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An easy-to-use liquid chromatography assay for the analysis of lamotrigine in rat plasma and brain samples using microextraction by packed sorbent: Application to a pharmacokinetic study

Sandra Ventura[a,b], Márcio Rodrigues[a,b], Sarah Pousinho[a], Amílcar Falcão[c,d], Gilberto Alves[a,c,e,*]

[a] CICS-UBI—Health Sciences Research Centre, University of Beira Interior, Av. Infante D. Henrique, 6200-506 Covilhã, Portugal
[b] UDI-IPG—Research Unit for Inland Development, Polytechnic Institute of Guarda, 6300-749 Guarda, Portugal
[c] CNC—Center for Neuroscience and Cell Biology, University of Coimbra, 3004-517 Coimbra, Portugal
[d] Laboratory of Pharmacology, Faculty of Pharmacy, University of Coimbra, Pólo das Ciências da Saúde, Azinhaga de Santa Comba, 3000-548 Coimbra, Portugal
[e] Laboratory of Pharmaco-Toxicology—UBIMedical, University of Beira Interior, 6200-284, Covilhã, Portugal

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ABSTRACT

A simple and rapid high-performance liquid chromatography method with diode-array detection (HPLC-DAD) using microextraction by packed sorbent (MEPS) during the sample preparation step was developed and validated to quantify lamotrigine (LTG) in rat plasma and brain samples. MEPS variables such as pH, number of draw-eject cycles, and washing and desorption conditions were optimized. The chromatographic resolution of LTG and chloramphenicol, used as internal standard (IS), was accomplished in less than 5 min on a C18 column, at 35 °C, using an isocratic elution with acetonitrile (13%), methanol (13%) and water-triethylamine (99.7:0.3, v/v; pH 6.0) pumped at a flow rate of 1 mL/min. Detection was performed at 215 nm. Calibration curves were linear over the range of 0.1–20 μg/mL ($r^2 \geq 0.9947$) for LTG in both rat plasma and brain homogenate samples. The intra and interday imprecision did not exceed 8.6% and the intra and interday inaccuracy ranged from −8.1 to 13.5%. LTG was extracted from rat plasma and brain homogenate samples with an average absolute recovery ranging from 68.0 to 86.7%, and its stability was demonstrated in the assayed conditions. No interferences were observed at the retention times of the analyte (LTG) and IS. To the best of our knowledge, this is the first bioanalytical assay that uses MEPS procedure for the determination of LTG not only in rat plasma but also in tissue (brain) samples. This novel method was successfully applied to a preliminary pharmacokinetic study in rats and it seems to be a cost-effective tool to support non-clinical pharmacokinetic-based studies involving LTG treatment.

1. Introduction

Lamotrigine (LTG; Fig. 1) is a second-generation antiepileptic drug (AED) exhibiting a broad spectrum of efficacy against several types of epilepsy seizures, and it is also effective as a mood stabilizer agent in bipolar syndromes [1–3].

LTG has a narrow therapeutic range, a large inter-individual variability in its pharmacokinetics and some side effects are concentration-dependent, justifying therapeutic drug monitoring (TDM) in many clinical circumstances. For instance, LTG undergoes extensive metabolism to an inactive glucuronide metabolite, and its own metabolism is characterized by an autoinduction phenomenon that appears to be complete within 2 weeks, resulting in a 17% reduction in LTG serum concentrations [4]. The biotransformation of LTG is also susceptible to heteroinduction and enzyme inhibition. Indeed, the metabolism of LTG is significantly affected by concomitant use of hepatic enzyme inducers such as classic AEDs (carbamazepine, phenytoin, primidone and phenobarbital) and oxcarbazepine, as well as others drugs such as rifampicin, ritonavir, acetaminophen and olanzapine [3–6]. Contraceptives containing estradiol can also reduce the serum concentration of LTG by 50% and in women on oral contraceptives this interaction results in different steady-state LTG concentrations between the days of pill intake compared with the pill-free interval [2,4]. On the other hand, the LTG metabolism is inhibited by valproic acid and

* Corresponding author at: CICS-UBI—Health Sciences Research Centre, University of Beira Interior, Av. Infante D. Henrique, 6200-506 Covilhã, Portugal.
E-mail address: gilberto@fcsaude.ubi.pt (G. Alves).

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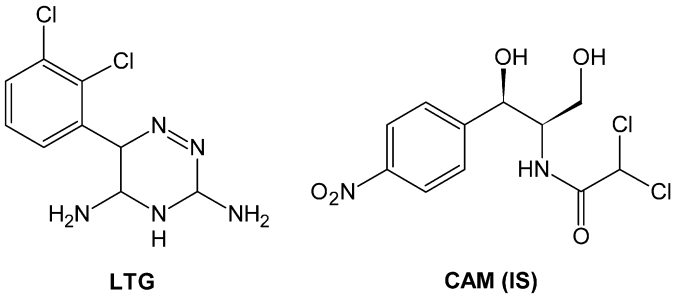

Fig. 1. Chemical structure of lamotrigine (LTG) and chloramphenicol (CAM) used as internal standard (IS).

sertraline. In fact, the inhibitory interaction with valproic acid was found to be clinically relevant and smaller doses of LTG as well as a slower titration rate should be used to minimize the risk of side effects [4]. The most serious adverse effect observed within the LTG therapeutic range (2.5–15 μg/mL) is skin rash, probably related to its aromatic ring and the formation of toxic metabolites [7]. Indeed, the incidence of toxic effects is significantly increased when serum or plasma concentrations exceed 15 μg/mL [4].

Over the years, rodents (rats and mice) have been largely employed as whole laboratory animal models to identify new anticonvulsant compounds and to obtain a better understanding of the pharmacokinetics of established AEDs at non-clinical level, and to study their involvement in drug-drug interactions [8–16]. Due to the fact that rodents eliminate most drugs much more rapidly than humans, anticonvulsant doses of AEDs are usually much higher in rodent models of seizures than effective doses in epilepsy patients. In spite of the pharmacokinetic differences observed between species, the effective plasma levels of AEDs are usually similar among rodents and humans [9,17]. Therefore, rodent models can be used to evaluate and predict plasma levels in humans by calculating the corresponding doses that will produce a similar anticonvulsant effect [15].

