Journal of Pharmaceutical and Biomedical Analysis 53 (2010) 1319–1323

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Determination of topiramate in human plasma by capillary electrophoresis with indirect UV detection

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ARTICLE INFO

Article history:
Received 28 September 2009
Received in revised form 2 March 2010
Accepted 4 March 2010
Available online 11 March 2010

Keywords:
Capillary electrophoresis
Human plasma
Indirect UV detection
Solid-phase extraction
Topiramate

ABSTRACT

A rapid capillary zone electrophoresis method with indirect UV detection for the determination of topiramate in human plasma was developed and validated. The analyses were carried out with a background electrolyte composed of 10 mM sulfamethoxazole as chromophore in phosphate buffer (25 mM, pH 12.0); gabapentin was selected as the internal standard. Application of a voltage of +15 kV led to an analysis time shorter than 5 min; indirect UV detection was operated at 256 nm. Isolation of topiramate from plasma was accomplished by a carefully implemented solid-phase extraction procedure on C18 cartridges. The method provided a linear response over the concentration range of 2–60 μg of topiramate per mL of plasma. The limit of detection (LOD) was 0.8 μg mL^{-1} and the limit of quantitation (LOQ) was 2.0 μg mL^{-1}. Precision, expressed as relative standard deviation, was always lower than 7.3%, extraction yields were always greater than 92%. The results obtained analysing plasma samples from epileptic patients undergoing therapy with topiramate were satisfactory in terms of precision and selectivity.

1. Introduction

Topiramate (2,3:4,5-bis-O-(1-methylethylidene)-D-fructopyranose sulfamate, TPR) is an antiepileptic drug with a monosaccharide structure, chemically unrelated to other classes of antiepileptics. It is currently used as an adjunctive agent for the control of partial and generalised seizures in both adults and children [1], but is also approved and frequently used for the prevention of migraine [2]. Moreover, there is growing evidence in the literature [3] to support the use of TPR in both the treatment of alcohol withdrawal and the prevention of alcohol relapse [4]. However, none of these indications have yet been approved by the FDA.

The exact mechanism of action is still not completely known; however, TPR influences multiple systems: it probably blocks voltage-dependent sodium channels, enhances GABAergic transmission, acts as antagonist at AMPA/kainate glutamatergic receptors and inhibits carbonic anhydrase [5].

TPR is administered as Topamax® tablets (sprinkle capsules and generic formulations also exist) at daily doses usually ranging from 200 to 400 mg, however up to 1600 mg day^{-1} can be applied when needed. The drug is rapidly absorbed (within 2 h) and bioavailability is about 80% [6]. Metabolism is scarce: no active metabolite is known and the parent drug is almost completely excreted unmodified in the urine. Therapeutic plasma levels are generally considered to be in the 5–20 μg mL^{-1} range [7]. Side effects are usually dose-dependent, thus more frequent and severe with daily doses above 400 mg; vertigo, drowsiness, ataxia, headache and difficulty in thinking and concentrating are the most frequent ones [8]. Since TPR also inhibits carbonic anhydrase, cases of nephrolithiasis and metabolic acidosis have been observed, especially in cases of polypharmacy with diuretics, such as acetazolamide [9].

As with several other antiepileptics, constant monitoring of TPR plasma levels is recommended to obtain optimal therapeutic results [10]. However, since the drug does not possess any significant chromophores (see structure in Fig. 1), its quantitation by conventional spectroscopic means is not straightforward.

Only one paper [11] reports a method, which makes use of HPLC and UV detection for the analysis of TPR in human serum, but it requires derivatisation using the fluorescent reagent 9-fluorenylmethyl chloroformate in order to make the molecule detectable. Another method by the same authors [12] adopts another fluorescent labelling agent (4-chloro-7-nitrobenzofurazan) and fluorescence detection, which enables the determination of TPR at concentrations down to 0.010 μg mL^{-1}.

