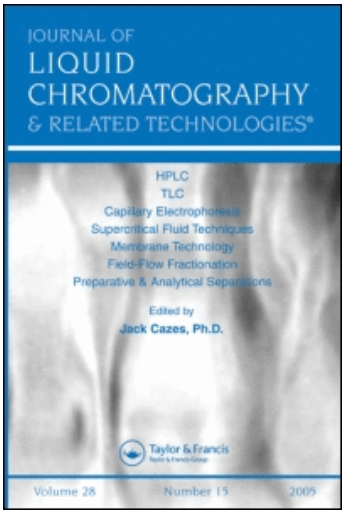

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Chemometric Approach to Simultaneous Chromatographic Determination of Paracetamol and Chlorzoxazone in Tablets and Spiked Human Plasma

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Chemometric Approach to Simultaneous Chromatographic Determination of Paracetamol and Chlorzoxazone in Tablets and Spiked Human Plasma

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Abstract: New chemometric approaches were introduced into the high performance liquid chromatographic (HPLC) determination of chlorzoxazone (CZX) and paracetamol (PAR) in the tablets and spiked human plasma. These chemometric approaches contain the application of classical least squares (CLS), principle component regression (PCR), and partial least squares (PLS) calibrations to the multiwavelength HPLC peak area obtained by plotting the chromatograms at the five wavelengths. The multichromatograms were obtained by using the photodiode array detector at 225 (A), 240 (B), 255 (C), 270 (D), and 285 (E) nm. The algorithms of CLS, PCR, and PLS were

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applied to the multichromatogram data to construct the HPLC-CLS, HPLC-PCR, and HPLC-PLS calibrations. A mixture of acetonitrile and 0.1 M ammonium carbonate (60:40, v/v) on a Waters Symmetry® C18 Column 5 μm 4.6 × 250 mm at a flow rate of 0.8 mL/min was used as a mobile phase to separate and determine CZX and PAR in samples. Hydrochlorothiazide (IS) was used as an internal standard in this chromatographic separation. The HPLC chemometric calibrations were successfully applied to the quantitative analysis of the investigated drugs in commercial pharmaceutical preparation and spiked human plasma samples with high precision and accuracy.

Keywords: HPLC-chemometric methods, Spiked human plasma, Tablets, Chlorzoxazone, Paracetamol

INTRODUCTION

Paracetamol (acetaminophen) is used for the relief of pain and as an antipyretic. It is rapidly absorbed from the gastrointestinal tract and is primarily metabolized by conjugation with glucuronic and sulphuric acid to form paracetamol glucuronide and paracetamol sulphate, respectively. These polar conjugates are subsequently cleared in the urine. Chlorzoxazone (CZX) is a centrally acting muscle relaxant used for the treatment of painful muscle spasms. In order to study pharmokinetics of these drugs in humans it is very important to develop a sensitive, simple, and specific method for the quantitative studies of the drugs in biological fluids like urine or plasma. Several methods describing the simultaneous determination of paracetamol and chlorzoxazone in dosage forms have been reported including UV spectrophotometry,[1,2] gas chromatography,[3] and HPLC.[4–6]

Nowadays, chemometric calibration techniques[6–10] based on mathematical logic to chemistry have been commonly used for the spectrophotometric determination of two or more active compounds in the multicomponent mixtures.[11–13]

Therefore, the same calibration techniques have been also applied to the analytical data obtained from near FTIR and electrochemical instruments. The chemometric techniques based on the above mentioned instruments have the advantage of simplicity, speed, and low cost to analyze multimixtures. In some cases, the chemometric calibration with spectrophotometric techniques is poor selectivity. Spectral multivariate calibrations cannot be applied to the multicomponent determinations due to interference coming from the sample matrix. In this context HPLC chemometric calibration techniques are a promising tool for solving the above drawbacks.

The target of this study is to develop new HPLC chemometric approaches to quantify CZX and PAR in tablets and spiked human plasma samples. The method validation with acceptable characteristics of suitability, reliability, and feasibility were done by using the guidelines of the International Conference on Harmonization (ICH).[14]

The assay results of the HPLC chemometric methods were statistically compared with each other, as well as those obtained by the classical single HPLC method.

THEORETICAL BASIS OF HPLC CHEMOMETRIC APPROACHES

In this HPLC chemometric study, the multivariate HPLC data were obtained by plotting the multichromatograms at five different wavelengths. Detector response was measured as peak area and then the chemometric calibration technique was applied to the multivariate HPLC data set consisting of the ratio of chromatographic peak area of analyte and IS. Chemometric calibrations and their application to the HPLC multivariate data are explained in the following subsections.[15,16]

HPLC-CLS

This approach is based on the application of multi linear regression (MLR) to the ratio of the peak area of each drug.

