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Simultaneous high-performance liquid chromatography determination of carbamazepine and five of its metabolites in plasma of epileptic patients

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Abstract

A high-performance liquid chromatographic method with UV detection for the simultaneous analysis of the antiepileptic drug carbamazepine and five of its metabolites in human plasma has been developed. The analysis was carried out on a reversed-phase column (C_8, 150×4.6 mm I.D., 5 μm) using acetonitrile, methanol and a pH 1.9 phosphate buffer as the mobile phase. Under these chromatographic conditions, carbamazepine and its metabolites 10,11-dihydro-10,11-epoxycarbamazepine, 10,11-dihydro-10,11-dihydroxycarbamazepine, 2-hydroxycarbamazepine, 3-hydroxycarbamazepine and 10,11-dihydro-10-hydroxycarbamazepine are baseline separated in less than 18 min. The extraction of the analytes from plasma samples was performed by means of an original solid-phase extraction procedure using Oasis HLB cartridges. The method requires only 250 μl of plasma for one complete analysis. The repeatability (RSD%<2.4), intermediate precision (RSD%<3.5) and extraction yield (84.8–103.0%) were very good for all analytes. The method is suitable for reliable therapeutic drug monitoring of patients undergoing chronic treatment with carbamazepine and for kinetic–metabolic studies of this drug.

Keywords: Metabolites; Solid-phase extraction; Carbamazepine

1. Introduction

Carbamazepine (5H-dibenzo[*b*,*f*]azepine-5-carboxamide, CBZ, Fig. 1) is a highly lipophilic, neutral tricyclic compound. It is widely used for the treatment of epileptic seizures, trigeminal neuralgia, and psychiatric disorders [1–3].

CBZ is usually administered at daily oral doses ranging from 200 to 1200 mg or more, which give rise to drug plasma levels of 4–12 μg ml^{-1} [4]. The relationship between the CBZ dose and its plasma concentration is often unpredictable and depends on the patient's metabolic state and age and might be affected by co-therapy [1,5].

In humans, CBZ is almost completely metabolised with only small traces excreted unchanged in the urine. The main metabolic pathway is oxidation to form carbamazepine 10,11-epoxide (CBZ-EP), a

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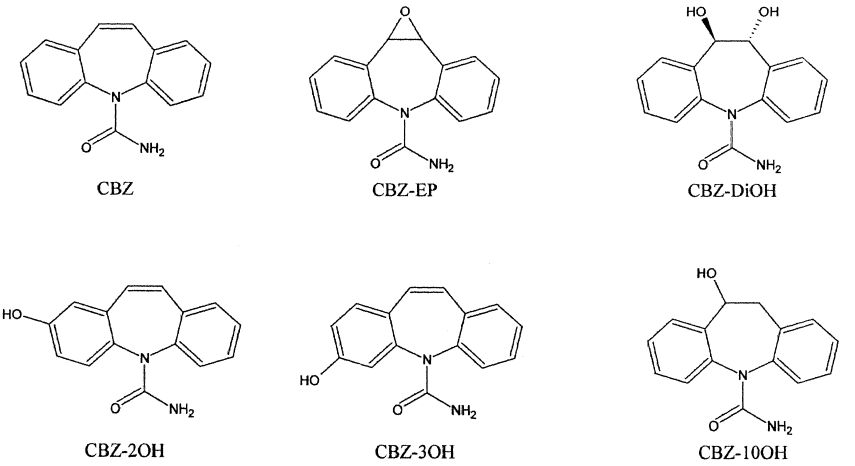

Fig. 1. Chemical structures of the analytes.

pharmacologically active compound with anticonvulsant properties. This reaction is catalysed by the enzymes of the cytochrome P450 system, and accounts for about half of the total metabolism [5,6]. Two other important CBZ metabolic pathways are the oxidation to form phenolic metabolites and direct *N*-glucuronidation. CBZ-EP is extensively metabolised by epoxide hydrolases to 10,11-dihydro-10,11-dihydroxycarbamazepine (carbamazepine *trans*-diol, CBZ-DiOH) which is then in part conjugated with glucuronic acid and excreted in the urine. Other metabolites found in the plasma of patients treated with CBZ are 10,11-dihydro-10-hydroxycarbamazepine (CBZ-10OH), 2-hydroxycarbamazepine (CBZ-2OH), 3-hydroxycarbamazepine (CBZ-3OH) and 9-carbamoylacridan [6]. Conjugation products (glucuronides, sulphates) are present as well [6]. CBZ is a strong inducer of microsomal enzymes and induces its own metabolism as well as that of other drugs [7,8]. At the same time, CBZ metabolism can be induced or inhibited by other drugs [1,5].

