Heta H. Desai and Anandkumari D. Captain. / Vol 4 / Issue 3 / 2014 / 168-177.

International Journal of Pharmaceutical Research & Analysis

e-ISSN: 2249 – 7781
Print ISSN: 2249 – 779X

www.ijpra.com

THREE SIMPLE VALIDATED UV SPECTROPHOTOMETRIC METHODS FOR THE SIMULTANEOUS ESTIMATION OF TIMOLOL MALEATE AND BRIMONIDINE TARTRATE AND THEIR COMPARISON USING ANOVA

Heta H. Desai* and Anandkumari D. Captain

Department of Pharmaceutical Chemistry, A.R College of Pharmacy & G.H Patel Institute of Pharmacy, Vallabh Vidyanagar, Anand, Gujarat, India-388120.

ABSTRACT

Timolol Maleate and Brimonidine Tartrate combination is used for the topical treatment of intra-occular pressure in patients of glaucoma. Three rapid, simple, accurate and precise UV spectrophotometric methods have been developed namely simultaneous equation method (Method 1), First Derivative Method (Method 2) and Ratio First Derivative Method (Method 3) for the simultaneous estimation of Timolol Maleate (TM) and Brimonidine Tartrate (BRT). In Method 1, Timolol Maleate and Brimonidine Tartrate exhibit good linearity over the concentration range of 2-50 µg/ml and 2-14 µg/ml respectively at 294 nm and 255 nm wavelengths. Method 2 is based on First Derivative Method, the ZCP of TM selected for the estimation of BRT is 294.3 nm and the ZCP of BRT selected for the estimation of TM is 302.3 nm. Linearity range is 2-50 µg/ml and 2-14 µg/ml for TM and BRT respectively. Method 3 is based on Ratio First Derivative Spectrophotometry. Linearity range is 2-50 µg/ml and 2-14 µg/ml at 290 nm and 244 nm for TM and BRT respectively. The proposed methods were validated according to the ICH guidelines for the evaluation of accuracy, precision and sensitivity. The three methods were compared using one-way ANOVA and the f_{cal} value was found to be less than f_{tab} value indicating that there is no significant difference in the assay results by the three methods. Thus, the proposed methods are rapid, accurate, precise and economically viable for the simultaneous estimation of TM and BRT respectively in pure form as well as in ophthalmic formulation.

Keywords: Timolol Maleate, Brimonidine Tartrate, Simultaneous Equation method, First Derivative Method, Ratio First Derivative Spectrophotometry, Validation, ANOVA.

INTRODUCTION

Timolol Maleate (TM), -)-1-(tert-butylamino)-3-[(4-morpholino-1,2,5-thiadiazol-3-yl)-oxy]-2-propanol maleate (1:1) (salt) is a non selective β-receptor blocker which reduces intra-ocular pressure in patients of open angle glaucoma. It is used topically to treat ocular hypertension (Fig.1a) [1]. Brimonidine Tartrate, 5-bromo-6-(2-imidazolidinylideneamino) quinoxaline L-tartrate is an α_2 receptor agonist which has a dual mechanism of action; it reduces aqueous humor production thereby reducing intraocular pressure and it also increases uveoscleral outflow. It is used topically to treat ocular hypertension (Fig.1b). Both the drugs are official in Merck Index and Martindale.[2,3] Timolol Maleate (TM) and Brimonidine Tartrate (BRT) are available in combination in eye-drop formulation for the topical treatment of open angle glaucoma and both the drugs have a synergistic effect with lesser side-effects and higher patient compliance.[4,5]

Literature review shows spectrophotometric methods, HPLC, HPTLC, Chemiluminescene, capillary electrophoresis and cyclic voltametry methods for the estimation of Timolol Maleate individually [6-12] and in combination with other drugs like Brinzolamide, Betaxolol, Latanoprost, Pilocarpine, oxprenolol and

Corresponding Author:-Heta Hemant Desai Email:- desaiheta.90@gmail.com

Dorzolamide. [12-23]. Stability indicating UV spectrophotometric method,GC-MS assay in plasma, HPLC method and Capilary Electrophoresis method in blood serum and aqueous humor, HPLC and HPTLC method on pure form and ophthalmic formulation, LC/MS/MS assay in ocular fluids and tissues, LC method for the estimation of pure drug and its related compounds, fluorimetric method and first derivative UV Spectrophotometric method have been reported for the estimation of Brimonidine Tartrate individually[24-35].

There is one TLC-densitometric method reported for the simultaneous estimation of Timolol Maleate and Brimonidine Tartrate in bulk and in formulation [36]. Literature review also suggests one RP-HPLC method for the simultaneous estimation of TM and BRT in nanoparticle formulation and one in conventional dosage form [37, 38]. Extensive Literature survey revealed one simultaneous equation method and q-absorbance ratio method for the simultaneous estimation of TM and BRT [39]. No first derivative and ratio first derivative method was found to be reported.

Hence the aim of the present work is to develop simple, rapid, accurate and precise simultaneous equation method, first derivative method and ratio derivative methods for the simultaneous estimation of TM and BRT in mixture form and comparing them statistically.

MATERIALS AND METHODS

Instrument

A dual beam UV visible Spectrophotometer (Shimadzu, Japan), model UV-1800, UV Probe software (version 2.35), Analytical balance (Shimadzu Electronic balance, Japan), model BL-220H were used for the study.

Materials

TM and BRT were kindly gifted by Astron Research Centre, Ahmedabad and FDC ltd., Mumbai respectively. Timolol Maleate and Brimonidine Tartrate eye drops were purchased from the local market. All other reagents and solvents used were of analytical grade.

