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Determination of lamotrigine in human plasma and saliva using microextraction by packed sorbent and high performance liquid chromatography–diode array detection: An innovative bioanalytical tool for therapeutic drug monitoring

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ABSTRACT

A ground-breaking high-performance liquid chromatography-diode array detection method based on microextraction by packed sorbent (MEPS) as sample preparation approach is described herein for determination of lamotrigine (LTG), a narrow therapeutic index drug, in human plasma and saliva. MEPS variables and chromatographic conditions were optimized to achieve appropriate selectivity and sensitivity using small sample volumes (100 μL). The chromatographic separation of LTG and chloramphenicol [internal standard (IS)] was accomplished in <5 min on a C18-column, at 35 °C, using a mobile phase composed by acetonitrile/methanol/water-triethylamine 0.3% at pH 6.0 (13:13:74, v/v/v) pumped isocratically at 1 mL/min. LTG and IS were detected at 215 nm. A good linearity was obtained for LTG ($r^2 \geq 0.9936$) in the range of 0.1–20 μg/mL in plasma and saliva, with the limit of quantification of 0.1 μg/mL. The method was shown to be precise (RSD ≤14.5%) and accurate (*bias* ± 13.4%), and the absolute recovery ranged from 64.9% to 73.6%. The stability of LTG was demonstrated in plasma and saliva samples in all studied conditions. The proposed assay was applied to the analysis of real human plasma and saliva samples from epilepsy patients under LTG therapy and the results support its usefulness for therapeutic drug monitoring.

1. Introduction

Lamotrigine (LTG) is a broad-spectrum antiepileptic drug (AED) used as monotherapy or in add-on therapy regimens in adults and children [1–4]. LTG is also approved for Lennoux-Gastaut, a rare and intractable form of childhood epilepsy, and for bipolar disorders [5–7]. Structurally, LTG is a 3,5-diamino-6-(2,3-dichlorophenyl)-1,2,4-triazine (Fig. 1), belonging to the phenyltriazine class, which is chemically unrelated to other existing AEDs [2,8]. The physicochemical and pharmacological properties of LTG determine its unique pharmacokinetic and pharmacodynamic profile [9].

Therapeutic drug monitoring (TDM) of LTG is of crucial interest in many clinical circumstances due to the large inter and intraindividual variability of its systemic drug concentrations, including under steady-state conditions, particularly in cotherapy with other AEDs such as phenytoin, carbamazepine and valproate [10]. Indeed, it is well-established that plasma/serum LTG concentrations should be monitored during its concomitant use with other drugs that are enzyme inhibitors (e.g. valproate, sertraline) or inducers (e.g., phenobarbital, phenytoin, carbamazepine, rifampicin, oral contraceptives) [11–14]. Although the therapeutic concentration range for LTG has been progressively modified over the years, the range of 2.5–15 μg/mL has been proposed for seizure control. However, there is a considerable overlap in serum concentrations among responders and nonresponders, and some refractory patients may need higher concentration levels [14]. TDM has also an important clinical value in pregnancy and in children taking LTG, because plasma drug concentrations are reduced throughout gestation and the clearance of LTG is higher in children compared to adults and the elderly [4].

The implementation of TDM for clinical management of LTG therapy requires the availability of suitable bioanalytical methodologies to support the LTG concentration measurements in the biological samples of interest in order to adjust the patient's medication regimen and achieve

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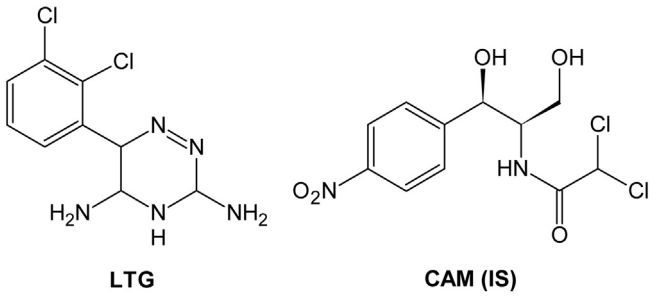

Fig. 1. Chemical structure of lamotrigine (LTG) and chloramphenicol (CAM), used as internal standard (IS).

