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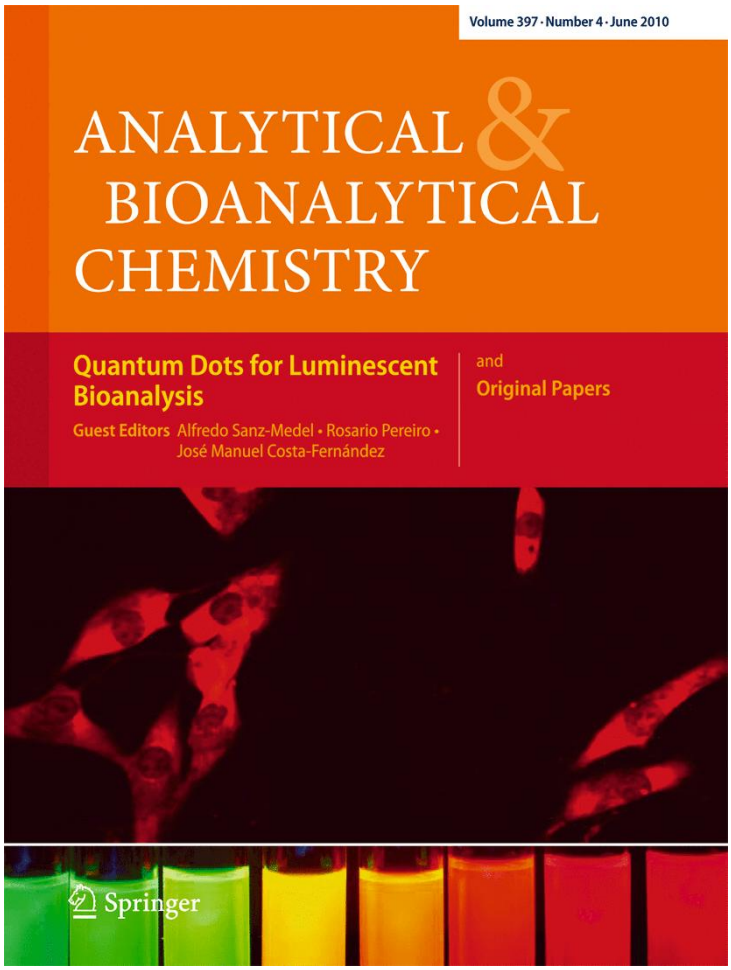

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ORIGINAL PAPER

Development and validation of an HPLC-UV method for the simultaneous quantification of carbamazepine, oxcarbazepine, eslicarbazepine acetate and their main metabolites in human plasma

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Abstract For the first time, a simple, selective and accurate high-performance liquid chromatography method with ultraviolet detection was developed and validated to quantify simultaneously three structurally related antiepileptic drugs; carbamazepine, oxcarbazepine, and the recently launched eslicarbazepine acetate and their main metabolites, carbamazepine-10,11-epoxide, 10,11-*trans*-dihydroxy-10,11-dihydro-carbamazepine, and licarbazepine. The method involves a solid-phase extraction and a reverse-phase C18 column with 5 cm length. The mobile phase consisting of water, methanol, and acetonitrile in the ratio 64:30:6 was selected as the best one and pumped at 1 mL/min at 40 °C. The use of this recent column and an aqueous mobile phase instead of buffers gives several advantages over the method herein developed; namely the fact that the chromatographic analysis takes only 9 min. The method was validated according to the guidelines of the Food and Drug Administration, showing to be accurate (bias within ±12%), precise (coefficient variation <9%), selective and linear (r^2>0.997) over the concentration range of 0.05–30 μg/mL for carbamazepine; 0.05–20 μg/mL for oxcarbazepine; 0.15–4 μg/mL for eslicarbazepine acetate; 0.1–30 μg/mL for carbamazepine-10,11-epoxide; 0.1–10 μg/mL for 10,11-*trans*-dihydroxy-10,11-dihydro-carbamazepine, and 0.1–60 μg/mL for licarbazepine. It was also shown that this method can adequately be used for the therapeutic drug monitoring of the considered antiepileptic drugs, carbamazepine, oxcarbazepine, eslicarazepine acetate, and their metabolites.

Keywords Carbamazepine · Oxcarbazepine · Eslicarbazepine acetate · Therapeutic drug monitoring · HPLC · Bioanalytical method validation

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Introduction

Epilepsy is one of the most common serious neurological disorders and is estimated to affect 50 million persons worldwide [1]. A wide variety of pharmacological treatments of different forms of epilepsy is available to provide a better quality of life for the majority of patients. Since the 1990s, the "Decade of the Brain", several new antiepileptic drugs (AEDs) were introduced into clinical practice, expanding considerably the therapeutic options beyond the traditional first-line medications such as carbamazepine

(CBZ), phenytoin, phenobarbital, and valproic acid. Those new AEDs, which include oxcarbazepine (OXC), topiramate, zonisamide, and lamotrigine, improved the therapeutic response and reduced side effects of epilepsy's treatment [2]. Nevertheless, there are still epileptic patients suffering from seizures and, consequently, the extensive research for the ideal AED continues currently. Eslicarbazepine acetate (ESL) is one of the newest AEDs [3]. This article focuses on CBZ, OXC, and ESL as they represent three generations of structurally related AEDs.

CBZ is one of the oldest and most frequently prescribed anticonvulsants for the treatment of tonic–clonic seizures and, in humans, it is oxidized by inducible hepatic cytochrome P-450-dependent enzymes to carbamazepine-10,11-epoxide (CBZ-E) which has antiepileptic properties and is the main metabolite responsible for the undesirable side effects of CBZ. This metabolite is further almost completely converted to the inactive 10,11-*trans*-dihydroxy-10,11-dihydrocarbamazepine (*trans*-diol). In routine clinical practice, the therapeutic drug monitoring (TDM) of CBZ is very frequent because of its narrow therapeutic range (4–12 μg/mL) and its complex pharmacokinetic properties [4]. Moreover, monitoring CBZ-E plasma levels is also valuable for optimizing the therapy individually.

