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Journal of Chromatography B, 828 (2005) 80–90
JOURNAL OF CHROMATOGRAPHY B
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Liquid chromatography–electrospray mass spectrometry determination of carbamazepine, oxcarbazepine and eight of their metabolites in human plasma

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Received 22 June 2005; accepted 12 September 2005
Available online 3 October 2005

Abstract

Carbamazepine (CBZ) and oxcarbazepine (OXCBZ) are both antiepileptic drugs, which are prescribed as first-line drugs for the treatment of partial and generalized tonic–clonic epileptic seizures. In this paper, a specific and sensitive liquid chromatography–electrospray ionization mass spectrometry method was described for the simultaneous determination of carbamazepine (CBZ), oxcarbazepine (OXCBZ) and eight of their metabolites [CBZ-10,11-epoxide (CBZ-EP), 10,11-dihydro-10,11-*trans*-dihydroxy-carbamazepine (DiOH-CBZ), 10-hydroxy-10,11-dihydroCBZ (10-OH-CBZ), 2-hydroxycarbamazepine (2-OH-CBZ), 3-hydroxycarbamazepine (3-OH-CBZ), iminostilbene (IM), acridone (AO) and acridine (AI)] in human plasma. The work-up procedure involved a simple precipitation with acetone. Separation of the analytes was achieved within 50 min using a Zorbax eclipse XD8 C8 analytical column. The mobile phase consisted of a mixture of acetonitrile–formate buffer (2 mM, pH 3). Detection was performed using a quadrupole mass spectrometer fitted with an electrospray ion source. Mass spectrometric data were acquired in single ion recording mode at *m*/*z* 237 for CBZ, *m*/*z* 180 for CBZ-EP and AI, *m*/*z* 236 for OXCBZ, *m*/*z* 237 for 10-OH-CBZ, *m*/*z* 253 for 2-OH-CBZ, 3-OH-CBZ and DiOH-CBZ, *m*/*z* 196 for AO and *m*/*z* 194 for IM. For all analytes, the drug/internal standard peak height ratios were linked via a quadratic relationship to plasma concentrations. The extraction recovery averaged 90% for CBZ, 80% for OXCBZ and was 80–105% for the metabolites. The lower limit of quantitation was 0.5 mg/l for CBZ, 0.4 mg/l for OXCBZ and ranged from 0.02 to 0.3 mg/l for the metabolites. Precision ranged from 2 to 13% and accuracy was between 86 and 112%. This method was found suitable for the analysis of plasma samples collected during therapeutic drug monitoring of patients treated with CBZ or OXCBZ.

Keywords: Liquid chromatography–mass spectrometry; Carbamazepine; Oxcarbazepine; Metabolites; Validation

1. Introduction

Carbamazepine (5H-dibenz/bf/azepine-5-carboxamide) (CBZ) is the most frequently prescribed first-line drug for the treatment of partial and generalized tonic–clonic epileptic seizures [1]. Of the adverse reactions associated with CBZ, 5% can be classified as idiosyncratic or hypersensitivity reactions. These can range from serious skin reactions, such as erythema multiforme [2], to severe haematological disorders, especially agranulocytosis and aplastic anemia [3,4]. Such reactions are unpredictable and are associated with high mortality rates. Although the mechanism of CBZ-induced adverse reactions is not clear, they are thought to result from the formation of reactive metabolites [5].

Carbamazepine is predominantly metabolized in the liver into various metabolites. At least 30 different metabolites have been

Abbreviations: CBZ, carbamazepine; OXCBZ, oxcarbazepine; CBZ-EP, carbamazepine-10,11-epoxide; DiOH-CBZ, 10,11-dihydro-10,11-*trans*-dihydroxy-carbamazepine; 10-OH-CBZ, 10-hydroxy-10,11-dihydro carbamazepine; 2-OH-CBZ, 2-hydroxycarbamazepine; 3-OH-CBZ, 3-hydroxycarbamazepine; IM, iminostilbene; AO, acridone; AI, acridine; QC, quality control

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doi:10.1016/j.jchromb.2005.09.019

