

# Development of an Analytical Method for the Plasma Determination of Five Anti-Epileptics and their Metabolites by HPLC: Comparison with Immunoanalytical Methods

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## Research Article

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### ABSTRACT

As bioanalysis emerged as a critical tool in the process of drug discovery and development, and it is desirable the availability of less expensive, faster and simpler analytical methodologies to support drug interaction screening studies with statistical validation over a wide concentration range, the aim of this work is to develop a simple, rapid and cost-effective High-Performance Liquid Chromatographic method for the simultaneous quantification of human plasma concentration of six antiepileptic drugs frequently used in clinical practice (phenobarbital, lamotrigine, oxcarbazepine, mono-hydroxy-carbazepine, carbamazepine and phenytoin). Clonazepam was used as an internal standard.

Sample preparation consisted of a deproteinization step with acetonitrile. After extraction, the analytes were separated on C<sub>18</sub> reversed-phase column thermostated at 25 °C. The mobile phase was composed of 50% phosphate buffer (pH=7.5) and 50% of methanol/acetonitrile (80:20, v/v), pumped isocratically at 1 mL/min. The UV detector was set at 215 nm. The total run time was only 10 min.

The extraction yield values were between 84% and 99%. All of these antiepileptics get separated with good peak shapes and resolution factor greater than 2. The new method proved to be simple and rapid. Calibration curves were linear with regression coefficients greater than 0.998. Intra- and interday precision CV% varied between 0.91% and 5.69%. Bias value for accuracy ranged from 2.42% to 1.27%. The mean absolute extraction recoveries at low, middle and high quality control sample levels ranged from 84.17% to 99.02%. This bioanalytical method was successfully applied to real plasma samples from 26 epileptic patients. Our results were compared to those obtained by immunoassay methods in biochemistry-toxicology FATTOUMA hospital laboratory and in Tunisia Pharmacovigilance Center. The developed method seems to be a suitable tool for routine therapeutic drug monitoring and also to support other clinical pharmacokinetic-based studies.

## INTRODUCTION

Epilepsy is a chronic neurological disorder, affecting approximately 50 million people world wide <sup>[1]</sup>. Multiple medications with anticonvulsant properties were developed. Historically, these AEDs can be classified into old generation: valproic acid, phenobarbital, phenytoin, carbamazepine... and new generation: oxcarbazepine, lamotrigine, levetiracetam <sup>[2]</sup>. The objective of these treatments is to reduce or prevent seizures. In general, the monotherapy is the preferred initial management approach in epilepsy care, but patient with more refractory epilepsy receive polytherapy with a combination of 2 or 3 drugs <sup>[3,4]</sup>. The antiepileptic therapy must be carefully optimized for each patient because the variability of the disease and the patient's patho-physiological states <sup>[5]</sup>. Also, such drugs are commonly prescribed for long periods of time making possible the cotherapy and, consequently,

the occurrence of drug-drug interactions with other kind of therapeutic agents used for usual comorbidities [6,7]. Actually, several comorbid health conditions are common among people with epilepsy, mainly psychiatric disorders (e.g., depression, psychosis, attention deficit hyperactivity, anxiety and panic disorder), increasing the likelihood of coprescription [8]. This fact added to the narrow therapeutic index of several AEDs and their marked effects on the activity of CYP isoenzymes (inhibition or induction) [6] make the patients with epilepsy especially susceptible to complex and unpredictable pharmacokinetic and also pharmacodynamic interactions [6,9-11].

It is therefore important to ensure that an individual's plasma concentration is within the therapeutic range. A number of factors may alter serum protein concentrations of AEDs including liver disease, old age and pregnancy. Concomitant medications or endogenous substances may displace drugs from serum protein-binding sites, potentially leading to higher free drug concentrations, higher side effects (drowsiness, sedation) and less effectiveness [12]. The widespread use of the new generation of AEDs, particularly in patients with psychiatric disorders, often for unlicensed indications, increases the risk of self-poisoning [13], suicide [14] and drug abuse [15-17]. For all these reasons, the drug levels and sometimes of its metabolites are required for therapeutic drug monitoring (TDM), studies of adverse events, drug interactions, acute poisoning and pharmacokinetics [18].

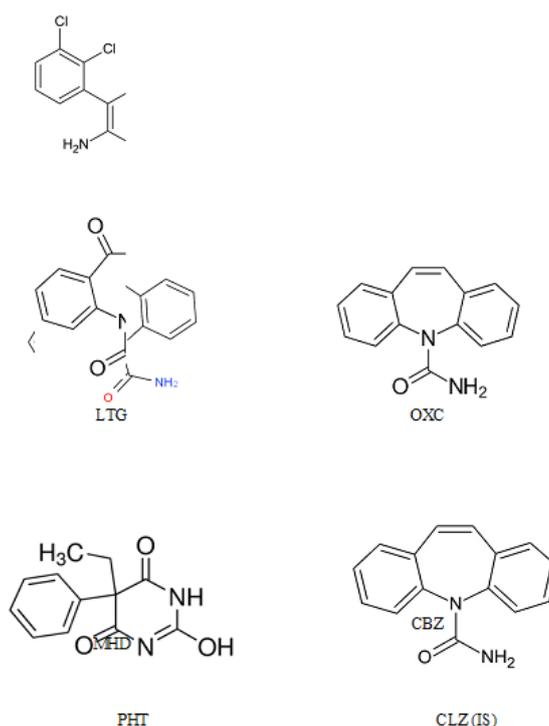
Various analytical tools have been developed for TDM of AEDs [19,20]. The most analytical methods described in literature are gas chromatography (GC) [21], HPLC [2,22] with UV detector [2,22-24] or evaporative light scattering detection [25], Ultra-High-Performance Liquid Chromatography (UHPLC) [26], LC-tandem mass spectrometry [27], Fluorescence Polarization Immunoassay [28] and Enzyme-Multiplied Immunoassay technique [29]. Automated immunoassay methods have been the most widely used methods for the determination of anticonvulsants for TDM since the 1980s [30].

