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Analysis of lamotrigine and its metabolites in human plasma and urine by micellar electrokinetic capillary chromatography

A reliable micellar electrokinetic capillary chromatographic method was developed and validated for the determination of lamotrigine and its metabolites in human plasma and urine. The variation of different parameters, such as pH of the background electrolyte (BGE) and Sodium dodecyl sulfate (SDS) concentration, were evaluated in order to find optimal conditions. Best separation of the analytes was achieved using a BGE composed of 10 mM borate and 50 mM SDS, pH 9.5; melatonin was selected as the internal standard. Isolation of lamotrigine and its metabolites from plasma and urine was accomplished with an original solid-phase extraction procedure using hydrophilic-lypophilic balance cartridges. Good absolute recovery data and satisfactory precision values were obtained. The calibration plots for lamotrigine and its metabolites were linear over the 1–20 μg/mL concentration range. Sensitivity was satisfactory; the limits of detection and quantitation of lamotrigine were 500 ng/mL and 1 μg/mL, respectively. The application of the method to real plasma samples from epileptic patients under therapy with lamotrigine gave good results in terms of accuracy and selectivity, and in agreement with those obtained with an high-performance liquid chromatography (HPLC) method.*

Keywords: Biological samples / Lamotrigine / Metabolites / Micellar electrokinetic chromatography / Solid-phase extraction
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1 Introduction

Lamotrigine (6-(2,3-dichlorophenyl)-1,2,4-triazine-3,5-diamine, LTG, Fig. 1), is a recent antiepileptic agent which belongs to the phenyltriazine class and is chemically unrelated to other anticonvulsants. Lamotrigine has shown to be effective against partial and secondarily generalized tonic–clonic seizures either on adjunctive treatment in patients with refractory epilepsy or when received as monotherapy [1]. The mechanism of action of LTG is, nevertheless, comparable to that of phenytoin and carbamazepine, which act by blocking the voltage-dependent sodium channels and thus prolonging their inactivated state and stabilizing the presynaptic membrane. Consequently LTG acts in particular to prevent the release of excitatory neurotransmitters [2]. In psychiatry, lamotrigine is characterized as a drug with a psychotropic and activating profile [3] and is used for the treatment of depression; it is also used as a mood stabilizer.

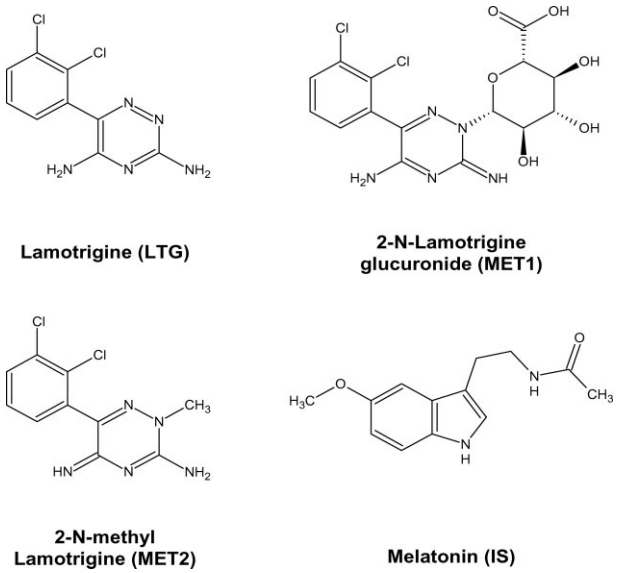

Figure 1. Chemical structures of lamotrigine and its metabolites.

Following oral absorption, maximum lamotrigine plasma concentrations are rapidly achieved with a bioavailability of approximately 98%, and its absorption is linearly dose-

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Abbreviations: AED, antiepileptic drug; **IS**, internal standard; **LTG**, lamotrigine; **MET 1**, N2-glucuronide lamotrigine metabolite; **MET 2**, N2-methylated lamotrigine metabolite

