

Optimization and validation of bioanalytical SPE – HPLC method for the simultaneous determination of carbamazepine and its main metabolite, carbamazepine-10, 11-epoxide, in plasma

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Abstract

Carbamazepine is widely used as an antiepileptic drug in the treatment of partial and generalized tonic-clonic seizures. Carbamazepine 10,11-epoxide is the most important metabolite of carbamazepine, because it is a pharmacologically active compound with anticonvulsant properties. According to that, the routine analysis of carbamazepine 10,11-epoxide along with carbamazepine may provide optimal therapeutic monitoring of carbamazepine treatment.

The aim of this study was to optimize and validate a simple and reliable solid - phase extraction method followed by RP-HPLC for the simultaneous determination of plasma levels of carbamazepine and carbamazepine-10,11-epoxide, in order to assure the implementation of the method for therapeutic monitoring.

The extraction of the analytes from the plasma samples was performed by means of a solid-phase extraction procedure. The separation was carried out on a reversed-phase column using isocratic elution with acetonitrile and water (35:65, v/v) as a mobile phase. The temperature was 30°C and UV detection was set at 220 nm.

The extraction yield values were more than 98% for all analytes, measured at four concentration levels of the linear concentration range. The method displayed excellent selectivity, sensitivity, linearity, precision and accuracy. Stability studies indicate that stock solutions and plasma samples were stable under different storage conditions at least during the observed period.

The method was successfully applied to determine the carbamazepine and carbamazepine-10,11-epoxide in plasma of epileptic patients treated with carbamazepine as monotherapy and in polytherapy.

In conclusion, the proposed method is suitable for application in therapeutic drug monitoring of epileptic patients undergoing treatment with carbamazepine.

Key words: carbamazepine, carbamazepine 10, 11-epoxide, plasma, solid-phase extraction, high-performance liquid chromatography, validation

Introduction

Carbamazepine (CBZ) (5-H-dibenze [*b,f*] azepine-5-carboxamide), a tricyclic lipophilic compound is a first line antiepileptic drug used in the treatment of partial and gen-

eralized tonic-clonic seizures. The main metabolic pathway of CBZ is epoxidation, which is catalyzed primarily by the cytochrome P450 enzyme CYP3A4 and results in the formation of carbamazepine-10,11-epoxide (CBZ-EP). From a clinical standpoint, CBZ-EP is the most important of the 33 metabolites of CBZ that have been isolated, because it is a pharmacologically active compound with anticonvulsant properties, as its parent compound (Sillanpaa

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et al., 2009).

Carbamazepine is highly bound (75% to 80%) to plasma proteins, including albumin and α_1 -acid glycoprotein, while the epoxide binding is less strong (50% to 60% bound). Since, the epoxide has a greater percentage in the free form (pharmacologically active fraction) and is equipotent to the parent drug, epoxide is assumed that contributes significantly to both therapeutic and adverse effects (Sillanpaa et al., 2009; Potter and Donnelly, 1998).

CBZ is usually administered at oral daily doses ranging from 400 to 1600 mg, which result in CBZ plasma concentrations in therapeutic range of 4-12 $\mu\text{g/ml}$. With monotherapy of CBZ, the steady-state of CBZ-EP plasma concentration is equivalent to 20%-25% of the parent drug (Sillanpaa et al., 2009; Shen et al., 2001; Liu and Delgado, 1999).

Carbamazepine has a narrow therapeutic index, and the relationship between dose and plasma concentrations of carbamazepine may be unpredictable because of differences in genetics, age, gender, absorption, autoinduction and disease state between individuals. Also, the presence of numerous clinically significant drug interactions supports the need of using therapeutic monitoring of carbamazepine as an essential tool in designing a safe and effective therapeutic regimen for patients with epilepsy (Sillanpaa et al., 2009; Patsalos et al., 2008).

CBZ-EP was not routinely measured, though there were patients for whom high concentrations of this metabolite could be responsible for otherwise unexplained toxicity (Patsalos et al., 2008).

Therefore, the routine analysis of CBZ-EP along with CBZ may provide optimal therapeutic monitoring of CBZ treatment (Potter and Donnelly, 1998).

Most commonly applied methods for the routine monitoring of CBZ alone in plasma were commercial reagent-based techniques like as fluorescence polarization immunoassay (FPIA) and enzyme multiplied immunoassay technique (EMIT), especially in clinical settings (Kang et al., 2011). However, the antibodies used in these procedures, may sometimes cross-react with CBZ metabolites, leading to an overestimation of CBZ plasma levels, and to the lower specificity of the immunoassays (Kang et al., 2011; Eadie, 1998; Wilson et al., 1992).

