Application:** | Atypical antipsychotic and a prokinetic agent |
| **Chemical name:** | N-[[(2S)-1-Ethylpyrrolidin-2- yl] methyl] -2- methoxy -5- sulfamoylbenzamide |
| **Molecular Formula:** | $C_{15}H_{23}N_3O_4S$ |
| **Molecular Weight:** | 341.00 gm/mole |
| **Melting point:** | 185 to 189 °C |
| **CAS Number:** | 23672-07-3 |
| **Appearance:** | A white to cream colour powder |
| **Solubility:** | Sparingly soluble in methanol, slightly soluble in alcohol and in methylene chloride. |

### 10.3 Reported methods

**Table No. 6: Review of literature of Levosulpiride**

| Sr. No. | Author/s | Method | Title | Ref. No. |
|---|---|---|---|---|
| 1. | Su-Eon Jin, Eunmi Ban, Yang-Bae Kim, Chong-Kook Kim | HPLC | Development of HPLC method for the determination of levosulpiride in human plasma | 44 |
| 2. | Jain Manu S, Agrawal Yogesh S, Chavhan Randhir B, Bari Manoj M, Barhate S D. | UV | UV Spectrophotometric methods for simultaneous estimation of levosulpiride and esomeprazole in capsule dosage Form | 45 |
| 3. | Ramakrishna Yadav, Avani Chokshi, Vijaykumar Parmar | UV | Development and validation of spectrophotometric methods for simultaneous estimation of levosulpiride and pantoprazole sodium | 46 |
| 4. | Chhalotiya Usmangani K., Bhatt Kashyap K, Shah Dimal A, Baldania Sunil L, Patel Jigar R. | HPLC | Development of stability indicating RP-HPLC method for determination of levosulpiride hydrochloride in bulk and pharmaceutical dosage form. | 47 |
| 5. | Nandakishore Agarwal and B.Jagadeesh | HPLC | Development and validation of stability indicating RP-HPLC method for simultaneous estimation of levosulpiride and rabeprazole sodium | 48 |
| 6. | Bijay Kumar Sahoo, Ayan Das, Jayanti Mukherjee, Soumendra Darbar and Tapan Kumar Pal | HPLC | Determination of levosulpiride in human plasma using HPLC method and its application to bioequivalence study | 49 |

| | | | | |
|---|---|---|---|---|
| 7. | Shobha Manjunath, Venkatesh Chouhan, S.Sandeep | UV | Spectrophotometric estimation of levosulpiride in bulk drug and formulations | 50 |
| 8. | Patel H, Shrivastava A K, Jindal D | HPLC | Analytical method development and validation of esomeprazole and levosulpiride in their combined capsule dosage form by RP-HPLC | 51 |
| 9. | A Sirisha, A Ravi Kumar | HPLC | Method development and validation of simultaneous estimation of levosulpiride and rabeprazole in bulk and pharmaceutical dosage form by RP-HPLC | 52 |
| 10 | Yogesh P Agrawal, Surya Prakash Gautam, Ajay Verma, Mona Y Agrawal and Arun K Gupta | UV | Simultaneous estimation of esomeprazole and levosulpiride in solid dosage form | 53 |

## 11 Esomeprazole:

It is a drug from antacid category indicated in the treatment of dyspepsia, peptic ulcer disease, gastro esophageal, and Zollinger-Ellison syndrome. It is S- enantiomer of omeprazole. It is a proton pump inhibitor. It reduces stomach acid secretion by inhibiting $H^+/K^+$ ATPase in the parietal cells of the stomach. It is also indicated in GERD. When gastric acid comes in contact with esophagus it causes irritation and gives burning sensation which is called as heartburn. Prolong contact may permanently damage esophagus. Esomeprazole by reducing the production of gastric acid minimizes chance of gastro esophageal reflux disease. Esomeprazole undergoes rapid degradation in gastric acidic conditions so it is always available with delayed release dosage form. In various countries it is available as delayed release capsule in the dose of 20 and 40 mg. It is also available as IV injections and infusion. Oral formulations are enteric coated. It is available in combinations with domperidon, ondansetron and levosulpiride.

### 11.1 Mechanism of action

Esomeprazole is a proton pump inhibitor that suppresses gastric acid secretion by specific inhibition of the $H^+/K^+$-ATPase in the gastric parietal cell. The S- and R-isomers of omeprazole are protonated and converted in the acidic compartment of the parietal cell forming the active inhibitor, the achiral sulphenamide. By acting specifically on the proton pump, esomeprazole inhibits the final step in acid production, thus decreasing gastric acidity.[54, 55]

## 11.2 Drug Profile:

**Esomeprazole**

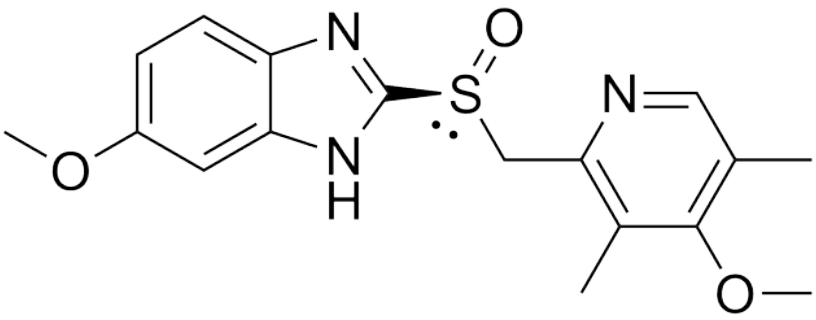


**Figure 7: Chemical structure of Esomeprazole**

| | |
|---|---|
| **Application:** | A gastric proton pump inhibitor, also used in gastro esophageal reflux disease (GERD) |
| **Chemical name:** | (*S*)-5-Methoxy-2-[(4-methoxy-3,5-dimethylpyridin-2-yl)methylsulfinyl]-3*H*-benzoimidazole |
| **Molecular Formula:** | $C_{17}H_{18}N_3O_3S$ |
| **Molecular Weight:** | 356.56 gm/mole |
| **Melting Point:** | 155 °C |
| **CAS Number:** | 217087-09-7 |
| **Appearance:** | White to slightly yellowish-white solid. |
| **Solubility:** | Soluble in methanol, DMSO, ethanol, water. |

### 11.3 Reported methods

**Table No. 7: Review of literature of Esomeprazole**

| Sr. No. | Author/s | Method | Title | Ref. No. |
|---|---|---|---|---|
| 1. | Santaji Uttam Nalwadea, B Vangala Ranga Reddya, Dantu Durga Raoa, Nagendra kumar Morisettia | UPLC | A validated stability indicating ultra-performance liquid chromatographic method for determination of impurities in esomeprazole magnesium gastro resistant tablets | 56 |
| 2. | S Sharma and M C Sharma | HPTLC | Densitometric method for quantitative determination of esomeprazole and domperidon in Dosage forms. | 57 |
| 3. | Tushar G Rukari and Ganesh V Ahire | UV | Formulation and evaluation of esomeprazole delayed release tablets | 58 |
| 4. | Jinesh A Doshi, Bhavna A Patel, Shraddha J Parmar | HPLC | Development and validation of HPLC method for simultaneous determination of aspirin and esomeprazole magnesium in binary mixture. | 59 |
| 5. | S Ashutosh Kumar, Manidipa Debnath, J V L N Seshagiri Rao | HPLC | Stability indicating simultaneous estimation of assay method for esomeprazole and naproxen in bulk as well as in pharmaceutical formulation by using RP-HPLC | 60 |
| 6. | Leo Zanitti, Rosella Ferretti, Bruno Gallinella, Francesco La Torre, Maria Luisa Sanna, Antonina Mosca, | HPLC | Direct HPLC enantioseparation of omeprazole and its chiral impurities: Application to the determination of enantiomeric purity of esomeprazole magnesium | 61 |

| | | | | |
|---|---|---|---|---|
| | Roberto Cirilli | | trihydrate | |
| 7. | Palavai Sripal Reddy, Shakil Saita, Gururaj Vasudevmurthya and Badri Vishwanatha, Vure Prasada and S. Jayapal Reddy | HPLC | Stability indicating simultaneous estimation of assay method for naproxen and esomeprazole in pharmaceutical formulations by RP-HPLC | 62 |
| 8. | S Lakshamana Prabhu, S Shirwaikar, Annie Shirwaikar, C Dinesh Kumar, A Joseph and R Kumar | UV | Simultaneous estimation of esomeprazole and domperidon by UV spectrophotometric method. | 63 |
| 9. | Putta Rajesh Kumar, Somashekar Shyale, Mallikarjuna Gouda M and S M Shanta Kumar | UV | Physico-chemical characterization, UV spectrophotometric method development and validation studies of esomeprazole magnesium trihydrate | 64 |
| 10 | Palavai Sripal Reddy, Kishore Kumar Hotha, Shakil Sait | HPLC | Complexity in estimation of esomeprazole and its related impurities stability in various stress conditions in low-dose aspirin and esomeprazole magnesium capsules | 65 |
| 11 | Deepak Kumar Jain, Nitesh Jain, Rita Charde, and Nilesh Jain | HPLC | The RP-HPLC method for simultaneous estimation of esomeprazole and naproxen in binary combination | 66 |

## 12 Rabeprazole

Drug belongs to same category of esomeprazole. It is a proton pump inhibitor used in Short-term treatment in healing and symptomatic relief of duodenal ulcers in dyspepsia disease, gastro esophageal, and Zollinger-Ellison syndrome. It is available in tablet dosage form, mainly in combination with itopride, domperidon, diclofenac sodium and levosulpiride.

### 12.1 Mechanism of action

Same as like Esomeprazole.

## 12.2 Drug Profile:

**Rabeprazole**

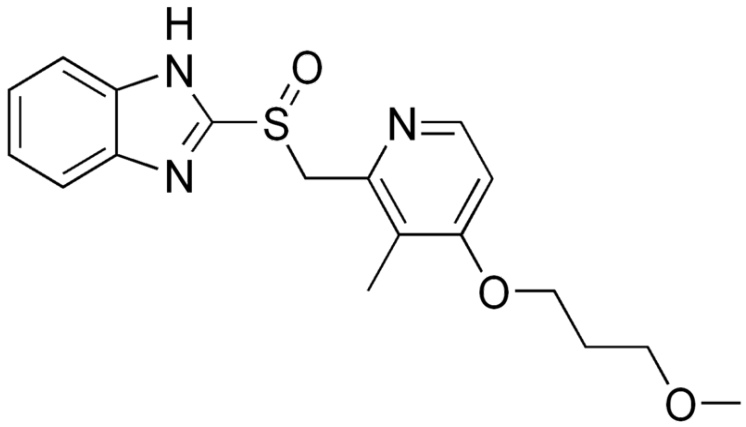


**Figure 8: Chemical structure of Rabeprazole**

| | |
|---|---|
| **Application:** | A gastric proton pump inhibitor |
| **Chemical name:** | (RS)-2-([4-(3-Methoxypropoxy) -3-methylpyridin -2- yl] methylsulfinyl) -1H- benzo[d]imidazole |
| **Molecular Formula:** | $C_{18}H_{21}N_3O_3S$ |
| **Molecular Weight:** | 359.444 gm/mole |
| **Melting Point:** | 140 to 141 °C |
| **CAS Number:** | 117976-89-3 |
| **Appearance:** | White to slightly yellowish-white solid. |
| **Solubility:** | Very soluble in water and methanol, freely soluble in ethanol, chloroform and ethyl acetate and insoluble in ether and n-hexane. |

## 12.3 Reported methods

**Table No. 8: Review of literature of Rabeprazole**

| Sr. No. | Author/s | Method | Title | Ref. No. |
|---|---|---|---|---|
| 1. | Arunadevi S Birajdar, Subramania Meyyanathan, Bhojraj Suresh | HPLC | A RP-HPLC method for determination of diclofenac with rabeprazole in solid dosage form | 67 |
| 2. | R Vasu Dev, G Sai Uday Kiran, B Venkata Subbaiah, B Suresh Babu, J Moses Babu, P K Dubeyc and K Vyasb | HPLC | Identification of degradation products in stressed tablets of rabeprazole sodium by HPLC-hyphenated techniques | 68 |
| 3. | Ren, Shan, Mi-Jin Park, Hongkee Sah, and Beom-Jin Lee. | HPLC | Effect of pharmaceutical excipients on aqueous stability of rabeprazole sodium | 69 |
| 4. | Ramakrishna N V S, Vishwottam K N, Wishu S, Koteshwara M, Suresh Kumar S | HPLC | High-performance liquid chromatography method for the quantification of rabeprazole in human plasma using solid-phase extraction. | 70 |
| 5. | Sabnis S S, Dhavale N D, Jadhav V Y, Gandhi S V | UV | Spectrophotometric simultaneous determination of rabeprazole Sodium and itopride hydrochloride in capsule dosage form | 71 |
| 6. | Miura M, Tada H, Satoh S, Habuchi T, Suzuki T | HPLC | Determination of rabeprazole enantiomers and their metabolites by high-performance liquid chromatography with solid-phase extraction | 72 |

| | | | | |
|---|---|---|---|---|
| 7. | El-Gindy A, El-Yazby F, Maher M M | UV HPLC | Spectrophotometric and chromatographic determination of rabeprazole in presence of its degradation products | 73 |
| 8. | Pimenta A M, Araujo A N, Montenegro | Fluorimetric | Simultaneous potentiometric and fluorimetric determination of diclofenac in a sequential injection analysis system | 74 |
| 9. | Bharekar Vishal | HPTLC | Validated HPTLC method for simultaneous estimation of Rabeprazole Sodium, Paraetamol and Aceclofenac in bulk drug and formulation | 75 |
| 10. | Suganthi, A, Sofiya John, and T K Ravi | HPTLC | Simultaneous HPTLC determination of rabeprazole and itopride hydrochloride from their combined dosage form | 76 |

## 13 Mometasone furoate

Mometasone furoate is used as topical anti-inflammatory agent. It is a glucocortico steroid. It is indicated for the treatment of inflammation in topical disorders like eczema and psoriasis. It is also prescribed in the treatment of allergic rhinitis such as hay fever. It is also used in the treatment of asthma for the patients who do not show response in less potent corticosteroids. In terms of order of potency from higher to lower side it rests between dexamethasone and hydrocortisone. It reduces inflammation by reversing the activated inflammatory proteins. It also activates anti-inflammatory proteins. It stabilizes cell membrane and decreases the influx of inflammatory cells. Mometasone furoate is mainly available as topical preparation like lotion, creams and gels. It is used in combination with some antifungal agents like terbinafine, fusidic acid, salicylic acid, miconazole etc.

### 13.1 Mechanisam of action

Mometasone furoate is a glucocorticosteriod. Corticosteriods are prescribed in allergic reactions. They have anti-inflammatory, antipruritic and vasoconstrictive properties. Corticosteriods reduce allergic reactions in various types of cells like mastocytes and eosinofiles which are responsible for allergeic reactions. Mometasone furoate and other corticosteroids can easily circulates in the blood and can easily pass cell membrane. After crossing cell membrane it binds with cytoplasmic receptors. It also inhibits the actions of enzymes cytochrome $P_{450}$ 2c8 which contributes in the activity of monooxygenase.

The glucocorticosteriods reduce inflammation by decreasing the release of hydrolase acid from leuckocyes. It also stops the accumulation of macrophages at the site of inflammation. It also interferes with the adhesion of leuckocytes with capillary walls. It reduces permeability of the capillary membrane and thus edema. The other responsible components like histamine and kinin liberation is also reduced. It interferes with scar tissue formation. The propagation of fibroblasts and collagen deposits are also reduced. The anti-inflammatory agents along with corticostreriods inhibits proteins of phospholipase A2 which are collectively called lipocortins. Lipocortins are involved in biosynthesis of potent mediators of inflammation as the prostaglandin's and leukotriens. It does so by inhibiting the liberation of the molecular

precursors of arachidonic acid. Topically it decreases inflammation related with acute and chronic dermatosis.[77-79]

## 13.2 Drug Profile:

**Mometasone furoate**

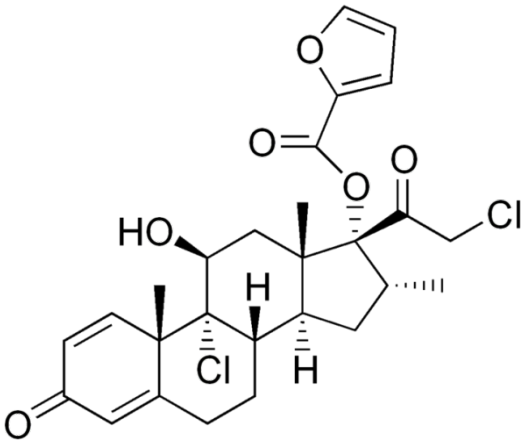


**Figure 9: Chemical structure of Mometasone furoate**

| | |
|---|---|
| **Application:** | An anti-inflammatory agent |
| **Chemical name:** | (11β,16α)-9,21-Dichloro-11-hydroxy-16-methyl-3,20-dioxopregna-1,4-dien-17-yl 2-furoate |
| **Molecular Formula:** | $C_{27}H_{30}Cl_2O_6$ |
| **Molecular Weight:** | 521.44 gm/mole |
| **Melting Point:** | 218 to 220 °C |
| **CAS Number:** | 83919-23-7 |
| **Appearance:** | White fine crystalline powder. |
| **Solubility:** | Soluble in Methanol. |

### 13.3 Reported methods

**Table No. 9: Review of literature of Mometasone furoate**

| Sr. No. | Author/s | Method | Title | Ref. No. |
|---|---|---|---|---|
| 1. | Saleem Shaikh, M S Muneera, O A Thusleem, Muhammad Tahir, and Anand V Kondaguli | RP-HPLC | A simple RP-HPLC method for the simultaneous quantitation of chlorocresol, mometasone furoate, and fusidic acid in creams | 80 |
| 2. | Chinmoy Roy and Jitamanyu Chakrabarty | RP-HPLC | Stability-indicating validated novel RP-HPLC method for simultaneous estimation of methylparaben, ketoconazole, and mometasone furoate in topical pharmaceutical dosage formulation | 81 |
| 3. | Rasha M Youssef, Mohamed A Koranya and Mostafa A Afify | HPLC-DAD | Development of a stability indicating HPLC-DAD method for the simultaneous determination of mometsone furoate and salicylic acid in an ointment matrix | 82 |
| 4. | Amol A Kulkarni, Rabindra K Nanda, Meenal N Ranjane, Poonam N Ranjane. | HPTLC | Simultaneous estimation of nadifloxacin and mometasone furoate in topical cream by HPTLC method | 83 |
| 5. | Patel Heta D, Patel Mehul M | UV | Development and validation of UV spectrophotometric method for simultaneous estimation of terbinafine hydrochloride and mometasone furoate in combined dosage form | 84 |

## 14 Terbinafine

An allyamine by chemical nature; terbinafine is categorized as antifungal agent. It is highly lipophilic in nature. It accumulates in different body tissue like skin, nails and fatty tissue. It is mainly indicated in fungal infections caused by dermatophyte. It is available in topical as well as oral formulations. The creams are in combinations with mometasone furoate as anti-inflammatory agent. The label claim is 1% w/w. the oral dosage forms are tablets available in the dose of 250 mg per tablet.

### 14.1 Mechanism of action

Squalene epoxidase is an enzyme involved in ergosterol synthesis which is a part of fungal cell membrane synthesis pathway. Terbinafine hydrochloride mainly inhibit ergosterol synthesis by preventing conversion of squalene to lanosterol and ergosterol. It does so by inhibiting the enzyme Squalene epoxidase involved in the synthesis. It leads to change in cell membrane permeability ultimately breakdown of fungal cell occurs.[85-86]

## 14.2 Drug Profile:

**Terbinafine**

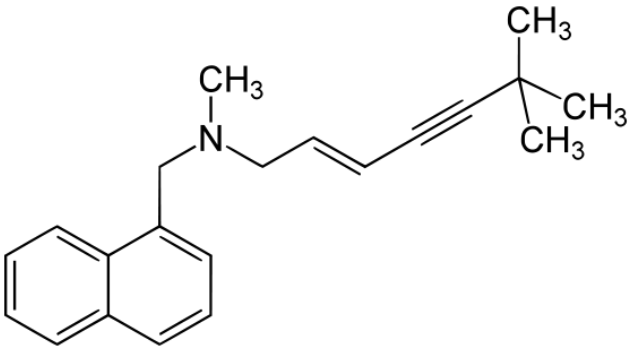


**Figure 10: Chemical structure of Terbinafine**

| | |
|---|---|
| **Application:** | Antifungal |
| **Chemical name:** | [(2E)-6,6-Dimethylhept-2-en-4-yn-1-yl](methyl)(naphthalen-1-ylmethyl)amine |
| **Molecular Formula:** | $C_{21}H_{25}N$ |
| **Molecular Weight:** | 291.43 gm/mole |
| **Melting Point:** | 195 to 198 °C |
| **CAS Number:** | 91161-71-6 |
| **Appearance:** | White fine crystalline powder. |
| **Solubility:** | Freely soluble in methanol and dichloromethane, soluble in ethanol, and slightly soluble in water. |

### 14.3 Reported methods

**Table No. 10: Review of literature of Terbinafine hydrochloride**

| Sr. No. | Author/s | Method | Title | Ref. No. |
|---|---|---|---|---|
| 1. | Pritam S Jain, Amar J Chaudhari, Stuti A Patel, Zarana N Patel, Dhwani T Patel | UV | Development and validation of the UV spectrophotometric method for determination of terbinafine hydrochloride in bulk and in formulation | 87 |
| 2. | Hamsa Kassem, Mohamed Amer Almardini | HPLC | High Performance Liquid Chromatography method for the determination of terbinafine hydrochloride in semi solids dosage form | 88 |
| 3. | Pushpa D Goswami. | HPLC | Stability-indicating RP-HPLC method for analysis of terbinafine hydrochloride in bulk and in tablet dosage form | 89 |
| 4. | Abdel-Moety E M, K O Kelani, and A M A Alamein | HPLC | Chromatographic determination of terbinafine in presence of its photodegradation products | 90 |
| 5. | Suma B V, Kannan K, Madhavan. V, Chandini R Nayar | HPTLC | HPTLC method for determination of terbinafine in the bulk drug and tablet dosage form | 91 |
| 6. | P D Goswami | UV | Validated spectrophotometric method for the estimation of terbinafine hydrochloride in bulk and in tablet dosage form using inorganic solvent | 92 |
| 7. | R Ramesh Raju, N Bujji Babu | HPLC | Simultaneous analysis of RP-HPLC method development and validation of terbinafine and bezafibrate drugs | 93 |

| | | | | |
|---|---|---|---|---|
| | | | in pharmaceutical dosage form | |
| 8. | Simone Goncalves Cardoso and E. E. Schapoval | UV | UV spectrophotometry and nonaqueous determination of terbinafine hydrochloride in dosage forms | 94 |

# *Aims and Objectives*

## Aims and Objectives

### 1. Need for the study

Due to increased drive for new chemical entities with specific pharmacological effect more potent molecules are developed; along with it many multicomponent formulations have come into market. The large number of drug candidates and strict quality control regulations for the analytical methods has increased the work load on the analytical department. To produce quality drug, quality assurance at every stage becomes the most important process.

Last few decades have seen significant advances and improvements in utilization of instrumental technology and analytical techniques. Efficacy and safety can be achieved by analytical monitoring of quality and by validated analytical procedures as per ICH guidelines and its updated convention. Therefore the development of reliable, accurate, precise, validated, stability indicating analytical method, suitable for routine determination for the quality control is both important and challenging for the analytical chemist. Pharmaceutical industries are also in a need of analytical methods for the determination of selected drug in different formulations with single or multi drug combinations. The developed method should be fast, accurate, precise and reproducible.

### 2. Objectives of the work

The objective of the work is to develop and validate an accurate, sensitive and selective analytical method for estimation of some active pharmaceutical ingredients in bulk and different dosage forms. It is also extended to the stability of some drugs in bulk towards acid and base hydrolysis, neutral hydrolysis, oxidative degradation, photolytic degradation processes. The objectives of the research work are enlisted below:

- To select some suitable drug candidates and their drug formulations.
- To develop suitable analytical technique/s for their analysis.
- To optimize the analytical technique/s employed.
- To validate the method/s as per ICH guidelines.

- To study forced degradation of drug/s using validated analytical method/s and identification of some of the degradants.
- To select some drug combinations and to develop analytical methodology for simultaneous estimation of the drugs.

# *Plan of work*

## Plan of Work

### 1 Procurement of materials, drugs and formulations

Extensive literature review will be carried out for the selection of drug candidates. The chemicals and reagents required for the study will be procured from available sources and marketed formulations will be purchased from local market.

### 2 Selection of suitable analytical techniques

Based on literature review appropriate chromatographic method will be selected which will mainly include HPLC and/or HPTLC. The selected chromatographic method will be used for the separation of selected drug candidate in combined dosage form and for their simultaneous quantification.

### 3 Plan of work for simultaneous analysis by HPLC and HPTLC

#### 3.1 Selection of drug candidates and formulations

Extensive literature review and marketed formulation survey will be carried out to select the suitable drug candidate and drug products.

#### 3.2 Selection of suitable analytical techniques

A suitable chromatographic technique like high performance thin layer chromatography or high performance liquid chromatography will be selected for separation of selected drug candidates. The chromatographic method selected will be used for quantification of selected drug candidate in the selected combined dosage forms.

#### 3.3 Solubility and stability studies

The selected drug candidates will be checked for their stability in different organic solvents with different polarities. The study will be carried out at room temperature.

#### 3.4 Selection of wavelength

The selected drug candidates will be analysed for their spectrophotometric behaviour. Their UV spectrum will be recorded and wavelength for considerable absorbance will be selected for the analysis.

### 3.5 Method development

Initially solvents with different organic solvents will be tried to achieve the separation of the selected drug candidates on high performance thin layer chromatographic plates. Later combinations of solvents will also be tried to achieve better separation with desired resolution. Mobile phase and experimental conditions from reported methods will also be considered. Finally, experimental conditions will be optimized for better separation and quantitation of selected drug candidates and combination.

To develop a high performance liquid chromatographic method combination of methanol water and acetonitrile water will be tried initially. Finally, experimental conditions will be optimized for better separation and quantitation of selected drug candidates and combination.

### 3.6 Validation of the method and statistical evaluation

The developed methods will be validated for various parameters as per ICH Q2 (R1) guidelines.

### 3.7 Analysis of pharmaceutical formulation

The developed analytical method will be applied for quantification of selected drug candidates and combinations in marketed formulations after its validation as per ICH guidelines.

## 4 Plan of work for stress degradation studies

The ICH Q1A (R2) guidelines necessitate that forced degradation testing be carried out on drugs to help recognize the probable degradation products, which can in return assist to establish the degradation pathways. It will help to establish the inherent stability of the molecule and then validation of a developed method for the stability check of the drug candidate. The stress testing depends on individual drug candidate as well as on the type of formulation involved. Attempts will also be made to identify some of the degradation products obtained under stress testing of these drugs and in formulations.

### 4.1 Solubility and stability studies

The solubility of selected drug candidates will be checked in different organic with different polarity. The stability of drug candidates will also be studied in the selected solvent.

### 4.2 Selection of wavelength

Ultra violet spectrum of the selected drug candidate will be recorded to select the wavelength of maximum absorbance.

### 4.3 Stress degradation studies

The intrinsic stability of the selected drug candidate in different stress condition will be carried out as per ICH guidelines.

### 4.4 Optimization of HPLC and HPTLC method

Experimental conditions to develop a stability indicating assay method will be set and optimized for both high performance liquid chromatography and high performance thin layer chromatography by selecting suitable mobile phase and parameters.

### 4.5 Validation of the method and statistical evaluation

Developed and optimized methods were validated as per ICH Q2 (R1) guidelines and have been statistically evaluated.

### 4.6 Analysis of pharmaceutical formulation

Developed stability indicating assay method will be applied for quantitation of selected drug in marketed formulations.

### 5 Identification of degradation products

An attempt will be made to identify some of the degradation products with the help of hyphenated techniques like LC-MS/MS and TLC-MS. The degradation pathway will be predicted from the results obtained from hyphenated techniques.

# *Results and Discussion*

## Results and Discussion

### 1 Development and validation of normal phase HPTLC method for simultaneous quantification of Levosulpiride and Esomeprazole in capsule dosage form.

#### 1.1 Optimization of HPTLC method

To obtain the desired *Rf* value range (0.2 - 0.8) and resolution (Rs ≥ 1.5) different mobile phases containing various ratios of toluene, dichloromethane, n-hexane, ethanol, methanol, water, ethyl acetate, and acetone were tried. The reported high performance thin layer chromatographic methods for individual drugs were also tried and resolution for both the drugs was studied. Both the drugs showed good resolution in ethyl acetate and methanol in the ratio of (9:1, v/v) but peak shape was not good. To improve the peak shape and to minimize tailing effect little quantity of ammonia was added. Finally, the mobile phase consisting of ethyl acetate: methanol: ammonia (9: 1: 0.5, v/v/v) was selected as it gave well resolved peaks. The retardation factor (*Rf*) for LSP and ESP were found to be 0.30 ± 0.02 and 0.64 ± 0.02, respectively (Figure 11).

#### 1.2 Selection of wavelength

At the time of trials for method development for LSP and ESP; after chromatographic development HPTLC plates were visualized under UV cabinet. Both the drugs showed absorbance in ultra violet range. Plates were scanned in the range of 200 to 400 nm. The ultra violet spectrum of both LSP and ESP were recorded and wavelength with considerable absorbance was selected. The optimum wavelength for detection and quantitation used was 216 nm, at this wavelength both the drugs showed comparatively more absorbance than the isoabsorptive point (Figure 12).

