**ABSTRACT**
Combination therapy of Montelukast (MNK) and Ebastine (EBA) provide anti-asthmatic effect by the maintenance treatment of asthma and relieve symptoms of allergies. The objective of this study is development of a new simple, accurate, sensitive, and reproducible RP-HPLC method for simultaneous estimation of MNK and EBA in pharmaceutical formulation (tablet) using Ofloxacin (OFL) as an internal standard and validate the same as per ICH guidelines. The chromatogram separation was achieved on Qualisil-5 BDS C8 column (250 mm × 4.6 mm, 5µm) column with mobile phase acetonitrile: water (pH 2.8 with TFA) in the composition of 84:16 v/v at a flow rate of 1 mL/min using PDA detector at 254 nm with ambient column temperature, keeping the injection volume 20 µL. The retention time of OFL, MNK, and EBA was observed to be 2.107 min, 2.517 min, and 3.819 min, respectively. All the criteria for the validation (linearity, accuracy, precision, and robustness) were observed to be within the acceptance range. The calibration plots were obtained between 5-60 µg/mL for MNK and 5-60 µg/mL for EBA with r² values of 0.999 in each case. The recovery of MNK and EBA was found to be 98.99% and 99.40%, respectively with a % RSD of <2. This RP-HPLC method was found to be rapid, specific, precise, and accurate and can be used for the routine analysis of MNK and EBA in bulk as well as in tablet dosage form. The separation was complete with a shorter analysis time along with well good resolved peak.

**Keywords:** RP-HPLC, Montelukast, Ebastine, Simultaneous, Estimation, Validation.

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**INTRODUCTION**
Antihistamines are a family of medicines that are frequently used to treat allergy symptoms. These medicines are used to treat diseases caused by an excess of histamine; a substance produced by the immune system. People who are allergic to pollen and other allergens are the most frequent users of antihistamines. They’re also used to treat a number of other issues including gastrointestinal issues, colds, anxiety, and so on. Histamine is a vital molecule that plays a part in a variety of physiological functions. It increases stomach acid production, contributes to inflammation, dilates blood vessels, alters intestinal and lung muscle contractions, and influences your heart rate. It also aids in the transmission of information between nerve cells and the movement of fluids through blood vessel walls. When your body is threatened by an allergen, histamine is produced. Allergy symptoms are caused by histamine, which causes vessels to expand and dilate. Broncho constriction as a component of an asthma treatment regimen (Figure 1A). The US FDA initially authorized MNK for clinical usage in 1998 under the trade name Singulair from Merck. The medicine belongs to the leukotriene receptor antagonist (LTRA) class of medications. Whilst LTRAs like MNK has been shown to be beneficial, they are usually used in conjunction with or in addition to inhaled corticosteroids or other asthma step treatment drugs. Nevertheless, there were FDA-led studies in 2008-2009 into the potential of MNK causing neuropsychiatric side effects such as agitation, hallucinations, suicidal conduct, and many others in people who used it. Despite the fact that these side effects are now listed in the authorized prescription instructions for MNK, the medication is still widely used globally, with millions of prescriptions written each year, and it is now accessible as both a generic and a brand name pharmaceutical.

Ebastine is a non-sedating H1-receptor antagonist from the second generation that is used to cure allergic rhinitis, hay fever, and...
urticaria (Figure 1B). It works by inhibiting the impacts of a signalling molecule in the body called histamine, which causes itching, swelling, and rashes in allergy sufferers. Sneezing, runny nose, itchy eyes, skin rashes, redness of the eyes, and other ailments are treated with it. It is under study for the management of Irritable Bowel Syndrome because it does not cross the blood-brain barrier and may simply prevent the H1 receptor in target tissues while generating no core adverse effects (IBS) [3].

Literature survey reveals that few instrumentation-based methods such as UV-spectroscopy [4], RP-HPLC [5], etc. are available for the determination of MNK and EBA in combination in tablet pharmaceutical formulation. Also, methods have been reported for the individual determination of MNK [6] and EBA [7] in pharmaceutical formulations.

Figure 1. Structure of drugs: (A) Montelukast and (B) Ebastine.

