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Simultaneous spectrophotometric determination of carbamazepine and phenytoin in serum by PLS regression and comparison with HPLC

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Abstract

Carbamazepine (CBZ) and phenytoin (PHT) are two antiepileptic drugs which are used simultaneously. In this paper a partial least-squares (PLS) calibration method is described for the simultaneous spectrophotometric determination of CBZ and PHT in plasma. Standard binary mixtures of CBZ and PHT have been resolved by application of PLS-1 to their UV spectra. Then, the binary standard solutions, spiked to plasma, were prepared and after the extraction of the drugs, their corresponding UV spectrum were analyzed by PLS regression to calculate the concentration of drugs in unknown plasma. A leave one out cross-validation procedure was employed to find the optimum numbers of latent variables using PRESS. A HPLC method was also applied for simultaneous determination of two drugs in the plasma and in methanol. The mean recoveries obtained by PLS were 98.4 and 98.2 for CBZ and PHT and those obtained by HPLC were 100.1 and 101.7, respectively. Although, the HPLC method showed better performance than PLS, it was found that the results obtained by PLS were comparable with those obtained by HPLC method.

Keywords: Carbamazepine; Phenytoin; PLS; Plasma; HPLC; Spectrophotometry

1. Introduction

Carbamazepine (CBZ) and phenytoin (PHT) (Fig. 1) are widely used in the treatment of epilepsy. CBZ is an iminostiblene derivative which is widely used in the treatment of epilepsy, frequently in combination with other anticonvulsants [1]. The pharmacokinetic characteristics of CBZ are complex; there is no simple relationship between the dose of CBZ and concentration of the drug in plasma. Therapeutic concentrations are reported to be 6–12 μg ml^{-1} although considerable variation occurs [2]. In several publications the therapeutic concentration for CBZ has been estimated at 4–12 μg ml^{-1} [3]. Factors such as age and simultaneous use of other drugs have been suggested to cause change in the plasma concentration of this drug [4].

PHT is effective against all types of seizures. No drug has a greater need for therapeutic drug concentration monitoring and individualized dosing than PHT. A good correlation usually is observed between the total concentration of PHT in plasma and the clinical effect. Therapeutic concentration of PHT is above 10 μg ml^{-1} [2]. Enzyme induction by phenytoin is well documented, even auto-induction by phenytoin should be considered during the treatment with phenytoin [5].

To minimize toxicity, monitoring of plasma anticonvulsant levels is a part of the routine management of patient in many clinics. To the best of our knowledge, almost all of the methods that were applied for determination of antiepileptic drugs in biological media are often chromatography, electrophoresis and immunoassay techniques [6–10]. In spite of the suitability of the proposed methods, these methods employ expensive instruments or materials, and need extra pure solvents which are hazardous material (i.e. HPLC method). In addition, chromatographic methods need a suitable compound as internal standard, which make the analytical procedure more complex. The analysis of the clinical samples demands simple and fast analytical methods and therefore, finding an alternative analytical technique is crucial.

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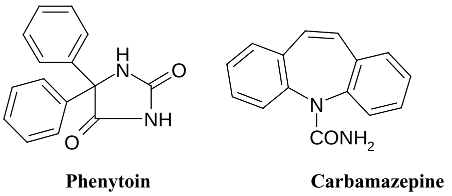

Fig. 1. Chemical structure of the drugs used in this study.

Spectrophotometry is a simple analytical method for quantitative analysis. However, this method is not selective and needs that the analytes present in a given sample have different spectrum with low overlapping. On the other hand, the introduction of the computer and statistical science in the chemistry help chemist to solve analytical problems for complex samples. Chemometrics, which is the result of the marriage of chemistry with computer, enable chemist to resolve the constituents of a complex system without need to prior separation step [11–13]. Hence, multivariate calibration methods are applied to the spectroscopic as well as the electrochemical data for simultaneous determination of several species in a given sample even in the presence of highly overlapping spectra or voltamograms [14–17].

