"DEVELOPMENT AND VALIDATION OF STABILITY INDICATING HPTLC METHOD FOR ESOMEPRAZOLE MAGNESIUM TRIHYDRATE"

A Thesis Submitted to

NIRMA UNIVERSITY

In Partial Fulfillment for the Award of the Degree of

MASTER OF PHARMACY

IN

PHARMACEUTICAL ANALYSIS

BY

KEWAL G. MODI (10MPH306), B.PHARM.

UNDER THE GUIDANCE OF

DR. CHARMY S. KOTHARI

Department of Pharmaceutical Analysis
Institute of Pharmacy
Nirma University
Ahmedabad-382481
Gujarat, India.

May 2012

CERTIFICATE

This is to certify that the dissertation work entitled "Development and Validation of Stability Indicating HPTLC Method for Esomeprazole Magnesium Trihydrate" submitted by Ms. Kewal G. Modi with Regn. No. (10MPH306) in partial fulfillment for the award of Master of Pharmacy in "Pharmaceutical Analysis" is a bonafide research work carried out by the candidate at the Department of Pharmaceutical Analysis, Institute of Pharmacy, Nirma University under our guidance. This work is original and has not been submitted in part or full for any other degree or diploma to this or any other university or institution.

Guide:

Dr. Charmy S. Kothari
M. Pharm., Ph.D.
Asst. Professor,
Department of Pharmaceutical Analysis
Institute of Pharmacy,
Nirma University.

Forwarded Through:

Dr. Priti J. Mehta
M. Pharm., Ph.D.
Head
Dept. of Pharmaceutical Analysis
Institute of Pharmacy
Nirma University.

Dr. Manjunath Ghate
M.Pharm., Ph.D.
Director
Institute of Pharmacy
Nirma University.

Date : 12th May, 2012.

DECLARATION

I hereby declare that the dissertation entitled "Development and Validation of Stability Indicating HPTLC Method for Esomeprazole Magnesium Trihydrate", is based on the original work carried out by me under the guidance of Dr. Charmy S. Kothari, Assistant professor, Department of Pharmaceutical Analysis, Institute of Pharmacy, Nirma University. I also affirm that this work is original and has not been submitted in part or full for any other degree or diploma to this or any other university or institution.

Ms. KEWAL G. MODI (10MPH306)
Department of Pharmaceutical Analysis
Institute of Pharmacy
Nirma University
Sarkhej – Gandhinagar Highway
Ahmedabad–382481
Gujarat, India.

Date:

ACKNOWLEDGEMENT

*I thank **Almighty** for making my dream come true. I have, for so many years, dreamt of the day that I would acquire my Master's Degree with some novel research and the day has finally come. I believe GOD is the best manager in the world; he manages everyone's life smoothly with ups to build the confidence in us and downs to prevent the penetration of arrogance in us. Every step, he comes to us in some or the other form, it may be a family or a friend or a teacher or a stranger, to help us out.*

*First and foremost I want to thank my guide **Dr. Charmy S. Kothari**, Dept. of Pharmaceutical Analysis, Institute of Pharmacy, Nirma University. It has been an honor to be her M.Pharm student. She has taught me, both consciously and un-consciously, how good experimental pharmacy research is done. I appreciate all her contributions of time and ideas to make my M.Pharm. experience productive and stimulating. The joy and enthusiasm she has for her research was contagious and motivational for me, even during tough times in the M.Pharm. pursuit. Her belief and trust in me was a source of determination for me which led to the conversion of an idea into a realistic method development on a novel technique.*

*I am also thankful to **Dr. Priti J. Mehta,** Head, Dept. of Pharmaceutical Analysis, for providing me with an excellent example a source of inspiration as a successful woman, an analyst and a professor, moreover a perfectionist.*

*I am highly thankful to **Dr. Manjunath Ghate** (Director, Institute of Pharmacy, Nirma University, Ahmedabad) for providing all necessary help and facility for my work and also for his constant support and encouragement.*

***Mr. Nrupesh R. Patel,** Assistant Professor, Dept. of Pharmaceutical Analysis, Institute of Pharmacy, Nirma University was a constant source of support and guidance with his endless patience, knowledge and depth on the subject matter. The members of the institute have contributed immensely to my personal and professional time at Nirma University. I am extremely grateful to **Omkar sir, Tejas sir,** Dept of Pharmaceutical Analysis, Institute of Pharmacy, Nirma University for their valuable suggestions, continuous encouragement and everlasting support throughout this*

dissertation work.

I would like to thank my colleagues ***Sweta, Margi, Ajay, Kartik, Shikha, Barkat, Jayesh, Chandni, Jay, Amisha, Krishna, Pavan, Sunil and Ankit.*** *I would also like to thank my all batchmates of all the departments as constant source of inspiration. Special words of gratitude to my dearest friend* ***Yesha and Jeel*** *who were always there besides with the hand of support to make my effort a successful task. I would like to thank my seniors* ***Lakshmikartik, Rakesh, Hiral and Pankti*** *and juniors* ***Upasana, Richa, Divya, Raghav, Harshal, Mitul, Suchi and Nirzari.***

I am also thankful to our Lab Assistant ***Satej Bhai, Shreyas Bhai*** *and also to* ***Bipin Bhai*** *for providing all the requirements and material as and when required. We would also like to thank the whole library staff to avail us with library facilities during project work.*

Lastly, I would like to thank ***my family*** *for all their love and encouragement. For my parents who raised me with a love of science, supported me in all my pursuits and provided me with a vision and a goal to achieve in future. And most of all for my loving, supportive, encouraging, and patient family including* ***my mom, my caring dad, my sister and my jiju*** *whose thorough support during the final stages of this M.Pharm. is very much appreciated. Thank you.*

Last, but not the least, I express my gratitude and apologize to anybody whose contributions, I could not mention in this page.

Date:

Kewal G. Modi

Institute of Pharmacy,

Nirma University,

Ahmedabad.

SIAM	:	Stability Indicating Assay Method
ICH	:	International Conference on Harmonization
FDA	:	Food and Drug Administration
USP	:	United States Pharmacopoeia
IP	:	Indian Pharmacopoeia
PUD	:	Peptic Ulcer Disease
GERD	:	Gaestroesophagal Reflux Disorder
PPI	:	Proton Pump Inhibitor
ESOM	:	Esomeprazole Magnesium Trihydrate
API	:	Active Pharmaceutical Ingredient
ppm	:	parts per million
pKa	:	Ionization constant
Log P	:	partition coefficient
UV	:	Ultra-Violet
IR	:	Infra-Red
FT-IR	:	Fourier Transfer Infra- Red
HPLC	:	High Performance Liquid Chromatography
RP-HPLC	:	Reverse Phase High Performance Liquid Chromatography
HPTLC	:	High Performance Thin Layer Chromatography
H_2O_2	:	Hydrogen Peroxide
HCl	:	Hydrochloric acid

NaOH	:	Sodium Hydroxide
R^2	:	Correlation coefficient
R.S.D.	:	Relative standard deviation
S.D.	:	Standard deviation
LOD	:	Limit of Detection
LOQ	:	Limit of Quantification

Index

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

ABSTRACT

A new, simple, precise, rapid, selective and specific stability indicating HPTLC method has been developed and validated for Esomeprazole Magnesium Trihydrate (ESOM).

Stability indicating HPTLC method was developed & validated for estimation of Esomeprazole Magnesium Trihydrate (ESOM). Stability indicating capability is established by forced degradation of ESOM to acid, alkali, oxidation, thermal, neutral and photo degradation as per ICH guidelines Q1A (R2) and Q1B. Separation was achieved on a Precoated Silica Gel 60_{F254} Plates with 250 μm thickness by using 1,4 - Dioxane : Toluene : Methanol (6:4:1, v/v/v) as mobile phase. Detection was carried out at 304 nm wavelength. The method was validated as per ICH guidelines Q2 (R1). The linearity of the proposed method was found to be in the range of 300-1900 ng/spot concentrations with correlation coefficient R^2=0.9989. For ESOM, Specificity of the developed method was established by determining the peak purity of the peaks of the drugs in a stress samples by scanning peak at peak start, peak apex and peak end. Correlation between them showed high level of purity and significant separation from pure ESOM peak and peak of degradation prodcts.

As the method could effectively separate the drug from its degradation products, it can be employed as a stability indicating method.

CHAPTER 1

INTRODUCTION

INTRODUCTION

CONTENTS

1.1. INTRODUCTION TO DISEASES[1,2]

1.1.1. PEPTIC ULCER :

A **Peptic Ulcer**, also known as **PUD or Peptic Ulcer Disease**, is an ulcer of an area of the gastrointestinal tract that is usually acidic and thus extremely painful. It results probably due to an imbalance between the aggressive (acid, pepsin and H. Pylori) and the defensive (gastric mucus and bicarbonate secretion, prostaglandins, nitric oxide) factors.

As many as 70-90% of ulcers are associated with Helicobacter pylori, a spiral-shaped bacterium that lives in the acidic environment of the stomach.

1.1.1.1. Classification : By Region/Location

- Duodenum (called duodenal ulcer)
- Oesophagus (called esophageal ulcer)
- Stomach (called gastric ulcer)

A peptic ulcer in the stomach is called a gastric ulcer. One that is in the duodenum is called a duodenal ulcer. A peptic ulcer also may develop just above your stomach in the esophagus, the tube that connects the mouth to the stomach. But most peptic ulcers develop in the stomach or duodenum.

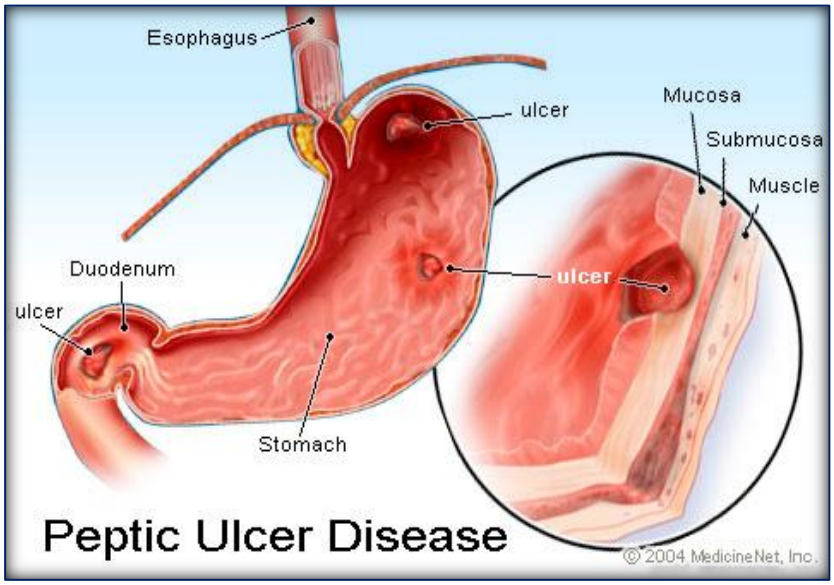

Figure 1.1.: Peptic Ulcer Disease[3]

1.1.1.2. Symptoms:

- Abdominal pain,
- Nausea and copious vomiting,
- Loss of appetite and weight loss;,
- Hematemesis (vomiting of blood); this can occur due to bleeding directly from a gastric ulcer, or from damage to the esophagus from severe/continuing vomiting,
- Melena (tarry, foul-smelling feces due to oxidized iron from hemoglobin),
- waterbrash (rush of saliva after an episode of regurgitation to dilute the acid in esophagus - although this is more associated with gastroesophageal reflux disease).

The timing of the symptoms in relation to the meal may differentiate between gastric and duodenal ulcers: A gastric ulcer would give epigastric pain during the meal, as gastric acid production is increased as food enters the stomach.

Duodenal ulcer pain would manifest mostly 2–3 hours after the meal, when the stomach begins to release digested food and acid into the duodenum.

1.1.1.3. Treatment:

- Antacids
  - Magnesium hydroxide,
  - Magnesium trisilicate,
  - Sodium bicarbonate etc.
- H_2 receptor antagonists
  - Cimetidine,
  - Ranitidine,
  - Famotidine etc.
- Antibiotics with proton pump inhibitor (PPI),
  - Clarithromycin, Amoxicillin, Tetracycline, Metronidazole.
  - Other proton pump inhibitors are omeprazole, esomeprazole, rabeprazole, pantoprazole, lansoprazole etc.

