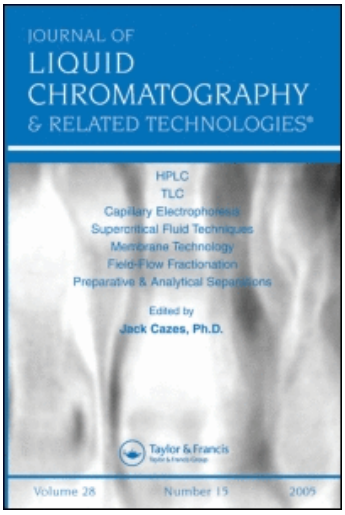

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Solid-Phase Extraction Study and RP-HPLC Analysis of Lamotrigine in Human Biological Fluids and in Antiepileptic Tablet Formulations

I. N. Papadoyannis[a]; A. C. Zotou[a]; V. F. Samanidou[a]
[a] Laboratory of Analytical Chemistry Chemistry Department, Aristotle University of Thessaloniki, Thessaloniki, Greece

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SOLID-PHASE EXTRACTION STUDY AND RP-HPLC ANALYSIS OF LAMOTRIGINE IN HUMAN BIOLOGICAL FLUIDS AND IN ANTIEPILEPTIC TABLET FORMULATIONS

I. N. PAPADOYANNIS*, A. C. ZOTOU, AND V. F. SAMANIDOU
Laboratory of Analytical Chemistry
Chemistry Department
Aristotle University of Thessaloniki
GR - 54006 Thessaloniki, Greece

ABSTRACT

An efficient off-line solid-phase extraction (SPE) of lamotrigine, 3,5-diamino-6-(2,3-dichlorophenyl)-1,2,4-triazine (LTG), a new antiepileptic drug, from human serum and urine, prior to high-performance liquid chromatographic analysis, was tested and optimized. High extraction recoveries were achieved from C_8 Bond Elut cartridges (200mg/3ml), using acidic acetonitrile for the elution of LTG and the internal standard, 3,5-diamino-6-(2-methoxyphenyl)-1,2,4-triazine. Isocratic reversed-phase high-performance liquid chromatographic (RP-HPLC) analysis on octylsilica, using a Lichrosorb RP-8, 5μm, 250x4.6 mm i.d. column and a mobile phase consisting of 0.05M acetate buffer pH5.6 and acetonitrile (72:28 v/v) proved to be sensitive and rapid. The identification of LTG was performed by UV detection at 306nm. The method detects approximately 0.9 ng of LTG on-column, using a 20-μl loop, and linearity holds from approximately 0.044 to 7.8 μg/ml in standard solutions. In plasma and urine, the limits of detection are 1.1 and 1.2ng respectively, while linearity holds from approximately 0.087 to 3.49μg/ml. The proposed method was also used for the direct analysis of antiepileptic tablets.

* To whom correspondence and reprint requests should be addressed.

INTRODUCTION

Lamotrigine, 3,5-diamino-6-(2,3-dichlorophenyl)-1,2,4-triazine (LTG), is a new anticonvulsant, which belongs to the triazine class of compounds and has a cationic character and a pK_a value of 5.5 [1], thus differing from the conventional antiepileptic drugs. It acts by blocking the sensitive sodium channels and inhibiting the release of the excitatory transmitter glutamate [2].

The pharmacokinetics of LTG after single and multiple oral doses, from 30-240 mg, in normal humans show good and rapid absorption. The mean t_{max} is less than 3 hours and after a single oral dose of LTG (120mg), at least 70% on average is excreted in the urine and of that total 90% is the glucuronide metabolite. A linear relationship between dose administered and maximal plasma concentration of LTG has been observed, which indicates that saturation of absorption or elimination mechanisms do not occur in the therapeutic dose range. LTG does not induce its own metabolism and inhibition or saturation of drug-metabolizing enzymes do not occur. The predicted therapeutic plasma concentration of LTG in humans is 1-3 μg/ml [3].

LTG is metabolized by glucuronidation. Its glucuronide is not detected in plasma but appears in urine at a rate equal to the elimination of LTG from plasma. Doig and Clare [4] have elucidated the structure of urinary metabolites of LTG by means of thermospray liquid chromatography-mass spectrometry and found that man produces two N-glucuronides of LTG, of which only the major one (the N-2-glucuronide) is cleaved by the action of β-glucuronidase. They concluded that the most likely structure of the minor metabolite is the N-5-glucuronide.

