ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Analytical Method Development and Validation of New RP-HPLC Method for Simultaneous Estimation of Brinzolamide and Timolol Maleate in Ophthalmic Solutions.

B Anusha, A Geetha Susmita*, and G Rajitha.

Institute of Pharmaceutical Technology, Sri Padmavathi Mahila Visvavidyalayam (women's university), Tirupati-517502, Andhra Pradesh, India.

ABSTRACT

A simple, rapid, sensitive, precise, accurate and economical reverse phase liquid chromatographic method was developed and validated for the simultaneous determination of Brinzolamide and Timolol maleate in ophthalmic preparations. Chromatographic separation was acheived on Inertsil ODS C18 column (250 X 4.6 mm, 5 µm particle size) with mobile phase consisting of Sodium dihydrogen phosphate buffer(0.2 M) : methanol (70:30 v/v) pH adjusted to 7.5 with sodium hydroxide solution at a flow rate of 1.0 ml/min and injection volume of 10µl. The analytes were detected at 279 nm using by UV detector. The method validated for accuracy, precision, linearity, LOD, LOQ, robustness and system suitability. The retention time of Brinzolamide and Timolol maleate was found to be 3.577 ± 0.01 and 7.206 ± 0.01 mins respectively. The linearity of the method ranged between and 0.0001 – 0.0018 mg/ml and 0.0001 – 0.0023 mg/ml for Brinzolamide and Timolol maleate respectively with correlation coefficient 0.999 for both the drugs. The pooled % RSD value for repeatability, intermediate precision, accuracy, robustness studies for proposed method was found to be less than 2. The mean percentage recovery in terms of accuracy was found to be in the range of 98.86 to 100.07% for both drugs. The developed method was validated according to the ICH guidelines and can be applied for the routine quality control analysis of Brinzolamide and Timolol maleate combined dosage forms.

Keywords: RP-HPLC, Brinzolamide, Timolol maleate, Eye drops, Method validation, Force degradation studies.

Corresponding author

ISSN: 0975-8585

INTRODUCTION

Brinzolamide[1] (BRZ), [(R)-(+)-4-Ethylamino-2-(3-methoxypropyl)-3, 4-dihydro-2H thieno[3,2-e]-1,2-thiazine-6-sulfonamide-1,1-dioxide] (Fig.1a), is useful for topical use in the treatment of glaucoma. 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[2]. Lowering high pressure inside the eye helps to prevent blindness. Timolol Maleate[2] (TM) (-)-1-(*tert*-butylamino)-3-[(4-morpholino-1,2,5-thiadiazol-3- yl)oxy]-2- propranol maleate (Fig.1b) 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. Now a days, BRZ has been marketed in combination with TM in eye drops for treatment of glaucoma, which have lesser side effects and patient specificity compared to previous eye drops, DORZOX-T (dorzolamide and timolol maleate).

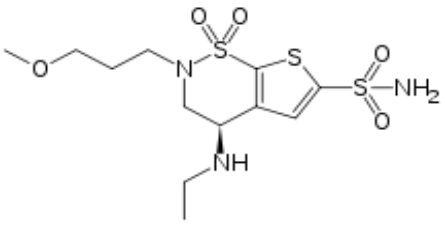


Fig 1a): Structure of Brinzolamide

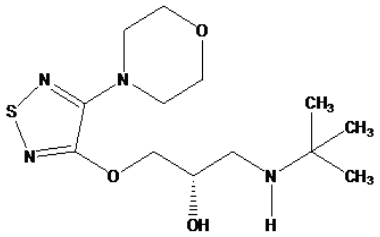


Fig 1b): Structure of Timolol Maleate

Recent literature review reveals that there are only few analytical methods like spectroscopic(11,12), HPLC(2,3) and HPTLC reported for the determination of Timolol Maleate and Brinzolamide individually and combination with other drugs like Dorzolamide and Brimonidine tartarate. A recent analytical method developed for simultaneous estimation of Brinzolamide and Timolol Maleate was found expensive and with high RT values.

