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Development of a spectrophotometric method for simultaneous determination of dorzolamide and timolol by partial least-squares and H-point standard addition method

Masoud Shariati-Rad*, Mohsen Irandoust, Maryam Karimi
Department of Analytical Chemistry, Faculty of Chemistry, Razi University, Kermanshah, (IRAN)
E-mail: mshariati_rad@yahoo.com

ABSTRACT

Simultaneous determination of dorzolamide (DOR) and timolol (TIM) by partial least squares (PLS) calibration and H-point standard addition method (HPSAM) is proposed. Due to the spectral interferences, simultaneous determination of DOR and TIM by using classical spectrophotometric analytical methods is difficult. PLS calibration model was based on the recording spectra in the range of 200–350 nm for 24 different mixtures of DOR and TIM. Simplex lattice design with a lattice degree of 3 was used for design of mixtures. Leave one out cross-validation method was used to select the optimum number of factors in PLS. The PLS method was validated by using 11 external test samples. The root mean square error of prediction (RMSEP) for DOR and TIM were 0.397 and 0.583, respectively. Moreover, the proposed methods were successfully used for determination of DOR and TIM in eye drop. The results of application of H-point standard addition method showed that DOR and TIM can be determined simultaneously with concentration ratios of 4:1, 8:2, 10:2.5 in the mixed sample. The results of application of two methods to the real eye drop samples showed the success of two methods.

KEYWORDS

Dorzolamide;
H-point standard addition method;
Partial least squares;
Spectrophotometric;
Timolol.

INTRODUCTION

Dorzolamide ((4-S trans)-4-ethylamino-5,6-dihydro-6-methyl-4H-thieno-[2,3-b]thiopyran-2-sulfonamide-7,7-dioxide monohydrochloride) (DOR) is a carbonic anhydrase inhibitor (CAI) used in the treatment of glaucoma. DOR was synthesized in the1980s[1]. Oral CAIs have been used to lower intraocular pressure (IOP) for the past 40 years.

Timolol maleate, (*S*)-1-[(1,1-dimethyl)amino]-3-[[4-(4 morpholinyl9-1,2,5-thiadiazol-3-yl]oxy]-2-propanol (TIM), is a nonspecific β-adrenergic blocker used in the treatment of hypertension, acute myocardial infarction, angina pectoris and has an important role as an antiglaucoma agent. TIM has shown a broad activity with differential effects of adrenergic and cholinergic blockades during experimental therapeutics. Structural formulas of TIM and DOR have been shown in Figure 1.

In the literature, very few methods appeared for the determination of DOR individually in human serum and urine which are based on HPLC assay with

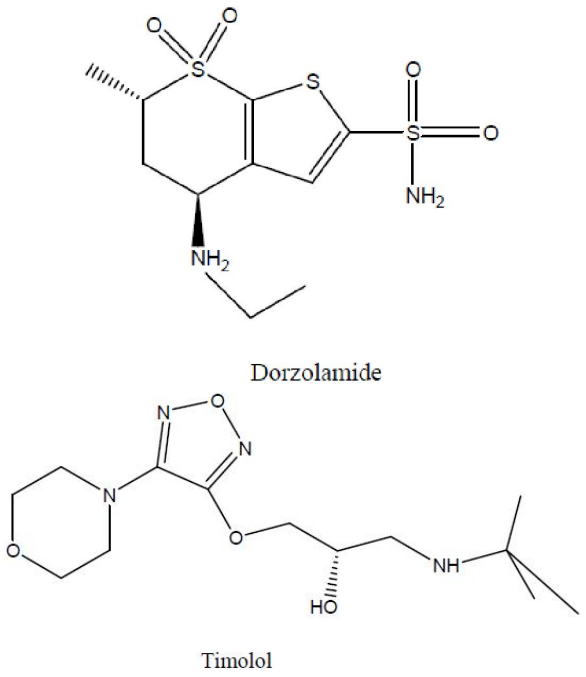

Figure 1 : Chemical structure of DOR and TIM

ultraviolet detection[2,3] and capillary electrophoresis[4]. On the other hand, various methods have been developed for the determination of TIM in drug formulations including UV spectrophotometry[5], and gas chromatography (GC) with different detection modes such as mass spectrometry (MS)[6] and capillary electrophoresis[7]. It must be mentioned that Santoro et al.[5] have used first-derivative of the UV spectral data for the determination of TIM in pharmaceutical ophthalmic solution.

