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Determination of carbamazepine in serum and pharmaceutical preparations using immobilization on a nylon support and fluorescence detection

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Abstract

A novel approach is presented for the spectrofluorimetric determination of the powerful anticonvulsant carbamazepine and its main metabolite in human serum. The strategy consists in the support of both compounds on a nylon membrane, and their subsequent determination through a solid-surface fluorescence methodology combined with a suitable chemometric analysis. The novelty of the present method lies in the fact that while carbamazepine does not fluoresce neither in solution nor supported on a variety of surfaces, significant emission signals are observed when it is supported on the nylon matrix, a property which has not been previously exploited by analysts. Multivariate calibration analysis was performed on three-way excitation-emission matrix data. The algorithms applied were: parallel factor analysis (PARAFAC), self-weighted alternating trilinear decomposition (SWATLD) and N-way partial least-squares regression (N-PLS). The results were compared with two-way calibration data analysed with partial least-squares regression (PLS-1). The methodology is highly specific, and it appears to be suitable for the routine monitoring of serum concentrations in patients receiving chronic therapy. In addition, the technique was satisfactorily applied to the determination of carbamazepine in pharmaceutical formulations.

Keywords: Carbamazepine; Spectrofluorimetry; Nylon membranes; Second-order calibration

1. Introduction

In recent years, our research group has been devoted to developing new analytical methods based on the emission of luminescence, with the aim of determining different drugs in pharmaceutical preparations and biological fluids [1–7]. Specifically, we have studied anti-inflammatory drugs in either cyclodextrin or micellar solutions, through the measurement of their fluorescence or phosphorescence signals. Due to the complexity of the analysed matrices, in several of these cases the determinations were accomplished with the aid of chemometric tools [4,6,7] or by combining the fluorimetric techniques with a solid-phase extraction [5,7].

In this paper, we present an original approach that allows the simultaneous determination of carbamazepine (CBZ), 5H-dibenzo[*b*,*f*]azepine-5-carboxamide (Fig. 1), and carbamazepine 10,11-epoxide (CBZ-EP, Fig. 1) in serum, and CBZ alone in pharmaceuticals, through a new fluorescence methodology. CBZ is a drug widely used for the treatment of epilepsy and psychiatric diseases [8]. It is almost completely metabolised in the body and only small traces are excreted unchanged in urine. The main CBZ metabolite, CBZ-EP, also possesses pharmacological activity as anticonvulsant [9] and therefore its determination in serum is as important as that for CBZ. Several methods have been proposed for the determination of both compounds, with special attention to those using chromatography [9–11]. In the strat-

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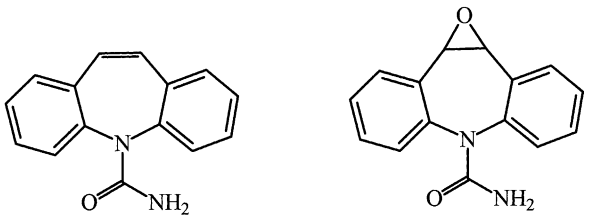

Fig. 1. Chemical structures of carbamazepine (CBZ) and carbamazepine 10,11 epoxide (CBZ-EP).

egy developed in the present report, CBZ and CBZ-EP are supported on a nylon membrane and are then spectrofluorimetrically determined on the surface of the solid substrate (CBZ is not fluorescent neither in solution nor supported on a variety of other surfaces). Since the emission spectra of both analytes are partially overlapped, their individual determination is performed by using three-way multivariate calibration applied to excitation-emission fluorescence matrices (EEFMs). In addition, and with the purpose of comparing the results provided by less complex spectroscopic information, two-way fluorescence emission data analysis is also applied.

To the extent of our literature search, this is the first report on a spectrofluorimetric method for the analysis of CBZ. It is also the first time that nylon is used as a support material for luminescence studies. With the exception of few recent reports, experimental applications of EEFMs in biological samples are scarce [4,6,7,12] and therefore this work represents an additional contribution which allows the comparison of the relative advantages and limitations of the different algorithms available for the resolution of complex samples.

2. Experimental

2.1. Reagents and solutions

Carbamazepine was obtained from Laboratorio Productor de Fármacos Medicinales (Santa Fe). Carbamazepine 10,11-epoxide was synthesized from carbamazepine and its purity was checked by standard methods. Hexane, benzene, cyclohexane, methanol, toluene and 1-pentanol were purchased from Merck (Darmstadt, Germany). All reagents were of high-purity grade and used as received. Aqueous solutions were made with doubly distilled water. Stock solutions of carbamazepine and carbamazepine 10,11-epoxide (c.a. 2000 $\mu g\,ml^{-1}$) were prepared in methanol. From these solutions, more diluted methanol working solutions were obtained.

The analysed pharmaceutical preparations were Carbamazepina (Denver Farma, Argentina), Tegretol tablets (Novartis, Argentina), and Tegretol oral suspension (Novartis, Argentina).

2.2. Apparatus

Fluorescence experiments were done on an Aminco Bowman Series 2 luminescence spectrometer equipped with a 150 W continuous xenon lamp, a 486 personal computer, a GPIB (IEEE-488) interface card for computer instrument communication. Data acquisition and data analysis were performed by use of the AB2 Series 2 software, running under OS/2 Version 4.0. Fluorescence measurements in solution were carried out with 1.00 cm quartz cells and slit widths of 8 nm.