In literature appears a limited number of publications dedicated to the analysis of LTG, since it is a relatively new antiepileptic drug and they almost exclusively describe HPLC methods. Only two publications describe different methods, namely a radioimmunoassay [5] and an immunofluorimetric assay for the determination of human plasma concentrations of LTG [6].

Normal-phase HPLC methods for the determination of LTG in human plasma and/or urine have been described in clinical reports [1-3,7]. More recent reports describe RP-HPLC methods for the quantitation of LTG in human plasma [8-10] or ion-pairing RP-HPLC methods in guinea pig blood and urine [11]. RP-HPLC is more appropriate for the direct quantitation of the highly polar glucuronide metabolite of LTG, when therapeutic monitoring of the LTG clearance is necessary.

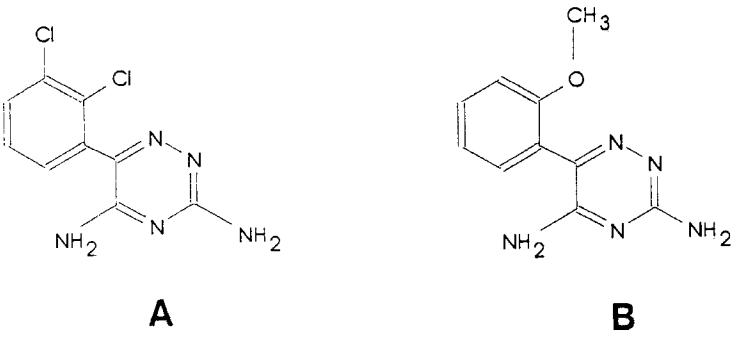

FIGURE 1. Molecular structures of (A) LTG and (B) the internal standard.

Most of the cited methods make use of liquid-liquid extraction for the isolation of LTG from endogenous compounds in the samples prior to HPLC analysis, except for one [11] that uses SPE. The objective of the present work was to find the optimum SPE conditions for achieving the highest possible recovery of LTG from biological matrices, therefore a comparative study of SPE conditions, appearing in the LTG literature for the first time, was performed. The developed RP-HPLC method is rapid, offers higher sensitivity compared to the existing methods and can be applied with high precision and accuracy to the direct analysis of antiepileptic drugs and to the analysis of extracted plasma and urine samples.

EXPERIMENTAL

Materials and Reagents

LTG, 3,5-diamino-6-(2,3-dichlorophenyl)-1,2,4-triazine, the internal standard, 5-diamino-6-(2-methoxyphenyl)-1,2,4-triazine (BW725C78), (Fig.1) and the LTG pharmaceutical preparations (Lamictal tablets) were obtained from Wellcome Foundation (London, UK). Stock solutions of LTG (109μg/ml), of the internal standard (55μg/ml) and of the tablet homogenate were prepared by dissolving the appropriate amounts in HPLC-grade methanol and their respective dilute solutions were prepared in mobile phase. All solutions were kept refrigerated until use.

HPLC-grade methanol and acetonitrile were obtained from Riedel-de-Haen AG (Hannover, Germany). The analytical-reagent grade glacial acetic acid 100% and NaOH, which were used for the preparation of the 0.05M acetate buffer pH5.6 were obtained from Merck (Darmstadt, Germany). After appropriate dilution of the reagents with double-deionized water, the buffer was prepared by mixing 56.0ml of 1M acetic acid solution with 50.0ml of 1M NaOH solution and diluting to 1 litre with double-deionized water [12]. The buffer was filtered through a 0.2μm filter before use.

The cartridges used for the SPE study were C_{18}, C_8 (200mg/3ml) and PH (100mg/ml) Bond Elut, obtained from Analytichem International a division of Varian (Harbor City, U.S.A.) and C_8 (200mg/3ml) Alltech, obtained from Alltech Associates (Deerfield, IL, U.S.A.).

Apparatus

A Shimadzu isocratic pump, model LC-9A (Kyoto, Japan), equipped with a Rheodyne 7125 injection valve (California, U.S.A.) and a 20-μl loop, an SSI, model 500, variable wavelength UV-visible detector (State College, PA, U.S.A.) and a Hewlett Packard, model HP 3396 II integrator (Hewlett Packard, Avondale, PA, U.S.A.) were employed for the analysis of LTG. The analytical column was a Lichrosorb RP-8, 5μm, 250x4.6mm i.d. column, obtained from MZ-Analysentechnik (Mainz, Germany).

A glass vacuum-filtration apparatus obtained from Alltech Associates, was employed for the filtration of the buffer solution, using 0.2μm membrane filters obtained from Schleicher and Schuell (Dassel, Germany).

