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DEVELOPMENT AND VALIDATION OF ANALYTICAL METHOD FOR SIMULTANEOUS ESTIMATION OF BRINZOLAMIDE AND TIMOLOL BY HPLC FROM OPHTHALMIC PREPARATION

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Keywords:

Brinzolamide, Timolol, RP-HPLC, validation, Ophthalmic Preparation

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ABSTRACT: A fast, sensitive and accurate reverse phase liquid chromatographic method has been developed and validated for the simultaneous determination of Brinzolamide and Timolol maleate from ophthalmic preparation. Chromatographic separation of these two drugs were achieved on Zorbax Eclipse Plus, Agilent Technology (150mm x 4.6mm, 5µm) as stationary phase with a mobile phase comprising of triethylamine phosphate buffer : Acetonitrile : Methanol (70:20:10(v/v) at a flow rate of 1.0ml / min and PDA detection was set at 274 nm. Injection volume was 10 µl. Column oven temperature was maintained at 40°C. The retention time of timolol, and brinzolamide were 4.0 (±0.5) min, and 10.5 (±0.5) min respectively. Theoretical plate for Brinzolamide and Timolol were 5832 and 4876 respectively. The tailing factor was 1.09 and 1.21 for Brinzolamide and Timolol respectively. Resolution between the two compounds was 16.12. The developed method was validated according to the ICH guidelines and the proposed method can be applied for the routine quality control analysis of Brinzolamide and Timolol maleate from combined dosage form.

INTRODUCTION: Brinzolamide is a carbonic anhydrase inhibitor used to lower intraocular pressure in patients with open-angle glaucoma or ocular hypertension by decreasing the amount of fluid produced by the eye. Lowering high pressure inside the eye helps to prevent blindness. Timolol maleate is a non-selective beta-adrenergic receptor antagonist indicated for treating glaucoma, heart attacks and hypertension. In its ophthalmic form, it is used to treat open-angle and occasionally secondary glaucoma by reducing aqueous humour production through blockage of the beta receptors on the ciliary epithelium [1, 2, 3, 4,5, 6, 7].

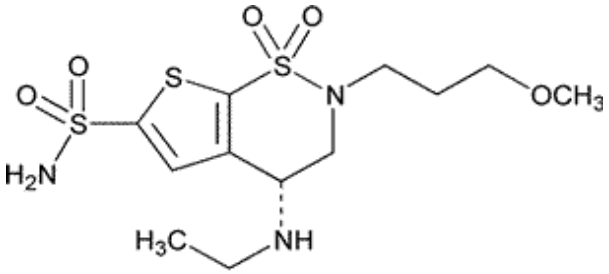

FIGURE 1: CHEMICAL STRUCTURE OF BRINZOLAMIDE

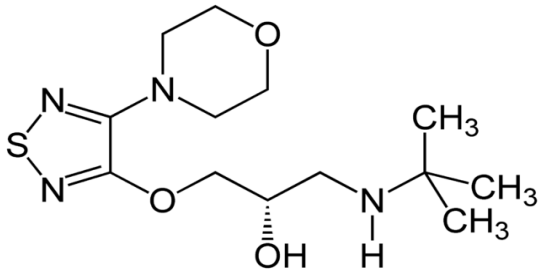

FIGURE 2: CHEMICAL STRUCTURE OF TIMOLOL MELEATE

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Glaucoma encompasses a wide clinical spectrum of disease, with the common pathophysiology of progressive optic neuropathy leading to visual field loss. Elevated intraocular pressure (IOP) is a key risk factor in disease progression. Treatment is aimed at reduction of IOP to minimize continued optic nerve head damage. Pharmacologic treatment with various classes of IOP-lowering medications is generally employed before more aggressive surgical interventions. Monotherapy is generally accepted as initial therapy for glaucoma, but at least half of patients may require more than one IOP-lowering medication. One option is the fixed combination of brinzolamide 1% and timolol maleate 0.5%, which is commercially available in some countries for treatment of glaucoma not adequately responsive to monotherapy. These agents may also be used in an unfixed fashion, but fixed combination therapy is generally more convenient for patients [8].

Literature survey reveals that there is no analytical method available for simultaneous estimation of Brinzolamide and Timolol. The absence of literature provides the need for developing a sensitive, new, economical, precise and accurate method for the simultaneous estimation of Brinzolamide and Timolol from combined ophthalmic preparation.

