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Development and validation of spectrophotometric methods for the simultaneous determination of Brimonidine Tartrate and Timolol Maleate in bulk and ophthalmic preparations

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Abstract

Three spectrophotometric methods have been developed and validated for the simultaneous estimation of a binary mixture of brimonidine tartrate (BRM) and timolol maleate (TML) in the presence of benzalkonium chloride (BNZ), namely derivative spectrophotometry (Method 1) calculated by numerical differentiation, Ratio spectra derivative zero crossing calculated by numerical differentiation (Method 2), and mean centering of ratio spectra (Method 3). Method 1 was applied to determine BRM at 286 nm, which shows zero crossing point with TML and BNZ, and TML was determined at 310 nm, which shows zero crossing point with BRM and BNZ. Method 2 was used to determine BRM and TML at 221.87 and 258.43 nm, respectively. Method 3 was applied for the determination of BRM at 221.9 nm and TML at 286.9 nm. BRM exhibited good linearity over the concentration range of 1.50 - 9.50 µg/mL for the three methods. TML was determined in the concentration range of 5.00 – 7.60 µg/mL, 5 – 10 µg/mL and 5.00 – 9.20 µg/mL for Methods 1, 2 and 3, respectively. The proposed methods have the requisite accuracy, selectivity and precision and were successfully applied for the determination of BRM and TML in the presence of BNZ in their pure forms, laboratory prepared mixtures and combined dosage forms. The results obtained for the analysis of both drugs in their pure forms by the proposed methods were statistically compared to those obtained by applying a reported HPLC method.

Keywords: Spectrophotometric Methods, Brimonidine Tartrate, Timolol Maleate and Benzalkonium Chloride.

1. Introduction:

Glaucoma is an eye disease characterized by a problem in the drainage of aqueous humor produced by the eye, causing an increase in the intraocular pressure, which in turn damages the optic nerve. Among the different classes that are used to decrease intraocular pressure for treatment of glaucoma are β-adrenergic blockers such as timolol, atenolol and metoprolol and α-adrenergic agonists such as brimonidine, clonidine and naphazoline, of which brimonidine and timolol

in combination at a set dose would be of interest for our study **(Lee et al., 2005)**.

Brimonidine tartrate *is 5-Bromo-N-(imidazolidin-2-ylidene) quinoxalin-6-amine (2R,3R)-2,3-dihydroxybutanedioate* **(Pharmacopeia. 2018)**. It is used for the treatment of chronic glaucoma or ocular hypertension as it is a selective α2-adrenergic receptor agonist. It works by reducing the intra-ocular pressure within 1 hour through a dual mechanism, reducing aqueous humor production upon short term treatment and stimulating aqueous humor outflow through the uveoscleral pathway upon long term treatment **(Cantor, 2006)**.

Timolol maleate is *(2S)-1-[(I,1-Dimethylethyl)amino]-3-[[4-(morpholin-4-yl)1,2,5-thiadiazol-3-yl]oxy]propan-z-ol(Z)-butenedioate* **(Pharmacopeia. 2018).** It's a non-selective β-adrenergic blocker used to treat open angle glaucoma and ocular hypertension with increased intraocular pressure. It functions by reducing the eye's aqueous fluid discharge. Its onset of action is considered to be fast as it appears after 20 minutes from its administration **(Pharmacopeia. 2016)**.

Derivative spectrophotometry is a technique that has been widely applied. It is based on derivatization of zero-order spectrum to separate overlapped signals. This results in more structured spectra of derivatives compared to zero-order ones that allow small differences from the original spectra to be amplified. By using this technique two or more analytes can be quantified with enhanced resolution without initial separation or purification **(O'Haver et al., 1982; Karpinska, 2004)**.

Derivative Calculation by Numerical Differentiation (ND) method:

Numerical differentiation method is considered to be the simplest method of derivative calculation. The derivative of a function can be explained graphically as the slope of this function.

For a separate spectrum x_i $(i = 1, \dots, n-1)$, if w_i $(i = 1, \dots, n-1)$ are its sampling wavelengths, where n is the number of data points in the spectrum.

The derivative calculation can be expressed as follows:

$y_1 = \frac{x_{i+1} - x_i}{w_{i+1} - w_i}$

Here, y_i $(i = 1, \dots, n-1)$ represent the series of derivatives of spectrum x_i

The main disadvantage of this method is increasing the noise effect in higher order derivatives.

Ratio spectra derivative is a method that exceeds the disadvantage of derivative method in the necessity of finding a zero-crossing for overlapping spectra in simultaneous determination of drugs in binary and ternary mixtures. The problem is that during selecting these critical wavelengths that would be used for measurements a significant loss of sensitivity and precision take place. Instead, in ratio spectra derivative calculation, the measurements are done easily on separate peaks and higher values of the analytical signals. The presence of a lot of maxima and minima solves the problem of ternary mixture through getting a zero-crossing point of two components corresponding to a maximum or minimum of the third one **(Erk et al., 2001)**.

Mean centering of ratio spectra is a very simple method used for resolving the problem of overlapping spectra in binary and ternary mixtures. Since no derivative steps are required in the method, the signal-to-noise ratio is enhanced **(Afkhami et al., 2005)**.

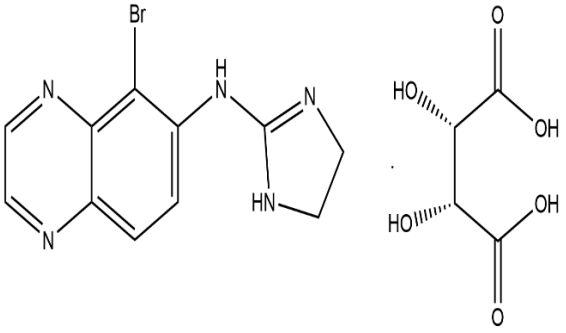
Mean centering theory is based on the fact that the absorbance of a ternary mixture consisting of components **X**, **Y** and **Z** is the sum of absorbances of each drug present in the ternary mixture, as illustrated in equation (1).

$$\boldsymbol{A_{mix} = \alpha_x C_x + \alpha_y C_y + \alpha_z C_z} \quad \textbf{(1)}$$ where

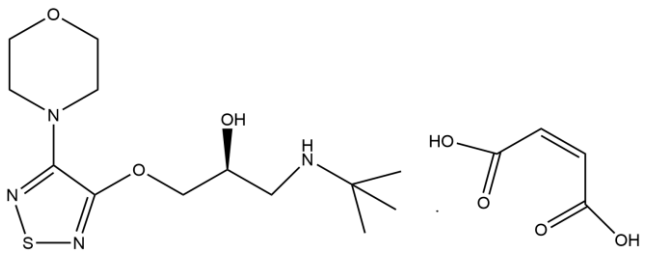
A_{mix} is the vector of the absorbances of the mixtures

α_x, α_y, α_z are the molar absorptivity vectors of **X**, **Y** and respectively.

C_x, C_y, C_Z are the concentrations of **X**, **Y** and **Z** respectively.



