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Spectrophotometric Methods for Determination of Binary Mixture of Dorzolamide Hydrochloride and Timolol Maleate in Bulk and Pharmaceutical Preparation

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ABSTRACT

Objectives: This study aimed to present and validate three simple, accurate, selective, sensitive and reproducible spectrophotometric methods for the determination of Dorzolamide hydrochloride in the presence of Timolol maleate without previous separation. **Methods:** (A) dual-wavelength method at 228.8 and 280 nm, (B) bivariate method at 220 and 250 nm and (C) area under the curve (AUC) method at wavelength ranges of (253-258 nm) and (272-277 nm). All the methods were applied in the range of (4-28 µg/mL). Furthermore, timolol maleate was determined directly at 297 nm. **Results**: These methods were validated according to the ICH guidelines where accuracy, precision, and repeatability were found to be within reasonable limits. The selectivity of the proposed methods was tested using laboratory prepared mixtures and assessed by applying the standard addition technique. **Conclusion**: The proposed spectrophotometric methods were presented as validated methods to resolve the interference. The proposed methods were simple with minimal manipulation steps, sensitive, precise and could be easily applied in quality control laboratories.

INTRODUCTION

Timolol maleate (TIM), [(S)-1-[(1,1-dimethyl)amino]-3-[[4-(4-morpholinyl9-1,2,5-thiadiazol-3-yl]oxy]-2-propanol] **Figure (1a)** is a non-selective beta blocker used in the management of glaucoma. It was the first blocker to be used as antiglaucoma agent [1].

Dorzolamide hydrochloride (DOR), [(4S,6S)-4-ethylamino-6-methyl-5,6-dihydro-4H thieno[2,3b]thiopyran-2-sulphonamide7,7-dioxide hydrochloride] **Figure (1b)** is a carbonic anhydrase inhibitor used in the management of open-angle glaucoma and ocular hypertension either alone or as an adjunct to a topical beta blocker [1].

Several methods have been reported for the determination of a mixture of DOR and TIM including spectrophotometric [2–4], capillary electrophoretic [5] and HPLC methods [6, 7] in the pharmaceutical dosage form.

A review of the described spectrophotometric methods shows that they require critical steps in handling the spectral data either through derivatization of the recorded spectral data with selection to zero crossing point or through dividing the spectral data with suitable divisor, which also may be followed by further derivatization step. Therefore, the aim of the present work was to develop three simple spectrophotometric methods handling the zero order spectra without the need for processing steps; namely dual wavelength [8], bivariate [9, 10] and area under curve [11, 12] for the determination of DOR in the presence of TIM in the pharmaceutical formulation. On the other hand, TIM can be determined directly at 297 nm [13] where there is no interference from DOR. The described methods seem to be zero order spectrophotometric methods that do not require any spectral processing through dividing or derivatization of the spectral data.

