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

Method Development and Validation of Brinzolamide and Brimonidine in Its Bulk and Ophthalmic Dosage Form 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 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 2.113 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 0.9979 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 a carbonic anhydrase inhibitor (specifically, carbonic anhydrase II). Carbonic anhydrase is found primarily in erythrocytes (but also in other tissues including the eye). It exists as a number of isoenzymes, the most active of which is carbonic anhydrase II (CA-II). (4R)-4-(ethy lamino)-2-(3-methoxypropyl)-1,1-dioxo 2H, 3H, 4H-1λ⁶ -thieno[3,2-e][1,2]thiazine-6-sulfonamide.

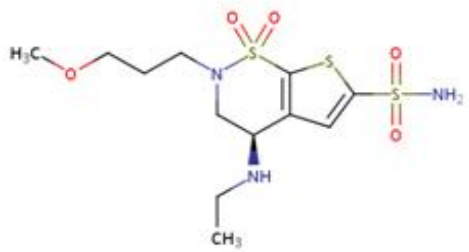

Fig 1: Structure of Brinzolamide

Brimonidine is an α2 adrenergic agonist.α2 agonists, through the activation of a G protein-coupled receptor, inhibit the activity of adenylate cyclase. This reduces cAMP and hence aqueous humour production by the ciliary body.Peripheral α2 agonist activity results in vasoconstriction of blood vessels (as opposed to central α2 agonist activity that decreases sympathetic tone, as can be seen by the medication clonidine). This vasoconstriction may explain the acute reduction in aqueous humor flow. The increased uveoscleral outflow from prolonged use may be explained by increased prostaglandin release due to α adrenergic stimulation. This may lead to relaxed ciliary muscle and increased uv eoscleral outflow5-bromo-N-(4,5-dihydro-1H-imidazol-2-yl)quinoxalin-6-amine.

Fig 2: Structure of Brimonidine

2. Materials and Methods

Chemicals

Methanol, Ortho phosphoric acid, Potassium di hydrogen ortho phosphate, Tri ethyl amine, Water.

Instrumentation

HPLC-auto sampler –UV detector, Separation module 2695, UV.detector2487,Empower-software version-2,Waters,U.V double beam spectrometer,UV 3000+,U.V win software, Lab India.

Chromatographic conditions

Table No 1: Optimized chromatographic conditions

Parameter	Description
Flow rate	1.0 ml/min
Column	Inertsil C18 Column (150mm x 4.6mm) 5µm.
Mobile phase	Phosphate buffer: Methanol PH4.0 (30:70 v/v)
Buffer	Potassium dihydrogen 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	10 min

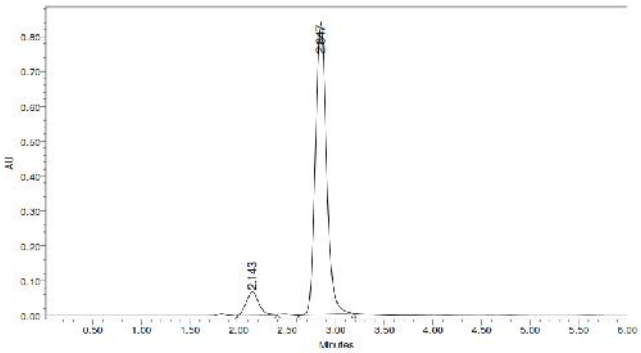

Fig 3: Optimized Chromatogram

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:

Thelinearityofananalyticalmethodisitsabilitytoelicittestresult sthataredirectly, or by a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range.

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.

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.

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.

a) 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.

b) Percentage method: For these assay method samples are prepared in three concentrations of 50%, 100%, and 150% respectively.

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 Discussions

Method development

Proper selection of the method depends upon the nature of the sample, its molecular weight and solubility. The drugs selected in the present study are polar in nature and hence reversed phase or ion-pair or ion exchange chromatography method may be used. The reversed phase HPLC was selected for the separation because of its simplicity and suitability.