optimal therapeutic outcomes. Therefore, several techniques have been developed and validated to quantify LTG in different human matrices (e.g. blood, plasma, serum, urine and saliva) through chromatography [15–26], immunoassay [23,27] and electrophoresis methods [28–33]. The predominant methodology for LTG bioanalysis is HPLC coupled to diode array detection (DAD) or UV detection [15,18–20,26,34–49]. On the other hand, considering all of these LC methods, the sample preparation/extraction processes employed involved SPME [50], SPE [19,22,26,45,46,51,52], protein precipitation (PP) [17,28,33–35,40,41,53,54], LLE [25,43,44,55–61] and microextraction by packed sorbent (MEPS) [15].

MEPS is indeed a novel sample preparation approach in the field of bioanalysis, directed towards miniaturization and automation, and it has been used for qualitative and quantitative bioanalysis of a vast number of drugs and metabolites [15,62–64]. Specifically, regarding the application of MEPS in the bioanalysis of LTG, up to date and to the best of our knowledge, no method was developed and validated for human saliva. Saliva was firstly investigated as an alternative biological fluid for TDM of AEDs in the 1970s [65]. The use of saliva instead of plasma/serum has several advantages: the collection of saliva is simple and non-invasive, avoiding discomfort or stress in patients, particularly in children and the elderly; in addition, the drug levels in saliva reflect the free non-protein bounded drug concentrations in blood [65]. Bearing in mind that some recent reports show a good relationship between salivary concentrations and plasma/serum concentrations, strengthening the idea that saliva represents a viable alternative sample to perform TDM [4,8,65–67], this work was planned to develop and validate a novel HPLC method for the quantification of LTG in human plasma and saliva 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) and chloramphenicol (CAM), used as internal standard (IS), was purchased from Sigma–Aldrich (St Louis, MO, USA). The chemical structures of these compounds are shown in Fig. 1. Methanol and acetonitrile, both of HPLC gradient grade, were purchased from Chem-Lab (Zedelgem, Belgium) 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 was acquired from Merck KGaA (Darmstadt, Germany) and the 85% *ortho*-phosphoric acid from Panreac Química SA (Barcelona, Spain). The MEPS 250 μL syringe and the MEPS BIN (barrel insert and needle) containing ~4 mg of solid-phase silica–C_{18} material (SGE Analytical Science, Australia) were purchased from ILC (Porto, Portugal). Blank human plasma from healthy blood donors was provided by the Portuguese Blood Institute after the written consent of each subject, and saliva was kindly obtained from a set of volunteers.

2.2. Stock solutions, calibration standards and QC samples

The LTG stock solution (1 mg/mL) and the working solution (100 μg/mL) were properly prepared in methanol and then adequately diluted in water-methanol (50:50, v/v) to afford six spiking solutions with final concentrations of 0.5, 1, 3.5, 15, 62.5 and 100 μg/mL. Each one of these solutions were daily used for spiking aliquots of blank human plasma and saliva in order to prepare six calibration standards in the concentration range of 0.1–20 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 in water-methanol (50:50, v/v). All stock, working and spiking solutions were stored at 4 °C and protected from light, with the exception of the IS working solution which was daily prepared.

QC samples at four concentration levels, representing the lowest (QC_{LOQ}) and the low (QC_1), medium (QC_2) and high (QC_3) ranges of the calibration curve, were also independently prepared. For that purpose, aliquots of blank human plasma and saliva were spiked to obtain final LTG concentrations of 0.1, 0.3, 10 and 18 μg/mL.

2.3. 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 the LC solution software (Shimadzu, Kyoto, Japan). The chromatographic separation of the analytes 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 water–triethylamine 0.3% (74%) at 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 a wavelength of 215 nm was selected for the detection of all compounds.

2.4. Sample preparation and extraction

The sample preparation procedure was optimized and the final conditions were as follows. It should be noted that saliva was collected without stimulation and was sonicated prior to sample extraction. Each aliquot (100 μL) of human plasma or saliva was spiked with 20 μL of the IS working solution, and then 400 μL of ice-cold acetonitrile was added for protein precipitation in order to minimize sample interferences in the MEPS step. The mixture was vortex-mixed for 30 s and centrifuged at 13,500 rpm for 10 min. Afterwards, the resulting supernatant was 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 6.0). This reconstituted sample was then submitted to MEPS.