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doi:10.1016/j.jpba.2010.03.007

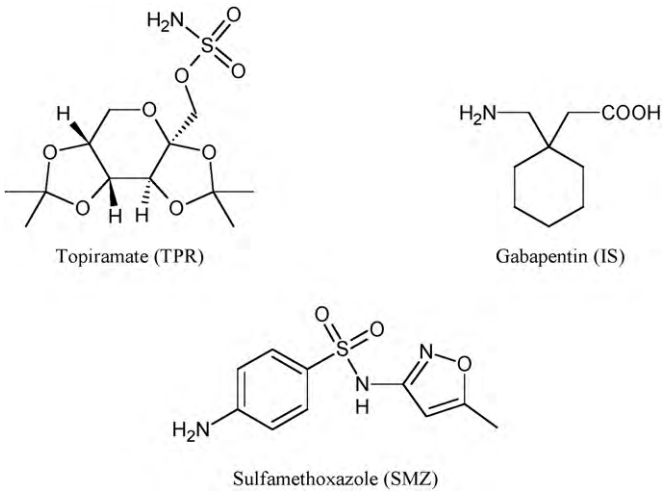

Fig. 1. Chemical structures of topiramate (TPR), gabapentin (IS) and sulfamethoxazole (SMZ).

Other papers avoid the derivatisation step and use mass spectrometric detection [13–15].

An alternative to chromatography for ionisable molecules is capillary electrophoresis (CE). TPR is a slightly acidic compound, and can thus potentially be analysed by CE at high pH. In order to avoid derivatisation when using a UV absorbance detector, we have applied indirect UV detection. Although the sensitivity of this detection method is not outstanding, it can be sufficient since the therapeutic concentration of TPR in biological fluids ranges in the tens of $\mu g\,mL^{-1}$. It was thus the aim of this paper to develop a fast, feasible and validated method for the quantitative analysis of TPR in human plasma based on capillary zone electrophoresis (CZE). No method currently exists for the analysis of TPR in biological fluids by CZE; only its impurities (sulphate and sulphamate) have been analysed in the pure compound with this technique [16].

2. Experimental

2.1. Chemicals and reagents

All chemicals and reagents were of analytical grade or better. Topiramate, gabapentin used as the internal standard (IS), sulfamethoxazole, potassium dihydrogen phosphate, and methanol were purchased from Sigma–Aldrich (St. Louis, MO, USA). Dibasic sodium phosphate, sodium chloride and potassium chloride were from Carlo Erba (Milan, Italy). Ultrapure water (18.2 MΩ cm), obtained by means of a Millipore (Billerica, MA – USA) Milli-Q apparatus, was used for the preparation of all solutions.

2.2. Instrumentation and electrophoretic conditions

All CZE experiments were carried out using a ^{3D}CE apparatus (Agilent Technologies, Palo Alto, CA, USA) equipped with a photodiode array (PDA) detector. Uncoated fused silica capillaries (75 μm I.D., 375 μm O.D., 48.5 cm total length, 40.0 cm effective length) from Composite Metal Services (Ilkley, UK) were used. The indirect UV determination of TPR was performed using 10 mM sulfamethoxazole as the chromophore in a phosphate buffer (25 mM, pH 12.0) as the background electrolyte (BGE). It was prepared as follows: 88.7 mg of potassium dihydrogen phosphate were dissolved in about 40 mL of water; this solution was then adjusted to pH 12.0 with 1 M NaOH and made up to 50 mL in a volumetric flask. Finally, 12.7 mg of sulfamethoxazole were weighed and dissolved in 5 mL of the phosphate buffer. The BGE was filtered through a cellulose acetate syringe filter (0.20 μm, Albet-Jacs-020-25) prior to use.

Injection was carried out by pressure at the anodic end of the capillary at 50 mbar for 5 s. The applied voltage was set at +15 kV, and the capillary was thermostatted at 25.0 °C. The detection wavelength was 256 nm.

Before use, the new capillary was conditioned by rinsing with 1 M sodium hydroxide, water, and then with the BGE for 10 min each. After each run the capillary was rinsed with BGE for 2 min. For storage overnight, the capillary was washed with water and additionally with 1 M sodium hydroxide, and again with water (rinsing time was 5 min each).

2.3. Preparation of standard solutions

The stock solutions ($2.00\,mg\,mL^{-1}$) of TPR and the IS were prepared by dissolving a suitable amount of pure TPR substance in 10 mL of methanol. Standard solutions of the individual compounds were prepared by diluting suitable amounts of each stock solution with water. The standard working solutions were prepared every day, while the stock solutions of the analyte and the IS in methanol were stable for at least three months when stored at −20 °C, as assessed by CE analysis.

2.4. Sample collection and preparation

Blood samples were collected from patients of the Division of Psychiatry, University of Parma (Italy) under polypharmacy with antipsychotics and TPR, and the blood put into vials containing EDTA as the anticoagulant. The blood was immediately centrifuged for 20 min at 3000 rpm and the supernatant plasma was frozen and maintained at −20 °C until analysis. The same procedure was used to obtain plasma from the blood of healthy volunteers ("blank" plasma).