However, to our knowledge, there are fewer methods described for the simultaneous quantification of the most commonly encountered AEDs in postmortem whole blood and clinical plasma/serum samples, with a statistical validation over a wide concentration range including those associated with toxicity levels. Thus, the aim of this work was to develop a simple, rapid and cost-effective HPLC method for the simultaneous quantification of six most widely used AEDs in human plasma. The method involves an easy sample preparation and relative short run time analysis for seven analytes including the internal standard. The results of the analytical validation of our method as well as its application to human plasma are presented and discussed. Our results are also compared to those found by immunoanalytic method.

## MATERIALS AND METHODS

### Chemicals and Reagents

Phenobarbital (PBB), lamotrigine (LTG), oxcarbazepine (OXC), mono-hydroxy-carbazepine (MHD), carbamazepine (CBZ) phenytoin (PHT), and Clonazepam (CLZ) were purchased from Sigma-Aldrich (St Louis, MO, USA). The chemical structures of these molecules are presented in **Figure 1**.



**Figure 1.** Chemical structures of phenobarbital (PBB), lamotrigine (LTG), oxcarbazepine (OXC), mono-hydroxy-carbazepine (MHD), carbamazepine (CBZ) phenytoin (PHT), and clonazepam (CLZ), which was used as internal standard (IS).

All solvents and reagents used in this study were of analytical grade (HPLC grade). Methanol and acetonitrile, were purchased from Sigma-Aldrich.

Ultra-pure water (HPLC grade, >18 MΩ cm) for solutions preparation was obtained in house by using a water purification system (Millipore Corporate Headquarters, Billerica, MA, USA).

Blank human plasma samples from healthy donors were gently provided from the department of hematology in Fattouma Bourguiba hospital, Monastir, Tunisia.

### HPLC-UV Instrumentation and Chromatographic Conditions

The chromatographic analysis was performed on a Perkin Elmer® liquid chromatography equipped with binary pump, a manual injector with 10 µL loop, an UV-visible detector, a solvent manager system and a data system interface (TC Navigator®). The chromatographic separation of all the six analytes and CLZ used as internal standard (IS) was carried out at room temperature on a reversed-phase Agilent® C18 column (150 mm × 4,6 mm; 5 µm particle size). Isocratic elution was employed using a mobile phase consisting of 50% phosphate buffer (pH adjusted to 7.5) and a mixture 50% of methanol-acetonitrile (80:20; v/v), at a flow rate of 1 mL/min. The wavelength detection was set at 215 nm. All the solutions were passed through 0.22 µm filter before injections. The mobile phase was filtered through a 0.2 µm filter and degassed ultrasonically for 15 min before use.

### Preparation of Standard Solutions, Calibration Standards and Quality Control Samples

Stock solutions of PBB (5 mg/5 mL), LTG (5 mg/5 mL), MHD (5 mg/5 mL), OXC (5 mg/5 mL), PHT (5 mg/5 mL), CBZ (5 mg/5 mL) and IS (5 mg/5 mL) were individually prepared by dissolving appropriate amount of each compound in methanol. Then, to obtain the corresponding working solutions, the stock solutions were then adequately diluted in methanol. Each one of these standard solutions was daily used for spiking aliquots of blank plasma to prepare the calibration curves at five calibration standards giving final concentrations of 12, 15, 20, 30 and 50 µg/mL for PBB; 2.5, 7.5, 10, 15 and 30 µg/mL for LTG; 10, 20, 25, 35 and 50 µg/mL for OXC; 10, 20, 25, 35 and 50 µg/mL for MHD; 8, 10, 15, 20 and 30 µg/mL for PHT and 5, 10, 15, 20 and 30 µg/mL for CBZ. The concentration range to be validated is chosen to cover the therapeutic index of each analyte.

Quality control (QC) samples at three concentration levels, representing the low (QC1), medium (QC2) and high (QC3) ranges of the calibration curves were independently prepared. With this purpose, aliquots of human plasma were spiked to attain final concentrations of 18, 25 and 40 µg/mL for PBB; 4, 16 and 25 µg/mL for LTG; 15, 30 and 40 µg/mL for MHD; 15, 30 and 40 µg/mL for OXC; 12, 16 and 25 µg/mL for PHT and 7, 17 and 25 µg/mL for CBZ. Bias and precision were assessed by analyzing in replicates these spiked QC samples. All the stock solutions, working solutions, and plasma samples were stored at 20 °C and protected from light before use.

### Sample Preparation and Extraction

Sample preparation was performed by protein precipitation. Each aliquot (200 µL) of human plasma was added to 10 µL of IS working solution and 200 µL of acetonitrile. Then, this mixture was vortex-mixed for 30 s and centrifuged at 13,500 rpm (3 min). Finally, an aliquot (10 µL) of the supernatant was injected into the chromatograph-system.

### Method Validation

The international accepted recommendations for bioanalytical method validation [31-33] were followed to the validation of the developed method. Accordingly, the acceptance criteria proposed for specific validation parameters including selectivity, linearity, precision, accuracy, limits of quantification and recovery were considered.

