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Solid-phase microextraction–liquid chromatography (SPME–LC) determination of lamotrigine simultaneously with carbamazepine and carbamazepine 10,11-epoxide in human plasma

A simple and specific analytical method is presented for the determination of lamotrigine (LTG) simultaneously with carbamazepine (CBZ) and carbamazepine 10,11-epoxide (CBZ-E) in human plasma by off-line solid-phase microextraction–liquid chromatography. The best analytical conditions for the SPME procedure were established by direct extraction on a 50 μm Carbowax™/TPR-100-coated fiber, employing 1.0 mL of sample plasma matrix modified with 30% NaCl and with 3 mL potassium phosphate buffer (pH 9.0); extraction at 22°C; stirring at a rate of 2500 rpm for 20 min; and then desorption of the drugs by exposure of the fiber to 50 μL of the mobile phase for 10 min. The method showed good linearity (0.05 to 10.0 μg mL^{-1} for LTG, 0.2 to 20.0 μg mL^{-1} for CBZ, and 1.0 to 20.0 μg mL^{-1} for CBZ-E), with regression coefficients ranging from 0.9947 to 0.9978 and coefficients of variation of the points of the calibration curve lower than 10%. The limit of quantification (LOQ) for the investigated drugs in plasma varied from 0.05 to 1.0 μg mL^{-1}.

Key Words: Solid-phase microextraction–liquid chromatography; Lamotrigine; Carbamazepine; Carbamazepine 10,11-epoxide; Plasma

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1 Introduction

Lamotrigine (LTG), 3,5-diamino-6-(2,3-dichrophenyl)-1,2,4-triazine, is a drug approved for several indications in epilepsy. The efficacy of LTG as an add-on therapy to existing antiepileptic drug regimens has been well established in adult patients and in children with refractory partial seizures with or without secondary generalization [1].

Wide individual differences exist in plasma LTG levels achieved at any given dose, largely because of pharmacokinetic interactions with concurrently prescribed antiepileptic drugs. In the presence of hepatic enzyme inducing agents such as carbamazepine (CBZ), the LTG's half-life is reduced and higher doses may be required. The recommended therapeutic plasma LTG levels are 1 to 4 μg mL^{-1} [2].

LTG has been determined by chromatographic methods with sample (biological fluids) preparation mainly performed by protein precipitation [3, 4], liquid-liquid extraction [5–8], and solid-phase extraction [9, 10].

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Solid-phase microextraction (SPME) integrates sampling, extraction, and concentration steps, and offers a simple, solvent-free alternative to traditional methods of sample preparation. SPME was introduced about a decade ago, being mainly applied to the analysis of organic compounds in aqueous sample matrices by gas chromatography (GC) [11, 12]. The preconcentration of pharmaceutical compounds present in biological fluids is one of the successful applications of SPME as sample pretreatment for GC analysis [13, 14]. However, only a limited number of reports on the coupling of this extraction method with liquid chromatography (LC) separations systems for drugs analysis have been published [15–19], mainly due to the difficulties imposed by the on-line coupling of these methods, as well as their subsequent operation [20]. Paschke et al. [21] have pointed out that due to the large volume (ca. 90 μL) of the commercially available SPME-LC interface, peak broadening is very difficult to avoid.

In the present work, we describe a specific, and very simple set up for off-line coupling of SPME and LC that does not require any interface, this being the system employed in the determination of LTG simultaneously with CBZ and carbamazepine 10,11-epoxide (CBZ-E, main metabolite of CBZ which has antiepileptic activity). Additionally, the proposed methodology was validated by investigating the limit of quantitation, linearity, precision, and application to human plasma samples from patients with epilepsy.

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2 Experimental

2.1 Reagents and analytical standards

CBZ and CBZ-E analytical standards were kindly donated by Ciba-Geigy (São Paulo, Brazil), and LTG by Glaxo Wellcome (São Paulo, Brazil). The internal standard, 4-methylprimidone (MPR), was purchased from Aldrich Chemical Co (São Paulo, Brazil). The working standard drug solutions were prepared by diluting the stock solutions of these compounds (1 mg mL^{-1} in methanol) with an appropriate volume of water previously purified in a Milli-Q system (Millipore, São Paulo, Brazil). Sodium chloride NaCl (Merck, analytical grade) was used after purification by heating at 300°C overnight; methanol and acetonitrile were HPLC grade from J.T. Baker (Phillipsburg, USA), monobasic and dibasic phosphate were purchased from Merck (Darmstadt, Germany).