2.3. Solid-surface fluorescence experiments

0.2 μm nylon membranes were obtained from Schleicher–Schuell (Dassel, Germany). These membranes were dissected into 8 mm disks and 7 μl of solutions containing either CBZ or CBZ and CBZ-EP in hexane were spotted. This volume completely covered the disk. After the solvent was evaporated by an air stream, the disk was placed in a laboratory-constructed solid substrate holder, and the fluorescence spectrum was collected at 90°. For the CBZ univariate determination, the excitation and emission wavelengths were adjusted at 280 and 400 nm, respectively. For CBZ-EP spectral measurements the optimum excitation and emission wavelengths were 256 and 430 nm, respectively. It was verified that the fluorescence intensity of CBZ-EP on the nylon membrane increases with the irradiation from the spectrofluorimeter xenon lamp at 256 nm, reaching a maximum after approximately three minutes and keeping constant for several days. It was also established that this irradiation does not affect the fluorescence emission of CBZ when both compounds are simultaneously present. Further investigations are required in order to fully explain this phenomenon, but they are not included in the scope of the present research. It is important to point out that reproducible spectra were obtained on each sample in all spectrofluorimetric experiments.

Prior to the determination of CBZ in pharmaceutical formulations, the corresponding calibration curve was constructed in order to obtain concentrations in the range 0–7 $\mu g\,ml^{-1}$. Solid-phase fluorescence measurements were subsequently carried out by the procedure described above.

2.4. Chemometric analysis

2.4.1. Theoretical considerations

Matrix data such as excitation-emission fluorescence matrices (EEFMs) can be adequately analysed by three-way trilinear methods [13]. Among the procedures normally used for the analysis of three-way trilinear data we selected those which yielded better results (according to our experience) [4,6]: parallel factor analysis (PARAFAC) [14,15], self-weighted alternating trilinear decomposition (SWATLD) [16] and N-way partial least-squares (N-PLS) [13].

The PARAFAC algorithm is based on a least-squares minimization [15], whereas SWATLD uses a procedure known as alternating trilinear decomposition [16]. The underlying theories have been recently reviewed [17]. These two multivariate methods allow relative concentrations of individual mixture components to be extracted directly, in the presence of any number of uncalibrated constituents. This property is known as the "second-order advantage" [18], a term referring to the tensor order of a single sample data matrix. In this work, these methods were implemented using an external calibration sample set, and the EEFM of the unknown was joined to this training sample set before decomposition of the data cube. In other words, the second-order advantage was obtained by constructing a joint model from both calibration and prediction instrumental responses.

On the other hand, in N-PLS the three-way array of calibration data is decomposed into a trilinear model, which however is not fitted in a least-squares sense [13]. According to the philosophy of the partial least-squares model, it intends to describe the covariance of the dependent (concentration) and the independent (response) variables. Although the N-PLS model is unique, the uniqueness in this case does not imply that real underlying phenomena like pure-analyte spectra can be recovered, because the model assumptions do not reflect any fundamental or theoretical model. Furthermore, N-PLS does not exploit the second-order advantage, because concentration information is unavailable for unknowns.

With the purpose of comparing the results obtained by using the above three-way algorithms with a method which uses first-order fluorescence emission data, partial least-squares regression (PLS-1) analysis was also applied [19]. The PLS-1 method involves a calibration step in which the relation between bi-dimensional emission spectra and analyte 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 unknown samples. Notice that no first-order method achieves a property comparable to the second-order advantage.

All calculations were done using MATLAB 5.3 [20]. A routine for PLS-1 was written in our laboratory following a previously known algorithm [21]. Those for N-PLS and PARAFAC are available on the internet (http://www.models.kvl.dk/source/) although a useful MATLAB graphical interface was developed in our laboratory for easy data manipulation and graphics presentation. This interface provides a simple mean of loading the data matrices into the MATLAB working space before running PARAFAC, SWATLD and N-PLS. The calculated excitation and emission profiles provided by the former two are separately plotted, in order to allow users to identify the analyte of interest. The pseudo-univariate calibration graph [22] corresponding to this particular component is then displayed. Once this is done, the results are conveniently shown in terms of predicted concentration and analytical figures of merit. The MATLAB interface code is available from the authors on request.

2.4.2. Experimental considerations

The experimental procedure corresponding to the three-way analysis was developed as follows: a calibration set of 11 samples was prepared by measuring different volumes of CBZ and CBZ-EP methanolic solutions into 1.00 ml calibrated flasks, evaporating the solvent with nitrogen and diluting with hexane to the mark. Nine of these samples corresponded to the concentrations provided by a full factorial design at three levels. This design is described as a 3^k-design, where the base 3 stands for the number of factor levels (the analysed concentrations) and k expresses the number of compounds. The three tested CBZ concentrations were: 2.24, 4.49 and 6.73 $\mu g\,ml^{-1}$, and those corresponding to CBZ-EP were: 2.30, 4.60 and 6.9 $\mu g\,ml^{-1}$. The remaining two samples contained only one of the studied analytes at an intermediate concentration ($C_{CBZ} = 4.49\,\mu g\,ml^{-1}$ and $C_{CBZEP} = 4.60\,\mu g\,ml^{-1}$). The nylon fluorescence measurements were carried out by the procedure described above. The EEFMs were then read and subjected to three-way data analysis. Excitation and emission wavelength ranges were 240–310 nm each 5 nm and 370–470 nm each 1 nm, respectively, i.e., a total of 3636 data points. The number of factors were selected as follows: by the so-called core consistency analysis in PARAFAC and SWATLD [13], and by leave-one-out cross-validation in N-PLS and PLS-1 [21]. In all cases two factors were employed. A test set of 10 validation samples, different from the calibration ones and following a random design, was prepared and processed in a similar way as the calibration samples.