MATERIALS AND METHODS:

Chemicals and reagents: Working standard of Brinzolamide with a potency of 99.53% was collected from Hanzhou Zhongchang Scientific Co. Ltd, China and Timolol as Timolol maleate with a potency of 99.50 was collected from Sifavitor SPA, Italy. Market sample of Brinzolamide 1% and Timolol 0.5% ophthalmic preparation 'Azarga' were collected from Alcon Laboratories (Australia) Pty Ltd. HPLC grade Acetonitrile and Methanol was purchased from Merck, Darmstadt, Germany, Triethylamine from Scharlab, Spain and Orthophosphoric acid were purchased from Merck, Darmstadt, Germany. HPLC grade water was obtained through Millipore water purification system (Model- Arium 611DI, Sartorious).

HPLC instrumentation and chromatographic condition: High performance liquid chromatographic system consisted of a Shimadzu LC-20 AT, prominence, equipped with an auto sampler (SIL-20AC HT, Shimadzu, Japan) and PDA detector (SPD- M20A, Japan) was used for the analysis. The data was recorded using LC-solution software. A Zorbax Eclipse Plus, Agilent Technology (150mm x 4.6mm, 5µm) column was used for the analysis. A powersonic 505 ultrasonic bath (Hwashin technology, Seoul, Korea) was used for degassing of the mobile phase.

In addition a pH meter (Mettler Toledo, Switzerland) and an electronic balance (Model-CP224S, Sartorious, Germany) were used in the present work.

The separation was carried out using a mobile phase consisting of buffer and organic phase. Triethylamine phosphate buffer was prepared using 4.0 ml of triethylamine in 1000 ml of water, pH was adjusted with phosphoric acid to a pH of 5.0 (±0.5). It was filtered with 0.45µ membrane filters and degassed in an ultrasonic bath for 10 minutes.

Organic phase consists of Acetonitrile: Methanol (20:10 v/v). The ratio of buffer, acetonitrile and methanol was 70:20:10, v/v. The column was maintained at a temperature of 40ºC with column oven (CTO-20AC) and the flow rate was 1.0 ml/min. Analysis was performed with injection volume of 10µl using PDA detection at 274nm.The run time was set for 15 minutes.

The typical retention time of Timolol peak is about 3.9 minutes and Brinzolamide peak is about 10.5 minutes. This is shown in **figure 3, 4 and 5**.

