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A New UPLC Method with Chemometric Design–Optimization Approach for the Simultaneous Quantitation of Brimonidine Tartrate and Timolol Maleate in an Eye Drop Preparation

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Abstract

A new ultra-performance liquid chromatography (UPLC) with photodiode array was proposed for the quantitation of Brimonidine Tartrate (BRI) and Timolol Maleate (TIM) in eye drop using experimental design and optimization methodology. A 3^3 full factorial design was applied to uncover the effects of the selected factors and their interactions on the chromatographic response function for the optimization of experimental conditions in the development of a new UPLC method. As a result, the optimal chromatographic conditions giving a better separation and short analysis time were found to be 49.2°C for column temperature; 0.38 mL/min for flow rate and 56.7 % (v/v) for 0.1 M CH_3COOH used in mobile phase. The elution of BRI and TIM was reported as 0.508 and 0.652 min within a short runtime of 1.5 min, respectively. Calibration graphs for BRI and TIM were obtained by the regression of the concentration on the peak area, which was detected at 246 and 298 nm, respectively. The method validation was performed by the analysis of the synthetic mixtures, intra-day and inter-day samples and standard addition samples. This study shows that the optimized and validated UPLC method is very promising and available for the quantification of BRI and TIM in an eye drop formulation.

Introduction

Brimonidine Tartrate (BRI), a highly selective and potent alpha$_2$-adrenergic receptor agonist, diminishes intraocular pressure by a dual mechanism of action: reduction in aqueous humor production and increase in uveoscleral outflow (1). Laboratory experimentation indicates brimonidine to be a more selective alpha$_2$ agonist than other ocular hypotensive medications in this class (2). Timolol Maleate (TIM), a beta-adrenergic-blocking agent, has conventionally been considered as a standard among intraocular pressure-lowering medications (3, 4).

In the literature survey, many analytical methods including HPLC (for BRI (5–8) and for TIM (9–11) and for BRI and TIM (12)), spectrophotometry (for BRI (13) and TIM (14)), first-derivative spectrophotometry and ratio spectra derivative spectrophotometry (for BRI and TIM (12)), flourimetric methods (for BRI (15, 16)), high-performance thin-layer chromatography (for BRI (17)), capillary electrophoresis (for BRI (18)), gas chromatography–mass spectrometry (for BRI (19)), chemiluminescence method (for TIM (20)), membrane electrodes method (for TIM (21)) and extraction-photometric method (for TIM (22)) have been reported for the quantitative analysis of individual compounds and their combinations in pharmaceutical preparations and biological fluids. Although some chromatographic methods have been applied to the quantitation of BRI and TIM either alone or in their combination, there are no references in the literature regarding the UPLC method application to the quantitative resolution of the same problem. In the practical applications, the UPLC approach

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provides some new opportunities in liquid chromatography, e.g., higher peak resolution, short analysis time, small mobile phase and low sample volume. Hence, it is necessary to develop a new reversed ultra-performance liquid chromatography (UPLC) method providing a good elution of the analyzed drugs in a short experimental period using experimental design and optimization methodology.

In the development of a new analytical method or procedure, usual way based on the optimization of one-experimental variable, when other experimental variables are constant, is used to be determined optimum experimental conditions. In the presence of the simultaneous effects of two or more experimental variables (or factors) on the results of an experiment, the optimization of one-experimental variable (factor) may not be appropriate to find exact optimal experimental conditions in the elaboration of a new technique (or method) or analytical procedure when effects of factors and their interactions on the analytical response are not considered. Moreover, one-variable optimization requires a very large number of experiments to be done, which would be very expensive and time consuming. Therefore, multivariate optimization methodologies based on considering the effects of all experimental variables (or factors) and their interactions on the experimental response are good enough to uncover real optimal experimental conditions.

In order to overcome drawbacks of the one-variable optimization technique, experimental design–optimization methodologies are very powerful chemometric tools to identify exact experimental condition with a small number of experiments. Therefore, chemometric experimental design–optimization methodology with advantages over one-variable optimization technique has been a popular approach for most of the application areas.

In previous studies, there is no chemometric optimization application that has been reported for the development of a new UPLC method providing the quantification of BRI and TIM in samples. Therefore, we focused mainly on the improvement of a new UPLC approach using a chemometric design–optimization tool that requires small mobile phase and low sample volume with short analysis time for the analysis of the related active compounds in this study.

