RP-HPLC method was developed and validated for the estimation of Ranolazine in bulk and dosage form

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1. INTRODUCTION

1.1 Analytical Chemistry [1-4]

Analytical chemistry is the branch of chemistry that deals with the analysis of chemical molecules, either in qualitative or quantitative manner. The former identifies the nature of substance, and if it is mixture, the nature of the components present, whereas the later determines the elemental composition of the substance and/or the quantitative distribution of each component.

Analytic method development and validation are key elements of any pharmaceutical development program. HPLC analysis method is developed to identify, quantity or purifying compounds of interest.

Effective method development ensures that laboratory resources are optimized, while methods meet the objectives required at each stage of drug development. Method validation, required by regulatory agencies at certain stages of the drug approval process, is defined as the “process of demonstrating that analytical procedures are suitable for their intended use”

To know information concerning the compound or analyte is worth. Understand its physical and chemical characteristics allow us to select the most appropriate HPLC method development from the vast literature. Information concerning sample, for example, molecular mass, structure and functionality, P^{Ka} values and UV spectra, solubility of compound(s) should be compiled, the

requirement of removal of insoluble impurities by filtration, centrifugation, dilution or concentration to control the concentration, extraction (liquid or solid phase), derivatization for detection etc., Should be checked. For pure compound, determine sample solubility whether it's organic soluble or water soluble, as this helps to select the best mobile phase and column to be used in the HPLC Method development.

Various detectors include: UV/Visible photodiode array detector, fluorescence detector, conductivity detector, refractive index detector, electrochemical detector, Mass spectrometer detector, evaporative light scattering detector. UV-Vis detectors are typical in many laboratories as they can detect a wide array of compounds.

Analytic methods are intended to establish the identity, purity, physical characteristics and potency of the drugs that we use. Methods are developed to support drug testing against specifications during manufacturing and quality release operations, as well as during long term stability studies. Methods may also support safety and characterization studies or evaluations of drug performance. Once a stability-indicating method is in place, the formulated drug product can then be subjected to heat and light in order to evaluate potential degradation of the API in the presence of formulation excipients.

The validation of an analytic method demonstrates the scientific soundness of the measurement or characterization. It is required to varying extents throughout the regulatory submission process. The validation practice demonstrates that an analytic method measures the correct substance, in the correct amount, and in the appropriate range for the intended samples. It allows the analyst to understand the behavior of the method and to establish the performance limits of the method. The goal is to identify

the critical parameters and to establish acceptance criteria for method system suitability.

1.2 High Performance Liquid Chromatography (HPLC)[5]

The acronym HPLC, coined by the late Prof.Csaba Horvath for his 1970 Pittcon paper, originally indicated the fact that high pressure was used to generate the flow required for liquid chromatography in packed columns. In the beginning, pumps only had a pressure capability of 500 psi. This was called high pressure liquid chromatography, or HPLC.

New HPLC instruments could develop up to 6,000 psi of pressure, and incorporated improved injectors, detectors, and columns. With continued advances in performance during this time (smaller particles, even higher pressure), the acronym HPLC remained the same, but the name was changed to high performance liquid chromatography.

HPLC is the method of choice in the field of analytical chemistry, since this method is specific, robust, linear, precise and accurate and the limit of detection is low and also it offers the following advantages.

- Speed(min)
- Greater sensitivity
- Improved resolution (wide variety of stationary phases)
- Reusable columns
- Needs a small sample with a high accuracy and precise
- Easy sample recovery, handling and maintenance.
- Reproducibility of +/- 1% (not so for LC)
- Non-destructed sample during operation compared to GC.

- Controls and automates chromatography instrumentation.
- Provides data management, security features, and reporting and instrument validation.

Major types of HPLC:

Normal Phase Chromatography

The IUPAC Compendium of Chemical Technology defines 'Normal Phase' as an elution procedure in which the stationary phase is more polar than the mobile phase. Adsorption chromatography, which was classified as a separation mode called "solid-liquid chromatography", is now considered to be normal-phase chromatography. In most cases, the solid phase used for normal-phase chromatography is an untreated porous silica-gel column (SIL column) or a column containing silica gel chemically bonded at the surface to polar functional groups, such as the aminopropyl group (NH2 column) or cyanopropyl group (CN column). The mobile phase used is generally ethanol or another polar solvent added to a non polar solvent such as n-hexane. However, a mobile phase containing water is sometimes used for the analysis of highly polar components. The separation of each component differs according to the distribution ratio between the solid phase and mobile phase. The interaction between the solid phase and target components during normal-phase chromatography are mainly hydrophilic interactions, such as hydrogen bond interactions and electrostatic interactions. Consequently, normal-phase chromatography generally offers different separation selectivity to reversed-phase

chromatography, which mainly involves hydrophobic interactions.

Normal-phase chromatography can easily separate tocopherol isomers that are difficult to separate by reversed-phase chromatography and sugars that are difficult to retain by reversed-phase chromatography. It can elute together all components with different alkyl chain lengths and branches during the analysis of alkyl benzene sulfonate. These properties arise as the regions involved in retaining compounds differ from those in reversed-phase chromatography. In addition, as the mobile phase used for normal-phase chromatography generally contains no water, this technique is ideal for the separation of easily hydrolyzed compounds, such as acid anhydride; concentration after fractioning; or preparative separation and purification that requires drying. Normal phase chromatography can also be advantageous from the viewpoints of quantum yield for fluorescence detection, molar absorptive and detection wavelength in absorption detection. With normal-phase chromatography, increasing the mobile phase polarity generally accelerates elution. For example, if an n-hexane/ ethanol mixture is used as the mobile phase, elution occurs faster if the proportion of ethanol, which has higher polarity, is increased. Care is required, as this is the reverse relationship to reversed-phase chromatography, whereby the rate of elution increases when the mobile phase polarity is decreased. Using a low-viscosity solvent in the mobile phase sometimes permits high flow rates and rapid column equilibration. If an NH2 column or CN column is used, some mobile phase compositions result in reversed polarity of the mobile phase and solid phase, such that the column functions as a reversed-phase chromatography column. Therefore, it is important to be aware of the possibility that the elution behavior may fluctuate wildly, especially if a mobile phase with a high proportion of water is used.

Reverse Phase Chromatography

The IUPAC definition understandably states that reversed phase chromatography is 'an elution procedure in which the mobile phase is significantly more polar than the stationary 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 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 partially charged. Acidic buffers such as acetic acid is 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 are 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 for the separation of polar molecules that either are insoluble in organic solvents or bind too strongly to the polar, normal-phase materials.

1.3 Instrumentation[6-10]

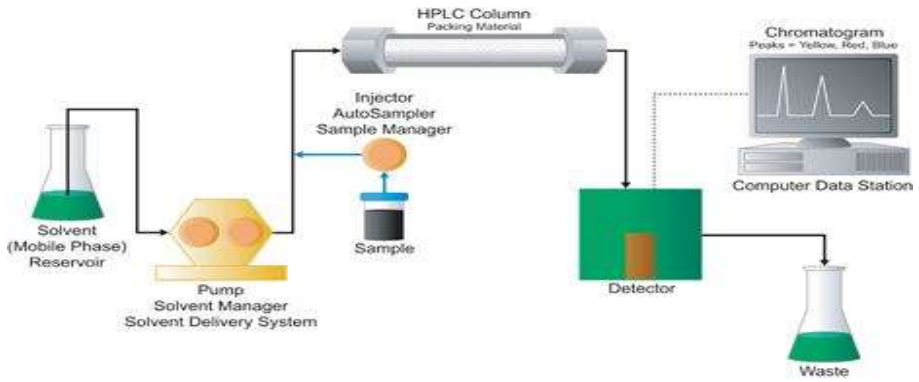


Fig No.1.1 High Performance Liquid Chromatography system

The basic components of a High Performance Liquid Chromatographic system are shown in Fig.1.1 The instrument consists of

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

Compounds are separated by injecting a plug of the sample mixture usually 5-micro liters onto the column. The different components in the mixture pass through the column at different rates due to differences in their partitioning behavior between the mobile liquid phase and the stationary phase.

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.

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 described for Normal-Phase Chromatography. Solvents with large dipole moments, such as methyel 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.

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 phase.

Gradient Elution Devices:

HPLC columns may run isocratically with constant eluent or they may 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.

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 up to 20 mL/min. The pumping systems used in HPLC can be categorized into three different types.

- Constant flow Pumps/ Reciprocating Pumps
- Constant displacement/ Syringe pumps
- Constant pressure/Pneumatic pumps

Reciprocating pump:

By definition, a reciprocating pump is a machine using reciprocating motion to cause fluid to be moved from one location to another. This type of pump traps a fixed volume of fluid and displaces it from suction conditions to discharge conditions by means of check valves placed in a series. It is electrically driven by a motor, which moves back and forth within a hydraulic chamber. On the backward stroke the piston sucks in eluent from the reservoir and due to check valves, the outlet to the separation column is closed. During the forward stroke the eluent is pushed on to the column and the inlet from the reservoir is closed. The pumping motion of the piston produces a pulsed flow that requires dampening. These pumps include a high output pressure with constant flow rates and the ability to be used for gradient elution.

- Advantages are small internal volumes (µL level), high pressures (up to 10000psi), constant flow rates and utility for use with solvent gradients.
- Main disadvantage is that flow is pulsed, leading to the need for a

dampener.

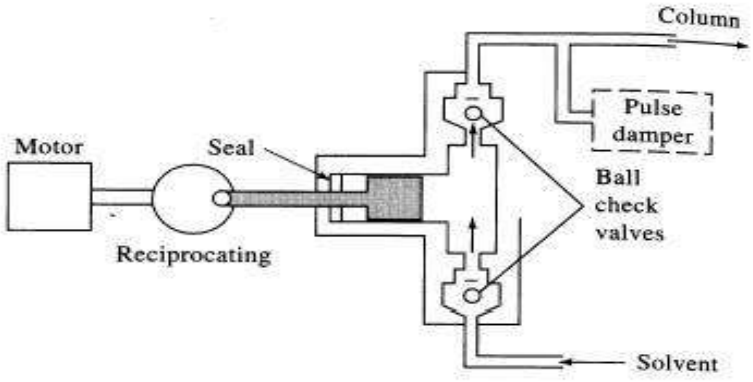


Fig No.1.2: Reciprocating pump

Syringe pump:

The syringe pump is a large, electrically operated simulation of a hypodermic syringe. It is rarely used today due to its design, it can provide only a limited pressure and the volume of mobile phase available for use is restricted to the pump volume. Unless the separation is stopped, the pump takes the form of a large metal syringe, the piston being propelled by an electric motor and driven by a worm gear. The speed of the motor determines the pump delivery. Another motor actuates the piston by a different system of gearing to refill the syringe rapidly when required. The solvent is sucked into the cylinder through a hole in the center of the piston and between the piston and the outlet there is a coil that acts as a dampener. It is also sometimes used for reagent delivery in post column derivitization as it can be made to deliver a very constant reagent supply at very low flow rates.

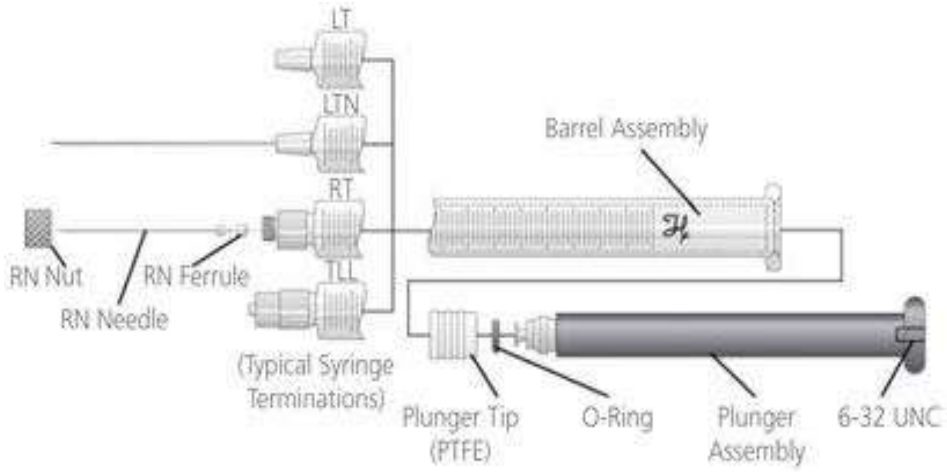


Fig No.1.3: Syringe pump

Pneumatic Pump:

Pneumatics is a branch of technology that uses the force of compressed gases to generate mechanical effects. Pneumatic pumps, in particular, use compressed air to create force that is used to move fluids through a piping system. Their system of operation is very similar to that of hydraulic pumps. Essentially, pneumatic pumps use air in the same way that hydraulic pumps use fluids. Both are capable of creating extremely amplified levels of pressure that can generate surprisingly large amounts of power. These pumps use a double piston system, with one piston having a substantially larger diameter than the other. Between the two pistons is an airtight chamber filled with either liquid or another compressed gas. The compressed gas from the outside pushes on the larger-diameter piston, which in turn pushes against

the gas or liquid in the intermediate chamber. Because there is no pressure lost between the larger piston and the smaller one, the smaller piston receives a highly amplified level of force, which can translate into powerful mechanical action.

Pneumatic pumps are used in only a few types of applications, most notably for moving slurry through chromatography columns. However, where they are used, these pumps are appreciated for the simplicity of their design, their low maintenance, and their relatively high safety ratings. The main disadvantage is the storage needed for the compressed gas tanks, and the fact that hydraulic pumps use very similar mechanisms to create even greater force.

Sample Injection Unit:

A sample is injected into the flow path for analysis. This is accomplished via a manual injector or an auto sampler. Each type is equipped with six port valves, so that a sample can be injected into the flow path at continuous pressure. For the manual injector, the knob is manually operated to deliver the sample to the column, as shown in Fig.1.4.

The knob is set at the “LOAD” position for sample injection, as shown in the left image. Using a micro syringe, the sample can be injected into the sample loop, which is separated from the flow path. The knob is turned to the “INJECT” position. The eluent travels through the loop from the pump then delivers the sample to the column. The autosampler can perform similar work automatically, enabling unmanned continuous operation.

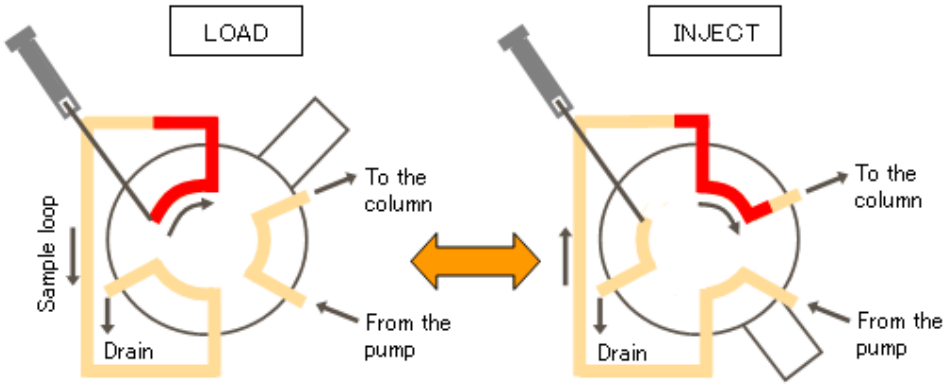


Fig No.1.4: Flow path of the manual injector.

Column:

HPLC column are made up of stainless steel or glass, which differs in length and inside diameter depending on the application. The two types of columns are analytical column and preparative column. Standard analytical columns are 4-5mm in internal diameter and 10-30 cm in length. The particle size used ranges from 5-10 micrometers. Preparative columns are 20-50 mm in internal diameter and 20-100 cm in length. The particle size used ranges from 37-50 micrometers.

Other types of columns include:

Guard column:

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. The y are dependable columns designed to filter or remove

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

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 analysis.

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.

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 the time of chromatographic analysis in order to avoid significant deviations in results.

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.

Requirements for an Ideal HPLC Column:

- Particles should be spherical and available in particle diameters ranging from 3 to10 µm. Particles should with stand typical pressures encountered during HPLC ((900-3000 psi) (6.1- 20.5 Moa) but ideally up to 4000 psi (27.2 Moa)) and should not swell or shrink with the nature of the eluent.
- Particles should have porosity in the range 50-70%, extending to 80 % for Size-Exclusion Chromatography.
- Particles should contain no pores smaller than ~60 A^0 in diameter and should have a uniform pore size distribution.
- Particles should be available with a range of mean pore diameters of 60-1000 A^0.
- The internal surface of the material should be homogeneous.
- The internal surface should be capable of modification to provide a range of surface functionalities.

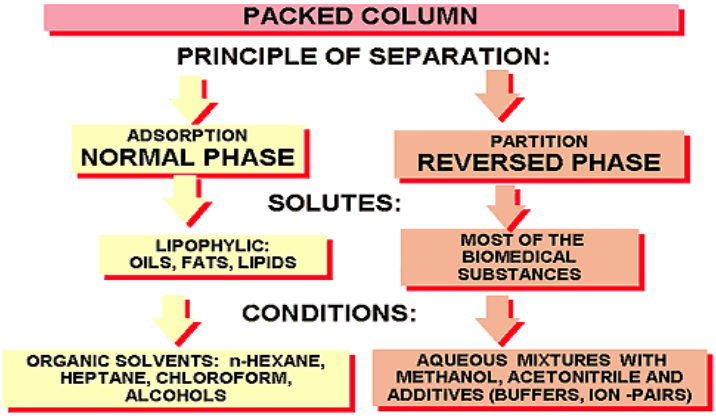


Fig No.1. 5: Packed column

Detectors:

Types of Detectors:

1. Bulk property detectors ⇒ respond to mobile phase property such as refractive index dielectric constant or density which is altered by the presence of analytes.
2. Solute property detectors ⇒ respond to properties of solute via UV-Vis absorbance fluorescence or current

Refractive Index Detector

Relatively versatile detector based on differences in refractive index for mobile phase with and without analyte present. Figure below shows a differential RI detector which compares RI of sample and reference streams. Presence of analyte in sample stream will lead to a deflection of the light beam on the photo detector, and a corresponding change in signal which is amplified and recorded. Main disadvantages are temperature sensitivity and relatively poor detection limits.

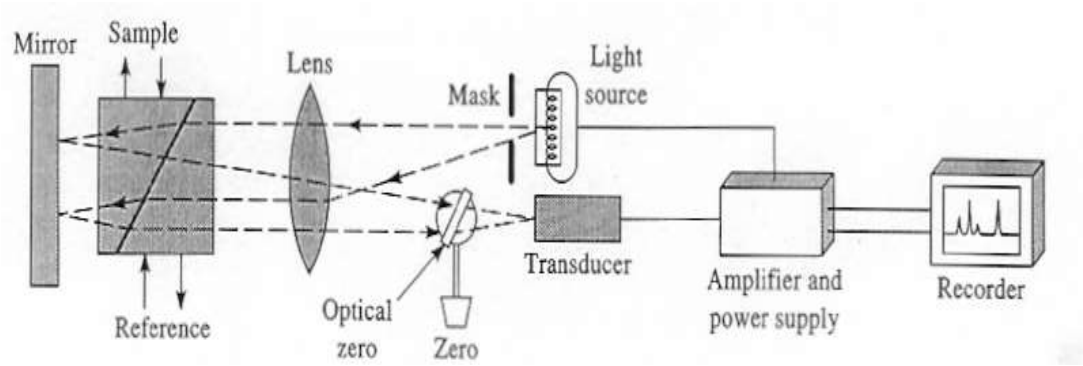


Fig No.1.6: **Refractive index detector**

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.

PDA Detector:

These are detectors which follow principle similar to UV detectors but the only advantage is higher sensitivity and measures the entire absorption range i.e. It gives scan of entire spectrum.

Evaporative Light Scattering Detector (ELSD):

The effluent from the column is nebulized and evaporated as it passes through the drift tube and particles of analyte are detected as they pass through the light scattering cell. Therefore the use of ELS detectors is restricted to non volatile analyzes and volatile mobile phases. However because of the ability to use ELS with gradient elution it is being used more frequently in these methods, especially for impurity analysis.

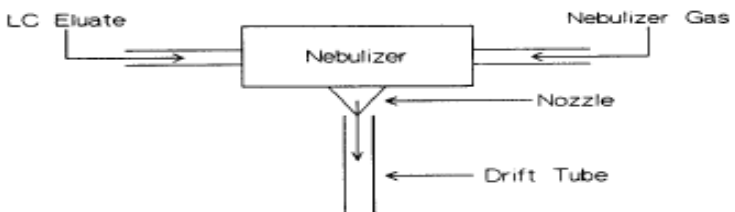


Fig No.1. 7: ELSD

Electro Chemical Detector:

This detector is especially 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.

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.

Fluorescence Detector:

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 samples.

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.

1.4 Applications of HPLC:

HPLC analysis in clinical diagnosis and health industry:

- Estimation of metabolites of purines, pyrimidines or other metabolites from plasma, cerebrospinal fluid and urine samples in patients.
- Estimation of corticoids from plasma in disorders of adrenal gland which secretes an endocrine hormone.

HPLC application in scientific research:

- To analyze and quantify the molecules.
- Components with similar chemistry and properties are easily distinguished by this method.

HPLC applications in pharmaceutical industry:

- In quality control, it is used to check if the prepared or manufactured products are in compliance with the specified standards as per the pharmacopoeia and other drug regulating bodies.
- In R & D as discussed in research before, it is used to identify the specific molecule or component in mixture under research
- It is used for bioavailability studies, drug release from the formulation, dissolution studies etc.

System Suitability Parameters [11]

Retention Time (R_t):

Retention time is the difference in time between the point of injection and appearance of peak maxima. Retention time is the time required for 50% of a component to be eluted from a column. R_t is measured in minutes or seconds.

Retention Volume (V_r):

Retention volume is the volume of carrier gas required to elute 50% of the component from the column. It is the product of retention time and flow rate.

Separation factor (S):

Separation factor is the ratio of partition co-efficient of the two components to be separated. It can be expressed and determined by using the following equation:

$$S=k_b/k_a=k'_a/k'_b= (t_b-t_o)/ (t_a-t_o)$$

Where,

T_o = Retention time of un retained substance

K_b, k_a = Partition coefficients of b and a

t_b, t_a = Retention time of substance b and a.

Resolution:

Resolution is a measure of the extent of separation of two components and the baseline separation achieved. It can be determined by using the following formula:

$$R = 2 (t2 - t1) / (w2 + w1)$$

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.

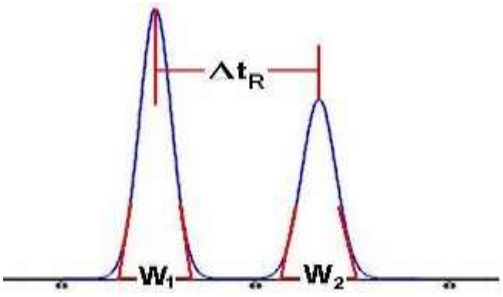


Fig No.1. 8: Resolution

Recommendations

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

Theoretical Plates:

A theoretical plate is an imaginary or hypothetical unit of a column where

distribution of solute between stationary phase and mobile phase has attained equilibrium. A theoretical plate can also be called as a functional unit of the column.

Efficiency (No. of theoretical plates):

Efficiency of a column is expressed by the number of theoretical plates.

N= 16Rt/w

Where, n = no. of theoretical plates

R_t = retention time

W = peak width at base.

Factor (T):

The chromatographic peak is assumed to have a Gaussian shape under ideal conditions, describing normal distribution of the velocity of the molecules populating the peak zone migrating through the stationary phase inside the column. Any deviation from the normal distribution indicates non-ideality of the distribution and the migration process. This is the reason why USP Tailing is a peak's parameter almost always measured in the system suitability step of the analysis.

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.

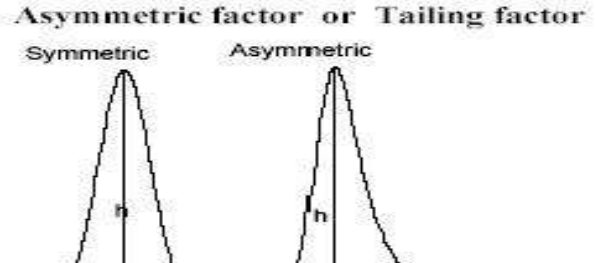


Fig No.1.9: Asymmetric Factor

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_O be the retention time for an unrestrained peak.

