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Chemometrics enhanced HPLC–DAD performance for rapid quantification of carbamazepine and phenobarbital in human serum samples

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ABSTRACT

This paper describes development and validation of a simple and efficient bioanalytical procedure for simultaneous determination of phenobarbital and carbamazepine in human serum samples using high performance liquid chromatography with photodiode-array detection (HPLC–DAD) regarding a fast elution methodology in less than 5 min. Briefly, this method consisted of a simple deproteinization step of serum samples followed by HPLC analysis on a Bonus-RP column using an isocratic mode of elution with acetonitrile/K_2HPO_4 (pH=7.5) buffer solution (45:55). Due to the presence of serum endogenous components as non-calibrated components in the sample, second-order calibration based on multivariate curve resolution–alternating least squares (MCR–ALS), has been applied on a set of absorbance matrices collected as a function of retention time and wavelengths. Acceptable resolution and quantification results were achieved in the presence of matrix interferences and the second-order advantage was fully exploited. The average recoveries for carbamazepine and phenobarbital were 89.7% and 86.1% and relative standard deviation values were lower than 9%. Additionally, computed elliptical joint confidence region (EJCR) confirmed the accuracy of the proposed method and indicated the absence of both constant and proportional errors in the predicted concentrations. The developed method enabled the determination of the analytes in different serum samples in the presence of overlapped profiles, while keeping experimental time and extraction steps at minimum. Finally, the serum concentration levels of carbamazepine in three time intervals were reported for morphine-dependents who had received carbamazepine for treating their neuropathic pain.

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1. Introduction

Measuring the drug concentration in human serum is one of the utmost important issues in pharmacokinetic study which is essential for various drugs including cardiovascular medications, antibiotics, anticonvulsants and anti-cancer drugs. Moreover, determination of drugs toxicity in therapeutic drug monitoring (TDM) is one of the other noticeable concerns [1]. Since, there is a significant relation between blood drug level and its effect, being aware of such relations would play significant role in strategizing treatment plans. Treatment coefficient for most anticonvulsants is low while their toxicity is high. Therefore, having the knowledge on the treatment levels, pharmacokinetic of the drugs and their toxicity is essential for efficient treatment of seizures [2].

Carbamazepine (CBZ), as an antiepileptic drug (AED) is a carboximide derivative of immunoacetylene which is extensively used in the treatment of seizures as well as in other psychiatric diseases, neuropathic pains and also for managing post-operative pain [3,4]. Due to its high segregation coefficient, the extraction of this substance into organic solvents is easy. Variable therapeutic concentration for carbamazepine in human serum has been reported [4,5]. These levels are generally considered safe and effective for therapy. Therefore, determination of CBZ at low levels in serum is important for the correction of doses of patients receiving CBZ as well as studying the pharmacokinetics of CBZ [6]. Research findings confirm that CBZ could have some negative effects on central nervous system including diplopia, dizziness, headache, nausea and motor incoordination which have been known as its side effects [7]. In addition, it causes toxic effects when the serum level exceeds 15 $\mu g\ mL^{-1}$ [8,9].

Phenobarbital (phenobarbitone; PB), that acts as a nonselective central nervous system depressant, is widely used as antiepileptic drug due to the advantages including reliability of supply,

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affordable cost, broad spectrum of action, and ease of use. It is also used for partial and generalized tonic–clonic seizures treatment. Its anticonvulsant properties were discovered serendipitously by Hauptmann, who originally used it as a hypnotic for his epilepsy patients [10]. Fatigue, weakness in adults and insomnia, hyperknesia and aggression in children (and sometimes in elderly) have been known as cognitive and behavioral side effects of phenobarbital. Furthermore, mild mood memory and learning disturbance can occur at both age groups [11].

During the last years, various sample preparations techniques, such as chemical deproteinization [12], liquid–liquid extraction [13–17], column switching [18], stir bar-sorbtion extraction (SBSE) [19], solid phase extraction (SPE) [13,20], dispersive liquid–liquid microextraction (DLLME) [21], solid phase microextraction (SPME) [22,23], solid phase extraction combined with dispersive liquid–liquid microextraction (SPE–DLLME) [24], have been used for extraction of organic analytes from biological fluids. Also, simultaneous determination of carbamazepine and other anticonvulsant, has been carried out with high-performance liquid chromatography (HPLC) coupled to ultraviolet detection (UV) or mass spectrometry detection (HPLC-MS and HPLC-MS/MS) [25–33], fluorescence polarization immunoassay (FPIA) and electro chemical methods such as, cyclic voltammetry [34], micellar electrokinetic capillary chromatography (MECC) [35], chemiluminescence [36] and capillary electrophoresis (CE) [37].