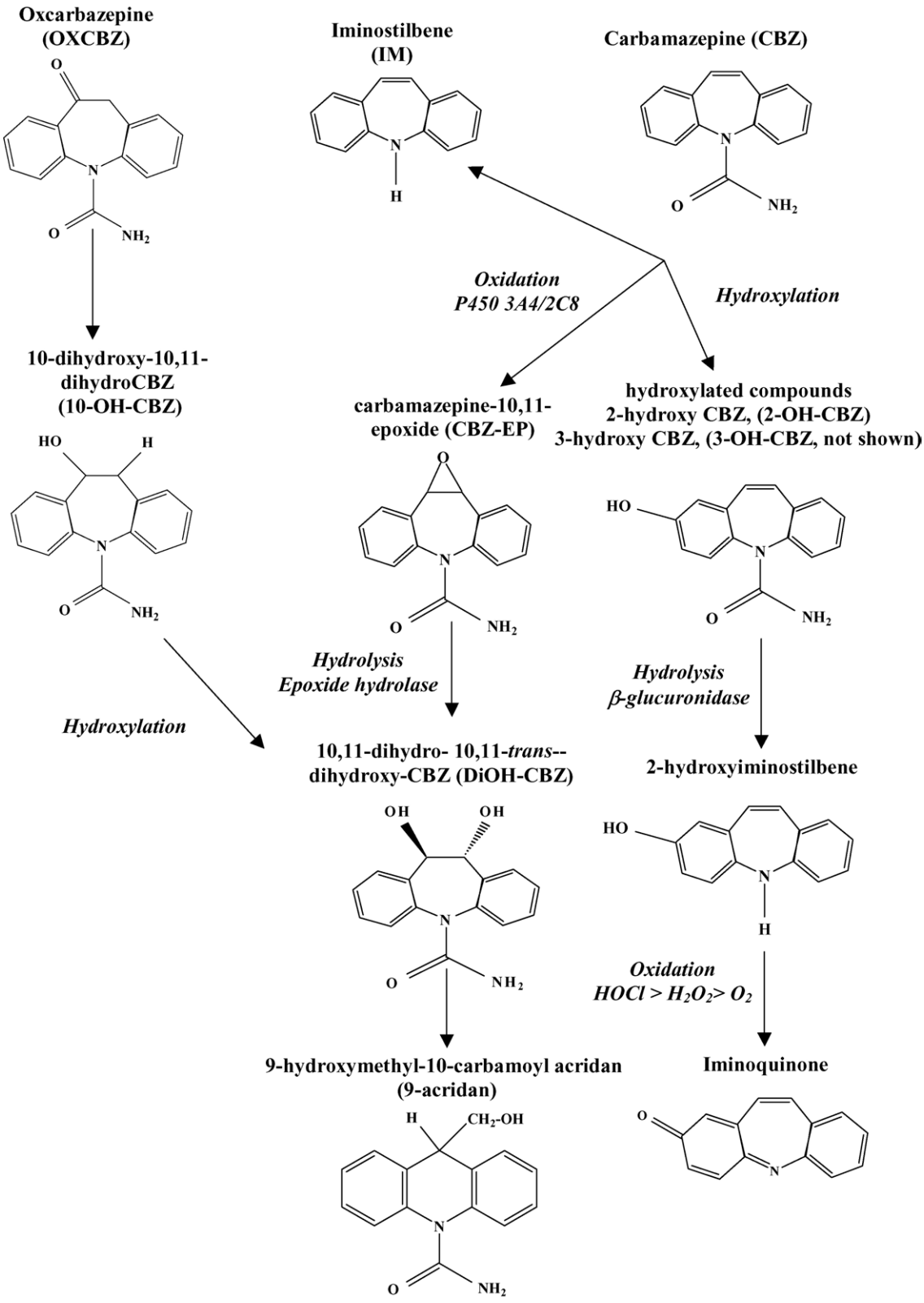

Fig. 1. Hepatic metabolism pathway of carbamazepine and oxcarbazepine. For the hydroxylated compounds, the hydroxylation in position 2 is only presented.

identified [6]. Three principal metabolic pathways have been described [7–13] (Fig. 1). The main route is the formation of the carbamazepine-10,11-epoxide (CBZ-EP), a pharmacologically active compound with anticonvulsant properties. A second route of metabolism concerns the production of hydroxylated compounds. The third minor route of metabolism leads to the formation of iminostilbene (IM). Although the liver is the major site of drug metabolism, the biological half-life of most reactive

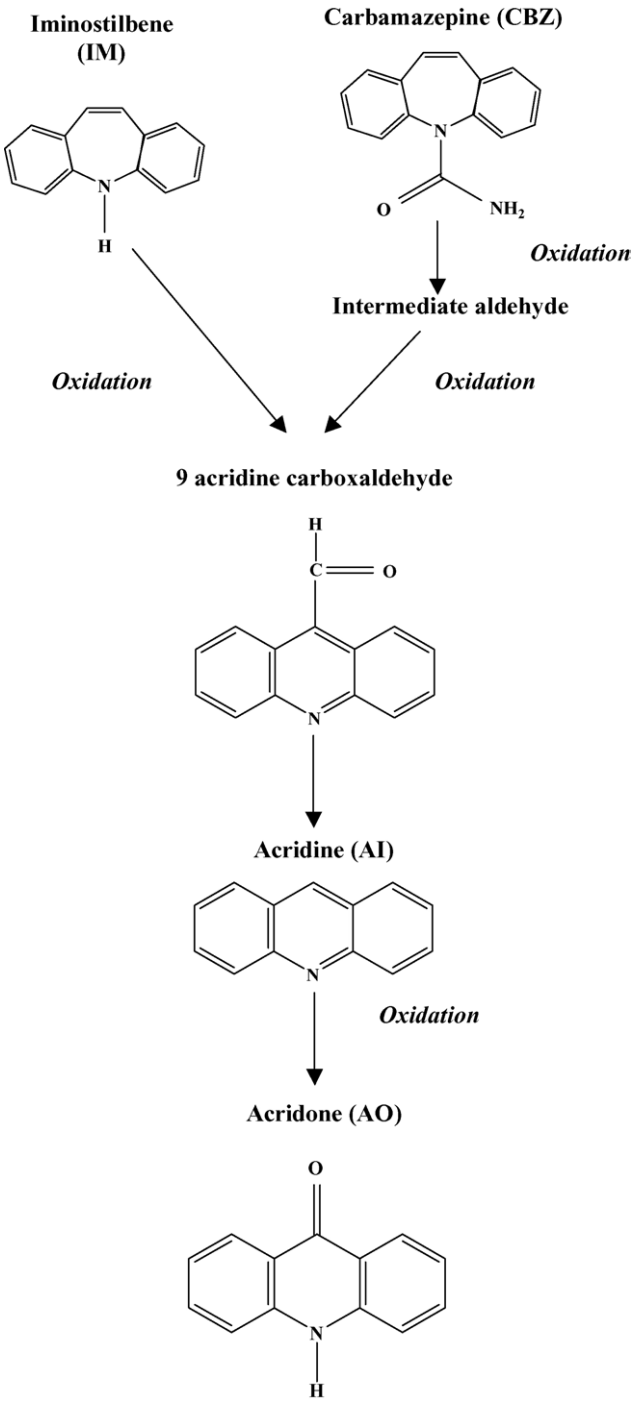

Fig. 2. Carbamazepine and iminostilbene oxidations by activated leucocytes.

metabolites is not sufficient to cause toxicity at distant sites. Leucocytes not only are the major cells involved in the induction of an immune response but also exhibit strong oxidizing properties. Into the leucocytes, CBZ is converted by the myeloperoxidase in a series of metabolites mainly to an intermediate aldehyde, 9-acridine carboxaldehyde, acridine (AI) and acridone (AO) [8] (Fig. 2).

During the last decade, CBZ-EP has been suspected to play a key role in CBZ-induced side-effects, especially severe cutaneous reactions such as Stevens–Johnson syndrome, toxic epidermolysis necrosis or drug rash with eosinophilia and systemic system syndrome. However, CBZ-EP's involvement as the reactive metabolite in idiosyncrasic reactions has been rule out in many studies showing the absence of relationship between this type of side-effects and the presence of CBZ-EP [14]. Furthermore, the initial hypothesis involving epoxyde hydrolase's polymorphism has been excluded [15]. Thus, minor metabolites produced by alternative pathways of metabolism could be considered as potential reactive metabolites. 9-acridine carboxaldehyde, IM and iminoquinone are reactive metabolites [9], whereas 9-hydroxymethyl-10-carbamoyl-acridan (9-acridan), AI and AO are only suspected to be reactive metabolites due to the structural similarity [8].

