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Research Paper

Development and Validation of RP-HPLC Methods for the Simultaneous Estimation of Brinzolamide, Brimonidine and Timolol in Combined Dosage Form

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ARTICLE DETAILS

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ABSTRACT

A precise and robust method was developed for the estimation of Brinzolamide (BRINZO), Brimonidine (BRIMO), and Timolol (TIMO) in bulk and formulations by RP-HPLC technique. The Method used Systronics LC138 model HPLC with UV multiwavelength detector and C18column with dimension 250 x 4.6 mm, 5 µm. The mobile phase combination used was methanol: acetonitrile: water in ratio of 50: 30 :20. Flow rate at 1.0 ml/min and wavelength at 231 nm with run time of 15 minutes. The retention time of BRINZO, BRIMO and TIMO peaks was at 3.212, 2.858 and 4.288 minutes respectively. The method was validated as per ICH guidelines. The instrument precision for BRINZO, BRIMO and TIMO had a %RSD of 0.4538%, 0.3787% and 0.4624% respectively. Method was linear and accurate for concentration rangefor Brinzolamide 10-50 µg/mL,Brimonidine 1-5 µg/mL and Timolol 10-30 µg/mL in mobile phase with regression coefficient of 0.9966, 0.9969 and 0.9983, respectively and % RSD for accuracy for BRINZO at 80%, 100% and 120% was found to be 99.75%, 98.60% and 99.75% respectively; for BRIMO at 80%, 100% and 120% was found to be 99.50%, 99.10% and 99.66% respectively; and for TIMO at 80%, 100% and 120% was found to be 99.25%, 99.20% and 99.16% respectively.

1. Introduction

Brinzolamide, chemically known as (4R)-4-(ethylamino)-3,4-dihydro-2-(3-methoxypropyl)-2H-thieno[3,2-e]-1,2-thiazine-6-sulfonamide 1,1-dioxide, is a carbonic anhydrase inhibitor primarily used in the management of glaucoma and ocular hypertension. Its therapeutic action centers on reducing intraocular pressure by inhibiting the enzyme carbonic anhydrase II, located in the ciliary processes of the eye. This enzyme plays a crucial role in the production of aqueous humor, the fluid within the eye. By inhibiting carbonic anhydrase II, Brinzolamide effectively decreases the rate of aqueous humor formation, leading to a reduction in intraocular pressure[1,2]. Brimonidine, with the chemical name 5-bromo-6-(2-imidazolidin-2-ylamino)quinoxaline tartrate, is an alpha-2 adrenergic agonist used to lower intraocular pressure in the treatment of open-angle glaucoma and ocular hypertension. Its mechanism of action involves activating alpha-2 adrenergic receptors in the ciliary body, leading to a dual effect: a reduction in aqueous humor production and an increase in uveoscleral outflow. This dual mechanism contributes to a significant decrease in intraocular pressure[3,4]. Timolol, chemically known as (S)-1-[(1,1-dimethylethyl)amino]-3-[4-(4-morpholinyl)-1,2,5-thiadiazol-3-yl]oxy]-2-propanol, is a beta-adrenergic blocker used to reduce intraocular pressure in the treatment of open-angle glaucoma and ocular hypertension. It exerts its therapeutic effect by blocking beta-adrenergic receptors in the ciliary epithelium, which leads to a decrease in aqueous humor production. This reduction in aqueous humor production results in a lowering of intraocular pressure[5,6].