A Glass-col, Terre Haute in 47802 small vortexer and a Hermle centrifuge, model Z 230 (B. Hermle, Gosheim, Germany) were employed for the pretreatment of biological samples.

The SPE study was carried out on a Vac-Elut system, obtained from Analytichem International, with a 10-cartridge capacity, equipped with Bond Elut luer stopcocks.

Chromatographic Conditions

The analysis of LTG was performed under isocratic conditions at ambient temperatures and at a detection wavelength of 306nm with the detector operating at

0.002 AUFS sensitivity range. The mobile phase consisted of acetate buffer pH5.6 - acetonitrile (72:28v/v) and the flow-rate was 1ml/min.

With the above mentioned conditions the retention times of the internal standard and LTG standard solutions diluted in mobile phase were approximately 3.5min and 6.5min respectively.

LTG Calibration Curve

Appropriate dilutions with mobile phase of the stock methanolic solutions of LTG (109μg/ml) and of the internal standard (55μg/ml) were made separately and from these dilute solutions, LTG working standards of 0.0436, 0.0872, 0.436, 0.872, 1.744, 3.488 and 7.848μg/ml were prepared in 25-ml volumetric flasks with mobile phase, all containing the internal standard at the same fixed concentration of 1.1μg/ml.

The analysis of each standard solution was repeated eight times and the calibration curve was constructed by plotting the mean value of the Peak Area ratios of LTG/internal standard at each concentration level versus the concentration of LTG.

Analysis of LTG Tablets

Ten tablets of 25mg (labelled amount) were weighed and their mean weight was found to be 0.0797g. The tablets were powdered in a porcelain mortar and a 0.0347g amount (equivalent to 0.0109g LTG) of the homogenized sample was transferred into a 100-ml volumetric flask and diluted to volume with methanol. The resulting solution was 109μg/ml in LTG according to labelled amount. The solution was sonicated for 5min and a portion of it was centrifuged at 3500g for 15min. A 5-ml volume of the supernatant was diluted to volume with mobile phase in a 25-ml volumetric flask, to give a 21.8μg/ml solution of LTG and a 10-fold dilution of this solution gave a 2.18μg/ml of LTG according to labelled amount.

Volumes of 1- and 5-ml of the 2.18μg/ml solution and 4-ml of the 21.8μg/ml solution of LTG were transferred into 25-ml volumetric flasks where 5ml of a 5.5μg/ml solution of the internal standard had already been added and the mixtures were diluted to volume with mobile phase to give solutions of 0.0872, 0.436 and 3.488μg/ml in LTG respectively and 1.1μg/ml in internal standard.

Five replicate injections (20μl) onto the HPLC column, of each of the above solutions were made and the mean value of the peak area ratios of LTG/internal

standard were employed for the quantitation of the LTG concentration using the regression equation for standard LTG solutions.

SPE and Determination of LTG in Biological Samples (Plasma, Urine)

The plasma and urine samples were free from LTG and collected from healthy volunteers. The plasma sample employed in the study was a pooled sample derived from the mixing of plasma from ten healthy volunteers and the urine sample was a pooled sample from three volunteers.

To five 40-µl aliquots of spiked plasma and five 100-µl aliquots of spiked urine samples, 80-µl and 200-µl volumes of acetonitrile were added respectively (for protein precipitation) in Eppendorf vials. Each plasma and urine aliquot had been spiked with a 200-µl volume of methanolic solutions containing respectively 0.0872, 0.436, 0.872, 1.744 and 3.488µg/ml of LTG and the same fixed concentration of the internal standard (1.1 µg/ml) before the addition of acetonitrile.

The samples were then centrifuged at 3500g for 15min and the supernatants were quantitatively transferred into clean Eppendorf vials. The organic solvents present were subsequently evaporated in a waterbath at 45°C under a stream of nitrogen and the remaining aqueous phase was applied to preconditioned C_8 Bond Elut cartridges (200mg/3ml) for the isolation of LTG from endogenous compounds by means of SPE. The cartridges and SPE conditions finally employed were selected after a complete optimization SPE study.

The C_8 Bond Elut cartridges, which were selected finally, were preconditioned with 1 volume of methanol followed by 1 volume of double-deionized water with use of vacuum and after application of the samples they were washed with 2 volumes of double-deionized water. The application of samples and the wash step was carried out under atmospheric pressure with no use of vacuum. LTG and the internal standard were finally eluted with 1 volume of acidic acetonitrile (0.01M in HCL) under slight vacuum and the eluates, collected in 3-ml cone vials, were evaporated to dryness at 45°C under nitrogen. The residues were reconstituted with 200µl mobile phase and aliquots of 20µl were analyzed by HPLC.