Sample pre-treatment was carried out by solid-phase extraction (SPE) using reversed-phase Varian (Harbor City, CA, USA) BondElut C18 cartridges (100 mg, 1 mL).

Before use, the cartridges were conditioned by flushing them 5 times with 1 mL of methanol and then equilibrated with 1 mL of 100 mM KH_2PO_4 5 times.

Plasma aliquots (500 μL) were spiked with the IS (100 μL) and mixed with 1 mL of 100 mM KH_2PO_4. The solution obtained was loaded onto the previously conditioned cartridge. The washing procedure consisted of 1 mL of 100 mM KH_2PO_4, followed by 1 mL of water.

Elution was carried out with 2 mL of methanol. The eluate was then dried by means of a rotary evaporator and reconstituted with 100 μL of ultrapure water.

2.5. Method validation

All assays were carried out according to USP XXIX [17] guidelines.

2.5.1. Linearity

A calibration curve was obtained by spiking blank plasma with seven different concentrations of TPR and constant concentration of the IS, followed by the SPE procedure and injection. The calibration graph was calculated from the measured TPR/IS area ratio values as function of the analyte concentrations added to blank plasma by means of the least square method.

2.5.2. Precision

The blank plasma was spiked with TPR at three different concentrations (with constant IS concentration) to give final TPR concentrations corresponding to the lower limit, a middle value

and the upper limit of the calibration curve. Sample pre-treatment and CE analysis were then carried out. The procedure was repeated six times within the same day to obtain repeatability values and six times over six different days to obtain intermediate precision values.

2.5.3. Extraction yield

Six different samples of blank plasma spiked with three different concentrations of TPR (as reported under Section 2.5.2) were subjected to the SPE procedure and analysed. The mean absolute recovery values of the analyte were calculated by comparing the peak area values obtained from the analysis of spiked plasma samples, to those obtained from the analysis of standard solutions at the same concentration (referred to the injected samples).

2.5.4. Accuracy

The accuracy of the method was evaluated by means of recovery studies. Appropriate amounts of the analyte (corresponding to the lower limit, a middle value and a high value of the calibration curve) were added to plasma samples containing known amounts of TPR (i.e. previously analysed samples). The spiked samples were submitted to the SPE procedure and analysed. Recovery values of the analyte were calculated by interpolating the peak area ratios on the calibration curve. The procedure was repeated three times in the same day to obtain standard deviation (SD) values.

3. Results and discussion

3.1. Indirect UV detection and CE conditions

Topiramate does not possess chromophores and thus cannot be detected with sufficient sensitivity using UV detectors; moreover, fluorescence labelling is not easily accomplished. Indirect UV detection would be an alternative to overcome these detection problems. It consists in the addition of a UV absorbing molecule (the probe) to the BGE, which is replaced by the analyte with a like charge during the electrophoretic run, and leads to a negative peak in the recorded electropherogram. In a previous paper, we applied this detection method for the determination of the antiepileptic agent valproate [18], using benzoic acid as the probe. It should be mentioned that the introduction of a probe into the BGE always leads to a multi-component electrolyte system, which can show an irregular behaviour with resonance effects, system peaks with non-zero mobilities and oscillation phenomena [19]. The first step was directed to obtain an at least approximate electrophoretic mobility of TPR, since peak symmetry (and in many cases sensitivity and detection limit) improves with the electrophoretic mobility of the probe approaching that of the analyte. Considering the chemical properties of topiramate, a BGE with a highly basic pH was taken into consideration. By using a phosphate buffer at pH 12 as the BGE and injecting high concentrations of topiramate, it was possible to identify the TPR peak by direct UV detection at 195 nm. TPR showed an effective anionic mobility of $2.1 \times 10^{-8}\ m^2\ V^{-1}\ s^{-1}$.

Several compounds were taken into account as potential probes, including benzoic acid, paracetamol and sulfamethoxazole (SMZ). SMZ was chosen as the most suitable compound: in fact, it possesses a sulphonamide function (see Fig. 1), with similar properties as the sulfamate group in TPR, has a mobility close to that of TPR and good absorptivity with a maximum at 256 nm. Indeed, with a BGE with SMZ added as chromophore, TPR was detected as a negative peak.

