

**A RP-HPLC METHOD DEVELOPMENT AND VALIDATION OF
TINIDAZOLE AND DILOXANIDE FUROATE IN PHARMACEUTICAL
FORMULATION AND ITS FORCED DEGRADATION STUDIES**

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1. INTRODUCTION

Chemistry is the study of matter, including its composition, structure, physical properties, and reactivity. There are many approaches to studying chemistry, but for convenience, we traditionally divide it into five fields: organic, inorganic, physical, biochemical, and analytical. Although this division is historical and arbitrary, as witnessed by the current interest in interdisciplinary areas such as bio analytical and organometallic chemistry, these five fields remain the simplest division spanning the discipline of chemistry. Analytical chemistry is often described as the area of chemistry responsible for characterizing the composition of matter, both qualitatively (what is present) and quantitatively (how much is present)¹.

Analytical chemistry may be defined as the “Science and art of determining the composition of materials in terms of the elements or compounds contained”. Pharmaceutical analysis plays a major role today, and it can be considered as an interdisciplinary subject. Pharmaceutical analysis derives its principles from various branches of science like Chemistry, Physics, Microbiology, Nuclear Science, Electronics, etc.

Analytical method is a specific application of a technique to solve an analytical problem. Analytical instrumentation plays an important role in the production and evaluation of new products and in the protection of consumers and the environment. This instrumentation provides the lower detection limits required to assure safe foods, drugs, water and air, generally used for drug analysis are spectral methods, chromatographic methods, electro analytical techniques, and miscellaneous techniques like conventional titrimetric, gravimetric and Polari metric methods.

Pharmaceutical analysis techniques are applied mainly in two areas traditionally:

Analytical chemistry has been split into two main types. They are qualitative and quantitative:

➤ **Qualitative**

Qualitative analysis seeks to establish the presence of a given element or compound in a sample.

➤ **Quantitative**

Quantitative analysis seeks to establish the amount of a given element or compound in a sample.

Specific Technologies and Instrumentation

A) Spectrometric Techniques:

1. Ultraviolet and visible Spectrophotometer
2. Fluorescence and phosphorescence Spectrophotometer
3. Atomic Spectrometry (Emission and Absorption)
4. Infrared Spectrophotometer
5. Raman Spectroscopy
6. X-Ray Spectroscopy
7. Radiochemical Techniques including activation analysis
8. Nuclear Magnetic Resonance Spectroscopy
9. Electron Spin Resonance Spectroscopy

B) Electrochemical Techniques:

1. Potentiometer
2. Voltametry
3. Volta metric Techniques
4. Amperometric Techniques

5. Colorimetry
6. Electrogravimetry
7. Conductance Techniques

C) Chromatographic Techniques:

1. Gas Chromatography
2. High performance Liquid Chromatography
3. Thin Layer Chromatography
4. Ultra performance Liquid Chromatography

D) Miscellaneous Techniques:

1. Thermal Analysis
2. Mass Spectrometry
3. Kinetic Techniques

1.1 ANALYTICAL METHOD DEVELOPMENT:

The number of drugs introduced into the market is increasing every year. These Drugs may be either new entities or partial structural modification of the existing one. Very often there is a time lag from the date of introduction of a drug into the market to the date of its inclusion in pharmacopeias. This happens due to the possible uncertainties in the continuous and wider usage of these drugs, reports of new toxicities (Resulting in their withdrawal from the market), development of Patient resistance and introduction of better drugs by competitors. Under these conditions, standards and analytical procedures for these drugs may not be available in the Pharmacopeia. Therefore it becomes necessary to develop newer analytical methods for such drugs².

Basic Criteria for New Method Development Of Drug Analysis:

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

Steps Involved In Method Development:

Documentation starts at the very beginning of the development process. A system for full documentation of development studies must be established. All data relating to these studies must be recorded in laboratory notebook or an electronic database³.

1. Analyte standard characterization:

- a) All known information about the analyte and its structure is collected i.e., physical and chemical properties.
- b) The standard analyte (100 % purity) is obtained. Necessary arrangement is made for the proper storage (refrigerator, desiccators and freezer).
- c) When multiple components are to be analyzed in the sample matrix, the number of components is noted, data is assembled and the availability of standards for each one is determined.

Only those methods (Spectroscopic, MS, GC, HPLC etc.,) that are compatible with sample stability are considered.

2. Method requirements:

The goals or requirements of the analytical method that need to be developed are considered and the analytical figures of merit are defined. The required detection limits, selectivity, linearity, range, accuracy and precision are defined.

3. Literature search and prior methodology:

The literature for all types of information related to the analyte is surveyed. For synthesis, physical and chemical properties, solubility and relevant analytical methods, books, periodicals, chemical manufacturers and regulatory agency compendia such as USP / NF are reviewed. Chemical Abstracts Service (CAS) automated computerized literature searches are convenient.

4. Choosing a method:

- a) Using the information in the literatures and prints, methodology is adapted. The methods are modified wherever necessary. Sometimes it is necessary to acquire additional instrumentation to reproduce, modify, improve or validate existing methods for in-house analytes and samples.
- b) If there are no prior methods for the analyte in the literature, from analogy, the compounds that are similar in structure and chemical properties are investigated and are worked out.
- c) There is usually one compound for which analytical method already exist that is similar to the analyte of interest.

5. Instrumental setup and initial studies:

The required instrumentation is setup. Installation, operational and performance qualification of instrumentation using laboratory standard operating procedures (SOP's) are verified. Always new consumables (e.g. solvents, filters and gases) are used. For example, method development is never started on a HPLC column that has been used earlier. The analyte standard in a suitable injection / introduction solution and in known concentrations and solvents are prepared. It is important to start with an authentic, known standard rather than with a complex

sample matrix. If the sample is extremely close to the standard (e.g., bulk drug), then it is possible to start work with the actual sample⁴.

6. Optimization:

During optimization, one parameter is changed at a time and set of conditions are isolated, rather than using a trial and error approach. Work has been done from an organized methodical plan, and every step is documented (in a lab notebook) in case of dead ends.

7. Documentation of analytical figures of merit:

The originally determined analytical figures of merit are Limit of Quantification (LOQ), Limit of Detection (LOD), linearity, time per analysis, cost, sample preparation etc., are documented.

8. Evaluation of method development with actual samples:

The sample solution should lead to unequivocal, absolute identification of the analyte peak of interest apart from all other matrix components.

9. Determination of percent recovery of actual sample and demonstration of quantitative sample analysis:

Percent recovery of spiked, authentic standard analyte into a sample matrix that is shown to contain no analyte is determined. Reproducibility of recovery (average + / - standard deviation) from sample to sample and whether recovery has been optimized or not has been shown. It is not necessary to obtain 100 % recovery as long as the results are reproducible and known with a high degree of certainty. The validity of analytical method can be verified only by laboratory studies. Therefore Documentation of the successful completion of such studies is a basic requirement for Determining whether a method is suitable for its intended applications⁵.

1.2 CHROMATOGRAPHY

Chromatography (Chroma means ‘color’ and graphein means to ‘write’) is the collective term for a set of laboratory techniques for the separation of mixtures. It involves passing a mixture dissolved in a "mobile phase" through a stationary phase, which separates the analyte to be measured from other molecules in the mixture based on differential partitioning between the mobile and stationary phases. Differences in compounds partition coefficient results in differential retention on the stationary phase and thus changing the separation. Chromatography is defined as a chemical analysis separation process which uses selective adsorption to segregate and identify components of complex mixtures such as solutions, liquids and vapors. Different types of Chromatographic techniques were summarized in table.1.3

Chromatography may be preparative or analytical. The purpose of preparative Chromatography is to separate the components of a mixture for further use (and is thus a form of purification). Analytical Chromatography is done normally with smaller amounts of material and is for measuring the relative proportion of analytes in a mixture⁶.

Table 1.3 Different Types of Chromatographic Techniques

Basic principle involved	Type of Chromatography
Techniques by Chromatographic bed shape	Column Chromatography
	Paper Chromatography
	Thin layer Chromatography
Techniques by physical state of mobile phase	Gas Chromatography
	Liquid Chromatography
Affinity Chromatography	Supercritical fluid Chromatography
Techniques by separation mechanism	Ion Exchange Chromatography
	Size Exclusion Chromatography
Special techniques	Reversed Phase Chromatography
	Two-dimensional Chromatography
	Simulated Moving-Bed Chromatography
	Pyrolysis Gas Chromatography
	Fast Protein Liquid Chromatography

High Performance Liquid Chromatography (HPLC):

In the modern pharmaceutical industry, High Performance Liquid Chromatography (HPLC) is the major and integral analytical tool applied in all stages of drug discovery, development, and production. Effective and fast method development is of paramount importance throughout this drug development life cycle. This requires a thorough understanding of HPLC principles and theory which lay a solid foundation for appreciating the many variables that are optimized during fast and effective HPLC method development and optimization. Chromatographic separations are based on a forced transport of the liquid (mobile phase) carrying the analyte mixture through the porous media and the differences in the interactions at analytes with the surface of this porous media resulting in different migration times for a mixture components.

High surface area of the interface between mobile and stationary phases is essential for space discrimination of different components in the mixture. Analyte molecules undergo multiple phase transitions between mobile phase and adsorbent surface.

Average residence time of the molecule on the stationary phase surface is dependent on the interaction energy. For different molecules with very small interaction energy difference the presence of significant surface is critical since the higher the number of phase transitions that analyte molecules undergo while moving through the chromatographic column, the higher the difference in their retention⁷.

The nature of the stationary and the mobile phases, together with the mode of the transport through the column, is the basis for the classification of Chromatographic methods¹⁰.

The four main types of HPLC techniques are

1. Normal-Phase Chromatography.
2. Reversed-Phase Chromatography.
3. Ion-Exchange Chromatography.
4. Size-Exclusion Chromatography.

Normal-Phase Chromatography:

The term "normal phase" is used to denote a chromatographic system in which a polar stationary phase is employed and a less polar mobile phase is used for elution of the analytes. In the normal-phase mode, neutral solutes in solution are separated on the basis of their polarity; the more polar the solute, the greater is its retention on the column. Since the mobile phase is less polar than the stationary phase, increasing the polarity of the mobile phase results in decreased solute retention. Normal-Phase chromatography is most commonly applied to the analysis of samples that are soluble in non-polar solvents, and it is particularly well suited to the separation of isomers and to class separations.

Although the separation mode has occasionally been misidentified as reversed phase, it is normal phase by virtue of the fact that increased aqueous levels of the mobile phase reduce carbohydrate retention, and elution order follows carbohydrate polarity. Normal-phase separations have occasionally been combined off-line with Reversed-phase chromatography to separate a wider range of species than could be accomplished by either technique alone. The feasibility of such a system, however, is contingent on the compatibility of the normal-phase eluent with that of the reversed-phase column⁸.

Reversed-Phase Chromatography:

As the name suggests, Reversed-Phase Chromatography is the reverse of Normal-Phase Chromatography in the sense that it involves the use of a non-polar stationary phase and a polar mobile phase. As a result, a decrease in the polarity of the mobile phase results in a decrease in solute retention. Modern Reversed-Phase Chromatography typically refers to the use of chemically bonded stationary phases, where a functional group is bonded to silica, for this reason, Reversed-Phase Chromatography is often referred to in the literature as Bonded-Phase Chromatography. Occasionally, however, polymeric stationary phases such as polymethacrylate or polystyrene, or solid stationary phases such as porous graphitic carbon, are used. Weak acids and weak bases, for which ionization can be suppressed, may be separated on reversed-phase columns by the technique known as ion suppression. In this technique a buffer of appropriate pH is added to the mobile

phase to render the analyte neutral or only partially charged. Acidic buffers such as acetic acid are used for the separation of weak acids, and alkaline buffers are used for the separation of weak bases. The analysis of strong acids or strong bases using reversed-phase columns is typically accomplished by the technique known as ion-pair chromatography (also commonly called paired-ion or ion-interaction chromatography). In this technique, the pH of the eluent is adjusted in order to encourage ionization of the sample; for acids pH 7.5 is used, and for bases pH 3.5 is common. Reversed-Phase Chromatography is the most popular mode for the separation of low molecular weight (<3000), neutral species that are soluble in water or other polar solvents. It is widely used in the pharmaceutical industry for separation of species such as steroids, vitamins, and β -blockers. Because of the mobile phase in Reversed-Phase Chromatography is polar, Reversed-Phase Chromatography is suited to the separation of polar molecules that either are insoluble in organic solvents or bind too strongly to the polar, normal-phase materials⁹.

Ion-Exchange Chromatography:

In Ion-Exchange Chromatography (IEC), species are separated on the basis of differences in electric charge. The primary mechanism of retention is the electrostatic attraction of ionic solutes in solution to "fixed ions" of opposite charge on the stationary phase support. The stationary phase or ion exchanger is classified as an anion-exchange material when the fixed ion carries a positive charge and as a cation exchanger when it carries a negative charge.

A specialized form of IEC is ion chromatography (IC), which is the name applied to the analysis of inorganic anions, cations, and low molecular weight, water-soluble organic acids and bases. Although any HPLC technique used to separate the above species can be termed ion chromatography, in general IC involves the use of ion-exchange columns and a conductivity detector. Ion chromatography itself can be sub classified. Suppressed IC involves the use of a membrane device, known as a suppressor, between the column and the detector to lower the response of the eluent and thereby enhance the signal from the solute; nonsuppressed or "single-column" IC does not contain a suppressor.

Size-Exclusion Chromatography:

Size-Exclusion Chromatography (SEC) is a convenient and highly predictable method for separating simple mixtures whose components are sufficiently different in molecular weight. For small molecules, a size difference of more than about 10% is required for acceptable resolution; for macromolecules a twofold difference in molecular weight is necessary¹⁵. Size-Exclusion Chromatography can be used to indicate the complexity of a sample mixture and to provide approximate molecular weight values for the components. It is an easy technique to understand, and SEC can be applied to the separation of delicate bio macromolecules as well as to the separation of synthetic organic polymers. Because SEC is a gentle technique, rarely resulting in loss of sample or reaction, it has become a popular choice for the separation of biologically active molecules. Each solute is retained as a relatively narrow band, which facilitates solute detection with detectors of only moderate sensitivity. One of the major applications of SEC is polymer characterization¹⁰.

1.4 INSTRUMENTATION:

The basic components of a High Performance Liquid Chromatographic system are shown in Fig.1. The instrument consists of

1. Mobile Phase Reservoir
2. A pump to move the eluent and sample through the system.
3. An injection device to allow sample introduction.
4. A Column(s) to provide solute separation.
5. A Detector to visualize the separated components.
6. A Data collection device to assist in interpretation and storage of results.

1. Mobile Phase Reservoir:

The most common type of solvent reservoir is a glass bottle. Most of the manufacturer's supply these bottles with special caps, Teflon tubing and filters to connect to the pump inlet and to the spurge gas (Helium) used to Remove Dissolved air. Filtration is needed to eliminate suspended Particles and organic impurities.

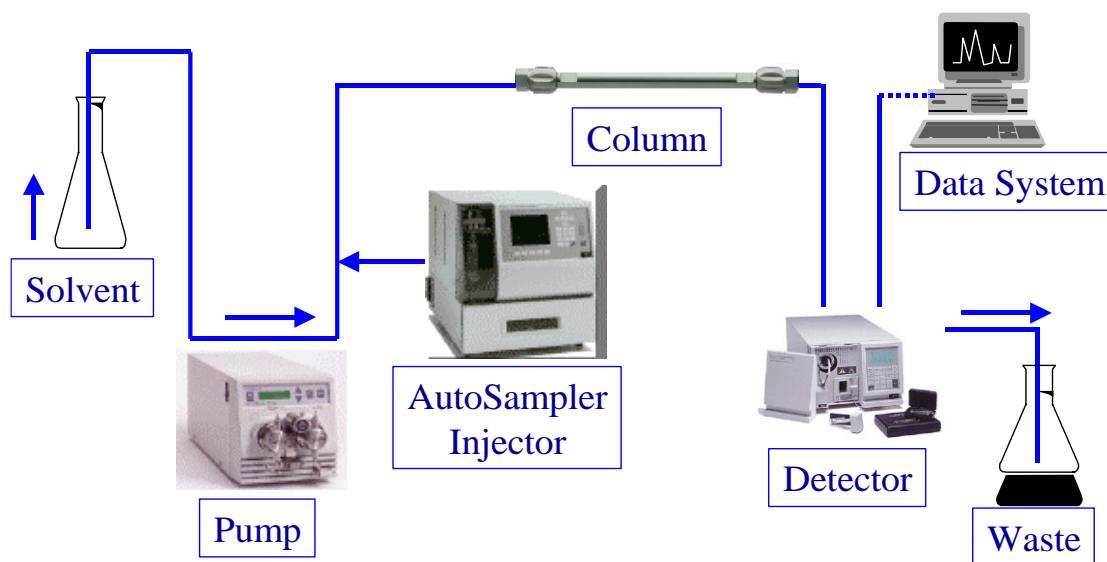


Fig. 1.5 Basic Components of HPLC System

Solvent System:

The mobile phases used in Reversed-Phase Chromatography are based on a polar solvent, typically water, to which a less polar solvent such as acetonitrile or methanol is added. Solvent selectivity is controlled by the nature of the added solvent in the same way as was described for Normal-Phase Chromatography. Solvents with large dipole moments, such as methylene chloride and 1, 2-dichloroethane interacts preferentially with solutes that have large dipole moments such as nitro-compounds, nitriles, amines, and sulfoxides. Solvents that are good proton donors such as chloroform, m-cresol, and water interact preferentially with basic solutes such as amines and sulfoxides and solvents that are good proton acceptors such as alcohols, ethers, and amines, tend to interact best with hydroxylated molecules such as acids and phenols. List of some useful solvents for use as mobile phases in Reversed-Phase Chromatography are listed below¹¹.

Table No 1.6 Mobile Phases in RP-HPLC

Solvent	Polarity/elution strength
Water	10.2
Dimethyl sulfoxide	7.2
Ethylene glycol	6.9
Acetonitrile	5.8
Methanol	5.1
Acetone	5.1
Dioxane	4.8
Ethanol	4.3
Tetrahydrofuran	4.0
2-Propanol	3.9

Solvent Degassing System:

The constituents of the mobile phase should be degassed and filtered before use. Several methods are employed to remove the dissolved gases in the mobile phase. They include heating and stirring, vacuum degassing with an aspirator, filtration through 0.45 filters, vacuum degassing with an air-soluble membrane, helium purging ultra sonification or purging or combination of these methods. HPLC systems are also provided an online degassing system, which continuously removes the dissolved gases from the mobile phase¹².

Gradient Elution Devices:

HPLC columns may be run isocratically, i.e., with constant eluent or they may be run in the gradient elution mode in which the mobile phase composition varies during run. Gradient elution over comes the problem of dealing with a complex mixture of solutes.

Stationary Phases:

In Liquid–Liquid Chromatography the stationary phase is a liquid film coated on a packing material consisting of 3–10 mm porous silica particles. The stationary phase may be partially soluble in the mobile phase, causing it to “bleed” from the column over time. To prevent this loss of stationary phase it is covalently bound to the silica particles. Bonded stationary phases are attached by reacting the silica particles with an organochlorosilane of the general form $\text{Si}(\text{CH}_3)_2\text{RCl}$, where R is an alkyl or substituted alkyl group. To prevent unwanted interactions between the solutes and any unreacted $-\text{SiOH}$ groups the silica frequently is “capped” by reacting it with $\text{Si}(\text{CH}_3)_3\text{Cl}$; such columns are designated as end-capped. The properties of a stationary phase are determined by the nature of the organosilane’s alkyl group. If R is a polar functional group then the stationary phase will be polar. Since the stationary phase is polar, the mobile phase is a nonpolar or moderately polar solvent. The combination of a polar stationary phase and a nonpolar mobile phase is called normal phase chromatography.

In reverse phase chromatography, which is the more commonly encountered form of HPLC, the stationary phase is nonpolar and the mobile phase is polar. The most common nonpolar stationary phases use an organochlorosilane for which the R group is an n-octyl (C_8) or n-octadecyl (C_{18}) hydrocarbon chain. Most reverse phase separations are carried out using a buffered aqueous solution as a polar mobile phase¹³.

Table 1.7 BONDED STATIONARY PHASES FOR HPLC

STATIONARY PHASE	FUNCTIONAL GROUP	APPLICATIONS
Silica	Si-OH	Normal phase material Pesticides, alkaloids
C18	Octadecyl	Reverse-phase material Fatty acids, PAH, Vitamins
C8	Octyl	Reverse-phase and ion pair, Peptides proteins
C6H5	Phenyl	Reverse-phase Polar aromatic fatty acids.
CN	Cyano	Normal and Reverse-phase, polar compounds
NO ₂	Nitro	Normal and Reverse-phase, PAH, Aromatic compounds
NH ₂	Amino	Normal, Reverse, weak ion exchange Carbohydrates, organic acids, chlorinated pesticides
OH	Diol	Normal, Reverse phase peptides, proteins.
SA	Sulphonic acid	Cation exchange, separation of cations.

2. PUMPS:

Pumps are used to flow mobile phase at high pressure and at controlled flow rates. The pumps must be capable of generating pressure of up to 5000 psi at flow rates up to 3ml/min for analytical purpose. Pumps used in preparative scale hplc may be required to pump at flow rates of upto 20ml/min.

Classification of pumps:

HPLC pump can be classified into the following groups according to the manner in which they operate:

- Constant flow rate pump (or) constant displacement pump
 - i) Reciprocating piston pump
 - ii) Syringe drive pump

- Constant pressure pump
 - i) Simple gas displacement pump
 - ii) Pneumatic amplifier pump

a) **Reciprocating pump**

Reciprocating pumps usually consist of a small chamber in which the solvent is pumped by the back and forth motion of a motor driven piston. Two check valves control the flow of solvent. Reciprocating pumps have a disadvantage of producing pulsed flow, which must be damped as its presence is manifested as base line noise on the chromatogram. Advantages of this pump include their small internal volume, high output pressure, ready adaptability to gradient elution, and independent of column backpressure and viscosity of solvent¹⁴.

b) **Displacement pump**

Displacement pumps usually consist of large syringe like chambers equipped with a plunger that is activated by a screw driven mechanism powered by stepping motor. Displacement pumps also produce a flow that tends to be independent of viscosity and backpressure. In addition, the output is pulse free. Disadvantages include limited solvent capacity (250 ml) and considerable inconvenience when solvents must be changed¹⁵.

c) **Pneumatic pumps**

In pneumatic pumps, the mobile phase is contained in a collapsible container housed in a vessel that can be pressurized by a compressor gas. Pumps of this kind are inexpensive and pulse free. They suffer from limited capacity, pressure output, dependence of flow rate on solvent viscosity and column backpressure. In addition, they are not amenable to gradient elution and are limited to pressures less than about 2000 psi¹⁶.

3. SAMPLE INJECTION SYSTEM:

Sample introduction can be accomplished in various ways. The simplest method is to use an injection valve. In more sophisticated LC systems, automatic sampling devices are incorporated where the sample is introduced with the help of auto samplers and microprocessors. In liquid chromatography, liquid samples may be injected directly and solid samples need only be dissolved in an appropriate solvent. The solvent need not be the mobile phase, but frequently it is judiciously chosen to avoid detector interference, column/component interference, loss inefficiency or all of these. It is always best to remove particles from the sample by filtering over a 5 μm filter, or centrifuging, since continuous injections of particulate material will eventually cause blockages in injection devices or columns. Sample sizes may vary widely¹⁷.

The availability of highly sensitive detectors frequently allows use of the small samples which yield the highest column performance.

Examples of injectors are shown in Fig 2 and Fig 3.

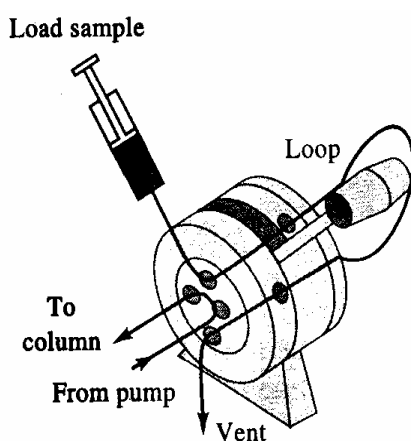


Fig 2.2: Load Sample

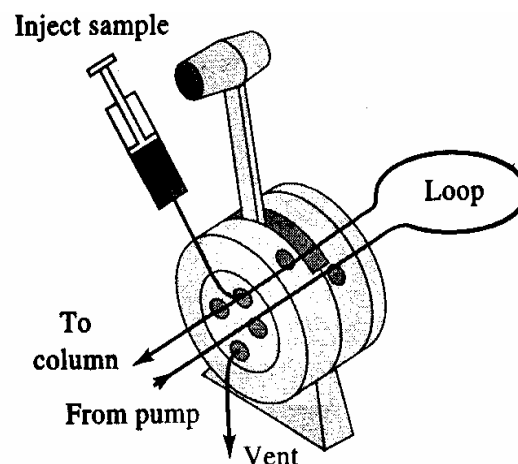


Fig 2.3 Inject Sample

4. COLUMNS:

The column is the heart of the chromatograph, providing the means for separating a mixture into components. The selectivity, capacity, and efficiency of the column are all affected by the nature of the packing material or the materials of construction¹⁸.

Requirements for an Ideal HPLC Column:

1. Particles should be spherical and available in particle diameters ranging from 3 to 10 μm .
2. Particles should withstand typical pressures encountered during HPLC ((900-3000 psi (6.1- 20.5 MPa) but ideally up to 4000 psi (27.2 MPa)) and should not swell or shrink with the nature of the eluent.
3. Particles should have porosity in the range 50-70%, extending to 80 % for Size-Exclusion Chromatography.
4. Particles should contain no pores smaller than $\sim 60 \text{ \AA}$ in diameter and should have a uniform pore size distribution.
5. Particles should be available with a range of mean pore diameters of 60-1000 \AA .
6. The internal surface of the material should be homogeneous.
7. The internal surface should be capable of modification to provide a range of surface functionalities.
8. Packing materials should be chemically inert under all conditions of pH and eluent composition.
9. The physico-chemical characteristics of the material should be reproducible from batch to batch and from manufacturer to manufacturer.
10. The material should be readily available and relatively inexpensive, and its chemical behavior should be well understood.

There are four different columns available

A. Guard Columns:

These columns are placed anterior to the separating column. This serves as a protective factor that prolongs the life and usefulness of the separation column. They are dependable columns designed to filter or remove.

- 1) Particles that clog the separation column.
- 2) Compounds and ions that could ultimately cause "baseline drift", decreased resolution, decreased sensitivity, and create false peaks.
- 3) Compounds that may cause precipitation upon contact with the stationary or mobile phase.

B. Derivatizing Columns:

Pre- or post-primary column derivatization can be an important aspect of the sample analysis. Reducing or altering the parent compound to a chemically related daughter molecule or fragment elicits potentially tangible data which may complement other results or prior analysis¹⁹.

C. Capillary Columns:

Advances in HPLC led to smaller analytical columns. Also known as micro columns, capillary columns have a diameter much less than a millimeter and there are three types: open-tubular, partially packed, and tightly packed. They allow the user to work with nanoliter sample volumes, decreased flow rate, and decreased solvent volume usage which may lead to cost effectiveness.

D. Fast Columns:

One of the primary reasons for using these columns is to obtain improved sample throughput (amount of compound per unit time). For many columns, increasing the flow or migration rate through the stationary phase will adversely affect the resolution and separation. Therefore, fast columns are designed to decrease time of the chromatographic analysis without forsaking significant deviations in results²⁰.

E. Preparatory Columns:

These columns are utilized when the objective is to prepare bulk (milligrams) of sample for laboratory preparatory applications. A preparatory column usually has a large column diameter which is designed to facilitate large volume injections into the HPLC system.

Types Of Column Packing:

- Pellicular
- Porous particle

Pellicular:

The former consist of spherical, non porous, glass or polymer beads with typical diameter 30 to 40 micrometer.

Porous particle:

The particles are composed of silica, alumina, and synthetic resin polystyrene divinyl benzene or ion exchange resin.

5. DETECTORS:

The detector converts a change in the column effluent into an electrical signal that is recorded by the data system. There are different types of detectors used in HPLC. Liquid chromatographic detectors are of two basic types.

Bulk Property detectors respond to a mobile-phase bulk property, such as refractive index, dielectric constant, or density. In contrast, solute property detectors respond to some property of solutes, such as UV absorbance, fluorescence, or diffusion current, that is not possessed by the mobile phase.

A) Refractive Index Detector: The detection principle involves measuring of the change in refractive index of the column effluent passing through the flow-cell. The greater the RI difference between sample and mobile phase, the larger the imbalance will become. Thus, the sensitivity will be higher for the higher difference in RI between sample and mobile phase. On the other hand, in complex mixtures, sample components may cover a wide range of refractive index values and some may closely match that of the mobile phase, becoming invisible to the detector²¹.

B) UV Detector: In these systems detection depends on absorption of UV ray energy by the sample. They are capable to detect very wide range of compounds. The sensitivity ranges till microgram quantity of estimation.

C) PDA Detector: These are detectors which follow principle similar to UV detectors but the only advantages are higher sensitivity and measure the entire absorption range i.e. It gives scan of entire spectrum.

D) Evaporative Light Scattering Detector (ELSD): In the ELSD, the mobile phase enters the detector is evaporated in a heated device and the remaining solute is finally detected by the way it scatters light. The intensity of the light scattered from solid suspended particles depends on their particle size. Therefore, the response is dependent on the solute particle size produced. This, in turn, depends on the size of droplets generated by the nebulizer and the concentration of solute in the droplets. The droplet size produced in the instrument nebulizer depends on the physical properties of the liquid and the relative velocity and flow-rates of the gas and liquid stream. The importance of all these parameters emphasizes the need for careful design and rigorous optimization of the instrument parts²².

E) Electro Chemical Detector: This detector is specially suitable to estimate oxidisable & reducible compounds. The principle is that when compound is either oxidized or reduced, the chemical reaction produces electron flow. This flow is measured as current which is the function of type and quantity of compound

F) Conductivity Detector: conductivity detector measures the conductivity of the mobile phase. There is usually background conductivity which must be backed-off by suitable electronic adjustments. If the mobile phase contains buffers, the detector gives a base signal that completely overwhelms that from any solute usually making detection impossible. Thus the electrical conductivity detector is a bulk property detector. And senses all ions whether they are from a solute or from the mobile phase.

G) Fluorescence Detectors: Fluorescence detectors are probably the most sensitive among the existing modern HPLC detectors. It is possible to detect even a presence of a single analyte molecule in the flow cell. Typically, fluorescence sensitivity is

10 -1000 times higher than that of the UV detector for strong UV absorbing materials. Fluorescence detectors are very specific and selective among the others optical detectors. This is normally used as an advantage in the measurement of specific fluorescent species in samples²³.

H) Mass Spectrometric Detection: The use of mass spectrometer for hplc detection is becoming common place, despite the high cost of such detector and need for a skilled operator. A mass spectrometer can facilitate hplc method development and avoid common problem by

- Tracking and identifying individual peaks in the chromatogram between experiments
- Distinguishing compounds of interest from minor compounds or interferences.
- Recognizing unexpected and overlapping interference peaks to avoid a premature finish to method development.