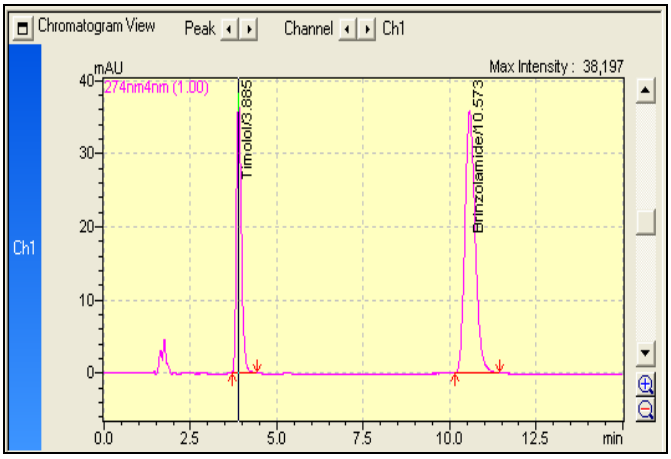

FIGURE 3: CHROMATOGRAM OF TIMOLOL AND BRINZOLAMIDE

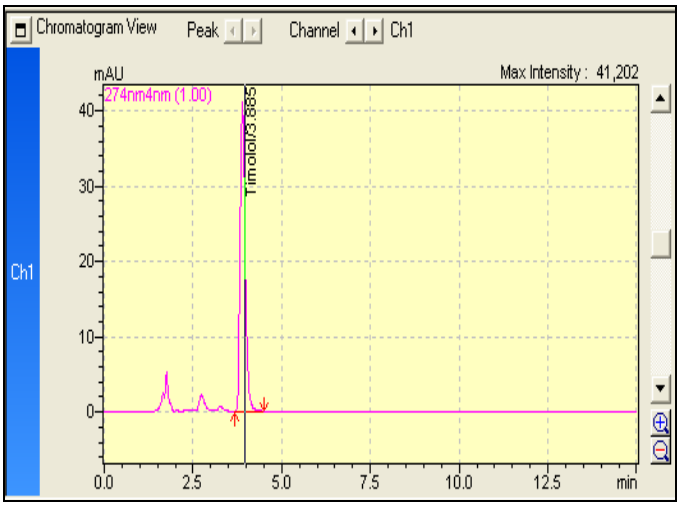

FIGURE 4: CHROMATOGRAM OF TIMOLOL IN SINGLE INJECTION

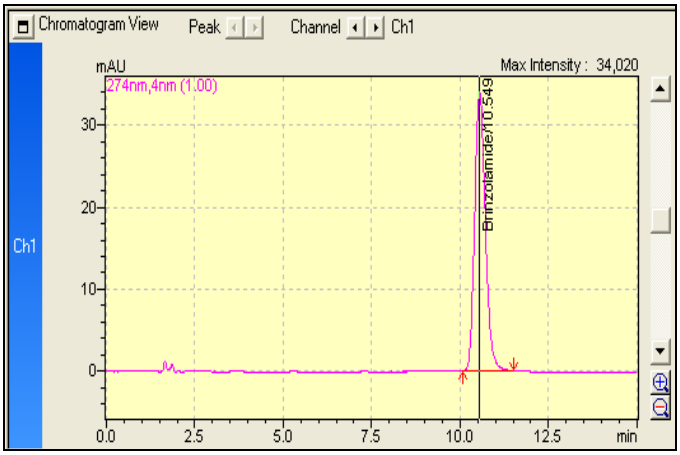

FIGURE 5: CHROMATOGRAM OF BRINZOLAMIDE IN SINGLE INJECTION

Preparation of Standard solution:

Preparation of Brinzolamide Stock solution: Accurately 50 mg of Brinzolamide working standard was taken into a 100 ml volumetric flask. It was diluted with about 25 ml of diluting solution (Buffer: Organic phase, 50:50).

About 60 ml of diluting solution was added to it and sonicated for 15 minutes. Cooled to room temperature and diluted to volume with the same and mixed well.

Preparation of Timolol Stock solution: Accurately 34 mg of Timolol Maleate equivalent to 25 mg Timolol was taken into a 100 ml volumetric flask. It was diluted with about 25 ml of diluting solution. 60 ml of diluting solution was added to it and sonicated for 15 minutes. Cooled to room temperature and diluted to volume with the same and mixed well.

Final standard solution preparation: 10 ml of stock solution Brinzolamide and 10 ml of stock solution Timolol was taken into a dried 50 ml volumetric flask. Volume was adjusted up to the mark with diluting solution. It was filtered through 0.45µ-disc filter.

Preparation of sample solution: Sample bottles were shaken well before use. Contents of 10 bottles were mixed and weight per ml of the sample was determined. 5 ml of ophthalmic suspension were taken into a dried 100 ml volumetric flask. About 60 ml of diluting solution was added to it and sonicated for 15 minutes. Cooled to room temperature and diluted to volume with the same and mix well. This solution was filtered through Whatman 1 filter paper. 10 ml of this solution was diluted to 50 ml with diluting solution and filtered through 0.45µ-disc filter.

Method validation: The present method of analysis was conducted to obtain a new, sensitive, convenient method for simultaneous estimation of Brinzolamide and Timolol by HPLC from ophthalmic preparation**.** The experimental method was validated according to the recommendations of ICH-1996 and USP-30 for the parameters like specificity, system suitability, accuracy, linearity, precision, robustness.

Specificity: The specificity of the method was evaluated to ensure that there is no interference of excipeints, diluting solution in the chromatogram of Brinzolamide and Timolol maleate. The specificity was studied by injecting the placebo, diluting solution and standard solution of Brinzolamide and Timolol maleate. Spectral purities of Brinzolamide and Timolol chromatographic peaks were evaluated for the interference of excipients and shown in the **figure 6 and 7**.

