WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 8.084
Volume 10, Issue 6, 1199-1251. Research Article ISSN 2277– 7105

STABILITY INDICATING RP-HPLC METHOD FOR ESTIMATION OF ITRACONAZOLE AND TERBINAFINE IN BULK AND TABLET DOSAGE FORMS

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Article Received on
09 April 2021,

Revised on 29 April 2021,
Accepted on 20 May 2021

DOI: 10.20959/wjpr20216-20571

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ABSTRACT

Background: Itraconazole is an Antifungal medication used to treat number of fungal infections. On set of action within an hour and Last upto twenty one hours. Terbinafine is an Antifungal medication used to treat pityriasis versicolor, fungal nail infections and ringworm. On Set of action within an hour and Last up to 36 hours. **Objective:** To develop and validate simple, fast, economical and eco-friendly RP-HPLC method for the estimation of Itraconazole and Terbinafine in bulk and tablet dosage form according to ICH guidelines. **Method:** This method achieved by Shimadzu LC-20A instrument with isocratic elution with the mobile phase of methanol and water in the ratio of (9.5:0.5v/v) on Zodiac C18 (250mm x 4.6mm, 5µm) with a flow rate of 1mL/min. at a wave length of 257nm with UV detector. Tablets were allowed to undergo different stress conditions like acid, base, oxidation, thermal degradation studies. **Results:** Retention time of Itraconazole and Terbinafine was found to be 4.288 and 2.551 respectively. The linearity of proposed method investigated in the range of 10-50µg/mL for both Itraconazole and Terbinafine. The Limit of Detection of Itraconzole and Terbinafine 1.25µg/mL and 8.00µg/mL respectively. The Limit of Quantification of Itraconazole and Terbinafine are 3.79µg/mL and 24.00µg/mL respectively. **Conclusion:** From the above results, it can be concluded that the developed RP-HPLC method represents a good technique for determination of Itraconazole and Terbinafine contents in bulk and tablet formulation with good sensitivity, precision, and reproducibility.

KEYWORDS: Itraconazole, Terbinafine, RP-HPLC, Forced degradation studies.

INTRODUCTION

1. Pharmaceutical Analysis

Pharmaceutical analysis is a method for determining the substance, its amount or impurity and plays a vital role in the pharmacy. Pharmaceutical analysis provides information on the identity, purity, content and stability of starting materials, excipients and Active Pharmaceutical Ingredients (APIs). A distinction is made between analysis of the pure active ingredients used to cure, alleviate, prevent or identify illness and disease (active ingredient analysis) and analysis of medicinal preparations.[1]

1.1 Methods of pharmaceutical analysis

A. Qualitative analysis

B. Quantitative analysis

A. Qualitative analysis

The analytical way deals with standards which are designed for products to have desirable efficacy of the medicines. Sample representing any batch are analysed for these standard and it is assumed that the drug which is having such standard having desired effect on use.

Recognition of chemical species by means of

- Colour, taste, viscosity and solubility
- Reaction producing a colour
- Reaction producing a precipitate
- Reaction involving a change of a physical parameter.

B. Quantitative analysis

Quantitative chemical analysis carried out by determining the volume of a solution of accurately known concentration which is required to react quantitatively with a measured volume of the substance to be determined.

C. Semi-quantitave analysis

It is the type of the analysis which only describes whether the quantity of impurity present in given sample is below or above the specified limits such as limit tests.

INSTRUMENTAL ANALYSIS

Instrumental methods are part of chemical analysis that interacts with all areas of chemistry

and with many other areas of pure and applied sciences. Analytical instrumentation plays an important role in the production and evaluation of new products and in the protection of consumers and environment. Instrumentation provides lower detection limits requires assuring safe foods, drugs, air and water.[2]

Most instrumental techniques fit into one of the four principle areas mentioned below.

i) Spectrophotometic techniques

UV-Visible Spectrophotometry

Fluorescence and Phosphorescence Spectrophotometry Atomic absorption & emission Spectrophotometry Infrared Spectrophotometry

Raman Spectrophotometry X-Ray Spectrophotometry

Nuclear Magnetic Resonance Spectroscopy Electron Spin Resonance Spectroscopy Mass Spectroscopy

ii) Electrochemical Techniques

Potentiomery Voltametry

Electrogravimetry Conductometry Amperomerty

iii) Chromatographic techniques Thin Layer Chromatography Gas Chromatography

High Performance Liquid Chromatography High Perforamance Thin Layer Chromatography

GC-MS (Gas Chromatography-Mass Spectroscopy) LC-MS (Liquid Chromatography-Mass Spectroscopy)

iv) Miscellaneous techniques

Thermal Analysis Kinetic Techniques

Chromatographic techniques are predominantly used in the pharmaceutical industry for a large variety of samples.

CHROMATOGRAPHY

The term chromatography (Greek kromatos –colour and graphos-written means colour writing) Twestt (1906) - invented the chromatography.

The IUPAC has defined chromatography as “a method used primarily for the separation of component of a sample, in which the component are distributed between two phases, one of which is stationary while the other moves .the stationary may be a solid or liquid supported

on a solid or a gel and may be packed in a column, spread as a layer or distributed as a film. The mobile phase may be gaseous or liquid".[3]

General classification of chromatographic methods are as below

1) Column chromatography
2) Paper chromatography
3) Thin-layer chromatography
4) Gas chromatography
5) High performance liquid chromatography
6) Ion-exchange chromatography
7) Gel filtration chromatography
8) Supercritical fluid chromatography

In the present investigation High Performance Liquid Chromatography was used as a tool for the determination of selected drugs.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

HPLC is the fastest growing analytical technique for the analysis of the drugs. Its simplicity, high specificity and wide range of sensitivity make it ideal for an analysis of many drugs in both dosage forms and biological fluids. HPLC is one of the chromatographic techniques is widely used for checking the purity of new drugs, monitoring changes or scale ups of synthetic procedures, evaluvating new formulations and scrutinizing quality control/assurance of final drug products.[4]

Types of HPLC Methods

Based on modes of chromatography: Normal phase chromatography Reverse phase chromatography.

Based on principle of separation: Partition chromatography Adsorption chromatography Ion exchange chromatrography Size exclusion chromatography Affinity chromatography Chiral phase chromatography.

Based on elution technique: Isocratic separation

Gradient separation

Based on the scale of operation

Analytical HPLC Preparative HPLC.

INSTRUMENTATION OF HPLC

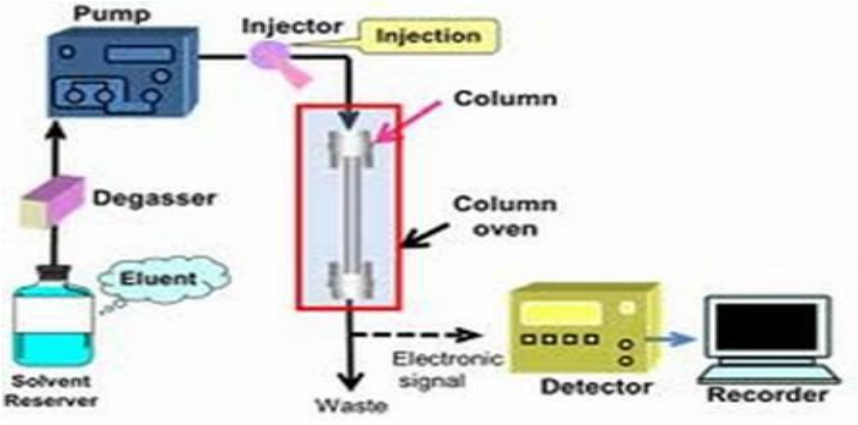

Fig 1: Instrumentation of HPLC.

A liquid chromatography consists of a reservoir containing the mobile phase, a pump to force the mobile phase through the system at high pressure, an injector to introduce the sample into the mobile phase, a chromatographic column (a column temperature controller may be also used) to attain retention, a detector to detect analyte response, and a data collection device such as a computer, integrator or recorder. Further, in some cases, degasser with vacuum pump and pre- column facility can implement in the modern HPLC.

Components of HPLC are as follows

1. Solvent reservoir
2. Pumps
3. Injectors
4. Columns
5. Detectors
6. Data Acquisition

A brief introduction of HPLC components

1. SOLVENT RESERVOIR

Mobile phase contents are contained in a glass reservoir. Different solvents are used for different types of HPLC. For normal-phase HPLC, the solvent is usually nonpolar, and, in reverse-phase HPLC, the solvent is normally a mixture of water and a polar organic solvent. The most common solvent reservoirs are as simple as glass bottles with tubing connecting them to the pump inlet.

Solvent degassing

This can be done by using following techniques:

a) Vacuum filtration
b) Helium purging
c) Ultrasonification

a) Vacuum filtration:- which can remove air bubbles, but it is not always reliable and complete.

b) Helium purging:- By passing helium through the solvent. This is very effective but expensive.

c) Ultrasonification:- By using Ultrasonicator, which converts ultra high frequency to mechanical vibrations and causes the removal of air bubbles.

2. PUMPS

The role of the pump is to force a liquid (called the mobile phase) through the liquid chromatography at a specific flow rate, expressed in milliliters per min (mL/min). Normal flow rates in HPLC are in the 1-2mL/min range. In general, the operable pressures in HPLC are 500 to 5000psi. During the chromatographic experiment, a pump can deliver a constant mobile phase composition (isocratic) or an increasing mobile phase composition (gradient). HPLC pumps generate pressure on the solvent so as to pass through the dense column. Since the column particles are very small, the surface area is high and even separation is efficient. But the pressure required for the mobile phase to flow is very high. So pumps able to generate such high pressure are needed in HPLC.

Types of pumps: Mainly three types of pumps are used

1. Constant flow reciprocating pump
2. Syringe type pump
3. Pneumatic pump

3. INJECTORS

After dissolution in mobile phase or suitable diluent, compounds to be chromatographed are injected into the mobile phase, either manually by syringe/loop injectors or automatically by autosampler.

Fig2: Rheodyne injector.

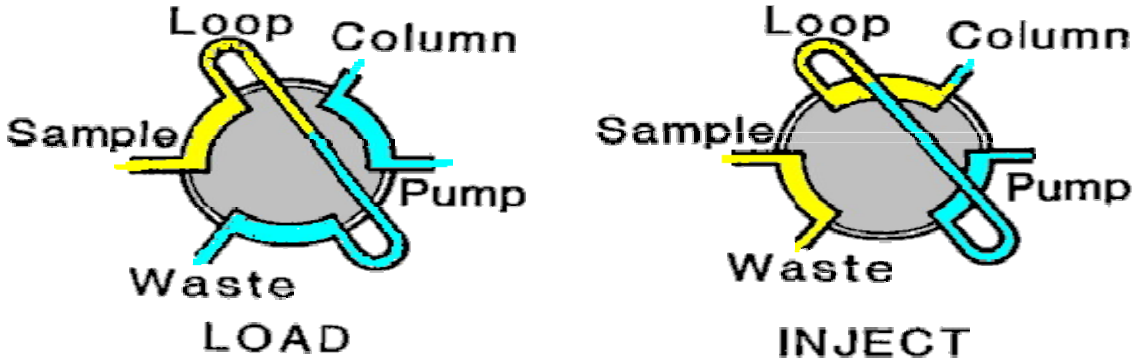

Fig 3&4: Rheodyne injector load and inject positions.

Figures above shows schematic drawings of a six-port Rheodyne valve in which the sample fills an external loop. Compared to shorter, wider i.d. sample loops, long, narrow loops are preferred when large sample volumes are required, because of lesser band-broadening effects. Alternatively, a specially designed syringe may be used to inject a small volume (e.g., <10 µl) into the loop when required, although in this case the precision in the sample introduction is dependent on the precision of syringe delivery. A clockwise rotation of the valve rotor places the sample-filled loop into the mobile-phase stream, with subsequent injection of the sample onto the top of the column through a low-volume, cleanly swept channel. Other valve types (e.g., Siemans and Valco) use an internal sample cavity consisting of an annular groove on a sliding rod that is thrust into the flowing stream. The minimum injection volume which can be made with the valve-type injectors is 60nl.

Auto samplers

Most of the autosamplers are microprocessor controlled and can serve as a master controller for the whole instrument.

Autosampler consist of a carousel or rack to hold sample vials with tops that have a pierceable septum or stopper and an injection device to transfer sample from the vials to a loop from which it is loaded into the chromatograph. Autosampler can be programmed to control sample volume, the number of injections, the interval between injections and rinse cycles.

COLUMNS

The HPLC column is the heart of HPLC system. It is usually made up of stainless steel to withstand high pressure. Columns used for analytical separations usually have 10-30cm length and 4-10mm inside diameter containing stationary phase having particle diameter of 3-10µm. Particles may range up to 50µm or more for preparative columns. Stationary phases for modern, reverse-phase liquid chromatography typically consist of an organic phase chemically bound to silica or other materials. The nature of the bonded phase is an important parameter for determining the separation properties of the chromatographic system.[5,6]

Commonly used bonded phases are shown below: Octadecyl (C_{18})=Si-$[CH_2]_{17}$-CH_3

Phenyl (C_6H_5)=Si-$[CH_2]_n$-C_6H_5

Cyanopropyl (CN)=Si-$[CH_2]_3$-CN Aminopropyl (NH_2)=Si-$[CH_2]_3$-NH_2

Diol (OH)=Si-$[CH_2]_3$-O-CH(OH)-CH_2-OH

Fig 5: columns.

HPLC Column Packings

Many of the types of packing used for gravity or low-pressure chromatography are not able to withstand the high pressures used in an HPLC system. Common packing materials in HPLC columns include silica or hydroxyapatite media and polymeric resins such as polystyrene divinylbenzene.

HPLC Column Types

A wide variety of HPLC column types are now available for various analytical applications. A few of the most commonly used types are described below, classified by separation mechanism.

Ion exchange HPLC columns have charged packing. An ion exchange column can be either cationic or anionic. This type of HPLC column separates polar molecules based on their

charge. The mobile phase is an aqueous buffer. Ion exchange HPLC columns can be used to separate many types of analytes and are commonly used for separations of carbohydrates, amino acids, and proteins.

Ion exchange and ligand exchange chromatography may be combined in a column. In these combined-mode columns, ion exchange is usually via metal ions, and the ligands are electron- donor molecules such as hydroxyl groups or amines. This type of HPLC column is frequently used for the separation of monosaccharides.

Reversed-phase HPLC columns have nonpolar packing. They are used with aqueous and water-miscible organic solvent mobile phases. The most common solvents are acetonitrile, methanol, and tetrahydrofuran (THF). Both isocratic (constant concentration) and gradient (increasing organic solvent concentration)

Elution are used with reversed-phase columns. Selectivity and retention times are dependent on a number of parameters including the pH of the mobile phase. The reversed-phase HPLC column is the most versatile and commonly used column type and can be used for a wide range of different types of analytes.

Normal-phase HPLC columns have polar packing. The mobile phase is nonpolar and therefore usually an organic solvent such as hexane or methylene chloride. This type of HPLC column includes a type of partition chromatography using hydrophilic interaction liquid chromatography (HILC), in which the mobile phase contains a low concentration of water. In an ion-moderated partition HPLC column, the addition of ionic compounds such as ammonium acetate to the mobile phase can both change the retention times of analytes and increase their polarity. This class of HPLC column is used for small molecules such as organic acids, some drugs, and a range of biomolecules including glycosylated proteins.

Size exclusion HPLC columns do not rely on the interaction of the analytes with the column packing but rather utilize a sieving effect based on molecular weight. The packing contains both mesopores and micropores. The size distribution of the pores determines the size of molecules in the sample that can diffuse into the pores. The extent to which molecules can diffuse into the pores determines the retention time and elution profile. Molecules that are too large to enter the pores pass through the column rapidly, eluting as a single peak after the void volumeSize exclusion HPLC columns are used primarily for the separation of proteins

and carbohydrates.

Other types of HPLC columns include affinity, ion exclusion, and displacement chromatography columns; a chiral HPLC column can be used to resolve racemic mixtures.

Guard columns

Guard columns, set between the injector and an analytical column, are used to protect analytical columns from chemical impurities in samples.

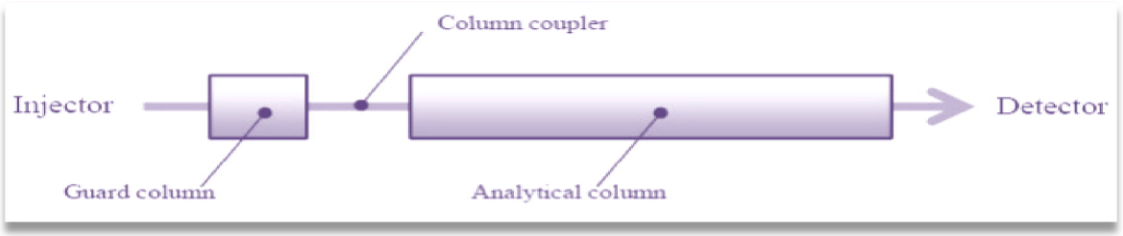

Fig 6: connection Guard column.

