STABILITY INDICATING RP-HPLC AND HPTLC METHODS FOR THE ESTIMATION OF ZOLMITRIPTAN

Dissertation Submitted to

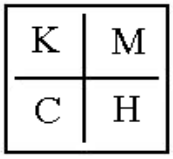
The TamilNadu Dr. M.G.R. Medical University, Chennai

In partial fulfillment for the requirement of the Degree of

MASTER OF PHARMACY

(Pharmaceutical Analysis)

April -2012



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CERTIFICATE

This is to certify that the dissertation work entitled**. 'STABILITY INDICATING RP-HPLC AND HPTLC METHODS FOR THE ESTIMATION OF ZOLMITRIPTAN',** is a bonafide research work carried out by **Tinu Thomas** to The TamilNadu Dr. M.G.R. Medical University, Chennai, in partial fulfillment for the Degree of **Master of Pharmacy in Pharmaceutical Analysis** at the Department of Pharmaceutical Analysis, KMCH College of Pharmacy, Coimbatore, TamilNadu under my guidance and supervision during the academic year 2011-2012.

Date: **Dr.A.Rajasekaran,M.Pharm., Ph.D.,**

Principal

DECLARATION

I do hereby declare that the dissertation work entitled **'STABILITY INDICATING RP-HPLC AND HPTLC METHODS FOR THE ESTIMATION OF ZOLMITRIPTAN'** submitted to The TamilNadu Dr. M.G.R. Medical University, Chennai, in partial fulfillment for the Degree of Master of Pharmacy in Pharmaceutical Analysis**,** was done by me under the guidance of **Prof. Dr. A.Rajasekaran, M.Pharm., Ph.D.,** at the Department of Pharmaceutical Analysis, KMCH College of Pharmacy, Coimbatore, during the academic year 2011-2012.

Date:

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EVALUATION CERTIFICATE

This is to certify that the dissertation work entitled **'STABILITY INDICATING RP-HPLC AND HPTLC METHODS FOR THE ESTIMATION OF ZOLMITRIPTAN'** submitted by ***Tinu Thomas*** University **Reg. No: 26107228** to The Tamil Nadu Dr. M.G.R. Medical University, Chennai, in partial fulfillment for the Degree of Master of Pharmacy in Pharmaceutical Analysis is a bonafide work carried out by the candidate at the Department of Pharmaceutical Analysis, KMCH College of Pharmacy, Coimbatore and was evaluated by us during the academic year 2011-2012.

Examination Center: KMCH College of Pharmacy, Coimbatore.

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TINU THOMAS

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ABBREVIATIONS

HPLC	High performance liquid chromatography
HPTLC	High performance thin layer chromatography
ICH	International conference on harmonization
API	Active pharmaceutical ingredients
ZMT	Zolmitriptan
USP	United State Pharmacopeia
SD	Standard deviation
RSD	Relative standard deviation
UV	Ultra violet
PDA	Photo diode array
NaOH	Sodium hydroxide
HCL	Hydrochloric acid
H_2O_2	Hydrogen peroxide
CONC	Concentration
Fig	Figure
Tab	Table
HETP	Height equivalent to theoretical plate

CHAPTER I

INTRODUCTION[1-26]

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Analytical chemistry has been defined as the science of determining the composition of materials in terms of the elements or compounds contained in the samples. The main goal of the analytical chemistry is to gather information about qualitative as well as quantitative compositions of chemical substances.

Pharmaceutical analysis deals with the analysis (qualification and quantification) of pharmaceutical substances. Pharmaceutical analyst in research and development (R&D) of pharmaceutical industry play an inevitable part in the development of a new drug product or a molecule and to follow up the activities to assure that, a new product meets the established standard and quality throughout its shelf life.

Before submitting a drug product for approval to the regulatory authority, all the batches of the products should comply with the specific standards. It is the duty of the pharmaceutical analyst in quality control (QC) and quality assurance (QA) departments to check whether it is complying with the standard or not. The methods are generally developed in the analytical R&D and to QC department or other departments.

Quality Assurance and Quality Control plays a key role in developing the safety and efficacy of the drugs. A highly specific and sensitive analytical technique holds the key to design, development, standardization and quality control of drug products. This is very much important in pharmacokinetics and metabolism of drugs, and is very much essential for the determination of bioavailability.

The pharmaceutical analyst plays a vital role in assuring the identity, safety, efficacy and quality of the drug products. Safety and efficacy study require that drug substances and drug product meet two critical appropriate requirements

- Established identity and purity
- Established bioavailability and dissolution

Common techniques for analysis[2-3]

The main techniques employed for quantitative analysis are based upon,

a) Suitable chemical reaction based on either the amount of reagent needed to complete the reactions or the amount of reaction product obtained.

Eg : Neutralization, Complex metric titration, Precipitation titration, Redo titration

b) Appropriate electrical measurements, which involves the measurement of current, voltage or resistance in relation to the concentration of a certain species in solution.

Eg : Voltammeter, Potentiometer, Conductometry

c) The measurement of certain optical properties which depends either upon

- Measurement of the amount of radiant energy of a particular wavelength of a particular wavelength absorbed by the sample
- On the emission of radiant energy and measurement of the amount of energy of a particular wavelength emitted.

Eg: Visible spectrophotometry, Ultraviolet spectrophotometry, Infrared spectrophotometry

Separation process employed for the separation of mixture of substances and for the identification of components

d) Chromatography

Eg: Gas chromatography, HPLC, HPTLC

e) X-ray methods

When high-speed electrons collide with a solid target, X-rays are produced. From the remittent x-ray emission, it is possible to identify certain emission peaks, which are characteristics of elements contained in the target. The wavelength of the peaks can be related to the atomic numbers of the elements producing them.

f) Radioactivity

The intensity of a radiation from a naturally radioactive substances or an induced radioactive substance arising from the exposure of the sample to a neutrons source is measured.

g) Mass spectroscopy

Sample to be determined are bombarded under a high energy electron beam. The formed fragments are separated by electric and/or magnetic field based on their m/e ratio and to determine their molecular mass.

h) Optical methods

I. Refractometer - refractive index of liquids are determined

II. Optical rotation- for the determination of optically active compounds

i) Thermal methods

It keeps temperature as a function to measure the changes

Eg : Thermogravimetry, Differential scanning calorimetry, Differential thermal analysis, Thermo mechanical analysis

Types of HPLC Methods[7]

- Normal phase Chromatography
- Reverse-Phase Chromatography
- Ion exchange Chromatography
- Affinity Chromatography
- Size exclusion Chromatography

Normal Phase Chromatography[10]

Normal phase chromatography is the technique where, the stationary phase is polar and the mobile phase is non-polar (organic solvents). In this phase the less polar component will elute faster than the more polar components. Normal phase chromatography is very much similar to thin layer chromatography or column chromatography. Even though this is termed as normal phase, it is not the most widely used chromatographic technique. The column should be filled with tiny silica particles and the solvents used are non-polar. A normal phase column usually has an internal diameter of 4.6 mm and a length of about 150 -200 mm. in this case more polar compounds will show more affinity towards stationary phase and will take more time to elute. The non-polar compounds will elute faster. The non-polar drugs are usually estimated by this normal phase chromatographic method.

Reverse-Phase Chromatography

RP-HPLC is the most widely used HPLC technique in the pharmaceutical analysis, because compounds will elute faster with high resolution. In this technique, the non-polar stationary phase and polar solvents are used. Usually polar drugs are analyzed by this

technique. Here the more polar compounds will elute faster because of its less affinity towards the stationary phase. The separation is based on the strength of the solvent, column temperature, pH etc. The RP-HPLC method is the most widely used mode of HPLC.

Normal Phase Chromatography Vs Reverse-Phase Chromatography

The adsorption of analyte to the stationary phase is by hydrophobic effect in RP-HPLC method. The reverse phase indicates that the stationary phase is less polar than the solvent and the normal phase indicates that the stationary phase is more polar than the solvent. In general, adsorption of a solute to reverse phase is by hydrogen bonding between the solute and stationary phase.

Normal phase – Non-polar Mobile Phase: Polar Stationary Phase
Reverse Phase- Polar Mobile Phase: Non-Polar Stationary Phase

In RP-HPLC, solute retention is mainly due to hydrophobic interaction between the solute and the non-polar hydrocarbon stationary phase. The non-polar components of the sample interact more with the relatively non-polar hydrocarbon column packing and thus elute than the polar components. The hydrogen bonding predominates than any other interactions in the normal phase. The elution of solutes is in the order of decreasing polarity in RP-HPLC method, i.e, increasing hydrophobicity, while in mobile phase the least polar compound will elute first. Before beginning with actual experimentation, it would be advantageous to view method development from a broader perspective. The method development process can be visualized from a high-level map perspective better to define the general steps encountered to achieving the end product, a stability-indicating method.

QUANTITATION

Quantitation methods in HPLC[25]:

From the detector signals, the peak height and peak area can be measured. This measurement will give an account about the concentration or mass of the compound. To establish this, some sort of calibration should be performed.

The primary techniques for quantitation are:

1. Normalized peak area method
2. External standard method
3. Internal standard method
4. Method of standard addition

1. Normalized peak area method

The peak area of any one of the peak is referred to the normalized peak area. To determine the degradation product or impurity in a product, this method is used. The response factor for each component is identified in this method.

2. External standard method

Both standard and unknown samples are injected inn this method. The unknown can be determined from a calibration graph or from calculating response factor.

The response factor Rf can be calculated from the following formula-

$$\text{Rf} = \frac{S\text{tan}dard\ Area\ (Peak\ height)}{S\text{tan}dard\ Concentration}$$

If there is no extensive sample preparation is needed. The external standard method id preferred. The chromatographic conditions should be maintained constant during the separation of all standards and samples for the better quantition using external standard method. External standard methods are mainly used to ensure that the total chromatographic system is performing properly and can provide reliable results.

3. Internal Standard Method

A compound which is differ from analyte is used as the internal standard. But that compound should be well resolved in the separation. The purpose of internal standard is just to mimic the nature and behavior of the sample. Mainly the internal standard is used for the compounds which needs a pre-treatment or preparation.

To determine the concentration of sample component in the original sample, the Response Factor is used. Response Factor is defined as the ratio of peak area of sample component (Ax) and the internal standard (AISTD). The formula is as follows,

$$Rf = \frac{A_x}{AI_{std}}$$

Based on the response factor and strength of the internal standard (N_{ISTD}), the amount of the analyte in the original sample can be calculated from the formula,

$$X = \frac{A_x}{R_F \bullet A_{ISTD}} \times N_{STD}$$

4. Method Of Standard Addition

The method of standard addition can be used to provide a calibration plot for the quantitative analysis. Trace analysis is making use of this method mostly. An important aspect of this method is that the response prior to spiking additional analytes should be high enough to provide a reasonable S/N ratio (>10), otherwise the result will have poor precision.

Reason for selecting HPLC[11]

1. A wide variety of packing material are available, which allows the separation of most of the compounds. The phases that are most commonly used for the drug substances of low molecular weight (< 1000) and their metabolites or degradants are the absorption systems based on silica and the reversed phase systems based on octyl silyl or octa decyl silyl bonded on silica.
2. A good choice of detectors are available which permit the sensitive detection of most chemical type, and the accuracy and precision with which eluted substances may be quantified give analytical data of the highest caliber.
3. Since the size of the packaging materials are in micron size, they will provide an excellent separation.
4. Since a standard analytical column provide about 5000-10000 theoretical plate, it provides adequate resolution for the effective separation of the compound.
5. For routine analysis, we can use short columns (3-10cm) which facilitates a faster separation within a short time. Combination of HPLC along with some spectrophotometric techniques(UV,IR,MS) can achieve a good range of separation.

The Detector[12]

The substances that are passed through a column can be detected by several ways. The UV-absorption method is the widely used method of detection. Many organic compounds absorb UV light of various wavelengths. The measurement of absorbed UV light can be directly determined from the UV detector. The amount of light absorbed is directly depend on the amount of particular compound passing through the beam at a specific time.

UV-Visible Detector (Diode Array Detector-DAD)

- In RP-HPLC method wide variety of detectors like UV-Visible spectrophotometers, refractive index detectors (RI), electrochemical detector (EC), evaporative light-scattering detector (ELS), fluorimeters and others.
- Due to the presence of grating assembly after the floe cell, the light of different wavelength can be measured simultaneously. DADs therefore provide more information on sample composition than provided by a single wavelength run.
- The optimum wavelength for the final HPLC is obtained from the UV spectrum of each separated peak. The peak purity can be evaluated from examining the UV spectrum from beginning to end. The use of DADs is also important for a peak tracking that contain the same compound between different experimental runs during method development.[7]

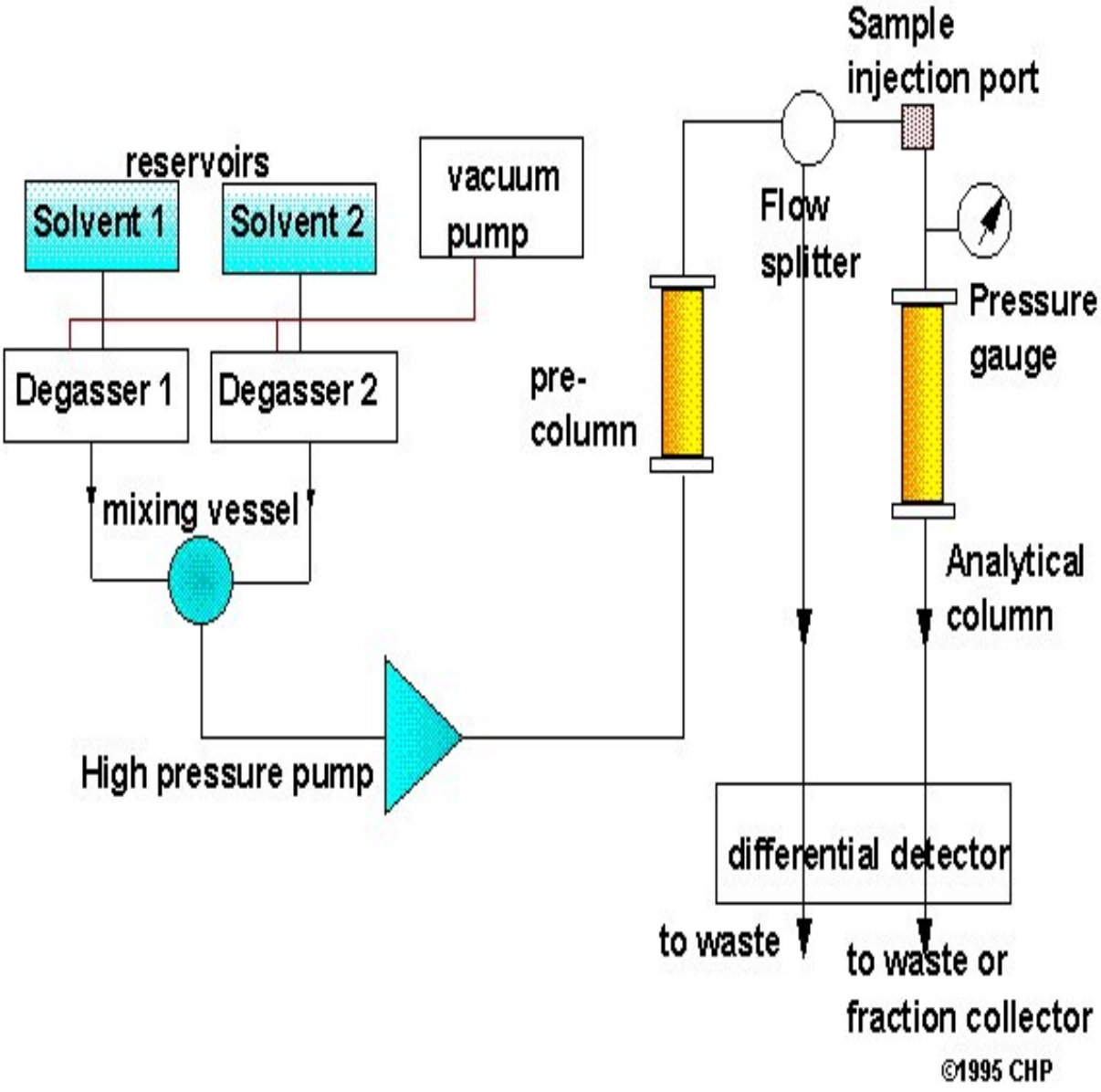

Fig 1: Schematic representation of HPLC

HPLC Vs Other Methods[14]

- The main objective of the pharmaceutical industry is to produce pure drug substances which are suitable for the human consumption in a cost effective manner. Inorder to check the purity of all pharmaceutical products, the separation methods like GC, TLC, HPLC etc are introduced. All these techniques are more sensitive than spectroscopic methods.
- If the compound shows good absorption in between 200-400 nm wavelength, the UV method is preferred. In this case, the degraded products or its excipients do not affect the absorption.
- Due to ease of separation, reproducibility and rapid separation, the HPLC method is preferred, also HPLC provides higher sensitivity and specificity than the UV method.
- Since the analyte needs to be neither volatile nor stable at elevated temperature, HPLC has an advantage over GC as an analytical technique.
- Since HPLC is highly accurate and assembled with universal detectors like UV-VIS, this makes the quantitation more easily than TLC.
- Internal and external standard calibration method made it possible to convert for structurally dependent differences in detector response.[8]

ANALYTICAL METHOD VALIDATION OF HPLC

The process of establishing documented evidence is the validation. Validation is an assurance that will consistently produce a product meeting its predetermined specifications and quality characteristics.

Validation methods are the process that, analytical procedures are suitable for their intended use and that they support the identity, potency, quality and purity of the drug products. In simple, the analytical method validation is the process of proving that an acceptable for its intended purpose. When both the technical and regulatory objectives of the analytical methods have been fulfilled, then only a developed method is said to be validated. The transfer of a method is best accomplished by a systemic method validation process. The primary aim of the validation process is to challenge the method and determine limits of allowed variability for the conditions needed to run the method[32,33].

Significance of Method Validation

The success of a drug development programme mainly depend on the fact, i.e, the quality of the analytical data. The process of method development and validation plays a key role in the quality of the datas[34].

- To trust the method
- Regulatory requirement.

When we are about to introduce a new product into the market or for the clinical trials, analytical method validation is one of the major facts that we has to submit before an international regulatory agency. A well developed method can throw out all the potential problems and a thoroughly validated method can be used with full confidence for the purpose it is proposed. In other words, a through validation can fulfill all the technical and regulatory aspects. The development of meaningful specifications can be predicted upon the use of validated analytical procedures that can assess changes in a drug substance or drug products during its shelf life[35].

Some of the analytical parameters that are to be validated for a well developed method are listed below. Al these parameters may or may not be applicable for all the compounds. The selection or validation of the parameters are mainly depend on the purpose for which the procedure is required, however, these following aspects of validation should be given due to importance.

Accuracy

The accuracy of an analytical method express 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.

If a method is said to be highly accurate, the measured value of the sample should be ideally identical to the true value. The recovery reports should give an idea about the accuracy. The usual range of % recovery should be 10 % above or below the expected range of claim.

The % recovery is calculating using the formula

$$\% \text{ Recovey} = \frac{(a+b)-a}{b \times 100}$$

where,

a – Amount of drug present in sample

b - Amount of standard added to the sample

Precision

If the measurement obtained from the multiple sampling of the same homogenous sample under same prescribed condition are close, then we say that the method is a precise one.

According to ICH guidelines, the precision can be classified into three.

- Repeatability
- Intermediate |Precision
- Reproducibility

Repeatability:

- It express the precision under the same operating conditions (with-in a short period of time, in same laboratory by the same analyst using same equipment)
- Injection repeatability/Measurement (System Precision)
- Method repeatability(Method Precison)

Intermediate Precision:

- Precision should be given under different laboratory condition (with variations in laboratory conditions on different days, with different analyst or equipment with-in the same laboratory).

Reproducibility:

- Indicates the precision among the laboratories (collaborative studies, usually applied to standardization of methodology)

Linearity:

The linearity may be defined as the ability of the analytical procedure to obtain test results, which are directly proportional to the concentration of the sample.

The linearity is determined from 50% of the ICH reporting level to 150% of the proposed shelf life specifications of the related substance as a minimum.

Range:

The linearity of the analytical procedure is its ability to obtain the test results which are directly proportional to the concentration of analyte in the sample. It is the upper and lower limit of the analyte which we are studied. The concentration interval over which acceptable accuracy, precision and linearity were obtained is referred to as the range of an analytical procedure. The data secured from the accuracy and precision studies are used to determine the range of an analytical method. The linearity studies alone will not give an idea about fixing the range of an analytical procedure.

Specificity:

The ability of the method to accurately measure the analyte response in the presence of all potential sample components is the specificity of the developed HPLC method. The response of the analyte in test mixtures containing the analyte and all potential sample like intermediates, degradants, excipients, impurities etc, is compared with the response of a solution containing only the analyte.

There were three methods proposed for the demonstration of specificity. In the first method, the HPLC methods were developed, namely, pH, ionic strength, percentage of organic solvent, flow rate etc. were changed and observed for the presence of any additional peaks.

The second method is by using diode array detector to determine the peak purity. The diode array spectrum and the diode array first derivative spectrum of the standard and sample drug peaks were recorded and compared.

The third method involves the absorbance ratio measurement of the drug peaks at two different wavelength.

Limit of Detection (LOD) and limit of Quantification (LOQ)

The limit of detection of an analytical procedure is defined as the lowest amount of analyte in a sample that can be detected, but not quantified. The LOD corresponds to a concentration that will give a signal-to-noise ratio of 3:1.

The limit of quantification is defined as the lowest concentration of an analyte in a sample that can be determined with acceptable accuracy and precision under the standard operational conditions of the method. Limit of quantification is the concentration of related substance in the sample that will give a signal-to-noise ratio of 10:1. LOD and LOQ may also be calculated from the standard deviation of the response (SD) and the slope of the calibration curve (S) at levels approximating the LOD of the formulae.

The LOD and LOQ may be expressed as

$$\mathbf{LOD = 3.3\ \sigma/S}$$

$$\mathbf{LOQ = 10\ \sigma/S}$$

Where, σ = 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

Ruggedness:

Analyzing of the sample under same experimental conditions by different analysts to check reproducibility of the method is referred to the ruggedness. This includes different analysts, laboratories, columns, instruments, sources of reagents, chemicals, solvents and so on[38].

Robustness:

Robustness is defined as the capacity to remain unaffected by small, but deliberate variations in method parameters and provides indication of its reliability during its normal usage. Small changes in parameters like mobile phase, pH, buffer concentration, temperature and injection volume are made and repeatability is checked. The chromatogram obtained with the varied parameters are compared with the chromatogram obtained with the fixed conditions[36,37].

System Suitability:

The system suitability studies of the methods were performed by determining various chromatographic parameters like column efficiency, resolution, peak asymmetry factor, capacity factor, peak area[39].

STRESS DEGRADATION STUDY

Stability-Indicating Method:

The stability-indicating method is the quantitative analytical methods that are based on the characteristic structural, chemical or biological properties of each ingredient of a product and that will distinguish each active ingredient from its degradation product so that the active ingredient content can be accurately measured.