### Selectivity

Selectivity was evaluated by comparing the chromatogram of blank human plasma with those of corresponding standards spiked with AEDs to ensure that there are no interfering peaks from endogenous substances presented in matrix at the respective retention times of the analytes.

### Linearity

The linearity of the developed method for each analyte was evaluated in the concentration ranges previously defined using three separate calibration curves per matrix prepared with five spiked plasma in AEDs working solutions, extracted according to the described procedure, and then assayed on three different days (n=3). All calibrations were prepared freshly in triplicate, so that we calculated the average of peaks areas. Calibrations were constructed by plotting the analyte-IS peak area ratio versus the corresponding nominal concentrations, using the simplest least-squares linear regression model and Agilent Workstation-Quantitative software. Blank matrix with IS was run with each batch but not included in the calibration curve. The correlation coefficient ( $R^2$ ) was calculated. The  $R^2$  values should be >0.99.

### Limit of detection and quantification

Limit of detection (LOD) and limit of quantification (LOQ) were determined with blank plasma samples spiked with decreasing concentrations of the analytes. The values of LOD and LOQ were calculated as three and ten times the baseline noise, respectively.

### Precision and accuracy

To investigate the interday precision, QC samples analyzed on three consecutive days (n=3) at the three concentration levels (QC1, QC2 and QC3) representative of the calibration range were used. On the other hand, the intraday precision and accuracy were assessed analyzing six sets of each QC samples in a single day (n=6). Taking into account the acceptance criterion defined by the bioanalytical method validation guidelines, the intra- and interday precision expressed as the coefficient of variation (% CV) should be equal to or lower than 15%; and accuracy expressed as percentage of bias (% bias) must be within  $\pm 15\%$  [31,32].

### Extraction recovery

Three QC samples (QC1, QC2 and QC3) were used to test the absolute recovery of the analytes from the samples submitted to the treatment previously described in the Sample preparation and extraction section. The recovery was calculated comparing the analytes' peak areas from extracted QC plasma samples with those obtained after direct injection of nonextracted solutions at the same nominal concentrations (n=5). Likewise, the calculation of the ratio of IS peak areas in extracted samples and nonextracted solutions, evaluated at the concentration used in sample analysis, was used to define its absolute recovery.

### Clinical Application

After development and validation of the present method, the clinical applicability of this method was verified by analyzing plasma samples from twenty six patients receiving mono or poly-therapy for epilepsy treatment. These samples had been previously quantified by immunoassay technique in both Tunisia Pharmacovigilance Center and in the biochemistry- toxicology laboratory of FATTOUMA hospital.

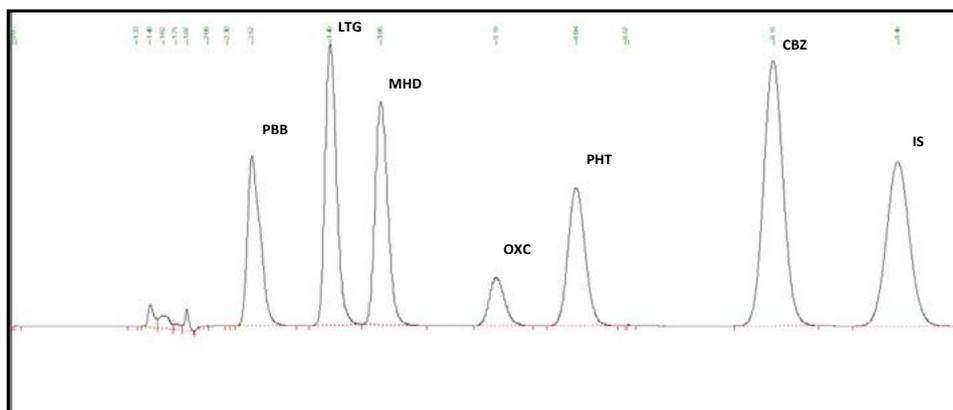
## RESULTS AND DISCUSSION

### Development and Optimization of Chromatographic Conditions

Spectral analysis of our analytes allowed us to choose 215 nm as the most suitable working wavelength since it is a compromise between all the AEDs. In order to achieve the best separation of the analytes within the shortest running time, a number of parameters were optimized. Preliminary experiments were carried out starting with a mobile phase composed of 40% phosphate buffer pH 7.5 and a mixture 60% of methanol/acetonitrile (80/20; v/v). This composition gave us a co-elution of PBB with LTG. Thus, we switched to other trials such as changing in the pH value of the buffer and in the organic phase percentage, with the aim of achieving good resolution, symmetrical peak shape and short analysis time for the drug substances spiked with its impurities.

The decrease of buffer pH didn't influence on the chromatographic behavior of the studied compounds except for PHT which we recorded a slight increase in its retention time. PBB, like PHT, should have the same behavior due to their weak acidic characteristic. Also, the pH changing did not improve the resolution between PBB and LTG. The co-elution between these two compounds always existed. The other compounds (LTG, MHD, OXC and CBZ) are weak basis and are partially ionized in this pH range (7.5-6.75). Their elution depends on their polarity, molecular weight and hydrophobicity ( $\log P$  (MHD)=1.26,  $\log P$  (OXC)=1.31,  $\log P$  (CBZ)=2.45,  $\log P$  (LTG)=2.5). In addition, the decrease of buffer pH from 7.5 to 6.75 showed a broadening of the peak relative to the MHD which results in a decrease of resolution between PBB, LTG and the MHD.

