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First HPLC–UV method for rapid and simultaneous quantification of phenobarbital, primidone, phenytoin, carbamazepine, carbamazepine-10,11-epoxide, 10,11-*trans*-dihydroxy-10,11-dihydrocarbamazepine, lamotrigine, oxcarbazepine and licarbazepine in human plasma

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ABSTRACT

A sensitive and fast high-performance liquid chromatographic method coupled with ultraviolet detection is herein reported for the simultaneous determination of human plasma concentration of six antiepileptic drugs frequently used in clinical practice [phenobarbital (PB), primidone (PRM), phenytoin (PHT), carbamazepine (CBZ), lamotrigine (LTG), oxcarbazepine (OXC)] and some of their main metabolites, carbamazepine-10,11-epoxide (CBZ-E), 10,11-*trans*-dihydroxy-10,11-dihydrocarbamazepine (*trans*-diol) and licarbazepine (Lic). Sample preparation consisted of a deproteinization step with methanol followed by a solid-phase extraction procedure. Chromatographic separation was achieved in approximately 15 min on a reversed-phase C_{18} column using a mobile phase composed by water–methanol–acetonitrile–triethylamine (68.7:25:6:0.3, v/v/v/v; pH 6.5) pumped isocratically at 1.0 mL/min. The detector was set at 237 nm. Calibration curves were linear with regression coefficients greater than 0.992 over the concentration ranges 0.25–100 μg/mL for PB, 0.4–50 μg/mL for PRM, 0.5–50 μg/mL for PHT, 0.1–50 μg/mL for CBZ, LTG and CBZ-E, 0.1–25 μg/mL for OXC, 0.25–10 μg/mL for *trans*-diol and 0.15–80 μg/mL for Lic. Inter- and intra-day imprecision did not exceed 12.15% and inaccuracy was within ±14.91%. Absolute mean recoveries ranged from 78.49 to 101.04% and no interferences were observed at the retention times of the analytes and internal standard (ketoprofen). This bioanalytical method was successfully applied to real plasma samples from epileptic patients and it seems to be a suitable tool for routine therapeutic drug monitoring and also to support other clinical pharmacokinetic-based studies.

1. Introduction

Epilepsy is a chronic neurological disorder that affects approximately 50 million people worldwide [1]. Among several therapeutic approaches, pharmacotherapy based on a wide variety of antiepileptic drugs (AEDs) is the first-line treatment option to achieve seizure control [2]. Indeed, since the introduction of phenobarbital (PB), several molecules with anticonvulsant properties were developed and, presently, more than twenty AEDs are patented in the world market, including older drugs such as phenytoin (PHT), primidone (PRM), ethosuximide, carbamazepine (CBZ) and valproic acid, and newer AEDs like lamotrigine (LTG), levetiracetam, oxcarbazepine (OXC), zonisamide and eslicarbazepine acetate.

Dealing with such a wide arsenal of AEDs does not make the clinical selection of the best pharmacotherapy an easy task and, therefore, several guidelines have been established [3,4]. Although monotherapy remains the preferred therapeutic approach in newly diagnosed epilepsy, it has failed in many patients even when two or more different AEDs are attempted and gradually titrated to the maximum tolerated dose [5]. Thus, if an epileptic condition is refractory to an initial monotherapy regimen, patients should

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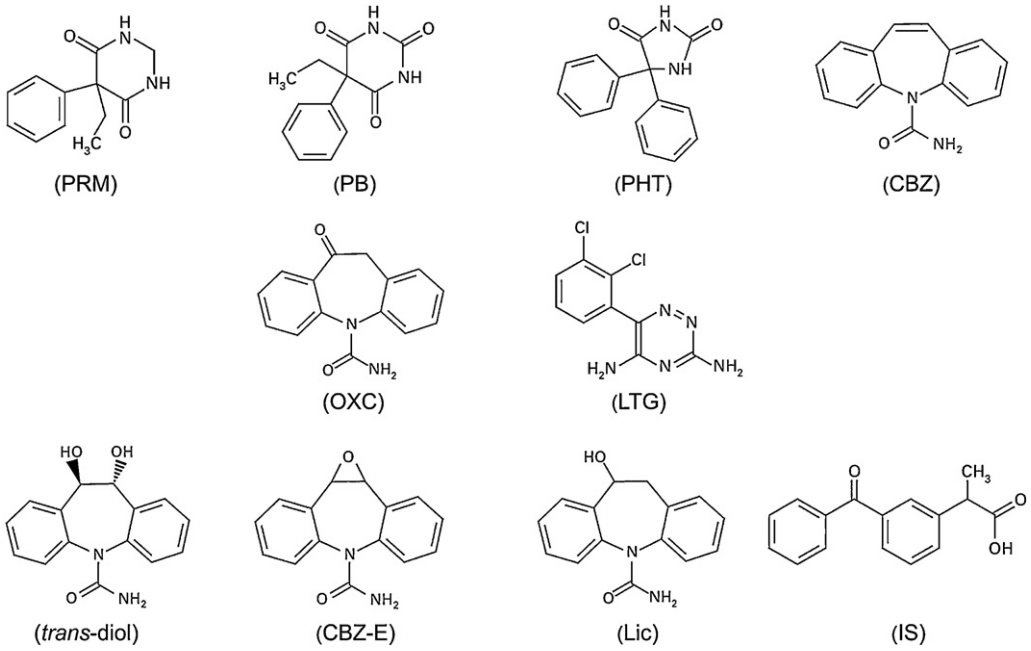

Fig. 1. Chemical structures of phenobarbital (PB), primidone (PRM), phenytoin (PHT), carbamazepine (CBZ), lamotrigine (LTG), oxcarbazepine (OXC), carbamazepine-10,11-epoxide (CBZ-E), 10,11-*trans*-dihydroxy-10,11-dihydrocarbamazepine (*trans*-diol), licarbazepine (Lic), and ketoprofen which was used as internal standard (IS).

be switched to monotherapy with another AED or to polytherapy [6,7]. However, to ensure that therapeutic failure is due to the inadequate seizure control of the selected AED monotherapy regimen rather than the inability to achieve the proper drug plasma concentrations in the patient, therapeutic drug monitoring (TDM) of plasma/serum levels becomes necessary. Considering the variable nature of epilepsy, patient's pathophysiological features, comorbidities and co-medications, AED therapy must be carefully optimized for each individual patient concerning both therapeutic and toxicological profiles. Hence, the TDM of AEDs has become a routine practice in the management of epilepsy [8] not only when AED polytherapy combinations are implemented, but also during the course of pharmacological conversion between different AED monotherapies.