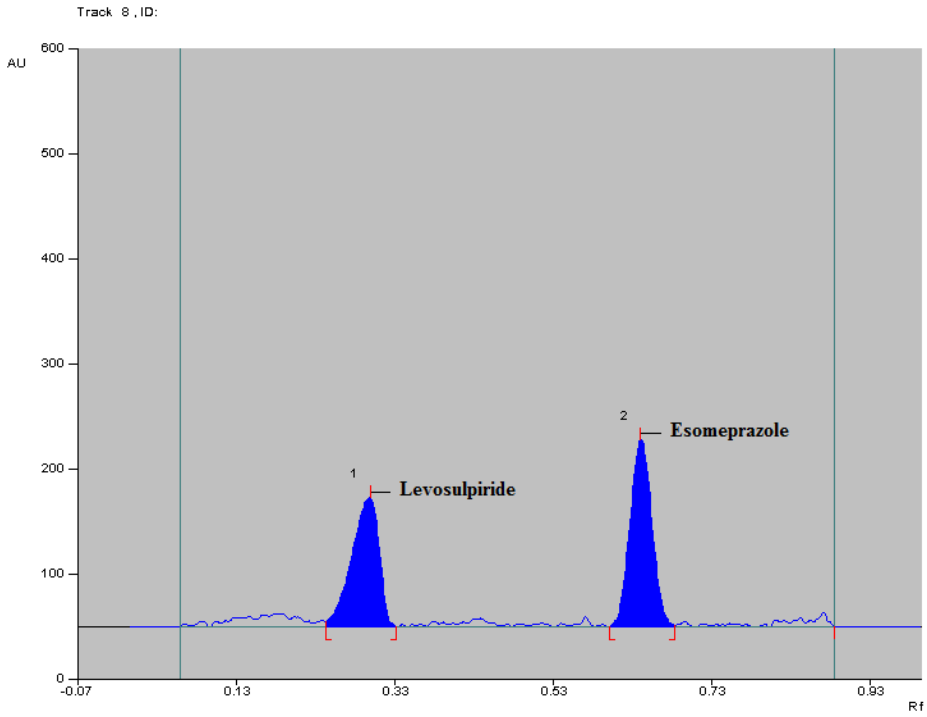


**Figure 11: Representative densitogram of LSP and ESP**

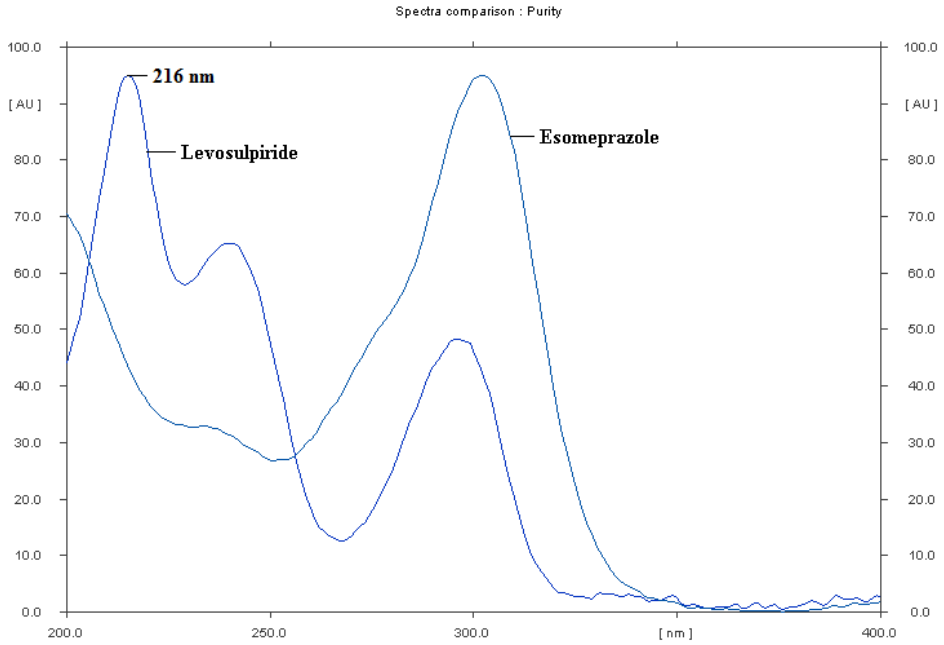


**Figure 12: Overlain UV spectrum of LSP and ESP**

### 1.3 Chromatographic conditions

The optimized high performance thin layer chromatographic conditions are as follows.

| | |
|---|---|
| **Stationary phase** | Aluminum plates precoated with silica gel 60 $F_{254}$ |
| **Mobile phase** | Ethyl acetate: methanol: ammonia (9: 1: 0.5, v/v/v) |
| **Plate size** | 20 X 10 cm |
| **Band size** | 6 mm (Distance between two bands: 10 mm) |
| **Development chamber** | Twin-trough glass chamber, 20 X 10 cm with stainless steel lid |
| **Saturation time** | 10 Min |
| **Migration distance** | 80 mm |
| **Room temperature** | 25 ± 2 °C |
| **Scanning mode** | Absorbance/reflectance |
| **Slit dimensions** | 5 × 0.45 mm |
| **Detection wavelength** | 216 nm |
| **R*f* values** | LSP - 0.30 ± 0.02 |
| | ESP - 0.64 ± 0.02 |

### 1.4 HPTLC method validation

The validation of the developed method was carried out as per ICH guidelines [Q2 (R1)]. The parameters studied for the validation were linearity, LOD, LOQ, precision, accuracy and robustness. Standard drug solution was used for the validation of the method and each time freshly prepared solutions were used.[25]

#### 1.4.1 Linearity and range

Calibration curves are mostly constructed for quantitation of unknown samples and for quality control of marketed formulations. It is mainly used to find out the concentration of unknown samples by comparing it with sets of known standards. It is plotted with signal response verses concentration. To study linearity of both the drugs stock solution of the standard drugs were prepared separately having concentration of 100 µg $mL^{-1}$. Bands of 6 mm from these solutions were spotted on prewashed and activated HPTLC plates in the range of 100 to 1000 ng $band^{-1}$. Volume of the solution spotted was 1, 2, 4, 6, 8 and 10µL having concentration of 100 µg $mL^{-1}$. After development plates were scanned. The detector response *i. e.* peak area was plotted against concentration (ng $band^{-1}$). The data obtained was subjected to statistical least square linear regression analysis. Slope, intercept and correlation coefficient was calculated from calibration curve. Calibration curves of standard drug concentrations and peak areas were found to be linear over a range of 100 to 1000 ng $band^{-1}$ for both LSP and ESP (Table No. 11). The linear regression data is mentioned in the table no. 12. Calibration curves are depicted in figures 13 and 15 for LSP and ESP respectively. And residual plot for both standard LSP and ESP showed no tendency in the plot supports linearity data (Figure 14 and 16).

**Table No. 11: Linearity data of Levosulpiride and Esomeprazole**

| **Concentration** (ng band$^{-1}$) | **Area** | |
|---|---|---|
| | **Levosulpiride** | **Esomeprazole** |
| **100** | 934 | 834 |
| **200** | 1595 | 1529 |
| **400** | 2705 | 3108 |
| **600** | 4068 | 4745 |
| **800** | 5277 | 6255 |
| **1000** | 6432 | 7659 |

**Table No. 12: Linear regression data for the calibration curves (n = 6)**

| **Parameters** | **Levosulpiride** | **Esomeprazole** |
|---|---|---|
| **Linearity range**[a] | 100 – 1000 | 100 - 1000 |
| **$r^2$** | 0.9994 | 0.9994 |
| **Slope** | 6.1366 | 7.6853 |
| **Intercept** | 331.24 | 50.92 |
| **LOD**[a] | 31.363 | 30.631 |
| **LOQ**[a] | 95.042 | 92.822 |
| **Sy.x** | 58.323 | 70.594 |

n - no of replicates, a = ng band$^{-1}$

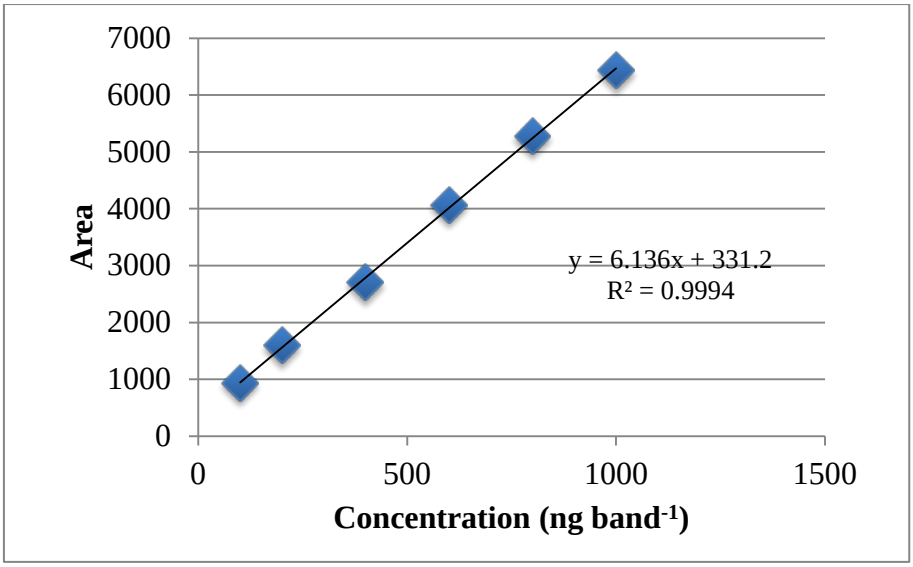


**Figure 13: Calibration curve of LSP**

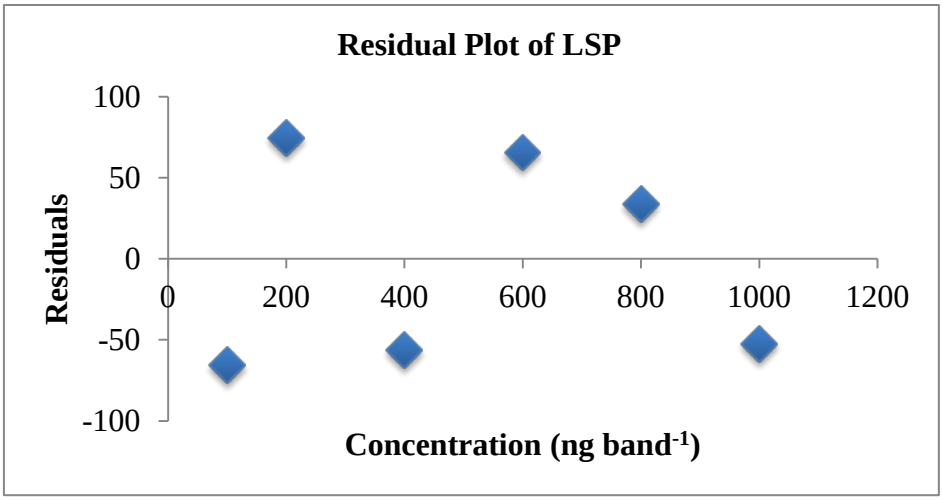


**Figure 14: Residual plot of LSP**

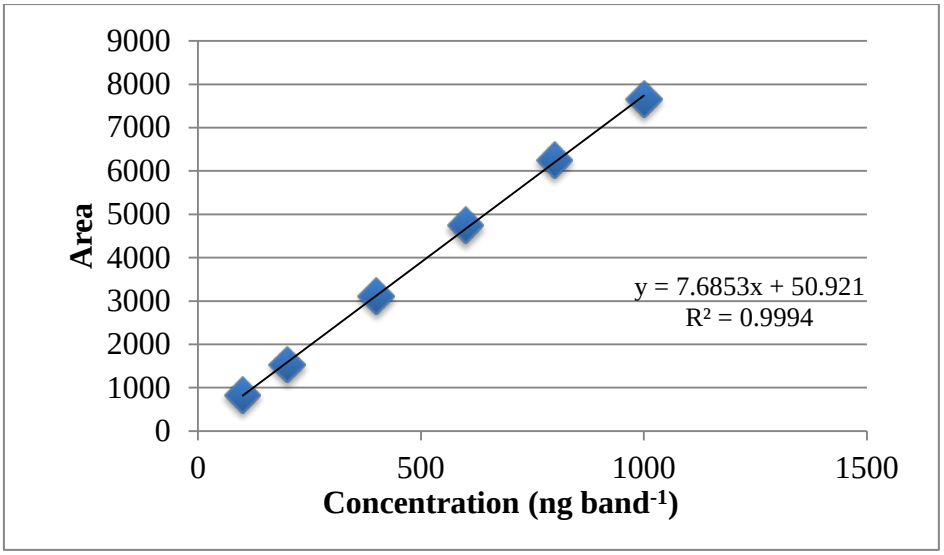


**Figure 15: Calibration curve of ESP**

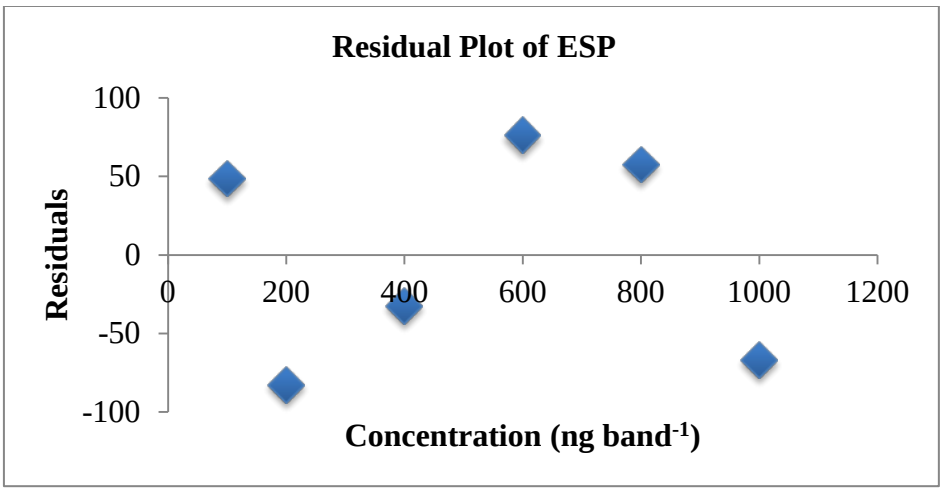


**Figure 16: Residual plot of ESP**

### 1.4.2 Sensitivity

Sensitivity of the developed method was established by determining limit of detection and limit of quantification. It was calculated using standard deviation of response and slope of the calibration curve. The LOD and LOQ for LSP and ESP were found to be 31.363 and 95.042 ng band$^{-1}$ and 30.631 and 92.822 ng band$^{-1}$, respectively (Table No. 12).

### 1.4.3 Specificity

After chromatographic development bands of samples were compared with standards for R*f* value. Specificity of the method was established by comparing standard and samples bands. The peak purity for LSP and ESP was assessed by comparing UV spectra acquired at the start (S), apex (M), and end (E) of the peak obtained from the scanning of band, that is, r(S, M) = 0.999, 0.998 and r(M, E) = 0.998, 0.999, respectively. Peak purity data showed that peaks obtained for LSP and ESP were pure.

### 1.4.4 Precision

Precision of the developed method was studied intra-day and inter-day. Three different concentrations were selected from linearity range and study was repeated three times on same day and three consecutive days. Results obtained were compared with freshly prepared samples. Intra-day precision, as % RSD was found to be 0.77 – 0.95 % for LSP and 0.98 -1.16 % for ESP. Inter-day variation, as % RSD was found to be 0.82 – 0.94 % for LSP and 0.80 – 0.94 % for ESP. As recommended by ICH guidelines, both intra and inter-day precision studies showed % RSD < 2, indicating good precision (Table No. 13).

**Table No. 13: Intra and inter day precision (n = 3)**

| Standard drugs | Concentration Taken [a] | Concentration obtained [a] | | Precision obtained [b] | |
|---|---|---|---|---|---|
| | | Intra day | Inter day | Intra day | Inter day |
| Levosulpiride | 400 | 399.485 | 412.575 | 0.95 | 0.82 |
| | 600 | 616.869 | 609.807 | 0.82 | 0.94 |
| | 800 | 812.688 | 840.011 | 0.77 | 0.88 |
| Esomeprazole | 400 | 393.272 | 398.52 | 0.98 | 0.94 |
| | 600 | 604.584 | 604.281 | 1.07 | 0.87 |
| | 800 | 796.639 | 807.396 | 1.16 | 0.80 |

a = ng band$^{-1}$, b = Precision as % RSD,

### 1.4.5 Accuracy

Recovery was established by standard addition method. Samples were spiked with 80, 100 and 120 % of standard drugs and recovered quantity was measured. Study was repeated six times. Recovery for LSP and ESP was found to be 98.13 - 100.4 %w/w and 98.08 - 101.86 %w/w, respectively indicating reliability of the method for simultaneous estimation of LSP and ESP in the marketed formulation used in the study (Table No. 14).

**Table No. 14: Results of recovery studies (n = 6)**

| Parameter | LSP | | | ESP | | |
|---|---|---|---|---|---|---|
| Amount Taken[a] | 375 | 375 | 375 | 200 | 200 | 200 |
| Amount Added[a] (%) | 300 (80) | 375 (100) | 450 (120) | 160 (80) | 200 (100) | 240 (120) |
| Amound Found[a] | 665.661 | 753.011 | 809.611 | 358.686 | 392.357 | 448.199 |
| % Recovery | 98.616 | 100.401 | 98.135 | 99.635 | 98.089 | 101.863 |
| SD | 70.193 | 63.921 | 31.139 | 49.131 | 39.513 | 55.608 |
| %RSD | 1.589 | 1.291 | 0.588 | 1.750 | 1.289 | 1.591 |

a = ng band$^{-1}$

### 1.4.6 Robustness studies

Robustness of the densitometric method was checked after deliberate alterations of the analytical parameters (Table No. 15). It showed that peak areas of interest

remained unaffected by small changes of the operational parameters (% RSD < 2) which indicate that the method is robust.

**Table No. 15: Results of robustness studies (n = 6, 600 ng band$^{-1}$)**

| Parameter | SD of concentration found | | % RSD | |
|---|---|---|---|---|
| | LSP | ESP | LSP | ESP |
| Mobile phase (ethyl acetate) composition (± 0.1 mL) | 2738.55 ± 6.43 | 3144.68 ± 11.76 | 0.23 | 0.37 |
| Amount of mobile phase (± 5 %) | 2678.92 ± 14.4 | 3066.79 ± 9.97 | 0.54 | 0.33 |
| Time from band application to chromatography (+ 10 min) | 2753.42 ± 6.06 | 3074.14 ± 12.09 | 0.22 | 0.39 |
| Time from chromatography to scanning (+ 10 min) | 2682.96 ± 9.5 | 3132.09 ± 9.07 | 0.35 | 0.29 |

### 1.4.7 Solution stability

Stability of standard solutions of LSP and ESP were assessed at room temperature for 48 hrs. The % RSD was found less than 2 indicate that the solutions were stable for 48 hrs. at room temperature when compared with freshly prepared samples.

### 1.4.8 Analysis of marketed formulations

Developed densitometric method was applied to the selected marketed formulations. Nexpro L was found to contain 98.65 ± 1.08 and 101.23 ± 1.59 % w/w of LSP and ESP, respectively and Sompraz L was found to contain 99.31 ± 0.99 and 100.47 ± 1.91 % w/w of LSP and ESP, respectively (Table No. 16).

**Table No. 16: Analysis of marketed formulations (n = 6)**

| Formulation | Label Claim mg per capsule | | Content Found mg per capsule | Recovery (% w/w) |
|---|---|---|---|---|
| Naxpro L | LSP | 75 | 73.987 | 98.65 |
| | ESP | 40 | 40.492 | 101.23 |
| Sompraz L | LSP | 75 | 74.482 | 99.31 |
| | ESP | 40 | 40.188 | 100.47 |

## 2 Development and validation of normal phase HPTLC method for simultaneous quantification of Levosulpiride and Rabeprazole in tablet dosage form.

### 2.1 Optimization of HPTLC method

To develop a method for separation of LSP and RBP, previously developed method for LSP and ESP was slightly modified. The drugs ESP and RBP are from same chemical category, so with slight modification in the previously developed method separation of LSP and RBP was achieved and desired R*f* value range (0.2 - 0.8) and minimum resolution of 1.5 obtained. Developed method was finally optimized as mobile phase consisting of ethyl acetate: methanol: ammonia (8.5: 1.5: 0.2, v/v/v). The retardation factors (R*f*) for LSP and RBP were found to be 0.25 ± 0.02 and 0.54 ± 0.02, respectively (Figure 17).

### 2.2 Selection of wavelength

After chromatographic development bands were scanned in the range of 200 to 400 nm and spectra were overlain. Considering the ratio of LSP and RBP in the marketed formulations which is almost 4:1 per tablet, the wavelength showing maximum absorbance for RBP was selected for densitometric analysis which was 287 nm. At this wavelength RBP showed maximum absorbance and LSP is showing considerable absorbance which is almost equivalent to their isoabsorptive wavelength. (Figure 18).

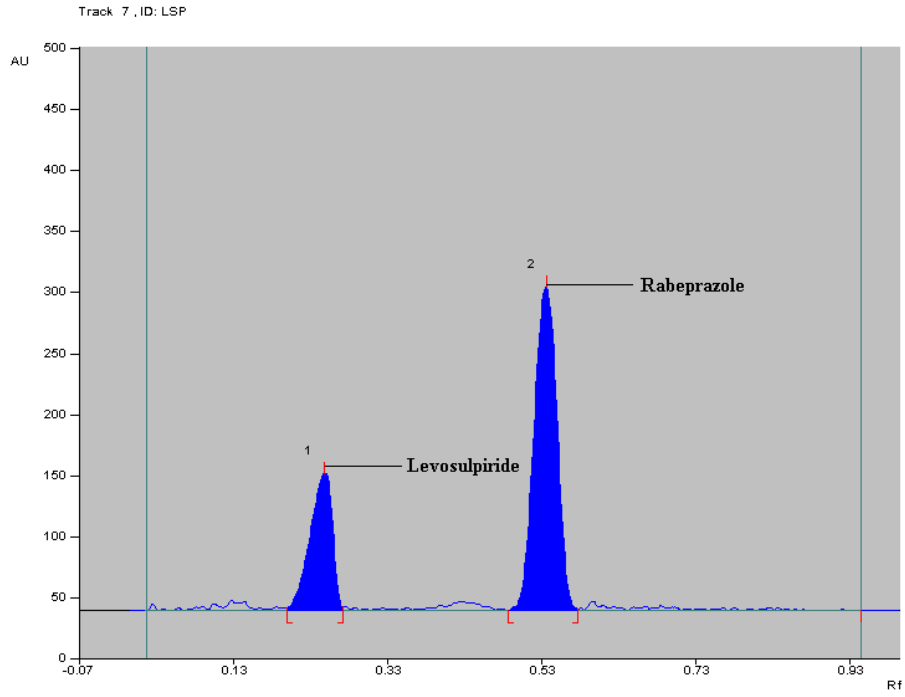


**Figure 17: Densitogram obtained from mixed standard solution of LSP and RBP**

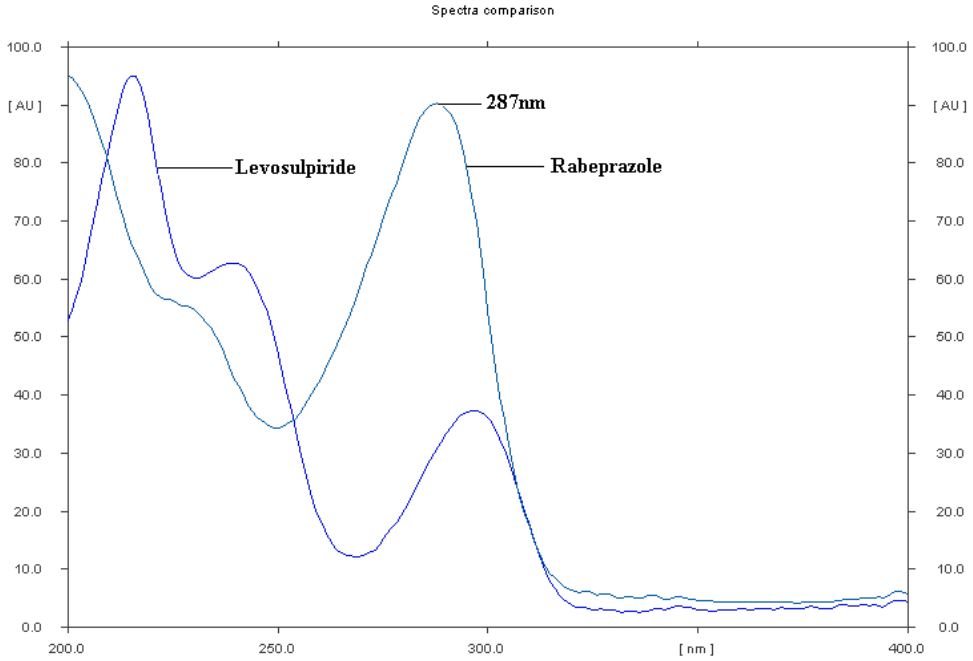


**Figure 18: Overlain UV spectrum of LSP and RBP**

### 2.3 Chromatographic conditions

The optimized high performance thin layer chromatographic conditions are as follows.

| | |
|---|---|
| **Stationary phase** | Aluminum plates precoated with silica gel 60 $F_{254}$ |
| **Mobile phase** | Ethyl acetate: methanol: ammonia (8.5: 1.5: 0.2, v/v/v) |
| **Plate size** | 20 X 10 cm |
| **Band size** | 6 mm (Distance between two bands: 10 mm) |
| **Development chamber** | Twin-trough glass chamber, 20 X 10 cm with stainless steel lid |
| **Saturation time** | 10 Min |
| **Migration distance** | 80 mm |
| **Room temperature** | 25 ± 2 °C |
| **Scanning mode** | Absorbance/reflectance |
| **Slit dimensions** | 5 × 0.45 mm |
| **Detection wavelength** | 287 nm |
| **Rf values** | LSP - 0.25 ± 0.02 |
| | RBP - 0.54 ± 0.02 |

## 2.4 HPTLC method validation

### 2.4.1 Linearity and range

To construct a calibration curve and to establish a relationship between detector response and know concentration of LSP and RBP; standard solutions of both having concentrations of 100 µg mL $^{-1}$ were spotted on HPTLC plates. The volume spotted was 1 to 10 µL spot$^{-1}$ of each to get the band of 100 to 1000 ng band$^{-1}$. After development; the area of the band was plotted against the concentration in terms of ng band$^{-1}$ to construct the calibration curve. The data obtained was subjected to statistical least square linear regression analysis. Slope, intercept and correlation coefficient was calculated from calibration curve. Calibration curves of standard drug concentrations and peak areas were found to be linear. Result showed that standard drugs concentration and peak areas were found to be linear in the range of 100 - 1000 ng band$^{-1}$ for LSP and 100 - 800 ng band$^{-1}$ for RBP (Table No. 17). The linear regression data is mentioned in the Table no. 18. Calibration curves are depicted in figures 19 and 21 for LSP and RSP respectively. The linearity of the calibration was tested by residual analysis (a non-numerical test). And residual plot for both standard LSP and RBP showed no tendency in the plot supports linearity data (Figure 20 and 22).