2.2 SPME Equipment

The SPME holder and the Carbowax™/TPR-100 (CW/TPR) coated fiber (50 μm film thickness) were obtained from Supelco (São Paulo, Brazil). The CW/TPR fiber was conditioned by soaking in methanol for 30 min.

2.3 Instrumentation

The LC system used was a Varian 9050 (Varian, California, USA). Signals were monitored by a Varian 9050 UV detector set at 220 nm in 0.01 AUFS. The chromatographic separation was achieved using a RP 18 LichroCART® column obtained from Merck (125 mm × 4 mm ID, 5-μm particles) with a guard column packed with the same material. The separation was performed at room temperature (ca. 25°C). The mobile phase used consisted of a mixture of potassium phosphate buffer (0.01 mol L^{-1}, pH 6.5)–acetonitrile–methanol (65:18:17 *v/v/v*) at a flow rate of 1.3 mL min^{-1}. The mobile phase was filtered and degassed prior to use.

2.4 Optimization of the SPME conditions

The first step was to evaluate the influence of pH on extraction. For that purpose, four different pH values of potassium phosphate buffers (pH 3.0, 7.0, 9.0, 11.0) were investigated. In a conical glass tube (5 mL), 100 μL internal standard (100 μg mL^{-1} MPR) and 3 mL of potassium phosphate buffer (0.01 mol L^{-1}) were added to 1 mL of the drug-free plasma spiked with working standards resulting at plasma levels, as follows: [CBZ (10.0 μg mL^{-1}), CBZ-E (10 μg mL^{-1}), and LTG (4 μg mL^{-1})]; the sample was vortexed for 10 s before extraction. The fiber (CW-TPR) was then immersed in the sample with stirring at room temperature for 10 min, and the drugs were further desorbed by exposure of the fiber to the mobile phase for 15 min. 10 μL of this extract was injected into the LC-UV system. Triplicate analyses were performed for all experiments.

The effect of ionic strength of the sample solution (addition of 10, 20, 30, 40% NaCl), the extraction temperature (22, 30, 40, 50°C), and the equilibrium time (5, 10, 20, 40, 50 min) on extraction efficiency were also investigated.

The parameters employed during the desorption procedure, such as the chemical composition of the desorption solvent, the solvent volume, and the duration of the fiber soak step were studied and optimized.

3 Results and discussion

3.1 Optimization of the SPME conditions

The optimum SPME desorption conditions were obtained by exposure of the fiber to 50 μL of the mobile phase for 10 min using a 0.1 mL volume glass vial (V-shape); this volume enabled the fiber coating to be completely immersed in the solvent. Those parameters were selected not only due to the higher quantitative desorption (maximum detector response) obtained, but also because of the minimum carryover observed under these experimental conditions.

The matrix pH effect on the extraction efficiency of the drugs using different pH values adjusted with potassium phosphate buffers is shown in **Figure 1.a**. The best overall results were obtained at pH = 9.0, and all subsequent analyses were performed with this pH value. In SPME it has been shown that adjusting the pH of a matrix solution will alter the dissociation constant (*K*) value for dissociable species, assuming that only the undissociated acid and base forms can be extracted by the coating [11].

As expected, the addition of NaCl to the samples had a significant influence on the amount of drugs absorbed on to the fiber. The addition of salt to the matrix, altering the ionic strength and consequently decreasing the solubility of the drugs in plasma solution, will favor adsorption of the drugs onto the fiber coating. The effect of NaCl concentration was investigated in the range 10% to 40% at pH 9.0 (**Figure 1.b**). For the conditions in which the solution was saturated with 40% NaCl, the mass extracted decreased for LTG and CBZ. Thus 30% NaCl was selected as the best condition.