In order to avoid the formation of CBZ-E, OXC was synthesized by adding a keto-group in the tenth position of CBZ's azepine group. This modification had no significant impact in the mechanism of action and spectrum of anticonvulsant efficacy of CBZ [5]; however, it resulted in important differences in metabolic and tolerability profiles of both drugs. Indeed, in humans, a minority (13%) of OXC remains as parent drug, whereas the majority (79%) is rapidly and extensively converted into its main and pharmacologically active metabolite, licarbazepine (Lic) [6], which appears in plasma as a 4:1 mixture of *S*-licarbazepine (*S*-Lic) and *R*-licarbazepine (R-Lic) [7].

ESL is the newest AED structurally related to CBZ and OXC and it is rapidly and almost entirely metabolized to *S*-Lic (~95%) and, to a minor extent, to *R*-Lic and OXC [3, 8]. Although the need for TDM of ESL or its main metabolite has not been fully established, as ESL is a new drug, the plasma concentrations of these compounds can be useful in assessing compliance and managing patients in particular physiological or pathological conditions [9, 10].

TDM is, undoubtedly, a powerful tool that is necessary to optimize the patient's clinical outcome, enabling individual dose adjustment. Thus, the availability of simple, accurate and reliable analytical methods is crucial for the successful use of TDM in clinical practice and also to support pharmacokinetic studies.

Several high-performance liquid chromatography (HPLC) methods for the determination of old and new AEDs in human plasma have been developed. Some of them are able to quantify CBZ and its main metabolites [11–13] while others quantify OXC and Lic [14–16]. Moreover, since polytherapy is commonly employed in the treatment of epilepsy, there are also methods to quantify simultaneously various AEDs [17–19], some of them including CBZ, OXC, and their main metabolites [20–22]. Obviously, to support the preclinical and clinical pharmacokinetic studies with ESL, bioanalytical methods to quantify ESL and its metabolites were also developed [9, 10, 23]. Although the simultaneous and selective evaluation of these compounds may be valuable in TDM during switching therapies and in some pharmacokinetic studies, to date, no method has yet been published that selectively and simultaneously quantifies all three structurally related drugs (CBZ, OXC, and ESL) and their main metabolites (CBZ-E, Lic and *trans*-diol; Fig. 1).

Thus, the present work aimed to develop and validate a reliable HPLC-UV method for measuring CBZ, OXC, ESL, CBZ-E, *trans*-diol, and Lic in human plasma. The application of this method to routine TDM was also assessed through the analysis of real plasma samples taken from epileptic patients undergoing therapy with CBZ, OXC, or ESL.

Materials and methods

Chemicals and reagents

ESL (lot number 2070-1-2), OXC (lot number FO40003), CBZ-E (lot number PC030916), *trans*-diol (lot number AB031014), Lic (lot number 2070-2-1), and 10,11-dihydrocarbamazepine (internal standard, IS) were kindly supplied by BIAL (Porto, Portugal), while CBZ (lot number 127 K1287) was obtained from Sigma-Aldrich (St Louis, MO, USA). Methanol (HPLC gradient grade) was purchased from Fisher Scientific (Leicestershire, UK) and acetonitrile (HPLC gradient grade) from Lab-Scan (Sowinskiego, Poland). Ultrapure water (HPLC grade, >15 MΩ) was obtained by means of a Milli-Q water apparatus from Millipore (Milford, MA, USA). Ethyl acetate, sodium dihydrogen phosphate dihydrate, di-sodium hydrogen phosphate dihydrate, and hydrochloric acid fuming 37% were purchased from Merck KGaA (Darmstadt, Germany). Human blank plasma samples from healthy donors were provided by the Portuguese Blood Institute after the written consent of each subject.

Preparation of stock solutions, calibration standards, and quality control samples

Stock solutions of CBZ (10 mg/mL), OXC (10 mg/mL), ESL (2 mg/mL), *trans*-diol (2 mg/mL), CBZ-E (10 mg/mL),

