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R E S E A R C H A R T I C L E

Analytical Method Development and Validation for the Determination of Brinzolamide and Brimonidine Using Reverse Phase HPLC Method in Bulk and Pharmaceutical Dosage Form

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A B S T R A C T

In RP-HPLC method, the conditions were optimized to obtain an adequate separation of eluted compounds. Initially, various mobile phase compositions were tried, to separate title ingredients. Mobile phase and flow rate selection was based on peak parameters (height, tailing, theoretical plates, capacity or symmetry factor), run time and resolution. The mobile phase containing mixture of Phophate buffer solution: Methanol (65:35v/v, pH 4) with a flow rate of 1.0 ml/min is quite robust. The optimum wavelength for detection was 260 nm at which better detector response for both the drugs was obtained. The retention times for Brinzolamide and Brimonidine tartrate was found to be 2.137 min and 2.844 min, respectively. To ascertain its effectiveness, system suitability tests were carried out on freshly prepared stock solutions. The calibration was linear in concentration range of 5 to 25 µg/ ml and 20 to100 µg/ml, with regression 0.9979 and 0.9999, Brinzolamide and Brimonidine tartrate respectively. The low values of % R.S.D indicate the method is precise and accurate. The mean recoveries were found above 99.3 % for both the drugs.

Keywords: Brinzolamide, Brimonidine tartrate, RP-HPLC, Validation.

A R T I C L E I N F O

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C O N T E N T S

1. Introduction

Brinzolamide is (4R)-4-(ethylamino)-2-(3-methoxypropyl)-1,1-dioxo-2H,3H,4H-1λ-thieno[3,2-e][1,2]thiazine-6-sulfonamide. It comes under the category of anti glaucoma agents. It is used in the treatment of eye disorders. It is a highly specific inhibitor of CA-II, which is the main CA isoenzyme involved in the secretion of aqueous humor. Inhibition of CA in the ciliary process of the eye slows the formation of bicarbonate, and reduces sodium and fluid transport. This results in a reduction in the rate of aqueous humor secretion and the intraocular pressure. Brinzolamide is absorbed systemically following topical ocular administration. Since it has a high affinity for CA-II, brinzolamide binds extensively to red blood cells, where CA-II is primarily found. As sufficient CA-II activity remains, adverse effects resulting from the systemic inhibition of CA by brinzolamide are not observed. The metabolite N-desethylbrinzolamide is also formed. This metabolite binds to CA and accumulates in red blood cells as well. In the presence of brinzolamide, the metabolite binds mainly to carbonic anhydrase I.

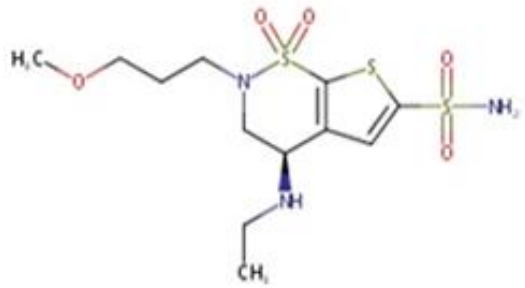

Figure 1: Structure of Brinzolamide

Brimonidine is 5-bromo-N-(4, 5-dihydro-1H-imidazol-2-yl) quinoxalin-6-amine. It comes under the category of Antihypertensive Agents, Adrenergic alpha-2 Receptor Agonists. It is an alpha adrenergic receptor agonist (primarily alpha-2). It has a peak ocular hypotensive effect occurring at two hours post-dosing. Fluorophotometric studies in animals and humans suggest that Brimonidine has a dual mechanism of action by reducing aqueous humor production and increasing uveoscleral outflow. The topical gel reduces erythema through direct vasoconstriction.

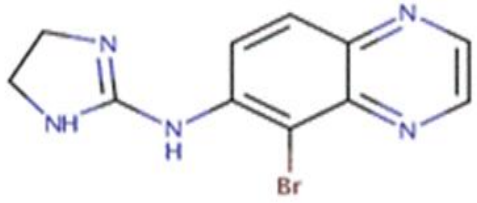

Figure 2: Structure of Brimonidine

The present work describes the development of a simple, precise, accurate, and reproducible HPLC method for the simultaneous estimation of Brinzolamide and Brimonidinein combined dosage form. The developed method was validated in accordance with ICH Guidelines [1&18] and successfully employed for the assay of Brinzolamide and Brimonidine combined dosage form.

2. Materials and Methods

Brinzolamide and Brimonidine were received under the brand names simbrinza, manufactured by Alcon, a Novartis company. It comes under the category of Anti glaucoma agents. Methanol, ortho phosphoric acid, potassium dihydrogenortho phosphate, water, and trim ethylamine were used which were of HPLC grade.