DOR has been marketed in combination with TIM in eye drops. For simultaneous determination of both drugs, TLC-densitometry, first-derivative UV-spectrophotometry and ratio derivative spectrophotometry have been used[8,9]. Derivative techniques are widely used in conjunction with spectrophotometric methods, especially in cases where improvements in selectivity are required[10]. However, one of the disadvantages of these data transformation procedures is that some loss of signal occurs during the transformation.

In 1988, Bosch–Reig and Campins–Falco[11] presented a new technique called the H-point standard addition method (HPSAM) based on the principle of dual-wavelength spectrophotometry and the standard addition method[12,13]. In the first publications, the HPSAM was applied to UV–visible spectrophotometry[11,14]. Later, it was also extended to liquid chromatography with diode array detection[15] and spectrofluorimetry[16].

Multivariate calibration methods such as partial least squares (PLS) are useful tool in the analysis of multicomponent mixtures[17-26]. These methods allow rapid and simultaneous determination of each component in the mixture with minimum sample preparation, reasonable accuracy and precision and without the need of lengthy separations. Moreover, the problems in selecting optimum wavelength encountered by derivative method and HPSAM are avoided.

This paper reports simple and rapid methods for the simultaneous quantitation of the two drugs in eye drops based on PLS and HPSAM.

THEORY

Partial least squares

PLS is used to correlate instrumental responses to chemical or physical properties[27-29]. Instrumental responses are included row-wise in matrix X and corresponding properties we are to predict them (e.g. concentration) construct vector y. The relation between X and y is constructed in calibration step through a vector of regression coefficients i.e. y = Xb. The algorithm used to find b can be found in the literatures[28,29].

H-point standard addition method

Theoretical background of HPSAM can be found elsewhere[11-13,30]. The method which requires the spectrum of the interferent to be known is based on the measurements of two standard addition lines at two wavelengths λ_1 and λ_2 where the interferent shows the same absorbance. The absorbance of the analyte at λ_1 and λ_2 should be different. Intersection of the two lines corresponds to the analyte concentration in the mixture.

MATERIALS AND METHODS

Reagents and solutions

All experiments were performed with pharmaceutical-grade DOR and TIM. Doubly distilled water was used for preparation of the solutions. Stock solutions containing 100 mg.L^{-1} of DOR and TIM were prepared by dissolving dorzolamide hydro-

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chloride and timolol maleate in doubly distilled water, respectively. Standard working solutions were made by appropriate dilution of these stock solutions as required. Fresh stock standard solutions were prepared every day.

For HPSAM, standard 500 mg.L-1 solutions of each of DOR and TIM were prepared by dissolving appropriate amounts of drugs in doubly distilled water. Furthermore, Zilomole (Iran, Sinadarou) and Co-Biosopt (Iran, Bakhtarbioshimi) pharmaceutical formulations were used as real samples. These formulations contain 2 g per 5 mL and 0.5 g per 5 mL DOR and TIM, respectively,

Apparatus, hardware and software

Spectrophotometric measurements were carried out with an Agilent 8453 spectrophotometer, employing a 1 cm path-length quartz cell. Spectra were acquired over the wavelength range of 200–350 nm at 1 nm intervals against a solvent blank. PLS multivariate calibration and other calculations were carried out using PLS Toolbox in the MATLAB 7.2 environment (The Mathworks Inc., Natick).

Procedure

Appropriate volumes of the stock solutions of DOR and TIM were diluted with doubly distilled water for preparation of standard calibration samples. The examined concentration range of the analytes in the univariate calibration was 0.1-100 $mg.L^{-1}$ for DOR and TIM.