Brimonidine, Brinzolamide, and Timolol are commonly prescribed in combination for the effective management of ocular hypertension and glaucoma. This therapeutic strategy leverages the synergistic action of these three drugs to control

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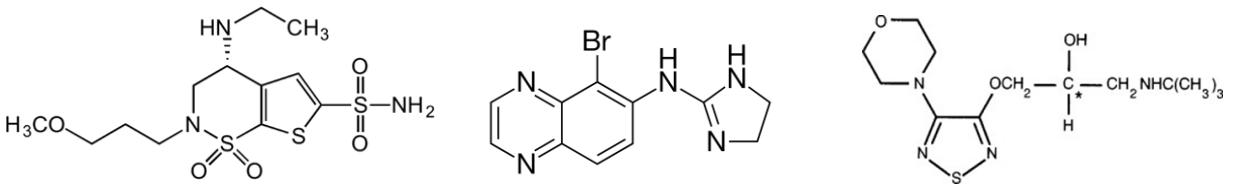
intraocular pressure. As combined dosage forms become increasingly vital in ophthalmic treatment, the necessity for accurate and reliable analytical methods to quantify these active pharmaceutical ingredients (APIs) is paramount. This project centers on the development and validation of RP-HPLC methods for the simultaneous estimation of Brimonidine, Brinzolamide, and Timolol in combined dosage form. Reverse-phase high-performance liquid chromatography (RP-HPLC) is chosen for its established efficacy in separating and quantifying pharmaceutical compounds, offering high resolution, sensitivity, and accuracy.[2,4,6,7,8].

Literature review suggests few HPLC determinations were performed.[9-21] The aim of the present study is to develop a simple, precise, accurate, sensitive HPLC method for the determination. In the present work we developed a simple, reliable and precise RP-HPLC method for analysis of Brimonidine, Brinzolamide, and Timolol. The developed method can be successfully used for analysis of these drugs.



Brinzolamide **Brimonidine** **Timolol**

Fig 1: Chemical structures of Brinzolamide (BRINZO), Brimonidine (BRIMO), and Timolol (TIMO).

2. Material and methods

2.1 Chemicals and reagents

Micro Labs Pvt. Ltd. provided a complimentary sample of Brinzolamide, Brimonidine and Timolol. Methanol and Acetonitrile was purchased from Merck in India and was of HPLC grade. Internal Milli-Q system provided water. All weighing was done using calibrated NABL scales. Samples were produced in Type A glassware and the analytical balance.

2.2 Apparatus

The chromatographic system (Systronics Corporation, India) consisted of LC-138 at prominence triplesolvent delivery module, a manual rheodyne injector with a 20 µL fixed loop and a UV-visible detector. The separation was performed on a Kromstar™ RP-Vertex C18 column (5 µm, 4.6mm* 250 mm) at an ambient temperature. Chromatographic data were recorded and processed using Clarify 2.0 software a Fast clean ultrasonicate cleaner (India) was used for degassing the mobile phase. Shimadzu UV 1800 double beam UV visible spectrophotometer and Sansui-vibra DJ-150S-S electronic balance were used for Spectrophotometric and weighing purposesrespectively.

2.3 Chromatography Conditions

Chromatographic separations of active substances were obtained by using Kromstar™ RP-Vertex C18 column (5 µm, 4.6mm* 250 mm). Mobile phase Methanol: Acetonitrile: Water (50:30:20 v/v) (PH 5.0 was adjusted with sodium acetate buffer) was prepared, filtered through a 0.2 µm nylon filter and degassed for 5 min in an ultrasonicator. The mobile phase was pumped through the column at flow rate of1.0 mL/min. Analyses were carried out at ambient temperature with detection at 231 nm. The injection volume was 20µL and each analysis required around 14 min.

2.4 Standard Solutions

Stock standard solutions of BRINZO 1mg/mL, BRIMO 1mg/mL and TIMO 1mg/mL were prepared by dissolving 10 mg BRINZO, 10mg BRIMO and 10 mg TIMO in methanol. Working standard solutions of BRINZO 40 µg/mL, BRIMO 4µg/mL and TIMO 20 µg/mL were prepared by diluting suitable aliquots of corresponding stock solutions with mobile phase.

