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Original Article

Development and validation of an HPLC method for determination of carbamazepine in human plasma and applications to a therapeutic drug monitoring study

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ABSTRACT

Background and Aims: Carbamazepine (CBZ) is an antiepileptic drug, which is prescribed as a first-line drug for the treatment of partial and generalized tonic–clonic epileptic seizures. The aim of this study was to develop and validate a simple, fast and reliable HPLC method for the determination of carbamazepine in human plasma.
Methods: Chlorpromazine (CPR) was used as an internal standard. The separation was conducted with a C18 reverse-phase column (150x3.9 mm, 5 µm) at 30°C, using a mobile phase prepared with 20 mM KH2PO4, acetonitrile and methanol (6:3:1, v/v/v) by isocratic elution.
Results: The method was linear between 0.5 and 40 µg/mL, determined by 10 individual calibration points. Total run time was ≤ 5 mins. Accuracy (RE%) values were determined between (-5.6) and 3.6%, and precision was determined at ≤4.2%. Limit of detection (LOD) was 0.04 µg/mL. The robustness test results of the method showed good values. Plasma CBZ of (n=30) those receiving CBZ quantities ranging from 0.2 to 1.2 g/day were measured with this method, and following analyses of their concentrations were found to be between 0.1 and 11.4 µg/mL (6.2±2.4 µg/mL). While all plasma sample analyses were applied properly, it was observed that 16 (53.3%) of the plasma samples had CBZ lower than the recommended range. In addition to that, female patient plasma-CBZ levels were found significantly higher than male plasma contents ($p<0.05$).
Conclusion: This method was found suitable for the analysis of plasma samples collected during the therapeutic drug monitoring (TDM) of patients treated with CBZ.

Keywords: Carbamazepine, plasma, therapeutic drug monitoring, validation, high-performance liquid chromatography, ultraviolet detection

INTRODUCTION

Carbamazepine (CBZ), (5H-Dibenz [b.f] azepine-5-carboxamide) (Figure 1) is used to treat bipolar disorder, especially (Chen & Lin, 2012) with geriatric patients' and individuals with multiple complaints (Punyawudho et al., 2012). But, it is extensively used for the management of epilepsy and also trigeminal neuralgia (Obermann 2010). In addition, it is also used as an anticholinergic agent. CBZ is considered a major antiepileptic drug which is used clinically to control different type of seizures. Its chemical structure is similar to tricyclic antidepressants. CBZ is a weakly acidic and non-polar aromatic ester. Since its chemical structure has a carbamoyl moiety at the 5th position (Figure 1), it is considered an iminostilbene derivative (Mittal & Das, 2012) which gives it anti-seizure activity.

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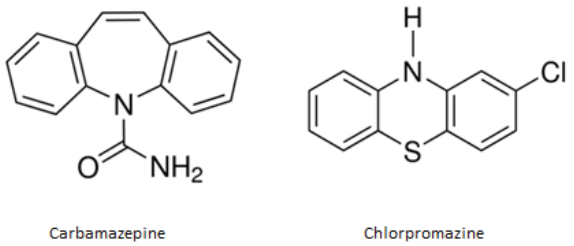

Figure 1. Chemical structures of CBZ and CPZ.

CBZ shows high oral bioavailability in humans (Marino et al., 2012). Since it is almost completely metabolized in the body, only a small part is excreted in the urine (Brunton, Knollman & Hilal-Dandan, 2018). The CBZ-10.11 epoxide is an active metabolite of CBZ, and also displays anticonvulsant properties similar to those of the parent compound (Chbili et al., 2017). Therapeutic concentrations of CBZ have been reported to range from 6 to 12 µg/mL (Brunton et al., 2018). CBZ is a potent inducer of the CYP3A4 and CYP2B6 enzymes, which are the main enzymes involved in the metabolism of this drug, and this fact is named auto-induction. Due to this property a significant decrease is observed in plasma drug levels during the first few weeks of ingestion (Ware, Tillery & Linder, 2016).

