



METHOD DEVELOPMENT AND VALIDATION OF RP – HPLC METHOD FOR DETERMINATION OF CETIRIZINE AND MONTELUKAST IN BULK AND PHARMACEUTICAL FORMULATION

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Abstract :

A precise, rapid, and reproducible Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) method was developed and validated for the simultaneous estimation of Cetirizine and Montelukast in both bulk drug substances and pharmaceutical formulations. The chromatographic separation was carried out using a Phenomenex Luna C18 column (4.6 × 150 mm, 5 µm) maintained at a temperature of 35°C. The optimized mobile phase consisted of Methanol and Water in the ratio of 70:30 v/v, delivered at a flow rate of 1.0 mL/min.

Detection was performed at a wavelength of 230 nm, with a 10 µL injection volume and a total run time of 10 minutes. Both drugs were effectively separated with sharp, symmetrical peaks and satisfactory retention times. The method was validated according to ICH guidelines, demonstrating acceptable results for linearity, accuracy, precision, robustness, specificity, and system suitability.

Index Terms - RP-HPLC, Cetirizine and Montelukast, simultaneous estimation, validation, Phenomenex Luna C18 column, ICH guidelines.

1. INTRODUCTION

Pharmaceutical analysis comprises those procedures necessary to determine “identity, strength, quality and purity of the drug substances and drug products. Pharmaceutical analyst plays a major role in all quality controlling divisions of industry. Analytical chemistry involves separating, identifying, and determining the relative amounts of components in a sample matrix. The number of new drugs is constantly growing. This requires new methods for controlling the quality. Modern pharmaceutical analysis must need the following requirements ¹.

1. The analysis should take a minimal time.
2. The accuracy of the analysis should meet the demands of the Pharmacopoeia.
3. The analysis should be performed with a minimal cost.
4. Precision and selectivity of the selected method should be good.

1.2 Typical Instrumental Techniques ^{2,3}:

The methods of estimation of drugs are divided into physical, chemical, physicochemical and biological ones of them, physical and physicochemical methods are used mostly. Physical methods of analysis involve the studying of the physical properties of a substance. They include determination of the solubility, transparency or degree of turbidity, colour density or specific gravity (for liquids), moisture content, melting, freezing and boiling points. Physicochemical methods are used to study the physical phenomenon that occurs as a result of chemical reactions. Among the physicochemical methods are optical refractometry, polarimetry, emission and fluorescent methods of analysis, photometry including photolorimetry, spectrophotometry, nephelometry and turbidometry, electrochemical (potentiometry, amperometry, coulometer, polarography) and chromatography (column, paper, thin layer, gas, high performance liquid) methods are generally preferable.

Methods involving nuclear reactions such as nuclear magnetic resonance (NMR) and paramagnetic resonance (PMR) are becoming more popular. The combination of mass spectroscopy with gas chromatography is one of the most powerful tools available. The chemical methods include the gravimetric and volumetric procedures, which are based on complex formation, acid-base and precipitation and redox reactions. Titrations in non-aqueous media and complexometry have been widely used in pharmaceutical analysis whenever the existing amounts are in milligram level and the interference is negligible. The methods (LC-MS,⁴ HPLC, GLC, NMR and Mass Spectroscopy) of choice for assay involve sophisticated equipment that are very costly and pose problems of maintenance. Hence, they are not in the reach of most laboratories and small-scale industries, which produce bulk drugs and pharmaceutical formulations.

The visible Spectrophotometric methods which fall in the wavelength region 400-800 nm and fluorimetric methods (may fall in UV & Visible regions) are very simple, cheap and easy to carry out estimations of drugs in bulk form and their formulations. The limitations of many colorimetric or fluorimetric methods of analysis lie in the chemical reactions upon which the procedures are based rather than the instruments available. Many of the reactions involve colour or fluorescence of a drug are quite selective or can be rendered selective through the introduction of masking agents, control of PH, use of solvent extraction technique, adjustment of oxidation states or by prior removal of interfering ingredients with the aid of chromatographic separation.

1. This is preferably followed by general methodology for UV-Visible and HPLC method developments.
2. Followed by literature of drugs used in Analysis

1.3 INTRODUCTION TO HPLC

Russian botanist Tswett invented chromatography as a separation technique. He describes in detail the separation of pigments, the colour substances by filtration through column, followed by developments with pure solvents.

High-performance liquid chromatography (HPLC) ⁵ is the fastest growing analytical technique for analysis of drugs. Its simplicity, high specificity and wide range of sensitivity make it ideal for the analysis of many drugs in both dosage forms and biological fluids.

According to IUPAC, chromatography ⁶ is a physical method of separation in which components will be separated or distributed between stationary and mobile phases. The importance of chromatography is increasing rapidly in pharmaceutical analysis for the exact differentiation, selective identification and quantitative determination of structurally closely related compounds. Another important field of application of chromatographic methods is the purity testing of final products and the intermediates. The reasons for the popularity of the method is its sensitivity, its ready adaptability to accurate quantitative determinations, its suitability for separating non-volatile species or thermally fragile ones and its wide spread applicability to substances that are of prime interest to the industry. Sensitive detectors have transformed liquid column chromatography into high speed, efficient, accurate and highly resolved method of separation.

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

- ❖ Speed (many analysis can be accomplished in 20 min or less)
- ❖ Greater sensitivity (various detectors can be employed)
- ❖ Improved resolution (wide variety of stationary phases)
- ❖ Reusable columns (expensive columns but can be used for many analysis)
- ❖ Ideal for the substances of low viscosity
- ❖ Easy sample recovery, handling and maintenance.
- ❖ Instrumentation leads itself to automation and quantification (less time and less labour)
- ❖ Precise and reproducible
- ❖ Integrator itself does calculations.

1.5 INSTRUMENTATION⁷

- ❖ The essential parts of the High Performance Liquid Chromatography are:
 - 1) Solvent reservoir and Treatment system
 - 2) Mobile phase
 - 3) Pump system¹⁷
 - 4) Sample Injection System

5) Column

6) Detector

1.6 HPLC METHOD DEVELOPMENT: ^{9, 10}

A good method development strategy should require only as many experimental runs as are necessary to achieved the desired final result. Finally, method development should be as simple as possible, and it should allow the use of sophisticated tools such as computer modelling. During initial method development, a set of initial conditions (detector, column, mobile phase) is selected to obtains the first “scouting” chromatograms of the sample. In most cases, these are based on reversed-phase separation on a C₁₈ column with UV detection. The important factors, which are to be taken into account to obtains reliable quantitative analysis, are

- ❖ Careful sampling and sample preparation.
- ❖ Appropriate choice of the column.
- ❖ Choice of the operating conditions to obtains the adequate resolution of the mixture.
- ❖ Reliable performance of the recording and data handling systems.
- ❖ Suitable integration/peak height measurement technique.
- ❖ The mode of calculation best suited for the purpose.
- ❖ Validation of the developed method.

Before beginning method development, it is need to review what is known about the sample in order to define the method goals.

STEPS IN HPLC METHOD DEVELOPEMENT²⁰

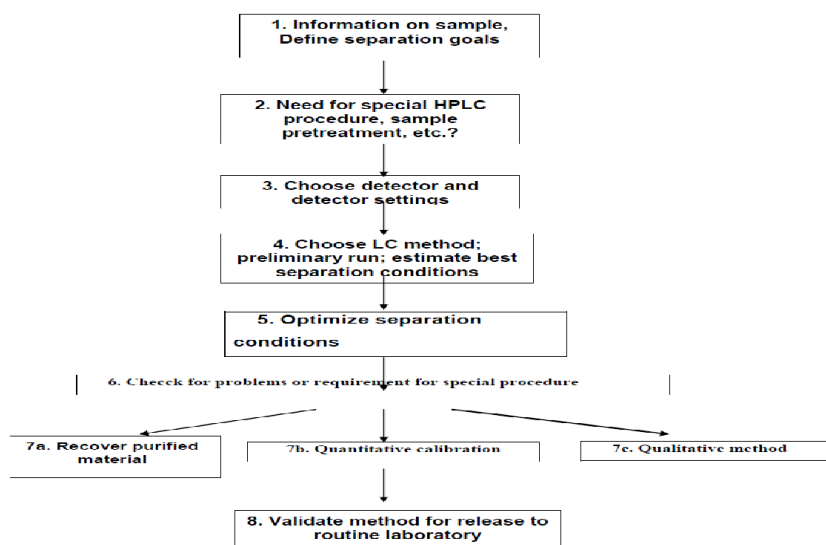


Fig-: Steps in HPLC Method Development

1.6.1 SAMPLE AND ANALYTE INFORMATION¹¹

This information is useful for the selection of appropriate sample preparation procedures as well as the initial detection and chromatographic modes. If critical data are not available (e.g., pKa, solubility), separate studies should be initiated as soon as possible. The chemical structure of the analyte furnishes data on molecular weight and the nature of the functional groups. Particular attention should be directed to acidic, basic, aromatic, or reactive functional groups from which estimates of pKa, solubility, chromophoric, or stability data can be inferred. If sufficient purified reference material is available, solubility studies of the analyte in common solvents such as water, alcohol, ether, and hexane should be conducted. Toxicity data and Material Safety Data Sheets (MSDS), COA including spectral data (MS, NMR, IR, and UV) should be ascertained. The chemical composition of the sample can provide valuable clues for the best choice of initial conditions for an HPLC separation.

1.6.2 SELECTION OF CHROMATOGRAPHIC MODE¹¹

Reversed-phase chromatography (RPC), the most common mode for small organic molecules. Note that ionisable compounds (acids and bases) are often separated by RPC with buffered mobile phases (to keep the analyte in a non-ionized state) or with ion-pairing reagents. In reverse phase mode, the mobile phase is comparatively more polar than the stationary phase. For the separation of polar or moderately polar compounds, the most preferred mode is reverse phase. The nature of the analyte is the primary factor in the selection of the mode of separation. A second factor is the nature of the matrix. The choice of chromatographic mode was given in table no 1.6.2a

Table no: 1.1. Guideline for Chromatographic Mode Selection

Sample type	Analytes type	Common mode
Macromolecules	Organic polymers	GPC
(MW > 2,000)	Bio molecules HILIC,	SEC, RPC, IEC,
Organics	Polar HILIC	RPC, NP,
(MW < 2,000)	Medium polarity	RPC
	Nonpolar	RPC, NARP, NP
Preparative	All	NP, RPC, GPC, IEC

1.6.3 SAMPLE PREPARATION¹²

Samples come in various forms

- ❖ Solutions ready for injection
- ❖ Solutions that require dilution, buffering, addition of an internal standard or other volumetric manipulation
- ❖ Solids must be dissolved or extracted

- ❖ Samples that require pre-treatment to remove interferences and/or protect the column or equipment from damage.

Most Samples for HPLC analysis require weighing and /or volumetric dilution before injection. Best results are often obtained when the composition of the sample solvent is close to that of the mobile phase since this minimizes baseline upset and other problems. Some sample require a particles separation (pre-treatment) prior to HPLC, because of need to remove interferences, concentrate sample analytes or eliminate “column killers”. Whereas special sample are better separate under customized conditions.

Table No: 1.2 Requirements of Sample Selection

Sample	Requirements
Inorganic ions	Detection is primary problem; use ion chromatography.
Isomers	Some isomers can be separated by reversed-phase HPLC and are then classified as regular samples; better separations of isomers are obtained using either (1) normal-phase HPLC or (2) reversed-phase separations with cyclodextrin-silica columns.
Enantiomers	These compounds require “chiral” conditions for their separation.
Biological	Several factors make samples of this kind “special”: molecular conformation, polar functionality, and a wide range of hydrophobicity.
Macromolecules	“Big” molecules require column packing’s with large pores ($>>10$ -nm diameters); in addition, biological molecules require special conditions as noted above.

1.6.4 CHOICE OF THE COLUMN

Selection of the column is the first and the most important step in method development. The appropriate choice of separation column indicates three different approaches.

- Selection of separation.
- The particle size and nature of the column packing.
- The physical parameters of the column i.e. the length and the diameter.

Some of the important parameters considered while selecting chromatographic columns are

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

- Size of the particles
- % of Carbon loading
- Pore volume
- Surface area
- End capping

In this case, the column selected had a particle size of 5µm and an internal diameter of 4.6mm. The column is selected depending on the nature of the solute and the information about the analyte. Reversed phase mode of chromatography facilities a wide range of columns covering wide range of polarities by cross linking silanol groups with alkyl chains like dimethyl silane (C₂), butylsilane (C₄), octylsilane (C₈), octadecylsane (C₁₈), base deactivated silane (C₁₈), phenyl, cyanopropyl (CN), nitro, amino etc. Silica based columns with different cross linkings in the increasing order of Polarity are as follows:

<..... Non-polar moderately polar..... Polar.>

C₁₈ < C₈ < C₆ < Phenyl < Amino < Cyano<nitrile < Silica

C₈ was chosen for this study since it is most retentive one. Generally longer columns provide better separation due to higher theoretical plate numbers. Columns with 5µm particle size give the best compromise of efficiency.

Table No: 1.3 Selection of Column

Sample		LC mode	Column choice
		Reverse Phase-ion pair (allows neutral and charged compounds to be simultaneously analyzed)	C ₁₈ , C ₈ , C ₆ , C ₄ , C ₂ , TMS, CN, amino (not for carbonyl compounds), phenyl, Hamilton PRP-1 (pH 1-13)
	Basic or Acidic		
		Ion suppression	C ₁₈ , C ₈ , C ₆ , C ₄ , C ₂ , TMS, CN, amino (not for carbonyl compounds), phenyl, Hamilton PRP-1 (pH 1-13)
Ionizable		Ion Exchange	
		Anionic	Strong Anion exchange

Sample		LC mode	Column choice
		Cationic	Strong Cation exchange
		Normal phase	Increasing polarity of bonded phases diol
			CN
Neutral			NH ₂
			Silica
		Reverse phase	Alumina
			Increasing polarity of bonding phase
			C-18
			C-8
			Phenyl

1.6.5 SELECTION OF COLUMN TEMPERATURE

Always carry out chromatographic separations at ambient temperature. The increase in column temperature generally will result in reduction of asymmetry and peak retention. The column temperature between 30°C – 80°C shall be adopted if necessary. If a column temperature above 80°C is required, packing material which can be with stand to that temperature was preferable.

1.6.6 SELECTION OF SOLVENT DELIVERY SYSTEM

Chromatographic separation with isocratic elution i.e. all constituents of the mobile phase is mixed and pumped together as a single solvent, is always preferable however, gradient elution is powerful tool in achieving separation between closely eluting compounds or compounds having widely differing in polarities.

The important feature of gradient elution is that the polarity and ionic strength of the mobile phase can be changed during run. The mobile phases are introduced into column by two different ways, low pressure and high pressure gradient systems. Low pressure gradient can be adopted when not more than 80% of organic phase is to be pumped or vice-versa. While optimizing gradient elution especially low viscous solvents like acetonitrile and phosphate buffer, it is recommended to mix about 10% aqueous portion preferably the same buffer used in mobile phase to avoid pumping problems.

The gradient programme which is rugged for organic phase up to 10% variation and up to 0.2 ml variation in flow rate was selected.

1.6.7 SELECTION OF MOBILE PHASE¹³

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

In liquid chromatography, the solute retention is governed by the solute distribution factor, which reflects the different interactions of the solute-stationary phase, solute-mobile phase, and mobile phase-stationary phase. For a given stationary phase, the nature and the composition of which has to be judiciously selected in order to get appropriate and required solute retention. The mobile phase has to be adapted in terms of elution strength (solute retention) and solvent selectivity (solute separation). Solvent polarity is the key word in chromatographic separations since a polar mobile phase will give rise to low solute retention in normal phase and high solute retention in reverse phase LC. The selectivity will be particularly altered if the buffer pH is close to the pKa of the analytes. The following are the parameters, which shall be taken into consideration while selecting and optimizing the mobile phase.

- Buffer and its strength
- pH of the buffer or pH of the mobile phase
- Mobile phase composition

A. BUFFERS IF ANY AND IT'S STRENGTH

Buffer and its strength play an important role in deciding the peak symmetries and separations. Some of the most commonly employed buffers are

- Phosphate buffers prepared using salts like KH_2PO_4 , K_2HPO_4 , NaH_2PO_4 , Na_2HPO_4 etc.
- Phosphoric acid buffers prepared using H_3PO_4 .
- Acetate buffers-Ammonium acetate, Sodium acetate etc.
- Acetic acid buffers prepared using CH_3COOH .

The retention also depends on the molar strengths of the buffer-molar strength is inversely proportional to the retention times. Ideally the strength of the buffer shall be opted between 0.01M-0.20M after selecting the strength of the buffer, it can be varied by about 10-20% and the effect of variation was studied and it should be rugged for at least 2% variation in strength. It is important to maintain the pH of the mobile phase in the range of 2.0 to 8.0 as most columns does not withstand to the pH which are outside this range. This is due to the fact that the siloxane linkages are cleaved below pH 2.0, while pH values above 8.0 the silica may dissolve.

1.6.8 SELECTION OF THE BUFFER

Optimum buffering capacity occurs at a pH equal to the pKa of the buffer. Almost all of the pH related change in retention occurs for pH values within ± 1.5 units of pKa value. Outside this range the compound is either ionized or unionized, and its retention doesn't change much with pH.

Table No: 1.4 COMMONLY USED BUFFERS FOR RPHPLC

Buffer	pKa	Buffer Range	UV Cutoff (nm)
Phosphate	2.1 7.2 12.3	1.1 – 3.1 6.2 – 8.2 11.3 – 13.3	200
Formic acid*	3.8	2.8 – 4.8	210
Acetic acid*	4.8	3.8 – 5.8	210
Citrate	3.1 4.7 5.4	2.1 – 4.1 3.7 – 5.7 4.4 – 6.4	230
Tris	8.3	7.3 – 9.3	205
Triethylamine*	11.0	10.0 – 12.0	200
Pyrrolidine	11.3	10.3 – 12.3	200

* Volatile buffers

Table No: 1.4 COMMONLY USED BUFFERS FOR RPHPLC

1.6.9 SELECTION OF FLOW RATE

Generally flow rate shall not be more than 2.0 ml/min. the flow rate shall be selected based on the following data.

- ✓ Retention time
- ✓ Column back pressure
- ✓ Resolution between the peaks
- ✓ Peak symmetries

The flow rate which gives least retention times, good peak symmetries, least back pressures and better separation will be selected.

1.6.10 SELECTION OF DETECTOR

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

- ✓ High sensitivity facilitating trace analysis
- ✓ Negligible baseline noise to facilitate lower detection
- ✓ Large linear dynamic range
- ✓ Low dead volume
- ✓ Ease in calibration and standardization

- ✓ Inexpensive to purchase and operate

Pharmaceutical ingredients do not absorb all UV light equally, so that selection of detection wavelength is important. An understanding of the UV light absorptive properties of the organic impurities and the active pharmaceutical ingredient is very helpful. Generally LC equipped with PDA detector was the first choice. UV source like mercury vapour lamp is most widely used because majority of the compounds of pharmaceutical interest absorb at 250 – 270 nm and this lamp has an intense line spectrum at this region.

For the greatest sensitivity λ_{max} should be used. Ultra violet wavelengths below 200nm should be avoided because detector noise increases in this region. Higher wavelengths give greater selectivity.

1.7 ANALYTICAL METHOD VALIDATION^{14, 15}

Method Validation can be defined as per ICH, “Establishing documented evidence, which provides a high degree of assurance that a specific activity will consistently produce a desired result or product meeting its predetermined specifications and quality characteristics”. For chromatographic methods used in analytical application there is more consistency in validation practice. The Q (2) R1 of ICH guidelines for validation for analytical procedures includes the following parameters.

1.7.1 SPECIFICITY

An investigation of specificity should be conducted during the validation of identification tests, the determination of impurities.

An ICH document defines specificity as the ability to assess unequivocally the analyte in the presence of compounds that may be expected to be present in products and matrix components.

The definition has the following implications:

Identification test: Suitable identification tests should be able to discriminate compounds of closely related structure which are likely to be present. Ensure identity of an analyte. The analyte should have no interference from other extraneous components and be well resolved from them.

Purity Test: To ensure that all the analytical procedures performed allow an accurate statement of the content of impurity of the content of impurity of an analyte i.e. related substances test, heavy metals, residual solvents etc.

Assay: To provide an exact result, this allows an accurate statement on the content or potency of the analyte in a sample.

1.7.2 LINEARITY

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. A linear relationship should be evaluated across the range of the analytical procedure. It may be demonstrated directly on the drug substance (by dilution of a standard stock solution) and/or separate weighing of synthetic mixtures of the drug product components, using the proposed procedure. Linearity should be evaluated by visual inspection of a plot of signals as a function of analyte concentration or content. If there is a linear relationship, test results should be evaluated

by appropriate statistical methods, for example, by calculation of a regression line by the method of least squares.

