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Chemometrics-assisted simple UV-spectroscopic determination of carbamazepine in human serum and comparison with reference methods

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Abstract

In the present report, carbamazepine is determined on serum samples of real patients by a procedure completely assisted by chemometric tools. First, a response surface methodology based on a mixture design was applied in order to select the best conditions for the extraction step. Finally, partial least squares multivariate calibration (PLS-1) was applied to second-derivative UV spectra, eliminating a shift baseline effect that originated in the extraction procedure. The performance assessment included: (a) a three-level precision study, (b) a recovery study analyzing spiked samples, and (c) a method comparison with high-performance liquid chromatography (HPLC) and fluorescence polarization immunoassay (FPIA) applied on real patient samples. The obtained results show the potentiality of the presently studied methodology for the monitoring of patients treated with this anticonvulsant.

Keywords: Carbamazepine; Serum samples; Chemometrics; Partial least squares regression; Systematic optimization

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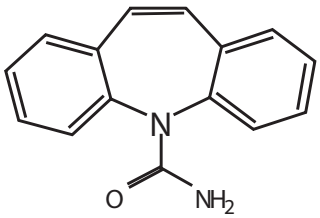
1. Introduction

Carbamazepine (CBZ) is an extensively used drug for the management of epilepsy and psychiatric diseases [1]. It is almost completely metabolized in the body and only small traces are excreted unchanged in urine [1]. CBZ is commonly used in combination with other antiepileptic drugs such as phenytoin or valproic acid, and its determination is very important for minimizing the toxicity in the treatment of patients. Therapeutic concentrations have been reported to be 6–12 μg ml^{-1}, although considerable variations may arise [1]. Its main metabolite, carbamazepine epoxide (CBZ-EP), also possesses pharmacological activity as anticonvulsant [2], though it reaches lower concentrations than CBZ.



Chemical structure of carbamazepine (CBZ)

Several methods have been proposed in the literature for the determination of CBZ, in particular those using chromatography [3–9]. High-performance liquid chromatography (HPLC) and fluorescence polarization immunoassay (FPIA) are usually employed as routine techniques for the determination of this and other anticonvulsants [10]. Other techniques such as micellar electrokinetic capillary chromatography (MECC) [11] and chemiluminescence [12] were used . Very recently, two approaches were presented in the literature. One of them uses spectrophotometry and multivariate calibration [13] for the simultaneous determination of CBZ and phenytoin. Very good results were obtained, although a tedious sample pre-treatment is needed and no real patient samples were analyzed when validating the method. The other method exploits the unusual fluorescence of the CBZ on a nylon membrane, while second-order excitation–emission data were modelled with parallel factor analysis (PARAFAC) [14]. Excellent results were obtained in the latter case.

Among the linear regression methods that have been proposed for multicomponent analysis, partial least squares (PLS) has become the most popular and the standard for multivariate calibration because of the quality of the calibration models, the ease of implementation and the availability of software [15–22]. PLS shows the advantage of using full spectra, which is critical for the spectroscopic resolution of complex mixtures of analytes. It allows for a rapid determination of components, usually with no need for prior separation. Additionally, when applying PLS, calibration can be performed by ignoring the concentrations of all other components except the analyte of interest. This fact may result especially significant when the analyte is immersed in a complex matrix such as serum.

In addition, chemometrics assists in the development of analytical methods through experimental design and systematic optimization [23]. The optimization of the extraction step of any analyte is critical in order to assure high recoveries. Mixture design associated

with response surface methodology is an excellent tool for successfully carrying out this step [24].

In the present report, CBZ is determined on real patient serum samples by using a procedure completely assisted by chemometric tools. First, a response surface methodology was applied based on a mixture design, in order to select the best conditions in the extraction step. Finally, multivariate calibration PLS-1 was applied to second derivative UV spectra, eliminating a shift baseline effect originated in the extraction procedure. A complete performance assessment showed the potentiality of the presently studied methodology for the monitoring of patients treated with this anticonvulsant.

2. Methods and materials

2.1. Apparatus

Absorbance measurements were carried out on a Perkin Elmer Lambda 20 spectrophotometer, using 1.00 cm quartz cells, 2 nm of slit width, a scan speed of 860 nm min^{-1} and a wavelength interval of 1.0 nm. UV spectra of working solutions were recorded in the range 280–350 nm. All spectra were saved in ASCII format, and transferred to a PC Athlon 2.2 microcomputer for subsequent manipulation. PLS-1 was implemented using the MVC1 MATLAB toolbox [21]. Design_Expert 6.0.10 was used for experimental design and optimization [25]. Derivative spectra were calculated with a Savitzky–Golay filter using a second-order polynomial and an 11-point window. HPLC was carried out on a liquid chromatograph Lachrom-Hitachi equipped with a detector UV L 7400.