Thirty percent of the patients with focal epilepsy do not respond to a maximum dose of CBZ, so there is a need for additional anti-epileptic drugs to control their seizures, but it is also known that this combination treatment has the risk of the causing drug interactions. CBZ is used in combination therapy with other antiepileptic drugs, such as valproic acid or phenytoin, if necessary. The monitoring of blood drug levels is crucial to minimize the risk of toxicity while the CBZ treatment is in progress. Multidrug therapy may result in unexpected blood-drug levels due to changes in the activity of enzymes responsible for the metabolism of drugs (Datar, 2015). It is well known that geriatric patients are generally very susceptible to multidrug therapy because of the decrease in enzyme activities responsible for drug metabolism.

Several methods have been published for the determination of CBZ in pharmaceutical preparations and human specimens, including spectrophotometry (Frag, Zayed & Omar, 2012), FT-Raman spectroscopy (Farias & Carneiro, 2014), spectrofluorimetry, micellar electrokinetic capillary chromatography (MECC) (Datar, 2015), fluorescence polarization immunoassay (FPIA) (Sánchez, Garcia & Abadín, 2010), chemiluminescence (Leite, Petersen & Lunardelli, 2009) and gas chromatography-mass spectrometry (GC-MS) (Rani & Malik, 2012). Many of these methods are complex, expensive, time-consuming, and may require special sample preparation techniques and instruments.

HPLC is a simple, precise, accurate and cost-effective method and provides excellent recovery with high precision for a wide range of pharmaceutical compounds (Deeb et al., 2014; Domingues et al., 2016). It is very successful in the simultaneous determination of chemicals or metabolic products in biological samples. This technique allows the separation, identification and quantitative measurement of each component that forms a mixture. The method includes a liquid sample being passed over a solid adsorbent packed into a column using a flow of liquid solvent known as the mobile phase. The different interactions of each analyte in the sample with the adsorbent cause the flow of analytes in the column to change. If the interaction is strong, the analytes flow off the column for an extended period of time and if the interaction is poor, the elution time is short (Datar 2015).

Methods of detection of CBZ and its impurities by HPLC are reported in the United States Pharmacopoeia (USP), Euro-Pharmacopoeia (EP), British Pharmacopoeia (BP) and Indian Pharmacopoeia (IP). Some pretreatment techniques such as liquid-liquid extraction (Juhacik & Jenkins, 2009; Lajeunesse et al., 2009), solid-phase extraction (Bugamelli et al., 2002; Fortuna, Sousa & Alves, 2010; Franceschi & Furlanut 2005; Vermeij and Edelbroek 2007), solid phase micro extraction (Ferreira et al., 2009), stir bar-sorptive extraction (Queiroz et al., 2008), and deproteinization (Leite et al., 2009) have been used for the determination of CBZ and its metabolites by HPLC in plasma. Therefore, HPLC became prominent as a reliable technique for the detection of these and other anticonvulsant drugs (Bugamelli et al., 2002; Fortuna et al., 2010; Franceschi & Furlanut 2005; Dordević, Kilibarda & Stojanović, 2009; Mowafy, Alanazi & Maghraby, 2012; Queiroz et al., 2008; Ezzeldin, Shahat & Basudan, 2013; Vermeij and Edelbroek 2007).

The aim of our study was to develop a rapid, accurate and reliable HPLC method, with a simple, repeatable and inexpensive technique for the quantitative determination of CBZ in human plasma. In order to prove the applicability of real samples for this developed and validated method, it was planned to be used for determining drug levels in the plasma of epilepsy patients who are using CBZ daily.

MATERIAL AND METHODS

Chemicals and reagents

Chemical standards of CBZ and chlorpromazine (CPZ) (Figure 1), used as an internal standard, were obtained from Sigma (Steinheim, Germany). HPLC grade methanol and acetonitrile were ordered from Sigma-Aldrich (Missouri, USA). Orthophosphoric acid, potassium dihydrogen phosphate and triethylamine were purchased from Merck (Darmstadt, Germany). Membrane filters (0.45 µm pore size) used for filtration of mobile phase were obtained from Millipore (Massachusetts, USA). The Elga Purelab Water Treatment System was used to obtain ultra-pure water (Lane End, UK).

