DEVELOPMENT AND VALIDATION OF REVERSE PHASE HPLC METHOD FOR THE SIMULTANEOUS ESTIMATION OF MONTELUKAST SODIUM AND EBASTINE IN ITS TABLET DOSAGE FORM

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ABSTRACT

Development and validation of reverse phase HPLC method for the simultaneous estimation of montelukast and ebastine in its tablet dosage form. The reverse phase HPLC includes stationary phase is non-polar hydrophobic packing with octyl (or) octa decyl functional group bonded to silica gel and mobile phase is polar solvent. Montelukast is used regularly to prevent the wheezing and anti-asthma. Ebastine is an anti histamines.

KEYWORDS: Reversephase, montelukast, ebastine, stationaryphase, non-polar, hydrophobic, octyl, octa decyl, mobile phase, wheezing, anti-ashtma.

1. INTRODUCTION

The ever expanding and broad horizon of pharmaceutical sciences invariably emphasizes one cardinal aspect that basically they are nothing but “Applied Sciences”. With the advent of newer drug molecules either partially synthesized totally synthesized or isolated from naturally occurring microbial and plant products- it has become absolutely necessary to ascertain and examine critically their physical characteristics, chemical equivalence, chemical impurities and their biological features. All these salient features of a “drug” help a researcher not only in planning a precise experimental design but also in interpretation of data.
in logical and scientific manner. Pharmaceutical scientists ought to achieve completeness in their scientific pursuit of knowledge.\(^1\)

**Analytical chemistry:** “The science which deals with the detection, identification and quantitation of chemical, biological and microbiological species in matrices of chemical, biological and environmental importance.”\(^2\)

Over the course of three decades analytical chemistry, has met with a fundamental change, which can be described as the development of instrumental analysis. These two words mean the physicochemical methods performed by instruments, which formerly were considered complicated machines, but which now have become familiar to chemists. Firstly, the analytical information can be obtained with much greater rapidity than by classical methods; secondly various types of information are obtained at one instance, which would be extremely difficult with chemical methods. These are the reasons why instrumental methods have developed fairly rapidly, despite the high cost of instrumental tools and the necessity for training people to use them properly.\(^3\)

**Analytical Chemistry And The Pharmaceutical Industry**

The impact of the pharmaceutical industry lies not only in social impact but also in its economic strengths accompanying the advances made in medical knowledge, hygiene and sanitation. Pharmaceuticals have helped reduced mortality and morbidity rate substantially over the past century adding to the potential productive life span of the world population and improving the quality of life for many. Furthermore, the industry is a powerful economic asset reducing health loss by lessening the need for extended care while generating substantial revenue and employment for world economy. R&D activity is the heart of modern pharmaceutical industry and its continuing success is essential for the continuous growth of the industry. The money and the time involved are necessary to ensure that knowledge of the product is complete before it is marketed. This knowledge refers to the understanding of possible side effects absorption rate, efficacy and all other possible effects the drug may have on the human body.\(^4\)

**Sample preparation** 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 pretreatment to remove interferences and/or protect the column or equipment from damage.

**Table 1. Selection of sample**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Requirements</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inorganic ions</td>
<td>Detection is primary problem; use ion chromatography.</td>
</tr>
<tr>
<td>Isomers</td>
<td>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.</td>
</tr>
<tr>
<td>Enantiomers</td>
<td>These compounds require “chiral” conditions for their separation.</td>
</tr>
<tr>
<td>Biological</td>
<td>Several factors make samples of this kind “special”: molecular conformation, polar functionality, and a wide range of hydrophobicity.</td>
</tr>
<tr>
<td>Macromolecules</td>
<td>“Big” molecules require column packings with large pores (&gt;10-nm diameters); in addition, biological molecules require special conditions as noted above.</td>
</tr>
</tbody>
</table>