To the best of our knowledge, none of the analytical method is available for simultaneous determination of combination of both the drugs in eye drops is economical. Hence, in the present article a simple, accurate, precise, robust and sensitive and economical RP - HPLC method for the simultaneous determination of BRZ and TM in their mixture form was developed and reported.

EXPERIMENTAL

Chemicals

The reference samples of Brinzolamide and Timolol Maleate, Sodium dihydrogen phosphate, HPLC grade methanol, HPLC grade water were obtained from Varma labs pvt. Limited, Vadlapudi, Visakhapatnam

Instrumentation

Chromatographic separation was performed on a Peak chromatographic system equipped with Shimadzu, LC-2010CHT, gradient pump, auto injector with 10µl fixed volume loop, variable wavelength programmable UV detector and SPDM20A. The output signal was monitored and integrated by Peak Chromatographic Software version Lc Solutions. Sonicator (3.5L) and Ultrasonicator were used for sonication of the mobile phase and samples. Standard and sample drugs were weighed by using Sartorius Analytical Balance (CPA225D) and pH of the mobile phase was adjusted by using Metsar (DPH 504) digital pH meter.

Preparation of standard stock solution

Standard stock solution was prepared by accurately weighing about 10.12 mg of Brinzolamide and 5.23 mg of Timolol Maleate pure drug and transferred into 10 ml volumetric flask containing 5ml methanol. The volumetric flasks were sonicated for 5min and then the final volume was made upto 10ml with methanol to get a concentration of 1mg/ml of BRZ and 0.5mg/ml of TM.

To 1.0ml solution from the above stock solution, 10 ml methanol was added and sonicated for 15 min and filtered through 0.45µ membrane sample filter paper so that it contains 0.1mg/ml of BRZ and 0.05mg/ml of TM. The standard calibration solutions of BRZ and TM having concentration range 10% - 200% were prepared by diluting appropriate aliquots of the standard stock solutions with the mobile phase.

Preparation of sample solution

Sample solution was prepared by taking 1.0 ml of sample, accurately weighed and quantitatively transferred into a 100 ml volumetric flask and 100 ml methanol was added to get concentration of 0.01mg/ml and the solution was sonicated for 15 min. Appropriate volumes of these solutions were further diluted with methanol to get appropriate concentrations.

Preparation of buffer solution (0.2 M NaH_2PO_4 Solution)

0.2M Sodium phosphate buffer was prepared by dissolving 7.798gms of Sodium dihydrogen phosphate 1000ml of HPLC grade water and the pH was adjusted to 7.5 with sodium hydroxide. It was kept in sonicator for complete dissolution, filter through 0.45µm nylon membrane filter and degassed.

Preparation of mobile phase

The mobile phase was prepared by mixing previously ultra sonicated and filtered sodium dihydrogen phosphate buffer and methanol in the ratio of 70:30(v/v). The solution was filtered through 0.45µ filter paper.

Preparation of samples for forced degradation studies

For acidic degradation study

To 2 ml of BRZ and TM sample solution, 2 ml of 0.1N hydrochloric acid was added. The zero hour sample solution has been prepared by taking immediately 2 ml of the above solution and neutralized with 2 ml of 0.1M sodium hydroxide solution and made upto 10 ml with methanol in volumetric flask. The acid stress study sample after 1 hour was prepared like zero hour solution. After 1 hr the samples were injected into the HPLC system.

For basic degradation study:

To 2 ml of BM and TM sample solution 2 ml of 0.1N sodium hydroxide was added. The zero hour sample solution has been prepared by taking immediately 2 ml of the above primary working standard solution and neutralized with 2 ml of 0.1N hydrochloric acid solution and made upto 10 ml with methanol in volumetric flask. The base stress study sample after 1 hour was prepared like zero hour solution. After 1 hr the samples were injected into the HPLC system.