The effect of temperature on SPME efficiency was examined in the range of 22 to 50°C (**Figure 1.c**). The increase in extraction temperature slightly enhanced the drug diffusion, which shortens the equilibrium time but decreases the distribution of drug between the fiber coating and the extraction mixture [11].

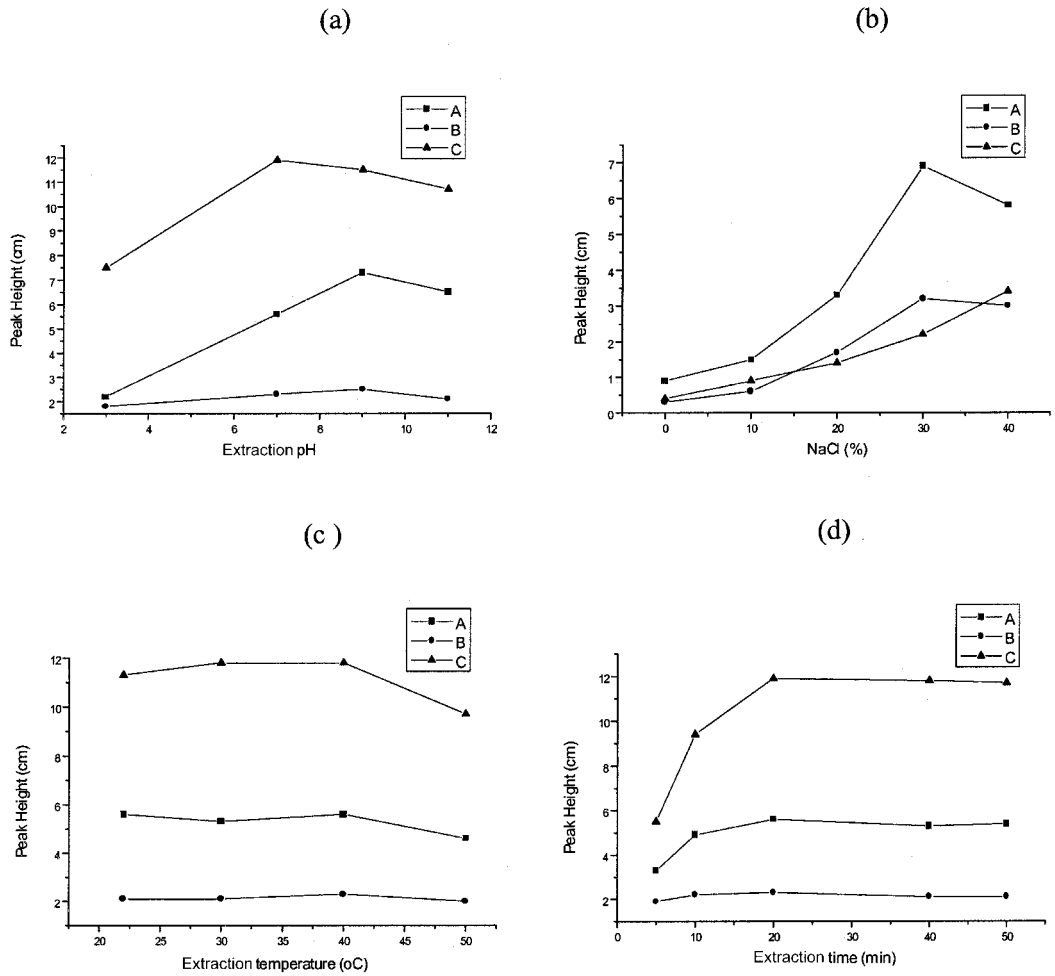

Figure 1. Effect of the experimental variables on the SPME extraction of drugs from human plasma. (a) matrix pH, (b) NaCl concentration, (c) temperature, and (d) extraction time. A: LTG, B: CBZ-E, C: CBZ.

Figure 1.d shows the time profile of the extraction for the drugs. The extraction equilibrium time was reached at 20 min for all investigated drugs. Based upon these data, we concluded that the best experimental conditions among those investigated for the SPME procedure were as follows: direct extraction with CW/TPR fiber (50 μm film thickness), 1.0 mL of sample plasma matrix containing 30% NaCl and with 3 mL potassium phosphate buffer (pH 9.0), extraction at 22°C at a stirring rate of 2500 rpm for 20 min, followed by desorption of the drugs by exposure of the fiber to 50 μL of the mobile phase for 10 min.

