



Development and Validation of Ultra Violet Spectrophotometric and Reversed-Phase High Performance Liquid Chromatography Techniques for Simultaneous Estimation of Brinzolamide and Brimonidine Tartrate in Ophthalmic Suspension Formulation

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ABSTRACT

A sensitive and fast UV spectrophotometric technique and feasible reverse phase liquid chromatographic method for the analysis of Brinzolamide and Brimonidine tartrate in ophthalmic formulation has been developed. Linearity ranges for both methods were 5 - 25 µg/ml and 1-5 µg/ml for brinzolamide and brimonidine tartrate respectively. The absorption maxima were observed at 232 and 257 nm for Brinzolamide and Brimonidine. The UV, ophthalmic formulation assay shows percentage purity ranging from 98.90 to 101.01% for Brinzolamide and 98.20 to 100.80% for Brimonidine. The mean percentage purity is 99.82% and 99.80% for Brinzolamide and Brimonidine respectively. The chromatographic retention time for Brinzolamide and Brimonidine was found to be 5.9 and 8.4 minutes respectively. The tailing factor was 0.808 and 0.860 for Brinzolamide and Brimonidine tartrate respectively. The method was validated according to the ICH guidelines and the method was found to be applicable for determination of Brinzolamide and Brimonidine tartrate from combined ophthalmic form.

Keywords: Brinzolamide (BRZ), Brimonidine (BRT), HPLC and UV.

INTRODUCTION

Glaucoma is a disorders characterized by a loss of visual associated with optic nerve damage and associated with intra-ocular pressure rise. Glaucoma is categorized into two forms primary open-angle glaucoma and primary angle closure glaucoma. The drugs which used to reduce intra-ocular pressure is

achieved by different types of mechanism¹⁻². The first choice of drug is topical beta-blocker or prostaglandin analogues. It is necessary to combine these drugs or by adding with other types like sympathomimetics miotics, or carbonic anhydrase inhibitors to maintain ocular pressure³. Brinzolamide chemically called as 4R)-4-(ethylamino)-2-(3-methoxypropyl)-1,1-dioxo-2H,3H,4H-1λ⁶-thieno[3,2-e][1,2]thiazine-6-

sulfonamide (Figure 1a) is a carbonic anhydrase inhibitor used to the patients with open-angle glaucoma to lower hypertension by decreasing the fluid amount produced by eyes. Blindness is prevented by lowering pressure inside the eye¹. Brimonidine chemically called as 5-Bromo-N-(4,5-dihydro-1H-imidazol-2-yl) quinoxalin-6-amine (Figure 1b), which is a α -adrenoceptor agonist, used in the reduction of intra-ocular pressure in open-angle glaucoma or ocular hypertension. Brimonidine is also used as adjunctive therapy when intra-ocular pressure is inadequate^{4,6}.

Literature review reveals several methods developed has been reported for Brinzolamide estimation in biological fluids and there are some methods reported by voltammetry⁷, spectroscopy⁸, HPTLC HPLC, UPLC and capillary electrophoresis⁹⁻¹¹. Two methods were only reported for estimation of this combination first is HPTLC¹² and the other is HPLC method¹³⁻¹⁵. The latter does not fulfil all requirements of validation. Method development of simultaneous UV and HPLC estimation for this combination is our new method will fulfil all requirements of validation according to ICH guidelines. It should be characterized by simplicity, fast analysis, sensitivity and suitability for routine pharmaceutical analysis.

MATERIALS AND METHODS

Chemicals and reagents

The working standard of Brinzolamide and Brimonidine was purchased from Sigma, UK. The Marketed sample of Brimonidine Tartrate 0.2% and Brinzolamide 1% ophthalmic preparation 'Simbrinza' were from Alcon Laboratories (UK) Ltd, purchased from the local Pharmacy, Jazan, KSA. Acetonitrile and Methanol HPLC grade was purchased from

Merck, Darmstadt, Germany, Triethylamine and Orthophosphoric acids purchased from Fisher Scientific (UK).

