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PREPARATION AND EVALUATION OF ECONAZOLE TRANSDERMAL DENDRIMER GEL

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1. INTRODUCTION[1]

Pharmaceutical Analysis plays a very vital role in the quality assurance and quality control of bulk drugs and their formulations. Pharmaceutical analysis is a specialized branch of analytical chemistry which involves separating, identifying and determining the relative amounts of components in a sample of matter. It is concerned with the chemical characterization of matter both quantitative and qualitative. In recent years, several analytical techniques have been evolved.

1.1 SPECTROPHOTOMETRIC METHODS[2-5]

Spectrophotometry is generally preferred especially by small-scale industries as the cost of the equipment is less and the maintenance problems are minimal. The method of analysis is based on measuring the absorption of a monochromatic light by colorless compounds in the near ultraviolet path of spectrum (200-380nm). The photometric methods of analysis are based on the Bouger-Lambert-Beer's law, which establishes the absorbance of a solution is directly proportional to the concentration of the analyte. The fundamental principle of operation of spectrophotometer covering UV region consists in that light of definite interval of wavelength passes through a cell with solvent and falls on to the photoelectric cell that transforms the radiant energy into electrical energy measured by a galvanometer.

The important applications are

- Identification of many types of organic, inorganic molecules and ions.
- Quantitative determination of many biological, organic and inorganic species.
- Monitoring and identification of chromatographic of effluents.

1.2 HPLC METHOD DEVELOPMENT

The term 'Chromatography' covers those processes aimed at the separation of the various species of a mixture on the basis of their distribution characteristics between a stationary and a mobile phase.

1.2.1MODES OF CHROMATOGRAPHY

Modes of chromatography are defined essentially according to the nature of the interactions between the solute and the stationary phase, which may arise from hydrogen bonding, Vander walls forces, electrostatic forces or hydrophobic forces or basing on the size of the particles (e.g. Size exclusion chromatography).

Different modes of chromatography are as follows:

- ♦ Normal Phase Chromatography
- ♦ Reversed Phase Chromatography
- ♦ Reversed Phase – ion pair Chromatography
- ♦ Ion-Exchange Chromatography
- ♦ Size Exclusion Chromatography

REVERSED PHASE CHROMATOGRAPHY

The objective was to make less polar or non polar so that polar solvents can be used to separate water-soluble polar compounds. Since the ionic nature of the chemically modified silica is now reversed i.e. it is non-polar or the nature of the phase is reversed. The chromatographic separation carried out with such silica is referred to as reversed- phase chromatography.

A large number of chemically bonded stationary phases based on silica are available commercially. Silica based stationary phases are still most popular in reversed phase chromatography however other adsorbents based on polymer (styrene-divinyl benzene copolymer) are slowly gaining ground.

Simple compounds are better retained by the reversed phase surface, the less water- soluble (i.e. the more non-polar) they are. The retention decreases in the following order: aliphatics > induced dipoles (i.e. CCl_4) > permanent dipoles (e.g.$CHCl_3$) > weak Lewis bases (ethers, aldehydes, ketones) > strong Lewis bases (amines) > weak Lewis acids (alcohols, phenols) > strong Lewis acids (carboxylic acids). Also the retention increases as the number of carbon atoms increases.

As a general rule the retention increases with increasing contact area between sample molecule and stationary phase i.e. with increasing number of water molecules, which are released during the adsorption of a compound. Branched chain compounds are eluted more rapidly than their corresponding normal isomers.

Chemically bonded octadecyl silane (ODS) an alkaline with 18 carbon atoms it is the most popular stationary phase used in pharmaceutical industry. Since most pharmaceutical compounds are polar and water soluble, the majority of HPLC methods used for quality

assurance, decomposition studies, quantitative analysis of both bulk drugs and their formulations use ODS HPLC columns. The solvent strength in reversed phase chromatography is reversed from a that of adsorption chromatography (silica gel) as stated earlier. Water interacts strongly with silanol groups, so that, adsorption of sample molecules become highly restricted and they are rapidly eluted as a result.

Exactly opposite applies in reversed phase system water cannot wet the non-polar (hydrophobic) alkyl groups such as C_{18} of ODS phase and therefore does not interact with the bonded moiety. Hence water is the weakest solvent of all and gives slowest elution rate. The elution time (retention time) in reversed phase chromatography increases with increasing amount of water in the mobile phase.

ADSORPTION CHROMATOGRAPHY OR NORMAL PHASE CHROMATOGRAPHY[6-10]

In normal phase chromatography, the stationary phase is a polar adsorbent and the mobile phase is generally a mixture of non-aqueous solvents.

The silica structure is saturated with silanol groups at the end. These OH groups are statistically disturbed over the whole of the surface. The silanol groups represent the active sites (very polar) in the stationary phase.

These situations arise when the molecule has one or several atoms with lone pair electron or a double bond. The adsorption strengths and hence k' values (elution series) increase in the following order. Saturated hydrocarbon < olefins < aromatics < organic halogen compounds < sulphides < ethers< esters < aldehydes and ketones < amines < sulphones < amides < carboxylic acids. The strength of interactions depends not only on the functional groups in the sample

molecule but also on steric factors. If a molecule has several functional groups, then the most polar one determines the reaction properties.

The aminopropyl and cyanopropyl phases provide opportunities for specific interactions between the analyte and the stationary phases and thus offer additional options for the optimizations of separations.

Resolution with water in weak mobile phase may be most conveniently achieved by drying the solvents and then adding a constant concentration of water or some very polar modifier such as acetic acid or trietylamine (TEA) to the mobile phase. The addition of such polar modifiers serves to deactivate the more polar shape as well as the reproducibility of the retention times.

Chromatographic methods can be classified most practically according to the stationary and mobile phases, as shown in the table 1.1

Table.1.1: Classification of Chromatographic Methods

Stationary phase	Mobile phase	Method
Solid	Liquid	Adsorption column, thin-layer, ion exchange, High performance liquid chromatography.
Liquid	Liquid	Partition, column, thin-layer, HPLC, paper chromatography.
	Gas	Gas–Liquid Chromatography

The modern form of column chromatography has been called high performance, high pressure, and high-resolution and high-speed liquid chromatography.

High-Performance Liquid Chromatography (HPLC) is a special branch of column chromatography in which the mobile phase is forced through the column at high speed. As a result the analysis time is reduced by 1-2 orders of magnitude relative to classical column chromatography and the use of much smaller particles of the adsorbent or support becomes possible increasing the column efficiency substantially.

The essential equipment consists of an eluent, reservoir, a high-pressure pump, and an injector for introducing the sample, a column containing the stationary phase, a detector and recorder. The development of highly efficient micro particulate bonded phases has increased the versatility of the technique and has greatly improved the analysis of multi component mixtures.

The various components of a HPLC system are herewith described.

Solvent container — Pump — damping unit — Injection port --Column

|

Recorder — Detector --- Effluent

1.2.2 SYSTEM COMPONENTS:

Solvent delivery system:

The mobile phase is pumped under pressure from one or several reservoirs and flows through the column at a constant rate. With micro particulate packing, there is a high-pressure drop across a chromatography column. Eluting power of the mobile phase is determined by its overall polarity, the polarity of the stationary phase and the nature of the sample components. For normal phase separations eluting power increases with increasing polarity of the solvent but for reversed phase separations, eluting power decreases with increasing

solvent polarity. Optimum separating conditions can be achieved by making use of mixture of two solvents. Some other properties of the solvents, which need to be considered for a successful separation, are boiling point, viscosity, detector compatibility, flammability and toxicity.

The most important component of HPLC in solvent delivery system is the pump, because its performance directly effects the retention time, reproducibility and detector sensitivity. Among the several solvent delivery systems (direct gas pressure, pneumatic intensifier, reciprocating etc.) reciprocating pump with twin or triple pistons is widely used, as this system gives less baseline noise, good flow rate reproducibility etc.

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

Sample introduction systems:

Two means for analyte introduction on the column are injection in to a flowing stream and a stop flow injection. These techniques can be used with a syringe or an injection valve. Automatic injector is a microprocessor-controlled version of the manual universal injector. Usually, up to 100 samples can be loaded in to the auto injector tray. **Liquid chromatographic detectors:** The function of the detector in HPLC is to monitor the

mobile phase as it emerges from the column. Generally, there are two types of HPLC detectors, bulk property detectors and solute property detectors.

1. Bulk property detectors:

These detectors are based on differential measurement of a property, which is common to both the sample and the mobile phase. Examples of such detectors are refractive index, conductivity and dielectric constant detectors.

2. Solute property detectors:

Solute property detectors respond to a physical property of the solute, which is not exhibited by the pure mobile phase. These detectors measure a property, which is specific to the sample, either with or without the removal of the mobile phase prior to the detection. Solute property detectors which do not require the removal of the mobile phase before detection include spectrophotometric (UV or UV-Vis) detector, fluorescence detectors, polarographic, electro-chemical and radio activity detectors, whilst the moving wire flame ionization detector and electron capture detector both require removal of the mobile phase before detection.

UV-Vis and fluorescent detectors are suitable for gradient elution, because many solvents used in HPLC do not absorb to any significant extent.

Column and Column-packing materials:

The heart of the system is the column. In order to achieve high efficiency of separation, the column material (micro-particles, 5-10 µm size) packed in such a way that highest numbers

of theoretical plates are possible. Silica (SiO_2, H_2O) is the most widely used substance for the manufacture of packing materials. It consists of a network of siloxane linkages (Si-O-Si) in a rigid three dimensional structure containing inter connecting pores. Thus a wide range of commercial products is available with surface areas ranging from 100 to 800 m^2/g. and particle sizes from 3 to 50 µm.

The most popular material is octadecyl-silica (ODS-Silica), which contains C_{18} chains, but materials with C_2, C_6, C_8 and C_{22} chains are also available. During manufacture, such materials may be reacted with a small mono functional silane (e.g. trimethyl chloro silane) to reduce further the number of silanol groups remaining on the surface (end-capping). The useful pH range for columns is 2 to 8, since siloxane linkages are cleaved below pH-2 while at pH values above eight silica may dissolve.

In HPLC, generally two types of columns are used, normal phase columns and reversed phase columns. Using normal phase chromatography, particularly of non-polar and moderately polar drugs can make excellent separation. It was originally believed that separation of compounds in mixture takes place slowly by differential adsorption on a stationary silica phase.

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structure containing inter connecting pores. Thus a wide range of commercial products is available with surface areas ranging from 100 to 800 m^2/g. and particle sizes from 3 to 50 μm.

The silanol groups on the surface of silica give it a polar character, which is exploited in adsorption chromatography using non-polar organic eluents. Silica can be drastically altered by reaction with organo chloro silanes or organo alkoxy silanes giving Si-O-Si-R linkages with the surface. The attachment of hydrocarbon change to silica produces a non-polar surface suitable for reversed phase chromatography where mixtures of water and organic solvents are used as eluents. The most popular material is octadecyl-silica (ODS-Silica), which contains C_{18} chains, but materials with C_2, C_6, C_8 and C_{22} chains are also available. During manufacture, such materials may be reacted with a small mono functional silane (e.g. trimethyl chloro silane) to reduce further the number of silanol groups remaining on the surface (end-capping). There is a vast range of materials which have intermediate surface polarities arising from the bonding to silica of other organic compounds which contain groups such as phenyl, nitro, amino and hydroxyl. Strong ion exchangers are also available in which sulphonic acid groups or quaternary ammonium groups are bonded to silica. The useful pH range for columns is 2 to 8, since siloxane linkages are cleaved below pH-2 while at pH values above eight, silica may dissolve.

In HPLC, generally two types of columns are used, normal phase columns and reversed phase columns. Using normal phase chromatography, particularly of non-polar and moderately polar drugs can make excellent separation. It was originally believed that separation of compounds in mixture takes place slowly by differential adsorption on a stationary silica phase. However, it now seems that partition plays an important role, with the compounds interacting with the polar silanol groups on the silica or with bound water molecules.

While normal phase seems the passage of a relatively non-polar mobile phase over a polar stationary phase, reversed phase chromatography is carried out using a polar mobile phase such as methanol, acetonitrile, water, buffers etc., over a non-polar stationary phase. Ranges of stationary phases (C_{18}, C_8, $-NH_2$, -CN, -phenyl etc.) are available and very selective separations can be achieved. The pH of the mobile phase can be adjusted to suppress the ionization of the drug and thereby increase the retention on the column. For highly ionized drugs ion-pair chromatography is used.

Derivatization:

In HPLC derivatization is used to enhance the sensitivity and selectivity of detection when available detectors are not satisfactory for the underivatized compounds. Both ultra violet absorbing and fluorescence derivatives have been widely used. Ultra violet derivatization reagents include N-succinimidyl p-nitro phenyl acetate, phenyl hydrazine and 3, 5-dinitro benzyl chlorides, while fluorescent derivatives can be formed with reagents such as dansyl chloride, 4-bromo methyl-7-methoxy-coumarin and fluorescamine. Derivative formation can be carried out before the sample is injected on to the column or by online chemical reactions between the column out let and the detector.

Gradient elution:

Gradient elution or solvent programming is the change of solvent composition during a separation in which the solvent strength increases from the beginning to the end of the separation. It is well suited to the analysis of samples of unknown complexity since good resolution is automatically provided for a wide range of sample polarities. There are two types of gradient systems: Low-pressure gradient mixtures and high- pressure gradient mixtures. In the

former the solvents are mixed at atmosphere pressure and then pumped to the column, where as in the later, solvents are pumped in to a mixing chamber at high pressure before going in to the column.

Performance calculations:

Calculating the following values (which can be include in a custom report) used to access overall system performance.

Relative retention, Theoretical plates, Capacity factor

Resolution, Peak asymmetry, Plates per meter

The parameters used to calculate these system performance values for the separation of two chromatographic components.

Relative retention (Selectivity):

$$\alpha = (t_2 - t_a) / (t_1 - t_a)$$

Theoretical plates:

$$n = 16 (t / W)^{2}$$

Capacity factor:

$$K' = (t_2 / t_a)^{-1}$$

Résolution:

$$\mathbf{R = 2\,(t_2 - t_1) / (W_2 + W_1)}$$

Peak asymmetry:

$$\mathbf{T = W0.05 / 2f}$$

Plates per meter:

$$\mathbf{N = n / L}$$

HETP: **L/n**

Where, α = Relative retention.

t_2 = Retention time of the second peak measured from point of injection.

t_1 = Retention time of the first peak measured from point of injection.

t_a = Retention time of an inert peak not retained by the column, measured from point of injection.

n = Theoretical plates.

t = Retention time of the component.

W = Width of the base of the component peak using tangent method.

K' = Capacity factor.

R = Resolution between a peak of interest (p2) and the peak preceding it (p1)

W_2 = Width of the base of component peak 2.

W_1 = Width of the base of component peak 1.

T = Peak asymmetry, or tailing factor.

W0.05 = Distance from the leading edge to the tailing edge of the peak,

Measured at a point 5 % of the peak height from the baseline.

f = Distance from the peak maximum to the leading edge of the peak.

L = length of column, in meters

N = Number of plates per meter

HPLC troubleshooting

Abnormal pressure

No pressure reading, no flow

Possible cause	Solution
Power off	Turn on power
Fuse blown	Replace fuse
Controller setting or failure	Verify proper settings, repair or replace controller
Broken piston	Replace piston
Air trapped in pump head	Degas mobile phase, bleed air from pump and prime pump

Insufficient mobile phase	Replenish reservoir, replace inlet frit if it is blocked

Now pressure reading, flow is normal

Possible cause	Solution
Faulty meter	Replace meter
Faulty pressure transducer	Replace transducer

High back pressure

Possible cause	Solution
Flow rate set too high	Adjust settings
Blocked column frit	Backflush column if it is permitted, replace frit according to the manufacturer's instructions and warranty conditions or replace column
Improper mobile phase, precipitated buffer	Use correct mobile phase, wash column
Improper column	Use proper column
Injector blockage	Clear blockage or replace injector

Column temperature too low	Raise temperature
Controller malfunction	Repair or replace controller
Blocked guard column	Remove/replace guard column
Blocked in-line filter	Remove/replace in-line filter

You should find out, what caused high back pressure - column or system? We recommend following procedure:

Remove column from the system and turn on pump. If high back pressure still appears, then the blockage is in the system:

- blocked or crimped tubing
- dirty pump frit
- or clogged injection valve

If the pressure is normal, there is a problem with the column:

- clogged or damaged pre-column filter, guard column or frit
- precipitation of sample or buffer in column
- Low back pressure

Possible cause	Solution
Flow set too low	Adjust flow rate

Leak in the system	Locate and correct
Improper column	Use proper column
Column temperature too high	Lower temperature
Controller malfunction	Repair or replace controller

- **Fluctuating pressure**

  See section *High back pressure*.

- **Pressure dropping to zero**

  See sections *No pressure reading, no flow* and No pressure reading, flow is normal

- **Pressure dropping, but not to zero**

  See section *Low pressure*

Pressure cycling

Possible case	Solution
Air in pump	Degas solvent and/or bleed air from the pump
Faulty check valve(s)	Replace check valve(s)
Pump seal failure	Replace pump seal
Insufficient degassing	Degas solvent and/or change degassing methods (e.g. use vacuum degasser)
Leak in system	Locate leak and correct it

Using gradient elution	Pressure cycling is normal due to viscosity change

- **Leaks**
- Leaks are usually stopped by tightening or replacing a fitting. Be aware, however, that overtightened metal compression fittings can leak and plasrtic fingertight fittings can wear out. If a fitting leak does not stop when the fitting is tightened a little, take the fitting apart and inspect for damage (e.g. distored ferrule, or particles on the sealing surface). If the fitting or ferrule is damaged, replace it with new one.
- **Leaky fittings**

Possible cause	Solution
Loose fitting	Tighten the fitting
Stripped fitting	Replace the fitting
Overtightened fitting	Loosen and retighten the fitting. If the fitting is damaged, replace it.
Overtightened fitting	Loosen and retighten the fitting. If the fitting is damaged, replace it.
Dirty fitting	Disassemble fitting and clean it. If the fitting is damaged, replace it.
Mismatched parts	Use all parts from the same brand/type.

Leaks at the pump

Possible cause	Solution

Loose check valves	Tighten check valve (do not overtighten) or replace check valve
Loose fittings	Tighten fittings (do not overtighten)
Mixer seal failure	Repair or replace
Pump seal failure	Repair or replace the seal
Pulse damper failure	Replace pulse damper
Proportionin valve failure	Check diaphragms, replace if leaky and/or check for fitting damage, replace
Purge valve	Tighten valve or replace it if it is faulty

Injector leaks

Possible cause	Solution
Rotor seal failure	Rebuild or replace rotor
Blocked loop	Clean or replace loop
Loose injector port seal	Adjust
Improper syringe needle diameter	Use correct syringe
Waste line siphoning	Keep waste line above surface vaste, with proper slope
Waste line blockage	Replace waste line

Column leaks

Possible cause	Solution
Loose end fitting	Tighten end fitting
Column packing in ferrule	Disassemble, rinse ferrule, reassemble
Improper frit thickness	Use proper frit(*)

**Note: When the particle size of stationary phase is 3 to 4 µm, use frit 0.5 µm. When particle size of stationary phase is 5 to 20 µm, use frit 2 µm.*

Detector leaks

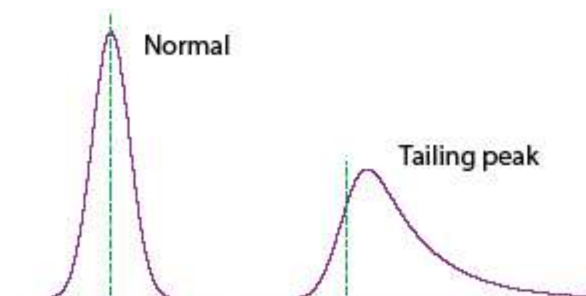
Possible cause	Solution
Cell gasket failure	Prevent excessive backpressure or replace gasket
Cracked cell window(s)	Replace cell window(s)
Leaky fittings	Tighten or replace fittings
Blocked waste line	Replace waste line
Blocked flow cell	Rebuild or replace flow cell

Problems with the chromatogram

Many issues in the LC system appear as changes in the chromatogram. Some of these can be solved by changes in the instrument, however, other problems require modification of the assay

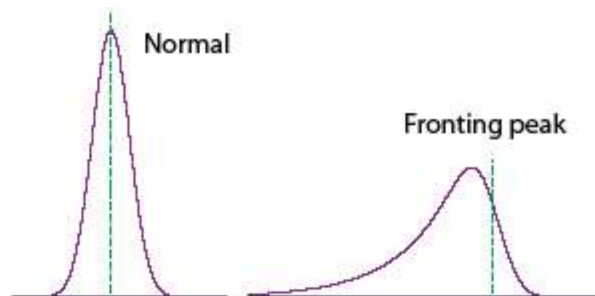
procedure. Setting the proper column type, pre-column or guard column, tubings, detector cell and mobile phase are keys to *good chromatography*.

Peak tailing



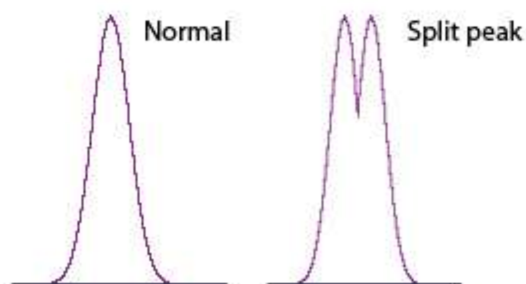
Possible cause	Solution
Blocked frit	Reverse flush column (if it is allowed) or replace frit (if it is allowed) or replace column
Column void	Fill void
Interfering peak	Use longer column or change mobile phase and/or column selectivity
Wrong mobile phase pH	Adjust pH. For basic compounds, lower pH usually provides more symmetric peaks
Sample reacting with active sites	Add ion pair reagent or volatile basic modifier or change column

Peak fronting



Possible cause	Solution
Low temperature	Increase column temperature
Wrong sample solvent	Use mobile phase for injection solvent
Sample overload	Decrease sample concentration
Bad column	Reverse flush column (if it is allowed) or replace inlet frit (if it is aalowed) or replace the column.