The CW/TRP fiber (partially crosslinked) proved to be very stable and more durable than polyacrylate (PA) fibers used in other studies.

In order to decrease the influence of proteins on the extraction yield, the plasma sample was diluted with buffer (1:3 *v/v*). The dilution procedure increases the diffusion coefficients of the drugs from plasma sample to the polymeric phase; compared with those obtained in the water matrices, diffusion coefficients are smaller in the more viscous protein solution. Bermejo et al. [22] 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. On evaluating the amount of lidocaine extracted from plasma after dilution with buffer pH 9.5 in different ratios, Koster et al. [23] showed that the extracted amount increases with a decreasing protein content.

Smaller amounts of drugs were extracted from plasma diluted with phosphate buffer than when the extraction was performed in water. This effect was more pronounced for CBZ, which shows high protein binding (75%) at physiological pH [24].

3.2 Validation of the method

The specificity of the method was demonstrated by representative chromatograms of human plasma in Figure 1

Table 1. Limit of quantification (LOQ), linearity of the method and relative retention time (t_r^{rel})[a] of selected AED drugs as determined by off-line SPME-LC.

AEDs	Limit of quantification (μg mL^{-1})	Relative retention time (t_r^{rel})	Linearity range (μg mL^{-1})	Linear regression	Correlation coefficient (r)
CBZ	0.2	2.07	0.2–20.0	$y = 0.02334 + 0.50341\ x$	0.9978
CBZ-E	1.0	1.24	1.0–20.0	$y = -0.06511 + 0.11473\ x$	0.9977
LTG	0.05	0.84	0.05–10.0	$y = -0.21782 + 0.60218\ x$	0.9947

[a] The relative retention time (t_r^{rel}) is defined as the retention time of the analyte over the retention time of the internal standard (MPR).

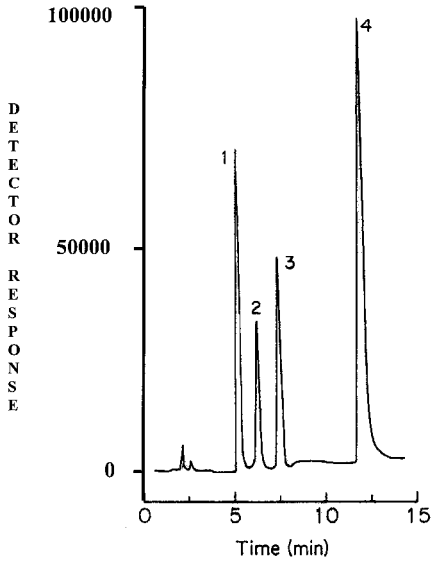

Figure 2. SPME-LC-UV (off-line) chromatogram of drug-free plasma spiked with drugs, resulting at the following plasma levels: 1: LTG (10 μg mL^{-1}); 2: Internal Standard; 3: CBZ-E (20 μg mL^{-1}); and 4: CBZ (20.0 μg mL^{-1}).

and **Figure 2**. The relative retention times t_r^{rel} of each drug with respect to the retention time of the internal standard (MPR) are listed in **Table 1**. Additional blank human plasma from several individuals was tested and showed no significant interference at the retention times of the drugs of interest.

The linearity of the assays was determined using drug-free plasma spiked with the antiepileptics: CBZ (0.2 to 20.0 μg mL^{-1}), CBZ-E (1.0 to 20.0 μg mL^{-1}), and LTG (0.05 to 10.0 μg mL^{-1}). The intervals evaluated were linear with the correlation coefficients better than 0.994 (Table 1) and CVs (coefficients of variation) of the points of the calibration curve were lower than 10% in all cases.

The precision (inter-assay coefficients) for the investigated drugs in plasma spiked with three different concentration of each analyte and subjected to the described procedure are shown in **Table 2**.

Table 2. Precision of inter assays obtained during the determination of drugs in plasma by off-line SPME-LC.