A number of HPLC methods for simultaneous determination of CBZ and its metabolites in plasma have been published, using pretreatment techniques such as liquid-liquid extraction (Oh et al., 2006; Moreno et al., 2003; Matar et al., 1999; Pienimaki et al., 1995; Miller and Vrandeick, 1993), solid-phase extraction (Vermeij and Edelbroek, 2007; Mandrioli et al., 2001), deproteinization (Leite et al., 2009; Yoshida et al., 2006) and stir bar-sorptive extraction (Querioz et al., 2008). However, with some of these techniques the obtained extraction yields were not satisfactory, most of them were time-consuming, some required expensive instrument, rendering them not appropriate for routine drug monitoring. Deproteinization is simple, fast and

costless technique, but offers minimal selectivity and sample cleanup as it only removes gross levels of protein from a sample prior to analysis. Oh et al. (2006) described a liquid-liquid extraction with methyl *tert*-butyl ether as sample pretreatment for simultaneous determination of CBZ and CBZ-EP in plasma. Even though the method showed excellent sensitivity, the extraction recoveries for CBZ-EP were less than 90%, the analysis was time-consuming, and also, the chromatographic run was 30 min. Another method for simultaneous determination of CBZ and CBZ-EP in plasma (along with phenytoin and phenobarbital) was described by Queiroz et al. (2008). They developed a stir bar-sorptive extraction, but this procedure required a very long analysis time (100 min), expensive instruments, and the analysis was cumbersome.

LC-MS methods (Sener et al., 2007; Zhu et al., 2005; Van Rooyen et al., 2002) have also been reported for the determination of CBZ and its metabolites in biological fluids, but although they provide improved sensitivity and specificity compare with other analytical methods, MS procedures were more sophisticated and more expensive than HPLC-UV.

Therefore, HPLC with UV detection was the method of choice for the simultaneous monitoring of CBZ and CBZ-EP in biological fluids, after suitable pretreatment.

The aim of this study was to optimize and validate a simple and reliable solid - phase extraction method followed by RP-HPLC with UV detection for the simultaneous determination of plasma levels of carbamazepine and its main metabolite, carbamazepine 10, 11-epoxide, in order to obtain results of adequate quality and reliability to assure the implementation of the proposed bioanalytical method for the therapeutic monitoring of carbamazepine and its active metabolite.

Material and methods

Chemicals and solutions

Carbamazepine (CBZ), its metabolite carbamazepine-10,11-epoxide (CBZ-EP) and nitrazepam used as the internal standard,(IS), were purchased from Sigma-Aldrich (St. Luis, MO, USA). Methanol and acetonitrile, HPLC grade, were obtained from Merck (Darmstadt, Germany). For all analysis HPLC grade water purified with a TKA_LAB Reinstwasser system (Niederelbert, Germany) was used. OASIS® HLB cartridges (30mg/1mL) used for the solid-phase extraction (SPE) procedure were supplied by Waters (Milford, MA, USA).

Stock solutions of CBZ (500 $\mu\text{g/ml}$), CBZ-EP (200 $\mu\text{g/ml}$) and the IS (500 $\mu\text{g/ml}$) were prepared by dissolving each compound in methanol. The stock solutions were stable for at least 3 months stored at 2-8 °C. Working solutions were prepared daily from stock solutions by dilution with purified water.

Apparatus and chromatographic conditions

The assay was carried out on Agilent 1100 HPLC system equipped with a vacuum degasser (G1322A Degasser), quaternary pump (G1311A QuatPump), autosampler (G1313A ALS), column compartment (G1316A COL-COM), diode array detector (G1315B DAD), and ChemStation for LC 3D software for data handling (Wilmington, DE).

Separation was performed on a reversed-phase column Zorbax Extend C18 (150 x 4,6 mm, 5 μ m) using isocratic elution with acetonitrile and water (35:65, v/v) as a mobile phase, at a flow rate of 1 ml/min. The temperature was 30°C, volume of injection 20 μ l and UV detection was set at 220 nm.

Human plasma sampling

Plasma samples from both, healthy volunteers (drug – free plasma for the method validation) and from the epileptic patients undergoing chronic CBZ therapy, were obtained from Clinic of Neurology, Faculty of Medicine, University “Ss Cyril and Methodius”, Skopje. The participation of each subject was voluntary and could be cancelled by any individual at any time during this study (according to the Helsinki II declaration). The Ethics Committee at the Faculty of Pharmacy and the Faculty of Medicine, Ss. Cyril and Methodius University – Skopje, approved the research protocol for this study and all volunteers signed the Study Informed Consent form.

Since CBZ has a relatively short half –life, sampling time in relation to dose ingestion was important for the interpretation of the drug concentration. Ideally, blood samples for TDM of CBZ should be drawn before the morning dose (Sillanpaa et al., 2009). Thus, blood samples were collected at 08.00 h in the morning, just before the first daily drug administration, into heparinised tubes (8-10 IU heparin/ml blood), and centrifuged at 3000 rpm for 10 min. The supernatant plasma was transferred into test tubes and frozen at - 20 °C until analysis.

Solid-phase extraction procedure (SPE)

Prior to analysis, plasma samples were thawed and allowed to equilibrate at room temperature. To 250 μ l of epileptic patient plasma, 200 μ l ultrapure water and 50 μ l of IS solution (5 μ g/ml) were added and the mixture was vortex-mixed for 30s.