Pharmaceutical preparation

Simbrinza eye drops; Alcon Laboratories (U.K) contains (Brimonidine Tartrate 0.2% and Brinzolamide 1%) B.NO: AMCM3111

Instrumentation

UV spectrophotometer

A double beam UV-visible spectrophotometer-Shimadzu, UV-1601 (Japan) matched with quartz cells corresponding to 1 cm path length.

HPLC instrumentation and chromatographic condition

HPLC system of Shimadzu LC-20 AT, with an auto sampler (SIL-20AC HT, Shimadzu, Japan) and SPD-10 detector (SPD-M20A, Japan) was used. For data recording the LC-solution software used. A Zorbax Eclipse Plus, Agilent Technology column (150mm x 4.6mm, 5 μ m was used,) Pore size of the column 95Å. For degassing mobile phase, power sonic 505 ultrasonic baths (Hwashin technology, Seoul, Korea) was used. By using oven (CTO-20AC) column was maintained at a temperature of 39°C and 1.0 ml/min was the flow rate. Analysis was carried over with 20 μ l injection volume using SPD-10 detection at 260nm. 10 minutes was set as run time.

Preparation of Standard Stock Solution for UV

The standard stock solution of Brinzolamide and Brimonidine were prepared by dissolving separately 3 mg of Brinzolamide and Brimonidine powder respectively in 100 ml 0.1 M HCl. Both

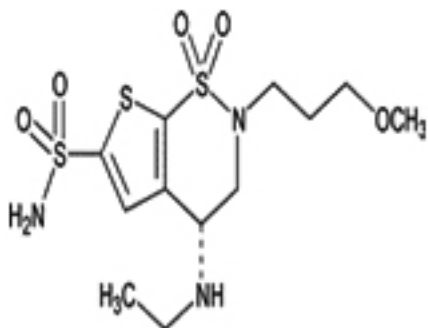


Fig. 1a: Chemical structure of Brinzolamide

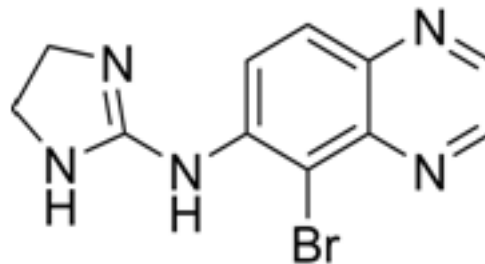


Fig. 1b: Chemical structure of Brimonidine

Brinzolamide and Brimonidine powder freely soluble in 0.1 M HCl and does not have any interference in the absorption peaks. Accurate volumes transferred to two sets of calibrated 10 ml flask.

Development of the method

The stock solution suitably diluted with 0.1M HCL so as to contain 5 - 25 µg/ml and 1-5

µg/ml Brinzolamide and Brimonidine respectively. The prepared solutions were scanned in the UV region between 200nm - 400nm. The Brinzolamide exhibited λ max at 232nm and Brimonidine exhibited λ max at 257nm. The individual drug concentration present in the mixture was determined against the calibration curve in quantization mode.