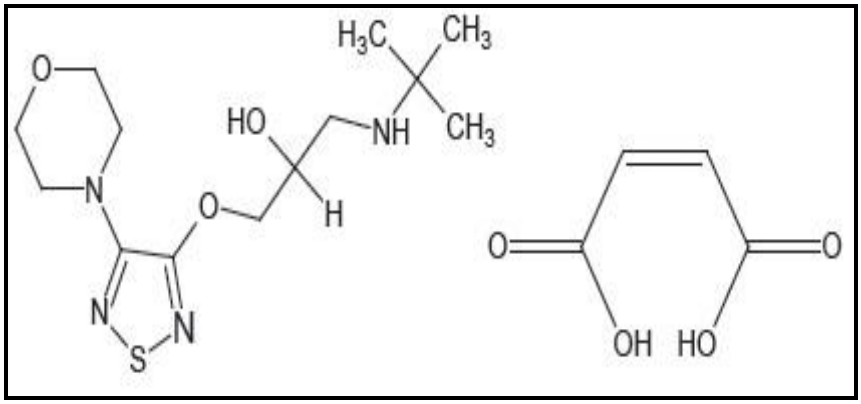

Figure (1a). Chemical structure of timolol maleate

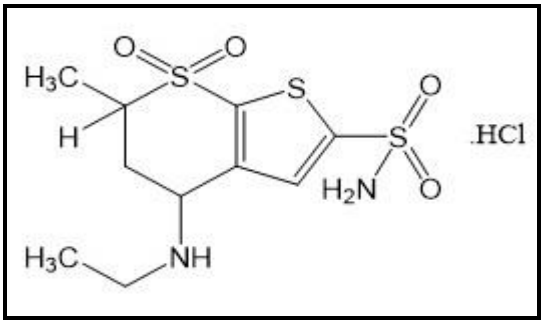

Figure (1b). Chemical structure of dorzolamide hydrochloride

EXPERIMENTAL

Instruments

SHIMADZU double beam UV–visible spectrophotometer (Kyoto/Japan), model UV-1800 PC linked to IBM compatible and an HP1020 laserjet printer. The bundled software, UV-Probe personal spectroscopy software version 2.43 (SHIMADZU) was used. The spectral band was 2 nm and scanning speed is 2800 nm/min with 1 nm interval.

MATERIALS AND REAGENTS

Standard

Standard DOR (certified to contain 99.92%) and TIM (certified to contain 99.96%) were kindly supplied by Orchidia for pharmaceuticals (Obour city, Egypt).

Pharmaceutical preparation

Twinzol ® eye drops: (batch number 0816149) each 1 ml is claimed to contain 22.26 mg dorzolamide hydrochloride and 6.83 mg timolol maleate, manufactured by Orchidia for pharmaceuticals (Obour city, Egypt), were purchased from local market.

Standard solutions

DOR and TIM standard solutions; 100 µg/mL in distilled water.

Procedure

Linearity and construction of calibration curves

Different aliquots equivalent to (4–28 µg) of DOR and (4–40 µg) of TIM were carefully transferred from their standard solutions (100 µg/mL) into two separate series of 10-mL volumetric flasks and completed to volume with distilled water. The absorption spectra (from 200 to 400 nm) of these solutions were recorded using distilled water as a blank. TIM was estimated directly by measuring the absorbance at the maximum wavelength at 297 nm without any interference from DOR **Figure (2)**. The calibration curve was constructed by plotting the absorbance at 297 nm against the corresponding concentrations. While the methods for DOR include the following.

Dual Wavelength Method

In zero order spectra of DOR, the difference absorbance at 280 and 228.8 nm was found to be zero for TIM **Figure (2)**. The calibration curve was constructed by plotting the difference absorbance values at 228.8 and 280 nm to the corresponding concentrations of DOR.

Bivariate Method

The absorbance of 20 µg/ml each of DOR and TIM **Figure (2)** were measured at 220 and 250 nm and then the corresponding regression equations were computed at the selected wavelengths for both DOR and TIM.

The area under the curve method (AUC)

The area under the curve (AUC) for the wavelength ranges selected for determination of DOR in the presence of TIM are (253–258) nm and (272-277) nm **Figure (3, 4)**. The absorptivity 'a' values of DOR and TIM were determined at each wavelength range. The concentrations of DOR in presence of TIM can be obtained from the following equation:

$$C_{(x)} = (A_{m1}\, a_{y2} - A_{m2}\, a_{y1}) / (a_{x1}\, a_{y2} - a_{x2}\, a_{y1})$$

Where $C_{(x)}$ is DOR concentration, A_{m1} and A_{m2} are the area under the curve of the mixture at the wavelength range (253 - 258) nm and (272 – 277) nm, respectively. Where a_{x1} = A_{x1}/conc. in µg/ ml, a_{x2} = A_{x2}/conc. in µg/ ml and a_{y1} = A_{y1}/conc. in µg/ ml, a_{y2} = A_{y2}/conc. in µg/ ml for DOR and TIM, respectively.

Application to laboratory prepared mixtures

Accurate aliquots of DOR and TIM were transferred from their standard solutions into a series of 10-mL volumetric flasks to provide mixtures containing different ratios of both drugs. The volumes were completed with the distilled water. The spectra of the prepared series from 200 to 400 nm were recorded and saved. The concentrations of DOR and TIM were calculated using the corresponding regression equation for each proposed method.