**Table No. 17: Linearity data of Levosulpiride and Rabeprazole**

| **Concentration**[a] | **Area** | |
|---|---|---|
| | **Levosulpiride** | **Rabeprazole** |
| **100** | 489.9 | 1217.7 |
| **200** | 751.3 | 2169.6 |
| **400** | 1442.9 | 3780 |
| **600** | 2052 | 5657.4 |
| **800** | 2660.1 | 7247.7 |
| **1000** | 3354.1 | - |

a = ng band$^{-1}$

**Table No. 18: Linear regression data for the calibration curves (n = 6)**

| **Parameters** | **Levosulpiride** | **Rabeprazole** |
|---|---|---|
| **Linearity range**[a] | 100 - 1000 | 100 – 800 |
| **$r^2$** | 0.9994 | 0.9993 |
| **Slope** | 3.181 | 8.627 |
| **Intercept** | 147.9 | 390.9 |
| **LOD**[a] | 32.29 | 29.71 |
| **LOQ**[a] | 97.86 | 90.03 |
| **Sy.x**[b] | 31.13 | 77.67 |

a = ng band$^{-1}$,

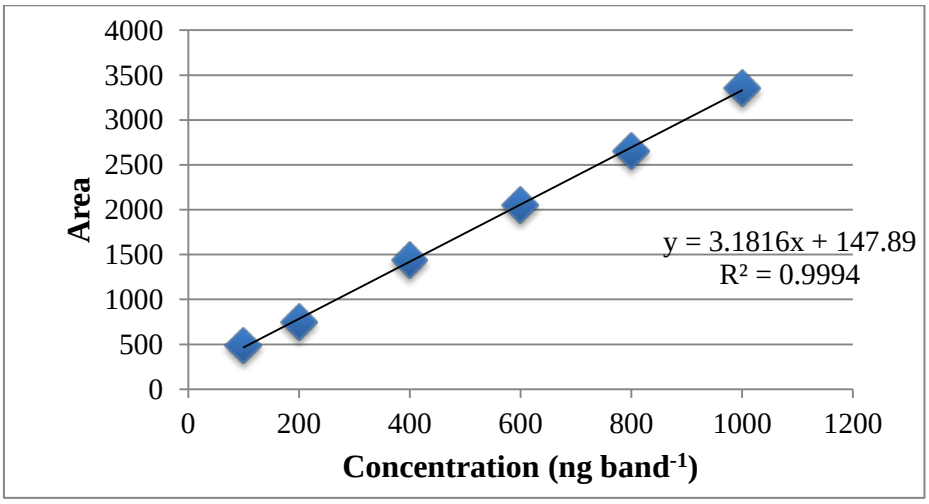


**Figure 19: Calibration curve of LSP**

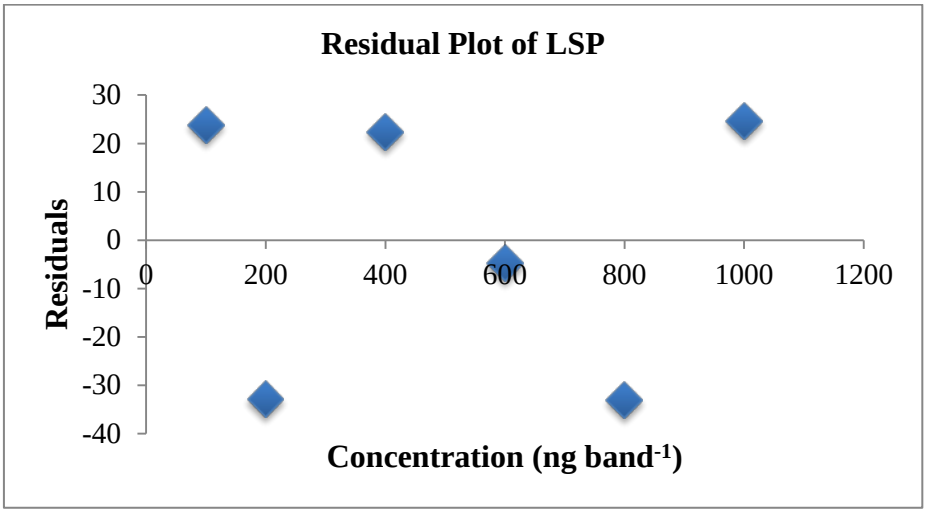


**Figure 20: Residual plot of LSP**

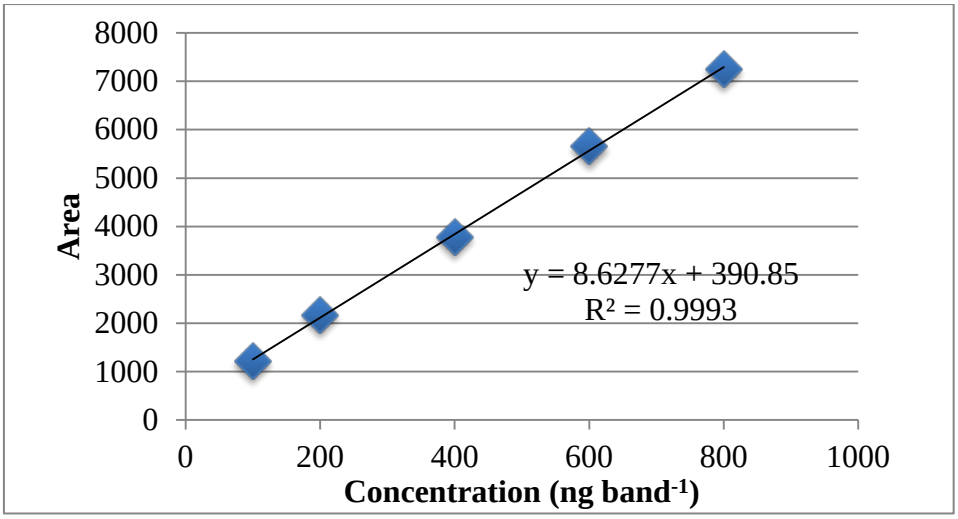


**Figure 21: Calibration curve of RBP**

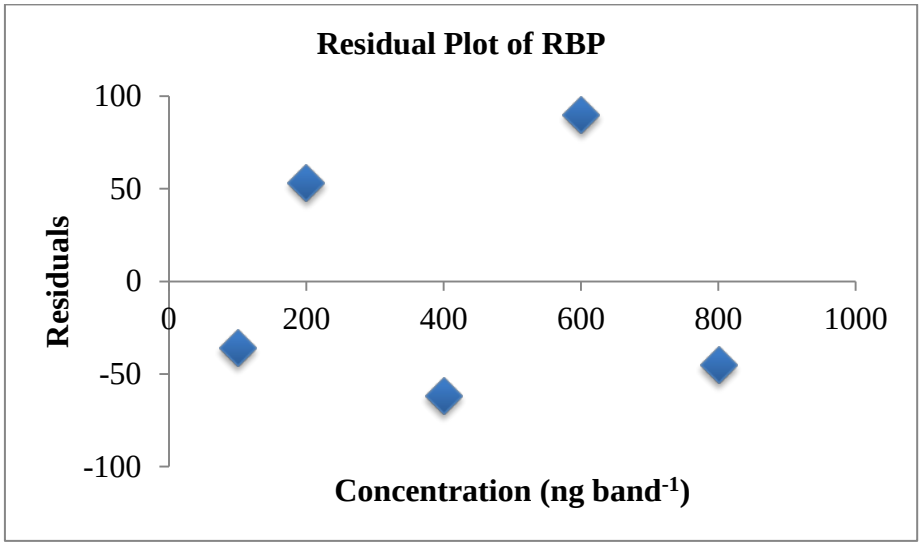


**Figure 22: Residual plot of RBP**

### 2.4.2 Sensitivity

Sensitivity of the method was established in terms of limit of detection and limit of quantification. It was calculated by slope of the calibration curve and standard deviation of response. The LOD and LOQ for LSP were found to be 32.29 and 97.86ng band$^{-1}$, respectively. The LOD and LOQ for RBP were found to be 29.71 and 90.03ng band$^{-1}$, respectively. (Table No. 18)

### 2.4.3 Specificity

The peak purity for LSP and RBP was assessed by comparing UV spectrum acquired at the start (S), apex (M), and end (E) of the peak obtained from the scanning of band, that is, r(S, M) = 0.998, 0.998 and r(M, E) = 0.998, 0.999, respectively. Peak purity data showed that peaks obtained for LSP and RBP were pure.

### 2.4.4 Precision

Three different concentrations from linearity range were selected and they were analyzed in triplicate on same day and three consecutive days to study the intra-day and inter-day precision. Each time they were compared with freshly prepared standard solutions for comparison of the area. Precision was established in terms of % RSD and was found to be in the range of 0.82 to 1.05 % and 1.13 to 1.43 % for LSP for intra-day and inter-day, respectively. RBP showed precision in the range of 0.52 to 0.79 % for intra-day and 1.05 to 1.41 % for inter-day. Both intra and inter-day precision studies showed % RSD < 2,as recommended by ICH guidelines, indicating good precision (Table No. 19).

**Table No. 19: Intra and inter day precision (n = 3)**

| **Drug** | **Concentration taken[a]** | **Concentration obtained [a]** | | **Precision obtained [b]** | |
|---|---|---|---|---|---|
| | | **Intra day** | **Inter day** | **Intra day** | **Inter day** |
| **Levosulpiride** | 400 | 396.89 | 395.12 | 0.89 | 1.33 |
| | 600 | 598.69 | 596.38 | 1.05 | 1.43 |
| | 800 | 782.25 | 779.41 | 0.82 | 1.13 |
| **Rabeprazole** | 400 | 400.78 | 399.93 | 0.54 | 1.28 |
| | 600 | 584.20 | 588.51 | 0.52 | 1.05 |
| | 800 | 758.15 | 748.58 | 0.79 | 1.41 |

a = ng band$^{-1}$, b = Precision as % RSD.

### 2.4.5 Accuracy

Accuracy was studied by standard addition method. The samples solutions were spiked with 80, 100 and 120% of standard solutions. Recovery for LSP and RBP was found to be 99.23 - 100.48 % w/w and 100.67 - 101.32 % w/w. It signifies reliability of the proposed densitometric method for concurrent estimation of LSP and RBP in the bulk as well as marketed formulation used in the study (Table No. 20).

**Table No. 20: Results of recovery studies (n=6)**

| **Parameter** | **LSP** | | | **RBP** | | |
|---|---|---|---|---|---|---|
| **Amount Taken[a]** | 400 | 400 | 400 | 200 | 200 | 200 |
| **Amount Added[a] (%)** | 320 (80) | 400 (100) | 480 (120) | 160 (80) | 200 (100) | 240 (120) |
| **Amount Found[a]** | 716.40 | 803.85 | 873.24 | 363.44 | 402.69 | 445.79 |
| **% Recovery** | 99.50 | 100.48 | 99.23 | 100.96 | 100.67 | 101.32 |
| **SD** | 19.12 | 16.88 | 32.73 | 43.73 | 29.68 | 28.28 |
| **%RSD** | 0.79 | 0.62 | 1.12 | 1.24 | 0.77 | 0.67 |

a = ng band$^{-1.}$

### 2.4.6 Robustness studies

Robustness study was carried out to check the effect of small but deliberate changes in experimental conditions on the results. Robustness of the proposed densitometric method showed that peak areas of interest remained unaffected by small but deliberate changes of the operational parameters (% RSD < 2) indicating robustness of the method (Table No. 21).

**Table No. 21: Results of robustness studies (n = 6, 600 ng band$^{-1}$)**

| Parameter Varied | Peak Area ± SD | | % RSD | |
|---|---|---|---|---|
| | LSP | RBP | LSP | RBP |
| **Mobile phase (ethyl acetate) composition (± 0.1 mL)** | 2068.70 ± 33.68 | 5617.75 ± 45.67 | 1.62 | 0.81 |
| **Amount of mobile phase (± 5 %)** | 2082.88 ± 21.89 | 5577.8 ± 91.09 | 1.05 | 1.63 |
| **Time from band application to chromatography(+ 10 min)** | 2063.98 ± 21.69 | 5627.66 ± 85.11 | 1.05 | 1.51 |
| **Time from chromatography to scanning (+ 15 min)** | 2085.91 ± 35.37 | 5714.28 ± 55.69 | 1.69 | 0.97 |

### 2.4.7 Solution stability

Stability of standard solutions of LSP and RBP were assessed at room temperature for 48 h. The % RSD < 2 indicates that the solutions were stable for 48 h at room temperature. Each time the area obtained was compared with the area obtained for freshly prepared standard solutions.

### 2.4.8 Analysis of marketed formulations.

Developed densitometric method was applied to the selected marketed formulations. Rabekind Plus was found to contain 99.02 ± 1.32 and 101.46 ± 0.32 % w/w of LSP and RBP, respectively. Rekool L was found to contain 99.41± 0.19 and 100.50 ± 0.38 % w/w of LSP and RBP, respectively. (Table No. 22).

**Table No. 22: Analysis of marketed formulations (n = 6)**

| Brand | Label Claim mg per tablet | | Content Found mg per tablet | Recovery (% w/w) |
|---|---|---|---|---|
| **Rabekind Plus** | LSP | 75 | 74.271 | 99.028 |
| | RBP | 20 | 20.292 | 101.46 |
| **Rekool L** | LSP | 75 | 74.559 | 99.41 |
| | RBP | 20 | 20.448 | 100.50 |

## 3 Development and validation of normal phase HPTLC method for simultaneous quantification of Mometasone furoate and Terbinafine hydrochloride in cream dosage form.

### 3.1 Optimization of chromatographic conditions

The mobile phase was optimized to get desired $R_f$ value range [0.2 - 0.8] with a resolution of > 1.5. It was achieved by trying different mobile phases containing solvents of different polarities in different ratios like toluene, n-hexane, ethanol, methanol, water and ethyl acetate. The solvent system containing n-hexane: ethyl acetate: acetic acid (7.5:3:0.5, v/v/v) was selected as it gave the desired $R_f$ values and resolution. The developing chamber was saturated with mobile phase for 20 min and development distance was 80 mm. The retardation factor for MTS and TBF was found to be 0.27±0.02 and 0.74±0.02, respectively (Figure 23).

### 3.2 Selection of the Wavelength

After chromatographic development bands were scanned in the UV range of 200 to 400 nm. Wavelength selected for the analysis was 258 nm which gave maximum absorbance for the MTS and considerable absorbance for TBF. The ratio of MTS and TBF in the formulation is 1:10; so considering the difference in the ratio the wavelength with maximum absorbance for MTS was selected (Figure 24). The results showed no variation due to selections of wavelength showing maximum absorbance for MTS.

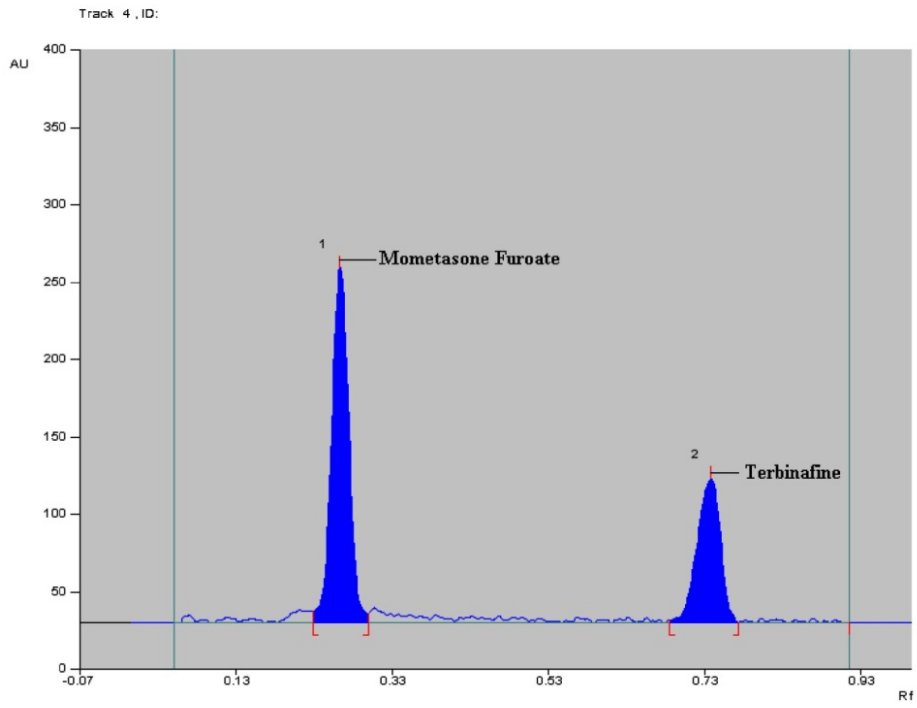


**Figure 23: Densitogram obtained from mixed standard solution of MTS and TBF**

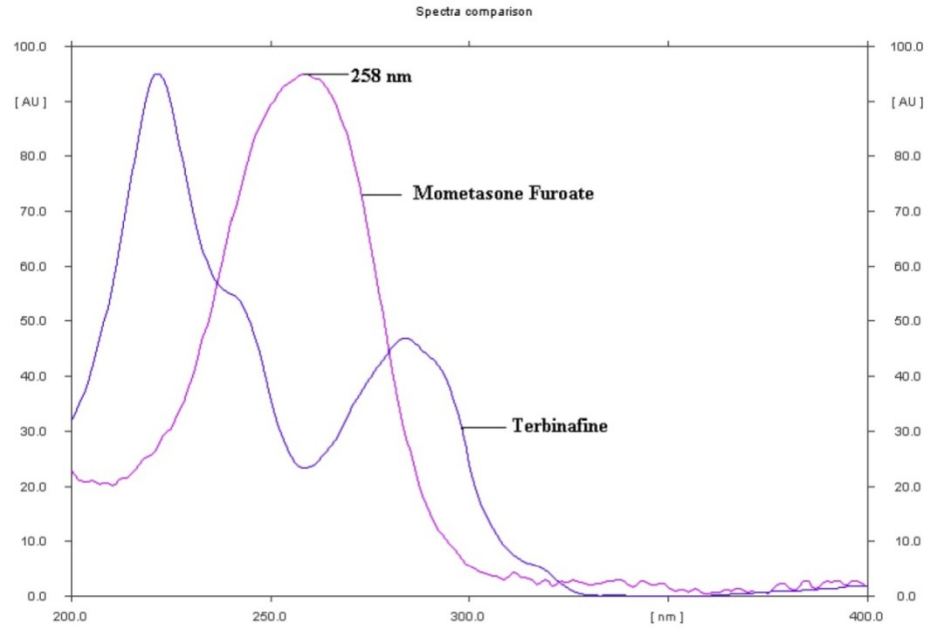


**Figure 24: Overlain UV spectrum of MTS and TBF**

### 3.3 Chromatographic conditions

The optimized high performance thin layer chromatographic conditions are as follows.

| | |
|---|---|
| **Stationary phase** | Aluminum plates precoated with silica gel 60 $F_{254}$ |
| **Mobile phase** | n-Hexane: ethyl acetate: acetic acid (7.5:3:0.5, v/v/v) |
| **Plate size** | 20 X 10 cm |
| **Band size** | 6 mm (Distance between two bands: 10 mm) |
| **Development chamber** | Twin-trough glass chamber, 20 X 10 cm with stainless steel lid |
| **Saturation time** | 20 Min |
| **Migration distance** | 80 mm |
| **Room temperature** | 25 ± 2 °C |
| **Scanning mode** | Absorbance/reflectance |
| **Slit dimensions** | 5 × 0.45 mm |
| **Detection wavelength** | 258 nm |
| **Rf values** | MTS - 0.27 ± 0.02 |
| | TBF - 0.74 ± 0.02 |

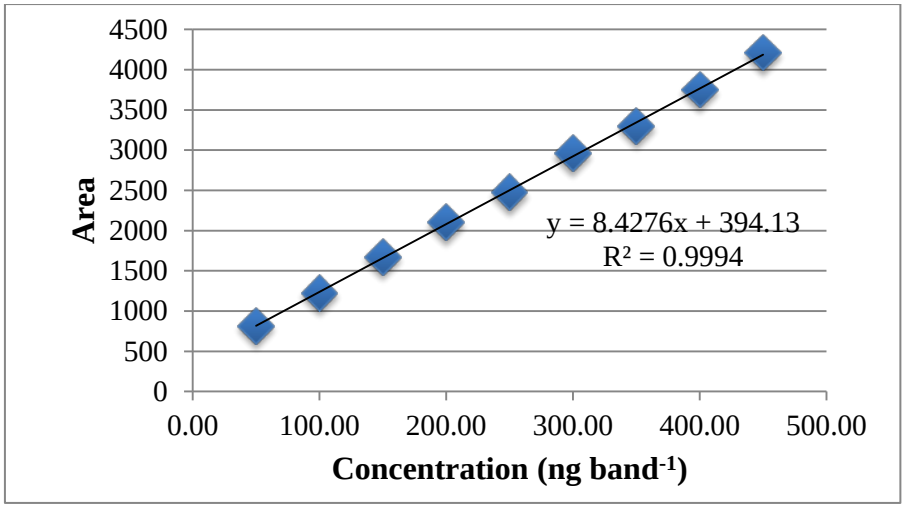


**Figure 25: Calibration curve of MTS**

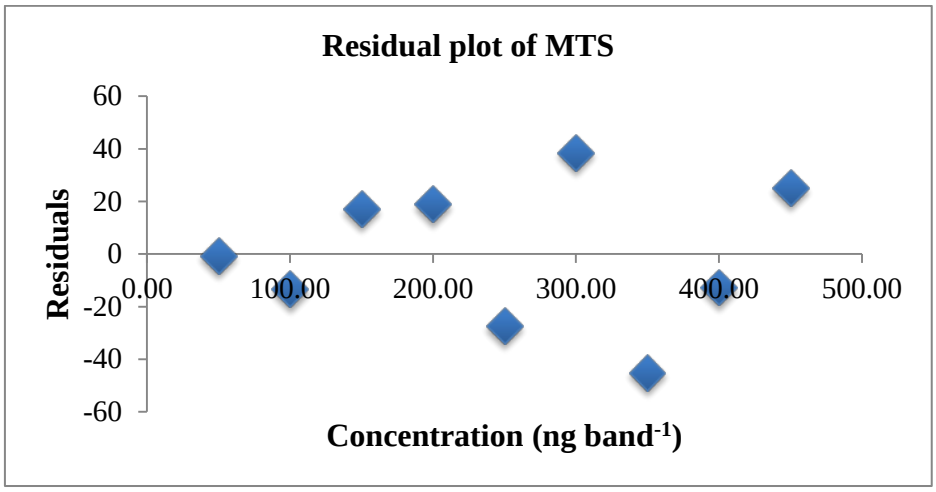


**Figure 26: Residual plot of MTS**

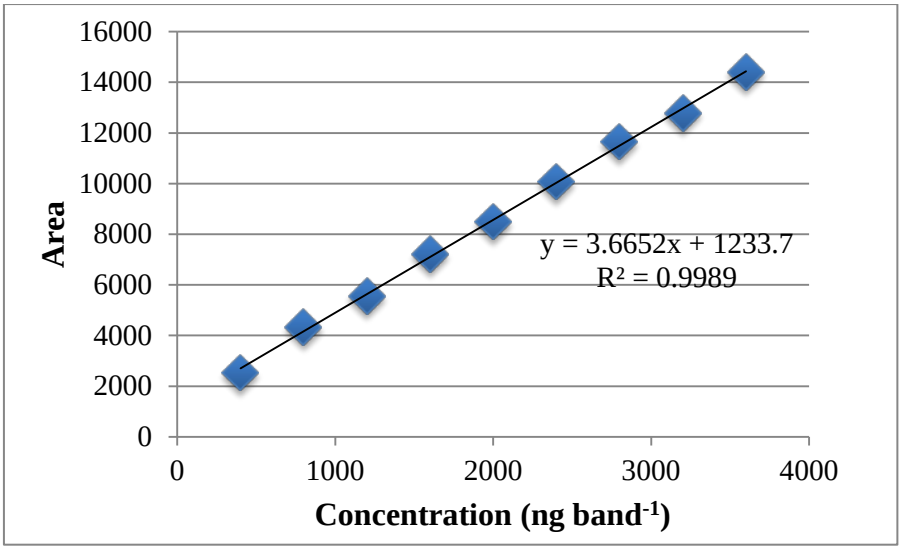


**Figure 27: Calibration curve of TBF**

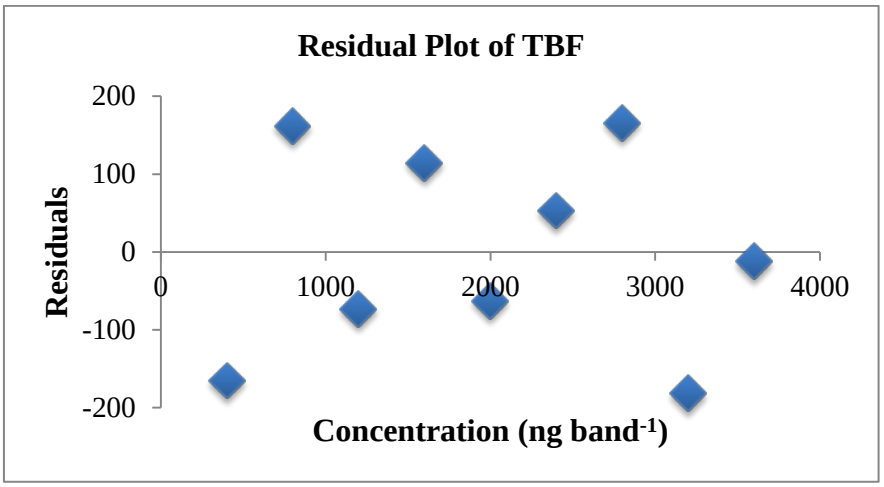


**Figure 28: Residual plot of TBF**

### 3.4 Method Validation

The validation of the developed method was carried out as per ICH guidelines [Q2 (R1)]. The parameters studied for the validation were linearity, LOD, LOQ, precision, accuracy and robustness. Standard drugs solution was used for the validation of the method and each time freshly prepared solutions were used.[25]

#### 3.4.1 Linearity and range

After chromatographic development plates were scanned and data obtained was statistically analyzed for least square linear regression analysis. A good linear relationship was observed for both the drugs (Table No. 23). Slope, intercept and correlation coefficient was calculated from calibration curve (Table No. 24). The linearity range for MTS was found to 50-450 ng band$^{-1}$ and for TBF it was found to be 400-3600 ng band$^{-1}$ (Table No. 23). Calibration curves are showed in figures 25 and 27 for MTS and TBF respectively. Linearity was also confirmed by residual analysis (Figure 26 and 28). The plot of concentration versus residuals showed no tendency supporting the linearity.

**Table No. 23: Linearity data of Mometasone furoate and Terbinafine hydrochloride**

| Name of drug | Concentration [a] | Area |
|---|---|---|
| **Mometasone furoate** | 50 | 814.83 |
| | 100 | 1223.5 |
| | 150 | 1675.28 |
| | 200 | 2098.55 |
| | 250 | 2473.67 |
| | 300 | 2960.85 |
| | 350 | 3298.68 |
| | 400 | 3752.4 |
| | 450 | 4211.6 |
| **Terbinafine hydrochloride** | 400 | 2534.33 |
| | 800 | 4327.43 |
| | 1200 | 5558.68 |
| | 1600 | 7212.58 |
| | 2000 | 8501.42 |
| | 2400 | 10083.37 |
| | 2800 | 11662.03 |
| | 3200 | 12780.45 |
| | 3600 | 14416.3 |

a = ng band$^{-1}$

**Table No. 24: Linear regression data for calibration curves (n=6)**

| **Parameters** | **Mometasone furoate** | **Terbinafine hydrochloride** |
|---|---|---|
| **Linearity range (ng band$^{-1}$)** | 50 - 450 | 400 – 3600 |
| **$r^2$** | 0.9994 | 0.9989 |
| **Slope** | 8.427 | 3.665 |
| **Intercept** | 394.13 | 1233.7 |
| **LOD (ng band$^{-1}$)** | 11.34 | 125.78 |
| **LOQ (ng band$^{-1}$)** | 34.38 | 381.15 |
| **Sy.x** | 28.98 | 141.1 |

### 3.4.2 Sensitivity

Sensitivity of the method was evaluated based on limit of detection (LOD) and limit of quantification (LOQ). The detection limit for MTS and TBF was found to be 11.34 and 125.78 ng band$^{-1}$ respectively. Quantitation limit for MTS and TBF was found to be 34.38 and 381.15 ng band$^{-1}$, respectively (Table No. 24).

### 3.4.3 Specificity

Specificity of the method was checked by assessing UV spectrum of the peak at start, middle and end positions of the band. It was found to be r(S, M)= 0.999, 0.999 and r(M, E)= 0.999, 0.998, respectively. It confirmed the purity of peaks.

### 3.4.4 Precision

Repeatability of the method was studies on same day as well as on different days. The repeatability and intermediate (intra and inter day) precision results showed % RSD values for both the drugs were less than 2, as recommended by ICH guidelines indicating good precision (Table No. 25).

**Table No. 25: Intra and inter day precision study (n=3)**

| Standard drugs | Nominal concentration (ng band$^{-1}$) | Concentration obtained (ng band$^{-1}$) | | Precision obtained (% RSD) | |
|---|---|---|---|---|---|
| | | Intra day | Inter day | Intra day | Inter day |
| Mometasone furoate | 100 | 99.34 | 99.93 | 1.03 | 1.72 |
| | 200 | 201.05 | 200.36 | 1.34 | 0.99 |
| | 300 | 300.16 | 303.71 | 1.53 | 1.47 |
| Terbinafine hydrochloride | 1200 | 1184.90 | 1167.43 | 0.67 | 0.58 |
| | 2000 | 1977.21 | 1986.30 | 0.29 | 0.88 |
| | 2800 | 2834.58 | 2823.66 | 0.47 | 0.89 |

### 3.4.5 Accuracy

Accuracy of the developed method was studied by standard addition method. The sample solution was spiked with 80, 100 and 120 % of the standard solution and total quantity recovered was calculated in terms of percent recovery. Recovery of MTS and TBF was found to be 99.40 to 101.61 % and 99.51 to 100.24 % respectively. These were within the limits which indicate reliability of the developed method for the simultaneous estimation of the MTS and TBF in pharmaceutical dosage form (Table No. 26).

**Table No. 26: Results of recovery studies (n=6)**

| Parameter | MTS | | | TBF | | |
|---|---|---|---|---|---|---|
| Amount Taken in ng band$^{-1}$ | 150 | 150 | 150 | 1500 | 1500 | 1500 |
| Amount Added in ng band$^{-1}$ (%) | 120 (80) | 150 (100) | 180 (120) | 1200 (80) | 1500 (100) | 1800 (120) |
| Amound Found ng band$^{-1}$ | 268.38 | 304.83 | 333.24 | 2701.70 | 3007.33 | 3283.93 |
| % Recovery | 99.40 | 101.61 | 100.98 | 100.06 | 100.24 | 99.51 |
| SD | 37.96 | 50.47 | 50.33 | 188.32 | 230.67 | 144.33 |
| % RSD | 1.43 | 1.70 | 1.57 | 1.69 | 1.88 | 1.09 |

### 3.4.6 Robustness studies

Small but deliberate changes in operational parameters showed no effect on peak areas which confirmed the robustness of the developed method [% RSD ≤ 2] (Table No. 27).

**Table No. 27: Results of robustness studies (n=6, MTS – 200 ng band$^{-1}$, TBF – 2000 ng band$^{-1}$)**

| Parameter | Peak Area ± SD | | % RSD | |
|---|---|---|---|---|
| | MTS | TBF | MTS | TBF |
| **Mobile phase (ethyl acetate) composition (± 0.1 mL)** | 2054.79 ± 21.61 | 8601.54 ± 103.76 | 1.05 | 1.21 |
| **Amount of mobile phase (± 5 %)** | 2104.86 ± 14.50 | 8565.30 ± 145.95 | 0.69 | 1.70 |
| **Time from band application to chromatography (+ 10 min)** | 2084.86 ± 31.99 | 8593.67 ± 107.98 | 1.53 | 1.26 |
| **Time from chromatography to scanning(+ 15 min)** | 2071.84 ± 37.05 | 8638.78 ± 126.35 | 1.79 | 1.46 |

### 3.4.7 Solution stability

Stability of the standard stock solution MTS and TBF were assessed at room temperature for 48 hrs. Area of the solution was compared with freshly prepared standard solution each time. The % RSD ≤ 2 indicates that solutions are stable at room temperature for 48 hrs.

### 3.4.8 Analysis of marketed formulation.

The developed and validated method was used to estimate the content of both the drugs in the marketed cream based formulation (MOMOZ T®). It was found to contain 0.098 ± 0.001 % (w/w) of MTS and 1.01 ± 0.005 % (w/w) of TBF. The recovery of the marketed formulation was found to be 98.79 and 101.05 % for MTS and TBF, respectively (Table No. 28).

**Table No. 28: Analysis of marketed formulation (n = 6)**

| Formulation | Label Claim | | Content Found | |
|---|---|---|---|---|
| | | | Mean % w/w | Recovery (%) |
| **MOMOZ T®** | MTS | 0.1 % w/w | 0.098 | 98.79 |
| | TBF | 1 % w/w | 1.01 | 101.05 |

## 4 Development and validation of reverse phase HPLC-DAD method for simultaneous quantification of Mometasone furoate and Terbinafine hydrochloride in cream dosage form.

### 4.1 Optimization of chromatographic conditions

Initially to get the desired separation and resolution mobile phase containing methanol water in 1:1 proportion were tried. It was giving comparatively very high RT for TBF. So proportion of methanol was decreased and optimized to 90:10. The flow rate was maintained at 1 mL $min^{-1}$. The chromatogram was recorded at 237 nm and quantification was carried out at 224 and 258 nm for TBF and MTS, respectively. The retention time was found to be 3.26 min for MTS and 6.67 min for TBF (Figure 29).