By testing many trials, the beneficial results were only obtained in the mobile phase composition mainly in organic phase percentage changing it from 60% to 50%. Hence, a mobile phase composed of 50% phosphate buffer (pH= 7.5) and 50% mixture of methanol/acetonitrile (80/20; v/v) was selected as it provided a good separation and peak sharpness for all analytes of interest (**Figure 2**).



**Figure 2.** Representative chromatogram of the analysis of spiked plasma sample with antiepileptic drugs (AEDs) in the optimized chromatographic conditions. PBB: 2.58 min, LTG: 3.40 min, MHD: 3.95 min, OXC: 5.19 min, PHT: 6.01 min, CBZ: 8.19 min and IS: 9.49 min.

The obtained conditions after optimization are presented in **Table 1**. Under these chromatographic conditions, the AEDs in the spiked plasma are separated with a running time of 10 min. The last eluting analyte was CBZ, with a retention time (RT) of approximately 8 min and the other analytes have the following RT: PBB: 2.58 min, LTG: 3.40 min, MHD: 3.95 in, OXC: 5.19 min, PHT: 6.01 min and CBZ: 8.19 min.

**Table 1.** Chromatographic optimised condition.

<b>Column thermostated at 25 °C</b>	Agilent® C18 column (150 mm × 4.6 mm; 5 µm particle size)
<b>Mobile phase</b>	50% phosphate buffer (pH adjusted to 7.5) and a mixture 50% of methanol-acetonitrile (80:20; v/v)
<b>Flow-rate (mL/min)</b>	1
<b>Injection volume (µL)</b>	10
<b>Detection. UV-VIS (nm)</b>	215

### Choice of the Internal Standard

In order to select the appropriate IS, a number of compounds including vinbarbital, caffeine, theophylline, ibuprofen, ketoprofen, diazepam and clonazepam were tested. The latter was preferred as IS as it presents the most adequate RT (9 min) and also displaying chromatographic behavior and absolute recovery values similar to those exhibited by AEDs under investigation. In addition, it absorbs around 211 nm and 243 nm. The wavelength 211 nm is very close to that of the studied analytes.

### Optimization of the Sample Preparation Procedures

Sample preparation was achieved by simple precipitation of macromolecules. Acetonitrile and perchloric acid were both tested. However, no profitable results were obtained using the perchloric acid. The acetonitrile was tested at different volumes. The precipitation with 200 µL acetonitrile was chosen. Satisfactory recoveries between 84% and 99% were found for all different compounds (**Table 2**). Thus, a plasma deproteinization with acetonitrile was chosen since it allows an effective elimination of interfering substances, and improves the selectivity of the analytical method. Furthermore, the sample precipitation methodology herein developed is less expensive and less complicated than solid phase extractions procedures reported in literature [2,22]. Liquid-liquid extraction has also been reported in literature by using ethyl acetate as solvent of extraction [34]. However, this method has some disadvantages for solvent consumption and long-time analysis. In addition, ethyl acetate is known for its toxicity and carcinogenicity.

**Table 2.** Inter- and intraday precision (% CV) and accuracy (% bias) obtained for analytes in human plasma at the Low (QC1), Middle (QC2) and High (QC3) concentrations representative of the calibration ranges for quality control (QC) samples.

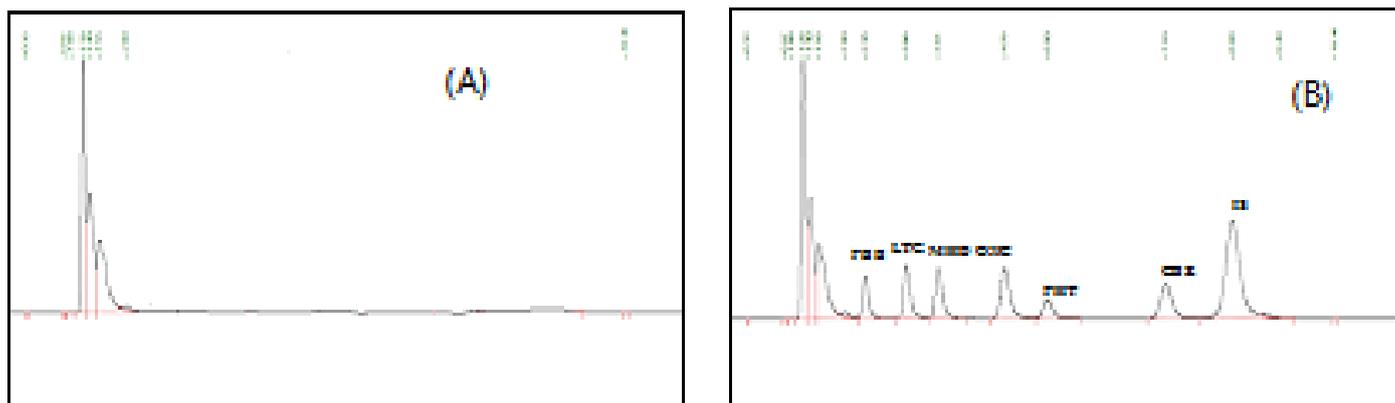
Analyte	Concentration (µg/mL)	Accuracy (% bias)	% CV		Absolute recovery (%) <sup>a</sup>
			Intra-day	Inter-day	
PBB	18	-0.51	1.33	1.51	95.35 ± 9.61
	25	1.27	2.62	2.76	90.71 ± 3.8
	40	-1.64	3.83	5.69	91.84 ± 3.82
LTG	4	-2.42	4.25	4.57	93.22 ± 12.4
	16	-0.74	2.85	2.97	92.94 ± 0.65
	25	-0.66	1.91	2.16	92.30 ± 1.25
MHD	15	-0.01	3.46	3.55	97.12 ± 5
	30	-0.94	3.56	3.95	89.85 ± 1.61
	40	-1.51	4.59	4.62	95.92 ± 3.12
OXC	15	-1.6	2.91	3.5	92.09 ± 1.83
	30	1.02	2.91	3.22	92.60 ± 2.5
	40	-0.99	2.21	4.24	99.02 ± 2.59
PHT	12	0.68	1.67	1.69	97.51 ± 5.4
	16	0.004	1.66	1.77	84.17 ± 1.35
	25	-0.13	0.91	0.99	84.33 ± 3.73
CBZ	7	-0.1	1.3	1.66	84.99 ± 3.1
	17	-0.004	1.5	1.59	87.49 ± 1.34
	25	-2.1	2.72	3.11	89.48 ± 0.7