More specifically, LTG efficacy has been extrapolated from pharmacological studies conducted in rats. However, like other AEDs, LTG needs to cross the blood–brain barrier to exert its therapeutic effect. Thus, the determination of LTG levels in plasma and brain tissue is essential to characterise its pharmacokinetic/pharmacodynamic relationship [17–19]. Likewise, information on the LTG concentrations achieved simultaneously in plasma/serum and brain (biophase) is also determinant to predict the impact of drug-drug or herb-drug interactions involving LTG as the victim (object) drug. Hence, the availability of suitable bioanalytical methodologies to support the measurement of LTG concentrations in these particular biological samples is imperative.

To date, only a few number of high performance liquid chromatography (HPLC) methods coupled to ultraviolet [20–24] or mass spectrometry [25] detection has been reported in literature for the quantification of LTG in rat plasma/serum and brain. However, in those methods, sample preparation has been mainly performed through classic procedures, such as solid-phase extraction [20], protein precipitation [22–25] and/or liquid-liquid extraction [21,23,24]. Nevertheless, in recent years several miniaturized sample preparation techniques have been developed whose importance in bioanalysis has been increasingly recognized, among them is microextraction by packed sorbent (MEPS). In fact, MEPS has been successfully applied to the quantitative analysis of several therapeutic agents, namely antibiotics, antihypertensives, antiarrhytmics, antidepressants, antipsychotics, and even antiepileptic drugs including LTG [26]. Nonetheless, as far as we know, no bioanalytical assay has been developed for the quantification of LTG specifically in rat plasma and brain tissue samples using MEPS. Therefore, the purpose of this work was to develop and validate a novel method for the quantification of LTG in rat plasma and brain homogenate using the innovative MEPS technology in sample preparation.

2. Material and methods

2.1. Materials and reagents

LTG was kindly provided by Bluepharma (Coimbra, Portugal). Chloramphenicol, used as internal standard (IS; Fig. 1), was purchased from Sigma–Aldrich (St Louis, MO, USA). Methanol and acetonitrile, both of HPLC gradient grade, were purchased from Fisher Scientific (Leicestershire, United Kingdom) and the ultra-pure water (HPLC grade, >18 MΩ cm) was prepared by means of a Milli-Q water apparatus from Millipore (Milford, MA, USA). Triethylamine, dihydrogen phosphate dehydrate and disodium hydrogen phosphate anhydrous were acquired from Merck KGaA (Darmstadt, Germany) and the 85% *ortho*-phosphoric acid from Fischer Scientific UK. Pentobarbital (Eutasil® 200 mg/ml, Ceva Saúde Animal) used as anaesthetic drug was commercially acquired. MEPS 250 μL syringe and MEPS BIN (barrel insert and needle) containing ~4 mg of solid-phase silica – C_{18} material (SGE Analytical Science, Australia) were supplied by ILC (Porto, Portugal).

2.2. Blank rat matrices

Healthy adult male Wistar rats (300–380 g, 10–12 weeks old) were obtained from local certified animal facilities (Faculty of Health Sciences of the University of Beira Interior, Covilhã, Portugal) and were used as source of blank matrices (plasma and brain tissue) required for the validation experiments. For that, rats not subjected to any other treatment were anesthetized with pentobarbital (60 mg/kg) and then decapitated. Blood samples were directly collected into heparinised tubes and after exsanguination the brain was quickly excised. The blood samples were centrifuged at 4000 rpm for 10 min (4 °C) and then the plasma was immediately separated from the blood cells and stored at −20 °C until to be used. The brain tissue was weighed and homogenized in 0.1 M sodium phosphate buffer, pH 5.5 (4 mL/g of tissue) using a Ultraturrax® tissue homogenizer. The brain tissue homogenates were centrifuged at 13,500 rpm for 10 min (4 °C) and the supernatants were collected and stored at −20 °C until used. The animal procedures were conducted in accordance with the European Directive (2010/63/EU).

2.3. Stock solutions, calibration standards and quality control samples

The LTG stock solution (1 mg/mL) and working solution (100 μg/mL) were prepared in methanol, and then adequately diluted in water-methanol (50:50; v/v) to afford six different spiking solutions at 0.5, 1, 3.5, 15, 62.5 and 100 μg/mL. Each one of these solutions were used daily for spiking aliquots of blank rat samples (plasma and brain homogenate; 20 μl spiking solution to 80 μl of blank sample) in order to prepare the corresponding calibration standards at six different concentrations (0.1, 0.2, 0.7, 3, 12.5 and 20 μg/mL). The stock solution of the IS was also prepared in methanol (1 mg/mL) and the working solution (250 μg/mL) was obtained after diluting an appropriate volume of the stock solution with water-methanol (50:50; v/v). All solutions were stored at 4 °C and protected from light, except the IS working solution which was daily prepared.

Quality control (QC) samples at four concentration levels were also prepared independently in the same biological matrices, representing the lowest (QC_{LLOQ}), low (QC_1), medium (QC_2) and high

(QC_3) ranges of the calibration curves. For that purpose, aliquots of blank rat plasma and brain homogenate samples were similarly spiked in order to obtain final LTG concentrations of 0.1, 0.3, 10 and 18 μg/mL.

2.4. Sample preparation and extraction

The optimal sample preparation and extraction conditions were set as follows. To aliquots (100 μL) of plasma or brain homogenate, spiked with 20 μL of the IS working solution (250 μg/mL), 400 μL of ice-cold acetonitrile were added; the final mixture was vortex-mixed for 30 s and centrifuged at 13,500 rpm for 10 min to precipitate proteins in order to minimize sample interferences. The resulting clear supernatant was collected and evaporated under a gentle nitrogen stream at 45 °C and the dry residue was reconstituted with 200 μL of 0.3% triethylamine-water solution (pH 6.5) and then submitted to MEPS procedure.

