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A Suitable Chromatographic Method for Simultaneous Estimation of Brimonidine and Brinzolamide in Glaucoma Treated Dosage Form: Influence of its Greenness with Use of Green Metric Tools

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ABSTRACT

In the present work, a RP-HPLC technique was used to separate the two drug moieties in simple run with the use of a Hypersil BDS C18 (100 mm x 4.6 mm; 5µ) P/N: 28105-104630 column and isocratic mobile phase with acetonitrile and 65:35 (v/v) 0.02M Octane-1-sulphonic acid sodium salt solution. A flow rate maintained at 1.0 mL/min was used. This stability indicating optimized method was validated considering ICH guideline parameter and found specific, accurate and robust. The observed resolution is greater than two and the retention periods for Brimonidine and Brinzolamide are 1.7 and 2.6 min respectively. Linearity and range was observed from 1-15 µg/mL and 5-75 µg/mL for Brimonidine and Brinzolamide respectively. The greenness of method was demonstrated by using AES, NEMI, MoGAPI and AGREE tools. The optimized method also shows stability indication by stress study in drastic conditions like acidic, basic, thermal and oxidative conditions.

Keywords: Glaucoma, Angular hypertension, RP-HPLC, Eco-friendly, Stability indication, Green matric tool.

INTRODUCTION

Glaucoma is an eye condition which irreversibly damages the optic nerve invariably leading to blindness. One of the major causes of glaucoma is increased intraocular pressure or IOP[1–4]. The treatment and management of glaucoma generally includes eye medications (drops), laser treatment or surgery. Eye medications focus on controlling/decreasing IOP. Reduction of IOP is the mainstay of treatment of open-angle glaucoma and ocular hypertension. This research work comprises of two drugs namely Brimonidine and Brinzolamide which are used as combination ophthalmic drops to treat ocular hypertension and glaucoma.

Brimonidine tartrate[1] is a nonselective alpha-adrenergic receptor agonist drug which is used as ophthalmic drops for the treatment of ocular hypertension and glaucoma[2]. Its IUPAC name is 5-Bromo-N-(4,5-dihydro-1H-imidazol-2-yl) quinoxalin-6-amine[2] and its structure is given in Fig. 1. It is currently present in USP monograph[3]. When it is used in the form of eye drop medication; it can be used as a short or long term drug lowering ocular pressure in eye treatment. When used in conjunction with some other medications it works more efficiently for minimising intraocular pressure, enhancing uveoscleral outflow in patients having ocular redness[4], ocular hypertension or open angle glaucoma[5].

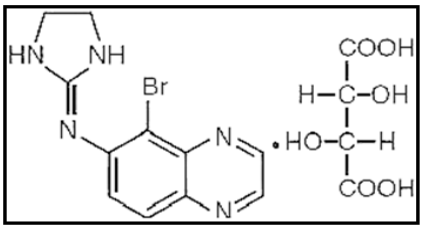


Fig. 1. Structure of Brimonidine Tartrate

Brinzolamide is a carbonic anhydrase II inhibitor[6]. When used as eye drops, it acts as an ocular hypertension reducing agent. Its IUPAC name is (4R)-4-(ethylamino)-2-(3-methoxypropyl)-1,1-dioxo-3,4-dihydrothieno[3,2-e] thiazine-6-sulfonamide[7] and its structure is given in Fig. 2. Ophthalmic form of Brinzolamide is also available in USP monograph[8]. It works in the eye drop dosage form by decreasing the formation of aqueous humor which causes a drop in the intraocular pressure in the interior part of the eyeball[9,10].

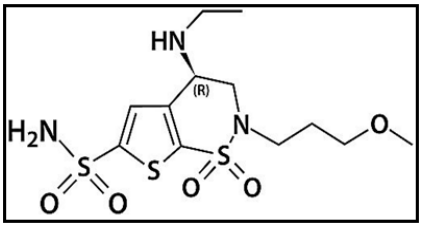


Fig. 2. Structure of Brinzolamide

Monotherapy drug treatment for glaucoma may be insufficient to reach target intraocular pressure (IOP) or tochyphylaxis effect. Single content drug may lose its potency over long periods and hence use of more than one drug exhibits tolerable and satisfactory effect for a long period[10]. Multi drug therapy consisting of Brimonidine and Brinzolamide has been found to be highly effective as anti-glaucoma medication. It has resulted in increased adherence by the patients[11-12] and is also considered to be safe for patients with heart ailments as these drugs do not contain β-blockers. Simultaneous multi drug evaluation techniques are highly favoured as they reduce analysis time and techniques used for simultaneous evaluation of Brimonidine and Brinzolamide have been reported; namely UV-spectroscopic method[13-17], HPTLC[18], RP-HPLC[19-21], UPLC[22], spectrofluorimetric[23], LC-UV[24], GC/MS[25] and Capillary electrophoresis[26] methods. The focus of this study is to develop and validate a novel, stability indicating[27-30], simple, specific, accurate, sensitive and environmental friendly RP-HPLC technique; for the concurrent evaluation of Brimonidine and Brinzolamide with a shorter runtime. This study has the potential to drastically reduce time needed to analyse pharmaceutical combination dose formulations of these two drugs, consequently making the analytical process economical and green.

MATERIALS AND METHODS

Chemicals and reagents

Brimonidine tartrate and Brinzolamide standards were received from Global Calcium Ltd as free samples. Combined product of Brimonidine tartrate and Brinzolamide ophthalmic suspension (Brinzolast), HPLC grade (Milli Q) water, phosphate buffer, 0.1% perchlorate buffer, octane-1-sulfonic acid sodium salt monohydrate (Make-Merck), orthophosphoric acid (Merck), acetonitrile (HPLC grade-Merck) and methanol (HPLC grade-Merck) were obtained from the open market.

Instrument and system

The analytical balance (Sartorius), pH meter (Labindia), ultrasonic bath (Precisonic), chromatographic system: Thermo Scientific HPLC with Chromeleon Software version 7.2 and separation module having PDA or UV-Visible Detector (Ultimate 3000) were the equipment used for the method analysis. The HPLC parameters are given in Table 1.

Mobile phase preparation

In order to prepare the mobile phase, 4.68 g octane-1-sulfonic acid sodium salt monohydrate was weighed and transferred into 1L of HPLC grade (Milli Q) water. The resultant solution pH was set to 3.0 with usage of diluted ortho-phosphoric acid. This 0.02M octane-1-sulfonic acid sodium salt monohydrate buffer was added to acetonitrile in

the ratio of 65: 35 v/v. Further, filtration through 0.45 µ nylon membrane filter and degassing yielded the mobile phase.

Standard preparation

Brimonidine tartrate (10 mg) and Brinzolamide standard (25 mg) were added to 100 mL and 50 mL standard volumetric flasks respectively. These medications on sonication underwent dissolution in the respective volumetric flasks with the addition of 30 mL and 60 mL of the mobile phase. By diluting with the same mobile phase, the appropriate concentrations of clear solutions were obtained. The same mobile phase was used to quantitatively fill 50 mL of the content from 5 mL of the resultant solutions into respective volumetric flasks. The final solutions contained 50 µg/mL of Brinzolamide (50 ppm) and 10 µg/mL of Brimonidine tartrate (10 ppm).

Sample preparation

The test sample drug of Brinzolamide and Brimonidine tartrate in the suspension form (Brinzolast eye drops) manufactured by Mankind Pharma was purchased from Mumbai open market. Density of ophthalmic suspension (weight per mL) was determined by a densitometer and this true value was utilized in the calculations. 0.5 g of this sample solution was taken in a 100 mL standard volumetric flask. Further, after addition of 60 mL of the diluent (mobile phase) this flask was kept for sonication for 5 min with intermediate shaking. Finally the solution was allowed to normalize at controlled room temperature and using the mobile phase further dilution was carried out. The resultant solution was subjected to thorough shaking and shaken well and on filtration through 0.45 µ nylon membrane syringe filter, it yielded 10 µg/mL of Brimonidine tartrate and 50 µg/mL of Brinzolamide respectively.

