VALIDATED SPECTROPHOTOMETRIC METHODS FOR SIMULTANEOUS DETERMINATION OF BRINZOLAMIDE AND TIMOLOL MALEATE IN THEIR PURE FORM AND OPHTHALMIC PREPARATION

BY

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Abstract

Four sensitive, simple and precise spectrophotometric methods for the simultaneous determination of brinzolamide (BRZ) and timolol maleate (TML) in their pharmaceutical formulation are developed and validated. Method A is the ratio difference spectrophotometric method (RDSM) which can measure the difference in amplitudes between 251 and 265 nm of ratio spectrum for brinzolamide and between 285 and 306 nm of ratio spectrum for timolol. Method B is the mean centering of ratio spectra method (MCR) by measuring the peak amplitude at 252 nm for brinzolamide and 304 nm for timolol. Method C is area under the curve (AUC) in which the area under the curves for brinzolamide and timolol were selected over the ranges of (260-265) and (282-288) nm. Method D is the bivariate method (BVM) which depended on the quantitative evaluation of the absorbances at 265 and 285 nm, respectively. Beer's Lambert's law for the adopted methods were obeyed over the concentration range of 6-36 µg/mL for Brinzolamide and 6-42 µg/mL for Timolol. The obtained results were

statistically compared with those obtained by the reported methods, showing no significant difference. The suggested methods were validated according to the ICH guidelines and were successfully applied for simultaneous determination of brinzolamide and timolol maleate in bulk drug and in their ophthalmic preparation.

Keywords: Brinzolamide; Timolol maleate; ratio difference; mean centering of ratio spectra; area under the curve; bivariate; spectrophotometry

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1. 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] **Fig. 1A [USP,2012]**, is a carbonic anhydrase inhibitor used to decrease intraocular pressure in patients with open-angle glaucoma or ocular hypertension by reducing the amount of fluid produced by the eye.

Timolol (TML), [(S)- 1-[(1,1-dimethylethyl)amino]-3-[[4-(4-morpholinyl)-1,2,5-thiadiazol-3-yl]oxy]-2 propanol (Z)-2 butenedioate(1:1)] **Fig. 1B [USP,2012]**, is a non-selective beta-adrenergic receptor antagonist used to treat glaucoma by decreasing the ocular pressure by reducing aqueous humur production through blockage of the beta receptors on the ciliary epithelium.

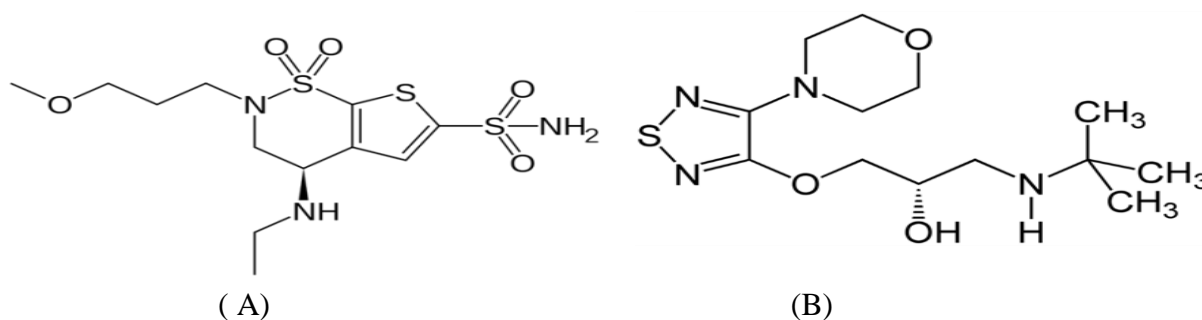(A)   (B)

Fig. (1): Chemical structure of Brinzolamide (A) and Timolol Maleate (B)

It was found that the combination of BRZ and TML gives better effect in treatment of glaucoma than each drug alone, this combination was marketed in the form of eye drops [**P.A.Shah et al. 2014**].

Literature survey reports that there are few analytical methods available for simultaneous estimation of BRZ and TML. Three spectrophotometric methods namely simultaneous equation method, Q-absorbance ratio method and ratio first derivative method for the simultaneous determination of BRZ and TML in their mixture form [**P.A.Shah et al. 2014**] and HPLC method [**R.Khatun et al. 2014**] were reported. In context to this, the aim of this research paper is to present four recent, sensitive, economical, precise and accurate UV spectrophotometric methods that present wide application for the simultaneous estimation of BRZ and TML in bulk drug and in their ophthalmic preparation without prior separation. Our work can be incorporated in the quality control analysis where time and coast of great value.

2. Experimental

2.1. Apparatus

JASCO dual beam (Japan) UV-visible spectrophotometer model V-630. The bundle software, spectra manager II was used. The spectral slit width was 2nm and the speed of scan is 1000 nm/min.

Sonicator: Bandelin Sonorex RK 510S, Donation of Alexander von Hum-boldt - Foundation, Bonn-Bad, Godesberg, Federal Republic of Germany.