The MEPS sorbent (C_{18}) inserted into a 250 μL gas-tight syringe was activated with methanol (3 × 200 μL) and then conditioned with ultra-pure water (3 × 200 μL) before use. Afterwards, the reconstituted sample (200 μL) was drawn up and down through the syringe three times in the same vial, at a flow rate of 10 μL/s. In the next step, the sorbent was washed once with ultra-pure water (200 μL) in order to remove interferences and then the compounds of interest (LTG and IS) were eluted with methanol (2 × 30 μL). The resulting methanolic extract was diluted with 90 μL of ultra-pure water, and 20 μL was injected into the chromatographic system. After each sample extraction, the MEPS sorbent was washed/reconditioned with 12 × 200 μL of methanol followed by 5 × 200 μL of ultra-pure water to avoid carry-over phenomena, and to allow the reutilization of the MEPS cartridge. Applying this protocol, each MEPS cartridge was reused for approximately 200 extractions before it was discarded.

2.5. Apparatus and chromatographic conditions

The chromatographic analysis was carried out using an HPLC system (Shimadzu LC-2010A HT Liquid Chromatography) coupled with a DAD (Shimadzu SPD-M20A). All instrumental parts were automatically controlled by LC solution software (Shimadzu, Kyoto, Japan). The chromatographic separation of LTG and the IS was carried out at 35 °C on a reversed-phase LiChroCART® Purospher Star column (C_{18}, 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 acetonitrile (13%), methanol (13%) and a mixture (74%) of water–triethylamine 0.3%, pH 6.0 adjusted with 85% *ortho*-phosphoric acid. The mobile phase was filtered through a 0.2 μm filter and degassed ultrasonically for 15 min before use. The injection volume was 20 μL and the wavelength of 215 nm was selected for the detection of both compounds (LTG and IS).

2.6. Method validation

The method validation procedures were carried out in agreement with the international guidelines on bioanalytical method validation [27,28]. Several specific validation parameters such as selectivity, linearity, lower limit of quantification (LLOQ), accuracy, precision, recovery and analyte stability were studied and assessed taking into account the corresponding acceptance criteria.

The selectivity of the method was evaluated by analysing blank plasma and brain homogenate samples obtained from six different rats in order to assess the existence of potential interference of endogenous compounds at the same retention times of the analyte (LTG) and IS. Additionally, the potential interference of exogenous compounds such as a set of anaesthetics (pentobarbital, xylazine and ketamine) commonly used in nonclinical in vivo studies was also tested, by injecting 20 μL of standard drug solutions with a concentration of 10 μg/ml.

The calibration curves for each biological matrix of interest (rat plasma and brain homogenate) were obtained after processing the six calibration standards, including the LLOQ, in the concentration range previously defined, on five different days ($n = 5$), and the LTG/IS peak area ratios obtained were plotted against the corresponding nominal concentrations. The data were subjected to a weighted linear regression analysis [29].

The LLOQ, defined as the lowest concentration of the calibration curve that can be measured with acceptable intra and interday precision and accuracy, was evaluated by analysing plasma and brain tissue homogenate samples prepared in replicate ($n = 5$). The LLOQ for LTG in both matrices was assessed considering as acceptance criteria a coefficient of variation (CV) not exceeding 20% and a deviation from nominal concentration (*bias*) within ±20%.

The interday precision and accuracy were evaluated after processing four QC samples (QCLLOQ, QC1, QC2, QC3) prepared in plasma and brain homogenate, which were tested on five consecutive days ($n = 5$), whereas the intraday precision and accuracy were tested by processing five sets of the corresponding QC samples in a single day (n = 5). The acceptance of inter and intraday precision criterion was defined by a CV value lower than or equal to 15% (or 20% for the LLOQ), and for the intra and interday accuracy a *bias* value lower than or equal to 15% (or ±20% for the LLOQ).

The absolute recovery of LTG and the IS from rat plasma and brain homogenate was estimated after the extraction of QC samples at three concentration levels (QC1, QC2 and QC3) in five replicates ($n = 5$), and comparing the resultant peak area with the peak area obtained by the direct injection of the corresponding non-extracted LTG and IS solutions at the same nominal concentrations. The values of absolute recovery for LTG and the IS were then obtained by the ratio of the peak areas of extracted and non-extracted samples.

The stability of LTG in rat plasma and brain homogenate samples was investigated for QC1 and QC3 ($n = 5$) in several experimental conditions to simulate the handling and storage of samples. Specifically, the stability of LTG was assessed in processed samples maintained in the autosampler during a period of 12 h; and also in unprocessed samples simulating the short-term and long-term stability conditions, particularly at room temperature for 4 h, at 4 °C for 24 h and at −20 °C for 30 days ($n = 5$). The stability was assessed by comparing the data of samples analysed before (reference samples) and after being exposed to the conditions for stability assessment (stability samples). A stability/reference samples ratio of 85–115% was accepted as the stability criterion ($n = 5$).

2.7. Method application and pharmacokinetic analysis

To demonstrate the applicability of the proposed method a pharmacokinetic study was conducted in a group of five Wistar rats ($n = 5$), which received a single oral dose of LTG (10 mg/kg). At several pre-defined post-dose time points (0.5, 1, 2, 4, 6, 8, 12, 24, 48 and 72 h), blood samples (~0.3 mL) were collected into heparinised tubes through a cannula introduced in the tail vein of rats or by decapitation at the end of the experiments. Brain tissue was also obtained from the same rats at two previously defined endpoints: at 24 h ($n = 1$) and at 72 h ($n = 4$) post-dosing; this procedure was designed to ensure the determination of LTG concentrations above the LLOQ in at least one brain sample (at 24 h post-dose), since LTG concentration levels in brain could be below the LLOQ of the method (BLQ) at 72 h post-dose. Blood samples and brain tissue were processed and stored until analysis as described in Section 2.2. Blank rat matrices. The obtained data were submitted to a