For serum analysis, an additional set of 10 samples was prepared by spiking normal human sera with both CBZ and CBZ-EP methanolic solutions, obtaining random concentration levels in a range between 1 and 14 $\mu g\,ml^{-1}$. The latter was slightly wider than the therapeutic one (4–12 $\mu g\,ml^{-1}$) [9]. The extraction of the studied analytes from serum into several solvents was tested. It was unfortunate to find that hexane, which is the best solvent for spotting CBZ and CBZ-EP over the nylon (see further), is however a poorly efficient extracting solvent. Instead, quantitative recovery was obtained with a mixture of benzene and 1-pentanol (60:40). Thus, a volume of 100 μl of a given spiked serum sample was placed in a capped flask and 100 μl of benzene:1-pentanol (60:40) were added. The tube was shaken for 5 min and briefly centrifuged (5 min at 2000 × g). An aliquot of 50 μl of the organic phase was transferred to a flask, evaporated by use of dry nitrogen, and 100 μl of hexane were added to the flask in order to obtain final concentrations for CBZ and CBZ-EP which were within the linear ranges for the analytes. In this way, the original concentrations of both compounds in spiked and real sera (1–14 $\mu g\,ml^{-1}$) yield concentrations below 7 $\mu g\,ml^{-1}$ (the upper limit of the linear range).

These series of operations took only a few minutes, and assured that the analytes were completely transferred to an

hexane solution, which is needed for achieving maximum emission intensity. Each serum sample was prepared in triplicate. The EEFMs corresponding to the serum samples were then read on the nylon surface and subjected to three-way data analysis.

For the PLS-1 method, the same sample sets used in the three-way methods were employed. In this case, only the corresponding two dimensional spectra were read, in the optimum nylon emission conditions for CBZ and CBZ-EP (see above).

2.5. Reference methods

With the purpose of validating the developed method, CBZ was determined by fluorescence polarization immunoanalysis (FPIA) [23,24] and CBZ-EP by high-performance liquid chromatography (HPLC) [25,26]. FPIA measurements were done with an Abbott FPIA-TDx equipment at Hospital Provincial de Santa Fe, Argentina. HPLC was carried out on a liquid chromatograph equipped with a Waters 515 HPLC pump.

3. Results and discussion

3.1. Fluorescent properties of CBZ and CBZ-EP

It was verified that CBZ produces fluorescence emission in its solid state. However, in solutions of different solvents the CBZ signal is too low to be detected. Apparently, the excited singlet state of CBZ is able to undergo an efficient nonradiative deactivation when their molecules are exposed to free interactions with the solvent. Taking into account this fact, we evaluated the possibility that the studied compound recovers its fluorescent properties when it is supported on a solid surface. Therefore, the first challenge was to find a material where the fluorescent emission of CBZ could be displayed. Different media, such as octadecyl (C18AR) disks, cellulose, chromatographic aluminium sheets of silica gel, sodium acetate and nylon membranes, were probed as potentially supporting materials. From these exploratory experiments, it was concluded that fluorescence signals were only detected in the nylon membrane (Fig. 2A). Different commercial lots of either the same or different brands were tested, but the obtained results were very similar. Nylon membranes of 0.45 μm pore size were also analyzed, but in these cases the adsorption was lower and therefore the measured signals were less intense. Nylon (Fig. 3) is a naturally hydrophilic membrane, compatible with both aqueous and organic solvents. It is mechanically durable and is also thermally stable up to 50 °C. The amide groups in nylon are similar to that present in the CBZ molecule, and thus a possible explanation for the observed phenomenon is that the analyte interacts with the support, by replacing some of the hydrogen bonds of the nylon disk. This suggests the existence of a structural requirement of the support for ad-