The matrix equation describing the systems is given as follows:

$$\begin{bmatrix} R_{11} & R_{12} & R_{13} & R_{14} & R_{15} & R_{16} \\ R_{21} & R_{22} & R_{23} & R_{24} & R_{25} & R_{26} \\ R_{31} & R_{32} & R_{33} & R_{34} & R_{35} & R_{36} \\ R_{41} & R_{42} & R_{43} & R_{44} & R_{45} & R_{46} \\ R_{51} & R_{52} & R_{53} & R_{54} & R_{55} & R_{56} \end{bmatrix} = \begin{bmatrix} K_{11} \\ K_{21} \\ K_{31} \\ K_{41} \\ K_{51} \end{bmatrix}$$

$$[C_{11} \quad C_{12} \quad C_{13} \quad C_{14} \quad C_{15} \quad C_{16}] \tag{1}$$

or in the compact form (1) becomes

$$R_{5x6} = K_{5x1}C_{1x6} \tag{2}$$

Here, R_{5x6} represents the matrix of the peak area responses (ratio of the peak area of analyte to the peak area of the internal standard). K_{5x1} is the matrix of the calibration coefficients and C_{6x1} denotes the concentration set of the investigated compound.

We mention that our PDA detector provides data collection at five wavelengths for six standard series (concentration set (C)) of the investigated drug. The equation (2) gives the form of the K_{5x1} as follows:

$$K_{5x1} = R_{5x6}C^{T}_{6X1}\left[C_{1x6}C^{T}_{6X1}\right]^{-1} \tag{3}$$

where, C^{T}_{6x1} is the transpose of C_{1x6} and $[C_{1x6}\ C^{T}_{6X1}]^{-1}$ represents the inverse of $C_{1x6}\ C^{T}_{6X1}$.

We implemented an algorithm inside the Matlab 7.0 software. In the step we determined Ka_{1x5} as

$$Ka_{1x5} = \frac{1}{[K^{T}_{1x5}K_{5x1}]_{x}K^{T}_{1x5}} \quad (4)$$

and in the second step Ka_{1x5} is introduced into the following equation

$$C_{prediction1xn} = Ka_{1x5} \times R_{sample5xn} \quad (5)$$

As a result the concentration of the content of analyte in the mixture is determined by multiplying Ka_{1x5} and $R_{sample5x}$.

HPLC-PCR Method

The ratio of the peak area of an individual drug and the drug concentration set was reprocessed by mean centering as R_o and C_o, respectively, and the covariance dispersion matrix of the centered matrix R_o was calculated. By using the square covariance matrix, the normalized eigenvalues and eigenvectors were computed. Identifying the highest values of the eigenvalues, we are able to select the number of the optimal principal components (eigenvectors (P)).

Therefore, the remaining eigenvalues and their corresponding eigenvectors are omitted. To follow this objective the coefficient b defined as $b = P \times q$ is calculated, where P is the matrix of eigenvectors and q is the C loadings given by $q = D \times T^{T} \times R_o$. T^{T} represents the transpose of the score matrix, T, and D is a diagonal matrix having the components the inverse of the selected eigenvalues. The drug content in samples was obtained by using the $C_{prediction} = b \times R_{sample}$. PLS toolbox 3.0 in Matlab 7.0 software was used for the data treatment.

HPLC-PLS Method

The PLS calibration using the orthogonalized PLS algorithm initiated by Wold,[3,4] and extensively discussed by Martens and Naes,[5] is based on the simultaneous use of both independent and dependent variables on the data compression and decomposition operations.

In the proposed data analysis, the HPLC-PLS calibration is obtained by decomposition of both concentration and the ratio of peak area matrix into latent variables, $R = T \times P^{T} + E$ and $C = U \times Q^{T} + F$. After that, the linear regression, $C_{prediction} = b \times R_{sample}$, is used for the estimation of thedrugs in the samples. The vector, b, has the expression $b = W \times (P^{T} \times W)^{-1} \times Q$, where W denotes a weight matrix.

PLS toolbox 3.0 in Matlab 7.0 software was used for the mathematical calculations.

EXPERIMENTAL

Instrumentation and Chromatography

Chromatography was performed with an Agilent 1100 series HPLC system (Agilent Technologies, Inc., California, and USA) provided with a binary pump, a thermostatted autosampler, a thermostatted column compartment, and a multiwavelength diode array detector (DAD). HPLC data were acquired and processed using HP Chem Station for LC (Rev. A0.01 [403]) software from Hewlett-Packard. The column used was a Waters Symmetry® C18 Column 5 μm 4.6 × 250 mm. The flow rate was maintained at 0.8 mL/min and the injection volume was 25 μL. The mobile phase was prepared daily, filtered through a 0.45 μm membrane filter, and degassed before use.

Chemical Reagents

Both CLX and PAR active compounds were kindly donated by Turkish pharmaceutical firms. The commercial pharmaceutical formulation, Parafon® Plus tablets (produced by Nobel Pharm., Turkey, Batch no. 2D008) containing 500 mg CLX, and 600 mg PAR, was purchased from a local drug store. All solvents and chemicals used were of HPLC or analytical grade. Hydrochlorothiazide (IS) was used as internal standard. A 0.1 M ammonium carbonate was used. Methanol HPLC grade was obtained from Merck. The mobile phase is a mixture of methanol 0.1 M ammonium carbonate.

