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New Validated RP-HPLC Method Development for the Simultaneous Estimation of Brinzolamide and Timolol in Pharmaceutical Dosage Form

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Abstract:
A new method was established for simultaneous estimation of Brinzolamide and Timolol by RP-HPLC method. The chromatographic conditions were successfully developed for the separation of Brinzolamide and Timolol by using C18 Inertsil ODS (200*4.6) 5um column, flow rate was 1 ml/min, mobile phase ratio was OPA (Orthophosphoric Acid) (0.1%) (80:20%v/v) ACN (detection wave length was 230 nm. The run times were found to be 3.401 min and 4.345 min. The % purity of Brinzolamide and Timolol was found to be 100.83% and 99.84% respectively. The system suitability parameters for Brinzolamide and Timolol such as theoretical plates and tailing factor were found to be 3677.56,1.5 and 4683.62,1.04 the resolution was found to be 6.76. The estimation of Brinzolamide and Timolol was done by RP-HPLC. The linearity of Brinzolamide and Timolol was found to be linear with a correlation coefficient of 0.999 and 0.999, which shows that the method is capable of producing good sensitivity. The acceptance criterion for LOD and LOQ is 3 and 10.The LOD and LOQ for Brinzolamide was found to be 2.98 and 10.00 and LOD and LOQ for Timolol was found to be 3.00 and 9.98. Compared to previous results the present method which was developed on Brinzolamide and Timolol there run time is less with the less concentration used. We recommend this method for the routine analysis of these drugs.
Keywords: Brinzolamide, Timolol, CAN, LOD and LOQ, RP-HPLC

1. Introduction

Brinzolamide: Brinzolamide is a carbonic anhydrase inhibitor used for the reduction of elevated intraocular pressure in patients with ocular hypertension or open-angle glaucoma

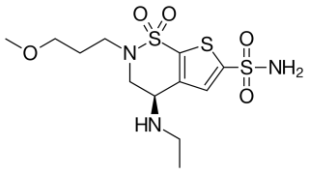

Fig.1: Structure-of Brinzolamide

Chemicalname:(4R)-4-(ethylamino)-2-(3-methoxypropyl)-1,1-dioxo-2H,3H,4H-1λ^6-thieno[3,2-e][1,2]thiazine-6-sulfonamide.
Molecular formula: $C_{12}H_{22}N_3O_5S_3$
Molecular Weight: 383.507 g/mol
Description: A white or almost white powder.
Solubility: Soluble in water; sparingly soluble in methanol R; practically insoluble in acetone R.
pKa: 8.19
Timolol: Timolol is a non-selective beta adrenergic blocker used in the treatment of elevated intraocular pressure in ocular hypertension or open angle glaucoma.

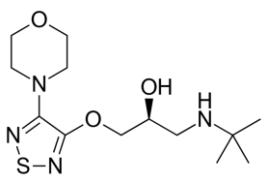

Fig.2: Structure of Timolol

Chemical Formula: $C_{13}H_{24}N_4O_3S$
IUPAC Name: (2S)-1-(tert-butylamino)-3-{[4-(morpholin-4-yl)-1, 2, 5-thiadiazol-3yl]oxy}propan-2-ol.
Solubility: Soluble in water and methanol and other organic solvents.
pKa : 9.76

2. Materials and Methods

Table 1: Instruments used

S.No	Instrument	Model
1	Instrument	WATERS, software: Empower, 2695 separation module, uv detector.
2	HPLC	LABINDIA UV 3000+
3	UV/VIS spectrophotometer	Adwa – AD 1020
4	pH meter	Afcoset ER-200A
5	Weighing machine	Borosil
6	Pipettes &Burettes	Borosil

Table 2: Chemicals used

S.No	Chemical	Brand
1	Timolol	Supplied by Ajantha Drugs
2	Brinzolamide	Supplied by Ajantha Drugs
3	KH_2PO_4	FINAR chemical LTD
4	Water and Methanol for HPLC	Standard solutions Ltd
5	Acetonitrile for HPLC	Standard solutions Ltd
	Water HPLC	MERCK

Optimized Chromatographic Conditions:
Instrument used : Waters HPLC with auto sampler and UV detector.

