REVERSE PHASE-HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR QUANTITATION OF EPINASTINE HYDROCHLORIDE IN EYE DROPS

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ABSTRACT
A specific RP-HPLC method has been proposed for the estimation of Epinastine Hydrochloride in marketed formulation (Eye Drops) in presence of Benzalkonium chloride (preservative). The method utilizes a Grace Smart RP 18 column  (250 × 4.6 mm, 5 µ) and a mixture of Acetonitrile: 1% Acetic acid buffer (pH 3 adjusted with 2% Triethanolamine) in ratio of 30:70 v/v as mobile phase maintained at a flow rate of 2.0 mL/min. Amlodipine was used as an internal standard to improve accuracy and precision of the method. The UV detection of analyte and internal standard was carried out at 240 nm. The retention time was found to be 4.17, 4.45 and 10.74 min for Epinastine hydrochloride, Benzalkonium chloride and Amlodipine respectively. The linearity was found in the concentration range of 2-100 µg/mL (r=0.999). The per cent mean recovery was found to be in range of 99.57-109.50. The LOD and LOQ were found to be 0.43326 µg/mL and 1.39291 µg/mL respectively. The proposed method was found to be specific, linear, accurate, precise, rugged and robust and found to be suitable to resolve Benzalkonium chloride commonly used preservative in eye drops.

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INTRODUCTION
Epinastine Hydrochloride (EPS) is a selective H₁ receptor antagonist and also has anti allergic and anti-histaminic effect by inhibiting the release of allergy inducing substance like histamine from mast cells and usually used in treatment of allergic conjunctivitis, bronchial asthma, urticaria and eczema [1]. The ophthalmic solution of EPS is widely prescribed for topical administration to eyes for allergic conjunctivitis. Chemically, it is 3-amino-9, 13 b-dihydro-1h-dibenz[c,f] imidazo[1,5-a] azepine hydrochloride (Figure 1). It is white, hygroscopic, crystalline powder, freely soluble in water and in methanol. The reported pKa value is about 11.2[2].

![Figure 1: Chemical structure of Epinastine Hydrochloride.](image)

Different analytical methods like RP-HPLC and stability indicating HPLC methods [3-5] have been reported for estimation of Epinastine Hydrochloride in bulk with enantiomers [6], in ophthalmic [7] and tablet formulation [8] and plasma [9-11]. An analytical report has been also found for determination of residual solvent in EPS by Headspace Gas Chromatography [12]. The difference spectroscopy, colorimetry [7] and derivative spectroscopy [13] have also been reported for quantification EPS.

There is no chromatographic method reported so far for the quantitation of Epinastine hydrochloride in eye drops which can separate Benzalkonium chloride, a commonly used preservative. Hence objective of present work is to develop and validate specific RP-HPLC method for quantitation of EPS with internal standard and which is also capable to separate Benzalkonium chloride.

MATERIALS AND METHODS
Instrument
A High Performance Liquid Chromatography Shimadzu LC 10AT VP having SPD 10A VP Detector model with Rheodyne injector of 100µL was used along with Grace Smart RP 18 column (250 x 4.6 mm, 5 µ) and connected to a PC computer running LC Solutions software.

Chemicals and Reagents
Acetonitrile (HPLC grade), Acetic acid (HPLC Grade), Triethanolamione (TEA), Millipore water were used as solvents. Epinastine hydrochloride and Benzalkonium chloride (50% solution) were obtained as gift samples from Bal Pharma Limited and Micro Labs Ltd., Bangalore respectively. EPINA Eye drops (Cipla Ltd.) and Amlodipine were procured.

Chromatographic Conditions
The chromatographic separation was carried on a Grace Smart RP 18 column (250 x 4.6 mm, 5 µ) using mixture of Acetonitrile and 1% Acetic Acid buffer, pH 3 (adjusted with 2% TEA) in the ratio of 30:70 as mobile phase. The standard and sample solutions were injected through Rheodyne injector of 100 µL loop. The flow rate was maintained at 2mL/min and detection was carried at 240 nm.

Preparation of Mobile Phase for RP-HPLC Method
Acetic acid buffer (1%) was prepared by adding 10 mL of acetic acid and making up the volume to 1000 mL with Millipore water, and pH was adjusted to 3 with 2% Triethanolamine (TEA). The acetonitrile and buffer were vacuum filtered through membrane filter (0.2 µ), sonicated for 15 min and stored in mobile phase reservoir bottles separately. The acetonitrile and buffer were mixed in 30:70 proportions by the system and pumped at flow rate of 2 mL/min. Analytes were detected by measuring the absorbance at 240 nm.