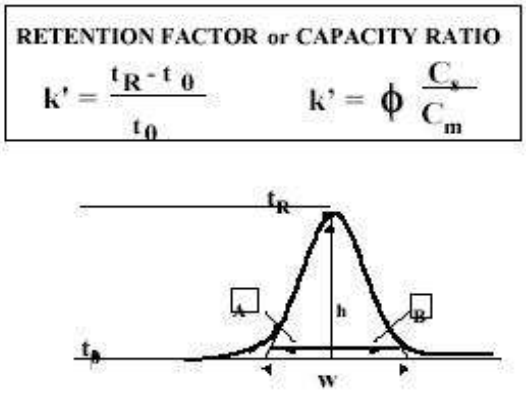


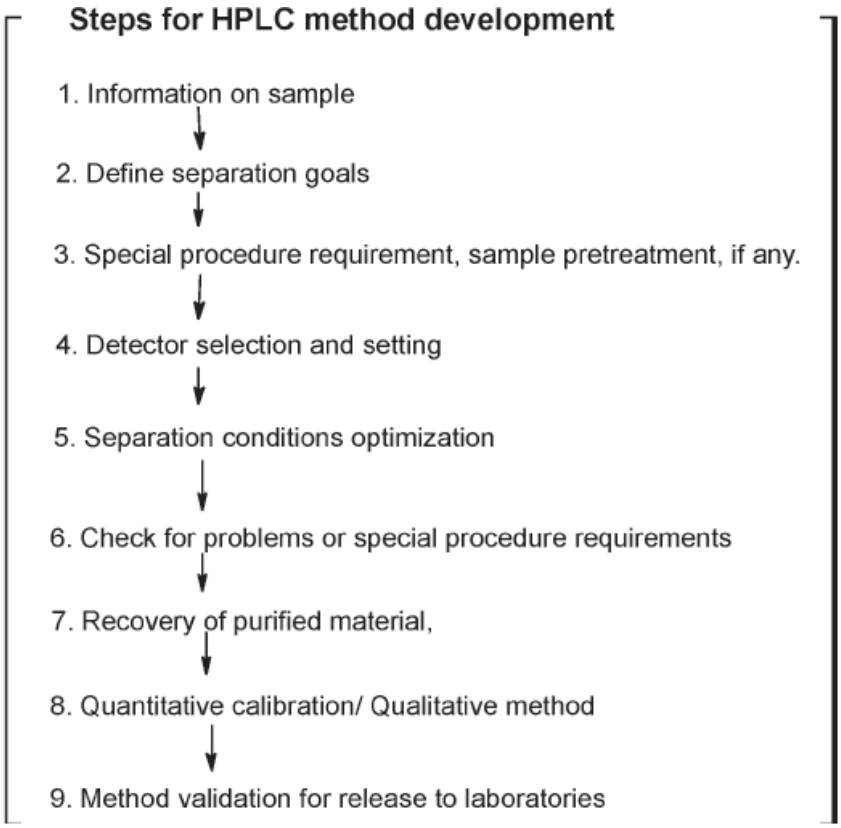
Fig No.1. 10: Capacity factor

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.

1.6 METHOD DEVELOPMENT [12-13]

The three critical components for a HPLC method are: sample preparation (% organic, pH, shaking/sonication, sample size, sample age), HPLC analysis conditions (%organic, pH, flow rate, temperature, wavelength, and column age) and standardization (integration, wavelength, standard concentration, and response factor correction). During the preliminary method development stage, all individual components should be investigated before the final method optimization. This gives the scientist a chance to critically evaluate the method performance in each component and streamline the final method optimization. The percentage of time spent on each stage is proposed to ensure the scientist will allocate sufficient time to different steps. In this approach, the three critical components for a HPLC method (sample preparation, HPLC analysis and standardization) will first be investigated individually. The degraded drug samples obtained are subjected to preliminary chromatographic separation to study the number and types of degradation products formed under various conditions. Scouting experiments are run and then conditions are chosen for further optimization. Resolving power, specificity, and speed are key chromatographic method attributes to keep in mind during method development Selectivity can be manipulated by combination of different factors like solvent composition, type of stationary phase, mobile phase, buffers and pH. Changing solvents and stationary phases are the most comfortable approaches to achieve the separation.



The proper range of pH is an important tool for separation of ionizable compounds. Acidic compounds are retained at low pH while basic compounds are more retained at higher pH. The neutral compounds remain unaffected. The pH range 4-8 is not generally employed because slight change in pH in this range would result in a dramatic shift in retention. However, by operating at pH extremes (2-4 or 8-10), not only is there a 10-30 fold difference in retention that can be exploited in method development but also the method can be made more robust which is a desirable outcome with validation in min.

Method development within the different pH range from 1 to 12 for better chromatographic resolution between two or more peak of an analyte depends upon three main factors that are -Column efficiency, Selectivity, Retention time. The ionizable analytes are either bases or acids and it effects above three factors dramatically with change in pH.

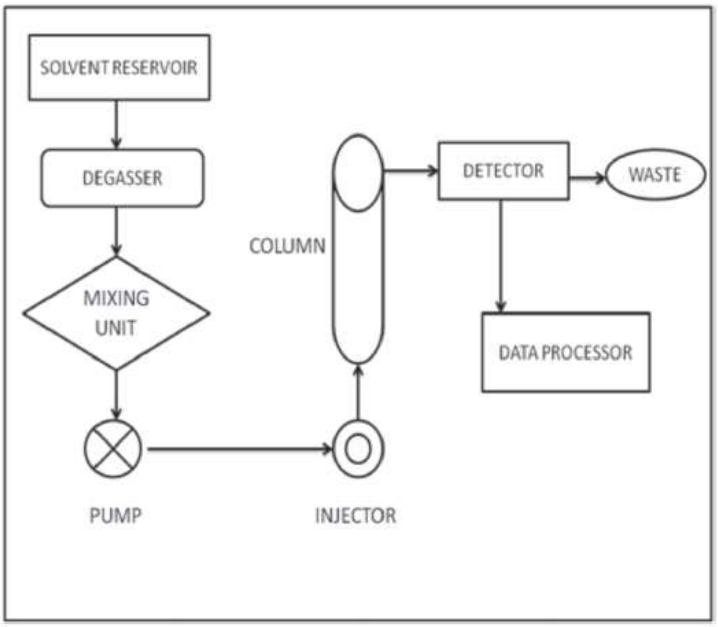


Fig No.1.11. HPLC system with its different components

Retention time can be improved by changing the pH that will lead to easy separation of ionizable analytes from non-ionized form. By changing the mobile phase pH can also improve column efficiency because it altered both the ionization of the analyte and the residual silanols and it also minimizes secondary interactions between analytes and the silica surface that will lead to poor peak shape. To achieve optimum resolution, it requires change in the pH of mobile phase. Method development proceeds by investigating parameters of chromatographic separations first at low pH and then at higher pH until optimum results are achieved.

Mobile phase composition (or solvent strength) plays an important role in RP-HPLC separation. Acetonitrile (ACN), methanol (MeOH) and tetrahydrofuran (THF) are commonly used solvents in RP-HPLC having low UV cut-off of 190, 205 and 212nm respectively. These solvents are miscible with water. Mixture of acetonitrile and water is the best initial choice for the mobile phase during method development. An HPLC column packed with stationary phase of C18-bonded silica (C18 Column) and C8-bonded silica (C8 Column) is used in RP-HPLC separation of a wide range of

organic compounds. The separation selectivity for certain components vary between the columns of different manufacturer as well as between column production batches from the same manufacturer. Column dimensions, silica substrate properties and bonded stationary phase characteristics are the main ones. The use of silica-based packing is favored in most of the present HPLC columns due to several physical characteristics. Silica substrates are available in spherical or irregular shapes and can be prepared with different surface areas, pore sizes and particle sizes, which make them suitable for most HPLC applications. Totally porous silica particles with 5 μm diameter provide the desired characteristics for most HPLC separations. Zirconia-based columns are revolutionary HPLC phases. Zirconia particles are mechanically stable, and have a porous structure similar to that of silica. However, zirconia's main advantage over silica is that it is very stable in a wide range of eluent pH; indeed the ZirChrom®-EZ and ZirChrom®-MS phases are stable over the pH range of 1-10.

Separation of many samples can be enhanced by selecting the right column temperature. Higher column temperature reduces system backpressure by decreasing mobile phase viscosity, which in turn allows use of longer columns with higher separation efficiency. However, an overall loss of resolution between mixture components in many samples occurs by increasing column temperature. The optimum temperature is dependent upon nature of the mixture components. The overall separation can be improved by simultaneous changes in column temperature and mobile phase composition. Recently, normal phase HPLC is back popular with the birth of HILIC technology that proved to improve reproducibility in separating polar and hydrophilic compounds such as peptides, carbohydrate, vitamins, polar drugs and metabolites. In order to develop a HPLC method effectively, most of the effort should

be spent in method development and optimization as that will improve the final method performance. Method validation is important to complete method development.

Reasons for new method development

- Existing methods may be unreliable (have poor accuracy or precision).
- Existing methods may be too expensive, time consuming or energy intensive or they may not be easily automated.
- Existing methods may not provide adequate sensitivity or analyte selectivity in sample of the interest.
- There may be a need for alternative methods to confirm, for legal or scientific reason, analytical data originally provided by existing methods.
- There may not be a suitable method for a particular analyte in the specific sample matrix
- In case of the patented molecules, innovator wouldn't publish the methods. In such cases for the use of public (Pharmacopoeia's) we can develop the method.

Steps for method development

Solubility studies:

Perform the solubility experiments to establish the solubility of the API in a number of aqueous and organic solvents like water, buffers, 0.1N sodium hydroxide, methanol, acetonitrile, chloroform, hexane, tetrahydrofuran etc. covering a range of polarities that are commonly used in the method development. The scientist should check the pH of the water soluble API to know its acidic or basic nature. The API should have good solubility in the selected diluent (preferably 1 mg/mL). Perform a

UV scan in the range of 200-400 nm in the selected solvent to ascertain the spectrophotometric properties of the drug. The solvent is selected as such, which covers the range of polarities that are commonly used in the method development. The typical pharmaceutical compounds are either soluble in water or organic solvent. The water soluble API is further differentiated as ionic and non-ionic. We should check the pH of the water soluble (preferably 1% solution in water) to know acidic/basic nature. The organic soluble API can be classified as polar and non-polar. The API should have good solubility in the selected diluents (preferably 1.0 mg/mL).

Selection of the mobile phase:

The selection of the mobile phase should be suitable for intended use of the method. The recipe for a mobile phase usually is determined empirically, and its complexity can range from a single solvent to a multi-component mixture of solvents and solutes.

The ideal mobile phase should have the following attributes:

- Dissolves the major analyte.
- Dissolves impurities and degradation products.
- Does not interfere with peak shape.
- Does not interfere with analyte response.
- Prevents analyte interaction with container surfaces.
- Does not promote analyte degradation.

There are various solvents commercially available in market having specific characteristics. The following table (Table 1) depicts the characteristics of various solvents commonly used in method development.

Selection of the wavelength:

The selection of the wavelength is a critical step in the method development. To select the wavelength, prepare the standard solution at the required concentration in the selected solvent and scan it on UV- Spectrophotometer. Based on the UV scan results, inject the test solution into HPLC system equipped with the Photo Diode Array Detector and collect the spectra. Select the wavelength, which gives the optimum response for the drug components.

Selection of isocratic or gradient mode:

Similar condition governs the design of both isocratic and gradient mode. Depending on the number of the active components to be separated, the more complex the separation, the gradient elution will be useful over the isocratic mode. Either isocratic or gradient mode is used to determine the initial conditions of the separations.

1.7 Optimization of parameters for HPLC method development:

Selection of buffer:

Efficient separation of the acidic or basic components is achievable by controlling the pH of the mobile phase. The buffer should be UV transparent to below or at the wavelength of the organic solvent. Other properties such as solubility and stability of the buffer and its reactivity to analyte and hardware components of the chromatographic systems should be taken into consideration. The buffer capacity is determined by pH, composition of the buffer and buffer concentration. Optimum buffering capacity occurs at a pH equal to the P^{Ka} of the buffer. In general, most

buffers provide the adequate buffering capacity for controlling mobile phase pH only within ±1 unit of their pKa. For liquid chromatographic separation a buffer concentration of 10 to 50mM is usually adequate. This assumes that the volume of injection is small and the sample is not too heavily buffered at a pH quite different from that of the mobile phase. Higher buffer concentration (> 50mM) provide increased buffer capacity but may not be soluble in the mobile phase for high % of the strong solvent in the mobile phase. Higher buffer concentration also may adversely affect the operation of HPLC system. Liquid Chromatography generally are carried out with C8 or C18 bonded-phase silica based columns that are less stable outside the pH range 2 to 8. Therefore, the buffer should be able to control pH between 2.0 to 8.0. Experiments should be conducted using the different buffers with the different strengths to obtain the required separation. Buffer that gives the separation of all the individual impurities form the API should be selected and effect of variation of buffer concentration should be studied. The selected buffer should be rugged for small changes in the strength say at least 2.0 %.

Selection of the pH of the buffer and mobile phase:

Depending on the P^{Ka} value of the drug molecules, pH of the mobile phase can change retention time e.g. acids shows an increase in the retention as the pH is reduced while base shows a decrease. Mostly, all of the pH caused changes in the retention occur within +1.5 pH units of the P^{Ka} value, it is best to adjust the mobile phase to pH values at least +1.5 pH units above or below the P^{Ka} to ensure practically 100% unionization for retention purposes. Generally, at the low pH (1-4) peak tailing is minimizes and method ruggedness is maximizes. On the other hand operating in the intermediate range (pH 4-8) offers an advantage in increased analyte retention and selectivity.

Selection of the mobile phase:

Most separations can be achieved by the choosing the optimum mobile phase compositions of the aqueous and organic portions. Most widely used solvent for the liquid chromatography are methanol and acetonitrile. Tetrahydrofuran is also used, but to lesser extent. Mobile phase with Tetrahydrofuran are known to be susceptible to oxidation. Experiments should be conducted with the mobile phase having buffers of different pH and different organic phases to check the best possible separation between the impurities with different mobile phase ratios. Alternatively, the stressed solution of the drug product can also be used. Mobile phase composition selected should be able to separate all the possible impurities from API and should be rugged for variation of both the aqueous and organic phase by at least 5%. If the sample is eluted with the mobile phase of 100% organic content, and there is no separation, the solvent strength should be decreased to get the retention. Generally the increase in organic content will shorten the run time but leads to increased band overlap. When the separations are complex, i.e. when many components are to be separated, and when solvent strength is decreased and still there is no resolution between two closely eluting peaks, another organic solvent of the different polarity or even a mixture of the two organic solvents may need to be tried to effect the separation.

Selection of column:

HPLC column is the heart of the instrument and critical in performing the separation. The following parameters of the columns should be taken into consideration while choosing the column for the HPLC method:

- Column Packing
- Size and Shape of the particle,

- Column length and diameter,
- End capping etc.

For liquid chromatography, a wide variety of the columns are available like C8, C18 , Cyano group –CN and amino group like –NH2 etc. It has to be remembered that no two column are same, they vary from manufacturer to manufacture with respect to above-mentioned parameters. The surface area of the bonded phase support is a major factor, as larger the surface area greater will be the retention. Sample retention normally increases for the bonded phases of greater length, C18>C8>C3>C1. As column length changes the column efficiency changes in direct proportion to the ratio of the column length. To select the type of column in the method, conduct the experiments using different columns with different mobile phase's to get best possible separation. Based on the experimental data, select the column which gives separation of all the possible impurities and principal peak and which is rugged for the variation in mobile phase.

Selection of the column temperature:

Generally it is preferable to optimize the chromatographic conditions with the column temperature ambient. However if the peak symmetry is not achieved with any combination of the column and mobile phase at the ambient condition, then the column temperature above ambient can be adopted. If the column temperature is increased, it generally results in the reduction in the peak symmetry and peak retention time. Sender reported that an increase in 1ºC would decrease retention by 1 to 2 %**.** When necessary, column temperature can be increased from 30ºC to 80ºC. Change in temperature may be more effective tool for the separations in the ionization of the sample and this changes pH and pKa values. If the column temperature is

necessary for the separation, then the packing material selected should be able to withstand such temperatures and robustness within +5ºC should be established.

Selection of test concentration and injection volume:

The selected test concentration depends upon the response of the API at the selected wavelength. The test concentration should be finalized only after it is proved that the API is completely extractable at the selected test concentration. Generally, the injection volume of the 5 µL to 10µL is recommended. Before selecting high injection volume, ensure that with the selected higher injection volume, column is not overloaded, resolution and peak symmetry are not compromised. After the finalization of sample concentration and the diluent, prepare the standard solution at the selected concentration and in the selected diluent and check it for the turbidly / precipitation for at least 24 hours, wherever applicable.

1.8 METHOD VALIDATION [12-14]

Validation is defined by the International Organization for Standardization (ISO) as “verification, where the specified requirements are adequate for an intended use”, where the term verification is defined as “provision of objective evidence that a given item fulfils specified requirements”.

The applicability and scope of an analytical method should be defined before starting the validation process. It includes defining the analytes, concentration range, description of equipment and procedures, validation level and criteria required. The validated range is defined by IUPAC as “the interval of analyte concentration within which the method can be regarded as validated”. This range does not have to be the highest and lowest possible levels of the analyte that can be determined by the method. Instead, it is defined on the basis of the intended purpose of the method. The

method can be validated for use as a screening (qualitative), semi-quantitative (e.g. 5-10ppm) or quantitative method. It can also be validated for use on single equipment, different equipment's in the laboratory, different laboratories or even for international use at different climatic and environmental conditions.

The criteria of each type of validation will of course be different with the validation level required. The various validation parameters include linearity, accuracy, precision, ruggedness, robustness, LOD, LOQ and selectivity or specificity.

Method Validation:

Accuracy:

Accuracy is the measure of exactness of an analytical method, or the closeness of agreement between the measured value and the value that is accepted either as a conventional, true value or an accepted reference value. Accuracy is measured as the percentage of analyte recovered by assay, by spiking samples in a blind study. For the assay of a drug substance, accuracy measurements are obtained by comparison of the results with those of a standard reference material, or by comparison to a second, well-characterized method. For the assay of a drug product, accuracy is evaluated by analyzing synthetic mixtures spiked with known quantities of components. For the quantization of impurities, accuracy is determined by analyzing samples (drug substance or drug product) spiked with known amounts of impurities. To document accuracy, the ICH guideline on methodology recommends collecting data from a minimum of nine determinations over a minimum of three concentration levels covering the specified range (for example, three concentrations with three replicates each). The data should be reported as the % recovery of the known, added amount, or as the difference between the mean and true value with confidence intervals.

Precision:

Precision can be defined as "the degree of agreement among individual test results when the procedure is applied repeatedly to multiple sampling of a homogenous sample". A more comprehensive definition proposed by the ICH divides precision into three types:

Repeatability:

Repeatability is the precision of a method under same operating conditions over a short period of time. One aspect of this is instrument precision. This is measured by the sequential, repetitive injection of the same homogenous sample (typically, 10 or more times), followed by the averaging of the peak area or peak-height values and determination of RSD of the all the injections. A second aspect is sometimes termed intra-assay precision and involves multiple measurements of the same sample (different preparation) by the same analyst under the same conditions.

Intermediate precision:

It 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 preparation of samples and standards.

Reproducibility:

It examines the precision between laboratories and is often determined in collaborative studies or method transfer experiments. The precision assessment during initial method validation often applies to the first two of these: repeatability and intermediate precision. Reproducibility is usually determined during method transfer or crossover to another laboratory or location. Precision often is expressed by the standard deviation (SD) or relative standard deviation (RSD) of a data set.

Specificity:

Specificity is the ability to measure accurately and specifically the analyte of interest in the presence of other components that may be expected to be present in the sample matrix. It is a measure of the degree of interference from such things as other active ingredients, excipients, impurities, and degradation products, ensuring that a peak response is only due to a single component; that is, that no co-elution exists. Specificity is measured and documented in a separation by the resolution, plate count (efficiency), and tailing factor. Specificity can also be evaluated with modern photodiode array detectors that compare spectra collected across a peak mathematically as an indication of peak homogeneity. ICH divides the term specificity into two separate categories: identification and assay/impurity tests. For identification purposes, specificity is demonstrated by the ability to discriminate between compounds of closely related structures, or by comparison to known reference materials. For assay and impurity tests, specificity is demonstrated by the resolution of the two closest eluting compounds. These compounds are usually the major component or active ingredient and an impurity. If impurities are available, it must be demonstrated that the assay is unaffected by the presence of spiked materials (impurities and/or excipients). If impurities are not available, the test results are compared to a second well-characterized procedure. For assay tests, the two results are compared; for impurity tests, the impurity profiles are compared head to head.

Limit of Detection (LOD):

The limit of detection is defined as the lowest concentration of an analyte in a sample that can be detected, though not necessarily quantitated. It is a limit test that specifies whether or not an analyte is above or below a certain value. It is expressed as a concentration at a specified signal-to noise ratio, usually a 2- or 3-to-1 ratio. The

ICH has recognized the signal-to-noise ratio convention but also lists two other options to determine LOD: visual no instrumental methods and a means of calculation. Visual no instrumental methods may include techniques such as thin-layer chromatography (TLC) or titrations. LODs may also be calculated based on the standard deviation (SD) of the response and the slope (S) of the calibration curve at levels approaching the LOD according to the formula: LOD = 3.3(SD/S). The standard deviation of the response can be determined based on the standard deviation of the blank, on the residual standard deviation of the regression line, or the standard deviation of y-intercepts of regression lines. The method used to determine LOD should be documented and supported, and an appropriate number of samples should be analyzed at the limit to validate the level.

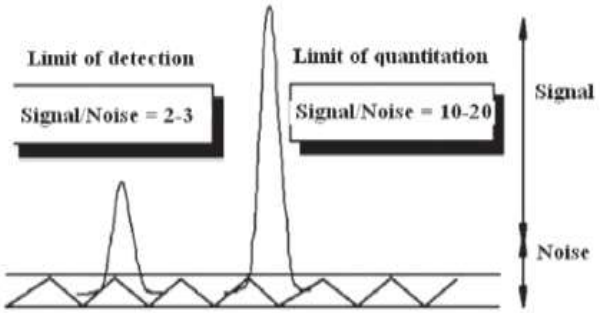


Fig No.1.12. Limit of Detection and Limit of Quantitation via Signal to Noise ratio (S/N)

Limit of Quantification (LOQ): The limit of quantification is defined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated operational conditions of the method. Like LOD, LOQ is expressed as a concentration, with the precision and accuracy of the measurement also reported. Sometimes a signal-to-noise ratio of 10-to-1 is used to

determine LOQ. This signal-to-noise ratio is a good rule of thumb, but it should be remembered that the determination of LOQ is a compromise between the concentration and the required precision and accuracy. That is, as the LOQ concentration level decreases, the precision decreases. If greater precision is required, a higher concentration must be reported for LOQ. This compromise is dictated by the analytical method and its intended use. The ICH has recognized the 10:1 signal-to-noise ratio as typical, and as for LOD, lists the same two additional options that can be used to determine LOQ: visual no instrumental methods and a means of calculation. The calculation method is again based on the standard deviation (SD) of the response and the slope (S) of the calibration curve according to the formula LOQ = 10(SD/S). Again, the standard deviation of the response can be determined based on the standard deviation of the blank, on the residual standard deviation of the regression line, or the standard deviation of y intercepts of regression lines. As with LOD, the method used to determine LOQ should be documented and supported, and an appropriate number of samples should be analyzed at the limit to validate the level. One additional detail should be considered: both the LOQ and the LOD can be affected by the chromatography. Sharper peaks result in a higher signal-to-noise ratio, resulting in lower LOQs and LODs. Therefore the chromatographic determination of LOQ and LOD should take into account both the type and age of the column, which is usually determined over the course of time as one gains experience with the method.

Linearity and Range:

Linearity is the ability of the method to elicit test results that are directly proportional to analyte concentration within a given range. Linearity is generally reported as the variance of the slope of the regression line. Range is the (inclusive) interval between the upper and lower levels of analyte that have been demonstrated to

be determined with precision, accuracy, and linearity using the method. The range is normally expressed in the same units as the test results obtained by the method. The ICH guidelines specify a minimum of five concentration levels, along with certain minimum specified ranges. For assay tests, the minimum specified range is 80–120% of the target concentration. For impurity tests, the minimum range is from the reporting level of each impurity to 120% of the specification. (For toxic or potent impurities, the range should be commensurate with the controlled level.) For content uniformity testing, the minimum range is 70–130% of the test or target concentration and for dissolution testing, ±20% over the specified range of the test.

Ruggedness:

Ruggedness, according to the USP, is the degree of reproducibility of the results obtained under a variety of contions, expressed as % relative standard deviation (RSD). These conditions include differences in laboratories, analysts, instruments, reagents, and experimental periods. In the guideline on definitions and terminology, the ICH does not address ruggedness specifically. This apparent omission is really a matter of semantics, however, as ICH chooses instead to cover the topic of ruggedness as part of precision, as discussed previously.

Robustness:

Robustness is the capacity of a method to remain unaffected by small deliberate variations in method parameters. The robustness of a method is evaluated by varying method parameters such as percent organic solvent, pH, ionic strength, or temperature, and determining the effect (if any) on the results of the method. As documented in the ICH guidelines, robustness should be considered early in the development of a method. In addition, if the results of a method or other measurements are susceptible to variations in method parameters, these parameters

should be adequately controlled and a precautionary statement included in the method documentation.