Upon variation of the SMZ amount in the BGE at concentrations between 1 and 20 mM it was found that the chromophore concentration influenced both the sensitivity and the peak shape (TPR concentration here was 50 μg mL^{-1}, or 0.15 mM). Peak shape improved with the increase of the SMZ concentration, with tailing factors lower than 1.3 for SMZ concentrations higher than 5 mM; however, at values above 10 mM a reduction of the sensitivity was observed. For this reason, a concentration of 10 mM was chosen as the best compromise between good peak shape and high sensitivity. The pH value of the BGE was varied between pH 10.0 and 12.5, since pH values above 12.5 are not favourable in CZE due to the high electric current caused by the high concentrations of OH^- ions with large mobility. As expected, the TPR mobility decreased when decreasing the pH of the BGE, the consequence of the reduced charge on the analyte (the calculated pK_a of TPR is 9.3), also reducing the resolution from the IS, which was <1.2 for pH values between 10 and 11. Increasing the pH, on the other hand, led to longer migration and analysis time. For these reasons, pH 12.0 was chosen as the best compromise in terms of resolution and analysis time.

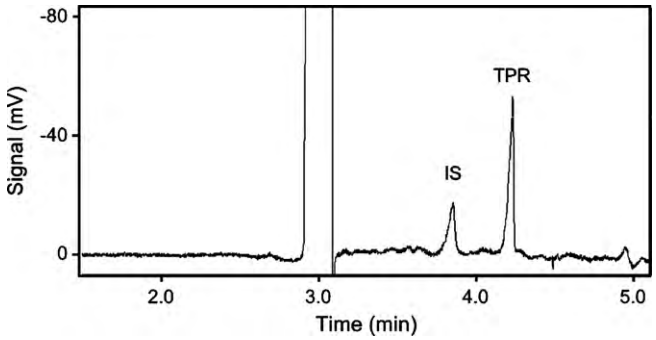

Fig. 2. Electropherogram of a standard solution containing 50 μg mL^{-1} of TPR and 25 μg mL^{-1} of the IS. Electrophoretic conditions: BGE, phosphate buffer (25 mM, pH 12.0) containing 10 mM SMZ; untreated fused silica capillary with 48.5 cm total length, 40.0 cm effective length, 75 μm i.d.; temperature, 25 °C; voltage, +15 kV; injection, pressure (50 mbar × 5 s); indirect detection at 256 nm.

Moreover, a tendency toward an improved peak shape upon reduction of the buffer concentration was observed. A concentration of the phosphate buffer corresponding to 25 mM (current was 50–60 μA) gave the best results. Lower concentrations were not investigated, as the buffering properties of the BGE could then be affected.

3.2. Choice of the internal standard

The choice of the internal standard is governed by the fact that it should have similar chemical and physical properties than the analyte. In case of indirect detection its mobility should also be in reasonable relation to that of the probe, otherwise strong peak distortion would result. Valproic acid, hydroxyisobutyric acid, vigabatrin and gabapentin were tested. The first two compounds could not be detected; in contrast, vigabatrin and gabapentin were both detected, but gabapentin was finally chosen because it migrates faster than the analyte, and has thus no influence on the total analysis time.

3.3. Analysis of standard solutions

The electropherogram obtained from a standard solution containing TPR (50 μg mL^{-1}) and the IS (25 μg mL^{-1}) is shown in Fig. 2. The analyte and the IS are migrating as anions after the EOF (which appears as a deep negative peak at 3 min). Complete resolution between the analyte and the IS is obtained. Migration times are 4.3 and 3.7 min for TPR and the IS, respectively, therefore a complete electrophoretic run is completed within 5 min.

Table 1
Extraction yield and precision results.

	Concentration ($\mu g\,mL^{-1}$)		Repeatability[a]		Intermediate precision[a]		Extraction yield (%)[b]
	Standard solutions	Spiked plasma	Standard solutions	Spiked plasma	Standard solutions	Spiked plasma	
TPR	10.0	2.00	3.7	3.7	5.4	7.2	93
	100	20.0	1.7	3.0	3.8	4.3	98
	300	60.0	0.3	4.0	2.3	3.3	94
IS	25.0		–	–	–	–	75

[a] RSD%, $n = 6$.
[b] Recovery percentage, average value.