In fact, when the extraction processes are not selective enough, a number of interfering components are co-extracted with the interested analytes and may cause serious problems in the separation process. So, during the recent years, different multiway and also multi-set data analysis methods have been utilized in combination with the second-order chromatographic systems for which appearance of peak overlaps is inevitable, especially for highly complex samples [38]. Interestingly, there are various second-order data analysis algorithms, such as generalized rank annihilation method (GRAM) [39], alternating trilinear decomposition (ATLD) [40], self-weighted alternating trilinear decomposition (SWATLD) [41], parallel factor analysis (PARAFAC) [42], PARAFAC2 [43], bilinear least squares/residual bilinearization (BLLS/RBL) [44], unfolded partial least squares/residual bilinearization (U-PLS/RBL) [45] and multivariate curve resolution alternating least squares (MCR–ALS) [46,47], that permit analyte quantification in samples containing unexpected components, a property which is called “second-order advantage” [48].

Among the mentioned algorithms, MCR–ALS and PARAFAC2 allow deviations to the trilinearity of three-dimensional data. MCR–ALS is an excellent tool in modeling of LC–DAD data for recovering distinct time profiles in each experimental sample. On the other hand, before applying the trilinear modeling algorithms, such as GRAM, ATLD and PARAFAC, the chromatographic peaks should be properly aligned. In fact, two important points should be considered while applying the synchronizing methods; the first is the presence of non-linear retention time shifts between different analytes during different chromatographic runs, and the other is the alignment of analyte peaks in the presence of matrix interferences [49]. Some applications of the mentioned algorithms for the analysis of biological fluids can be found in the literature [37,38,50,51].

Regarding that today the simple and low cost sample preparation and clean-up steps are preferred in bio-analytical methods and knowing this fact that most of these procedures are not selective enough, long HPLC (as the most widely used techniques in this regard) run times are needed to avoid appearing coelution problems, arising from matrix components. So, in this study we developed an approach for rapid quantification of serum levels of carbamazepine and phenobarbital in the presence of overlapping serum interferences by means of HPLC–DAD combined with MCR–ALS as a powerful chemometrics tool, with the aim of using very simple sample preparation step, reducing analysis time and consequently, decreasing the cost per analysis.

2. Experimental section

2.1. Chemicals and solvents

Analytical grade standards (purity higher than 98%) of carbamazepine and phenobarbital were donated by pharmaceutical companies, Daroopakhsh and Sobhandaroo (Tehran, Iran), respectively. HPLC-grade methanol (MeOH), acetonitrile (ACN) and ethyl acetate (EA) were from Merck (Germany). Phosphoric acid, dipotassium hydrogen phosphate and sodium hydroxide were of analytical grade from Merck. Ultrapure water was provided by a Milli-Q purification system from Millipore (USA). Solvents, calibration and real samples used to perform the HPLC application were filtered through 0.22 μm nylon filter membranes filter paper (Varian, USA).

2.2. Serum samples

Drug-free human serum samples were received from Taleghani Medical Center (Tehran, Iran). For the analysis of real samples, we did need the epileptic patients who were on duotherapies with carbamazepine/phenobarbital which were difficult to access. So, serums of the patients administered with CBZ (as oral) as a pre-operational pain treatment drug, were investigated. These samples which were received from Faculty of Medicine, Shahid Beheshti University of Medical Sciences, were taken from 27 patients belonging to three groups of morphine-dependent patients who had received carbamazepine before surgery. Then, the patients' serum samples were collected before surgery, 2 and 12 hours after surgery, respectively. If the analysis was delayed, the serum samples were frozen at -80 °C.

2.3. Instrumentation and chromatographic conditions

The HPLC system was an Agilent 1200 Series system (Agilent Technologies Inc., USA), consisted of a Rheodyne 7725 manual injector, a degasser, a quaternary pump, a column oven compartment, a Hewlett-Packard 1200 series photo diode-array detector (DAD) and Chemstation software package (version B.03.01) to control the instrument, data acquisition and data analysis. Chromatographic separation was carried out on a Bonus-RP column (15 cm × 0.46 cm, 5 μm particle size, Agilent). The column oven temperature was set at 25 °C. An isocratic mobile phase consisted of acetonitrile (45%, v/v) and 0.005 mol L^{-1} K_2HPO_4 (pH = 7.5) buffer solution (55%, v/v) was optimized and implemented for all analyses. Flow rate of the mobile phase and injection volume were 1.0 mL min^{-1} and 20 μL, respectively. The total run time was less than 5 min. The solvents were filtered daily through a 0.2 μm Nylon membrane filter. Photometric detection using DAD detector was performed between 210 and 400 nm with the spectral resolution of 1.5 nm and integration period of 0.4 s per spectrum.

In the extraction procedure, a 320R Hettich centrifuge (Germany) and a Sonorex Digital 10P ultrasonic bath (Germany) were used. Screw capped glass test tubes (used as extraction vessels) with a conical bottom were purchased from Varian.