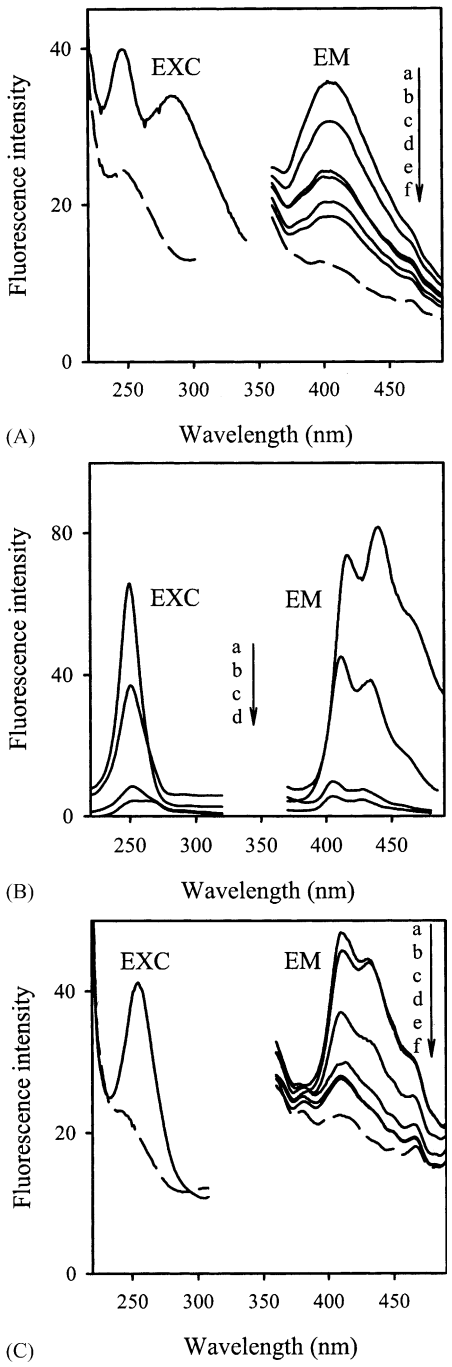

Fig. 2. (A) Excitation spectrum on a nylon membrane for carbamazepine in hexane, and emission spectra on a nylon membrane for carbamazepine in (a) hexane, (b) benzene, (c) cyclohexane, (d) methanol, (e) water and (f) toluene. $\lambda_{ex} = 280$ nm, $\lambda_{em} = 400$ nm. Photomultiplier tube (PMT) sensitivity = 310 V. (B) Excitation and emission spectra for 4.5 μg ml^{-1} carbamazepine 10,11-epoxide solution in (a) water, (b) methanol, (c) cyclohexane, (d) hexane. $\lambda_{ex} = 256$ nm, $\lambda_{em} = 430$ nm. PMT sensitivity = 600 V. (C) Excitation spectrum on a nylon membrane for carbamazepine 10,11-epoxide in hexane, and emission spectra on a nylon membrane for carbamazepine 10,11-epoxide in (a) cyclohexane, (b) hexane, (c) water, (d) methanol, (e) benzene and (f) toluene. $\lambda_{ex} = 256$ nm, $\lambda_{em} = 430$ nm. PMT sensitivity = 310 V. In the cases of (A) and (C), the nylon membranes were spotted with 7 μl of 11 μg ml^{-1} carbamazepine or carbamazepine 10,11-epoxide, respectively, and the dashed lines are the background membrane signals.

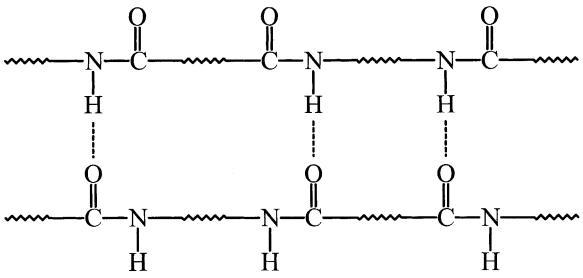

Fig. 3. Scheme of the nylon structure.

sorption and fluorescence emission from CBZ. Under the assumption that a single flatly adsorbed CBZ molecule occupies approximately 36 Å^2 of the nylon surface (estimated from molecular models), a volume of 7 μl containing an 8 μg ml^{-1} CBZ solution would be necessary to completely cover the 8 mm diameter disk. This concentration is larger than the 7 μg ml^{-1} limit (upper linear range of the calibration curve). If the porosity of the membrane is taken into account (leading to an increasing surface area) it seems unlikely that π-bonding between CBZ molecules occurs.

Several solvents were used to prepare CBZ solutions to be brought in contact with the nylon surface. As can be concluded from Fig. 2A, the solvent employed has a significant incidence in the intensity of the produced emission. This behavior has also been observed in other surfaces with different compounds [27–29]. Work is continuing to explain this fact.

In contrast to the CBZ system, intense fluorescence signals were observed in CBZ-BP solutions. Fig. 2B shows the fluorescence spectra for CBZ-EP in different solvents. As can be observed, the aqueous solution produces the highest intensity among the investigated systems, while fluorescence was not observed from neither benzene nor toluene solutions. CBZ-EP, like CBZ, bears an amide group in its structure and also shows fluorescent properties in nylon (Fig. 2C). The comparison of these spectra with those measured in solution (Fig. 2B) indicates that the nylon surface does not promote significant changes in the fluorescent behaviour of CBZ-EP.

3.2. Simultaneous determination of CBZ and CBZ-EP

Once the fluorescent properties of both CBZ and CBZ-EP in nylon were established, our aim was their simultaneous spectrofluorimetric determination in this solid matrix. Hexane solutions of CBZ and CBZ-EP were used for spotting the nylon membrane, because the former solvent offered the best signals in the studied systems (see Fig. 2A and C). Analyzing these figures, a partial spectral overlapping between both compounds is clearly observed, hence the need of employing a chemometric tool for data analysis.