Experimental work:

Chromatographic conditions

The HPLC system (LC Waters, Milford, MA, USA) consisted of quaternary gradient system (600 Controller), in-line degasser (Waters, model AF), photodiode array detector (Water, 2998 model) and auto sampler (Waters, model 717 plus). Data was processed using Empower Pro software (Waters, Milford, MA, USA). Isocratic elution of the mobile phase 0.1 M Dipotassium Phosphate buffer(P^H 4) and Methanol in the ratio of 65:35 v/v with the flowrate of 1 ml/min. Separation was performed on a Waters C_{18} (250 x 4.6 mm i.d, 5 µ particle size) analytical column and a pre-column to protect the analytical column from strongly bonded material. Integration of the detector output was performed using the Waters Empower software to determine the peak area. The contents of the mobile phase were filtered through a 0.45 µm membrane filter and degassed by sonication before use. Mobile phase was used as diluents.

The flow rate of the mobile phase was optimized to 1 ml/min which yields a column back pressure of 110–112 kg/cm. The run time was set at 6 min and a column temperature was maintained at 35°C. The volume of injection was 10 µl, prior to injection of the analyte, the column was equilibrated for 30–40 min with the mobile phase. The eluents were detected at 260 nm. The developed method was validated in terms of specificiy, linearity, accuracy, limit of detection (LOD), limit of quantification(LOQ), intra-day and inter-day precision and robustness for the assay of Brinzolamide and Brimonidine as per ICH guidelines.

Preparation of standard solutions:

About10 mg of Brinzolamide Working Reference Standard and 15mg of Brimonidine tartrate Working Reference Standard were taken and transferred in to 100ml volumetric flask and then it was dissolved and diluted to volume with mobile phase up to the mark. After that 50ml of the above solution was taken into 100ml standard flask and made up with mobile phase. (Stock solution) Further pipetted out 0.5ml of the above stock solution in to a 10ml volumetric flask and diluted up to the mark with diluent.

Preparation of sample solution:

Equal volumes of sample solutions such that 10 mg Brinzolamide and 15 mg Brimonidine were taken. A volume of 70ml of mobile phase was added and sonicated for 30min.Then the solution was cooled to room temperature and diluted to volume with mobile phase and filtered through 0.45µm membrane filter. (Stock solution)
Further pipetted out 0.25ml of Brinzolamide and Brimonidine tartrate of the above stock solution in to a 10ml volumetric flask and diluted up to the mark with diluent.

3. Results and Discussion

Method Development: Number of mobile phase and their different proportions were tried and finally was selected as 0.1 M Dipotassium Phosphate buffer (pH 4) and Methanol in the ratio of 65:35 v/v appropriate mobile phase which gave good resolution and acceptable system suitability parameters. The results of system suitability parameters were shown in table 2. The chromatogram of working standard solution is shown in Fig 3. The summary of Chromatographic conditions was given in table 1.

Method Validation:

Accuracy

Recovery assessment was obtained by using standard addition technique which was by adding known quantities of pure standards at three different levels in 50%, 100%and 150% to the pre analysed sample formulation. From the amount of drug found, amount of drug recovered and percentage recovery were calculated which sense to conformation that the proposed method was accurate. The results were tabulated in Table 3, 4**.**

Precision

The intraday and interday precision of the proposed method was determined by analyzing mixed standard solution of Brinzolamide and Brimonidine at concentration 15µg/mL& 60µg/mL, 3 times on the same day and on 3 different days. The results shown in table 4 were reported in terms of relative standard deviation.

Linearity

Calibration graphs were constructed by plotting peak area vs concentration of Brinzolamde and Brimonidine and the regression equations were calculated. The calibration graphs were plotted over 5different linear concentrations in the range of 5-25µg/ml for Brinzolamide and 20-100 µg/ml for Brimonidine. Aliquots (10 µl) of each solution were injected under the operating chromatographic condition described above [Number of replicates (n =6)]. The linearity graphs were shown in fig 4 & 5.

Limit of detection (LOD) and limit of quantitation (LOQ): The limit of detection (LOD) and limit of quantitation (LOQ) of ASP and OMP were determined by calculating the signal-to-noise(S/N) ratio of 3:1 and 10:1, respectively according to International Conference on Harmonization guidelines.LOD values for Brinzolamideand Brimonidine were found to be 0.021and 0.025µg/mL respectively. LOQ values for the same were found to be0.063 and 0.075µg/mL respectively.