Recommendations

The linearity range for examination depends on the purpose of the test method. For example, the recommended range for an assay method for content would be $\text{RSD} \pm 2\%$ and the range for an assay/impurities combination method based on area % (for impurities) would be $\pm 2\%$ of target concentration down to the limit of quantification of the drug substance or impurity. Under most circumstances, regression coefficient (r) is 0.999. Intercept and slope should be indicated

1.7.3 RANGE

Range is the interval between the upper and lower concentration of the analyte in the sample for which it has a suitable level of precision, accuracy and linearity.

Range is derived from linearity studies in the method validation procedure. Some parameters with their range are shown in table No: 1.5

Table No: 1.5 Range parameters

Parameters	Range
Assay (Drug substance and finished product)	80-120%
Content uniformity	70-130%
Dissolution testing	$\pm 20\%$

1.7.4 ACCURACY

The accuracy is the closeness of the measured value to the true value for the sample. The ICH documents recommended that accuracy should be assessed using a minimum of nine determinations over a minimum of three concentrations levels the specified range (i.e, three concentrations and three replicates of each concentration)

Accuracy was tested (% Recovery and % RSD of individual measurement) by analyzing samples at least in triplicate, at each level (80,100 and 120 % of label claim) is recommended. For each determination fresh samples were prepared and assay value is calculated. Recovery was calculated from regression equation obtained in linearity study. Accuracy was determined from the mean relative error for a set of replicate analysis (i.e. the difference between measured and nominal concentration) for spiked samples.

Recommendations:

Accuracy should be assessed using a minimum of 9 determinations over a minimum of 3 concentration levels covering the specified range (e.g. 3 concentrations /3 replicates each of the total analytical procedure). Accuracy should be reported as percent recovery by the assay of known added amount of analyte in the sample or as the difference between the mean and the accepted true value together with the confidence intervals.

1.7.4 PRECISION

Precision is the measure of how close the data values are to each other for a series of measurement under the same analytical conditions obtained from multiple sampling of the same homogeneous sample. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility.

A. Repeatability

1. Injection repeatability

The sensitivity or precision as measured by multiple injections of a homogeneous sample (prepared solution) indicates the performance of the HPLC instrument under the chromatographic conditions and day tested. The information is provided as part of the validation data and as a system suitability test. The specification, as the coefficient of variation in % or relative standard deviation (RSD), set here will determine the variation limit of the analysis. The tighter the value, the more precise or sensitive to variation one can expect the results. This assumes that the chromatograph does not malfunction after the system suitability testing has been performed. The set of four duplicate samples were injected sequentially. Variation in peak area and drift of retention times are noted.

Recommendations

As part of methods validation, a minimum of 10 injections with an RSD of 2% is recommended. With the methods for release and stability studies, an RSD of 2% for precision of the system suitability tests for at least five injections ($n=5$) for the active drug either in drug substance or drug product is desirable. For low-level impurities, higher variation may be acceptable.

2. Analysis repeatability

Determination, expressed as the RSD, consists of multiple measurement of a sample by the same analyst under the same analytical conditions. For practical purpose, it is often combined with accuracy and carried out as a single study.

B. Intermediates precision

Intermediates precision was previously known as part of ruggedness. The attribute evaluates the reliability of the method in a different environment other than that used during development of the method. The objective is to ensure that the method will provide the same results when similar samples are analyzed once the method development phase is over. Depending on time and resources, the method can be tested on multiple days, analyst, instruments, etc.

Intermediates precision in the test method can be partly assured by good system suitability specifications. Thus, it is important to set tight, but realistic, system suitability specifications.

Recommendations

As a minimum, data generated as described under accuracy, for two separate occasions, is recommended to indicate the intermediate precision of the test method.

C. Reproducibility

As defined by ICH, reproducibility expresses the precision between laboratories as in collaborative studies. Multiple laboratories are desirable but not always attainable because of the size of the firm.

Recommendations

It is not normally expected if intermediate precision is accomplished.

1.7.5 LIMIT OF DETECTION

These limits are normally applied to related substances in the drug substance or drug product. Specifications on these limits should be provided by the regulatory authorities.

Limit of detection is the lowest concentration of analyte in a sample which can be detected, but not necessarily quantitated, as an exact value under the stated experimental conditions. With UV detectors, it is difficult to assured the detection precision of low-level compounds due to potential gradual loss of sensitivity of detector lamps with age, or noise level variation by detector manufacturer. At low levels, assurance is needed that the detection and quantification limits are achievable with the test method each time. With no reference standard for a given impurity or means to assured detectability, extraneous peak(s) could "disappear/appear." A crude method to evaluate the feasibility of the extraneous peak detection is to use the percentage claimed for detection limit from the area counts of the analyte. Several approaches for determining the detection limit are possible, depending on whether the procedure is a non-Instrumental or Instrumental.

A. Based on Visual Evaluation

Visual evaluation may be used for non-Instrumental methods but may also be used with Instrumental methods. The detection limit is determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected.

B. Based on Signal-to-Noise

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

C. Based on the Standard Deviation of the Response and the Slope

The detection limit (DL) may be expressed as:

$$DL = \frac{3.3 \sigma}{S}$$

Where,

σ = the standard deviation of the response

S = the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte.

Recommendations:

The detection limit and the method used for determining the detection limit should be presented. If DL is determined based on visual evaluation or based on signal to noise ratio, the presentation of the relevant chromatograms is considered acceptable for justification.

In cases where an estimated value for the detection limit is obtained by calculation or extrapolation, this estimate may subsequently be validated by the independent analysis of a suitable number of samples known to be near or prepared at the detection limit.

1.7.6 LIMIT OF QUANTIFICATION

Limit of quantification is the lowest concentration of analyte in a sample which can be quantitatively determined with acceptable precision and accuracy under the stated experimental conditions. Several approaches for determining the quantification limit are possible, depending on whether the procedure is a non-Instrumental or Instrumental. Approaches other than those listed below may be acceptable.

A. Based on Visual Evaluation

Visual evaluation may be used for non-Instrumental methods but may also be used with Instrumental methods. The quantification limit is generally determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision.

B. Based on Signal-to-Noise Approaches

This approaches can only be applied to analytical procedures that exhibit baseline noise. Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and by establishing the minimum concentration at which the analyte can be reliably quantified. A Typical signal-to-noise ratio is 10:1.

C. Based on the Standard Deviation of the Response and the Slope

The quantification limit (QL) may be expressed as:

$$QL = \frac{10 \sigma}{S}$$

Where,

σ = the standard deviation of the response

S = the slope of the calibration curve the slope S may be estimated from the calibration curve of the analyte

Recommendations:

The limit should be subsequently validated by the analysis of a suitable number of samples known to be near or prepared at the quantification limit. Otherwise the information that is expressed as percentage area or height of the drug substance peak from the same HPLC chromatogram will be biased. It should also be noted that the extraneous peak using area count does not consider the detection response that depends on the UV extinction coefficient or absorptivity of the compound.

1.7.7 ROBUSTNESS

Robustness of an analytical method is measure of its capacity to remain unaffected small but deliberate variation in method parameters and provides an indication of its reliability during normal usage.

Testing varying some or all condition:

- Column temperature
- PH of buffer in mobile phase
- Reagents and flow rate

Recommendations:

- Data obtained from studies for robustness, though not usually submitted, are recommended to be included as part of method validation

1.7.8 SYSTEM SUITABILITY

System suitability tests are based on the concept that the equipment, electronics, analytical operations and samples constitute an integral system that can be evaluated as a whole.

According to USP system suitability are an integral part of chromatographic methods. These tests verify that the resolution and reproducibility of the system are adequate for the analysis to be performed. One consequence of the evaluation of robustness and ruggedness should be that a series of system suitability parameters is established to ensure that the validity of the analytical method is maintained whenever used.

The parameters that are affected by the changes in chromatographic conditions are,

- Capacity factor (k')
- Peak asymmetry / tailing factor (A_s)
- Column efficiency (N) and
- Selectivity (α)

A. Capacity factor (k')

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

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

Recommendations

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

B. Tailing factor (T)

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

A measure of the symmetry of a peak, given by the following equation where $W_{0.05}$ is the peak width at 5% height and f is the distance from peak front to apex point at 5% height. Ideally, peaks should be Gaussian in Shape or totally symmetrical.

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

Recommendations

T of ≤ 2

C. Theoretical plate number / Efficiency (N)

A measure of peak band spreading determined by various methods, some of which are sensitive to peak asymmetry. The most common are shown here, with the ones most sensitive to peak Shape shown first:

$$N = \left(\frac{t_R}{\sigma} \right)^2 = \left(\frac{4t_R}{w_b} \right)^2 = 16 \left(\frac{t_R}{w_b} \right)^2$$

Theoretical plate number is a measure of column efficiency. Theoretical plate number is a measure of column efficiency, that is, how many peaks can be located per unit run-time of the chromatogram, where t_R is the retention time for the sample peak and W is the peak width.

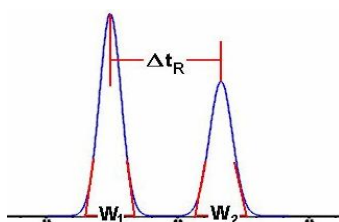
N is fairly constant for each peak on a chromatogram with a fixed set of operating conditions. H , or HETP, the height equivalent of a theoretical plate, measures the column efficiency per unit length (L) of the column. Parameters which can effect N or H include

Recommendations

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

D. Resolution (R_s)

Ability of a column to separate chromatographic peaks, Resolution can be improved by increasing column length, decreasing particle size, increasing temperature, changing the eluent or stationary phase. It can also be expressed in terms of the separation of the apex of two peaks divided by the tangential width average of the peaks.



$$R_s = \Delta t_R / (W_1/2 + W_2/2)$$

Where, $\Delta t_R = t_2 - t_1$ For reliable quantitation, well-separated peaks are essential for quantitation.

Recommendations

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

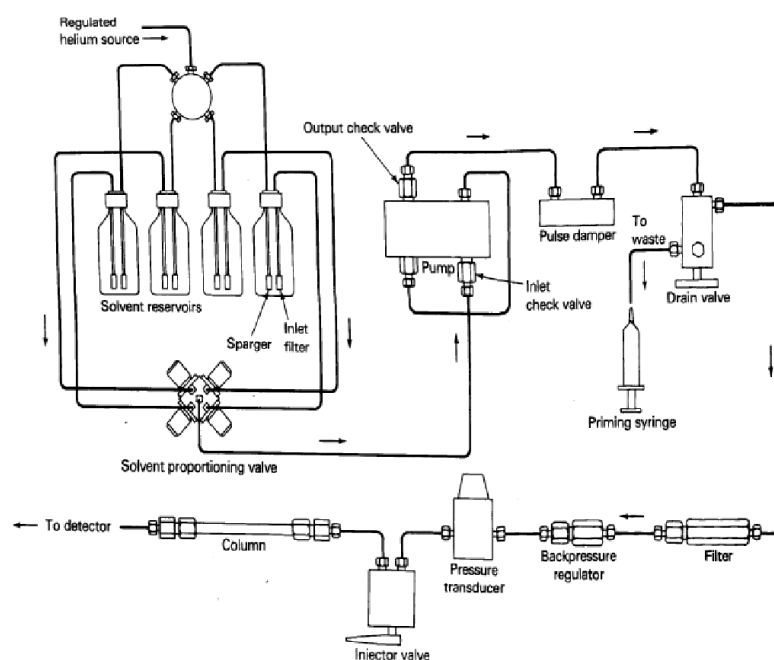


Fig:- Typical diagram of HPLC

2. LITERATURE REVIEW

Chetan Vilasrao Patil et al., (2023) Stability Indicating Rp-Hplc Method Development And Validation For Simultaneous Estimation Of Bilastine And Montelukast In Bulk And Tablet Dosage Form. In the present research work, a successful attempt was made for determination of Bilastine and Montelukast in Bulk and dosage form by high performance liquid chromatography. The method was developed by experimentation, based on literature survey. The simplicity, rapidity, reproducibility and economy of the proposed method completely fulfil the objective of this research work. The HPLC method was developed and validated for simultaneous estimation of Bilastine and Montelukast. The mobile phase was consisting of pH 5.5 Acetate: methanol. The linearity range of Bilastine was found to be 50-250 μ g/ml and Montelukast 25-125 μ g/ml. The calibration curve was plotted and regression equation of Bilastine was found to be $y = 9153.17x + 1658869.30$ with correlation coefficient (r^2) of 0.9996 and Montelukast $y = 22933.18x + 2251569.30$ with correlation coefficient (r^2) of 0.9991. Detection was done at 225 nm and the retention time of Bilastine was found to be 4.3 min and Montelukast 11.3 min with the flow rate of 1.0 ml/min. From accuracy study % recovery of Bilastine was found in the range of 99.57- 100.07% and Montelukast is 99.667- 100.45 % which is in the limits accordingly the ICH guidelines.

Dadi Vasudha et al., (2023) RP-HPLC method for quantification of bilastine and Montelukast sodium in pharmaceutical dosage form. A simple, accurate, precise and robust reverse phase high performance liquid chromatographic (RP-HPLC) method was developed and validated for the simultaneous estimation of Bilastine and Monteleukast Sodium in bulk and pharmaceutical dosage form. Chromatographic separation

was performed on an Zodiac sil RP C18 column (100mm × 4.6mm, 3µm) with mobile phase consist a mixture of 75:25% v/v acetonitrile: phosphate Buffer pH adjusted to 5.2 with ortho-phosphoric acid in an isocratic elution mode, at a flow rate of 1 ml/min. The detection was monitored at 272nm. The retention time of Monteleukast Sodium and Bilastine were found to be 3.668 min and 2.746 min respectively. Linearity range was found between 20-100µg/ml for Bilastine with correlation coefficient of 0.999 and 5-25µg/ml for Montelukast Sodium with correlation coefficient of 0.999. This method was validated with respect to linearity, precision, accuracy, ruggedness, limit of detection, limit of quantification and robustness. This method was successfully applied for the simultaneous estimation of Bilastine and Monteleukast Sodium in bulk and pharmaceutical formulation.

Sudhakar B. et al., (2023) Analytical Method Development and Validation for the Simultaneous Estimation of Bilastine and Montelukast by RP-HPLC. A new, simple, rapid and precise reverse phase high performance liquid chromatographic method has been developed for the validation of Bilastine and Montelukast in its pure form as well as in combined marketed formulation. Chromatography was carried out on a Phenomenex Luna C18 (4.6mm×250mm) 5µm particle size column using a mixture of Methanol: Phosphate Buffer (pH-4.2) (37:63% v/v) as the mobile phase at a flow rate of 1.0ml/min, the detection was carried out at 260nm. The retention time of the Bilastine and Montelukast was found to be was 2.133, 3.692±0.02 min respectively. The method was validated according to ICH guidelines for linearity, sensitivity, accuracy, precision, specificity and robustness. The method produce linear responses in the concentration range of 20-60mg/ml of Bilastine and 10-30mg/ml of Montelukast. The inter-day and intra-day precisions were found to be within limits. The method precision for the determination of assay was below 2.0% RSD. The method is useful in the quality control of bulk and pharmaceutical formulations.

Shabnam Khan et al., (2022) Stability Indicating Rp-Hplc Method Development And Validation For Simultaneous Quantification Of Antihistaminic & Asthmatic Drug In Bulk And Tablet Dosage Form. This research describes the stability indicating RP-HPLC method in pharmaceutical tablet dosage forms for simultaneous estimation of Levocetirizine dihydrochloride (LEV) and Montelukast Sodium (MON). The proposed RP-HPLC method was developed using separation module Waters 2695 with PDA detector and chromatographic separation was performed at a flow rate of 1 mL / min using column Hypersil BDS C18 (250/4.6 mm, 5 µm) with a run time of 10 min. The mobile phase consisted of a 40:60% v / v Phosphate buffer and acetonitrile, pH with orthophosphoric acid was adjusted to 7.0 and the eluents were scanned at 230 nm using a PDA detector. Retention times for LEV and MON were 3.06 min, and 6.76 min, respectively. A linearity response was observed with a concentration range of 12.56–37.68 µg / mL for LEV and 23.78–71.20 µg / mL for MON. Limit of detection and limit of quantification for LEV are 0.079 µg/mL and 0.239 µg/mL and for MON are 0.156 µg/mL and 0.473 µg/mL, respectively. The stability indicating method was developed by subjecting the drugs to stress conditions such as acid, base hydrolysis, oxidation, neutral, photo- and thermal degradation, and the degraded products produced from the samples were successfully solved.

Pranav Vyas et al.,(2022) Development and Validation of RP-HPLC based Analytical method for Simultaneous Estimation of Montelukast and Bilastine in Tablet Dosage Form. A new RP-HPLC method was developed for the estimation of bilastine and montelukast in tablets and it was validated as per ICH guidelines. The chromatogram for was found to be satisfactory on symmetry C-18 (4.6×150mm, 5µ Thermosil column) using phosphate buffer (pH 4.5) and acetonitrile in the ratio of 30:70 v/v at a flow rate of 0.8 ml/min. The retention time of montelukast and bilastine were found to be 7.03 min and 9.50 min respectively. The system suitability parameters proved that the proposed method is suitable for simultaneous estimation of bilastine and montelukast. Tailing factor for the peak was found to be 1.003 and 1.258 for montelukast and bilastine respectively and the theoretical plates for separation were found to be 3409 and 7365 respectively montelukast and bilastine. The method was found to be linear in the range of 10-50µg/ml for both the drugs. The precision of the method was good and the recovery of drugs is well within the acceptance limits of 80-120%. The LOD was found to be 0.003 µg/ml for montelukast and 0.09 µg/ml for bilastine while the LOQ ws found to be 0.012 µg/ml for montelukast and 0.3 µg/ml for bilastine.

Shinde Ankita et al., (2021) Simultaneous HPLC method development and validation of bilastine and montelukast in bulk and formulation. A new, simple, rapid, selective, precise and accurate reverse phase high performance liquid chromatography assay has been developed for simultaneous estimation of Bilastine, and Montelukast in tablet formulations. The separation was achieved by using Phenomenax Kinetex XB C-18 column (150 x 4.6mm, 5/i.) using mobile phase Methanol: 0.1% TFA water (80:20). Injection volume was 10µl. The flow rate was 1.0mL.min⁻¹ and the separated drugs were detected using UV detector at the wavelength of 270nm. The retention time of Bilastine and Montelukast was noted to be 1.27, and 4.86 respectively, indicative of rather shorter analysis time. The method was validated as per ICH guidelines. The proposed method was found to be accurate, reproducible, and consistent. It was successfully applied for the analysis of these drugs in marketed formulations and could be effectively used for the routine analysis of formulations containing any one of the above drugs, or a combination, without any alteration in the chromatographic conditions.

3. AIM AND OBJECTIVE

Aim:

To develop and validate an RP-HPLC method for the simultaneous determination of Cetirizine and Montelukast in bulk and pharmaceutical formulations, ensuring accuracy, precision, and reproducibility for quality control.

Objectives:

1. Method Development:

○ Selection of Chromatographic Conditions:

- Choose an appropriate stationary phase (e.g., C18 column) to separate Cetirizine and Montelukast.

- Optimize mobile phase composition, flow rate, and detection wavelength to ensure maximum resolution and sensitivity for both drugs.
- Determine optimal chromatographic parameters such as column temperature, injection volume, and sample concentration.

2. Method Validation:

- **Specificity:** Ensure the method can separate Cetirizine and Montelukast from excipients and other components in pharmaceutical formulations.
- **Linearity:** Establish the linear relationship between concentration and peak area over a specific concentration range for both drugs.
- **Accuracy:** Conduct recovery studies to assess the method's accuracy by spiking known amounts of drugs into formulations and determining the recovery rate.
- **Precision:** Evaluate intra-day precision (repeatability) and inter-day precision (ruggedness) to assess the method's consistency.
- **LOD and LOQ:** Calculate the Limit of Detection (LOD) and Limit of Quantification (LOQ) for both drugs to determine the method's sensitivity.
- **Robustness:** Assess the robustness of the method by testing slight variations in chromatographic conditions (e.g., mobile phase composition, flow rate, and temperature).
- **System Suitability:** Perform system suitability tests (resolution, tailing factor, theoretical plates, and capacity factor) to ensure the method's reliability.