The SPE procedure was repeated 3 times for the plasma and urine samples at all the above-mentioned concentration levels of LTG and the reconstituted residues from each extraction were then analyzed 5 repeated times each. The mean

concentration values obtained from the 5 replicate analyses, at each concentration level, were calculated for each extraction performed. Subsequently, the concentration overall means, i.e. means of the 3 values obtained from each extraction, were calculated and their RSDs and recoveries were estimated in order to establish a comprehensive study of the SPE precision and accuracy, covering the whole range of the calibration curves. For the calculation of the LTG measured concentrations in plasma and urine, the respective pooled calibration curves were employed. The pooled calibration curves were constructed by plotting the overall mean value of the peak area ratios of eluted LTG to eluted internal standard, at each concentration level versus the concentration of LTG.

SPE Optimization Study - Extraction Recovery

Different cartridges and various washing and elution conditions were tested in order to establish the best extraction conditions for LTG. For this purpose, aliquots of the pooled plasma (40µl) and urine samples were spiked with a methanolic solution containing both LTG (0.872µg/ml) and the internal standard (1.1µg/ml). The samples were then processed as mentioned earlier and subsequently subjected to SPE.

The objective of this study was to find those SPE conditions which yield high extraction recoveries and ensure that LTG and the internal standard are eluted to almost the same extent, when both compounds are present during extraction. This latter criterion must be satisfied if the internal standard is to be used as a recovery one, i.e. added together with LTG from the beginning of sample treatment. If the extraction recoveries of analyte and internal standard differ considerably, erroneously high or low peak area ratios are obtained, which would affect the calibration curve's slope and hence the sensitivity of the method.

The absolute extraction recoveries of LTG and of the internal standard were calculated as percentage ratios of peak areas of the eluted compounds from plasma and urine, to the respective peak areas of the compounds in a non-extracted standard solution. The standard solution, containing the same concentrations of LTG and of the internal standard as the extracted samples, was analyzed alternatively with the samples during the same day.

RESULTS AND DISCUSSION

A chromatogram of a LTG standard solution with its internal standard is shown in Fig.2. The selectivity of the proposed method, with respect to the analysis of pharmaceutical preparations and extracted plasma and urine pooled samples, is shown in the chromatograms of Figs. 2-4, where it can clearly be seen that at the retention time of LTG no interferences from endogenous compounds occur.

In standard solutions the ratios of LTG peak area to internal standard peak area were linearly related to concentrations ranging from 0.0436 to 7.848μg/ml (or 0.872 to 156.96ng injected on-column). The limit of detection (LOD) defined as that quantity which produces a signal of a peak height twice the size of background noise, was found to be approximately 0.9ng (on-column) and the same quantity was found from the statistical treatment of the regression equation data [13]. The limit of quantitation (LOQ), defined as that quantity which gives a signal equal to that of the blank $\pm$ 10SD (based on the calibration curve) [13] was found to be approximately 1.7ng (on-column).

In the processed plasma and urine samples, the peak area ratios of eluted LTG to eluted internal standard were linearly related to LTG concentrations ranging from 0.0872 to 3.488μg/ml. The LOD calculation, based on the statistical treatment of the regression equation data [13], revealed values of approximately 1.1 and 1.2ng for LTG determination in plasma and urine samples respectively. The LOQs, calculated as mentioned above, were approximately 2.8 and 3.0ng of LTG in plasma and urine respectively.

The linear regression equations with their confidence limits at 95% confidence level (for n-2 degrees of freedom) and the correlation coefficients for LTG in standard solutions and spiked plasma and urine samples are presented in Table 1.

The intra- and inter-day precision and accuracy of measurements was assessed by analyzing LTG standard solutions of 0.0872, 0.872 and 3.488μg/ml (or 1.744, 17.44 and 69.76ng injected on-column) eight repeated times each during the same day and five times each per day during a period of twelve days, respectively. During this period the standard solutions were kept refrigerated and no change in LTG concentration was observed. The intra-day Relative Standard Deviations (RSDs) and recoveries ranged from 0.98 to 3.0% and from 100.7 to 101.7% respectively, while the

