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Liquid chromatographic determination of oxcarbazepine and its metabolites in plasma of epileptic patients after solid-phase extraction

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Abstract

A method based on high-performance liquid chromatography with UV detection in combination with solid-phase extraction for sample pretreatment has been developed for the simultaneous analysis of the antiepileptic drug oxcarbazepine and its main metabolites in human plasma. The extraction of the analytes from plasma samples was carried out by means of a selective solid-phase extraction procedure using hydrophilic–lipophilic balance cartridges. The separation was obtained on a reversed-phase column (C_{18}, 150×4.6 mm I.D., 5 μm) using a phosphate buffer–acetonitrile–methanol–triethylamine mixture (final apparent pH* 3.5) as the mobile phase. Under these chromatographic conditions, oxcarbazepine and its metabolites 10,11-dihydro-10-hydroxycarbamazepine, 10,11-dihydro-10,11-dihydroxycarbamazepine and the internal standard are baseline separated in less than 9 min. The extraction yield values were >94% for all analytes and the precision, expressed by the RSD%, was in the low percentage range. For the entire method, including sample pre-treatment and HPLC determination, the linearity of the calibration lines, expressed by the linear correlation coefficient, was better than 0.995; the limit of quantitation was 15 ng ml^{-1}. The method was applied to plasma samples from patients undergoing chronic treatment with oxcarbazepine, both in monotherapy and in polytherapy. Based on the analytical parameters precision, accuracy, limit of quantitation and analysis time the method is suitable for routine application in therapeutic drug monitoring.

Keywords: Oxcarbazepine

1. Introduction

Oxcarbazepine (10,11-dihydro-10-oxo-5H-dibenzo-[*b*,*f*]azepine-5-carboxamide, OXCBZ, Fig. 1a) is a recent antiepileptic drug, introduced onto the market during the Nineties. OXCBZ is structurally related to carbamazepine (5H-dibenzo[*b*,*f*]azepine-5-carboxamide, CBZ, Fig. 1b), of which it is a keto-derivative [1]. These drugs share many chemical and pharmacological properties: both are highly lipophilic and neutral at most pH values, and both are used mainly for the treatment of partial seizures and generalised tonic–clonic seizures [2,3]. However, OXCBZ is often used for patients who are intolerant to CBZ

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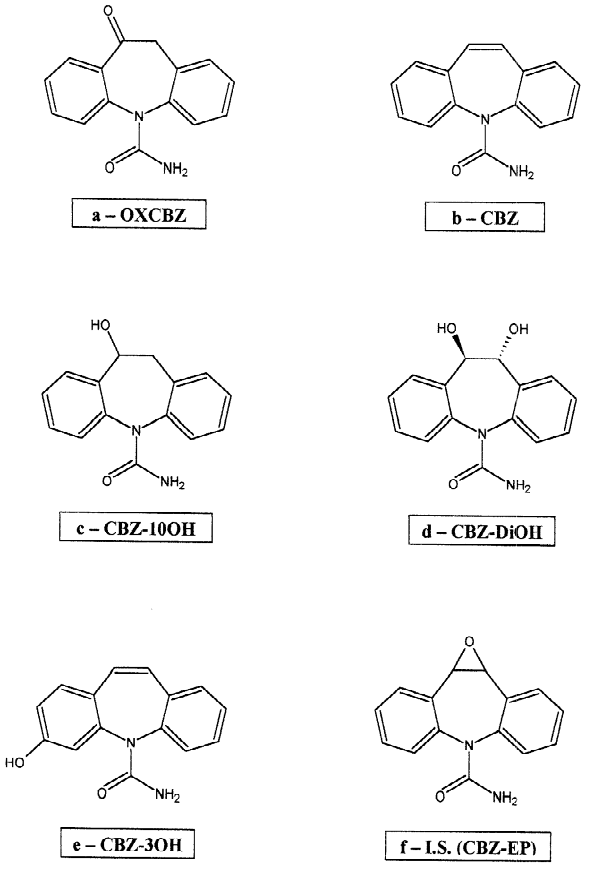

Fig. 1. Chemical structures of OXCBZ (a), CBZ (b), CBZ-10OH (c), CBZ-DiOH (d), CBZ-3OH (e) and CBZ-EP (f) used as the I.S.

treatment, because it seems to be safer and less prone to cause undesired effects. In fact, although OXCBZ tends to cause clinical side effects similar to those of CBZ (such as diplopia, ataxia, somnolence, equilibrium impairment), their incidence and severity are reduced [4,5]. Idiosyncratic reactions seem to be less common with OXCBZ than with CBZ [6]. Moreover, OXCBZ is relatively free of clinically relevant drug interactions [7], thus facilitating its use during polytherapy.

