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

Analytical Method Development for Simultaneous Estimation of Brimonidine and Brinzolamide by Using RP-HPLC

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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 Phosphate buffer: Methanol PH 4.0 (30:70 v/v) with a flow rate of 1ml min-1 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 2.113 min and 3.560 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 to 100 µg/ml, with regression 0.9999 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 (trade names Azopt, Alcon Laboratories, Befardin, Fardi Medicals,) is a carbonic anhydrase inhibitor used to lower intraocular pressure in patients with open-angle glaucoma or ocular hypertension.

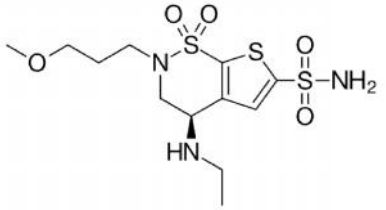

Figure 1: Structure of Brinzolamide

Brimonidine is a drug, used as eye drops under the brand names Alphagan and Alphagan-P to treat open-angle glaucoma or ocular hypertension, as a gel, Mirvaso, for facial skin redness in rosacea, and in tartrate formulation, beginning in July 2018, as an ophthalmic vasoconstrictor, sold by Bausch and Lomb under the brand name Lumify, also as eye drops, in 0.025% solution.

Figure 2: Structure of Brimonidine

2. Materials and Methods

Instrumentation

System Shimadzu LC-20 AD Pump Analytical HPLC isocratic pump Detector SPD-M20A diode array detector, Software LC 2 software, Sonicator, Analytical Technologies Limited- Ultrasonic cleaner

Chemicals

Methanol, Ortho Phosphoric Acid, Potassium Dihydrogen Ortho Phosphate, Tri Ethyl Amine, Water.

Chromatographic Conditions

Table 1: Chromatographic condition

Parameters	Description
Flow rate	1ml min^{-1}
Column	Inertsil C_{18} Column (4.6mm x 150mm)5µm.
Mobile Phase	Phosphate buffer: Methanol P^H 4.0 (30:70 v/v)
Buffer	Potassium di hydrogen orthophosphate PH 4.0 adjust with Orthophosphoric acid
Detector	PDA
Column temperature	Ambient
Type of elution	Isocratic
Wavelength	260 nm
Injection volume	10µl
Run time	10min

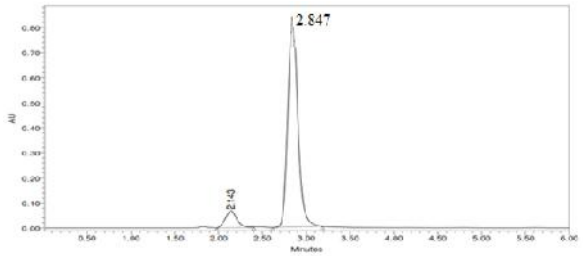

Figure 3: Optimized Chromatogram

Observation: The separation was good, peak shape was good, so we conclude that there is no required for reduce the retention times of peaks, so it is taken as final method.

Standard preparation:

Weigh accurately 10mg Brinzolamide Working Reference Standard and 15mg of Brimonidine tartrate Working Reference Standard is taken 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 pipette 0.5ml of the above stock solution in to a 10ml volumetric flask and dilute up to the mark with diluent.

Sample preparation:

Take an equal volume of sample solution such that 10 mg Brinzolamide and 15 mg Brimonidine. A volume of 70ml of mobile phase was added and sonicate for 30min.Then the solution was cooled and diluted to volume with mobile phase and filtered through 0.45µm membrane filter. (Stock solution)

Further pipette 0.25ml of Brinzolamide and Brimonidine tartrate of the above stock solution in to a 10ml volumetric flask and dilute up to the mark with diluent.

Method Validation

System Suitability:

A Standard solution of Brinzolamide and Brimonidine tartrate working standard was prepared as per procedure and was injected five times into the HPLC system. The system suitability parameters were evaluated from standard Chromatograms obtained by calculating the % RSD of retention times, tailing factor, theoretical plates and peak areas from five replicate injections.