Application to the pharmaceutical preparation

1 ml of Twinzol ® eye drops was transferred into 100-mL volumetric flask and completed to volume with distilled water to obtain a concentration of 222.6 and 68.3 µg/mL of DOR and TIM, respectively. The proposed methods were repeated using aliquots covering the working concentration ranges. The concentrations of DOR and TIM were calculated from the corresponding regression equation of each proposed method.

Reported method [3]

The reported method depends on the first derivative at 250.3 nm for dorzolamide hydrochloride and 315.8 nm for timolol maleate.

RESULTS AND DISCUSSION

The UV spectra of DOR and TIM show the certain degree of overlapping **Figure (2)**, which creates difficulty in the analysis of DOR by direct UV absorbance measurements. TIM can be determined directly at its maximum wavelength at 297 nm without interference from DOR.

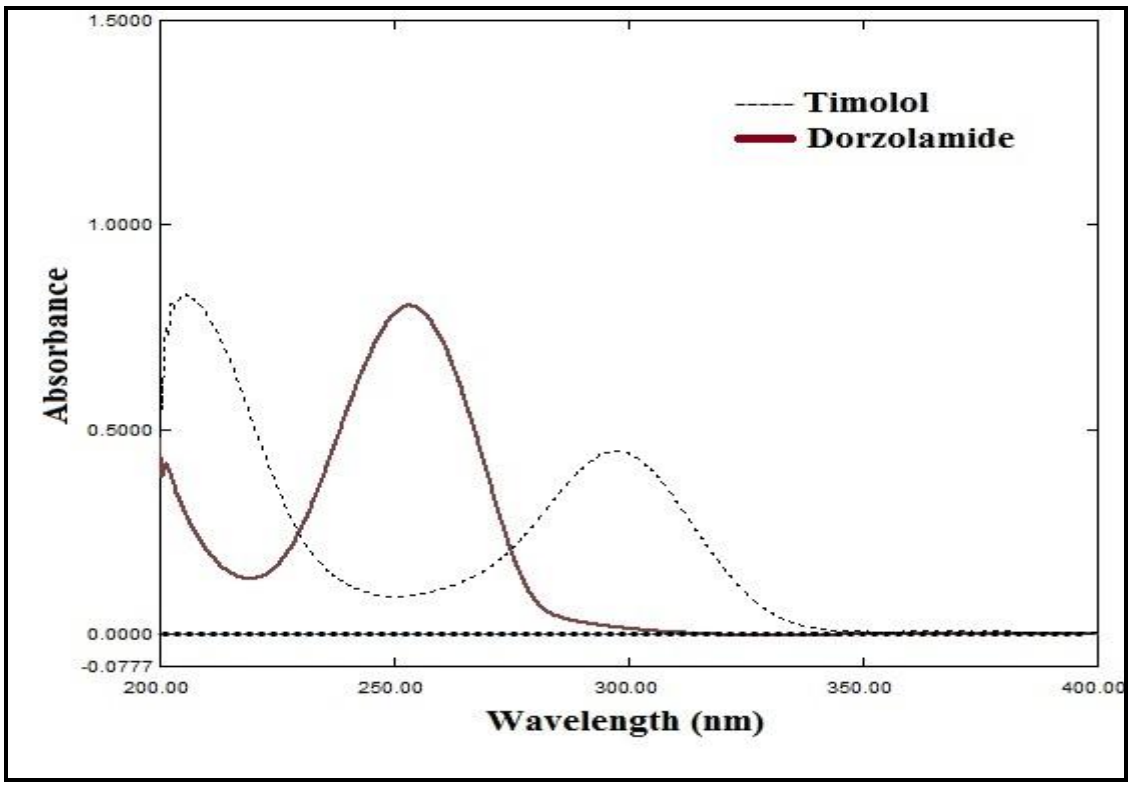

Figure (2): Zero-order absorption spectra of DOR (20 µg/ml) and TIM (20 µg/ml).