In order to develop a new UPLC method for the simultaneous quantification of BRI and TIM, first aim of this investigation was to optimize the experimental variables (or factors), i.e., flow rate, column temperature and 0.1 M CH_3COOH% (v/v) in mobile phase, which influence chromatographic response function. Second aim was to validate the UPLC method with the analysis of the validation samples consisting of synthetic mixtures, intra-day and inter-day samples, and standard addition samples. Third objective of the study was to apply the optimized and validated UPLC method to the simultaneous quantitation of BRI and TIM in a commercial eye drop formulation.

Theory

Chemometric design–optimization methodology has been used for the analytical method development and chemical process control in analytical chemistry and related fields as well as science and industry. The purpose is to optimize a response (dependent variable), which is influenced by two or more factor variables (independent variables). Shortly, chemometric design–optimization procedure is a very useful way to identify main factors' effects and their interactions on response to select effective factors and to eliminate ineffective factors in design matrices, and also to optimize experimental conditions. In design–optimization methodology, second-order polynomial quadratic model obtained by the multiple regression of the factor variables with their interaction on the response variable is expressed in the equation:

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i \geq j}^{k} \sum_{i=1}^{k} \beta_{ij} x_i x_j + \mathcal{E} \qquad (1)$$

where y is the predicted response, β_0 is the intercept term; β_i is the linear coefficient indicating linear effect, β_{ii} is the quadratic coefficient representing squared effect, β_{ij} is the cross-product coefficient indicating interaction effect. x_i and x_j are coded factor variables (or independent variables), and $\mathcal{E}$ is the error of experimental model.

Experimental

Apparatus and software

The chromatograms of the calibration set and samples containing BRI and TIM drugs were obtained by using Waters ACQUITY UPLCH-Class instrument with a quaternary solvent manager, photodiode array (PDA) detector, cooling autosampler and an oven allowing control at the column temperature. Waters UPLC BEH C_{18} column (50 mm × 2.1 mm intradermally (i.d.), 1.7 µm) was utilized for the elution of the related active compounds. Waters®Empower2 software was used for the data acquisition. Linear regression analysis and related computations were done by means of in-house software implemented in MATLAB 7.12 and Microsoft Excel Software.

Experimental conditions

In order to develop a new UPLC approach, the 27 experimental runs of a 3^3 full factorial design matrix were performed on a Waters UPLC BEH C_{18} (50 mm × 2.1 mm i.d., 1.7 µm) column system at the different flow rates (between 0.20 and 0.40 mL/min), different column temperature (between 35°C and 65°C) and different volume of 0.1 M CH_3COOH (between 35% and 75%) in mobile phase.

Under optimized chromatographic conditions, the calibration and quantitation steps of analyzed compounds, separation was carried out by using the mentioned analytical column system with the column temperature of 49.2°C, the flow rate of 0.38 mL/min and mobile phase consisting of acetonitrile and 0.1 M CH_3COOH (43.3:56.7, v/v). BRI and TIM in UPLC were detected by measuring the peak areas at the wavelength of 246 and 298 nm, respectively.

Standards and chemicals

HPLC grade acetonitrile and acetic acid were supplied by Sigma-Aldrich (USA). Chromatographic grade double-distilled water was used throughout the analysis. Standards of BRI (99.7 % purity) and TIM (99.6 % purity) was kindly obtained from World Medicine (Istanbul, Turkey). A pharmaceutical dosage form (Combigan Eye Drop produced by Abdi İbrahim Pharm. Ind., Istanbul, Turkey) containing 2 mg BRI and 5 mg TIM per milliliter was purchased from a regional pharmacy, Ankara, Turkey.

Standard and calibration solutions

Stock solutions of BRI and TIM were individually prepared by dissolving 10 mg of each active substance in 100-mL volumetric flask in methanol. From the stock solutions, the calibration set of BRI

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and TIM using a 5^2 full factorial design was prepared between 5.0–25.0 µg/mL and 8.0–72.0 µg/mL, respectively.

In order to validate the optimized UPLC method, an independent validation set consisting of 11 different mixture solutions of BRI and TIM within the mentioned concentration ranges was daily prepared. In addition to this, intra-day and inter-day samples containing BRI and TIM at three different concentration levels were freshly prepared. The proposed UPLC method was validated according to the US FDA guidelines in terms of its selectivity, linearity, precision, accuracy, matrix effect and recovery (23), and the validation was described and discussed in the literature (24, 25).

All the calibration and sample solutions were prepared in methanol during the chromatographic analysis.

