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Quantification of carbamazepine, carbamazepine-10,11-epoxide, phenytoin and phenobarbital in plasma samples by stir bar-sorptive extraction and liquid chromatography

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ABSTRACT

A sensitive and reproducible stir bar-sorptive extraction and high-performance liquid chromatography-UV detection (SBSE/HPLC-UV) method for therapeutic drug monitoring of carbamazepine, carbamazepine-10,11-epoxide, phenytoin and phenobarbital in plasma samples is described and compared with a liquid:liquid extraction (LLE/HPLC-UV) method. Important factors in the optimization of SBSE efficiency such as pH, extraction time and desorption conditions (solvents, mode magnetic stir, mode ultrasonic stir, time and number of steps) assured recoveries ranging from 72 to 86%, except for phenytoin (62%). Separation was obtained using a reverse phase C_{18} column with UV detection (210 nm). The mobile phase consisted of water:acetonitrile (78:22, v/v). The SBSE/HPLC-UV method was linear over a working range of 0.08–40.0 $\mu g\,mL^{-1}$ for carbamazepine, carbamazepine-10,11-epoxide and phenobarbital and 0.125–40.0 $\mu g\,mL^{-1}$ for phenytoin, The intra-assay and inter-assay precision and accuracy were studied at three concentrations (1.0, 4.0 and 20.0 $\mu g\,mL^{-1}$). The intra-assay coefficients of variation (CVs) for all compounds were less than 8.8% and all inter-CVs were less than 10%. Limits of quantification were 0.08 $\mu g\,mL^{-1}$ for carbamazepine, carbamazepine-10,11-epoxide and phenobarbital and 0.125 $\mu g\,mL^{-1}$ for phenytoin. No interference of the drugs normally associated with antiepileptic drugs was observed. Based on figures of merit results, the SBSE/HPLC-UV proved adequate for antiepileptic drugs analyses from therapeutic levels. This method was successfully applied to the analysis of real samples and was as effective as the LLE/HPLC-UV method.

1. Introduction

Therapeutic drug monitoring (TDM) of antiepileptic drugs is necessary to optimize patients' clinical outcome by managing their medication regimen with the assistance of measured drug concentrations [1]. Plasma concentration monitoring is widely used for the clinical management of patients with epilepsy receiving phenytoin (PHT), phenobarbital (PHB) and carbamazepine (CBZ). CBZ is metabolized to carbamazepine-10,11-epoxide (CBZ-E) and other metabolites in the liver by the CYP3A4 and CYP2C8 subtypes of the cytochrome P450 system. From a clinical standpoint, CBZ-E is the most important of the 33 metabolites of CBZ that have been isolated, because CBZ-E appears to show pharmacological activity, as does its parent compound (CBZ) [2].

Several methods have been published for the determination of one or more antiepileptic drugs in biological fluids for TDM or for toxicology purposes. There are several high-performance liquid chromatography (HPLC) methods for determination of CBZ and its metabolites, including CBZ-E, in biological fluids and drug products [2–6] and there are various HPLC methods for the simultaneous determination of PHT, PHB and CBZ [7–13].

The advantages of HPLC for antiepileptic analysis are its versatility and simplicity of sample preparation, as well as a broad linearity in detectors, making HPLC the method of choice for TDM of the antiepileptic [2–16]. Liquid:liquid extraction (LLE) [2–6,10–13]

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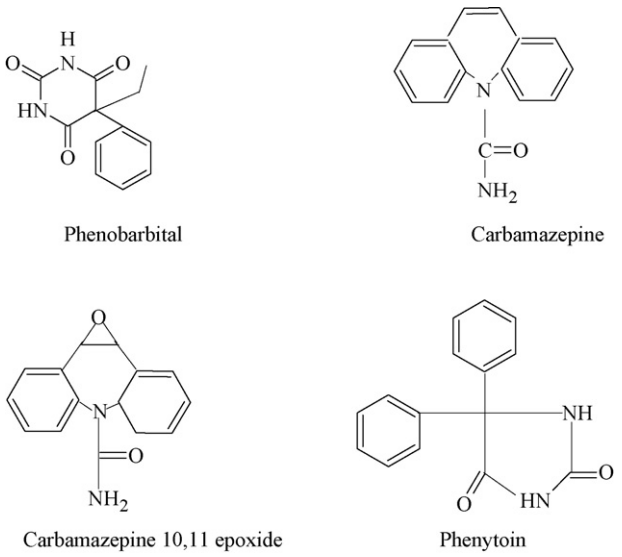

Fig. 1. Chemical structures of antiepileptic drugs.