1.1.2. DYSPEPSIA:

Dyspepsia is also known as **upset stomach** or **indigestion**, refers to a condition of impaired digestion characterized by chronic or recurrent pain in the upper abdomen, upper abdominal fullness and feeling full earlier than expected when eating.

Dyspepsia is a common problem, and is frequently associated with, gastroesophageal reflux disease (GERD) or gastritis.

1.1.2.1. Symptoms:

- Upper abdominal pain,
- Bloating, fullness and tenderness on palpation,
- Nausea, difficulty swallowing,
- Gastrointestinal bleeding (vomit containing blood),
- Loss of appetite, unintentional weight loss,
- Abdominal swelling and persistent vomiting.

1.1.2.2. Treatment:

- H_2 receptor antagonists
  - Cimetidine,
  - Ranitidine,
  - Famotidine etc.
- Proton pump inhibitors
  - Omeprazole,
  - Lansoprazole,
  - Pantoprazole,
  - Esomeprazole etc.

1.1.3. GASTROESOPHAGAL REFLUX DISEASE (GERD):

Gastroesophageal reflux disease (GERD), gastro-oesophageal reflux disease (GORD), gastric reflux disease, or acid reflux disease is a chronic symptom of mucosal damage caused by stomach acid coming up from the stomach into the esophagus.

1.1.3.1. Symptoms:

- Heartburn,
- Regurgitation,
- Trouble swallowing (dysphagia),
- Pain with swallowing (odynophagia),
- Increased salivation (also known as water brash),
- Nausea, Chest pain.

1.1.3.2. Prevention:

- Sleep on the left side, or with your upper body raised,
- Eat smaller meals,
- Lose weight,
- Avoid acidic and rich foods.

1.1.3.3. Treatment:

1) Healthy diet, lifestyle modification and proper exercise.

2) Medications.

A number of drugs are approved to treat **PUD / Dyspepsia / GERD**. They are

- Proton pump inhibitors.
  - Omeprazole,
  - Esomeprazole,
  - Pantoprazole etc.
- Gastric H_2 receptor blockers.
  - Ranitidine, Famotidine and Cimetidine.
- Antacids.
- Sucralfate.

1.2. INTRODUCTION TO DRUG and DRUG PROFILE:

ESOMEPRAZOLE MAGNESIUM TRIHYDARTE[4,5,6,7,8,9,10,11]

- **Official status:** Official in IP-2010, USP- 2010.

- **Description:** Esomeprazole magnesium trihydrate is the S-enantiomer of Omeprazole and is used in treatment of PUD, Dyspepsia, GERD.

- **Category:** Proton Pump Inhibitor (PPI)

- **Chemical name:** (S)-5-methoxy-2-[(4-methoxy-3,5-dimethylpyridin-2-yl) methylsulfinyl]-3H-benzoimidazole.

- **CAS Registry number:** 217087-09-7.

- **Molecular weight:** 767.2 g/mol as a trihydrate and 713.1 g/mol on anhydrous basis.

- **Molecular formula:** $C_{34}H_{36}MgN_6O_6S_2.3H_2O$

- **Structural formula:**

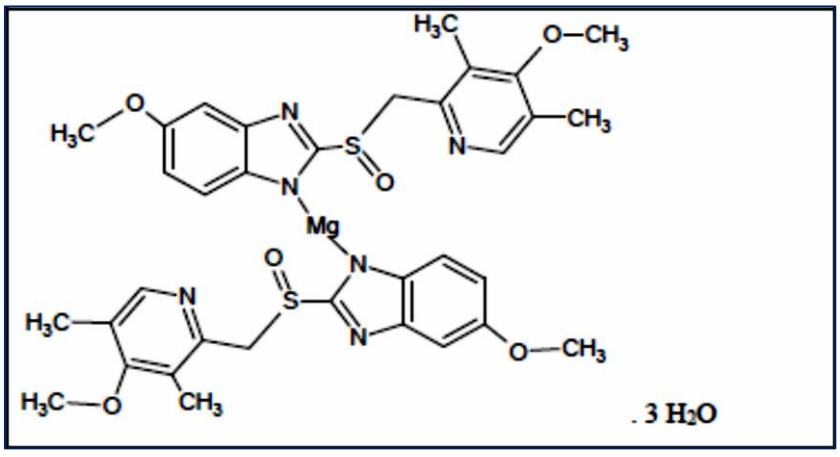

Figure 1.2.: Structure of Esomeprazole Magnesium Trihydrate

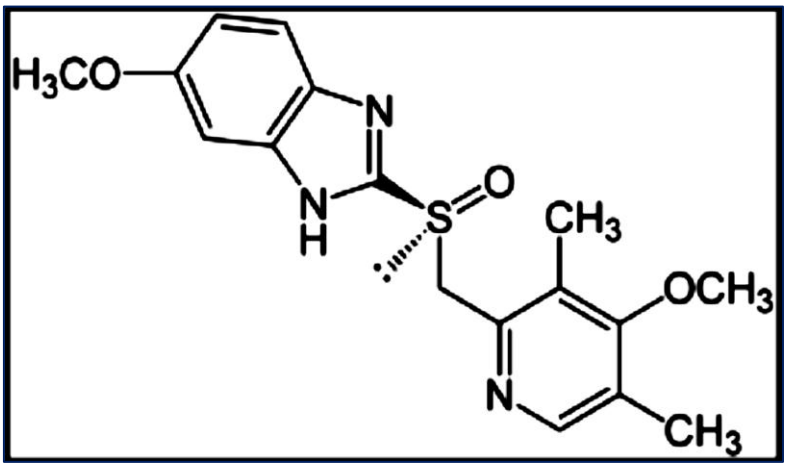

Figure 1.3.: Structure of Esomeprazole

❖ Physical and chemical Properties:

- **Appearance:** White to off-white Powder

❖ Solubility:

- Slightly soluble : Water, Acetone.
- Soluble: Chloroform, Methanol, Ethanol and Acetonitrile.

❖ Melting point: 176- 179°C

❖ BCS Class: Class-II (High Permeability and Low Solubility)

❖ Ionisation Constant

- pKa: 4.0

❖ Pharmacological Profile:

- **Therapeutic Category:** Proton Pump Inhibitor (PPI)

- **Mode of Action:**

  The first proton pump inhibitor was the substituted benzimidazole **omeprazole** , which irreversibly inhibits the H^+/K^+ ATPase (the proton

pump), the terminal step in the acid secretory pathway. Both basal and stimulated gastric acid secretion is reduced.

The drug is a weak base, and accumulates in the acid environment of the canaliculi of the stimulated parietal cell where it is activated. By inhibiting the functioning of this enzyme, the drug prevents formation of gastric acid.

This preferential accumulation means that it has a specific effect on these cells. Other proton pump inhibitors include **esomeprazole** (*S*-isomer of omeprazole), **lansoprazole**, **pantoprazole** and **rabeprazole**.

❖ Pharmacokinetic Profile:

- **Absorption**: Esomeprazole is completely absorbed after oral administration. The bioavailability of esomeprazole is about 50-90%.
- **Biotransformation:** The drug is rapidly cleared from the body, largely by urinary excretion of pharmacologically-inactive metabolites such as 5-hydroxymethylesomeprazole and 5-carboxyesomeprazole. The drug is metabolized by liver.
- **Elimination:** Half life is around 1-1.5 hours. The drug is excreted 80% by Renal and 20% by feacal excretion.

❖ Indication:

- For the treatment of Peptic Ulcer Disease (PUD), Dyspepsia, GERD/GORD and Zollinger syndrome.

❖ Dosage and Dosage forms:

- Adult: 20mg and 40 mg daily as single dose orally.

❖ Formulation:

- **Neksium** (Astra Zeneca Pharma India Ltd., Mumbai) – 20 mg and 40 mg strength enteric coated tablets.

1.3. INTRODUCTION TO STABILITY INDICATING ASSAY METHODS (SIAM) [12,13,14,15,16,17]

Stability testing of drug products is a requirement from regulatory as well as from industrial point of view, owing to increasing concerns for drug product safety, efficacy and quality.

Availability of a suitable stability-indicating assay method (SIAM) with degradation mechanisms is necessary (1) To study stability behavior of drug substances and drug products, (2) Isolation and characterization of degradation products is required for validation of SIAM and to investigate mechanisms of degradation, (3) Regulatory requirements specify long term, intermediate and accelerated stability testing.

But degradation products formed during long term and accelerated testing may not be present in substantial amounts for isolation and development of SIAM. These drawbacks (long time, less quantity) necessitate forced decomposition by stress testing.

1.3.1 STABILITY INDICATING ASSAY METHODS (SIAM) [12,13,14,15,16,17]

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

The method is expected to allow analysis of individual degradation products.
SIAM is a validated quantitative analytical procedure that can detect changes with time in the pertinent properties of the drug substance and drug product under defined storage conditions.

It accurately measures the API without interference from other substances and is

sensitive enough to detect and quantify even small amounts of degradation products/ impurities.

Stability-indicating methods according to United States-Food and Drug Administration (US-FDA) stability guideline of 1987 were defined as the "Quantitative analytical methods that are based on the characteristic structural, chemical or biological properties of each active ingredient of a drug product and that will distinguish each active ingredient from its degradation products so that the active ingredient content can be accurately measured."

To develop a SIAM, stress testing in the form of forced degradation should be carried out at an early stage so that impurities and degradation products can be identified and characterized. SIAM must be discriminating and properly validated to ensure the accuracy of the long term stability testing study.

1.3.2 TYPES OF STABILITY INDICATING ASSAY METHODS (SIAM)[13]

a) Specific Stability Indicating Assay Method

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

b) Selective Stability Indicating Assay Method

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

1.3.3 TECHNIQUES USED IN DEVELOPMENT OF SIAM

Titrimetric, spectrophotometric, and chromatographic techniques have been commonly employed in analysis of stability samples. There are sporadic reports of the use of miscellaneous techniques (Table 1.1).

Table 1.1.: Analytical methods used for SIAM [15]

Method	Advantage / Disadvantage	Examples
Spectrophotometric	Low cost Simplicity (Non specific)	Guanabenz (derivative UV)
TLC	Simplicity (Variability,Non quantitative)	Ranitidine HCl
HPTLC	Rapid Accurate Small quantity of mobile phase needed	Nifedipine
GC	Rapid Accurate (Unsuitable for non volatile and thermo labile compounds)	Fluconazole
HPLC	High resolution capacity Specificity Sensitivity Best for compounds which are non volatile, ionic and/or unstable at high temperatures	Zidovudine
LC-MS/ LC-NMR/ CE-MS/ LC-MS/MS	Quantitation + Characterization	Losartan (LC-MS)

1.4. DEVELOPMENT OF VALIDATED STABILITY INDICATING ASSAY METHOD (SIAM) [15,16,17,18,19,20,21,22]

A stability-indicating method is defined as an analytical method that accurately quantitates the active ingredients without interference from degradation products, process impurities, excipients, or other potential impurities.

Forced degradation should be the first step in method development. Method development and identification of primary degradation products and unknown impurities is next.

Step I: Critical study of the drug structure to assess the likely decomposition route(s)

This should be the first step in establishment of a SIAM. Much information can simply be gained from the structure, by study of the functional groups and other key components.

There are defined functional group categories like amides, esters, lactams, lactones, etc. that undergo hydrolysis. Others like thiols, thioethers, etc. undergo oxidation and olefins, aryl halo derivatives, aromatic nitro groups, N-oxides undergo photo decomposition.

Step II: Collection of information on physicochemical parameters

Before method development is taken up, it is very important to know various physicochemical parameters like pKa, log P, solubility, absorptivity and wavelength maximum of the drug in question.

The knowledge of pKa is important as most of the pH- related changes in retention occur at pH of the buffer to be used in the mobile phase. The knowledge of log P for the drug and the identified degradation products provides good insight into the separation behavior likely to be obtained on a particular stationary phase.

