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Simultaneous determination of dorzolomide and timolol in aqueous humor: A novel salting out liquid–liquid microextraction combined with HPLC

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ABSTRACT

A Snovel method for the simultaneous separation and determination of two antiglaucoma drugs namely, dorzolamide hydrochloride (DOR) and timolol maleate (TIM) in aqueous humor samples (AH) was developed by using salting-out assisted liquid–liquid microextraction (SALLME) combined with HPLC–UV method. Box–Behnken experimental design and response surface methodology were employed to assist the optimization of SALLME conditions, including salt concentration, the pH of sample solution and vortex time as variable factors. The optimal extraction conditions were as follows: to 50 μL of AH sample, 100 μL of phosphate buffer (100 mmol L^{-1}, pH 11.9), 90 μL of acetonitrile (ACN) and 0.11 g of $(NH_4)_2SO_4$ salt were added into an Eppendorf vial (1 mL) then vortexed for 1.1 min. As an effort to miniaturize SALLE system, a 1 mL syringe adapted with a capillary tube was employed as the phase separation device. Once the phase separation occurred, the upper layer could be narrowed into the capillary tube by pushing the plunger; thus, the collection of the upper layer solvent was simple and convenient. By miniaturization, the consumption of the organic solvent was decreased as low as possible. The chromatographic separation was achieved on Gemini C_{18} column using a mobile phase of ACN: 30 mmol L^{-1} potassium dihydrogen phosphate buffer containing 0.1% triethylamine, pH 3.5 (20:80, v/v) at a flow rate of 1 mL min^{-1} and UV detection at 254 and 295 nm for DOR and TIM, respectively. Mepivacaine hydrochloride was used as an internal standard. The described method showed better separation with enhanced sensitivities than the previously reported methods with limits of quantitation of 8.75 and 10.32 ng mL^{-1} in aqueous solution and 15.97 and 23.53 ng mL^{-1} in AH for DOR and TIM, respectively. The simple, rapid and eco-friendly SALLME–HPLC method has been successfully applied for the simultaneous pharmacokinetic studies of DOR and TIM in rabbit AH.

1. Introduction

Timolol maleate (TIM), Fig. 1, is a nonselective β-adrenergic receptor antagonist which acts by lowering the intraocular pressure (IOP) primarily by reducing the production of aqueous humor (AH) by the ciliary epithelium [1]. It is considered the first-line drug for the treatment of glaucoma [1–3].

Dorzolamide hydrochloride (DOR), Fig. 1, is the first topical carbonic anhydrase inhibitor (CAI) used for treatment of glaucoma by lowering IOP through inhibition of CA isoenzyme involved in AH production [3].

In many cases, there is a need for more than one type of medication. Hence, to improve compliance, fixed combinations of different drugs have been introduced. A combination therapy including TIM and DOR has a scientific bearing as the two drugs have complementary mechanisms of action [4]. Therefore, a fixed combination of DOR and TIM (FCDT) has been approved by the FDA and is widely used for treatment of glaucoma. Generally, it is instilled by patients themselves for months or years. Therefore, a better understanding of the pharmacokinetics of this combination in AH will help decreasing the incidence of any adverse effects.

Abbreviations: ACN, acetonitrile; AH, aqueous humor; BBD, Box–Behnken design; DOR, dorzolamide hydrochloride; CE, capillary electrophoresis; FCDT, fixed combination of dorzolamide and timolol; HPLC, high-performance liquid chromatography; IS, internal standard; LLE, liquid–liquid extraction; PPT, protein precipitation; RSM, response surface methodology; SALLME, salting-out assisted liquid–liquid microextraction; TEA, triethylamine; TIM, timolol maleate; TLC, thin layer chromatography

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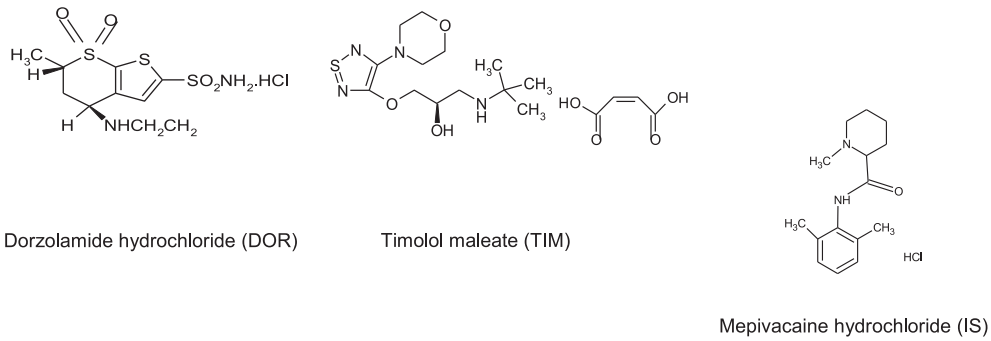

Fig. 1. Chemical structures of the studied antiglaucoma drugs and the internal standard.

In addition, recently, several preparations of this combination with novel drug delivery systems (DDS) have been developed [5,6]. Thus, analysis of their pharmacokinetics in AH becomes more important since it can help in evaluating the properties of the new DDS preparations and determining dosage schedules.

The literature survey revealed that there are few methods reported for the simultaneous analysis of DOR and TIM including spectrophotometric [7–9], thin layer chromatographic (TLC) [7], capillary electrophoretic (CE) [10] and high-performance liquid chromatographic (HPLC) [11–16] methods. All these methods lack the enough sensitivity which enables the determination of small concentrations of DOR and TIM in complex matrices such as AH in which they are typically found. Meanwhile, these methods are applied only for the analysis of the studied drugs in their pharmaceutical formulations. Moreover, although both drugs were determined in the USP 35 [17] and BP 2013 [18], there is no pharmacopeial method for their simultaneous determination so far. Therefore, the present work aimed to develop a sensitive HPLC method for their simultaneous analysis in AH samples.

Furthermore, pre-treatment and enrichment processes are crucial steps in analysis of AH samples because of the very low concentrations of the antiglaucoma drugs typically found, besides the complexity of this matrix. Methods reported for either DOR or TIM extraction from AH samples are either protein precipitation (PPT) [19–23] or liquid–liquid extraction (LLE) [24–28]. Although PPT is simple, the obtained extract still contains a significant amount of impurities which could result in relatively high background in the chromatogram and column deterioration. At the same time, although LLE provides much cleaner extracts than PPT and is the most popular choice, it is time-consuming, tedious and uses large amounts of potentially toxic organic solvents. These organic solvents pose a threat to the environment and human health and their disposal is also extremely expensive. Recently, a novel extraction technique termed as salting-out assisted liquid–liquid microextraction (SALLME) has been developed and applied for the determination of various target analytes from water, food and biological matrices [29–32]. SALLME integrates sample cleanup and preconcentation in a single step. This method is based on the extraction of analytes from the aqueous phase with water miscible organic solvent at high salt concentrations (salting-out phenomena).

