DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR THE SIMULTANEOUS ESTIMATION OF AMBROXOL HYDROCHLORIDE AND LORATADINE IN TABLET DOSAGE FORM

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1. INTRODUCTION

INTRODUCTION TO ANALYTICAL CHEMISTRY [1, 2, 3]

Analytical chemistry is often described as the area of chemistry responsible for characterizing the composition of matter, both qualitatively (what is present) and quantitatively (how much is present). Analytical chemistry is not a separate branch of chemistry, but simply the application of chemical knowledge.

Pharmaceutical Analysis

Pharmaceutical Analysisis the branch of chemistry involved in separating, identifying and determining the relative amounts of the components making up a sample of matter. It is mainly involved in the qualitative identification or detection of compounds and quantitative measurements of the substances present in bulk and pharmaceutical preparation.

The technique employed in quantitative analysis is based upon the quantitative performance of suitable chemical reactions and either measuring the amount of reagent needed to complete the reaction, or ascertaining the amount of reaction product obtained.
Quality is important in every product or service but it is vital in medicine as it involves life. Unlike ordinary consumer goods there can be no “second quality” in drugs. Quality control is a concept, which strives to produce a perfect product by series of measures designed to prevent and eliminate errors at different stages of production.

Physico-chemical methods are used to study the physical phenomenon that occurs as a result of chemical reactions. Among the Physico-chemical methods, the most important are optical (Refractometry, Polarimetry, Emission, Fluorescencemethods of analysis, Photometry including PhotoColorimetry and Spectrophotometry covering UV-Visible and IR regions and Nephelometry or Turbidimetry) and chromatographic (Column, Paper, TLC, GLC, HPLC) methods. Methods such as Nuclear Magnetic Resonance and Para Magnetic Resonance are becoming more and more popular.

The combination of Mass Spectroscopy with Gas Chromatography and Liquid Chromatography are the most powerful tools available. The chemical methods include the gravimetric and volumetric procedures which are based on complex formation; acid-base, precipitation and redox reactions. Titrations in non-aqueous media and complexometric have also been used in pharmaceutical analysis.

The number of new drugs is constantly growing. This requires new methods for controlling their quality. Modern pharmaceutical analysis must need the following requirements.

1. The analysis should take a minimal time.
2. The accuracy of the analysis should meet the demands of pharmacopeia
3. The analysis should be economical.
4. The selected method should be precise and selective.

These requirements are met by the Physico-chemical methods of analysis, a merit of which is their universal nature that can be employed for analyzing organic compounds with a diverse structure. Of them, Visible Spectrophotometry is generally preferred especially by small scale industries as the cost of the equipment is less and the maintenance problems are minimal.

INTRODUCTION ON CHROMATOGRAPHY:

Chromatography was originally developed by the Russian botanist Michael Tswett in 1903 for the separation of colored plant pigments by percolating a petroleum ether extract through a glass column packed with powdered calcium carbonate. It is now, in general, the most widely used separation technique in analytical chemistry having developed into a number of related but quite different forms that enable the components of complex mixtures of organic or inorganic components to be separated and quantified. A chromatographic separation involves the placing of a sample onto a liquid or solid stationary phaseand passing a liquid or gaseous mobile phase through or over it, a process known as elution. Sample components, or solutes, whose distribution ratiosbetween the two phases differ will migrate (be eluted) at different rates, and this differential rate of migration will lead to their separation over a period of time and distance.

Chromatographic techniques can be classified according to whether the separation takes place on a planar surfaceor in a column. They can be further subdivided into gasand liquid chromatography, and by the physical form, solid or liquid, of the stationary phase and the nature of the interactions of solutes with it, known as sorption mechanisms.

Table1. Classification of the principle chromatographic techniques

Technique	**Stationary Phase**	**Mobile Phase**	**Format**	**Principle sorption mechanism**
Paper chromatography (PC)	Paper (cellulose)	Liquid	Planar	Partition (adsorption, ion-exchange, exclusion)
Thin-layer chromatography (TLC)	Silica, cellulose, ion-exchange resin, controlled porosity solid	Liquid	Planar	Adsorption (partition, ion-exchange, exclusion)
Gas chromatography (GC)				
Gas-liquid chromatography (GLC)	Liquid	Gas	Column	Partition
Gas-solid chromatography	Solid	Gas	Column	Adsorption
Liquid chromatography (LC)				
High performance liquid chromatography (HPLC)	Solid or bonded-phase	Liquid	Column	Modified partition (adsorption)
Size-exclusion chromatography (SEC)	Controlled porosity solid	Liquid	Column	Exclusion
Ion-exchange chromatography (IEC), Ion chromatography (IC)	Ion-exchange resin or bonded-phase	Liquid	Column	Ion-exchange
Chiral chromatography (CC)	Solid chiral selector	Liquid	Column	Selective adsorption

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY: [4]

In the modern pharmaceutical industry, HPLC is a major analytical tool applied at all stages of drug discovery, development and production. Fast and effective development of rugged analytical HPLC methods is more efficiently undertaken with a thorough understanding of HPLC principles, theory and instrumentation.

Liquid Chromatography (LC), which is one of the forms of Chromatography, is an analytical technique that is used to separate a mixture in solution into its individual components. The separation relies on the use of two different "phases" or "immiscible layers," one of which is held stationary while the other moves over it. Liquid Chromatography is the generic name used to describe any chromatographic procedure in which the mobile phase is a liquid. The separation occurs because, under an optimum set of conditions, each component in a mixture will interact with the two phases differently relative

to the other components in the mixture. HPLC is the term used to describe Liquid Chromatography in which the liquid mobile phase is mechanically pumped through a column that contains the stationary phase. An HPLC instrument, therefore, consists of an injector, a pump, a column, and a detector.

Table: 2 List of solvents used in HPLC

Solvent Adsorption energy(e^0) on Al_2O_3		**Solvent Adsorption energy(e^0) on Al_2O_3**	
n-Pentane	0.00	Acetone	0.56
Isooctane	0.01	Ethyl Acetate	0.58
Cyclohexane	0.04	Dimethylamine	0.63
Carbon Tetrachloride	0.18	Acetonitrile	0.65
Toluene	0.29	Ethanol	0.88
Benzene	0.32	Methanol	0.95
Chloroform	0.40	Acetic Acid	Large
Methyl Ethyl Ketone	0.51	Water	
		Very large	

HPLC METHOD DESIGN AND DEVELOPMENT [4]

Set the analytical objective first that may be quantification or qualitative identification or separation of two components / multicomponent mixtures or optimization of analysis time before starting HPLC. Method for analyzing drugs by HPLC demands primary knowledge about the nature of the sample, structure, polarity, volatility, stability and the solubility parameter. An exact recipe for HPLC cannot be provided because method development involves considerable trial and error procedures. The most difficult problem usually is where to start, with what kind of mobile phase.

Analytes are detected using absorbance mode. But if the analytes are not detected perfectly than it need change of column or mobile phase or need the help of pre or post chromatographic derivatization.

Optimization can be started only after a reasonable Chromatogram which can be done by slight change in mobile phase composition. This leads to a reasonable Chromatogram which has all the desired peaks in symmetry and well separated.

VALIDATION: [5,6]

Validation may be viewed as the establishment of an experimental data base that certifies an analytical method performs in the manner for which it was intended and is the responsibility of the method development laboratory. Method transfer, on the other hand, is the introduction of a validated method into a designated so that it can be used in the same capacity for which it was originally developed. .

Validation is defined as:

Food and Drug Administration (FDA):

Provides a high degree of assurance that specific process will consistently produce a product meeting its predetermined specification and quality attributes.

Analyticalmethod validation:

Method validation is the process to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Methods need to be validated or revalidated.

Before their introduction into routine use

- Whenever the conditions change for which the method has been validated, e.g., instruments with different characteristics.
- Whenever the method is changed, and the change is outside the original scope of the method. The International Conference on Harmonization (ICH) of Technical Requirements for the Registration of Pharmaceutical for human use has developed a consensus text on the validation of analytical procedures. The document includes definitions for eight validation characteristics.

The parameters as defined by the ICH and by other organizations are;

Precision:

"The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels; repeatability, intermediate precision and reproducibility."

Precision should be obtained preferably using authentic samples. As parameters, the standard deviation (SD), the relative standard deviation (coefficient of variation) and the confidence interval should be calculated for each level of precision.

Repeatability expresses the analytical variability under the same operating conditions over a short interval of time (within-assay, intra-assay). At least nine determinations covering the specified range or six determinations at 100 % test concentration should be performed. Intermediate precision includes the influence of additional random effects within laboratories, according to the intended use of the procedure, for example, different days, analysts or equipment, etc.

Reproducibility, i.e., the precision between laboratories (collaborative or interlaboratory Studies), is not required for submission, but can be taken into account for standardization of analytical procedures.

Specificity:

"Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc. Lack of specificity of an individual procedure may be compensated by other supporting analytical procedure(s)".

With respect to identification, discrimination between closely related compounds likely to be present should be demonstrated by positive and negative samples. In the case of chromatographic assay and impurity tests, available impurities / degradants can be spiked at appropriate levels to the corresponding matrix or else degraded samples can be used. For assay, it can be demonstrated that the result is unaffected by the spiked material. Impurities should be separated individually and/or from other matrix components. Specificity can also be demonstrated by verification of the result with an independent In the case of chromatographic separation, resolution factors should be obtained for critical separation. Tests for peak homogeneity, for example, by diode array detection (DAD) or mass spectrometry (MS) are recommended.

Accuracy:

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

Accuracy can be demonstrated by the following approaches:

- Inferred from precision, linearity and specificity
- Comparison of the results with those of a well characterized,
  Independent procedure
- Application to a reference material (for drug substance)
- Recovery of drug substance spiked to placebo or drugproduct (for drug product)
- Recovery of the impurity spiked to drug substance or drug product (for impurities)

Linearity:

"The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample".

It may be demonstrated directly on the analyte, or on spiked samples using at least five concentrations over the whole working range. Besides a visual evaluation of the analyte signal as a function of the concentration, appropriate statistical calculations are recommended, such as a linear regression. The parameters slope and intercept, residual sum of squares and the coefficient of correlation should reported. A graphical presentation of the data and the residuals is recommended.

Range:

"The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity."

Limit of detection (LOD):

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

Limit of Quantification (LOQ):

The quantification limit is the lowest level of analyte that can be accurately and precisely measured. This limit is required only for impurity methods and is determined by reducing the analyte concentration until a level is reached where the precision of the method is unacceptable. If not determined experimentally, the quantification limit is often calculated as the analyte concentration that gives S / N = 10. An example of quantification limit criteria is that the limit will be defined as the lowest concentration level for which an RSD 20 % is obtained when an intra-assay precision study is performed.

Robustness:

According to ICH Q2A "the robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage".

Furthermore, it is stated in ICH Q2B "The evaluation of robustness should be considered during the development phase and depends on the type of procedure under study. It should show the reliability of an analysis with respect to deliberate variations in method parameters. If measurements are susceptible to variations in analytical conditions, the analytical conditions should be suitably controlled or a precautionary statement should be included in the procedure. One consequence of the evaluation of robustness should be that a series of system suitability parameters (e.g., resolution test) is established to ensure that the validity of the analytical procedure is maintained whenever used".

Ruggedness:

"The ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of conditions, such as different laboratories, different analysts, different instruments, different days, etc. Ruggedness is normally expressed as the lack of influence on test results of operational and environmental variables of the analytical method. Ruggedness is a measure of reproducibility of test results under the variation in conditions normally expected from laboratory to laboratory and from analyst to analyst". The degree of reproducibility is then evaluated by comparison of the results obtained under varied conditions with those under standard conditions.

2. DRUG PROFILE

A) AMBROXOL HCL :

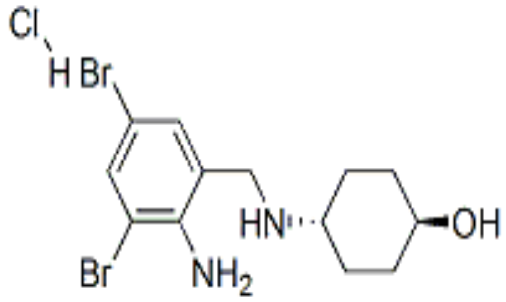

Figure:1 Structure of Ambroxol Hcl

Chemical name : 1 ({[2 – Amino – 3, 5 dibromo phenyl] methyl} amino) Cyclohexanolmonohydrochloride

Molecular formulae: $C_{13}H_{19}Br_2ClN_2O$

Molecular Weight : 414.56

Category : Mucolytic Agents

Dose : oral: Mucolytic: Adult: 60-120mg daily

Description : white crystalline powder

Solubility : slightly soluble in water and soluble in Methanol.

Storage conditions: Store at a temperature not exceeding 30°C.