<sup>a</sup>Mean ± standard deviation, n=5.

### Method Validation

#### Selectivity

By applying the same extraction procedure to blank human plasma as that used to extract the overloaded plasma with our six analytes, no endogenous interference was observed in drug-free matrix at the retention times of the target drugs (**Figure 3**).



**Figure 3.** Typical chromatograms of extracted human plasmas: (A) blank plasma; (B) plasma spiked with IS and analytes at intermediate concentrations of the calibration ranges.

Also, no interference was observed at the retention times of the analytes and internal standard. Good resolution and peak shapes of every component can be seen. The resolution factor for the drug peak from the nearest resolving peak was also checked to confirm the separation behavior.

### Linearity

Calibration curves were set up for all analytes over the concentration ranges established. A consistent correlation between analyte-IS peak area ratios was obtained showing a good linear relationship between concentrations and peak area, so that the method is linear with a correlation coefficient  $R^2$  greater than 0.998.

The regression equations of the calibration curves and the corresponding correlation coefficients for each analyte are summarized in **Table 3**.

**Table 3.** Calibration range, regression equation and correlation coefficient for the antiepileptic drugs (AEDs) used in the study.

Analyte	Calibration Range ( $\mu\text{g/mL}$ )	Equation* $Y=aX+b$	Correlation Coefficient ( $R^2$ )
PBB	[12-50]	$Y=0.005X+0.004$	0.9987
LTG	[2.5-30]	$Y=0.01X+0.046$	0.9997
MHD	[10-50]	$Y=0.008X-0.02$	0.9996
OXC	[10-50]	$Y=0.007X+0.044$	0.9994
PHT	[8-30]	$Y=0.005X+0.011$	0.9999
CBZ	[5-30]	$Y=0.015X+0.037$	0.9988

\*Y represents analyte-IS peak area ratio; X represents analyte concentration ( $\mu\text{g/mL}$ )

**Table 4** reports the statistical calculations of linearity validation study for each analyte. These results show well that the assay method of each analyte in the mixture is linear over the wide range of concentrations tested with  $R^2 > 0.99$ .

### Precision, accuracy

The results of precision (intra- and interday) and accuracy analyses are presented in **Table 2**. All of the data fulfilled the acceptance criteria. The intra- and interday precision CV% values were between 0.91% and 5.69% ( $\leq 15\%$ ). For accuracy, % bias varied between 2.42% and 1.27% (within  $\pm 15\%$ ). These data clearly demonstrate that the HPLC-UV method herein developed is reliable, accurate and reproducible.

### Extraction recovery

Five repeated analysis ( $n=5$ ) at the three concentration levels (QC1, QC2 and QC3) were used to determine the overall absolute recovery of each analyte. As presented in **Table 4**, the mean absolute recoveries at low, middle and high QC sample levels ranged from 84.17% to 99.02% with CV values  $< 15\%$ , which are within the acceptable range for all the drugs and the internal standards.

**Table 4.** Statistical results for linearity study of our six analytes.

Test	Tabulated value	Calculated value					
		PBB	LTG	MHD	OXC	PHT	CBZ
Homogeneity of the variances: Cochran ©	$C_{tab} : 0.68$	$C_{cal} : 0.65$	$C_{cal} : 0.65$	$C_{cal} : 0.58$	$C_{cal} : 0.20$	$C_{cal} : 0.57$	$C_{cal} : 0.57$
Existence of a significant slope: Fischer (F1)	$F_{tab} : 4.67$	$F_{cal} : 491$	$F_{cal} : 1287$	$F_{cal} : 2112$	$F_{cal} : 1142$	$F_{cal} : 122$	$F_{cal} : 2366$
Validity of the regression line (F2)	$F_{tab} : 3.71$	$F_{cal} : 0.56$	$F_{cal} : 2.30$	$F_{cal} : 0.036$	$F_{cal} : 3.70$	$F_{cal} : 2.97$	$F_{cal} : 0.83$
Comparison of the ordinates at the origin: Student (t)	$T_{tab} : 2.16$	$T_{cal} : -0.083$	$T_{cal} : -0.942$	$T_{cal} : 0.207$	$T_{cal} : -0.54$	$T_{cal} : -0.38$	$T_{cal} : -0.36$

### Limit of detection and quantification

The LOD was established at 3 µg/mL for PBB, 0.75 µg/mL for LTG, 1.5 µg/mL for MHD, 2.99 µg/mL for OXC, 2.4 µg/mL for PHT and 1.91 µg/mL for CBZ.

