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Article

Development and Validation of a Stability-Indicating Micellar Liquid Chromatographic Method for the Determination of Timolol Maleate in the Presence of Its Degradation Products

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A stability-indicating micellar liquid chromatographic (MLC) method was developed and validated for the quantitative determination of timolol maleate (TM) in the presence of its degradation products resulting from accelerated degradation in a run time not more than 8 min. TM was subjected to stress conditions of hydrolysis (including alkaline, acidic and thermal hydrolysis) and oxidation. An isocratic, rapid and mobile phase saving the micellar LC method was developed with a BioBasic phenyl column (150 × 1.0 mm, 5 μm particle size) and a micellar mobile phase composed of 0.1 M sodium dodecyl sulfate, 10% of 1-propanol and 0.1% of triethylamine in 0.035 M *ortho*-phosphoric acid. The flow rate of the mobile phase was 0.1 mL/min. UV detection was adjusted at 298 nm and performed at room temperature. The method has been validated according to the International Conference on Harmonisation guidelines. The method is successfully applied for the determination of TM in bulk powder and pharmaceutical dosage form.

Introduction

Timolol maleate (TM) is a nonselective β-adrenergic receptor antagonist indicated orally for treating heart attacks and hypertension, and topically for treating glaucoma by reducing aqueous humor production through blockage of the β-receptors on the ciliary epithelium (1). Several methods have been reported for the determination of TM including titrimetric official methods of USP (2) and BP (3), spectrophotometric determination for TM alone (4–6) or in mix with other drugs (7–10), densitometric determination (11–13), high-performance liquid chromatography (HPLC) determination with other drugs (14–20), stability-indicating HPLC (21), chiral liquid chromatography for enantiomer separation (22, 23), capillary electrophoresis (24, 25), chemiluminescence determination (26) and voltammetric determination (27, 28). Micellar liquid chromatographic (MLC) is an alternative to conventional reversed-phase liquid chromatography with aqueous organic mobile phase in which the mobile phase is aqueous solution of a surfactant at a concentration above the critical micelle concentration. Micellar LC has many advantages over RPLC, as it enhances the separation selectivity; it has the ability of separation of hydrophilic and hydrophobic analytes in the same run without gradient elution and enables the direct injection of untreated physiological fluids.

The aim of this work is to develop a novel stability study for TM under stress condition of hydrolysis (including alkaline, acidic and thermal hydrolysis) and oxidation of TM. There are no reported HPLC methods for stability study of TM in pure form or in pharmaceutical dosage form under simulating Egyptian summer climate.

Experimental

Reagents and chemicals

(i) TM pure sample was kindly supplied by Sigma Pharmaceuticals Industries, Egypt, and its purity was found to be 100.0 ± 0.697 according to a comparison method (7).

(ii) Timolol® 0.5% eye drops, labeled to contain 6.8 mg/mL TM equivalent to 5.0 mg/mL Timolol, manufactured by Egyptian International Pharmaceuticals Industries Co. (EIPICO), Egypt, were purchased from a local market.

(iii) *Ortho*-phosphoric acid (85%, w/w) (HPLC grade), triethylamine (TEA) and sodium dodecyl sulfate (SDS, 99%) were purchased from Riedel-de Häen (Seelze, Germany). 1-Propanol (HPLC grade) and sodium hydroxide were purchased from Sigma-Aldrich (Steinheim, Germany). Hydrochloric acid (30%, w/v) and hydrogen peroxide (30%, w/v) were purchased from Prolabo (West Chester, PA, USA). Deionized water was obtained from Purelab flex (Marlow, UK).

Instrumentation and chromatographic conditions

A Thermo Fisher Scientific HPLC Accela autosampler system equipped with an HPLC Accela photodiode array (PDA) detector (80 Hz version) (CA, USA) and an Accela 600 pump (CA, USA) was used. The injection volume was 20 μL. Analytical data were stored in a computer equipped with Chromoquest version 5 software. The pH was measured with Jenway pH meter 3510 (Essex, UK). The mobile phase was filtered through Charles Austen Pumps Ltd filter (model-B100 SE; UK), using a nylon membrane of 0.2 μm (Waters Corporation; Cork, Ireland). Ultrasonic used was Power Sonic 410 (Treviglio, Italy). The analyses were carried out on a BioBasic phenyl column (150 × 1.0 mm, 5 μm particle size; Thermo Corporation, CA, USA), as a stationary phase. The mobile phase was prepared by transferring 28.84 g of SDS (0.1 M), 100 mL of 1-propanol (10%) and 1 mL of TEA (0.1%) into a 1-L volumetric flask and then completed to volume with 0.035 M *ortho*-phosphoric acid. The apparent pH was adjusted to 2.8 if necessary with *ortho*-phosphoric acid. The mobile phase was filtered and sonicated for 30 min before use. The flow rate of the mobile phase was 0.1 mL/min. The wavelength of the PDA detector was set at 298 nm to detect TM and its degradation products. All analyses were carried out at room temperature.