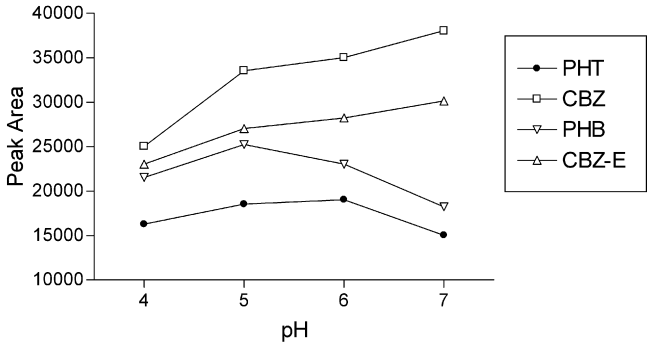

Fig. 2. Effect of the matrix pH on the SBSE efficiency of antiepileptics in a plasma sample.

or solid-phase extraction [8–9,15–18] have been used for sample clean-up followed, in some cases, by column-switching [19] and deproteinization [9]. Solid-phase microextraction (SPME) has been successfully applied to analyze drugs in biological fluids by chromatography techniques. More recently, stir bar-sorptive extraction (SBSE) [18,20–27], a sample-preparation technique based on the same principles as SPME, the partitioning coefficient of the solutes between the silicone phase and the aqueous phase, has been evaluated for the enrichment of organic solutes from biological fluids [27–29].

In SBSE, a stir bar coated with a polydimethylsiloxane (PDMS) layer is stirred for a given time in the sample solution. After this concentration step, the stir bar is placed in a small vial and solvent desorption is performed followed by liquid chromatographic analysis. In SBSE the amount of PDMS typically coated, 24–126 μL is substantially higher than on an SPME fibre, for which the maximum volume is usually 0.5 μL (100 μm film thickness). Consequently the sensitivity is increased by a factor of 50 and 250, reducing detection limits to sub-ng L^{-1} levels. The analytical methods described in the literature to analyze antiepileptics in biological fluids usually use conventional sample pre-treatment techniques that is laborious, time-consuming and require large amounts of organic solvents. Sorptive extraction has proven to be interesting and environmental friendly alternative to liquid extraction [21]. Benanou et al. demonstrated that loaded stir bars can be stored at 4 °C for a week without loss of solutes. This opens interesting prospects for on-site sampling and extraction. The loaded stir bars are sent to the laboratory for analysis, not the samples [30].

A very interesting application area is described by Soini et al. [31]. SBSE was used to characterize chemical signal compounds in animal urine samples and gland excretion. Based on the detailed profiles obtained, gender and age could be differentiated using chemometric data processing [31]. Wahl et al. [32] described the analysis of barbiturates in urine [32] and phthalates and the metabolites 2-ethyl-hexanol and 4-heptanone in body fluids and infusates [32]. Other applications of SBSE for the determination of environmental contaminants in biological samples include the determination of PCBs in sperm [33], the determination of phenols and chlorophenols in urine [34], the determination of pesticides in breast milk [35], the determination of aroma compounds in vinegar [36], the determination of brominated flame retardants in environmental samples and pyrethroids [37–38], phenols, pharmaceuticals and herbicides [39] in water samples.

As LLE, SBSE can also be combined with prior enzymatic hydrolysis, as is often used for the analysis of organic compounds in biological samples. Overviews of different *in situ* derivatization techniques for the determination of steroids, drugs of abuse and pharmaceuticals in biological samples have been described [21] Neng et al. reported a determination of glyoxal and methylglioxal in beer, biological fluids and in water samples by SBSE with *in situ* derivatization [40].

The aim of this study was to evaluate SBSE and compare it with the LLE method followed by HPLC analysis for the determination of CBZ, CBZ-E, PHT and PHB (Fig. 1) in plasma samples.

2. Experimental

2.1. Reagents and analytical standards

CBZ and CBZ-E, PHB, PHT, analytical standards were purchased from Sigma–Aldrich Inc., St. Louis, USA and 5-ethyl-5-*p*-tolylbarbituric acid (IS) from Aldrich Chemical Company Inc., USA.