The stability indicating method is an analytical method that separates the active pharmaceutical ingredient from any degradation (decomposition) products formed under defined storage conditions during the stability evaluation period. This method is very much essential to detect and quantify one or more degradation products. This analytical method is also used for resolving any other potential interfering peaks such as an internal standard. Considering all these criteria, the nature of this method indicates the method to be stability-indicating as well as stability-specific. Stressed testing under forced conditions of oxidation, photolysis, hydrolysis and varying pH values may from some decomposition products that are unlikely to form under accelerated or long-term stability storage conditions. The degraded products may be useful in developing and validating a suitable stability-indicating analytical method for the analysis of the drug substances.

Stress testing is defined as the stability testing of the drug substances and the drug products under conditions exceeding those used for accelerated testing[40].

According to ICH guidelines, there are three main purpose for the stress testing[41].

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

Pharmaceutical companies performs forced degradation studies during pre-formulation to help select compounds and excipients for further development, to provide optimization and produce samples for developing stability-indicating analytical methods.

Degradation study provides information about the degradation mechanism and potential degradation products. This information can be used to develop manufacturing processes or to select proper packaging. It may also help in preparing reference material of identified degradation products[42].

Selected stress testing directly on a drug product may be accepted for the purpose of establishment of the validated stability-indicating assay method, if it is proved that no physical change occurs in the drug product over the stress period. The stress testing should be done on a single batch of the product, which must be of the same composition and the quality as the marketing batch, including packaging. The stress tests are normally conducted for a period of six months. The samples are observed for physical changes at particular intervals. Stress degradation studies are carried out to identify the possible degradation products. Stress degradation studies are carried out by solid as well as liquid state using different degradation conditions. The container should be chemically inert while performing the degradation study and the container for photolysis should be transparent. If the drug substance is insoluble in aqueous, acidic or alkaline medium, then the organic solvents can be used to dissolve the material. For oxidation purpose a hydrogen peroxide can be used. At the end of the exposure period, the samples should be examined for the change of any physical properties. Selectivity of the analytical method can be known from the stress degradation study. It can be determined from the test results of analysis of test samples with-out any degradation product[43].

Factors Affecting Drug Stability:

- Stability of the API from storage
- Interaction between the API and the excipients-during Formulation Development
- Selection of dosage form
- Manufacturing process of drug product
- Selection of container closure packaging system
- Effect of storage
- Selection of marketing image
- Handling of finished product

ICH Guidelines[41, 44] :

The ICH guidelines indicates that stress testing is designed to determine the intrinsic stability of the molecule by establishing degradation pathway in order to identify the degradation products and to validate the stability indicating power of the analytical procedure used. The ICH guidelines stability testing of new drug substances and products requires that stress testing should be carried out to reveal the substance. The degradation products that are formed under the variety of conditions should include the effect of temperature, humidity where appropriate oxidation, photolysis and susceptibility to hydrolysis across a wide range of pH value.

Selection of Stress Condition:

In forced degradation conditions, more severe conditions that accelerated studies are used[45]. The stress conditions should be selected such that, it should be consistent with the products decomposition under normal manufacturing, storage and use conditions which are specific in each case[46]. The choice of forced degradation studies conditions should be based on the data from accelerated pharmaceutical studies and sound scientific understanding's of the product decomposition mechanism under typical use conditions. The factors that are suggested for the stress degradation study includes acid and base hydrolysis, thermal degradation, photolysis, oxidation and may include freeze-thaw cycles and shear[49, 50, 51].

Degradation product that is produced during the manufacturing and storage should be identified, tested and monitored against appropriately established acceptance criteria. The forced degradation studies should be designed as a part of the impurity studies. Examination of some degradation products formed under stress conditions may not be necessary for certain degradants if it has been demonstrated they are not formed under accelerated or long-term storage conditions.

HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY[99]

The power of thin layer chromatography has been enhanced by considering the chromatographic principles to improve the speed and efficiency of separation by development of instrumentation to automate sample application, development of chromatogram and detection including accurate and precise quantification. As the particle size of the layer decreases, the separation will be faster and efficient. The particle size of stationary phase has a narrower distribution range with an average size of 5μm, instead of the average 20μm for conventional **TLC**. The use of mechanical applicators provides reproducible application and reduces the diameter of the starting spots. Compared to conventional TLC, only very small volume of samples are used in HPTLC, i.e.; about one-tenth of the sample. The separation time also reduced to the same. In addition to precoated silica gel layers, a range of chemically bonded phases, similar to those used in normal- and reversed- phase high performance liquid chromatography, are available.

Modern thin layer chromatography can be complementary to **HPLC**. It allows the processing of many samples in parallel, providing low cost analysis of simple mixtures for which the sample workload is high. The **TLC** plates acts as a "Storage detectors" of the analyte if they are saved.

IMPORTANT APPLICATION OF PLANAR CHROMATOGRAPHY

Pharmaceutical industry

- Quality control
- Content Uniformity Test
- Identity/purity checking
- Phytopharmaceuticals

Food analysis

- Quality control
- Additives
- Pesticides
- Stability testing
- Clinical applications

Lipids

- Metabolic studies
- Drug screening
- Stability testing

Industrial applications

- Process development and optimization, In-process control
- Cleaning validation

Forensic

- Detection of document false cations, Poisoning investigations
- Dye stuff analysis
- Doping control

Environmental analysis

- Water
- Soil
- Residue analysis

Sample application[12]

The samples are applied on to the separation layer, either as spots through capillary tube or as narrow bands using the spray-on technique. The criteria like precision of applied volume, small size of application zone and exact positioning of sample are essential for the quality of the analysis. Band wise sample application as available with Linomat 5 Applicator or Automatic **TLC** Sampler 4 offers the best separation regarding sample solvents and application volumes. By using spray-on technique the applied volume can be easily adjusted to match the required detection limit of the analytical task.

Chromatogram development

Chromatogram is developed by capillary force. The developing solvent (mobile phase) migrates through the layer (stationary phase) over a defined distance called solvent front. During this process the sample is separated into fractions/bands (components). After evaporation of the mobile phase by keeping the layer /plate in hot air oven or at room temperature, all fractions remain stored on the layer.

Derivatization

It is a special advantage of planar chromatography that fractions are stored on the plate and can be derivatized after chromatography. By derivatization, substances that do not respond to visible or UV light can be rendered detectable. In case of certain compounds, substances or classes of substances can be identified by using specific reagents.

Eg: Ninhydrin, Dragandroffs reagent.

Chromatogram evaluation[20]

The chromatogram is evaluated under ultraviolet or white light. Options range from visual inspection, electronic image processing, video densitometry and documentation to quantitative determination by means of monochromatic light in a classical densitometer, which additionally facilitates measurement of spectral information. Spectral information's are available through the densitometer.

Two types of ultraviolet light are required for inspecting thin-layer chromatograms:

Long-wave UV light 366nm

At 366 nm, substances may inherent or reagent induced fluorescence appear as bright spots, often differently colored, on a dark background. The sensitivity of this detection method is proportional with the intensity of the long-wave UV light and also as more visible light is eliminated. A fluorescent indicator F254 contained in the layer neither contributes to nor interferes with this detection method.

Short-wave UV light 254nm

At 254 nm UV substances absorbing at that wavelength become visible, provided the TLC layer contains a fluorescent indicator F 254. These substances appear as dark spots on a bright background. For this detection method, UV intensity and complete elimination of visible light are less critical.

- **In-House Preparation of TLC Plates**

Only for economic or logistic reason, now a day's in-house preparation of TLC plates is selected. The self-coating of **TLC** plates should also be considered when special layers are required that are not available in the form of precoated plates, e.g. layers containing silver nitrate, buffer substances or other reagents, layers consisting of adsorbent mixtures, or in the exceptional cases where the binder contained in commercial precoated plates might interfere with detection.

*Adsorbents for In-House Preparation of **TLC** Layers*

For the self preparation of chromatographic layers, adsorbents in the form of powder are mixed with water or with aqueous solutions of salts or buffer solutions to form thick slurry which is spread onto glass plates by means of a coating device.

Adding calcium sulfate as a "binder" makes it easier to achieve the correct consistency of the slurry for coating; the calcium sulfate contributes very little to the mechanical strength of the layer.

A fluorescence indicator is required to visualize substances which absorb UV light of a wavelength (254 nm), by which the indicator is excited to emit visible light. These substances appear as dark spots on a bright background. The fluorescence indicator neither interferes with the chromatographic separation, with any derivatization reactions, nor with densitometric evaluation. On contact with acids, most of these types of indicators lose their fluorescence. Only very few precoated layers are available, which with stand its fluorescence property against acid.

- **Precoated Layers**

The modern planar chromatography utilizes precoated plates. They are more convenient and their quality is superior to that of layers available for self-coating. Several types of TLC phases, HPTLC layer, etc. are only available in the form of precoated plates.

HPTLC Analysis[26]

High performance thin layer chromatography (**HPTLC**) is a quality assessment tool for the evaluation of a wide variety materials. It allows for the analysis of a broad number of compounds both efficiently and cost effectively. In addition to that, numerous samples can be run at a single analysis thereby dramatically reducing analytical time. With HPTLC, the same analysis can be viewed at different wavelengths of light thereby providing a more complete profile of the compound than is typically observed with more specific types of analyses.

QUANTITATIVE ANALYSIS[100]

The primary goal of the method is to provide validated methods to be used for the quantitation of the compounds most correlated with pharmacological activity or qualitative markers as determined by the primary pharmacological literature, product labeling and a survey of experts. The method will be selected from the primary analytical literature by a Methods Selection Committee with priority given to compendial methods when available. In this context, validation consists minimally of a two-lab validation using the same procedures, samples, and reference standards. Primary factors for considering a method as appropriate include accuracy of the findings, speed, basic ruggedness, applicability to a large segment of the manufacturing community, and avoidance of the use of toxic reagents and solvents. In an attempt to promote harmonization, primary consideration is given to those methods which are already accepted in official pharmacopoeias or by AOAC International. When necessary, comparative tests shall be conducted to determine which of the available method(s) is most appropriate.

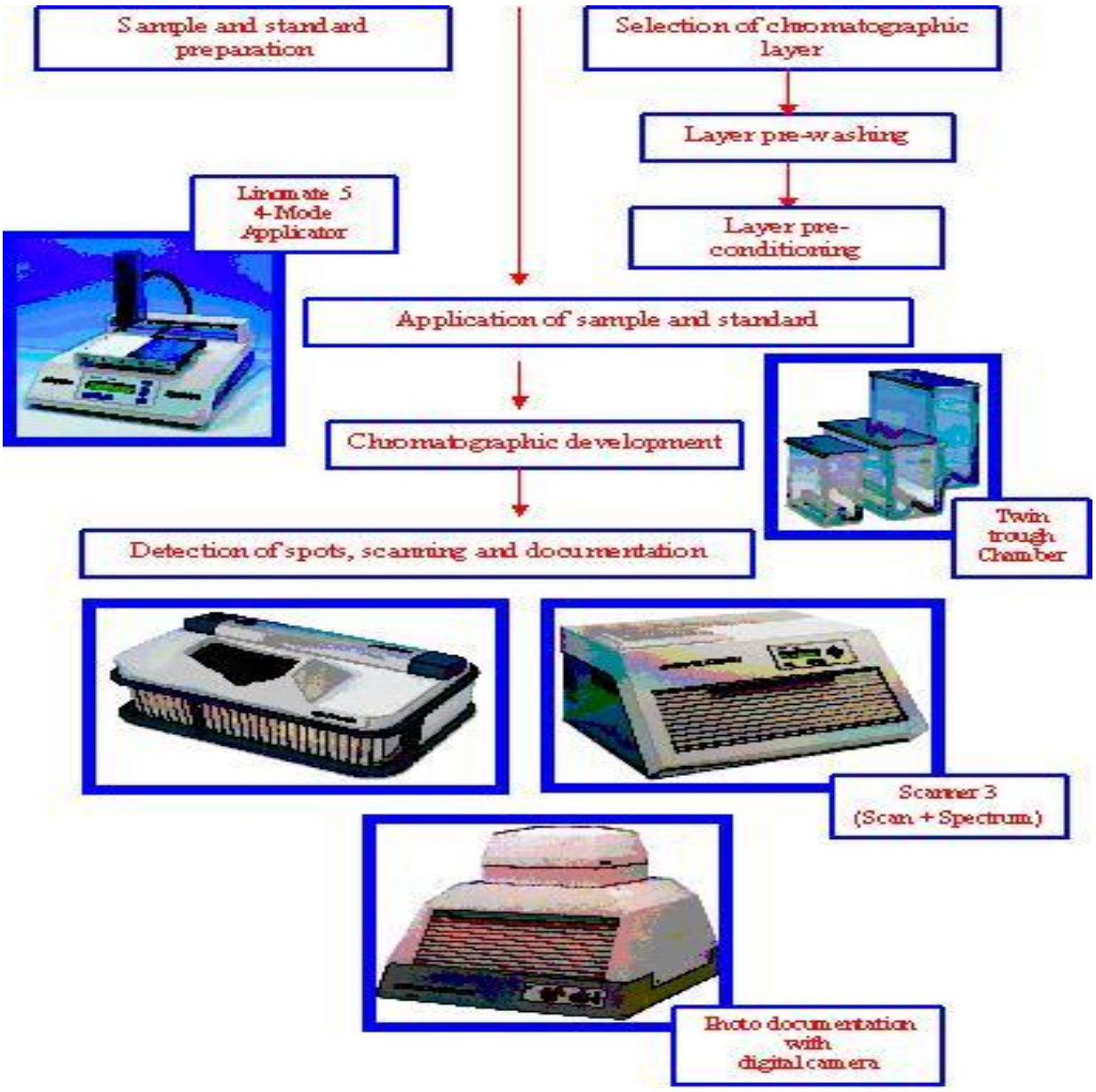

Fig 2: Schematic representation of HPTLC instrument

ANALYTICAL METHOD VALIDATION (AMV) FOR HPTLC[6, 103,102]

The validation of analytical procedures, i.e. the proof of its suitability for the intended purpose, is an important part of the registration application for a new drug**.** The International Conference on the Harmonization (**ICH)** of the Technical Requirements for Registration of Pharmaceuticals for Human Use has harmonized the requirements in two guidelines Validation is a process of establishing documented evidence, which provides a high degree of assurance that a specific activity will consistently produce a desired result.

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. The real goal of validation process is to challenge the method and determine limits of allowed variability for the conditions needed to run the method.

1. Specificity
2. Accuracy (Trueness)
3. Precision
   - Repeatability
   - Intermediate Precision
   - Reproducibility
4. Limits of detection and quantitation
5. Linearity
6. Range
7. Robustness
8. Ruggedness
9. Stability of analytical solutions

STRESS DEGRADATION STUDY

Stability-Indicating Method: [40-43]

The stability-indicating method is the quantitative analytical methods that are based on the characteristic structural, chemical or biological properties of each ingredient of a product and that will distinguish each active ingredient from its degradation product so that the active ingredient content can be accurately measured.

The stability indicating method is an analytical method that separates the active pharmaceutical ingredient from any degradation (decomposition) products formed under defined storage conditions during the stability evaluation period. This method is very much essential to detect and quantify one or more degradation products. This analytical method is also used for resolving any other potential interfering peaks such as an internal standard. Considering all these criteria, the nature of this method indicates the method to be stability-indicating as well as stability-specific. Stressed testing under forced conditions of oxidation, photolysis, hydrolysis and varying pH values may from some decomposition products that are unlikely to form under accelerated or long-term stability storage conditions. The degraded products may be useful in developing and validating a suitable stability-indicating analytical method for the analysis of the drug substances.

Stress testing is defined as the stability testing of the drug substances and the drug products under conditions exceeding those used for accelerated testing.

According to ICH guidelines, there are three main purpose for the stress testing.[45, 46]

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

Pharmaceutical companies performs forced degradation studies during pre-formulation to help select compounds and excipients for further development, to provide optimization and produce samples for developing stability-indicating analytical methods.

Degradation study provides information about the degradation mechanism and potential degradation products. This information can be used to develop manufacturing processes or to select proper packaging. It may also help in preparing reference material of identified degradation products.

Selected stress testing directly on a drug product may be accepted for the purpose of establishment of the validated stability-indicating assay method, if it is proved that no physical change occurs in the drug product over the stress period. The stress testing should be done on a single batch of the product, which must be of the same composition and the quality as the marketing batch, including packaging. The stress tests are normally conducted for a period of six months. The samples are observed for physical changes at particular intervals. Stress degradation studies are carried out to identify the possible degradation products. Stress degradation studies are carried out by solid as well as liquid state using different degradation conditions. The container should be chemically inert while performing the degradation study and the container for photolysis should be transparent[45]. If the drug substance is insoluble in aqueous, acidic or alkaline medium, then the organic solvents can be used to dissolve the material. For oxidation purpose a hydrogen peroxide can be used. At the end of the exposure period, the samples should be examined for the change of any physical properties. Selectivity of the analytical method can be known from the stress degradation study. It can be determined from the test results of analysis of test samples with-out any degradation product.

ICH Guidelines[41, 44]:

The ICH guidelines indicates that stress testing is designed to determine the intrinsic stability of the molecule by establishing degradation pathway in order to identify the degradation products and to validate the stability indicating power of the analytical procedure used. The ICH guidelines stability testing of new drug substances and products requires that stress testing should be carried out to reveal the substance. The degradation products that are formed under the variety of conditions should include the effect of temperature, humidity where appropriate oxidation, photolysis and susceptibility to hydrolysis across a wide range of pH value.

Selection of Stress Condition: [49, 50, 51]

In forced degradation conditions, more severe conditions that accelerated studies are used. The stress conditions should be selected such that, it should be consistent with the products decomposition under normal manufacturing, storage and use conditions which are specific in each case. The choice of forced degradation studies conditions should be based on the data from accelerated pharmaceutical studies and sound scientific understanding's of the product decomposition mechanism under typical use conditions. The factors that are suggested for the stress degradation study includes acid and base hydrolysis, thermal degradation, photolysis, oxidation and may include freeze-thaw cycles and shear.

Degradation product that is produced during the manufacturing and storage should be identified, tested and monitored against appropriately established acceptance criteria. The forced degradation studies should be designed as a part of the impurity studies. Examination of some degradation products formed under stress conditions may not be necessary for certain degradants if it has been demonstrated they are not formed under accelerated or long-term storage conditions.

The degradation products can be put into two categories, involving physical instability and chemical instability. Chemical instability yields a new chemical molecule including modification of the protein. Physical instability denotes the changes in the higher order structure. This can lead to denaturation, precipitation and adsorption to surfaces.

CHAPTER 2

LITERATURE REVIEW

HPLC method

Mallikarjuna Rao[72] et al developed a RP-HPLC method for the quantitative determination of ZMT. The developed method is also applicable for the related substances determination in bulk drugs. The chromatographic separation was achieved on a Waters X Terra RP18 column. The mobile phase, solution A contained a mixture of phosphate buffer pH 9.85 methanol: acetonitrile (70:20:10, v/v/v) and solution B contained a mixture of phosphate buffer, pH 9.85: acetonitrile (30:70). The detection wavelength was 225 nm. In the developed HPLC method, the resolution between ZMT and its potential impurities, namely Imp-1, Imp-2 and Imp-3 was found to be greater than 3. The drug was subjected to stress conditions of hydrolysis, oxidation, photolysis and thermal degradation. Considerable degradation was observed in alkaline medium and oxidative stress conditions. Degradation product formed during base hydrolysis was found to be Imp-3.

Mathrusri Annapurna[80] et al described a simple, precise and accurate RP-HPLC validated method for the analysis of ZMT. The separation and quantization were achieved on a 250 mm reversed phase column with a hydrophilic linkage between silica particles and hydrophobic alkyl chains. The mobile phase was constituted of methanol and aqueous tetra butyl ammonium hydrogen sulphate, pH 3.4; 10 mM using isocratic elution with, UV detection at 224 nm. The method showed good linearity for ZMT in the 1–100 μg mL-1 range. The limit of quantitation (LOQ) and limit of detection (LOD) were found to be 0.8134 and 0.2687 μg mL-1 respectively. The applicability of the method was validated according to ICH guidelines and can be applicable for the analysis of commercial dosage forms.

Xiaoyan Chen[71] et al described a sensitive and selective liquid chromatography–tandem spectrometry method for the determination of ZMT in 0.5 ml of plasma with a linearity range of 0.05–30 ng/ml using diphenhydramine as the internal standard. Liquid–liquid extraction using a mixture of diethyl ether and dichloromethane was used to extract the drug and the internal standard from plasma. The mass spectrometer was operated under the

selected reaction monitoring (SRM) mode using the atmospheric pressure chemical ionization (APCI) technique. The mobile phase consisted of acetonitrile: water: formic acid (70:30:0.5), at a flow rate of 0.5 ml/min. In positive mode, ZMT produced a protonated precursor ion at *m*/*z* 288 and a corresponding product ion at *m*/*z* 58. Internal standard produced a protonated precursor ion at *m*/*z* 256 and a corresponding product ion at *m*/*z* 167. The method had a lower limit of quantification of 0.05 ng/ml for ZMT, which offered increased sensitivity and selectivity of analysis, compared with existing methods. The method was successfully applied to a pharmacokinetic study of ZMT after an oral administration of 5 mg ZMT to 20 healthy volunteers.

Srinivasu[73] et al developed an accurate chiral liquid chromatographic for the enantiomeric separation of ZTR-5 [(4*S*)-4-(4-aminobenzyl) - 2-oxazolidinone, (*S*)-isomer], a key intermediate of ZMT in bulk drugs. The enantiomers of ZTR-5 were baseline resolved on a Chiralpak AD-H (250 mm×4.6 mm, 5 µm) column using a mobile phase system containing hexane: ethanol (70:30, v/v). The resolution between the enantiomers was not less than four and interestingly distomer was eluted prior to eutomer. The limit of detection and limit of quantification of (4*R*)-4-(4-aminobenzyl)-2-oxazolidinone [(*R*)-isomer] were found to be 250 and 750 ng/ml, respectively, for 10 µl injection volume. The percentage recovery of (*R*)-isomer ranged from 92.0 to 105.6 in the bulk drug samples of ZTR-5. The validated method yielded good results regarding precision, linearity, accuracy and ruggedness. The proposed method was found to be suitable and accurate for the quantitative determination of (*R*)-isomer in bulk drug samples of ZTR-5.

Zunjian Zhanla[70] et al proposed a sensitive and specific liquid chromatography electrospray ionization mass spectrometry (LC–ESI–MS) method has been developed and validated for the identification and quantification of ZMT in human plasma. After the addition of the internal standard and 1 M sodium hydroxide solution, plasma samples were extracted with methylene chloride: ethyl acetate mixture (20:80, v/v). The organic layer was evaporated under a stream of nitrogen at 40ºC. The residue was reconstituted with 100 µl mobile phase. The compounds were separated on a prepacked Lichrospher CN column using a mixture of methanol: water (pH 4.0) = 78:22 as mobile phase. Detection was performed on a single quadrupole mass spectrometer by selected ion monitoring (SIM) mode via

electrospray ionization (ESI) source. The method was proved to be sensitive and specific by testing six different plasma batches. Linearity was established for the range of concentrations 0.30–16.0 ng/ml with a coefficient of determination (R^2) of 0.9998 and good back-calculated accuracy and precision. The intra and inter-day precision (RSD. %) were lower than 15% and accuracy ranged from 85 to 115%. The lower limit of quantification was identifiable and reproducible at 0.30 ng/ml. The proposed method enables the unambiguous identification and quantification of ZMT for pharmacokinetic, bioavailability or bioequivalence studies.