Brimonidine Tartrate



Timolol Maleate

Fig. 1. Chemical Structures of Brimonidine Tartrate and Timolol Maleate

Upon dividing Equation **(1)** by a spectrum of a standard solution of Z that is demonstrated by αZ, the first ratio of spectra is obtained as shown in Equation **(2)**
Mean centering of Equation **(2)** results in Equation **(3)**, in which Cz is eliminated as mean centering of constants is zero.
Then, Equation **(3)** is divided by the mean centered ratio of a spectrum of Y to a spectrum of Z. This results in the second ratio spectra as illustrated in Equation **(4).**
Mean centering of Equation **(4)** yields to omission of C_y whose mean centering is equal to zero, as illustrated in Equation **(5)**
The resulted spectra show peak maxima and minima at several wavelengths, whose amplitudes are dependent on component **X** concentration without any interference from the other two components in the ternary mixture, as illustrated in Equation **(5).**
The constructed calibration curve is between the amplitudes of peak maxima or minima at a selected wavelength and the corresponding concentrations of component **X**. A regression equation is calculated for method validation and prediction. The same procedure is applied for component **Y** in the ternary mixture.
By reviewing the literature, it was found that several UV-spectrophotometric methods have been reported for simultaneous determination of BRM and TML but few of them consider BNZ in their calculation **(Desai et al., 2014; Rizk et al., 2014; Dinc, 2017; Annapurna et al., 2017; Annapurna et al., 2021)**.

$$First\ ratio\ of\ spectra = \frac{A_{mix}}{\alpha_z} = \frac{\alpha_x C_x}{\alpha_z} + \frac{\alpha_y C_y}{\alpha_z} + C_z \quad \textbf{(2)}$$

$$Mean\ Centering\ of\ first\ ratio\ of\ spectra = MC\left[\frac{\alpha_x C_x}{\alpha_z}\right] + MC\left[\frac{\alpha_y C_y}{\alpha_z}\right] \quad \textbf{(3)}$$

$$Second\ ratio\ of\ spectra = \frac{MC(first\ ratio\ of\ spectra)}{MC(\alpha_y/\alpha_z)} = \frac{MC(\alpha_x C_x/\alpha_z)}{MC(\alpha_y/\alpha_z)} + C_y \quad \textbf{(4)}$$

$$Mean\ Centering\ of\ second\ ratio\ of\ spectra = MC\left[\frac{MC(\alpha_x C_x/\alpha_z)}{MC(\alpha_y/\alpha_z)}\right] \quad \textbf{(5)}$$

2. Experimental

2.1. Chemicals and drugs

BRM, TML and BNZ solution of 5% were kindly supplied as gift samples by EIPICO, Egypt. BRM and TML were certified to contain 99.38 % (w/w) and 99.76% (w/w) according to the manufacturer's method, respectively. Methanol (HPLC grade) and hydrochloric acid (analytical grade) were purchased from Sigma-Aldrich, Germany. Combigan® ophthalmic solution produced by Allergan for pharmaceutical industries, USA. Batch no. E88023 was obtained from the local market, where each 1 mL of ophthalmic solution is labeled to contain 0.2% brimonidine tartrate, 0.683% timolol maleate (equivalent to 0.5% timolol) and the inactive ingredient benzalkonium chloride as 0.005%.

2.2. Instrumentation and software

A Shimadzu UV 1650 double-beam spectrophotometer connected to a computer with Shimadzu software UV probe 2.10 was used, (Hiroshima, Japan). UV spectra were recorded using a 1-cm quartz cell; the scan range was 200 – 400 nm with 0.1 nm intervals. The computations were done using the Matlab® 7.0 software.

2.3. Preparation of stock and standard solutions

- **Stock solutions:**

The stock solutions were prepared by accurately weighing 50 mg of each of BRM and TML into a 50-mL volumetric flask, then dissolving each of them in 10 mL of methanol, and completing to volume with distilled water, to obtain a final concentration of 1.00 mg/mL for each of BRM and TML. For BNZ, 1 mL was taken from the 5% v/v stock solution, and transferred to 100-mL volumetric flask then completed to volume with distilled water to obtain a final concentration 0.05% v/v.

- **Working solutions:**

Suitable aliquots were taken from each stock solution and diluted using 0.05M hydrochloric acid to obtain two working solutions having concentrations of 100.00 µg/mL and 10.00 µg/mL for each of BRM and TML.

2.4. Pharmaceutical dosage form preparation

Stock solutions were prepared by accurately transferring 1 mL of Combigan® drops, expected to contain 2 mg of BRM and 6.83 mg TML into 100-mL volumetric flasks, to which 10 mL of methanol were added and the volumes were adjusted using distilled water. Suitable aliquots were taken from each stock solution and further diluted using 0.05M hydrochloric acid to obtain samples expected of having concentrations of 2.00 µg/mL and 6.83µg/mL for each of BRM and TML, respectively.

2.5. Method validation

Method 1: Derivative spectrophotometry calculated by numerical differentiation (ND)

- Linearity:

Aliquots were accurately transferred from the working solutions into 10-mL volumetric flasks and diluted with 0.05 M hydrochloric acid to obtain final concentrations of 1.50-9.50 µg /mL for BRM and 5.00-7.60 µg/mL for TML. The absorption spectra were recorded in the range 200 – 400 nm. The spectra were transferred to Matlab® 7.0 software for subsequent signal processing and analysis.

First derivatives (D^1) were then calculated for the scanned spectra by ND. The D^1 amplitudes were then recorded at 286 nm for BRM and 310 nm for TML. The results were plotted against corresponding concentrations of BRM and TML and the regression equations were then computed.

- Accuracy:

The procedure mentioned under linearity for each method was applied for different individual concentrations of pure BRM and TML in triplicates. The % recoveries, mean recovery and standard deviation were calculated.

- Precision:

Repeatability:

Freshly prepared solutions of concentrations 1.50, 2.50 and 4.00 µg/mL of BRM and 5.50, 6.60 and 7.40 µg/mL of TML were assayed in triplicates within the same day and relative standard deviation was calculated.

Intermediate precision:

It was estimated by measuring samples having the same concentrations of repeatability in triplicates for three successive days and relative standard deviation was then calculated.

- Specificity:

Aliquots of the studied compounds were transferred from their working solutions to prepare binary laboratory mixtures. They were analyzed using the procedure mentioned under linearity. The % recoveries, mean recovery and standard deviation were calculated.

Method 2: Ratio spectra derivative zero crossing calculated by numerical differentiation

- Linearity:

Aliquots were accurately transferred from the working solutions into 10-mL volumetric flasks and diluted with 0.05 M hydrochloric acid to obtain final concentrations of 1.50-9.50 µg/mL for BRM and 5.00-10.00 µg/mL for TML. The absorption spectra were recorded in the range 200 – 400 nm. The spectra were transferred to Matlab® 7.0 software for subsequent signal processing and analysis.

The zero order spectra of BRM (1.50-9.50 µg/mL) and TML (5.00-10.00 µg/mL) were divided by BNZ, TML and BRM respectively. First derivatives of ratio spectra (DD^1) were then calculated by the ND method. The DD^1 amplitude were then recorded at 221.87 nm for BRM and 258.43 nm for TML, using ND method. The recorded amplitudes were then plotted against the corresponding concentrations of BRM and TML, and the regression equations were then computed.

- Accuracy:

The procedure mentioned under linearity was applied for different individual concentrations of pure BRM and TML in triplicates. The % recoveries, mean recovery and standard deviation were calculated.