2.5 Sample Solution

The eyedrop quantity equivalent to BRINZO (40 mg), BRIMO (4 mg) and TIMO (20 mg) was measured accurately,then it was transferred to a 100 mL volumetric flask containing methanol (50mL). Then the content was ultrasonicated for 20 min. and volume was made up to the mark using methanol. The above solution was filtered through Whatmann filter paper No.1. This solution was again filtered through 0.45µm millipore membrane filter. From this solution (1mL) was diluted to 10mLusing mobile phase to get BRINZO (40 µg/mL), BRIMO (4µg/mL), TIMO (20 mg) solution. The content was ultrasonicated for 20 min.

2.6 Selection of Detection wavelength

All the three concentrations of given samples are scanned separately. Overly spectra clearly denote optimum wavelength at 231nm with all selected analyte with possible maximum absorbance as shown in figure 2.

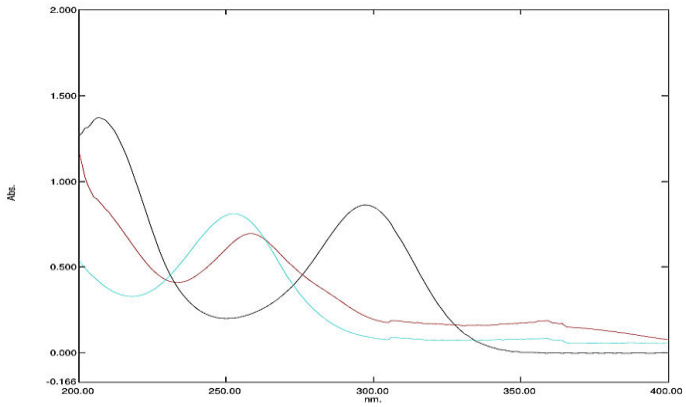


Fig 2. Overly spectra of drugs

3. Validation of Proposed Method

3.1 Calibration curve (linearity)

Accurately measured aliquots of working standard solutions equivalent to Brinzolamide 10-50 µg/mL, Brimonidine 1-5 µg/mL and Timolol 10-30 µg/mL were transferred to series of 10 mL volumetric flasks and the contents of the flasks were diluted to volume with mobile phase. A 20 µL aliquot of each solution was injected in triplicate into the liquid chromatography. The conditions including the flow rate of mobile phase at 1.0 mL/min, detection at 231 nm and run time program for 12 min, were adjusted. A calibration curve for each drug was obtained by plotting area under the peak versus concentration. The graphs of area vs concentration were recorded for all the drugs and are shown in (Figure 4, 5 and 6).

3.2 Accuracy (% recovery)

Recovery studies were carried out by adding a known number of pure drugs BRINZO, BRIMO and TIMO to a pre analyzed sample solution. These studies were carried out by spiking 80%, 100% and 120% respective drug.

3.3 Method precision (repeatability)

The precision of the developed method was assessed in terms of repeatability, intraday and inter-day precision by analyzing six replicate standard samples. The standard deviation values of the results corresponding to the peak area and retention time were expressed for intra-day precision and on 3 days for inter-day precision.

3.4 Intermediate precision (reproducibility)

The intra-day and inter-day precisions of the proposed method were determined by estimating the corresponding responses 5 times on the same day and on 5 different days for present method. The results are reported in terms of relative standard deviation (RSD).

3.5 Limit of detection (LOD) and limit of quantitation (LOQ)

LOD and LOQ of the drug were calculated using the equations according to International Conference on Harmonization (ICH) guidelines.

3.6 Robustness

Robustness of the method was determined by making slight changes in chromatographic conditions, theeffect of % of methanol (49, 50 and 51%) in mobile phase on the retention time and slight changes in flow rate were applied as variable parameters. Flow rate varied at three levels (0.9, 1, 1.1). One factor at the time was changed to estimate the effect.Thus standard solution at varied pH (pH 4.9, 5 and 5.1) three pH levels was performed.

3.7 Specificity

Specificity is the ability of the analytical method to measure analyte response in presence of interferences including degradation products and related substances. Specificity was checked by determining BRINZO, BRIMO and TIMO in laboratory prepared triple mixture and in triple mixture containing different degradation products.