Temperature:

Room temperature is the first choice. Elevated temperatures are sometimes used to reduce column pressure are enhancing selectivity. Typically, temperatures in excess of 60⁰ C are not used²⁴.

Retention Time:

Due to a number of samples assayed in the course of preformulation study, it is advisable to have as short a retention time as far as possible. However, the retention time should be long enough to ensure selectivity. While choosing the optimum mobile phase, considerations should be given to the retention time of degradation products. So that these compounds do not elute in the solvent front and remain in the column.



Fig 1.7.1 Block Diagram Of HPLC

1.8 APPLICATIONS OF HPLC:

1.0 Preparative HPLC refers to the process of isolation and purification of compounds. This differs from analytical HPLC, where the focus is to obtain includes identifications, quantification, and resolution of a compound.

- 1. Chemical separations** can be accomplished using HPLC by utilizing the fact that certain compounds have different migration rates given a particular column and mobile phase. Thus the chromatography can separate compounds from each other using HPLC; the extent or degree of separation is mostly determined by the choice of stationary phase and mobile phase²⁵.
- 2. Purification refers** to the process of separating or extracting the target compound from other (possibly structurally related) compounds or contaminants. Each compound should have a characteristic peak under certain chromatographic condition. The migration of the compounds and contaminants through the column need to differ enough so that the pure desired compound can be collected or extracted without incurring any other undesired compound.
- 3. Identification of the compounds by HPLC** is a crucial part of any HPLC assay. The parameters of this assay should be such that a clean peak of the known sample is observed from the chromatograph. The identifying peak

should have a reasonable retention time and should be well separated from extraneous peaks at the detection levels, in which the assay would be performed.

4. **Quantification of compounds by HPLC** is the process of determining the unknown concentration of a compound in a solution. It involves injecting a series of known concentration of the standard compound solution onto the HPLC for detection.
5. The chromatograph of these known concentrations will give a series of peaks that correlate to the concentration of the compound injected²⁶.

ADVANTAGES:

HPLC separations can be accomplished in a matter of minutes, in some cases, even in seconds. High resolution of complex sample mixture into individual components can be obtained.

- Rapid growth of HPLC is also because of its ability to analyse substances that are unsuitable for gas liquid chromatographic (GLC) analysis due to non-volatility or thermal-instability.
- Quantitative analysis is easily and accurately performed and errors of less than 1 % are common to most HPLC methods.
- Depending on sample type and detector used it is frequently possible to measure 10^{-9} g or 1 ng of sample. With special detectors, analysis down to 10^{-12} g has been reported²⁷.

DISADVANTAGES:

- HPLC instrumentation is expensive and represents a major investment for many laboratories.
- It requires a proficient operator to handle the instrument.
- HPLC cannot handle gas samples.

- HPLC is poor identifier. It provides superior resolution but it does not provide the information that identifies each peak.
- Sample preparation is often required.
- Only one sample can be analyzed at a time.
- Finally there is at present time no universal and sensitive detector.

1.9 GUIDELINES FOR ANALYTICAL METHOD VALIDATION:

For pharmaceutical method guidelines are prescribed by

- United States Pharmacopoeia (USP)
- Food and Drug Administration (FDA)
- World Health Organization (WHO)
- International Conference on Harmonization (ICH)

These Guidelines provide a framework for performing validation. In general, methods for routine analysis, standardization or regulatory submission must include studies on specificity, linearity, accuracy, precision, range detection limit, quantitations limit and robustness²⁸.

United States Pharmacopoeia (USP) :

USP defines analytical method validation as “The process by which it is established by laboratory studies that performance characteristics of method meet the requirement for intended analytical application”

Food and drug Administration (FDA):

FDA defines validation as “Establishing documented evidence, which provides a high degree of assurance that a specific process will consistently produce meeting its pre- determined specification and quality attributes”.

World Health Organization (WHO) :

WHO defines validation as “Process of providing documented evidences that a system /procedure does what it is supposed to do precisely and reliably”.

Objective of Validation:

The primary objective of validation is to form a basis for written procedures for production and process control which are designed to assure that the drug products have the identity, strength, quality and purity they purport or are represented to possess. Quality, safety and efficacy must be designed and built into the products. Each step of the manufacturing process must be controlled to maximize the probability that the finished product meets all quality and design specifications²⁹.

Types of Validation:

Prospective Validation: This is performed for all new equipments, products and processes. It is a proactive approach of documenting the design, specifications and performance before the system is operational. This is the most defensible type of validation.

Concurrent Validation: This is performed in two instances, i.e., for existing Equipment, verification of proper installation along with specific Operational tests is done. In case of an existing, infrequently made Product, data is gathered from at least three successful trials³⁰.

Retrospective Validation:

This is establishing documented evidence that the Process is performed satisfactorily and consistently over time, based on review and analysis of historical data. The source of such data is production and QA/QC records. The issues to be addressed here are changes to equipment, process, specifications and other relevant changes in the past.

ANALYTICAL METHOD VALIDATION:

Analytical monitoring of a pharmaceutical product or of specific ingredients within the product is necessary to ensure its safety efficacy throughout all phases of its shelf life. Such monitoring is in accordance with the specifications elaborated during product development. Analytical validation is the corner stone of process validation without a proven measurement system it is impossible to confirm whether the manufacturing process has done what it purports to do. All new methods developed are validated.

Steps followed for validation procedures

1. Proposed protocols or parameters for validations are established.
2. Experimental studies are conducted.
3. Analytical results are evaluated
4. Statistical evaluation is carried out.
5. Report is prepared documenting all the results.

Objective:

The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose. According to ICH, typical analytical performance characteristics that should be considered in the validation of the types of methods are:

- Accuracy
- Precision
- Specificity
- Detection limit
- Quantitation limit

- Linearity and Range
- Ruggedness
- Robustness
- System suitability

International conference on Harmonization (ICH) :

ICH is tripartite agreement between European community, USA and Japan. Its purpose is to provide a forum for constructive dialogue between regulatory authorities and Pharmacy industry on real and perceived differences in technical requirements for product registration in European community USA and Japan³¹.

Objective is lying down of minimum standards applicable uniformly, irrespective of where the product is manufactured or marketed in the three regions.

The ICH documents give guidance on the necessity for revalidation in the following circumstances.

- Changes in the synthesis of the drug substances
- Changes in the composition of the drug product and
- Changes in the analytical procedures

Although there is general agreement about what type of studies should be done, there is great diversity in how they are performed. The literature contains diverse approaches to performing validations. This approach should be viewed with the understanding that validation requirements are continually changing and vary widely, depending on the type of drug being tested the stage of drug development and the regulatory group that will review the drugs application. For our purposes, we will discuss validation studies as they apply to Chromatography method, although the same principles apply to other analytical technique³².

The process of validating a method cannot be separated from the actual development of the method conditions, because the developer will not know whether the method conditions are acceptable until validation studies are performed.

The development and validation of a new analytical method may therefore be an iterative process. Results of validation studies may indicate that a change in the procedure is necessary, which may then require revalidation. During each validation study, key method parameters are determined and the n used for all subsequent validation steps. To minimize repetitious studies and ensure that the validation data are generated under conditions equivalent to the final procedure, we recommend the following sequence of studies.

1.10 METHOD VALIDATION PARAMETERS:

They have been defined in different working groups of national and international committees and are described in literature. The parameters as defined by the ICH and by other organization and authors are summarized below. They are

A.SPECIFICITY / SELECTIVITY:

Specificity, which can be defined as the ability to measure accurately the concentration of analyte in the presence of all other sample materials. If specificity is not assured, method accuracy, precision and linearity all are seriously compromised. Assuring specificity is the first step in developing and validating good method. The determination of method specificity can be achieved in two ways, first most desirable all potential interfering compounds can be tested to demonstrate their separation from the peaks of interest with a specified Resolution Second method for achieving specificity is the use of selective detector, especially for co-eluting compounds, for e.g. a selective detector (e.g. electrochemical, radioactive will respond to some compounds but not others Specificity of a developed method often is difficult to ensure. However, there are a number of techniques that can be used in method validation experiments that will increase confidence in specificity³³

1. Spiking of known interferants.
2. Sample degradation studies.
3. Peak collection with subsequent analysis by other techniques.
4. Use of another chromatographic method.

5. Changing the conditions of the HPLC method (alternative solvents or different gradient slopes).

B) PRECISION:

Precision can be defined as “the degree of agreement among the individual test results when the Procedure is applied repeatedly to multiple samplings of homogenous sample

ICH divides Precision into three types

1. Repeatability.
2. Intermediate precision
3. Reproducibility

Repeatability

Repeatability is the precision of a method under same operating conditions over a short period of time. This is measured by the sequential repetitive injections of the same homogenous sample (typically, 10 or more times), followed by averaging of the peak height (or) peak area values and determination of relative standard deviation of all injections.

Intermediate precision:

Is the agreement of complete measurements (including standards) when the same method is applied many times within the same laboratory. This can include full analysis on different days, Instruments or analysts, but would involve multiple preparations of samples and standards³⁴.

Reproducibility:

Examines the Precision between laboratories and is often determined in collaborative studies or method transfer experiments. Precision often is expressed by the S.D and RSD data set.

C) ACCURACY:

The accuracy of a measurement is defined as the closeness of measured value to the true value. In a method with a high accuracy, a sample (“Whose true value” is known) is analyzed and the measured value should ideally be identical to the true value. Typically accuracy is represented and determined by recovery studies but there are three ways to determine accuracy

1. Comparison to a reference standard
2. Recovery of analyte spiked into blank matrix.
3. Standard addition of analyte.

Comparison to Reference Standard:

Determining accuracy by direct comparison to a reference standard (a standard reference material is the preferred technique for an analyte.(e.g.; Purified drug substance)that is not in a complex sample matrix. if the analyte is widely assayed, a certified standard may be obtained from an external source as the national institute for standards and technology (NIST).

Accuracy determination for an hplc method should be carried out with a minimum of nine measurements using at least three concentrations. (Include separate weighing plus preparation for each sample).This approach minimizes any variability and or bias in sample preparation technique and analysis or one sample at only one concentration. An example would be three replicate measurements each of three replicate measurements each of three different concentration preparations. All nine values are averaged and used for the final accuracy determination³⁵.

Analyte recovery:

It can be determined by analyte reference standard is added to a blank matrix (sometimes called a placebo) at various levels the blank matrix could take many forms. For e.g. in an analysis of a drug formulation it would include all formulation ingredients except analyte to be measured.

The recovery at each level is determined by comparison to the known amount added. For major component assay, spiked levels typically should be at 50%, 75%, 100%, 125% and 150% of the level is expected for the analyte in a normal assay. A minimum of three replicate measurements should be performed at each level.

Method of standard addition:

In this method, known amounts of an analyte are spiked at different levels into a sample matrix that already contains some (unknown) quantity of the analyte. The concentration of analyte in the original sample may then be determined mathematically. In general, for standard addition of, a good approach is to add 25, 50 and 100% of the expected analyte concentration to the matrix in different experiments. The unspiked sample and each of the spiked samples should be analyzed (usually in triplicate) and the measured amounts reported vs. the amount added. This method is used when it is difficult or impossible to prepare blank matrix without analyte. An example would be the analysis of insulin in a normal blood sample, where background levels of insulin always are present³⁶.

D) LINEARITY:

The linearity of a method is a measure of how well a calibration plot of response Vs concentration approximates a straight line. Linearity can be assessed by performing single measurements at several analyte concentrations. The data are then processed through linear least square regression. The resulting plot on slope, intercept and correlation coefficient gives the desired information on linearity. A linearity correlation coefficient above 0.999 is acceptable for most methods, especially for a major component in assay methods, methods with linearity poorer than this may have to be treated as non-linear and use more complicated multipoint calibrations or non linear response modeling³⁷.

The least squares method of determining linearity can have serious shortcomings if response must be measured over one or more orders of magnitude. Here the slope, intercept, and correlation coefficient can be unduly influenced by data at low or high concentrations. Small changes in the calculated value of either the slope or

intercept can lead to errors in estimating the true value for a sample, Therefore a better method of assessing linearity is desired.

A generally superior method for determining method linearity over wide concentration ranges. This approach involves determining the response factor at each measured concentration and plotting this response factor vs. analyte concentration.

$$R_F = D_R / C$$

Where D_R is the detector response and C is the concentration of the analyte. Ideally the response factor should be independent of concentration if the method is truly “linear” the response factor is independent of concentration for ranges of 1.2 to 10.0 $\mu\text{g/ml}$. At lower concentration this relationship deviates, and the assumed linearity no longer holds.

E) RANGE:

The range of a method can be defined as the lower and upper concentrations for which the analytical method has adequate accuracy, precision, and linearity, while a desired concentration on range is often known before starting the validation of a method, the actual working range results from data generated during validation studies. The range of concentration examination will depend on the type of method and its use. For a major component assay, the concentration range should encompass values expected in samples to be measured. A good strategy is to perform at 50%, 75%, 100%, and 125% and 150% of target levels. This range also has potential to demonstrate that the method is linear outside the limits of expected use. (Typically 90 to 110%)

Major component assays of pharmaceuticals often are used to measure content uniformity for a dosage unit. The USP definition of content uniformity allows a single value to deviate from the target by as much as $\pm 25\%$ even if the assay is expected to fall within ± 5 to 10% of the target value. Furthermore, drug stability data (especially those in accelerated studies) can generate values outside the anticipated specification range. This requires that the validation extend well beyond

the expected specification level or Target values for the assay of unstressed product .In case where the sample concentration is above the calibration range, dilution of the sample to the appropriate concentration is recommended³⁸.

Methods for the determining impurities, degradants and other related substances can generate concentrations that vary over several orders of magnitude, depending on method sensitivity. A recommended range to be examined in validation studies in Pharmaceutical and related samples should start at the limit of quantitation and extend up to at least 5% of the concentration of the major component. Measurements beyond this range typically are not needed since related substances are rarely tolerated at higher levels in a raw materials or finished product .For application to other types of sample, this recommended range may need to be adjusted: however, the key point is to validate the expected range of all potential samples³⁹.

F) LIMIT OF DETECTION (LOD):

LOD is defines as lowest concentration of analyte that can be detected, but not necessarily quantified, by the analytical method. The limit of detection is the point at which a measured value is larger than the uncertainty associated with it. In chromatography the detection limit is the injected amount that results in a peak with a height at least twice or three times as high as the baseline noise level. Usually expressed as concentration of analyte generating an instrument response and is equivalent to three times the noise (S / N ratio~3).

Based on S / N ratio:

This approach can be applied to analytical procedures that exhibit baseline noise. Determination of the S / N ratio is performed by comparing measured signals from samples and establishing the minimum concentration at which the analyte can be reliably detected. The S / N ratio between 3 or 2:1 is generally considered acceptable for estimating the detection limit.

G) LIMIT OF QUANTIFICATION (LOQ):

LOQ is defined as the lowest concentration of analyte that can be determined with acceptable accuracy and precision by the analytical method. Usually expressed as concentration of analyte generating an instrument response and is equivalent to ten times the noise (S / N ratio~10). Several approaches for determining the detection limit are possible, depending on whether procedure is non-instrumental or instrumental

Based on Signal-to-Noise ratio:

This approach can be applied to analytical procedures that exhibit baseline noise. Determination of the signal-to-noise ratio is performed by comparing measured signals from with known low concentration of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected. A signal-to-noise ratio between 3 or 2:1 is generally considered acceptable for estimating the detection limit⁴⁰.

The LOD and LOQ values determined during method validation are affected by the separation conditions : columns, reagents, and especially instrumentation and data systems .Instrumental changes .Particularly pumping systems and detectors ,or the use of contaminated reagents can results in large changes in S/N ratio .

H) ROBUSTNESS:

It can be defined as measure of its capacity to remain unaffected by small but deliberate variations in method parameters. Robustness tests examine the effect operational parameters have on the analysis results. Factors internal to the method: mobile phase pH, mobile phase composition, temperature, flow rate, injector / detector temperatures etc. the robustness of a method is its ability to remain unaffected by the small changes in the parameters such as percent organic content and pH of the mobile phase, buffer concentration, temperature, flow rate and injection volume. These method parameters may be evaluated one factor at a time or simultaneously as part of factorial experiment. Obtaining data on the effects of these

parameters may allow a range of acceptable values to be included in the final method procedure⁴¹.

Attention to the foregoing considerations will significantly improve the quality of the final method. The one exception, however, is the column. There is the possibility that a column from a different manufacturing lot will not give reproducible retention of all sample components, possibly resulting in an unacceptable separation. For this reason it is important to evaluate columns from at least three different columns can be obtained. If significant lot to lot variations in sample retention are observed, appropriate steps should be taken to avoid future problems. One approach is to stockpile enough columns from a good batch for all future uses of the method. Another approach is to determine whether small changes in condition (%B, temperature-pH, etc) can be used to minimize or correct any undesirable changes in retention from lot to lot.

SYSTEM SUITABILITY PARAMETERS

Prior to the analysis of samples each day, the operator must establish that the HPLC system and procedure are capable of providing data of acceptable quality. This is accomplished with system suitability experiments and can be defined as tests to ensure that the method can generate results of acceptable accuracy and precision. The requirements for system suitability are usually developed after method development and validation have been completed. The criteria selected will be based on the actual performance of the method as determined during its validation. For example, if sample retention times form part of the system suitability criteria, their validation can be determined. System suitability might then require that retention times fall within ± 3 SD range during routine performance of the method⁴².

The USP defines parameters that can be used to determine system suitability prior to analysis. These parameters include plate number (N), Tailing factor k and Resolution (Rs) and Relative Standard Deviation (RSD) of Height or peak area for repetitive injections. Typically, at least two of these criteria are required to demonstrate system suitability for any method. The RSD of Peak height or area of five repetitive injections of a standard solution is normally accepted as one of the standard criteria. For an assay method of a major component, the RSD should

typically be less than 1% for these repetitive injections. For the measurements of a compound at trace levels, such as an impurity standard run at or near the limit of quantitation, a higher RSD (5 to 15%) is acceptable⁴³.

Commonly used system suitability parameters are as follows: -

Retention Time (R_T):

Retention time is the time of elution of peak maximum after injection of compound.

Theoretical Plates (N):

It is also called as column efficiency. A column can be considered as being made of large number of theoretical plates where distribution of sample between liquid-liquid / solid-liquid phase occurs. The number of theoretical plates in column is given by relationship⁴⁴.

$$N = 16 (t_R / w)^2$$

Where ' t_R ' is the retention time and ' w ' is the width at the base of peak. Theoretical Plates should be more than 2000

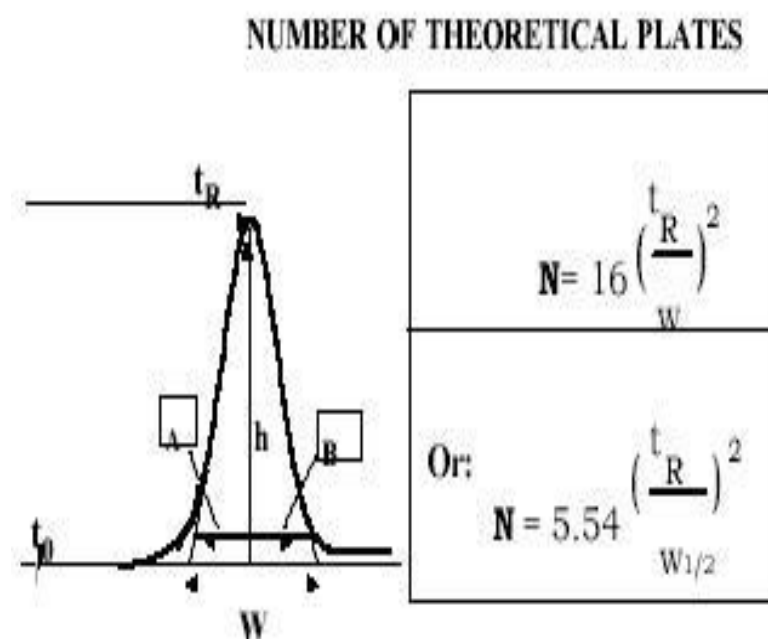


Fig2. 5-Theoretical Plates

How many peaks can be located per unit run-time of the chromatogram, where t_R is the retention time for the sample peak and W is the peak width?

N is fairly constant for each peak on a chromatogram with a fixed set of operating conditions. H , or HETP, the height equivalent of a theoretical plate, measures the column efficiency per unit length (L) of the column. Parameters which can affect N or H include Peak position, particle size in column, flow-rate of mobile phase, column temperature, viscosity of mobile phase, and molecular weight of the Analyte⁴⁵.

The theoretical plate number depends on elution time but in general should be > 2000 .

Resolution (R):

It is a function of column efficiency and is specified to ensure that closely eluting compounds are resolved from each other to establish the general resolving power of the system. For the separation of the two components in mixture the resolution is determined by equation.

$$R = 2 (t_2 - t_1) / (w_2 + w_1)$$

Where t_2 and t_1 are the retention time of second and first compounds respectively, where as W_1 and W_2 are the corresponding widths at the bases of peak obtained by extrapolating straight sides of the peaks to baselines. R' should be more than 2 between peak of interest and the closest eluted peak for potential interferences⁴⁶ (impurities, Excipients, degradation products or internal standard)

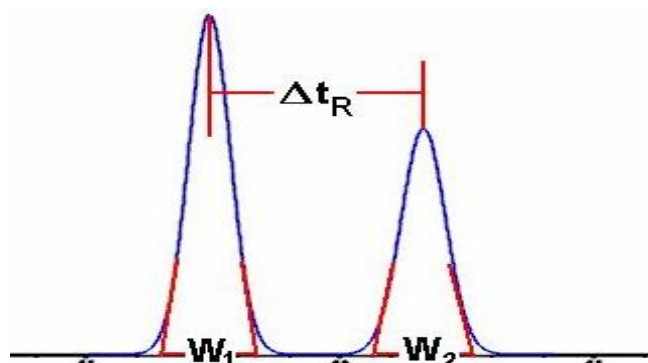


Fig.2.6 Resolution

For reliable Quantitation, well-separated peaks are essential for Quantitation.

Recommendations:

R of > 2 between the peak of interest and the closest potential interfering peak (impurity, excipient, degradation product, internal standard, etc.) are desirable.

Tailing Factor (T):

It is a measure of peak symmetry, and is unity for perfectly symmetrical peaks and its value increases as tailing become more pronounced.

$$T = W_{0.05} / 2F$$

Where $W_{0.05}$ is the width of peak at 5% height and 'F' is the distance from the peak maximum to the leading edge of the peak height forms the baseline.

Tailing factor should be less than 2

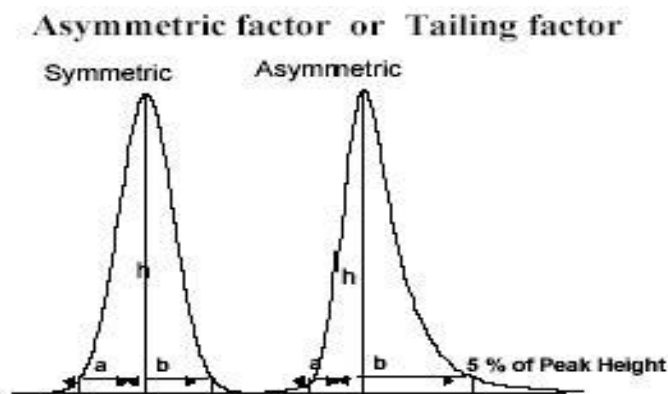


Fig.2.7- Tailing Factor

The accuracy of Quantitation decreases with increase in peak tailing because of the difficulties encountered by the integrator in determining where/when the peak ends and hence the calculation of the area under the peak. Integrator variables are preset by the analyst for optimum calculation of the area for the peak of interest⁴⁷.

Recommendations T of ≤ 2

Capacity Factor (k'):

The capacity factor is a measure of the degree of retention of an analyte relative to an unrestrained peak, where t_R is the retention time for the sample peak and t_0 be the retention time for an unrestrained peak⁴⁸.

RETENTION FACTOR or CAPACITY RATIO	
$k' = \frac{t_R - t_0}{t_0}$	$k' = \phi \frac{C_s}{C_m}$

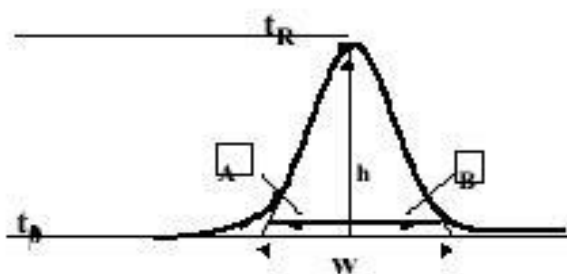


Fig.2.8- Capacity factor

$$k' = (t_R - t_0) / t_0$$

Recommendations:

The peak should be well-resolved from other peaks and the void volume. Generally the value of k' is > 2 .

Precision / Injection repeatability (RSD) of $< 1\%$ for ' n ' > 5 is desirable.

Selectivity (α), Separation factor:

It is a measure of peak spacing and expressed as,

$$\alpha = (k'_2 / k'_1).$$

Table 1.11 System Suitability Parameters and Recommendations

Parameter	Recommendation
Capacity Factor (k')	The peak should be well-resolved from other peaks and the void volume, generally k' > 2.0.
Repeatability	RSD ≤ 1% for N ≥ 5 is desirable.
Relative retention	Not essential as long as the resolution is stated.
Resolution (R _s)	R _s of >2 between the peak of interest and the closest eluting potential interferent (impurity, excipient, degradation product, internal standard, etc.)
Tailing factor (T)	T of ≤ 2
Theoretical plates (N)	N ≤ 2000

1.12 STATISTICAL ANALYSIS:

The consistency and suitability of the developed method are substantiated through the statistical analysis like standard deviation, relative standard deviation and theoretical plates per meter⁴⁹.

For Accuracy:

$$\text{Standard deviation} = \sigma = \sqrt{\frac{\sum (x - x_i)^2}{n - 1}}$$

Where, \bar{x} = sample.

x_i = mean value of samples.

n = number of samples.

Relative Standard Deviation = $\sigma/\bar{x} \times 100$

Molar extinction coefficient ($\text{mol}^{-1} \text{cm}^{-1}$) = $A/C \times L$

Where, A = Absorbance of drug.

C = concentration of drug.

L = Path length.

Sandell's sensitivity ($\mu\text{g} / \text{cm}^2 / 0.001$ absorbance units) = $C/A \times 0.001$

Where, C = concentration of drug

A = Absorbance of drug

Unit = $\mu\text{g} / \text{cm}^2 = 0.001$ absorbance)

Coefficient of variance (σ) :

Coefficient of variance = $\sum(x - \bar{x})^2 / n - 1$

Régressions éqution, $y = a + bx$

Slope = y/x

Where, x = Concentration

y = Absorbance

a = Intercept

Limit of detection (D_L):

Limit of Detection = $3.3 \times \sigma \backslash S$

Units - ($\mu\text{g} / \text{ml}$)

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.

The estimation of σ may be carried out in a variety of ways.

Limit of quantitation (Q_L):

Limit of Quantitation = $10 \times \sigma \backslash S$

Unit- ($\mu\text{g} / \text{ml}$)

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.

The estimation of σ may be carried out in a variety of way.

2. LITERATURE REVIEW

1. **P. N. S Pai et., al.**⁴⁶ reported a precise method in which Tinidazole and diloxanide furoate have been simultaneously determined by spectrometric methods. For the proposed method all the chemicals of analytical reagent grade, solvents of HPLC grade and distilled water (Millipore) were used. The LC system consisted of LC-10AT pump (Shimadzu), SS Wakosil-II C-18, 250×4.6 mm, 5 µm column, Rheodyne injector equipped with a 100 µl sample loop and UV detector (Shimadzu SPD-10A VP) set at 282 nm. The output signal was monitored and integrated using CZ-RA software (Shimadzu). The standard solution of diloxanide furoate 500 µg/ml and tinidazole 500 µg/ml were prepared separately by dilution of tinidazole and diloxanide furoate respectively in mobile phase of acetonitrile, methanol and 0.2M potassium dihydrogen phosphate pH 5.0 in the ratio 2:3:2. The retention time for tinidazole and diloxanide furoate at a flow rate of 1ml/min were recorded as 3.4 and 5.2 min respectively. From the respective peak areas obtained in standard and sample chromatogram.
2. **P. Parinoo et., al**⁴⁷ A differential spectrophotometric procedure has been developed for the simultaneous determination of Tinidazole (TD) and Diloxanide furoate (DF) in tablet preparations. The method comprised the measurement of absorbance of a solution of the tablet extract in pH 2.0 buffer solution relative to that of an equimolar solution in pH 13.0 buffer at the wavelengths of 282nm and 240nm. The presence of identical isoabestic points for pure drug samples and tablet extract solutions indicated the non-interference of excipients in the absorption at these wavelengths. The compliance of Beer's Law was obtained in the concentration range of 20–40µg/ml for TD and DF at these wavelengths.
3. **Chiranjeevi bodepudi et.,al.**⁴⁸ was reported a precise and feasible high-performance liquid chromatographic (HPLC) method for the analysis of the Fluconazole and Tinidazole in a combined tablet dosage form has been developed. The analysis was carried out on a Kromasil stainless steel C18 (250 x 4.6 mm, 5 µ) reversed-phase column, using a mixture of Acetonitrile: Water (55:45% v/v) as the mobile phase using a low pressure gradient mode with flow

rate at 1ml/min. The injection volume was 20 μ l..The retention time of the drug was 2.5 for Fluconazole and 3.1 for Tinidazole. The method produced linear responses in the concentration range of 10 to 50 μ g/ml for both Fluconazole and Tinidazole. The Tailing factors of Fluconazole and Tinidazole were found to be 1 and 1.3 respectively. The method was found to be applicable for determination of the drug in tablets.

4. **Nada Sayed Abdelwahab et.,al** ⁴⁹ Was reported a work which is concerned with development and validation of chromatographic and spectrophotometric methods for analysis of Mebeverine HCl (MEH), Diloxanide furoate (DF) and Metronidazole (MET) in tablets spectrophotometric and RP-HPLC methods using UV detection. The developed spectrophotometric methods depend on determination of MEH and DF in the combined dosage form using the successive derivative ratio spectra method which depends on derivatization of the obtained ratio spectra in two steps using methanol as a solvent and measuring MEH at 226.4-232.2 nm (peak to peak) and DF at 260.6-264.8 nm (peak to peak). While MET concentrations were determined using first derivative (1D) at $\lambda = 327$ nm using the same solvent. The chromatographic method depends on HPLC separation on ODS column and elution with a mobile phase consisting water: methanol: triethylamine (25: 75: 0.5, by volume, orthophosphoric acid to pH =4). Pumping the mobile phase at 0.7 ml min⁻¹ with UV at 230 nm. Factors affecting the developed methods were studied and optimized, moreover, they have been validated as per ICH guideline and the results demonstrated that the suggested methods are reproducible, reliable and can be applied for routine use with short time of analysis. Statistical analysis of the two developed methods with each other using F and student's-t tests showed no significant difference
5. **Divya Patel et., al** ⁵⁶ was developed precise and accurate Stability indicating RP-HPLC method for simultaneous estimation of Diloxanide Furoate and Ornidazole in Their Combined Dosage Form has been developed. The separation was achieved by LC- 20 AT C18 (250mm x 4.6 mm x 2.6 μ m) column and Buffer (pH 4.5): Acetonitrile (40:60) as mobile phase, at a flow rate of 1 ml/min. Detection was carried out at 277 nm. Retention time of Ornidazole and Diloxanide Furoate were found to be 4.620 min and 7.633 min, respectively. The

method has been validated for linearity, accuracy and precision. Linearity observed for Ornidazole 5-15 µg/ml and for Diloxanide Furoate 7.5-22.5 µg/ml. The percentage recoveries obtained for Ornidazole and Diloxanide Furoate were found to be in range of 100.88 ± 0.60 and 100.85 ± 0.20 respectively.