Solution Stability:

Generate reproducible and reliable results, the sample, standards, and reagents used for the HPLC method must be stable for reasonable time (e.g. one day, one week, one month, depending on need.) for e.g., the analysis of even a single sample may require ten or more chromatographic runs to determine system suitability, including standard concentrations to create a working analytical curve and duplicate or triplicate injections of the sample to be assayed. Therefore, a few hours of standard and sample solution stability can be required even for a short separation. Buffer mobile phases may cause problems; for e.g., phosphate and acetate provide good media for microbial growth. Sodium amide (0.1%) is often added mobile phase buffer to inhibit such growth; adding more than 5% of organic solvents is also effective.

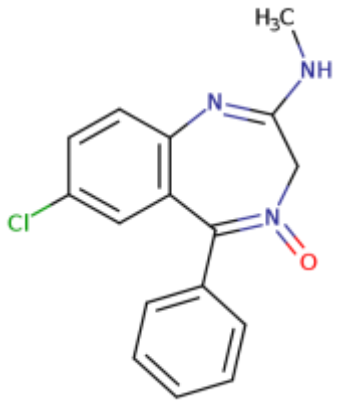
System Suitability:

It can be defined as tests to ensure that the method can generate results of acceptable accuracy and precision. The USP defines parameters that can be used to determine the system suitability prior to analysis. These parameters include: - plate no. (n), tailing factor, k and / or, resolution (Rs), and relative standard deviation (RSD) of peak height or peak area or repetitive injections. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such.

DRUG PROFILE

Chlorodipoxide[15]

Chemical structure :



IUPAC name : 7-chloro-2-(methyl amino)-5-phenyl-3H-1,4λ^5-benzodiazepine 4-one

Chemical formula : $C_{16}H_{14}ClN_3O$

Molecular weight : 299.755gm/mol

Category : Anti-Anxiety Agents
Hypnotics and Sedatives
Benzodiazepines

Solubility : Soluble in water

Melting point : 236-236.5^{O}C

Mechanism of Action :

Chlorodipoxide binds to stereospecific benzodiazepine (BZD) binding sites on GABA (A) receptor complexes at several sites within the central nervous system, including the limbic system and reticular formation. This results in an increased binding of the inhibitory neurotransmitter GABA to the GABA(A) receptor.

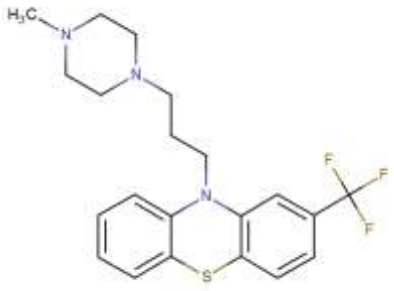
BZDs, therefore, enhance GABA-mediated chloride influx through GABA receptor channels, causing membrane hyperpolarization. The net neuro-inhibitory effects result in the observed sedative, hypnotic, anxiolytic, and muscle relaxant properties.

Generic Name : Chlorodipoxide Hydrochloride

Uses : An anxiolytic benzodiazepine derivative with anticonvulsant, sedative, and amnesic properties. It has also been used in the symptomatic treatment of alcohol withdrawal.

Trifluoperazine[16]

Chemical Structure :



IUPAC Name : 10-[3-(4-METHYL-piperazin-1-yl)-propyl]-2-trifluoromethyl-10H-phenothiazine.

Chemical formula : $C_{21}H_{24}F_3N_3S$

Molecular weight : 407.496

Category : Antipsychotic Agent

Dopamine Antagonist

Antiemetic

Psycholeptics

Solubility **:** soluble in organic solvents

Melting Point **:** 158-160^{o}C

Mechanism of action :

Trifluoperazine blocks postsynaptic mesolimbic dopaminergic D1 and D2 receptors in the brain; depresses the release of hypothalamic and hypophyseal hormones and is believed to depress the reticular activating system thus affecting basal metabolism, body temperature, wakefulness, vasomotor tone, and emesis.

Uses **:** It is used as an antipsychotic and an antiemetic.

AIM AND OBJECTIVE

Literature review reveals that there is few analytical method reported for the analysis of Chlorodipoxide and Trifluoperazine by simultaneous estimation by RP-HPLC. Spectrophotometer, HPLC and HPTLC are the reported analytical methods for compounds either individually or in combination with other dosage form. Hence, it was felt that, there is a need of new analytical method development for the simultaneous estimation of Chlorodipoxide and Trifluoperazine in pharmaceutical dosage form.

Present work is aimed to develop a new, simple, fast, rapid, accurate, efficient and reproducible RP-HPLC method for the simultaneous analysis of Chlorodipoxide and trifluoperazine of the developed method will be validated according to ICH guidelines.

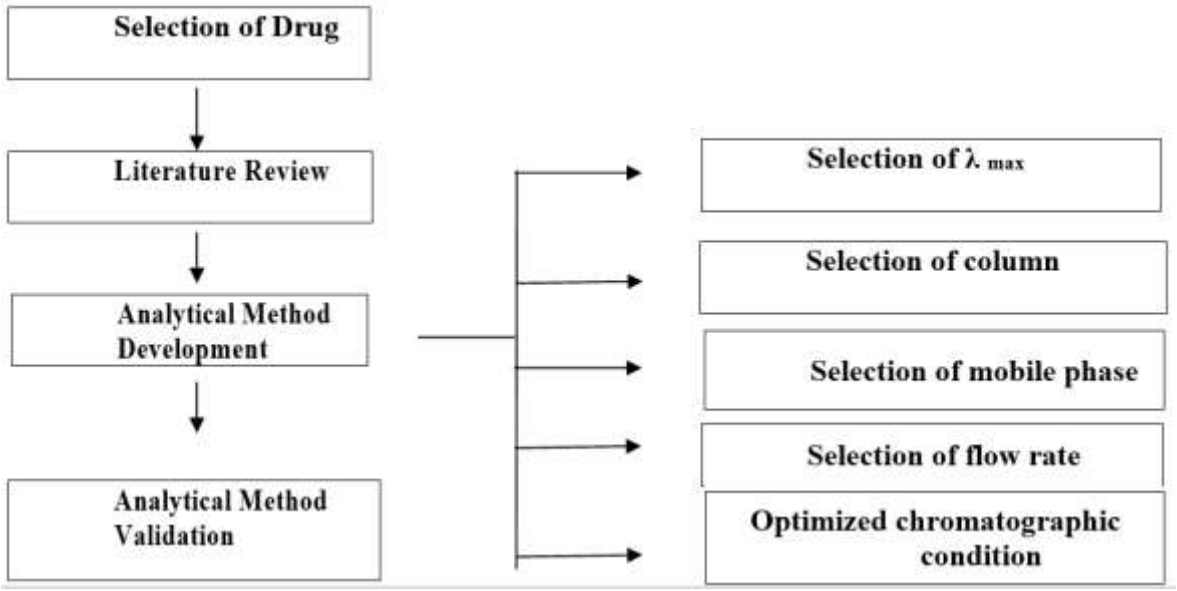
Objective of the work

- The analytical method for the simultaneous estimation of Chlorodipoxide and Trifluoperazine will be developed by RP-HPLC method by optimizing the chromatographic conditions.
- The developed method is validated according to ICH guidelines for various parameters specified in ICH guidelines, Q2 (R1).

PLAN OF WORK

To develop a new analytical method for the simultaneous estimation Chlorodipoxide and Trifluoperazine of by RP-HPLC method.

The dissertation work has been carried out in the following steps:



LITERATURE REVIEW

1.Shashi Daksh et al,[17] RP-HPLC method in which determination of Trifluoperazine hydrochloride and Trihexy phenidyl hydrochloride was carried out by reverse phase C-18 column (Inertsil ODS-3,250*4.6mm) using a mobile phase consisting of acetonitrile: water: triethyelamine (68: 31.8: 0.2v/v) with pH 4 adjusted by using ortho-phosphoric acid. The mobile phase was pumped at rate of 1.0 ml/min and the detection was carried out at 210 nm. The linearity was found in the range of 10-150 µg/ml and 4-60 µg/ml with regression coefficient (r=0.999 for both). The peaks obtained were sharp having clear baselines separation with a retention time of 2.78±5.68 and 2.31 ± 4.72 min for Trifluoperazine hydrochloride and Trihexyphenidyl hydrochloride respectively.

2. Aravind.Doki et al,[18] The study describes method development and subsequent validation of RP-HPLC method for simultaneous estimation of Clidinium bromide (CDB), Chlordiazepoxide (CDZ) and Dicyclomine hydrochloride (DICY) in bulk and combined tablet dosage forms. Chromatographic separation was achieved on a Kromasil C18 (250 mm × 4.6 mm id, 5µm) column using a mobile phase ratio consisting of (40:30:30) Methanol: Acetonitrile: Potassium di hydrogen phosphate buffer (0.05M, PH 4.0 adjusting with 0.5% Ortho phosphoric acid) at flow rate 1.0 ml/min. The detection wavelength is 270 nm. The retention times of Clidinium bromide, Chlordiazepoxide and Dicyclomine hydrochloride were found to be 7.457 min, 4.400 min and 3.397 min respectively. The developed method was validated as per ICH guidelines using the parameters such as accuracy, precision, linearity, LOD, LOQ, ruggedness and robustness. The developed and validated method was successfully used for the quantitative analysis of Clidinium bromide,

Chlorodizepoxide and Dicyclomine hydrochloride in bulk and combined tablet dosage forms.

3. Navya Sri D et al,[19] A simple, precise, rapid, specific and accurate reverse phase high performance liquid chromatography method was developed for simultaneous estimation of Trifluoperazine and Isopropamide in pharmaceutical dosage form. Chromatographic separation was performed on Agilent zorbax SB-C18, 4.6 x 250mm, 5micros column, with mobile phase comprising of mixture of buffer (pH 6.0, adjusted with Ortho phosphoric acid, Acetonitrile in the ratio of 80:20v/v), at the flow rate 0.8ml/min. The detection was carried out at 227nm. The retention times of Trifluoperazine and Isopropamide were found to be 2.4 and 3.6mins respectively with a run time of 10mins, theoretical levels for Trifluoperazine and Isopropamide were 5194 and 6738 respectively, with a resolution of 7.6. As per ICH guidelines the method was validated for linearity, accuracy, precision, limit of detection and limit of quantitation, robustness and ruggedness. Linearity of Trifluoperazine and Isopropamide was found in the range of 30-130µg/ml and that for Isopropamide was found to be 150-250µg/ml. The correlation coefficient for Trifluoperazine and Isopropamide were 0.999 and 1 respectively. The LOD values for Trifluoperazine and Isopropamide were 2.963 and 2.9851 respectively. The LOQ values for Trifluoperazine and Isopropamide were and 9.877µg/ml and 9.9502µg/ml respectively. This demonstrates that the developed method is simple, precise, rapid, selective, accurate and reproducible for simultaneous estimation of Trifluoperazine and Isopropamide tablet dosage form.

4. A. Ashok Kumar et al,[20] To develop an accurate, precise and linear Reverse Phase High Performance Liquid Chromatographic (RP-HPLC) method for simultaneous quantitative estimation of Mebeverine hydrochloride and

Chlorodizepoxide in MEVA C capsules and validate as per ICH guidelines. The optimized method uses a reverse phase column, Enable Make C18G (250 X 4.6 mm; 5µ), a mobile phase of triethyl ammonium phosphate buffer (pH 2.5): acetonitrile in the proportion of 60:40 v/v, flow rate of 1.0 ml/min and a detection wavelength of 240 nm using a UV detector. The developed method resulted in Mebeverine eluting at 5.8 min and Chlordiazepoxide at 3.9 min. Mebeverine exhibited linearity in the range 250-750µg/ml, while Chlordiazepoxide exhibited linearity in the range 9.25-27.75µg/ml. The precision is exemplified by relative standard deviations of 1.45% for Mebeverine and 1.58% for Chlordiazepoxide. Percentage Mean recoveries were found to be in the range of 98-102, during accuracy studies. The limit of detection (LOD) for Mebeverine hydrochloride and Chlordiazepoxide were found to be 91.67µg/ml and 2.23µg/ml respectively, while limit of quantitation (LOQ) for Mebeverine hydrochloride and Chlordiazepoxide were found to be 277.81µg/ml and 6.77µg/ml respectively. A simple, accurate, precise, linear and rapid RP-HPLC method was developed for simultaneous quantitative estimation of Mebeverine hydrochloride and Chlordiazepoxide in MEVA C capsules and validated as per ICH guidelines. Hence it can be used for the routine analysis of Mebeverine and Chlordiazepoxide in capsules in various pharmaceutical industries.

5. Ameen Q. Waleed et al,[21] A new method was developed for the determination of trifluoperazine hydrochloride (TFPH) at trace levels in pharmaceuticals. This method involved formation of a TFPH-Pt(IV) complex at a specific pH, and extraction of this complex into an organic solvent. Flame atomic absorption spectrometry was used to measure the absorbance of platinum in the complex, and indirectly determine the concentration of TFPH. Under the optimized conditions, the linear dynamic range, detection limit, relative standard deviation (n = 5), and the recoveries of the standard

addition method were 2−60 μg mL^{-1}, 0.085 μg mL^{-1}, 1.58−2.03, and 102.24 ± 0.43, respectively. The proposed method was applied to the determination of TFPH in the drug Stelazine (5 mg of TFPH per tablet) by both direct and standard addition procedures and gave results of 4.89 and 4.88 mg of TFPH per tablet. This method was compared statistically with the determination of TFPH by UV–Vis spectroscopy, and the difference in the precision from these methods was insignificant at the 95% confidence level.

6. Sree Vidya Parvataneni1 et al,[22] A simple reverse phase liquid chromatographic method has been developed and subsequently validated for simultaneous determination of Trifluoperazine hydrochloride and Trihexyphenidyl hydrochloride in combination. The separation was carried out using a mobile phase consisting of acetonitrile: 0.02 M sodium dihydrogen orthophosphate dihydrate (60:40), pH adjusted to 4.5 with orthophosphoric acid. The column used was Supelco 516 C-18, 25 cm × 4.6 mm i.d., with flow rate of 1 ml / min using PDA detection at 210 nm. The described method was linear over a concentration range of 32-96μg/ml and 4-12 μg/ml for the assay of Trifluoperazine hydrochloride and Trihexyphenidyl hydrochloride respectively. The retention times of Trifluoperazine hydrochloride and Trihexyphenidyl hydrochloride were found to be 5.4 and 3.5mins respectively. Results of analysis were validated statistically and by recovery studies. The limit of detection (LOD) and the limit of quantification (LOQ) for Trifluoperazine hydrochloride and Trihexyphenidyl hydrochloride were found to be 0.0015μg/ml and 0.0010μg/ml, 0.0045and 0.0032μg/ml respectively. The results of the study showed that the proposed RP-HPLC method is simple, rapid, precise and accurate, which is useful for the routine determination of Trifluoperazine hydrochloride and Trihexyphenidyl hydrochloride bulk drug and in its pharmaceutical dosage form.

7. N. Ananda Kumar Reddy et al,[23] A new simple, rapid, accurate, sensitive and precise spectrophotometric method for the determination of trifluoperazine hydrochloride in bulk and capsule dosage form .pharmaceutical formulations is described. The method is based on the simple solubility of trifluoperazine hydrochloride in methanol. The absorbance maximum of trifluoperazine hydrochloride measured at wave length 265 nm. The drug obeys Beer's Law in the concentration range 2-45 µg/ml employed for this method. Accuracy and reproducibility of the proposed method was statistically validated by recovery studies. The method is easily be employed in the laboratory for the routine estimation of drug and it's extended to the analysis of trifluoperazine hydrochloride in pharmaceutical formulations.

8. Safwan Ashour et al,[24] A selective, sensitive and simple reversed-phase HPLC method for the determination of risperidone in bulk powder and pharmaceutical formulations was developed and validated. Risperidone can be separated on a Supelcosil LC_8 DB column (250 mm × 4.6 mm i.d., 5 µm particle size) at 40°C with a mobile phase of methanol and 0.1 M ammonium acetate pH 5.50 (60:40, *v/v*), pumped at flow rate 1.0 mL min^{-1} and detected at 274 nm. Chlordiazepoxide hydrochloride was used as internal standard. The retention time of risperidone and chlordiazepoxide hydrochloride was found to be 5.89 and 7.65 min, respectively. The validation of the proposed method was carried out for specificity, linearity, accuracy, precision, limit of detection, limit of quantitation and robustness. The linear range was 4.0-275.0 µg mL^{-1} (r^2=0.9998) with limits of detection and quantification values of 0.48 and 1.59 µg mL^{-1}, respectively. The precision of the method was demonstrated using intra- and inter-day assay RSD values which were less than 3.27%, while the recovery was 99.00-101.12% (*n*=5). According to the validation results, the proposed method was

found to be specific, accurate, and precise and could be applied to the quantitative analysis of risperidone in raw material and tablets.

9. Sejalk.patel et al,[25] A binary mixture of trifluoperazine HCl and chlordiazepoxide was determined using reversed-phase liquid chromatography method using methanol:water (97:03, v/v) pumped at a flow rate of 1.0 ml/min. Quantification was achieved with ultraviolet detection at 262 nm over concentration ranges of 0.1-1 and 0.5-5 μg/ml; mean accuracies were 101.05±0.47 and 98.97±0.33 %, respectively. The method was successively applied to tablet dosage forms as no chromatographic interferences from the tablet excipients were observed. The method retained its accuracy and precision when the standard addition technique was applied.

10. P. Shetti et al.[26] A new, simple, precise, rapid, selective and stability indicating reversed-phase high performance liquid chromatographic (HPLC) method has been developed and validated for simultaneous quantification of trihexyphenidyl hydrochloride, trifluoperazine hydrochloride and chlorpromazine hydrochloride from combined tablet formulation. The method is based on reverse-phase using C-18 (250×4.6) mm, 5 μm particle size column. The separation is achieved using isocratic elution by methanol and ammonium acetate buffer (1% w/v, pH 6.5) in the ratio of 85:15 v/v, pumped at flow rate 1.0 mL/min and UV detection at 215 nm. The column is maintained at 30 °C throughout the analysis. This method gives baseline resolution. The total run time is 15 min. Stability indicating capability is established buy forced degradation experiment. The method is validated for specificity, accuracy, precision and linearity as per International conference of harmonisation (ICH). The method is accurate and linear for quantification of trihexyphenidyl hydrochloride,

trifluoperazine hydrochloride and Chlorpromazine hydrochloride between 5 - 15 µg/mL, 12.5- 37.5µg/ ml and 62.5 - 187.µg/ml respectively.

11. Nief Rahman Ahmed et al,[27] A simple, accurate, precise, rapid, economical and sensitive UV spectrophotometric method has been developed for the determination of trifluoperazine Hydrochloride in pharmaceutical preparations and environmental wastewater samples, which shows maximum absorbance at 257 nm in distilled water. Beer's law was obeyed in the range of 10 -100 µg/ ml, with molar absorptive of 5.284×103 L.mol-1.cm-1, relative standard deviation of the method was less than 1.6%, and accuracy (average recovery %) was 100 ± 1.2. No interference was observed from common excipients and additives often accompany with trifluoperazine Hydrochloride in pharmaceutical preparations .The method was successfully applied to the determination of trifluoperazine Hydrochloride in some pharmaceutical formulations (tablets) and industrial wastewater samples. The proposed method was validated by sensitivity and precision which proves suitability for the routine analysis of trifluoperazine Hydrochloride in true samples.

12. Bharath Rathna Kumar. P et al,[28] A simple, rapid, accurate, specific and sensitive reverse phase-HPLC method has been developed and validated for the simultaneous estimation of Trifluoperazine and Chlordiazepoxide in pharmaceutical dosage form. The chromatographic separation was performed on InertsilODS-3V C18 Column (150mm×4.6mm, 5µm particle size) using a mobile phase of Mixed phosphate buffer: Acetonitrile (55:45 v/v), at a flow rate of 1.0ml/min at an ambient temperature with the detection wave length at 252nm. The retention times of Trifluoperazine and Chlordiazepoxide were 5.001 min and 3.058 min respectively. The linearity was performed in the concentration range of 2.5-15µg/ml (Trifluoperazine) and 2.5-15µg/ml (Chlordiazepoxide) with a correlation coefficient

of 0.9997 and 0.9983 for Trifluoperazine and Chlordiazepoxide respectively. The percentage purity of Trifluoperazine and Chlordiazepoxide was found to be 99.46 and 99.13% w/v respectively. The proposed method has been validated for specificity, linearity, precision, accuracy and robustness were within the acceptance limit according to ICH guidelines and the developed method was successfully employed for routine quality control analysis in the combined pharmaceutical dosage forms.

MATERIALS AND METHODS

6.1 MATERIALS:

The list of instruments used in the course of experimental work is as follows:

Table No. 6.1 List of Instruments

S. No	Equipments	Source
1	HPLC	Shimadzu Separation Module LC- 20AT Prominence Liquid chromatograph
2	P^H meter	Eli co.
3	Vacuum pump	Gloney LC 10AT.
4	Sonicator	Winsor.
5	Detector	SPD 10A UV-Detector.
6	Colum	C_{18}, 150x4.6mm,5µ BDS Hypersil
7	Injector	Rheyodone

The experimental work involves several chemicals. Chemicals used presently are listed below:

Table No. 6.2 List of Chemicals:

S.No.	Chemical	Manufacturer	Grade
1	Water	Merck	HPLC Grade
2	Methanol	Merck	HPLC Grade
3	Acetonitrile	Merck	HPLC Grade
4	Potassium di hydrogen phosphate	Merck	A.R
5	Chlorodipoxide and Trifluoperazine	MNS	A.R

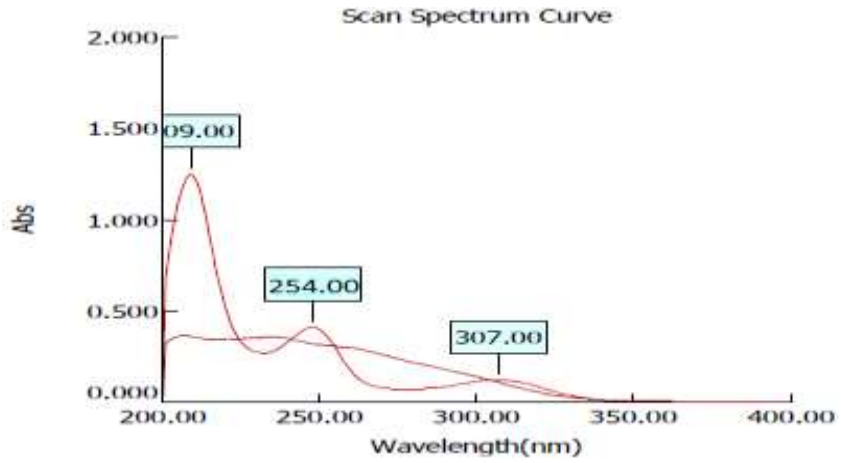
6.2 METHOD DEVELOPMENT:

Method development for simultaneous estimation of Chlorodizepoxide and Trifluoperazine in Pharmaceutical dosage forms includes the following steps:

1. Selection of detection wavelength (λ_{max})
2. Selection of column
3. Selection of mobile phase
4. Selection of flow rate
5. Preparations and procedures

1. Selection of Detection wavelength:

10 mg of Chlorodizepoxide and Trifluoperazine was dissolved in mobile phase. The solution was scanned from 200-400 nm the spectrum was obtained. The overlay spectrum was used for selection of wavelength for Chlorodiazepoxide and Trifluoperazine. The isobestic point was taken as detection wavelength.



2. Selection of column:

Column is selected based on solubility, polarity and chemical differences among Analytes [Column: C_{18}, 150x4.6mm,5µ BDS Hypersil].

3.Selection of mobile phase:

ACN : Buffer (45:55%v/v) has been selected as mobile phase. If any buffer selected buffer pH should be between 2 to 8. If the buffer pH is below 2 siloxane linkages are cleaved. If the buffer pH is above 8 dissolution of silica takes place. pH controls the elution properties by controlling the ionization characteristics. It also decreases the retention and improves separation. Good Response, Area, Tailing factor, Resolution will be achieved.