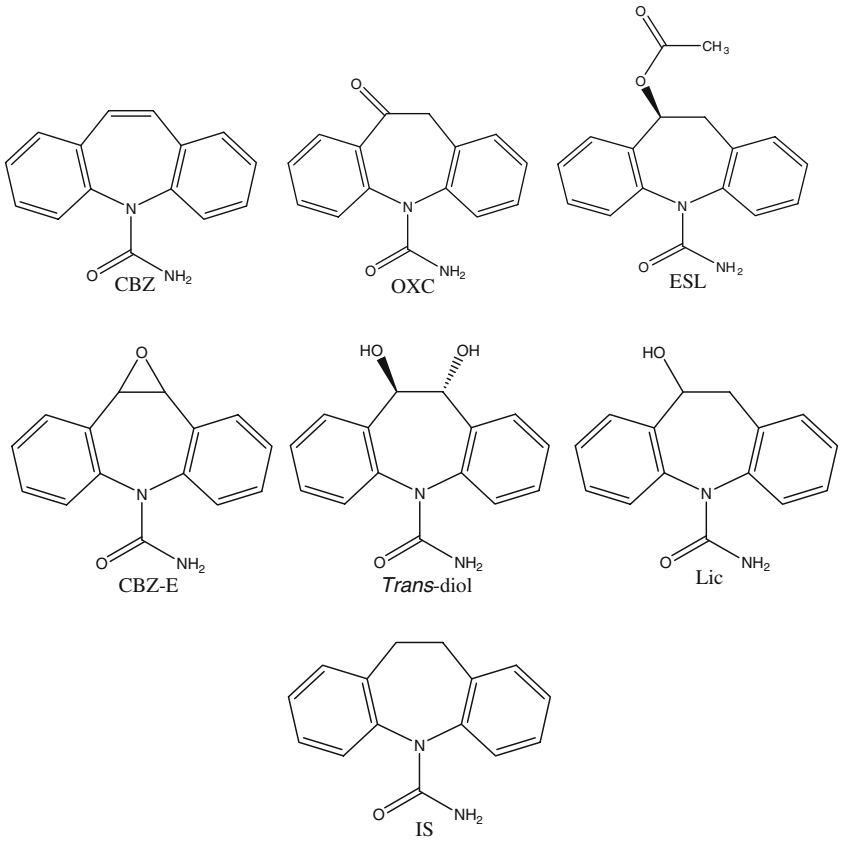

Fig. 1 Chemical structures of carbamazepine (*CBZ*), oxcarbazepine (*OXC*), eslicarbazepine acetate (*ESL*), carbamazepine-10,11-epoxide (*CBZ-E*), 10,11-*trans*-dihydroxy-10,11-dihydro-carbamazepine (*trans-diol*), licarbazepine (*Lic*) and 10,11-dihydro-carbamazepine used as internal standard (*IS*)

Lic (20 mg/mL), and IS (1 mg/mL) were prepared by dissolving appropriate amounts of each compound in acetonitrile. The stock solution of IS was daily diluted with water–acetonitrile (95:5, *v*/*v*) to prepare the working solution (200 μg/mL) while stock solutions of all drugs and metabolites were also appropriately diluted with acetonitrile. Thereafter, stock and diluted solutions of the compounds were appropriately mixed to afford six combined spiking solutions of all six compounds with final concentrations of 2.5, 5, 20, 100, 500, and 1,500 μg/mL for CBZ and OXC; 7.5, 15, 30, 60, 120, and 200 μg/mL for ESL; 5, 15, 50, 250, 500, and 1,500 μg/mL for CBZ-E; 5, 15, 50, 200, 350, and 500 μg/mL for *trans*-diol and 5, 15, 90, 300, 1,000, and 3,000 μg/mL for Lic. Then, 10 μL of each of these combined solutions were individually added to human blank plasma samples in order to construct the calibration curves. All solutions were stored at 4 °C and protected from light. Calibration standards were daily prepared by adding known amounts of the appropriate combined spiking solution to blank human plasma. In the same matrix, quality control (QC) samples were prepared independently at three concentration levels, representing low (QC_1), middle (QC_2), and high (QC_3) points of the calibration curves. Aliquots of 500 μL of blank plasma were spiked to attain CBZ, OXC, ESL, CBZ-E, *trans*-diol, and Lic, respectively, at the following concentrations: 0.14, 0.14, 0.44, 0.24, 0.24, and 0.24 μg/mL in QC_1; 15, 10, 2, 15, 5, and 30 μg/mL in QC_2, and 27, 18, 3.6, 27, 9, and 54 μg/mL in QC_3.

Extraction procedure

Aliquots of human plasma (500 μL) were added to 500 μL of 0.1 M sodium phosphate buffer (pH 5) spiked with 10 μL of the IS working solution. The samples were mixed and loaded into Oasis® HLB (30 mg, 1 mL) cartridges (Waters, Milford, MA, USA), which were previously conditioned with 1 mL of methanol, 1 mL of acetonitrile, and 1 mL of water–acetonitrile (95:5, *v*/*v*). After sample elution, the loaded cartridges were submitted to −30 kPa and washed four times with 1 mL of water–methanol (90:10, *v*/*v*). After drying the sorbent under airflow for 5 min, analytes were eluted with 1 mL of ethyl acetate under gentle vacuum. The eluates were evaporated to dryness under a nitrogen stream at 45 °C and the residues reconstituted in 100 μL of HPLC mobile phase, vortexed for approximately 1 min and placed in an ultrasonic bath at room temperature for approximately 1 min. Following this, the reconstituted extracts were transferred to a 0.22 μm Spin-X centrifugal filter, centrifuged at 13,400 rpm for 2 min and 20 μL of the final filtered samples were injected into the HPLC system.

Chromatographic equipment and conditions

The chromatographic analysis was carried out using a BAS-480 liquid chromatograph equipped with a PM-80 pump, a Rheodyne manual injector with a 20 μL loop, a BAS UV-116 UV–vis detector, a BAS LC-22C temperature controller, a BAS DA-5 chromatography control and a data system interface (all from Bioanalytical Systems, West Lafayette, IN, USA). Data collection and integration were achieved by means of BAS Chromgraph Control and Chromgraph Report software version 2.30.

All three drugs, metabolites and IS were separated at 40 °C by isocratic elution with water–methanol–acetonitrile (64:30:6, *v*/*v*/*v*), at a flow rate of 1 mL/min, on a reversed-phase column LiChroCART® Purospher® Star (C_{18}, 3 μm, 55 mm×4 mm) purchased from Merck KGaA (Darmstadt, Germany). The mobile phase was filtered through a 0.45 μm filter and degassed ultrasonically for 15 min