2.4. Sample handling and extraction procedure

An aliquot of 500 μL from spiked serum samples was transferred to a glass tube, and then 500 μL of 0.1 M NaOH and 3 mL of ethyl acetate were added. Then, the tube was capped and shaken

on a vortex mixer for 30 s and afterwards centrifuged at 4000 rpm for 10 min. The upper organic layer was transferred to a clean tube and 1 mL of ethyl acetate was added to the sample and was centrifuged again for 5 min. The supernatant organic layer was added to previously collected organic layer. Then, the extract was evaporated to dryness under a stream of nitrogen. The residual was redissolved in 500 μL of mobile phase in ultrasonic bath, filtered through a 0.22 μm PTFE syringe filter and 20 μL of the final solution was injected into the HPLC system.

2.5. Preparation of calibration and spiked serum samples

Individual stock standard solutions of CAZ and PB at the 200 mg L^{-1} concentration level were prepared by exact weighting and dissolution in methanol and stored at −4 °C in dark glass vials. These solutions were stable for at least one month. Standard working mixtures, at different concentrations, were prepared daily by appropriate dilution of the stock solutions with mobile phase. A set of five samples, each of them in triplicate, containing 0.1, 1.2, 3.6, 5.5 and 9.1 μg mL^{-1} of CBZ and 0.6, 1.6, 4.3, 7.2 and 9.8 μg mL^{-1} of PB was prepared in pure solvent and was used as the calibration set. As validation samples, a set of thirteen blank serum samples were taken from two different pools and fortified with CBZ and PB at concentration levels ranging from 0 to 8.15 μg mL^{-1} and 0 to 9.3 μg mL^{-1}, respectively. The concentration levels of drugs were selected considering the levels usually found in serum of different patients for therapeutic drug monitoring purposes. Also, one of the most complex patients' serum samples was spiked with 1 μg mL^{-1} of CBZ.

2.6. Data generation and software

HPLC–DAD data which was gathered by Chemstation software (B.03.01), exported as Microsoft Excel® file for further processing. Routines for MCR–ALS were available at (http://www.ub.edu/mcr/welcome.htm) and all algorithms were written in MATLAB (version 7.2.0.232 R2006a, The Mathworks, Natick, MA).

3. Results and discussion

3.1. General considerations

In the present study, a simple mobile phase of acetonitrile-0.005 mol L^{-1} K_2HPO_4 (pH=7.5) buffer solution was selected. Then, different ratios of the solvents were tested, taking into account the run time and the peak profiles of the analytes. Finally a ratio of acetonitrile-0.005 mol L^{-1} K_2HPO_4 (pH=7.5) solution (45:55, v/v) with total run time of 5 min was chosen. With this mobile phase composition, the retention times of PB and CBZ were 2.33 and 2.97 min, respectively. On the other hand, considering chemometrics analysis of chromatographic data, it was possible to analyze the serum samples without preparation step, but a simple deproteinization step was performed with the purpose of preserving the useful life of the chromatographic column.

Fig. 1 shows the chromatographic profiles recorded at 241 nm (blue line, curve a) for a standard mixture solution containing 1.2 and 1.6 μg $mL^{-1}$of CBZ and PB, respectively. This figure also shows the obtained chromatogram corresponding to two serum samples; the first is a sample spiked with analytes of interest (red line, curve b) and the second is a patient's serum sample containing CBZ (green line, curve c). The complexity of the analytical problem under study can be easily appreciated from this figure. As can be seen, there is obvious coelution problem in the prediction of serum samples, because of the interferences from the sample matrix. In the first serum sample, the large peak from serum

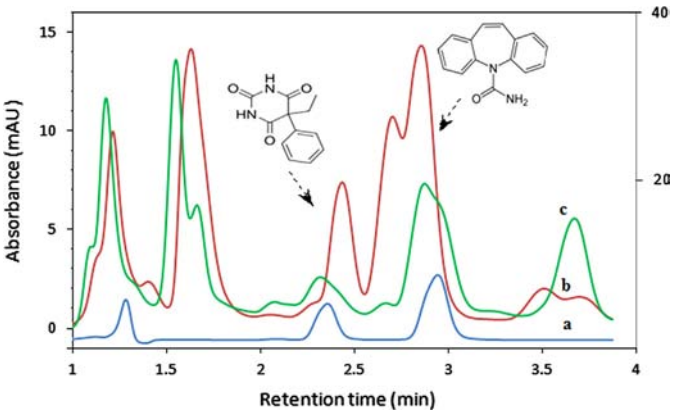

Fig. 1. Chromatographic profile of a typical calibration sample of CBZ and PB at 1.2 and 1.6 μg mL^{-1}, (blue line, curve a), blank serum sample spiked (s13) with 1.8 and 2.75 μg mL^{-1} of CBZ and PB (red line, curve b) and patient's serum sample10 (green line, curve c) at 241 nm. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of this article.).

Table 1
Regions in which the chromatographic data were divided for building MCR–ALS models.