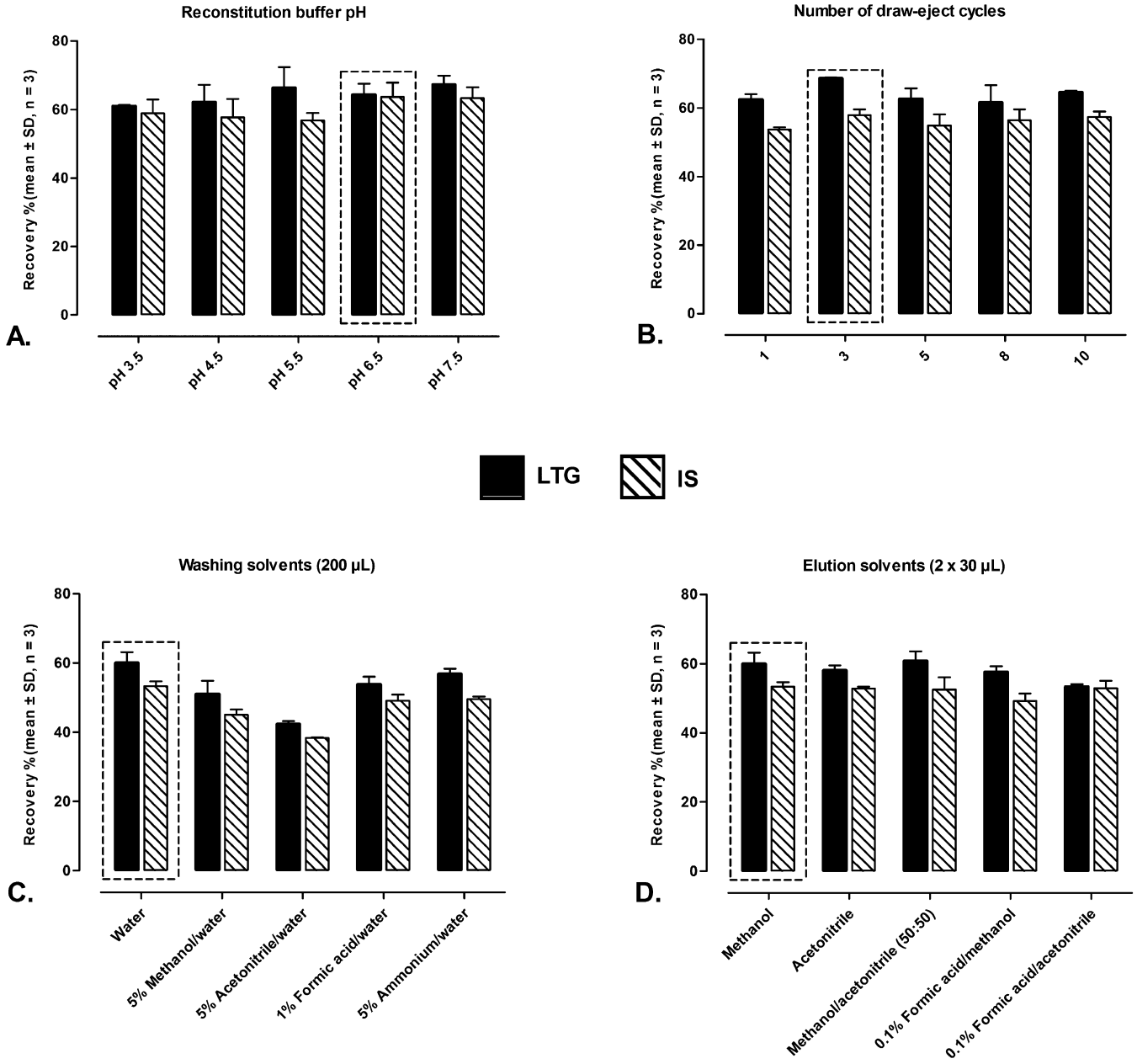

Fig. 2. Effect of different MEPS conditions on the extraction efficiency of lamotrigine (LTG) and internal standard (IS): influence of the reconstitution buffer pH (A), number of draw-eject cycles at pH 6.5 (B), different washing solutions (C) and elution solvents (D).

non-compartmental pharmacokinetic analysis using WinNonlin® version 5.2 (Pharsight Co., Mountain View, CA, USA).

3. Results and discussion

3.1. Optimization of chromatographic conditions

The chromatographic conditions were optimized according to the experience of the in-house developed techniques for the determination of AEDs and in order to achieve a symmetric peak shape and a good chromatographic resolution for LTG and the IS, within the shortest running time. Since LTG is a UV-absorbing compound with weak basic properties, containing a hydrophobic moiety, the use of reverse-phase liquid chromatography coupled to a DAD detector was considered to be appropriate for the quantification of LTG in both rat plasma and brain samples. Different conditions were tested in order to find the best mobile phase, the most appropriate wavelength values bearing in mind a good relationship between selectivity and sensitivity, and the selection of the IS was also carefully studied.

In what concerns the composition of the mobile phase, and considering the reversed-phase (C_{18}) retention mechanisms, different percentages of acetonitrile and methanol were tested as organic modifiers and a mobile phase composed by acetonitrile (13%), methanol (13%) and a mixture (74%) of water–triethylamine 0.3% was selected. Although the use of amine additives is not consensual, in this case the addition of a small amount of triethylamine was favourable perhaps due to the saturation of free silanol groups on the stationary phase, reducing the peak asymmetry and peak tailing phenomenon [30]. In addition, the aqueous component of the mobile phase (water–triethylamine 0.3%) was also tested at different pH values. The most favourable retention times and the best