Step III: Stress (forced decomposition) studies

The next step in the development of SIAM is the conduct of forced decomposition studies to generate degradation products of the drug. The ICH guideline Q1A suggests the following conditions to be employed:

(i) 10 °C increments above the accelerated temperature
(ii) humidity
(iii) hydrolysis across a wide range of pH values
(iv) oxidation and
(v) photolysis.

However, the guideline provides no details on how hydrolytic, photolytic and oxidative studies have to be actually performed. The selection of the right type of stress condition in a minimum number of attempts is the main objective. Dependent upon the results, decision is taken on whether to increase the strength of the reaction conditions.

Step IV: Preliminary separation studies on stressed samples

The stress samples so obtained are subjected to preliminary analysis to study the number and types of degradation products formed under various conditions. The simplest way is to start with a reversed-phase octadecyl column (ODS) which is more preferred.

Step V: Final method development and optimization

For separation of close or co-eluting peaks, the method is optimized by changing the parameters such as the mobile phase ratio, pH, gradient, flow rate, temperature, solvent, the column and its type.

Step VI: Identification and characterization of degradation products

Before moving to the validation of a SIAM, it is necessary to identify the drug degradation products and if possible try to arrange for their standards. These are required to establish specificity/selectivity of the method. Peak purity of the active substance is checked by photo-diode array detector to verify that the method is selective.

Step VII: Validation of Stability Indicating Assay Methods (SIAM)

Validation has been extensively covered in the ICH guidelines Q2A and Q2B, the FDA guidance and by USP.

There are two stages in the Validation of a Stability Indicating Assay Method (SIAM):

First stage is when drug substance is subjected to forced decomposition studies and the SIAM is established based on the knowledge of drug degradation behavior. The main focus of validation at this stage is on establishment of specificity/selectivity, accuracy, precision, linearity, range, robustness, LOD, LOQ etc.

The second stage, when the SIAM so developed is extended to formulations or other matrices, the emphasis gets limited to just prove the pertinence of the established validation parameters in the presence of excipients or other formulation constituents.

1.5. INTRODUCTION TO METHOD OF ANALYSIS (HPTLC)[23,24]

Planar chromatography (PC) is a multistage distribution process. It is a form of liquid chromatography in which the stationary phase is supported on a planar surface than a column.

Thin layer chromatography (TLC), also known as planar chromatography (PC), is one of the oldest methods in analytical chemistry still in use.

In TLC, the different components of the sample are separated by their interaction with the stationary phase (bonded to the glass, aluminium, or plastic support) and the liquid mobile phase that moves along the stationary phase.

High Performance Thin Layer Chromatography (HPTLC) has developed to the extent that separation and quantification can provide results that are comparable with other analytical methods such as High Performance Layer Chromatography (HPLC).

HPTLC technique is most suited technique for content uniformity test and impurity profiling of the drugs as per compendial specification.

HPTLC uses the same type of silica gel 60 layers as TLC, with a thickness of 0.20-0.25 mm. however the particle size is much smaller, typically ranging from 4-8 μm, with optimum 5-6 μm.

Table 1.2.: Difference between HPTLC and TLC

Parameters	**HPTLC**	**TLC**
Layer of Sorbent	100μm	250μm
Particle size	5-6 μm	10-12 μm
Pore diameter	60 Å	60-100 Å
Sensitivity limit	Upper pg	ng
Efficiency	High due to smaller particle size generated.	less
Separations	3-5 cm	10-15 cm
Analysis Time	Shorter migration distance and the analysis time is greatly reduced.	Slower

Solid support	Wide choice of stationary phase like silica gel for normal phase and C8, C18 for reversed phase modes	Silica gel, alumina and kiesulguhr
Development chamber	New type that require less amount of mobile phase	More amount
Sample spotting	Auto sampler	Manual spotting
Scanning	Use of UV/Visible/ Fluoroscence scanner scans the entire chromatogram qualitatively and quantitatively and the scanner is an advanced type of densitometer	Not possible

1.5.1. MECHANISM OF HPTLC SEPARATION:

- Adsorption
- Partition
- Ion-exchange

1.5.2. FEATURES OF HPTLC:

- Simultaneous processing of sample and standard - better analytical precision and accuracy
- Simultaneously several samples of even divergent nature and composition can be studied
- Less need for internal standard
- Several analysts work simultaneously
- Lower analysis time and less cost per analysis
- Low maintenance cost
- Simple sample preparation- handle samples of divergent nature
- No prior treatment for solvents like filtration and degassing
- Low mobile phase consumption per sample
- No interference from previous analysis- fresh stationary and mobile phases for each analysis- no contamination
- Visual detection possible- open system

- Non UV absorbing compounds detected by post- chromatographic derivatization
- Possibility of multiple evaluation of the plate with different parameters because all fractions of the sample are stored on the plates.

1.5.3. STEPS INVOLVED IN HPTLC:

1) Selection of chromatographic layer
2) Sample and standard preparation
3) Chromatographic plate pre-washing
4) Chromatographic plate pre-conditioning
5) Application of sample and standard
6) Chromatographic development
7) Detection of spots
8) Scanning
9) Documentation of chromatographic plate

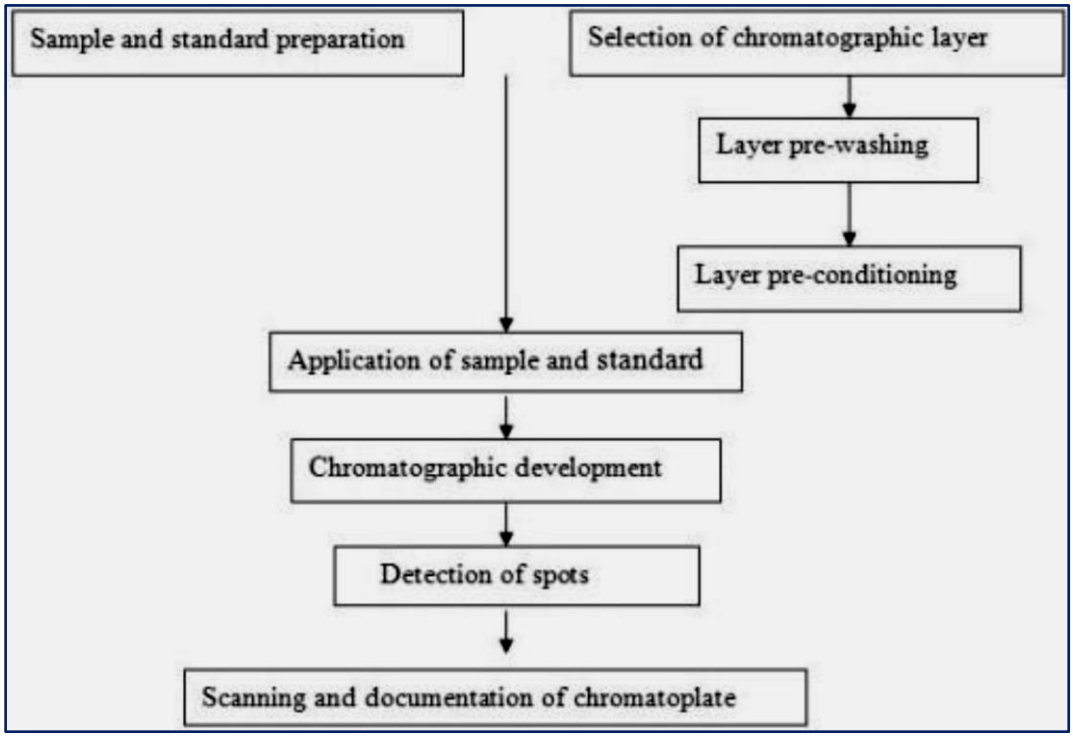

Figure 1.4.: Steps involved in HPTLC

❖ **Selection of chromatographic layer**

- Precoated plates with different support materials and different sorbents are available.
- 80 % of analysis is done on silica gel GF.

❖ **Pre-washing and activation of pre-coated plates:**

- Plates exposed to high humidity or kept on hand for long time requires pre-washing.
- Pre-washing is mainly done by dipping the plate in methanol.
- Activation of pre-coated plates is done by placing them in an oven at 110-120ºC for 30 min prior to spotting.
- Aluminum sheets should be kept in between two glass plates and placing in oven at 110- 120ºC for 15 minutes.
- Hot plates can also be used for HPTLC plates with aluminium as support material.

❖ **Application of sample and standard:**

- The solvent used to apply the sample to the TLC plate can have a decisive influence on the spot size.
- The least polar single solvent or mixture of solvents in which the analyte(s)are completely soluble or completely extracted from the sample matrix can be used.
- Usual concentration range is 0.1-1µg / µl - above this causes poor separation.
- Automatic applicators are available wherein N2 gas sprays sample and standard from syringe on HPTLC plates as bands.
- Band wise application can be more accurately accomplished and provides better separation with high response to densitometer.

❖ **Selection of mobile phase:**

- Selectivity of separation is greatly influenced by the choice of solvent or solvent mixture. Selection can be based on
  - Trial and error
  - One's own experience and literature

- When the mobile phase is polar, polar compounds would be eluted first because of lower affinity with stationary phase while non-polar compounds retained because of higher affinity with the stationary phase.
- Components of mobile phase should be mixed thoroughly and before introduction to the chamber.
- Multi component mobile phase once used not recommended for further use.

❖ **Development chambers:**

- There are a variety of different types of chambers, each designed with particular features to control to the greater or lesser extent the parameters of chromatogram development reproducibility.
- As solvent vapour saturation, sorbent vapour adsorbed, solvent vapour "demixing" and solvent front and edge effects on the chromatographic layer can have a bearing on separation achieved, it is important to eliminate unwanted effects and to utilize those features that will improve resolution.
- The types of HPTLC chambers are
  - Nu- chamber
  - Ns- chamber
  - Twin-trough chamber
  - Su- chamber
  - Ss-chamber
  - Horizontal chamber
  - U- chamber
  - Automatic Development chamber (ADC)
  - Vario chamber
  - Forced flow development chamber (OPLC)
- Amongst these Twin-trough chamber is most commonly used and it requires only 10-15 ml of mobile phase.

❖ **Pre-conditioning (Chamber saturation):**

- Unsaturated chamber takes longer time for run and may lead to high R_f values.

- Saturation of chamber is done by lining with filter paper for approximately 30 minutes prior to development which allows uniform distribution of solvent vapors in the chamber, so less solvent is required for the sample to travel.

❖ **Chromatographic development and drying**

- After development, plate is removed from the chamber and mobile phase is removed from the plate.
- Drying can be done either at room temperature or at alleviated temperatures if solvents like water or acids are used.