For oxidation studies:

2ml of 3% hydrogen peroxide was added to 2ml prepared stock solution of Brinzolamide and Timolol Maleate. From above solution 2 ml was then transferred to 10 ml volumetric flasks and diluted to final volume with methanol. After 1 hrs the samples were injected into the HPLC system.

For Thermal degradation:

2ml of prepared stock solution of Brinzolamide and Timolol maleate was placed into the petridish and kept in oven at 60^0 C upto 1 hr. After 1 hr the thermally exposed sample was further used to prepare the testing solution and injected into the liquid chromatography.

For Photo Degradation study:

2ml sample is kept in open petri dish at sunlight. Samples were checked for initial degradation and after exposure for 1 hour. The samples were removed from the sun light cabinet. The light exposed samples

ISSN: 0975-8585

were further used to prepare the testing solution by dilution with methanol and injected into the liquid chromatography.

RESULTS AND DISCUSSION

Optimization of Chromatographic Conditions

A gradient, rapid and simple RP-HPLC method was developed and validated for the simultaneous estimation of Brinzolamide and Timolol Maleate. Mobile phase consisting of 0.2M sodium dihydrogen phosphate buffer and methanol was taken in the ratio of 70:30 set with gradient programming for 20 min. Chromatographic conditions were optimized for mobile phase using Inertsil ODS C18 (150 × 4.6 mm, 5 µm) column at a flow rate of 1 ml/ min. Effluents were detected at 279nm in UV detector. Column compartment temperature was maintained at 25°C.The optimized chromatogram was show in **Fig 2.** Sample Chromatogram and Standard chromarogram are shown in **Fig 3** and **Fig 4** respectively.