Oxcarbazepine (OXCBZ), the 10-keto analogue of CBZ, is a more recently marketed antiepileptic drug which has chemical and therapeutic similarities to CBZ. CBZ is frequently substituted by OXCBZ. OXCBZ is mainly metabolized to 10-hydroxy-10,11-dihydroCBZ (10-OH-CBZ) (Fig. 1) probably through a non-inducible aldo-keto-reductase [16]. This therapeutically active metabolite gives the 10,11-dihydro-10,11-*trans*-dihydroxy-carbamazepine (carbamazepine *trans*-diol, DiOH-CBZ) a common metabolite with CBZ. Another potential common metabolite is the hydroxylated compound, 3-hydroxycarbamazepine (3-OH-CBZ), found in plasma of patients treated with OXCBZ, but the enzyme system involved is not identified yet [17–19]. OXCBZ cause undesired side-effects similar to those of CBZ; however, their incidence and severity are reduced [20].

The thermal instability of CBZ and OXCBZ makes high-performance liquid chromatography (HPLC) more attractive to use than gas chromatography [6,21–23], which in addition requires a complicated derivatisation procedure as sample preparation. To date, some HPLC methods with ultra-violet [17–19,24–34] or mass spectrometry [10,11,35,36] detection for determining CBZ and/or OXCBZ in biological fluids (plasma, urine) have been published. Some of them involved both the quantitation of CBZ or OXCBZ and their main metabolites (CBZ-EP, 10-OH-CBZ, DiOH-CBZ [19,24,26–31,33,34]. Other methods allow to quantify either CBZ, CBZ-EP, 10-OH-CBZ, DiOH-CBZ and the 9-acridan [25,32], or the hydroxylated compounds (2-hydroxycarbamazepine (2-OH-CBZ); 3-OH-CBZ) [18]. One method allows to quantify CBZ, OXCBZ, CBZ-EP, 10-OH-CBZ, DiOH-CBZ and the 9-acridan [17]. Four liquid chromatography–mass spectrometry (LC–MS) methods have been published; two of them were focused mainly to the determination of the 9-acridan [10,11]. Two methods have recently been described, the first one for the determination of CBZ and CBZ-EP by tandem liquid chromatography–mass spectrometry with electrospray ionisation [35] and the second one for the determination of CBZ and 10-OH-CBZ by atmospheric pressure chemicalionization liquid chromatography/mass spectrometry [36]. All these methods involved tedious sample pretreatment (liquid–liquid or solid-phase extraction) of the biological samples.

To date, no bioanalytical assay has been described for the simultaneous determination of CBZ, OXCBZ and most of their metabolites from both hepatic and leucocyte metabolism path-

ways. The aim of this study was to develop a reliable, specific and sensitive LC–MS method for the simultaneous quantitation of the parent drugs (CBZ and OXCBZ) and eight identified metabolites (CBZ-EP, 10-OH-CBZ, DiOH-CBZ, 2-OH-CBZ, 3-OH-CBZ, IM, AO, AI) in human plasma. This method involved a simple precipitation of the samples with acetone allowing rapid therapeutic drug monitoring of patients treated with CBZ or OXCBZ. This method has an enhanced precision due to the use of an internal standard (2-methyl CBZ). It was validated according to validation procedures, parameters and acceptance criteria based on USP XXIII guidelines and FDA guidance [37–40].

2. Experimental

2.1. Materials and reagents

CBZ, CBZ-EP, 2-methyl CBZ (internal standard), IM, AO and AI were purchased from Sigma (Saint Quentin Fallavier, France). OXCBZ, 10-OH-CBZ, DiOH-CBZ, 2-OH-CBZ, 3-OH-CBZ were kindly supplied by Novartis Pharma (Basel, Switzerland). The reagents were of analytical-reagent grade and the acetonitrile was of HPLC grade, all of them were obtained from Merck (Nogent/Marne, France). The formate buffer solution (pH 3) consisted of formic acid salt (126 mg/l) in purified water. Purified water was generated by a Milli-Q reagent water system (Millipore corporation, Bedford, MA).

For the validation of the method, blood samples from healthy volunteers (Etablissement Français du sang, Montpellier, France) were collected in heparinized tubes and plasma was obtained by centrifugation at 1000 × *g* for 10 min. Pooled drug-free plasma samples were aliquoted, frozen at −20 °C, and then used during the study for the preparation of calibration standards and quality control (QC) samples.

Stock solutions (1 mM) of the analytes and of the internal standard (0.2 mM) were prepared by dissolving each compound in acetonitrile. Stock solutions could be stored at 4 °C protected from light for 1 month. For each compound, two separate stock standard solutions were prepared; one which was used for the preparation of calibration curve standards and the second, which was used for the preparation of quality control samples. The working solutions were prepared fresh daily by diluting stock solutions with acetonitrile–water (50:50, v/v).

2.2. Equipment and chromatographic conditions

The LC–MS analysis was performed using a Hewlett Packard (Les Ulis, France) Agilent 1100 mass spectrometer equipped with an electrospray interface and a data acquisition station (08.04 version). The mass spectrometer was coupled to a Hewlett Packard LC system equipped with a quaternary pumping unit and an autosampler with a loading valve fitted with a 100-μl sample loop (Interchim, Montluçon, France) and set at 4 °C. Separation of the analytes was performed on a Zorbax eclipse XD8 C8 column 150 mm × 4.6 mm, i.d., packed with particles of 4 μm-size. The eluent mixture used consisted of acetonitrile (solvent A) and formate buffer (2 mM, pH 3, solvent B) delivered at a flow rate of 0.8 ml/min. The mobile phase used was a linear gradient over 30 min from 15 to 60% (solvent A) followed by a linear gradient over 10 min from 60 to 70% (solvent A). The column was thermostated at 25 °C. The column was then washed for 5 min with the final gradient solution (70% of solvent A), brought back to the initial condition over 1 min and re-equilibrated for 4 min. The total cycle was therefore 50 min. The injection volume was 20 μl.

Table 1
Selected ions for each compound

Drugs	Molecular ions ($M + H^+$), (*m*/*z*)	Specific fragments (*m*/*z*)
CBZ	237	194
CBZ-EP	236	180
OXCBZ	253	236
10-OH-CBZ	255	237
DiOH-CBZ	271	253
9-Acridan[a]	255	180
2-OH-CBZ	253	210
3-OH-CBZ	253	210
AI	180	152
AO	196	167
IM	179	194

[a] Selected ions were identified from the literature [10,11].