**Table .2 Experimental Conditions For HPLC Separations**

<table>
<thead>
<tr>
<th>Separation Variable</th>
<th>Preferred Initial Choice</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Column</strong></td>
<td></td>
</tr>
<tr>
<td>Dimensions (length, ID)</td>
<td>15 X 0.46 cm</td>
</tr>
<tr>
<td>Particle size</td>
<td>5 μm²</td>
</tr>
<tr>
<td><strong>Stationary phase</strong></td>
<td>C₈ or C₁₈</td>
</tr>
<tr>
<td><strong>Mobile phase</strong></td>
<td></td>
</tr>
<tr>
<td>Solvents A and B</td>
<td>Buffer- acetonitrile</td>
</tr>
<tr>
<td>% B</td>
<td>80-100%</td>
</tr>
<tr>
<td>Buffer (compound, PH, concentration)</td>
<td>25mM potassium phosphate, 2.0&lt;pH&lt;3.0</td>
</tr>
<tr>
<td>Additives (e.g., amine modifiers, ion-pair reagents)</td>
<td>Do not use initially</td>
</tr>
<tr>
<td>Flow rate</td>
<td>1.5-2.0 mL/min</td>
</tr>
<tr>
<td>Temperature</td>
<td>35-45°C</td>
</tr>
<tr>
<td><strong>Sample Size</strong></td>
<td></td>
</tr>
<tr>
<td>Volume&lt;sup&gt;d&lt;/sup&gt;</td>
<td>&lt; 25μL</td>
</tr>
<tr>
<td>Weight</td>
<td>&lt;100 μg</td>
</tr>
</tbody>
</table>

**SELECTION OF MOBILE PHASE:** The following are the parameters, which shall be taken into consideration while selecting and optimizing the mobile phase.

- Buffer
- PH of the buffer
- Mobile phase composition
VALIDATION  “While accuracy of results is the goal for a particular analysis under development, the ultimate goal of validation is to ensure that something does what it is intended to do, precisely and reliably.”

2.DRUG PROFILE

Montelukast Drug Structure

Chemical name

(\(S,E\))\(-2-(1-(1-(3-(2-(7\text{-}chloroquinolin\text{-}2\text{-}yl})\text{vinyl})\text{phenyl})-3-(2-(2\text{-}hydroxypropan\text{-}2\text{-}yl})\text{phenyl})\text{propylthio})\text{methyl})\text{cyclopropyl} \text{acetic acid.}

Chemical data

Formula: C35H36ClNO3
Mol.mass:586.184g/mol

Drug category: Antiasthamatic Agent(Leukotriene receptor Antagonist)

Characteristics: Montelukast sodium is a hygroscopic, optically active, white to off white Powder.

Solubility: Freely soluble in methanol, ethanol and water, slightly soluble in alcohol, insoluble in acetonitrile.

Ebastine Drug structure
Chemical name
1[4(1,1-dimethylethyl)-phenyl]-4-[4-{diphenyl methoxy}-1-piperidinyl]-1-butanone.

Chemical data
Formula: C32H39NO2
Mol.mass: 469.658g/mol

Molecular weight: 496.66

Category: Anti-histamine

Characteristics: White to almost white, crystalline powder.

Solubility: Sparingly soluble in methanol, insoluble in water, very soluble in methylene chloride.

3. ANALYTICAL METHOD
ANALYTICAL METHOD DEVELOPMENT
A. Selection of wavelength
A solution of 10µg/ml of Montelukast and Ebastine were prepared in milliQ water. The resulting solutions were scanned individually on HPLC PDA detector from 190 to 400 nm. The optimal response for three of them was obtained at 242 nm. Hence the complete method was processed at the wavelength of 242 nm.

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

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

Preparation of standard solution: 10 mg of Montelukast and 10mg of Ebastine were accurately weighed and transferred into a 10 ml clean dry volumetric flask, about 7 ml of
diluent was added, sonicated to dissolve it completely and the volume was made up to the mark with the same solvent to give a concentration of 1000 µg/ml. (Stock solution) Further 0.2 ml were pipetted out from the above stock solution into a 10 ml volumetric flask and diluted up to the mark with diluent to give a concentration of 20 µg/ml and 20 µg/ml respectively.

**Preparation of sample solution**

Take injection vial of Montelukast and Ebastine and pipet out the sample quantity equivalent to 10 mg of active ingredient present in Montelukast and Ebastine was transferred into a 10 ml clean dry volumetric flask, 7 ml of diluent was added to it and was shaken by mechanical stirrer and sonicated for about 30 minutes by shaking at intervals of five minutes each and was diluted up to the mark with diluent to give a concentration of 1000 µg/ml and allowed to stand until the residue settles before taking an aliquot for further dilution (stock solution). 0.2 ml of upper clear solution was transferred to a 10 ml volumetric flask and diluted with diluent up to the mark to give the respective concentrations as par with standard solution. The solution was filtered through 0.45 µm filter before injecting into HPLC system.