No one article employing Ofloxacin (OFL) as the internal standard (IS) was found when searching the major global databases for literature on any analytical RP-HPLC method for the routine simultaneous determination of MNK and EBA drug combinations in a pharmaceutical tablet formulation. To solve the issue, a simple, long-lasting, precise, low-cost, and accurate solution was developed. The purpose of this research is to develop a new validated RP-HPLC method for calculating MNK and EBA in tablet formulations utilizing OFL IS.

EXPERIMENTAL

Chemical

MNK and EBA were obtained as a generous gift sample by Micro Labs Ltd., Sikkim, India. Ebast-M® tablet (containing 10 mg of MNK and 10 mg of EBA) manufactured by Micro Labs Ltd., Sikkim, India was procured from the local Pharmacy shop. HPLC grade acetonitrile (ACN) and analytical grade trifluoroacetic acid (TFA) were obtained from Merck Life Science Ltd., Bengaluru, India. Ofloxacin (OFL) was obtained as a generous gift sample by Ebastine.

Instrumentation

The HPLC system consisted of solvent delivery module LC-20AD Shimadzu liquid chromatography pump with 10 μL loop having PDA detector model SPD-M20A, controlled by LCMSolution software. Qualisil-5 BDS C5 column (250 mm × 4.6 mm; 5μm) was used for separation. The weighing was carried out using a Shimadzu® (Kyoto, Japan) AUW220D balance. A VSI® VSI-1B (Mohali, India) digital pH meter was used to determine the pH. Transonic Digital S (Mumbai, India) sonicator was used for sonication.

Mobile Phase

Selection of the mobile phase

The mobile phase must be carefully selected for the elution of the solutes. The mobile phase was selected based on theoretical plates, peak purity index, and peak symmetry. The study began with buffer systems and an eluant like methanol, acetonitrile, or other solvents. Low-intensity peaks with a lot of tailing were produced by elution with an identical combination of buffer KH2PO4 and methanol. Although this was an improvement over the previous experiment, the combination of KH2PO4 buffer (pH 4.8) with acetonitrile resulted in the formation of a broad peak with tailing. The peak symmetry improved considerably and tailing was reduced when the buffer was replaced with water, but it was still inadequate to elute the solutes. To obtain a sharp peak with a good Gaussian peak, ACN was combined with water (adjusted to pH 2.8 with 0.1 percent TFA). The 84:16 v/v ratio generated the most theoretical plates as well as the greatest peak purity index. The mobile phase was degassed under vacuum before being filtered using a 0.45 μm membrane filter. Allowing the mobile phase to equilibrate until it achieved a steady baseline was permitted.

Preparation trifluoroacetic acid (0.1%) buffer

1 mL TFA was measured out and diluted with 1000 mL HPLC grade water before being sonicated to eliminate any gas.

Preparation of Mobile Phase

ACN was completely mixed with water (adjusted to pH 2.8 with 0.1 percent TFA) at a ratio of 84:16 v/v. The aforementioned solution was degassed for 5 minutes with sonication before being filtered under vacuum through a 0.45 μm membrane filter.

Preparation of Diluent

Throughout the research, HPLC grade ACN: methanol (50: 50 v/v) was produced and utilized as a diluent.

Chromatographic Condition

Qualisil-5 BDS C5 column (250 mm × 4 mm, 5 μm) was used to develop the technique. The mobile phase, which included ACN and water in a ratio of 84:16 v/v (adjusted to pH 2.8 with 0.1% TFA), was supplied at a flow rate of 1.0 mL/min, with detection at wavelength 254 nm. The injection volume was 10 μL, and the assay was carried out at room temperature.
Standard stock solution
MNK and EBA were accurately weighed (100 mg) and put into two separate 100 mL volumetric flasks containing 50 mL of diluent. Sonication for 5 mins was used to dissolve the contents. The volume was diluted to the required concentrations after being brought up to the mark using diluent.

Preparation of Internal standard
OFL was accurately weighed (100 mg) and transferred to a 100 mL volumetric flask with 50 mL of diluent. The content was dissolved for 10 minutes using sonication. With diluent, the volume was increased to 100 mL, and the aliquot was further diluted to a concentration of 10 μg/mL.