Preparation of Working Standard Solutions

10 mg each of TM and BRT were accurately weighed and transferred to 100 ml volumetric flasks individually. The volume was made up to the mark with distilled water to produce 100µg/ml concentration of each of the drugs. These solutions were used as the working standard solutions.

CONSTRUCTION OF CALIBRATION CURVES

Calibration curves for Simultaneous equation method (Method I)

Standard solutions of both TM and BRT in the range of 2-50 µg/ml and 2-14 µg/ml respectively were separately prepared by appropriate dilutions of their respective working standard solutions in distilled water and then scanned in the range of 200–400 nm against distilled water as blank to determine the wavelength of maximum absorption for both drugs.

Two wavelengths 255nm (λ_{max} of BRT) and 294nm (λ_{max} of TM) were selected for the formation of simultaneous equation. The linearity curves of both the drugs are shown in fig. 3a and Fig.3b respectively. The absorptivity coefficients of each drug at both wavelengths were determined and substituted in their equation to obtain concentration of both drugs. The concentration of each compound in the mixture was calculated from the following simultaneous equations [40].

$C_{BRT} = A_2ay_1 - A_1ay_2 / ax_2ay_1 - ax_1ay_2$

$C_{TM} = A_1ax_2 - A_2ax_2 / ax_2ay_1 - ax_1ay_2$

Where, A_1 and A_2 are the absorbances of mixture at 255.0 nm and 294 nm respectively,

ax_1 and ax_2 are absorptivities of BRT at 255.0 nm and 294 nm respectively,

ay_1 and ay_2 are absorptivities of TM at 255.0 nm and 294 nm respectively,

C_{BRT} is concentration of BRT, C_{TM} is concentration of TM.

Calibration Curves for First Derivative Method (Method 2)

Standard solutions of both TM and BRT in the range of 2-50 µg/ml and 2-14 µg/ml respectively were separately prepared by appropriate dilutions of their respective working standard solutions in distilled water and then scanned in the range of 200–400 nm against distilled water as blank to determine the wavelength of maximum absorption for both drugs.

These spectrums were converted to first order derivative spectra by using derivative mode traced with interval of $\Delta\lambda$= 5 nm. For this method, 302.3 nm (ZCP of BRT) and 294.3 nm (ZCP of TM) were selected as wavelengths of measurements for TM and BRT respectively. There was proportionate increase in amplitude at 302.3 and 294.3 nm for TM and BRT respectively. The overlain spectrum of first order derivative of both the drugs is shown in the fig.4.

Calibration Curves for Ratio First Derivative Method (Method 3)

Standard solutions of both TM and BRT in the range of 2-50 µg/ml and 2-14 µg/ml respectively were separately prepared by appropriate dilutions of their respective working standard solutions in distilled water and then scanned in the range of 200–400 nm against distilled water as blank to determine the wavelength of maximum absorption for both drugs.

The ratio spectra of different TM standards at increasing concentration in distilled water were obtained by dividing each with the stored spectrum of the standard solution i.e. 2 µg/mL BRT using computer aid as divisor spectra; these ratio spectra are shown in Fig.5a. The first derivatives of this spectrum traced with interval of $\Delta\lambda$= 5

nm are illustrated in Fig.5b. As seen in Fig 6b, two minimum (216 nm & 311 nm) and one maximum (290 nm) exist and we found that both were suitable for determination of TM in TM and BRT eye drops. The wavelength 290 nm was selected for the determination of this compound in the assay of tablets, due to its lower RSD values and more suitable mean recovery compared with other wavelengths.

For the determination of BRT, the ratio spectra of different BRT standards at increasing concentrations in distilled water, obtained by dividing each with stored spectrum of the standard solution of 2 µg/ml of TM as divisor spectra by aid of computer software, are demonstrated in Fig.6a. The first derivatives of this spectrum traced with intervals of $\Delta\lambda$= 5 nm are illustrated in Fig.6b. As seen in Fig 6b, there exist one minimum (262 nm) and one maximum (244 nm) and in this both were suitable for the determination of BRT in TM and BRT eyedrops. The peak at wavelength 244 nm was selected because of its lower RSD and more suitable mean recoveries.

Validation of the methods

The proposed methods were validated as per the ICH guidelines Q2 (R1) for the evaluation of linearity, accuracy, precision, limit of detection and limit of quantitation.

Linearity of the calibration curves

The linearity of the methods was assessed by preparing 6 replicates each of standard solutions of TM and BRT in the concentration range of 2-50 µg/ml and 2-14 µg/ml for all the three methods. Calibration curves were constructed by plotting absorbance vs concentration and regression analysis was performed. (Table 1)

Accuracy

Accuracy was determined at three different level 80 %, 100 % and 120 % of the target concentration 13.6 µg/ml of TM and 4 µg/ml of BRT in triplicate. (Tables 2a, 2b & 2c).

Precision

Precision studies included the following studies:

Repeatability

The precision of the analytical method was studied by analysis of multiple sampling of homogeneous sample. Precision was estimated by repeatability and the repeatability was assessed by analyzing six determination of a homogeneous sample of 10 µg/ml of TM and 10µg/ml of BRT. (Table 3a).

Intra and Inter day Precision

Intra-day and inter day using three different concentrations 20µg/ml, 30 µg/ml and 40 µg/ml of TM intriplicate for three consecutive days and the value of precision of repeatability along with intra-day and inter day using three different concentrations 10µg/ml, 12 µg/ml and 14 µg/ml of BRT in triplicate for three consecutive days and %RSD was assessed. (Table 3b,3c& 3d).