### 4.2 Chromatographic conditions

The optimized high performance thin layer chromatographic conditions are as follows.

| | |
|---|---|
| **HPLC column** | SyncronisaQ $C_{18}$ (5 µm, 250mm × 4.6 mm i. d.) |
| **Mobile phase** | Methanol: water (90: 10, v/v) |
| **UV detection** | 237 nm |
| **Injection volume** | 20 µL |
| **Run time** | 10 Min |
| **Flow rate (mL $min^{-1}$)** | 1 mL $min^{-1}$ |
| **Retention time** | MTS - 3.26 Min |
| | TBF - 6.67 Min |

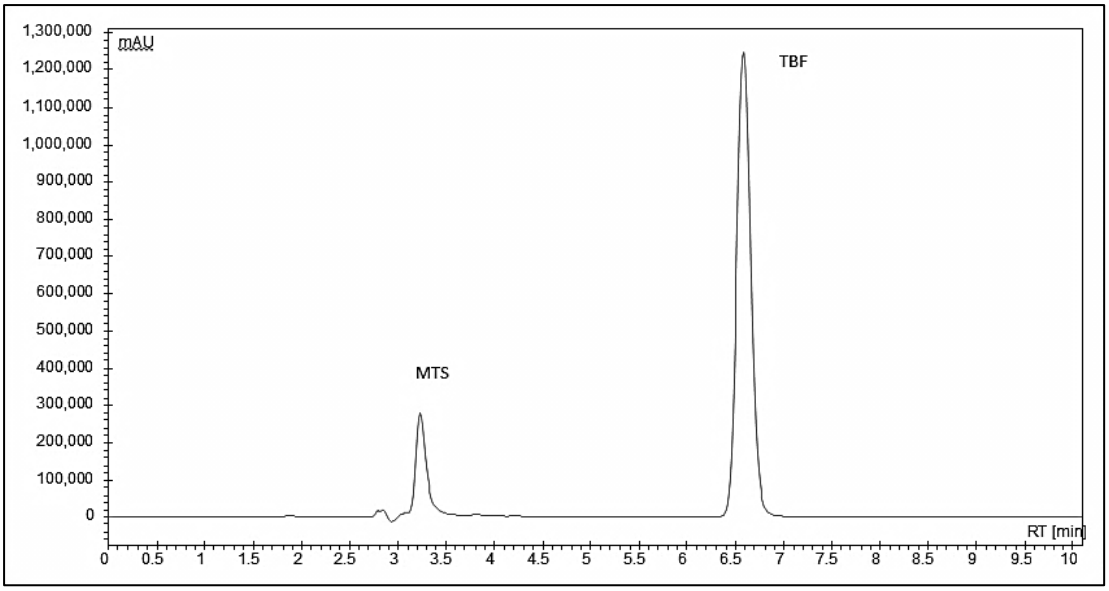


**Figure 29: Chromatogram obtained from sample solution of MTS and TBF recorded at 237 nm.**

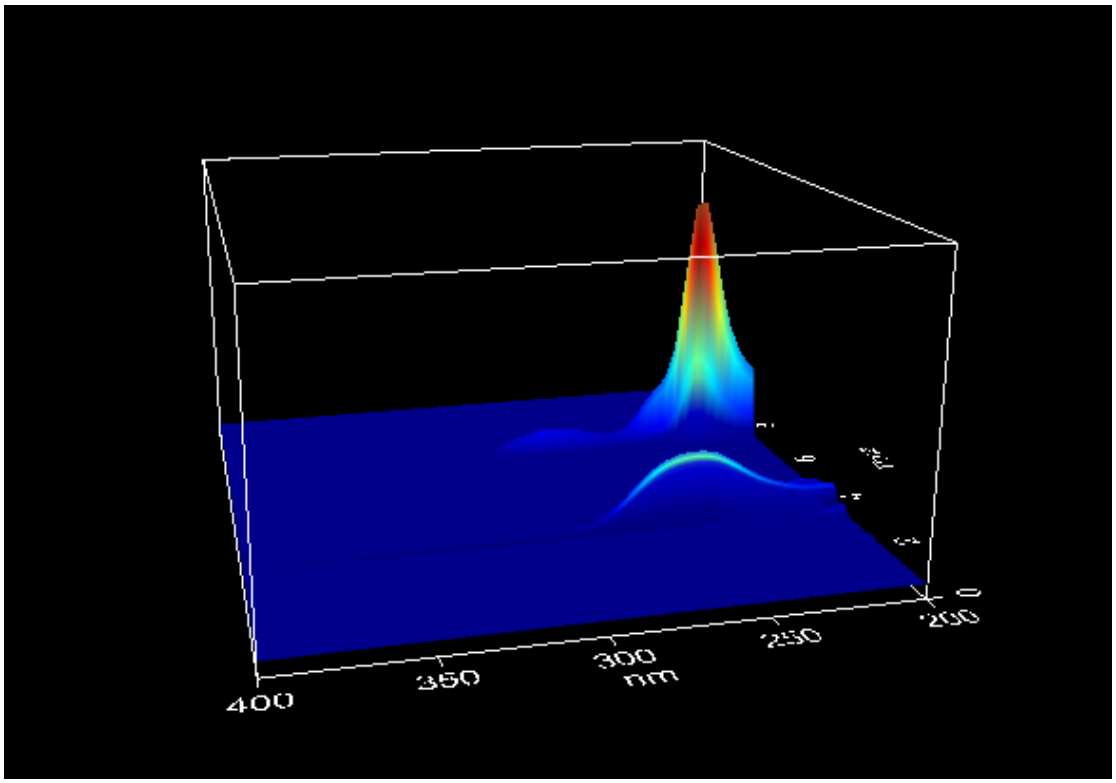


**Figure 30: 3D UV spectrum of MTS and TBF**

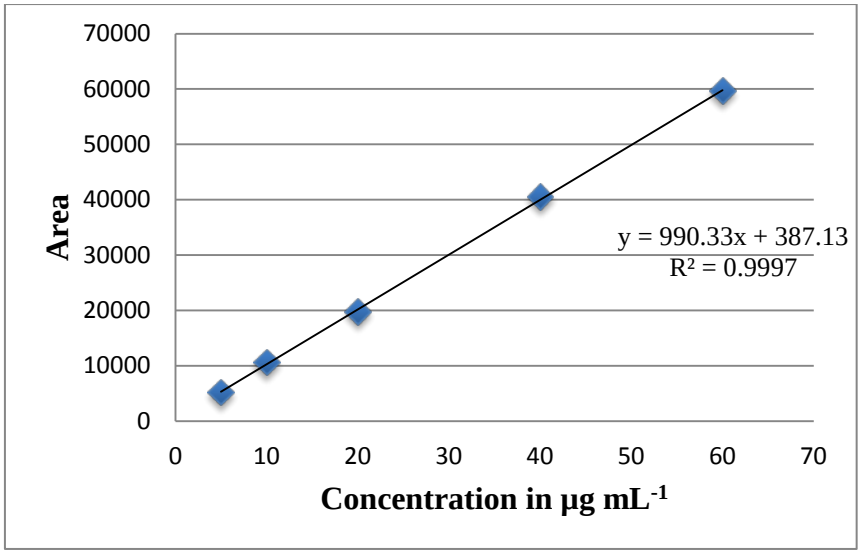


**Figure 31: Calibration curve of MTS**

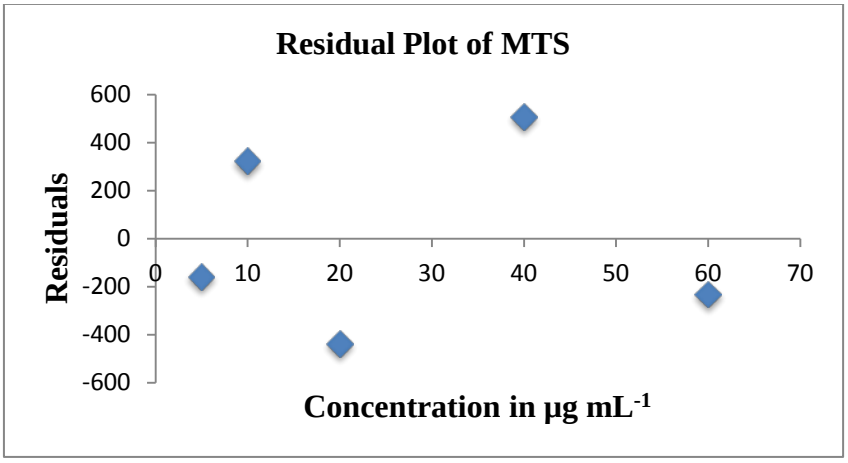


**Figure 32: Residual plot of MTS**

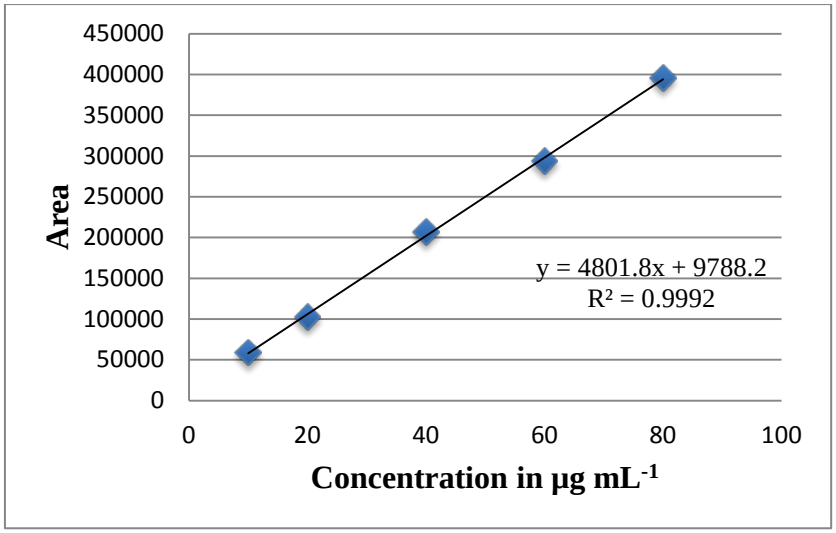


**Figure 33: Calibration curve of TBF**

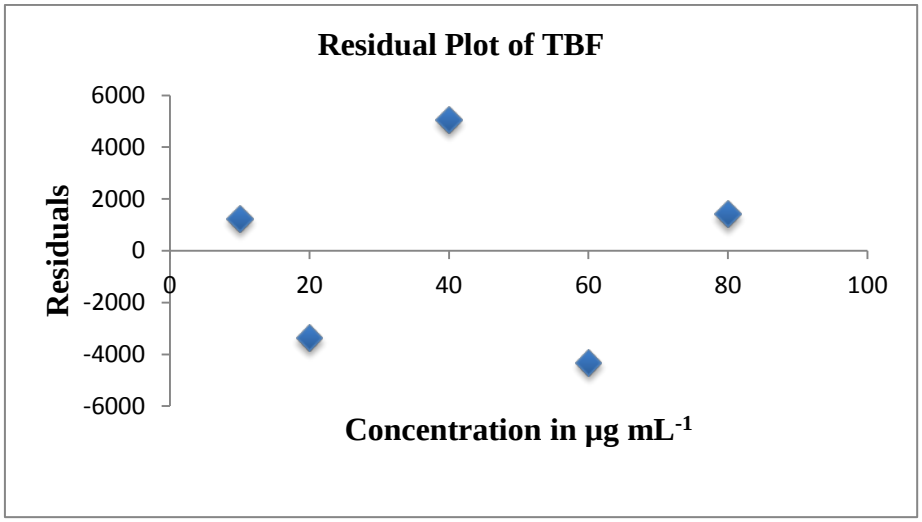


**Figure 34: Residual plot of TBF**

### 4.3 Method Validation

The validation of the developed method was carried out as per ICH guidelines [Q2 (R1)]. The parameters studied for the validation were linearity, LOD, LOQ, precision, accuracy and robustness. Standard drugs solution was used for the validation of the method and each time freshly prepared solutions were used.[25]

#### 4.3.1 Linearity and range

A good linear relationship was observed for both the drugs (Table No. 29). Slope, intercept and correlation coefficient was calculated from calibration curve (Table No. 30). The linearity range for MTS was found to be 5-60 µg $mL^{-1}$ and for TBF it was found to be 10-80 µg $mL^{-1}$ (Table No. 29). Calibration curves are showed in figures 31 and 33 for MTS and TBF respectively. Linearity was also confirmed by residual analysis (Figure 32 and 34). The plot of concentration versus residuals showed no tendency supporting the linearity.

**Table No. 29: Linearity data of Mometasone furoate and Terbinafine hydrochloride**

| Name of drug | Concentration [a] | Area (mAU.min) |
|---|---|---|
| **Mometasone furoate** | 5 | 5179.817 |
| | 10 | 10612.42 |
| | 20 | 19756.12 |
| | 40 | 40507.77 |
| | 60 | 59574.13 |
| **Terbinafine hydrochloride** | 10 | 59047.58 |
| | 20 | 102446 |
| | 40 | 206913.1 |
| | 60 | 293569.8 |
| | 80 | 395333.4 |

a = µg $mL^{-1}$

**Table No. 30: Linear regression data for calibration curves (n=6)**

| **Parameters** | **MTS** | **TBF** |
|---|---|---|
| **Linearity range** [a] | 5 - 60 | 10 – 80 |
| **$r^2$** | 0.9997 | 0.9992 |
| **Slope** | 990.33 | 4801.8 |
| **Intercept** | 387.13 | 9788.2 |
| **LOD** [a] | 1.53 | 3.05 |
| **LOQ** [a] | 4.63 | 9.24 |
| **Sy.x** | 459 | 4441 |

a = µg $mL^{-1}$

### 4.3.2 Sensitivity

Detection limit and quantitation limits were evaluated for the establishment of sensitivity of the developed analytical method. The detection limit for MTS and TBF was found to be 1.53 and 3.05 µg $mL^{-1}$ respectively. Quantitation limit for MTS and TBF was found to be 4.63 and 9.24 µg $mL^{-1}$, respectively (Table No. 30).

### 4.3.3 Specificity

Purity of the peak was evaluated for the establishment of specificity of the developed analytical method. Apex peak purity was found to be 997 and 999 for MTS and TBF respectively. The visual assessment of the peak also showed that peaks were pure (Figure 35 and 36).

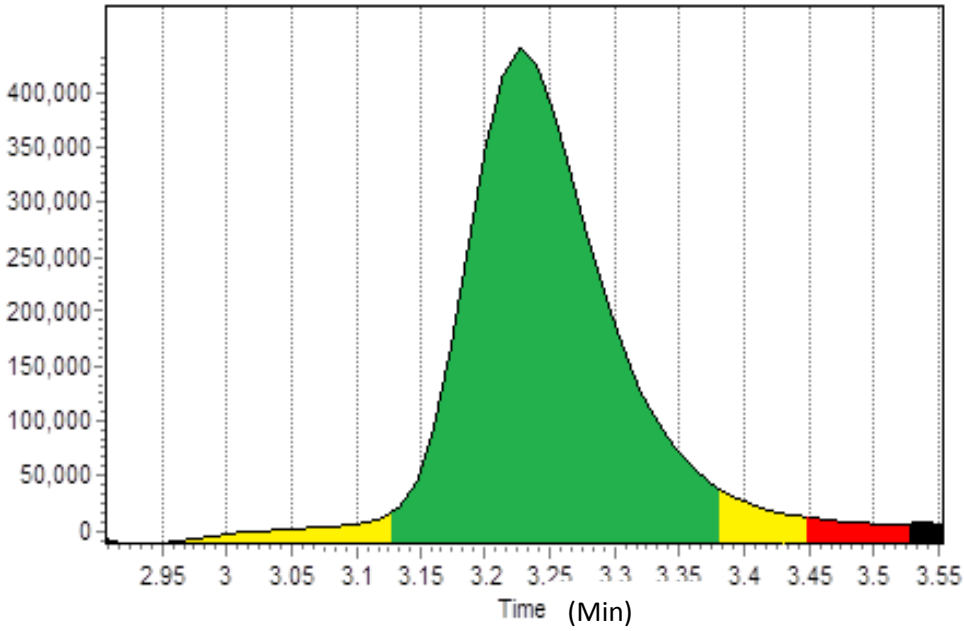


**Figure 35: Chromatogram showing peak purity of MTS**

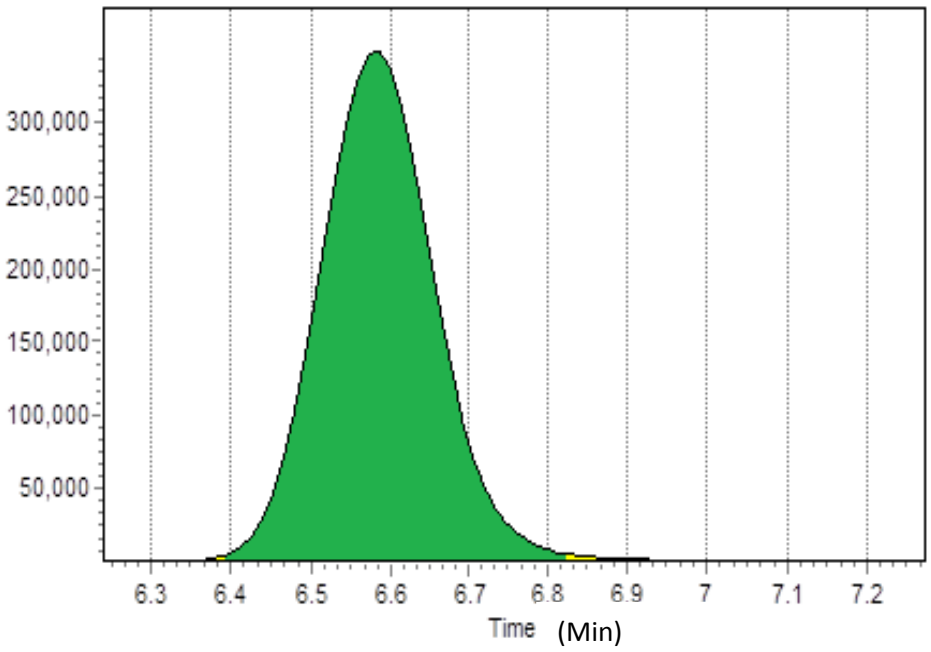


**Figure 36: Chromatogram showing peak purity of TBF**

### 4.3.4 Precision

Repeatability of the method was studies on same day as well as on different days. The repeatability and intermediate (intra and inter day) precision results showed % RSD values for both the drugs were less than 2, as recommended by ICH guidelines indicating good precision (Table No. 31).

**Table No. 31: Intra and inter day precision study (n=3)**

| Standard drugs | Nominal concentration [a] | Concentration obtained [a] | | Precision obtained [b] | |
|---|---|---|---|---|---|
| | | Intra day | Inter day | Intra day | Inter day |
| Mometasone Furoate | 10 | 10.27 | 10.12 | 1.02 | 1.62 |
| | 20 | 19.24 | 20.23 | 1.40 | 0.35 |
| | 40 | 40.50 | 40.55 | 0.26 | 0.57 |
| Terbinafine hydrochloride | 20 | 19.49 | 19.50 | 0.85 | 1.05 |
| | 40 | 40.59 | 40.64 | 1.00 | 0.88 |
| | 60 | 59.41 | 59.11 | 0.74 | 0.35 |

a = $\mu g\ mL^{-1}$, b = Precision obtained as % RSD

### 4.3.5 Accuracy

Standard addition method was used to evaluate the accuracy of developed analytical method. The sample solution was spiked with 80, 100 and 120 % of the standard solution and total quantity recovered was calculated in terms of percent recovery. Recovery of MTS and TBF was found to be 100.27 to 101.01 % and 100.57 to 100.96 % respectively. These were within the limits which indicate reliability of the developed method for the simultaneous estimation of the MTS and TBF in pharmaceutical dosage form (Table No. 32).

**Table No. 32: Results of recovery studies (n=6)**

| **Parameter** | **MTS** | | | **TBF** | | |
|---|---|---|---|---|---|---|
| **Amount Taken[a]** | 10 | 10 | 10 | 20 | 20 | 20 |
| **Amount Added[a] (%)** | 8 (80) | 10 (100) | 12 (120) | 16 (80) | 20 (100) | 24 (120) |
| **Amound Found[a]** | 18.05 | 20.20 | 22.13 | 36.20 | 40.38 | 44.28 |
| **% Recovery** | 100.27 | 101.01 | 100.59 | 100.57 | 100.96 | 100.63 |
| **SD** | 77.89 | 113.25 | 169.78 | 2104.37 | 1205.74 | 1692.93 |
| **%RSD** | 0.43 | 0.56 | 0.76 | 1.15 | 0.59 | 0.76 |

a = µg $mL^{-1}$

### 4.3.6 Robustness

Small but deliberate changes in operational parameters showed no effect on peak areas which confirmed the robustness of the developed method [% RSD ≤ 2] (Table No. 33).

**Table No. 33: Results of robustness studies (n=3, MTS – 20 µg $mL^{-1}$, TBF – 40 µg $mL^{-1}$)**

| **Parameter varied** | **MTS** | | **TBF** | |
|---|---|---|---|---|
| | **Mean Area ± SD** | **% RSD** | **Mean Area ± SD** | **% RSD** |
| **Mobile phase composition (Methanol) (±1%)** | 19762.80 ± 80.95 | 0.41 | 205736.99 ± 1918.47 | 0.93 |
| **Elution flow rate (±0.1 mL $min^{-1}$)** | 19583.73 ± 245.86 | 1.26 | 205425.23 ± 1846.08 | 0.90 |
| **Detection wavelength (± 2 nm)** | 19590.10 ± 211.70 | 1.08 | 205171.87 ± 2097.32 | 1.02 |

### 4.3.7 System suitability parameters

To ensure quality performance of the developed HPLC method system suitability parameters were studied. It was assessed in terms of theoretical plates (≥ 2000), peak symmetry (≤ 2), resolution between the peaks (≥ 2.0), and proper retention time. System suitability parameters for the developed method were studied and found to be within limits (Table No. 34).

**Table No. 34: System suitability parameters**

| Parameter | MTS | TBF |
|---|---|---|
| **Retention time (Min)** | 3.26 | 6.67 |
| **Resolution** | - | 3.89 |
| **Tailing factor** | 1.31 | 1.11 |
| **No. of theoretical plates** | 3934 | 9805 |

### 4.3.8 Solution stability

Stability of the standard stock solutions of MTS and TBF were assessed at room temperature for 48 hrs. Area of the solution was compared with freshly prepared standard solution each time. The % RSD $\leq$ 2 indicates that solutions are stable at room temperature for 48 hrs.

### 4.3.9 Analysis of marketed formulation

The developed and validated method was used to estimate the content of both the drugs in the marketed cream based formulation (MOMOZ T®). It was found to contain 0.099 % (w/w) of MTS and 1.003 % (w/w) of TBF. The recovery of the marketed formulation was found to be 99.74 and 100.3 % for MTS and TBF, respectively (Table No. 35).

**Table No. 35: Analysis of marketed formulation (n = 6)**

| Formulation | Label Claim | | Content Found | |
|---|---|---|---|---|
| | | | Mean % w/w | Recovery (%) |
| **MOMOZ T®** | MTS | 0.1 % w/w | 0.099 | 99.74 |
| | TBF | 1 % w/w | 1.003 | 100.30 |

## 5 Development of stability indicating high performance thin layer chromatographic method for quantitation of Levosulpiride in pharmaceutical dosage form and application of TLC-MS for identification of degradation product.

### 5.1 Optimization of stability indicating HPTLC method

With the primary aim of development of stability indicating assay method it is intended to quantify the drug in presence of its degradation products. Stability indicating assay methods are mainly used to identify and quantify the drug in presence of its degradation product. To achieve desired separation ($Rs \geq 1.5$) and R*f* value (0.2 - 0.8) different mobile phases of various combinations and ratios of toluene, dichloromethane, n-hexane, ethanol, methanol, ethyl acetate, water and acetone were tried. Along with this, previously developed method for quantification of LSP in presence of ESP and RBP was slightly modified and tried to separate the standard drugs from its degradation products. The modified method gave good separation from degradation products of the LSP. Finally the mobile phase containing ethyl acetate: methanol: ammonia (7:3:0.5 v/v/v) was selected as it gave well resolved peaks of standard LSP and its degradation product. The retardation factor for standard LSP was found to be $0.44 \pm 0.02$ (Figure 37).

### 5.2 Selection of wavelength

After chromatographic development plate was scanned in the range of 200 to 400 nm. The optimum wavelength selected for detection and quantitation was 216 nm.

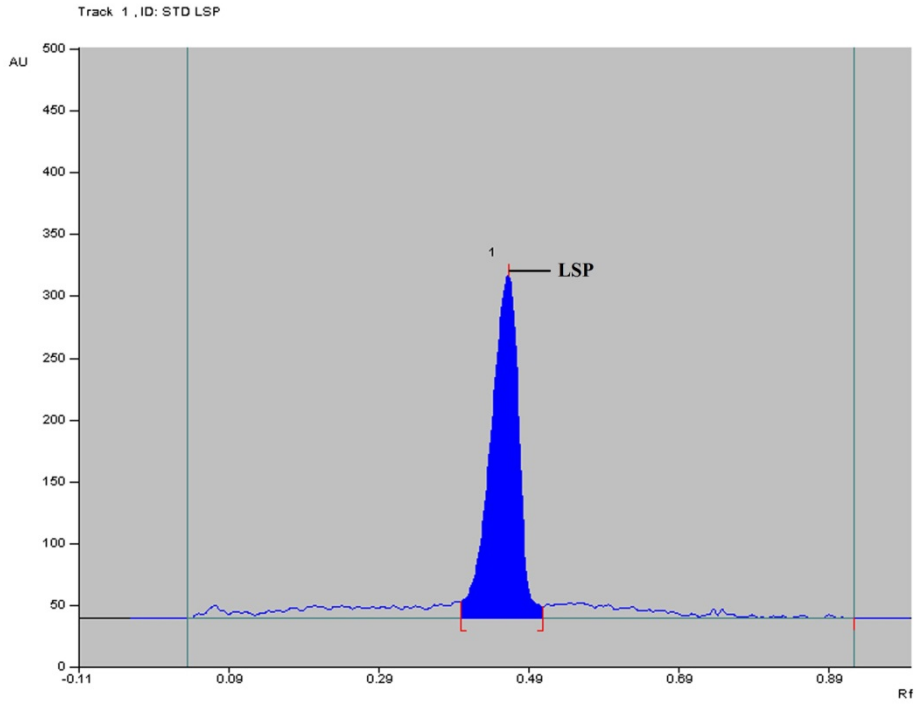


**Figure 37: Representative densitogram of LSP**

### 5.3 Chromatographic conditions

The optimized high performance thin layer chromatographic conditions are given follows.

| | |
|---|---|
| **Stationary phase** | Aluminum plates precoated with silica gel 60 $F_{254}$ |
| **Mobile phase** | Ethyl acetate: methanol: ammonia (7:3:0.5 v/v/v) |
| **Plate size** | 20 X 10 cm |
| **Band size** | 6 mm (Distance between two bands: 10 mm) |
| **Development chamber** | Twin-trough glass chamber, 20 X 10 cm with stainless steel lid |
| **Saturation time** | 10 Min |
| **Migration distance** | 80 mm |
| **Room temperature** | 25 ± 2 °C |
| **Scanning mode** | Absorbance/reflectance |
| **Slit dimensions** | 5 × 0.45 mm |
| **Detection wavelength** | 216 nm |
| **Rf Values** | LSP- 0.44 ± 0.02 |

## 5.4 Forced Degradation studies

As per recommendations of ICH guidelines bulk sample of LSP was subjected to forced degradation study. The sample was subjected to various stress conditions like acidic and basic hydrolysis, oxidation, photo degradation and thermal conditions. After getting sufficient degradation, samples were analyzed with the developed method and resolution of degradation products and drugs was studied.

### 5.4.1 Acid induced degradation product

According to the schemes mentioned by Bakshi and Singh[33, 37]; sample was exposed to the acidic conditions. Initially LSP was subjected to acid degradation in 0.1 N HCl at 80 °C. Sample was withdrawn and neutralized with 0.1 N NaOH. The sample was spotted on HPTLC plates and analyzed using developed method. It did not show any degradation. It was then refluxed up to 8 hrs but still no degradation product was obtained. Hence strength of acid was increased to 1 N and degradation pattern was studied. It showed 12.37 % degradation in 2 hr. The peak for degradation product was well resolved from the peak of standard LSP. The retardation factor of the degraded product was found to be 0.38 ± 0.02 and that of standard LSP was 0.44 ± 0.02 (Figure 38).

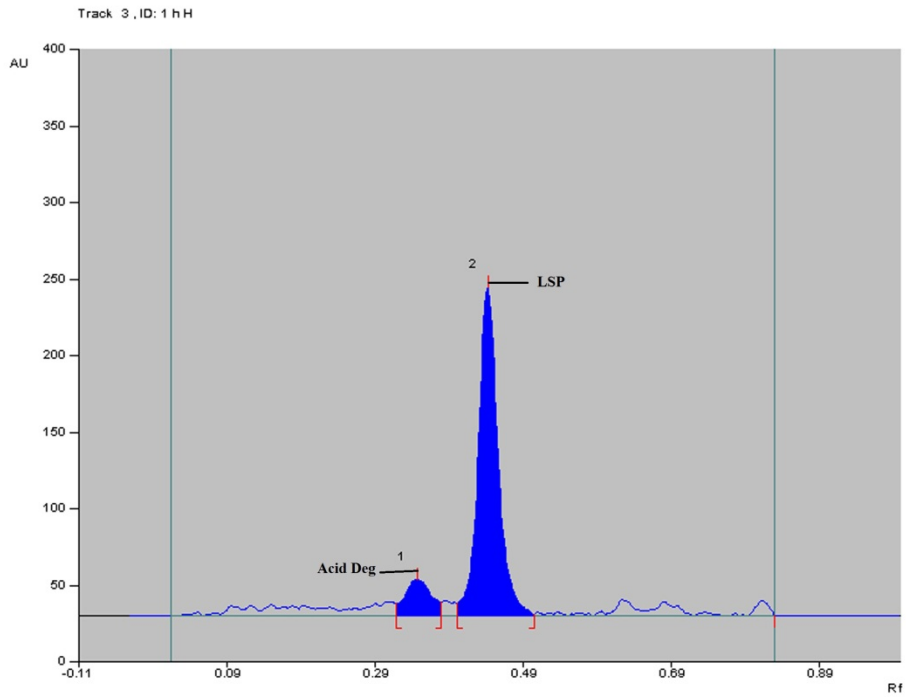


**Figure 38: Densitogram obtained from forced degraded product of LSP in 1N HCl**

### 5.4.2 Base induced degradation product

The sample was also subjected to base degradation. LSP is more prone to base degradation. As like acid degradation standard LSP was first subjected to base degradation with 0.1 N NaOH at 80 °C for 8 hrs. Sample was withdrawn and neutralized with 0.1 N HCl and spotted on HPTLC plate and analyzed with developed method. It did not show any degradation product. Therefore strength of base was increased up to 1 N in which it showed 22.72% degradation in 2 hrs. The retardation factor for the degraded product was found to be 0.37 ± 0.02 and that of standard LSP was 0.44 ± 0.02 (Figure 39).