4. PLAN OF WORK

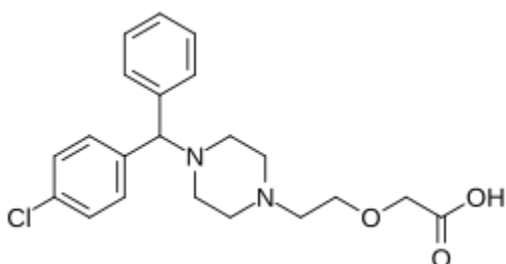
1. Collection of literature for the selected drug.
2. Extensive literature survey for selection of appropriate solvents to dissolve respective selected drug and preparation of stock solution.
3. Study of drug profile.
4. Procurement of samples, standards and other chemicals.
5. Selection of chromatographic conditions.
6. Selection of mobile phase.
7. Method trials on HPLC by using different solvents and columns.
8. Development of RP-HPLC method which is different from the finished articles.
9. Optimization of the developed method by varying mobile phase conditions, temperature.
10. Validation of the developed method for the following parameters:

- Accuracy
- Precision
- Specificity
- Limit of Detection
- Limit of Quantitation
- Linearity
- Robustness

5. DRUG PROFILE

CETIRIZINE

- **Drug:** Cetirizine
- **Synonym:** Cetirizine dihydrochloride
- **Drug category:** Antihistamine (second-generation)
- **Structure:**



-
- **Chemical name/ Nomenclature / IUPAC Name:** (R)-(4-(4-(p-chlorobenzhydryloxy) phenyl)-1-piperazineethanol)
- **Molecular Formula:** C₂₁H₂₅ClN₂O₃
- **Molecular Weight:** 388.89 gm/mole
- **Official Pharmacopoeia:** USP

PHYSICOCHEMICAL PROPERTIES:

- **Description (Physical State):** White crystalline powder
- **Solubility:** Soluble in water, ethanol; practically insoluble in hexane
- **Stability:** Stable under normal conditions
- **Storage Conditions:** Store at room temperature, away from moisture
- **Dosage:** 5-10 mg once daily
- **Melting point:** 105-110 °C
- **pKa (strongest acidic):** 5.5

- **Log P:** 3.1

PHARMACOKINETIC PROPERTIES:

- **Bioavailability:** 70%
- **Half-life:** 8 hrs
- **Absorption:** Rapidly absorbed from the gastrointestinal tract
- **Volume of Distribution:** 0.5 L/kg
- **Protein binding:** 93%
- **Metabolism:** Hepatic (minor)
- **Metabolites:** Cetirizine is excreted unchanged
- **Time of peak action:** 1 hr
- **Excretion:** 67% via urine
- **Adverse effects/Side effects:** Drowsiness, dry mouth, fatigue, dizziness

PHARMACODYNAMICS:

- **Mechanism of action:** Selective H1 receptor antagonist
- **Therapeutic efficacy/ Indications:** Allergic rhinitis, urticaria
- **Adverse reactions:** Rarely includes sedation, gastrointestinal disturbances
- **Contraindications:** Hypersensitivity to cetirizine or piperazine derivatives

INTERACTIONS:

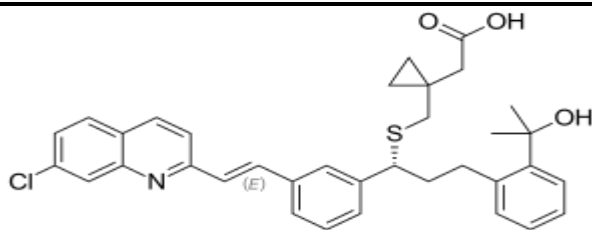
- **Drug interactions:** May enhance CNS depressants
- **Food interactions:** Food does not significantly affect the absorption of cetirizine

DRUG FORMULATION:

S.No	Drug Name	Label Claim	Brand Name	Company
1	Cetirizine	10 mg Tablet	Zyrtec	Johnson & Johnson

MONTELUKAST

- **Drug:** Montelukast
- **Synonym:** Montelukast sodium
- **Drug category:** Leukotriene receptor antagonist
- **Structure:**



- **Chemical name/ Nomenclature / IUPAC Name:** (E)-N-[(1R)-1-(3-(2-(7-chloro-2-quinolinyl)ethenyl)-phenyl)-3-methyl-1-piperidinyl]propionamide
- **Molecular Formula:** C₃₅H₃₆ClN₂O₃S
- **Molecular Weight:** 608.24 gm/mole
- **Official Pharmacopoeia:** USP

PHYSICOCHEMICAL PROPERTIES:

- **Description (Physical State):** White to off-white powder
- **Solubility:** Soluble in water, methanol; slightly soluble in ethanol
- **Stability:** Stable under normal conditions
- **Storage Conditions:** Store at room temperature, protect from light
- **Dosage:** 10 mg once daily
- **Melting point:** 197-198 °C
- **pKa (strongest acidic):** 4.2
- **Log P:** 3.3

PHARMACOKINETIC PROPERTIES:

- **Bioavailability:** 64%
- **Half-life:** 2.7-5.5 hrs
- **Absorption:** Rapidly absorbed from the gastrointestinal tract
- **Volume of Distribution:** 8-12 L/kg
- **Protein binding:** 99%
- **Metabolism:** Primarily hepatic via CYP3A4
- **Metabolites:** Active and inactive metabolites
- **Time of peak action:** 3-4 hrs
- **Excretion:** 86% via feces, 12% via urine
- **Adverse effects/Side effects:** Headache, abdominal pain, fatigue, mood changes

PHARMACODYNAMICS:

- **Mechanism of action:** Blocks leukotriene receptors (CysLT1)
- **Therapeutic efficacy/ Indications:** Asthma, allergic rhinitis
- **Adverse reactions:** Rare neuropsychiatric events
- **Contraindications:** Hypersensitivity to montelukast

INTERACTIONS:

- **Drug interactions:** May decrease the effectiveness of certain medications (e.g., phenobarbital)
- **Food interactions:** Food does not significantly affect the absorption of montelukast

DRUG FORMULATION:

S.No	Drug Name	Label Claim	Brand Name	Company
1	Montelukast	10 mg Tablet	Singulair	Merck

6. EXPERIMENTAL METHODS**INSTRUMENTS USED****Table 6.1: Instruments used**

S. No	Instruments And Glass wares	Model
1	HPLC	WATERS Alliance 2695 separation module, Software: Empower 2, 996 PDA detector.
2	pH meter	Lab India
3	Weighing machine	Sartorius
4	Volumetric flasks	Borosil
5	Pipettes and Burettes	Borosil
6	Beakers	Borosil
7	Digital ultra sonicator	Labman

CHEMICALS USED:**Table 6.2: chemicals used**

S. No	Chemical	Brand names
1	Cetirizine	provided by Sura Pharma labs
2	Montelukast	provided by Sura Pharma labs
3	Water and Methanol for HPLC	LICHROSOLV (MERCK)

HPLC METHOD DEVELOPMENT:**TRAILS****Preparation of standard solution:**

Accurately weigh and transfer 10 mg of Cetirizine and Montelukast working standard into a 10ml of clean dry volumetric flasks add about 7ml of Methanol and sonicate to dissolve and removal of air completely and make volume up to the mark with the same Methanol.

Further pipette 2.25ml of the above Cetirizine and 0.45ml of the Montelukast stock solutions into a 10ml volumetric flask and dilute up to the mark with Methanol.

Procedure:

Inject the samples by changing the chromatographic conditions and record the chromatograms, note the conditions of proper peak elution for performing validation parameters as per ICH guidelines.

Mobile Phase Optimization:

Initially the mobile phase tried was Methanol: Water, Acetonitrile: Water with varying proportions. Finally, the mobile phase was optimized to Methanol and water in proportion 70:30 v/v respectively.

Optimization of Column:

The method was performed with various columns like C18 column, X- bridge column, Xterra. Phenomenex Luna C18 (4.6 x 150mm, 5µm) was found to be ideal as it gave good peak shape and resolution at 1ml/min flow.

VALIDATION**PREPARATION OF MOBILE PHASE:****Preparation of mobile phase:**

Accurately measured 700ml (70%) of HPLC Methanol and 300ml of Water (30%) were mixed and degassed in a digital ultrasonicator 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**SYSTEM SUITABILITY**

Accurately weigh and transfer 10 mg of Cetirizine and Montelukast working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 2.25ml of the above Cetirizine and 0.45ml of the Montelukast stock solutions into a 10ml volumetric flask and dilute up to the mark with Methanol.

Procedure:

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

SPECIFICITY STUDY OF DRUG:**Preparation of Standard Solution:**

Accurately weigh and transfer 10 mg of Cetirizine and Montelukast working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 2.25ml of the above Cetirizine and 0.45ml of the Montelukast stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

Preparation of Sample Solution:

Take average weight of the Tablet and crush in a mortar by using pestle and weight 10 mg equivalent weight of Cetirizine and Montelukast sample into a 10mL clean dry volumetric flask and add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

Further pipette 2.25ml of Cetirizine and Montelukast above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Procedure:

Inject the three replicate injections of standard and sample solutions and calculate the assay by using formula:

%ASSAY =

$$\frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity}}{100} \times \frac{\text{Weight of tablet}}{\text{Label claim}} \times 100$$

PREPARATION OF DRUG SOLUTIONS FOR LINEARITY:

Accurately weigh and transfer 10 mg of Cetirizine and Montelukast working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Preparation of Level – I (5ppm of Cetirizine and 15ppm of Montelukast):

Pipette out 0.5ml of the Cetirizine and 0.15ml of the Montelukast from the above stock solutions in to a 10ml of volumetric flask and dilute the solution. Performes sonication for 10minutes.

Preparation of Level – II (10ppm of Cetirizine and 30ppm of Montelukast):

Pipette out 0.10ml of the Cetirizine and 0.30ml of the Montelukast from the above stock solutions in to a 10ml of volumetric flask and dilute the solution. Performes sonication for 10minutes.

Preparation of Level – III (15ppm of Cetirizine and 45ppm of Montelukast):

Pipette out 0.15ml of the Cetirizine and 0.45ml of the Montelukast from the above stock solutions in to a 10ml of volumetric flask and dilute the solution. Performes sonication for 10minutes.

Preparation of Level – IV (20ppm of Cetirizine and 60ppm of Montelukast):

Pipette out 0.20ml of the Cetirizine and 0.6ml of the Montelukast from the above stock solutions in to a 10ml of volumetric flask and dilute the solution. Performes sonication for 10minutes.

Preparation of Level – V (25ppm of Cetirizine and 75ppm of Montelukast):

Pipette out 0.25ml of the Cetirizine and 0.75ml of the Montelukast from the above stock solutions in to a 10ml of volumetric flask and dilute the solution. Performs sonication for 10 minutes.

Procedure:

Inject each level into the chromatographic system and measure the peak area.

Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient.

PRECISION**REPEATABILITY****Preparation of Cetirizine and Montelukast Product Solution for Precision:**

Accurately weigh and transfer 10 mg of Cetirizine and Montelukast working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 2.25ml of the above Cetirizine and 0.45ml of the Montelukast stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

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

INTERMEDIATE PRECISION:

To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different days by maintaining same conditions.

Procedure:**DAY 1:**

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

DAY 2:

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

Accuracy:**For preparation of 50% Standard stock solution:**

Accurately weigh and transfer 10 mg of Cetirizine and Montelukast working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 1.12ml of the above Cetirizine and 0.225ml of the Montelukast stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

For preparation of 100% Standard stock solution:

Accurately weigh and transfer 10 mg of Cetirizine and Montelukast working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 2.25ml of the above Cetirizine and 0.45ml of the Montelukast stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

For preparation of 150% Standard stock solution:

Accurately weigh and transfer 10 mg of Cetirizine and Montelukast working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 3.37ml of the above Cetirizine and 0.675ml of the Montelukast stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

Procedure:

Inject the Three replicate injections of individual concentrations (50%, 100%, 150%) were made under the optimized conditions. Recorded the chromatograms and measured the peak responses. Calculate the Amount found and Amount added for Cetirizine and Montelukast and calculate the individual recovery and mean recovery values.

ROBUSTNESS:

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

For preparation of Standard solution:

Accurately weigh and transfer 10 mg of Cetirizine and Montelukast working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 2.25ml of the above Cetirizine and 0.45ml of the Montelukast stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

Effect of Variation of flow conditions:

The sample was analyzed at 0.9 ml/min and 1.1 ml/min instead of 1ml/min, remaining conditions are same. 10 μ l of the above sample was injected and chromatograms were recorded

Effect of Variation of mobile phase organic composition:

The sample was analyzed by variation of mobile phase i.e. Methanol: Water was taken in the ratio and 60:40, 70:30 instead of 70:30, remaining conditions are same. 10 μ l of the above sample was injected and chromatograms were recorded.

7. RESULTS AND DISCUSSION

Trails

Trail 1:

Column	: Symmetry C18 (4.6 $\times$ 250mm) 5 μ
Column temperature	: Ambient
Wavelength	: 220nm
Mobile phase ratio	: Methanol: Water (95:5 v/v)
Flow rate	: 1ml/min

Injection volume : 20µl

Run time : 10minutes

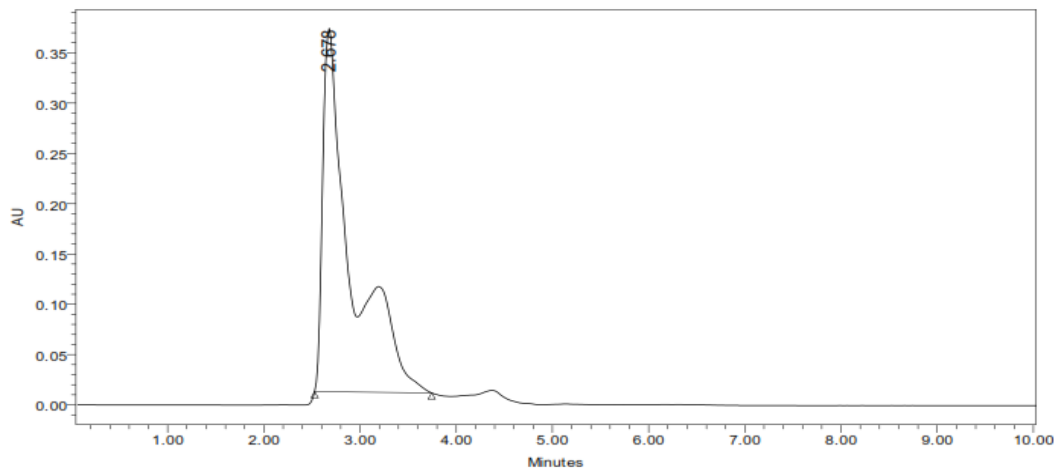


Figure 7.1: chromatogram for trail 1

Table7.1: peak results for trail 1

S. No	Peak Name	R _t	Area	Height	USP Tailing	USP Plate count
1	Cetirizine	2.678	215471	38742	1.6	745

Observation:

In this trial it shows less plate count and improper separation of two peaks in the chromatogram. so, its required more trials to obtain good peaks.

Trail 2:

Column : ODS C18 (4.6×250mm) 5µ

Column temperature : 30°C

Wavelength : 220nm

Mobile phase ratio : Methanol: Water (85:15 v/v)

Flow rate : 0.5ml/min

Injection volume : 10µl

Run time : 8minutes

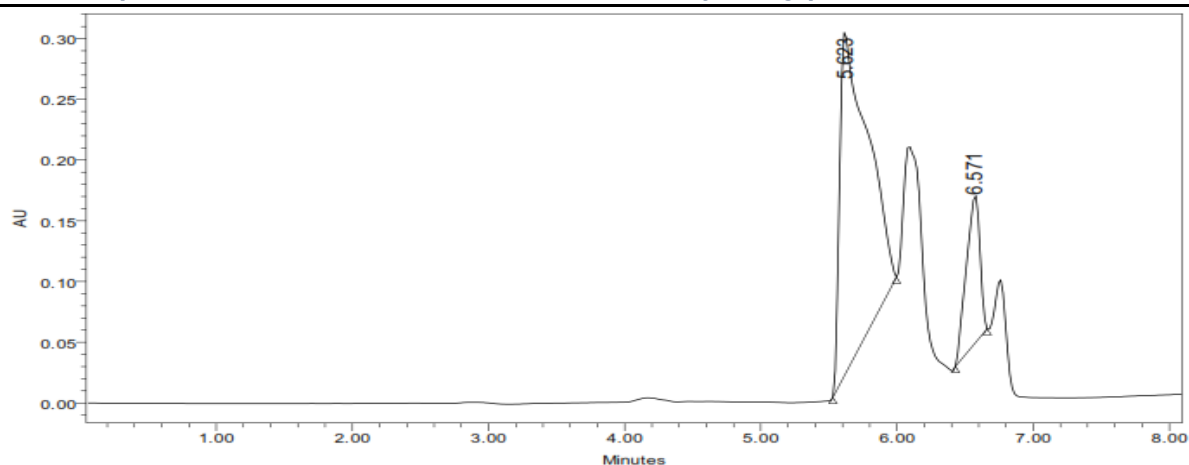


Figure 7.2: chromatogram for trail 2

Table 7.2: peak results for trail 2

S. No	Peak name	R _t	Area	Height	USP Tailing	USP plate count
1	Cetirizine	5.623	46523	29816	1.8	1937
2	Montelukast	6.571	5366	1947	1.6	1183

Observation:

In this trail it shows improper separation of two peaks, shows less plate count and improper baseline in the chromatogram. Its required more trails to get optimized peaks.

Trail 3:

Column : ODS C18 (4.6×250mm) 5μ

Column temperature : 30°C

Wavelength : 220nm

Mobile phase ratio : Methanol: Water (80:20 v/v)

Flow rate : 1ml/min

Injection volume : 10μl

Run time : 9minutes

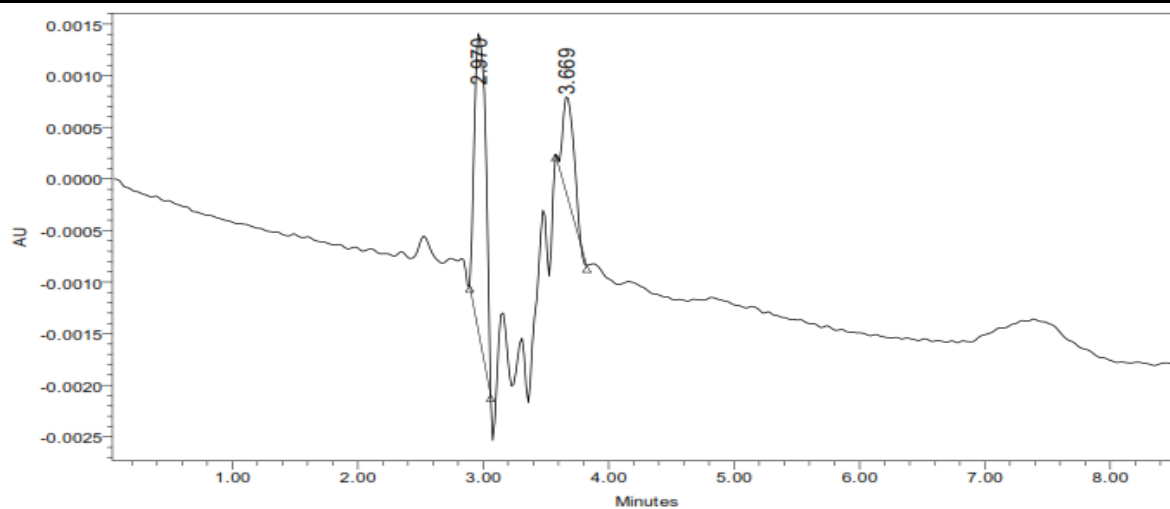


Figure 7.3- chromatogram for trail 3

Table 7.3: - peak results for trail 3

S. No	Peak name	R _t	Area	Height	USP Tailing	USP plate count
1	Cetirizine	2.970	38261	17463	2.4	284
2	Montelukast	3.669	6452	6482	1.9	645

Observation:

From the above chromatogram it was observed that it shows less plate count and more tailing, improper separation of two sample peaks in the chromatogram. More trails required to get optimized chromatogram.