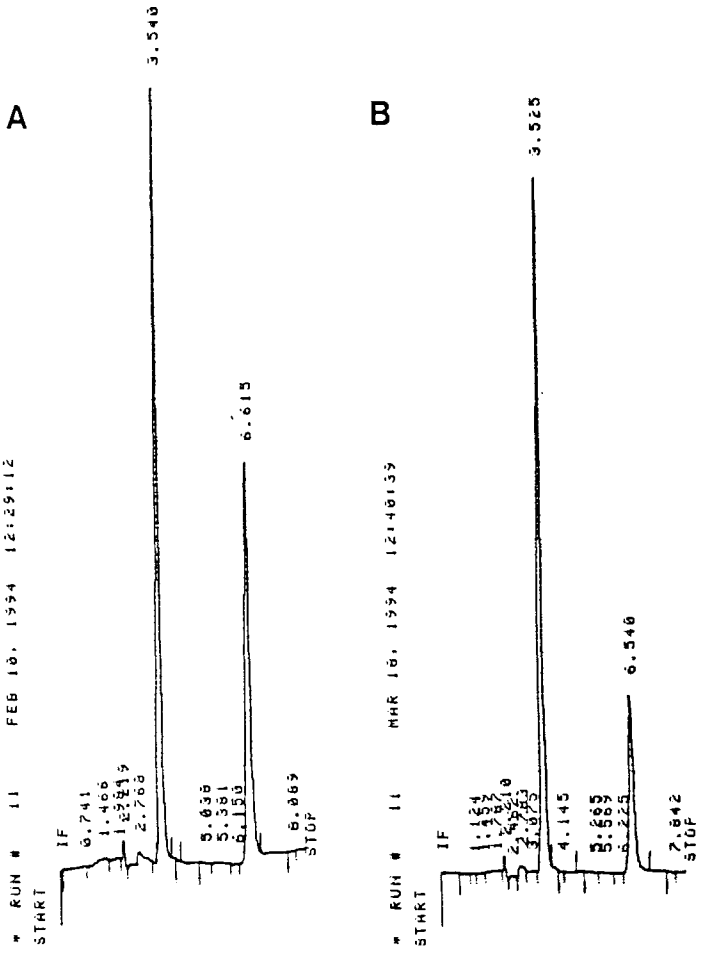

FIGURE 2. Chromatograms of (A) a standard 0.872µg/ml LTG solution and (B) a Lamictal tablet solution containing 0.436µg/ml of LTG (according to labelled amount). Concentration of internal standard 1.1µg/ml. Chromatographic conditions as described in Experimental. Chart speed: 0.5cm/min. Peaks in (A): 3.540min=internal standard, 6.615min=LTG; peaks in (B): 3.525min=internal standard, 6.540min=LTG.

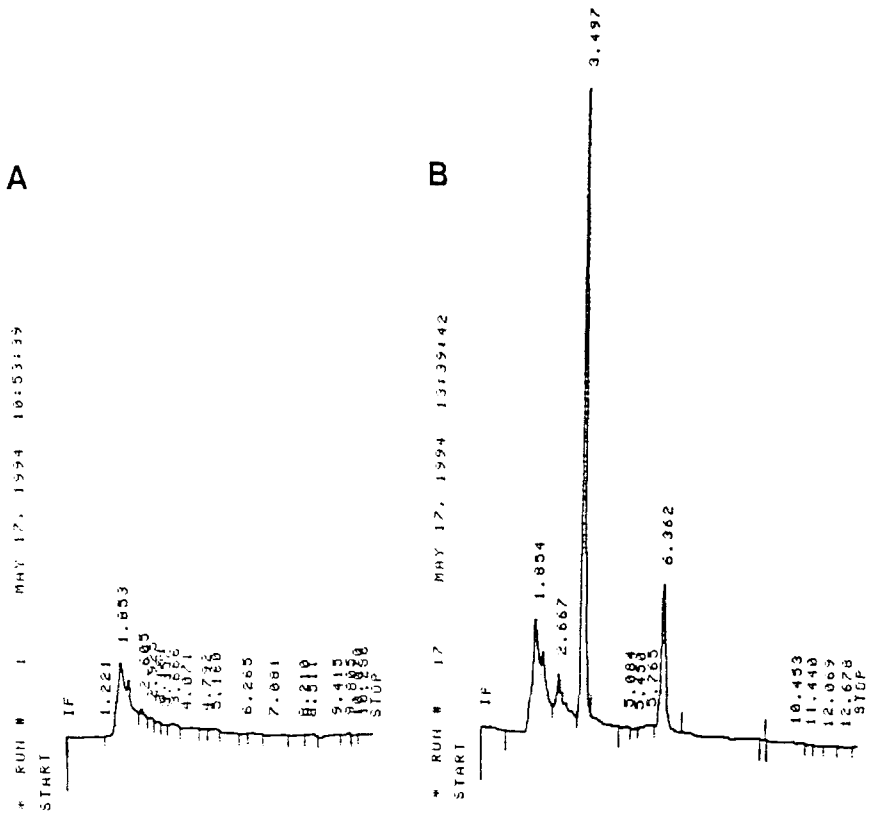

FIGURE 3. Chromatograms of (A) extracted blank plasma sample and (B) extracted plasma sample spiked with 0.436µg/ml of LTG and 1.1µg/ml of the internal standard. Chromatographic conditions as described in Experimental. Chart speed: 0.5cm/min. Peaks in (B): 3.497min=internal standard, 6.362min=LTG.

inter-day RSDs and recoveries ranged from 0.78 to 3.0% and from 94.4 to 102.2% respectively, as can be seen in Table 2.