Preparation of solutions

Preparation of standard solutions

Standard stock solutions. Standard stock solution of TM (1.0 mg/mL): An accurately weighed 100.0 mg of TM was transferred into a 100-mL volumetric flask, dissolved and completed to volume with mobile phase.

Standard working solutions. Standard working solution of TM (0.1 mg/mL): A portion of 10 mL of the previously prepared standard stock solution was transferred into a 100-mL volumetric flask and then completed to volume with mobile phase.

Preparation of eye drop solutions

Eye drop stock solution. Stock solution equivalent to 136 μg/mL of TM was prepared by transferring 1 mL of Timolol® 0.5% eye drops into a 50-mL volumetric flask and then completed to volume with mobile phase.

Eye drop working solutions. Portions of 2.5, 3.75 and 5 mL of the previously prepared eye drops stock solution were accurately transferred separately into three 10-mL volumetric flasks and then completed to volume with mobile phase.

Forced degradation conditions

Oxidative degradation

An accurately weighed 20.0 mg of TM powder was transferred into a 250-mL conical flask and 2 mL of 0.3% H_2O_2 was added and then evaporated until dryness (~2 min). The residue was dissolved and quantitatively transferred into a 50-mL volumetric flask using deionized water and then the volume was completed with it. Triplicate 20 μL from a 40 μg/mL solution were injected into the column after filtration.

Alkaline degradation

An accurately weighed 20.0 mg of TM powder was transferred into a 250-mL conical flask, and 50 mL of 0.1 N NaOH was added and refluxed for 5 min at 100°C. After cooling, 1 mL of the solution was neutralized with HCl and then the volume was completed with mobile phase in a 10-mL volumetric flask. The solution was filtered through a disposable syringe filter (0.2 μm), and triplicate 20 μL were injected into the column.

Acidic degradation

Accurately weighed amounts of (20.0 mg) TM powder were transferred into five 250-mL conical flasks, and 50 mL of 0.1 N HCl was added in each flask and refluxed for time intervals of 5, 30, 60, 90 and 120 min at 100°C, respectively. After cooling, 1 mL from each solution was transferred separately into a 10-mL volumetric flask and neutralized with NaOH, and then the volume was completed with mobile phase. The solution was filtered through a disposable syringe filter (0.2 μm), and triplicate 20 μL were injected into the column.

Thermal degradation

Accurately weighed amounts of (20.0 mg) TM powder were transferred into five 250-mL conical flasks, and 50 mL of double distilled water was added and refluxed for time intervals 5, 30, 60, 90 and 120 min at 100°C, respectively. After cooling, 1 mL from each solution was transferred separately into a 10-mL volumetric flask and then the volume was completed with mobile phase. The solution was filtered through a disposable syringe filter (0.2 μm), and triplicate 20 μL were injected into the column.

Degradation of eye drops under conditions simulating the Egyptian summer ambient conditions

The dosage form was exposed to 40°C and 65% relative humidity in an incubator for 2 months. Timolol® 0.5% dosage form was kept in a jar containing saturated solution of sodium nitrite, which gives 65% relative humidity (29), then the jar was kept in an incubator that was adjusted to 40°C, taking samples at time intervals of 10, 30 and 60 days. At each time interval, 1 mL of the dosage form was taken into a 50-mL volumetric flask and completed to volume with mobile phase and then 2.94 mL was transferred from it into a 10-mL volumetric flask and the volume was completed with mobile phase. The solution was filtered through a disposable syringe filter (0.2 μm), and triplicate 20 μL were injected into the column.

Procedure

Linearity

The linearity of the method was evaluated at six concentration levels. Aliquots equivalent to 50–2,500 μg of TM from its respective standard working solutions (0.1 mg/mL for TM) were transferred separately into a series of 25-mL volumetric flasks and completed to volume with mobile phase. The solutions were filtered through a disposable syringe filter (0.2 μm) before column injection. Then, 20 μL aliquots of each solution were injected and eluted with the mobile phase under the previously described chromatographic conditions. The peak areas were recorded and then the relative peak areas were calculated for each concentration relative to the external standard (40 μg/mL) of TM. The average of the relative peak areas of TM was plotted versus the corresponding concentrations in μg/mL to obtain the calibration graph. Alternatively, the corresponding regression equation was derived.

