Determination of Lamotrigine Simultaneously with Carbamazepine, Carbamazepine Epoxide, Phenytoin, Phenobarbital, and Primidone in Human Plasma by SPME–GC–TSD

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Abstract

A simple and rapid analytical method is presented for the determination of lamotrigine simultaneously with primidone, carbamazepine, carbamazepine epoxide, phenobarbital, and phenytoin in human plasma using solid-phase microextraction (SPME) and gas chromatography with thermionic specific detection. The best conditions for the SPME procedure is established as following: direct extraction on a 65-µm Carbowax–divinylbenzene fiber; 1.0 mL of a sample plasma matrix modified with 15% NaCl and 3 mL of a potassium phosphate buffer (pH 7.0); extraction temperature at 30°C; and stirring at a rate of 2500 rpm for 15 min. The method shows good linearity between 0.05 and 40.0 µg/mL with regression coefficients ranging between 0.9965 and 0.9995 and a coefficient of variation of the points of the calibration curve lower than 10%. The lowest limit of quantitation for the plasma-investigated drugs varies from 0.05 to 0.20 µg/mL, according to the drug. The proposed method is sensitive enough to work into subtherapeutic and therapeutic concentrations, being that it is applied in pharmacokinetic studies and patient routine therapeutic drug monitoring.

Introduction

Lamotrigine (LTG), a phenyltriazine derivative, is unrelated chemically to other anticonvulsant drugs in current use. The efficacy of LTG as an add-on therapy to existing antiepileptic drug (AED) regimens has been well-established in adult patients and children with refractory partial seizures with or without secondary generalization. When used as monotherapy, LTG is as effective as carbamazepine (CBZ) and phenytoin (PHT) against partial-onset seizures and idiopathic generalized tonic–clonic seizures (1).

The dosage of this drug depends on the type of concomitantly administered drugs. In the presence of hepatic-enzyme-inducing agents such as CBZ, phenobarbital (PB), PHT, and primidone (PRM), the LTG's half-life is reduced and higher doses may be required. In comedication with valproic acid (enzyme inhibitors), a much lower dose of LTG may be required as a result of the drug's decreasing elimination (2).

In general, LTG has been determined by chromatographic methods with a pretreatment of the biological fluids being done by protein precipitation (3,4), liquid–liquid extraction (5–8), and solid-phase extraction (9,10).

Many of these methods, however, are time-consuming, require complex procedures, are difficult to be automated, require the use of a large volume of solvents, and very often require a preconcentration of the extract prior to instrumental analysis.

In order to overcome these problems, solid-phase microextraction (SPME) has been evaluated as a suitable sampling technique. SPME has been demonstrated to be a very effective, highly sensitive solvent-free sampling technique (11). Although this technique was introduced for the extraction of organic compounds from environmental samples (12), since 1995 it has also been applied to various biological matrices such as urine, plasma, and hair (13).

In this study, we established the optimum conditions for the determination of LTG simultaneously with PRM, PB, PHT, CBZ, and CBZ epoxide (CBZ-E). Additionally, the proposed method-

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ology was validated by investigating the limit of quantitation (LOQ), linearity, accuracy, and precision, after which it was applied to the analysis of human plasma samples from patients with epilepsy.

Experimental

Reagents and analytical standards

CBZ and CBZ-E analytical standards were kindly given by Ciba-Geigy (São Paulo, Brazil); LTG by Glaxo Wellcome (São Paulo, Brazil); and PHT, PB, PRM, and nitrazepan (NTZ) were obtained from Sigma-Aldrich (São Paulo, Brazil). The working standard drug solutions were prepared by diluting the stock solutions of these compounds (1 mg/mL in methanol) with water treated through a Milli-Q system (Millipore, São Paulo, Brazil).