The extraction of the analytes from the plasma samples was performed by means of a solid-phase extraction (SPE). For this purpose, OASIS® HLB cartridges (30mg/1ml) were used. The SPE procedure was carried out according to the following steps: a) conditioning with 1ml methanol; b) equilibration with 1 ml water; c) loading the plasma sample (of patients as described above and for validation purpose as described under the section *Calibration curves*); d) washing with 1ml 5% methanol. Cartridge dry-

ing for 30 s at - 20 kPa; e) elution with 500 μ l methanol. 20 μ l of the eluate was injected into the HPLC system.

Bioanalytical method validation

Method validation was conducted following the recommendations for validation of bioanalytical methods of EMA guideline (EMA, 2009).

According to the guideline, a complete method validation should be performed for any analytical method, new or based upon literature.

Selectivity

Selectivity was assessed by comparing the chromatograms of drug-free plasma (blank plasma) from six sources and those obtained from plasma samples spiked with analytes and internal standard.

Calibration curves

The calibration standards consist of drug free plasma samples spiking with known concentration of the analyte. Aliquots of 50 μ l of analytes working solutions (CBZ and CBZ-EP) at 7 different concentrations, 50 μ l of IS working solution, and 100 μ l ultrapure water were added to 250 μ l of blank plasma. The resulting plasma concentrations were: 0.25 μ g/ml, 0.5 μ g/ml, 1.0 μ g/ml, 5.0 μ g/ml, 10.0 μ g/ml, 20.0 μ g/ml and 25.0 μ g/ml for CBZ and 0.1 μ g/ml, 0.25 μ g/ml, 0.5 μ g/ml, 1.0 μ g/ml, 2.5 μ g/ml, 5 μ g/ml and 10 μ g/ml for CBZ-EP, containing the IS at constant concentration of 5 μ g/ml. Calibration standards were subjected to the SPE procedure and injected into the HPLC. The obtained analyte - IS peak area ratios were plotted versus the respective analyte concentrations and the calibration curves for CBZ and CBZ-EP were constructed by means of the least-squares method.

Accuracy and precision

The quality control samples (QC samples) were used to assess the accuracy and precision of the method. Four levels of QC samples were prepared at the concentrations of 0.25 μ g/ml (lower limit of quantitation), 1.0 μ g/ml (low QC sample), 5.0 μ g/ml (medium QC sample) and 20.0 μ g/ml (high QC sample) for CBZ and at the concentrations of 0.1 μ g/ml (lower limit of quantitation), 0.5 μ g/ml (low QC sample), 2.5 μ g/ml (medium QC sample) and 5.0 μ g/ml (high QC sample) for CBZ-EP, in same way as described above for the calibration standards. Accuracy and precision were evaluated for the values of the QC samples obtained within a single run (the within run) and in different runs (the between-run). Within-run accuracy and precision were determined for five samples per concentration level at LLOQ, low, medium and high QC samples in a single run. Between-run accuracy and precision were assessed by

five determination per concentration per run at LLOQ, low, medium and high QC samples from three runs analyzed on two different days.

Recovery

Extraction yields were assessed at four concentration levels corresponding to the lower limit, low, medium and high point of each calibration curve (i.e. plasma concentration of 0.25 µg/ml, 1.0 µg/ml, 5.0 µg/ml and 20 µg/ml for CBZ, and 0.1 µg/ml, 0.5 µg/ml, 2.5 µg/ml and 5.0 µg/ml for CBZ-EP).

Stability

Stability tests were performed on three replicates of low and high QC samples after 24 h at room temperature (short term stability), after three freeze-thaw cycles, autosampler stability for 12 h, and after 90 days on samples stored at -20 °C (long term stability). Stability tests were also performed on stock solutions of analytes after 24 h at room temperature and after 3 months at 2-8 °C.

Results and discussion

Optimization of chromatographic condition

Many studies were conducted with the aim of developing new bioanalytical methods or improve existing methods for TDM of antiepileptic drugs.

In order to establish the chromatographic conditions for the determination of CBZ and CBZ-EP in plasma, number of literature data were reviewed and many experiments were made. During the optimization of the chromatographic conditions different mobile phases (different types of organic solvents and different organic to aqueous phase ratio) were evaluated, in order to ascertain whether a faster separation is possible, which is an important fact for routine control. The experiments were made using the mixture of methanol/water, the mixture of methanol/acetonitrile/water, and the mixture of acetonitrile/water, in different ratio compared the organic and water phase.

The best results of good separation of all analytes were obtained with the mixture of acetonitrile-water (35 : 65, v/v), using a Zorbax Extend C18 (150 x 4,6 mm, 5µm) column at temperature of 30 °C and flow rate of 1ml/min.

Under these chromatographic conditions, CBZ, its active metabolite CBZ-EP and the internal standard were baseline separated in less than 7 min, in contrast to 30 min reported by Oh et al. (2006) and 20 min reported by Leite et al. (2009).