Methanol, HPLC grade, was obtained from J.T. Baker (Phillipsburg, USA), acetonitrile and dichrometane, HPLC grade, were purchased from Merck (Darmstadt, Germany). The reagents used for drug extraction were analytical grade and were purchased from Merck (Darmstadt, Germany). The water used was deionised and filtered with a Milli-Q water processing system (Millipore, São Paulo, Brazil), acetic acid and sodium acetate were obtained from Merck (Damstadt, Germany).

2.2. Calibration curve

Separated stock solutions of each antiepileptic were prepared by dissolving accurately weighed amounts of each reference compound in methanol to yield a 1 mg mL^{-1} drug concentration. A 1 mg mL^{-1} stock solution of (IS) in methanol was prepared and further diluted in methanol to give a 0.1 mg mL^{-1} working solution. All stock solutions were stored at −20 °C and they were stable for 6 months.

Routine daily calibration curves were prepared by the addition of 25 μL of each standard solution at concentrations of 0.01, 0.04, 0.08, 0.16, 0.8 and 1.6 mg mL^{-1} of methanol to 1 mL of blank plasma ((plasma from a patient not exposed to any drug for at least 2 months) aliquots, resulting in plasma drug concentrations of 0.25, 1.0, 2.0, 4.0, 20 and 40 μg mL^{-1}. Each spiked plasma was processed as described in sample preparation.

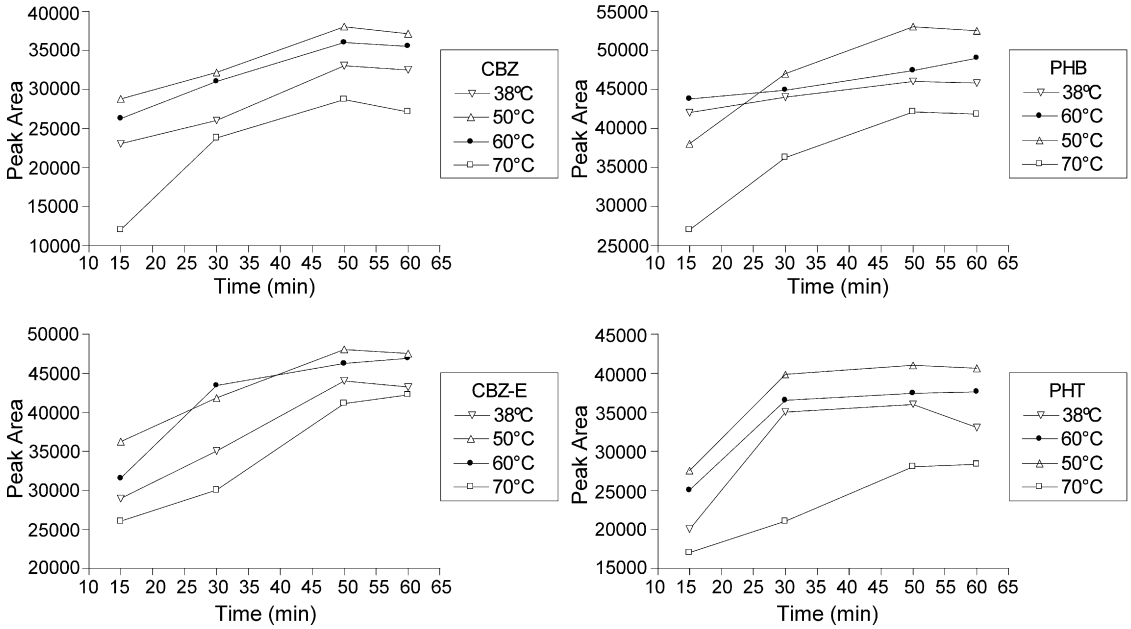

Fig. 3. SBSE time extraction profiles of antiepileptics at different temperatures values.

2.3. SBSE accessories

The commercial stir bar Twister for sorptive extraction was obtained from Gerstel (Gerstel GmbH, Mulheim an der Ruhr, Germany). It consists of a 10 mm long glass-encapsulated magnetic stir bar, externally coated with 22 μg of PDMS. This layer is 0.5 mm thick, corresponding to a volume of 24 μL of PDMS. Prior to the first use, the stir bars were placed in a vial containing an acetonitrile:methanol solution (80:20, v/v) and conditioned for 24 h. Among the successive extractions, the used stir bars were cleaned in methanol for 30 min at 50 °C, under magnetic stirring rate of 1200 rpm, followed by a drying step using a lint-free tissue.