Preparation of Standard Stock Solution of Epinastine Hydrochloride (EPS)
Accurately weighed 100 mg of EPS was transferred into a clean dry 100 mL volumetric flask and dissolved in Millipore water and made up the volume to get a concentration of 1000 µg/mL. This solution was further diluted to get a concentration of 100 µg/mL.

Preparation of Standard Stock Solution of Amlodipine (AML)
Accurately weighed 100 mg of AML was transferred to a clean dry 100 mL volumetric flask and dissolved and made up volume with Millipore water to get a concentration of 1000 µg/mL. Further dilution was made to get a concentration of 10 µg/mL.
Working Standard Solution
The working standard solution was prepared by transferring 0.5 mL of standard stock solution of EPS (100 µg/mL) and 1mL of standard stock solution of AML (10 µg/mL) to a clean dry 10 mL volumetric flask and diluted with mobile phase to 10 mL, to get concentration of 5:1 µg/mL of EPS:AML. This working standard solution was injected (100 µL) to the chromatograph.

Analysis of eye drops
Accurately 2 mL of Eye Drops (EPINA containing 0.05% Epinastine Hydrochloride and 0.01% Benzalkonium chloride) was transferred to a clean and dry 10 mL volumetric flask dissolved in millipore water and volume is made to mark with Millipore water, to get a concentration of 100 µg/ml. This resultant mixture was sonicated and filtered through Whatman filter. Accurately 0.5 mL of this solution was transferred into 10 mL volumetric flask along with 1mL of standard stock solution of AML and volume was made with mobile phase to get concentration of 5:1 µg/ml of EPS: AML. This solution was filtered through Millipore filter (0.2µ).

The sample solution and working standard solution were analysed by injecting 100 µL into chromatograph .The concentration of EPS in eye drops was determined by comparing the ratio of peak area under curve (AUC) of EPS to AML of sample with that of standard using single point analysis. The chromatogram of sample was as shown in figure 2.

![Chromatogram showing Epinastine Hydrochloride (4.171 min) and Amlodipine (10.74 min) peak resolution with Acetonitrile: 1%Acetic acid (adjusted to pH-3 with 2% TEA) in 30:70 ratio mobile phase](image)

**Figure 2:** Chromatogram showing Epinastine Hydrochloride (4.171 min) and Amlodipine (10.74 min) peak resolution with Acetonitrile: 1%Acetic acid (adjusted to pH-3 with 2% TEA) in 30:70 ratio mobile phase

**VALIDATION OF HPLC METHOD**
The proposed RP-HPLC method for the quantitative analysis of EPS was validated as per the recommended method of ICH Guidelines Q2(R1) for parameters like linearity, range, accuracy, precision, LOD, LOQ, and robustness.

**Linearity**
Linearity of the method was determined by analyzing six sets of the solutions in the concentration range of 2-100 µg/ml and by plotting the graph of ratio of AUC of EPS to AML versus concentration of EPS while the range for linearity was determined by plotting ratio of response (ratio of drug to internal standard) to concentration versus log concentration.

**LOD and LOQ**
The LOD (Limit of Detection) and LOQ (Limit of Quantification) were determined by formulae method after taking mean of slopes and standard deviation of intercepts from the calibration curves.
Accuracy
The accuracy of the proposed method was performed by standard addition method at three different levels (50%, 100% and 150%). A known amount of standard EPS is added to the sample solution and percent recoveries were determined. The results are as shown in table 3.

Precision
The precision studies of the method was determined by measuring % RSD of sample solutions (n=3) at different time intervals on the same day (intraday) and three consecutive days (interday) and by two different analysts for intermediate reproducibility.

Specificity
The placebo was prepared by diluting 50% benzalkonium chloride solution to 1 and 100 μg/ml. The chromatograms were analysed for retention time.

Robustness
The robustness of the developed system was determined by % RSD of retention time, AUC, Ratio of AUC of drug to internal standard on increasing or decreasing flow rate and pH by 3%.

System Suitability
The working standard solution was injected six times successively and parameters like retention time, tailing factor, theoretical plates and resolution were studied.