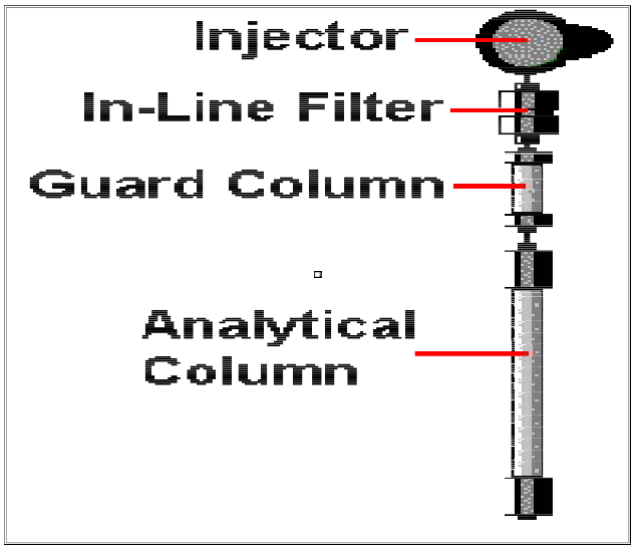

Fig 7: Guard column.

There are two types of guard columns.

Cartridge type - which can be changed easily by hand.

Packed type - which are packed in stainless cartridge like analytical columns

In order to obtain maximum protection, it is recommended to use a guard column that contains the same packing material as the analytical column.

4. DETECTORS

Detectors for HPLC are designed to take advantage of some physical or chemical attribute of either the solute or mobile phase in the chromatographic process in one of four ways:

1. A bulk property or differential measurement.
2. Analyte specific properties.
3. Mobile phase modification.
4. Hyphenated techniques.

Bulk property detectors are the most universal detectors for HPLC as they measure properties common to all analytes by measuring differences in the mobile phase with and without the sample. One of the most common bulk property detectors is the refractive index detector. Given the universal nature of bulk property detectors, they respond to all analytes, placing more emphasis on the selectivity of the chromatographic column.

Analyte specific property detectors respond to a characteristic that is unique to an analyte. The UV detector is the most common example of an analyte specific property detector, responding to analytes that absorb UV light at a particular wavelength. UV detectors are usually thought of as somewhat specific, responding only to compounds with chromophores, but at low UV wavelengths (< 210 nm), where just about every organic compounds absorb, UV detectors are actually somewhat universal. Other analyte specific detectors include fluorescence, conductivity, and electrochemical.

Mobile phase modification detectors change the mobile phase post-column to induce a change in the properties of the analyte, for example, by creating particles suspended in a gas phase. Evaporative light scattering and corona discharge detectors fit into this category. Pre- or postcolumnderivatization of the analyte is also sometimes considered to fit into this category, but is outside the scope of this review and will not be discussed here.

Hyphenated techniques refer to the coupling of a separate independent analytical technology to an HPLC system. The most common is mass spectrometry (LC-MS), and technologies such as infrared spectrometry (LC-IR) and nuclear magnetic resonance (LC-NMR) have also been used.

UV Absorbance Detectors

Over 75% of all of the HPLC detectors are UV absorbance detectors. The mobile phase is

passed through a small flow cell, where the radiation beam of a UV/visible photometer or spectrophotometer is located. Only UV-absorbing compounds, such as alkenes, aromatics, and compounds that have multiple bonds between C and O, N, or S are detected. The mobile phase components should be selected carefully so that they absorb little or no radiation.

5. Data Acquisition/Display Systems

Since the detector signal is electronic, use of modern data acquisition techniques can aid in the signal analysis. The data acquisition system of most HPLC systems is a computer. The computer integrates the response of the detector to each component and places it into a chromatograph that is easy to read and interpret. Other more advanced features can also be applied to a chromatographic system. These features include computer-controlled automatic injectors, multi- pump gradient controllers and sample fraction collectors.

SEPARATION TECHNIQUES

1. **Isocratic elution:** A separation that employs a single solvent or solvent mixture of constant composition.
2. **Gradient elution:** Here two or more solvent systems that differ significantly in polarity are employed. After elution is begun, the ratio of the solvents is varied in a programmed way, sometimes continuously and sometimes in a series of steps. Separation efficiency is greatly enhanced by gradient elution.

Derivatization

Derivatization of samples involves a chemical reaction that alters the molecular structure of the analyte of interest to improve detection. In HPLC, derivatization of a drug is usually unnecessary to achieve satisfactory chromatography. Derivatization is used to enhance the sensitivity and selectivity of detection when available detectors are not satisfactory for the underivatized compounds.

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SOME ADVANCED HPLC INSTRUMENTS USED IN INDUSTRY

Fig: 8 Shimadzu LCMS-8050. *Triple Quadrupole Liquid Chromatograph Mass Spectrometer (LC-MS/MS) (p/n 225-19610-44) complete with Edwards XDS46i vacuum pump (p/n A731-01-983), and Lab Solutions LCMS PC workstation with license.*

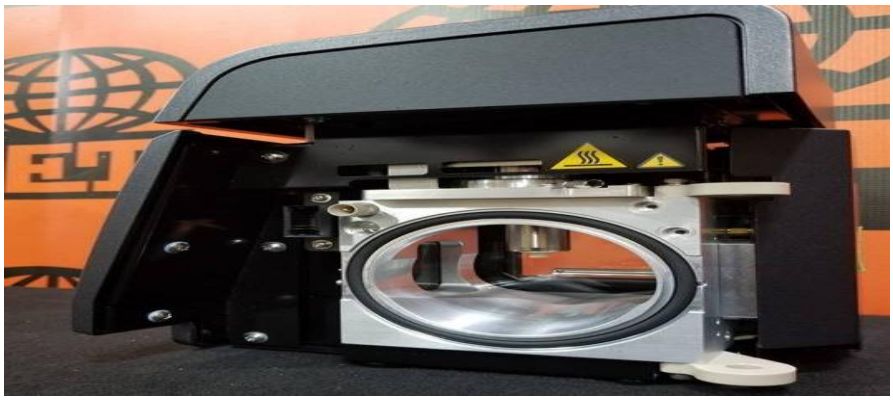

Fig:9 Shimadzu APCI probe *(p/n 225-19601-58) for the LCMS-8050 Triple Quadrupole Liquid Chromatograph Mass Spectrometer (LC-MS/MS).*

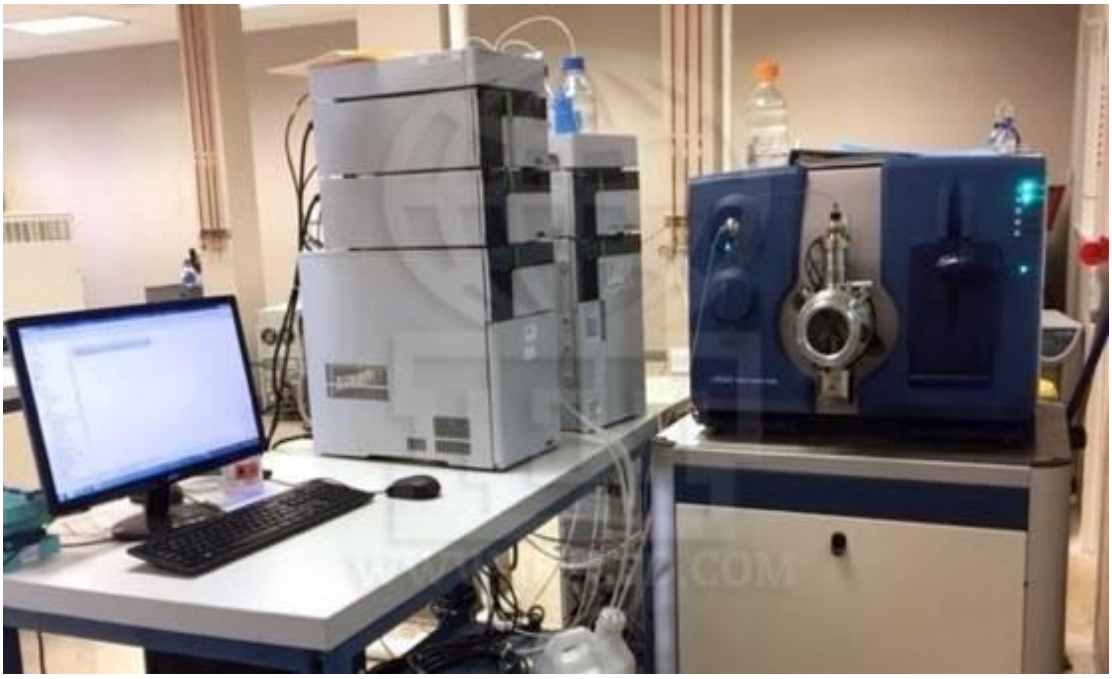

Fig:10 *Sciex Triple Quad 4500 LC/MS/MS mass spectrometer system (p/n 5020102) complete with turbo V source, turboionspray probe, vacuum pump, custom table, Windows 7 Analyst 1.6.2 PC workstation.*

ANALYTICAL METHOD DEVELOPMENT Analytic method development andvalidation are key elements of any pharmaceutical of pharmaceuticals. Method is developed to identified, quantify or purifying compounds of interest.

ANALYTICAL METHOD VALIDATION[7]

According to ICH guideliness, validation can be defined as Establishment of documented evidence which provided high degree of assurance that specific process will consistently produce a product with predetermined specification and quality attributes. The validity of a specific method should be demonstrated in laboratory experiments using samples or standards that are similar to the unknown that can be samples that can be considered such as sampling, sample preparation, chromatographic separation, detection, and data evaluation, using the same matrix as that of the same the intended sample. The proposed procedures must go through a rigorous process of validation. All validation experiments should be documented in a formal report

- ✓ Specificity
- ✓ Linearity and range
- ✓ Accuracy
- ✓ Precision

- Repeatability
- Intermediate precision
- Reproducibility

- ✓ Limit of detection
- ✓ Limit of Quantification
- ✓ Robustness
- ✓ System suitability

SPECIFICITY/SELECTIVITY

Specificity is the ability to measure accurately the concentration of the an analyte in the presence of all other sample materials. Assuring specificity is the step in developing and validating a good method. The most selective analytical method involve a chromatographic separation. Detection methods can be ranked according to their selectivity.

The analyte should have no interference from other extraneous components and well resolved from them. A representative HPLC chromatography should be generated and submitted to that the extraneous peaks either by addition of known compounds or samples from stress testing are baseline resolved from the parent analyte.

Recommendations: Representative HPLCchromatograms should be submitted for stress and nonstress samples that include impurities test method, preservatives, etc. Representative HPLC chromatogram to show selectivity by the addition of known extraneous compounds should be submitted.

LINEARITY AND RANGE

The linearity of thean analytical procedure is the ability to obtain test results which are directly proportional to the concentrationofanalyte in the sample.

Linearity may be evaluated by visual inspection of a plot of signals as a function of analyte concentration. If there is linear relationship, test result should be evaluated by appropriate statistical methods. For the establishment of linearity, a minimum 5 concentrations are required as the lower and upper concentrations for which the analytical methods has adequate accuracy, precision and linearity.

The range of a method can be defined as the lower and the upper concentrations for which the analytical method has adequate accuracy, precision and linearity. The range is normally expressed in the same units as the test results obtained by the method.

The ICH guidelines specify a minimum of 5 concentration levels, along with certain minimum specified ranges. For assay, the minimum specified range is from 80-120% of the target concentration. For an impurity test, the minimum range is from the reporting level of each impurity to 120% of the specification (for toxic or more potent impurities, the range should be commensure with controlled level). For content in uniformity testing the minimum range is from 70-130% of the test or target concentrations and for dilution testing ±20% over the specified range of the test.

Recommendation: The linearity range for examination depends on the purpose of the test method. The minimum specified range is from 80-120% of the target concentration. The correlation coefficient should not be less than 0.999.

ACCURACY

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

Accuracy can be assessed by applying the analytical method to samples or mixtures of sample matrix compound to which known amount of the analyte has been added, above and below the normal levels expected in the sample. Method accuracy is the agreement between the difference in the measured analyte concentrations of the spiked and unspiked samples and the amount analyte to the spiked sample.

Recommendation: An acceptancecriteria for accuracy is that the percentage recovery should be within 98-102%.

Accuracy is calculated in terms % recovery which is given as,

%recovery=(b-a/c) x100 Where,

a=response of test solution b=response of spiked solution c=response of standard solution

PRECISION

The precision of an analytical procedure expresses the closeness of agreement between a series of measurement obtained from multiple sampling of the homogenous sample under the

prescribed condition. Precision may be considered at 3 levels:

Repeatability Intermediate precision Reproducibility

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

- **Repeatability**

Repeatability is obtained when the analysis is carried under the some operating conditions over a short time interval. It is also termed as intra assay precision. It is determined from at least six replications measured at 100% of the test target concentration or from at least three replications covering the complete specified range.

- **Intermediate precision**

Intermediate precision is a term that has been defined by ICH as the long term variability of the measurements process. It is determined by comparing the results of method run within a single laboratory over a number of weeks. A method's run within a single laboratory over a number of weeks. A methods intermediate precision may reflect discrepancies in results obtained.

- From different operators
- From inconsistent working practice of the operator
- From different instruments
- With standards and reagents from different suppliers
- With columns from different batches or a combination of these

Reproducibility

Reproducibility is defined as the precision results obtained between laboratories. The objective is to verify that the method will provide the same in different laboratories. The reproducibility of an analytical method is determined by analyzing aliquots from homogenous lots in different analysts and by using operational and environmental conditions that may differ from, but are still within, the specified parameters of the method is to be used in different laboratories.

Recommendations

%RSD of the peak area obtained from 6 replicates injections of the solution not be more than 2.0

LIMIT OF DETECTION

It is the smallest amount of an analyte which can be detected by a particular method. several approaches for determining the detection limit are possible, depending on whether the procedure is on a non instrumental or instrument.

- **Based on visual evaluation**

visual evaluation may be used for non instrumental methods but may also be used with instrumental methods.

- **based on signal to noise ratio**

This approach can only be applied to analytical procedures which exhibit baseline noise. Determination of the signal to noise ratio is performed by comparing measured signals from samples with known low concentration of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliable detected. A signal to noise ratio between 3 or 2:1 is generally considered acceptable for estimating the detection limit.

- Based on the standard deviation of the respone and the slope The limit of detection (LOD) may be expressed as

$LOD = 3.3 \times \sigma/s$

Where,

σ = the standard deviation of the respone

S = the slope the calibration curve

LIMIT OF QUANTIFICATION

The limit of quantification is defined as the smallest amount of analyte which can be quantified reliably.

- **Based on visual evaluation**

Visual evaluation may be used for non instrumental methods but may also be used with instrumental methods. The quantification limit is generally determined by the analysis of sample with known concentration of analyte and by establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision.

❖ **Based on signal to noise ratio**

This approach can only be applied to noise is performed by comparing measured siganals from samples with known low concentration of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliable quantified. A signal to noise ratio between 10:1.

❖ **Based on the standard deviation of the response and the slope**

The limit of detection (LOD) may be expressed as

LOD =10Xσ/s

Where

σ= the standard deviation of the respone

S= the slope of the calibration curve

ROBUSTNESS

ICH defines robustness as a measure of the capability specifications. If measurements are susceptible to variations in analytical condition, the analytical conditions should be suitably controlled or a precautionary statement should be included in the procedure. The typical variations are:

- Variations of PH of the mobile phase ± 0.2
- Influence of variations in mobile phase compositions ±2ml of organic and aqueous phases
- Different columns
- Temperature ±5 °C
- Flow rate ±0.1ml
- Wavelength ±3nm

Recommendations: Data obtained from studies of robustness, through not usually submitted, are recommended to be included as part of method validation.

SYSTEM SUTABILITY

Capacity factor (k')

It measures how many times the analyte is retained to an unretained components. K=(t2/ta)-1

A k' valve zero means that the compound is not retained and elutes with the solvent front. A k' valves of I mean that the component is slightly retained by the column while k' valve of 20

means that components is highly retained and spends much time in interacting with the stationary phase.

b. Retention Factor

Retention factor means of measuring the retention of analyte on the chromatographic column

$K=t_R-t_{0/}t_0$

A high k valve indicaing that the sample is highly retained and highly retained and has spent a signified amount of time interacting with the stationary phase. The retention factor is equal to the ratio of the retention time of the analyte on the column to the retention time of a non retained compound.