Sample preparation

For the preparation of commercial samples, 1.0 mL of the commercial eye drop was transferred into the 50-mL volumetric flasks and made up to 50 mL with methanol. This solution was sonicated for 5 minutes and then filtered through 0.2 µm hydrophilic PTFE syringe filters. The filtrate was diluted in the ratio of 1:2.5 with methanol. Then the sample solution was transferred into the UPLC vial and put on autosampler. For the chromatographic detection and analysis, 1.0 µL of the final sample solution was injected into the UPLC system. This procedure was repeated 10 times.

Results

Chemometric method optimization

In this study, prior to starting the chemometric design–optimization procedure for developing a new UPLC method, three-factor variables, column temperature, flow rate and 0.1 M CH_3COOH% in mobile phase were chosen according to preliminary experiments and then the factors levels were quantified to optimize a response (dependent variable) corresponding to improved chromatographic response function (ICRF). After this, a 3^3 full factorial design was constructed. As described earlier, 0.1 M CH_3COOH (x_1, %, v/v), flow rate (x_2, mL/min) and column temperature (x_3, °C) were selected as three factors of the design matrix for the development and optimization of UPLC method to determine BRI and TIM in commercial eye drop preparation. Three levels of the selected factors, which correspond to low (−1), medium (0) and high (+1) were identified and illustrated in Table I. In the implementation of the 3^3 full factorial design, the ICRF values were calculated from UPLC chromatograms of the 27 run samples (Figure 1). Their numerical values were used as response vectors expressed by ICRF. For the multivariate optimization of the response in chromatographic analysis, Jančić-Stojanović *et al.* recently proposed a new chromatographic response function, called newly elaborated chromatographic response function (26). In another study, a new ICRF to reach effective chromatographic peak elution with short runtime in a given chromatogram was firstly improved by Rakić and co-workers (27).

Table I. Selected factors with their corresponding levels in investigated experimental space

Level	Temperature °C (X_3)	Flow rate (mL/min) (X_2)	0.1 M CH_3COOH (%) (X_1)
−1	35	0.2	35
0	50	0.3	55
+1	65	0.4	75

Low level (−1), medium level (0) and high level (+1).

In this article, for the experimental design–optimization procedure, ICRF was considered as a dependent variable (or response variable). ICRF values were calculated from the following equation:

$$\mathrm{ICRF} = \left(a\left(1 - \frac{\sum_{i=1}^{N-1}\theta_{s,l}}{N-1}\right) + 1\right)\left(1 + \left(\frac{t_f}{t_{opt}}\right)^b\right)\left(1 + \frac{\sum w_i}{N}\right)^c \quad (2)$$

where N is the number of analyzed compound peaks in a chromatogram, t_f is the elution time of the last peak, t_{opt} corresponds to the selected optimal overall elution time, a, b and c are the coefficients of the equation, w_i is the peak width at baseline. $\theta_{s,l}$ is a function for the estimation of peak elution given the following equation. The peak elution criterion explained in the literature (28) is expressed as:

$$\theta_{s,l} = 1 - \frac{H_v\left|t_{R,l} - t_{R,s}\right|}{\left|t_{R,v} - t_{R,s}\right|\left(H_l - H_s\right) + H_s\left(t_{R,l} - t_{R,s}\right)} \quad (3)$$

where H_s and H_l are the height of the peaks, H_v is the valley height, $t_{R,s}$ and $t_{R,l}$ are the retention time of the peaks and $t_{R,v}$ is the time position of the valley. If $\theta_{s,l}$ is placed in Equation (2), ICRF can be obtained.

According to the selected factors and their levels, three-factor three-level full factorial design matrix was constructed to quantify optimum chromatographic conditions for our new UPLC method providing short runtime and a good elution of the analyzed compounds. The chromatograms of the 27 run samples, which correspond to the 3^3 full factorial design illustrated in Table II, were recorded and presented in Figure 1. From equation (2), the ICRF

