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RESEARCH ARTICLE

DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR SIMULTANEOUS DETERMINATION OF DORZOLAMIDE AND TIMOLOL MALEATE IN PHARMACEUTICAL DOSAGE FORMS

[a*]M Mathrusri Annapurna, A Narendra[b], D Deepika[c]

[a]Department of Pharmaceutical Analysis & Quality Assurance,
GITAM Institute of Pharmacy, GITAM University, Visakhapatnam-530045, India
[b] Roland Institute of Pharmaceutical Sciences, Berhampur, Orissa-760010, India
[c] Royal College of Pharmacy and Health Sciences, Berhampur, Orissa, India.
* *Corresponding author's E mail: mathrusri2000@yahoo.com Tel: 91-9010476308*

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ABSTRACT

A fast, sensitive and accurate reverse phase liquid chromatographic method was developed and validated for the simultaneous determination of Dorzolamide and Timolol maleate in ophthalmic preparations. Chromatographic separation was achieved on Inertsil ODS 3V C18 column (250 X 4.6 mm, 5 µm particle size) with mobile phase consisting of Acetonitrile and 1-Octane Sulphonic acid buffer (0.02M) pH adjusted to 3.5 ± 0.05 with o-phosphoric acid (36:64 V/V) at a flow rate of 1.0 mL/min. The analytes were detected at 254 nm and 295 nm for Dorzolamide and Timolol maleate respectively by PDA detector. Brimonidine was used as internal standard (IS). The retention time of Dorzolamide, Timolol maleate and Brimonidine were found to be at 6.020 ± 0.02, 8.254 ± 0.01 and 4.636 ± 0.01 mins respectively. The linearity of the method ranged between 4-720 and 1-180 µg/mL for Dorzolamide and Timolol maleate respectively with correlation coefficient 0.999 for both the drugs in binary mixture. The LOD was found to be 0.6951 µg/mL and 0.2489 µg/mL for Dorzolamide and Timolol maleate respectively and LOQ was found to be 2.3214 µg/mL and 0.8317 µg/mL for Dorzolamide and Timolol maleate respectively.

Key words: RP-HPLC, Dorzolamide, Timolol maleate, Brimonidine, Eye drops

INTRODUCTION

Dorzolamide [1] (DRZ) chemically [(4*S*, 6S) -4-(Ethylamino) -6-methyl- 5, 6- dihydro-4*H* thieno [2,3*b*] thiopyran- 2-sulphonamide 7, 7-dioxide hydrochloride] has a molecular formula $C_{10}H_{16}N_2O_4S_3$ and molecular weight 324.44. Dorzolamide (Figure 1) is a carbonic anhydrase inhibitor. It is a sulfonamide and a highly specific carbonic anhydrase II inhibitor, which is the main carbonic anhydrase iso-enzyme involved in aqueous humor secretion. Inhibition of carbonic anhydrase -II in the ciliary processes of the eye decreases aqueous humor secretion, presumably by slowing the formation of bicarbonate ions with subsequent reduction in sodium and fluid transport. It is indicated for the reduction of elevated intra-ocular pressure in patients with open-angle glaucoma or ocular hypertension who are insufficiently responsive to beta-blockers. Timolol maleate (TML) (Figure 2) chemically (*S*)-1-(*tert*-butylamino)-3-[(4-morpholin-4-yl-1,2,5-thiadiazol-3-yl)oxy]propan-2-ol is a non-selective beta-adrenergic receptor blocker [2] and has a molecular formula $C_{13}H_{24}N_4O_3S$ with molecular weight 316.421 g/mol. 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. The pharmacological mechanism by which it actually does this is still unknown. Brimonidine (BMD) (Figure 3) was used as internal standard (IS).