Table 1: Summary of Chromatographic conditions

S. No	Parameter	Description/Value
1.	Stationary Phase	Inertsil C_{18} Column (150mm x 4.6mm)5µm.
2	Mobile Phase	Methanol: Phosphate buffer P^H 4.0 (35:65 v/v)
3	Flow rate	1 ml/min
4	Detection Wavelength	260 nm
5	Detector	Photo diode array
6	Injection	auto sampler -Waters, model 717 plus
7	Rt's	Brinzolamide 2.137 Min Brimonidine tartrate 2.844 Min
8	Injection volume	10 µl
9	Column Temperature	35 °C
10	Run time	6 mins
11	Diluent	Mobile Phase

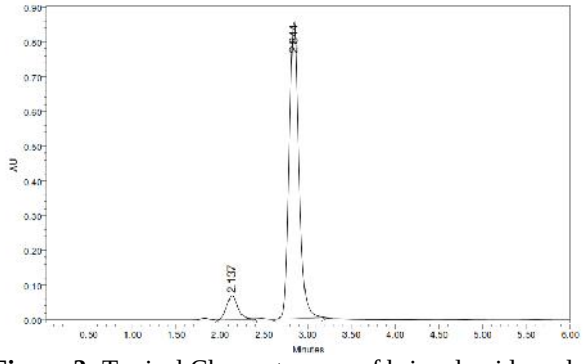

Figure 3: Typical Chromatogram of brinzolamide and brimonidine

Table 2: System suitability parameters

S.No	Parameter	Result	
		Brinzolamide	**Brimonidine**
1	Retention Time	2.137 min	2.844 min
2	Tailing	1.079	1.189
3	Theoretical Plates (n)	5076	7837
4	Resolution factor (R)	3.08	
5	Similarity Factor	1.0124 (Limit: 0.98 – 1.2)	

Assay of the tablet dosage form

The proposed validated method was successfully applied to determine brinzolamide and Brimonidine in Pharmaceutical dosage form. The result obtained for Brinzolamide and Brimonidine were comparable with corresponding labeled amounts. The results were tabulated in table 4.

Table 3: Results of Accuracy

	Brinzolamide				Brimonidine		
S. No	**% Concentration (at specific level)**	**Amount added (µg)**	**Amount found (µg)**	**Mean % Recovery**	**Amount added (µg)**	**Amount found (µg)**	**Mean % Recovery**
		5	5.01	100*	5	5	100*
1	50	10	10	99.13 **	10	9.96	100**
2	100	15	14.84	99.69 *	15	14.96	99*

Table 4: Results of Precision (%Assay)

Sample No.	Brinzolamide		Brimonidine	
	Sample Area - 1	% Assay - 1	Sample Area - 2	% Assay - 2
1	596886	100.06	6423669	100
2	597766	99.49	6418299	100
3	600318	99.14	6435957	98
4	600832	100.27	6426016	99
5	600884	100.27	6425928	99
6	599337	100.39	6425974	99
Average Assay:		100	Average Assay:	99
STD		0.51	STD	0.82
% RSD		0.51	% RSD	0.83

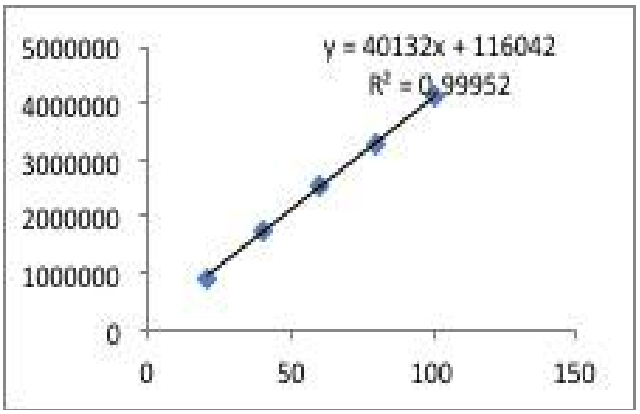

Figure 4: Linearity of Brinzolamide

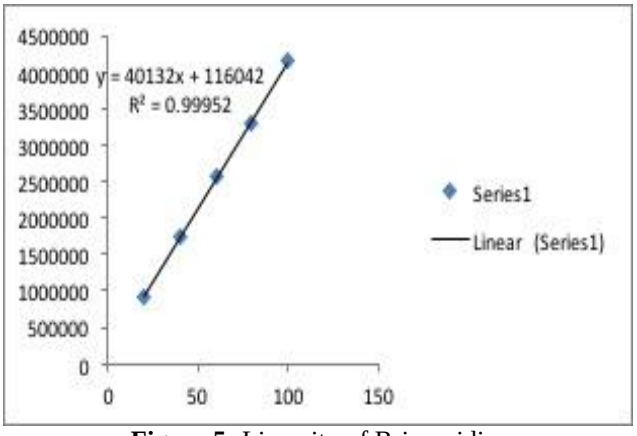

Figure 5: Linearity of Brimonidine

4. Conclusion

The proposed method has advantage of simplicity and convenience for the separation and quantitation of Brinzolamide and Brimonidine in the combination which can be used for the assay of their dosage form. Also, the low solvent consumption and short analytical run time lead to environmentally friendly chromatographic procedure. The method is accurate, precise,rapid and selective for simultaneous estimation of Brinzolamide and Brimonidine in suspension dosage form. Hence itcan be conveniently adopted for routine analysis.

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