Standard Solutions and Chromatography

Stock solutions of 25 mg/100 mL CLX and PAR were prepared in a mixture of 0.1 M ammonium carbonate and methanol (v/v, 60:40). A standard series of the solutions containing 3–8 μg/mL CLX and PAR was obtained from the stock solutions. A validation set consisting of 12 synthetic mixture solutions in the working range of 3–8 μg/mL CLX and PAR was prepared. For the standard addition method, six solutions using the stock solutions and tablet solutions were prepared. In all of the chromatographic study, 25 μg/mL HCT as internal standard was added into each solution. All the solutions were prepared fresh and protected from light.

The HPLC separations were conducted at ambient temperature (25°C). Aliquots of 25 μL were injected by auto sampler into the instrument.

Tablet Analysis

Accurately weighed 20 tablets were powdered in a mortar and an amount equivalent to one tablet was dissolved in the solvent consisting of 0.1 M

ammonium acetate and methanol (v/v, 60 : 40). The solution was filtered in a 100 mL volumetric flask by a 0.45 μm membrane filter. Tablet solutions were diluted to the working concentration range of 3–8 μg/mL for CLX and PAR in a 25 mL-calibrated flask.

Plasma Analysis and Calibration

Extraction of drugs from human plasma was carried out in 25 mL volumetric flasks. Drug free plasma of 1 mL was spiked with known amounts of the drug from the standard solutions of CLX and PAR in the concentration ranges of 3–8 μg/mL. IS solutions of 20 μg/mL is added to every prepared solution. For the extraction of drugs from plasma, mobile phase containing (v/v, 60 : 40) acetonitrile-buffer solution was added into the volumetric flask and vortexed for 5 min.

After precipitation of all proteins that come from the plasma content, 10 mL of solution was added to the 100 mm × 16 mm screwed capped polyethylene tubes and centrifuged for 15 min at 10000 rpm. The supernatant of solution was put into 2 mL vials for automatic sampling into the HPLC instrument. Calibration graphs in the presence of human plasma, were constructed by using the peak area ratio of drug/internal standard. For the HPLC method, linear regression analysis was used to determine slope, intercepts, and correlation coefficient of the calibration curve. The concentrations of CLX and PAR in the test samples were calculated using regression parameters obtained from the standard curves. The chemometric calibrations were done by using the same peak area ratio obtained for each concentration.

RESULTS AND DISCUSSION

Method Development and Optimization

Various chromatographic conditions including mobile phase composition, flow rate, column temperature, e.g., were tested for good chromatographic separation between CLX and PAR in the presence of the IS. In our case, optimal chromatographic conditions were found by using a Waters Symmetry® C18 Column 5 μm 4.6 × 250 mm and a mobile phase consisting of acetonitrile and 0.1 M ammonium carbonate (60 : 40,v/v), at a flow rate of 0.8 mL/min (at room temperature).

Introduction of chemometric methods to the HPLC data is a new approach for the quantitative determinations of drugs in tablet dosage forms and biological liquids. This application completely depends on the detector response of the instrument that can provide simultaneous monitoring of multiwavelengths. The multiwavelength absorption monitoring of drugs provides different chromatogram profiles that give different peak areas for the same concentration of drugs. Simultaneous data collection at multiwavelength

points provides an opportunity for the application of chemometric methods CLS, PCR, and PLS to the HPLC data.

For the HPLC chemometric calibrations, the obtained data needs to be processed as in single wavelength HPLC calibrations, that contains peak area ratio of each drug to the IS peak area. A relationship between training set and calculated peak area ratios were used to construct the multivariate calibrations as HPLC-CLS, HPLC-PCR, and HPLC-PLS (see Tables 1 and 2). The data handling of each method is different from each other and produces different calibration sets, depending on their specific algorithms. Although, the single wavelength HPLC method uses only one point, multivariate methods use all the data points that are simultaneously collected by the PDA detector for each concentration.

The use of multiwavelengths or chemometric calibrations will remove some unwanted information and will lead to better results. In this study, results of those obtained by basic HPLC methods were compared with the results of those obtained by chemometric HPLC methods for samples, namely, spiked plasma, synthetic mixtures, and tablets. In this study, the main aim is to show applicability of multivariate methods to the HPLC data to eliminate or to reduce the errors that comes from experimental setup and environmental effects. Also, the method can be used to resolve the overlapped HPLC peaks for the quantitative studies of drugs. In here, the second situation was not simulated, but the method can be applied in the situations where it is not possible to resolve the peaks.

Multivariate HPLC Data Analysis

A training set of the mixture solution over a concentration range of $3-8\,\mu g/mL$ PAR and CLX using $25\,\mu g/mL$ IS, were prepared in the mobile phase. Chromatograms were plotted at the five wavelengths 225 (A), 240 (B), 255 (C), 270 (D), and 285 (E) nm and their peak area for calibrations in both spiked and unspiked mediums were separately measured for the training set at the selected five wavelengths (Figs. 1 and 2). Retention times for the tablet analysis (unspiked plasma) and spiked plasma analysis were observed as 3.05 and 3.12 min for PAR, 4.26 and 4.34 min for CLX, and 3.50 and 3.60 min for IS, respectively. The training set and corresponding multivariate HPLC data set in unspiked human plasma and in spiked human plasma are listed in Table 1 and 2. Multivariate calibrations, CLS, PCR, and PLS were obtained by using the relationships between training set and multivariate HPLC peak area. These multivariate HPLC approaches were applied to the spiked and unspiked samples.