Height equivalent to theoretical plate (HETP)

A theoretical plate can be any height, which decided the efficiency of separation.

If HETP IS less it means column is more efficient, if HETP more it means column is less efficient.

HETP= Length of the column/ no. of theoretical plates

FORCED DEGRADATION STUDIES

Forced degradation is a degradation of new drug substance and drug product at conditions more severe than accelerated conditions. It is required to demonistrate specificity of stability indicating method and also provides an insight into degradation pathways and degradation pathways and degradation products of the drug substance and helps in elucidation of the structure of the degradation products. Forced degradation studies show the chemical behavior of the molecule which in turns helps in the development of formulation and package.

Strategy for selection of degradation conditions

Forced degradation is carried out to produce representative samples for developing stability-indicating methods for drug substances and drug products. The choice of stress conditions should be consistent with the product's decomposition under normal manufacturing storage, and use conditions which are specific in each case. A general protocol of degradation conditions used for drug substance and drug product is shown in figure.

Hydrolytic conditions

Hydrolysis is one of the most common degradation chemical reactions over a wide range of ph. Hydrolysis is a chemical process that includes decomposition of a chemical compound by

reaction with water. Hydrolytic study under acidic and basic conditions involves catalysis of ionizable fuctional group is present in the molecule. Acid or basic stress testing involves forced degradation studies of a drug substance by exposure to acidic or basic conditions which generates primary degradants in desirable range. The selection of the type and concentration of acid or sulfuric acid (0.1-1M) for acid hydrolysis and sodium hydroxide or potassium hydroxide (0.1-1M) for base hydrolysis are suggested as suitable reagents for hydrolysis. If the component for stress testing are poorly soluble in water, the solvents can be used to dissolve then in HCl or NaOH. The selection of co-solvent is based on the drug substance structure. Stress testing trial is normally started at room temperature and if there is no degradation, elevated temperature (50- 70°C) is applied. Stress testing should not exceed more than 7 days. The degraded sample is then neutralized using suitable acid. Base or buffer to avoid futher decomposition.

2. oxidation condition

Hydrogen peroxide is widely used for oxidation of drug substances in forced degradation studies but other oxidizing agents such as metal ions, oxygen, and radical initiators (e.g., azobisisobutyronitrile, AIBN) can also be used. Selection of an oxidizing agent, its concentration, and conditions depends on the drug substance. It is reported that subjecting the solutions to 0.1-3% hydrogen peroxide at neutral pH and room temperature for radaition seven days or up to a maximum 20% degradation could potentionally generate relevant degradation products. The oxidative degradation of drug substance involves an electron transfer mechanism to form reactive anions and cations. Amines, sulfides and phenols are susceptible to electron transfer oxidation to give N- oxides, hydroxylamine, sulfones and sulfoxide. The functional group with labile hydrogen like benzylic carbon, allylic carbon, and tertiary carbon or α- positions with respect to hero atom is susceptible, to form hydrogen peroxides, hydroxide or ketone.

3. Photolytic conditions

The photo stability testing of drug substances must be evalvated to demonstrate that a light exposure does not results in unacceptable change. Photo stability studies are performed to generate primary degradants of drug substance by exposure to UV or fluorescent conditions. Some recommended conditions for photo stability testing are described in ICH guidelines. Sample of drug substances by exposure to UV or fluorescent conditions. Some recommended conditions for stability testing are described in ICH guidelines. Samples of drug substances

and solid/liquid drug product should be exposed to a minimum of 1.2 millions 1x h and 200 W h/ m2 light. The most commonly accepted wavelength of light is in the range of 300-800nm to cause the photolytic degradation. The maximum illumination recommended is 6 millions 1x h. Light. stress conditions can induce photo oxidation by free radical mechanism.

4. Thermal conditions

Thermal degradation (e.g., dry heat and wet heat) should be carried out at more strenuous conditions than recommended ICH Q1 A accelerated testing conditions. Samples of solid-state drug substances and drug products should be exposed to dry and wet heat, while liquid drug products should be exposed to dry heat. Studies may be conducted at higher temperature for a shorter period. Effect OF temperature on thermal degradation of a substances is studied is through the Arehenius equation:

$K=Ae^{-(Ea/RT)}$

Where

K is specific reaction rate, A is frequency factor,

Ea is energy of activation

R is gas content (1.987 cal/ deg mole)

And T is absolute temperature, thermal degradation study is carried out at 40-80°C.

DRUG PROFILE ITRACONAZOLE DRUG[8]

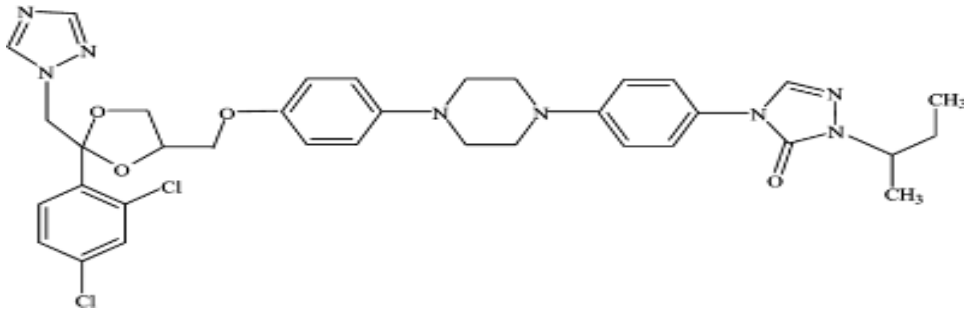

Fig 11: ITRACONAZOLE.

- **IUPAC NAME**

(±)-1-[(RS)-sec-butyl]-4-[p-[[(2R,4S)-2-(2,4dichlorophenyl)-2-2(1H-1,2,4-triazol-ylmethyl)-methoxy) phenyl]-1-piperazinyl]phenyl]-2-1,2,4-triazolin-5-one

- **Molecular formula :** $C_{35}H_{38}Cl_2N_8O_4$
- **Molecular wieght** : 705.64g/mol.
- **Category** : Antifungal

- **Description** : White
- **Solubility** : Methanol and Ethanol
- **Melting point** : Between 166° and 170°C
- **Half time** : 21hrs
- **Bioavailability** : 55%
- **Dose** : 200mg

Mechanism of action

It inhibits the fungal mediated synthesis of ergosterol via inhibition of lanosterol 14αdemethylase. This enzyme is involved in the conversion of lansoprozol to ergosterol.

DRUG PROFILE TERBINAFINE DRUG[8]

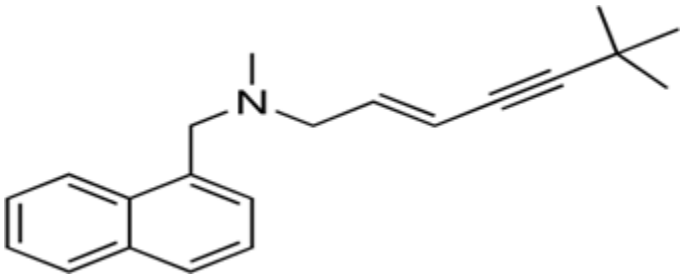

Fig 12: TERBINAFINE.

- **IUPAC NAME**

[(2E)-6,6-dimethylhept-2-en-4-ynyl)-N-methyl-1-napthalene methanamine

- **Molecular formula :** $C_{21}H_{25}N$
- **Molecular weight** : 291.438g/mol
- **Category** : Antifungal
- **Description** : White
- **Solubility** : Ethanol, DMSO, DMF
- **Melting point** : 202°C
- **Half time** : 36hrs
- **Bioavailability** : 70-90%
- **Dose** : 250mg

Mechanism of action

It is highly lipophilic in nature and tends to accumulate in skin, nails and fatty tissues like other allylamines, terbinafine inhibits ergosterol synthesis by inhibiting the fungal squalenemonooxygenase [squalene 2,3 - epoxidase] an enzyme that is part of the fungal cell

wall synthesis pathway.

LITERATURE REVIEW

1. **Devyani *et al.*, 2019**[9], The objective of the present work was to develop and validate the stability-indicating method for the simultaneous estimation of Itraconazole and Terbinafine HCl in bulk and pharmaceutical tablet dosage form by reversed-phase high-performance liquid chromatography (HPLC). This combination of drugs is not reported for simultaneous HPLC analysis as of now. Methods: The analysis of the developed method was carried on Shimadzu LC Prominence-i 2030 model with Lab Solution software and the separation was done on Shim-pack C18 GIST (250 mm×50 mm, 5 µm) column with a flow rate of 1.2 mL/min and run time of 12 min. The injection volume was 10 µl and mobile phase consisted of acetonitrile and 0.1% triethylamine in the ratio of 90:10 and 225 nm was used as a detection wavelength. Results: The retention time was found to be 3.464 min and 8.705 min for Itraconazole and Terbinafine HCl, respectively. The calibration curve was found to be linear and r2 values were 0.9989 and 0.9995 for Itraconazole and Terbinafine HCL, respectively.

2. **Prachi *et al.*, 2019**[10]**,** The proposed method is simple, rapid, precise and accurate and can be used for the reliable quantitation of Itraconazole and Terbinafine hydrochloride in pharmaceutical formulation. An UV Spectrophotometric method for the quantitative determination of Itraconazole and Terbinafine hydrochloride a highly potent antimycotic in tablet was developed in present work. The UV Spectroscopic determination was carried out at an absorption maximum of 247 nm using methanol as solvent. In the UV spectroscopic method linearity over the concentration range of Itraconazole was found to be 1-5µgm/ml with a correlation coefficient 0.9978. And for Terbinafine hydrochloride was found to 2.5-10µgm/ml with a correlation coefficient 0.9986. A RP-HPLC method has been developed and validated to determine Itraconazole and Terbinafine hydrochloride in tablet dosage form. The chromatography was performed on c18 column and a mobile phase consisting of methanol and 0.1% OPA in water(65:35) where monitored by UV photo diode array detector at wavelength of 230 nm. The flow rate was 1ml. The method was validated for linearity, precision, specificity, accuracy, Limit of detection, limit of quantification and robustness.

3. **Nirmal M kasekar*et al.*, 2017**[11]**,** Reported A simple, accurate, rapid, selective and robust high pressure liquid chromatography (HPLC) method was developed and validated

for estimation of Itraconazole in bulk and marketed formulation, Acetonitrile and double distilled water was used as mobile phase for chromatographic separation and estimation on HIQSIL C18- HS(250x4.6mn) in the ratio of 90:10v/v at flow rate of 1.0mL/min. The detection was carried out with UV detector set at 263nm. The retention time for Itraconazole was found to be 7.75minutes.The linearity range for Itraconazole was found to be 5-60µglml with coefficient of linear regression 0.991. The LOD for Itraconazole was found to be 0.3356µg/mL. The LOQ for Itraconazole was found to be 1.1657µg/mL respectively.

4. **Verma vikrant *et al.*, 2016**[12], Reported a simple, economic, selective, precise and accurate reverse phase high performance liquid chromatography method for analysis of Itraconazole and related substances and validated according to ICH guidelines. Itraconazole was well separated using Thermo Hypersil BDS C18, 150mm X4.6mm, 5µm column for assay qualification in isocratic mode with mobile phase comprisingof buffer. Acetonitrile (65:35) with a flow rate of 1.5ml/min and Thermo Hyper BDS C18, 100mm x 4.6mm, 3µm column for related substances quantification in gradient mode with mobile phase comprising of 0.08M tetra butyl ammonium hydrogenSulphate : acetonitrile with a flow rate of 1.5ml/mL. The retention time was found out to be 6.2min and percentage assay was found to be 99.9%. The percentage recovery was found to be 99.6 to 101.2%. The LOD of Itraconazole was found to be 0.85µg/mL. The LOD for Itraconazole was found to be 2.60µg/mL respectively.

5. **Troy Purvis *et al.*, 2015**[13], Reported a reverse phase high performance liquid chromatography which uses pH buffer phosphate solution and acetonitrile to create a gradient to separate the compounds contained in the formulation. The column used was phenomenex* Gemini 150x4.6mm C_{18} 5µg with a Flow rate of 1.0mL/min at a λmax 220nm. and the Retention time was found to be 25.4min and Linerity was in the range of 50-150% The percentage recovery was found to be 99-101%. The LOD for Itraconazole was foumd to be 0.00029mg/mL and the LOQ for Itraconazole was found to be 0.00030mg/mL respectively.

6. **Mahomed rizk *et al.*, 2014**[14], Reported a liquid chromatographic method was developed for determination of Itraconazole in bulk, dosage form and human plasma using µboundpack cyano column and a mobile phase consisting of 0.1M sodium dodecyl sulphate, 20% 1-propanol, 0.3% triethylamine in 0.02 M orthophosphoric acid (P^H = 3.5).

The UV detection was achieved at 258nm. Various chromatographic parameters were studied, e.g. types of columns, P^H of mobile phase, concentration of sodium dodecyl sulphate, 1-proponal, trietylamine, etc.

7. **Thangabalan *et al.*, 2014**[15], A simple fast and precise RP-HPLC method was developed for the quantification of Itraconazole in pure and pharmaceutical dosage forms the quantification was carried out using DIONEX C_{18} 4.6x250mm, 5µm enhanced polar selectivity column and mobile phase comprised of methanol and pH 7.5 potassium hydrogen phosphate in the ratio of 40:60 and degassed under the ultrasonication. The flow rate was found to be 1.5mL/min and the effluent was monitored at 306nm. The retension time of Itraconazole was found to be 5.2min. Linearity of Itraconazole was found to be 200-600µg/mL. The Relative Standard Deviation (%RSD) was found to be 0.097. LOD was found 6to be 1.8549µg/mL. LOQ was found to be 6.197µg/mL. Recovery was found to be 99.33% and 99.66% respectively.

9. **Sarvana paruchuri *et al.*, 2013**[16], The present investigation reveals about a simple, economic, selective, precise and accurate reserve phase high performance liquid chromatography method for analysis of Itraconazole and related substances of Itraconazole was developed and validated according to ICH guidliness. Itraconazole was well separated using thermohypersil BDS C18, 100 mm x 4.6 mm, 3µm column for related substances qualification in gradient mode with mobile phase comprising of 0.08M tetra butyl ammonium hydrogen sulphate: Acetonitrile with a flow rate of 1.5ml/min. the retention time was found to be 6.2min and the percentage recovery was found to be 99.9%. The percentage recovery was found to be 99.6 to 101.2%.

10. **Chinmoyroy *et al.*, 2012**[17], The present study describe a reverse phase ultra performance liquid chromatographic(RP-UPLC) method development and validation for determination Itraconazole in capsule formulation. Accquity BEH C18 Column was used as a stationary phase while mobile phase A was buffer tetrahydrofuran 95:05v/v (Buffer=0.1%v/v triethylamine in water pH 2.5 by orthophosphoric acid) and mobile phase B as acetonitrile. Method was developed in isocratic binary mode with 8minutes run time. Flow rate of 0.40mL/min. eluent was monitored at260mn. The developed methods separates Itraconazole from its seven related compounds by 8min run time. Recovery was found in the range of 98-101%.

11. **M.V Kundanavalli *et al.*, 2011[18],** Reported isocratic RP-HPLC, UV method development and validation of Itraconazole in capsule dosage form. An inertsil C_{18} (5µm, 250x 4.6mm) column with mobile phase containing tetra butyl ammonium hydrogen sulphate buffer solution and acetonitrile in the ratio of (40:60 v/v) was used. The flow rate was found to be 1.5mL/min and effluets were monitored at 225nm. The retention time for Itraconazole was 5.6min, LOD for Itraconazole were found to be 0.85µ/mL. LOQ was found to be 2.60µg/mL respectively. Recovery of Itraconazole from capsule formulation was found to be 98.3% to 100.3%.

12. **Zeynep *et al.*, 2010[19],** Reported a method far estimation of itraconazole and its metabolites from human plasma by high performance liquid chromatography-Tandem mass(LC-MS) spectrometry. The HPLC system of Agilent 1100 series with micro API detector, G1311A Pump, G1329A Autosampler mass Lynx V4, X-Terra RP18(3.5µm,50x4.6mm) at 20^{o}C. The range of calibration concentration 0.5-263ng/mL for ITRA and 0.49-256.