In human, OXCBZ is reduced rapidly after oral absorption to its therapeutically active metabolite 10,11-dihydro-10-hydroxy-5H-dibenzo[*b*,*f*]azepine-5-carboxamide (CBZ-10OH, Fig. 1c) [8,9]. This metabolic pathway probably depends on a non-inducible aldo-keto-reductase [10]; CBZ-10OH is mainly eliminated into the urine after glucuronidation [11,12]. Both OXCBZ and CBZ-10OH have anticonvulsant efficacy similar to CBZ in standard animal seizure models [13]. A small percentage of CBZ-10OH is metabolised to *trans*-10,11-dihydro-10,11-dihydroxy-5H-dibenzo[*b*,*f*]azepine-5-carboxamide (CBZ-DiOH, Fig. 1d) which does not show pharmacological activity [14]. 3-Hydroxycarbamazepine (CBZ-3OH, Fig. 1e) has been also reported to be a minor metabolite of OXCBZ [12].

The exact mechanism of action of the active species is currently unknown; however, electrophysiological studies evidenced that it blocks voltage-dependent sodium channels and consequently stabilises excitable membranes. Calcium channel modulation could contribute to the antiepileptic action [15,16].

Oxcarbazepine is administered orally as filmed tablets (Trileptal®, Tolep® in Italy) containing 150–600 mg of the drug. The usual daily doses range from 600 to 1500 mg, which result in OXCBZ plasma levels in the hundreds of nanograms per millilitre range and in CBZ-10OH plasma levels in the tens of micrograms per millilitre range [17]. Consequently, CBZ-10OH represents the main active compound during chronic OXCBZ therapy in humans.

Chronic antiepileptic treatment may last for years or even for the whole patient's life; therefore, in order to minimise unwanted long-term effects, the therapy must be carefully individualised in each patient, particularly in pediatric patients and in the aged. Even if OXCBZ is usually well tolerated, adverse effects are still possible during treatment [18,19], and its recent introduction in therapy means that its pharmaco-toxicological profile is not fully understood. For these reasons, therapeutic drug monitoring (TDM) of patients undergoing treatment with OXCBZ, and especially pediatric patients, is advisable.

In the past, the most common methods for OXCBZ determination in biological fluids were gas chromatographic methods [20,21]. Currently, HPLC with spectrophotometric detector is much more widespread [12,22–32]; some HPLC–UV methods analyse only CBZ-10OH [27,29], or both OXCBZ and CBZ-10OH [22,31], and some are enantioselective [28–30]. These last methods, however, require expensive chiral columns and lengthy analysis times;

HPLC methods with microcolumns require special instrumentation as well [24,25], some other methods have poor sensitivity [26,31]. Two of our recent papers demonstrate the separation of OXCBZ and its metabolites by capillary electrokinetic chromatography using octakis-6-sulfato-γ-cyclodextrin as an additive [33] and by microemulsion micellar electrokinetic chromatography [34]. These papers, however, do not deal with the determination of the analytes in biological fluids.

The aim of this investigation was the implementation of a reliable HPLC method with UV detection for the simultaneous determination of OXCBZ and its metabolites, namely CBZ-10OH, CBZ-DiOH and CBZ-3OH, in the plasma of epileptic patients. The method should be suitable for therapeutic drug monitoring of patients subjected to either monotherapy or polytherapy. Solid-phase extraction (SPE) was chosen for the sample pre-treatment, because this technique is more feasible and less polluting than the traditional liquid–liquid extraction used in other methods [12,28] and allows for high extraction yields with good selectivity.

2. Experimental

2.1. Chemicals and solutions

Oxcarbazepine (OXCBZ), its metabolites 10,11-dihydro-10-hydroxycarbamazepine (CBZ-10OH), 10,11-dihydro-10,11-dihydroxycarbamazepine (CBZ-DiOH) and 3-hydroxycarbamazepine (CBZ-3OH), and 10,11-dihydro-10,11-epoxycarbamazepine (CBZ-EP, Fig. 1f) used as the internal standard (I.S.) were kindly provided by Novartis Pharma (Basel, Switzerland). The purity of these standard compounds was higher than 99%.

Acetonitrile and methanol for HPLC, 85% (w/w) phosphoric acid and 37% (w/w) hydrochloric acid, pure for analysis, were purchased from Carlo Erba (Milan, Italy). Triethylamine, analytical grade, was purchased from Fluka (Buchs, Switzerland), dibenzepine (pure compound) was from Sigma (St. Louis, MO, USA).

Ultrapure water (18.2 MΩ cm) was obtained by means of a Milli-Q apparatus from Millipore (Milford, MA, USA).