Application to pharmaceutical dosage form and degradable solutions of TM

The selectivity of the proposed MLC method was evaluated by injecting the previously prepared solutions that exposed to different forced degradation conditions. Furthermore, the prepared working solutions of Timolol® 0.5% eye drops were analyzed as mentioned under "chromatographic conditions". The concentrations of TM were calculated from their corresponding regression equation, and system suitability parameters were calculated.

Sample solution and mobile phase stability

Evaluation of the stability of TM solutions was achieved by quantification of TM on seven successive days and comparison with freshly prepared solutions. Similarly, the stability of the mobile phase was checked.

Results

The current International Conference on Harmonisation (ICH) guidelines require that stability analysis should be done by using stability-indicating assay methods, developed and validated after stress testing on the drug under a variety of conditions, including hydrolysis (at various pH values), oxidation and thermal degradation (30).

Optimization of chromatographic conditions

To investigate the chromatographic performance, different columns had been tried that include Hypersil Gold amino (100 × 1.0 mm, 5 μm particle size), Biobasic cyano (100 × 2.1 mm, 5 μm particle size), Biobasic C_{18} (100 × 1.0 mm, 5 μm particle size), Biobasic C_8 (100 × 1.0 mm, 5 μm particle size), Phenyl Hypersil (250 × 4.6 mm, 5 μm particle size) and Biobasic phenyl (150 × 1.0 mm, 5 μm particle size) columns. Experimental trials revealed that all tried columns showed bad separation and bad resolution of peaks except Phenyl Hypersil that showed good separation but within long analysis time (~15 min) and peak broadening. The BioBasic phenyl column (150 × 1.0 mm i.d., 5 μm particle size, Thermo Corporation) was the most suitable one giving narrower symmetric peaks and highest number of theoretical plates within a reasonable analytical time (~8 min) (Figure 1). The mobile phase composition was optimized to provide sufficient selectivity and sensitivity in a short separation