Vivek Sagar[78] et al proposed a simple reverse phase HPLC method for the simultaneous estimation of Rizatriptan, Sumatriptan and ZMT in bulk form. Chromatography was performed by gradient reverse phase separation on a Stainless steel C18 column with mobile phase Acetonitrile: Sodium Phosphate buffer. The separation was monitored at 280 nm. The retention times were 7.215 min, 8.432 and 9.185 min for of Rizatriptan, Sumatriptan and ZMT respectively. The standard curve was linear over a working range of 1–10 µg/ml and gave an average correlation coefficient of 0.9996, 0.9992, and 0.9992 for Rizatriptan, Sumatriptan and ZMT respectively. The limit of quantitation (LOQ) of this method was 2 µg/ml for rizatriptan Sumatriptan and ZMT. The absolute recovery was 101.84 for rizatriptan, 101.492 for sumatriptan and 101.44 for ZMT. This method can be easily and conveniently adopted for routine analysis of Rizatriptan, Sumatriptan and ZMT in pure form and can also be used for dissolution or similar studies.

Koti Reddy[77] et al developed a new sensitive, precise, rapid, specific, and linear and stability indicating isocratic HPLC method was developed for the analysis of related substances in ZMT. The mobile phase consisted of a mixture of 0.01 M ammonium dihydrogen phosphate adjust pH 9.5±0.02 with ammonia solution and acetonitrile in 83:17 v/v, which was filtered through a 0.22 µ nylon membrane and degassed by sonication before use. The chromatographic column used was Waters acquity BEH C18. The column temperature was maintained at 40° and the detection wavelength was 225 nm. The potential known related substances are impurity-1, impurity-2 and impurity-3. The method can be used for the detection and quantification of known and unknown impurities and degradants in the drug substance ZMT during routine analysis and also for stability studies in view of

its capability to separate degradation products. ZMT was subjected to the stress conditions of oxidative, acid, base and photolytic degradation. The degradation product was found to be at alkaline hydrolysis and oxidative conditions. The degradation products were well resolved from main peak and its impurities. The method was validated with perfect linearity, accuracy, precision, robustness and ruggedness.

Zeynep Aydogmus[76] et al proposed a simple and sensitive extractive spectrophotometric methods for determination of ZMT (ZTP) in tablets. These methods are based on the formation of yellow ion-pair complexes between ZTP and tropaeolin OO (TPOO) and bromothymol blue (BTB) in citrate–phosphate buffer of pH 4.0 and 6.0, respectively. The formed complexes were extracted with dichloromethane and measured at 411.5 and 410 nm for TPOO and BTB, respectively. Beer's law was obeyed in the concentration ranges of 2–20 and 1.5–17 µg/mL with molar absorptivities of 1.42×10^4 and 1.60×10^4 L/mol/cm for the TPOO and BTB methods, respectively. Correlation coefficients were 0.9998 and 0.9999 for TPOO and BTB methods, respectively. Limits of detection of the TPOO and BTB methods were 0.341 and 0.344 µg/Ml , respectively and the limits of quantitation were 1.034 and 1.051 µg/mL, respectively. Sandell's sensitivity and stability constant were also calculated. The proposed methods have been applied successfully for the analysis of the drug in its dosage forms. No interference was observed from excipients present in tablets. Statistical comparison of the results with those obtained by a high-performance liquid chromatography method showed excellent agreement and indicated nosignificant differences in accuracy and precision.

Madhusudhanareddy Induri[79] et al proposed a simple and rapid RP-HPLC method for quantification of ZMT in tablet dosage form. A reversed phase C18 column with dual wavelength absorbance detection at 229 nm with a Diode Array Detector was used for the study. The mobile phase consisted of acetonitrile and phosphate buffer (pH adjusted to 3.5 using ortho phosphoric acid) in the ratio of 10:90 % v/v at flow rate of 1.5 mL/min. The linearity range was found to be 10-50 µg/mL. The method was validated and it was concluded that the developed method was accurate, sensitive, precise, robust and useful for the quality control of ZMT in pharmaceutical preparations.

Jun Chen[75] et al developed a simple, rapid and sensitive high-performance liquid chromatographic (HPLC) method to quantify ZMT in plasma using an isocratic system with fluorescence detection. The method included a single-step liquid–liquid extraction with methyl tertiary butyl ether. HPLC separation was carried out by reversed phase chromatography with a mobile phase composed of 0.05% (v/v) triethylamine in water (adjusting to pH 2.75 with 85% phosphoric acid) and acetonitrile (92:8, v/v). Fluorescence detection was performed at 225 nm (excitation) and 360 nm (emission). The calibration curve for ZMT was linear from 0.2 to 40 ng/ml. The values of the limit of detection (LOD) and limit of quantification (LOQ) were 20 and 40 pg respectively. The method described in this report is able to determine low levels of ZMT in human plasma. The detection limit of this method for ZMT is 20 pg, which is enough to detect terminal phase concentrations of ZMT after oral administration of 5 mg dose of ZMT to healthy volunteers. The validation method yielded good results regarding linearity, precision, accuracy, specificity and recoveries. In addition, this method has a short chromatographic run (<7 min), so the method is more suitable for high-through quantitative analysis such as human pharmacokinetic studies. The method was sensitive, simple and repeatable enough to be used in pharmacokinetic studies.

Clement[74] et al proposed that, ZMT, *N*-desmethyl ZMT, ZMT N-oxide and an internal standard (an analogue of ZMT) were extracted from plasma by a solid-phase extraction (SPE). Chromatography was performed using isocratic reversed-phase high-performance liquid chromatography (HPLC) with coulometric end-point detection. The mobile phase consisted of 0.05 *M* potassium phosphate buffer (pH 3.5) in acetonitrile (87:13, v/v). The standard curves were linear over the range 2–20 ng/ml for ZMT and its metabolites in plasma. The mean inter- and intra-assay coefficients of variation over the range of the standard curves were less than 11%. The absolute recovery averaged 87, 58 and 77% for ZMT, *N* desmethylZMT and ZMT N-oxide, respectively. The assay sensitivity was 0.5 ng for each analyte. This method is reasonably cheap to run and can easily be set up I analytical as well as clinical laboratory.

Mallikarjuna Rao[72] et al suggested a gradient, reversed-phase liquid chromatographic (RP-LC) assay method for the quantitative determination of ZMT. The developed method is also

applicable for the related substances determination in bulk drugs. The chromatographic separation was achieved on a Waters X Terra RP18 column. The gradient LC method employs solutions A and B as mobile phase. The solution A contains a mixture of phosphate buffer pH 9.85 methanol: acetonitrile (70:20:10, v/v/v) and solution B contains a mixture of phosphate buffer, pH 9.85: acetonitrile (30:70). The detection wavelength was 225 nm. In the developed HPLC method, the resolution between ZMT and its potential impurities, namely Imp- 1, Imp-2 and Imp-3 was found to be greater than 3. The drug was subjected to stress conditions of hydrolysis, oxidation, photolysis and thermal degradation. Considerable degradation was found to occur in alkaline medium and oxidative stress conditions. Degradation product formed during base hydrolysis was found to be Imp-3. The stress samples were assayed against a qualified reference standard and mass balance was found close to 99.5%. The developed RP-LC method was validated with respect to linearity, accuracy, precision and robustness.

Srinivasua[73] et al developed a new, accurate and reliable chiral HPLC method was developed for the determination of ZMT and its potential impurities namely (4*R*)-4-[[3-[2-(dimethylamino)ethyl]- 1*H*-indol-5-yl] methyl]-2-oxazolidinone [(*R*)-enantiomer] and (4*S*)-4-(4-aminobenzyl)-2-oxazolidinone (Imp-1) in pharmaceutical formulations and in bulk drugs. HPLC separation was carried out by normal phase chromatography with a mobile phase composed of hexane: isopropanol: methanol: diethylamine in the ratio (75:10:15:0.1, v/v/v/v) run on a Chiralpak AD-H column. The presence of diethylamine in the mobile phase has played a key role in achieving chromatographic resolution between the enantiomers and also in enhancing chromatographic efficiency. The values of the limit of detection (LOD) and limit of quantification (LOQ) of (*R*)-enantiomer and Imp-1 were 100, 250 ng/ml and 30, 1000 ng/ml, respectively, for 10 µl injection volume. The validated method yielded good results regarding selectivity, linearity, precision, accuracy, robustness and ruggedness. ZMT sample solution and mobile phase are found to be stable for at least 24 h. The proposed method was found to be suitable and accurate for the quantitative determination of ZMT and its impurities namely (*R*)-enantiomer and Imp-1 in bulk drugs and commercial formulations.

Sasmita Kumari[108] et al proposed a new method for the determination of ZMT in bulk and pharmaceutical formulation by UV, first derivative, and AUC-spectrophotometry. The UV-spectrophotometry was measured at 283.0 nm in UV spectrophotometry. The linearity ranges were found to be 0.5–100 μg/mL in 0.1M HCl and the regression equation was A=2.02×10−2C + 4.6×10−4 (R^2=0.9999). The response (dA/dλ) of standard solutions was measured at 298.0 nm for first derivative method. Calibration curve was constructed by plotting dA/dλ values against concentrations, 1–100 μg/mL of ZMT. Regression equation of linear calibration graph was calculated as D1= -1.14×10−3C-2.00×10−5 (R^2=0.9999). The AUC-spectrophotometric method was based on calculation of area under curve (AUC) for analysis of ZMT in the wavelength range of 278.0–288.0 nm. Calibration curve was constructed by plotting AUC values against concentrations, 0.5–100.0 μg/mL of ZMT. Regression equation of linear calibration graph was calculated as AUC=1.963×10−1C+1.34×10−3 (R^2=0.9999). The methods were validated with ICH guidelines. The developed methods were successfully applied to estimate the amount of ZMT in pharmaceutical formulations.

Maria Puchalska[109] et al developed an HPLC method for the analysis of ZMT of Pharmaceutical purity. The chromatographic separation was achieved on a Waters XTerra RP Column using linear gradient solutions. The mobile phase chosen was 20 mM ammonium hydrogen orthophosphate and acetonitrile. In the developed HPLC method, the resolution between ZMT and its potential impurities, ZL3, ZL4, ZL5, ZL7, were found to be greater than 3. According to ICH guidelines, the obtained product, as pharmaceutical substance, should contain less than 0.5% of total impurities and no more than 0.10 % of an individual unidentified impurities. The detection limit (0.5 mg mL^{-1}) for compoud ZL7 obtained using the developed HPLC method with spectrophotometric detection is unsatisfactory.

Vishwanathan[107] et al developed a sensitive and selective method for the determination of antimigraine drugs from human serum, for understanding the pharmacokinetics of these drugs when administered concurrently. The drugs (sumatriptan, naratriptan, ZMT and rizatriptan) where extracted with solid phase extraction (SPE) using Oasis HLB .The internal standard was bufotenine from serum. A liquid chromatography/tandem mass spectrometry

(LC/MS/MS) method was developed and validated for the simultaneous quantification of these antimigraine drugs from human serum. The major product ions of the analytes were monitored on a triple quadrupole mass spectrometer with positive ion electrospray ionization (ESI) with multiple reaction monitoring (MRM) mode. The base peak in all the analytes is formed by alpha cleavage associated with protonation of the secondary amine. Mechanisms for the formation of the collision-induced dissociation products of these antimigraine compounds are proposed. The calibration curves were obtained with 1-100 ng/mL and it was linear with all coefficients of determination greater than 0.99. The inter- and intraday precision (%RSD) were less than 9.3% and accuracy (%error) was less than 9.8% for all components. The limits of detection (LOD) for the method were 250 pg/mL for sumatriptan and 100 pg/mL for the remaining analytes based on a signal-to-noise ratio of 3.

Yao Jin-cheng[105] et al established a new rapid and sensitive HPLC-MS method for the determination of ZMT in human plasma and they extended this study to determine the pharmacokinetics of ZMT in healthy volunteers. A single oral dose of 5 mg of ZMT tablet was given to 20 healthy male volunteers. After the administration, blood samples were collected for a period of 24 h, and the concentration of drug in plasma was analyzed by HPLC-MS. The plasma concentration-time course fitted well a two-compartment open model with a lag time, giving the following pharmacokinetic parameters: *T*max 1.60±0.24 h, *C*max 9.73±1.43 ng·mL^{-1}, *T*1/2α1.72±0.46 h, *T*1/2β4.52±0.97 h and *AUC*0-t 55.59±5.12 ng·mL^{-1}·h. The improved analytical method for ZMT is rapid, sensitive and suitable for application to pharmacokinetic studies and routine determination of numerous samples.

Hu YZ[106] et al developed an analytical method and quality control for the determination of ZMT and related substances. ZMT and related substances were separated on a shimadzu CLC-C (8) column (150 mm x 6 mm, 10 micron). Acetonitrile-10 mmol/L and phosphate buffer pH 7.5 in the ratio 25:75 was optimized as the mobile phase and a flow-rate of 1 ml/min; was kept. The detection wave length was found to be at 229 nm. The limit of detection for the related substances was 0.5 ng on the ZMT basis (S/N >3). The calibration curve was drawn from 4 - 40 mcg/ml. The correlation coefficient was found at 0.9999. The recovery rate of ZMT was 99.1% with a standard deviation of 0.2%. The results of HPLC method were consistent with those of non-aqueous titration method. The developed HPLC

method is rapid sensitive and accurate method for the determination of ZMT and its related substances.

Vijayakumar[104] et al proposed a new stability indicating, precise, specific, linear isocratic HPLC method for the analysis of related substances in ZMT. The potential known related substances are (S)-4-(4-aminobenzyl)-1, 3-oxazolidin-2-one (impurity 1) and (S)-4-(4-hydrazinobenzyl)-1, 3-oxazolidin-2-one (impurity 11). This method can be used for the quantition and detection of both known and unknown impurities as well as for the stability studies and for the identification of degradation products. The forced degradation method was developed to establish the stability of this method. The detection wave length was fixed at 225 nm. The mobile phase consisted of a mixture of 0.02M ammonium formate containing 0.1% n-propylamine and acetonitrile in 80:20 v/v. The retention time of ZMT, impurity 1 and 11 are found to be at 11.0, 4.7, 27.6 mins respectively with a resolution of 13.5 between ZMT and impurity 1 and 24.1between ZMT and impurity 11. The degradation was found under the alkaline hydrolysis and oxidative conditions.

HPTLC Method

Himani Agrawal[95]et al developed a sensitive, accurate, precise and stability indicating high-performance thin layer chromatographic method of analysis of clopidogrel bisulphate both as a bulk drug and in formulations was developed. The method employed TLC aluminium plates precoated with silica gel 60F-254 as the stationary phase. The solvent system consisted of carbon tetrachloride: chloroform: acetone (6:4:0.15, v/v/v). This system was found to give compact spots for clopidogrel bisulphate with Rf value of 0.309. Clopidogrel bisulphate was subjected to acid and alkali hydrolysis, oxidation, photo degradation and dry heat treatment. Also the degraded products were well separated from the pure drug. The detection wavelength was 230 nm. The linear regression data for the calibration plots showed good linear relationship with R^2 0.9999 in the concentration range of 200_ 1000 ng. The method was validated according to ICH guidelines. The drug undergoes degradation under acidic and basic conditions, oxidation and dry heat treatment. All the peaks of degraded product were resolved from the standard drug with significantly different Rf values.

Ansari[94] et al proposed a simple, selective, precise and stability-indicating high-performance thin-layer chromatographic method of analysis of curcumin both as a bulk drug and in formulations was developed and validated. The method employed TLC aluminium plates precoated with silica gel 60F- 254 as the stationary phase. The solvent system consisted of chloroform: methanol (9.25:0.75 v/v). Rf value was found at 0.48. The detection wavelength was fixed at 430 nm. The linear regression analysis data for the calibration plots showed good linear relationship with R^2 = 0.996 and 0.994 with respect to peak height and peak area, respectively, in the concentration range 50–300 ng per spot. The method was validated for precision, recovery and robustness. Curcumin was subjected to acid and alkali hydrolysis, oxidation and photo degradation. The drug undergoes degradation under acidic, basic, light and oxidation conditions.

Kotiyan[93] et al developed a rapid, selective and precise stability indicating high performance thin layer chromatography method was developed and validated for the estimation of ESD in bulk and pharmaceutical dosage forms. The method employed TLC aluminium plate precoated with silica gel 60F254 as the stationary phase. The solvent system employed consisted of chloroform–acetone–isopropyl alcohol–glacial acetic acid (9:1:0.4:0.1, v: v: v: v). Rf value was found to be at 0.409. The drug on intentional degradation gave two products with *Rf* values of 0.5290 and 0.5890 respectively. Spectrodensitometric scanning-integration was performed on a Camag system using a wavelength of 286 nm. The polynomial regression data for the calibration plots exhibited good linear relationship (R^2=0.9947) over a concentration range of 1–8 mg. The proposed method was found to be stability indicating. Statistical analysis proves that the method is precise, accurate and reproducible, hence can be employed for the routine analysis of the drug.

Kohli[110] et al introduced a simple, sensitive, selective, and precise and stability indicating high-performance thin-layer chromatographic method for determination of gatifloxacin both as a bulk drug and from polymeric nanoparticles was developed and validated as per the International Conference on Harmonization (ICH) guidelines. The method employed thin-layer chromatography (TLC) aluminium plates precoated with silica gel 60F-254 as the stationary phase and the mobile phase consisted of *n*-propanol: methanol: concentrated

ammonia solution (25%) (5:1:0.9, v/v/v). This solvent system was found to give compact spots for gatifloxacin *R*f value of 0.6. Densitometric analysis of gatifloxacin was carried out in the absorbance mode at 292 nm. The linear regression analysis data for the calibration plots showed good linear relationship with R^2 = 0.9953 with respect to peak area in the concentration range of 400–1200 ng/spot. The mean value (±S.D.) of slope and intercept were 9.66±0.05 and 956.33±27.67, respectively. Gatifloxacin was subjected to acid and alkali hydrolysis, oxidation, photo degradation and dry heat treatment. The drug undergoes degradation under acidic and basic conditions and upon wet and dry heat treatment. The degraded products were well separated from the pure drug. As the method could effectively separate the drug from its degradation products, it can be employed as stability-indicating one.

Vader[90] et al proposed a simple, selective, precise and stability-indicating high-performance thin-layer chromatographic method of analysis of imatinib mesylate both as a bulk drug and in formulations. The method employed HPTLC aluminium plates precoated with silica gel 60F-254 as the stationary phase. The solvent system consisted of chloroform: methanol (6:4, v/v). *R*f value was fixed at 0.53.Densitometric analysis of imatinib mesylate was carried out in the absorbance mode at 276 nm. The linear regression analysis data for the calibration plots showed good linear relationship with R^2 = 0.9966±0.0013 with respect to peak area in the concentration range 100–1000 ng per spot. The method was validated for precision, recovery and robustness. Imatinib mesylate was subjected to acid and alkali hydrolysis, oxidation and thermal degradation. The drug undergoes degradation under acidic, basic, oxidation and heat conditions. This indicates that the drug is susceptible to acid, base hydrolysis, oxidation and heat. The proposed developed HPTLC method can be applied for identification and quantitative determination of imatinib mesylate in bulk drug and dosage forms.

Neeraj Kaul[89] et al developed a sensitive, selective, precise and stability-indicating high-performance thin layer chromatography (HPTLC) method for analysis of indinavir sulphate both as a bulk drug and in formulations was developed and validated. The method employed TLC aluminium plates precoated with silica gel 60F-254 as the stationary phase. The solvent system consisted of carbon tetrachloride/chloroform/methanol/10% v/v ammonia

(4:4.5:1.5:0.05, v/v/v/v). The wavelength was fixed at 260 nm. Rf value was found at 0.63. Indinavir sulphate was subjected to acid and alkali hydrolysis, oxidation, dry and wet heat treatment, and photo degradation. The drug undergoes degradation under acidic and basic conditions, oxidation, dry and wet heat treatment, and photo degradation. Also the degraded products were well resolved from the pure drug with significantly different *R*f values. The method was validated for linearity, precision, robustness, limit of detection (LOD), limit of quantitation (LOQ), specificity and accuracy. Linearity was found to be in the range of 100–6000 ng/spot. As the method could effectively separate the drug from its degradation products, it can be employed as a stability-indicating one.

Nilu Jain[88] developed a simple, stability-indicating high-performance thin-layer liquid chromatographic (HPTLC) method for analysis of minocycline was developed and validated. The densitometric analysis was carried out at 345 nm. The mobile phase was fixed as methanol: acetonitrile: isopropyl alcohol: water (5:4:0.5:0.5, v/v/v/v). The method employed TLC aluminium plates pre-coated with silica gel 60F-254 as the stationary phase. To achieve good result, plates were sprayed with a 10% (w/v) solution of disodium ethylene diamine tetra acetic acid (EDTA), the pH of which was adjusted to 9.0. Compact spots of minocycline were found at *R*f = 0.30±0.02. The correlation coefficient, R^2 = 0.9997. The drug undergoes acidic and basic degradation, oxidation and photo degradation. All the peaks of degradation products were well resolved from the pure drug with significantly different *R*f values. The acidic and alkaline degradation kinetics of minocycline, evaluated using this method, is found to be of first order.

Sanjay K. Motwani[87] et al proposed simple, sensitive, selective, precise and stability-indicating HPTLC method for densitometric determination of moxifloxacin both as a bulk drug and from pharmaceutical formulation. The method employed TLC aluminium plates pre-coated with silica gel 60F-254 as the stationary phase and the mobile phase consisted of *n*-propanol–ethanol–6M ammonia solution (4:1:2, v/v/v). Densitometric analysis of moxifloxacin was carried out in the absorbance mode at 298 nm. Compact spots for moxifloxacin were found at *R*f value of 0.58±0.02. The linear regression analysis data for the calibration plots showed good linear relationship with R^2 = 0.9925 in the working concentration range of 100–800 ng spot−1. The method was validated for precision,

accuracy, ruggedness, robustness, specificity, recovery, LOD and LOQ. Drug was subjected to acid and alkali hydrolysis, oxidation, dry heat, wet heat treatment and photo degradation. All the peaks of degradation products were well resolved from the standard drug with significantly different *R*f values. As the method could effectively separate the drug from its degradation products, it can be employed as stability-indicating one. Moreover, the proposed HPTLC method was utilized to investigate the kinetics of the acidic and alkaline degradation processes at different temperatures.

Neeraj Kaul[86] et al developed a sensitive, selective, precise and stability indicating HPTLC method of analysis of nelfinavir mesylate both as a bulk drug and in formulations. The method employed TLC aluminium plates precoated with silica gel 60F-254 as the stationary phase. The solvent system consisted of toluene:methanol:acetone (7:1.5:1.5, v/v/v). This system was found to give compact spots for nelfinavir mesylate (*R*f value of 0.45±0.02). Nelfinavir mesylate was subjected to acid and alkali hydrolysis, oxidation, dry heat treatment and photo degradation. The wavelength was fixed at 250 nm. The linear regression analysis data for the calibration plots showed good linear relationship with R^2=0.999±0.002 in the concentration range of 1000–6000 ng per spot. The method was validated for precision, robustness and recovery. As the method could effectively separate the drug from its degradation products, it can be employed as a stability indicating one.