A set of 35 mixtures were prepared for PLS multivariate calibration. These samples were prepared by mixing convenient volumes of stock solutions of DOR and TIM and diluting by doubly distilled water to the final concentrations in the range of 10-28 $mg.L^{-1}$ for DOR and 3-11.5 $mg.L^{-1}$ for TIM. The mixtures were designed based on the simplex lattice design with a lattice degree of 3. The design was conducted based on the linear ranges in univariate calibration and the real sample content. These mixtures have been reported in TABLE 1. These 35 samples were divided into 24 calibration and 11 external test samples based on the Kennard-Stone algorithm[31]. External test samples are used to check the stability of each calibration model and the ability to predict samples which have not been included in calibration set.

For the evaluation of HPSAM in determination of DOR and TIM, synthetic mixtures with different ratios of DOR/TIM were designed and prepared in which DOR was either considered as the analyte (and TIM the interferent) or as the interferent (and TIM the analyte). The added concentrations of DOR to these mixtures varied from 0 to 20 $mg.L^{-1}$ ($n = 10$) for determination of DOR. TIM was added in the range of 0 to 10 $mg.L^{-1}$ ($n = 10$) for determination of TIM.

For determination of DOR and TIM in ophthalmic eye drops, the added concentrations to the solutions of ophthalmic eye drops were performed as for the synthetic mixtures of DOR and TIM.

Real sample preparation

1 mL of the commercial ophthalmic eye drop solution (containing 20 mg DOR and 5 mg TIM per 5 mL) was transferred into a 1000 mL volumetric flask and diluted to the volume with doubly distilled

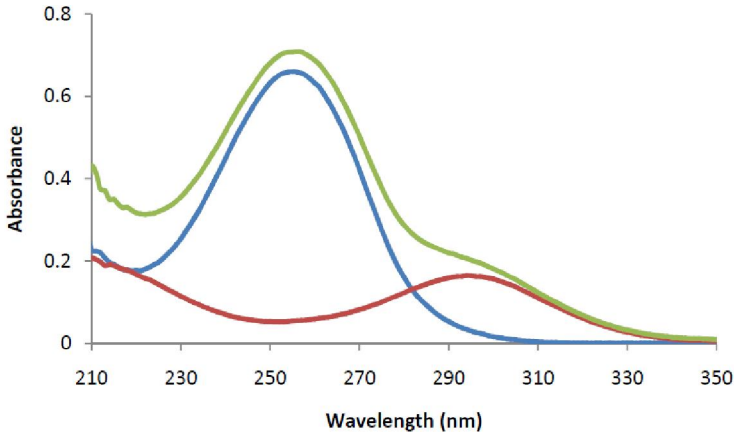

Figure 2 : Absorption spectra of (a) DOR (20 $mg.L^{-1}$), (b) TIM (5 $mg.L^{-1}$) and (c) mixture of them

water. Therefore, the resulted solution is 20 and 5 $mg.L^{-1}$ in DOR and TIM, respectively. The resulted solution was then used to analysis by HPSAM and PLS.

RESULTS AND DISCUSSION

Figure 2 shows the absorption spectra of DOR (20 $mg.L^{-1}$), TIM (5 $mg.L^{-1}$) and a mixture of them in doubly distilled water. TIM shows maxima at 212 and 297 nm while DOR exhibits a maximum absorbance at 258 nm. It is evident that spectra strongly overlap which make difficult the simultaneous determination of drugs by classical methodology. Therefore, we expected that using multivariate calibration could be a better resource to circumvent spectral overlapping and mutual interference problems. These methods allow the resolution of the mixtures of the analytes without the need for their previous separation.

TABLE 2 summarizes the most relevant results of the univariate calibration. The squares of correlation coefficients (r^2), which indicate the quality of the straight lines that fit the absorbance-concentration data, were 0.999 and 0.989 for DOR and TIM, respectively. Linear range for DOR is longer than TIM and detection limit for DOR is smaller.