Fig. 4 shows a three-dimensional plot of the EEFM, read over the nylon membrane, for a typical sample containing both CBZ and CBZ-EP, and Fig. 5A illustrates the screen

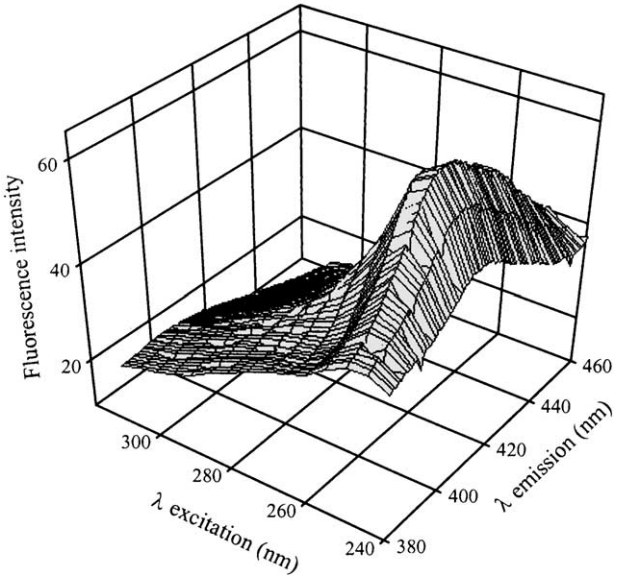

Fig. 4. Three-dimensional plot of the EEFM for a nylon membrane spotted with 7 μl of 5.30 μg ml^{-1} carbamazepine and 2.60 μg ml^{-1} carbamazepine 10,11-epoxide in hexane.

output of the computational program used in the present report, showing the individually calculated emission and excitation profiles. A comparison of the emission and excitation profiles obtained by decomposition of a particular data cube (Fig. 5A) with the experimental ones (Fig. 5B) shows a satisfactory agreement.

As was indicated in the experimental section, these spectra assist in matching the correct sample components when they are compared with those experimentally obtained. In our case, the similarity between the calculated and experimental spectra is apparent. In Table 1, the results are shown corresponding to a preliminary quantitative study, carried out in the validation set of synthetic samples (see Section 2). A rapid inspection of the values obtained by applying both two and three-way data seems to indicate that all methods yield accurate results. However, the dispersion of those corresponding to PLS-1 is very large: the standard deviation of the average recovery (reported in Table 1) is in this case ca. 10%.

3.3. Serum samples

Table 2 shows the nominal and predicted CBZ and CBZ-EP concentrations in spiked serum samples, after the treatment indicated in the Experimental Section, and Table 3 displays the corresponding statistical analysis. An examination of both tables indicates that the recoveries and the statistical parameters are good, especially those obtained by PARAFAC and SWATLD. While the three-way calibration procedure using PARAFAC and SWATLD takes into account the effect of constituents not present in the calibration set (recall that this gives rise to the second-order advantage), N-PLS and PLS-1 are sensitive to compounds potentially present in the serum extract and which produce

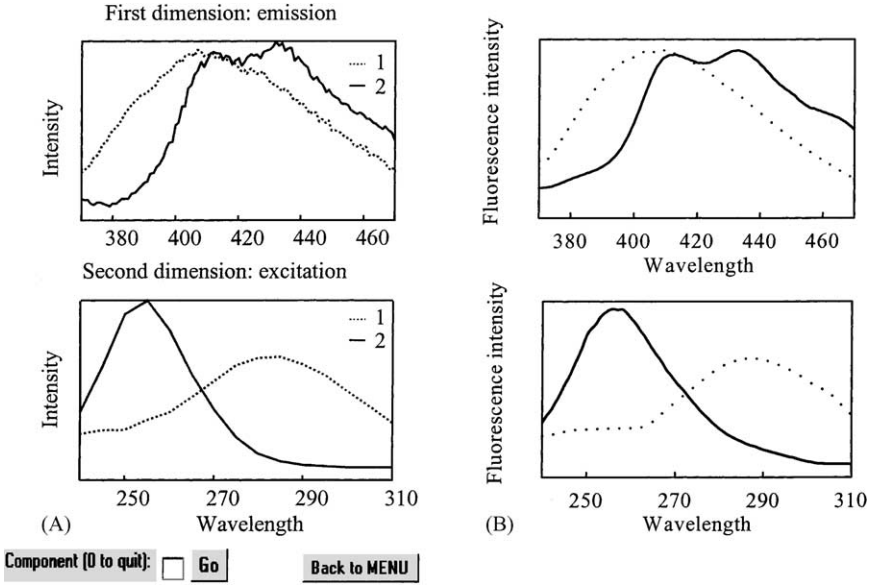

Fig. 5. (A) Screen output for the individually calculated emission and excitation normalized profiles for carbamazepine (1) and carbamazepine 10,11-epoxide (2). (B) The corresponding experimental background-corrected emission (top) and excitation (bottom) spectra, normalized to unit length.

