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A New RP-HPLC Method for the Simulatenous Estimation of Rabeprazole &Itopride in Bulk & Capsule Dosage Form

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Abstract

Reversed-phase high-performance liquid chromatography (RP-HPLC) involves the separation of molecules on the basis of hydrophobicity. The separation depends on the hydrophobic binding of the solute molecule from the mobile phase to the immobilized hydrophobic ligands attached to the stationary phase, i.e., the sorbent.Rabeprazole is an antiulcer drug in the class of proton pump inhibitors. It is a prodrug – in the acid environment of the parietal cells it turns into active sulphenamide form. Rabeprazole inhibits the H+, K+ATPase of the coating gastric cells and dose-dependent oppresses basal and stimulated gastric acid secretion. Rabeprazole belongs to a class of antisecretory compounds (substituted benzimidazole proton-pump inhibitors) that do not exhibit anticholinergic or histamine H2-receptor antagonist properties, but suppress gastric acid secretion by inhibiting the gastric H^+/K^+ATPase (hydrogen-potassium adenosine triphosphatase) at the secretory surface of the gastric parietal cell. Because this enzyme is regarded as the acid (proton) pump within the parietal cell, rabeprazole has been characterized as a gastric proton-pump inhibitor. Rabeprazole blocks the final step of gastric acid secretion. In gastric parietal cells, rabeprazole is protonated, accumulates, and is transformed to an active sulfenamide. When studied in vitro, rabeprazole is chemically activated at pH 1.2 with a half-life of 78 seconds. Itopride increases acetylcholine concentrations by inhibiting dopamine D2 receptors and acetylcholinesterase. Higher acetylcholine increases GI peristalsis, increases the lower esophageal sphincter pressure, stimulates gastric motility, accelerates gastric emptying, and improves gastro-duodenal coordination. Itopride given as a single dose study found that it also raises levels of motilin, somatostatin and lowers levels of cholecystokinin, as well as adrenocorticotropic hormone.

Keywords:RP-HPLC 1, Enzyme Inhibitors,Anti-Ulcer Agents2, Cholinesterase Inhibitor3

1. Introduction

Analytical chemistry is a scientific discipline used to study the chemical composition, structure and behaviour of matter. The purposes of chemical analysis are together and interpret chemical information

that will be of value to society in a wide range of contexts. Quality control in manufacturing industries, the monitoring of clinical and environmental samples, the assaying of geological specimens, and the support of fundamental and applied research are the principal applications. Analytical chemistry involves the application of a range of techniques and methodologies to obtain and assess qualitative, quantitative and structural information on the nature of matter.

- **Qualitative analysis** is the identification of elements, species and/or compounds present in sample.
- **Quantitative analysis** is the determination of the absolute or relative amounts of elements, species or compounds present in sample.

Structural analysis is the determination of the spatial arrangement of atoms in an element or molecule or the identification of characteristic groups of atoms (functional groups). An element, species or compound that is the subject of analysis is known as analyte. The remainder of the material or sample of which the analyte(s) form(s) a part is known as the matrix.

The gathering and interpretation of qualitative, quantitative and structural information is essential to many aspects of human endeavour, both terrestrial and extra-terrestrials. The maintenance of an improvement in the quality of life throughout the world and the management of resources heavily on the information provided by chemical analysis. Manufacturing industries use analytical data to monitor the quality of raw materials, intermediates and finished products. Progress and research in many areas is dependent on establishing the chemical composition of man-made or natural materials, and the monitoring of toxic substances in the environment is of ever increasing importance. Studies of biological and other complex systems are supported by the collection of large amounts of analytical data. Analytical data are required in a wide range of disciplines and situations that include not just chemistry and most other sciences, from biology to zoology, butte arts, such as painting and sculpture, and archaeology. Space exploration and clinical diagnosis are two quite desperate areas in which analytical data is vital. Important areas of application include the following.

Quality control (**QC**) in many manufacturing industries, the chemical composition of raw materials, intermediates and finished products needs to be monitored to ensure satisfactory quality and consistency. Virtually all consumer products from automobiles to clothing, pharmaceuticals and foodstuffs, electrical goods, sports equipment and horticultural products rely, in part, on chemical analysis. The food, pharmaceutical and water industries in particular have stringent requirements backed by legislation for major components and permitted levels of impurities or contaminants. The electronic industry needs analyses at ultra-trace levels (parts per billion) in relation to the manufacture of semi-conductor materials. Automated, computer-controlled procedures for process-stream analysis are employed in some industries.

Monitoring and control of pollutants The presence of toxic heavy metals (e.g., lead, cadmium and mercury), organic chemicals (e.g., polychlorinated biphenyls and detergents) and vehicle exhaust gases (oxides of carbon, nitrogen and sulphur, and hydrocarbons) in the environment are health hazards that need to be monitored by sensitive and accurate methods of analysis, and remedial action taken. Major sources of pollution are gaseous, solid and liquid wastes that are discharged or dumped from industrial sites, and vehicle exhaust gases.

Clinical and biological studies The levels of important nutrients, including trace metals (e.g., sodium, potassium, calcium and zinc), naturally produced chemicals, such as cholesterol, sugars and urea, and administered drugs in the body fluids of patients undergoing hospital treatment require monitoring.

Speed of analysis is often a crucial factor and automated procedures have been designed for such analyses.

Geological assays The commercial value of ores and minerals are determined by the levels of particular metals, which must be accurately established. Highly accurate and reliable analytical procedures must be used for this purpose, and referee laboratories are sometimes employed where disputes arise.

Fundamental and applied research The chemical composition and structure of materials used in or developed during research programs in numerous disciplines can be of significance. Where new drugs or materials with potential commercial value are synthesized, a complete chemical characterization maybe required involving considerable analytical work. Combinatorial chemistry is an approach used in pharmaceutical research that generates very large numbers of new compounds requiring confirmation of identity and structure.

Analytical techniques There are numerous chemical or physico-chemical processes that can be used to provide analytical information. The processes are related to a wide range of atomic and molecular properties and phenomena that enable elements and compounds to be detected and/or quantitatively measured under controlled conditions. The underlying processes define the various *analytical techniques*. The more important of these are listed in Table.No.1 together with their suitability for qualitative, quantitative or structural analysis and the levels of analyte(s) in a sample that can be measured. *Atomic, molecular spectrometry* and *chromatography*, which together comprise the largest and most widely used groups of techniques, can be further subdivided according to their physico-chemical basis. *Spectrometric techniques* may involve either the *emission or absorption* of *electromagnetic radiation* over a very wide range of energies, and can provide qualitative, quantitative and structural information for analytes from major components of a sample down to ultra-trace levels. The most important atomic and molecular spectrometric techniques and their principal applications are listed in Table.No.2.

Chromatographic techniques provide the means of separating the components of mixtures and simultaneous qualitative and quantitative analysis, as required. The linking of chromatographic and spectrometric techniques, called *hyphenation*, provides a powerful means of separating and identifying unknown compounds.

Analytical methods

Method development forms a significant part of the work of most analytical laboratories, and *method validation and* periodic revalidation is a necessity. Selection of the most appropriate analytical method should take into account the following factors:

- The purpose of the analysis, the required time scale and any cost constraints;
- The level of Analyte(s) expected and the detection limit required;
- The nature of the sample, the amount available and the necessary sample preparation procedure;
- The accuracy required for a quantitative analysis;
- The availability of reference materials, standards, chemicals and solvents, instrumentation and any special facilities;
- Possible interference with the detection or quantitative measurement of the analyte(s) and the possible need for sample clean-up to avoid matrix interference;
- The degree of selectivity available − methods may be selective for a small number of analytes or specific for only one.

- Quality control and safety factors.