Indications : All forms of tracheobronchitis

Brand name : **ACOREX, AMBOLYT, AMBOSIL, AMBOTEN, AMBROXOL, AMBROSOL, AMBROX.**

B) LORATADINE

Chemical structure:

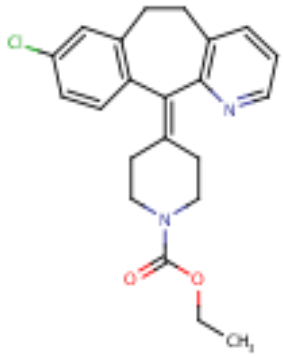

Figure:2 Structure of Loratadine

Chemical name : 4-[8-chloro-5, 6- dihydro-11Hbenzo [5, 6] cyclohepta [1, 2-b] pyridin-11-ylidene]-1-piperidinecarboxylic Acid

Molecular formulae : $C_{22}H_{23}ClN_2O_2$

Molecular Weight : 382.883

Category : Antipruritics, Anti-Allergic Agents, Antihistamines,Histamine H1 Antagonists, Non-Sedating

Dose : 30, 40, 5, 10 mg tablets

Description : white colored powder

Solubility : soluble in water and Methanol

Storage conditions : Store at 25°C (77°F); excursions permitted to 15°-30°C (59°-86°F)

Brand name : Aerotina, Alarin, Alavert, Allertidine, boloina, Civeran.

3. LITERATURE REVIEW

Chebrolu .Sunil Narendra Kumar et al[11] **(2011)** reported a validated reverse phase HPLC method for simultaneous determination of AmbroxolHcl and Loratadine in pharmaceutical dosage form.The method employs measurement of absorbance at two wavelengths,308nm and 245nm, of ambroxol and loratadine respectively. Beer´s law obeyed in the concentration range of 10-50µg/ml for Ambroxol and Loratadine respectively.

Krishna VeniNagappanet al[12] **(2010)** A RP- Hplc method for Simultaneous estimation of Ambroxol HCL and Loratadine in pharmaceutical dosage forms. The method was carried out on a Phenomenex Gemini C18 (25 cm x 4.6 mm i.d., 5 µ) column with a mobile phase consisting of acetonitrile: 50mM Ammonium Acetate (50:50 v/v) at a flow rate of 1.0 mL/min. Detection was carried out at 255 nm

Hejnanenet al[13] **(2011)** developed HPLC method for the Simultaneous estimation of Ambroxol HCL and Loratadine in tablet dosage form. Column Symmetry Shield RPC8, 5 microm 250 x 4.6 mm, and methanol/(H(3)PO(4) 8.5 mM/triethylamine pH=2.8) 40:60 v/v. Validation was performed using standards and the pharmaceutical preparation which contains the compounds.

Pradeep Kumar T[1]et al[14]**(2010)** reported A method for Simultaneous Determination of CitirizineHcl and Ambroxol Hcl in bulk and pharmaceutical formulations. The separation was achieved on a phenomenex C18 column (150 × 4.6 mm i.d, particle size of 5µ) using a mixture of methanol, acetonitrile and water in the ratio of (30:30:40v/v)as mobile phase in an isocratic elution mode, at a flow rate of 1 ml/min. The detection was monitored at 230 nm.

RakeshKotkar P et al[15] (**2010**) Development and Validation of RP-HPLC Method forSimultaneous Estimation of CefpodoximeProxetil and Ambroxol Hydrochloride in Bulk and in TabletsThe method was carried out on a Qualisil RP C-8 (250 mm x 4.6 mm, 5 µm) column with a mobile phase consisting of acetonitrile: 0.025 M potassium dihydrogen phosphate buffer (70:30 v/v) pH adjusted to 4.0 with orthophosphoric acid and flow rate of 1.0 mL/min. Detection was carried out at 248 nm.

GeorgetaPavalacheet al[16] (**2009**)The development of a high-performance liquid chromatography (HPLC) method for the determination of loratadine in dosage forms is described. The method involved liquid - phase extractionof loratadine using methanol as solvent. Separation was achieved with a C18 column (250 mm x 4.6 mm) 5µm XDB - C18 Agilent (Zorbax Eclipse XDB-18) employing detection λ= 264 nm.

Malathiet al [17]**(2009)** developed for the simultaneous estimation of cefpodoximeproxetil and Ambroxol HCl from pharmaceutical dosage forms. The method was carried out on a Zorbax Eclipse XDB 5 µ C 18 (150×4.6 mm) column with a mobile phase consisting of acetonitrile:50mM potassium dihydrogen phosphate buffer (pH 3.0, 70:30 v/v) at a flow rate of 1.0 ml/min. Detection was carried out at 228 nm.

G.Abiramiet al[18] **(2012)** the simultaneous determination of Cefpodoximeproxetil (CEF) and Ambroxol hydrochloride (AMB) in tabletdosage form were described. The method A involves simultaneous equation, using methanol as common solvent showing absorption maxima at 235nm and 308nm.The linearity for both CEF and AMB hydrochloride in method. A at the range of 5-30µg/ml and 3-18µg/ml respectively. The method B Derivative Spectrophotometric method,was based on the principle that both CEF and AMB hydrochloride spectra was derivatised into first order and thederivative spectra showed λ max at 279nm and 235 nm .

M Patel et al[19]**(2012)** developed RP-HPLC method was developed and validated for determination of CPD and AMB in tablets. Isocratic RP-HPLC separation was achieved on a ACE C18 column (150 × 4.6 mm id, 5 µm particle size) using the mobile phase Acetronitrile:Phosphate buffer (pH 6.0) :Methanol(25:35:40v/v) at a flow rate of 0.8 mL/min. The retention time of CefpodoximeProxetil and Ambroxol hydrochloride was 3.5 and 6.5 min. The detection was performed at 248 nm.

M. Senthil Raja1 et al[20] **(2013)**developed and subsequently validated for simultaneous determination of Azithromycin and Ambroxol Hydrochloride in combined dosage form. Theseparation was carried out using a mobile phase consisting of acetonitrile and mono basic potassium phosphate buffer ofpH 8.5 in the ratio of 65:35 v/v. The column used was C18 phenomenex Gemini 5m, 250cm x 4.6mm id with flow rate of 2ml/min using PDA detection at 220nm.

4.AIM AND OBJECTIVE OF THE STUDY

The literature review reveals few HPLC methods for the estimation of Ambroxol HCL and Loratadine alone and in combination with other drugs. Few methods are also reported for estimation of both drugs from formulation so.We intend to develop a Stability indicating RP-HPLC method by simultaneous determination with simple, rapid, greater sensitivity and faster elution with an objective to develop a HPLC method for analysis of both the drugs and validate the method using formulations.

5. EXPERIMENTAL WORK

S.No	INSTRUMENT	MODEL
1	HPLC	WATERS, software: Empower, 2695 separation module.996PDA detector.
2	UV/VIS spectrophotometer	LABINDIA UV 3000^{+}
3	pH meter	LABINDIA
4	Weighing machine	Sartorius BSA 224s
5	Pipettes and Burettes	Borosil
6	Beakers	Borosil

Table: 3 List of Instruments used

S.No	CHEMICAL	BRAND
1	Ambroxol HCL	Dr. Reddy`s
2	Loratadine	Dr. Reddy`s
3	KH_2PO_4	FINER chemical LTD
4	Water and Methanol for HPLC	SD FINE-CHEM
5	Acetonitrile for HPLC	SD FINE-CHEM
6	HCl, H_2O_2, NaOH	FISHER

Table 4: List of chemicals used

HPLC METHOD DEVELOPMENT:

Mobile Phase Optimization:

Initially the mobile phase tried was methanol: Water and acetonitrile: phosphate buffer with various combinations of pH as well as varying proportions. Finally, the mobile phase was optimized to potassium dihydrogen phosphate with buffer (pH 5.0), acetonitrile in proportion 30: 70 v/v respectively.

Wave length selection:

UV spectrum of 10 µg / ml Ambroxol and Loratadine in diluents (mobile phase composition) was recorded by scanning in the range of 200nm to 400nm. From the UV spectrum wavelength selected as 245. At this wavelength both the drugs show good absorbance.

Optimization of Column:

The method was performed with various columns like C18 column, hypersil column, lichrosorb, and inertsil ODS column. Symmetry C18 (4.6 x 250mm, 5µm, Make: Waters) was found to be ideal as it gave good peak shape and resolution at 1.0ml/min flow.

OPTIMIZED CHROMATOGRAPHIC CONDITIONS:

Instrument used : Waters HPLC with auto sampler and DAD or PDA detector.

Temperature : Ambient

Column : Symmetry C18 (4.6 x 250mm, 5µm, Make: Waters) or equivalent

Buffer : 7.0 grams of potassium dihydrogen ortho phosphate in 1000 ml water pH adjusted with Potassium Hydroxide.

pH : 5.0

Mobile phase : 30% buffer 70% acetonitrile

Flow rate : 1.0 ml per min

Wavelength : 245 nm

Injection volume : 20 µl

Run time : 10min.

Optimized Chromatogram is shown in the **figure 6.1(i)** and blank is shown in the **figure 6.1(b).** System suitability parameters are shown in **figure 6.2** and theresults are shown in **Table 1**

PREPARATION OF BUFFER AND MOBILE PHASE:

Preparation of Phosphate buffer:

Accurately weighed 7.0 grams of KH_2PO_4 was taken in a 1000ml volumetric flask, dissolved and diluted to 1000ml with HPLC water and the volume was adjusted to pH 5.0 with Potassium Hydroxide.

Preparation of mobile phase:

Accurately measured 300 ml (30%) of above buffer and 700 ml of Acetonitrile HPLC (70%) were mixed and degassed in an ultrasonic water bath for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Diluent Preparation:

The Mobile phase was used as the diluent.

VALIDATION PARAMETERS:

Precision:

Preparation of Standard Solution:

Accurately weighed amount of 60mg Ambroxol and 5 mg Loratadine were taken to a 25 ml cleaned and dried volumetric flask. This was then diluted with 20ml of diluent and was sonicated. The volume was made to25 ml with the same solvent. This was marked and labeled as Stock solution. Further, an amount of 0.4 ml Ambroxol and Loratadine each were pipette from the above stock solution into a 10ml volumetric flask and diluted up to the mark with diluents to get 96 µg/ml of Ambroxol and 8 µg/ml of Loratadine.

Preparation of Sample Solution:

Accurately weighed amount of 191.6mg Ambroxol and Loratadine were taken to a 100 ml cleaned and dried volumetric flask. This was then diluted with 70ml of diluent and was sonicated. The volume was made to100 ml with the same solvent. This was marked and labeled as Stock solution. Further, an amount of 1.6 ml Ambroxol and Loratadine each were pipette from the above stock solution into a 10ml volumetric flask and diluted up to the mark with diluents to get 96 µg/ml of Ambroxol and 8 µg/ml of Loratadine.The standard and sample solutions of 96 µg/ml of Ambroxol and 8 µg/ml of Loratadine was injected for five times and the peak areas were recorded

The mean and percentage relative standard deviation were calculated from the peak areas.

Intermediate Precision/Ruggedness:

96 µg/ml of Ambroxol and 8 µg/ml of Loratadine of the above sample solution were injected for five times in five different days and peak areas were recorded.

Accuracy:

For accuracy determination, three different concentrations were prepared separately i.e. 50%, 100% and 150% for the analyte and Chromatograms are recorded for the same.

Preparation Sample solutions:

Preparation of 50% solution (18µg/ml of Ambroxol and 9 µg/ml of Loratadine):

About 30mg of Ambroxol 2.5mg of Loratadine were weighed and transferred to 25ml volumetric flask, it was dissolved with diluent and the volume was made up to the mark with same solvent. Further 0.4 ml of above solution was diluted to 10ml with the diluent to get 48 µg/ml of Ambroxol and 4 µg/ml of Loratadine.

Preparation of 100% solution (30 µg/ml of Ambroxol and 15 µg/ml of Loratadine):

About 60mg of Ambroxol 5mg of Loratadine were weighed and transferred to 100ml volumetric flask, it was dissolved with diluent and the volume was made up to the mark with same solvent. Further 0.4 ml of above solution was diluted to 10ml with the diluent to get 96 µg/ml of Ambroxol and 8 µg/ml of Loratadine.

Preparation of 150% solution (46.8 µg/ml of Ambroxol and 23.4 µg/ml of Loratadine):

About 90mg of Ambroxol 7.5mg of Loratadine were weighed and transferred to 100ml volumetric flask, it was dissolved with diluent and the volume was made up to the mark with same solvent. Further 0.4 ml of above solution was diluted to 10ml with the diluent to get 144 µg/ml of Ambroxol and 12 µg/ml of Loratadine**.** These solutions were filtered through 0.45µ membrane and then each concentration; three replicate injections were made under the optimized conditions. Recorded the Chromatograms and measured the peak responses.