3.4. Sample pre-treatment

To remove biological interference and to reach appropriate LOD and LOQ values, a sample pre-treatment and pre-concentration procedure was implemented. Protein precipitation was not considered, because it has the main drawback of diluting the sample. The liquid/liquid extraction initially tested out resulted not being appropriate, because of the different behaviour of TPR and the IS. Therefore, a solid-phase extraction (SPE) procedure was evaluated. Different cartridges were investigated, including C2, C8, C18, HLB (hydrophilic–lipophilic balance), cyanopropyl and MAX (mixed-mode reversed-phase and anionic exchange) sorbents. Among these, only the C18 phase gave satisfactory results in terms of sample clean-up and extraction yields.

An aqueous KH_2PO_4 solution (100 mM) gave the best results for sample loading and washing, as previously observed by other authors for the IS [20,21]. Finally, elution with 2 mL of methanol granted good extraction yields of both the analyte and the IS.

Samples were then dried and reconstituted in order to obtain sample pre-concentration and reach LOQ values required to analyse therapeutic TPR levels. Water turned out to be the best solvent to re-dissolve the samples, because it is a good solvent for both the analyte and the IS and also granted good peak shapes, which were strongly influenced by the solvent used for sample dilutions. The developed SPE procedure was tested out on blank plasma samples from healthy volunteers. A representative electropherogram is shown in Fig. 3a. A complete removal of the biological interferences was obtained. Fig. 3b shows the electropherogram from the analysis of a blank plasma sample, spiked with TPR ($10\,\mu g\,mL^{-1}$) and the IS. This concentration corresponds to $50\,\mu g\,mL^{-1}$ in the injected sample, due to the sample pre-concentration obtained with the SPE procedure. The electropherogram does not show remarkable differences if compared to that obtained from the analysis of a standard solution (compare with Fig. 2).

3.5. Method validation

The *extraction yield* results, expressed as absolute recovery values, are reported in Table 1. Extraction yields were always ≥93% for TPR and ≥75% for the IS.

The *linearity range* was assessed on blank plasma spiked at six different concentrations: 2.00, 5.00, 10.0, 20.0, 40.0 and $60.0\,\mu g\,mL^{-1}$, while the IS was maintained constant at the concentration value $25.00\,\mu g\,mL^{-1}$. Linearity in the range between 2.00 and $60.0\,\mu g\,mL^{-1}$, expressed by the correlation coefficient, was $r^2 = 0.9972$. The corresponding equation was $y = 0.0459x - 0.169$ (the slope of the line corresponds to the detection sensitivity, here given in μg^{-1} mL of TPR).

The *limit of quantitation* (LOQ) and *limit of detection* (LOD) corresponded to 2.0 and $0.8\,\mu g\,mL^{-1}$ TPR, respectively.

The precision of the method was evaluated both as *repeatability* and *intermediate precision* and expressed as the relative standard deviation percentage (R.S.D.%); the results are reported in Table 1. The reproducibility of the method is always better than 7.3% R.S.D.

Selectivity assays were carried out by analysing several potentially interfering compounds: all tested compounds are listed in Table 2. Apart from the antiepileptic agents, tested compounds were those, which can be negatively charged, a requirement for detection using the present conditions. The separation selectivity of the method is appropriate. Several compounds are not detected and, among the detected ones, only aspartic acid co-migrates with the analyte. However, experiments showed that the SPE procedure adopted completely removes this compound; it can therefore be considered as non-interfering.

3.6. Analysis of plasma samples obtained from patients

After validation, the method was applied to the analysis of plasma samples obtained from patients. The samples were subjected to the described sample clean-up procedure and then analysed. The TPR/IS peak area ratios were interpolated in the calibration curve obtained by analysing spiked plasma samples and the corresponding concentration of TPR was derived.

Fig. 4 shows a representative electropherogram obtained from the analysis of a plasma sample from a patient under treatment with TPR ($50\,mg\,day^{-1}$). Despite the patient being subjected to polypharmacy (risperidone, brotizolam, biperidene and calcitriol), TPR is detected without interference from the biological matrix or the other co-administered drugs. The concentration of TPR resulted