Limit of Detection & Limit of Quantitation

LOD and LOQ of the drug were derived by calculating the signal-to-noise (i.e. 3.3 for LOD and 10 for LOQ) ratio using the following equations as per ICH guideline. (Table 1)

$LOD = 3.3 \times \sigma/S$

$LOQ = 10 \times \sigma/S$

Where, σ = the standard deviation of the response.

S = slope of the calibration curve.

Analysis of BRT and TM in eyedrop formulation

1 ml eyedrop solution containing 2 mg/ml BRT and 6.8 mg/ml TM was transferred into 100 ml volumetric flask containing 50 ml of distilled water and volume was made up to the mark with the same solvent to obtain a solution containing 200 µg/ml BRT and 680 µg/ml TM . From this solution , 2 ml aliquot was transferred into 10 ml volumetric flask and volume was made up to mark with distilled water to obtain the concentration of 13.6 µg/ml TM and 4µg/ml of BRT. Sample solution was prepared in triplicate and analyzed according to the proposed methods (Table 4).

RESULTS AND DISCUSSION

Method Development and Selection of Wavelength

Simultaneous Equation Method

To determine wavelength for measurement, standard spectra of BRT and TM were scanned between 200-400 nm against distilled water as blank. Absorbance maxima were obtained at 255 nm and at 294 nm for BRT and TM respectively. Overlain spectra of BRT and TM are presented in figure 2.

Linearity was obtained between 2-50 µg/ml for TM and 2-14 µg/ml for BRT at the selected wavelengths for each drug. The absorptivity coefficients were determined from the calibration curve graphs and substituted in the simultaneous equation to obtain the concentration of each drug. The linearity curves for both the drugs are shown in fig.3a and fig. 3b respectively.

First Derivative Method

Standard solutions of TM (2-50 µg/ml) and BRT (2-14 µg/ml) were scanned separately in the range of 200-400 nm. These spectrums were converted to first order derivative spectra by using derivative mode traced with interval of $\Delta\lambda$= 5 nm.First order derivative spectrum for TM showed zero crossing points: 220.2 nm,251.8 nm, and 294.3 nm. The wavelength selected for estimation of BRT was 294.3 nm because it showed adequate absorbance at this wavelength in mixture.

Similarly, first order derivative spectrum for BRT was taken and it showed zero crossing points; 216 nm, 231.5 nm, 255.07 nm and 302.3 nm. The wavelength selected for estimation of TM was 302.3 nm because it showed adequate absorbance at this wavelength in mixture. The overlain first derivative spectra of both the drugs is shown in fig.4.

Ratio First Derivative Method

This method works on two mechanisms viz. (1) Ratio and (2) Derivatization. In this method, the mixture spectra are divided with the divisor and first derivative spectra of these ratio spectra are generated. The main advantage of the ratio-spectra derivative spectrophotometry is the chance of doing easy measurements in correspondence of peaks so it permits the use of the wavelength of highest value of analytical signals (a maximum or a minimum). Moreover, the presence of a lot of maxima and minima is another advantage by the fact that these wavelengths give an opportunity for the determination of active compounds in the presence of other compounds and excipients which possibly interferes the assay.

The ratio spectra of different TM standards at increasing concentration in distilled water were obtained by dividing each with the stored spectrum of the standard solution of 2 µg/mL BRT using computer aid as divisor spectra; these ratio spectra are shown in Fig 5a. The first derivatives of this spectrum traced with interval of $\Delta\lambda$= 5 nm are illustrated in Fig. 5b. As seen in Fig 5b, two minimum (216 nm & 311 nm) and one maximum (290 nm) exist and we found that both were suitable for determination of TM in TM and BRT eye drops. The wavelength 290 nm was selected for the determination of this compound in the assay of tablets, due to its lower RSD values and more suitable mean recovery compared with other wavelengths.For the determination of BRT, the ratio spectra of different BRT standards at increasing concentrations in distilled water, obtained by dividing each with stored spectrum of the standard solution of 2 µg/mL of TM as divisor spectra by the aid of computer software, are demonstrated in Fig.6a. The first derivatives of this spectrum traced with intervals of $\Delta\lambda$= 5 nm are illustrated in Fig.6b. As seen in Fig 6b, there exist one minimum (262 nm) and one maximum (244 nm) and in this both were suitable for the determination of BRT in TM and BRT eyedrops. The peak at wavelength 244 nm was selected because of its lower RSD and more suitable mean recoveries.

VALIDATION OF THE PROPOSED METHODS

The proposed methods were validated for parameters like linearity, accuracy, and precision, limit of detection and limit of quantitation. The calibration curves were constructed for the proposed methods according to the concentration ranges and were found to be linear over the concentration range for TM and BRT with acceptable regression coefficient values as shown in the table 1 for the three proposed methods.

The table 1 also shows the limit of detection (LOD) and limit of quantitation (LOQ) values for TM and BRT for the three proposed methods.

The tables 2(a), 2 (b) & 2(c) depict respectively the percent recovery data at three concentration levels 80%, 100% and 120% for TM and BRT by the three proposed methods. The percent recovery is within the acceptable limits of 98-102% as per ICH guidelines and the %RSD is < 2%, thus indicating that the proposed methods are accurate.

The table 3(a) & 3 (b) depict the precision studies. Repeatability, Intra-day and inter-day precision was carried out and results showed %RSD <2 %, thus indicating that the proposed methods are precise.