2.2. Software

All computations of mean centering method were performed in Matlab® version 7.10 **[Matlab 2010].**

2.3. Materials and reagents

Methanol (Piochem®) spectroscopic grade and distilled water.

2.4. Pure standard:

Brinzolamide standard was purchased from Sigma-Aldrich Chemie (GmbH, Germany) and Timolol was kindly supplied by Epico (Cairo, Egypt) ; their purity was reported to be 99.7 % and 99.92 % , respectively according to the reported HPLC method **[R.Khatun etal 2014]**.

2.5. Pharmaceutical formulations:

Azarga eye drops, manufactured by ALCON-COUVREUR (Belgium). Batch number 180416, labeled to contain 10 mg/mL BRZ and 5 mg/mL TML, purchased from local market.

2.6. Stock standard solution

1- Stock standard solution of BRZ (0.6 mg/mL)

An accurately weighed 6 mg of BRZ was dissolved in 10 mL methanol in 10-mL volumetric flask and completed to the volume with the same solvent.

2- Stock standard solution of TML (0.6 mg/mL)

An accurately weighed 6 mg of TML was dissolved in 10 mL methanol in 10-mL volumetric flask and completed to the volume with the same solvent.

Aliquots of the prepared stock solutions were further diluted with methanol to a final volume of 10 mL. The diluted solutions were used as the working solutions for BRZ and TML.

2.7. Construction of calibration curves:

Standard solutions of both BRZ and TML in the range of (6-36 µg/mL) and (6-42 µg/mL), respectively, were separately prepared by appropriate dilutions of their respective stock standard solutions in methanol. The absorption spectra of the resulting solutions were measured in the range of 200-400 nm using methanol as blank and stored in the computer.

2.7.1. Ratio difference spectrophotometric method (RDSM)

The stored spectra of BRZ were divided by the spectrum of 12 µg/mL of TML to obtain ratio spectra, as well as the spectra of TML were divided by the spectrum of 12 µg/mL of BRZ. A two calibration curves were constructed relating the difference between the amplitudes of ratio spectra of BRZ and TML at (251 and 265 nm) and (285 and 306 nm), respectively, versus the corresponding concentrations of BRZ and TML, respectively, and the regression equations were computed.

2.7.2. Mean centering of ratio spectra method (MCR)

The stored spectra of BRZ were divided by the spectrum of 12 µg/mL of TML to obtain ratio spectra, as well as the spectra of TML were divided by the spectrum of

12 µg/mL of BRZ, the obtained ratio spectra of both mentioned drugs were mean centered. Two calibration curves were constructed relating the mean center values of BRZ at 252 nm and TML at 304 nm, respectively, versus the corresponding concentrations of BRZ and TML, respectively, and the regression equations were computed.

2.7.3. Area under the curve method (AUC)

The AUC of BRZ was measured between (260 -265) nm for BRZ and between (282 -288) nm for TML. The calibration curve of the mentioned drugs was plotted relating area under the curve versus the corresponding concentrations of BRZ and TML, and then the regression equations and the absorptivity constants were computed.

2.7.4. Bivariate method (BVM)

The absorption spectra of the prepared solutions were measured at 265, 270, 275, 280 and 285 nm. Four calibration curves; 2 calibrations for each component at 2 wavelengths selected using method of Kaiser at 265 and 285 nm, were constructed against the corresponding concentrations of BRZ and TML, and the corresponding regression equations were computed.

2.8. Analysis of laboratory prepared mixtures different ratios of BRZ and TML using the proposed methods

Six laboratory prepared mixtures containing different ratios of BRZ and TML were analyzed using the suggested methods. The concentrations of BRZ and TML were calculated from their corresponding regression equations.

2.9. Assay of pharmaceutical formulations

AZARGA eye drops bottle each one mL is claimed to contain 10 mg of brinzolamide and 5 mg of timolol maleate equivalent to 5 mg timolol mixed with reported excipients like 0.1% benzalkonium chloride, 0.1 mg mannitol, and 0.1 mg sodium chloride.

One ml of Azarga eye drop was transferred to 10 ml volumetric flask, 8 mL methanol were added and sonicated for 10 minutes then filtered. The volume was completed to the mark with methanol to get 1000µg/mL BRZ and 500µg/mL TML. From the above prepared solution, further dilutions were performed in the obtained linearity ranges using methanol for the adopted spectrophotometric methods. The procedure of each of the suggested methods was applied to determine the concentration of the cited drugs in the prepared dosage form solution. The determination was performed in triplicate for each concentration. Concentrations of BRZ and TML in the prepared samples were calculated from their corresponding regression equations.

3. Results and discussion

The main target of this paper is to develop four sensitive, simple and precise spectrophotometric methods for the simultaneous determination of (BRZ) and (TML) in bulk drugs and in their pharmaceutical formulation. Since there are few analytical methods available for the simultaneous estimation of Brinzolamide and Timolol in their combined pharmaceutical formulation.