Neeraj Kaul[97] et al proposed a precise, sensitive, selective and stability-indicating high-performance thin-layer chromatographic method for the analysis of nevirapine both as a bulk drug and in formulations was developed and validated. The mobile phase consisted of toluene–carbon tetrachloride–methanol–acetone–ammonia (3.5:3.5:2.0:1.0:0.05, v/v/v/v/v). Absorbance mode was fixed at 289 nm. *R*f value of nevirapine was found at 0.44 ± 0.02. Nevirapine was subjected to acid and alkali hydrolysis, oxidation, dry heat and wet heat treatment and photodegradation. The drug undergoes degradation under acidic, basic conditions and oxidation. Also the degraded products were well resolved from the pure drug with significantly different *R*f values. Linearity was found to be in the range of 30–1000 ng/spot with good value of correlation coefficient. The linear regression analysis data for the calibration plots showed good linear relationship with R^2 = 0.998 ± 0.002. The method was validated for precision, robustness and recovery. The LOD and LOQ were 5 and 10 ng/spot

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

Puthli[85] et al suggested a rapid and sensitive HPTLC method was developed and validated for the estimation of Piroxicam. The wavelength was fixed at 360 nm. To justify the suitability, accuracy and precision of the proposed method, recovery studies were performed at three concentration levels. One of the degradation products of piroxicam is 2-aminopyridine (2AP). A TLC aluminium plate precoated with silica gel 60F-254 was used as the stationary phase. The solvent system consisted of toluene–acetic acid (8:2 v:v),which gave a compact spot of piroxicam with a Rf value of 0.5890.01 which was well separated from 2AP (Rf 0.2390.01). The calibration plots exhibited good linear relationship coefficient of correlation R^2=0.9982 over a concentration range of 400–800 ng. Statistical analysis proves that the proposed method is accurate and reproducible. The method is stability indicating and being economical can be employed for the routine analysis in bulk drug as well as pharmaceutical formulations.

Sapna[84] et al developed rapid, selective and stability indicating high performance thin layer chromatographic method was developed and validated for their simultaneous estimation in pharmaceutical dosage forms. The method employed TLC aluminium plates precoated with silica gel 60F-254 as the stationary phase and solvent system consisted of ethyl acetate–methanol– ammonia (7:1.5:1, v/v/v). This system was found to give compact spots for both pseudoephedrine (*Rf* value of 0.69_0.01) and cetirizine (*Rf* value of 0.38_0.01). The detection wavelength was fixed at 240 nm. The calibration plots showed good linear relationship with R^2=0.9947 in the concentration range of 10–26 ng for pseudeophedrine and 200–1200 ng for cetirizine with R^2=0.9973. The method was validated for precision, accuracy, ruggedness and recovery. Both the drugs do not undergo degradation under acidic and basic conditions. The samples degraded with hydrogen peroxide showed additional peaks at *Rf* values of 0.75 and 0.28 for pseudoephedrine and cetirizine, respectively. This indicates that both the drugs are susceptible to oxidation. Statistical analysis proves that the method is reproducible and selective for the simultaneous estimation of pseudoephedrine and cetirizine. As the method could effectively separate the drugs from their degradation products, it can be employed as a stability indicating one.

Sanjivani[83] et al proposd a simple, rapid ,selective and sensitive HPTLC method for the estimation of timolol in bulk drug and formulations. The mobile phase selected was ethyl acetate–methanol–isopropyl alcohol–ammonia (25%) (80:20:2:1, v:v:v:v). The calibration curve of the drug was linear in the range of 100–600 ng. The spectrodensitometric analysis was carried out at 294 nm. The system precision and the method precision were excellent with an RSD of 2.8 and 1.004, respectively. The limits of detection and quantitation were 10 and 40 ng, respectively. The mean percent recovery was found to be 98.6. Timolol maleate was degraded by exposing the drug to heat, acid and base. The degraded products were found to be well separated from the pure drug with significantly different *R*f values. The reported method is simple, selective, precise, accurate, time saving and economic as compared to reported HPLC methods. Hence this method can be employed as a stability indicating analysis method for quantification of timolol maleate in pharmaceutical preparations and as bulk drug.

Mahadik[82] et al suggested a simple, selective, precise and stability-indicating high-performance thin-layer chromatographic method of analysis of tizanidine hydrochloride both as a bulk drug and in formulations.The method employed TLC aluminium plates precoated with silica gel 60F-254 as the stationary phase. The solvent system consisted of toluene: acetone:ammonia (5:5:0.1, v/v/v). The Rf value of tizanidine was found at 0.329 0.01). Tizanidine hydrochloride was subjected to acid and alkali hydrolysis, oxidation and photodegradation. Also, the degraded product was well separated from the pure drug. Densitometric analysis of tizanidine hydrochloride was carried out in the absorbance mode at 315 nm. The linear regression analysis data for the calibration plots showed good linear relationship with R^2=0.9922 in the concentration range 300 - 1000 ng per spot. The method was validated for precision, recovery and robustness. The limits of detection and quantitation were 88 and 265 ng per spot, respectively. The samples degraded with hydrogen peroxide showed additional peak at Rf value of 0.12. This indicates that the drug is susceptible to oxidation. Statistical analysis proves that the method is repeatable and selective for the estimation of tizanidine. As the method could effectively separate the drug from its degradation product, it can be employed as a stability-indicating one.

Simmy[81] et al proposed a simple,precise and stability-indicating high-performance thin-layer chromatographic method of estimation of trimetazidine hydrochloride both as a bulk drug and in formulations. The mobile phase composition was n-butanol-water-methanol-ammonia (20%) (14:0.2:0.2:2, v:v:v:v). Densitometric detection of trimetazidine hydrochloride was carried out in the absorbance mode at a wavelength of 254 nm. The calibration curve of trimetazidine hydrochloride in methanol was linear in the range 400 – 2400 ng. The mean value of correlation coefficient was 0.99815,which shows a good correlation. The limits of detection and quantitation were 50 and 80 ng respectively. The recovery of trimetazidine hydrochloride was about 98 – 100%. The drug was subjected to different degradation conditions and the degraded products could be easily separated from the drug. Hence this developed method can be employed as a stability indicating one.

Ibrahim[111] et al developed a validated sensitive and highly selective stability indicating methods are adopted for simultaneous quantitative determination of sulpiride and mebeverine hydrochloride in presence of their reported impurities and hydrolytic degradates whether in pure forms or in pharmaceutical formulation. The first method is High Performance Liquid Chromatography, where the mixture of sulpiride and mebeverine hydrochloride together with metopimazine as internal standard are separated on a reversed phase cyano column (5 mm ps, 250 mm X4.6 id) using acetonitrile: water (70:30 v/v) adjusted to pH ¼ 7 as a mobile phase. The detection wavelength was fixed at 221 nm over a concentration range of 5-40 mg m^{-1} and 5-60 mg ml^{1} with mean percentage recoveries 99.75% and 99.99% for sulpiride and mebeverine hydrochloride respectively. The second method is High Performance Thin Layer Chromatography, where sulpiride and mebeverine hydrochloride are separated on silica gel HPTLC F254 plates using absolute ethanol:methylene chloride: triethyl amine (7:3:0.2 by volume) as mobile phase and scanning of the separated bands at 221 nm over a concentration range of 0.4-1.4 and 0.2-1.6 mg $band^{-1}$with mean percentage recoveries 101.01% and 100.40% for sulpiride and mebeverine hydrochloride respectively.

Neeraj kaul[97] et al proposed a precise, sensitive, selective and stability-indicating high-performance thin-layer chromatographic method of analysis of nevirapine both as a bulk drug and in formulations was developed and validated. The mobile phase consisted of

toluene–carbon tetrachloride–methanol–acetone–ammonia (3.5:3.5:2.0:1.0:0.05,v/v/v/v/v). Densitometric analysis of nevirapine was carried out in the absorbance mode at 289 nm. This system was found to give compact spots for nevirapine at *R*f value of 0.44 ± 0.02. Nevirapine was subjected to acid and alkali hydrolysis, oxidation, dry heat and wet heat treatment and photodegradation. The drug undergoes degradation under acidic, basic conditions and oxidation. The degraded products were well resolved from the pure drug with significantly different *R*f values. Linearity was found to be in the range of 30–1000 ng/spot with significantly high value of correlation coefficient of R^2 = 0.998 in the working concentration range of 300 ng/spot to 1000 ng/spot. The method was validated for precision, robustness and recovery. The limit of detection and quantitation were 5 and 10 ng/spot, respectively. As the method could effectively separate the drug from its degradation products, it can be employed as a stability indicating one.

CHAPTER III

DRUG PROFILE

- Drug Name : ***Zolmitriptan***
- Molecular formulae : $C_{16}H_{21}N_3O_2$
- Molecular weight : 287.36

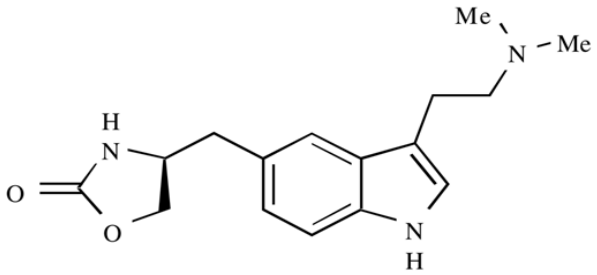

Fig:3 Chemical structure of ZMT

- Chemical name : 4(*S*)-4-[3-(2-dimethyl aminoethyl)-1H-5- indolyl-methyl]-1,3- oxazolan-2-one
- Color and nature : White to almost powder
- Solubility : Freely Soluble in methanol, soluble in ethanol, Sparingly soluble in Dichloromethane and insoluble in water
- Melting point : 136 - 141ºC
- Category : ZMT is a selective serotonine 5-HT receptor agonist
  Used for the treatment of migrane.
- Dosage Forms : Available as,
  Tablet 2.5 and 5.0 mg
  Orally disintegrating tablet 2.5 and 5.0 mg.
  Nasal spray 5.0 mg.
- Half life : 2-3 hours

Chemistry and pharmacology:

ZMT is a selective $5HT_{1D}$ and $5HT_{1B}$ receptor drug. It is converted to an active N-desmethyl metabolite which has several fold higher affinity for $5HT_{1D}$ and $5HT_{1B}$ receptors than the parent drug. Two hypothesis have been proposed to explain the efficacy of the drug in migrane therapy. One hypothesis implicates the ability of receptors to cause the constriction of intracranial blood vessels including arteriovenous anastomoses and thus restore the blood flow to the brain. Second hypothesis relates to the observation that both $5HT_{1D}$ and $5HT_{1B}$ receptors serve as presynaptic autoreceptors, modulating neurotransmitter relief from neuronal terminal. ZMT may block the release of proinflammatory neuropeptides at the level of nerve terminal in the perivascular space.

CHAPTER IV

AIM AND OBJECTIVE OF THE WORK

- ZMT is a synthetic triptamine derivative, chemically known as (s)-4-{[3-(2-dimethylaminoethyl)-1H-indol-5-yl] methyl} 1, 3-oxazolidin-2one. It is an oral, selective serotonin receptor agonist of the serotonin receptor and is used for the treatment of acute migraine attacks. It causes constriction of the blood vessels there by relieving the pain due to migraine headache.

- It is a Non-Pharmacopoeial drug.

- Several analytical HPLC methods were reported in literature for the quantitative determination of ZMT and its metabolites in human plasma and other biological fluids.

- Very few achiral and chiral HPLC methods were reported for the detection and quantification of the related impurities and degradation products in the drug substance ZMT.

- Since there is no HPTLC method reported for the stress degradation studies of this drug and some of the reported HPLC stress-degradation methods presented unsatisfactory peak symmetry, it was proposed to develop, validate and perform forced degradation studies for the determination and quantification of degradation products of ZMT by HPLC and HPTLC methods.

CHAPTER V

PLAN OF WORK

HPLC & HPTLC

- Selection of suitable method for the study
- Selection of suitable wavelength
- Method development
    - Selection of suitable mobile phase
- Quantification of drugs by selected method
- Validation of RP-HPLC and HPTLC method
    - Accuracy
    - Precision
    - Linearity
    - Robustness
    - Ruggedness
    - Solution stability
    - Limit of detection (LOD)
    - Limit of quantification (LOQ)
- Stress degradation studies
- Determination of degradants

STRESS DEGRADATION STUDY

1. Liquid state degradation study

- Neutral hydrolysis
- Acid hydrolysis
- Base hydrolysis
- Oxidation
- Photolysis

2. Solid state degradation study

- Dry heat degradation study (120° C)

CHAPTER VI

EXPERIMENTAL

HIGH PRESSURE LIQUID CHROMATOGRAPHY (HPLC)

METHOD DEVELOPMENT AND OPTIMIZATION

Method Development

Bulk drug: ZMT supplied by Orchid Health Care Chennai

Chemicals and Solvents:

- HPLC grade Acetonitrile
- HPLC grade Methanol
- Analytical reagent grade sodium hydroxide
- Analytical reagent grade hydrochloric acid
- Analytical reagent grade hydrogen peroxide
- Ortho phosphoric acid
- HPLC grade water was prepared by using Millipore MilliQ water purification system.

Instruments:

- HPLC : Shimadzu Prominance
- Detector : SPD-M20A Prominance-diode array detector
- Pump : LC-20AT Prominance –liquid chromatogram (Rheodyne 7725i with 20µL loop injector. The output signal was monitored and processed using LC Solution software on HCL computer).
- Sonica ultrasonic cleaner sonicator was used
- pH meter (Elico LI 127)
- Hot air oven was used for thermal stability studies.

OPTIMIZATION

Selection of wavelength

The wave length for the analysis of ZMT was selected from the UV spectrum absorbance of ZMT by scanning in the range of 200-400nm. From this spectrum, the wavelength of 226 nm was selected for the final method since the drug has shown good absorbance.

Selection of mode of separation

The selection of the method depends on the nature of the sample, its molecular weight and solubility. The drug selected for the present study is polar in nature and hence RP-HPLC method was preferred because of its simplicity and suitability.

Initial chromatographic conditions

Trial 1

Stationary phase	:	C-18 column
Mobile phase	:	70:30
Aqueous phase	:	Water (pH 3.0)
Organic phase	:	Acetonitrile
Detection wavelength	:	226 nm
Flow rate	:	1 ml/min
Temperature	:	Room temperature of 25 ±2º c

Trial 2

Stationary phase	:	C-18 coumn
Mobile phase	:	80:20
Aqueous phase	:	phosphate buffer (pH 3.0)
Organic phase	:	Acetonitrile:Methanol (50:50)
Detection wave length:		226 nm
Flow rate	:	1 ml/min
Temperature	:	Room temperature of 25±2ºc

Effect of pH:

With the initial chromatographic conditions, the standard solution were chromatographed for 20 min using 30% acetonitrile in water with pH ranging from 2.5 to 4.5 at 1 ml/min flow rate. There was found no significant change in retention time. Then the standard solution was chromatographed with 50:50 ratio of acetonitrile and methanol as organic solvent of different pH from 3.0 to 4.0 at the same flow rate of 1 ml/min. It was observed that the increase in pH decreases the retention time. For the present study, pH 3.5 was selected.

Effect of flow rate:

The flow rate shall be selected depends on the following data ;

- Retention time
- Peak symmetry
- Column back pressure

  Preferably the flow rate shall not be more than 2.5 ml/min. the selection of flow rate was done as flow rate which gives the least retention time and good peak symmetry.

Fixed chromatographic conditions

Stationary phase	:	C-18 column
Mobile phase	:	Solvent A (Phosphate buffer, pH 3.5)
	:	Solvent B (Acetonitrile: Methanol, 50:50)
pH	:	3.5 (adjusted with Orthophosphoric acid)
Solvent ratio	:	85 : 15
Detection wavelength	:	226 nm
Flow rate	:	1 ml/min
Temperature	:	Room temperature of 25±2ºC
Injection volume	:	20 µL

VALIDATION OF THE METHOD

System suitability

System suitability of method was performed by calculating the chromatographic parameters namely, column efficiency, resolution, peak symmetry factor and capacity factor on the repetitive of injection of standard solution.

Linearity and Range

A stock solution of the drug was prepared at a strength of 1 mg/ml. it was diluted to prepare solution containing 2-10 µg/ml in bulk sample of ZMT; the percentage of recoveries were calculated.

Precision

The precision of the developed method was determined in terms of intermediate precision (intra-day and inter-day). Three different concentrations of ZMT were analyzed in six independent series during the same day(intra-day precision) and six consecutive days (inter-day precision); with each series every sample was injected in triplicate. The %RSD values of intra-day and inter-day studies for ZMT showed that the precision of the method was satisfactory.

Specificity and Selectivity

The specificity of the method was established through study of resolution factors of the drug peak from the nearest resolving peak, and also among all other peaks.

Limit of Detection (LOD) and limit of Quantification (LOQ)

LOD is the smallest concentration of the analyte that can be detected and gives the measurable response (signal to noise ratio of 3). The signal to noise ratio were performed by comparing by measured signal of known low concentration of drug. LOQ is the smallest concentration of the analyte that can be accurately quantified (signal to noise ratio 10).

Solution stability

The solution stability of ZMT was carried out by leaving the test solution in a tightly capped volumetric flask at room temperature for 48 hours. The same sample solution was assayed for 24hr interval up to a study period against freshly prepared solution of ZMT. The

%RSD of assay of ZMT was calculated for the subject period during solution stability experiments.

Ruggedness and Robustness of the method

The ruggedness and robustness of the methods were determined by analyzing the sample at normal operating conditions and also by changing some operating analytical conditions such as column make, mobile phase compositions, flow rate, instrument and analyst.

STRESS DEGRADATION STUDY

Types of stress degradation study:

1. Liquid state degradation study
2. Solid state degradation study

Liquid state degradation study:

1. Base hydrolysis (0.1 M NaOH)
2. Acid hydrolysis (0.1M and 1M HCl)
3. Oxidation (3% H_2O_2)
4. Photolysis

Liquid state degradation study

Acid hydrolysis:

Ten ml of the 10 µg/ml of sample solution was transferred to a round bottomed flask and then 10 ml of the 0.1 M and 1 M HCl was added to the above and refluxed for 8 h in a boiling water bath. At the end of the exposure, the solution was cooled and neutralized with 10 ml NaOH.

Base hydrolysis:

Ten ml of the 10 µg/ml of sample solution was transferred to a round bottomed flask and then 10 ml of the 0.1 M NaOH was added to the above and refluxed for 8 h in a boiling water bath. At the end of the exposure, the solution was cooled and neutralized with 10 ml HCl.

Oxidation:

Ten ml of the 10 µg/ml of sample solution was transferred to a round bottomed flask and then 10 ml of 3% H_2O_2 was added to the above and refluxed for 8 h in a boiling water bath. At the end of the exposure, the solution was cooled.

Photolysis:

Fifty ml of 10 µg/ml of the sample solution was taken in a standard flask and kept at sunlight for 24 h.

Solid state degradation study:

Dry heat degradation studies:

Hundred mg of the drug sample was kept in an oven at 60ºC for 8 h to study the heat degradation. A solution of 10 µg/ml of the dry heat degraded sample solution was prepared and the chromatogram was run.

HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHT (HPTLC)

METHOD DEVELOPENT AND OPTIMIZATION

MEHOD DEVELOPEMNT

Bulk drug : ZMT supplied by Orchid health care Chennai

Selection of solvent : The selected solvent must give some ideal properties like, the drug should be stable in the selected solvent. Hence methanol was selected as the solvent for the drug.

Selection of detection wavelength:

The selection of proper wavelength is an important fact in the result of an HPTLC. The sensitivity of the HPTLC is depend on the wavelength selected and the UV detector. An ideal wavelength is the one that gives maximum absorbance and good response for the drug detected at lower concentration also. The drug was scanned under UV and 226 nm was selected as detection wavelength for the selected mobile phase.

Selection of Mobile Phase:

Various mobile phase systems were tried for selecting the best mobile phase.

Mobile Phase	Rf value	Observation
Methanol:Water	0.0714	Spot migrated with solvent
Ethyl acetate:Benzene	0.2727	Broad peak
Ethylacetate:Methanol:Water	0.1	Spot did not moved
Chloroform:Methanol	0.285	Symmetrical peak
Ethyl acetate:Chloroform:Methanol	0.197	Spot did not moved
Chloroform:Acetic acid	0.05	Spot did not moved
Chloroform:Acetone	0.2	Asymetrical peak

Optimization:

Fixed experimental parameters:

Injection	:	Linomat 5
Detection	:	CAMAG TLC scanner

Information:

Stationary phase	:	pre - coated silica gel GF aluminium sheets TLC plate
Mobile phase	:	chloroform: methanol: water (5:4:1 V/V/V)
Chamber saturation	:	15 min
Band length	:	6 mm
Application position	:	10 mm
Solvent front position	:	80 mm

Instrument:

Number of track	:	8
Position of first track X	:	15 mm
Distance between tracks	:	10 mm
Scan start position Y	:	5.0 mm
Scan end position	:	85 mm
Slit dimensions	:	6 x 0.3 mm micro
Optimize optical system	:	light
Scanning speed	:	20 mm/S
Data resolution	:	100 µm/steps

Measurement table:

Wavelength	:	226 nm
Lamp	:	D2 & W
Measurement type	:	Remission
Measurement mode	:	Absorption
Optical filter	:	Second order
Detector mode	:	Automatic

Preparation of standard solution:

ZMT 2 mg is weighed accurately and transferred to a 10 ml standard flask. Dissolve it in few ml of methanol and made up the volume with the same. The final concentration of the standard solution was kept at 200 ng/ µL.

VALIDATIOD OF THE METHOD

Linearity and range

A stock solution of the drug was prepared at strength of 2 mg/10 ml. It was diluted to prepare solutions containing 200-1000 ng/spot of the drug. The solutions were injected in triplicate into the **HPTLC**, keeping the injection volume constant (200 ng/ µL).

Accuracy

The accuracy of the method was evaluated in triplicate in different concentration levels 200-1000 ng/spot in bulk sample of ZMT.

Precision

Precision of the method was determined by

a) Intra-day precision
b) Inter-day precision
c) Repeatability
    i) Repeatability of sample application
    ii) Repeatability of measurement

a*) Intra-day Precision*

Intra-day precision was found out by carrying out the analysis of the standard drug solution and one of sample to be analyzed at concentration 600 ng/spot and 800 ng/spot for three times on the same day. % RSD were calculated.

b*) Inter-day precision*

Inter-day precision was found out by carrying out the analysis of the drug solution at a concentration 600 ng/spot for two days and % RSD were calculated.

c) *Repeatability*

i) *Repeatability of sample application*

Repeatability of the sample application was assayed by spotting 800 ng/spot of drug solution three times on a precoated TLC plate followed by the development and scanning, the % RSD was calculated

ii) *Repeatability of measurement*

Repeatability of measurement of the peak area was determined by spotting 600 ng/spot of the drug solution on a pre-coated TLC plate followed by the development. The separated spots were scanned three times without changing the position of the plate and the %RSD were calculated.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

In order to estimate the LOD and LOQ, blank methanol alone was spotted six times . Then the signal to noise ratio was determined. The LOD was found to be 3:1 and the LOQ was 10:1.

Stability Studies

When the developed chromatographic plate is exposed to atmosphere, the analyte is likely to decompose. Hence it is necessary to conduct stability studies.

Ruggedness and robustness of the method

Method robustness and ruggedness were determined by analyzing same sample at normal operating conditions and also by changing some operating analytical conditions such as mobile phase composition, instrument and analyst.

STRESS DEGRADATION STUDY

Types of stress degradation proposed to study:

- Liquid state degradation study.
- Solid state degradation study.