Temperature : Ambient (25° C)
Mode of separation : Isocratic mode
Column : Inertsil ODS, column (150 x 4.6mm, 5µm)
Buffer : Water
Mobile phase : 0.1% OPA: Acetonitrile (80: 20)
Flow rate : 1.0 ml per min
Wavelength : 274 nm
Injection volume : 10 µl
Run time : 10 min.

Preparation of phosphate buffer:
Take 1ml of ortho phosphoric acid in 1000ml volumetric flask and make up with HPLC water and degassed in an ultrasonic water bath for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Preparation of mobile phase:
Accurately measured 800 ml (80%) of above Buffer and 200 ml (20%) of Acetonitrile were mixed and degassed in an ultrasonic water bath for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Diluent Preparation: The Mobile phase was used as the diluent.

Standard Solution Preparation:
Accurately weigh and transfer 20 mg of Brinzolamide and 10 mg of Timolol working standard into a 100 ml clean dry volumetric flask add about 70 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 1.5 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Sample Solution Preparation:
Accurately transfer of the Opthalmic solution equivalent to 20 mg of Brinzolamide and 10 mg of Timolol sample into a 100 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 1.5 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Procedure:
Inject 20µL of the standard, sample into the chromatographic system and measure the areas for Timolol and Brinzolamide peaks and calculate the %Assay by using the formulae.

System suitability:
Tailing factor for the peaks due to Timolol and Brinzolamide in Standard solution should not be more than 2.0. Theoretical plates for the Timolol and Brinzolamide peaks in Standard solution should not be less than 2000.
Resolution for the Timolol and Brinzolamide peaks in standard solution should not be less than 2.

Calculation:

$$\%\ Assay = \frac{AT}{AS} * \frac{WS}{DS} * \frac{DT}{WT} * \frac{Average\ weight}{Label\ Claim} * \frac{P}{100} * 100$$

Where:
AT = average area counts of sample preparation.
AS = average area counts of standard preparation.
WS = Weight of working standard taken in mg.
P = Percentage purity of working standard
LC = Label Claim mg/ml.

Table 3: Chemicals used

S. No.	Samples
1	Timolol & Brinzolamide Opthalmic Solution 1% w/v &0.5%w/v respectively
2	Timolol & Brinzolamide

Preparation of stock solution:
Accurately weigh and transfer 20 mg of Brinzolamide and 10 mg of Timolol working standard into a 100 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Preparation of Level – I:
0.5 ml of above stock solutions has taken in 10ml of volumetric flask, dilute up to the mark with diluent.

Preparation of Level – II:
1ml of above stock solutions has taken in 10ml of volumetric flask, dilute up to the mark with diluent.

Preparation of Level – III:
1.5 ml of above stock solutions has taken in 10ml of volumetric flask, dilute up to the mark with diluent.

Preparation of Level – IV:
2ml of above stock solutions has taken in 10ml of volumetric flask, dilute up to the mark with diluent

Preparation of Level – V:
2.5ml of above stock solutions has taken in 10ml of volumetric flask, dilute up to the mark with diluent

Procedure:
Inject each level into the chromatographic system and measure the peak area. Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient.

Acceptance Criteria: Correlation coefficient should be not less than 0.999.

Precision:

Preparation of stock solution:
Accurately weigh and transfer 20 mg of Brinzolamide and 10 mg of Timolol working standard into a 100 ml clean dry volumetric flask add about 70 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution). Further pipette 1.5 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Procedure:
The standard solution was injected for six times and measured the area for all six. Injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

Acceptance Criteria: The % RSD for the area of six standard injections results should not be more than 2%**.**

Intermediate Precision/Ruggedness:
To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different day.

Preparation of stock solution:
Accurately weigh and transfer 20 mg of Brinzolamide and 10 mg of Timolol working standard into a 100 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 1.5 ml of the

above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Procedure:

The standard solutions prepared in the precision was injected on the other day, for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

Acceptance Criteria: The % RSD for the area of six standard injections results should not be more than 2%.

Specificity:

For Specificity Blank and Standard are injected into system. There is no any interference of any peak in blank with the retention time of the analytical peaks.