Analytical-grade sodium chloride (Merck, Rio de Janeiro, Brazil) was used after purification by heating at 300°C overnight. The methanol used was HPLC grade and obtained from J.T. Baker (Phillipsburg, NJ), and monobasic and dibasic phosphate were purchased from Merck (Darmstadt, Germany).

SPME equipment

The SPME holder and the Carbowax (CW)–divinylbenzene (DVB) fiber with a 65-µm film thickness were both obtained from Supelco (São Paulo, Brazil). The CW–DVB fiber was conditioned by heating it in the injection port of the chromatographic system for 0.5 h at 250°C.

Instrumentation

GC analyses were performed on a Varian Star 3400 Cx gas chromatograph equipped with a thermionic specific detector (TSD). Drugs were separated on a DB 1 column from J&W Scientific (Folson, CA) (30-m × 0.25-mm i.d., 0.1-µm film thickness). The split–splitless injector was operated in the split mode. The injector port and detector temperatures were 250°C and 300°C, respectively. The column temperature was held at 90°C for 4 min, increased to 170°C at 15°C/min, increased to 240°C at 6°C/min, and then increased to 300°C at 15°C/min and held for 1 min. Nitrogen (White Martins, Ribeirão Preto, Brazil) was used as the carrier gas, being that the column head pressure settled at 12 psi. Injections were made in the split mode at a split ratio of 1:20 and a septum purge rate of 2 mL/min.

Table I. LOQ, Linearity of the Method, and Relative Retention Time

AEDs	LOQ (µg/mL)	Relative retention time*	Linearity range (µg/mL)	Linear regression equation (y)	Correlation coefficient
PB	0.20	0.603	0.20–40.0	$y = -0.0530 + 0.0700x$	0.9965
PRM	0.20	0.627	0.20–25.0	$y = 0.0270 + 0.0750x$	0.9965
CBZ	0.06	0.731	0.06–20.0	$y = 0.9928 + 0.1872x$	0.9980
PHT	0.05	0.787	0.05–25.0	$y = 1.5147 + 0.5227x$	0.9982
CBZ-E	0.20	0.798	0.20–12.0	$y = 0.0263 + 0.0331x$	0.9975
LTG	0.20	0.859	0.20–10.0	$y = -0.2676 + 0.6912x$	0.9995

* Relative to NTZ (the internal standard).

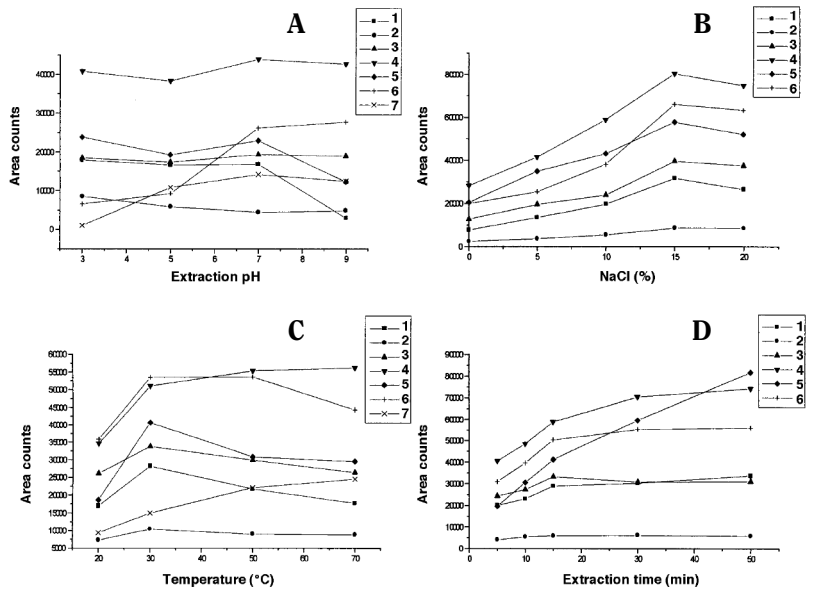

Figure 1. Effect of the experimental variables (A) pH, (B) NaCl concentration, (C) temperature, and (D) extraction time on the SPME efficiency: PB, 1; CBZ-E, 2; PRM, 3; CBZ, 4; PHT, 5; LTG, 6; and NTZ, 7.