LINEARITY:

Preparation of sample stock solution:

About 60 mg of Ambroxol and 5mg of Loratadine samples was weighed in to 25ml volumetric flask, it was dissolved with diluent and the volume was made up to the mark with same diluent (2400µg/ml of Ambroxol and 200µg/ml of Loratadine).

Preparation of Level – I (48µg/ml of Ambroxol &4µg/ml of Loratadine)

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

Preparation of Level – II (72 µg/ml of Ambroxol &6 µg/ml ofLoratadine)

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

Preparation of Level – III (96 µg/ml of Ambroxol &8 µg/ml of Loratadine)

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

Preparation of Level – IV (120 µg/ml of Ambroxol &10µg/ml of Loratadine)

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

Preparation of Level – V (144 µg/ml of Ambroxol &12 µg/ml of Loratadine)

0.6ml of stock solution had taken in 10ml of volumetric flask diluted up to the mark with diluent.

10µl of each 48% of Ambroxol and 4% of Loratadine, 72% of Ambroxol and 6% of Loratadine, 96% of Ambroxol and 8% of Loratadine, 120% of Ambroxol and 10% of Loratadine 144% of Ambroxol and 12% of Loratadine were injected in triplicate and recorded the peak response.

LIMIT OF DETECTION (for Ambroxol):

Preparation of 96µg/ml solution:

Pipette 0.4ml of the stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Preparation of 0.63% solution At Specification level (0.06µg/ml solution):

Pipette 1ml of the stock solution into a 10ml volumetric flask and diluted up to the mark with diluent. Pipette 1ml of the stock solution into a 10ml volumetric flask and diluted up to the mark with diluent.

Further Pipette 0.63ml of 1µg/ml solution into a 10 ml of volumetric flask and dilute up to the mark with diluent.

LIMIT OF QUANTIFICATION:

Preparation of 96µg/ml solution:

Pipette 0.4ml of the stock solution into a 10ml volumetric flask and diluted up to the mark with diluent.

Preparation of 2.1% solutions At Specification level (0.2µg/ml solution):

Pipette 1ml of the stock solution into a 10ml volumetric flask and diluted up to the mark with diluents. Pipette 1ml of the stock solution into a 10ml volumetric flask and diluted up to the mark with diluent. Further Pipette 2.1ml of 1µg/ml solution into a 10 ml of volumetric flask and diluted up to the mark with diluents.

LIMIT OF DETECTION: (for Loratadine)

Preparation of 8µg/ml solution:

Pipette 0.4 ml of the above stock solution into a 10ml volumetric flask and diluted up to the mark with diluent.

Preparation of 3.0% solution At Specification level (0.02µg/ml solution):

Pipette 1ml of the above stock solution into a 10ml volumetric flask and diluted up to the mark with diluentsPipette 1ml of the above stock solution into a 10ml volumetric flask and diluted up to the mark with diluent Further Pipette 3.0ml of 1µg/ml solution into a 10 ml of volumetric flask and diluted up to the mark with diluent.

LIMIT OF QUANTIFICATION:

Preparation of 8µg/ml solution:

Pipette 0.4ml of the above stock solution into a 10ml volumetric flask and diluted up to the mark with diluent.

Preparation of 1.0% solution At Specification level (0.08µg/ml solution):

Further pipetted 1ml of the above stock solution into a 10ml volumetric flask and diluted up to the mark with diluent. Pipetted 1.0ml of 1µg/ml solution into a 10 ml of volumetric flask and diluted up to the mark with diluent.

ROBUSTNESS:

The analysis was performed in different conditions to find the variability of test results. The following conditions are checked for variation of results. .

Preparation of sample solution (96µg/ml of Ambroxol 8 µg/ml of Loratadine)

About 60mg of Ambroxol and 5 mg of Loratadine were weighed and transferred to 100ml volumetric flask, it was dissolved with diluent and the volume was made up to the mark with same solvent. Further 0.4 ml of above solution was diluted to 10ml with the diluent to get 60µg/ml of Ambroxol 8 µg/ml of Loratadine.

Effect of Variation of flow:

The sample was analyzed at 0.8 ml/min and 1.2 ml/min instead of 1.0 ml/min, remaining conditions are same. 20µl of the above sample was injected twice and Chromatograms were recorded.

Effect of Variation of mobile phase organic composition:

The sample was analyzed by variation of mobile phase i.e. phosphate buffer: acetonitrile was taken in the ratio 35: 65 and 25:75 instead of 30:70, remaining conditions are same. 20µl of the above sample was injected twice and Chromatograms were recorded.

6. RESULTS AND DISCUSSION

Optimized Chromatogram Is Obtained By Following Conditions

Trial 1:

Column	:	Symmetry C18 (4.6 x 250mm, 5µm, Make: Waters) or equivalent
Buffer pH	:	-
Mobile phase	:	50% Water 50% methanol
Flow rate	:	0.8 ml per min
Wavelength	:	245 nm
Temperature	:	ambient.
Run time	:	9min.

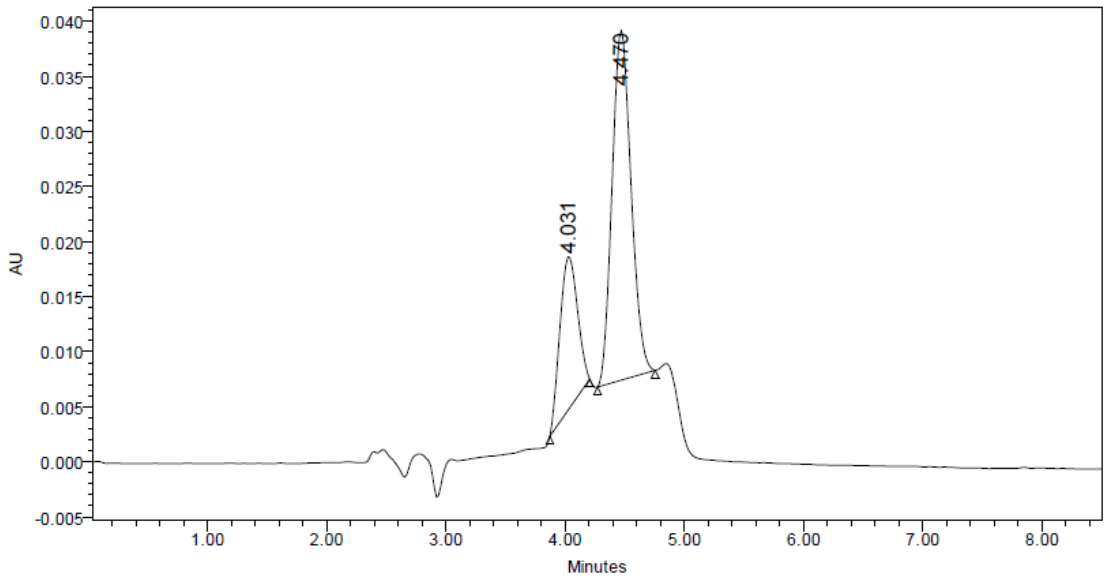

Figure:3 Trial Chromatogramfor Ambroxol(4.031) and Loratadine(4.470)

From the above Chromatogram it was observed that the Ambroxol and Loratadine peak was not good.

Trial 2:

Column	:	Symmetry C18 (4.6 x 250mm, 5µm, Make: Waters) or equivalent
Buffer pH	:	3.0.
Mobile phase	:	30% buffer 30% Methanol 40%Water
Flow rate	:	1ml per min
Wavelength	:	245 nm
Temperature	:	ambient.
Run time	:	10min.

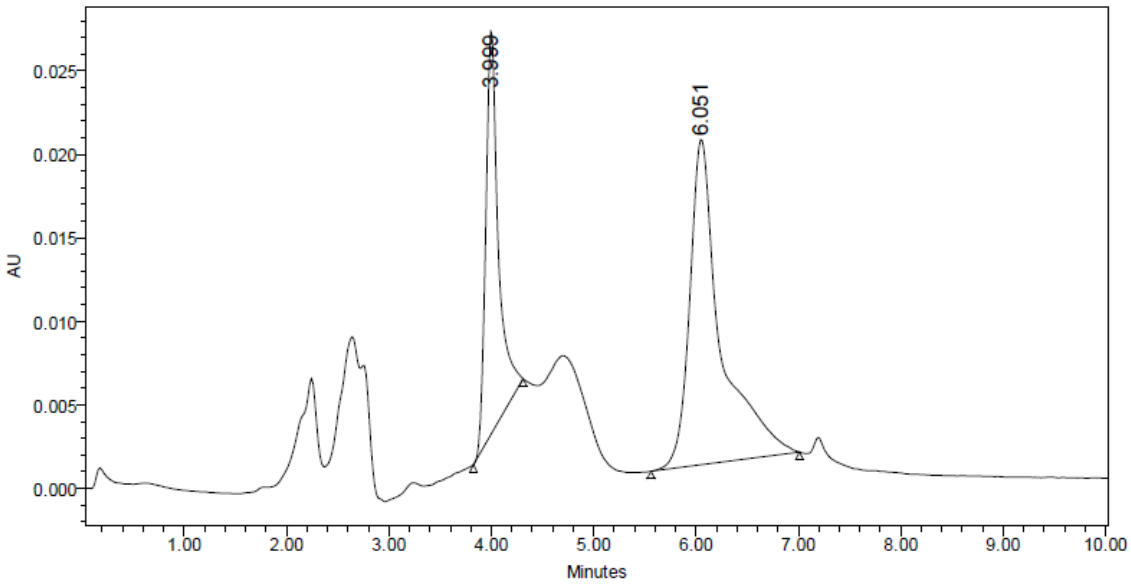

Figure:4 TrialChromatogram for Ambroxol(3.999) and Loratadine(6.051)

From the above Chromatogram it was observed that the Ambroxol Peak was splitted and Loratadine peak was tailing.

Trial 3:

Column	:	Symmetry C8 (4.6 x 250mm, 5µm, Make: Waters) or equivalent
Buffer pH	:	3.9
Mobile phase	:	30% buffer 70% Acetonitrile
Flow rate	:	1.0ml per min
Wavelength	:	245 nm
Temperature	:	ambient.
Run time	:	10min.

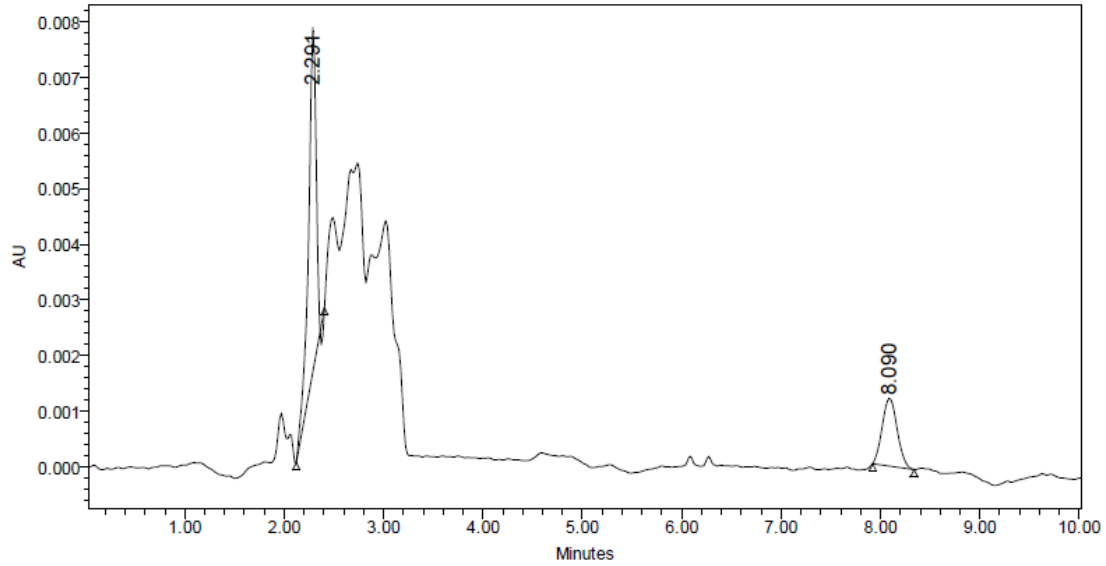

Figure:5 TrialChromatogram forAmbroxol(2.291) and Loratadine (8.090)

From the above Chromatogram it was observed that the Ambroxol peak was not good.

Trial 4:

Column	:	Symmetry C8 (4.6 x 250mm, 5µm, Make: Waters) or equivalent
Buffer pH	:	3.5
Mobile phase	:	30% buffer 70% Methanol
Flow rate	:	1.0ml per min
Wavelength	:	245 nm
Temperature	:	ambient.
Run time	:	10min.