Split peaks



Possible cause	Solution
Contamination on guard or analytical column inlet	Remove guard column and attempt analysis. Replace guard column if necessary. If analytical column is obstructed, reverse and flush (if it is allowed). If problem persists, column may be fouled with strongly retained contaminants. Use appropriate restoration procedure (see column care information). If problem persists, inlet is probably plugged. Change frit or replace column.
Sample solvent incompatible with mobile phase	Change solvent. whenever possible, inject samples in mobile phase.

Distortion of larger peaks

The peak distortion can be caused by sample overload. Reduce the sample size.

Distortion of early peaks

The distortion of early eluting peaks can be caused by wrong injection solvent. Reduce the injection volume, or use weaker injection solvent.

Tailing, early peaks more than later ones

Possible cause	Solution
Extra-column effects	Replumb the system (shorter, narrower tubing), use smaller volume detector cell

Increased tailing as k' increases

Possible cause	Solution
Secondary retention effects, reversed-phase mode	Add triethylamine (basic samples) or add acetate (acidic samples) or add salt or buffer (ionic samples) or try a different column.

Acidic or basic peaks tail

Possible cause	Solution
Inadequate buffering	Use 50 to 100 mM buffer concentration, use buffer with pKa equal to pH of mobile phase

Extra peaks

Possible cause	Solution
Ghost peaks	Impurities in the sample, reagents or material used. Change clean-up procedure and/or check possible source of contamination (glassware, vials, used reagents, solvents, etc...)
Late eluting peak from previous injection	Increase run time or gradient slope and/or increase flow rate

METHOD OPTIMISATION:

Selection of stationary phase / column: Selection of the column is the first and the most important step in method development.

Some of the important parameters considered while selecting chromatographic columns are:

- Length and diameter of the column.
- Packing material.
- Shape of the particles.
- Size of the particles..

The column is selected depending on the nature of the solute and the information about the analyte. Reversed phase mode of chromatography facilitates a wide range of columns like dimethyl silane (C_2), butylsilane (C_4), octylsilane (C_8), octadecylslane (C_{18}), base deactivated silane (C_{18}) BDS phenyl, cyanopropyl (CN), nitro, amino etc. C_{18} was chosen for this study since it is most retentive one. The sample manipulation becomes easier with this type of column

Generally longer columns provide better separation due to higher theoretical plate numbers. Columns with 5-µm particle size give the best compromise of efficiency, reproducibility and reliability. In this case, the column selected had a particle size of 5 µm and a internal diameter of 4.6 mm

A useful and practical measurement of peak shape is peak asymmetry factor and peak tailing factor. Peak asymmetry is measured at 10% of full peak height and peak tailing factor at 5%. Reproducibility of retention times and capacity factor is important for developing a rugged and repeatable method.

Selection of mobile phase: The primary objective in selection and optimization of mobile phase is to achieve optimum separation of all the individual impurities and degradants from each other and from analyte peak

In liquid chromatography, the solute retention is governed by the solute distribution factor, which reflects the different interactions of the solute – stationary phase, solute – mobile phase and the mobile phase – stationary phase .For a given stationary phase, the retention of the given solute depends directly upon the mobile phase, the nature and the composition of which has to be judiciously selected in order to get appropriate and required solute retention. The mobile has to be adapted in terms of elution strength (solute retention) and solvent selectivity (solute separation) Solvent polarity is the key word in chromatographic separations since a polar mobile phase will give rise to low solute retention in normal phase and high solute retention in reverse phase LC. The selectivity will be particularly altered if the buffer pH is close to the pKa of the analytes; the solvent strength is a measure of its ability to pull analyte from the column. It is generally controlled by the concentration of the solvent with the highest strength.

The following are the parameters, which shall be taken into consideration while selecting and optimizing the mobile phase.

- Buffer,
- pH of the buffer
- Mobile phase composition.

Selection of detector:

The detector was chosen depending upon some characteristic property of the analyte like UV absorbance, fluorescence, conductance, oxidation, reduction etc. characteristics that are to be fulfilled by a detector to be used in HPLC determination are,

- High sensitivity, facilitating trace analysis
- Negligible baseline noise. To facilitate lower detection
- Low dead volume
- Non destructive to sample
- Inexpensive to purchase and operate

For the greatest sensitivity λ_{max} should be used. Higher wavelengths give greater selectivity.

1.3 METHOD VALIDATION (11-17)

Method validation can be defined as (ICH) "estabilishing documented evidence which provides a high degree of assurance that specific activity will consistenty produce a desired result or product meeting its predetermined specifications and quality characteristics.

Method validation is an integral part of the method development; it is the process of demonstrating that analytical procedures are suitable for their intended use and that they support the identity, quality, purity, an and drug products. Simply, method validation is the process of proving that and potency of the drug substances analytical method is acceptable for its intended purpose.

For chromatographic methods used in analytical applications there is more consistency in validation practice with key analytical parameters

(a) Recovery (b) Response function (c) Sensitivity (d) Presicion (e) Accuracy (f) limits of detection (g) Limit of quantitation (h) Ruggedness (i) Robustness (j) stability (k) system suitability

(a) Recovery:

The absolute recovery of analytical method is measured as the response of a processed spiked matrix standard expressed as a percentage of the response of pure standard which has not been subjected to sample pre treatment and indicates whether the method provides a response for the entire amount of analyte that is present in the sample.

$$Absolute\ recovery = \frac{Response\ of\ an\ analyte\ spike\ into\ matrix\ (processed)}{Response\ of\ analyte\ of\ pure\ standard(unprocessed)} \times 100$$

b) Sensitivity:

The method is said to be sensitive if small changes in concentration cause large changes in response function. The sensitivity of an analytical method is determined from the slope of the calibration line. The limits of quantification (LOQ) or working dynamic range of bio analytical method are defined as the highest and lowest concentrations, which can determined with acceptable accuracy. It is suggested that, this be set at ± 15% for both the upper and lower limit of quantitation respectively. Any sample concentration that falls outside the calibration range cannot be interpolated from the calibration line and extrapolation of the calibration curve is discouraged. If the concentration is over range, the sample should be diluted in drug-free matrix and re-assayed.

c) Precision:

The purpose of carrying out a determination is to obtain a valid estimate of a 'true' value. When one considers the criteria according to which an analytical procedure is selected, precision and accuracy are usually the first time to come to mind. Precision and accuracy together determine the error of an individual determination. They are among the most important criteria for judging analytical procedures by their results.

Precision refers to the reproducibility of measurement within a set, that is, to the scatter of dispersion of a set about its central value. The term 'set' is defined as referring to a number (n) of independent replicate measurements of some property. One of the most common statistical terms employed is the standard deviation of a population of observation. Standard deviation is the square root of the sum of squares of deviations of individual results for the mean, divided by one less than the number of results in the set. The standard deviation S, is given by

$$S = \sqrt{\frac{1}{n-1}\sum_{i-1}^{n}\left(x_i - \overline{x}\right)^2}$$

Standard deviation has the same units as the property being measured.

The square of standard deviation is called variance (S^2). Relative standard deviation is the standard deviation expressed as a fraction of the mean, i.e., S/x. It is sometimes multiplied by 100 and expressed as a percent relative standard deviation. It becomes a more reliable expression of precision.

% Relative standard deviation = S x 100 / x

d) Accuracy:

Accuracy normally refers to the difference between the mean x****, of the set of results and the true or correct value for the quantity measured. According to IUPAC accuracy relates to the difference between results (or mean) and the true value. For analytical methods, there are two possible ways of determining the accuracy, absolute method and comparative method.

Accuracy is best reported as percentage bias, which is calculated from the expression

$$\%Bias = \frac{(\text{measured value} - \text{true value})}{\text{true value}} \times 100$$

The accuracy of analytical method is then determined at each concentration by assessing the agreement between the measured and nominal concentrations of the analytes in the spiked drug – free matrix sampler.

e) Limit of detection (LOD):

The limit of detection (LOD) of an analytical method may be defined as the concentration, which gives rise to an instrument signal that is significantly different from the

blank. For spectroscopic techniques or other methods that rely upon a calibration curve for quantitative measurements, the IUPAC approach employs the standard deviation of the intercept (Sa), which may be related to LOD and the slope of the calibration curve, b, by

LOD = 3 Sa/ b

f) Limit of quantitation (LOQ)

The LOQ is the concentration that can be quantitate reliably with a specified level of accuracy and precision. The LOQ represent the concentration of analyte that would yield a signal-to-noise ratio of 10.

LOQ = 10 Sa/ b

Where, Sa- the estimate is the standard deviation of the peak area ratio of analyte to IS (5 injections) of the drugs. b -is slope of the corresponding calibration curve.

g) Ruggedness

Method Ruggedness is defined as the reproducibility of results when the method is performed under actual use conditions. This includes different analysts, laboratories, columns, instruments, source of reagents, chemicals, solvents etc. Method ruggedness may not be known when a method is first developed, but insight is obtained during subsequent use of that method.

h) Robustness

The concept of robustness of an analytical procedure has been defined by the ICH as "a measure of its capacity to remain unaffected by small but deliberate variations in method parameters". The robustness of a method is the ability to remain unaffected by small changes in parameters

such as pH of the mobile phase, temperature, %organic solvent strength and buffer concentration etc. to determine the robustness of the method experimental conditions were purposely altered and chromatographic characters were evaluated.

i) System suitability

System suitability experiments can be defined as tests to ensure that the method can generate results of acceptable accuracy and precision. The requirements for system suitability are usually developed after method development and validation have been completed. (or) The USP (2000) defines parameters that can be used to determine system suitability prior to analysis.

The criteria selected will be based on the actual performance of the method as determined during its validation. For example, if sample retention times form part of the system suitability criteria, their variation (SD) during validation can be determined system suitability might then require that retention times fall within a ±3 SD range during routine performance of the method.

DRUG PROFILE 1[18]

Name: Ethambutol

IUPAC: (2S)-2-[(2-{[(2S)-1-hydroxybutan-2-yl] amino} ethyl) amino] butan-1-ol.

Molecular Formula: $C_{10}H_{24}N_2O_2$

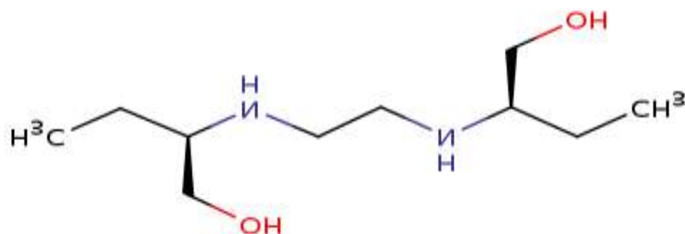
Molecular Weight: 204.3098 g/mole

PHYSIOCHEMICAL PROPERTIES:

Nature: white crystalline powder, odorless, has bitter taste.

Solubility: soluble in water, soluble in methanol, slightly soluble in ether and in chloroform.

Structure:



Mechanism of action: Ethambutol inhibits arabinosyl transferase which is involved in cell wall biosynthesis. By inhibiting this enzyme, the bacterial cell wall complex production is inhibited. This leads to an increase in cell wall permeability.

Category: Anti Tubercular agent.

Storage condition: Ethambutol should be stored at room temperature (below 25°C) in the original pack.

Brand used: Myambutol.

DRUG PROFILE 2 [19]

Name: Isoniazid

IUPAC: pyridine-4-carbohydrazide

Molecular formula: $C_6H_7N_3O$

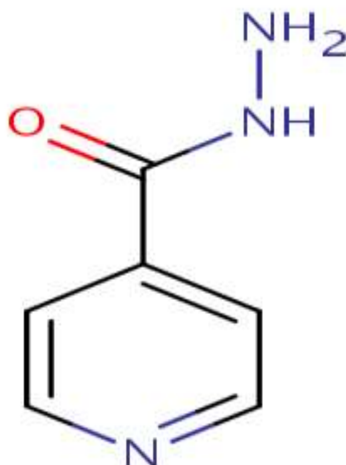
Molecular Weight: 137.1393 g/mole

PHYSIOCHEMICAL PROPERTIES:

Nature: white crystalline powder, taste is sweet at first and then bitter.

Solubility: Soluble in water, methanol, and ethanol. Freely soluble in 0.1n hcl.

Structure:



Mode of action: Isoniazid is a prodrug and must be activated by bacterial catalase. Specifically, activation is associated with reduction of the mycobacterial ferric KatG catalase-peroxidase by hydrazine and reaction with oxygen to form an oxyferrous enzyme complex. Once activated, isoniazid inhibits the synthesis of mycoloic acids, an essential component of the bacterial cell wall. At therapeutic levels isoniazid is bactericidal against actively growing intracellular and extracellular Mycobacterium tuberculosis organisms. Specifically isoniazid inhibits InhA, the enoyl reductase from Mycobacterium tuberculosis, by forming a covalent adduct with the NAD cofactor. It is the INH-NAD adduct that acts as a slow, tight-binding competitive inhibitor of InhA.

Category: Anti-tubercular agents.

Storage: Store it at room temperature, and keep away from children.

Brand used: Hydra, Isovit,Laniazid

2. AIM AND SCOPE OF PRESENT WORK

1. To estimate Isoniazid and ethambutol simultaneously in tablet dosage forms by RP-HPLC method.

2. To validate the method according to ICH guidelines.

Plan of work

The experimental work has been planned as follows:

Review of the literature for Isoniazid and ethambutol regarding their physical and chemical properties, various analytical methods that were conducted for Isoniazid and ethambutol forms the basis for development of new analytical RP-HPLC method Isoniazid and ethambutol combination.

Development of the method by RP- HPLC

1. Selection of the solvent to be used as diluents and mobile phase:

Choosing the suitable solvent in which the drug is soluble and stable.

They must be easily available, economical and of the HPLC grade

2. Selection of Mobile phase:

For the mobile phase, the first variable to be decided is whether an organic or aqueous eluent should be used. With the RP-HPLC analysis, either an aqueous eluent or a very polar organic solvent such as methanol or Acetonitrile should be fixed. If the K’ values are too large with an

aqueous solvent, organic solvent should be tried. If the K' value are too low with organic solvent the separation should be attempted using a mixture of two solvents with various properties.

- K'-capacity factor is a measurement of the degree where the peak of the interest is located with respect to void volume, i.e. Elution time of non-retained components. Generally the value of K' is > 2.
- If a buffer is used, the p^H as well as ionic strength of the buffer can be tried.

3. In order to select the wavelength to carry out the analysis, critical examination of the Ultraviolet absorbance spectra of the drug should be done.

4. A perfect study of the structure of drug and its physicochemical properties; to select the chromatographic parameters.

5. Selection of method for quantitative chromatographic analysis. Determination of working concentration range.

6. Validation of the developed method by following ICH guidelines.

3. REVIEW OF LITERATURE

1. Ali .J et al [21] in this method the drug combination, isoniazid and rifampicin was analysed by developing HPTLC technique. Analysis was done in both bulk drugs and formulations. In this method compounds were separated by using aluminium backed silica gel 60 F_{254} plates. Densitometric analysis of isoniazid and rifampicin was performed at 254 nm. The developed method was validated and accuracy, specificity, linearity, range, quantitation limit were determined. They reported this method is easy, sensitive, simple method.

2. Kumar P et al [22] they developed a new technique which was a stability indicated RP-HPLC method for the analysis of Prothionamide. It is an anti tubercular drug. The column used was a RP C18 column and mobile phase used was Methanol: Buffer solution and PH was adjusted to 4.5. As drug undergoes degradation in different conditions like acidic, basic, photochemical degradation and thermal degradation all the peaks of -degradation product were differentiated by retention time. This method was validated for robustness, recovery, ruggedness and precision.

3. Mohan B et al [23] In this method they determined the ability of a recently notified USP HPLC method quantitative determination of rifampicin, isoniazid and pyrazinamide in fixed dose combination (FDC) formulations to resolve major degradation products of rifampicin. Resolving power depends on the make of the column. This study gave a good insight into the performance of the proposed USP method for the assay of Rifampicin, Isoniazid and pyrazinamide in pharmaceutical FDC formulations.

4. Karthikeyan K et al [24] In this article a new RP-HPLC method was developed by using chiral derivatization reagents like o-phthalaldehyde and N-acetyl-l-cysteine. In this Zorbax SB Phenyl HPLC column was used for resolving diastereomers. A mobile phase of 95:05 (v/v), 20mMNa2HPO4 (pH 7), and acetonitrile was used and the flow rate was maintained at 1.0ml/min. The above developed method was validated and suitability was determined[5]. Comparison of the data regarding two HPLC methods for the determination of isoniazid. In this method they developed two HPLC methods for determination of Isoniazid. Waters 2695 liquid chromatography and a UV - Waters 2489 detector were used for this experiment. The first method (I) used a Nucleosil 100-10 C18 column (250 x 4.6 mm), a mobile phase formed by a mixture of acetonitrile/10^{-2} M oxalic acid (80/20) and a flow rate of 1.5 ml/ min; detection was done at 230 nm. The second method (II) used a Luna 100-5 C18 column (250 x 4.6 mm), a mobile phase formed by a mixture of methanol/acetate buffer, pH = 5.0 (20/ 80), and a flow rate of 1 ml/min; detection was done at 270 nm. Both methods were validated and applied for INH determination.

5. Lui J et al [25] developed a HPLC method by using c18 monolithic column. In this method they analysed rifampicin and four related compounds. They used methanol-acetonitrile-monopotassium phosphate (0.075 M)-citric acid (1.0M) as the mobile phase at a flow rate of 2 ml/min. The total run time was less than 11 min. The developed method was validated and accuracy and precision were determine Review of literature reveals the determination of contents and dissolution of ethambutol hydrochloride in fixed dose combinations for anti tuberculosis drugs by high performance liquid chromatography with pre-column derivatization. In this RP-HPLC method derivatisation should be carried out in non aqueous environment. ethambutol was dervatised with phenylethylisocynate in a molar ratio of 1:6. Active recovery of the sample was 98.7% and standard deviation was 0.70%. the linear regression of standard calibration curve was good. The method was good rapid, specific and sensitive, so it was successfully applied for dissolution and quantitisation of ethambutol in fixed dose combinations (FDCs).

6. **E Calleri** et al [26] carried out by simple accurate liquid chromatographic method was developed and validated for the estimation of isoniazid, pyrazinamide and rifampicin in combined dosage forms. The drugs were chromatographed on a reverse phase c18 column using a mobile phase gradient and monitored at corresponding maximum peaks with rention time compared to standards and confirmed with charcterstic spectra of diode array detector solutions. Concentrations were measured on weight basis to avoid using of internal standard. The method does not require any sample preparation except preparation of guard column. The method is simple rapid specific. Due to its simplicity, and accuracy the method is used in routine quality control of anti tuberculosis combined dosage forms.

7. **Butterfield AG** et al [27] An aliquot of a diluted aqueous tablet extract was introduced onto a micro particulate cyanopropyl bonded-phase a valve-loop injector was used as a column and acetonitrile-0.01 M, pH 3.5 aqueous acetate buffers (5:95) used as a mobile phase. By this method the compound can be determined at levels as low as 0.5% of the isoniazid label claim.

8. Gaitonde CD et al [28] In this method the sample was dissolved in the mobile phase and the mixture was filtered. A portion of the column analysed by HPLC by using column (25 cm × 4.6 mm) of Excalibur ODS-CN (5 µm). In this method they used 5mN-tetrabutyl ammonium hydroxide (adjusted to pH 3 with phosphoric acid) - methanol (1:4) as the mobile phase and detection was carried out at 265 nm. The calibration graph was rectilinear for 0.1 to 0.5 mg/ml of isoniazid, pyrazinamide and rifampicin.

9. Padmarajaiah Nagaraja et al [29] A simple, rapid and sensitive spectrophotometric method for the determination of isoniazid (INH) and ritodrine hydrochloride (RTH) in pure form as well as dosage forms is described. The method is based on the diazotisation of 4,40 -sulphonyldianiline (dapsone, DAP) followed by a coupling reaction with either INH or RTH in sodium hydroxide medium. Beer's law is obeyed in the concentration range of 0.5-20 µg ml−1 for INH at 440 nm and 0.5-18 µg ml−1 for RTH at 460 nm. The method is successfully employed for the determination of INH/RTH in pharmaceutical preparations and the results agree favourably with the official and reported methods. Common excipients used as additives in pharmaceuticals do not interfere in the proposed method. The method offers the advantages of simplicity, rapidity and sensitivity without the need for extraction or heating. Limit of detection (LOD) and limit of quantification (LOQ).

10. S.A Benetton et al [30] Two methods are described for the determination of rifampicin and isoniazid in mixtures by visible spectrophotometry and first-derivative ultraviolet spectrophotometry. The absorbance at 475 nm in buffer solution pH 7.4 was employed to determine rifampicin after applying the three-point correction technique between 420 and 520 nm, while the amplitude of the first-derivative spectrophotometric spectrum at 257 nm in HCl 0.012 M was selected for the determination of isoniazid. The methods are rapid, simple and do not require any separation step. The recovery average was 99.03% for rifampicin and 100.01% for isoniazid. The methods were applied to determine the two compounds in commercial capsules and compared with the official method of the USP XXIII with good agreement between the results.

11. S Furlanetto et al [31] A simple and accurate liquid chromatographic method was developed and validated for estimation of isoniazid (ISN), pyrazinamide (PYR) and rifampicin (RIF) in combined dosage forms. Drugs were chromatographed on a reverse phase C18 column using a mobile phase gradient and monitored at the corresponding maximum of each compounds. Peaks were identified with retention time as compared with standards and confirmed with characteristic spectra using diode-array detector. Solution concentrations were measured on a weight basis to avoid the use of an internal standard. The method does not require any specific sample preparation except the use of a guard column. The method is linear (r^2>0.999), precise (RSD%: 0.50% for ISN, 0.12% for PYR and 0.98% for RIF), accurate (overall average recovery yields: 98.55% for ISN, 98.51 for PYR and 98.56% for RIF) and selective. Due to its simplicity and accuracy the method is suitable for roustine quality control analysis of antitubercolosis combination dosage form.