- Precision:

Repeatability:

Freshly prepared solutions of concentrations 6.50, 8.00 and 9.00 µg/mL of BRM and 6.80, 8.20 and 8.40 µg/mL of TML were assayed in triplicates within the same day and relative standard deviation was calculated.

Intermediate precision:

It was estimated by measuring samples having the same concentrations of repeatability in triplicates for three successive days and relative standard deviation was then calculated.

- Specificity:

Aliquots of the studied compounds were transferred from their working solutions to prepare binary laboratory mixtures. They were analyzed using the procedure mentioned under linearity. The % recoveries, mean recovery and standard deviation were calculated.

Method 3: Mean centering of ratio spectra

- **Linearity:**

Aliquots were accurately transferred from the working solutions into 10-mL volumetric flasks and diluted with 0.05 M HCL to obtain final concentrations of 1.50-9.50 µg/mL for BRM and 5.00-9.20 µg/mL for TML. The absorption spectra were recorded in the range 200 – 400 nm. The spectra were transferred to Matlab® 7.0 software for subsequent signal processing and analysis.

The scanned absorption spectra of BRM were divided by the normalized spectrum of TML **(TML`)** to produce the first ratio spectra. The second step was mean centering the obtained ratio spectra and dividing them by a mean centered ratio of a normalized spectrum of BNZ **(BNZ`)** to **TML`.** The resulted spectra represent the second ratio spectra that were mean centered then. For TML estimation, spectra of TML were divided by a normalized spectrum of BRM **(BRM`)**, to obtain the first ratio spectra. The obtained ratio spectra were mean centered and divided by a mean centered ratio of **BNZ` to BRM`.** The resulted spectra represent the second ratio spectra that were mean centered then.

Linear regression analysis was performed by plotting the peak amplitudes of mean-centered second ratio spectra at 222 nm and 286.9 nm for BRM and TML,

respectively versus corresponding concentrations. Calibration equations and correlation coefficients were calculated.

- **Accuracy:**

The previously mentioned procedure under linearity was applied to different concentrations of BRM and TML. The concentrations were calculated from the corresponding regression equations. The % recoveries, the mean recovery and the standard deviation were then calculated.

- **Precision:**

Repeatability:

Freshly prepared solutions of concentrations 4.00, 8.00 and 9.00 µg/mL of BRM and 5.20, 5.60 and 6.60 µg/mL of TML were assayed in triplicates within the same day and relative standard deviations were calculated.

Intermediate precision:

It was estimated by measuring samples having the same concentrations of repeatability in triplicates for three successive days and relative standard deviations were then calculated.

- **Specificity:**

Aliquots of the studied compounds were transferred from their working solutions to prepare binary laboratory mixtures. They were analyzed using the procedure mentioned under linearity. The % recoveries, mean recovery and standard deviation were calculated.

2. 6. Application to pharmaceutical preparation:

The procedure mentioned under linearity for each of the three methods was applied to the solutions of pharmaceutical preparations mentioned under (2.4). The concentrations were determined from the corresponding regression equations. The % recoveries, mean recovery and standard deviation were then calculated. The accuracy of the method was further assessed by applying the standard addition technique.

3. Results and discussion:

This work presents three simple spectrophotometric methods for the analysis of BRM and TML in the presence of BNZ. The literature review revealed several spectrophotometric methods for the analysis of BRM and TML but none of them considered the interference caused by the presence of BNZ that is added as a preservative in ophthalmic dosage forms. The absorption spectra of BRM, TML and BNZ show obvious spectral overlap in the wavelength range of 200 – 400 nm as presented in (**Fig. 2**) which hinder their simultaneous determination by direct spectrophotometry. Thus, the proposed methods were successfully applied to resolve this overlap and determine BRM and TML without prior separation.

Method 1: Derivative spectrophotometry calculated by ND

In derivative calculation by ND method, signal to noise ratio (SNR) is degenerated due to the noise that distorts the experimental signals. To control this noise and enhance SNR, wide $\Delta\lambda$ values can be used. They reduce the noise but at the same time they diminish the resolution to some extent. Many $\Delta\lambda$ values were tried to equilibrate between high resolution and good SNR and 16 nm difference was found to be optimal to guarantee data smoothing. D^1 spectra of BRM and TML in presence of BNZ were calculated. In **(Fig. 3)**, BRM shows a zero crossing with TML and BNZ at wavelength 286 nm and TML shows a zero crossing with BRM and BNZ at wavelength 310 nm. D1 spectra of BRM and TML serial dilutions were then calculated using ND method **(Fig.4)**.

The linear regression analysis was done by measuring the amplitudes at the selected wavelengths against the corresponding concentrations of BRM and TML. Linearity was in the range of 1.50 - 9.50 µg/mL and 5.00 – 7.60 µg/mL for BRM and TML, respectively (Fig.5).

$P = 0.0313\,C - 0.004 \qquad r = 0.9998$

$P = 0.0535\,C - 0.0178 \qquad r = 0.9998$

Where, "**P**" is the D^1 peak amplitude at the selected wavelength, "**C**" is the corresponding concentration in µg/mL, and "**r**" is the correlation coefficient.

Method 2: Ratio spectra derivative zero crossing calculated by ND

The same value of $\Delta\lambda$ (16 nm) was also found to be optimal for data smoothing. DD^1 of BRM and TML using BNZ as a divisor, DD^1 of BRM and BNZ using TML as a divisor and DD^1 of TML and BNZ using BRM as divisor were calculated. The first derivative ratio spectra using BRM and TML as divisors resulted in smoother points and less noise. In the DD^1 of BRM and BNZ using TML as a divisor **(Fig. 6)**, a zero-crossing point of BNZ was noticed at wavelength 221.87 nm, where BRM shows absorbance at which calibration and measurement could take place. DD^1 of TML and BNZ using BRM as a divisor was calculated (**Fig. 7**) and a zero crossing of BNZ was found at wavelength 258.43 nm, while TML exhibits absorbance at which calibration and measurement could take place.

DD^1 were calculated for serial dilutions of BRM (**Fig. 8)** and TML (**Fig. 9)** at the chosen wavelengths using ND method.

The linear regression analysis was done for the adopted method by plotting the amplitudes at the chosen wavelengths against the corresponding concentrations. Linearity was obvious in the range of 1.50-8.50 µg/mL for BRM and 5.00-10.00 µg/mL for TML (**Fig 10)**.