The present work reports, for the first time, the development and applicability of a new vortex-assisted SALLME method for the rapid and efficient extraction of DOR and TIM from AH samples. Moreover, a 1-mL syringe adapted with a capillary tube was employed as the phase separation device to reduce the consumption of organic solvent as much as possible, while ensuring a convenient and simple operation. In addition, Box–Behnken design (BBD) approach and response surface methodology (RSM) were employed to assist finding optimal extraction conditions, quickly and reliably. To the best of our knowledge, this is the first demonstration of SALLME optimization by virtue of experimental design for antiglaucoma drug analysis. The SALLME coupled with HPLC with the aid of experimental design was developed, validated and successfully applied for the simultaneous pharmacokinetic studies of DOR and TIM in rabbit AH samples.

2. Experimental

2.1. Chemicals and reagents

DOR and TIM were obtained as a gift from Jamjoom Pharmaceuticals (Jeddah, Kingdom of Saudi Arabia). All solvents were of HPLC grade (Merck, Darmstadt, Germany) and all other materials were of analytical grade. Pharmaceutical dosage forms (Xolamol® eye drops) were kindly supplied by Jamjoom Pharmaceuticals, Egypt Scientific Office and were claimed to contain 2% and 0.5% of DOR and TIM, respectively. Double distilled water was used throughout the work.

2.2. Chromatographic system

The HPLC system consisted of a Knauer HPLC system (Knauer, Berlin, Germany), which consisted of K-500 solvent delivery pump, injector valve with a 20 μL loop and K-2600 UV detector. The HPLC system control and data processing were performed by computer integration software (EuroChrom 2000® Knauer). Digital microtransfer pipettes 5–250 μL were used (Acura, Socorex, Switzerland).

Analytes were separated using Gemini RP-C_{18} column (250 × 4.6 mm^2, 5 μm) (phenomenex, USA) protected with a precolumn (guard column with Gemini C_{18} precolumn inserts) (Phenomenex, USA). Isocratic mobile phase consisted of ACN: 30 mmol L^{-1} potassium dihydrogen phosphate buffer containing 0.1% triethylamine (TEA) at pH 3.5 (20: 80, v/v). The mobile phase was degassed in an ultrasonic cleaner (Cole-Parmer, Chicago, IL, USA) and was filtered through a 0.45 mm membrane filter (Gelman Instrument) using vacuum filtration unit (Phenomenex, USA) and delivered at a flow rate of 1 mL min^{-1}. The injection volume was 20 μL and the detector was set at 254 and 295 nm for DOR and TIM, respectively. The chromatography was performed at room temperature using mepivacaine hydrochloride as internal standard (IS).

2.3. Standard and quality control solutions

Standard stock solutions of DOR and TIM were prepared separately in water at concentrations of 50 μg mL^{-1}. Working solutions containing both drugs were prepared from the stock solutions by appropriate mixing and dilution with water. Working solution of mepivacaine HCl (IS) was prepared in ACN at a concentration of 2 μg mL^{-1}. AH standards for the calibration daily

Table 1
Box–Behnken design chart including factors, levels, matrix with three factors and the observed and predicted extraction recoveries.

Independent variables				Factors			Levels		
							−1	0	+1
A				$(NH_4)_2$ SO_4 conc. (g)			0.05	0.0875	0.125
B				pH			9	11	13
C				Time (min)			0.5	1	1.5
				DOR			TIM		
Run	A	B	C	Observed Y_{DOR} [a] %	Predicted Y_{DOR} %	% Er[b]	Observed Y_{TIM} [a]%	Predicted Y_{TIM} %	% Er[b]
1	−1	−1	0	52.5	51.6	1.87	32.8	31.7	3.49
2	−1	+1	0	62.6	62.9	0.67	65.6	65.7	0.15
3	+1	−1	0	66.2	65.8	0.64	47.6	47.5	0.21
4	+1	+1	0	95.5	96.5	1.00	95.9	96.9	1.14
5	−1	0	−1	63.5	64.6	1.74	59.2	60.3	1.76
6	−1	0	+1	69.1	68.6	0.85	64.8	64.7	0.08
7	+1	0	−1	84.2	84.8	0.68	80.2	80.3	0.07
8	+1	0	+1	97.2	96.1	1.17	92.9	91.8	1.15
9	0	−1	−1	60.2	60.0	0.26	41.7	41.7	0.10
10	0	−1	+1	65.5	67.1	2.31	46.2	47.4	2.45
11	0	+1	−1	82.1	80.5	1.92	82.3	81.1	1.43
12	0	+1	+1	88.5	88.7	0.17	91.5	91.5	0.04
13	0	0	0	99.3	98.2	1.09	95.3	94.4	1.01
14	0	0	0	98.2	98.2	3.0×10^{-3}	93.1	94.4	1.29
15	0	0	0	97.2	98.2	1.09	94.6	94.4	0.28

[a] Extraction recoveries, average of triplicate extractions.
[b] % Error.

curve were prepared by spiking 50 μL of blank rabbit AH with 10 μl of mixed standard solutions to obtain concentrations of 10, 25, 50, 75, 100, 250 and 500 ng mL^{-1} and stored at −20 °C until assay. The quality control (QC) samples were prepared in the same way to obtain the following concentration levels of both drugs: low-QC (25 ng mL^{-1}), middle-QC (100 ng mL^{-1}) and high-QC (250 ng mL^{-1}).

2.4. Experimental design for optimization of extraction parameters

The preliminary ranges of the extraction variables, $(NH_4)_2SO_4$ concentration (A), sample pH (B) and vortex time (C), were evaluated by traditional one-factor analysis. Afterwards, a three-level, three-factorial BBD was used to determine the best combination of extraction variables for the recovery of both DOR and TIM. Table 1 represents the coded values of the experimental variables and 15 experimental runs. A multiple regression analysis was done to obtain the coefficients and the response. The experimental plan, data analysis and RSM were performed using the Unscrambler® X 10.3 software package (CAMO AS, USA).

2.5. SALLME procedure

Figure 2 illustrates the SALLME steps. Briefly, 50 μL of AH sample was placed into an Eppendorf vial (1 mL) (Fig. 2A). Followed by addition of 10 μL of IS (2 μg mL^{-1}), 100 μL of phosphate buffer (100 mmol L^{-1}, pH 11.9), 90 μL of ACN and 0.11 g of $(NH_4)_2SO_4$ salt and vortexed for 1.1 min. Afterwards, all the solution was withdrawn into a 1 mL syringe (Fig. 2B), and left to stand statically upside down, (Fig. 2C). A capillary tube (1.7 mm × 13.3 cm, Teflon, sterile, BD Angiocath) was attached onto the syringe tip. In this step, two separate phases could be easily observed. Subsequently, the plunger was slowly pushed to move the upper layer phase to the narrow capillary tube, which helped to concentrate the organic solvent in a narrow place, enabling the convenient collection of it (Fig. 2D). The upper solvent was sucked using a 100 μL micro-syringe into an Eppendorf vial (Fig. 2E) and dried under a gentle stream of nitrogen. The residue was reconstituted with 50 μL of the mobile phase (Fig. 2F) and 20 μL was injected into the HPLC–UV system.