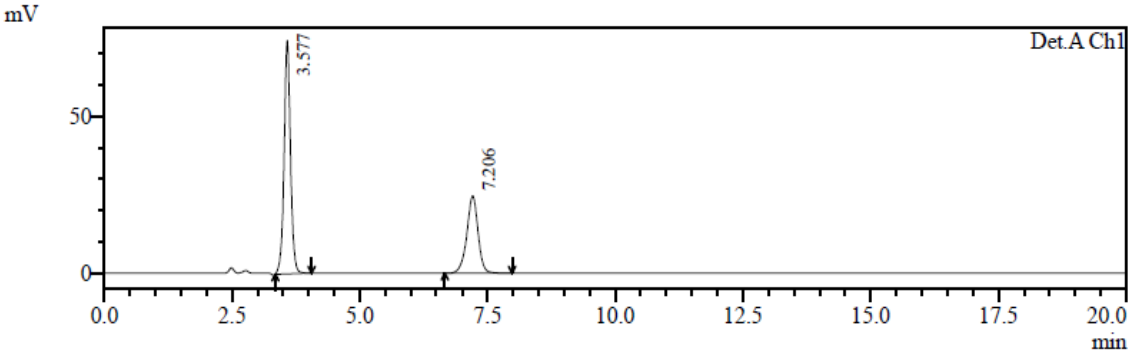


Fig 2: Optimized Chromatogram of Brinzolamide and Timolol Maleate

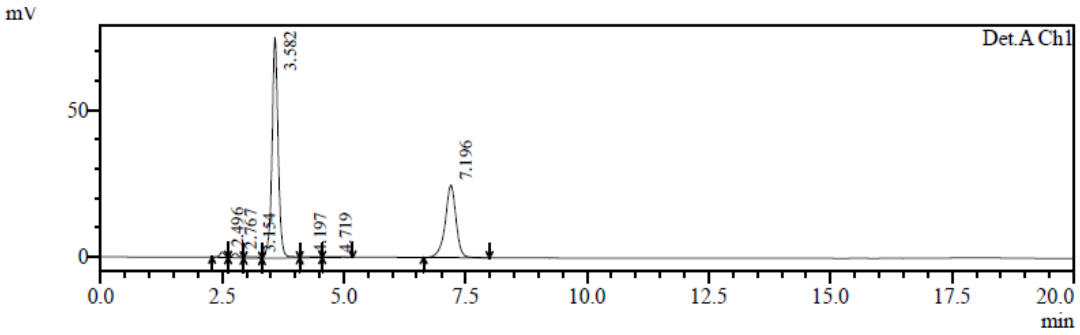


Fig 3: Sample chromatogram

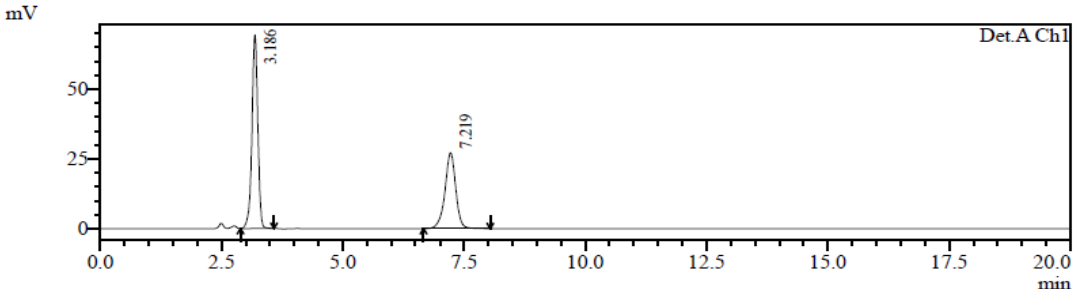


Fig 4: Standard chromatogram

Method Validation

The experimental method was validated according to the recommendations of ICH- guidelines for the parameters like specificity, system suitability, accuracy, linearity, precision, robustness, LOD & LOQ and also forced degradation studies for stability testing.

ISSN: 0975-8585

Specificity

The specificity of the method was evaluated to ensure that there was no interference of excipeints, diluting solution in the chromatogram of Brinzolamide and Timolol maleate. The specificity was studied by injecting the placebo, BKT, 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 the results were shown in the **Fig 5, 6 & 7.**