12. A Manna et al [32]A simple spectrophotometric method for the simultaneous estimation of rifampicin and isoniazid in their combined pharmaceutical dosage form has been developed. The method does not require any extraction or isolation procedure. The method is simple, rapid, specific and reproducible. Recovery studies were also found to be satisfactory.

13. Khuhawar MY et al[33] have Isoniazid (INH) has been quantitated after precolumn derivatization with 5-methylfuran-2-carboxaldehyde (MFA). Chromatography was performed on a 150 mm × 4.6 mm i.d. YMC-ODS column with water–methanol–tetrahydrofuran, 59:39:2 (v/v) as a mobile phase at a flow rate of 2 mL min−1 . Detection was performed at 328 nm. By adoption of this procedure pyrazinamide (PZA) and indomethacin (IM) separated completely and could be determined together with INH. Linear calibration plots were obtained between 1.4 and 5.5 µg mL−1 for INH, between 6.2 and 30.8 µg mL−1 for PZA, and between 10.7 and 64.08 µg mL−1 for IM. The method of analysis was used for quantitation of INH, PZA, and IM in pharmaceutical preparations with a coefficient of variation.

14. Chilukuri et al [34] A simple and sensitive method for the spectrophotometric determination of bio-active compounds (drugs and non-nutritive sweeteners) containing reactive functional groups, viz., aromatic primary amine (drugs: Dapsone, Sulphamethoxazole), aromatic secondary amine (drug: Pindolol), aliphatic secondary amine (non-nutritive sweetener: Cyclamate), acid hydrazide (drug: Isoniazid) and thiol (drug: Captopril), is proposed. The method involves the addition of excess of sodium nitrite to the compound in the presence of 0.25 M hydrochloric acid solution and the unreacted nitrous acid is determined by the measurement of corresponding decrease in the absorbance of cresyl fast violet acetate (Δ_{max} : 555 nm), the most suitable one out of several dyes tested. This method was applied for the determination of bio-active compounds

in commercial samples. (drugs: pharmaceutical formulations; non-nutritive sweetener (cyclamate): foodstuffs). The newly proposed method enabled the determination of the bio-active compounds in microgram quantities (0.1 - 0.5 μg/ml). Standard deviation values evaluated through replicate determinations were found to be < 0.5 mg per dose (RSD : 0.5 - 1.2%). The common excipients do not effect the determination of the drugs in pharmaceutical formulations. Many of the usually occurring additives in food stuffs are tolerated to a very high level in the determination of cyclamate in beverages, syrup, ice candy and ice cream. The validity of the method was tested against the reference method. Recoveries to the tune of 99.2 - 101.1% were observed by adopting this method.

4. MATERIALS AND METHODS

1. MATERIALS

Table No: 4.1. Materials used in present research work

S. No	Chemical Name	Make	GRADE
1	KH_2PO_4 (di-sodium hydrogen phosphate and potassium dehydrogenate phosphate)	RANKEM	HPLC Grade
2	Methanol	MERCK	HPLC Grade
3	Acetonitrile	MERCK	HPLC Grade
4	Water	MERCK	HPLC Grade

2. EQUIPMENTS

Table No: 4.2. Equipments used in present research work

S. No	Instruments	MAKE
1.	HPLC Model No: 2690/5 Series	WATERS
2.	Column	INERTSIL – C18 ODS COLUMN
3.	Electronic balance	SARTORIOUS
4.	Sonicator	FAST CLEAN
5.	Degasser	DISTEK

4.1 METHOD DEVELOPMENT FOR HPLC:

The objective of this experiment was to optimize the assay method for simultaneous estimation of Isoniazid and ethambutol on the literature survey made. So here the trials mentioned describes how the optimization was done.

Preparation of Standard Solution:

Weigh down 10mg's of Isoniazid and ethambutol drugs and dissolved in 10ml of Mobile phase taken in two 10ml of volumetric flasks seperately and sonicated for 20 minutes to get 1000ppms and 1 ml was taken from each solution into a 10ml volumetric flask and diluted to 10 ml with mobile phase.

Trial: 1

Chromatographic Conditions:

Mobile Phase	: Degassed Acetonitrile 100%.
Flow rate	: 1.0ml/min
Column	: Inertsil - C18, ODS column
Detector wavelength	: 254nm
Column temp	: Ambient
Injection volume	: 20µl
Run time	: 10min
Retention time	: 3.8min for Isoniazid and 4.1 for ethambutol.

Observation: Two peaks are merged and not separated completely. The trial 1 chromatogram result was shown in Fig: 1.

Mobile Phase: Degassed Acetonitrile and methanol in the ratio of 90:10 V/V.

Trail: 2

Chromatographic Conditions:

Flow rate	: 1ml/min
Column	: Inertsil -C18, BDS column
Detector wavelength	: 274nm
Column temp	: Ambient
Injection volume	: 20µl
Run time	: 10min
Retention time	: 3.7 min for Isoniazid and 4.0 min for ethambutol

Observation: The two peaks are separated completely but peak shapes are not good. The trial 2 chromatogram result was shown in Fig: 2.

Trail: 3

Mobile Phase: Degassed Acetonitrile and Methanol in the ratio of 80:20 V/V.

Chromatographic Conditions:

Flow rate : 1.0ml/min

Column : Inertsil - C18, BDS column

Detector wavelength : 274 nm

Column temp : Ambient

Injection volume : 20µl

Run time : 10min

Retention time : 1.7 min for Isoniazid and 2.1 min for ABCVR.

Observation: ethambutol got peak fronting and base line between two peaks is not straight. The trial 3chromatogram result was shown in Fig: 3

4.2 OPTIMIZED METHOD

Mobile Phase: Degassed Methanol and Buffer in the ratio of 55:45 V/V.

Preparation of (KH_2PO_4 0.1M) buffer: Weight 3.8954g of di-sodium hydrogen phosphate and 3.4023g of potassium dehydrogenate phosphate in to a beaker containing 1000ml of distilled Water and dissolve completely. Then ph is adjusted with orthophosphoric acid and then filtered through 0.45µm membrane filter.

Preparation of stock solution:

Reference solution: The solution was prepared by dissolving 20.0 mg of accurately weighed Isoniazid and 25.0 mg ethambutol in Mobile phase, in two 100.0 mL volumetric flasks separately and sonicate for 20min. From the above solutions take 10.0 mL from each solution into a 50.0 mL volumetric flask and then makeup with mobile phase and sonicate for 10min.

Preparation of working standard solution:

The stock solutions equivalent to 20ppm to 80ppm with respect to both drugs were prepared in combination of Isoniazid and ethambutol above, sonicated and filtered through 0.45μ membrane.

Preparation of sample drug solution for pharmaceutical formulations:

Twenty tablets were weighed accurately and a quantity of tablet powder equivalent to 20 mg Isoniazid and 20 mg ethambutol was weighed and dissolved in the 70 mL mobile phase with the aid of ultrasonication for 20 min. The content was diluted to 100 mL with mobile phase to furnish a stock test solution. The stock solution was filtered through a 0.45 μm Nylon syringe filter and 10.0 mL of the filtrate was diluted into a 50.0 mL volumetric flask to give a test solution containing 40 μg/mL Isoniazid and 40 μg/mL ethambutol.

Procedure for calibration curve:

The contents of the mobile phase were filtered before use through 0.45micron membrane and pumped from the respective solvent reservoirs to the column at a specified flow rate. Prior to injection of the drug solutions, the column was equilibrated for at least 30min with the mobile phase flowing through the system. The chromatographic separation was achieved using a mobile phase consisting of Acetonitrile : Buffer at 55:45V/V the eluent was monitored

using Pda detector at a wavelength of 274nm .The column was maintained at ambient temperature (27^0c) and an injection volume of 20l of each of standard and sample solutions were injected into the HPLC system to get the chromatograms. The retention time, peak areas of drug was recorded graph was plotted by taking concentration of the drug on x-axis and peak area on y-axis. A typical chromatogram of Isoniazid and ethambutol combination was shown in Fig 4.

Optimized chromatographic conditions:

Parameters	**Method**
Stationary phase (column)	Inertsil -ODS C_{18}(250 x 4.6 mm, 5 µ)
Mobile Phase	Methanol : Buffer (55:45)
Flow rate (ml/min)	1.0 ml/min
Run time (minutes)	8min
Column temperature (°C)	Ambient
Volume of injection loop (l)	20
Detection wavelength (nm)	274nm
Drug RT (min)	2.7min for Isoniazid, 3.4 for ethambutol

Calculation:

The amount of drugs present in each pharmaceutical formulation was calculated by using the standard calibration curves (concentration in ppm was taken on x-axis and peak area on y-axis).

A typical chromatogram of Isoniazid and ethambutol (100ppm) (formulation) were shown in Fig: 5

4.3 METHOD VALIDATION:

4.3.1 System suitability:

A Standard solution was prepared by using Isoniazid and ethambutol working standards as per test method and was injected Five times into the HPLC system.

The system suitability parameters were evaluated from standard chromatograms by calculating the % RSD from five replicate injections for Isoniazid and ethambutol**,** retention times and peak areas.

Acceptance criteria:

1. The % RSD for the retention times of principal peak from 5 replicate injections of each Standard solution should be not more than 2.0 %

2. The % RSD for the peak area responses of principal peak from 5 replicate injections of each standard Solution should be not more than 2.0%.

3. The number of theoretical plates (N) for the Isoniazid and ethambutol peaks is NLT 3000.

4. The Tailing factor (T) for the Isoniazid and ethambutol Peaks is NMT 2.0

Observation:

The %RSD for retention times and peak areas were found to be within the limit. Refer table: 1 As shown in fig 6 – 10.

4.3.2 Specificity:

Isoniazid and ethambutol:

Solutions of standard and sample were prepared as per the test method are injected into chromatographic system.

Acceptance criteria:

Chromatograms of standard and sample should be identical with near Retention time.

Observation:

The chromatograms of Standard and Sample were same identical with same retention time. As shown in fig: 12 and fig: 13

4.3.3 Precision:

4.3.3.1 Repeatability:

- System precision: Standard solution prepared as per test method and injected five times.
- Method precision: Prepared six sample preparations individually using single as per test method and injected each solution.

Acceptance criteria: The % relative standard deviation of individual Isoniazid and ethambutol, from the six units should be not more than 2.0%.

The individual assays of Isoniazid and ethambutol should be not less than 98% and not more than 102.0%.

Observation:

Test results are showing that the test method is precise. Refer tables 2 and 3 for system precision and for method precision.

4.3.3.2 Intermediate precision (analyst to analyst variability):

A study was conducted by two analysts as per test method

Acceptance criteria:

The individual assays of Isoniazid and ethambutol should be not less than 98% and not more than 102% and %RSD of assays should be NMT2.0% by both analysts.

Observation:

Individual %assays and %RSD of Assay are within limit and passes the intermediate precision, Refer table: 4

4.3.4 Accuracy (recovery):

A study of Accuracy was conducted. Drug Assay was performed in triplicate as per test method with equivalent amount of Isoniazid and ethambutolinto each volumetric flask for each spike level to get the concentration of Isoniazid and ethambutol equivalent to 50%, 100%, and 150% of the labeled amount as per the test method. The average % recovery of Isoniazid and ethambutol were calculated.

Acceptance criteria:

The mean % recovery of the Isoniazid and ethambutolat each spike level should be not less than 98.0% and not more than 102.0% for both the drugs separately.

Observation:

$$\% \text{ Recovery} = \frac{\text{Amount found}}{\text{Amount added}} \times 100$$

The recovery results indicating that the test method has an acceptable level of accuracy. Refer table: 5

4.3.5 Linearity of test method:

A Series of solutions are prepared using Isoniazid and ethambutol working standards at concentration levels from 20ppm to 80 ppm of target concentration .Measure the peak area response of solution at Level 1 and Level 6 six times and Level 2 to Level 5 two times.

Acceptance criteria:

Correlation Coefficient should be not less than 0.9990.

% of y- Intercept should be ±2.0.

% of RSD for level 1 and Level 6 should be not more than 2.0%.

Observation:

The linear fit of the system was illustrated graphically. The results are presented in table6.

4.3.6 Ruggedness of test method:

a) System to system variability:

System to system variability study was conducted on different HPLC systems, under similar conditions at different times. Six samples were prepared and each was analyzed as per test method.

Comparison of both the results obtained on two different HPLC systems, shows that the assay test method are rugged for System to system variability.

Acceptance criteria:

The % relative standard deviation of Isoniazid and ethambutol from the six sample preparations should be not more than 2.0%

The % assay of Isoniazid and ethambutol should be between 98.0%-102.0%.

Observation:

The % RSD was found within the limit. Ref tables: 3 &7.

b) column to column variability:

Column to column variability study was conducted by using different columns. Six samples were prepared and each was analysed as per test method

Acceptance criteria:

The %RSD of Isoniazid and ethambutol tablets should be NMT2.0%. The %assay of Isoniazid and ethambutol should be between 98.0% and 102.0% for individual drugs.

Observation:

The results obtained by comparing with both two types were within limit. Refer tables: 3 &9

4.3.7 Robustness:

a) Effect of variation of flow rate:

A study was conducted to determine the effect of variation in flow rate. Standard solution prepared as per the test method was injected into the HPLC system using flow rates, 1.0ml/min and 1.2ml/min. The system suitability parameters were evaluated and found to be within the limits for 1.0ml/min and 1.2ml/min flow.

Isoniazid and ethambutol and was resolved from all other peaks and the retention times were comparable with those obtained for mobile phase having flow rates 1.0ml/min.

Acceptance criteria:

The Tailing Factor of Isoniazid and ethambutol standards should be NMT 2.0 for Variation in Flow.

Observation:

The tailing factor for Isoniazid and ethambutol was found to be within the limits. As shown in table 10.

b) Effect of variation of temperature:

A study was conducted to determine the effect of variation in temperature. Standard solution prepared as per the test method was injected into the HPLC system at 20ºC temperature. The system suitability parameters were evaluated and found to be within the limits for a temperature change of 20ºc.

Similarly sample solution was chromatographed at 25ºC temperature. Isoniazid and ethambutol were resolved from all other peaks and the retention times were comparable with those

Acceptance criteria:

The Tailing Factor of Isoniazid and ethambutol standard and sample solutions should be NMT 2.0 for Variation in temperature.

Observation:

The tailing factor for Isoniazid and ethambutol

x is found to be within the limits. As shown in table 11.

4.3.8 Limit of detection and quantitation (LOD and LOQ):

From the linearity data calculate the limit of detection and quantitation, using the following formula.

$$LOD = \frac{3.3\,\sigma}{S}$$

σ = standard deviation of the response

S = slope of the calibration curve of the analyte.

$$LOQ = \frac{10\,\sigma}{S}$$

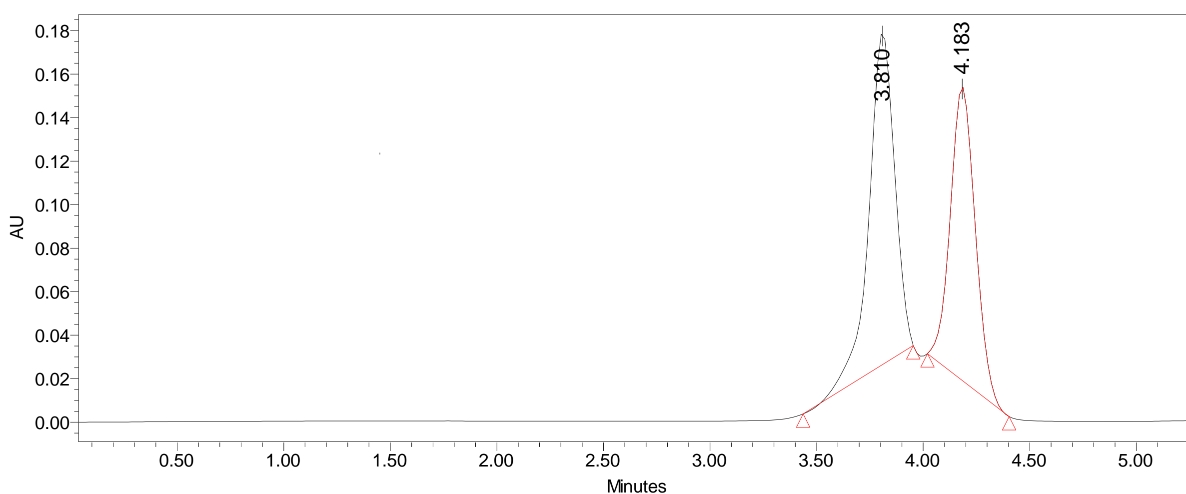
σ = standard deviation of the response

S = slope of the calibration curve of the analyte.

5. RESULTS AND DISCUSSION

5.1 Method development:

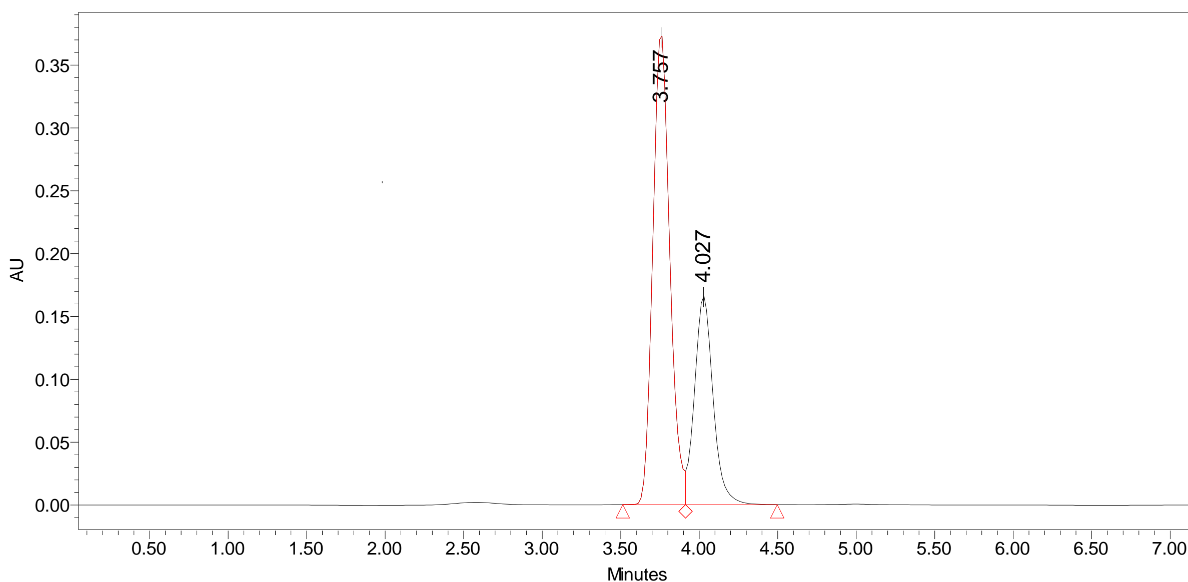
Fig1: Chromatogram of Trial 1



Inference : Two peaks are not seperated, completely merged.

S.NO	Name of the peak	Retention time(min)
1	**Isoniazid**	3.810
2	**Ethambutol**	4.183

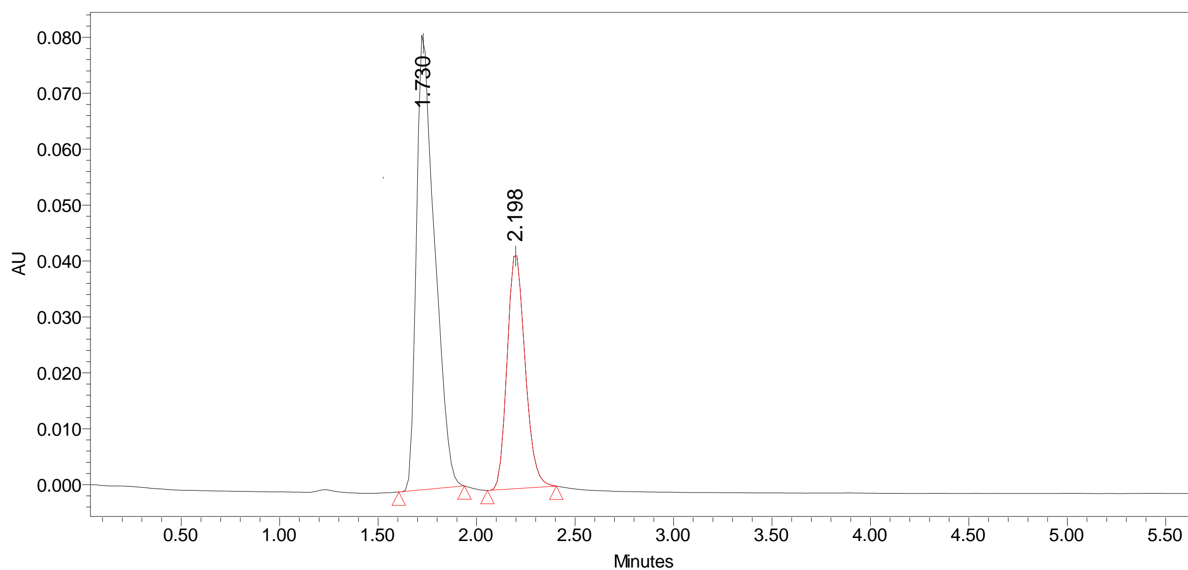
Fig 2: Chromatogram of Trial 2:



Inference: Peaks shapes are not good.