Optimization of the SPME conditions

The first step was to evaluate the influence of pH on the extraction. For that purpose, potassium phosphate buffers at four different pH values (3.0, 5.0, 7.0, and 9.0) were investigated. In a conic glass tube (5 mL), 100 µL of the internal standard (IS) (10 µg/mL NTZ) and 3 mL of a potassium phosphate buffer (0.1M) were added to 1 mL of the drug-free plasma spiked with the working standards resulting in the following plasma levels: 20.0 µg/mL for PB, 20.0 µg/mL for PRM, 10.0 µg/mL for CBZ, 12 µg/mL for CBZ-E, 6 µg/mL for LTG, and 6 µg/mL for PHT. The sample was vortexed for 10 s. The fiber was immersed in the sample by stirring at room temperature for 10 min, and then it was directly transferred to the hot injector and desorbed for 4 min. Triplicate analyses were performed for all experiments.

The effect of the ionic strength of the sample solution (addition of 5%, 10%, 15%, and 20% NaCl), the extraction temperature (20°C, 30°C, 50°C, and 70°C), and the equilibrium time (5, 10, 15, 30, and 50 min) on the extraction efficiency were also investigated.

The desorption conditions were evaluated by testing the main variables involved: injection temperature, desorption time, and depth of fiber into the injector liner.

Results and Discussion

Optimization of the SPME conditions

The optimum SPME desorption conditions were found as 250°C for the injection temperature and 4 min for the desorption time. Those parameters were selected not only because of the quantitative desorption (maximum detector response), but also because it causes a minimum of carryover.

Table II. Precision of the Interassay for the Determination of Drugs in Plasma

Drug	Spiked amount (µg/mL)	Found amount ± SD* (µg/mL)	CV†
PB	2.0	2.05 ± 0.15	7.73
	10.0	10.42 ± 0.65	6.31
	30.0	28.67 ± 1.88	6.57
PRM	5.0	5.88 ± 0.45	7.75
	10.0	11.67 ± 0.97	8.35
	20.0	22.53 ± 1.41	6.27
CBZ	4.0	3.96 ± 0.14	3.56
	10.0	9.67 ± 0.47	4.91
	20.0	19.31 ± 0.62	3.22
PHT	3.0	3.82 ± 0.22	5.81
	10.0	11.23 ± 0.76	6.77
	20.0	19.47 ± 0.82	4.23
CBZ-E	4.0	3.60 ± 0.26	7.34
	8.0	7.53 ± 0.44	5.95
	12.0	12.30 ± 0.97	7.93
LTG	0.2	0.25 ± 0.018	7.25
	1.0	1.44 ± 0.08	6.10
	10.0	10.40 ± 0.71	6.88

* SD, standard deviation.
† $n = 10$.

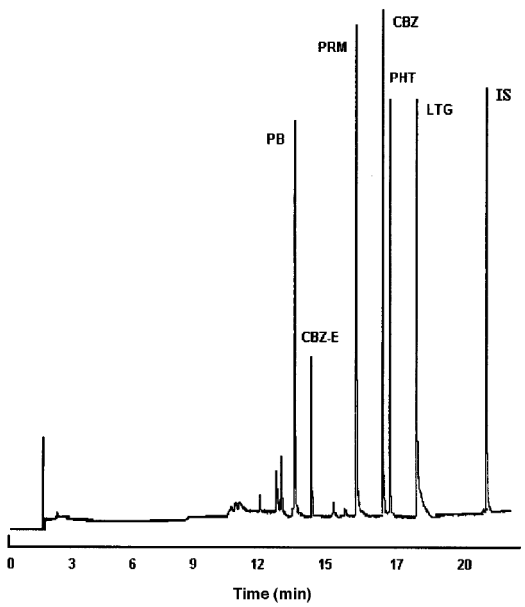

Figure 2. Capillary GC–TSD chromatogram for the SPME extracts of free-drug plasma from healthy volunteers spiked with AEDs.