AIM:

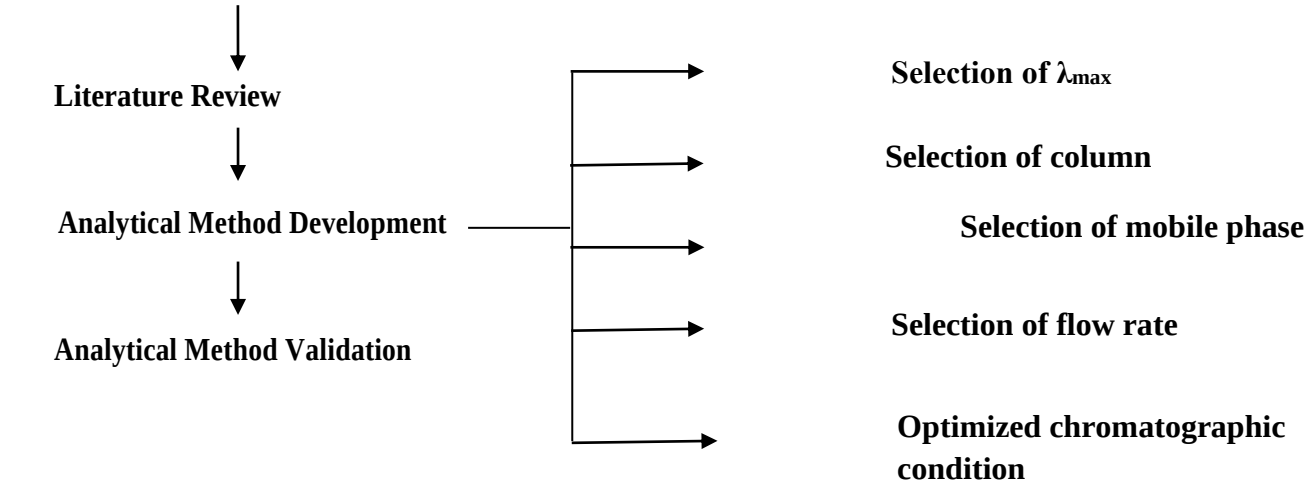
The literature review reveals t few HPLC methods for the estimation of Rabeprazole and Itopride alone and in combination with other drugs. Few methods are also reported for estimation of both drugs from formulation .we intend to develop a Stability indicating RP-HPLC method by simultaneous determination with simple, rapid, greater sensitivity and faster elution.

OBJECTIVE:

- Development of a HPLC method for analysis of both the drugs.
- Validation of the method using formulations.

PLAN OF WORK

- To develop a new analytical method for the simultaneous estimation of Rabeprazole and Itopride by RP-HPLC.
- The dissertation work has been carried out in the following steps



ANALYTICAL METHOD DEVELOPMENT

A. Selection of wavelength

A solution of 10 µg/ml of Rabeprazole and Itopride were prepared in milliQ water. The resulting solutions were scanned individually on HPLC PDA detector from 200 to 400 nm and also in UV-Visible spectrophotometer. The optimal response for three of them was obtained at 240 nm. Hence the complete method was processed at the wavelength of 240nm.

B. Selection of chromatographic condition

Proper selection of the method depends up on the nature of the sample (ionic/ ionisable/neutral molecule), its molecular weight and solubility. The drugs selected in the present study, were polar in nature. Thus reverse phase HPLC was selected for the initial separation because of its simplicity, suitability, ruggedness and its wider usage.

C. Initial separation condition

The mobile phase selected to elute the drug from the stationary phase was milliQ water and HPLC methanol, because of its favorable UV transmittance, low viscosity and low back pressure.

Preparation of standard solution : 10 mg of Rabeprazole and 10mg of Itopride were accurately weighed and transferred into a 10 ml clean dry volumetric flask, about 7 ml of diluent was added, sonicated to dissolve it completely and the volume was made up to the mark with the same solvent to give a concentration of 1000 µg/ml. (Stock solution) Further 0.3 and 1.8 ml were pipetted out fromthe above stock solution into a 10ml volumetric flask and diluted up to the mark with diluent to give a concentration of 30 µg/ml and 180µg/ml respectively.

Preparation of sample solution:

10 Tablets of contents were weighed and triturated in glass mortar. The quantity of powder equivalent to 10 mg of active ingredient present in Rabeprazole and Itopride was transferred into a 10 ml clean dry volumetric flask, 7 ml of diluent was added to it and was shaken by mechanical stirrer and sonicated for about 30 minutes by shaking at intervals of five minutes each and was diluted up to the mark with diluent to give a concentration of 1000 µg/ml and allowed to stand until the residue settles before taking an aliquot for further dilution (stock solution). 0.3 ml of upper clear solution was transferred to a 10 ml volumetric flask and diluted with diluent up to the mark to give the respective concentrations as par with standard solution.The solution was filtered through 0.45 µm filter before injecting into HPLC system.

Preparation of Placebo:

The amount of powdered inactive ingredient supposed to be present in 10 tablets were accurately weighed and transferred in to 10 ml volumetric flask, 7 ml of diluent was added and shaken by mechanical stirrer and sonicated for about 30 minutes by shaking at intervals of five minutes and was diluted up to the mark with diluent and allowed to stand until the residue settles before taking an aliquot for dilution. 0.1 ml of upper clear solution was transferred to a 10 ml volumetric flask and diluted with diluent up to the mark and the solution was filtered through 0.45 µm filter before injecting into HPLC system.

TRIALS

TRIAL 1:

Method development for the drugs was initiated based on the individual chemical characteristics' and their methods given in individual journals.

Mobile phase: Methanol: Phosphate buffer P^H3 (70:30)

Diluent: Methanol

Chromatographic conditions:

Flow rate **:**1ml/min

Column **:**Thermosil C_{18} (4.6 x 150mm, 5µm)

Detector wavelength **:**240 nm

Column oven **:** Ambient

Injection volume **:** 10µl

Observation:

There was no proper peak separation. The Chromatogram for trial 1 was shown in **Fig: 6.**

TRAIL 2:

Inorder to improve resolution and remove fronting of the peak and avoid unwanted peaks interfering, mobile phase was changed and again the same experiment was performed.

Mobile phase: Methanol: Sodium acetate P^H 4 (60:40)

Diluent: methanol

Chromatographic conditions:

Flow rate **: 0.8**ml per min

Column **:**Thermosil C_{18} (4.6 x 150mm, 5µm)

Detector wavelength **:**240 nm

Column oven **:** Ambient

Injection volume **:**10µl

Observation:

Peaks are separated but tailing has been observed. The Chromatogram for trial 2 was shown in **Fig: 7.**

TRIAL-3

In order to avoid poor response and tailing mobile phase was changed i.e organic phase was changed

Mobile phase: Methanol: Ammonium acetate P^H3 (70:30)

Diluent: methanol.

Chromatographic conditions:

Flow rate **: 1.0** ml per min

Column **:**Inertsil C_{18} (4.6 x 150mm, 5µm)

Detector wavelength **:**240 nm

Column oven **:**Ambient

Injection volume **:** 20µl.

Observation:

Peaks were eluted but with less resolution peaks were seen. . The chromatogram for trial 3 was shown in **Fig: 8.**

OPTIMIZED METHOD

Preparation of mobile phase:

Take 24 gm of Ammonium acetate into 1000ml volumetric flask dissolved inHplc grated water and adjust Phupto 3 with ortho phosphoric acid.From the above prepared buffer take 300 ml(30%)and 700ml Methanol(70%) HPLC were mixed and degassed in ultrasonic water bath for 5 minutes and was filtered through 0.45 µ filter under vacuum filtration.

Diluent Preparation: Mobile phase was used as Diluent.

Chromatographic conditions:

Flow rate **:**1.0 ml per min

Column **:**Agilent C18 (4.6 x 150mm, 5µm)

Detector wavelength **:** 256 nm

Column oven **:** Ambient

Injection volume **:** 10µl

Run time **:** 6 min

Test Procedure:

20 µl of the Standard, Sample and Blank preparations in duplicate were injected separately into HPLC system and the peak responses for Rabeprazole and Itopride were measured. The quantities from the peak area in mg of Rabeprazole and Itopride were calculated per tablet taken.

The developed RP-HPLCmethod for the simultaneous estimation of Rabeprazole and Itopride were carried out On Agilent C_{18} (4.6 x 150mm), 5µm column in gradient mode using mobile phase composition of Methanol: Phosphate buffer Ph 3 [60 : 40, v / v] with flow rate of 1.0ml / min at 256nm.

Observation: Resolution between two analytes were good. No peak asymmetry was observed. No other impurity interference was seen. All the results were found to be with in the acceptance criteria. Hence the method was considered to be optimized.

Calculation: The amount of drug present was calculated by using the following formula:

For Rabeprazole:

$$\text{Assay\%} = \frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \frac{\text{Avg. Wt}}{\text{Label Claim}} \times 100$$

Where:

AT = average area counts of sample preparation.

As= average area counts of standard preparation.

WS = Weight of working standard taken in mg.