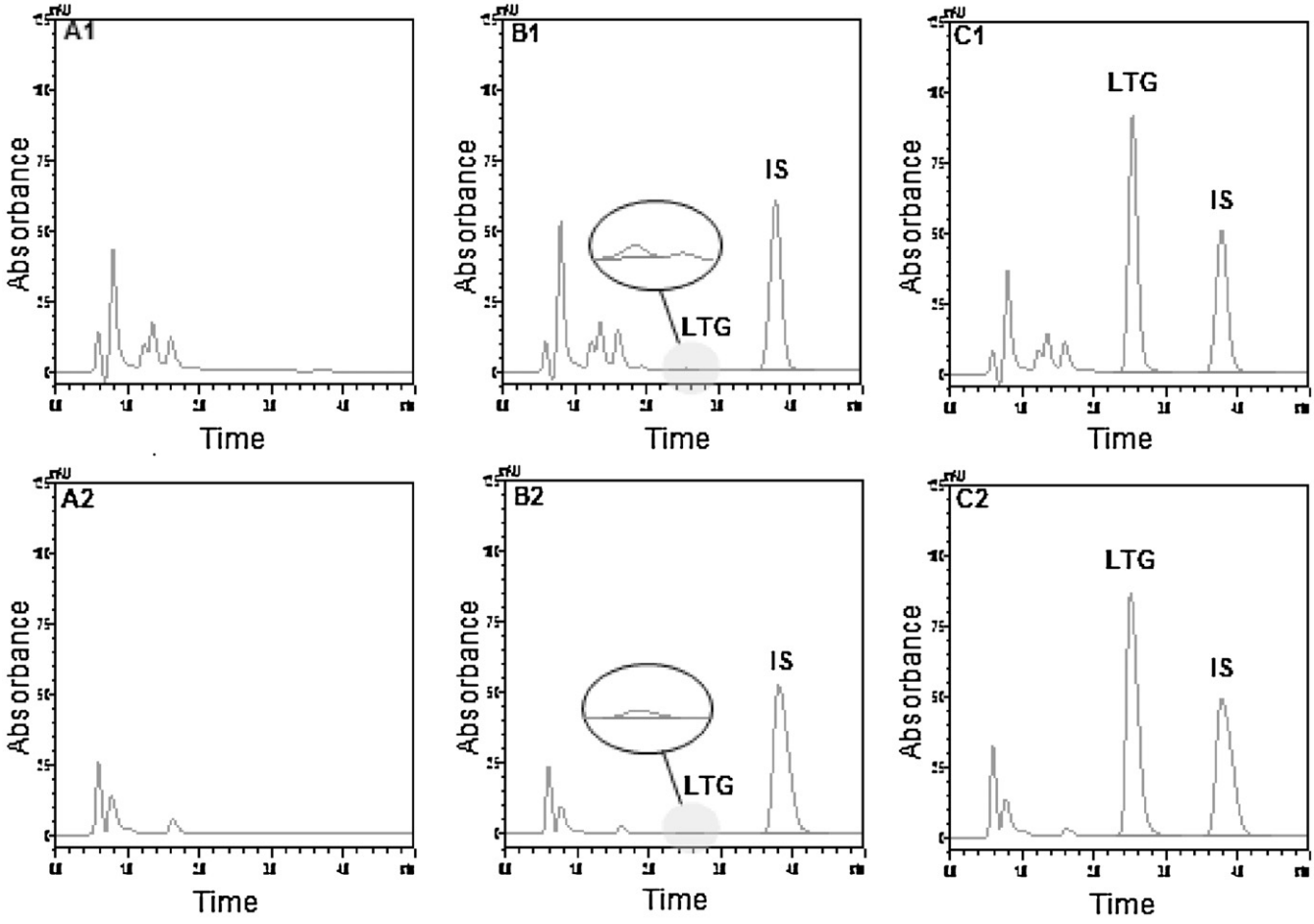

Fig. 3. Typical chromatograms of extracted rat plasma and brain homogenate samples obtained by the method developed: blank plasma (A1) and blank brain homogenate (A2); plasma (B1) and brain homogenate (B2) spiked with the internal standard (IS) and lamotrigine (LTG) at the lower limit of quantification (0.1 μg/mL); and plasma (C1) and brain homogenate (C2) spiked with the IS and LTG at the concentrations of the upper limit of calibration range (20 μg/mL).

peak separation and shapes were achieved with an aqueous component of water–triethylamine 0.3% at pH 6.0, adjusted with 85% *ortho*-phosphoric acid. Regarding detection conditions, although different wavelengths were tested considering the absorption of the two chromophores that compose LTG, the best compromise in terms of sensitivity and selectivity was achieved at 215 nm. Moreover, in HPLC analysis, the reliable quantification of any analyte requires the use of an adequate IS. The selection of chloramphenicol as IS was made according to reported experiments [31–33] and also due to its favourable behaviour in the selected chromatographic conditions in comparison with other tested compounds (e.g., levamisole). Under these bioanalytical conditions, the LTG and IS peaks showed a symmetric shape and were well separated in a running time shorter than 5 min, enabling a faster chromatographic analysis than the previously reported methods [23–25]. The chromatographic instrumentation required and the simple bioanalytical conditions established enable the easy implementation of this assay in any analytical laboratory.

Table 1
Intra and interday precision (% CV) and accuracy (% *bias*) values obtained for lamotrigine (LTG) in rat plasma and brain homogenate samples at the lower limit of quantification (QC_{LLOQ}), and at low (QC_1), middle (QC_2) and high (QC_3) concentration levels representative of the calibration ranges ($n = 5$).

Matrix	Cnominal (μg/mL)		Interday			Intraday		
			$C_{experimental}$ (Mean ± SD) (μg/mL)	Precision (% CV)	Accuracy (% bias)	$C_{experimental}$ (Mean ± SD) (μg/mL)	Precision (% CV)	Accuracy (% bias)
Plasma	QC_{LLOQ}	0.1	0.106 ± 0.003	1.9	6.0	0.098 ± 0.004	2.6	−2.0
	QC_1	0.3	0.315 ± 0.010	2.8	5.1	0.276 ± 0.028	8.6	−8.1
	QC_2	10	10.451 ± 0.509	4.8	4.5	10.253 ± 0.110	1.1	2.5
	QC_3	18	18.222 ± 0.637	3.5	1.2	17.866 ± 0.196	1.1	−0.7
Brain	QC_{LLOQ}	0.1	0.105 ± 0.008	6.2	5.2	0.104 ± 0.007	5.3	3.9
	QC_1	0.3	0.321 ± 0.017	5.0	6.9	0.341 ± 0.021	5.8	13.5
	QC_2	10	10.117 ± 0.164	1.6	1.2	9.780 ± 0.195	2.0	−2.2
	QC_3	18	17.610 ± 0.275	1.6	−2.2	17.368 ± 0.175	1.0	−3.5

$C_{experimental}$, experimental concentration; $C_{nominal}$, nominal concentration; CV, coefficient of variation; SD, standard deviation.

Table 2
Comparison of key bioanalytical aspects (sensitivity, extraction efficiency/recovery and run time) between the current method and previous methods used for the bioanalysis of lamotrigine in rat plasma/serum and brain homogenate samples.