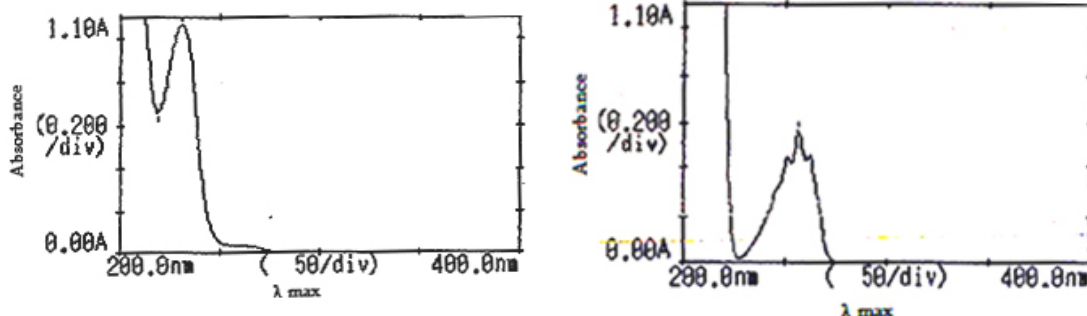


Fig. 2: UV spectrum of raw Brinzolamide and Brimonidine

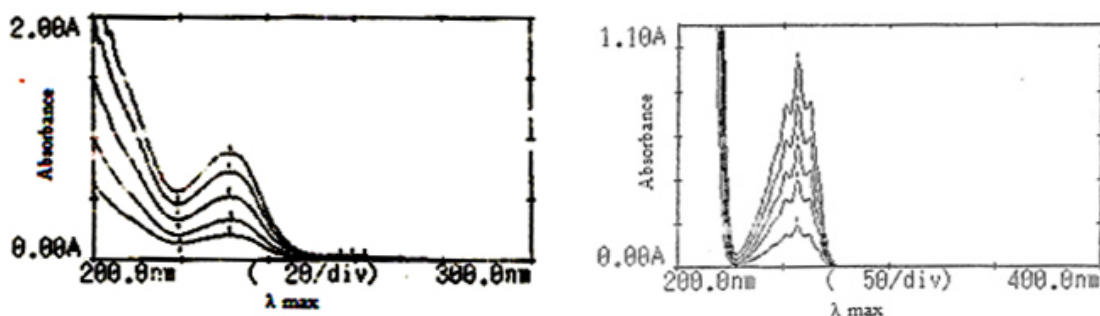


Fig. 3: UV spectrum Linearity of raw Brinzolamide and Brimonidine

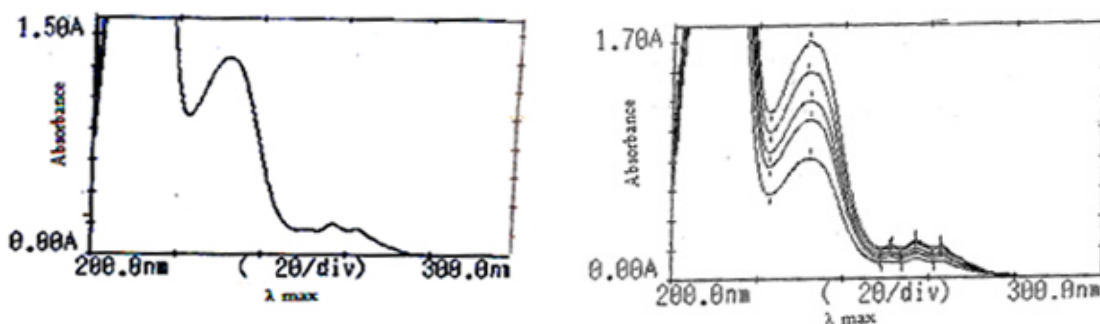


Fig. 4: Quantitative estimation of combined Brinzolamide and Brimonidine in ophthalmic formulation

Analysis of Ophthalmic Suspension

1 ml suspension was taken from formulation and transferred to volumetric flask 100 ml and diluted up to 100 ml with 0.1 M HCl, which is equivalent to 20

$\mu\text{g/ml}$ of Brimonidine and 100 ig/ml of Brinzolamide. From this stock, 1 ml of solution was diluted with 0.1 M HCl to 10 ml, which contains 2 ig/ml of Brimonidine and 10 ig/ml of Brinzolamide. The mixture kept stand