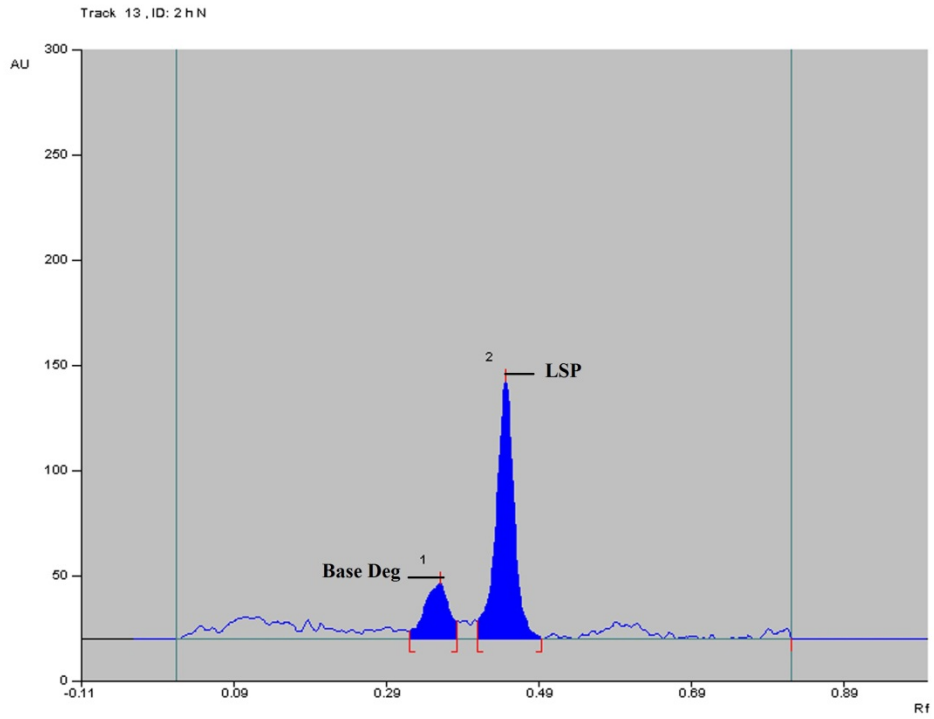


**Figure 39: Densitogram obtained from forced degraded product of LSP in 1N NaOH**

### 5.4.3 Oxidative degradation product

To study degradation due to oxidative stress conditions sample was treated with $H_2O_2$ solution at room temperature. Oxidative degradation was carried out first in 6 % $H_2O_2$ at room temperature up to 24 hrs. Sample was later boiled to remove oxygen and sample was diluted with methanol. The diluted sample was later spotted on HPTLC plate and analyzed with developed method; but showed no degradation. Using 30 % $H_2O_2$ degradation was observed under same conditions. It showed 20.35

% degradation in 2 hrs. The retardation factor for degraded product was found to be 0.77 ± 0.02 and that of standard LSP was 0.44 ± 0.02 (Figure 40).

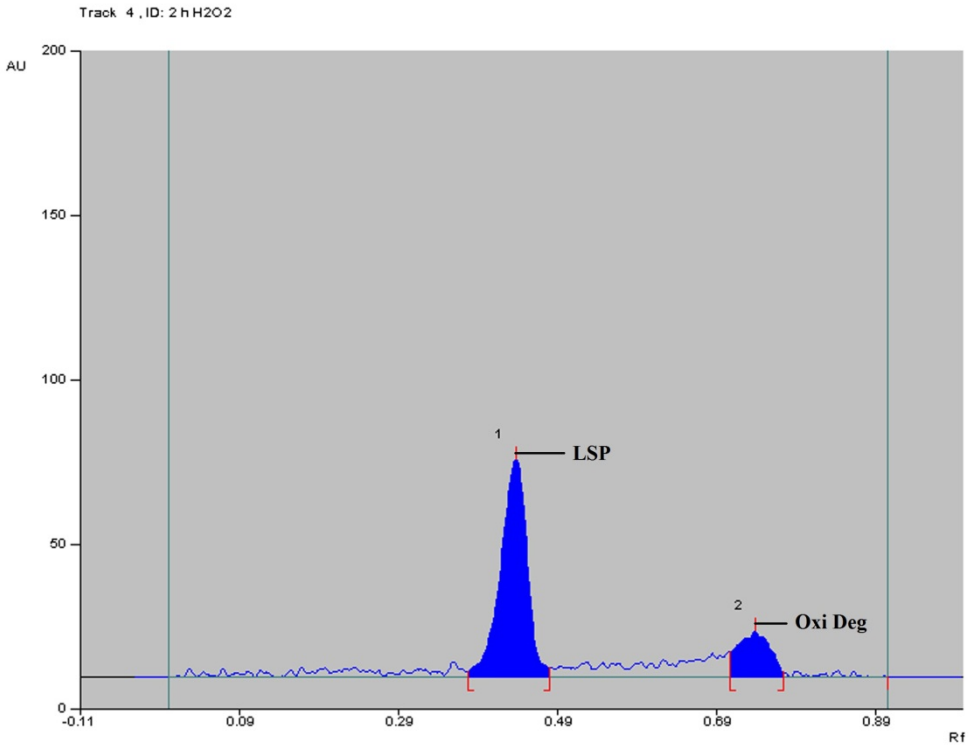


**Figure 40: Densitogram obtained from forced degraded product of LSP in 30 % $H_2O_2$**

### 5.4.4 Photochemical degradation

Stability of the sample to photo degradation was studied by exposing sample to direct sunlight as well as keeping in photostability chamber. LSP was found to be stable for photochemical degradation. Stock solution was exposed to direct sun light up to 1 week as well as stock solution was kept in the photo stability chamber for 7 days. Both the samples were analyzed with developed method and showed no degradation product. Only one band with retardation factor of 0.44 ± 0.02 for standard LSP was observed (Figure 41).

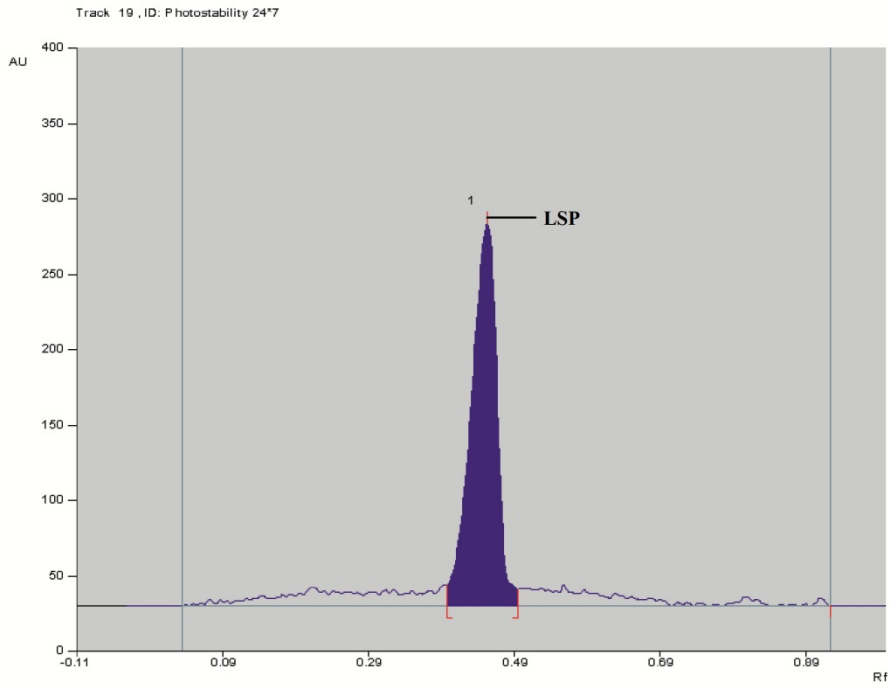


**Figure 41: Densitogram obtained from photo degraded sample of LSP**

### 5.4.5 Thermal conditions.

Standard LSP was also exposed to dry heat and wet heat conditions to study thermal stability. The chromatogram showed no peak for degradation product. The drug was found to be stable in dry and wet heat conditions.

### 5.4.6 Neutral hydrolysis.

The drug was refluxed in double distilled water for five days to study neutral hydrolysis. The samples withdrawn showed no degradation indicating that LSP is stable under neutral hydrolysis conditions.

## 5.5 HPTLC method validation

The stability indicating high performance thin layer chromatographic method was validated as per ICH guidelines and results were statistically analyzed.[25]

### 5.5.1 Linearity and range

Calibration curve of standard drug concentrations and peak areas was found to be linear over a range of 100 to 1000 ng band$^{-1}$ for LSP (Table No. 36). The data obtained was statistically analyzed for linear regression analysis to calculate slope, intercept and correlation coefficient. The calibration curve is shown in Figure 42. The

linear regression data is given Table no 37. The linearity data was also subjected to non-numerical residual analysis. The residual plot showed no tendency in the plot which supports the linearity results (Figure 43).

**Table No. 36: Linearity data of Levosulpiride**

| **Concentration [a]** | **Area** |
|---|---|
| 100 | 885.44 |
| 200 | 1646.78 |
| 400 | 2881.11 |
| 600 | 4117.33 |
| 800 | 5489.33 |
| 1000 | 6747.00 |

a = ng band$^{-1}$

**Table No. 37: Linear regression data for the calibration curves (n = 6)**

| **Parameters** | **Levosulpiride** |
|---|---|
| **Linearity range[a]** | 100 - 1000 |
| **$r^2$** | 0.9996 |
| **Slope** | 6.465 |
| **Intercept** | 287.54 |
| **LOD[a]** | 25.71 |
| **LOQ[a]** | 77.91 |
| **Sy.x** | 50.37 |

a = ng band$^{-1}$.

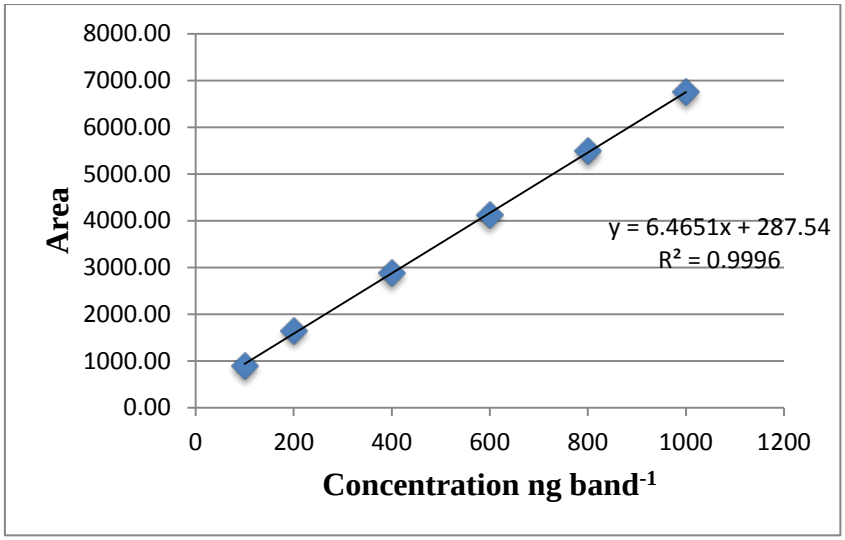


**Figure 42: Calibration curve of LSP**

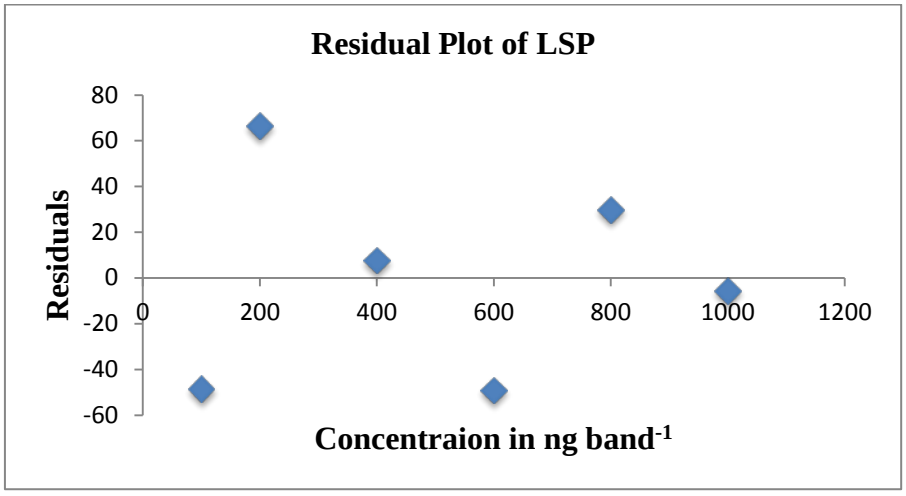


**Figure 43: Residual plot of LSP**

### 5.5.2 Sensitivity

The sensitivity of the developed method in terms of the limit of detection and limit of quantitation was determined by slope of calibration curve and standard deviation of response. LOD and LOQ for LSP were found to be 25.71 and 77.91 ng band$^{-1}$, respectively (Table No. 37).

### 5.5.3 Specificity

The specificity of the method expressed in terms of peak purity. The peak purity for LSP was assessed by comparing UV spectra acquired at the start (S), apex (M), and end (E) of the peak obtained from the scanning of band, that is, r (S, M) = 0.998, 0.999 and r (M, E) = 0.998, 0.999, respectively. Peak purity data showed that peaks obtained for LSP were pure (Figure 44).

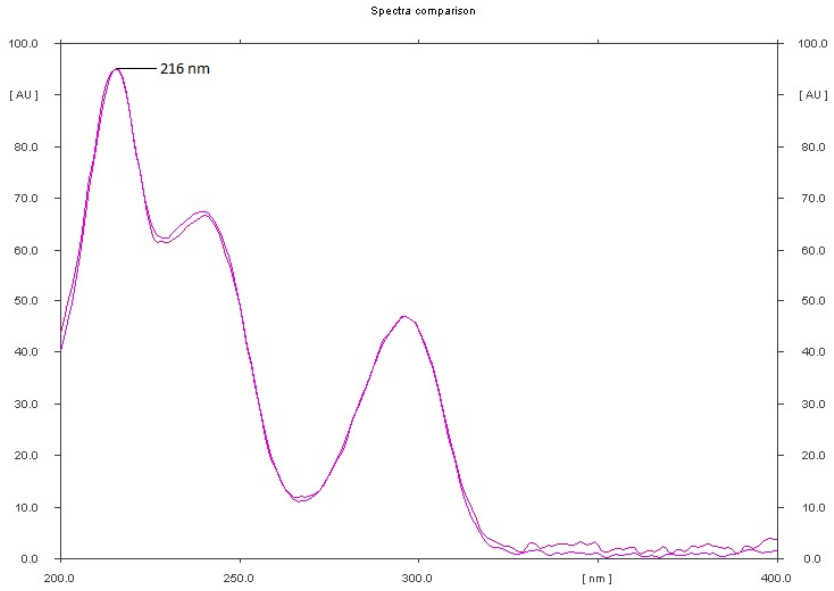


**Figure 44: Comparison of UV spectrum of standard and samples of LSP**

To confirm that no excipients are interfering with the peaks of standard and degradation product; extract of commonly used tablet excipients were spotted along with the degraded sample on the HPTLC plate and plate was analyzed with developed method. The absence of interfering peaks was confirmed which supported specificity of the developed method (Figure 45).

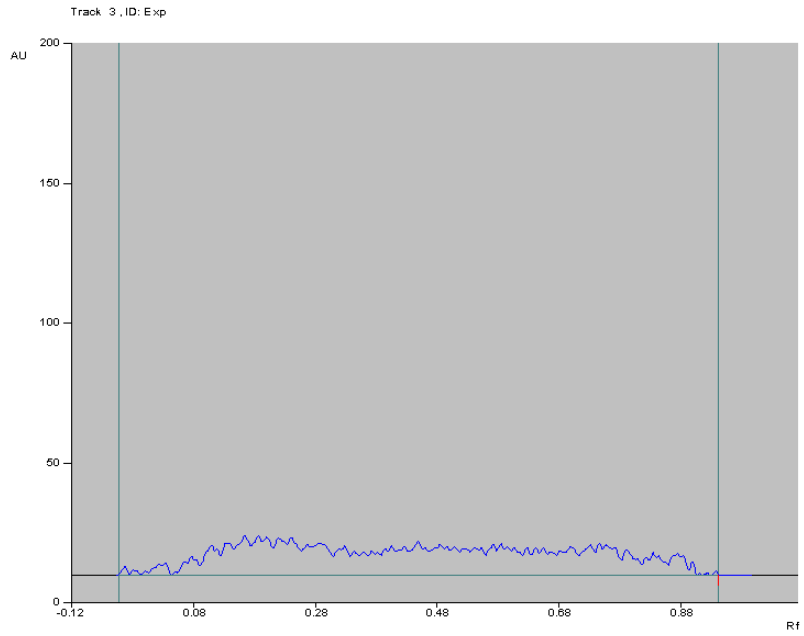


**Figure 45: Densitogram of extract of commonly used tablet excipients**

### 5.5.4 Precision

Intra-day and inter day precision of LSP was studied and expressed as % RSD and was found to be 0.20 – 0.43 % and 0.95 – 1.65 %, respectively. As recommended by ICH guidelines, both intra and inter-day precision studies showed % RSD < 2, indicating good precision (Table No. 38).

**Table No. 38: Intra and inter day precision (n = 3)**

| Standard drug | Nominal concentration [a] | concentration obtained [a] | | Precision obtained [b] | |
|---|---|---|---|---|---|
| | | Intra day | Inter day | Intra day | Inter day |
| Levosulpiride | 400 | 393.88 | 394.55 | 0.43 | 1.65 |
| | 600 | 607.86 | 593.37 | 0.73 | 0.95 |
| | 800 | 802.39 | 790.64 | 0.20 | 1.09 |

a = ng band$^{-1}$, b = Precision as % RSD

### 5.5.5 Accuracy

Recovery for LSP was found to be 99.39 - 100.44 %w/w. (Table No. 39)

**Table No. 39: Results of recovery studies (n = 3)**

| **Parameter** | **Levosulpiride** | | |
|---|---|---|---|
| **Amount Taken[a]** | 250.00 | 250.00 | 250.00 |
| **Amount Added[a] (%)** | 200 (80) | 250 (100) | 300 (120) |
| **Amound Found[a]** | 447.26 | 502.20 | 551.82 |
| **% Recovery** | 99.39 | 100.44 | 100.33 |
| **SD** | 2.00 | 2.40 | 2.34 |
| **%RSD** | 0.45 | 0.48 | 0.42 |

a = ng band$^{-1}$

### 5.5.6 Robustness studies

Small but deliberate changes were carried out in analytical parameters to study the robustness (Table No. 40). The robustness study showed no alteration in the peak areas of standard LSP due to deliberate changes in the analytical parameters. Relative standard deviation is less than 2 which indicate that the method is robust.

**Table No. 40: Results of robustness studies (n = 3, 500 ng band$^{-1}$)**

| **Parameter varied** | **Levosulpiride** | | | |
|---|---|---|---|---|
| | **Area** | **SD** | **% RSD** | **Rf** |
| **Mobile phase (ethyl acetate) composition (± 0.1 mL)** | 3525.89 | 16.57 | 0.47 | 0.44 |
| **Amount of mobile phase (± 5 %)** | 3580.49 | 16.27 | 0.45 | 0.44 |
| **Time from band application to chromatography (+ 10 min)** | 3564.83 | 34.76 | 0.98 | 0.44 |
| **Time from chromatography to scanning (+ 10 min)** | 3570.90 | 11.69 | 0.33 | 0.44 |

### 5.5.7 Solution stability

Stability of standard solution of LSP was studied at room temperature for 48 hrs. The % RSD was found less than 2 indicate that the solutions were stable for 48 hrs at room temperature in methanol.

### 5.5.8 Analysis of marketed formulation

Developed densitometric method was applied to the selected marketed formulations. Formulations A and B were found to contain 25.21 ± 0.27 mg per tablet and 12.55 ± 0.13 mg $mL^{-1}$of LSP with recovery of 100.88 and 100.42 % respectively (Table No. 41).

**Table No. 41: Analysis of marketed formulations (n = 6, 250 ng $band^{-1}$)**

| **Formulation** | **Label claim** | **Content Found (Mean ± SD)** | **Recovery (%)** |
|---|---|---|---|
| **Formulation A** | 25 mg per tablet | 25.21 ± 0.27 mg per tablet | 100.88 |
| **Formulation B** | 12.5 mg $mL^{-1}$ | 12.55 ± 0.13 mg $mL^{-1}$ | 100.42 |

## 5.6 Identification of degradation product

### 5.6.1 TLC-MS

The separated degradation products were analyzed by TLC-MS for its m/z values. The mass spectrum of LSP separated from base degradation product and oxidative degradation product were recorded. The mass spectrum of LSP showed base peak at m/z 342.1 in positive ion mode. The peak at m/z 364 is due to addition of sodium ion (Figure 46). Due to limitation of the instrument which do not record m/z value below 250, the acid and base degradation product could not be identified by TLC-MS. They were identified by LC-MS/MS.

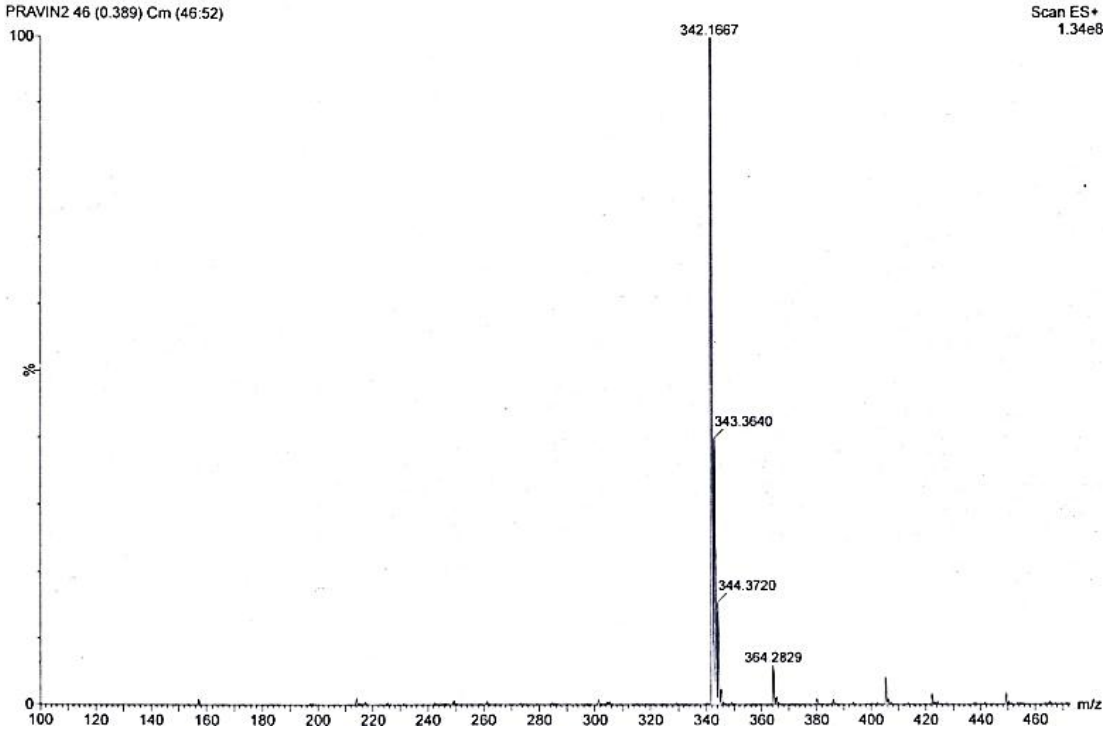


**Figure 46: Mass spectrum of LSP separated from base degradation product obtained from TLC-MS**

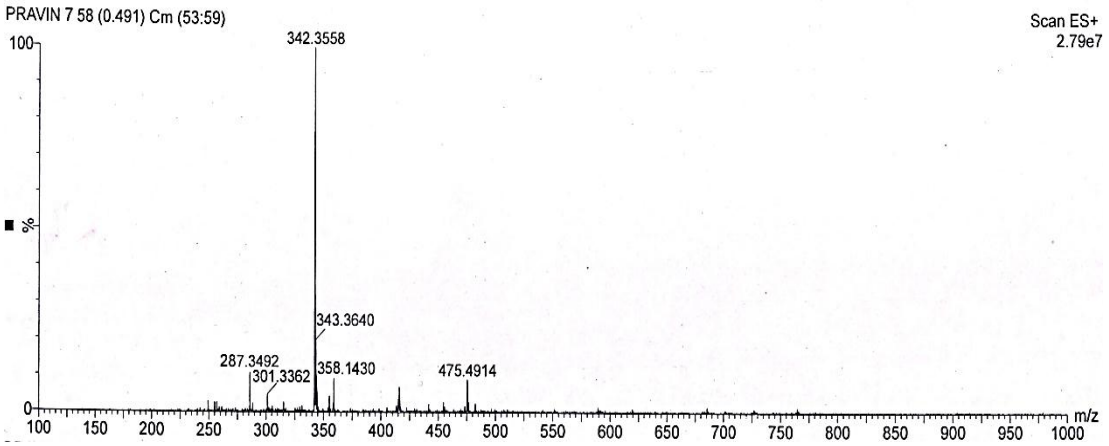


**Figure 47: Mass spectrum of oxidative degradation product obtained from TLC-MS**

The oxidative degradation was achieved by exposing sample to 30% $H_2O_2$. The spots obtained were analyzed for its m/z values. The oxidative degradation product showed m/z at 358.1 and base peak at m/z 342.3. The daughter ions are recorded at m/z 287.3

and 301.3 (Figure 47). The peak at m/z 358.1 is due to N-oxidation of pyrrolidine ring. The further fragmentation is giving the peak at m/z 287.3 (Figure 48).

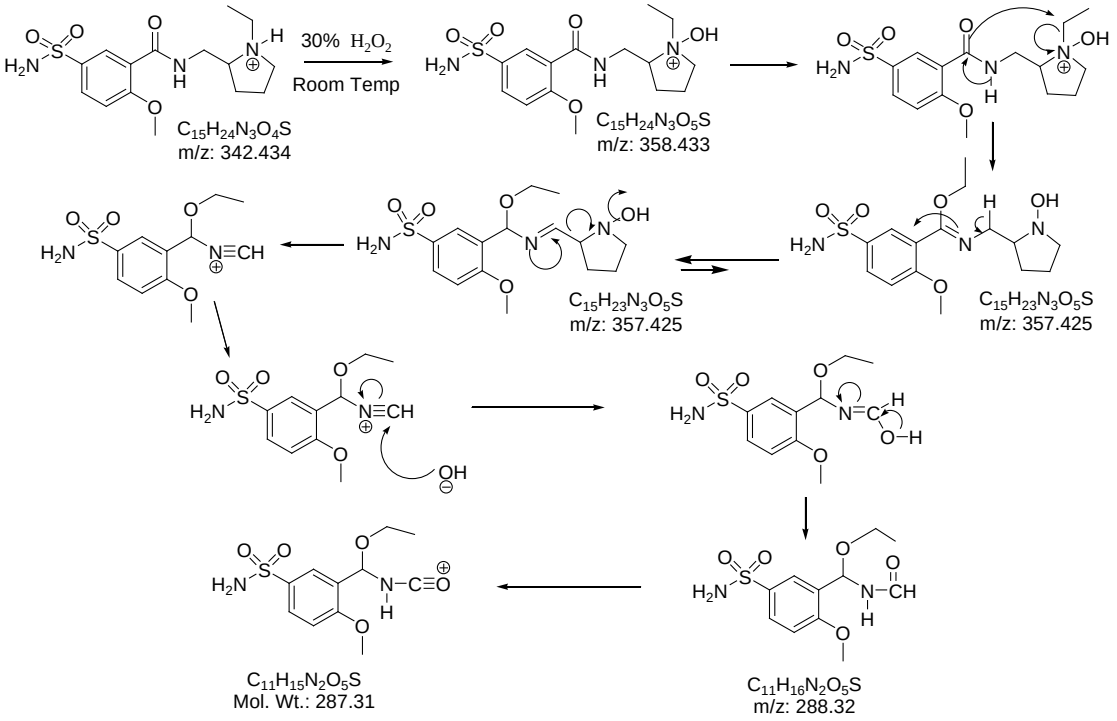


**Figure 48: Pathway for oxidative degradation**

# 6 Development of stability indicating high performance liquid chromatographic method for quantitation of Levosulpiride in pharmaceutical dosage form and application of LC-MS/MS for identification of degradation product.

## 6.1 Development and optimization of stability indicating HPLC method

The method was developed with a view to separate degradation product from LSP. Method development started with the selection of mobile phase containing methanol and water. The high percentage of methanol was giving peaks of standard LSP in dead volume. Hence percentage of methanol was decreased. Mobile phase consisting of methanol: water (20:80) gave good results. The optimized flow rate of the mobile phase was 0.7 mL $min^{-1}$. The UV spectrum was recorded using standard solution of LSP and wavelength selected for analysis was 216 nm (Figure 49). The retention time was found to be 3.408 min for standard LSP (Figure 50). The method gave acceptable retention, theoretical plates and good resolution for all degradation products from standard LSP.

