SIMULTANEOUS DETERMINATION OF BRINZOLAMIDE AND TIMOLOL MALEATE USING THREE DIFFERENT SPECTROPHOTOMETRIC METHODS

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ABSTRACT

Three simple, rapid and precise UV spectroscopic methods namely simultaneous equation (method 1), Q-absorbance ratio (method 2) and ratio first derivative (method 3) have been developed for the simultaneous determination of brinzolamide (BRZ) and timolol maleate (TM). In method 1, both the drugs exhibit good linearity over the concentration range of 5 to 30µg/ml at 254 and 294 wavelengths. Method 2 involves the formation of Q-absorbance equation using the absorptivity values at 272 nm (isoabsorptive point) and 254 nm (λ\text{max} of BRZ) and Beer’s Lambert’s law was obeyed over the concentration range of 5-30 µg/ml with regression coefficient 0.9985 and 0.9964 respectively. The third method is based on ratio first derivative spectrophotometry, and at 242.9 and at 296 nm, linear concentration range for BRZ and TM was 5 to 25 µg/ml with regression coefficient 0.9985 and 0.9944 respectively. The proposed methods were validated according to ICH guidelines for evaluation of accuracy, precision, sensitivity etc. In conclusion, the proposed methods are novel, simple, accurate, precise, sensitive, rapid and economically viable methods that do not require any prior separation procedure. The proposed three methods hold potential for simultaneous determination of BRZ and TM in ophthalmic formulation.

Keywords: Brinzolamide, Timolol maleate, Simultaneous equation method, Q-absorbance ratio method and Ratio first derivative method, validation.
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 a new active substance which is useful only for topical use in the treatment of glaucoma (Fig. 1a). Timolol maleate (TM), [(S)-1-[(1,1-dimethylethyl)amino]-3-[[4-(4-morpholiny)-1,2,5-thiadiazol-3-yl]oxy]-2 propanol (Z)-2 butenedioate(1:1)], is a non-selective beta-adrenergic receptor blocking agent that lowers the ocular pressure in open angle glaucoma and ocular hypertension hence acts as most effective β blocker as an anti-glaucoma agent (Fig. Ib) (11). 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 previous eye drops, DORZOX-T (dorzolamide and timolol maleate) (12).

Scientific literature reports that there are many analytical methods like spectrophotometry, HPLC, HPTLC, Chemiluminescence, capillary electrophoresis and cyclic voltammetry reported for the determination of TM individually (3-9) and spectrophotometry and RP-HPLC in combination with other drugs like Latanoprost, betaxolol, pilocarpine, Brimonidine and dorzolamide (10-18) while only one method for the determination of BRZ (19). To the best of our knowledge, none of the analytical method is available for simultaneous determination of combination of both the drugs in eye drop. In context to this, this research paper describes UV spectrophotometric methods that find wide application for the determination of BRZ and TM in ophthalmic formulation.

The aim of paper is to report three spectrophotometric methods namely simultaneous equation method (method 1), Q-absorbance ratio method (method 2) and ratio first derivative method (method 3) for the simultaneous determination of BRZ and TM in their mixture form. The proposed methods are simple, accurate, precise, economically viable, more rapid and novel methods that do not require any prior separation procedure.
MATERIALS AND METHODS

Instruments
A dual beam UV-Visible spectrophotometer (Shimadzu, Japan), model UV-1800 and UV-1650 having two matched quartz cells of 1 cm light path and fixed slit width (2 nm), UV probe software (Shimadzu version 2.34), electronic analytical balance (AUX-220D, Shimadzu) and Ultrasonic cleaner (USC 100) Toshniwal Process Instrument Pvt. Ltd. were used in the study.

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

Preparation of standard solutions
Stock solutions of 1000 µg /ml of BRZ and TM were prepared by dissolving accurately weighed quantity of 100 mg mentioned drugs in to 100 ml volumetric flasks, dissolved and diluted up to mark with water to obtain a final concentration of 1000 µg/ml. Further dilution was performed with water, to prepare working standard solutions containing 100 µg/ml of BRZ and TM each.