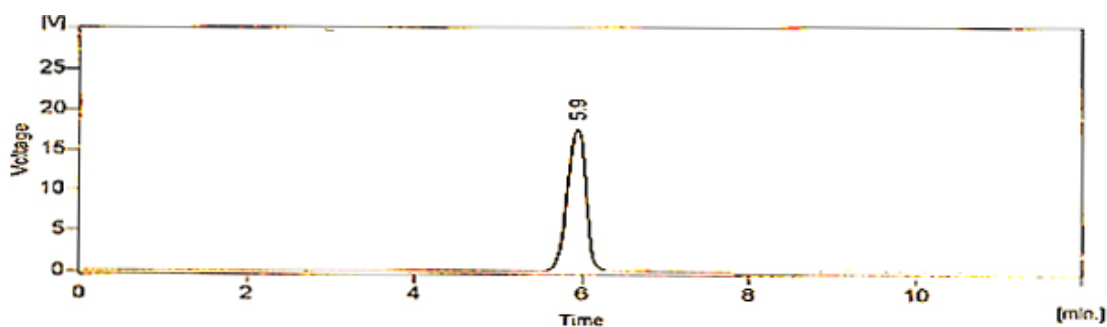


Fig. 5: A Typical Chromatogram of Brinzolamide Standard

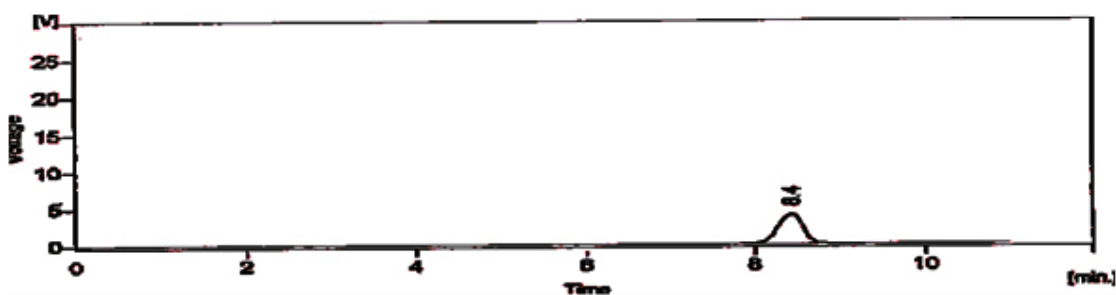


Fig. 6: A Typical Chromatogram of Brimonidine Standard

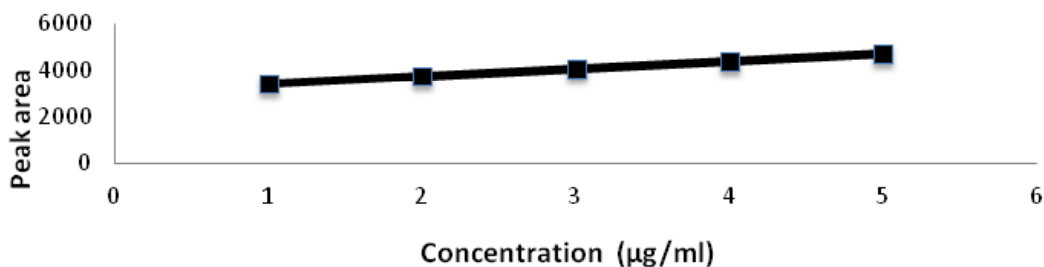


Fig. 7: Calibration graph of Brimonidine 2-5 $\mu\text{g/ml}$ precision

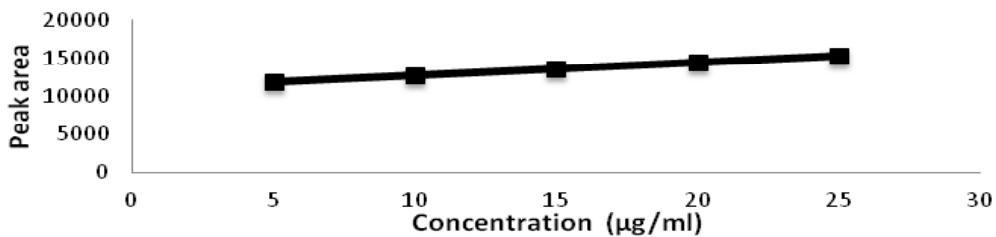


Fig. 8: Calibration graph of Brinzolamide 10 - 25 $\mu\text{g/ml}$ precision

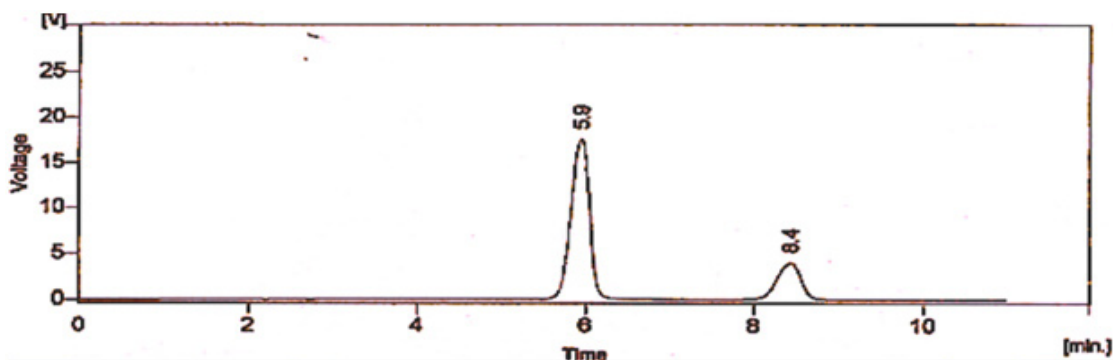


Fig. 9: Chromatogram of Brinzolamide and Brimonidine in ophthalmic formulation

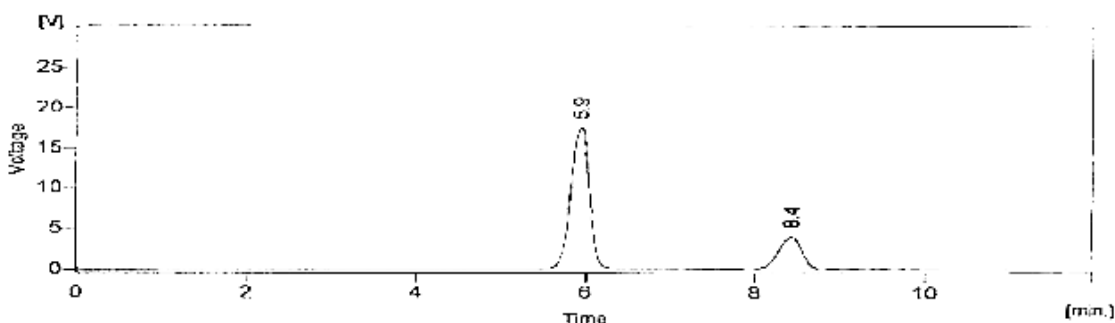


Fig. 10: Quantitative estimation (Assay) of Brinzolamide and Brimonidine in ophthalmic formulation

for 15 min, to ensure complete solubility of the drug by intermittent sonication, and filtered through a 0.45 μ m membrane filter. The concentrations were determined by measuring the absorbance of the sample at Brimonidine 257nm and Brinzolamide 232 nm. The results were calculated against the calibration curve in quantization mode.

Preparation of Standard solution for HPLC

Preparation of Mobile phase

Triethylamine phosphate buffer was prepared using 4.0 ml of triethylamine in 1000 ml of water by using phosphoric acid pH adjusted to a 5.0 (± 0.5). It was filtered with 0.45 μ m membrane filters and degassed in an ultrasonic bath for 10 minutes. The ratio of buffer, acetonitrile and methanol was 75:20:05, v/v.

Preparation of Brinzolamide Stock solution

Accurately 5 mg of Brinzolamide working standard was taken in 100 ml volumetric flask and dissolved by mobile phase and diluted to 25 ml of

mobile phase and sonicated for 10 minutes and 75ml of mobile phase was added to the mark and cooled to room temperature. 1 ml of standard stock solution was diluted to 10ml with mobile phase to obtain a concentration of 5 μ g/ml.