Accuracy:

Preparation of Standard stock solution:

Accurately weigh and transfer 20 mg of Brinzolamide and 10 mg of Timolol working standard into a 100 ml clean dry volumetric flask add about 70 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 1.5 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Preparation Sample solutions:

For preparation of 50% solution (With respect to target Assay concentration):

Accurately weigh and transfer 10 mg of Brinzolamide and 5 mg of Timolol working standard into a 100 ml clean dry volumetric flask add about 70 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 1.5 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

For preparation of 100% solution (With respect to target Assay concentration):

Accurately weigh and transfer 20 mg of Brinzolamide and 10 mg of Timolol working standard into a 100 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 1.5 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

For preparation of 150% solution (With respect to target Assay concentration):

Accurately weigh and transfer 30 mg of Brinzolamide and 15 mg of Timolol working standard into a 100 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 1.5 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Procedure:

Inject the standard solution, Accuracy -50%, Accuracy -100% and Accuracy -150% solutions. Calculate the Amount found and Amount added for Timolol & Brinzolamide and calculate the individual recovery and mean recovery values. **Acceptance Criteria:** The % Recovery for each level should be between 98.0 to 102.0%

Detection Limit

Limit of Detection: (for Brinzolamide)

Preparation of 0.15 µg/ml solution:

Accurately weigh and transfer 20 mg of Brinzolamide working standard into a 100 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 1.5 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent. Further pipette 1ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent. Further pipette 0.1ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Calculation of S/N Ratio:

Average Baseline Noise obtained from Blank

Signal Obtained from LOD solution

S/N =160/52 = 3.08

Acceptance Criteria:

S/N Ratio value shall be 3 for LOD solution.

Limit of quantification:

Preparation of 0.50 µg/ml solution:

Accurately weigh and transfer 20 mg of Brinzolamide working standard into a 100 ml clean dry volumetric flask add about 70 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 1.5 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent. Further pipette 1 ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent. Further pipette 0.33 ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Calculation of S/N Ratio:

Average Baseline Noise obtained from Blank

Signal Obtained from LOQ solution

S/N = 525/52 = 10.10

Acceptance Criteria:

S/N Ratio value shall be 10 for LOQ solution.

Limit of Detection: (for Timolol)

Preparation of 0.11 µg/ml solution:

Accurately weigh and transfer 10 mg of Timolol working standard into a 100 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 1.5 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent. Further pipette 1 ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent. Further pipette 0.15 ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent

Calculation of S/N Ratio:

Average Baseline Noise obtained from Blank

Signal Obtained from LOD solution

S/N = 156/52 = 3.00

Acceptance Criteria:

S/N Ratio value shall be 3 for LOD solution.

Limit of quantification:

Preparation of 0.36 µg/ml solution:

Accurately weigh and transfer 10 mg of Timolol working standard into a 100 ml clean dry volumetric flask add about 70 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 1.5 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent. Further

pipette 1 ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent. Further pipette 0.48 ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Calculation of S/N Ratio:

Average Baseline Noise obtained from Blank

Signal Obtained from LOQ solution

S/N = 521/52 = 10.02

Acceptance Criteria:

S/N Ratio value shall be 10 for LOQ solution.

Procedure for LOD and LOQ:

The LOD and LOQ solutions was prepared injected, for three times and measured the area for all three injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

Robustness:

As part of the Robustness, deliberate change in the Flow rate, Mobile Phase composition, Temperature Variation was made to evaluate the impact on the method.

A. The flow rate was varied at 0.9 ml/min to 1.1 ml/min

Standard solution 30 ppm of Brinzolamide & 15 ppm of Timolol was prepared and analysed using the varied flow rates along with method flow rate. On evaluation of the above results, it can be concluded that the variation in flow rate affected the method significantly. Hence it indicates that the method is robust even by change in the flow rate ±10%.

B. The Organic composition in the Mobile phase was varied from ±10%: Standard solution 30 ppm of Brinzolamide & 15 ppm of Timolol was prepared and analysed using the varied Mobile phase composition along with the actual mobile phase composition in the method. On evaluation of the above results, it can be concluded that the variation in 10%. Organic composition in the mobile phase affected the method significantly. Hence it indicates that the method is robust even by change in the Mobile phase ±10

Degradation studies:

The International Conference on Harmonization (ICH) guideline entitled stability testing of new drug substances and products requires that stress testing be carried out to elucidate the inherent stability characteristics of the active substance. The aim of this work was to perform the stress degradation studies on the Brinzolamide and Timolol using the proposed method.