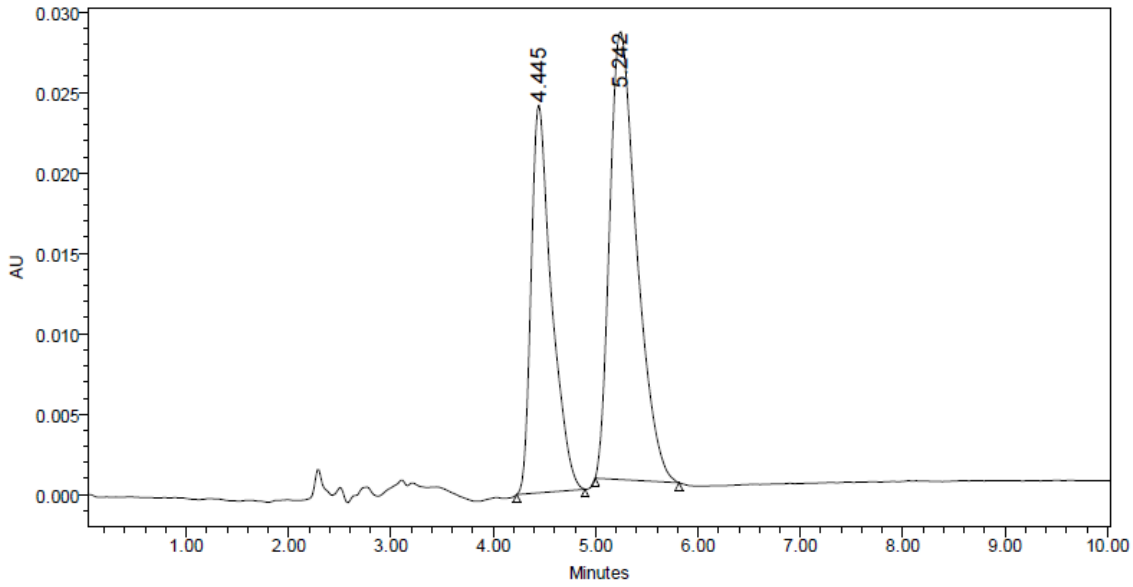

Figure:6 TrialChromatogram for Ambroxol(2.291) andLoratadine (8.090)TrialChromatogram for Ambroxol(4.445) and Loratadine(5.242)

From the above Chromatogram it was observed that the Ambroxol and Loratadine peaks are merged.

Trial 5:

Column	:	Symmetry C8 (4.6 x 250mm, 5µm, Make: Waters) or equivalent
Buffer pH	:	3.5
Mobile phase	:	30% buffer 70% Acetonitrile
Flow rate	:	0.6ml per min
Wavelength	:	245 nm
Temperature	:	ambient.
Run time	:	10min.

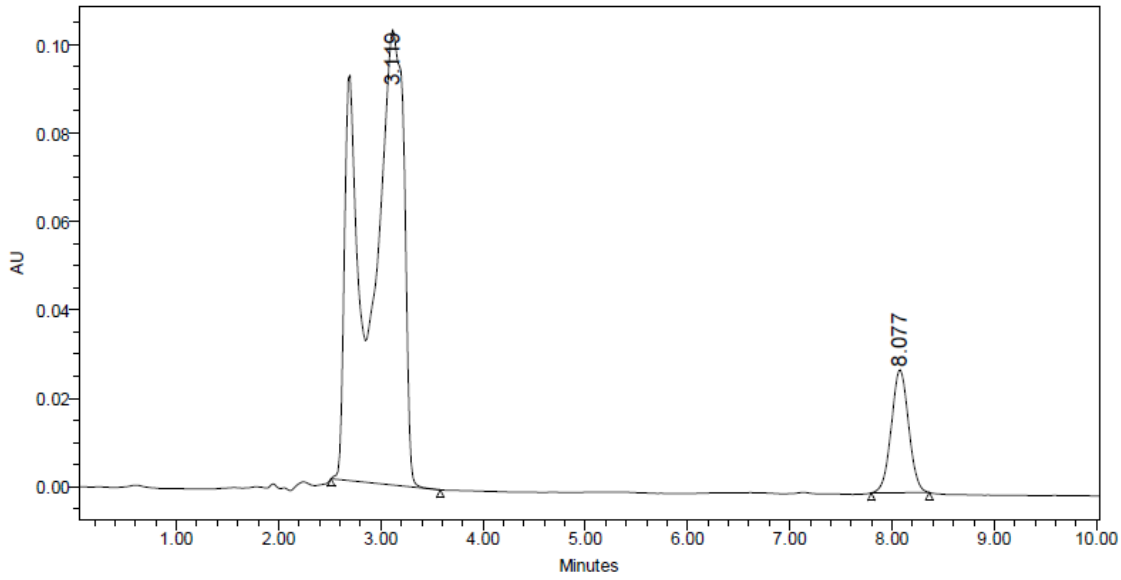

Figure: 7 TrialChromatogram for Ambroxol(2.291) andLoratadine (8.090) TrialChromatogram for Ambroxol(3.119) and Loratadine (8.077)

From the above Chromatogram it was observed that the Ambroxol peak was splitted.

Trial 6:

Column	:	Symmetry C8 (4.6 x 250mm, 5µm, Make: Waters) or equivalent
Buffer pH	:	-
Mobile phase	:	40% Water 60% Acetonitrile
Flow rate	:	0.8ml per min
Wavelength	:	245 nm
Temperature	:	ambient.
Run time	:	10min.

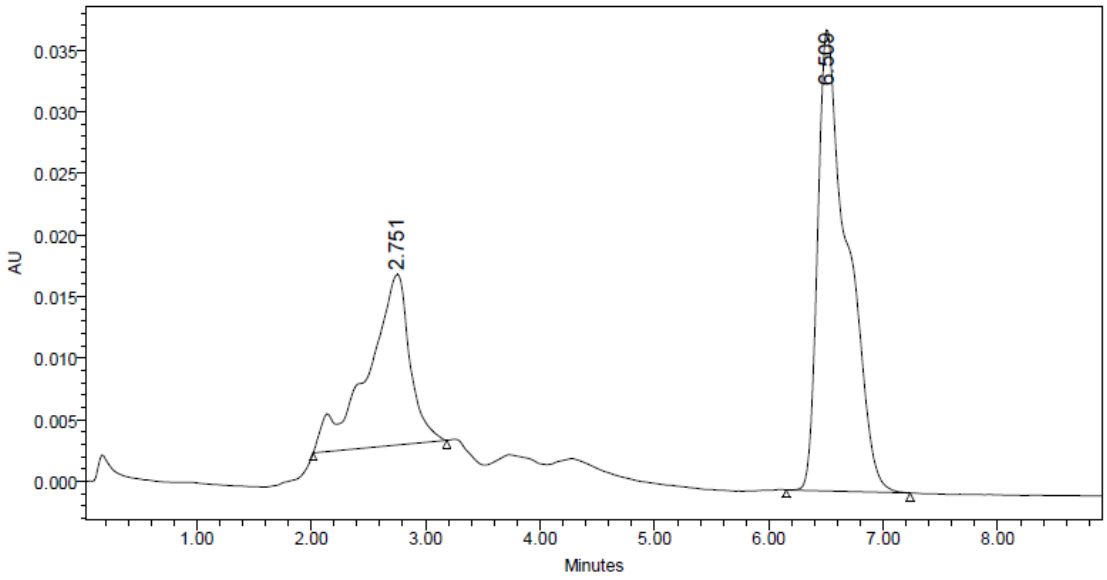

Figure:8 TrialChromatogram for Ambroxol(2.291) andLoratadine (8.090)TrialChromatogram for Ambroxol (2.751) and Loratadine(6.509)

From the above Chromatogram it was observed that the Ambroxol and Loratadine peak shape was not good.

Trial 7:

Column	:	Symmetry C18 (4.6 x 250mm, 5µm, Make: Waters) or equivalent
Buffer pH	:	-
Mobile phase	:	50% Water 50% Acetonitrile
Flow rate	:	0.8ml per min
Wavelength	:	245 nm
Temperature	:	ambient.
Run time	:	10min.

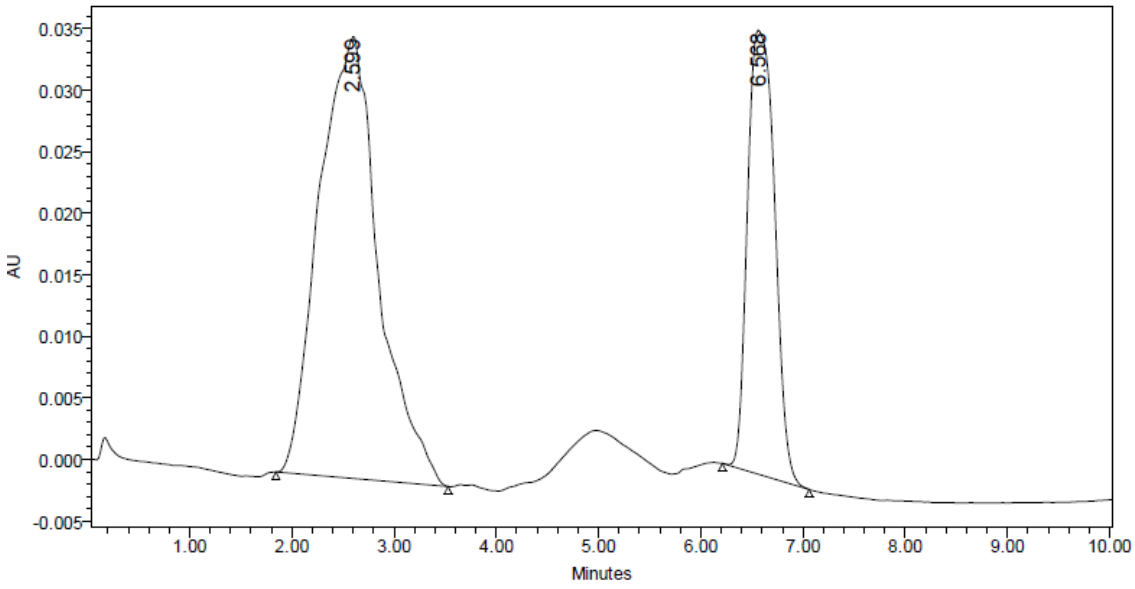

Figure: 9 TrialChromatogram for Ambroxol(2.291) andLoratadine (8.090)TrialChromatogram for Ambroxol (2.599) and Loratadine (6.568)

From the above Chromatogram it was observed that the Ambroxol and Loratadine peak shape was not good.

Trial 8:

Column	:	Symmetry C18 (4.6 x 250mm, 5µm, Make: Waters) or equivalent
Buffer pH	:	3.0
Mobile phase	:	45% buffer 55% Acetonitrile
Flow rate	:	0.8ml per min
Wavelength	:	245 nm
Temperature	:	ambient.
Run time	:	10min.

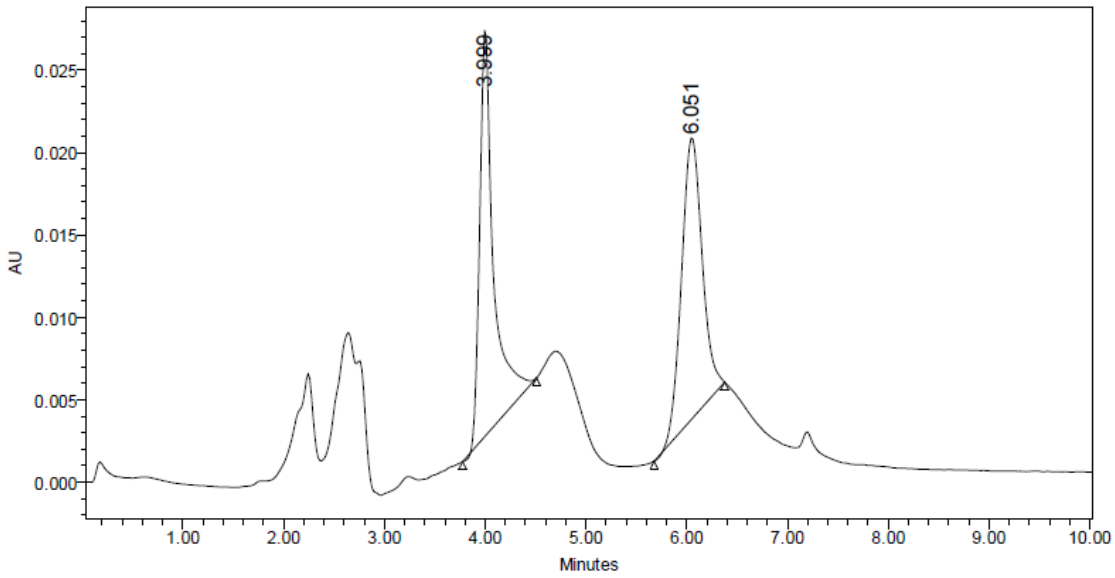

Figure: 10 TrialChromatogram for Ambroxol (2.291) andLoratadine (8.090) TrialChromatogram for Ambroxol (3.999) and Loratadine (6.051)

From the above Chromatogram it was observed that the Ambroxol peak was splitted.