Table 1
Determination of CBZ and CBZ-EP by two- and three-way data analysis of the validation set of synthetic samples[a]

	Actual values ($\mu g\,ml^{-1}$)	Two-way data				Three-way data			
		PLS-1		PARAFAC		SWATLD		N-PLS	
		Found	Recovery	Found	Recovery	Found	Recovery	Found	Recovery
CBZ	1.99	2.04 (7)	102	1.9 (1)	95	2.0 (1)	101	2.2 (1)	108
CBZ-EP	6.50	6.28 (4)	97	6.56 (6)	101	6.57 (5)	101	6.49 (7)	100
CBZ	2.65	2.64 (6)	100	2.7 (1)	102	2.79 (8)	105	2.6 (1)	100
CBZ-EP	3.90	3.50 (3)	90	3.87 (5)	99	3.88 (4)	99	3.82 (6)	98
CBZ	6.12	6.26 (7)	102	5.97 (9)	98	5.97 (8)	98	5.9 (1)	97
CBZ-EP	6.10	5.60 (4)	92	5.53 (5)	91	5.52 (5)	90	5.40 (7)	89
CBZ	5.30	5.15 (7)	97	5.3 (1)	100	5.23 (8)	99	5.4 (1)	102
CBZ-EP	2.60	3.14 (3)	121	2.96 (5)	114	2.95 (5)	113	3.02 (5)	116
CBZ	6.63	4.99 (7)	75	7.7 (1)	116	7.7 (1)	116	7.7 (1)	116
CBZ-EP	1.95	2.43 (4)	125	2.11 (6)	108	2.12 (5)	109	2.11 (6)	108
CBZ	1.84	1.4 (2)	76	2.07 (8)	112	2.07 (7)	112	2.18 (8)	118
CBZ-EP	5.70	6.0 (2)	105	6.65 (7)	117	6.65 (6)	116	6.48 (9)	114
CBZ	5.76	5.8 (2)	101	5.72 (8)	99	5.72 (7)	99	5.85 (9)	102
CBZ-EP	1.82	2.0 (2)	110	1.70 (7)	93	1.69 (6)	93	1.90 (8)	104
CBZ	3.46	3.6 (1)	104	3.59 (8)	104	3.60 (6)	104	3.54 (7)	102
CBZ-EP	3.42	3.1 (2)	91	3.44 (6)	101	3.41 (5)	100	3.29 (7)	96
CBZ	1.38	1.5 (2)	109	1.5 (1)	109	1.54 (8)	112	1.63 (9)	118
CBZ-EP	3.42	3.4 (1)	99	3.74 (6)	109	3.76 (5)	110	3.58 (7)	105
CBZ	2.02	1.8 (5)	89	2.2 (1)	109	2.21 (8)	109	2.1 (1)	104
CBZ-EP	2.04	1.76 (5)	86	2.26 (6)	111	2.31 (5)	113	2.16 (7)	106
CBZ average recovery[b]		95 ± 9		104 ± 5		105 ± 5		107 ± 6	
CBZ-EP average recovery[b]		102 ± 10		104 ± 6		104 ± 7		104 ± 6	

[a] The found values and recoveries are given in $\mu g\,ml^{-1}$ and percent, respectively. Standard deviations (in parenthesis) were calculated according to references [35] (PLS-1), [34] (PARAFAC and SWATLD) and [36] (N-PLS).

[b] These values are given in percent and the confidence intervals (CI) are also indicated. The CI values were calculated as: $CI = ts/\sqrt{N}$, where t is the Student coefficient, s the standard deviation and N the number of data.

Table 2
Serum recovery results[a]

	Actual values ($\mu g\,ml^{-1}$)	Two-way data				Three-way data			
		PLS-1		PARAFAC		SWATLD		N-PLS	
		Found	Recovery	Found	Recovery	Found	Recovery	Found	Recovery
CBZ	3.84	3.4 (9)	89	3.4 (2)	89	3.2 (2)	83	5.0 (2)	130
CBZ-EP	1.58	1.4 (2)	89	1.7 (1)	108	1.7 (1)	108	1.8 (1)	114
CBZ	12.1	13.4 (2)	111	12.1 (1)	100	11.8 (2)	98	12.4 (2)	102
CBZ-EP	9.00	10.1 (1)	112	9.6 (1)	107	9.92 (8)	110	9.6 (1)	107
CBZ	9.00	8.8 (6)	98	9.2 (1)	102	8.9 (1)	99	9.0 (2)	100
CBZ-EP	11.9	11.4 (1)	96	10.7 (1)	90	10.8 (1)	91	10.5 (1)	88
CBZ	12.5	10.8 (4)	86	13.4 (2)	107	12.3 (1)	98	13.4 (2)	107
CBZ-EP	12.4	12.7 (1)	102	13.1 (1)	106	13.4 (1)	108	12.9 (1)	104
CBZ	12.5	10.1 (1)	81	11.9 (2)	95	11.7 (1)	94	12.4 (2)	99
CBZ-EP	6.22	8.12 (6)	131	5.2 (1)	84	5.2 (1)	84	5.2 (2)	84
CBZ	9.80	9.1 (1)	93	11.0 (2)	112	10.9 (2)	111	11.2 (2)	114
CBZ-EP	6.00	8.56 (6)	143	5.9 (1)	98	5.9 (1)	98	6.0 (2)	100
CBZ	6.52	5.9 (1)	90	6.9 (2)	106	6.7 (1)	103	7.1 (2)	109
CBZ-EP	9.70	9.78 (6)	101	8.9 (1)	92	8.90 (8)	92	8.9 (1)	92
CBZ	12.5	11 (1)	88	11.9 (1)	95	11.3 (2)	90	12.8 (2)	102
CBZ-EP	3.10	2.36 (6)	76	3.1 (1)	100	2.0 (1)	65	3.1 (1)	100
CBZ	3.14	2.2 (1)	70	3.0 (2)	96	3.1 (1)	99	3.2 (2)	102
CBZ-EP	12.4	15.8 (1)	127	10.9 (1)	88	10.9 (1)	88	10.6 (1)	85
CBZ	3.40	4.2 (8)	124	3.5 (1)	103	3.2 (2)	94	6.2 (2)	182
CBZ-EP	3.36	4.62 (8)	138	3.3 (1)	98	3.4 (1)	101	3.2 (1)	95