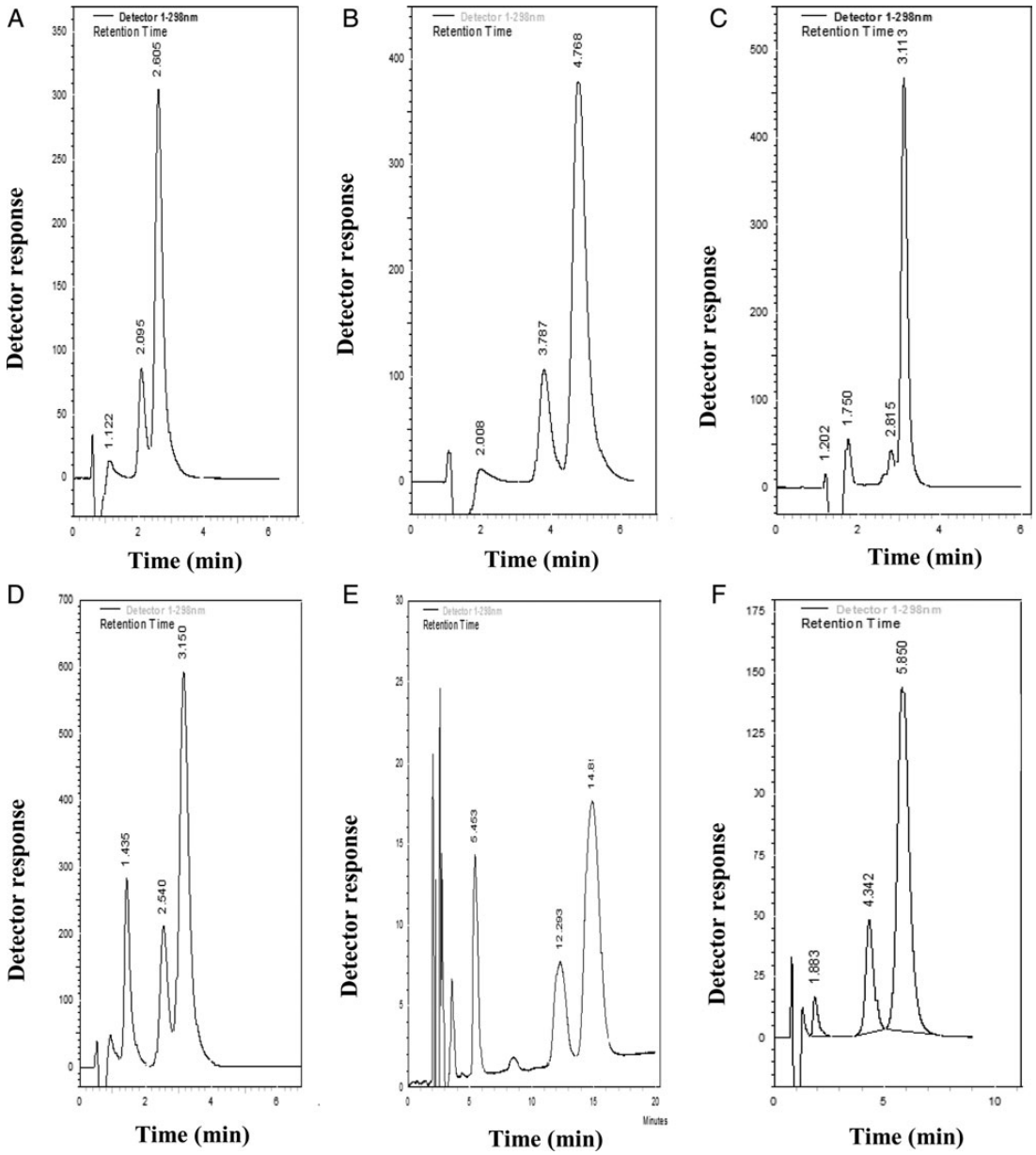

Figure 1. ML Chromatograms of TM and its alkaline degradation products separated on (A) BioBasic C_8 column (100 × 1.0 mm, 5 μm particle size), (B) BioBasic C_{18} (100 × 1.0 mm, 5 μm particle size), (C) BioBasic CN column (100 × 2.1 mm, 5 μm particle size), (D) Hypersil Gold amino (100 × 1.0 mm, 5 μm particle size), (E) Phenyl Hypersil (250 × 4.6 mm, 5 μm particle size) and (F) BioBasic phenyl column (150 × 1.0 mm, 5 μm particle size).

time. To select the optimum pH value for the analysis of TM in the presence of its degradation products, the pH of the mobile phase was studied over the range of 2.5–7. The mobile phase with pH values 2.8 ± 0.2 provided suitable peak symmetry and better peak shape. Increasing the pH of the mobile phase offered bad separation of the degradates and broadening of the TM peaks. SDS concentration was varied over the range of 0.08–0.15 M. Using mobile phase containing 0.15 M SDS, high efficiency and decreasing the retention time was obtained but overlapping between peaks of TM and one of its degradate was occurred, while lower concentration of SDS below 0.1 M, peak broadening and longer time of analysis was observed. The best compromise in terms of run time, efficiency and peak symmetry was achieved upon using a mobile phase containing 0.1 M SDS. To study the influence of the concentration of 1-propanol on the peak of TM, it was varied over the range of 5–15%. As expected, the retention of TM decreases as percentage of organic modifier increases. In addition, peak broadening was observed at low concentrations of 1-propanol. A concentration of 10% of 1-propanol was chosen as the optimal concentration, where it offers a good separation between TM and its degradates, good peak symmetry and reasonable analysis time. TEA concentration was varied over