Determination of the plasma levels of the various CBZ metabolites is required in pharmacokinetic and metabolic studies, and the routine analysis of CBZ-EP along with CBZ may provide optimal therapeutic monitoring of CBZ treatment.

Gas chromatography has often been used for the determination of CBZ [9,10]; however, this technique often needed a complicated derivatisation procedure. Two interesting papers on GC determination of CBZ and other drugs without derivatisation have been published [11,12]. The most widely used means of analysis for CBZ is now liquid chromatography with UV [13–24] or mass spectrometry [25] detection. Two papers have recently been published which determine CBZ by means of micellar electrokinetic chromatography [26,27].

Most authors only consider the two main metabolites of CBZ (CBZ-EP and CBZ-DiOH) [14–23,26] and/or matrices different from plasma: urine [14,25] or hair [17]. Pienimäki et al. [24] described the simultaneous determination of CBZ-10OH, CBZ-2OH and CBZ-3OH as well; however, the analysis is laborious and time-consuming: it requires a liquid–liquid extraction with methyl-*tert*.-butyl ether and a 30-min chromatographic run.

Immunochemical techniques are also used for the routine therapeutic drug monitoring of CBZ alone in plasma, especially in clinical settings. The antibodies used in these procedures, however, may sometimes cross-react with one or more CBZ metabolites, leading to an overestimation of CBZ plasma levels [28,29].

The aim of this study is the implementation of a new, simple and selective HPLC–UV method for the determination of plasma levels of CBZ and five of its metabolites in human plasma. The sample pretreatment is constituted by a careful solid-phase extraction (SPE) procedure, which is a fast and feasible alternative to liquid–liquid extraction.

2. Experimental

2.1. Chemicals and solutions

Carbamazepine (5H-dibenzo[*b*,*f*]azepine-5-carboxamide) and its metabolites (10,11-dihydro-10,11-epoxycarbamazepine; 10,11-dihydro-10,11-dihydroxycarbamazepine; 10,11-dihydro-10-hydroxycarbamazepine; 2-hydroxycarbamazepine and 3-hydroxycarbamazepine) were kindly provided by Novartis Pharma (Basel, Switzerland).

Amoxapine, used as the internal standard (I.S.) for the control of retention times, was kindly provided by Wyeth Pharmaceuticals (St. Davids, PA, USA).

Acetonitrile, methanol and tetrahydrofuran (THF) were HPLC grade from Carlo Erba (Milan, Italy). Potassium dihydrogen phosphate, disodium hydrogen phosphate, phosphoric acid, potassium chloride and sodium chloride were analytical grade from Carlo Erba.

Triethylamine analytical grade was from Fluka (Buchs, Switzerland). Bovine serum albumin was purchased from Sigma (St. Louis, MO, USA).

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

Stock solutions of CBZ, metabolites and the I.S. were 1 mg ml^{-1} in methanol and were stable for at least 3 months when stored at −20°C. Standard working solutions were prepared by diluting each stock solution with the mobile phase.

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

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 set at $\lambda =$ 237 nm. CBZ and its metabolites were separated on a Varian (Harbor City, CA, USA) Microsorb MV Rainin (C_8, 150×4.6 mm, 5 μm) reversed-phase column with a mobile phase composed of a 15 mmol l^{-1} phosphate buffer (pH adjusted to 1.9 with HCl), methanol and acetonitrile (62.5:20.0:17.5, v/v/v) containing 18 mmol l^{-1} triethylamine, flowing at 1.2 ml min^{-1}. The column was maintained at room temperature. The mobile phase was filtered through a Sartorius (Göttingen, Germany) nylon membrane filter (diameter 47 mm, pore size 0.2 μm). The samples were injected into the chromatographic system by means of a 20-μl injection loop.

2.3. Analytical procedures

2.3.1. Sample pretreatment

For the solid-phase extraction (SPE) procedure Waters (Milford, MA, USA) Oasis® HLB (30 mg, 1 ml) cartridges were used. Conditioning was carried out by passing 1 ml of methanol twice through the cartridge; equilibration was carried out by passing 1 ml of water twice through the cartridge. An aliquot of 500 μl of water and a known amount of I.S. solution were added to 250 μl of blank or patient plasma, which was then loaded onto the already equilibrated cartridge. After washing twice with 1 ml of water and once with 100 μl of methanol, the cartridge was dried under vacuum (−40 kPa) for 30 s and the analytes eluted with 1 ml of THF.