Trial 9:

Column	:	Symmetry C18 (4.6 x 250mm, 5µm, Make: Waters) or equivalent
Buffer pH	:	5.0
Mobile phase	:	30% buffer 70% Acetonitrile
Flow rate	:	1.0ml per min
Wavelength	:	245 nm
Temperature	:	ambient.
Run time	:	10min.

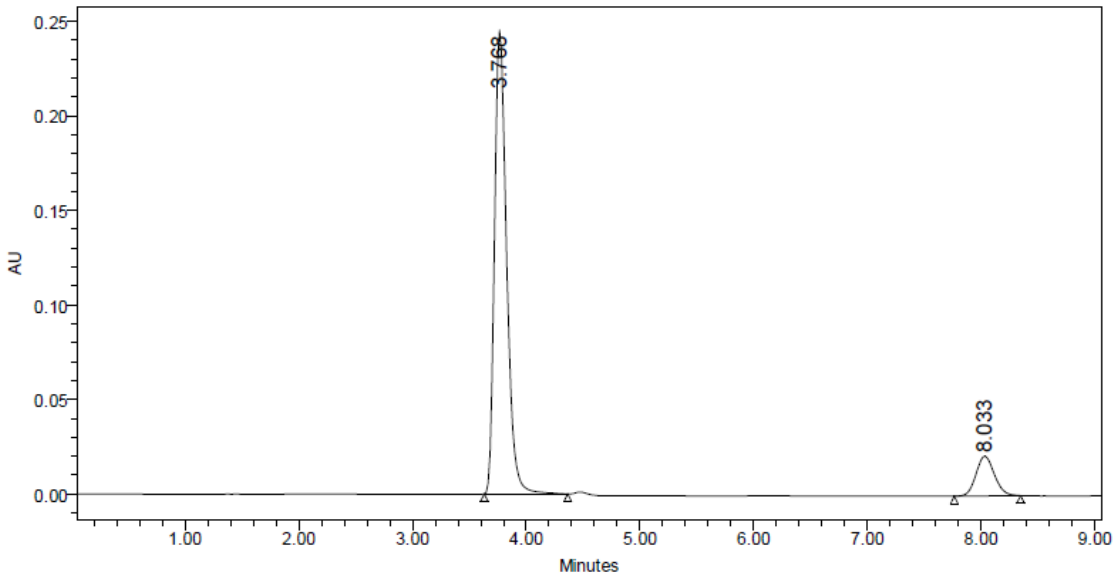

Figure:11 TrialChromatogram for Ambroxol(3.768) and Loratadine(8.033)

From the above Chromatogram it was observed that the Ambroxol and Loratadine peaks was good.

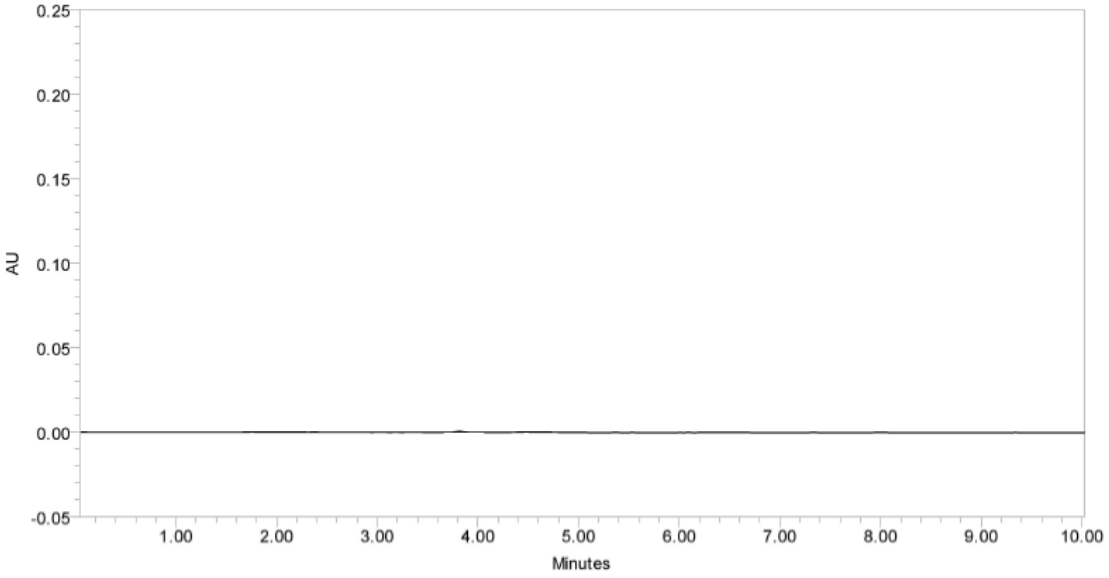

Figure: 12Chromatogram for blank

From the above Chromatogram it was observed that there are no interferences

6.2: SYSTEM SUITABILITY:

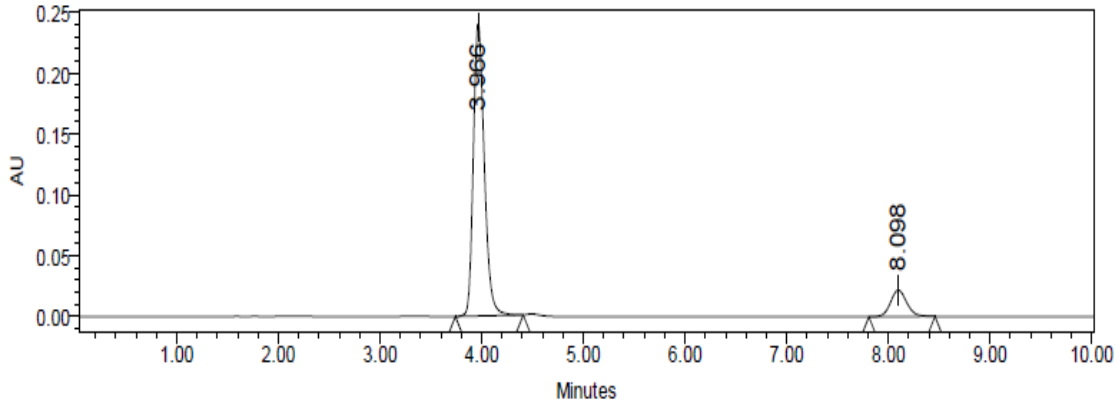

Figure: 12 Chromatogram for system suitability

Table 5: Results of system suitability parameters for Ambroxol and Loratadine

S.No	Name	Retention time(min)	Area (µV sec)	Height (µV)	USP resolution	USP tailing	USP plate count
1	Ambroxol	4.059	1805645	220971		1.2	5708.3
2	Loratadine	8.098	238977	21777	16.8	1.1	12604.0

Acceptance criteria:

- Resolution between two drugs must be not less than 2
- Theoretical plates must be not less than 2000
- Tailing factor must be not less than 0.9 and not more than 2.
- It was found from above data that all the system suitability parameters for developed method were within the limit.

VALIDATION PARAMETERS:

Precision:

Precision of the method was carried out for both sample and standard solutions as described under experimental work. The corresponding Chromatograms and results are shown below.

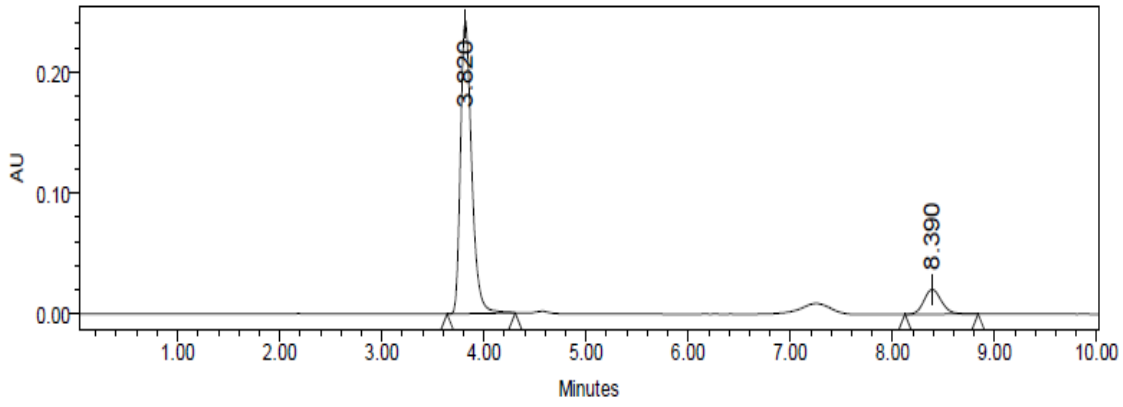

Figure:13Chromatogram for Ambroxol (3.820) and Loratadine (8.390)

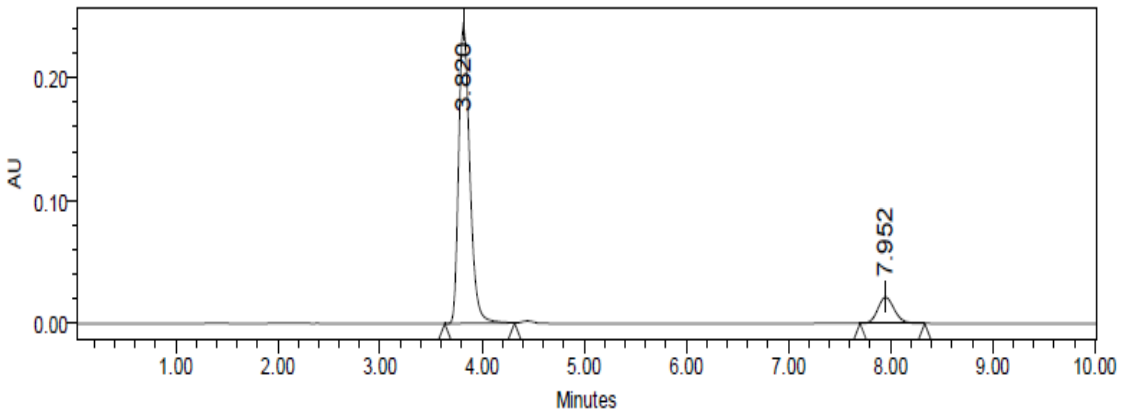

Figure: 14Chromatogram forAmbroxol (3.820) and Loratadine (7.952)

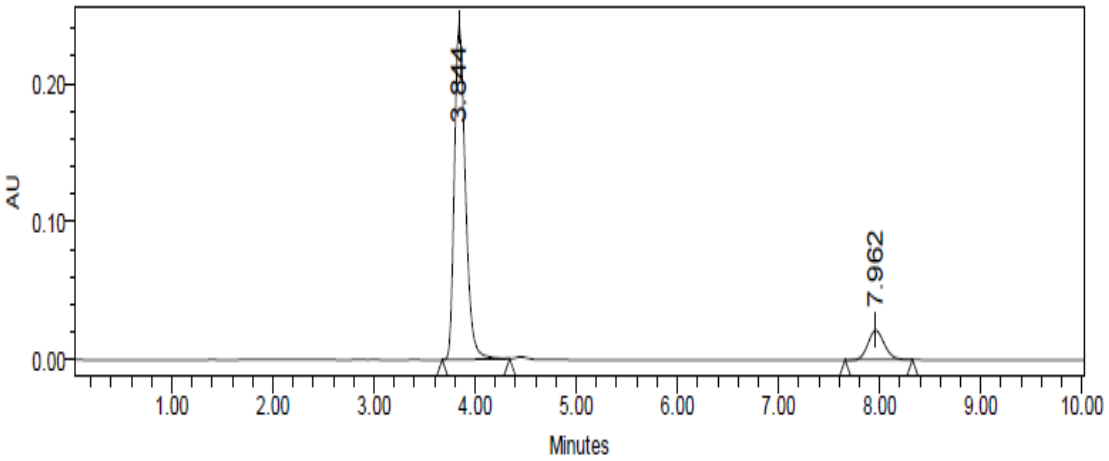

Figure: 15Chromatogram forAmbroxol (3.820) and Loratadine (7.952) Chromatogram forAmbroxol (3.844) and Loratadine (7.962)