P = Percentage purity of working standard

LC = label claim of Rabeprazole mg/ml.

For Itopride:

$$\text{Assay\%} = \frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \frac{\text{Avg. Wt}}{\text{Label Claim}} \times 100$$

Where:

AT = average area counts of sample preparation.

As= average area counts of standard preparation.

WS = Weight of working standard taken in mg.

P = Percentage purity of working standard

LC = label claim of Itopride mg/ml.

The Standard, Sample and Blank Chromatograms for optimized method were shown in **Fig: 9, 10&11**

METHOD VALIDATION

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

1. System suitability.
2. Linearity.

3. Precision.
4. Accuracy.
5. Specificity.
6. Robustness.
7. Ruggedness.
8. Limit of detection.
9. Limit of quantification.

1. SYSTEM SUITABILITY

Sample solution of Rabeprazole and Itopride were injected three times into HPLC system as per test procedure. The system suitability parameters were evaluated from standard Chromatograms obtained, by calculating the % RSD of retention times, tailing factor, theoretical plates and peak areas from three replicate injections.

Acceptance criteria

1. The % RSD for the retention times of principal peak from 3 replicate injections of each Standard solution should be not more than 2.0 %
2. The number of theoretical plates (N) for the Rabeprazole and Itopride peaks should be not less than 2000.
3. The Tailing factor (T) for the Rabeprazole and Itopride peaks should be not more than 2.0.

From the system suitability studies it was observed that all the parameters were within limit. Hence it was concluded that the Instrument, Reagents and Column were suitable to perform the Assay.

The results were expressed in **Table: 4** and Chromatogram was shown in **Fig: 13.**

2.LINEARITY

Preparation of sample stock solution:

About 10 mg of Rabeprazole and 60 mg of Itopride samples was weighed in to 10ml volumetric flask, it was dissolved with diluent and the volume was made up to the mark with same diluent (1000µg/ml of Rabeprazole and 6000µg/ml of Itopride).

Preparation of Level – I (10µg/ml of Rabeprazole &60µg/ml of Itopride)

0.1ml of stock solution had taken in 10ml of volumetric flask diluted up to the mark with diluent.

Preparation of Level–II (20 µg/ml of Rabeprazole &120 µg/ml of Itopride)

0.2ml of stock solution had taken in 10ml of volumetric flask diluted up to the mark with diluent.

Preparation of Level–III (30 µg/ml of Rabeprazole &180 µg/ml of Itopride)

0.3ml of stock solution had taken in 10ml of volumetric flask diluted up to the mark with diluent.

Preparation of Level–IV (40 µg/ml of Rabeprazole &240 µg/ml of Itopride)

0.4ml of stock solution had taken in 10ml of volumetric flask diluted up to the mark with diluent.

Preparation of Level–V (50 µg/ml of Rabeprazole &300 µg/ml of Itopride).

0.5ml of stock solution had taken in 10ml of volumetric flask diluted up to the mark with diluent.

10µl of each level were injected into the system and recorded the peak response.

The chromatograms were recorded as show in **Figure 6.3.4 (a) – 6.3.4 (e)** and results are shown in **Table- 7 and 8.**

Procedure:

Each level solution was injected into the chromatographic system and the peak area was measured. A graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) was plotted and the correlation coefficient was calculated.

The linearity of the method was demonstrated over the concentration range of 10-100µg / ml. Aliquots of five levels were prepared from sample solution and labeled as solution 1, 2, 3, 4 and 5 respectively. The solutions were injected in to HPLC system as per test procedure. The Chromatograms were given in **Fig: 14,15,16,17 and 18.**

A calibration curve was plotted for concentration v/s peak area and was given in the **Fig: 19, 20&21**. The results were discussed in **Table: 5, 6& 7.**

Acceptance criteria

1. Correlation Coefficient should be not less than 0.9990.
2. % RSD of peak areas for Solution 1, 2, 3, 4 and 5 should be not more than 2.0 %.

3.PRECISION

Preparation of stock solution:

10 mg of Rabeprazole and 10mg of Itopride were accurately weighed and transferred into a 10 ml clean dry volumetric flask, about 7 ml of diluent was added and sonicated to dissolve it completely. The volume was made up to the mark with the same solvent to give the concentration of 1000 µg/ml. (Stock solution) .Further 0.3 ml and 1.8 ml was pipette out from the above stock solutions into a 10ml volumetric flask and diluted up to the mark with diluent to give the concentration of 30 µg/ml and 180 µg/ml respectively.

Procedure:

The standard solution was injected for five times and the areas for all five injections were measured in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits.

The Chromatograms were presented as **Fig: 22.**

The results were given in **Table: 8.**

Acceptance criteria

1. % Relative standard deviation of peak areas and R_t should not be more than 2.0 %.

Intermediate Precision/Ruggedness

To evaluate the intermediate precision (also known as Ruggedness) of the methodPrecision was performed on different day by using different make column of sameDimensions.

Preparation of stock solution:

10 mg of Rabeprazole and 10mg of Itopride accurately weighed and transferred into a 10 ml clean dry volumetric flask, about 7 ml of diluent was added ,sonicated to dissolve it completely and the volume was made up to the mark with the same solvent to give the concentration of 1000 µg/ml. (Stock solution) Further 0.3ml and 1.8ml were pipette out fromthe above stock solution into a 10ml volumetric flask and diluted up to the mark with diluent to give the concentration of 30µg/ml and 180 µg/ml respectively.

Procedure:

The standard solution was injected for five times and the area was measured for all five injections in HPLC. The %RSD for the area and R_t of five replicate injections was found to be within the specified limits.

Two analysts as per test method conducted the study. For Analyst-1 the results are under precision (Repeatability) results and the results for Analyst-2 were discussed in **Table: 10.**Chromatograms were shown in **Fig: 23,24,25,26&27.**

Acceptance criteria

1. % Relative standard deviation of peak areas and R_t should not be more than 2.0 %

4.ACCURACY

Assay was performed in triplicate for various concentrations ofRabeprazole and Itopride equivalent to 50, 100, and 150 % of the standard amount was injected into the HPLC system as per the test procedure.

Preparation of Standard stock solution:

10 mg of Rabeprazole and 10mg of Itopride accurately weighed and transferred into a 10 ml clean dry volumetric flask, about 7 ml of diluent was added, sonicated to dissolve it completely and volume was made up to the mark with the same solventto give the concentration of 1000 µg/ml. (Stock solution)

Preparation Sample solutions:

Preparation of 50% solution (15 µg/ml of Rabeprazole and 90µg/ml ofItopride):

From the above stock solutions take 0.15 ml and 0.9 ml into 10 ml dry volumetric flask, make up to the mark with diluent**.**

Preparation of 100% solution (30 µg/ml of Rabeprazole and 180 µg/ml of Itopride):

From the above stock solutions take 0.3 ml and 1.8 ml into 10 ml dry volumetric flask, make up to the mark with diluent

Preparation of 150% solution (45 µg/ml of Rabeprazole and 270 µg/ml of Itopride):

From the above stock solutions take 0.45 ml and 2.7 ml into 10 ml dry volumetric flask, make up to the mark with diluent.

These solutions were filtered through 0.45µ membrane and then each concentration; three replicate injections were made under the optimized conditions

Procedure:

The standard solution, Accuracy -50%, Accuracy -100% and Accuracy -150% solutions were injected. The amount found and amount added for Rabeprazole and Itopride individual recovery and mean recovery values were calculated and the results were summarized in **Table:11,12&13 .**The chromatograms were represented in**Fig: 28,29,30 and 31.**

Acceptance criteria

The mean % recovery of the Rabeprazole and Itopride each spike level should be not less than 98.0 % and not more than 102.0 %.

5.SPECIFICITY

A) Rabeprazole and Itopride identification:

Solutions of Standard and Sample were prepared as per test procedure and injected into the HPLC system. The recorded Chromatograms were shown in **Fig: 32 and 33**.

Acceptance criteria

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

B) Placebo interference:

A study to establish the interference of placebo was conducted. A sample of placebo was injected into the HPLC system as per the test procedure. The Chromatogram of placebo was represented as **Fig:34**.

Acceptance criteria

Chromatogram of placebo should not show any peak at the retention time of analyte peak. There is no interference due to placebo at the retention time of analyte. Hence the method is specific.