Stock solutions (1 mg ml^{-1}) of the analytes and the I.S. were prepared by dissolving each compound in methanol. The stock solutions were stable for at least 2 months at −20 °C. Standard solutions were prepared daily from stock solutions by dilution with the mobile phase.

2.2. Apparatus and chromatographic conditions

The chromatographic apparatus consisted of a Jasco (Tokyo, Japan) PU-980 isocratic pump and a Jasco UV-975 spectrophotometric detector. Data were elaborated by means of Varian (Harbor City, CA, USA) Star Chromatography software.

The stationary phase was a Varian Microsorb MV Rainin reversed-phase column (C_{18}, 150×4.6 mm I.D., 5 μm) with a Varian C_{18} precolumn (30×4.6 mm I.D., 5 μm). The mobile phase (flow-rate, 1 ml min^{-1}) was a 15 m*M* phosphate buffer–methanol–acetonitrile–triethylamine (62.25:20.0:17.5:0.25, v/v/v/v) mixture, brought to pH* 3.5 with 1 *M* HCl. The detector was set at 237 nm.

2.3. Human plasma sampling

To validate the method, anonymous residual plasma samples from patients chronically treated with OXCBZ were obtained from the Laboratory of Clinical Neuropharmacology, Department of Neurological Sciences, University of Bologna. Patients' attendance at the TDM service was strictly dependent on clinical needs, and no extra blood sampling was performed for this study.

Blood samples were collected at 08.00 h in the morning, just before the first daily drug administration, into heparinised tubes (8–10 IU heparin ml^{-1} blood), and centrifuged at 1400 *g* for 20 min. The supernatant plasma was transferred into test tubes and frozen at −20 °C until analysis.

Frozen, drug-free, plasma for calibration curves was obtained from the hospital blood bank and thawed at room temperature before use.

2.4. Solid-phase extraction procedure (SPE)

The analytes were extracted from plasma samples by means of SPE. For this purpose, Waters (Milford, MA, USA) Oasis® HLB cartridges (30 mg, 1 ml) were used.

The SPE procedure was carried out on a Varian

VacElut apparatus according to the following steps. (i) Conditioning: 1 ml of methanol twice. (ii) Equilibration: 1 ml of water twice. (iii) Loading: 200 μl of plasma + 500 μl of water + 50 μl of 8.0 μg ml^{-1} I.S. solution (or 50 μl of analytes and I.S. solution, for plasma spiking). (iv) Washing: 1 ml of 100 m*M*, pH 11.0, phosphate buffer–methanol (80:20, v/v) mixture, twice; then 1 ml of water. (v) Cartridge drying for 30 s at −60 kPa. (vi) Elution: 500 μl of methanol. (vii) Cartridge drying for 30 s at −60 kPa.

The eluate was then dried under vacuum (rotary evaporator), redissolved in 800 μl of the mobile phase and injected into the HPLC.

2.5. Method validation

2.5.1. Calibration curves

Aliquots of 50 μl of analyte standard solutions at 10 different concentrations containing the I.S. at constant concentration were added to 200 μl of blank plasma. The resulting plasma concentration ranges were: 100–4000 ng ml^{-1} for OXCBZ, 1.0–40.0 μg ml^{-1} for CBZ-10OH and 0.3–12.0 μg ml^{-1} for CBZ-DiOH; 2.0 μg ml^{-1} (constant) for the I.S. (corresponding to on-column concentration ranges of 25–1000 ng ml^{-1} for OXCBZ, 0.25–10.00 μg ml^{-1} for CBZ-10OH and 75–3000 ng ml^{-1} for CBZ-DiOH; 500 ng ml^{-1} for the I.S.). The resulting mixture was subjected to the sample pre-treatment procedure and injected into the HPLC. The procedure was carried out in triplicate for each concentration, the analyte–I.S. peak area ratios obtained were plotted against the corresponding concentrations of the analytes (expressed as ng ml^{-1}) and the calibration curves constructed by means of the least-squares method. One stock solution was used for each replicate; different working solutions were prepared from the stocks and added to the blank plasma samples to obtain the different concentrations.

The values of LOQ and LOD were calculated according to the Crystal City [35] guidelines as 10 and three times the baseline noise, respectively.

2.5.2. Extraction yield (absolute recovery)

The procedure was the same as that described in Section 2.5.1, except the points were at three different concentrations, corresponding to the upper limit, lower limit and middle point of each calibration curve (i.e., plasma concentrations of 100, 1000 and 4000 ng ml^{-1} for OXCBZ, 1.00, 10.0 and 40.0 μg ml^{-1} for CBZ-10OH and 300, 3000 and 12 000 ng ml^{-1} for CBZ-DiOH, corresponding to on-column concentrations of 25, 250 and 1000 ng ml^{-1} for OXCBZ, 0.25, 2.50 and 10.0 μg ml^{-1} for CBZ-10OH and 75, 750 and 3000 ng ml^{-1} for CBZ-DiOH). The analyte–I.S. peak height ratio was compared to the ratio obtained injecting standard solutions at the same theoretical concentrations, and the percent recovery calculated.