Selection of detection wavelength:

The sensitivity of method that uses UV- Vis detector depends upon the proper selection of wavelength. An ideal wavelength is that gives maximum absorbance and good response for both the drugs to be detected. Standard solutions of Brinzolamide and Brimonidine tartrate were scanned in the UV range (200-400nm) and the spectrums obtained were overlaid and the overlain spectrum was recorded. From the overlain spectrum, 260 nm was selected as the detection wavelength for the present study.

Linearity:

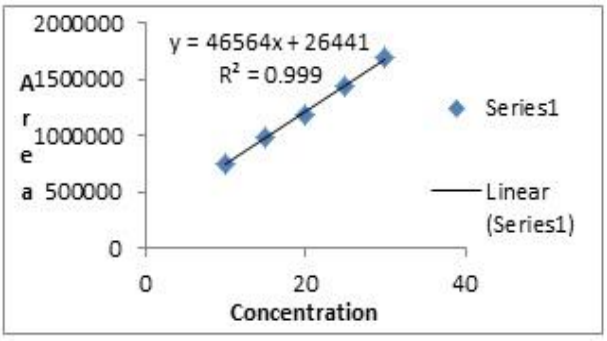

Fig 4: Linearity Graph of Brimonidine Tartrate

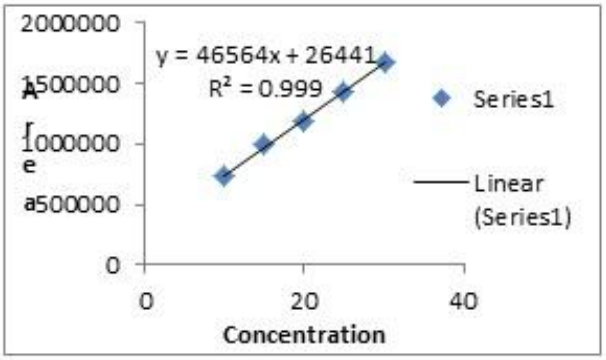

Fig 5: Linearity Graph of Brinzolamide

Robustness:

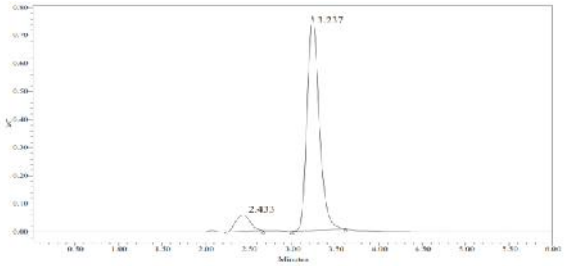

Fig 6: Representative Chromatogram at Flow rate of 0.8 ml/min

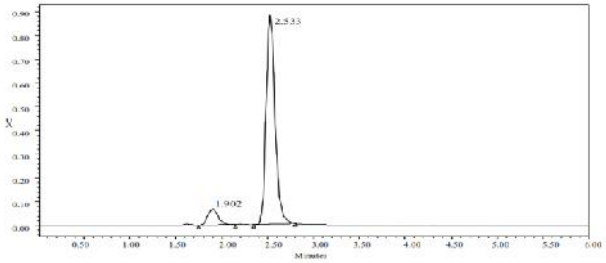

Fig 7: Representative Chromatogram at Flow rate of 1.2 ml/min

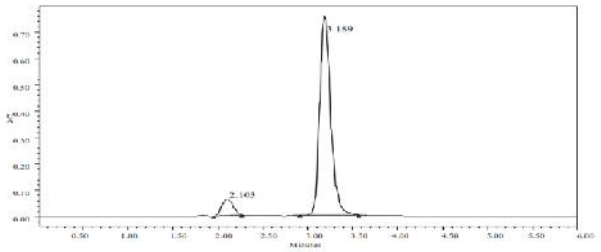

Fig 8: Representative Chromatogram for Mobile phase composition (Buffer: Methanol: 40:60)