Analysis of drugs in marketed formulation
A total of twenty tablets were weighed and powdered suitably. 0.1649 mg of tablet powder (equal to 10 mg of MNK and 10 mg of EBA) was accurately weighed and transferred to a 100 mL volumetric flask with 50 mL of diluent. The sample was dissolved for 15 mins with sonication, and the volume was brought up to the required level using diluent. The resultant solution was filtered through a membrane filter with a 0.22 μm pore size. The filtrate was then diluted to provide MNK and EBA concentrations of 20 μg/mL and 20 μg/mL, respectively. HPLC was used to examine the diluted sample.

Method validation
The technique was verified using the Q2A and Q2B guidelines from the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH), as well as guidance from the USFDA.

Linearity and Range
For the MNK and EBA assays, the method's linearity was tested using seven different concentrations of the solutes, ranging from 5 to 60 g/mL. The solutions were prepared using the diluent and an equal quantity was injected into the HPLC instrument to determine the peak area. On a linearity graph, the concentration and average area of each solute were plotted. The $r^2$ value of the regression coefficient was computed as well.[9]

Accuracy
The accuracy of the proposed method was tested by spiking the reference drug solutions at concentrations of 50 percent, 100 percent, and 150 percent (recovery). The experiment was repeated three times, with the results given as percent recovery. The percent relative error based on the concentrations was determined and suitably concluded.[9]

Precision
The accuracy of the suggested method was tested in terms of inter-day and intra-day variability by spiking concentrations of 40%, 60%, and 80% six times in a single day (intra-day) and on three different days (inter-day). In addition, two analysts worked on the ruggedness research. Percentage relative error precision was used to describe the data.[10]

Robustness
The robustness of the method was tested by altering the flow rate by 0.2 mL/min (from 0.8 mL/min to 1.2 mL/min) while retaining all other chromatographic parameters unchanged.[11]

Systems suitability parameters
The analytical method's repeatability profile was determined by injecting five times the standard solution and monitoring data such as retention length, peak area, theoretical plates, and tailing factor.[12]

Limit of detection and quantification
Although it is not necessary to define the exact amount, the limit of detection (LOD) is the lowest concentration that any analytical method can detect.[13]

The limit of detection (LOD) was determined by the formula:

$$\text{LOD} = 3.3 \left( \frac{\sigma}{S} \right)$$

Where, $\sigma =$ standard deviation of response; $S =$ slope of the calibration curve. The slope $S$ may be estimated from the calibration curve of the analyte.

The limit of quantification is the smallest amount that can be measured with a given degree of accuracy and precision using any analytical method (LOQ).[14]

The limit of quantification (LOQ) is determined by the formula:

$$\text{LOQ} = 10 \left( \frac{\sigma}{S} \right)$$

Where, $\sigma =$ standard deviation of response; $S =$ slope of the calibration curve. The slope $S$ may be estimated from the calibration curve of the analyte.

RESULTS AND DISCUSSION
Method development and optimization of chromatographic conditions
The new technique was completely based on trial since there had been no prior comparable procedures. Nonetheless, previous studies had a significant impact on the choice of the stationary phase. By maintaining the mobile phase at a low pH, peak tailing was reduced and the analytical method's robustness was much improved. High basic pH-induced dissolution in silica-based reversed-phase columns, the use of acidic pH was justified to a larger degree. The pH of the mobile phase and the pKa of the solute were also discovered to be quite similar, allowing them to stay in the unionized state. As a result, the pH value was determined using two units.

The elution was carried out on the HPLC method that was developed using Qualisil-5 BDS C8 column (250 mm × 4 mm, 5 μm). ACN and water in a ratio of 84:16 v/v (adjusted to pH 2.8 with 0.1% TFA), was supplied at a flow rate of 1.0 mL/min, with detection at wavelength 254 nm at ambient column temperature, keeping the injection volume 10 μL. The blank solution comprising of the mobile phase showed no such peak with a prominent baseline which shows a stable system for elution (Figure 2A). The retention time(s) of sample
solutions OFL, MNK, and EBA were observed to be 2.107 min, 2.517 min, and 3.819 min, respectively (Figure 2C) which matched closely with the retention time(s) of the standard solution (Figure 2B). The % label claim for the marketed product Ebast-M® was found to be 98.99% for MKN and 99.40% for EBA, respectively (Table 1). This clearly showed that the suggested analytical method for routine medication combination analysis in tablets was exact, accurate, and robust.