System suitability

The verifying tests for brimonidine and brinzolamide were carried out through the assessment of the performance parameters. Respectively, the performance parameters were having at least countable theoretical plates of 1000, tailing factors not higher than 2.0 and relative standard deviations (RSD) lower than 2.0%.

Method development

To establish the greatest distinction in Brimonidine and Brinzolamide RT[31-33] several researchers have used various combinations of mobile phase buffers alongside differing stationary phases[34-35]. Since both peaks were resolved with decent separation, a flow rate set at 1.0 mL/min was recommended using mobile phase as 0.02 M octane -1-sulfonic acid sodium salt pH 3.0: acetonitrile (65:35) v/v. The stationary phase for the column was also selected based on the resolution, clarity of dual peaks and sharpness of both medications of regions of interest. A hypersil BDS C18 (100 mm × 4.6 mm; 5 µ) P/N: 28105-104630 was chosen. A wavelength of 254 nm was used as it was optimal for both of the medications and provided great absorbance[36-37]. Brimonidine and Brinzolamide retention times were recorded.

Table 1: Optimized HPLC Chromatographic condition

Parameters	Description
Column Dimension	C18, 100 mm×4.6 mm; 5µm
Column Used	Hypersil BDS
Flow (mL/min)	1.0 mL/min
Volume of Injection	10 µL
Run Period	5 min
Wavelength of Detection	254 nm
Mobile Phase	0.02 M octane -1-sulfonic acid sodium salt pH 3.0:acetonitrile (65:35) v/v
System	Isocratic
Temperature of Column	40°C
Auto sampler temperature	25°C

Developed method validation

As per ICH guideline[38] Q2(R2) and USP chapter <1225> developed method validation parameters are evaluated. The brief validation parameters outcome is shown along with data and final outcome.

Specificity

As per ICH guidelines, analytical method specificity is defined as the ability to demonstrate the analyte in the presence of matrix component without any obstruction. This was elucidated by the injection of the diluent, inactive matrix, standard and individual content solution finding the obstruction at the Brimonidine and Brinzolamide retention times.

Preparation of the each solution began with use of diluent as such. For inactive matrix solution, the same procedures were were applied as those

used for sample preparation. After proper injection in HPLC, it was observed for any obstruction affecting the active content.

Precision

According to the prescribed criterion, in standard preparation both active contents were injected into the HPLC system with six injections each. The relative area percentage deviation of Brimonidine and Brinzolamide area was calculated.

Method precision

The intraday precision of the developed analytical method was followed by standard precision with injection of six individual sample preparations and the percentage RSD of six assay results was calculated accordingly.

Intermediate precision

The inter day precision of the developed analytical method was adhered by performing intraday precision on another day by a different individual using same experimental scenario. The individual performed the variation by use of different chemical lots, column and chromatographic system. After the performance of inter day and intraday precision both the sets were calculated for absolute difference.

Linearity and Range

An analytical method is said to be linear if it is demonstrated that the data from the test is exactly proportional to the analyte concentration within the sample's predetermined range. A line of best fit was plotted, with the concentration of the test substance (in micrograms per mL) graphed along the horizontal axis, against the measured area on the vertical axis. The coefficient of determination, the percentage of Y-intercept, the slope of the regression line, residual sum of squares, and the %RSD of the areas of Brimonidine and Brinzolamide were all determined. Standard solutions were prepared at concentrations of 10%, 50%, 80%, 100%, 120%, and 150% of target concentrations. For Brimonidine these are 10 µg/mL and for Brinzolamide these are 50 µg/mL. These six levels were set to include the lower and upper limit of the method range.

Limit of detection and quantification:

The lowest concentration of analyte (LOD) and the lowest concentration of analyte which can be quantified (LOQ) are parameters which are detected by signal to noise ratio or through a formula derived from standard deviation and slope of linearity curve.

Accuracy

To determine accuracy, three target concentration values were chosen. A normal stock solution was prepared and in triplicate condition spiked with a placebo at distinct concentrations level: 50%, 100% and 150%. In accordance with the methodology, these three levels were injected. All levels showed their recovery within the 98%-102% range.

Intermediate precision (Inter-day precision):

The inter day precision parameter established by performing analysis on another day with different instrument using different lot of chemicals. This performance indicates that there is no significant impact on results set. The percentage RSD of 6 sample results set was observed to be 1.56 and 1.22 for Brimonidine and 1.27 and 0.47 for Brinzolamide performed on different days on different instruments using different chemical lots.

Robustness

The robustness is the analytical method parameter which was evaluated by making little but intentional adjustment in the analytical method performance which did not affect the reliance approach of the method. The standard and sample analysis was carried out as per the methodology. The adjustment parameters of the method such as mobile phase buffer pH ±0.2 (pH 2.80 and pH 3.20), column oven temperature ±5°C (35°C and 45°C) and flow change ± 0.1 mL of 1.0 mL (0.9 mL/ min and 1.1 mL/min) were carried out.

Solution stability

The solution stability of reference as well as test solution is determined by showing result reliability over the period time. This parameter gives importance after the preparation and injection of both the preparations in the chromatographic system.

Evaluation of greenness of proposed analytical method

An AES tool, which is based on Van Aken *et al.,* Eco-Scale for organic synthesis[39], is a methodology that analyzes the “greenness” of a process or a method. The penalty point of chemicals based on number of pictogram present in material safety datasheet (MSD)x score for the signal (safe shown as 1 and danger shown as 2). Penalty point is also assigned for the solvent used (<10 mL=1, between 10-100 mL=2 and >100 mL=3). So the total score for acetonitrile is (2 Pictogram x 2

(danger) x 2 (10-100 mL)) that is 8. Similarly penalty point for octane-1-sulphonic acid sodium salt and ortho-phosphoric acid is calculated. In the penalty point instrumental consumption of energy is also shown (0 point for energy less than 0.1 kWh per sample, 1 point for 0.1-1.5 kWh and 2 point for more than 1.5 kWh). In our case HPLC energy consumption is 1 for 0.1-1.5kWh. The waste penalty point is calculated by considering the formula-Run time x flow rate+processing score. In our study, the run time is 5 min. flow rate is 1 mL/min and processing score is 3 (no treatment); so the formula became (5 x 1+3) = 8.

The score of the optimized method of our study is 79, an indicator of good greenness. With real green analysis the consumption of reagents, energy and waste is reduced. All the points deducted from the overall penalty score are summated as 100 which signifies an ideal approach[40].

The second metric tool is NEMI, which incorporates a pictogram with four sections. One of the sections is PBT solvents, which stands for toxic, persistent and bio accumulative. This takes us to the next section which indicates dangers, the next is corrosiveness (usage of solution pH 2-12) and the last is waste. Out of the four parts, the suggested method shows three green sections, which indicates waste, non PBT solvents and non-corrosive conditions. On the other hand, the inclusions of harmful solvents like acetonitrile and ortho-phosphoric acid as stipulated by the Code of Federal Regulations and Environmental Protection 201641 represents the blank danger section.

AES Evaluation NEMI Evaluation

Parameters	Penalty points
Octane-1-sulphonic acid sodium salt	2
Ortho-phosphoric acid	2
Acetonitrile	8
Instrument - HPLC	1
Occupational hazards	0
Waste	8
Total penalty point	21
AES score	79
Evaluation	Excellent green analysis

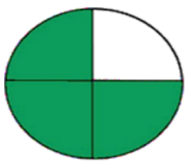


Fig. 3. Evaluation of greenness of method using 2 different semi quantitative metric tools

Metric tools application

AES evaluation: >75 represents excellent green analysis, >50 represents acceptable green analysis, <50 represents inadequate green analysis.

For the green metric assessment[42], MoGAPI employed five pentagrams which range from one to five. The colours red, yellow and green represents score of three, two and one–indicating high, medium and low environmental impact respectively. Taking into account three red, seven yellow and five green parts of the suggested HPLC method, it could be said that this method has a rather moderate to medium environmental impact. The fourth metric tool AGREE[43] employs software that can be downloaded which in turn allows it to use its findings on the 12 principles of green analytical chemistry. The findings were represented as a 12 sectioned colored pictogram on a range between zero to one. A number closer to one and colored dark green indicates a greener approach. It must be noted, however, that AGREE is instant friendly, all rounded and quite simple to use. In relation to their overall greenness, the suggested methods range in colour from pale to dark green. The results obtained from all the green metric tools are detailed of in Figure 4.