Multivariate calibration

The proposed multivariate calibration method is based on the PLS analysis of UV-Vis spectral data. Optimum number of PLS latent variables was selected by searching the minimum *RMSECV* (root mean square error of cross validation, a measure of the predictive ability of the model) with the leave-one-out cross-validation[32]. In order to perform the

TABLE 1 : Composition of the samples used for PLS calibration and prediction

Calibration set samples			External test samples		
Solutions	**Dorzolamide ($mg.L^{-1}$)**	**Timolol ($mg.L^{-1}$)**	**Solutions**	**Dorzolamide ($mg.L^{-1}$)**	**Timolol ($mg.L^{-1}$)**
S1	19.0	6.0	S1	19.0	5.5
S2	27.0	8.0	S2	14.5	5.5
S3	20.0	5.0	S3	16.5	8.5
S4	18.5	6.5	S4	26.5	8.5
S5	23.5	11.5	S5	14.0	5.5
S6	21.0	9.0	S6	11.5	3.5
S7	13.5	6.5	S7	15.5	4.5
S8	18.0	7.0	S8	15.0	5.0
S9	22.0	8.0	S9	12.0	3.0
S10	24.5	10.5	S10	24.0	6.0
S11	11.0	5.0	S11	25.5	9.5
S12	10.5	4.5			
S13	23.0	7.0			
S14	17.5	7.5			
S15	14.0	6.0			
S16	21.5	8.5			
S17	28.0	7.0			
S18	11.5	4.0			
S19	11.0	4.0			
S20	25.0	10.0			
S21	20.0	10.0			
S22	10.0	5.0			
S23	16.0	4.0			
S24	22.5	7.5			

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TABLE 2 : The results of univariate calibration for DOR and TIM

Parameter	DOR	sTIM
λ_{max} (nm)	258	213
Number of samples	28	17
Linear range (mg.L^{-1})	0.8-80.0	3.3-50.0
Slope	0.029	0.025
Standard error of slope	0.001	0.001
Intercept	0.002	0.019
Standard error of intercept	0.035	0.034
Correlation coefficient	0.999	0.989
Standard error of correlation coefficient	0.147	0.092
Detection Limit (mg.L^{-1})	0.052	0.333

TABLE 3 : Statistical parameters of the PLS models

Parameters	DOR	TIM
Factors	3	5
RMSEP[a]	0.397	0.583
RMSECV[b]	0.336	0.35
Q^2	0.995	0.985
R^2	0.995	0.963

$RMSEP = (\sum(C - \hat{C})^2) / (n - 1))$. C and $\hat{C}$ are real and predicted concentrations, respectively. n is the number of the external test samples; *RMSECV* with same formulation of *RMSEP* for calibration set; $Q^2 = 1-(\sum(C - \hat{C})^2) / (\sum(C - \bar{C})^2)$, where $\bar{C}$ and $\bar{\hat{C}}$ are average of the real concentration and predicted concentration with PLS, respectively.

analysis, a calibration was built and validated. The results of calibration and prediction with PLS models are shown in TABLE 3. *RMSEP* for DOR and *RMSECV* for TIM is smaller. PLS model for DOR performs better in prediction (Q^2 is higher). Values of Q^2 and R^2 are near to 1. These show the good predictivity of the PLS models.

Analysis of real samples

The validated PLS calibration models were applied to the simultaneous determination of DOR and TIM in commercial ophthalmic eye drop formulations. The concentrations obtained for DOR and TIM in the Zilomole and Co-Biosopt eye drop formulations are shown in TABLE 4.

Calculated RSD% and Recovery% for DOR show that the PLS method for DOR is better. Higher accuracies in determination of DOR can be attributed to its higher concentration in real samples (four times). The precision of the method in prediction indicated by RSD% is very good (all of the calculated RSD% are lower than 1).

H-point standard addition method (HPSAM) for synthetic mixtures

Synthetic mixtures containing DOR and TIM in three different ratios of DOR to TIM (4:1, 8:2 and 10:2.5) were prepared. To each mixture, increasing amounts of the analyte were added to apply the HPSAM. Results of HPSAM for determination of DOR and TIM in these mixtures have been reported in TABLE 5. The good agreement between these results and known values indicates the successful applicability of HPSAM for simultaneous determination of DOR and TIM.