Analytes	Region	Time region (min)	Retention time (min)	Wavelength range (nm)
PB	1	2.19–2.59	2.33	225–320
CBZ	2	2.59–3.15	2.97	225–370

matrix between CBZ and PB produced a significant interference. On the other hand, the patient's serum profile showed completely different chromatographic pattern, so that there was not such interference as mentioned for the first sample. Instead, a sever coelution problem can be seen in the right hand side of CBZ peak. In fact, different complexity problems have been faced during analysis of serum samples, which is completely evident, because of endogenous and exogenous components present in serum sample of people, depending on their health, sex, administrated dose, route of drug administrated and probable consumption of other drugs. Almost all chromatographic patterns confirmed this fact that classical univariate calibration is not a suitable method of quantification in such condition. In fact, lots of time, great effort and resources must be devoted to experimentally remove the interferences and/or to optimize the chromatographic conditions for resolving the interferences from the analytes of interest, which the latter may be not necessarily the optimized condition for another sample. Alternatively, second-order multivariate calibration methods, exploiting second-order advantage, can be resorted in such cases for mathematical separation of serum interferents. Among the different second-order calibration methods which do not require the trilinear structure of data array and hence, chromatographic alignment, MCR/ALS was selected for data processing. In this work, the matrix augmentation was performed in the time direction because of the presence of retention time shifts between different chromatographic runs.

3.2. MCR/ALS analysis of spiked serum samples

In order to simplify the analysis, two time domains from raw data, in the ranges of 2.19–2.59 (containing PB) and 2.59–3.15 (containing CBZ) were adopted for MCR/ALS analysis (see Table 1), so that determination of the analytes was exactly a single target determination in their own elution time region. Thus, there was a model for each drug in each of serum type, containing one analyte

and the corresponding co-eluted interferents. In spectral dimension, a wavelength range of 225–320 nm and 225–370 nm with a spectral resolution of 1.5 nm and the integration period of 0.4 s per spectrum were selected for PB and CBZ, respectively. Through the mentioned two-way subset selection and considering the number of calibration matrices augmented (in column wise) with each test sample, a global matrix of **D** with dimensions $(16\times 81)\times 63$ and $(16\times 51)\times 96$ for PB and CBZ were constructed, respectively. In fact, because of the retention time shifts and also band shape changes between the calibration and serum samples, much more mathematical rank would be necessary to model the different bilinear components in matrix **D** in row-wise augmentation. So, the matrix augmentation in the spectral direction was inconvenient. That was not what actually happened in matrix augmentation in retention time direction, because of similarity of component spectra from matrix to matrix.

The obvious aim in this work was quantification of the analytes in serum sample, mainly without signal pre-treatment, with a rapid chromatographic elution. So, the constructed data matrix **D** was fed to the algorithm without any pre-processing step. Before starting resolution, the number of components to each data matrix D was estimated using singular value decomposition (SVD). Typically, the plot of singular values as a function of principal component number is visually inspected, locating a number for which the plot stabilizes. Then, the spectra type initial estimates were built based on the selected component number using SIMPLISMA [52].

Then, the bilinear decomposition of the augmented matrix **D** was performed considering the predefined number of components and a spectral matrix of initial estimates, according to the following expression:

$$\mathbf{D}=\mathbf{C}\mathbf{S}^{\mathbf{T}}+\mathbf{E} \quad (1)$$

where the number of rows in matrix **D** equals the total number of recorded elution times in different chromatographic runs for test and standard addition samples ($(I+1)K$, I=number of training samples and K=number of elution times in each sample) and the number of columns is equal to the considered number of wavelengths (J). Given **D** and $\mathbf{S}^{\mathbf{T}}$, appropriate constraints (i.e. correspondence criterion, non-negativity in both modes and unimodality in chromatographic mode) were implemented to drive the iterative optimization to the right solution. Then, according to the appropriateness of the solutions, such as reasonable least-square fit and physically recognizable profiles, initially selected component was increased or decreased. It is necessary to notice here, that the number of components in almost the entire test samples had a good accordance with their SVD plot. This number was different between two validation samples and also between various patients' serum samples. Finally, the convergence criterion for MCR/ALS was set to 0.1% (equal to relative change in fit values for successive iterations). The number of iterations on which the mentioned convergence criterion achieved was below 90 for all test samples.

Fig. 2a shows the chromatograms of a typical serum sample spiked with CBZ and PB in multiple wavelengths. The chromatographic and spectral profiles, corresponding to the analyte and the interferences, extracted by MCR/ALS has been shown in Fig. 2b and Fig.3, respectively. As can be seen from Fig. 2b, there are one and two serum peaks coeluted with PB and CBZ, respectively. Fig. 3 also, shows a perfect match between the recovered spectra (the red solid line) and the normalized pure spectra in each region, which is indicative of that the extracted profiles correspond to the analytes of interest in each region. Similar acceptable results were obtained for other spiked serum samples (serum 1) and also for the other spiked serum samples (serum 2). In all cases the number of factors was 2 or 3, but never 1, which is normally required and presupposed for univariate calibration. In this manner, the quantification of the isolated analytes was done using estimated relative peak areas for the target analytes and so a pseudo-univariates calibration curve was built accordingly.