Metric tool application

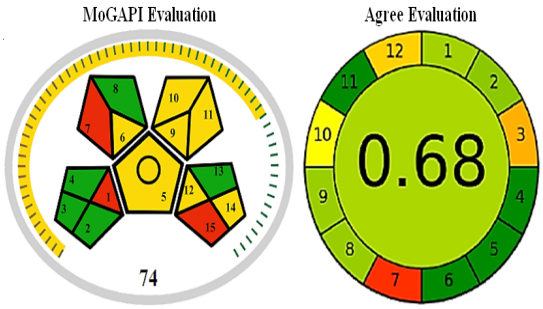


Fig. 4. Evaluation of greenness of method using 2 different quantitative metric tools

MoGAPI : excellent green (≥75), acceptable green (50–74), and inadequately green (<50)

AGREE : excellent green (≥0.75), acceptable green (0.5–0.74), and inadequately green (<0.50)

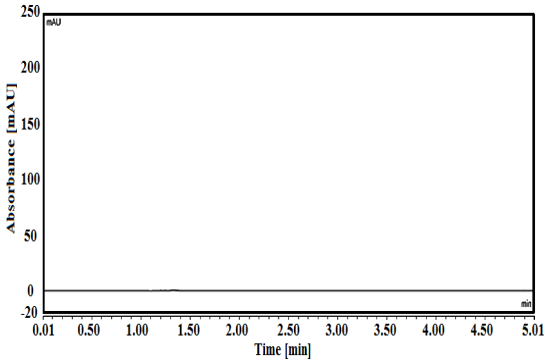
RESULTS AND INTERPRETENTION

Specificity

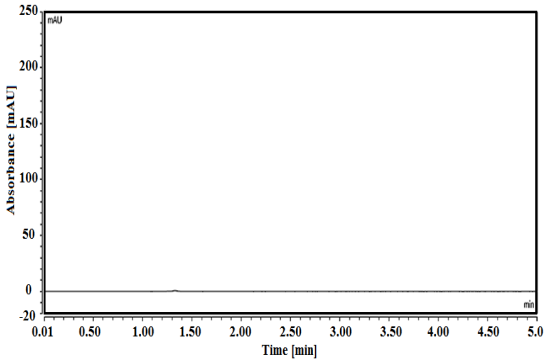
The specificity of method was tested to ensure placebo, other matrix, brimonidine and brinzolamide diluent solution (M. Phase) do not intervene at the retention times for Brimonidine (Rt-1.7 minutes) and Brinzolamide (Rt- 2.6 minutes) respectively as per Figure 5

Chromatogram

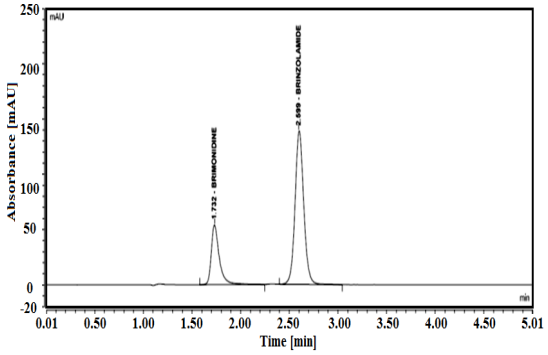
a. Blank Chromatograph



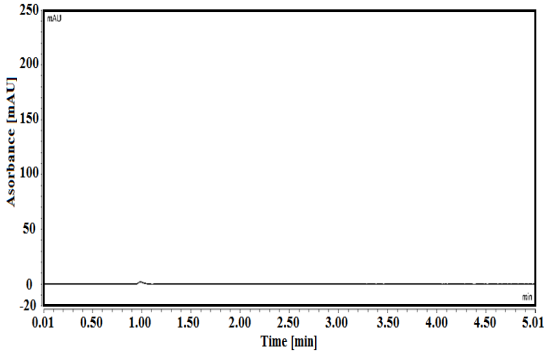
b. Placebo Chromatograph



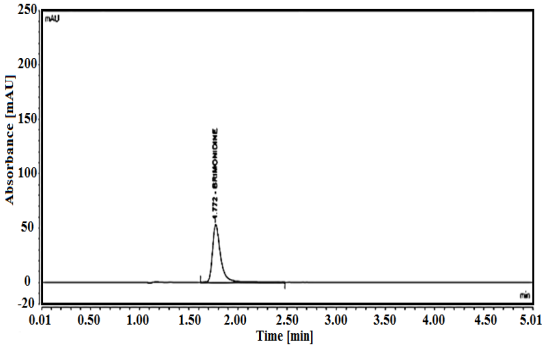
c. Standard Chromatograph



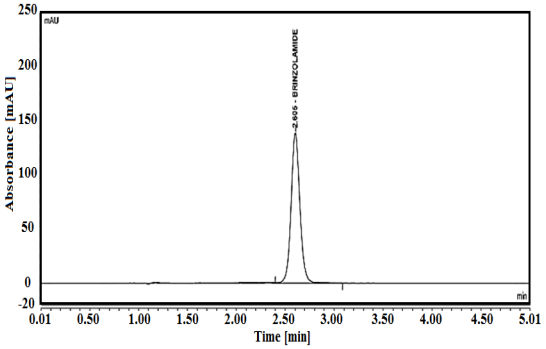
d. Individual Tartaric acid Chromatograph



e. Individual Brimonidine Chromatograph



f. Individual Brinzolamide Chromatograph



g. Sample Chromatograph

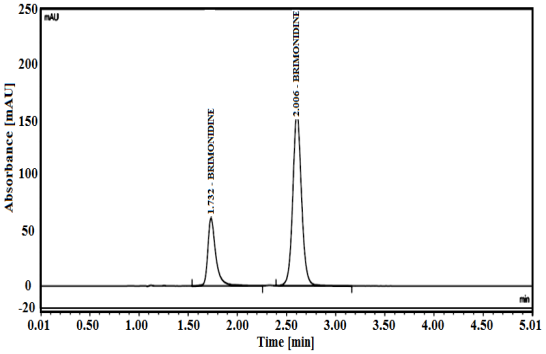


Fig. 5. Specificity chromatograph of BRT and BRZ

Chromatogram overlay

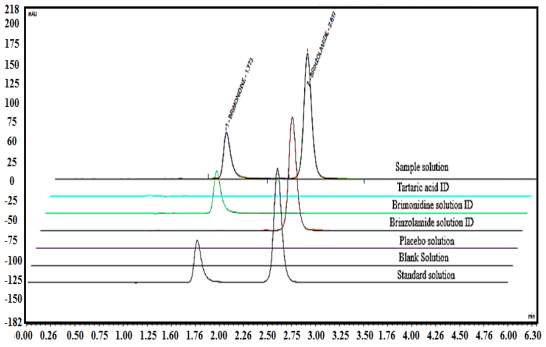


Fig 6: Overlay chromatograph of specificity

Linearity and Range

After the insertion of standard concentrations of Brinzolamide and Brimonidine; the peak area correlation coefficients (R^2) of these two drugs were established which indicates the linearity of the chromatographic procedure. BRT and BRZ stock solutions were injected in HPLC system in concentration levels of 10, 50, 80, 100, 120 and 150 percent, while considering 10 ppm BRT and 50 ppm BRZ respectively. For these two drugs the detector response was observed to be linear from 10 to 150 percent of the concentration. Fig. 7 represents overlay chromatograph of linearity, Fig. 8 and Fig. 9 represent the linearity curves of BRT and BRZ along with its residual graph Fig. 10 and Figure 11.