Principal component regression (PCR) and partial least-squares (PLS) are two full spectrum multivariate calibration methods that have received considerable attention in the chemometrics literature [18–21]. It was found that these factor analysis [22] based methods can overcome spectral overlapping, while the PLS has a better performance than PCR. The use of PLS method for chemical applications was initiated by Wold [23] and the number of literature references have been extensively increasing in recent years [24–30]. PLS regression as a full spectrum multivariate calibration method requires that only the concentration of the analyte of interest should be known in the calibration samples and knowing the concentration of other analytes are not necessary. PLS represents by two modifications, known as PLS-1 and PLS-2. The former performs the decomposition and regression for only one component at a time whereas the latter calculates latent variables based on all of the concentrations simultaneously and only one calibration matrix is necessary. In a past few years, PLS has been frequently used in the spectrophotometric-multicomponent analysis of drugs in the biological samples [31–34] as well as the pharmaceutical formulations [35–38]. For example, Goicoechea and Olivieri reported the simultaneous determination of phenobarbital and phenytoin in tablet preparations using PLS regression [39]. We have recently reported the application of PLS regression to quantitative structure activity/property relationships (QSAR/QSPR) [40,41] and simultaneous determination of metal cations [42], phenothiazine derivatives [43] and potentiometric multicomponent titration of organic acids [44].

In this study, we present a simple spectrophotometric method for simultaneous determination of CBZ and PHT in plasma using PLS regression. According to the best of our knowledge, the simultaneous determination of these drugs by PLS regression has not been reported. An extraction procedure was used to extract both drugs from the plasma. In order to evaluate the results obtained by PLS regression, a HPLC methods was also employed and the results of two methods were compared.

2. Experimental

2.1. Reagents

Phenytoin, carbamazepine, benzimidazole, methanol (HPLC grade), dichloromethane and acetone were taken from Merck. Acetonitrile (HPLC grade) was prepared from Fluka. Stock solutions of both drugs were prepared as 1000 $\mu g\,ml^{-1}$ in methanol.

2.2. Apparatus and software

The UV absorbance data was recorded by a Shimadzu (UV-1650 PC) UV–vis spectrophotometer combined with a 10 mm quartz cell. The chromatographic system consisted of Shimadzu LC-10AD, SCL-10A (system controller) and a SPD-10V UV–vis detector.

A Pentium IV personal computer with Windows XP operating system was used. A home-made program written in MATLAB (Math work Inc.) was used for PLS modeling and the other statistical analysis.

2.3. Procedures

2.3.1. One component calibration

In order to find the linear dynamic concentration range of each drug, one component calibration was performed. Different volumes of a 50 $\mu g\,ml^{-1}$ solution of each drug were added into different 5.0-ml volumetric flasks and diluted to the mark with methanol. The absorbance spectra was recorded over the 190–300 nm spectral range versus a solvent blank. The linear dynamic range for each drug was determined by plotting the absorbance at its λ_{max} (284 nm for CBZ and 210 nm for PHT) versus sample concentration.

2.3.2. PLS determination of drugs in pure solvent

Two sets of standard solutions were prepared. The calibration set contained 12 standard solutions and the validation set contained 6 standard solutions, so that the concentrations of each drug in the resulting solutions were in its own linear dynamic range. The composition of calibration set was chosen by orthogonal array design and the composition of validation set was randomly chosen. For preparation of each solution, the required volumes of its standard solution (50 $\mu g\,ml^{-1}$) were added into a 5.0-ml volumetric flask and diluted to the mark with methanol. The absorption spectra of the mixtures were then recorded versus a solvent blank between 190 and 300 nm.

The digitized absorbance data, at 1.0 nm intervals, were collected in a data matrix whose rows were the absorbance data of a specified solution and the concentration of drugs were collected in two different column vectors. The PLS regression was run for each drug separately (PLS-1). The PLS model was build using the calibration data. Leave one out cross-validation procedure was used to obtain the optimum number of latent variables using PRESS and Fisher *F*-ratio [45]. The concentrations of drugs in validation set were calculated at the optimum number of factors using the designed PLS model. The singular value decomposition-based PLS algorithm was used here [41–43,46].

A Pentium IV personal computer with Windows XP operating system was used. A home-made PLS algorithm, written in MATLAB version 5.0 (Mathwork Inc.), was used. This program performed PLS based on SVD and other statistical analysis.