The mass spectrometer was calibrated in the positive ion mode (ESI+) using a mixture of NaI and CsI. The drying gas temperature and flow were maintained at 350 °C and 10 l/min, respectively and the nebulizer pressure was set at 15 psi. The mass spectra obtained from the ion fragmention of each analyte were acquired between 105 and 170 V. The precise value of each cone voltage selected for each compound is not done since it should be kept in mind that the use of the same fragmenter voltage in different apparatus might result in different abundances of the fragments formed. From the full-scan spectra (Fig. 3), for each compound, two specific ions were selected: the protonated molecular ion ($M + H^+$) and the daughter ion (Table 1). Mass spectrometric data were acquired in simple ion recording mode at *m*/*z* 237 for CBZ, *m*/*z* 180 for CBZ-EP and AI, *m*/*z* 236 for OXCBZ, *m*/*z* 237 for 10-OH-CBZ, *m*/*z* 253 for 2-, 3-OH-CBZ and DiOH-CBZ, *m*/*z* 196 for AO and *m*/*z* 194 for IM.

2.3. Preparation of calibration standards and QC samples

The calibration set consisted of seven concentrations, prepared by adding 10 μl of the appropriate working solutions into 0.3 ml of drug-free human plasma. Concentration ranges were 0.5–20 mg/l for CBZ, 0.1–5 mg/l for CBZ-EP, 0.4–20 mg/l for OXCBZ and DiOH-CBZ, 0.3–20 mg/l for 10-OH-CBZ, 0.023–1.25 mgl/l for 2-OH-CBZ, 3-OH-CBZ and AI, and 0.024–1.25 mg/l for AO and IM. The volume added was always less than or equal to 4% of total volume of the samples, so that the integrity of the matrix was maintained.

QC samples used in the validation study were prepared in the same way as the calibration standards, by mixing drug-free human plasma with appropriate volumes of working solutions to obtain three different concentrations, one near to the lower limit of quantitation (C1), one near to the center (C2) and one near to

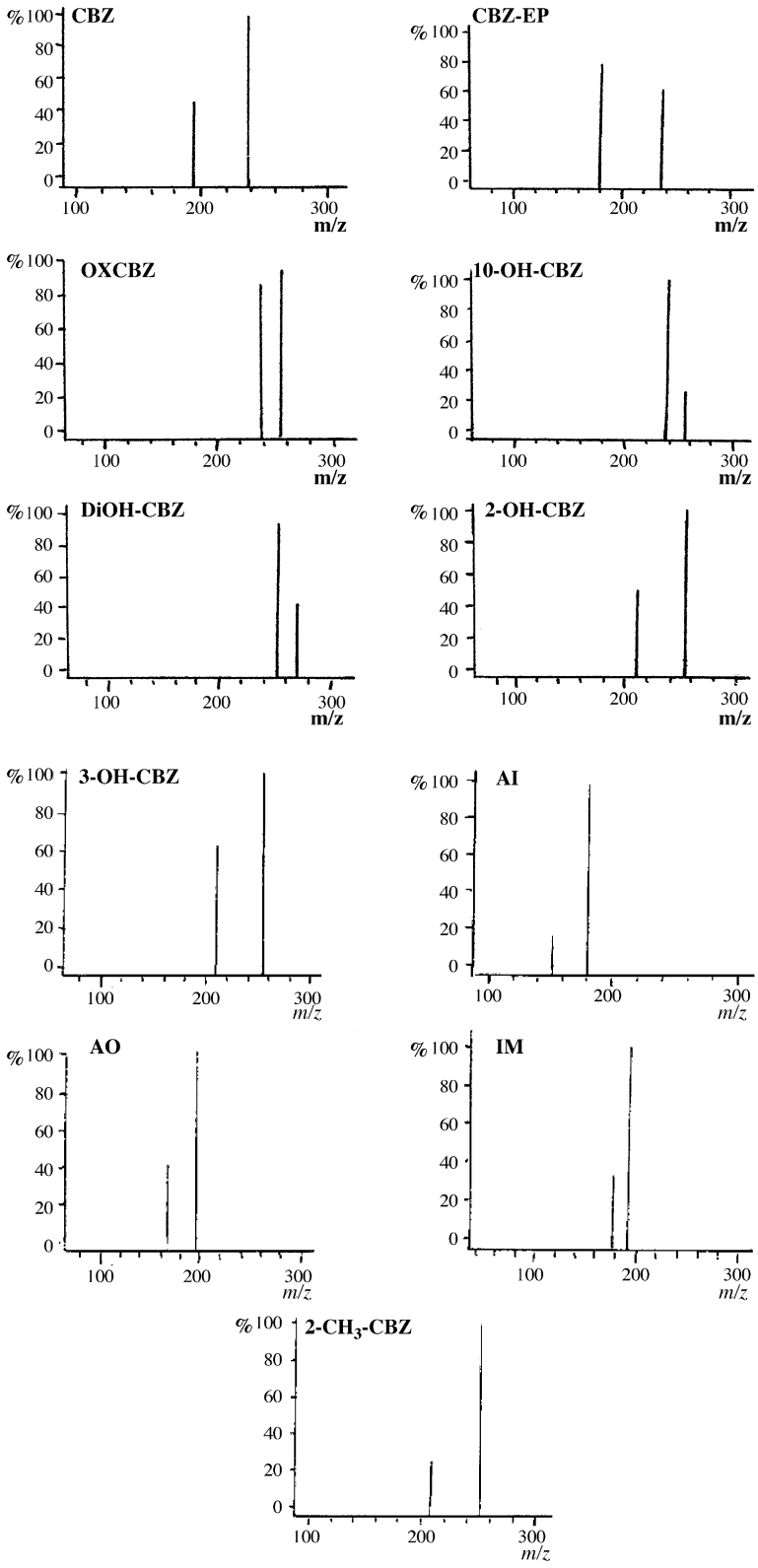

Fig. 3. ESI mass spectra (scan mode) for all compounds.

the upper boundary of the standard curve (C3), (0.7, 5.5, 15 mg/l for CBZ, OXCBZ, 10-OH-CBZ, DiOH-CBZ; 0.16, 1.5, 4 mg/l for CBZ-EP and 0.04, 0.35, 1 mg/l for 2-OH-CBZ, 3-OH-CBZ, AI, AO, IM). Before the sample pretreatment procedure, standards and QC samples previously vortex-mixed were incubated at 25 °C for 15 min, to allow a steady-state with the matrix components. A calibration curve and 3 QC samples were run with every set of eight unknown samples.