Previous to MEPS procedures, the sorbent (C_{18}) was activated with methanol (3 × 200 μL) and passed through ultra-pure water (3 × 200 μL). Then, the reconstituted sample was drawn through the needle into the syringe and ejected at a flow rate of approximately 10 μL/s and three draw-eject cycles were applied on the same sample aliquot. After discarding the sample, the sorbent was washed with 200 μL of ultra-pure water in order to remove matrix interferences and, at the end, the analytes were eluted with methanol (2 × 30 μL) and diluted with 90 μL of ultra-pure water. An aliquot (20 μL) of the final sample extract was injected into the chromatographic system. After the extraction of each sample, the MEPS device was reconditioned with 12 × 200 μL of methanol followed by 5 × 200 μL of ultra-pure water to avoid transferring the analyte to the next sample (carryover effect). Each MEPS cartridge was reused for approximately 200 times before being discarded.

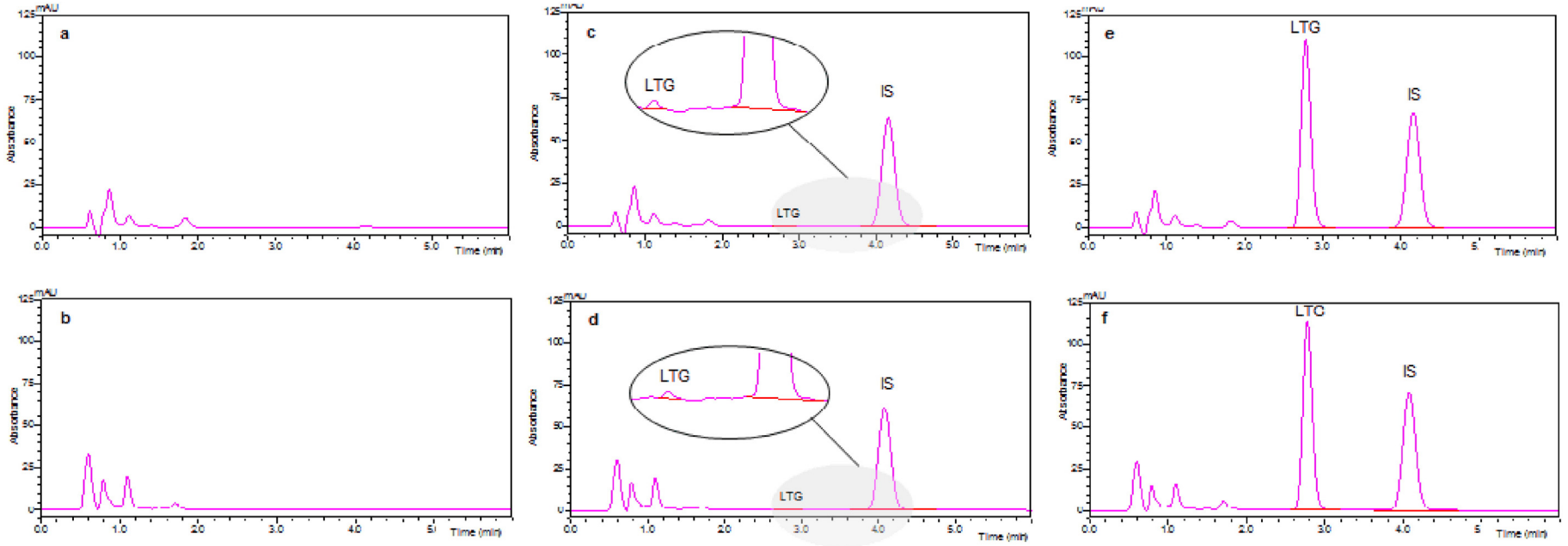

Fig. 2. Typical chromatograms of extracted human plasma and saliva samples obtained by the MEPS/HPLC-DAD method developed: blank plasma (a) and saliva (b); plasma (c) and saliva (d) spiked with the internal standard (IS) and lamotrigine (LTG) at the LOQ (0.1 μg/mL); and plasma (e) and saliva (f) spiked with the IS and LTG at the concentration of the upper limit of the calibration range (20 μg/mL).