Chromatogram overlay

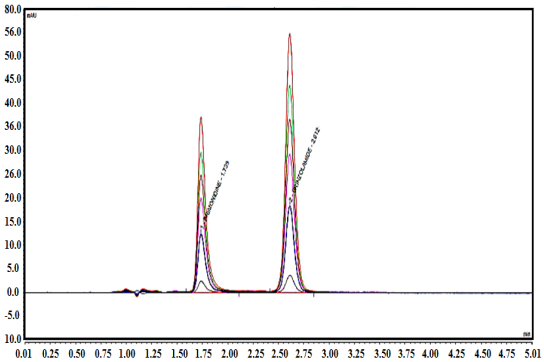


Fig. 7. Overlay chromatograph of linearity concentration

As per the linearity requirements, the R^2 value (correlation of coefficient) for Brimonidine and Brinzolamide was within the range (not less than 0.995) at 0.9999 and 0.9996 respectively. The graph of linearity is shown in the Fig. 8 and Figure 9.

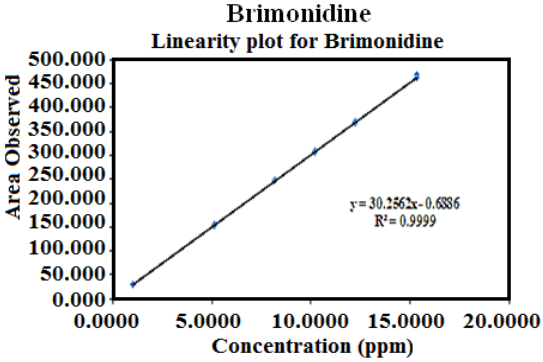


Fig. 8. Calibration curve of Brimonidine

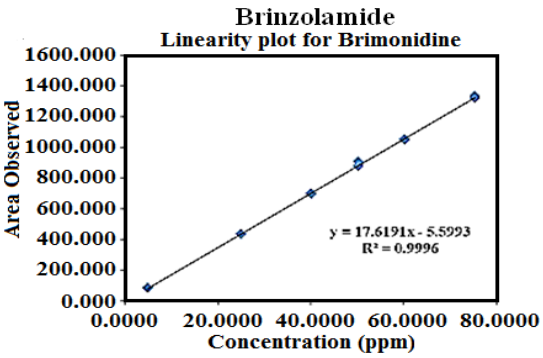


Fig 9: Calibration curve of Brimonidine

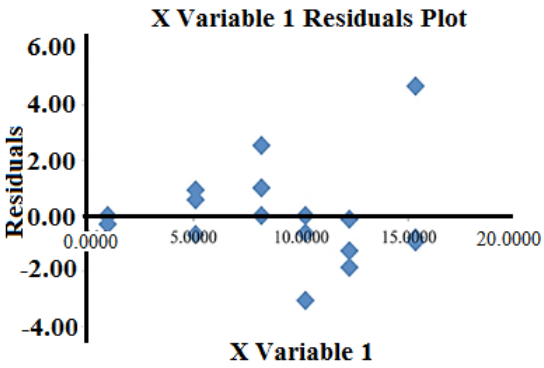


Fig. 10. Residual plot of Brimonidine

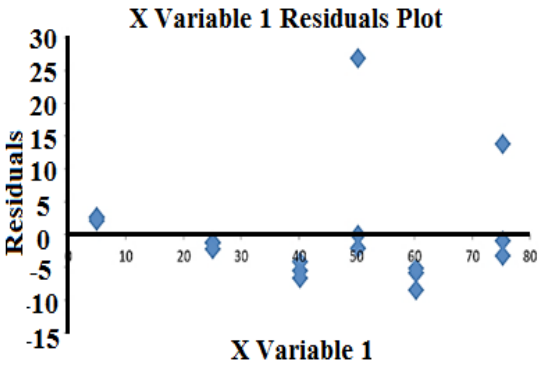


Fig. 11. Residual plot of Brinzolamide

Table 2: Linearity summary for BRT and BRZ

Parameter for Linearity	Brimonidine	Brinzolamide	Criteria
Correlation coefficient R^2	0.9999	0.9996	> 0.999
%Y–axis intercept	-0.28	-0.80	≤ ±3 %
Slope of regression line	30.26	1181.99	To be reported
Residual sum of squares	47.8	80.3	To be reported

Limit of detection and quantification

LOD and LOQ were determined by considering slope of the linear calibration curve and standard deviation of the response. The method sensitivity of the established analytical method was evaluated by the LOD and the LOQ values. Data is shown in Table 3.

Table 3: LOD & LOQ values of Brimonidine and Brinzolamide

Molecules	LOD	LOQ
Brimonidine	0.19	0.57
Brinzolamide	1.61	4.88

Accuracy

The method's accuracy as measured by percentage recovery fell well within the range of 98-102% (Table 4 and 5).

Table 4: Accuracy for Brimonidine

Accuracy level	Standard concentration added (µg/mL)	Standard concentration recovered (µg/mL)	%Recovery	Average	%RSD
50%-1	5.1080	5.1336	101	100	0.62
50%-2	5.1080	5.1222	100		
50%-3	5.1080	5.0802	99		
100%-1	10.2160	10.1290	99	100	0.60
100%-2	10.2160	10.2102	100		
100%-3	10.2160	10.2313	100		
150%-1	15.3240	15.5148	101	100	0.79
150%-2	15.3240	15.3325	100		
150%-3	15.3240	15.3277	100		

Table 5: Accuracy for Brinzolamide

Accuracy level	Concentration of standard added (µg/mL)	Concentration of standard recovered (µg/mL)	%Recovery	Average	%RSD
50%-1	25.1080	25.2744	101	101	0.02
50%-2	25.1080	25.2800	101		
50%-3	25.1080	25.2807	101		
100%-1	50.2160	51.0812	102	102	0.20
100%-2	50.2160	50.9065	101		
100%-3	50.2160	51.0220	102		
150%-1	75.3240	76.3470	101	102	0.23
150%-2	75.3240	76.5159	101		
150%-3	75.3240	76.6491	102		

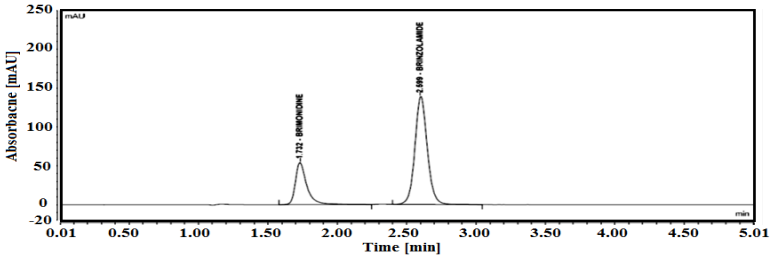
Precision

The percentage RSDs of system precision for Brimonidine and Brinzolamide peak values were 0.2 and 0.1 for the standard solution area, which was within the limit of 2.0%. To investigate method precision, six different sample preparations were utilized and the percentage RSD values for the Brimonidine and Brinzolamide content were 1.6 and 1.3 respectively within the limit of 2.0%. In the same manner, six sample preparations were done for intermediate precision and the results indicated that the content measured was 1.2% and 0.5% which fits into the 2% RSD limit range. The measured RSD and the absolute difference between intraday and inter day precision fell within the specified range. Hence, this method was reported as accurate with respect to system precision; inter day precision and intraday precision; refer Table 6 and 7.

Table 6: System precision

Parameter	Brimonidine	Brinzolamide	Acceptance Limit
Tailing factor	1.49	1.12	NMT 2.0
Theoretical plates	2562	4296	NLT 1000
%Relative standard deviation (RSD)	0.20	0.10	NMT 2.0%
Retention time (in minutes)	1.72	2.59	-

Chromatogram



Peak No.	Peak Name	Ret. Time min	Area mAU*sec	RRT	Asymmetry (EP)	Plates (EP)	Resolution (EP)
1	BRIMONIDINE	1.732	309.159	1.00	1.49	2562	5.88
2	BRINZOLAMIDE	2.599	866.221	1.50	1.12	4296	n.a.