C) Blank interference:

A study to establish the interference of blank was conducted. Diluent was injected into HPLC system as per the test procedure. The Chromatogram of blank was shown in **Fig: 35.**

Acceptance criteria

Chromatogram of blank should not show any peak at the retention time of analyte peak. There is no interference due to blank at the retention time of analyte. Hence the method is specific.

RUGGEDNESS

The simultaneous estimation of Rabeprazole and Itopride was performed by different analysts on different days. The Chromatogram for Day-1, Analyst-1 was presented in **Fig: 36** and the results for were illustrated in **Table: 14**. TheChromatogram for Day-2, Analyst-2 was given in **Fig: 37** and the results were discussed in **Table: 15**.

Acceptance criteria

1. % Relative standard deviation of peak areas and R_t should not be more than 2.0 %.

6. ROBUSTNESS

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

a) Effect of variation of flow rate*:*

A study was conducted to determine the effect of variation in flow rate. The flow rate was varied at 1.0 ml/min to 1.4 ml/min. Standard solution 30 ppm Rabeprazole and 180 ppm Itopride were prepared and analysed using the varied flow rates along with method flow rate.

7. RUGGEDNESS

The results are summarizedOn evaluation of the above results, it can be concluded that the variation in flow rate affected the method significantly. Hence it indicates that the method is robust even by change in the flow rate ±10%.The method is robust only in less flow condition.The effect of variation of flow rate was evaluated. The Chromatograms were shown in **Fig: 338 and 39**. The results were discussed in the **Table: 16,17and 18**.

Acceptance criteria

1. The tailing factor for Rabeprazole and Itopride should be not more than 2.0 for Variation in flow.
2. The % RSD of Asymmetry and retention time for Rabeprazole and Itopride should be not more than 2.0 % for variation in flow.

b) Effect of variation of mobile phase composition:

A study was conducted to determine the effect of variation in mobile phase ratio by changing the ratio of mobile phase. The Organic composition in the Mobile phase was varied from30 % to 70 %.

Standard solution 30 µg/ml of Rabeprazole and 180 µg/ml Itopride were prepared and analysed using the varied mobile phase composition along with the actual mobile phase composition in the method.Standard solution was prepared and injected into the HPLC system and the Chromatograms which were recorded and shown in **Fig: 40**and **41.** The retention time values were measured and are given in **Table: 19,20**and **21.**

Acceptance criteria

1. Tailing Factor of Rabeprazole and Itopride drugs should not be more than 2.0 for Variation in composition of mobile phase.
2. The % RSD of tailing factor and retention times of Rabeprazole and Itopride drugs should be not more than 2.0 for Variation in composition of mobile phase.

8. LIMIT OF DETECTION

For RABEPRAZOLE:

Preparation of 10 µg/ml solution:

10mg of Rabeprazole was accurately weighed and transferred into a 10 ml clean dry volumetric flask, about 7 ml of diluent was added , sonicated to dissolve it completely and volume was made up to the mark with the same solvent to give the concentration of 1000 µg/ml. (Stock solution).

Further 0.1ml of the above stock solution was pipetted out into a 10ml volumetric flask and dilute up to the mark with diluent to give the concentration of 10 µg/ml.

Preparation of 0.15% solution at Specification level (0.004 µg/ml solution):

Further 1ml of the above stock solution was pipette into a 10ml volumetric flask and diluted up to the mark with diluentto give the concentration of 10 µg/ml.

0.1 ml of 10 µg/ml solution was pipette into a 10 ml of volumetric flask and diluted up to the mark with diluentto give the concentration of 0.004 µg/ml.

For Itopride

Preparation of 10 µg/ml solution:

10mg of Itoprideworking standard was accurately weighed and transferred into a 10 ml clean dry volumetric flask, about 7 ml of diluent was added, sonicated to dissolve it completely and the volume was made up to the mark with the same solventto give the concentration of 1000 µg/ml. (Stock solution)

Further 0.1 ml of the above stock solution was pipette into a 10ml volumetric flask and diluted up to the mark with diluent to give the concentration of 10 µg/ml.

Preparation of 0.22% solution At Specification level (0.006 µg/ml solution):

Further 1ml of the above stock solution was pipette into a 10ml volumetric flask and diluted up to the mark with diluentto give the concentration of 10 µg/ml

0.22 ml of 1µg/ml solution was pipette into a 10 ml of volumetric flask and diluted up to the mark with diluentto give the concentration of 0.006 µg/ml.

LOD sample:

From the 10 µg/ml of Rabeprazole and 10 µg/ml of Itopride of the indiduval stock solution we prepared 0.004 µg/ml and 0.006µg/ml injected into the system.

The LOD is determined by the formula

LOD = **S**/N

Where

N = Average Baseline Noise obtained from Blank

S = Signal Obtained from LOD solution (0.25% of target assay concentration).

Chromatograms are represented in **Fig.no:42**

Acceptance Criteria: S/N Ratio value shall be not more than 3 for LOD solution.

9. LIMIT OF QUANTIFICATION (LOQ)

For Rabeprazole:

Preparation of 10 µg/ml solution:

10 mg of Rabeprazole was accurately weighed and transferred into a 10 ml clean dry volumetric flask, about 7 ml of diluent was added, sonicated to dissolve it completely and make volume up to the mark with the same solventto give the concentration of 1000 µg/ml. (Stock solution).
Further 0.1ml of the above stock solution was pipette into a 10ml volumetric flask and diluted up to the mark with diluentto give the concentration of 10 µg/ml.

Preparation of 0.05% solution At Specification level (0.015 µg/ml solution):

Further 1ml of the above stock solution was pipette into a 10ml volumetric flask and dilute up to the mark with diluent to give the concentration of 1 µg/ml.
0.05 ml of 10 µg/ml solution was pipetted into a 10 ml of volumetric flask and diluted up to the mark with diluentto give the concentration of 0.015 µg/ml.

For Itopride:

Preparation of 10 µg/ml solution:

10mg of Itopride was accurately weighed and transferred into a 10 ml clean dry volumetric flask, about 7 ml of diluent was added, sonicated to dissolve it completely and make volume up to the mark with the same solventto give the concentration of 1000 µg/ml. (Stock solution).
Further 0.1ml of the above stock solution was pipetted into a 10ml volumetric flask and diluted up to the mark with diluentto give the concentration of 10 µg/ml.

Preparation of 0.06% solution At Specification level (0.02 µg/ml solution):

Further 1ml of the above stock solution was pipetted into a 10ml volumetric flask and diluted up to the mark with diluentto give the concentration of 10 µg/ml**.**
0.06 ml of 10 µg/ml solution was pipetted into a 10 ml of volumetric flask and diluted up to the mark with diluentto give the concentration of 0.02 µg/ml.

LOQ SAMPLE:

From the 10 µg/ml of Rabeprazole and 10 µg/ml of Itopride of the indiduval stock solution we prepared 0.015 µg/mland0.1µg/ml injected into the system.
LOQ is determined by the following formula:
LOQ =S/N
where
N =Average Baseline Noise obtained from Blank.
S=Signal Obtained from LOQ solution (1% of target assay concentration).
Acceptance Criteria: S/N Ratio value shall be 10 for LOQ solution.
Chromatograms are represented in **Fig. No: 43.**