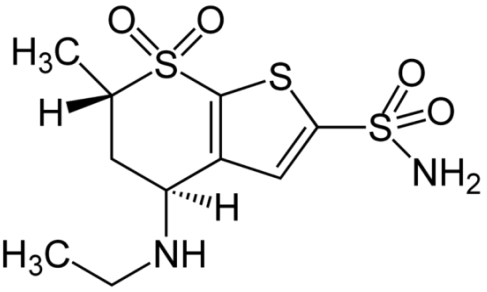

Figure 1: Chemical Structure of Dorzolamide (DRZ)

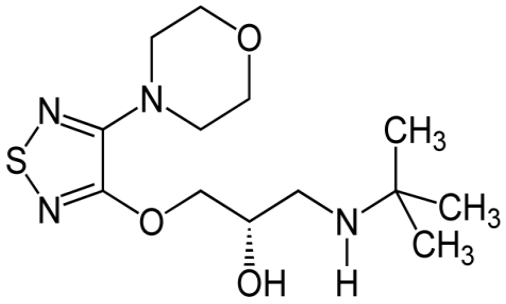

Figure 2: Chemical Structure of Timolol maleate (TML)

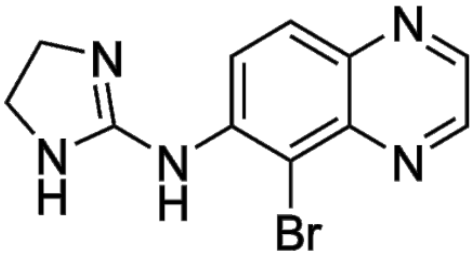

Figure 3: Chemical Structure of Brimonidine (BMD)

Both Dorzolamide and Timolol maleate have been analysed by various techniques either alone or in combination with other drugs. Several methods have been applied to determine Dorzolamide involving spectrophotometry [3], LC/MS [4], HPLC [5], Capillary electrophoresis [6] and voltametry [7] where as Timolol maleate was determined by voltametry [8], HPTLC [9] and HPLC [10].

Dorzolamide and Timolol maleate have been simultaneously analysed by spectrophotometry [11] and TLC / ratio derivative spectrophotometry [12]. Nevin Erk [13] et al and Nagori [14] developed HPLC methods with narrow linearity range for both the drugs. In this paper we reported a sensitive liquid chromatographic method with wide linearity range, good resolution where running time is less than 10 mins. The method has been validated as per the ICH guidelines [15-16] and can be successfully applied to pharmaceutical formulations.

MATERIALS AND METHODS

Reagents and Materials

Reference standards of Dorzolamide, Timolol maleate and Brimonidine were supplied by Micro Vision (India) with purity of 99.94%, 99.88 and 99.91% respectively. HPLC grade Acetonitrile (Merck), 1-Octane Sulphonic acid (Spectrochem), *o*-phosphoric acid and Milli Q water were used throughout the analysis. All other reagents were of analytical grade and were used without further purification. Mobile phase was filtered using 0.45µ membrane filters by Millipore (USA).

Instrumentation

The HPLC system is a binary gradient HPLC with Waters Alliance (Waters Corporation, MA, USA) equipped with a Waters 2695 separations module and a Waters 2695 PDA detector. Data acquisition was performed by the Empower pro-software operated on a Pentium® IV microprocessor. The experimental conditions were optimized with an Inertsil ODS 3V column (250 x 4.0 mm, 5 µm particle size) at room temperature.

Chromatographic conditions

The mobile phase consist of a mixture of (0.02M) 1-Octane Sulphonic acid buffer (pH 3.5) and Acetonitrile (64:36 V/V) respectively. The 1-Octane Sulphonic acid buffer was prepared by dissolving 4.35 grams of 1-Octane Sulphonic acid in a 1000ml of volumetric flask with pH adjustment to 3.5 ± 0.05 with *o*-phosphoric acid. Flow rate of the mobile phase was 1.0 mL/min.

Preparation of stock solution

Standard stock solution was prepared by dissolving accurately 200 mg Dorzolamide and 50 mg Timolol maleate in 100 ml mobile phase. 5 ml of standard stock solution was diluted to 25 ml with mobile phase to obtain 400 µg/mL Dorzolamide and 100 µg/mL Timolol maleate. The solution was filtered through 0.45µ nylon filter before analysis. 10 mg of Brimonidine was accurately weighed and dissolved in a 100 ml volumetric flask (100 µg/mL) with mobile phase. A series of solutions containing Dorzolamide and Timolol maleate (4:1) were prepared along with Brimonidine (20 µg/mL) as internal standard were prepared with mobile phase and 10 µl of these solutions were injected in to the HPLC system.