3.8 System suitability Test (SST)

In the system suitability test tertiary solution of BRINZO (40µg/ml), BRIMO (4µg/ml) and TIMO (20µg/ml) (n=5) was prepared and injected. Then the system suitability parameters like retention time, theoretical plates, tailing factor and resolution were calculated from the chromatogram.

3.9 Analysis of Brinzolamide, Brimonidine and Timolol in Combined Opthalmic Dosage Form

Eye drops containing BRINZO(10 mg), BRIMO (2 mg) and TIMO (5 mg) of the brand Trisopt from Micro Labs. Ltd. India, were purchased from the local pharmacy. The responses of the opthalmic dosage form were measured at 231 nm for quantification of Brinzolamide, Brimonidine and Timolol by using LC method above. The amounts of Brinzolamide,

Brimonidine and Timololpresent in sample solutions were determined by adjusting the responses into the regression equations for BRINZO, BRIMO and TIMO.

4. Results and discussion

The absorption spectra of Brinzolamide, Brimonidine and Timolol greatly overlap so conventional determination of these compounds in mixture is not possible. To optimize the LC parameters, several mobile phase compositions were tried. A satisfactory separation and good peak symmetry for Brinzolamide, Brimonidine and Timolol were obtained with a mobile phase consisting of Methanol: Acetonitrile: Water (50:30:20 v/v), pH 4.9 adjusted using Ortho-phosphoric acid buffer. Quantification of the drugs was performed at 231 nm. Resolution of the components with clear baseline separation was obtained.

4.1 Validation of the Proposed Method[22]

Linearity

Linear correlation was obtained between peak areas and concentrations of Brinzolamide, Brimonidine and Timolol in range of Brinzolamide 10-50 µg/mL, Brimonidine 1-5 µg/mL and Timolol 10-30 µg/mL respectively. The linearity of calibration curves was found to be acceptable over the concentration ranges of for BRINZO, BRIMO and TIMO with a R^2 0.9966, 0.9969 and 0.9983 values respectively (Table 1, Figure 4, 5 and 6). The results show that good correlation existed between the peak area and concentration of the analysts.

Accuracy

The recovery experiments were performed by the standard addition method. The recoveries obtained were 98.60%,99.10% and 99.20% for BRINZO, BRIMO and TIMO respectively (Table 2). The high values indicate that the method was accurate. The recovery studies showed that the results were within acceptable limits, above 99.5% and below 100.5%.

Method precision

Precision study was carried out using parameter like method repeatability study which showed that results were within acceptable limit 99.79%, 99.87% and 99.68%with the % RSD below 2.0 indicating that the method is reproducible. The results are shown in (Table No.3)

Intermediate precision

The intra-day standard deviation values for BRINZO, BRIMO and TIMO were 0.7674, 0.4703 and 0.2222 %, respectively. The inter-day RSD values for BRINZO, BRIMO and TIMO were 0.5296, 0.5317 and 0.5154 % respectively. The % RSD (< 2%) values indicate that the method was sufficiently precise (Table 2).

LOD and LOQ

LOD values for BRINZO, BRIMO and TIMO were found to be 3.50508957 µg/mL, 0.333963256 µg/mL and 1.23303µg/mL respectively. LOQ values for BRINZO, BRIMO and TIMO were found to be 10.62148354 µg/mL, 1.012009867 µg/mL and 3.73645 µg/mL respectively (Table 1). These data showed that the method was sensitive enough for the determination of BRINZO, BRIMO and TIMO.

Specificity

Specificity is the ability of the analytical method to measure analyte response in presence of interferences including degradation products and related substances. Specificity was checked by determining BRINZO, BRIMO and TIMO in laboratory prepared triple mixture and in triple mixture containing different degradation products (Table 1,2 and 3).

Robustness

The method was found to be robust with no significant changes on test result upon change of analytical conditions like different flow rate, amount methanol in mobile phase and pH of mobile phase with the standard deviation was found to be Below 1 and % RSD is less than 2 for all results. It was found that under small deliberate changes of chromatographic factors, there was no considerable change in under study parameters (Table 4).