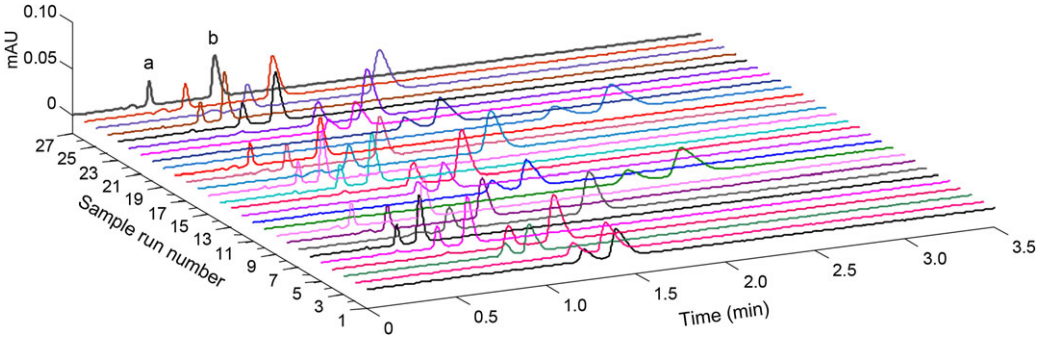


Figure 1. Three-dimensional illustration of the UPLC chromatograms of the 27 run samples, which correspond to the 3^3 full factorial design matrix (a) BRI and (b) TIM.

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Table II. Three-factor three-level full factorial design and ICRF with its predicted values

Run no.	0.1 M CH_3COOH (%)	Flow rate	Temperature	ICRF	
	X_1	X_2	X_3	Measured	Predicted
1	−1	−1	−1	7.7910	7.2499
2	−1	0	−1	2.9334	3.3233
3	−1	1	−1	1.8358	1.8725
4	0	−1	−1	2.3727	3.1460
5	0	0	−1	1.3733	0.9279
6	0	1	−1	1.2341	0.5767
7	1	−1	−1	3.8838	3.3996
8	1	0	−1	1.8646	1.8658
9	1	1	−1	1.4223	2.3493
10	−1	−1	0	7.4782	7.7706
11	−1	0	0	3.4176	3.7958
12	−1	1	0	1.9860	2.0593
13	0	−1	0	2.4452	3.4080
14	0	0	0	1.4662	0.9787
15	0	1	0	1.2398	0.3937
16	1	−1	0	3.7727	3.2835
17	1	0	0	1.8633	1.5572
18	1	1	0	1.3982	1.8204
19	−1	−1	1	11.4599	9.5580
20	−1	0	1	3.7880	4.6094
21	−1	1	1	2.1663	2.6175
22	0	−1	1	2.5348	4.0386
23	0	0	1	1.5072	1.3836
24	0	1	1	1.2583	0.5783
25	1	−1	1	3.6448	3.5289
26	1	0	1	1.8361	1.6082
27	1	1	1	1.3861	1.6591

Table III. Results of the statistical *t*- and *F*-tests of the second-order polynomial quadratic model parameters

	Coefficients	Statistical *t*	*P*-value
Intercept	0.8491	7.2388	0.0000
X_1	−1.2102	−22.2881	0.0000
X_2	−1.7476	−32.1842	0.0000
X_3	0.2706	4.9832	0.0001
X_1^2	1.8369	19.5318	0.0000
X_2^2	1.0672	11.3478	0.0000
X_3^2	0.2310	2.4562	0.0251
X_1X_2	1.1372	17.0997	0.0000
X_1X_3	−0.4298	−6.4631	0.0000
X_2X_3	−0.2728	−4.1021	0.0007

values were computed as response variables and then their numerical values were listed in Table II.

Second-order polynomial quadratic function was calculated by regressing the independent variables x_1, x_2, and x_3 (0.1 M CH_3COOH% (v/v), flow rate and column temperature) with factor interactions ($x_1.x_2$, $x_1.x_3$, $x_2.x_3$) on the dependent variable (ICRF). The model was obtained in the form of the following equation:

$$y = 0.8491 - 1.2102x_1 - 1.7476x_2 + 0.2706x_3 + 1.8369x_1^2 + 1.0672x_2^2 + 0.2310x_3^2 - 1.1372x_1x_2 - 0.4298x_1x_3 - 0.2728x_2x_3 \quad (4)$$

In the second-order polynomial quadratic model, the coefficient terms were evaluated by the significance testing. The significance test results are shown in Table III. It was concluded from this table that the effects and interactions are significant due to high *t*-values and low *P*-values with 95% significance level. This showed that the regression coefficients were significant to justify the model from our experiments to optimize the chromatographic conditions providing short runtime with better elution of BRI and TIM in UPLC analysis.