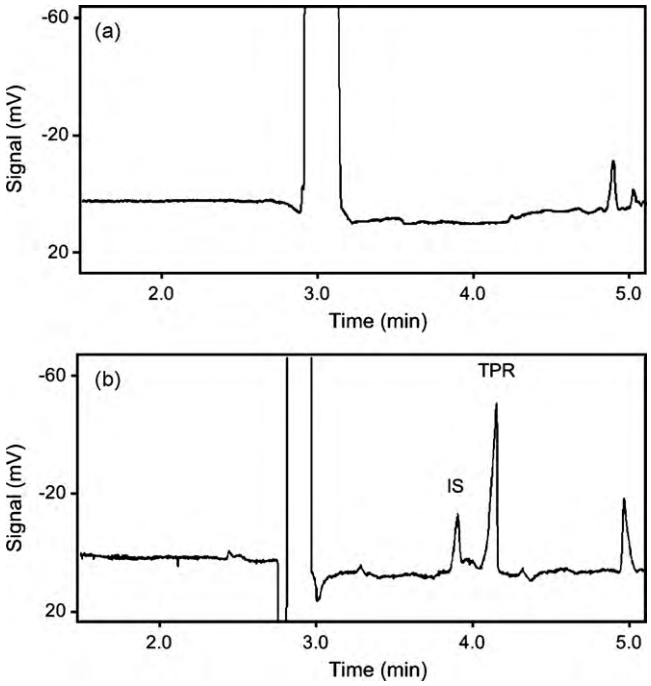

Fig. 3. Electropherogram of (a) a blank plasma sample; (b) a blank plasma sample spiked with $10\,\mu g\,mL^{-1}$ of TPR and $25\,\mu g\,mL^{-1}$ of the IS. Electrophoretic conditions: as in Fig. 2.

Table 2
Compounds tested for selectivity assays.

Compound	t_m (min)[a]	Interference[b]
Topiramate (analyte)	*4.3*	
Gabapentin (IS)	*3.7*	
Antiepileptic agents		
Valproic acid	4.8	–
Carbamazepine	n.d.	–
Vigabatrin	5.1	–
Primidone	n.d.	–
Levetiracetam	n.d.	–
Oxcarbamazepine	n.d.	–
Others		
Diclofenac	4.5	–
Indomethacin	4.5	–
Acetyl salicylic acid	n.d	–
Ascorbic acid	n.d	–
Succinic acid	n.d.	–
Glutamic acid	4.5	–
Aspartic acid[c]	*4.3*	–
Glycyrrhetic acid	n.d.	–
2-hydroxybuthyric acid	n.d.	–
N-acetylcysteine	n.d.	–
Lactic acid	4.8	–

[a] n.d.= not detected
[b] interference: –, no; +, yes.
[c] removed by the sample pre-treatment procedure.

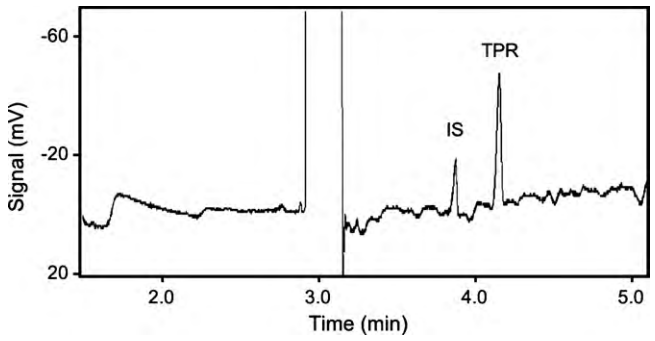

Fig. 4. Electropherogram of a plasma sample from a patient subjected to polypharmacy with risperidone, brotizolam, biperiden and calcitriol and undergoing treatment with 50 mg day^{-1} of TPR. Plasma was spiked with 25 μg mL^{-1} of the IS. Electrophoretic conditions: as in Fig. 2.

to be 9.7 μg mL^{-1}, in good agreement with the expected concentration for the administered dose.

Accuracy was evaluated by means of recovery assays. Patient plasma samples were added to the same plasma samples, which were analysed again. The recovery of the added TPR was then calculated. The mean recovery value was 90% (SD = 3), thus accuracy was satisfactory.

4. Concluding remarks

A method based on CZE was developed for the analysis of the antiepileptic agent TPR in human plasma samples. As the molecule does not possess sufficient UV absorbance, indirect UV detection was carried out, thus enabling the determination of the analyte without any requirement for sample derivatisation or mass spectrometric detection. CZE analysis is carried out within less than 5 min. The sample pre-treatment procedure based on SPE enables the removal of biological interference and satisfactory extraction yields. The method is suitable for the therapeutic drug monitoring of TPR in patients under treatment with Topamax®.

Acknowledgments

This study was supported by grants from MIUR (Ministero dell'Istruzione, dell'Università e della Ricerca, Italy), RFO (ex-60%) funds.

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