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related and unaffected by food [1]. Lamotrigine undergoes extensive metabolism, primarily by conjugation, and its main metabolite is lamotrigine N2-glucuronide (MET1). The N2-methylated (MET2) metabolite has been found to have cardio-active properties [4]. The elimination half-life of LTG depends on other antiepileptic drugs (AEDs) coadministered, indeed the LTG plasma concentrations are substantially reduced by hepatic enzyme-inducing AEDs (phenytoin, carbamazepine, phenobarbital, and primidone), while comedication with sodium valproate increases its half-life [5]. Due to these peculiar characteristics of lamotrigine, there is a clinical need to individualize patient therapy by means of plasma level determination [5]. Firstly, lamotrigine plasma concentrations increase linearly with increasing dosage. Secondly, there is a large inter-patient variability in the plasma concentration at which an optimum response is achieved. Thirdly, different lamotrigine dosages are required, depending on interacting AED comedication. However, the most common daily doses (25–400 mg/day) usually correspond to maximum LTG plasma levels of 4–16 μM [6]. Clinical experience, however, suggests that this range is too low [7], while a target range of 4–60 μM is more appropriate, which corresponds to a 1.02–15.37 μg/mL concentration range.

Several chromatographic methods for the determination of LTG concentration in human serum or plasma have been reported [8–24]. On the contrary, only a few methods based on capillary electrophoresis have been described in the literature for the determination of LTG [25–28]. The method described by Shihabi and Oles [25] was the first CZE approach used for the determination of LTG in human plasma, which was then employed for therapeutic drug monitoring by Thormann *et al.* [26, 27]. This assay is based upon deproteinization of plasma samples with acetonitrile and injection of the acidified supernatant. The running buffer was composed of 130–mM sodium acetate adjusted to pH 4.5 with acetic acid.

To our best knowledge, only one paper describes the determination of lamotrigine in patients' plasma by means of MEKC [28]. The determination of lamotrigine is accomplished simultaneously with carbamazepine, carbamazepine-10,11-epoxide, primidone, phenytoin, phenobarbital, and 2-phenyl-2-ethyl-malonamide. MEKC analysis was performed using a solution consisting of 10 mM monobasic sodium phosphate, with 6 mM sodium tetraborate, and 75 mM SDS at pH 9.0 as the running buffer. The isolation of lamotrigine from plasma samples was carried out by means of a simple liquid-liquid extraction procedure using ethyl acetate. After the extraction the organic layer was evaporated and reconstituted with 40 μL of 50% methanol in water for injection into the MEKC system. However, no CE method has been described so far in the literature for simultaneous determination of LTG and its metabolites in biological fluids. In this study, we have developed a rapid micellar electrokinetic capillary chromatography method with diode-array detector. An original and efficient solid-phase extraction (SPE) procedure was also implemented for the pretreatment of the biological samples, which provided a high extraction yield and precision. The reliable, simultaneous determination of LTG and its N2-glucuronide and N2-methylated metabolites in human plasma and urine for therapeutic drug monitoring purposes has been thus obtained.

2 Materials and methods

2.1 Chemicals and solutions

Lamotrigine (6-(2,3-dichlorophenyl)-1,2,4-triazine-3,5-diamine) and its metabolites (N2-glucuronide and N2-metilated metabolites) were kindly provided by Glaxo-SmithKline (Stevenage, UK). Sodium dodecyl sulfate and melatonin (internal standard, IS), all analytical grade, were purchased from Sigma Chemical (St. Louis, MO, USA). Sodium hydroxide and boric acid were pure for analysis from Carlo Erba (Milan, Italy). Methanol and acetonitrile were of HPLC-grade from Fluka (Buchs, Switzerland). Ultrapure water (18.2 MΩ cm), obtained by means of a Millipore (Bedford, MA, USA) Milli-Q apparatus, was used for the preparation of all solutions. The stock solutions (1 mg/mL) of lamotrigine and its metabolites were made by dissolving a suitable quantity of each standard compound in 10 mL methanol. The stock solutions of the IS, melatonin (1 mg/mL), were made by dissolving 10 mg melatonin in 10 mL methanol. Standard working solutions were prepared by diluting suitable amounts of each stock solution with water. The standard working solutions were prepared every day, while the stock solutions of the analytes in methanol were stable for at least three months when stored at −20°C.

2.2 Apparatus and capillary handling

All MEKC experiments were carried out using a ^{3D}CE apparatus (Agilent Technologies, Palo Alto, CA, USA). An uncoated, fused-silica capillary (50 μm ID, 375 μm OD, 48.5 cm total length, 40.0 cm effective length) from Composite Metal Services (Hallow, UK) was used as separation column. The MEKC analysis of lamotrigine was performed using a borate buffer (10 mM, pH 9.5), containing 50 mM SDS. The BGE was prepared as follows: a suitable amount of boric acid was dissolved in water, to this solution the suitable amount of SDS was added; the mixture was then brought to pH 9.5 with 2 M sodium hydroxide.