RESULTS AND DISCUSSION
Literature survey reveals that many HPLC methods and few UV –Visible spectroscopic methods have been reported for quantitation of EPS in bulk, formulation and Plasma. But no specific chromatographic method has been reported so far for the estimation of EPS that separates commonly used preservative, BZL. Hence attempts were made to develop and validate specific RPHPLC method which resolves BZL from EPS.

Various mobile phase compositions like Acetonitrile-orthophosphoric acid (OPA) buffer, Methanol-OPA buffer, Sodium and Potassium phosphate buffer-Acetonitrile were used in different proportions between pH 3 to 5 to resolve EPS and BZL effectively. EPS was found to retain optimum in mobile phase composition of Acetonitrile:1% Acetic acid buffer (30:70 v/v, pH 3) on Grace Smart RP 18 column (250 x 4.6 mm, 5 μ) at flow rate of 2 mL/min.

The same chromatographic system was found to be capable to detect BZL and AML with optimum resolution where amlodipine was used as internal standard to improve precision and accuracy. The retention time was found 4.17 and 10.74 min for EPS and AML in the finalized chromatographic system with resolution 13.63 min with theoretical plates of 2897 (EPS) and 4119 (AML) respectively and passes all the system suitability parameters.

The relative standard deviation was found to be decreased for the results of assay and all validation parameters when ratio of drug to IS was used for analysis. Hence it was decided to use internal standard and AML was found to be suitable as internal standard with good resolution and theoretical plates compare to Paracetamol, Ticlopidine HCl and Tolperison HCl.

The standard solution of EPS, BZL and AML were prepared in mobile phase and scanned in the region of 190-350 nm. EPS and AML were found to be absorbing at 240 nm whereas optimum absorption of BZL was observed at 260 nm. Hence the analysis was performed at 240 nm while specific study was performed on both 240 and 260 nm.

The method was applied for EPINA eye drops containing 0.05% Epinastine hydrochloride and 0.01% Benzalkonium chloride and the results of assay were found to be in the range of 96.541 – 99.957% as given in table 1.
Table 1: Assay of Epinastine Hydrochloride Marketed Formulation

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>EPS Peak area *</th>
<th>AML Peak area*</th>
<th>RATIO</th>
<th>STD RATIO</th>
<th>% Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>162253</td>
<td>89866</td>
<td>1.805499</td>
<td>1.80626</td>
<td>99.95787</td>
</tr>
<tr>
<td>2</td>
<td>169394</td>
<td>88520</td>
<td>1.913624</td>
<td>1.952466</td>
<td>98.01062</td>
</tr>
<tr>
<td>3</td>
<td>174439</td>
<td>93523</td>
<td>1.865199</td>
<td>1.932024</td>
<td>96.54119</td>
</tr>
</tbody>
</table>

*Average of three readings.

The validation was performed as per ICH Guidelines Q2 R1 for various parameters. Linearity was found to be 0.9986 ($r^2$) for the concentration range of 2-100 µg/ml as shown in Fig.3. The LOD and LOQ were found to be 0.43326 µg/mL and 1.39291 µg/mL respectively (table2).

![Calibration Linearity curve for Epinastine Hydrochloride.](image)

Figure 3: Calibration curve of EPS

Table 2: Linearity report of Epinastine hydrochloride

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Epinastine hydrochloride</th>
<th>Acceptance Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity Range</td>
<td>2-100 µg/ ml</td>
<td></td>
</tr>
<tr>
<td>Regression Equation</td>
<td>$Y = 0.3937x - 0.2959$</td>
<td></td>
</tr>
<tr>
<td>Correlation Co-efficient</td>
<td>0.9986</td>
<td>NLT 0.997</td>
</tr>
<tr>
<td>Percentage Curve Fitting</td>
<td>99.86%</td>
<td>NLT 99.7%</td>
</tr>
<tr>
<td>LOD (µg/mL)</td>
<td>0.43326</td>
<td></td>
</tr>
<tr>
<td>LOQ (µg/mL)</td>
<td>1.39291</td>
<td></td>
</tr>
</tbody>
</table>

Accuracy was determined by standard addition method and percentage recoveries were found to be 99.569 -109.495% as given in table 3. Relative standard deviation was used to analyse precision of developed method for inter and intraday studies and intermediate reproducibility and found to be less than 2% (table 4).