Preparation of sample solution

Commercial eye drops MISOPT (2%, 5 ml) and OCUDOR (2%, 5 ml) were purchased from the local market and 2 ml of sample solution was diluted to obtain Dorzolamide and Timolol maleate as 400 and 100 µg/ml with mobile phase. The solution was filtered through 0.45µ nylon filter before analysis. A series of diluted solutions containing the mixture of Dorzolamide and Timolol maleate of the extracted formulation were prepared along with the internal standard (20 µg/mL) were prepared with mobile phase and 10 µl of these solutions were injected in to the HPLC system.

Method Validation

Linearity

Linearity of the method was evaluated at different concentration levels by diluting the standard Dorzolamide and Timolol maleate (4:1) solutions with IS (20 µg/mL) to give solutions over the range 4-720 µg/mL and 1-180µg/mL respectively. These were injected in triplicate and the peak area ratio value of Dorzolamide to that of IS as well as Timolol maleate to that of IS against their respective concentration were inputted into a Microsoft Excel® spreadsheet program to plot calibration curves.

Limit of detection and limit of quantitation

To calculate Limits of Detection (LOD) and Limits of Quantification (LOQ) values, sequential dilutions prepared and analyzed by the proposed method. The LOD and LOQ established by evaluating the level (signal to noise ratio of 3:1 and 10:1 respectively) at which the analytes can be readily detected and quantified with accuracy.

Precision

Precision was evaluated in terms of intra-day repeatability and inter-day reproducibility. The intra-day repeatability was investigated using three separate sample solutions each at three different levels (200, 400 and 600 µg/mL) prepared as reported above, from the freshly reconstructed eye drops formulations. Each solution was injected in triplicate (n=3) and the peak area followed by peak ratio was calculated. The inter-day reproducibility was checked on three different days by analyzing the sample solutions at three different levels (n=3) and the % RSD values were calculated.

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Accuracy

To demonstrate the accuracy of the proposed method was ascertained by carrying out recovery studies employed by standard addition method. Known quantities of Dorzolamide and Timolol maleate along with the internal standard (20 µg/mL) were supplemented to pre-quantified sample solution and then experimental and true values compared i.e. freshly prepared placebo of the combined pharmaceutical formulation (Eye drops) were spiked with various amounts of pure Dorzolamide and Timolol maleate at 50, 100 and 150%. Each solution was injected in triplicate and the peak area ratio of Dorzolamide to that of IS as well as Timolol maleate to that of IS were calculated.

RESULTS AND DISCUSSION

Reverse phase liquid chromatography using silica-base column is successfully applied in many separations of pharmaceuticals. Asymmetric peaks, irreproducible retention and non-robust separation methods can be obtained. In this work we proposed a simple, sensitive and accurate HPLC method for simultaneous determination of Dorzolamide and Timolol maleate. To obtain symmetrical peaks with better resolution the chromatographic conditions i.e. eluent optimization (pH, silanol blockers) were optimised. Various chromatographic conditions such as mobile phase composition, analytical columns with different packing materials (C8, C18, phenyl, cyano), and configurations (10, 15, 25cm columns) tested to obtain sharp peaks with reduced tailing and better resolution. Finally a Inertsil ODS 3V C18 column (250 X 4.6mm 5 µm particle size) column selected which provided reduced peak tailing. Mobile phase selected from peak parameters (symmetry, tailing etc.), run time, ease of preparation and cost. The most suitable mobile phase composition found to be (0.02M) 1, Octane Sulphonic acid buffer (pH 3.5) and Acetonitrile in the ratio of (64:36 V/V). Typical chromatograms of Dorzolamide (254 nm), Timolol maleate (295 nm) and Brimonidine (254 nm) alone under the above mentioned conditions were shown in Figure 4, 5 and 6.