However, each pair of the selected factors versus ICRF was plotted as surface graphs and contour maps for the quantitative estimation of factors' interactions. Figures 2a–c show 3D plots (or surface graphs) and their contour maps of flow rate–column temperature vs. ICRF, column temperature–0.1 M CH_3COOH% vs. ICRF and flow rate–0.1 M CH_3COOH% vs. ICRF, respectively. As it was pointed out in Figures 2a–c, significant interactions of the studied factors on ICRF were reported.

Figure 2a shows the higher ICRF at the low levels of the relevant factors, flow rate and 0.1 M CH_3COOH%. In another case, the plot of column temperature and 0.1 M CH_3COOH% vs. ICRF indicates to be at the high values of column temperature and 0.1 M CH_3COOH%, which give high ICRF (Figure 2b). As can be seen from the surface graphs and contour map illustrated in Figure 2c, the interaction of low flow rate and high column temperature results in higher ICRF. From Figures 2c, a small change was observed in both low and high levels of the column temperature. These factors at the low level of flow rate and high level of temperature give us high ICRF values.

As a result, the optimal chromatographic conditions giving good peak elution and short runtime were calculated from Equation (4)

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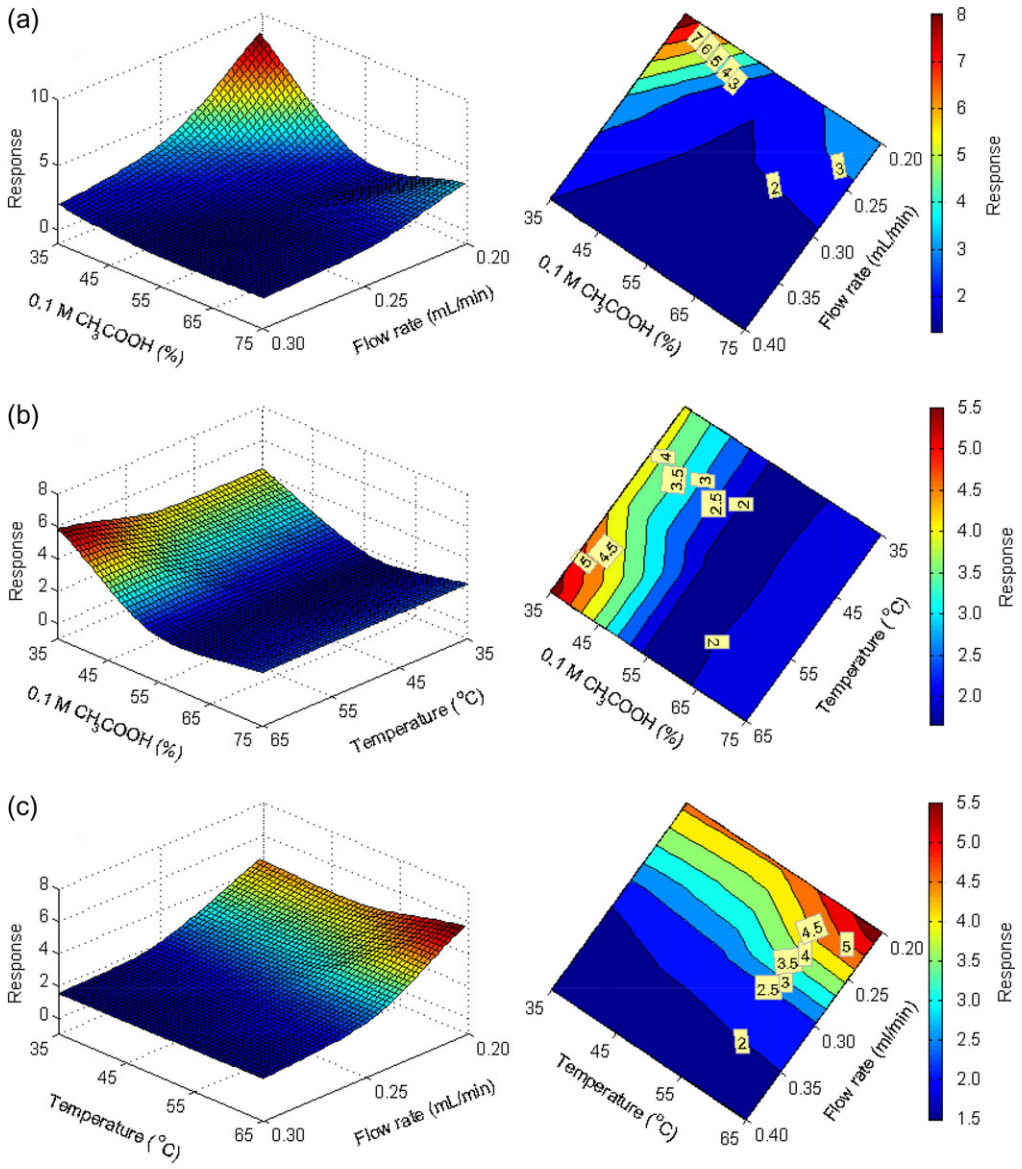