Instrumentation and chromatographic conditions

Agilent 1100 series HPLC system equipped with a degasser (G1322A), a gradient pump (G1311A, QuadPump), a manual injector (Rheodyne 7725i) with a 20 µL loop volume, a column oven (G1316A), and an ultraviolet detector (G1314A) was used in this study. The analytical separation was performed by a stainless steel C_{18} analytical column (150 mm x 3.9 mm I.D. 5 µm p.s.) packed with ODS particle-NovaPak (Waters, Japan).

Optimum analytic conditions were set after an optimization procedure was performed for the column selection, content

of mobile phase and wavelength. Prior to optimization, an assay for the CBZ determination was chosen using the literature data, and each parameter was adjusted or fixed. UV detection was adjusted to 220 nm. The mobile phase was prepared with 20 mM phosphate buffer (0.1% triethylamine, pH 3.0), acetonitrile, and methanole (60:30:10, v/v/v). Before each use, the mobile phase was filtered and then degassed in the ultrasonic bath for 30 minutes. The mobile phase solution was isocratically applied to the column by 1.0 mL/min flow rate, at 30°C.

The unknown concentrations of CBZ were quantified using linear regression of response (drug/ISTD peak area) versus CBZ concentrations. Chemstation®, 08.03 version (California, USA) was used for the control of system equipment and data integration.

Preparation of chemical standards

Main stock solutions of CBZ (2 mg/mL) were prepared in methanol. The working solutions of CBZ were prepared weekly from the main stock solution with methanol as 25, 50, 100, 250, 500 and 1000 µg/mL concentrations. Working standards were prepared weekly, and used to spike blank plasma samples daily, prior to analysis of freshly prepared CBZ dilution in drug-free human plasma to provide concentrations of 0.5, 1, 2, 5, 10 and 20 µg/mL. Fresh human blood samples were collected, to act as a negative control, from the Faculty of Medicine Blood Center. The blood was centrifuged at 3000 rpm for 5 min to separate the plasma. Plasma samples, and all the working solutions were stored at -20˚C until the analyses were carried out. Working solutions were checked chromatographically for purity before the experiments, utilized as quality control specimens and were checked for stability before and after the injections of every sample set.

The ISTD main stock solution (1 mg/mL) was diluted weekly with methanol to yield a 100 µg/mL working solution. Plasma quality control standards, spiked with 1, 5 and 20 µg/mL of CBZ, were prepared to measure the repeatability values of the method. The same protocol was used in the preparation of the limit of detection (LOD), quantification (LOQ), robustness, recovery and stability samples.

All standards were stored at -20°C until use. It was observed that they were stable for at least 1 month.

Sample preparation

As an ISTD, 10 µL CPZ (100 µg/mL) and 10 µL CBZ as a working solution (performed on validation test samples only) were both added into the 250 µL of blank plasma sample, and then 200 µL methanol was added. After the sample tube was mixed by vortex at 1200 rpm for 2 min, it was centrifuged at 10000 rpm for 7 min to precipitate the plasma proteins. Finally, the upper clear phase was collected and loaded to the liquid chromatograph as 20 µL volume manually.

Selection of ISTD

In order to determine the ISTD; fluphenazine, opipramol, imipramine, sildenafil, and CPR were tested. Some of the tested chemicals did not demonstrate the acceptable ultraviolet intensity, and some did not show appropriate retention times. Finally, CPR was selected as the ISTD as its separation sharpness and retention time was acceptable in the chromatogram. In addition, CPR showed a very good intensity in low concentrations. The obtained extraction recovery values were shown to be acceptable and reproducible for ISTD.

Method validation

The developed chromatographic technique was validated for selectivity, linearity, accuracy, precision, the limit of detection (LOD) and limit of quantification (LOQ), recovery and robustness. In agreement with theInternational Conference on Harmonization (ICH) guideline, the intraday and inter-day validation protocol were applied considering the reproducibility of method in order to obtain accurate and precise measurements (ICH, 2005).

