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Two Stability Indicating Chromatographic Methods: TLC Densitometric versus HPLC Method for the Simultaneous Determination of Brinzolamide and Timolol Maleate in Ophthalmic Formulation in the Presence of Probable Carcinogenic Oxidative Degradation Product of Timolol Maleate

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Abstract: A comparative study between two stability-indicating chromatographic methods for the assay of brinzolamide and timolol maleate in the co-existence of the probable carcinogenic oxidative degradation product of timolol maleate in their ophthalmic formulation was demonstrated. The first method established the thin-layer chromatography coupled with the densitometric determination of the analyzed spots, using silica gel TLC aluminum plates F254 and a developing system of chloroform: methanol: ammonia (6:1:0.1, in volumes) at room temperature to give good separation for the three investigated components, where retardation factors for the oxidative degradation product of timolol maleate, brinzolamide and timolol maleate were (Rf 0.21), (Rf 0.46), and (Rf 0.55), respectively. The linear ranges were 2–10 and 3–16 µg/band for brinzolamide and timolol maleate, respectively. In the second method, high performance liquid chromatography (HPLC), photo diode array detection was used on a Eurospher 5 µm C18 100 Å (4.6 × 250 mm) column, using triethylamine pH 3.5, adjusted by glacial acetic acid: acetonitrile (20:80, *v/v*) at a rate of 0.5 mL per minute. An acceptable separation was achieved, where the retention times for timolol maleate, the oxidative degradation product of timolol maleate and brinzolamide, were (Rt 3.6), (Rt 4.7), and (Rt 5.6), respectively. Linearity covered a range of 20–120 µg/mL for both drugs. It has been proved previously that timolol maleate is liable to oxidation, giving a high-probability carcinogenic product in female mice. The validation for the new proposed stability-indicating methods was optimized in line with the ICH guidelines with good outcomes. It is worth noting that the HPLC-DAD method showed superior separation, economic and time-saving results, while TLC method was more sensitive.

Keywords: brinzolamide; timolol maleate; TLC; HPLC-DAD; oxidative degradation

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1. Introduction

Brinzolamide (BRZ) (4R)-4-(ethylamino)-2-(3-methoxypropyl)-1,1-dioxo-3,4-dihydro-thieno[3,2-e] thiazine-6-sulfonamide (Figure 1a) is a carbonic anhydrase inhibitor used for the management of elevated intraocular pressure in patients with open-angle glaucoma or ocular hypertension [1–3]. The timolol maleate drug (TM) (-)-1-(tert-Butylamino)-3-[(4-morpholino-1, 2, 5-thiadiazol-3-yl) oxy]- 2-propanol maleate (1:1) (Figure 1b) is one of the

non-selective beta-adrenergic blockers administered in eye drops to reduce intraocular pressure [1,2]. It is also used as tablets for elevated blood pressure treatment and has high efficacy in the treatment of open-angle glaucoma [4]. Monotherapy is generally used as an initial therapy for glaucoma. However, most patients required combined therapy for glaucoma treatment, as they were not adequately responsive to monotherapy. Nowadays, brinzolamide is present in the market, combined with timolol maleate in AZARGA® eye drops for glaucoma treatment.

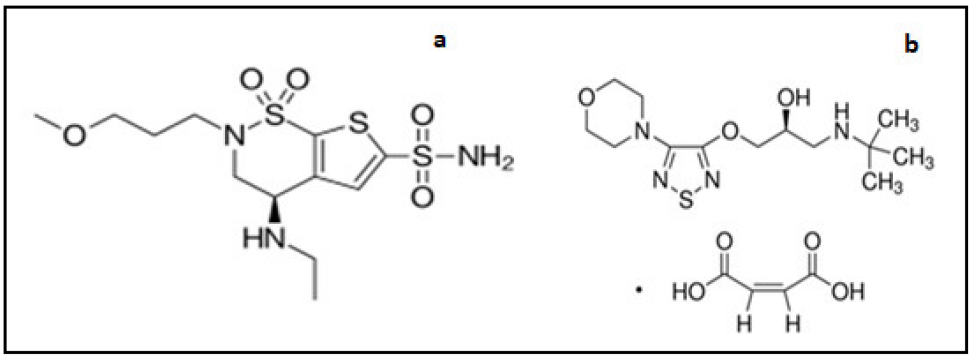


Figure 1. Chemical formula of brinzolamide (**a**) and chemical formula timolol maleate (**b**).

It was observed from the literature surveys that some analytical techniques have been investigated for the analysis of brinzolamide and timolol maleate separately [5–7], or in their mixture [8–12], or in mixture with other medicines [13–20], using high performance liquid chromatography (HPLC) analytical methods or thin-layer chromatography (TLC) methods [21–23], where the recorded linearity in the method [8] was in the range 0.001–0.018 µg/mL and 0.001–0.023 µg/mL for brinzolamide and timolol maleate, respectively, while in the method [9], linearity ranges were at 40–140 µg/mL and 20–70 µg/mL for both drugs with retention times of more than 10 min. In addition, in the method [10], the two drugs were analyzed in the rectilinear ranges of 1.25–25 µg/mL and 5–50 µg/mL for timolol and brinzolamide, respectively, in the presence of dorzolamide and brimonidine. In method [11], the recorded ranges were 5–25 µg/mL and 20–100 µg/mL, for brinzolamide and timolol maleate, respectively, and a UV detector was used at 260 nm. In the method [12], the more sensitive HPLC-MS method was efficiently applied for the detection of both drugs in the range of (50–5000 ng/mL) in rabbit aqueous humor. However, none of these methods determines the drugs in the co-existence of the oxidative degradation product of timolol maleate.