2.6. Pharmacokinetic study

The developed SALLME–HPLC method has been clinically applied for the pharmacokinetic studies of DOR and TIM in rabbit AH following ocular instillation of 1 drop of FCDT eye drop. The protocol of the study was approved by the Animal Care and Use Committee of Teaching Veterinary Hospital, Faculty of veterinary medicine, Assiut University and conformed to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research as well as the EU Directive 2010/63/EU for animal experiments.

Five (New Zealand albino) rabbits of both sexes (weighing 2–2.5 kg) were housed in an air conditioned room and fed a standard pellet diet and water ad libitum. Alternate night and day cycles, 12 h each, were provided with artificial fluorescent light. The animals were checked by a veterinarian to check their health and ensure the absence of clinical observable abnormalities. One drop of FCDT eye drop was instilled onto the cul-de-sac of both eyes of the rabbits without touching the eyes or irritating the corneal surface. To minimize the runoff of the instilled dose, the eyelids were closed gently for few seconds after dosing. The rabbits were systemically anesthetized with i.m. injections of ketamine hydrochloride (50 mg kg^{-1}) in combination with a relaxing agent xylazine (10 mg kg^{-1}) and locally anesthetized with benoxinate hydrochloride (0.4%, w/v, 2 drops). AH samples (50 μL) were withdrawn by anterior chamber paracentesis using an insulin syringe (1.0 mL) fitted with a 29 G needle at the following time points: predose 0.25, 0.5, 1, 2, 4 and 6 h. The samples were collected and stored at −20°C until analysis. The obtained concentration–time data were analyzed using WinNonlin™ Standard Version 1.5 software (Science consulting, Apex, NC, USA).

3. Results and discussion

In order to obtain well-defined symmetrical peaks with maximum extraction efficiency for the simultaneous analysis of the antiglaucoma drugs in AH samples, a strategic experimental approach was implemented. Where, parameters influencing chromatographic separation,

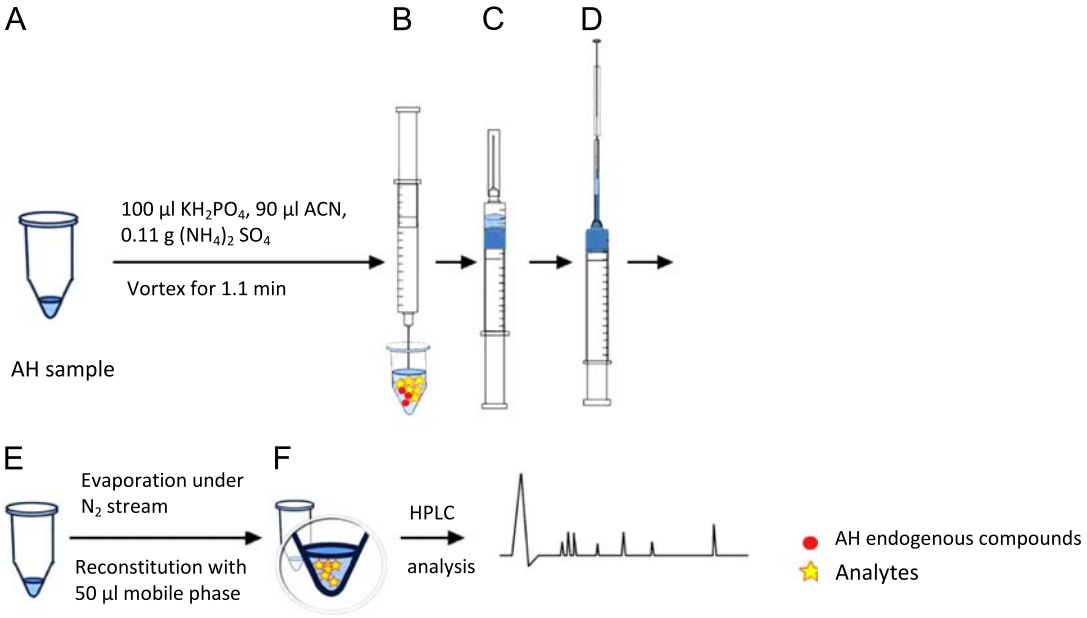

Fig. 2. Schematic illustration of the SALLME procedure.

extraction efficiency as well as the calibration features of the proposed method and its application were systematically investigated.

3.1. Chromatographic performances

For the good separation of DOR, TIM together with an IS in a single chromatographic run with the following experimental parameters: stationary phase, mobile phase, flow rate and IS selection were optimized as follows.

3.1.1. Detection

DOR and TIM were detected at their corresponding λ_{max}, 254 and 295 nm, using UV detector.

3.1.2. Stationary phase

Three different columns namely Luna C_8, Luna C_{18} and Intersil CN were tested. The experimental studies revealed that, CN column gave very broad overlapping peaks with excessive tailing. While, the other 2 columns could separate the analytes without any overlapping but the broadening and tailing still persist. Hence, a need arises for manipulating another C_{18} column with higher efficiency for the separation, which is Gemini C_{18}. Where, sharp peaks with good resolution could be achieved; therefore, Gemini C_{18} column was chosen. This is because during the final stage of silica manufacturing a unique silica–organic layer was grafted to create a completely new composite particle which protects the silica particles from chemical attack. As a result, it retains its mechanical strength and rigidity along with an excellent efficiency.

3.1.3. Mobile phase

3.1.3.1. Organic modifier. ACN and MeOH were tested as the organic modifiers in the mobile phase. It was found that MeOH gave slight broad peak for TIM with extensive tailing. On the other hand using ACN resulted in improvement of peak symmetry for both drugs. Different percents of ACN were tested to select the most suitable one for retention and peak shape. It was found that, peak shape improved dramatically by increasing ACN percent in the mobile phase from 15 to 20; after that upon increasing the ACN percent over 22, DOR peak started to overlap with the solvent front. Therefore, a mobile phase containing 20% of ACN was chosen. However, the eluted peaks had low sensitivity. So the effect of addition of buffer system instead of water had to be investigated.