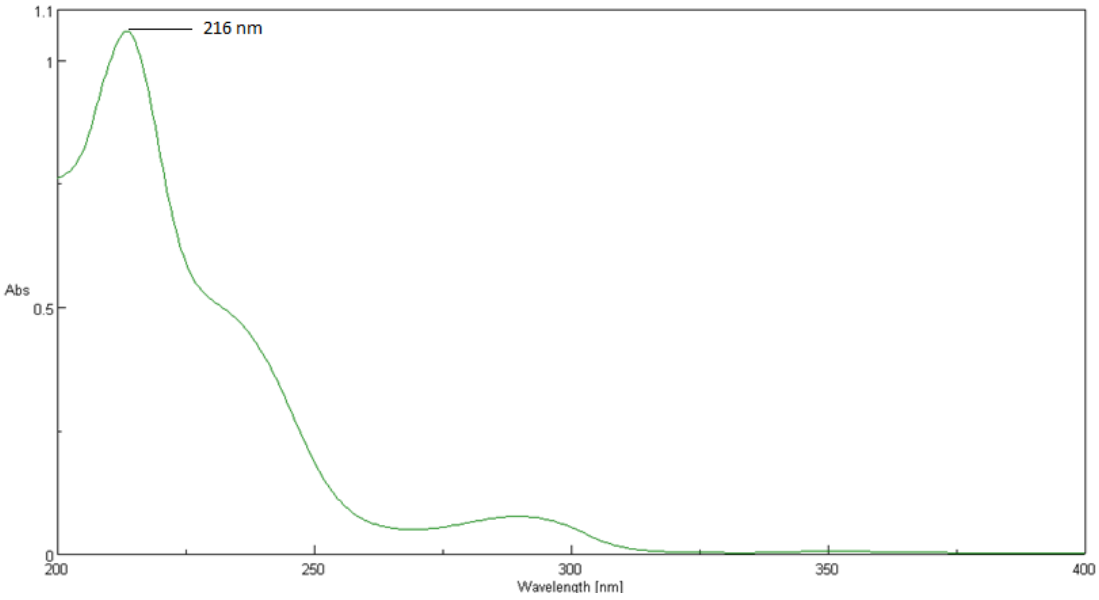


**Figure 49: UV spectrum of standard solution of LSP**

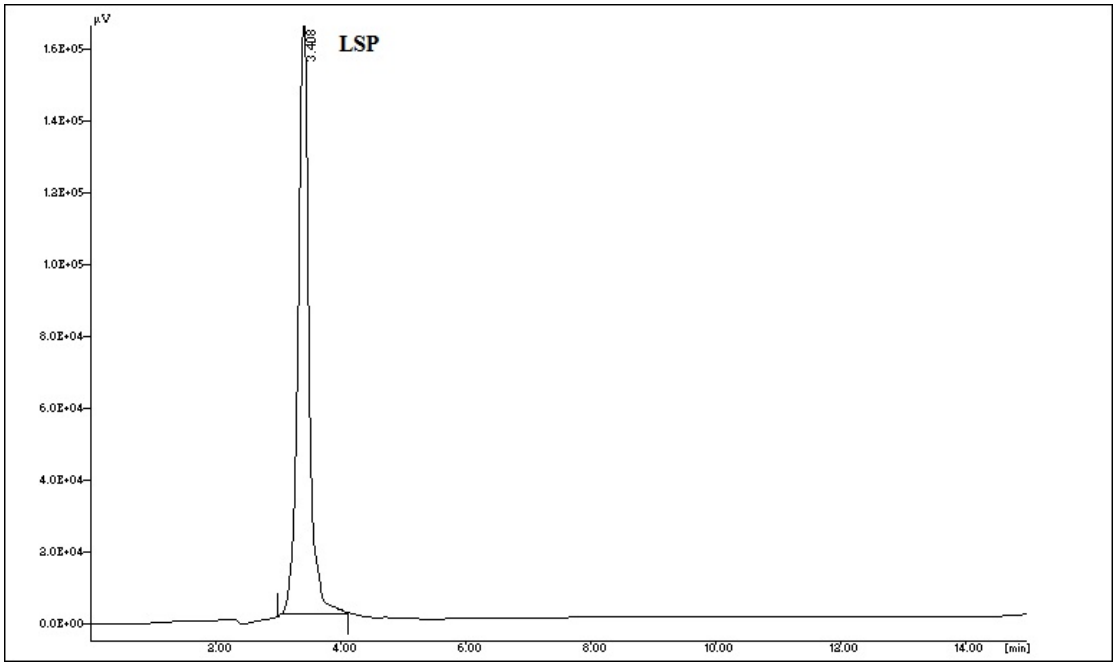


**Figure 50: Representative chromatogram of standard LSP at 216 nm**

## 6.2 Chromatographic conditions

The optimized high performance thin layer chromatographic conditions are as follows.

| | |
|---|---|
| **HPLC column** | Syncronis aQ $C_{18}$ (5 µm, 250 × 4.6 mm i. d.) |
| **Mobile phase** | Methanol: water (20: 80, v/v) |
| **UV detection** | 216 nm |
| **Injection volume** | 20 µL |
| **Run time** | 10 Min |
| **Flow rate (mL $min^{-1}$)** | 0.7 mL $min^{-1}$ |

### 6.3 Forced Degradation studies

As per recommendation of ICH guidelines bulk sample of LSP was subjected to forced degradation study. The sample was subjected to hydrolytic, oxidative, photolytic and thermal conditions. After getting sufficient degradation samples were analyzed with the developed method and resolution of degradation products and drugs was studied.

#### 6.3.1 Acid induced degradation

As per schemes mentioned by Bakshi and Singh[33, 37] bulk samples of LSP was initially exposed to 0.1N HCl. Sample was mixed with 0.1 N HCl and refluxed for 8 hrs. At the end sample was withdrawn and neutralized with 0.1 N NaOH and diluted with methanol. The diluted sample was injected on the HPLC column and analyzed with the developed method. It did not show any degradation peak. So strength of the acid was increased up to 1N and procedure was repeated under same experimental conditions. The samples exposed to 1N HCl showed 12.50 % degradation in 2hrs. Standard LSP gave a peak with retention time of 3.350 min and the acid degradation product showed peak at 3.993 min. Both peaks were well resolved from baseline with resolution factor of 2.42 (Figure 51).

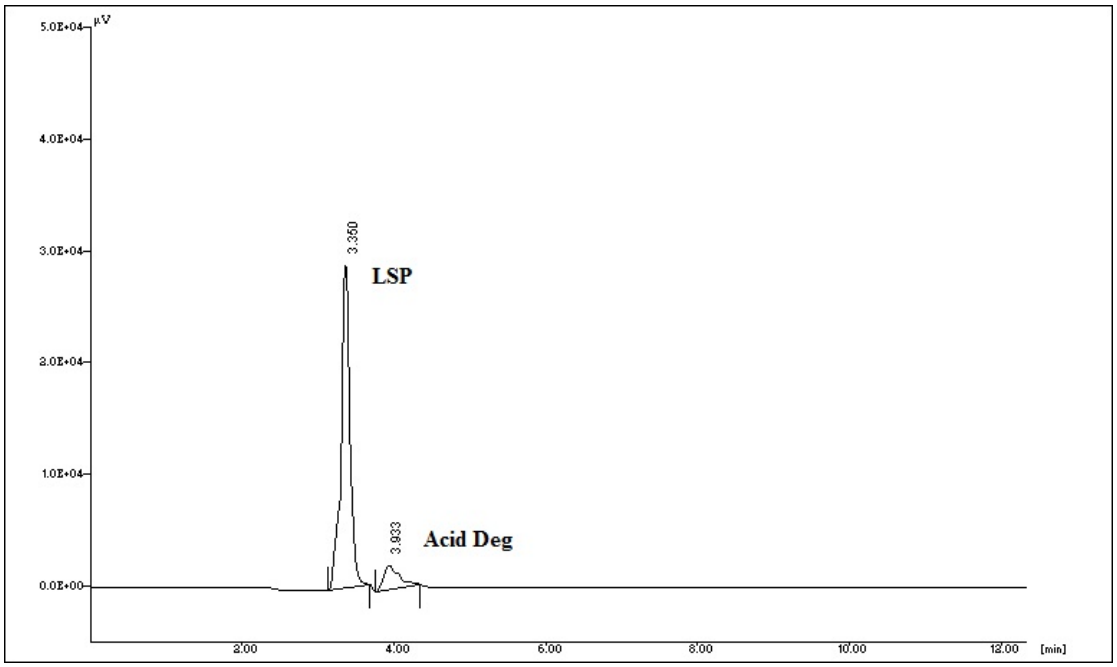


**Figure 51: Chromatogram of acid degraded LSP**

### 6.3.2 Base induced degradation

The degradation in basic condition was initiated with 0.1 N NaOH. Sample was mixed with 0.1 N NaOH and refluxed for 8 hrs. After 8 hrs of reflux sample was withdrawn and neutralized with 0.1 N HCl and diluted with methanol. The diluted sample was injected on the HPLC column and analyzed with the developed method. It showed only one peak for LSP. Hence strength of the base was increased to 1 N. Same procedure was repeated under the same experimental conditions. The samples exposed to 1 N NaOH showed around 20.58 % degradation in 2 hrs. The degradation product showed a peak at retention time of 3.925 and LSP showed a peak at 3.350 min. The peaks were well resolved and the resolution factor was 3.24 (Figure 52).

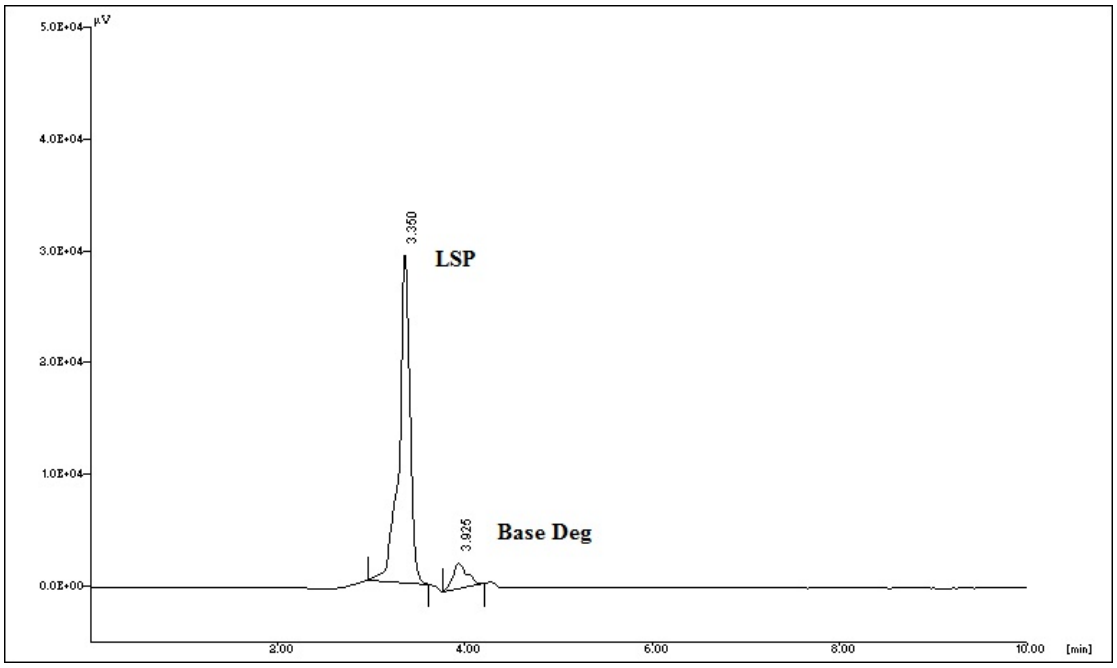


**Figure 52: Chromatogram of base degraded LSP**

### 6.3.3 Oxidation degradation product

To study degradation due to oxidative stress, sample was exposed to $H_2O_2$ solution at room temperature. Oxidative degradation was carried out first in 6 % $H_2O_2$ at room temperature up to 24 hrs. Sample was later boiled and sample was diluted with methanol. The diluted sample was injected on the HPLC column and analyzed with developed method; it showed no degradation. Hence strength of hydrogen peroxide solution was increased. Using 30 % $H_2O_2$ degradation was observed under same

conditions. The chromatogram obtained after injecting the oxidative degraded product showed three peaks with retention times 1.817, 2.592 and 3.450 min. The peak at 1.817 min was identified as solvent peak. The peak at 2.592 min was due to peak of oxidative degradation product and peak at 3.450 min was due to LSP. The sample showed 20.35 % degradation in 2 hrs. the peaks were well resolved from baseline with resolution factor of 3.21(Figure 53).

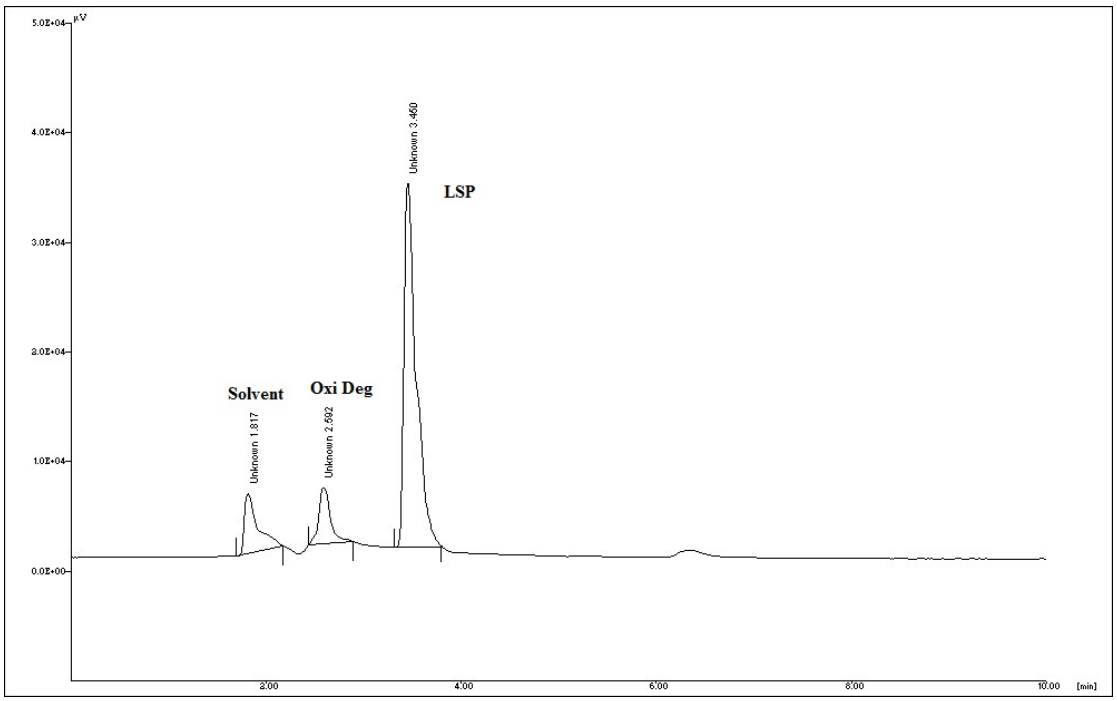


**Figure 53: Chromatogram of oxidative degradation LSP**

### 6.3.4 Photochemical degradation

The photo stability of the sample was studied by exposing sample to direct sunlight as well as keeping in photostability chamber. LSP was found to be stable towards photochemical degradation. Stock solution was exposed to direct sun light up to 1 week as well as stock solution was kept in the photo stability chamber for 7 days. Both the samples were analyzed with developed method and showed no degradation product. The chromatogram showed only one peak for LSP.

### 6.3.5 Thermal conditions.

Standard LSP was also exposed to dry heat and wet heat condition to study thermal stability. The sample was injected onto the HPLC column and analyzed using

developed method. The chromatogram showed no peak for degradation product. The drug was found to be stable towards dry and wet heat conditions.

### 6.3.6 Neutral hydrolysis.

The drug was refluxed in double distilled water for five days to study neutral hydrolysis. The sample was injected onto the HPLC column and analyzed for degradation product. The samples withdrawn showed no degradation indicating that LSP is stable under neutral hydrolysis conditions.

## 6.4 Validation of the stability indicating method

The developed method was validated as per the ICH Q2 (R1) guidelines. The parameters studied were linearity, LOD, LOQ, range, precision, accuracy and specificity.[25]

### 6.4.1 Linearity

Linearity was performed by preparing standard solutions in the range of 10 to 100 µg $mL^{-1}$. The solutions were injected onto the HPLC column and detector response was recorded. The study was repeated six times. The mean detector response of six replicates was plotted against concentrations of the standard solutions. The data obtained was statistically analyzed and subjected to least square regression analysis to determine slope, intercept and correlation coefficient for the calibration curve (Figure 54). The data was found to be linear in the range of 10 to 100 µg $mL^{-1}$. The correlation coefficient was found to be 0.9996 (Table No. 43). The regression analysis data is given in Table no. 43. The data obtained subjected to residual analysis. The residual plot showed no tendency in the plot supports linearity results (Figure 55).

**Table No. 42: Linearity data of Levosulpiride**

| **Concentration [a]** | **Area (µV.sec)** |
|---|---|
| **10** | 403292 |
| **20** | 821414.83 |
| **40** | 1603663.66 |
| **60** | 2430725.83 |
| **80** | 3179345.5 |
| **100** | 4064924.33 |

a = µg $mL^{-1}$

**Table No. 43: Linearity regression data for calibration curve (n=6)**

| **Parameters** | **Levosulpiride** |
|---|---|
| **Linearity range[a]** | 10 - 100 |
| **$r^2$** | 0.9996 |
| **Slope** | 40321 |
| **Intercept** | 659.24 |
| **LOD[a]** | 2.47 |
| **LOQ[a]** | 7.5 |
| **Sy.x** | 30260 |

a = µg $mL^{-1}$

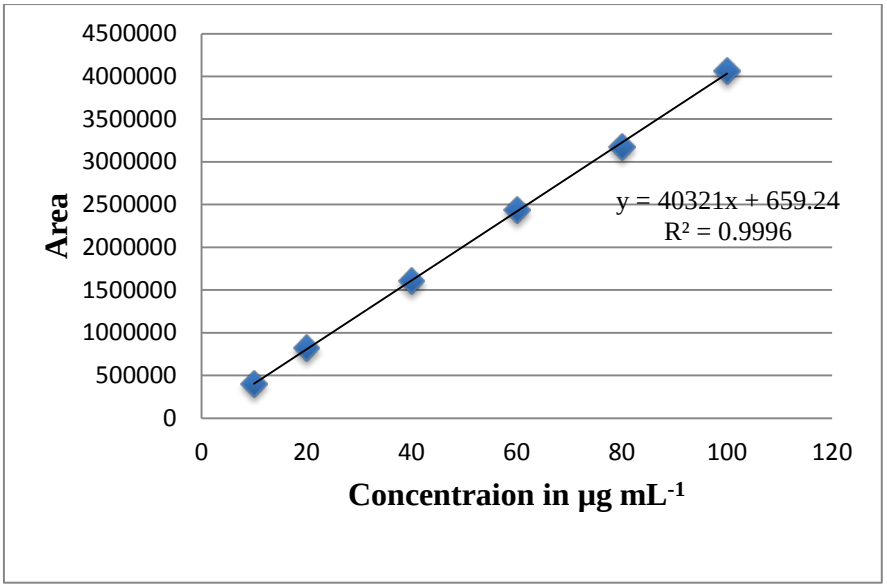


**Figure 54: Calibration curve of LSP**

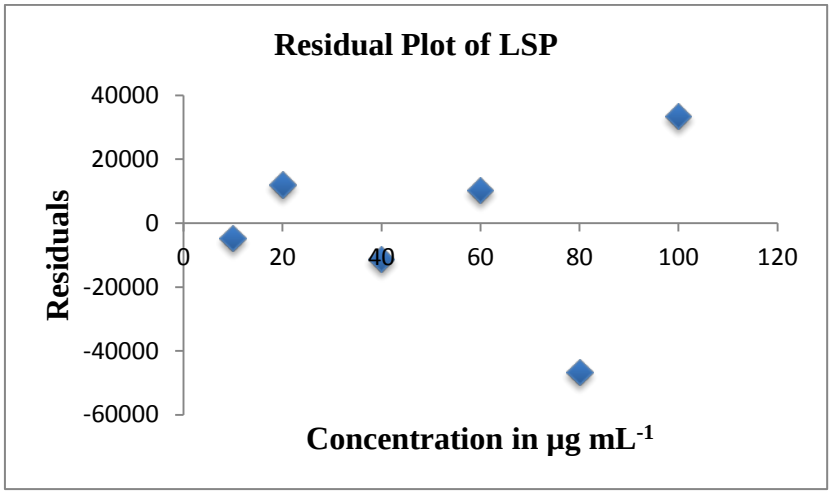


**Figure 55: Residual plot of LSP**

### 6.4.2 Precision

The precision of the developed RP-HPLC method was studied by intra-day and inter-day precision. Intra-day precision was performed by analysis of three different concentrations from the linearity range and three replicates were studied. Inter-day was precision study was carried out by using same concentration but carried out on three consecutive days.

The results for repeatability and intermediate precision are summarized in Table no. 44. The % RSD was found to be less than 2 as per recommendation of ICH guidelines indicating good precision. The % RSD for intra and inter day precision was found to be 0.30 to 0.96 % and 0.32 to 1.11%, respectively.

**Table No. 44: Intra and inter day precision (n=3)**

| Standard drug | Nominal Concentration [a] | Concentration obtained [a] | | Precision obtained [b] | |
|---|---|---|---|---|---|
| | | Intra day | Inter day | Intra day | Inter day |
| Levosulpiride | 10 | 10.00 | 9.99 | 0.96 | 0.53 |
| | 40 | 40.43 | 42.46 | 0.54 | 1.11 |
| | 80 | 79.10 | 79.44 | 0.30 | 0.32 |

n- no of replicates, a = µg $mL^{-1}$,b = Precision as % RSD,

### 6.4.3 Sensitivity

Sensitivity of the method was established by determining LOD and LOQ. The LOD and LOQ was determined from slope and standard deviation of the response. The limit of detection and quantitation was found to be 2.47 and 7.5 µg $mL^{-1}$, respectively (Table No. 43).

### 6.4.4 Robustness

Small but deliberate changes were made in the analytical conditions from the optimized conditions to study the robustness of the developed method. One factor was changed at a time and each time fresh samples were prepared. The data was analyzed and results were expressed in terms of % RSD. The method was found to be robust as it gave % RSD values less than 2 (Table No. 45).

**Table No. 45: Results of robustness studies (n=3, 50µg $mL^{-1}$)**

| Parameter Varied | Levosulpiride | | | | |
|---|---|---|---|---|---|
| | **Rt (Min)** | **N** | **Peak Area** | **Mean Area ± SD** | **% RSD** |
| **Mobile phase (methanol) composition (± 1%)** | 3.278 | 3052 | 2016709 | 2021310.3 ± 5491.81 | 0.27 |
| | 3.326 | 3103 | 2019832 | | |
| | 3.409 | 2973 | 2027390 | | |
| **Elution flow rate (± 0.1 mL $min^{-1}$)** | 3.332 | 3118 | 2019320 | 2024264 ± 13680.29 | 0.67 |
| | 3.401 | 3087 | 2039729 | | |
| | 3.481 | 2914 | 2013743 | | |
| **Detection wavelength (± 2 nm)** | 3.416 | 3089 | 2013732 | 2024891 ± 11775.04 | 0.58 |
| | 3.41 | 3101 | 2023743 | | |
| | 3.412 | 3092 | 2037198 | | |

n- no of replicates, N- No. of theoretical plates,

### 6.4.5 Specificity

The specificity of the method was assessed by studying the resolution factor for the peaks for the drug and degradation products. The peaks obtained for standard and degradation product were sharp and with clear baseline separation. The resolution factor was found to be greater than 2.4 indicating good resolution between the peaks. Extract of commonly used tablet excipients was injected and no interference of the peaks with peaks of the standard and degradants was observed (Figure 56).

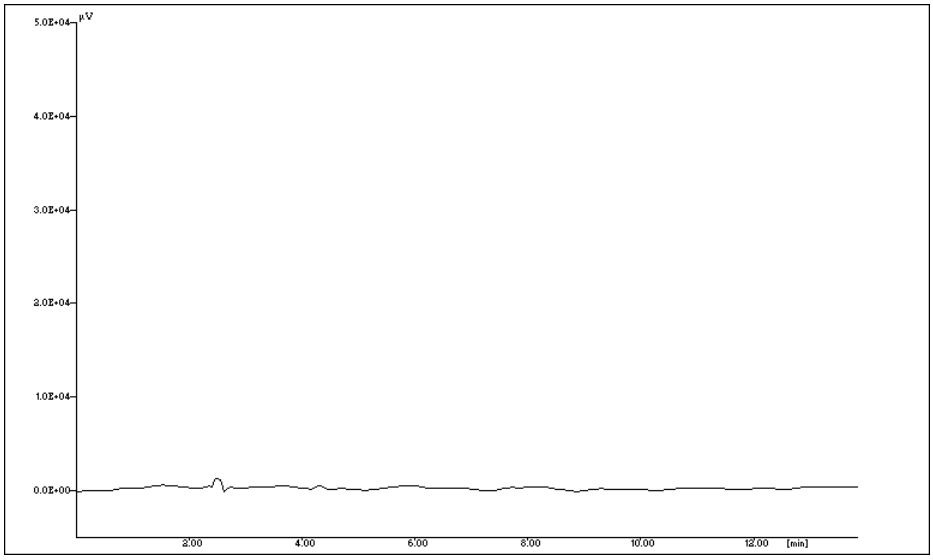


**Figure 56: Chromatogram of the extract of commonly used tablet excipients**

### 6.4.6 Accuracy

Accuracy of developed method was studied by standard addition method. The sample solution was spiked with 80, 100 and 120 % solution of standard solution. The study was repeated three times and total amount recovered was calculated. The results for recovery study were within the limits. Recovery for the drug was observed in the range of 100.48 to 100.64 % (Table No. 46).

**Table No. 46: Results of recovery studies (n=3)**

| **Parameter** | **Levosulpiride** | | |
|---|---|---|---|
| **Amount Taken[a]** | 25 | 25 | 25 |
| **Amount Added[a] (%)** | 20(80) | 25(100) | 30(120) |
| **Amount Found[a]** | 45.287 | 50.239 | 55.293 |
| **SD** | 0.279 | 0.273 | 0.258 |
| **% Recovery** | 100.64 | 100.48 | 100.53 |
| **%RSD** | 0.62 | 0.54 | 0.46 |

a = $\mu g\ mL^{-1}$

### 6.4.7 System suitability

System suitability confirms quality performance of the developed HPLC method. It was assessed by number of theoretical plates (≥ 2000), peak symmetry (≤ 2), resolution between the peaks (≥ 2.0), and proper retention time. System suitability

parameters for the developed method were studied and found to be within limits. The retention time, theoretical plates and asymmetry for standard LSP was found to be 3.40, 3052 and 0.98, respectively. The resolution of LSP from acid, base and oxidative degradants was found to be 2.42, 3.43 and 3.21, respectively.

### 6.4.8 Solution stability

The relative standard deviation of the area obtained was found to be less than 2% which indicates the stability of the standard and sample solution of LSP up to 48 hrs at room temperature.

### 6.4.9 Analysis of Marketed formulations

Two different formulations of LSP were analyzed and the assayed content was found to be 25.24 ± 0.23 mg per table in formulation A and 12.49 ± 0.04 mg $mL^{-1}$ in formulation B with recovery of 100.98 and 99.96 %, respectively as with respect to the labeled claim (Table No. 47).

**Table No. 47: Analysis of marketed formulations (n=6, 25 µg $mL^{-1}$)**

| Formulation | Label Claim | Content Found (Mean ± SD) | Recovery (%) |
|---|---|---|---|
| **Formulation A** | 25 mg per tablet | 25.24 ± 0.23 mg per tablet | 100.98 |
| **Formulation B** | 12.5 mg $mL^{-1}$ | 12.49 ± 0.04 mg $mL^{-1}$ | 99.96 |

## 6.5 Identification of major degradation product

### 6.5.1 Acid and base hydrolysis degradation product

LSP was prone to acid and base hydrolysis. The rate of hydrolysis was more in basic conditions compared to acidic conditions. The chromatogram of acid hydrolysis reaction mixture had showed only one extra peak at 3.933 min (Figure 51). The sample was subjected to LCMS analysis to obtain the m/z value.

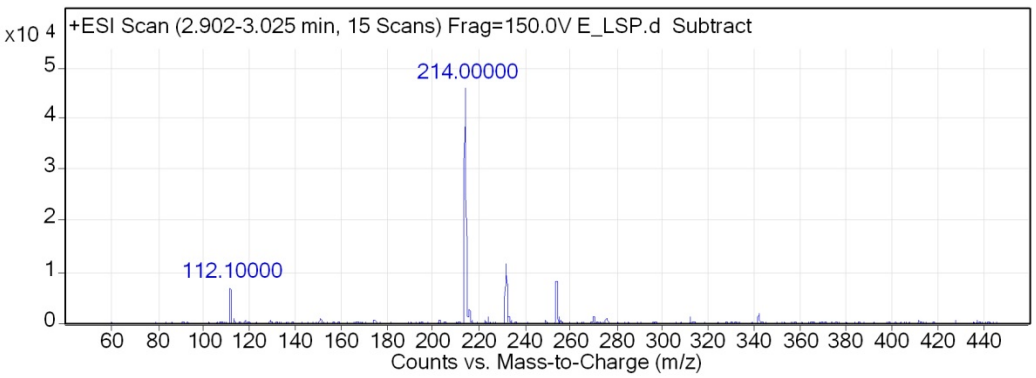


**Figure 57: Mass spectrum of acidic degradation product of LSP**

The chromatogram of base hydrolysis reaction mixture had showed only one extra peak at and 3.925 min (Figure 52). The sample was subjected to LCMS analysis to obtain the m/z value.

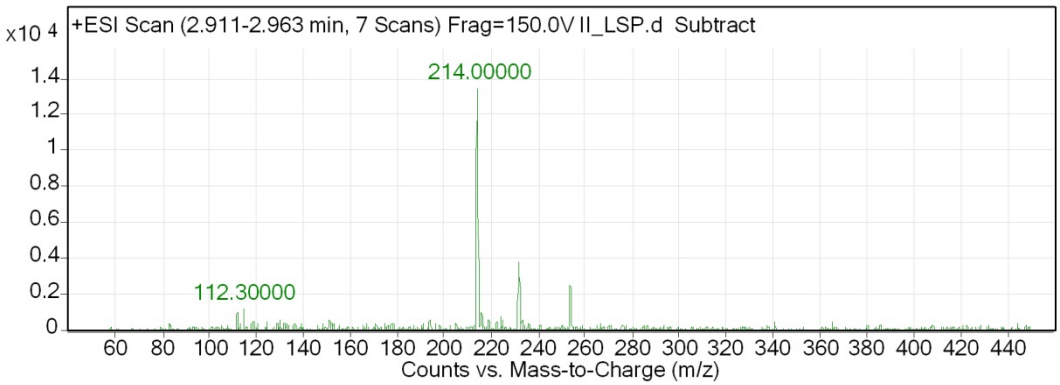


**Figure 58: Mass spectrum of basic degradation product of LSP**

The LCMS analysis of acid degraded product showed m/z value 254. Daughter ions which are observed in the mass spectrum of degraded product were at m/z 232 and m/z 112.1 with a base peak of 214 (Figure 57). The LCMS analysis of base degraded product had showed m/z value of 253.9. Product ions observed in the mass spectrum of degraded product were m/z 232 and m/z 114.4 with a base peak of m/z 214 (Figure 58).