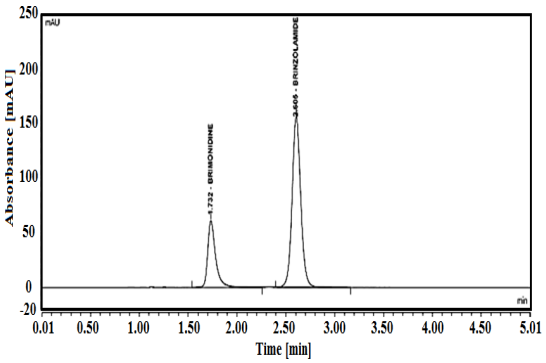
Fig 12: System precision Chromatograph

Table 7: Intermediate and method precision

Sr. No	Repeatability		Intermediate precision	
	Brimonidine	Brinzolamide	Brimonidine	Brinzolamide
1	100.0	102.3	101.6	101.3
2	103.5	100.5	103.9	101.0
3	102.8	99.3	103.5	100.6
4	100.3	102.1	103.1	100.2
5	101.9	102.2	101.4	101.1
6	103.7	100.1	104.5	101.5
Mean	102.0	101.1	103.0	101.0
S. Dev.	1.5921	1.2844	1.2522	0.4764
%RSD	1.56	1.27	1.22	0.47

Chromatogram

a. Method precision Chromatograph



b. Intermediate precision Chromatograph

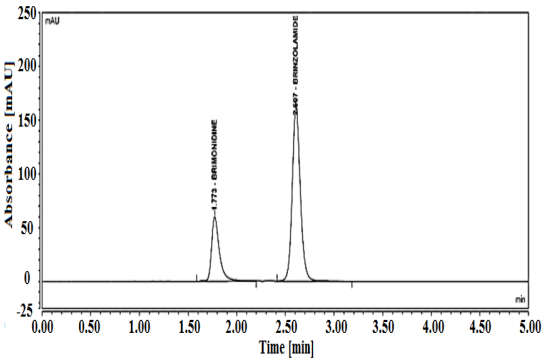


Fig. 13. Method precision/intermediate precision chromatograph of test

Robustness

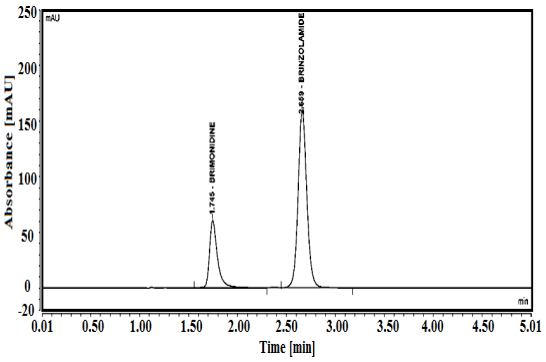
The robustness parameters such as column oven temperature (±5°C), flow rate (±0.1mL), mobile phase buffer pH (±0.2) were performed and their percentage RSD was calculated (Table 8) which was found within the limit. No significant changes were observed in the results even after changing the above parameters and hence we have concluded that the above method is robust as per ICH validation guideline.

Table 8: Evaluation of Robustness Parameter

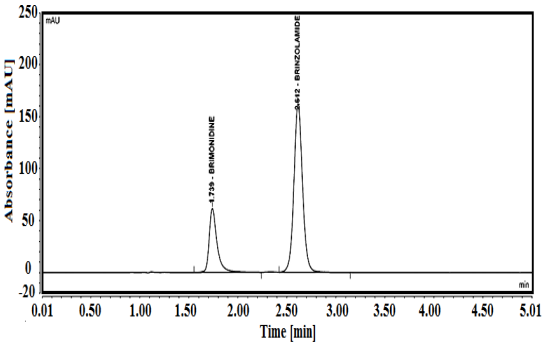
Sr. No	Parameter change	Absolute Difference	Brimonidine		Brinzolamide	
			RT	Assay	RT	Assay
1	Column Temperature 35°C	2.0%	1.74	101.6	2.65	102.1
2	Column Temperature 45°C		1.73	101.4	2.61	102.0
3	Buffer pH 2.8		1.70	102.1	2.51	102.5
4	Buffer pH 3.2		1.73	101.9	2.58	102.4
5	Flow Rate (0.9 mL/Min)		1.92	101.8	2.89	102.2
6	Flow Rate (1.1 mL/Min)		1.58	101.6	2.39	102.2

Chromatogram

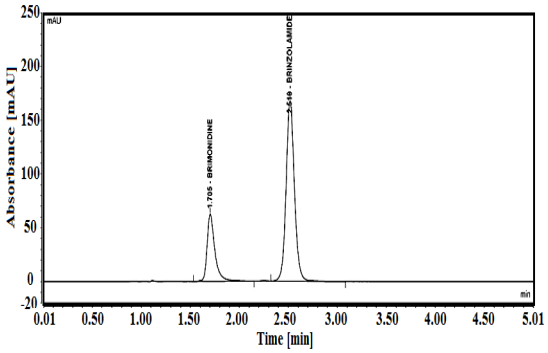
a. Column Temp 35°C



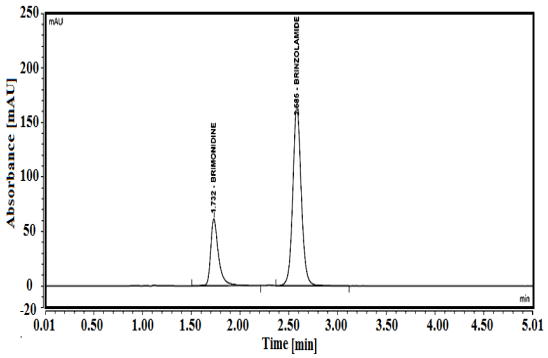
b. Column Temp 45°C



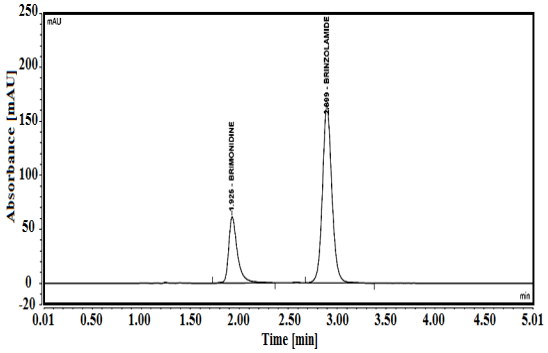
c. Buffer pH 2.80



d. Buffer pH 3.20



e. Flow Rate (0.9 mL/min)



f. Flow Rate (1.1 mL/min)

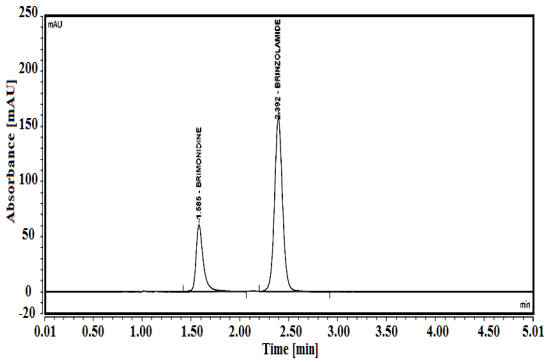


Fig. 14. Robustness parameter chromatograph of test

Chromatogram overlay

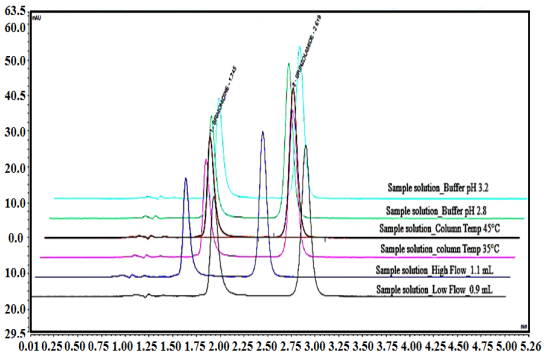


Fig. 15. Overlay graph of test robustness parameter

Stability of resulting solution

Solution stability is a key indicator of method validation. To establish the stability of analyte content in solution over a period of time under specific conditions, the sample solution was analyzed from the initial time after preparation at different time intervals. It was observed that standard as well test solutions in our method were stable upto 24 hours. This emphasizes the method consistency and results reliability over the time period of method application; refer Table 9.