Solid-phase extraction (SPE) procedure

Application of a SPE technique was chosen for the sample pre-treatment, because it makes sample preparation more feasible, simple and accurate, less polluting than the

liquid-liquid extraction, and allows high extraction yields with good selectivity.

Mandrioli et al. (2001) performed a SPE procedure for extraction of CBZ and its five metabolites from human plasma using tetrahydrofuran for elution. However, the usage of this strong solvent caused peak broadening and interference, so its completely elimination from injection mixture was necessary.

In order to simplify the SPE procedure and considering the good solubility of CBZ and CBZ-EP in methanol, elution of the analytes from the cartridge was performed with methanol (instead of tetrahydrofuran), thus making the method less prone to interference. Furthermore, the last step of procedure, the dryness of the eluate, was avoided.

The optimized SPE procedure included loading the conditioned cartridge with 250 µl of plasma, washing with 1 ml of 5% methanol and eluting the analytes with 500 µl of methanol. The extraction yield values were higher than 98% for CBZ and CBZ-EP, which were better than those reported in papers using liquid-liquid extraction (Oh et al., 2006) or SPE procedure (Mandrioli et al., 2001). The chromatograms of a blank plasma sample and of a blank plasma spiked with standard solutions of analytes and IS, subjected to the described SPE procedure, were presented in Fig. 1. No interference from endogenous plasma components was present. Moreover, the SPE procedure gave excellent results when was applied to plasma samples of patients undergoing chronic treatment with CBZ, both in monotherapy and polytherapy. No interfering peaks due to the co-administered drugs were eluted at the retention times of analytes (Fig. 3.).

Bioanalytical method validation

Validation of the bioanalytical method is a compulsory step to estimate the ability of the developed method to provide accurate results for its routine application (Rozet et al., 2011).

Selectivity

The selectivity of the method was assessed by analyzing six different drug free plasma samples and drug free plasma spiked with CBZ, CBZ-EP and nitrazepam as internal standard. The chromatograms of blank plasma and blank plasma spiked with analytes were presented in Fig. 1. The retention times for CBZ, CBZ-EP and IS were 2.8 min, 4.7 min and 6.6 min, respectively. No interfering peaks for endogenous compounds were observed in the retention times of CBZ, CBZ-EP and IS.

Calibration curves

Prior to carrying out the validation of the bioanalytical method the concentration range should be justified based on scientific information. The range should be covered by