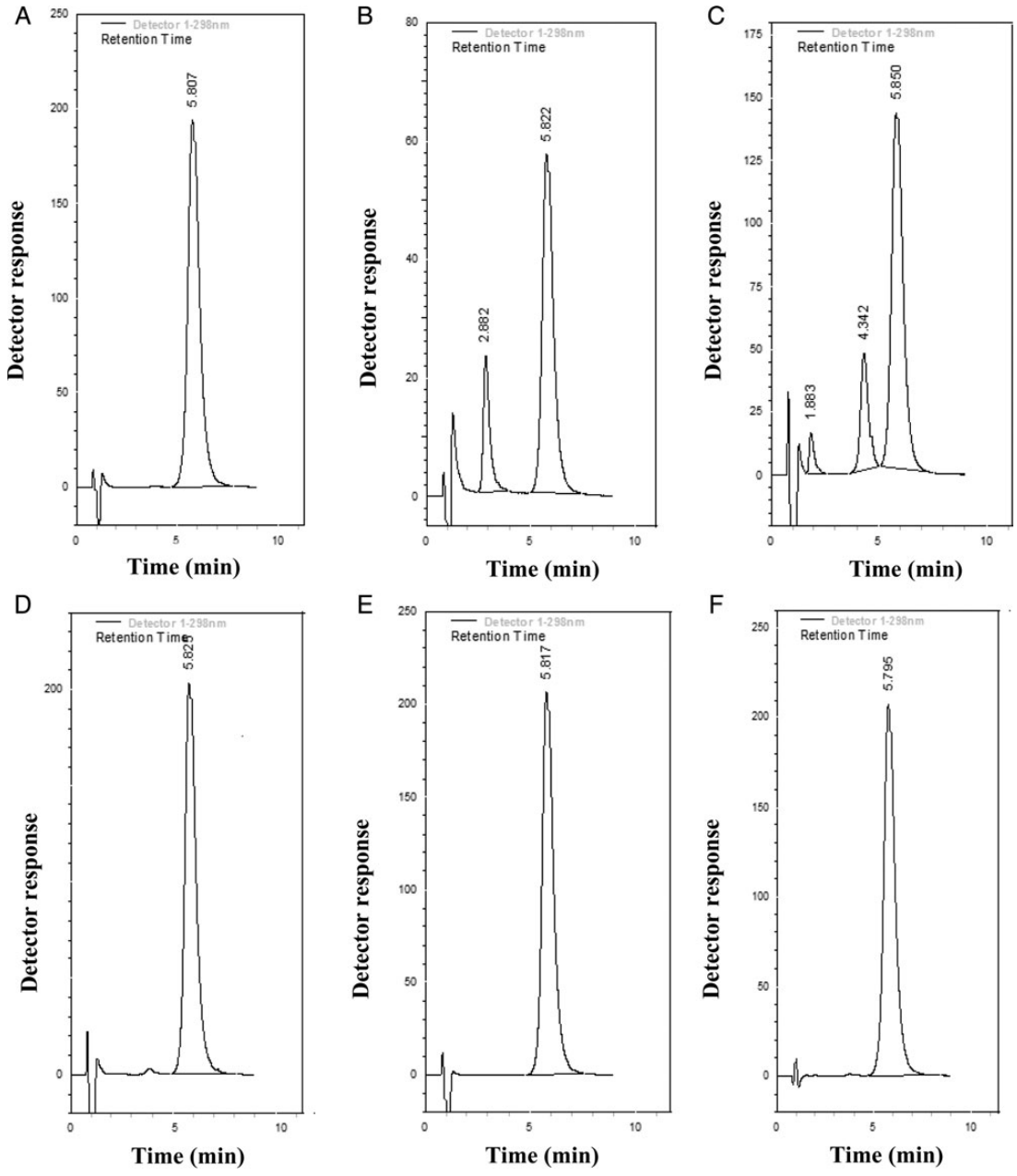

Figure 2. ML Chromatograms under optimum chromatographic conditions represent 40 μg/mL of TM under various stress conditions: (A) TM standard, (B) TM and its oxidative degradation product, (C) TM and its alkaline degradation products, (D) TM after acidic degradation, (E) TM after thermal degradation and (F) 40 μg/mL of Timolol® 0.5% eye drops after 2 months under 40°C and 65% humidity.

the range of 0.05–0.3%. It was found that the peak tailing, peak asymmetry and number of theoretical plates were better with 0.1% TEA. The effect of flow rate of the mobile phase on the retention of TM was investigated over the range of 0.05–0.1 mL/min. A flow rate of 0.1 mL/min was chosen because it provides better peak shape within a reasonable time. For the flow rate higher than 0.1 mL/min, the column pressure became a problem.

After optimization of these variables, the best peak shape and the lowest peak tailing were achieved with well-defined peaks and good sensitivity within a reasonable analytical run time. It was observed that satisfactory resolution of TM (retention time 5.8 min) and its degradation products (retention time of 1.8 and 4.3 in addition to 2.8 min for alkaline and oxidative degradates) formed under various stress conditions was achieved when analysis of stressed samples was performed on an HPLC system using a BioBasic phenyl column (150 × 1.0 mm i.d., 5 μm particle size) and a mobile phase, composed of 0.1 M SDS, 10% of 1-propanol and 0.1% of TEA in 0.035 M *ortho*-phosphoric acid. The detection was carried out at 298 nm. The mobile phase flow rate was 0.1 mL/min. Typical retention time of TM was ~5.8 min (Figure 2), and peak asymmetry was 1.25.