13. **Gholam Ali., 2005[20],** Has developed and reported stability indicating high performance thin layer chromatographic method for analysis of Itraconazole in bulk drug and in pharmaceutical dosage form. Separation was achieved on aluminium plate pre-coated with silica gel 60F254 using toluene: chloroform: methanol (5:5:1.5 v/v) as mobile phase. Densitometric analysis was performed at 260nm. Compact bands of ITZ were obtained at R_f0.52±0.02, linearity (R^2 = 0.997), LOD was found to be 180.29ng/band. LOQ were found to be 546.34ng/band respectively.

14. **Dipak *et al.*, 2019[21]**, The simple, sensitive, rapid, specific, precise, and accurate RP-HPLC method for simultaneous determination of Terbinafine hydrochloride and Mometasone furoate was developed and validated. Chromatographic separation was achieved on C18 column (250×4.6 mm-5µm p.s) using methanol and water in a ratio (85:15 v/v) as a mobile phase at flow rate of 0.8 ml/min. UV detection was done at 244 nm and injection volum was 20 µl. The peaks obtained were sharp with retention time for Terbinafine hydrochloride and Mometasone furoate as 6.20 min and 9.38 min respectively. The percentage recoveries obtained were 99.87 % and 99.71% respectively.

15. **Sireesha *et al.*, 2018[22]**, To develop a validated analytical RP-HPLC method for estimation of antifungal drug Terbinafine HCL in bulk and pharmaceutical dosage form.

INTERSILODS (50X4.6mm, 5µm). The sample was estimated using Postassium dihydrogenphospthate:acetonitrile (65:35v/v) as mobile phase at a Flow rate of 1.5mL/min and detection at 220nm. Linearity was in the range of 50-150g/mL and LOD for Terbinafine was found to be 0.0585µg/mL LOQ for Terbinafine was found to be was found 0.1950µg/mL and %RSD was found to be 0.001411% respectively.

16. **Murli manohar *et al.*, 2017[23],** To develop a validated analytical RP-HPLC method for simultaneous estimation of containing Terbinafine hydrochloride and Mometasone furoate for pharmaceutical formulation. The sample was estimated using acetonitrile and 0.1% ortho phosphoric acid PH 3 in the ratio (67:33)(v/v) as mobile phase at a flow rate of 1.0 mL/min and detection at 242 nm. The retention time for Terbinafine hydrochloride and Mometasone furoate was found to be 2.2 min and 3.5 min respectively. The linearity of the developed method was tested in the range of 0.5µg/mL for Terbinafine hydrochloride and 1-32µg/mL for Mometasone furoate. The limit of detection was 0.0189µg/mL for Terbinafine hydrochloride and 0.327µg/mL for Mometasone furoate, the % recoveries Was found in the range of 99.98- 100.07%.

17. **Mehul *et al.*, 2016[24],** A new simple, precise, accurate, specific and selective high performance thin layer chromatographic (HPTLC) method has been developed for the simultaneous estimation of Terbinafine hydrochloride (TH) and Mometasone furoate (MF) in cream dosage form. The chromatographic separation was achieved on Merck precoated silica gel aluminium plate 60 F254 using Toluene: Ethyl acetate: Glacial acetic acid (8: 4: 0.1 v/v) as mobile phase. The densitometric scanning was carried out at 248 nm. Response was found to be linear in the concentration range of 1000-3000 ng/band with correlation coefficient (r2 = 0.999) for Terbinafine hydrochloride and 100-300 ng/band with correlation coefficient (r2 = 0.998) for Mometasone furoate respectively.

18. **Priyanka *et al.*, 2014[25]**, To develop a Validated stability indicating RP-HPLC method for simultaneous determination of ofloxacin, ornidazole, clobetasol propionate, terbinafine HCL, Methyl paraben propyl paraben in bulk and pharmaceutical dosage forms. Zodiac C18(250mmx 4.6mm,5µm). Mobile phase Orthophosphoric acid buffer :acetonitrile (82:18) (v/v) at a Flow rate of 1min/mL and detection at 255nm. Retention time was found to be 7.032min. Linearity is in the range of 0.999 LOD 4.37g/mL and LOQ:13.26g/mL %RSD was found to be 0.316-1.08 and Recovery 99.8-101.3% respectively.

19. **Mehul patel *et al.*, 2013**[26], To develop a validated analytical RP-HPLC method for simultaneous estimation of containing Terbinafine hydrochloride and M ometasone furoate for pharmaceutical formulation. The sample was estimated using methanol and water in the ratio (95:5) (v/v) as mobile phase at a flow rate of 1.2 mL/min and detection at 248 nm. The retention time for Terbinafine hydrochloride and Mometasone furoate was found to be 6.9 min and 3.2 min respectively. The linearity of the developed method was tested in the range of 20- 200µg/mL for Terbinafine hydrochloride and 2-20µg/mL for Mometasone furoate. The limit of detection was 5.57µg/mL for Terbinafine hydrochloride and 0.07µg/mL for Mometasone furoate, the % recoveries obtained were 101.18% and 99.67% respectively.

20. **Puspa D *et al.*, 2013**[27], Terbinafine Hydrochloride (TH) is a new potent antifungal agent. Several HPTLC, non-aqueous voltametric, spectrometric methods, ion-pair RP chromatography and Stability-indicating HPTLC methods have been published till now. The aim of the present study is to develop and validate simple, precise, specific and sensitive stability indicating reversed-phase HPLC (RP-HPLC) method for analysis of Terbinafine Hydrochloride in bulk and in tablet dosage form. Terbinafine hydrochloride was analysed on a Neosphere C18 (250 x4.6 mm, 5□m) with a mobile phase comprising of methanol: 0.5% Triethanolamine. 0.5% Triethanolamine was added to pure methanol to reduce tailing problem. Wavelength of detection was 250 nm. Linear regression study revealed a good linear relationship (R2 = 0.997) between peak area and concentration in the range of 2-12 □g/mL. The Linearity was found to be2-12g/mL LOD of Terbinafine was found to be0.22g/mL and LOQ of Terbinafine was found to be 0.66g/mL and %RSD was less than 2 respectively.

21. **Ramesh *et al.*, 2011**[28]**,** A simple, sensitive and accurate reversed phase high performance liquid chromatographic method for Terbinafine and Bezafibrate drugs. This method is developed for Terbinafine and Bezafibrate drugs. Reversed phase chromatographic separation of the two drugs was performed a C18 column is used with different mobile phases of methanol, water, ammonium dihydrogen phosphate and methanol, acetonitrile, orthophosphoric acid respectively. The detection of wave length is 225 nm for Terbinafine and 232 nm for Bezafibrate. The percentage of recovery 99.51% for terbinafine and 99.94% for bezafibrate Retention time was found to be (T):5.1min and for Bezafibrate was found to be 6.0 min LOD for Terbinafine was found to be 0.5g/mL

and for Bezafibrate was found to be 0.01ppm LOQ for Terbinafine was found to be 0.15g/mL and for Bezafibrate 0.04ppm %RSD for Terbinafine was found to be 0.751 and for Bezafibrate was 0.410 respectively.

22. **Pasumarthy *et al.*, 2008[29],** a rapid and sensitive high performance liquid chromatographic method was developed for the estimation of Terbinafine in pharmaceutical dosage forms. Terbinafine was chromatographed on a reverse phaseC18 column in a mobile phase containing buffer:acetonitrile in the ratio 65:35 v/v. The mobile phase was pumped at a flow rate of 1.8 mL/min and the eluents were monitored at 220 nm. The calibration curve was linear in the range of 20-1000 ng/mL. The intra- and inter-day variation was found to be less than 2 % showing high precision of the assay method. The mean recovery of the drug from the solution containing20 ng/mL was 99.1 ± 0.73 % indicating high accuracy of the proposed HPLC method may be used for determining Terbinafine in bulk drug samples or in pharmaceutical dosage forms.

23. **Mehul *et al.*, 2008[30],** To develop a validated analytical RP-HPLC method for simultaneous estimation of containing Terbinafine hydrochloride and Mometasone furoate for pharmaceutical formulation. The sample was estimated using methanol and water in the ratio 95:5 as mobile phase at a flow rate of 1.2 mL/min and detection at 248 nm. The retention time for Terbinafine hydrochloride and Mometasone furoate was found to be 6.9 min and 3.2 min respectively. The linearity of the developed method was tested in the range of 20-200µg/mL for Terbinafine hydrochloride and 2-20µg/mL for Mometasone furoate. The limit of detection was 5.57µg/mL for Terbinafine hydrochloride and 0.07µg/mL for Mometasone furoate, the % recoveries obtained were 101.18% and 99.67% respectively.

NEED OF WORK

The literature review reveals that the:

- Various publications are available regarding determination method of Itraconazole and Terbinafine but most of the methods are applicable for the analysis of Itraconazole and Terbinafine alone or in combination with other drugs in pharmaceutical dosage forms.
- There is no open literature available for analysis of both the compounds in simple HPLC method.
- Itraconazole and Terbinafine being more polar and non polar with different functional groups and varying chemical properties hard to separate for different studies related to

them. To our present knowledge, no stability indicating analytical method for determination Itraconazole and Terbinafine in combine dosage form has been published consequently, the focus in the present study was to develop a validated stability indicating RP-HPLC method for the combination, by degrading the drugs under various stress conditions like acid hydrolysis, base hydrolysis, oxidation, Thermal stress which is recommended by ICH guidelines.

- The method is highly specific, these compounds can be used for routine analysis and R & D.

AIMAND OBJECTIVES

- Based on the literature survey, Stability indicating RP-HPLC method for estimation of Itraconazole and Terbinafine in bulk and pharmaceutical formulation has been designed.

Objectives

- To develop an analytical method of Itraconazole and Terbinafine tablets in combination
- To validate a simple, accurate and precise analytical method of Itraconazole and Terbinafine in bulk and in pharmaceutical dosage forms by stability Indicating RP- HPLC Method.
- The developed methods was optimized and validated as per the guidelines of ICH.
- To monitor degradation behaviour of the drugs under different ICH prescribed stress conditions.
- For the first time, the experimental values of ITRA and TERB was determined using RP-HPLC method.

MATERIALS AND METHODS

INSTRUMENTS

Various instruments used for the present work are given as

Table 1: List of Equipments.

S.NO	INSTRUMENT	MODEL	COMPANY
1.	Weighing / electronic balance	AUY220	Shimadzu
2.	Ultra sonicator water bath	1.5L50	Ultrasonics
3.	UV- VIS spectrophotometer	UV-1800	Shimadzu
4.	HPLC	SPD-20A	Shimadzu

CHEMICAL USED

Various chemicals and reagents used in the present work are given in table.

Table 2: List of chemicals.

S.NO	NAME OF THE CHEMICAL	GRADE	MANUFACTURED
1.	Methanol	HPLC	Research lab fine chem industries
2.	Water	HPLC	Avantor performance material india limited
3.	Itraconazole	AR	K.P. Labs
4.	Terbinafine	AR	K.P. Labs

CHROMATOGRAPHIC CONDITIONS

The mobile phase consists of methanol and water. The chromatograph was operated in isocratic mode starting at a mobile phase of methanol: water (95:5%v/v). Eluent wasdelieverd at a flow rate 0.5 mL/min. Absorbance was monitored at.

PREPARATION OF MOBILE PHASE

Mix the 95 mL of HPLC grade methanol with the 5mL of HPLC grade water and degas in ultrasonic water bath for 15 minutes.

VEHICLE

Methanol and water (95:5%v/v) used as solvent.

PREPARATION OF STANDARD SOLUTIONS

Accurately weighed 10mg Itraconazole and Terbinafine transferred into a 100mL of clean and dry volumetricflask, add about 30 mL of mobile phase and sonicate to dissolve and degas completely and make volume up to the mark with the mobile phase. Further dilutions like 3, 5, 10, 20,25, 30,40, 50, 70, 90, 100 µg/mL(10-100µg/mL) make up with mobile phase.

PREPARATION OF TABLET SAMPLE SOLUTION

Tweenty tablets of mytra-T (250mg of TERB, 100mg of ITRA) were taken and powdered. A quantity of powder equivalent to 10mg of Itraconazole (equivalent to 20mg of terbinafine)

taken in to 10mg volumetric flask and make the volume by using methanol. The solution was filtered through 0.45µm filter. From the filtrate 0.1mL was taken in 10mL volumetric flask and made up the mark with methanol to get a sample solution concentration of 10µg/mL itraconazole (20mg of terbinafie).

VALIDATION[7]

1. LINEARITY

Five different concentrations (10-50µg/mL) of Itraconazole and Terbinafine were prepared for linearity studies. The calibration curve obtained by plotting peak area against concentrations showed linearity in accordance to bear's law.

2. PRECISION

The precision of the method was determined by intraday studies. Prepare 20µg/mL solutions from a standard solution and inject five times in a day on to analytical column. The percentage relative standard deviation (%RSD) was calculated and lower % RSD indicates that there are less variation and there are high precision in the valves.

%RSD= (S.DX100)/mean

3. LIMIT OF DETECTION

The limit of detection (LOD) is the smallest concentration of the analyte, that gives the measurable response. LOD was calculated using the following formula:

LOD= 3.3X(Standard deviation / slope of calibration curve)

4. LIMIT OF QUANTIFICATION

The limit of quantification (LOQ) is the smallest concentration of the analyte, which is gives a response that can be accurately quantified. LOQ was calculated using the formula:

LOQ= 10X (Standard deviation/ slope of calibration curve)

5. ACCURACY

The accuracy of the method was determined by recovery experiments. The recovery studies were performed by the regular addition method. At 50%, 100%, 150% level, the percentage recovery was calculated. For both the drugs, recovery was performed in the same way. The recovery studies were performed in triplicate.

6. ROBUSTNESS

Robustness of the method was studied by making slight changes in chromatographic conditions, such as mobile phase ratio and mobile phase flow rate.

FORCED DEGRADATION STUDIES ACID DEGRADATION STUDIES

Accuratelyweighed 1mg of each Itraconazole and Terbinafinewere taken in to a volumetric flask, add 10mL, of 0.1N of hydrochloric acid and it is kept a side for 24 hours at room temperature.

Take 1mL, mobile phase and inject on to analytical column of HPLC.

BASE DEGRADATION

Accurately weighed 1mg each Itraconazole and Terbinafine were taken into a volumetric flask, add 10mL, of 0.1N of sodium hydroxide and it is kept a side for 24 hrs at room temperature.

Take 1mL of above solution, dilute with 10 ml mobile phase and inject on to analytical column of HPLC.

OXIDATION STUDIES

Accurately weighed 1mg of each Itraconazole and Terbinafine were taken into a volumetric flask, add 10ml of 0.1N of hydrogen peroxide and it is kept a side for 24hours at room temperature. Take 1mL of above solutions, dilute with 10mL mobile phase and inject on to analytical column of HPLC.

TEMPERATURE STRESS STUDIES

1gm of Itraconazole and Terbinafine were taken in two petridishes and kept in hot air oven at 60°C for 24hr. 1mg of this sample was taken into a 10ml volumetric flask, dissolved in mobile phase and inject on to analytical column.

RESULTS AND DISCUSSIONS

Determination of absorbance maxima(λ_{max})

Small amounts of drugs were dissolved in the HPLC grade methanol and the absorbance maxima was determined in shimadzu UV spectrophotometer using methanol as blank **λ.**

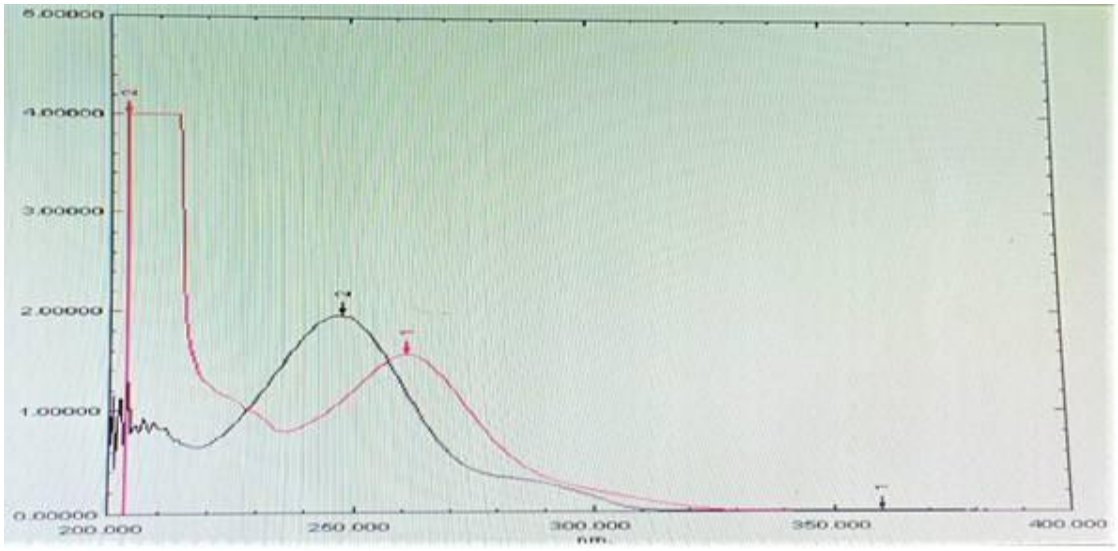

Fig: 13 Isobestic point of the absorption curves of Itraconazole and Terbinafine (20µg/ml)

METHOD OPTIMIZATION

To optimize the operation conditions for isocratic RP- HPLC detection of Itraconazole and Terbinafine a number of parameters such as the column type, mobile phase composition and the flow rate were varied.