Preparation of stock:

Accurately weigh and transfer 20 mg of Brinzolamide and 10 mg of Timolol working standard into a 100 ml clean dry volumetric flask add about 70 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Hydrolytic degradation under acidic condition: Pipette 3 ml of above solution into a 10ml volumetric flask and 3 ml of 0.1N HCl was added. Then, the volumetric flask was kept at 60ºC for 24 hours and then neutralized with 0.1 N NaOH and make up to 10ml with diluent. Filter the solution with 0.44 microns syringe filters and place in vials.

Hydrolytic degradation under alkaline condition

Pipette 3 ml of above solution into a 10ml volumetric and add 3ml of 0.1N NaOH was added in 10ml of volumetric flask. Then, the volumetric flask was kept at 60ºC for 24 hours and then neutralized with 0.1N HCl and make up to 10ml with diluent. Filter the solution with 0.44 microns syringe filters and place in vials.

Thermal induced degradation

Timolol and Brinzolamide sample was taken in petridish and kept in Hot air oven at 110^0 C for 3 hours. Then the sample was taken and diluted with diluents and injected into HPLC and analyzed.

Oxidative degradation

Pipette 3 ml above stock solution into a 10ml volumetric flask and 1ml of 30% w/v of hydrogen peroxide added in 10 ml of volumetric flask and the volume was made up to the mark with diluent. The volumetric flask was then kept at room temperature for 15 min. Filter the solution with 0.45 microns syringe filters and place in vials.

Photo degradation:

Pipette 3 ml above stock solution into a 10ml volumetric flask and expose to sunlight for 24hrs and the volume was made up to the mark with diluent. Filter the solution with 0.45 microns syringe filters and place in vials.

3. Results and Discussion

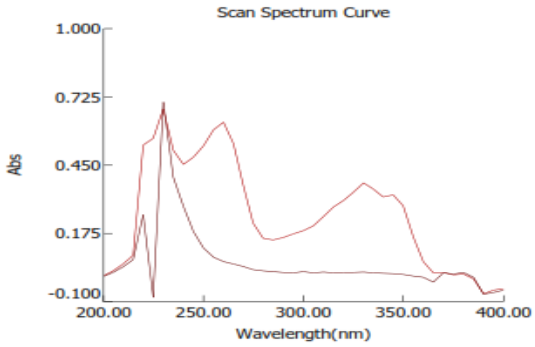

Fig.3: Isobestic Point

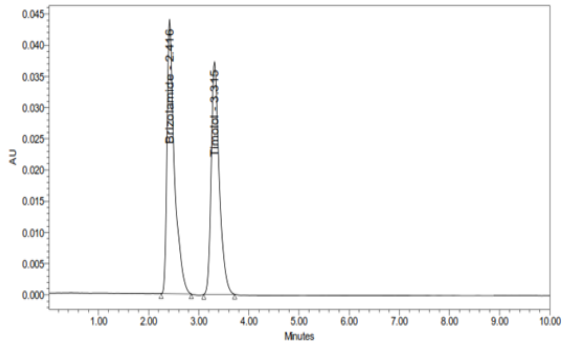

Fig.4: Chromatogram for Standard

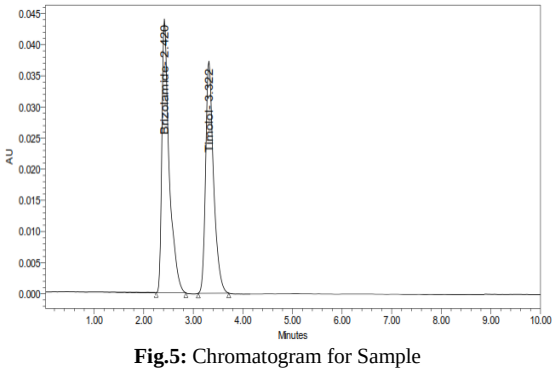

Fig.5: Chromatogram for Sample

Assay Results: (For Brinzolamide)

$$\frac{1607240}{1602701.7} * \frac{50}{100} * \frac{3}{10} * \frac{100}{262} * \frac{10}{3} * \frac{262}{50} * \frac{99.8}{100} * 100 = 100.08\%$$