Since the safety and efficacy of therapeutic products are strongly related to their stability, impurities and related substances may affect the pharmacological effect, or may in some cases cause the progression of adverse reactions [24]. It is therefore helpful to evaluate these active constituents qualitatively and quantitatively using a stability-indicating method.

At the time of writing, no stated HPLC or TLC method was found for the determination of BRZ and TM in the existence of the degradation product of timolol maleate due to oxidation (OXD) in their mixture. In order to improve the quality, efficiency and safety of active therapeutic formulations, the establishment of a stability-indicating method that separates and identifies the most possible degradation products produced by forced degradation is required. The objective of the present study is to set up and validate—in a comparative way—two accurate, reliable and reproducible stability-indicating analytical TLC-densitometric and HPLC methods for the simultaneous estimation and quantification of BRZ and TM with OXD in bulk powders or pharmaceutical ophthalmic formulation, according to ICH guidelines.

2. Experiment

2.1. Reagents and Materials

2.1.1. Standards

Standard BRZ was provided by PHARCO Pharmaceuticals, Cairo, Egypt. Standard TM was provided by EPICO Company, Cairo, Egypt. Their purities were described as 99.7% and 99.92%, respectively, as reported in their HPLC method [9].

2.1.2. Pharmaceutical Formulation

AZARGA® eye drops: each 1 mL was labeled as having 10.00 mg BRZ and 5.00 mg TM (Batch no: 27001), and was manufactured by ALCON-COUVREUR, Belgium, and purchased from a community pharmacy in Cairo, Egypt.

2.2. Chemicals and Solvents

Acetonitrile, methanol hydrogen peroxide 10%, glacial acetic acid, triethylamine, chloroform and 33% ammonia solution were all HPLC grade and obtained from Sigma-Aldrich, Germany. Ultra-pure water was provided from an Elga Ultrapure Quest.

2.3. Instruments and Software

(a) Pre-coated silica gel TLC AL plates F254 (20 cm × 20 cm), of layer thickness 0.20 mm (Buchs, Switzerland) were purchased. Camag autosampler (Muttens, Switzerland) equipped with a Camag micro syringe (100µL) was used for samples at a constant addition frequency of 10 µL per second and a band of 6 mm thickness. The Camag TLC-scanner model 3S/N 130419 in the reflectance absorption mode (Muttens, Switzerland) with the speed of 20 mm per second was used for scanning, and the winCATS software was used for densitometric evaluation (Muttens, Switzerland). The dimension of the slit was 6.00 mm ×0.30 mm. An ultraviolet lamp of short wavelength 254 nm (United States) was used for the visualization of the plates.

(b) An Agilent ultra-performance 1290 infinity was used, supported with a diode array detector, quaternary pump VL, column oven TCC and 1290 Thermostat. Agilent Chemstation software (B.04.03) was used for the data acquisition. Separation was carried out using a Eurospher 5 µm C 18 column [100 Å, 4.6 × 250 mm)]. pH adjustment was performed using a Jenway 3505 pH meter (Staffordshire, UK).

2.3.1. TLC Conditions

The best mobile phase system was chloroform: methyl alcohol: ammonia (6: 1: 0.1, *in volumes*) at normal room temperature. This developing system allows for the best resolution of the 3 components and quantitative determination without any interference.

2.3.2. HPLC Conditions

Separation was carried out on a Eurospher 5µm C18 100 Å (4.6 × 250 mm) column, using triethylamine pH 3.5, adjusted by glacial acetic acid: acetonitrile (20:80, *v/v*) at a rate of 0.5 mL per minute.

2.4. Standard Solutions

- For the TLC-densitometric method (Method I, MI)

Standard stock liquid solutions of 2.00 mg/mL BRZ, TM were prepared by weighing 100 mg of each drug in two distinct 50-mL flasks and the final volume was achieved using methanol.

- For PR-HPLC method (Method II, MII)

Standard stock solutions of 1.00 mg/mL BRZ and TM were prepared by the addition of 50 mg of each in two distinct 50 mL flasks, and the volume was made up with methanol.

- Degradation stock solution

Preparation of oxidative degradation product of timolol maleate.

Twenty milliliters of 10% H_2O_2 was added to 100 mg of TM dissolved in 10 mL water, reflux at 70 °C for 30 min, then allowed to evaporate until dry. The residue was dissolved in methyl alcohol and quantitatively poured into 50-mL flask. The final volume was achieved with methyl alcohol.

The stock solution of the oxidative degradation product (OXD) derived from the complete degradation of standard solution of TM (2 mg/mL) was used for MI, while a diluted solution equivalent to 0.4 mg/mL in methanol was used for MII.