S.NO	Name of the peak	Retention time(min)
1	**Isoniazid**	3.757
2	**Ethambutol**	4.024

Fig 3: Chromatogram of Trial3

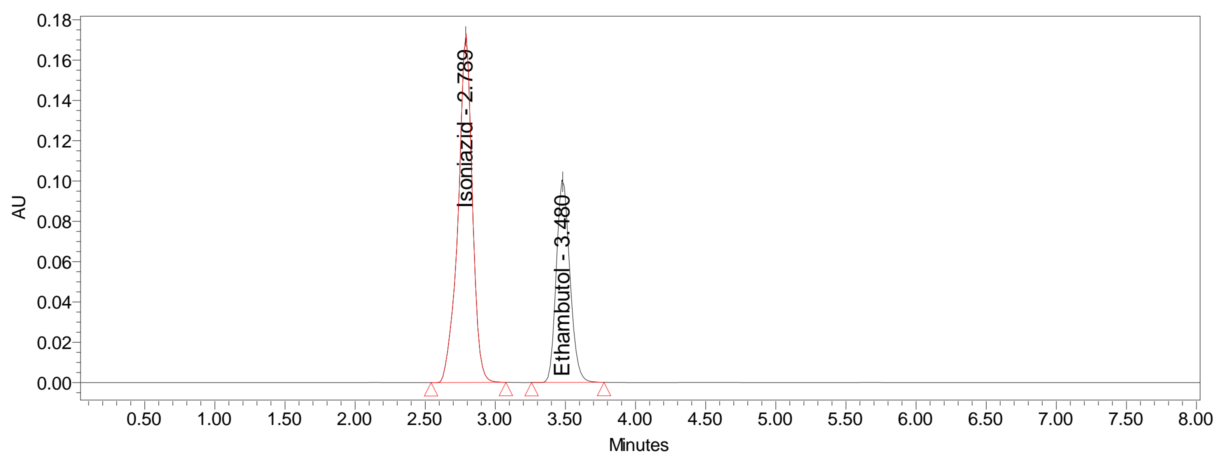


Inference : peaks are not seperated completely.

S.NO	Name of the peak	Retention time(min)
1	**Isoniazid**	1.730
2	**Ethambutol**	2.198

5.2 Optimized method

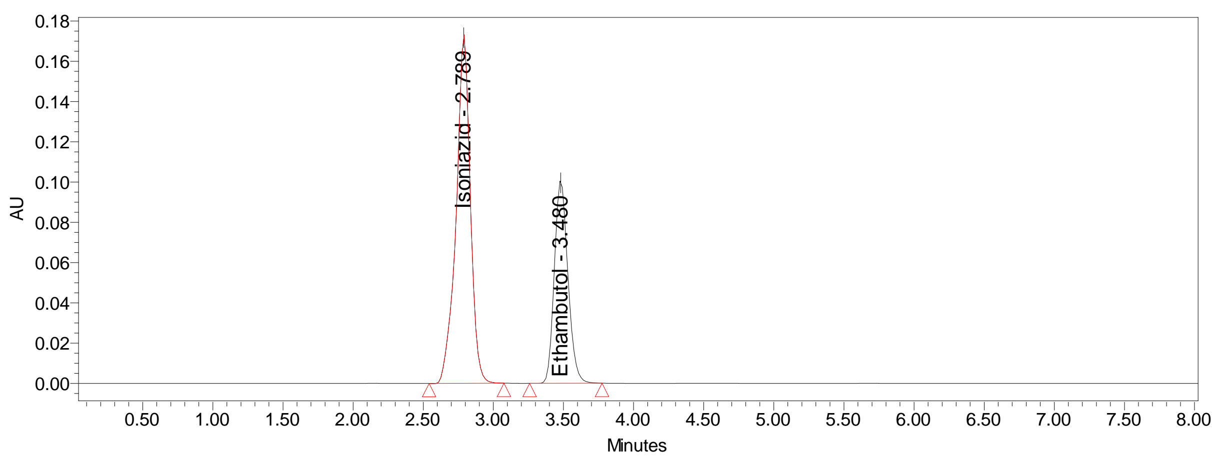
Fig 4: Chromatogram of standard



Inference: Got chromatogram at RT's of 2.9min to Isoniazid and 4.1min to Ethambutol

S.NO	Name of the peak	Retention time(min)
1	**Isoniazid**	2.789
2	**Ethambutol**	3.480

Fig5: Chromatogram of sample



Inference: Got same chromatogram with same RT values as of standard.

S.NO	Name of the peak	Retention time(min)
1	**Isoniazid**	2.789
2	**Ethambutol**	3.480

5.3 Validation data

5.3.1 System suitability:

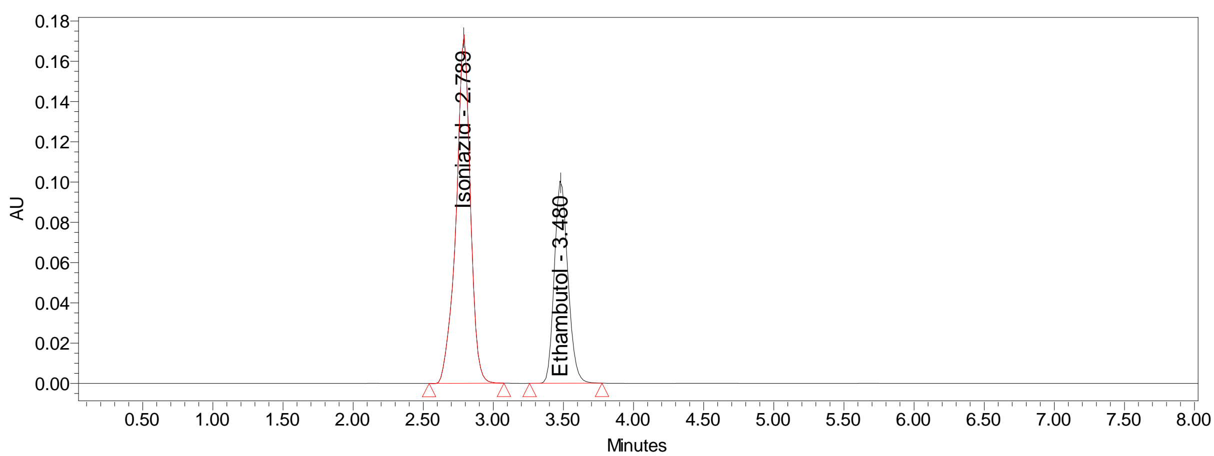
TABLE- 1(a): Data of System Suitability for Isoniazid

Injection	RT	Peak Area	USP Plate count	USP Tailing
1	2.789	1188272	56720.964	1.239715
2	2.790	1134892	5875.423	1.234137
3	2.789	1138301	5904.796	1.247458
4	2.788	1134767	5936.253	1.236195
5	2.789	1148124	5913.814	1.248473
Mean	2.789	1138711	5934.251	1.231296
SD	0.00727	56140.015	-------	-------
% RSD	0.025353	0.213538	-------	-------

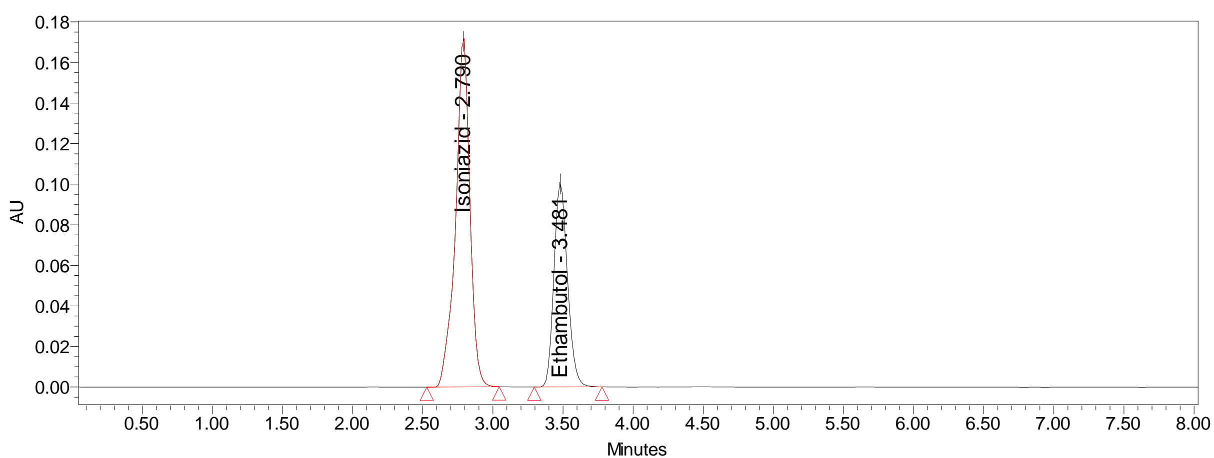
TABLE-1 (b): Data of System Suitability for Ethambutol

Injection	RT	Peak Area	USP Plate count	USP Tailing
1	3.480	807564	8426.113	1.089100
2	3..481	792138	8713.641	1.116929
3	3.481	797985	8536.937	1.111477
4	3.477	812669	8930.182	1.117660
5	3.478	761249	8965.629	1.116904
Mean	3.4794	784201	8480.398	1.111334
SD	0.001817	2074.413	-------	-------
% RSD	0.001817	0.266484	-------	-------

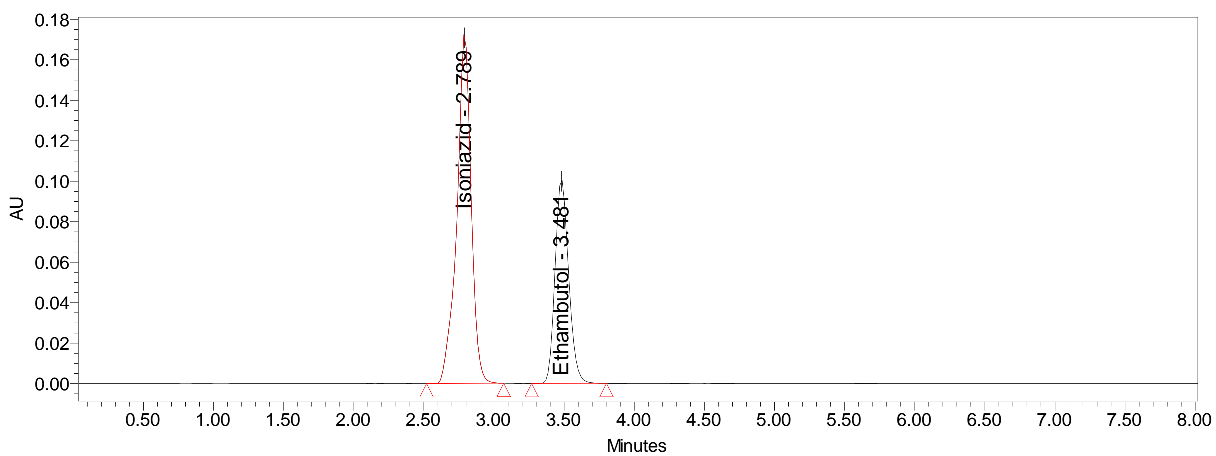
Fig: 6-10 Chromatograms of system suitability (standards 1-5)



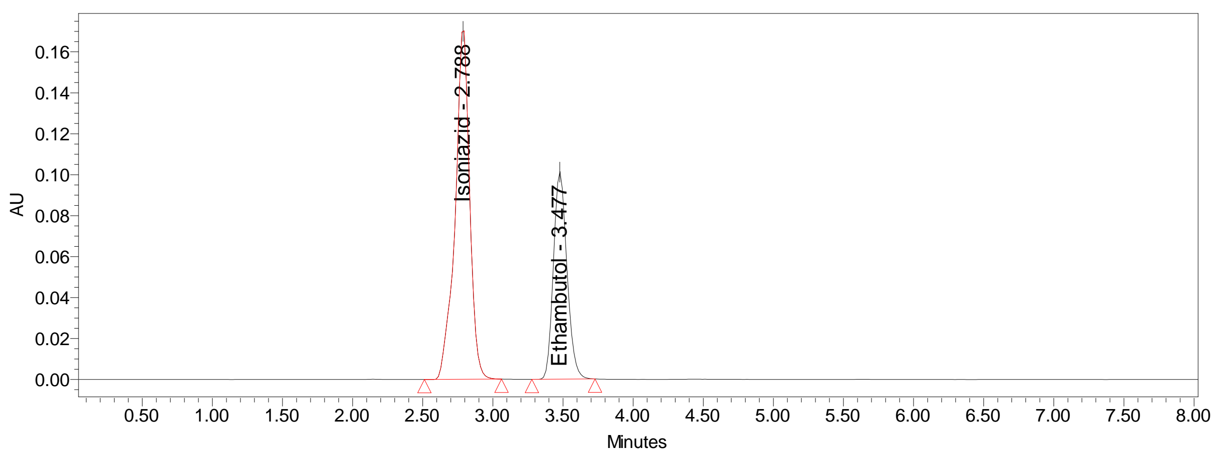
Inference: System suitability Chromatogram for standard – 1



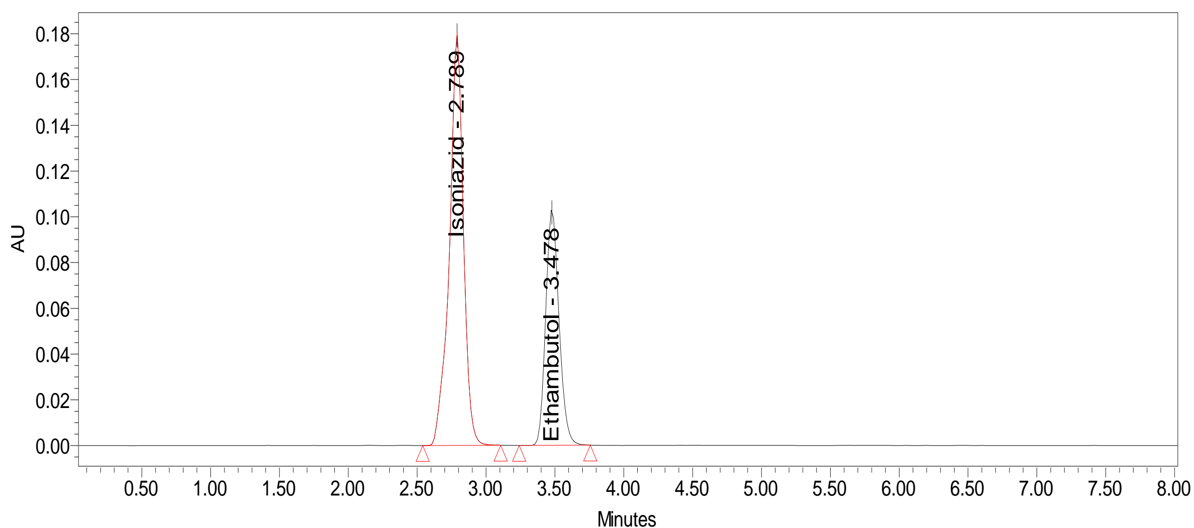
Inference: System suitability Chromatogram for standard – 2



Inference: System suitability Chromatogram for standard - 3



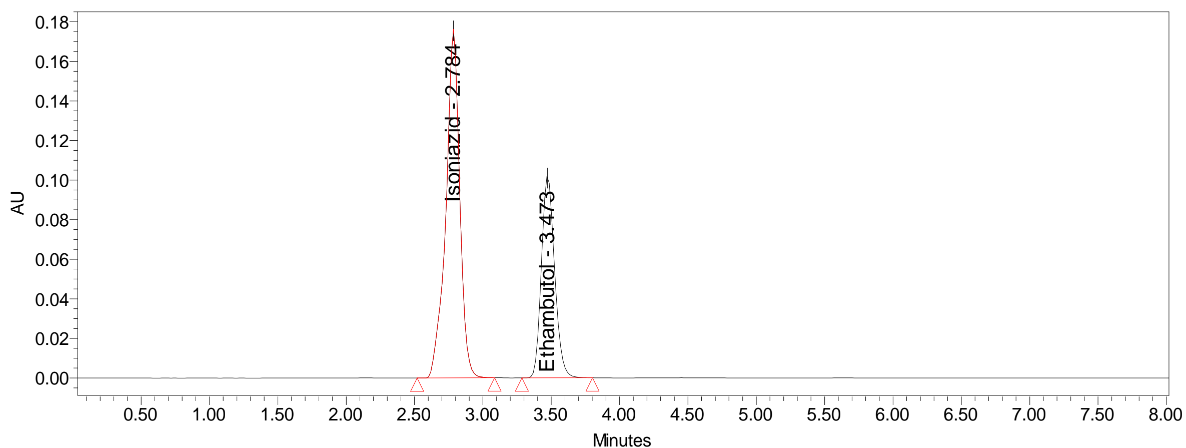
Inference: System suitability Chromatogram for standard - 4



Inference: System suitability Chromatogram for standard – 5

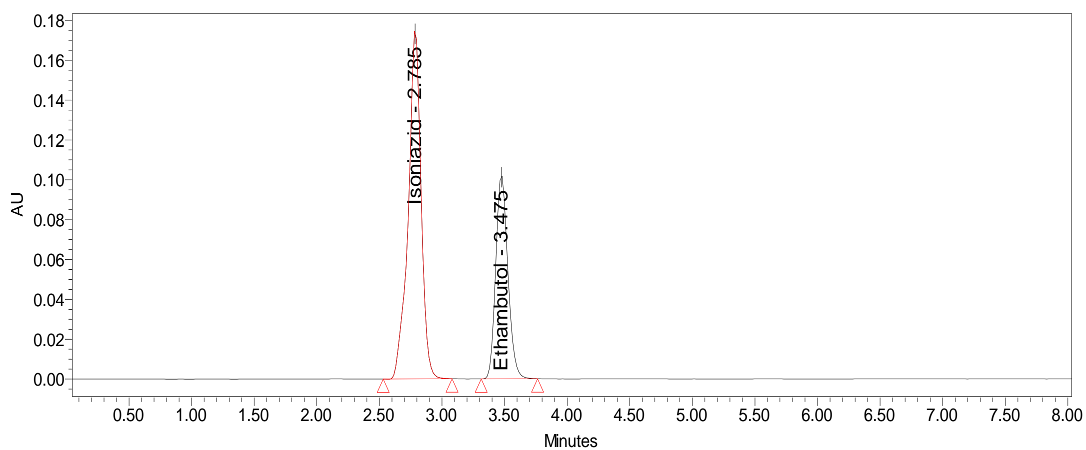
5.3.2: Specificity:

Fig 11: Chromatogram of standard



Inference: Got a peak for standard at an Rt of 2.784min for Isoniazid and 3.473 min for Ethambutol

Fig 12: Chromatogram of sample



Inference: Got a peak for sample at an Rt of 2.785min for Isoniazid and 3.475min for Ethambutol

5.3.2: PRECISION:

5.3.2.1Repeatability:

(a) System precision:

TABLE-2(i): Data of Repeatability (System precision) for Isoniazid

	Injection	**Peak Areas of Isoniazid**	**%Assay**
Concentration 40ppm	1	1176923	99.89
	2	1143416	100.08
	3	1158753	99.86
	4	1159283	99.04
	5	1152423	100.25
Statistical Analysis	**Mean**	1144127	100.78
	SD	5825.027	0.435569
	% RSD	0.382472	0.35152

TABLE-2(ii): Data of Repeatability (System precision) for Ethambutol

	Injection	**Peak Areas of Ethambutol**	**%Assay**
Concentration 40ppm	1	814690	99.84
	2	799531	100.69
	3	816383	99.05
	4	801573	100.41
	5	793432	100.47
Statistical Analysis	**Mean**	815306.4	99.13
	SD	3270.034	0.964103
	% RSD	0.447297	0.935987

Fig13-17 Chromatograms of system precision

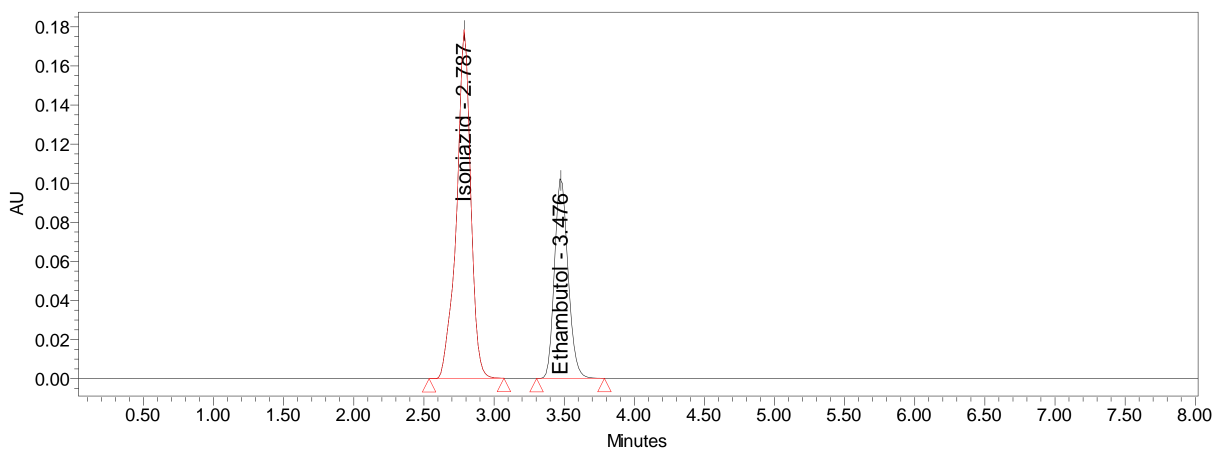


Fig13 Inference: Chromatogram for system precision (standard - 1)