2.4. Instrumentation

The analysis was performed on HPLC system consisting of a Shimadzu Model (Japan) LC 10 AD pump, a Shimadzu Model SPD 10 A ultraviolet detector, a chromatopac C-R6A integrator (Shimadzu, Japan). Chromatographic separation was achieved at room temperature on a LiChrospher 100 RP-18 column (125 mm × 4 mm, 5 μm particle size (Merck, Damstadt, Germany). The mobile phase consisted of water:acetonitrile (78:22, v/v). Flow-rate was 1.0 mL min^{-1}. The ultraviolet detector was set at 220 nm.

2.4.1. Optimization of SBSE process

The influence of the pH matrix on antiepileptic drug extractions was the first step evaluated, investigating different pH values from 4.0 to 8.0 (sodium acetate buffer, 0.75 mol L^{-1}). In a glass vial (5 mL) sealed with a silicone septum, 25 μL of IS and 4 mL of 0.75 mol L^{-1} sodium acetate buffer were added to 1 mL of plasma sample spiked with the standard solutions. The vial was heated up to 50 °C on a hotplate, the stir bar was then immersed into the sample, and the extraction was performed at a magnetic stirring rate of 1200 rpm for 50 min.

The influence of ionic strength of the matrix solutions (NaCl addition), extraction time (10–60 min) and temperature (38, 50, 60 and 70 °C) in the SBSE process were also investigated.

To determine the best desorption conditions: solvents (acetonitrile and mobile phase), modes (magnetic stir and ultrasonic), desorption time (5, 15, 30 and 60 min), number of desorption steps and the control of the carryover were all individually evaluated. For the desorption, the stir bars were removed with clean tweezers, rinsed lightly with Milli-Q water (1.0 mL), dried with lint-free tissue, and placed in a glass vial containing 1.0 mL of solvent, ensuring total immersion. Desorption was performed by ultrasonic treatment for 15 min at room temperature (25 °C) or by magnetic agitation for the same period at the same temperature. After the desorption process, the stir bars were removed by means of a magnetic rod and the solvent was evaporated until dryness. The dry residues were re-dissolved in 200 μL of the mobile phase, and 100 μL of this extract were injected into the HPLC-UV system.

2.4.2. Sample preparation for LLE

The extraction consisted of the addition of 25 μL of IS, 1 mL of sodium acetate buffer 0.75 M (pH 5.0) and 5 mL dichrometane to 1 mL of plasma. After shaking in a vortex-type shaker for 1 min and centrifugation at 1800 × *g* for 5 min, an aliquot (4.4 mL) of the organic phase was transferred to conic tubes and was evaporated under a constant air-flow at room temperature. The dry residue was

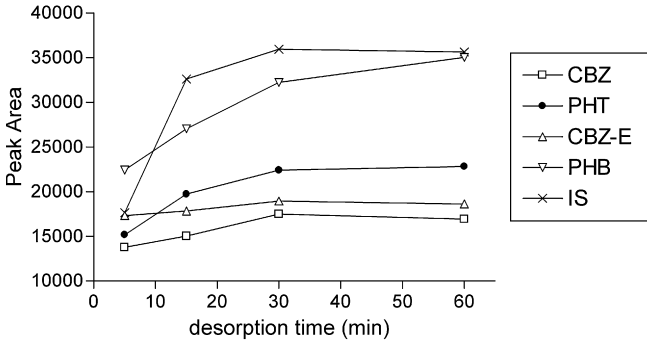

Fig. 4. SBSE desorption time profile of antiepileptics in plasma samples.