2.5. Method validation

The international guidelines on bioanalytical method validation include several criteria for specific validation parameters that should be considered in the validation of any quantitative method. Such parameters are selectivity, linearity, LOQ, accuracy, precision, recovery and stability [68,69].

The selectivity of the method was evaluated by analysing six blank plasma and saliva samples from different subjects to evaluate the existence of matrix endogenous substances in retention times that could interfere with LTG and the IS. Additionally, the interference of other drugs that can potentially be co-administered with LTG was evaluated, by injecting standard drug solutions at 10 μg/mL. The drugs tested in this selectivity assay included other AEDs (carbamazepine, phenytoin, phenobarbital, phosphenytoin, oxcarbazepine, primidone, valproic acid), analgesics/antipyretics/anti-inflammatory drugs (acetylsalicylic acid, ketoprofen, ibuprofen, acetaminophen, piroxicam), antidepressants (amitriptyline, escitalopram, fluoxetine, mirtazapine, paroxetine, sertraline, trazodone, venlafaxine), antihypertensives (atenolol, furosemide), anxiolytics/sedatives/hypnotics (clorazepate, mexazolam) and many other drugs such as sulpiride, hydrocortisone, omeprazole, caffeine and nicotine.

The calibration curves for each biological matrix (plasma and saliva) were constructed after preparation of six calibration standards, including the LOQ, in the concentration range previously defined, on five distinct days ($n = 5$), and plotted according to the LTG/IS peak area ratio against the corresponding nominal concentrations. The data were subjected to a weighted linear regression analysis [70].

The LOQ, defined as the lowest concentration of the calibration curve that can be measured with adequate intra e interday precision and accuracy, was evaluated by analysing plasma and saliva samples prepared in five replicates ($n = 5$). The LOQ for LTG in both matrices was assessed considering relative standard deviation (RSD) values ≤ 20% and deviation from nominal concentration (*bias*) within ±20%.

The interday precision and accuracy were evaluated after processing four QC samples (QC_{LOQ}, QC_1, QC_2, QC_3) prepared in plasma and saliva, 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 criteria for interday and intraday precision is a RSD value lower than or equal to 15% (or 20% in the LOQ) and for accuracy, a *bias* value lower than or equal to 15% (or ±20% in the LOQ).

The absolute recovery of LTG and the IS from human plasma and saliva samples was determined after the extraction of the corresponding QC samples at three concentration levels (QC_1, QC_2 and QC_3) in five replicates ($n = 5$), and by comparing the resultant peak area with the peak area obtained after the direct injection of non-extracted LTG and IS solutions at the same nominal concentrations, also in five replicates. 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 human plasma and saliva was investigated for QC_1 and QC_3 ($n = 5$) in different experimental conditions. On the one hand, in processed samples maintained in the autosampler during a period of 12 h; and on the other hand, in unprocessed samples, simulating the short-term and long-term stability conditions, at room temperature for 4 h, at 4 °C for 24 h, and at −20 °C for 30 days ($n = 5$). Additionally, the effect of three freeze-thaw cycles on the stability of the LTG in human plasma and saliva samples was also studied at −20 °C. For that purpose, aliquots of spiked plasma and saliva samples (QC_1 and QC_3) were stored at −20 °C for 24 h, thawed unassisted at room temperature, and when completely thawed, the samples were frozen again for 24 h under the same conditions until completing the three freeze-thaw cycles.

2.6. Clinical application

Blood and saliva samples were obtained from two volunteer patients after the written consent of each subject. Plasma and saliva aliquots were analysed to demonstrate the clinical applicability of this bioanalytical method. Four blood and saliva samples were collect from each patient, who were under continuous long-term treatment with LTG, at predefined time-points (2 h, 4 h, 8 h and 12 h) after the first daily dose of the drug. The period between 0 and 2 h after the oral drug administration was not considered for sampling because some residual drug could be retained in the mouth (saliva) during this period of time [71]. Therefore, the collection of blood and saliva samples was only initiated at 2 h post-dose in order to obtain more reliable values for the saliva to plasma ratio observed for LTG concentrations.