2.5. Method Validation

2.5.1. Chromatographic Conditions of Applied Methods

TLC-Densitometry (MI)

Different aliquots of 2–10 mg of BRZ and 3–16 mg of TM were transferred from their equivalent standard stock solutions into two distinct series of 10-mL flasks, then filled to 10 mL with methyl alcohol. A volume of 10 µL from each standard working solution was applied three times to 20 × 10 cm TLC Al plates (the width for the band was 6 mm; the space between bands was 14 mm; 10 mm and 15 mm from the sides and from the plate bottom edge, respectively) using a Camag Linomat autosampler. The chromatographic development was carried out in a glass tank that was pre-saturated for 30 min with a mobile phase consisting of chloroform: methyl alcohol: ammonia (6:1:0.1, in volumes) at normal temperature. The plates obtained were air dried and measured at 260 nm. The linearity was achieved by plotting the average integrated peak areas versus the corresponding concentrations. The regression equation was then calculated.

RP-HPLC Method (MII)

Concentrations of 0.2–1.2 mg of either TM or BRZ were distinctly prepared in sequences of 10 mL flasks and diluted with methyl alcohol. Then, 5.00 µL from each drug solution was inserted by the aid of an Agilent® auto-sampler ALS into reversed phase Eurospher 5µm C 18 as a stationary phase column (100 Å, (4.6 × 250 mm)) stationary phase and eluted using triethylamine pH 3.5 adjusted by glacial acetic acid and acetonitrile (20:80, *v/v*) as a movable liquid system. A 0.2 µm millipore filter, a white nylon membrane was employed for filtration process. After that, an ultrasonic bath was processed for 10 min for degassing before final use. The system was operated at ambient temperature and the speed of flow was 0.5 mL per minute. The UV detector was adjusted at 260 nm for both BRZ and TM. Chromatograms, peak areas, calibration curves, and regression equations were determined and computed for TM and BRZ automatically.

2.5.2. Selectivity/Specificity

Specificity was tested by achieving complete baseline separation between the 3 components BRZ, TM and OXD, as shown in Figures 2 and 3, with good resolution. LOD and LOQ were more sensitive to MI than MII, while the time needed for complete separation was shorter in MII than MI. (Table 1).

2.5.3. Accuracy

Pure samples for both TM and BRZ were analyzed to check the accuracy. The overall results of percent recoveries of both drugs in pure drug were calculated from its regression equation. Relevant results are shown in Table 1, indicating the accuracy of the proposed method. Furthermore, they were tested in pharmaceutical dosage form by applying the standard addition technique to which known amounts of TM and BRZ standards had been added in different concentrations (Table 2).

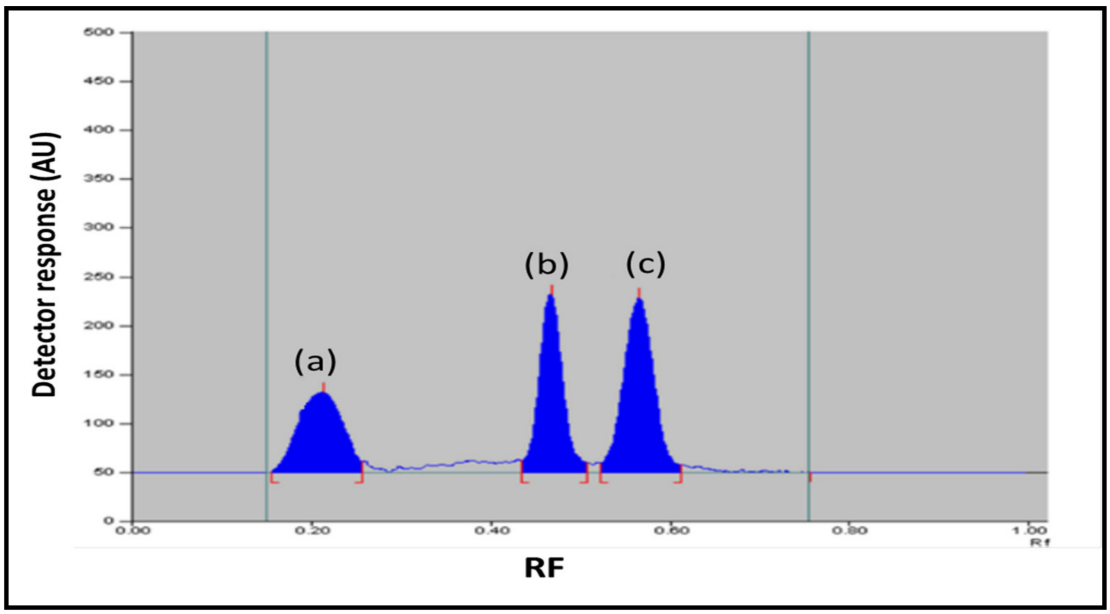


Figure 2. TLC densitogram of (**a**) 3 µg/band oxidative degradation product of timolol maleate (Rf 0.21), (**b**) 6 µg/band brinzolamide (Rf 0.46) and (**c**) 3 µg/band timolol maleate (Rf 0.55) using a developing system consisting of chloroform: methanol: ammonia (6:1:0.5, by volume), at 260 nm.