The present work aimed at developing a sensitive and fast bioanalytical method able to simultaneously determine the plasma concentrations of PB, PRM, PHT, CBZ, LTG and OXC as they represent the main frontline of old and new AEDs in the current clinic [9], but also some of their main metabolites [carbamazepine-10,11-epoxide (CBZ-E), 10,11-*trans*-dihydroxy-10,11-dihydrocarbamazepine (*trans*-diol), and licarbazepine (Lic)] (Fig. 1). Regarding the metabolic and pharmacotoxicological properties of such metabolites, it is clear that they should also be quantified in several circumstances. Specifically, PRM is mainly metabolized in the liver to PB, its active metabolite, and both exhibit anticonvulsant activity. In humans, CBZ is mainly oxidized to CBZ-E which is the main responsible for the toxic effects of the parent compound; then, the epoxide undergoes microsomal hydrolysis and is converted to the inactive *trans*-diol [4]. Although structurally similar to CBZ, OXC is extensively reduced to the pharmacologically active metabolite Lic.

Bearing in mind that PB, PRM, PHT and CBZ are mainly metabolized by the hepatic cytochrome P450 system, and that LTG and OXC are second-generation AEDs frequently used when switching those first-generation AEDs, it is expected that during these conversion processes drug–drug interactions may occur. Therefore, TDM becomes crucial because abruptly stopping an existing baseline AED increases the risk of breakthrough seizures, while introducing a new adjunctive AED too quickly can cause an exacerbation of adverse effects [7]. Hence, the availability of rapid, simple, sensible, accurate and reliable analytical techniques is primordial to support TDM of these drugs in clinical routine.

During the last years, several high-performance liquid chromatographic methods coupled to ultraviolet detection (HPLC–UV) or mass spectrometry detection (HPLC–MS and HPLC–MS/MS) for the simultaneous determination of AEDs and some of their metabolites have been reported in literature [10–26]. Notwithstanding, to the best of our knowledge, this paper describes the development and validation of the first HPLC–UV for the simultaneous quantification of PB, PRM, PHT, CBZ, LTG, OXC and three of their main metabolites (CBZ-E, *trans*-diol and Lic) in human plasma.

2. Materials and methods

2.1. Chemicals and reagents

PRM, PHT, CBZ, CBZ-E, OXC and ketoprofen, used as internal standard (IS), were purchased from Sigma–Aldrich (St Louis, MO, USA). Lic and *trans*-diol were kindly supplied by BIAL-Portela & C^{a} S.A. (S. Mamede do Coronado, Portugal). LTG was kindly provided by Bluepharma (Coimbra, Portugal). PB was commercially obtained from Labesfal (Campo de Besteiros, Portugal). Methanol (HPLC gradient grade) was purchased from Fisher Scientific (Leicestershire, UK), acetonitrile (HPLC gradient grade) from Lab-Scan (Sowinskiego, Poland) and ultrapure water (HPLC, >15 MΩ) was prepared by means of a Milli-Q water apparatus from Millipore (Milford, MA, USA). Ethyl acetate and triethylamine (TEA) were acquired from Fisher Scientific (Leicestershire, UK) and Merck KGaA (Darmstadt, Germany) respectively, while *ortho*-phosphoric acid (85%) was purchased from Panreac Química SA (Barcelona, Spain).

Blank human plasma samples from healthy donors were gently provided by the Portuguese Blood Institute after written consent of each subject.

2.2. Preparation of standard solutions

Stock solutions of PB (20 mg/mL), PRM (10 mg/mL), PHT (10 mg/mL), CBZ (10 mg/mL), LTG (10 mg/mL), OXC (5 mg/mL),

CBZ-E (10 mg/mL), *trans*-diol (10 mg/mL), Lic (20 mg/mL) and IS (1 mg/mL) were individually prepared by dissolving appropriate amounts of each compound in methanol. These solutions were then adequately diluted with methanol to obtain the corresponding working solutions. Afterwards, stock and working solutions of drugs and metabolites were properly mixed to afford six combined spiking solutions with final concentrations of 6.25, 12.5, 50, 250, 100 and 2500 μg/mL for PB; 10, 20, 50, 125, 500 and 1250 μg/mL for PRM; 12.5, 25, 50, 125, 500 and 1250 μg/mL for PHT; 2.5, 5, 12.5, 50, 250 and 1250 μg/mL for CBZ, LTG and CBZ-E; 2.5, 5, 12.5, 25, 125 and 625 μg/mL for OXC; 6.25, 12.5, 25, 50, 100 and 250 μg/mL for *trans*-diol and 3.75, 7.5, 20, 100, 500 and 2000 μg/mL for Lic. Each combined solution was daily used for spiking aliquots of blank human plasma in order to prepare six calibration standards in the concentration ranges 0.25–100 μg/mL for PB, 0.4–50 μg/mL for PRM, 0.5–50 μg/mL for PHT, 0.1–50 μg/mL for CBZ, LTG and CBZ-E, 0.1–25 μg/mL for OXC, 0.25–10 μg/mL for *trans*-diol and 0.15–80 μg/mL for Lic.