4. Selection of flow rate:

Flow rate selected was 1.0ml/min

Flow rate is selected based on

1. Retention time
2. Column back pressure
3. Peak symmetry
4. Separation of impurities

Trial:1

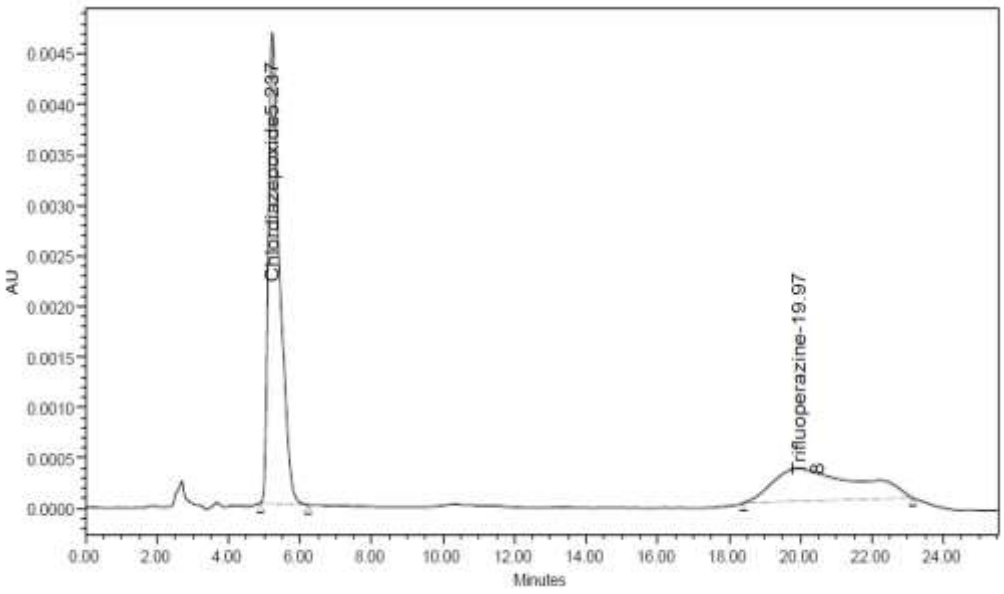


Fig No. 6.1 Chromatogram of Trial-1

1. Preparation of Buffer:

0.01M potassium di hydrogen phosphate was dissolved in 1000ml of water and the pH was adjusted to 3 using diluted.

2. Preparation of mobile phase:

Filter and degas mixture of acetonitrile and water in the ratio of 20:80 and filter through 0.45µmicron membrane filter.

Chromatographic Parameters:

Equipment	:	HPLC Shimadzu Separation Module LC- 20AT Prominence Liquid Chromatograph
Mobile phase	:	Water : Acetonitrile
Ratio	:	20:80
Column	:	BDS Hypersil C18 (250×4.6, 5µ)
Wave length		236nm
Flow rate		1ml/min
Injection volume	:	20 µl
Column oven	:	Ambient
Run Time	:	12 min

Observation: Chlorodipoxide and Trifluoperazine were separated and two individual peaks are displayed.

Trail-2

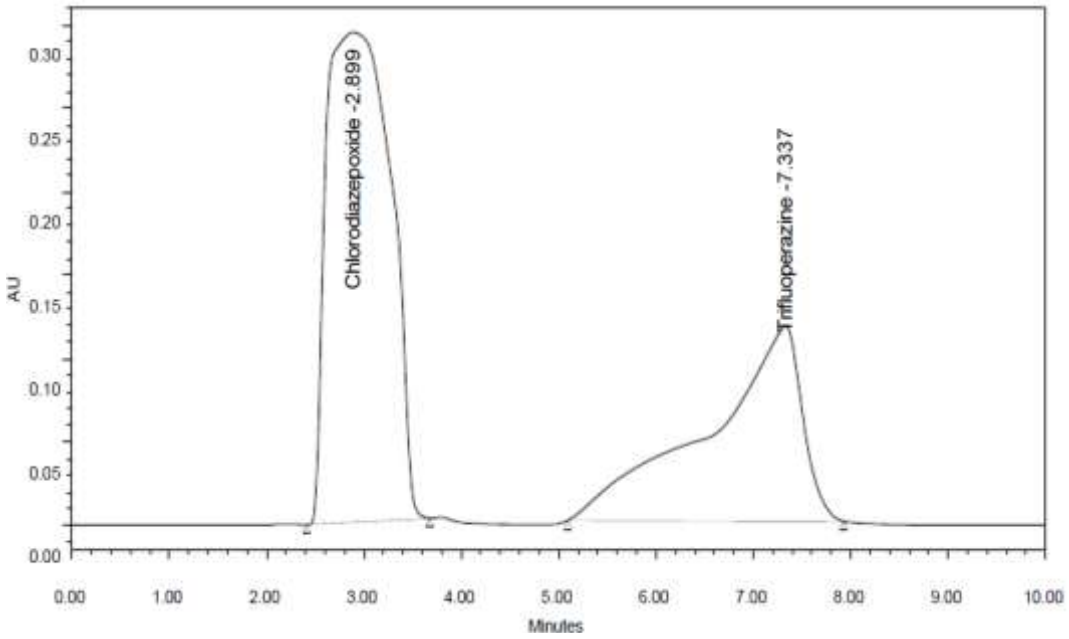


Fig No 6.2 Chromatogram of Trial-2

Preparation of mobile phase:

Filter and degas mixture of Methanol and water in the ratio of 70:30 and filter through 0.45 micron membrane filter.

Chromatographic Parameters:

Equipment	: HPLC Shimadzu Separation Module LC-20AT Prominence Liquid Chromatograph
Mobile phase	: Methanol : water
Ratio	: 30:70
Column	: Symmetry C18 (150×4.6 ,5µ)
Wave length	: 236nm
Flow rate	: 1ml/min
Injection volume	: 20µl
Column oven	: ambient
Run Time	: 12min

Observation:

Peak S symmetry is being improved when compared to the previous trial. Further trials are conducted for better resolution.

Trial-3:

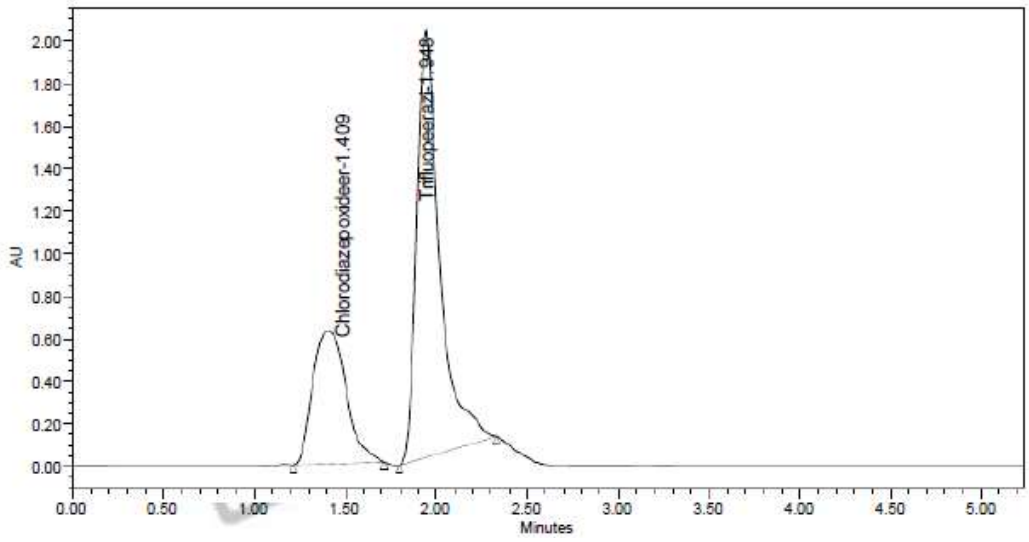


Fig No 6.3 Chromatogram of Trial-3

Preparation of Buffer:

0.1 M of sodium di hydrogen orthophosphate was dissolved in 1000ml of water, sonicate and then filter.

Preparation of mobile phase:

Filter and degas mixture of Methanol and buffer in the ratio of 60:40 and filter through 0.25 micron membrane filter.

Chromatographic Parameters:

Equipment	:	HPLC Shimadzu Separation Module Prominence Liquid Chromatograph
Mobile phase	:	0.1M Sodium di hydrogen Ortho Phosphate Buffer : Methanol
Ratio	:	60:40
Column	:	Inertsil ODS C18 (150×4.6,5µ)
Wave length	:	236nm
Flow rate	:	1ml/min
Injection volumes	:	:20µl
Column oven	:	Ambient
Run Time	:	12min

Observation:

There is noticeable improvement in resolution. But peak symmetry is not achieved.

Trial-4:

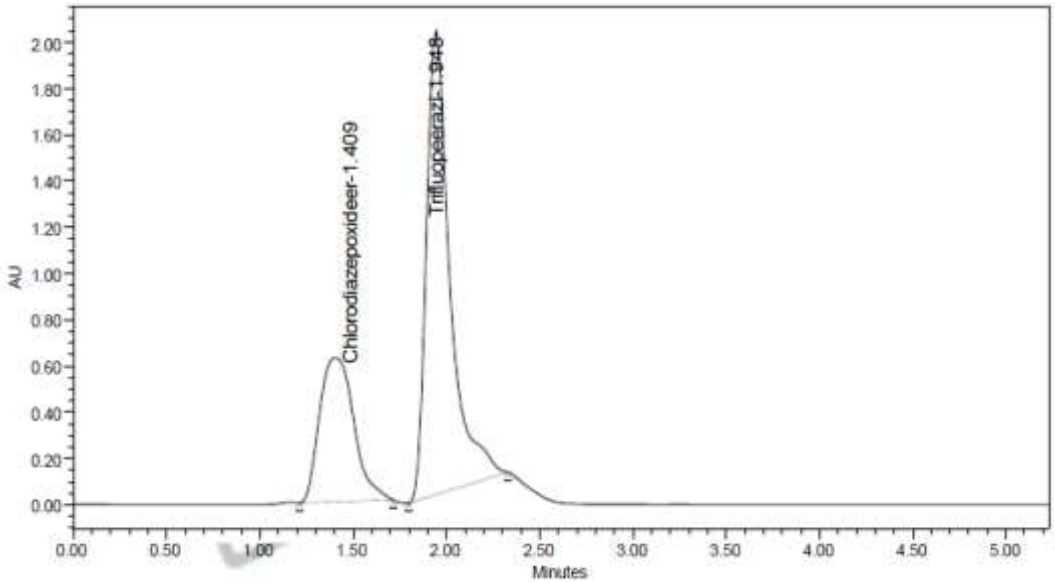


Fig No 6.4: Chromatogram of Trial-4

Preparation of Buffer :

0.02 M of Ammonium Acetate was dissolved in 1000ml of water, sonicate and then filter.

Preparation of mobile phase :

Filter and degas mixture of methanol and buffer in the ratio of 70:30 and filter through 0.25 micron membrane filter.

Chromatographic Parameters:

Equipment	:	HPLC Shimadzu Separation Module LC- 20AT Prominence Liquid Chromatograph
Column	:	Symmetry C8 (150×4.6,3.5µ)
Mobile Phase	:	0.02M Ammonium Acetate buffer : Methanol.
Ratio	:	30:70
Flow rate	:	1.5ml/min
Wavelength	:	236nm
Injection volume	:	20µl
Column oven	:	Ambient

Observation: Chlorodipoxide was eluted clearly and Trifluoperazine peak was not merged and peak shape was not good.

OPTIMIZED METHOD

Preparation of Buffer:

Dissolve 1.4969gm of Potassium di hydrogen phosphate (0.02M), in 550 ml HPLC water and sonicate for 10 min. Filtered through 0.25 microns membrane filter.

Preparation of mobile phase:

Filtered and degassed mixture of Acetonitrile and buffer in the ratio of 45:55 was made and filtered through 0.25 micron membrane filter.

Table 6.3: Optimized Chromatographic Condition

Parameters	**Description**
Mode of operation	Isocratic
Diluents	Methanol
Column	C_{18}, 150x4.6mm,5µ BDS Hypersil
Mobile phase	Acetonitrile: buffer (45:55)
Flow rate	1.0 ml/min
Detection	236 nm
Temperature	25^0 C
Injection Volume	20 µl
Run time	9 min
Detector	UV detector
Excitation Wave length	236nm

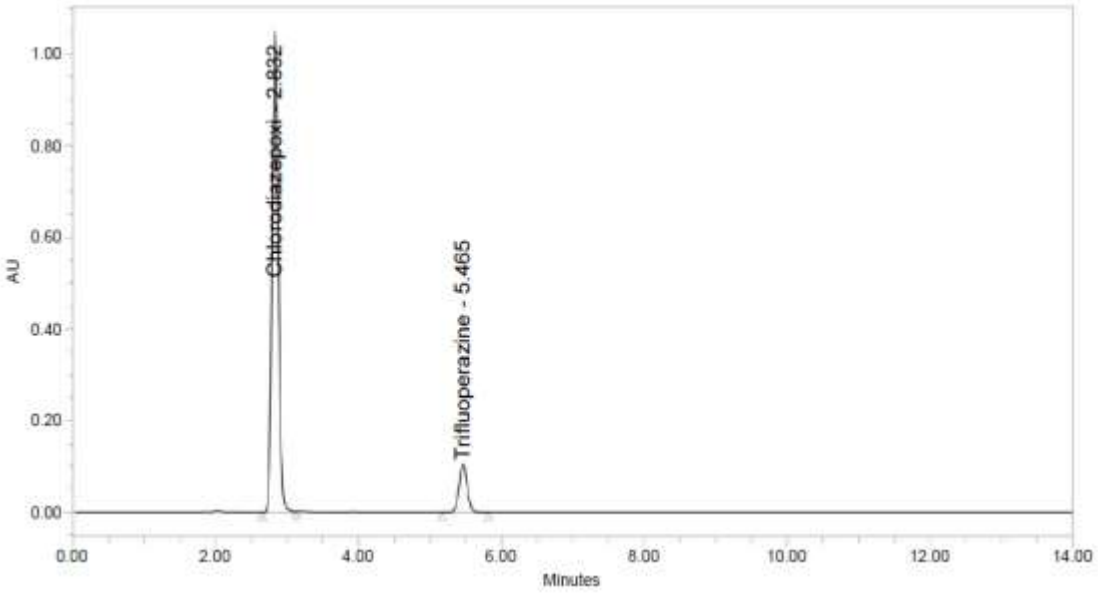


Fig No.6.5: Chromatogram of optimized method

Observation:

The chromatogram is perfect with clear separation of components. The peak symmetry and system suitability parameters are within the limits. Hence this method is chosen as optimized one.

6.4. Preparations and procedures:

Preparation of mobile phase:

A mixture of ACN 700ml (70%), 300mL of Buffer (30%) are taken and degassed in ultrasonic water bath for 5 minutes. Then this solution is filtered through 0.45 µ filter under vacuum filtration.

Diluent Preparation:

Methanol is used as Diluent**.**

Preparation of the individual Chlorodipoxide standard:

10mg of Chlorodipoxide working standard was accurately weighed and transferred into a 10ml clean dry volumetric flask and about 2ml of Methanol is added. Then it is sonicated to dissolve it completely and made volume up to the mark with the diluents (Stock solution). Further 10.0 ml from the above stock solution is pipette into a 100 ml volumetric flask and was diluted up to the mark with diluents.

Preparation of the individual Trifluoperazine standard preparation:

10mg of Trifluoperazine working standard was accurately weighed and transferred into a 10ml clean dry volumetric flask and about 2ml of Methanol is added. Then it is sonicated to dissolve it completely and made volume up to the mark with the diluent (Stock solution). Further 10.0 ml from the above stock solution is pipette into a 100 ml volumetric flask and was diluted up to the mark with diluents.

Preparation of Sample Solution:

Accurately 10 tablets are weighed and crushed in mortar and pestle and weight equivalent to 10 mg of Chlorodipoxide and Trifluoperazine (marketed formulation) sample into a 10mL clean dry volumetric flask and about 7mL of Diluents is added and sonicated to dissolve it completely and made volume up to the mark with the same solvent. (Stock solution) Further 3 ml of above stock solution was pipetted into a10ml volumetric flask and diluted up to the mark with diluents.

Procedure:

20µL of the standard, sample are injected into the chromatographic system and the areas for Chlorodipoxide and Trifluoperazine peaks are measured and the %Assay are calculated by using the formulae.

ASSAY CALCULATION:

The assay study was performed for the Chlorodipoxide and Trifluoperazine. Each three injections of sample and standard was inject into chromatographic system. The chromatograms are shown in Fig. No. 6.6 – 6.9.

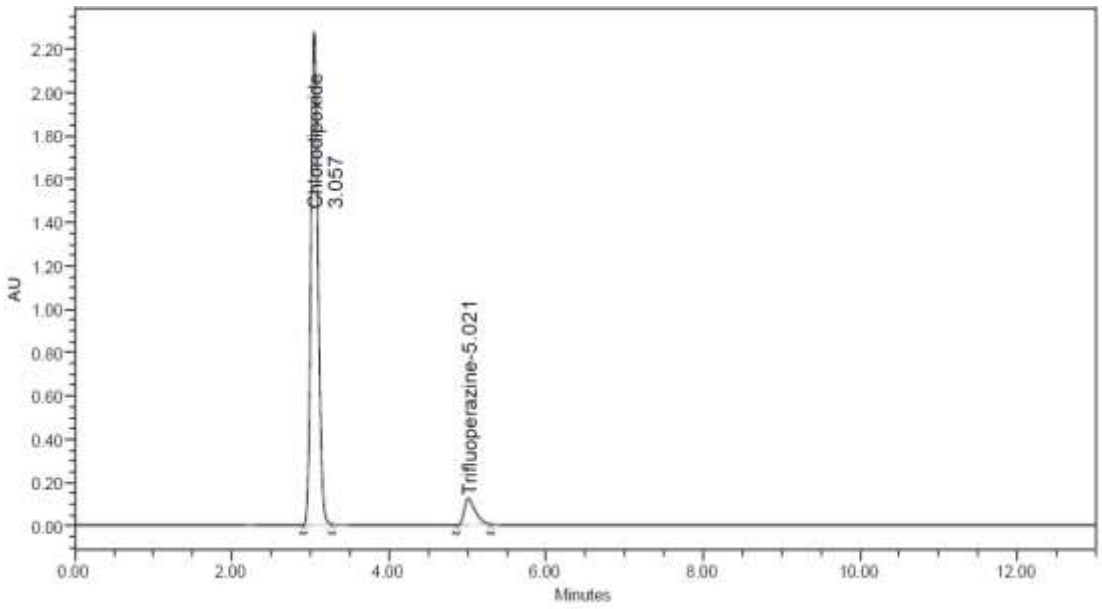


Fig No. 6.6: Chromatogram showing sample injection-1

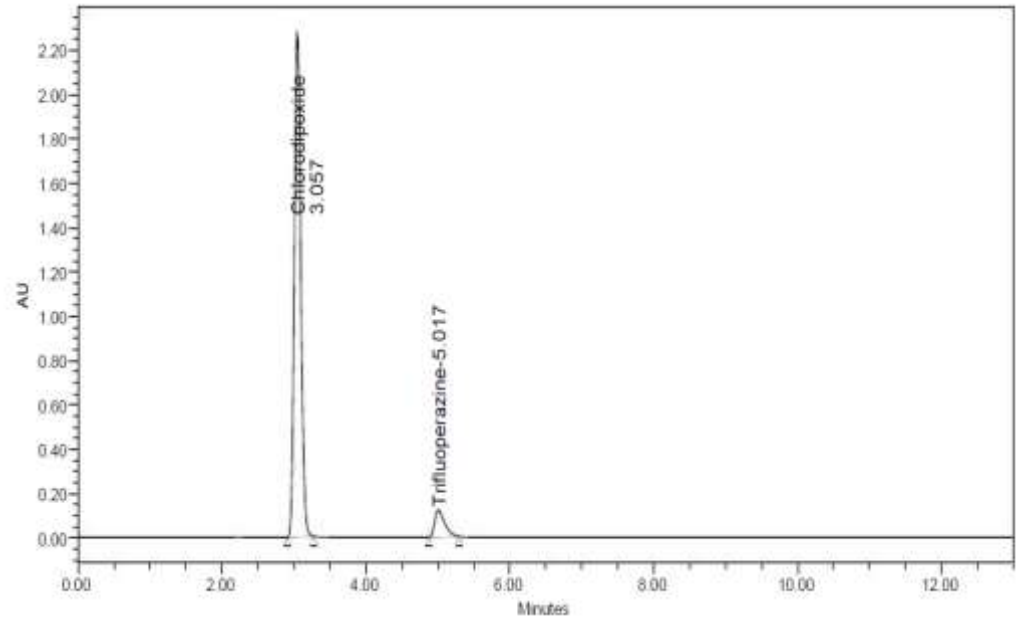


Fig No. 6.7: Chromatogram showing sample injection-2

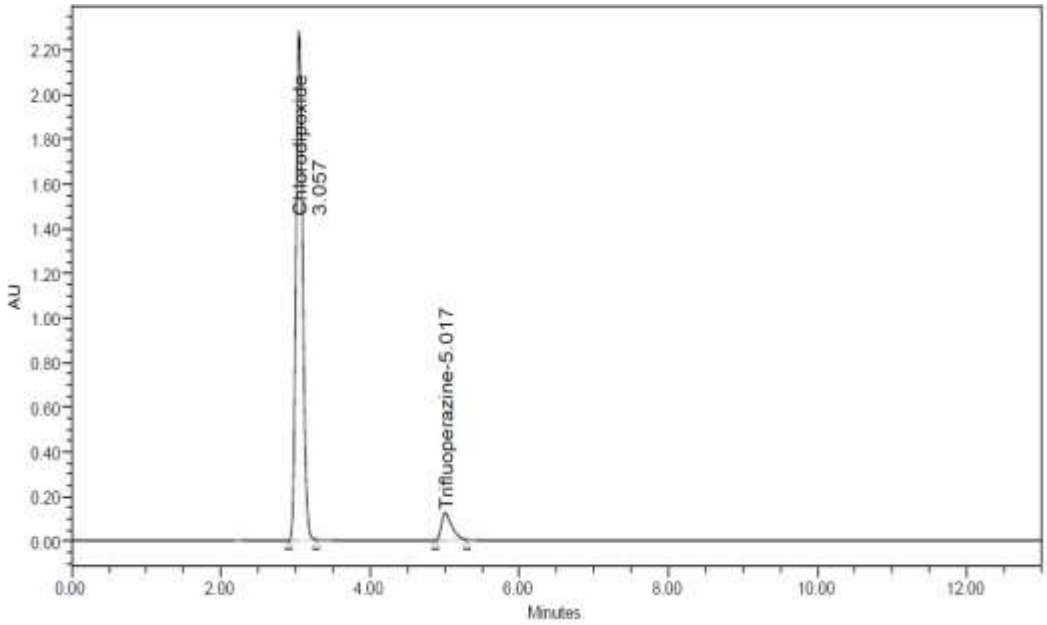


Fig No. 6.8: Chromatogram showing standard injection-1

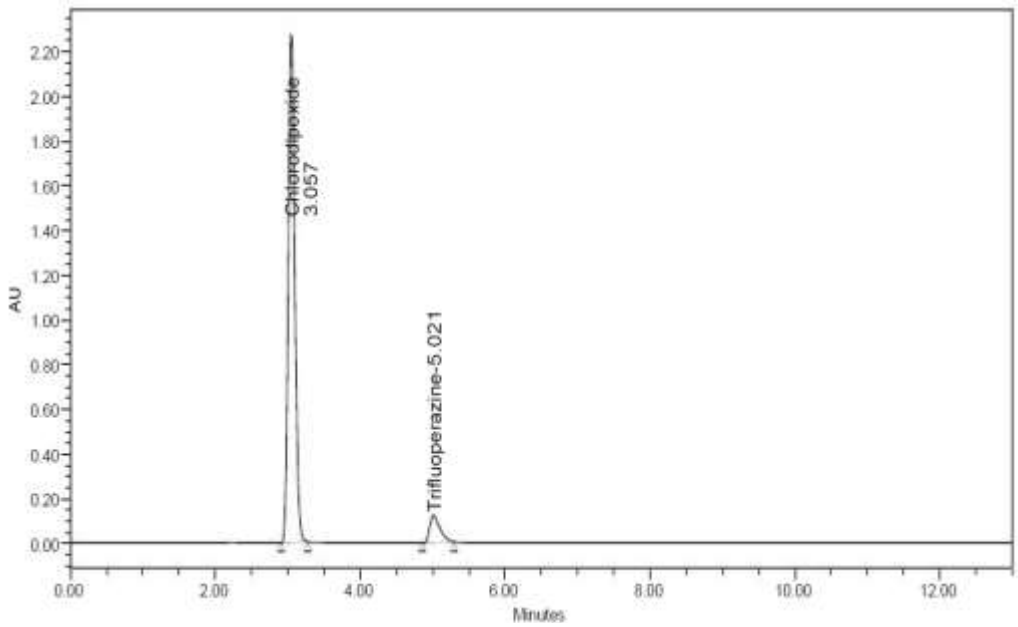


Fig No. 6.9: Chromatogram showing standard injection-2

Table No .6.4: Observation for Assay

S No		Chlorodipoxide		Trifluoperazine	
		Retention Time (Min)	Peak Area	Retention Time (Min)	Peak Area
1	Standard-1	3.057	14280015	5.017	1171564
2	Standard-2	3.057	14287892	5.017	1175466
Average		3.057	14283954	5.017	1173515
1	Assay-Sample	3.057	14374879	5.021	1172043
2	Assay-Sample	3.057	14364785	5.021	1171988
Average		3.057	14369832	5.021	1172016

VALIDATION RESULTS

1. ACCURACY:

The accuracy study was performed for 50%, 100% and 150 % for Each level was injected in triplicate into chromatographic system. The area of each level was used for calculation of % recovery. Chromatograms are shown in Figs. 7.1-7.12 and results are tabulated in Tables 7.1-7.4

Preparation of standard solution (Chlorodipoxide and Trifluoperazine):

Accurately weighed 10 mg of Chlorodipoxide and 10 mg of Trifluoperazine working standard were transferred into a 10mL and 100ml of clean dry volumetric flasks. About 7mL and 70ml of Diluents are added and sonicated to dissolve it completely and made volume up to the mark with the same solvent. (Stock solution) Further 0.3ml and 0.3ml of the above stock solution was pipetted into a 10ml volumetric flask and diluted up to the mark with diluents.