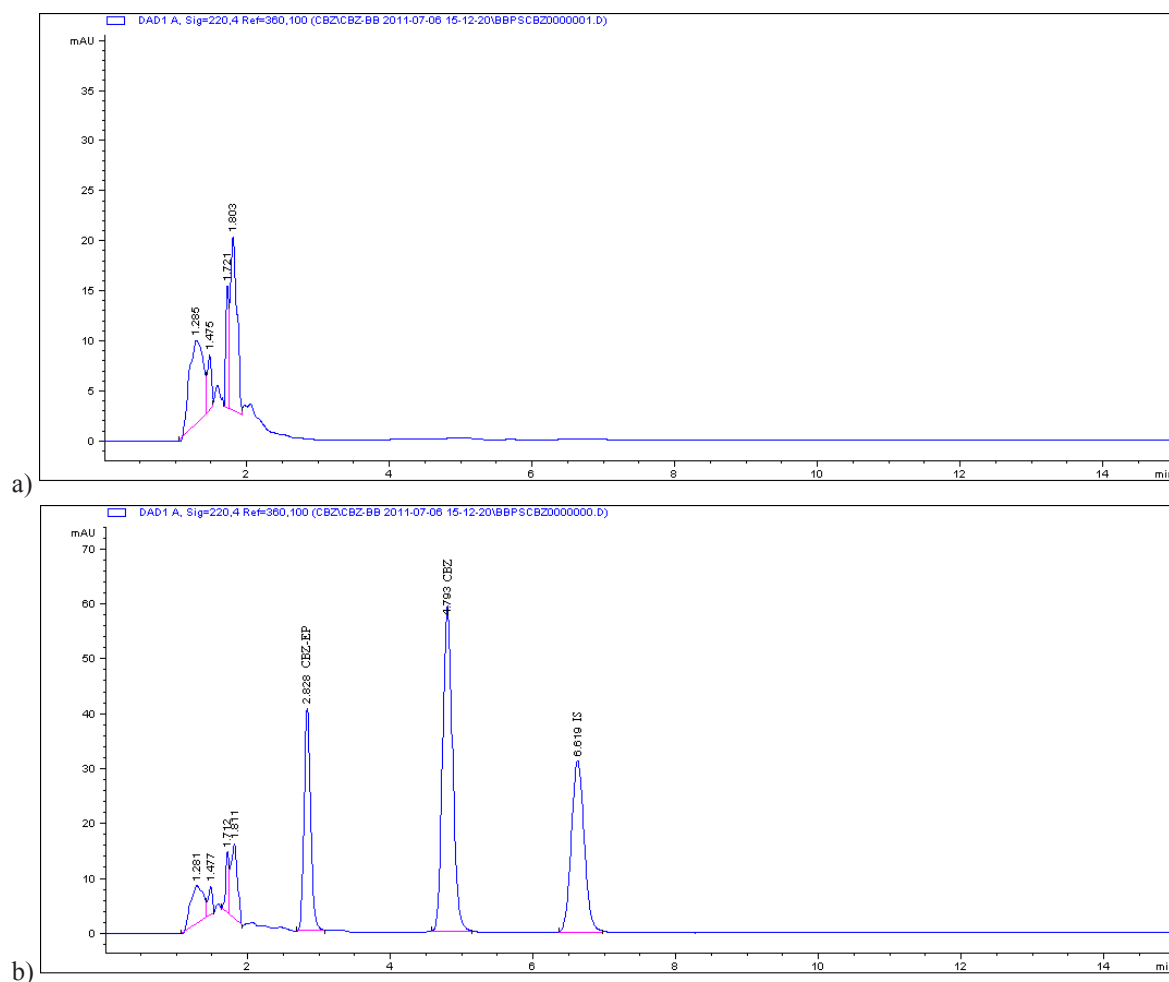


Fig. 1. Chromatograms of: a) blank plasma, and b) blank plasma spiked with 5.0 µg/ml CBZ, 2.5 µg/ml CBZ-EP and 5.0 µg/ml nitrazepam (IS), after the SPE procedure.

the calibration curve range, defined by the lower limit of quantitation (LLOQ) and the upper limit of quantitation (ULOQ). LLOQ is the lowest amount of analyte in a sample (plasma) which can be quantified reliably and should be adapted to expected concentrations and to the aim of the study.

The acceptable criterion for each standard concentration was $\pm 15\%$ of the nominal value, except for the LLOQ which was $\pm 20\%$.

Seven-point calibration curves were set up for CBZ and CBZ-EP within the concentration range of 0.25 - 20.0 µg/ml for CBZ and 0.1 - 10.0 µg/ml for CBZ-EP. The calibration curves were linear over the defined concentration ranges according to the levels of each compound expected in patients' plasma samples, including LLOQ.

The regression equations were $y = 154.87x - 19.828$ with a coefficient of determination $r^2 = 0.9989$ for CBZ and $y = 117.96x + 1.219$ with a coefficient of determination $r^2 = 0.9991$ for CBZ-EP.

Accuracy and precision

The within – run and between – run accuracy and precision were shown in Table 1. All the results of the tested samples were within recommended limits. Within-run assay precision ranged from 0.6% to 1.2% for CBZ and from 0.7% to 2.0% for CBZ-EP, while within-run assay accuracy ranged from 98.4% to 101.3% and 97.0% to 100.6%, for CBZ and CBZ-EP, respectively. The between-run precision and accuracy, ranged from 0.9% to 2.2% and 97.9% to 102.1% for CBZ, while for CBZ-EP the ranges were 0.9% to 3.1% and 96.7% to 102.7%, respectively.

Recovery (Extraction yield)

Recovery describes the extraction efficiency of the analytical process. The recovery was calculated by comparison of the peak areas of analytes obtained from the SPE processed blank plasma sample previously spiked with analytes versus peak areas obtained from a post – extracted spiked blank plasma sample, which represent 100% recovery (Matuszewski et al., 2003).

Table 1. Accuracy and precision of carbamazepine and carbamazepine-10,11-epoxide in human plasma

Analyte	Nominal conc. (µg/ml)	Within-run assay (n=5)		Between-run assays (n=30)	
		accuracy (%)	precision (CV%)	accuracy (%)	precision (CV%)
CBZ	20.0	99.2	1.2	98.6	2.2
	5.0	99.5	0.8	98.5	1.1
	1.0	101.3	1.0	102.1	0.9
	0.25	98.4	0.6	97.9	1.8
CBZ-EP	5.0	100.6	1.1	100.9	1.0
	2.5	99.1	2.0	97.6	3.1
	0.5	97.3	0.8	96.7	1.7
	0.1	97.0	0.7	102.7	0.9

Table 2. Recoveries of carbamazepine and carbamazepine-10,11-epoxide from plasma (n=5)

Analyte	Nominal conc. (µg/ml)	Found conc. (µg/ml)	Recovery (%)
CBZ	20.0	19.70	98.5
	5.00	4.98	99.6
	1.00	0.99	99.0
	0.25	0.247	98.8
CBZ-EP	5.00	4.98	99.6
	2.50	2.48	99.2
	0.50	0.497	99.4
	0.10	0.099	99.1

Table 3. Stability of carbamazepine and carbamazepine-10,11-epoxide in human plasma under various conditions (n=3)