Blood samples were collected into heparinised tubes, centrifuged at 4000 rpm (4 °C) for 10 min and the plasma was transferred to eppendorfs and stored at −20 °C until analysis. After mouth flushing, saliva samples were collected without stimulation into falcon tubes immediately after blood sampling, and stored at −20 °C until analysis. Ingestion of food, coffee and tobacco was not permitted within the two hours preceding saliva collection.

3. Results and discussion

A set of preliminary studies were carried out to optimize the bioanalytical process in order to validate an efficient method for the quantitative analysis of LTG in both human plasma and saliva. The final chromatographic and sample preparation/extraction conditions established were those previously mentioned in Sections 2.3 and 2.4, respectively. Actually, proper sample preparation is a key step and a prerequisite for most bioanalytical procedures. The introduction of MEPS as microextraction procedure brought several advantages namely, good recovery and enough sensitivity, and the use of more reduced sample and solvent volumes when compared to SPE. Under the defined bioanalytical conditions the LTG and IS peaks showed a symmetric shape and were well separated in a running time shorter than 5 min (Fig. 2). Hence, the analytical instrumentation required, as well as the simple experimental conditions established, enable the easy implementation of this assay in most hospital settings interested in the TDM of LTG.

3.1. Method validation

3.1.1. Selectivity

The chromatograms of blank and spiked human plasma and saliva samples are presented in Fig. 2. The analysis of blank human plasma and saliva samples from six healthy volunteers confirmed the absence of endogenous interferences in the retention times of LTG and the IS. Most of the tested drugs potentially co-administered with the AED under investigation (LTG) were also not found to interfere using the

Table 1
Retention times (RT) in minutes (min) of tested drugs potentially co-prescribed with lamotrigine (LTG).

Drugs	RT (min)	Drugs	RT (min)	Drugs	RT (min)
LTG	2.803	Acetylsalicylic acid	nd	Furosemide	3.381
IS	4.174	Ketoprofen	6.530	Escitalopram	18.278
Carbamazepine	12.991	Ibuprofen	23.382	Atenolol	0.839
Phenytoin	12.277	Paracetamol	nd	Mirtazapine	2.056
Phenobarbital	3.784	Piroxicam	3.778	Amitriptyline	nd
Phosphenytoin	3.480	Omeprazole	nd	Sertraline	1.694
Oxcarbazepine	6.521	Hydrocortisone	12.456	Trazadone	2.111
Primidone	2.013	Theophylline	nd	Venlafaxine	4.859
Valproic acid	1.986	Glibenclamide	1.700	Mexazolam	1.995
Valerian	nd	Caffeine	1.116	Nicotine	nd

IS, internal standard; nd, not detected in the analytical conditions used.

Table 2
Intra and interday precision (% RSD) and accuracy (% *bias*) values obtained for lamotrigine (LTG) in human plasma and saliva at the limit of quantification (QC_{LOQ}) concentration and at low (QC_1), medium (QC_2) and high (QC_3) concentrations representative of the calibration ranges ($n = 5$).

Matrix	$C_{nominal}$ (μg/mL)		Interday			Intraday		
			$C_{experimental}$ (mean ± SD) (μg/mL)	Precision (% RSD)	Accuracy (% *bias*)	$C_{experimental}$ (mean ± SD) (μg/mL)	Precision (% RSD)	Accuracy (% *bias*)
Plasma	QC_{LOQ}	0.1	0.109 ± 0.010	14.5	9.3	0.103 ± 0.005	8.8	3.1
	QC_1	0.3	0.294 ± 0.014	5.6	−2.1	0.278 ± 0.014	5.9	−7.5
	QC_2	10	9.357 ± 0.372	4.0	−6.4	9.260 ± 0.536	5.8	−7.4
	QC_3	18	17.208 ± 0.408	2.4	−4.4	16.197 ± 0.713	4.4	−10.0
Saliva	QC_{LOQ}	0.1	0.106 ± 0.005	6.3	6.0	0.106 ± 0.013	12.7	5.6
	QC_1	0.3	0.294 ± 0.011	4.1	−1.9	0.287 ± 0.006	2.2	−4.5
	QC_2	10	10.333 ± 0.231	2.2	3.3	11.236 ± 0.933	8.3	12.4
	QC_3	18	18.863 ± 1.369	7.3	4.8	20.420 ± 0.893	4.4	13.4

$C_{experimental}$, experimental concentration; $C_{nominal}$, nominal concentration.

established chromatographic and detection conditions. However, some drugs such as furosemide, phenobarbital, fosphenytoin and piroxicam eluted around the retention time of LTG and/or IS and, therefore, they can interfere in the quantification of LTG. The retention times observed for the tested drugs that potentially may be prescribed with the LTG are indicated in Table 1.