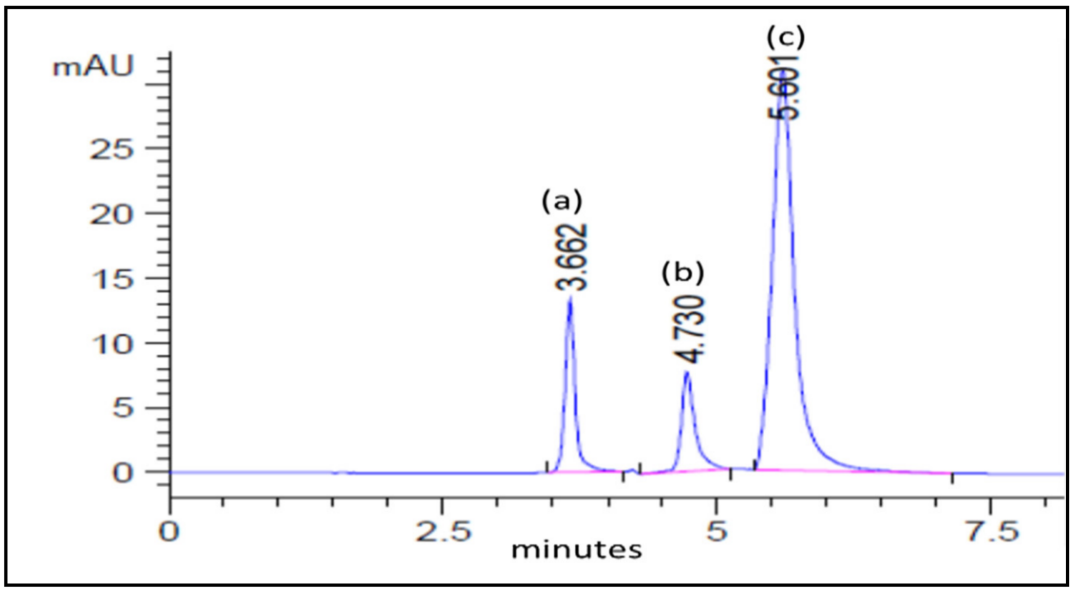


Figure 3. RP-HPLC chromatogram for the investigated mixture of (**a**) 40 µg/mL timolol maleate (Rt 3.6) (**b**) 20 µg/mL oxidative degradation product of timolol maleate (Rt 4.7) and (**c**) 60 µg/mL brinzolamide (Rt 5.6) on a Eurospher C18 stationary system, with a mobile phase composed of triethylamine pH 3.5: acetonitrile (20:80, in volumes).

2.5.4. Precision

The interday and intraday precision of the investigated methods were estimated by evaluating three repeated injections of mixture of concentrations 3.5, 5.5, 6.5 for BRZ and 6, 10, 13 for TM for MI and 30, 50, 70 for BRZ and TM for MII the same day and on three successive days, respectively.

Precision was estimated by repeatability. The values of precision (RSD %) for BRZ and TM for MI and MII are shown in Table 1.

Table 1. Items for the Validation for TLC-densitometric (MI) and RP-HPLC (MII) methods for the analysis of BRZ and TM.

Parameter	(MI)		(MII)	
	BRZ	**TM**	**BRZ**	**TM**
Wavelength (nm)	260		260	
Time of analysis (minutes)	10 min		6 min	
Regression parameters				
range	2.00–10.00 (µg/band)	3.00–16.00 (µg/band)	20.00–120.00 µg/mL	20.00–120.00 µg/mL
Linearity				
Intercept	+7659.2	+721.85	−38.577	−0.3853
Slope	640.87	293.65	13.886	0.7505
Correlation Coefficients	0.9996	0.9997	0.9998	0.9996
Accuracy				
Mean ± SD	99.51 ± 0.78	99.22 ± 1.08	101.31 ± 0.64	100.16 ± 0.62
Precision data (± %RSD)				
Intraday precision [a]	±0.43	±0.71	±0.19	±0.67
Intermediate Precision [b]	±0.93	±1.05	±0.21	±0.98
Specificity [c] **(Mean ± SD)**	99.92 ± 0.66	100.18 ± 1.35	99.92 ± 1.08	100.04 ± 0.39
Robustness	±0.99	±0.76	±0.16	±0.90
LOD [d]	0.70 µg/band	0.99 µg/band	5.11 µg/mL	6.45 µg/mL
LOQ [d]	2.13 µg/band	3.00 µg/band	15.49 µg/mL	19.57 µg/mL

[a] Intraday precisions: the %RSD for three various concentration levels (3.5, 5.5, 6.5 for BRZ and 6, 10, 13 for TM) for TLC- densitometric and (30, 50, 70 for BRZ and TM) for RP-HPLC / three times each, within a day. [b] Interday precisions: the %RSD for three dissimilar concentrations (3.5, 5.5, 6.5 for BRZ and 6, 10, 13 for TM) for TLC-densitometric and (30, 50, 70 for BRZ and TM) for RP-HPLC / three times each, repeated on three consecutive days. [c] Recovery of FP and CL in laboratory equipped mixtures having an oxidative degradation product of TM. [d] Using the equations [LOQ = 10 (S.D/S); LOD = 3.3 (S.D/S), where S is the slope for the proposed technique, and S.D is the residual standard deviation for the slope.