Preparation of Sample solutions:

For preparation of 50% solution (With respect to target Assay concentration):

Accurately 5mg of Chlorodipoxide and 5mg Trifluoperazine working standard were weighed and transferred into a 10mL and 100ml of clean dry volumetric flask and about 7mL of Diluents was added and sonicated to dissolve it completely and made volume up to the mark with the same solvent (Stock Solution). Further 3ml and 0.3ml of the above Chlorodipoxide and Trifluoperazine stock solution were pipetted into a 10ml volumetric flask and diluted up to the mark with diluents.

For preparation of 100% solution (With respect to target Assay concentration):

Accurately 10mg of Chlorodipoxide and 10 mg of Trifluoperazine working standard were weighed and transferred into a 10mL and 100ml of clean dry volumetric flask and about 7mL of Diluents was added and sonicated to dissolve it

completely and made volume up to the mark with the same solvent (Stock Solution). Further 0.3ml and 0.3ml of the above Chlorodipoxide and Trifluoperazine stock solution were pipetted into a 10ml volumetric flask and diluted up to the mark with diluents.

For preparation of 150% solution (With respect to target Assay concentration):

Accurately 15mg of Chlorodipoxide and 15 mg of Trifluoperazine working standard were weighed and transferred into a 10mL and 100ml of clean dry volumetric flask and about 7mL of Diluents was added and sonicated to dissolve it completely and made volume up to the mark with the same solvent (Stock Solution). Further 0.3ml and 0.3ml of the above Chlorodipoxide and Trifluoperazine stock solution were pipetted into a 10ml volumetric flask and diluted up to the mark with diluents.

Procedure:

1. Accuracy:

The standard solution, Accuracy -50%, Accuracy -100% and Accuracy -150% solutions were injected. The Amount found and Amount added for Chlorodipoxide and Trifluoperazine and the individual recovery and mean recovery values were calculated.

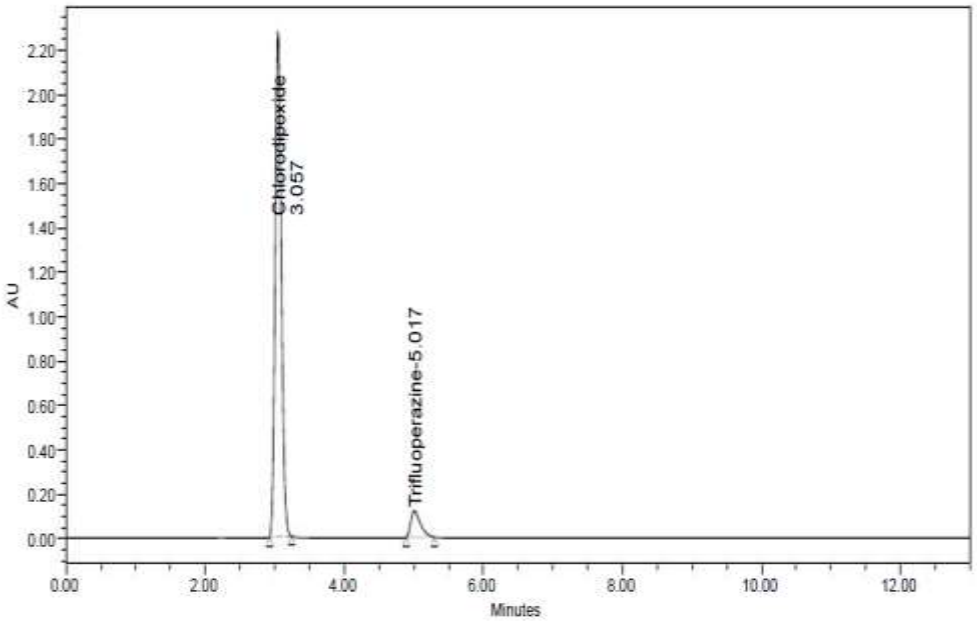


Fig No: 7.1. Chromatogram for Accuracy standard injection-1

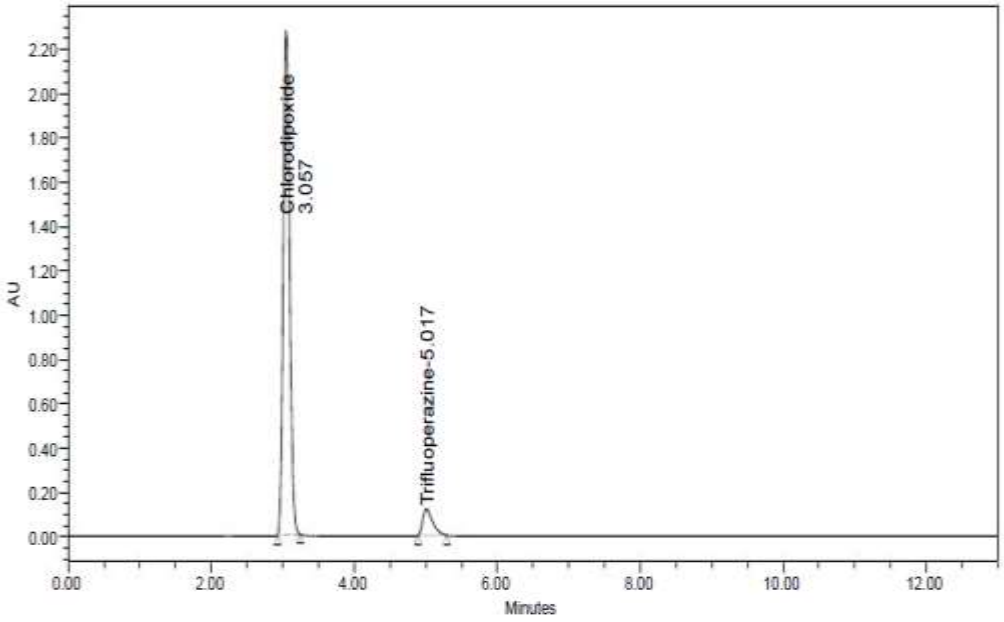


Fig No. 7.2: Chromatogram for Accuracy standard injection-2

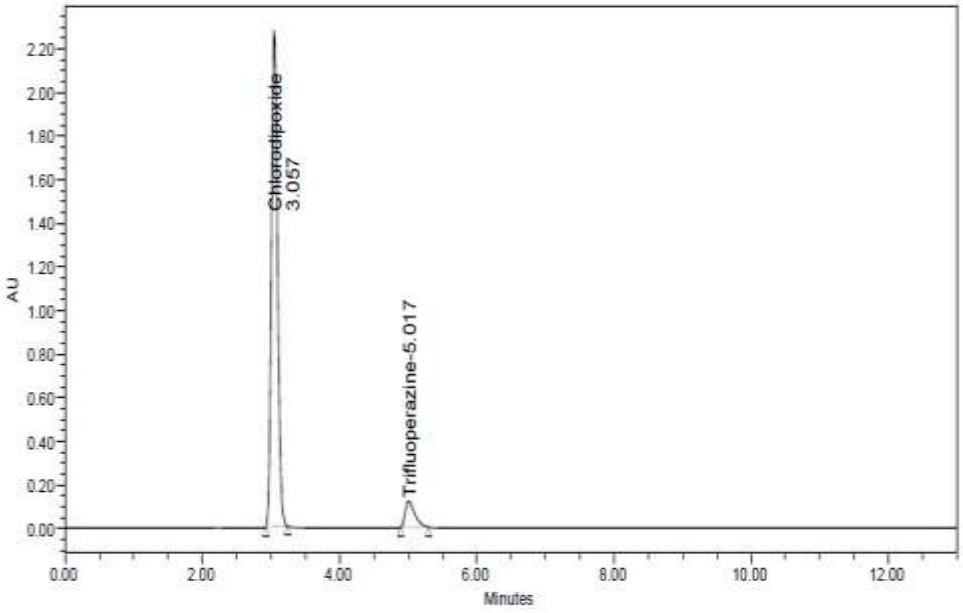


Fig No 7.3 :Chromatogram for Accuracy standard injection-3

Table No. 7.1: Observation for Accuracy standard

S.NO	Injections	Peak area	
		Chlorodipoxide	**Trifluoperazine**
1	Set-1	14211779	1149025
2	Set-2	14218756	1148648
3	Set-3	14220875	1147859
	AVG	14217137	1148511
	SD	4759.30	595
	%RSD	0.03	0.05

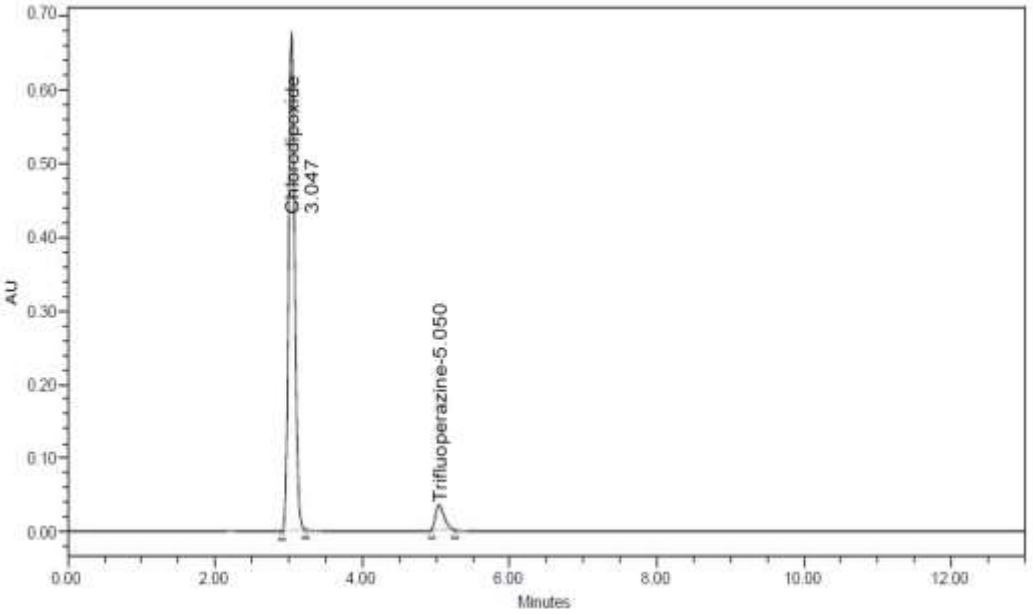


Fig No 7.4 :Chromatogram for Accuracy 50%injection-1

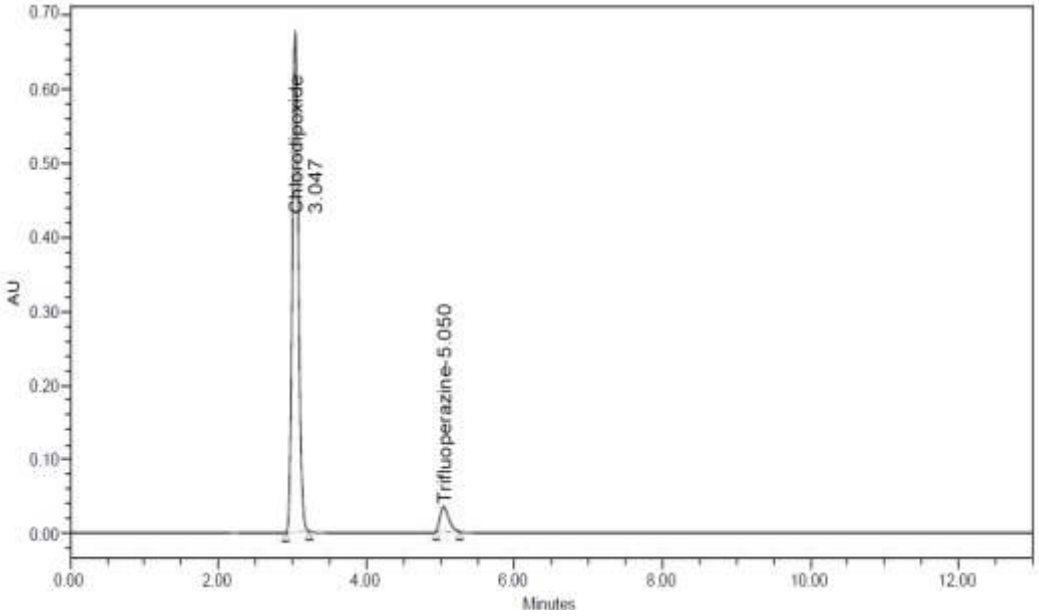


Fig No7.5: Chromatogram for Accuracy 50% injection-2

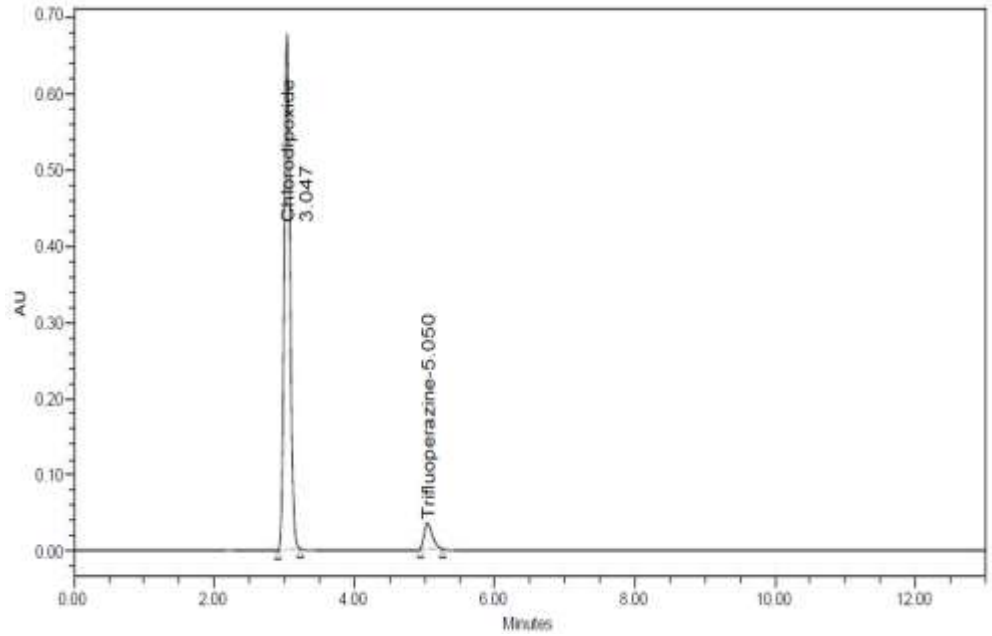


Fig No 7.6: Chromatogram for Accuracy 50% injection-3

Table No.7.2: Observation for accuracy (50%)

S.NO	Injections	Peak Area	
		Chlorodipoxide	Trifluoperazine
1	Set-1	3514719	287197
2	Set-2	3513785	286145
3	Set-3	3509874	286478
	AVG	3512793	286607
	Result	24.71	24.95
	%Rec	49.4	49.9
	SD	2570.4	537.6
	%RSD	0.07	0.18

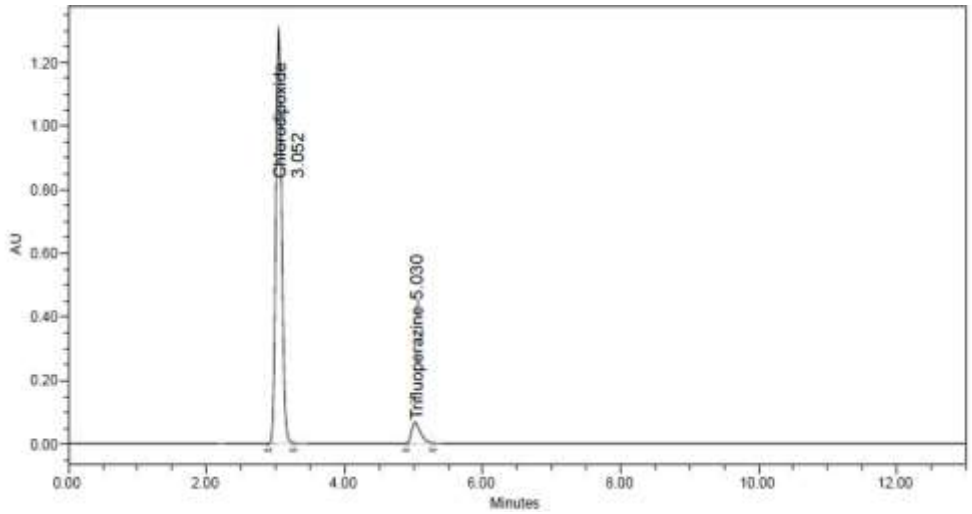


Fig No 7.7: Chromatogram for Accuracy 100%injection-1

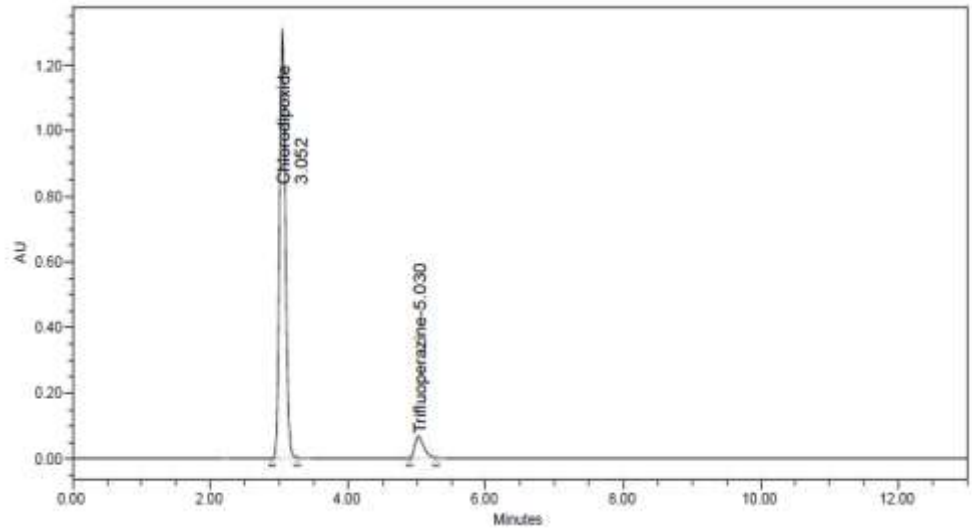


Fig No 7.8: Chromatogram for Accuracy 100%injection-2

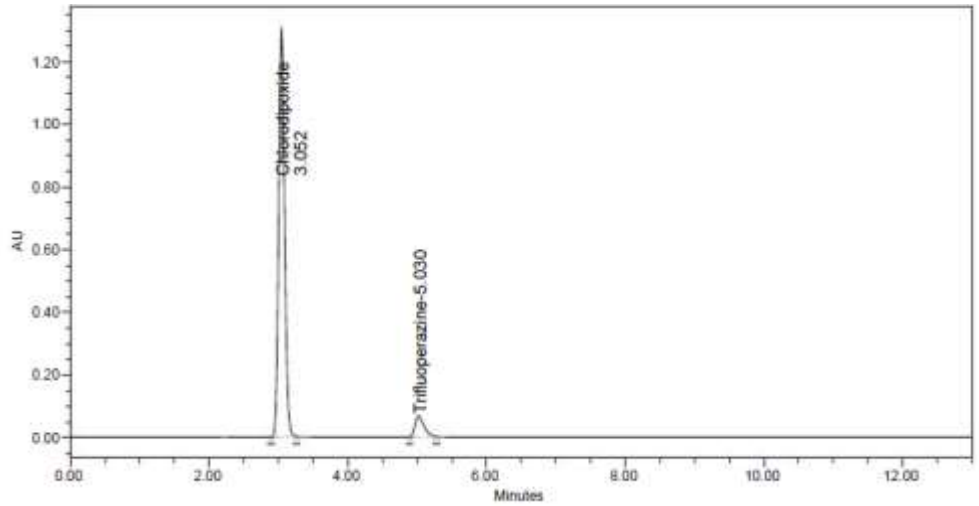


Fig No 7.9 : Chromatogram for Accuracy 100%injection-3

Table No.7.3: Observation for Accuracy 100%

S.NO	Injections	Peak Area	
		Chlorodipoxide	**Trifluoperazine**
1	Set-1	14181414	1138115
2	Set-2	14192458	1138524
3	Set-3	14190785	1137985
	AVG	14188219	1138208
	Result	99.8	99.10
	%Rec	99.8	99.1
	SD	5952.3	281.2
	%RSD	0.04	0.024

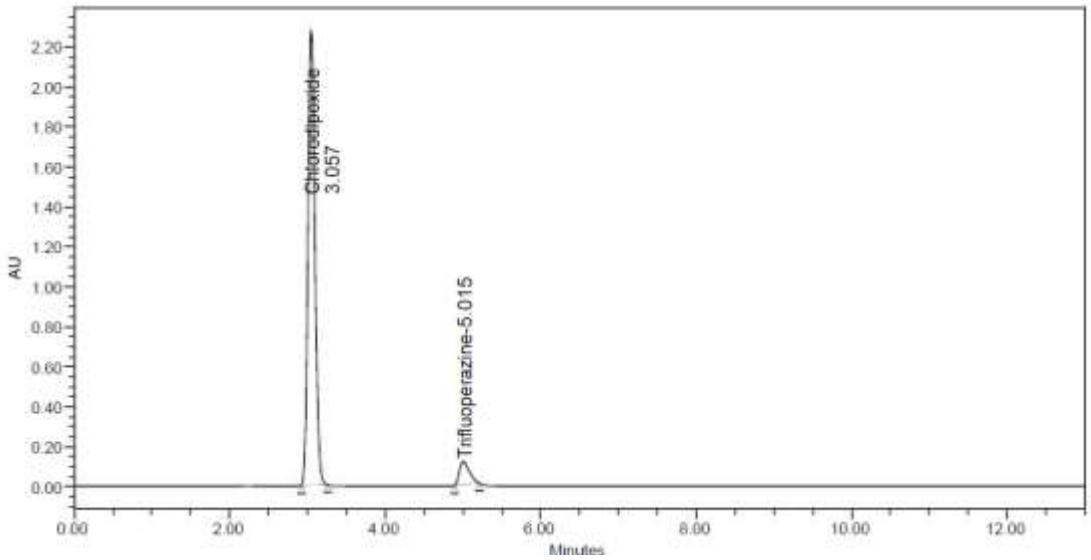


Fig No 7.10: Chromatogram for Accuracy 150%injection-1

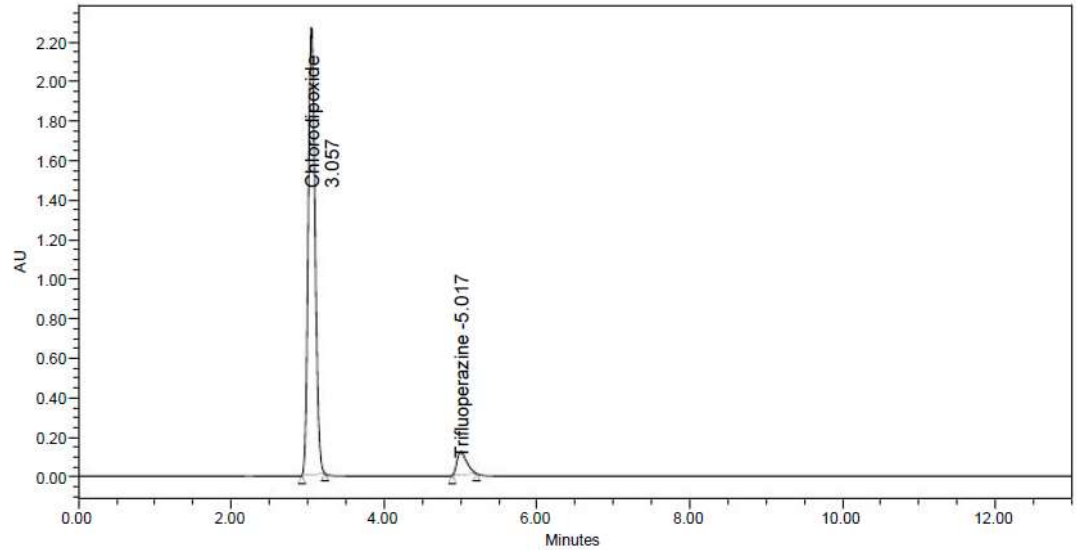


Fig No 7.11: Chromatogram for Accuracy 150%injection-2

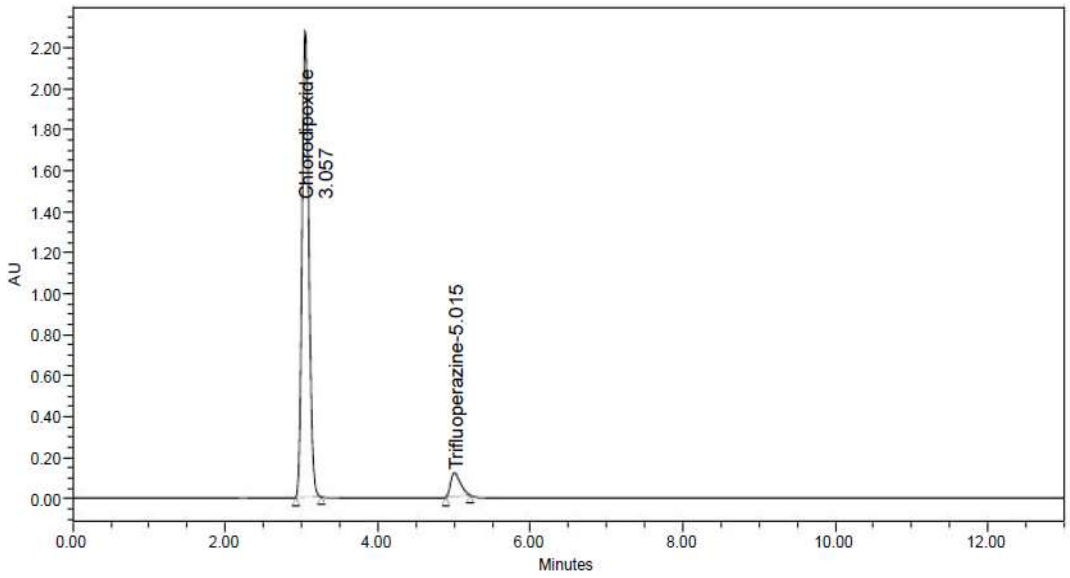


Fig No 7.12: Chromatogram for Accuracy 150%injection-3

Table 7.4: Observation for Accuracy 150%

S.NO	Injections	Peak Area	
		Chlorodipoxide	Trifluoperazine
1	Set-1	21284578	1716546
2	Set-2	21283469	1715875
3	Set-3	21247852	1716524
	AVG	21271966	1716315
	Result	149.62	149.44
	%Rec	99.7	99.6
	SD	20890.9	381.2
	%RSD	0.09	0.02

2. PRECISION:

The precision of the analytical method was studied by analysis of multiple sampling of homogeneous sample. The precision expressed as standard deviation or relative standard deviation.