TRIAL 1

Chromatographic conditions:

Mobile phase : Methanol: Water (97:3%v/v) Column: C_{18} (250mL x4.6mm i.d.,5µm)

Flow rate : 0.5mL/min

Wavelength : 257nm

Detector : UV detector

Injector : Rheodyne injector Injector volume : 20µL

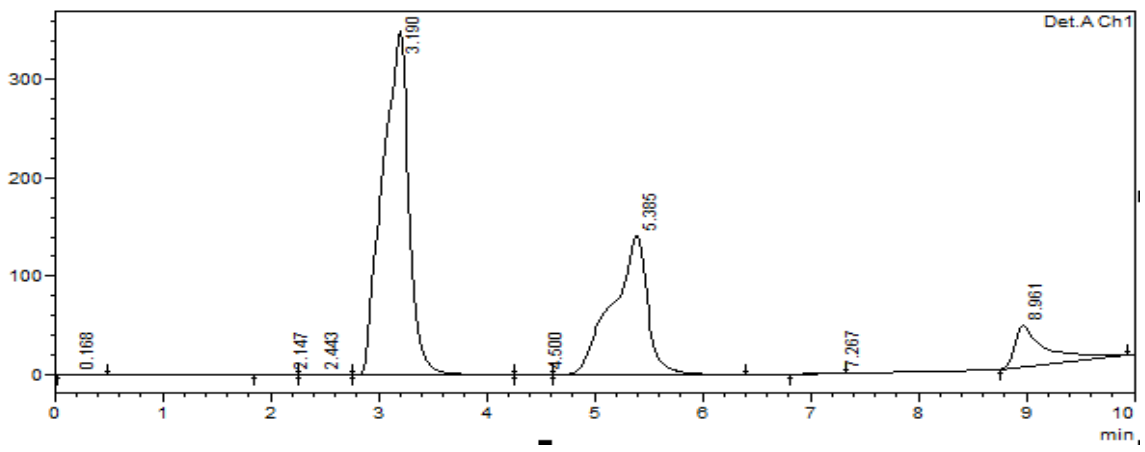

Fig14: Chromatogram of trial 1.

DISCUSSION

Fronting observed in the peaks.

TRIAL-2

Chomatographic conditions:

Mobile phase : Methanol: Water (96:4%v/v)

Solvent : Mobile phase used as a solvent

Flow rate : 0.8mL/min

Column : C_{18} (250mLx4.6mm i.d., 5µm)

Wavelength : 257nm

Detector : UV detector

Injector : Rheodyne injector Injector volume : 20µL

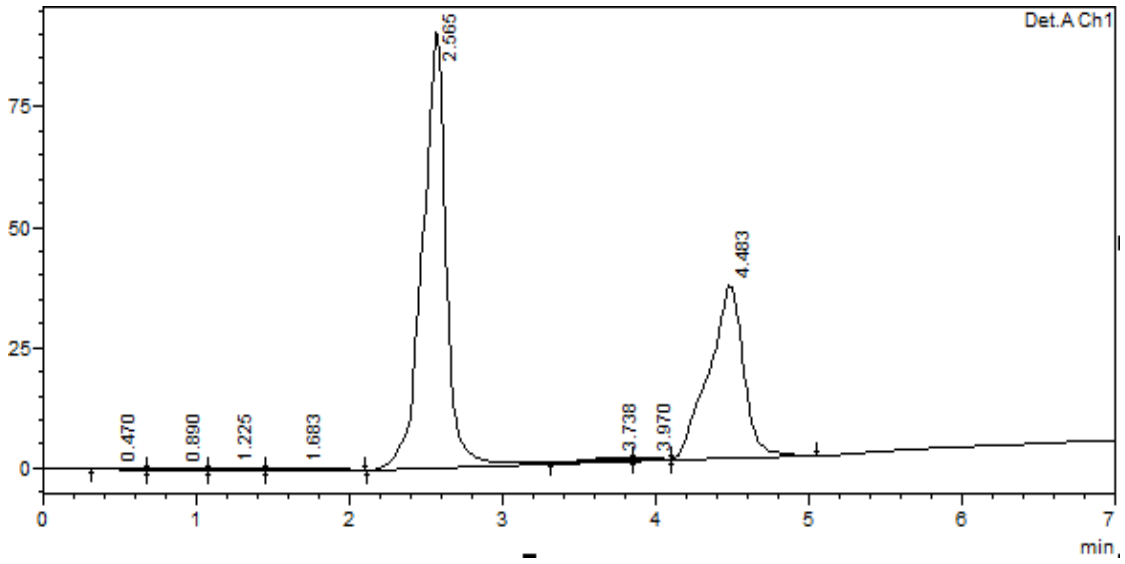

Fig 15*: *chromatogram of trial 2.

DISCUSSION

Broadening of peak observed.

OPTIMIZED METHOD

Table 3: Chromatographic conditions of optimized method.

Instrument	**HPLC SHIMADZU**
Column	HYPERSILC18(250x4.6mm i.d., 5µm)
Detection	257nm
Mobile phase	Methanol:Water (95:5%)
Flow rate	1mL/min
Injection volume	20µL

Run time	10mints
Type of elution	Isocratic

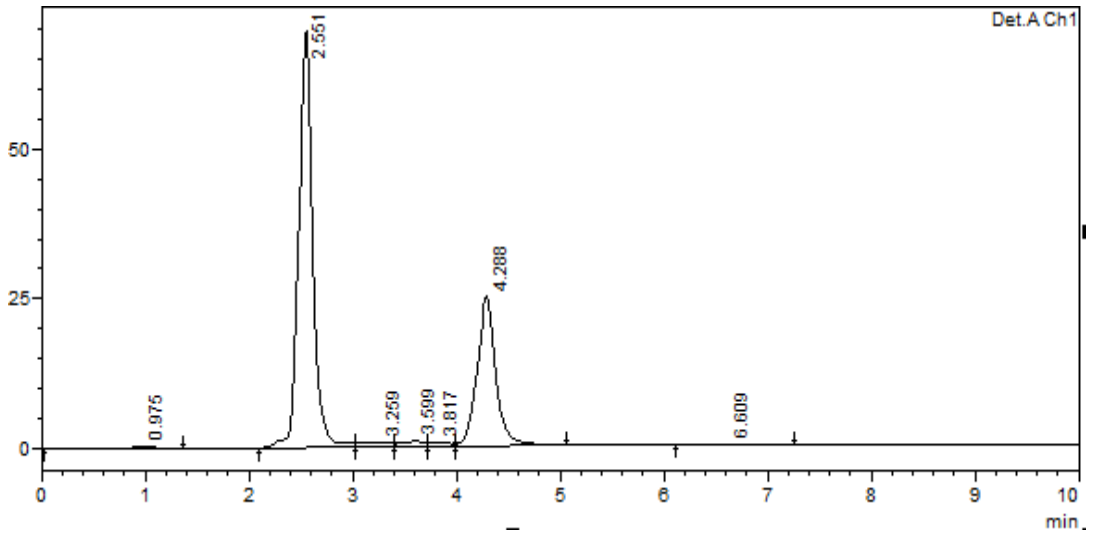

Fig 16: Optimized chromatogram.

METHOD OF VALIDATION LINEARITY

TABLE 4: Linearity results for Terbinafine and Itraconazole.

S.NO	CONCENTRATION	PEAK AREAS OF TERBINAFINE	PEAK AREAS OF ITRACONAZOLE
1	10	1948427	1172032
2	20	3712036	2032206
3	30	5176156	2971042
4	40	6886397	3939981
5	50	8635108	4999870
Correlation Coeffecient (R^2)		0.999	0.998

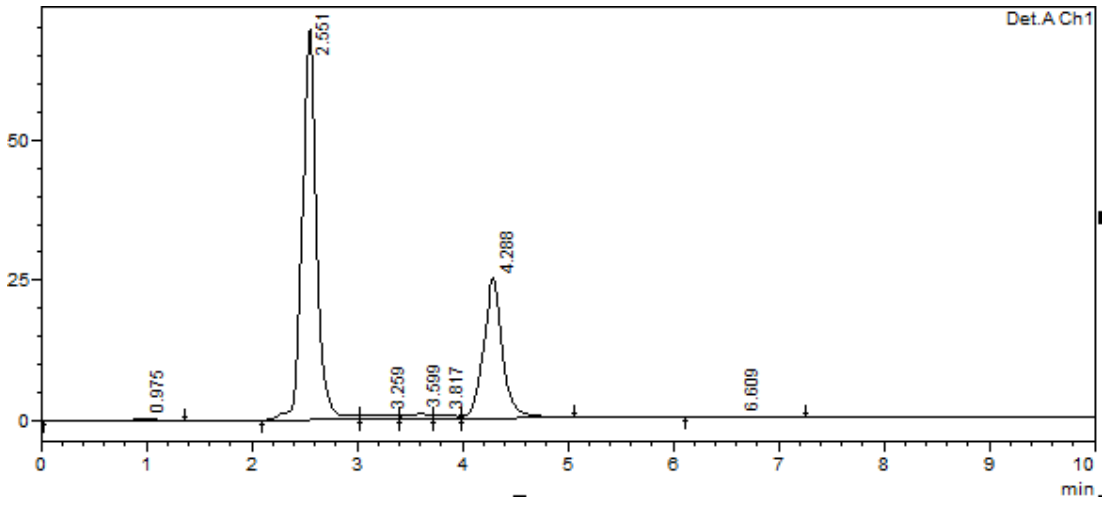

Fig 17: Linearity chromatogram (10µg/mL)

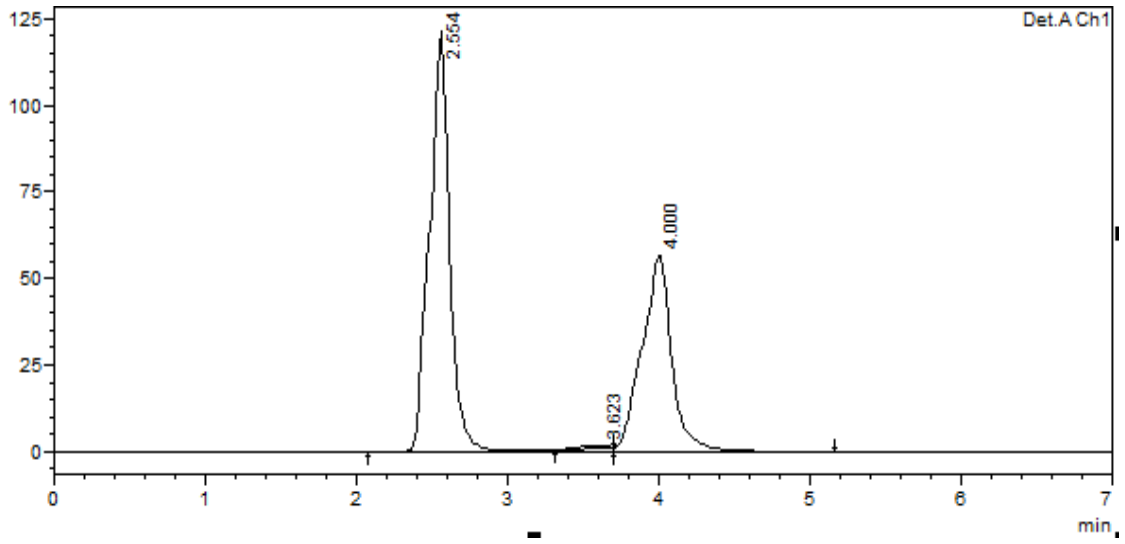

Fig18: Linearity chromatogram(20µg/mL)

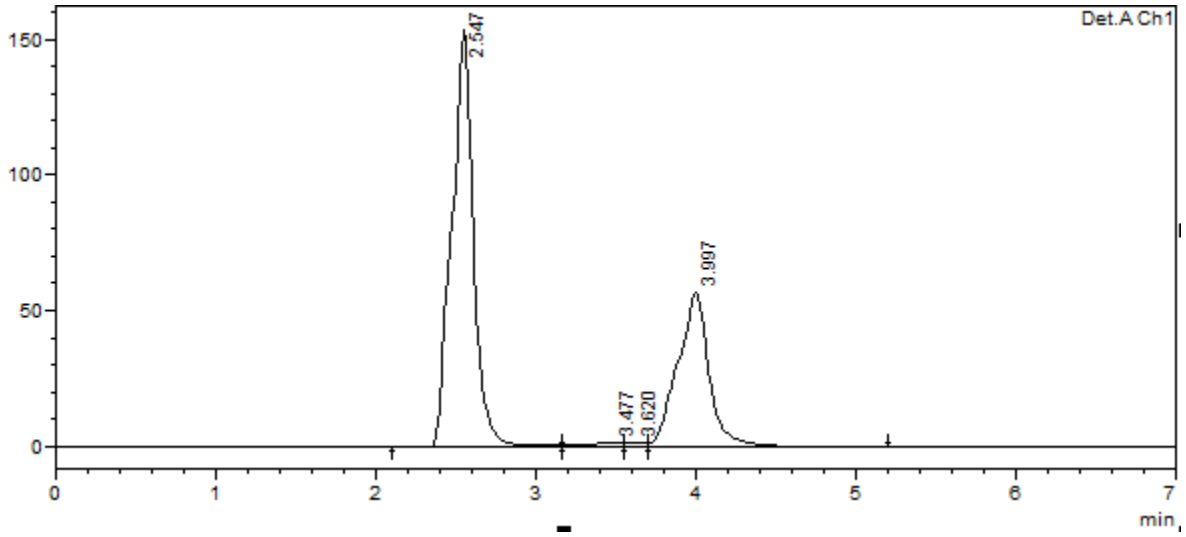

Fig 19: Linearity chromatogram (30µg/mL)

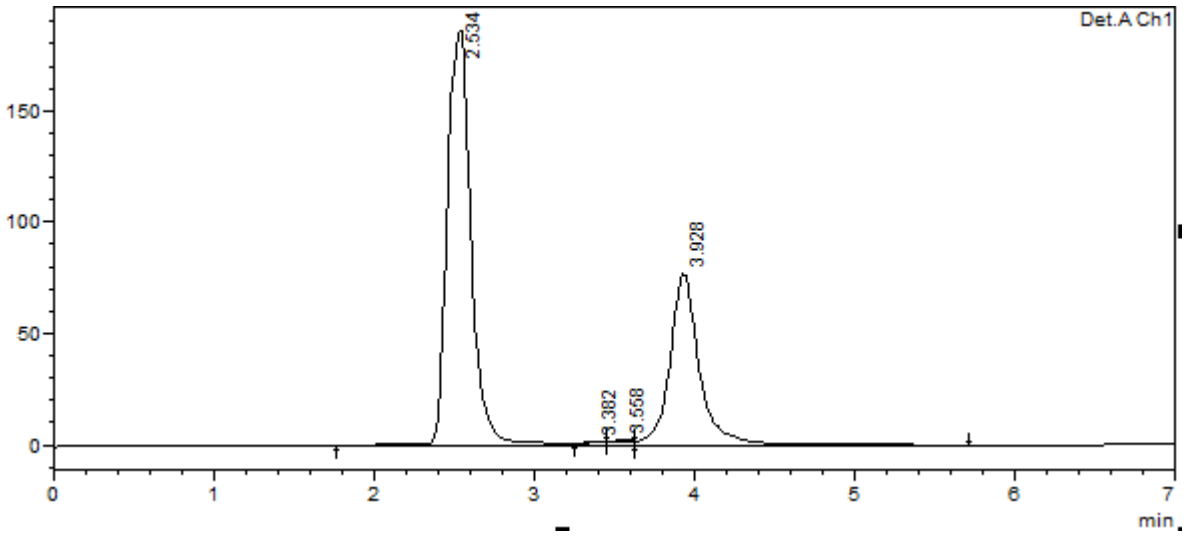

Fig 20: Linearity chromatogram (40µg/ml)