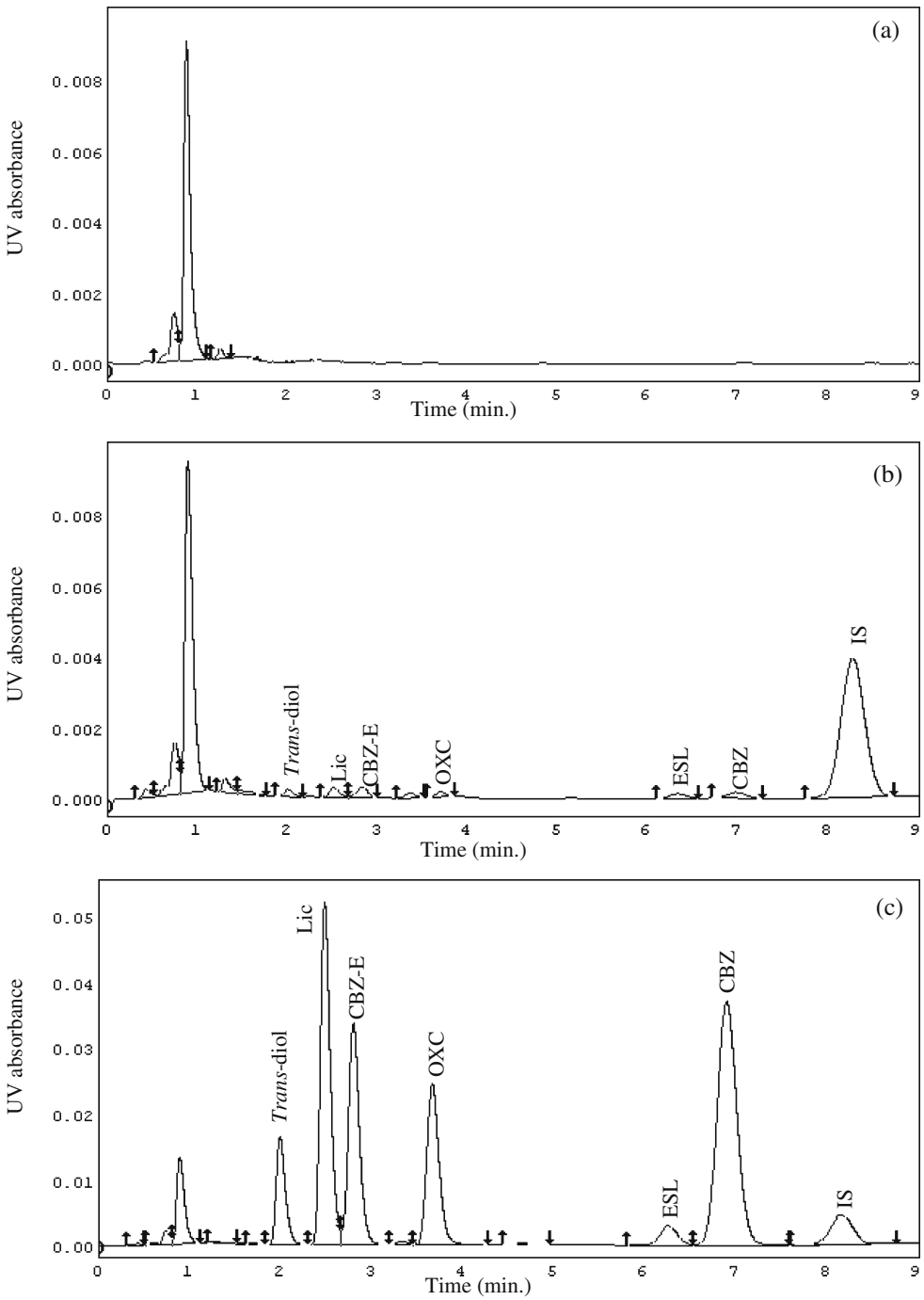

Fig. 2 Typical chromatograms of extracted human plasma: blank sample (**a**), sample spiked at levels of the limit of quantification (**b**), and sample spiked at levels of the higher limit of the calibration ranges (**c**). *Trans*-diol, 10,11-*trans*-dihydroxy-10,11-dihydro-carbamazepine; *Lic*, licarbazepine; *CBZ-E*, carbamazepine-epoxide; *OXC*, oxcarbazepine; *ESL*, eslicarbazepine acetate; *CBZ*, carbamazepine; *IS*, internal standard

Table 1 Retention time of some drugs potentially co-prescribed with the three antiepileptic drugs examined for possible interferences

Drugs	Retention Time (min)
Acetylsalicilic acid	1.10
Amitriptyline	ND
Clomipramine	ND
Diazepam	30.0
Fluoxetine	ND
Furosemide	1.48
Hydrochlorothiazide	0.72
Ibuprofen	ND
Imipramine	ND
Ketoprofen	5.15
Lamotrigine	12.0
Maprotiline	ND
Naproxen	10.0
Nicardipine	ND
Paracetamol	0.72
Phenytoin	5.75
Risperidone	ND
Theophylline	0.72
Topiramate	ND
Trazodone	ND

ND not detected within 30 min after the injection

before use. A sample volume of 20 μL was injected and the analytes were detected at 235 nm.

Method validation

The described method was validated according to internationally accepted recommendations for bioanalytical method validation [24, 25].

Selectivity

Six different blank plasma samples were analyzed for peaks of endogenous substances interfering with those of the analytes. In addition, several drugs potentially co-administered to epilepsy patients with the tested AEDs were injected to check possible interferences at the retention time of each analyte.

Calibration curves

The linearity of the method was assessed by using calibration standards at six different concentrations at the defined plasma concentration ranges of 0.05–30 μg/mL, 0.05–20 μg/mL, 0.15–4 μg/mL, 0.1–30 μg/mL, 0.1–10 μg/mL, and 0.1–60 μg/mL for CBZ, OXC, ESL, CBZ-E, *trans*-diol, and Lic, respectively. The calibration curves were prepared on five different days and were constructed by plotting analyte–IS peak area ratio against the corresponding concentration in plasma. The data were subjected to a weighted linear regression analysis using $1/x^2$ as weighting factor, which was chosen taking the plots and the sums of absolute percentage relative error into account [26].

Precision and accuracy

The precision of the method was assessed using the three QC samples repeated five times within the same day to obtain the repeatability (intra-day precision) and five times over different days to obtain the intermediate precision (inter-day precision), both expressed by coefficient variation in percentage (CV %). The acceptance criterion for precision was a CV lower than 15%.