Selectivity

The method showed good chromatographic (in plasma matrix) without interference at the retention times of 2.6 and 4.0 min for CBZ and CPZ respectively. Representative chromatograms of blank plasma (Figure 2a), spiked plasma (Figure 2b) and patient plasma samples (Figure 2c) illustrate the high reso-

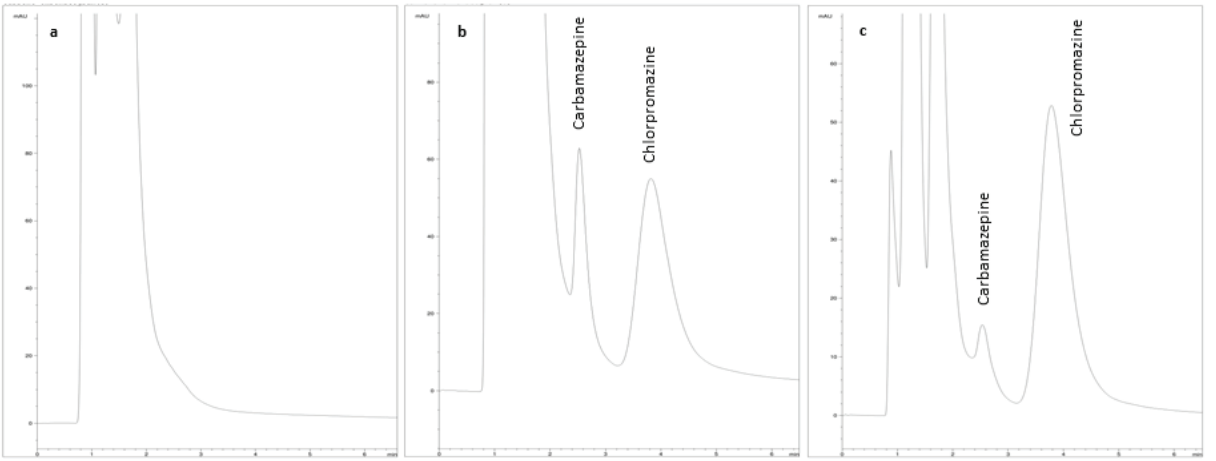

Figure 2a. The chromatogram of blank plasma used in validation tests. **b.** The chromatogram of plasma sample that contained CBZ as 5 µg/mL which is prepared by standard addition method used as quality control sample. **c.** The chromatogram of real patient plasma sample.

lution in a short 6 minute separation time. Although the 210, 214, 237 and 285 nm ultraviolet values were recommended in the literature, the highest peak yield for both the internal standard and analyte was obtained from 220 nm. Therefore, the ultraviolet detector (UV) was set to 220 nm.

Linearity

After chromatographic conditions were established, matrix-based calibration curves of CBZ were plotted over the range 0.5 - 40 µg/mL versus peak-area ratios to the ISTD. The calibration points (n=7), which were 0.5, 1, 2, 5, 10, 20 and 40 µg/mL composed of 3 individual replicates, were prepared by the standard addition method in plasma and injected into HPLC.

Accuracy and precision

The accuracy, defined as the relative error (RE%), was calculated as the percentage difference between the added and found CBZ quantity by 5 separate replicates both intraday and inter-day. The precision, defined as relative standard deviation (RSD%), was calculated by five separate replicates of CBZ both intraday and inter-day. Five replicate spiked samples were assessed both intraday and inter-day at the three different concentrations (1, 5 and 20 µg/mL).

Sensitivity

The concentration of 0.5 µg/mL, as the lowest calibration point, was used in the sensitivity tests of CBZ. Individually, 10 quality control (QC) samples were prepared on the same day and applied to sequential analyses. Obtained chromatograms' analyte peaks belong to CBZ and CPZ were calculated with calibration data prepared in the linearity test.