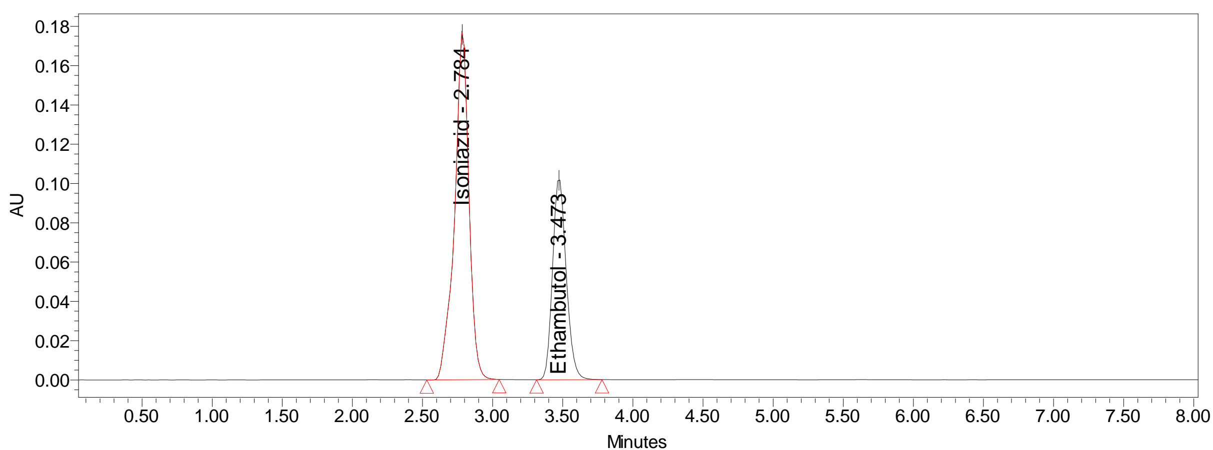


Fig14 Inference: Chromatogram for system precision (standard - 2)

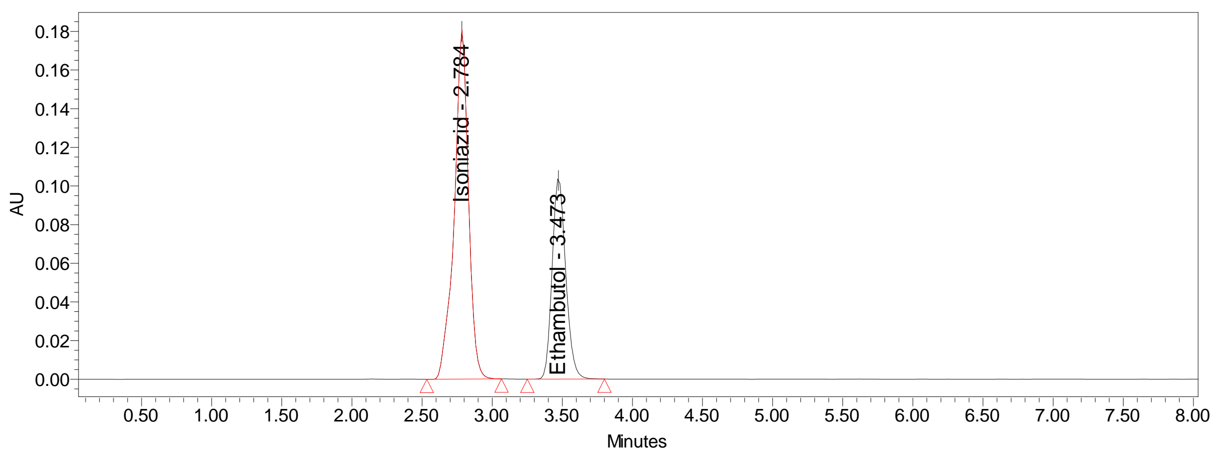


Fig15 Inference: Chromatogram for system precision (standard - 3)

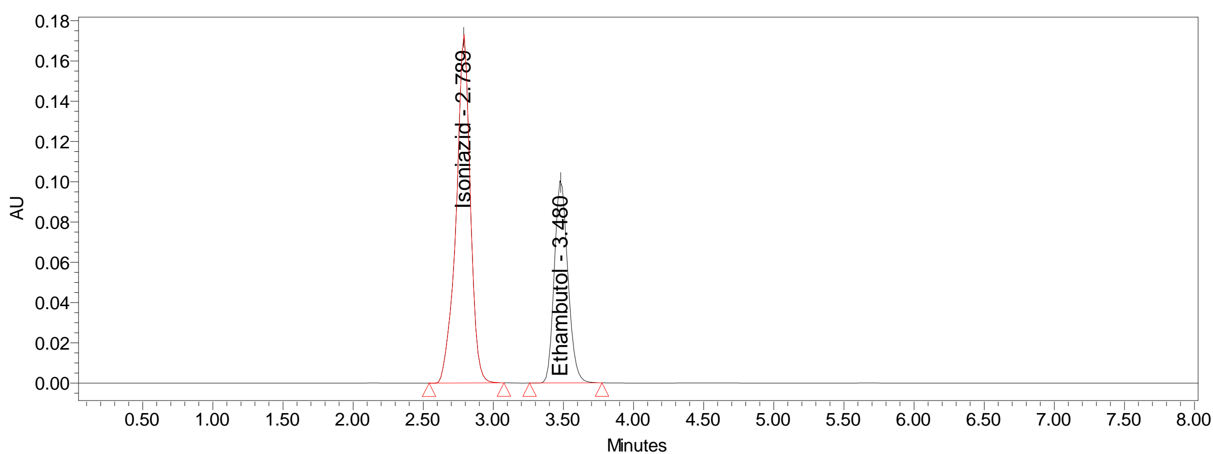


Fig16 Inference: Chromatogram for system precision (standard - 4)

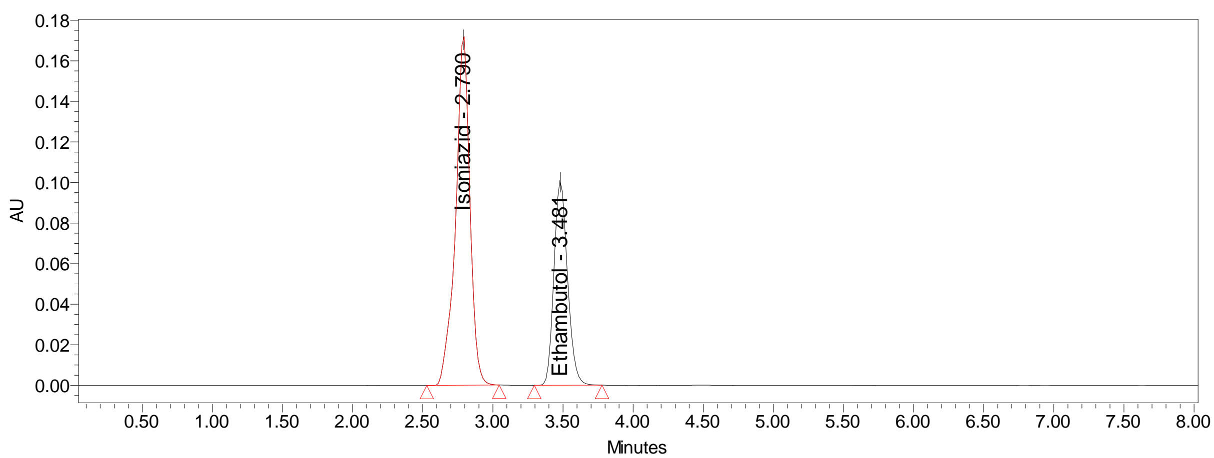


Fig17 Inference: Chromatogram for system precision (standard - 5)

(b)Method precision:

TABLE-3 (i): Data of Repeatability (Method precision) for Isoniazid

	Injection	**Peak Areas of Isoniazid**	**%Assay**
Concentration 40ppm	1	1152324	99.85
	2	1139217	99.38
	3	1257183	99.72
	4	1153870	99.61
	5	1134851	99.66
	6	1137210	99.17
Statistical Analysis	**Mean**	1146528	99.83
	SD	4732.471	0.2589213
	% RSD	0.425228	0.236113

TABLE-3 (ii): Data of Repeatability (Method precision) for Ethambutol

	Injection	Peak Areas of Ethambutol	%Assay
Concentration 40ppm	1	793853	99.36
	2	819390	100.96
	3	798396	99.13
	4	817332	100.40
	5	725854	100.94
	6	802496	99.53
Statistical Analysis	**Mean**	801593	100.69
	SD	4712.714	0.293017
	% RSD	0.274614	0.264173

Fig 18-23: Chromatograms of Repeatability

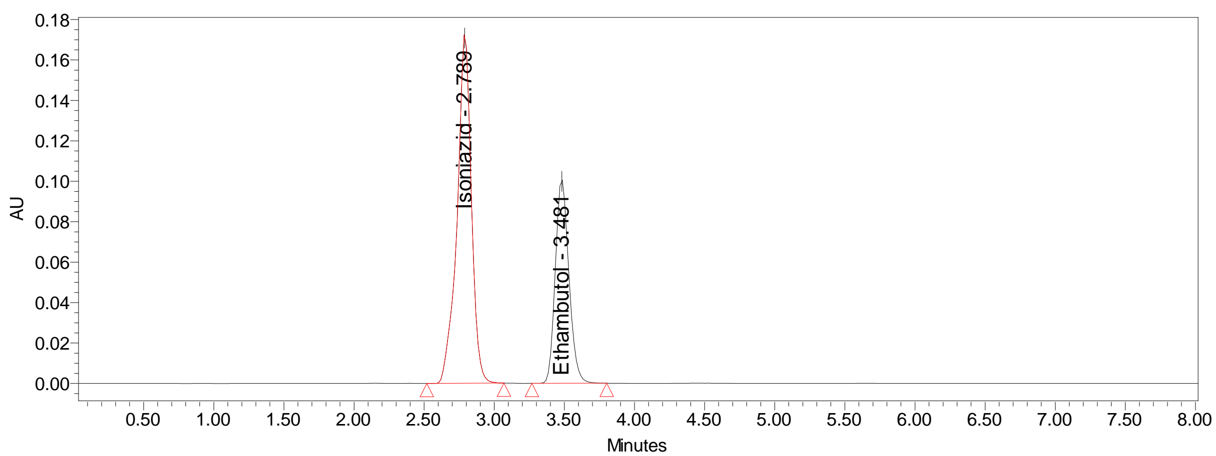


Fig18 Inference: Chromatogram for Repeatability (standard - 1)

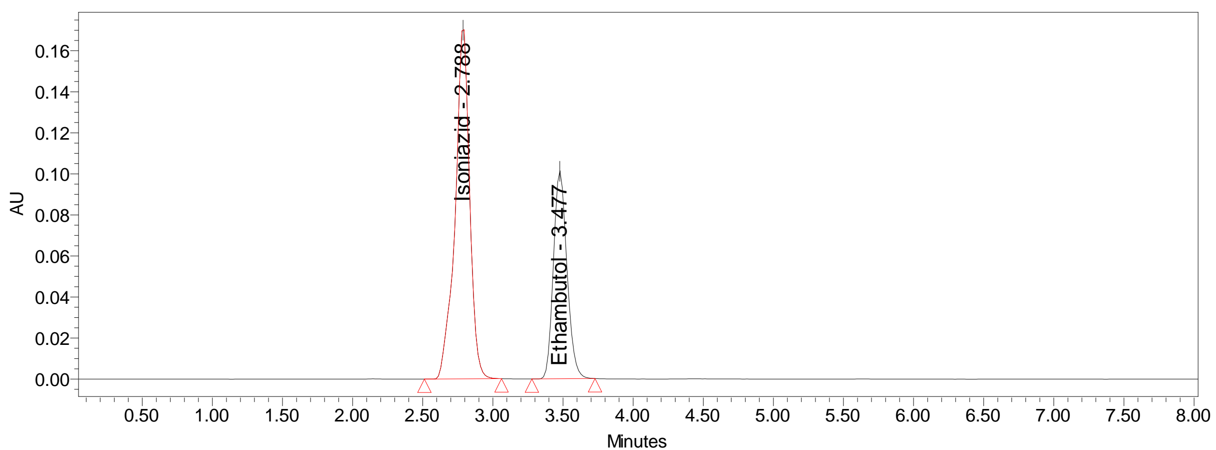


Fig19 Inference: Chromatogram for Repeatability (standard - 2)

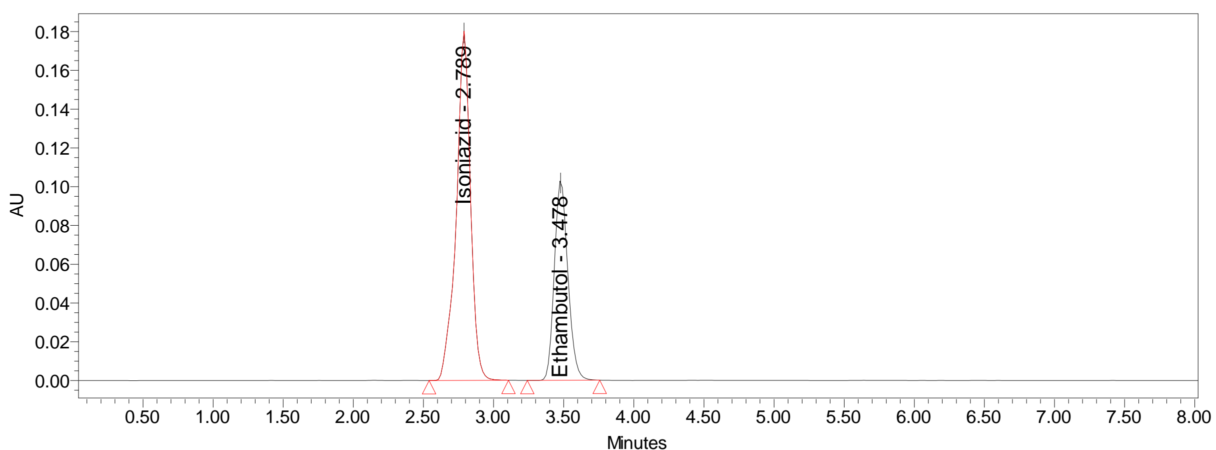


Fig20 Inference: Chromatogram for Repeatability (standard - 3)

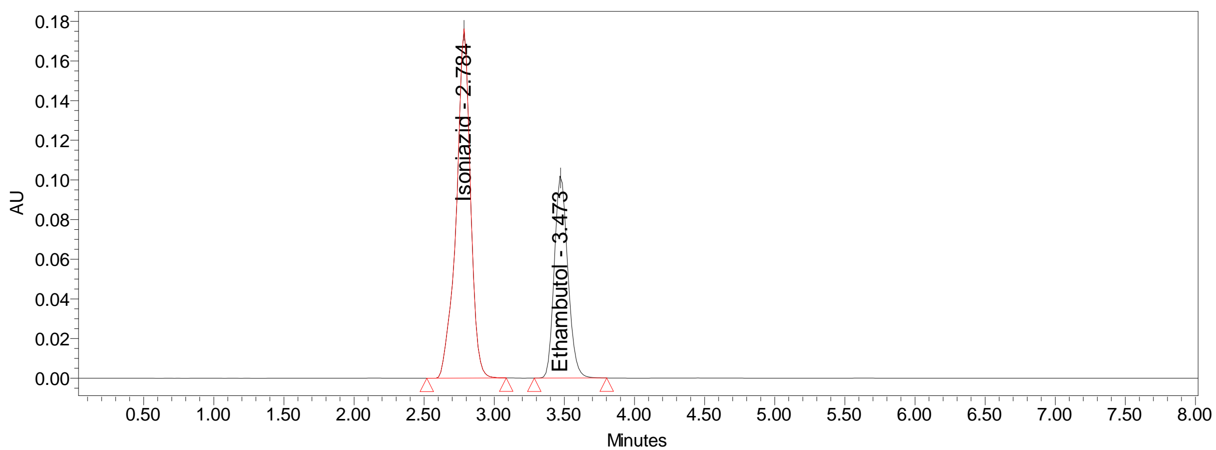


Fig21 Inference: Chromatogram for Repeatability (standard - 4)

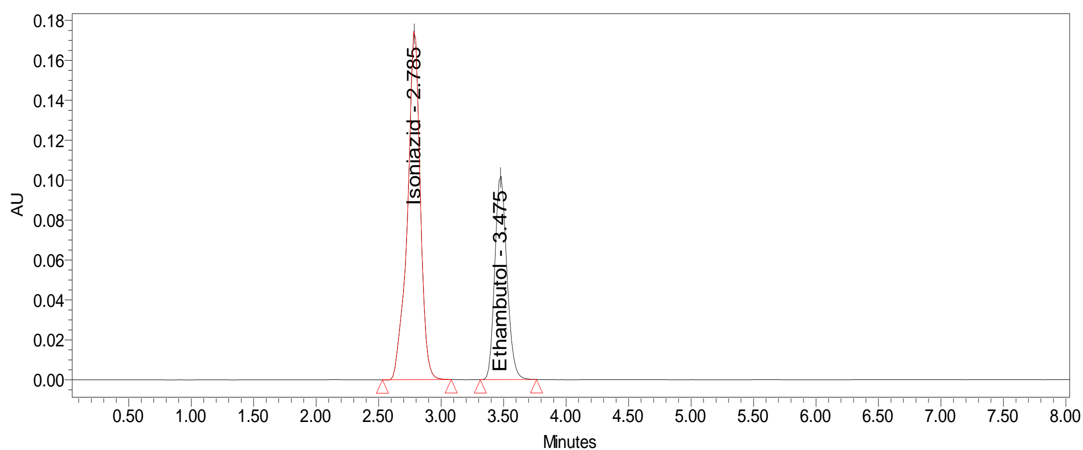


Fig22 Inference: Chromatogram for Repeatability (standard - 5)

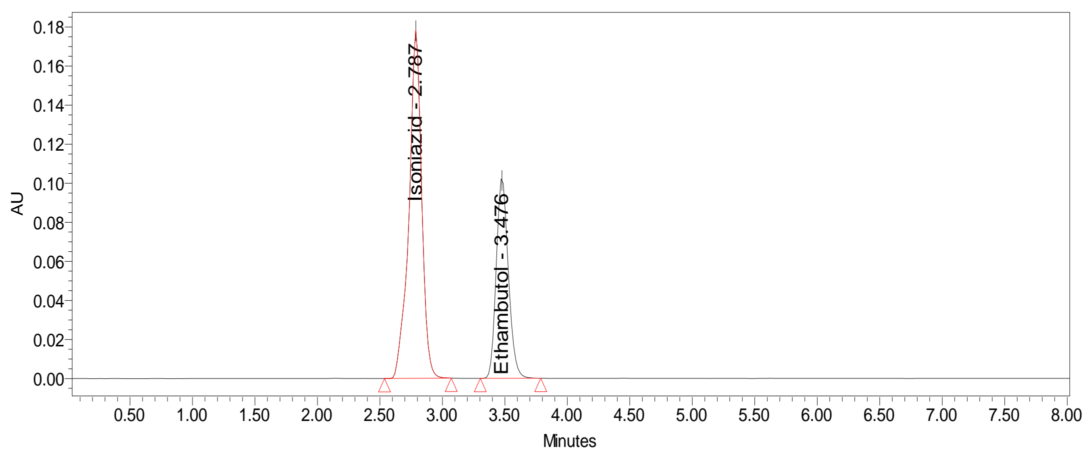


Fig23 Inference: Chromatogram for Repeatability (standard - 6)

5.3.2.2 Intermediate precision:

For Analyst 1 ref: Table3.