2.2. Reference methods

With the purpose of validating the developed method, CBZ was determined by both HPLC [6–9] and by FPIA [10]. Chromatographic separation was performed on a LiChrocart® 4-4, RP-18, 5 μm pre-column, and a LiChrocart® 125-4, RP-18, 5 μm column, both at ambient temperature. The mobile phase consisted of a filtered and degassified mixture of methanol, water, acetonitrile (25/65/10). The analysis was done under isocratic conditions at a flow rate of 1 ml min^{-1} and the effluent was monitored by UV measurements at 214 nm. An internal standard was used. It was prepared by adding 0.30 ml of a solution 10 μg l^{-1} of methylcarbamazepine in acetonitrile to 0.20 ml of sample. All solutions were filtered through 0.22 μm Millipore filter before injected. FPIA measurements were done with an Abbott FPIA-TDx equipment at Hospital Provincial de Santa Fe, Argentina.

2.3. Experimental considerations

A calibration set of 9 samples following a central composite design was prepared by spiking a pool of normal human sera with both CBZ and CBZ-EP methanolic solutions, obtaining concentration levels in a range between 0 and 14.0 μg ml^{-1} for CBZ and 0–4.2

for CBZ-EP. These ranges were previously checked for linearity. The latter component was taken into account in the calibration set because it is the main metabolite of CBZ, and is always present in the serum, and thus extracted together with CBZ. The CBZ range was slightly wider than the CBZ therapeutic one (4–12 μg ml^{-1}) [1]. A validation set (number 1) was prepared in the same way, but with different concentrations than those chosen for the calibration set. Fig. 1 displays the analyte concentrations in both sets.

The extraction of the studied analyte from serum into three solvents was tested, based on the results presented by Escandar et al. [14]. These authors postulated a mixture of benzene and 1-pentanol (60:40) as the most efficient one. In order to improve these results, a simplex centroid mixture design with 15 experiments was used to test the three pure solvents (benzene, 1-pentanol and toluene) and combinations of two or three of them. Table 1 shows the design and the recoveries obtained when performing each experiment.

The extraction procedure was as follows: a volume of 500 μl of a given spiked serum sample was placed in a 2.00 ml capped flask and 500 μl of the studied solvent were added. The tube was shaken for 3 min and briefly centrifuged (5 min at 2000×g). An aliquot of 300 μl of the organic phase was transferred to a micro-cuvette, and the spectrum was obtained. These series of operations took only a few minutes, and assured us that the analytes were completely transferred to the organic solution (or at least the maximum for each solvent or mixture). Each serum sample was prepared in triplicate.

For the performance assessment, the following samples were prepared: (1) validation set number 2: three different pool serum samples were spiked with three levels of carbamazepine: 3.0, 9.9 and 13.8 μg ml^{-1}; these samples were used for a precision study; (2) validation set number 3: four different serum samples spiked with four levels of carbamazepine in order to span the range 3.0–14.3 μg ml^{-1}; these samples were used for a recovery study; and (3) validation set number 4: 10 real patients treated with

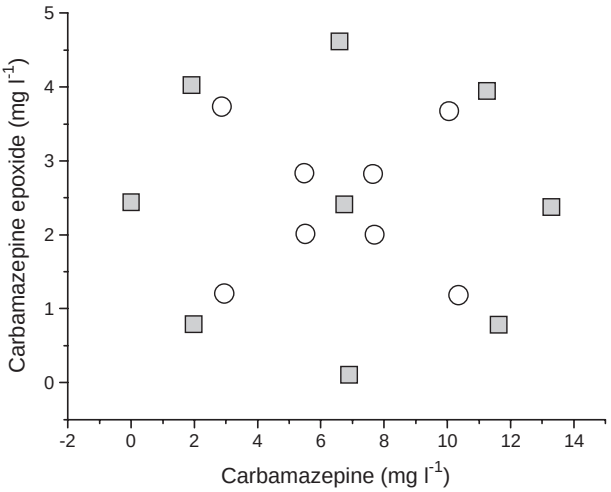


Fig. 1. Experimental designs for building both calibration (squares) and validation (circles) sets.

