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Simultaneous Estimation of Brinzolamide and Timolol Maleate Using Chromatographic Methods.

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ABSTRACT

Two simple, accurate, precise, robust and economical chromatographic methods viz. RP-HPLC and HPTLC have been developed and validated according to ICH guideline for the simultaneous determination of Brinzolamide (BRZ) and Timolol maleate (TM). The separation of BRZ and TM in HPLC method was carried out using Inertsil C_{18}, (5 µm, 150 mm x 4.6 mm) column using isocratic condition, whereas in HPTLC using precoated silica gel $60F_{254}$ aluminium plates. The optimized mobile phase comprised of methanol: 0.05M phosphate buffer (70: 30, v/v) pH adjusted to 7.5 with 1.0 ml/min flow rate in HPLC while toluene: methanol: ethyl acetate: acetone (7: 3: 0.1: 0.1, v/v/v/v) in HPTLC at 279 nm. The linear concentration range for HPLC method was 50-250 µg/ml and 25-125 µg/ml; and for HPTLC method was 800-1800 ng/band and 300-800 ng/band for BRZ and TM respectively. The pooled % RSD value for repeatability, intermediate precision, accuracy, robustness studies for proposed methods were found to be less than 2. The mean percentage recoveries in terms of accuracy were found to be in the range of 98.04 to 101.94 % (for both drugs) in proposed methods. In conclusion, proposed chromatographic methods may be successfully applied for routine quality control testing of BRZ and TM in bulk and ophthalmic formulation.

Keywords: Brinzolamide, Timolol maleate, HPLC, HPTLC, method validation

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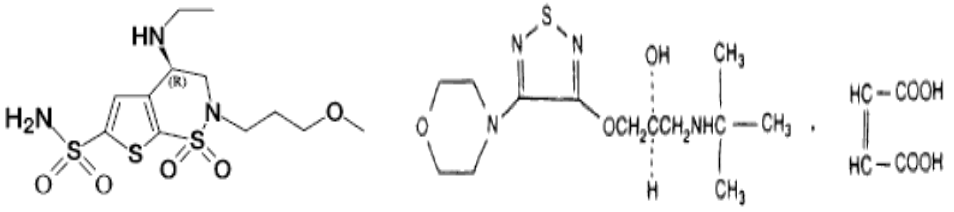
INTRODUCTION

Brinzolamide (BRZ) (R)-(+)-4-Ethylamino-2-(3-methoxypropyl)-3, 4-dihydro-2H thieno [3,2 e]-1,2-thiazine-6-sulfonamide-1,1-dioxide, is useful for topical use in the treatment of glaucoma (Figure 1a). Timolol maleate (TM) (S)-1-[(1,1-dimethylethyl)amino]-3-[[4-(4-morpholinyl)-1,2,5-thiadiazol-3-yl]oxy]-2 propanol (Z)-2 butenedioate (1:1) is a non-selective beta-adrenergic receptor blocking agent (Figure 1b). It lowers the pressure in the eye for various conditions such as open angle glaucoma and ocular hypertension. It is most effective β blocker as an antiglaucoma agent [1]. Nowadays, 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 the combination available as eye drop, DORZOX-T (dorzolamide and timolol maleate) [2].



(a) (b)

Figure 1: Chemical structures of (a) Brinzolamide, (b) Timolol maleate

Literature reports various analytical methods for determination of TM like Spectrophotometric, HPTLC, chemiluminescene, cyclic voltammetry in pharmaceutical formulation [3-7]; bioanalytical methods like HPLC, GC, Capillary column GC-MS and LC-MS methods [8-13], as well as Chiral-HPLC and capillary electrophoresis [14-16] for the enantioselective analysis whereas only one method reported for the determination of BRZ alone [17]. Moreover, HPLC and spectrophotometric methods have been reported for the simultaneous determination of BRZ and TM in pharmaceutical formulation in literature [18-19].

To the best of our knowledge, none of the HPTLC method is available for simultaneous determination of combination of both the drugs. Although, HPLC method for simultaneous determination of BRZ and TM has been reported [18**].** But the drawback of this reported method is complexity in the composition of mobile phase and higher amount of buffer that can affect column performance. In context to this, chromatographic methods viz. HPLC and HPTLC that find wide application in research and industry were developed for the simultaneous estimation of BRZ and TM. Hence, in the present article, simple, accurate, precise, robust and sensitive HPLC (Method I) and HPTLC (Method II) methods for the simultaneous determination of BRZ and TM in their mixture form was reported.