A. Method precision

B. System precision

Preparation of standard stock solution:

Accurately 10 mg of Chlorodipoxide and 10 mg of Trifluoperazine working standard were weighed and transferred into a 10mL and 100ml of clean dry volumetric flasks and about 7mL and 70ml of Diluent was added and sonicated to dissolve it completely and made volume up to the mark with the same solvent. (Stock solution) Further it was pipette (0.3ml and 0.3ml) into a 10ml volumetric flask and diluted up to the mark with diluent.

The precision study was performed for five injections of Chlorodipoxide and Trifluoperazine Each standard injection was injected in to chromatographic system. The area of each Standard injection was used for calculation of % RSD. The chromatograms are shown in Fig.7.13 – 7.18 and results are tabulated in Table 7.5

A. **Method precision:**

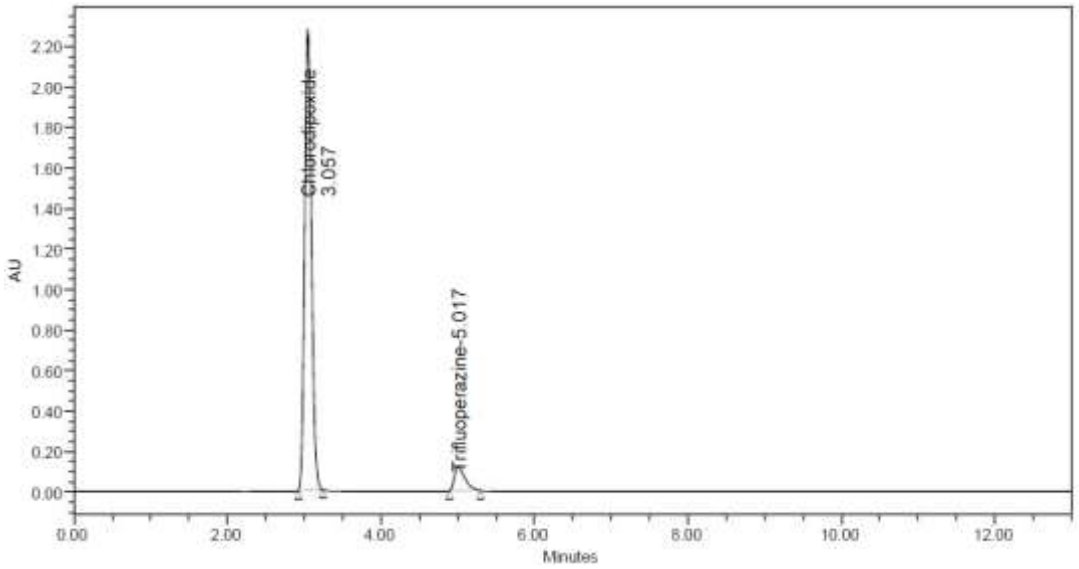


Fig No: 7.13:Chromatogram for Method Precision (standard-1)

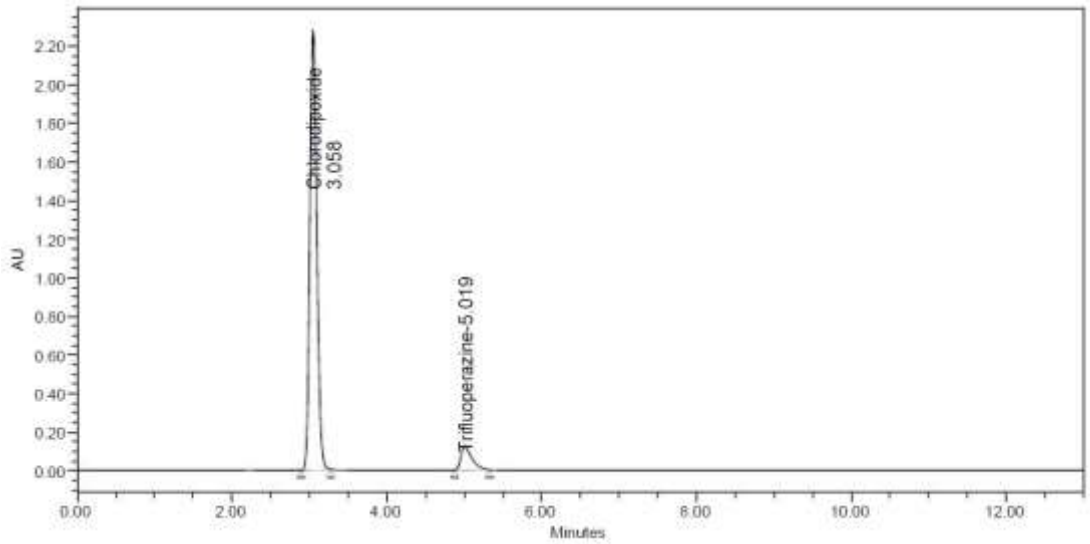


Fig No:7.14:Chromatogram for Method Precision (standard -2)

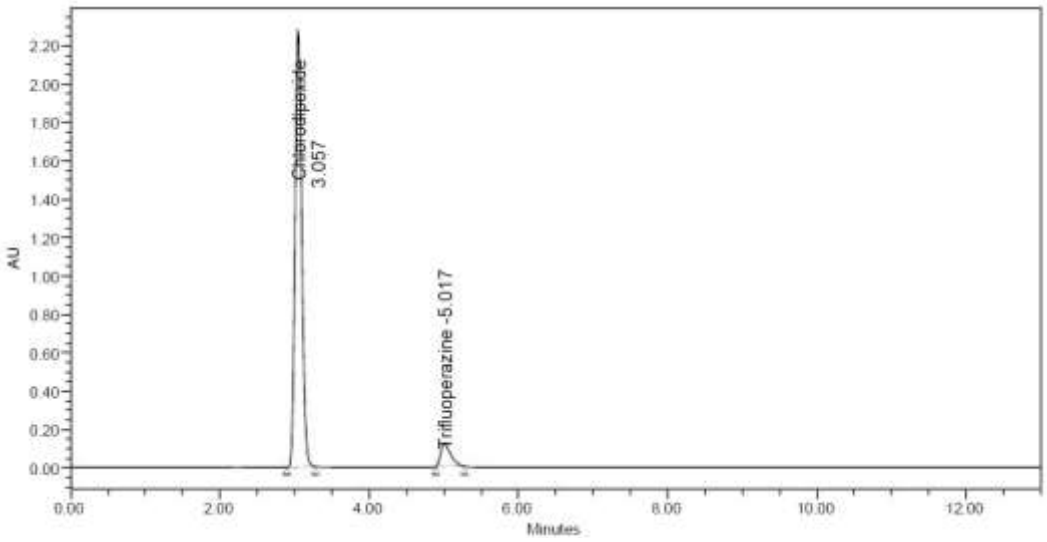


Fig No: 7.15:Chromatogram for Method Precision (standard -3)

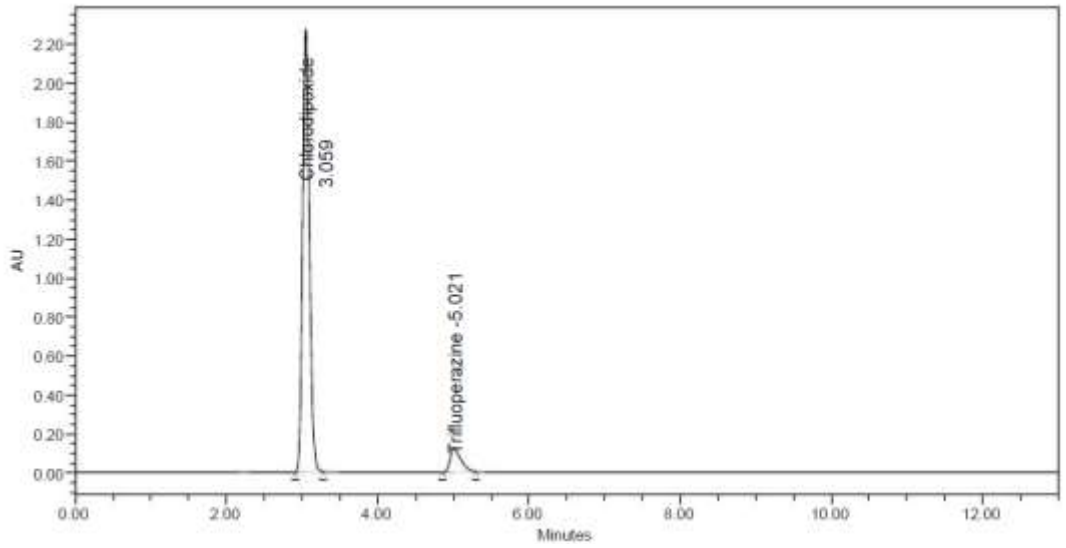


Fig No:7.16: Chromatogram for Method Precision (standard -4)

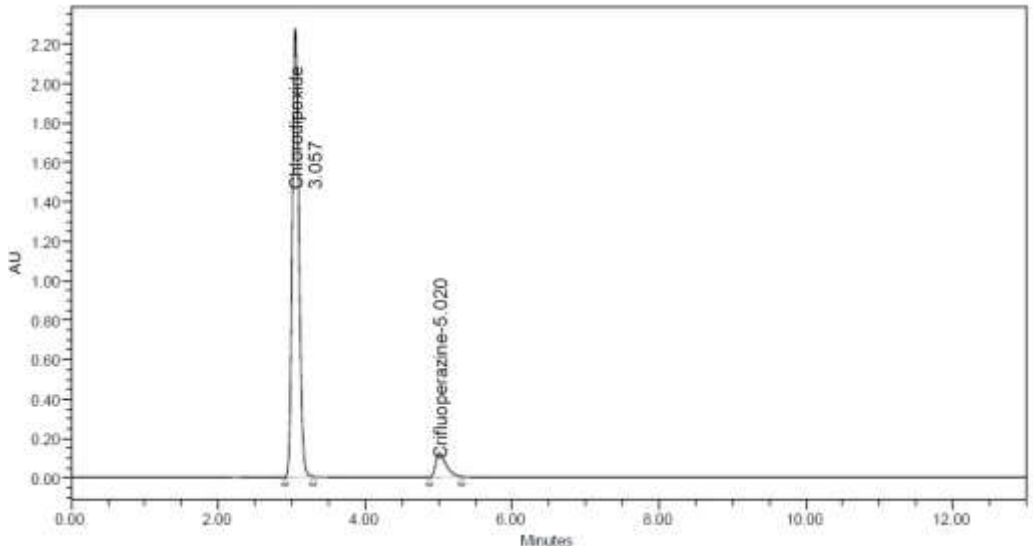


Fig No: 7.17:Chromatogram for Method Precision (standard -5)

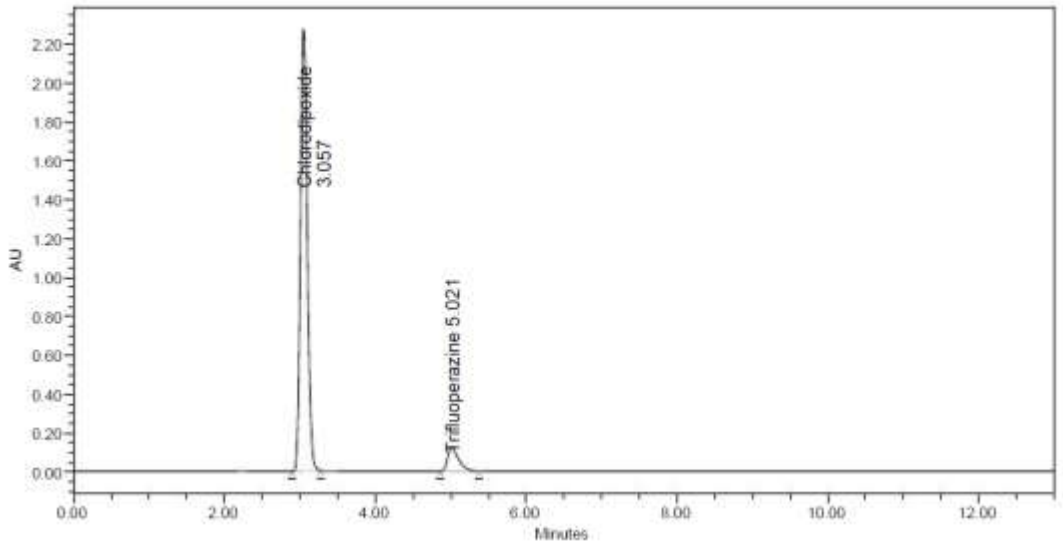


Fig No: 7.18:Chromatogram for Method Precision (standard -6)

Table No .7.5: Method Precision Result for Chlorodipoxide and Trifluoperazine

S.No	Chlorodipoxide		Trifluoperazine	
	Retention Time (min)	Peak Area	Retention Time(min)	Peak Area
1	3.057	14211779	5.017	1189025
2	3.058	14279960	5.019	1183777
3	3.057	14279960	5.017	1184535
4	3.059	14374879	5.021	1172043
5	3.057	14387095	5.02	1186300
6	3.057	14374755	5.021	1182384
AVG	3.058	14318071	5.019	1183011
SD	0.0008	71284.53	0.0018	5840.98
%RSD	0.027	0.498	0.037	0.494

B. System precision

Preparation of standard stock solution:

Accurately 10 mg of Chlorodipoxide and 10 mg of Trifluoperazine working standard were weighed and transferred into a 10mL and 100ml of clean dry

volumetric flasks and about 7mL and 70ml of Diluents was added and sonicated to dissolve it completely and made volume up to the mark with the same solvent. (Stock solution) Further this Stock was pipette (3ml and 0.3ml) into a 10ml volumetric flask and dilute up to the mark with diluents.

Procedure:

The intermediate precision study was performed for five injections of Chlorodipoxide and Trifluoperazine. Each standard injection was injected into chromatographic system. The area of each standard injection was used for calculation of % RSD. The chromatograms are shown in Fig.7.19-7.24 and results are tabulated in Table 7.6

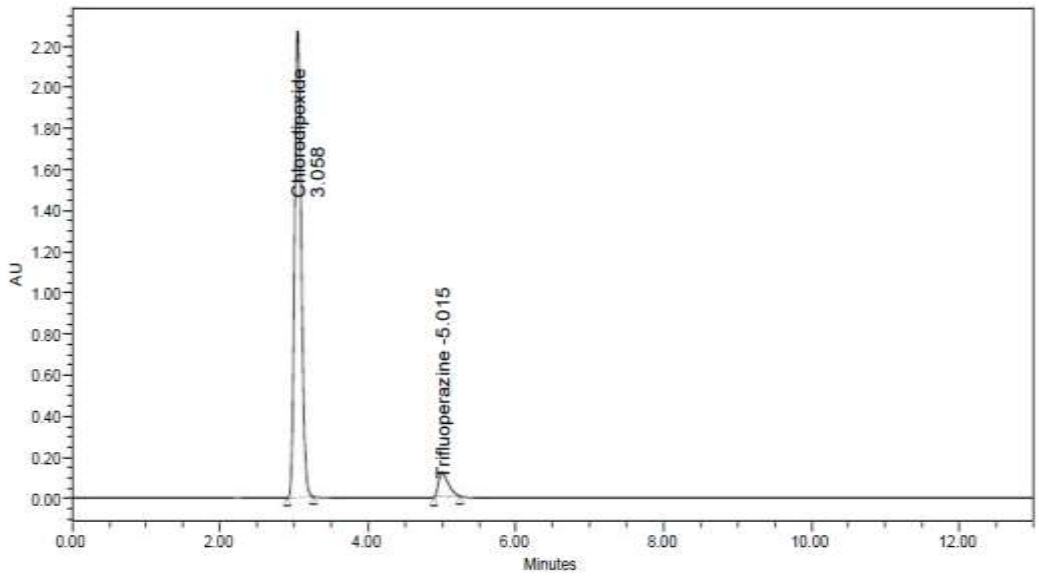


Fig.No.7.19: Chromatogram for System precision (standard -1)

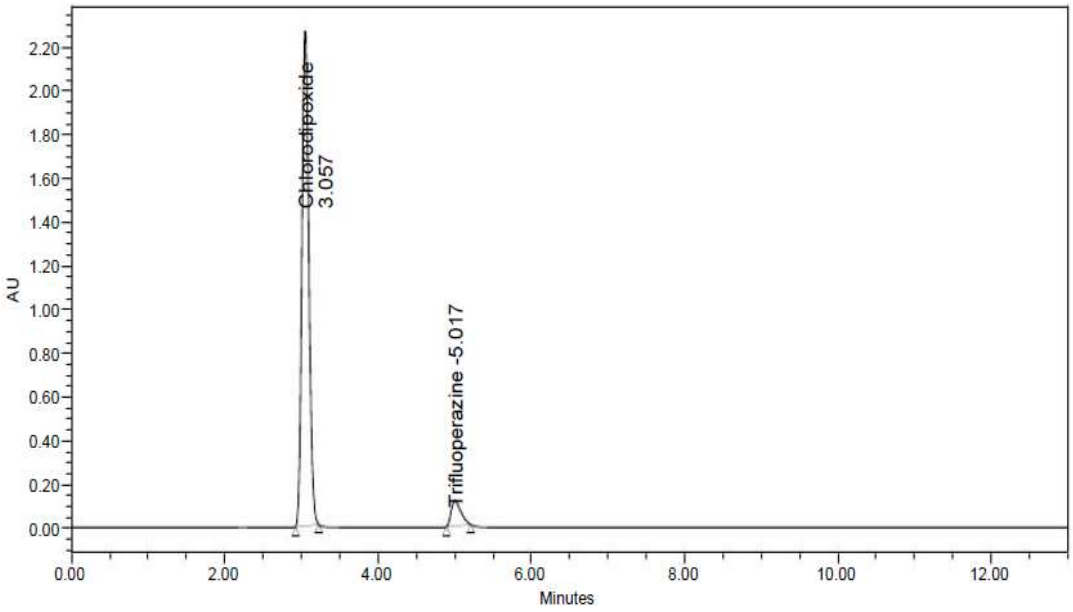


Fig.No.7.20: Chromatogram for System precision (standard -2)

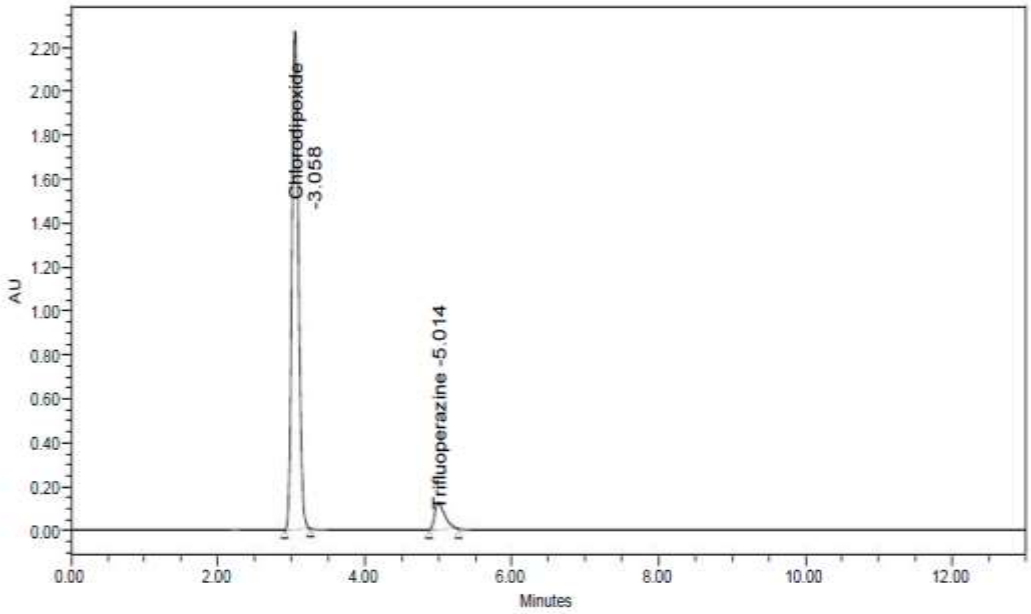


Fig.No.7.21: Chromatogram for System precision (standard -3)

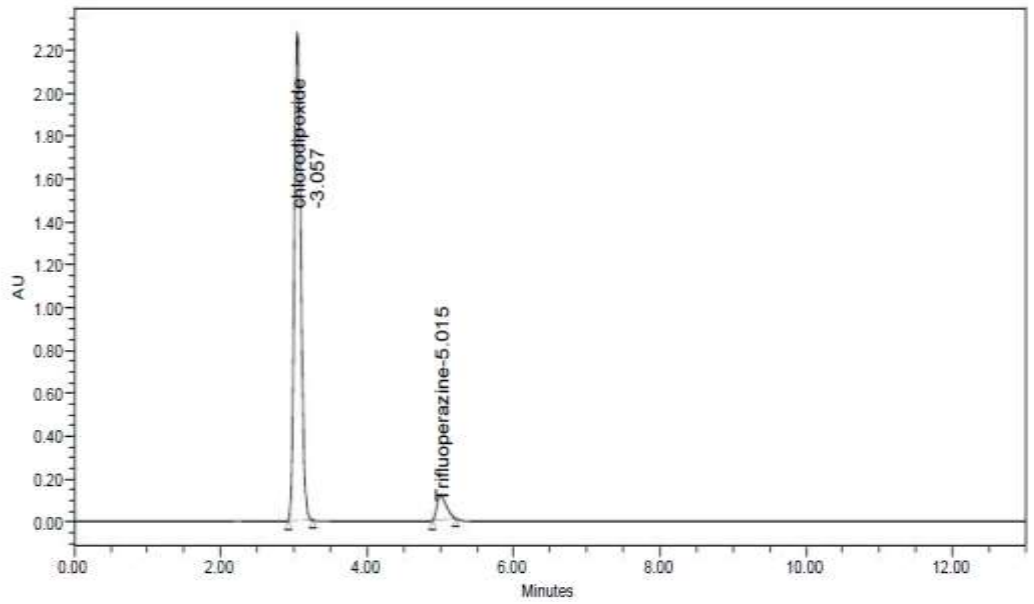


Fig.No.7.22: Chromatogram for System precision (standard -4)