RESULTS

Fig Chromatogram Of Trial 1

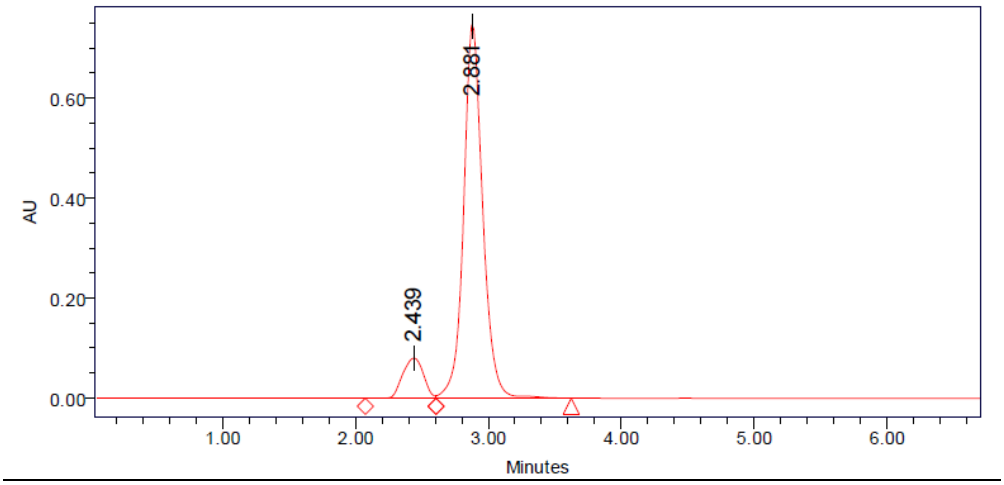


Fig 7: Chromatogram Of Trial 2

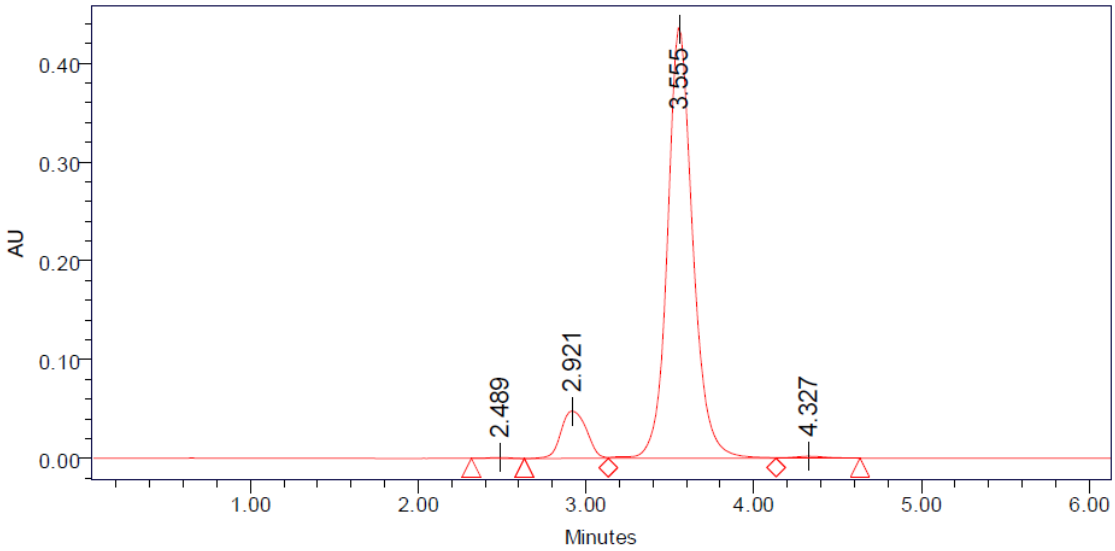


Fig 8: Chromatogram Of Trial 3

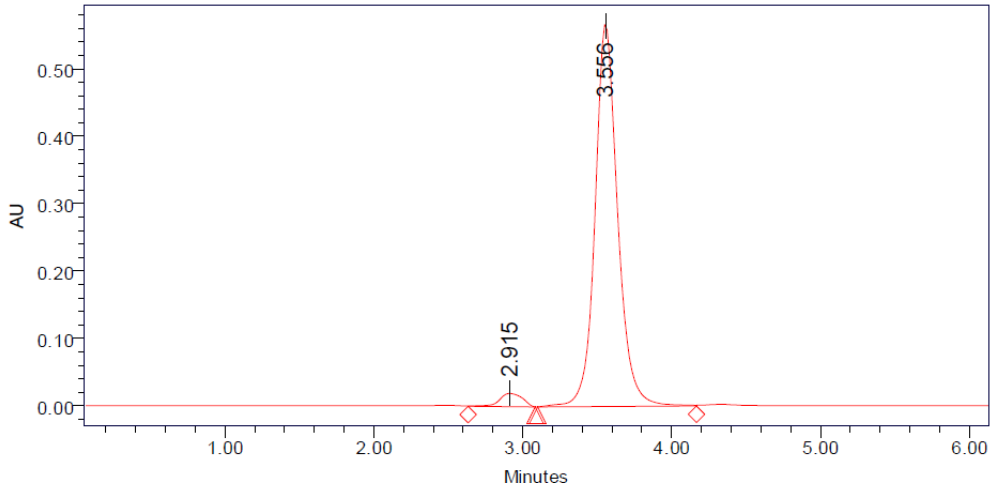


Fig 9: Chromatogram Of Trial 4:

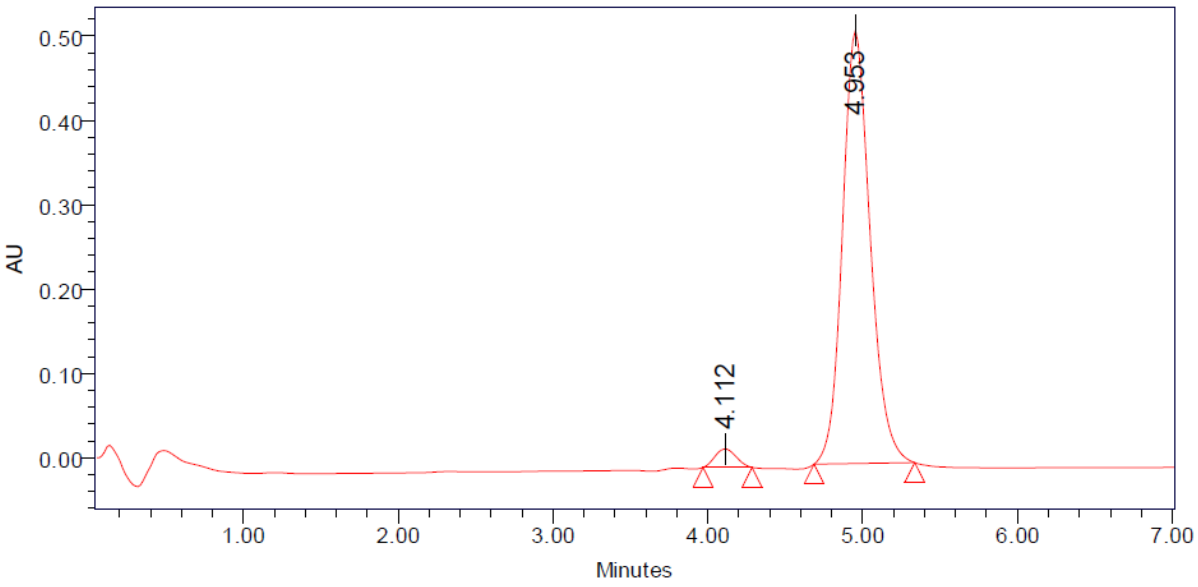


Fig 9: Standard Chromatogram for Optimised Method

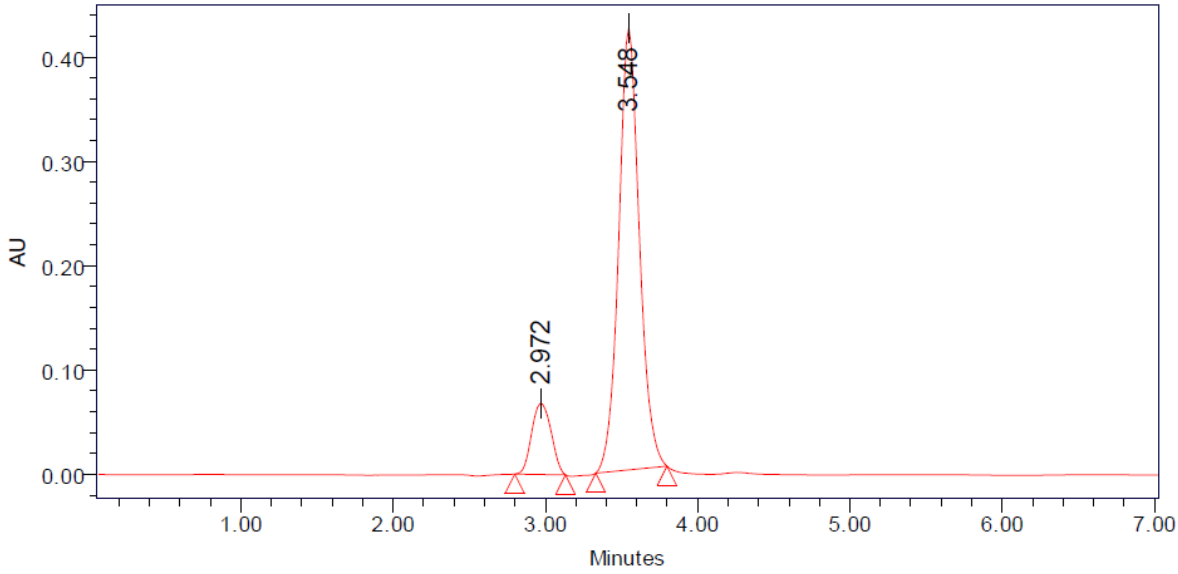


Fig 10:Chromatogram of Blank

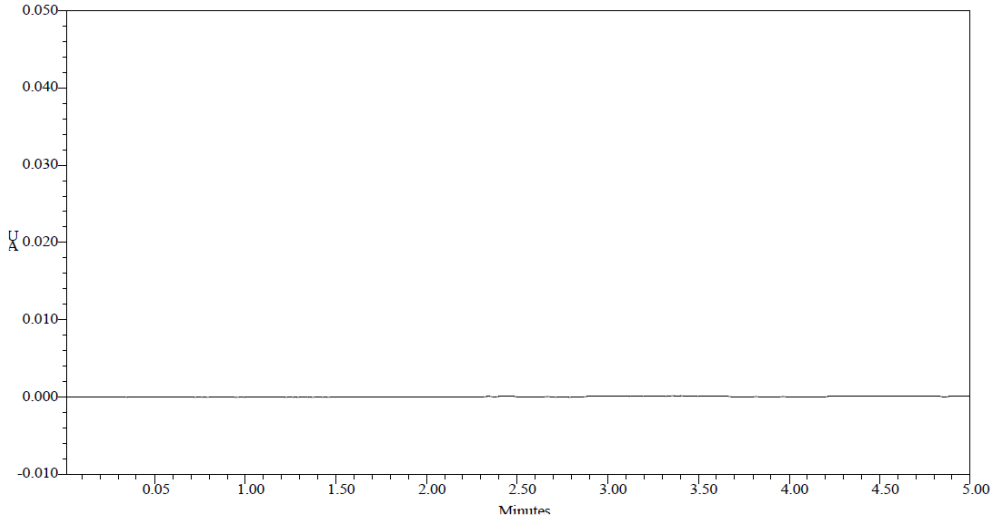
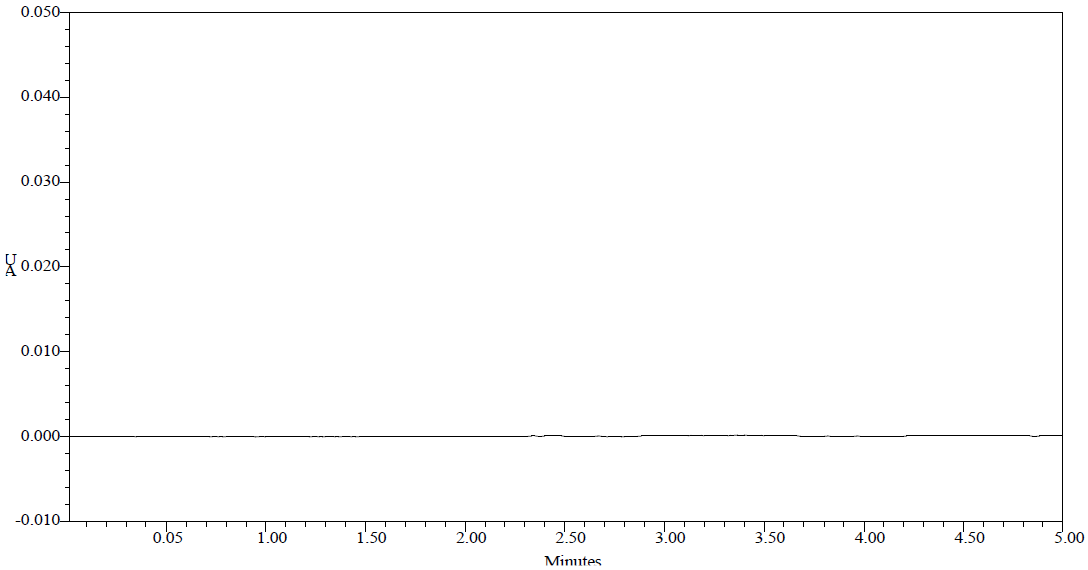


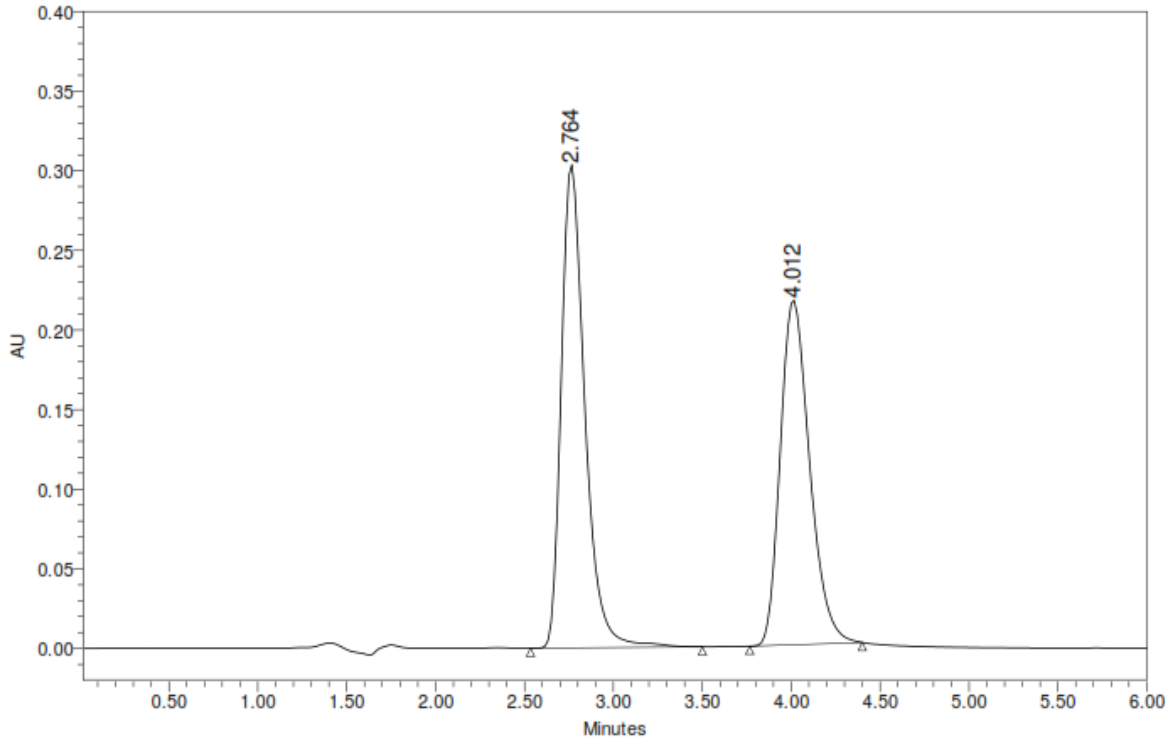
Fig 12: Chromatogram for Placebo



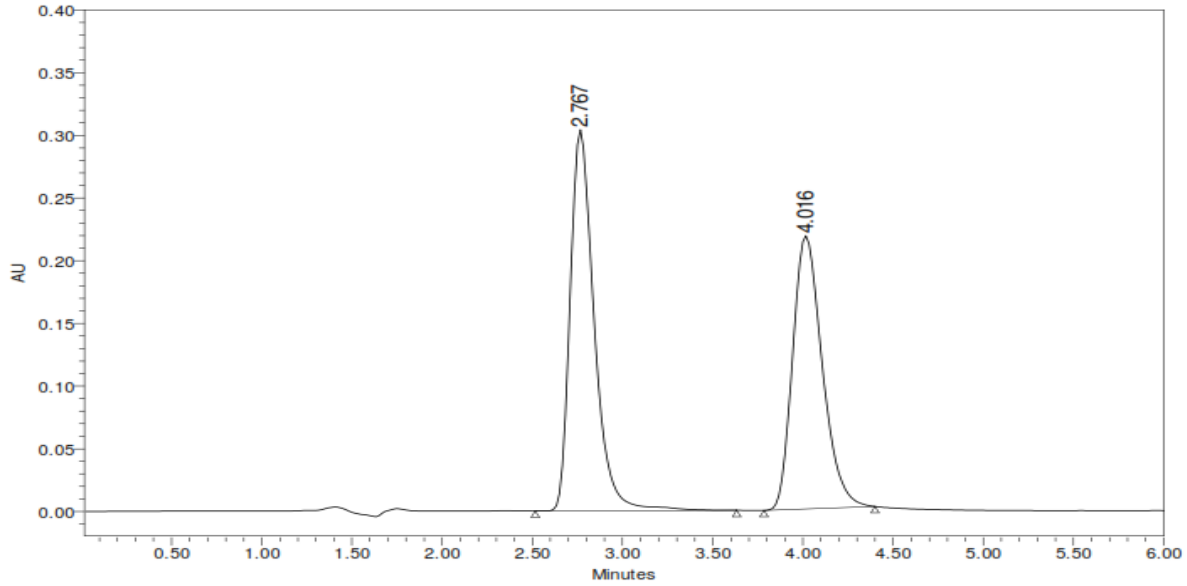
METHOD VALIDATION

1. SYSTEM SUITABILITY

Fig 13: Chromatograms for System suitability



	Name	Retention Time (min)	Area (µV*sec)
1	Rabeprazole	2.764	2794115
2	Itopride	4.012	2478642