Table 9: Stability results of test solution

Time interval	%Brimonidine	%Brinzolamide	Limit
Initial	101.8	103.0	%Difference
4 h	101.5	102.5	NMT 2.0
12 h	101.8	103.2	
24 h	101.4	102.4	

Degradation study

Hydrolysis degradation study

The hydrolysis stress level of the test sample was checked by refluxing the test sample quantity at 60°C for 6 h under suitable conditions. The above hydrolytic solution was diluted up to the mark and inserted in the HPLC system in order to verify the stability of the sample. It does not show impact by producing any impurity.

Chromatogram

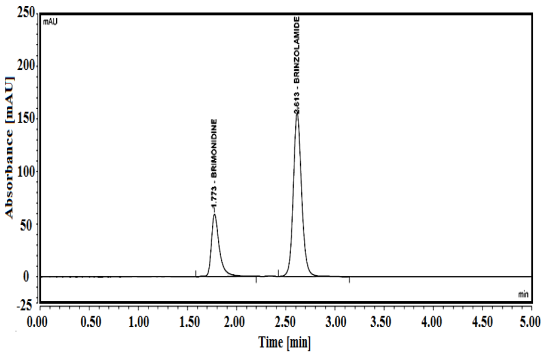


Fig 16: Hydrolytic degradation graph of test solution

Thermal degradation study

In order to conduct the thermal degradation study, the test sample was subjected to a temperature of 105°C for 6 h in the hot oven. It was allowed to cool to room temperature. Subsequently the test sample was diluted to obtain the desired concentration and injected in the chromatographic device so as to evaluate its thermal stability. This condition does not show impact on assay results by producing any unknown impurity.

Chromatogram

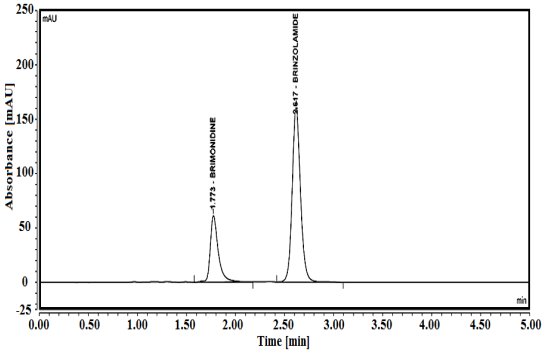


Fig. 17. Thermal degradation graph of test solution

Photolytic stability study

The stability of the photolytic test sample was studied by irradiating the same to ultraviolet-visible light by placing it in a glass beaker in UV photo chamber for 1.2 million Lux hours or 200 watt hrs/m^2. The resulting diluted desired solution was then inserted into the HPLC chromatographic system. It shows sensitivity of Brimonidine peak by generating unknown at 1.2 and 2.8 RT.

Chromatogram

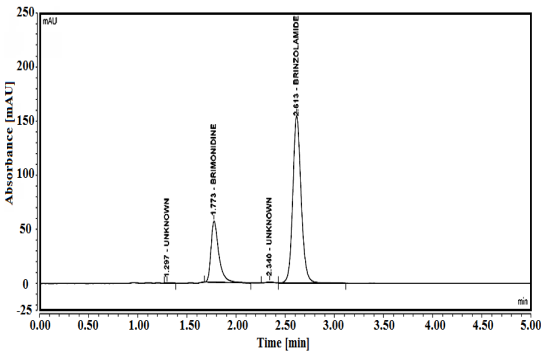


Fig. 18. Photolytic degradation graph of test solution

Acid degradation study

Stress level of the acidic sample was performed by addition of not more than 1 mL of 2N hydrochloric acid into the sample and subjected to boiling at 60°C for 30 min duration. The resulting solution on cooling and neutralization with 1 mL of sodium hydroxide solution (2 N) was diluted to obtain the desired concentration. HPLC chromatographic system was used to evaluate its acidic stress level at 60°C which shows not much impact on drug product.

To observe temperature impact on the acidic stress level we further kept the test solution with addition of same acid and volume for 75°C for 3 hours. However no such temperature impact was observed on both drug moieties.

Chromatogram

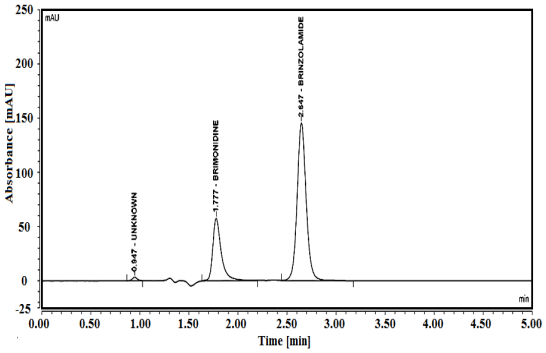


Fig. 19. Acidic degradation graph of test solution at 75°C for 3 hours

Base degradation study

Alkali base degradation of the sample was performed by addition of 1 mL of sodium hydroxide (2 N) into the sample and refluxing it for 30 min duration at 60°C. The resulting solution on cooling and neutralization with 1 mL of hydrochloric acid solution (2N) was diluted to obtain the desired concentration. The resulting solution was injected in the HPLC system to evaluate its alkali base stability. The resulting chromatograph shows unknown impurity at 1.1 retention time. This condition shows Brinzolamide peak reduction drastically.

We further degraded the test sample by applying same chemical but keeping sample at 75°C for 3 h and the resulting desired test solution after dilution was injected in the chromatographic system (Ref Fig. 20). The chromatograph shows drastic degradation of both the contents by generating peak at 0.9 and 1.1 RT.

Chromatogram

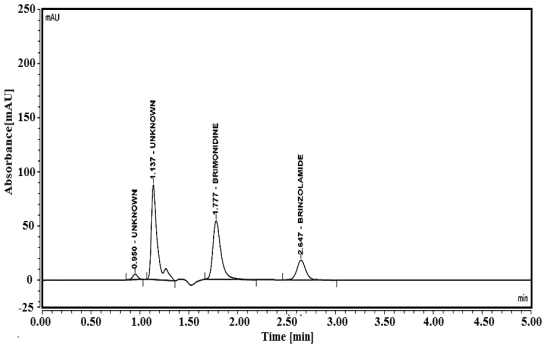


Fig. 20. Basic degradation graph of test solution at 75°C for 3 hours

Oxidation degradation study

Oxidative degradation of sample was evaluated by addition of 1 mL of 3% hydrogen peroxide solution into the sample and refluxing for 30 min at 60°C. After cooling the resulting solution 1 mL of reducing agent sodium meta-bisulfate solution was added and diluted to obtain the desired concentration. The resulting solution was injected in the HPLC device for peroxide degradation study.

We degraded the sample solution by storing the same reagent in the same quantity at 75°C for an hour in order to observe the effects of the temperature increase on both medications. The resulting solution shows half drastic impact on Brimonidine peak. (Ref Figurs 21)

Chromatogram

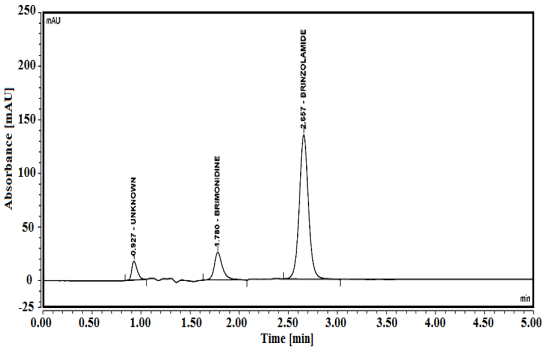


Fig. 21. Oxidative degradation graph of test solution at 75°C for 1 hours

The degradation pathways are shown in Table 8.