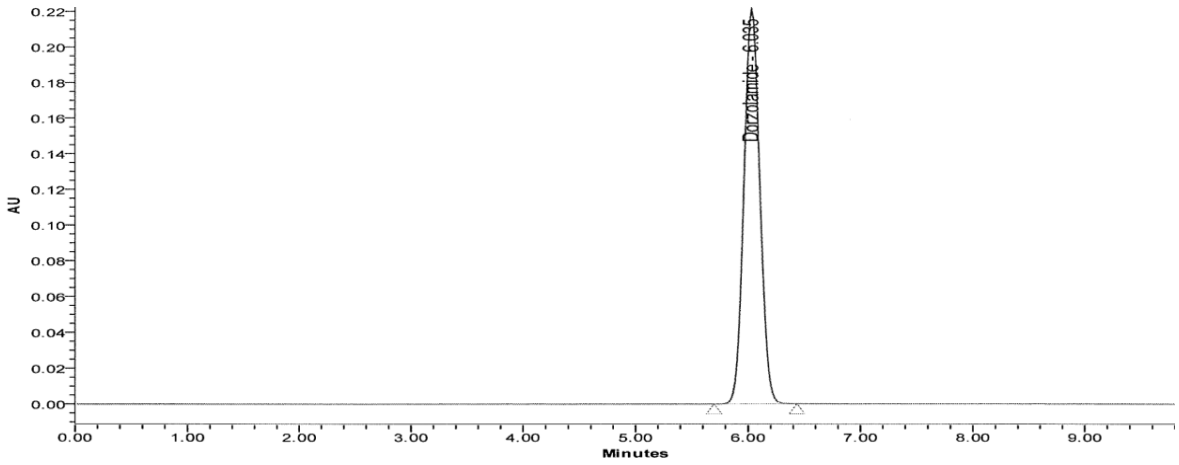

Figure 4: Typical chromatogram of Dorzolamide (100 µg/mL) at 254 nm

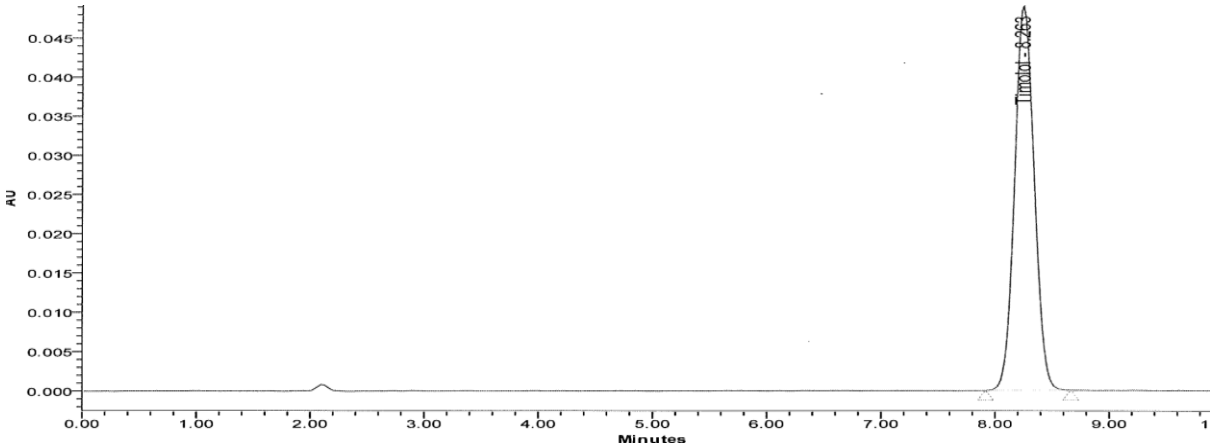

Figure 5: Typical chromatogram of Timolol maleate (50 µg/mL) at 295 nm

(Maleic acid eluted at 2.152 mins)