6. **Hiradeve S.M et.,al**⁵⁷ had did a reverse phase high performance liquid chromatography method was developed for the simultaneous estimation of diloxanide furoate and metronidazole in formulation. The separation was achieved by octadecyl C8 column and a mixture of methanol: acetonitrile: 0.05M phosphate buffer at pH 4.0 (45:25:30 v/v) as eluent, at a flow rate of 1 ml/min. detection was carried out at 277 nm. Quantitation was done by external standard method. The retention time of metronidazole and diloxanide furoate was found to be 3.28 and 6.42 min, respectively. The method has validated for linearity, accuracy and precision. Linearity of metronidazole and diloxanide furoate were in the range of 5-50 µg/ml for both the drugs The mean recoveries obtained for metronidazole and diloxanide furoate were 100.01% and 99.71%, respectively
7. **RK Maheshwarin et.,al**⁵² was developed a safe and sensitive method of spectrophotometric estimation in the ultraviolet region has been developed using 1M lignocaine hydrochloride (an economic drug) as a hydrotropic solubilizing agent for the quantitative determination of tinidazole, a sparingly water-soluble antiprotozoal drug in tablet dosage form. Beer's law was obeyed in the concentration range of 5-25 mg/ml. Lignocaine hydrochloride does not interfere above 280 nm. There was more than a six-fold enhancement in aqueous solubility of tinidazole in 1M lignocaine hydrochloride solution as compared with the solubility in distilled water. Commonly used tablet excipients and lignocaine hydrochloride did not interfere in spectrophotometric estimation,
8. **Minal Rohit et.,al**⁵³ have been developed a HPTLC method for simultaneous determination of clotrimazole and tinidazole in tablet and cream. The developed method is more sensitive than the reported method. Chromatographic separation was carried out on aluminum-backed silica gel 60 GF254 TLC plates with mobile phase comprising of toluene: ethyl acetate: methanol: triethylamine

(5.5:1.0:1.0:0.1, v/v). The validated calibration ranges were 200-700 ng/spot ($r=0.9960$ and 0.9960 by height and area respectively) and 500-1750 ng/spot ($r=0.9990$ and 0.9975 by height and area respectively) for clotrimazole and tinidazole respectively. Quantitation was achieved with UV detection at $\lambda=220$ nm.

9. **Swati Bantu et.,al**⁵⁵ was developed in precise and feasible high-performance liquid chromatographic (HPLC) method for the analysis of the Fluconazole and Tinidazole in a combined tablet dosage form has been developed. The analysis was carried out on a Kromasil stainless steel C18 (250 x 4.6 mm, 5 μ) reversed-phase column, using a mixture of Acetonitrile: Water (55:45% v/v) as the mobile phase using a low pressure gradient mode with flow rate at 1ml/min. The injection volume was 20 μ l. The retention time of the drug was 2.5 for Fluconazole and 3.1 for Tinidazole. The method produced linear responses in the concentration range of 10 to 50 μ g/ml for both Fluconazole and Tinidazole. The Tailing factors of Fluconazole and Tinidazole were found to be 1 and 1.3 respectively.
10. **Nirav Patel B et.,al**⁵⁸ research manuscript describes simple, sensitive, accurate, precise and repeatable RP-UPLC method for the simultaneous determination of Ciprofloxacin HCl (CH) and Tinidazole (TZ) in tablet dosage form. The sample was analyzed by reverse phase C18 column (Purospher Star 100 \times 2.1 mm, 2 μ m) as stationary phase and Phosphate Buffer: Acetonitrile (80:20) as a mobile phase and pH 3.0 was adjusted by ortho-phosphoric acid at a flow rate of 0.3 ml/min. Quantification was achieved of Ciprofloxacin HCl at 278.5 nm and of Tinidazole at 317.5 nm with PDA detector. The retention time for Ciprofloxacin HCl and Tinidazole was found to be 1.71 and 2.22 minute respectively. The linearity for Ciprofloxacin HCl and Tinidazole was obtained in the concentration range of 3.125-43.75 μ g/ml and 3.75-52.5 μ g/ml with mean accuracies of 99.77% and 99.75% respectively.
11. **R. Adinarayana et., al**⁵⁴ was developed in precise stability indicating RP-HPLC method was developed and validated for the simultaneous determination of Tinidazole and Diloxanide furoate in pharmaceutical dosage forms.

Chromatography was carried out on kromasil C 18 (150 mm x 4.6 mm, 5 μ particle size) column using a mobile phase of phosphate buffer (adjusted to pH 3.3 with 0.1% orthophosphoric acid): acetonitrile (45:55 % v/v) at a flow rate of 1.0 ml/min. The analyte was monitored using PDA detector at 278 nm. The retention time was found to be 2.443 min and 3.653 min for Tinidazole and Diloxanide furoate respectively. The proposed method was found to be having linearity in the concentration range of 30-180 μ g/ml for Tinidazole and 25-150 μ g/ml for Diloxanide furoate respectively. The mean % recoveries obtained were found to be 99.7-100.08% for Tinidazole and 99.8-100.02% for Diloxanide furoate respectively.

12. **Atul Sayare et.,al**⁵⁰ was developed a simple, accurate and reproducible spectrophotometric methods have been developed for the simultaneous estimation of norfloxacin and Tinidazole in pharmaceutical dosage forms. The first method involves determination using the Vierodt's Method (Simultaneous Equation Method); the sampling wavelengths selected are 273 nm and 319 nm over the concentration ranges of 2.5- 20 μ g/mL and 5-40 μ g/mL for Norfloxacin and Tinidazole respectively. The second method involves determination using the Multicomponent Mode Method; the sampling wavelengths selected are 273 nm and 319 nm over the concentration ranges of 2.5-20 μ g/mL and 5-40 μ g/mL for Norfloxacin and Tinidazole respectively.

13. **Nabil .A .F. Alhemiarya. b et., al**⁵¹ was developed as two simple sensitive and reproducible spectrophotometric methods have been developed for the determination of tinidazole either in pure form or in their tablets. The proposed methods are based on the reduction of the nitro group to amino group of the drug. The reduction of tinidazole was carried out with zinc powder and zinc dust and concentrated HCl at 90 ± 5 °C for 15 min in water. Method A is based on Schiff's basses reaction used ethyl vanillin reagent and measurement of the yellow coloured species (λ_{max} : 470 nm),whereas method B is based on oxidative coupling reaction used promethazine hydrochloride reagent and sodium hypochlorite oxidation agent in alkaline medium to form red colored measurable at 525 nm.The working conditions of both methods have been optimized. Regression analysis of Beer 's law plots showed good correlation in

the concentration ranges 5-65 and 2-50 $\mu\text{g ml}^{-1}$ for methods A and B, respectively. The apparent molar absorptivity and Sandell sensitivity values are calculated to be 3.214×10^3 and 1.028×10^4 $\text{l. mol}^{-1} \text{ cm}^{-1}$, and 0.0769 and $0.0267 \mu\text{g cm}^{-2}$, with LOD $0.552 \mu\text{g ml}^{-1}$ and $0.285 \mu\text{g ml}^{-1}$, LOQ $1.840 \mu\text{g ml}^{-1}$ and $0.942 \mu\text{g ml}^{-1}$, respectively.

3. OBJECTIVE AND PLAN OF WORK

OBJECTIVE

According to literature survey, various analytical methods like UV, HPLC, etc. were reported for the simultaneous analysis of Tinidazole and Diloxaide Furoate. Stability indicating method studies were not available as per the existing literature survey. Hence studies were under taken to develop and validate stability indicating assay for estimation of these two drugs. It is felt worthwhile to develop a simple, rapid, accurate, precise and more economical stability indicating high performance liquid chromatographic method for Tinidazole and Diloxanide Furoate combination in bulk and its combined dosage form. Some RP-HPLC methods were not economical in terms of mobile phase composition, column dimensions and run times. Hence there is need for the development of newer method for estimation of Tinidazole and Diloxanide Furoate present in tablet to overcome above discussed hurdles.

Develop and validate the stability indicating assay method for Tinidazole and Diloxanide Furoate using RP-HPLC technique:

Hence the present work is aimed to develop and validate the stability indicating assay method for Tinidazole and Diloxanide Furoate using RP-HPLC technique. The Present study was planned to develop a faster gradient elution for estimation of Tinidazole and Diloxanide Furoate. The developed method was planned to validate as per ICH and USP guide lines in terms of accuracy, precision, specificity, and limit of detection, limit of quantitation, linearity, range robustness.

PLAN OF WORK

- Study of physicochemical properties of the drug.
- Review of literature.
- Procurement of drug substances and related substances for study.
- Determination of isobestic point of Tinidazole and Diloxanide Furoate.

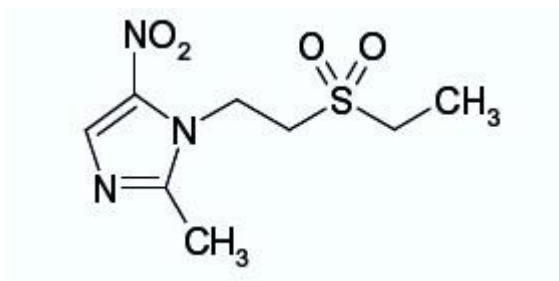
- Selection of chromatographic conditions like column, mobile phase, and flow rate in RP-HPLC.
- Optimization of the method.
- Validation of the developed method as per ICH guidelines and conduct forced degradation studies.

4. DRUG PROFILE

4.1 TINIDAZOLE

Structure

Fig No. 4.1.1 Structure of Tinidazole



Chemical Name : 1-[2-(ethylsulfonyl)ethyl]-2-methyl-5-nitroimidazole.

Molecular Formula : C₈H₁₃N₃O₄S

Molecular Weight : 247.27

Melting point : 127-128 °C.

PKa Value : 3.1 (strong basic)

Drug Category : Anti protozoal, anti Infective.

Solubility : Soluble in most organic solvents, and insoluble in water.

Description : White powder.

Storage : Store at controlled room temperature at 20-25°C.

Dosage Forms : Tablets, pills.

Available dose : 250mg, 500mg.

CLINICAL PHARMACOLOGY

Mechanism of action

Tinidazole is an antiprotozoal, antibacterial agent. The nitro- group of tinidazole is reduced by cell extracts of *Trichomonas*. The free nitro- radical generated as a result of this reduction may be responsible for the antiprotozoal activity. Chemically reduced tinidazole was shown to release nitrites and cause damage to purified bacterial DNA *in vitro*. Additionally, the drug caused DNA base changes in bacterial cells and DNA strand breakage in mammalian cells. The mechanism by which tinidazole exhibits activity against *Giardia* and *Entamoeba* species is not known.

Pharmacokinetic Parameter:

Bioavailability	:	50 to 90%
Metabolism	:	Hepatic (CYP2C19, CYP3A4)
Half-life	:	12-14 hours
Excretion	:	20-25% Renal 12% Faecal

Contraindications

Contraindications for treatment with olmesartan include biliary obstruction (BNF). Another major contraindication is pregnancy; reports in the scientific literature reveal fetal malformations for pregnant women taking sartan-derived drugs. Tinidazole may enhance the effect of warfarin and other coumarin anticoagulants, resulting in a prolongation of prothrombin time.

Drug Interactions:

- In patients with a previous history of hypersensitivity to tinidazole reported reactions have ranged in severity from urticaria to Stevens-Johnson syndrome.

- During first trimester of pregnancy
- In nursing mothers: Interruption of breast-feeding is recommended during tinidazole therapy and for 3 days following the last dose
- CYP3A4 Inducers and Inhibitors: Simultaneous administration of tinidazole with drugs that induce liver microsomal enzymes, i.e., CYP3A4 inducers such as phenobarbital, rifampin, phenytoin, and fosphenytoin (a pro-drug of phenytoin), may accelerate the elimination of tinidazole, decreasing the plasma level of tinidazole. Simultaneous administration of drugs that inhibit the activity of liver microsomal enzymes, i.e., CYP3A4 inhibitors such as cimetidine and ketoconazole, may prolong the half-life and decrease the plasma clearance of tinidazole, increasing the plasma concentrations of tinidazole.
- Tinidazole may enhance the effect of warfarin and other coumarin anticoagulants, resulting in a prolongation of prothrombin time.

SIDE EFFECTS:

- Fever, chills, body aches, flu symptoms.
- Numbness, burning pain, or tingly feeling.
- Seizure (convulsions).
- Vaginal itching or discharge.
- Nausea, vomiting, loss of appetite, indigestion.
- Constipation, diarrhea, stomach cramps.
- Feeling weak.

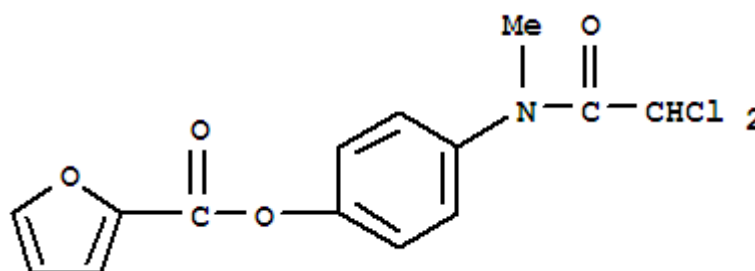
USES:

- Tinidazole is an antibiotic that fights bacteria in the body.
- Tinidazole is used to treat certain infections caused by bacteria, such as infection of the intestines or vagina. It is also used to treat certain sexually transmitted infections.

- Tinidazole may also be used for purposes other than those listed in this medication guide
- Is used to treat certain types of vaginal infections (bacterial vaginosis, trichomoniasis). It is also used to treat certain types of parasite infections (giardiasis, amebiasis).

BRAND NAMES:

Brand name	Manufacturer	Strength	Type of dosage form
Fasigyn	Pfizer	500 mg	Tablets
Tindamax	Mission pharma	250mg, 500mg	Tablets and pills
Tindazole	Roxane laboratories	500mg	Tablets

4.2 DILOXANIDE FURATE**Structure:****Fig no 4.2.1 Structure of Diloxanide Furoate**

Chemical Name : 4-[(d ichloroacetyl)(methyl)amino]phenyl
furan-2-carboxylate

Molecular Formula : C₁₄H₁₁ClNO₄

Molecular Weight : 328.2 g/mol.

Melting point : 114-116

PKa Value : 4.7 (strong basic) and 13.09 (strong acidic)

- Drug Category** : Antiprotozoal.
- Solubility** : Diloxanide furoate is very slightly soluble in water, soluble to the extent of 1 in 100 in alcohol, 1 in 25 of chloroform, and 1 in 130 of ether.
- Description** : White or almost white crystalline powder
- Storage** : Store at room temperature between 59 and 86 degrees F (15 to 30 degrees C) away from heat and light.
- Dosage Forms** : Tablets
- Available does** : 500 mg.

CLINICAL PHARMACOLOGY

Mechanism of action

Dioxanide furoate luminal amebicide. The mechanism of action of diloxanide is unknown. This agent destroys the trophozoites of *E. histolytica* that eventually form into cysts. The cysts are then excreted by persons infected with asymptomatic amebiasis.

Pharmacokinetic Parameters:

- Bioavailability : 90%.
- Metabolism : hydrolyze to furoic acid and diloxanide which under goes extensive glucuronidation.
- Half-life : 3 hours.
- Excretion : Renal (90%) fecal (10 %).

Contraindications:

- Contraindicated in hypersensitivity.
- It is contraindicated in children below 2 years.

Drug Interactions:

No interactions of Diloxanide furoate with other has not been fully studied or established.

SIDE EFFECTS:

The side effects of Diloxanide Furoate include

- Flatulence
- Nausea
- Vomiting
- Anorexia
- Diarrhea
- Abdominal cramps
- Urticaria
- Esophagitis
- Paresthesia
- Albuminuria

USES:

Diloxanide is used to treat an infection caused by a parasite in your bowel. Infection occurs after drinking water contaminated by infected faeces, or eating food prepared or washed using contaminated water. People most at risk of amoebiasis include travellers to areas where amoebiasis is common, such as countries with poor sanitation. Treatment includes medication such as diloxanide to kill the parasite, and drinking plenty of fluids to prevent dehydration.

BRAND NAMES:

Brand Name	Manufacturer	Strength	Type of form
Amiciline	Franco Indian pharmaceuticals	500 mg	Tablet
Furamide	Abbott health care (p) ltd	500 mg	Tablet
Dilamide	Pharmadesh laboratories ltd	500mg	Tablet

5. MATERIALS AND METHODS

The various materials and equipment's used for the present study are summarized as follows.

CHEMICALS/REAGENTS AND SOLVENTS

S.No.	Materials	Grade	Company
1	Potassium dihydrogen orthophosphate	AR	MERCK
2	Methanol	HPLC	MERCK
3	Acetonitrile	HPLC	MERCK
4	Water	HPLC	MERCK
5	Ortho phosphoric acid	AR	MERCK
6	Hydrochloric acid	AR	MERCK
7	Sodium Hydroxide	AR	MERCK
8	Hydrogen Peroxide	AR	MERCK

Table No: 5.1 List of Various Chemicals and Solvents Used

S.No	Working standard	Potency
1	Tinidazole	99.6 %
2	Diloxanide Furoate	99.6 %

Table No. 5.2 Working Standard / Reference Standard/ API

Brand name	Composition
METROQUIN	Tinidazole - 300 mg + Diloxanide Furoate - 250 mg

Table No. 5.3 Test Sample

EQUIPMENTS:

S.NO	Name of equipment	Software	Model	Company
1.	HPLC	Empower	Waters 2695	Waters
2.	UV – spectrophotometer	Spectral treats	SL 159	ELICO
3.	pH meter	N/A	LI615	ELICO
4.	Weighing balance	N/A	A X 200	SHIMADZU
5.	Sonicator	N/A	ISO 9001- 2000	PCI
6.	Vaccum filter	N/A	PDF2-75	PROMIVAC

Table No. 5.4 List o f Various Equipments Used

6. METHOD DEVELOPMENT & VALIDATION

6.1 METHOD DEVELOPMENT

Instrument and Materials

An UV-SL 159 Series UV / Visible double beam Spectrophotometer with 1 cm matched quartz cells was used for all spectral measurements. All chemicals used were of Analytical Reagent (AR) grade from, Merck, Fischer scientific and Qualigen.

Solvent Selection

In order to select suitable solvent for determination of Tinidazole and Diloxanide Furoate various solvents were selected for the solubility studies and it was found that they were freely soluble in methanol. Hence in the present work, methanol was used for all the dilutions.

Preparation of Standard Stock Solution of Tinidazole

Accurately weighed and transferred 10 mg of Tinidazole working standard into a 100 ml clean dry volumetric flask and it was dissolved by using methanol and the volume was made up to the mark with methanol (100 µg/ml).

1 ml of the above standard stock solution was pipetted into 10 ml volumetric flasks and diluted up to the mark with methanol to get concentration of 10 µg/ml.

Preparation of Standard Stock Solution of Diloxanide Furoate

Accurately weighed and transferred 10 mg of Diloxanide furoate working standard into a 100 ml clean dry volumetric flask and it was dissolved by using methanol and volume was made up to the mark with methanol (100 µg/ml).

1 ml of the above standard stock solution was pipetted into 10 ml volumetric flasks and diluted up to the mark with methanol to get concentration of 10µg/ml.

Optimization of UV- conditions

Initially method development work was done in UV by taking UV-visible spectra from the wavelength range of 200-400 nm for Tinidazole and Diloxanide furoate standard solutions. Results revealed the isobestic point of Tinidazole and Diloxanide furoate standard was 278 nm. The isobestic point of two drugs shows maximum absorbance at 278 nm. Hence this λ_{max} of 278 nm was utilized for HPLC method development.

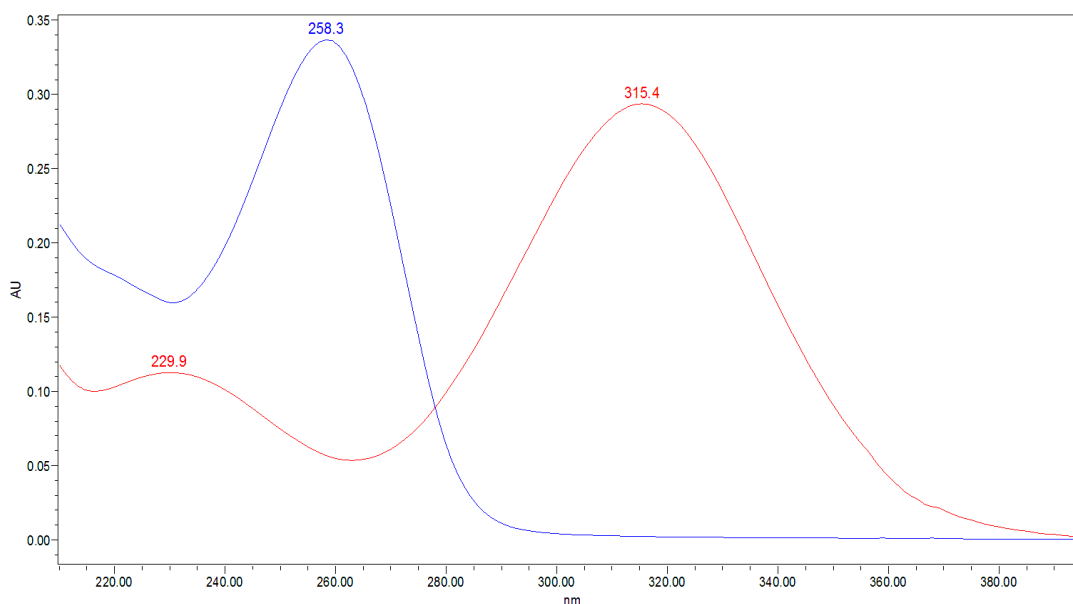


Fig No 6.1 Isobestic point of Tinidazole and Diloxanide furoate. (278 nm)

6.2 HPLC METHOD DEVELOPMENT TRAILS

Trail: 1

Preparation of Phosphate buffer:

Transferred an accurately weighed 1.36gm of Potassium di-hydrogen Ortho phosphate into a 1000 ml volumetric flask, 900 ml of milli-Q water was added and degas to sonicate and finally make up the volume with milli –Q water and pH adjusted to 5.0 with dil.Orthophosphoric acid solution

Mobile phase:

Buffer and Acetonitrile taken in the ratio 30:70 % v/v.

Diluent preparation: ACN: Water (50:50 % v/v)

Accurately 50 ml of acetonitrile and 50 ml of milli-Q water were measured and mixed well.

Chromatographic conditions:

Flow rate	: 1.0 ml/min
Column	: Hypersil BSD C18 (250 x 4.6 mm, 5 μ .particle size)
Detector wave length	: 278nm
Column temperature	: 30°C
Injection volume	: 10 μ L
Run time	: 15 min

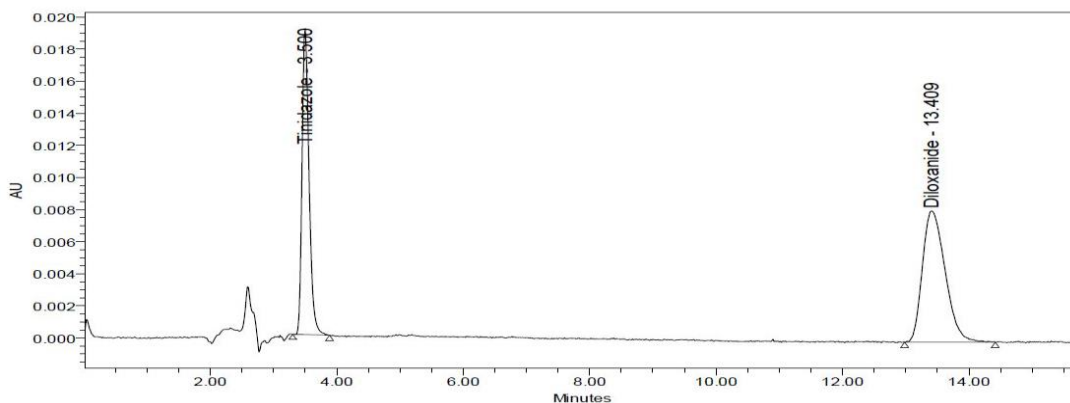


Fig no.6.2 Chromatogram of tail (1)

S.No	Sample name	Retention time(min)	Peak Area	% peak Area	USP Tailing	USP Plate Count	Resolution
1	Tinidazole	3.5	136055	40.22	1.30	4771	22.74
2	Diloxanide furoate	13.409	225442	56.15	1.35	6534	

Observation: Retention time and Resolution were found to be more.

Trail: 2

Preparation of buffer:

Transferred an accurately weighed 1.36gm of Potassium di-hydrogen Ortho phosphate into a 1000 ml volumetric flask, 900 ml of milli-Q water was added and degas to sonicate and finally make up the volume with milli -Q water and pH adjusted to 5.0 with dil.Orthophosphoric acid solution

Mobile phase:

Buffer and Acetonitrile taken in the ratio 50:50 % v/v.

Diluent preparation: ACN: Water (50:50 % v/v)

Accurately 50 ml of acetonitrile and 50 ml of milli-Q water were measured and mixed well.

Chromatographic conditions :

Mobile phase : KH_2PO_4 : Acetonitrile (50:50 ml)

Flow rate : 1.0 ml/min

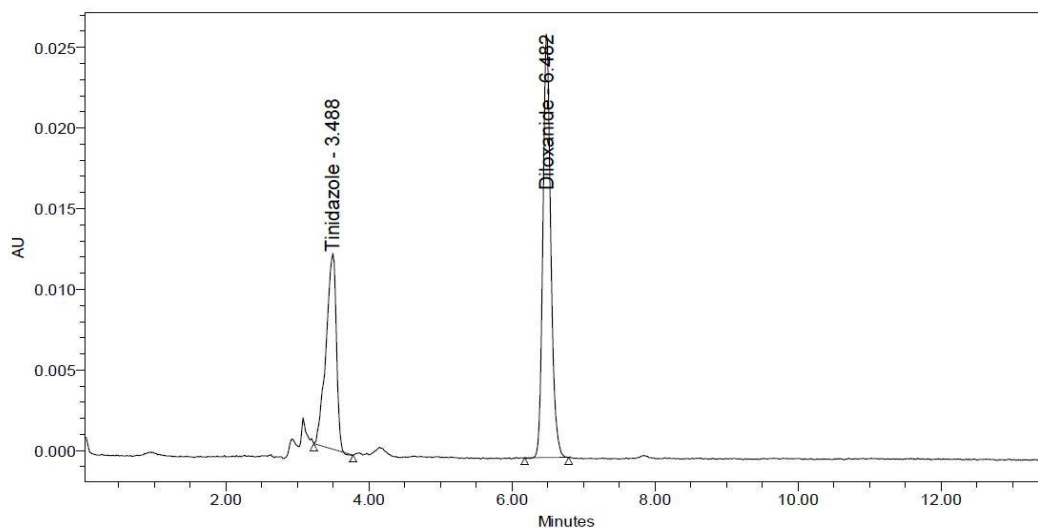
Column : Hypersil BDS C18 (250 x 4.6 mm, 5 μ .)

Detector wavelength : 278 nm

Injection volume : 10 μ l

Run time : 15 min

Temperature : 30°C

**Fig no 6.3 Chromatogram of tail (2)**

S.No	Sample name	Retention time(min)	Peak Area	% peak Area	USP Tailing	USP Plate Count	Resolution
1	Tinidazole	3.488	121946	37.75	0.79	2820	12.90
2	Diloxanide furoate	6.482	201090	62.25	1.17	17324	

Observation: Resolution was found to be more.

Trail: 3**Preparaion of buffer:**

Transferred an accurately weighed 1.36gm of Potassium di-hydrogen Ortho phosphate into a 1000 ml volumetric flask, 900 ml of milli-Q water was added and degassed. Finally make up the volume with water the added 1ml of triethylamine and then pH adjusted to 3.3 with dil.Orthophosphoric acid solution

Mobile phase:

Buffer and Acetonitrile taken in the ratio 40:60 % v/v.

Diluent preparation: ACN: Water (50:50 % v/v)

Accurately 50 ml of acetonitrile and 50 ml of milli-Q water were measured and mixed well.

Chromatographic conditions:

Mobile phase	:	KH ₂ PO ₄ : Acetonitrile (40:60 V/V)
Flow rate	:	1.1 ml/min
Column	:	Hypersil BDS C18 (250 x4.6 mm 5 μ particle size)
Detector wavelength	:	278 nm
Injection volume	:	10 μl
Run time	:	6 min
Temperature	:	30°C

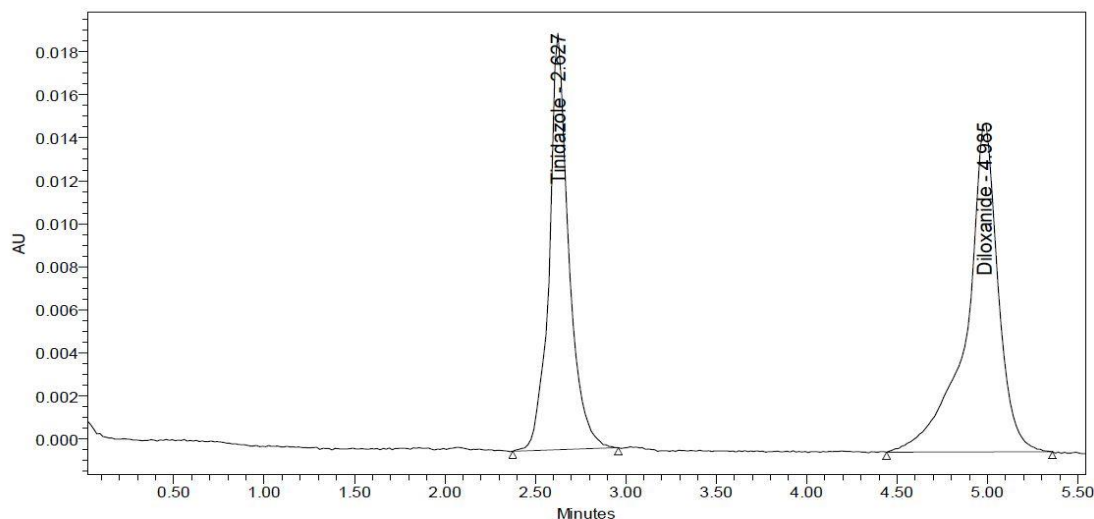


Fig no 6.4 Chromatogram of tail (3)

S.No	Sample name	Retention time(min)	Peak Area	% peak Area	USP Tailing	USP Plate Count	Resolution
1	Tinidazole	2.627	144621	42.61	1.14	3416	10.02
2	Diloxanide furoate	4.985	183418	55.39	0.77	5798	

Observation: Retention time was found to be more.