LTG was determined with highly reproducible and accurate results in Lamictal tablets of 25mg. Since no interference from the tablet extract occured, as can be confirmed by comparing the chromatograms A (of a standard solution) and B (of the tablet extract) in Fig.2, which are identical, there was no need to apply a standard

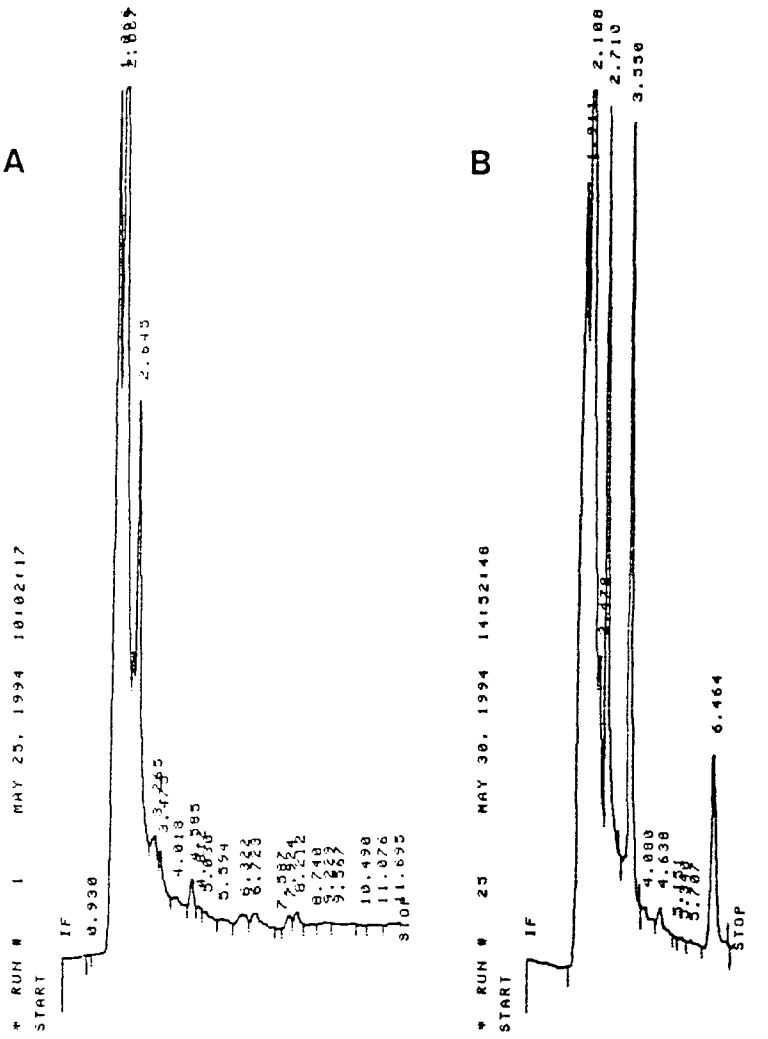

FIGURE 4. Chromatograms of (A) extracted blank urine sample and (B) extracted urine sample spiked with 0.436µg/ml of LTG and 1.1µg/ml of the internal standard. Chromatographic conditions as described in Experimental. Chart speed: 0.5cm/min. Peaks in (B): 3.550min=internal standard, 6.464min=LTG.

TABLE 1

Statistical Evaluation of Regression Equations

LTG Samples	Regression Equation $Y = (a \pm t_a S_a) + (b \pm t_a S_b)X$	Correlation Coefficient
Standard Solutions	$Y^* = (0.02450 \pm 0.01943) + (0.87210 \pm 0.00583)X$	0.99998
Plasma	$Y^{**} = (0.01344 \pm 0.03761) + (0.83361 \pm 0.02092)X$	0.99991
Urine	$Y^{**} = (0.05560 \pm 0.04041) + (0.83509 \pm 0.02247)X$	0.99989

Y=peak area ratio of LTG to internal standard, X=concentration in μg/ml, a=intercept, b=slope, S_a,S_b=standard deviations of intercept and slope respectively, t_a=student's t-test for n-2 degrees of freedom and 95% confidence level.