3.1.2. Calibration curves and LOQ

The calibration curves obtained for human plasma and saliva were linear within the concentration range previously defined and showed a consistent correlation between the analyte-IS peak area ratios and the corresponding nominal concentrations. A weighted linear regression analysis was performed due to the wide calibration range and to compensate for heteroscedasticity. The calibration curves were subjected to weighted linear regression analysis using $1/\chi^2$ as the weighting factor. The regression equations of the calibration curves and the corresponding determination coefficients (r^2) achieved for LTG in human plasma were $y = 0.05954 \times -0.00246$ ($r^2 = 0.9945$) and in human saliva were $y = 0.05725 \times -0.00127$ ($r^2 = 0.9936$). The calibration curves were defined within the range of 0.1–20 μg/mL in order to largely cover the therapeutic range of LTG (2.5–15 μg/mL) [14].

The LOQ was experimentally defined as 0.1 μg/mL, in both human plasma and saliva, with acceptable precision and accuracy (Table 2). Of note, the LOQ value obtained in plasma using our method is lower than those achieved by many other HPLC–UV/DAD techniques reported in the literature, even though a smaller volume of plasma is employed herein [24,37,44,47]. In the case of human saliva, the LOQ obtained with the current method is similar to those reported in previous works [8,66,67] (Table 3).

Table 3
Comparison of determinant bioanalytical aspects between the current method and previous methods used for the bioanalysis of lamotrigine in human plasma and saliva samples.

Sample	Bioanalytical method	Sample volume	Sample extraction	LOQ (μg/mL)	Recovery	Reference
Plasma	HPLC-DAD	100 μL	MEPS	0.10	72%	Current method
	HPLC-UV	50 μL	SPE	0.20	98%	[46]
	HPLC-DAD	50 μL	SPE	0.25	99%	[42]
	HPLC-UV	50 μL	LLE	0.50	97%	[44]
	HPLC-UV	1000 μL	LLE	0.10	82%	[57]
	HPLC-UV	500 μL	PP	0.50	100%	[40]
	HPLC-UV	250 μL	LLE	1.0	96%	[25]
	HPLC-UV	500 μL	PP	0.10	97–98%	[66]
	HPLC-UV	200 μL	LLE	0.10	95%	[8]
	HPLC-UV	500 μL	LLE	0.02	88%	[71]
Saliva	HPLC-DAD	100 μL	MEPS	0.10	71%	Current method
	HPLC-UV	100 μL	PP	0.10	106%	[8]
	HPLC-UV	500 μL	PP	0.10	105%	[66]
	HPLC-UV	500 μL	LLE	0.01	ND	[71]

DAD, diode array detection; HPLC, high performance liquid chromatography; LLE, liquid-liquid extraction; MEPS, microextraction by packed sorbent; ND, not determined; PP, protein precipitation; SPE, solid-phase extraction; UV, ultraviolet.

3.1.3. Precision and accuracy

The results for intra and interday precision and accuracy obtained from QC samples of human plasma and saliva at the four different concentration levels (QC_{LOQ}, QC_1, QC_2 and QC_3) are presented in Table 2. In human plasma, the intra and interday RSD values did not exceed 14.5%, and the intra and interday *bias* values varied between −10.0 and 9.3%. Likewise, in human saliva, the intra and interday RSD values did not exceed 12.7%, and the intra and interday *bias* values varied between −4.5 and 13.4%.

3.1.4. Recovery

Overall, the results for LTG absolute recovery from both human plasma and saliva samples, tested at three different concentration levels (QC_1, QC_2 and QC_3), ranged from 64.9% to 73.6% with RSD values equal or lower than 7.0%; the detailed data are available in Table 4. The absolute recovery for the IS in human plasma was 65.8% with a RSD value of 6.8% and in human saliva was 63.9% with a RSD value of 14.7%.