Quality control (QC) samples at three representative concentration levels (low, medium and high) of the calibration curves were independently prepared in the same biological matrix. Thus, aliquots of blank human plasma were spiked to attain final concentrations of 0.75, 50 and 90 μg/mL for PB; 1.2, 25 and 45 μg/mL for PRM; 1.5, 25 and 45 μg/mL for PHT; 0.3, 25, 45 μg/mL for CBZ, LTG and CBZ-E; 0.3, 12.5 and 22.5 μg/mL for OXC; 0.75, 5 and 9 μg/mL for *trans*-diol and 0.45, 40 and 72 μg/mL for Lic.

A methanolic IS working solution at 250 μg/mL was also prepared by dilution of an appropriate volume of the respective stock solution.

All stock, working and combining solutions were stored at 4 °C and protected from light, with exception of the IS working solution which was daily prepared.

2.3. Sample preparation

An aliquot of human plasma (500 μL) added of 20 μL of the IS working solution was mixed with 1 mL of methanol in order to precipitate plasma proteins. After centrifuging at 13,400 rpm for 10 min, the resulting supernatant was evaporated under a gentle nitrogen stream at 80 °C for 10 min; then, the residual volume of supernatant was diluted with 1.5 mL of water and vortex-mixed for 30 s. Afterwards, the pre-treated sample was subjected to a solid-phase extraction (SPE) on the Oasis® HLB (30 mg, 1 mL) cartridge (Waters, Milford, MA, USA), which was previously conditioned with 1 mL of methanol, 1 mL of acetonitrile and 1 mL of water–acetonitrile (95:5, v/v). The loaded cartridge was subsequently submitted to −60 kPa and washed four times with 1 mL of water. After drying the sorbent under airflow for 5 min, analytes were eluted with 1 mL of ethyl acetate using gentle vacuum. The eluate was evaporated to dryness at 45 °C under a gentle stream of nitrogen gas and reconstituted with 500 μL of mobile phase by vortexing and ultrasonication. At last, 20 μL of the final mixture were injected into the chromatographic system.

2.4. HPLC–UV instrumentation and chromatographic conditions

The chromatographic analysis was performed on 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 DA-5 chromatography control and a data system interface (Bioanalytical Systems, West Lafayette, IN, USA). Data acquisition was achieved by means of BAS Chromgraph Control and Chromgraph Report software version 2.30.

The chromatographic separation of all the six drugs, metabolites and IS was carried out at room temperature on a reversed-phase LiChroCART® Purospher Star® C_{18} column (55 mm × 4 mm; 3 μm particle size) purchased from Merck KGaA (Darmstadt, Germany). An isocratic elution was applied at a flow rate of 1.0 mL/min with a mobile phase composed of water–methanol–acetonitrile–TEA (68.7:25:6:0.3, v/v/v/v) adjusted at pH 6.5 with *ortho*-phosphoric acid 85%. The wavelength detection was set at 237 nm.

2.5. Method validation

The method was validated according to the international recommendations for bioanalytical method validation [27,28] regarding selectivity, linearity, precision, accuracy, limits of quantification and detection, recovery and stability.

Blank human plasma samples from six different subjects were used in order to assess method selectivity, analysing the eventual interference of matrix endogenous substances at the retention times of PB, PRM, PHT, CBZ, LTG, OXC, CBZ-E, *trans*-diol, Lic and IS. Interferences from several commonly co-administered drugs were also tested and included amitriptyline, chlorpromazine, clonazepam, dexamethasone, diazepam, digoxin, fluoxetine, haloperidol, hydrochlorothiazide, ibuprofen, levetiracetam, lorazepam, mirtazapine, naproxen, omeprazole, paracetamol, ranitidine, salicylic acid, sertraline, theophylline, and venlafaxine.

The linearity of the method was evaluated within the defined plasma concentration ranges, using calibration curves prepared on five different days ($n = 5$), constructed with six spiked plasma calibration standards and plotting analyte-IS peak height ratio *versus* the corresponding plasma nominal concentrations. The data were subjected to a weighted linear regression analysis using $1/x^2$ as weighting factor, taking the plots and the sums of absolute percentage of relative error into account [29].

Inter-day precision and accuracy were investigated analysing each QC sample on five consecutive days of the assay ($n = 5$), while the intra-day data were obtained by analysing five sets of QC samples in a single day ($n = 5$). According to bioanalytical method validation guidelines, the acceptance criterion for precision, which is expressed as percentage of coefficient of variation (% CV), must be equal to or lower than 15%; whereas accuracy, which is expressed as the deviation of experimental from nominal concentration values in percentage (% bias), must be within ±15%.

The limit of quantification (LOQ) was defined as the lowest concentration of the calibration curve that can be measured with adequate precision and accuracy. The LOQ was stipulated analysing plasma samples intra- and inter-daily ($n = 5$) and absolute deviations lower than 20% for both CV and bias values were accepted. The limit of detection (LOD) was determined for all the compounds by analysing spiked plasma samples with known concentrations after successive dilutions.

Table 1
Mean calibration parameters ($n = 5$) of primidone (PRM), lamotrigine (LTG), 10,11-*trans*-dihydroxy-10,11-dihydrocarbamazepine (*trans*-diol), phenobarbital (PB), licarbazepine (Lic), carbamazepine-10,11-epoxide (CBZ-E), oxcarbazepine (OXC), phenytoin (PHT) and carbamazepine (CBZ) in human plasma.