The suggested methods; Ratio difference, Mean centering of ratio spectra, Area under the curve and Bivariate spectrophotometric methods, were applied to resolve the spectral overlap of BRZ and TML in their binary mixture without prior separation steps.

3.1. Method development and optimization

3.1.1. Ratio difference spectrophotometric method (RDSM)

Ratio difference method is simple and accurate method that has the ability for solving severely overlap spectra without prior separation at the same time it does not require any sophisticated apparatus or computer programs. It can determine the unknown concentration of a component of interest present in a mixture containing both the component of interest and an interfering component **[H.M.Lotfy et al. 2014]**.

The zero order absorption spectra of the laboratory-prepared mixtures of BRZ and TML are divided by a concentration of 12μg/mL of BRZ and 12μg/mL of TML as a divisor for the determination of BRZ and TML, respectively separately to get the ratio spectra shown in(**Fig. 2and 3).** Different pairs of wavelengths were selected to choose the optimum wavelengths at which measurements were done, the amplitudes at 251 nm and 265 nm were selected for determination of BRZ and 285 and 306 nm for determination of TML which provide the best selectivity. The concentration of BRZ and TML were obtained using their corresponding regression equations obtained by plotting the difference in the amplitudes at 251-265 nm and 285-306 nm respectively, of the ratio spectra against their corresponding concentrations:

$RDSM_{BRZ}=0.4020\ C-0.4088$ $r=0.9993$

$RDSM_{TML}=0.2827C-0.0934$ $r=0.9999$

Where C is the concentrations of cited drugs μg/mL, RDSM is the difference in peak amplitudes of the ratio spectrum curves and r is the correlation coefficient.

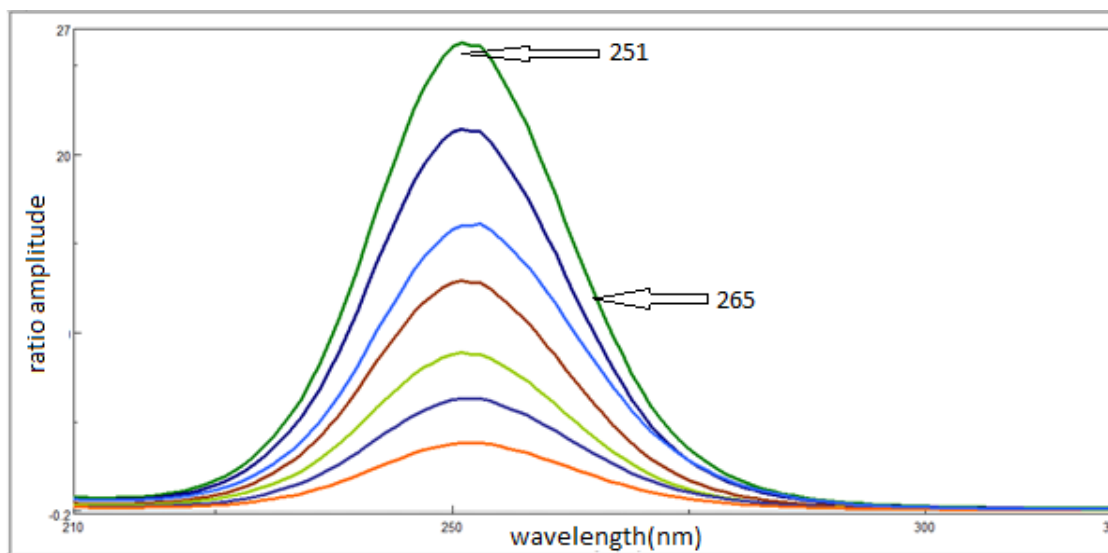**Fig. (2):** Ratio spectra of (6-36μg/mL) brinzolamide using the spectrum of 12μg /mL timolol as divisor.
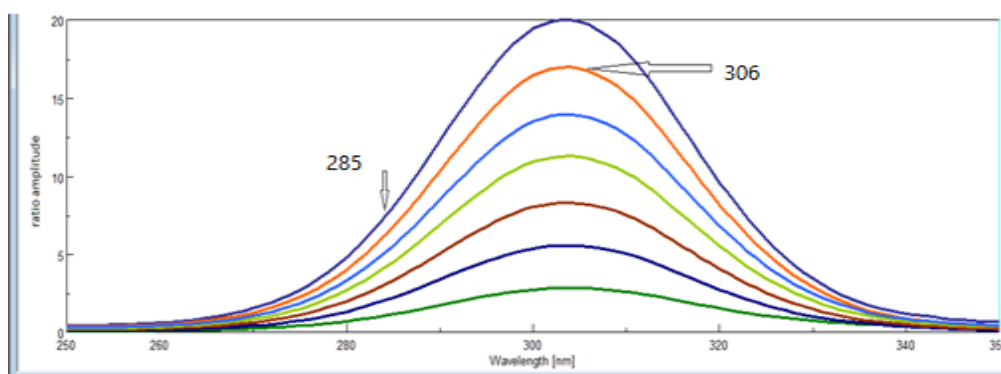
Fig.(3): Ratio spectra of timolol (6-42µg/mL) using the spectrum of 12µg /mL brinzolamide as divisor.