EXPERIMENTAL

Chemicals

Pharmaceutical grade of BRZ and TM were obtained from Biocon Pharmaceutical Pvt. Ltd., Bangalore, India and Marck Bioscience Pvt. Ltd., Kheda, Gujarat respectively as a gift sample for proposed study. All solvents and chemicals used were of analytical grade, purchased from Merck Specialities Pvt. Ltd., India.

Instrumentation

HPLC system, with LC solutions data handling system (Shimadzu-LC 2010-CHT), with PDA detector and an auto sampler was used for the analysis. The data was recorded using LC 2010 solutions software version 1.25. A. Camag HPTLC system (Switzerland) comprising of Camag Linomat V applicator; Camag TLC scanner IV; ultraviolet (UV) cabinet with dual wavelength UV lamp; Camag flat bottom and twin trough chamber (10 × 20 cm); Camag winCATS version 1.4.6 software; Hamilton syringe, 100 µL (Linomat syringe 659.0014, Hamilton-Bonaduz Schweiz, Camag, Switzerland); and pre-coated silica gel 60F254 aluminium plates (10 × 10 cm,100 µm thickness; Merck, Darmstadt, Germany) were used during the study.

Preparation of solutions

Preparation of phosphate buffer

6.8 g of potassium dihydrogen phosphate was dissolved in double distilled water and diluted with the same up to 1000 ml to make 0.05 M phosphate buffer. 0.2 M sodium hydroxide was added to the prepared solution to adjust pH 7.5.

Preparation of mobile phase

30 ml of phosphate buffer, pH 7.5 was taken in a clean and calibrated 100 ml measuring cylinder, 70 ml of methanol (HPLC grade) was added, sonicated for 10 min and then filtered through Whatman filter paper.

Preparation of combined standard stock solution

A combined stock solution of mentioned drugs was prepared by dissolving accurately weighed quantity of 100 mg BRZ and 5 mg TM in to 100 ml volumetric flasks, dissolved and diluted up to mark with methanol to obtain a final concentration of 1000 µg/ml of BRZ and 500 µg/ml of TM. These stock solutions were appropriately diluted to make working standard solution that contains 100 µg/ml of BRZ and 50 µg/ml of TM.

Chromatographic procedure

Method I

The chromatographic determination was performed on a reversed-phase stainless steel column, filled with octadecylsilane chemically bonded to porous silica particles (Inertsil C_{18}, 5 µm, 150 mm x 4.6 mm) with the mobile phase containing methanol:0.05 M phosphate buffer (70:30,v/v), pH adjusted to 7.5 with 2M NaOH. The mobile phase was prepared daily, filtered using 0.45 µm nylon filter, and degassed in sonicator for 10 min before use. The flow rate was adjusted to 1.0 ml/ min and the elution was monitored at 279 nm. Standard or sample solution of BRZ and TM were injected in to column and mobile phase was used as a diluents.

Method II

Suitable volume of standard and sample solution was spotted in the form of bands having band width of 8 mm on precoated silica gel 60 F254 HPTLC plate, 10 mm from the bottom and 10 mm from the side edges in the form of bands using a Camag Linomat 5 sample applicator. Chromatographic run was carried out by linear ascending development technique in twin trough glass chamber with an optimized chamber saturation time of 25 min at room temperature. The optimized mobile phase consisted of toluene-methanol-ethyl acetate-acetone (7:3:0.1:0.1, v/v/v/v). The length of chromatographic run was 80 mm, and all measurements were made in the reflectance–absorbance mode at 279 nm; slit dimension of 6.00 × 0.45 mm, micro; scanning speed of 20 mm/s; and data resolution of 100 µm/step. The source of radiation was deuterium lamp emitting a continuous UV spectrum between 190 and 400 nm.

Method validation

The method was validated in accordance with ICH guidelines Q2 (R1) for evaluation of various parameters: linearity, precision, accuracy, LOD, LOQ, specificity, and robustness for both methods [20].

System suitability test

System suitability in HPLC was determined from six replicate injections of the working standard solution of BRZ (100 µg/ml) and TM (50 µg/ml) prepared daily using the combined stock solution.