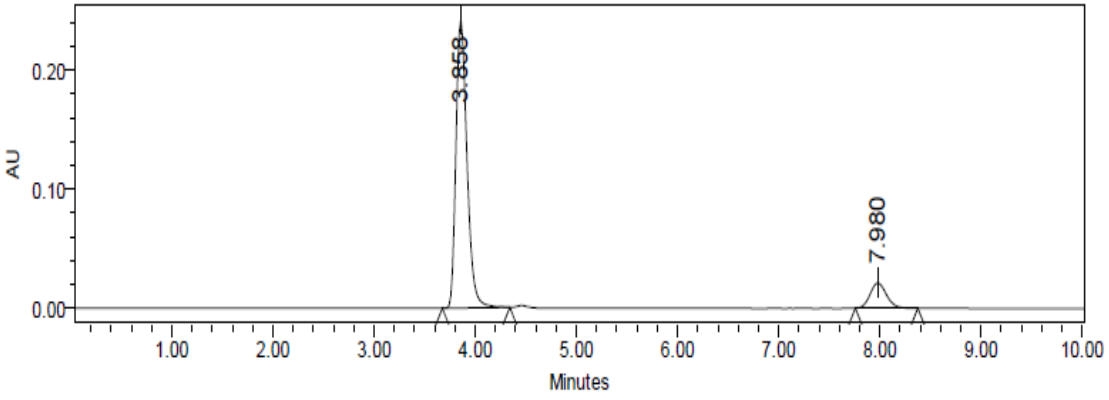

Figure:16Chromatogram forAmbroxol (3.858) and Loratadine (7.980)

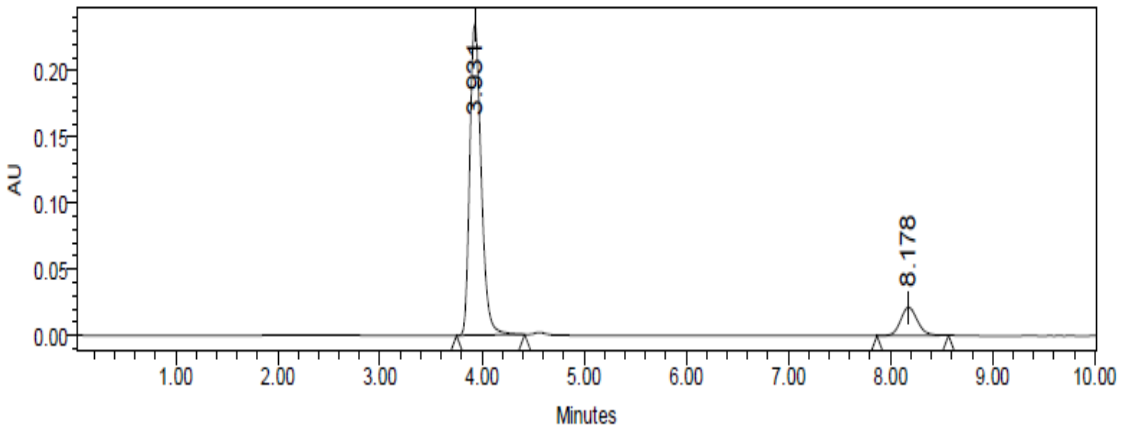

Figure: 17Chromatogram forAmbroxol (3.858) and Loratadine (7.980)
Chromatogram forAmbroxol (3.931) and Loratadine (8.178)

Table 6: Results of precession for Ambroxol

S. No	Standard Area
1	1833968
2	1834448
3	1834848
4	1837718
5	1839859
Average	1836168.1
Std.dev	2528.1
%RSD	0.1

Table 7: Results of method precession for Loratadine

S. No	Standard Area
1	238740
2	239348
3	240459
4	240476
5	240777
Average	239960
Sta.Dev	872.8
%RSD	0.4

Acceptance criteria:

- %RSD for sample should be NMT 2
- The %RSD for the standard solution is below 1, which is within the limits hence method is precise.

INTERMEDIATE PRECESSION (ruggedness)

There was no significant change in assay content and system suitability parameters at different conditions of ruggedness like day to day and system to system variation.

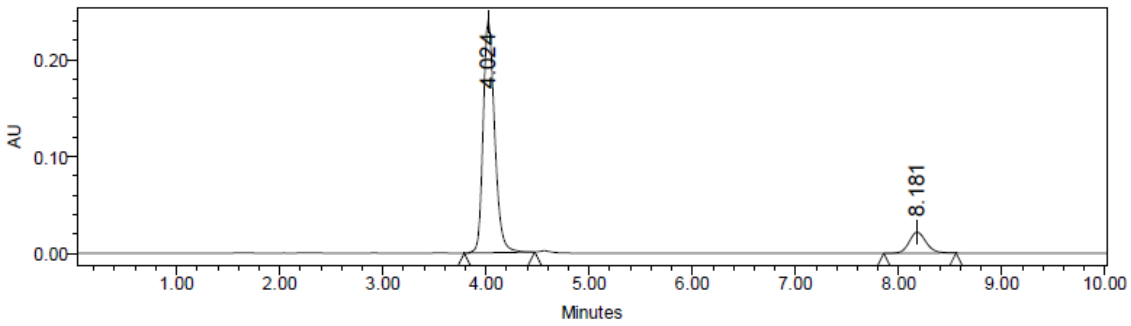

Figure: 18 Chromatogram forAmbroxol (4.024) and Loratadine (8.181)

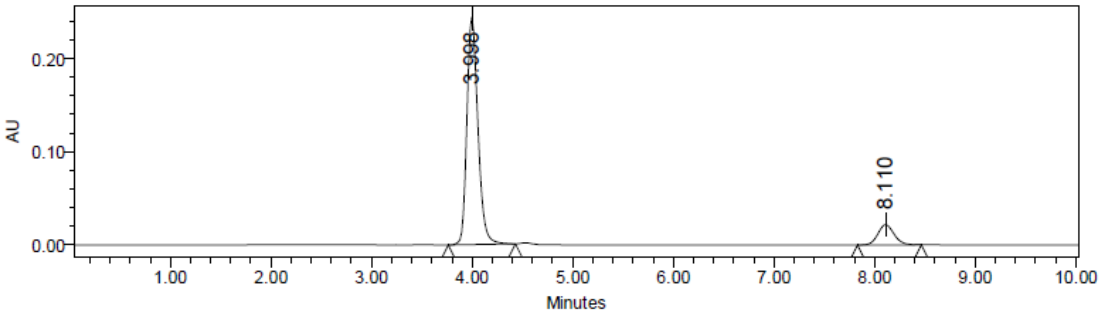

Figure: 19 Chromatogram forAmbroxol (3.996) and Loratadine (8.110)

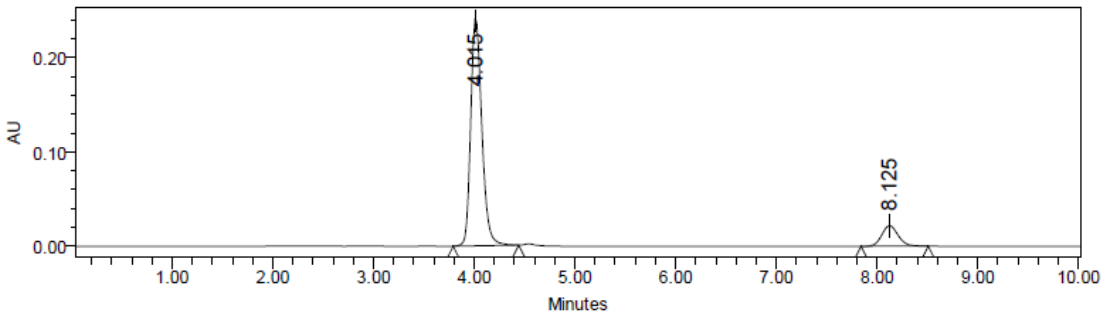

Figure 20: Chromatogram forAmbroxol (4.015) and Loratadine (8.125)

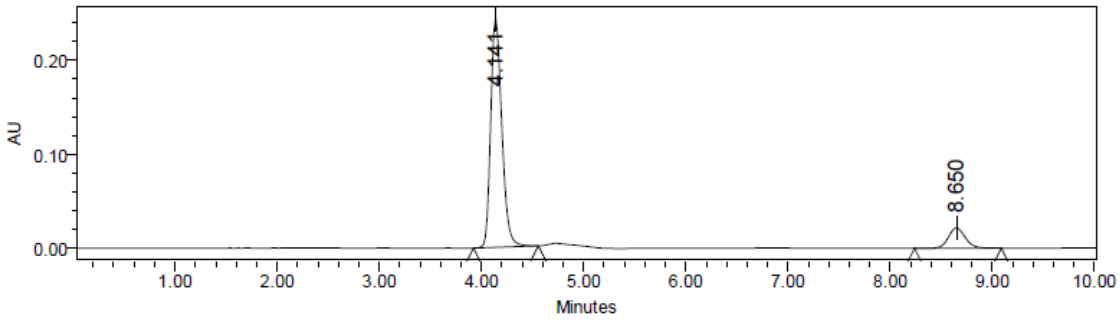

Figure 21: Chromatogram forAmbroxol (4.141) and Loratadine (8.650)

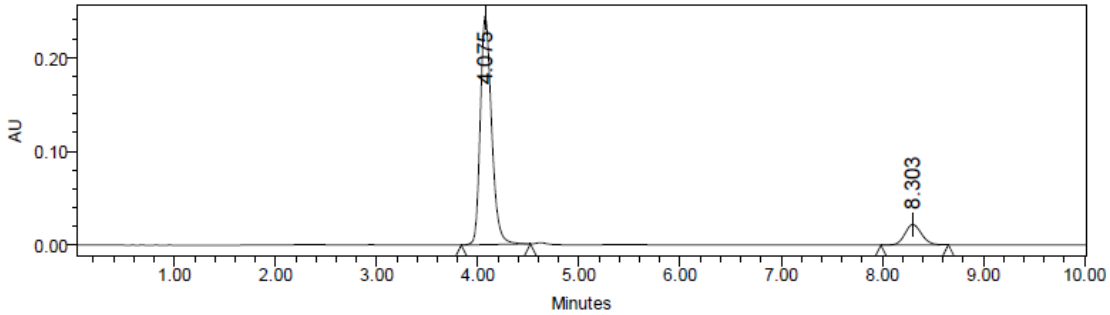

Figure 22: Chromatogram forAmbroxol (4.075) and Loratadine (8.303)

Table 8: Results of Intermediate precision for Ambroxol:

S.No	Standard area
1	1831421
2	1853810
3	1856703
4	1858323
5	1890693
Average	1858189.8
Std.dev	21180.3
%RSD	1.1

Table 9: Results of Intermediate precision for Loratadine

S. No	Standard area
1	242817
2	244893
3	245184
4	245392
5	249068
Average	245470.9
Std.dev	2258.6
%RSD	0.9

Acceptance criteria:

- %RSD of five different sample solutions should not more than 2
- The %RSD obtained is within the limit, hence the method is rugged.

ACCURACY:

Sample solutions at different concentrations (50%, 100%, and 150%) were prepared and the % recovery was calculated.