Nominal concentration (µg/ml) CBZ / CBZ-EP	Initial concentration t = 0 min (µg/ml) CBZ / CBZ-EP	Accuracy (%) CBZ / CBZ-EP
Short term stability (24h at room temperature)		
1.0 / 0.5	1.02 / 0.51	99.0 / 98.6
20.0 / 5.0	19.98 / 5.03	101.2 / 99.3
Autosampler stability (after 12h)		
1.0 / 0.5	1.01 / 0.51	97.1 / 96.0
20.0 / 5.0	20.04 / 4.99	98.8 / 97.4
Three freeze-thaw cycles		
1.0 / 0.5	1.02 / 0.49	94.8 / 93.2
20.0 / 5.0	20.02 / 4.97	97.7 / 94.1
Long term stability (90 days at -20 °C)		
1.0 / 0.5	0.99 / 0.50	98.8 / 99.1
20.0 / 5.0	19.89 / 5.10	100.6 / 99.3

The extraction yields were evaluated at four concentration levels (LLOQ, low QC, medium QC and high QC sample) of the linear concentration range for both, CBZ and CBZ-EP. The obtained values were in the range of 98.5% - 99.6% for CBZ and 99.1% - 99.7% for CBZ-EP (Table 2), while the recovery value for IS was 98.9%. These results indicate excellent recoveries for all analytes which means that the proposed SPE procedure is appropriate to be ap-

plied to the determination of CBZ and its active metabolite in plasma taken from epileptic patients.

Stability

Stability experiments were performed through evaluation the stability of CBZ and CBZ-EP, and also IS stock

solutions and plasma samples under different conditions. Stock solutions of analytes were stable at room temperature for 24 h (accuracy: 99.6%, 99.4% and 100.3% for CBZ, CBZ-EP and IS, respectively) and at 2 - 8 °C for 3 months (accuracy: 101.7%, 98.3% and 98.1% for CBZ, CBZ-EP and IS, respectively). It was shown that the human plasma samples spiked with CBZ and CBZ-EP were stable after three freeze-thaw cycles, after 12 h in autosampler and at room temperature for 24 h. The study indicated that the plasma samples could be stored at - 20 °C for 90 days. The results of stability were presented in Table 3.

The obtained results indicate that the analytes were stable under all storage conditions described above and

that no stability related problems would be expected during the routine plasma sample analysis.

Application to patient plasma

The proposed method was applied to the simultaneous determination of CBZ and its active metabolite, CBZ-EP, in plasma samples taken from epileptic patients under chronic CBZ therapy (400 – 1200 mg/day). The chromatogram of a plasma sample from a patient, who received 400 mg/day of CBZ, taken 12 h after the last drug intake was reported in Fig. 2. By interpolation on the respective calibration curves, the following concentrations were ob-

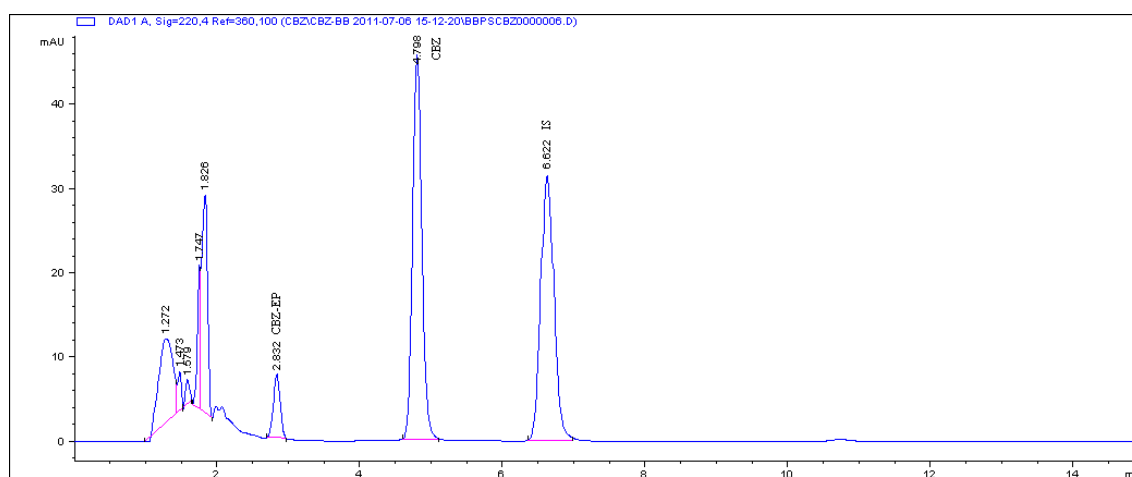


Fig. 2. Chromatogram of plasma sample from a patient treated with 400 mg/day of CBZ, subjected to the developed SPE procedure.