The eluate was then dried by means of a rotary evaporator, the residue redissolved in 125 μl of mobile phase and injected into the HPLC.

2.3.2. Calibration curves on blank plasma

The calibration curves were constructed by adding known amounts of analyte standard solutions to blank plasma. The resulting mixture was subjected to the SPE procedure. The peak areas of each analyte were plotted against the respective concentrations (expressed as ng ml^{-1}), and the curves were obtained by means of the least-squares method.

2.3.3. Analysis of carbamazepine and metabolites in human plasma

Plasma samples were taken from epileptic patients under therapy with CBZ at the Neurologic Clinic of the University of Bologna and put in vials containing EDTA as the anticoagulant. The blood was immediately centrifuged for 20 min at 1400 *g* and the supernatant plasma frozen and maintained at −20°C until analysis.

To 250 μl of plasma, 500 μl of ultrapure water and 25 μl of I.S. solution (5 μg ml^{-1}) were added. The mixture was then subjected to the above-de-

scribed pretreatment procedure and injected into the HPLC.

3. Results and discussion

3.1. Preliminary studies

The spectrum of CBZ shows three absorbance bands in the UV region, with maxima at 213, 237 and 286 nm, respectively; all metabolites show also the first two absorbance bands. For this reason, all subsequent chromatographic assays were carried out setting the UV detection at 237 nm, in order to have good sensitivity while avoiding as much as possible the strong interference, which could be expected at lower wavelengths.

3.2. Development and validation of the HPLC method

3.2.1. Chromatographic conditions

Preliminary assays for the analysis of CBZ and metabolites were carried out using chromatographic conditions similar to those of our previous paper on the determination of the antipsychotic drug clozapine [30], namely a mixture of acetonitrile, methanol and a pH 1.9, 10.4 mol l^{-1} phosphate buffer containing triethylamine (17.5:20:62.5, v/v/v) as the mobile phase, and a C_8 column as the stationary phase. Amoxapine was used as the internal standard (I.S.) only for the control of retention times. This can be a critical issue when six or seven analytes are determined, as in this case. The use of amoxapine allows for the reliable identification of peaks without need for the addition of analyte standard solutions to the plasma samples. The column was a C_8 Microsorb Rainin instead of a C_8 Varian as used in the previous paper. This kind of column offered better separation efficiency and thus better analyte separation.

Under these leading conditions and with a flow-rate of 1.0 ml min^{-1}, the last-eluting analyte was CBZ, with a retention time of about 20 min. To shorten retention times, the phosphate buffer concentration was increased from 10 to 15 mol l^{-1} and the flow-rate was increased from 1.0 to 1.2 ml min^{-1}. With these modifications, all analytes were baseline separated and eluted within 17 min. Retention times are: 3.8 min for CBZ-DiOH; 5.3 min for CBZ-10OH; 6.3 min for CBZ-2OH; 7.1 min for CBZ-EP; 8.9 min for CBZ-3OH; 12.9 min for amoxapine (I.S.) and 16.5 min for CBZ. Calibration curves were set up in the 0.5–15.0 μg ml^{-1} range for CBZ, in the 12.5–15 000.0 ng ml^{-1} range for CBZ-EP and in the 12.5–2500.0 ng ml^{-1} range for the other metabolites. The different concentration ranges were selected considering the amounts of the analytes found in real plasma samples. Repeatability, expressed as relative standard deviation (RSD%) intra-day, was always between 0.4 and 1.3%, while intermediate precision, expressed as RSD% inter-day, was between 0.5 and 2.1%; each value was assessed on six repeated assays at three different concentrations for each analyte.

3.2.2. Implementation of the SPE procedure on human plasma

For this procedure Oasis HLB cartridges were used. The cartridges contain a polymeric stationary phase with different kinds of functional groups; this leads to a ''hydrophilic/lipophilic balance'' (HLB). This characteristic should allow for the retention of compounds with different chemical/physical properties, and their elution, with a relatively simple and standardised procedure (according to the manufacturer [31]). Furthermore, the polymeric matrix allows for the use of very high- and low-pH buffers (pH values from 1 to 14). The procedure used was at first similar to that used for the analysis of clozapine [30], however the Oasis HLB cartridges were loaded with 250 μl of plasma instead of 100 μl. In order to find the best elution solvent, several trials were made. At first, an elution with 1 ml of methanol was tried; CBZ metabolites were eluted with satisfactory recovery; however, CBZ was completely retained by the cartridge. Similar results, but with lower recovery, were obtained using pure acetonitrile or an acetonitrile–methanol (30:70, v/v) mixture. Elution with THF gave good extraction yields; however, the direct injection of this strong solvent caused peak broadening and baseline interference. In order to completely eliminate THF from the injection mixture, the eluate was dried under vacuum and the residue redissolved with 125 μl of the mobile phase (thus obtaining a 2:1 concentration of the sample). Moreover, another washing step (with 100 μl of methanol) was introduced in the SPE procedure to completely eliminate aqueous residues from the