2.5.5. Robustness

The chromatographic method robustness was investigated by testing the outcome of minimal variations in the nominated conditions on the parameters for system suitability. Regarding the TLC-densitometric technique, BRZ, TM and the degradation product of TM were analyzed in many settings as development distance (10.00 ± 0.50 cm), developing system amount (50.00 ± 3.00 mL) and duration of saturation of chromatographic tank (30.00 ± 1.00 min). The values for retardation factors of the measured peaks were the same, and the calculated resolution (Rs) data were usually within the accepted range, confirming optimum separation (Table 3). Concerning the HPLC analytical method, the combination of the three chemicals was efficiently analyzed in various settings via the usage of dissimilar flow speeds (0.5 ± 0.1 mL/min), diverse pH values (3.5 ± 0.1) and wavelength (260 ± 0.5). When making a small change in pH range and in wavelength, the observed retention times for the detected peaks remained relatively similar, while changing the flow speed caused minor deviations in retention times for all detected peaks. However, Table 4 shows that the calculated resolution values were at all times above 2 and the accepted results for tailing factor (T) and capacity factor (K′).

Table 2. Standard Addition Technique for the analysis of BRZ and TM in AZARGA® eye drops by MI and MII.

Product	Drugs	MI (Standard Addition)					MII (Standard Addition)				
		Claimed Taken (µg/band)	Added (µg/band)	Total Found [b] (µg/band)	Standard Found [b] (µg/band)	%Recovery of Added [b]	Claimed Taken (µg/mL)	Added (µg/mL)	Total Found [b] (µg/mL)	Standard Found [b] (µg/mL)	%Recovery of Added [b]
AZARGA Drops [a] (B.N 27001)	BRZ	4.00	——	3.94	——	——	50.00	-	50.40	-	-
		4.00	2.00	5.92	1.98	99.00	50.00	25.00	75.72	25.32	101.28
		4.00	4.00	7.91	3.97	99.25	50.00	50.00	100.91	50.51	101.02
		4.00	5.00	9.01	5.07	101.40	50.00	60.00	111.00	60.60	101.00
		Mean ± SD [b]				99.88 ± 1.31	**Mean ± SD [b]**				101.10 ± 0.15
	TM	5.00	——	4.90	——	——	25.00	-	25.30	-	-
		5.00	3.00	7.93	3.03	101.00	25.00	20.00	45.42	20.12	100.60
		5.00	5.00	10.00	5.10	102.00	25.00	25.00	49.98	24.68	98.72
		5.00	6.00	10.92	6.02	100.33	25.00	50.00	75.53	50.23	100.46
		Mean ± SD [b]				100.11 ± 0.84	**Mean ± SD [b]**				99.92 ± 1.04

[a] Labeled to contain 5 mg TM and 10 mg BRZ. [b] average for three estimations.

Table 3. Data for robustness for the TLC-Densitometric Method (MI).

Analyzed Drug	Parameters		T[a]	K′ [a]	Rs [b]	% Assay [c]
BRZ	Amount of the developing system	50 + 3 mL	0.87	1.22	3.15	100.51
		50 − 3 mL	1.0	1.19	3.10	99.72
	Duration of saturation of chromatographic tank	30 + 1 min	0.83	1.20	3.23	98.55
		30 − 1 min	1.0	1.21	3.20	100.10
	Development distance	10 + 0.5 cm	1.0	1.19	3.3	98.82
		10 − 0.5 cm	1.0	1.17	3.28	101.17
TM	Developing system amount	50 + 3 mL	0.90	0.89	1.22	100.95
		50 − 3 mL	1.02	0.82	1.18	99.92
	Duration of saturation of chromatographic tank	30 + 1 min	0.87	0.85	1.17	99.23
		30 − 1 min	0.90	0.89	1.21	98.96
	Development distance	10 + 0.5 cm	1.0	0.82	1.3	99.57
		10 − 0.5 cm	0.83	0.79	1.23	100.52

[a] Tailing and capacity factors estimated for individual peaks. [b] Resolution factor calculated based on the difference between each two adjacent peaks. [c] Assay percentage was computed via regression equations.

Table 4. Data for robustness for the HPLC Method (MII).

Drug	Parameters		T [a]	K′ [a]	Rs [b]	% Assay [c]
TM	Flow speed	0.5 + 0.1 mL/minute	1.20	16.61	—	99.25
		0.5 − 0.1 mL/minute	1.00	16.62	—	100.50
	pH values	3.5 + 0.1 units	1.10	16.58	—	101.40
		3.5 − 0.1 units	1.10	16.56	—	101.13
	Wavelength	260 + 0.5 nm	1.10	16.61	—	100.48
		260 − 0.5 nm	1.10	16.61	—	101.81
BRZ	Flow speed	0.5 + 0.1 mL/minute	1.30	25.63	2.98	98.62
		0.5 − 0.1 mL/minute	1.10	25.62	3.07	98.81
	pH values	3.5 + 0.1 units	1.30	25.59	3.06	98.63
		3.5 − 0.1 units	1.30	25.59	3.09	98.45
	Wavelength	260 + 0.5 nm	1.30	25.63	2.98	98.76
		260 − 0.5 nm	1.30	25.63	2.97	98.91

[a] Tailing and capacity factors estimated for separate peaks. [b] Resolution factor calculated based on the difference between each two adjacent peaks. [c] Assay percentage was computed via regression equations.