H-point standard addition method (HPSAM) for pharmaceutical samples

When DOR or TIM is selected as the analyte, it is possible to select several pairs of wavelengths where they present the same absorbance for interferent. The results for DOR and TIM considered as the analyte in the Zilomole are given in TABLE 6.

TABLE 6 shows that the best results for DOR and TIM have been obtained for wavelength pair 270:314 and 239:268 nm, respectively. Using the wavelength pair 211:276 nm has resulted in a good prediction for TIM, too. Acceptable results have been obtained only when measurements are performed at two wavelengths where the analyte absorbance is not too small and the difference in slopes of the addition lines is larger.

Comparison by the other methods

This section reports the results of the proposed

TABLE 4 : The results of analysis of the real samples by PLS

	Dorzolamide			Timolol		
	Found[a] ($mg.L^{-1}$)	RSD%	Recovery%	Found[a] ($mg.L^{-1}$)	RSD%	Recovery%
Zilomole	19.43	0.80	97.20	4.73	0.70	94.70
Co-Biosopt	19.69	0.06	98.46	4.79	0.19	95.84

The results have been obtained by 6 times determination of the real samples

TABLE 5 : Results obtained by HPSAM in simultaneous determination of DOR and TIM in synthetic mixtures

Mixture	Analyte	Interferent	Ratio	DOR Found	TIM
1	DOR	TIM	4:1	4.083±0.118	
	TIM	DOR			1.000
2	DOR	TIM	8:2	8.048	
	TIM	DOR			1.842
3	DOR	TIM	10:2.5	10.226±0.022	
	TIM	DOR			2.475

TABLE 6 : Results of HPSAM for DOR and TIM in Zilomole and Co-Biosopt pharmaceutical products

Pharmaceutical product	DOR			TIM		
	Found ($mg.L^{-1}$)	RSD (%)	Relative error (%)	Found ($mg.L^{-1}$)	RSD (%)	Relative error (%)
Zilomole						
	20.22	3.65	1.10	5.07	4.07	1.40
Co-Biosopt						
	19.35	1.01	-3.25	4.86	4.20	-2.80

TABLE 7 : Results of different methods for simultaneous determination of DOR and TIM

Methods	DOR		TIM	
	RSD%	Recovery%	RSD%	Recovery%
TLC [19]	0.41	100.50	0.37	99.53
First-derivative UV-spectrophotometry [19]	0.30	101.25	0.12	99.90
Ratio derivative spectrophotometry [19]	0.31	99.87	0.51	99.84
PLS (Zilomol)	0.80	97.20	0.70	94.70
PLS (Co-Biosopt)	0.06	98.46	0.19	95.84
HPSAM (Zilomol)	0.74	101.07	0.21	101.40
HPSAM (Co-Biosopt)	1.01	96.77	4.20	97.20

method and other methods in simultaneous determination of DOR and TIM. The results have been collected in TABLE 7.

The results obtained by HPSAM for Zilomol pharmaceutical product are comparable with those obtained by TLC and derivative methods. However, it must be mentioned that if matrix effect is present derivative methods cannot be used. Moreover, HPSAM performs better compared with PLS for this product. This can be related to the some matrix effect in the real ophthalmic eye drops. HPSAM can solve the matrix effect and the known interference synchronously. In general, DOR has been predicted by lower errors. This can be attributed to its lower amounts in the pharmaceutical products.

CONCLUSIONS

Simultaneous determination of DOR and TIM in mixtures is difficult due to the high spectral overlapping between the absorption spectra of the components. Methods based on the use of electronic ab-

sorption measurements in conjunction with PLS multivariate calibration and HPSAM were developed for the simultaneous determination of DOR and TIM in eye drops and synthetic binary mixtures. These techniques are simple, fast and precise. Moreover, the proposed methods do not need separation of dorzolamide hydrochloride and timolol maleate before the analysis. HPSAM performed better than the PLS method which can be attributed to the presence of some matrix effect in the real ophthalmic eye drop samples.

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