Table 3: Per cent Recovery of Epinastine Hydrochloride in Ophthalmic Formulation (EPINA)

<table>
<thead>
<tr>
<th>ACCURACY</th>
<th>ASSAY</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>50% spiking</td>
</tr>
<tr>
<td>1</td>
<td>99.95</td>
<td>109.495</td>
</tr>
<tr>
<td>2</td>
<td>98.0106</td>
<td>109.453</td>
</tr>
<tr>
<td>3</td>
<td>96.5411</td>
<td>104.777</td>
</tr>
</tbody>
</table>
Table 4: Precision Data for Epinastine Hydrochloride

<table>
<thead>
<tr>
<th>Precision Parameters</th>
<th>% RSD</th>
<th>Acceptance Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method Precision (n=6)</td>
<td>1.581633</td>
<td></td>
</tr>
<tr>
<td>Intra-Day Precision (n=3)</td>
<td>1.006308</td>
<td></td>
</tr>
<tr>
<td>Inter-Day Precision (n=3)</td>
<td>0.477983</td>
<td>NMT 2%</td>
</tr>
<tr>
<td>Reproducibility (n=9)</td>
<td>1.482051</td>
<td></td>
</tr>
</tbody>
</table>

Intermediate reproducibility was performed by analysing three sets of sample by two different analysts and compared by T-test and F-test. The F-test value was found to be 0.9033 and is less than the standard value at degree of freedom (υ) 5, 2 at 10% probability. Similarly t-test value was found to be 2.958 and is less than standard at degree of freedom (υ) 7. Hence there is no significant difference found among the results produced by two analysts.

Specificity was analysed by injecting 1 µg/mL of BZL solution at 240 nm. Because of low concentration and weak UV detectability of BZL, peak was not detectable. Hence BZL peak was confirmed by injecting 100 µg/mL solution at 240 nm and 263 nm as shown in Fig.4 and 5. The retention time of BZL was found to be about 4.45 min. Hence it can be concluded that the developed method was specific and suitable to resolve EPS and BZL, commonly used preservative in eye drops.

![Chromatogram](image)

**Figure 4:** Chromatogram of Benzalkonium chloride (BZL, 4.49 min) at 240 nm
Figure 5: Chromatogram of Benzalkonium chloride BZL (4.435 min) at 263 nm

The developed method was validated for robustness on varying pH and flow rate by 3%. The Relative Standard Deviation for ratio of AUC for drug to internal standard and retention time were found to be less than 3% (table 5).

Table 5: Report of Epinastine hydrochloride with Change in Flow rate and pH

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Flow rate 2.06 ml/min</th>
<th>Flow rate 1.94 ml/min</th>
<th>pH 2.91</th>
<th>pH 3.09</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Assay*</td>
<td>102.59</td>
<td>101.34</td>
<td>101.72</td>
<td>102.34</td>
</tr>
<tr>
<td>(% RSD)</td>
<td>(1.752)</td>
<td>(1.765)</td>
<td>(1.647)</td>
<td>(1.95)</td>
</tr>
</tbody>
</table>

*n=6

The system suitability of the developed method was established by injecting standard solution six times and evaluated on the basis of theoretical plates, tailing factor and resolution and results were found to be in the limit and satisfactory (table 6).

Table 6: Data for System Suitability parameters of Epinastine Hydrochloride

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Name of the drug/IS</th>
<th>*tR</th>
<th>AUC</th>
<th>N</th>
<th>Tailing Factor</th>
<th>Resolution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>EPINASTINE</td>
<td>4.123</td>
<td>166567.7</td>
<td>2897.608</td>
<td>1.8155</td>
<td>_</td>
</tr>
<tr>
<td>2</td>
<td>AMLODIPINE</td>
<td>10.823</td>
<td>88482.5</td>
<td>4119.333</td>
<td>1.7796</td>
<td>13.638</td>
</tr>
</tbody>
</table>

The developed RPHPLC method was found to be accurate, precise, specific (capable to resolve BZL and EPS) with linearity range 2-100 µg/mL. This method can be further modified to quantify BZL in the eye drops. This was not possible by the present method as concentration of sample solution required was very high (outside the linearity range).

CONCLUSION

The developed and validated RP-HPLC method was found to be specific, accurate and precise to estimate Epinastine Hydrochloride in Eye drops containing Benzalkonium chloride as preservative and can be used for routine estimation Epinstine Hydrochloride in pharmaceutical Industry.
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