Trial 4:

Mobile phase : Methanol: Water (60:40%v/v)
 Column : X-Bridge (4.6 ×150mm, 5µm particle size) Make; waters
 Flow rate : 1.0ml/min
 Wavelength : 262nm
 Column temp : 34°C
 Injection Volume : 10µl

Run time : 10 minutes

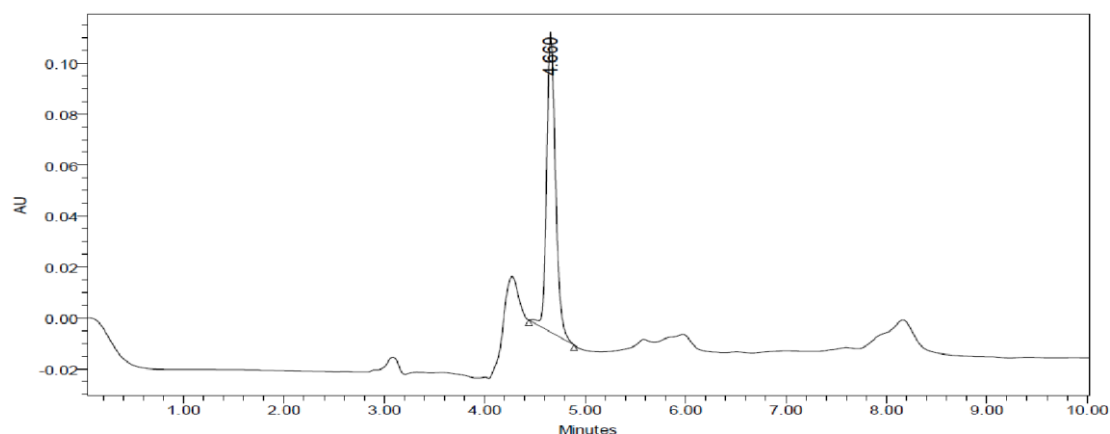


Fig 7.4: Chromatogram of trial 4

Table 7.4: Observation of trial 4 chromatogram

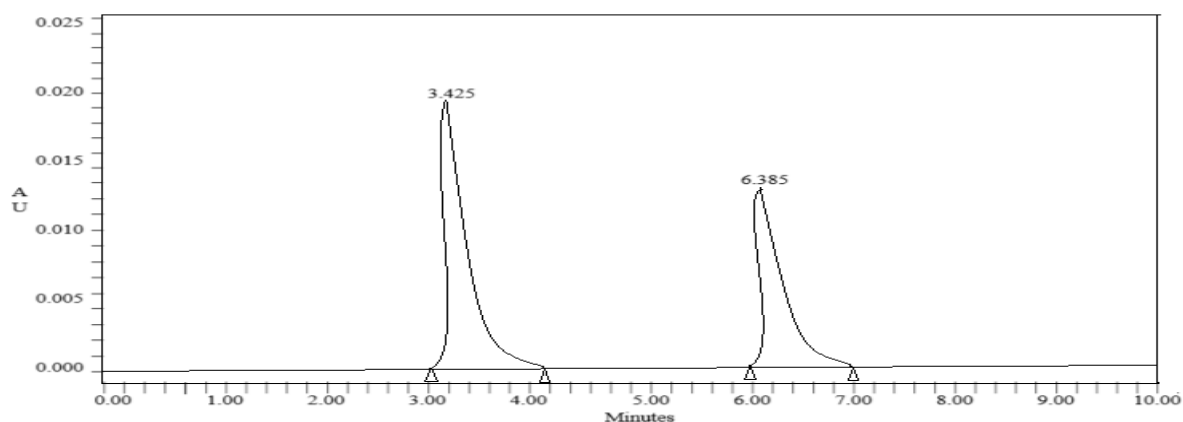
S. No	Peak Name	Retention Time	Area	Height	USP Tailing	USP Plate Count
1	Cetirizine	4.660	721543	118542	1.4	12546.2

Inference:

The Retention Time observed from chromatogram was 4.66, only one peak was eluted.

Trail 5 (Optimized chromatogram) (Standard):

Temperature : 35°C
 Column : Phenomenex Luna C18 (4.6 x 150mm, 5µm)
 Mobile phase : Methanol and water (70:30 v/v)
 Flow rate : 1ml/min
 Wavelength : 230 nm
 Injection volume : 10 µl
 Run time : 10 min

**Figure 7.5: Optimized Chromatogram (Standard)****Table 7.5: Optimized Chromatogram (Standard)**

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count	USP Resolution
1	Cetirizine	3.425	2391746	39726	1.2	9028	
2	Montelukast	6.385	194627	8497	1.1	7398	7.4

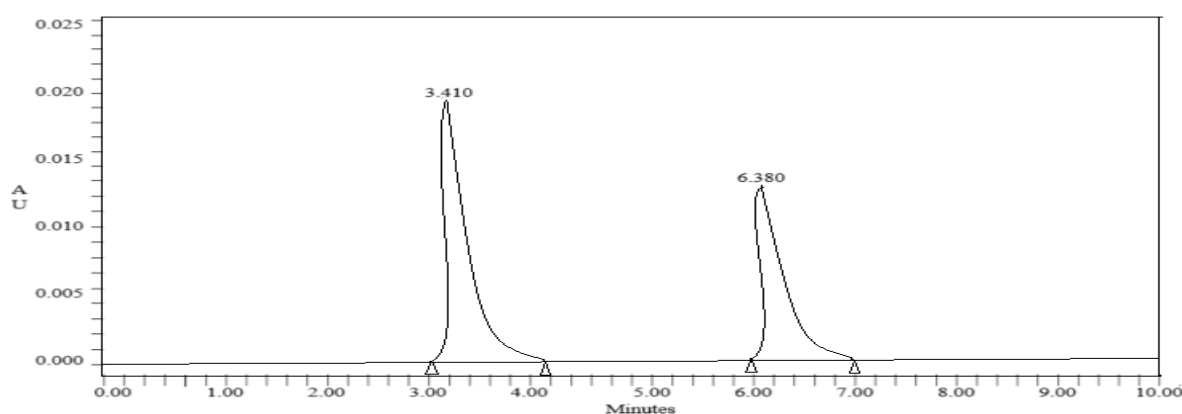
Observation:

This trial shows improper separation sample peaks, baseline and show very less plate count in the chromatogram. So it's required more trials to obtain good peaks.

From the above chromatogram it was observed that the Cetirizine and Montelukast peaks are well separated and they shows proper retention time, resolution, peak tail and plate count. So it's optimized trial.

Optimized Chromatogram (Sample)

Temperature : 35°C
 Column : Phenomenex Luna C18 (4.6 x 150mm, 5µm)
 Mobile phase : Methanol and water (70:30 v/v)
 Flow rate : 1ml/min
 Wavelength : 230 nm
 Injection volume : 10 µl
 Run time : 10 min

**Figure 7.6: Optimized Chromatogram (Standard)****Table 7.6: Optimized Chromatogram (Sample)**

S. No	Name	RT	Area	Height	USP Tailing	USP Plate Count	USP Resolution
1	Cetirizine	3.410	2381649	391846	1.2	9472	
2	Montelukast	6.380	1910575	8104	1.1	8936	7.5

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

Blank:

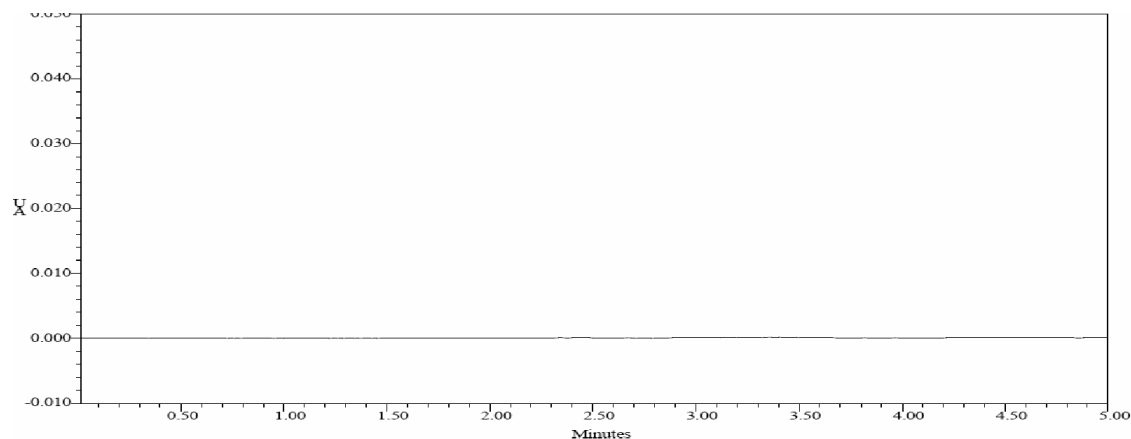


Fig7.7: Chromatogram showing blank (mobile phase preparation)

System suitability:

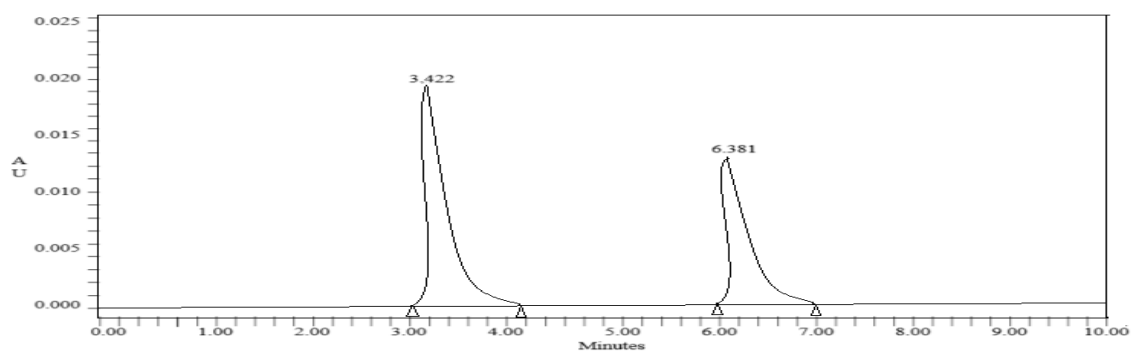


Fig7.8: Chromatogram showing injection -1

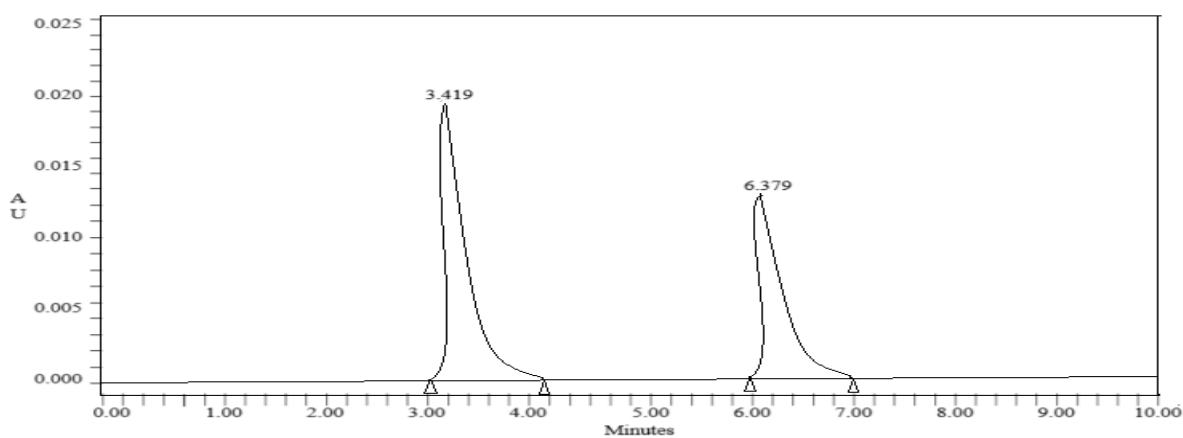


Fig7.9: Chromatogram showing injection -2

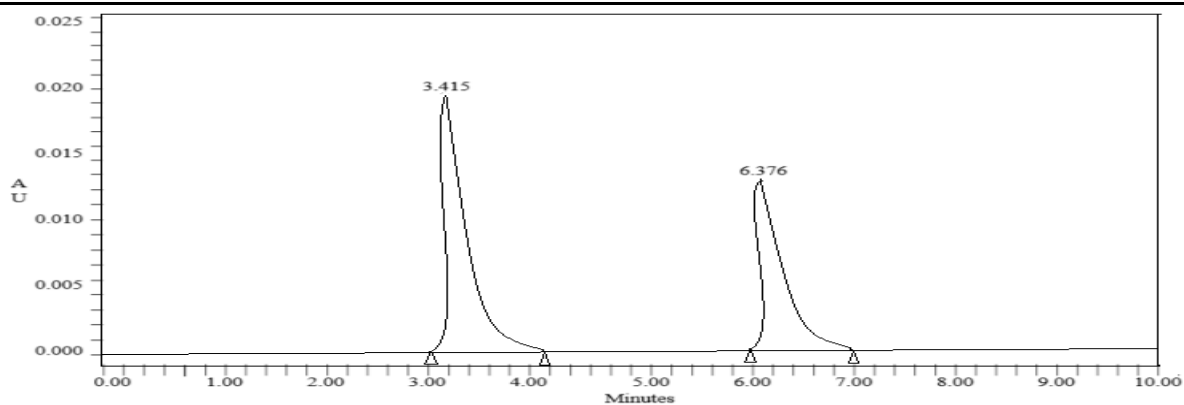


Fig7.10: Chromatogram showing injection -3

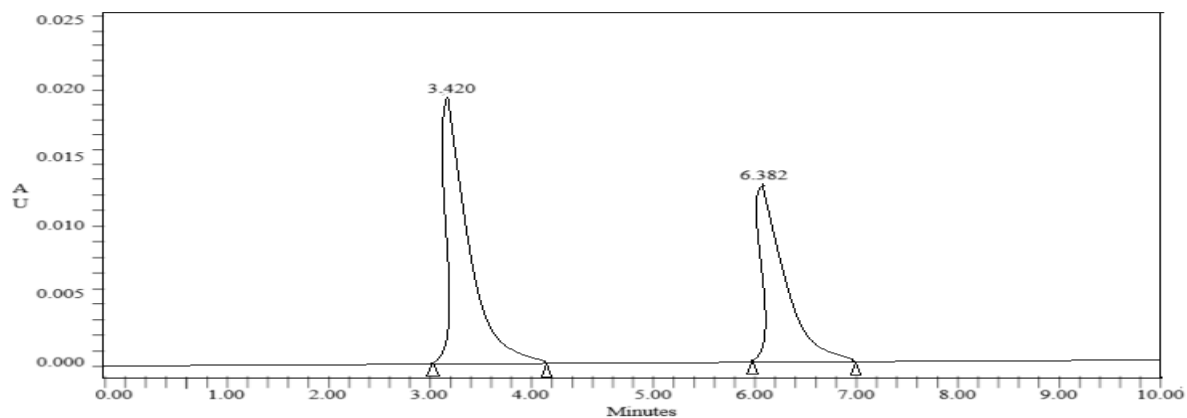


Fig7.11: Chromatogram showing injection -4

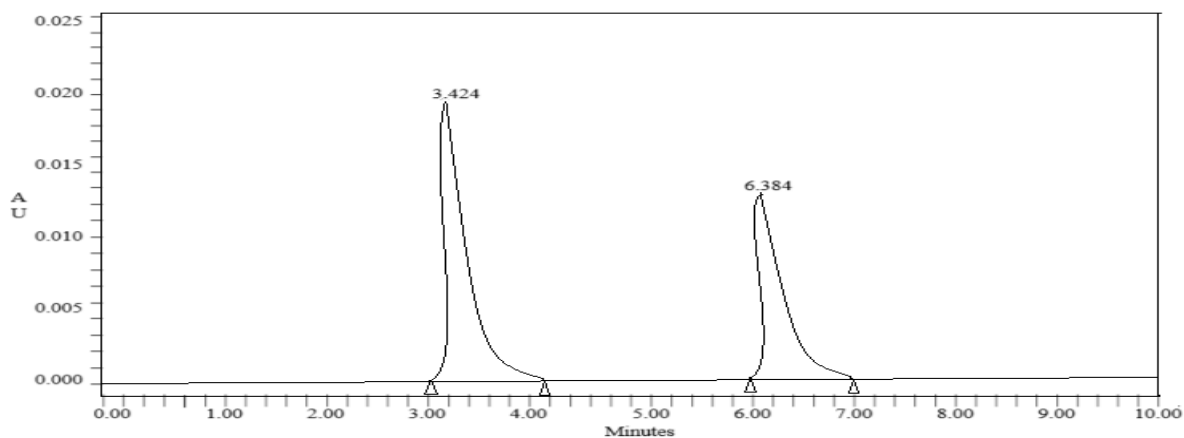


Fig7.12: Chromatogram showing injection -5

Table:7.7 Results of system suitability for Cetirizine

S.No	Peak Name	RT	Area ($\mu\text{V}\cdot\text{sec}$)	Height (μV)	USP Plate Count	USP Tailing
1	Cetirizine	3.422	2391746	394171	8952	1.2
2	Cetirizine	3.419	2391647	381946	9561	1.2
3	Cetirizine	3.415	2381647	391746	6572	1.2

4	Cetirizine	3.420	2385631	386562	6452	1.2
5	Cetirizine	3.424	2385635	389164	7452	1.2
Mean			2387261			
Std. Dev.			4363.771			
% RSD			0.182794			

Acceptance criteria:

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

Table:7.8 Results of system suitability for Montelukast

S.No	Peak Name	RT	Area ($\mu\text{V}\cdot\text{sec}$)	Height (μV)	USP Plate Count	USP Tailing
1	Montelukast	6.381	198362	7917	5272	1.1
2	Montelukast	6.379	197486	7486	6291	1.1
3	Montelukast	6.376	198354	7859	6184	1.1
4	Montelukast	6.382	197352	7926	7145	1.1
5	Montelukast	6.384	198453	7946	6946	1.1
Mean			198001.4			
Std. Dev.			535.1774			
% RSD			0.27029			

Acceptance criteria:

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

SPECIFICITY

The ICH documents define specificity as the ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation products, and matrix components.

Analytical method was tested for specificity to measure accurately quantitate Cetirizine and Montelukastin drug product.

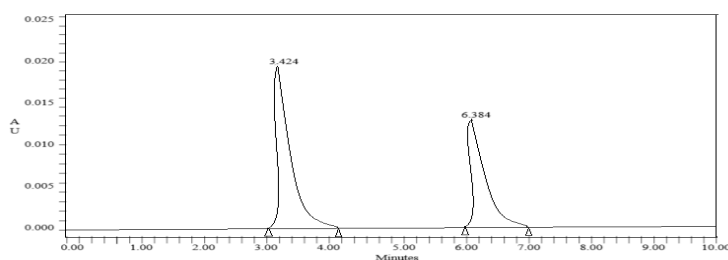
Assay (Standard):