Application of the proposed methods in the analysis of marketed formulation (eye drop)

The marketed eye drop formulation was analyzed in triplicate using the proposed methods. The content of TM was found to be in the range of 101.24-101.35 % and 100.9-101.08 % for BRT indicating the applicability of the proposed methods in the analysis of marketed ophthalmic formulation. The result of analysis of marketed formulation is shown in table 4. Fig. 7 shows the zero order spectra of eye-drop formulation.

Statistical Comparison of the methods using One-way ANOVA

Method 1, Method 2 and Method 3 were compared using one-way ANOVA and no significant difference was found between them as the F_{cal}value is less than F_{tab}. The results of one-way ANOVA are shown in table 5a and 5b.

Table 1. Linearity Data and Sensitivity (LOD & LOQ)

Parameters	Method 1				Method 2		Method 3	
	BRT	TM	BRT	TM	BRT	TM	BRT	TM
Wavelength (nm)	255	294	294	255	294.3	302.3	244	290
Linearity Range (µg/ml)	2-14	2-50	2-14	2-50	2-14	2-50	2-14	2-50

Regression Equations	Y=0.06x +0.034	Y=0.02x +0.042	Y=0.01x +0.012	Y=0.005x +0.013	Y=0.0005x-0.0006	Y=0.0005x+0.0012	Y=0.192x +0.062	Y=0.021x+0.041
Correlation Coefficient (r^2)	0.999	0.998	0.996	0.997	0.995	0.996	0.998	0.996
LOD (µg/ml)	0.272	0.24	0.369	0.5	0.167	0.14	0.114	0.091
LOQ (µg/ml)	0.824	0.727	1.118	1.515	0.506	0.424	0.347	0.276

Accuracy Studies

Table 2a. Recovery Studies for Method 1

Method-1						
Conc. of sample taken (µg/ml)	**Level**	**Conc. of pure API spiked(µg/ml)**	**Total Conc. (µg/ml)**	**Mean Total Conc. Found(µg/ml)(n=3)**	**% Recovery (n=3)**	**% RSD**
TM 13.6	80%	10.88	24.48	24.22	98.94	0.844
	100%	13.6	27.2	27.24	100.73	0.136
	120%	16.32	29.92	30.16	100.80	0.611
BRT 4	80%	3.2	7.2	7.24	100.55	1.12
	100%	4	8	8.12	101.5	0.83
	120%	4.8	8.8	8.7	98.86	0.56

Table 2a. Recovery Studies for Method 2

Method-2						
Conc. of sample taken (µg/ml)	**Level**	**Conc. Of pure API spiked(µg/ml)**	**Total Conc. (µg/ml)**	**Mean TotalConc.found (µg/ml) (n=3)**	**% Recovery Mean(n=3)**	**% RSD**
TM 13.6	80%	10.88	24.48	24.56	100.32	0.45
	100%	13.6	27.2	27.5	101.10	0.89
	120%	16.32	29.92	29.84	99.73	0.93
BRT 4	80%	3.2	7.2	7.31	101.52	0.52
	100%	4	8	8.15	101.87	0.65
	120%	4.8	8.8	8.9	101.13	0.43

Table 2(c). Recovery Studies for Method 3

Method-3						
Conc. of sample taken (µg/ml)	**Level**	**Conc. of pure API spiked(µg/ml)**	**Total Conc. (µg/ml)**	**Mean Total Conc. found(µg/ml)(n=3)**	**% Recovery (n=3)**	**% RSD**
TM 13.6	80%	10.88	24.48	24.5	100.08	0.36
	100%	13.6	27.2	27.3	100.36	0.49
	120%	16.32	29.92	29.7	99.26	0.71
BRT 4	80%	3.2	7.2	7.12	98.88	0.54
	100%	4	8	7.9	98.75	0.32
	120%	4.8	8.8	8.87	100.79	0.92

Table 3a. Repeatability Studies

	Method 1				**Method 2**		**Method 3**	
	TM (10µg/ml)		**BRT (10µg/ml)**		**TM (10µg/ml)**	**BRT (10 µg/ml)**	**TM (10µg/ml)**	**BRT (10 µg/ml)**
Wavelength	255 nm	294 nm	255 nm	294 nm	303.3	294.3	290 nm	244 nm
Mean Abs.	0.0703	0.279	0.7176	0.1606	0.00703	0.00503	0.26733	1.94533
±SD	0.000516	0.00103	0.001033	0.00103	0.0000516	0.0000516	0.001862	0.004179
%RSD	0.734	0.369	0.144	0.642	0.734	1.02	0.696	0.214

Table 3b. Intra-day and Inter-day Precision Studies for Method-1

Sr. no	Conc (µg/ml)TM	λ (nm)	Abs. of TM (n=3)	% RSD of TM	Conc. (µg/ml)BRT	λ (nm)	Abs. of BRT (n=3)	% RSD of BRT
Method-1: Intra- Day Precision								
1	20	255	0.114	0.503	10	255	0.719	0.212
2	30		0.163	0.613	12		0.873	0.238
3	40		0.211	0.722	14		1.151	0.132
1	20	294	0.524	0.190	10	294	0.161	0.358
2	30		0.695	0.219	12		0.203	0.752
3	40		0.925	0.165	14		0.256	0.449
Method-1: Inter- Day Precision								
1	20	255	0.115	0.869	10	255	0.717	0.489
2	30		0.162	0.940	12		0.87	0.526
3	40		0.213	0.938	14		1.148	0.495
1	20	294	0.523	0.764	10	294	0.160	1.25
2	30		0.696	0.438	12		0.204	1.296
3	40		0.923	0.329	14		0.2573	0.977