The novel methods were successfully used for the determination of BRZ and TM in eye drops. The outcomes were very satisfactory, with proper alignment with the claimed amounts. In addition, no interferences due to excipients were noted when the standard addition procedures were used, as shown in Table 2.

2.5.6. Application to AZARGA® Eye Drops Pharmaceutical Formulation

One milliliter of AZARGA® eye drops (labeled as containing 10 mg of BRZ and 5 mg of TM) was transferred into 10 mL flask, then the volume was made up with methyl alcohol, followed by sonication for 10 min. The concentration levels in the linear range were obtained with dilutions with methyl alcohol and analyzed following the aforementioned settings.

3. Results and Discussion

The stability analysis is a significant step in production processes. The purpose of stability testing is to determine the variation of drug quality with time intervals by the influence of different environmental issues such as temperature, light and humidity, and to provide commendations for optimal storage environments, retest periods and establish shelf life [25]. The assay of drug products in stability test samples needs to be examined using a stability-indicating assay, as recommended by USP [1] and the International Conference on Harmonization (ICH) strategies [26]. The efficiency of the stability indicating chromatographic methods has been well stated for many drugs [27–32]. The published methods of assay for TM and BRZ in the pharmaceutical formulation as eye drops have not been yet validated using degradation studies and specificity [8–12,21].

TM is susceptible to oxidation with H_2O_2 at normal room temperature into its OXD, which was characterized by LC/MS. The electron impact outcomes showed mass ion peak at *m/z* [m^{+H}] = 317 for the intact drug, while the OXD mass ion peak was at m/z 411, which is due to the oxidation of timolol and also the oxidation of maleate to epoxide form [33], as shown in Figure 4. It can therefore be suggested that the implementation of the oxidation of TM can be demonstrated in Scheme 1. This illustration coincides with previous results, as the obtained OXD was reported to be one of the degradation products suggested by Devrukhakar et al. [34]. As OXD has a toxicity indication in the NTP Carcinogenicity Call in Female Mice [34]. The analysis for combinations of BRZ and TM in pure powder or in their pharmaceuticals without interference of OXD was therefore an analytical duty of great importance and up to now there has been no stability-indicating assay methods for the analysis of BRZ and TM in the co-existence of the oxidative degradation product using TLC and HPLC for quantitative assay of BRZ and TM.