Table4:

(i) Data of Intermediate precision (Analyst 2) for Isoniazid

Concentration 40ppm	**Injection**	**Peak Areas of Isoniazid**	**%Assay**
	1	1147272	99.80
	2	1144192	100.54
	3	1142701	99.62
	4	1138067	100.63
	5	1136024	99.08
	6	1143792	99.17
Statistical Analysis	**Mean**	1148125	100.82
	SD	2315.417	0.741601
	% RSD	0.271678	0.718512

(ii) Data of Intermediate precision (Analyst 2) for Ethambutol

	Injections	**Peak Areas of Ethambutol**	**%Assay**
Concentration 40ppm	1	802564	99.96
	2	804138	100.83
	3	795326	101.08
	4	807529	101.01
	5	805149	100.52
	6	809185	99.08
Statistical Analysis	**Mean**	801948.3	99.37
	SD	1851.386	0.341886
	% RSD	0.274155	0.341599

Fig24-25: Chromatograms of Intermediate precision

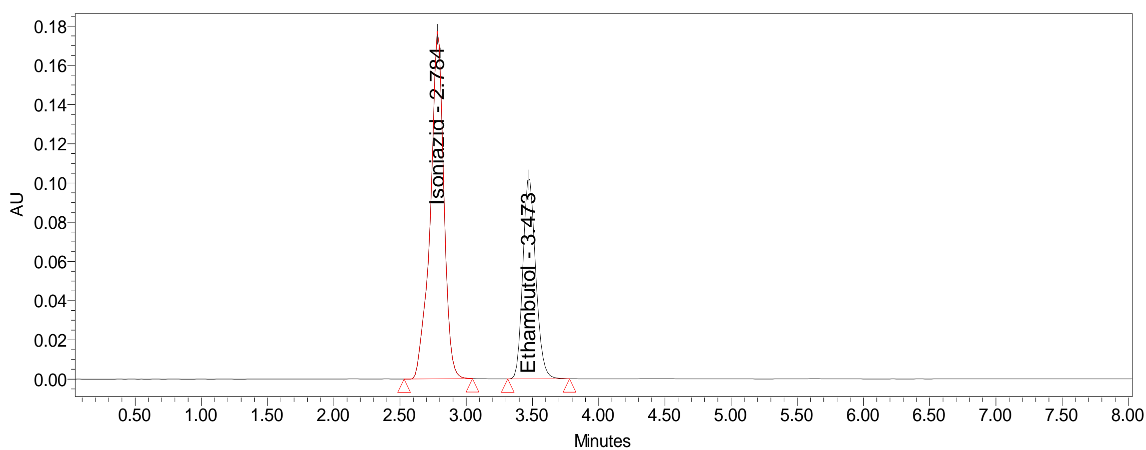


Fig24 Inference: Chromatogram for Intermediate Precision

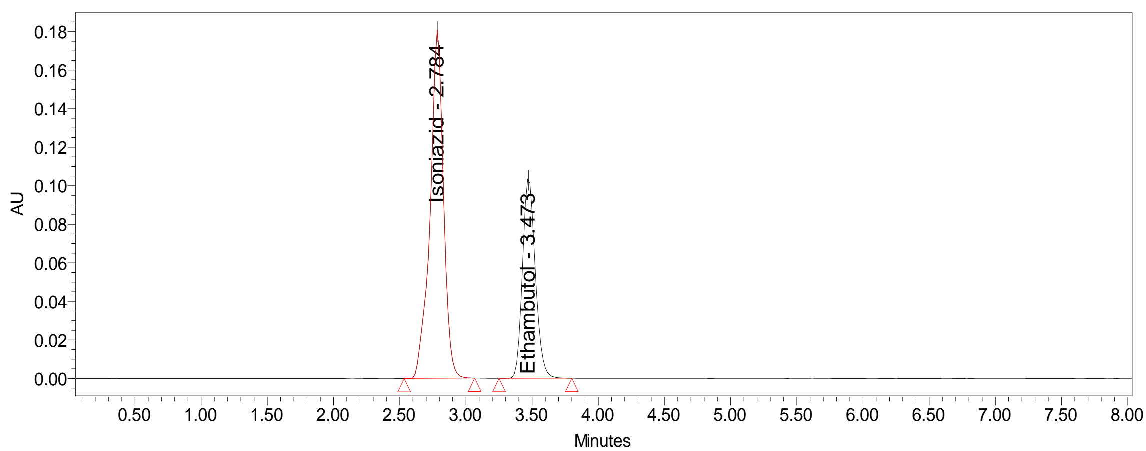


Fig25 Inference: Chromatogram for Intermediate Precision

5.3.4 ACCURACY (RECOVERY)

TABLE-5:

(i) Data of Accuracy for Isoniazid

S.No	Amount added(ppm)	Amount found(ppm)	% Recovery	Statistical Analysis of % Recovery	
50% Injection 1	20	20.14	100.25	**MEAN**	99.88
50% Injection 2	20	19.87	99.80		
50% Injection 3	20	20.03	100.05	**%RSD**	0.67
100 % Injection 1	40	39.89	99.79	**MEAN**	99.81
100 % Injection 2	40	40.16	100.57		

100% Injection 3	40	40.19	100.62	**%RSD**	0.399
150% Injection 1	60	60.04	100.40	**MEAN**	99.19
150% Injection 2	60	59.47	99.73		
150% Injection 3	60	59.69	99.87	**%RSD**	0.72

(ii) Data of Accuracy for Ethambutol

Concentration % of spiked level	Amount added (ppm)	Amount found (ppm)	% Recovery	Statistical Analysis of % Recovery	
50% Injection 1	20	19.86	99.30	**MEAN**	99.46
50% Injection 2	20	19.98	99.90		
50% Injection 3	20	19.84	99.20	**%RSD**	0.38
100 % Injection 1	40	39.54	98.85	**MEAN**	99.76
100 % Injection 2	40	39.82	99.55		
100%	40	39.96	99.9	**%RSD**	0.189

Injection 3					
150% Injection 1	60	59.92	99.86	**MEAN**	100.0067
150% Injection 2	60	60.08	100.13		
150% Injection 3	60	60.02	100.03	**%RSD**	0.136

Fig26 -27Chromatograms for accuracy (50%)

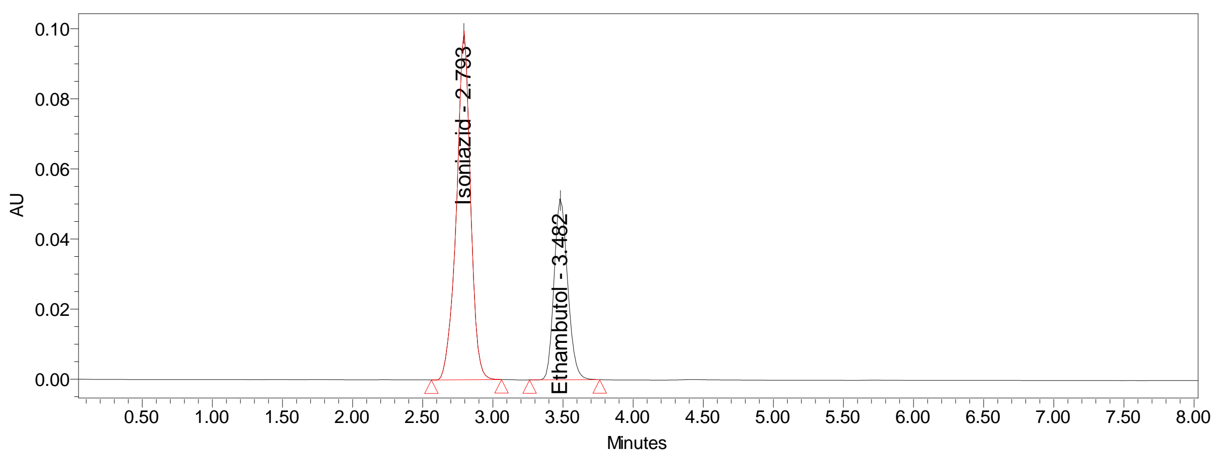


Fig26 Inference: Chromatogram for standard 1

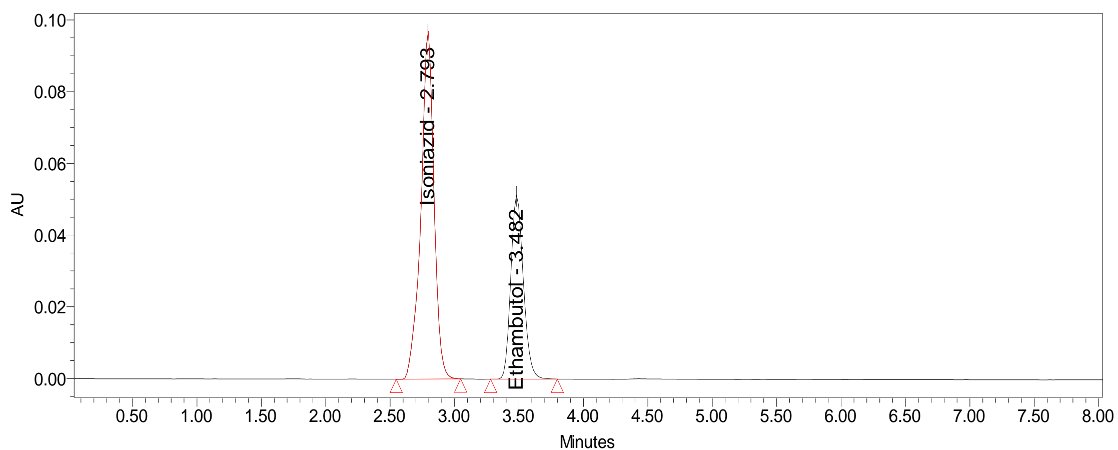


Fig27 Inference: Chromatograms for accuracy (50%)

Fig 28-29: Chromatograms for accuracy (100%)

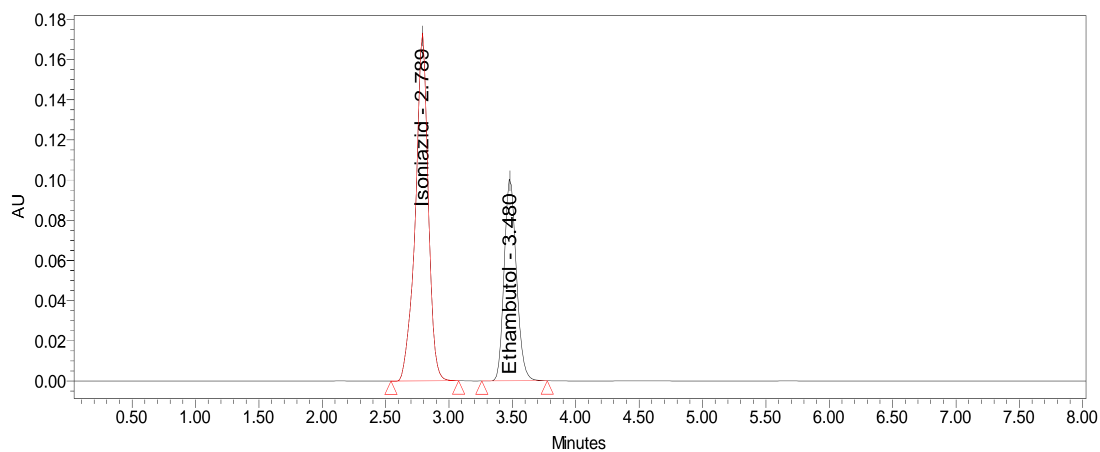


Fig28 Interference: chromatogram for standard 1

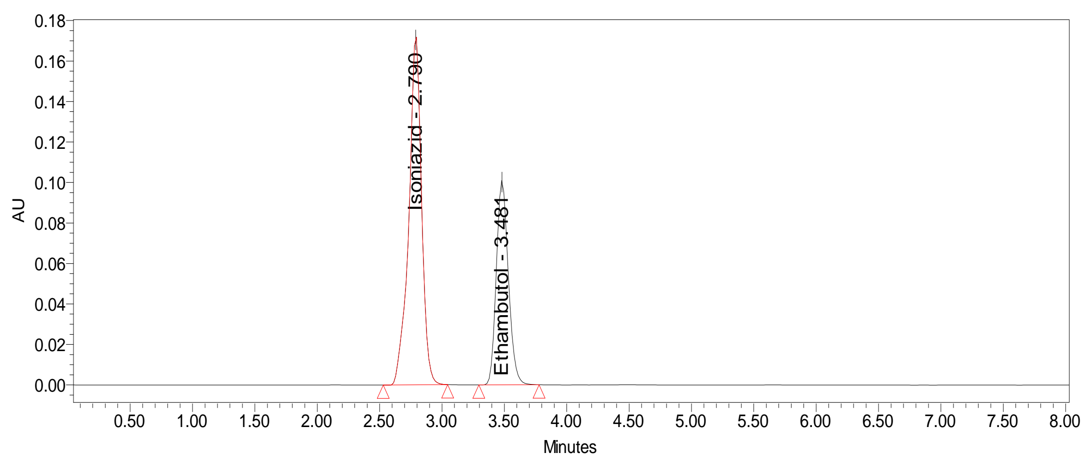


Fig29 Inference: Chromatogram for standard 2

Fig 30-31: chromatograms For Accuracy (150%)

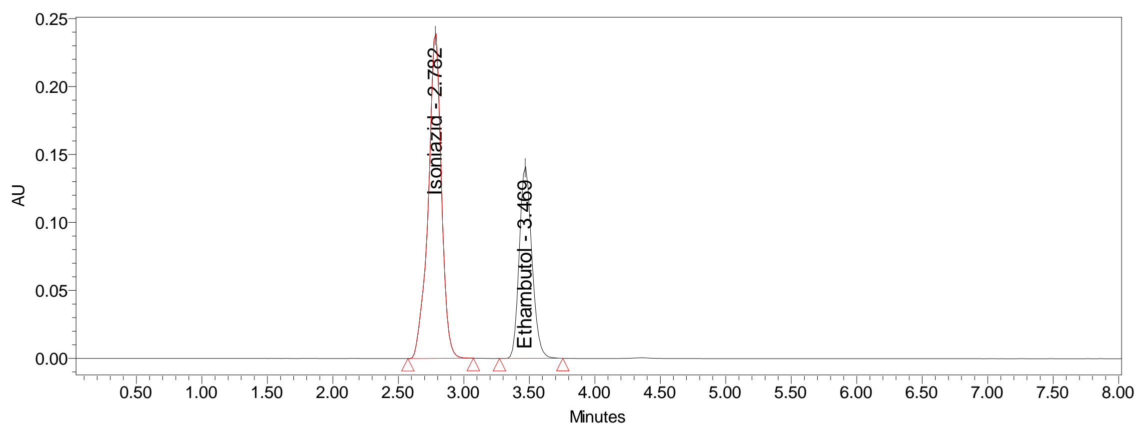


Fig30 Inference: Chromatogram for standard 1

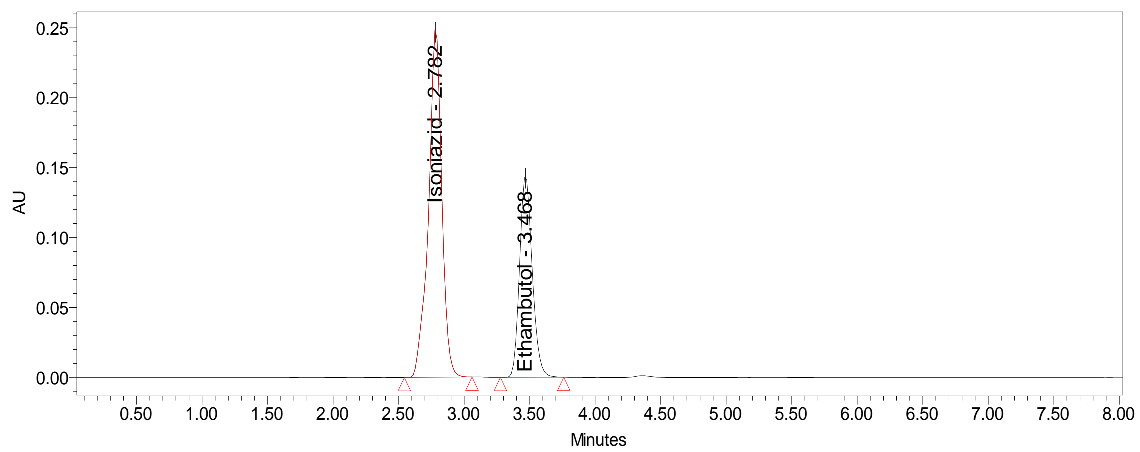


Fig31 Inference: Chromatogram for standard 2

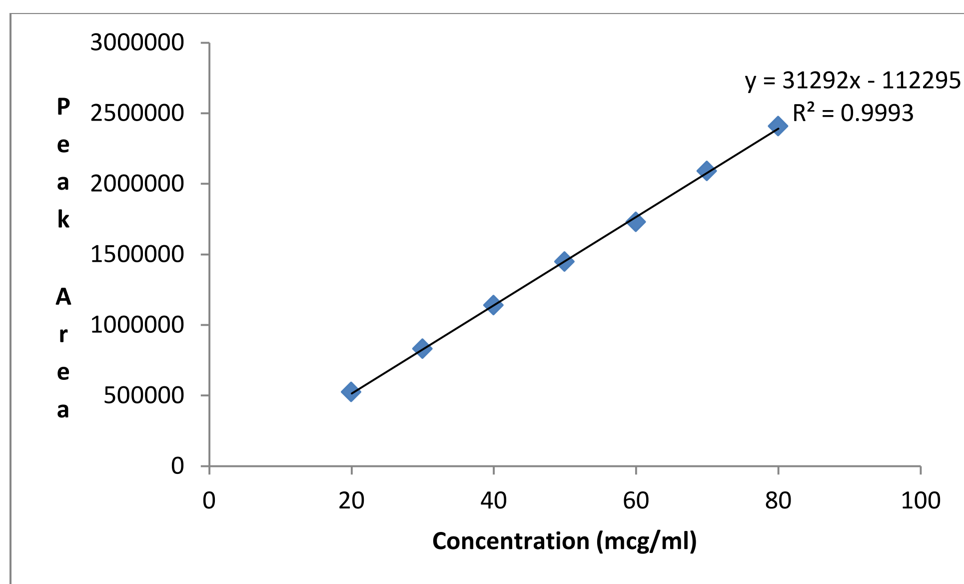
5.3.5 LINEARITY:

TABLE 6:

(i) Data of Linearity (Isoniazid)

S.No	Concentration (µg/ml)	Peak Area
1	20	523467
2	30	829544
3	40	1139272
4	50	1448018
5	60	1728926
6	70	2089505
7	80	2407574

Fig: 41(a) Linearity Plot (Concentration Vs Response) of **Isoniazid**



(ii) Data of Linearity (Ethambutol)

S.No	Concentration (µg/ml)	Peak Area
1	20	412977
2	30	605369
3	40	807564
4	50	1007428
5	60	1210925
6	70	1409560
7	80	1627087

Fig: 41(b) Linearity Plot (Concentration Vs Response) **of Ethambutol**

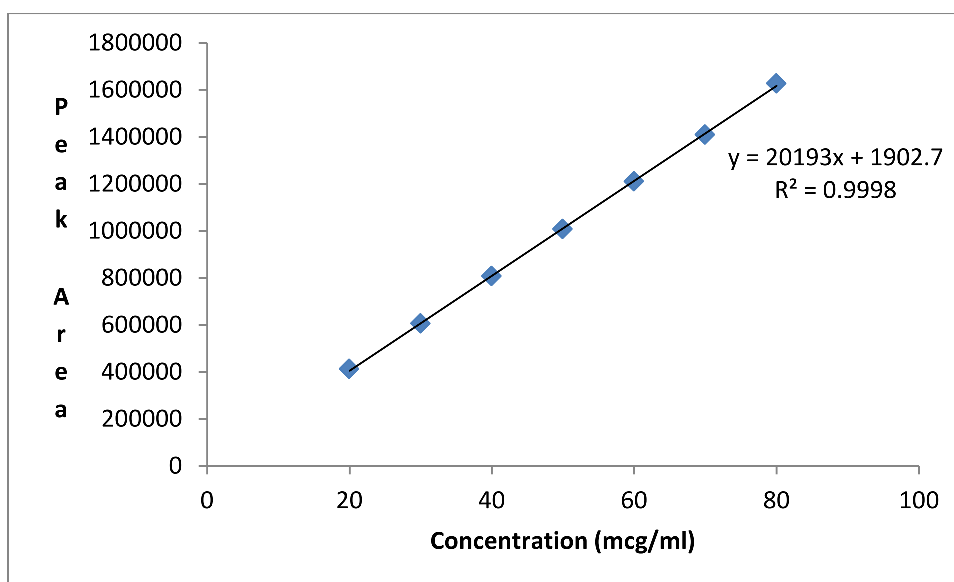


Fig: 32-38 Chromatograms for 20 ppm:

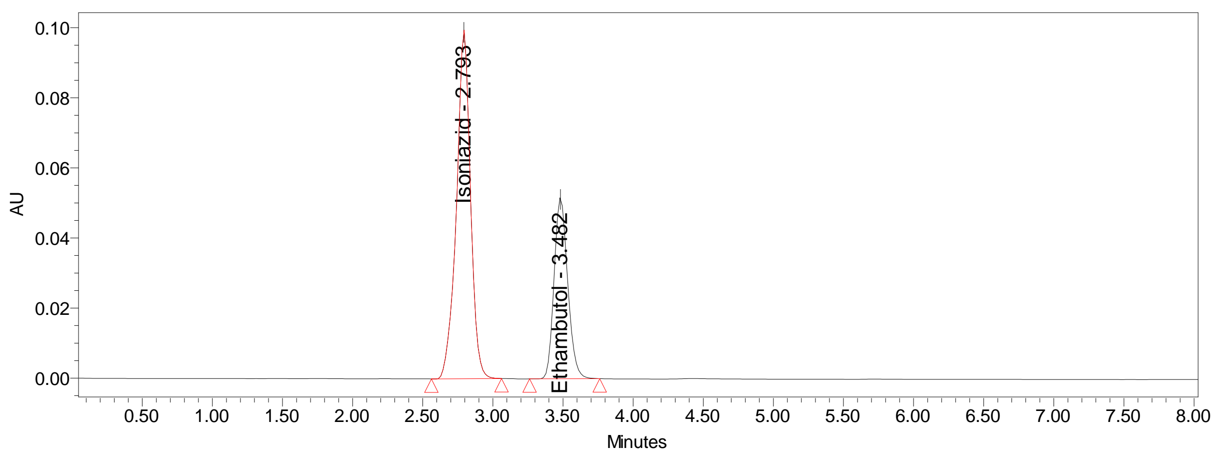


Fig32 Inference: Chromatogram for 20 ppm standard 1

chromatograms for30ppm, 40 ppm

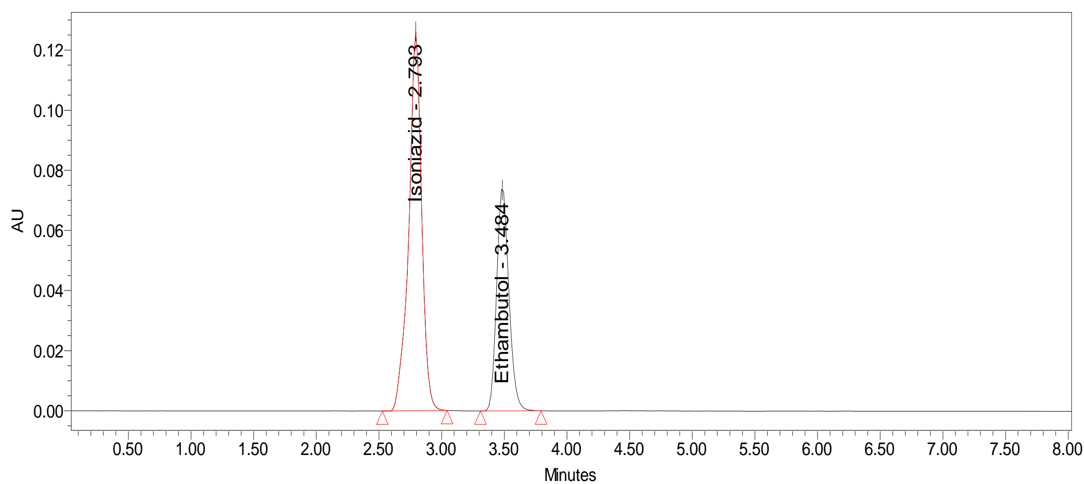


Fig33 Inference: Chromatogram for 30 ppm standard 1

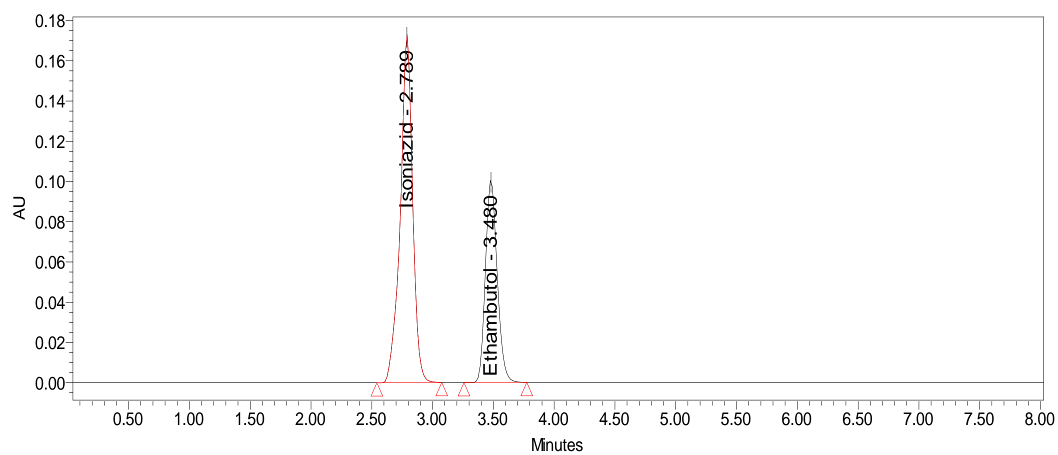


Fig34 Inference: Chromatogram for 40 ppm standard 1

Chromatograms for 50 ppm, 60 ppm

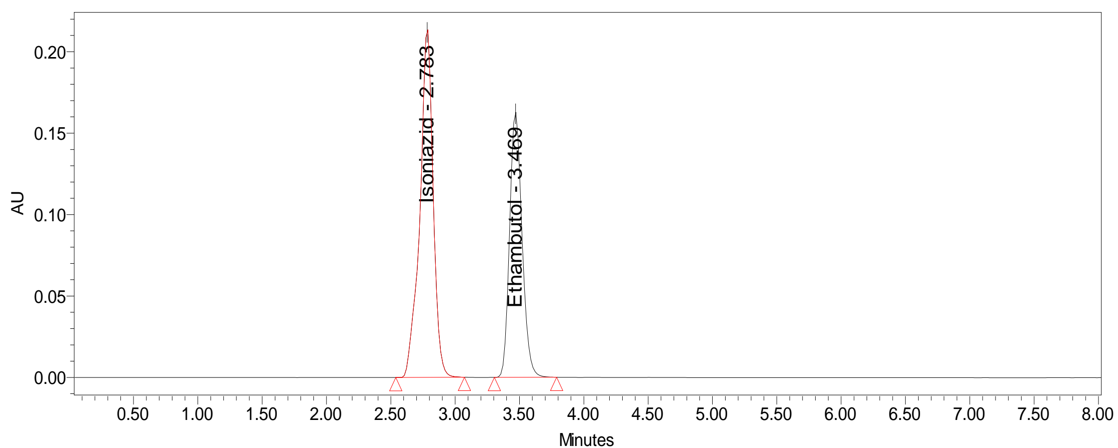


Fig35 Inference: Chromatogram for 50 ppm standard 1

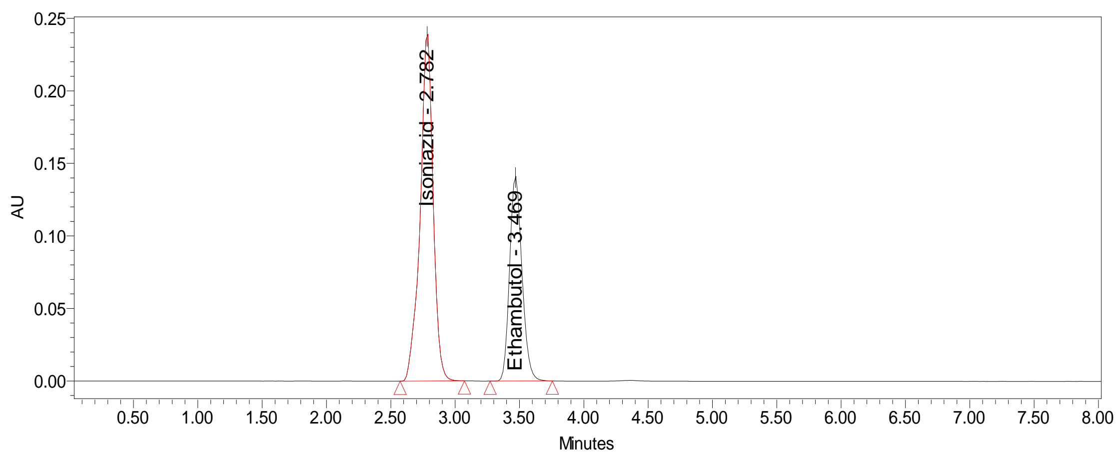


Fig36 Inference: Chromatogram for 60 ppm standard 1

Chromatograms for 70 ppm, 80 ppm

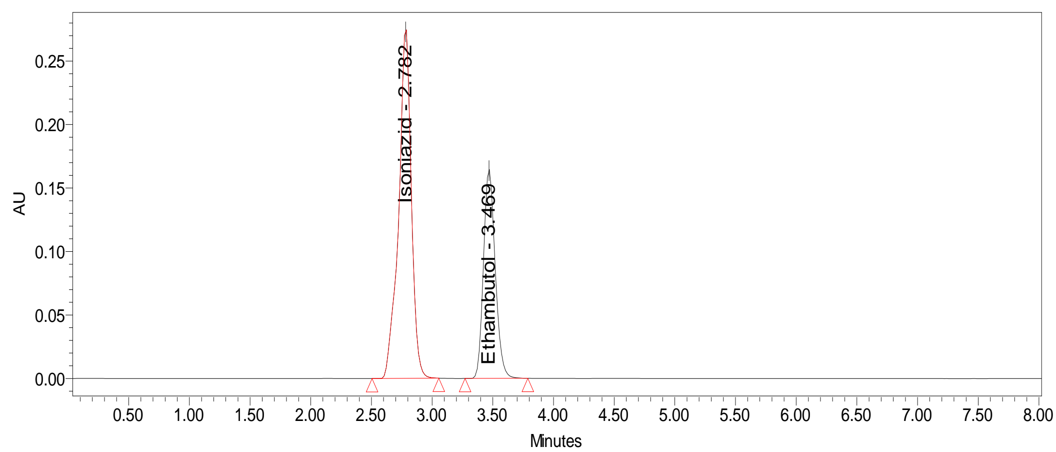


Fig37 Inference: Chromatogram for 70 ppm standard 1

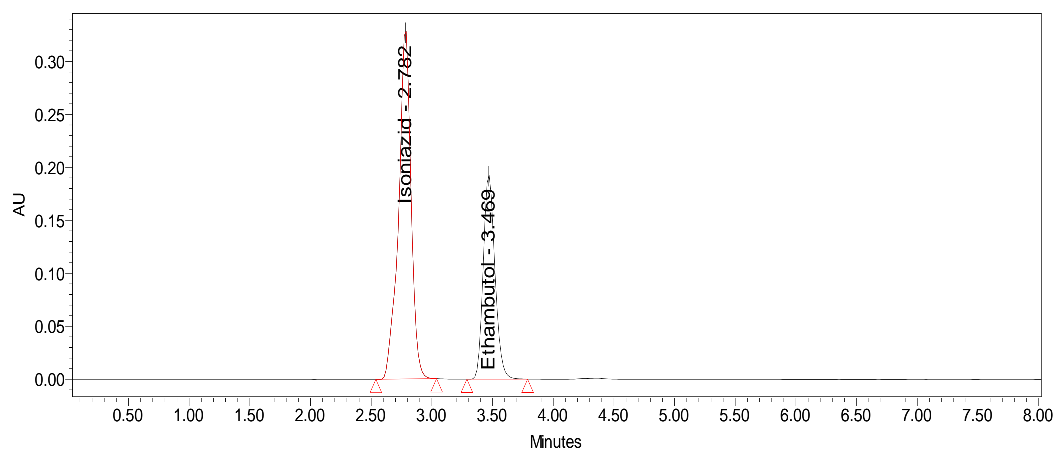


Fig38 Inference: Chromatogram for 80 ppm standard 1

5.3.6 Ruggedness:

a) System to System variability:

For system 1 Refer: Table3

TABLE: 7

(i) Data of system to system variability (Isoniazid)

System-2

S.NO:	Peak area	Assay % of Isoniazid
1	1155923	100.65
2	1153996	100.38
3	1143793	99.88
4	1158183	99.14
5	1148490	100.63
6	1143093	99.47
Mean	1149246	100.78
%RSD	0.540725	0.38252

(ii) Data of system to system variability (Ethambutol)

System-2

S.NO:	Peak area	Assay % of Ethambutol
1	819390	100.84
2	786331	99.69
3	818383	99.05
4	875296	101.06
5	806232	99.96
6	808369	101.31
Mean	802746.8	100.15
%RSD	0.413454	0.752768

39-44 Chromatograms of system to system variability

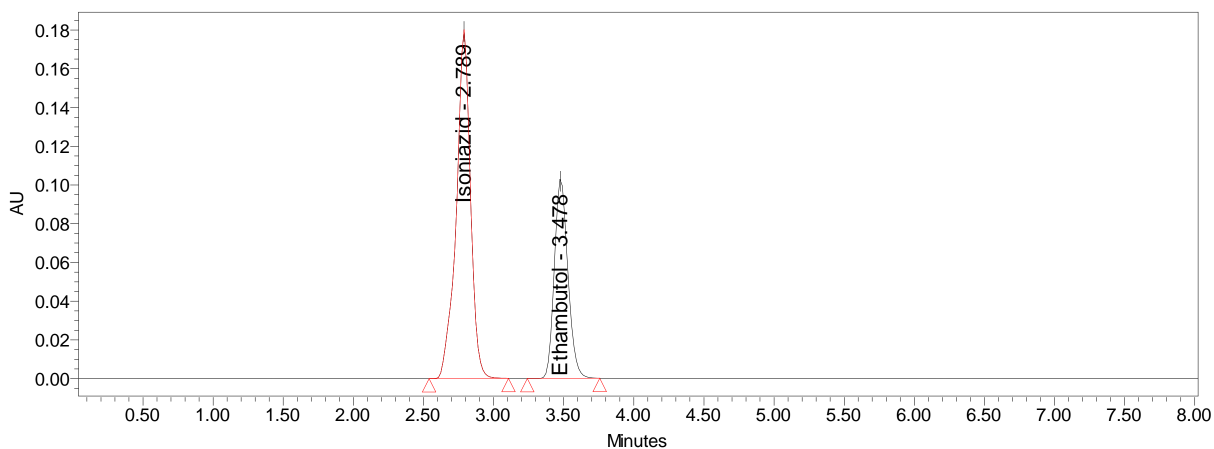


Fig 39 Inference: Chromatogram of system to system variability std- 1

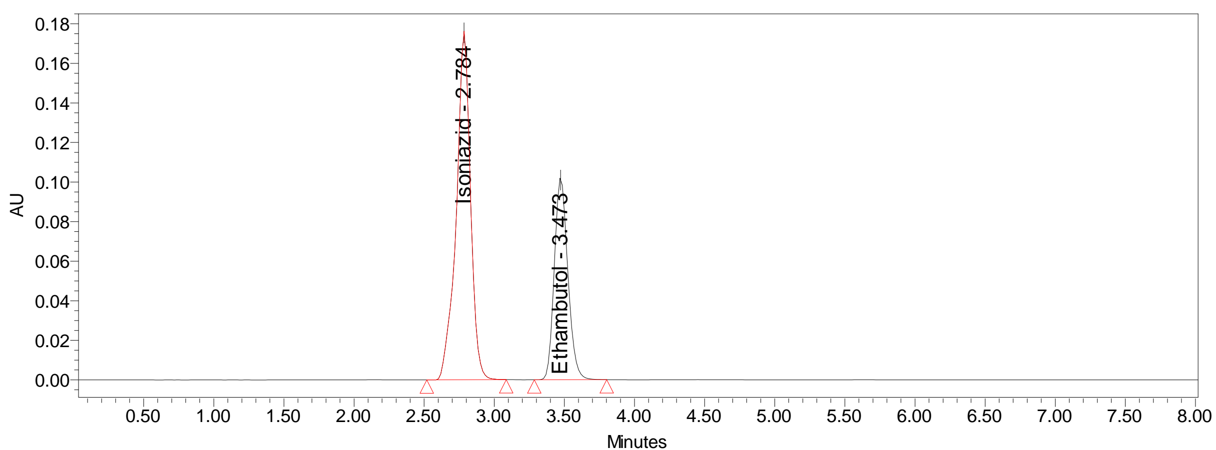


Fig 40 Inference: Chromatogram of system to system variability std- 2

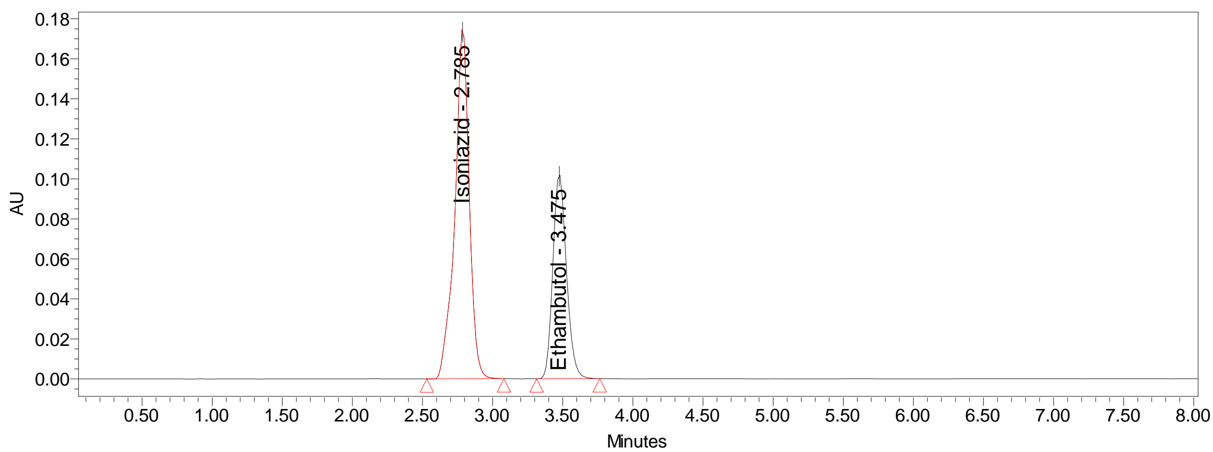


Fig 41 Inference: Chromatogram of system to system variability std- 3

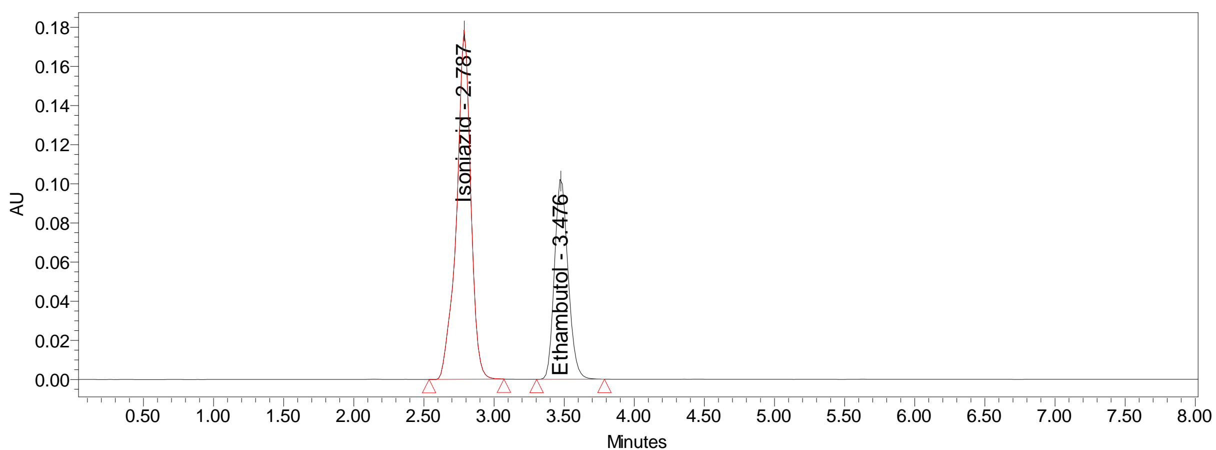


Fig 42 Inference: Chromatogram of system to system variability std- 4

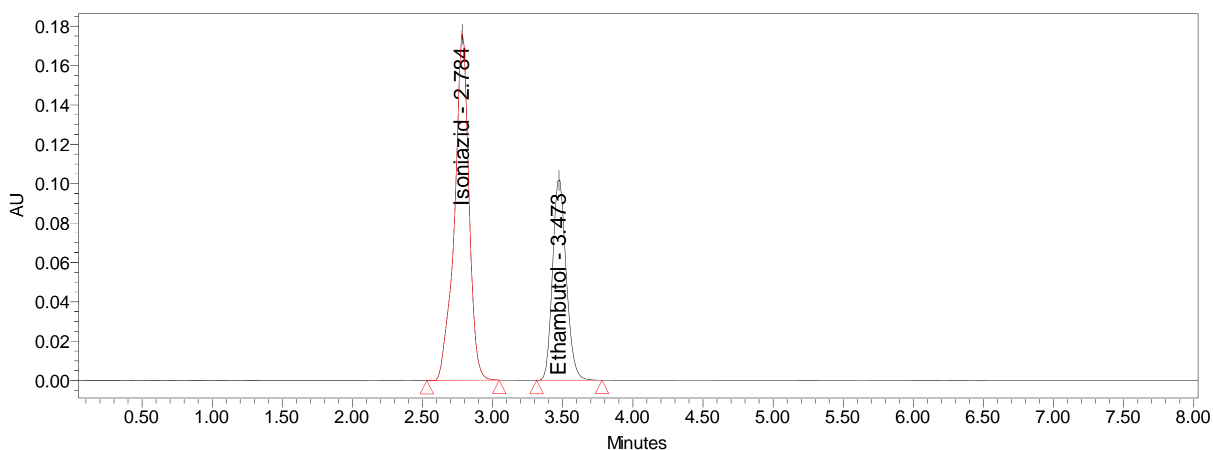


Fig 43 Inference: Chromatogram of system to system variability std- 5

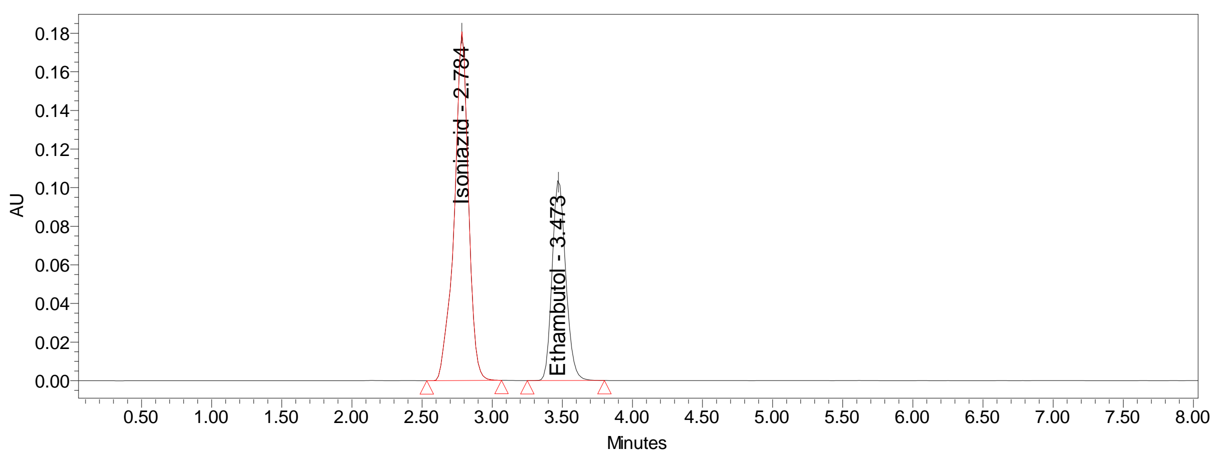


Fig 44 Inference: Chromatogram of system to system variability std- 6

5.3.7 Robustness:

TABLE: 10

(i) Data for Effect of variation in flow rate (Isoniazid):

Flow 0.8 ml	Std Area	Tailing factor	Flow 1.0 ml	Std Area	Tailing factor	Flow 1.2 ml	Std Area	Tailing factor
	1139272	1.238915		1146923	1.251658		1152293	1.262464
	1140892	1.230637		1143596	1.245435		1146923	1.251658
	1136301	1.240858		1158293	1.262464		1147283	1.237018
	1141067	1.238995		1147283	1.237018		1152490	1.239010
	1136024	1.241073		1152490	1.239010		1139272	1.238915
Avg	1138711	1.23649	**Avg**	1149717	1.24711	**Avg**	1148852	1.245813
SD	2431.578	0.005254	**SD**	5754.015	0.01032	**SD**	7076.84	0.010984
%RSD	0.213538	0.424907	**%RSD**	0.50047	0.00828	**%RSD**	0.61599	0.0088171

TABLE: 10

(ii) Data for Effect of variation in flow rate (Ethambutol)