The effect of fiber depth into the liner showed that the deeper the fiber was exposed to the hottest part of the injector (closer to the column entrance), then the higher the peak areas were that were obtained. The CW–DVB fiber showed a high extraction efficiency for all drugs.

The matrix pH effect on the extraction efficiency of the drugs using potassium phosphate buffers (pH 3.0, 5.0, 7.0, and 9.0) is shown in Figure 1A. In SPME it has been shown that by adjusting the pH of a matrix solution the dissociation constant of the dissociable species will alter, assuming that only the undissociated form of the acidic and alkaline analytes can be extracted by the coating (11).

Most AEDs (which are weak acids) are present in the undissociated forms at a pH value between acidic to neutral, resulting in a higher extraction efficiency in this pH range. As a function of the results obtained in this work, a pH of 7.0 was selected to continue the SPME optimization.

Buffer solutions were used for pH control because in an unbuffered extraction mixture the ratio of undissociated to dissociated forms of the drug may vary. Therefore, one does not achieve the continual transfer of a drug from the dissociated to the undissociated form and then to the fiber coating. Lord and Pawliszyn (14) reported that a significant deviation from linearity in the amounts extracted as the drug increases in the nonbuffered system can be observed.

As expected, the addition of NaCl to the samples had a significant influence on the amount of analyte absorbed by the fiber. The addition of salt to the matrix (altering the ionic strength and consequently decreasing the solubility of the drugs in the plasma solution) will favor the drugs absorbing onto the fiber coating. The effect of NaCl concentration was investigated in the ranges

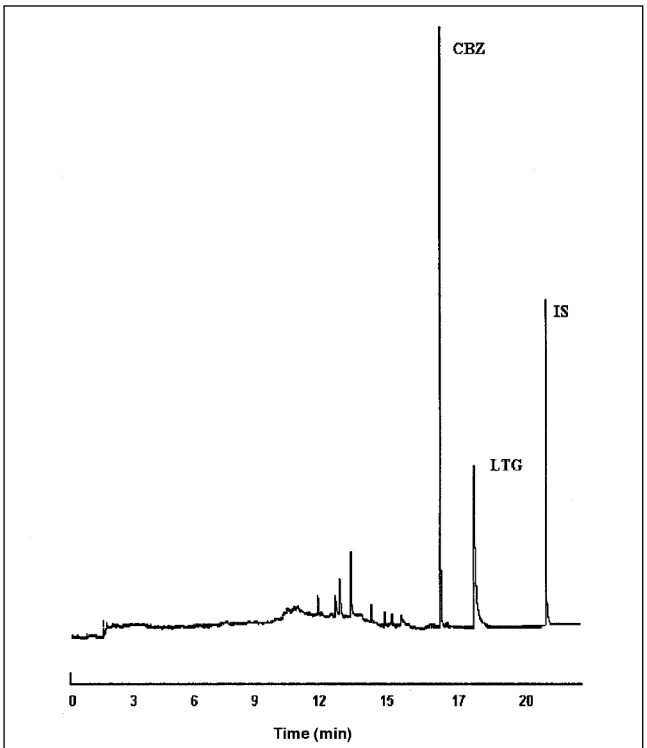

Figure 3. Capillary GC-TSD chromatogram for the SPME extracts of human plasma from patients with epilepsy.

of 0%, 5%, 10%, 15%, and 20% at pH 7.0 (Figure 1B). The addition of amounts of NaCl higher than 15% did not increase the efficiency of the drug extraction.