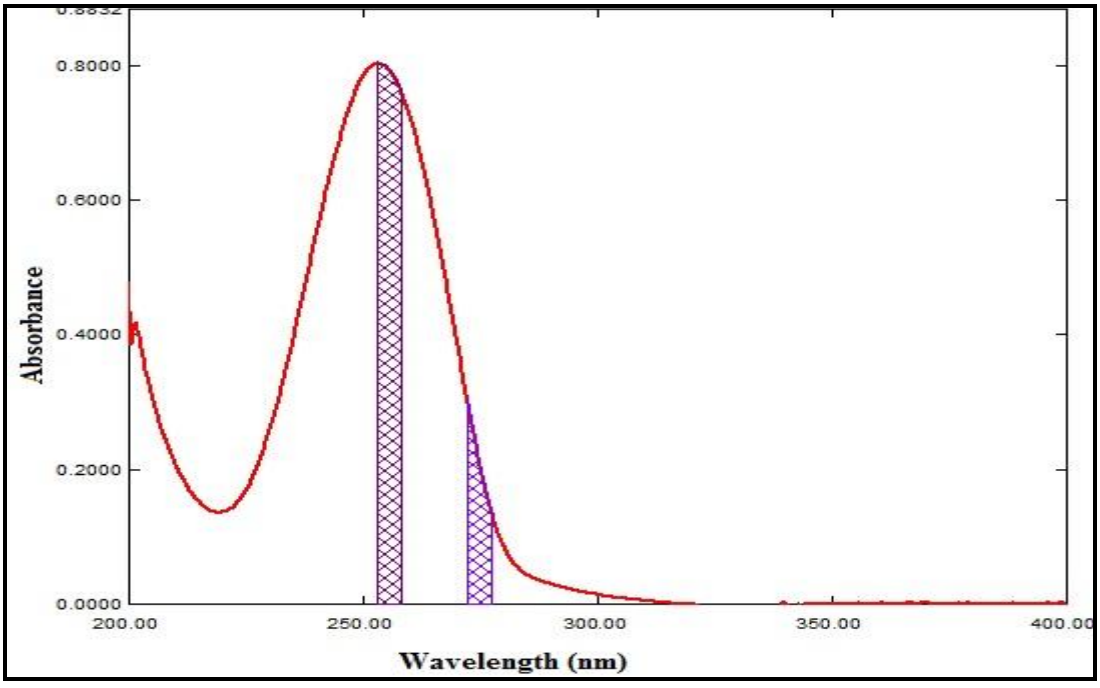

Figure (3): Wavelength ranges selected for AUC method of dorzolamide hydrochloride (20 µg/ml).

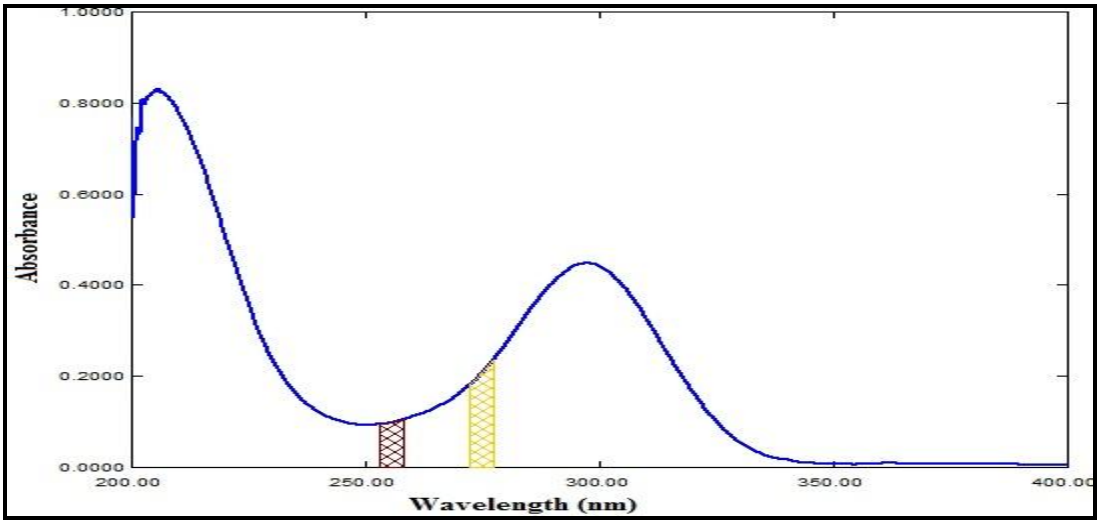

Figure (4): Wavelength ranges selected for AUC method of timolol maleate (20 µg/ml).