Recovery

The recovery of extraction procedures from human plasma was determined by comparing pre-extraction spikes with the post-extraction spiked ISTD. Five individual replicates of spiked samples at low, middle and high concentrations (1, 5, 20 µg/mL, respectively) of CBZ were prepared with and without ISTD. The extraction procedure was carried out as described in the sample preparation step.

Robustness

The response of the method of changes in UV wavelength (±3 nm), mobile phase flow rate (±0.1 mL/min), mobile phase organic solvent content (±5%) and column temperature (±4°C) was observed.

Collection of plasma samples

1.5 mL of whole blood samples were taken from the patients which had a steady-state concentration of CBZ in the plasma. Generally, blood samples were taken from patients at 9.00 a.m. in the morning. The vacuum tube containing Na_2EDTA was used for collection of the samples. The whole blood was centrifuged at 3500 rpm for 5 minutes, and the plasma obtained was stored at -18°C until analyzed.

The volunteer blood samples were obtained from 30 patients who were under the CBZ treatment at the Medical School, Department of Neurology. Plazma CBZ levels were measured within 15 days after blood samples were taken from the patients.

Statistical analysis

All statistical analyses were done using the IBM SPSS software version 22. The analysis of plasma-CBZ data produced from the developed method was performed by Independent-samples T test. Paired-sample T test was used for comparison of the plasma-CBZ results, which were obtained by two different analysis methods. The statistical significance level was accepted as $p<0.05$ for all analyses performed. Results were presented as the mean±SD.

RESULTS AND DISCUSSION

Linearity

The calibration curve of CBZ was drawn by the standard addition method at 7 points (n=3) between 0.5-40 µg/mL concentration versus the area of CPZ as ISTD. The obtained correlation of coefficiency was calculated as r^2=0.9983, (Table 1). The linearity study was designed to cover sub-therapeutic, therapeutic, overdose and toxic levels of the CBZ in plasma.

Table 1. Chromatographic characteristics and system suitability parameters of the method.

Analyte	Wavelength (nm)	Retention time TR (min)	LOD (µg/mL)	LOQ (µg/mL)	Linear Range (µg/mL)	Calibration equation	Correlation coefficient (r^2)	Capacity factor (k')	Theoretical plate number
CBZ	220	2.6	0.040	0.122	0.5-40	y=0.0519x+0.023	0.9983	1.6	3972
CPR	220	4.0	-	-	-	-	-	2.8	

Sensitivity

The limit of quantification (LOQ) and limit of detection (LOD) were calculated according to the ICH recommendations based on standard deviation of the response and the slope of the calibration graph. Equations were given as: LOQ= 10σ/S; LOD= 3.3σ/S (σ: The standard deviation of the response; S: The slope of the calibration curve) (ICH, 2005).

The results of LOD and LOQ values are shown in Table 1. The obtained LOD and LOQ values were sufficient for both identification and quantitative analysis of CBZ.

Precision and accuracy

The data obtained from the accuracy and precision tests (Table 2) performed in intraday and inter-day with quality control standards established in the blank plasma samples by standard addition method showed a low RSD% value ≤4.2 and also low RE% values between (-5.6%) and 3.6%. The obtained intraday and inter-day repeatability values support that the method can be applied safely in real blood samples.

Table 2. Confidence parameters of the method that including intraday and inter-day precision and accuracy.

Conc. (µg/mL)	Intraday				Inter-day				Average Recovery (%)
	No. Obs.	Estimated conc. $\bar{X}$±SD (µg/mL)	Precision (RSD%)	Accuracy (RE%)	No. Obs.	Estimated concen. $\bar{X}$±SD (µg/mL)	Precision (RSD%)	Accuracy (RE%)	
1	5	0.96±0.03	3.6	-3.7	5	0.94±0.04	4.2	-5.6	82.4
5	5	4.84±0.13	2.8	-3.2	5	4.85±0.18	3.8	-2.8	88.4
20	5	20.69±0.26	1.3	3.6	5	20.60±0.39	2.0	3.0	105.7

Recovery

Recovery test results were between 82.4% and 105.7% (92.2% ±12.1), and are given in Table 2. The recovery values that were obtained in the extraction procedure, demonstrated efficiency. It was observed that the extraction procedure was not complicated and did not require a sophisticated instrument to carry out the method. The recovery data of this method was reliable, and was produced after firstly carrying out protein precipitation followed by sample the preparation protocol based on HPLC application.