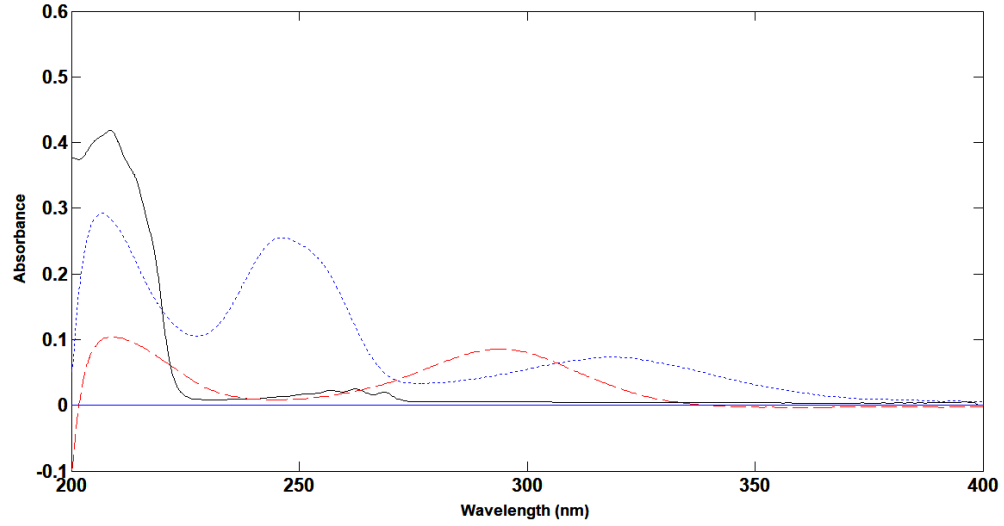


Fig.2. Absorption spectra of BRM (4.00 µg/mL ...), TML (5.00 µg/mL - -) and BNZ (0.01 µg/mL –) in 0.05 M hydrochloric acid.

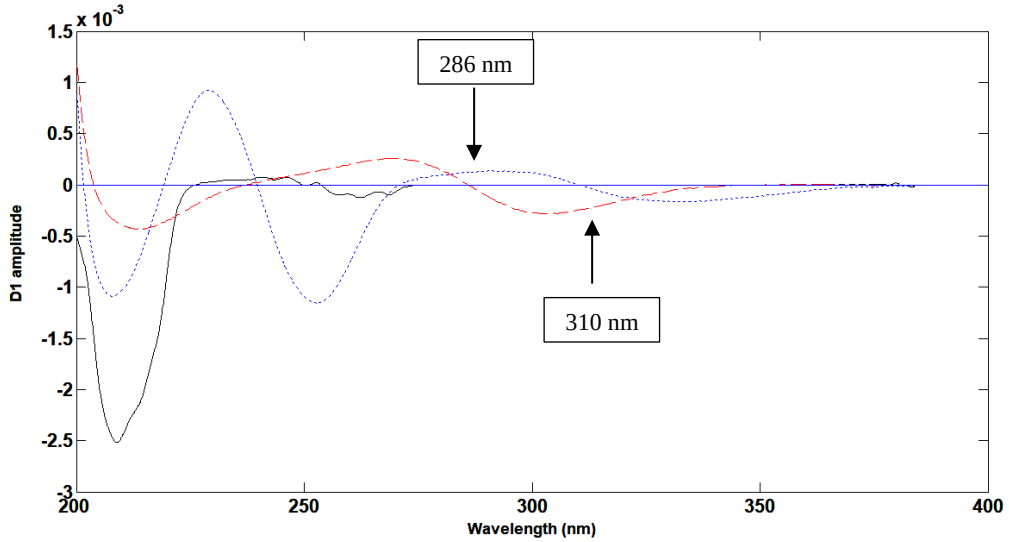


Fig.3. D^1 spectra of BRM (4.00 µg/mL …) and TML (5.00 µg/mL - -) in presence of BNZ (0.01 µg/mL –), using 0.05 hydrochloric acid as solvent, calculated by ND.

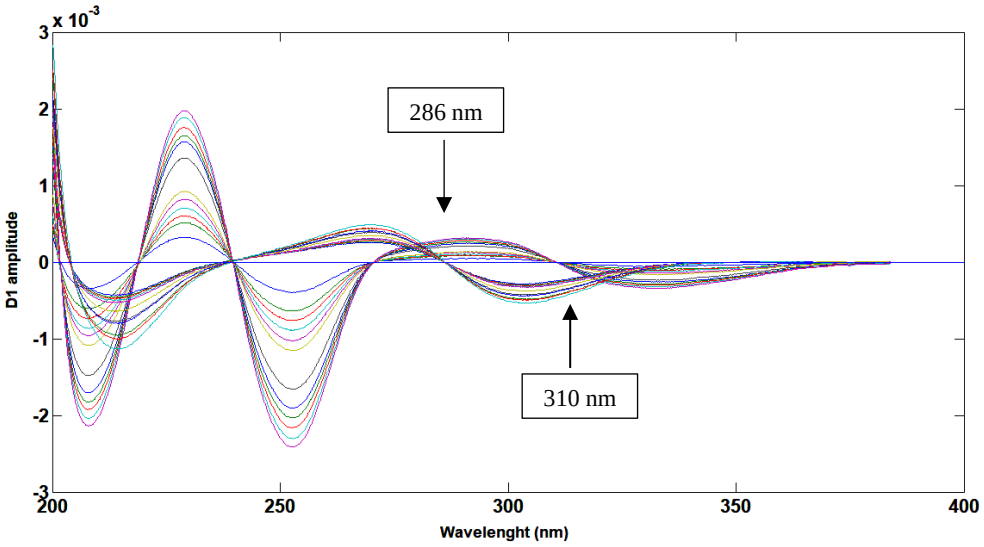
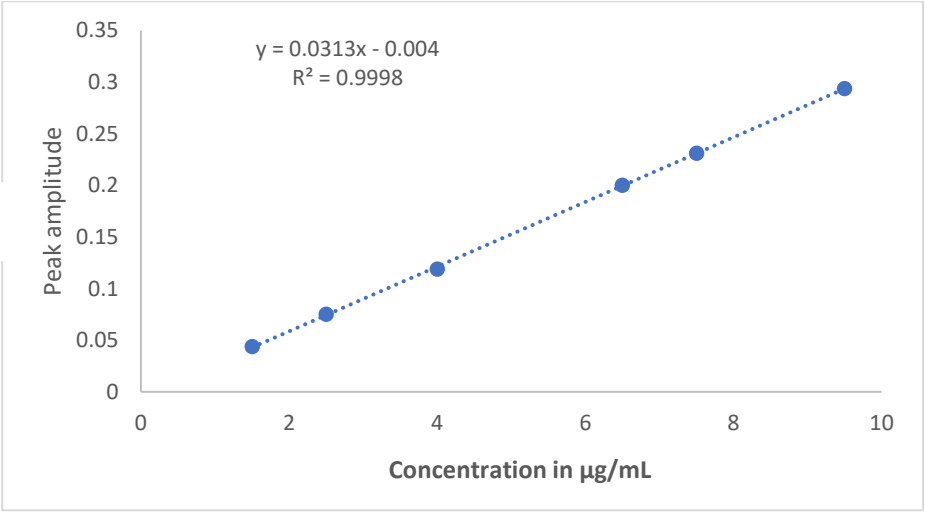


Fig.4. D[1] spectra of BRM, (1.50-9.50 µg/mL) and TML (5.00-7.60 µg/mL) using 0.05 M hydrochloric acid as solvent, calculated by ND.