Matrix	Sample volume	Extraction method	Analytical instrumentation	Sensitivity (LLOQ)	Recovery	Run time	Reference
Plasma	100 μL	PP + MEPS	HPLC-DAD	0.1 μg/mL	68.0–73.5%	5 min	Current method
Brain	100 μL	PP + MEPS	HPLC-DAD	0.1 μg/mL	71.7–86.7%	5 min	Current method
Plasma	20 μL	SPE	HPLC-UV	–	–	–	[20]
Serum	50 μL	LLE	HPLC-UV	–	–	20 min	[21]
Brain	100 μL	LLE	HPLC-UV	–	–	20 min	[21]
Serum	50 μL	PP	HPLC-UV	–	–	–	[22]
Brain	1000 μL	PP + LLE	HPLC-UV	0.1 μg/mL	74.27–98.60%	10 min	[23]
Plasma	100 μL	LLE	HPLC-UV	0.5 μg/mL	82.2–93.1%	≈11 min	[24]
Brain	100 μL	PP	HPLC-UV	0.25 μg/g	81.3–89.5%	≈11 min	[24]
Plasma	100 μL	PP	HPLC–MS	0.01 μg/mL	90.4–94.5%	12 min	[25]

DAD, Diode array detection; HPLC, High performance liquid chromatography; LLE, liquid-liquid extraction; LLOQ, lower limit of quantification; MEPS, microextraction by packed sorbent; MS, Mass spectrometry; PP, protein precipitation; SPE, solid-phase extraction; UV, ultraviolet.

Table 3
Recovery (values in percentage) of lamotrigine (LTG) from rat plasma and brain homogenate samples at low (QC_1), middle (QC_2) and high (QC_3) concentrations of the calibration ranges ($n = 5$).

Matrix	$C_{nominal}$ (μg/mL)		Recovery (%)	
			Mean ± SD (n = 5)	CV (%)
Plasma				
	QC1	0.3	73.5 ± 4.6	6.3
	QC2	10.0	68.0 ± 2.7	4.0
	QC3	18.0	71.4 ± 3.0	4.3
Brain				
	QC1	0.3	86.7 ± 3.5	4.1
	QC2	10.0	71.7 ± 3.4	4.8
	QC3	18.0	74.6 ± 1.6	2.1

$C_{nominal}$, nominal concentration; CV, coefficient of variation; SD, standard deviation.

Table 4
Stability (values in percentage) of lamotrigine (LTG) at low (QC_1) and high (QC_3) concentrations of the calibration ranges, in unprocessed rat plasma and brain homogenate samples at room temperature for 4 h, at 4 °C for 24 h, and at −20 °C for 30 days; and in processed rat plasma and brain homogenate samples left in the HPLC autosampler for 12 h ($n = 5$).

Stability (values in percentage) of LTG				
Analyte	Plasma		Brain	
	QC_1	QC_3	QC_1	QC_3
$C_{nominal}$ (μg/mL)	0.3	18.0	0.3	18.0
Unprocessed samples				
Room temperature (4 h)	107.7	106.0	110.3	104.7
4 °C (24 h)	102.0	102.3	88.1	97.0
−20 °C (30 days)	107.9	99.4	88.0	97.2
Processed samples				
Autosampler (12 h)	97.9	99.4	101.0	100.1

$C_{nominal}$, nominal concentration.

3.2. Development and optimization of sample extraction procedure

Proper sample pre-treatment is a key step and a prerequisite for most bioanalytical procedures. The introduction of MEPS as microextraction procedure brought several advantages in comparison with solid-phase extraction (SPE), enabling a good recovery and sensitivity, using smaller sample and solvent volumes [26].

The sample extraction steps were optimized from a validated MEPS procedure used in a previous work of the research group [34], in order to reach suitable MEPS efficiency for the extraction of LTG and the IS in both samples (rat plasma and brain homogenate). Taking into account our practical experience with MEPS protocols [34–36] and as Abdel-Rehim [37] also highlighted, the rat samples were deproteinized with acetonitrile before sample loading to avoid the rapid clogging of the MEPS cartridges. Then, due to the high percentage of acetonitrile in the sample supernatant, which strongly impairs the retention of the compounds of interest (LTG and IS) in the MEPS sorbent, the supernatant was collected and evaporated to dryness and the residue was reconstituted in an aqueous buffer before MEPS loading. Specifically, the pH of the aqueous reconstitution solution (0.3% triethylamine-water) was the first experimental variable to be evaluated during the optimization of the MEPS protocol, and it was assessed in the pH range of 3.5–7.5; considering the similarity in the obtained results concerning the influence of the pH of the reconstitution buffer on the recovery of LTG and the IS (Fig. 2A), the pH value of 6.5 was selected. In addition, other MEPS variables such as the number of draw-eject cycles and washing and elution conditions were also investigated. Considering the overall results of this set of experiments (Fig. 2B–D) and in order to streamline the MEPS protocol, three draw-eject cycles were selected in the sample loading stage, 200 μL of water was used in the washing step and the desorption (elution) of the compounds of interest (LTG and IS) was efficiently accomplished with methanol (2 × 30 μL). Moreover, to ensure the total removal of LTG, the IS and other endogenous compounds from the packed sorbent before the next sample extraction, the MEPS cartridge was cleaned/reconditioned with 12 × 200 μL of methanol and 2 × 200 μL of water between each extraction. All these experiments were carried out with aliquots (100 μL) of rat plasma samples spiked with LTG at 20 μg/mL and added with 20 μL of IS solution at 250 μg/mL. Indeed, the sample extraction procedure was formally developed and optimized using rat plasma matrix, but in parallel some assays were also conducted using brain homogenate samples in order to anticipate its applicability to both rat matrices.

3.3. Method validation

3.3.1. Selectivity

The chromatograms of blank and spiked rat plasma and brain homogenate samples are shown in Fig. 3. The analysis of blank rat plasma and brain samples from six rats confirmed the absence of endogenous interferences in the retention times of LTG and the IS, using the established chromatographic and detection conditions. Furthermore, considering the chromatographic behaviour of the anaesthetic drugs tested as potentially exogenous interferences, only xylazine was found to interfere in the retention of LTG. Thus, in future pharmacokinetic studies involving the determination of LTG is desirable to avoid anaesthetic procedures with xylazine.