3.1.2. Mean centering method (MCR)

This method depends on the mean centering of ratio spectra. It eliminates the derivative step so the signal-to-noise ratio is enhanced **[A.Afkham et al. 2004 - A.Afkham et al. 2005 - M.Bahram et al. 2007]**. Mean centering method was applied to determine concentration of BRZ in presence of TML and vice versa. For determination of BRZ, the absorption spectra of BRZ were divided by the absorption spectrum of 12µg/mL of TML as a divisor and for determination of TML, the absorption spectra of TML were divided by the absorption of 12µg/mL of BRZ as a divisor. The obtained ratio spectra were mean centered and the concentrations of BRZ and TML were determined by measuring the amplitude at 252 nm and at 304 nm, respectively **(Fig.4and5).** The linear regression equation is found to be:

$MCN_{BRZ}=0.6292\ C-0.6595$ r=0.9995

$MCN_{TML}=0.3829\ C+0.2796$ r=0.9997

Where C is the concentrations of the cited drugs, MCN is the peak amplitude of the mean centered ratio spectrum curve and r is the correlation coefficient.
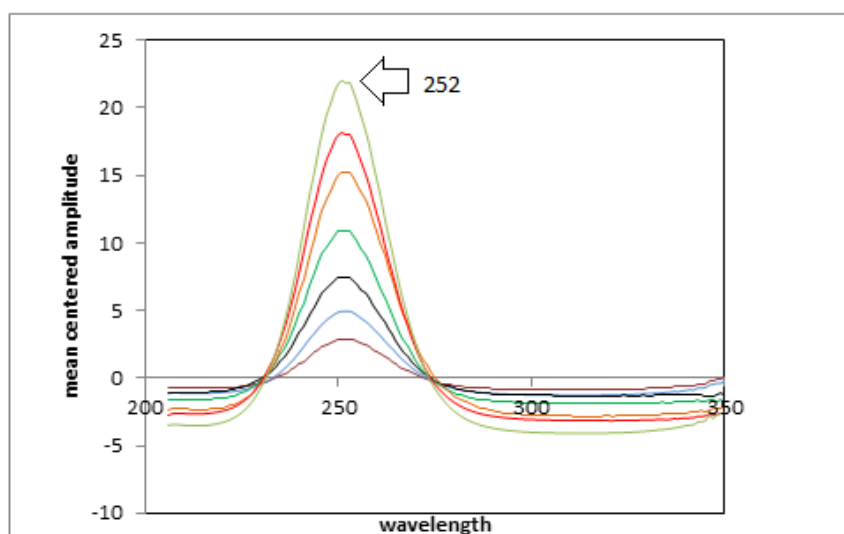

Fig.(4). Mean centered ratio spectra of brinzolamide(6–30µg/mL) using 12
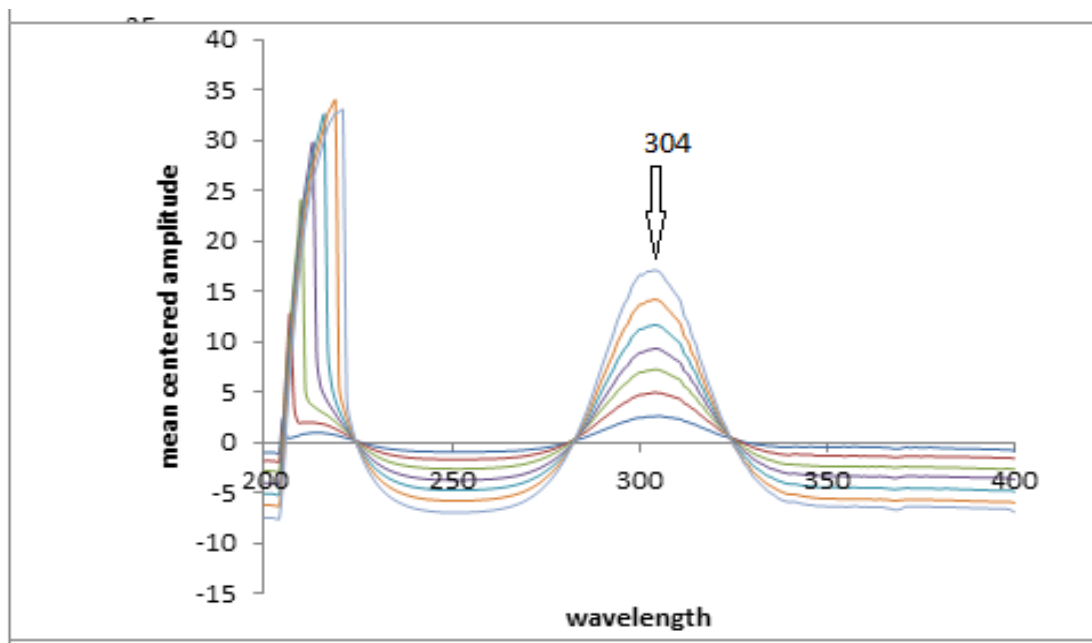
µg/mL of timolol as a divisor.