The accuracy of the method was determined by comparing the means of the calculated concentrations at

Table 2 Calibration parameters (mean values of $n=5$) of 10,11-*trans*-dihydroxy-10,11-dihydro-carbamazepine (*trans*-diol), licarbazepine (Lic), carbamazepine-10,11-epoxide (CBZ-E), oxcarbazepine (OXC), eslicarbazepine acetate (ESL) and carbamazepine (CBZ) and values of precision, given by coefficient variation (CV %), and accuracy, given by bias %, in limit of quantification (LOQ)

Analyte	Calibration parameters			LOQ			
	Range (μg/mL)	Equation	r^2	Nominal value (μg/mL)	Experimental concentration[a] (μg/mL)	CV %	Bias %
trans-diol	0.10–10	$Y=0.2006x-0.0016$	0.9993	0.10	0.1002±0.006	6.97	0.23
Lic	0.10–60	$Y=0.2387x+0.0060$	0.9993	0.10	0.1003±0.013	10.0	0.32
CBZ-E	0.10–30	$Y=0.3360x-0.0004$	0.9992	0.10	0.1003±0.009	9.32	0.35
OXC	0.05–20	$Y=0.4561x+0.0014$	0.9989	0.05	0.0509±0.004	8.58	1.76
ESL	0.15–4.0	$Y=0.1941x-0.0042$	0.9974	0.15	0.1542±0.009	7.05	2.82
CBZ	0.05–30	$Y=0.6702x-0.0029$	0.9983	0.05	0.0496±0.004	8.68	−0.89

[a] Mean ± standard deviation, $n=5$

the three plasma concentrations (QC_1, QC_2, and QC_3) with the nominal concentrations after the analysis of five replicates in the same day (intra-day accuracy) and in five different days (inter-day accuracy). Both are expressed by the deviation from nominal value in percentage (bias %) which must be within ±15%.

Recovery

The recovery of the analytes from human plasma was calculated using the three QC samples and the procedure of extraction described previously. The recoveries of the analytes were calculated by comparing the analytes peak area from extracted samples with the corresponding areas obtained injecting standard solutions at the same theoretical concentrations. The recovery of the IS was determined at the concentration used in sample analysis by calculating the peak area ratio of the IS in extracted samples and non-extracted standards.

Limit of quantification and limit of detection

The sensitivity was evaluated by determining the limit of quantification (LOQ), which is defined as the lowest concentration of the calibration curve that can be measured with acceptable inter- and intra-day precision and accuracy, assessed respectively by CV values lower than 20% and bias values within ±20%.

Limit of detection (LOD), defined as the lowest concentration that can be distinguished from the noise level, was determined for all the compounds by analyzing plasma samples with known concentrations, after successive dilutions, and it was established by visual evaluation of the minimum level at which the analytes can be reliably detected.

Stability

Stability was assessed by comparing the data of QC_1 and QC_3 samples analyzed before (reference samples) and after

Table 3 Intra- and inter-day precision and accuracy (n=5), given by coefficient variation (CV %) and bias % respectively, for 10,11-*trans*-dihydroxy-10,11-dihydro-carbamazepine (*trans*-diol), licarbazepine (Lic), carbamazepine-10,11-epoxide (CBZ-E), oxcarbazepine (OXC), eslicarbazepine acetate (ESL) and carbamazepine (CBZ)

Nominal concentration (μg/mL)	Intra-day			Inter-day		
	Experimental concentration[a] (μg/mL)	CV %	Bias %	Experimental concentration[a] (μg/mL)	CV %	Bias %
trans-diol						
0.24	0.246±0.014	5.73	2.76	0.238±0.017	7.47	−0.66
5	4.955±0.219	4.42	−0.91	4.969±0.297	5.99	−0.62
9	9.194±0.485	5.27	2.16	9.480±0.552	5.82	5.33
Lic						
0.24	0.241±0.016	6.11	0.43	0.245±0.013	4.77	2.15
30	31.11±1.576	5.06	3.69	30.54±0.967	3.16	1.80
54	54.44±3.025	5.55	0.81	54.53±1.748	3.20	0.97
CBZ-E						
0.24	0.241±0.013	5.25	0.31	0.245±0.017	6.89	2.25
15	15.15±0.781	5.16	0.98	14.85±0.486	3.27	−0.99
27	27.25±1.538	5.65	0.92	27.17±0.852	3.14	0.61
OXC						
0.14	0.140±0.012	8.36	0.09	0.138±0.010	7.01	−1.65
10	9.766±0.449	4.59	−2.34	9.599±0.306	3.19	−4.01
18	18.08±1.009	5.58	0.45	17.97±0.531	2.96	−0.19
ESL						
0.44	0.472±0.035	7.81	7.32	0.460±0.035	7.99	4.64
2	1.983±0.118	6.04	−0.85	2.041±0.034	1.70	2.07
3.6	3.501±0.165	4.74	−2.76	3.724±0.93	2.52	3.44
CBZ						
0.14	0.157±0.004	2.74	11.8	0.147±0.012	8.40	5.30
15	15.51±0.774	4.99	3.41	15.17±0.449	2.96	1.14
27	27.51±1.481	5.38	1.90	27.56±0.857	3.11	2.07

[a] Mean ± standard deviation, n=5

being exposed to the conditions for stability assessment (stability samples). A stability/reference samples ratio of 85–115% was accepted as stability criterion.