Table 3c. Intra-day & Inter-day Precision Studies for Method 2

Method-2 : Intra-day Precision								
Sr.No.	**Conc.(µg/ml) TM**	**Λ (nm)**	**Abs. of TM (n=3)**	**% RSD**	**Conc. (µg/ml) BRT**	**Λ (nm)**	**Abs. of BRT (n=3)**	**% RSD**
1.	10	302.3	0.00701	0.335	10	294.3	0.00503	1.03
2.	20		0.01303	0.361	12		0.006017	0.48
3.	30		0.01703	0.276	14		0.007467	0.77
Method-2: Inter-day Precision								
1.	10	302.3	0.00703	0.603	10	294.3	0.005033	1.14
2.	20		0.0131	0.721	12		0.00603	0.86
3.	30		0.0172	1.373	14		0.007433	1.55

Table 3d. Intra-day & Inter-day Precision Studies for Method 3

Method-3 : Intra-day Precision								
Sr.No.	**Conc.(µg/ml) TM**	**Λ (nm)**	**Abs. of TM (n=3)**	**% RSD**	**Conc. (µg/ml) BRT**	**Λ (nm)**	**Abs. of BRT (n=3)**	**% RSD**
1.	10	290	0.267	0.374	10	244	1.942	0.263
2.	20		0.511	0.298	12		2.328	0.429
3.	30		0.662	0.379	14		2.823	0.896
Method-3: Inter-day Precision								
1.	10	290	0.268	0.646	10	244	1.945	0.178
2.	20		0.515	0.388	12		2.33	0.333
3.	30		0.663	0.870	14		2.806	0.541

Table 4. Result of Analysis of Marketed formulation

Parameters	**Eye drop Formulation**					
	Method 1		**Method 2**		**Method 3**	
	BRT	**TM**	**BRT**	**TM**	**BRT**	**TM**
Theoretical Conc. (µg/ml)	4	13.6	4	13.6	13.6	4
Practically Obtained conc. (µg/ml) (n=6)	4.072	13.77	4.036	13.76	4.042	13.78
%Purity	101.08	101.29	100.9	101.24	101.05	101.35
%RSD	0.670	0.537	0.627	0.694	0.712	0.434

Table 5a. One-way ANOVA for TM

Sourceof variation	Sumof square	Degreeof freedom(d.f.)	Mean squares	F_{cal}	P-value	F_{tab}(at5 % significant level)
Between Methods	0.040	2	0.020	0.0639	0.938	3.682
Withinsamples	4.802	15	0.320			
Total	4.843	17				

Table 6a. One-way ANOVA for BRT

Sourceof variation	Sumof square	Degreeof freedom(d.f.)	Mean squares	F_{cal}	P-value	F_{tab}(at5 % significant level)
Between Methods	0.133	2	0.057	0.126	0.881	3.682
Within samples	6.754	15	0.450			
Total	6.887	17				