Figure 2. Response surfaces and corresponding contour maps as functions of two factors: flow rate–0.1 M CH_3COOH vs. ICRF (a), temperature–0.1 M CH_3COOH vs. ICRF (b), flow rate–temperature vs. ICRF (c).

for the application of a new RP-UPLC method. The optimal UPLC conditions for the analysis of BRI and TIM in their mixture were found to be 49.2 °C for column temperature, 0.38 mL/min for flow rate and 56.7% for 0.1 M CH_3COOH.

Discussion

Validation of the optimized method

As described in the section *Standard and calibration solutions*, a calibration set of the mixture solutions for BRI and TIM were prepared between 5.0–25.0 µg/mL and 8.0–72.0 µg/mL, respectively. Under optimal chromatographic conditions determined from our optimization approach, the UPLC chromatograms of the calibration set were recorded by PDA detection at 246.0 and 298.0 nm for BRI and TIM, respectively. The chromatograms of the calibration set containing BRI-TIM mixtures were illustrated in Figures 3A1 and B1. As can be seen from these figures, the retention times of BRI and TIM were reported as 0.508 and 0.652 min, respectively. Calibration curves for BRI and TIM were obtained by using the linear regression of the drug concentration on the peak area calculated from the UPLC chromatograms. The linear regression analysis and its statistical results, i.e., concentration ranges, slope, intercept and correlation coefficients of the linear regression lines, limit of detection (LOD) and limit of quantification (LOQ) are listed in Table IV. BRI and TIM in eye drop samples were analyzed using the calibration graphs.

From experimental results obtained for the quantitation of BRI and TIM, it was observed that the elaborated and optimized UPLC method gave us better elution with short runtime of 1.5 min. It is clear that the developed and optimized UPLC approach is very useful to perform the fast and reliable analysis of the active compounds.

An independent validation set of 11 different mixture solutions of BRI and TIM within working concentration ranges of the

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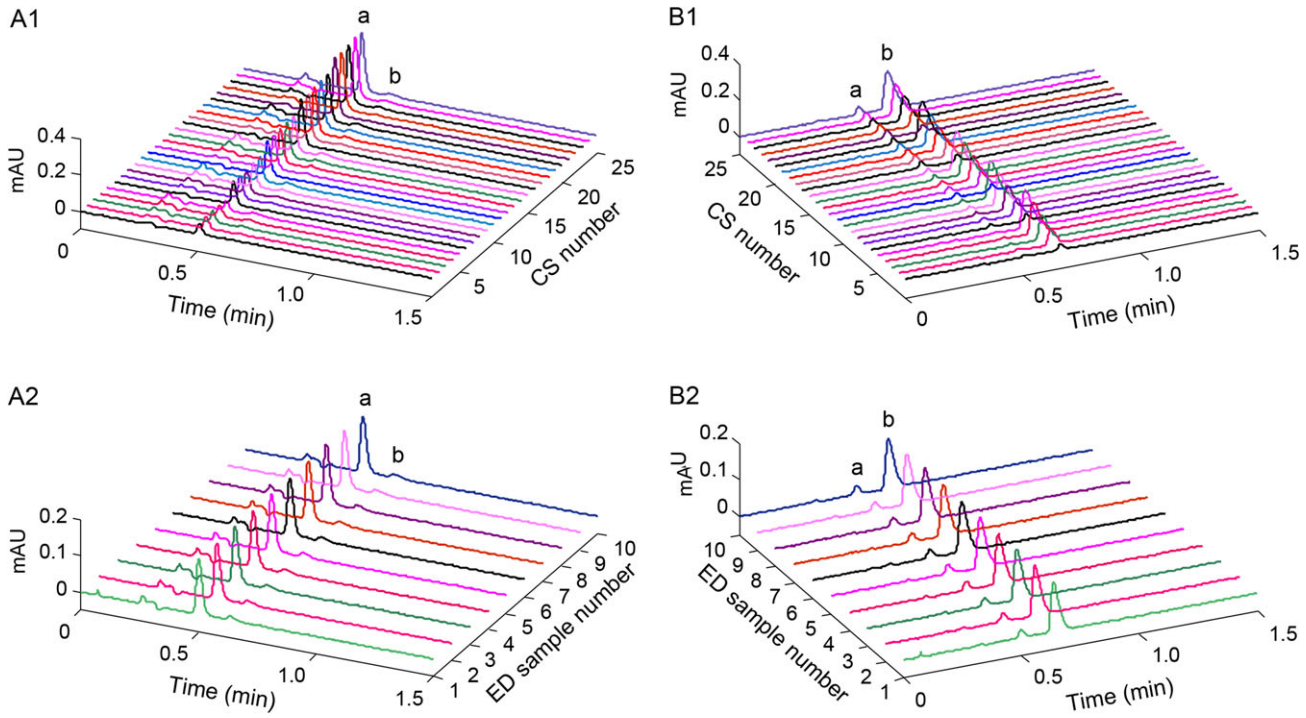