2.3.3. *PLS determination of drugs in plasma*

For determination of drugs in the plasma, an extraction procedure [9] was used to extract the drugs from plasma, before recording the absorbance spectra. For this purpose, two set of standard solutions (calibration 12 samples and validation 6 samples) were prepared. For preparation of each solution, into a 5.0-ml volumetric flask were added the required volumes of standard solution (50 μg ml^{-1}) of each drugs and 1.0 ml of the plasma solution. The resulted solutions were diluted to the mark with distilled water. Resulted solution (2.0 ml) was mixed with 5.0 ml dichloromethane, shaked for 5.0 min and after that centrifuged at 3000 min^{-1} for 30.0 min. The organic phase was separated from the aqueous phase and another 5.0 ml of dichloromethane was added to the aqueous phase and the extraction procedure was repeated. The resulted organic phases were mixed and their solvent was evaporated under the nitrogen gas. The residue was dissolved in 2.0 ml methanol. The same extraction procedure was applied for blank. The absorbance spectrum of each resulted solutions was recorded against the blank. The PLS modeling was the same as that discussed in the previous section.

2.3.4. *HPLC method*

The HPLC method was also employed for analysis of the mixtures of CBZ and PHT in the pure solvent and in plasma. The procedures for preparation of the solutions and extraction of drugs from plasma were the same as those discussed for PLS except that after the preparation of the solutions, a 20 μl aliquot of the samples was injected into HPLC column. The chromatographic condition was chosen according to the procedure discussed by Mei and Williams [9]. An 1100 PSI with C18 column and a mobile phase comprising acetonitrile/methanol/water (20/50/30) at a flow rate of 1.0 ml min^{-1} were operated. The absorbance measurement was performed at 214 nm for both drugs.

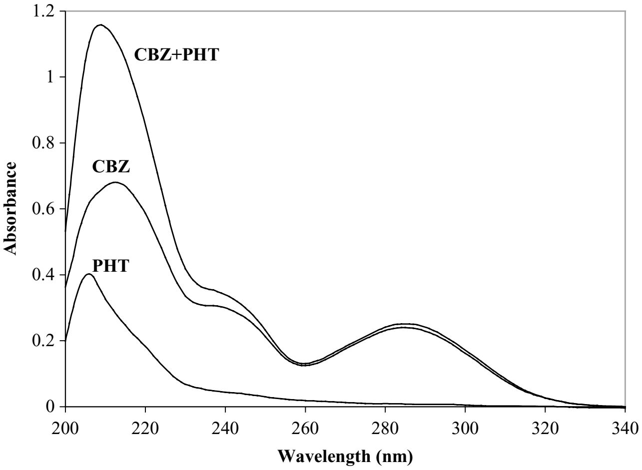

Fig. 2. The UV absorbance spectra of 5 μg ml^{-1} of each one of the drugs and their mixtures.

3. Results and discussion

Fig. 2 shows the electronic absorbance spectra of the methanolic solutions of both studied compounds and their equimolar mixture. As can be seen, the PHT spectrum significantly overlapped with the spectrum of CBZ. Thus, these compounds can not be determined in the presence of each other by a simple calibration procedure.

3.1. *PLS modeling*

Before application of the PLS for simultaneous determination of CBZ and PHT in plasma, this method was applied to analysis of these compounds in their binary mixtures in the absence of serum. In each case, two set of standard solutions were prepared. The calibration set contained 12 standard mixtures and 6 mixtures were used in the validation set. The concentrations of drugs in the calibration and validation sets, which were in their linear dynamic range (0.40–30.00 μg ml^{-1} for CBZ and 0.65–50.00 μg ml^{-1} for PHT), are represented in Tables 1 and 2, respectively.

The PLS model was developed in the PLS-1 mode (i.e. PLS was run for each drug separately). Meanwhile, PLS-1 was run for each drug two times; one in the pure solvent and the second in the presence of plasma. In order to select the number of factors, a cross-validation procedure, leaving out one (LOO) sample at a time, was used [47]. The absorbance data was auto-scaled before any PLS modeling. Given the set of 12 calibration spectra, the PLS-1 calibration was performed on 11 calibration spectra and, using this calibration, the concentration of the compound in the sample left out during calibration was predicted. This process was repeated 12 times until each calibration sample had been left out once. Then, the predicted concentrations were compared with the known concentrations of the reference sample and the prediction error sum of squares (PRESS) of LOO was calculated (PRESS$_{\mathrm{LOO}}$). The PRESS$_{\mathrm{LOO}}$ was calculated in

Table 1
The composition of the calibration sets mixtures for simultaneous determination of drugs in the pure solvent and plasma[a]