Table 2 shows the predicted concentrations when applying MCR/ALS to a set of 13 serum samples from two different pools (without and with spiked drugs). Taking into account the simple sample treatment, the analytical results were reasonably good, with recoveries ranging from 73.8% to 98.2% for PB and 78.8% to 106.3% for CBZ, respectively. The relative standard deviations (RSD%) of predicted concentration values for three replicates of samples s5 and s13 were less than 9.0% which can be considered acceptable considering this fact that no attempt has been performed to remove the interfering compounds before HPLC analysis. Moreover, in order to acquire further insight into the accuracy of the proposed method, a linear regression analysis of nominal versus found concentration values was applied. The estimated intercept and slope were compared with their ideal values of 0 and 1 using the elliptical joint confidence region (EJCR) test [53]. As can be seen in Fig. 4, EJCR plot contains the ideal point (0,1) and further proved the accuracy for the estimated concentrations of CBZ and PB in human serum samples. In fact, the good recovery values obtained through application of MCR/ALS, in addition to proving the applicability of this algorithm for the present problem, confirmed further that the use of external calibration was an adequate calibration strategy and matrix effects were not significant.

Table 3 shows some statistical parameters such as root-mean-square-error of prediction (RMSE) and the figures of merit obtained through application of MCR/ALS on serum samples, which demonstrate the good performance of the calibration. Linear pseudo-univariate calibration curves were obtained with R^2 values of 0.9984 and 0.9986 for CBZ and PB, respectively. Also, the linearity of the models for both analytes in their mentioned concentration ranges was confirmed using lack of fit test (Statgraphics Centurion XVI, V 16.1.11). The p-values for lack-of-fits in the ANOVA tables were greater than 0.05, so the linear models appeared to be adequate for the recovered areas at the 95.0% confidence level.

The limits of detection (LODs) and limits of quantification (LOQs) values [54] obtained in the serum samples were acceptable, considering that a very simple methodology was applied to a complex real system. Taking into account the typical values which can be found in serum samples, the proposed method could be directly applied without a pre-concentration step.

3.3. Quantitative analysis of CBZ in real samples

With the purpose of testing the applicability of the proposed method to clinical samples, a set of 27 serum samples belonging to three groups of morphine-dependent patients who had received carbamazepine before surgery, were analyzed using second-order methodology based on MCR/ALS, in three time intervals of before surgery, 2 h and 12 h after surgery. In Fig. 5 the contour plot of the absorbance signals in retention time range of 2.7–3.4 min, corresponding to serum sample of a patient and a standard sample of CBZ, is shown. The overlapping between the signals for this drug and serum components is clear. It was found that patients' serum matrices of the first two groups (before surgery and 2 h after surgery) contained different number of interfering compounds while the third serum group did not produce any interfering compound in the retention time region of CBZ. So, the number of factors responsible for the variations in these test samples was one. The analysis of CBZ was done by applying MCR/ALS to the sub-matrix containing its peak. Although this could also be done using a univariate calibration, we preferred to quantify CBZ in these samples using MCR/ALS for consistency. In fact, because of