Table 1
Experimental design used for the optimization of the extraction step

Experiment	Benzene (%)	Toluene (%)	1-Pentanol (%)	Recovery (%)
1	0.0	50.0	50.0	48.0
2	0.0	0.0	100.0	12.0
3	0.0	0.0	100.0	13.0
4	50.0	50.0	0.0	93.6
5	50.0	50.0	0.0	91.8
6	50.0	0.0	50.0	38.0
7	0.0	100.0	0.0	89.9
8	66.7	16.7	16.7	75.8
9	100.0	0.0	0.0	103.0
10	0.0	100.0	0.0	87.5
11	16.7	16.7	66.7	34.9
12	0.0	50.0	50.0	46.0
13	100.0	0.0	0.0	108.0
14	16.67	66.67	16.7	66.5
15	33.3	33.3	33.3	70.0

carbamazepine samples that were analyzed by reference FPIA and HPLC methods and the one developed herein.

3. Results

Several spectra of human sera, together with aqueous solution spectra of CBZ and CBZ-EP both at 10 μg ml^{-1} are shown in Fig. 2. As can be seen, both the strong spectral overlapping and the intrinsic variability displayed by basal sera hinder the resolution of the mixture by conventional spectrophotometry. Considering the complexity of the presently

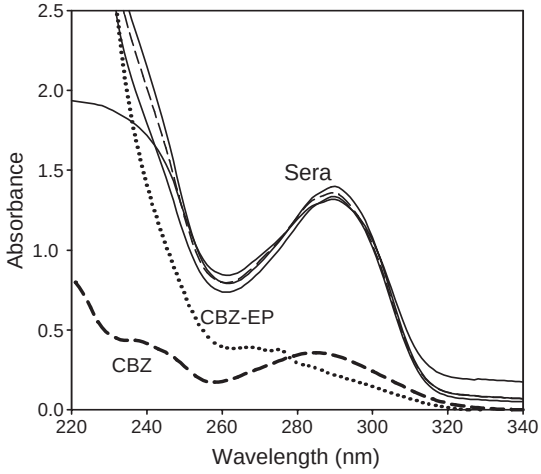


Fig. 2. Several spectra of human sera, together with aqueous solution spectra of CBZ (dashed) and CBZ-EP (dotted) both at 10 μg ml^{-1}.

studied sample, building a multivariate calibration model would need a large number of calibration samples to span all the variability sources [26]. On the other hand, if a previous extraction step to alleviate the interference originated by both endogenous and exogenous serum components is performed, the chemometric model will need less samples in the calibration step. The latter procedure was implemented in the present work.

3.1. Optimization of the solvent extraction step by using a simplex centroid mixture design

In order to optimize the extraction step, a mixture experimental design was used. It is a special class of surface response experiments, in which the factors are the mixture components, and the response is a function of the proportions of each component [24]. The design used (simplex centroid) comprises $(2^q - 1)$ experiments, where q is the number of factors being analyzed. In this case, $q = 3$, thus 10 experiments should be done. Table 1 shows the solvent combinations for the 15 experiments performed, that include five replicates.

A second-order Scheffé polynomial function was postulated with the aim of obtaining a response map. The design allowed us to obtain the response surface, fitting the data to the following polynomial model:

$$y = \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_1 x_2 + \beta_5 x_1 x_3 + \beta_5 x_2 x_3 + \varepsilon \quad (1)$$

where x_i are the analyzed factors (x_1 = benzene, x_2 = toluene and x_3 = 1-pentanol) and β_i are the regression coefficients.

The organic phase obtained in the extraction procedure was transferred to a cuvette, and the UV spectrum was obtained using the corresponding solvent (or mixture) as a blank. This procedure was repeated for each of the 15 extraction experiments. The absorbance value obtained at 290 nm was compared with the one read for a standard solution prepared in the same solvent (or mixture) used in the extraction step. The latter absorbance value was taken as 100%. Then the recovery was computed. Table 1 shows the recoveries obtained.

After least-squares fitting, the parameters obtained were the following:

$$y = 1.05x_1 + 0.87x_2 + 0.13x_3 - 0.12x_1x_2 - 0.66x_1x_3 - 0.07x_2x_3. \quad (2)$$

According to the obtained fitting and the associated Eq. (2), the variable values corresponding to maximum response (recovery = 104.9%) were: benzene = 100%, toluene = 0% and 1-pentanol = 0%. Fig. 3 shows the contour plot corresponding to this response surface, in which the selected coordinates can be easily seen.