Method validation

The MLC method was validated according to the ICH Q2 (R1) recommendations (31). The method was validated for parameters such as system suitability, linearity, limit of detection (LOD), limit of quantitation (LOQ), accuracy, precision and selectivity. Stability of sample solution and mobile phase were also determined.

As the system suitability test is an integral part of chromatographic method development and it is used to verify that the system is adequate for the analysis to be performed, the parameters for TM were evaluated. The suitability of the chromatographic system was determined according to USP guidelines (2) and with acceptance of the obtained parameter values (32) (Table I).

Under the optimum chromatographic conditions, the concentrations of TM were proportional to the relative peak areas, in the concentration range of 2–100 μg/mL, by adopting the external standard method for calibration.

The regression equation was computed

$$R_{PA} = 0.025C + 0.0044 \quad r = 0.9999,$$

where R_{PA} is the relative peak area, C is the concentration of TM in μg/mL and r is the correlation coefficient.

Table I
Parameters Required for System Suitability of MLC Method of TM

Parameter	Obtained value for TM	Reference value (32)
Number of theoretical plates, *N*	626	Increases with efficiency of the separation
Capacity factor, *K′*	2.75	1–10 acceptable
α (relative retention time)	$\alpha_{1,2} = 1.35$ $\alpha_{1,3} = 2.02$	$\alpha > 1$
Resolution factor, R_s	$R_{1,0} = 6.45$ $R_{1,2} = 1.91$ $R_{1,3} = 4.01$	$R > 1.5$
Tailing factor, *T*	1.25	$T = 1$ for a typical symmetric peak

0, 1, 2 and 3 are the mobile phase peak, TM peak, TM alkaline degradate at 4.34 min (its R_s from the first alkaline degradate = 4.39) and oxidative degradate, respectively.

The accuracy of bulk powder was found to be 100.4 ± 1.196 for TM, using the proposed MLC method. Statistical comparison of the results obtained by the proposed MLC method with those obtained by the comparison method revealed no significant differences between the performances of the two methods (33) (Table II).

Precision of the assay was determined in relation to repeatability (intraassay) and intermediate precision (interassay). The relative standard deviation (RSD) values were <2%, demonstrating that the method was precise. Good recoveries were obtained for each concentration, confirming that the method was accurate (Table III).

The selectivity was examined for nondegraded and degraded samples [the solutions of TM after stress conditions of alkaline hydrolysis (0.1 M NaOH refluxed at 100°C), acid hydrolysis (0.1 M HCl refluxed at 100°C), oxidation (0.3% H_2O_2) and thermal degradation (100°C)]. The MLC method for the determination of TM was found to be selective in the presence of degradation products as shown in Figure 2. Peaks were symmetrical, clearly separated from each other (Figure 2). Furthermore, the selectivity of the proposed MLC method was established by its ability to determine TM in eye drops without interference from common eye drop additives, indicating selectivity of the method.

The LOD and LOQ for TM were calculated practically from the signal-to-noise ratio and it was found that LOD = 0.56 μg/mL and LOQ = 1.86 μg/mL.

Table II
Statistical Analysis of the Results Obtained by the Proposed MLC Method and the Comparison Method (7) for TM Pure Bulk Powder

Value	MLC proposed method	Comparison method[b] (7)
Mean	100.4	100.0
± SD	1.196	0.697
% RSD	1.191	0.697
n	6	7
Variance	1.430	0.486
Student's *t*- test	0.752 (2.201)[a]	
F-test	2.94 (4.39) [a]	

[a]The values between parenthesis are the corresponding theoretical values of *t* and *F* at $P = 0.05$ (33).
[b]The comparison method involving spectrophotometric determination by the ratio difference method by measuring the peak amplitudes at $\Delta P = 260–290$ nm for BT and at $\Delta P = 295–330$ nm for TM.

Table III
Assay Parameters and Method Validation for TM by the Proposed MLC Method

Parameter	TM
Validation of regression equation	
Slope[a]	0.025
Intercept[a]	0.0044
Correlation coefficient (*r*)	0.9999
Validation of response	
Concentration range (μg/mL)	2–100
LOD (μg/mL)	0.56
LOQ (μg/mL)	1.86
Average accuracy (%)	100.4
± SD (precision)	1.196
Percentage RSD (SD × 100/*X*)	1.191
Percentage Error (% RSD/$\sqrt{n}$)	0.486
Repeatability[b] ± %RSD	100.8 ± 0.892
Intermediate precision[b] ± %RSD	99.5 ± 1.406

[a]Average of three determinations.
[b]3 × 3 (concentrations 20, 40 and 60 μg/mL of TM were measured three times).