* Mean value of 8 replicate measurements at each concentration level; based on 7 data points.

**Overall mean: Mean value of 3 extractions; the value obtained for each extraction is the mean of 5 replicate measurements at each concentration level; the pooled calibration curves are based on 5 data points.

TABLE 2

Within-day and Between-day Precision and Accuracy of LTG Determination in Standard Solutions

Injected Quantity (ng)	Within-day			Between-day		
	Mean Measured Quantity[a] ± SD* (ng)	RSD** (%)	Recovery (%)	Mean Measured Quantity[b] ± SD* (ng)	RSD** (%)	Recovery (%)
1.744	1.771±0.054	3.0	101.5 ± 3.1	1.646±0.050	3.0	94.4 ± 2.8
17.44	17.73±0.20	1.1	101.7 ± 1.2	17.83±0.17	0.95	102.2 ± 1.0
69.76	70.24±0.69	0.98	100.7 ± 0.99	70.44±0.55	0.78	101.0 ± 0.79

[a] Mean of values obtained from the linear regression equation for standard solutions for n=8 measurements during the same day.

[b] Mean of values obtained from the linear regression equation for standard solutions for n=5 measurements / day over a period of 12 days.

* Standard Deviation; ** Relative Standard Deviation.

TABLE 3

Precision and Accuracy of LTG Determination in Lamictal Tablets of 25mg

Injected Quantity[a] (ng)	Mean Measured Quantity[b] ± SD* (ng)	Found in Tablets[c] ± SD* (mg)	RSD** (%)	Recovery (%)
1.744	1.707±0.026	24.49±0.37	1.5	98.0 ± 1.5
8.720	8.533±0.184	24.49±0.53	2.2	98.0 ± 2.1
69.76	71.95±0.21	25.81±0.07	0.27	103.2 ± 0.28

[a] According to labelled amount.

[b] Mean of values obtained from the linear regression equation for standard solutions for n=5 measurements.

[c] According to the procedure described under Experimental.

* Standard Deviation; ** Relative Standard Deviation.

TABLE 4

SPE Recoveries of LTG and of the Internal Standard in Plasma using Various Types of Cartridges

Cartridge Type[a]	Absolute Extraction Recovery[b] ± SD* (%)	
	LTG (0.872µg/ml)	Internal Standard (1.1µg/ml)
C_{18} Bond Elut (200mg/3ml)	68.0±2.5	61.3±2.2
C_8 Bond Elut (200mg/3ml)	87.1±3.3	88.4±2.8
C_8 Alltech (200mg/3ml)	67.9±3.3	67.6±2.4
PH Bond Elut (100ml/1ml)	80.1±4.2	61.9±2.4

[a] Preconditioning of cartridges with 1 volume methanol + 1 volume double-deionized water; application of sample; washing of cartridges with 2 volumes double-deionized water; elution of the compounds with 1 volume methanol.

[b] Calculated as described under Experimental; Mean value of three determinations.

* Standard Deviation.

TABLE 5
SPE Recoveries of LTG and of the Internal Standard in Plasma using C_8 Bond Elut Cartridges (200mg/3ml) at Different Conditions

SPE Conditions	Absolute Extraction Recovery[a] ± SD* (%)	
	LTG (0.872μg/ml)	Internal Standard (1.1μg/ml)
(1)	77.5±0.75	84.9±0.79
(2)	78.0±2.3	27.2±1.1
(3)	88.7±0.89	95.6±4.8
(4)	74.0±1.7	8.0±0.78
(5)	89.0±4.6	89.4±1.2
(6)	No-elution	No-elution
(7)	86.8±2.1	95.7±2.1

[a] Calculated as described under Experimental; Mean value of three determinations.
* Standard Deviation
(1) Preconditioning of cartridges with 1 volume methanol + 1 volume double-deionized water; application of sample; washing of cartridges with 2 volumes double-deionized water; elution of the compounds with 1 volume acidic methanol (0.01M in HCl).
(2) Preconditioning with 1 volume methanol + 1 volume acetate buffer pH5.6; application of sample; washing with 1 volume acetate buffer pH5.6 + 1 volume double-deionized water; elution with 1 volume methanol.
(3) As in (2), but elution with 1 volume acidic methanol (0.01M in HCl).
(4) As in (1), but elution with 1 volume acetonitrile.
(5) As in (1), but elution with 1 volume acidic acetonitrile (0.01M in HCl).
(6) As in (2), but elution with 1 volume acetonitrile.
(7) As in (2), but elution with 1 volume acidic acetonitrile (0.01M in HCl).