Robustness

No significant changes were observed in the analytical signals upon changing the UV wavelength value (±3 nm), mobile phase flow rate (±0.1 mL/min), mobile phase organic solvent ingredient (±5%) or column temperature (±4°C). Not only changes in analysts, columns, sources of chemicals and/or solvents, but did not lead to significant changes in chromatographic signals. Robustness experimental results demonstrated that the method is able to create data with acceptable precision and accuracy. Changing the analysts, columns, and sources of chemicals/solvents did not lead to significant changes in chromatographic signals.

Stability

The stability of QC samples (1, 5 and 20 µg/mL) and analytes in the stock solutions of the analytes were assessed under several conditions. Firstly, the stability of the stock solutions of CBZ and CPR were evaluated at the end of each week for 4 weeks, using the same analysis method. In this test, stocks were stored at 4°C. A stability test for freeze-thawing was executed for three QC samples (1, 5 and 20 µg/mL of CPZ) after carrying out five repeated freeze-thaw actions. The stability test for long-term CPZ storage was carried out at 1, 2 and 3 months using QC samples kept at -20°C. Neither a significant decrease nor degradation was observed in the concentration of CBZ in the different storage times. The relative standard deviation (RSD) was less than 4% for all samples.

There are some studies for analysing CBZ levels in blood samples. In the HPLC-based study performed by Mowafy et al. (2012), a new CBZ determination method was developed and validated for rabbit plasma (Mowafy et al., 2012). In this study, separation was performed with µ-Boundpak C18 (150 mm x 4.6 mm i.d.) column by mobile phase consisting of methanol and water (1:1, v/v. Propylparaben was used as an internal standard. Detection was accomplished by a UV detector set at 285 nm. The retention time of CBZ and propylparaben were 7.7 and 11.4 min, respectively. Total run time was 15 min . Intra day and inter-day accuracy were reported between 97.5 to 103.6%. Intraday and interday precision were found to be ≤3.7%. The calibration curve was found to be linear in the range of 0.5–40 µg/mL.

In the study conducted by Vermeij & Edelbroek (2007), the plasma levels of 7 antiepileptic drugs were analyzed simultaneously, using the HPLC-DAD method. Acetonitrile, methanol and phosphate buffers were used as mobile phase. A 150 mm x 4.6 mm C18 analytical column was used for analytical separation. The calibration range is 0 to 14.8 µg/mL. The retention time of CBZ was 18.3 minutes, and the total analysis time was 20 minutes. A volume of 0.1 mL serum sample was extracted by solid phase extraction. The sample volume loaded onto HPLC after extraction was 50 µL. The intraday precision was <1.9% and the LOQ was <0.065 µg/mL.

In the HPLC-UV-based study performed by Franceschi & Furlanut (2005), 3 different antiepileptic drugs and 2 metabolites with CBZ were observed in the blood (Franceschi & Furlanut 2005). The solid phase extraction method was used, and analysis was performed with 0.5 mL volume of serum. The UV detector is set to 214 nm. Cyeptamide was used as an internal standard. The 250 mm x 4.6 mm analytical column was used for separation. Water, acetonitrile, methanol, acetic acid and triethylamine (72.5:15:125/0.1/0.06, v/v/v/v/v) were used as the mobile phase. The flow was 1.2 mL / min and the retention time of CBZ was 10.5 min, so the total analysis time was 15 minutes. Between 0 - 60 µg/mL CBZ was used for calibration. The accuracy was determined between 99 and 105%. The LOQ was 0.2 µg/mL, and the recovery was determined between 95.78 - 102.84%.