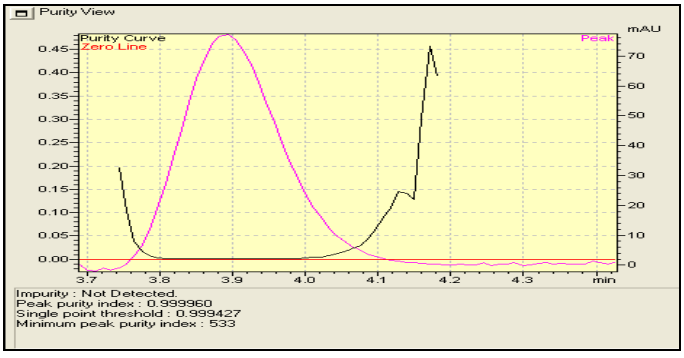

FIGURE 6: PURITY CURVE FOR TIMOLOL

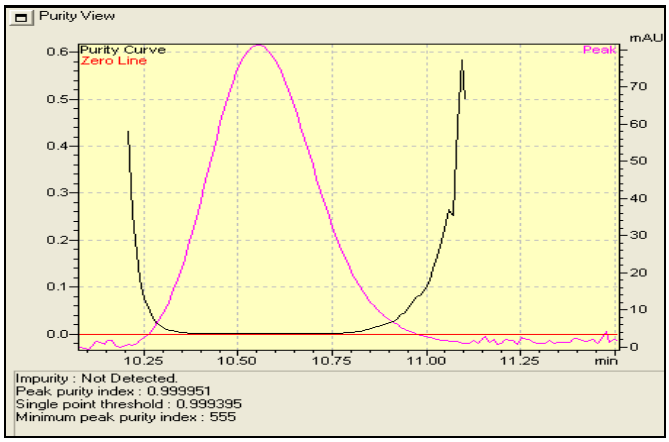

FIGURE 7: PURITY CURVE FOR BRINZOLAMIDE

System suitability: System suitability was performed by injecting six replicates of standards and two replicates of sample preparation at a 100% level to verify the accuracy and precision of the chromatographic system. This method was evaluated by analyzing the repeatability of retention time, tailing factor, theoretical plates of the column.

Linearity: The linearity of the chromatographic method was established by plotting a graph to concentration vs. peak area of Brinzolamide and Timolol maleate standard and determining the correlation coefficients (R^2) of the two compounds. Linearity of Brinzolamide and Timolol maleate standard solution at a concentration level of 40%, 60%, 80%, 100%, 120%, and 140% were injected into the HPLC system. The detector response was found to be linear form 40% to 140% of test concentration for both the standard solutions. Before injection of the solutions, the column was equilibrated for at least 60 minutes with the mobile phase. The linearity curves of Brinzolamide and Timolol are shown in **table 1, 2 and figure 8, 9**.

TABLE 1: LINEARITY OF BRINZOLAMIDE

SI no.	% Test concentration	Concentration(µg/ml)	Average peak area
1	40	40	292584
2	60	60	435975
3	80	80	595265
4	100	100	743619
5	120	120	890582
6	140	140	1036964

TABLE 2: LINEARITY OF TIMOLOL

SI no.	% Test concentration	Concentration(µg/ml)	Average peak area
1	40	20	162358
2	60	30	245463
3	80	40	326120
4	100	50	410689
5	120	60	492968
6	140	70	575162