Drug	Spiked amount (μg mL^{-1})	Amount found (μg mL^{-1}) ± SD	CV ($n = 6$)
CBZ	0.8	0.88 ± 0.01	1.02
	10.0	8.68 ± 0.63	7.34
	20.0	22.8 ± 1.5	6.60
CBZ-E	2.0	1.92 ± 0.16	8.55
	10.0	9.84 ± 0.49	5.00
	20.0	22.8 ± 1.5	6.61
LTG	0.3	0.29 ± 0.01	5.68
	5.0	4.66 ± 0.43	9.30
	10.0	11.3 ± 0.53	4.76

CV: Coefficient of variation.
SD: Standard deviation.

The recoveries of the drugs from plasma were compared to the direct injection of equivalent drug concentrations of the analytical standards dissolved in methanol and injected into the LC system. The results obtained were 6.0, 1.2, 1.6% for CBZ, CBZ-E, and LTG, respectively.

The limit of quantification (LOQ) of the investigated drugs in plasma varied from 0.05 to 1.0 μg mL^{-1} (Table 1) depending upon the analyte. Those LOQ were determined as the lowest concentration on the calibration curve in which the CVs were lower than 10%.

Analyses of the selected drugs using the described method (SPME/LC) were compared with analogous determinations by liquid-liquid extraction (LLE/LC) [6]. Comparison of the concentrations obtained by those two methods in spiked samples for CBZ and LTG at two different concentrations (5 μg mL^{-1} and 10 μg mL^{-1}) showed a very good agreement of the results. By using the unpaired t test, both the mean of the concentrations (95% confidence interval of the difference) and the CVs showed no statistically significant difference between these two investigated methods.

To demonstrate the SPME efficiency the method was further applied to the detection of antiepileptics in human plasma from patients with epilepsy in a steady state. Fig-

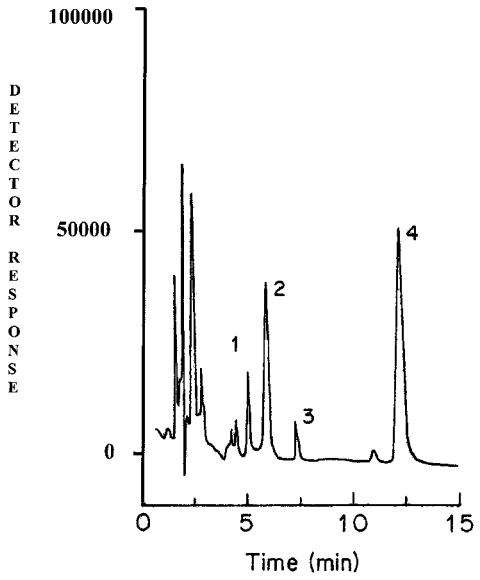

Figure 3. SPME-LC-UV (off-line) chromatogram of a human plasma extract from a patient with epilepsy. The drugs were orally administrated at concentrations of 4.44 mg $kg^{-1}day^{-1}$ (LTG) and 31.10 mg $kg^{-1}day^{-1}$ (CBZ), resulting in plasma levels of 1.99 μg mL^{-1} of LTG, 9.10 μg mL^{-1} of CBZ, and 3.96 μg mL^{-1} of CBZ-E. (1) LTG, (2) Internal Standard, (3) CBZ-E, (4) CBZ.

ure 3 shows a typical LC-UV chromatogram of a SPME extract of human plasma from a patient on an oral dosage of 4.44 mg $kg^{-1}day^{-1}$ of LTG and 31.10 mg $kg^{-1}day^{-1}$ of CBZ, resulting in plasma levels of 1.99 μg mL^{-1} of LTG, 9.1 μg mL^{-1} of CBZ, and 3.96 μg mL^{-1} of CBZ-E.

The established method showed that the off-line coupling of SPME and LC-UV without the use of an interface is a useful tool for determining LTG plasma concentrations in patients receiving therapeutic dosages. The method may be also applied to evaluate plasma LTG levels in patients taking CBZ as co-medication; this aspect is particularly important because LTG is used mostly as an add-on therapy to existing antiepileptic drug regimens.

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