2.5.3. Precision

The assays described in Section 2.5.2 were repeated six times within the same day to obtain the repeatability (intra-day precision) and six times over different days to obtain the intermediate precision (inter-day precision) of the method, both expressed as RSD% values.

2.5.4. Accuracy

Accuracy was evaluated by means of recovery assays. The assays described in Section 2.5.2 were carried out adding standard solutions of the analytes and the I.S. to plasma samples taken from patients subjected to treatment with Tolep® tablets instead of blank plasma. The amounts of analytes added were the same as those for absolute recovery assays, except the plasma high-level concentrations were 2.00 μg ml^{-1} for OXCBZ, 20.0 μg ml^{-1} for CBZ-10OH and 6.00 μg ml^{-1} for CBZ-DiOH (corresponding to on-column concentrations of 500 ng ml^{-1} for OXCBZ, 5000 ng ml^{-1} for CBZ-10OH and 1500 ng ml^{-1} for CBZ-DiOH). The assays were repeated six times during the same day to obtain the mean recovery and the precision.

3. Results and discussion

3.1. Chromatographic conditions

Since OXCBZ (Fig. 1a) has chemical structure and properties rather similar to those of CBZ (Fig. 1b), our recent paper on the determination of CBZ and five of its metabolites in human plasma [36] was

the starting point for the development of the present HPLC method. The CBZ method used a C_8 reversed-phase column (150×4.6 mm I.D., 5 μm) and a mobile phase composed of acetonitrile–methanol–15 m*M* phosphate buffer containing 18 m*M* triethylamine adjusted to pH 1.9 (17.5:20:62.5, v/v/v); final pH* was 2.5. UV detection was carried out at 237 nm. Under these conditions the chromatographic analysis of standard solutions containing the present analytes showed a good separation of OXCBZ from its metabolites CBZ-10OH and CBZ-DiOH, with the following retention times: OXCBZ, 10.8 min; CBZ-10OH, 6.5 min, CBZ-DiOH, 5.0 min. The corresponding capacity factors k', were: 7.64 for OXCBZ; 4.22 for CBZ-10OH; and 3.00 for CBZ-DiOH.

In order to ascertain whether a faster separation is possible (an important fact for routine analysis), several variations to the mobile phase were undertaken: changes in the concentration of the buffer, in the relative percentages of aqueous buffer and organic modifier, in the methanol–acetonitrile ratio, in the apparent pH* value. The only beneficial variations were those regarding the pH* of the mobile phase: the effect of pH* values ranging from 2.5 to 4.5 on peak resolution were studied, and the results are shown in Fig. 2. As can be seen, highest resolution values are obtained at an apparent pH value of 3.5, which has the added advantage of reducing the retention times of the analytes and was thus chosen for all further assays. However, none of these modifications allowed to completely separate CBZ-3OH from OXCBZ. Since CBZ-3OH is reported as a metabolite of OXCBZ [12] only at very low concentrations it should not interfere with the quantitation of OXCB (usually present at levels higher than 100 ng ml^{-1}).

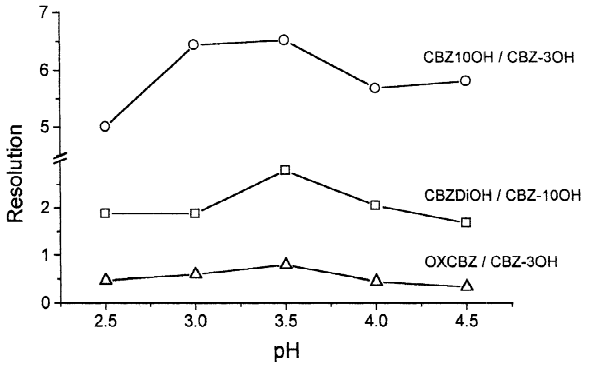

Fig. 2. Resolution values of the pairs of analytes as a function of pH. Resolution was calculated as $R_S = 2\ (t_2 - t_1)/(W_1 + W_2)$ for completely resolved peaks and as $R_S = 1.18\ (t_2 - t_1)/(w_1 + w_2)$ for partially unresolved peaks, where t_i is the retention time, W_i is the baseline width and w_i is the width at half height of peak *i*, all expressed in seconds.