An HPLC-based method was developed by Bugamelli et al. (2002) to determine oxcarbamazepine, CBZ, lamotrigine, phenobarbital, primidone, phenytoin and two metabolites in human plasma. The total analysis time was 11.5 minutes. The C18 column (150×4.0 mm, i.d. 4.5 µm) was used for separation. The mobile phase was 15 mM phosphate buffer (0.63% triethylamine), methanol and acetonitrile (64.0: 19.2: 16.8, v/v/v). The flow rate was 1 mL/min, and the DAD detector was set at 237 nm. Plasma samples were first precipitated with perchloric acid, and then

extracted using the solid phase method. The total analysis time was 13 min, and the retention time of CBZ was 11.5. minutes. The LOQ was 60 ng/mL. The recovery was 91%. Precision was determined between 8.1 and 5.0%.

Fortuna et al. (2010) developed an HPLC-UV method for determining the concentrations of CBZ, oxcarbamazepine, eslicarbazepine acetate and metabolites in human blood. A C18 column (55 mm×4 mm) was used for separation. Water, methanol and acetonitrile (64:30:6, v/v/v) mobile phase flow rate was 1 mL/min, and the column temperature was 40°C. Total analysis time was 9 min, and the retention time of CBZ was 7 min. The linearity test was performed between 0.05-30 µg/mL. The accuracy was 12%, the LOQ was 0.05 µg/mL and the precision was 8.68% (RSD%). The ultraviolet detector was set at 235 nm.

Dordević et al. (2009) established a new HPLC-UV method for CBZ in saliva and serum. Separation was achieved by a reversed phase C_{18} column with a mobile phase of methanol, water and acetic acid (65:34:1, v/v/v). Flow was 1.0 mL/min. UV was set at 285 nm. In the method, the liquid-liquid extraction method was used for the extraction of alkalinized samples with chloroform. Total run time was 5 min. The method was linear in the range of 0.1–5 µg/mL for both types of sample. The average recovery for serum was found as 97.59%. The method precision for serum was found between 2.10% to 4.03%. LOQ of the method was detected as 0.237 µg/mL for serum sample. Method accuracy was found at 0.52.

Queiroz et al. (2008) developed an HPLC-UV method for the monitoring CBZ, its main metabolite, phenytoin and phenobarbital in human blood samples. Chromatographic separation was performed using the C8 column, the UV detector was adjusted to 210 nm. The calibration range was 0.08 - 40 µg/mL. The LOQ was 0.125 µg/mL. The accuracy was between 3.3 and 9.2%.

Ezzeldin et al. (2013) described an HPLC-UV method for the determination of CBZ in human plasma. Diclofenac was used as an internal standard. Separation was conducted by a C8 (250 x 4.6 mm, 5 µm) column with a mobile phase prepared with buffer (pH:3.0), acetonitrile and isopropyl alcohol (49:36:15, v/v/v). Chromatographic determination was achieved at 220 nm. LOQ was 0.1 µg/mL. Total run time was 20 min. Precision was ≤12.8%. Intraday and interday accuracy were determined between 85 to 100%. Average recovery was 87.0%. All these methods, published in the literature, were summarized in Table 3.

Table 3. The validation parameters and chromatographic properties of CBZ determination methods published in literature.