Human plasma stability of CBZ, OXC, ESL and their main metabolites was assessed during 4 h at room temperature, 24 h at 4 °C and 30 days at −30 °C to simulate sample handling and storage time in the freezer before analysis (n=5). Stability of the analytes was also studied after extraction in mobile phase at 4 °C during 24 h to simulate the time that sample can be in the autosampler. Finally, the analytes stability was evaluated after three freeze and thaw cycles. Aliquots of QC_1 and QC_3 were stored at -30 °C for 24 h, thawed unassisted at room temperature and, when completely thawed, the samples were refrozen for 24 h under the same conditions until completing the three cycles.

Clinical application of the method

This method was used to identify and quantify the concentrations of CBZ, CBZ-E and *trans*-diol in plasma samples of two epileptic patients treated with CBZ and in one patient treated with OXC, who were being monitored in the University Hospital of Coimbra. Moreover, four plasma samples of epileptic patients treated with ESL were kindly provided by BIAL to determine ESL's metabolites.

Results

Choice of assay conditions

In order to achieve the best chromatographic conditions to separate all the analytes in a LiChroCART® Purospher® Star C_{18} column, drug individual and combined solutions were directly injected in HPLC system and several mobile phases were tested. At room temperature and using a water–methanol (70:30, *v*/*v*) solution as mobile phase, CBZ-E, *trans*-diol, and Lic were not separated. Although decreasing the methanol percentage to 25% was enough to separate them, the resolution of ESL and CBZ was compromised and the run time enhanced significantly. Therefore, the authors established that 30% of methanol should be used in the mobile phase, but other strategies for achieving the optimum resolution of CBZ-E, *trans*-diol, and Lic had to be considered. As several articles use phosphate buffer in mobile phase [14, 15, 17, 19], the influence of mobile phase pH on peaks shape, resolution, and retention times was evaluated. No differences were observed in the pH range of 3.9–9.0 and, consequently, water was used for the following studies, avoiding the incorporation of salts in the mobile phase. The retention times were reduced enhancing the temperature to 40 °C but, although the resolution of peaks of *trans*-diol, Lic, and CBZ-E was better, it was not yet acceptable. Then acetonitrile, in a percentage range of 2–10%, was also included as a component of the mobile phase and it was found that the mobile phase composed by water, methanol, and acetonitrile (64:30:6, *v*/*v*/*v*) allowed the complete separation of the six compounds in study. After that, several compounds were tested for potential use as IS. Chloramphenicol, BIA 2-265 and 10,11-dihydro-carbamazepine appeared to be appropriate. However, since BIA 2-265 is not commercially available and chloramphenicol is structurally less related to the analytes than 10,11-dihydro-carbamazepine, the latter was selected to be used as IS.

Before using the solid-phase extraction (SPE) procedure to extract the analytes from human plasma, precipitation of proteins was tested with methanol, acetonitrile, and trichloroacetic acid 20%. The use of methanol and acetonitrile was not possible because of their low selectivity power, resulting in the appearance of several peaks of endogenous substances at the retention time of the drugs and their metabolites. The use of trichloroacetic acid 20% was also not possible because the acidic environment easily degraded the CBZ-E. Thus, SPE was developed and, at first, the conditions reported by Alves et al. [23] were tested. Under this conditions, the recovery of *trans*-diol was

Table 4 Absolute recovery of 10,11-*trans*-dihydroxy-10,11-dihydro-carbamazepine (*trans*-diol), licarbazepine (Lic), carbamazepine-10,11-epoxide (CBZE), oxcarbazepine (OXC), eslicarbazepine acetate (ESL) and carbamazepine (CBZ) from human plasma

Analyte	Nominal concentration (μg/mL)	Recovery[a] %	CV %
Trans-diol	0.24	78.21 ± 4.18	5.35
	5	81.44 ± 5.61	6.88
	9	78.44 ± 3.16	4.03
Lic	0.24	88.67 ± 8.31	9.38
	30	91.16 ± 6.15	6.74
	54	86.02 ± 2.06	2.39
CBZ-E	0.24	85.72 ± 2.34	2.72
	15	91.52 ± 6.05	6.61
	27	86.08 ± 1.99	2.31
OXC	0.14	84.34 ± 5.32	6.31
	10	91.03 ± 5.87	6.45
	18	85.98 ± 1.80	2.10
ESL	0.44	73.77 ± 4.98	6.77
	2	84.76 ± 7.08	8.35
	3.6	79.54 ± 3.21	4.03
CBZ	0.14	79.99 ± 2.82	3.52
	15	91.87 ± 4.90	5.32
	27	85.72 ± 2.29	2.67

[a] Mean ± standard deviation

low (<50%) and, therefore, different washing solutions and eluting solvents (acetonitrile, methanol, and diethyl ether) were tried. The best extraction conditions were obtained when washing the loaded cartridges four times with 1 mL of water–methanol (90:10, *v*/*v*) and eluting it with 1 mL of ethyl acetate.

Method validation

When blank plasma samples from six healthy volunteers were analyzed, no interferences appeared at the retention times of the analytes (Fig. 2a). Figure 2b shows a representative chromatogram of a blank plasma sample spiked with the IS (4 μg/mL) and all analytes at concentration levels of the LOQ, while Fig. 2c represents a chromatogram obtained after spiking a plasma sample with IS and all analytes at concentration levels of the upper limit of the calibration range. The run time was less than 9 min and the retention times of *trans*-diol, Lic, CBZ-E, OXC, ESL, CBZ, and IS were, respectively, 1.98, 2.49, 2.80, 3.68, 6.31, 6.98, and 8.10 min. In addition, the method was tested for possible interferences from co-prescribed AEDs and other drugs (Table 1), and no interferences were found.

The linearity of the method in the range stated previously was demonstrated for all the compounds ($r^2 > 0.997$). The regression equations of calibration curves and the corresponding regression coefficients obtained for each AED and metabolites are summarized in Table 2. Under the experimental conditions described, the LOQ of the assay was set at 0.1 μg/mL for *trans*-diol, Lic, and CBZ-E, 0.15 μg/mL for ESL and 0.05 μg/mL for CBZ and OXC with acceptable precision and accuracy as shown in Table 2. The LOD was defined as 0.010 μg/mL for *trans*-diol, Lic and CBZ-E, 0.020 μg/mL for ESL and 0.005 μg/mL for CBZ and OXC.