Under these chromatographic conditions the analytes have the following retention times (k' values): OXCBZ, 7.9 min ($k' = 7.68$); CBZ-10OH, 5.0 min ($k' = 4.49$); CBZ-DiOH, 3.9 min ($k' = 3.29$); CBZ-3OH, 7.6 min ($k' = 7.35$).

3.2. Choice of the internal standard

A number of compounds were tested for potential use as internal standard (I.S.) for this assay; among these, antidepressants, antipsychotics and other active substances, such as CBZ-EP, glycyrrhizin, glycyrrhetic acid, adenosine, triprolidine and melatonin, not usually coadministered with OXCBZ.

The compounds with the most suitable retention factors were: melatonin ($t_R = 4.6$ min, $k' = 4.05$), risperidone ($t_R = 7.1$ min, $k' = 6.80$), dibenzepine ($t_R = 5.9$ min, $k' = 5.01$) and CBZ-EP ($t_R = 6.6$ min, $k' = 6.25$); of these, dibenzepine and CBZ-EP (Fig. 1f) have chemical structures similar to those of the analytes. However, the peak of dibenzepine is somewhat broad and asymmetric, and it is eluted close to the peak of CBZ-10OH. For these reasons, CBZ-EP was preferred as I.S.

It should be noted that CBZ-EP is the main metabolite of CBZ; as such, it cannot be used as the I.S. if a patient is treated simultaneously with CBZ and OXCBZ. However, this is an extremely rare occurrence in clinical practice, and the interference should not be a problem for most routine analyses. In those rare instances when OXCBZ is coadministered with CBZ, dibenzepine could be used as a reliable, albeit not optimal, I.S. (data not shown).

3.3. Analysis of standard solutions

The method was applied to the analysis of standard solutions at different concentrations (containing the I.S. at a constant concentration of 500 ng ml^{-1}) according to the relative amounts of the analytes expected in real plasma samples. Calibration curves

were set up by plotting the analyte–I.S. peak area ratios versus the respective analyte concentrations. Linear correlation coefficient were between 0.9997 and 0.9999 for concentration ranges of 25–1000 ng ml^{-1} for OXCBZ, 0.25–10.00 μg ml^{-1} for CBZ-10OH and 75–3000 ng ml^{-1} for CBZ-DiOH. Repeatability and intermediate precision assays gave good results, with RSD% values always lower than 2.1 for repeatability (0.9 for the I.S.) and lower than 3.7 for intermediate precision (1.4 for the I.S.). The values of LOQ and LOD, calculated according to the Crystal City [35] guidelines (as 10 and three times the baseline noise, respectively), were 15 and 5 ng ml^{-1}, respectively, for all analytes.

3.4. SPE procedure

The structural similarities between CBZ and OXCBZ prompted us to use the same SPE pretreatment procedure already used for the extraction of CBZ and metabolites from human plasma. However, since OXCBZ is less lipophilic than CBZ, it was decided to elute the analytes from the cartridge with methanol (instead of tetrahydrofuran), thus making the method less prone to interference. The modified SPE procedure included loading the conditioned cartridge with 200 μl of plasma (spiked with 50 μl of I.S. standard solution), washing with 1 ml of water twice and eluting the analytes with 1 ml of methanol. The eluate was then brought to dryness and redissolved in 800 μl of the mobile phase (resulting in a 1:4 dilution of the analytes with respect to the initial plasma concentration). This SPE procedure gave high yields when applied to plasma samples of patients undergoing monotherapy with OXCBZ. Unfortunately, the application to plasma of patients undergoing polytherapy was problematic in some cases. Indeed, an interfering peak was always present in chromatograms of plasma samples of patients treated with phenobarbital or primidone. The interfering peak was found to correspond to that of phenobarbital (which is also a metabolite of primidone), as can be seen in Fig. 3a from the chromatogram of a blank plasma sample spiked with the analytes and phenobarbital; the peak of phenobarbital is clearly visible at $t_R = 5.5$ min. This interference hinders the accurate determination of CBZ-10OH; for this reason, its elimination was necessary. Since