Construction of calibration curves
Calibration curves for simultaneous equation method (method 1)
Standard solutions of both TM and BRZ in the range of 5-30 µg/ml 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 254nm (λ\text{max} of BRZ) and 294nm (λ\text{max} of TM) were selected for the formation of simultaneous equation. 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 (120).

\[ C_{BRZ} = \frac{A_1 \alpha_2 y_1 - A_2 \alpha_1 y_2}{\alpha_2 \alpha_1 y_1 - \alpha_1 \alpha_2 y_2} \]  
\[ C_{TM} = \frac{A_1 \alpha_2 x_1 - A_2 \alpha_1 x_2}{\alpha_2 \alpha_1 x_1 - \alpha_1 \alpha_2 x_2} \]

Where, \( C_{BRZ} \) and \( C_{TM} \) are concentration of BRZ and TM respectively; \( A_1 \) and \( A_2 \) are absorbance of mixture at 294 nm and 254 nm respectively; \( \alpha_1 \) and \( \alpha_2 \) are absorptivity
coefficient of BRZ at 294 nm and 254 nm respectively; $a_y_1$ and $a_y_2$ are absorptivity coefficient of TM at 294 nm and 254 nm respectively.

**Calibration curves for Q absorbance Ratio method (method 2)**

Standard solutions of both TM and BRZ in the range of 5-30 µg/ml were separately prepared by appropriate dilutions of their respective working standard solutions in distilled water and then were scanned in the range of 200–400 nm. Then the absorbance values at 272 nm ($\lambda_{iso}$) and 254 nm ($\lambda_{max}$ of BRZ) were measured from which the absorptivity values for both drugs at the selected wavelengths were calculated. This method employs Q values and the concentrations of mentioned drugs in the prepared solutions were carried out by using following equations:

$$C_{BRZ} = \frac{(Q_m - Q_y)}{(Q_x - Q_y)} \times \frac{A}{a_x}$$

$$C_{TM} = \frac{(Q_x - Q_m)}{(Q_x - Q_y)} \times \frac{A}{a_y}$$

Where, $C_{BRZ}$ and $C_{TM}$ are concentrations of BRZ and TM in µg/ml respectively; $A$ is absorbance of sample at $\lambda_{272}$; $Q_x$ and $Q_y$ are ratio of absorptivity's of BRZ and TM at $\lambda_{254}$ and $\lambda_{272}$, respectively; $Q_m$ is ratio of absorbance sample at $\lambda_{254}$ and $\lambda_{272}$, respectively; $a_x$ is the absorptivity of BRZ at $\lambda_{272}$ and $a_y$ is the absorptivity of TM at $\lambda_{272}$.

**Calibration curves for ratio first derivative method (method 3)**

Samples were prepared into 10 ml separate volumetric flasks containing 5-25 µg/ml of BRZ and TM in distilled water. Absorption spectra of each solution for both drugs were recorded in the scanning range from 400 to 200 nm. Then absorption spectra of BRZ were divided by the spectrum of the standard solution of TM (10 µg/ml). By the same way, absorption spectra of TM were divided by the standard spectrum of BRZ (10 µg/ml). All spectra were stored in the IBM-PC. The first derivative of the ratio spectra of both drugs were calculated by taking $\Delta\lambda = 8$ nm.

**Validation of the methods**

Proposed methods were validated in accordance with ICH guidelines Q2 (R1) for evaluation of various parameters; linearity, limit of detection, limit of quantification, precision, accuracy and specificity ($^{[21]}$).

**Linearity of calibration curves:** Linear relationship between absorbance and concentration of both drugs were evaluated over the concentration range expressed by making five replicate
measurements in the concentrations range of 5-30 µg/ml in method I and II, while 5-25 µg/ml in method III for both BRZ and TM. Calibration plots were constructed by plotting the absorbance versus the concentration and treated using the method of ordinary least squares regression analysis. Moreover, linearity was also validated by applying “Bartlett’s test” for homoscedasticity of variance \(^{(22)}\). Although, the homoscedasticity requirement was fulfilled for the two regression lines, the slope and the intercept with their 95% confidence intervals were calculated using ordinary least squares. The linearity was evaluated visually and statistically by using the F-test for lack-of-fit (LOF) \(^{(23)}\).