Table 4. Results of analysis on patient plasma samples

Patient no.	Dosage (mg/day)	Co-administered drugs	Determined concentration ($\mu\text{g/ml}$)		
			FPIA method	HPLC method	HPLC method
			CBZ	CBZ	CBZ-EP
1	400	-	4.96	4.69	0.92
2	400	-	8.98	5.71	0.88
3	600	-	6.83	6.54	1.11
4	400	-	8.04	7.32	0.74
5	400	-	4.26	4.02	0.40
6	400	-	4.73	4.71	0.55
7	800	-	7.05	6.49	1.00
8	400	-	4.73	4.02	0.81
9	800	Levetiracetame	8.09	7.55	1.33
10	800	-	6.93	6.34	0.83
11	800	Phenobarbital	3.31	2.89	0.67
12	600	-	8.27	7.52	1.92
13	1200	Lamotrigine	8.51	8.06	1.78
14	800	Valproate	10.40	7.90	2.90
15	1200	Levetiracetame, Lamotrigine	7.73	6.37	1.45
16	800	-	6.86	6.14	1.09
17	400	-	4.66	4.65	0.92
18	1200	Levetiracetame, Lamotrigine, Topiramate	8.27	5.59	0.95
19	400	-	4.73	4.05	1.14
20	800	-	7.80	7.01	1.68

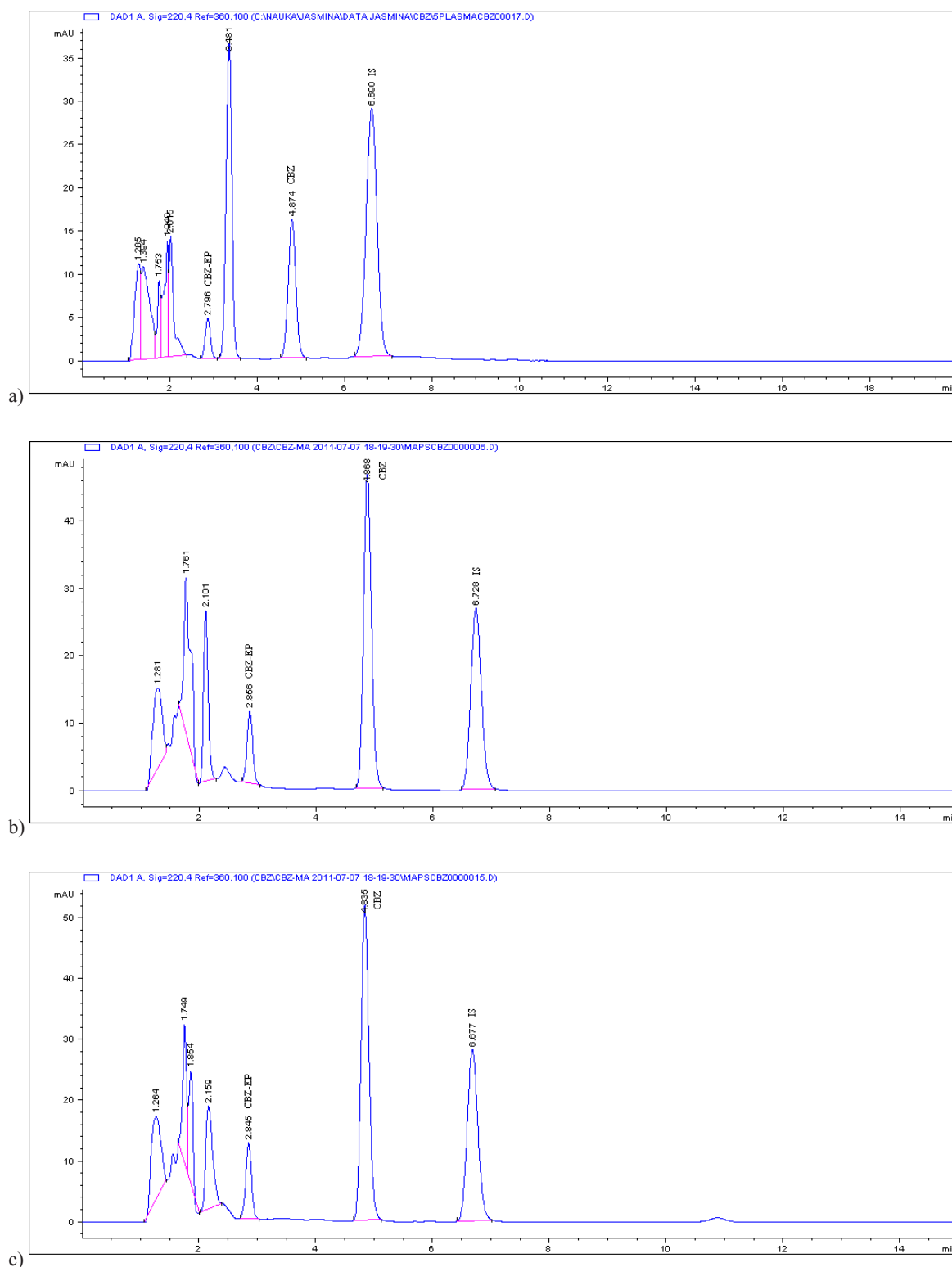


Fig. 3. Chromatograms of plasma samples from patients treated with: a) carbamazepine and phenobarbital; b) carbamazepine and valproate; c) carbamazepine, lamotrigine, topiramate and levetiracetam, subjected to the developed SPE procedure.

tained: 4.69 µg/ml for CBZ and 0.92 µg/ml for CBZ-EP. The results obtained from the assays on 20 patient plasma samples were reported in Table 4.