Table 8: Evaluation of Forced degradation

Sr. No	Type of degradation	Brimonidine %Assay	Brimonidine %Releasew.r.t digit	Brimonidine %Deg	Brinzolamide %Assay	Brinzolamide %Releasew.r.t digit	Brinzolamide %Deg
1	Neutral	102.2	100.0	0.0	101.2	100.0	0.0
2	Hydrolysis at 60°C for 6 hours	101.7	99.5	0.5	98.5	97.3	2.7
3	Thermal at 105°C for 6 hours	102.7	100.5	0.5	98.8	97.6	2.4
4	Photolytic 1.2 Million Lux hours	97.2	95.1	4.9	100.9	99.7	0.3
5	Acid at 60°C for 30 minute	99.0	96.9	3.1	99.2	98.0	2.0
6	Base at 60°C for 30 minute	98.6	96.5	3.5	92.1	91.0	9.0
7	Oxidative at 60°C for 15 minute	102.4	100.2	0.2	94.4	93.4	6.6
8	Acid at 75°C for 3 hours	102.0	99.8	0.2	99.8	98.6	1.4
9	Base at 75°C for 3 hours	92.8	90.8	9.2	12.4	12.3	87.7
10	Oxidative at 75°C for 1 hours	45.6	44.6	55.4	93.7	92.6	7.4

The given optimised method shows sensitivity towards stress conditions in the range of 5 to 20% of degradation. Brimonidine tartrate shows its sensitivity towards photolytic, acid and base zconditions. Similarly Brinzolamide exhibits sensitivity to base and oxidative conditions. The above data shows clear stability indication for the given optimised method.

SUMMARY

- A new, fast, sensitive and specific methodology has been developed to assess the ocular dosage forms of Brimonidine tartrate and Brinzolamide.
- Now it is possible to quantify Brinzolamide and Brimonidine tartrate in their ocular dosage form using a new, fast, precise, and dependable approach. Using a novel isocratic analytical method, both active ingredients have been rapidly located and measured.
- Both active components were rapidly located and measured using a new isocratic analytical method.
- The retention times for Brimonidine and Brinzolamide were found to be 1.7 and 2.6 minutes, respectively, with good resolution.
- The estimation of Brimonidine and

Brinzolamide has been demonstrated to be within ICH guideline Q2(R2) and USP <1225>.

- This method, indicating stability, is applicable in systematic stability as all validation parameters were found to be within acceptable bounds. Hence it can be applied for both qualitative and quantitative purposes.

CONCLUSION

The analytical method that was put forward in this work has been successfully validated for measuring of Brimonidine and Brinzolamide in accordance with the relevant regulations of the USP <1225> and the ICH guideline Q2(R2). This technology has been determined to be robust, linear, accurate, precise and particular. This approach will serve for both qualitative and quantitative analysis of brimonidine and brinzolamide combination throughout the process as well as in the final formulation. The greenness of the method was explained by using various quantitative green metric such as AES, NEMI, MoGAPI and AGREE tools which reiterates the optimized method is eco-friendly. This indication will be useful as an alternative to many available published methods for the estimation of Brimonidine tartrate and Brinzolamide both single and in combine dosage form for incoming stage testing and stability testing in defined formulation.

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Conflict of interest

The authors disclose that there is no conflict of interest.

REFERENCES

1. Weiser, P. Brimonidine ophthalmic (Alphagan P, Lumify, Qoliana): Drug and medication information. Drug and Medication Information.
2. Oh, D. J.; Chen, J. L.; VaJaranant, T. S.; Dikopf, M. S. Brimonidine tartrate for the treatment of glaucoma. Expert Opin., *Pharmacother.*, **2019**, *20*(1), 1–9. https://doi.org/10.1080/14656566.2018.1561465.
3. United States Pharmacopeia. Brimonidine tartrate USP 43 NF 38; United States Pharmacopeial Convention; **2023**.
4. Torkildsen, G. L.; Sanfilippo, C. M.; DeCory, H. H.; Gomes, P. J. Evaluation of efficacy and safety of brimonidine tartrate ophthalmic solution, 0.025% for treatment of ocular redness., *Curr. Eye Res.*, **2018**, *43*(1), 43–51. https://doi.org/10.1080/02713683.2017.1338339.
5. Reddy, E. R.; Maguire, C. J.; Hastings, R. H.; L'Hommedieu, T.; Dennis, M. C.; Grady, H. L.; Peddapatla, S.; Haley II, M. A. Review of the Synthesis of Timolol Maleate and Brimonidine Tartrate Drug Substances Used for the Treatment of Open-Angle Glaucoma. Org., *Process Res. Dev.*, **2025**, *29*(1),https://doi.org/10.1021/acs.oprd.4c00280.
6. Tiwari, B.; Shirsat, M. K.; Kulkarni, A. Analytical method development and validation for the determination of brinzolamide by RP-HPLC., *J. Drug Delivery Ther.*, **2020**, *10*(1), 92–96. https://doi.org/10.22270/jddt.v10i1.4025
7. Mandour, A. A.; Nabil, N.; Zaazaa, H. E.; Abdelkawy, M. Review on analytical studies of some pharmaceutical compounds containing heterocyclic rings: Brinzolamide, timolol maleate, flumethasone pivalate, and clioquinol., *Future J. Pharm. Sci.*, **2020**, *6*, 52. https://doi.org/10.1186/s40502-020-00212-5
8. Vo, A.; Feng, X.; Patel, D.; Mohammad, A.; Patel, M.; Zheng, J.; Kozak, D.; Choi, S.; Ashraf, M.; Xu, X. In Vitro Physicochemical Characterization and Dissolution of Brinzolamide Ophthalmic Suspensions with Similar Composition., *Int. J. Pharm.*, **2020**, *588*, 119761.https://doi.org/10.1016/j.ijpharm.2020.119761
9. Agrawal, V. P.; Desai, S. S.; Jani, G. K. Development of RP-HPLC method for simultaneous determination of brimonidine tartrate and brinzolamide by QbD approach and its validation., *Eurasian J. Anal. Chem.,* **2016**, *11*(2), 63–78. https://doi.org/10.29333/ejac.2016.11.2.63.