Trail: 4

Preparaion of buffer:

Transferred an accurately weighed 1.36gm of Potassium di-hydrogen Ortho phosphate into a 1000 ml volumetric flask, 900 ml of milli-Q water was added and degassed. Finally make up the volume with water the added 1ml of triethylamine and then pH adjusted to 5.0 with dil.Orthophosphoric acid solution

Mobile phase:

Buffer and Acetonitrile taken in the ratio 60:40 % v/v.

Diluent preparation: ACN: Water (50:50 % v/v)

Accurately 50 ml of acetonitrile and 50 ml of milli-Q water were measured and mixed well.

Chromatographic conditions:

Mobile phase : KH_2PO_4 : Acetonitrile (60:40 ml)
 Flow rate : 1.0 ml/min
 Column : Hypersil BSD C18 (250 x4.6 mm, 5 μ particle size).
 Detector wavelength : 278 nm
 Injection volume : 10 μ l
 Run time : 15 min
 Temperature : 30°C

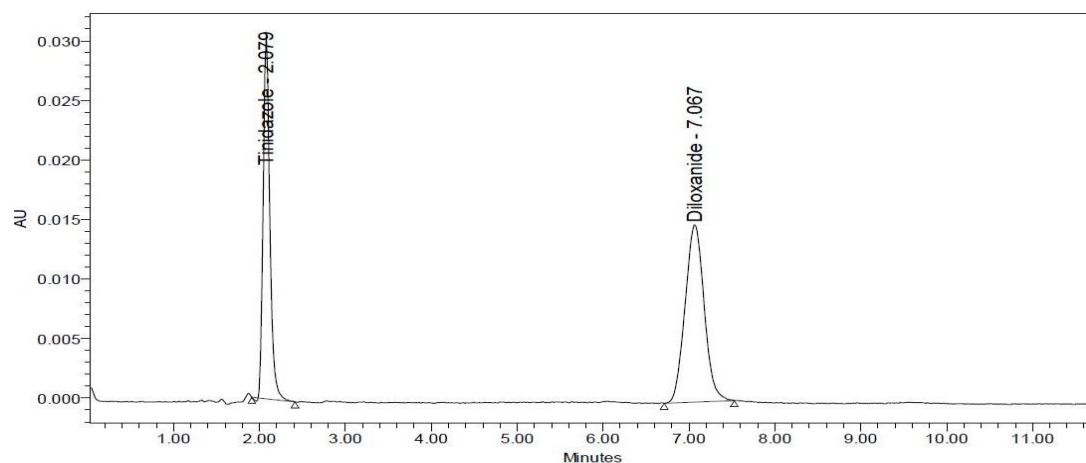


Fig No 6.5 Chromatogram for trail (4)

S.No	Sample name	Retention time(min)	Peak Area	% peak Area	USP Tailing	USP Plate Count	Resolution
1	Tinidazole	2.079	166758	41.99	1.26	3323	17.66
2	Diloxanide furoate	7.067	230392	58.01	1.05	4767	

Observation: Retention time was found to be more.

Trail: 5**Preparaion of buffer:**

Transferred an accurately weighed 1.36gm of Potassium di-hydrogen Ortho phosphate into a 1000 ml volumetric flask, 900 ml of milli-Q water was added and degassed. Finally make up the volume with water the added 1ml of triethylamine and then pH adjusted to 3.3 with dil.Orthophosphoric acid solution

Mobile phase:

Buffer and Acetonitrile taken in the ratio 55:45 % v/v.

Diluent preparation: ACN: Water (50:50 % v/v)

Accurately 50 ml of acetonitrile and 50 ml of milli-Q water were measured and mixed well.

Chromatographic conditions:

Mobile phase	: KH ₂ PO ₄ : Acetonitrile (55:45 ml)
Flow rate	: 1.0 ml/min
Column	: Hypersil OSD C18 (250 x4.6 mm, 5 μ particle size.)
Detector wavelength	: 278 nm
Injection volume	: 10 μl
Run time	: 10 min
Temperature	: 30°C

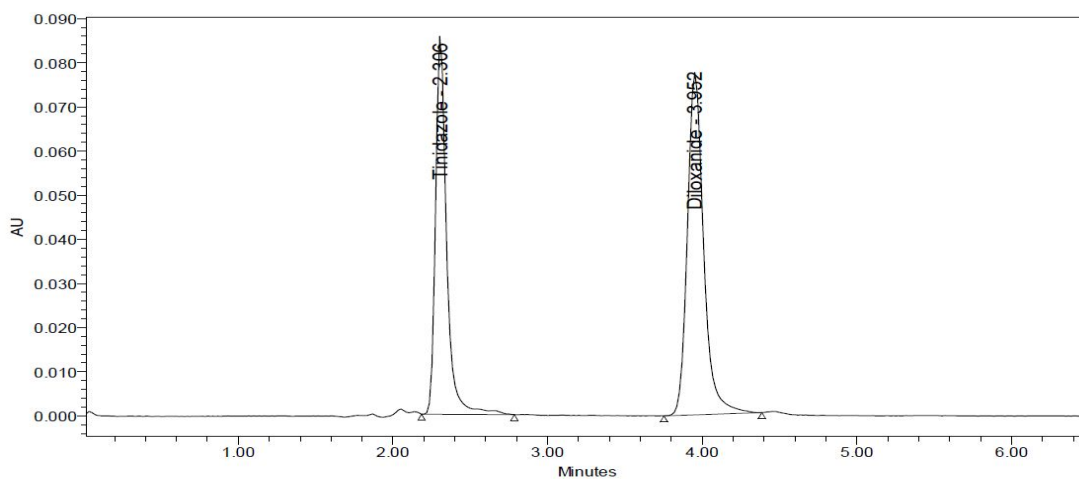


fig No 6.6 Chromatogram for trail (5)

S. No.	Sample name	Retention time(min)	Peak Area	% peak Area	USP Tailing	USP Plate Count	Resolution
1	Tinidazole	2.306	427856	42.23	1.35	5468	10.41
2	Diloxanide furoate	3.952	566834	54.65	1.21	7437	

Observation: Retention time and resolution were found to be more.

OPTIMIZED METHOD**Preparaion of buffer:**

Transferred an accurately weighed 1.36gm of Potassium di-hydrogen Ortho phosphate into a 1000 ml volumetric flask, 900 ml of milli-Q water was added and degassed. Finally make up the volume with water the added 1ml of triethylamine and then pH adjusted to 3.3 with dil.Orthophosphoric acid solution

Mobile phase:

Buffer and Acetonitrile taken in the ratio 45:55% v/v.

Diluent preparation: ACN: Water (50:50 % v/v)

Accurately 50 ml of acetonitrile and 50 ml of milli-Q water were measured and mixed well.

Chromatographic conditions:

Mobile phase	:	Phosphate Buffer (KH_2PO_4) : Acetoitrile (45:55V/V)
Flow rate	:	1.0 ml/min
Column	:	Hypersil OSD C18 (250 x 4.6 mm, 5 μ particle size).
Detector wavelength	:	278 nm
Injection volume	:	10 μ l
Run time	:	10 min
Temperature	:	30°C

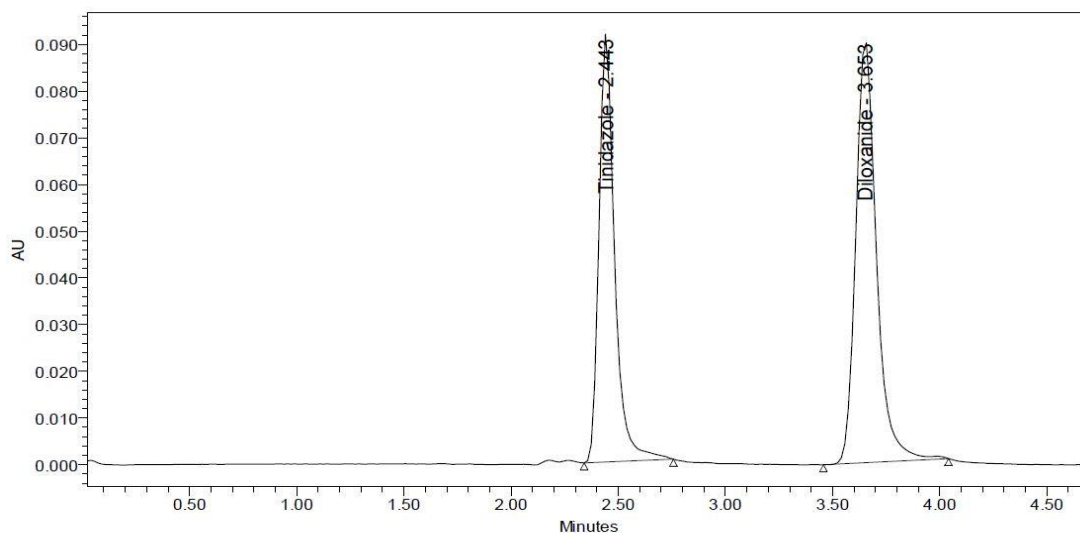


Fig No 6.7 Chromatogram for optimized method

S. No.	Sample name	Retention time(min)	Peak Area	%peak Area	USP Tailing	USP Plate Count	Resolution
1	Tinidazole	2.443	464001	43.40	1.32	5630	7.89
2	Diloxanide furoate	3.653	605076	56.60	1.24	7362	

Observation: Retention time was less and Peak shapes were good

6.8 METHOD VALIDATION

Method validation was performed as per the ICH guidelines. The developed method was validated for the following parameters.

1. System Suitability
2. Specificity
3. Linearity and Range
4. Accuracy
5. Precision

6. LOD & LOQ
7. Robustness
8. Ruggedness

1) SYSTEM SUITABILITY:

System suitability test should be carried out to verify that the analytical system is working properly and can give accurate and precise results. Standard solutions were prepared as per the test method and injected into the chromatographic system. The system suitability parameters were evaluated from tailing factor, retention times and theoretical plates of standard chromatograms.

Procedure:

Standard solution preparations were injected into the chromatograph and system suitability parameters were recorded. The results obtained are tabulated in table no.7.9

2) SPECIFICITY:

Specificity is the ability to assess unequivocally the analyte in the presence of compounds that may be expected to present, such as impurities, degradation products and matrix components. The specificity of the method was assessed by comparing the chromatograms obtained from the drug standards with that of obtained from the tablet solution. The retention times of the drug standards and the drug from sample solutions were found to be same. The method was also specific and selective because there was no interference from excipients in the Tablets.

3) LINEARITY:

The linearity of an analytical method was carried out to check its ability to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range. Different levels of standard solutions were prepared and injected into the HPLC and the chromatograms were recorded.

Procedure:**Preparation of Standard Stock Solution: (12000 μ g/ml of Tinidazole and 1000 μ g/ml of Diloxanide Furoate)**

Accurately weighed and transferred 12mg of Tinidazole and 10mg of Diloxanide working Standards into a 10 ml clean dry volumetric flask, add 7ml of diluent, sonicated for 5 minutes and make up to the final volume with diluents.

Preparation of working standard solution: (120 μ g/ml of Tinidazole and 100 μ g/ml of Diloxanide Furoate)

From the above two stock solutions, 1ml was Pipetted out into a 10ml volumetric flask and made up to final volume with diluent.

Preparation of Sample Solution: (120 μ g/ml of Tinidazole and 100 μ g/ml of Diloxanide Furoate)

20 tablets were weighed and calculate the average weight of each tablet then the weight equivalent to 120 mg of Tinidazole and 100 mg Diloxanide Furoate transferred into a 100mL volumetric flask, 70mL of diluent added and sonicated for 25 min, further the volume made up with diluent and filtered. From the filtered solution 1 ml was Pipetted out into a 10 ml volumetric flask and made up to 10ml with diluent.

Procedure: Standard and sample solutions were injected into the chromatogram and results obtained are shown in table no 7.17

Preparation of serial dilutions

Different levels of standard solution were prepared by pipetting out known volume of stock solution and made up to volumes with the diluent to get different analyte concentrations. Injected above solutions into the chromatograph (Injected higher levels five times for precision). Recorded the area response for each level and calculated the slope, intercept, correlation coefficient and regression coefficient (R square). Test the intercept for statistical equivalence to zero. A graph of concentration (ppm) on X-axis and area on Y axis was plotted.

Table no. 6.3 Preparation of standard serial dilutions for linearity of Tinidazole and Diloxanide Furoate:

S.No	Pipetted from stock (ml)	Volume of flask (ml)	Concentration in ppm(Tinid)	Concentration in ppm(Dilo)	%Linearity Level
1	0.25	10	30	25	25
2	0.5	10	60	50	50
3	0.75	10	90	75	75
4	1	10	120	100	100
5	0.25	10	150	125	125
6	0.50	10	180	150	150

4) ACCURACY:

The accuracy of an analytical method is 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.

The study was performed by making three different standard concentrations at 50%,100% and 150% levels of known amounts of studied drugs. The accuracy of an analytical method should be established across its range. Finally, the final volume made up with diluent (ACN: Water 50:50 % v/v) and mixed well. The resulting mixtures were analyzed by the proposed HPLC method at 278 nm.

Preparation of Standard Stock Solution: (12000µg/ml of Tinidazole and 1000µg/ml of Diloxanide Furoate)

Accurately weighed and transferred 12mg of Tinidazole and 10mg of Diloxanide working Standards into a 10 ml clean dry volumetric flask, add 7ml of diluent , sonicated for 5 minutes and make up to the final volume with diluents.

Preparation of working standard solution: (120µg/ml of Tinidazole and 100µg/ml of Diloxanide Furoate)

From the above two stock solutions, 1 ml was pipetted out into a 10 ml volumetric flask and made up to final volume with diluent.

Preparation of Sample Solution: (120µg/ml of Tinidazole and 100µg/ml of Diloxanide Furoate)

20 tablets were weighed and calculate the average weight of each tablet then the weight equivalent to 120 mg of Tinidazole and 100 mg Diloxanide Furoate transferred into a 100 mL volumetric flask, 70 mL of diluent added and sonicated for 25 min, further the volume made up with diluent and filtered. From the filtered solution 1 ml was pipetted out into a 10 ml volumetric flask and made up to 10 ml with diluent.

Preparation of 50 % Standard Addition Sample

To 0.5 ml of sample stock solution present in 10 ml volumetric flask, 0.25 ml of standard stock solution was added and diluted up to the mark with the mobile phase.

Preparation of 100 % Standard Addition Sample

To 0.5 ml of sample stock solution present in 10 ml volumetric flask, 0.5 ml of standard stock solution was added and diluted up to the mark with the mobile phase.

Preparation of 150 % Standard Addition Sample

To 0.5 ml of sample stock solution present in 10 ml volumetric flask, 0.75 ml of standard stock solution was added and diluted up to the mark with the mobile phase.

Acceptance criteria:

The % of recovery should be between 98 to 102%.

5) PRECISION:

The precision of an analytical method is the degree of agreement among individual test results when the method is applied repeatedly to multiple sampling of homogeneous sample. The precision of analytical method is usually expressed as the standard deviation or relative standard deviation (coefficient of variation) of series of measurements.

$$\% RSD = \frac{\text{standard deviation}}{\text{mean}} \times 100$$

a) System Precision:

The system precision was carried out to ensure that the analytical system is working properly by Injecting standard preparation six times into the HPLC and calculated the %RSD for Tiniazole and Diloxanide furoate. The results obtained are tabulated in table no. 8.6 The retention time and area of six determinations are measured and % RSD were calculated.

Preparation of Standard Stock Solution: (12000µg/ml of Tinidazole and 1000µg/ml of Diloxanide Furoate)

Accurately weighed and transferred 12mg of Tinidazole and 10mg of Diloxanide working Standards into a 10 ml clean dry volumetric flask, add 7ml of diluent , sonicated for 5 minutes and make up to the final volume with diluents.

Preparation of working standard solution: (120µg/ml of Tinidazole and 100µg/ml of Diloxanide Furoate)

From the above two stock solutions,1ml was Pipetted out into a 10ml volumetric flask and made up to final volume with diluent.

Acceptance criteria:

The % RSD for the area of six standard injections results should not be more than 2 % as shown in the table no. 8.6.

b) Method Precision:

In method precision, a homogenous sample of a single batch should be analyzed six times and was checked whether the method is giving consistent results for a single batch. The samples of Tinidazole and Diloxanide furoate were analyzed six times. The % RSD was calculated for the sample.

Preparation of Sample Solution: (120µg/ml of Tinidazole and 100µg/ml of Diloxanide Furoate)

20 tablets were weighed and calculate the average weight of each tablet then the weight equivalent to 120 mg of Tinidazole and 100 mg Diloxanide Furoate transferred into a 100mL volumetric flask, 70mL of diluent added and sonicated for 25 min, further the volume made up with diluent and filtered. From the filtered solution 1 ml was Pipetted out into a 10 ml volumetric flask and made up to 10ml with diluent.

Acceptance criteria

The % RSD for the area of six sample injections results should not be more than 2 % as shown in table no.8.7.

6) Detection Limit and Quantitation Limit (LOD and LOQ):

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value and the quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy .The detection limit and the quantitation limit can be calculated based on the Standard Deviation of the Response and the Slope. .

Limit of Detection (LOD):

The parameter LOD was determined on the basis of response and slope of the regression equation.

The Detection Limit (DL) may be expressed as:

$$DL = 3.3 F/S$$

Where,

F = Residual Standard deviation of the response,

S = Slope of the calibration curve.

The LOD for this method was found to be 0.25 µg/ml and 0.20 µg/ml for Tinidazole and Diloxnide Furoate respectively.

Limit of Quantification (LOQ):

The parameter LOQ was determined on the basis of response and slope of the regression equation.

The Quantitation Limit (QL) may be expressed as:

$$QL = 10 F/S$$

Where,

F = Residual Standard deviation of the response,

S = Slope of the calibration curve.

The LOQ for this method was found to be 0.76 µg/ml and 0.59 µg/ml for Tinidazole and Diloxanide furoate respectively.

7) ROBUSTNESS:

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

The robustness of the proposed method was determined by analysis of aliquots from homogenous lots by differing physical parameters like mobile phase composition, flow rate and temperature which may differ but the responses were still

within the specified limits of the assay. The standard solution, sample solution and sample solution spiked with impurities were injected into the chromatograph at varied conditions of flow rate $\pm 0.1\%$ ml/min, mobile phase composition by $\pm 10\%$ organic phase buffer pH ± 0.2 units and temperature by $\pm 5^\circ\text{C}$

a) Effect of variation of flow rate

A study was conducted to determine the effect of variation in flow rate. Standard solution was prepared and injected into the HPLC system by keeping variation in flow rate + or $- 10\%$ (0.1 ml/min). The effect of variation of flow rate was evaluated.

b) Effect of variation of Temperature

A study was conducted to determine the effect of variation in temperature. Standard solution was prepared and injected into the HPLC system by keeping variation in temperature + or $- 5^\circ\text{C}$. The effect of variation of temperature was evaluated.

ASSAY OF FORMULATION

Preparation of Standard Stock Solution: (12000 $\mu\text{g/ml}$ of Tinidazole and 1000 $\mu\text{g/ml}$ of Diloxanide Furoate)

Accurately weighed and transferred 12mg of Tinidazole and 10mg of Diloxanide working Standards into a 10 ml clean dry volumetric flask, add 7ml of diluent, sonicated for 5 minutes and make up to the final volume with diluents.

Preparation of working standard solution: (120 $\mu\text{g/ml}$ of Tinidazole and 100 $\mu\text{g/ml}$ of Diloxanide Furoate)

From the above two stock solutions, 1ml was pipetted out into a 10ml volumetric flask and made up to final volume with diluent.

Preparation of Sample Solution: (120µg/ml of Tinidazole and 100µg/ml of Diloxanide Furoate)

20 tablets were weighed and calculate the average weight of each tablet then the weight equivalent to 120 mg of Tinidazole and 100 mg Diloxanide Furoate transferred into a 100mL volumetric flask, 70mL of diluent added and sonicated for 25 min, further the volume made up with diluent and filtered. From the filtered solution 1 ml was Pipetted out into a 10 ml volumetric flask and made up to 10ml with diluent.

Inject 10µl of filtered portion of the sample and standard into the chromatograph. Record the responses for the major peaks. Calculate the amount of Tinidazole and Diloxanide Furoate per each tablet from the following expression.

Calculation:

$$\text{Amount present in each tablet} = \frac{\text{Spl}_{\text{area}} \times \text{Std}_{\text{dil}} \times \text{potency}}{\text{Std}_{\text{area}} \times \text{Spl}_{\text{dil}}} \times \text{Avg Wt}$$

$$\% \text{ Assay} = \frac{\text{Amount present (mg)}}{\text{Label claim}} \times 100$$

Where,

Avg wt = average weight of tablets.

Spl_{area} = Sample area

Std_{area} = Standard area

Std_{dil} = Standard dilution

Spl_{dil} = Sample dilution

Assay of Tinidazole:

$$\text{Assay \%} = \frac{\text{-----}}{\text{-----}} \times 100$$

Assay of Diloxanide Furoate :

$$\text{Assay \%} = \frac{\text{-----}}{\text{-----}} \times 100$$

Acceptance criteria

The percentage purity of Tinidazole and Diloxanide Furoate should be not less than 90% and not more than 110%.

DEGRADATION STUDIES:**Acid Degradation Studies:**

To 1.0 ml of working solution of Tinidazole and Diloxanide, 1 ml of 2N Hydrochloric acid was added and refluxed for 30mins at 60⁰c .The resultant solution was diluted to obtain 120µg/ml&100µg/ml solution and 10 µl solutions were injected into the system and the chromatograms were recorded to assess the stability of sample.

Alkali Degradation Studies:

To 1.0 ml of working solution of Tinidazole and Diloxanide, 1 ml of 2N sodium hydroxide was added and refluxed for 30mins at 60⁰c. The resultant solution was diluted to obtain 120µg/ml&100µg/ml solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Oxidation:

To 1.0 ml of working solution of Tinidazole and Diloxanide, 1 ml of 20% hydrogen peroxide (H₂O₂) was added separately. The solutions were kept for 30 min at 60⁰c. For HPLC study, the resultant solution was diluted to obtain 120µg/ml&100µg/ml solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Dry Heat Degradation Studies:

The standard drug solution was placed in oven at 105⁰c for 6 h to study dry heat degradation. For HPLC study, the resultant solution was diluted to 120µg/ml&100µg/ml solution and 10µl were injected into the system and the chromatograms were recorded to assess the stability of the sample.

Photo Stability studies:

The photochemical stability of the drug was also studied by exposing the solution to UV Light by keeping the beaker in UV Chamber for 7days or 200 Watt hours/m² in photo stability chamber. For HPLC study, the resultant solution was diluted to obtain 120µg/ml&100µg/ml solutions and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Neutral Degradation Studies:

The photochemical stability of the drug was also studied by exposing the solution to UV Light by keeping the beaker in UV Chamber for 7days or 200 Watt hours/m² in photo stability chamber. For HPLC study, the resultant solution was diluted to obtain 120µg/ml&100µg/ml solutions and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

7. RESULTS AND DISCUSSION

7.1 SYSTEM SUITABILITY:

Fig:1 Chromatogram of System suitability Standard Solution

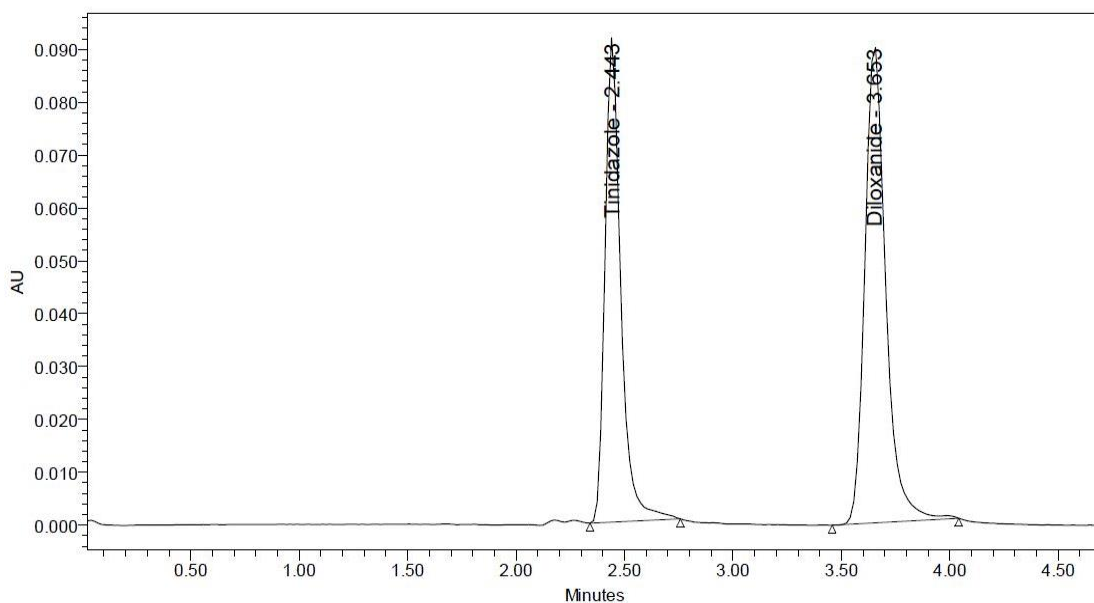


Table No.7.1 System Suitability:

S.No	Sample name	Retention time(min)	Peak Area	% peak Area	USP Tailing	USP Plate Count	Resolution
1	Tinidazole	2.443	464001	43.40	1.32	5630	7.89
2	Diloxanide furoate	3.653	605076	56.60	1.24	7362	

Data interpretation:

It is observed from the tabulated above, that system suitability parameters are within the acceptable limit. Hence it can be concluded that the system suitability parameters met the requirements of method validation.

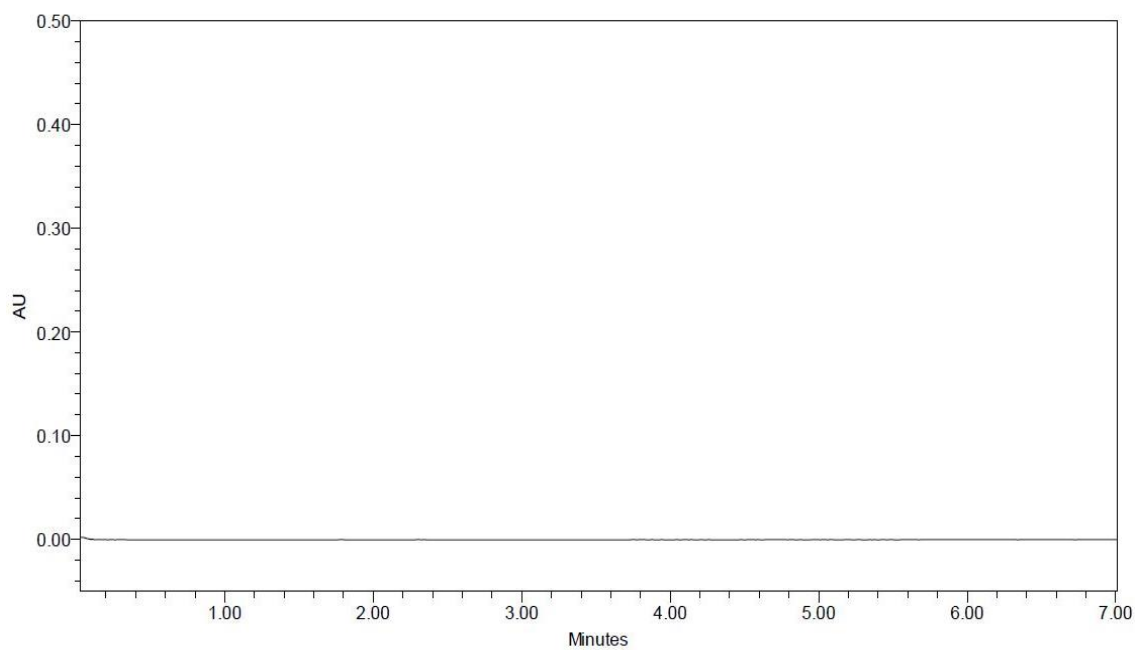
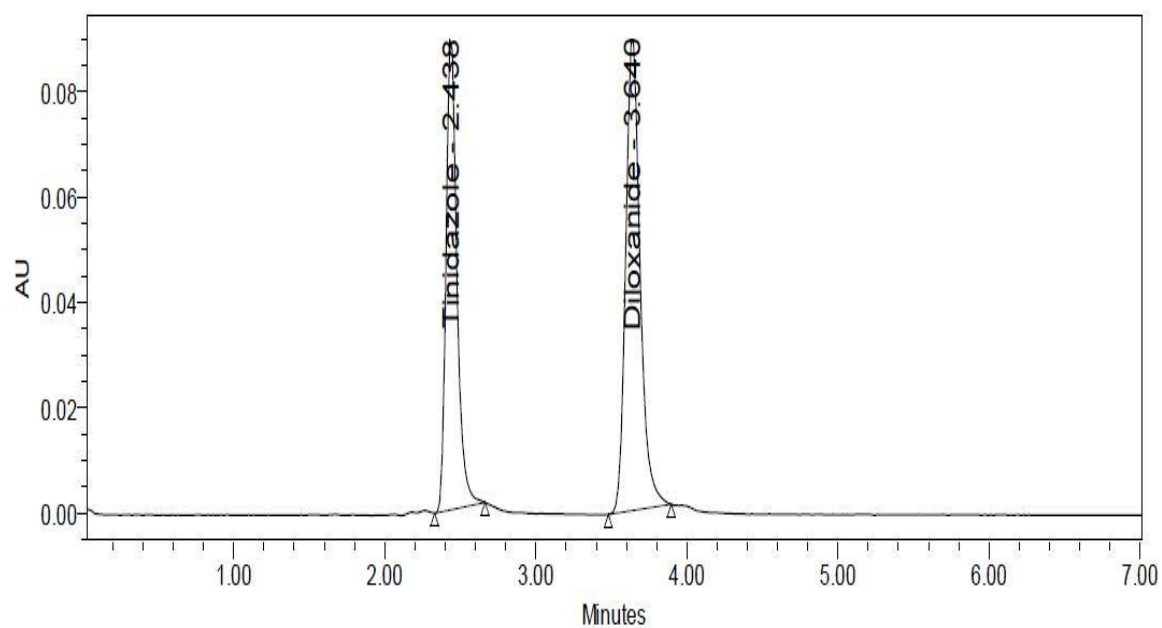
7.2 SPECIFICITY:**Fig: 2 Chromatogram of Blank solution :****Fig: 3 Chromatogram of Standards solution:**

Fig: 4 Chromatogram of Sample

solution:

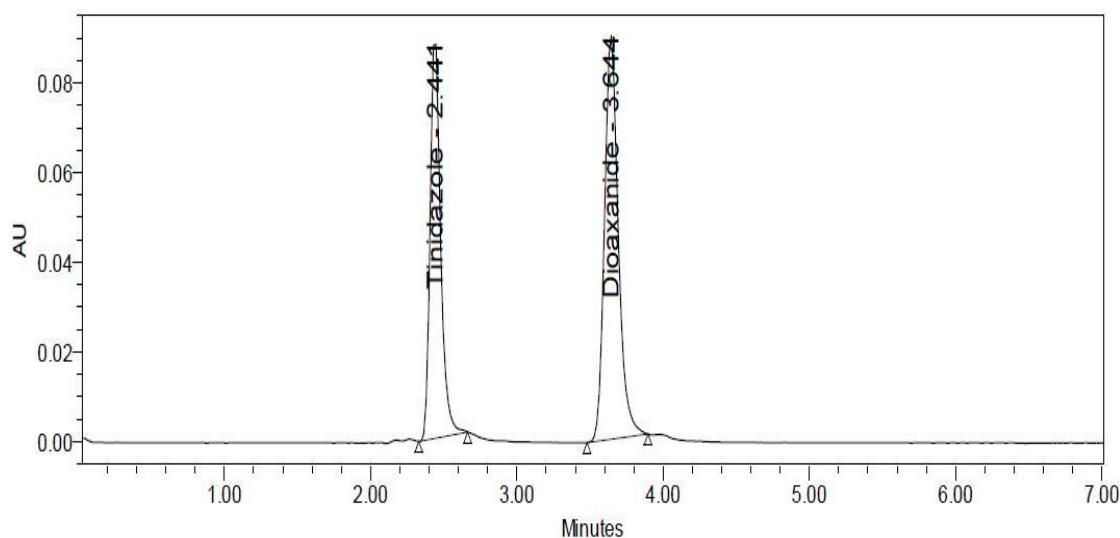


Table No. 7.2 Specificity Results

S.No	Compound name	Retention time	
		Tinidazole	Diloxanide furoate
1	Blank	-	-
2	Standard	2.438	3.640
3	Sample	2.441	3.644

Data interpretation

1. The selectivity of the method was checked by injecting solutions of both Tinidazole and Diloxanide Furoate. It was observed that two sharp peaks for each drug were obtained at retention times of 2.441 and 3.644 min respectively. The found R_t of test and standard peaks was almost equal and diluents did not interfere with the peaks.
2. The retention times of drug standards and drug samples were almost same. Hence the method was specific.
3. The method was also found to be specific & selective because there was no interference from excipients in the tablets.