[a] The found values and recoveries are given in $\mu g\,ml^{-1}$ and percent, respectively. Standard deviations (in parenthesis) were calculated according to references [35] (PLS-1), [34] (PARAFAC and SWATLD) and [36] (N-PLS).

fluorescence emission under the working conditions. Due to the possible affinity of the nylon membrane for other molecules containing NH groups, a possible interference from molecules which bear this type of group should be analyzed. For example, molecules such as amino acids should, in principle, be considered. Among the amino acids present in serum, only tryptophan and tyrosine show appreciable fluorescence emission [30]. According to our results, both amino acids were able to fluoresce in the nylon membrane when spotted from aqueous solutions. The concentrations tested were those usually found in healthy subjects (between 1×10^{-4} and 1×10^{-5} M) [31,32]. Emission bands on the solid surface were detected at wavelengths considerably far from those of CBZ and CBZ-EP (about 330 and 300 nm for tryptophan and tyrosine, respectively). In addition, these amino acids are not completely extracted during the treatment of the serum with the organic solvent, and therefore their possible interference for the PLS-1 methods seems unlikely. However, the presence of other interferences cannot be discarded due to the complexity of human serum.

In order to get further insight into the accuracy and precision of the different algorithms herein analysed, linear regression analysis of nominal versus found concentration values 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 [33]. Fig. 6 shows the EJCR plots for the employed chemometric methods. As can be seen, although the ellipses contain the theoretical ($a = 0$, $b = 1$) point, that corresponding to PLS-1 and N-PLS show a large (and undesirable) size. The conclusion is that these latter algorithms produce less precise results. This result may be due to the effect of the leverage parameter, which gives the position of the unknown sample in the calibration space, i.e., relative to the training set of samples. Low-leverage samples, i.e., those which are close to the center of the calibration, are

Table 3
Validation statistical results for the simultaneous determination of CBZ and CBZ-EP in serum by two-and three-way data analysis[a]

	PLS-1			PARAFAC			SWATLD			N-PLS		
	RMSEP ($\mu g\,ml^{-1}$)	REP (%)	R^2	RMSEP ($\mu g\,ml^{-1}$)	REP (%)	R^2	RMSEP ($\mu g\,ml^{-1}$)	REP (%)	R^2	RMSEP ($\mu g\,ml^{-1}$)	REP (%)	R^2
CBZ	1.3	15	0.893	0.61	7.1	0.976	0.66	7.7	0.973	1.2	14	0.913
CBZ-EP	1.7	22	0.830	0.83	11	0.958	0.95	12	0.945	0.92	12	0.949

[a] RMSEP (root mean square error of prediction) $= [(1/I-1)\sum_1^I (c_{act} - c_{pred})^2]^{1/2}$ where I is the number of prediction samples, c_{act} and c_{pred} are the actual and predicted concentrations, respectively. REP (relative error of prediction) $= (100/\bar{c}) \times$ RMSEP where $\bar{c}$ is the average component concentration. R^2 (square correlation coefficient) $= 1 - (\sum_1^I (c_{act} - c_{pred})^2 / \sum_1^I (c_{act} - \bar{c}))$.

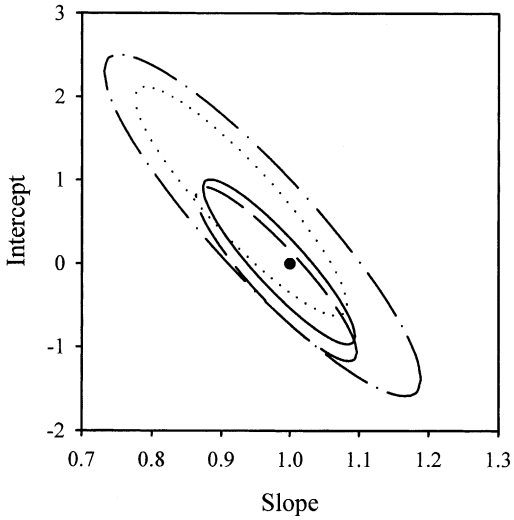

Fig. 6. Elliptical joint confidence regions for the slope and intercept corresponding to regressions of predicted vs. actual concentrations of carbamazepine and carbamazepine 10,11-epoxide, applying PARAFAC (solid lines), SWATLD (dashed lines), N-PLS (doffed lines) and PLS-1 (solid-dotted lines). The black circle marks the theoretical ($a = 0$, $b = 1$) point.

more precisely determined than high-leverage samples. In PARAFAC the leverage does only depend on the concentration of the analyte of interest in the unknown and in the calibration samples, regardless of the concentrations of other components [34]. In contrast, both in PLS-1 and N-PLS, the leverage depends on all sample constituents simultaneously [34]. Thus, PARAFAC leverages are correspondingly smaller than in PLS, leading to an improved precision when the former methodology is applied.