3.1.4. Buffer type, pH and ionic strength

Firstly, considering buffer type, three different types namely acetate, citrate and phosphate at pH 3.5 were investigated. It was obviously seen that, the highest enhancement of the sensitivity was achieved with phosphate buffer. Secondly, different phosphate buffer solutions (30 mmol L^{-1}) covering the pH range of 2.5–5 were studied. Both analytes showed good peak shapes with high sensitivities at pH range of 3–4, but at higher pH values DOR gave broad peak of low sensitivity. Therefore, pH 3.5 was selected where, both drugs are completely ionized. Finally, it was found that increasing phosphate buffer concentration enhanced the sensitivity, retention time as well as the baseline of the eluted peaks till reaching a concentration of 25 mmol L^{-1}, after which the effect remained constant. Therefore, 30 mmol L^{-1} phosphate buffer was selected. However, there was a slight tailing in TIM peak. Therefore, a competing amine was tested to improve peak shape.

3.1.5. TEA concentration

Adding different percents of TEA, up to 0.08%, enhanced the peak symmetry and tailing of the eluted peaks, after which the effect remained constant. Therefore, 0.1% TEA was added to minimize peak tailing and enhance peak symmetry.

3.1.6. Flow rate

Different flow rates (0.5–1.5 mL min^{-1}) were studied. It was found that 1.0 mL min^{-1} was the optimum flow rate for good separation and resolution of the analytes and in a reasonable time.

3.1.7. IS

Five different drugs, namely atenolol, Levobunolol, metoprolol, mepivacaine hydrochloride and sotalol that are structurally close to the analytes, have been tested as IS. Mepivacaine hydrochloride (Fig. 1) is the most proper one to be used as IS. Hence, it gave a sharp symmetrical peak and well separated from the analytes peaks under the optimized chromatographic conditions.

Ultimately from these comprehensive investigations the mobile phase used was a mixture of ACN and 30 mmol L^{-1} potassium

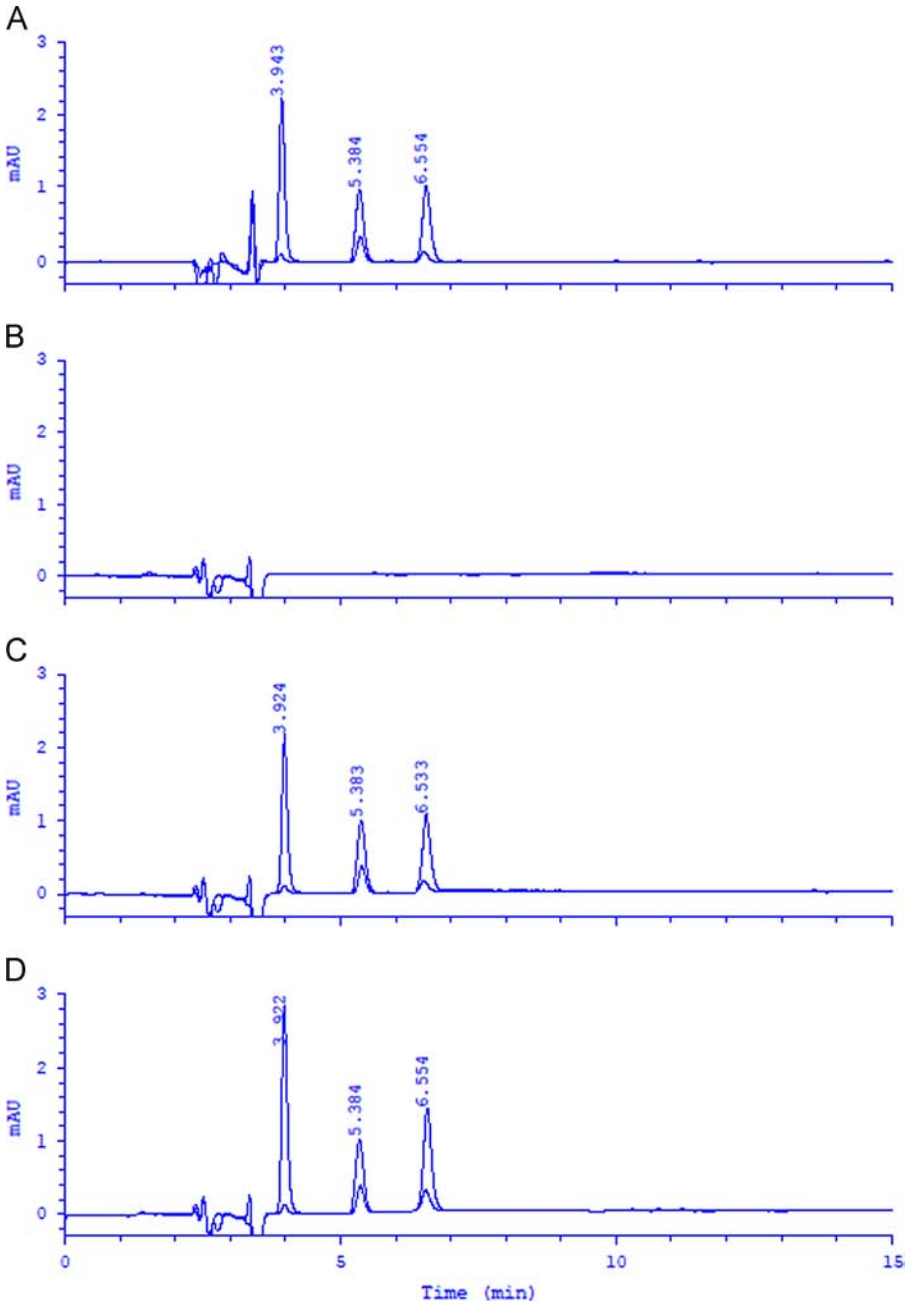

Fig. 3. Typical HPLC–UV chromatograms of (A) standard aqueous mixture of the studied antiglaucoma drugs and IS; (B) blank and (C) spiked AH samples; the aqueous and spiked AH concentration of the antiglaucoma standards was 250 ng mL^{-1}; each and 400 ng mL^{-1} of IS and (D) AH sample 0.5 h after ocular instillation of 1 drop of FCDT eye drop spiked with 400 ng mL^{-1} IS. SALLME conditions: 50 μL AH sample; 100 μL phosphate buffer (100 mmol L^{-1}, pH 11.9); 90 μL ACN; 0.11 g $(NH_4)_2SO_4$ salt and vortex for 1.1 min. HPLC–UV conditions: column, Gemini C_{18}; mobile phase, ACN and 30 mmol L^{-1} potassium dihydrogen phosphate buffer containing 0.1% TEA, pH 3.5 (20:80, v/v); flow rate, 1 mL min^{-1}; detection wavelengths, 254 and 295 nm for dorzolamide and timolol, respectively; IS, mepivacaine hydrochloride; (dorzolamide at 3.9 min, IS at 5.3 min, timolol at 6.5 min).

dihydrogen phosphate containing 0.1% TEA at pH 3.5, adjusted with orthophosphoric acid, in a ratio of 20:80 (v/v). Where, symmetric peaks with suitable short retention times were obtained for all the analytes. As shown in Fig. 3 DOR, IS and TIM were eluted at 3.9, 5 and 6.5 min, respectively.