Table 1
Comparison of linearity, limit of quantification and recovery of antiepileptic drugs by stir bar-sorptive extraction vs. liquid:liquid extraction followed by HPLC-UV analysis in plasma samples

Linearity SBSE /LLE (μg mL^{-1})	Linear regression r^2 SBSE vs LLE	LOQ (μg mL^{-1}) SBSE vs LLE	Recovery SBSE vs LLE Concentration (μg mL^{-1})	Results (%) (n=5)
Phenobarbital	y = 0.1372 x - 0.0117 0.999		20	78.2 vs 89.1
(0.08 - 40)		0.08/0.06	8	72.0 vs 90.0
vs (0.05 - 40)	y = 0.00109x + 0.03189 0.998		0.5	76.0 vs 88.3
Carbamazepine	y = 0.3159x - 0.1157 0.999		20	86.7 vs 95.3
(0.08 – 40)		0.08/0.06	8	86.9 vs 95.2
vs (0.05 – 40)	y = 0.00148x + 0.105 0.999		0.5	86.2 vs 93.1
CBZ-E			20	80.3 vs 90.3
(0.0.8 -40)	y = 0.2014x + 0.0366 0.999	0.08/0.06	8	81.6 vs 91.0
vs (0.05 -40)	y = 0.00107x + 0.04896 0.999		0.5	84.9 vs 91.3
Phenytoin			20	62.9 vs 76.8
(0.125 -40) vs (0.09 -40)	y = 0.0423x - 0.0104 0.999	0.125/0.08	8	65.6 vs 76.3
	y = 0.00078x +0.01877 0.999		0.5	63.3 vs 78.6

LLE, liquid:liquid extraction; vs., versus; SBSE, stir bar-sorptive extraction; LOQ, limit of quantification.

Table 2
Comparison of intra- and inter-day precision and accuracy of the antiepileptic drugs by stir bar-sorptive extraction vs. liquid:liquid extraction followed by HPLC-UV analysis in plasma samples

Drugs (μg mL^{-1})	Precision intra-assay CV (%) $n = 10$; SBSE vs. LLE	Precision inter-assay CV (%) $n = 5$; SBSE vs. LLE	Accuracy error (%); SBSE vs. LLE
Phenobarbital			
20	6.3 vs. 3.5	6.5 vs. 7.7	6.4 vs. 4.2
4	5.2 vs. 4.5	6.8 vs. 6.6	6.0 vs. 3.5
1	7.6 vs. 7.8	6.3 vs. 5.8	8.2 vs. 8.2
Carbamazepine			
20	3.7 vs. 5.4	7.8 vs. 6.9	4.3 vs. 3.3
4	6.8 vs. 5.2	9.5 vs. 8.9	3.7 vs. 5.2
1	8.8 vs. 6.3	9.0 vs. 9.6	9.2 vs. 8.6
CBZ-E			
20	6.4 vs. 4.2	9.5 vs. 8.9	9.2 vs. 8.5
4	7.7 vs. 6.2	7.1 vs. 8.8	8.6 vs. 9.1
1	8.5 vs. 5.8	8.4 vs. 7.8	9.2 vs. 8.9
Phenytoin			
20	5.9 vs. 3.6	7.2 vs. 9.8	9.3 vs. 8.5
4	5.9 vs. 6.6	5.0 vs. 5.9	6.5 vs. 8.2
1	8.3 vs. 9.7	9.7 vs. 8.3	9.4 vs. 9.6

CV, coefficient of variation; LLE, liquid:liquid extraction; SBSE, stir bar-sorptive extraction.