No significant changes were observed in standard solution or mobile phase responses, relative to freshly prepared ones. The results obtained in both cases proved that the sample solution and mobile phase used during the assay were stable up to 7 days.

Table IV
Determination of TM in Timolol® 0.5% Eye Drops by the Proposed MLC and Application of Standard Addition Technique

Product	Proposed MLC method	Standard addition			
		Claimed (μg/mL)	Added (μg/mL)	Found (μg/mL)	Recovery[a] (%)
	TM in Timolol®		10.00	9.82	98.2
	0.5% eye drops[b]	20.00	20.00	19.89	99.5
	B. no. 1202133		40.00	39.59	99.0
Mean	100.9				98.9
± SD	0.513				0.656
Percentage RSD	0.508				0.663

[a]Each result is the average of three separate determinations.
[b]Timolol® 0.5% labeled to contain 5.0 mg timolol (6.8 mg TM) per milliliter.

The validation sheet of the proposed MLC method, according to the ICH Q2 (R1) recommendations (31) of linearity and range, accuracy, precision, LOD and LOQ, is presented in Table III.

Application to pharmaceutical formulation

Satisfactory results were obtained for the determination of TM in Timolol® 0.5% eye drops by the proposed MLC method, which proved to be valid and applicable for the analysis without any interference of the additives or preservatives. The accuracy of the proposed procedure was assessed by applying the standard addition technique, and the results are shown in Table IV.

Investigation of stability of TM by the proposed chromatographic method

During stability studies, 20–80% degradation of the substance to be examined is to be achieved to qualify the assay method as able