(a)



(b)

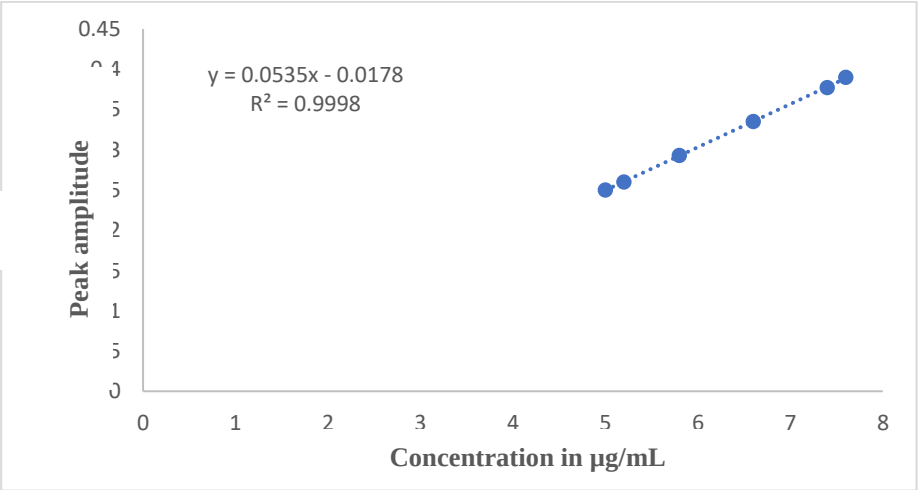


Fig.5. Calibration curves correlating the D[1] amplitudes to the corresponding concentrations of (a) BRM (1.50-9.50 µg/mL) and (b) TML (5.00-7.60 µg/mL), calculated by ND method at 286 nm and 310 nm, respectively.

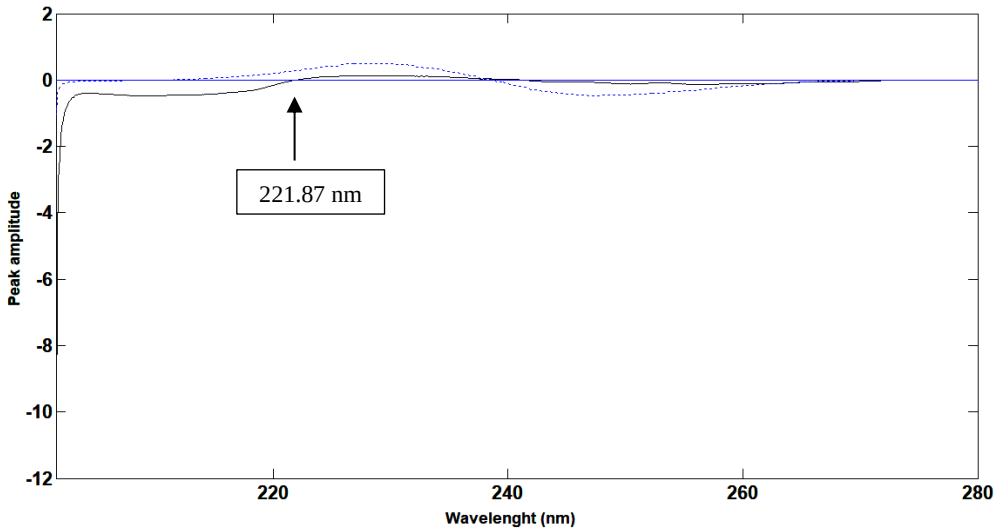


Fig.6. DD[1] of BRM (4.00 µg/mL…) and BNZ (0.01µg/mL –) using TML as a divisor in 0.05 M hydrochloric acid as solvent, calculated by ND method.

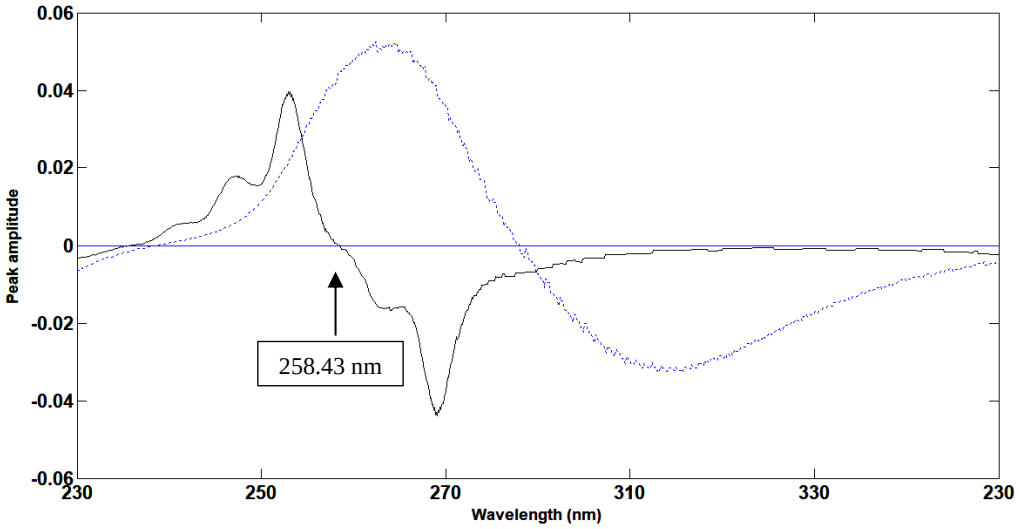


Fig.7. DD[1] of TML (5.00 µg/mL…) and BNZ (0.01µg/mL –) using BRM as a divisor in M 0.05 hydrochloric acid as solvent, calculated by ND method.

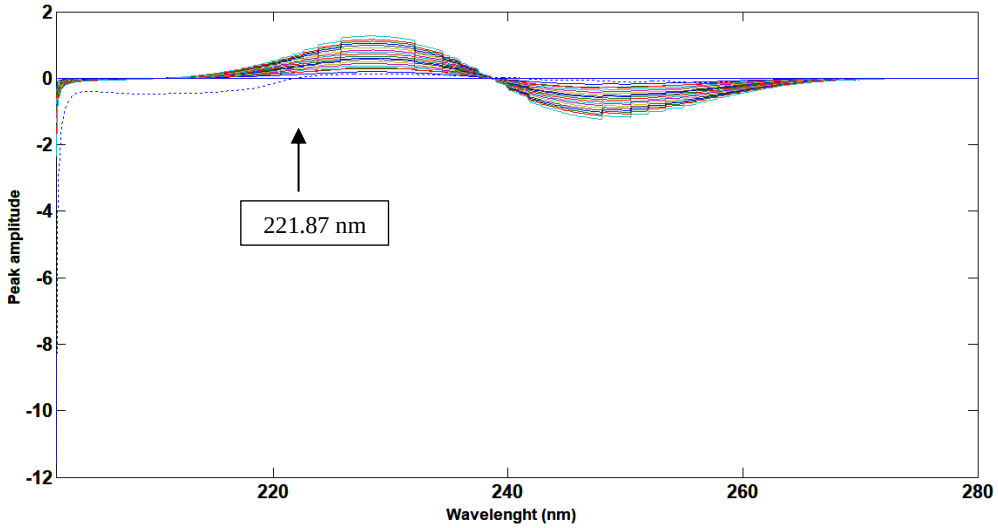


Fig.8. DD[1] of BRM (1.50-9.50 µg/mL) and BNZ (0.01µg/mL…) using TML as a divisor in 0.05 hydrochloric acid as solvent, calculated by ND method.