❖ **Detection and Visualization**

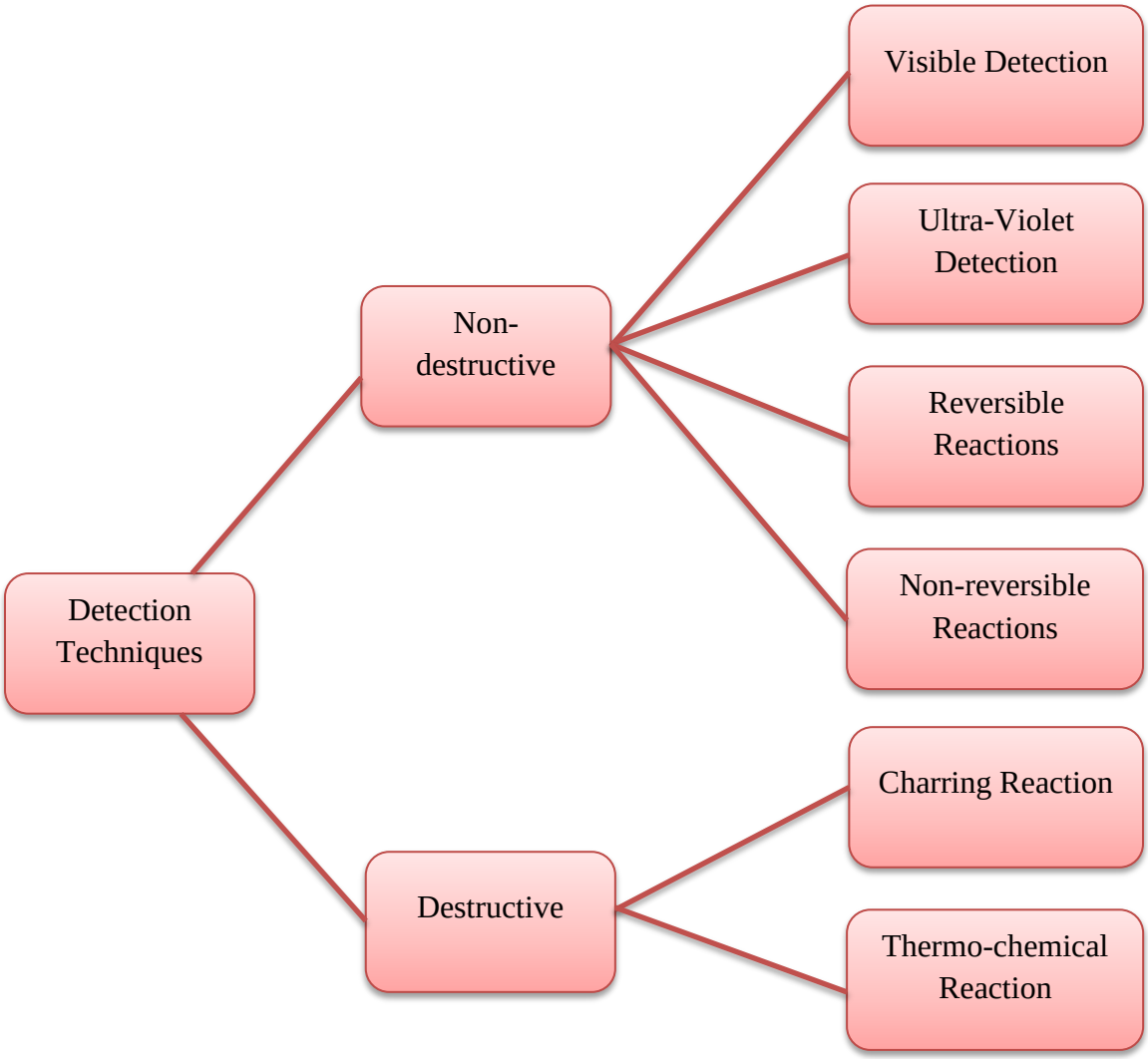

Figure 1.5.: Detection methods in HPTLC

- Spots of fluorescent compounds can be seen at 254 nm (short wave length) or at 366 nm (long wave length).
- Spots of non-fluorescent compounds can be seen when fluorescent stationary phase is used like as silica gel GF.
- Non UV absorbing compounds can be visualized by dipping the plates in 0.1 % iodine solution.
- When individual component does not respond to UV- derivatization required for detection.

❖ **Quantification:**

1) Densitometry

Densitometry is a means of measuring the concentration of the chromatographic zones on the developed HPTLC layer without damaging the separated substance. There are three possible scanning modes, single beam, single wavelength, double beam using a beam splitter and dual wavelength, double beam combined into a single beam.

The single beam format is most popular, as the beam of electromagnetic radiation hits the chromatographic layer, some passes into and through the layer whilst the remainder is reflected back from the surface.

Reflectance occurs due to the opaqueness of the layer. This reflected radiation is measured by the photomultiplier unit or photoelectric cell in the instrument. The spectrodensitometric scanner scan separate tracks and wavelength produces vast amount data.

These data includes peak heights and areas, and position of zones (start, middle and end) for every resolved component on every chromatographic track on the HPTLC plate.

A baseline adjustment is applied so that all peaks can be accurately integrated ready for possible quantification. Calibration of single and multiple levels with linear or non-linear regressions are possible when target values are to be verified

such as stability testing and dissolution profile single level calibration is suitable. Statistics such as RSD or CV are reported automatically.

2) Video imaging and densitometry

The developed chromatogram is illuminated from above with visible, 254nm (UV) or 366 nm (UV) light, depending on the radiation required to visualize the analytes.

Illumination from below the plate can often improve the brightness of the image. With the plate suitably lit, an image acquisition device, usually CCD (changed coupled device) camera with zoom attachment is positioned vertically above. The CCD camera transmits a digital signal to a computer and video printer.

1.5.4. ADVANTAGES OF HPTLC:[25,26,27,28]

- HPTLC is an off-line process. A number of samples are chromatographed simultaneously, side by side.
- Samples may be directly compared, often as they are running.
- The stationary phase is used only once. So the sample preparation must be performed only to the extent necessary to ensure that extraneous material in the samples applied to the plate does not interfere with the separation or retain any of the analyte.
- Carryover of the material from one sample to another is not a problem.
- Separation can be followed throughout the whole process and stopped when desired or when the solvent systems are changed.
- In the off-line TLC process all fractions are stored on the plate. Their optical properties are measured by densitometric evaluation, including the recording of in-situ spectra. All such measurements can be repeated without the need to repeat chromatography. The decision to measure with different parameters, or to interpose a derivatization step, can be made upon reviewing first results.
- Solvents and other reagents are required in very small volumes.

1.5.5. LIMITATIONS OF HPTLC:[28]

The limitation of TLC is its restricted separation efficiency. Separation efficiency of a chromatographic system can be expressed as the number of theoretical plate (N). HPLC yields a maximum of 10,000 to 15,000 plates, whereas the maximum N numbers for modern planar chromatography (HPTLC) are around 5000.

The reason for the lower performance of TLC is the fact that, because of its capillary flow behavior, the length of a TLC bed cannot be extended at will, whereas the permissible length of a column is limited only by the pressure available in the system.

1.6. INTRODUCTION TO METHOD VALIDATION [14,15,18,19,20,21,22]

"Doing thorough method validation can be tedious, but the consequences of not doing it right are wasted time, money, and resources."

Method validation is the process of demonstrating that analytical procedures are suitable for their intended use and that they support the identity, quality, purity, and potency of the drug substances and drug products. A successful validation guarantees that both the technical and regulatory objectives of the analytical methods have been fulfilled.

1.6.1. TYPE OF ANALYTICAL PROCEDURES TO BE VALIDATED

- Validation of analytical procedures is directed to the four most common types of analytical procedures.
- Identification test.
- Quantitative test for impurities content.
- Limit test for the control of impurities.
- Quantitative test of the active moiety in samples of drug substance on drug product on other selected components in the drug product.

1.6.2. OBJECTIVE OF VALIDATION

The objective of validation of analytical procedure is to demonstrate that it is suitable for its intended purpose. Any developed method may be influenced by variables like different days, reagents lots, instruments, equipments, environmental conditions like temperature etc. Thus, it is necessary that after the method has been developed, it is properly validated and the result of validity tests reported.

For analytical method validation of pharmaceuticals, guidelines from the International Conference on Harmonization (ICH), United States Food and Drug Administration (US FDA), American Association of Official Analytical Chemists (AOAC)United States Pharmacopoeia (USP) provide a framework for performing such validations in a more efficient and productive manner.

1.6.3. METHOD VALIDATION IS REQUIRED WHEN:

1. A new method is been developed

2. Revision established method

3. When established methods are used in different laboratories and different analysts etc.

4. Comparison of methods

5. When quality control indicates method changes.

The different parameters which are to be considered in analytical method validation as per USP (1225), CDER and ICH guidelines can be summarized as follows.

Table 1.3.: Method Validation Parameters[14,15,19]

USP (1225)	ICH	CDER
Accuracy	Accuracy	Accuracy
Precision	Precision	Precision
Repeatability	Repeatability	Repeatability
---------	Intermediate precision	Intermediate precision
Reproducibility	Reproducibility	Reproducibility
Specificity	Specificity	Specificity
Limit of Detection	Limit of Detection	Limit of Detection
Limit of Quantitation	Limit of Quantitation	Limit of Quantitation
Linearity	Linearity	Linearity
Range	Range	Range
Ruggedness	----------	----------
Robustness	Robustness	Robustness
----------	----------	Stability of solution

Data Elements Required for Assay Validation

Both the USP and ICH recognize that is it not always necessary to evaluate every analytical performance parameter.

The type of method and its intended use dictates which parameters needed to be investigated, as illustrated in Table.

Table 1.4.: ICH Validation Guideline[15]

Type of analytical procedure characteristics	Identification	Testing for impurities quantitative limit		Assay- dissolution (measurement only)- content/potency
Accuracy	-	+	-	+
Precision				
Repeatability		+	-	+
Intermed precision	-	+(1)	-	+(1)
Reproducibility	-	-(2)	-	-(2)
Specificity(3)	+	+	+	+(4)
Detection Limit	-	-	+	-
Quantitation Limit	-	+	-	-
Linearity	-	+	-	+
Range	-	+	-	+

- Signifies that this characteristic is not normally evaluated.

+ Signifies that this characteristic is normally evaluated.

(1) Intermediate precision is not needed in some case, when reproducibility is checked.

(2) May be needed in some cases.

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

(4) May not be needed in some cases.

1.6.4. VALIDATION PARAMETERS:

The different parameters of analytical method development are discussed below as per ICH guideline:-[4,5,6]

1) SPECIFICITY

Definition

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present.

Acceptance criteria: No interference peaks from blank or placebo at the R.T. of the drug and the peak purity should pass.

(Purity angle should be less than the purity threshold).

2) LINEARITY

Definition

The linearity of an analytical procedure is its ability (within given range) to obtain test results, which are directly proportional to the concentration (amount) of analyte in the sample.

Acceptance criteria: Correlation coefficient should not be less than 0.999.

3) RANGE

Definition

The range of analytical procedure is the interval between the upper and lower concentration of analyte in the sample which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

4) ACCURACY

Definition

The accuracy of analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. This is sometimes termed trueness.

Acceptance criteria: % Recovery should be in the range of 98-102%.

5) PRECISION

Definition

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

Acceptance criteria: % Relative standard deviation should be less than 2.

6) DETECTION LIMIT

Definition

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample, which can be detected but not necessarily quantitated under stated experimental conditions.

Acceptance criteria

- S/N ratio > 3 ; not specified in other cases

7) QUANTITATION LIMIT

Definition

The quantitation limit of an individual analytical procedure is defined as the lowest amount of analyte in a sample, which can be quantitatively determined with suitable precision and accuracy.

Acceptance criteria

- S/N ratio > 10:1; not specified in other cases

8) ROBUSTNESS

Definition

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

Acceptance criteria

- The method must be robust enough to withstand slight changes and allow routine analysis of sample.