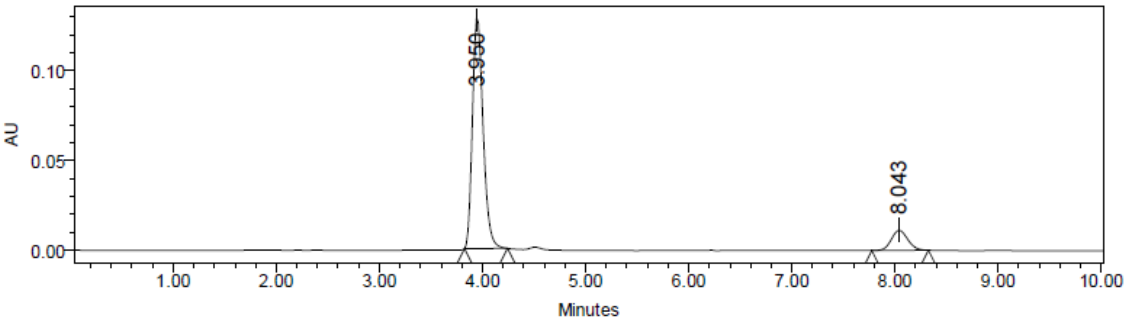

Figure 23: Chromatogram for sample concentration-50%
Ambroxol (3.950) and Loratadine (8.043)

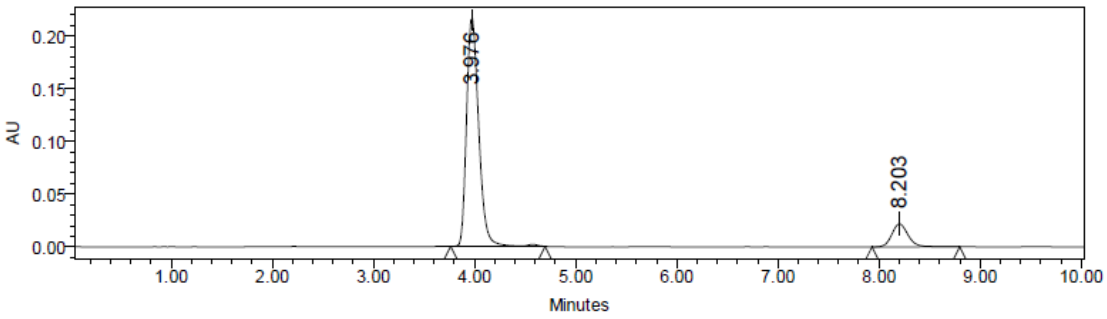

Figure 24: Chromatogram for sample concentration-100% Ambroxol (3.976) and Loratadine (8.203)

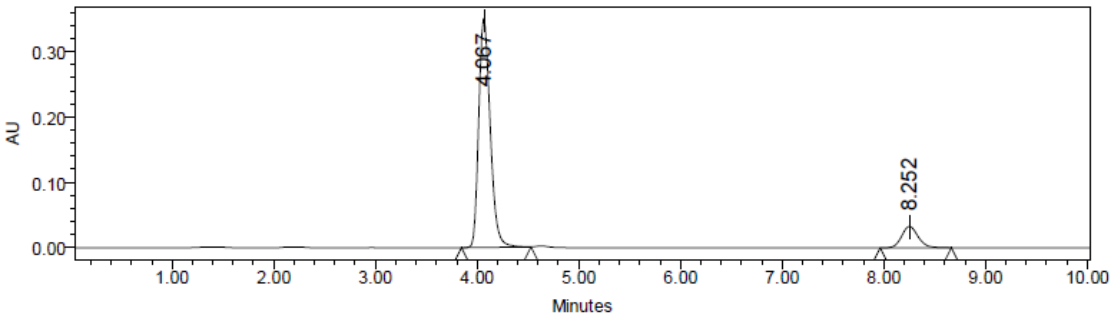

Figure 25: Chromatogram for sample concentration-150% Ambroxol (4.067) and Loratadine (8.252)

Table 10: accuracy (recovery) data for Ambroxol

%Concentration	Area	Amount present (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	925815	30	30.4	101.4%	100.0%
100%	1811753	60	59.5	99.2%	
150%	2727300	90	89.6	99.5%	

Acceptance Criteria:

- The % Recovery for each level should be between 98.0 to 102.0%.

Table 11: accuracy (Recovery) data for Loratadine

%Concentration	Area	Amount present (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	122622.6	2.5	2.5	101.2%	100.4%
100%	241255	5.0	5.0	100.0%	
150%	362274.7	7.5	7.5	100.1%	

Acceptance Criteria:

- The percentage recovery was found to be within the limit (98-102%).

The results obtained for recovery at 50%, 100%, 150% are within the limits. Hence method is accurate

LINEARITY:

The linearity range was found to lie from 48µg/ml to 144µg/ml of Ambroxol, 4µg/ml to 12µg/ml 0f Loratadine and Chromatograms are shown below.

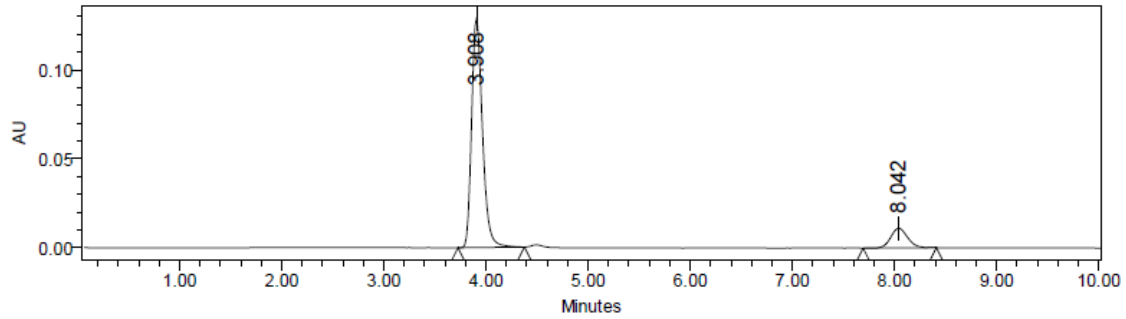

Figure 26: Chromatogram for linearity concentration-48µg/ml of Ambroxol (3.908) & 4 µg/ml of Loratadine (8.042)

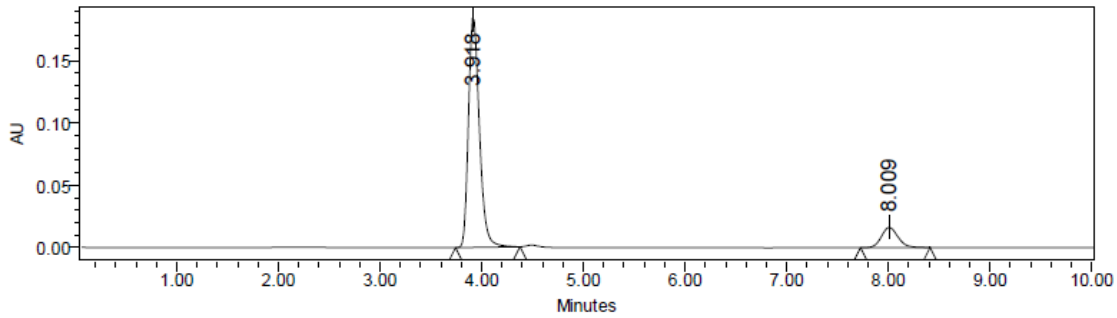

Figure 27: Chromatogram for linearity concentration-72 µg/ml of Ambroxol (3.918) & 6 µg/ml of Loratadine (8.009)

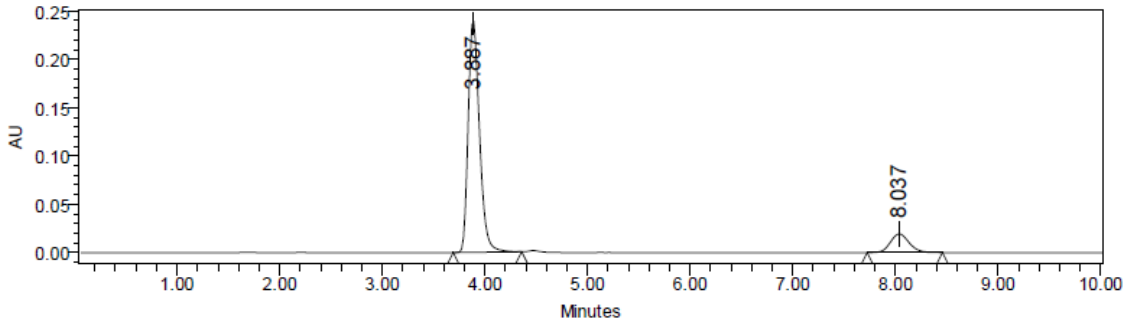

Figure 28: Chromatogram for linearity concentration-96 µg/ml of Ambroxol (3.887) & 8 µg/ml of Loratadine (8.037)

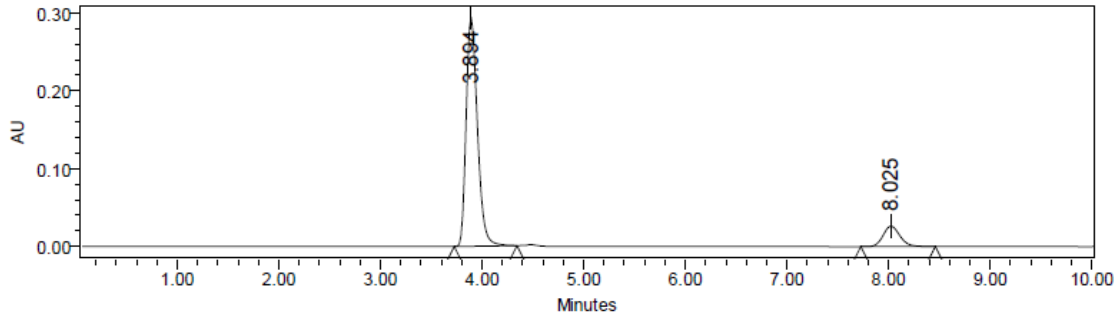

Figure 29: Chromatogram for linearity concentration-120ppm of Ambroxol (3.894) & 10ppm of Loratadine (8.025)

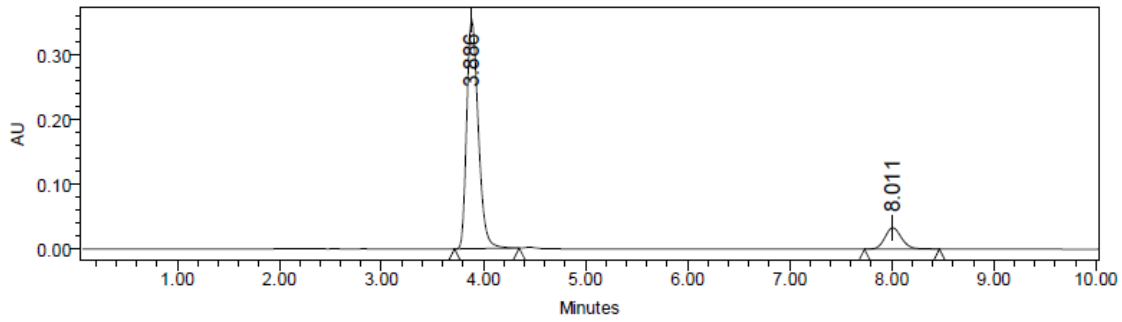

Figure 30: Chromatogram for linearity concentration-144 µg/ml of Ambroxol (3.886) & 12 µg/ml of Loratadine (8.011)

Table 12: Area of different concentration of Ambroxol and Loratadine

Concentrations (µg/ml)	Ambroxol area	Loratadine area
48,4	950849	125627
72,6	1379832	182900
96,8	1833407	246067
120,10	2266343	300717
144,12	2796252	370589

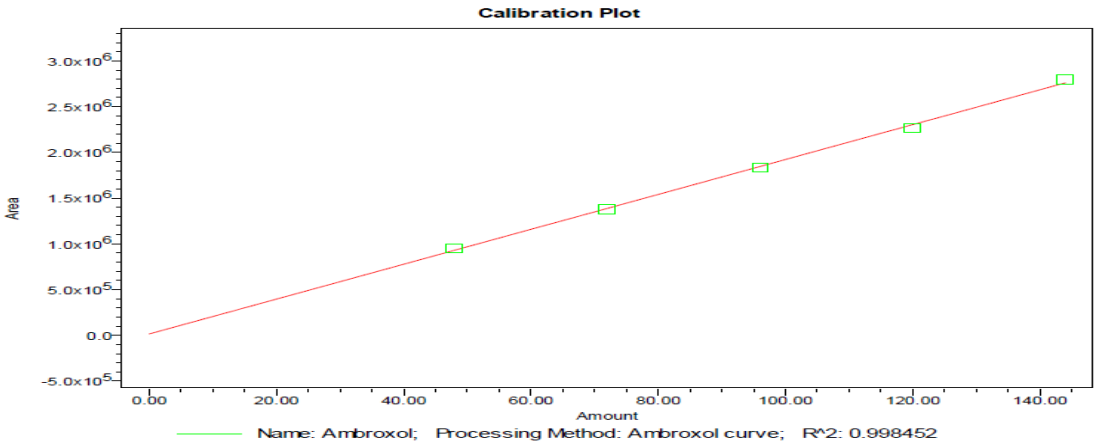

Figure 31: Calibration graph for Ambroxol at 245 nm

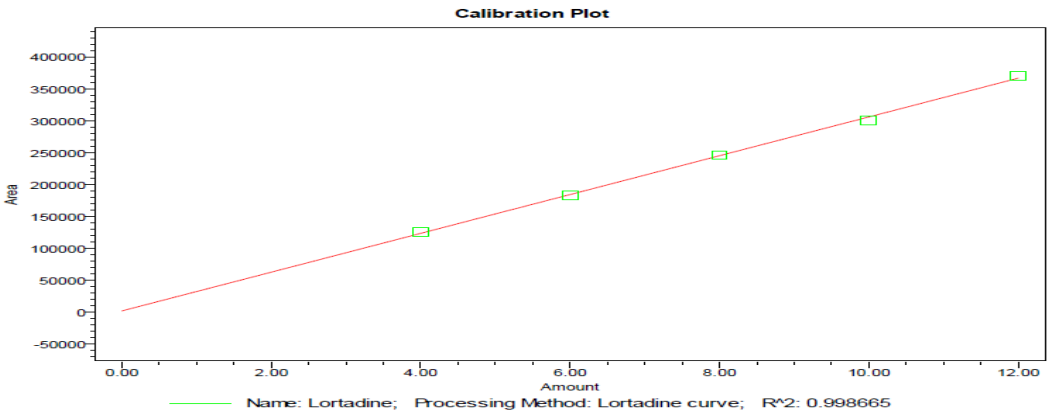

Figure 32: Calibration graph for Loratadine at 245 nm

Table 13: Analytical performance parameters of Ambroxol and Loratadine

Parameters	Ambroxol	Loratadine
Correlation coefficient (R^2)	0.999	0.999

Acceptance criteria:

Correlation coefficient (R^2) should not be less than 0.999

- The correlation coefficient obtained was 0.999 which is in the acceptance limit. The linearity was established in the range of 48% to 144% of Ambroxol and 4% to 12% of Loratadine

LIMIT OF DETECTION FOR AMBROXOL AND LORATADINE

The lowest concentration of the sample was prepared with respect to the base line noise and measured the signal to noise ratio.