Figure 2: Chromatograms: (A) Blank solution, (B) Standard solution, (C) Sample solution, and (D) Systems suitability parameter

![Chromatograms](image)

Table 1: Assay of the marketed formulation.

<table>
<thead>
<tr>
<th>Marketed formulation</th>
<th>Drug</th>
<th>Label claim (mg/tablet)</th>
<th>Estimated amount (mg/tablet)</th>
<th>% label claim</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBAST-M</td>
<td>Montelukast</td>
<td>10</td>
<td>9.89</td>
<td>98.99% ± 0.0132</td>
</tr>
<tr>
<td></td>
<td>Ebastine</td>
<td>10</td>
<td>9.94</td>
<td>99.40% ± 0.0147</td>
</tr>
</tbody>
</table>

Method validation

Linearity and range

Both MKN and EBA had very high linearity between concentration and peak area in the range of 5-60 μg/mL, with linear regression equations of $y = 11.899x - 0.3196$ and $y = 0.156x - 0.043$, respectively. The regression coefficient values were all 0.999, suggesting that there was a high degree of linearity in all cases (Figure 3).

![Linearity plots](image)

Accuracy

The percent recovery characteristic of the proposed method for simultaneous analysis by utilizing the calibration curve
was determined in part by the Y-intercept and slope of the graph. In all three concentrations, the observed percent RSD values for MNK were 0.2234, 0.0761, and 0.1948. In the instance of EBA, the percent RSD values in all three concentrations were 0.2772, 0.0728, and 0.1667. All of them met the US Pharmacopeia’s acceptance criterion of less than 2% (Table 2). Ultimately, the method revealed that the data retrieved was correct.

Table 2: % Recovery for Montelukast and Ebastine.

<table>
<thead>
<tr>
<th>% Recovery</th>
<th>50%</th>
<th>100%</th>
<th>150%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amount present (mg)</td>
<td>MNK</td>
<td>EBA</td>
<td>MNK</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Amount of Std. added (mg)</td>
<td>4.9</td>
<td>4.7</td>
<td>9.7</td>
</tr>
<tr>
<td>4.6</td>
<td>4.4</td>
<td>9.7</td>
<td>9.3</td>
</tr>
<tr>
<td>Amount of Recovered (mg)</td>
<td>14.70</td>
<td>14.55</td>
<td>19.60</td>
</tr>
<tr>
<td>% Recovery</td>
<td>99.08</td>
<td>99.35</td>
<td>99.80</td>
</tr>
<tr>
<td>99.52</td>
<td>99.05</td>
<td>99.26</td>
<td>99.16</td>
</tr>
<tr>
<td>Mean</td>
<td>99.31</td>
<td>99.33</td>
<td>99.65</td>
</tr>
<tr>
<td>SD</td>
<td>0.2218</td>
<td>0.2753</td>
<td>0.0786</td>
</tr>
<tr>
<td>% RSD</td>
<td>0.2234</td>
<td>0.2772</td>
<td>0.0761</td>
</tr>
</tbody>
</table>

Precision

The technique was shown to be very accurate throughout the tested ranges for MNK and EBA in both intra-day and inter-day variability tests for precision data. In all instances, the peak area of the sample solution matched that of the standard solution, with a % RSD of less than 2%. The % RSDs in terms of intraday precision for MNK in the concentration range of 10-30 μg/mL lies in the range of 0.83% to 0.91% whereas the % RSDs in terms of intraday precision for EBA in the concentration range of 10-30 μg/mL falls in the range of 0.83% to 0.92% (Table 3). In the case of inter day precision for both the drugs MNK and EBA in the concentration range of 10-30 μg/mL, the % RSDs were observed to be in the range 0.7668-0.8494% and 0.4130-0.8303%, respectively (Table 4). When the ruggedness test was conducted between two analysts for both the

Robustness

The intentional change of one of the most critical chromatographic factors, flow rate, resulted in a substantial shift in the chromatogram for both medicines. A minor change (5%) in peak area, theoretical plates, retention duration, and tailing factor was seen when the flow rate was raised or reduced by 0.2 mL/min for both MNK and EBA (Table 6). The ability of the suggested method to withstand deliberate changes in the flow rate for all of the medicines was shown by the intentional adjustment of the flow rate for all of the pharmaceuticals.

Table 6. Robustness study.