Figure 3. The representative UPLC chromatograms of the calibration set consisting of the 25 run samples (A1 and B1) and the commercial eye drop samples (A2 and B2) (a) BRI and (b) TIM.

Table IV. Regression analysis results of the calibration graphs

	BRI	TIM
m	0.8096	0.3591
n	−0.6122	−0.3440
r	0.9993	0.9993
SD (*m*)	0.0065	0.0029
SD (*n*)	0.1073	0.1333
SD (*r*)	0.0017	0.0017
LOD (μg/mL)	0.3976	1.1138
LOQ (μg/mL)	1.3254	3.7125

m = slope of the linear regression equation; *n* = intercept of the linear regression equation; *r* = correlation coefficient of the linear regression equation; SE (*m*) = standard error of slope; SE (*n*) = standard error of intercept; SE (*r*) = standard error of correlation.

analyzed compounds were prepared and analyzed by the optimized UPLC method. Recovery results with relative standard deviations are illustrated in Table V. These results indicate that the proposed UPLC method have adequate accuracy and precision for the UPLC analysis of BRI and TIM.

Selectivity/specificity of the optimized UPLC method was evaluated by observing the presence and absence of the excipient effect on the analysis of BRI and TIM from eye drop formulation. As explained in section Standard and calibration solutions, the standard addition samples were obtained by adding the stock solutions BRI (at three different concentration levels; 6.0, 12.0 and 16.0 μg/mL) and TIM (at three different concentration levels; 8.0, 30.0 and 40.0 μg/mL) to the sample solution of eye drop preparation. From the analysis results shown in Table VI, it was reported that there is no interference of the commercial formulation's excipients on the analysis results of BRI and TIM in eye drop samples.

Table V. Recovery results of BRI and TIM in their mixtures by the optimized and validated UPLC method

Recovery mixture						
Added (μg/mL)		Found (μg/mL)		Recovery (%)		
Mix no.	BRI	TIM	BRI	TIM	BRI	TIM
M1	5.0	40.0	5.1	40.4	101.5	101.1
M2	10.0	40.0	10.4	40.0	103.7	100.1
M3	15.0	40.0	14.9	39.2	99.2	98.0
M4	20.0	40.0	19.7	40.1	98.7	100.2
M5	25.0	40.0	25.0	38.7	100.0	96.7
M6	16.0	8.0	15.2	8.0	95.3	99.7
M7	16.0	24.0	16.6	24.9	104.0	103.9
M8	16.0	40.0	15.7	39.2	98.1	97.9
M9	16.0	56.0	15.9	55.1	99.4	98.3
M10	16.0	72.0	16.0	73.0	99.8	101.3
M11	20.0	50.0	19.7	48.5	98.7	97.0
				Mean	99.9	99.5
				SD	2.50	2.14
				RSD	2.50	2.15

SD = standard deviation; RSD = relative standard deviation.

The high values of correlation coefficients show the good linearity of calibration curves for BRI and TIM in the concentration range of 5.0–25.0 μg/mL and 8.0–72.0 μg/mL, respectively (Table IV). LOD and LOQ were computed from the intercepts of linear regression lines and their slopes. The numerical values of LOD and LOQ for both compounds were given in Table IV.