The LOQ was 10 µg/mL for PBB, 2.5 µg/mL for LTG, 7.5 µg/mL for MHD, 10 µg/mL for OXC, 8 µg/mL for PHT and 4 µg/mL for CBZ. The LODs and LOQs are higher than those described in literature<sup>[2,22]</sup>. This difference can be explained by the low injected volume. The injection of 100 µL of sample would improve the limits of detection and quantification by a factor of 10. Nevertheless, our limits of quantification are sufficient to meet the use for which our method is intended, namely the therapeutic follow-up.

### Stability

Regarding stability, we have contented ourselves with the detailed bibliographic data presented in **Table 5**<sup>[35-37]</sup>. The stability study shows well that no significant degradation in drugs was observed in any of the storage tested conditions.

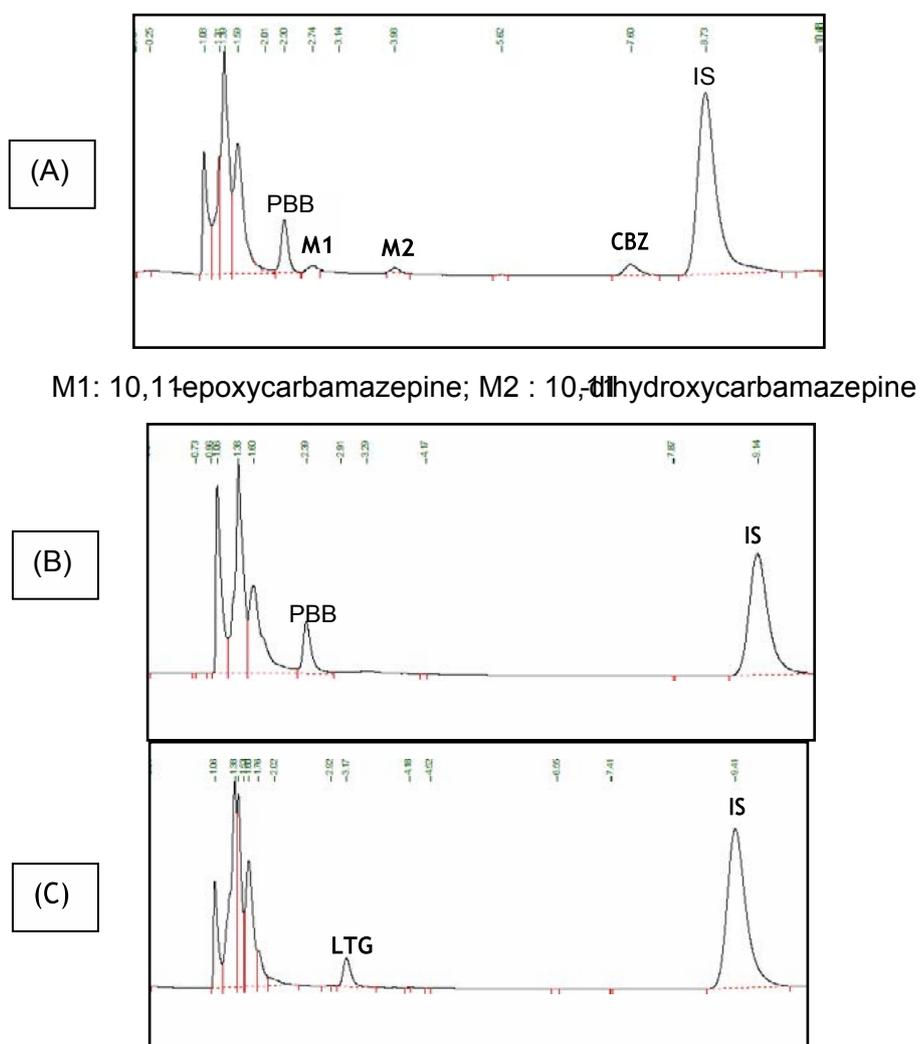
**Table 5.** Stability of our six analytes in plasma and after extraction.

	Room temperature	+ 4 °C	- 20 °C	Freeze-thaw cycle	Post-preparative stability of extracts
<b>PBB</b>	4h (Ferreira et al.)	24h (Ferreira et al.) 24h (Serralheiro et al.)	30 days (Ferreira et al.)		
	4h (Serralheiro et al.)		30 days (Serralheiro et al.)	3 cycles (Ferreira et al.) 3 cycles (Serralheiro et al.)	24h at + 4 °C (Serralheiro et al.)
	6h (Shibata et al.)		3 months (Shibata et al.)		12h on the automatic sampler (Ferreira et al.)
<b>PHT</b>	6h (Kim et al.)	24h (Ferreira et al.) 24h (Serralheiro et al.)			
	4h (Ferreira et al.)		30 days (Ferreira et al.)	3 cycles (Kim et al.)	24h at 15 °C (Kim et al.)
	4h (Serralheiro et al.)		30 days (Serralheiro et al.)	3 cycles (Ferreira et al.) 3 cycles (Serralheiro et al.)	24h at + 4 °C (Serralheiro et al.)
	6h (Shibata et al.)		3 months (Shibata et al.)		12h on the automatic sampler (Ferreira et al.)
<b>CBZ</b>		24h (Ferreira et al.) 24h (Serralheiro et al.)	30 days (Ferreira et al.)	3 cycles (Kim et al.)	24h at 15 °C (Kim et al.)
	6h (Kim et al.)		30 days (Serralheiro et al.)	3 cycles (Ferreira et al.) 3 cycles (Serralheiro et al.)	24h at + 4 °C (Serralheiro et al.)
	4h (Ferreira et al.)				
	6h (Shibata et al.)		3 months (Kim et al.)		12h on the automatic sampler (Ferreira et al.)
<b>LTG</b>	4h (Ferreira et al.)	24h (Ferreira et al.)			
	6h (Kim et al.)		30 days (Ferreira et al.)		12h on the automatic sampler (Ferreira et al.)
			3 months (Kim et al.)	3 cycles (Ferreira et al.)	
<b>OXC</b>	4h (Ferreira et al.)	24h (Ferreira et al.)			
	6h (Kim et al.)		30 days (Ferreira et al.)		12h on the automatic sampler (Ferreira et al.)