	Name	Retention Time (min)	Area (µV*sec)
1	Rabeprazole	2.767	2805673
2	Itopride	4.016	2481848

Assay Results: (Rabeprazole)

1250595 10 0.3 100 10 99.9 1754.5
---------- x ----------x --------- x -------- x------- x --------- x100 x --------- =99.83%
1251360 10 10 1754.5 0.1100 300

Assay Results:(Itopride**)**

928829 10 0.3 10010 99.8 1754.5
---------- x -------- x--------- x ----------x------- x -------- x --------- x100 =98.89%
937364 10 10 1754.5 0.1100 300

2. LINEARITY

Fig:14 Chromatogram for Linearity level 1

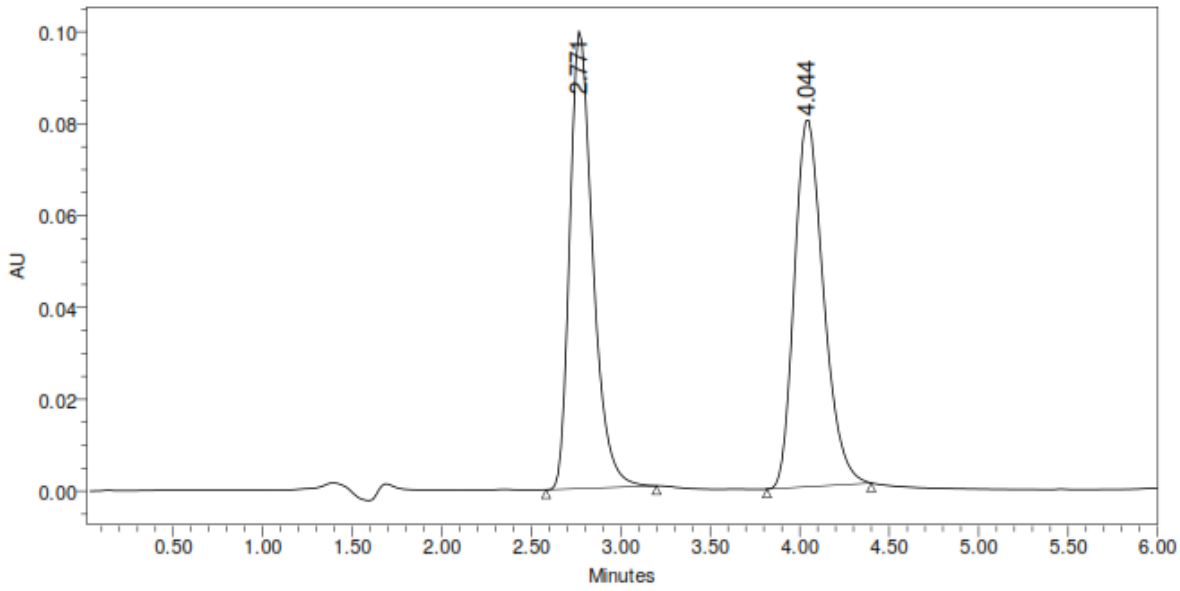


Fig:15 Chromatogram for Linearity level 2

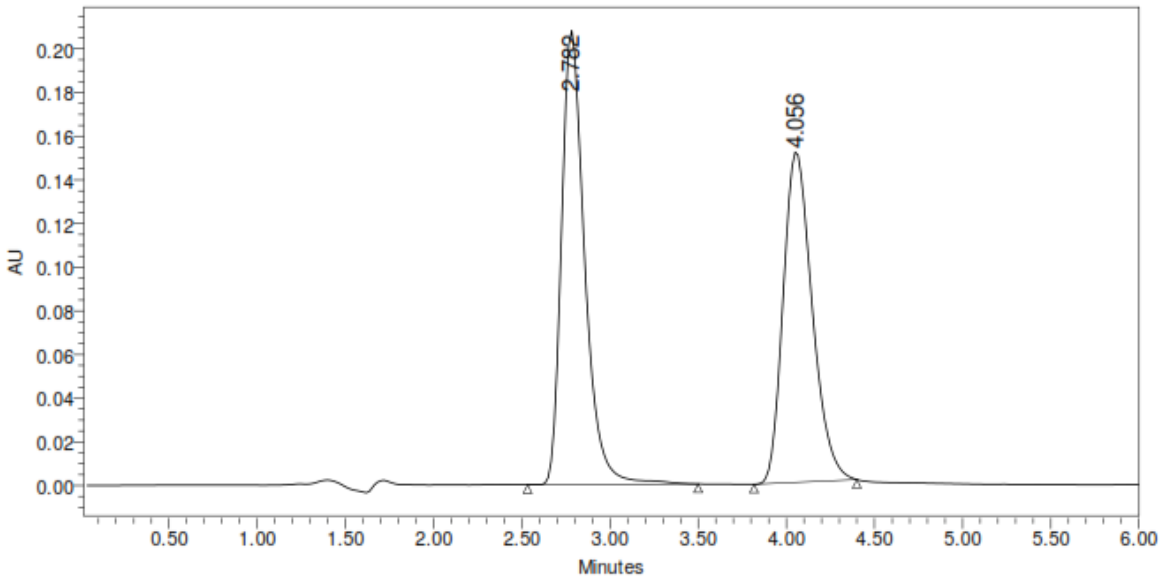


Fig:16 Chromatogram for Linearity level 3

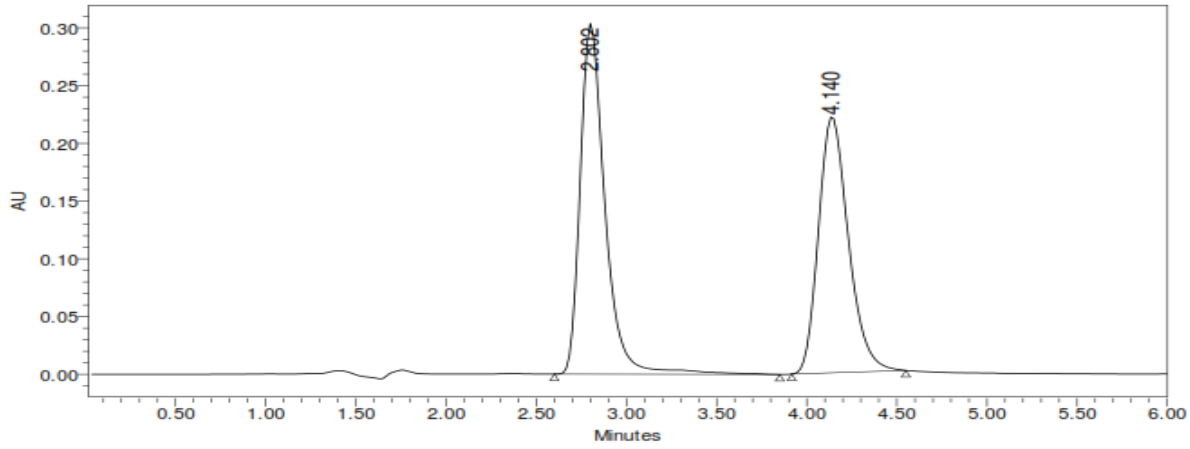


Fig: 17. Chromatogram for Linearity level 4

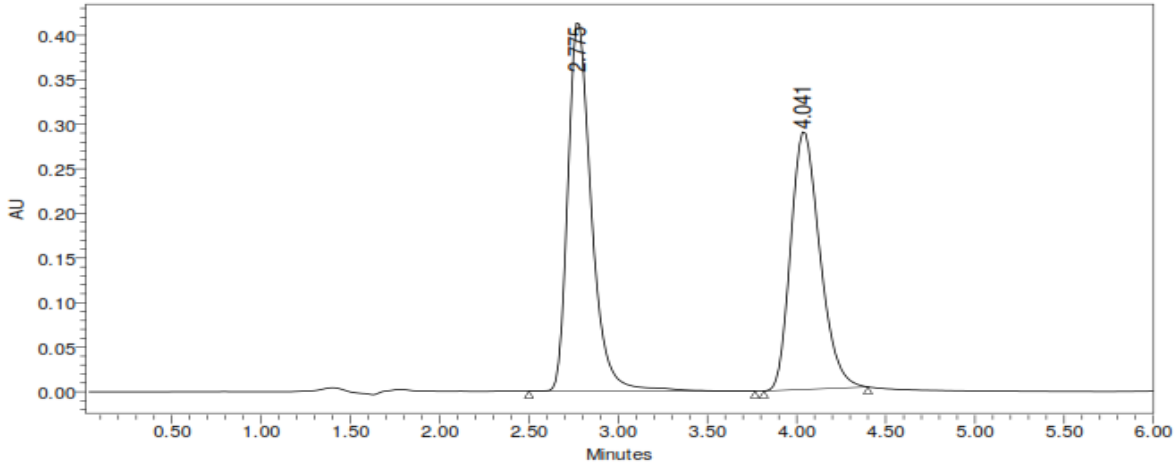
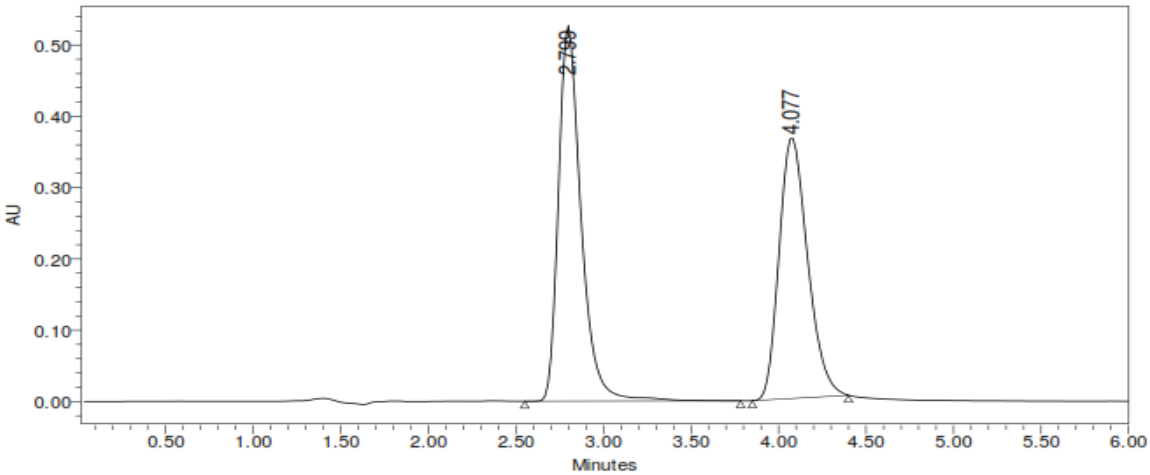


Fig: 18 Chromatogram for Linearity level 5



	Name	RT	Area	Height (µV)
1	Rabeprazole	2.771	892464	99758
2	Itopride	4.044	907953	80072
3	Rabeprazole	2.782	1904884	207723