TIM can be easily determined directly at 297 nm without previous separation. The calibration graph for the method was established by plotting the absorbance values at 297 nm versus drug concentrations in µg/ml. The regression plot was found to be linear over the range of 4-40 µg/ml**.** The linear regression equation for the graph was:

y = 0.0237 x - 0.0101 (r = 0.9998).

The statistical results are shown in **Table 2.**

Dual wavelength Method

From the spectra of DOR and TIM, two specific wavelengths are chosen. The absorbance at 280 and 228.8 nm was found to be zero for TIM, while it is directly proportional to the concentration of DOR.

The calibration graph for the method was established by plotting the difference in absorbance at 280 and 228.8 nm, against the corresponding concentration of DOR. The regression plot was found to be linear over the range of 4-28 µg/ml. The linear regression equation for the graph was:

y = 0.0073 x + 0.0014 (r = 0.9999).

The statistical results are shown in **Table 3.**

Bivariate method

In order to apply the bivariate method in the resolution of DOR and TIM, the absorbance of the two components at seven different selected wavelengths was recorded in the region of overlapping; (220-280 nm). The calibration curve equations and their respective linear regression coefficients were obtained directly to confirm that there was a linear relationship between the absorbance and the corresponding concentration. All of the calibration curves at the chosen wavelengths displayed satisfactory linear regression coefficient ($r > 0.9981$).

According to Kaiser Method [14], the slope values of the linear regression equations for both DOR and TIM at the chosen wavelengths were used to calculate the sensitivity matrices K to get the optimum pair of the wavelength at which the binary mixtures were recorded as shown in **Table 1.** For the bivariate determination of DOR and TIM, 220 and 250 nm were found to yield the maximum value of K and thus can be used for the analysis**.** The statistical results are shown in **Table 3.**

The area under the curve method (AUC)

The area under the curve values of the absorption spectra in the wavelength ranges 253–258 nm (λ_1–λ_2) and 272-277 nm (λ_3–λ_4) for DOR and TIM in the concentration range of (4-28) and (4-40) µg /mL, respectively were calculated. The absorptivity 'a' values of DOR and TIM were calculated at each wavelength range. We get the concentrations of DOR from the following equation:

$$C_{(x)} = (A_{m1}\, a_{y2} - A_{m2}\, a_{y1}) / (a_{x1}\, a_{y2} - a_{x2}\, a_{y1})$$

Where C(x) is DOR concentration, A_{m1} and A_{m2} are the area under the curve of the mixture at the wavelength range (253 - 258) nm and (272 – 277) nm, respectively. Where a_{x1} = A_{x1}/conc. in µg/mL, a_{x2} = A_{x2}/conc. in µg/mL and a_{y1} = A_{y1}/conc. in µg/mL, a_{y2} = A_{y2}/conc. in µg/mL for DOR and TIM, respectively. The statistical results are shown in **Table 3.**

So, the proposed area under the curve method was successfully applied for estimation of DOR in presence of TIM in their laboratory prepared mixtures.

Methods validation

The proposed methods were validated according to ICH recommendations [15]

Linearity and range

The regression analysis data for the method was checked **Table (1)**. The linearity was found to obey *Beer's law* in the range of 4-40 µg/mL for TIM and 4-28 µg/mL for DOR.

Limits of detection and quantitation

The limits of detection (LOD) and the limits of quantitation (LOQ) were determined according to ICH guidelines from the following equations:

$$LOD = 3.3\ \sigma / S$$

$$LOQ = 10\ \sigma / S$$

Where σ is the standard deviation of y-intercepts of regression lines and S is the slope of the calibration graphs as mentioned in **Table 2, 3.**

Accuracy

The accuracy of the proposed methods was calculated by the average of three determinations for three concentrations for TIM or DOR repeated three times as mentioned in **Table 2, 3**.