The acid and base hydrolysis is mainly observed at amide linkage of LSP. The possible reaction of acid and base hydrolysis and fragmentation pattern is shown in

the Figure 60. The degradation products observed in acid and base hydrolysis were found to be same as 2-methoxy-5-sulfamoyl-benzoic acid having m/z value 231.2 showed m/z 232 in +ESI mode which was confirmed by the mass spectrum. The daughter ion observed at m/z 254 was due to addition of sodium ion scanned in +ESI mode (Figure 57 and 58).

### 6.5.2 Oxidative degradation product

Oxidative degradation was observed when LSP was exposed to 30% $H_2O_2$ at room temp. The chromatogram had showed two extra peaks at retention time 1.817 and 2.592 min (Figure 53). The peak at retention time at 1.817 was later identified as the peak of $H_2O_2$ when blank was injected. The sample was subjected to LCMS analysis.

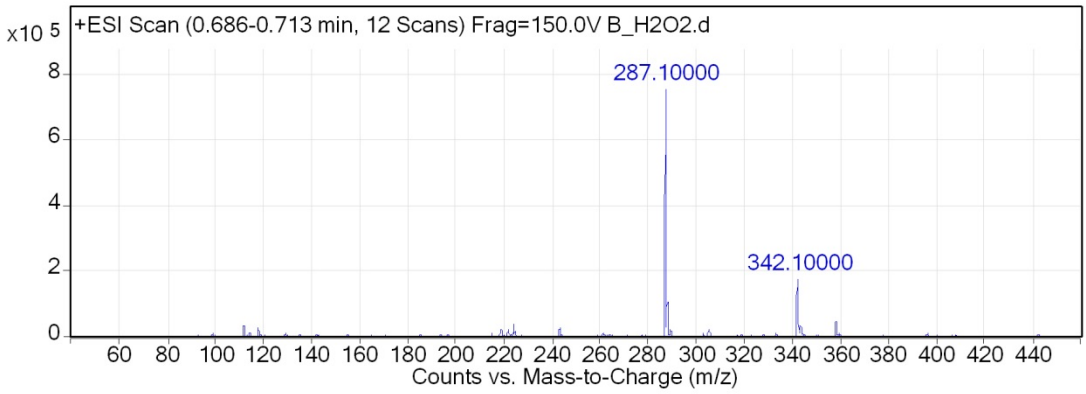


**Figure 59: Mass spectrum of oxidative degradation product of LSP**

The LCMS analysis of oxidative degradation product showed parent ion peak at m/z 358.1. The daughter ions observed are m/z 342.1 with a base peak of m/z 287.1 (Figure 59). From the data of mass spectrum of oxidative degradation product it looks like oxidation is taking place at nitrogen of the pyrrolidine ring of the LSP and further fragmentation is giving the base peak of m/z 287.1. The oxidative degradation product is suggested to be 1-ethyl-1-hydroxy-2-[(2-methoxy-5-sulfamoyl-benzoylamino)-methyl]-pyrrol (Figure 61).

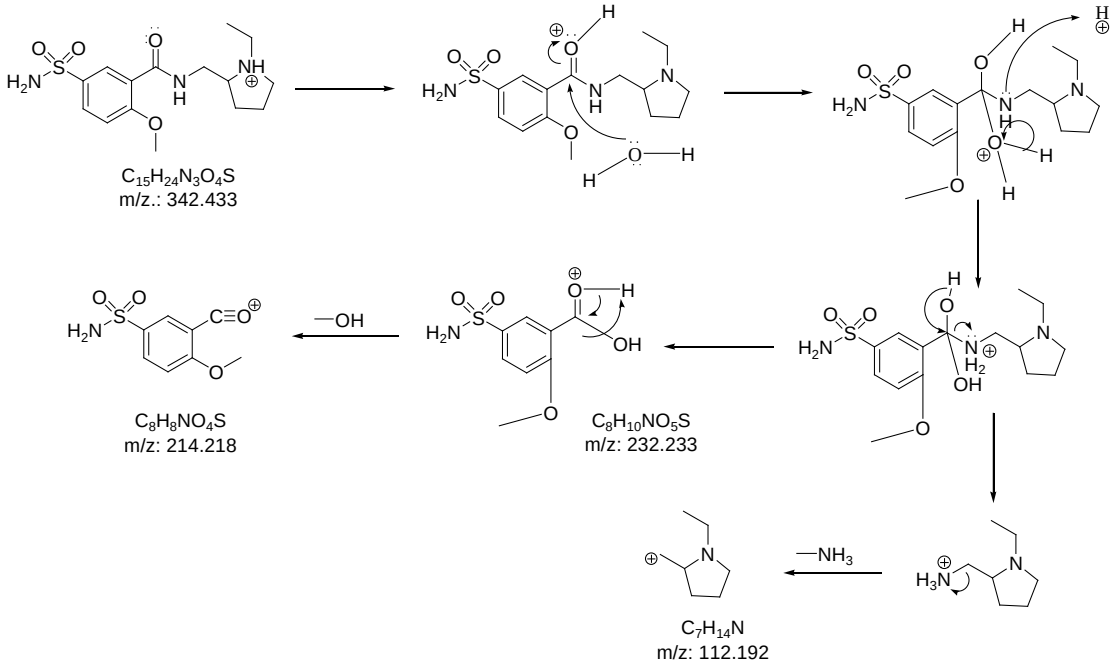


**Figure 60: Degradation pathway for acid and base hydrolysis**

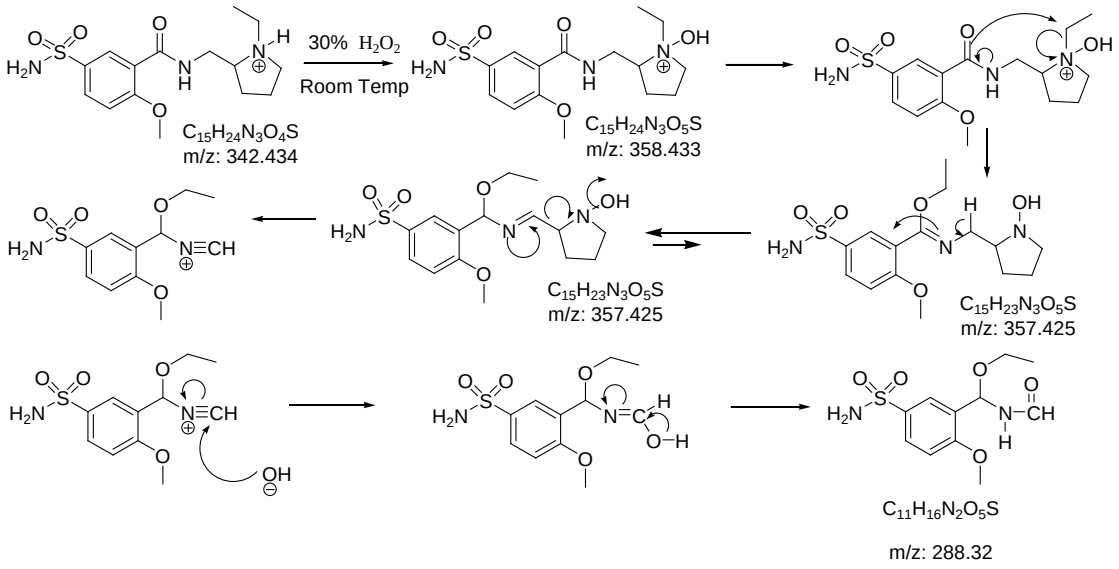


**Figure 61: Pathway for oxidative degradation.**

# *Experimental*

## Experimental

Standard drugs were obtained as a gift samples from various manufacturers from India.

Standard Levosulpiride was procured as a gift sample by Wanbury Ltd, Mumbai. (Batch no. 4602052009). Esomeprazole was obtained as a gift sample from Cipla Ltd, Kurkumbh (Batch no. CW130110). Rabeprazole was obtained from Lupin Ltd, Tarapur (Batch no. WS/GA/R04/06). Mometasone furoate was obtained from Glenmark Pharmaceuticals Ltd, Nashik (Batch no. NRM1400130). Terbinafine hydrochloride was obtained as gift sample from Dr. Pritam S. Jain, Professor, R. C. Patel Institute of Pharmaceutical Education and Research, Shirpur which was provided by Dr. Reddy's Lab, Hyderabad.

The pharmaceutical dosage forms used for the assay study were Nexpro L (Batch no. MYAW07) (Torrent Pharmaceuticals Ltd.) and Sompraz L (Batch no. BSN2746) (Sun Pharmaceutical Industries Ltd.) purchased from local market. Both contain Levosulpiride 75 mg and Esomeprazole 40 mg per capsule. Rabekind Plus (Batch no. F7FZ0006) (Mankind Pharm Ltd.) and labeled to contain 75 mg of Levosulpiride and 20 mg of Rabeprazole per tablet. Momoz T (Batch no. 035) manufactured by Helios Pharmaceuticals, Malpur and marketed by Unichem Laboratories Ltd, Mumbai containing 1% w/w Terbinafine hydrochloride and 0.1% w/w Mometasone furoate.

Recovery of Levosulpiride in stability indicating assay methods was carried out on marketed formulation purchased from local market. The tablet dosage form selected was Lesuride tablet (Batch no. BSN0482A) and injection formulation selected was Lesuride injection (Batch no. HKM0078). Both were manufactured by Sun Pharm Laboratories Ltd. Tablet was labeled to contain 25 mg of Levosulpiride per tablet and injection was labeled to contain 12.5 mg $mL^{-1}$.

The high performance thin layer chromatography method development and analysis was carried out on system of Camag (Camag, Muttenz, Switzerland). Sample application was carried out by Camag Linomat V sample applicator and 100µL Camag syringe. Densitometric scanning was performed on Camag TLC scanner 3.

Stationary phase used in the study was silica gel 60 $F_{254}$ (E. Merck, Darmstadt, Germany) precoated on aluminium HPTLC plates having dimensions of 20 × 10 cm with 250 µm thickness. HPTLC plates were prewashed with methanol and dried in oven at 120 °C for 15 min prior to spotting and development. Development of chromatogram was carried out in Camag twin trough chamber (20 × 10 cm). The band length of applied spot was 6mm and slit width was 5 × 0.45 mm. Scanning was performed at a speed of 10 mm $s^{-1}$.

The High Performance Liquid Chromatographic method development and validation was carried out on a Jasco Inc. (Easton MD) Model PU 2080 Intelligent LC Pump with autosampler programmed at 20 µL injection volume with Jasco Ultra Violet detector Model UV 2075. Recording and processing of chromatographic data was carried out using the Jasco Borwin version 1.5 LC-Net II /ADC system. The column used was Syncronis aQ $C_{18}$ (5 µm, 250 × 4.6 mm i. d.) from Thermo Fisher Scientific India Ltd, Mumbai.

Simultaneous estimation of MTS and TBF was carried out on High Performance Liquid Chromatographic method development and validation was carried out on a Jasco Inc. (Easton MD) Model PU 2089plus Intelligent LC Pump with manual injector with a loop of 20 µL injection volume with Jasco PDA detector Model MD 2010plus. Recording and processing of chromatographic data was carried out using the Jasco ChromPass version 1.8 LC-Net II /ADC system. The column used was Syncronis aQ $C_{18}$ (5 µm, 250 × 4.6 mm i. d.) from Thermo Fisher Scientific India Ltd, Mumbai.

The liquid chromatography mass spectrometry analysis was carried out on Agilent 6460 LCMS/MS (ESI). The LC system used was Agilent 1260 Infinity Series along with Triple Quadrupole mass analyzer. The system parameters were set to positive polarity mode with capillary voltage of 4000 V. The column used was Poroshell 120 EC-$C_{18}$ (2.7 µm, 50 × 4.6 mm i. d.) manufactured by Agilent Technologies. The data processing was carried out by using Mass Hunter 6.0 software.

The identification of the degradants was carried out by recording its m/z by using TLC-MS hyphenated system. The TLC-MS interface (CAMAG) was used for mass spectrometric analysis of the separated degradants on TLC plates. The elution of the

separated compounds was carried out by using Acetonitrile at flow rate of 0.3 mL $min^{-1}$. The m/z values of eluted compounds were recorded by using Waters® ACQUITY UPLC® SQ (single quadrupole) detector. The ionization of the compound was achieved by ESI technique in positive mode. When source temperature and desolvation temperature were kept at 120 °C and 350 °C, respectively and capillary voltage was 2.21 kV. Desolvation gas flow was maintainted at 5 L/hrs. The mass spectra were recorded from the minimum range of instrument *i. e.* from m/z 250-1000 by using single quadrupole mass analyser. The data acquisition and handling was carried out by MassLynx™ mass spectrometry software.

All chemicals and reagents used in the study were of analytical grade. HPLC analysis was carried out on HPLC grade solvents and reagents. They were purchased from Merck Specialities Private Limited, India. Double distilled water was generated in the lab and filtered through 0.45 µ filter paper and same was used in the research work. pH Meter of HANNA instruments, model-PHEP was used. The ultrasonicator used was Toshcon SW-4.5.

1. **Development and validation of normal phase HPTLC method for simultaneous quantification of Levosulpiride and Esomeprazole in capsule dosage form.**

### 1.1 Optimized Chromatographic conditions

The separation of LSP and ESP was achieved on Merck aluminium plates precoated with silica gel 60 $F_{254}$. The mobile phase was optimized to ethyl acetate: methanol: ammonia (9: 1: 0.5, v/v/v). Chromatographic development was carried out in 20 cm × 10 cm twin trough glass chamber with stainless steel lid previously saturated with mobile phase for 10 min. at room temperature (25 ± 2 $^{o}$C). The solvent front was 80 mm and 20 mL mobile phase was used per development. Plates were dried in the current of air. Densitometric scanning was performed in reflectance-absorbance mode at 216 nm.

### 1.2 Preparation of Standard stock solutions

Standard stock solutions of LSP and ESP were prepared separately by dissolving 10 mg of standard drug in sufficient quantity of methanol in 10 mL volumetric flask and volume was made up to mark with methanol. One mL of the resulting solution was further diluted to 10 mL with methanol to get final concentration of 100 μg $mL^{-1}$.

### 1.3 Preparation of standard solution for recovery study

For recovery study standard solution was prepared by dissolving 15 mg of standard LSP and 8 mg of standard ESP in sufficient quantity of methanol in a 10 mL volumetric flask and finally diluted up to the mark with methanol. The final concentration of LSP and ESP in the solution was 1500 and 800 μg $mL^{-1}$, respectively.

### 1.4 Selection of detection wavelength

After chromatographic development bands were scanned in the range of 200 to 400 nm and spectra were overlain. LSP and ESP showed considerable absorbance at 216 nm and hence was selected for densitometric analysis.

### 1.5 Preparation of sample solution

Content of twenty capsules were weighed accurately; the average weight was calculated and finely powdered. Powder equivalent to 75 mg of LSP and 40 mg of ESP was weighed and transferred into 50 mL volumetric flask containing 30 mL of

methanol. The solution was sonicated for 15 min. and finally diluted up to mark with methanol to obtain the final concentration 1500 and 800 μg $mL^{-1}$ of LSP and ESP, respectively. The solution was filtered through Whatman filter paper no.41 and first few drops of filtrate were discarded.

### 1.6 Method validation

The developed method was validated for linearity, range, precision, accuracy, specificity, LOD and LOQ as per ICH Q2 (R1) guidelines.[25]

#### 1.6.1 Linearity and range

Linearity was evaluated by applying six different concentrations six times to the HPTLC plate in the range of 100 - 1000 ng $band^{-1}$ for both LSP and ESP. Mean of six replicates of area obtained for each peak was recorded. Calibration curve of peak area versus concentration was plotted and data was subjected to least square linear regression analysis and the slope, intercept and correlation coefficient for the calibration curve were estimated.

#### 1.6.2 Sensitivity

Limit of detection (LOD) and limit of quantification (LOQ) were calculated to determine sensitivity as 3.3 σ/S and 10 σ/S, respectively, where σ is the standard deviation of the response (y-intercept) and S is the slope of the linearity plot.

#### 1.6.3 Specificity

In specificity studies, LSP and ESP standard solutions and the marketed sample solutions were applied on a HPTLC plate. The plate was developed in the mobile phase and scanned. The peak purity of LSP and ESP were assessed by comparing the UV spectra of drugs at peak start, peak apex and peak end positions of the band i.e., r (start, middle) and r (middle, end).

#### 1.6.4 Precision

Precision of the method was analyzed by intra and inter-day variation studies. To study intra-day variation, sets of three different drug sample concentrations (400, 600 and 800 ng $band^{-1}$) of LSP and ESP in triplicates were spotted and analyzed on the same day. To study inter-day variation study, triplicates of above mentioned three different drug concentration were analyzed on three consecutive days.

### 1.6.5 Accuracy

The accuracy of the method was evaluated by standard addition method. Samples of LSP and ESP were spiked with 80, 100 and 120 % of standard LSP and ESP.

### 1.6.6 Robustness

Robustness was studied by carrying out small but deliberate changes in the analytical conditions. The analytical conditions varied were mobile phase combination (± 0.1 mL), amount of mobile phase (± 5 %), time from band application to chromatographic development and time from chromatography to scanning (+ 10 min). One factor was varied at a time to study the effect. The robustness of the densitometric method was studied six times at concentration of 600 ng band$^{-1}$ for both LSP and ESP. The standard deviation of peak areas and % relative standard deviation (% RSD) were calculated for each variable factor.

### 1.6.7 Solution stability

Solution stability of LSP and ESP standard solutions (100 ng band$^{-1}$) was studied at an interval of 6 hrs up to 48 hrs. when stored at room temperature and estimated by comparing peak areas at each time interval against freshly prepared standard solution.

## 2 Development and validation of normal phase HPTLC method for simultaneous quantification of Levosulpiride and Rabeprazole in tablet dosage form.

### 2.1 Optimized Chromatographic conditions

The separation of LSP and RBP was achieved on Merck aluminium plates precoated with silica gel 60 $F_{254}$. The mobile phase was optimized to ethyl acetate: methanol: ammonia (8.5:1.5:0.2, v/v/v). Chromatographic development was carried out in 20 cm × 10 cm twin trough glass chamber with stainless steel lid previously saturated with mobile phase for 10 min. at room temperature (25 ± 2 $^{o}$C). The solvent front was 80 mm and 20 mL mobile phase was used per development. Plates were dried in the current of air. Densitometric scanning was performed in reflectance-absorbance mode at 287 nm.

### 2.2 Preparation of standard solution for linearity

A standard stock solution of LSP and RBP was prepared separately by dissolving 10 mg of standard drug in 10 mL methanol and 1 mL of the resulting solution was further diluted to 10 mL with methanol to get final concentration of 100 μg $mL^{-1}$.

### 2.3 Preparation of standard solution for recovery study

Standard solution was prepared separately by dissolving 10 mg of standard RBP and 20 mg of standard LSP in methanol in a 50 mL volumetric flask and finally diluted up to the mark with methanol. The final concentration of LSP and RBP in the solution was 400 and 200 μg $mL^{-1}$, respectively.

### 2.4 Preparation of sample solution for recovery of LSP

Average weight of twenty tablets was calculated and tablets were finely powdered. Powder equivalent to 20 mg of LSP was weighed and transferred into 50 mL volumetric flask containing approximately 30 mL of methanol. Solution was sonicated for 15 min and diluted up to mark with methanol to obtain the final concentration 400 μg $mL^{-1}$ of LSP. The solution was filtered through Whatman filter paper no.41 and resulting solution was used for the study.

### 2.5 Preparation of sample solution for recovery of RBP

Average weight of twenty tablets was calculated and tablets were finely powdered. Powder equivalent to 10 mg of RBP was weighed and transferred into 50 mL

volumetric flask containing approximately 30 mL of methanol. Solution was sonicated for 15 min and diluted up to mark with methanol to obtain the final concentration 200 μg $mL^{-1}$ of RBP. The solution was filtered through Whatman filter paper no.41 and resulting solution was used for the study.

### 2.6 Method validation

The developed method was validated for linearity, range, precision, accuracy, specificity, LOD and LOQ as per ICH Q2 (R1) guidelines.[25]

#### 2.6.1 Linearity and range

Linearity was evaluated by applying different concentrations six times each to the HPTLC plate in the range of 100 - 1200 ng $band^{-1}$ for both LSP and RBP. Calibration curve of peak area versus concentration was plotted and data was subjected to statistical analysis such as residual analysis least square linear regression analysis and the slope, intercept and correlation coefficient for the calibration curve were estimated.

#### 2.6.2 Sensitivity

Limit of detection (LOD) and limit of quantification (LOQ) were calculated to determine sensitivity as 3.3 σ/S and 10 σ/S, respectively, where σ is the standard deviation of the response (y-intercept) and S is the slope of the linearity plot.

#### 2.6.3 Specificity

In specificity studies, LSP and RBP standard solutions and the marketed sample solutions were applied on a HPTLC plate. The plate was developed in the mobile phase and scanned as mentioned above. The peak purity of LSP and RBP was assessed by comparing the UV spectra of drugs at peak start, peak apex and peak end positions of the band i.e., r (start, middle) and r (middle, end).

#### 2.6.4 Precision

Precision of the method was analyzed by intra and inter-day variation studies. To study intra-day variation sets of three different drug sample concentration of LSP and RBP in triplicates (400, 600 and 800 ng $band^{-1}$) were spotted and analyzed on the same day. To study inter-day precision study, triplicates of above mentioned three different drug concentrations were analyzed on three successive days.

### 2.6.5 Accuracy

The accuracy of the method was evaluated by standard addition method. Samples of LSP and RBP were spiked with 80, 100 and 120 % of standard LSP and RBP.

### 2.6.6 Robustness

Robustness was studied by carrying out small, deliberate changes in the analytical conditions. The analytical conditions varied were mobile phase combination (± 0.1 mL), amount of mobile phase (± 5 %), time from band application to chromatographic development and time from chromatography to scanning (+ 10 min). One factor was varied at a time to study the variation. The robustness of the proposed HPTLC method was studied six times at concentration of 600 ng band$^{-1}$ for both LSP and RBP as it lies within the range of linearity. The standard deviation of peak areas and % relative standard deviation (% RSD) were calculated for each variable factor.

### 2.6.7 Solution stability

Solution stability of LSP and RBP standard solutions (200 ng band$^{-1}$) was studied at an interval of 6 hrs. up to 48 hrs. when stored at room temperature and estimated by comparing peak areas at each time interval against freshly prepared standard solution.

## 3 Development and validation of normal phase HPTLC method for simultaneous quantification of Mometasone furoate and Terbinafine hydrochloride in cream dosage form.

### 3.1 Optimized Chromatographic conditions

Chromatographic development of standard and samples were carried out on Merck aluminium plates precoated with silica gel 60 F254 as the stationary phase. Separation of MTS and TBF was achieved by using n-hexane: ethyl acetate: acetic acid (7.5:3:0.5 v/v/v). Chromatographic development was carried out in 20 cm × 10 cm twin trough glass chamber with stainless steel lid previously saturated with mobile phase for 20 min. at room temperature (25 ± 2 $^{o}$C). The solvent front was 80 mm and 20 mL mobile phase was used per development. UV wavelength selected for the detection was 258 nm in reflectance/absorbance mode. The retardation factors were found to be 0.27 ± 0.02 and 0.74 ± 0.02 for MTS and TBF, respectively.

### 3.2 Preparation of Standard stock solutions

Stock solution of MTS was prepared by dissolving 10 mg of standard drug in sufficient quantity of methanol in 10 mL volumetric flask. Final volume was made up to mark with methanol and 1 mL of the resulting solution was diluted to 10 mL with methanol to get the final concentration of 100 µg mL$^{-1}$.

Stock solution of TBF was prepared by dissolving 10 mg of standard drug in sufficient quantity of methanol in 10 mL volumetric flask. Final volume was made up to mark with methanol to get the final concentration of 1000 µg mL$^{-1}$.

### 3.3 Preparation of sample solution for recovery study and assay

The dosage form used for the analysis was cream. Sample solution was prepared by following liquid-liquid extraction procedure. To determine the percentage of MTS and TBF in commercial dosage form, sample solution was prepared by dissolving cream equivalent to 2.5 mg of TBF and 0.25 mg of MTS in 20 mL petroleum ether in a separating funnel. The petroleum ether layer was extracted with methanol [2 × 5 mL]. Methanol layer was transferred to 25 mL volumetric flask. Petroleum ether layer was again washed with 5 mL portion of methanol and this washing was also added to the 25 mL volumetric flask. Finally volume was made up to the mark with methanol. The final concentration of TBF in sample solution was 100 µg mL$^{-}$

[1] and that of MTS was 10 µg $mL^{-1}$. This solution was used for the recovery study and assay. Chromatogram of petroleum ether layer sample was run and scanned to ensure complete extraction of MTS and TBF from the organic layer. It showed complete absence of the drugs. (Data not shown).

### 3.4 Selection of detection wavelength

The ratio of MTS and TBF in the formulation is 1:10; so considering the difference in the ratio the wavelength with maximum absorbance for MTS was selected. Wavelength selected for the analysis was 258 nm which has given maximum absorbance for the MTS.

### 3.5 Method validation

The developed method was validated for linearity, range, precision, accuracy, specificity, LOD and LOQ as per ICH Q2 (R1) guidelines.[25]

#### 3.5.1 Linearity and range

Linearity of MTS and TBF was evaluated by applying nine different concentrations six times to the HPTLC plate in the range of 50 to 450 ng $band^{-1}$ and 400 to 3600 ng $band^{-1}$, respectively. The final concentration of MTS was 100 µg $mL^{-1}$. From this nine different volumes (0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 µL) of stock solution were applied on the TLC plate to get the range of 50 – 450 ng $band^{-1}$. Final concentraion of TBF was 1000 µg $mL^{-1}$. From this nine different volumes of (0.4, 0.8, 1.2, 1.6, 2, 2.4, 2.8, 3.2, 3.6 µL) of stock solution were applied on the plate to get the range of 400 – 3600 ng $band^{-1}$. Mean of six replicates of area obtained for each peak was recorded. Calibration curve of peak area versus concentration was plotted and data was subjected to least square linear regression analysis and the slope, intercept and correlation coefficient for the calibration curve were estimated.

#### 3.5.2 Sensitivity

Limit of detection (LOD) and limit of quantification (LOQ) were calculated to determine sensitivity as 3.3 σ/S and 10 σ/S, respectively, where σ is the standard deviation of the response (y-intercept) and S is the slope of the linearity plot.

#### 3.5.3 Specificity

In specificity studies, MTS and TBF standard solutions and the marketed sample solutions were applied on a HPTLC plate. The plate was developed in the mobile

phase and scanned. The peak purity of MTS and TBF were assessed by comparing the UV spectra of drugs at peak start, peak apex and peak end positions of the band i.e., r (start, middle) and r (middle, end).

### 3.5.4 Precision

Precision of the method was analyzed by intra and inter-day variation studies. Each level of precision was investigated by three sequential replicates of injections of MTS and TBF. Precision of MTS was studied at 100, 200 and 300 ng band$^{-1}$ and that of TBF was studied at 1200, 2000 and 2800 ng band$^{-1}$.

### 3.5.5 Accuracy

The accuracy of the method was evaluated by standard addition method. Samples of MTS and TBF were spiked with 80, 100 and 120 % of standard MTS and TBF.

### 3.5.6 Robustness

Robustness was studied by carrying out small but deliberate changes in the analytical conditions. The analytical conditions varied were mobile phase combination (± 0.1 mL), amount of mobile phase (± 5 %), time from band application to chromatographic development and time from chromatography to scanning (+ 10 min). One factor was varied at a time to study the effect. The study was repeated six times at concentration of 200 ng band$^{-1}$ and 2000 ng band$^{-1}$ for MTS and TBF, respectively. The standard deviation of peak areas and % relative standard deviation (% RSD) were calculated for each variable factor.

### 3.5.7 Solution stability

Solution stability of the standard solution of MTS and TBF was analyzed by spotting 200 and 2000 ng band$^{-1}$, respectively from 6hrs to 48hrs. The areas were measured and compared with the areas of the spots applied from freshly prepared standard solutions after chromatographic development with optimized conditions.

## 4 Development and validation of reverse phase HPLC-DAD method for simultaneous quantification of Mometasone furoate and Terbinafine hydrochloride in cream dosage form.

### 4.1 Optimized Chromatographic conditions

A method was developed to resolve the MTS and TBF. The developed method was validated as per ICH guidelines and results were found to be within the limits. The successful separation was achieved on Syncronis aQ $C_{18}$ (5 µm, 250 × 4.6 mm i. d.) column with mobile phase of methanol: water (90:10) at a flow rate of 1 mL $min^{-1}$. The method was developed by using HPLC-PDA system. The chromatogram was recorded at 237 nm. For quantification detector response was recorded at 258 nm for MTS and at 224 nm for TBF.

### 4.2 Preparation of Standard stock solutions

Stock solution of MTS and TBF were prepared separately by dissolving 10 mg of standard drug in sufficient quantity of methanol in 10 mL volumetric flask. Final volume was made up to mark with methanol and 1 mL of the resulting solution was diluted to 10 mL with methanol to get the final concentration of 100 µg $mL^{-1}$. The solution was later serially diluted to get the solutions in having concentrations of 0.5, 1, 5, 10, 20, 40, 60, 80 and 100 µg $mL^{-1}$.

### 4.3 Preparation of sample solution for recovery study and assay

The dosage form used for the analysis was cream. Sample solution was prepared by following liquid-liquid extraction procedure. To determine the percentage of MTS and TBF in commercial dosage form, sample solution was prepared by dissolving cream equivalent to 2.5 mg of TBF and 0.25 mg of MTS in 20 mL petroleum ether in a separating funnel. The petroleum ether layer was extracted with methanol [2 × 5 mL]. Methanol layer was transferred to 25 mL volumetric flask. Petroleum ether layer was again washed with 5 mL portion of methanol and this washing was also added to the 25 mL volumetric flask. Finally volume was made up to the mark with methanol. The concentration of TBF in sample solution was 100 µg $mL^{-1}$and that of MTS was 10 µg $mL^{-1}$. The solution was marked as (A). Solution (A) was used for the recovery study of MTS. From the resulting solution 2 mL was again transferred to 10 mL volumetric flask and volume was made up to mark with methanol. The final concentration of TBF in the solution was 20 µg $mL^{-1}$ and that of MTS was 0.2 µg $mL^{-}$

[1]. This solution was labeled as (B). This solution (B) was used for the recovery study of TBF. The solution (A) was again diluted to 1:2 proportion to get the final concentration of 50 µg $mL^{-1}$ of TBF and 5 µg $mL^{-1}$ of MTS and marked as (C). The solution (C) was used for assay.