Liquid state degradation proposed to study:

1. Base hydrolysis. (0.1M NaOH)
2. Acid hydrolysis. (0.1M and 1M HCl)
3. Oxidation. (3% H_2O_2)
4. Photolysis

Solid state degradation proposed to study:

1. Dry heat degradation study (100°C).

Forced degradation studies

A stock solution containing 10 µg/ml of ZMT in methanol was prepared. Further diluted was made with Methanol. This solution was used for forced degradation to provide an indication of the stability indicating property and specificity of the proposed method. In all degradation studies the average peak area of ZMT after application (2000 ng/spot) obtained. In order to study the degradation products of ZMT using the HPTLC method most of the study was carried out by single development of the HPTLC plate in order to prevent the movement of the non-polar degradation products to the extreme end of the plate.

1. Acid and base induced degradation studies:

Acid decomposition studies were performed by refluxing the solution of drug 1M and 0.1M hydrochloric acid at 80°C for 8 h. The studies under alkaline conditions were carried out in 0.1M sodium hydroxide and the solution was refluxed for 8 h at 80°C. These were repeated at a lower temperature of 40°C keeping all other conditions constant. The resulting solutions were applied to HPTLC plate in such a way that final concentration achieved was 2000 ng/spot for both acid and degradation products and the chromatograms were run as described in section.

2. Hydrogen peroxide-induced degradation

To study hydrogen peroxide induced degradation. Initial studies were performed in 3% hydrogen peroxide at 80ºc for 8 h. For the HPTLC study, the resultant solutions were applied to TLC plate in such a way that final concentration achieved was 2000 ng/spot and the chromatograms were run as described in section.

3. Dry heat- and wet heat-degradation

The standard drug in solid form was placed in an oven at 100°C for 8 h to study the dry heat-degradation. The sample is then made into solution and a 2000 ng is spotted and the chromatogram were run as described.

4. Photochemical degradation

Ten µg/ml of the solution was kept at direct sunlight for 24 h and then a 2000 ng spot is made on TLC plate and the chromatogram were run as mentioned.

CHAPTER VII

RESULTS

HIGH PRESSURE LIQUID CHROMATOGRAPHY (HPLC)

Table 1: System Suitability Parameters

Parameters	ZMT
Number of theoretical plates	12863
HETP	11.66
Tailing factor	1.09
Capacity factor	2.372
Limit of detection LOD	0.54
Limit of quantification LOQ	1.82
Resolution	4.294
Linearity range	2-10 µg / ml

Table 2: Linearity and range

Sl.No	Concentration (µg/ml)	Peak area
1	2.0	281620
2	4.0	552172
3	6.0	837773
4	8.0	1134687
5	10.0	1395703

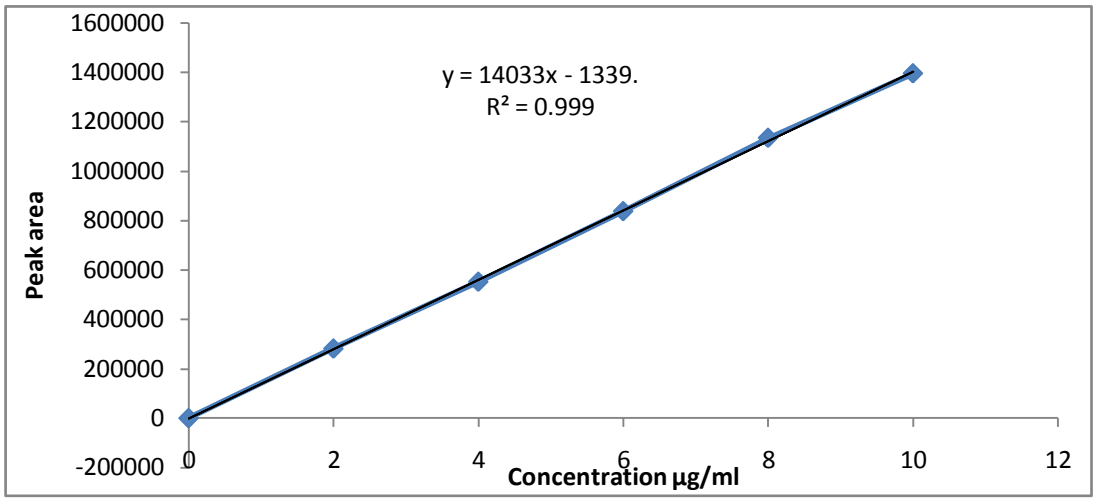

Fig 4: Linearity curve of ZMT

Table 3: Interday precision

Drug	Concentration (µg/ml)	Interday precision		
		Peak area	SD	%RSD
ZMT	4	546254	3365.15	0.6124
	6	829900	4468.16	0.5388
	8	1227985	7641.22	0.6222

Table 4: Intraday precision

Drug	Concentration (µg/ml)	Intraday precision		
		Peak area	SD	%RSD
ZMT	4	567355	4386.00	0.7730
	6	846865	5010.37	0.5916
	8	1235532	11174.43	0.9044

Table 5: Solution stability

Sl.No	Drug	Days	%Assay
1	Standard ZMT	Initial	99.42
2		After 24 hr	99.08
3		After 48 hr	98.74

Limit of detection and limit of quantification

Signal to noise ratio of 3:1 and 10:1 were obtained for the LOD and LOQ respectively.

Table 6: LOD and LOQ of ZMT

Parameter	ZMT (ng/ml)
LOD	0.54
LOQ	1.83

Table 7: Ruggedness (Day - 1, Analyst - 1)

Method Precision	Peak area	Average area	%Drug content	Average	SD	%RSD
Standard	837528					
Sample 1	826482	832626	98.68	99.41	4456.74	0.5352
Sample 2	835176		99.71			
Sample 3	834872		99.68			
Sample 4	829476		99.03			
Sample 5	837124		99.95			

Table 8: Ruggedness (Day-2, Analyst – 2)

Method Precision	Peak area	Average area	%Drug content	Average	SD	%RSD
Standard	846865					
Sample 1	824719	838187.2	97.38	98.50	13193.74	1.57408
Sample	842731		99.51			
Sample 3	837246		98.86			
Sample 4	842476		99.48			
Sample 5	823764		97.27			

Table 9: Robustness testing of the method

Parameters	Modifications	ZMT Recovery (%)
pH	3.0	96.43
	4.0	98.18
Detection wavelength (nm)	224	98.74
	230	99.82
Flow rate (ml/min)	0.8	97.46
	1.2	98.42

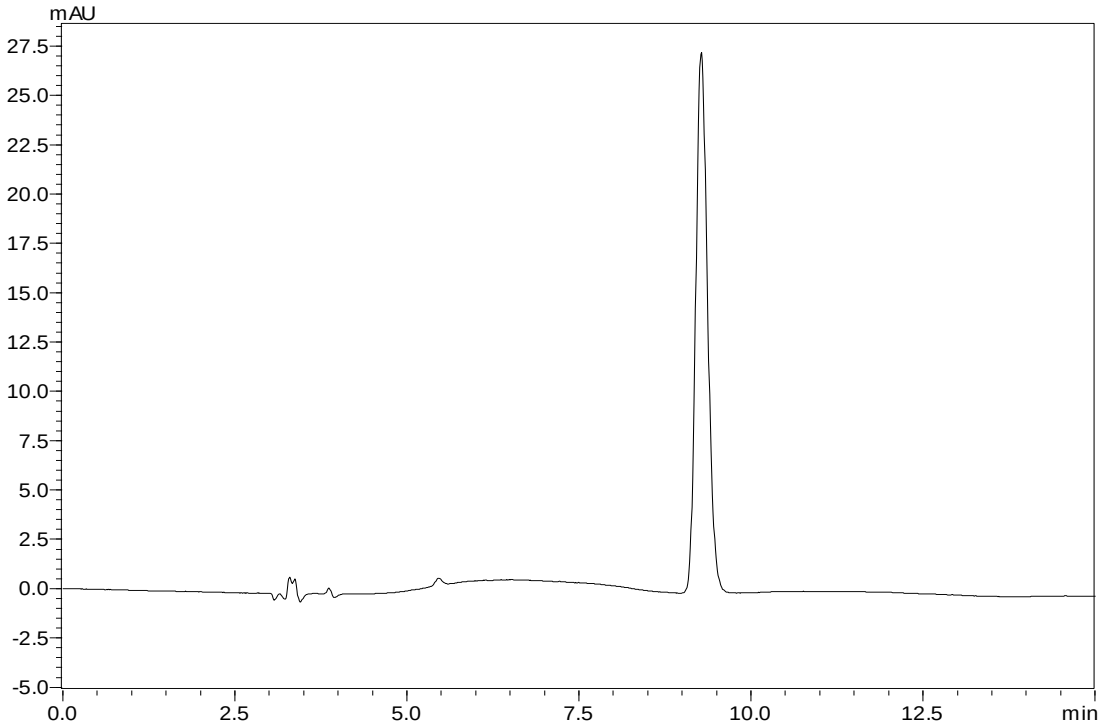

Fig 5: Chromatogram of 2 µg/ml of ZMT

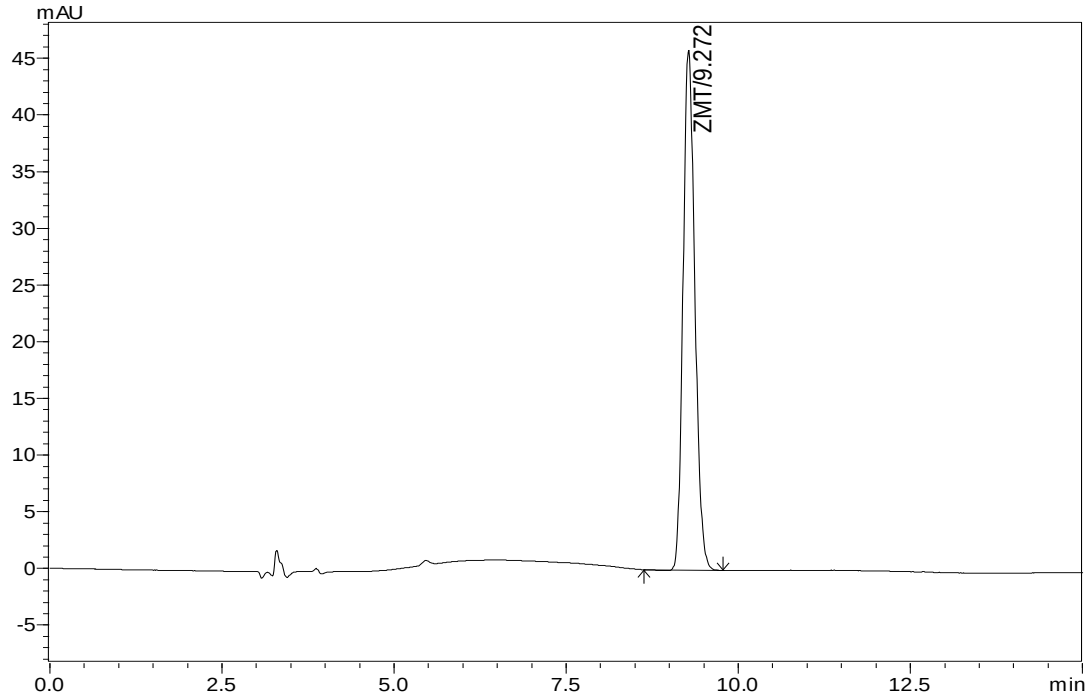

Fig 6: Chromatogram of 4 µg/ml of ZMT

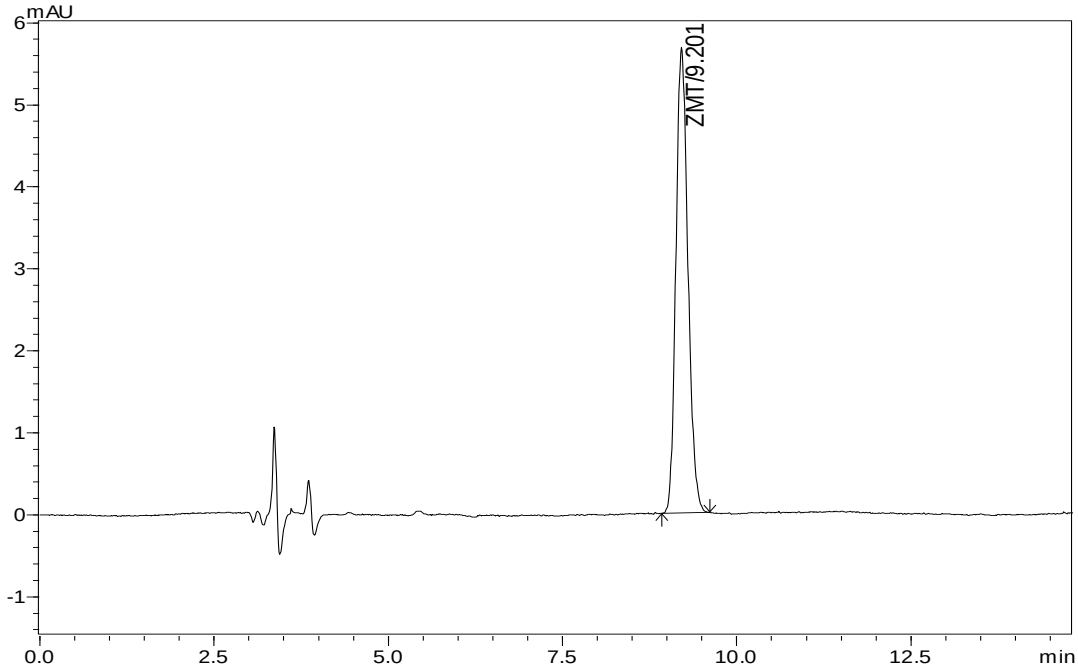

Fig 7: Chromatogram of 6 µg/ml of ZMT

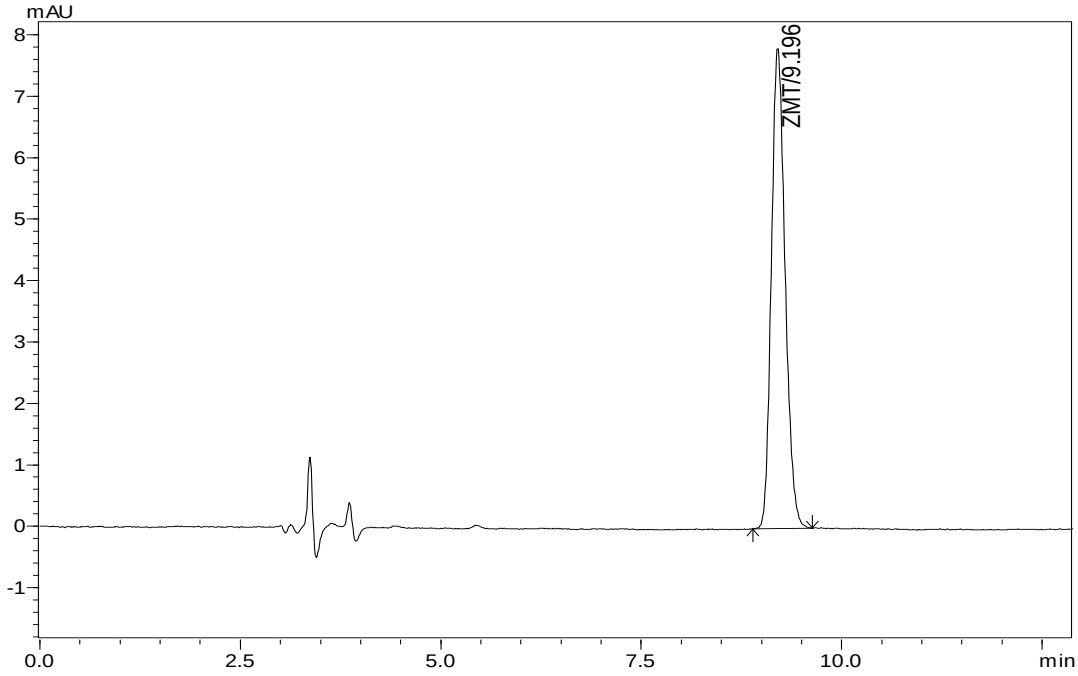

Fig 8: Chromatogram of 8 µg/ml of ZMT

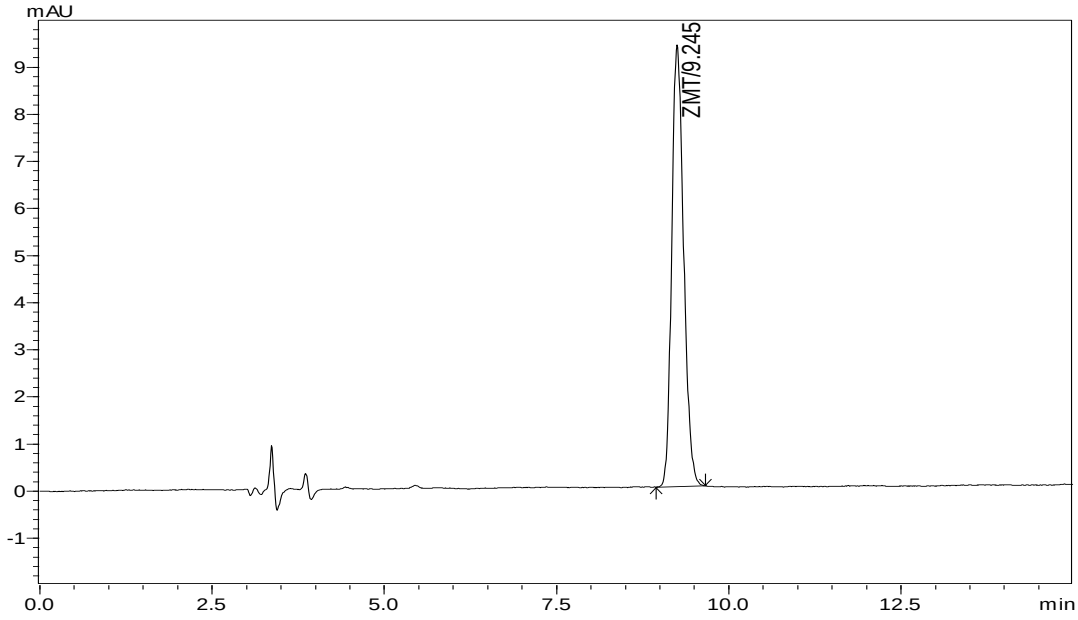

Fig 9: Chromatogram of 10 µg/ml of ZMT

Table 10: Result of forced degradation studies of ZMT samples using the proposed method

Stress condition/Duration/State	Degradation (%)
Acidic/1M HCl/8 h/Solution/80ºc	98.61
Acidic/ 0.1M HCl/8 h/Solution/80ºc	84.18
Basic/0.1M NaOH/8 h/Solution/80ºc	59.03
Oxidation/3% H_2O_2 /8 h/Solution/80ºc	97.68
Photolysis/Solution/Direct sunlight	74.37
Dry heat/Solid/100ºc in oven	32.23