Sample no.	Pure solvent		Plasma	
	PHT	CBZ	PHT	CBZ
1	8.0	8.0	12.0	2.5
2	8.0	2.5	4.0	15.0
3	20.0	5.0	6.0	10.0
4	5.0	20.0	8.0	6.0
5	20.0	8.0	10.0	4.0
6	20.0	2.5	2.5	8.0
7	8.0	5.0	12.0	5.0
8	5.0	8.0	2.5	12.0
9	20.0	20.0	4.0	10.0
10	2.5	20.0	10.0	3.0
11	5.0	2.5	7.5	4.0
12	2.5	2.5	6.0	8.0

[a] The concentrations are in μg ml^{-1}.

the same manner each time a new factor was added to the PLS-1 model. In addition, the concentration of the drugs in the validation set and consequently the PRESS of validation ($PRESS_{VAL}$) were calculated in the presence of different added factors. To select the optimum number of factors, the criterion of Halland and Thomas [45] was used by monitoring the $PRESS_{LOO}$ and $PRESS_{VAL}$. It was found that for each drug 3 latent variables was needed to find the optimum results for determination of drugs in pure solvent. The first three PLS loadings of drugs are plotted in Fig. 3. As it is observed, the first loadings of both drugs are the same, and the major differences are between the second and third loadings. The profile of the first loadings is relatively the same as the absorbance spectra of the mixture of CBZ and PHT (Fig. 2). The second as well as the third loadings of the CBZ and PHT have reverse directions (i.e. those of CBZ and PHT are orthogonal). The third loading of CBZ has positive values at the wavelength intervals greater than 220 nm, whereas that of PHT has negative values at these regions. Besides, In contrast to the positive values of the third loading of the PHT at wavelength intervals lower than 220 nm, CBZs third loading has negative values. These observations may be due to the differences between spectra of the two drugs at the specified wavelength regions, as shown in Fig. 2.

Table 2
The composition of the validation sets mixtures for simultaneous determination of drugs in the pure solvent and plasma[a]

Sample no.	Pure solvent		Plasma	
	PHT	CBZ	PHT	CBZ
1	10.0	10.0	8.0	4.0
2	12.0	3.0	6.0	8.0
3	2.0	12.0	10.0	3.0
4	5.0	5.0	4.0	10.0
5	2.5	5.0	12.0	5.0
6	8.0	15.0	3.0	12.0

[a] The concentrations are in μg ml^{-1}.

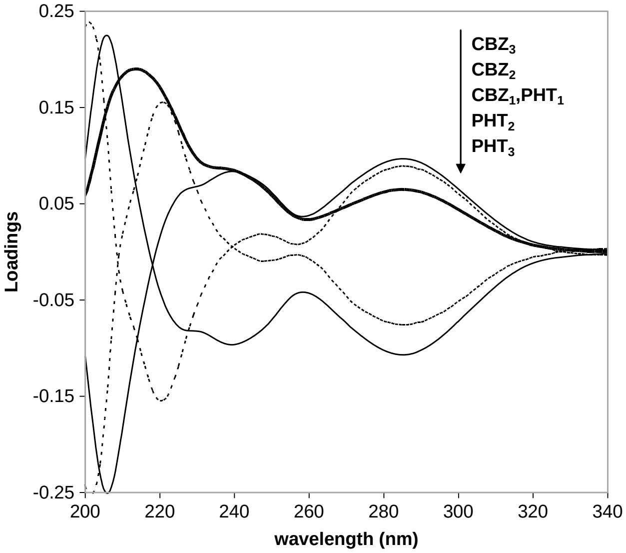

Fig. 3. Plots of the first three PLS loadings for the drugs in the pure solvent.

The predicted concentrations of drugs at optimum number of factors are listed in Table 3 for validation samples. The percent of recovery are also represented in this table. As it is observed, there is a good agreement between the calculated and actual concentrations of drugs. The respective mean recovery for the PHT and CBZ is 103.0 and 101.2, which confirm the high prediction power of the resulted PLS models.

In order to analyze the drugs in the plasma, separate PLS calibrations were made. For this purpose, both the calibration and validation samples were prepared in the plasma solution and the drugs were separated from the plasma by an extraction procedure. The absorbance spectra of the extracts were subjected to PLS regression by the same manner as discussed in the previous paragraphs. The optimum numbers of factors, which obtained for the simultaneous determination of drugs in the plasma, were 3 and 4 for CBZ and PHT, respectively. Because of the complexity of the samples in the plasma, the numbers of factors used were more than that found in the pure solvent. The predicted concentrations and the respected percent of recovery of the drugs in plasma are listed in Table 3. The data supports the success of the proposed PLS models for simultaneous determination of CBZ and PHT. The mean recoveries were 98.2 and 98.4 for PHT and CBZ, respectively.