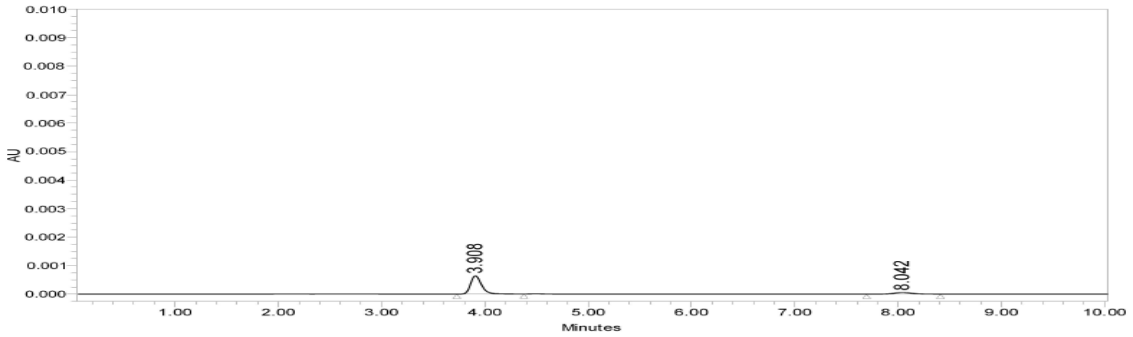

Figure 33: Chromatogram of Ambroxol(3.908) and Loratadine(8.042) showing LOD

Table 14: Results of LOD

Drug name	Baseline noise(µV)	Signal obtained (µV)	S/N ratio
Ambroxol	55	175	3.18
Loratadine	55	158	2.87

- Signal to noise ratio shall be 3 for LOD solution
- The result obtained is within the limit.

LIMIT OF QUANTIFICATION FOR AMBROXOL AND LORATADINE:

The lowest concentration of the sample was prepared with respect to the base line noise and measured the signal to noise ratio.

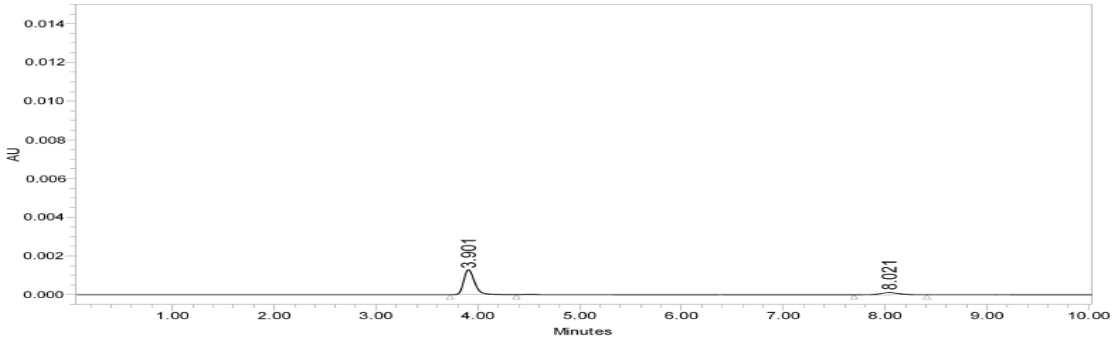

Figure 34: Chromatogram of Ambroxol(3.901) and Loratadine(8.021) showing LOQ

Table 15: Results of LOQ

Drug name	Baseline noise(µV)	Signal obtained (µV)	S/N ratio
Ambroxol	55	569	10.3
Loratadine	55	536	9.74

- Signal to noise ratio shall be 10 for LOQ solution
- The result obtained is within the limit.

ROBUSTNESS:

The standard and samples of Ambroxol and Loratadine were injected by changing the conditions of chromatography. There was no significant change in the parameters like resolution, tailing factor, asymmetric factor, and plate count.

Variation in flow

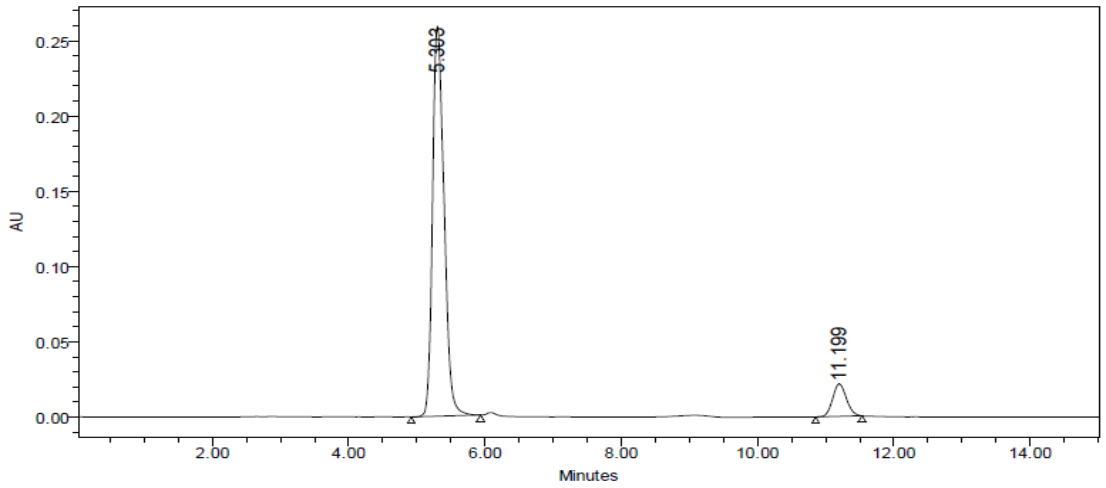

Figure 36: Chromatogram showing less flow of 0.8ml/min

Ambroxol(5.303) and Loratadine(11.199)

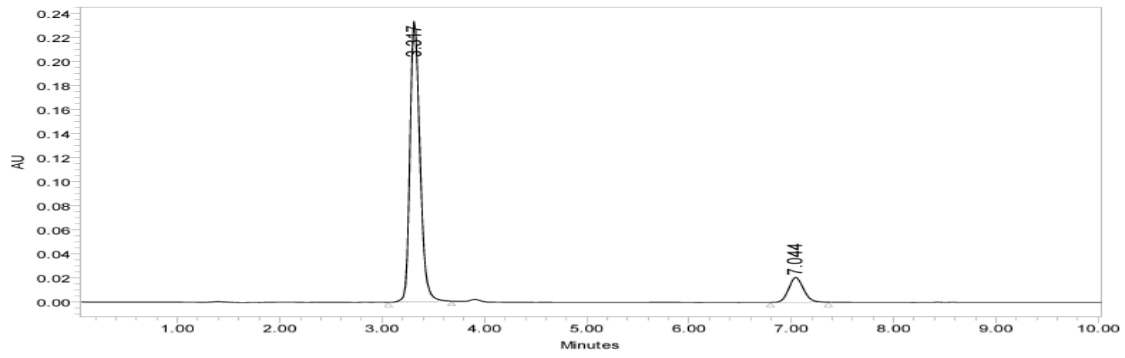

Figure 37: Chromatogram snowing more flow of 1.2ml/min
Ambroxol(3.317) and Loratadine(7.044)

Variation of mobile phase organic composition:

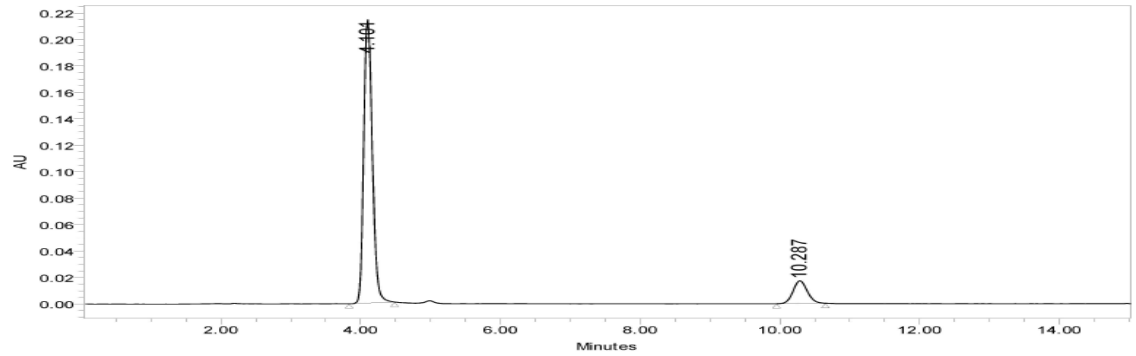

Figure 38: Chromatogram showing less organic composition Ambroxol (4.101) and Loratadine(10.287)

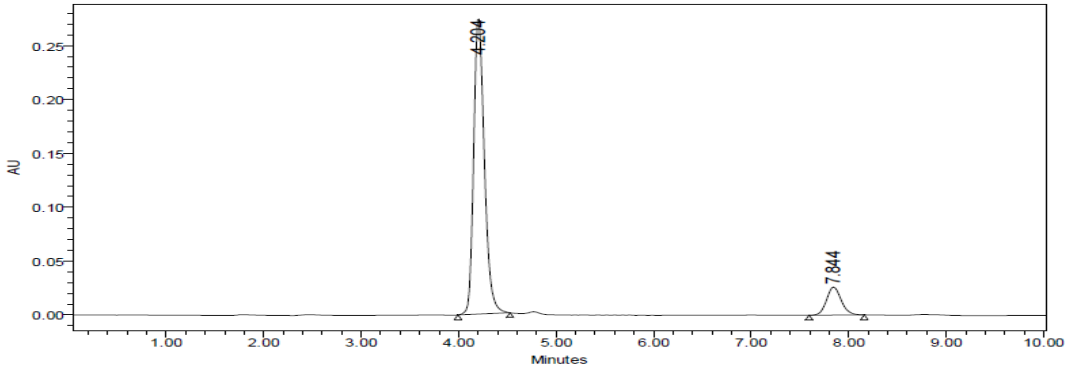

Figure 39: Chromatogram showing more organic composition Ambroxol (4.204) and Loratadine (7.844)

Table 16: Results for variation in flow

S.No	Less flow (0.8 ml/min) peak area		More flow (1.2 mi/min) peak area	
	Ambroxol	Loratadine	Ambroxol	Loratadine
1	3039946	301754	1587014	206493

Table 17: Results for variation in mobile phase composition

S.No	Less organic(65%) peak area		More organic (75%) peak area	
	Ambroxol	Loratadine	Ambroxol	Loratadine
1	1825803	240231	2098504	269544

Acceptance criteria:

Percentage RSD should be below 2.

- The %RSD obtained for change of flow rate, variation in mobile phase was found to be below 1, which is within the acceptance criteria. Hence the method is robust.

7. SUMMARY AND CONCLUSION

High performance liquid chromatography is at present one of the most sophisticated tool of the analysis. The estimation of Ambroxol Hcl and Loratadine was done by RP-HPLC. The Phosphate buffer was p^H 5.0 and the mobile phase was optimized with consists of Acetonitrile: Phosphate buffer mixed in the ratio of 70:30 % v/ v. A C_{18} column C18 (4.6 x 250mm, 5µm, Make: Waters) or equivalent chemically bonded to porous silica particles was used as stationary phase. The detection was carried out using UV detector at 245 nm. The solutions were chromatographed at a constant flow rate of 1.0 ml/min. the linearity range of Ambroxol Hcl and Loratadine were found to be from 96-192 µg/ml.of Ambroxol Hcl and 8-16µg/ml of Loratadine. Linear regression coefficient was not more than 0.999.The values of % RSD are less than 2% indicating accuracy and precision of the method. The percentage recovery varies from 97-102% of Ambroxol Hcl and Loratadine. LOD and LOQ were found to be within limit.The results obtained on the validation parameters met ICH and USP requirements .it inferred the method found to be simple, accurate, precise and linear. The method was found to be having suitable application in routine laboratory analysis with high degree of accuracy and precision.

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