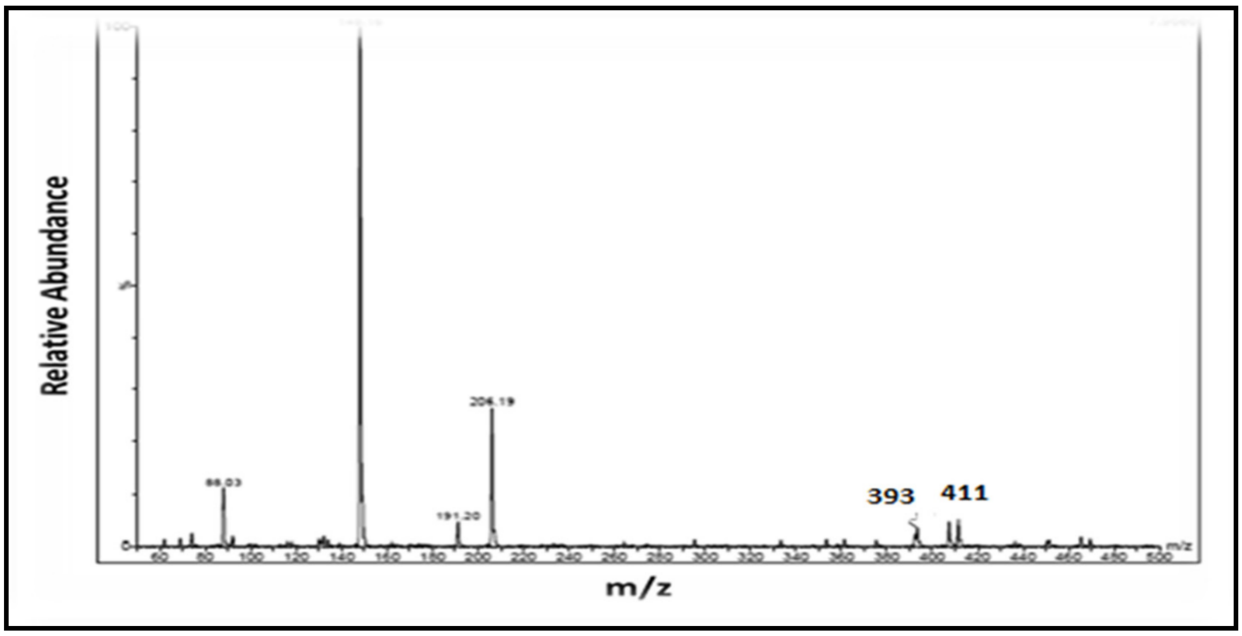
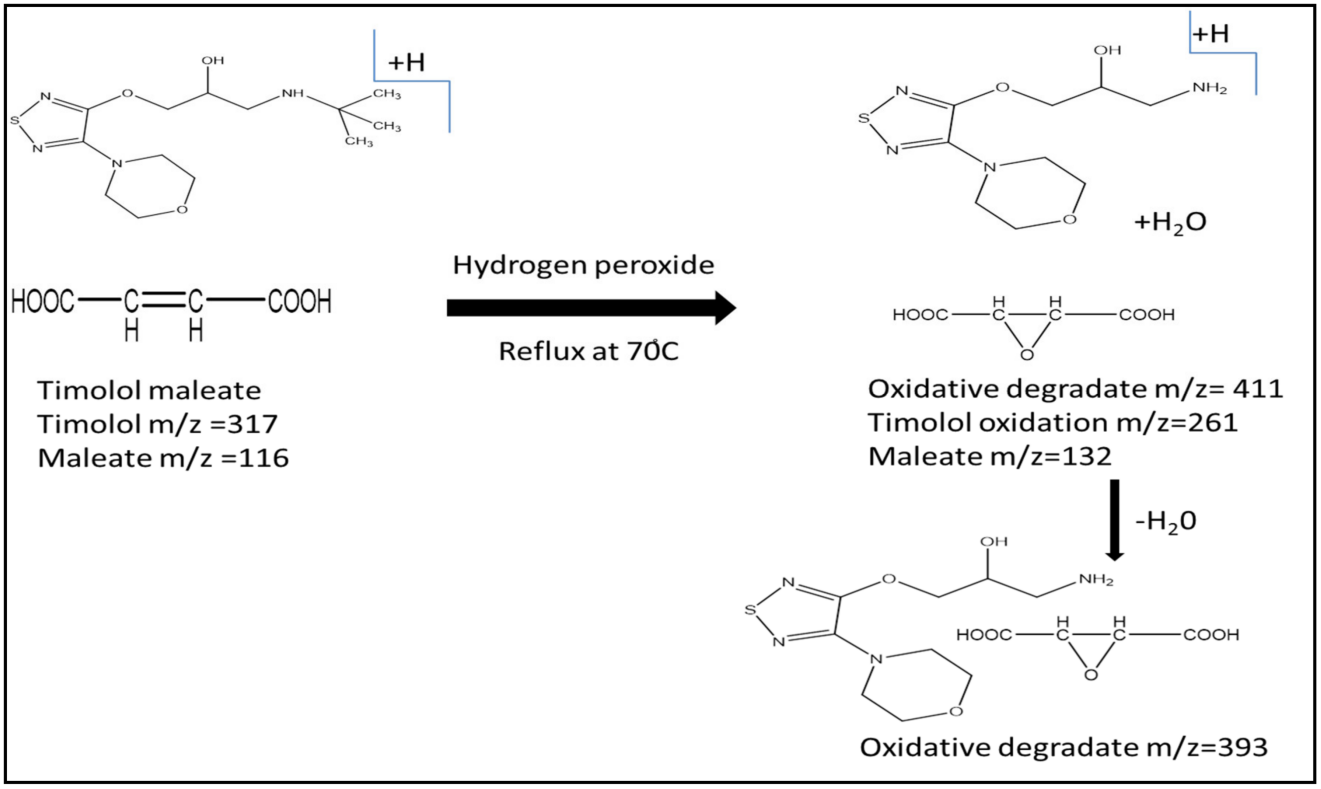


Figure 4. Mass spectrum of the oxidative degradation product of timolol maleate.



Scheme 1. Oxidation mechanism of timolol maleate to its oxidative degradation product.

Thus, this study aimed to set up and compare stability-indicating methods for the selective estimation of BRZ and TM in the co-existence of the latter oxidative degradation product.

3.1. Method Optimization

3.1.1. TLC-Densitometry (MI)

Dissimilar developing liquid phases of different components and percentages were tested to separate the aforementioned drugs from OXD, such as chloroform: methanol: ethyl acetate: ammonia and ethyl acetate: methanol: ammonia or acetic acid in different ratios, but no satisfactory separation was obtained.

The finest liquid mobile phase was chloroform: methanol: ammonia (6:1:0.1 in volumes). This liquid phase allowed for the best resolution of the three components and quantitative determination without any interference (Figure 3). By testing different band dimensions, 6 mm was found to be the optimum band dimension as it allows sharp and symmetrical separated peaks to be obtained. Various wavelengths were tested; detection at 260 nm was the optimum as it gives the finest sensitivity with minimal noise. An optimum separation with good resolution and acceptable system suitability parameters was obtained, as shown in Table 5.

Table 5. Data for Suitability of the System for the TLC-Densitometric Method (MI).

Parameters	Degradation of TM	BRZ	TM	Reference Value [35]
K′ "capacity factor"	3.76	1.17	0.82	**The higher the K value for a compound, the less retardation factor is observed**
α "Relative retention"	3.21	1.43		>1
Resolution	3.31	1.23		>1
Factor of Symmetry	1.00	1.00	1.00	**1 for symmetrical peak**

3.1.2. HPLC (MII)

First, Zobax C18 1.8µm (2.1 × 50 mm) stationary phase was tried with many liquid phases with various ratios, but poor separation was obtained, with non-symmetric broad peaks. Exchanging it for a Eurospher 5µm C18 100 Å (4.6×250 mm) column gave symmetrical, sharp and well resolved peaks of the three components. Varied mobile phase compositions were tested, such as mixtures of many organic solvents with water, but very poor resolution was obtained. Also, phosphate buffers in many pH ranges with diverse non-aqueous solvents, e.g., acetonitrile and methyl and ethyl alcohols in various percentages were tried until good separation with satisfactory results was obtained by using a mobile phase consisting of triethyl amine pH 3.5, adjusted by glacial acetic acid: acetonitrile (20:80, *in volumes*). The optimum flow speed was 0.5 mL/min and maximum sensitivity was obtained at 260 nm.