3.3.2. Calibration curves and LLOQs

The calibration curves obtained in rat plasma and brain homogenates were linear within the concentration ranges previously defined and showed a consistent relationship between analyte-IS peak area ratios and the corresponding nominal con-

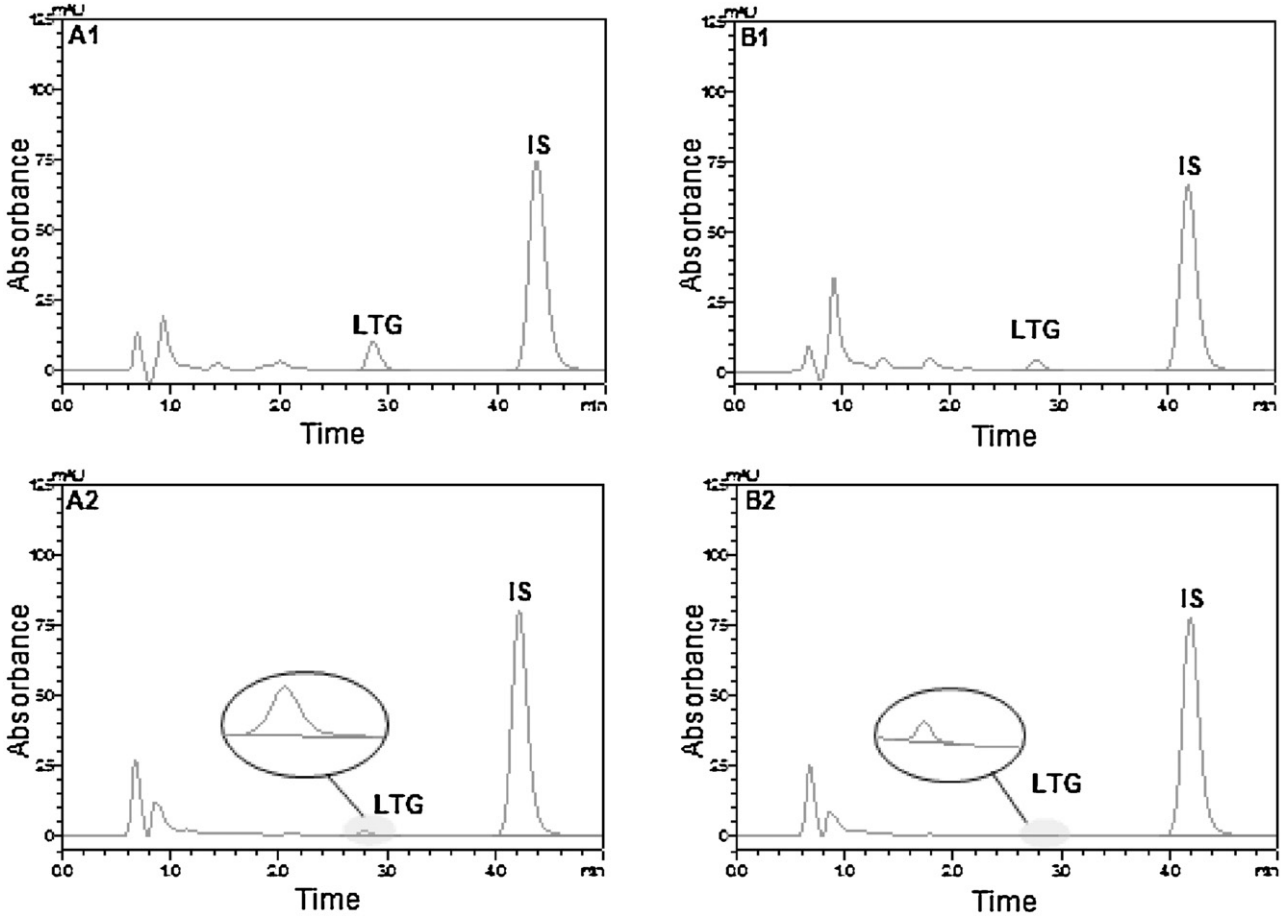

Fig. 4. Representative chromatograms of the analysis of real samples of rat plasma and brain homogenate: at 24 h post-dose in plasma (A1) and brain homogenate (A2), and at 72 h post-dose in plasma (B1) and brain homogenate (B2).

Table 5
Pharmacokinetic parameters estimated by non-compartmental analysis of the individual plasma concentration-time profiles of lamotrigine (LTG) obtained in rats ($n = 5$) after a single oral dose of LTG (10 mg/kg).

Pharmacokinetic parameters	Rat 1[c]	Rat 2	Rat 3	Rat 4	Rat 5	Mean	Ref. [20]	Ref. [38]
Plasma								
t_{max} (h)	2.0	24.0	2.0	24.0	2.0	2.0[a]	3.4	2.0
C_{max} (μg/mL)	1.543	3.423	3.719	3.474	4.317	3.295	2.84	4.24
AUC_{0-t} (μg h/mL)	NC	161.00	142.92	149.98	165.02	154.73	81.2[b]	–
$AUC_{0-\infty}$ (μg h/mL)	NC	241.67	168.78	171.79	184.14	191.60	–	–
k_{el} (h^{-1})	NC	0.0179	0.0272	0.0329	0.0329	0.0277	–	–
$t_{1/2el}$ (h)	NC	38.6	25.5	21.1	21.1	26.6	25.1	12
MRT (h)	NC	64.5	38.7	37.4	32.8	43.3	–	33

t_{max}, time to reach peak concentration; C_{max}, peak concentration; AUC_{0-t}, area under the concentration-time curve from time zero to the last sampling time with measurable concentration; $AUC_{0-\infty}$, area under the concentration-time curve from time zero to infinite; k_{el}, apparent terminal elimination rate constant; MRT, mean residence time; NC, not calculated; $t_{1/2el}$, apparent terminal elimination half-life. C_{max} and t_{max} are experimental values; AUC_{0-t}, $AUC_{0-\infty}$, k_{el}, $t_{1/2el}$ and MRT values were calculated by non-compartmental analysis.

[a] Median.

[b] AUC_{0-48h}.

