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Original Paper

Rapid HPLC analysis of the antiepileptic lamotrigine and its metabolites in human plasma

A liquid chromatographic method with diode array detection (DAD) has been developed for the analysis of the antiepileptic agent lamotrigine (LTG) and its metabolites, lamotrigine 2-*N*-glucuronide and 2-*N*-methylated in plasma samples. The analytes were separated on a C8 RP column, using a mobile phase composed of methanol and a 0.45 mM, pH 3.5 phosphate buffer containing 0.17% triethylamine (24:76 v/v). Melatonin was used as the internal standard (IS). The DAD detector was set at 220 nm for the detection of all the analytes. A simple protein precipitation with methanol guaranteed high extraction yield values (>90%) and good purification from matrix interference. Good linearity was obtained in the 0.1 – 15.0 μg/mL range for LTG and lamotrigine 2-*N*-glucuronide and in the 0.1 – 2.0 μg/mL range for lamotrigine 2-*N*-methylated. The analytical method was validated in terms of precision, extraction yield, and accuracy. These assays gave RSD% values for precision always lower than 4.3% and mean accuracy higher than 80%. The method seems to be suitable for the analysis of plasma samples from patients treated with Lamictal.

Keywords: Biological fluids / DAD detection / Lamotrigine / Liquid chromatography / Metabolites

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1 Introduction

Lamotrigine (LTG, 6-(2,3-dichlorophenyl)-1,2,4-triazine-3,5-diamine, Fig. 1a) is an antiepileptic agent which belongs to the phenyltriazine class and is effective against partial and secondary generalized tonic – clonic seizures as well as idiopathic (primary) generalized epilepsy [1, 2]. LTG blocks the voltage-dependent sodium channels and thus prevents the release of excitatory neurotransmitters [3]. Being a drug with a psychotropic and activating profile [4], LTG is used for the treatment of bipolar depression, and also as a mood stabilizer.

The LTG daily dose usually ranges from 25 to 400 mg (700 mg without sodium valproate) and the drug is rapidly absorbed with an elimination half-life of about 30 h. Steady-state plasma levels range from 4 to 60 μM LTG (which corresponds to a 1.02 – 15.37 μg/mL range) with pronounced interindividual variability. Peak plasma concentrations occur anywhere from 1.4 to 4.8 h [1]. LTG undergoes extensive metabolism, primarily by glucuronidation, and its main inactive metabolite is lamotrigine 2-*N*-glucuronide [5]. The minor 2-*N*-methylated lamotrigine metabolite seems to cause dose-dependent cardiovascular effects that are not anticipated in humans because only trace amounts of this metabolite (<0.6% of LTG dose) have been found in human urine [6]. However, it is conceivable that plasma concentrations of this metabolite could be increased in patients with a reduced capacity to metabolize LTG (*i.e.*, in patients with liver disease). The most commonly observed adverse effects in monotherapy are dizziness, ataxia, somnolence, headache, double vision, blurred vision, nausea, vomiting, and rash. Clinical data suggest a higher incidence of rash, including serious rash, in patients receiving concomitant valproate than in patients not receiving it [7].

Carbamazepine, phenytoin, phenobarbital, and primidone have been shown to increase the apparent clearance of LTG, which on the contrary decreases with valproate; in fact, the valproate doubles the elimination half-life of LTG [8]. Recent papers suggest that LTG plasma levels can be decreased by oral contraceptives [9, 10] as well as pregnancy [11, 12] with a consequent increased risk of seizures.

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Abbreviations: DAD, diode array detection; **IS,** internal standard; **LTG,** lamotrigine; **MET1,** 2-*N*-lamotrigine glucuronide; **MET2,** 2-*N*-methyl lamotrigine