Fig.(5). Mean centered ratio spectra of timolol (6–36μg/mL) using 12

μg/mL of brinzolamide as a divisor.

3.1.3. Area under the curve method (AUC)

AUC **[M.M.Abdelrahman et al. 2013]** is an accurate, rapid and selective method for the simultaneous determination of components having overlain spectra in binary mixtures by calculating the areas under the spectra in relation to their concentrations. Careful choice of the wavelength ranges at which calculations were done was of great importance, so different pairs of wavelength ranges were selected to choose the optimum wavelength range.

Area under the curves obtained from the scanned spectra over the ranges of wavelengths (260 - 265) and (282 - 388) nm **(Fig.6 and 7)** were recorded and then the corresponding regression equations were obtained for both BRZ and TML.

-For BRZ:

$Y_{(260-265)} = 0.1499\,x - 0.0161$ $(r = 0.9990)$.

$Y_{(282-388)} = 0.0323\,x + 0.0306$ $(r = 0.9988)$.

-For TML:

$Y_{(260-265)} = 0.0359\,x - 0.0689$ $(r = 0.9991)$.

$Y_{(282-388)} = 0.1297x - 0.0689$ $(r = 0.9998)$.

Where **y** is the area under curve over the ranges (260-265) and (282-388) respectively, **x** is the concentration in μg/mL and **r** is the correlation coefficient

Areas under the curve and the absorptivity values at the selected wavelength ranges are used for calculating the concentration of BRZ in presence of TML and vice versa using the following equations:

$$C^X = \frac{(a^Y{}_{\lambda1-\lambda2} \; AUC_{\lambda3-\lambda4}) - (a^Y{}_{\lambda3-\lambda4} \; AUC_{\lambda1-\lambda2})}{(a^Y{}_{\lambda1-\lambda2} \; a^X{}_{\lambda3-\lambda4}) - (a^Y{}_{\lambda3-\lambda4} \; a^X{}_{\lambda1-\lambda2})}$$

$$C^Y = \frac{(a^X{}_{\lambda1-\lambda2} \; AUC_{\lambda3-\lambda4}) - (a^X{}_{\lambda3-\lambda4} \; AUC_{\lambda1-\lambda2})}{(a^Y{}_{\lambda1-\lambda2} \; a^X{}_{\lambda3-\lambda4}) - (a^Y{}_{\lambda3-\lambda4} \; a^X{}_{\lambda1-\lambda2})}$$

- Where C^X, C^Y are the concentrations of the cited drugs.

- $a^X{}_{\lambda1-\lambda2}, a^X{}_{\lambda3-\lambda4}$ are the absorptivity of drug X at $(\lambda1-\lambda2)$ nm and at $(\lambda3-\lambda4)$nm

  , Respectively.

- $a^Y{}_{\lambda1-\lambda2}, a^Y{}_{\lambda3-\lambda4}$ are the absorptivity of drug Y at $(\lambda1-\lambda2)$nm and at $(\lambda3-\lambda4)$nm, respectively.

- $AUC_{\lambda1-\lambda2} \; AUC_{\lambda3-\lambda4}$ are the area under the curves at $(\lambda1-\lambda2)$ nm and at $(\lambda3-\lambda4)$ nm, respectively.

  The calibration graphs that relate the measured areas under the curve to the concentration of each component in µg/mL were constructed.