Table 1.5.: System Suitability Parameters and their recommended limits[14,22]

PARAMETER	RECOMMENDATION
Capacity factor (k')	The peak should be well-resolved from other peaks and the void volume, generally K' > 2
Repeatability	RSD ≤ 2% (N ≥ 5 is desirable)
Relative retention	Not essential as the resolution is stated.
Resolution(r_s)	R_s of > 2 between the peak of interest and the closest eluting potential interferent (impurity, excipients, degradation product, internal standard, etc.)
Tailing factor(t)	T ≤ 2
Theoretical plates(n)	In general should be > 2500

Table 1.6. : Characteristics to be validated in HPTLC[15]

CHARACTERISTICS	ACCEPTANCE CRITERIA
Accuracy/trueness	Recovery 98-102% (individual)
Precision	RSD < 2%
Repeatability	RSD < 2%
Intermediate precision	RSD < 2%
Specificity/ selectivity	No interference
Detection limit	S/N > 3
Quantitation limit	S/N > 10
Linearity	Correlation coefficient r^2 > 0.999
Range	80 –120 %
Stability	> 24 h or >12 h

Table 1.7. : Suggested outline for performing Forced Degradation Studies[13,20,21]

Decide / Select matrix for degradation							
		Degradation Conditions					
Product/ Matrix	**Degradation**	**Acid**	**Base**	**Peroxide**	**Bisulfite**	**Photo stability**	**Temperature**
Drug Product	Yes	✓	✓	✓	✓	✓	✓
Placebo/Vehicle	Yes	✓	✓	✓	✓	✓	✓
API/ Raw material	Yes	✓	✓	✓	✓	✓	✓

Decide / Select degradation conditions/ agents	
Medium	**Conditions (Strive for 10 – 30% degradation)**
1 N HCl, 10 ml	Reflux 30 Minutes, Neutralize with base
0.1 N Sodium Hydroxide, 10 ml	Reflux 30 Minutes, Neutralize with acid
3 % Hydrogen Peroxide, 10 ml	15-30 Minutes
Light	Light Chamber, (1.2 million lux.hours)
Temperature	105°C

CHAPTER 2

LITERATURE REVIEW

3. LITERATURE REVIEW OF ESOMEPRAZOLE MAGNESIUM TRIHYDRATE

3.1. HPLC Methods:

Sr. no	Matrix	Column	Mobile phase	Conditions	Remarks	Reference
1	Tablets (with naproxen)	Phenomenex C18 column 150 x 4.6mm ID; 5µm	Acetonitrile phosphate buffer (50:50) pH 7	**Flow rate:** 0.5 ml/min **Detector:** UV detection at 300 nm	**Linear range**: 2-10 µg/ml	29
2	Tablets	C18 phenomenex column 250 x 4.6mm ID; 5µm	Acetonitrile phosphate buffer (60:40) pH 7	**Flow rate:** 1.0 ml/min **Detector:** UV detection at 205 nm	**Linear Range**: 100-1000 ng/ml.	30
3	Tablets (with pantoprazole, rabeprazole and domperidone)	Hypersil BDS C18	0.05 M, potassium dihydrogen phosphate buffer - acetonitrile (720:280 *v/v*) pH 4.70	**Flow rate:** 1.0 ml/min **Detector:** UV detection at 210 nm	**Linear Range**: 400-4000 ng/ml for ESOM, LOD= 131.27 ng/ml.	31

Sr. no	Matrix	Column	Mobile phase	Conditions	Remarks	Reference
4	Capsules	Packing L7 (L1 may be used). 12.5cm x 4mm ID; 5µm.	ACN : Sodium Phosphate buffer pH 7.6 (7:13)	**Flow rate** : 1.0 ml/min **Detector** : UV detection at 280 nm	**Linear Range**: 10-60 µg/ml	32
5	Tablets	Kromasil 100 C18 250 x 4.6 mm ID; 5 µm	Acetonitrile phosphate buffer (50:45)	**Flow rate:** 1 ml/min **Detector:** UV detection at 301 nm	**Linear Range**: 0.781-200 µg/ml	33

3.2. HPTLC Methods:

Sr. no	Matrix	Stationary phase	Solvent system	Conditions	Remarks	Reference
1	Tablets (with domperidone)	Silica gel 60 GF_{254} thin layer chromatographic plates	Dioxane : formic acid (4: 0.4 v/v)	**Detector** UV detection at 286 nm	**R_f Value:** 0.80 **Linear range** 800-5600 ng/spot	34

3.3. UV Methods:

Sr. no	Matrix	Method	Conditions	Remarks	Reference
1	Tablets	UV spectrophotometric method	λmax of ESOM : 203.5nm	**Linear range :** 2-10 µg/ml (R^2 = 0.9998)	35
2	Capsules (with domperidone)	Absorption ratio method	Absorption was measured at 301nm (λmax of ESOM) and 290 nm (iso-absorptive point)	**Linear range :** 1-11 µg/ml (R^2 = 0.9998)	36
3	Esomeprazole (API and Tablets)	UV spectrophotometric method	solvent used: methanol and chloroform (80:20) using Indigo Carmine reageants, λ max : 577 and 617 nm	**Linear range :** 5-35 µg/ml with (R^2 = 0.9997 and 0.9989).	37

4	Capsules (with domperidone)	Simultaneous estimation by UV Spectrophotometry	λmax : 301 nm for ESOM and 284 for DOM	**Linearity: Range**: 5-20 µg/ml for ESOM and 8-30 µg/ml for DOM	38
5	Tablets	Spectrophotometric method (method A) First order derivative spectra (method B) Area under curve (method C)	λmax : 303 nm for ESOM (method A) 303 nm and 292nm (method B) 294nm-310nm (method C)	**Linearity range :** 5 - 40 µg/ml for ESOM in all three methods (R^2 =0.9999, 0.9996)	39
6	Capsule (with domperidone)	First derivative zero crossing spectrophotometric method	λmax : 302 nm for ESOM (iso absorptive point = 290 nm)	**Linear range:** 3-12 µg/ml (R^2 =0.9997)	40

3.4. LC-MS/MS Methods:

Sr. no	Matrix	Column	Method	Conditions	Remarks	Reference
1	Human plasma, rat plasma and dog plasma	Reversed – phase LC colum	Atmospheric pressure positive ionization MS.	Extracted from plasma into methyl *tert*-butyl ether - dichloromethane (3:2, v/v).	**Flow rate:** 1.0 ml/min **Linear Range:** 20–20000 nmol/L	41

3.5. MISCELLANEOUS Methods:

Sr. no	Method	Conditions	Remarks	Reference
1.	Colourimetric methods (in pure form and in tablets)	**Method A-** The yellow colour formed due to reaction between ESOM and 5-sulfosalicylic acid in methanol. Absorbance measured at 365nm. **Method B-** Reaction of ESOM with N-bromosuccinamide in chloroform-acetone mixture. Absorbance measured at 380nm.	**Linear range:-** 2-48µg/ml (method A) 10-100µg/ml (method B)	42

CHAPTER 3

AIM OF PRESENT WORK

ESOM is a proton pump inhibitor (PPI), recently approved by the USFDA, for the treatment of Dyspepsia, Peptic Ulcer Disease (PUD), Gastroesophageal reflux disease (GORD/GERD) and Zollinger-Ellison syndrome.

Omeprazole is the most widely used proton pump inhibitor (PPI) that is used for the treatment of PUD/GERD/GORD. But Omeprazole is highly unstable at lower pH or acidic pH. Omeprazole is also unstable at room temperature.

ESOM has better activity and efficacy as compared to Omeprazole for treatment of PUD/GERD and other acid disorders. Thus, it has succeeded Omeprazole as the most widely used PPI in recent years.

The activity and efficacy of ESOM is dependent on its stability. The activity and efficacy of ESOM is dependent on its conversion into the active sulfonamide moiety inside the acidic pH of the GI tract. Thus, it is necessary to develop a stability indicating assay method (SIAM) for ESOM to check for the stability at various conditions like acidic and basic pH, oxidation etc.

Literature review shows that several spectrophotometric and chromatographic methods are reported for the estimation of ESOM alone and/or in combination with other drugs. Also acid hydrolysis degradation profile (acid pH profile) has been reported for Omeprazole but no SIAM by HPTLC has yet been reported in literature for estimation of ESOM.

Thus, it was endeavoured to develop a complete degradation profile for ESOM using HPTLC method.

OBJECTIVE OF PRESENT WORK

- To develop and validate a simple, accurate, precise, specific and selective Stability indicating HPTLC method for estimation of ESOM in presence of degradation products.

CHAPTER 4

IDENTIFICATION OF DRUG

4. IDENTIFICATION OF DRUG

Identification of drug Esomeprazole Magnesium Trihydrate (ESOM) was carried out by Melting point, UV-Visible spectroscopy and FT-IR spectroscopy methods.

4.1. INSTRUMENTATION

4.1.1 Melting point apparatus

Melting point apparatus with model: T0603160; Manufactured by EIE Instruments PVT Ltd.

4.1.2 UV-Visible spectrophotometer

UV visible spectrophotometer with model: UV-1800 PC series; Manufactured by Shimadzu Inc., Japan was used.

4.1.3 FT IR spectrophotometer

FT IR spectrophotometer with model: IR-Affinity, 7800-350cm^{-1}; manufactured by Shimadzu Corporation Inc., Japan was used.

4.2. Determination of Melting Point

Melting point of Esomeprazole Magnesium Trihydrate has been determined using the open capillary method.

A small amount of drug sample was transferred in to capillary tube. Then capillary tube was placed in melting point test apparatus and the temperature at which the drug started melting and was completely melted was noted down.

Table 4.1.: Melting Point of ESOM

Drug	Reported melting point (°C)[43]	Observed melting point (°C)
Esomeprazole Magnesium Trihydrate	176-180 °C	179-182 °C

4.3. Determination by UV Visible spectrophotometer

Table 4.2.: Data of UV Spectra of ESOM (10 µg/ml)

Drug	Reported peak (nm)[43]	Observed peak (nm)
Esomeprazole Magnesium Trihydrate	205 nm, 302 nm	204.6 nm, 302.8 nm

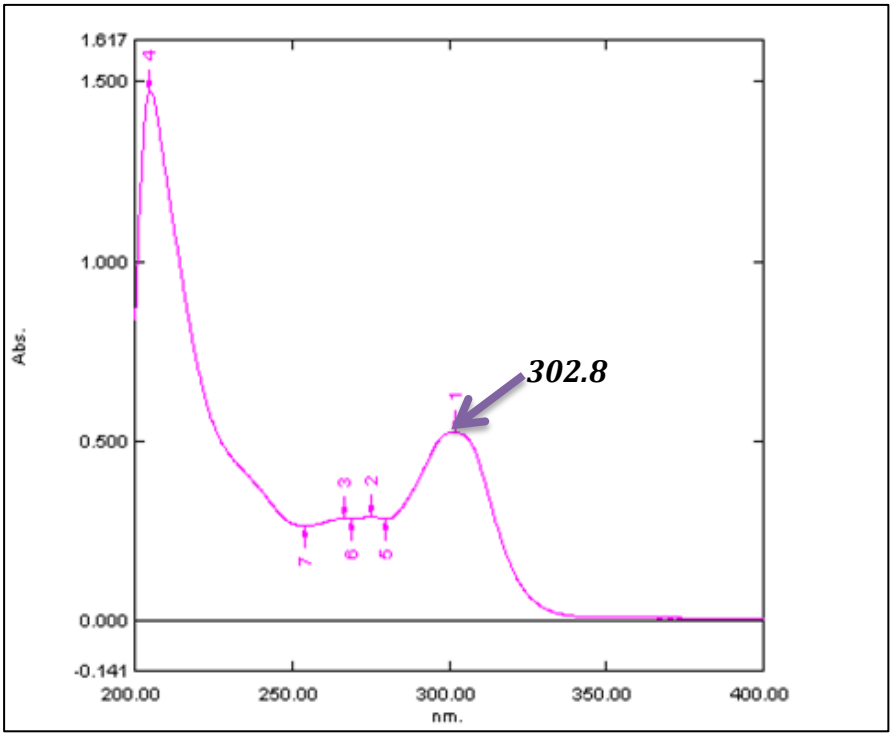

Figure 4.1.: UV Spectra of ESOM (10 µg/ml) in methanol

4.4. Determination by FT-IR

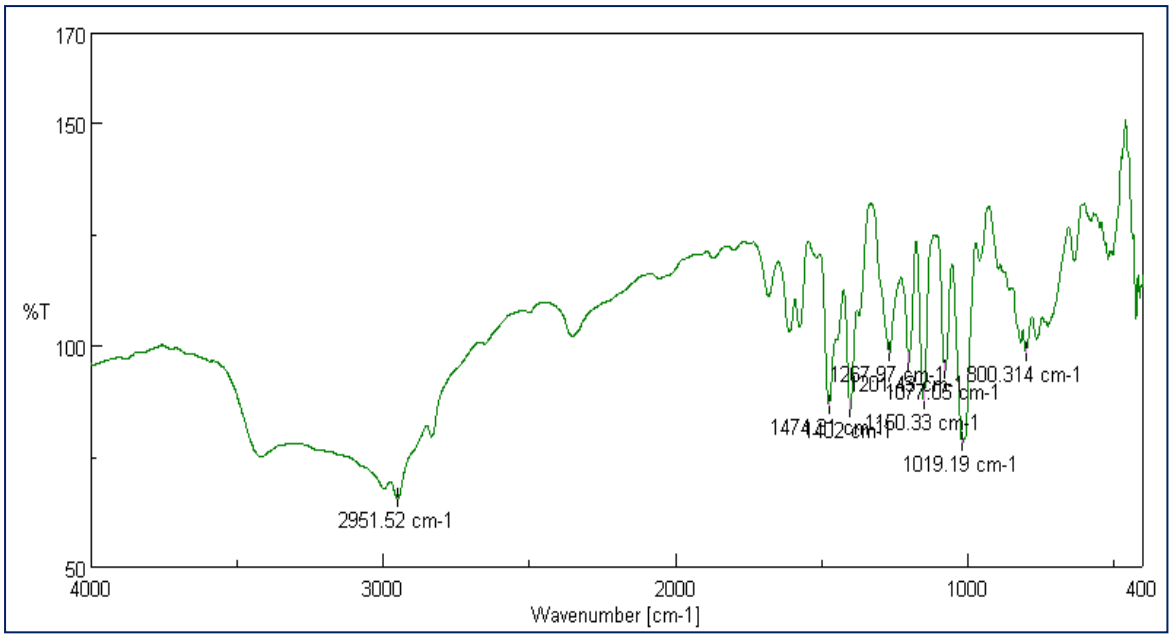

Figure 4.2.: FT- IR spectra of ESOM

Table 4.3.: Specifications of IR peaks for ESOM

Sr. No.	Peak (cm^{-1})	Intensity	Functional Group
1	2951.52	65.3372	Aliphatic CH stretching
2	1402	85.5756	C=C stretching
3	1267.97	98.1441	C-N stretching
4	1150.33	87.4924	C-O/Ether
5	1077.05	94.2529	C-O/Ether
6	800.314	98.3065	=C=H bending