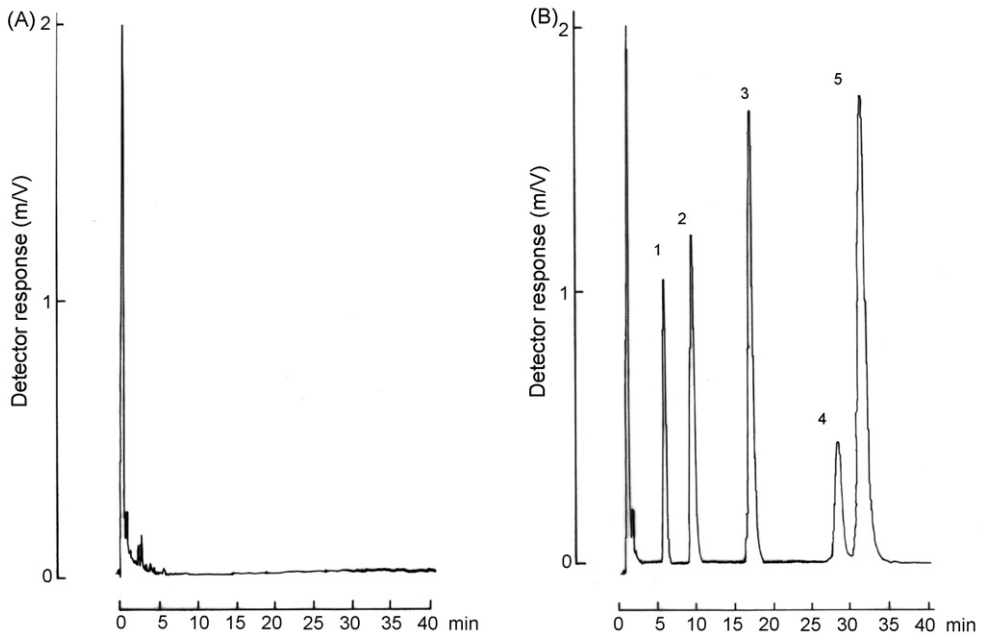

Fig. 5. (A) Chromatogram obtained from a blank (without internal standard) and (B) chromatogram obtained from a plasma spiked with 20 μg mL^{-1}. (1) Phenobarbital, (2) carbamazepine-10,11-epoxide, (3) internal standard (5-ethyl-5-p-tolylbarbituric acid), (4) phenytoin and (5) carbamazepine.

re-dissolved in 200 μL of the mobile phase and 100 μL of hexane. After shaking for 10 s in a vortex-type shaker, 100 μL of the mobile phase was injected into the HPLC-UV system.

2.4.3. Validation of the method

The recovery of the drugs was determined at three different concentrations in blank plasma. Plasma samples with the drugs were extracted in triplicate according to the procedure proposed. The recoveries were calculated by comparing the UV-peak areas of the spiked samples with the direct injection of standards solutions of equal concentrations.

To determine the intra-assay precision, aliquots ($n = 10$) of blank plasma containing the standard solutions of the drugs at three concentrations were analysed by the method proposed. To determine the inter-assay precision, blank plasma containing the standard solutions at the same concentrations were analysed on 10 consecutive days. Linearity was obtained by analysing blank plasma samples ($n = 3$) containing standard solutions of drugs at concentrations of 0.01–40 μg mL^{-1}. The concentration range was estimated on the basis of the regression curve ($y = ax + b$) and correlation coefficient (r^2).

The limit of detection (LOD) was determined as the lowest concentration giving a response of three times the average of the baseline noise defined from five determinations. The limit of quantification (LOQ) was considered the lowest concentration quantified with a coefficient of variation less than 10% obtained from five determinations. The selectivity of the method was evaluated by analysing several drugs normally combined with these antiepileptic drugs.

2.5. Blood samples

Blood samples from patients receiving antiepileptic drugs were collected after filling out a protocol containing the patients name, sex, age, weight, prescribed medication, dose and combined medications. Blood samples from patients in steady-state plasma concentrations of antiepileptic drugs were obtained in the morning with heparin (Liquemine®) immediately before drug administration. After centrifugation, plasma samples were stored at −20 °C until analysis. Stability studies carried out directly on plasma indicated that samples were stable for at least 3 months when stored at −20 °C.

Pooled blank plasma samples used for development and validation of the procedure were obtained from a local blood bank. The principles embodied in the Helsinki Declaration were adhered to, and the Ethics Committee at the University of São Paulo in Ribeirão Preto, Brazil approved the study.

3. Results and discussion

PDMS, homogeneous polymer coating, extract analytes *via* absorption, where the analytes dissolve in the coating and diffuse into the bulk of it during the extraction process. This process is non-competitive (compared to adsorption) and the amount of analyte extracted from a sample is independent of the matrix composition. This interaction is much weaker and the degradation processes of unstable analytes are significantly less than adsorption on an active surface. Furthermore, the retaining capacity of the PDMS material is not influenced by other analytes because each analyte has its own partition equilibrium in the PDMS phase [21].

The SBSE variables, such as time, temperature, pH matrix, ionic strength and desorption conditions were optimized to reach drug partition equilibrium in shorter analysis time, and to obtain adequate sensitivity to work in the therapeutic interval. The sample volume, stirring speed and stir bar dimensions were maintained constant throughout optimization.