2.4. Sample preparation procedure

A 300 μl aliquot of plasma was mixed with 10 μl of internal standard (0.2 mM,); the mixture was vortex-mixed (15 s) then 300 μl of acetone were added and proteins were denatured during 30 s of full speed Vortex mixing. After centrifugation at 1400 × *g* for 10 min, the supernatant was collected and concentrated by evaporation under a stream of nitrogen for 30 min at 37 °C. The dried residue was reconstituted with 40 μl of acetone–water (50:50, v/v), then vortex-mixed to homogenize. A 20 μl volume was injected onto the HPLC column.

2.5. Method validation

2.5.1. Data analysis

From recorded peak heights, the ratio of each analyte to internal standard was calculated. The peak-height ratios were linked to the concentrations of each analyte in plasma according to a quadratic process as: $Y = aX^2 + bX + c$, in which Y is the peak height ratio and X is the concentration of the analyte. The regression curve was not forced through zero. The resulting a, b, c parameters were used to calculate back-calculated concentrations for the calibration curves, which were then statistically evaluated. The normal distribution of the residuals (the difference between nominal and back-calculated concentrations) was verified. Moreover, the mean residual values were computed and compared with zero (Student's t-test); the 95% confidence intervals were also determined.

Due to the lack of reference substance available, 9-acridan, a suspected reactive metabolite, was estimated in plasma from the peak height ratio, 9-acridan/DiOH-CBZ, DiOH-CBZ being the direct precursor of 9-acridan. For a specific patient, its concentration was expressed as a percent of the concentration of its direct precursor, as follows:

$$\left[\frac{\text{Peak height of 9-acridan}}{\text{peak height DiOH-CBZ}}\right] \times 100$$

The 9-acridan was identified using the predominant ions at m/z 255 and m/z 180; the two common ions described in the literature [10,11].

2.5.2. Precision and accuracy

Inter- and intra-day precision and accuracy of the assay were assessed by performing replicate analyses of QC samples (C1, C2, C3) in plasma against a calibration curve. The procedure was repeated on different days ($n = 5$) to determine inter-day repeatability. Intra-day repeatability was determined by treating spiked samples in replicate ($n = 5$) the same day.

The accuracy was evaluated as [mean found concentration/theoretical concentration] × 100; precision was given by the percent relative standard deviation (R.S.D.).

2.5.3. Extraction efficiency

Different solvents (methanol, acetonitrile and acetone) were used to precipitate the proteins from the matrix. Extraction recoveries of the different analytes from plasma were measured three times at each QC sample concentration by calculating the percentage difference between the peak heights of treated QC samples and those of untreated reference standard solutions containing the corresponding concentrations.

2.5.4. Determination of the lower limit of quantitation (LLOQ)

The LLOQ estimated on spiked samples was defined as the lowest concentration which can be determined with a precision ≤20% and an accuracy between 100 ± 20% on a day to day basis. [37–40].

2.5.5. Ion suppression and specificity studies

The absence of ion suppression was demonstrated by the method of Matuszewski et al. [41]. To investigate potential ion suppression effects attributable to the matrix, six different batches of drug-free plasma were treated as described above. The reconstituted extracts (40 μl containing acetone–water, 50:50, v/v) were then enriched with all analytes at three different concentrations (C1, C2, C3). A reference solution containing 40 μl of acetone–water (50:50, v/v) was also enriched with all drugs to the same nominal concentrations. The reconstituted extracts and reference solutions were injected into the LC–MS system. Peak heights obtained from the reconstituted extracts were compared with the corresponding peak heights produced by the reference solutions. The mean height ratios (reconstituted extract in plasma/reference solutions) are presented in Table 2. Thus, no ion-suppression was observed.

The specificity of the method was investigated by analyzing six different batches of drug-free human plasma to determine whether endogenous constituents coeluted with the different analytes. The retention times of endogenous compounds in the matrix were compared with that of the compounds of interest.

Plasma samples from patients receiving other drugs, which might be taken concomitantly with the test drugs, were analyzed for interference. Thus, for drugs that were metabolized in the body, the possible interference with the metabolites was also tested. The following drugs were checked: ethosuximide, lamotrigine, phenobarbital, phenytoïne, and valproïc acid.

Table 2
Results of ion suppression studies

Compounds	Mean height ratios (reconstituted extract in plasma/reference solutions)	R.S.D. (%)
CBZ	0.974	3.41
CBZ-EP	1.04	4.22
OXCBZ	0.96	2.11
10-OH-CBZ	1.05	3.32
DiOH-CBZ	1.04	4.43
2-OH-CBZ	1.03	1.80
3-OH-CBZ	0.937	2.02
AI	1.04	1.90
AO	1.01	1.75
IM	0.977	3.27

2.6. Therapeutic drug monitoring of patient treated with CBZ or OXCBZ

To validate this method, plasma samples from therapeutic drug monitoring of patients chronically treated with CBZ or OXCBZ were analyzed. The recommended daily CBZ, and OXCBZ adult doses were 10–15 and 8–10 mg/kg/day, respectively. This dosage regimen allowed to obtain drug plasma levels at steady-state of 4–12 mg/l for CBZ and 5–20 mg/l for the sum of OXCBZ and 10-OH-CBZ. During therapeutic drug monitoring, blood samples were collected into heparinised tubes at 8:00 h in the morning, just before the first daily drug administration. Immediately after collection blood samples were centrifuged at 1400 × *g* for 10 min; the plasma was transferred into polypropylene tubes and immediately frozen at −20 °C until assay.