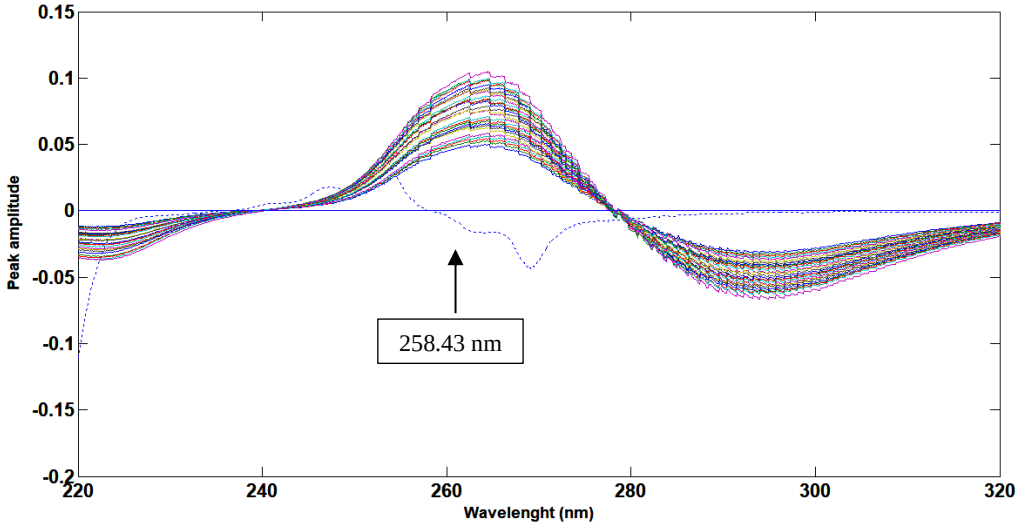


Fig.9. DD[1] of TML (5.00-10.00 µg/mL) and BNZ (0.01µg/mL…) using BRM as a divisor in 0.05 hydrochloric acid as solvent, calculated by ND method.

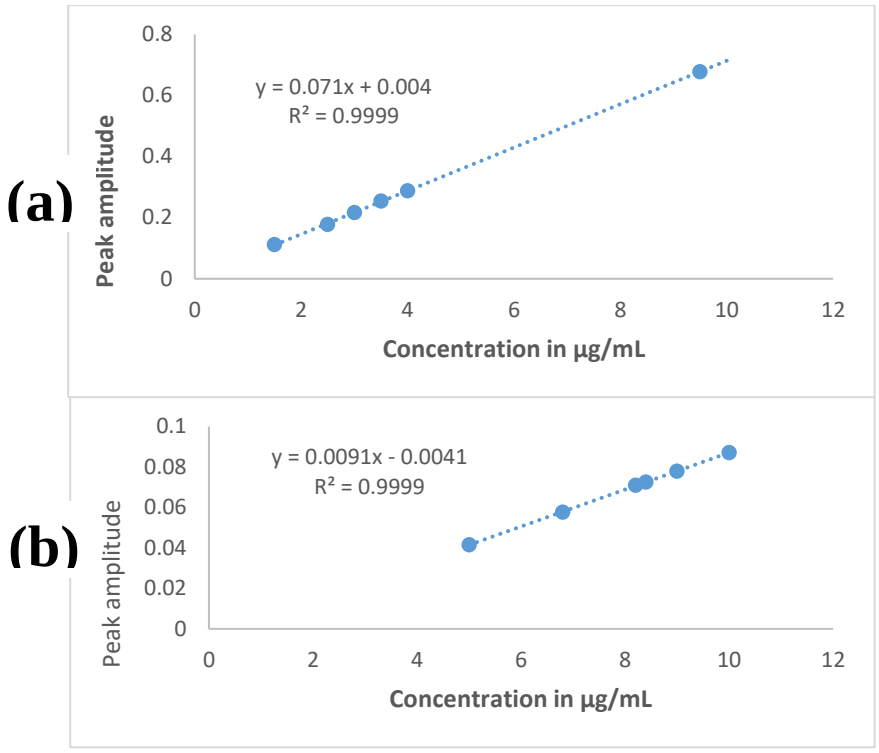


Fig.10. Calibration curves correlating the DD[1] amplitudes to the corresponding concentrations of (a) BRM (1.50 – 8.50 µg/mL) and (b) TML (5.00 – 10.00 µg/mL) at the chosen wavelengths, calculated by ND method.

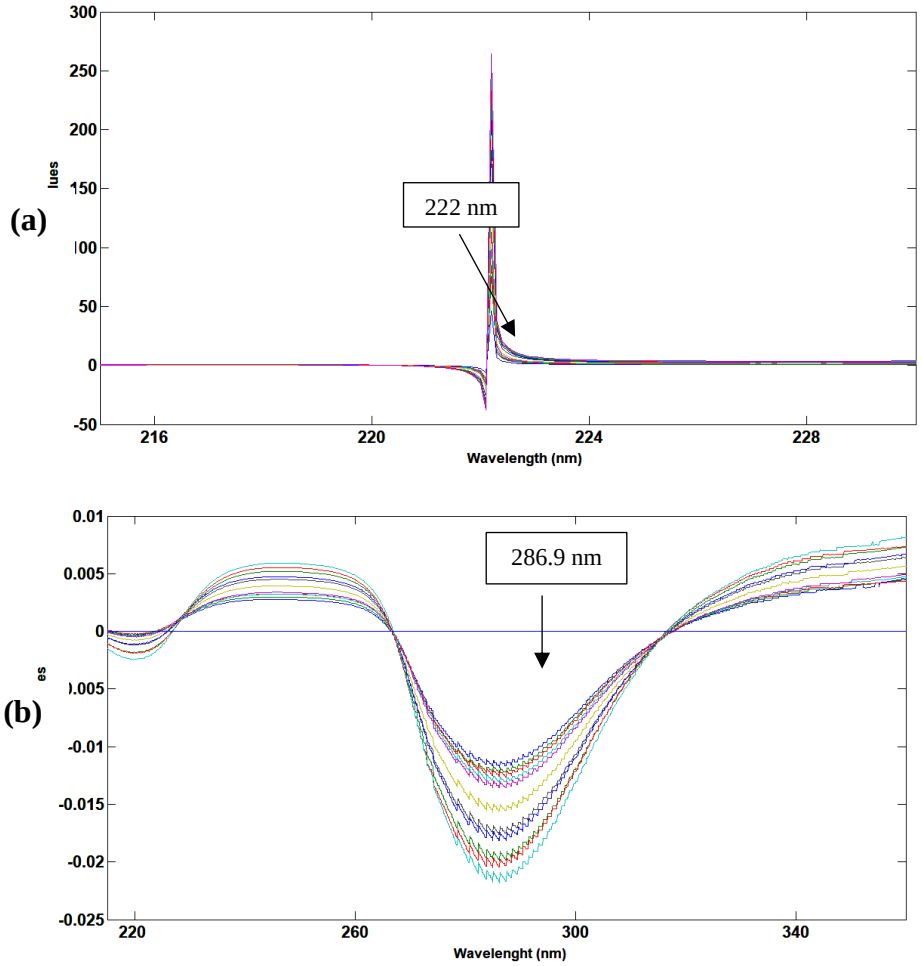


Fig. 11. The mean centring of the second ratio spectra of (a) 1.50 – 9.5 µg/mL of BRM and (b) 5.00 – 9.20 µg/mL of TML in 0.05 M hydrochloric acid.