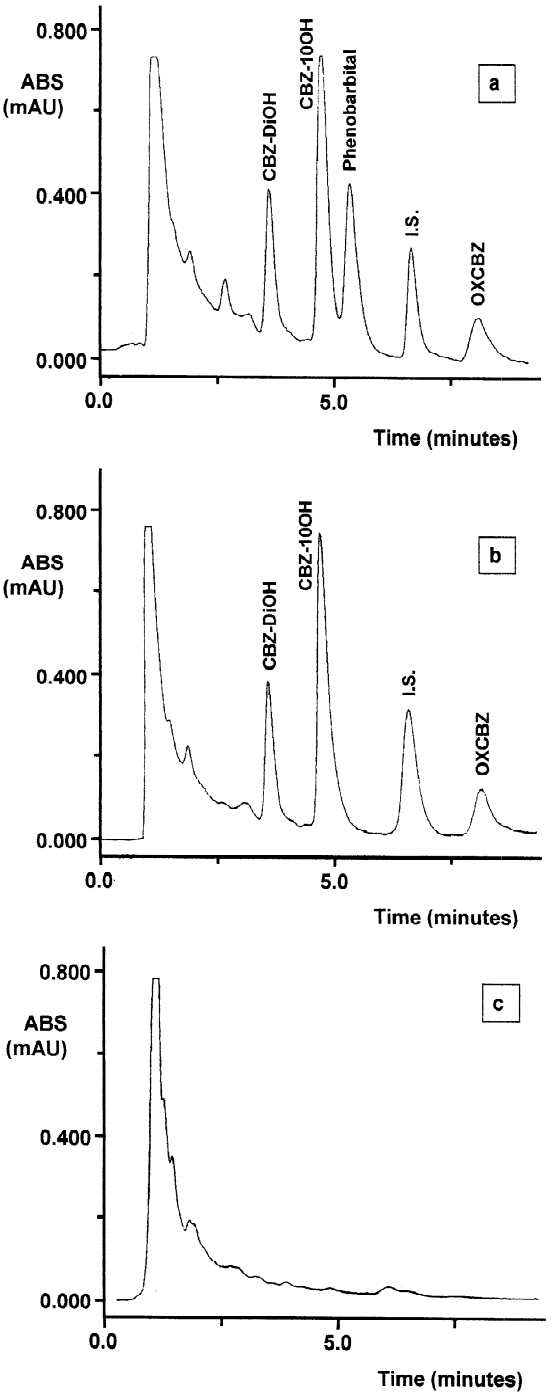

Fig. 3. Chromatogram of a blank plasma sample spiked with OXCBZ, its metabolites and phenobarbital, after SPE with water washing (a) and the same sample after SPE with the additional phosphate–methanol washing steps (b). A blank plasma sample (c) is shown for comparison.

phenobarbital has acidic properties, a washing step with a mixture of a basic (pH 11.0) buffer and methanol was introduced into the SPE procedure. Different kinds of buffer were tried, namely borate, carbonate and phosphate buffers, mixed with different percentages (10–30%) of methanol. It was found that the maximum percentage of organic solvent that could be used without reducing the quantitative extraction of OXCBZ and metabolites was 20% methanol. All buffers were successful in eliminating the interfering peak; however, the use of borate or carbonate buffers caused a sharp decrease in the absolute recovery of OXCBZ. Consequently, a phosphate buffer (pH 11.0, 100 m*M*)–methanol (80:20, v/v) mixture was used in the subsequent assays as the most favourable washing step.

Fig. 3b reports the chromatogram of the same plasma sample shown in Fig. 3a, subjected to the SPE procedure including the new washing step: as can be seen, the interfering peak corresponding to phenobarbital has been completely eliminated, and CBZ-10OH can be reliably quantified. The chromatogram of a blank plasma sample without any analyte spiking is shown in Fig. 3c: no interference from endogenous compounds is present.

3.5. Selectivity

Several compounds, both antiepileptics and other CNS drugs, were injected under the selected leading conditions in order to check for possible interferences. The complete list of the substances tested is reported in Table 1. As can be seen, none of these drugs interferes with the determination of the analytes, apart from phenobarbital, which however is completely eliminated during the washing step of the SPE procedure (see Section 3.4).

3.6. Method validation

Ten-point calibration curves were set up for all analytes, in different concentration ranges according to the levels of each compound expected in patients' plasma samples. The analyte–I.S. peak area ratio was plotted against the corresponding analyte concentration (expressed as ng ml^{-1}), and the calibration curves were obtained by means of the least-square method.

Table 1
Compounds tested as possible interference

Pharmacological class	Compound	t_R (min)
Antiepileptics	Phenobarbital	5.5
	Primidone	2.9
	Gabapentin	n.d.
	Valproate	n.d.
	Clobazam	n.d.
	Phenytoin	12.3
	Ethosuximide	n.d.
	Levetiracetam	n.d.
	Carbamazepine	14.0
Antipsychotics	Clozapine	11.2
	Levomepromazine	>20
	Haloperidol	16.0
Antidepressants	Reboxetine	16.3
	Maprotiline	18.0
	Protriptiline	13.2
	Fluoxetine	>20.0

Linear correlation coefficients were >0.995 in all cases for the concentration range chosen.

Detectability in plasma was assessed according to the ''Crystal City'' [35] guidelines (see above): the LOQ was 15 ng ml^{-1} and the LOD was 5 ng ml^{-1} for all analytes. All data are reported in Table 2.