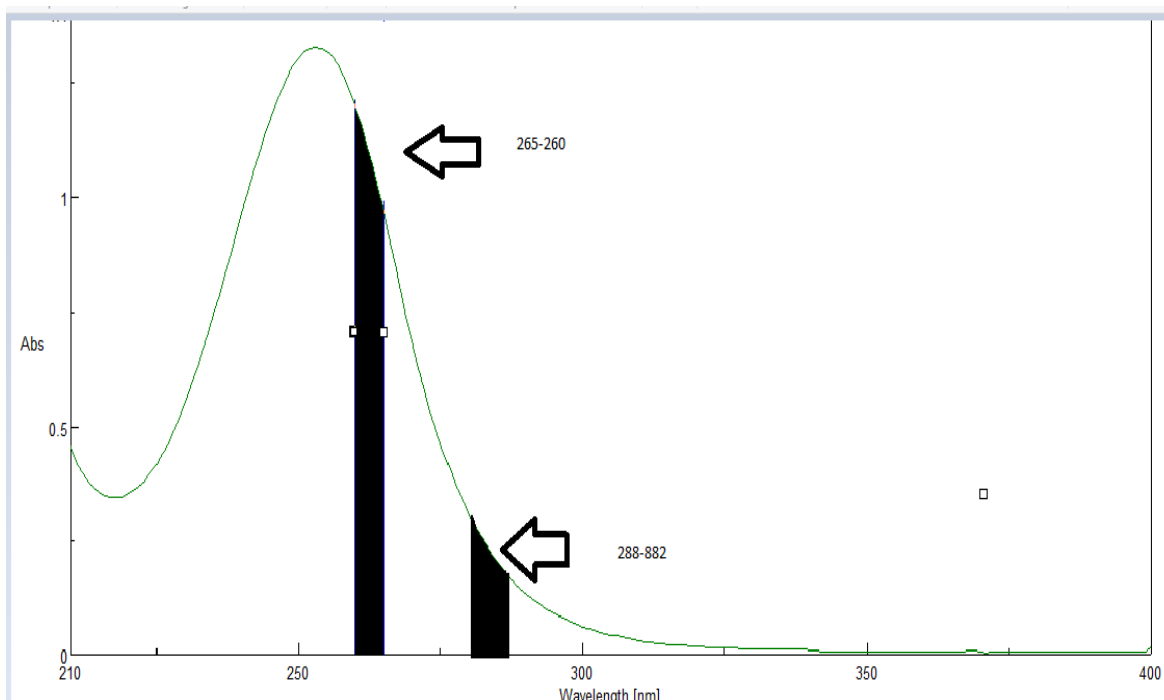**Fig. (6):** Zero order absorption spectrum of brinzolamide (36µg/ml) showing area under the curve over the ranges (260-265) and (282-288) nm.
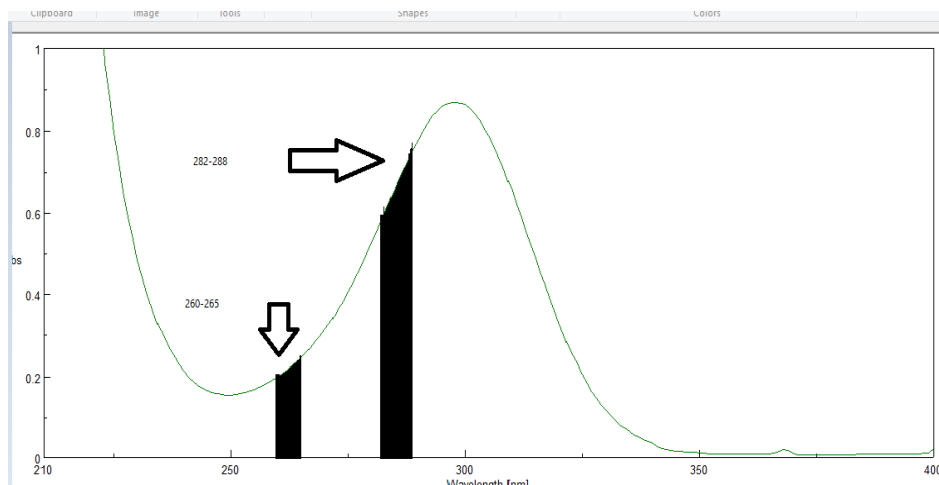
Fig. (7): Zero order absorption spectrum of timolol (36μg/ml) showing area under the curve over the ranges (260-265) and (282-288) nm**.**

3.1.4. Bivariate method (BVM)

This method depends on a simple mathematical algorithm, in which the data ara used from four regression equations, two calibrations for each component at two selected wavelengths using Kaiser method **[D.Massart et al. 1988]**. The absorbance was measured at the selected wavelengths and the corresponding regression equations were calculated for both BRZ and TML. The calculated slope and intercept values were used for obtaining concentration of each compound using the following equation:

$$C_A = \frac{A_{AB1} - e_{AB1} - m_{B1}\, C_B}{m_{A1}}$$

$$C_B = \frac{m_{A2}\,(A_{AB1} - e_{AB1}) + m_{A1}\,(e_{AB2} - A_{AB2})}{m_{A2}\, m_{B1} - m_{A1}\, m_{B2}}$$

- Where C_A, C_B are the concentrations of the analytes.

-A_{AB1}, A_{AB2} are the absorbances of the drug mixture at the two selected wavelengths (1 and 2), respectively.

- e_{AB1}, e_{AB2} are the sum of the intercepts of the linear calibration at two wavelengths ($e_{AB1} = e_{A1} + e_{B1}$).

-$m_{A1,\,2}$, $m_{B1,\,2}$ are the slopes of the linear regression.

For BRZ and TML mixture, five wavelengths (265, 270, 275, 280, 285 nm) were chosen and Kaiser Method is used to select two wavelength at which four regression equations, two calibrations for each component were obtained. The selected wavelengths are 265.0 nm and 285.0 nm. At these selected wavelengths, the one-component calibration curves were obtained in the range of (6 – 36 μg/mL) for BRZ and (6 – 42 μg/mL) for TML. The linear regression equations were found to be:

$A_{265} = 0.02162\ C - 0.0025$ $r = 0.9998$ at 265 nm for BRZ

$A_{285} = 0.0049\ C - 0.0119$ $r = 0.9993$ at 285 nm for BRZ

$A_{265} = 0.0067\ C - 0.0020$ $r = 0.9994$ at 265 nm for TML

$A_{285} = 0.0182C - 0.0012$ $r = 0.9998$ at 285 nm for TML

Where A is the absorbance value at 265 nm and 285 nm, C is the concentration in µg/mL and r is the correlation coefficient.