As shown in Table 3, the intra-day and inter-day precision, expressed by CV, were below 9% at all concentration levels and for all the compounds. Similarly, intra- and inter-day accuracy, expressed by bias, was within ±12% for all analytes.

The recovery values from human plasma are presented in Table 4. The mean recoveries, taking CBZ and its derivatives into account, ranged from 73.77% to 91.87% and showed low CV values. The recovery of the IS was 91.73%, with a CV of 8.75%.

Finally, from the stability data obtained at QC_1 and QC_3 concentration levels (Table 5), no significant loss was

Table 5 Stability (values in percentage) of 10,11-*trans*-dihydroxy-10,11-dihydro-carbamazepine (*trans*-diol), licarbazepine (Lic), carbamazepine-10,11-epoxide (CBZ-E), oxcarbazepine (OXC), eslicarbazepine acetate (ESL) and carbamazepine (CBZ) in plasma after 4 h at room temperature (RT), 24 h at 4 °C, 30 days at −30 °C, three freeze and thaw cycles and in mobile phase at 4 °C during 24 h

Analyte concentration (μg/mL)	Stability/reference analyte concentrations (%)				
	Stability conditions				
	Human plasma				Mobile Phase
	RT 4 h	4 °C 24 h	-30 °C 30 days	Three cycles freeze/thaw	4 °C 24 h
trans-diol					
0.24	96.07	99.83	92.60	91.17	98.64
9	105.3	107.5	96.35	99.13	89.00
Lic					
0.24	91.35	112.5	96.49	93.34	104.8
54	103.5	110.1	95.39	86.55	102.1
CBZ-E					
0.24	99.82	109.9	93.21	92.54	103.3
27	103.1	110.0	95.87	86.71	102.6
OXC					
0.14	98.30	93.22	91.81	90.75	98.18
18	101.7	108.0	93.54	88.5	101.9
ESL					
0.44	97.66	97.10	86.71	90.06	92.86
3.6	101.8	105.8	94.90	89.40	101.3
CBZ					
0.14	96.96	101.18	100.7	91.01	96.17
27	102.3	109.2	94.08	87.40	101.2

observed when spiked plasma samples were stored for 24 h at 4 °C or for 1 month at −30 °C. Moreover, the analytes also demonstrated to be stable in mobile phase at 4 °C during 24 h. Because of the possible freezing of patient's plasma samples before their processing and analysis, the stability of the analytes was also evaluated after three freeze and thaw cycles and relevant differences were not found.

Clinical application of the method

The method was shown to be suitable for monitoring CBZ, CBZ-E, and *trans*-diol in patients treated with CBZ. A representative chromatogram obtained for these patients is depicted in Fig. 3a and it is clearly seen that peak shape and resolution are very similar to those obtained using spiked blank plasma with no interferences. The same results were