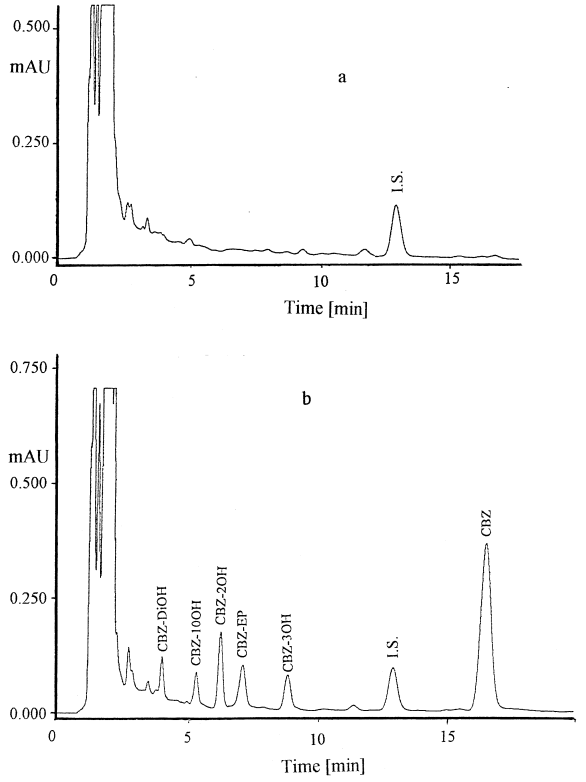

Fig. 2. Chromatogram of (a) a blank plasma sample spiked with 500 ng ml^{-1} of the I.S. and (b) the same blank plasma sample spiked with 1250 ng ml^{-1} of CBZ, 250 ng ml^{-1} of each metabolite and 500 ng ml^{-1} of the I.S., after the SPE procedure.

cartridges before the elution, thus facilitating the subsequent drying of the eluate.

The use of larger cartridges (Oasis HLB, 60 mg, 1 ml) was tried but did not significantly improve extraction yields and required larger volumes of THF for elution.

Typical chromatograms of a drug-free plasma sample spiked with I.S. and the same sample spiked with standard solutions of analytes and I.S. are reported in Fig. 2. No interference is present in either chromatograms and all analyte peaks are well resolved, with retention times similar to those obtained injecting standard solutions.

3.3. Method validation

Calibration curves were set up on blank plasma spiked with standard solutions; good linearity was found in the same concentration ranges already considered for standard solutions (i.e., 0.5–15.0 μg ml^{-1} for CBZ, 12.5–15 000.0 ng ml^{-1} for CBZ-EP, 12.5–2500.0 ng ml^{-1} for the other metabolites). The limit of quantification (LOQ) was 6 ng ml^{-1} for CBZ and CBZ-2OH, 12 ng ml^{-1} for all other analytes; the limit of detection (LOD) was 2 ng ml^{-1} for CBZ and CBZ-2OH, 4 ng ml^{-1} for all other analytes (Table 1). These values were calculated according to USP XXIV Edition guidelines [32]; i.e., LOD as the concentrations which give a 3:1 signal:noise ratio and LOQ as the concentrations which give a 10:1 signal:noise ratio.

Absolute recovery and precision assays were carried out. To 250 μl of blank plasma, three different concentrations of each analyte were added. The procedure was repeated 6 times for each concentration within the same day and over different days.

The results were very good: the mean absolute recovery (extraction yield) values range from 84.8 to 103.0%, while the RSD% values for precision range from 0.53 to 2.32% (repeatability) and from 0.92 to 3.44% (intermediate precision) (Table 2).