The regression equations were calculated by ND method and were found to be:

BRM at 221.87 nm
$P = 0.071C + 0.004$ $r = 0.9999$
TML at 258.43 nm:
$P = 0.0091C - 0.0041$ $r = 0.9999$

Where "P" is the DD^1 peak amplitude at the chosen wavelengths, "C" is the corresponding concentration in µg/mL, and "r" is the correlation coefficient.

Method 3: Mean centering of ratio spectra

The absorption spectra of BRM were scanned in the wavelength range of 200 – 400 nm. The spectra were then divided by a normalized spectrum of TML (**TML`)** to obtain the first ratio spectra. Normalized spectrum is always preferred to be used as a divisor to avoid the negative effects of divisor concentration on analytical parameters such as detection limits, slope, intercept and correlation coefficient. Mean centering of the obtained ratio spectra and dividing it by a mean centered ratio of **BNZ`** to **TML`** was then performed. The resulting spectra represent the second ratio spectra which were mean centered subsequently. The mean centered second ratio spectra show peak maxima at 222.1, 226.89 and 264.3 nm and peak minima at 222, 237 and 264.2 nm. Calibration curve was constructed after selecting the optimum wavelength at which the calculated correlation coefficient was satisfactory. The BRM mean centered ratio of spectra with the selected wavelength for calibration which is 221.9 nm are demonstrated in (**Fig. 11.a**). The same procedure was applied for prediction of samples of precision as well as those of laboratory prepared mixtures and pharmaceutical preparations.

The absorption spectra of TML were scanned in wavelength range (200 – 400 nm). The spectra were then divided by **BRM`** to produce the first ratio spectra. The resulted ratio spectra were mean centered and divided by a mean centered ratio of **BNZ`** to **BRM`**. The obtained spectra that represent the second ratio spectra were mean centered. The mean centered second ratio spectra show peak maxima at 247.9 nm and 378.1 nm and peak minima at 286.9 nm and 378.2 nm. (**Fig.11.b)** illustrates the TML mean centered second ratio of spectra with the selected wavelength for calibration which is 286.9 nm.

Linearity was obtained in the range of 1.50-9.50 µg/mL and 5.00 - 9.20 µg/mL for BRM and TML, respectively (**Fig. 12)**.

The regression equations were calculated and found to be:

For BRM at 222 nm:
$MCN = -1.6283\ C - 0.1941$ r = 0.9999

For TML at 286.9 nm:
$MCN = 0.0024\ C - 0.0005$ r = 0.9999

Where, "MCN" are the mean centered values at the selected wavelengths, "C" is the concentration in µg/mL and "r" is the correlation coefficient.

Method validation of the three proposed methods was done according to the ICH guidelines **(ICH. 2005)**. The regression parameters for the determination of BRM and TML by the proposed methods, as well as the results of accuracy, precision, specificity, limit of detection and limit of quantification are demonstrated in (**Table 1**). Accuracy results obtained by each of the adopted methods are illustrated in (**Table 2**). Specificity of the proposed methods was assessed through analyzing laboratory prepared mixtures containing different ratios of BRM and TML as shown in (**Table 3**).

The adopted methods were also applied for the simultaneous determination of BRM and TML in Combigan® ophthalmic solution. Standard addition technique was also applied. Results obtained by applying each method are shown in (**Table 4**).

The results obtained for accuracy by the proposed methods were statistically compared to those of a reported method **(Ibrahim et al., 2019)** and no significant difference was found between the results of Method 1 and Method 3 as the calculated t and F values are less than the tabulated ones at confidence limit of 95%. The results of Method 2 showed a significant difference when compared to those of the reported metod. (**Table 5**).

4. Conclusion

The proposed spectrophotometric methods are simple, selective, accurate and precise. They were successfully applied for the simultaneous determination of BRM and TML in their pure forms and in pharmaceutical dosage forms. We recommend the application of Method 1 and Method 3 for the analysis of a combination of the two drugs as the results obtained by Method 2 and the reported method were significantly different.

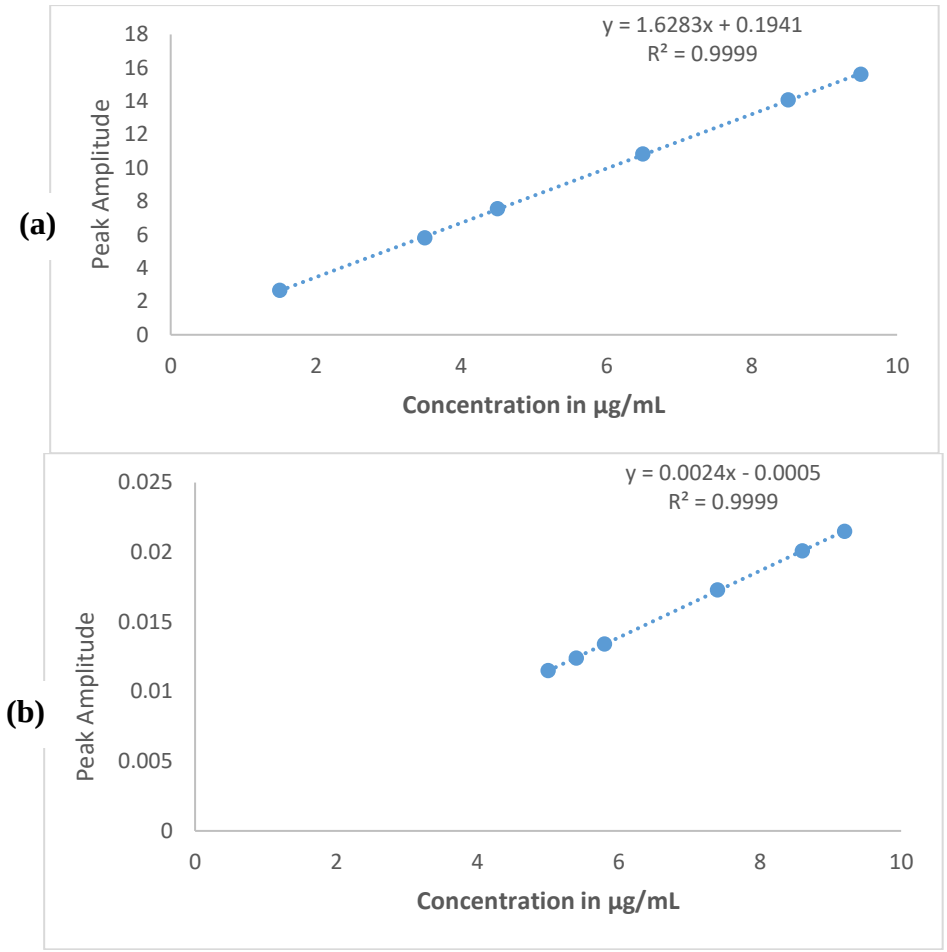


Fig.12. Calibration curves between the mean centered values of the second ratio spectra to the corresponding concentrations in the ranges of: (a) 1.50 – 9.50 µg/mL for BRM at 221.9 nm and (b) 5.00 – 9.20 µg/mL for TML at 286.9 nm.

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Table 1: Regression and validation parameters for the determination of BRM and TML in their pure forms by the proposed methods.