There are different approaches for determination of the detection limits (DL) in the multivariate calibration methods [42,48–51]. In the present study, DL was calculated by the net analyte signal calculation [51] method, proposed by Lorber [50]. The calculated DLs for PHT and CBZ in pure solvent were 0.73 and 0.58 μg ml^{-1} and in plasma were 0.89 and 0.84 μg ml^{-1}, respectively. The reproducibility of the proposed PLS regression models was examined by using five replicate measurements on the validation samples at the first, second and third days and first and second weeks. The

Table 3
The predicted concentrations (Pred) of drugs in the validation sets and the corresponding percent of recoveries (%Rec) obtained by PLS in two media[a,b]

No.[c]	Pure solvent				Plasma			
	PHT		CBZ		PHT		CBZ	
	Pred	%Rec	Pred	%Rec	Pred	%Rec	Pred	%Rec
1	10.02 (±0.19)	100.2	10.09 (±0.31)	100.9	8.30 (±0.47)	103.7	3.78 (±0.27)	94.5
2	13.00 (±0.19)	108.3	2.85 (±0.27)	95.0	5.63 (±0.41)	93.8	7.91 (±0.46)	98.9
3	2.10 (±0.22)	105.0	12.11 (±0.28)	100.9	9.62 (±0.70)	96.2	3.16 (±0.17)	105.3
4	5.06 (±0.19)	101.2	5.08 (±0.25)	101.6	4.28 (±0.16)	107.0	9.26 (±0.44)	92.6
5	2.70 (±0.21)	108.0	5.10 (±0.23)	102.0	11.33 (±0.81)	94.4	5.19 (±0.42)	103.8
6	7.63 (±0.24)	95.37	16.07 (±0.23)	107.3	2.82 (±0.21)	94.0	11.43 (±0.75)	95.3
Mean		103.0		101.2		98.2		98.4

[a] The concentrations are in $\mu g\,ml^{-1}$.
[b] The values in the parentheses are the standard deviation of five replicates at first, second and third days and first and second weeks.
[c] The numbers refer to the samples of Table 2.

data are included in Table 3. The results indicate the good reproducibility of the results. The relative standard deviations are varied between 1.5 for concentrated solutions and 8.5 for diluted solutions.

3.2. Analysis of the synthetic mixtures

It should be noted that because of the complexity of the human serum and the probability of the contamination of the plasma with some absorbing species, the multivariate calibration methods can not be used for analysis of the real human serum at this time and further development are necessary. Almost all of the proposed multivariate calibration methods for simultaneous determination of drugs in biological fluids used synthetic spiked sample solutions for validation of the methods [31–34,52–54]. Thus, in order to complete our investigation around the performance of the optimized model, the proposed PLS models were used to determine the concentration of CBZ and PHT in some synthetic mixtures (prediction set). The results are shown in Table 4. Obviously, the PLS method could determine the concentrations of the spiked PHT and CBZ in the plasma, accurately.

3.3. Comparison of the results of PLS with HPLC

In order to evaluate the results of the PLS regression, a HPLC procedure was also employed to analyze these drugs in their binary mixtures. Previously, Mei and Williams reported the simultaneous determination of CBZ and PHT in hair samples [9]. We used the same procedure in this study, except that benzimidazole was used as internal standard. Accordingly, the composition of the mobile phase was varied to resolve the chromatogram of benzimidazole from that of drugs. The mobile phase solvent comprising acetonitrile/methanol/water with the volume ratio of 20/50/30 produced better separation of the chromatograms. At the optimized conditions, the retention times for benzimidazole, CBZ and PHT were 4.70, 9.20 and 10.3 min, respectively. One component analysis showed that the chromatographic responses were linear in the concentration ranges, which were used in the PLS procedure.