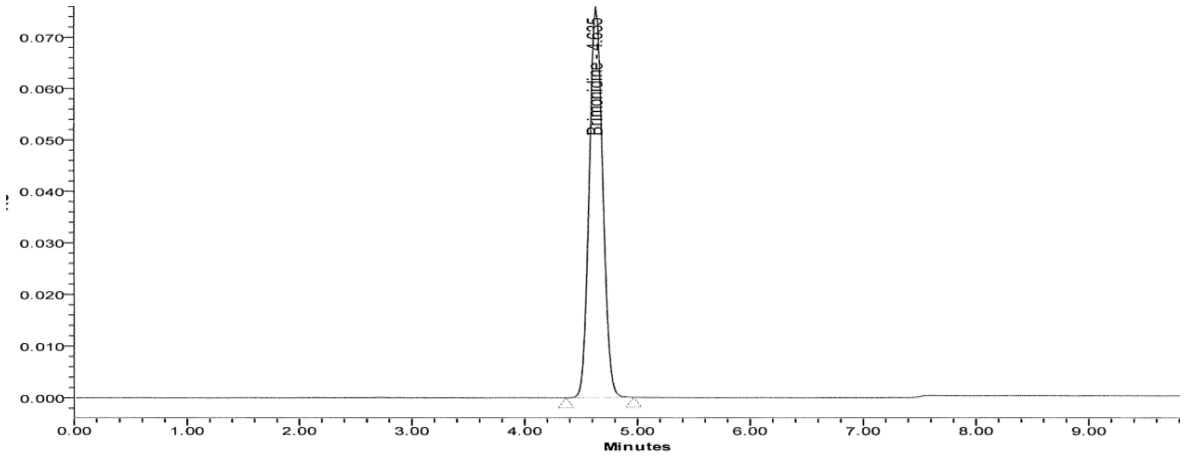

Figure 6: Typical chromatogram of Brimonidine (20 µg/mL) at 254 nm

Method Validation

Linearity

The proposed chromatographic method validated using ICH guidelines. Validation parameters performed include linearity, accuracy, precision, limit of detection and quantitation. Linear calibration plots for the proposed method were obtained in the concentration range of 4-720 µg/mL for Dorzolamide and 1 to 180 µg/mL for Timolol maleate in presence of Brimonidine as internal standard (Table 1).

The linear regression equation for Dorzolamide was found to be Y = 0.035X + 0.044 with correlation coefficient 0.999 and the linear regression equation for Timolol maleate found to be Y = 0.016X - 0.002 with correlation coefficient 0.999.

Table 1: Linearity for the simultaneous analysis of Dorzolamide and Timolol maleate in presence of Brimonidine (n=3)

Conc. DRZ (µg/mL)	Peak area ratio DRZ / BMD	Conc. TML (µg/mL)	Peak area ratio TML / BMD
4	0.1533	1	0.0164
8	0.287	2	0.032
20	0.7505	5	0.0859
40	1.4576	10	0.1656
80	2.9295	20	0.3318
160	5.8163	40	0.6641
200	7.2106	50	0.8259
240	8.715	60	1.0005
320	11.592	80	1.3339
400	14.3428	100	1.6548
480	17.0474	120	1.9698
560	19.8324	140	2.3006
600	21.5454	150	2.5033
720	25.8419	180	3.025

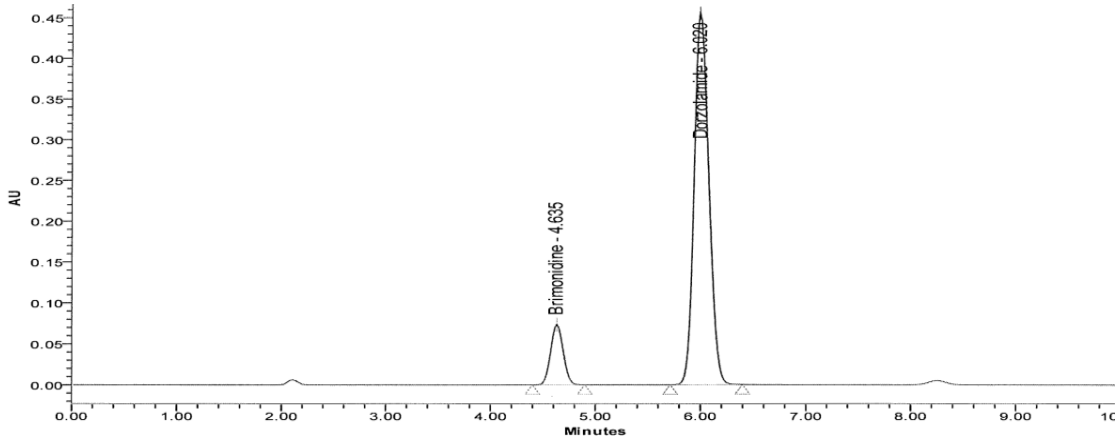

Figure 7: Representative chromatogram of Dorzolamide (200 µg/mL) with Brimonidine (IS) (20 µg/mL) at 254 nm