Parameters	Method 1		Method 2		Method 3	
	BRM	**TML**	**BRM**	**TML**	**BRM**	**TML**
Linearity range (µg/mL)	1.50 – 9.50 µg/mL	5.0 – 7.60 µg/mL	1.50 – 9.50 µg/mL	5.00 - 10.00 µg/mL	1.50 – 9.50 µg/mL	5.00-9.20 µg/mL
Regression:						
• Slope.	0.0313	0.0535	0.072	0.0091	1.628	0.0024
• SE of the slope.	0.0002	0.0004	0.0003	0.0001	0.0100	0.0001
• Intercept.	-0.0040	- 0.0178	0.0040	-0.0041	0.1941	-0.0005
• SE of the intercept.	0.0012	0.0022	0.0018	0.0004	0.0621	0.0001
• Correlation coefficient (r).	0.9998	0.9998	0.9999	0.9999	0.9999	0.9999
LOD (µg/mL)	0.1184	0.1357	0.0887	0.0591	0.1108	0.0515
LOQ (µg/mL)	0.3947	0.4112	0.2957	0.1971	0.3693	0.1715
Accuracy: Mean ± SD	**99.92 ± 1.479**	**100.27 ± 0.632**	**100.77 ± 1.294**	**99.28 ± 0.809**	**100.02 ± 1.319**	**100.73 ± 0.838**
Precision:						
• Repeatability (% RSD)	**100.51 ± 1.634**	**100.37 ± 0.634**	**100.09 ± 1.356**	**100.42 ± 0.308**	**100.27 ± 0.668**	**100.07 ± 0.342**
• Intermediate precision (%RSD)	**100.04 ± 1.788**	**99.83 ± 0.030**	**100.12 ± 1.323**	**100.09 ± 0.389**	**100.27 ± 0.698**	**100.85 ± 0.776**
Specificity: Mean ± SD	**99.77 ± 1.089**	**100.69 ± 0.245**	**100.08 ± 0.942**	**100.05 ± 0.308**	**100.32 ± 0.511**	**100.06 ± 0.770**

Table 2: Accuracy of the proposed methods for the determination of BRM and TML in their pure form.

Method	Compound	Concentration (μg/mL)	Recovery%	Mean ± SD	RSD%
Method 1	BRM	3.00	99.00	99.92 ± 1.479	1.480
		6.00	101.83		
		7.00	98.71		
		8.00	98.87		
		9.00	101.22		
	TML	5.4	100.92	100.27 ± 0.632	0.630
		5.6	99.28		
		7.00	100.71		
		8.40	100.23		
		9.20	100.21		
Method 2	BRM	4.50	101.90	100.77 ± 1.294	1.284
		6.50	98.67		
		8.00	101.26		
		8.50	101.59		
		9.00	100.45		
	TML	5.60	98.39	99.28 ± 0.809	0.815
		5.80	98.44		
		6.20	100.00		
		7.00	99.57		
		9.20	100.00		
Method 3	BRM	3.00	98.00	100.02 ± 1.319	1.319
		4.00	99.47		
		7.50	101.33		
		8.00	100.57		
		9.00	100.77		
	TML	5.20	101.76	100.73 ± 0.838	0.832
		5.60	100.44		
		6.60	100.37		
		7.60	101.42		
		8.40	99.70		

Table 3: Results of simultaneous determination of BRM and TML in laboratory mixtures by the proposed methods.

Method	Compound	Concentration (μg/mL)	Recovery%	Mean ± SD	RSD%
Method 1	BRM	6.00	98.66	99.77 ± 1.089	1.092
		7.00	98.71		
		7.50	100.19		
		8.50	100.11		
		9.00	101.22		
	TML	6.00	100.33	100.69 ± 0.245	0.243
		6.20	100.80		
		7.00	100.71		
		7.80	100.64		
		8.00	101.00		
Method 2	BRM	3.00	99.90	100.08 ± 0.942	0.941
		4.00	100.14		
		6.50	98.67		
		8.00	101.26		
		9.00	100.45		
	TML	5.00	100.40	100.05 ± 0.308	0.308
		6.20	100.00		
		7.00	99.57		
		9.00	100.11		
		10.00	100.20		
Method 3	BRM	4.00	99.47	100.32 ± 0.511	0.509
		6.50	100.55		
		8.00	100.57		
		8.50	100.25		
		9.50	100.77		
	TML	5.40	99.53	100.06 ± 0.770	0.769
		5.80	99.85		
		7.60	101.42		
		8.40	99.70		
		8.60	99.80		

Table 4: Determination of BRM and TML in Combigan® by the proposed methods and application of standard addition technique.

Method	Pharmaceutical Preparation	Compound	Mean recovery ± SD [b]	Standard addition technique [a]			
				Pure added (μg/mL)	Pure found (μg/mL)	% Recovery [c]	Mean ± SD
Method 1	**Combigan®** B.N. E88023 0.2% BRM, 0.5% TML, and 0.005% BNZ per 1 mL ophthalmic solution	**BRM**	**99.33 ± 0.763**	1.60	1.58	98.75	**98.94 ± 0.565**
				2.00	1.97	98.50	
				2.40	2.39	99.58	
		TML	**99.80 ± 0.302**	5.46	5.41	99.08	**99.27 ± 0.504**
				6.83	6.82	99.85	
				8.19	8.10	98.90	
Method 2		**BRM**	**99.17 ± 0.007**	1.60	1.57	98.12	**98.59 ± 0.526**
				2.00	1.99	98.50	
				2.40	2.38	99.16	
		TML	**100.04 ± 0.080**	5.46	5.47	100.18	**100.05 ± 0.175**
				6.83	6.82	99.85	
				8.19	8.20	100.12	
Method 3		**BRM**	**100.08 ± 0.151**	1.60	1.59	98.37	**99.31 ± 0.846**
				2.00	2.00	100.00	
				2.40	2.39	99.58	
		TML	**100.34 ± 0.355**	5.46	5.48	100.36	**100.07 ± 0.262**
				6.83	6.82	99.85	
				8.19	8.19	100.00	

[a] Amount taken is 2.00 μg/mL for BRM and 6.83μg/mL for TML.
[b] SD of 3 determinations.
[c] Mean of three determinations.

Table 5: Statistical comparison for the results obtained by the proposed methods and the reported method[15] for the determination of BRM and TML.

Parameters	BRM				TML			
	Method 1	**Method 2**	**Method 3**	**Reported method****	**Method 1**	**Method 2**	**Method 3**	**Reported method****
Mean	99.93	98.73	100.03	100.74	100.27	99.28	100.74	101.03
SD	1.479	0.806	1.319	0.835	0.623	0.809	0.838	0.853
Variance	2.187	0.650	1.741	0.698	0.400	0.655	0.703	0.727
F value (5.32)*	1.1425	14.9022	1.0335		2.5617	11.0827	0.2981	
Student's t test (2.306)*	1.0689	3.8604	1.0167		1.6001	3.3391	0.5461	

* Values between parenthesis are the theoretical values of t and F at P = 0.05 and n = 5.

****** HPLC method using C_{18} (250 mm x 4.6 mm), 1 mL/min., mobile phase composed of (0.05M sodium dihydrogen phosphate buffer - acetonitrile, 70:30, v/v) at pH 3.5 and UV detection at 220 nm.

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