3.2. SALLME procedure

3.2.1. Preliminary studies

Several parameters that may influence the SALLME efficiency, including the extraction solvent (type and volume), salt (type and amount), pH of aqueous sample and vortex time should be investigated. Therefore, preliminary experiments were done using a one-factor analysis of these variables. Different water-miscible organic solvents (ACN, Acetone, Isopropanol and Tetrahydrofurane) and different salts (NaCl, $ZnSO_4$, $MgSO_4$ and $(NH_4)_2SO_4$) were investigated. The best combination that exhibited the highest extraction efficiency was ACN and $(NH_4)_2SO_4$. Moreover, salt concentrations of 0.05–0.125 g $(NH_4)_2SO_4$, sample pH values of 9–13 and vortex times of 0.5–1.5 min were found favorable for this study.

3.2.2. Box–Behnken design and response surface for SALLME condition optimization

After a one-factor analysis of variables preliminary experiments, the concentration of $(NH_4)_2SO_4$ (A: 0.05–0.125 g), sample

pH (B: 9–13) and vortex time (C: 0.5–1.5 min) were found to potentially affect the extraction efficiency considered in BBD [33]. The responses of the experiment were the extraction efficiencies of DOR and TIM. It should be noted that, the number of experiments needed to investigate these three parameters at three levels would be 27 $(3)^3$. Whereas, this number was reduced using the BBD to only 15 experimental runs. The low, medium and high levels of each variable were coded as -1, 0 and $+1$, respectively. The results of this limited number of experiments provided a statistical model that was used to identify trends in high yield for the extraction process. The BBD matrix involving the extraction conditions for the 15 runs together with the actual and predicted recovery values of the two analytes is listed in Table 1.

As shown in Table 1, the experimental results were close to the theoretical values with low values of percent error (% Er). Furthermore, statistical testing of the model was carried out in the form of analysis of variance (ANOVA) (Table 2). From the statistic data of R^2, F and p-values in Table 2, it was concluded that the final models were considered to be satisfactory. Moreover, a high degree of precision and a good deal of reliability of the conducted experiments were explained by low values of the coefficient of variation (CV). In addition, the non-significant values of lack of fit ($p > 0.05$) revealed that the quadratic models were statistically significant for the responses. The 3 replicated center points in the BBD made it possible to assess the pure error of the experiments and enabled the model's lack of fit to be checked [34]. Based on the sum of squares obtained from the ANOVA, the percentage contribution (PC) for each term was calculated (Table 3). Obviously, the pH of the sample, its quadratic term $(pH)^2$ and salt concentration showed the highest levels of significance for DOR extraction efficiency with contributions of 28.17%, 25.57% and 21.95%, respectively. While for TIM, only the pH of the sample and its quadratic term $(pH)^2$ showed the highest levels of significance with contributions of 47.91% and 23.16%, respectively.

The RSM was used to determine the optimum response for DOR and TIM extractions using the proposed SALLME method. Three-dimensional (3D) surface plots and contour plots for DOR and TIM extraction were constructed. Figure 4 shows the estimated response surfaces generated by the quadratic model showing visually the effects and interaction of two independent variables on the response as the third independent variable was fixed at the central experimental level of zero [34–36]. Meanwhile, using the computer optimization process and the response surface plots the optimal conditions were depicted regarding the factor interactions and balancing the high extraction recoveries for both drugs as follows: $(NH_4)_2SO_4$ concentration of 0.11 g, pH of 11.9 and time of 1.17 min.

These observations are consistent with the fact that, although the concentration of $(NH_4)_2SO_4$ used for protein precipitation is low, the supernatant starts to separate into two layers only at higher concentrations, which is preferred for salting-out [37]. Therefore, 0.11 g of $(NH_4)_2SO_4$ was consistent with the theoretical rationale. Moreover, this SALLME method acts as a triple stacked sample extraction technique of double PPT and salting-out. Where, addition of ACN into a biological sample precipitates more than 90% of endogenous proteins [38]. Afterwards, addition of $(NH_4)_2SO_4$ will precipitate more proteins in the supernatant. This justifies why stacked PPT of ACN and $(NH_4)_2SO_4$ depletes

Table 2
ANOVA analysis for SALLME of dorzolamide and timolol.

Source	SS[a]		DF[b]		MS[c]		*F*-ratio		*P*-value	
	DOR	TIM	DOR	TIM	DOR	TIM	DOR	TIM	DOR	TIM
Regression	3837.86	7029.12	9	9	426.42	781.01	168.63	392.73	1.14×10^{-5}	1.39×10^{-6}
Linear	2141.31	4725.33	3	3	713.77	1575.11	282.27	792.04	5.37×10^{-6}	4.11×10^{-7}
Interaction	106.88	77.62	3	3	35.62	25.87	14.08	13.01	7.12×10^{-3}	8.48×10^{-3}
Quadratic	1794.17	2478.53	3	3	598.05	826.17	236.51	415.44	8.32×10^{-6}	2.05×10^{-6}
Lack of fit	10.33	7.45	3	3	3.44	2.48	2.98	1.99	0.26	0.35
Pure error	2.31	2.48	2	2	1.15	1.24				
R^2	0.99	0.99								
Adj-R^2	0.99	0.99								
% CV[d]	2.01	1.95								

[a] SUM of squares.
[b] Degree of freedom.
[c] Mean square.
[d] Coefficient of variation.

Table 3
Multiple regression results and significance of the components for the quadratic models.

Factor (coded)	Parameter	Coefficient		*t*-Value		*p*-Value		SS[a]		PC (%)[b]	
		DOR	TIM	DOR	TIM	DOR	TIM	DOR	TIM	DOR	TIM
Intercept	β_0	98.23	94.37								
A	β_1	11.93	10.49	12.99	14.46	4.31×10^{-6}	2.53×10^{-6}	1138.83	1109.44	28.17	15.24
B	β_2	10.53	19.59	11.47	25.64	7.98×10^{-6}	1.46×10^{-7}	887.25	3488.21	21.95	47.91
C	β_3	3.79	2.71	4.13	4.90	1.08×10^{-3}	4.89×10^{-4}	115.21	127.68	2.85	1.75
AB	β_{12}	4.82	2.04	5.25	4.73	1.07×10^{-3}	2.78×10^{-3}	93.21	59.52	2.31	0.82
AC	β_{13}	1.82	−0.03	1.99	2.18	0.07	0.053	13.39	12.67	0.33	0.17
BC	β_{23}	0.26	−0.64	0.28	1.43	0.75	0.16	0.27	5.42	6.69×10^{-3}	0.07
AA	β_{11}	−12.28	−14.41	−13.37	−15.39	2.51×10^{-5}	1.26×10^{-5}	557.05	579.88	13.78	7.96
BB	β_{22}	−16.73	−23.25	−18.22	−26.24	5.47×10^{-6}	8.95×10^{-7}	1033.81	1686.52	25.57	23.16
CC	β_{33}	−7.42	−9.46	−8.08	−9.31	2.88×10^{-4}	1.46×10^{-4}	203.30	212.12	5.03	2.91

[a] Sum of squares.
[b] Percentage contribution (%).