			3 months (Kim et al.)	3 cycles (Ferreira et al.)	
<b>MHD</b>	4h (Ferreira et al.)	24h (Ferreira et al.)	30 days (Ferreira et al.)	3 cycles (Ferreira et al.)	12h on the automatic sampler (Ferreira et al.)

**Clinical Application**

The method was verified with a set of 26 plasma samples from patients treated with AEDs during routine case analysis using immunoassay methods. The obtained chromatograms (**Figure 4**) differ from those obtained from the plasmas overloaded with reference substances. Such differences were expected for several reasons. The main one is that plasmas from patients treated with AEDs contained metabolites corresponding to the researched analytes. The most metabolized molecule is CBZ which has at least two metabolites. Although we were unable to integrate them into our study because of their unavailability, our method proved to be rather selective and allows their separation. As shown in **Figure 4A**, the chromatogram has two additional peaks that we did not find during the analysis of overloaded plasmas. Their retention times are respectively 2.74 and 3.98 minutes. These two peaks would most probably correspond to the two major metabolites of CBZ, namely the 10,11-epoxycarbamazepine ( $t_R=2,74$  min) and the 10,11-dihydroxycarbamazepine ( $t_R=3,98$  min) [38]. **Figures 4B and 4C** show the chromatograms of two patients respectively under PBB and LTG.



**Figure 4.** Representative chromatograms of plasma samples obtained from epileptic patients treated with: (A) PBB+CBZ; (B) PBB; (C) LTG.

The results obtained by applying our method ( $C_M$ ) on 26 plasma samples as well as those obtained from biochemistry-toxicology laboratory in FATTOUMA hospital ( $C_{M\text{BTL}}$ ) and from Tunisia pharmacovigilance center ( $C_{\text{PvCT}}$ ) are presented in **Table 6**.

**Table 6.** Antiepileptic drugs (AEDs) Levels: comparison of results obtained by application of the validated method to those obtained in other laboratories.

N° of sample and requested AEDs		Concentrations (µg/mL)		
		PBB [15-30 µg/mL]*	CBZ [5-12 µg/mL]*	LTG [2.5-15 µg/mL]*
VPA+CBZ 268	CMBTL	–	8.8	–
	C <sub>M</sub>	–	7.69	–
VPA+CBZ 273	CMBTL	–	7.1	–
	C <sub>M</sub>	–	5.75	–
VPA+CBZ 4599	CMBTL	–	6.6	–
	C <sub>M</sub>	–	6.1	–
PBB+CBZ 4620	CMBTL	14.9	5.6	–
	C <sub>M</sub>	16	5.05	–
VPA+CBZ 4584	CMBTL	–	10	–
	C <sub>M</sub>	–	6.01	–
PBB 4625	CLBTM	29.9	–	–
	C <sub>M</sub>	25.65	–	–
VPA+CBZ 4599	CMBTL	–	6.6	–
	C <sub>M</sub>	–	5.02	–
PBB 4404	CMBTL	35.7	–	–
	C <sub>M</sub>	36.07	–	–
PBB+CBZ 4571	CMBTL	17.5	5.7	–
	C <sub>M</sub>	16.4	5.15	–
PBB+CBZ 4365	CMBTL	7.6	6.78	–
	C <sub>M</sub>	7.56	5.67	–
LTG 1	CPVCT	–	–	5.8
	C <sub>M</sub>	–	–	4.36
LTG 2	CPVCT	–	–	4
	C <sub>M</sub>	–	–	3.2
LTG 3	CPVCT	–	–	7.7
	C <sub>M</sub>	–	–	6.41
LTG 4	CPVCT	–	–	5.4
	C <sub>M</sub>	–	–	3.95
LTG 5	CPVCT	–	–	< LOQ
	C <sub>M</sub>	–	–	<LOQ
LTG 6	CPVCT	–	–	7.8
	C <sub>M</sub>	–	–	6.02
LTG 7	CPVCT	–	–	< LOQ
	C <sub>M</sub>	–	–	<LOQ
LTG 8	CPVCT	–	–	< LOQ
	C <sub>M</sub>	–	–	<LOQ
LTG 9	CPVCT	–	–	5.8
	C <sub>M</sub>	–	–	4.35
LTG 10	CPVCT	–	–	3.4
	C <sub>M</sub>	–	–	4.04
LTG 11	CPVCT	–	–	3.2
	C <sub>M</sub>	–	–	2.8
LTG 12	CPVCT	–	–	3.8
	C <sub>M</sub>	–	–	2.9
LTG 13	CPVCT	–	–	12.1
	C <sub>M</sub>	–	–	11.01

LTG 14	CPVCT	–	–	6.8
	C <sub>M</sub>	–	–	5.5
LTG 15	CPVCT	–	–	<LOQ
	C <sub>M</sub>	–	–	<LOQ
LTG 16	CPVCT	–	–	<LOQ
	C <sub>M</sub>	–	–	<LOQ

C<sub>M</sub>: concentrations obtained by applying our method; C<sub>MBTL</sub>: concentrations obtained by the biochemistry- toxicology laboratory in FATTOUMA hospital; C<sub>CPVCT</sub>: concentrations obtained by Tunisia pharmacovigilance center. \*Therapeutic range

The application of our method to 26 plasma samples from epileptic patients treated with AEDs shows that five patients under LTG were under-dosed, corresponding to the following codes: LTG 5, LTG 7, LTG 8, LTG 15 and LTG 16. The patient with code 4365 was under- dosed to PBB and the one with code 4404 was over-dosed to PBB. The variability of these results confirms well the need for therapeutic monitoring of these molecules.