Analyte	Calibration parameters Concentration range (μg/mL)	Equation[a]	r^2
PRM	0.40–50	$Y = 0.0485x - 0.0012$	0.992
LTG	0.10–50	$Y = 0.5412x + 0.0031$	0.994
trans-diol	0.25–10	$Y = 0.2163x - 0.0073$	0.994
PB	0.25–100	$Y = 0.0995x + 0.0095$	0.996
Lic	0.15–80	$Y = 0.1898x + 0.0108$	0.994
CBZ-E	0.10–50	$Y = 0.2088x - 0.0005$	0.996
OXC	0.10–25	$Y = 0.2410x - 0.0032$	0.993
PHT	0.50–50	$Y = 0.0384x - 0.0030$	0.992
CBZ	0.10–50	$Y = 0.2132x - 0.0021$	0.994

[a] Y represents analyte-IS peak height ratio; x represents analyte concentration (μg/mL).

The absolute recovery of the compounds from human plasma samples submitted to the treatment previously described was investigated at the three concentration levels (low, medium and high). It was calculated comparing the analytes peak heights from extracted QC plasma samples with those obtained after direct injection of non-extracted aqueous solutions at the same nominal concentrations ($n = 5$). The absolute recovery of the IS was also determined by calculating its peak height ratio

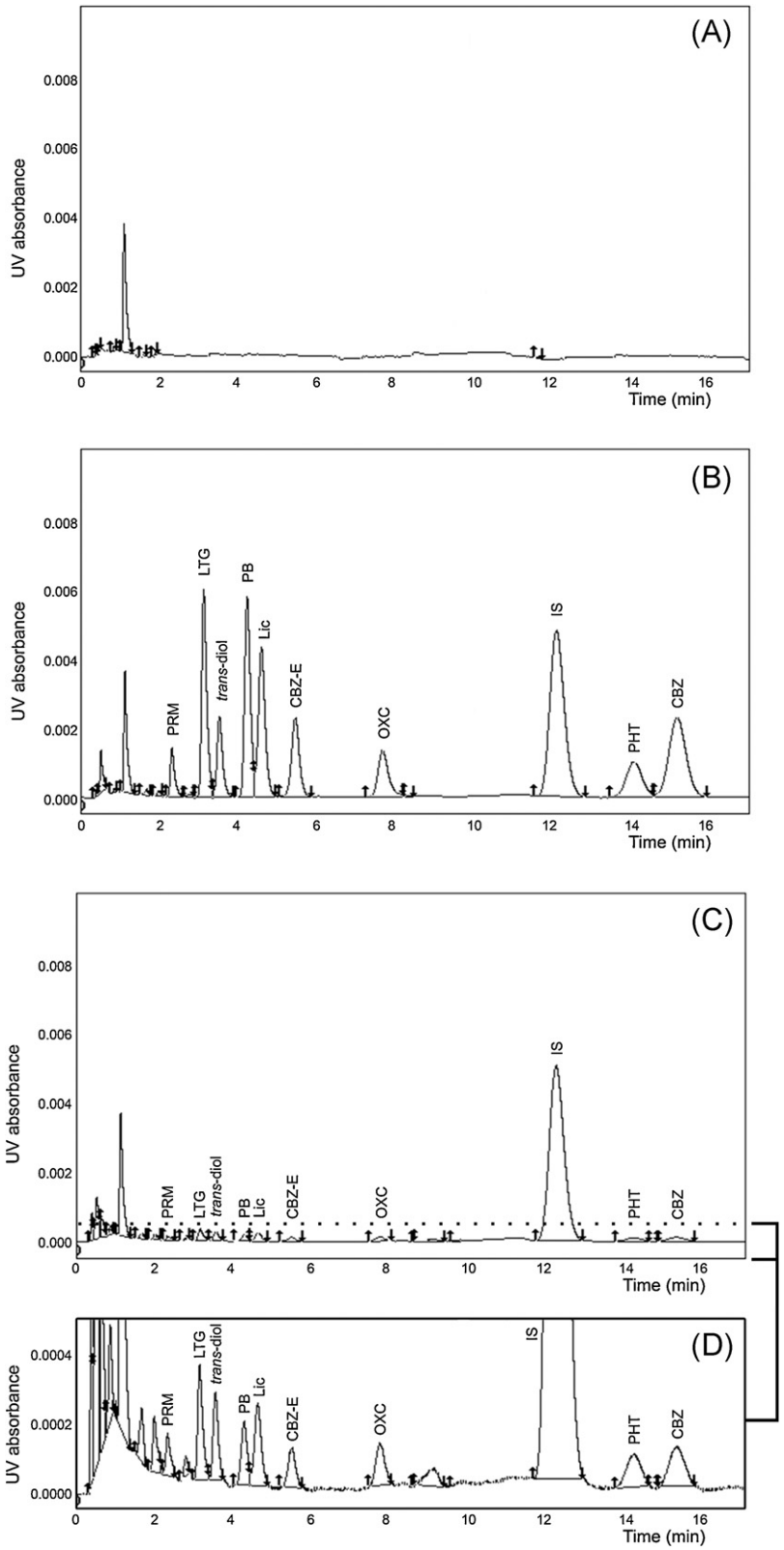

Fig. 2. Typical chromatograms of extracted human plasma: (A) blank plasma; (B) plasma spiked with IS and analytes at intermediate concentrations of the calibration ranges; (C) plasma spiked with the IS and analytes at concentrations of the limit of quantification; (D) expanded partial representation of the chromatogram depicted in (C). CBZ, carbamazepine; CBZ-E, carbamazepine-10,11-epoxide; IS, internal standard; LTG, lamotrigine; Lic, licarbazepine; OXC, oxcarbazepine; PB, phenobarbital, PHT, phenytoin; PRM, primidone; *trans*-diol, 10,11-*trans*-dihydroxy-10,11-dihydrocarbamazepine.

Table 2
Inter- and intra-day precision (% CV) and accuracy (% bias) of the analytes in human plasma samples at the limit of quantification (LOQ) ($n = 5$).