Linearity

The linearity of both methods was evaluated by linear regression analysis in the concentration range of 50-250 µg/ml of BRZ and 25-125 µg/ml of TM for HPLC as well as 800-1800 ng/spot BRZ and 300-800 ng/spot TM for HPTLC in five replicate measurements. Furthermore, the homoscedasticity of both the drugs along the regression line was verified using the Bartlett's test in both the method [21].

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

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: LOD = 3.3 x σ/S and LOQ = 10 x σ/S.

Precision

Precision of proposed methods was evaluated by performing repeatability on same day (three times a day) and intermediate precision on three different days and results were expressed as percent relative standard deviation (% RSD). For the same, 50-250 µg/ml of BRZ and 25-125 µg/ml of TM in method I whereas 800-1800 ng/spot of BRZ and 300-800 ng/spot TM in method II were analyzed by taking three replicate measurement of each and peak area measured was expressed in terms of % RSD.

Accuracy

To demonstrate the accuracy of the proposed methods, the recovery studies were carried out by standard addition method at three different levels 50%, 100% and 150% in triplicate. This was done to check the recovery of the drug at different levels in the formulations by optimized methods. In method I, recovery studies was carried out by spiking three different amounts of BRZ standard (50, 100 and 150 µg/ml) to the synthetic mixture (100 µg/ml) and TM standard (25, 50 and 75 µg/ml) to the synthetic mixture (50 µg/ml). Moreover, recovery studies in method II was carried out by spiking three different amounts of BRZ standard (300 ng, 600 ng, and 900 ng) to the synthetic mixture (600 ng/band) and TM standard (150 ng, 300 ng, and 450 ng) to the synthetic mixture (300 ng/band) by standard addition method.

Robustness

Robustness of method was determined by deliberate changes in various parameters such as flow rate, mobile phase composition, and wavelength for HPLC analysis and mobile phase ratio, saturation time, solvent front and wavelength for HPTLC analysis. For the robustness study, small deliberate changes of various factors in method I includes: flow rate (1.0 ± 0.1 ml/minute; ratio of mobile phase composition (methanol: Buffer, 70:30, v/v ± 5), and wavelength scan (279 nm ± 2). In HPTLC, the effect of deliberate variations in method parameters were the composition of the ratio of methanol in mobile phase (Toluene: methanol: ethyl acetate: acetone,7:3:0.1:0.1,v/v/v/v $\pm$ 0.25); saturation time (25 min $\pm$ 5); development distance (8 cm $\pm$ 1) and wavelength scan (279 nm ± 2). The effect of these changes on both the R_f or R_t values and peak areas was evaluated by calculating the % RSD for each parameter.

Application of proposed methods for simultaneous estimation of BRZ and TM

In HPTLC method, accurately weighed quantities BRZ (10 mg) and TM (5 mg) were mixed with reported excipients like 0.1% benzalkonium chloride, 0.1 mg mannitol, and 0.1 mg sodium chloride and dissolved in 2 ml methanol. This synthetic mixture was then transferred to 10 ml volumetric flask and diluted with 5 ml mobile phase. The mixture was sonicated for 10 min and further diluted up to the mark with mobile phase to get 1000 µg/ml BRZ and 500µg/ml TM, followed by filtration through Whatman filter paper no. 42 wetted with mobile phase. Further dilution with mobile phase was performed to obtain BRZ (100 µg/ml) and TM (50 µg/ml). The method described above was then applied for determination of peak area, and triplicate analysis was performed by following the above procedure.

In method II, accurately weighed quantities BRZ (10 mg) and TM (5 mg) were mixed with reported excipients like 0.1% benzalkonium chloride, 0.1 mg mannitol, and 0.1 mg sodium chloride and dissolved in 2 ml

methanol. This synthetic mixture was then transferred to 10 ml volumetric flask and diluted with 5 ml methanol, followed by sonication for 10 min and further diluted up to the mark with methanol. The resulting solution was filtered through Whatman filter paper no. 42 wetted with methanol. Further dilution with methanol was performed to obtain BRZ (100 µg/ml) and TM (50 µg/ml). Ten microliter of the filtered solution (1000 ng of BRZ and 500 ng of TM per band) was applied on the HPTLC plate followed by development and scanning; the analysis was repeated in triplicate according to method procedure.