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

**TRIALS**

**Trial 1:** Method development for the drugs was initiated based on the individual chemical characteristics’ and their methods given in individual journals.

**Mobile phase:** TEA: Methanol pH6 (30:70)

**Diluent:** Methanol

**Chromatographic conditions:**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow rate</td>
<td>1 ml/min</td>
</tr>
<tr>
<td>Column</td>
<td>Symmetry C18 (4.6 x 150mm, 5µm)</td>
</tr>
<tr>
<td>Detector wavelength</td>
<td>220 nm</td>
</tr>
</tbody>
</table>
Column oven : Ambient
Injection volume : 10µl
Observation: There was no proper peak separation.

Trail 2: Inorder to improve resolution and remove fronting of the peak and avoid unwanted peaks interfering, mobile phase ratios were changed and again the same experiment was performed.
Mobile phase: TEA: Methanol pH 6 (20:80)
Diluent: methanol
Chromatographic conditions
Flow rate : 1ml per min
Column : Symmetry C18 (4.6 x 150mm, 5µm)
Detector wavelength : 220 nm
column oven : Ambient
Injection volume : 10µl
observation:
Peaks are separated but tailing has been observed.

Trial-3: In order to avoid poor response and tailing mobile phase was changed i.e organic phase was changed.
Mobile phase: TEA: Methanol pH 6 (30:70)
Diluent: methanol.
Chromatographic conditions
Flow rate : 1 ml per min
Column : Symmetry C18 (4.6 x 150mm, 5µm)
Detector wavelength : 318 nm
Column oven : Ambient
Injection volume : 10µl

Observation
Peaks were not separated in this trail. So next go for another trail.
Fig-1 Chromatogram of Trial 1

Fig-2 Chromatogram of Trial 2

Fig-3 Chromatogram of Trial 3
Objective
Validation of Ebastine and Montelukast preparation by RP HPLC method.

Assay of Ebastine and Montelukast (By RP HPLC)
Chromatographic parameters
Column: C18 [Thermo Hypersil-BDS], 4.6 x 250 mm, 5 µ
Flow rate: 1.0 ml / minute
Wavelength: 254 nm
Injection volume: 20 µl
Mobile phase: Buffer: Acetonitrile (200 : 800), Mix, filter and degas it.
Buffer preparation: Add 3.0 ml of Triethylamine in 1000ml water and pH adjust to 3.5 with diluted orthophosphoric acid.

Standard preparation: Weigh accurately about 50 mg of Ebastine WS and 52 mg of Montelukast sodium WS into two separate 250ml volumetric flask. Add 150ml of mobile phase. Sonicate to dissolve. Make up to volume with mobile phase.

Sample preparation: Weigh accurately sample powder equivalent to about 50 mg of Ebastine and 52 mg of Montelukast sodium into two separate 250 ml volumetric flask. Add 150 ml of mobile phase. Sonicate for 10 minutes. Cool the content, make up to volume with mobile phase. Filter the solution through Whatman No.42 filter paper. Discard first 5ml of filtrate. Use this solution as sample.

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System suitability: Inject Standard solution five times and calculate the % RSD. It should not be more than 2 %.

Procedure: Inject standard and sample solution and record the chromatograms.

Calculation: Ebastine
Sample AUC Std wt (mg) 250 % purity of std Average wt
----------------------------------------- x----------------------------------------- x 100 = %
Std AUC 250 Spl wt (mg) 100 Lable claim

Montelukast
Sample AUC Std wt (mg) 250 % purity of std Average wt
------------------------------- x------------------------------- x------------------------------- x,....... x100 = %
Std AUC 250 Spl wt (mg) 100 Lable claim

4. RESULTS AND DISCUSSION
Analytical Parameters
1. System Precision
2. Method Precision
3. Accuracy
4. Linearity
5. Specificity
6. Range
7. Stability of the Solutions
8. Repeatability
9. Reproducibility

1. System Precision

The system precision is determined by analysing replicates [ten replicates] of the Standard Solution of Median Concentration of the Range [50-150%] of the theoretical quantity of analytes under similar conditions of operation and composition and analyzing as per the test procedure.