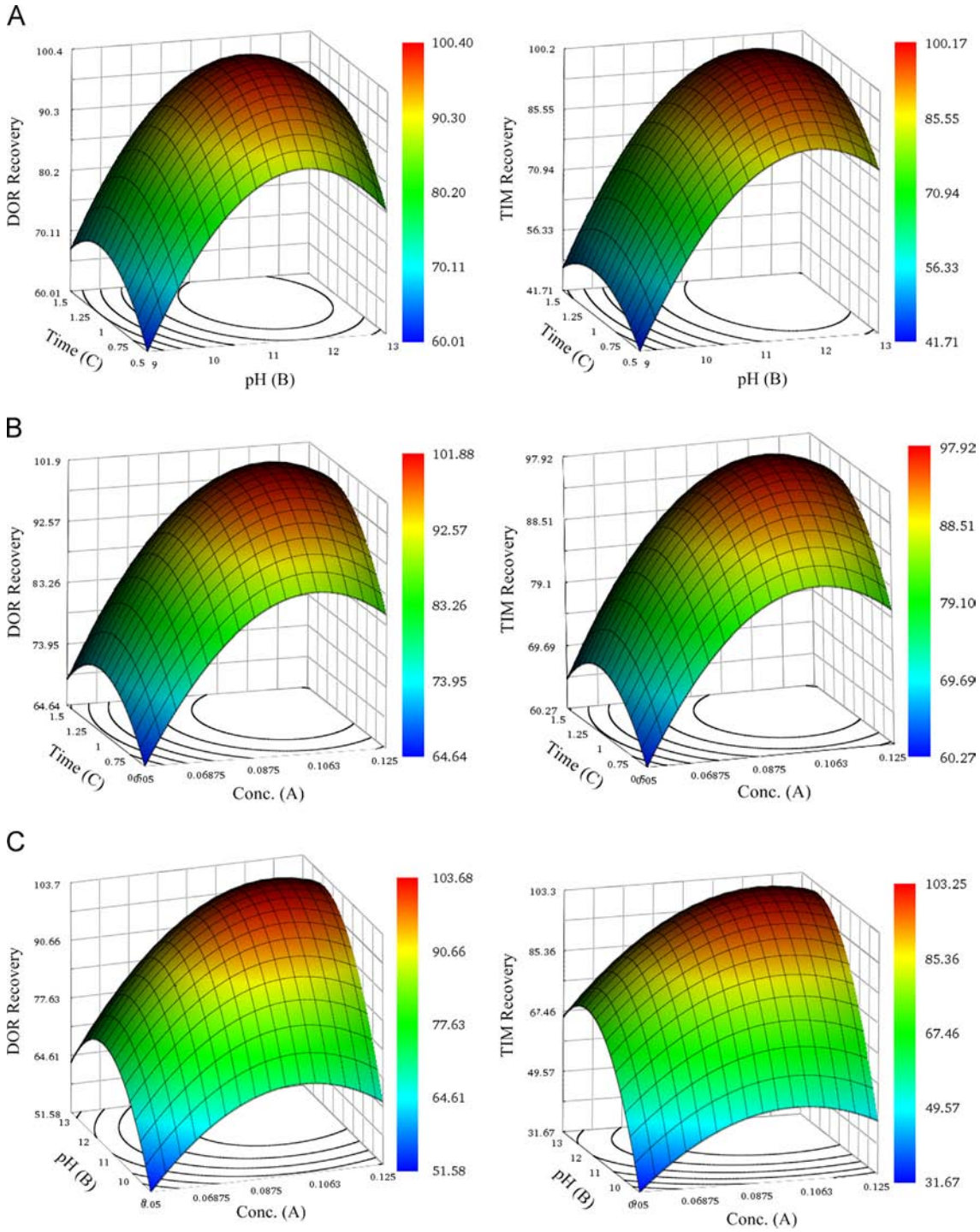

Fig. 4. Response surfaces (in recovery) for the Box–Behnken design of the studied antiglaucoma drugs. (A) $(NH_4)_2 SO_4$ kept constant at 0.1 g; (B) pH kept constant at 11; and (C) time kept constant at 1 min.

more than 99% of the endogenous proteins [38] which imparts high efficiency to the developed SALLME method.

In addition, DOR and TIM are weak basics having p*K*a values of 6.35 and 8.5 for DOR and 9.2 for TIM [3]. Hence, it is necessary to keep the analytes in completely unionized forms where, their solubility in the aqueous solution will be reduced and facilitating their extraction into the organic solvent. Therefore, achieving high extraction recoveries at pH 11.9 was consistent with the theoretical rationale. As a result, the present BBD and the optimized computation were found to be suitable and applicable for this work.

3.3. Method validation

The developed procedure was fully validated according to US-FDA guidance for bioanalytical method validation [39].

3.3.1. Linearity, limit of detection (LOD) and limit of quantification (LOQ)

Good linearities were obtained over the concentration ranges of 9–500 and 10.5–500 ng mL^{-1} in aqueous solution and 16–500 and 23.5–500 ng mL^{-1} in AH for DOR and TIM, respectively.

Table 4
Summary of the linear regression data for calibration curves of dorzolamide and timolol in aqueous solution and rabbit aqueous humor analyzed by the developed HPLC method.

Compound	Linearity[a] range (ng mL^{-1})	Correlation coefficient (r)	Intercept ± SD[b]	Slope ± SD[b]	LOD (ng mL^{-1})	LOQ (ng mL^{-1})
Aqueous solution						
DOR	9–500	0.9999	0.003 ± 0.005	0.006 ± 2.55 × 10^{-5}	2.89	8.75
TIM	10.5–500	0.9999	0.009 ± 0.004	0.004 ± 1.98 × 10^{-5}	3.41	10.32
Aqueous humor						
DOR	16–500	0.9998	0.005 ± 0.009	0.006 ± 4.59 × 10^{-5}	5.27	15.97
TIM	24–500	0.9997	0.014 ± 0.009	0.004 ± 4.35 × 10^{-5}	7.76	23.53

[a] Peak area ratio of the analyte/IS versus concentration (ng mL^{-1}).
[b] Standard deviation, average of five experiments.

Table 5
Inter-day and intra-day precision and accuracy for dorzolamide and timolol in aqueous solution and rabbit aqueous humor analyzed by the developed HPLC method.