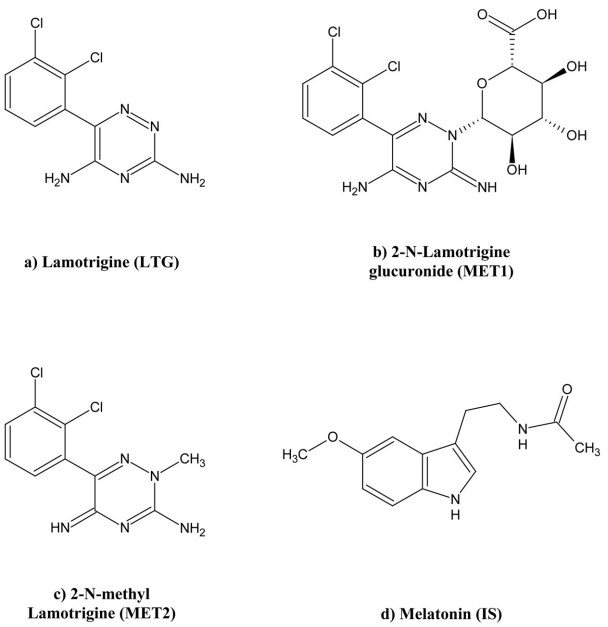

Figure 1. Chemical structures of (a) LTG, (b) MET1, (c) MET2, and (d) melatonin (IS).

For these reasons, the determination of the plasma concentrations of the drug and its metabolites seems to be necessary in patients at risk such as the elderly or patients with renal insufficiency or hepatic disease and in the presence of some other comedications. Some papers can be found in the literature regarding the determination of LTG as a single analyte or together with other drugs in biological fluids by means of HPLC ([13 – 17]; M. A. Saracino *et al.*, *Ther. Drug Monit.*, submitted for publication) or CE methods [2]. Only three papers describe the determination of LTG and its metabolites in biological fluids by means of an MEKC [18], an automated sequential trace enrichment of dialysates HPLC [19], and of an HPLC-MS [15]. The pretreatment of biological samples is usually carried out by means of deproteinization by solvents [2, 13, 15, 16], liquid – liquid extraction [14], and SPE [17, 18]. The aim of this study was the development of an easy and reliable HPLC method with diode array detection (DAD) for the simultaneous analysis of LTG and its 2-*N*-glucuronide and 2-*N*-methylated metabolites in human plasma from patients using a rapid sample pretreatment procedure.

2 Experimental

2.1 Chemicals

LTG (Fig. 1a) and its metabolites (2-*N*-lamotrigine glucuronide (MET1) and 2-*N*-methyl lamotrigine (MET2), Figs. 1b and c) were kindly provided by GlaxoSmithKline (Stevenage, UK). Melatonin (internal standard (IS), Fig. 1d) was purchased from Sigma Chemical (St. Louis, MO, USA). Methanol was of HPLC grade from Fluka (Buchs, Switzerland); potassium dihydrogen phosphate, 85% w/w orthophosphoric acid, and triethylamine were pure for analysis from Carlo Erba (Milan, Italy). 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 the solutions.

2.2 Solutions

The stock solutions (1 mg/mL) of LTG, its metabolites, and the IS were made by dissolving a suitable amount of each standard compound in 10 mL of methanol. Standard working solutions were prepared by diluting suitable amounts of each stock solution with the mobile phase. The standard working solutions were prepared every day, while the stock solutions of the analytes in methanol were stable for at least 3 months when stored at −20°C.

2.3 Human plasma sampling

The blood samples were collected from patients of the "Santa Maria delle Croci" Hospital (Ravenna, Italy) and of the Psychiatric Division of Parma University (Parma, Italy) who were subjected to therapy with LTG, at daily doses between 25 and 400 mg. Blood samples were drawn 12 h after the last drug administration and were stored in glass tubes containing EDTA. Then, all the samples were centrifuged at 1400 × *g* for 15 min at 5°C; the supernatants (plasma) were transferred to polypropylene tubes and stored frozen at −20°C until the analysis.

Blood samples from healthy volunteers (whose plasma was used as blank plasma) were treated in the same way.

2.4 Chromatographic apparatus and conditions

Chromatographic analysis was carried out on an Agilent (Waldbronn, Germany) 1100 Series chromatographic system equipped with a DAD. The detector wavelength was set at 220 nm for quantitative assays.

The chromatographic separation was achieved on a Microsorb Rainin C8 RP column from Varian (150 × 4.6 mm id, 5 μm) with a C8 cartridge precolumn (4 × 3 mm id, 5 μm). The mobile phase was composed of a mixture of methanol and a 0.45 mM, pH 3.5 phosphate buffer containing 0.17% triethylamine (24:76 v/v). The mobile phase was filtered through a Phenomenex (Torrance, CA, USA) membrane filter (47 mm membrane, 0.2 μm, NY) and degassed by an ultrasonic apparatus. The flow rate was 1.3 mL/min. The samples were injected into the HPLC system by means of a 20 μL loop.