Flow 0.8 ml	**Std Area**	**Tailing factor**	**Flow 1.0 ml**	**Std Area**	**Tailing factor**	**Flow 1.2 ml**	**Std Area**	**Tailing factor**
	797564	1.099100		801690	1.122813		805783	1.121321
	795138	1.103929		797631	1.112181		801690	1.122813
	795685	1.111477		805783	1.121321		801496	1.124805
	800569	1.117660		801496	1.124805		806432	1.123373
	797049	1.119004		806432	1.123373		797564	1.099100
Avg	797201	1.110234	**Avg**	802606.4	1.120899	**Avg**	801593	1.118282
SD	2124.413	0.008622	**SD**	3590.034	0.00503	**SD**	3613.298	0.047969
%RSD	0.500472	0.77655	**%RSD**	0.447297	0.004488	**%RSD**	0.450203	0.965376

Fig: 45-50 Chromatograms of robustness

a) Effect of variation of flow rate(for 0.8 ml/min flow)

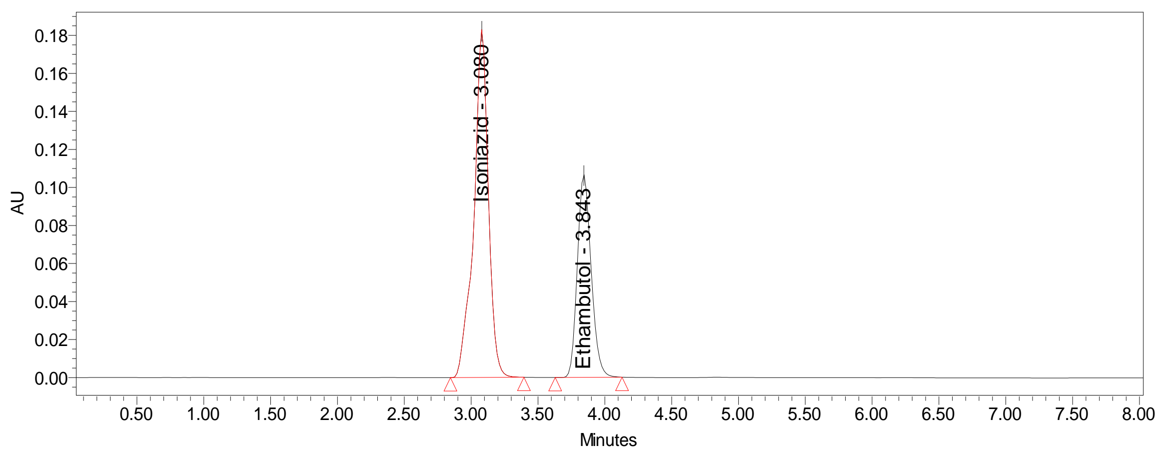


Fig 45 Inference: Chromatogram for robustness standard - 1

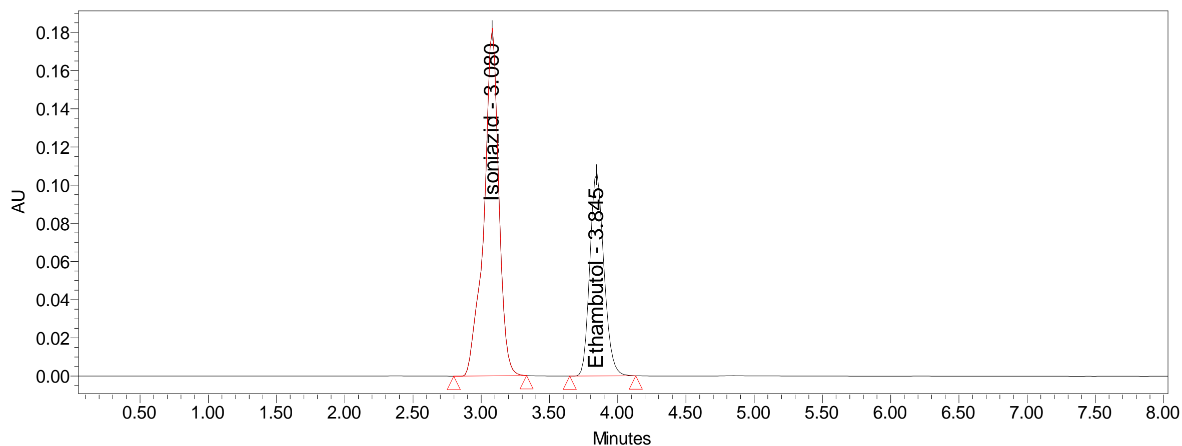


Fig 46 Inference: Chromatogram for robustness standard - 2

Fig47-48: chromatograms for 1ml/min

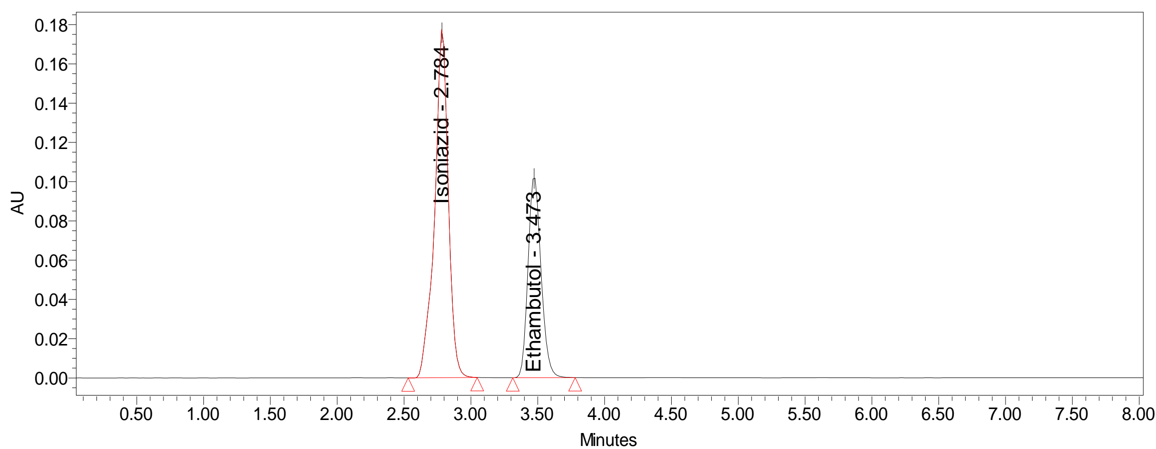


Fig 47 Inference: Chromatogram for robustness standard – 1

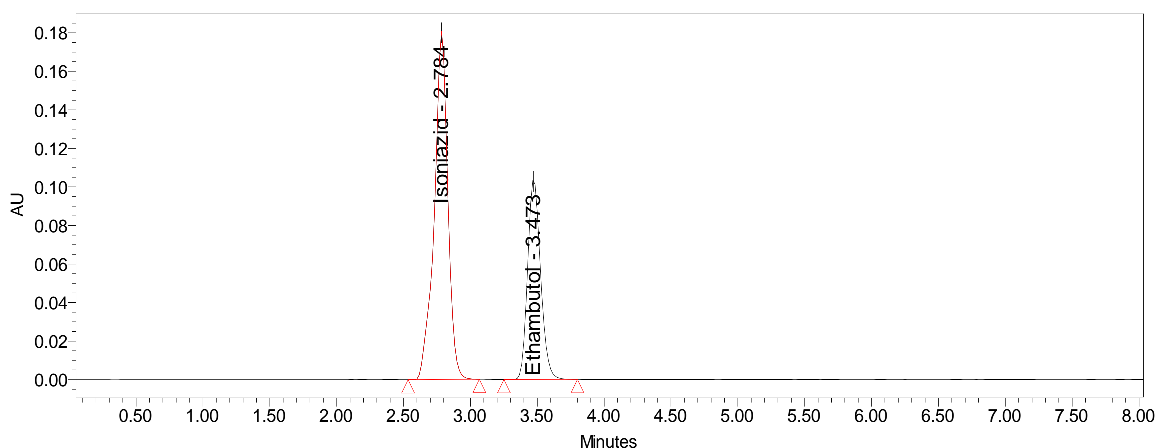


Fig 48 Inference: Chromatogram for robustness standard – 2

Fig49-50: Chromatograms for 1.2ml/min

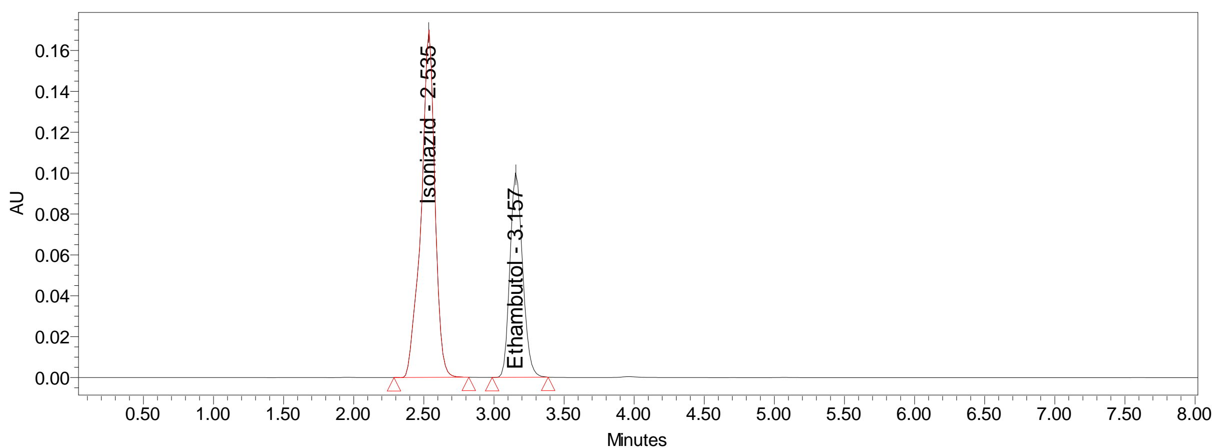


Fig 49 Inference: Chromatogram for robustness standard – 1

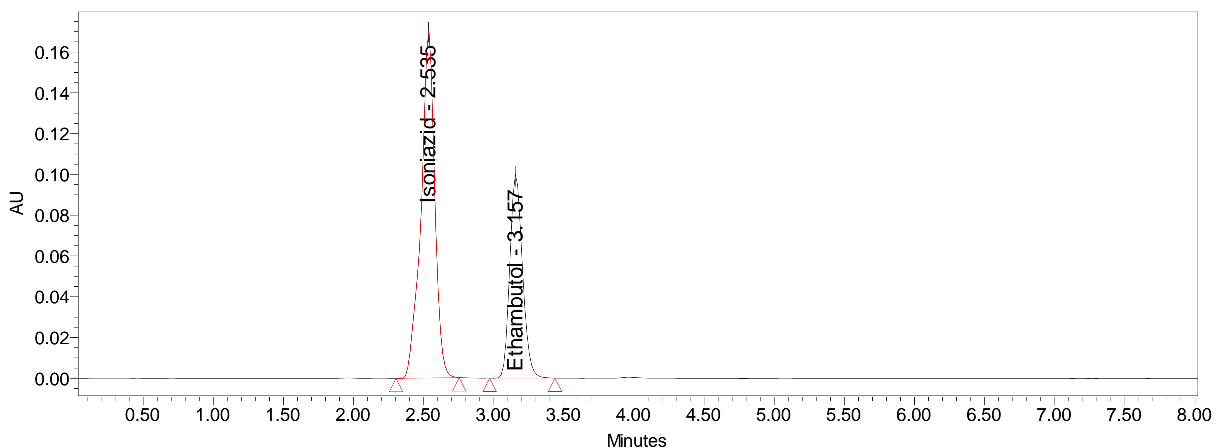


Fig 50 Inference: Chromatogram for robustness standard – 2

5.3.8 LIMIT OF DETECTION AND LIMIT OF QUANTITATION (LOD and LOQ):

Isoniazid:

From the linearity plot the LOD and LOQ are calculated:

$$LOD = \frac{3.3\,\sigma}{S}$$

$$= \frac{3.3 \times 2431.578}{31282} = 0.25$$

$$LOQ = \frac{10\,\sigma}{S}$$

$$= \frac{10 \times 2431.578}{31282} = 0.77$$

Ethambutol ;

$$LOD = \frac{3.3\,\sigma}{S}$$

$$= \frac{3.3 \times 2124.4}{20193} = 0.34$$

$$LOQ = \frac{10\,\sigma}{S}$$

$$= \frac{10 \times 2124.413}{20193} = 1.05$$

Fig 51-55 Chromatograms of LOD & LOQ:

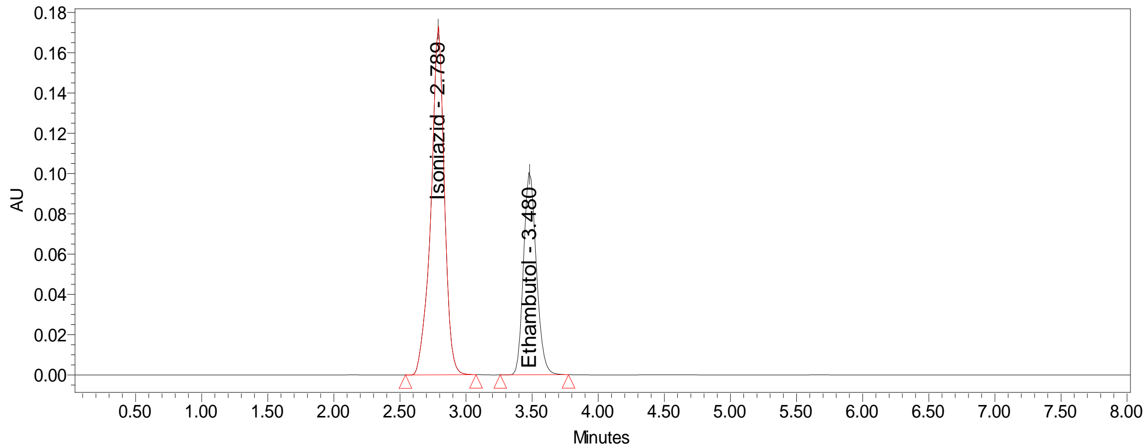


Fig 51 Interference: chromatogram for LOD and LOQ;

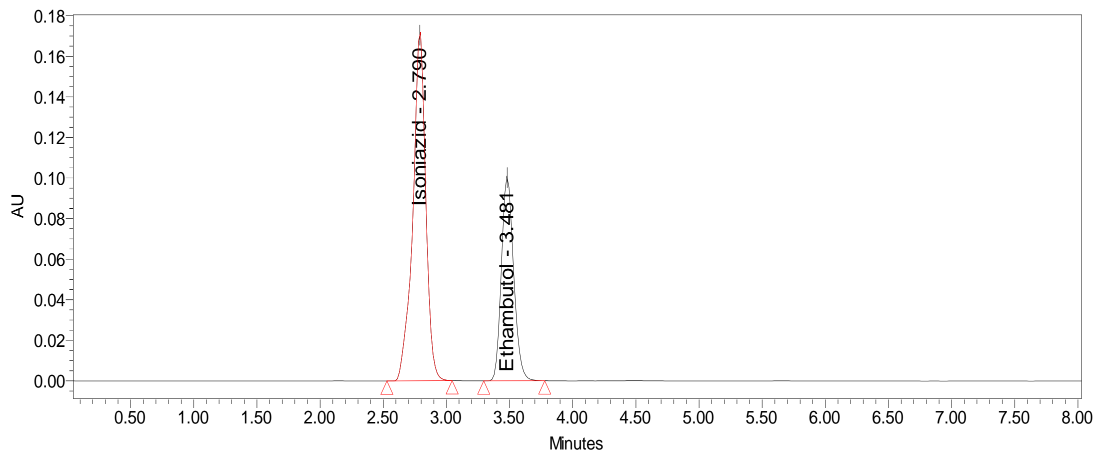


Fig 52 Interference: chromatogram for LOD and LOQ

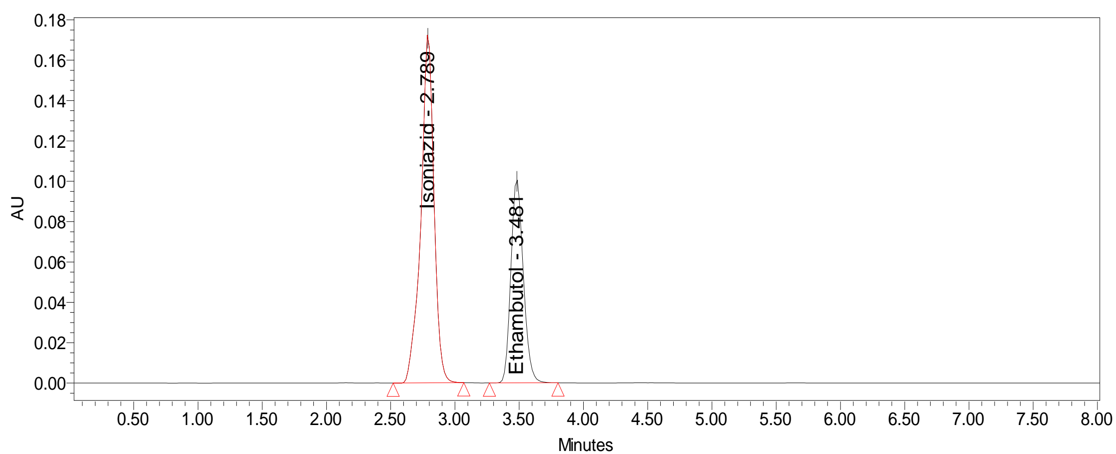


Fig 53 Interference: chromatogram for LOD and LOQ

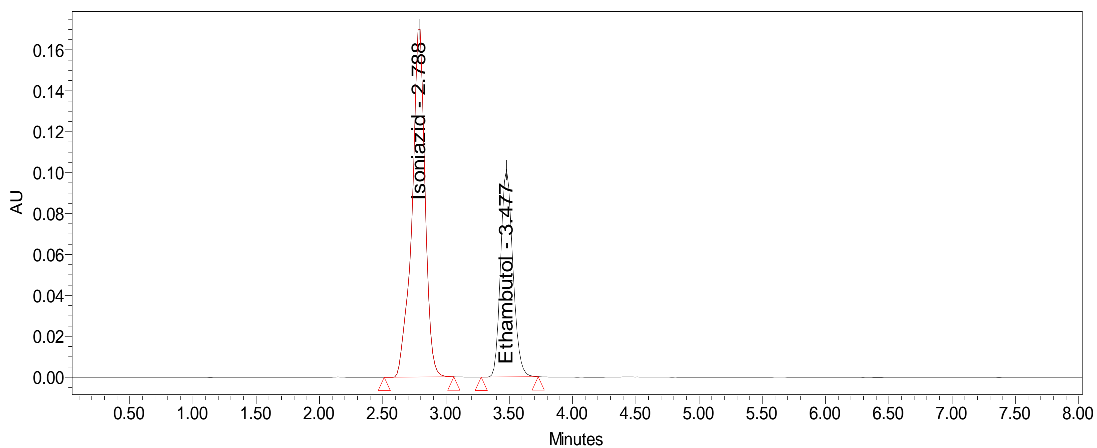


Fig 54 Interference: chromatogram for LOD and LOQ

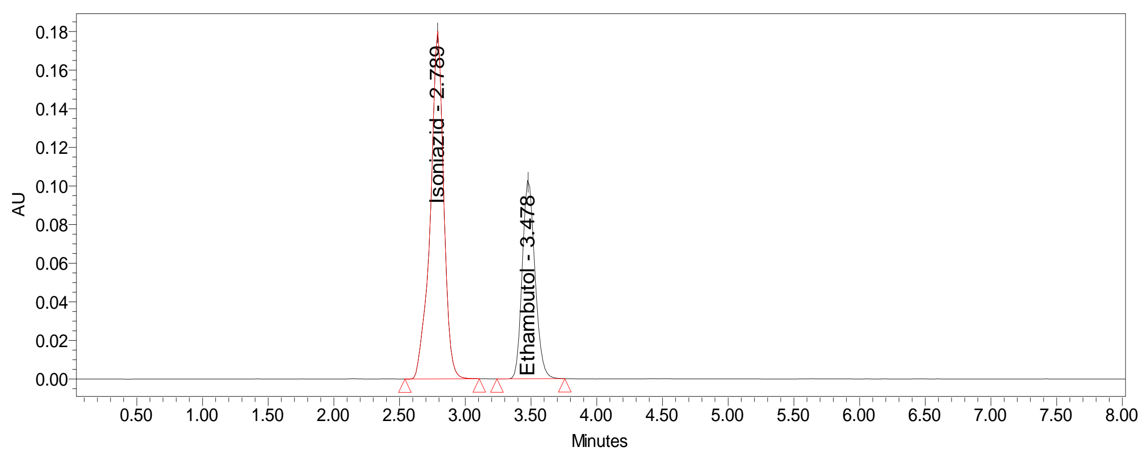


Fig 55 Interference: chromatogram for LOD and LOQ

6. SUMMARY AND CONCLUSION

The analytical method was developed by studying different parameters. First of all, maximum absorbance was found to be at 280nm Isoniazid for and 270nm for Ethambutol. Common wavelength will be 254nm and the peaks purity was excellent. Injection volume was selected to be 20µl which gave a good peak area. The column used for study was Inertsil C_{18}, ODS chosen good peak shape. Ambient temperature was found to be suitable for the nature of drug solution. The flow rate was fixed at 1.0ml/min because of good peak area, satisfactory retention time and good resolution. Different ratios of mobile phase were studied, mobile phase with ratio of 55:45 Methanol: Buffer was fixed due to good symmetrical peaks and for good resolution. So this mobile phase was used for the proposed study.

The present recovery was found to be 98.0-101.50 was linear and precise over the same range. Both system and method precision was found to be accurate and well within range. Detection limit was found to be 2.789 Isoniazid and 3.480 for Ethambutol. Linearity study was, correlation coefficient and curve fitting was found to be. The analytical method was found linearity over the ssrange of 20-80ppm of the target concentration for both the drugs. The analytical passed both robustness and ruggedness tests. On both cases, relative standard deviation was well satisfactory.

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