3.2. Method validation

The applied methods had been validated according to ICH guidelines **[ICH 2005]** .

3.2.1. Linearity & Range:

The linearity of the methods was evaluated by analyzing six concentrations of BRZ and seven concentrations of TM ranging from 6–36 µg/ml and 6–42 µg/mL, respectively. The linear regression parameters were listed in **Table1**.

3.2.2. Accuracy

The accuracy of suggested method was checked by analyzing five different concentrations of each drug in triplicate within the linearity range where percentage recoveries suggested satisfactory results of the accuracy of the performed methods as shown in **Table 1**. The validity of the method was checked by applying the standard addition technique, where satisfactory results are obtained **Table 2**.

3.2.3. Selectivity

The selectivity of the methods was assessed by the analysis of laboratory prepared mixtures of BRZ and TM in different ratios within the linearity range where good results were obtained as shown in **Table 3**.

3.2.4. Precision

Precision of the adopted methods was evaluated by performing intra-day and inter-day precision studies. The intra- and inter-day precision were assessed using three different concentrations of each of BRZ and TML in triplicate on the same day and on three successive days using the proposed methods, respectively. The relative standard deviations were obtained **Table 1.**

3.2.5. Limit of detection (LOD) and Limit of quantification (LOQ):

The LOD and LOQ of the suggested methods were checked and calculated from the standard deviation (σ) of the response and the slope of the calibration curve (S) according to the equations: LOD = 3.3 x σ/S , LOQ = 10 x σ/S and the results were obtained as shown in **Table1**.

3.3. Application of the methods in assay of eye drops

The suggested methods are applicable for the analysis of BRZ and TM in their combined pharmaceutical formulation Azarga eye drops with no interference from the excipients; the results are shown in **Table 2**.

3.4. Statistical analysis

Statistical comparison between the results obtained by the suggested methods and those obtained by the reported HPLC method **[R.Khatun etal 2014]** showed no significant differences as shown in **Table 4**.

Table1. Regression parameters and results of determination of pure samples of BRZ and TML by the proposed methods.

Parameter	RDSM		MC		AUC				Bivariate			
	BRZ	TML	BRZ	TML	BRZ		TML		BRZ		TML	
λ (nm)	P251-265	P285-306	252	304	260-265	282-288	260-265	282-288	265	285	265	285
Range (µg/ml)	6-36	6-42	6-36	6-36	6-36	6-36	6-36	6-36	6-30	6-30	6-36	6-36
Slope	0.4020	0.2827	0.6292	0.3829	0.1499	0.0323	0.0359	0.1297	0.026161	0.0049	0.0067	0.0182
intercept	-0.4088	-0.0934	-0.659	0.2796	-0.016	0.0306	0.0689	0.0689	0.0025	0.0119	0.0020	-0.001
Correlation Coefficient (r)	0.9993	0.9999	0.9995	0.9997	0.9990	0.9988	0.9991	0.9998	0.9998	0.9993	0.9994	0.9998
LOD (µg/ml)	1.41	0.53	1.17	0.96	1.72	1.86	1.64	0.78	0.78	1.43	1.30	0.71
LOQ (µg/ml)	4.27	1.59	3.53	2.92	5.22	5.63	4.97	2.36	2.37	4.36	3.94	2.14
Accuracy (mean ± SD)	100.07 ±	99.86 ±	99.90 ±	99.69 ±	99.70 ±		99.42 ±		100.29 ±		100.08 ±	
RSD%[a]	0.886	0.492	0.795	0.459	0.348		0.296		0.494		0.318	
RSD%[b]	1.101	0.630	0.944	1.625	0.826		0.523		0.623		0.466	

RSD%[a] : the intra-day relative standard deviation of 12 , 24 and 36 µg/mL of BRZ and TML by the proposed methods.

RSD%[b] : & inter-day respectively relative standard deviation of 12 , 24 and 36 µg/mL of BRZ and TML by the proposed methods.

Table2. Determination of BRZ and TML in Azarga eye drop by the proposed methods and application of standard addition technique.

Sample	BRZ			
Method	RDSM	MC	AUC	Bivariate
Azarga R*% ± SD	101.28 ± 0.477	102.10 ± 0.596	101.05 ± 0.530	100.83 ± 1.553
Standard Addition R**% ± SD	100.50 ± 0.670	99.65 ± 1.628	100.38 ± 0.405	100.42 ± 1.457
Sample	TML			
Method	RDSM	MC	AUC	Bivariate
Azarga R*% ± SD	101.26 ± 0.628	100.96 ± 1.583	101.13 ± 0.983	100.63 ± 1.748
Standard Addition R**% ± SD	101.12 ± 0.610	100.71 ± 0.669	99.84 ± 1.326	99.50 ± 0.785

*Average of six determinations.

**Average of nine determinations over three concentration levels.

Table3. Determination of the studied drugs in the laboratory prepared mixtures.