The BGE was filtered through a cellulose acetate syringe filter (0.20 μm, Albet-Jacs-020-25) prior to use. Injection was carried out by pressure at 50 mbar for 10 s. The applied voltage was set at 30 kV and the capillary temperature was thermostated at 25°C. The detection was carried out using a diode array detector operating at 214 nm. Before use, the new capillary was conditioned with 2 M sodium hydroxide, water, and then with BGE for 10 min each. After each run the capillary was rinsed with water (2 min), 2 M sodium hydroxide (2 min) and then again water for 2 min. For storage overnight, the capillary was washed with water.

2.3 Sample pretreatment

Blood samples were taken from patients of the Ravenna Hospital (Ravenna, Italy) under therapy with lamotrigine, and the blood put into vials containing EDTA as the anticoagulant. The blood was immediately centrifuged for 20 min at 3000 rpm and the supernatant plasma was frozen and maintained at −20°C until analysis. The same procedure was used to obtain plasma from the blood of healthy volunteers ("blank" plasma).

For the SPE procedure, Oasis HLB (30 mg, 1 mL) cartridges (Waters, Milford, MA, USA) were used. Conditioning and equilibration were carried out by passing 1 mL methanol through the cartridge followed twice by 1 mL water. A volume of 250 μL blank or patient plasma or urine (spiked with 30 μL of IS standard solution) was diluted with 500 μL water, and the mixture was then loaded onto the already equilibrated cartridge. After washing twice with 1 mL water, the cartridge was dried under vacuum (−40 kPa) for 30 s and the analytes eluted with 1 mL methanol. The eluate was then dried by means of a rotary evaporator, the residue redissolved in 250 μL of water, and filtered through a syringe filter of 0.2 μm pore size (Whatman, Clifton, NJ, USA) prior to injection into the HPCE instrument.

2.4 HPLC procedure

The patient plasma samples analyzed by means of the HPLC method were purified from the biological matrix with protein precipitation with zinc sulfate, according to the following procedure. In particular, 300 μL plasma was treated with a methanolic solution of 0.4 mM $ZnSO_4$ and ethylen glycol in a 1:2 volume ratio. The mixture was vortexed for 3 min and then centrifuged at 5000 rpm for 5 min. Then the supernatant was injected into the HPLC instrument. The chromatographic analysis was carried out isocratically at a flow rate of 1 mL/min on a reversed-phase C18 column (250 × 4.0 mm ID, 5 μm) using a mixture of 0.01 M phosphate buffer, pH 7.5, and acetonitrile (65/35 v/v) as the mobile phase, and ultraviolet detection at 220 nm.

2.5 Method validation

Absolute recovery: Different samples of spiked blank plasma and urine were purified from the biological matrix as previously described and analyzed in the HPCE system. The mean absolute recovery values of the analytes were calculated by interpolating the ratios between the area of the analytes and that of the IS on the calibration curve obtained from the analysis of standard solutions. Linearity: The calibration curves for LTG and its two metabolites (MET1 and MET2) were obtained in the 1–20 μg/mL concentration range by spiking 250 μL blank plasma/urine with suitable amounts (*e.g.*, 30 μL) of standard solution of the analytes and IS, followed by SPE and analytical MEKC procedures as described above. The calibration graphs were obtained by means of the least square method, by plotting the ratio values between the area of analytes and that of the IS (melatonin) against the analytes concentrations added to blank plasma/urine. Precision: The blank plasma/urine was spiked with LTG and its metabolites at three different concentrations (and IS at constant concentration) to give final LTG and metabolite concentrations of 1-10-20 μg/mL (and 10 μg/mL of IS). After thorough mixing, sample pretreatment and MEKC analysis were carried out. The procedure was repeated at least six times within the same day to obtain the repeatability and at least six times over different days to obtain the intermediate precision. Accuracy: Appropriate amounts of the analytes (namely 1, 5, 10 μg/mL) were added to plasma samples containing known amounts of LTG and metabolites (*i.e.*, previously analyzed samples). The spiked samples were submitted to the sample pretreatment procedure described above and analyzed by MEKC. Recovery values of the analytes added to human plasma were calculated by interpolating the peak area ratios on the calibration curves. The procedure was repeated six times on the same day to obtain repeatability values. The precision and recovery assays were carried out according to the United States Pharmacopoeia (USP) XXVI [29] guidelines.