In this table the CBZ plasma concentrations obtained with the proposed HPLC method were also compared with those obtained from the same samples by FPIA method pre-

formed in the laboratory at Clinic of Neurology, Faculty of Medicine, Skopje. Statistical analysis was performed using GraphPad Prism software (version 5.0). A significant linear correlation was obtained ($r = 0.8898$, $p < 0.0001$). Comparison of the values by paired t -test indicated that the FPIA concentrations were significantly greater ($p < 0.001$) than those obtained by HPLC method. The probable reason for this was interference by CBZ metabolites, mainly by CBZ-EP, with FPIA technique (Kang et al., 2011; Wilson et al., 1992).

Given that epileptic patients are often treated with polytherapy, it was important to assess probable chromatographic interferences from potentially co-administered antiepileptic drugs. To evaluate the selectivity of proposed method, plasma samples taken from patients simultaneously treated with valproate, lamotrigine, topiramate, phenobarbital and levetiracetam (commonly prescribed drugs along with CBZ), were analysed. The chromatograms of plasma samples of patients treated with polytherapy (patient 11: phenobarbital along with CBZ; patient 14: valproate along with CBZ, and patient 18: lamotrigine, topiramate and levetiracetam along with CBZ) were presented in Fig.3.

It was evident that no chromatographic interference from co-administered drugs were observed in the retention times of CBZ, CBZ-EP and IS, hence the method could be applied for TDM of CBZ and CBZ-EP in patients undergoing chronic treatment with CBZ, either as monotherapy or in polytherapy with other antiepileptic drugs.

Conclusion

A simple and reliable bioanalytical HPLC method has been optimized and validated for the simultaneous determination of carbamazepine and its active metabolite, carbamazepine-10, 11-epoxide in human plasma after solid-phase extraction.

The SPE procedure applied gave excellent recovery values for carbamazepine and carbamazepine-10, 11-epoxide. All analytes were baseline separated in less than 7 min.

The validation data demonstrate that the proposed bioanalytical method is selective, sensitive, linear, precise and accurate.

The method was successfully applied to separate and determine the CBZ and CBZ-EP in plasma obtained from epileptic patients treated with CBZ as monotherapy and in polytherapy.

According to all obtained results, it can be concluded that the proposed method is suitable for a reliable therapeutic drug monitoring of patients undergoing therapy with carbamazepine, both in monotherapy and in polytherapy. Since, it requires only 250 μ l of plasma for one complete analysis, the proposed method could be particularly advantageous when multiple blood sampling were needed, as in pharmacokinetic studies.

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Резиме

Оптимизација и валидација на биоаналитички SPE-HPLC метод за истовремено определување на карбамазепин и неговиот главен метаболит, карбамазепин-10,11-епоксид, во плазма

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Клучни зборови: карбамазепин, карбамазепин-10,11-епоксид, плазма, цврсто-фазна екстракција (SPE), HPLC, валидација

Карбамазепинот е најчесто употребуван антиепилептичен лек во третман на парцијални и генерализирани тонично-клонични напади. Карбамазепин-10,11-епоксидот е најзначаен метаболит на карбамазепинот, бидејќи е фармаколошки активна супстанција со антиконвулзивни особини. Според тоа, рутинската анализа на карбамазепин-10,11-епоксид заедно со карбамазепин, може да даде оптимални резултати во терапевтското следење на третманот со карбамазепин.

Целта на овој труд е да се оптимизира и валидира едноставен и сигурен SPE-HPLC метод за истовремено определување на карбамазепин и неговиот главен метаболит, карбамазепин-10,11-епоксид во плазма, со цел да се добијат резултати со соодветен квалитет и сигурност со што ќе се обезбеди примена на предложениот биоаналитички метод во следење на терапевтските концентрации на карбамазепин и неговиот активен метаболит.

Екстракцијата на анализите од примероците на плазма е изведена со помош на цврсто-фазна екстрактивна постапка. Разделувањето на екстрахираните анализи е спроведено на реверзно фазна колона со изократско елуирање со мобилна фаза составена од ацетонитрил и вода (35:65, v/v), на температура од 30 °C. Детекцијата е изведена на бранова должина од 220 nm.

Добиените вредности за приносот на екстракција се повисоки од 98% за сите анализи, определени во четири концентрациски точки од линеарниот концентрациски опсег. Воспоставениот метод покажува одлична селективност, осетливост, линеарност, прецизност и точност. Студиите за стабилност укажуваат дека работните раствори и примероците од плазма, чувани под различни услови, се стабилни во тек на целиот период потребен за изведување на анализите. Методот е успешно применет за определување на карбамазепин и карбамазепин-10,11-епоксид во плазма добиена од пациенти третирани со карбамазепин како монотерапија или во политерапија.

Како заклучок, воспоставениот биоаналитички метод е соодветен за примена во следење на терапевтските концентрации на карбамазепин и на неговиот активен метаболит, карбамазепин-10,11-епоксид, кај пациенти болни од епилепсија кои се под терапија со карбамазепин.