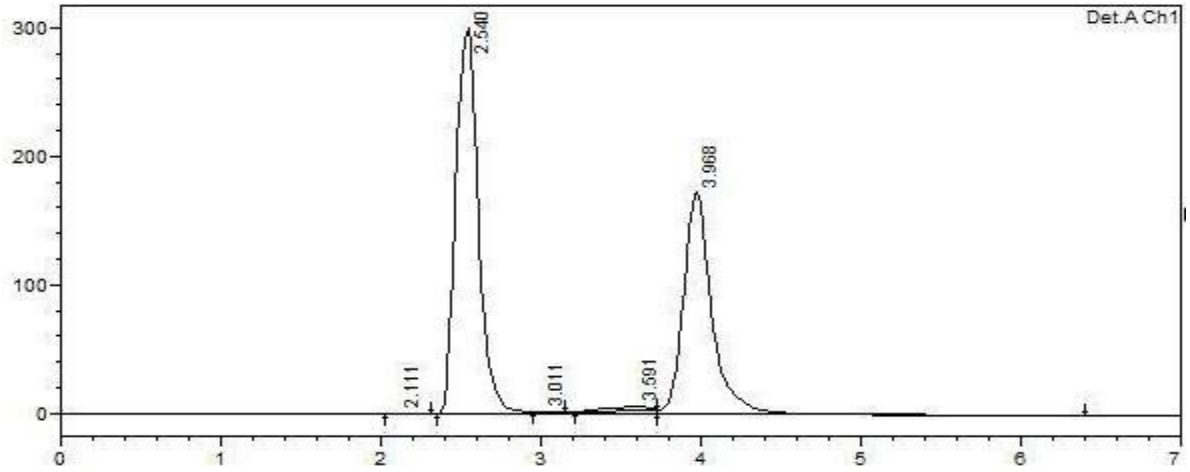

Fig 21*: *Linearity chromatogram(50µg/ml)

STANDARD CALIBRATION CURVE OF TERBINAFINE

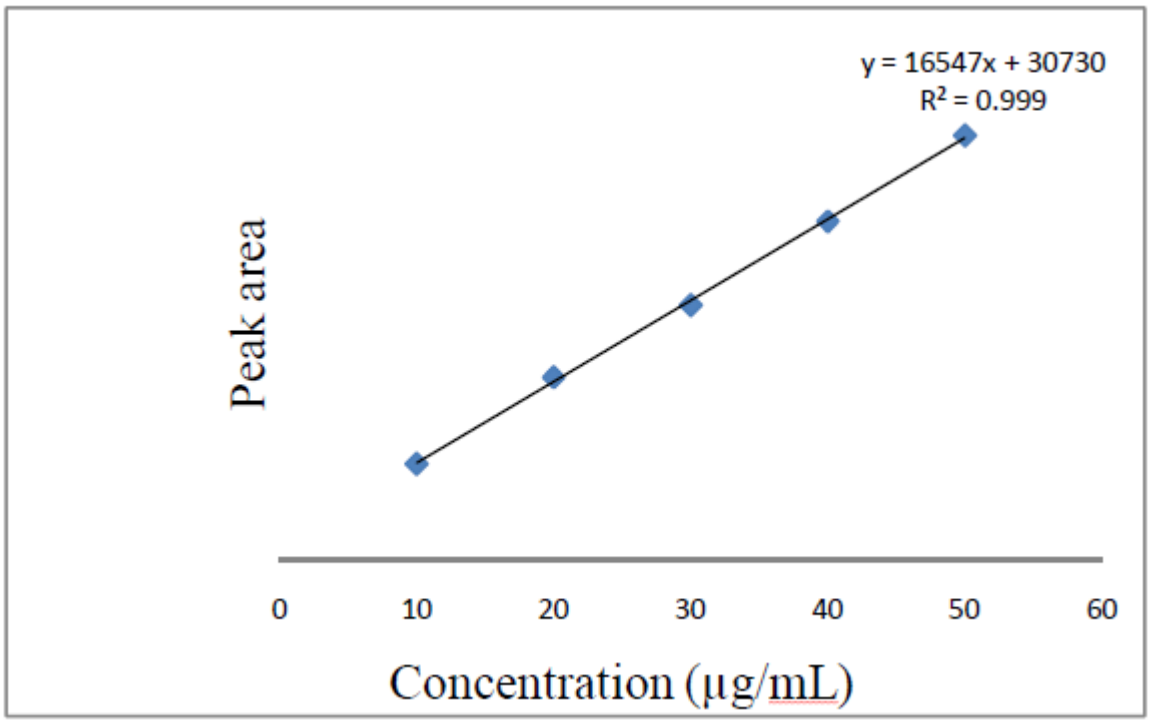

Fig 22*: *Standard graph of Terbinafine.

STANDARD CALIBRATION CURVE OF ITRACONAZOLE

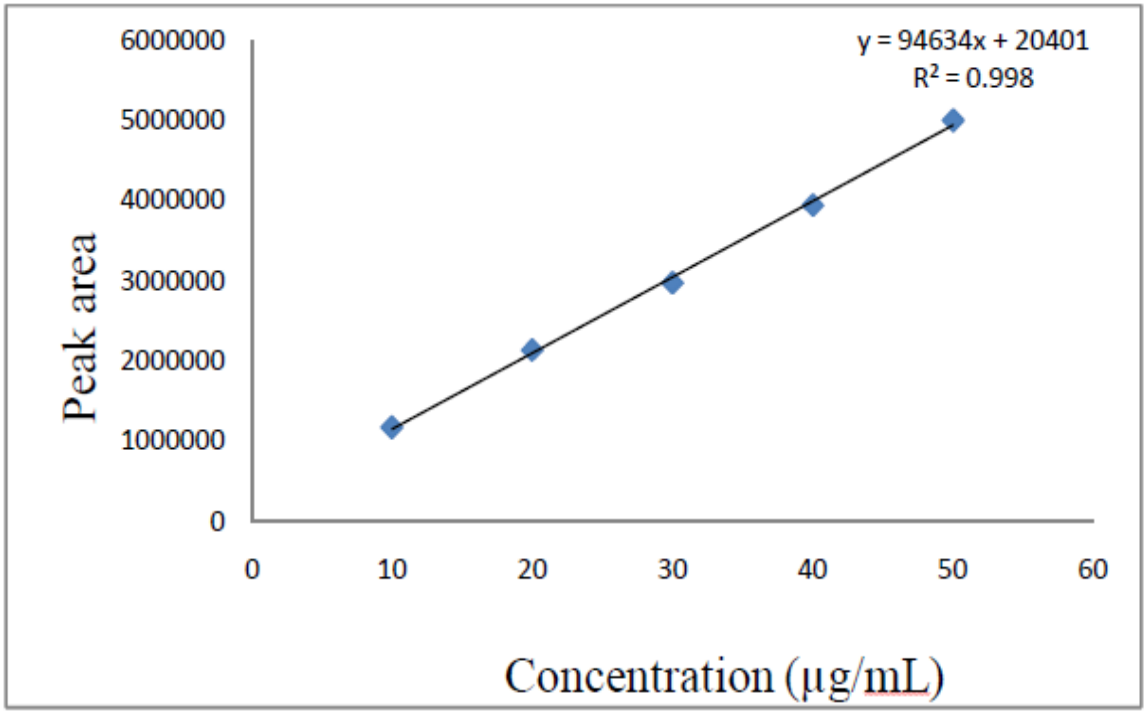

Fig 23: Standard graph of Itraconazole.

DISCUSSION

Under the optimized experimental conditions, linear correlation between the peak and applied concentration was found to be in the concentration range of 10-100µg/ mL, as conformed by the correlation coefficient of 0.999. The peak area (y) is proportional to the concentration of Itraconazole and Terbinafine (x) followings three regression equation y=94634x and y=16547x.

PRECISION

Table 5: Results for the Intra-day precision of Terbinafine and Itraconazole.

S.NO	Peak area of Terbinafine	Peak area of Itraconazole
1	3712036	2132263
2	3613420	1970843
3	3784862	2016349
4	3615412	2153834
Average	3726433	2143324
S.D	40135	35185
R.S.D	1.007	1.64

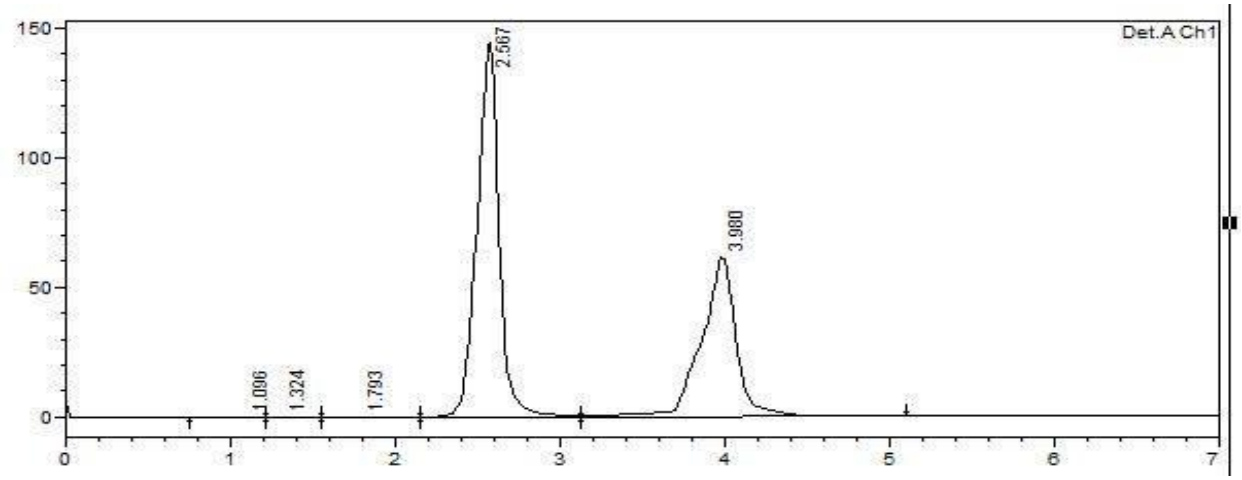

Fig 24: chromatogram of Intra day precision-1(20µg/mL)

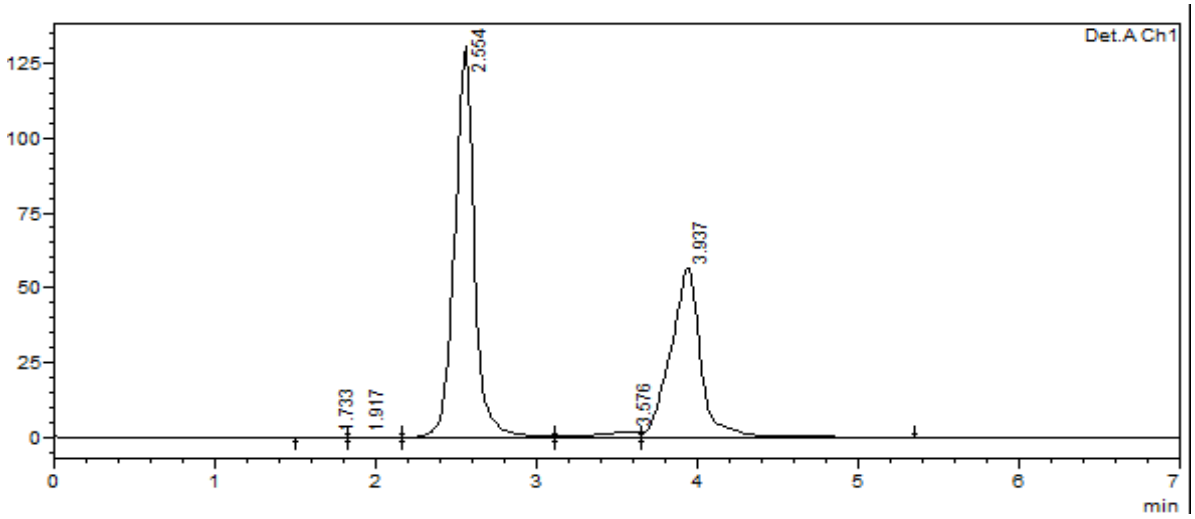

Fig25: chromatogram of Intra day precision-2 (20µg/mL)

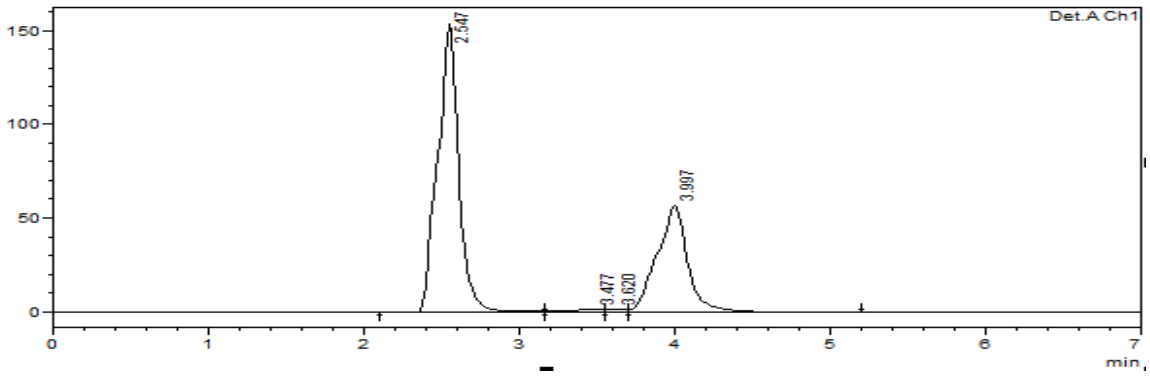

Fig 26: chromatogram of Intra day precision-3 (20µg/mL)

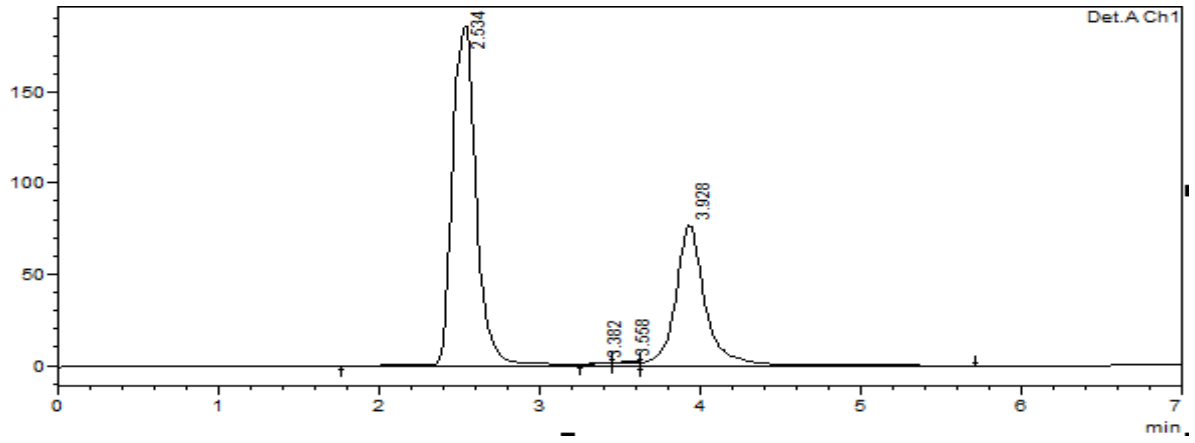

Fig 27: chromatogram of Intra day precision-4 (20µg/mL)

DISCUSSION

Precision data on the Intra day variations for single concentrations levels (20µg/mL) are summarized and %RSD was found to be less than 2.

LIMIT OF DETENTION & LIMIT OF QUANTIFICATION

Table No. 6: Limit of detection and limit of quantification.

Drug	LOD	LOQ
Terbinafine	8.00	24.00
Itraconazole	1.25	3.79

DISCUSSION

The LOD and LOQ values of Itraconazole and Terbinafine has show in the Table 5. These data shows that the method was highly sensitive and specific.

Accuracy

Terbinafine and Itraconazole recovery from pharmaceutical active ingredients after spiking with 50%, 100%, 150%.

Table 7: Accuracy Data of Terbinafine and Itraconazole.