Analyte	LOQ					
	Nominal Concentration (μg/mL)	Inter-day			Intra-day		
		Experimental Concentration (μg/mL)[a]	% CV	% bias	Experimental Concentration (μg/mL)[a]	% CV	% bias
PRM	0.40	0.43 ± 0.055	13.48	7.38	0.41 ± 0.014	3.31	1.94
LTG	0.10	0.11 ± 0.017	14.95	5.78	0.12 ± 0.019	14.29	16.43
trans-diol	0.25	0.26 ± 0.022	9.60	5.46	0.27 ± 0.006	2.42	6.09
PB	0.25	0.25 ± 0.062	18.27	−1.58	0.22 ± 0.026	8.09	−13.93
Lic	0.15	0.14 ± 0.036	17.93	−5.28	0.16 ± 0.019	7.62	5.47
CBZ-E	0.10	0.11 ± 0.011	10.22	9.24	0.11 ± 0.011	10.41	10.87
OXC	0.10	0.11 ± 0.012	12.40	6.26	0.10 ± 0.007	7.04	0.43
PHT	0.50	0.51 ± 0.052	12.05	1.22	0.50 ± 0.021	4.66	-0.17
CBZ	0.10	0.11 ± 0.012	12.31	10.88	0.11 ± 0.017	14.85	14.74

PRM, primidone; LTG, lamotrigine; *trans*-diol, 10,11-*trans*-dihydroxy-10,11-dihydrocarbamazepine; PB, phenobarbital; Lic, licarbazepine; CBZ-E, carbamazepine-10,11-epoxide; OXC, oxcarbazepine; PHT, phenytoin and CBZ, carbamazepine.
[a] Mean ± standard deviation, $n = 5$.

Table 3
Inter- and intra-day precision (% CV) and accuracy (% bias) of the analytes in human plasma samples at the low, middle and high concentrations of the calibration ranges ($n = 5$).

Analyte Nominal Concentration (μg/mL)	Inter-day			Intra-day		
	Experimental Concentration (μg/mL) [a]	% CV	% bias	Experimental Concentration (μg/mL) [a]	% CV	% bias
PRM						
1.2	1.14 ± 0.110	9.90	−4.90	1.22 ± 0.133	10.76	1.43
25	25.54 ± 3.002	11.76	2.18	26.22 ± 1.167	4.45	4.88
45	42.49 ± 2.600	6.12	−5.57	43.95 ± 2.126	4.84	−2.34
LTG						
0.3	0.28 ± 0.028	9.92	−6.42	0.27 ± 0.031	10.67	−8.96
25	24.83 ± 2.565	10.33	−0.66	24.46 ± 0.699	2.86	−2.16
45	45.96 ± 3.601	7.83	2.13	48.49 ± 2.890	5.96	7.76
***trans*-diol**						
0.75	0.70 ± 0.067	9.99	−6.56	0.69 ± 0.032	4.80	−8.62
5	4.95 ± 0.443	9.00	−0.94	4.89 ± 0.229	4.70	−2.11
9	9.40 ± 0.821	8.76	4.49	10.13 ± 0.431	4.27	12.55
PB						
0.75	0.70 ± 0.096	12.08	−6.41	0.66 ± 0.051	6.67	−11.80
50	48.74 ± 4.300	8.80	−2.52	47.52 ± 1.496	3.14	−4.95
90	87.45 ± 7.313	8.35	−2.83	94.91 ± 6.040	6.36	5.46
Lic						
0.45	0.45 ± 0.047	9.38	−0.33	0.46 ± 0.039	7.06	1.91
40	40.91 ± 4.061	9.91	2.28	40.54 ± 1.669	4.11	1.36
72	73.53 ± 5.700	7.75	2.12	76.63 ± 3.847	5.01	6.44
CBZ-E						
0.3	0.27 ± 0.023	8.55	−8.97	0.27 ± 0.016	6.16	−10.92
25	25.28 ± 2.151	8.51	1.11	24.60 ± 1.004	4.08	−1.61
45	44.98 ± 2.833	6.30	−0.04	45.65 ± 2.469	5.41	1.45
OXC						
0.3	0.27 ± 0.022	8.43	−9.63	0.27 ± 0.015	6.62	−10.57
12.5	13.61 ± 1.204	8.86	8.85	13.30 ± 0.523	3.93	6.43
22.5	25.57 ± 1.481	5.80	13.63	24.50 ± 1.471	6.01	8.90
PHT						
1.5	1.34 ± 0.086	6.81	−10.79	1.32 ± 0.102	8.11	−12.22
25	24.46 ± 2.765	11.34	−2.15	23.72 ± 0.730	3.08	−5.14
45	46.99 ± 5.700	12.15	4.43	51.08 ± 4.370	9.13	13.50
CBZ						
0.3	0.27 ± 0.027	10.57	−10.39	0.26 ± 0.025	9.76	−14.91
25	25.12 ± 2.590	10.31	0.47	24.94 ± 0.781	3.13	−0.25
45	46.16 ± 3.957	8.57	2.58	48.82 ± 3.266	9.69	8.48

PRM, primidone; LTG, lamotrigine; *trans*-diol, 10,11-*trans*-dihydroxy-10,11-dihydrocarbamazepine; PB, phenobarbital; Lic, licarbazepine; CBZ-E, carbamazepine-10,11-epoxide; OXC, oxcarbazepine; PHT, phenytoin and CBZ, carbamazepine.
[a] Mean ± standard deviation, $n = 5$

between extracted samples and non-extracted aqueous solutions.