![Fig-4 Chromatogram of system precision for standard](image)

Table 3: Results of system precision for standard

<table>
<thead>
<tr>
<th>Name</th>
<th>RT</th>
<th>Area</th>
<th>% Area</th>
<th>Height (µA)</th>
<th>USP Resolution</th>
<th>USP Tailing</th>
<th>USP Plate Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Ebastine</td>
<td>5.321</td>
<td>8820999</td>
<td>59.61</td>
<td>579691</td>
<td>1.8</td>
<td>2502</td>
<td></td>
</tr>
<tr>
<td>2 Montelukast</td>
<td>8.279</td>
<td>5976787</td>
<td>40.39</td>
<td>506221</td>
<td>7.9</td>
<td>11325</td>
<td></td>
</tr>
</tbody>
</table>

Inference: The system precision is acceptable as the % RSD is 0.892 & 0.866 for Ebastine and Montelukast respectively, which is less than 2.

2. Method Precision

The Method Precision is determined by using six standard solutions [not replicates from same solution] at the median concentration used for checking the Linearity of the Method and analysing as per the test procedure.
Inference
The method precision is acceptable as the % RSD is **0.211 & 0.228** for Ebastine and Montelukast respectively, which is less than 2%.

3. Accuracy of the Method
**Spiked Placebo Method:** The accuracy of the Method is determined by % recovery data obtained by spiking the placebo with 80, 90, 100, 110 and 120% of the declared content of the active ingredients and analyzing according to the test procedure. At each of the concentration.

6 Chromatogram of accuracy-100% for unknown sample
Inference  The % recovery of [Table 14 & 15] lies between 99.21 to 100.48 % & 99.38 to 101.39% for Ebastine & Montelukast respectively, which is with in the acceptance limit

4. Linearity of the Method  The linearity of the peak area [y] / concentration [x] of a dilution series in the range of 50 to 150% of the theoretical concentration is determined by analyzing as per the test procedure. The linearity is checked by the Linear Regression Analysis using the Method of Least Squares. The concentration points shall be approximately 50, 75,100,125 and 150% of the theoretical concentration. Injecting the Standard Solutions has checked the linearity [5 concentrations] for three consecutive day n level three replicate samples has been analysed.

7 Chromatogram of accuracy-100% for unknown sample

Table 5 Results for accuracy 100

<p>| Peak Results |</p>
<table>
<thead>
<tr>
<th>Name</th>
<th>RT</th>
<th>Area</th>
<th>% Area</th>
<th>Height (µV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Ebastine</td>
<td>5.357</td>
<td>8882563</td>
<td>59.65</td>
<td>578150</td>
</tr>
<tr>
<td>2 Montelukast</td>
<td>8.306</td>
<td>6008546</td>
<td>40.35</td>
<td>503167</td>
</tr>
</tbody>
</table>

Inference
The correlation coefficients of the regression equation are 0.999 indicating the high level of correlation between the peak area and concentrations.

5. Specificity Specificity of the method is determined by adding 80,90,100,110 and 120 percent of the theoretical quantity of placebo to the analytes at the median concentration of the range and analyzing as per the test procedure. Each concentration is analysed three times.
A standard solution and a pure placebo solution has also been analysed separately to ascertain the peak areas of Ebastine and Montelukast and to check the interference, if any, by the placebo ingredients in the estimation of Ebastine and Montelukast respectively.

![Chromatogram](autoScaledChromatogram.png)

**Fig-8: Chromatogram for specificity of standard**

**Table 6: Results for specificity of standard**

<table>
<thead>
<tr>
<th>Name</th>
<th>RT</th>
<th>Area</th>
<th>% Area</th>
<th>Height (μV)</th>
<th>Purifyl Angle</th>
<th>Purifyl Threshold</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Ebastine</td>
<td>5.357</td>
<td>8914398</td>
<td>59.62</td>
<td>578379</td>
<td>0.038</td>
<td>0.239</td>
</tr>
<tr>
<td>2 Montelukast</td>
<td>8.294</td>
<td>6037792</td>
<td>40.38</td>
<td>500247</td>
<td>0.071</td>
<td>0.244</td>
</tr>
</tbody>
</table>

**Inference**
1. There is no interference by the placebo in the determination of Ebastine and Montelukast, as the placebo does not show any peak at the Retention times of Ebastine and Montelukast peak.
2. The % recovery during the determination of specificity lies within acceptance limits
3. The % RSD value is below 2.

6. **Range:** The range of the analyte concentration shall be 50 to 150% of the theoretical median concentration. This is the concentration interval within which the analyte may be estimated with defined level of accuracy and precision.