S.NO	Concentration	%Recovery of Terbinafine	%Recovery of Itraconazole	Mean Recovery of Terbinafine	Mean Recovery of Itraconazole
1	50%	90.0%	106%	94.33%	101.3%
2	100%	95.7%	98.4%		
3	150%	97.3%	99.6%		

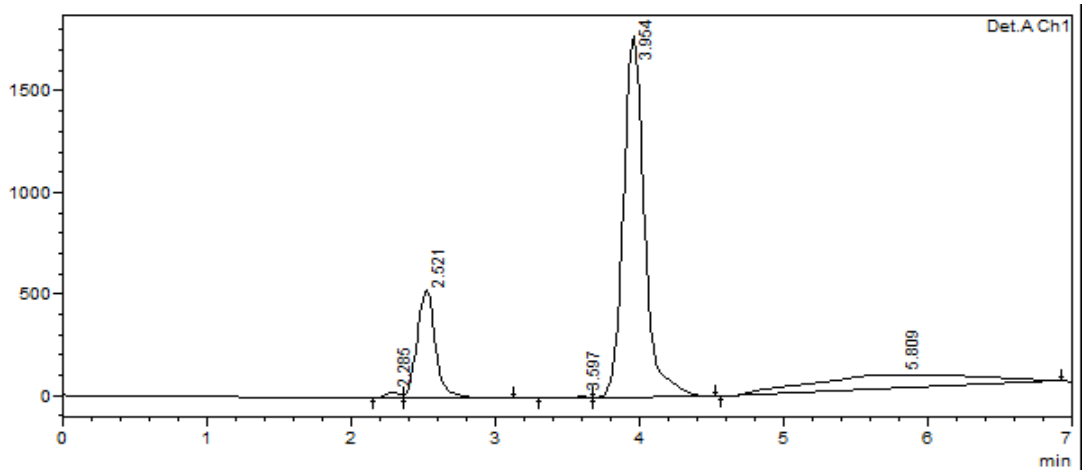

Fig 28: Chromatogram for accuracy 50%

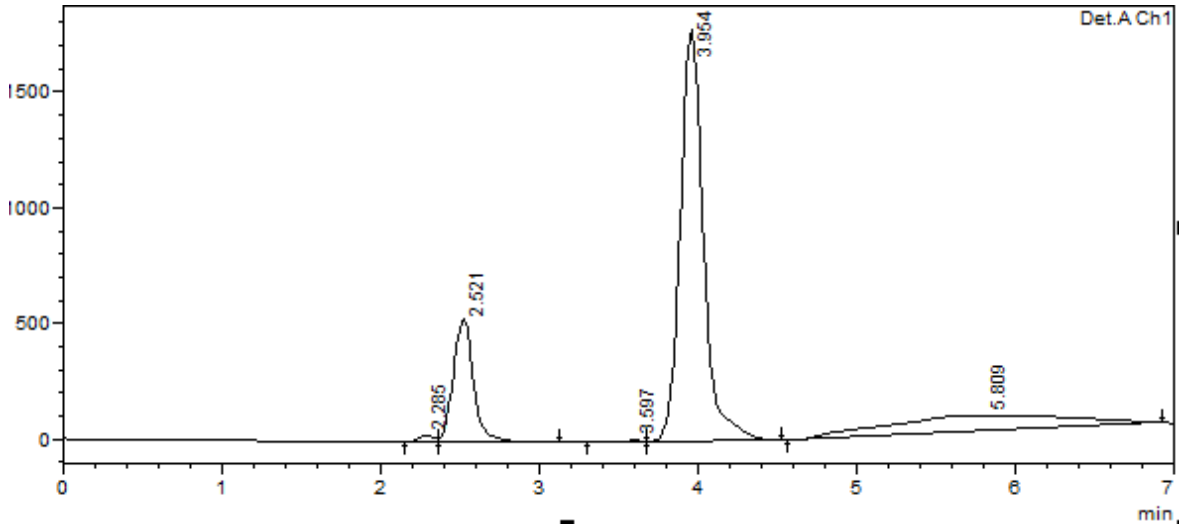

Fig 29: Chromatogram for accuracy 100%

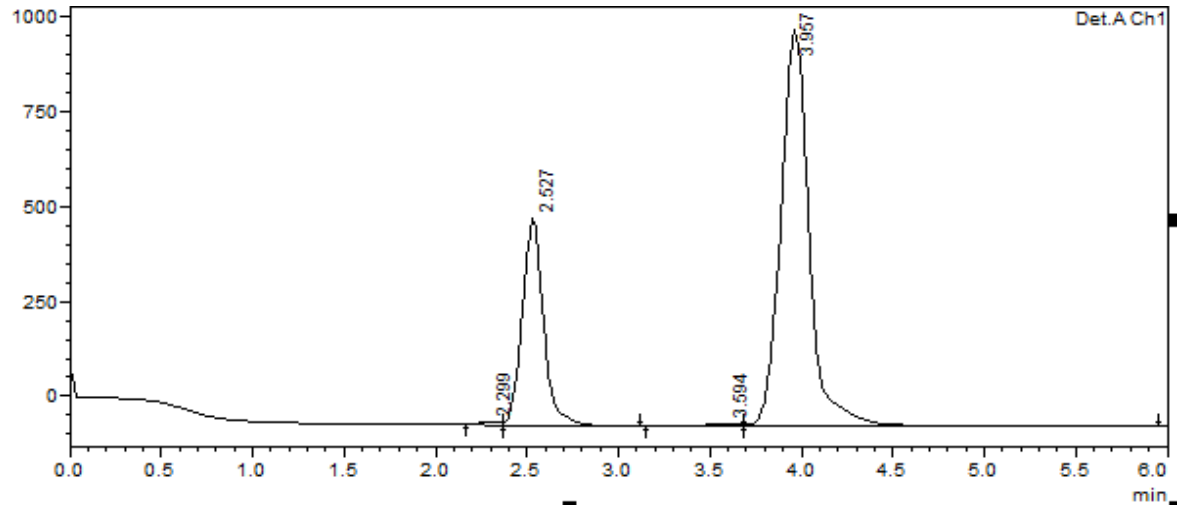

Fig 30: Chromatogram for accuracy 150%

DISCUSSION

The recovery studies were performed in triplicate. Recovery was found to be with in the range of 98-101.3%. The results of recovery studies were found to be satisfactory.

Robustness

The robustness of the proposed method was determined by analysis of aliquots from homogenous lots by differing parameters like flow rate, mobile phase, composition.

Table 9: Robustness results of Terbinafine and Itraconazole.

S.NO	MOBILE PHASE	FLOW RATE (mL/min)	Max(nm)	%RSD OF TERBINAFINE	%RSD OF ITRACONAZOLE
1	97:3	1	257	1.09	1.03
2	96:4	1	257	1.61	1.34
3	95:5	0.8	257	0.99	1.50
4	95:5	1.2	257	1.42	1.77

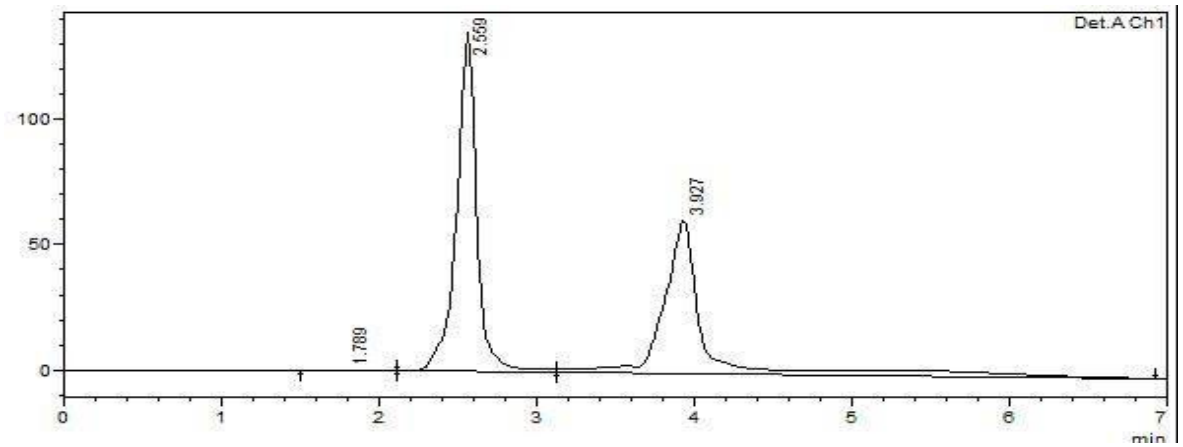

Fig 31: Chromatogram for Robustness(mobile phase 97:3)

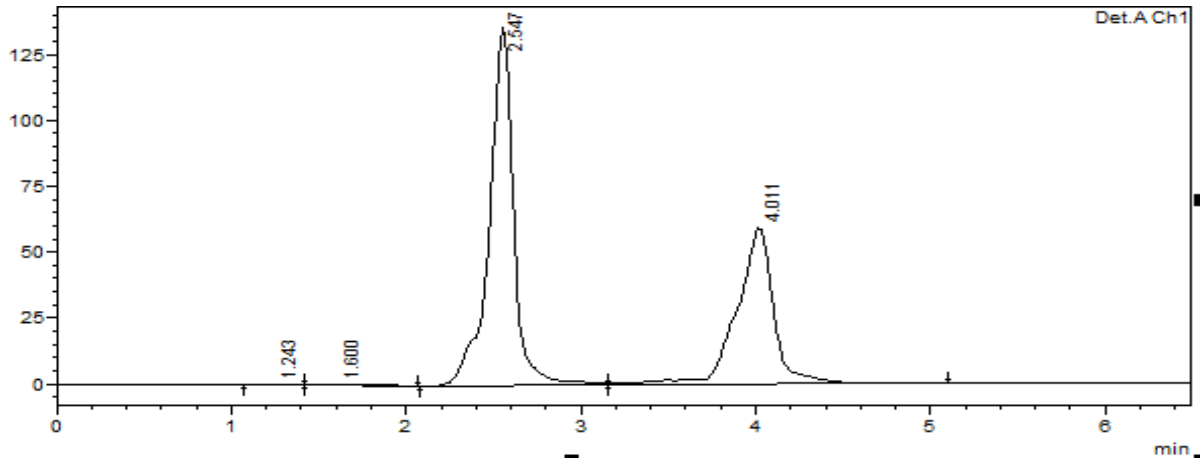

Fig 32: Chromatogram of Robustness (mobile phase 96:4v/v)

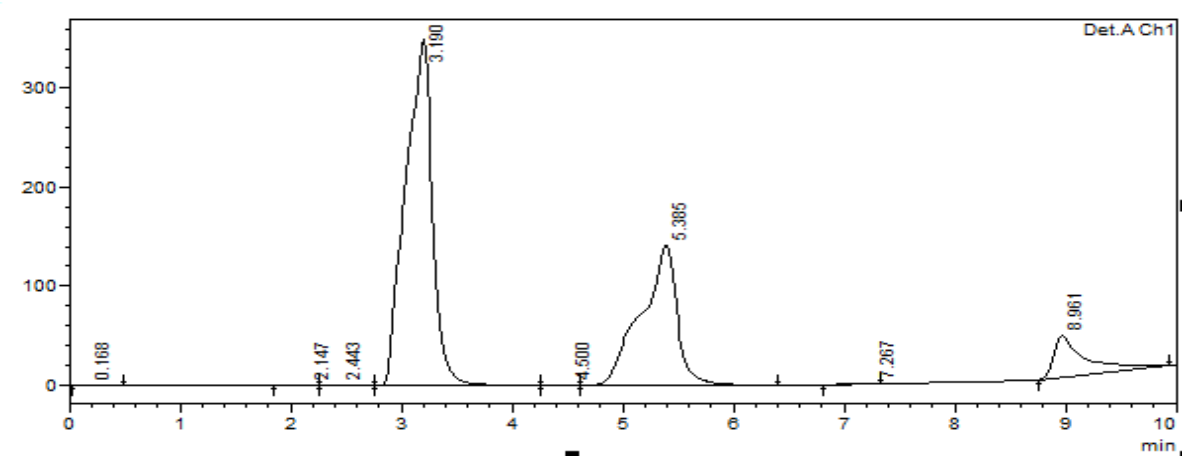

Fig 33: Chromatogram of Robustness (Flow Rate-0.8)

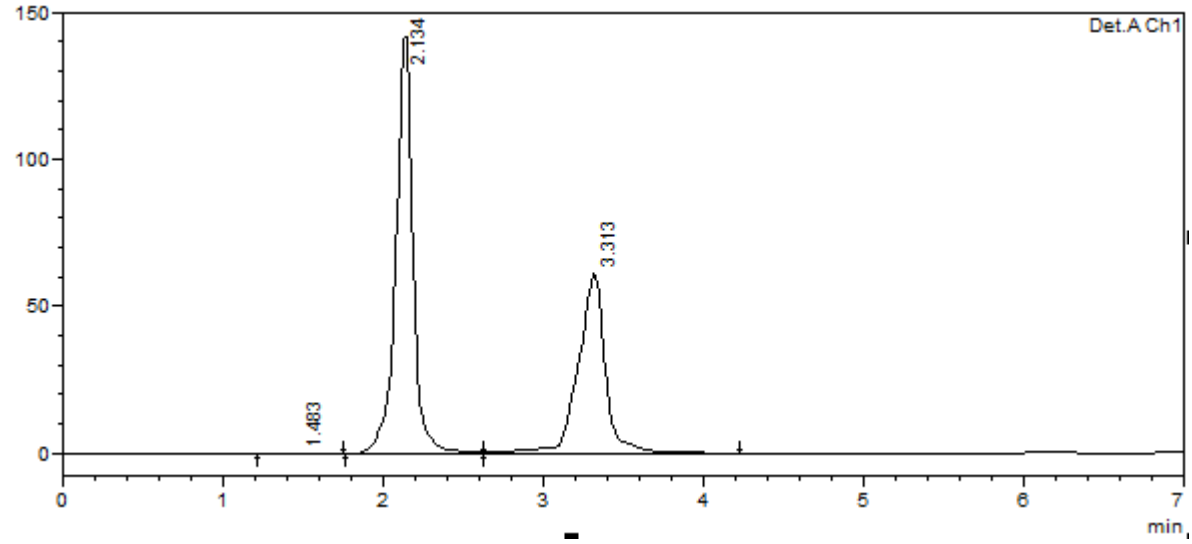

Fig 34: Chromatogram of Robustness (Flow Rate-1.2v/v)

DISCUSSION

The effect of selected parameters were evaluated over a range of conditions by determining the maximum Terbinafine and Itraconazole response. The retention time will be change with the changing of mobile phase. Flow rate and column temperature. The robustness of Terbinafine and Itraconzole indicates there is no change observed in retention times of drug with changing of the flow rate and mobile phase ratio. The %RSD was less than 2.

DETERMINATION OF TABLET

Table 8: Results for Determination of tablet.

Drug	Retention time(min)	Peak area
Terbinafine	2.555	2795039
Itraconazole	3.960	5207109

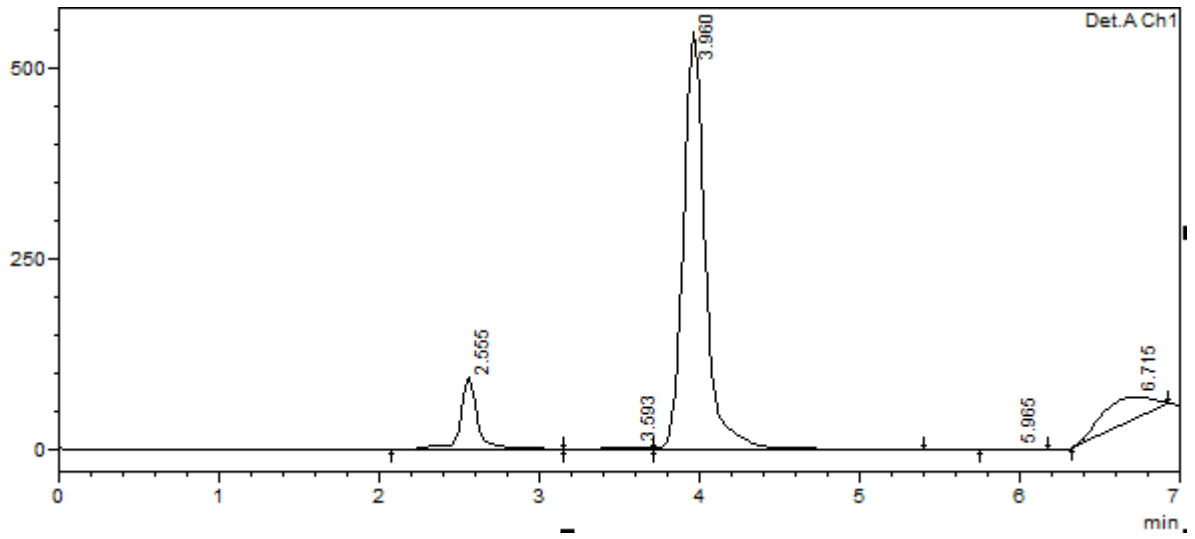

Fig 35: Chromatogram of Terbinafine and Itraconazole in tablet.