CHAPTER 5

EXPERIMENTAL WORK

5.1. INSTRUMENTATION

- Pre-coated silica gel aluminium Plate 60F–254 (20 x 20 cm with 250 µm thickness),
- Camag – 100µl Applicator syringe (Hamilton, Bonaduz, Schweiz),
- Camag Applicator-Linomat V,
- Camag – Twin trough chamber (10 x 20 cm) with stainless steel lid,
- Camag TLC scanner III,
- UV cabinet with dual wavelength UV lamp (254 nm and 366 nm),
- Balances Model: Citizen Cx-220, Citizen Pvt. Ltd,
- Ultra Sonicator, Trans-o-sonic, India,
- Digital pH Meter, Analab Scientific Instruments Pvt. Ltd,
- Hot air oven, EIE Instruments Pvt. Ltd,
- Blower: ORPAT®, Ajanta Ltd,

5.2. MATERIALS AND REAGENTS

5.2.1. Material

Pure Esomeprazole magnesium trihydrate (purity-99.7%) was obtained as a gift sample from Torrent Research Centre, Bhat, Gujarat, India.

5.2.2. Reagents and Chemicals

- Methanol, 1,4- dioxane, toluene, sodium hydroxide, hydrochloric acid, hydrogen peroxide (30% v/v) were used of AR grade (S.D. Fine chemicals Ltd., Mumbai, India) and distilled water were used.
- Marketed tablet formulation "Neksium" (Astra Zeneca Pharma India Ltd., Mumbai), containing ESOM 40 mg was purchased from local market.

5.3. METHOD

5.3.1. Optimized chromatographic conditions

- Stationary phase: Precoated silica gel on aluminum plate 60_{F254}, (10cm x 10cm), prewashed by methanol and activated at 60 °C for 20 min prior to chromatography.
- Mobile phase: 1,4-Dioxane: Toluene: Methanol (6:4:1, v/v/v)

- Quantity of mobile phase: 10 ml
- TLC chamber saturation time: 20 min
- Run length: 80 mm
- Development Time: 17 min
- Application rate: 0.1 µl/s
- Scanner band width: 5 mm
- Slit dimension: 4 mm x 0.45 mm (micro)
- Scanning speed: 20 mm/s
- Detection: Densitometrically using a UV detector at 304 nm
- Temperature: Ambient
- Evaluation mode: Absorbance mode

5.3.2. Preparation of Standard Stock Solution

Standard ESOM (25 mg) was accurately weighed and transferred to 25 ml volumetric flask. It was dissolved properly and diluted up to mark with Methanol to obtain stock solution of concentration 1000 µg/ml.

From this solution, working standard solution was prepared by taking 1ml standard stock solution and diluting it with methanol up to 10 ml to obtain concentration of 100 µg/ml.

5.3.3. Preparation of Sample Solution

Quantity of tablet powder (305.67 mg) equivalent to 25 mg of ESOM was weighed and transferred to a 25 ml volumetric flask containing about 10 ml of methanol, ultrasonicated for 5 min, filtered by whattman filter paper No. 44, filter paper was washed with methanol and washing was transferred to flask and volume was made up to the mark with methanol to get sample stock solution of 1000 µg/ml.

From this solution, working sample stock solution was prepared by taking 1ml and diluting it to 10 ml with methanol to get concentration 100 µg/ml.

5.3.4. Preparation of Mobile Phase

1,4- Dioxane (6 ml) and toluene (4 ml) were mixed and then methanol (1 ml) was added and was mixed properly.

5.4. GENERATION OF STRESS SAMPLE

5.4.1. Optimization of stress conditions

Acid hydrolysis studies were carried out in different conc. (0.05N, 0.1N, 1N) of HCl in atmospheric conditions for 5 min to get sufficient degradation. Aliquots were withdrawn periodically and subjected to analysis after dilution with methanol to obtain concentration of 100µg/ml.

Similarly, the studies in alkaline conditions were carried out in different conc. (0.1N, 1N, 3N) of NaOH in atmospheric conditions and by refluxing at 95 °C for 3 hours to get sufficient degradation. Aliquots were withdrawn periodically and subjected to analysis after dilution with methanol to obtain concentration of 100µg/ml.

Neutral hydrolysis studies were carried out in water by heating at 80 °C for 4 hours to get sufficient degradation. Aliquots were withdrawn periodically and subjected to analysis after dilution with methanol to obtain concentration of 100µg/ml.

The oxidative stress studies were carried out in 3% H_2O_2 for 24 hours at room temperature (ambient) to get sufficient degradation. Aliquots were withdrawn periodically and subjected to analysis after dilution with methanol to obtain concentration of 100µg/ml.

Photolytic studies were done in solid state by spreading a thin layer of drug in a petri-plate as well as in solution state by preparing 1 mg/ml solution in Water and exposing them directly to sunlight for 6 hours to get sufficient degradation.

For thermal stress testing, the drug powder was subjected to dry heat at 100ºC for 96 hours to get sufficient degradation.

5.4.2. Preparation of Stress Sample

ESOM (25 mg) was taken in 25 ml. volumetric flask and 25 ml of stressor was added. Solution was sonicated and transferred in round bottom flask. For zero time study, 1 ml sample was pipetted out. Solution was heated and refluxed for respective temperature used in various stress conditions. At different time interval, 5 ml sample was pipetted out. For acid-base hydrolysis sample, solutions were neutralized using pH meter. 1 ml of solution was taken in 10 ml volumetric flask and diluted with methanol up to the mark having final concentration of 100µg/ml.

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

Table 5.1.: Optimized Degradation Condition Parameters

Degradation type	Stressor	Time period	Temperature
Acid hydrolysis	0.05N HCl	5 min	Ambient
Base hydrolysis	0.1N NaOH	3 hours	Reflux at 95 °C
Neutral hydrolysis	Water	4 hours	Heat 80 °C
Oxidation	3% H_2O_2	24 hours	Ambient
Thermal degradation	Dry heat	96 hours	100 °C
Photolytic degradation (aqueous condition)	Direct sunlight	6 hours	-
Photolytic degradation (solid form)	Direct sunlight	24 hours	-

5.5. SELECTION OF WAVELENGTH FOR QUANTIFICATION

Standard ESOM solution of 100 µg/ml (7 µl volume) wwas applied on TLC plate. The plate was developed, dried and scanned by Scanner III using UV detector in the range of 200-400 nm. The UV scanned spectra is shown in figure 5.1. The spectrum of ESOM showed maximum absorbance at 304 nm and hence 303 nm was selected as detection wavelength.

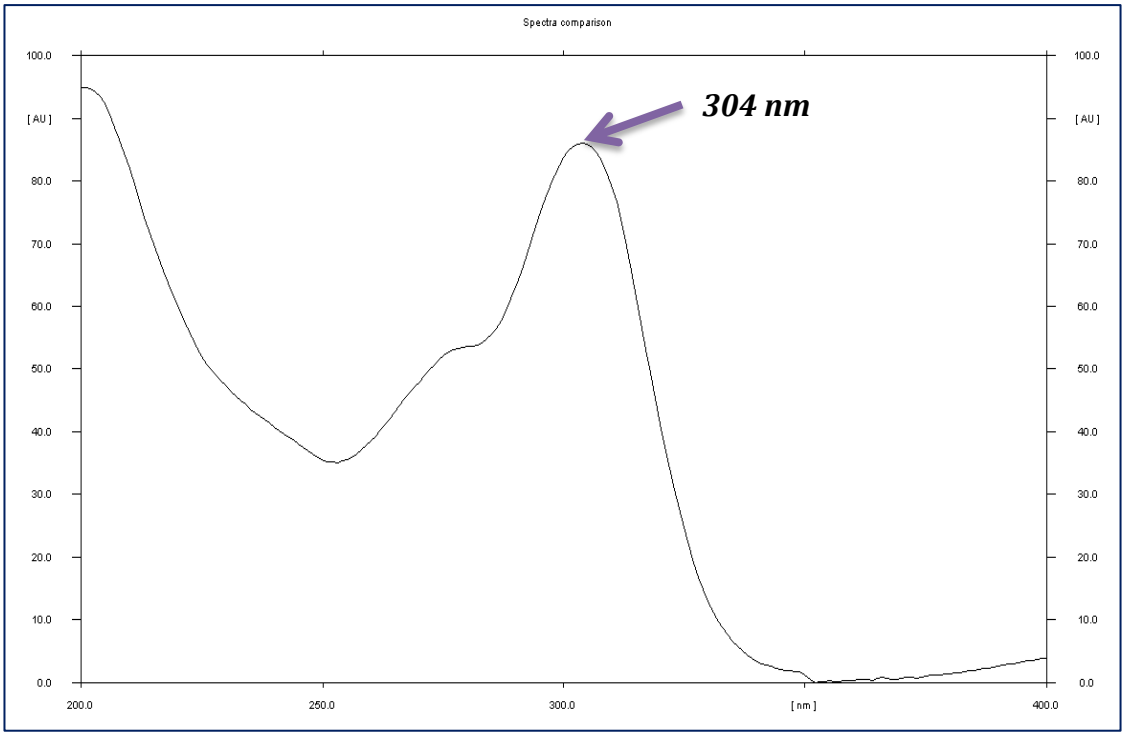

Figure 5.1.: UV scanned spectra of ESOM (100 µg/ml) in methanol

5.6. METHOD VALIDATION

5.6.1. Preparation of Calibration curve of ESOM

From working standard ESOM solution, appropriate volumes (3, 7, 9, 11, 13, 15, 17, 19 µl) were spotted on the TLC plate to obtain final concentration 300-1900 ng/spot of ESOM. Each concentration was spotted 3 times on the TLC plate. The plate was developed in optimized conditions as per section 5.3.1. Peak areas were plotted against corresponding concentrations to obtain the calibration graph.

5.6.2. Linearity

The linearity was taken in the range of 300-1900 ng/spot of ESOM. The solution for linearity was prepared as per section 5.6.1. Peak areas of each concentration were measured and plot of peak area vs. concentration were plotted. The regression equation was determined.

5.6.3. Precision

5.6.3.1. Repeatability of Sample Scan

Repeatability of sample scan was determined by scanning 700 ng/Spot, 6 times and peak area was recorded. Repeatability of sample scan was measured in terms of %RSD.

5.6.3.2. Repeatability of Sample Application

Repeatability of sample application was determined by spotting the standard solution (700 ng/spot) 6 times on the same plate and peak area was recorded. Repeatability of sample application was measured in terms of %RSD.

5.6.3.3. Intermediate Precision (Intraday Precision and Interday Precision)

The intermediate precision of the method was checked by repeating studies on three different days and 3 times on single day for 3 different concentrations (700, 1200, 1700 ng/spot) and were applied in three replicate manner.

5.6.4. Accuracy

Accuracy of the method was determined by recovery study by standard addition method at three different levels (80%, 100% and 120%).