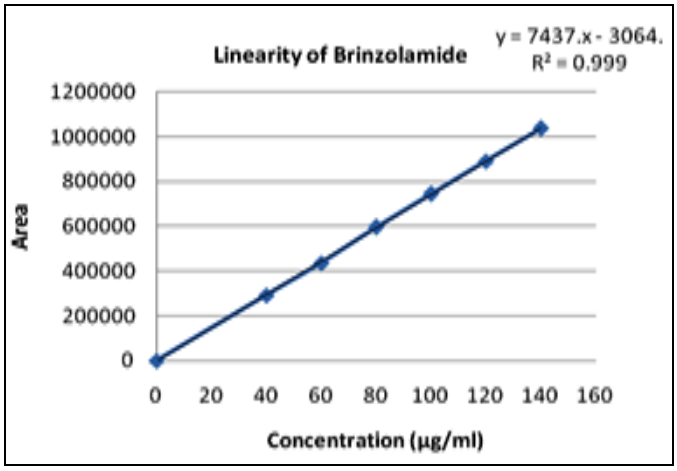

FIGURE 8: LINEARITY CURVE OF BRINZOLAMIDE CONCENTRATION VS AREA

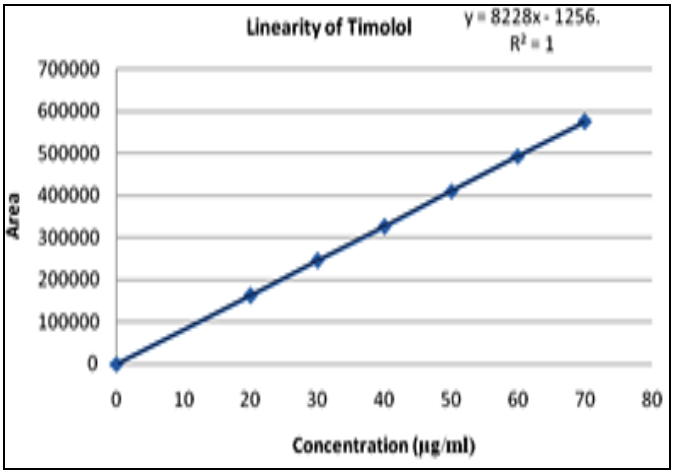

FIGURE 9: LINEARITY CURVE OF TIMOLOL CONCENTRATION VS AREA

Accuracy: The accuracy of the method is the nearness of the result obtained to the true value. Accuracy of the method was determined by recovery experiments. The recovery was performed by adding Brinzolamide and Timolol maleate working standards to placebo (excipients mixture) in the range of test concentration (40%, 60%, 80%, 100%, 120%, and 140%) and expressed as percent (%) recovered. Three samples were prepared for each recovery level. The recovery value for Brinzolamide and Timolol maleate ranged from 99.46 to 100.38% and 99.34 to 100.35%. Results are shown in table 3 and 4.

TABLE 3: RESULTS OF ACCURACY EXPERIMENT USING SPIKED PLACEBO METHOD FOR BRINZOLAMIDE.

Level (%)	Amount of drug spiked (mg)	Found (mg)	Recovery (%) (n=3)
40	39.76	39.68	99.79
60	60.04	59.92	99.8
80	79.98	80.21	100.29
100	100.0	99.46	99.46
120	120.24	119.78	99.62
140	139.80	140.34	100.38
		Average	99.89
	SD		0.3677
	% RSD		0.3681

TABLE 4: RESULTS OF ACCURACY EXPERIMENT USING SPIKED PLACEBO METHOD FOR TIMOLOL.

Level (%)	Amount of drug spiked (mg)	Found (mg)	Recovery (%)(n=3)
40	19.91	19.78	99.34
60	30.12	29.99	99.57
80	39.79	39.63	99.6
100	49.98	50.04	100.12
120	59.97	60.18	100.35
140	69.94	69.62	99.54
		Average	99.7533
	SD		0.3909
	% RSD		0.3918

Stability of Analytical solution: The stability of analytical solutions was established by injecting the standard solution and sample solution at different time intervals up to 24 hours (0, 4, 8, 12, 16, 18 and 24 hours) by keeping the auto sampler temperature at room temperature (25ºC). The response of standard solution and sample solution were measured and % differences of peak area were calculated. The values are presented in the **table 5 and 6**.

TABLE 5: STABILITY OF STANDARD AND SAMPLE SOLUTION OF BRINZOLAMIDE

Time Interval	Standard		Sample	
	Standard peak area	% Difference	Sample peak area	% difference
0 hour	745544	-	744193	-
4 hour	747930	0.32	743532	0.09
8 hour	744567	0.13	743596	0.08
12 hour	743785	0.24	744976	0.11
16 hour	746253	0.10	743243	0.13
18 hour	743389	0.29	744082	0.01
24 hours	744895	0.09	743961	0.03

TABLE 6: STABILITY OF STANDARD AND SAMPLE SOLUTION OF TIMOLOL

Time Interval	Standard		Sample	
	Standard peak area	% Difference	Sample peak area	% difference
0 hour	410824	-	411688	-
4 hour	411209	0.09	412086	0.10
8 hour	410678	0.04	411502	0.05
12 hour	409956	0.21	412098	0.10
16 hour	410724	0.02	412304	0.15
18 hour	410167	0.16	411845	0.04
24 hours	410504	0.08	411372	0.08

Precision: The precision of an analytical method is the degree of agreement among individual test results where the method is applied repeatedly to multiple samplings. Precision of the assay was assessed with respect to repeatability, reproducibility and intermediate precision by estimating the assay for six different sample preparations of same batch. Statistical analysis for repeatability, intermediate precision and reproducibility of Brinzolamide and Timolol are given in the **table 7**.