DISCUSSION

The retention time for the Terbinafine and Itraconazole were found to be 2.55 min and 3.96 min.

FORCED DEGRADATION STUDY

Forced degradation of Terbinafine and Itraconazole in various conditions like acidic, basic, oxidation and thermal degradation was observed. Results of forced degradation study.

1. BASE DEGRADATION

The base degradation study observed with 0.1M of sodium Hydrochloride solution. The study has been observed for 1,3,5 days time intervals on HPLC.

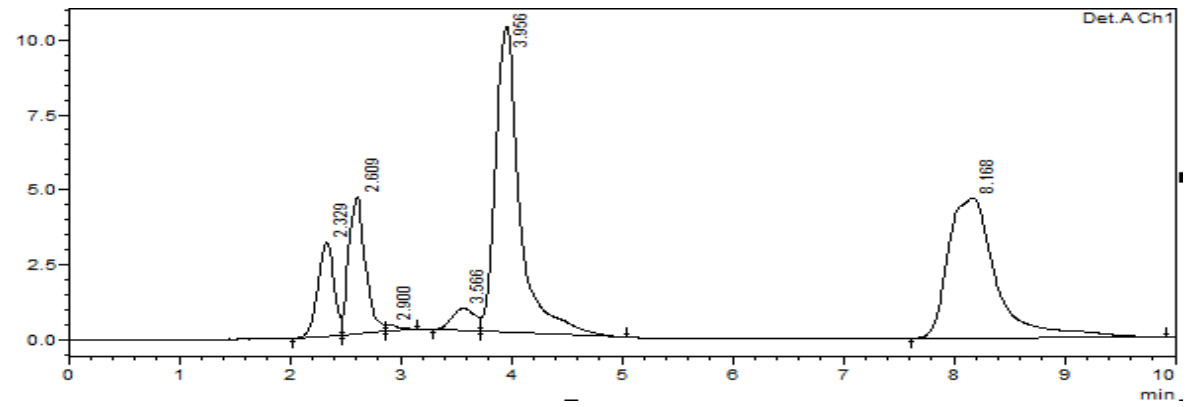

Fig 36: chromatogram of Terbinafine hydrochloride and Itraconazole for base degradation 1 day.

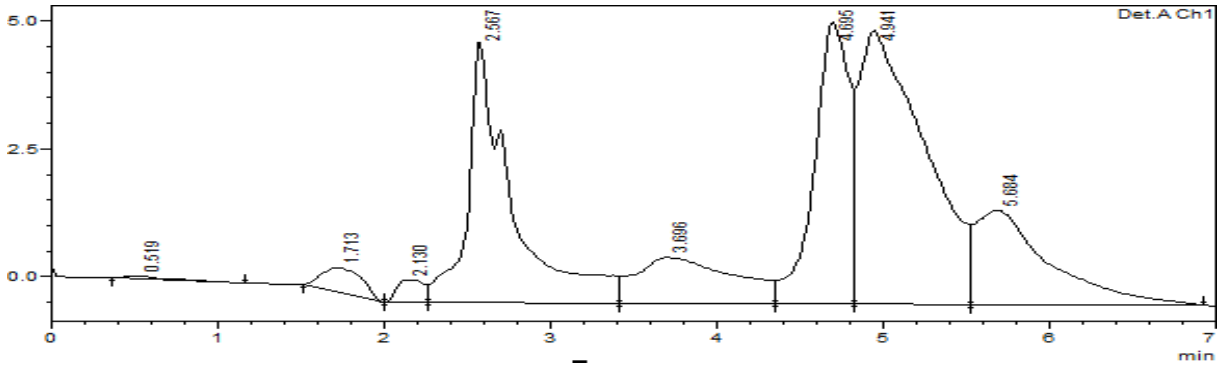

Fig 37: chromatogram of Terbinafine and Itraconazole for base degradation 3 day.

2. ACID DEGRADATION

The base degradation study observed with 0.1M of Hydrochloric acid solution. The study has been observed for 1,3,5 days time intervals on HPLC.

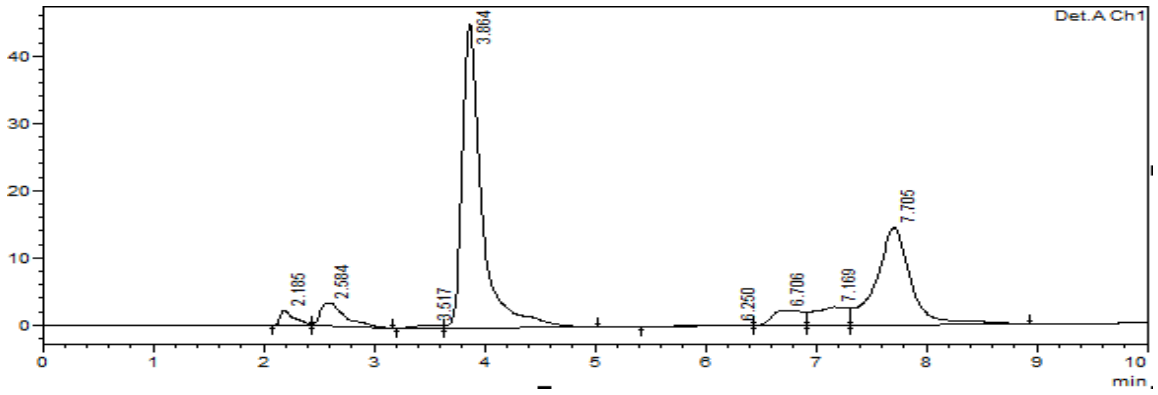

Fig 38: chromatogram of Terbinafine and Itraconazole for acid degradation 1 day.

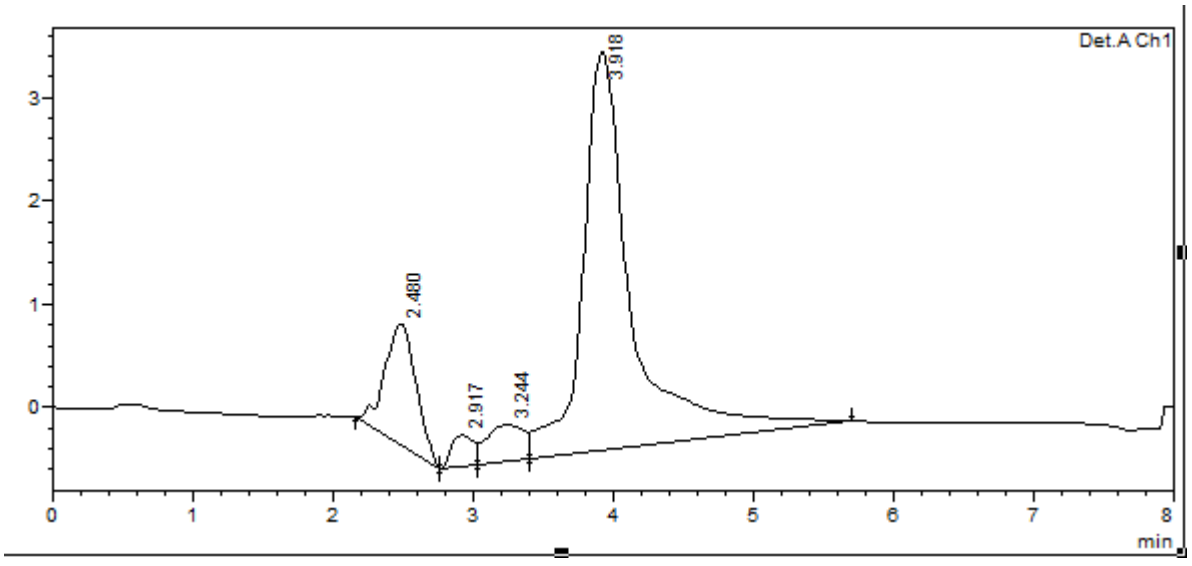

Fig 39: chromatogram of Terbinafine and Itraconazole for acid degradation 3 day.

3. OXIDATIVE DEGRADATION

The base degradation study observed with 0.1M of Hydrogen peroxide solution. The study has been observed for 1,3,5 days time intervals on HPLC.

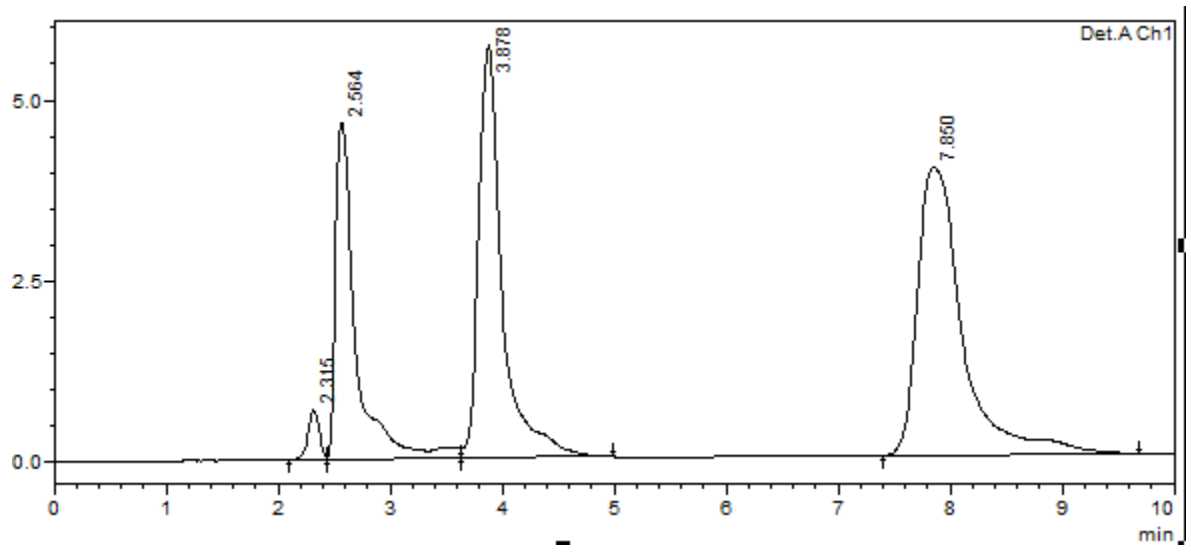

Fig 40: chromatogram of Terbinafine and Itraconazole for hydrogen peroxide degradation 1 day.

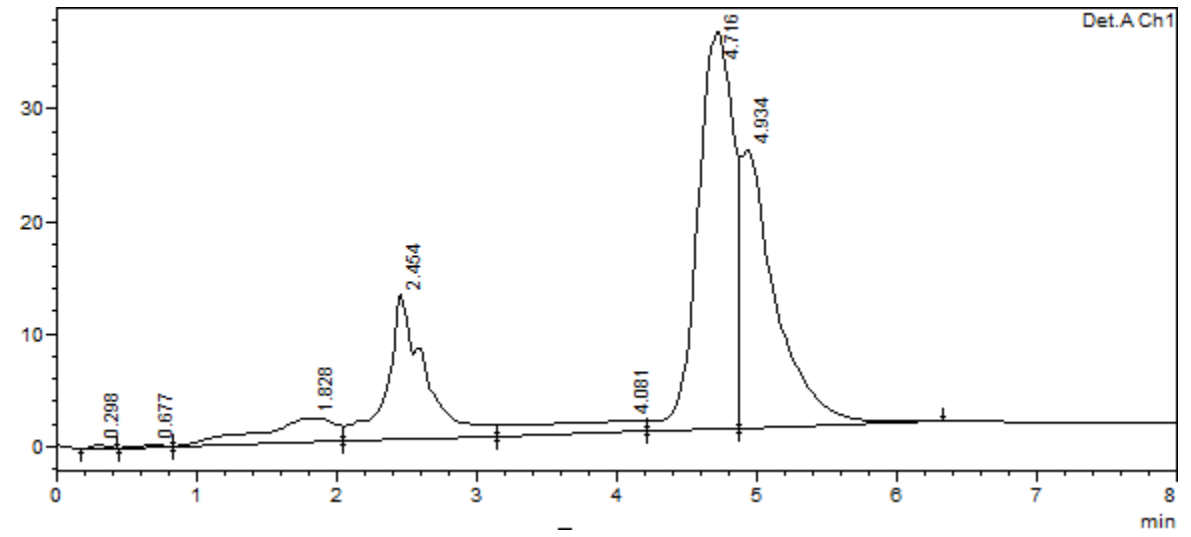

Fig 41: chromatogram of Terbinafine and Itraconazole for hydrogen peroxide degradation 3 day.

DISCUSSION

Forced degradation of Terbinafine and Itraconazole tablet dosage form in various conditions like acidic, alkaline, oxidation and thermal degradation were performed. Drugs degradation was confirmed by observing the additional peaks formed by degraded products in chromatogram of Terbinafine and Itraconazole. Degradation of drugs were observed in only 1

days for acidic and alkaline conditions. In oxidation stress degradation was observed in after 3 days**.**

4. THERMAL DEGRADATION

The thermal degradation was observed with methanol. The study has been performed for 1,3,5 days.

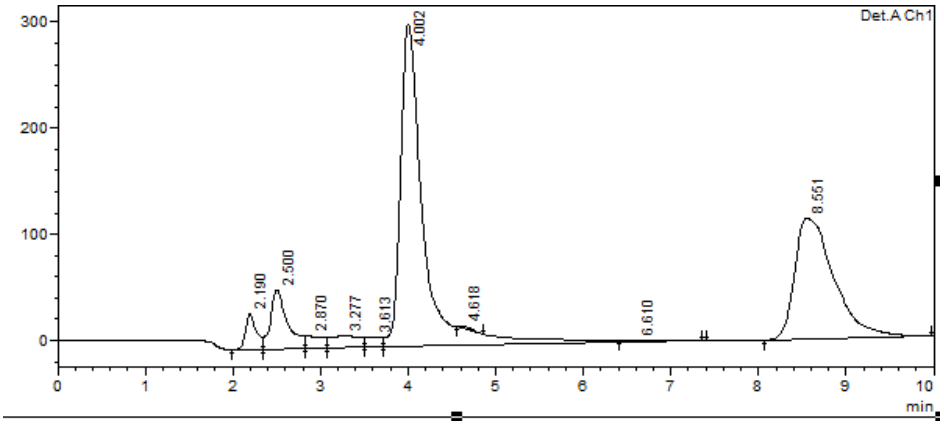

Fig 42: Chromatogram of Terbinafine and Itraconazole for thermal degradation on 5 day.

DISCUSSION

Forced degradation of Terbinafine and Itraconazole in tablet dosage form in thermal degradation was performed. Additional peaks observed in the chromatograms.

SUMMARY AND CONCLUSION

A new sensitive method was developed and optimized, the following parameters were validated according to the ICH guidelines.

1. Mobile phase –water : methanol (95:5%v/v)
2. Limit of detection (LOD)- 1.2 , 8.0 µg/mL for Itraconazole and Terbinafine respectively
3. Limit of quantification (LOQ)-3.79, 24.00 µg/mL for Itraconazole and Terbinafine respectively.
4. Linearity-10-50µg/mL
5. Precision, accuracy and robustness were performed.
6. From the above results, it can be concluded that the developed RP-HPLC method represents a good technique for the determination of Itraconazole and Terbinafine content in tablet formulation with good sensitivity, precision and reproducivity. The sample preparation involving ultrasonic extraction is simple and cost effective. Further more, the method can be used in the determination of Itraconazole and Terbinafine in other

pharmaceutical preparations and in routine quality control of formulations. Quantification was achived of terbinafine and itraconazole at 257nm.the retention time for Terbinafine and itraconazole was found to be 2.551 and 4.228 min respectively.

7. Linear correlation was obtained between peak area versus concentration of Terbinafine and Itraconazole in the concentration ranges of 10-50µg/mL.
8. The mean recovery obtained were of Terbinafine and Itraconazole, was 94.33% and 101.3%, which indicates the accuracy of the proposed method.
9. The %RSD valve of terbinafine and Itraconazole was found to be less than 2, which indicates the accuracy of the proposed method.
10. Forced degradation of Terbinafine and Itraconazole in various conditions like alkaline, acidic, oxidation and thermal degradation was observed in this investigation. The contents of degradation of the drug was quantitavively analysed by HPLC. The Terbinafine and Itraconazole is very sensitive drug it was degraded in various conditions.

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