Preparation of standard solution:

ESOM standard (25 mg) was weighed and transferred accurately in 25 ml volumetric flask and 15 ml methanol was added. It was sonicated for 2 min and volume was made up to mark with methanol to get concentration of 1000 µg/ml. 1 ml was taken in 10ml volumetric flask and volume was made up to mark with methanol to obtain concentration of 100µg/ml.

Level-1 (80%):

From standard stock solution, take 0.8 ml solution in 10 ml volumetric flask to prepare 180 µg/ml solution. Then spike this solution with 1 ml of sample solution. Make up the volume with methanol up to the mark and mix it well to obtain final concentration of 180 µg/ml. Sonicate the solution.

Level-2 (100%):

From standard stock solution, take 1 ml solution in 10 ml volumetric flask to prepare 200 µg/ml solution. Then spike this solution with 1 ml of test solution. Make up the volume with methanol up to the mark and mix it well to obtain final concentration of 200 µg/ml. Sonicate the solution.

Level-3 (120%):

From standard stock solution, take 1.2 ml solution in 10 ml volumetric flask to prepare 220 µg/ml solution. Then spike this solution with 1 ml of test solution. Make up the volume with methanol up to the mark and mix it well to obtain final concentration of 220 µg/ml. Sonicate the solution.

5.6.5. Specificity

Interference from degradation products is formed by stress condition, was checked by specificity study.

Purity of the ESOM and degradation products were checked by scanning in the range of 200-400 nm with the help of spectra scanning mode of the WinCATS software. The peak purity of ESOM and degradation products were determined by comparing the spectrum at three different regions of the spot i.e. peak start (S), peak apex (M), and peak end (E) positions which confirms that the peak represents pure single component for ESOM and also for its degradation products.

5.6.6. Robustness

Change following parameters one by one and observe their effect on system suitability.

- *Change in the Mobile phase composition (±5%)*

  Change in mobile phase composition was done by ±5% volume of 1,4 – dioxane with toluene and volume of methanol was kept constant.
- *Change in Run length (± 5 mm)*
  - *Optimum run length : 80 mm*
- *Change in Saturation time (± 5 min)*
  - *Optimum saturation time :20 min*
- *Change in Wave length (± 2 nm)*
  - *Optimum wave length: 304 nm*

5.6.7. Limit of Detection and Limit of Quantification

For determination of LOD/LOQ, all linearity range solution was prepared in triplicate as described in section of linearity. Chromatograms of all these solutions are recorded. 3 linearity curves of Peak area v/s concentration (ppm) were plotted. From these curves, mean slop value was calculated from 3 intercept value, SD of intercept was calculated. From all these values, LOD/LOQ were calculated and reported.

5.6.8. Analysis of ESOM in Marketed Formulation

From the working sample stock solution containing 100 µg/ml of ESOM, the volume of 7 µl was applied on the TLC plate followed by development and scanning as described under section 5.3.1. The amount of drug was calculated from regression equation.

CHAPTER 7

COMPARISON

7.1. LINEARITY

ESOM (25 mg) was weighed and dissolved in the diluent, Acetonitrile : Phosphate buffer (50:50, v/v) and volume was made up to the mark with the diluent to get concentration of 1000 µg/ml. From this, aliquots of 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 ml were transferred to the 10 ml of volumetric flasks and the volume was made up to the mark with the diluent to get the concentration of 2, 4, 6, 8, 10 and 12 µg/ml. Linearity range was found to be 2-12 µg/ml with correlation coefficient 0.9992.

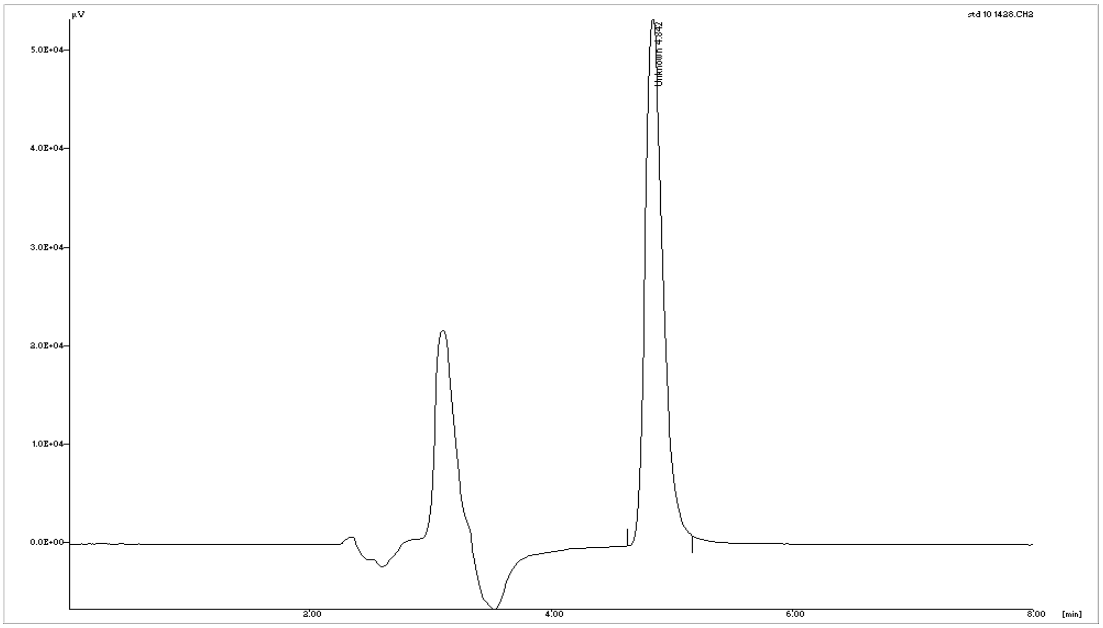

Figure 7.1.: HPLC Chromatogram of standard ESOM (10 µg/ml)

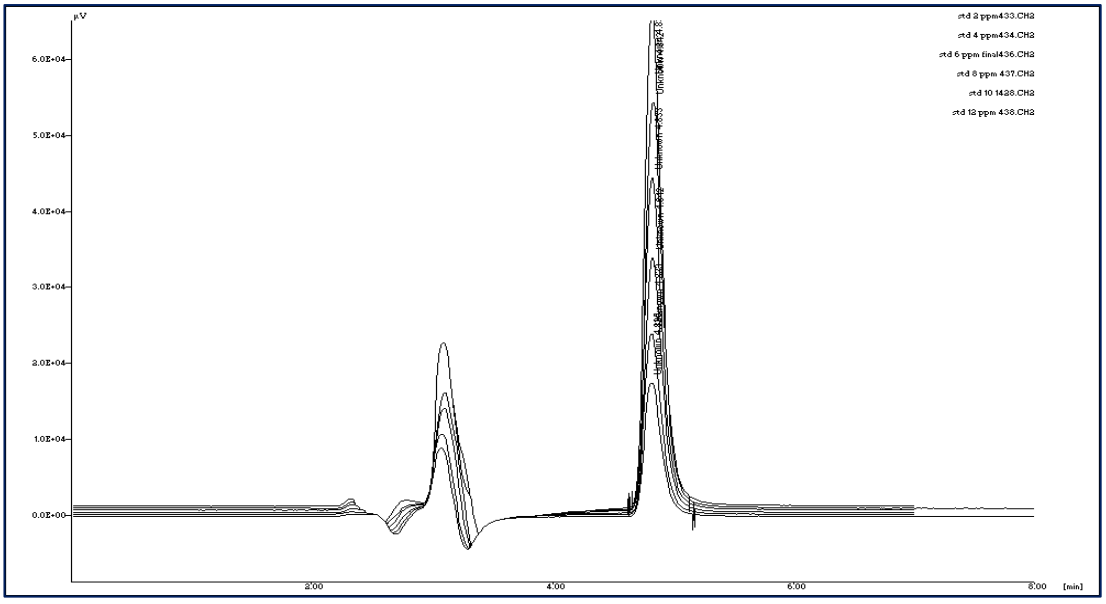

Figure 7.2.: Linearity of ESOM standard (2-12 µg/ml)

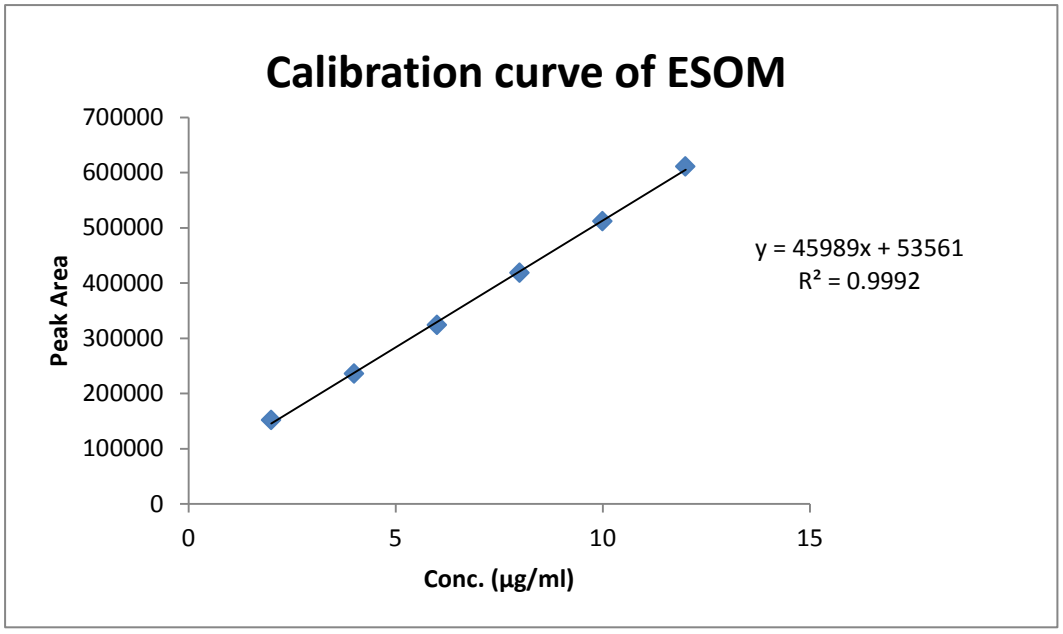

Figure 7.3.: Calibration curve of ESOM (2-12 µg/ml)

7.2. ASSAY OF MARKETED FORMULATION

The assay of the marketed dosage form was carried out with the five determinations of the brand name of "Neksium". The result of percentage assay is shown in following table.

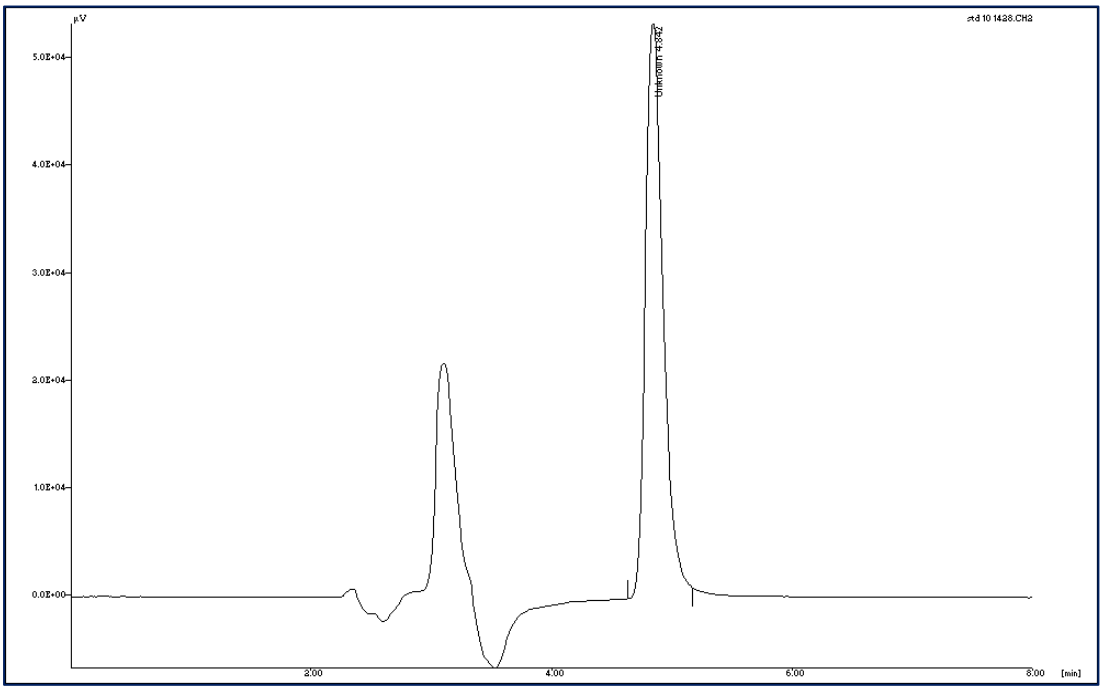

Figure 7.4.: HPLC chromatogram of marketed formulation of ESOM (10 µg/ml)

7.3. STATISTICAL COMPARISON OF DEVELOPED HPTLC AND REPORTED RP-HPLC METHOD

Linearity was performed for reported RP-HPLC method and % assay was calculated. Result of % assay was found acceptable. RP-HPLC and developed HPTLC methods were compared by % assay value.