4	Itopride	4.056	1730043	151678
5	Rabeprazole	2.802	2906620	303486
6	Itopride	4.140	2553693	222173
7	Rabeprazole	2.775	3800672	416391
8	Itopride	4.041	3283876	289820
9	Rabeprazole	2.799	4738193	525995
10	Itopride	4.077	4144232	365940

Table:5Linearity results for Rabeprazole

S.No	Linearity Level	Concentration	Area
1	I	10 ppm	892464
2	II	20 ppm	1904884
3	III	30 ppm	2906620
4	IV	40 ppm	3800672
5	V	50 ppm	4738193
Correlation Coefficient			0.99932

Table:6 Linearity results for Itopride

S.No	Linearity Level	Concentration	Area
1	I	10 ppm	907953
2	II	20 ppm	1730043
3	III	30 ppm	2553693
4	IV	40 ppm	3283876
5	V	50 ppm	4144232
Correlation Coefficient			0.99916

Fig:19Calibration curve ofRabeprazole

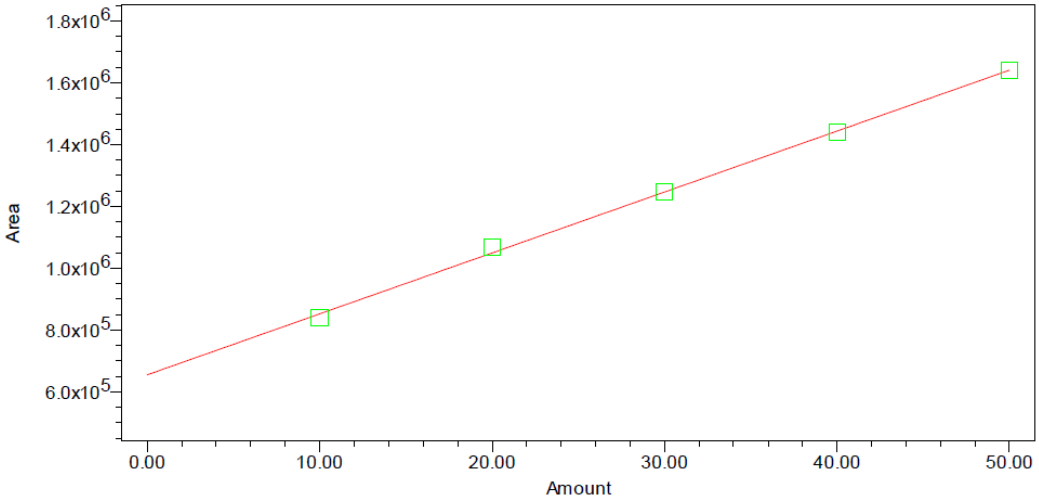
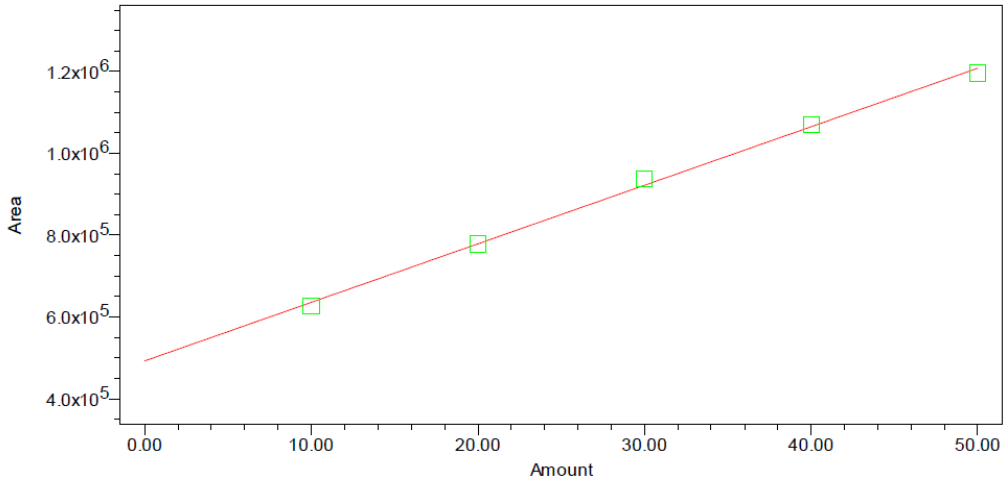


Fig: 20 Calibration curve ofItopride



Calibration parameters for Rabeprazole and Itopride

Parameter	Results for Rabeprazole	Results for Itopride
Slope	19718	14311
Intercept	65498	49120
Correlation co-efficient	0 .9993	0.99916

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