Two sets of standard solutions were also used in HPLC experiment. The concentrations of drugs in the mixture were the same those used in PLS method (Tables 1 and 2). The calibration set was used to build the calibration curve for each drug separately, and the resulted calibration curve was used to predict the concentration of the CBZ

Table 4
The result of the analysis of the synthetic mixtures (prediction set) spiked in the plasma[a]

No.	Composition		Predicted by PLS				Predicted by HPLC			
	CBZ	PHT	CBZ		PHT		CBZ		PHT	
			Pred	%Rec	Pred	%Rec	Pred	%Rec	Pred	%Rec
1	15.0	16.0	14.5	96.7	17.3	108.1	15.3	102.0	16.3	103.1
2	14.0	8.0	14.3	102.1	8.3	103.8	14.4	102.9	7.8	96.3
3	6.0	5.0	5.7	95.0	5.2	104.0	6.2	103.3	5.1	104.0
4	2.0	10.0	2.2	110.0	10.5	105.0	2.0	98.9	9.9	97.0
5	16.0	4.0	15.3	95.6	3.75	93.8	15.6	97.5	3.9	95.0
6	5.0	18.0	5.25	105.0	17.4	96.7	5.1	102.0	18.2	103.3

[a] The concentrations are in $\mu g\,ml^{-1}$.

Table 5
The predicted concentrations (Pred) of drugs in the validation sets and the corresponding percent of recoveries (%Rec) obtained by HPLC in two media[a,b]

No.[c]	Pure solvent				Plasma			
	PHT		CBZ		PHT		CBZ	
	Pred	%Rec	Pred	%Rec	Pred	%Rec	Pred	%Rec
1	9.83 (±0.23)	98.3	10.08 (±0.32)	100.8	7.95 (±0.50)	99.4	3.92 (±0.57)	98.0
2	12.23 (±0.14)	102.0	3.01 (±0.37)	100.3	6.20 (±0.31)	103.3	8.40 (±0.54)	105.0
3	2.06 (±0.14)	103.0	11.90 (±0.31)	99.2	9.94 (±0.38)	99.4	2.96 (±0.21)	98.7
4	5.09 (±0.11)	101.8	5.06 (±0.34)	101.2	4.08 (±0.20)	102.0	10.02 (±0.38)	100.2
5	2.43 (±0.21)	97.2	5.19 (±0.23)	103.8	11.94 (±0.41)	99.5	4.95 (±0.46)	99.0
6	8.01 (±0.24)	100.1	14.98 (±0.25)	99.8	3.20 (±0.31)	106.7	11.93 (±0.32)	99.4
Mean		103.0		100.9		101.7		100.04

[a] The concentrations are in $\mu g\,ml^{-1}$.

[b] The values in the parentheses are the standard deviation of three replicates at first, second and third days.

[c] The numbers refer to the samples of Table 2.

and PHT in the validation set. The results are summarized in Table 5, which confirm the high prediction ability of the employed HPLC method. The reproducibility of the HPLC was nearly the same as that of PLS. However, the calculated theoretical DL of HPLC (0.42 and 0.51 $\mu g\,ml^{-1}$ in pure solvent and 0.49 and 0.61 $\mu g\,ml^{-1}$ in plasma for CBZ and PHT, respectively) was lower than those of PLS.

The statistical parameters for the resulted PLS models and the HPLC method are summarized in Table 6. In this table, the PRESS values for LOO cross-validation of the calibration set and PRESS of validation set are included. In addition, relative error of calibration, validation and prediction sets (REC, REV and REP, respectively) and root mean square errors of calibration, validation and prediction (RMSC, RMSV and RMSP, respectively) are shown in Table 6. The data supports that the prediction ability of the HPLC method is higher than that of the PLS procedure, but, the differences are not very significant. In order to evaluate the results of the PLS regression method, they were compared with the results of HPLC as the reference method. To do this, the predicted values of concentrations by PLS were plotted against the calculated value by HPLC for the validation set (Fig. 4). In an ideal case, these types of plots must give a straight line with respective slope and intercept equal to 1 and zero. As it is obvious from the plots shown in Fig. 4, there is a good agreement between the predicted values of concentrations by PLS and HPLC methods. Meanwhile, this agreement is higher in the case of pure solvent. In addition, the student *t*-test indicated that there is no significant differences between the results of PLS and HPLC. Therefore, it can be concluded that PLS method give results that are very close to HPLC method. The simplicity of the PLS procedure in combination with its lowest cost are the great advantages of this chemometrics methods for simultaneous determination of the anticonvulsant drugs in comparison with the chromatographic or electrophoresis methods. In the other hand, the more accurate results obtained by