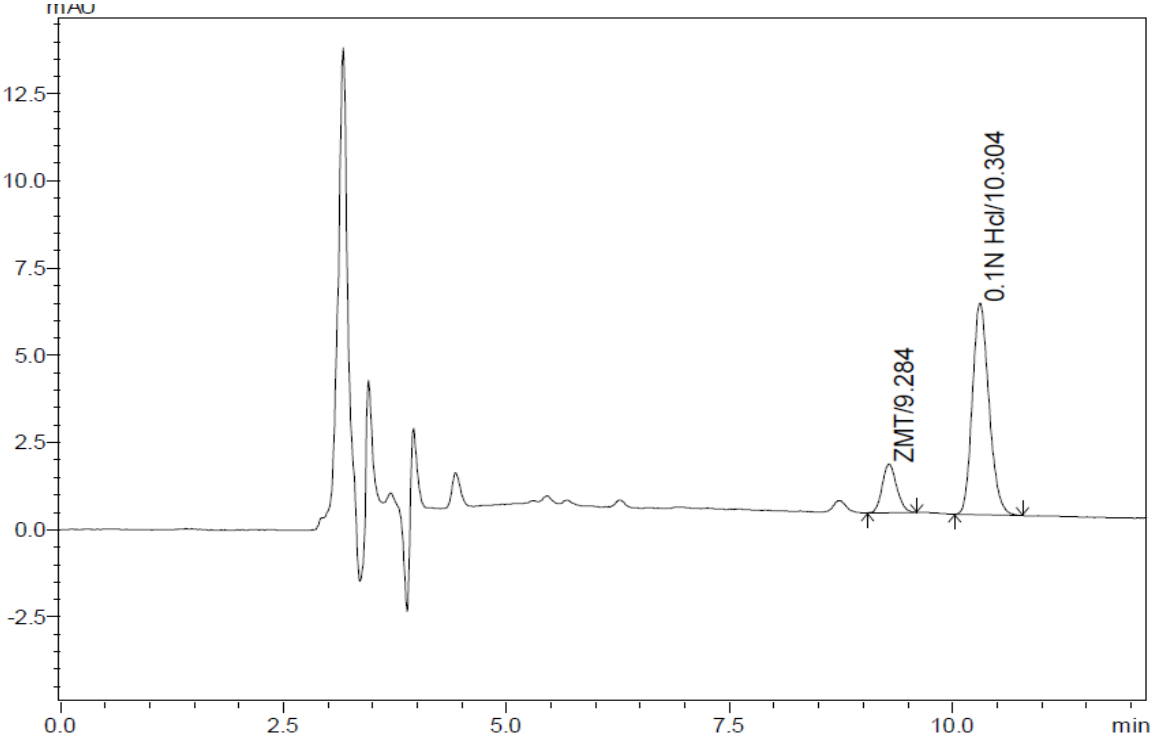

Fig 10: Chromatogram of ZMT subjected to acid degradation in 0.1M HCl

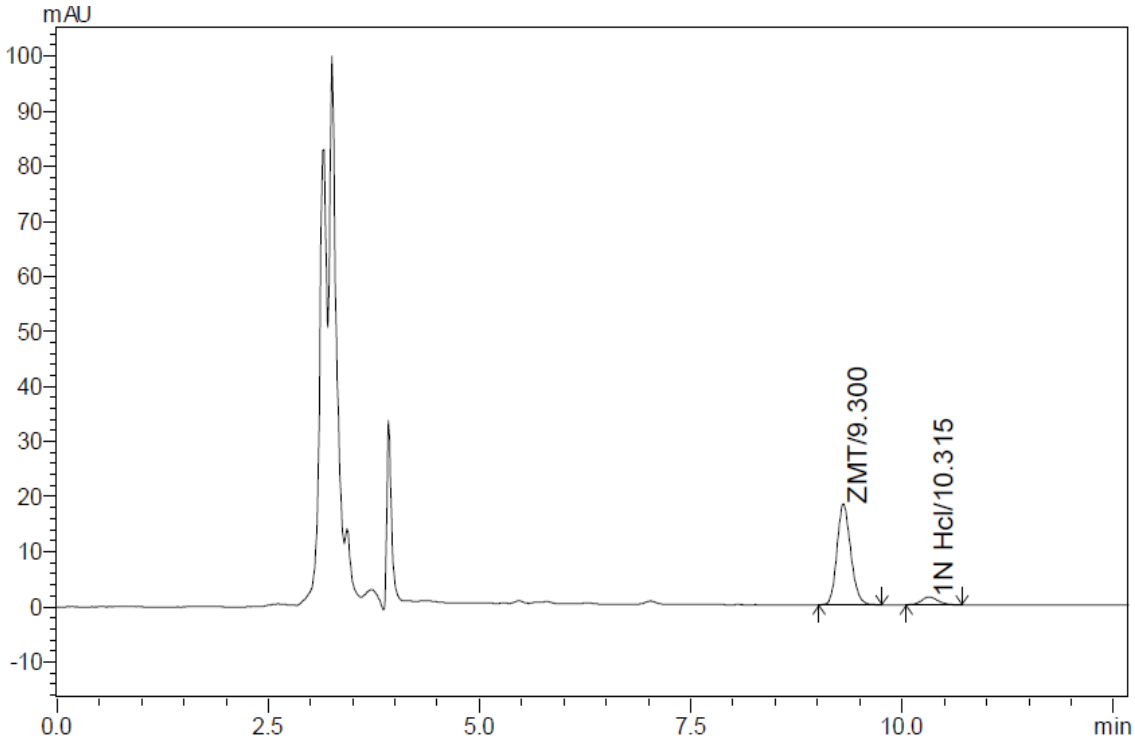

Fig 11: Chromatogram of ZMT subjected to acid degradation in 1M HCl

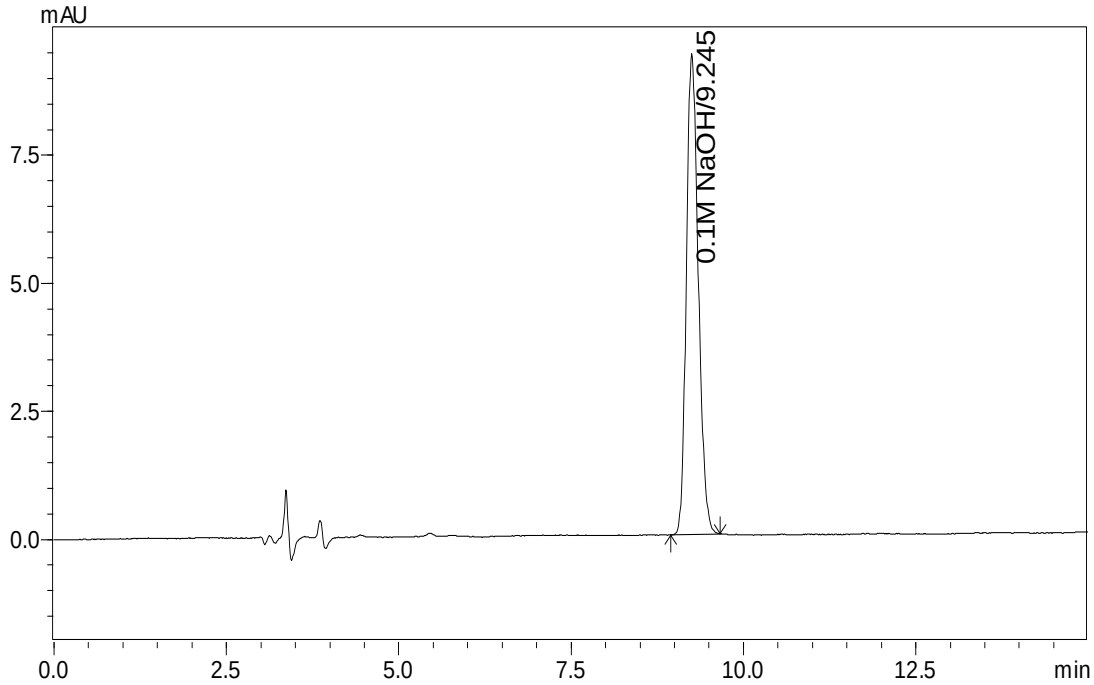

Fig 12: Chromatogram of ZMT subjected to alkaline degradation in 0.1M NaOH

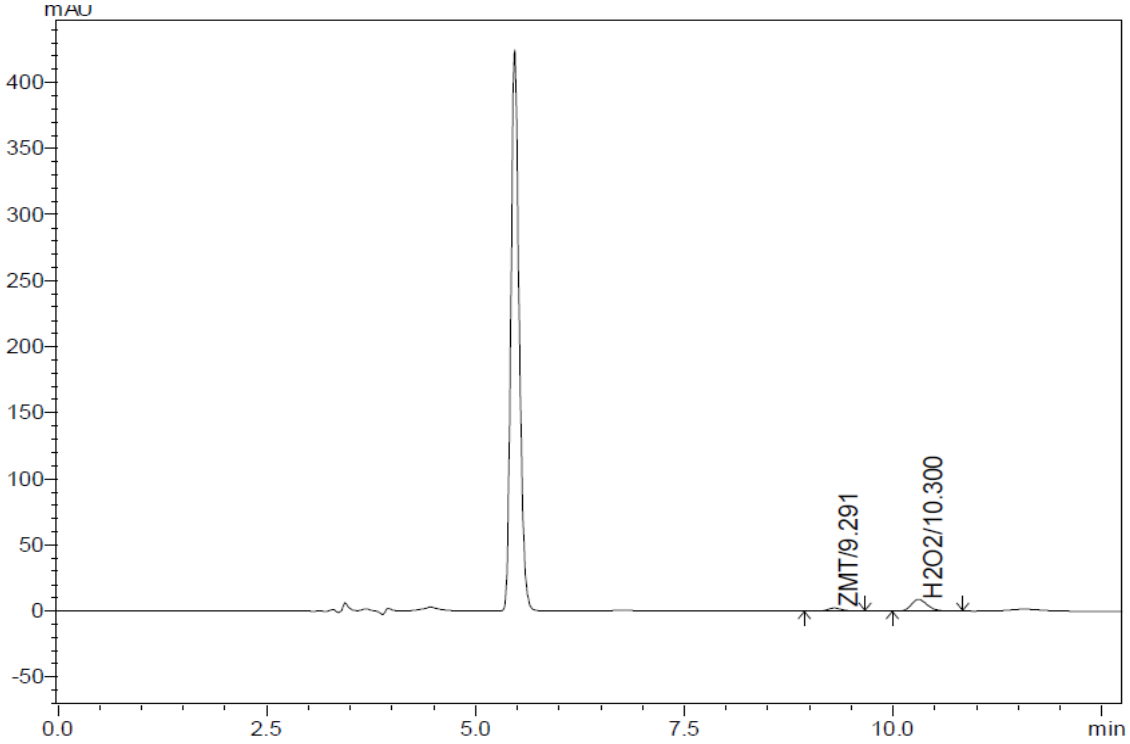

Fig 13: Chromatogram of ZMT subjected to oxidative degradation in 3% H_2O_2

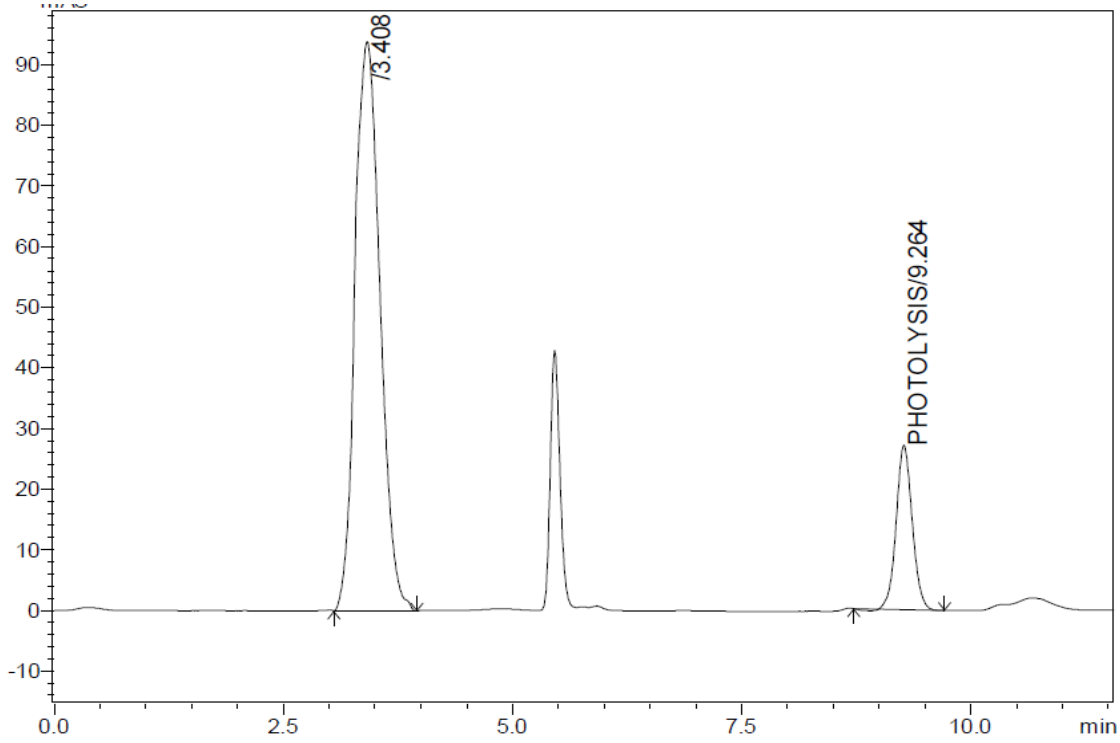

Fig 14: Chromatogram of ZMT subjected to photolytic degradation

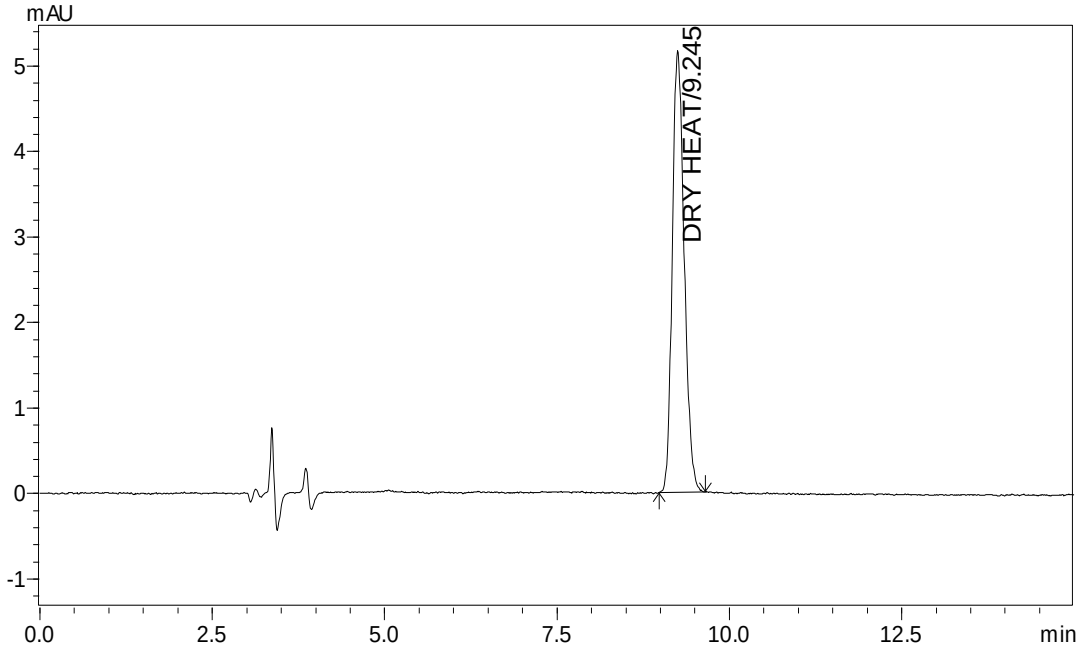

Fig 15: Chromatogram of ZMT subjected to dry heat degradation

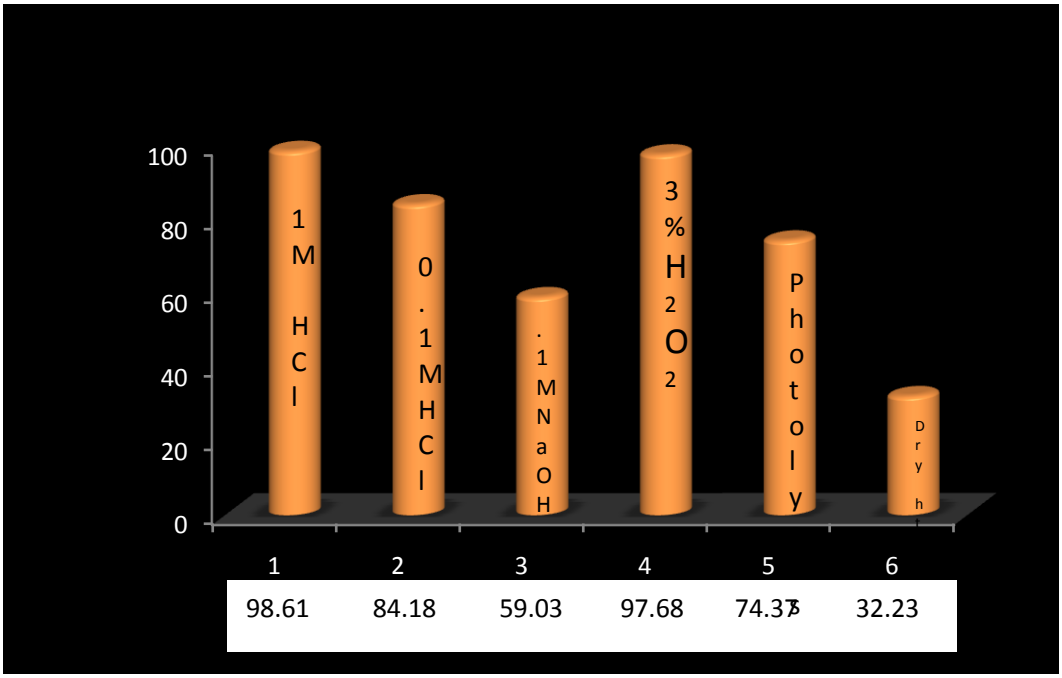

Fig 16: Bar diagram of force degradation study

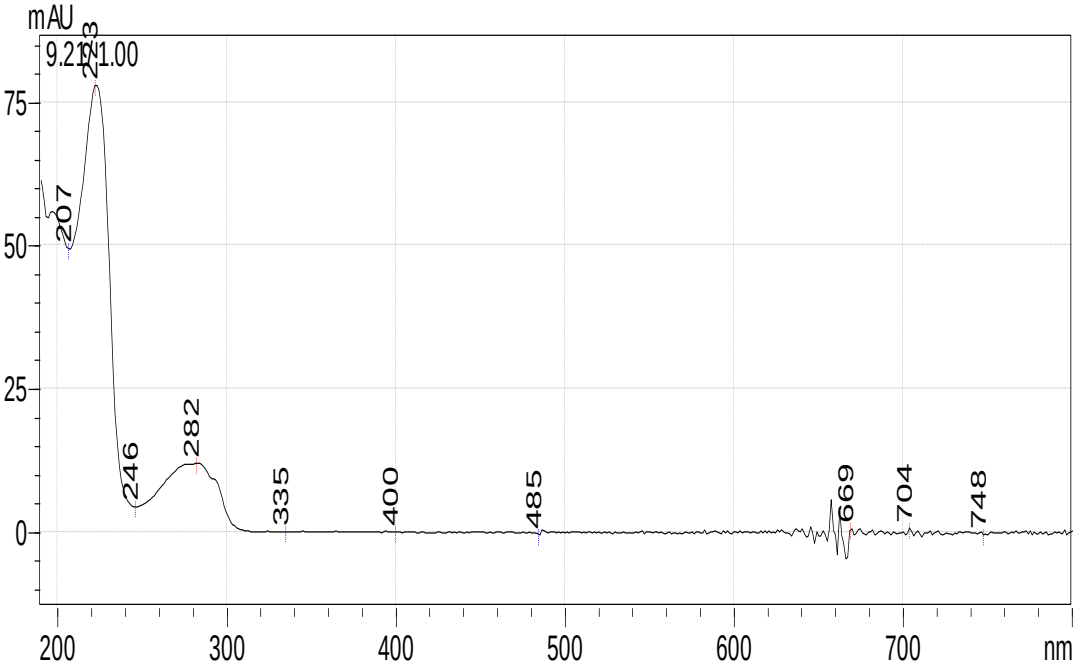

Fig 17: Spectrum index for ZMT

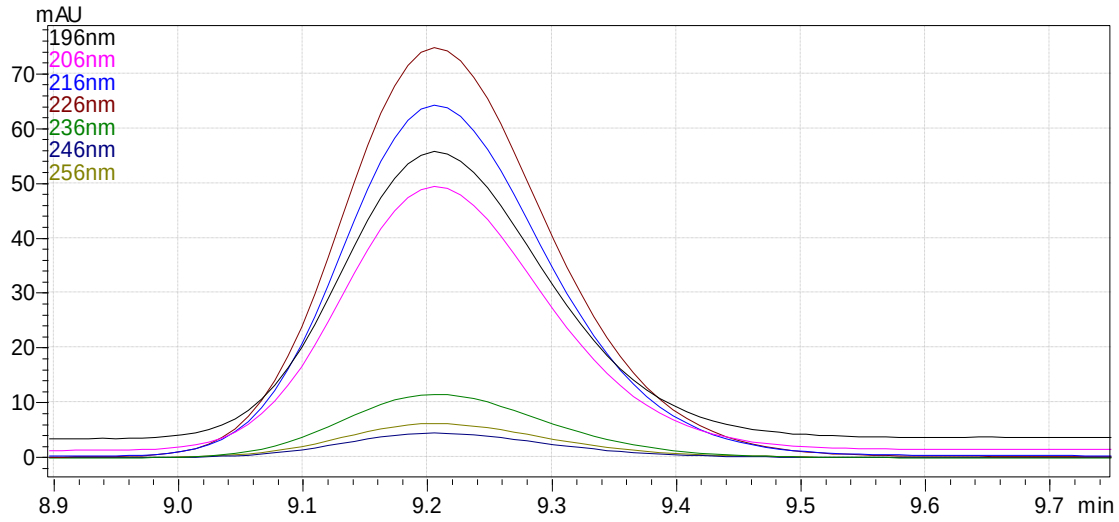

Fig 18: Peak purity profile for ZMT

Retention Time : 9.2 min

Impurity : Non Detected

Peak purity index : 1.00000

Single point threshold : 0.999218

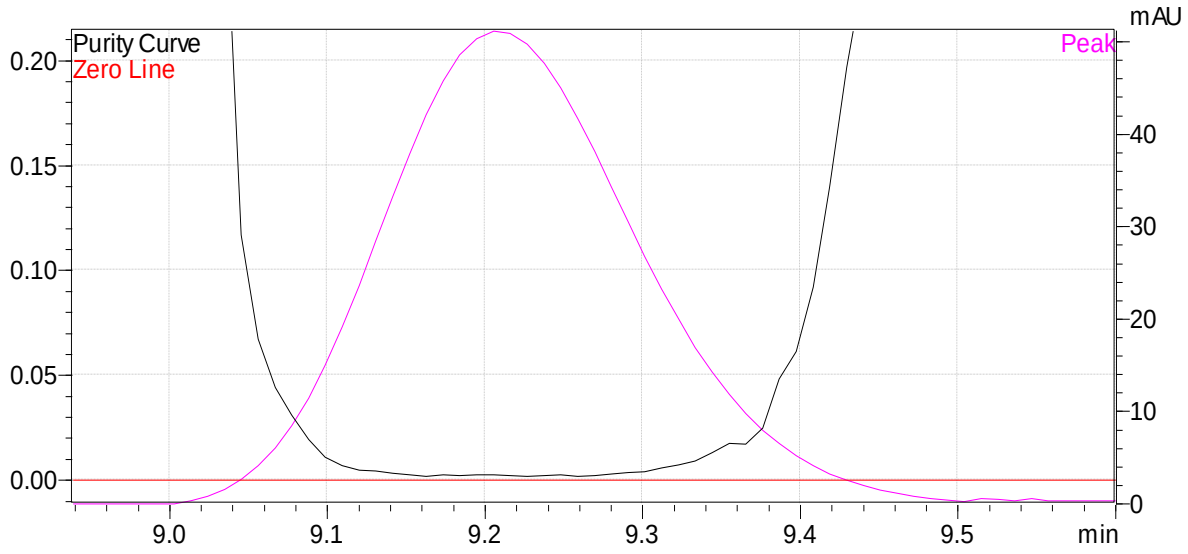

Fig 19: Peak purity curve for ZMT

Retention Time : 9.2 min

Impurity : Non Detected

Peak purity index : 0.999659

Single point threshold : 0.998673

HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY (HPTLC)

Table 11: LINEARITY AND RANGE

Concentration (ng/spot)	ZMT	
	Rf value	Peak Area
200	0.29	1759
400	0.30	3917
600	0.30	5297
800	0.30	6670
1000	0.30	7715

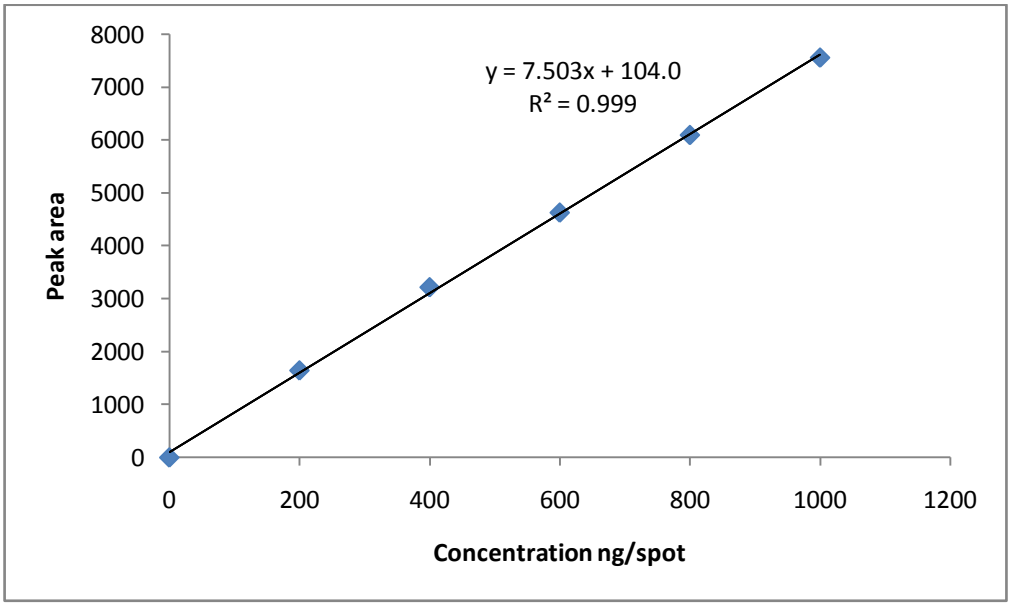

Fig 20: Calibration curve for ZMT

Inter Day and Intraday assay Precision (n=6)

Table 12: Intra Day Precision

Drug	Concentration (ng/spot)	Intraday precision		
		Peak area	SD	%RSD
ZMT	600	1854	29.0057	1.5639
	800	3922	70.4651	1.8064
	1000	5261	63.8513	1.1979

Table 13: Interday precision

Drug	Concentration (ng/spot)	Inter day precision		
		Peak Area	SD	%RSD
ZMT	600	1956	30.6648	1.5891
	800	3692	71.5052	1.9747
	1000	6492	71.1641	1.0932

Limit of detection and limit of quantification

Signal to noise ratio of 3:1 and 10:1 were obtained for the LOD and LOQ respectively.