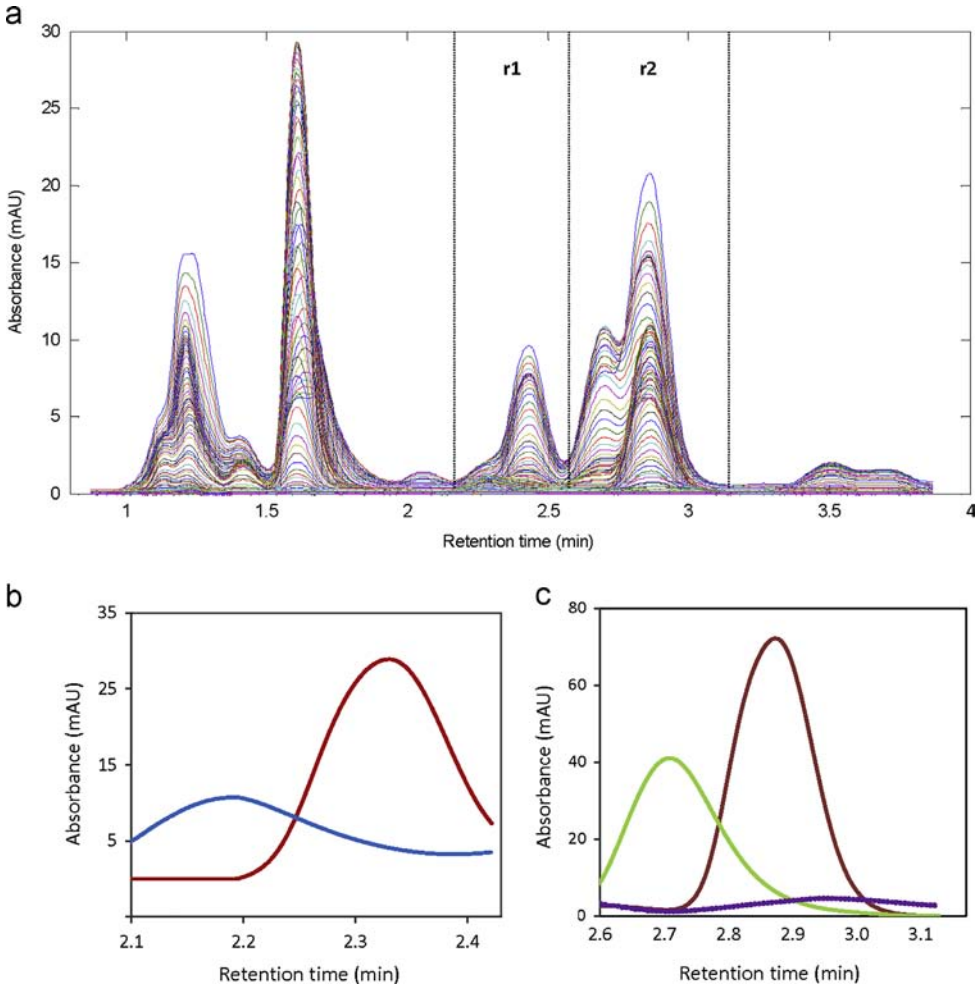

Fig. 2. HPLC–DAD chromatograms, each at a single wavelength (225–370 nm), of a serum sample (serum 2) spiked with two analytes (s13, Table 2). The analytes of interest are indicated, (a). Estimated elution time profiles retrieved by MCR/ALS analysis for regions 1 containing PB (red solid line) and one interfering compound (blue dot line) (b) and for region 2 containing CBZ (red solid line) and two interfering compounds (green solid line and purple dot line) (C). (For interpretation of the references to color in this figure caption, the reader is referred to the web version of this article.).

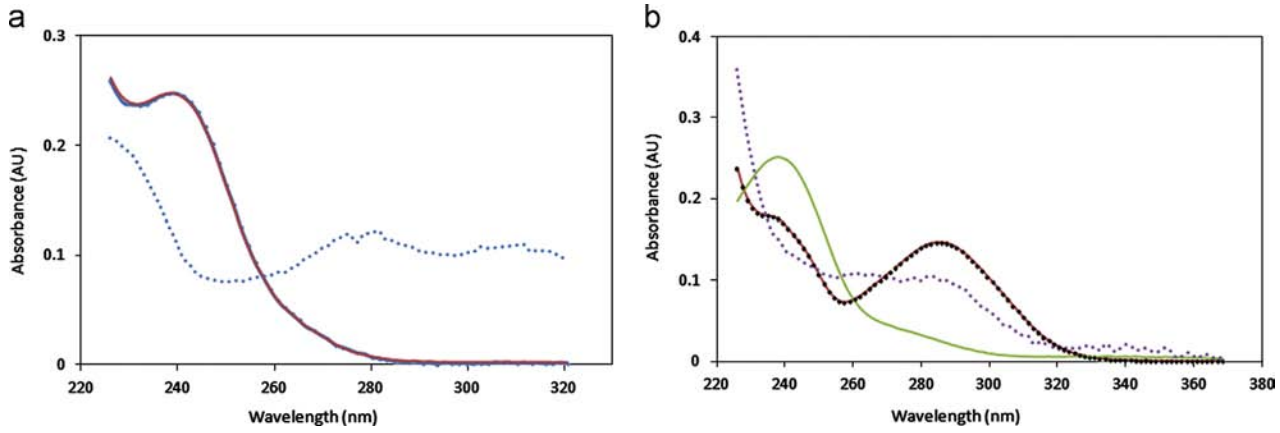

Fig. 3. Spectral profiles recovered by MCR–ALS modeling for PB (a) and CBZ (b). Comparison between the normalized pure analytes spectra for PB (blue round dot line) and CBZ (black round dot line) and the spectra reconstructed by the MCR/ALS model (red solid line). The interfering components have been shown (purple dot line) for PB and (green solid and purple dot lines) for CBZ, respectively. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of this article.).

keeping all serum samples at −80 °C and non possibility of degradation of sample matrix, these phenomena can be ascribed to elimination of those specific endogenous components in the serum samples coeluted with CBZ, 12 h after surgery. However, when the determination was carried out in the presence of these interfering components for the rest of the samples, achievement of the second-order advantage for accurate quantification was necessary. The recovered time profiles by MCR/ALS, corresponding to the analysis of patient's serum sample (serum 18) are shown in Fig. 6. In addition to retention time shift of CBZ, it can be clearly

Table 2
Predicted concentrations using MCR–ALS on two different serum samples spiked with different amount of analytes.