TABLE 7: STATISTICAL ANALYSIS FOR REPEATABILITY, INTERMEDIATE PRECISION AND REPRODUCIBILITY OF BRINZOLAMIDE AND TIMOLOL

Sample ID	Assay (% labeled amount) of Brinzolamide			Assay (% labeled amount) of Timolol		
	Repeatability (Analyst 1)	Intermediate precision (Analyst 2)	Reproducibility (Analyst 3)	Repeatability (Analyst 1)	Intermediate precision (Analyst 2)	Reproducibility (Analyst 3)
S-1	99.03	99.59	98.32	98.67	99.12	99.92
S-2	99.67	99.26	99.85	99.37	99.42	99.18
S-3	100.2	98.56	99.02	99.45	98.21	98.77
S-4	98.99	98.88	100.53	98.99	98.26	99.31
S-5	98.29	100.31	99.08	98.89	99.82	100.42
S-6	99.31	99.17	99.26	99.23	98.48	98.92
Average	99.248	99.295	99.34	99.100	98.885	99.42
SD	0.651	0.608	0.76	0.301	0.667	0.631
% RSD	0.656	0.612	0.766	0.304	0.675	0.635

Robustness: The robustness is the ability of method to remain unaffected by small changes in parameters. The robustness of the method was determined by purposely altering experimental conditions and % assay of Brinzolamide and Timolol, peak tailing, theoretical plates, % RSD were calculated. To study the effect of flow rate, it was changed to 0.2 units from 1.0 ml/min to 0.8 ml/min and 1.2ml/min. The effect of column temperature was studied at 38ºC and 42ºC instead of 40ºC. The effect of mobile phase ratio change was studied by changing the buffer concentration from 70% to 68% and 72%. Results are shown in **table 8**.

TABLE 8: RESULTS OF ROBUSTNESS STUDY

SI. No.	Parameter	Variation	Assay % of Brinzolamide (n=3)	Assay % of Timolol (n=3)
1.	Flow rate (± 20% of the set flow)	a) at 0.8 ml/min b) at 1.2 ml/min	a) 99.10 b) 99.32	a) 99.28 b) 99.75
2.	Mobile phase ratio (± 2% of the set ratio)	a) at buffer 68% b) at buffer 72%	a) 98.96 b) 99.67	a) 99.88 b) 99.16
3.	Column oven temperature (± 2ºC of set temperature)	a) at 38ºC b) at 42ºC	a) 99.56 b) 99.24	a) 99.66 b) 99.09

RESULTS AND DISCUSSION: The developed method was validated as per ICH guidelines. The method was specific because there was no interference of excipients, diluting solution and impurity in the chromatogram of Brinzolamide and Timolol (purity curve shown in figure 6 and 7). The method showed linearity of detector response and produces linear calibration curve over the range of 20-70µg/ml for Timolol (Figure 9, table 2) and 40-140µg/ml for Brinzolamide (Figure 8, table 1).

Results of accuracy were proven by the table 3 and 4 and % RSD of both the compounds was below 2%., which is within the acceptable limit. The % difference of peak area of Standard solution and Sample solution that were injected at periodic intervals were found to be within the specified limit (Table 5 and 6). Results for precision and robustness evaluation for Brinzolamide and Timolol (Table 7 and 8) were also satisfactory. So, this method is applicable for determination of Brinzolamide and Timolol simultaneously from its ophthalmic preparation.

CONCLUSION: The validation study showed that the developed method was accurate, rapid, precise, reproducible and convenient with acceptable correlation co-efficient and standard deviations which make the proposed RP-HPLC method valuable for simultaneous determination of Brinzolamide and Timolol from ophthalmic preparations. So the developed method can be used conveniently for analysis of quality control, stability and further studies.

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