Table 14: LOD and LOQ of ZMT

Parameter	ZMT (ng/spot)
LOD	40.8
LOQ	138.2

Table 15: Solution stability

Sl no	Drug	Days	% Assay
1	ZMT	Initial	99.24
		After 24 h	99.09
		After 48 h	98.51

Table 16: Ruggedness (Day-1, Analyst-1)

Method Precision	Peak area	Average area	% Drug content	Average	SD	%RSD
Standard	3997					
Sample 1	3917	3911.6	98.20	99.01	0.7286	0.7358
Sample 2	3856		98.41			
Sample 3	3992		99.68			
Sample 4	3920		99.82			
Sample 5	3873		98.96			

Table 17: Ruggedness (Day-2, Analyst-2)

Method Precision	Peak area	Avg area	% Drug content	Average	SD	%RSD
Standard	5412					
Sample 1	5297	5262.4	99.15	98.86	0.4335	0.4384
Sample 2	5316		99.28			
Sample 3	5237		98.84			
Sample 4	5198		98.16			
Sample 5	5264		98.89			

Table 18: Robustness

Parameters	Modification	% Recovery
Mobile phase ratio	5:3.5:1.5	96.83
	5:3.8:1.2	92.21
Development distance	20 mm	95.65
Detection wavelength	230 nm	97.52
Slit dimension	5.00 X 0 .30m micro	97.28

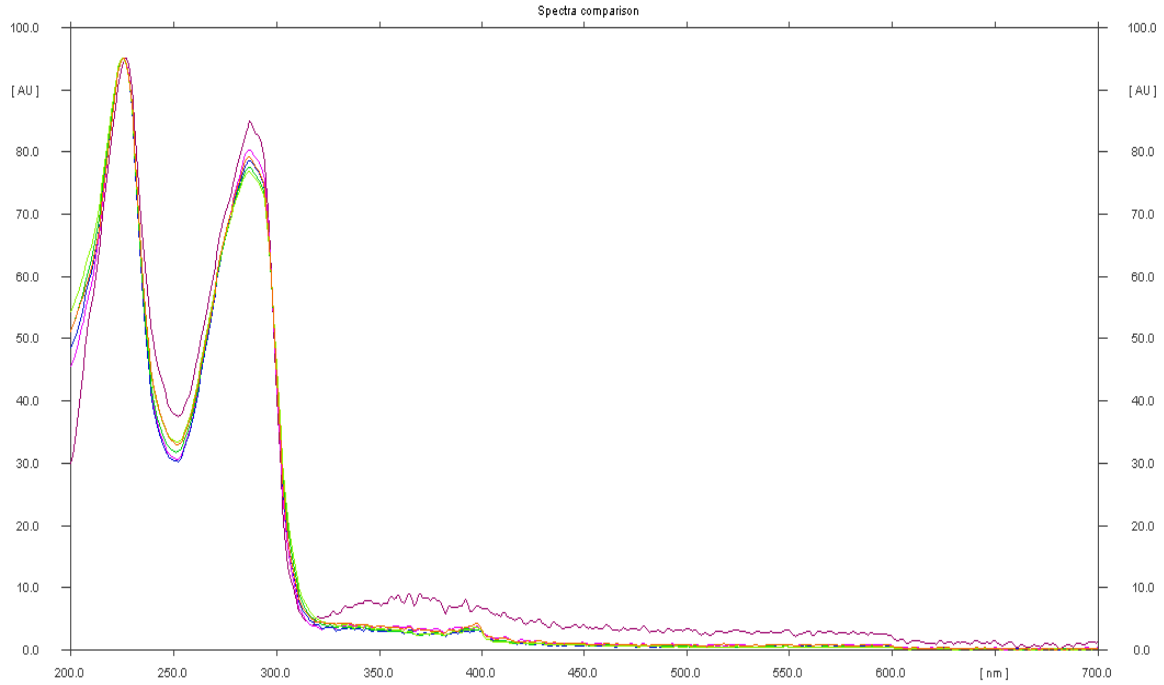

Fig 21: Overlay spectrum of ZMT at 226 nm

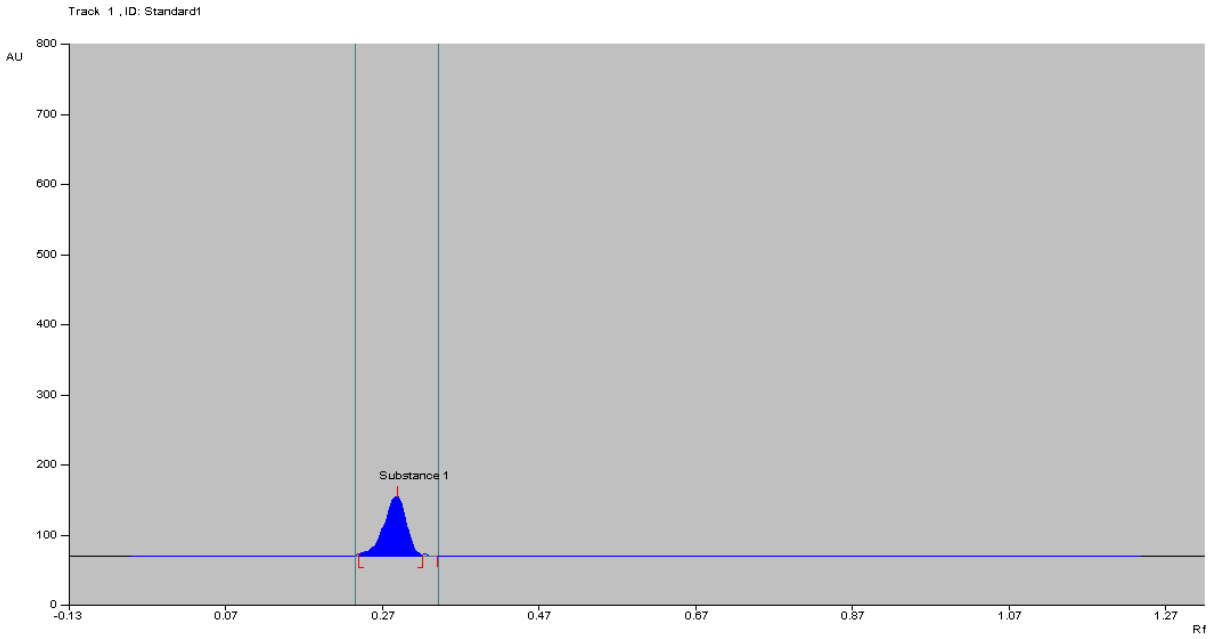

Fig 22: Densitogram of ZMT 200 ng/spot

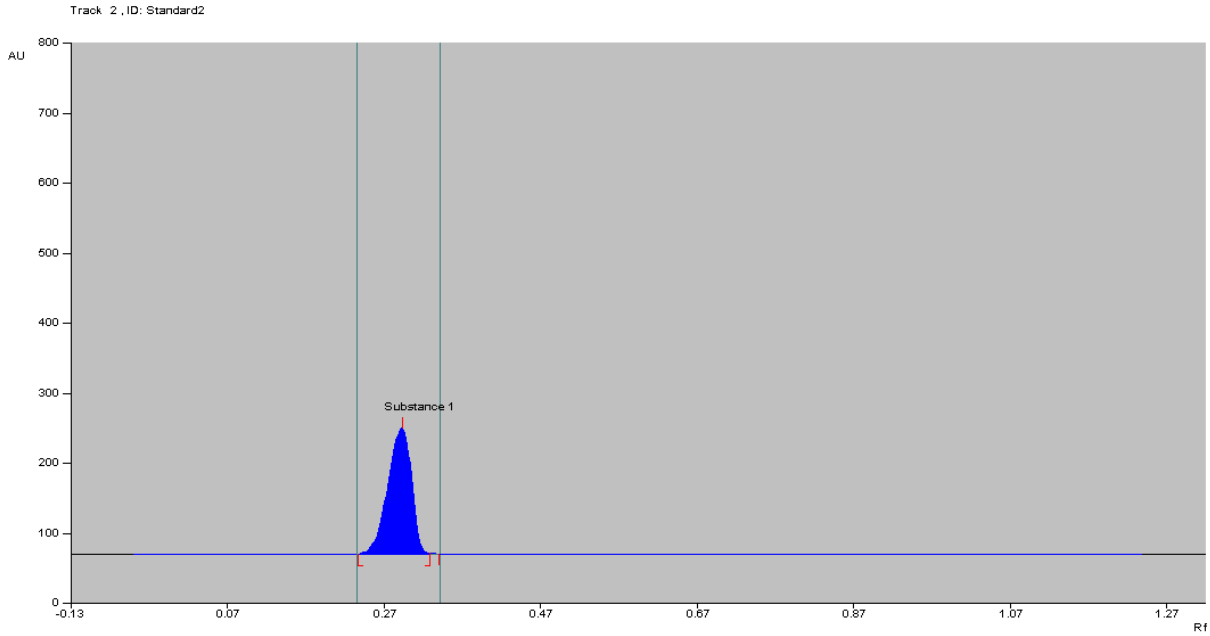

Fig 23: Densitogram of ZMT 400 ng/spot

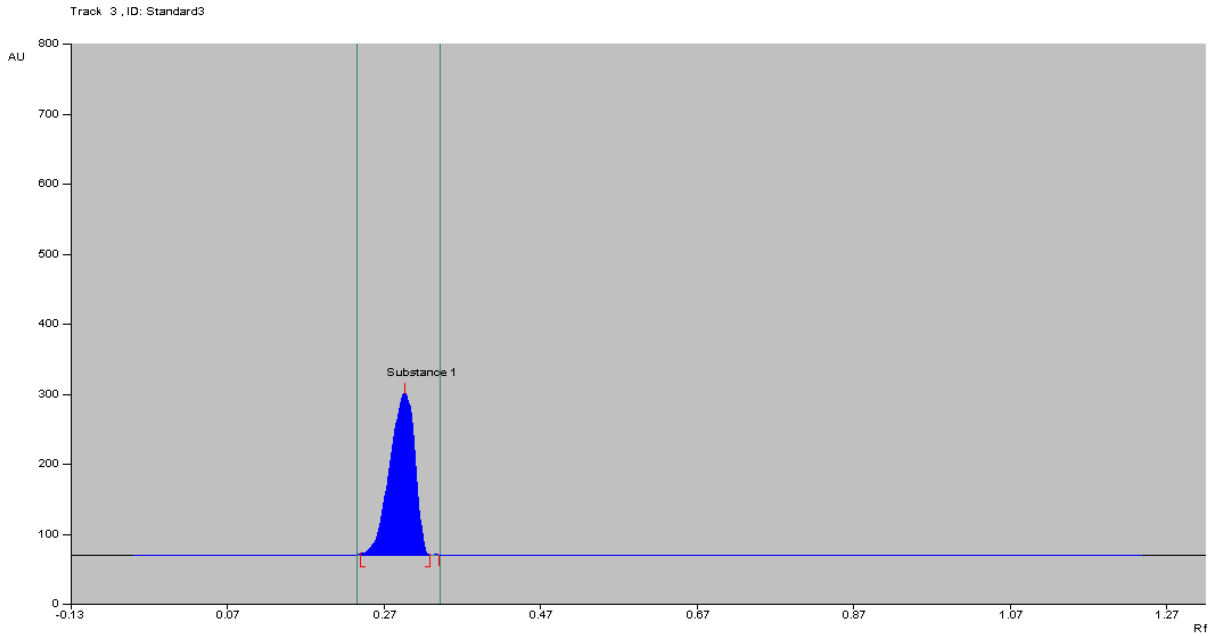

Fig 24: Densitogram of ZMT 600 ng/spot

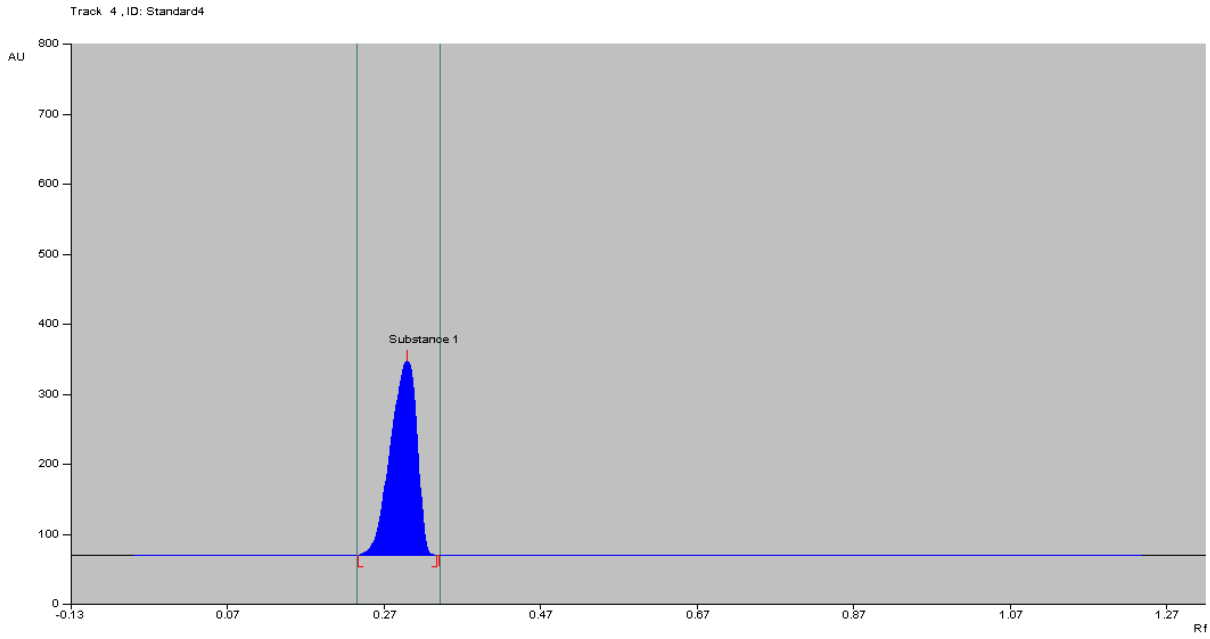

Fig 25: Densitogram of ZMT 800 ng/spot

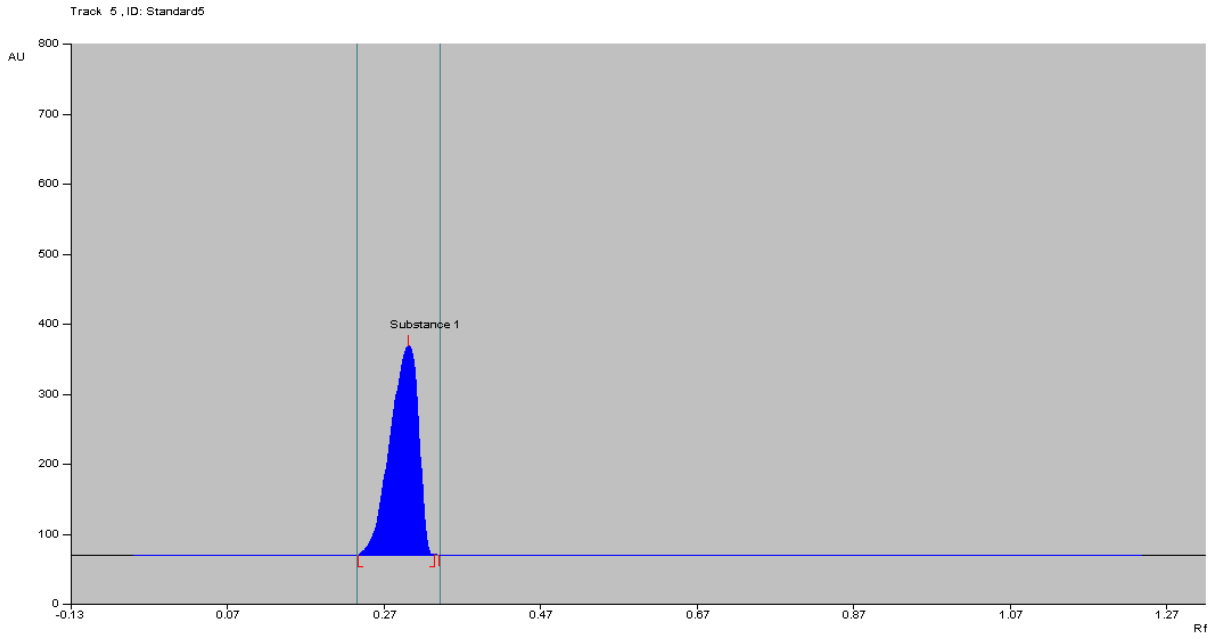

Fig 26 : Densitogram of ZMT 1000 ng/spot

Table 19: Result of Force Degradation Studies on ZMT

Stress condition/Duration/State	Degradation (%)
Acidic / 1M HCl / 8 h / solution / 80ºc	97.42
Acidic/0.1M HCl/8 h/solution/80ºc	86.64
Basic / 0.1M NaOH / 8 h/solution / 80ºc	62.27
Oxidation / 3% H_2O_2/ 8 h/solution / 80ºc	94.41
Photolysis / 10µg/ml solution / direct sun	76.83
Elevated temperature/ solid / 100ºc	33.59