Fig 1a. Timolol Maleate

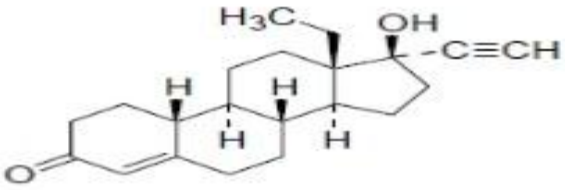


Fig 1b. Brimonidine Tartrate

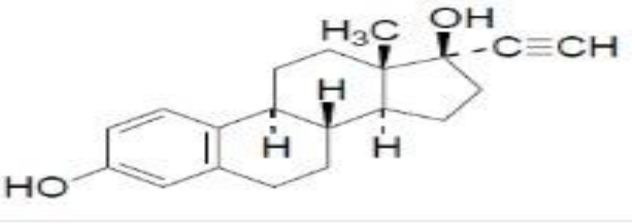


Fig 2. Overlain Spectra of TM and BRT

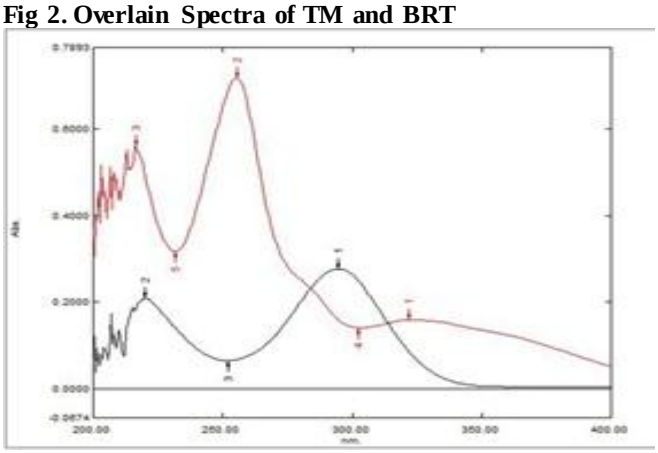


Fig 3a. Linearity Spectra of TM

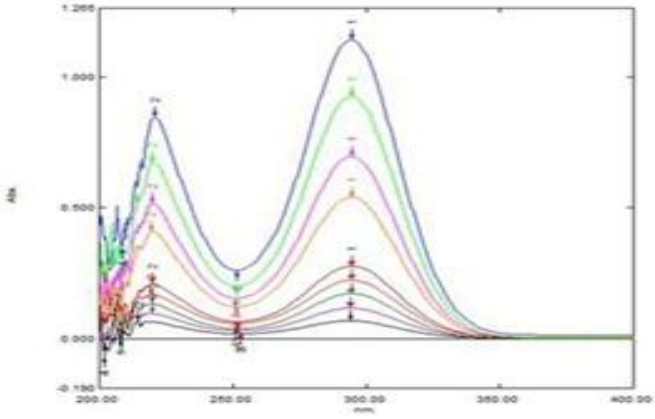


Fig 3b. Linearity Spectra of BRT

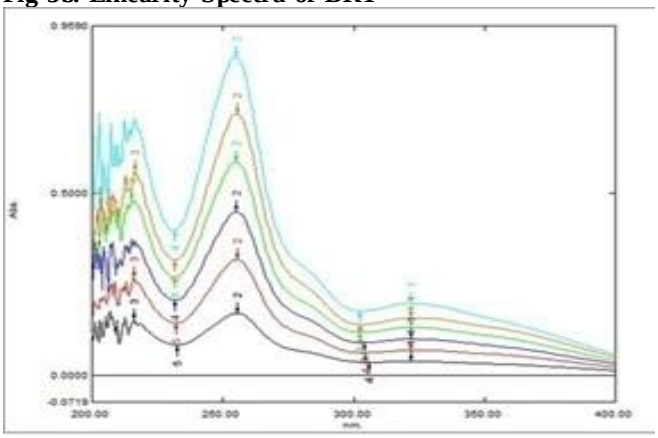


Fig 4. Overlain First Derivative Spectra of TM and BRT

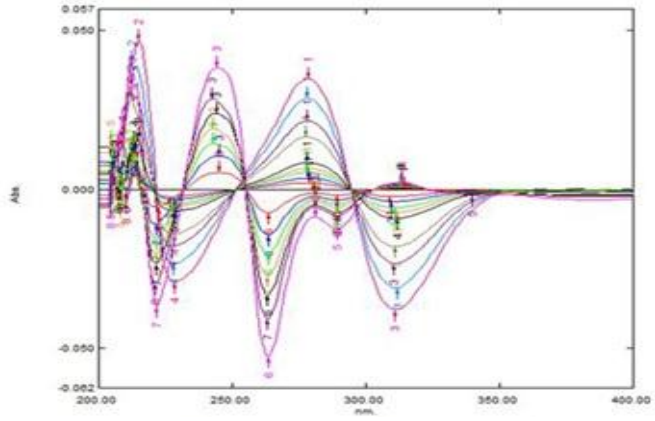


Fig 5a. Ratio Spectra of TM

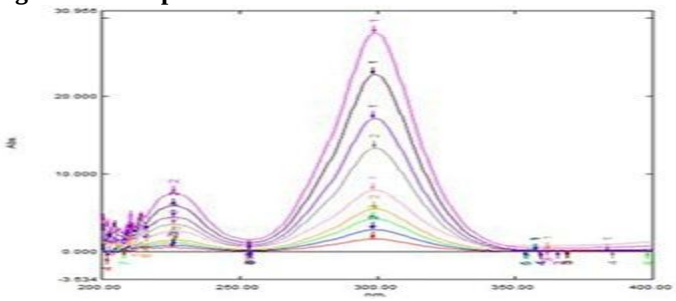


Fig 6a. Ratio Spectra of BRT

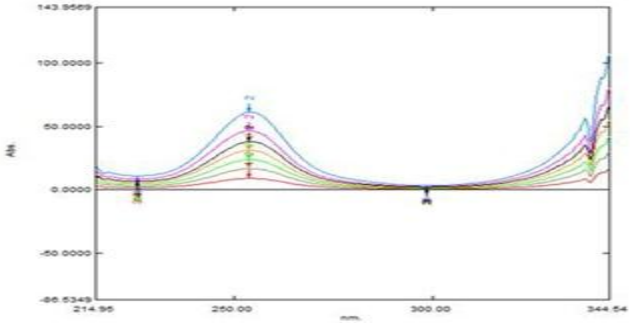


Fig 5b. Ratio First Derivative Spectra of TM

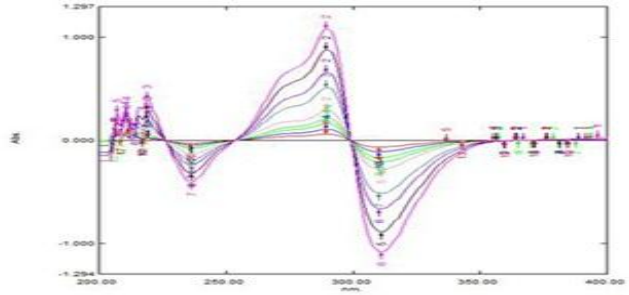


Fig 6b. Ratio First Derivative Spectra of BRT

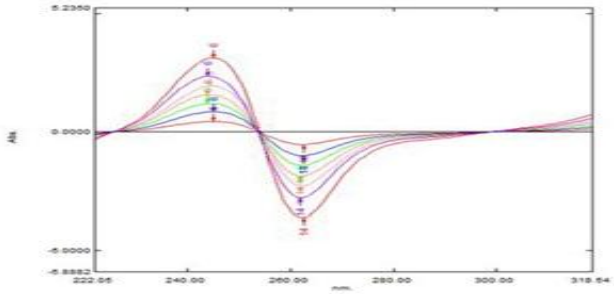
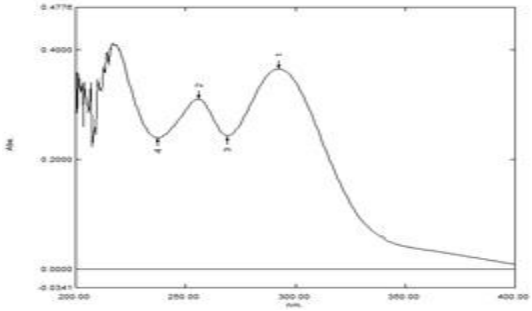


Fig 7. Zero order Spectra of eye-drop formulation



CONCLUSION

Three UV spectrophotometric methods were developed and validated as per the ICH guidelines. The methods were found to be accurate and precise with %RSD <2 % for the simultaneous estimation of TM and BRT. From the above results we can conclude that the proposed methods are simple, rapid, accurate, precise and economically viable as no prior separation procedure is required. The one-way ANOVA results show that there is no significant difference between the assay results obtained from these three methods.Hence theproposed methods can be used in routine analysis of TM and BRT with relatively less expensive and simple to operate instrumentation.