Precision

Precision was evaluated by calculating intraday (repeatability) and interday (Intermediate precision) after repeating measuring of the three different concentrations three times in the same day and in three successive days using the proposed methods. The calculated RSD% values were listed as mentioned in **Table 2, 3** indicating the acceptable precision of the proposed methods.

The selectivity of the proposed procedures was assessed by the analysis of laboratory synthetic mixtures of both drugs as mentioned in **Table 4, 5**. Moreover, the standard addition technique was applied to check the accuracy and specificity of the described methods. It was done by adding known quantities of the studied drugs in its pure form to already analyzed pharmaceutical preparation and the percent recovery of the pure added concentrations was

calculated. The data listed in **Table 6, 7** proved that the proposed methods could selectively analyze the drugs without any interference from any excipients.

Robustness

The robustness of the proposed methods was evaluated by repeating the general procedure of each method with slight changes in the optimum conditions such as the selected wavelengths (±0.2 nm). Under these slight changes, no marked changes were observed in the results, confirming the robustness of the described methods.

Statistical analysis

To test the capability of the proposed methods for the determination of each drug separately in the pharmaceutical preparation. Statistical comparison of the results obtained by these methods and reported method (first derivative) [3]. The calculated student's t-test and F values were less than the theoretical ones indicating that there were no significant differences as mentioned in **Table 8**. Another statistical comparison of the results obtained by the proposed methods and the reported method for the determination of DOR in pharmaceutical preparation using one-way ANOVA test as mentioned in **Table 9**. The results obtained by applying these methods show no significant differences between all of them.

Table 1. Application of Kaiser method for the selection of the wavelength set for the determination of DOR-TIM mixture

λ_2 \ λ_1	220	230	240	250	260	270	280
220	0	-237.28	-643.69	**-944.72**	-854.12	-414..36	2.3
230		0	-232.49	-376.24	-325.48	-110.84	121.5
240			0	-95.01	-46.08	105.31	327.35
250				0	58.44	215.72	480.1
260					0	169.4	434.2
270						0	211.1
280							0

Table 2. Regression and analytical parameters of the proposed spectrophotometric methods for determination of TIM.

Parameter	
Wavelength (nm)	297
Range (µg /mL)	(4-40)
Slope	0.0237
Intercept	-0.0101
Correlation coefficient (r)	0.9998
LOD	0.587
LOQ	1.779
Accuracy[a]	100.43
Precision	
Repeatability (RSD)[b]	0.763
Intermediate precision (RSD)[c]	1.015

a, Average of three determinations for three concentrations (8, 16 and 36 µg/mL), for TIM repeated three times.

b, The intraday (n=3), an average of three concentrations (8, 16 and 36µg/mL), for TIM repeated three times within the day.

c, The interday (n=3), an average of three concentrations (8, 16 and 36µg/mL), for TIM repeated three times in three days.

Table 3. Regression and analytical parameters of the proposed spectrophotometric methods for determination of DOR.

Parameter	Dual wavelength	Bivariate		Area under curve	
Wavelength (nm)	228.8 and 280	220	250	253-258	272-277
Range (µg /mL)	(4-28)				
Slope	0.0073	0.0051	0.0377	0.1882	0.0485
Intercept	0.0014	0.0222	0.0092	0.0437	0.0626
Correlation coefficient (r)	0.9999	0.9931	0.9998	0.9998	0.9995
LOD	0.329	0.963	0.465	0.362	0.737
LOQ	0.998	2.918	1.408	1.097	2.235
Accuracy[a]	99.56	100.04		100.06	
Repeatability(RSD)[b]	0.855	1.127		0.832	
Intermediate precision(RSD)[c]	1.035	1.219		1.011	

a, Average of three determinations for three concentrations (10, 20 and 24 µg/mL), for DOR repeated three times.

b, The intraday (n=3), an average of three concentrations (10, 20 and 24µg/mL), for DOR repeated three times within the day.

c, The interday (n=3), an average of three concentrations (10, 20 and 24µg/mL), for DOR repeated three times in three days.