3.2. Application of PLS-1 to second-derivative UV-spectra

PLS has become a routine multivariate method for resolving uncomplicated secondary and ternary component mixtures. However, more and more complicated systems such as the presently studied problem are being analyzed by multivariate methods. The PLS method involves a calibration step in which the relation between spectra and component concentrations is estimated from a set of reference samples, and a prediction step in which the results of the calibration are used to estimate the component concentrations in an

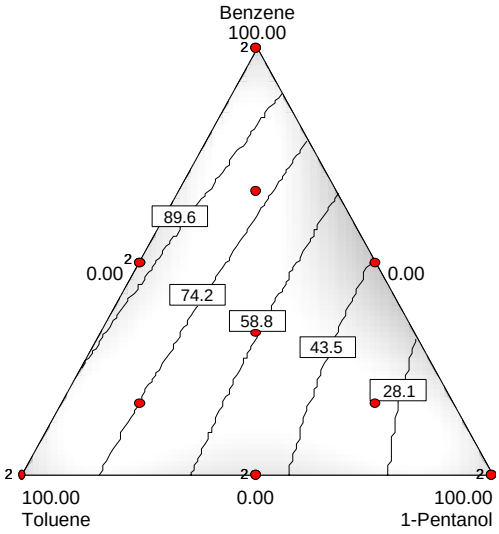


Fig. 3. Contour plot of the simplex centroid mixture design.

unknown sample spectrum [16]. Herein, we implemented the PLS-1 version that is optimised for the determination of a single analyte of interest (carbamazepine). The first step consists in decomposing the calibration data as [15]:

$$\mathbf{R} = \mathbf{T}\mathbf{P}^{\mathrm{T}} + \mathbf{E}_{\mathrm{R}} \tag{3}$$

where $\mathbf{R}$ is an $I \times J$ matrix containing the spectra of I calibration samples obtained at J wavelengths. These calibration spectra contain not only the variability associated to carbamazepine but also those corresponding to serum and CBZ-EP, and other serum components that can be extracted in the first step. $\mathbf{P}$ is a $J \times A$ matrix containing the full spectrum vectors (loadings), $\mathbf{T}$ is an $I \times A$ matrix of intensities (or scores) in the new co-ordinate system defined by the A loading vectors, and $\mathbf{E}_{\mathrm{R}}$ is the $I \times J$ matrix of spectral residual not fitted by the optimal PLS-1 model. The loading vectors contained in $\mathbf{P}$ are usually determined by an iterative algorithm, which also provides a set of orthogonal weight loading factors which form the $J \times A$ matrix $\mathbf{W}$. The matrix $\mathbf{T}$ is related to concentration by an inverse regression step:

$$\mathbf{c}_k = \mathbf{T}\ \mathbf{v} + \mathbf{e}_{\mathrm{c}} \tag{4}$$

where $\mathbf{c}_k$ is the $J \times 1$ vector of the concentrations of analyte k in the calibration samples, $\mathbf{v}$ is the $A \times 1$ vector of coefficients relating the score to the concentrations and $\mathbf{e}_{\mathrm{c}}$ collects the corresponding concentration residuals.

After the calibration is performed, and the optimal conditions are obtained, prediction is the next step. For prediction, the spectrum $\mathbf{r}$ registered for an unknown sample is transformed into the sample score $\mathbf{t}$ by:

$$\mathbf{t} = \left(\mathbf{P}^{\mathrm{T}}\mathbf{W}\right)^{-1}\mathbf{W}^{\mathrm{T}}\mathbf{r} \tag{5}$$

from which the concentration can be calculated as:

$$\mathbf{c}_{k,\mathrm{un}} = \mathbf{t}^{\mathrm{T}}\mathbf{v}. \quad (6)$$

The optimum number of factors to be used within the PLS-1 algorithm is an important parameter to achieve better performance in prediction. This allows one to model the system with the optimum amount of information, avoiding overfitting. The well-known cross-validation procedure was applied in the present work. The optimum numbers of factors are shown in Table 2. As can be seen, three latent variables are suggested, indicating the variability sources number in the presently studied system. When the second-derivative spectra are used, the number of factors decreases to 2, while the baseline shift problem is eliminated. This fact suggests that the third variability source corresponds to this phenomenon, and that it is corrected by using the derivative signal. Fig. 4A shows the zero-order calibration spectra, while Fig. 4B shows the second-derivative calibration spectra. It can be clearly observed how the effect was corrected.