Multivariate HPLC Calibrations

Two HPLC-CLS calibrations described in the methodology section were calculated from the relationship between training set and its corresponding

Table 1. Multi wavelengths-HPLC data set corresponding to the training set in the tablet analysis

Training set (μg/mL)		Peak areas-ratio (PAR/IS)					Peak areas-ratio (CLX/IS)				
PAR	CLX	225	240	255	270	285	225	240	255	270	285
3	3	0.4627	10.2498	5.2474	0.6054	0.8652	0.5781	3.8530	1.7689	0.3750	2.2306
4	4	0.6125	14.2339	6.8947	0.8014	1.1413	0.7590	5.1153	2.2956	0.4895	2.9150
5	5	0.7607	18.1604	8.4706	0.9900	1.4170	0.9278	6.2011	2.7972	0.5997	3.5589
6	6	0.8306	21.9520	10.2914	1.1840	1.7119	1.0187	7.4057	3.2671	0.7163	4.1919
7	7	1.0575	26.1029	11.7983	1.3811	1.9948	1.2726	8.5496	3.8333	0.8243	4.8959
8	8	1.2446	29.7878	13.8248	1.6001	2.3135	1.5002	9.9080	4.5221	0.9711	5.6783

Table 2. Multi wavelengths-HPLC data set corresponding to the training set in spiked human plasma

Training set (μg/mL)		Peak areas-ratio (PAR/IS)					Peak areas-ratio (CLX/IS)				
PAR	CLX	225	240	255	270	285	225	240	255	270	285
3	3	0.5053	8.4930	5.0574	0.6566	0.9457	0.5408	2.8448	1.6474	0.3650	2.1049
4	4	0.6424	11.2690	6.6826	0.8505	1.1920	0.6952	3.6949	2.1274	0.4698	2.7571
5	5	0.8123	14.2335	8.3877	1.0612	1.5202	0.9020	4.7307	2.7645	0.6133	3.5331
6	6	0.9422	16.6061	9.9864	1.2565	1.7613	1.1063	5.6889	3.2518	0.7531	4.1845
7	7	1.0855	19.2493	11.3926	1.4130	2.0305	1.1965	6.4969	3.7544	0.8144	4.8987
8	8	1.2275	21.9187	13.0746	1.6407	2.2955	1.4109	7.3692	4.2499	0.9562	5.5100