Finally, with the purpose of evaluating the proposed method in real samples, serum of patients administered with CBZ were investigated. The values obtained are statistically comparable to those provided by the reference techniques (Table 4), when a paired *t*-test is applied at a 95% confidence level. These results suggest that the presence of endogenous or exogenous compounds in serum causes no serious interference when applying the proposed method.

Table 5
Calibration results for CBZ[a]

Parameters	
Linear range ($\mu g\,ml^{-1}$)	1.2–7.0
Correlation coefficient	0.996
Intercept (*h*)	19.3 (0.1)
Slope (*m*)	1.96 (0.04)
γ[b] ($\mu g\,ml^{-1}$)	5.3
L.O.D[c] ($\mu g\,ml^{-1}$)	0.4
L.O.Q[d] ($\mu g\,ml^{-1}$)	1.2
R.S.D[e] (%)	7.4 (1.6 $\mu g\,ml^{-1}$)
	2.8 (4.4 $\mu g\,ml^{-1}$)
	2.1 (6.0 $\mu g\,ml^{-1}$)

[a] The number of data for each calibration curve corresponds to 7 different concentration levels, with three replicates for each level ($N = 21$).
[b] Analytical sensitivity: $\gamma = m/S_s$ where S_s is the standard deviation of the regression residuals [37].
[c] Limit of detection: calculated according to Clayton [38] using 0.05 as assurance probabilities.
[d] Limit of quantification: calculated as 3 × L.O.D.
[e] Relative standard deviation. The CBZ concentrations are indicated between parenthesis. In all cases three replicates were measured.

3.4. Determination of CBZ in pharmaceuticals

CBZ can be easily determined in commercial formulations using the method herein described. This univariate spectrofluorometric determination of CBZ in nylon involves the construction of the corresponding calibration curve. The equation for the calibration graph is: $F = h + mC$, where F is the fluorescence intensity (in arbitrary units) and C the concentration of CBZ. The figures of merit obtained are displayed in Table 5, which demonstrate the good performance of the calibration. The pharmaceutical preparations investigated were those presented in Table 6, and the values obtained indicate that the applied method yields satisfactory results in all studied cases. The absence of interference from the excipients was confirmed by the very good recoveries, all in the range recommended by Pharmacopoeias for this type of analyses (see Table 6).

Table 4
Results of analyses on patient serum samples[a]

Serum		Reference method[b]	Two-way data	Three-way data		
			PLS-1	PARAFAC	SWATLD	N-PLS
I	CBZ	8.73	8.6 (5)	7.8 (1)	5.2 (1)	8.0 (1)
	CBZ-EP	1.60	1.4 (1)	1.70 (7)	1.36 (6)	1.86 (7)
II	CBZ	7.23	6.8 (5)	6.0 (1)	5.4 (1)	7.2 (1)
	CBZ-EP	1.36	1.4 (1)	1.42 (7)	1.46 (7)	1.56 (7)
III	CBZ	5.88	6.0 (5)	5.9 (1)	5.7 (1)	7.0 (1)
	CBZ-EP	1.12	0.8 (1)	1.18 (7)	1.32 (7)	1.42 (8)

[a] The values are given in $\mu g\,ml^{-1}$.
[b] FPIA for CBZ and HLPC for CBZ-EP (see Section 2).

Table 6
Determination of CBZ in pharmaceutical preparations[a]

Preparation	Composition	CBZ found[b]
Carbamazepina, Denver Farma (tablet)	Carbamazepine 200 mg, excipients	201 (8) mg [101%]
Tegretol, Novartis (tablet)	Carbamazepine 200 mg, excipients	208 (1) mg [104%]
Tegretol, Novartis (oral suspension)	Carbamazepine 2.00 g/100 ml, excipients	2.03 (0.05) mg/100 ml [102%]

[a] The United States Pharmacopoeia recommends the content of CBZ in the ranges 90.0–110.0% (oral suspension) and 92.0–108.8% (tablet) [39]. For the British Pharmacopoeia the range is 95.0–105.9% for tablets [40].

[b] Average of three determinations. Standard deviations are given between parenthesis and recoveries between square brackets. Recoveries were calculated considering that the preparations contain the amount reported by the manufacturing laboratories.

4. Conclusions

A simple method for the determination of carbamazepine based on its fluorescence emission when it is supported on a nylon membrane has been developed. In serum samples, carbamazepine was simultaneously determined together with its main metabolite, carbamazepine 10,11-epoxide. In comparing the performances of the multivariate algorithms used for their quantification, one may conclude that better results were obtained by using three-way calibration data. Among these algorithms, both PARAFAC and SWATLD yielded the most precise results. Carbamazepine alone was also determined in pharmaceutical preparations using a univariate calibration, and satisfactory results were obtained in all investigated cases. The approach herein developed was conceived as an alternative for the widely used chromatographic methods. In this case, the use of a large amounts of organic solvents is not required, and the experimental procedure, even including an extraction step, is very simple. In comparison with other supporting materials, nylon has the following advantages: (1) it is easily available and cheap, (2) a small amount of support is used in each experimental determination and (3) it does not require pre-treatments. Therefore, the use of nylon considerably reduces the analysis cost for numerous samples. As a consequence of the phenomenon herein studied (the affinity of nylon for the amide groups) a new line of investigation including the determination of relevant compounds bearing this type of group can be started and further developed.

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