3. Results and discussion

3.1. Retention times and specificity

Representative chromatograms are shown in Fig. 4. Under the chromatographic conditions described above, peaks

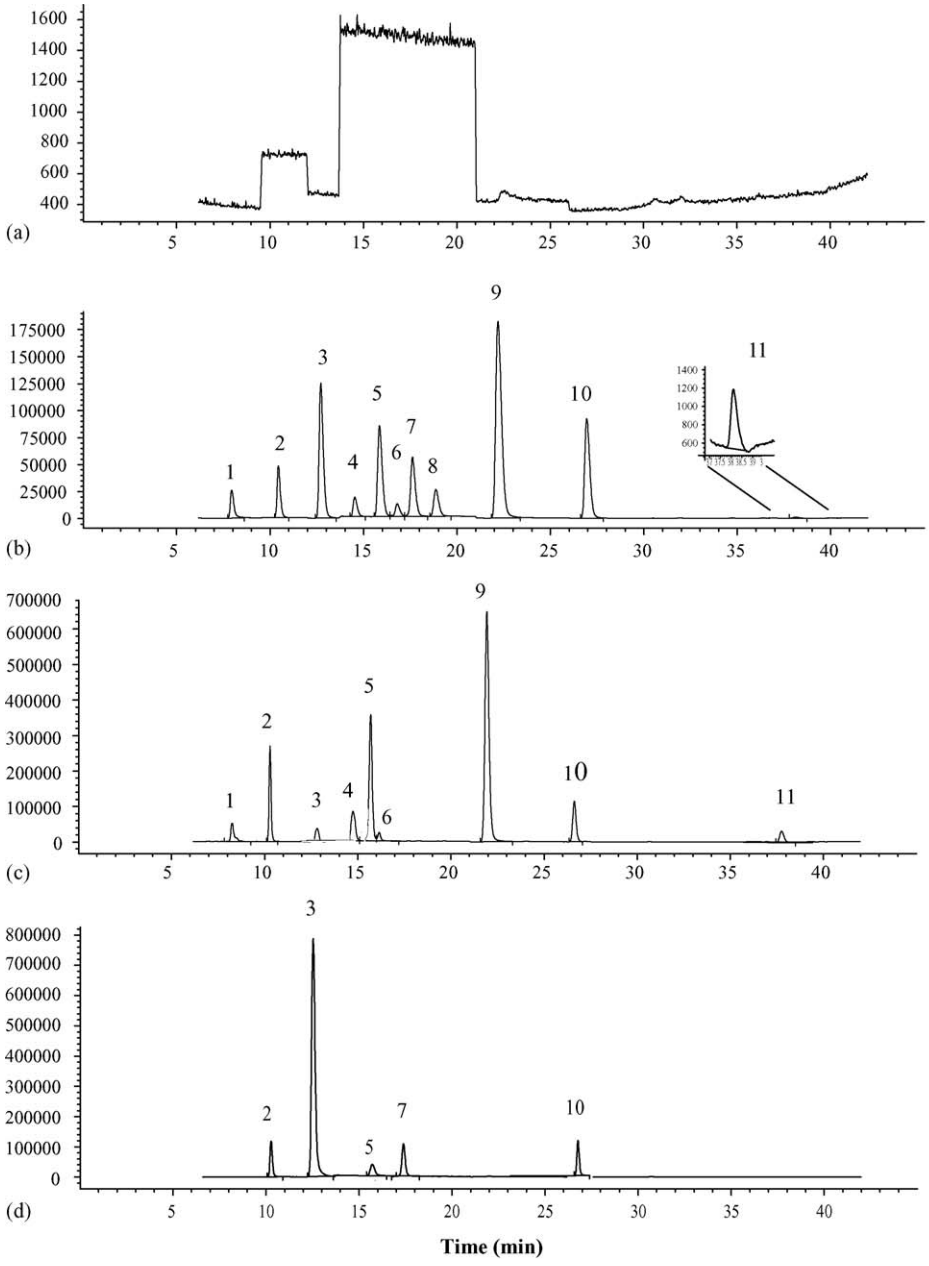

Fig. 4. Typical chromatograms (single-ion recording mode) of drug-free human plasma (a), of drug-free human plasma enriched with AI, 2-OH-CBZ, 3-OH-CBZ, AO, IM at 0.0391 mg/l, DiOH-CBZ, 10-OH-CBZ, OXCBZ, CBZ at 0.625 mg/l and CBZ-EP at 0.156 mg/l (b), of plasma from an authentic epileptic patient treated with CBZ (200 mg twice a day) (concentrations of each compound: 0.034, 5.67, 0.60, 0.037, 3.62, 0.023, 10.9 and 0.031 mg/l for AI, DiOH-CBZ, 10-OH-CBZ, 2-OH-CBZ, CBZ-EP; 3-OH-CBZ, CBZ and IM, respectively) (c) and of plasma from an authentic epileptic patient treated with OXCBZ (600 mg the morning, 300 mg the evening) (concentration of each compound: 1.84, 3.24, 0.023 and 2.98 mg/l for DiOH-CBZ, 10-OH-CBZ, 3-OH-CBZ and OXCBZ, respectively) (d). 1: AI; 2: DiOH-CBZ; 3: 10-OH-CBZ; 4: 2-OH-CBZ; 5: CBZ-EP; 6: 3-OH-CBZ; 7: OXCBZ; 8: AO; 9: CBZ; 10: SI; 11:IM.

were adequately separated. During the 6 months of validation, observed retention times were: AI, 7.7 ± 0.4 min; DiOH-CBZ, 10.3 ± 0.2 min; 10-OH-CBZ, 12.5 ± 0.2 min; 2-OH-CBZ, 14.3 ± 0.25 min; CBZ-EP, 15.7 ± 0.15 min; 3-OH-CBZ, 16.6 ± 0.3 min; OXCBZ, 17.5 ± 0.2 min; AO, 18.3 ± 0.15 min; CBZ, 21.9 ± 0.25 min;; I.S., 26.9 ± 0.2 min; IM, 38.1 ± 0.15 min. Under the chromatographic conditions used, the number of theoretical plates (calculated from the 10-OH-CBZ peak) was approximately 88,500. The column was replaced when the number of theoretical plates decreased below 49,000 (i.e., after 800 analyses).

As shown in Fig. 4a, no peaks due to the matrix interfered at the retention time of the analytes. No interference was found with all tested drugs.