3.1.5. Stability

The results for LTG stability in human plasma and saliva achieved in the different conditions studied are presented in Table 5. According to the results obtained, LTG was stable in unprocessed and processed human plasma and saliva samples in the different handling and storage conditions.

3.1.6. Clinical application

The plasma and saliva samples from the two volunteer patients were analysed to demonstrate the clinical usefulness of the method validated herein. The patient ID1 was receiving LTG 100 mg (p.o.) once-daily, whereas the other patient (ID2) was taking LTG twice-daily: 150 mg (p.o.) in the morning and 200 mg (p.o.) at night co-administrated with

Table 4
Recovery (values in percentage) of lamotrigine (LTG) from human matrices (plasma and saliva) at low (QC_1), medium (QC_2) and high (QC_3) concentrations of the calibration range ($n = 5$).

Matrix	$C_{nominal}$ (μg/mL)		Recovery (%)	
			Mean ± SD ($n = 5$)	RSD (%)
Plasma	QC_1	0.3	72.1 ± 5.0	7.0
	QC_2	10.0	73.6 ± 2.4	3.2
	QC_3	18.0	70.3 ± 4.8	6.9
Saliva	QC_1	0.3	70.9 ± 4.0	5.7
	QC_2	10.0	67.8 ± 4.2	6.2
	QC_3	18.0	64.9 ± 3.3	5.0

$C_{nominal}$, nominal concentration.

Table 5
Stability (values in percentage) of lamotrigine (LTG) at low (QC_1) and high (QC_3) concentrations of the calibration range in unprocessed and processed human plasma and saliva samples ($n = 5$).

Stability (values in percentage) of LTG				
Analyte	Plasma		Saliva	
	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)	106.0	94.4	87.6	114.9
4 °C (24 h)	105.6	111.5	85.2	106.3
Freeze-thaw (3 cycles; −20 °C)	89.9	103.2	99.6	98.8
−20 °C (30 days)	113.3	105.1	112.5	103.2
Processed samples				
Autosampler (12 h)	95.2	102.7	105.6	97.4

$C_{nominal}$, nominal concentration.

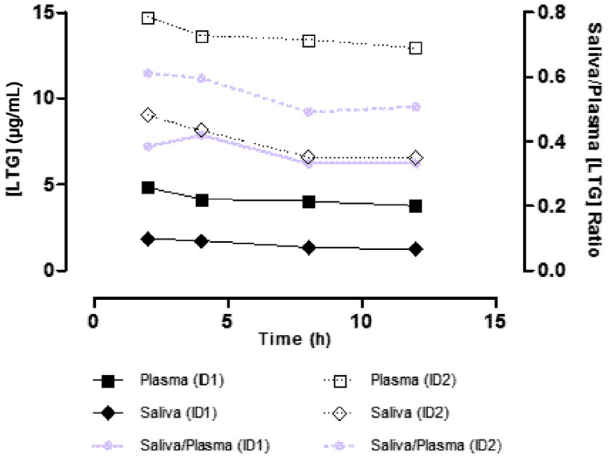

Fig. 4. Concentration-time profiles of lamotrigine (LTG) obtained from plasma and saliva samples collected at 2, 4, 8 and 12 h post-dose (taking as reference the morning dose) in two patients (ID1 and ID2) under oral LTG therapy (ID1, 100 mg once-daily in the morning; ID2, 150 mg in the morning, and 200 mg at night in cotherapy with valproic acid). The corresponding salivary to plasma LTG concentration ratios were also calculated at 2, 4, 8 and 12 h post-dose and graphically represented for both patients.

valproic acid. Given that the use of morning drug levels is a standard practice for TDM of AEDs [72], this aspect was adopted in the sample collection protocol for these two patients.