System Suitability Test

A sample solution of BRINZO (40µg/ml), BRIMO (4µg/ml) and TIMO (20µg/ml) (n=5) was prepared and same was injected, then the system suitability parameters were calculated from the chromatogram. The parameters, retention times, resolution factor, tailing factor and theoretical plates were evaluated. The results (Table 6) obtained from system suitability tests are in agreement with the official requirements.

5.Conclusion

The developed RP-HPLC method offers a straightforward, accurate, and practical approach for the simultaneous separation and quantification of Brinzolamide, Brimonidine, and Timolol within a combined formulation. Its ease of use and precision make it a valuable tool for routine analysis of these active pharmaceutical ingredients in their dosage forms. Furthermore, the method's ability to assess the stability of these compounds in the presence of potential degradation

products establishes it as a robust stability-indicating assay. Consequently, this validated method is well-suited for reliable quality control analysis of Brinzolamide, Brimonidine, and Timolol in standard laboratory.

Table 1. Regression analysis of the calibration curves for Brinzolamide, Brimonidine and Timolol in the proposed HPLC Method

Parameter	Brinzolamide	Brimonidine	Timolol
Linearity Range (µg/mL)	10-50	1-5	10-30
Detection Wavelength (nm)		231	
Slope ± SD	29.108	21.355	9.4258
Intercept ± SD	14.11	0.379	4.033
Correlation coefficientR^2	0.9966	0.9969	0.9983

SD- Standard deviation

Table 2. Summary of the validation parameters for the proposed HPLC method

Parameter	Brinzolamide	Brimonidine	Timolol
LOD	3.50508957	0.333963256	1.23303
LOQ	10.62148354	1.012009867	3.73645
Accuracy	99.60	99.10	99.20
Repeatability (%RSD, n = 5)	0.213	0.258	0.561
Precision (%RSD)	0.4538	0.3787	0.4624
Inter-day, n =5	99.57 (0.5296)	99.07 (0.5317)	99.14 (0.5154)
Intra-day, n = 5	99.52 (0.7674)	99.04 (0.4703)	99.10 (0.2222)

LOD = Limit of detection; LOQ = Limit of quantification; RSD = Relative standard deviation.

Table 3. Results of Precision Study Repeatability study of Brinzolamide, Brimonidine and Timolol

Replicate	Brinzolamide	Brimonidine	Timolol
Mean Peak Area	1204.39	83.89	196.42
S.D	0.2548	0.8954	0.4542
% RSD	0.8562	0.2895	0.6895

**Mean of six Observations*

Table 4. Results of Robustness Study

Factor Level	Retention Time			Theoretical Plates		
Flow Rate (mL/min)	BRINZO	BRIMO	TIMO	BRINZO	BRIMO	TIMO
Mean ± S.D	3.201 ±0.0199	2.843 ±0.0262	4.2626 ± 0.0438	2819.66 ± 0.5773	7707.33 ± 1.5270	14258.33 ±1.1547
	% of Methanol in the Mobile Phase (v/v)					
Mean ± S.D	3.201 ±0.0199	2.843 ± 0.0268	4.2645 ± 0.0424	2819.66 ± 1.5275	7706.66 ± 0.5773	14259.33 ±1.5275
	pH of Mobile Phase					
Mean ± S.D	3.200667 ± 0.0213	2.845 ±0.0225	4.2633 ± 0.0427	2819.66 ± 1.5275	7706.66 ± 0.5773	14258.67 ± 0.5773

Table 5. Assay results for the combined dosage form using the proposed HPLC method

Formulation	Brinzolamide	Brimonidine	Timolol
Trisopt Eye Drops	99.76 ± 0.0864	97.38 ± 1.2001	99.46 ± 0.2435

SD =Standard deviation, 6 determinations

Table 6. System suitability test parameters for Brinzolamide, Brimonidine and Timolol for the proposed HPLC method