Sample	Analyte concentrations (μg mL^{-1})[a]			
	CBZ		PB	
	Taken	Found	Taken	Found
Serum 1				
Unspiked	0.00	n.d.[b]	0.00	n.d.
s1	0.48	0.42 (87.5)	6.15	5.35 (85.3)
s2	0.95	0.77 (81.0)	1.45	1.07 (73.8)
s3	7.60	6.29 (82.8)	9.30	7.72 (83.0)
s4	5.45	4.73 (86.7)	0.00	n.d.
s5	2.35	2.50 (106.3)[7.1] [c]	3.20	2.65 (82.8)[5.5]
Serum 2				
s6	0.85	0.85 (100.8)	7.50	7.10 (94.6)
s7	8.15	6.86 (84.1)	0.75	0.63 (84.0)
s8	4.12	3.25 (78.8)	3.46	3.29 (95.1)
s9	6.45	5.54 (85.6)	6.45	6.02 (93.3)
s10	3.20	2.97 (92.8)	0.00	n.d.
s11	0.50	0.43 (86.0)	4.45	3.05 (68.5)
s12	0.00	n.d.	1.40	1.24 (88.9)
s13	1.80	1.89 (105.0) [6.2]	2.75	2.70 (98.2)[8.4]

[a] Recoveries in parenthesis.
[b] Not detected.
[c] RSD (%) for three replicates of s5 and s8 in square brackets.

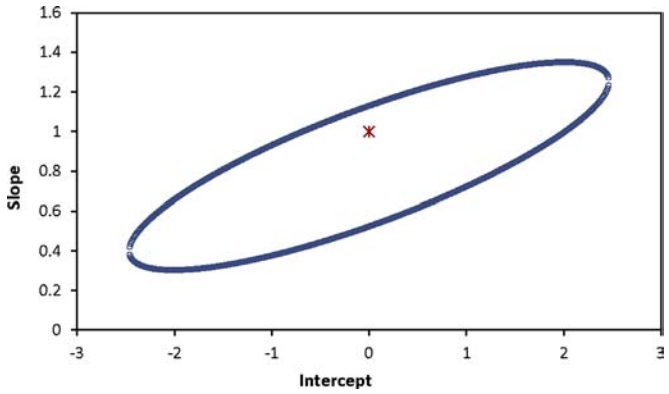

Fig. 4. Elliptical joint confidence region (EJCR) plot at 95% confidence limit, obtained through regression of found versus added concentration levels of analytes in the global data set. The asterisk in the elliptical plot shows the theoretical (intercept= 0, slope =1) point. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of this article.).

Table 3
Statistical parameters and figures of merit for determination of analytes in human serum by MCR–ALS.

	CBZ	PB
RMSE (μg mL^{-1})[a]	0.19	0.22
SEN (mL μg^{-1})[b]	1040	240
R^2 [c]	0.9984	0.9986
LOD (μg mL^{-1})[d]	0.006	0.11
LOQ (μg mL^{-1})[e]	0.02	0.37

[a] Root mean square error of prediction, RMSEP (μg L^{-1})$=[\frac{1}{n}\sum_{n=1}^{n}(c_{add.}-c_{pred.})^2]^{1/2}$where n is the number of unknown samples, $c_{add.}$ and $c_{pred.}$ are the added and predicted concentrations, respectively.
[b] Sensitivity is defined as the slope of the pseudo-univariate calibration curve.
[c] Determination coefficient.
[d] LOD, limit of detection calculated according to Ref. [54].
[e] LOQ, limit of quantification calculated as LOD × (10/3.3).

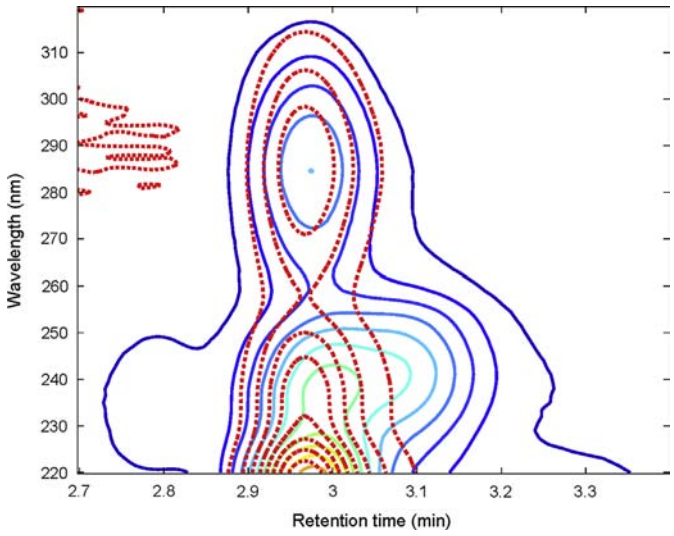

Fig. 5. Two-dimensional contour plot of retention times/absorbance matrix of a patient's serum sample (blue solid lines) superimposed on the matrix corresponding to a typical calibration sample of CBZ (red dot lines). (For interpretation of the references to color in this figure caption, the reader is referred to the web version of this article.).