		Intra-day assay (n=6)			Inter-day assay (n=6)		
Matrix	Concentration (ng mL^{-1})	% Recovery ± SD[a]	Precision (CV)[b]	Accuracy (%Er)[c]	% Recovery ± SD[a]	Precision (CV)[b]	Accuracy (% Er)[c]
Aqueous solution							
DOR	25 (LQC)	100.1 ± 0.45	0.45	0.14	99.8 ± 0.52	0.52	0.17
	100 (MQC)	99.8 ± 0.69	0.69	0.19	99.8 ± 0.61	0.61	0.14
	500 (HQC)	100.1 ± 0.70	0.70	0.14	100.1 ± 0.70	0.70	0.12
TIM	25 (LQC)	99.9 ± 0.57	0.57	0.12	99.8 ± 0.50	0.50	0.17
	100 (MQC)	99.9 ± 0.67	0.67	0.12	99.9 ± 0.62	0.62	0.11
	500 (HQC)	99.8 ± 0.70	0.71	0.16	100.1 ± 0.74	0.74	0.13
Aqueous humor							
DOR	25 (LQC)	99.7 ± 0.85	0.85	0.28	99.7 ± 1.00	1.00	0.28
	100 (MQC)	99.6 ± 1.08	1.08	0.34	99.7 ± 1.12	1.12	0.31
	500 (HQC)	100.4 ± 0.95	0.95	0.40	100.3 ± 0.21	1.20	0.34
TIM	25 (LQC)	99.6 ± 0.94	0.94	0.36	100.3 ± 0.89	0.89	0.32
	100 (MQC)	100.3 ± 1.01	1.00	0.30	99.8 ± 0.95	0.96	0.23
	500 (HQC)	99.7 ± 0.96	0.96	0.31	99.6 ± 0.95	0.95	0.37

[a] Standard deviation, n=6.
[b] Coefficient of variation.
[c] % Error.

Table 6
Recoveries of dorzolamide, timolol and IS from spiked rabbit aqueous humor analyzed by the developed SALLME–HPLC method.

Analyte	Added concentration (ng mL^{-1})	% Recovery ± SD[a]
DOR	25	100.11 ± 0.853
	100	98.91 ± 0.736
	250	99.34 ± 0.933
TIM	25	98.74 ± 0.732
	100	100.34 ± 0.622
	250	99.65 ± 1.332
IS	400	99.34 ± 0.7381

[a] Standard deviation, average of five determinations.

Table 4 summarizes the parameters for the calibration curves used for the linearity studies. The LOQs that produced the requisite precision and accuracy were 8.75 and 10.32 ng mL^{-1} in aqueous solution and 15.97 and 23.53 ng mL^{-1} in AH for DOR and TIM, respectively. Therefore, the sensitivity of the proposed method was found to be much better than that of all the previously reported methods for simultaneous quantification of the studied drugs [7–16]. Moreover, it is superior than almost all other methods reported for determination of either DOR or TIM in AH [19–28].

3.3.2. Accuracy, precision and recovery

The intra-day precision and accuracy were evaluated by analyzing six replicates of aqueous and AH QC samples at three different concentration levels: low-QC, middle-QC and high-QC of both DOR and TIM in a single day. The inter-day precision and accuracy were estimated by analysis of all QC samples over the six consecutive days. Results are summarized in Table 5; the intra- and inter-day accuracy and precision values were well within acceptable limits stated for bioanalytical method validation [39]. Moreover, the method showed good recovery results for both DOR and TIM along with the IS from rabbit AH (Table 6). In comparison with the reported methods, the obtained recoveries were much better than those obtained by most of the reported methods [22,23,25,26]. Taking into account that, the reported methods did not extract the studied drugs simultaneously from AH samples. From these results, the method was considered to be reliable, reproducible and accurate.

3.3.3. Selectivity and stability studies

The selectivity of the method was confirmed since the time intervals where DOR, TIM and IS eluted were free from interferences in all the drug-free AH samples (10 different batches). Representative chromatograms showed complete separation of the studied drugs and IS from endogenous AH constituents (Fig. 3). Meanwhile, using an efficient method of extraction (the proposed SALLME) removed any possible interferences from AH components and granted good method selectivity. In addition, it was found that, there was no interference from different antihypertensive drugs (hydrochlorothiazide and lisinopril) or diuretics (acetazolamide, amiloride, epithiazide, furosemide,

Table 7
Stability studies of dorzolamide, timolol and IS analyzed by the developed SALLME–HPLC method.

Condition	Percentage of initial concentration (%) ± SD[a]				
	DOR		TIM		IS
	LQC (25 ng mL^{-1})	HQC (250 ng mL^{-1})	LQC (25 ng mL^{-1})	HQC (250 ng mL^{-1})	400 ng mL^{-1}
Aqueous humor stability					
Three freeze–thaw cycles (−20 °C)	99.6 ± 1.08	99.7 ± 1.33	99.2 ± 0.84	98.8 ± 1.32	99.3 ± 0.83
Room temperature (12 h)	99.3 ± 0.71	99.8 ± 1.09	99.1 ± 0.55	99.1 ± 0.58	99.4 ± 1.12
Room temperature (24 h)	99.6 ± 1.25	98.9 ± 0.79	98.9 ± 0.80	99.3 ± 1.00	99.1 ± 0.46
Refrigeration for 24 h (4 °C)	99.5 ± 0.97	100.0 ± 0.72	99.2 ± 0.89	100.1 ± 1.07	99.1 ± 0.64
Freezer at −20 °C for 1 month	98.6 ± 0.44	99.5 ± 1.44	98.8 ± 0.66	99.1 ± 1.20	99.5 ± 0.91
Aqueous solutions' stability					
Refrigeration for 12 h (at 4 °C)	100.0 ± 0.27	100.0 ± 0.38	99.6 ± 0.46	99.8 ± 0.33	100.2 ± 0.69
Refrigeration for 24 h (at 4 °C)	99.5 ± 0.24	99.7 ± 0.42	99.9 ± 0.57	99.3 ± 0.19	99.6 ± 0.44

[a] Standard deviation, average of three determinations.

Table 8
Chromatographic performance parameters of the developed HPLC method.

Drug	Chromatographic parameter[a]						
	t_R	K'	α	R_s	$T_{0.05}$	N	HETP
DOR	3.9	0.56	1.78	6.20	1.09	7765	310
TIM	6.5	1.6	2.85	9.94	1.12	11 600	464

[a] t_R: retention time, K': capacity factor, α: selectivity coefficient, R_s: resolution, $T_{0.05}$: tailing factor, N: number of theoretical plates; and HETP: height equivalent to theoretical plate.