A Crison (Barcelona, Spain) MicropH 2000 pH meter and a Hettich (Tuttlingen, Germany) Universal 32 R centrifuge were used.

2.5 Sample pretreatment

The plasma sample pretreatment was carried out by means of a rapid purification procedure which consisted in deproteinization by methanol. An aliquot of 150 μL of blank or patient plasma sample, previously treated with 50 μL of IS working solution (and analytes' standard solution for blank plasma samples) was added to 250 μL of methanol in a polypropylene tube. The sample was vigorously mixed for 1 min and then centrifuged for 10 min at 5°C. An aliquot of 300 μL of the resulting supernatant was brought to dryness under vacuum (rotary evaporator); the residue was redissolved in 100 μL of mobile phase and injected into the HPLC system.

2.6 Method validation

2.6.1 Extraction yield (absolute recovery) and precision

Standard solutions at three different concentrations of LTG, MET1, and MET2 were added to 150 μL of blank plasma (in order to obtain final analyte concentrations of 0.1, 7.5, and 15.0 μg/mL for LTG and MET1 and 0.1, 1.0, and 2.0 μg/mL for MET2), subjected to the deproteinization procedure and injected into the HPLC.

The analyte/IS peak area ratios thus obtained were compared to the ratios obtained by injecting standard solutions at the same theoretical concentrations, and the percentage recovery was calculated. The assays described above were repeated six times within the same day to obtain the repeatability (intraday precision) and six times over six different days to obtain the intermediate precision (interday precision) of the method, expressed as RSD% values.

2.6.2 Calibration curves, LOQ, LOD

Analyte standard solutions (50 μL) at ten different concentrations, containing the IS at a constant concentration were added to 150 μL of blank plasma. The resulting mixtures were subjected to the sample pretreatment procedure and injected into the HPLC. The procedure was carried out in triplicate for each concentration and the calibration curves were constructed by means of the least-square method. The LOQ and the LOD were calculated as the analyte concentrations which gave rise to chromatographic peaks whose height was equal to ten and three times the baseline noise, respectively, according to official guidelines [20, 21].

2.6.3 Accuracy

Analyte standard solutions at three different concentrations (*i.e.*, analyte additions of 1.0, 3.0, and 5.0 μg/mL for LTG and MET1 and 0.1, 0.3, and 0.5 μg/mL for MET2) were added to 150 μL of plasma from patients subjected to treatment with Lamictal®, and the mixture was subjected to the sample pretreatment procedure as described above. Recovery values were calculated according to the following formula: $100 \times$ ([after spiking] − [before spiking])/[added].

3 Results and discussion

3.1 Choice of chromatographic conditions

Starting from our previous paper on the plasma analysis of LTG together with other antiepileptic drugs by HPLC with UV detection [22], different mobile and stationary phases were investigated in order to obtain the simultaneous determination of LTG, MET1, and MET2 in human plasma. Separation and analysis are not easy due to the very different lipophilicity of the analytes. Using a C18 column (150 × 4.0 mm id, 4.5 μm), a mobile phase composed of methanol, ACN, and a pH 3.0, 15 mM phosphate buffer containing 0.63% triethylamine (19.2:16.8:64.0 by volume) was used, flowing at 1 mL/min (Fig. 2a). Under these conditions, MET1 was rather close to the injection peak; the consequent increase in the percentage of aqueous phase, to obtain a higher retention of the analyte, caused a prolongation of the run times, above all for MET2. Furthermore, the wide range of polarity covered by the analytes required the use of a gradient elution on the C18 RP column with the disadvantages of distortion in the gradient shape, low accuracy and precision, and baseline shift.

In order to overcome these problems, a mixture of a pH 3.5 phosphate buffer containing 0.17% triethylamine and methanol (76:24 v/v), at the flow rate of 1.3 mL/min, was used on a C8 RP column. In fact, C8 column allowed a good separation of all the analytes in isocratic elution; the percentage of triethylamine was studied in the 0.17–0.63% range and the amount of 0.17% was sufficient to reduce the silanol interactions and to improve the peak shape. These conditions proved to be suitable for the separation of LTG and its metabolites within an acceptable run time (10 min) without using a gradient elution. A detection wavelength of 220 nm was chosen for the quantitative analysis of LTG and its metabolites. Several compounds were tested as possible ISs; the most suitable was found to be melatonin. In fact, melatonin has a relatively short retention time and chemical–physical properties similar to the analytes.