A statistical analysis was performed to determine whether the difference between our results and those obtained in the other refereed laboratories using immunoassay methods are well significant. We applied the Student t test for paired samples. Statistical calculation was done with by using IBM® SPSS® software. The results are shown in **Table 7**.

**Table 7.** Results of the Student t test analysis.

Student t test for paired samples								
	Average	Standard Deviation (SD)	Average standard error	95% confidence interval of the difference				Sig (Bilateral)
				Inferior	Superior	t	ddl	
Pair 1 CBZ_M/CBZ_MBTL	1.617	1.069	0.378	0.723	2.511	4.276	7	0.004
Pair 2 PBB_M/PBB_MBTL	0.78	2.094	0.936	-1.81	3.384	0.837	4	0.45
Pair 3 LTG_M/LTG_PVCT	1.023	0.669	0.201	0.573	1.473	5.071	10	0.0005

We notice that difference between the concentrations obtained by our method and those obtained in biochemistry-toxicology laboratory is not significant for PBB ( $t_{\text{calculated}} < t_{\text{table}}$  with  $p=0.05$ ), whereas it is significant for CBZ ( $t_{\text{calculated}} > t_{\text{table}}$  with  $p=0.05$ ). With regard to LTG, the difference between the concentrations obtained by our method and those by the national center of pharmacovigilance is significant ( $t_{\text{calculated}} > t_{\text{table}}$  with  $p=0.05$ ). The observed difference in CBZ concentrations could be explained by the fact that the biochemistry-toxicology laboratory uses an immunoanalytical method based on CEDIA involving monoclonal antibodies. However, the relative specificity of these antibodies does not make it possible to selectively determine CBZ when it is in the presence of its metabolites such as CBZ-E and CBZ-(OH)<sub>2</sub>. The contents of these metabolites were reached in some patients to 10.2% of total concentration expressed as CBZ and metabolites<sup>[39]</sup>. Moreover, in the instructions of the assay kit, the manufacturer indicates a cross-reactivity towards the CBZ-E estimated at 7.4%<sup>[40]</sup>. Similarly for LTG, the difference observed in LTG concentrations could be explained by the fact that the pharmacovigilance center uses an immunoanalytical method based on PETINIA involving polyclonal antibodies. The use of these antibodies does not allow the selectivity assay of LTG in presence of its metabolites which the two main ones are 2-N-Lamotrigine glucuronide and 2-N-methyl Lamotrigine. These results are comparable to those found by Baldelli et al.<sup>[41]</sup>. As for PBB, our results were in accordance to those obtained in biochemistry-toxicology laboratory. Indeed, metabolites of PBB are rapidly eliminated in glucuroconjugated form and are not detectable in plasma<sup>[42,43]</sup>. The analysis cost and time in addition to the ability to afford state of art techniques can be obstacles, which may affect the number of requested tests. Therefore, the development of the simultaneous analysis of AEDs using a small sample volume and a simple extraction procedure may improve the TDM of these drugs and can play an important role in enhancing the quality of life for epileptic people. With increased awareness of poly-AED use, the incidence of over-dose could be reduced. Finally, by monitoring all the medications taken by the patients and observing any changes in the concentrations, which might result from the drugs interaction, pharmacogenetic variations or from other AEDs taken concomitantly by the patients with or without prescription for non-epileptic medications or for abuse purposes, greater clinical care can be given to these patients.

## CONCLUSION

As bioanalysis emerged as a critical tool in the process of drug discovery and development, being essential for pharmacokinetic/pharmacodynamics characterization of a drug compound, and it is desirable the availability of less expensive, faster and simpler analytical methodologies to support drug interaction screening studies, with statistical validation over a wide concentration range, thus the scarcity of HPLC methodologies fully validated to support in vitro studies. Notwithstanding, the HPLC assay herein reported to quantify six AEDs (PBB, LTG, OXC, CBZ, PHT and CLZ) in human plasma was validated taking into account the international criteria of the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) guidelines. The

developed method is the most rapid one giving only 10 min for analysis time when compared to previously reported quantification methods TDM. AEDs can be analyzed in one step using a very cheap and simple protein precipitation. Validation results showed well that our method is selective, precise and accurate. Good linearity for each calibration range was observed. Taking into account that other important tool in analysis such as LC-MS-MS methods with high level of confidence have been published for AEDs analysis, it should not be neglected the lack of HPLC assays fully validated for the quantification of these drugs in culture media or even in supernatants of cell cultures.

Our developed method has been successfully verified using authentic case samples, and it seems to be a suitable tool for routine TDM, to support clinical pharmacokinetic, bioequivalence, toxicology, and genetic polymorphism-based studies, and in following AEDs metabolism, especially for most metabolized molecules (LTG and CBZ).

### CONFLICT OF INTEREST

None of the authors of this paper has financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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