**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.

**Accuracy:** To ascertain the accuracy of the proposed methods, recovery studies were carried out by standard addition method at three different levels 50%, 100% and 150% and had analyzed by the proposed method, in triplicate. This was done to check the recovery of the drug at different levels in the formulations by optimized method.

**Precision:** Precision of the developed methods was studied by performing intra-day and inter-day precision studies. The intra-day precision was determined by performing three measurements (in morning, afternoon and evening) on the same day. The inter-day precision of method was checked by repeating the study on three consecutive days. Intermediate precision was also performed by different analysts and by using different instruments. Results of intra-day, inter-day and intermediate precision are expressed in % RSD.

**Specificity:** Method specificity was achieved by analysis of different laboratory prepared mixtures of BRZ and TM by addition of reported excipients at various concentrations within their linearity range.

**Application of the proposed methods for the simultaneous determination of BRZ and TM**

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 to the mark with distilled water to get 1000 µg/ml BRZ and 500µg/ml TM. Working sample solutions of BRZ (10 µg/ml) and TM (5 µg/ml) were prepared by diluting suitable volumes of the stock sample solution with water. Sample solutions were prepared in triplicate and analyzed according to the above method procedure.

RESULTS AND DISCUSSION
The proposed methods, simultaneous equation method, Q-absorbance ratio method and ratio first derivative spectroscopic methods were developed and applied for the simultaneous determination of BRZ and TM in the binary mixture without prior separation steps. These methods were found to be simple, accurate, precise, economic and rapid for routine analysis of mentioned drugs.

Method development and selection of wavelength

Simultaneous equation method
The zero-order absorption spectra of BRZ and TM showed wavelength of maximum absorption for BRZ and TM at 254 nm and 294 nm respectively that was selected for formation of simultaneous estimation of both the drugs in method 1 (Figure II). Linearity of the both drugs was obtained in the concentration range of 5-30 µg/ml at both selected wavelength. The absorptivity coefficients of each drug at both wavelengths were determined and substituted in their equation to obtained concentration of both drugs.

Q-absorbance ratio method
In this method, the absorbances of BRZ and TM at concentration range of 5-30 µg/ml were measured at 272 nm (λiso) and 254 nm (λmax of BRZ) wavelength from which the
absorptivity values for both drugs at the selected wavelengths were calculated. The method employs Q values and the concentrations of the studied drugs in the prepared standard drug solutions. From the calculated regression equation, concentration of both drugs in sample solution was determined in µg/ml.

**Ratio first derivative spectrophotometric method**

This proposed method was applied for the simultaneous determination of BRZ and TM in the laboratory prepared binary mixture without interference of each other. The third method ratio first derivative method has various advantages of easy measurements on separate peaks, higher values of analytical signals, and no need to work at zero cross over point. The effect of divisor concentration on the analytical parameters such as slope, intercept and correlation coefficient was also tested. The chosen divisor concentration gave good results for the slope, intercept and correlation coefficient of calibration graphs as well as for selectivity.

The ratio spectra of different BRZ standards at increasing concentrations obtained by dividing each with the stored zero order spectrum of standard solution of 10 µg/ml solution of TM are shown in Figure III(a) and the first derivative of these spectra traced with the interval of Δλ= 8 nm are illustrated in Fig. III(b). Similarly, the ratio derivative spectra of the solutions of TM in different concentrations traced with the interval of Δλ= 8 nm by using the zero order spectra of standard solution of 10 µg/ml of BRZ as divisor by computer aid is demonstrated in Fig. IV. The Δλ found as optimum for the first derivative of their ratio spectra was 8 nm. From the Fig. III (b) and Fig. IV (b), wavelength maxima 242.90 nm and 294.90 nm were selected for the determination of the BRZ and TM respectively for quantitative determination of both drugs, due to its lower R.S.D. value and more suitable mean recovery.