The chromatogram of a standard solution containing 3.5 μg/mL LTG and MET1, 0.5 μg/mL MET2, and 1.0 μg/mL melatonin (IS) is reported in Fig. 2b. As can be seen, the peaks are neat and well resolved and the chromatographic run lasts for 10 min. Retention times (t_R) are: MET1, t_R = 2.4 min; LTG, t_R = 6.1 min; IS, t_R = 7.4 min; MET2, t_R = 9.2 min.

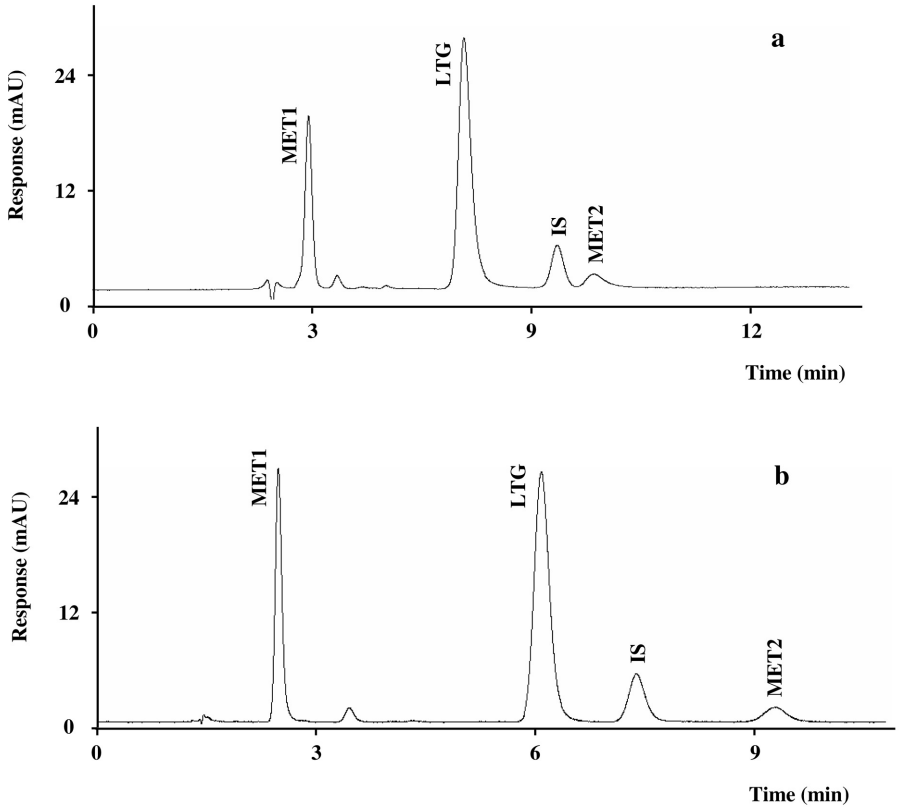

Figure 2. Chromatograms of a standard solution containing (a) 3.5 μg/mL LTG and MET1, 0.5 μg/mL MET2, and 1.0 μg/mL IS, obtained with a C18 column and (b) 3.5 μg/mL LTG and MET1, 0.5 μg/mL MET2, and 1.0 μg/mL IS, obtained with a C8 column.

3.2 Sample pretreatment

Different kinds of sorbents were tested for the pretreatment procedure: diol, octyl, octadecyl, ethyl, and hydrophilic–lipophilic balance (HLB). HLB cartridges did not allow an appropriate purification of the biological samples; hydrophilic (diol) and lipophilic (ethyl, octyl, octadecyl) cartridges, gave very low recoveries of the analytes. Thus, a protein precipitation procedure was developed. Several agents such as perchloric acid, ACN, and methanol were investigated. The addition of a suitable amount of methanol proved to be very efficient for the purification of the biological matrix. Moreover, the effects of occlusion of the analytes on the denatured protein were minimized by slowly adding the plasma to the methanol, rather than the opposite way. This simple strategy led to an improved extraction yield of all the analytes (from 70 to 90%). After drying the supernatant, the residue was redissolved in the mobile phase. The best plasma/mobile phase analyte concentration ratio was found to be 1:1. Taking into account that MET2 levels were often at plasma concentrations lower than the LOQ, it was necessary to try a 2:1 plasma/mobile phase analyte concentration ratio, which gave satisfactory results and was used when necessary.