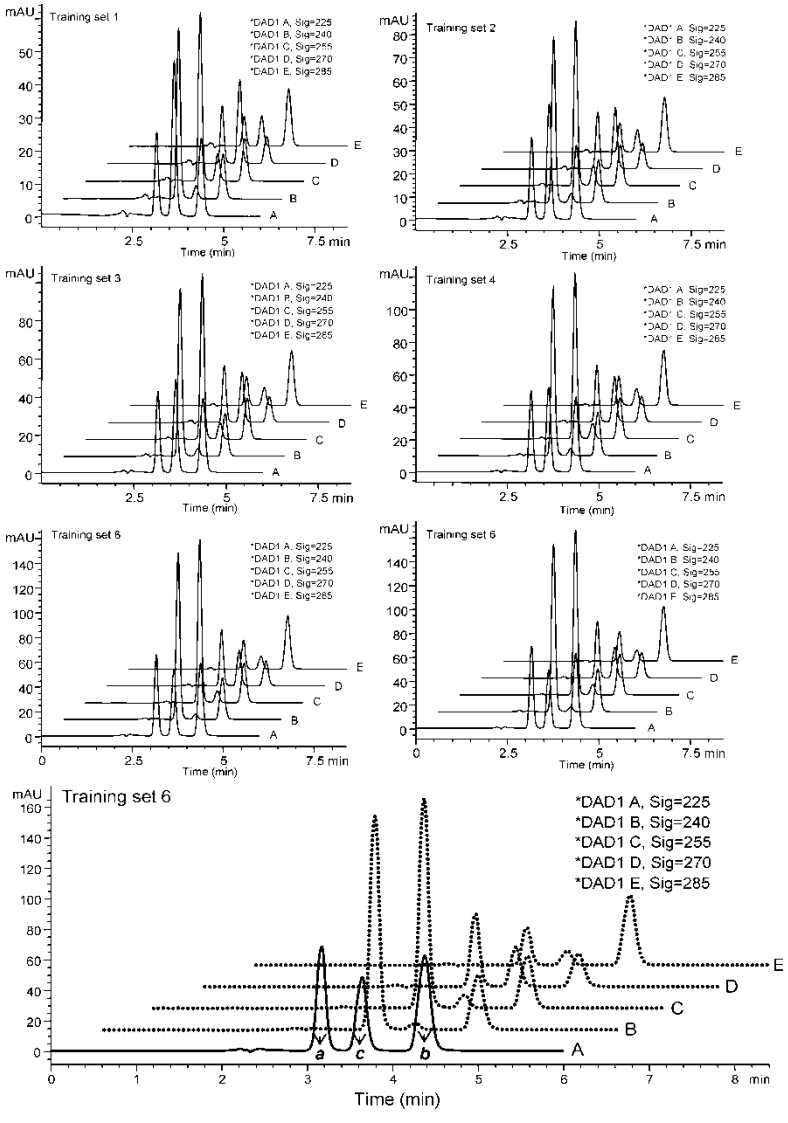

Figure 1. HPLC 3D chromatograms of concentration set 6 containing 8 μg/mL PAR (a) and 8 μg/mL CLX (b) and 25 μg/mL IS (c) at five different wavelengths in tablet analysis. The small chromatograms represent concentrations sets 1–6.

HPLC peak area data set obtained in spiked and unspiked medium. By introducing coefficients (K) into linear equation systems, these calibrations were obtained. The calibrations were applied to the quantitative analysis of PAR and CLX in spiked and unspiked samples. All the data treatment and calculations were done by writing small m-file algorithms in Matlab 7.0 software.

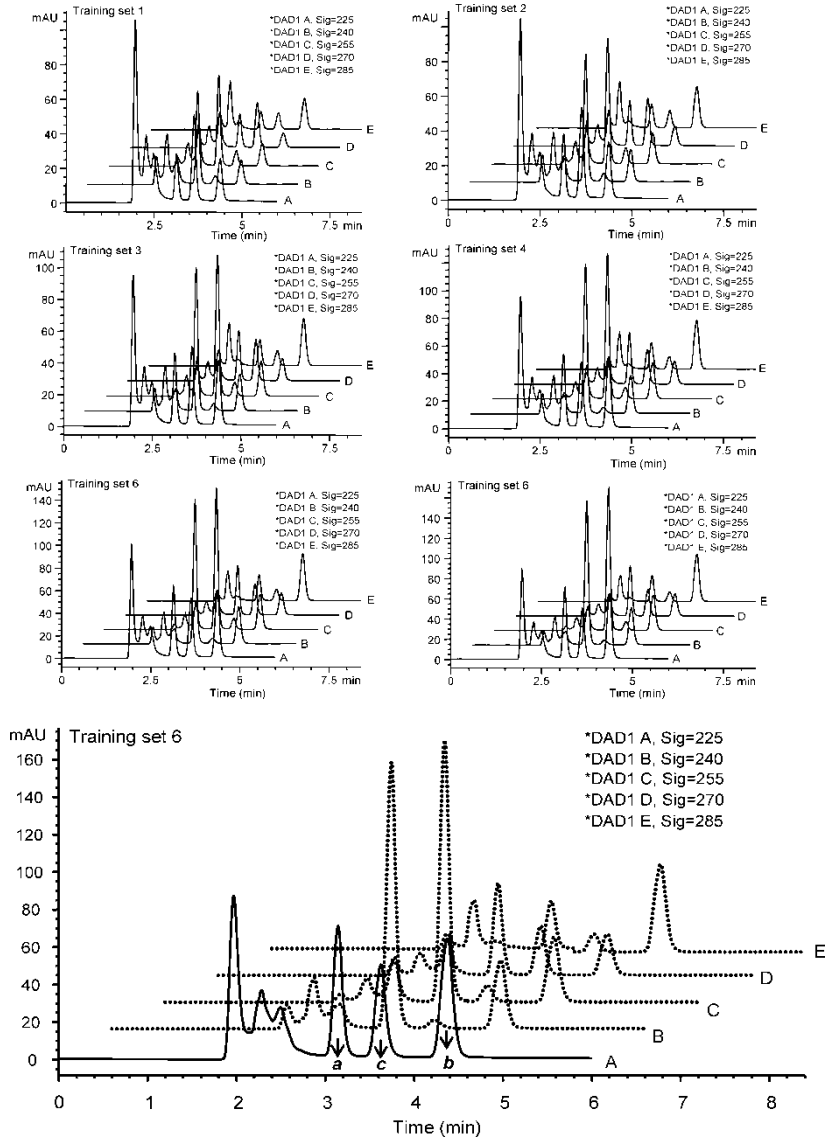

Figure 2. HPLC 3D chromatograms of concentration set 6 containing 8 μg/mL PAR (a) and 8 μg/mL CLX (b) and 25 μg/mL IS (c) at five different wavelengths in spike human plasma medium. The small chromatograms describe concentrations sets 1–6.

Secondly, HPLC-PCR algorithms explained in the methodology section were used to obtain spiked and unspiked calibrations from training set and HPLC peak area data set presented in Tables 1 and 2. The amount of PAR and CLX in spiked and unspiked samples was determined by using the

constructed HPLC-PCR calibrations. For the data treatments and calculations, PLS toolbox 3.5 integrated in Matlab 7.0 was used.

The described HPLC-PLS algorithms were applied to the obtained data sets which contain concentrations versus peak area. The subjected data set was decomposed and then HPLC-PLS calibration was obtained. The obtained calibrations were used to predict the amount of PAR and CLX compounds in spiked and unspiked samples. PLS calculations were done by using toolbox 3.0 integrated in Matlab 7.0.