Table 1
Linearity parameters

Analyte	Linearity ($y = a + bx$)				LOQ (ng ml^{-1})	LOD (ng ml^{-1})
	Range (ng ml^{-1})	a (SE)	b (SE)	r_c		
CBZ	500–15 000	−5115.2 (873)	54.52 (0.29)	0.9990	6	2
CBZ-EP	12–15 000	1434.9 (1072)	20.43 (0.47)	0.9997	12	4
CBZ-DiOH	12–2500	912.9 (453)	17.32 (0.20)	0.9997	12	4
CBZ-2OH	12–2500	−97.7 (88)	65.85 (0.44)	0.9993	6	2
CBZ-3OH	12–2500	171.4 (201)	31.86 (0.28)	0.9998	12	4
CBZ-10OH	12–2500	421.5 (342)	19.12 (0.15)	0.9996	12	4

Table 2
Method characteristics

Analyte	Conc. (ng ml^{-1})	Precision (RSD%)[a]		Absolute recovery (%)[a]	Accuracy[a]	
		Repeat-ability	Intermediate precision		Conc. added (ng ml^{-1})	Recovery (%)
CBZ	500	1.22	1.29	94.0	4000	96.7
	1250	1.17	1.09	97.4		
	5000	0.83	0.92	99.8		
CBZ-EP	100	1.92	1.98	103.0	4000	90.8
	250	0.87	1.83	102.6		
	1000	0.53	1.77	102.4		
CBZ-DiOH	100	1.66	2.36	95.0	200	89.8
	250	1.33	1.57	92.8		
	1000	0.94	1.20	94.6		
CBZ-2OH	100	2.32	3.44	84.8	200	85.2
	250	1.32	2.69	86.4		
	1000	0.92	1.77	90.8		
CBZ-3OH	100	1.27	2.34	89.4	200	97.7
	250	0.90	1.16	88.0		
	1000	0.77	0.98	92.0		
CBZ-10OH	100	1.90	2.39	87.8	200	87.5
	250	1.70	1.91	88.6		
	1000	1.39	1.43	93.6		

[a] $n=6$.

3.4. Analysis of patient plasma

The validated method was applied to plasma samples taken from epileptic patients under oral chronic CBZ therapy.

To 250 μl of a plasma sample, 12.5 μl of 10 μg ml^{-1} I.S. solution were added; the mixture was then subjected to the SPE procedure and injected into the HPLC.

The chromatogram of a plasma sample from a patient who receives 1200 mg day^{-1} of CBZ, taken 12 h after the last drug intake, is reported in Fig. 3. As can be seen, all analyte peaks are well separated and no interference is present, even if other peaks (probably due to endogenous compounds or other simultaneously administered drugs) are present in the chromatogram. The following concentrations of the analytes were found in this sample by interpolating on the calibration curves: 7.1 μg ml^{-1} of CBZ, 7.0 μg ml^{-1} of CBZ-EP, 1.04 μg ml^{-1} of CBZ-DiOH, 215 ng ml^{-1} of CBZ-10OH and 21 ng ml^{-1} of CBZ-3OH. In this case, the concentration of CBZ-2OH resulted to be less than 2 ng ml^{-1}, which is the LOD of the method.

Results of the assays of plasma samples on other

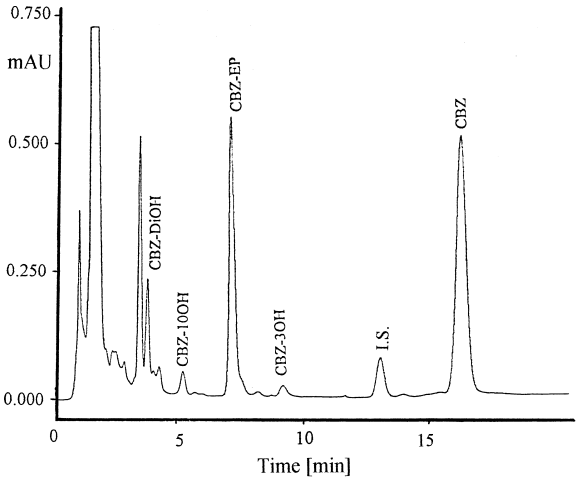

Fig. 3. Chromatogram of a plasma sample from a patient treated with 1200 mg day^{-1} of CBZ, drawn 12 h after the last administration. The following concentrations of the analytes were found in this sample: 7.1 μg ml^{-1} of CBZ, 7.0 μg ml^{-1} of CBZ-EP, 1.04 μg ml^{-1} of CBZ-DiOH, 215 ng ml^{-1} of CBZ-10OH, 21 ng ml^{-1} of CBZ-3OH, CBZ-2OH$<$LOD.