3 Results and discussion

Preliminary spectrophotometric assays demonstrated that all the analytes (LTG, MET1, and MET2) show an intense absorbance band in the 210–220 nm wavelength range. For this reason, a λ of 214 nm was chosen for the MEKC analysis.

3.1 Characteristics and optimization of the BGE

At first experiments were carried out using an MEKC system with phosphate buffer (10 mM, pH 7.5) as BGE containing 20 mM SDS. Under these conditions, the peak of lamotrigine was not fully resolved from those of its metabolites and from that of the EOF, and moreover a broad and asymmetric peak shape was detected. For this reason, the effect of the pH was studied and several measurements were carried out using buffer in the 6.5–9.5 pH range. An improvement in the peak shape and symmetry was achieved using a 10 mM borate buffer, pH 9.5, however, the separation was still not suitable for the accurate determination of all the analytes. For a further improvement of the separation the concentration of SDS was changed in the 20–60 mM range. The experimental assays showed that the migration time and the resolution increased when increasing the concentration of SDS in the BGE. It was observed that a good separation with satisfactory resolution for all analytes was obtained using 50 mM SDS concentration.

The result of the adjustment of the BGE composition to the analytical demands can be seen from Fig. 2: baseline separation of all analytes is obtainable within 4 min in an uncoated fused-silica capillary of 48.5 cm total length (effective length, 40 cm) and 50 μm ID, using a BGE containing 50 mM SDS in a 10 mM, pH 9.5 borate buffer. A constant voltage of 30 kV was applied and the injection was made from the anodic side of the capillary by 50 mbar pressure for 10 s. This system was taken further for the determination of the analytes in standard solutions and in biological fluids.

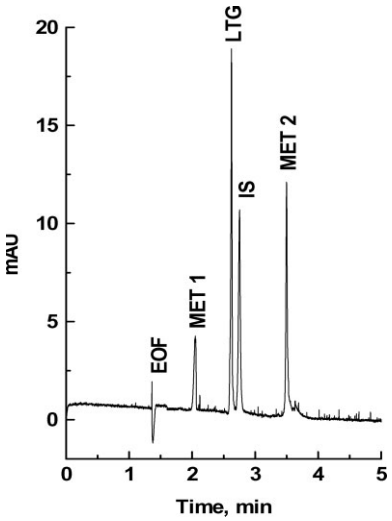

Figure 2. MEKC of a standard solution containing 10 μg/mL lamotrigine, its metabolites, and IS. Conditions: BGE borate buffer (10 mM, pH 9.5) with 50 mM SDS; capillary length, 48.5/40.0 cm, 50 μm ID; voltage, 30 kV; temperature, 25.0°C; detector wavelength, 214 nm.

3.2 MEKC analysis of standard solutions

The optimized methodology was applied to the analysis of aqueous solutions of lamotrigine and its metabolites. Standard working solutions in the 1–20 μg/mL concentration range were prepared as described in Section 2. Standard calibration curves were established by plotting the ratio between the peak area of analytes and that of the IS against the analyte concentration. Good linearity was obtained in the 1–20 μg/mL concentration range for all the analytes. The regression equation of LTG, obtained by means of the least square method, was $y = -0.003 + 0.125\,x$, where y is the ratio between the peak area of LTG and that of the IS, and x is the concentration of LTG, expressed as μg/mL. The linear correlation coefficient was 0.9999. The regression equation of MET1 was $y = -0.004 + 0.044\,x$, while the linear correlation coefficient was 0.9998. The regression equation of MET2 was $y = 0.006 + 0.091\,x$, while the linear correlation coefficient was 0.9999. The limit of detection (LOD) was 500 ng/mL, while the limit of quantitation (LOQ) was 1 μg/mL for all the analytes. Detection and quantitation limits were calculated according to the official requirements [30].

Precision data expressed by the mean relative standard deviation (RSD%) values, ranged from 0.8 to 1.9 for repeatability and from 1.1 to 2.1 for intermediate precision.