<table>
<thead>
<tr>
<th>Robustness parameter</th>
<th>MNK</th>
<th>EBA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retention Time (Rt)</td>
<td>0.8</td>
<td>3.79</td>
</tr>
<tr>
<td>1</td>
<td>3.8</td>
<td>0.895</td>
</tr>
<tr>
<td>1.2</td>
<td>3.86</td>
<td>0.896</td>
</tr>
<tr>
<td>Avg.</td>
<td>3.8166</td>
<td>0.8943</td>
</tr>
<tr>
<td>SD</td>
<td>0.0378</td>
<td>0.0200</td>
</tr>
<tr>
<td>% RSD</td>
<td>0.9919</td>
<td>0.2327</td>
</tr>
</tbody>
</table>

Systems suitability parameters

The proposed method’s system suitability characteristics showed a high degree of repeatability and may be used for regular medication combination analysis. The proposed technique for MKN resulted in an average retention time (Rt) of 3.826 mins and a mean theoretical plate (TP) of 7532 (Figure 2D). In the case of EBA, the Rt and TP were 2.511 mins and 6981, respectively (Table 7). In all instances, a tailing value of less than 2% revealed no particular tailing. In an ideal Gaussian peak with perfect peak symmetry (asymmetric factor = 1), both symmetric and asymmetric components are of equal size. The idea that the proposed technique fulfilled the
US Pharmacopoeia monograph’s minimal criteria (minimum theoretical plates of 2000 and tailing factor of less than 2%), indicating that it has excellent resolution, isolation, column effectiveness, and repeatability. The separation factor (α) and resolution factor (Rs) were considerably higher than the ICH limits and requisite guidelines of 1 and 1.5, indicating that the suggested analytical method produces greater isolation of both peaks with much less tailing and greater resolution than the ICH limits and necessary guidelines of 1 and 1.5, respectively. Because of its great precision, repeatability, and accuracy, the technique may be used for routine analysis.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Montelukast</th>
<th>Ebastine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Theoretical plates</td>
<td>7532</td>
<td>6981</td>
</tr>
<tr>
<td>Resolution</td>
<td>9.8</td>
<td>2.8</td>
</tr>
<tr>
<td>Asymmetric factor</td>
<td>0.947</td>
<td>1.347</td>
</tr>
<tr>
<td>Retention time (min)</td>
<td>3.826</td>
<td>2.511</td>
</tr>
<tr>
<td>Linearity range (µg/mL)</td>
<td>5-60</td>
<td>5-60</td>
</tr>
<tr>
<td>Regression equation</td>
<td>y = 11.899x - 0.3196</td>
<td>y = 15.677x - 0.043</td>
</tr>
<tr>
<td>Slope (m)</td>
<td>11.899</td>
<td>15.677</td>
</tr>
<tr>
<td>Intercept (c)</td>
<td>0.3196</td>
<td>0.043</td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>0.999</td>
<td>0.999</td>
</tr>
<tr>
<td>LOD (µg/mL)</td>
<td>0.041</td>
<td>0.0211</td>
</tr>
<tr>
<td>LOQ (µg/mL)</td>
<td>0.1271</td>
<td>0.0641</td>
</tr>
</tbody>
</table>

Limit of detection and quantification

The LOD and LOQ for MNK were 0.041 µg/mL and 0.1271 µg/mL, respectively, while the LOD and LOQ for EBA were 0.0211 µg/mL and 0.0641 µg/mL, demonstrating the method’s great capacity to detect the lowest possible concentration of the solute concurrently from the combination or formulation.

CONCLUSIONS

Using a C8 column with acetonitrile and water pH adjusted to 2.8 as mobile phase and 0.1% trifluoroacetic acid as internal standard, the new RP-HPLC technique for simultaneous quantification of Montelukast and Ebastine from the combination dosage form at 254 nm was created. The medication components’ run duration was slightly under 5 mins, enabling quick daily quality assurance use in industries. In terms of linearity, accuracy, specificity, intraday and interday, precision, and robustness, the technique was verified according to ICH guidelines. The percent RSD, theoretical plates, and tailing values all met the minimum requirements of the US Pharmacopoeia. Chemists will find this very helpful for quality control and assurance. The method may be utilized for routine analysis because of its high precision, reproducibility, and accuracy.

REFERENCES


How to cite this article