Table 2 also gives other important statistical parameters and figures of merit such as the root mean square error of cross-validation (RMSECV), the relative error of prediction (REP%), the sensitivity (SEN_k), the selectivity (SEL_k), the analytical sensitivity (γ_k) and the limits of detection (LOD_k) and quantification (LOQ_k). These latter figures of merit can be calculated and used for method comparison or to study the quality of a given analytical technique. SEN for a given analyte k has been defined as

$$\mathrm{SEN}_k = \frac{1}{\| \mathbf{b}_k \|} \quad (7)$$

where $\| \ \|$ indicates the Euclidean norm and $\mathbf{b}_k$ is the vector of final regression coefficients appropriate for component k, which can be obtained by any multivariate method. Better insight is furnished by the analytical sensitivity, defined by

$$\gamma_k = (\mathrm{SEN}_k / \| \delta r \|) \quad (8)$$

where $\| \delta r \|$ is a measure of the instrumental noise. It allows comparing analytical methods, regardless the specific technique equipment and scale employed. Moreover, it

Table 2
Spectral region, factors and statistical parameters corresponding to both PLS-1 models built

Statistical parameters	Zero-order spectra	Second-derivative spectra
Region	285–350	285–350
Factors[a]	3	2
RMSECV (μg ml^{-1})[b]	0.34	0.24
REP (%)[c]	5.00	3.90
SEN	0.13	0.00043
SEL	0.73	1.00
γ^{-1} (μg ml^{-1})[d]	0.08	0.12
LOD (μg ml^{-1})[d]	0.25	0.40
LOQ (μg ml^{-1})[d]	0.77	1.21

[a] Factors were selected following the Haaland criterion [15].

[b] RMSECV $= \sqrt{\frac{\sum (x_{\mathrm{act}} - x_{\mathrm{pred}})^2}{I}}$, where I is the number of calibration simples, x_{act} is the actual concentration in calibration samples and x_{pred} is the predicted concentration with the PLS models.

[c] REP(%)=RMSECV × 100/$\bar{x}_{\mathrm{act}}$, where $\bar{x}_{\mathrm{act}}$ is the average concentration in the calibration set.

[d] Calculated according to Eqs. (8), (10) and (11), respectively. $\| \delta r \|$ is a measure of the instrumental noise and equal to 0.01 for zero-order spectra and 5×10^{-5} for second-derivative spectra.

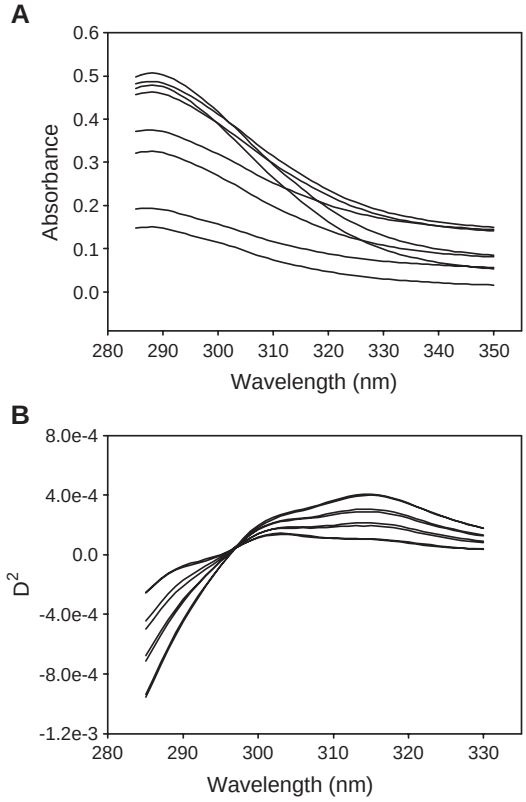


Fig. 4. (A) Zero-order calibration spectra. (B) Second-derivative calibration spectra.

establishes the minimum concentration difference (γ_k^{-1}) which is statistically discernible by the method along the dynamic range [27].