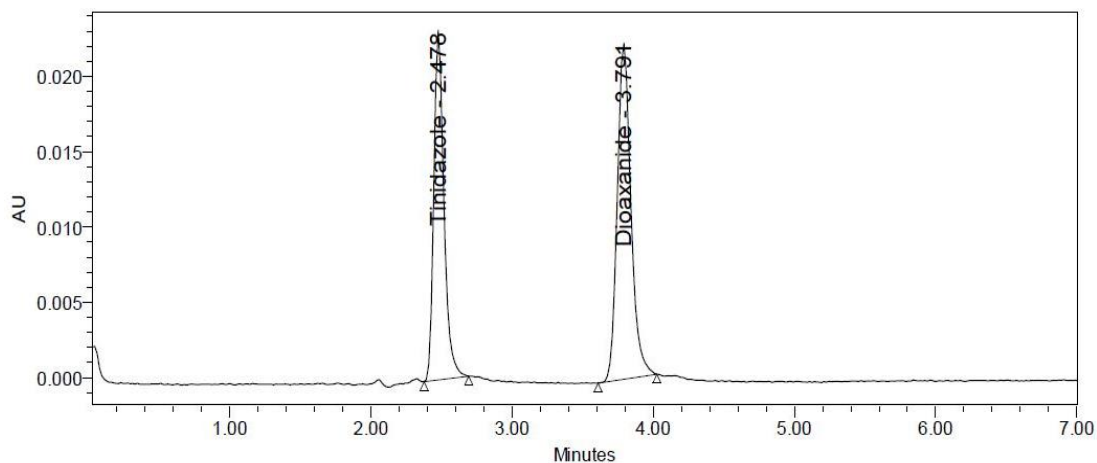
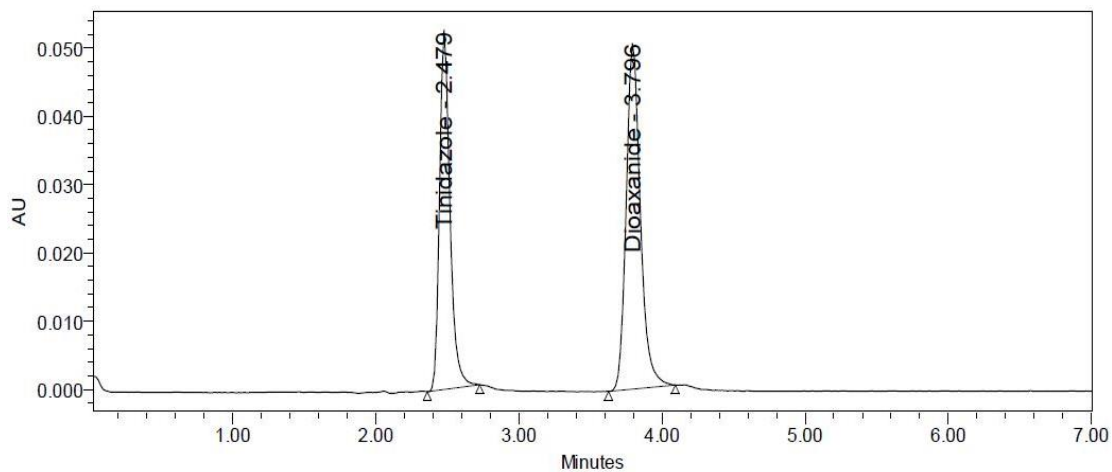
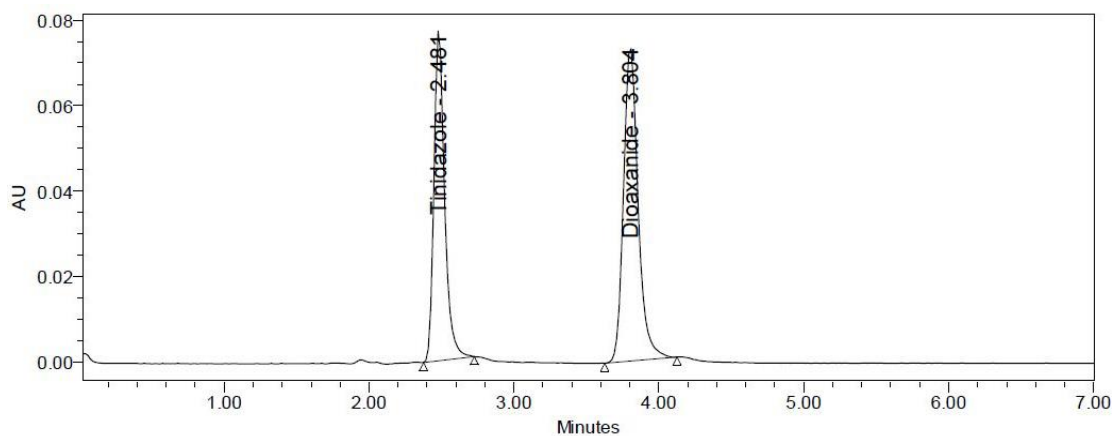
7.3 LINEARITY:**Fig: 5 Chromatogram of Linearity 25% :****Fig: 6 Chromatogram of Linearity 50 % :****Fig: 7 Chromatogram of Linearity 75 % :**

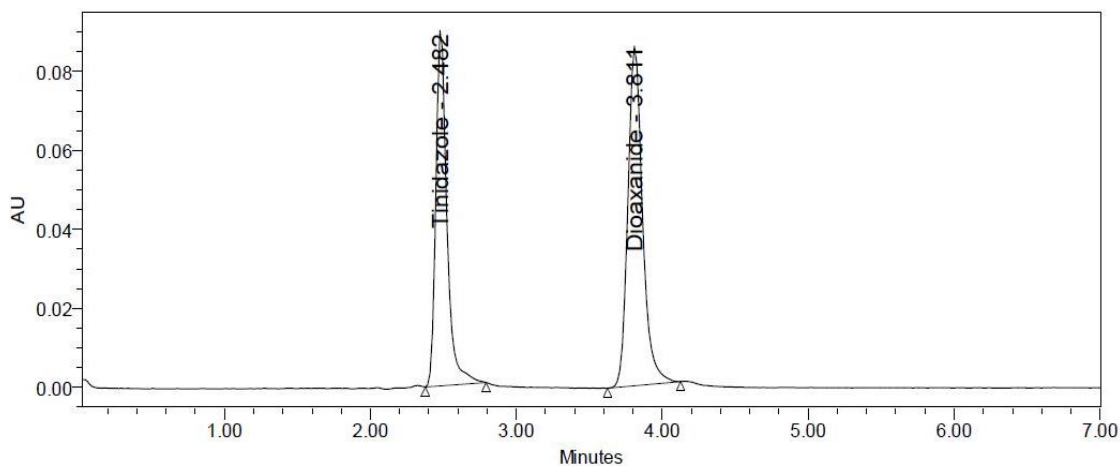
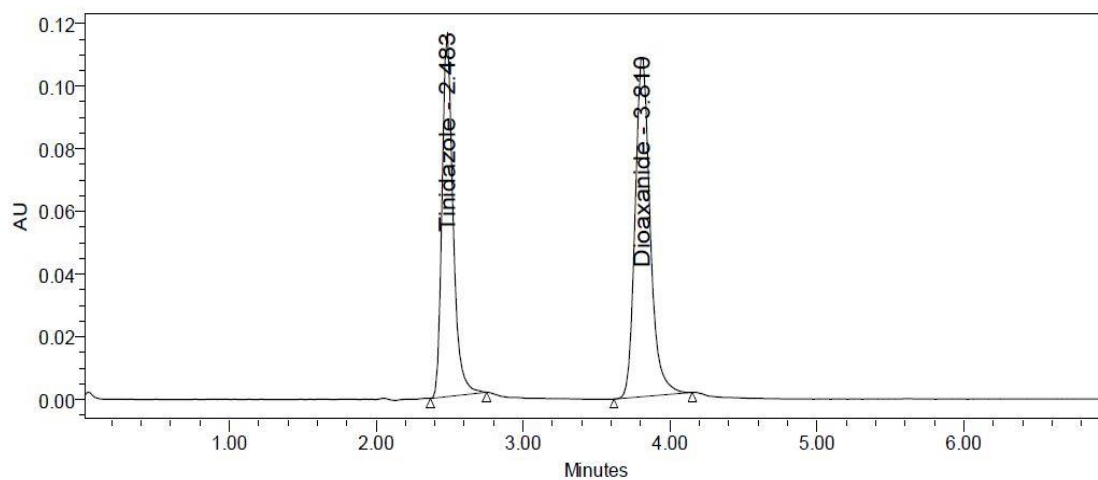
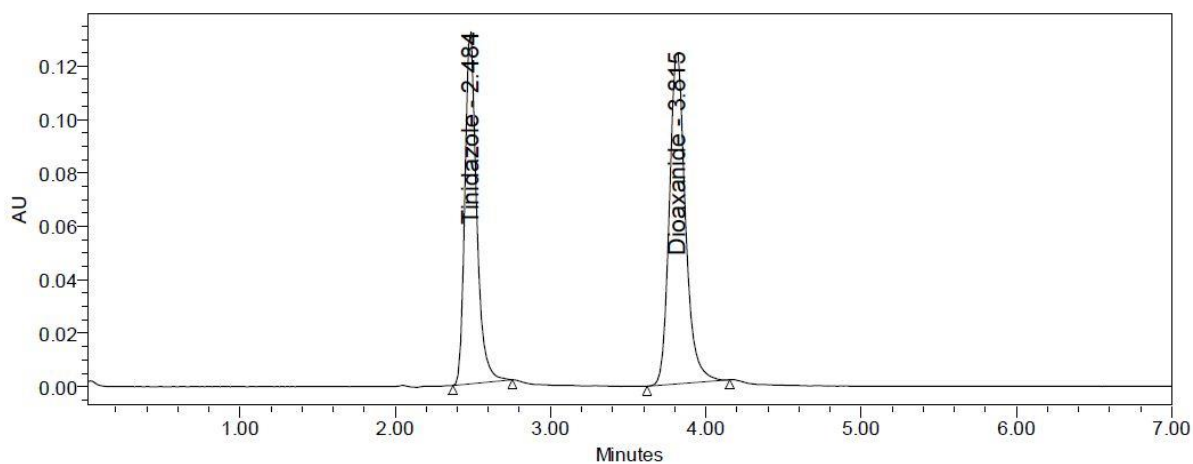
Fig: 8 Chromatogram of linearity 100% :**Fig: 9 Chromatogram of Linearity of 125 %:****Fig: 10 Chromatogram of Linearity of 150 % :**

Table No.7.3 Linearity studies of Tinidazole

S.NO	Concentration ($\mu\text{g/ml}$)	Peak Area
1	30	115108
2	60	235766
3	90	355006
4	120	474538
5	150	600806
6	180	697209
Correlation coefficient(r^2)		0.999
Slope (m)		3930
Intercept		298.1

Fig. No 7.1 Linearity Graph for Tinidazole

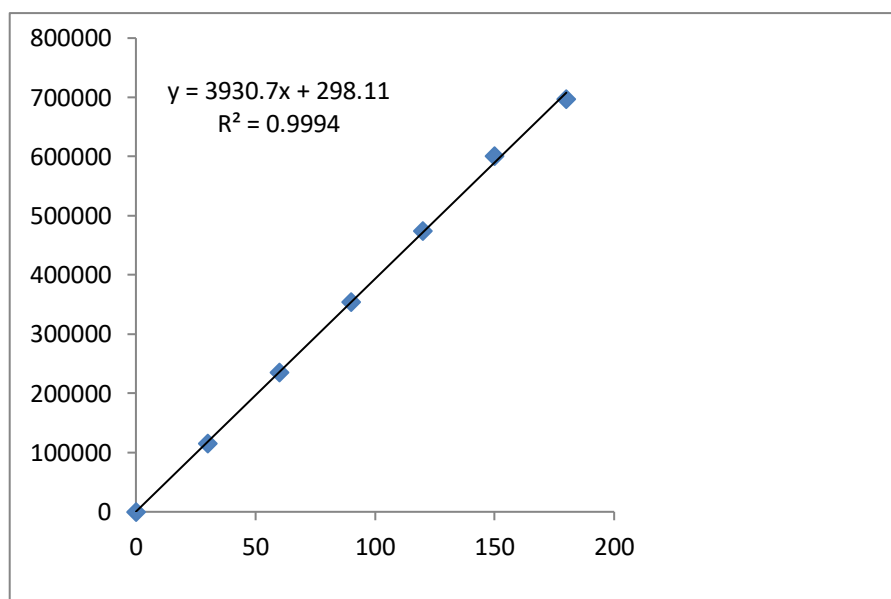
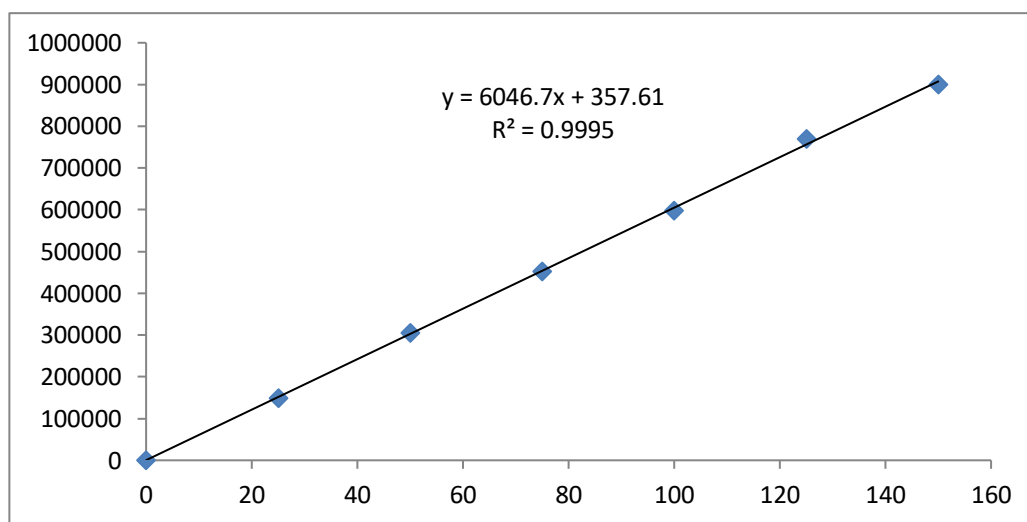


Table.no- 7.4 Linearity of Diloxnide Furoate

S.NO	Concentration ($\mu\text{g/ml}$)	Peak Area
1	25	149734
2	50	305874
3	75	452938
4	100	598634
5	125	770138
6	150	899711
Correlation coefficient(r^2)		0.999
Slope (m)		6046
Intercept		357.6

Fig. No.7.2 Linearity studies for Diloxanide Furoate

**Data interpretation:**

The correlation coefficient (or) regression coefficient should not be less than 0.999. Calculate the correlation coefficient (r^2), y-intercept and slope. From the statistical treatment of linearity data of Tinidazole and Diloxanide Furoate it was clear that the response is linear between lower levels to higher levels. The correlation coefficient was found to be 0.999.

7.4 ACCURACY:

Fig: 11 Chromatogram of Sample 50% (1):

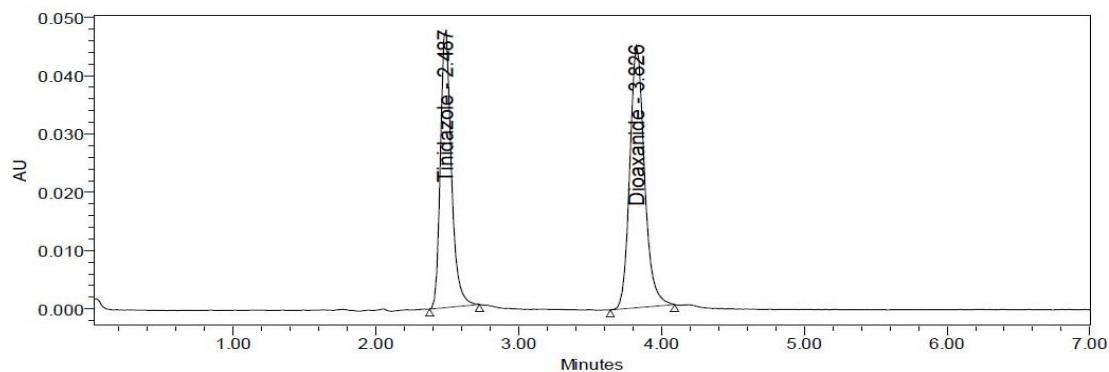


Fig:12 Chromatogram of Sample 50%(2):

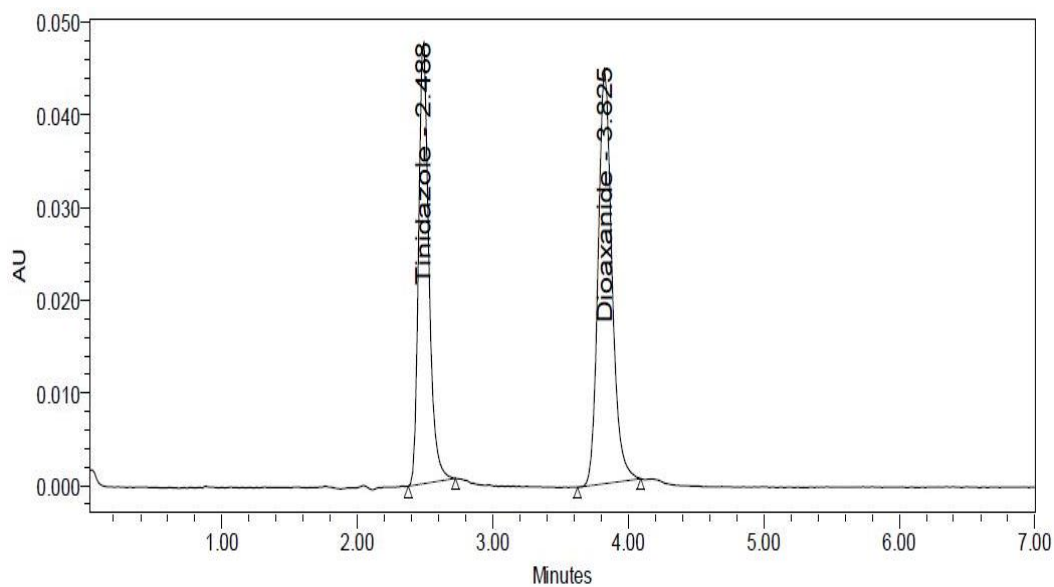


Fig: 13 Chromatogram of Sample 50% (3):

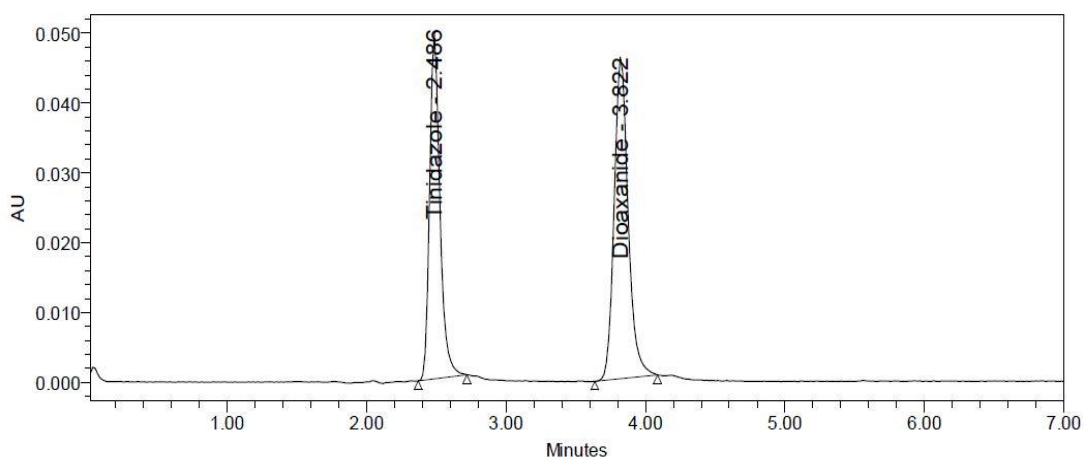


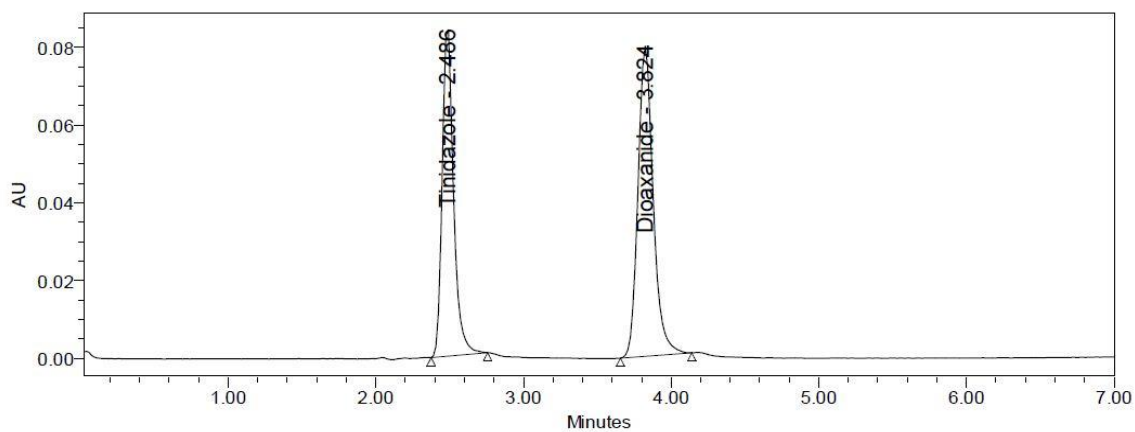
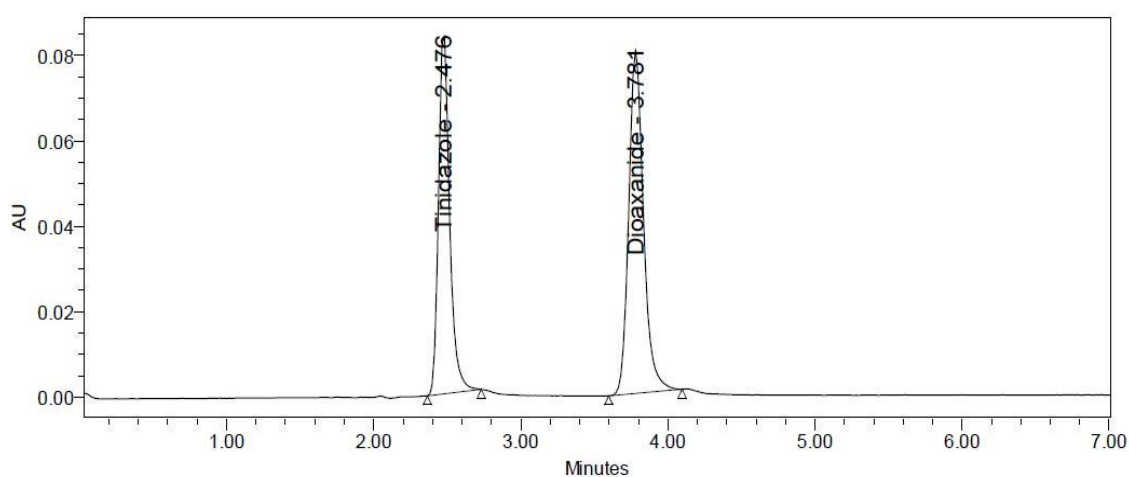
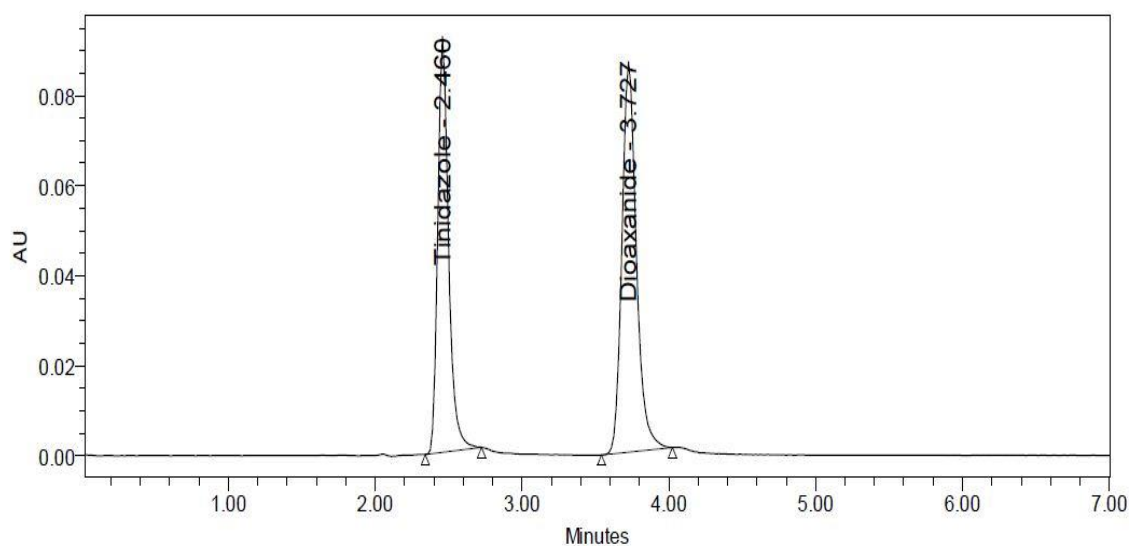
Fig: 14 Chromatogram of Sample 100% (1):**Fig: 15 Chromatogram of Sample 100% (2):****Fig: 16 Chromatogram of Sample 100%(3):**

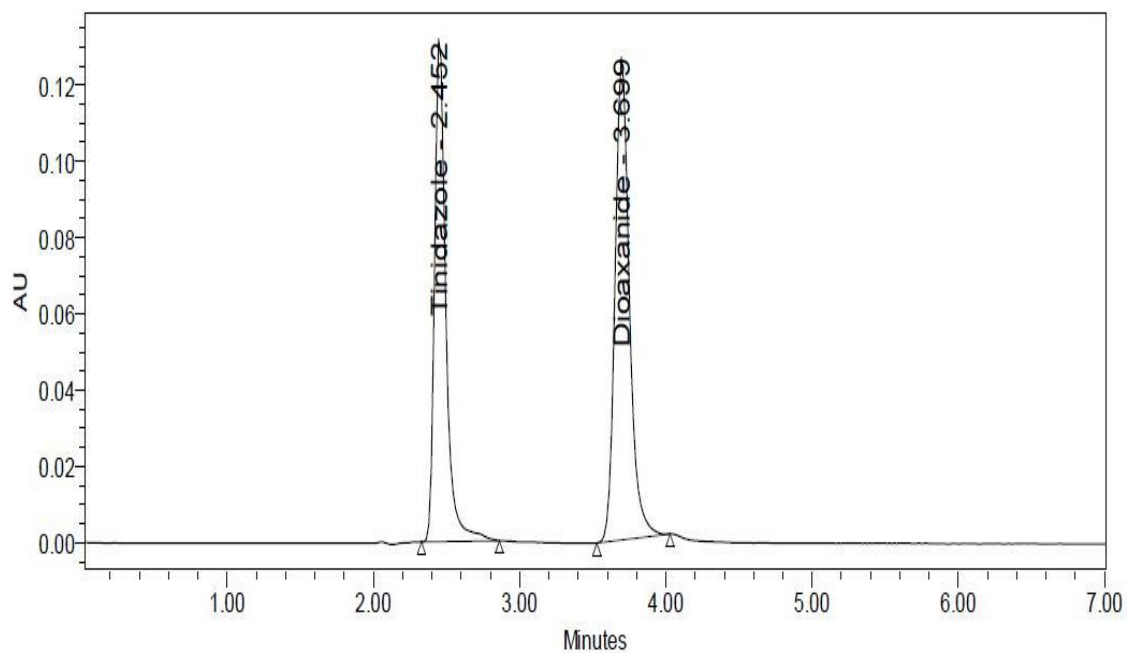
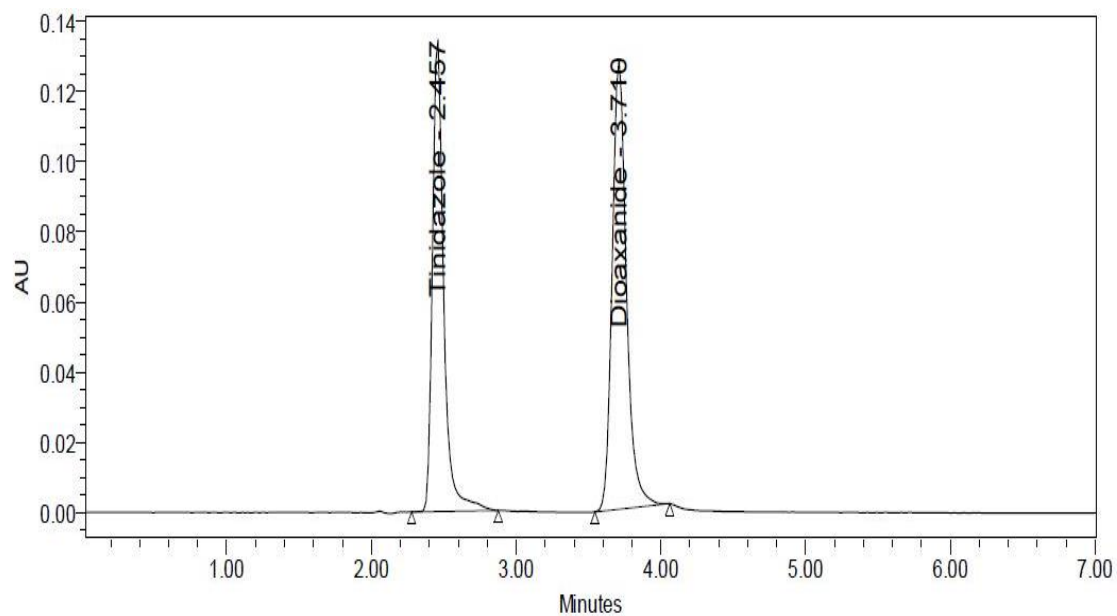
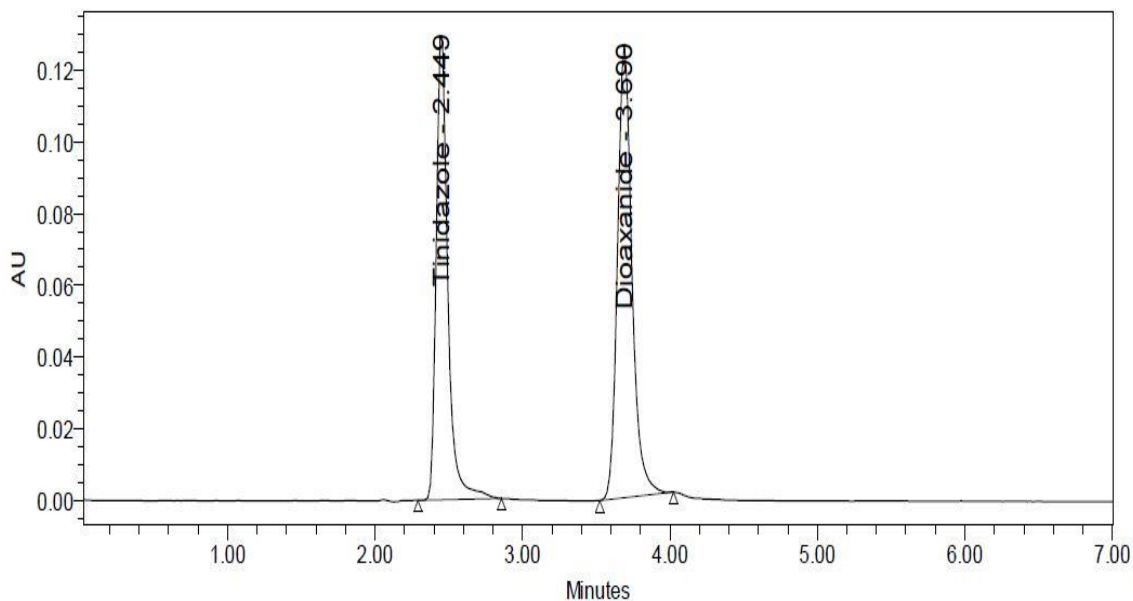
Fig: 17 Chromatogram of Sample 150%(1):**Fig: 18 Chromatogram of Sample 150% (2):**

Fig: 19 Chromatogram of Sample 150% (3):



Sample	Accuracy	Peak Area	% Recovery	Mean % Recovery	Overall Mean %Recovery
Tinidazole	50%	225551	99.75	MEAN=99.85	MEAN= 99.89 S.D = 0.165 %RSD = 0.165
	50%	226374	100.11	S.D = 0.22	
	50%	225424	99.69	%RSD = 0.22	
	100%	454713	100.55	MEAN=100.08	
	100%	451882	99.92	S.D = 0.40	
	100%	451282	99.79	%RSD = 0.39	
	150%	674477	99.43	MEAN=99.76	
	150%	677286	99.84	S.D = 0.30	
	150%	678582	100.03	%RSD = 0.30	
Diloxanide Furoate	50%	300874	100.66	MEAN=100.21	MEAN= 100.11 S.D = 0.095 %RSD = 0.09
	50%	297250	99.45	S.D = 0.66	
	50%	300525	100.54	%RSD = 0.65	
	100%	593292	99.24	MEAN = 100.02	
	100%	600461	100.44	S.D = 0.67	
	100%	600065	100.38	%RSD = 0.66	
	150%	899800	100.34	MEAN=100.12	
	150%	899510	100.31	S.D = 0.34	
	150%	894341	99.73	%RSD = 0.33	

Table No 7.5 Accuracy for Tinidazole and Diloxanide Furoate

Data interpretation

The Mean % Recovery at 50%,100% and 150% at higher levels for Tinidazole and Diloxanide Furoate were found to be between 99.89 % and 100.11% which were within the acceptance criteria limit.