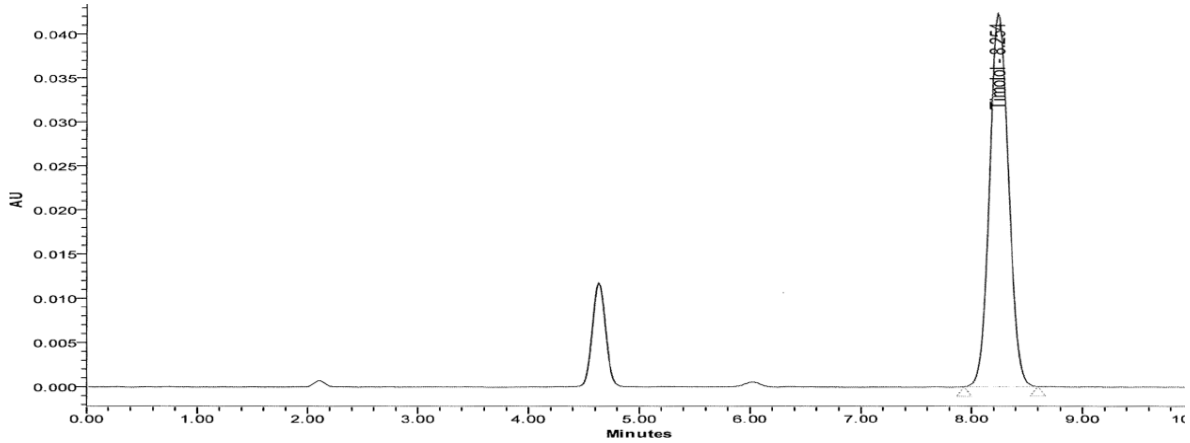

Figure 8: Representative chromatogram of Timolol maleate (50 µg/mL) with Brimonidine (IS) (20 µg/mL) at 295 nm

The representative chromatograms of Dorzolamide and Timolol maleate together in presence of IS at UV detection 254 and 295 nm (PDA detector) were shown in Figure 7 and Figure 8 respectively.

Limit of Detection and Limit of Quantification

LOD found to be 0.6951µg/mL and 0.2489 µg/mL for Dorzolamide and Timolol maleate respectively (signal to noise ratio of 3:1). LOQ found to be 2.3214 µg/mL and 0.8317 µg/mL for Dorzolamide and Timolol maleate respectively (signal to noise ratio of 10:1).

Precision

Within-day precision determined by injecting five standard solutions of three different concentrations on the same day (n=5) and between-day precision determined by injecting the same solutions for consecutive days. Relative standard deviation (RSD %) of the peak area calculated to represent precision. The results of within-day and between-day precision presented in Table 2.

Table 2: Intra-Day and Inter-Day Precision for the simultaneous analysis of Dorzolamide and Timolol maleate in presence of Brimonidine (n=3)

Drug	Conc. (µg/mL)	Intra-day Precision		Inter-day precision	
		Mean peak area ratio ± SD	RSD (%)	Mean peak area ratio ± SD	RSD (%)
DRZ	200	7.2193 ± 0.0317	0.439	7.91227 ± 0.0497	0.628
	400	14.3793 ± 0.0549	0.382	15.9748 ± 0.0809	0.507
	600	21.4301 ± 0.1787	0.834	21.5478 ± 0.2413	1.12
TML	50	0.8269 ± 0.00373	0.449	0.9256 ± 0.00631	0.682
	100	1.6594 ± 0.00581	0.349	1.9934 ± 0.00993	0.498
	150	2.4896 ± 0.02163	0.868	2.5034 ± 0.0313	1.25

SD = Standard deviation; RSD = Relative standard deviation

Accuracy

Accuracy of the method was performed by the standard addition technique. Three levels of solutions (50, 100 and 150%) of the nominal analytical concentrations prepared. The recovery and relative standard deviation for each analyte has given in Table 3. Recovery studies showed the method to be highly accurate and suitable for intended use.

Table 3: Accuracy for the simultaneous analysis of Dorzolamide and Timolol maleate in presence of Brimonidine (n=3)

Drug	Sample level%	Conc. (µg/ml)			Amount recovered (µg/ml)	% Recovery	% RSD
		Pure	Formulation	Total			
DRZ	50	20	40	60	59.91	99.85	0.23
	100	40	40	80	79.45	99.31	0.36
	150	60	40	100	99.76	99.76	0.39
TML	50	5	10	15	14.84	98.93	0.83
	100	10	10	20	19.83	99.15	0.76
	150	15	10	25	24.75	98.99	0.69

RSD = Relative standard deviation

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Assay of the Tablet Dosage Forms

Application of the proposed method checked by analyzing the Dorzolamide and Timolol maleate in commercially available pharmaceutical formulations (MISOPT and OCUDOR eye drops). The results provided in Table 4 showed high percentage recoveries and low RSD (%) values for both analytes.