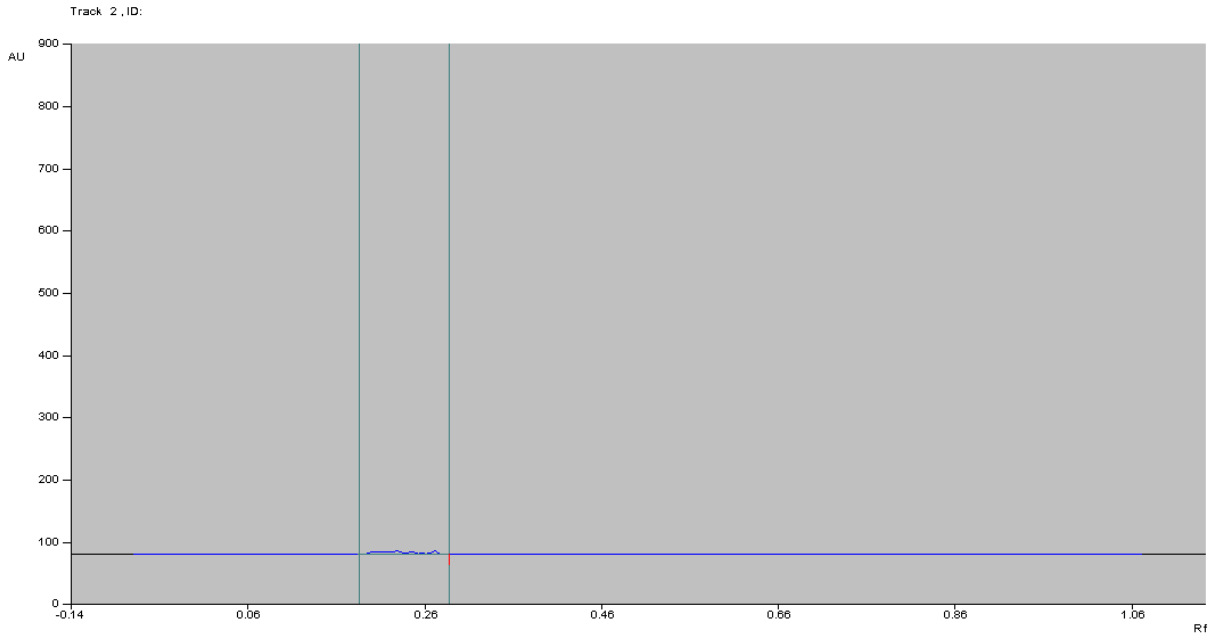

Fig 27: Densitogram of ZMT subjected to acid degradation in 1M HCl

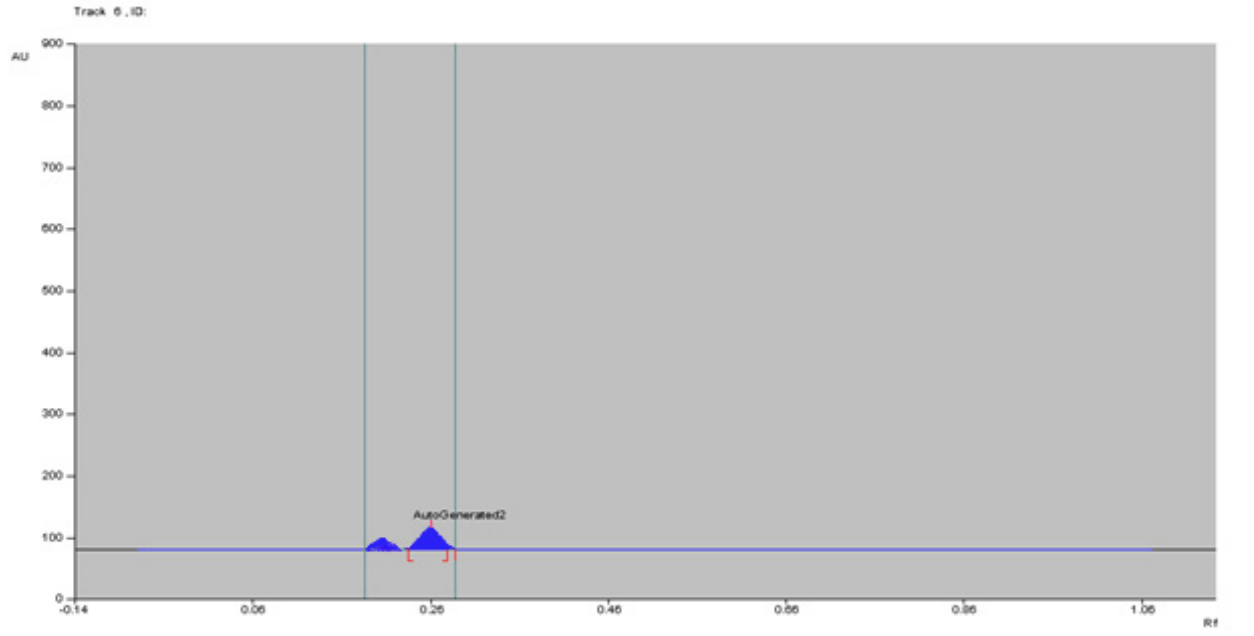

Fig 28: Densitogram of ZMT subjected to acid degradation in 0.1M HCl

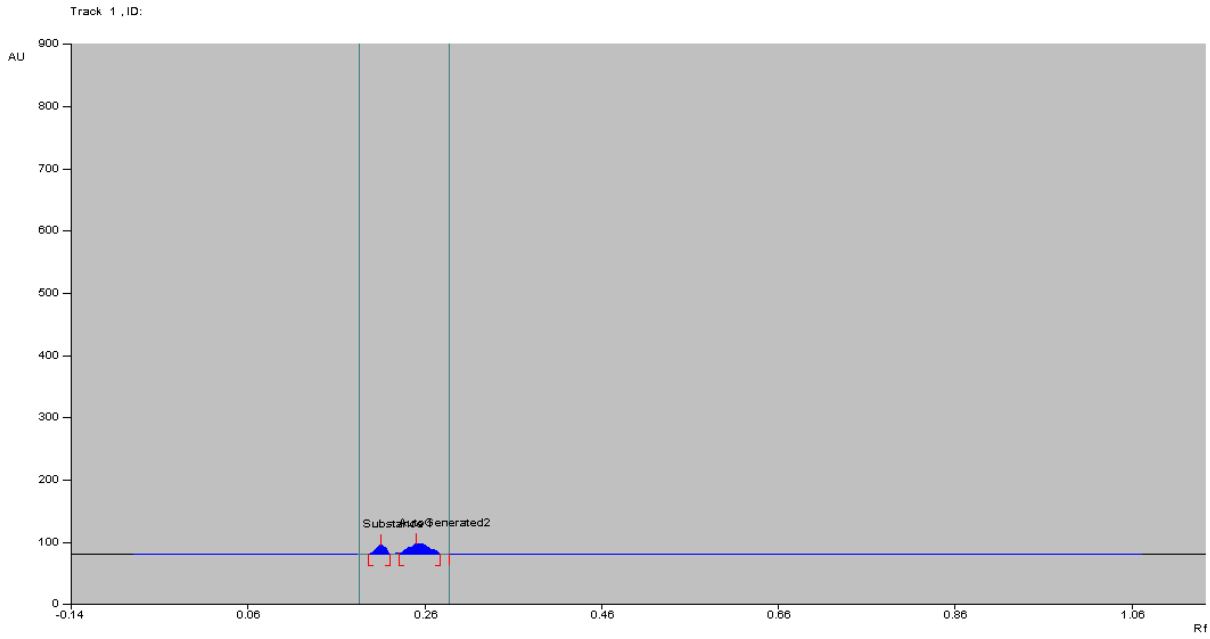

Fig 29: Densitogram of ZMT subjected to base degradation in 0.1M NaOH

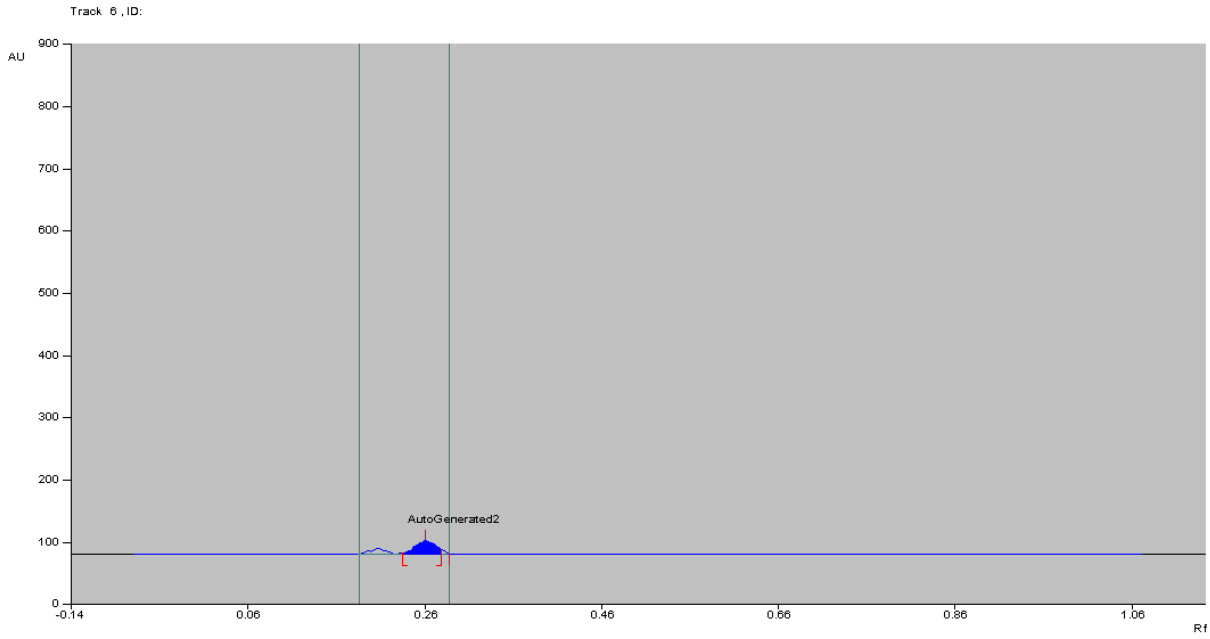

Fig 30: Densitogram of ZMT subjected toxidtaive degradation in 3% H_2O_2

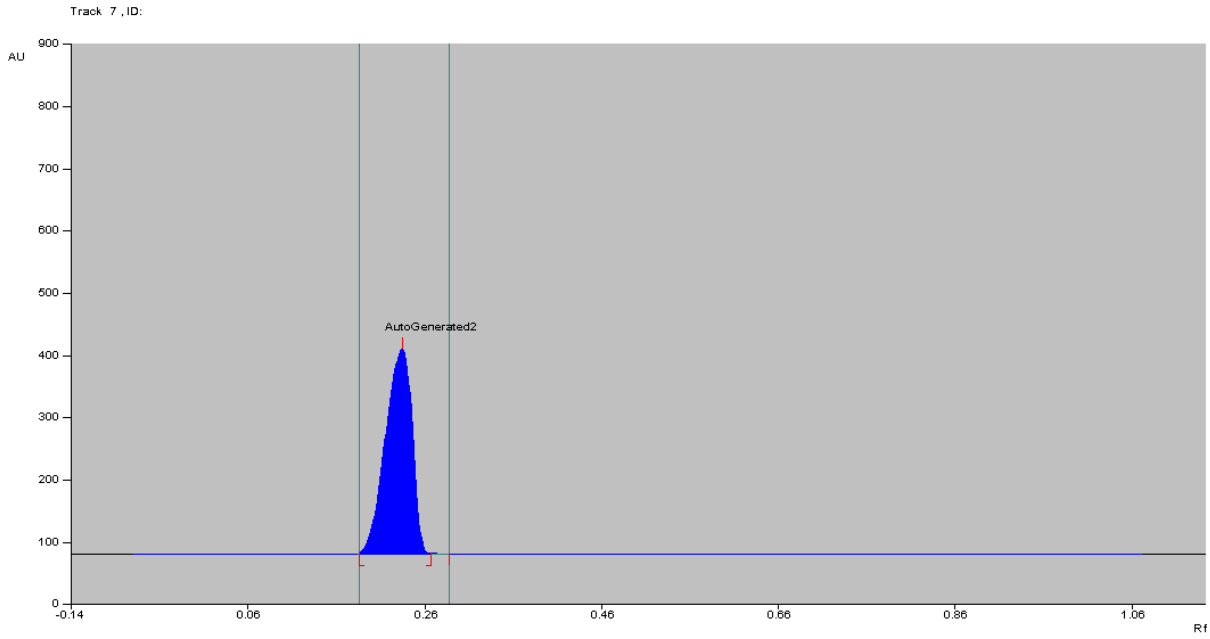

Fig 31: Densitogram of ZMT subjected to photochemical degradation (direct sunlight)

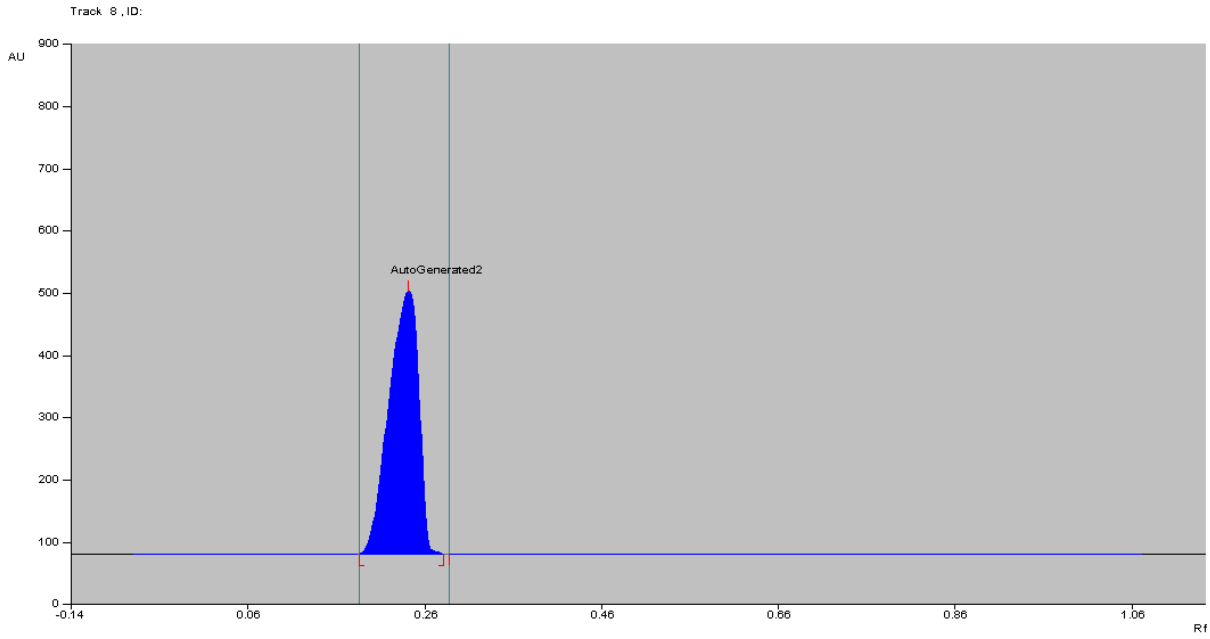

Fig 32: Densitogram of ZMT subjected to dry heat degradation (100ºC)

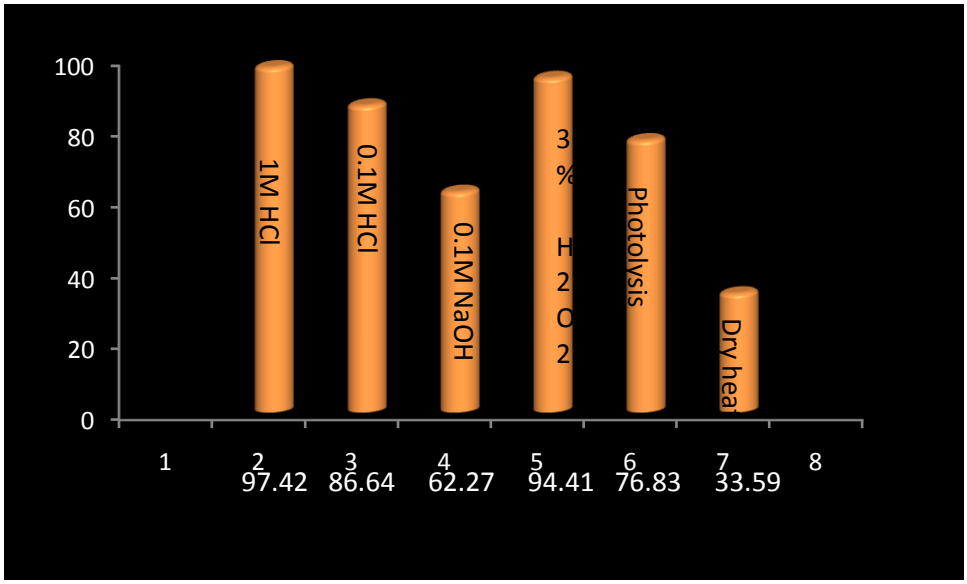

Fig 33: Bar diagram of forced degradation study

CHAPTER VIII

DISCUSSION

HIGH PRESSURE LIQUID CHROMATOGRAPHY (HPLC) DEVELOPEMNT AND OPTIMIZATION OF THE STABILITY INDICATING METHOD

The stability-indicating RP-HPLC method for the determination of ZMT in the presence of degradation products was developed. ZMT was completely resolved from degradation products and impurities in a C-18 column (250 X 4.0 mm id, 5 µm particle size) using phosphate buffer (pH 3.5) and acetonitrile: methanol (50:50) as mobile phase (85:15) with a flow rate of 1 ml/ min and detected at a wavelength of 226 nm. A Phenomenex C-18 column was chosen because it has high carbon loading with very closely packed material to give high resolution compared to other C-18 column to develop a specific, accurate, precise and stability indicating RP-HPLC method for the estimation of ZMT. Different mobile phase systems were attempted to detect and quantify ZMT and its degradation product. The results of optimised mobile phase system (consisted of solvent A, phosphate buffer (pH 3.5), solvent B, acetonitrile: methanol (50:50) in the ratio of 85:15) was found to be satisfactory with respect to location and resolution of the peaks. The observed retention time for ZMT was found to be 9.2 min. The mobile phase saturation time was given about 15 min. Regression analysis of the calibration data for ZMT showed that the dependent variable (peak area) and the independent variable (concentration) were represented by the equation Y=14033x + (-1339). The correlation of coefficient (R^2) obtained for ZMT was 0.9990. Thus a good linear relationship was observed in the concentration range of 2 – 10 µg/ml for ZMT. The assay of ZMT was found to be 99.42, which indicated high accuracy of the method. The absence of additional peaks in chromatogram indicated the non- interference of impurities.

METHOD VALIDATION

System Suitability

In order to determine the adequate resolution and repeatability of the proposed method, system suitability parameters including retention factor, selectivity, resolution and asymmetry factor were investigated and reported in Table 1.

Linearity and range

The calibration curves were linear in the range of 2-10 µg/ml, with a mean correlation coefficient, (R^2) of 0.9990 and the mean of regression equation of Y=14033 X - 1339, which shows a perfect correlation which is reported in Table 2.

Precision

The intraday and interday variation of the method was evaluated at different concentration levels. The %RSD values of intraday and interday were <2% for the ZMT revealed that the proposed method is precise and is reported in Table 3 and 4.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD was determined based on the signal-to-noise ratio and was determined using an analytical response of three times the background noise. The LOD for ZMT was found to be 0.54 µg/ml. The LOQ was determined as the minimum amount of analyte that was quantified above the baseline noise following the triplicate injection. The LOQ for ZMT was found to be 1.83 µg/ml, and the data are reported in Table 6.

Solution Stability

Stability of the solution was determined from the % assay of the solutions at different intervals. The study shows that the %RSD at different time was less than 2. Hence the sample solutions was found to be stable over the time (Table 5).

Ruggedness and Robustness of the method

According to ICH Guidelines, ruggedness and robustness assessment was performed during the development of an analytical procedure. By comparing interday and intraday precision results done by two analyst, the ruggedness of the method can be assessed. The %RSD value less than 2 for intraday and interday assay of ZMT, performed in the same

laboratory by two different analysts, indicated the ruggedness of the developed method as shown in Table 7 and 8. As shown in Table 9, robustness data of the mean obtained (n=6) for each factor studied, including that the selected factors remained unaltered by small variations of these parameters.

FORCED DEGRADATION STUDIES

A stock solution containing 10 µg/ml of ZMT was used for forced degradation to provide an indication of specificity of the proposed method. In all degradation studies like acid and base hydrolytic degradation, hydrogen peroxide (oxidation), dry heat and photolytic degradation, the average peak area of ZMT were obtained. ZMT were subjected to different ICH prescribed stress conditions. RP-HPLC analyses to identify the ZMT degradation products formed under stress conditions of hydrolysis, oxidation and photolysis.

The degradation of ZMT was found to occur in hydrolytic, oxidative and to some extend in photolytic conditions. A stability-indicating RP-HPLC method was developed for the estimation of the ZMT in the presence of its degradation products. Hence a more complete degradation pathway of the drug was established than what known at present, by using a stress degradation technique and employing a RP-HPLC method shown in Table 10.

Acid hydrolysis

Acid hydrolysis of ZMT was done with 0.1 M and 1 M HCl at 80ºC for 8 h, where severe hydrolytic degradation was observed (Fig 9 and 10) with one of the degraded product eluted at a retention time of 10.3 min. The hydrolysis of ZMT is intense when compared to that of alkali.

Base Hydrolysis

On treatment with 0.1M NaOH for 8 h at 80ºC which resulted in the degradation of the ZMT. No degradation peak (Fig 11) was found along with the main ZMT peak.

Oxidation

Oxidative degradation was performed for ZMT with 3% hydrogen peroxide at 80ºC. One degraded peaks was observed at a retention time of 10.3 min (Fig 12). Oxidative treatment gives an intense degradation of ZMT.

Photolysis

Photolytic degradation was done by exposing the ZMT to direct sunlight for 24 h, which results in the degradation of ZMT. There were no other co-eluting peaks were found along with the main peak (Fig 13).

Dry heat-degradation

The solid state studies revealed that the ZMT stable even after the exposure to elevated temperature of 100ºC for 8 h in oven. No degradation peaks were formed at this temperature and ZMT undergo degradation to a less extend only (Fig 14).

HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY (HPTLC) DEVELOPEMNT AND OPTIMIZATION OF THE STABILITY INDICATING METHOD

A HPTLC method was developed for the determination of ZMT in the presence of their degradation products generated during forced degradation studies. Use of HPTLC aluminium plates pre-coated silica gel 60_{F254} with chloroform: methanol: water in the volume ratio of 5:4:1 (v/v) resulted in good separation of ZMT and degradation products. A typical densitogram of ZMT is represented in Fig 25. Different mobile phases compositions were attempted to detect and quantify ZMT and its degradation product. The optimised mobile phase system consisted of Chloroform: Methanol: Water in the ratio of 5:4:1 (v/v/v). The observed Rf value was found to be 0.25. The complete separation of their degradation products and impurities was done at ambient temperature. Regression analysis of the calibration data for ZMT showed that the dependent variable (peak area) and the independent variable (concentration) were represented by the equation Y=7.503 X + 104.0. The correlation of coefficient (R^2) obtained for ZMT was 0.9990. Thus a good linear relationship was observed in the concentration range of 200-1000 ng/spot for ZMT. The assay of ZMT was found to be 99.24, which indicates high accuracy of the method. The absence of additional peaks in chromatogram indicates non-interference of impurities.

METHOD VALIDATION

Linearity and range

The calibration curves were linear in the range of 200-1000 ng/spot for each analyte, with a mean correlation coefficient (r^2) of more than 0.999 and the mean of regression equations of Y=7.503 X + 104.0, which shows a perfect correlation. The mean values (± SD *n* = 6) of correlation coefficient, slope, and intercept were calculated and given in Table 11 and Fig 19.

Precision

The intraday and interday variation of the method was evaluated by analyzing 5 replicate samples of each at 3 different concentration levels (600, 800, and 1000 ng/spot). The %RSD values of within-day and day-to-day study were <2% for the ZMT revealed that the proposed method is sufficiently precise and is reported in Table 12 and 13.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD was determined based on signal-to-noise ratio and was determined using an analytical response of three times the background noise. The LOD of ZMT was found to be 40.8 ng/spot. The LOQ was determined as the lowest amount of analyte that was reproducibly quantified above the baseline noise following triplicate injections. The resultant %RSD for these studies was ≤ 0.65%. The LOQ that produced the requisite precision and accuracy was found to be 138.2 ng/spot for ZMT and is reported in Table 14.

Solution Stability

Stability of the solution was determined from the % assay of the solutions at different intervals. The study shows that the %RSD at different time was less than 2. Hence the sample solutions was found to be stable over the time (Table 15).

Ruggedness and robustness of the method

According to ICH Guidelines, ruggedness and robustness assessment was performed during the development of an analytical procedure. By comparing interday and intraday precision results done by two analyst, the ruggedness of the method can be assessed. The %RSD value less than 2 for intraday and interday assay of ZMT, performed in the same laboratory by two different analysts, indicated the ruggedness of the developed method as

shown in Table 16 and 17. As shown in Table 18, robustness data of the mean obtained (n=6) for each factor studied, including that the selected factors remained unaltered by small variations of these parameters such as mobile phase ratio, slit dimension, pH and detection wavelength.

FORCED DEGRADATION STUDIES

A stock solution containing 2 mg ZMT in 10 ml methanol was prepared. This solution was diluted with methanol to get the Final concentration was 100 µg/µl. ZMT is used for forced degradation to provide an indication of specificity of the proposed method. In all degradation studies (acid- and base-hydrolytic degradation, hydrogen peroxide, dry heat and photolytic degradation) the average peak areas of ZMT after injection of six replicates were obtained. ZMT subjected to different ICH prescribed stress conditions. HPTLC analyses to identify the ZMT drug degradation products formed under stress conditions of hydrolysis, oxidation and photolysis. Hence a more complete degradation pathway of the drug was established than what known at present, by using a stress degradation technique and employing a HPTLC method shown in Table 19.

Acid Hydrolysis

ZMT undergoes severe hydrolytic degradation in acidic (0.1 M HCl and 1M HCl) condition at 80°C for 8 h. Complete degradation of ZMT was found on hydrolysis with 1M HCl. On degradation with 0.1M HCl, ZMT undergone degradation to a greater extend, which shows one additional peak with an Rf value of 0.21. The hydrolysis ZMT in acid was more intense as compared to that of alkali (Fig 26 and 27).

Base hydrolysis

On with 0.1M NaOH for 8 h at 80˚C which resulted in partial degradation of ZMT and two peaks were observed (Fig 28) at an Rf value of 0.25 and 0.21. The degradation peak (Fig 11) was well resolved from the main ZMT peak.

Oxidation

Oxidative degradation was performed for ZMT with 3% hydrogen peroxide at 80ºC for 8 h. The degradation was happened to a greater extend under oxidative condition. One degraded peak was observed at Rf value 0.21 (Fig 29).

Dry heat- degradation

The solid state studies revealed that the ZMT is not stable after the exposure to elevated temperature of 100ºC for 8 h in oven. Degradation was happened to a lesser extend at this temperature and ZMT was found to be not stable. No co-eluting peaks found along with the main peak (Fig 31).

Photolysis

No other co eluting peak was found with the main peaks in photolytic degradation which was kept in direct sunlight for 24 h, suggesting the specificity of the method for the estimation of ZMT in the presence of degradation products (Fig 30). The degradation of ZMT was happened to a greater extend in photolytic degradation.

CHAPTER IX

CONCLUSION

- The method was designed to validate the stability-indicating RP- HPLC and HPTLC method for the determination of ZMT in bulk drug.

- The methods were validated by determining system suitability, specificity, precision, linearity, accuracy, stability, LOD, LOQ ruggedness and robustness parameters and found to be satisfactory.

- The stability indicating features of the methods were demonstrated by the forced degradation of the active ingredient by acid hydrolysis, base hydrolysis, oxidative degradation, dry heat degradation and photolysis.

- Forced degradants were shown to be non-interfering with the R_f value and Retention time of active ZMT. This reveals that the method is specific and selective.

- The standard preparations in the range of 2 µg/ml to 10 µg/ml of the assay concentration were linear (Correlation coefficient, R^2 =0.999, n = 6) in the developed RP-HPLC method.

- The standard preparations in the range of 200 ng/spot to 1000 ng/spot of the assay standard concentration were linear (Correlation coefficient, R^2 = 0.9989, n=6) in the developed HPTLC method.

- The ruggedness of both the RP-HPLC and HPTLC method demonstrated that different operational and environmental variables had only a minimal influence on the test results.

- It can be concluded that there is no other co eluting peak with the main peaks and hence both the RP-HPLC and HPTLC methods are specific for the estimation of ZMT in the presence of degradation products. Although no attempt was made to

identify the degradation products, the described method can be used as stability indicating method for the assay of ZMT in bulk drug form.

- The method was completely validated showing satisfactory data for all the method validation parameters that were tested. The method can be employed as a stability indicating one, as the described method is capable of separating the drug from its degradation products.

- It was concluded that, the developed RP-HPLC and HPTLC method is specific, accurate, precise, linear, and it may be used for the routine application for the determination of ZMT in the bulk drug forms in the presence of their degradation products.

CHAPTER X

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