3.2. Drug/response relationship

For all compounds, the quadratic regressions indicated a mean coefficient of determination higher than 0.996. For each compound, mean parameters of the quadratic equation are presented in Table 3. For each point on the calibration curves, the concentrations were back-calculated from the corresponding quadratic equation and mean ± S.D. values were calculated. The results are presented in Table 4. For each analyte, the goodness of fit between back-calculated concentrations and nominal concentrations was statistically evaluated (i) by comparing the regression line of back-calculated versus nominal concentrations to the reference line of slope = 1 and intercept = 0; no significant different was observed; (ii) by studying the frequency distribution histogram of the residuals, which were normally distributed and centred around zero, the number of positive and negative values being approximately equal; and (iii) by comparing the bias to zero; a *t*-test showed that this parameter was not statistically different from zero; moreover, the 95% confidence interval included the zero value.

3.3. Extraction efficiency, precision, accuracy and LLOQ

Most of the published methods involved tedious liquid–liquid [17,25,27,29,32,35,36] or solid-phase [18,19,26,28] extraction of the biological samples. In other published methods, acetonitrile was added to plasma samples to precipitate the plasma proteins [31,33,34]. In our study, protein precipitation was obtained by modification of the dielectric constant by using either methanol, acetonitrile and acetone. However, the highest extraction recoveries were obtained with acetone (data no shown). Extraction recoveries were determined at concentrations of QC samples (0.7, 5.5, 15 mg/l for CBZ, OXCBZ, 10-OH-CBZ, DiOH-CBZ; 0.16, 1.5, 4 mg/l for CBZ-EP and 0.04, 0.35, 1 mg/l for 2-OH-CBZ, 3-OH-CBZ, AI, AO, IM). They were 90 ± 6.7% for CBZ and 80 ± 8.2% for OXCBZ. For the metabolites, the extraction recoveries ranged from 80 to 105%: CBZ-EP (98 ± 5.4%), DiOH-CBZ (80 ± 7.2%), 10-OH-CBZ (105 ± 9.1%), 2-OH-CBZ (89 ± 6.5%), 3-OH-CBZ (92 ± 8.4%), AI (91 ± 4.9%), AO (89 ± 7.4%), IM (80 ± 11.6%).

In regard to previously published methods, the present method involving a simple precipitation of the samples with acetone is neither time-consuming nor expensive, with good extraction recoveries.

Inter- and intra-day precision and accuracy results are presented in Table 5.

Using 0.3 ml of plasma, the LLOQs were 0.5 mg/l for CBZ, 0.1 mg/l for CBZ-EP, 0.4 mg/l for OXCBZ and DiOH-CBZ, 0.3 mg/l for 10-OH-CBZ and 0.023 mg/l for the minor metabolites: 2-OH-CBZ, 3-OH-CBZ, AI, AO and IM (Table 3). Similar results were obtained by Martens and Banditt [27] for CBZ and EP-CBZ and by Pienimäki et al. [17] and Mandrioli et al. [18] for 2-OH-CBZ and 3-OH-CBZ. Higher LLOQ values were reported from published studies involving a simple precipitation with acetonitrile [33,34] or liquid–liquid extraction [32]. However, other workers reached lower LLOQ values (0.7 μg/l to 0.05 mg/l for CBZ [17,18,25,31,35]; 0.012 to 0.055 mg/l for OXCBZ [17-19,28,36]; 5 μg/l to 0.05 mg/l for EP-CBZ [17,18,25,31,35]; 0.012 to 0.025 mg/l for 10-OH-CBZ [17–19,28,36] and 0.01 to 0.025 mg/l for DiOH-CBZ [17,19,25,28]. The first reason to explain a higher LLOQ in our study is the harsh validation procedure employed [37–40]. The second reason is the mode of detection; indeed, most of these methods are based on the spectrophotometric detection around 210 nm and one of them used LC–MS–MS [35] or atmospheric pressure chemical ionization LC–MS [36]. The last reason is the human plasma sample needed (300 μl in this study instead of 500–1000 μl in most of other studies).

Table 3
Results of calibration curves ($n = 8$)[a]

Drugs	Range (mg/l)	*a* (mean)	*b* (mean)	*c* (mean)	r^2	LLOQ (mg/l)
CBZ	0.5–20	−0.0389	2.04 (R.S.D. = 17.6%)	0.973	0.996	0.5
CBZ-EP	0.1–5	−0.317	5.25(R.S.D. = 15.2%)	0.539	0.998	0.1
OXCBZ	0.4–20	−0.0021	0.414(R.S.D. = 18.0%)	0.0737	0.999	0.4
10-OH-CBZ	0.3–20	−0.0401	1.92 (R.S.D. = 16.9%)	0.887	0.999	0.3
DiOH-CBZ	0.4–20	−0.0137	0.732 (R.S.D. = 8.92%)	0.0867	0.999	0.4
2-OH-CBZ	0.023–1.25	−0.842	3.12 (R.S.D. = 10.4%)	0.0753	0.999	0.023
3-OH-CBZ	0.023–1.25	−0.555	2.57 (R.S.D. = 11.7%)	0.0546	0.998	0.023
AI	0.023–1.25	−1.94	9.85 (R.S.D. = 14.6%)	0.162	0.999	0.023
AO	0.024–1.25	−3.06	12.1 (R.S.D. = 15.1%)	0.322	0.999	0.024
IM	0.024–1.25	0.0130	0.257 (R.S.D. = 13.6%)	0.0085	0.999	0.024

n: number of replicates.
[a] $Y = aX^2 + bX + c$.

Table 4
Back-calculated concentrations from calibration curves performed in human plasma

Theoretical concentration (mg/l)	CBZ		OXCBZ		DiOH-CBZ		10-OH-CBZ		CBZ-EP		
	R.S.D. (%)	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)	Recovery (%)	Theoretical concentration (mg/l)	R.S.D. (%)	Recovery (%)
0.5 (CBZ); 0.4 (OXCBZ, DiOH-CBZ); 0.3 (2-OH-CBZ)	7.22	117	18.4	117	17.6	80	16.2	81	0.1	17.2	119
0.625	3.87	103	3.02	102	10.3	89	12.5	89	0.15	12.9	109
1.25	3.14	102	13.2	112	2.43	101	5.72	105	0.31	7.23	108
2.5	5.74	94	9.67	91	1.79	102	3.41	104	0.625	6.51	108
5	8.02	110	4.89	96	7.37	106	7.14	105	1.25	4.24	103
10	5.92	105	2.48	102	3.68	96	4.82	96	2.5	2.55	96
20	2.45	101	1.98	99	2.28	101	1.35	100	5	3.23	100

	R.S.D. (%)					Recovery (%)				
	1	2	3	4	5	1	2	3	4	5
0.023	17.1	16.0	15.2	14.2	16.1	85	118	118	89	84
0.039	11.4	3.89	4.23	8.65	13.3	87	99	98	94	85
0.078	4.68	3.46	5.47	4.11	6.58	107	94	103	107	105
0.15	1.87	9.82	6.05	7.93	4.72	100	109	107	106	98
0.31	1.45	3.45	3.78	3.89	5.85	103	104	103	105	103
0.625	3.92	7.21	2.34	6.02	3.45	98	95	96	96	98
1.25	1.77	2.53	3.41	1.56	2.31	100	101	100	103	100

(1) AI; (2) 2-OH-CBZ; (3) 3-OH-CBZ; (4) AO; and (5) IM. The first concentration level corresponds to the LLOQ defined by a precision $\leq$20% and an accuracy of 100 ± 20%.