The chromatogram of a typical blank plasma sample is reported in Fig. 3a, while the chromatogram of the same blank plasma sample spiked with 3.5 μg/mL LTG, 3.5 μg/mL MET1, 0.5 μg/mL MET2, and 1.0 μg/mL IS is reported in Fig. 3b.

As can be seen, peak shapes and resolution are good and sample clean-up is satisfactory.

3.3 Method validation

Once the sample pretreatment procedure was developed, blank plasma from healthy volunteers was used for method validation in terms of extraction yield, linearity, and precision.

Extraction yield assays at three different concentrations of the analytes gave good results. The mean absolute recovery was higher than 90% for all the analytes. Good linearity (correlation coefficient r_c >0.9992) was obtained on spiked blank plasma samples in the following concentration ranges: 0.1 and 15.0 μg/mL for LTG and MET1 and 0.1 and 2.0 μg/mL for MET2. The LOQ was 0.1 μg/mL for all the analytes, while the LOD was 0.05 μg/mL for all the analytes. Precision values were very satisfactory: RSD% values for repeatability were lower than or equal to 4.0; RSD% values for intermediate precision were lower than or equal to 4.3. Mean extraction yield for the IS was 90%, with an RSD% value lower than or equal to 3.0. The details of extraction yield and precision assays are reported in Table 1.

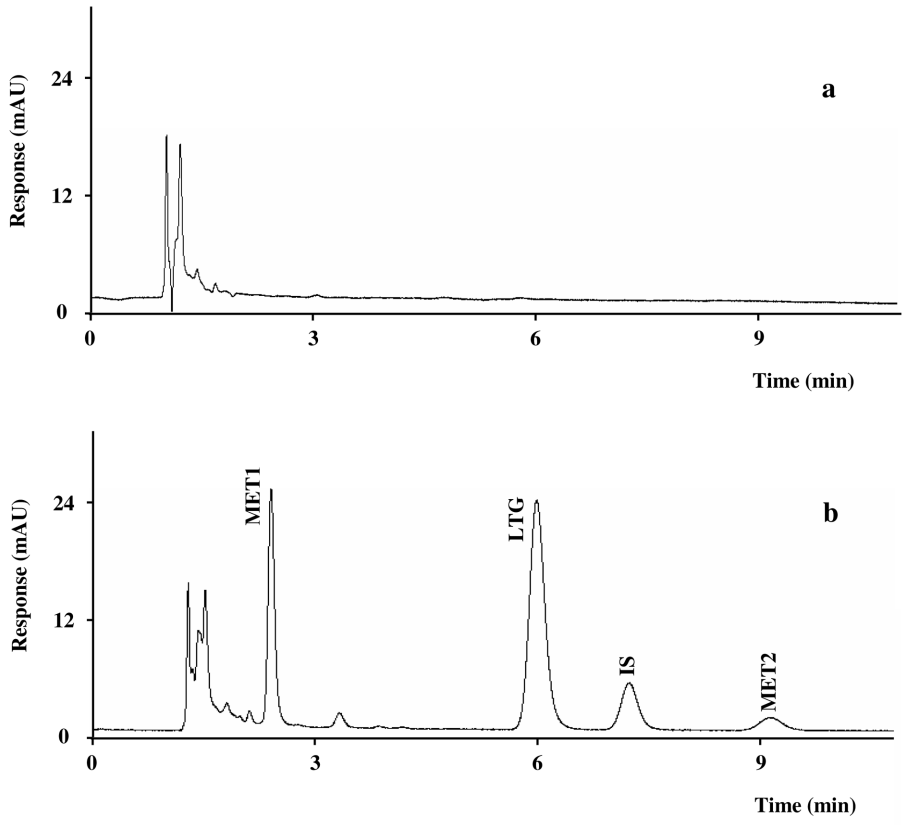

Figure 3. Chromatograms of (a) a blank plasma sample and of (b) the same blank plasma sample spiked with 3.5 μg/mL LTG, 3.5 μg/mL MET1, 0.5 μg/mL MET2, and 1.0 μg/mL IS.