Assay Results: (For Timolol)

$$\frac{1225797.7}{1224118} * \frac{25}{100} * \frac{3}{10} * \frac{100}{262} * \frac{10}{3} * \frac{262}{25} * \frac{99.8}{100} * 100 = 99.94\%$$

Table 5: Results of Assay for Timolol and Brinzolamide

	Label Claim (mg)	% Assay
Brinzolamide	10	100.08
Timolol	5	99.94

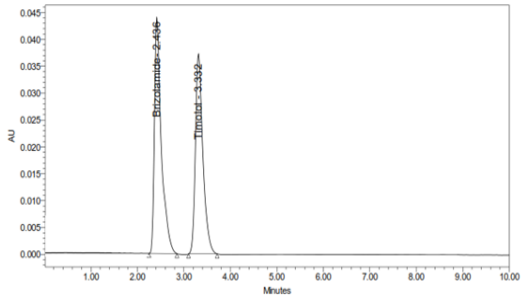

Fig.6: Chromatogram for system suitability

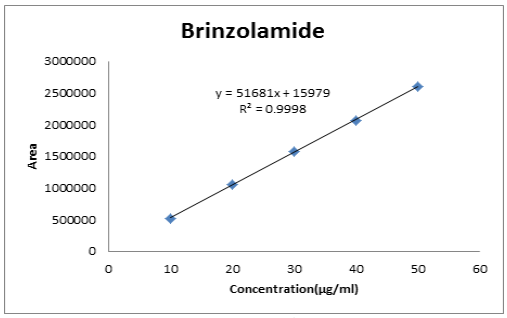

Fig.7: Calibration graph for Brinzolamide

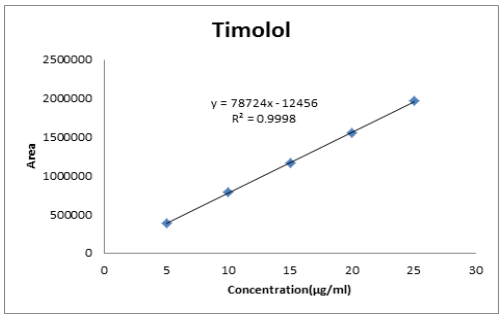

Fig.8: Calibration graph for Timolol

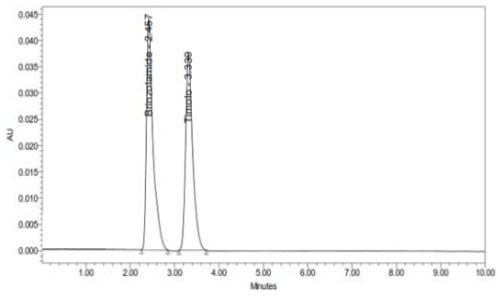

Fig.9: Chromatogram for Precision -6

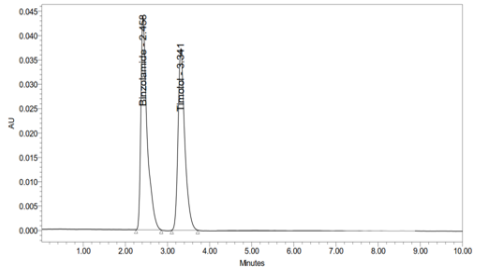

Fig.10: Chromatogram for ID Precision -6

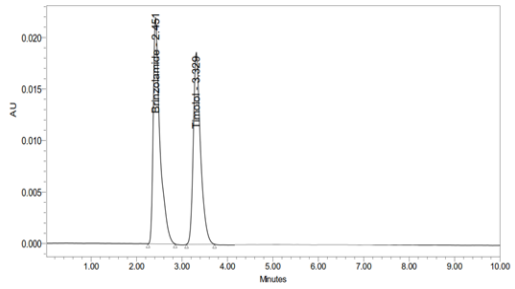

Fig.11: Chromatogram for Accuracy 50%-3

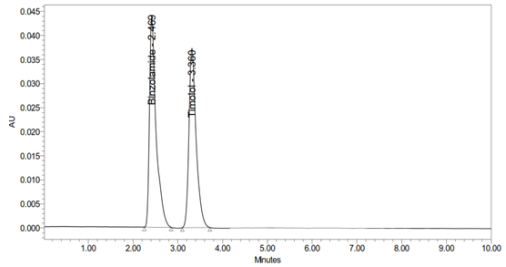

Fig.12: Chromatogram for Accuracy 100%-3

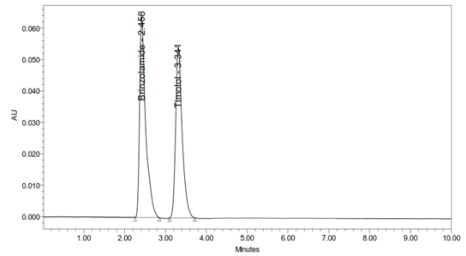

Fig.13: Chromatogram for Accuracy 150%-3

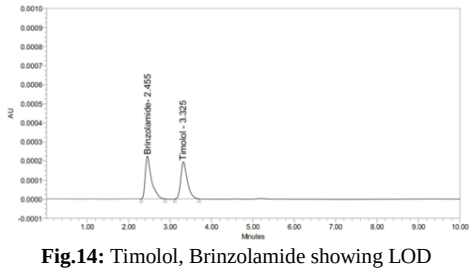

Fig.14: Timolol, Brinzolamide showing LOD

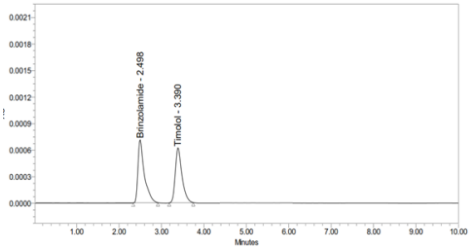

Fig.15: Timolol, Brinzolamide showing LOQ

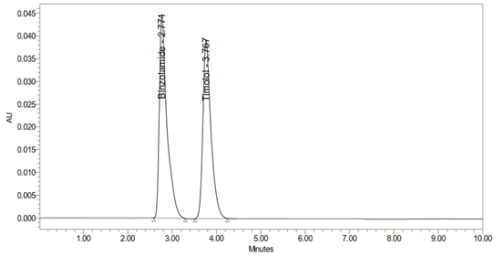

Fig.16: Chromatogram showing less flow

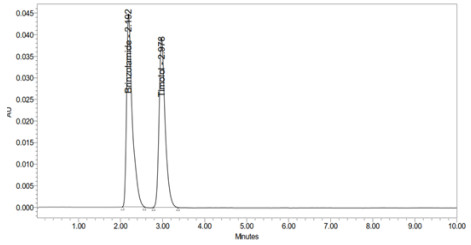

Fig.17: Chromatogram showing more flow

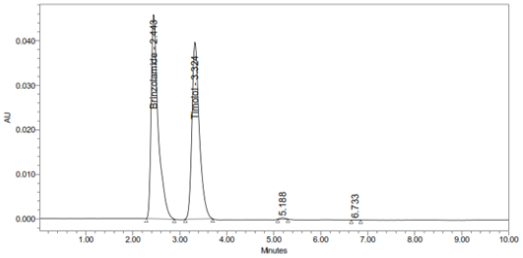

Fig.18: Chromatogram showing Acid degradation

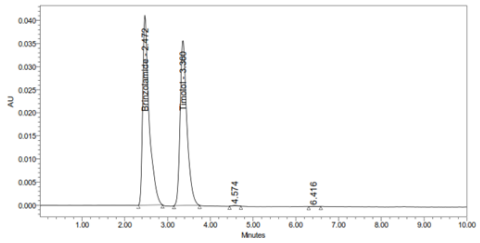

Fig.19: Chromatogram showing Base degradation

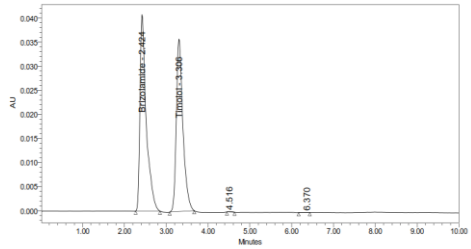

Fig.20: Chromatogram showing Peroxide degradation