The Mean % Recovery for Tinidazole at 50%,100% and 150% levels was found to be 99.85%, 100.08% and 99.76% respectively and are within the limits.

The Mean % Recovery for Diloxanide furoate at 50%,100% and 150% levels was found to be 100.21%, 100.02% and 100.12% respectively and are within the limits.

The excellent mean recoveries and standard deviation suggested that the good accuracy of the proposed method.

7.6 PRECISION:

i) System Precision:

Fig: 20 Chromatogram of System Precision (1):

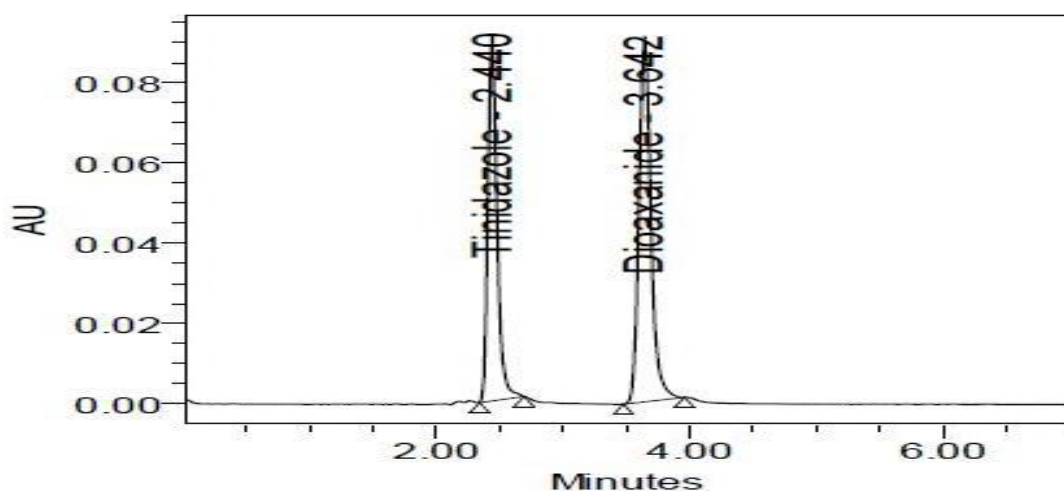


Fig: 21 Chromatogram of System Precision (2):

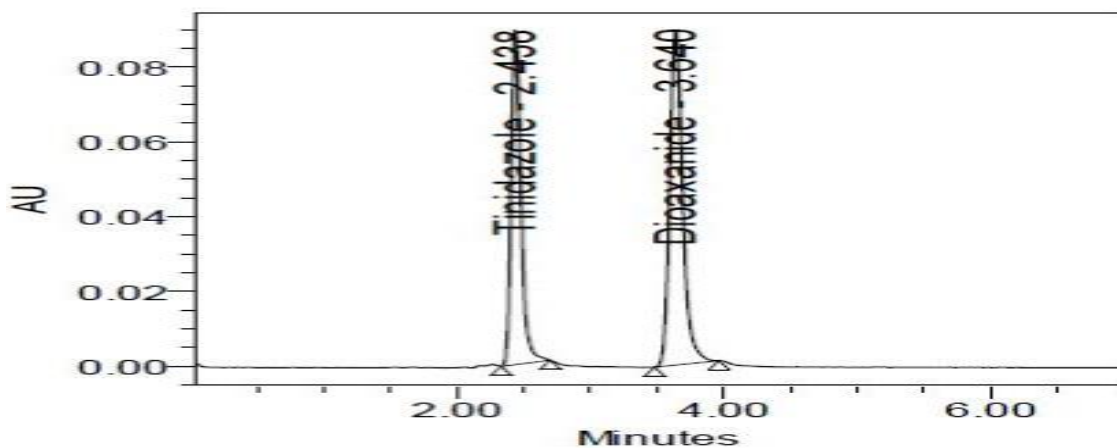


Fig: 22 Chromatogram of System Precision (3):

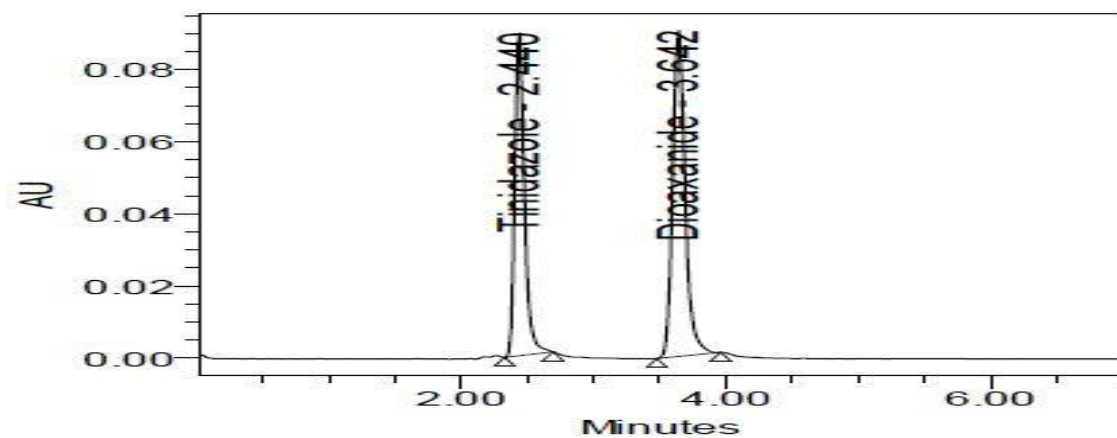


Fig: 23 Chromatogram of System Precision (4):

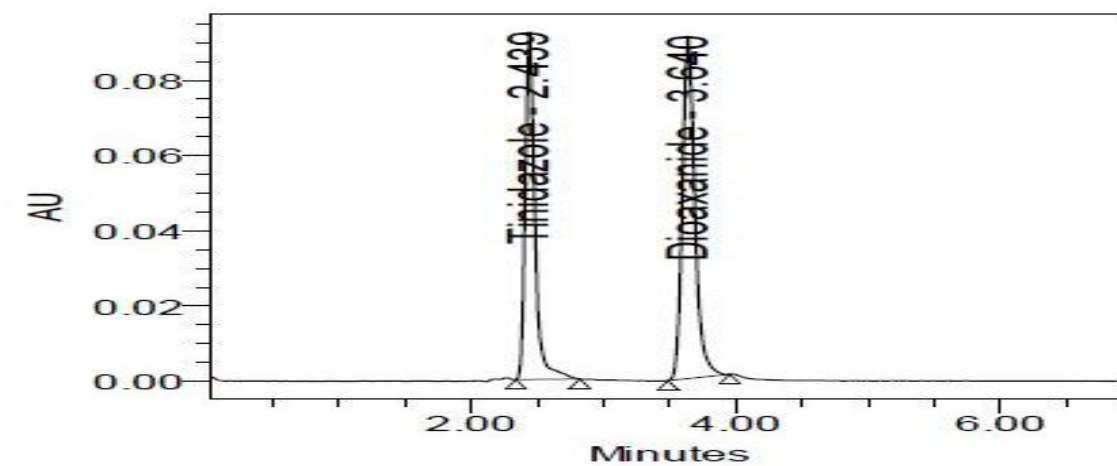


Fig: 24 Chromatogram of System Precision (5):

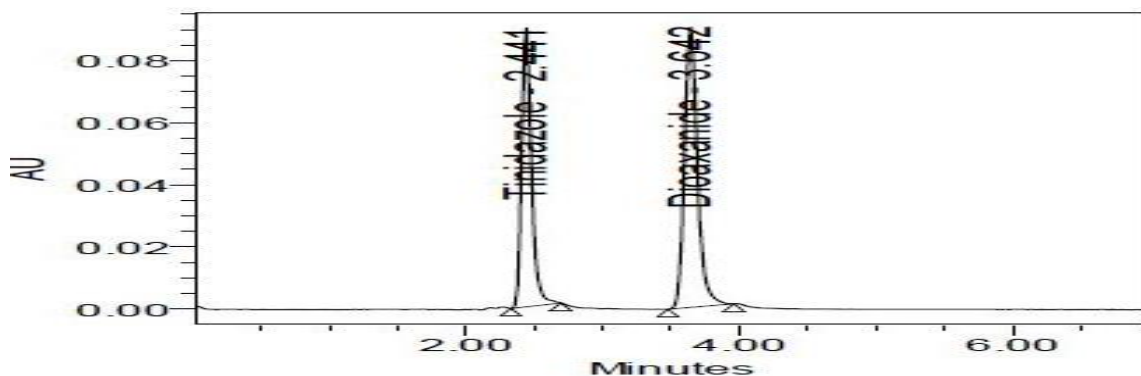


Fig: 25 Chromatogram of System Precision (6):

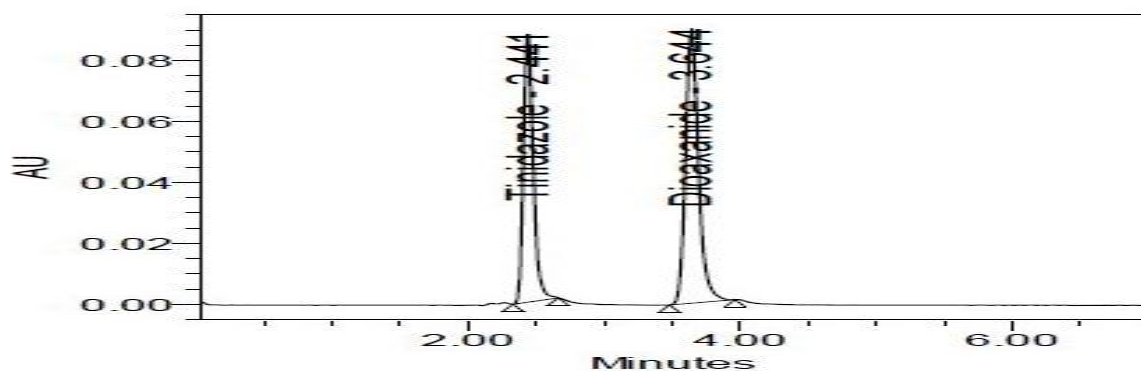


Table No.7.7 System Precision of Tinidazole and Diloxanide furoate:

S No	Tinidazole		Diloxanide Furoate	
	RT	Area	RT	Area
1	2.438	453961	3.640	593318
2	2.439	450640	3.640	600062
3	2.440	455090	3.642	594119
4	2.440	450337	3.642	599686
5	2.441	444217	3.642	592891
	2.441	448218	3.644	592273
Avg		450441		595392
Std Dev		3942.8		3526
RSD		0.9		0.6

Data interpretation:

The retention time and area for Tinidazole and Diloxanide furoate peaks obtained from six replicate injections are consistent as evidenced by the values of relative standard deviation. Hence it can be concluded that the system precision parameter meets the requirement of method validation.

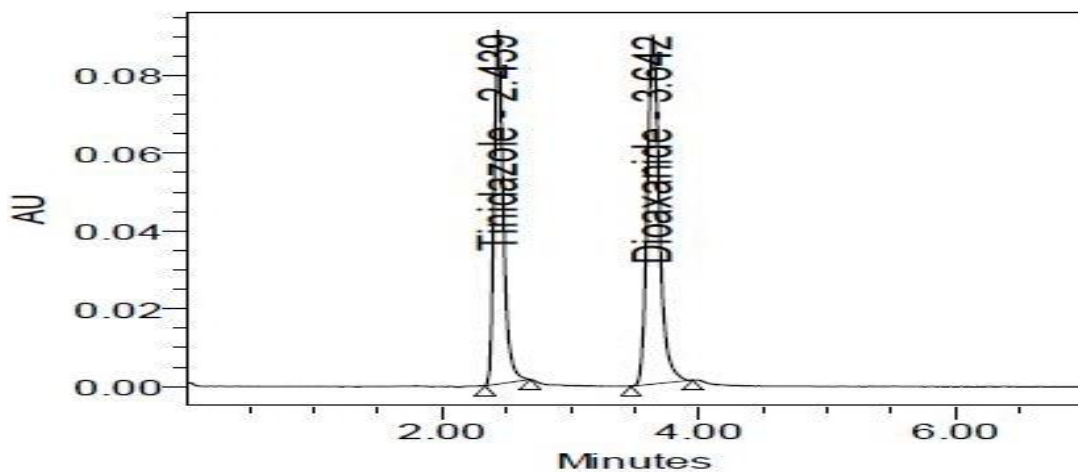
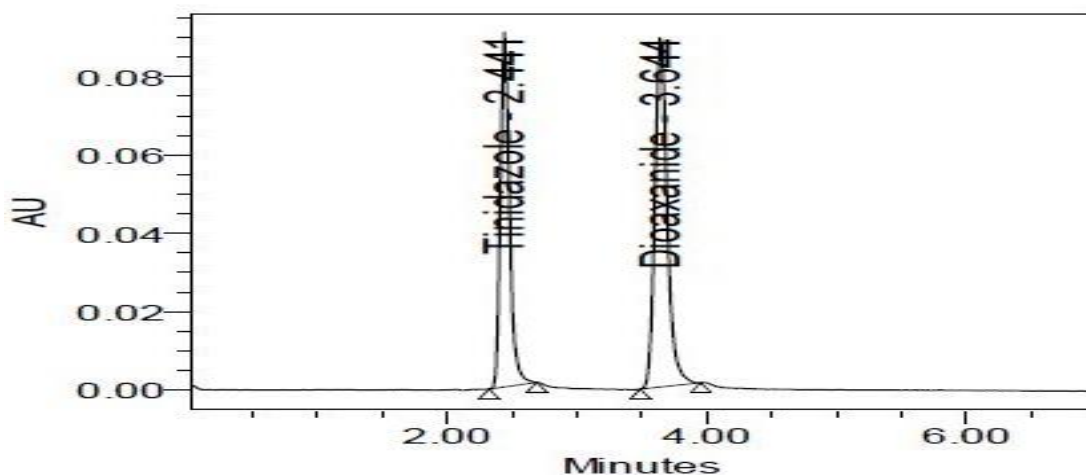
ii) Method Precision:**Fig: 26 Chromatogram of Method Precision (1):****Fig: 27 Chromatogram of Method Precision (2):**

Fig: 28 Chromatogram of Method Precision (3):

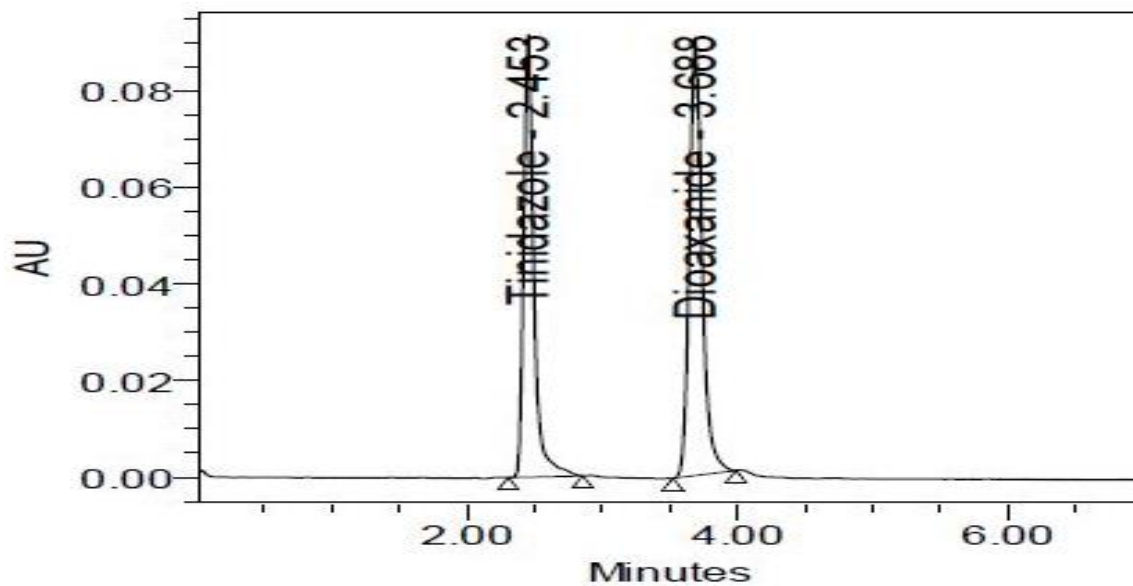


Fig: 29 Chromatogram of Method Precision (4):

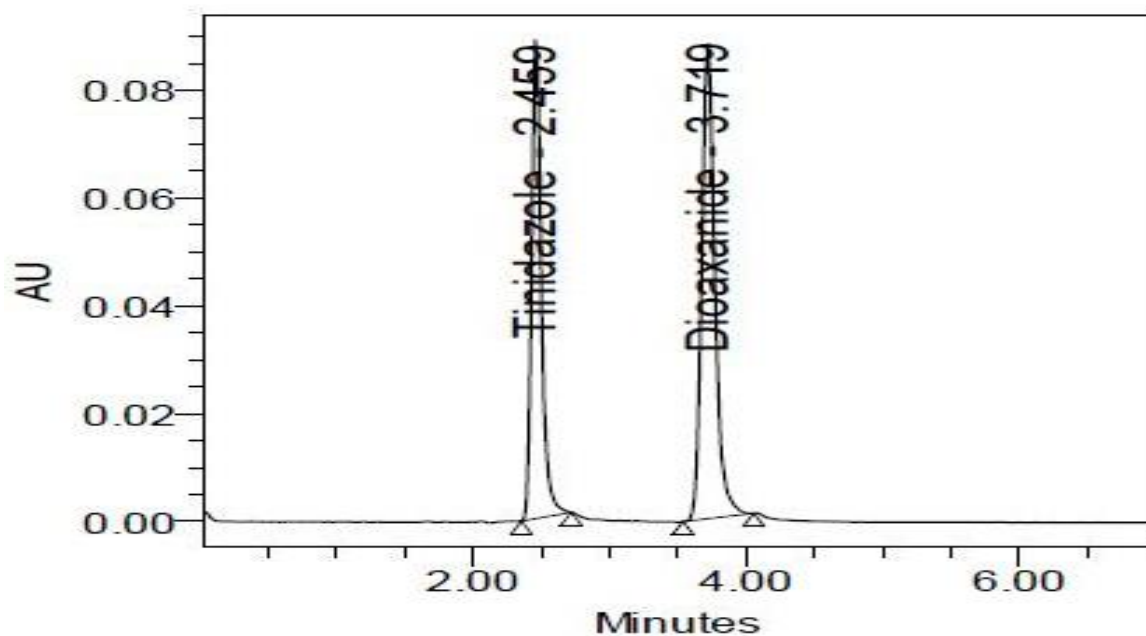


Fig: 30 Chromatogram of Method Precision (5):

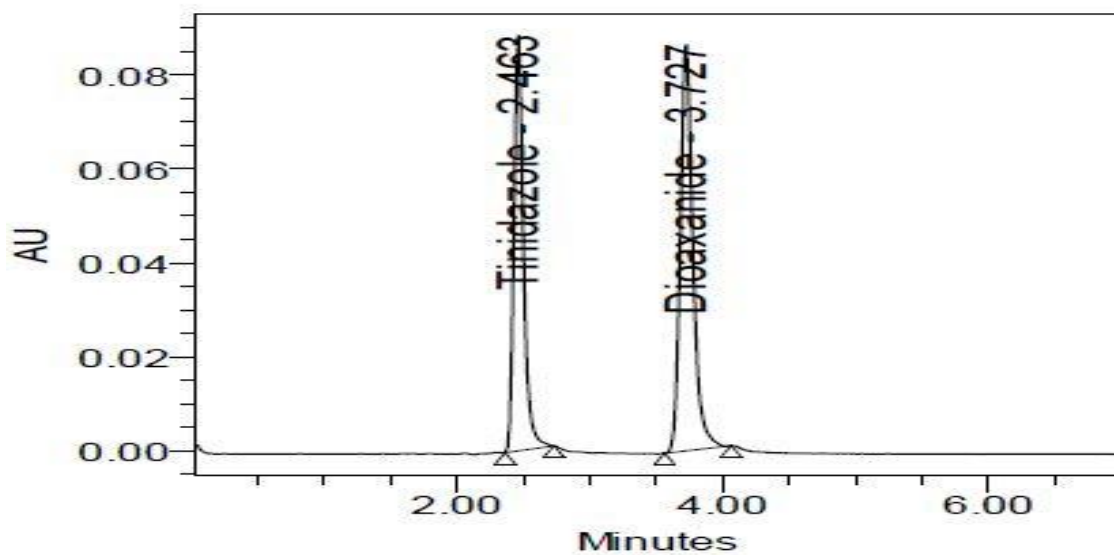


Fig: 31 Chromatogram of Method Precision 6:

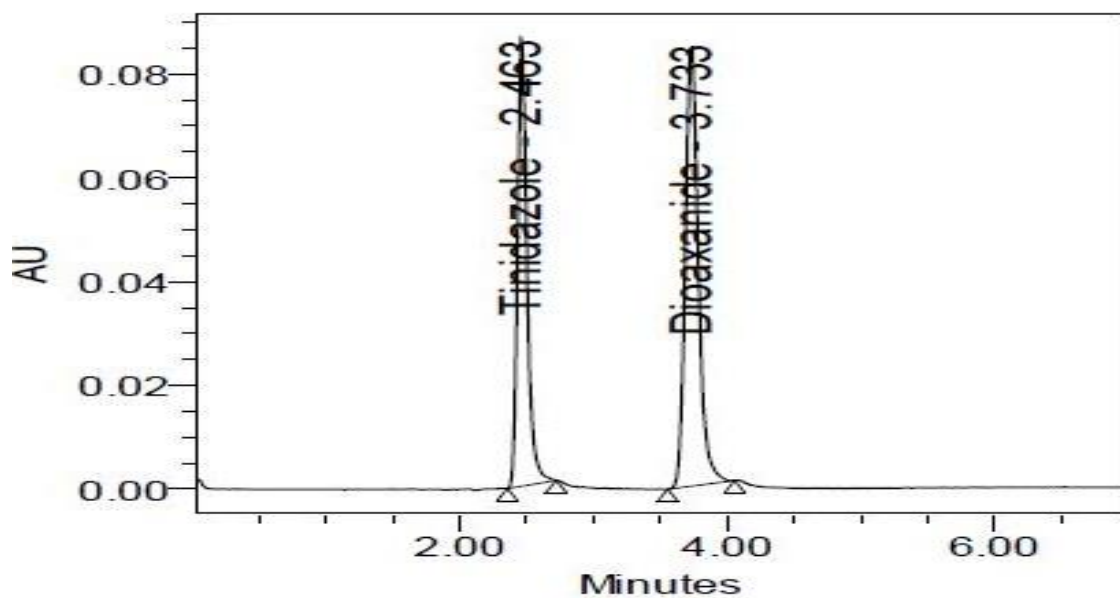


Table No.7.8 Method Precision of Tinidazole and Diloxanide Furoate :

S No	Tinidazole		DiloxanideFuroate	
	RT	Area	RT	Area
1	2.439	447916	3.642	594845
2	2.441	448928	3.644	595903
3	2.453	454769	3.688	601566
4	2.459	448153	3.719	591800
5	2.463	450212	3.727	592833
6	2.463	452618	3.733	598582
Avg		450433		595922
Std Dev		2736		3650
% RSD		0.6		0.6

Data interpretation:

Test results Tinidazole and Diloxanide Furoate were shown that the % RSD of peak areas and retention times are within limits. Hence it can be concluded that the method is precise.