In the classical HPLC method, the ratio of peak area of analyte to IS was plotted against the concentration of PAR and CLX for their calibration graphs. Tables 1 and 2 indicate the data of the ratio peak area obtained at the five wavelength set 225 (A), 240 (B), 255 (C), 270 (D), and 285 (E). At these wavelength points, HPLC data were used to obtain five straight lines for each drug. Two equations having acceptable regression coefficients and big slope values at 240 nm among the calculated calibration equations, were randomly chosen for analyzed procedures of PAR and CLX (see Tables 3 and 4).

Tables 3 and 4 for both spiked and unspiked randomly spiked medium plasma represent the calculated straight lines and their statistical parameters. The computed correlation coefficients of regression equations are bigger than 0.99. At the assigned wavelength points, the calibration equations give good linearity and satisfactory results for the analysis of PAR and CLX concentrations in samples.

For the tablet analysis, the HPLC method validation was evaluated by applying the standard addition technique for six replicates. The obtained results were shown in Table 5. The standard addition technique proves that no interference comes from the excipients used in the tablet formulations. A similar procedure was repeated in spiked human plasma medium, and the obtained recovery results were presented in Table 5.

Statistical Evaluation

The performance of multivariate HPLC calibrations can be expressed in several ways. One of the most used parameters is the standard error of calibration (SEC) in the calibration step for both spiked and unspiked samples. In this study, six chromatograms for the concentration set with IS were used in calibration steps for both drugs at spiked and unspiked medium. The SEC values of PAR and CLX were calculated by the data obtained from the difference between actual and predicted concentrations in the calibration steps of both drugs. The linear regression analysis and its other statistical results, based on the relationship between actual and predicted concentrations, were obtained. Their statistical results with SEC values were given in Table 6. According to the cross validation procedure, the first two factors for HPLC-PCR and HPLC-PLS were to be found reliable for the prediction of both

Table 3. Regression analysis and its results in tablet analysis

Drug	λ	Regression equation	r	SE (m)	SE (n)	SE (r)	LOD (μg/mL)	LOQ (μg/mL)
PAR	225	$A = 0.1518C_{PAR} - 0.00706$	0.9912	0.0101	0.0582	0.0423	0.80	2.64
	240	$A = 3.9168C_{PAR} - 1.4614$	0.9999	0.0239	0.1380	0.1002	0.25	0.364
	255	$A = 1.6977C_{PAR} + 0.0840$	0.9993	0.0317	0.1825	0.1325	0.32	1.06
	270	$A = 0.1973C_{PAR} - 0.0083$	0.9997	0.0023	0.0134	0.0097	0.20	0.66
	285	$A = 0.2884C_{PAR} - 0.0127$	0.9997	0.0034	0.0198	0.0144	0.21	0.66
CLX	225	$A = 0.1783C_{CLX} + 0.0285$	0.9920	0.0113	0.0652	0.0474	0.75	2.47
	240	$A = 1.1938_{CLX} + 0.2730$	0.9996	0.0172	0.0989	0.0718	0.23	0.76
	255	$A = 0.5385C_{CLX} - 0.1187$	0.9980	0.0173	0.0994	0.0722	0.57	1.87
	270	$A = 0.1171C_{CLX} - 0.0181$	0.9989	0.0027	0.0157	0.0114	0.38	1.27
	285	$A = 0.6804C_{CLX} - 0.1696$	0.9994	0.0122	0.0700	0.0509	0.31	1.02

Table 4. Linear regression analysis and its statistical results in spiked human plasma

Drug	λ	Regression equation	r	SE (m)	SE (n)	SE (r)	LOD (μg/mL)	LOQ (μg/mL)
PAR	225	$A = 0.1449C_{PAR} - 0.0725$	0.9995	0.0022	0.0129	0.0093	0.26	0.88
	240	$A = 2.6697C_{PAR} + 0.6111$	0.9996	0.0373	0.2146	0.0093	0.39	1.10
	255	$A = 1.5947C_{PAR} + 0.3264$	0.9997	0.0203	0.1170	0.0093	0.58	1.91
	270	$A = 1.943C_{PAR} - 0.0774$	0.9992	0.0038	0.0221	0.0093	0.42	1.39
	285	$A = 0.2716C_{PAR} - 0.1305$	0.9993	0.0049	0.0283	0.0093	0.53	1.75
CLX	225	$A = 0.1731C_{CLX} + 0.0232$	0.9962	0.0076	0.0439	0.0093	0.76	2.52
	240	$A = 0.9139_{CLX} + 0.1112$	0.9993	0.0167	0.0963	0.0093	0.49	1.47
	255	$A = 0.5253C_{CLX} - 0.0775$	0.9992	0.0105	0.0605	0.0093	0.98	2.94
	270	$A = 0.1171C_{CLX} - 0.0131$	0.9960	0.0053	0.0306	0.0093	0.39	1.29
	285	$A = 0.6886C_{CLX} - 0.0438$	0.9996	0.0100	0.0574	0.0093	0.57	1.88