The effect of temperature on SPME efficiency was examined at 20°C, 30°C, 50°C, and 70°C on samples at pH 7.0 with the addition of 15% NaCl and stirred at a rate of 2500 rpm for 15 min (Figure 1C).

The increase in extraction temperature enhanced the drug diffusion, which shortened the equilibrium time but decreased the drug distribution between the fiber coating and the extraction mixture (11).

Figure 1D shows the time profile of the extraction for the AEDs. The extraction equilibrium time was reached at 15 min for most AEDs investigated (a shorter extraction time is one of the advantages of the SPME method). Therefore, we concluded that the best experimental conditions among those investigated for the SPME procedure was established as following: direct extraction with a CW–DVD fiber (65-µm film thickness), 1.0 mL of the sample plasma modified with 15% NaCl and a 3-mL potassium phosphate buffer (pH 7.0), extraction temperature at 30°C, and stirring at a rate of 2500 rpm for 15 min.

The plasma sample was diluted with a buffer (1:3, v/v) to decrease the influence of proteins on the extraction yield. The dilution increased the diffusion coefficients of the drugs from the plasma sample to the polymeric phase. Compared with water, diffusion coefficients are smaller in the more viscous protein solution. Bermejo et al. (15) also diluted the plasma solution (1:4, v/v, with buffer) in order to optimize the SPME conditions for the determination of methadone in plasma.

Koster et al. (16) evaluating the amount of lidocaine extracted from plasma after dilution with a buffer (pH 9.5) in different ratios showed that the extracted amount increases with a decreasing protein content.

The AEDs extracted from the plasma that was diluted with phosphate buffer showed smaller areas (amount extracted) than the values obtained when the extraction was performed in water. This effect was more pronounced in AEDs that were high protein binding at physiologic pH (CBZ (75%), PHT (90%), NTZ (87%)) than other PBs (40 to 60%), LTGs (55%), PRMs (19%), and CBZ-Es (not found).

When the plasma samples were spiked with AEDs after protein precipitation (remaining aqueous phase from plasma), the peak areas obtained were comparable with those values obtained by spiking pure water. Protein precipitation has been used to release drugs from plasma protein binding prior to sampling by SPME (16–18).

Validation of the method

The relative retention times obtained for all drugs are listed in Table I. No interfering peaks appeared in the chromatograms of drug-free plasma from healthy volunteers spiked with AEDs (Figure 2).

The linearity of the assays was determined using drug-free plasma spiked with the drugs PB (0.20 to 40.0 µg/mL), PRM (0.20 to 25.0 µg/mL), CBZ (0.06 to 20.0 µg/mL), PHT (0.05 to 25.0 µg/mL), CBZ-E (0.20 to 12.0 µg/mL), and LTG (0.20 to 10.0 µg/mL). The intervals evaluated were linear with the correlation coefficients better than 0.996 in all cases (Table I), and the coefficients of the variation (CVs) of the points used to prepare the calibration curve were below 10% (Table II).

The LOQ of the plasma drugs varied from 0.05 to 0.20 µg/mL (Table I). These LOQs were determined as the lowest concentration on the calibration curve in which the CVs were lower than 10%.

The proposed method is sensitive enough to work into subtherapeutic and therapeutic concentrations and has application in pharmacokinetic studies and patient routine therapeutic drug monitoring.

In order to show the efficiency of the SPME methodology described, the method was further applied for the detection of AEDs in human plasma from patients with epilepsy in steady-state. Figure 3 shows the capillary GC–TSD chromatogram of the SPME extract of human plasma from a patient who orally administrated 3.75 mg/kg of LTG and 16.2 mg/kg of CBZ per day resulting in plasma levels of 3.31 µg/mL and 10.79 µg/mL, respectively. As can be seen, an excellent separation was achieved with a very clean chromatographic profile.

In short, the development and validation of a new method using SPME is described and proposed for the determination of lamotrigine in plasma in the presence of several other drugs. The method has been successfully applied to the analysis of plasma from patients with epilepsy.

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