RESULTS AND DISCUSSION

Method development

Method I

Various preliminary trials were performed using polar solvents, methanol and acetonitrile and, with and without addition of buffer for optimization of mobile phase. Among various trials, methanol with 0.05 M phosphate buffer in the ratio of 70: 30 produced well resolved peaks of BRZ and TM free from tailing. Finally, the best results were obtained by adjusting the pH of buffer and the final mobile phase composition optimized was a mixture of methanol: 0.05 M phosphate buffer (70:30, v/v) adjusted to pH 7.5 with 2 M NaOH. Under these optimized chromatographic conditions, the retention time of BRZ and TM was 3.41 and 6.92 min, respectively, at a flow rate of 1 ml/min (Figure 2). From the overlain spectra of BRZ and TM, 279 nm wavelength was selected for detection and quantification (Figure 3).

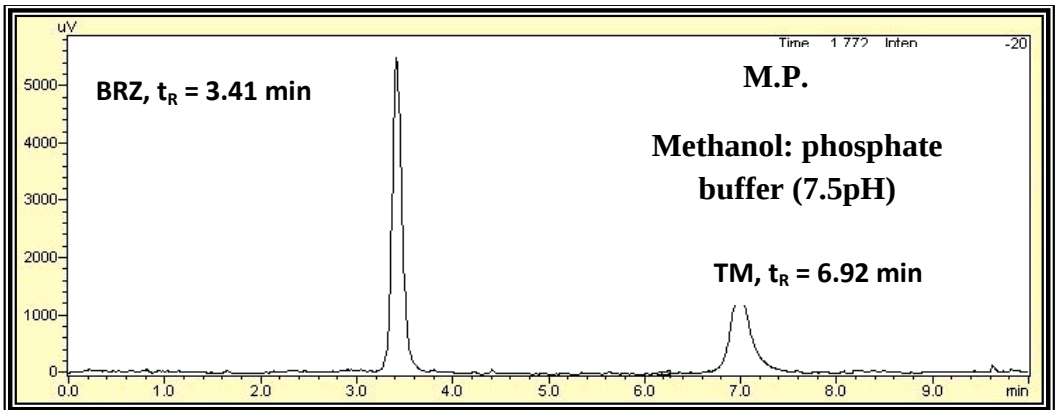


Figure 2: HPLC chromatogram under optimized conditions showing R_t = 3.41 min for BRZ and R_t = 6.92 min for TM

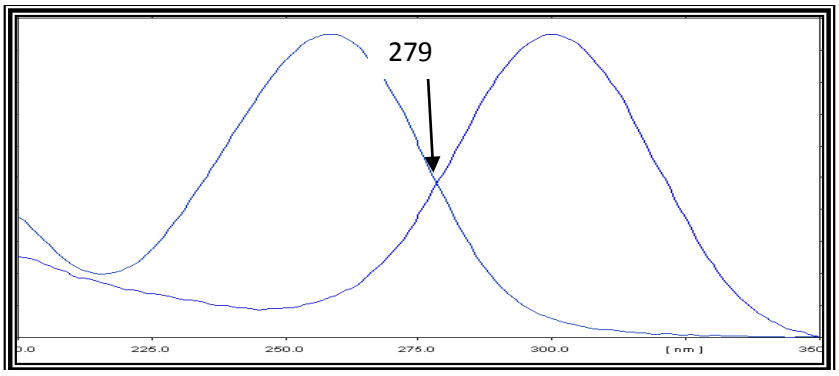


Figure 3: Overlain absorption spectra for simultaneous estimation of BRZ and TM at 279

Method II

From through literature review, HPTLC method had been reported for TM alone and with other combination, in which selected mobile phase comprised of Toluene, methanol, ethyl acetate, isopropyl alcohol and ammonia. Hence, such solvents were used in different combinations and proportions for optimization of

mobile phase. Toluene: methanol: ethyl acetate (7:3:0.1, v/v/v) gave acceptable R_f value with improper peak shape. Hence, acetone was added further to improve peak shape of both drugs showing acceptable R_f value. Finally, the optimized mobile phase consisted of toluene-methanol-ethyl acetate-acetone (7:3:0.1:0.1, v/v/v/v) showing good resolution of both the drugs at 279 nm with R_f value of TM and BRZ was 0.35 and 0.64 (Figure 4).