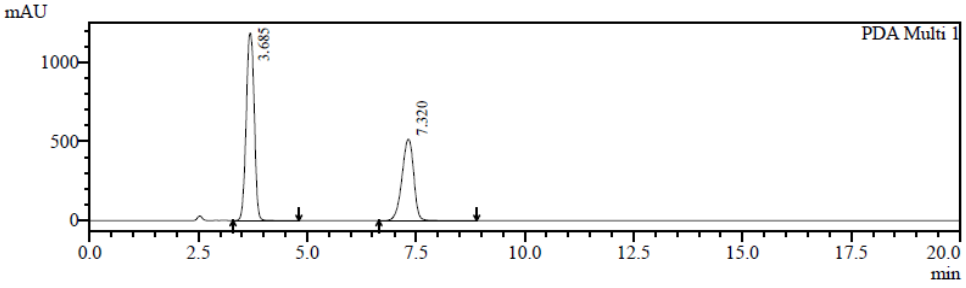


Fig 5: Specificity standard sample

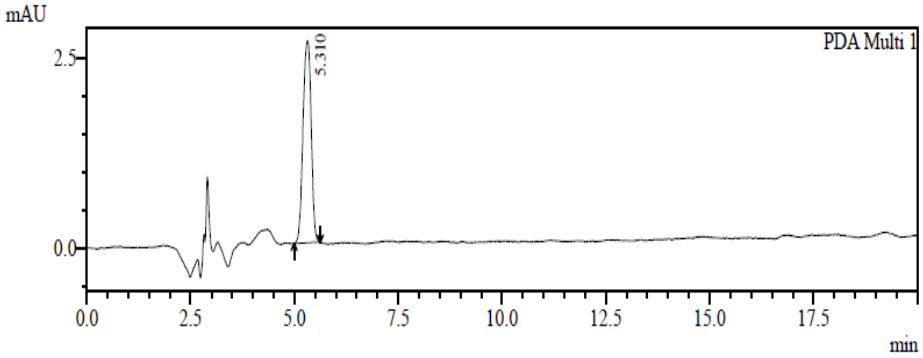


Fig 6: Specificity/BKT sample

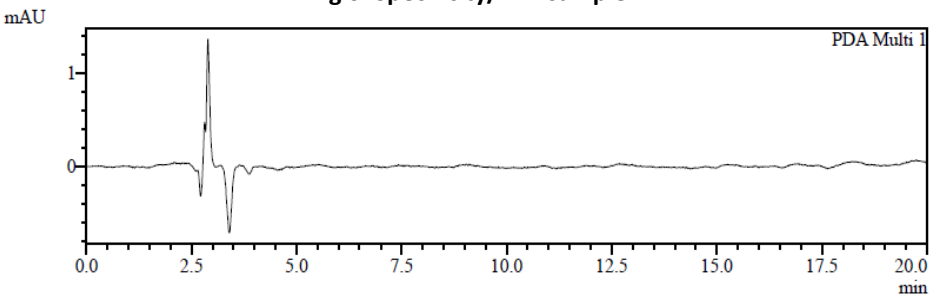


Fig 7: Specificity Placebo sample

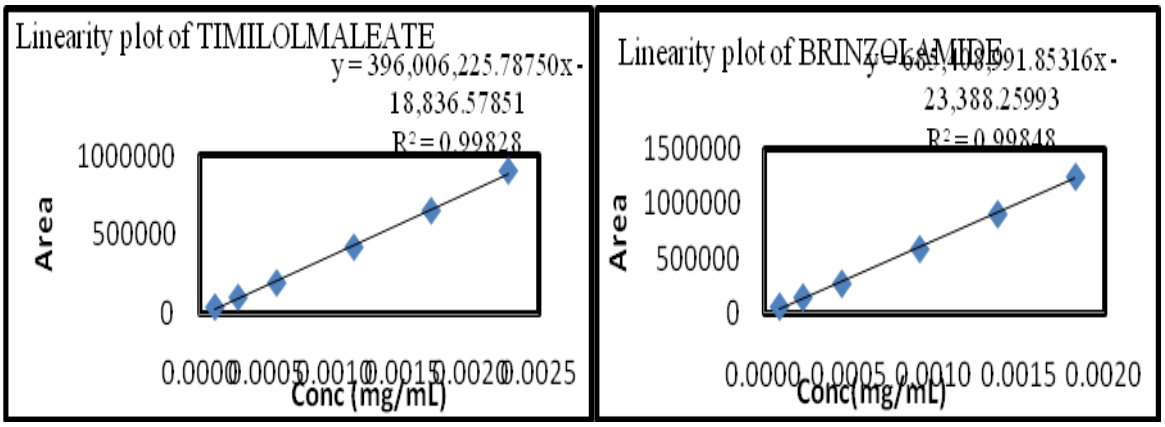
Linearity

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 concentration levels of 10%, 25%, 50%, 100%, 150%, and 200% were injected into the HPLC system. The detector response was found to be linear form 10% to 200% of test concentration for both the standard solutions. The linearity curves of Brinzolamide and Timolol are shown in **Table 1.**

ISSN: 0975-8585

Table 1: Linearity data of Brinzolamide and Timolol Maleate

S. NO.	% of concentration	Concentration (mg/ml)		Avg. peak area	
		BRZ	TM	BRZ	TM
	10%	0.0001	0.0001	58957	40457
1.	25%	0.0002	0.0003	142665	101310
2.	50%	0.0005	0.0006	268806	192795
3.	100%	0.0009	0.0011	586562	418855
4.	150%	0.0014	0.0017	906622	648861
5.	200%	0.0018	0.0023	1249401	899946



Accuracy

The accuracy of the method was established by recovery studies. The known amount of standard was added at three different levels to pre-analyzed sample. Each determination was performed in triplicate at three different concentration levels 50%, 100% and 150%, taking in to percentage purity of added drug sample. The amount of Brinzolamide and Timolol maleate was estimated by applying obtained values to the respective regression lines equation. Each concentration was analyzed 3 times and avg. recovery was measured. The results were shown in **Table 2.**