[c] Rat 1 was sacrificed at 24 h post-dose to collect an earlier brain sample.

centrations. A weighted linear regression analysis was used due to the wide calibration range and to compensate for heteroscedasticity. The calibration curves were subjected to weighted linear regression analysis using $1/x^2$ as the weighting factor. The regression equations of the calibration curves and the corresponding determination coefficients (r^2) achieved for LTG in rat plasma and brain homogenates were $y = 0.06499x + 0.00272$ ($r^2 = 0.9947$) and $y = 0.06647x + 0.00187$ ($r^2 = 0.9952$), respectively. The LLOQs were experimentally defined as 0.1 μg/mL in both rat plasma and brain homogenate with acceptable precision and accuracy (Table 1). In addition, as shown in Table 2, the sensitivity (LLOQ) achieved with our method is similar or even better than that obtained by other HPLC-UV techniques reported in the literature and, comparatively, a quicker chromatographic analysis is accomplished.

3.3.3. *Precision and accuracy*

The intraday and interday precision and accuracy results obtained in rat plasma and brain homogenates at four different concentration levels (QC_{LLOQ}, QC_1, QC_2 and QC_3) are presented in Table 1. In plasma, the intra and interday CV values did not exceed

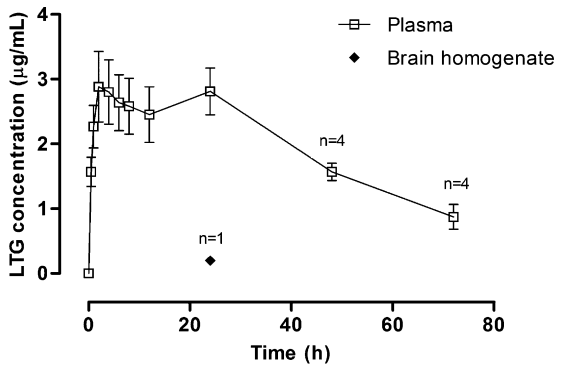

Fig. 5. Mean plasma concentration-time profile of lamotrigine (LTG), over a period of 72 h, obtained from rats treated with a single dose of LTG (10 mg/kg) administered by oral gavage. Symbols represent the mean values ± standard error of the mean (SEM) of five determinations per time point ($n = 5$, unless otherwise indicated). The concentration of LTG in a brain homogenate sample collected at 24 h post-dose ($n = 1$) is also represented; at 72 h post-dose the brain concentrations of LTG were found at BLQ levels in all rats analysed ($n = 4$).

8.6%, and the intra and interday *bias* values ranged from −8.1 to 6.0%. Likewise, in brain homogenate, the intra and interday CV values did not exceed 6.2%, and the intra and interday *bias* values varied between −3.5 and 13.5%. All results fulfilled the acceptance criteria of the international guidelines; therefore, the developed method is precise and accurate for the quantification of LTG in the rat matrices studied.

3.3.4. Recovery

The LTG recovery results in both rat plasma and brain homogenates tested at three different concentration levels (QC_1, QC_2 and QC_3) are provided in Table 3. The absolute mean recovery values ranged from 68.0 to 73.5% in rat plasma with CV values equal or lower than 6.3%, and ranged from 71.7 to 86.7% in brain homogenates with maximal CV values of 4.8%. The absolute recovery of the IS in rat plasma was 61.0% with a CV value of 3.7% and in brain homogenate was 64.2% with a CV value of 4.5%. The extraction efficiency estimated for the present bioanalytical assay is within the values usually achieved when MEPS is used as a sample preparation procedure [26]. Moreover, a comparison of extraction efficiency (recovery) between the current method and previous methods used for the bioanalysis of LTG in rat brain homogenate and plasma samples is presented in Table 2.

3.3.5. Stability

The results for LTG stability in rat plasma and brain homogenate are shown in Table 4. According to the data obtained, no significant loss of LTG was observed in unprocessed and processed rat plasma and brain homogenate samples in the different handling and storage conditions studied.

3.4. Method application and pharmacokinetics

The validated MEPS/HPLC-DAD method was applied to the analysis of LTG concentration levels in plasma and brain homogenate samples obtained from Wistar rats ($n = 5$) treated with a single oral dose of LTG (10 mg/kg). Representative chromatograms of the analysis of real samples of rat plasma and brain homogenate are shown in Fig. 4, and no matrix interferences were observed during the sample analysis. In general, the plasma concentration–time profiles of LTG were obtained over a period of 72 h post-dose ($n = 4$) in order to appropriately characterize the terminal elimination phase of the drug. In contrast, in one rat ($n = 1$) the LTG plasma concentration–time profile was obtained only up to 24 h post-dose because an early collection of a brain sample was considered to be important to ensure LTG concentration levels above the LLOQ of the method. Whenever possible, the corresponding individual pharmacokinetic profiles were analysed and the estimated pharmacokinetic parameters are summarized in Table 5. Globally, these results are in agreement with the results obtained in other works, in which the same dose of LTG was administered [20,38] (Table 5). The mean plasma concentration-time profile of LTG ($n = 5$, unless otherwise indicated) is depicted in Fig. 5, as well as the concentration of LTG quantified in brain homogenate at 24 h post-dose (0.197 μg/mL); as expected, at 72 h post-dose the brain concentrations of LTG were found at BLQ levels in all rats ($n = 4$). At this point, it is worthy to mention that the low concentrations of LTG measured in brain tissue homogenate do not compromise the application of the method; however, it is suggested that shorter post-dose sampling time points should be considered in future pharmacokinetic studies designed to assess the brain disposition of LTG.

4. Conclusion

This new MEPS/HPLC-DAD method developed for the quantification of LTG in rat plasma and brain homogenate was successfully validated. The sample microextraction procedure involving MEPS seems to be cost-effective because each MEPS cartridge can be reused for the extraction of a high number of samples before being discarded. One important aspect that should be emphasised is that MEPS was applied for the determination of LTG in brain tissues. Indeed, MEPS has frequently been applied for the determination of drugs in serum, plasma and other biological fluids but has rarely been applied in tissues. Another important aspect of this method is the small sample volume (100 μL) required, allowing the collection of several blood samples from the same animal during pharmacokinetic studies and, therefore, reducing the number of animals used. The reported bioanalytical method was also successfully applied to quantify LTG in real biological samples. Therefore, it can be concluded that this MEPS/HPLC-DAD method is a useful tool to support future pharmacokinetic and biodisposition studies in rats involving LTG administration.

Conflict of interest statement

The authors have declared no conflict of interest.

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