Human plasma stability of the nine analytes was investigated at low and high QC concentration levels comparing the data of samples analyzed before (reference samples) and after being exposed to the conditions for stability assessment (stability samples). Stability/reference samples ratio between 85 and 115% was defined as stability criterion ($n = 5$) according to the guidelines. Short-term and long-term stability were evaluated at room temperature for 4 h, at 4 °C for 24 h and at −30 °C for up to 30 days in order to simulate sample handling and storage time in the refrigerator and freezer before analysis ($n = 5$). The effect of three freeze–thaw cycles on the stability of the compounds in human plasma samples was additionally studied. For that, aliquots of spiked plasma samples were stored at −30 °C for 24 h, thawed unassisted at room temperature and then refrozen for 24 h under the same conditions until completing the three cycles. In order to assess the post-preparative stability of processed samples in usual autosampler conditions, the stability of the analytes on the reconstituted extracts was also assessed at 4 °C for 24 h.

2.6. Clinical application

After development and validation of the method, it was applied for the quantification of parent compounds and main metabolites in real plasma samples of epileptic patients treated with PB, PHT or CBZ who were being monitored at the Coimbra University Hospital (HUC).

3. Results and discussion

3.1. Development and optimization of chromatographic conditions

During the development and optimization phase of the assay, several chromatographic conditions were tested in order to achieve the best separation of the compounds within the shortest running time. In the early stages, a mobile phase composed by a mixture of water–methanol–acetonitrile in the proportion of 64:30:6 (v/v/v) was tested. However, the poor peak resolution of LTG concerning both shape and symmetry demanded the incorporation of TEA to the mixture. Indeed, TEA saturates the free silanol groups of the stationary phase, allowing the decrease of the asymmetry and peak tailing phenomenon [30]. The influence of mobile phase pH in the separation and retention time of the analytes was also evaluated in the range 3–10. Hence, a mixture of water–methanol–acetonitrile–TEA in the proportion of 68.7:25:6:0.3 (v/v/v/v) and pH at 6.5 was chosen, as it provided a good separation and peak shape for all compounds of interest (including AEDs, metabolites and IS). Under these analytical conditions, the last-eluting analyte was CBZ, with a retention time of approximately 15 min, and the order of elution of the compounds was PRM, LTG, *trans*-diol, PB, Lic, CBZ-E, OXC, IS, PHT and CBZ (Fig. 2).

The development of a practical and accessible HPLC method that could be broadly applied in hospitals and clinical pharmacokinetic departments was our main concern. Therefore, the use of UV detection and isocratic elution were preferred to mass spectrometry [12,24,26,31] and gradient elution [26,32,33], which involve more complex and expensive equipments, and thus cannot be easily adopted by the majority of clinical laboratories. Several wavelength values ranging from 210 nm to 250 nm were assessed and the best compromise in terms of sensitivity and selectivity was achieved at 237 nm.

In order to select the appropriate IS, some compounds including amitriptyline, chloramphenicol, naproxen, 10,11-dihydrocarbamazepine and ketoprofen were tested. The latter was selected as it presented the most adequate retention time and it also displayed chromatographic behaviour and absolute recovery values similar to those exhibited by the AEDs under investigation.

3.2. Optimization of the sample preparation procedure

Sample pre-treatment methodology was initially investigated by plasma precipitation with acetonitrile and methanol; however, no profitable results were achieved because the resulting processed samples were relatively unclean, still presenting matrix endogenous substances that interfered with the retention times of the analytes. As previous studies [18,23] revealed that CBZ-E is easily degraded in acid environments, the use of strong precipitation agents like perchloric and trichloroacetic acids was herein excluded. Then, since SPE procedures are usually associated to high and reliable extraction of AEDs from human plasma [23,34], several SPE conditions were tested, including washing steps and eluting solvents, but also without success. Thus, the combination of a plasma deproteinization with methanol followed by a SPE procedure was tested and demonstrated to be the best option as it allowed a more effective elimination of interfering substances and avoided sample dilution, improving the selectivity and sensitivity of the analytical method. Furthermore, the sample preparation methodology herein developed is faster, less cumbersome, less pollutant and affords greater recovery values than liquid–liquid extraction procedures reported in literature [19,35].

Table 4
Absolute recovery of primidone (PRM), lamotrigine (LTG), 10,11-*trans*-dihydroxy-10,11-dihydrocarbamazepine (*trans*-diol), phenobarbital (PB), licarbazepine (Lic), carbamazepine-10,11-epoxide (CBZ-E), oxcarbazepine (OXC), phenytoin (PHT) and carbamazepine (CBZ) from human plasma ($n = 5$).

Analyte	Nominal concentration (μg/mL)	Absolute recovery (%)[a]	Precision (%CV)
PRM	1.2	92.24 ± 3.99	4.33
	25	80.17 ± 2.84	3.54
	45	94.47 ± 2.80	2.96
LTG	0.3	84.87 ± 5.89	6.94
	25	84.16 ± 3.20	3.81
	45	85.96 ± 2.41	2.80
trans-diol	0.75	83.57 ± 3.94	4.71
	5	83.13 ± 2.57	3.06
	9	88.68 ± 2.48	2.79
PB	0.75	89.77 ± 4.77	5.32
	50	81.35 ± 2.60	3.19
	90	83.80 ± 2.60	3.10
Lic	0.45	101.04 ± 3.83	3.79
	40	87.45 ± 3.05	3.49
	72	91.42 ± 2.67	2.92
CBZ-E	0.3	89.43 ± 6.02	6.73
	25	85.27 ± 3.05	3.57
	45	90.46 ± 2.67	2.95
OXC	0.3	82.88 ± 1.95	2.36
	12.5	86.96 ± 2.98	3.43
	22.5	89.01 ± 2.47	2.77
PHT	1.5	84.19 ± 7.42	8.81
	25	82.81 ± 3.63	4.39
	45	78.49 ± 2.60	3.31
CBZ	0.3	88.89 ± 9.37	10.54
	25	88.46 ± 3.35	3.78
	45	86.37 ± 2.57	2.97

[a] Mean ± standard deviation, $n = 5$.