7. **Stability**
   Standard Solution  The standard solution of median concentration at room temperature was analysed at zero hour and after 8 hours by the test procedure and the stability shall be
determined. Concentration of Solution 200 mcg /ml of Ebastine & 200 mcg/ml of Montelukast.

**9 Chromatogram for standard stability**

**Results for standard stability**

<table>
<thead>
<tr>
<th>Peak Results</th>
<th>Name</th>
<th>RT</th>
<th>Area</th>
<th>% Area</th>
<th>Height (µV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ebastine</td>
<td>5.333</td>
<td>8886075</td>
<td>59.67</td>
<td>579450</td>
</tr>
<tr>
<td>2</td>
<td>Montelukast</td>
<td>8.281</td>
<td>6006510</td>
<td>40.33</td>
<td>504881</td>
</tr>
</tbody>
</table>

**Inference:** The % RSD values of peak areas in standard solution at zero hour and after 8 hours are about **0.811 & 0.618** and in sample solution are about **1.081 & 0.940**, for Ebastine and Montelukast respectively which is below the 2%. This indicates the solution is stable up to 8 hours.

8. Repeatability [Robustness]

The repeatability of the method shall be determined by varying the appropriate conditions

8.1 Mobile Phase [2% ± analytical concentration of Acetonitrile, i.e.80% ± 2%]

8.2 Wavelength [3nm ± Actual Wavelength i.e., 254 ± 3nm]

8.3 Flow Rate [0.2 mL ± Actual i.e., 1.0 ± 0.2 mL]

9. Reproducibility [Ruggedness]: The reproducibility of the method shall be determined by varying the appropriate conditions. This was determined by analysing the same set of samples by different analysts. Five replicates were analysed by each analyst.

The results have been presented in Table 27 & 28,
Table 8. Data for Reproducibility of the Method with different analysts Ebastine

<table>
<thead>
<tr>
<th>Analyst</th>
<th>Conc. of Ebastine [Added] [mcg/ml]</th>
<th>Conc. of Ebastine [Recovered] [mcg/ml]</th>
<th>% Recovery</th>
<th>Mean</th>
<th>Relative Standard Deviation</th>
<th>% Relative Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>200.05</td>
<td>200.15 199.96 200.06 200.04 199.94</td>
<td>100.08 99.98 100.03 100.02 99.97</td>
<td>100.02</td>
<td>0.044</td>
<td>0.044</td>
</tr>
<tr>
<td>II</td>
<td>200.02</td>
<td>200.44 200.06 199.92 200.13 200.02</td>
<td>100.22 100.03 99.96 100.07 100.01</td>
<td>100.06</td>
<td>0.099</td>
<td>0.099</td>
</tr>
</tbody>
</table>

Table 9. Data for Reproducibility of the Method with different analysts Montelukast

<table>
<thead>
<tr>
<th>Analyst</th>
<th>Conc. of Montelukast [Added] [mcg/ml]</th>
<th>Conc. of Montelukast [Recovered] [mcg/ml]</th>
<th>% Recovery</th>
<th>Mean</th>
<th>Relative Standard Deviation</th>
<th>% Relative Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>200.04</td>
<td>200.26 199.99 200.74 201.02</td>
<td>100.13 100.06 100.00 100.37 100.51</td>
<td>100.21</td>
<td>0.217</td>
<td>0.217</td>
</tr>
<tr>
<td>II</td>
<td>200.05</td>
<td>200.02 199.84 200.06 199.99 200.46</td>
<td>100.51 99.92 100.03 100.00 100.23</td>
<td>100.04</td>
<td>0.115</td>
<td>0.115</td>
</tr>
</tbody>
</table>

Inference
1. Refer Table 27 & 28. The % recovery of Ebastine and Montelukast lies between to 99.96 to 100.22 %

5. CONCLUSION
A method is developed for the estimation of ebastine and montelukast in tablets dosage form using High performance Liquid chromatograph. The developed method is validated for various parameters as per ICH guidelines like accuracy, precision, linearity, specificity, ruggedness and robustness. The results obtained are within the acceptance criteria.
The proposed method is applied for determination of ebastine and montelukast in marketed tablet formulations. The assay results confirmed with the label claim of the formulation. Hence the proposed method is found to be satisfactory and could be used for the routine analysis of ebastine and montelukast in tablets dosage form.

6. ACKNOWLEDGEMENT
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7. BIBLIOGRAPHY