3.3 Pretreatment of biological samples

3.3.1 SPE procedure for plasma samples

Sample pretreatment for quantitative analysis of lamotrigine and its metabolites in human plasma and serum was carried out using a simple SPE procedure. The novel SPE procedure was based on the use of Oasis HLB cartridges (see Section 2); only 250 μL blank plasma was loaded on the cartridges and after suitable washing the analytes were eluted with methanol, the eluate was brought to dryness and the residue redissolved with 250 μL of water. The electropherograms of a blank plasma sample and the same sample spiked with the analytes (10 μg/mL LTG, MET1, and MET2) are shown in Fig. 3 (detection wavelength, 214 nm). Comparison of the two electropherograms shows that no interference peak appears at the retention times of the analytes and thus a reliable identification of lamotrigine and its two metabolites can be obtained.

Calibration curves from spiked blank plasma samples were set up for the particular analytes in the 1–20 μg/mL concentration range. The parameters of the linear regression (obtained from six different concentrations) equations for all analytes are given in Table 1. It can be

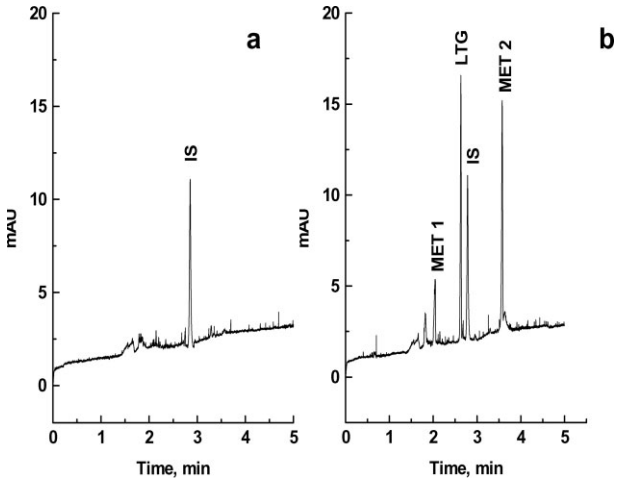

Figure 3. MEKC of (a) a blank plasma sample spiked with 10 μg/mL IS after the extraction procedure and (b) the blank plasma sample spiked with 10 μg/mL LTG, MET1, MET2, and 10 μg/mL IS. Conditions as in Fig. 2.

Table 1. Assay characteristics

Compound	Range	Parameters[a)]		
		a	*b*	r_c
LTG in plasma	1–20 μg/mL	−0.005	0.135	0.9999
LTG in urine		−0.014	0.106	0.9995
MET1 in plasma		−0.011	0.040	0.9989
MET1 in urine		−0.016	0.036	0.9990
MET2 in plasma		−0.005	0.115	0.9990
MET2 in urine		0.006	0.085	0.9999

a) Calibration curve: $y = a + bx$, where x is the analyte concentration, expressed as μg · mL^{-1}, and y is the peak area, expressed as arbitrary units; r_c is the linear correlation coefficient.

seen that good linearity is achieved and sensitivity as well. The limits of detection (LODs) and quantification (LOQs) were 0.5 and 1 μg/mL, respectively, for all analytes.

The extraction yield or absolute recovery was evaluated on blank plasma samples spiked with three different concentrations of the analytes. The yield was calculated from the measured analyte concentrations in these samples, compared with those obtained from pure aqueous reference solutions (after application of the same analytical procedure). The results are reported in Table 2. The mean extraction yield was 92.8% for LTG, 90.9% for MET1, and 91.3% for the metabolite MET2. Good repeatability and intermediate precision resulted as well (Table 2): the mean RSD% of peak areas was less than 2.3% for LTG and 2.7% for the metabolites.

Table 2. Absolute recovery data of spiked blank plasma samples

Compound	Amount added (μg/mL)	Extraction yield (%)[a)]	Repeatability (RSD%)	Interday precision (RSD%)
LTG	1	89.7	1.7	2.3
	10	93.5	1.3	1.7
	20	95.2	0.9	1.3
MET1	1	86.5	1.8	2.7
	10	92.6	1.5	2.1
	20	93.8	1.1	1.6
MET2	1	90.7	1.5	2.3
	10	91.2	1.3	2.1
	20	92.0	1.0	1.7

a) Each value is the mean of six independent assays. The extraction yield was calculated from peak areas from spiked samples compared to peak areas of the same analyte concentration in standard solutions.