Table 5
Stability (values in percentage) of primidone (PRM), lamotrigine (LTG), 10,11-*trans*-dihydroxy-10,11-dihydrocarbamazepine (*trans*-diol), phenobarbital (PB), licarbazepine (Lic), carbamazepine-10,11-epoxide (CBZ-E), oxcarbazepine (OXC), phenytoin (PHT) and carbamazepine (CBZ) under different conditions of sample handling and storage.

Analyte concentration (μg/mL)	Stability conditions				
	Human Plasma				Mobile Phase
	RT[a] 4 h	4 °C 24 h	−30 °C 30 days	Three freeze/thaw cycles	4 °C 24 h
PRM					
1.2	100.23	98.18	101.45	97.18	103.68
45	103.28	103.82	94.89	91.47	106.33
LTG					
0.3	98.02	96.01	104.30	102.30	101.06
45	103.07	103.31	95.50	90.12	104.73
***trans*-diol**					
0.75	103.47	102.98	101.64	100.49	100.65
9	105.86	97.19	95.15	89.84	105.28
PB					
0.75	105.78	100.78	102.05	104.53	99.87
90	103.88	105.08	95.30	94.97	105.31
Lic					
0.45	107.49	98.14	104.20	93.70	101.39
72	104.60	101.15	95.06	89.62	105.30
CBZ-E					
0.3	96.52	94.32	103.51	97.27	99.86
45	105.50	100.96	95.10	88.98	105.01
OXC					
0.3	106.22	100.53	94.14	107.41	98.32
22.5	106.06	99.21	95.87	88.26	104.64
PHT					
1.5	96.47	100.47	104.14	95.91	100.32
45	105.53	101.03	95.38	87.16	103.15
CBZ					
0.3	99.69	100.39	104.73	98.52	100.64
45	106.56	99.82	95.64	87.18	103.73

[a] RT, room temperature.

3.3. Method validation

3.3.1. Selectivity

Representative chromatograms of blank and spiked human plasma samples are depicted in Fig. 2. The analysis of blank plasma samples from six healthy volunteers confirmed the absence of interfering peaks from matrix endogenous substances at the retention times of the studied compounds.

In addition, none of the tested drugs potentially co-administered with the considered AEDs were found to interfere with peaks of the analytes or IS.

3.3.2. Linearity, LOQ and LOD

The calibration curves prepared in human plasma for all the compounds at the concentration ranges defined in Section 2.2 were linear ($r^2 \geq 0.992$) and showed a consistent correlation between analyte-IS peak height ratios and corresponding plasma concentrations. The regression equations of the calibration curves and the corresponding regression coefficients attained for each AED and metabolites are summarized in Table 1. At this point, it is important to highlight that the concentration range for each compound is much wider than its respective therapeutic window [4], allowing the applicability of this assay to both pharmacokinetic and toxicological determinations.

The LOQ was experimentally defined as 0.50 μg/mL for PHT, 0.40 μg/mL for PRM, 0.25 μg/mL for PB and *trans*-diol, 0.15 μg/mL for Lic and 0.10 μg/mL for OXC, CBZ, CBZ-E and LTG with acceptable precision (CV ≤ 18.27%) and accuracy (bias varied from −13.93% to 16.43%) as depicted in Table 2. Interestingly, the LOQs obtained with this method are often lower than those achieved with other HPLC–UV techniques reported in literature [10,13,16–20,25].

After successive dilutions of the lowest calibration standard, the LOD was reliably established at 0.050 μg/mL for PHT, 0.040 μg/mL for PRM, 0.025 μg/mL for PB and *trans*-diol, 0.015 μg/mL for Lic and 0.010 μg/mL for OXC, CBZ, CBZ-E and LTG.

3.3.3. Precision and accuracy

The results of intra- and inter-day precision and accuracy analyses are reported in Table 3. The acceptance criteria were fulfilled for all the compounds at the three concentration levels assessed, since the overall inter- and intra-day CV values were below 12.15% and bias varied between −14.91 and 13.63%. These data clearly demonstrate that the HPLC–UV method herein developed is reliable, accurate and reproducible.

3.3.4. Recovery

The overall recovery values obtained using the sample preparation methodology herein developed are presented in Table 4. The absolute mean recoveries determined for AEDs and metabolites ranged from 78.49% to 101.04% and showed CV values lower than 10.54%; while the mean recovery of IS was of 69.07% and CV was lower than 7.57%. These results undoubtedly demonstrate that the sample preparation methodology employed is consistent, precise and reproducible.

3.3.5. Stability

The stability of the six AEDs and metabolites was assessed under the conditions previously stated in Section 2.5 and the