Preparation of Brimonidine Stock solution

Accurately 1 mg of Brimonidine working standard was taken in 100 ml volumetric flask and dissolved by mobile phase and diluted to 25 ml of mobile phase and sonicated for 10 minutes and 75ml of mobile phase was added to the mark and cooled to room temperature. 1 ml of standard stock solution was diluted to 10ml with mobile phase to obtain a concentration of 1 μ g/ml.

Preparation of sample solution

Sample bottles were shaken well before use. 10 ml of ophthalmic suspension were taken into a dried volumetric flask 100 ml. About 50 ml of mobile phase was added to dilute and stand for 15 minutes and diluted to mark with mobile phase. The resulting

solution was filtered through Whatman 1 filter paper. 10 ml of the resulting solution was diluted to 50 ml with mobile phase and filtered by 0.45 μ disc filter.

Method validation

The present method was conducted to obtain a new, sensitive and convenient method for

simultaneous estimation by HPLC from ophthalmic formulation. The experimental method was validated according to the ICH guidelines recommendations and USP-30 for parameters such as, system suitability, accuracy, precision, linearity and specificity.

Table 1: Quantitative Estimation Of Brinzolamide And Brimonidine In Ophthalmic Formulation

| Ophthalmic sample | Label claim (mg/ml) | Amount present | Percentage Label claim | Percentage deviation |
|-------------------|---------------------|----------------|------------------------|----------------------|
| Brinzolamide | 10 | 9.96 | 99.20 | -0.80 |
| Brimonidine | 2 | 2.21 | 100.19 | +0.19 |

*Each value is a mean of six readings

Table 2: UV Statistical Data

| Ophthalmic sample | Standard deviation (S.D) | Relative Standard deviation (RSD) | Relative Standard deviation % RSD | Standard Error of mean (S.E) |
|-------------------|--------------------------|-----------------------------------|-----------------------------------|------------------------------|
| Brinzolamide | 0.006519 | 0.00627 | 0.627 | 0.0797 |
| Brimonidine | 0.0010 | 0.001048 | 0.1048 | 0.1311 |

* Each value is a mean of six readings

Table 3: Results Of UV Limit Of Detection (LOD) & Limit Of Quantification LOQ

| Parameters | Brinzolamide | Brimonidine |
|-------------------|--------------|-------------|
| LOD (μ g/ml) | 0.51 | 0.41 |
| LOQ (μ g/ml) | 1.57 | 1.21 |

System suitability

System suitability parameters like resolution, retention time, tailing factor and column theoretical plates was performed by injecting six replicates of standards and two replicates of sample preparation at a 100% level to cross verify the accuracy and precision of the chromatographic system.

Table 4: HPLC Linearity Data For Brimonidine

| SNo | Concentration (mg/ml) | Peak area |
|-----|-----------------------|-----------|
| 1 | 1 | 3436.94 |
| 2 | 2 | 3760.64 |
| 3 | 3 | 4090.95 |
| 4 | 4 | 4427.75 |
| 5 | 5 | 4759.29 |

Table 5: HPLC Linearity Data For Brinzolamide

| SNo | Concentration (mg/ml) | Peak area |
|-----|-----------------------|-----------|
| 1 | 5 | 11903.78 |
| 2 | 10 | 12763.24 |
| 3 | 15 | 13628.45 |
| 4 | 20 | 14493.95 |
| 5 | 25 | 15358.19 |

Linearity

The linearity of chromatographic method was established by plotting a graph to concentration vs peak area of Brinzolamide and Brimonidine standard and determining the correlation coefficients (R²) of the two compounds. For the linearity studies of Brinzolamide and Brimonidine the specific range was determined at 5 - 25 µg/ml and 1-5 µg/ml for Brinzolamide and Brimonidine tartrate respectively were injected into the HPLC system. For 60 minutes column was equilibrated with the mobile phase before injection of the solutions.