Accuracy and precision of the optimized UPLC method were estimated by analyzing the intra-day and inter-day samples. These experiments were performed by using the samples containing BRI (at three different concentrations low: 6.0 μg/mL medium: 15.0 μg/mL

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Table VI. Assay results obtained from the standard addition samples by UPLC

	Added (μg/mL)		Found (μg/mL)		Recovery (%)		RSD (%)	
	BRI	TIM	BRI	TIM	BRI	TIM	BRI	TIM
Formulation	6	8	6.2	7.7	103.0	95.8	0.17	1.30
Formulation	12	30	11.7	29.0	97.5	96.8	0.15	0.74
Formulation	16	40	16.3	41.2	101.7	103.0	0.24	1.12

RSD = relative standard deviation.

Table VII. Analysis results for inter-day and intra-day samples

	Added (μg/mL)		Found (μg/mL)		Recovery (%)		RSD (%)		RSE (%)	
	BRI	TIM	BRI	TIM	BRI	TIM	BRI	TIM	BRI	TIM
Inter-day	6	10	5.7	9.5	95.1	94.7	6.42	9.68	−4.86	−5.34
	15	40	14.8	40.1	98.9	100.2	0.93	0.88	−1.13	0.24
	24	70	24.1	71.5	100.6	102.1	0.57	1.24	0.56	2.11
Intra-day	6	10	5.8	10.5	96.8	105.1	6.38	8.05	−3.17	5.11
	15	40	14.5	39.9	97.0	99.7	0.95	0.44	−3.02	−0.34
	24	70	24.1	71.4	100.4	101.9	0.72	0.85	0.39	1.94

SD = standard deviation; RSD = relative standard deviation.
*Recovery results for each level are the average of five replications ($n = 5$).

Table VIII. Experimental results of BRI and TIM in real samples by UPLC

	mg/mL		
	Experiment number	BRI	TIM
	1.0	1.94	5.06
	2.0	1.94	5.01
	3.0	1.98	4.97
	4.0	1.94	4.93
	5.0	2.00	5.15
	6.0	2.03	5.08
	7.0	2.05	5.29
	8.0	2.06	5.17
	9.0	1.99	5.01
	10.0	1.96	4.96
Mean		1.99	5.06
SD		0.05	0.11
RSD		2.37	2.23

SD = standard deviation; RSD = relative standard deviation.

and high: 24.0 μg/mL) and TIM (at three different concentrations low: 10.0 μg/mL medium: 40.0 μg/mL and high: 70.0 μg/mL). Measurements of intra-day and inter-day solutions were done at different three times and at different 3 successive days with three replications, respectively. Percent mean recoveries; relative standard deviations and relative standard errors are presented in Table VII. These results show that the newly developed UPLC method is appropriate chromatographic approach for the analysis of the related drugs in samples with desirable precision and accuracy.

Analysis of eye drop samples

After method optimization and validation steps, the proposed UPLC method was used for the simultaneous quantification of BRI and TIM in eye drop samples. The chromatograms of these commercial samples were recorded under optimized conditions. The peak areas of BRI and TIM at the elution times, 0.508 and 0.652 min were calculated and replaced into the related calibration equations of BRI and TIM to quantify their amounts in eye drops, respectively.

Assay results obtained from eye drop samples are shown in Table VIII. As can be seen from the experimental results given in Table VIII, the optimized and validated UPLC method is very suitable for the quantitative estimation of BRI and TIM in marketing eye drop formulation.

Conclusions

In this study, a new optimized and validated UPLC method was developed for the simultaneous estimation of the content of BRI and TIM in commercial eye drop formulation with short analysis time and small number of experiments using experimental design and optimization methodology. For the UPLC analysis of the related compounds, a 3^3 full factorial design matrix with response expressed by ICRF was applied to the optimization of chromatographic conditions, which correspond to column temperature, flow rate and 0.1 M CH_3COOH in mobile phase. For developing selective, precise, accurate and reliable UPLC method, this chemometric optimization approach based on ICRF provided a balance between good elution and short runtime. In this way, analyses by UPLC developed in our study were performed within a short runtime of 1.5 min, with a good elution of the TIM and BRI peaks in a chromatogram. Compared to HPLC (12), the newly proposed UPLC is more inexpensive with desirable chromatographic elution of TIM and BRI in a commercial formulation. The optimized and validated UPLC method implemented in this study was a very powerful, cheap, fast and reliable approach for the simultaneous quantification and routine analysis of the related drugs. It was concluded that the newly developed UPLC method optimized and validated in this study could be very useful for the quantitative evaluation of both BRI and TIM in real eye drop samples with a good selectivity and sensitivity.

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