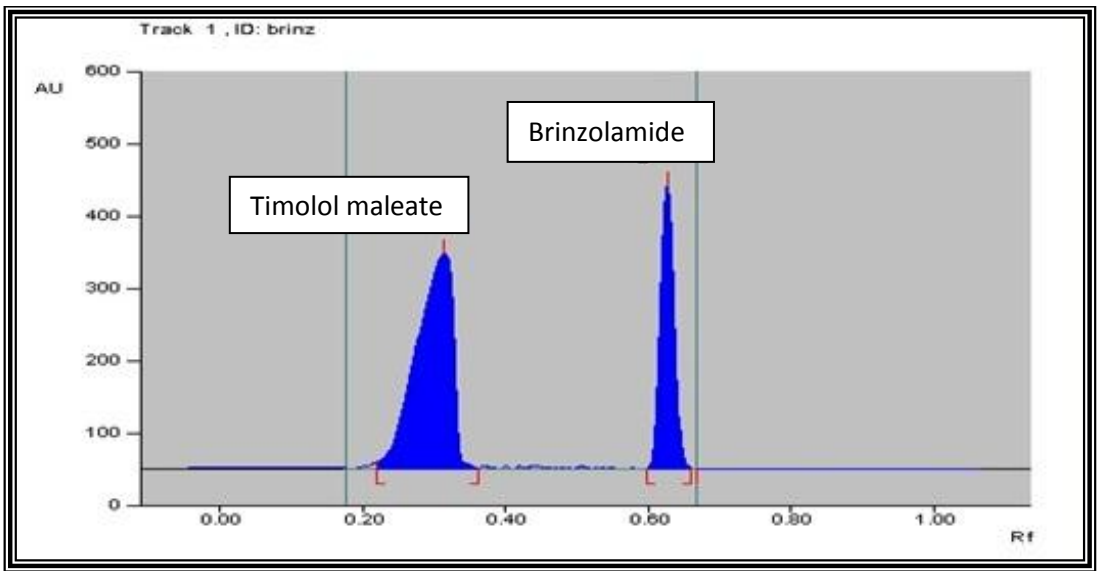


Figure 4: HPTLC densitogram under optimized conditions showing R_f = 0.35 for TM and R_f =0.64 for BRZ

Method validation

The proposed methods have been validated for linearity, precision, accuracy, limit of detection (LOD), limit of quantification (LOQ), and robustness evaluated in accordance with ICH guidelines Q2 (R1) as described below

System Suitability testing

System suitability testing in HPLC method showed that the method was suitably performed under the optimized conditions and % RSD was found less than 2%, for system suitability parameters; R_t (for BRZ, 3.41 min ± 0.01, for TM, 6.92 min ± 0.02), tailing factor (less than 1.4), number of theoretical plates (more than 4000) and resolution (12.18 ± 0.07).

Linearity

The linear relationship of BRZ and TM were found to be in the concentration range of 50-250 µg/ml and 25-125 µg/ml for HPLC and 800-1800 ng/band and 300-800 ng/band for HPTLC respectively. The linearity of calibration graphs and adherence of the system to Beer's law was evident from correlation coefficient greater than 0.99 in proposed methods. Moreover, linearity was also validated by applying "Bartlett's test" for homoscedasticity of variance on the data of linearity, peak area with respect to concentration range for both the methods [21]. The results showed that the calculated $\chi 2$ value was smaller than the critical value, $\chi 2_{(0.05, 5)}$ = 9.488; thus indicating that the variance of response is homogeneous (Table 1).

Sensitivity

The sensitivity of proposed methods were expressed in terms of LOD and LOQ. LOD and LOQ for BRZ was found to be 0.902 µg/ml and 2.734 µg/ml while 0.768 µg/ml and 2.328µg/ml for TM in Method I. Similarly, in Method II, LOD and LOQ of BRZ was found to be 52.09 µg/ml and 22.11 µg/ml whereas 157.85 µg/ml and 67.00 µg/ml for TM respectively (Table 1).

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Precision

The results of precision study of proposed methods expressed in terms of %RSD are depicted in Table 1. % RSD (less than 2) value reveals that the proposed method provides acceptable intraday and interday variation of BRZ and TM thus indicating acceptable repeatability and reproducibility of the proposed methods.

Accuracy (Recovery study)

Recovery study by spiking the standard at three concentration levels, 50, 100 and 150 %, showed acceptable percent recovery of 98.04 to 101.94 % with % RSD value less than 2, indicating that the proposed methods are accurate and suitable for routine analysis of bulk and formulation (Table 1).