additions' method here and the calibration curve for standard solutions was employed directly for the quantitation of LTG content in the tablet extract. For obtaining a comprehensive study of the precision and accuracy of LTG determination in Lamictal tablets, three different dilutions of the tablet extract were prepared yielding nominal concentrations of 0.0872, 0.436 and 3.488μg/ml or 1.744, 8.720 and 69.76ng on-column respectively, which cover representative concentrations of the standard calibration curve. LTG was determined with RSDs ranging from 0.27 to 2.2%, while the recoveries ranged from 98.0 to 103.2% of the true value, indicating good agreement with the labelled amount as shown in Table 3.

Tables 4-6 show the extraction recoveries obtained for LTG and the internal standard, when various types of cartridges and different SPE washing and elution conditions are used for spiked plasma and urine samples. The final selection of

TABLE 6

SPE Recoveries of LTG and of the Internal Standard in Urine using Various Types of Cartridges and Different Elution Conditions

Cartridge Type[a]	Absolute Extraction Recovery[b] ± SD* (%)			
	Elution with 1 Volume Methanol		Elution with 1 Volume Acidic Acetonitrile	
	LTG (0.872µg/ml)	Internal Standard (1.1µg/ml)	LTG (0.872µg/ml)	Internal Standard (1.1µg/ml)
C_{18} Bond Elut (200mg/3ml)	71.3±0.29	148.0±3.5	87.4±2.7	71.4±2.3
C_8 Bond Elut (200mg/3ml)	No elution	No elution	96.9±0.28	97.2±1.3
PH Bond Elut (100mg/1ml)	No elution	No elution	109.0±2.5	88.5±2.3

[a] Preconditioning with 1 volume methanol + 1 volume double-deionized water; washing with 2 volumes double-deionized water after application of the sample.

[b] Calculated as described under Experimental; Mean value of three determinations.

* Standard Deviation.

TABLE 7

Precision and Accuracy of SPE in Spiked Plasma and Urine Samples

Injected Quantity (ng)	In Plasma			In Urine		
	Mean Measured Quantity[a] ± SD* (ng)	RSD** (%)	Recovery (%)	Mean Measured Quantity[b] ± SD* (ng)	RSD** (%)	Recovery (%)
1.744	1.785±0.016	0.92	102.4 ± 0.95	1.833±0.006	0.33	105.1 ± 0.35
8.720	8.878±0.039	0.44	101.8 ± 0.44	8.264±0.066	0.80	94.8 ± 0.75
17.44	17.11±0.04	0.23	98.1 ± 0.20	17.46±0.13	0.74	100.1 ± 0.73
34.88	34.41±0.27	0.78	98.6 ± 0.78	35.49±0.48	1.3	101.7 ± 1.4
69.76	70.04±0.81	1.2	100.4 ± 1.2	69.50±1.36	2.0	99.6 ± 1.9

[a,b] Overall mean of values obtained from the pooled regression equations, i.e. mean of values obtained from each of the three extractions performed, which in turn are the means of values obtained from 5 replicate measurements at each concentration level.

* Standard Deviation; **Relative Standard Deviation.

cartridges and SPE conditions was made on the basis of the following criteria: 1) high extraction recovery for both LTG and its internal standard and 2)if possible, same percentage of LTG and the internal standard eluted. As can be seen from Tables 4-6, the use of C_8 Bond Elut (200mg/3ml) cartridges, preconditioned with one volume of methanol plus one volume of double-deionized water and washed with two volumes of double-deionized water after application of the samples, combined with the use of one volume of acidic acetonitrinile (0.01M in HCl) for the elution of the compounds fulfill the criteria mentioned above.

The results of LTG determination in spiked plasma and urine samples are given in Table 7. For concentrations of LTG between 0.0872 and 3.488μg/ml (or 1.744 to 69.76ng on-column respectively) RSDs ranging from 0.23 to 1.2% and recoveries ranging from 98.1 to 102.4% were achieved for the analysis of plasma spiked samples, while for the same concentrations of LTG in urine spiked samples the RSDs ranged from 0.33 to 2.0% and the recoveries from 94.8 to 105.1%.

CONCLUSION

The satisfactory results obtained from the SPE study which were confirmed by the determination of LTG in spiked plasma and urine samples, as well as the simplicity, rapidity, sensitivity and the satisfactory precision and accuracy of the HPLC assay, make the proposed method suitable for the analysis of LTG in pharmaceutical preparations and in extracted human biological fluids.

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