Fig.7.13 Chromatogram showing assay of standard injection -1

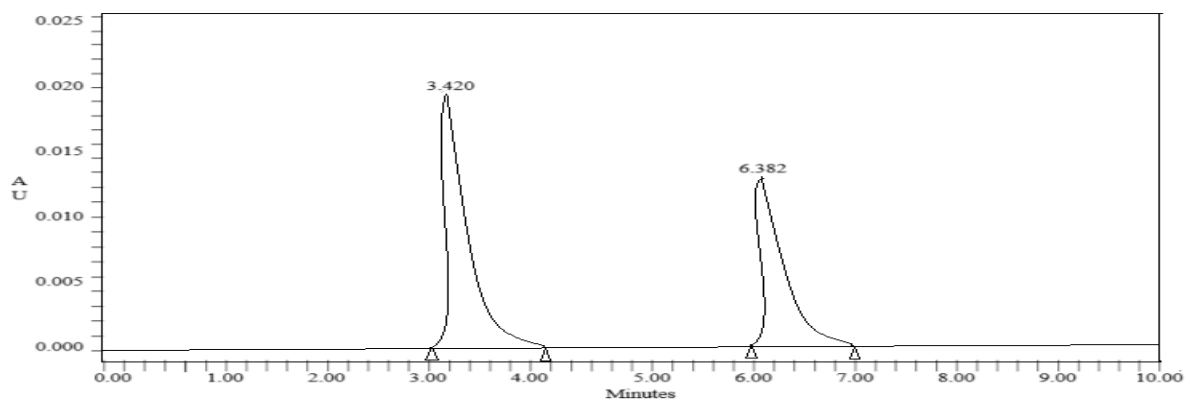


Fig.7.14 Chromatogram showing assay of standard injection -2

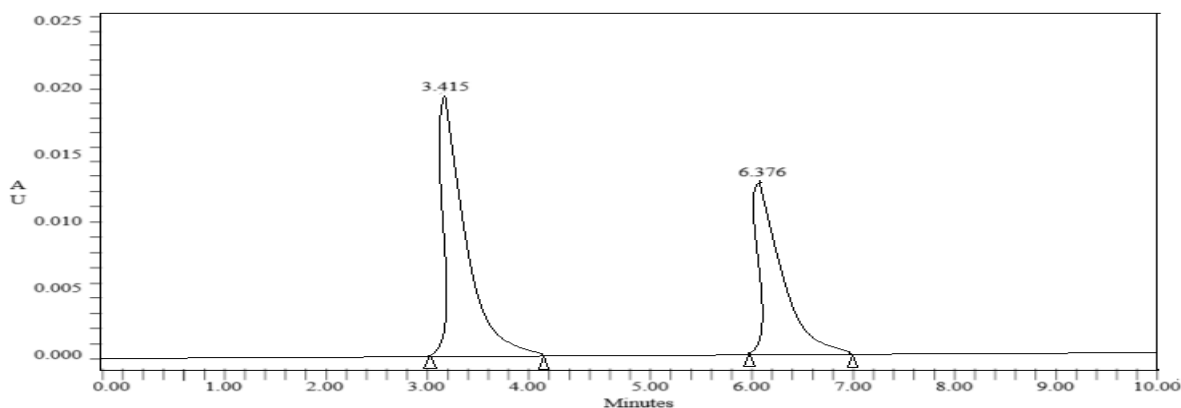


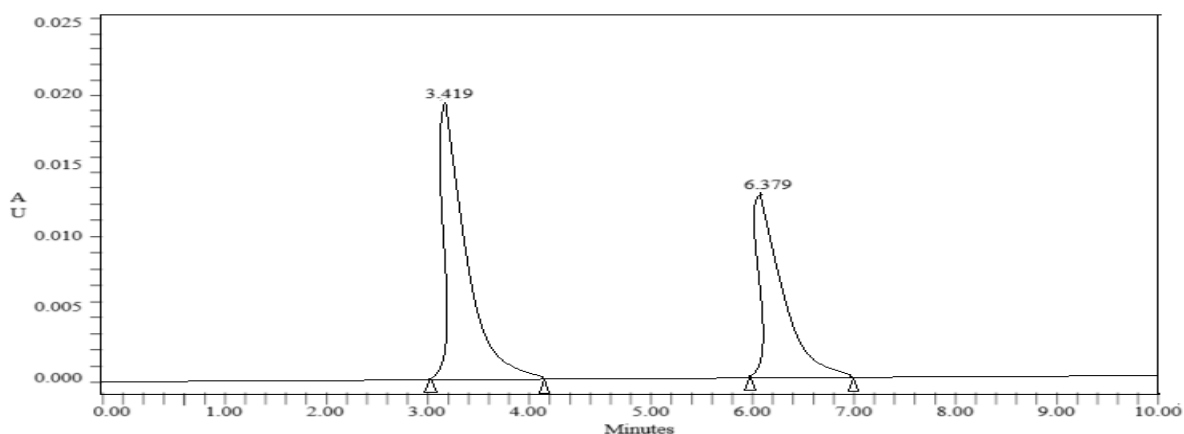
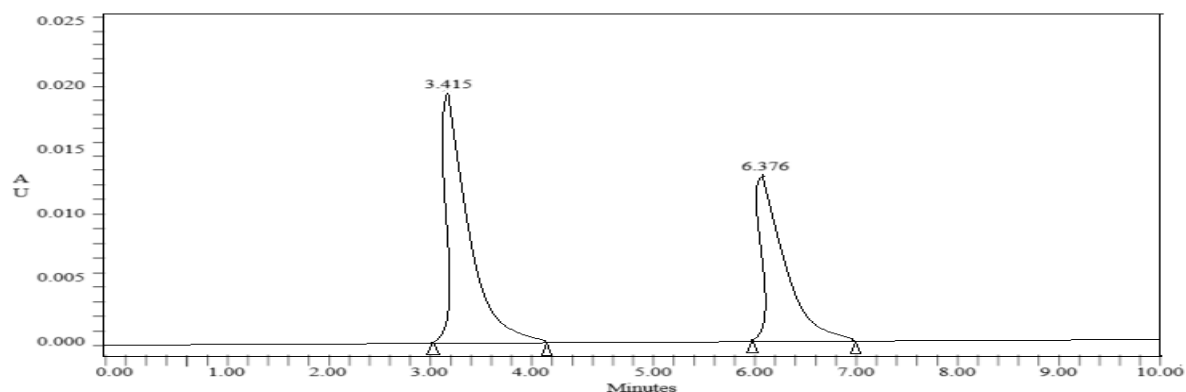
Fig.7.15 Chromatogram showing assay of standard injection -3

Table:7.9 Peak results for assay standard**Cetirizine**

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count
1	Cetirizine	3.424	2397162	397161	1.2	9472
2	Cetirizine	3.420	2394721	389173	1.2	9745
3	Cetirizine	3.415	2389461	391723	1.2	8917

Montelukast

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count	Resolution
1	Montelukast	6.384	198462	7811	1.1	8492	7.49
2	Montelukast	6.382	198472	8193	1.1	8916	7.52
3	Montelukast	6.376	198735	7972	1.1	9372	7.44

Assay (Sample):**Fig:7.16 Chromatogram showing assay of sample injection-1****Fig.7.17 Chromatogram showing assay of sample injection-2**

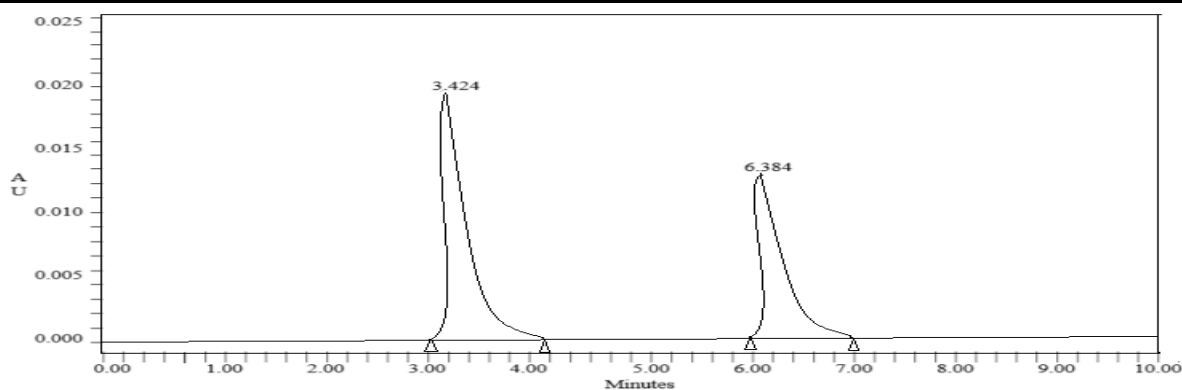


Fig:7.18 Chromatogram showing assay of sample injection-3

Table:7.10 Peak results for Assay sample

Cetirizine

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count
1	Cetirizine	3.419	2391741	381612	1.2	9472
2	Cetirizine	3.415	2389166	391746	1.2	8927
3	Cetirizine	3.424	2361731	381634	1.2	9017

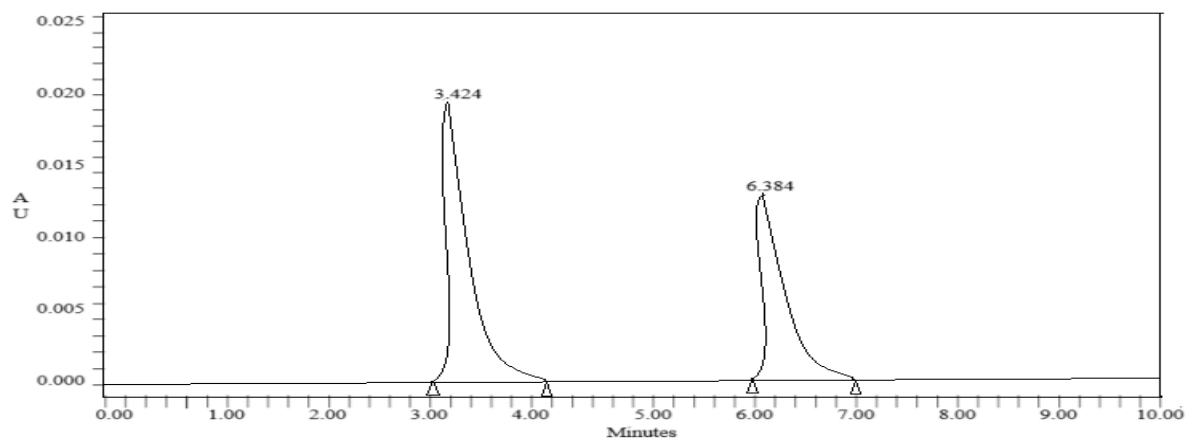
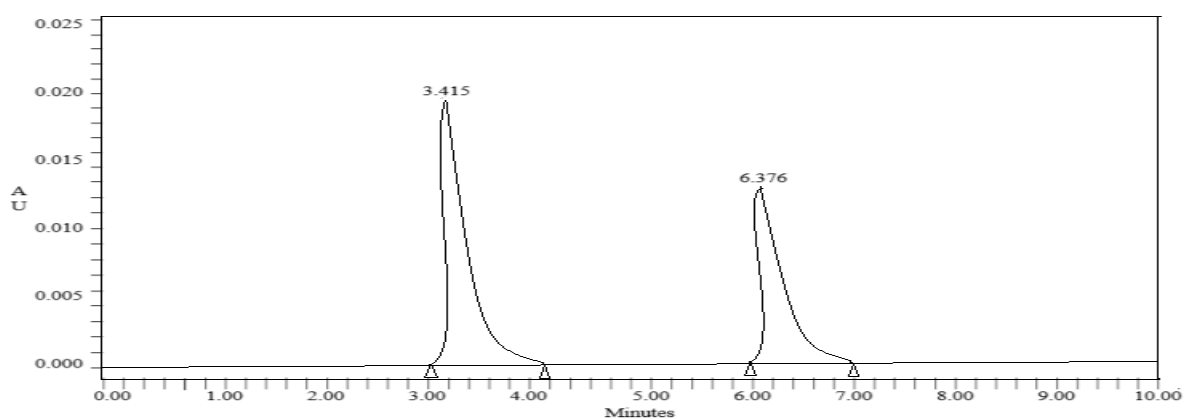
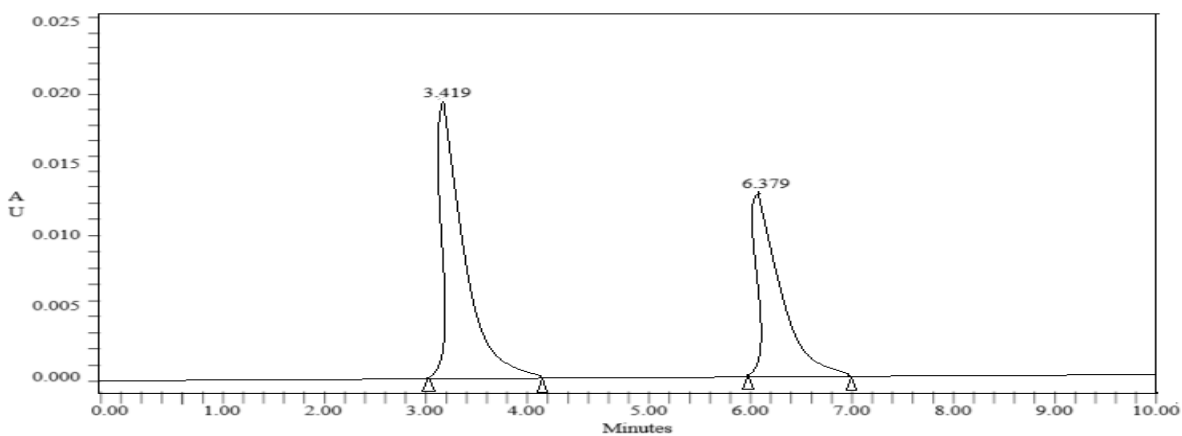
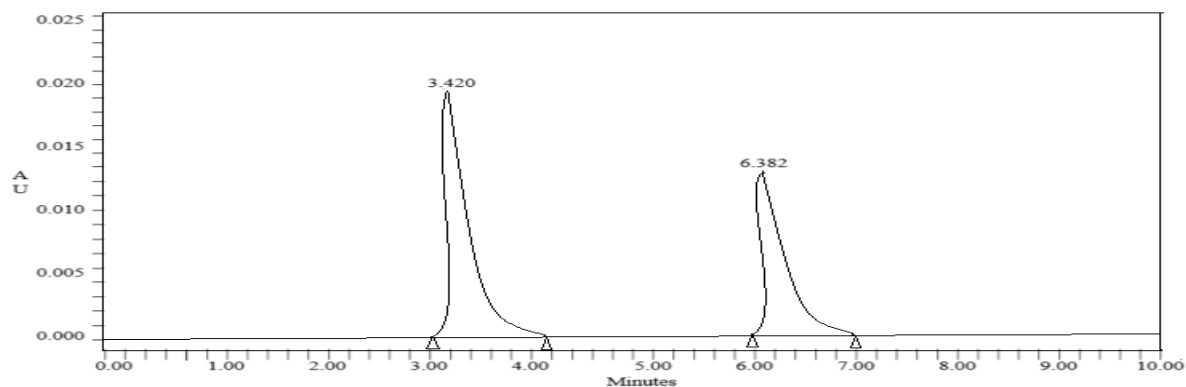
Montelukast

S. No	Name	RT	Area	Height	USP Tailing	USP Plate Count	Resolution
1	Monteluka st	6.379	198641	8174	1.1	9284	7.18
2	Monteluka st	6.376	196547	8942	1.1	8974	7.44
3	Monteluka st	6.384	194027	7294	1.1	9017	7.38

%ASSAY =

$$\frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity}}{100} \times \frac{\text{Weight of tablet}}{\text{Label claim}} \times 100$$

The % purity of Cetirizine and Montelukastin pharmaceutical dosage form was found to be 99.5%

LINEARITY**Fig.7.19 Chromatogram showing linearity level-1****Fig.7.20 Chromatogram showing linearity level-2****Fig.7.21 Chromatogram showing linearity level-3****Fig.7.22 Chromatogram showing linearity level-4**

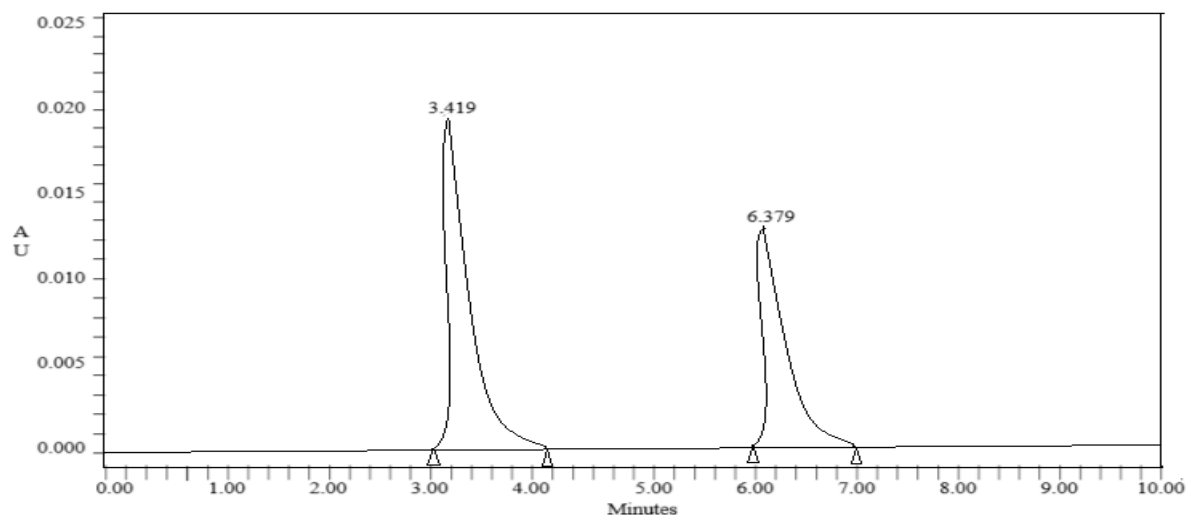
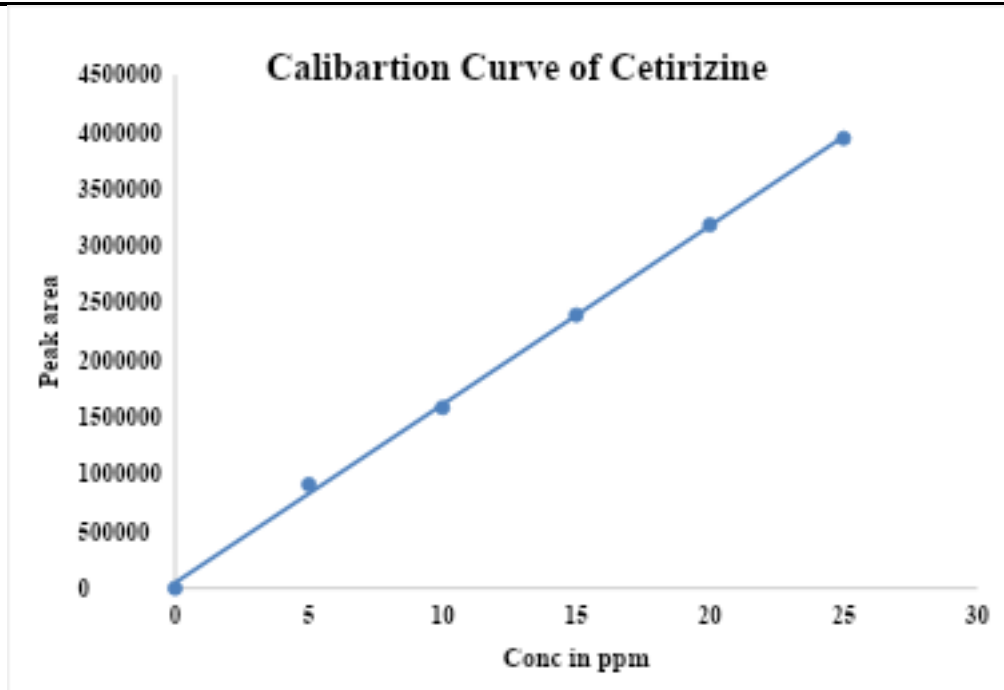


Fig.7.23 Chromatogram showing linearity level-5

CHROMATOGRAPHIC DATA FOR LINEARITY STUDY:

Cetirizine

Concentration µg/ml	Average Peak Area
0	0
5	909889
10	1583641
15	2395378
20	3185089
25	3943725



LINEARITY PLOT:

The plot of Concentration (x) versus the Average Peak Area (y) data of Cetirizine is a straight line.

$$Y = mx + c$$

$$\text{Slope (m)} = 156320$$

$$\text{Intercept (c)} = 48956$$

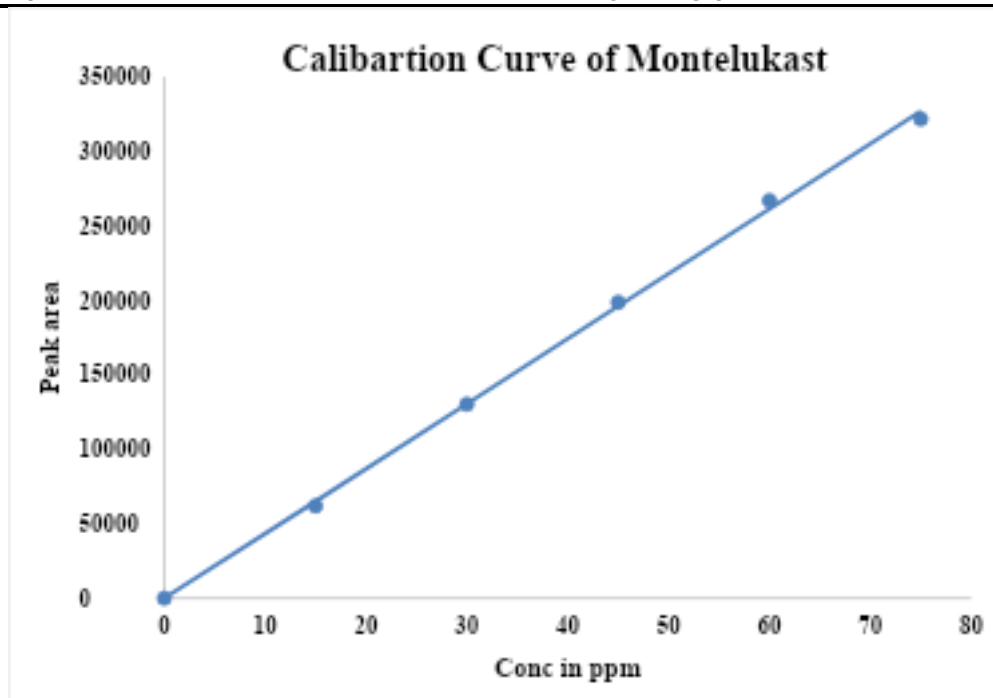
$$\text{Correlation Coefficient (r)} = 0.999$$

VALIDATION CRITERIA: The response linearity is verified if the Correlation Coefficient is 0.99 or greater.

CONCLUSION: Correlation Coefficient (r) is 0.99, and the intercept is 48956. These values meet the validation criteria.

Montelukast

Concentration µg/ml	Average Peak Area
0	0
15	61953
30	130213
45	198697
60	267002
75	321658



LINEARITY PLOT:

The plot of Concentration (x) versus the Average Peak Area (y) data of Montelukast is a straight line.

$$Y = mx + c$$

$$\text{Slope (m)} = 4365.6$$

$$\text{Intercept (c)} = 454.8$$

$$\text{Correlation Coefficient (r)} = 0.999$$

VALIDATION CRITERIA: The response linearity is verified if the Correlation Coefficient is 0.99 or greater.