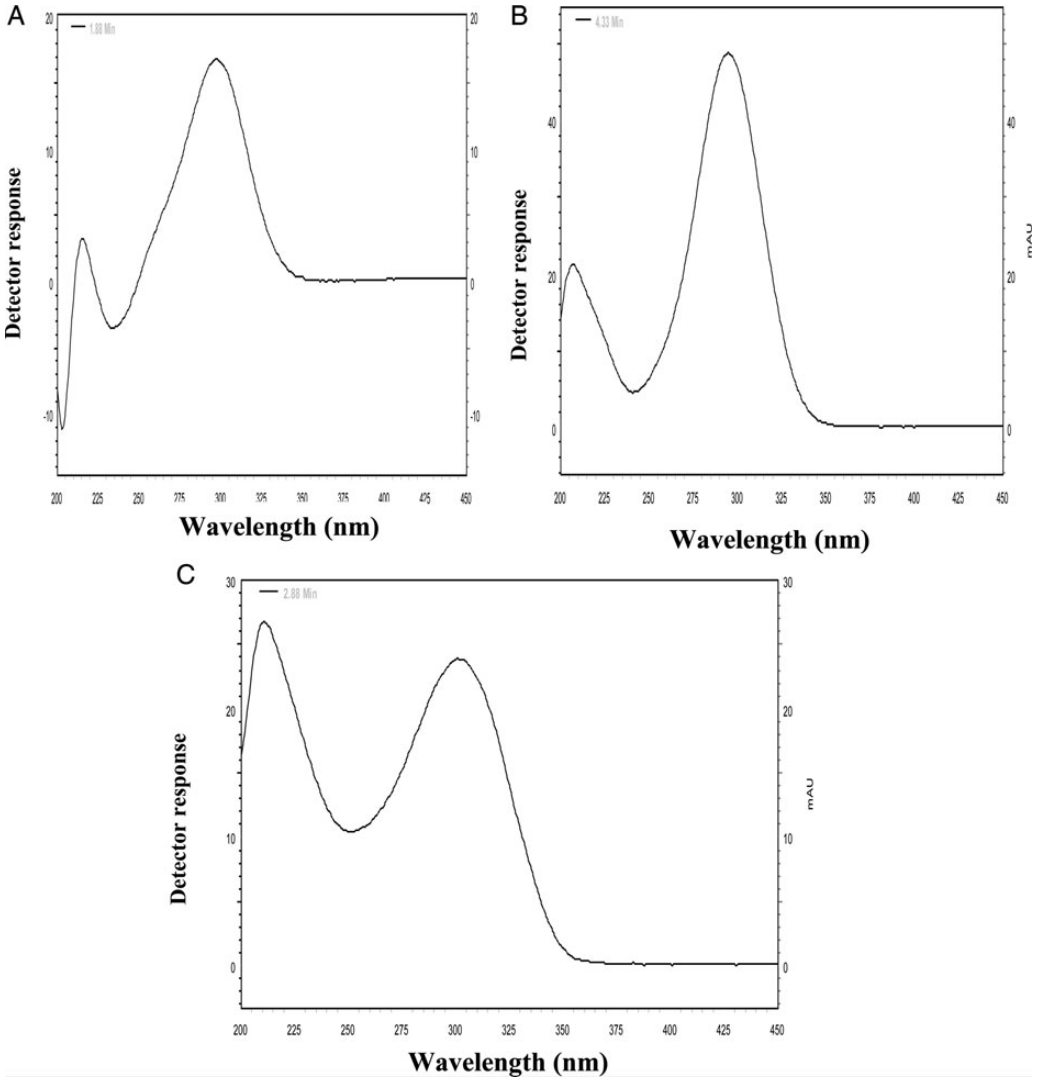

Figure 3. Spectra of separated TM degradates measured by PDA: (A) TM alkaline degradate at 1.88 min. Maximum wavelength at 298 nm. (B) TM alkaline degradate at 4.34 min. Maximum wavelength at 295 nm. (C) TM oxidative degradate at 2.88 min. Maximum wavelength at 302 nm.

Table V
Recovery and Degradation % of TM after Stress Testing by the Proposed MLC Method

Stress condition	Time	Degradation[a] (%)	Recovery[a] (%)
Oxidative[b] (2 mL, 0.3% H_2O_2 at 100°C)	Evaporation until dryness	72.4	27.6
Alkaline[b] (50 mL, 0.1 N NaOH refluxed at 100°C)	5 min	30.1	69.9
Acidic[b] (50 mL, 0.1 N HCl refluxed at 100°C)	5–120 min	No degradation	99.7
Thermal[b] (50 mL H_2O refluxed at 100°C)	5–120 min	No degradation	100.0
Simulating Egyptian summer ambient[c] (40°C and 65% humidity)	10–60 days	No degradation	99.8

[a]Each result is the average of three separate determinations, and the percentage degradation was calculated using the peak area of TM.
[b]Stress conditions performed on bulk powder.
[c]Stress condition performed on dosage form.

to indicate stability (34). TM was observed to be significantly liable to oxidative degradation more than alkaline one. It was found to be resistant to acidic hydrolysis and thermal hydrolysis even in high temperatures (100°C). Chromatograms of solutions obtained after degradation under various conditions are shown in Figure 2. The main degradation products had retention times of ~1.8, 2.8 and 4.3 min. Figure 3 shows the spectra of the separated degradates scanned by PDA. The results of degraded dosage form, under conditions simulating the Egyptian summer ambient conditions, show stability of TM for 2 months. The results of forced degradation experiments on TM under various stress conditions are summarized in Table V.

Discussion

To the best of our knowledge, there are only two methods reporting the determination of TM in the presence of its degradation products. Kulkarni and Amin (13) reported a stability-indicating HPTLC determination of TM as bulk drug and in pharmaceutical preparations, but under more drastic conditions (0.2 M HCl and 0.2 M NaOH and the solutions were refluxed for 3 h, the solid state of drug was also degraded by dry heat at 190°C for 10 min) and they did not perform the forced oxidative degradation procedure. They obtained three degradation products on alkaline degradation, two on acidic degradation and one on thermal degradation. Although an ion-exchange HPLC method was reported by Mazzo and Snyder (21) for the determination of TM in the presence of three expected degradation products, this method was time consuming and suffered from high mobile phase consumption in comparison with our study, as their run time was >17 min at a flow rate of 1 mL/min. Moreover, they did not perform the procedure under forced degradation stress conditions. Further, our method has another advantage over other methods as we used a low concentration of organic solvents, which decrease the environmental hazards.

Conclusion

The proposed method is proved to be a selective, precise and accurate stability-indicating MLC–UV method for the determination of TM in the presence of alkaline and oxidative degradates either in bulk powder or in eye drops. The complete separation of the analytes was accomplished in <8 min and so it is useful for routine analysis studies of TM in bulk powder and dosage form in quality control laboratories, which is our aim.

From the chromatographic point of view, the proposed method is considered to be an economical method, due to the very low flow rate (0.1 mL/min), and a cheap HPLC method, due to low mobile phase consumption (>10 times), in comparison with other HPLC methods.

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