Table 5
Accuracy and precision of the method ($n = 5$)

Drugs	Theoretical concentrations mg/l	Precision (%)		Accuracy (%)	
		Intra-assay	Inter-assay	Intra-assay	Inter-assay
CBZ	0.7	7.60	7.29	94	100
	5.5	4.47	12.5	102	107
	15	5.41	5.23	98	99
CBZ-EP	0.16	7.04	10.2	87	87
	1.5	7.84	13.0	97	105
	4	9.21	7.28	112	103
OXCBZ	0.7	8.72	13.0	95	106
	5.5	7.83	10.4	107	86
	15	10.2	12.0	88	100
10-OH-CBZ	0.7	4.39	13.0	87	99
	5.5	3.39	9.42	93	104
	15	5.06	6.24	102	95
DiOH-CBZ	0.7	5.94	10.6	88	89
	5.5	3.44	5.70	94	109
	15	2.79	7.85	92	90
2-OH-CBZ	0.04	4.58	11.8	104	99
	0.35	3.52	8.94	91	104
	1.0	2.91	2.31	98	95
3-OH-CBZ	0.04	2.90	9.97	90	95
	0.35	2.44	3.46	103	102
	1.0	2.71	2.36	97	95
AI	0.04	4.39	6.93	107	112
	0.35	2.74	10.5	89	103
	1.0	2.21	6.49	93	94
AO	0.04	9.75	11.6	86	90
	0.35	7.63	12.6	93	92
	1.0	8.55	7.65	92	99
IM	0.04	7.25	8.08	88	93
	0.35	3.24	5.54	97	94
	1.0	4.99	3.75	104	108

Table 6
Concentration of the parent drug and its metabolites in plasma from patients treated with CBZ or OXCBZ

Compound	CBZ monotherapy (30 patients)		OXCBZ monotherapy (15 patients)	
	Concentration (mg/l)	*n*	Concentration (mg/l)	*n*
CBZ	0.92–16	30	Trace levels (<LLOQ)	15
OXCBZ	–	–	0.4–3.32	13
CBZ-EP	0.12–4.74	30	Trace levels (<LLOQ)	5
10-OH-CBZ	Trace levels (<LLOQ)	20	0.3–10.6	15
DiOH-CBZ	0.4–7.56	30	0.4–2.96	15
2-OH-CBZ	0.023–0.057	9	–	–
3-OH-CBZ	0.023–0.72	25	0.023–0.03	3
AI	0.023–0.067	28	Trace levels (<LLOQ)	3
AO	0.024–0.12	9	Trace levels (<LLOQ)	11
IM	0.024–0.03	3	–	–
9-Acridan[a]	2.5–69%	26	3.9–15%	7

n: number of patients for whom the metabolite was detected.
[a] Results are expressed as a percent of the concentration of its direct precursor: DiOH-CBZ using the formula: [Peak height of 9-acridan/peak height DiOH-CBZ] × 100.

3.4. Therapeutic drug monitoring of patient treated with CBZ or OXCBZ

This LC–MS method was used to analyse plasma samples from epileptic patients treated with CBZ ($n = 30$) or OXCBZ ($n = 15$). Results are reported in Table 6. Measured concentrations were obtained from blood samples drawn immediately before a new drug administration. Fig. 4c shows the chromatogram of a plasma sample from a patient treated with CBZ (400 mg/day) and Fig. 4d shows a chromatogram of a plasma sample from a patient treated with OXCBZ (1200 mg/day). In the present study, plasma levels ranged from 0.92 to 26 mg/l for CBZ and from 0.04 to 3.32 mg/l for OXCBZ (Table 6).

In patients under CBZ therapy, the main metabolites found in plasma were CBZ-EP, DiOH-CBZ, AI and 9-acridan with a great interindividual variability in plasma concentrations. With regard to minor pathways, 2-OH-CBZ and 3-OH-CBZ were identified in 9 and 25 patients, respectively. In only few patients, AO and IM were present. Surprisingly, 10-OH-CBZ, the main OXCBZ metabolite, was found at very low concentrations in only 20 CBZ treated patients (Table 6).

In patients treated with OXCBZ, in addition to the expected metabolites, 3-OH-CBZ, a typical CBZ hydroxylated metabolite, was identified in plasma (Table 6); AI and AO were only detected at trace levels.

4. Conclusion

A specific and sensitive LC–MS method for the simultaneous determination of carbamazepine (CBZ) and eight metabolites: CBZ-10,11-epoxide (CBZ-EP), 10,11-dihydro-10,11-*trans*-dihydroxy-CBZ (DiOH-CBZ), 10-hydroxy-10,11-dihydro-CBZ (10-OH-CBZ), 2-OH-CBZ, 3-OH-CBZ, iminostilbene (IM), acridone (AO), acridine (AI) in human plasma was described. Moreover, under the chromatographic conditions described, the 9-acridan, a suspected reactive metabolite, was well separated from the other compounds. This method was developed to study the potential relationship between reactive metabolites and the onset of idiosyncrasic reactions to CBZ and/or OXCBZ. The AI, AO and IM compounds have reactive properties and could be considered as potential candidates involved in idiosyncrasic reactions. The relationship between side effects and metabolite exposure will be explored.

Acknowledgments

This study was supported, in part, by the CHU of Montpellier with an internal research program and by the French Fondation Against Epilesia (LFCE). The authors would like to acknowledge Novartis pharma Laboratories for kindly providing part of the reference substances of metabolites and Prof. M Audran, Biophysical laboratory, Faculty of Pharmacy, Montpellier, for its analytical assistance.

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