Table 7.1.: Comparison of developed HPTLC and reported RP-HPLC Data

Brand name	Parameters	HPTLC method	HPLC method
Neksium tablets	Linearity	300-1900 ng/spot	2-12 µg/ml
	Regression Equation	y = 7.9857x + 5624.7	y = 45989x + 53561
	Correlation Coefficient (R^2)	0.9989	0.9992
	%Assay	100.80%	101.15%
		R_f = 0.59 ± 0.02	R.T. = 5.05 ± 0.09
	Mean recovery	100.46%	98.76%
	%R.S.D.	< 2	< 2

Table 7.2.: Statistical Comparison of developed HPTLC and reported HPLC methods by Student unpaired t-test

Parameter	Developed HPTLC Method	Reported HPLC Method
Mean	100.80	101.15
Variance	1.5	1.44
No. of Observation	3	
Degree of Freedom	4	
t-cal value	0.00284	
t-table value	2.132	

Inference from t-test:

Comparison of developed HPTLC method with reported RP-HPLC method was performed by student unpaired t-test. T-calculated value was found to be less than T-table value. This shows that there is no significant difference between developed HPTLC and reported RP-HPLC methods.

7.4. CONCLUSION

The developed HPTLC method was simple, rapid, accurate, precise, specific and has the ability to separate ESOM and other degradation products formed during different stress conditions. Hence the method is stability indicating assay method.

The results of validation parameters are satisfactory which indicates the reliability of proposed method for estimation of ESOM in routine analysis.

The method allows for the application in the quality control laboratory for routine analysis as well as for the stability studies of the drug substance.

The results of t-test reveals that HPTLC method could be used as an alternative to conventional HPLC method for analysis of bulk drugs and pharmaceutical dosage forms as it is relatively faster than RP-HPLC.

CHAPTER 8

SUMMARY

A stability indicating HPTLC method was developed and validated for determination of ESOM in presence of its degradation products. The optimization of the mobile phase was done by doing different trials using various solvents individually and also in combinations. Optimized mobile phase was 1,4 - Dioxane : Toluene : Methanol (6:4:1, v/v/v). The method is based on high performance thin layer chromatography using Precoated Silica Gel 60_{F254} Plates with 250 μm thickness. The separation in all the degradation peaks were achieved by using 1,4 - Dioxane : Toluene : Methanol (6:4:1, v/v/v) as mobile phase. Detection was carried out at 304 nm wavelength. The R_f of ESOM was 0.59 ± 0.02.

Forced degradation was carried out according to ICH Guidelines Q1A (R2) and Q1B. The objective of the study was to find out the likely degradation products which in turn help in predicting the degradation pathway and intrinsic stability of molecule.

Stability indicating capability is established by forced degradation of ESOM to acid, alkali, oxidation, thermal and photo degradation.
The peaks of degraded products were well separated. The linear regression analysis data for the calibration plots showed good linear relationship with concentration range of 300-1900 ng/spot with the linearity equation y = 7.9857x + 5624.7 with the correlation co-efficient of 0.9989.

The method is validated for specificity, accuracy, precision, linearity, repeatability and robustness as per ICH guideline Q2 (R1) method validation. The limit of detection and quantitation were 47.05 ng/spot and 121.16 ng/spot with the signal to noise ratio of 3 and 10 respectively.

As the method could effectively separate the drug from its degradation products, it can be employed as a stability indicating method.

CHAPTER 9

FUTURE SCOPE

- For ESOM, newer and simpler methods like quantification using Raman spectroscopy, near IR spectroscopy could be developed.
- Impurity profiling analytical method (HPLC, HPTLC) for ESOM can be developed.
- The combination of ESOM and domperidone has been in market since many years. Conventional methods like UV, HPLC and HPTLC have been reported for this combination.
- Stability indicating HPLC and HPTLC method for simultaneous determination of ESOM and domperidone in their combined dosage form has not been reported till date, hence it can be further developed.

CHAPTER 10

REFERENCES

1. Rang H. P., Dale M.M.; Ritter J.M., Textbook of Pharmacology, Churchil Livingstone Publiations, 5th Edition, 2006, 370-372.

2. Tripathi K. D., Essentials of Medical Pharmacology, 2008, 6th Edition, Jaypee Publication, 587-589, 591-593.

3. http://www.medicinenet.com/peptic_ulcer/article.htm (Accessed on 25th Feb 2012).

4. Indian Pharmacopeia; Government of India, Ministry of Health and Family Welfare; The Indian Pharmacopeial Commission; 2010, 2, 1295.

5. United States Pharmacopeia; USP33-NF28; United States Pharmacopoeial Convention; 2010, 2, 2095.

6. http://www.chemicalbook.com/ProductChemicalPropertiesCB9740619_EN.htm (Accessed on 25th Feb 2012).

7. The Merck Index. 14th Edition, Merck & Co., Inc., Whitehouse Station, NJ, pp. 6845.

8. http://www.rxlist.com/nexium-drug.htm (Accessed on 4th Feb 2012).

9. http://www.health.gov.au/internet/main/publishing.nsf/Content/pbac-psd-esomeprazole-nov05 (Accessed on 2nd March 2012).

10. http://www.chemblink.com/products/217087-09-7.htm (Accessed on 2nd Mar 2012).

11. http://en.wikipedia.org/wiki/Esomeprazole (Accessed on 24th Feb 2012).

12. Josyula L., Development and Validation of Stability Indicating RP-HPLC Method for Esomeprazole Magnesium Trihydrate in Bulk Fom, May 2011.

13. Singh S.S., Bakshi M., Development of Validated Stability Indicating Assay Methods: Critical Review. J. Pharm. Biom. Anal. 2002, 28, 1011 – 1040.

14. Food and Drug Administration. Guidance for Industry: Analytical Procedures and Methods Validation (Draft guidance). 2000, 501 – 4.

15. ICH, Q2 (R1). Validation of Analytical Procedures: Text and Methodology: 2005.

16. FDA, Guidance for Industry Q1A (R2) Stability Testing of New Drug Substances and Products. 2003.

17. FDA, Guidance for Industry ICH Q1B: Stability Testing: Photostability Testing of New Drug Substances and Products.

18. Green J.M., A Practical Guide to Analytical Method Validation. . Anal Chem. 1996, 68, 305A – 309A.

19. Chandran S., Singh RSP.: Comparison of various international guidelines for analytical method validation. Pharmazie 2007, 62, 4 – 14.

20. Giddings J. C., Unified Separation Science, Willey & Sons Ltd, New York, USA, 1991.

21. Poole C.F., and Poole K., Chromatography Today, Elsevier, Amsterdam, The Netherlands ,1991.

22. US FDA. Technical Review Guide: Validation of Chromatographic Methods. 1993.

23. Wall PE. Thin Layer Chromatography- A Modern practical approach, Royal chemical society, 7th Edition, 126-175.

24. Sethi PD. HPTLC- Quantitative Analysis of Pharmaceutical Formulations, 1st Edition, CBS publisher.

25. Practical Thin-Layer Chromatography A Multidisciplinary Approach. CRC Press: 1996, 231.

26. Handbook of Thin-Layer Chromatography. Second ed.; Marcel Dekker, Inc.: Vol. 71.

27. Handbook of Instrumental Techniques for Analytical Chemistry. Prentice-Hall, Inc.: 1997.

28. Mendham J., D. R. C., Barnes J. D., Thomas M., Vogel's Textbook of Quantitative Chemical Analysis. 6th edition.; Pearson Education.

29. Jain D., Jain N., Charde R., Jain N., The RP-HPLC Method For Simultaneous Estimation of Esomeprazole and Naproxen in Binary Combination, A Pharmaaceutical Analysis Journal by Inpharm Association, Pharmaceutical Methods, 2011, 2(3), 167-172.

30. Önal A., Development and Validation of High Performance Liquid Chromatographic Method for The Determination of Esomeprazole in Tablets, Journal of Food and Drug Analysis, 2006, 14(1), 12-18.

31. Patel B. H., Suhagia B. N., Patel M. M. and Patel J. R., Determination of Pantoprazole, Rabeprazole, Esomeprazole, Domperidone and Itopride in Pharmaceutical Products by Reversed Phase Liquid Chromatography Using Single Mobile Phase, Chemistry and Material science Chromatographia,65 (11-12), 743-748.

32. United States Pharmacopeia, USP33-NF28,United States Pharmacopoeial Convention, 2010, 2, 3365.

33. Rathi G. G., Singh R. K., Patel P. S., Singh R. K. And Kumar B., RP-HPLC Method For The Estimation of Esomeprazole Magnesium in Bulk and Its Pharmaceutical Dosage Forms, International Journal Of Pharmaceutical Sciences And Research, 2010, 1(6), 75-81.

34. Dalindre H.N., Thorve R.R., Bugdane P.M., Validated HPTLC Method For Simultaneous Estimation of Esomeprazole And Domperidone In Tablets, Journal of Analytical Chemistry, 2008, 7(6).

35. Putta R. K., Shyale S., Physico-chemical characterization, UV spectrophotometric method development and validation studies of Esomeprazole Magnesium Trihydrate, J.Chem.Pharm.Res., 2010, 2(3), 484-490.

36. Trivedi P. D., Maheshwari D. G,, Estimation of Esomeprazole and Domperidone by absorption ratio method in Pharmaceutical Dosage Forms, International Journal of ChemTech Research, 2010, 2(3), 1598-1605.

37. Sharma M. C., Sharma S., Spectrophotometric Methods for the Estimation of Esomeprazole magnesium trihydrate in Pharmaceutical Formulations Using

Indigo Carmine Reagent, International Journal of PharmTech Research, 2011, 3(2), 1186-1190.

38. Lakshmana Prabu S., Shirwaikar A., Shirwaikar A., Kumar C. D., Simultaneous estimation of esomeprazole and domperidone by UV spectrophotometric method, Indian J Pharm Sci 2008, 70, 128-31.

39. Patil S. S., Dhabale P. N. and Kuchekar B. S., Development and Statistical Validation of Spectrophotometric Method for Estimation of Esomeprazole in Tablet Dosage Form, Asian J. Research Chem., 2009, 2(2), 154-156.

40. Solanki S., Captain A and Patel V. B., Simultaneous determination of Domperidone and Esomeprazole magnesium in Pharmaceutical Capsule Formulation by Derivative Spectrophotometric Method, International Journal of ChemTech Research, 2011, 3(4), 1747-1750.

41. Hultman Ia, Stenhoff H. and Liljeblad M., Determination of esomeprazole and its two main metabolites in human, rat and dog plasma by liquid chromatography with tandem mass spectrometry, Journal of Chromatography B, 2007 848(2), 317-322.

42. Rahman N., Bano Z. and Azmi S. N. H., Spectrophotometric Determination of Esomeprazole Magnesium in Commercial Tablets Using 5-Sulfosalicylic Acid and N-Bromosuccinimide, Journal of the Chinese Chemical Society, 2008, 55 (3), 557-566.

43. http://www.chemicalbook.com/ProductChemicalPropertiesCB9740619_EN.htm (Accessed on 25th Dec 2011).