3.3.2 Determination of the analytes in spiked urine samples

Both blank and spiked blank urine samples were analyzed and subjected to the same extraction procedure used for the purification of plasma samples. The results demonstrated that this novel extraction procedure gave satisfactory extraction yield and eliminated matrix interference, thus was suitable also for urine samples. Figure 4a shows the electropherogram of a blank urine spiked with the IS (10 μg/mL) and subjected to the SPE procedure, while Fig. 4b shows the electropherogram of the same blank urine sample spiked with 10 μg/mL of LTG, MET1, and MET2. As can be seen, the analytes are detected as symmetrical electrophoretic peaks at a retention time of 2.1 min for MET1, 2.7 min for LTG,

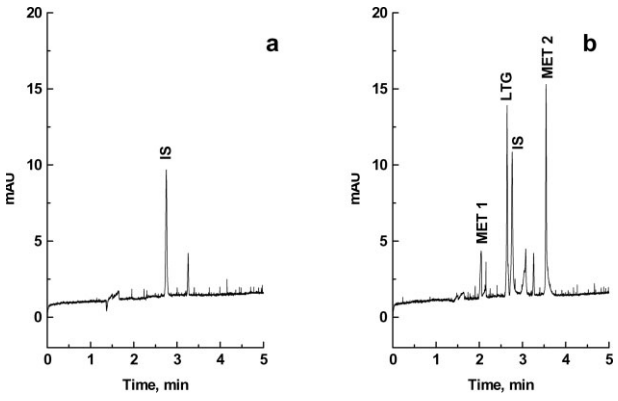

Figure 4. MEKC of (a) a blank urine sample spiked with 10 μg/mL IS after the extraction procedure and (b) the blank urine sample spiked with 10 μg/mL LTG, MET1, MET2, and 10 μg/mL IS. Conditions as in Fig. 2.

2.9 min for IS, and 3.7 min for MET2, and no significant interference is present at the retention times corresponding to the analyte peaks. Calibration curves were obtained for spiked blank urine samples in the 1–20 μg/mL concentration range for all analytes. The parameters of the regression equation are reported in Table 1. The results are very similar and comparable to those obtained analyzing plasma samples. The LOQ was 1 μg/mL and the LOD value was 0.5 μg/mL for all analytes.

Intraday and interday precision assays were carried out to evaluate the precision of the method (extraction and chromatography). The extraction yield obtained at different concentrations of the analytes, and the precision data are shown in Table 3. The results show that the mean extraction yield values ranged from 91.9 to 94.2%. RSDs were lower than 1.9 for repeatability (RSD% intraday) and lower than 2.8 for the intermediate precision (RSD% interday). The obtained results show that the proposed method is suitable for the urine lamotrigine analysis, in terms of sensitivity and precision.

Table 3. Absolute recovery data of on spiked blank urine samples

Compound	Amount added (μg/mL)	Extraction yield (%)[a)]	Repeatability (RSD%)	Interday precision (RSD%)
	1	89.6	1.3	2.4
LTG	10	93.8	1.0	1.8
	20	99.2	1.1	1.3
	1	90.1	1.9	2.7
MET1	10	92.2	1.7	2.3
	20	93.4	1.4	2.1
	1	93.1	1.6	2.8
MET2	10	90.8	1.3	2.3
	20	92.7	1.1	2.0

a) $n = 6$

3.4 Application to patient plasma

Having thus validated the method, it was successfully applied to the analysis of some plasma samples from epileptic patients undergoing therapy with lamotrigine. Polypharmacy with other antiepileptic drugs during treatment of patients could be the source of potential interference for the determination of lamotrigine. The method selectivity was evaluated analyzing several antiepileptic agents. The tested drugs were namely: carbamazepine, carbamazepine epoxide, primidone, gabapentin, ethosuximide, valproic acid, levetiracetam, and clobazam. The method showed good selectivity; in fact, none of the tested drugs was detected, thus no interference from them can occur. The electropherogram of a plasma sample from a patient under therapy with Lamictal® is shown in Fig. 5. The concentration of LTG, obtained by interpolation on the corresponding calibration curve, was 3.4 μg/mL, that of MET1 and MET2 were 5.9 and 3.5 μg/mL, respectively. The accuracy of the method was evaluated by means of recovery studies. Known amounts of the standard analyte solutions were added to known amounts of plasma samples from patients treated with Lamictal®, and the resulting mixture analyzed. The recovery of the added analytes was then calculated. The mean accuracy data were satisfactory, being 91.8% for LTG and 89.3% for the metabolites.