Table 4: Assay results of Dorzolamide and Timolol maleate in commercial eye drops (Total volume = 5 ml)

Brand Names	Ingredient	Label claim (%)	Drug Found (%)	% Recovery
MISOPT	Dorzolamide	2.0	1.9965	99.83
	Timolol maleate	0.5	0.4947	98.94
OCUDOR-T	Dorzolamide	2.0	1.9985	99.93
	Timolol maleate	0.5	0.4949	98.98

The representative chromatograms of Dorzolamide and Timolol maleate in presence of IS at UV detection 254 and 295 nm (PDA detector) were shown in Figure 9, 10, 11 and 12 respectively. The % recovery was found to be 99.83-99.93 for Dorzolamide and 98.94-98.98 for Timolol maleate respectively.

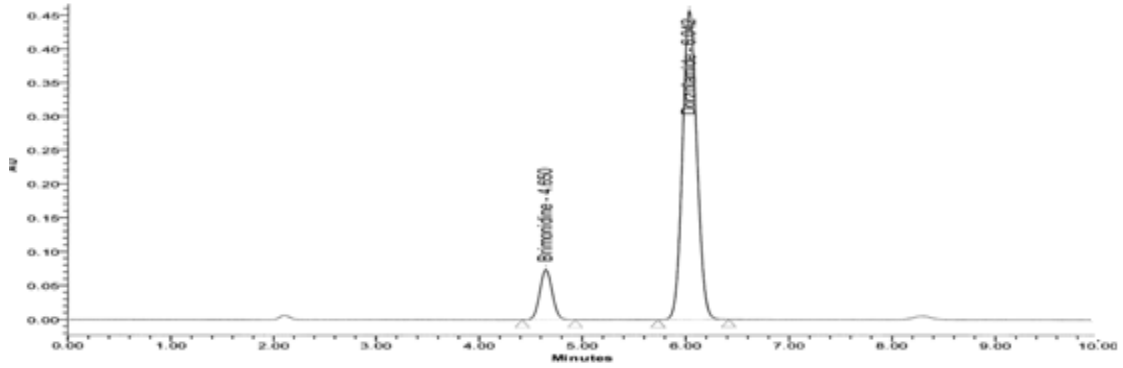

Figure 9: Typical chromatogram of DRZ (200 µg/mL) with BMD (20 µg/mL) with UV detection at 254 nm (MISOPT ® Eye Drops)

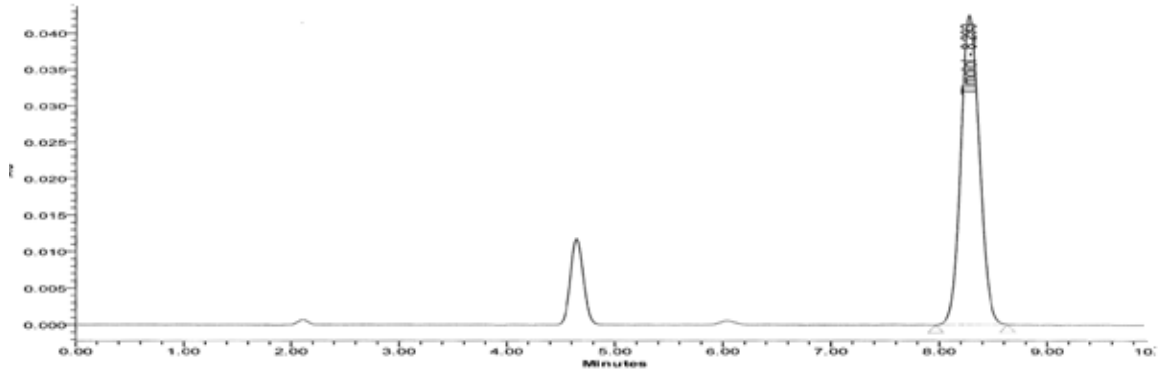

Figure 10: Typical chromatogram of TML (50 µg/mL) with BMD (20 µg/mL) with UV detection at 295 nm (MISOPT ® Eye Drops)