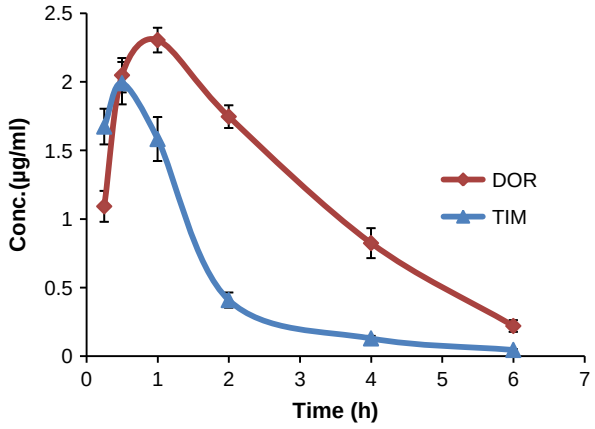

Fig. 5. Dorzolamide and timolol concentration–time profiles (mean ± SD) in aqueous humor of 5 rabbits following ocular instillation of 1 drop of FCDT eye drop.

indapamide) that could be co-administered. Also, there was no interference from different ß-blockers (atenolol, betaxolol, bisoprolol, Levobunolol, metipranolol, propranolol and sotalol) or from the preservative commonly used in FCDT eye drops (benzalkonium chloride). Where, all of these compounds either had retention times different from the studied drugs and IS or not detected at all.

DOR, TIM and IS were considered stable in rabbit AH and aqueous solutions under the different studied conditions as shown in Table 7.

3.3.4. System suitability and robustness

As shown in Table 8, the chromatographic performance parameters of both DOR and TIM were of acceptable values. Therefore, the results confirm the capability of the developed method for efficient separation of the studied drugs with good peak symmetry and within about 7 min. The method was claimed to be robust, since small changes of the chromatographic conditions did not affect the method precision or recovery. This provides an indication of the reliability of the proposed method during normal usage.

Table 9
Pharmacokinetic parameters (mean ± SD) of dorzolamide and timolol in aqueous humor of 5 rabbits following ocular instillation of 1 drop of FCDT eye drop.[a]

Pharmacokinetic parameter[b]	Value (mean ± SD)[c]	
	DOR	TIM
C_{max} (µg mL^{-1})	2.30 ± 0.078	2.03 ± 0.133
T_{max} (h)	1.01 ± 0.053	0.51 ± 0.015
K_a (h^{-1})	1.81 ± 0.238	2.28 ± 0.407
$t_{1/2K_a}$ (h)	0.38 ± 0.047	0.31 ± 0.049
K_{el} (h^{-1})	0.47 ± 0.044	1.68 ± 0.273
$t_{1/2}$ (h)	1.46 ± 0.132	0.42 ± 0.080
AUC (µg h mL^{-1})	7.85 ± 0.403	2.89 ± 0.232
V_d/F (L kg)	269.15 ± 16.33	211.57 ± 39.33
Cl/F (mL h^{-1} kg)	127.57 ± 6.417	347.31 ± 28.289

[a] Pharmacokinetic parameters (mean ± SD) were assessed by fitting individual concentration–time data to a one-compartment model.

[b] C_{max}, maximum concentration; T_{max}, time of maximum concentration achieved after ocular instillation; K_a, absorption rate constant; $t_{1/2K_a}$, absorption half-life; K_{el}, elimination rate constant; $t_{1/2}$, elimination half-life; AUC, area under the concentration–time curve; V_d/F, apparent volume of distribution, divided by the bioavailable fraction; and Cl/F, clearance, divided by bioavailabe fraction.

[c] Standard deviation, $n=5$.

3.4. Pharmacokinetic studies

With this simple and highly sensitive HPLC method combined with the rapid and efficient SALLME method, monitoring of the studied drugs in AH up to 6 h was achieved. Representative chromatograms obtained from analysis of blank and spiked AH samples and also AH sample 0.5 h after ocular instillation of 1 drop of FCDT eye drop are shown in Fig. 3B–D. Based on the measured concentrations of DOR and TIM in rabbits' AH, individual concentration–time profiles were constructed. Mean concentration–time courses (± SD) in AH are presented in Fig. 5. The concentration–time profiles were fitted with a one-compartment model with first-order absorption and elimination. Mean (± SD) values for the pharmacokinetic parameters in AH are listed in Table 9. The data demonstrated that the drugs are rapidly absorbed and eliminated from the rabbits' AH. It is worth observing that the pharmacokinetic profiles of DOR and TIM in AH are comparable to that reported for each drug [20,28]; thus suggesting that no drug–drug interaction occurs when DOR and TIM are administered together.

Table 10
Performance comparisons for dorzolamide and/or timolol determination in aqueous humor with other reported analytical methods.

Technique	Extract. method[a]	Vol. of AH sample (μL)	Vol. of extract. solvent	LOQ (ng mL^{-1})	Extract. time (min)	Recovery (%)	Ref.
DOR							
HPLC–UV	LLE, back LLE	150	10.3 mL	100	15	–	[27]
HPLC–UV	PPT	100	100	30	8	98.4–102	[21]
HPLC–MS	PPT	150	100 μl	125	45	98.6–98.9	[22]
HPLC–MS	PPT	100	400 μl	25	15	71.6–81.7	[23]
TIM							
HPLC–UV	LLE, back LLE	300	6 mL	–	15	–	[24]
HPLC–UV	LLE, back LLE	150	100 μl	125	15	88–90	[26]
HPLC–UV	PPT	200	300 μl	600	20	–	[19]
HPLC–UV	PPT	200	200 μl	216	15	–	[20]
DOR, TIM							
HPLC–UV	SALLME	50	90 μl	15.97 for DOR 23.53 for TIM	2	98.91–100.11 for DOR 98.74–100.34 for TIM	This work

[a] LLE: liquid–liquid extraction, PPT: protein precipitation, SALLME: salting out assisted liquid liquid microextraction.

3.5. Method performance comparison

Analytical performances of the developed SALLME–HPLC–UV method were compared with different published methods for determination of either DOR or TIM in AH. As can be seen from Table 10, the present method has better performance in the sample consumption, quantity of solvent used, extraction time and detection limits when compared with other reported methods [19–24,26,27]. Moreover, it utilizes water-miscible organic solvent which has low toxicity as extractants and most of all, it enabled the simultaneous extraction and determination of the studied drugs in AH. Therefore, it was demonstrated to be a simple, fast, cost-effective and eco-friendly option for simultaneous determination of the two antiglaucoma drugs.

4. Conclusion

For the first time, a simple, fast and sensitive SALLME–HPLC method has been developed with the aid of BBD and RSM for the simultaneous determination of DOR and TIM in rabbit AH. The developed SALLME–HPLC with simple UV detector offered a number of features including enhanced sensitivities, high recoveries, simple operation process and short analysis time as well as low cost and eco-friendly than the reported methods. Taking into account, there is no method reported for the simultaneous determination of both drugs in AH. Therefore, the proposed method not only enabled the simultaneous determination of both drugs in AH, but also with higher sensitivities than the reported methods of each drug alone in AH.

In addition, the developed method has been successfully applied, for the simultaneous pharmacokinetic studies of DOR and TIM in rabbit AH after their ocular instillation. Finally, the method could be useful for simultaneous therapeutic monitoring of levels of both DOR and TIM in biological samples and could have clinical applications for patients receiving these drugs.

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