The extraction yield was evaluated on blank plasma spiked with known concentrations of the analytes, corresponding to the low and high limit and the middle point of the respective calibration curves. Extraction yield values were always higher than 94.5%, as can be seen from Table 3. Precision assays were carried out at three different levels for each analyte, with six replicates for each level. The results are summarised in Table 3 as well: RSD% was always between 0.5 and 2.8% for repeatability or inter-day precision (1.5% for the I.S.) and between 0.9 and 3.7% for intermediate (inter-day) precision (2.1% for the I.S.).

CBZ-3OH, the minor metabolite of OXCBZ, is extracted almost quantitatively with this SPE procedure (mean extraction yield, 89.9%); it was found in only one sample, at a concentration lower than the LOQ. However, the metabolite could be identified and determined (at least semiquantitatively) in plasma in particular instances, e.g., when CBZ is administered simultaneously with OXCBZ (in this case

Table 2
Calibration line, $y = a + bx$, as derived from assays on blank plasma samples spiked with the analytes

Analyte	Range (ng ml^{-1})[a]	$a \pm$SE	$b \pm$SE	r_c	LOQ (ng ml^{-1})[a]	LOD (ng ml^{-1})[a]
OXCBZ	25–1000	−0.014±0.003	3.434±0.008	0.997	15	5
CBZ-10OH	250–10000	−0.016±0.004	1.960±0.006	0.998	15	5
CBZ-DiOH	75–3000	0.063±0.006	1.559±0.005	0.995	15	5

x = analyte concentration (ng ml^{-1}); y = analyte–I.S. peak area ratio; a is dimensionless; b is expressed as ml ng^{-1}. SE, standard error.
[a] On-column concentration.

CBZ-3OH is produced by CBZ metabolism), and in samples from patients whose particular metabolic pattern causes the formation of unusually high levels of this metabolite.

3.7. Application to patient plasma

The method was applied to the determination of plasma concentrations of OXCBZ and its metabolites in plasma of patients undergoing chronic treatment with OXCBZ (300–2100 mg day^{-1}), either as monotherapy or in association with other antiepileptic drugs (primidone, phenobarbital, valproate, clobazam, ethosuximide). The example in Fig. 4 shows the chromatogram of a patient treated simultaneously with OXCBZ (1500 mg day^{-1}) and phenobarbital: it is seen that no interference from the matrix constituents, nor from phenobarbital, occurs. By interpolation on the respective calibration curves, the following concentrations were obtained: 412 ng ml^{-1} of OXCBZ, 20.8 μg ml^{-1} of CBZ-10OH and 2.29 μg ml^{-1} for CBZ-DiOH.

The results obtained from the assays on 12 patient plasma samples are reported in Table 4. The analyte concentrations varied between 104 and 522 ng ml^{-1} for OXCBZ, 8.0 and 24.7 μg ml^{-1} for CBZ-10OH and 0.54 and 3.01 μg ml^{-1} for CBZ-DiOH. As expected, CBZ-10OH concentrations were much higher than OXCBZ ones in all cases. Intermediate values were obtained for the inactive metabolite CBZ-DiOH. Only one sample shows a small peak

Table 3
Method characteristics, as derived from assays on blank plasma samples spiked with the analytes

Analyte	Conc. (ng ml^{-1})	Repeatability		Intermediate precision	
		Extraction yield (%)	Precision (RSD%)	Extraction yield (%)	Precision (RSD%)
OXCBZ	25	95.3	2.8	94.7	3.7
	250	96.6	2.6	95.8	2.9
	1000	97.0	2.3	97.2	2.4
CBZ-10OH	250	97.6	1.8	97.4	3.3
	2500	97.8	1.8	96.9	2.3
	10000	97.8	0.5	98.8	0.9
CBZ-DiOH	75	95.5	2.1	97.1	3.9
	750	98.3	1.5	97.1	1.6
	3000	98.2	1.0	97.1	1.3
I.S. (CBZ-EP)	500	96.4	1.5	97.0	2.1
I.S. (Dibenzepine)	500	88.8	1.9	90.2	3.3

Data are the results of six replicated assays.