An acceptable separation with good resolution and acceptable system suitability parameters, as shown in Table 6, and appropriate chromatographic time was achieved using these optimum settings, where the retention times for TM, degradation product of TM, BRZ were 3.6, 4.7, 5.6 min., respectively (Figure 4).

Table 6. System Suitability Results for the RP-HPLC method (MII).

Parameters	Obtained Value			Reference Value [36]
	TM	Degradation of TM	BRZ	
Resolution	7.29		3.49	**R > 2**
α "relative retention"	1.31		1.18	**>1**
K' "capacity factor"	16.62	21.74	25.62	**K' > 2**
N "column efficiency"	10,196	15,650	5425	**The higher the value, the more efficient separation**
Tailing factor	1.10	1.10	1.20	**T = 1 for typical symmetric peak**

3.2. Validation of the Applied Method

The novel chromatographic methods were exposed to validation protocols as per ICH approach [26], and acceptable results were obtained (Table 1).

3.2.1. Linearity

Linear relationships were achieved between the integrated peak areas of the two medicines and their equivalent concentrations in the ranges of 2.00–10.00 µg/band and 3.00–16.00 µg/band for BRZ and TM, respectively, for MI. Also, linear relationships were obtained between the peak areas and the equivalent concentrations of each component in the ranges of 20.00–120.00 µg/ mL for both TM and BRZ in MII (Table 1).

3.2.2. Selectivity/Specificity

Specificity was tested by achieving complete baseline separation between the three components BRZ, TM and OXD with good resolution, as shown in Figures 2 and 3. LOD and LOQ were more sensitive to MI than MII, while the time needed for complete separation was shorter in MII than MI. (Table 1).

3.2.3. Accuracy

The accuracy of the established methods was validated by analyzing pure concentrations of both TM and BRZ. The overall results of percent recoveries of both drugs in the pure drug were considered using the calculated regression equations. Accepted results are shown in Table 1, indicating good accuracy of the proposed method, and also were tested in pharmaceutical dosage form by applying the standard addition technique on the dosage form, to which known amounts of TM and BRZ standards had been added in different concentrations (Table 2).

3.2.4. Precision

The interday and intraday precision for the novel methods were estimated by evaluating three repeated injections of a mixture of concentrations 3.5, 5.5, 6.5 for BRZ and 6, 10, 13 for TM for MI and 30, 50, 70 for BRZ and TM for MII the same day and on three successive days, respectively.

Precision was estimated by repeatability. The values of precision (RSD %) for BRZ and TM for MI and MII are shown in Table 1.

3.2.5. Robustness

Analytical method robustness was evaluated by testing the outcomes of minor variations in the investigational settings on the parameters of system suitability. Concerning the TLC densitometric method, combinations of BRZ, TM and the degradation product of TM were analyzed in various environments such as mobile phase amount (50.00 ± 3.00 mL), development distance (10.00 ± 0.50 cm), and duration of saturation of chromatographic tank (30.00 ± 1.00 min). Table 3 shows that the Rf values are constant and the values for resolution ensure complete separation between the separated peaks. System suitability parameters were studied for separated peaks such as tailing factor, capacity factor and selectivity, where acceptable results were obtained (Table 5). Regarding the HPLC analytical method, the combination of the three chemicals was efficiently analyzed in various settings using dissimilar flow speeds (0.5 ± 0.1 mL/min), diverse pH values (3.5 ± 0.1) and wavelength (260 ± 0.5). When making a small change in pH range and in wavelength, the observed retention times for the detected peaks are relatively similar, while changing the flow speed causes minor deviations in retention times for all detected peaks. However, Table 4 shows that the calculated resolution values were at all times above 2 and accepted results for the tailing factor (T) and capacity factor (K′). The items for system suitability for the RP-HPLC method are shown in Table 6.

The novel methods were successfully used for the determination of BRZ and TM in eye drops. The outcomes were very satisfactory, with proper alignment with the claimed amounts. In addition, no interferences due to excipients were noted when the standard addition procedures were used, as shown in Table 2.

4. Conclusions

For the first time, the interference of OXD impurity was removed and the simultaneous analysis for brinzolamide (BRZ) and timolol maleate (TM) was successfully applied in real ophthalmic formulations. Two novel and validated stability indicating chromatographic methods were presented for the concurrent quantitation of BRZ and TM in the co-existence of the probable carcinogenic oxidative degradation product of timolol maleate in bulk and in ophthalmic preparation. Also, a precise comparison between the two chromatographic methods was demonstrated, where the TLC-densitometric method was more sensitive when comparing numerical values for LOD and LOQ for both analytes. However, the merits recommend the HPLC-DAD method for its fast separation and automation. The new suggested chromatographic methods could be applied conveniently for the routine quality control analysis for BRZ and TM without any interference of OXD impurity in pharmaceuticals. By contrast, LC-MS is still more sensitive and reliable in pharmacokinetics studies for both investigated drugs.

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