The selectivity quantifies the amount of analyte signal that is overlapped with the interferences, and is calculated as

$$\mathrm{SEL}_k = \frac{||\mathbf{s}_k^*||}{||\mathbf{s}_k||} \tag{9}$$

where $\mathbf{s}_k$ is the vector of spectral sensitivities of component k in pure form and $\mathbf{s}_k^*$ is the corresponding projection onto the net analyte signal space [28].

At last, usual definitions for the limits of detection and quantification are [29]:

$$\mathrm{LOD} = 3.3\ ||\delta r||_{||\mathbf{b}_k||} \tag{10}$$

$$\mathrm{LOQ} = 10\ ||\delta r||_{||\mathbf{b}_k||}. \tag{11}$$

Tables 2 and 3 present the results corresponding to calibrations and prediction on the validation set number 1.

Table 3
Results obtained when applying both PLS-1 models on the validation set number 1

Validation sample	CBZ spiked (μg ml^{-1})	Zero-order spectra		Second-derivative spectra	
		CBZ found (μg ml^{-1})	Recovery (%)	CBZ found (μg ml^{-1})	Recovery (%)
1	2.87	1.91	66.41	2.75	95.84
2	2.95	2.24	75.78	2.71	91.75
3	10.06	9.68	96.19	10.36	102.97
4	10.36	9.78	94.44	10.15	97.93
5	5.48	5.17	94.39	5.42	98.99
6	5.52	5.21	94.36	4.87	88.21
7	7.66	8.01	104.55	7.74	101.05
8	7.71	7.54	97.80	7.38	95.76
Average recovery			90.49		96.56
REP(%)			8.64		5.35

3.3. Performance study

3.3.1. Precision

Excellent results were obtained for repeatability (intra-assay variability) for the three studied levels when five replicates were processed (validation set number 2). The predictions, standard deviations (between parenthesis) and the coefficients of variation were the following: (a) level 1: 3.2 (1) μg ml^{-1} and 2.6%; (b) level 2: 9.4 (2) μg ml^{-1} and 1.8%; and (c) level 3: 13.7 (2) μg ml^{-1} and 1.2%.

3.3.2. Recovery on spiked samples

Table 4 shows the obtained results when three replicates of samples corresponding to validation set number 3 were analyzed. Recoveries near to 100% were obtained for all the four assayed levels. A statistical Student's *t*-test was performed in order to compare the average values with the known spiked carbamazepine concentration [23].

Table 4
Results obtained for the recovery study performed on validation set number 3

	Carbamazepine concentration (μg ml^{-1})							
	Level 1		Level 2		Level 3		Level 4	
	Spiked	Found	Spiked	Found	Spiked	Found	Spiked	Found
Replicate 1	2.97	2.88	8.74	8.65	11.54	11.10	14.28	13.73
Replicate 2	2.97	3.03	8.74	8.78	11.54	11.28	14.28	14.25
Replicate 3	2.97	2.97	8.74	8.48	11.54	11.48	14.28	14.11
Average		2.96		8.64		11.29		14.03
Standard deviation		0.08		0.15		0.14		0.27
Student's *t*-probability (p)[a]		0.829 ND		0.300 ND		0.080 ND		0.183 ND
Mean recovery		99.7		101.7		98.0		98.3

[a] ND: no statistical differences were found between the spiked value and the mean of the three replicates.

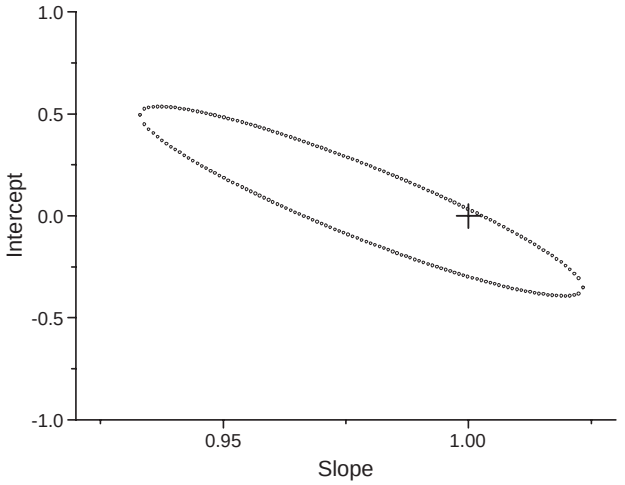


Fig. 5. Elliptical joint confidence regions for the slope (b) and intercept (a) corresponding to regressions of the nominal vs. chemometric-assisted predicted concentrations of carbamazepine. The black cross marks the theoretical ($a=0$, $b=1$) point.