System Suitability Parameters	Brinzolamide	Brimonidine	Timolol
Retention Time (tR)	3.212	2.858	4.288
Capacity Factor (k)	3.288	1.858	2.212
Theoretical Plate Number (N)	2820	7707	14259
Asymmetry Factor	1.221	0.938	0.787
Resolution Factor (R)	4.235	0.000	2.978

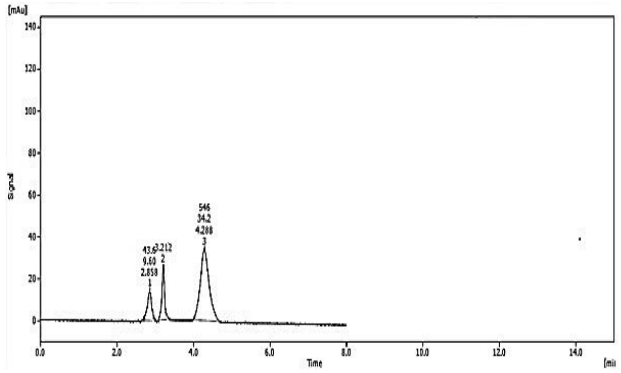


Fig 3. Typical liquid chromatogram obtained for a 20 µL injection of eye drop of Brinzolamide, Brimonidine and Timolol

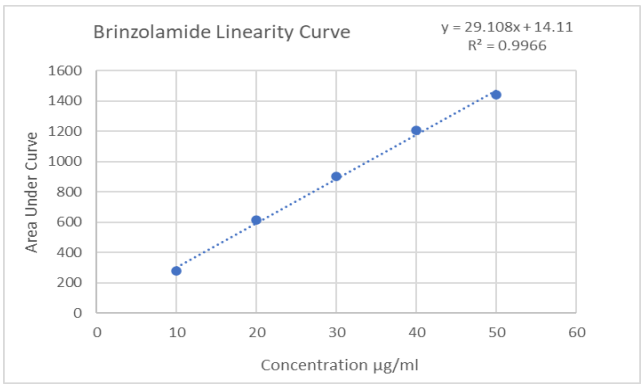


Fig 4. Calibration Curve for Brinzolamide

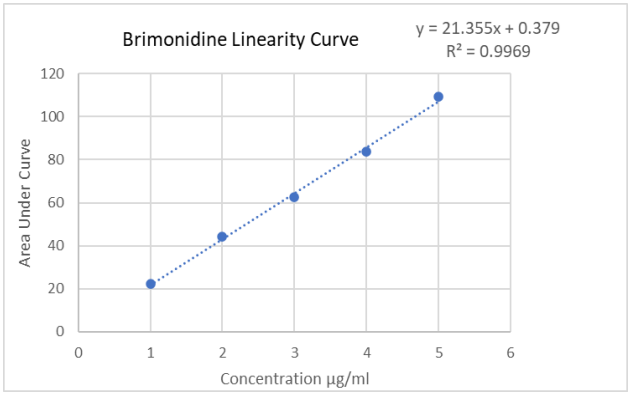


Fig 5. Calibration Curve for Brimonidine

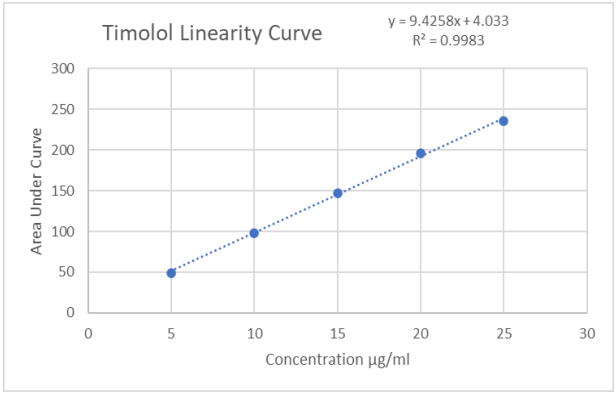


Fig 6. Calibration Curve for Timolol

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