![Fig. III: (a) Ratio derivative spectra of BRZ, 10 µg/mL TM as divisor and (b) Ratio first order derivative spectra of BRZ (λ_max 242.9 nm, Δλ = 8)](image-url)
Fig. IV: Ratio derivative spectra of TM, 10 µg/mL BRZ as divisor and (b) Ratio first order derivative spectra of TM (λmax 296 nm, Δλ = 8)

Validation of proposed methods
The proposed methods have been validated for linearity, precision, accuracy, limit of detection (LOD) and limit of quantification (LOQ), and specificity. The calibration curves were constructed for the proposed methods according to their respective concentration ranges and were found to be linear over the concentration range for BRZ and TF with acceptable regression coefficient as shown in Table I for three proposed methods. Furthermore, the homoscedasticity of the calibration standards was verified using a Bartlett’s test before performing regression. The results showed that the calculated χ² value is less than the critical value at 95% confidence interval, χ² (0.05, 5) = 9.488; thus proving the homogeneity of the variances. The lack-of-fit test results for the calibration data of mentioned drugs were presented in Table I in which F_{calc} values were smaller than the critical value 4.26. Thus, straight lines were considered adequate to describe the relationships between the absorbance and the concentrations for each drug.

Table I: Linear regression parameters of BRZ and TM for proposed methods

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Method 1</th>
<th>Method 2</th>
<th>Method 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BRZ</td>
<td>TM</td>
<td>BRZ</td>
</tr>
<tr>
<td>Wavelength (nm)</td>
<td>254</td>
<td>294</td>
<td>254</td>
</tr>
<tr>
<td>Linearity range (µg/ml)</td>
<td>5-30</td>
<td>5-30</td>
<td>5-30</td>
</tr>
<tr>
<td>Regression equations (Y)</td>
<td>0.0259x+0.028</td>
<td>0.002x-0.001</td>
<td>0.001x+0.01</td>
</tr>
<tr>
<td>Correlation coefficient (r)</td>
<td>0.9964</td>
<td>0.9968</td>
<td>0.9979</td>
</tr>
<tr>
<td>S.D. of intercept</td>
<td>0.0031</td>
<td>0.0033</td>
<td>0.0007</td>
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</table>
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<table>
<thead>
<tr>
<th>S.D. of slope</th>
<th>0.00048</th>
<th>0.0009</th>
<th>0.0008</th>
<th>0.0002</th>
<th>0.00048</th>
<th>0.0007</th>
<th>0.00032</th>
<th>0.00031</th>
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<tbody>
<tr>
<td>Bartlett’s test $^b$ ($\chi^2$)</td>
<td>0.0284</td>
<td>0.0035</td>
<td>0.073</td>
<td>0.0077</td>
<td>0.0284</td>
<td>2.7838</td>
<td>0.0057</td>
<td>0.0011</td>
</tr>
<tr>
<td>LOD (µg/ml)</td>
<td>0.39</td>
<td>0.070</td>
<td>0.39</td>
<td>0.42</td>
<td>0.25</td>
<td>0.17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LOQ (µg/ml)</td>
<td>1.20</td>
<td>0.213</td>
<td>1.20</td>
<td>1.27</td>
<td>0.76</td>
<td>0.51</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Average of five determinations, $^b$ $\chi^2$ critical value = 9.488 at $\alpha=0.05$, $^c$ F-ratio critical value= 4.26 at $\alpha=0.05$

Table I also depicts the LOD and LOQ value of BRZ and TM. Intra-day; Inter-day and intermediate precision (by using different analyst and instrument) studies showed % RSD < 2, thus demonstrating precision of the proposed methods (Table II). Recovery study by spiking the standard at 3 concentration levels, 50, 100 and 150 %, showed % RSD of less than 2% with acceptable percent recovery, indicating that the proposed method is accurate and can be applicable for routine analysis of formulation (Table III).