### 4.4 Selection of detection wavelength

The wavelength selected for quantification was the maximum wavelength of absorption for the selected drugs. Quantification of MTS was carried out at 258 nm and that of TBF was carried out at 224 nm.

### 4.5 Method validation

The developed method was validated for linearity, range, precision, accuracy, specificity, LOD and LOQ as per ICH Q2 (R1) guidelines.[25]

#### 4.5.1 Linearity and range

Linearity of MTS and TBF was evaluated by injecting nine different concentrations six times to the HPLC column in the range of 0.5 to 100 µg $mL^{-1}$. Mean of six replicates of area obtained for each peak was recorded. Calibration curve of peak area versus concentration was plotted and data was subjected to least square linear regression analysis and the slope, intercept and correlation coefficient for the calibration curve were estimated.

#### 4.5.2 Sensitivity

Limit of detection (LOD) and limit of quantification (LOQ) were calculated to determine sensitivity as 3.3 σ/S and 10 σ/S, respectively, where σ is the standard deviation of the response (y-intercept) and S is the slope of the linearity plot.

#### 4.5.3 Specificity

The peak purity data was obtained from the system. In specificity studies, MTS and TBF standard solutions and the marketed sample solutions were injected in HPLC column. At the time of elution of peaks UV spectrum of the peaks were recorded, compared and peak purity data was generated.

#### 4.5.4 Precision

Precision of the method was analyzed by intra and inter-day variation studies. Each level of precision was investigated by three sequential replicates of injections of MTS

and TBF. Precision of MTS was studied at 10, 20 and 40 µg $mL^{-1}$ and that of TBF was studied at 20, 40 and 60 µg $mL^{-1}$.

### 4.5.5 Accuracy

The accuracy of the method was evaluated by standard addition method. Samples of MTS and TBF were spiked with 80, 100 and 120 % of standard MTS and TBF. The concentration selected for accuracy study was 10 µg $mL^{-1}$ for MTS and 20 µg $mL^{-1}$ for the TBF.

### 4.5.6 Robustness

Robustness was studied by carrying out small but deliberate changes in the analytical conditions. The analytical conditions varied were mobile phase combination (± 1 %), elution flow rate (± 0.1 mL $min^{-1}$) and detection wavelength (± 2 nm). One factor was varied at a time to study the effect. The study was repeated three times at concentration of 20 µg $mL^{-1}$ and 40 µg $mL^{-1}$ for MTS and TBF, respectively. The standard deviation of peak areas and % relative standard deviation (% RSD) were calculated for each variable factor.

### 4.5.7 System suitability parameters

System suitability parameters were studied to measure the system performance. The parameters studied were retention time, resolution, asymmetry, theoretical plates.

### 4.5.8 Solution stability

Solution stability of the standard solution of MTS and TBF was analyzed by spotting 20 and 40 µg $mL^{-1}$, respectively from 6hrs to 48 hrs. The areas were measured and compared with the areas of the spots applied from freshly prepared standard solutions after chromatographic development with optimized conditions.

# 5 Development of stability indicating high performance thin layer chromatographic method for quantitation of Levosulpiride in pharmaceutical dosage form and application of TLC-MS for identification of degradation product.

## 5.1 Optimized Chromatographic conditions

The method was developed by using aluminium plates precoated with silica gel 60 $F_{254}$ as stationary phase. LSP was subjected to forced degradation under acidic, alkaline, oxidative and photolytic conditions. The mobile phase optimized for better resolution of LSP along with its degradation products was ethyl acetate: methanol: ammonia (7: 3: 1 v/v/v). Chromatographic development of plates was carried out in 20 cm × 10 cm twin trough glass chamber previously saturated with 20 mL of mobile phase for 10 min (optimized chamber saturation time) at room temperature (25 ± 2 $^{o}$C) by linear ascending method. The solvent front was 80 mm. Plates were dried in the current of air. The selected mobile phase resolved the degradation products peaks from LSP and gave significant difference in the Rf values. Rf value for standard LSP was found to be 0.44 ± 0.02. The densitometric analysis of separated bands was carried out in reflectance - absorbance mode at 216 nm.

## 5.2 Preparation of stock solution

A stock solution of LSP was prepared by dissolving 25 mg of standard LSP in sufficient quantity of methanol in 25 mL volumetric flask and final volume was made up to mark with methanol. The final concentration of the stock solution was 1000 µg mL$^{-1}$. From the resulting solution 1 mL was further diluted to 10 mL with methanol to get final concentration of 100 µg mL-1.

## 5.3 Analysis of marketed formulation

Two different formulations were studied. Formulation A (Tablet Lesuride) was a tablet dosage form labeled to contain 25 mg of LSP per tablet and formulation B (Lesuride Injection) was an injection labeled to contain 12.5 mg mL$^{-1}$ of LSP.

Formulation A: Twenty tablets were weighed accurately; the average weight was calculated and tablets were finely powdered. Powder equivalent to 25 mg of LSP was weighed and transferred into 10 mL volumetric flask, sonicated for 15 min. and diluted up to mark with methanol. The solution was filtered through Whatman filter paper no.41 and first few drops of filtrate were discarded. From the resulting solution

1 mL was further diluted to 10 mL with methanol to obtain the final concentration 250 µg $mL^{-1}$ of LSP. This solution was used further for the recovery study and assay of formulation.

Formulation B: Contents of ampule was transferred to a 10 mL volumetric flask and volume was made up to mark with methanol. From the resulting solution 1 mL was further diluted to 10 mL with methanol to give final concentration of 250 µg $mL^{-1}$. This solution was further used for assay study.

## 5.4 Forced Degradation studies[33, 37]

### *5.4.1* Preparation of acid induced degradation product

Forced degradation study was first carried out in 0.1 N HCl at 80 °C for 8 hrs but no degradation was observed so degradation study was carried out by using 1 N HCl. It showed considerable degradation. Forced degradation study in acidic condition was carried out by refluxing 20 mg of standard LSP with 20 mL of 1 N HCl at 80 °C for 8 hrs. From refluxed solution 1 mL was neutralized with 1 mL of 1 N NaOH and volume was made up to 10 mL with methanol to get the final concentration of 100 µg $mL^{-1}$. The resulting solution was used for the spotting on aluminium plates.

### 5.4.2 Preparation of base induced degradation product

Forced degradation study was first carried out in 0.1 N NaOH at 80 °C for 8 hrs but no degradation was observed so degradation study was carried out by using 1 N NaOH. It showed considerable degradation. Forced degradation study in basic condition was carried out by refluxing 20 mg of standard LSP with 20 mL of 1 N NaOH at 80 °C for 8 hrs. From refluxed solution 1 mL was neutralized with 1 N HCl and volume was made up to 10 mL with methanol to get the final concentration of 100 µg $mL^{-1}$. The resulting solution was used for the spotting on aluminium plates.

### 5.4.3 Preparation of oxidative degradation product

Forced degradation study in oxidative condition was carried out by hydrogen peroxide (30% w/v) at room temperature for 24 hrs. Standard LSP was dissolved in sufficient quantity of methanol and volume was made up with 30 % hydrogen peroxide to achieve concentration of 1mg $mL^{-1}$. Samples were withdrawn at an interval of 6 hrs till 24 hrs and boiled on a water bath for complete removal of oxygen. For

chromatographic development the resulting solution was applied to achieve concentration of 1000 ng band$^{-1}$.

Standardization of hydrogen peroxide solution: Ten milliliter of hydrogen peroxide solution was transferred to the 100 mL volumetric flask and volume was made up to the mark with distilled water. Twentry milliliter of the resulting solution was titrated against 0.02M $KMnO_4$ in the cold after adding sulphuric acid (50% v/v, 5 mL).[95]

### *5.4.4* Preparation of photochemical degradation product

Photochemical degradation was also induced by keeping stock solution and standard LSP in stability chamber as well as in direct sunlight on terrace on a wooden plank (overall illumination of 1.2 million lux hr and an integrated near UV energy of not less than 200 Whm$^{-2}$) for 7 days. The standard LSP sample was further diluted to get a final concentration of 100 µg mL$^{-1}$. The resultant solutions were applied on HPTLC plate and the concentration achieved was 1000 ng band$^{-1}$.

### 5.4.5 Thermal degradation

For dry heat stress test, standard was kept in a petri plate in an oven at 60 °C for five days and sampling was carried out at first, third and fifth day. The collected samples were dissolved in methanol and further diluted to get the concentration of 100 µg mL$^{-1}$. Samples were analysed with the developed method for presence of degradation product.

For wet heat stress test, standard was kept in petri plate in a humidity chamber at 50 °C up to three months. Sampling was carried out in every after 15 days. The collected samples were dissolved in methanol and further diluted to get the concentration of 100 µg mL$^{-1}$. Samples were analyzed with the developed method for presence of degradation product.

### 5.4.6 Neutral hydrolysis

For neutral hydrolysis the 20 mg of standard LSP was weighed and mixed with 20 mL of water in a 50 mL round bottom flask and refluxed for five days. The sampling was carried out at first, third and fifth day. The samples were further diluted with methanol to get the final concentration of 100 µg mL$^{-1}$.

### 5.5 Preparation of standard solution for linearity

A standard stock solution of LSP was prepared by dissolving 10 mg of standard drug in sufficient quantity of methanol in a 10 mL volumetric flask and volume was made up to the mark with methanol to give a concentration of 1000 μg $mL^{-1}$. From the resulting solution 1 mL was further diluted to 10 mL with methanol to get final concentration of 100 μg $mL^{-1}$.

### 5.6 Preparation of standard solution for recovery study

Standard solution was prepared by dissolving 25 mg of standard LSP in methanol in a 10 mL volumetric flask and diluted up to the mark with methanol. From the resulting solution 1mL was further diluted to 10 mL with methanol. The final concentration of LSP in the solution was 250 μg $mL^{-1}$.

### 5.7 Selection of detection wavelength

After chromatographic development bands were visualized in both UV and visible region. It showed no spot in the visible region. So bands for standard LSP and degradation product was scanned in the range of 200 to 400 and spectra were overlain. LSP and degradation product showed considerable absorbance at 216 nm and hence was selected for densitometric analysis.

### 5.8 Preparation of sample solution

Formulation 1: Twenty tablets were weighed accurately; the average weight was calculated and finely powdered. Powder equivalent to 25 mg of LSP was weighed and transferred into 10 mL volumetric flask, sonicated for 15 min. and diluted up to mark with methanol. The solution was filtered through Whatman filter paper no.41 and first few drops of filtrate were discarded. From the resulting solution 1 mL was further diluted to 10 mL with methanol to obtain the final concentration 250 μg $mL^{-1}$ of LSP. This solution was used further for the recovery study and assay of formulation.

Formulation 2: Contents of ampule was transferred to a 10 mL volumetric flask and volume was made up to mark with methanol. From the resulting solution 1 mL was further diluted to 10 mL with methanol to give final concentration of 250 μg $mL^{-1}$. This solution was further used for recovery and assay study.

## 5.9 Method validation

The developed method was validated for linearity, range, precision, accuracy, specificity, LOD and LOQ as per ICH Q2 (R1) guidelines.[25]

### 5.9.1 Linearity and range

Six different volumes (0.1, 0.2, 0.4, 0.6, 0.8 and 1 µL $spot^{-1}$) of standard LSP having concentration of 1000 µg $mL^{-1}$ were applied on HPTLC plate to study the linearity in the range of 100 to 1000 ng $band^{-1}$. After developing the plate peaks were scanned and area was plotted against concentration. The data obtained was statistically analyzed for least square regression analysis and slope, intercept and correlation coefficient for the curve was calculated. Data was also studied for its residual analysis and checked for any tendency in the data.

### 5.9.2 Sensitivity

Sensitivity of the method was studied in terms of detection and quantitation limits. Limit of detection (LOD) and limit of quantification (LOQ) were calculated by 3.3 σ/S and 10 σ/S, respectively, where σ is the standard deviation of the response (y-intercept) and S is the slope of the linearity plot.

### 5.9.3 Specificity

Specificity of the LSP was studied in terms of peak purity. The standard and sample solutions were applied on HPTLC plate, after development in mobile phase plate was scanned. Peak purity was assessed by comparing the UV spectra of drug at peak start, peak apex and peak end positions of the band i. e., r (start, middle) and r (middle, end). Along with this extract of commonly used tablet excipients was also spotted and its interference with peaks of standard and degradation product was observed.

### 5.9.4 Precision

Precision of the method was studied by intra and inter-day variation. Sets of three different drug concentrations of LSP in triplicates (400, 600 and 800 ng $band^{-1}$) were spotted and analyzed on the same day to study intra-day variation. Same concentrations were analyzed on three consecutive days to study inter day precision.

### 5.9.5 Accuracy

The accuracy was evaluated by standard addition method. Samples solutions of LSP were spiked with 80, 100 and 120 % of standard LSP. Concentration taken for the study of recovery was 250 ng band$^{-1}$.

### 5.9.6 Robustness

Robustness was studied by carrying out small but deliberate changes in the analytical conditions. The analytical conditions varied were mobile phase composition (± 0.1 mL), amount of mobile phase (± 5 %), time from band application to chromatographic development and time from chromatography to scanning (+ 10 min). One factor was varied at a time to study the effect. The robustness of the densitometric method was studied six times at concentration of 500 ng band$^{-1}$ for LSP. The standard deviation of peak areas and % relative standard deviation (% RSD) were calculated for each variable factor.

### 5.9.7 Solution stability

Stability of standard solution of LSP (400 ng band$^{-1}$) at room temperature was studied at an interval of 6 hrs up to 48 hrs. The peak area was compared each time with freshly prepared standard solution.

## 6 Development of stability indicating high performance liquid chromatographic method for quantitation of Levosulpiride in pharmaceutical dosage form and application of LC-MS/MS for identification of degradation product.

### 6.1 Optimized chromatographic method

A method was developed to resolve the standard LSP from its degradants. The developed method was validated as per ICH guidelines and results were found to be within the limits. The successful separation was achieved on Syncronis aQ $C_{18}$ (5 µm, 250mm × 4.6 mm i. d.) column with mobile phase of methanol: water (20:80) at a flow rate of 0.7 mL $min^{-1}$. The response was recorded at 216 nm. The developed method was used for LCMS/MS analyses of the degraded sample on Agilent 1260 Infinity LC model along with an Agilent Triple Quad LC/MS 6460 mass spectrometer from Agilent Technologies.

### 6.2 Optimized method for LC-MS/MS analysis of the degradation products

The mobile phase developed for the resolution of degradation product and standard LSP was compatible for LC-MS/MS analysis and was used without further modification. The m/z values for the degradation products were obtained from LC-MS/MS analysis. The MS data was studied and possible structures for degradation products with possible degradation pathways are proposed.

### 6.3 Preparation of stock solution

A stock solution of LSP was prepared by dissolving 25 mg of standard LSP in sufficient quantity of methanol in 25 mL volumetric flask and final volume was made up to mark with methanol. The final concentration of the stock solution was 1000 µg $mL^{-1}$.

### 6.4 Analysis of marketed formulation

Two different formulations were studied. Formulation A (Tablet Lesuride) was a tablet dosage form labeled to contain 25 mg of LSP per tablet and formulation B (Lesuride Injection) was an injection labeled to contain 12.5 mg $mL^{-1}$ of LSP.

Formulation A: Average weight of twenty tablets was calculated and tablets were finely powdered. Powder equivalent to 25 mg of LSP was weighed and transferred to 10 mL volumetric flask, sonicated to 15 min and diluted up to mark with methanol.

The solution was later filtered through Whatmann filter paper no 41 and first few drops of filtrate were discarded. From the resulting solution 1 mL was further diluted to 10 mL with methanol to obtain the concentration of 250 µg $mL^{-1}$ of LSP. Form the resulting solution 1 mL was again diluted to 1 mL to get final concentration of 25 µg $mL^{-1}$. This solution was used for the assay and recovery studies.

Formulation B: Content of ampule was transferred to a 10 mL volumetric flask and volume was made up to mark with methanol. From the resulting solution 1 mL was further diluted to 10 mL with methanol to get the solution of having concentration of 250 µg $mL^{-1}$. From the resulting solution 1 mL was again diluted to 10 mL to get the final concentration of 25 µg $mL^{-1}$ of LSP. This solution was further used for assay study.

## 6.5 Forced degradation studies[33, 37]

### 6.5.1 Acid induced degradation

Degradation behavior under acidic condition was studied by force degradation of LSP in 1N HCl. Study was initiated by refluxing LSP with 0.1 N HCl at 80 °C for 8 hrs but no degradation was observed. Samples were withdrawn after 60 min and neutralized with 0.1 N NaOH. Later it was diluted with methanol and analyzed with the developed method. The samples showed no degradation hence strength of acid and base was increased to 1N.

Acid degradation study was carried out by refluxing 20 mg of standard LSP with 20 mL of 1N HCl for 8 hrs at 80 °C. After each of 60 mins, 1mL of refluxed solution was withdrawn and neutralized with 1 mL of 1 N NaOH and volume was made up to 10 mL with methanol. The final concentration of the solution was 100 µg $mL^{-1}$. This sample was further used for analysis with developed method.

### 6.5.2 Base induced degradation

Degradation behavior under basic condition was studied by force degradation of LSP in 1N NaOH. Study was initiated by refluxing LSP with 0.1 N NaOH at 80 °C for 8 hrs. Samples were withdrawn after every 60 min and neutralized with 0.1 N HCl. Later is was diluted with methanol and analyzed with the developed method. The samples showed no degradation hence strength of acid and base was increased to 1N.

Base degradation study was carried out by refluxing 20 mg of standard LSP with 20 mL of 1N NaOH for 8 hrs at 80 °C. After each of 60 mins, 1mL of refluxed solution

was withdrawn and neutralized with 1 mL of 1 N HCl and volume was made up to 10 mL with methanol. The final concentration of the solution was 100 µg $mL^{-1}$. This sample was further used for analysis with developed method.

### 6.5.3 Hydrogen peroxide induced degradation

Oxidative degradation study was carried out by exposing standard LSP to hydrogen peroxide solution at room temperature. Initially degradation was attempted with 6 % hydrogen peroxide but sufficient degradation was not observed hence 30 % hydrogen peroxide was used for the study. Standard LSP was dissolved in sufficient quantity of methanol and volume was made up to 10 mL with 30 % hydrogen peroxide to achieve concentration of 1mg $mL^{-1}$. After getting sufficient degradation whole content was boiled to remove the excess hydrogen peroxide and 1 mL of the solution was further diluted with methanol to get the final concentration of 100 µg $mL^{-1}$.

Standardization of hydrogen peroxide solution: Ten milliliter of hydrogen peroxide solution was transferred to the 100 mL volumetric flask and volume was made up to the mark with distilled water. Twentry milliliter of the resulting solution was titrated against 0.02M $KMnO_4$ in the cold after adding sulphuric acid (50% v/v, 5 mL).[95]

### 6.5.4 Photochemical degradation

Photochemical degradation was studied by exposing the standard LSP and stock solution to direct sunlight kept on a wooden plant for 7 days. Photochemical degradation was also studied by keeping standard LSP in photo stability chamber for 7 days. (Overall illumination of 1.2 million lux hr and an integrated near UV energy of not less than 200 $Whm^{-2}$). After seven days the solutions as well as standard LSP was further diluted to get the concentration of 100 µg $mL^{-1.}$

### 6.5.5 Dry heat degradation

For dry heat stress test, standard was kept in a petri plate in an oven at 60 °C for five days and sampling was carried out at first, third and fifth day. The collected samples were dissolved in methanol and further diluted to get the concentration of 100 µg $mL^{-1}$. Samples were analyzed with the developed method for presence of degradation product.

For wet heat stress test, standard was kept in petri plate in a humidity chamber at 50 °C up to three months. Sampling was carried out in every after 15 days. The

collected samples were dissolved in methanol and further diluted to get the concentration of 100 µg $mL^{-1}$. Samples were analyzed with the developed method for presence of degradation product.

### 6.5.6 Neutral hydrolysis

For neutral hydrolysis the 20 mg of standard LSP was weighed and mixed with 20 mL of water in a 50 mL round bottom flask and refluxed for five days. The sampling was carried out at first, third and fifth day. The samples were further diluted with methanol to get the final concentration of 100 µg $mL^{-1}$.

## 6.6 Validation of method

Validation of the developed method was carried out as per ICH (Q2) R1 guidelines.[25] The following parameters were studied separately to validate the method.

### 6.6.1 Linearity and range

Linearity of the method was studied by preparing six different concentrations in the range of 10 to 100 µg $mL^{-1}$ separately. All these solutions were injected in the column to observe the detector response. A plot of concentration versus detector response was plotted to get the calibration curve.

### 6.6.2 Precision

Repeatability and intermediate precision were studied by selecting three different concentrations from the linearity range. The concentrations selected were 10, 40 and 80 µg $mL^{-1}$. Three freshly prepared replicates of each concentration were studied for repeatability. The same procedure was followed on three different days to study the intermediate precision.

### 6.6.3 Limit of detection and limit of quantitation.

Detection limit and quantitation limit was calculated by 3.3 σ/S and 10 σ/S, respectively, where σ is the standard deviation of the response (y-intercept) and S is the slope of the linearity plot.

### 6.6.4 Robustness of method

Robustness of the developed method was studied by observing the change in the detector response and retention time by small but deliberate changes in the parameters of the methods. Different parameters of the method like flow rate, mobile phase

composition and wavelength were changed to study the robustness of the method. Freshly prepared standard stock solution was used each time and one parameter was changed at a time. Concentration taken for the analysis was 50 µg $mL^{-1}$.

### 6.6.5 Specificity

The specificity of the method for LSP was established by studying the resolution factor of the drug from the nearest resolved peak. It is also established checking interference of the peaks due to excipients. Extract of commonly used excipients was injected to establish the specificity of the method.

### 6.6.6 Accuracy

Standard addition method was used to study the accuracy. The sample solutions of LSP were spiked with the 80, 100 and 120 % standard LSP. The concentration selected for the study was 25 µg $mL^{-1}$.

### 6.6.7 System suitability

System suitability parameters like retention time, theoretical plates, asymmetry and resolution of the developed method were studied to confirm the efficiency of the developed method for the quantitation of LSP.

### 6.6.8 Solution stability

Stability of the LSP in methanol at room temperature was studied from 0 to 48 hrs. Each time area was compared with freshly prepared standard LSP solution.

# *Summary and Conclusion*

## Summary and Conclusion

Productivity of the method and processes used; leads to success of the commercial accomplishments. In the era of emerging economics of the pharmaceuticals, productivity depends on many factors including the time of analysis. The analytical chemist possess important role in the discovery, development and quality establishment of the formulations. A well-developed method, with adjectives like precise, robust and simple analyst can perform rapid analysis of the selected drugs and combinations with accurate results.

The thesis includes four simultaneous estimation methods and two stability indicating methods. The developed method can be utilized for quality control and routine analysis of the combinations.

The first densitometric method was about simultaneous estimation of Levosulpiride and Esomeprazole in capsule dosage forms. The developed method was validated as per ICH Q2 (R1) guideline. The results obtained were within limits as per guidelines. The separation of standard LSP and ESP was achieved on Merck aluminum plates precoated with silica gel 60 $F_{254}$, using ethyl acetate: methanol: ammonia (9: 1: 0.5, v/v/v). Both the drugs were UV active showed considerable absorbance at 216 nm; so the wavelength selected for the densitometric analysis. The retardation factor (*Rf*) for LSP and ESP were found to be 0.30 ± 0.02 and 0.64 ± 0.02, respectively. All parameters for validation were studied. Both the drugs found to be linear over the range of 100 to 1000 ng band$^{-1}$ with LOD and LOQ of 31.363 and 95.042 ng band$^{-1}$ and 30.631 and 92.822 ng band$^{-1}$ for LSP and ESP, respectively. The developed method is successfully applied to analysis marketed formulations available and found to give recovery in acceptable range.

The second densitometric method was about simultaneous estimation of Levosulpiride and Rabeprazole in tablet dosage forms. The developed method is validated as per ICH Q2 (R1) guideline for all mentioned parameters and results found to be within limits. The separation was carried out on Merck aluminum plates precoated with silica gel 60 $F_{254}$, using mobile phase as ethyl acetate: methanol: ammonia (8.5: 1.5: 0.2, v/v/v). The retardation factors (R*f*) for LSP and RBP were found to be 0.25 ± 0.02 and 0.54 ± 0.02, respectively. The densitometric scanning was carried out at 287 nm. Both the drugs showed considerable absorbance at this

wavelength. Standard drugs concentration and peak areas were found to be linear in the range of 100 - 1000 ng band$^{-1}$ for LSP and 100 - 800 ng band$^{-1}$ for RBP. The LOD and LOQ for LSP were found to be 32.29 and 97.86 ng band$^{-1}$, respectively and for RBP it was found to be 29.71 and 90.03 ng band$^{-1}$, respectively. The developed method was sucessfully applied to marketed formulation.

The third densitometric method was about simultaneous estimation of Mometasone furoate and Terbinafine hydrochloride in cream dosage form. The separation was carried out on Merck aluminum plates precoated with silica gel 60 $F_{254}$ as stationary phase and mobile phase optimized as n-hexane: ethyl acetate: acetic acid (7.5:3:0.5, v/v/v). Wavelength selected for the analysis was 258 nm. The retardation factor for MTS and TBF was found to be 0.27±0.02 and 0.74±0.02, respectively. The developed method was validated as per ICH Q2 (R1) guideline and results were within the range. The linearity range for MTS was found to 50-450 ng band$^{-1}$ and for TBF it was found to be 400-3600 ng band$^{-1}$. The detection limit for MTS and TBF was found to be 11.34 and 125.78 ng band$^{-1}$ respectively. Quantitation limit for MTS and TBF was found to be 34.38 and 381.15 ng band$^{-1}$, respectively. The developed method was applied to marketed formulation and gave recovery in acceptable range. The marketed formulation selected was cream dosage form, the extraction of the drugs was carried out by liquid-liquid extraction procedure. The remainder was checked for presence of the drugs showed negative results which confirms complete extraction of drugs.

A HPLC-DAD method was developed and validated as per ICH guidelines for simultaneous estimation of MTS and TBF in cream dosage form. The method was developed with a column of SyncronisaQ $C_{18}$ (5 µm, 250mm × 4.6 mm i. d.) when mobile phase optimized as Methanol: water (90: 10, v/v). The flow rate was 1 mL min$^{-1}$. Injection volume is 20 µL and runtime was 10 min. The wavelength used for recording of chromatogram was 237 nm and quantification of MTS and TBF was carried out at 258 and 224 nm, respectively. The retention time for MTS and TBF was observed to be 3.26 and 6.67 min, respectively. The developed method was validated as per ICH guidelines and results were within limits. The linearity range for MTS was found to be 5-60 µg mL$^{-1}$ and that of TBF 10-80 µg mL$^{-1}$. The quantitation limit was found to be 4.63 and 9.24 µg mL$^{-1}$ for MTS and TBF, respectively. The analysis of marketed formulation was carried out by using developed method. It gave recovery in

acceptable limits. The marketed formulation selected was cream dosage form, the extraction of the drugs was carried out by liquid-liquid extraction procedure. The use of diode array detector gave freedom in quantification of both the active ingredients at their maximum wavelength of absorption.

Stability of drug substance and drug product in pharmaceutical world is the most prior concern in turn to maintain its efficacy and safety. The FDA and ICH guidance state the importance and regulations in terms of maintaining, testing and documenting the stability profile The remaining methods reports two stability indicating assay methods for determination and quantitation of Levosulpiride in presence of degradation products. A high performance thin layer chromatographic and high performance liquid chromatography methods were developed.

The stability indicating methods helps to determine the conditions which cause degradation through the degradation pathways, it also helps to identify the impurities and degradants from excipients. The forced degradation study helps to improve shelf life and helps in deciding the physical state of the dosage form.

The stability indicating HPTLC method was developed to quantify LSP in presence of its degradation products. The sample was subjected to forced degradation under various conditions like hydrolytic, oxidative, photolytic and thermal conditions. The developed method well resolved degradation products for standard Levosulpiride. Merck aluminum plates precoated with silica gel 60 $F_{254}$ were used as stationary phase when mobile phase was optimized as ethyl acetate: methanol: ammonia (7:3:0.5 v/v/v). The wavelength selected for detection was 216 nm. The retardation factor for standard LSP was found to be 0.44 ± 0.02. The degradation products showed retardation factors as be 0.38 ± 0.02, 0.37 ± 0.02 and 0.77 ± 0.02 for acid, base and oxidative stress conditions respectively. The developed method was validated as per ICH guidelines and results found within limits. The validated method was used to analyze marketed formulations and gave acceptable recovery of the drugs in the formulations.

An attempt was made to identify the major degradation products by mass spectrometry. Newer interface TLC-MS has simplified the identification by recording its m/z values of the degradation products. The interface has wide applications in

various fields of pharmaceuticals and advantages over LC-MS. The limitations of buffers in the use of LC-MS methods can be overcome by TLC-MS. The degradation products were subjected to TLC-MS analysis and m/z values were recorded and degradation pathways predicted.

A high performance liquid chromatography method was also developed for separation and quantification of Levosulpiride from its degradation products. The stability indicating assay methods developed with a column of SyncronisaQ $C_{18}$ (5 µm, 250mm × 4.6 mm i. d.) when mobile phase optimized as Methanol: water (20: 80, v/v). The flow rate was 0.7 mL $min^{-1}$. Injection volume is 20 µL and runtime was 10 min. The developed method was validated as per ICH guidelines and results found within limits. The samples were subjected to LC-MS/MS analysis. The developed method with no modification was subjected to LC-MS/MS as developed method was suitable for it. The m/z values were recorded and possible degradation pathways were predicted. The m/z values showed that acid and base degradation products were same; formed due to acid and base hydrolysis.

The developed chromatographic methods verified to be simple, fast, accurate, precise and robust thus can be anticipated for routine analysis of mentioned drugs and its marketed formulations used in the study.

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# *Publications*

## Publications

- "Validated Normal Phase HPTLC Method For Simultaneous Quantification Of Levosulpiride And Esomeprazole In Capsule Dosage Form." International Journal of Pharmacy and Pharmaceutical Sciences 6.2 (2014): 347-350.
- "Simultaneous quantification of Levosulpiride and Rabeprazole in tablet dosage form by validated normal phase high performance thin layer chromatographic method." Journal of Chemical & Pharmaceutical Research 6.5 (2014): 1193-1199.
- “Development and validation of normal phase HPTLC method for simultaneous quantification of Mometasone furoate and Terbinafine hydrochloride in cream dosage form.” Der Pharmacia Lettre, 6.6 (2014):239-245.

# *Errata*

## Errata

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