Table 2: Accuracy data for Brinzolamide and Timolol Maleate

Drug	Addedconcentration (mg/ml)	Measuredconcentration (mg/ml)	% Recovery	Mean % recovery
Brinzolamide	0.05	0.049	97.8	98.86
	0.1	0.102	102.0	
	0.15	0.145	96.8	
TimololMaleate	0.05	0.048	96.5	100.07
	0.1	0.103	103.3	
	0.15	0.150	100.4	

Precision

Precision of proposed method was evaluated by performing repeatability on same day and intermediate precision of two different days and by estimating the assay for six different sample preparations of same batch, each sample analyzed by taking two replicates and results were expressed as % RSD. The results was shown in **Table 3.**

ISSN: 0975-8585

Table 3: Method precision and Intermediate precision data for Brinzolamide and Timolol Maleate

No. of Preparation (100%)	% Assay of Brinzolamide		% Assay of Timolol Maleate	
	Repeatebility (Analyst 1)	Intermediate Precision(Analyst 2)	Repeatebility (Analyst 1)	Intermediate precision(Analyst 2)
1	101.4	101.3	101.8	101.3
2	102.3	102. 3	102.3	101.5
3	102.3	100.3	103.0	99.5
4	101.5	100.2	101.6	99.5
5	102.5	101.6	102.4	100.8
6	101.2	101.7	102.7	100.4

Limit of detection (LOD) and Limit of quantification (LOQ)

Limit of detection and Limit of quantification were calculated from linearity plot. The LOD and LOQ of the proposed methods were calculated from the standard deviation (σ) of the response and the slope of the calibration curve (S) in accordance to the equations. The results were shown in **Table 4.**

$$LOD = 3.3 \times \sigma/S \text{ and } LOQ = 10 \times \sigma/S.$$

Table 4: LOD & LOQ data for Brinzolamide and Timolol Maleate

	Brinzolamide			Timolol Maleate		
Preparations	Standard concentration	Standard area	Stadard average area	Standard concentration	Standard area	Stadard average area
10% solution	0.0001	58305	58957	0.0001	40437	40457
		59608			40477	
25%solution	0.0002	142233	142665	0.0003	101793	101310
		143097			100827	
50%solution	0.0005	268675	268806	0.0006	192898	192795
		268937			192691	
100%solution	0.0009	585913	586562	0.0011	418259	418855
		587210			419450	
150%solution	0.0014	905826	906622	0.0017	649422	648861
		907418			648300	
200%solution	0.0018	1247550	1249401	0.0023	900798	899946
		1251252			899093	
Standard deviation of intercept			23388.25993	Standard deviation of intercepts		18836.57
Slop of calibration curve			685408991.85	Slop of calibration curve		396006225.8
Limit of detection			0.00011	Limit of detection		0.00016
Limit of quantification			0.00034	Limit of quantification		0.00048

Robustness

The robustness is the ability of method to remain unaffected by small changes in parameters. The robustness of the method was 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 i.e, 0.8 ml/min and 1.2ml/min. The effect of column temperature was studied at 30ºC than 25^{0}C. the results were shown in **Table 5.**

Table 5: Robustness data for Brinzolamide and Timolol Maleate

Flow rate 0.8 ml/min data:

Drug	Retention Time	Tailing Factor	Plate Count
Brinzolamide	4.508	1.028	4284
Timolol Maleate	9.194	0.926	5554

Flow rate 1.2 ml/min data

Drug	Retention Time	Tailing Factor	Plate Count
Brinzolamide	3.020	1.053	3144
Timolol Maleate	6.133	0.941	3973

Temperature 30^{0}c

Drug	Retention Time	Tailing Factor	Plate Count
Brinzolamide	3.529	0.996	3590
Timolol Maleate	7.406	0.909	48190