ACKNOWLEDGEMENT

We are thankful to Astron Research Centre, Ahmedabad and FDC ltd., Mumbai for providing gift sample of TM and BRT respectively. We also thank Department of Pharmaceutical Sciences, S.P University, Anand for their support and guidance.

REFERENCES

1. Indian Pharmacopoeia. Ministry of Health and Family Welfare; The Indian Pharamacopoeial Commission; Ghaziabad ;IP monographs. 3, 2010, 2224-2225.
2. The Merck Index. An encyclopedia of chemicals, drugs and biological. 14th Ed. Merck and Co. Inc.; 1996, 225, 1623.
3. Sweetman SC. Martindale, The Complete drug reference, 35th ed., Pharmaceutical Press, 2006, 1269-1718.
4. Schwartz GF, Burk C, Bennett T, Patel VD, Adherence and Persistence with Glaucoma Therapy: Brimonidine/Timolol versus Dorzolamide/ Timolol and Various Two-Bottle Combinations. *Journal of Clinical Experimental Ophthalmology.*, 3, 2012,248.

5. Lee Anne J, McCluskey P. "Fixed combination of topical brimonidine 0.2% and timolol 0.5% for glaucoma and uncontrolled intraocular pressure *Clinical Ophthalmology*, 2, 2008,545-555.
6. Kulkarni SP, Amin PD. Stability indicating HPTLC determination of Timolol Maleate as bulk drug and in pharmaceutical preparations, *Journal of Pharm Bio Analysis*, 23, 2000, 983–987.
7. Hamdan I.I, Qurani H.Development and Validation of a HPLC Method for Determination of Potential Residual Cortisone Compounds in Timolol Maleate Eye Drops), *Journal of Liq. Chrom.& Related Tech.*, 32, 2008, 449-467.
8. Mohammedi H, Shyale S, Shanta Kumar SM. Physico-Chemical Characterization, UV Spectrophotometric Method Development and Validation studies of Timolol Maleate., *International Journal of Pharm Sciences*, 6, 2011, 163–166.
9. Quan J, Wang Y. Chemiluminescence determination of timolol maleate by gold nanoparticles-catalyzed luminol – N-bromosuccinimide system. *Talanta*, 90, 2012, 117– 122.
10. Marini RD, Rozet E, Heyden YV, Ziemons E, Boulanger B, Bouklouze A. Nonaqueous capillary electrophoresis method for the enantiomeric purity determination of *S*-timolol using heptakis(2,3-di-*O*-methyl-6-*O*-sulfo)-β-cyclodextrin: Validation using the accuracy profile strategy and estimation of uncertainty. *Journal of Chrom A*, 1120, 2006, 102–111.
11. Norouzi P, Ganjali MR, Sepehri A, Ghorbani M. Novel method for fast determination of ultra-trace amounts of timolol maleate by continuous cyclic voltammetry at Au microelectrode in flowing injection systems. *Sensorrs Actuators B*, 110, 2005, 239–245.
12. Marini RD *et al*., Interlaboratory study of a NACE method for the determination of *R*-timolol content in *S*-timolol maleate: Assessment of uncertainty. *Electrophoresis*, 27, 2386–2399.
13. Nejadi R, Kazemipour M, Ansari M, Mehdipour M, Ahmadi R. Simultaneous determination of timolol maleate and Latanoprost tartrate in ophthalmic preparations by derivative spectrophotometry. *Research in Pharm Sci*, 2012, 7.
14. Maria LS, Juan CR, Héctor CG, Alejandro CO. Simultaneous Determination of Timolol Maleate and Pilocarpine Hydrochloride in Ophthalmic Solutions by First Derivative UV Spectrophotometry and PLS-1 Multivariate Calibration. *German natinal lib sci tech*, 32, 1999, 2019-2033.
15. Chiao-His C, Jiin-Long C, Ching-Tzy T. High pressure liquid chromatographic analysis oftimolol and betaxolol in eye drops. *J Med Sci*, 12, 1991, 86-95.
16. Erk N. Rapid and sensitive HPLC method for the simultaneous determination of dorzolamide hydrochloride and timolol maleate in eye drops with diode-array and UV detection. *International Journal of Pham Sci*, 58, 2003, 491-493.
17. Mathrusri AM, Narendra A.(2012) Development and Validation of RP-HPLC method for Simultaneous Determination of Dorzolamide and Timolol Maleate in Pharmaceutical dosage forms. *Journal drug deli Ther*, 2, 2012, 81–87.
18. Erk N. Determination of dorzolamide HCL and Timolol maleate in eye drops by two different spectroscopic Methods. *Journal of Pharm Bio Ana*, 2002, 391-397.
19. Bebawy L. Application of TLC-densitometry, first-derivative UV-spectrophotometry and ratio derivative spectrophotometry for the determination of dorzolamide hydrochloride and timolol maleate. *J Pharm Bio Ana*, 27, 2002, 737–746.
20. Shadoul WA, Gad Kariem EA, Adam ME, Ibrahim KE. Simultaneous Determination of Dorzolamide hydrochloride and Timolol maleate in ophthalmic solutions using HPLC. *Elixir Pharm*, 38, 2011, 4060–4063.
21. Maguregui MI, Jimenez RM, Alonso RM, Akesolo U. Quantitative determination of oxprenolol and timolol in urine by capillary zone electrophoresis. *Journal of Chr A*, 949, 2002, 91-97.
22. Shah Purvi A, KadikarAnkita S, KatiraRiddhi M, Patel Kalpana G, Gandhi Tejal R. Simultaneous Determination of Brinzolamide and Timolol Maleate using Three Different Spectrophotometric Methods. *World Journal of Pharmacy &Pharmaceutical Sciences*, 3, 2014, 1955-1967.
23. Khatun R and Islam Ashraful SM. Development and Validation of Analytical Method for Simultaneous Estimation of Brinzolamide and Timolol by HPLC from Opthalmic Preparation. *International Journal of PharmSci& Research*, 5, 2014, 1001-1007.
24. Bhagav P, Deshpande P, Pandey S, Chandran S. Development and Validation of Stability Indicating UV Spectrophotometric Method for the Estimation of Brimonidine Tartrate in Pure Form, Formulations and Preformulation Studies. *Scholar Research Library*, 2, 2010, 106-122.
25. Acheampong A1, Tang-Liu DD. Measurement of Brimonidine Concentration in human plasma by a highly sensitive GC-MS Assay. *Journal of Pharm Biomed Anal*, 13, 1995, 995-1002.
26. Ali MS, Khatri AR, Munir MI, Ghori M. A Stability-Indicating Assay of Brimonidine Tartrate Ophthalmic Solution and Stress Testing Using HILIC. *Chromatographia*, 70, 2009, 539-544.
27. Tzovolou DN, Lamari F, Mela EK, Gartaganis SP, Karamanos NK. Capillary electrophoretic analysis of brimonidine in aqueous humor of the eye and blood sera and relation of its levels with intraocular pressure. *Biomed Chr*, 14, 2000, 301-305.
28. Shirke RR, Pai N. Rp-hplc determination of brimonidine tartrate in brimonidine tartrate eye drops. *Ind Drugs*, 39,2002, 484-486.