Table 6
The statistical parameters obtained by the PLS and HPLC methods

Parameter	PLS				HPLC			
	Pure solvent		Plasma		Pure solvent		Plasma	
	PHT	CBZ	PHT	CBZ	PHT	CBZ	PHT	CBZ
f	3	3	4	3	–	–	–	–
$\mathrm{PRESS_{LOO}}$	1.832	1.774	2.017	2.011	–	–	–	–
$\mathrm{PRESS_{VAL}}$	0.931	0.997	1.191	1.204	0.360	0.378	0.495	0.403
R^2	0.991	0.997	0.990	0.995	0.999	0.999	0.999	0.998
REC	0.321	0.211	0.204	0.250	0.123	0.107	0.121	0.132
REV	0.347	0.228	0.252	0.276	0.129	0.115	0.127	0.149
REP	0.369	0.265	0.375	0.317	0.202	0.123	0.129	0.151
RMSC	0.417	0.398	0.346	0.351	0.127	0.194	0.111	0.154
RMSV	0.451	0.429	0.389	0.380	0.148	0.198	0.127	0.185
RMSP	0.492	0.433	0.554	0.431	0.149	0.204	0.179	0.282

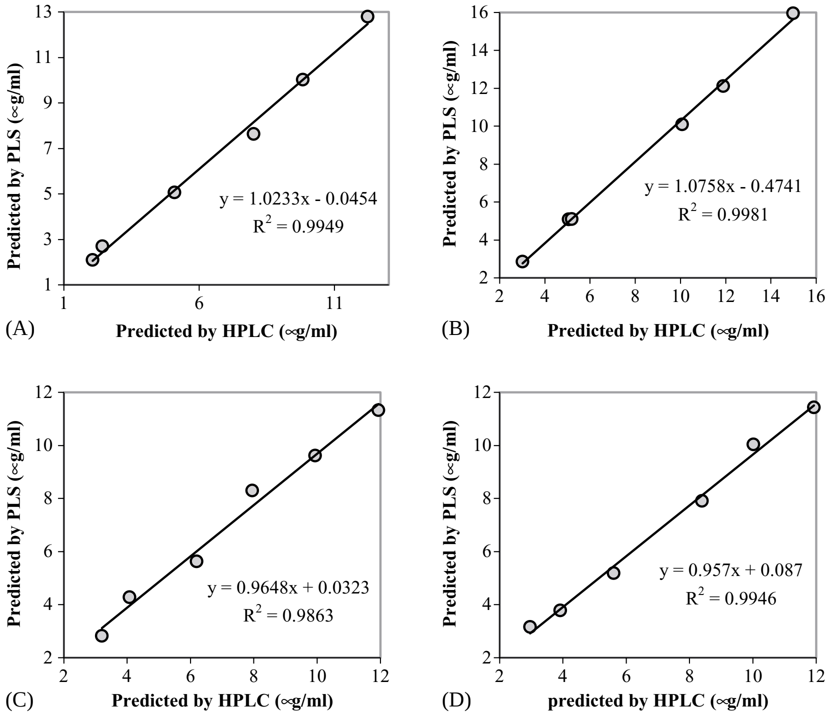

Fig. 4. Plot of the predicted concentrations by PLS procedures against those predicted by HPLC: (A) PHT in pure solvent; (B) CBZ in pure solvent; (C) PHT in serum; (D) CBZ in serum.

HPLC method indicates the advantage of this technique over PLS.

4. Conclusion

A partial least-square regression was employed to analyze the binary mixtures of carbamazepine and phenytoin in the spiked plasma using their UV absorbance data. It was found that the proposed method, after extraction of the drugs from serum by a suitable method, could predict the concentration of drugs with average percent of recovery equal to 98.4 and 98.2 for CBZ and PHT, respectively. In order to evaluate the results obtained by this method, a HPLC procedure was also used. The results revealed that the HPLC method produced better results than PLS, however, the difference were not significant. Plot of the predicted values by PLS methods against the predicted values by HPLC procedure produced a straight line with the slope equal to 0.957 and 0.986 and intercept equal to 0.087 and 0.032 for CBZ and PHT, respectively. This indicates the closeness of the results of PLS to HPLC. However, because of the complexity of the human plasma, we do not advise the application of this PLS method for the analysis of the clinical samples at this moment and therefore, further development will be achieved. In the next, we will perform multivariate calibration methods for simultaneous determination of the drugs and their metabolites. Meanwhile, efforts will be made to remove the plasma variability from the absorbance data of mixtures using net analyte signal calculations.

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