CONCLUSION: Correlation Coefficient (r) is 0.99, and the intercept is 454.8. These values meet the validation criteria.

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.

REPEATABILITY

Obtained Five (5) replicates of 100% accuracy solution as per experimental conditions. Recorded the peak areas and calculated % RSD.

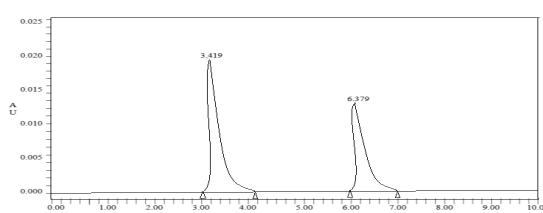


Fig7.24. Chromatogram showing precision injection -1

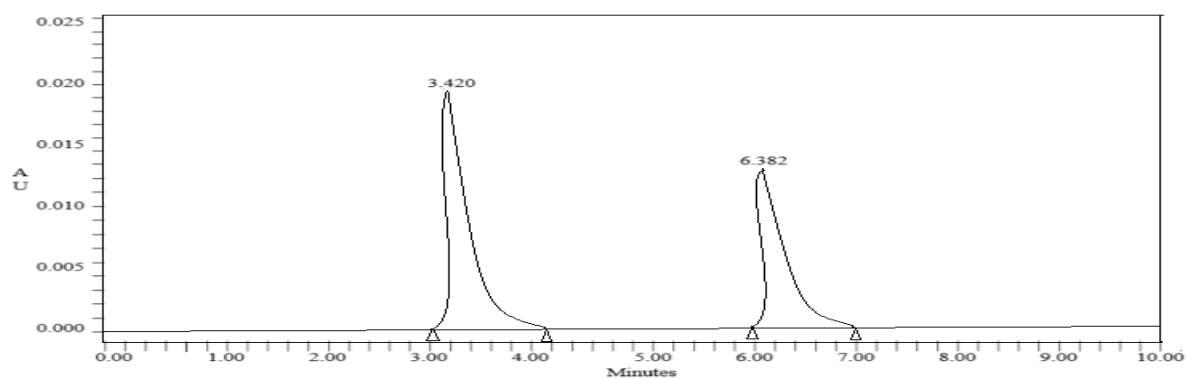
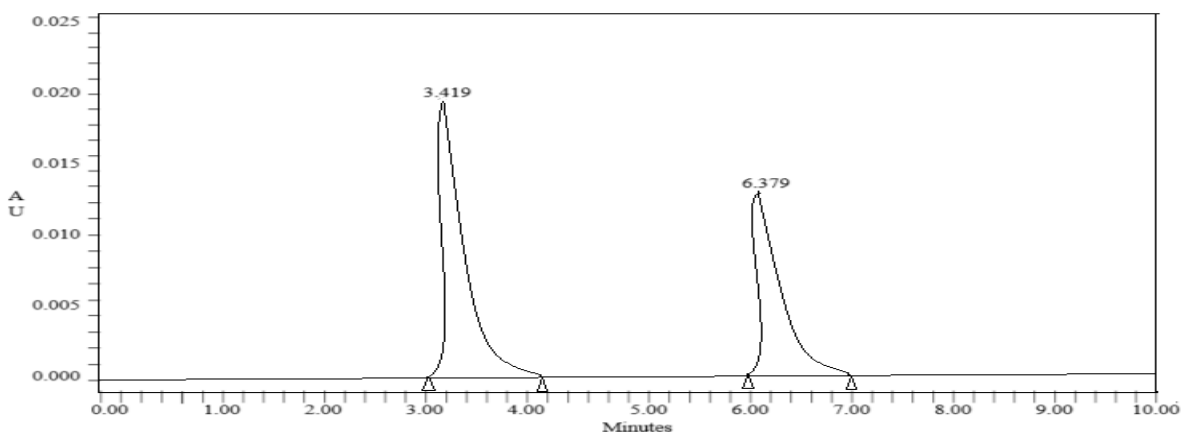
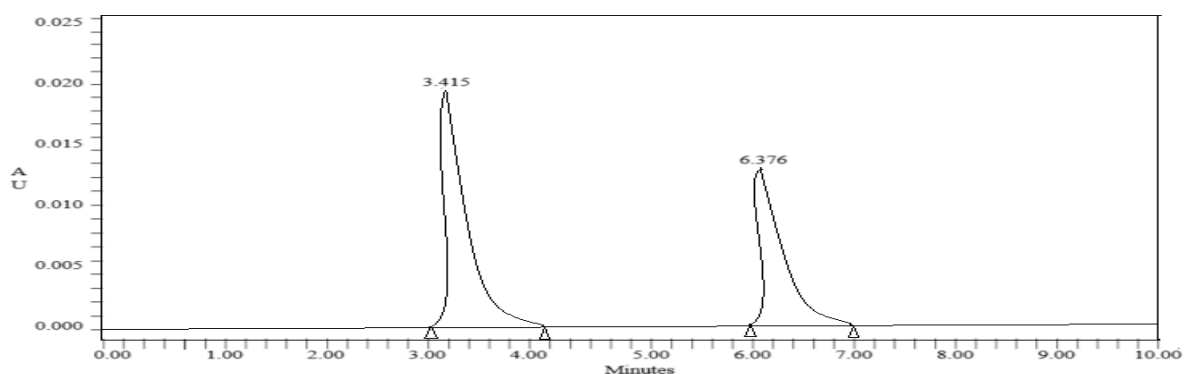
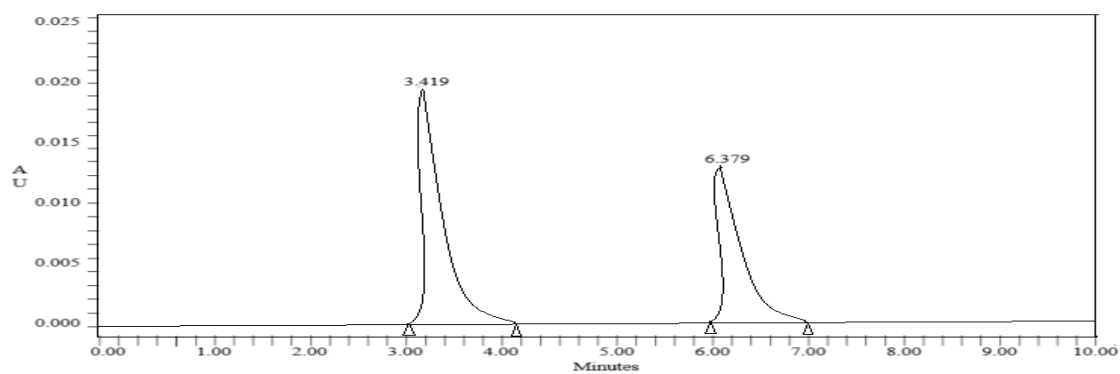
**Fig.7.25 Chromatogram showing precision injection -2****Fig.7.26 Chromatogram showing precision injection -3****Fig.7.27 Chromatogram showing precision injection -4****Fig. 7.28 Chromatogram showing precision injection -5**

Table: 7.11 Results of repeatability for Cetirizine:

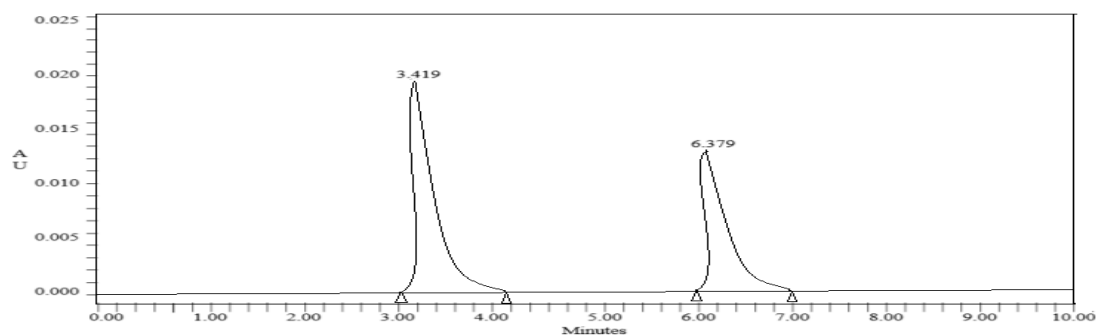
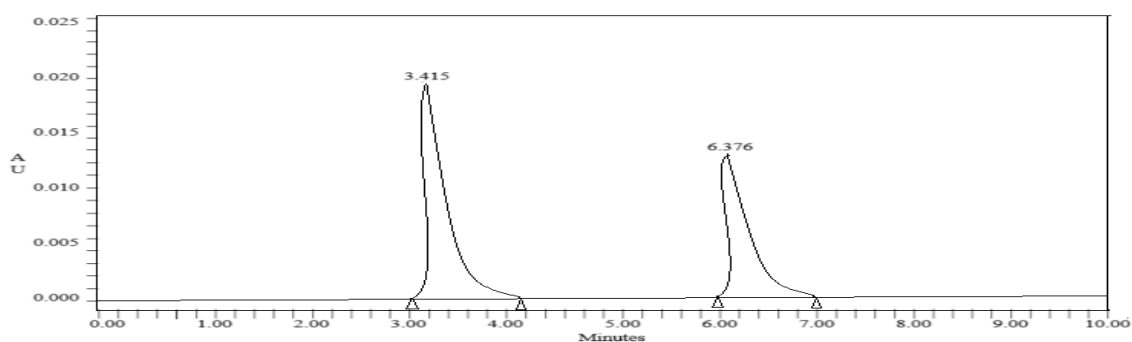
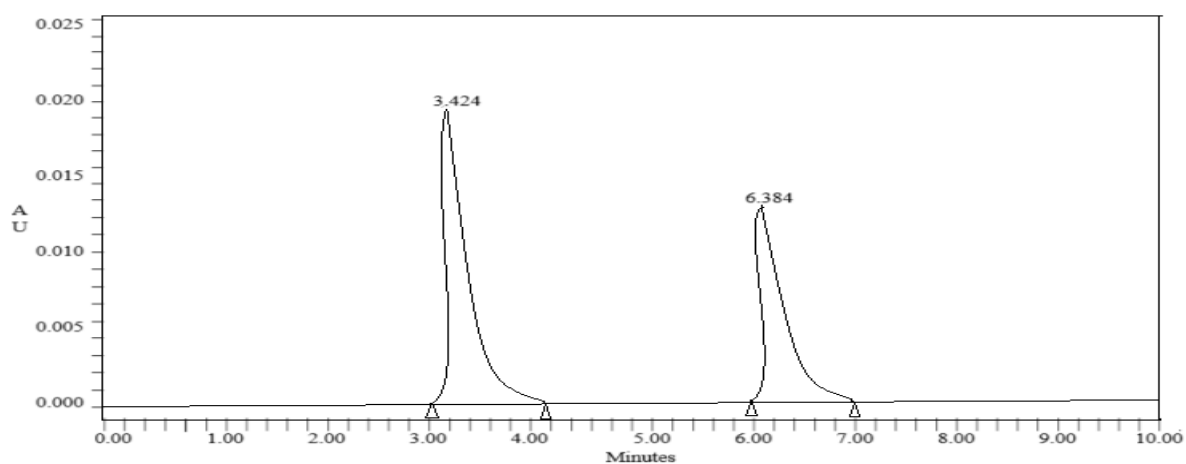
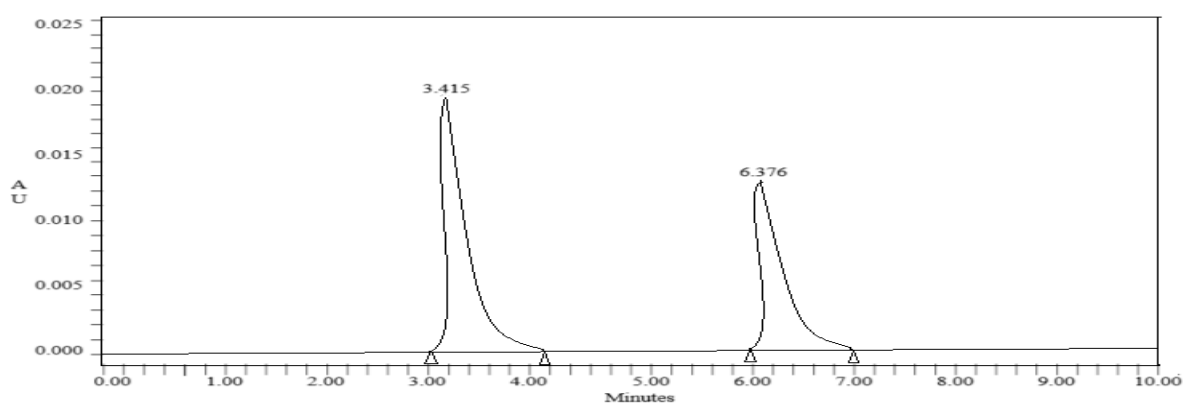
S. No	Peak name	Retention time	Area($\mu\text{V}^*\text{s}$ ec)	Height (μV)	USP Plate Count	USP Tailing
1	Cetirizine	3.419	2397164	381741	8155	1.2
2	Cetirizine	3.420	2391741	371742	9174	1.2
3	Cetirizine	3.419	2371846	391746	7154	1.2
4	Cetirizine	3.415	2361748	391847	9917	1.2
5	Cetirizine	3.419	2371649	384622	9247	1.2
Mean			2378830			
Std.dev			14958			
%RSD			0.628797			

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.

Table:7.12 Results of repeatability for Montelukast:

S. No	Peak name	Retention time	Area($\mu\text{V}^*\text{s}$ ec)	Height (μV)	USP Plate Count	USP Tailing
1	Montelukast	6.379	198464	7291	6274	1.1
2	Montelukast	6.382	193643	7219	6592	1.1
3	Montelukast	6.379	196462	7194	6028	1.1
4	Montelukast	6.376	194644	8174	6927	1.1
5	Montelukast	6.379	198464	8653	5920	1.1
Mean			196335.4			
Std.dev			2190.191			
%RSD			1.115536			

Intermediate precision:**Day 1:****Fig:7.29 Chromatogram showing Day1 injection -1****Fig:7.30 Chromatogram showing Day1 injection -2****Fig:7.31 Chromatogram showing Day1 injection -3****Fig:7.32 Chromatogram showing Day1 injection -4**

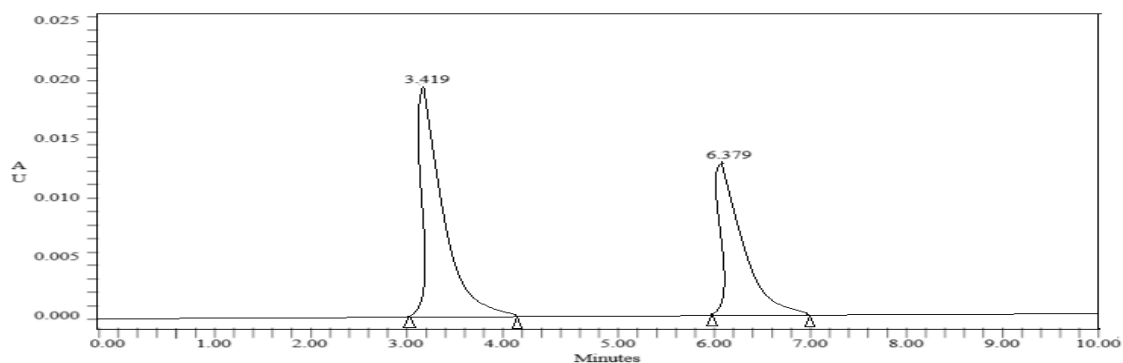


Fig:7.33 Chromatogram showing Day1 injection -5

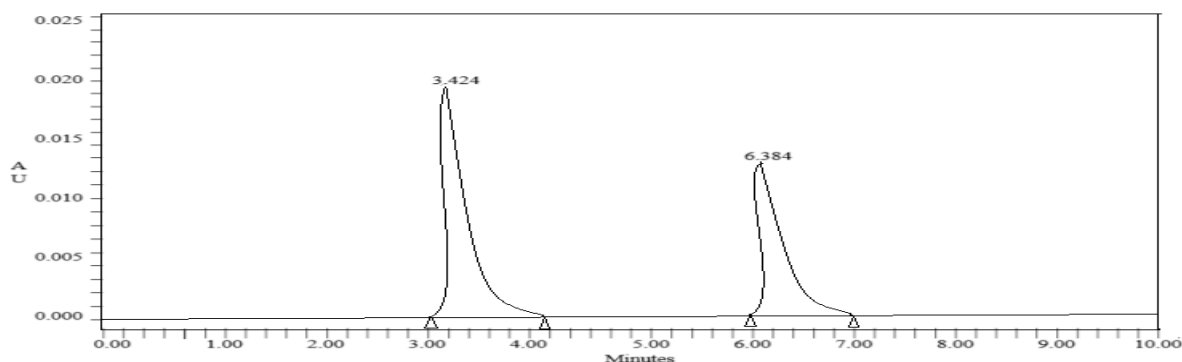


Fig:7.34 Chromatogram showing Day1 injection -6

Table:7.13 Results of Intermediate precision for Cetirizine

S. No	Peak Name	RT	Area ($\mu\text{V}\cdot\text{sec}$)	Height (μV)	USPPlate count	USPTailing
1	Cetirizine	3.419	2389572	395275	9375	1.2
2	Cetirizine	3.415	2391847	392175	9275	1.2
3	Cetirizine	3.424	2319472	312947	8265	1.2
4	Cetirizine	3.415	2306842	310585	6254	1.2
5	Cetirizine	3.419	2375972	310694	9028	1.2
6	Cetirizine	3.424	2396746	358373	8928	1.2
Mean			2363409			
Std. Dev.			39730.83			
% RSD			1.681082			

Acceptance criteria:

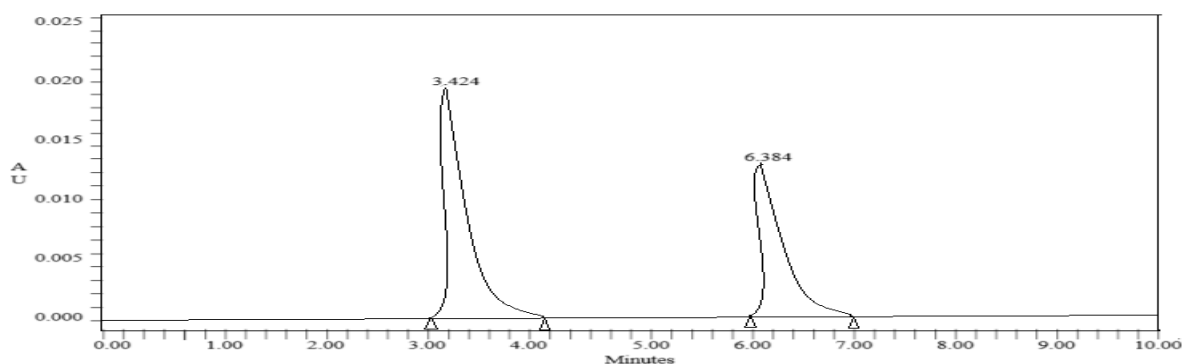
- %RSD of six different sample solutions should not more than 2