29. Ni J, Rowe J, Heidelbaugh T, Sinha S, Acheampong A. Characterization of benzimidazole and other oxidative and conjugative metabolites of brimonidine*in vitro* and in rats *in vivo* using on-line H/D exchange LC-MS/MS and stableisotope tracer techniques. *Xenobiotica*, 2007, 37, 205-220.
30. Kvapil L, Grepl M, Hradil P. Potential Impurity InBrimonidine-Their Synthesis And Mass Spectrometry Identification.*ActaUnivPalackiOlomucFacrernatChemica*, 42, 2003, 19-26.
31. Jiang S, Chappa AK, Proksch JW. A rapid and sensitive LC/MS/MS assay for the quantitation of brimonidine in ocular fluids and tissues.*J of Chr B*, 877, 2009, 107-114.
32. Madhvi A, Naidu A, SubbaRao DV, Srinivasu P. Development and Validation of a New LC Method for Analysis of Brimonidine Tartrate and Related Compounds. *Chromatographia*, 69, 2009, 1413-1419.
33. Bhagav P, Upadhyay H, ChandranS.Brimonidine tartrate-eudragit long-acting nanoparticles: formulation, optimization, *in vitro* and *in vivo* evaluation. *AAPS PharmSciTech*, 2011**,** 12.
34. Jain P, Khatal R, Surana S. Development and Validation of First Order Derivative UV Spectrophotometric Method For Determination of Brimonidine Tartrate in Bulk and in Formulation.*Asian J Phar Biol Res*., 3, 2011, 323-329.
35. Sunitha G, Bhagirath R, Alapati VR, Ramakrishna K., Subrahmanyam CVS and Anumolu PD. Fluorimetric Quantification of Brimonidine Tartrate in Eye drops. *Indian J Pharm Sci*, 75, 2013, 730-732.
36. Jain PS, Khatal RN, Jivani HN, Surana SJ, Development and validation of TLC- densitometry Method for the Simultaneous estimation of Brimonidine Tartrate and Timolol Maleate in Bulk and Pharmaceutical dosage form. *J Chromatograph SeparatTechniq*, 2, 2011, 113.
37. Arun P, Murugesan SK, Nanjaian M. Simultaneous Estimation of Brimonidine Tartrate and Timolol Maleate in Nanoparticles Formulation by RP-HPLC. *International Journal Recent Adv Pharm Res*, 3, 2011, 31-36.
38. Nagaraju P, Kiran Kumar S, Poornima CH. Development and Validation of RP-HPLC Method for the Simultaneous Estimation of Brimonidine Tartrate and Timolol Maleate in combined dosage form" Pharma Research Library, 2014.
39. Popaniya*et al*., Simultaneous Determination of Brimonidine Tartrate and Timolol Maleate in Combined Pharmaceutical Dosage Form using two different Green Spectrometric Methods. *World Journal of Pharmacy and Pharmaceutical Sciences*, 3, 2014, 1331-1340.
40. Bechett AH, Stenlake JB. Practical pharmaceutical chemistry, 4 ed, Part-2., CBS publishers and distributors, New Delhi, 2002, 275-337.