The sensitivity of the SBSE/HPLC-UV method was improved by diluting the samples with the acetate buffer solution, to pH 5.0, in which the drugs (phenobarbital and phenytoin, p*K*a 7.3 and 7.4 and carbamazepine, p*K*a 13.4) were partially in the non-ionic form that enable them to be extracted by the PDMS phase (Fig. 2). The sample dilution favours the stirring SBSE process. The addition of NaCl did not alter the efficiency of the SBSE process.

Fig. 3 shows representative time extraction profiles (15–60 min) in different temperature values (38–70 °C). We observed that an

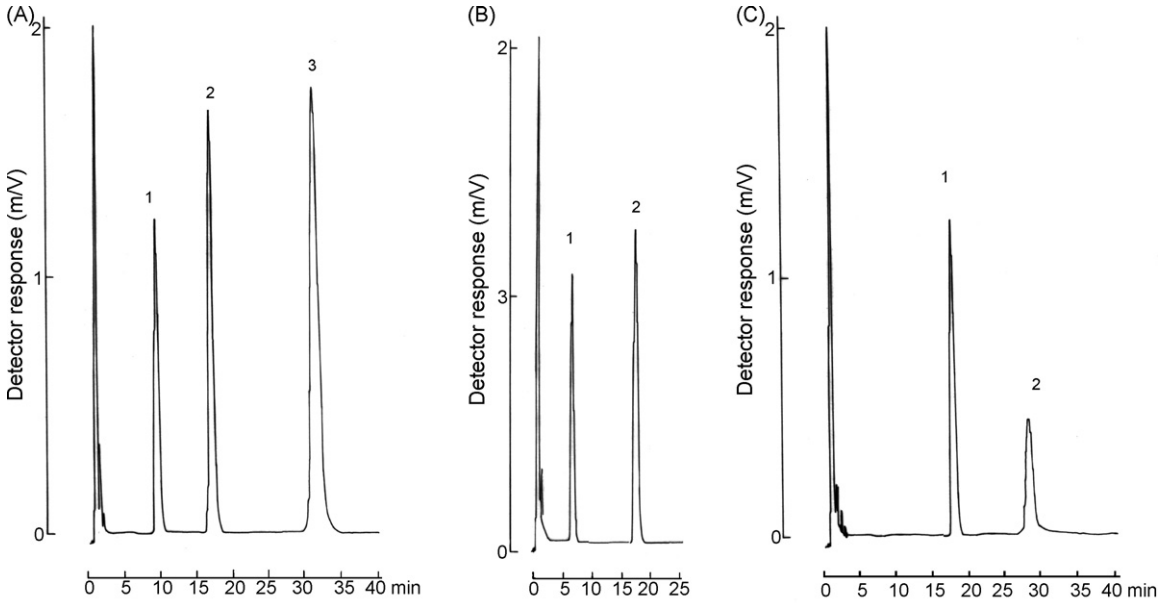

Fig. 6. Clinical plasma samples containing (A) (1) carbamazepine-10,11-epoxide, (2) IS (5-ethyl-5-*p*-tolylbarbituric acid) and (3) carbamazepine; (B) (1) Phenobarbital and (2) IS (5-ethyl-5-*p*-tolylbarbituric acid); (C) (1) IS (5-ethyl-5-*p*-tolylbarbituric acid) and (2) phenytoin.

increase in extraction temperature from 38 to 50 °C results in an increased amount of the extracted drugs. This occurs because at lower temperature, extraction is further from equilibrium, and therefore, a low level of analyte is extracted. At higher temperature under the same extraction time, however, the absorption-time profile will be closer to equilibrium, and therefore, the amount extracted is generally greater. The results obtained at 50 and 60 °C were very similar for some drugs, and followed by a lowering of extraction level at 70 °C. As a result, the SBSE conditions of temperature at 50 °C and time extraction of 50 min were selected.

Rinsing the stir bar slightly with 1.0 mL of the Milli-Q grade water to remove adsorbed proteins did not cause drug loss because the sorbed drugs are present in the PDMS phase.

The conditions of desorption were tested to ensure effective removal of the extracted analytes from the SBSE device. Acetonitrile yielded the best results of the desorption solvent investigated (dichrometane and mobile phase). The time of desorption was varied from 5 to 60 min (Fig. 4). The peak areas increased from 5 to 15 min, but remained nearly constant for desorption time of 15–60 min that corresponds to the complete desorption drugs from the SBSE bar (magnetic stirring), as no detectable carryover was observed. The magnetic stirring desorption (t = 70 °C), using acetonitrile was more effective than sonication performance in the same period (15 min, ambient temperature, t = 25 °C). It is likely that desorption process was factored at higher temperature. Therefore, thermal magnetic stirring was selected for desorption process.