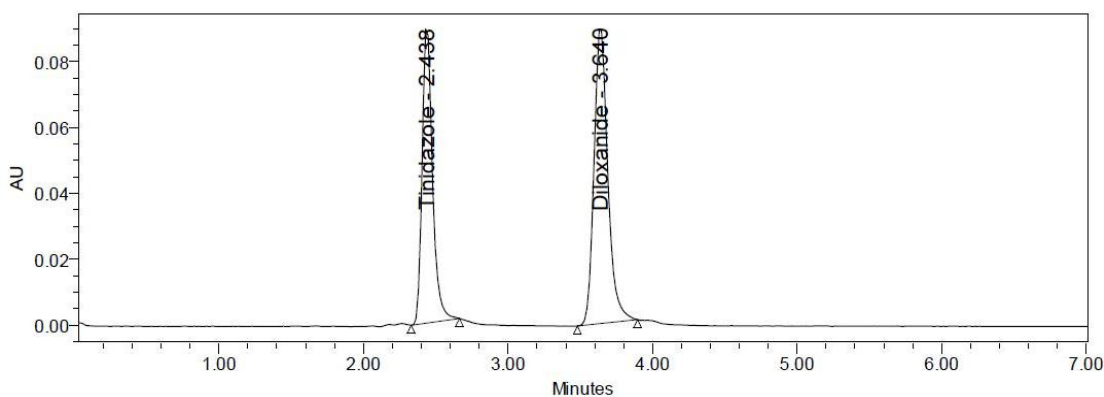
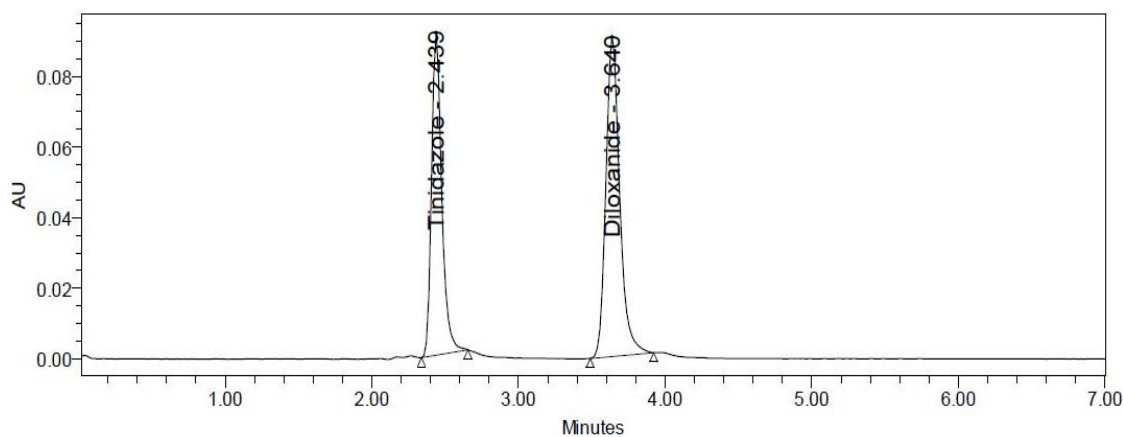
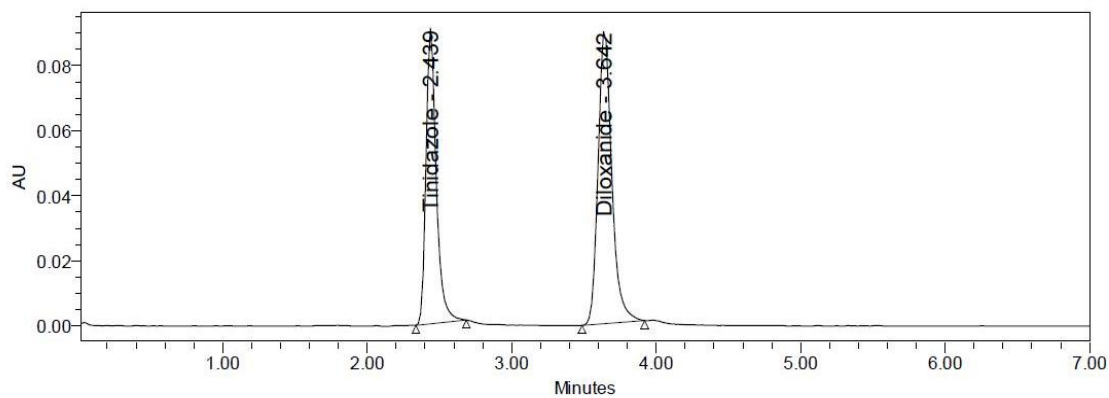
7.9 Intermediate Precision:**Intermediate Precision Day - 1:****Fig: 32 Chromatogram Intermediate Precision Day - 1: (1)****Fig: 33 Chromatogram Intermediate Precision Day - 1: (2)****Fig: 34 Chromatogram Intermediate Precision Day - 1: (3)**

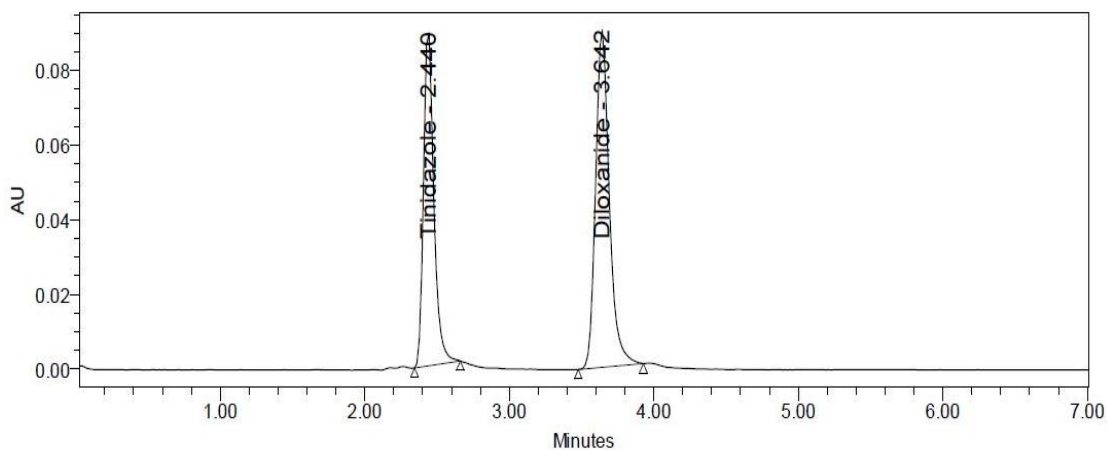
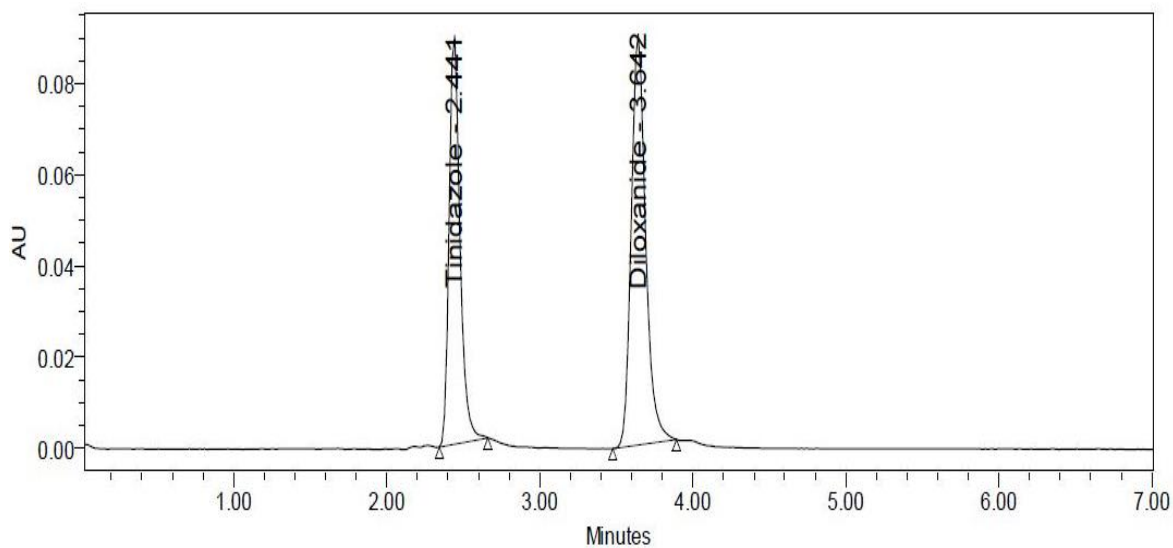
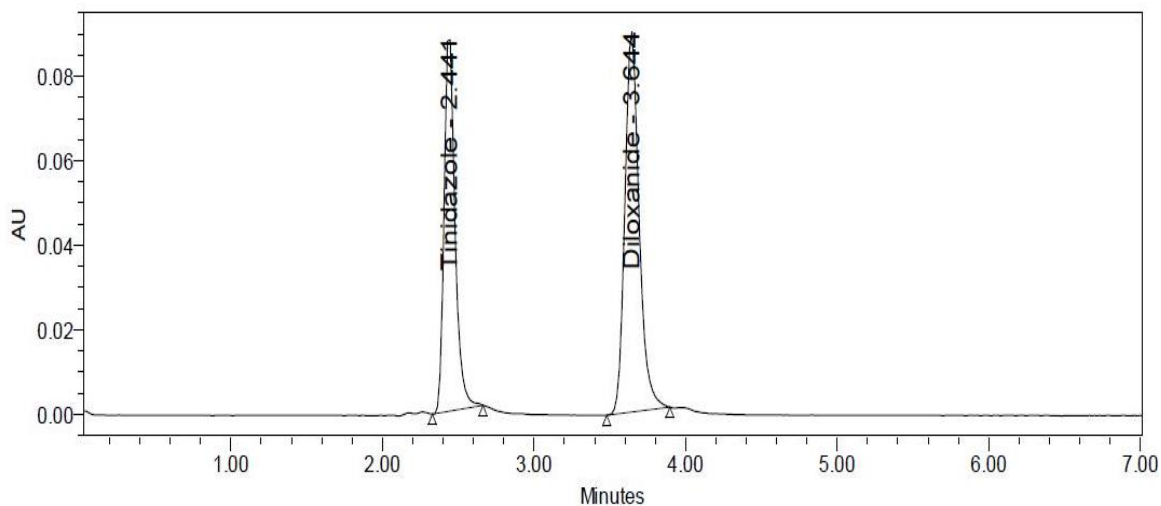
Fig: 35 Chromatogram Intermediate Precision Day - 1: (4)**Fig: 36 Chromatogram Intermediate Precision Day - 1:(5)****Fig: 37 Chromatogram Intermediate Precision Day - 1: (6)**

Table No 7.10 Intermediate Precision Day – 1 :

S No	Tinidazole		Diloxanide Furoate	
	RT	Area	RT	Area
1	2.438	448247	3.640	592102
2	2.439	447916	3.640	593877
3	2.439	442242	3.642	593144
4	2.440	444679	3.642	593671
5	2.441	444217	3.642	586570
	2.441	442305	3.644	587384
Avg		444934		591125
Std Dev		2631.0		3281.3
% RSD		0.6		0.6

Chromatogram of Intermediate Precision Day – 2:

Fig: 38 Chromatogram of Intermediate Precision Day – 2 (1)

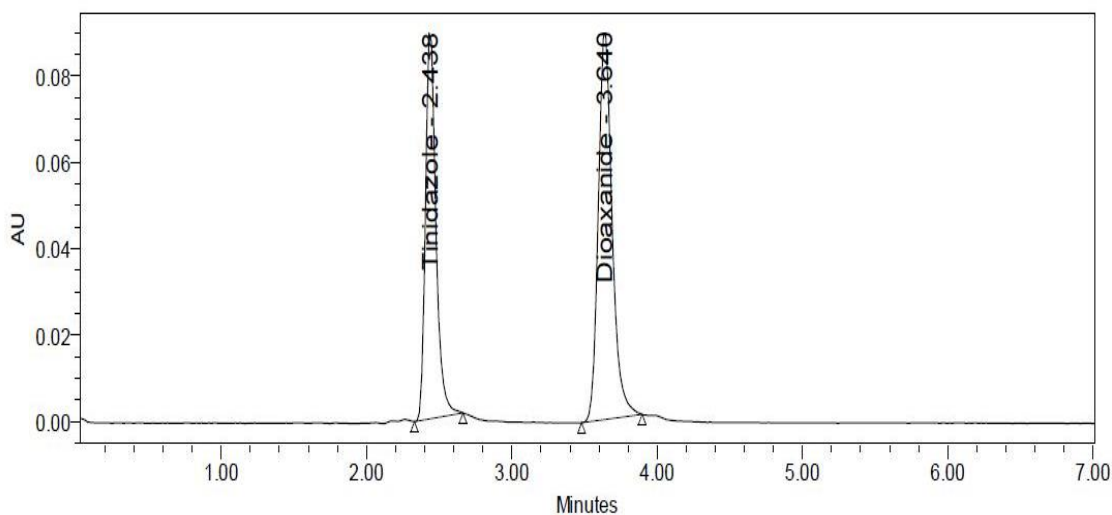


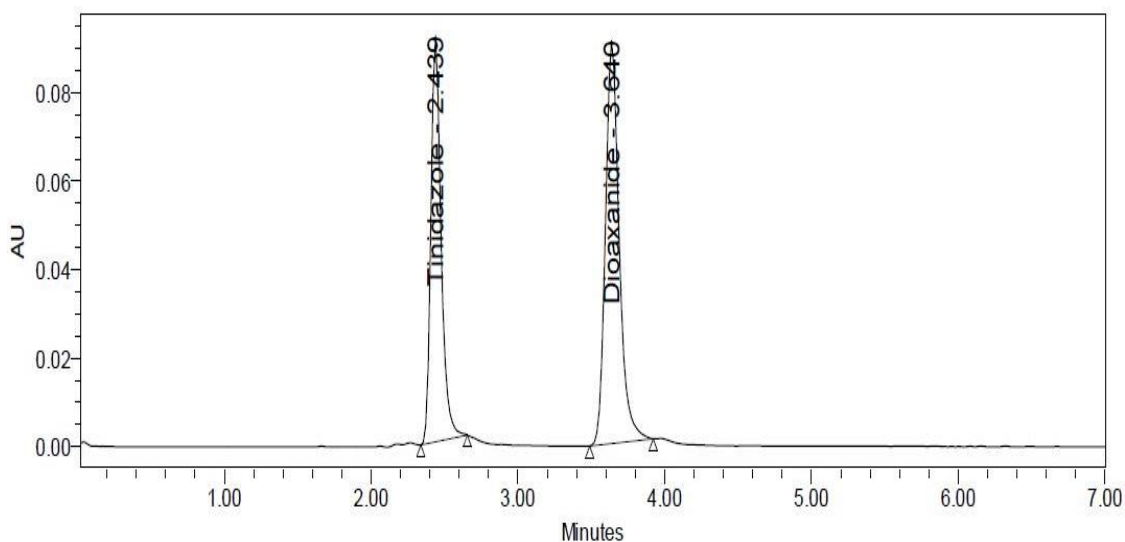
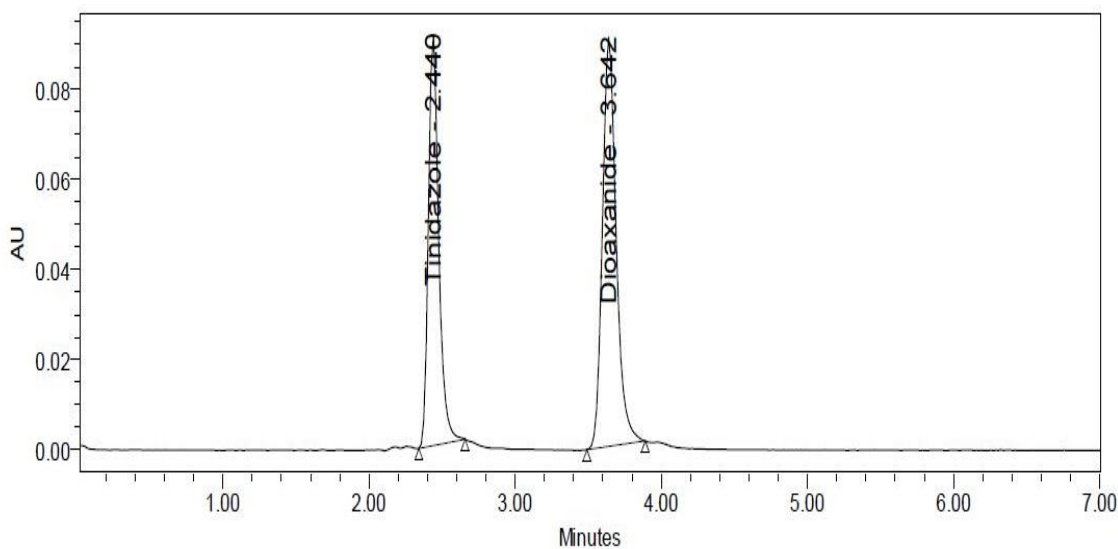
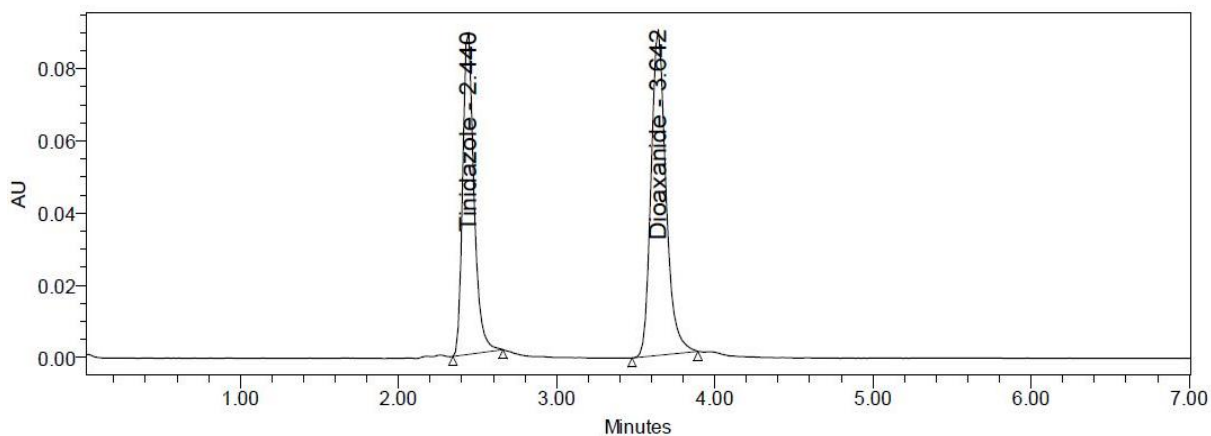
Fig: 39 Chromatogram of Intermediate Precision Day – 2 (2)**Fig: 40 Chromatogram of Intermediate Precision Day – 2 (3)****Fig: 41 Chromatogram of Intermediate Precision Day – 2 (4)**

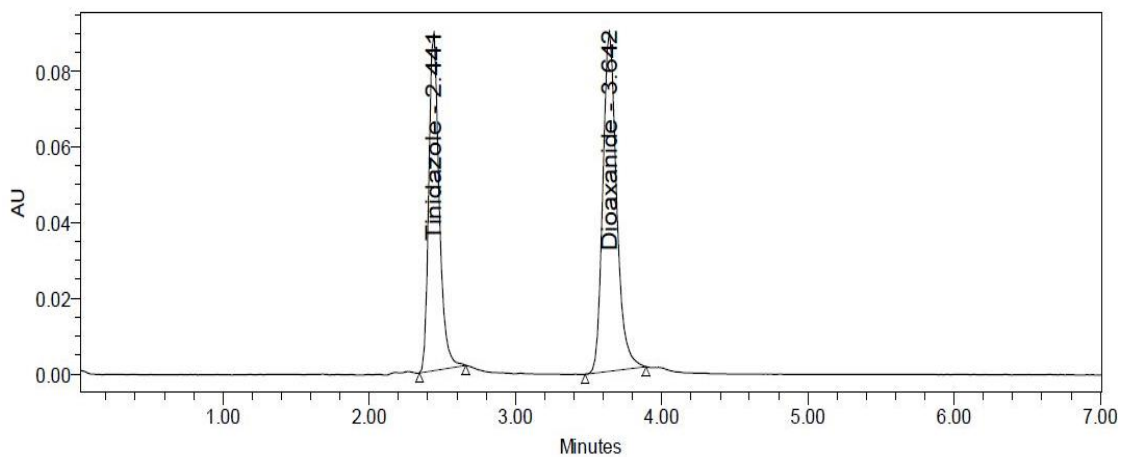
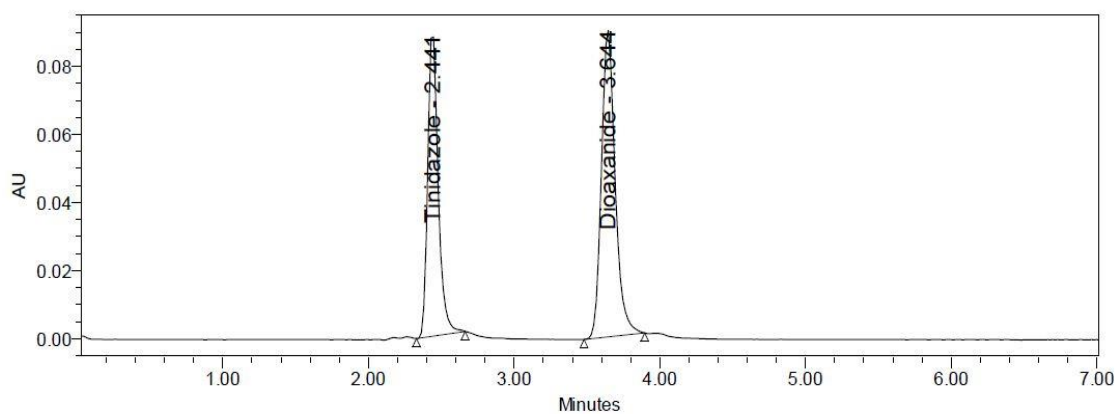
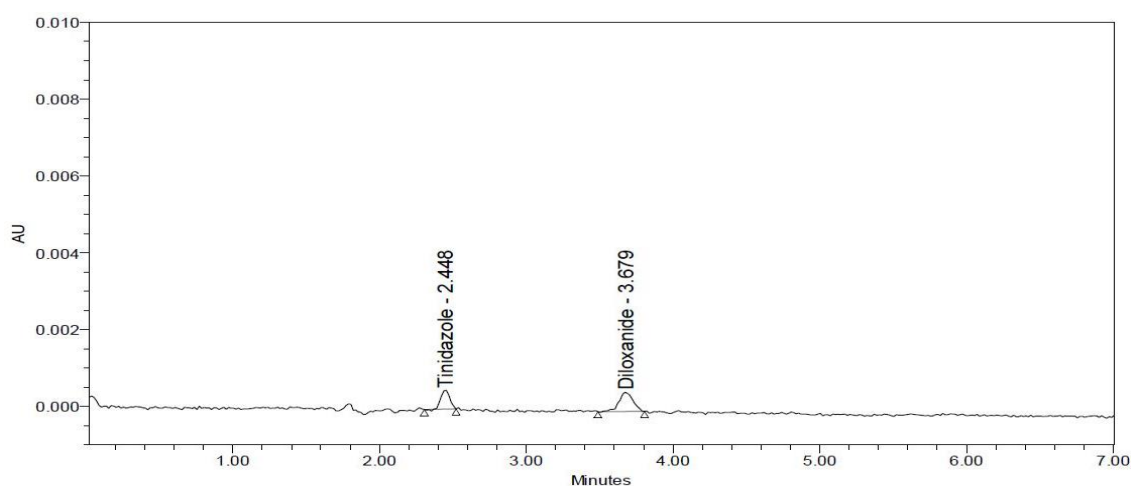
Fig: 42 Chromatogram of Intermediate Precision Day – 2 (5)**Fig: 43 Chromatogram of Intermediate Precision Day – 2 (6)**

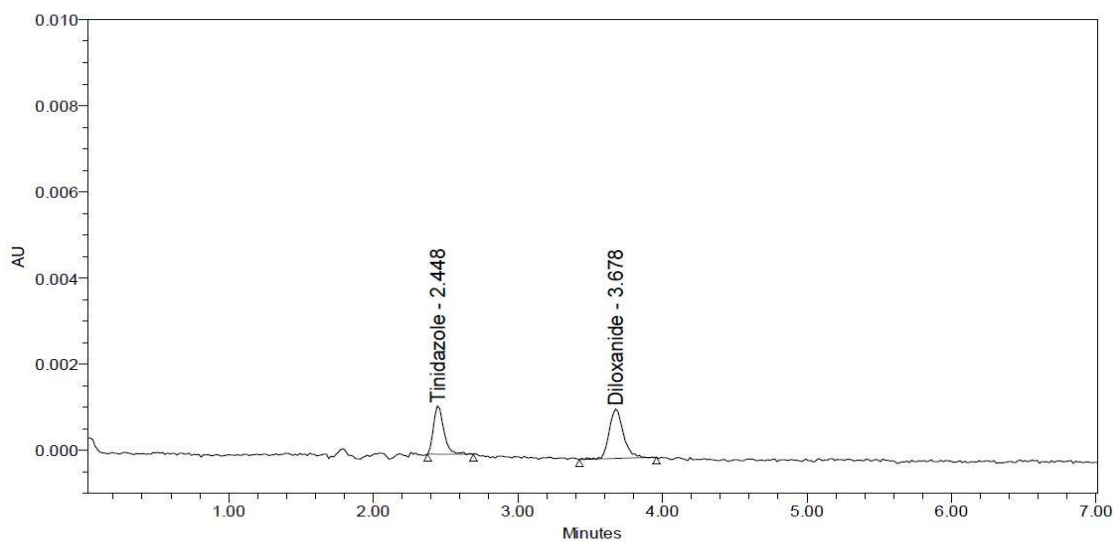
Table No 7.11 Intermediate Precision Day – 2 :

S No	Tinidazole		Diloxanide Furoate	
	RT	Area	RT	Area
1	2.438	448247	3.640	592102
2	2.439	442242	3.640	593877
3	2.440	449008	3.642	589066
4	2.440	444679	3.642	592811
5	2.441	444217	3.642	586570
	2.441	442305	3.644	587384
Avg		445116		590302
Std Dev		2902.1		3042.6
% RSD		0.7		0.5

7.12 LIMIT OF DETECTION:

Fig 44 Chromatogram of Standard:



7.13 LIMIT OF QUANTIFICATION:**Fig 45 Chromatogram of Standard:****Tinidazole:****LOD = 3.3 F/S**

$$= 3.3 \times 298.1/3930 = 0.25$$

LOQ = 10 F/S

$$= 10 \times 298.1/3930 = 0.76$$

Diloxanide Furoate:**LOD = 3.3 F/S**

$$= 3.3 \times 357.6/6046 = 0.20$$

LOQ = 10 F/S

$$= 10 \times 357.6/6046 = 0.59$$

7.14 ROBUSTNESS:

Fig 46 Chromatogram of Flow 1 (0.9 ml/min):

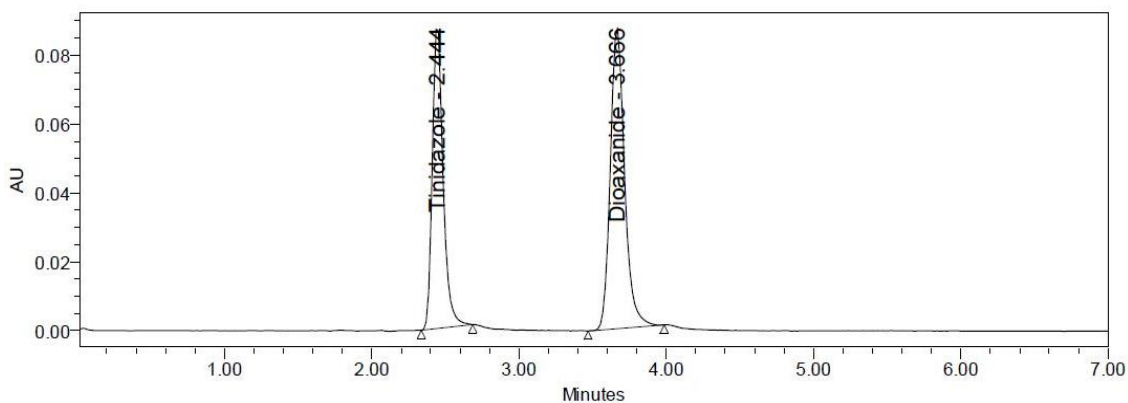


Fig 47 Chromatogram of Flow 2 (1.1 ml/min):

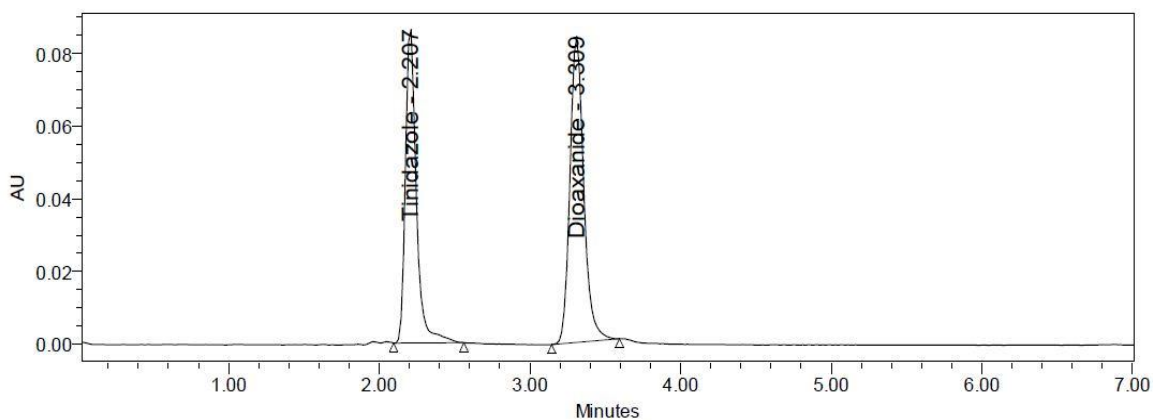
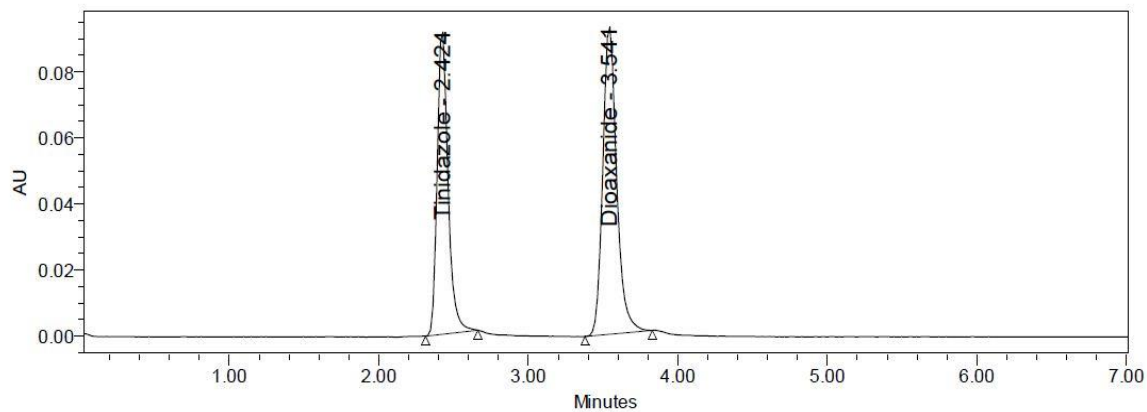
Fig 48 Chromatogram of Temp 1 (25⁰c):

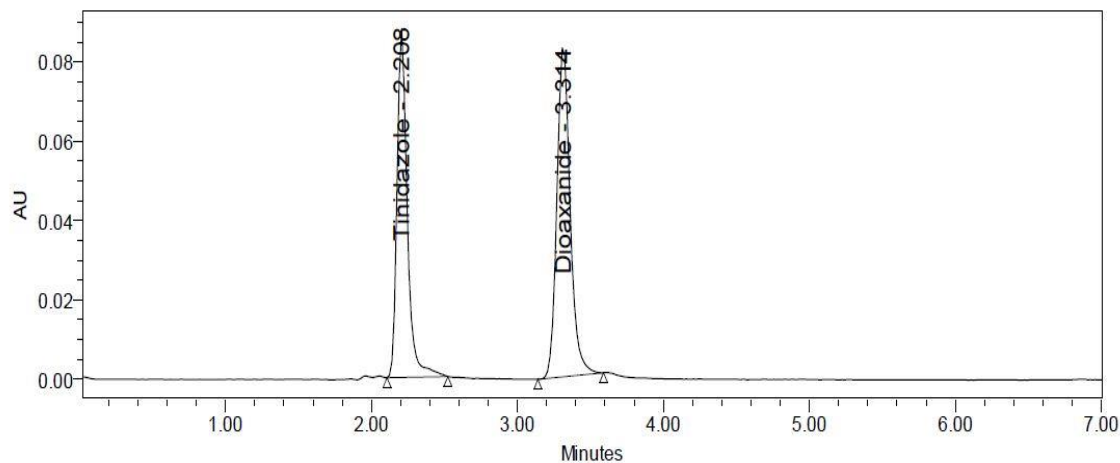
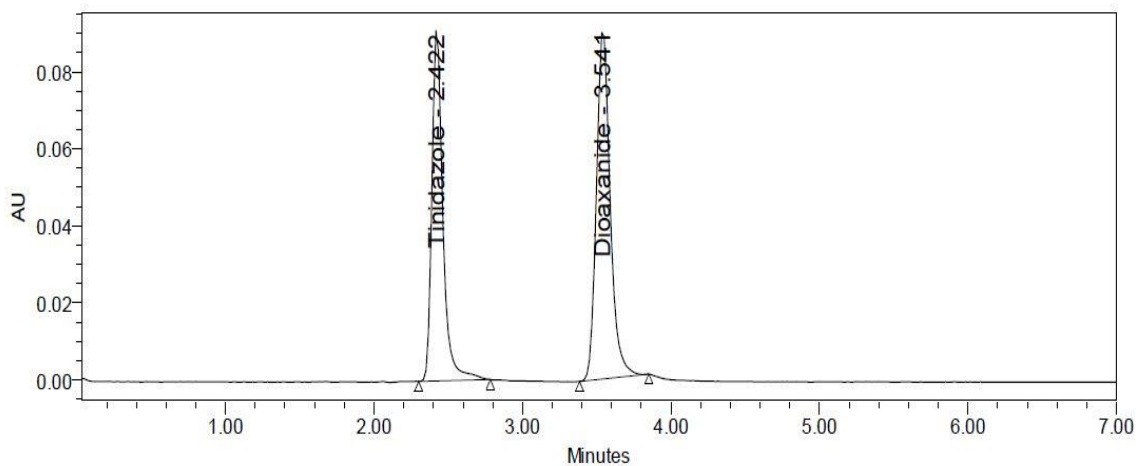
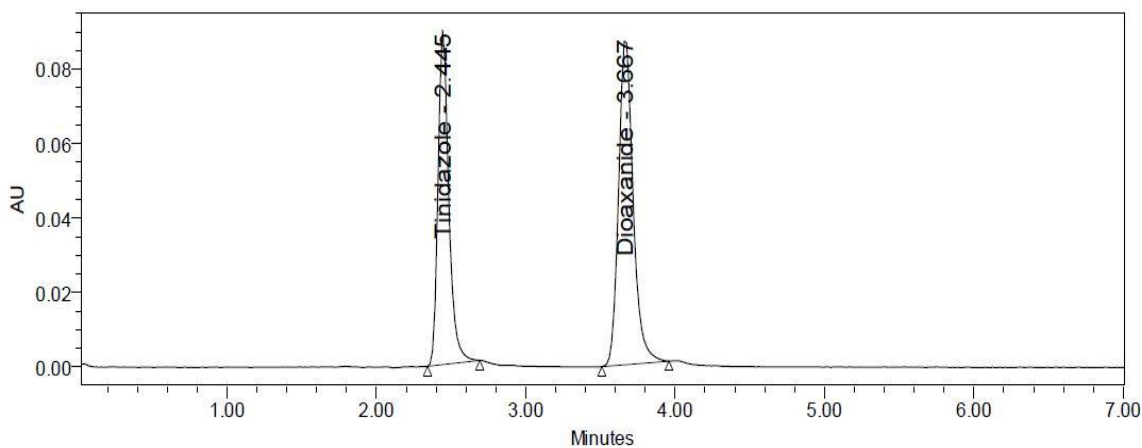
Fig 49 Chromatogram of Temp 2 (35° c):**Fig 50 Chromatogram of mobile phase 1 (50:50 % V/V):****Fig 51 Chromatogram of mobile phase 2 (40:60 % V/V):**

Table. No 7.15 Robustness of Tinidazole and Diloxanide Furoate :

S No	Parameter	Tinidazole			Diloxanide Furoate		
		RT	Area	Tailing factor	RT	Area	Tailing factor
1	Standard						
2	Robustness-Flow-1	2.444	445197	1.25	3.666	586483	1.16
3	Robustness-Flow-2	2.207	434640	1.28	3.309	533051	1.15
4	Robustness- change in mobile phase_1	2.422	466627	1.33	3.541	581740	1.19
5	Robustness-change in mobile phase_2	2.445	449894	1.28	3.666	592388	1.18
6	Robustness-change in temp _1	2.424	466627	1.33	3.541	581740	1.19
7	Robustness-change in temp _2	2.208	434640	1.28	3.314	533051	1.16

Data interpretation:

It was found that the system suitability parameters were within the limits at all variable conditions. From the results obtained it can be concluded that, this method is robust towards small variations in method parameters.