Table 3
Results of analyses on patient plasma samples

Patient	Hours from last administ.	Coad-ministered drugs	CBZ dosage (mg day^{-1})	I.E. Kit CBZ	Concentration (ng ml^{-1}): HPLC method					
					CBZ	CBZ-EP	CBZ-DiOH	CBZ-2OH	CBZ-3OH	CBZ-10OH
B.S.	12	Phenobarbital	1200	7200	7100	7000	1035	n.d.	21	605
	4	Lamotrigine		9000	8600	6300	1070	n.d.	127	91
D.F.P.	12	Lamotrigine	1100	7700	7100	1050	1600	20	n.d.	1280
	4			8300	8300	780	1380	17	n.d.	950
T.S.	4	Phenobarbital	1000	4400	4100	11 200	2000	n.d.	n.d.	n.d.
N.G.	4	Phenytoin	800	5900	5400	1580	1090	49	15	69
M.V.	4	–	600	8100	7900	1390	1030	n.d.	17	n.d.
S.M.	4	Olanzapine Clonazepam	600	n.e.	5650	1040	2330	n.d.	43	155
B.R.	4	Phenobarbital	400	4700	4300	7900	1440	n.d.	325	30
R.P.	4	Phenobarbital	400	5600	5600	4500	320	31	23	340

n.d., not determined, under LOQ; n.e., analysis not carried out.

patients are reported in Table 3. In this table the CBZ plasma concentrations obtained with the proposed method are also compared to those obtained from the same samples by means of a commercial immunochemical kit (EMIT®, DADE-Boehringer, USA). As one can see from Table 3, the results are in good agreement; results by the immunochemical kit, however, were slightly higher, possibly because of the well-known [28,29], cross-reactions of CBZ and CBZ metabolites (mainly CBZ-EP) with this technique.

3.5. Method accuracy

Method accuracy was evaluated by means of recovery studies. Known amounts of analyte standard solutions were added to 250 μl of plasma from patients whose analyte levels were already known. The mixture was then analysed. The assay was repeated 6 times, and the mean recovery resulted to be: 96.7% for CBZ (4.0 μg ml^{-1}), 90.8% for CBZ-EP (4.0 μg ml^{-1}), 89.8% for CBZ-DiOH (200 ng ml^{-1}), 87.5% for CBZ-10OH (200 ng ml^{-1}), 85.2% for CBZ-2OH (200 ng ml^{-1}) and 97.7% for CBZ-3OH (200 ng ml^{-1}), as reported in Table 2.

3.6. Interference

Epileptic patients are often treated with polytherapy and, furthermore, antiepileptic drugs are sometimes used together with antipsychotics as mood stabilisers. To evaluate the selectivity of our method, we analysed plasma samples taken from patients treated with different drugs: phenobarbital, lamotrigine, olanzapine, clonazepam and phenytoin. None of these drugs showed any interference in the chromatograms (because some of them were almost not retained and were eluted with the injection peak, while others had retention times longer than 30 min, and were eliminated by column washing). Thus, the method seems to be selective enough for the therapeutic monitoring of patients undergoing therapy with CBZ, even when other drugs are coadministered.

4. Conclusion

The chromatographic method proposed herein allows for the separation, identification and simultaneous quantification of CBZ, its main metabolites (CBZ-EP and CBZ-DiOH) and some of its minor metabolites (CBZ-2OH, CBZ-3OH and CBZ-10OH). No chromatographic interference was observed when analysing samples from patients treated with multiple drugs. Hence the method can be used both for the therapeutic monitoring of epileptic patients under chronic therapy with CBZ and for the study of CBZ pharmacokinetic and metabolic transformation in humans. The method is advantageous thanks to the new SPE procedure which is more rapid and feasible

than the liquid–liquid extraction procedures reported in the literature [13,15,19] and gives good recovery, better than those reported in other papers [15,17], and optimal selectivity.

Another advantage is the short analysis time: CBZ and five metabolites are determined in less than 17 min, whereas the same analytes require at least 30 min in other papers [23].

If only CBZ and CBZ-EP are to be determined, analysis time can be further reduced, by suitably modifying the mobile phase and/or the flow-rate.

The I.S. employed is a marketed antidepressant drug; however, it is used only to confirm retention times, and the possible presence of the compound in a patient's plasma would not impede the use of the present method.

For these reasons, and because it requires only 250 μl of plasma for an entire analysis, we think the proposed method is suitable for the analysis of CBZ and its metabolites, and may be particularly advantageous when multiple blood samplings are needed as in pharmacokinetic studies.

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