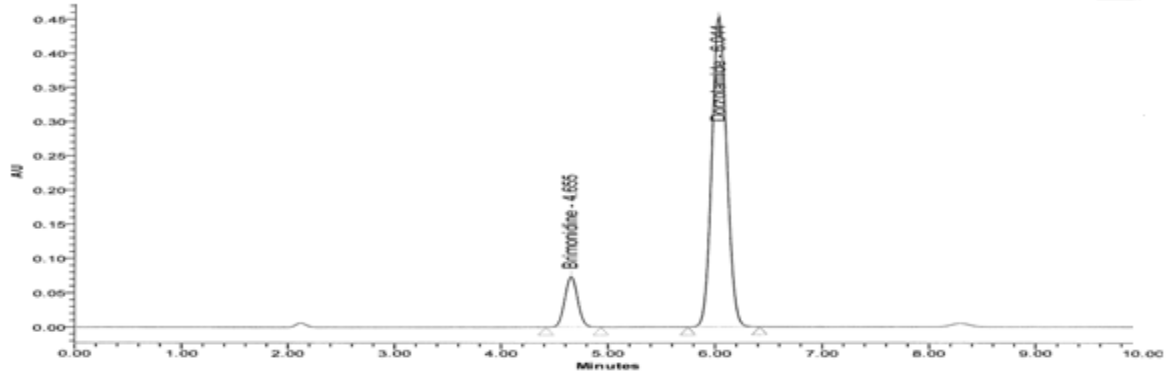

Figure 11: Typical chromatogram of DRZ (200 µg/mL) with BMD (20 µg/mL) with UV detection at 254 nm (OCUDOR ® Eye Drops)

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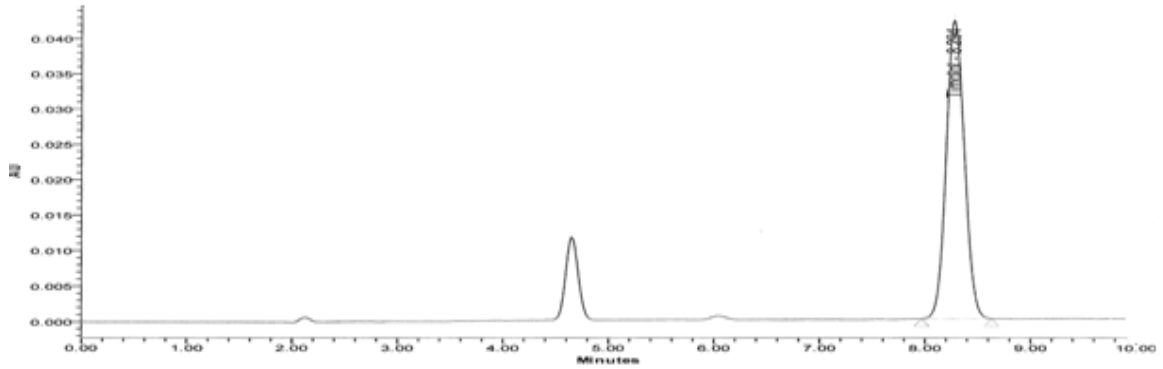

Figure 12: Typical chromatogram of TML (50 µg/mL) with BMD (20 µg/mL) with UV detection at 295 nm (OCUDOR ® Eye Drops)

System suitability

As system suitability test is an integral part of chromatographic methods development and it is used to verify that the system is adequate for the analysis to be performed, the parameter for Dorzolamide and Timolol maleate were evaluated. The theoretical plates were found to be 8676 for DRZ and 10707 for TML (N >2000) respectively. The resolution was >1.5. The tailing factor was found to be 1.05 and 1.03 for DRZ and TML respectively.

CONCLUSIONS

The advantages of the proposed method involve a simple procedure for sample preparation and relatively short time of analysis. Apart from this, it can be used for assays of Dorzolamide and Timolol maleate in biological fluids or in pharmacokinetic investigations. The proposed method was validated by testing its linearity, accuracy, precision, limits of detection and quantitation. The results of the analysis of pharmaceutical dosage forms by the proposed methods are highly reproducible, reliable, and are in good agreement with the label claims of the drug. The additives usually present in the pharmaceutical formulations of the assayed samples did not interfere with Dorzolamide and Timolol maleate. It may be said that the proposed methods are precise, sensitive, and accurate, so that these can be used as standard pharmacopoeial methods for the simultaneous determination of Dorzolamide and Timolol maleate using the HPLC systems with PDA detector.

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