Table II: Precision study for BRZ and TM by proposed methods

<table>
<thead>
<tr>
<th>Precision $^a$ (%RSD)</th>
<th>Method 1</th>
<th>Method 2</th>
<th>Method 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BRZ</td>
<td>TM</td>
<td>BRZ</td>
</tr>
<tr>
<td><strong>Intra-day</strong> $^a$</td>
<td></td>
<td></td>
<td>0.35</td>
</tr>
<tr>
<td><strong>Inter-day</strong> $^a$</td>
<td></td>
<td></td>
<td>0.62</td>
</tr>
<tr>
<td>Precision by using different analyst $^a$</td>
<td></td>
<td></td>
<td>0.68</td>
</tr>
<tr>
<td>Analyst 1</td>
<td></td>
<td></td>
<td>1.66</td>
</tr>
<tr>
<td>Analyst 2</td>
<td></td>
<td></td>
<td>1.66</td>
</tr>
<tr>
<td>Precision by using different instrument $^a$</td>
<td></td>
<td></td>
<td>0.68</td>
</tr>
<tr>
<td>UV 1650</td>
<td></td>
<td></td>
<td>0.68</td>
</tr>
<tr>
<td>UV 1800</td>
<td></td>
<td></td>
<td>0.68</td>
</tr>
</tbody>
</table>

$^a$ Average of three determinations for each concentration

Table III: Recovery study at three concentration levels for BRZ and TM by proposed methods

<table>
<thead>
<tr>
<th>% Level</th>
<th>Amt taken (µg/ml)</th>
<th>Amt. of drug added (µg/ml)</th>
<th>Amt. of drug recovered (µg/ml) Mean ± S.D $^a$</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BRZ</td>
<td>TM</td>
<td>BRZ</td>
<td>TM</td>
</tr>
<tr>
<td><strong>For method 1 and 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>10</td>
<td>5</td>
<td>5</td>
<td>7.5</td>
</tr>
<tr>
<td>100</td>
<td>10</td>
<td>5</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

$^a$ Average of five determinations, $^b$ BRZ critical value = 9.488 at $\alpha=0.05$, $^c$ TM critical value= 4.26 at $\alpha=0.05$
Application of the proposed methods for the simultaneous determination of BRZ and TM

When laboratory prepared mixture analyzed in triplicate using the developed methods, no interference of the added excipients was observed. The content of BRZ was in the range of 98.90-99.50% and for TM in the range of 97.40-97.60%, which proves applicability of the developed methods in routine analysis of ophthalmic formulation (Table IV).

Table IV: Application of the proposed methods for the simultaneous determination of BRZ and TM in laboratory prepared synthetic mixture

<table>
<thead>
<tr>
<th>Method</th>
<th>Amount found (mg)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BRZ</td>
<td>TM</td>
</tr>
<tr>
<td>Method 1</td>
<td>9.89 ±0.102</td>
<td>4.87 ±0.022</td>
</tr>
<tr>
<td>Method 2</td>
<td>9.89 ±0.102</td>
<td>4.87 ±0.022</td>
</tr>
<tr>
<td>Method 3</td>
<td>9.95 ±0.141</td>
<td>4.88 ±0.052</td>
</tr>
</tbody>
</table>

\(^a\)mean of three determinations, \(^b\)at 294 nm, \(^c\)at 254 nm, \(^d\)at 242.9 nm, \(^e\)at 296 nm

CONCLUSION

Three UV Spectroscopic methods were developed and validated as per ICH guidelines. The proposed methods are found to be precise as revealed from % RSD less than 2, for simultaneous estimation of BRZ and TM in mixture over the applied range. From the obtained results, proposed methods are simple, rapid, sensitive, accurate, precise and does not require any prior separation procedure of BRZ and TM. Hence, the proposed method could be regarded as economically viable techniques in the routine quality control analysis of BRZ and TM either alone or in combination with a relatively inexpensive instrumentation.
ACKNOWLEDGEMENT
We are highly thankful to Biocon Pharmaceutical Pvt. Ltd, Bangalore, India and Marck Bioscience Pvt. Ltd, Kheda, Gujarat for providing gratis sample of BRZ and TM respectively.

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