Concentration [µg/mL]		RDSM		MC		AUC		Bivariate	
		Recovery %		Recovery %		Recovery %		Recovery %	
BRZ	TML	BRZ	TML	BRZ	TML	BRZ	TML	BRZ	TML
30	12	100.02	99.8	99.40	99.73	100.75	99.05	99.55	100.31
24	18	102.06	100.13	100.16	97.90	101.20	98.66	99.87	99.81
24	12	99.89	100.13	100.24	99.45	99.78	101.19	100.08	100.10
24	6	99.96	96.57	99.89	98.38	99.98	99.95	100.04	100.20
12	12	102.06	100.01	98.91	99.89	99.65	97.65	100.10	99.88
18	12	99.71	99.9	101.62	99.33	99.82	102.00	99.43	101.03
Mean		100.62	99.42	100.04	99.11	100.20	99.75	99.84	100.22
SD		1.123	1.404	0.923	0.794	0.628	1.629	0.289	0.439
RSD		1.116	1.412	0.923	0.801	0.627	1.633	0.290	0.438

Table4. Statistical analysis of the proposed methods and the reported method of BRZ and TML in their pharmaceutical dosage form.

Parameter	BRZ				
	RDSM	MCR	AUC	Bivariate	Reported method[1][c]
Mean%[a]	101.28	102.10	101.05	100.83	101.95
SD	0.477	0.596	0.530	1.553	0.863
N	6	6	6	6	6
Variance	0.228	0.355	0.281	2.412	0.745
t-test (2.23)[b]	1.66	0.35	2.18	1.54	
F (5.05)[b]	3.27	2.10	2.65	3.24	
	TML				
	RDSM	MCR	AUC	Bivariate	Reported method[1][c]
Mean %[a]	101.26	100.96	101.13	100.63	101.98
SD	0.628	1.583	0.983	1.748	0.832
N	6	6	6	6	6
Variance	0.394	2.506	0.966	3.055	0.692
t-test (2.23)[b]	1.69	1.40	1.62	1.71	
F (5.05)[b]	1.76	3.62	1.40	4.41	

4. Conclusion

From the previous discussion, it could be concluded that the applied methods were simple with minimum manipulation steps, accurate, don't need any sophisticated apparatus and could be easily applied in quality control laboratories.

The developed methods were validated as per ICH guidelines and were found to be precise as revealed from % RSD less than 2, for simultaneous estimation of BRZ and TML in mixture over the applied range. Finally the suggested method could be successfully applied for the routine analysis of BRZ and TML in their pure bulk powders and in their combined ophthalmic preparation without prior separation procedure steps.

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طرائق طيف ضوئية لتحليل خليط ثنائي مكون من البرينزولاميد و التيمولول في الحاله النقيه وفي المستحضر الدوائي

للساده الدكاتره

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الملخص:

اربع طرق طيفيه بسيطه وحساسه لتحليل خليط ثنائي من البرينزولاميد والتيمولول تم تطويرها والتحقق من صحتها في صورتها النقيه وفي المستحضر الدوائي. الطريقه(أ) طريقة فرق منحني الامتصاص الطيفي والتي تستطيع حساب الفرق في الاطوال الموجيه بين ٢٥١ و ٢٦٥ نانومتر لتحديد تركيز برينزولاميد و ويتم حساب الفرق في الاطوال الموجيه بين ٢٨٥ و ٣٠٦ نانومتر لتحديد تركيز تيمولول. الطريقه (ب) طريقة حساب المتوسط المركزي حيث تم تعيين البرينزولاميد عند الطول الموجي٢٥٢ نم وتم تعيين التيمولول عند الطول الموجي٣٠٤ نم .الطريقه (ج) طريقة المنطقه تحت المنحني حيث تم قياس المنطقة تحت المنحنى للبرينزولاميد والتيمولول على مدى الاطوال الموجيه مابين (٢٦٠-٢٦٥) نانومترو(٢٨٢-٢٨٨) نانومتر.الطريقه(د) طريقة ذات المتغيرين الطيفيه والتي تعتمد علي التقيم الكمي للامتصاص عند الطولين الموجيين ٢٦٥ نم و ٢٨٥ نم.وتم تطبيق قانون بير لامبر للطرق المعتمده للتركيزات (٦-٣٦) ميكروغرام / مل للبرينزولاميد و(٦-٤٢) ميكروغرام / مل للتيمولول .وقد تم مقارنه النتائج احصائيا بنتائج طريقه مذكوره حيث لاتوجد اي اختلافات كبيره .وقد تم تقييم الطرق المقترحه طبقا لقواعد منظمه ICH وتم طبيقها بنجاح لتعيين البرينزولاميد والتيمولول في الصوره النقيه وفي المستحضر الدوائي.

الكلمات الداله : برينزولاميد – تيمولول- طريقة فرق منحني الامتصاص الطيفي- طريقة حساب المتوسط المركزي - طريقة المنطقه تحت المنحني- طريقة ذات المتغيرين الطيفيه.