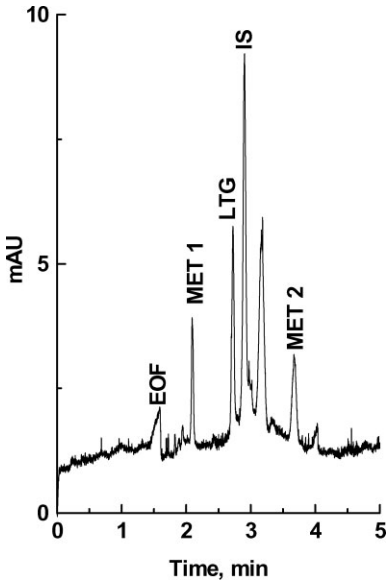

Figure 5. MEKC of plasma sample from a patient undergoing therapy with lamotrigine after the SPE procedure. The peaks correspond to the following concentrations: 10 μg/mL IS, 3.4 μg/mL LTG, 5.9 μg/mL MET1, and 3.5 μg/mL MET2. Conditions as in Fig. 2.

3.5 Comparison with an HPLC method

The results obtained using the MEKC method described herein were compared to those obtained by means of an HPLC method with UV detection. Preliminary assays were carried out using chromatographic conditions already reported in the literature for the HPLC analysis of lamotrigine and other AEDs [19]. Under these conditions the retention time of lamotrigine was of 8.3 min. In order to decrease the analysis run time, suitable variations in the mobile phase were introduced, in particular, only acetonitrile was used as organic modifier (instead of acetonitrile and methanol), which allowed the elution of lamotrigine in 4.8 min. Moreover, the plasma samples were purified from the biological matrix, by means of protein precipitation

Table 4. Comparison of the two methods for the analysis of lamotrigine in the plasma of epileptic patients treated with Lamictal®

Sample	LTG plasma level	
	MEKC	HPLC
1	2.21 μg/mL	2.16 μg/mL
2	3.40 μg/mL	3.44 μg/mL
3	2.06 μg/mL	2.01 μg/mL

with methanolic zinc sulfate solution, which guaranteed high extraction yield values (mean extraction yield of 101%). The results are reported in Table 4. As can be seen, the plasma levels of lamotrigine obtained by means of the two methods are in good agreement. However, it should be noted that the HPLC method allowed only for the determination of lamotrigine, while the two metabolites were not detected.

Considering the obtained data, it is possible to affirm that both proposed methods are suitable for the accurate determination of lamotrigine in human plasma. The electrokinetic chromatographic method in particular is advantageous allowing the simultaneous determination of LTG and its two metabolites. Moreover, due to the new SPE procedure, which is highly feasible and gives good absolute recovery and optimal selectivity, the MEKC method can be used for plasma and urine as well. Another advantage is the short analysis time; the MEKC determination of LTG is carried out in less than 3 min, whereas the HPLC analysis of LTG alone requires more than 4 min. Furthermore, when compared to the HPLC method, the proposed MEKC method is less expensive and non-polluting.

4 Concluding remarks

A micellar electrokinetic capillary chromatographic method for the simultaneous analysis of lamotrigine and its two main metabolites has been developed, using 10 mM borate buffer, pH 9.5 and 50 mM SDS as BGE. The method is suitable in terms of accuracy and precision for the determination of lamotrigine and its metabolites in human plasma. The sample pretreatment of human plasma carried out by means of an SPE procedure was very simple and rapid assuring high absolute recoveries and good selectivity. Furthermore, the extraction procedure proposed for the plasma samples is also suitable for the pretreatment of urine samples. The MEKC method and the extraction procedure were also validated for the urine samples, obtaining the same satisfactory results, in terms of extraction yields, sensitivity, and LOD observed for the plasma samples. The method has been successfully applied to the determination of lamotrigine in human plasma samples of epileptic patients undergoing a monotherapy with Lamictal®.

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