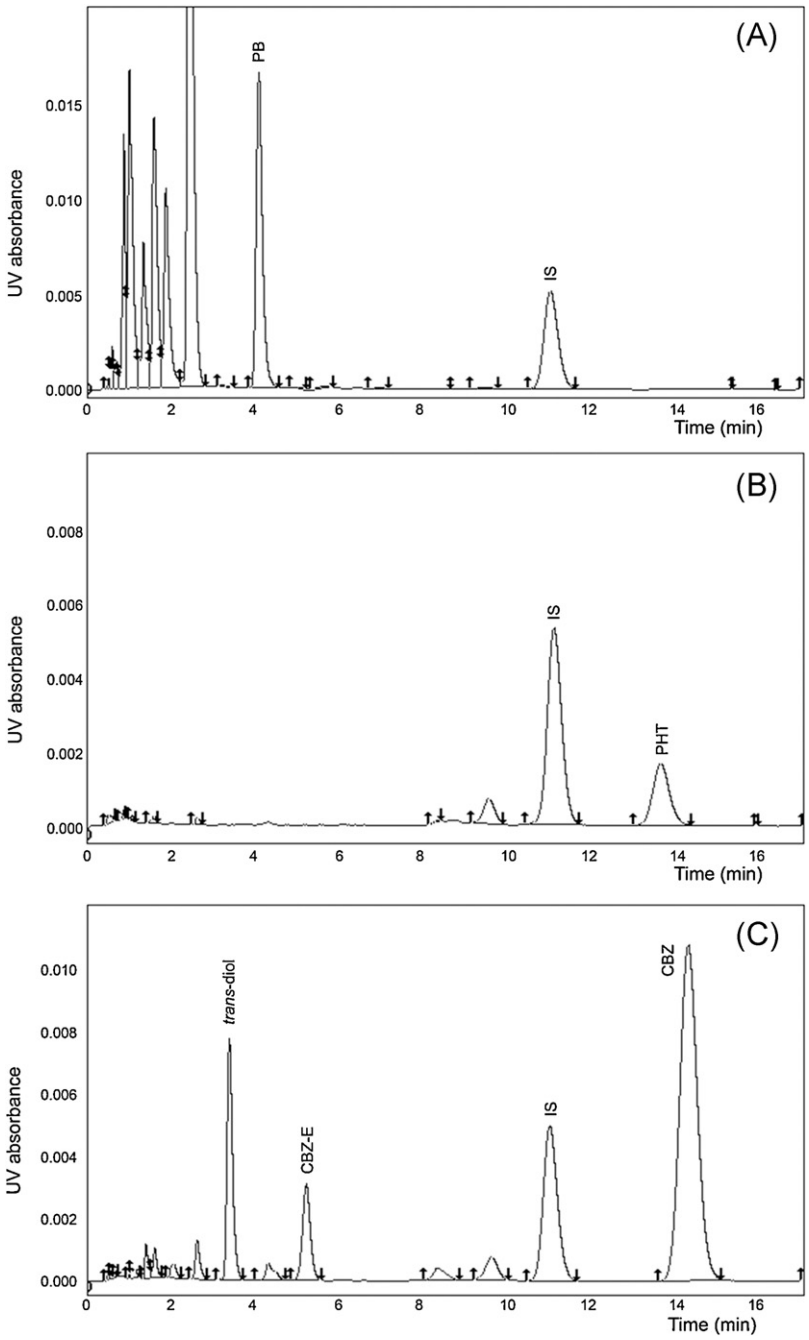

Fig. 3. Representative chromatograms of plasma samples obtained from epileptic patients treated with: (A) phenobarbital, PB; (B) phenytoin, PHT; (C) carbamazepine, CBZ. CBZ-E, carbamazepine-10,11-epoxide; *trans*-diol, 10,11-*trans*-dihydroxy-10,11-dihydrocarbamazepine.

results are reported in Table 5. Accordingly, all the analytes demonstrated to be stable in unprocessed plasma samples for up to 4 h at room temperature, for 24 h at 4 °C, for 1 month at −30 °C and after three freeze–thaw cycles. Stability of processed plasma samples was also ensured for at least 24 h at 4 °C for all the compounds.

3.4. Clinical application

This new analytical technique was mainly developed to be applied for TDM in hospitals and clinical pharmacokinetic departments. Accordingly, the method was employed in the analysis of some plasma samples from patients treated with PB, PHT or CBZ. Representative chromatograms are shown in Fig. 3. Analytical results revealed good peak shape and resolution and no interferences from endogenous matrix constituents were observed. Drug plasma concentrations were calculated by the interpolation on the corresponding calibration curves and the experimental levels obtained were within the concentration range established in the chromatographic technique (Table 6). Furthermore, although the patients were co-treated with other drugs, no interfering peaks were detected (Table 6 and Fig. 3).

Therefore, the method showed to be highly reliable and selective and thus it seems to be suitable for monitoring plasma concentrations of the selected AEDs and metabolites.

Table 6
Characteristics of drug therapy instituted to the epileptic patients whose plasma samples were analyzed by the HPLC–UV method herein developed and the respective measured concentrations obtained for phenobarbital (PB), phenytoin (PHT), carbamazepine (CBZ) and metabolites.

Patient	AED therapy	Concentration (μg/mL)	Concomitant administered drugs
A	PB	PB – 32.34	Azitromycin; acetylcisteine; enoxaparin sodium; ceftriaxone; furosemide; ipatropium bromide; pantoprazole; paracetamol; theophylline; salbutamol
B	PHT	PHT – 8.24	Amoxicillin; azitromycin; bromazepam; clonazepam; chlorpromazine; ipatropium bromide; lysine acetylsalicylate; olanzapine; omeprazole; metilprednisolone; paracetamol; risperidone; salbutamol; theophylline; valproic acid
C	CBZ	*trans*-diol – 7.22 CBZ-E – 2.97 CBZ – 10.18	Amoxicillin; azitromycin; acetylcisteine; diazepam; enoxaparin sodium; lactulose; pantoprazole; paracetamol; piracetam; valproic acid

4. Conclusions

A novel isocratic reversed-phase HPLC–UV method associated to a sample pre-treatment methodology based on protein precipitation followed by SPE was herein developed and fully validated for the simultaneous determination of PB, PRM, PHT, CBZ, LTG, OXC and some of their main metabolites CBZ-E, *trans*-diol and Lic in human plasma. The experimental results demonstrated that the reported bioanalytical method is selective, precise, accurate and sensitive, yielding also an efficient and reproducible sample extraction. Therefore, it can be applied in clinical settings as a useful tool for routine TDM of patients treated with such AEDs either in monotherapy, transitional or chronic polytherapy regimens, as well as in other pharmacokinetic-based studies involving these drugs.

Conflict of interest

The authors have declared no conflict of interest.

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