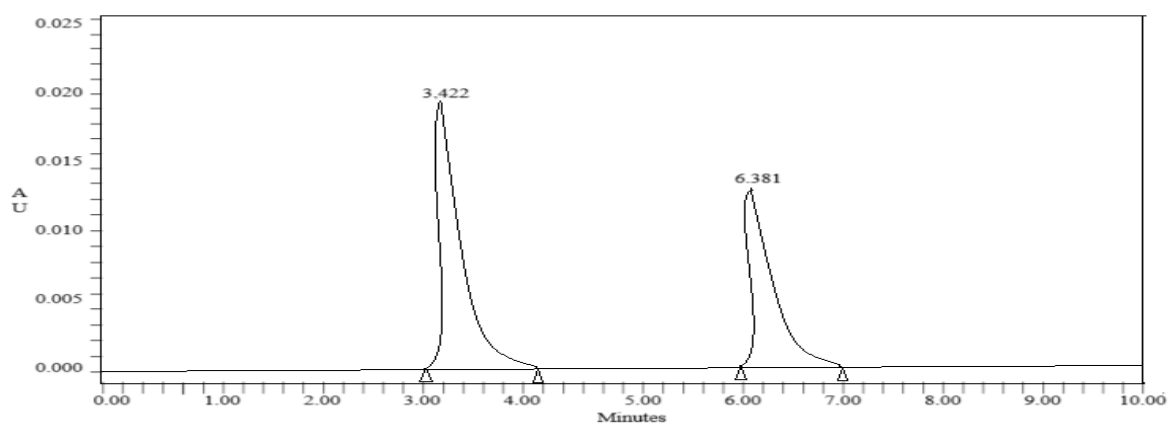
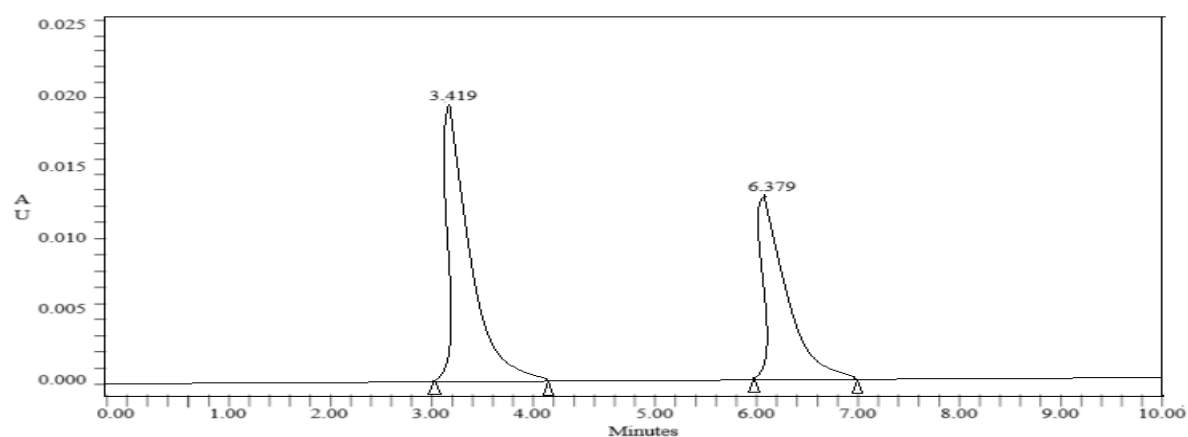
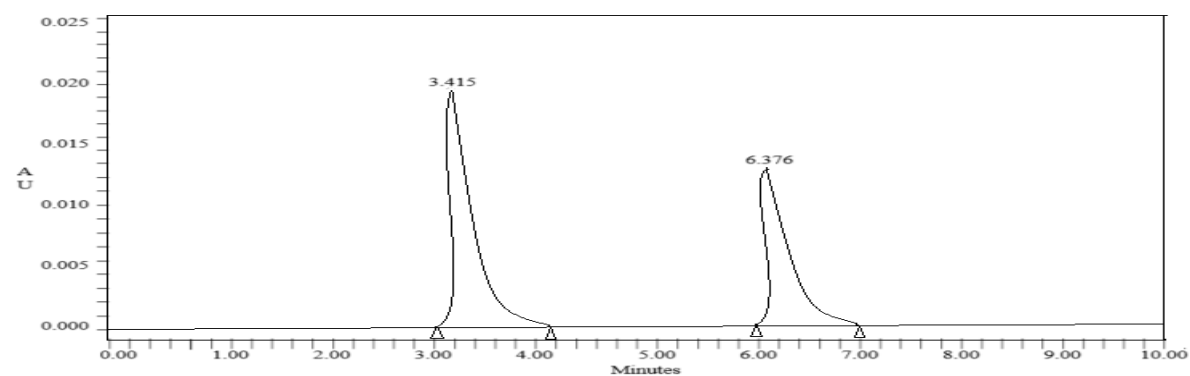
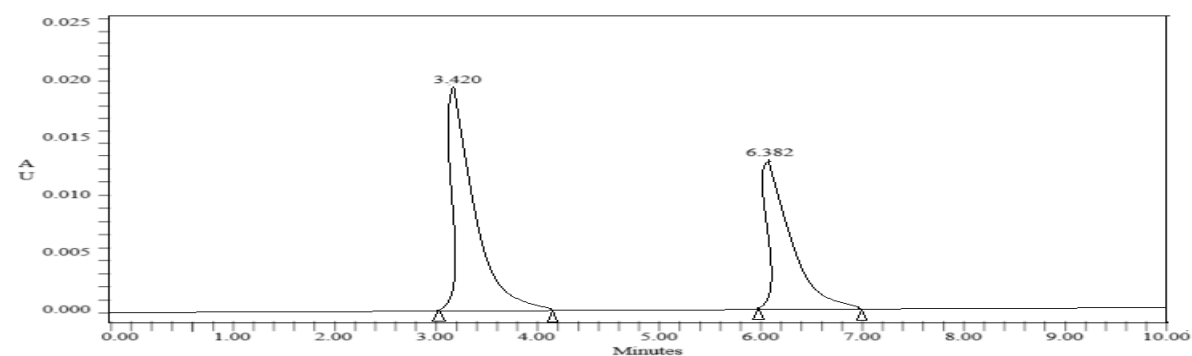
Table:7.14 Results of Intermediate precision for Montelukast

S. No	PeakName	RT	Area ($\mu\text{V}\cdot\text{sec}$)	Height (μV)	USPPlate count	USPTailing
1	Montelukast	6.379	197284	7194	8264	1.2
2	Montelukast	6.376	197849	7294	9174	1.2
3	Montelukast	6.384	196572	7147	9164	1.2
4	Montelukast	6.376	195028	7927	9733	1.2
5	Montelukast	6.379	199474	8238	9194	1.2
6	Montelukast	6.384	197482	7638	8973	1.2
Mean			197281.5			
Std. Dev.			1466.354			
% RSD			0.74328			

Acceptance criteria:

- %RSD of six different sample solutions should not more than 2

Day 2:**Fig:7.35 Chromatogram showing Day 2 injection -1**

**Fig:7.36 Chromatogram showing Day 2 injection -2****Fig:7.37 Chromatogram showing Day 2 injection -3****Fig:7.38 Chromatogram showing Day 2 injection -4****Fig:7.39 Chromatogram showing Day 2 injection -5**

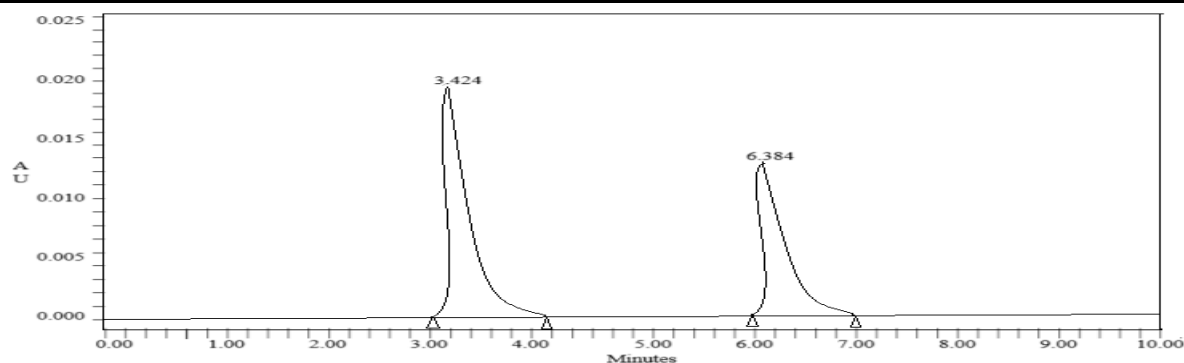


Fig:7.40 Chromatogram showing Day 2 injection -6

Table:7.15 Results of Intermediate precision Day 2 for Cetirizine

S.No	PeakName	RT	Area ($\mu\text{V}\cdot\text{sec}$)	Height (μV)	USPPlate Count	USPTailing
1	Cetirizine	3.424	2389562	391741	9264	1.2
2	Cetirizine	3.422	2381654	391047	9746	1.2
3	Cetirizine	3.419	2381946	391748	9816	1.2
4	Cetirizine	3.415	2391741	391746	9917	1.2
5	Cetirizine	3.420	2386452	381641	9742	1.2
6	Cetirizine	3.424	2374763	381645	9017	1.2
Mean			2384353			
Std. Dev.			6183.339			
% RSD			0.25933			

Acceptance criteria:

- %RSD of six different sample solutions should not more than 2

Table:7.16 Results of Intermediate precision Day 2 for Montelukast

S.No	PeakName	RT	Area ($\mu\text{V}\cdot\text{sec}$)	Height (μV)	USPPlate Count	USPTailing
1	Montelukast	6.384	197486	7582	6272	1.1
2	Montelukast	6.381	197486	7184	6174	1.1

3	Montelukast	6.379	196746	7456	5184	1.1
4	Montelukast	6.376	195862	7814	6194	1.1
5	Montelukast	6.382	196582	7194	6292	1.1
6	Montelukast	6.384	198463	7745	6191	1.1
Mean			197104.2			
Std. Dev.			903.542			
% RSD			0.458408			

Acceptance criteria:

- %RSD of six different sample solutions should not more than 2

ACCURACY:

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

Accuracy50%:

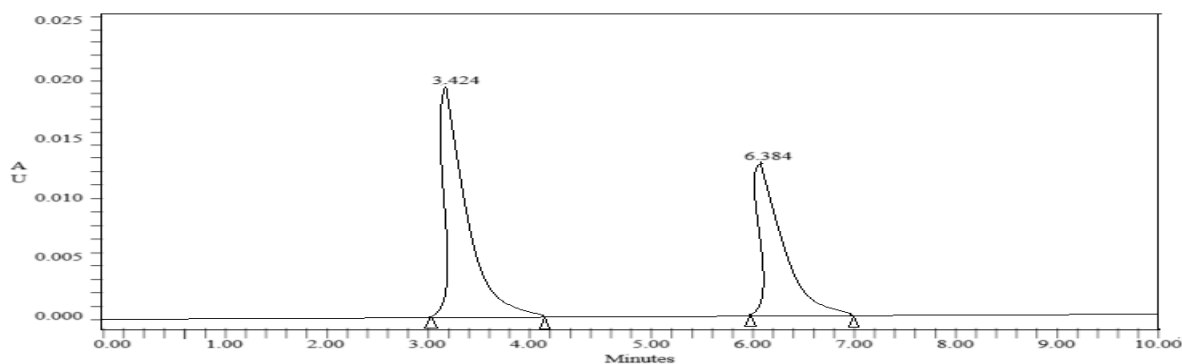


Fig.7.41 Chromatogram showing accuracy-50% injection-1

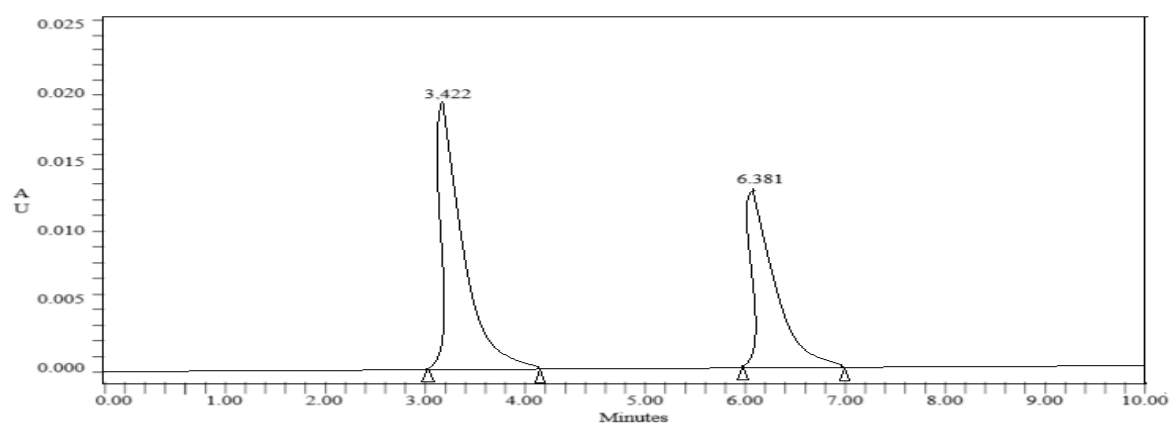


Fig.7.42 Chromatogram showing accuracy-50% injection-2

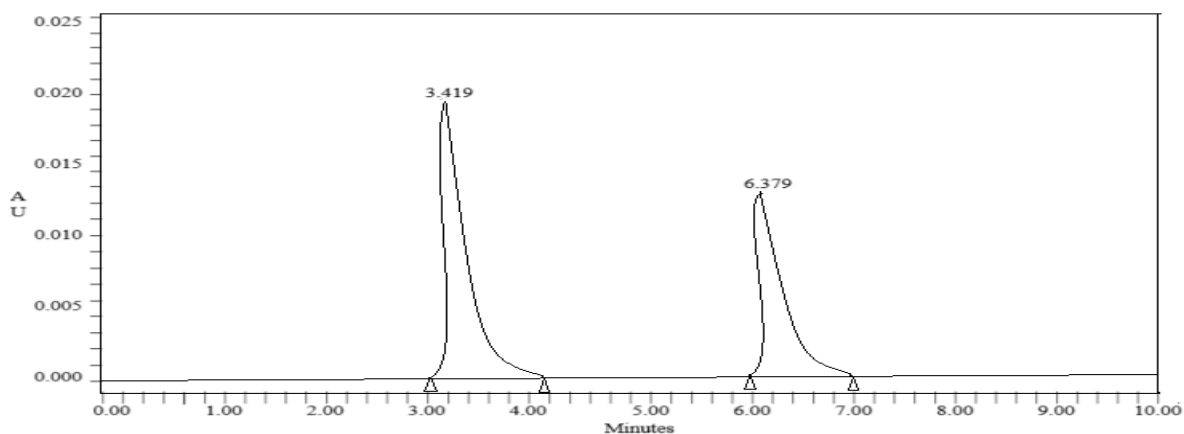


Fig.7.43 Chromatogram showing accuracy-50% injection-3

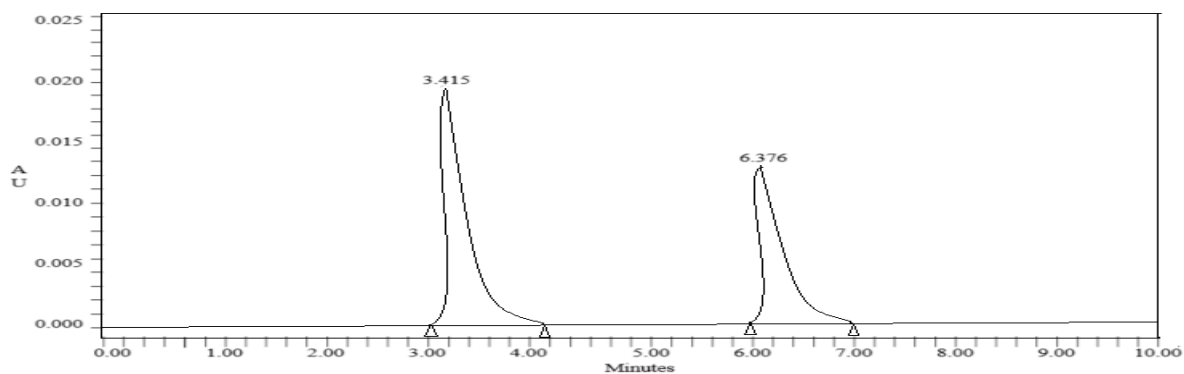
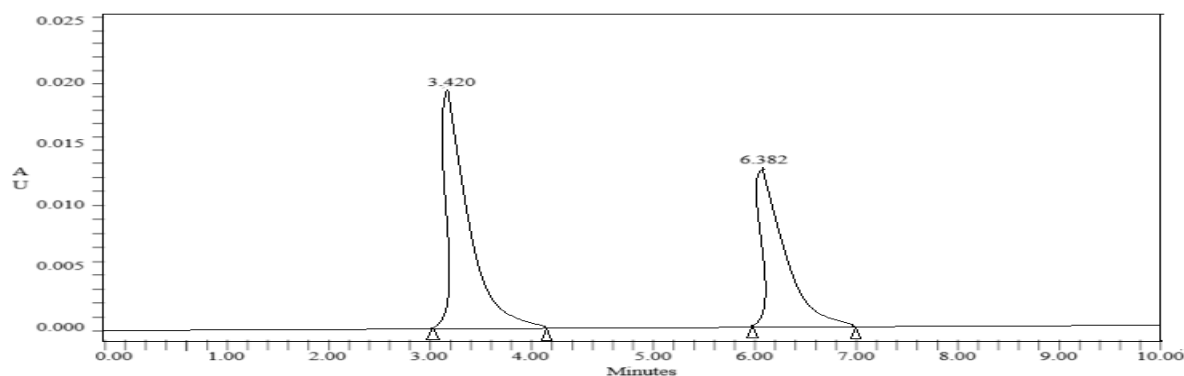
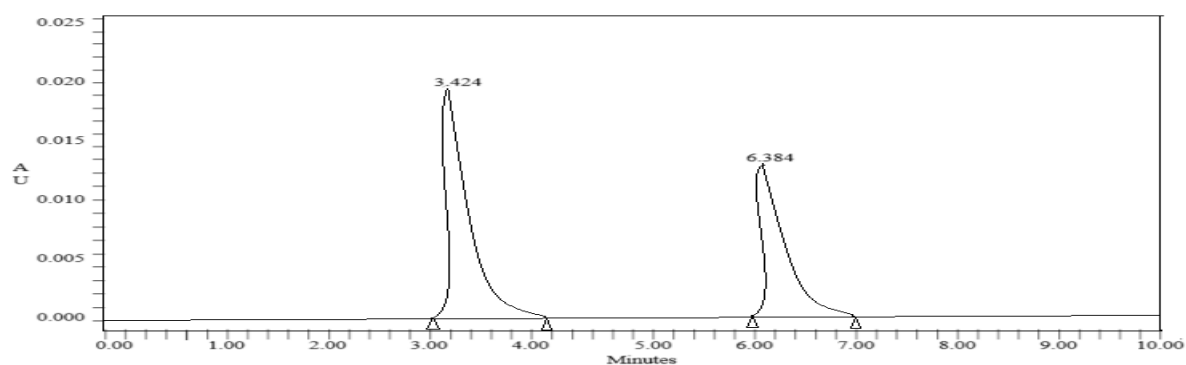
Table:7.17 Results of Accuracy for concentration-50%

Cetirizine:

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count	Injection
1	Cetirizine	3.424	1214719	156568	1.1	6353	1
2	Cetirizine	3.422	1218462	164774	1.2	8632	2
3	Cetirizine	3.419	1218472	159664	1.1	7554	3

Montelukast:

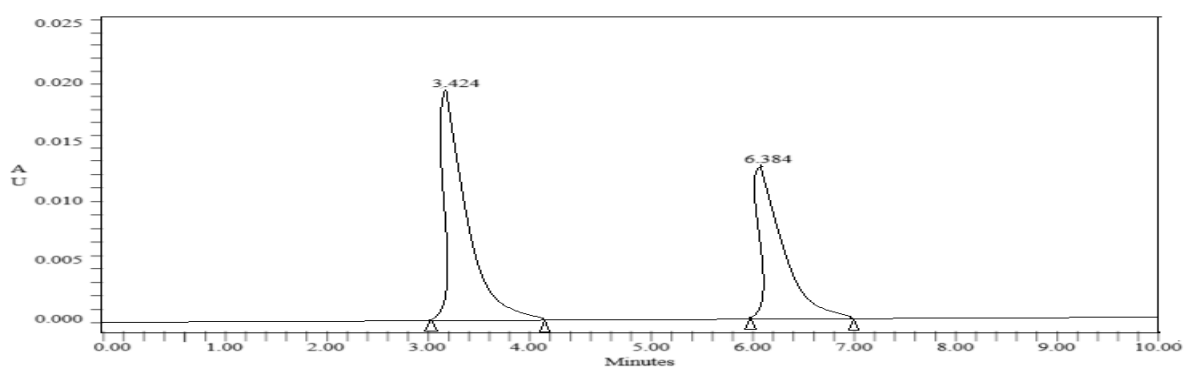
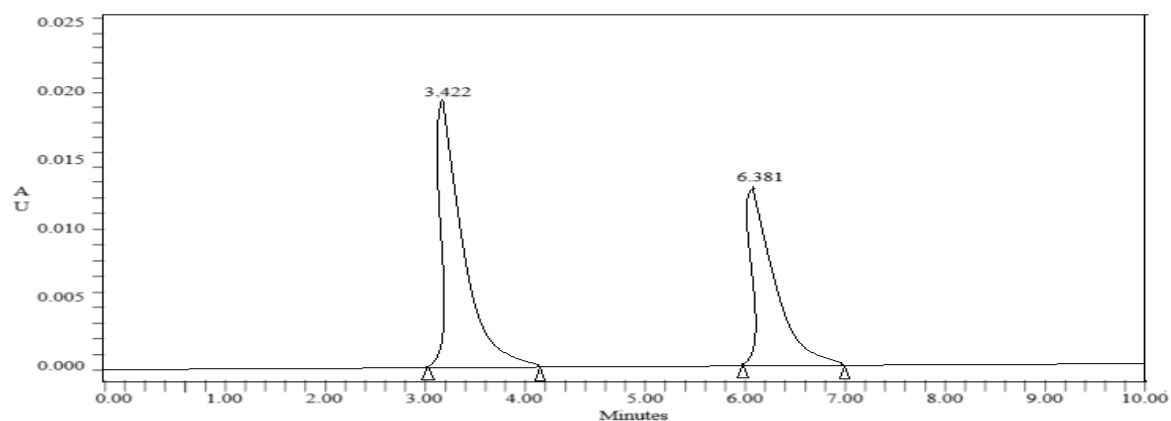
S. No	Name	RT	Area	Height	USP Tailing	USP Plate Count	Injection
1	Montelukast	6.384	98627	7462	1.1	7833	1
2	Montelukast	6.381	98634	7642	1.1	8264	2
3	Montelukast	6.379	98535	7814	1.1	7642	3