Table 1: Analytical Validation Parameters for the proposed HPLC and HPTLC method

Parameters	HPLC		HPTLC	
	BRZ	TM	BRZ	TM
Linearity[a,b]				
Calibration range	50-250	25-125	800-1800	300-800
Correlation coefficient (r^2)	0.9999	0.9992	0.9964	0.9978
Slope ± SD (S_b)	0.003	0.003	0.018	0.058
Intercept ± SD (S_a)	0.601	0.274	32.040	35.051
Bartlett's test[c] (χ^2)	0.0001375	0.0003221	3.108	2.837
Sensitivity				
LOD[b]	0.902	0.768	52.09	157.85
LOQ[b]	2.734	2.328	22.11	67.00
Precision (%RSD)[d]				
Repeatability	0.02-0.31	0.17-0.46	0.11-1.37	0.18-1.45
Intermediate precision	0.12-1.15	0.32-1.69	0.30-1.45	0.23-1.72
Accuracy[e]				
50%	99.04 ± 1.00	98.04 ± 0.01	101.02±0.07	99.32±0.03
100%	98.27 ± 0.11	100.70 ± 0.06	101.38±0.12	100.20±0.03
150%	98.75 ± 0.04	99.95 ± 0.01	100.21±0.10	101.94±0.14

[a]Average of five determinations, [b]µg/ml for HPLC and ng/band for HPTLC method; [c]Calculated value less than tabulated value, 9.488 at 95% confidence interval, [d]Average of three determinations for each concentration, [e]Average of three determinations at each level ± SD

Robustness

Robustness of proposed methods were evaluated by performing deliberate changes in various parameters such as flow rate, mobile phase composition, and wavelength scan for HPLC analysis and mobile phase ratio, saturation time, solvent front and wavelength scan for HPTLC analysis as mentioned in Table 2. From the obtained results, it is evident that the response remained unaffected by small variations of these parameters. % RSD less than 2 (Table 2) indicates the robustness of the proposed methods**.** Moreover, insignificant differences in peak areas and less variability in retention time and retention factor were observed.

Table 2: Robustness study for proposed HPLC and HPTLC methods

Method	Parameters	%RSD[a]	
		BRZ	TM
HPLC	Change in flow rate (1.0 ml/min ± 0.1)	0.018-0.069	0.175-0.201
	Change in mobile phase ratio (Methanol: Buffer, 70: 30, v/v ± 5)	0.067-0.084	0.078-0.176
	Change in wavelength scan (279 nm ± 2)	0.078-0.167	0.022-0.141
HPTLC	Change in methanol ratio of Mobile phase composition (Toluene: methanol: ethyl acetate: acetone, v/v/v/v; 7: 3: 0.1: 0.1 ± 0.25)	0.996-1.104	0.820-0.480
	Saturation time (25 min ± 5)	0.260-1.360	0.120-1.070
	Development distance (8 cm ± 1)	0.420-1.500	0.370-0.90
	Change in wavelength scan (279 nm ± 5)	1.300-1.830	0.920-1.780

[a] Average of three determinations

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Application of proposed methods for the simultaneous estimation of BRZ and TM

Ophthalmic formulation of BRZ and TM (Azarga eye drops) is not available in India. Hence, laboratory prepared synthetic mixture was prepared in similar composition and analyzed in triplicate using the proposed methods. Experimental results for the analysis of mixture were expressed in mean recovery of drugs that indicates no interference from any excipients by both the methods. The content of BRZ was in the range of 99.55-100.20 % and for TM in the range of 99.00- 99.20 % with low % RSD proves applicability of proposed methods in routine analysis of ophthalmic formulation (Table 3).

Table 3: Application of proposed methods for the simultaneous estimation of BRZ and TM

Method	Amount of drug taken (mg)		% Mean recovery of drug [a] ±%RSD	
	BRZ	TM	BRZ	TM
HPLC	10	5	100.2±1.37	99.20±0.98
HPTLC	10	5	99.55±1.86	99.00± 0.04

[a] Average of three determinations

CONCLUSION

Two chromatographic methods viz. HPLC (Method I) and HPTLC (Method II) were developed and validated as per ICH guidelines. From the obtained results, proposed methods are simple, accurate, precise, robust and economical chromatographic methods for simultaneous estimation of BRZ and TM. Further, proposed chromatographic methods may be successfully applied for routine quality control testing of BRZ and TM in bulk and ophthalmic formulation without any interference from the excipients.

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