Table 1. Validation parameters

Compound	Amount added (μg/mL)	Extraction yield (%)[a)]	Repeatability (RSD%)	Interday precision (RSD%)
LTG	0.1	97	4.01	4.33
	7.5	96.5	3.11	3.35
	15	98	2.44	2.81
MET1	0.1	95.2	3.59	3.7
	7.5	97.3	2.49	3.54
	15	94	2.3	2.8
MET2	0.1	90	3.57	4.27
	1	94	2.56	3.8
	2	95	2.35	2.15
IS	1	90	2.8	3

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

3.4 Selectivity assays

Polypharmacy with other antiepileptic drugs during the treatment of patients could be the source of potential interference in the determination of LTG and its metabolites. The method selectivity was evaluated analyzing several antiepileptic agents, namely: carbamazepine, oxcarbazepine, phenobarbital, primidone, phenytoin, ethosuximide, gabapentin, valproic acid, and levetiracetam (Table 2). The method showed a good selectivity; in fact, no interference was found.

Standard solutions of other compounds belonging to different therapeutic classes were injected: antidepressants, sedative–hypnotics, neuroleptics, and atypical antipsychotics. Some of these drugs had retention times different to those of the analytes or the IS (Table 2), except for diazepam which interfered with LTG peak and for delorazepam which interfered with MET1 peak; thus, the method has proven to be almost selective.

3.5 Application to patient plasma

Having thus validated the method, it was applied successfully to the analysis of some plasma samples from patients undergoing therapy with LTG, obtaining good results; none of these samples posed any problem for the quantitation of the analytes, and no interference from the matrix was found in any of them.

The chromatogram obtained by injecting a plasma sample from a patient under therapy with Lamictal (100 mg/day) for at least 4 wk is shown in Fig. 4a, while Fig. 4b shows a chromatogram of a plasma sample from a

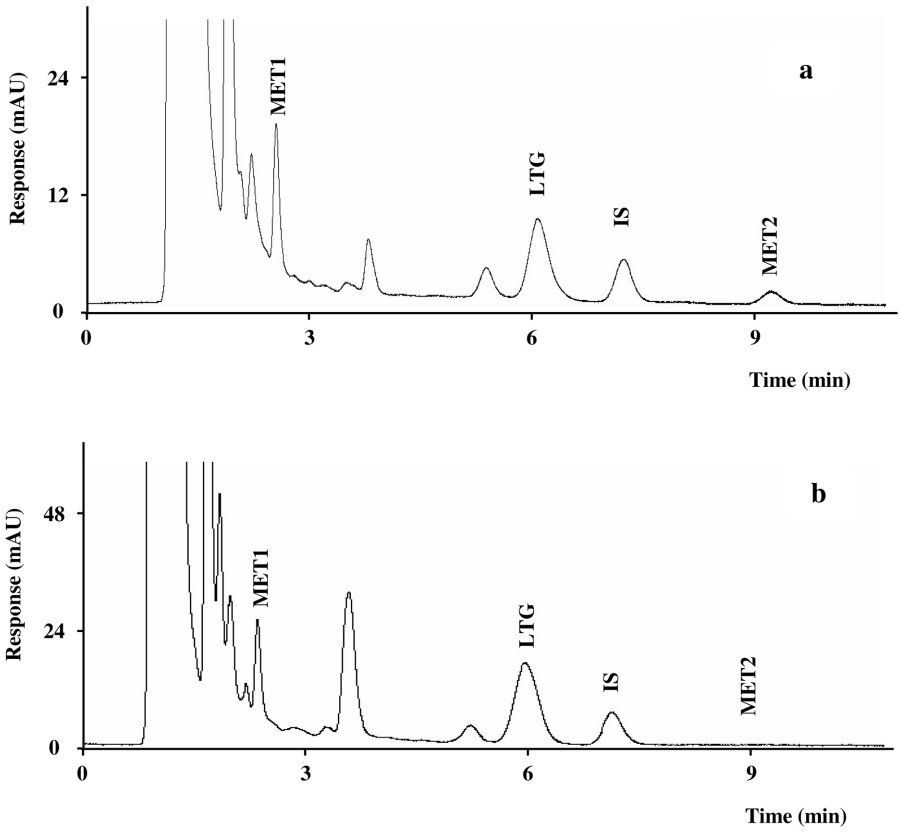

Figure 4. Chromatograms of a plasma sample from a patient taking (a) 100 mg/day of LTG and (b) 250 mg/day of LTG.