Accuracy

The method accuracy was determined by recovery experiments. The recovery was performed by adding Brinzolamide and Brimonidine working standards to placebo (excipients mixture) in the range of test concentration (60%, 80% and 100 %) and expressed as percent (%) recovered. Three samples were prepared for each recovery level. The recovery statistical results are

Precision

The intraday and interday precision of the analyzed method was determined by analyzing the sample responses 4 repeats on the same day and 4 different days of a week for 4 different concentrations of standard solutions of Brinzolamide and Brimonidine. 10 - 25 µg/ml and 2-5 µg/ml for brinzolamide and brimonidine tartrate respectively, and results are represented in terms of % RSD.

Specificity

The analytical method specificity is to measure the compound accurately in presence of interferences like excipients, degradants and matrix components. The UV spectrum and HPLC of standard mixture and formulation shows specificity of method. The UV spectrophotometric and HPLC method is able to access the analyte in presence of excipients.

Statistical Parameters

The results of assay obtained are subjected to the following statistical analysis, standard

Table 6: Results Of Statistical Parameters

| SNo | Parameters | Brinzolamide | Brimonidine |
|-----|--------------------------------------|----------------------------|----------------------------|
| 1 | Standard deviation (SD) | 4.353 | 13.933 |
| 2 | Relative standard deviation (RSD) | 0.00574 | 0.0101 |
| 3 | % RSD | 0.574 | 1.011 |
| 4 | Standard error (SE) | 0.02347 | 0.0836 |
| 5 | Correlation Coefficient (r) | 0.9983 | 0.9996 |
| 6 | Slope (a) | 33.1181 | 86.395 |
| 7 | Intercept (b) | 1503.26 | 11037.67 |
| 8 | Regression equation Y = (a X + b) | Y = 33.1181 X + 1503.26 | Y = 86.395 X + 11037.67 |

Table 7: Results Of System Suitability Parameters

| SNo | Parameters | Brinzolamide | Brimonidine |
|-----|--|--------------|-------------|
| 1 | Theoretical plates | 8948 | 8303 |
| 2 | Tailing factor | 0.808 | 0.860 |
| 3 | Resolution factor | 8.33 | 8.33 |
| 4 | Retention time | 5.9 | 8.4 |
| 5 | Calibration range or Linear dynamic range | 1-5 | 5-25 |

deviation, relative standard deviation, coefficient of variation and standard error.

RESULT AND DISCUSSION

The UV spectrum of Brinzolamide and Brimonidine are showed in figure 2. The maxima absorption was observed at 232 and 257 nm for Brinzolamide and Brimonidine. Brimonidine attached to chromophoric molecule, increase both its wave length and intensity of absorption maximum. Increase in conjugation, increase absorbance of light to higher I, bathochromic shift with hyperchromic effects of Brimonidine are showed in figure 2.

The linearity of calibration curve of Brinzolamide and Brimonidine shows that obedience of Beer's law was confirmed, which are represented in figure 3. Brinzolamide linearity concentration range of 5-25 µg/ml and Brimonidine linearity range of 1-5 µg/ml. The overlying spectra for the linearity of Brinzolamide and Brimonidine and spectrum of sample formulation are presented in

Table 8: Results Of Limit Of Detection (LOD) & Limit Of Quantification LOQ

| Parameters | Brinzolamide | Brimonidine |
|-------------|--------------|-------------|
| LOD (ng/ml) | 0.430 | 0.530 |
| LOQ (ng/ml) | 1.310 | 1.610 |