Accuracy100%:**Fig:7.44 Chromatogram showing accuracy-100% injection-1****Fig:7.45 Chromatogram showing accuracy-100% injection-2****Fig:7.46 Chromatogram showing accuracy-100% injection-3****Table:7.18 Results of Accuracy for concentration-100%****Cetirizine:**

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count	Injection
1	Cetirizine	3.415	2397481	311422	1.1	7462	1
2	Cetirizine	3.420	2397471	316154	1.1	6726	2
3	Cetirizine	3.424	2396471	311973	1.1	5756	3

Montelukast:

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count	Injection
1	Montelukast	6.376	198371	7112	1.1	8464	1
2	Montelukast	6.382	198353	7148	1.1	7364	2
3	Montelukast	6.384	198355	7191	1.1	7733	3

Accuracy150%:**Fig.7.47 Chromatogram showing accuracy-150% injection-1****Fig.7.48 Chromatogram showing accuracy-150% injection-2**

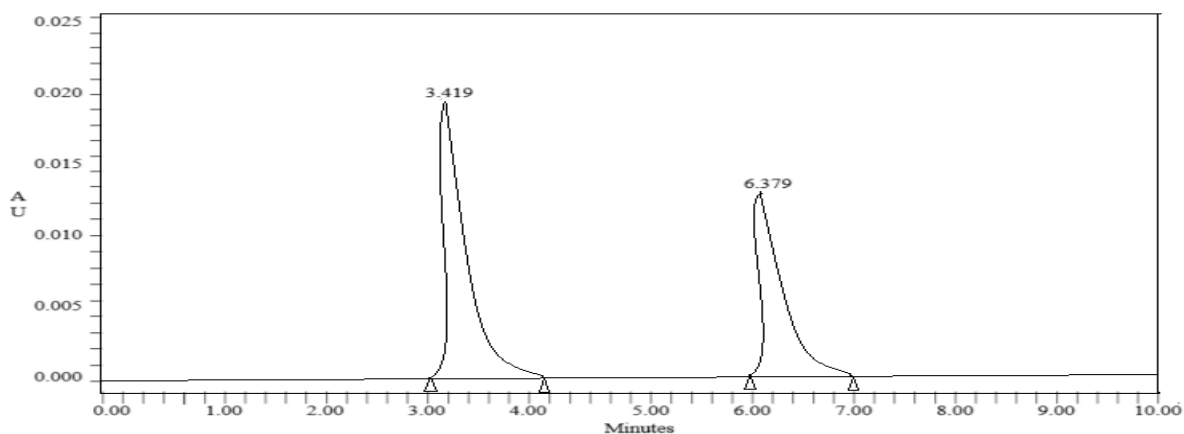


Fig.7.49 Chromatogram showing accuracy-150% injection-3

Table:7.19 Results of Accuracy for concentration-150%

Cetirizine:

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count	Injection
1	Cetirizine	3.442	3512923	311841	1.1	7622	1
2	Cetirizine	3.422	3518846	316452	1.1	8462	2
3	Cetirizine	3.419	3511871	318644	1.1	7483	3

Montelukast:

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count	Injection
1	Montelukast	6.384	291461	7192	1.2	7464	1
2	Montelukast	6.381	291431	7111	1.1	9375	2
3	Montelukast	6.379	291645	7125	1.1	6733	3

The accuracy results for Cetirizine

% Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	1217218	112.5	112.4	99.6	99.3
100%	2397141	225	225	100	
150%	3514547	337.5	332.5	98.5	

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.

The accuracy results for Montelukast

% Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	98598.67	22.5	22.4	99.9	99.6
100%	198359.7	45	45	100	
150%	291512.3	67.5	66.8	99	

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.

LIMIT OF DETECTION

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.

$$\text{LOD} = 3.3 \times \sigma / s$$

Where

σ = Standard deviation of the response

S = Slope of the calibration curve

Cetirizine :**Result:**

$$=3.3 \times 39762 / 10421$$

$$=12.5 \mu\text{g/ml}$$

Montelukast:**Result:**

$$=3.3 \times 5008 / 4365$$

$$=3.7 \mu\text{g/ml}$$

Quantitation limit

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined.

$$\text{LOQ} = 10 \times \sigma / S$$

Where

σ = Standard deviation of the response

S = Slope of the calibration curve

Cetirizine :**Result:**

$$=10 \times 39762 / 10421$$

$$=38.1 \mu\text{g/ml}$$

Montelukast:**Result:**

$$=10 \times 5008 / 4365$$

$$=11.4 \mu\text{g/ml}$$

ROBUSTNESS

The robustness was performed for the flow rate variations from 0.9 ml/min to 1.1ml/min and mobile phase ratio variation from more organic phase to less organic phase ratio for Cetirizine and Montelukast. The method is robust only in less flow condition and the method is robust even by change in the Mobile phase $\pm 5\%$. The standard sample of Cetirizine and Montelukast were injected by changing the conditions of chromatography. There was no significant change in the parameters like resolution, tailing factor and plate count.

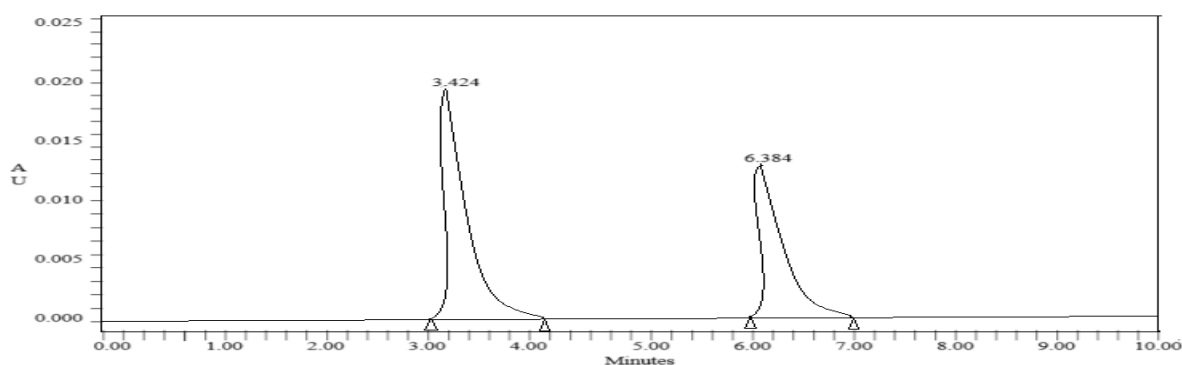
Variation in flow

Figure:7.50 chromatogram showing less flow of 0.9ml/min

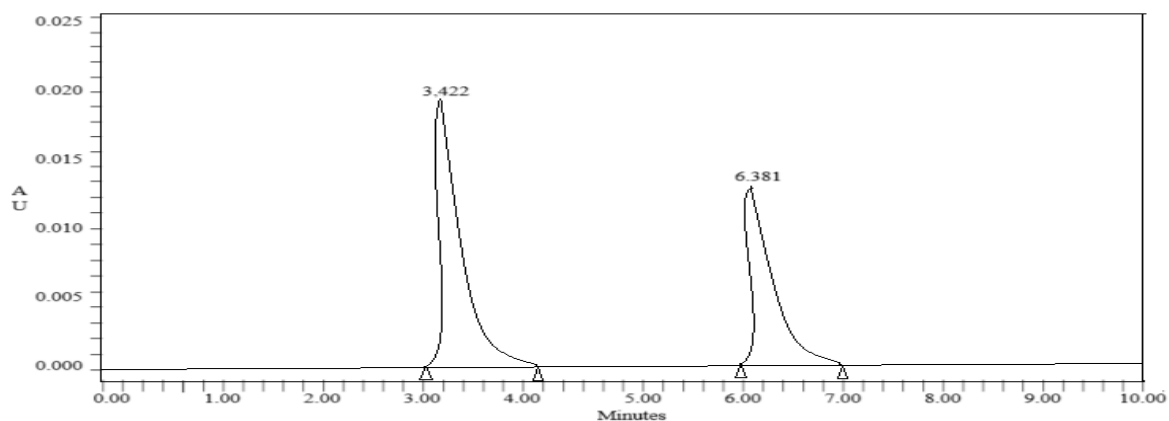


Figure:7.51 chromatogram showing more flow of 1.1 ml/min

Variation of mobile phase organic composition

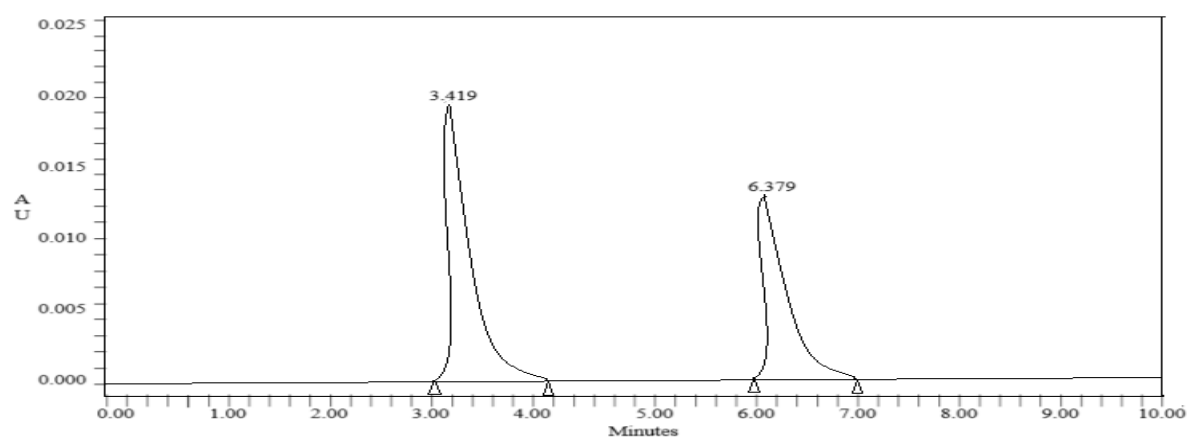


Figure:7.52 chromatogram showing less organic composition

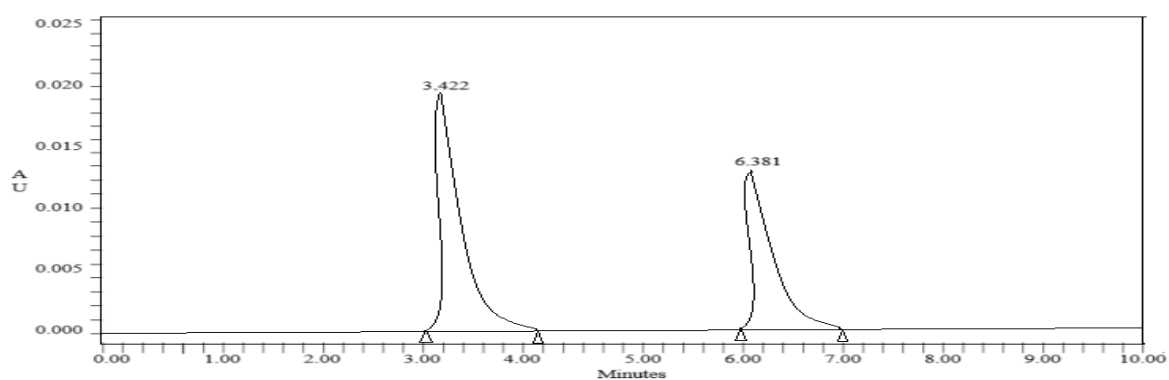


Figure:7.53 chromatogram showing more organic composition

Table:7.20 Results for Robustness**Cetirizine**

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 1.0mL/min	2391746	3.418	9028	1.2
Less Flow rate of 0.9mL/min	2371831	3.424	7381	1.2
More Flow rate of 1.1mL/min	2218319	3.422	9311	1.1
Less organic phase (about 5 % decrease in organic phase)	2294821	3.419	7462	1.2
More organic phase (about 5 % Increase in organic phase)	2394811	3.422	6817	1.1

Acceptance criteria:

The tailing factor should be less than 2.0 and the number of theoretical plates (N) should be more than 2000.

Table:7.21 Results for Robustness**Montelukast**

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 1.1mL/min	194627	6.463	7398	1.1
Less Flow rate of 0.9mL/min	183738	6.384	6883	1.1
More Flow rate of 0.8mL/min	198373	6.381	9917	1.2
Less organic phase (about 5 % decrease in organic phase)	178471	6.379	8372	1.1
More organic phase (about 5 % Increase in organic phase)	189462	6.381	7716	1.2

Acceptance criteria:

The tailing factor should be less than 2.0 and the number of theoretical plates (N) should be more than 2000.

8. SUMMARY AND CONCLUSION

A reliable and accurate Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) method was developed and validated for the simultaneous estimation of Cetirizine and Montelukast in both bulk drug and pharmaceutical formulations. The separation was achieved on a Phenomenex Luna C18 column (4.6 × 150 mm, 5 µm) maintained at a temperature of 35°C.

The mobile phase used was a mixture of Methanol and Water in the ratio of 70:30 v/v, with a flow rate of 1.0 mL/min. Detection was carried out at a wavelength of 230 nm, using a 10 µL injection volume, and the total run time was 10 minutes.

Both Cetirizine and Montelukast were effectively separated with well-resolved, sharp, and symmetrical peaks. The method was validated according to ICH guidelines for parameters including linearity, accuracy, precision, specificity, robustness, and system suitability, all of which were within acceptable limits, confirming the method's reliability and reproducibility.

The developed RP-HPLC method is simple, rapid, and sensitive for the simultaneous determination of Cetirizine and Montelukast in bulk and pharmaceutical formulations. The optimized chromatographic conditions enabled efficient separation within 10 minutes, making the method suitable for routine quality control and analytical testing. The successful validation as per ICH guidelines demonstrates that this method can be confidently used in pharmaceutical laboratories for consistent and accurate results.

9. BIBLIOGRAPHY

1. Dr. Kealey and P.J Haines, Analytical Chemistry, 1st edition, Bios Publisher, (2002), PP 1-7.
2. A. Braithwait and F.J. Smith, Chromatographic Methods, 5th edition, Kluwer Academic Publisher, (1996), PP 1-2.
3. Andrea Weston and Phyllis. Brown, HPLC Principle and Practice, 1st edition, Academic press, (1997), PP 24-37.
4. Yuri Kazakevich and Rosario Lofrutto, HPLC for Pharmaceutical Scientists, 1st edition, Wiley Interscience A John Wiley & Sons, Inc., Publication, (2007), PP 15-23.
5. Chromatography, (online). URL: <http://en.wikipedia.org/wiki/Chromatography>.
6. Meyer V.R. Practical High-Performance Liquid Chromatography, 4th Ed. England, John Wiley & Sons Ltd, (2004), PP 7-8.
7. Sahajwalla CG a new drug development, vol 141, Marcel Dekker Inc., New York, (2004), PP 421–426.
8. D. H. Shewiy, E. Kaale, P. G. Risha, B. Dejaegher, J. S. Verbeke, Y. V. Heyden, Journal Pharmaceut. Biomed. Anal, 66, 2012, 11-23.
9. M. D. Rockville, General Tests, Chapter 621 – Chromatography System Suitability, United States Pharmacopeial Convention (USP), USP 31, 2009.
10. FDA Guidance for Industry-Analytical Procedures and Method Validation, Chemistry, Manufacturing, and Controls Documentation, Centre for Drug Evaluation and Research (CDER) and Centre for Biologics Evaluation and Research (CBER), 2000.

11. Korany MA, Mahgoub H, Ossama TF, Hadir MM. Application of artificial neural networks for response surface modelling in HPLC method development. J Adv Res, 3, 2012, 53-63.
12. Swartz ME, Jone MD, Fowler P, Andrew MA. Automated HPLC method development and transfer. Lc Gc N. Am, 75, 2002, 49-50.
13. Snyder LR, Kirkland JJ, Glajach JL. X. In Practical HPLC Methods Development. John Wiley, New York, 295, 1997, 643-712.
14. Swartz M, Murphy MB. New Fronties in Chromatography. Am Lab, 37, 2005, 22-27.
15. Dolan JW. Peak tailing and resolution. Lc Gc N. Am, 20, 2002, 430-436.
16. Chan CC, Leo YC, Lam H. Analytical method validation and Instrument Performance Validation. Vol-I, Wiley Interscience, 2004.
17. Doserge, Wilson and Gisvold's text book of organic medicinal and pharmaceutical chemistry, 8thed, Lippincott Company, 1982, P.183-197.
18. Michael e. S., Ira s. K., "analytical method development and validation", Marcel Dekker, inc., New York, 1997; 25-29.
19. Becket and stenlake, practical pharmaceutical chemistry, part 24th edition CBS publications and distributors, 2005, 157-168.
20. Practical HPLC method development Lloyd R.Snyder, Joseph J. Kirkland, Joseph L. Glajch, second edition, 1, 420-430, 686-704.
21. International conference on harmonization: ICH Q 2 (R1) Validation of Analytical Procedures: Text and Methodology 1995.
22. Connors K.A, "a text book of pharmaceutical analysis", Wiley-inter science, Singapore, 1999; 175.
23. Willard-H.H., Lynnel. M. Jr., john a., dean F.A., "instrumental methods of analysis", 7th edn. CBS publishers and distributors, New Delhi; 1-12, 580-610, 614-652.
24. Davidson A.G, "basis of Spectrophotometry", 4th ed., part-2, CBS publishers, New Delhi, 2002; 264-74.
25. Fronka. S., "Handbook of instrumental techniques for analytical chemistry", 1st edn., Pearson education, 2004 ; 7.
26. Kalsi P.S., "Spectroscopy of organic compounds", 5thed, new age international publishers New Delhi, 2002; 7.
27. Braun R.d., "Introduction to instrument analysis", Pharma book syndicate, Hyderabad, 2005; 261.
28. Beckett, A.H., Stenlakej. B., "Practical Pharmaceutical Chemistry", (1997), 4th edition, part 2, CBS publishers and distributors; 275-337.
29. Sethip. D., "High Performance Liquid Chromatography: Quantitative Analysis of Pharmaceutical Formulation", 2001; 1st edn; 5 – 11, 141.
30. Chetan Vilasrao Patil; Shabnam Khan; Ramakant Sharma; Jeevan Patel; Dr. Rakesh Patel. Stability Indicating Rp-Hplc Method Development and Validation For Simultaneous Estimation Of Bilastine And Montelukast In Bulk And Tablet Dosage Form. International Journal of Pharmaceutical Sciences & Medicine (IJPSM), Vol.8 Issue. 3, March- 2023

31. Bhanu Mohammed Sameera, Dadi Vasudha, Rao Yarraguntla Srinivasa, Rao Vara Prasad K. RP-HPLC method for quantification of bilastine and Montelukast sodium in pharmaceutical dosage form. Research Journal of Pharmacy and Technology, Year: 2023, Volume: 16, Issue: 3.
32. Sudhakar B., Akshaya Karipe, Sri S Ramya. Analytical Method Development and Validation for the Simultaneous Estimation of Bilastine and Montelukast by RP-HPLC. Research Journal of Pharmacy and Technology, Year: 2023, Volume: 13, Issue: 2.
33. Aishwarya Wagh; Shabnam Khan; Ramakant Sharma; Dr. Rakesh Patel. Stability Indicating Rp-Hplc Method Development And Validation For Simultaneous Quantification Of Antihistaminic & Asthmatic Drug In Bulk And Tablet Dosage Form. International Journal of Pharmaceutical Sciences & Medicine (IJPSM), Vol.7 Issue. 11, November- 2022.
34. Pranav Vyas, Vivek Singh Thakur, Deepak Basedia, and Balkrishna Dubey. “Development and Validation of RP-HPLC Based Analytical Method for Simultaneous Estimation of Montelukast and Bilastine in Tablet Dosage Form”. Current Research in Pharmaceutical Sciences, Vol. 12, no. 1, Apr. 2022.