Table 5. Recovery result obtained by the standard addition technique in both unspiked and spiked human plasma mediums

	PLS		PCR		CLS		HPLC	
	PAR	CLX	PAR	CLX	PAR	CLX	PAR	CLX
Tablet analysis								
Mean	99.4	98.4	99.4	98.4	99.4	99.4	98.4	100.1
SD	1.13	0.85	1.12	0.69	0.98	0.98	3.35	1.11
RSD	1.14	0.86	1.12	0.70	0.98	0.98	3.41	1.11
SE	0.40	0.30	0.39	0.25	0.35	0.35	1.19	0.39
Spiked human plasma								
Mean	98.1	98.2	98.1	98.1	97.5	97.9	98.8	97.9
SD	0.41	0.27	0.41	0.25	0.39	0.02	0.42	0.70
RSD	0.42	0.28	0.42	0.25	0.40	0.02	0.43	0.72
SE	0.15	0.10	0.15	0.09	0.14	0.01	0.15	0.25

drugs. The above SEC values and other statistical values, correlation coefficient (r), slope (m), and intercept (n) were computed by the HPLC-PCR and HPLC-PLS calibrations using the first two factors.

In a similar way, the standard error of prediction (SEP) and their statistical values were calculated according to the difference between actual and predicted concentrations in the prediction step. The obtained results, SEP, correlation coefficient (r), slope (m), and intercept (n) were presented in Table 6.

All the statistical data indicate that the minimum values of SEC and SEP give us acceptable results under optimized conditions in the calibration and prediction steps.

Method Validation

Linearity

All the methods were tested for linearity by using the HPLC data and training set given in Tables 1 and 2. For the classical HPLC method, linear regression equations were obtained by analyzing a series of different concentrations of each compound. In accordance with the International Conference on Harmonization (ICH),[14] six concentrations between 3 and 8 $\mu g/mL$ for both compounds were used. The regression plots showed that there is a linear dependence of the analytical response for the peak area ratios on the concentration for HPLC methods. Analysis of each concentration was repeated three times. According to the ICH, the limits of detection and quantitation (LOD and LOQ) were calculated from the standard deviation of the response and

Table 6. Statistical results for both tablet analysis and spiked human plasma

Parameter	PLS		PCR		CLS		HPLC	
	PAR	CLX	PAR	CLX	PAR	CLX	PAR	CLX
Tablet analysis								
PRESS	0.0001	0.0017	0.0001	0.0018	0.0089	0.0089	0.0041	0.0120
SEP	0.0134	0.0646	0.0135	0.0609	0.0540	0.0605	0.0227	0.0616
SEC	0.1159	0.2542	0.1164	0.2468	0.2324	0.2460	0.1505	0.2482
R	0.9999	0.9987	0.9999	0.9988	0.9998	0.9989	0.9999	0.9988
N	0.0130	−0.0396	0.0124	−0.0280	0.2143	0.0809	0.0241	0.0376
M	0.9977	1.0081	0.9978	1.0055	0.9614	0.9849	0.9990	0.9953
Spiked human plasma								
PRESS	0.0017	0.0025	0.0017	0.0028	0.0059	0.0041	0.0019	0.0047
SEP	0.0134	0.0646	0.0135	0.0609	0.0540	0.0605	0.0227	0.0616
SEC	0.1159	0.2542	0.1164	0.2468	0.2324	0.2460	0.1505	0.2482
R	0.9999	0.9987	0.9999	0.9988	0.9998	0.9989	0.9999	0.9988
N	0.0130	−0.0396	0.0124	−0.0280	0.2143	0.0809	0.0241	0.0376
M	0.9977	1.0081	0.9978	1.0055	0.9614	0.9849	0.9990	0.9953

the slope of the calibration. Tables 3 and 4 also show the slopes, the intercepts, and the correlation coefficients obtained by the linear least square treatment of the results, along with standard deviation of slopes S(m) and intercept S(r) on the ordinate, and the standard deviation of the residuals S(y/x).

Accuracy and Precision

The accuracy and precision of the methods were tested by analyzing different synthetic mixtures in spiked and unspiked mediums. As shown in Tables 7 and 8, the repeatability and reproducibility of proposed methods is satisfactory as indicated by the low values of S.D. and R.S.D.%, respectively. The specificity of all methods was proven by observing no interference encountered from the excipients of the tablets and the matrix of plasma.