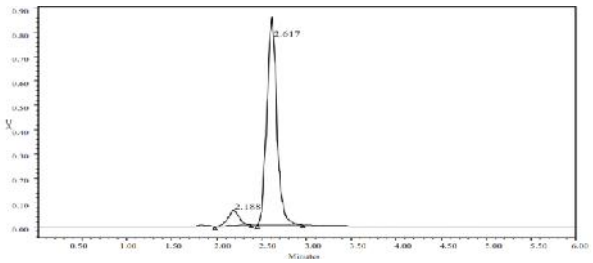

Fig 9: Representative Chromatogram for Mobile phase composition (Buffer: Methanol: 30:70)

Table No 2: 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	1324140	20	940046
40% of operating concentration	10	1395681	40	990204
60% of operating concentration	15	1392966	60	1083023
80% of operating concentration	20	1356546	80	1139886
100% ofoperating concentration	25	1397214	100	1082302
Correlation Coefficient	0.999		0.999	

Table No 3: Precision data for Brinzolamide &Brimonidine tartrate

	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 No 4: Intermediate Precision data for Brinzolamide and Brimonidine tartrate

	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 No 5: 50% Accuracy Result

	Name	Rt	Area
1	Brinzolamide	2.136	499058
2	Brinzolamide	2.139	499571
3	Brinzolamide	2.137	499134
Mean			499254
Std.dev			276.86
%RSD			0.05
	Name	**Rt**	**Area**
1	Brimonidine	2.839	5236293

2	Brimonidine	2.842	5279758
3	Brimonidine	2.840	5281105
Mean			5265719
Std.dev			25492.2
%RSD			0.48

Table No 6: 100% Accuracy Result

	Name	**Rt**	**Area**
1	Brinzolamide	2.140	618309
2	Brinzolamide	2.144	612974
3	Brinzolamide	2.138	615199
Mean			615492
Std.dev			2676.8
%RSD			0.43
	Name	**Rt**	**Area**
1	Brimonidine	2.844	6573904
2	Brimonidine	2.845	6569701
3	Brimonidine	2.841	6580470
Mean			6574692
Std.dev			5427.5
%RSD			0.08

Table No 7: 150% Accuracy Result

	Name	**Rt**	**Area**
1	Brinzolamide	2.144	747032
2	Brinzolamide	2.144	746977
3	Brinzolamide	2.143	746043
Mean			746684
Std.dev			555.8
%RSD			0.07
	Name	**Rt**	**Area**
1	Brimonidine	2.847	7828352
2	Brimonidine	2.847	7828794
3	Brimonidine	2.847	7827962
Mean			7828369
Std.dev			416.2
%RSD			0.005

Table no 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	99.86	**%RSD= 0.506**
50%	5	4.86	98.2		
50%	5	4.89	98.8		
100%	10	10.0	100	99.8	**%RSD=0.64**
100%	10	9.82	98.4		
100%	10	9.86	98.4		
150%	15	14.84	97.8	99.4	**%RSD=1.42**
150%	15	14.74	98.2		
150%	15	15.02	100.1		

Table No 9: Accuracy Study of Brimonidine tartrate

Sample Id	**Concn Obtained(µg/ml)**	**%Recovery of drug**	**Mean accuracy**	**%RSD**
50%	4.94	98.2		
50%	4.92	99.4		**1.4**

50%	5.01	100.5	**100.1**	
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 No 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 LOD: 0.021µg/ml LOQ: 0.024µg/ml	20	1641	S = 38092 c =359381 LOD:0.025 µg/ml LOQ: 0.025µg/ml
5	4126		20	5568	

Table No 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 No 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

4. Conclusion

The proposed HPLC method was found to be simple, specific, precise, accurate, rapid and economical for simultaneous estimation of Brinzolamide and Brimonidine Tartrate in tablet dosage form. 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 65:35 were set (Buffer PH 2.45 adjusted with ortho phosphoric acid), Agilent C18 (250×4.6mm, 5µ) Column, Flow rate 1.0 ml/min and temperature was ambient, eluent was scanned with PDA detector in system and it showed maximum absorbance at 254 nm. As the methanol content was increased Brinzolamide and Brimonidine Tartrategot 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 between50% 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.

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