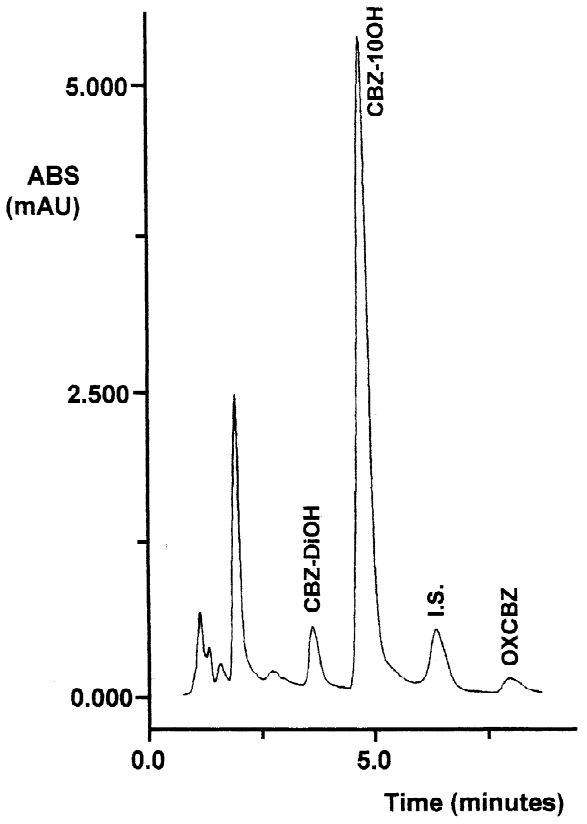

Fig. 4. Chromatogram of a plasma sample from a patient treated with 1500 mg day^{-1} of OXCBZ and 100 mg day^{-1} of phenobarbital, subjected to the developed SPE procedure.

corresponding to CBZ-3OH, however it is not quantifiable, since the concentration is lower than the LOQ. No interference from coadministered drugs was found.

The accuracy of the method was assessed by means of recovery studies, by adding known concentrations of the analytes (at three different levels) to already analysed patient plasma samples. These spiked samples were then subjected to SPE, re-analysed, and the recovery of the added analytes calculated. All accuracy assays gave recovery values always higher than 95% for OXCBZ and CBZ-10OH, and higher than 97% for CBZ-DiOH. Repeatability values (expressed as RSD) are in the range of $\leq$2%. All results are reported in Table 5.

4. Conclusion

The method worked out allows for the simultaneous quantification of OXCBZ and its main metabolites (CBZ-10OH and CBZ-DiOH) in TDM.

The LOQs reached (15 ng ml^{-1}) are below the concentrations expected in patients. The SPE procedure appropriately adopted for sample pre-treatment enabled avoidance of chromatographic interference when analysing samples from patients treated with multiple drugs. Furthermore, the SPE procedure

Table 4
Analytical results on patient plasma samples (taken 12 h after the last OXCBZ administration)

Patient no.	Dosage (mg day^{-1})	Coadministered drugs	OXCBZ (ng ml^{-1})	CBZ-10OH (μg ml^{-1})	CBZ-DiOH (μg ml^{-1})
1	300	Primidone	117	8.0	1.34
2	600	Valproate Clobazam	164	15.9	3.01
3	600	Phenobarbital Valproate	331	15.0	0.75
4	1200	Phenobarbital Valproate	104	12.7	1.22
5	1500	–	448	22.1	2.39
6	1500	–	144	15.4	0.31
7	1500	–	489	24.7	2.77
8	1500	Phenobarbital	412	20.8	2.29
9	1500	Valproate Ethosuximide	173	19.3	1.52
10	1800	Valproate	522	22.3	2.81
11	1800	Clobazam	335	18.7	1.86
12	2100	–	421	18.6	0.54

Table 5
Method performance

Analyte	Conc. added (ng ml^{-1})	Recovery (%)	Precision (RSD%)
OXCBZ	25	98.8	1.8
	250	95.1	1.1
	500	95.0	1.0
CBZ-10OH	250	95.5	0.8
	2500	98.5	0.6
	5000	99.2	0.6
CBZ-DiOH	75	97.7	2.0
	750	98.6	1.9
	1500	99.7	1.4

Data obtained analysing plasma samples from epileptic patients, undergoing chronic therapy with OXCBZ, spiked with the analytes. Data are the results of six replicated assays.

gives extraction yield values higher than 90%, better than those reported in other papers which use liquid–liquid extraction [22,26] or SPE [32] procedures. In addition, it is faster and less cumbersome than the liquid–liquid extraction procedures reported in the literature [12,22,28]. Although the minor metabolite CBZ-3OH is not fully baseline separated from OXCBZ, this is not significant for the quantitation of OXCBZ, because the metabolite concentration is two orders of magnitude smaller than that of OXCBZ. The I.S. used is a metabolite of the antiepileptic CBZ, which is almost never administered simultaneously with OXCBZ. In case of coadministration, dibenzepine can be used as an alternative I.S.

Summarising, it can be concluded that the proposed method is suitable for a reliable therapeutic drug monitoring of patients undergoing therapy with OXCBZ. Since it requires only 200 μl of plasma for one complete analysis, the method could be also suitable for pharmacokinetic studies; assays are in progress in this field.

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