The efficiency of the desorption process (magnetic stirring) was also confirmed by two consecutive acetonitrile desorptions, in which a unique step provides maximum yield. Furthermore, no evidence of interference was found during blank assays and the PDMS phase of the stir-bars was highly stable, with no evidence of deterioration. The robustness of the stir bar was confirmed by Frank and Sandra with over 50 extractions with a minimum loss of extraction efficiency [21]. Lambert et al. observed some degradation of the restricted access material stir bar coating, after 30 desorption cycles, using sonication process [41].

Although it is possible to re-use stir bars without additional clean up, a cleaning procedure was implemented using methanol for 30 min at 50 °C, under magnetic stirring rate of 1200 rpm, between extractions, to assure efficient protein removal.

Based upon these data, we concluded that the best SBSE experimental conditions, among those investigated for antiepileptic drugs assays (Figs. 2–4) were as follow: 1.0 mL of plasma sample modified with 4 mL acetate buffer (pH 5.0), extraction temperature at 50 °C, under magnetic stirring during 50 min, followed by the drugs off-line liquid desorption by immersion of the PDMS bar on acetonitrile at 50 °C, under magnetic stirring during 50 min.

The average recovery and linearity of the SBSE and LL/HPLC methods were determined with plasma samples spiked with analytical standards that result in a concentration ranging from the limit of quantification up to 40.0 μg mL^{-1}. The regression equations and corresponding correlation coefficients for all drugs are given in Table 1.

The accuracy and intra- and inter-days precision of the SBSE and LLE/HPLC methods were assessed by replicate analysis of plasma samples spiked with standards in three different concentrations (Table 2). The specificity (selectivity) of the developed method is demonstrated by representative chromatograms from drug-free human plasma sample, and the same sample spiked with antiepileptic drugs in therapeutic interval concentrations (Fig. 5A and B), which showed the ability of the method to measure the drugs unequivocally in the presence of endogenous plasma components. Additional drug-free human plasma samples from several individuals were tested and showed no significant interference at the retention times of the analytes.

Antiepileptic drugs may be prescribed in combination with different psychotropic agents and other drugs, so it was important to assess probable interferences from potentially co-administered compounds. No interference was observed after the extraction procedures (SBSE and LLE) of the drugs normally associated with antiepileptic drugs in patients with epilepsy: primidone, lamotrigine, zonizamide, haloperidol, chropromazine, risperidone, moclobemide and several benzodiazepines (diazepam, lorazepam, flurazepam, triazolam, clonazepam and alprazolam). The same was true for methyldopa, captopril, furosemide, hydrochlorothiazide, methoclopramide, acetaminophen, caffeine, salicylic acid, diclophenac, indomethacin, sulphamethazaxol,

metoprolol, propanolol, amiodarone, cimetidine, ranitidine and prednisone.

4. Clinical application of the method

The method was used for therapeutic drug monitoring of epileptic patients under the treatment of different antiepileptic drugs (CBZ, CBZ-E, PHB and PNT). Peak shapes and resolution were very similar to those obtained using spiked blank plasma and no interference was apparent. Drug concentrations found in these samples were 10.4 $\mu g\,mL^{-1}$ for carbamazepine, 10.6 $\mu g\,mL^{-1}$ for carbamazepine-10,11-epoxide, 13.8 $\mu g\,mL^{-1}$ for phenobarbital and 13.6 $\mu g\,mL^{-1}$ for phenytoin (Fig. 6). The plasma samples were colleted from patients with epilepsy in therapy with Tegretol® (400 mg/day), Gardenal® (140 mg/day) and Hidantal® (400 mg/day), respectively.

5. Conclusion

The SBSE/HPLC-UV methodology developed presents high sensitivity and enough reproducibility to permit the quantification of carbamazepine, carbamazepine-10,11-epoxide, phenytoin and phenobarbital in human plasma. The method has been successfully applied to analysis of real samples demonstrating that it works equally as well as the routine extraction method for therapeutic drug monitoring of antiepileptic drugs.

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