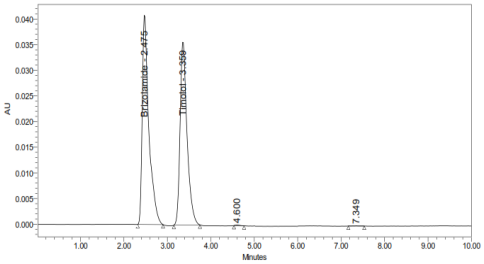

Fig.21: Chromatogram showing Thermal degradation

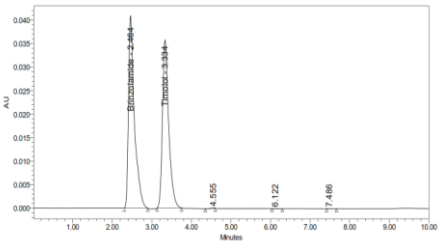

Fig.23: Chromatogram showing Photo degradation

Table 4: Results of Precision for Brinzolamide and Timolol

Injection	Area for Brinzolamide	Area for Timolol
Injection-1	1610934	1228406
Injection-2	1609985	1223300
Injection-3	1619309	1213803
Injection-4	1608645	1201667
Injection-5	1610885	1228897
Injection-6	1618951	1220372
Average	1613118.2	1219407.5
Standard Deviation	4731.4	10327.1
%RSD	0.3	0.8

Table 5: Results of Intermediate precision for Brinzolamide and Timolol

Injection	Area for Timolol	Area for Brinzolamide
Injection-1	1604507	1214125
Injection-2	1594158	1210517
Injection-3	1591505	1212127
Injection-4	1601953	1211539
Injection-5	1598025	1219177
Injection-6	1604821	1203992
Average	1599161.5	1211912.8
Standard Deviation	5538.0	4950.5
%RSD	0.3	0.4

Table 6: Results of LOD

Drug name	Baseline noise (µV)	Signal obtained (µV)	S/N ratio
Brinzolamide	52	160	3.08
Timolol	52	156	3.00

Table 7: Results of LOQ

Drug name	Baseline noise (µV)	Signal obtained (µV)	S/N ratio
Brinzolamide	52	525	10.10
Timolol	51	521	10.02

Table 8: Results for variation in mobile phase composition for Brinzolamide

S.N	Change in Organic Composition in the Mobile Phase	System Suitability Results	
		USP Plate Count	USP Tailing
1	10% less	2569.17	1.39
2	*Actual	2657.20	1.42
3	10% more	2526.40	1.38

4. Conclusion