The peaks obtained from the patients' processed samples revealed symmetry and good resolution, similarly to those obtained in the analysis of spiked human plasma and saliva samples (Fig. 3). The drug concentrations determined in plasma samples were within the therapeutic range (2.5–15 mg/L) defined for LTG [14,19,65] and, as expected, the LTG concentrations measured in saliva (free drug concentration) were lower than those determined in plasma (Fig. 4). Since the total LTG daily dose received by patient ID1 was less than one third of the total daily dose taken by patient ID2 (100 mg versus 350 mg), as it could be anticipated, the LTG concentration levels were substantially lower in the both samples (plasma and saliva) of patient ID1. Nevertheless, by normalizing the LTG concentrations obtained by the total daily dose administered, a similar proportion was found. In addition, the salivary to plasma concentration ratio was found to be higher in patient ID2 (0.55 versus 0.37), which was receiving adjunctive therapy (Fig. 4). These results led us to anticipate the existence of a good relationship between the LTG concentrations in saliva and plasma in both subjects. Some previous studies reported saliva/serum LTG ratios of 0.46 in healthy subjects receiving a single oral dose of LTG, and of 0.64 in patients receiving adjunctive therapy [65,71]. Ryan et al. [67] also studied the relationship between serum and salivary concentrations of LTG in paediatric and adult patients and demonstrated a good saliva/serum correlation with LTG concentration ratios ranging from 0.40 to 1.19; Mallayasamy et al. [8] also reported an identical salivary to serum LTG concentration ratio (0.683). Moreover, observing the LTG concentration-time profiles (2 − 12 h) found in plasma and saliva in both subjects, the high stability of drug concentrations over time and the high parallelism between salivary and plasma levels should be highlighted, which clearly supports the use of saliva as a promising and alternative sample for TDM of patients under LTG treatment.

4. Conclusion

This MEPS/HPLC-DAD method for LTG quantification was successfully validated in both plasma and saliva matrices with high sensitivity, selectivity, precision and accuracy. The small sample volume needed for MEPS processing, the absence of significant chromatographic interferences from the biological matrices, together with the short running time in the LTG analysis and the low LOQ achieved, all enhance the clinical interest of this assay. The use of MEPS as microextraction procedure also has several important advantages, which are usually associated with the miniaturization and automation of bioanalytical procedures.

In spite of the lack of information on a consensual and specific therapeutic range for LTG in saliva, the saliva/plasma correlation achieved in this study indicates a good relationship between salivary and plasma drug concentrations, and it is expected that in the near future the use of saliva samples for TDM of patients under LTG therapy will be a reality in routine clinical practice.

List of the non-standard abbreviations

AED antiepileptic drug
CAM chloramphenicol

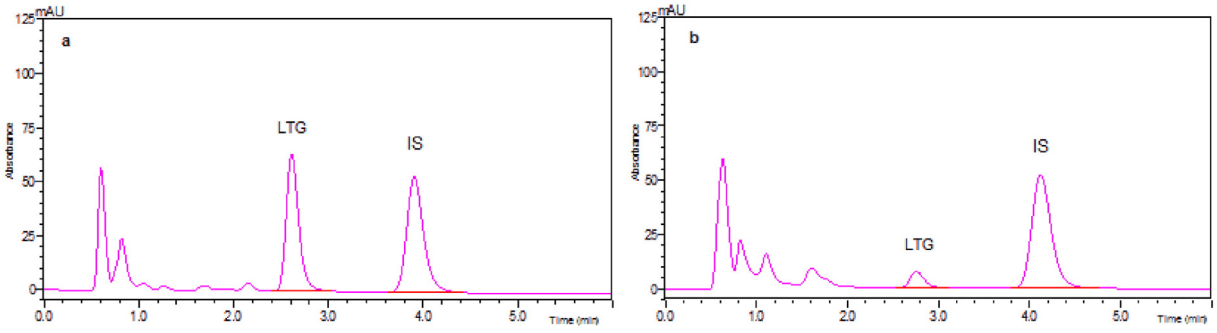

Fig. 3. Representative chromatograms of the analysis of real plasma (a) and saliva (b) samples at 2 h post-dose obtained from the patients treated with lamotrigine (LTG). IS, internal standard.

DAD	diode array detection
IS	internal standard
LTG	lamotrigine
MEPS	microextraction by packed sorbent
PP	protein precipitation
TDM	therapeutic drug monitoring

Conflict of interest statement

The authors have declared no conflict of interest.

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