Linearity:

The linearity of an analytical method is its ability to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range.

Precision:

The precision of the method was demonstrated by intra-day and inter-day precision studies. Intra-day studies were performed by injecting three (3) repeated injections within a day. Peak area and %RSD were calculated and reported.

Method Precision:

Method precision also called as repeatability/Intra-day precision indicates whether a method gives consistent results for a single batch. Method precision was demonstrated by preparing six test solutions at 100%

concentration as per the test procedure & recording the chromatograms of six test solutions.

Intermediate Precision:

Intermediate precision of the analytical method was determined by performing method precision on another day by different analysts under same experimental condition. Assay of all six replicate sample preparations was determined and mean %assay value, standard deviation & %RSD was calculated.

Accuracy:

Accuracy of the method was determined by recovery experiments. There are mainly 2types of recovery studies are there.

Standard addition method: To the formulation, the reference standard of the respective drug of known concentration was added, analyzed by HPLC and compared with the standard drug concentration.

Specificity:

ICH defines specificity as "the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically this might include impurities, degradants, matrix, etc.

Limit of Detection and Limit of Quantification:

The Sensitivity of measurement of Brinzolamide and Brimonidine tartrateby use of the proposed method was estimated in terms of the Limit of Detection (LOD) and the Limit of Quantitation (LOQ). The LOD and LOQ were calculated by the use of the equations

Robustness:

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage. For the determination of a method's robustness, deliberate change in the Flow rate was made to evaluate the impact on the method.

3. Results and Discussion

Linearity:

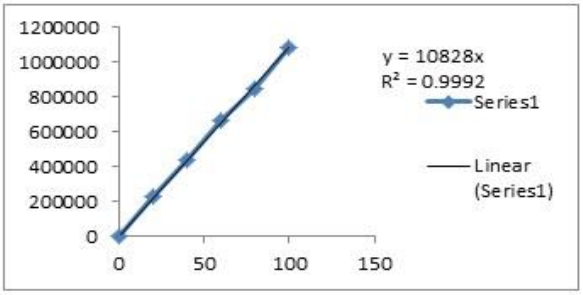

Figure 4: Linearity Graph of Brinzolamide

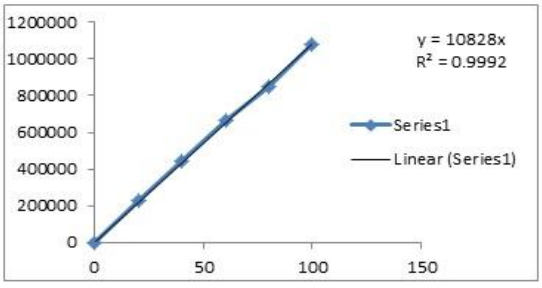

Figure 5: Linearity Graph of Brimonidine Tartrate

Robustness:

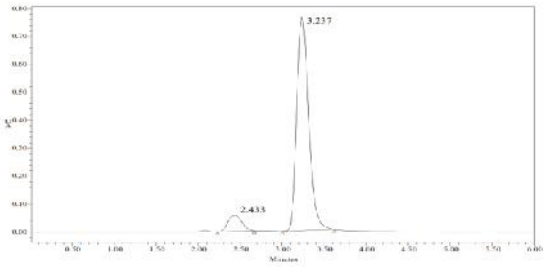

Figure 6: Representative Chromatogram at Flow rate of 0.8 ml/min

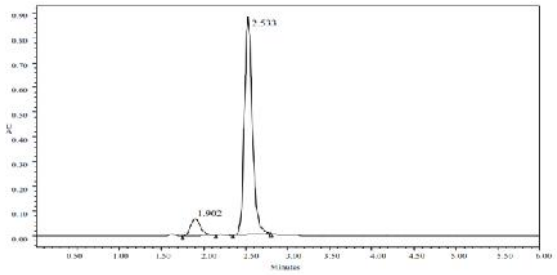

Figure 7: Representative Chromatogram at Flow rate of 1.2 ml/min

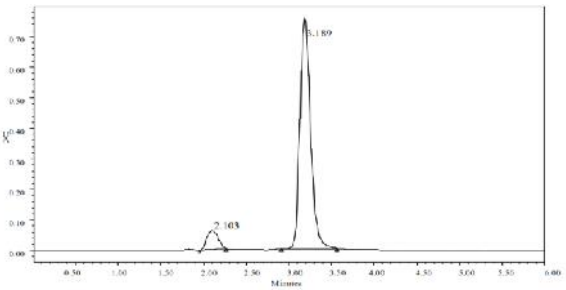

Figure 8: Representative Chromatogram for Mobile phase composition (Buffer: Methanol: 40:60)

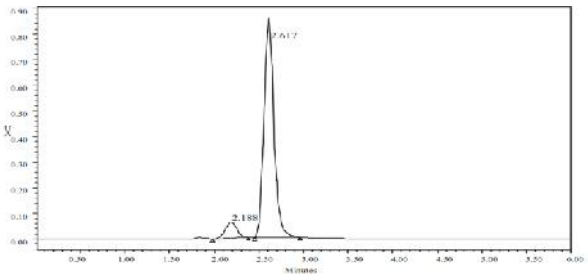

Figure 9: Representative Chromatogram for Mobile phase composition (Buffer: Methanol: 30:70)

4. Conclusion

The developed method was validated in terms of accuracy, precision, linearity, robustness and ruggedness, and results will be validated statistically according to ICH guidelines. The Sample recoveries in all formulations were in good agreement with their respective label claims. From literature review and solubility analysis initial chromatographic conditions Mobile phase Phosphate buffer: Methanol PH 4.0 (30:70 v/v) were set (Potassium dihydrogen orthophosphate PH 4.0 adjust with Ortho phosphoric acid), Inertsil C18 Column (150mm x

4.6mm)5µm. Column, Flow rate 1ml min-1and temperature was ambient, eluent was scanned with PDA detector in system and it showed maximum absorbance at 260nm. As the methanol content was increased Brinzolamide and Brimonidine Tartrate got eluted with good peak symmetric properties. The retention times for Brinzolamide and Brimonidine Tartrate was found to be 2.113 min and 3.560 min respectively.System suitability parameters were studied by injecting the standard five times and results were well under the acceptance criteria.Linearity study was carried out between 50% to150 % levels, R2 value was found to be as 0.999.By using above method assay of marketed formulation was carried out, 100.7% was present.

Table 2: System suitability results for Brinzolamide and Brimonidine

S.No	Peakname	R_t	Area	Height	USP Plate count	USP Tailing	USP Resolution
1	Brinzolami	2.14	936123	156428	5125	1.2	7.1
2	Brimonidin	2.84	125541	13439	3748	1.3	

Table 3: Peak results of Standard & Test Chromatograms for Assay

Parameter	Standard		Test	
	Brinzolamide	**Brimonidine tartrate**	**Brinzolamide**	**Brimonidine tartrate**
Retention time	2.137	2.844	2.143	2.847
Peak Area	628509	6595964	614738	6607226
USP Plate Count	5177	5369	5742	5167
Tailing Factor	1.3	1.3	1.4	1.2
USP Resolution	-	6.3	-	8.2

Table 4: Results of Assay

Parameters	Brinzolamide	Brimonidine tartrate
Standard peak area	628509	6595964
Test peak area (mean)	614738	6607226
% Purity of Standard	98.54	99.68
% Assay	99.86%	100.10%

Table 5: Preparation of working standard solutions for Linearity

Sample ID	Brinzolamide		Brimonidine tartrate	
	Concentration (mcg/ml)	**Area**	**Concentration (mcg/ml)**	**Area**
20% of operating concentration	5	324140	20	**230046**
40% of operating concentration	10	655681	40	**440204**
60% of operating concentration	15	992966	60	**663023**
80% of operating concentration	20	1295546	80	**849886**
100% of operating concentration	25	1677214	100	**1082302**
Correlation Coefficient		**0.999**		**0.999**