10. Rastogi, P. S.; Jain, K.; Chander, A.; Ansari, M. A comparative study of topical fixed-dose combination of brimonidine (0.2%) plus brinzolamide (1%) versus brimonidine (0.2%) plus timolol (0.5%) in patients of primary open-angle glaucoma., *Indian J. Clin. Exp. Ophthalmol.*, **2021**, *7*(1), 112–117.
11. Topouzis, F.; Goldberg, I.; Bell, K.; Tatham, A. J.; Ridolfi, A.; Hubatsch, D.; Nicolela, M.; Denis, P.; Lerner, S. F. Brinzolamide/ Brimonidine Fixed-Dose Combination BID as an Adjunct to a Prostaglandin Analog for Open-Angle Glaucoma/Ocular Hypertension., *Eur. J. Ophthalmol.,* **2021**, *31*(1), 103-111. https://doi.org/10.1177/1120672119878044.
12. Onoe, H.; Hirooka, K.; Nagayama, M.; Mochizuki, H.; Hirota, A.; Suzuki, K.; Sagara, T.; Kiuchi, Y. The Efficacy, Safety, and Satisfaction Associated with Switching from Brinzolamide or Brimonidine to Brinzolamide/ Brimonidine in Open-Angle Glaucoma Patients., *J. Pers. Med.*, **2022**, *12*(12), 2057. https://doi.org/10.3390/jpm12122057.
13. Tiwari, B.; Shirsat, M. K.; Kulkarni, A. Analytical Method Development and Validation for the Determination of Brinzolamide by RP-HPLC., *J. Drug Deliv. Ther.*, **2020**, *10*(1), 92-96. https://doi.org/10.22270/jddt.v10i1.3845
14. Vijya, P.; Patel, D.; Desai, S.; Meshram, D. Development and Validation of Derivative Spectrophotometric Method for Simultaneous Estimation of Brimonidine Tartrate and Brinzolamide in Combined Dosage Form., *Indo Am. J. Pharm. Res.*, **2014**, *4*(3), 1472–1478.
15. Jadhav, V. L.; Patil, A. S.; Chaudhari, S. R.; Shirkhedkar, A. A. UV-Spectrophotometry-Multicomponent Mode of Analysis for Simultaneous Estimation of Brinzolamide and Brimonidine Tartrate in Bulk and Ophthalmic Formulation., *J. Pharmac. Technol., Res. Manage.*, **2019**, *7*(1), 31-35.
16. Agrawal, V. P.; Desai, S. S.; Jani, G. K. Development of ultraviolet spectrophotometric method for the simultaneous estimation of brimonidine tartrate and brinzolamide., *Indian Drugs.,* **2015**, *52*(11), 29–34.
17. Manoharan, G.; Al-Bratty, M. Development and validation of ultraviolet spectrophotometric and reversed-phase high-performance liquid chromatography techniques for simultaneous estimation of brinzolamide and brimonidine tartrate in ophthalmic suspension formulation., *Orient. J. Chem.*, **2016**, *32*(2), 1111–1120. https://doi.org/10.13005/ojc/320236.
18. Patil, A. S.; Mali, B. J.; Deore, B. L.; Mahajan, D.; Chaudhari, S.; Shirkhedkar, A. A.; Haswani, N. G. An integrated pharmaceutical analysis utilizing dual HPTLC for simultaneous estimation of brinzolamide and brimonidine tartrate in bulk and ophthalmic formulation., *Revista Electronica de Veterinaria.,* **2024**, *24* (1).
19. Rao, K. N.; Doonaboyina, R.; Hema, R. Method development and validation of brinzolamide and brimonidine in its bulk and ophthalmic dosage form by using RP-HPLC., *Int. J. Chem. Pharm. Sci.*, **2018**, *6*(11), 306–312.
20. Vani, R.; Samreen, A.; Tabassum, H.; Sunita, M. Analytical method development and validation for the determination of brinzolamide and brimonidine using reverse phase HPLC method in bulk and pharmaceutical dosage form., *Int. J. Med. Pharm. Res.*, **2018**, *6*(1), 38–43.
21. Patel, P.; Darji, V. C.; Patel, B. R. Analytical Method Development and Validation of Stability-Indicating RP-HPLC Method for Estimation of Brinzolamide and Brimonidine Tartrate in an Ophthalmic Suspension., *Int. J. Res. Appl. Sci. Eng. Technol.*, **2019**, *6*(1), 150–156.
22. Sonanis, M. C.; Rajput, A. P. Development and validation of a new stability indicating analytical method for the determination of related components of brimonidine tartrate in drug substances and drug product using UPLC., *Int. J. Pharm. Pharm. Sci.*, **2011**, *3*(1), 145–150.
23. Sunitha, G.; Bhagirath, R.; Alapati, V. R.; Ramakrishna, K.; Subrahmanyam, C. V.; Anumolu, P. D. Fluorimetric quantification of brimonidine tartrate in eye drops., *Indian J. Pharm. Sci.*, **2013**, *75*(6), 730.
24. Wahab Mubarak, M. A.; Mohammad, M. A.-A.; El-Bagary, R. I.; Elkady, E.; Abo-Talib, N. F. A Simple Validated LC-UV Method for the Simultaneous Determination of Brimonidine and Brinzolamide in the Presence of Brinzolamide's Degradation Product (Major Metabolite) in Rabbit Aqueous Humor., *J. Chromatogr. Sci.*, **2025**, *63*(2), bmae040. https://doi.org/10.1093/chromsci/bmae040.
25. Acheampong, A.; Diane, D. S. Measurement of brimonidine concentrations in human plasma by a highly sensitive gas chromatography/ mass spectrometric assay., *J. Pharm. Biomed. Anal.*, **1995**, *13*(8), 995–1002.

26. Tzovolou, D. N.; Lamari, F.; Mela, E. K.; Gartaganis, S. P.; Karamanos, N. K. Capillary electrophoretic analysis of brimonidine in aqueous humor of the eye and blood sera and relation of its levels with intraocular pressure. Biomed., *Chromatogr.*, **2000**, *14*(5), 301–305.
27. Krishna, M. R.; Gupta, M. K. Development and stability indicating RP-HPLC method for the determination of brinzolamide in bulk and pharmaceutical dosage forms., *Pharmaceutical Sciences.*, **2023**, *3*(2).
28. Ghodse, S. M.; Gawde, K. K., *Stability indicating analytical method development and validation of brinzolamide in bulk drugs.*, **2023**, *12*(10).
29. Ranga, M.; Modini, A. K.; Doddipilla, R.; Kaliyaperumal, M.; Goswami, A. Oxidative stress-induced degradation of brinzolamide: Isolation and in-depth characterization of unique hydroxylamine and oxime degradation products., *J. Pharm. Biomed. Anal.*, **2025**, 252.
30. Peris-Vicente, J.; Esteve-Romero, J.; Carda-Broch, S. Validation of Analytical Methods Based on Chromatographic Techniques: An Overview., *Anal. Sep. Sci.*, **2015**, *5*, 1757–1808. https://doi.org/10.1002/9783527678129.assep064
31. Patel, P.; Darji, V. C.; Patel, B. R. Analytical method development and validation of stability indicating RP-HPLC method for estimation of brinzolamide and brimonidine tartrate in ophthalmic suspension., *Int. J. Res. Anal. Rev.*, **2019**, *6*(1), 2348–1269.
32. Christian, J. R.; Patel, K.; Gandhi, T. R. Validation and Experimental Design Assisted Robustness Testing of RPLC Method for the Simultaneous Analysis of Brinzolamide and Brimonidine Tartrate in an Ophthalmic Dosage Form., *Indian J. Pharm. Sci.*, **2016**, *78*(5), 631–640.
33. García-Ferrer, D.; Peris-Vicente, J.; Bose, D.; Durgbanshi, A.; Carda-Broch, S. Hybrid Micellar Liquid Chromatography Separation of Brimonidine Tartrate and Brinzolamide: Retention Study and Quantification in Fixed Dose Ophthalmic Suspensions., *Microchem. J.*, **2024**, *207*, 112176. https://doi.org/10.1016/j.microc.2024.112176.
34. Deeb, S. A.; Parr, K.; Whiter, M. K. Greener RP-HPLC method for simultaneous determination of dorzolamide, brinzolamide, and timolol using isopropanol as a sustainable organic solvent in the mobile phase., *Separations.*, **2024**, *11*, 83.
35. Khatun, R.; Islam, S. M. A. Development and validation of analytical method for simultaneous estimation of brinzolamide and timolol by HPLC from ophthalmic preparation., *IJPSR.*, **2014**, *5*(3), 1001–1007.
36. Agrawal, V. P.; Desai, S. S.; Jani, G. K. Development of RP-HPLC Method for Simultaneous Determination of Brimonidine Tartrate and Brinzolamide by QbD Approach and Its Validation., *Eurasian J. Anal. Chem.*, **2016**, *11*(2), 63–78.
37. Sandhya, D.; Sowndarya, G.; Swathi, V. Stability indicating RP-HPLC method for simultaneous estimation of brinzolamide and brimonidine in pharmaceutical dosage forms., *Int. J. Adv. Pharm. Biotech.*, **2020**, *6*(3), 15–18.
38. International Conference on Harmonization (ICH). Method validation guidelines; Geneva, **1996**.
39. Dicks, A.; Hent, A.; Koroluk, K. The EcoScale as a Framework for Undergraduate Green Chemistry Teaching and Assessment., *Green Chem. Lett. Rev.*, **2018**, *11*, 29–35. https://doi.org/10.1080/17518253.2018.1431313.
40. Herbatallah, M. E.; Saad, M. N.; Elzanfaly, E. S.; Amer, S. M. Optimization and validation of eco-friendly RP-HPLC and univariate spectrophotometric methods for the simultaneous determination of fluorometholone and tetrahydrozoline hydrochloride., *Acta Chromatographica* ., **2021**, *33*(3), 216–227.
41. Code of Federal Regulations, Protection of Environment, Subchapter I-Solid Wastes, Part 261 Identification and Listing of Hazardous Waste, Environmental Protection Agency (EPA). **2016**, available at https://www.govinfo.gov/app/details/CFR-2016-title40-vol28/CFR-2016-title40-vol28-part261.
42. Kowtharapu, L. P.; Katari, N. K.; Muchakayala, S. K.; Marisetti, V. M. Green metric tools for analytical methods assessment: Critical review, case studies, and crucify., *Trends Anal. Chem.*, **2023**, *166*, 117196. DOI: https://doi.org/10.1016/j.trac.2023.117196.
43. Pena-Pereira, F.; Wojnowski, W.; Tobiszewski, M. AGREE—Analytical Greenness Metric Approach and Software., *Anal. Chem.*, **2020**, *92*(14), 10076–10082.