In order to get additional insight into the accuracy and precision of the method herein analyzed, linear regression analysis of nominal versus chemometric method found concentration values when analyzing validation set number 3 was applied. The estimated intercept and slope ($\hat{a}$ and $\hat{b}$, respectively) were compared with their ideal values of 0 and 1 using the elliptical joint confidence region (EJCR) test, in this case by using an ordinary least squares fitting as recommended in Ref. [30]. Fig. 5 shows the corresponding EJCR plot.

3.3.3. Comparative study with HPLC and FPIA on real patients serum samples

Finally, Table 5 shows the results obtained when 10 real patients serum samples (validation set number 4) were analyzed by reference methods and the one herein proposed.

Table 5
Comparative study of the results obtained when applying HPLC or FPIA and PLS-1 on real patients samples

Sample	Carbamazepine predicted (μg ml^{-1})		
	HPLC	FPIA	Chemometric assisted
Patient 1	4.67	–	4.65
Patient 2[a]	6.02	6.18	6.34
Patient 3	9.12	9.34	9.66
Patient 4	3.70	3.88	4.06
Patient 5	6.92	–	6.12
Patient 4	8.32	8.52	8.86
Patient 7	4.88	4.93	4.82
Patient 8	7.41	7.60	8.56
Patient 9	6.40	6.56	6.48
Patient 10	8.37	8.54	8.14

[a] Phenytoin was found to be 10.0 μg ml^{-1} by applying the FPIA method.

4. Discussion

According to the optimization of the extraction step, benzene was selected as the most convenient solvent. Although it is a highly toxic solvent, only 300 μl of the organic phase is transferred to the micro-cuvette for measurements.

As regard the results presented in Tables 2 and 3, an increase in the predictive ability is obtained when the derivative spectra are used for building the chemometric model. On one hand, lower errors in both calibration and prediction are estimated. On the other, a loss in sensitivity is experienced and consequently lower detection and quantification limits are obtained by using the less-sensitive derivative spectra. Finally, the higher selectivity obtained by using derivative spectra can be understood on inspection of Fig. 4B. Here, small differences between spectra are magnified, obtaining richer information from spectra.

Analyzing the performance study results, the precision improves as the carbamazepine concentration becomes higher. Remarkably, comparable precision with the reference methods was found in all the assayed concentration levels. Moreover, as can be observed in Table 4, no statistical differences were found ($p > 0.05$ in all the cases), and it can be concluded that systematic errors are not present. Additionally, the EJCR plot of Fig. 5 contains the theoretical ($a = 0$, $b = 1$) point, indicating that constant and proportional bias are absent. On the other hand, the good agreement found between the concentration values obtained when analyzing real patient samples with HPLC, FPIA and the studied methods (Table 5), is indicative of the excellent performance of the chemometric-assisted spectrophotometric method. A visual inspection of Table 5 shows relative errors ranging from 0.4% to 6% for most of the analyzed samples (only sample 8 presents a large relative error of 15.5%). Interestingly, sample 2 also contains phenytoin (10.0 mg ml^{-1} measured by FPIA), for which 5% is the maximum relative error found (when comparing results obtained by applying the HPLC method and the proposed one). This fact and the lack of absorbance for valproic acid in the studied spectral region allow us to conclude that other antiepileptic drugs commonly used in combination with carbamazepine do not present interference when applying the present methodology. Finally, once the calibration is performed, measurements can be made in a few minutes, leading to benefits in both cost and time over more conventional techniques.

5. Conclusions

The combination of UV spectrophotometry coupled to both optimized-analyte-extraction and multivariate calibration (PLS-1) leads to a powerful tool to be applied to drug monitoring. The results obtained by applying the developed method on real patient serum samples and by comparing it with reference methods show the enormous potentiality of this analytical strategy. Carbamazepine was determined with high accuracy and precision by using a very simple, quick and inexpensive method.

6. Simplified description of the method and its (future) applications

A simple procedure completely assisted by chemometric tools is herein presented. The method consists in a simple extraction step in which 500 μl of serum sample are treated with 500 μl of benzene. Then, 300 μl of the organic phase is transferred to a micro-cuvette and a UV-derivative spectrum is obtained and subsequently undergone in a chemometric analysis. The method can be recommended for pharmacokinetic studies owing to its simplicity, speediness and low cost.

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