Samples Analysis

Three HPLC chemometric calibrations and a classical HPLC method were successfully applied to the unspiked (commercial tablet analysis) and spiked human plasma samples. Both assay results were summarized in Tables 9 and 10. It was observed that our proposed methods can be used for the

Table 7. Recovery data obtained by applying the proposed methods to the synthetic mixtures for tablet analysis

Added		Recovery (%)							
		PLS		PCR		CLS		HPLC	
PAR	CLX	PAR	CLX	PAR	CLX	PAR	CLX	PAR	CLX
3	5	102.3	99.8	102.3	99.9	97.3	97.8	102.3	98.2
4	5	102.7	100.3	102.8	100.3	103.0	98.4	101.6	101.2
5	5	100.5	101.1	100.6	101.1	99.6	99.6	102.5	101.7
6	5	100.2	100.5	100.2	100.5	100.2	102.0	100.2	101.4
7	5	98.3	100.8	98.3	100.7	98.9	102.4	99.1	102.0
8	5	99.3	98.4	98.0	98.6	96.4	96.2	97.7	96.2
6	3	99.3	100.4	97.6	100.5	97.3	96.1	101.8	101.7
6	4	99.1	102.6	99.2	104.1	99.1	98.7	101.4	100.1
6	5	101.9	102.0	101.9	101.8	102.0	102.4	96.7	97.1
6	6	100.1	100.9	100.1	100.7	100.1	100.1	100.1	97.5
6	7	99.5	98.9	99.5	97.4	95.9	97.9	102.8	101.2
6	8	101.7	98.1	98.5	97.8	96.5	97.3	98.8	101.2
	Mean	100.4	100.3	99.9	100.3	98.9	99.1	100.4	100.0
	SD	1.43	1.35	1.72	1.80	2.27	2.25	1.98	2.11
	RSD	1.42	1.35	1.72	1.79	2.29	2.27	1.98	2.11

Table 8. Recovery data obtained by applying the proposed methods to the synthetic mixtures in spiked human plasma

Added		Recovery (%)							
		PLS		PCR		CLS		HPLC	
PAR	CLX	PAR	CLX	PAR	CLX	PAR	CLX	PAR	CLX
3	5	100.1	100.8	100.4	100.8	103.7	101.0	99.3	101.8
4	5	100.6	100.0	101.3	99.9	102.1	100.1	99.1	99.1
5	5	99.8	98.7	99.8	98.7	100.3	99.1	98.7	99.0
6	5	100.4	98.7	100.4	98.6	100.1	97.0	101.0	98.8
7	5	100.0	99.9	100.9	99.8	99.5	97.7	100.9	98.0
8	5	100.0	99.2	100.5	99.2	99.1	99.4	101.1	99.5
6	3	99.9	100.8	99.9	100.7	99.4	102.3	101.3	100.3
6	4	100.2	98.1	100.2	98.4	100.1	98.7	101.1	100.3
6	5	100.0	101.4	98.3	103.1	99.6	102.6	101.5	103.0
6	6	100.0	100.6	100.0	100.7	99.6	101.1	101.7	101.3
6	7	99.7	99.8	99.7	99.6	99.0	99.0	102.1	99.4
6	8	99.6	100.5	99.6	99.8	99.0	99.2	100.3	100.4
	Mean	100.0	99.9	100.1	100.0	100.1	99.8	100.7	100.1
	SD	0.27	1.02	0.74	1.29	1.39	1.72	1.09	1.41
	RSD	0.27	1.02	0.74	1.29	1.39	1.73	1.08	1.41

determination of CLX and PAR in their unspiked and spiked human plasma samples.

CONCLUSIONS

An HPLC method based on a single wavelength point represents a comparison method for the analysis of samples. In this paper, the HPLC method was considered as a classical HPLC method. As it is known, to find a chromatographic

Table 9. Assay results obtained from the commercial tablet analysis

	PLS		PCR		CLS		HPLC	
	PAR	CLX	PAR	CLX	PAR	CLX	PAR	CLX
Mean	594.7	494.5	591.6	492.2	589.8	493.2	589.9	488.5
SD	3.75	2.48	4.06	3.68	6.85	4.39	5.61	4.19
RSD	0.63	0.50	0.69	0.75	1.16	0.89	0.95	0.86
SE	1.53	1.01	1.66	1.50	2.80	1.79	2.29	1.71
CL (0.05)	3.00	1.98	3.25	2.95	5.48	3.51	4.49	3.35

Label claimed: Parafon® tablet, 600 mg PAR and 500 CLX mg per tablet.

Table 10. Assay results obtained from spiked human plasma

	PLS		PCR		CLS		HPLC	
	PAR	CLX	PAR	CLX	PAR	CLX	PAR	CLX
Mean	5.98	4.96	5.95	4.93	5.92	4.91	5.98	4.95
SD	0.07	0.02	0.10	0.04	0.13	0.08	0.08	0.06
RSD	1.19	0.31	1.69	0.80	2.15	1.71	1.39	1.29
SE	0.03	0.01	0.04	0.01	0.05	0.03	0.03	0.02
CL (0.05)	0.05	0.01	0.07	0.03	0.09	0.06	0.06	0.04

condition and optimization isn't possible in every case. For this reason, the HPLC chemometric calibration technique has an important role for the evaluation of multichromatograms at the multiwavelengths using PDA responses.

In our HPLC chemometric study, HPLC-CLS, HPLC-PCR, and HPLC-PLS calibration models were proposed for the simultaneous prediction of unknown concentrations of compounds in samples. A good agreement was observed for the results of the HPLC chemometric approaches. This new application of the chemometric calibration technique to the HPLC data set is an alternative model for the minimization or reduction of the experimental errors in chromatographic analysis.

The HPLC chemometric calibration techniques can be successfully applied to the quantitative analysis of the subject compounds both spiked and unspiked samples.

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