Table 6: Precision data for Brinzolamide & Brimonidine tartrate

S.No	Peak name	RT	Area	Peak name	RT	Area
1	Brinzolamide	2.138	596886	Brimonidine	2.860	6423669
2	Brinzolamide	2.137	597766	Brimonidine	2.860	6418299
3	Brinzolamide	2.135	600318	Brimonidine	2.860	6435957
4	Brinzolamide	2.136	600832	Brimonidine	2.852	6426016
5	Brinzolamide	2.138	600884	Brimonidine	2.846	6425928
Mean			599337			6425974
Std.dev			1875.2			6400.9
%RSD			0.31			0.10

Table 7: Intermediate Precision data for Brinzolamide and Brimonidine tartrate

S.No	Peak name	RT	Area	Peak name	RT	Area
1	Brinzolamide	2.138	628573	Brimonidine	2.845	6609089
2	Brinzolamide	2.138	624731	Brimonidine	2.842	6625558
3	Brinzolamide	2.143	619076	Brimonidine	2.843	6633630
4	Brinzolamide	2.140	622317	Brimonidine	2.843	6643244
5	Brinzolamide	2.139	625203	Brimonidine	2.845	6628255
Mean			623980			6627945
Std.dev			3534.5			12545.9
%RSD			0.57			0.19

Table 8: Accuracy Study of Brinzolamide

Sample Id	Conc found (µg/ml)	Concn Obtained (µg/ml)	% Recovery	Mean recovery	Statistical Analysis
50%	5	5.01	100.2		
50%	5	4.86	98.2	99.86	
50%	5	4.89	98.8		**%RSD= 0.506**
100%	10	10.0	100		
100%	10	9.82	98.4	99.8	
100%	10	9.86	98.4		**%RSD=0.64**
150%	15	14.84	97.8		
150%	15	14.74	98.2	99.4	
150%	15	15.02	100.1		**%RSD=1.42**

Table 9: Accuracy Study of Brimonidine tartrate

Sample Id	Concn Obtained(µg/ml)	%Recovery of drug	Mean accuracy	%RSD
50%	4.94	98.2	100.1	1.4
50%	4.92	99.4		
50%	5.01	100.5		
100%	9.94	99.6	99.6	0.3
100%	9.92	99.2		
100%	9.96	99.4		
150%	14.79	98.2	99.2	0.520
150%	14.96	99.4		
150%	14.86	98.9		

Table 10: LOD and LOQ Data of Brinzolamide and Brimonidine tartrate

Brinzolamide			Brimonidine tartrate		
Conc.(x) (µg/ml)	**Peak Areas (y)**	**Statistical Analysis**	**Conc.(x) (µg/ml)**	**Peak Areas (y)**	**Statistical Analysis**
5	1296	S = 38092 c = 608048	20	1641	S = 38092 c =359381
5	4126	LOD: 0.021µg/ml LOQ: 0.024µg/ml	20	5568	LOD:0.025 µg/ml LOQ: 0.025µg/ml

Table 11: Robustness data for Brinzolamide

Std. Replicate	Variation in flow rate		Variation in Mobile phase composition	
	Flow Rate 0.8ml/min	**Flow Rate 1.2ml/min**	**Buffer: Methanol (40:60)**	**Buffer: Methanol (30:70)**
1	6514049	536403	553546	554027
Retention time	2.433	1.902	2.103	2.188
Tailing factor	1.1	1.1	1.1	1.0

Theoretical plates	2430.1	2369.7	2195	2170.2

Table 12: Robustness data for Brimonidine tartrate

Parameter	Variation in flow rate		Variation in Mobile phase composition	
Standard	**Flow Rate 0.8ml/min**	**Flow Rate 1.2ml/min**	**Buffer: Methanol (40:60)**	**Buffer: Methanol (30:70)**
1	7526136	5870230	6528717	6644985
Retention time	3.237	2.533	3.189	2.167
Tailing factor	1.2	1.2	1.2	1.1
Theoretical plates	3543.0	3226.9	3187.8	2569.0

5. References

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