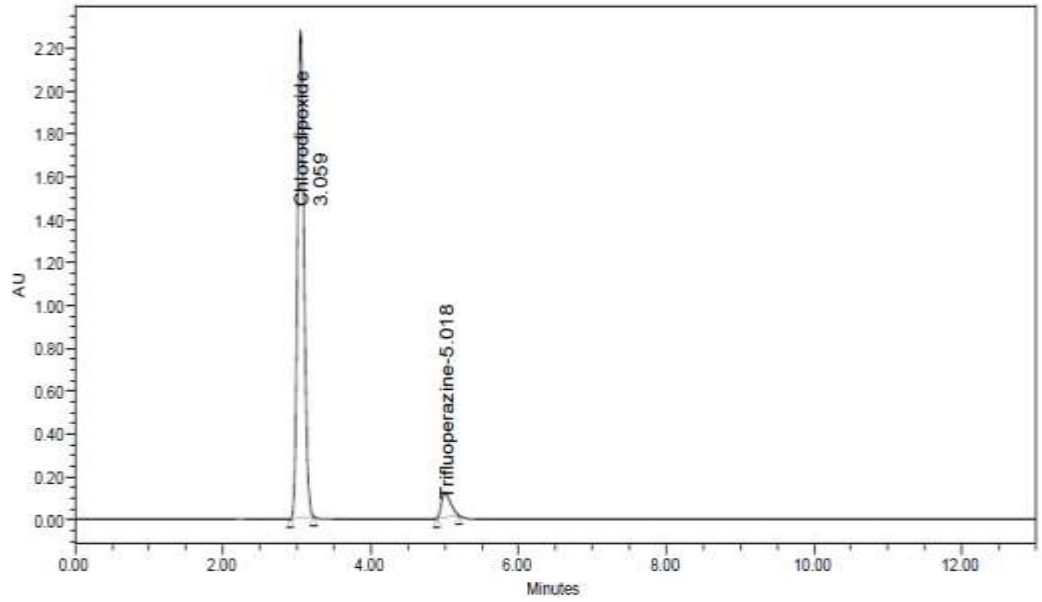


Fig.No.7.23:Chromatogram for System precision (standard -5)

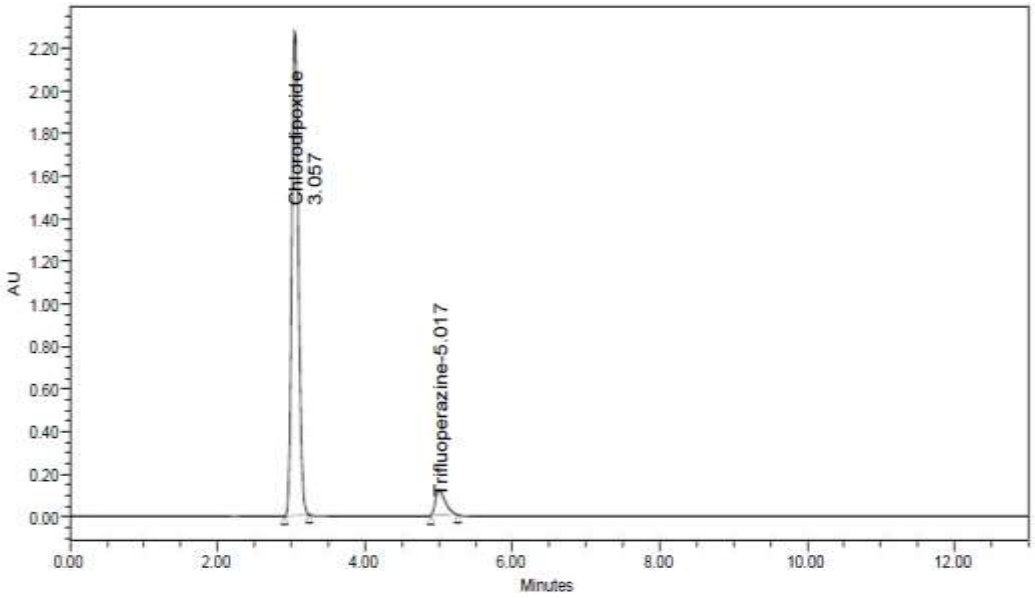


Fig.No.7.24: Chromatogram for System precision (standard -6)

Table No 7.6: System precision Result for Chlorodipoxide and Trifluoperazine

S.No	Chlorodipoxide		Trifluoperazine	
	Retention Time(min)	**Peak Area**	**Retention Time(min)**	**Peak Area**
1	3.057	14378212	5.015	1192027
2	3.057	14279579	5.017	1189089
3	3.058	14378212	5.014	1192020
4	3.057	14281414	5.015	1198115
5	3.059	14228980	5.018	1185539
6	3.057	14276872	5.017	1194360
AVG	3.058	14303878	5.016	1191858
SD	0.0008	60803.28	0.0015	4314121
%RSD	0.027	0.425	0.031	0.362

3. Linearity:

Preparation of stock solution:

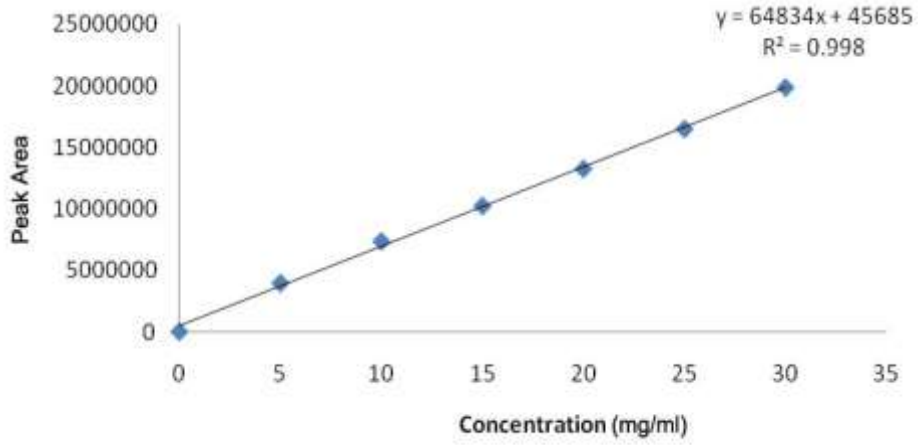
Accurately 10 tablets were weighed & crushed in mortar and pestle and weight equivalent to 10 mg of Chlorodipoxide and Trifluoperazine (marketed formulation)

sample were transferred into a 10 ml clean dry volumetric flask and about 7mL of Diluents was added and sonicated to dissolve it completely and made volume up to the mark with the same solvent. (Stock solution)

From the above stock solution Chlorodipoxide of dilutions of 5, 10, 15,20, 25,30. µg/ml were prepared.

From the above stock solution Trifluoperazine of dilutions of 1, 2, 3, 4, 5, 6µg/ml were prepared.

Linearity curve



FigNo7.25: Calibration curve for Chlorodipoxide

Linearity curve

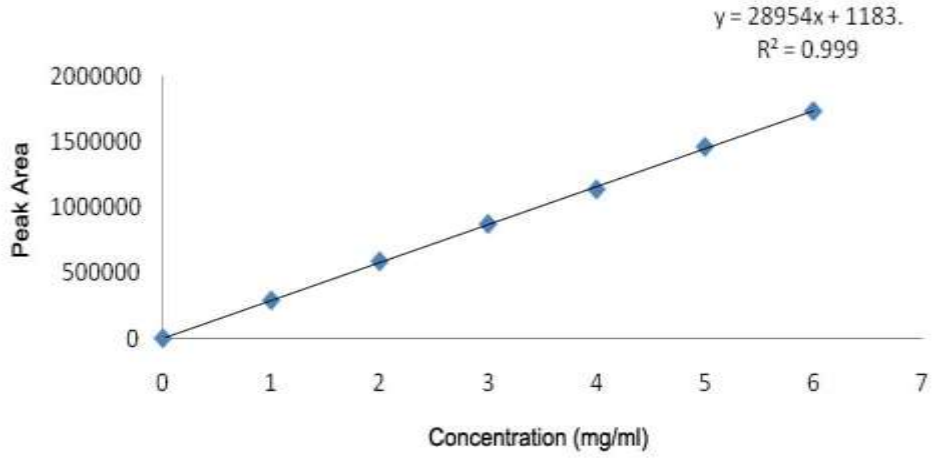


Fig No7.26: Calibration Curve for Trifluoperazine

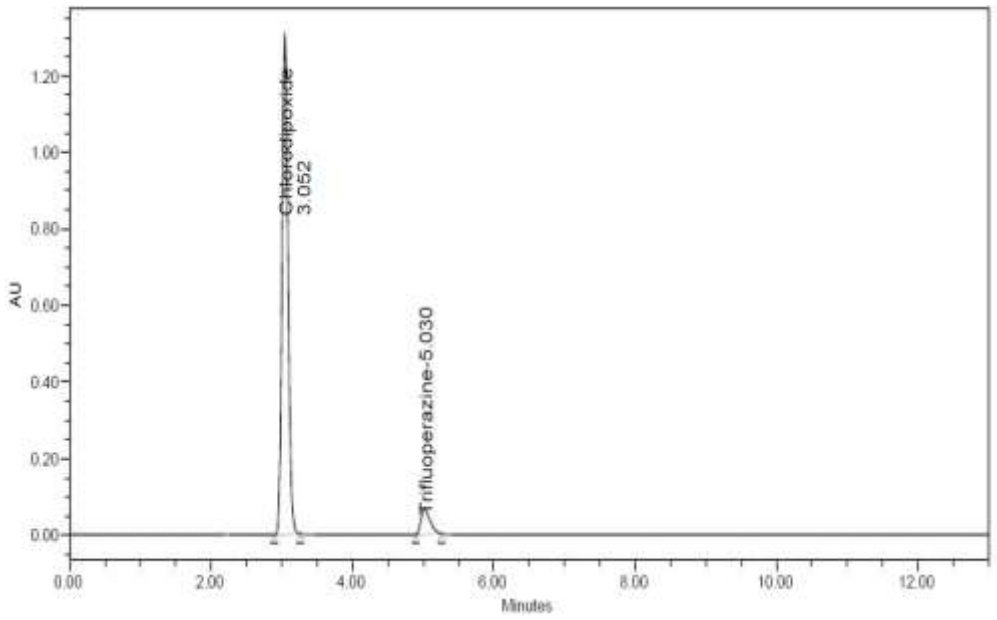


Fig No. 7.27: Chromatogram for Injection Level-I

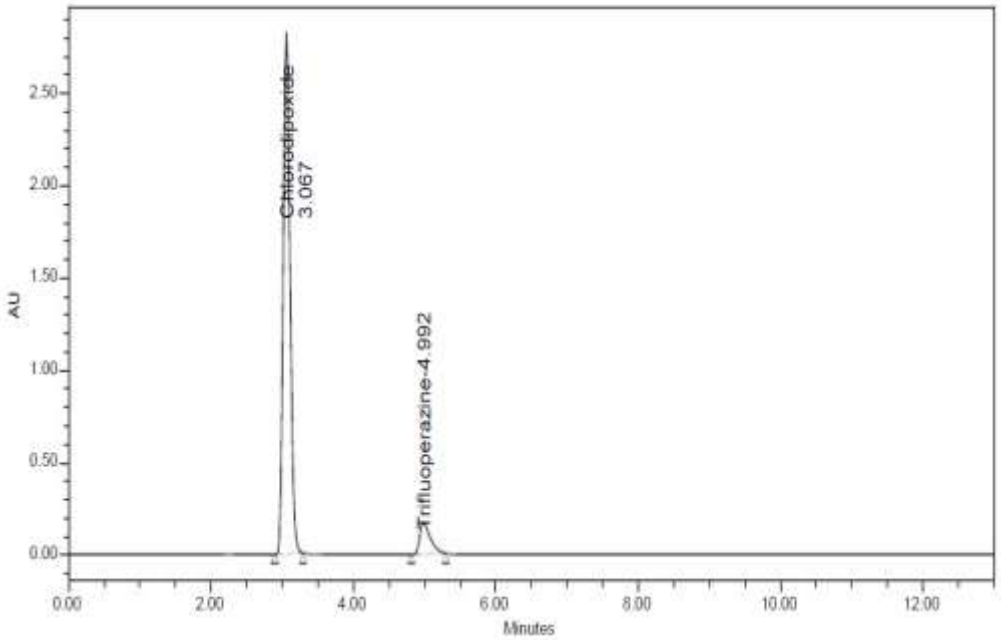


Fig No. 7.28: Chromatogram for Injection Level-II

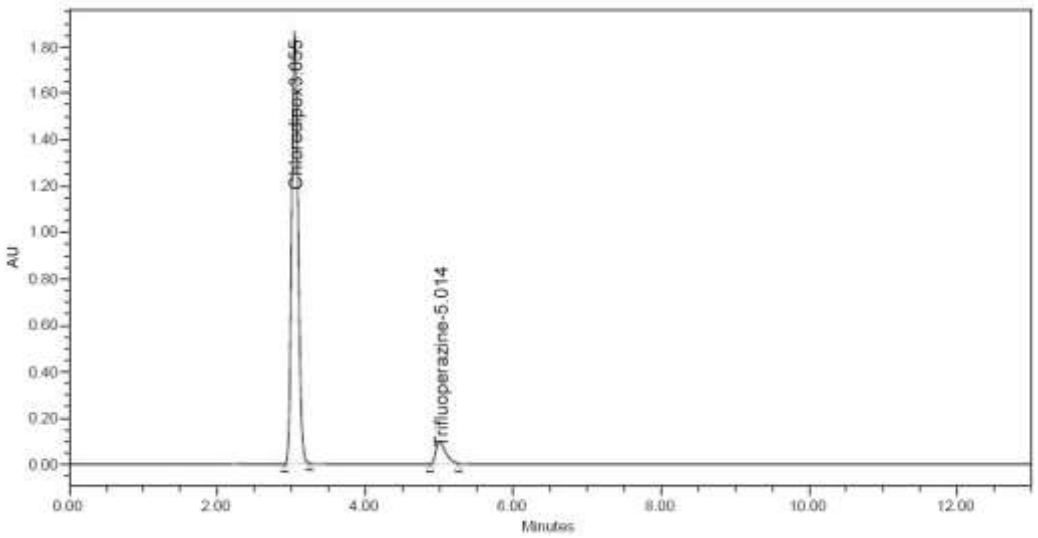


Fig No7.29: Chromatogram for Injection Level-III

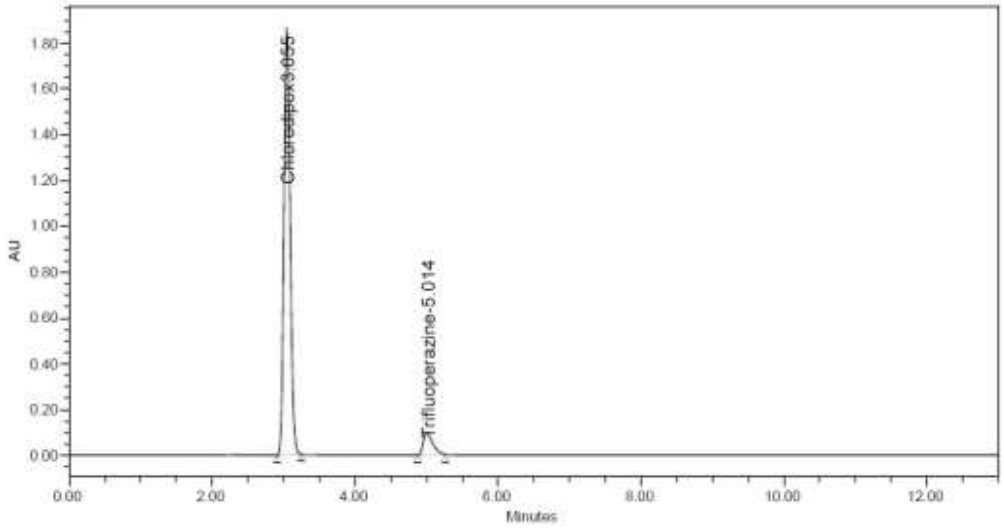


Fig No 7.30: Chromatogram for Injection Level-IV

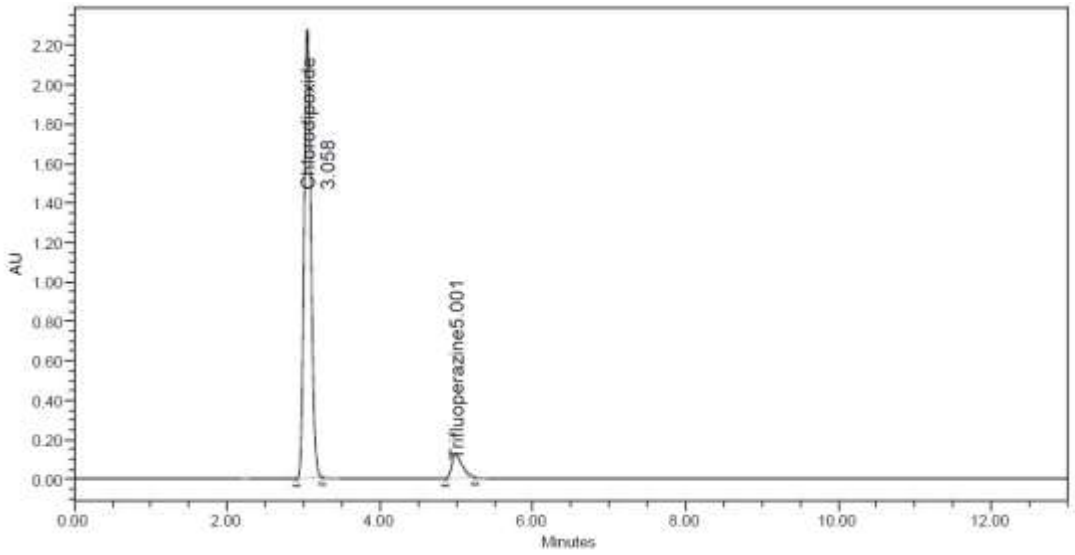


Fig No 7.31: Chromatogram for Injection Level-V

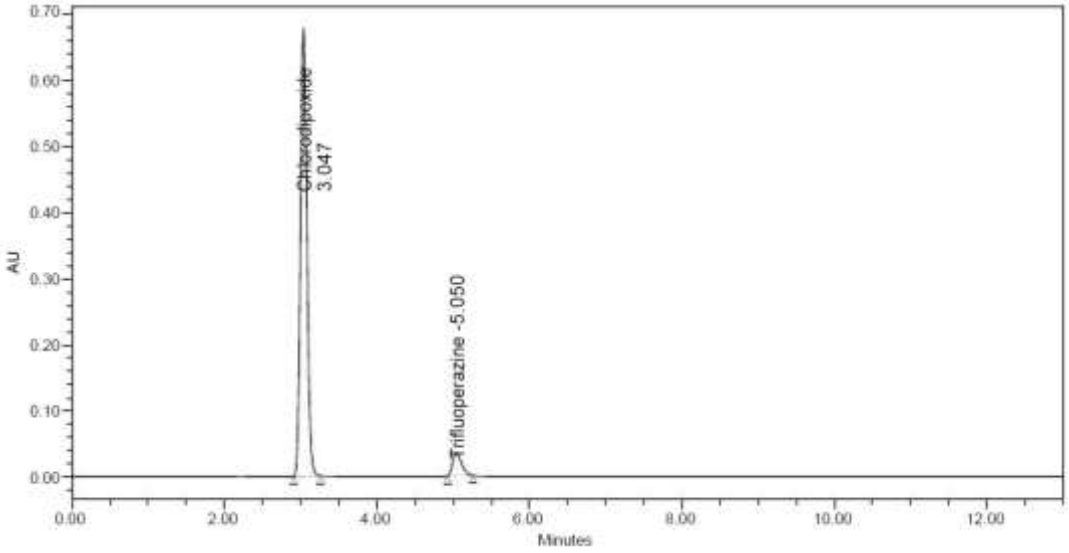


Fig No 7.32: Chromatogram for Injection Level-VI

Table No 7.7: Linearity Results Chlorodipoxide

S. No	Concentration(µg/ml)	Peak area
1	0	0
2	5	3957807
3	10	7361399
4	15	10247854
5	20	13275617
6	25	16525102
7	30	19906547

Table No 7.8: Linearity Results for Trifluoperazine

S. No	Concentration(µg/ml)	Peak area
1	0	0
2	1	289760
3	2	587503
4	3	873352
5	4	1138225
6	5	1463447
7	6	1736351

4.SENSITIVITY:

Limit of detection (LOD) and limit of quantitation (LOQ) were determined from standard deviation and slope method as per ICH guidelines.

Limit of Detection (LOD):

LOD's can be calculated based on the standard deviation of the response (SD) and the slope of the calibration curve (S) at levels approximating the LOD according to the formula. The standard deviation of the response can be determined based on the standard deviation of y-intercepts of regression lines.

Formula:

$$LOD = 3.3 \text{ X } \frac{\sigma}{S}$$

Where

σ - Standard deviation (SD)

S – Slope

Table No.7.9: Observation of Limit of detection for Chlorodipoxide

S .No	Slope	SD of precision	LOD
1	648346	60803.28	0.309

Table No.7.10: Observation of Limit of detection for Trifluoperazine

S .No	Slope	SD of precision	LOD
1	289542	4314.21	0.049

Limit of Quantitation (LOQ):

LOQ=10x σ/s

Where

σ - Standard deviation (SD)

S – Slope

Table6 No 7.11: Observation of Limit of Quantitation for Chlorodipoxide

S. No	Slope	SD of precision	LOQ
1	648346	60803.28	0.937

Table6 No 7.12: Observation of Limit of Quantitation for Trifluoperazine

S. No	Slope	SD of precision	LOQ
1	289542	4314.21	0.149

5. RUGGEDNESS:

The ruggedness of an analytical method is determined by analysis of aliquots from homogenous lots by different analysts using operational and environmental conditions that may differ but are still within the specified parameters of the assay. The assay of Chlorodipoxide and Trifluoperazine was performed by different analyst and on different dates (days).

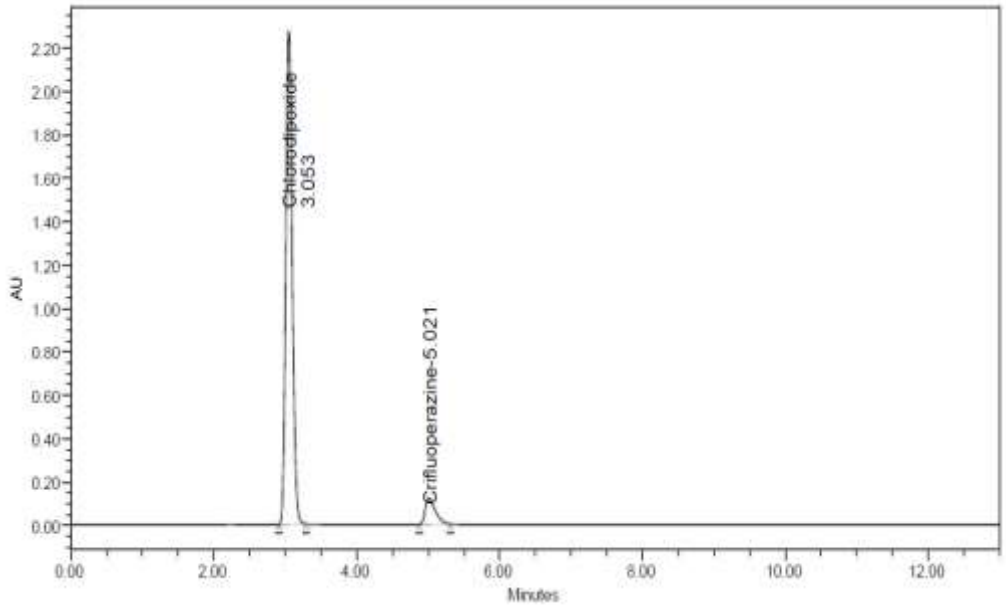


Fig.No.7.33: Chromatogram for Ruggedness (Analyst-1)

Table No 7.13: Observation of Ruggedness for Chlorodipoxide and Trifluoperazine (Analyst-1)

S.No	Injection	Chlorodipoxide		Trifluoperazine	
		RetetionTime(min)	PeakArea	RetentionTime(min)	PeakArea
1	**1**	3.051	14387099	5.021	1186308
2	**2**	3.052	14387089	5.019	1186302
3	**AVG**	0.0014	14387094	5.02	1186305
4	**SD**	0.004	7.07	0.0014	4.24
5	**%RSD**		0.00004	0.027	0.0003

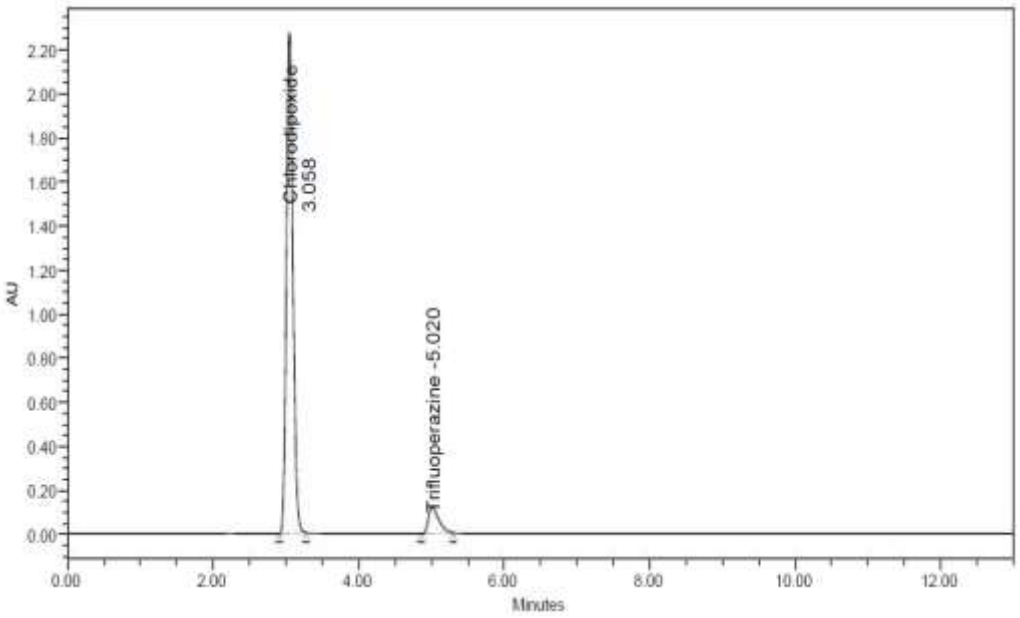


Fig.No.7.34. Chromatogram for Ruggedness

Table No 7.14: Observation of Ruggedness for Chlorodipoxide and Trifluoperazine (Analyst-2)

Injection	Chlorodipoxide		Trifluoperazine	
	RetentionTime(min)	Peak Area	RetentionTime(min)	Peak Area
1	3.058	14374882	5.020	1172052
2	3.052	14374876	5.018	1172046
AVG	3.055	14374870	5.019	1172049
SD	0.004	8.48	0.0014	4.24
%RSD	0.13	0.0005	0.027	0.0036

6. Robustness:

As part of the Robustness, deliberate change in the Flow rate, Mobile Phase composition, Temperature Variation was made to evaluate the impact on the method.