A new method was established for simultaneous estimation of Brinzolamide and Timolol by RP-HPLC method. The chromatographic conditions were successfully developed for the separation of Brinzolamide and Timolol by using C18 Inertsil ODS (200*4.6) 5um column, flow rate was 1 ml/min, mobile phase ratio was OPA (Orthophosphoric Acid) (0.1%) (80:20%v/v) ACN (detection wave length was 230nm. The linearity of Brinzolamide and Timolol was found to be linear with a correlation coefficient of 0.999 and 0.999, which shows that the method is capable of producing good sensitivity. The

acceptance criteria of precision is RSD should be not more than 2.0% and the method show precision 0.6 and 0.5 for Brinzolamide and Timolol which shows that the method is precise. The acceptance criteria of intermediate precision is RSD should be not more than2.0% and the method show precision 0.6 and 0.2 for Brinzolamide and Timolol which shows that the method is repeatable when performed in different days also. The acceptance criterion for LOD and LOQ is 3 and 10.The LOD and LOQ for Brinzolamide was found to be 2.98 and 10.00 and LOD and LOQ for Timolol was found to be 3.00 and 9.98. The robustness limit for mobile phase variation and flow rate variation are well within the limit, the % degradation results are in limits which shows that the method is having good system suitability and precision under given set of conditions. Compared to previous results the present method which was developed on Brinzolamide and Timolol there run time is less with the less concentration used. We recommend this method for the routine analysis of these drugs.

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