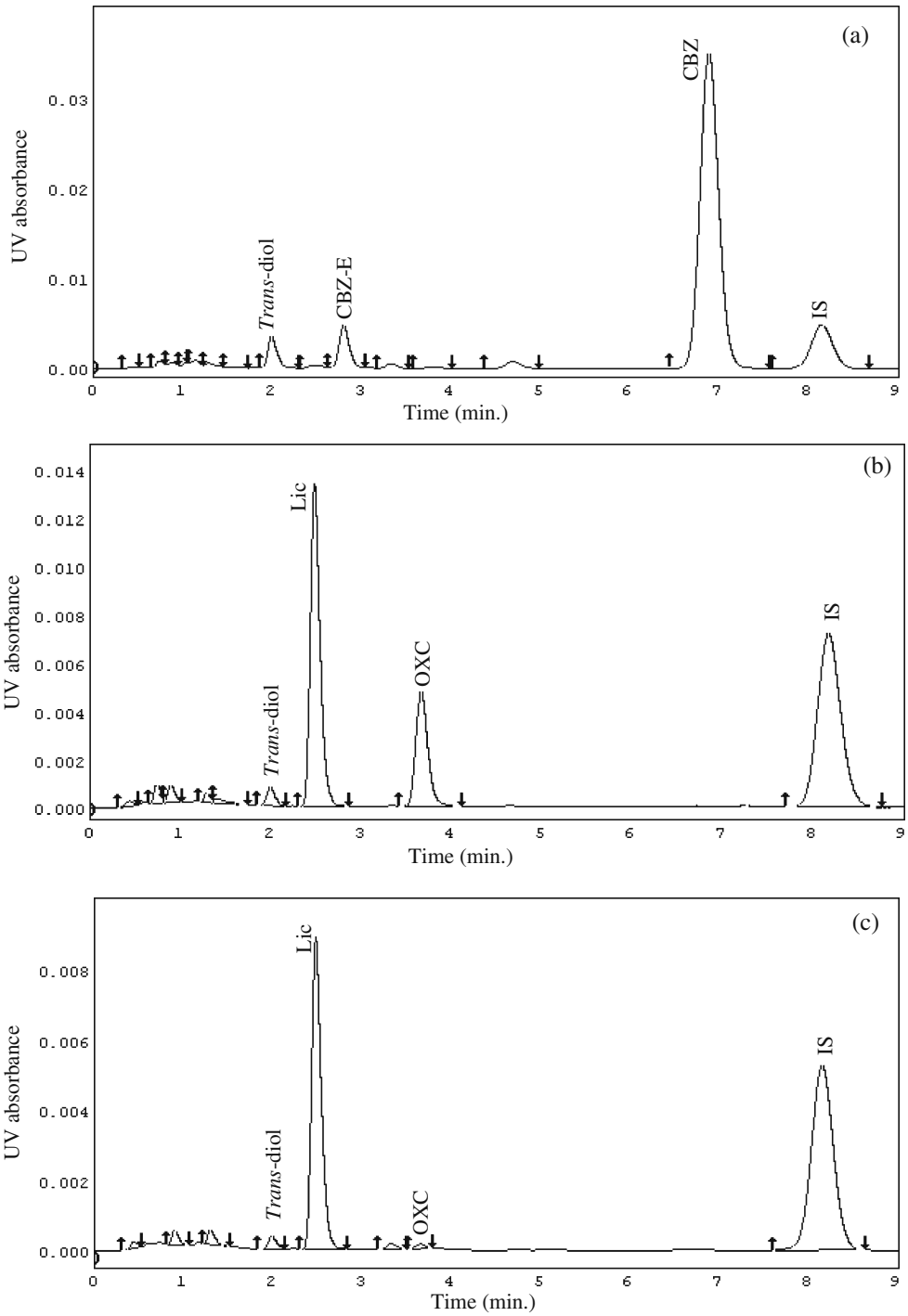

Fig. 3 Representative chromatograms of plasma samples obtained from epileptic patients treated with **a** carbamazepine (*CBZ*); **b** oxcarbazepine (*OXC*), and **c** eslicarbazepine acetate. In **a**, the concentrations of 10,11-*trans*-dihydroxy-10,11-dihydrocarbamazepine (*trans-diol*), carbamazepine-epoxide (*CBZ-E*), and CBZ are, respectively, 1.50; 1.41 and 10.63 μg/mL. In **b**, *trans*-diol, Lic, and OXC concentrations are 0.2; 3.21, and 0.74 μg/mL, respectively. In **c**, the concentration of *trans*-diol, licarbazepine (*Lic*) and OXC are, respectively, 0.29, 6.18, and 0.06 μg/mL, and eslicarbazepine acetate was not detected (prodrug)

found for patients treated with OXC and ESL as shown in Fig. 3b and c, respectively.

Discussion

The aim of this study was to develop and validate an HPLC-UV method for simultaneous quantification of CBZ, OXC, ESL, *trans*-diol, CBZ-E and Lic in human plasma samples and to demonstrate its use in TDM of epileptic patients. The therapeutic efficacy of AEDs depends on attaining and maintenance of well-defined plasma concentrations. A validated analytical method is essential to yield results that satisfactorily allow the monitoring of patients during therapy. Furthermore, a routine laboratory, where a large number of samples have to be analyzed every day, requires short analysis times, simple instrumentation, and easy chromatographic conditions. Thus, herein, a rapid, easy, selective, accurate, precise, and sensitive HPLC-UV method to quantify simultaneously CBZ, OXC, ESL, CBZ-E, *trans*-diol and Lic in human plasma is presented for the first time.

For sample pre-treatment, SPE was chosen, because it allows a simple, rapid, and accurate sample preparation. The use of Oasis® HLB extraction cartridges also contributes to its simplicity because flow rates are critical only during sample application and elution. In contrast to the deproteinization with methanol, acetonitrile, and trichloroacetic acid, the SPE procedure proved to be able to extract the analytes with negligible chromatographic interferences. In addition, the SPE procedure developed is faster, less cumbersome and gives better extraction yields than the liquid–liquid extraction procedures reported in literature [22, 27].

On the other hand, we chose to develop a new HPLC method employing the most usual detection system and the simplest chromatography elution conditions found in clinical laboratories and clinical pharmacokinetic departments. Therefore, detection with mass spectrometry [28] and gradient elution [29, 30] were avoided. In fact, our method provided, with high precision, accuracy and sensitivity, a good separation between the seven compounds using UV detection and isocratic conditions. In routine analysis, the main advantage of isocratic conditions is that it is not necessary to wait between the end of one run and the next one. Furthermore, the use of the C_{18} reverse-phase chromatographic column with 5 cm length allows a run time of 9 min to elute all the compounds.

The validation of our method assured that, whatever the drug, a linear response can be obtained for a concentration range larger than the therapeutic window and, thereby, it can be used in TDM. The LOQ of compounds is in agreement with literature, although there are articles where the LOQ of Lic is lower than our values [27, 31]. Nevertheless, this can be explained by the different wavelength used in both techniques since the methods described in literature are based on detection around 210 nm. Although this allows a lower LOQ for Lic (and also for the other compounds), its application was not possible in our method because endogenous interferences were detected. The choice of the 235 nm was a compromise to obtain a correct selectivity for all analytes evaluated. It is extremely important to state that the LOQ of the analytes obtained with the method developed herein is often lower than those that are reported in literature [15, 18, 19, 23, 32], including those involving more sensitive and expensive techniques such as (tandem) mass spectrometric detections were used [16, 30].

The LOQ values and concentration range obtained with our method for each analyte demonstrate its successful application for TDM of epileptic patients undergoing treatment with CBZ or OXC or ESL (Fig. 3).

Conclusion

We present a simple, rapid, fully validated HPLC-UV method that, for the first time, determines simultaneously CBZ, OXC, ESL, *trans*-diol, CBZ-E, and Lic in human plasma. Using this procedure, the sample pre-treatment is fast, chromatographic analysis takes only 9 min and, the stability of analytes in samples is guaranteed during sample management, chromatographic system and the freezing time. The possibility of determining plasma levels of each drug and its metabolite at the same time as other drugs are co-administered is far-reaching for drug monitoring, especially during the switching of AEDs, for instance, from CBZ to OXC or CBZ to ESL or OXC to ESL. It was demonstrated that the proposed method is suitable for a reliable TDM of patients undergoing therapy with CBZ, OXC, or ESL.

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