7.16 CHROMATOGRAMS OF ASSAY:

Fig 52 Chromatogram of standard 1:

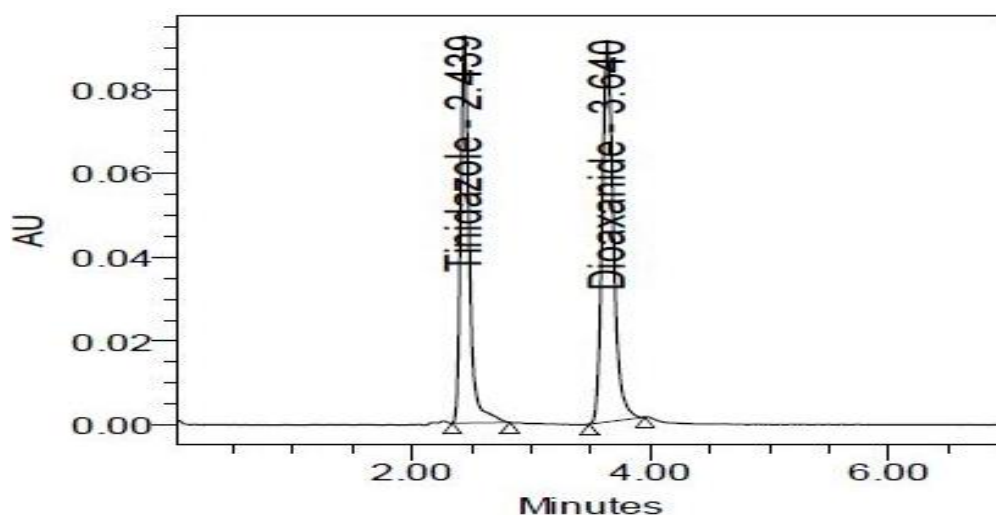


Fig 53 Chromatogram of standard 2:

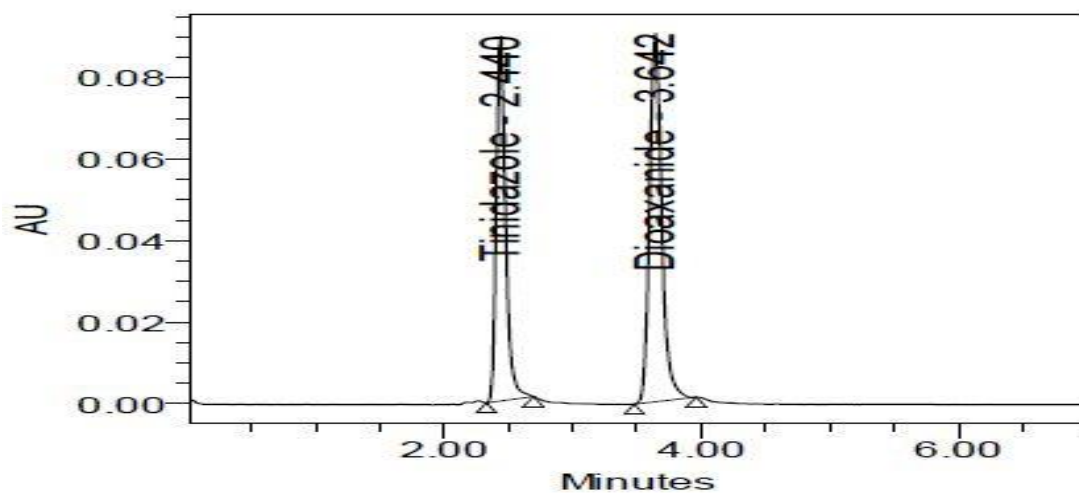


Fig 54 Chromatogram of sample 1:

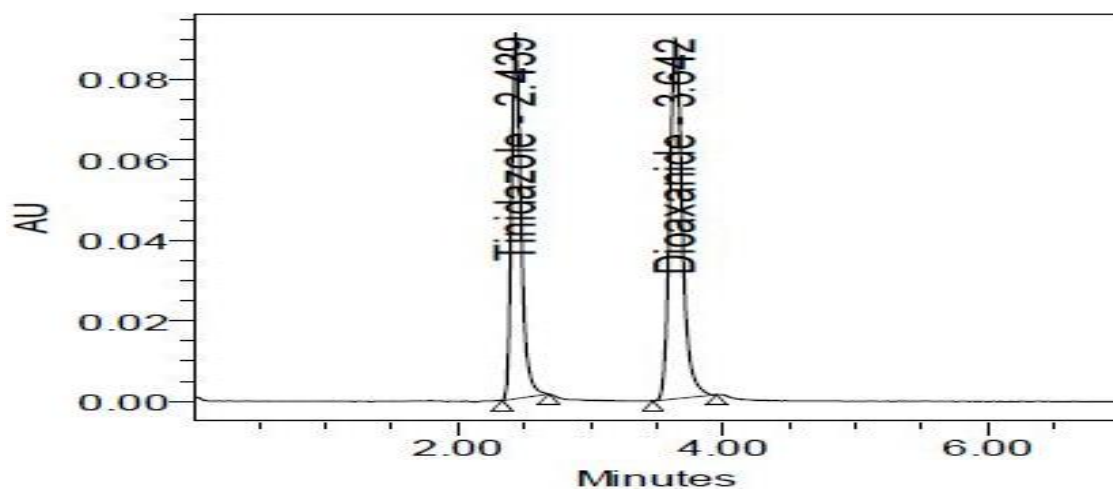


Fig 55 Chromatogram of sample 2:

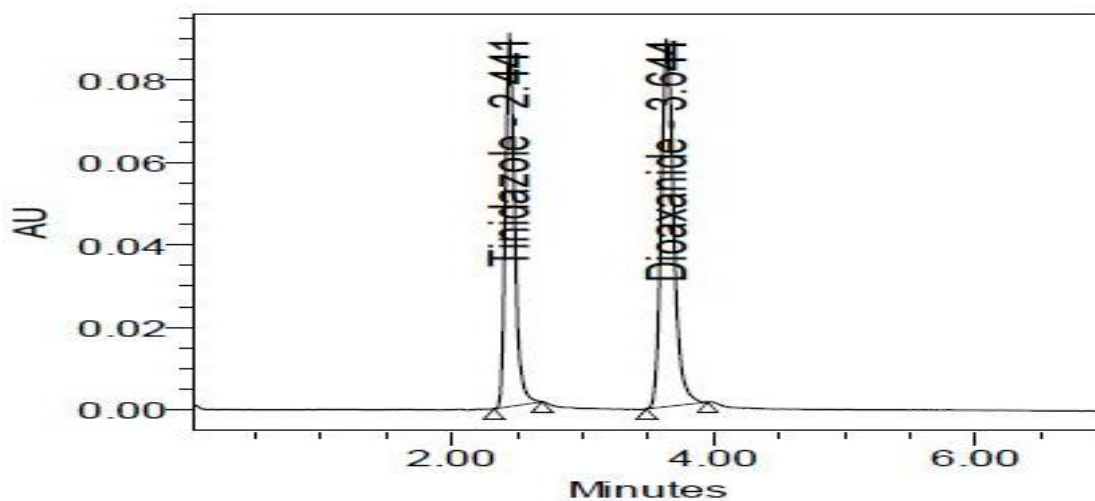


Table 7.17 Assay of standard & sample chromatogram

S No		TINIDAZOLE		DILOXANNIDE FUROATE	
		RT	Area	RT	Area
1	Standard-1	2.439	450640	3.640	600062
2	Standard-2	2.440	455090	3.642	592891
	Average		452865		596476.5
1	Assay-Sample	2.439	447916	3.642	595854
2	Assay-Sample	2.441	448928	3.644	595903
	Average		448422		595878.5

Calculation:

$$\% \text{ Assay} = \frac{\text{Spl area} \times \text{Std dil} \times \text{potency} \times \text{Avg Wt}}{\text{Std area} \times \text{Spl dil} \times \text{weight taken} \times \text{Label claim}} \times 100$$

Where,

Avg wt = average weight of tablets.

Spl area = Sample area

Std area = Standard area

Std dil = Standard dilution

Spl dil = Sample dilution

Assay of Tinidazole

$$\begin{aligned} \text{Assay \%} &= \frac{448422 \times 12 \times 1 \times 100 \times 10 \times 99.6 \times 315}{45286.5 \times 10 \times 10 \times 124.68 \times 1 \times 100 \times 300} \times 100 \\ &= 99.64\% \end{aligned}$$

Assay of Diloxanide furoate :

$$\text{Assay \%} = \frac{595878.5 \times 10 \times 1 \times 100 \times 10 \times 99.6 \times 315}{596476.5 \times 10 \times 10 \times 124.68 \times 1 \times 100 \times 250} \times 100$$
$$= 100.45\%$$

Data interpretation

The percentage purity of Tinidazole and Diloxanide furoate was found to be 99.64 % and 100.45 % were within limit. From the results obtained it can be concluded that, this method is applied for routine analysis of simultaneous estimation of tinidazole and Diloxanide Furoate in their combined tablet dosage form.

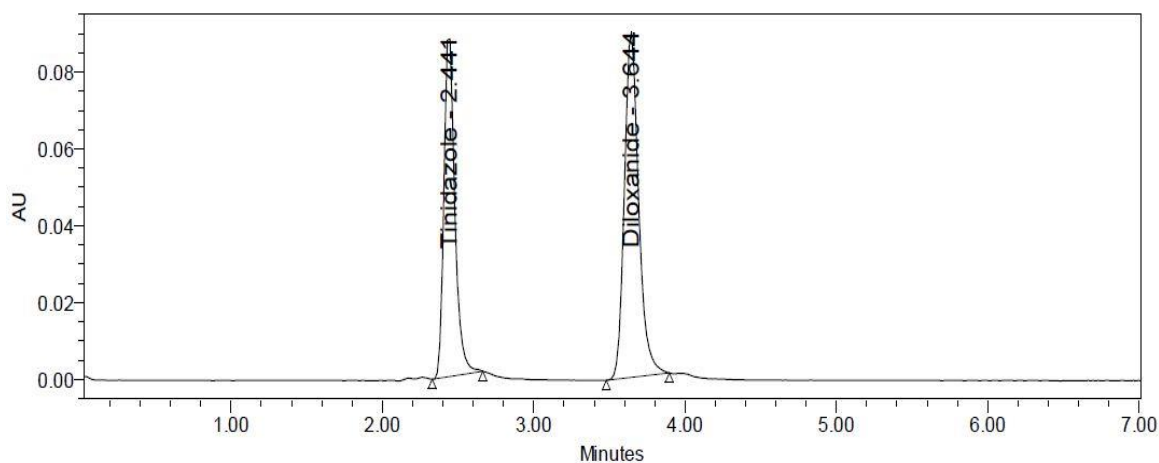
7.18 DEGRADATION STUDIES**Fig 56 Chromatogram of Standard:**

Fig 57 Chromatogram of Acid Hydrolysis:

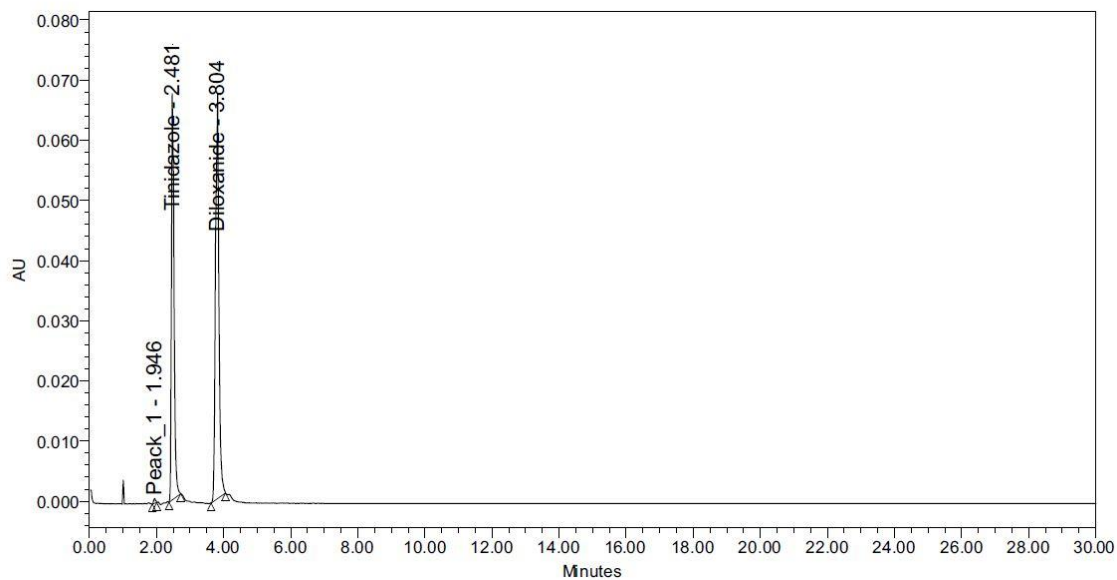


Fig 58 Chromatogram of Base Hydrolysis:

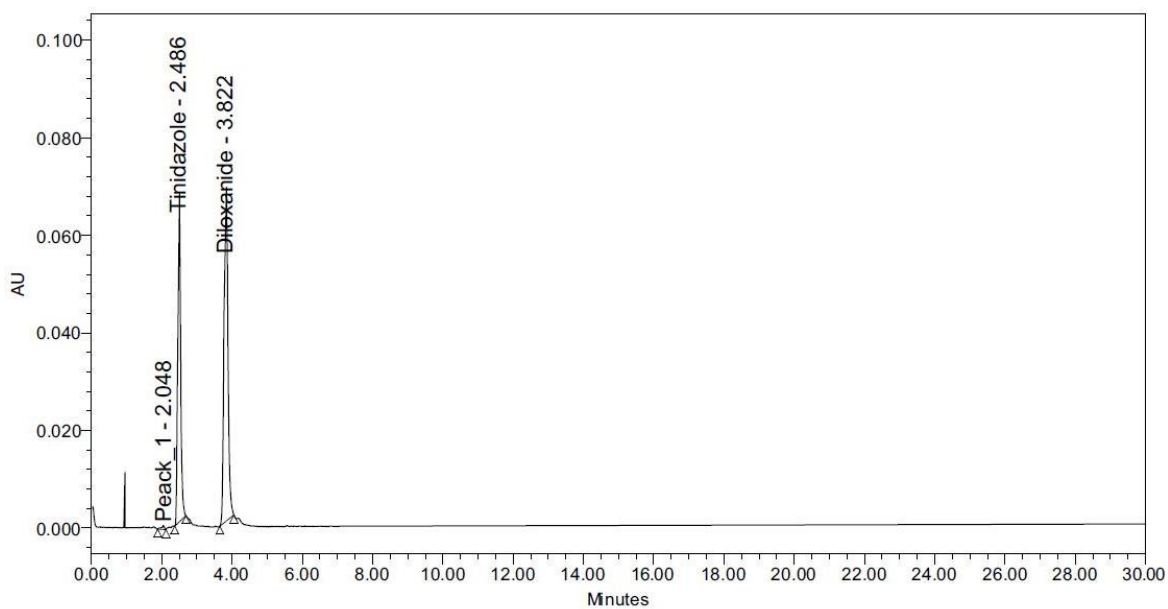


Fig 59 Chromatogram of Oxidation (Peroxide):

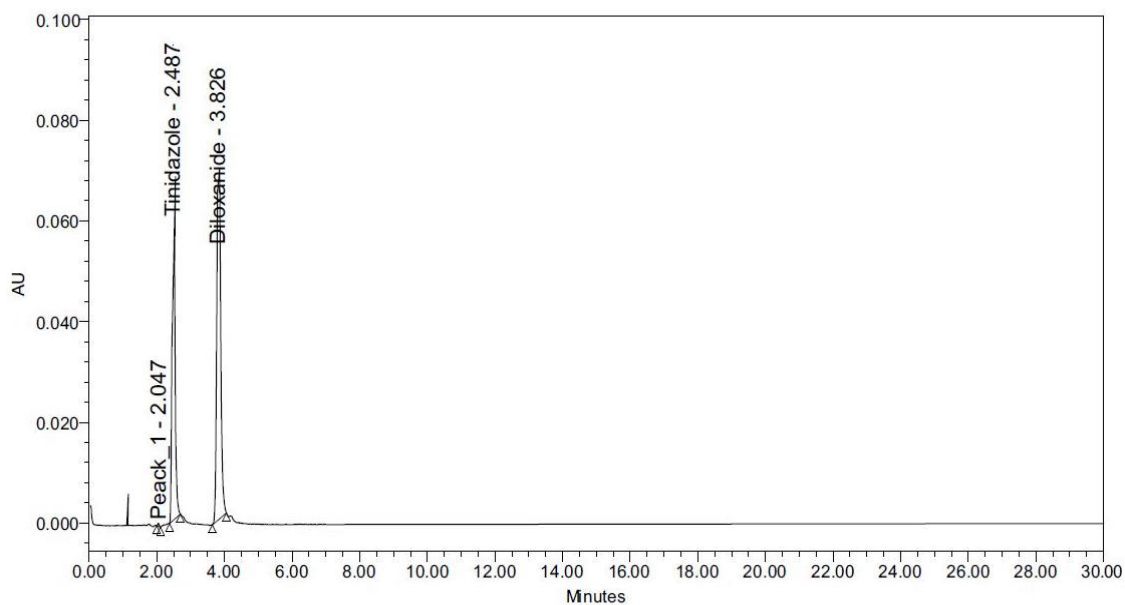


Fig 60 Chromatogram of Heat Exposure:

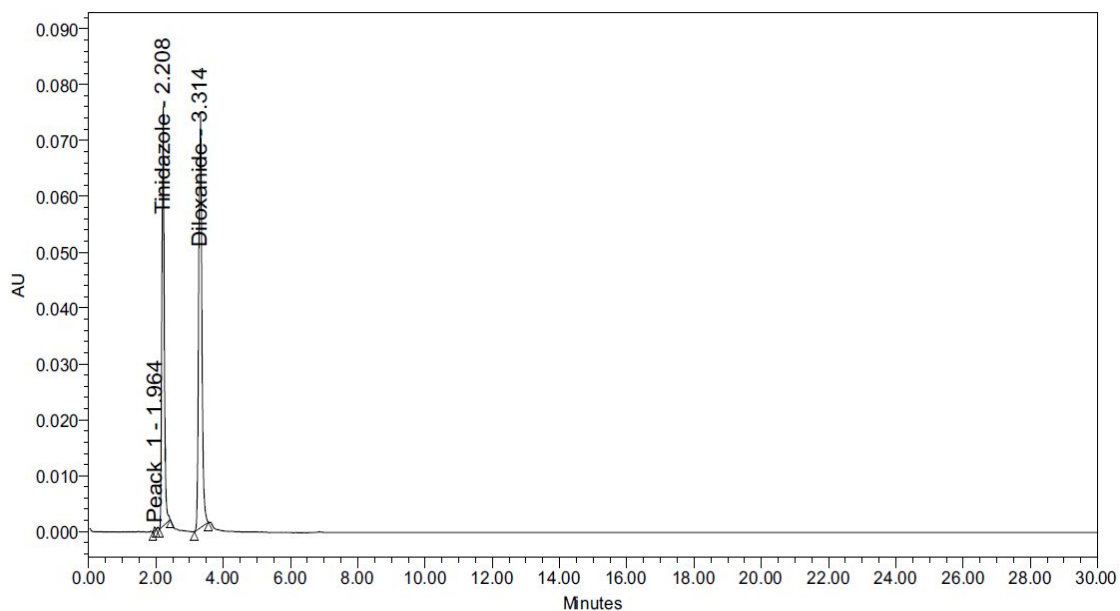


Fig 61 Chromatogram of UV Exposure:

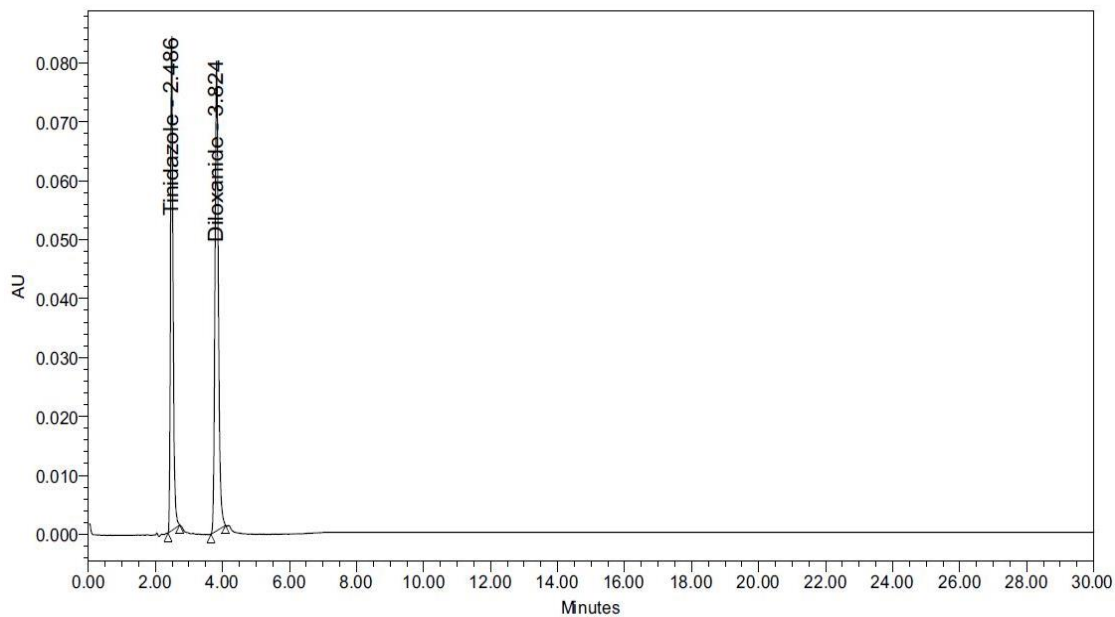


Fig 62 Chromatogram of neutral hydrolysis:

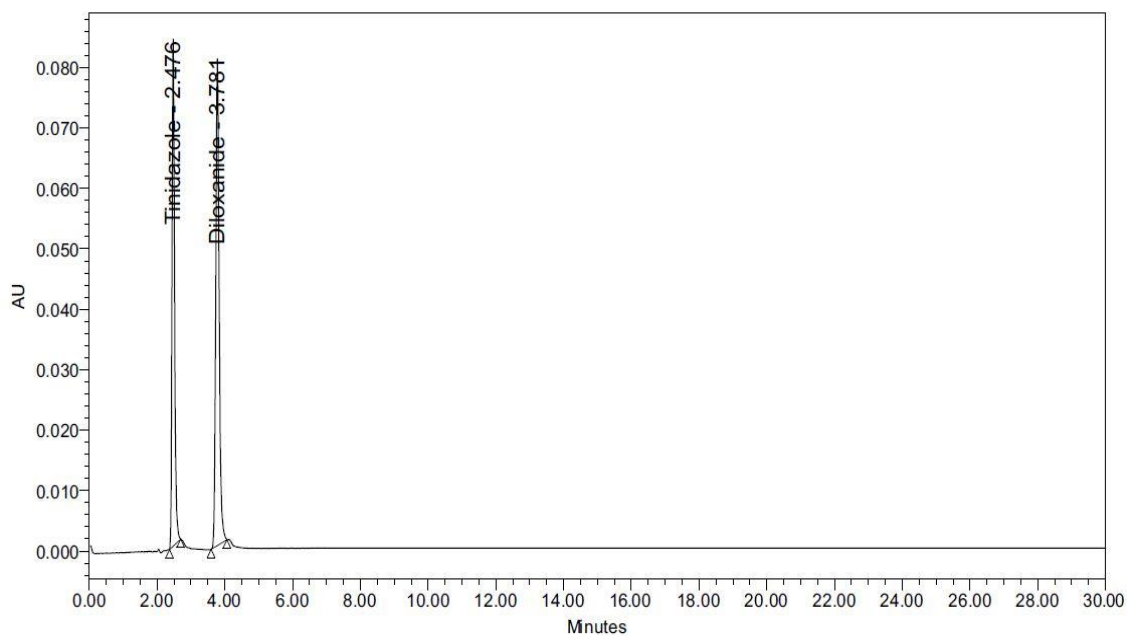


Table. No 7.19 Degradation study of Tinidazole

S.No	Name	Peak Area	Degradation Assay	% Net Degradation
1	Acid Hydrolysis	415006	92.90	6.74
2	Base Hydrolysis	418710	93.72	5.92
3	Heat Exposure	425486	95.24	4.40
4	Oxidation (peroxide)	421280	94.30	5.34
5	UV Exposure	438180	98.08	1.56
6	Neutral	443619	99.30	0.34

Table No.7.20 Degradation study of Diloxanide Furoate

S.No	Name	Peak Area	Degradation Assay	% Net Degradation
1	Acid Hydrolysis	5467557	92.12	8.33
2	Base Hydrolysis	556333	93.73	6.72
3	Heat Exposure	567746	95.66	4.79
4	Oxidation (peroxide)	561104	94.54	5.91
5	UV Exposure	585874	98.71	1.74
6	Neutral	588589	99.17	1.28

8. SUMMARY AND CONCLUSION

Mobile phase	Potassium dihydrogen phosphate and Acetonitrile(45:55 V/V)
Stationary phase	Kromasil C18 (150 x 4.6 mm, 5 μ)
Wave length	278 nm
Run time	20 min
P.H of mobile phase	3.3
Flow rate	1 ml/min
Injection volume	10 μ l
Temperature	30 ⁰ c

Table No 8.1 Chromatographic Conditions

System suitability parameters	RESULTS	
	TINIDAZOLE	DILOXANIDE FUROATE
Retention time	2.438	3.653
Area	464001	605076
Theoretical plates	5630	73622
Tailing factor	1.32	1.24

Table No 8.2 System Suitability Parameters Of Assay

Table No 8.3 Validation Parameters of Assay

S.No	Parameter	RESULT					
		Tinidazole			Diloxanide Furoate		
1	Linearity	30-180 µg/ml Correlation coefficient = 0.999			25-150 µg/ml Correlation coefficient = 0.999		
2	System precision %RSD	0.9			0.6		
3	Method precision %RSD	0.6			0.6		
4	Intermediate precision %RSD	0.6			0.6		
5	Accuracy	Mean recovery =			Mean recovery =		
	50%	99.85%			100.21%		
	100%	100.08%			100.02%		
	150%	9.76%			100.12%		
6	Robustness	RT	Area	Tailing factor	RT	Area	Tailing factor
	Flow-1	2.444	445197	1.25	3.666	586483	1.16
	Flow-2	2.207	434640	1.28	3.309	533051	1.15
	change in mobile phase_1	2.422	466627	1.33	3.541	581740	1.19
	change in mobile phase_2	2.445	449894	1.28	3.666	592388	1.18
	change in temp _1	2.422	466627	1.33	3.541	581740	1.19
	change in temp _2	2.208	434640	1.28	3.314	533051	1.16

Table No 8.4 Degradation study results:

S. No	Compound Name	Acid hydrolysis	Alkali hydrolysis	Oxidation	Heat	UV	Neutral hydrolysis
1	Tinidazole	6.74	5.92	5.34	4.40	1.56	0.34
2	Diloxanide Furoate	8.33	6.72	5.91	4.79	1.74	1.28

CONCLUSION

A simple, sensitive, rapid and economical stability indicating RP-HPLC method was developed and validated for the assay of Tinidazole and Diloxanide Furoate in combined tablet formulation. This method yielded high recoveries with good linearity and precision. It can be concluded that the proposed method is a good approach for obtaining reliable results and found to be suitable for the routine analysis of Tinidazole and Diloxanide Furoate combined tablet formulation.

From these studies we reported the % of degradation products in various conditions like Acid hydrolysis, Alkali hydrolysis, Oxidation, Heat, UV and Neutral hydrolysis.

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