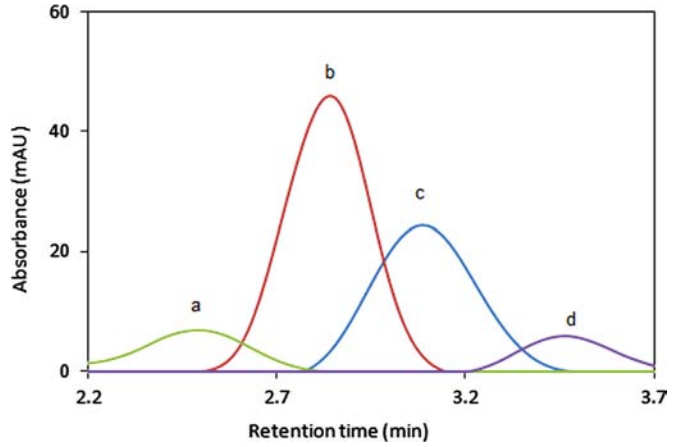

Fig. 6. Estimated chromatographic profiles by MCR/ALS modeling for region 2 of a morphine-dependent serum sample (serum 18) which includes CBZ (red line, curve b) and three interfering components (curves a, c and d). (For interpretation of the references to color in this figure caption, the reader is referred to the web version of this article.).

seen that the three components are eluting in the selected time region and the two interferences coeluted with the CBZ. Table 4 shows the quantification results of CBZ obtained through the analysis of 27 serum samples belonging to morphine-dependent patients. The average determined CBZ in each group together with its standard deviation is clearly confirmed by the one that really happened for the patients in hospital, so that the concentration of CBZ increased in the time range of before surgery until 2 h after that and then remained nearly constant until 12 h after surgery. The tests of repeatability and recovery were further performed for two of the most complex samples with two matrix components interfering with CBZ peak. So, the RSD% of 5.1% was obtained for serum17 with three replicates and recovery value of 81.5% was obtained for serum 18 with spiked concentration of 1 μg mL^{-1} which could be considered as acceptable, compared with the above mentioned validation results and complexity of the samples. All these results demonstrated the applicability of the proposed method to human serum samples.

Table 4
Results of quantification of CBZ on three groups of morphine-dependent patients' serum samples using second-order calibration by MCR/ALS.

Group1 samples[a]	Concentration level ($\mu g\ mL^{-1}$)	Group 2 samples	Concentration level ($\mu g\ mL^{-1}$)	Group 3 samples	Concentration level ($\mu g\ mL^{-1}$)
Serum 1	0.11	Serum 10	0.64	Serum 19	0.18
Serum 2	0.06	Serum 11	0.74	Serum 20	0.22
Serum 3	0.06	Serum 12	0.49	Serum 21	0.62
Serum 4	0.02	Serum 13	0.40	Serum 22	0.94
Serum 5	0.10	Serum 14	0.16	Serum 23	0.20
Serum 6	0.11	Serum 15	0.19	Serum 24	0.78
Serum 7	0.08	Serum 16	0.34	Serum 25	0.40
Serum 8	0.16	Serum 17	0.96 [5.1][b]	Serum 26	0.38
Serum 9	0.06	Serum 18	0.19 (81.5)[c]	Serum 27	0.33
Mean [d]	0.08 ± 0.04		0.45 ± 0.27		0.45 ± 0.27

[a] Groups 1–3 samples, correspond to serum samples of morphine-dependent patients, before surgery, 2 h and 12 h after surgery, respectively.
[b] RSD (%) for three replicates analysis of sample 17 in square bracket.
[c] Recovery value calculated by spiking the concentration of 1 $\mu g\ mL^{-1}$ of CBZ into the sample 18.
[d] Mean concentration values and standard deviation obtained for determination of CBZ in each group of patients.

4. Conclusion

In the present study, second-order calibration of HPLC–DAD data using MCR/ALS for quantification of carbamazepine and phenobarbital in serum samples has been developed. Since the coelution problems of the analytes with the matrix constituents were resolved mainly using "mathematical separation" instead of conventional optimization of physico-chemical parameters, the chromatographic run time was dramatically reduced and small amounts of organic solvents was used while compared to the univariate methodologies. In addition, considering the inherent capability of MCR/ALS for handling chromatographic data with retention time shift, no preprocessing step was necessary in this work. The good quality of recovery and precision values in validation samples confirmed that the proposed method can be used efficiently for rapid, simple, sensitive and direct determination of carbamazepine and phenobarbiatal in different serum samples.

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