System suitability:

System suitability was performed by injecting six replicates of standards solution of BRZ (0.1mg/ml) and TM (0.05mg/ml) prepared by using stock solution. This method was evaluated by analyzing the repeatability of retention time, tailing factor, theoretical plates of the column. The results were shown in **Table 6.**

Table 6: Data for system suitability

Drug	Retention Time	Tailing Factor	Plate Count
Brinzolamide	3.529	0.996	3590
Timolol Maleate	7.406	0.909	48190

Forced degradation of Brinzolamide Tartrate and Timolol Maleate

To determine the proposed method as a stability-indicating method, Brinzolamide and Timolol Maleate were stressed under different conditions in forced degradation studies. Stock solutions of Brinzolamide and Timolol Maleate used to forced degradation studies - were prepared by dissolving it in methanol. The results were shown in T**able 7.**

Table 7: Force degradation studies for Brinzolamide and Timolol Maleate

Samples	Brinzolamide		Timolol Maleate	
	% assay	% degradation	% assay	% degradation
Control sample	102.1	-	99.4	-
Dark control	103.0	-0.9	100.8	-1.4
Light exposed	102.6	-0.5	100.3	-0.9
Acid stress	56.9	45.9	69.6	29.8
HEAT	102.8	-0.7	100.3	-0.9
Base stress	96.3	5.8	102.2	-2.8
Peroxide stress	102.7	-0.6	100.1	-0.7

CONCLUSION

The validation study showed that the developed method was accurate, rapid, precise, reproducible, economical 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.

ACKNOWLEDGEMENT

The authors thank Varma Labs Pvt. Ltd, Visakapatnam for providing gift samples of Brinzolamide and Timolol Maleate and providing facilities to carry out this work.

REFERENCES

[1] Purvi AS, Ankita SK, Riddhi MK, Kalpana GP, Tejal RG, W J of Pharm and P'ceutical Sci, 2014,Vol 3(2),1955 – 1967.
[2] Purvi AS, Ankita SK, Niketa RG, Kalpana GP, R J of Pharm, Bio and Chem Sci, 2014,5(5),1010-1017.
[3] Rahima Khatun, Ashraful Islam SM, Int J of Pharm Sci and Res; 2014, Vol 5(3)1001-1007.
[4] Mathrusri Annapurna M, Narendra A, Deepika D, J of Drug Delivery and Therapeutics, 2012, Vol 2(2),81-87.
[5] Nagori Bp, Amit M, Pankaj M and Subhash Gupta, J.Chem. Pharm. Res, 2011,3(4), 866-874.
[6] Abdullah A.E, Lobna M.A, Mustafa S.M and Hany M.H, Chromatograph Separat Techniq, 2014,Vol 5(3),
[7] Shadoul WA, Elixir pharmacy 2011,38,4060-4063.
[8] Suketa KM, Dr. Dilip GM, J of Pharma Sci and Biosci Res vol 4(6), 2014, 351-356.
[9] Pritam S.J, Rahul N.K, Hardik N.J and Sanjay J.S, J Chromat0graph Separat Techniq Vol 2(3), 2011.
[10] Rajeshsree M, Bharat S, Asian J of Pharm and Life Sci,vol.4 (2), 2014,16-20.
[11] Hete H.D, Anandkumari D.C, Int J of Pharma Res & Anal, 2014 Vol 4(3) 168-177.
[12] Hiral S. Popaniya, Hemant M.P, World J of Pharm and Pceutical Sci, 2014. Vol3(3), 1330-1340.
[13] Charde M.S, Jitendra Kumar, Welankiwar A.S, Chakole R.D, Int J of Adv in Pceutics, 2013,2(3),34-39.
[14] ICH guidelines, Q1A(R2), Stability Testing of New Drug Substances and Products (revision2), International Conference on Harmonization.
[15] International Conference on Harmonization, "Q2A" Text on Validation of Analytical Procedures, 1995, 11260 – 11262.