Table 4. Determination of TIM in laboratory prepared mixtures

Conc. of TIM (µg/ml)	Conc. of DOR (µg/ml)	TIM found (µg/ml)	Recovery % of TIM
4	12	4.02	100.51
5	15	4.97	99.39
6	18	6.05	100.83
8	24	7.92	99.01
Mean			99.93
RSD%			0.873

Table 5. Determination of DOR in laboratory prepared mixtures

	Conc. of DOR (µg/ml)	Conc. of TIM (µg/ml)	DOR found (µg/ml)	Recovery % of DOR
Dual wavelength	12	4	12.10	100.83
	15	5	14.88	99.19
	18	6	17.95	99.72
	24	8	24.21	100.87
	Mean ± RSD			100.15±0.831
Bivariate	12	4	12.15	101.25
	15	5	15.14	100.93
	18	6	17.85	99.16
	24	8	24.05	100.21
	Mean ± RSD			100.38±0.921
Area under curve	12	4	11.91	99.25
	15	5	15.01	100.06
	18	6	17.65	98.05
	24	8	24.07	100.29
	Mean ±RSD			99.41±1.017

Table 6. Determination of TIM in Twinzol ® eye drops by the proposed method and application of standard addition technique

Pharmaceutical took (µg/ml)	Found (µg/ml)	Pure added (µg/ml)	Pure found (µg/ml)	Recovery %
10	9.96	4	3.95	98.75
		8	8.15	101.87
		20	19.75	98.75
		24	24.22	100.91
Mean ± RSD				100.07±1.575

Table 7. Determination of DOR in Twinzol ® eye drops by the proposed methods and application of standard addition technique

	Pharmaceutical took (µg/ml)	Found (µg/ml)	Pure added (µg/ml)	Pure found (µg/ml)	Recovery %
Dual wavelength	10	10.12	4	3.93	98.25
			8	8.05	100.62
			12	12.14	101.16
			20	20.09	100.45
	Mean ± RSD				100.12±1.283
Bivariate	10	9.93	4	4.06	101.50
			8	8.15	101.87
			12	12.05	100.41
			16	15.97	99.81
	Mean ± RSD				100.90±0.944
Area under curve	10	10.06	8	7.95	99.37
			10	10.14	101.40
			12	12.05	100.41
			16	16.15	100.93
	Mean ±RSD				100.53±0..865

Table 8. The statistical comparison for the results obtained by the proposed and the reported methods for the analysis of TIM and DOR in Twinzol ® eye drops.

Parameters	Proposed methods				Reported method	
	Zero-order	**Dual wavelength**	**Bivariate**	**Area under curve**	**TIM**	**DOR**
N	5	5	5	5	5	5
Mean	100.18	99.88	100.06	99.72	99.93	99.83
SD	0.956	0.955	1.597	1.420	0.882	0.865
Variance	0.914	0.911	2.551	2.017	0.778	0.749
Student- t-test*	0.419 (2.306)	0.092 (2.306)	0.290 (2.306)	0.144 (2.306)	____	____
F-value*	1.174 (6.388)	1.216 (6.388)	3.406 (6.388)	2.017 (6.388)	____	____

*The values in the parenthesis are the corresponding theoretical values of *t* and *F* at (P = 0.05)

Table 9. One-way ANOVA testing for the different proposed methods used for the determination of DOR in Twinzol® eye drops.

Source of variation	**Degree of freedom**	**Sum of squares**	**Mean square**	***F* value**
Between exp.	3	0.307	0.102	0.066 (3.238)
Within exp.	16	24.914	1.557	

The values in parentheses are the theoretical *F* values. The population means are not significantly different.

CONCLUSION

The proposed spectrophotometric methods were presented as robust methods to resolve the interference. The proposed methods were simple with minimal manipulation steps, sensitive, precise and could be easily applied in quality control laboratories as they have equal accuracy and precision compared to the reported first derivative spectrophotometric method for the determination of the studied drugs without the previous separation step.

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