A) Flow Rate:

The robustness was performed for the flow rate variations from 0.9 ml/min to 1.1ml/min. Standard solution 300 µg /ml of Trifluoperazine& 3µg/ml of Chlorodipoxide was prepared and analyzed using the varied Mobile phase composition along with the actual mobile phase composition in the method.

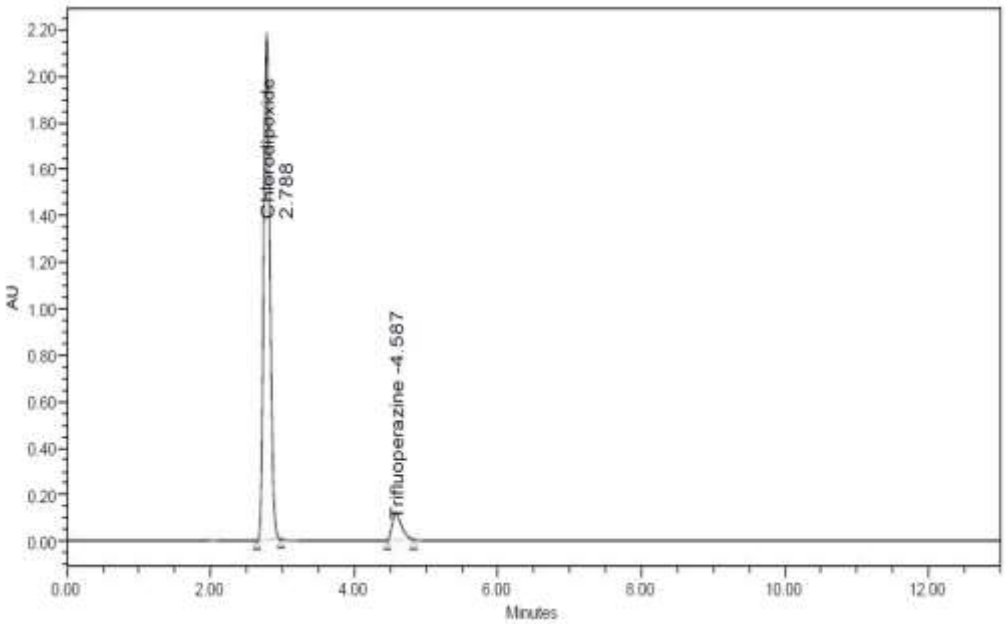


Fig No 7.35: Chromatogram for Robustness (0.9 ml flow)

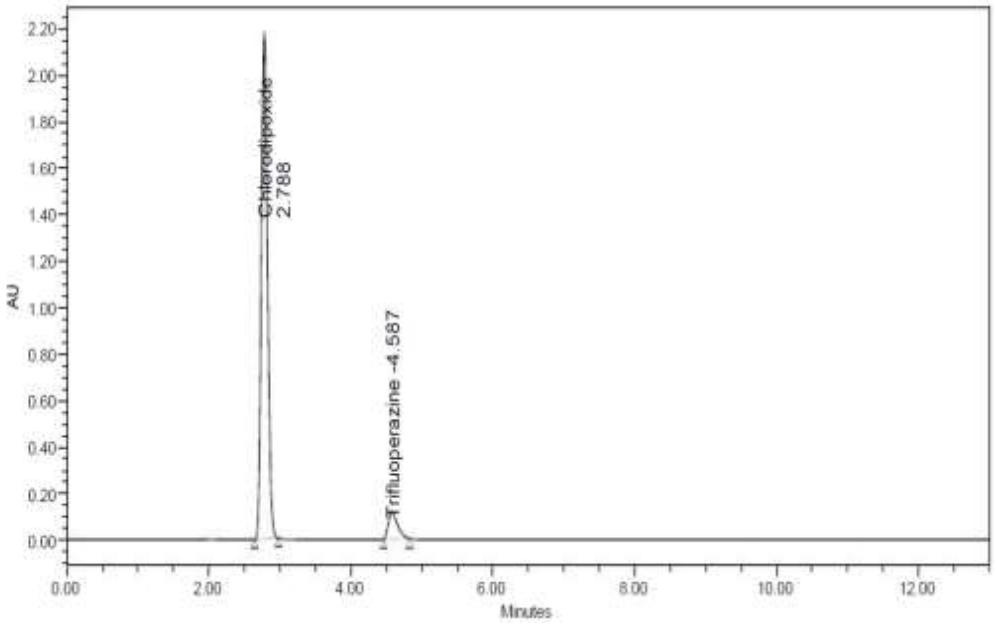


Fig No. 7.36 :Chromatogram for Robustness (1.1ml flow)

Table No 7.15: Observation of Robustness for Chlorodipoxide and Trifluoperazine (Flow rate-1)

Injection	Chlorodipoxide		Trifluoperazine	
	RetentionTime(min)	**Peak Area**	**RetentioinTime(min)**	**Peak Area**
1	3.386	15934436	5.56	1253798
2	3.379	15934610	5.52	1253789
AVG	3.382	15934523	5.54	1253793.5
SD	0.004	123.03	0.028	11248.6
%RSD	0.11	0.0007	0.50	0.89

Results for actual flow (1.1 ml/min) have been considered from Assay standard.

Table No 7.16: Observation of Robustness Chlorodipoxide and Trifluoperazine (Flow rate-2)

Injection No.	Chlorodipoxide		Trifluoperazine	
	RetentionTime(min)	**Peak Area**	**RetentioTime(min)**	**Peak Area**
1	2.788	13061927	4.587	1049598
2	2.780	13061917	4.590	1049599
AVG	2.784	1306192	4.588	1049598.5
SD	0.005	7.07	0.002	0.70
%RSD	0.17	0.00005	0.04	0.00006

Buffer:

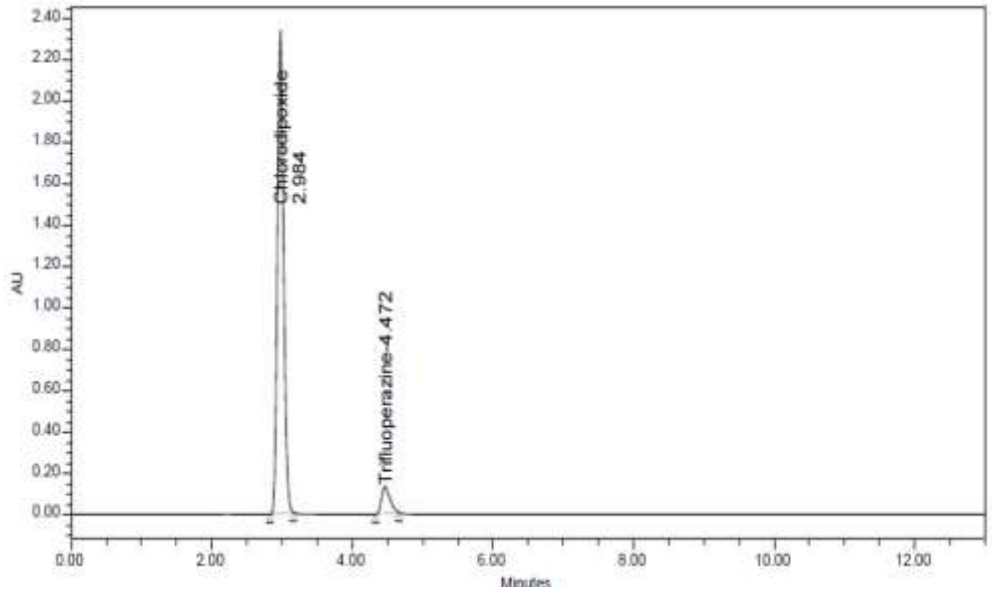


Fig No. 7.37Chromatogram for Robustness (Buffer-1)

Table No 7.17: Observation of Robustness For Chlorodipoxide and Trifluoperazine (Buffer-1)

Injection	Chlorodipoxide		Trifluoperazine	
	RetentionTime(min)	**Peak Area**	**RetentionTime(min)**	**Peak Area**
1	3.151	14427114	5.965	1167352
2	3.149	14427089	5.959	1167340
AVG	3.15	14427101.5	5.962	1167346
SD	0.001	17.67	0.004	8.485
%RSD	0.03	0.00012	0.067	0.0007

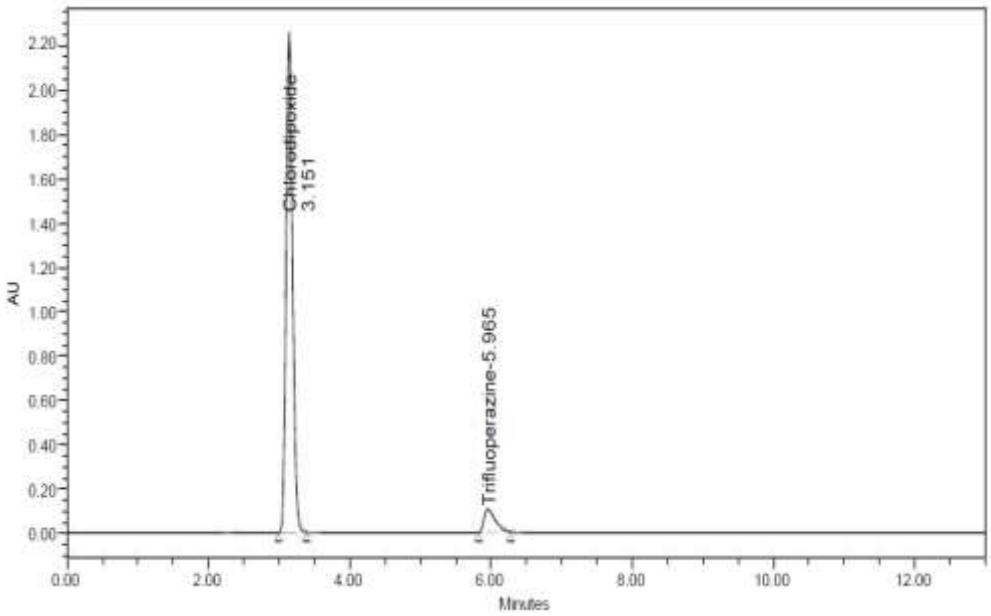


Fig No 7.38 Chromatogram for Robustness (Buffer-2)

Table No 7.18: Observation of Robustness for Chlorodipoxide and Trifluoperazine (Buffer-2)

Injection	Chlorodipoxide		Trifluoperazine	
	RetentionTime(min)	**Peak Area**	**RetentionTime(min)**	**PeakArea**
1	2.984	14339419	4.472	1076621
2	2.980	14339418	4.473	1076624
AVG	2.982	14339418	4.4725	1076622.5
SD	0.02	6.36	0.0007	2.121
%RSD	0.67	0.00004	0.015	0.000019

7.SPECIVICITY:

Mixed Standard:

Transfer 1 mL of each standard stock solution into 10 mL volumetric flask and dilute to volume with diluent.

Sample Solution:

Transfer sample quantitatively equivalent to 10 mg of Chlorodipoxide and 10 mg of Trifluoperazine in to 50 mL volumetric flask add 25 mL of diluent, sonicate to dissolve for 10 minutes and dilute to volume with diluent. Further filter the solution through filter paper. Dilute 1 ml of filtrate to 10 ml with mobile phase.

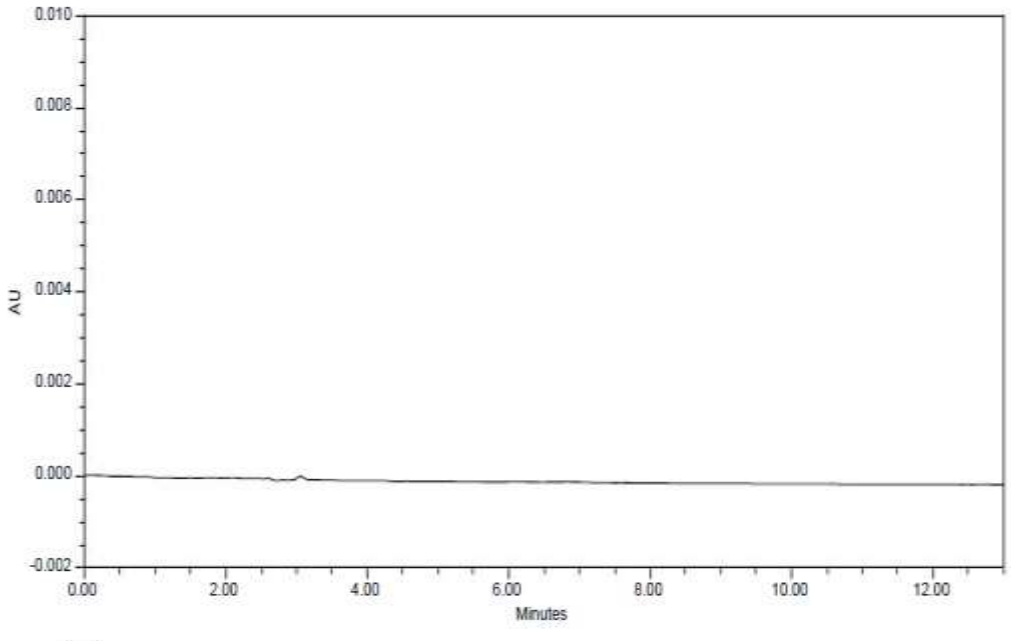


Fig.No.7.39: Chromatogram for specificity(blank)

Acid:

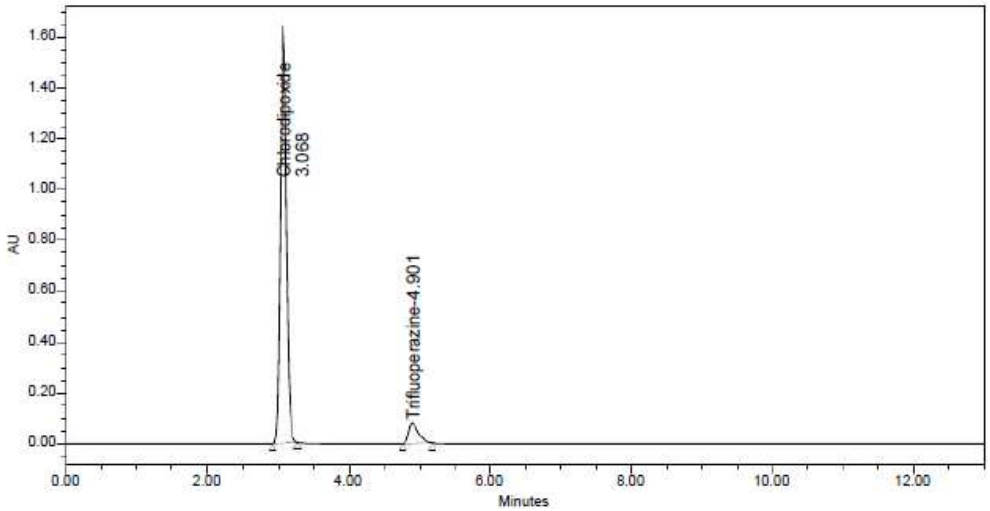


Fig No 7.40: Chromatogram for Acid

Base:

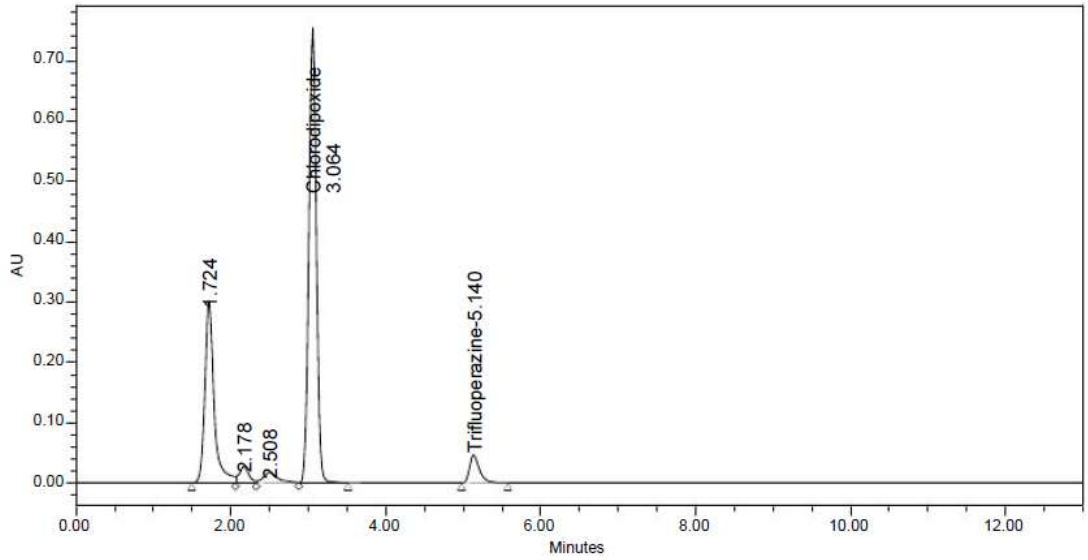


Fig No 7.41: Chromatogram for Base

Heat:

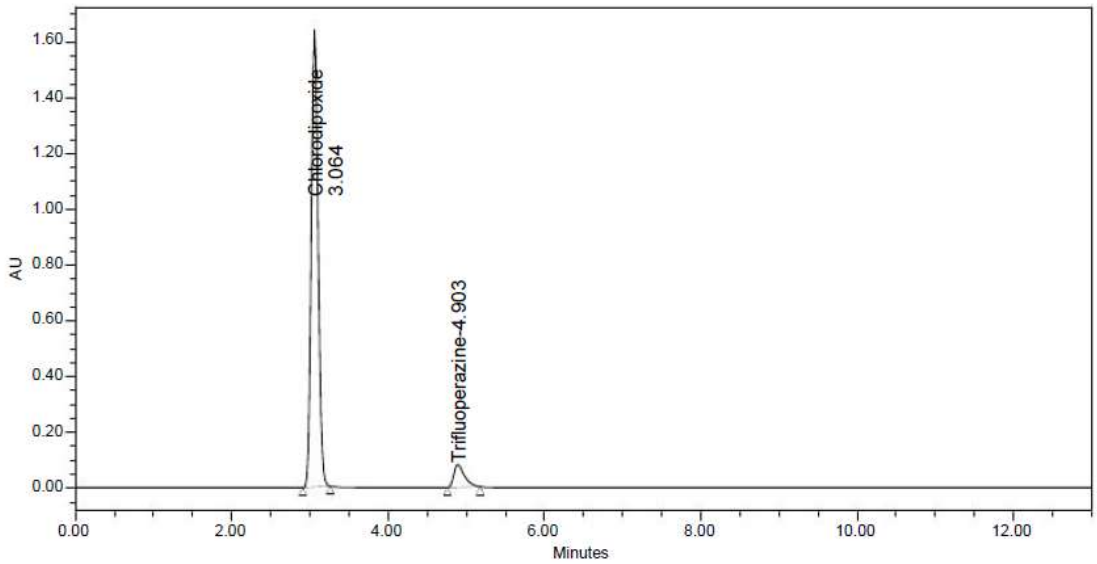


Fig No 7.42: Chromatogram for heat

placebo:

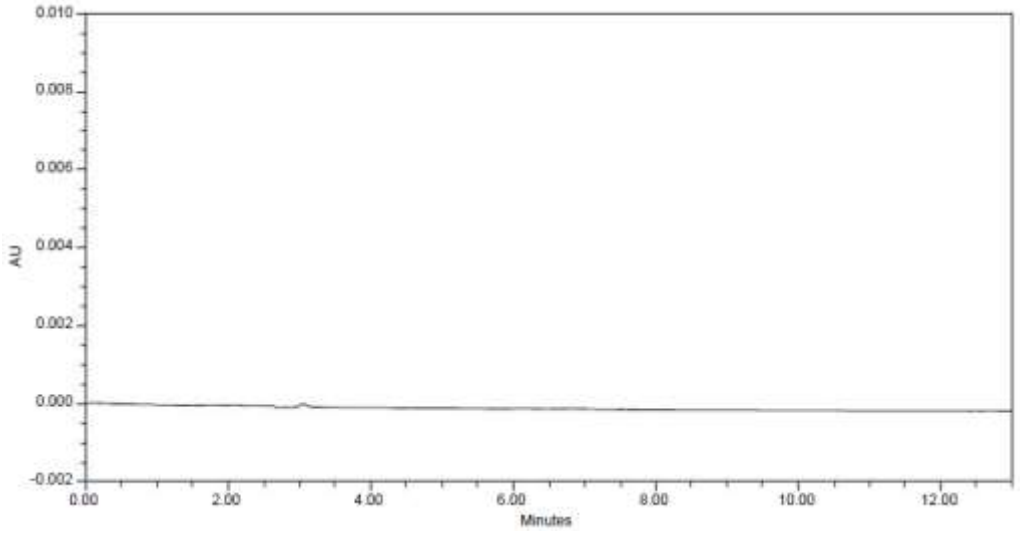


Fig.No.7.43: Chromatogram for specificity placebo

Table No 7.19: Observation of specificity for Chlorodipoxide and Trifluoperazine

S.No	Name	**Chlorodipoxide**		**Trifluoperazine**	
		Retention Time (min)	Peak Area	Retention Time (min)	Peak Area
1	Blank	_	_	_	_
2	Chlor	3.057	14283954	5.017	1173515
3	Placebo	_	_	_	_
4	Sample	3.057	14369832	5.021	1172015

SUMMARY AND CONCLUSION

A new method was established for simultaneous estimation of Chlorodipoxide and Trifluoperazine by RP-HPLC method. The chromatographic conditions were successfully developed for the separation of Chlorodipoxide and Trifluoperazine by using Agilent C_{18}, 150x4.6mm,5µ BDS Hypersil, flow rate was 1ml/min, mobile phase ratio was ACN : Buffer (45:55%v/v), detection wave length was 236nm. The instrument used was WATERS HPLC Auto Sampler, Separation module 2695, PDA Detector 996, Empower-software version-2.

The retention times were found to be 2.832min and 5.462min. The % purity of Chlorodipoxide and Trifluoperazine was found to be 99.13% and 99.46%.The analytical method was validated according to ICH guidelines (ICH, Q2 (R1)). The linearity study for Chlorodipoxide and Trifluoperazine was found in concentration range of 5µg-30µg and 1µg-6µg and correlation coefficient (r2) was found to be 0.9983 and 0.997% .Mean recovery was found to be 102.5% and 101.0%. %RSD for retention time of System Precision was 0.027 and 0.031. % RSD for retention time and area of Method precision was 0.027 and 0.037 respectively. The precision study was precise, robust, and repeatable. LOD value was 0.309 and 0.049, and LOQ value was 0.937 and 0.149 respectively. Hence the suggested RP-HPLC method can be used for routine analysis of Chlorodipoxide and Trifluoperazine in API and Pharmaceutical dosage form.

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