Table 2. Compounds tested for interference

Compound	Retention time (min)	Compound	Retention time (min)
Antipsychotics		*Antidepressants*	
Olanzapine	4.9	Paroxetine	n.d.
Clotiapine	n.d.	Fluoxetine	n.d.
Clozapine	n.d.	Venlafaxine	n.d.
Risperidone	n.d.	Fluvoxamine	n.d.
Chlorpromazine	n.d.	Duloxetine	n.d.
Levomepromazine	n.d.	*Sedatives – hypnotics*	
Haloperidol	n.d.	Delorazepam	2.3
Quetiapine	n.d.	Lorazepam	n.d.
Antiepileptics		Flurazepam	n.d.
Primidone	4.8	Clonazepam	n.d.
Carbamazepine	34	Diazepam	6.3
Oxcarbazepine	19.8	*Others*	
Levetiracetam	n.d.	Omeprazole	n.d.
Valproic acid	n.d.	Levosulpiride	3.3
Phenytoin	24.2	*Analytes*	
Ethosuximide	n.d.	LTG	6.1
Phenobarbital	15.8	MET1	2.4
Gabapentin	n.d.	MET2	9.2

n.d., Not detected within 35 min.

patient under early therapy with Lamictal (250 mg/day). As one can see, no interference from the matrix is apparent, analyte separation is good, and peaks are neat and symmetric. LTG, MET1, and MET2 levels in these samples were found to be: 1.9, 2.5, and 0.144 μg/mL, respectively, for patient 1 and 2.7 μg/mL, 3.1 μg/mL, and n.d. (nondetected) value, respectively, for patient 2. In fact, MET2 plasma level in patient 2 is lower than LOQ probably because the patient is at the beginning of the therapy with LTG. Accuracy was assessed on patient plasma samples by means of recovery assays. The results of these assays were satisfactory: mean accuracy was about 80% for three analytes.

Furthermore, the comparison of this method with our previous method based on an SPE procedure using phenyl cartridges (M. A. Saracino *et al.*, *Ther. Drug Monit.*, submitted for publication) was performed only for LTG determination in nine patient plasma samples. In fact, the SPE procedure did not allow the analysis of LTG metabolites. The results obtained with the SPE procedure were plotted as a function of the results obtained with the proposed procedure (protein precipitation) and a straight line was interpolated by the least-square method. The equation of the line had a slope of 1.02 and a correlation coefficient of $r_c = 0.96$ (Fig. 5); thus, the two methods produced comparable results.

4 Concluding remarks

An analytical method based on the use of a simple HPLC-DAD apparatus has been developed for the simultaneous analysis of LTG and its two main metabolites in the

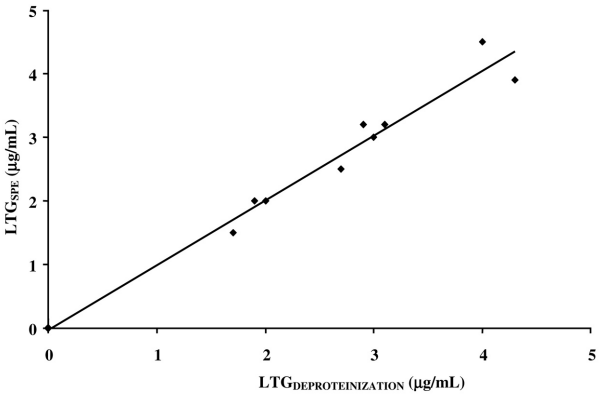

Figure 5. Comparison of results for LTG levels obtained from nine patient samples using SPE (*Y*-axis) and deproteinization (*X*-axis) procedures.

plasma of patients. The proposed HPLC method is simpler and more precise than the HPLC method in the literature which used gradient elution [13]; moreover, the present method gives a more rapid chromatographic analysis (10 min instead of 17 min) than gradient elution chromatography. It is a feasible procedure based on deproteinization with methanol and grants appropriate sample purification and good extraction yields with satisfactory precision. Compared to previously published pretreatment procedures based on liquid – liquid extraction [14], the pretreatment procedure presented here has demonstrated to be superior in terms of rapidity, feasibility, precision (4.3% instead of 8.1%), and extraction yield (90% instead of 80%). Moreover, the HPLC apparatus is less expensive and more readily available in clinical laboratories than other apparatus such as the HPLC-MS [15], reported in literature for the analysis of LTG together with its metabolites. Thus the method seems to be suitable for the therapeutic drug monitoring of LTG and its metabolites in the plasma of patients undergoing therapy with Lamictal and could also be useful for pharmacokinetic studies.

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