Table 9: Quantitative Estimation (Assay) of Data of Brinzolamide and Brimonidine From Ophthalmic Formulation

| S. No | Drug | Label claim (mg/ml) | Amount found | Mean amount | Percentage purity | Mean percentage | % Deviation |
|-------|--------------|---------------------|--------------|-------------|-------------------|-----------------|-------------|
| 1 | Brinzolamide | 10 | 9.97 | 9.95 | 99.40 | 99.80 | -0.6 |
| | | | 9.91 | | 98.22 | | -1.8 |
| | | | 10.02 | | 100.44 | | -0.4 |
| | | | 10.04 | | 100.80 | | +0.8 |
| | | | 10.01 | | 100.20 | | +0.2 |
| 2 | Brimonidine | 2 | 1.94 | 1.95 | 98.90 | 99.82 | -1.1 |
| | | | 1.96 | | 99.20 | | -0.8 |
| | | | 2.05 | | 101.01 | | +1.1 |
| | | | 2.02 | | 100.40 | | +0.4 |
| | | | 1.98 | | 99.62 | | -0.4 |

figure 4. The concentration of Brinzolamide 15µg/ml and Brimonidine 3µg/ml was taken for quantitative estimation.

The ophthalmic formulation shows percentage purity value ranging from 98.60-100.20 % for Brinzolamide and 99.98-100.16 % for Brimonidine. The % deviation was found to lie between -1.4 - +0.2 for Brinzolamide and -0.1 - +0.2 for Brimonidine. The relative standard deviation values are found to be below 2% indicating the methodology precision and low standard error values show the method accuracy. The quantitative estimation of Brinzolamide and Brimonidine in ophthalmic formulation is shown in table 1. The statistical data is given in table 2. The limit of detection and limit of quantification for Brinzolamide and Brimonidine were calculated and values are given in table in table 3.

The chromatogram of Brinzolamide and Brimonidine are presented in figure 5 and 6. Wavelength was selected by scanning all standard drugs over a wide range of wavelength 200-400nm. Both the components show reasonably good response at 260 nm. Linearity was evaluated by plotting peak area as a functional of analyte concentration for both Brinzolamide and Brimonidine. The graphical representation was given in figure 7 and 8; data is presented in table 4 and 5.

The specific range was determined from linearity studies, for both drugs and found to be

1-5 mg/ml for Brimonidine and 5-25 mg/ μ l for Brinzolamide. The data was analyzed by linear regression least square fit method. The slope, intercept, correlation coefficient and regression equation were also determined and the data are presented in table 6.

The suitability parameters of the system like resolution, tailing factor, retention time and theoretical plates for the developed RP-HPLC method are presented in figure 9; the data are presented in table 7.

The chromatographic retention time for Brinzolamide and Brimonidine was found to be 5.9 and 8.4 minutes respectively. This is well within the specific limits of 10-15 minutes. The high – resolution value of 8.33 Brimonidine indicates complete separation of the drugs. The tailing factor was found to be 0.808 and 0.860 for Brinzolamide and Brimonidine respectively. The peaks are symmetrical and theoretical plates for Brinzolamide and Brimonidine were 8948 and 8308 respectively which shows the column efficient performance. The limit of detection and limit of quantification for Brinzolamide and Brimonidine are presented in table 8. The quantitative estimation of Brinzolamide and Brimonidine ophthalmic formulation was carried out by RP-HPLC method using Triethylamine phosphate buffer, acetonitrile and methanol was 75:20:05, v/v using C18 column as the stationary phase. The quantitative estimation of the suspension formulation

is presented in table 9 and graphically presented in figure 10.

The ophthalmic formulation assay shows percentage purity ranging from 98.90 to 101.01% for Brinzolamide and 98.20 to 100.80% for Brimonidine. The mean percentage purity is 99.82% and 99.80% for Brinzolamide and Brimonidine respectively. The percentage deviation was found to be -1.1 to +1.1% and - 1.8 to +0.8 for Brinzolamide and Brimonidine respectively. The RSD values are below 2% indicating the method precision and the accuracy of the method shown by the low standard error values. This shows a good index of accuracy and reproducibility of the developed method. All the parameters including flow rate, detection wavelength sensitivity was maintained constant.

CONCLUSION

The proposed and developed UV and RP-HPLC method is precise, accurate, and sensitive. The method is rapid, reproducible, and economical and does not have any interference due to the excipients in the pharmaceutical preparations.

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