Study	Instrument	Linear range (µg/mL)	LOQ	Recovery (%)	Mobile phase	Flow (mL/min)	Retention time (min)	Total analysis time (min)	Detection wavelength	Column	Precision (RSD%)	Accuracy	Column oven temperature (°C)
Franceschi & Furlanut, 2015	HPLC-UV	0–60.0	2.0 µg/mL	95.8-102.8	Water, ACN, MeOH, Acetic acid, TEA (725:150:125: 0.1, 0.06, v/v/v/v/v)	1.2	10.5	15.0	214 nm	CN, 250 mm x 4.6 mm, 5 µm	-	99.0 - 105.0 %	50.0
Bugamelli et al., 2002	HPLC-DAD	2–40 µg/mL	60.0 ng/mL	91.0	PB, TEA, MeOH, ACN (64.0: 19.2: 16.8, v/v/v)	1.0	11.5	13.0	237 nm	C18, 150 mm x 4.0 mm , 4.5 µm	5.0 - 8.1	-	-
Fortuna et al., 2010	HPLC-UV	0.05–30.0 µg/mL	0.05 µg/mL	80.0–91.9	Water, MeOH, ACN (64:30:6, v/v/v)	1.0	7.0	9.0	235 nm	C18, 55 mm x 4.0 mm	8.7	12.0 (RE%)	40.0
Dordević et al., 2009	HPLC-UV	0.1–5 µg/mL	0.2 µg/mL	95.2–106.0	MeOH, water, acetic acid (65:34:1, v/v/v)	1.0	3.9	5.0	285 nm	C18, 250 x 4.0 mm, 5 µm	2.1 – 4.0	0.5 – 1.9 (RE%)	-
Queiroz et al., 2008	HPLC-UV	0.08–40.0 µg/mL	0.08 µg/mL	72.0–86.0	Water: ACN (78:22, v/v)	1.0	32.0	40.0	210 nm	C8, 125 mm × 4 mm, 5 µM	8.8 – 10.0	3.3 – 9.2 (RE%)	-
Ezzeldin et al., 2013	HPLC-UV	0.1–8.0 µg/mL	0.1 µg/mL	87.0	ACN: IPA: PB (36:15:49, v/v/v)	1.2	11.4	14.0	220 nm	C8 250 x 4.6 mm, 5 µm	4.2 - 12.8	85.0 – 100.0 %	-

ACN: Acetonitrile, MeOH: methanol, TEA: Triethylamine, IPA: isopropyl alcohol, PB: phosphate buffer

CBZ is susceptible to temperature changes, so its analysis by gas chromatography is not suitable. For this reason, before analysis by gas chromatographic method, it is derivatized with some agents (Minkova & Getova, 2001). In the study conducted by Auer et al. (2003), carbamazepin stability was observed in a commercial dosage form. As a result of this study, it was found to be stable. Matar et al. (1999) investigated the stability of CBZ in their HPLC based analysis study, which was conducted after being stored 4 weeks at -20°C, and according to obtained result, CBZ was found to be stable (Matar, Nicholls & Tekle, 1999). Tonic-Ribarska et al. (2012) investigated the stability of CBZ in their study, and they conducted this study on stock solutions of CBZ in 24 h at room temperature and 3 months at 2–8°C, and they found it as stable (Tonic-Ribarska, Haxhiu & Sterjev, 2012). In our study, CBZ and CPR as internal standards were clearly observed to be stable at +4°C for 4 weeks, and at -20°C for 3 months. In the stability test applications, no degradation or decrease was observed in the areas of peaks of CBZ and CPR.

We have developed and validated the application in 30 epilepsy patients with blood samples as well as the practical application of our study. It was observed that the validation data contains efficient results. It has been found that the calibration range of 0.5 to 40 µg/mL can be used successfully at different concentration levels of CBZ. The reproducibility test results of the method performed at 3 different concentration points were found to be reliable when compared to the methods in the literature. The accuracy was ≤4.2% during the same day and between days. The accuracy was between 5.6 and 3.6. Lower retention times than in the literature were obtained using a 150 mm C_{18} column. İt is a simple, fast and reliable analysis method with high recovery values obtained with this protein precipitation method. This is an advantage of the method as it allows for routine analysis. Plasma (250 µL) was used in the analysis. The resulting LOQ value was 0.122 µg/mL. This value is below the calibration range, so it proves that the method is suitable for highly sensitive analyses.

Patient plasma CBZ levels

CBZ levels in blood samples taken from patients receiving CBZ oral treatments as 200, 300, 400, 600, 800 and 1200 mg/day for epilepsy treatment were monitored with the developed and validated HPLC method. Analyses were performed with a 0.25 mL plasma sample taken from patients with a steady-state concentration of the drug in their blood. None of these samples showed any problem for the quantification of the analytes. Additionally, peak purity showed that no analytical interference was encountered from endogenous substances. The obtained blood CBZ level results are given in Table 4 and Table 5, and comparision of the CBZ blood levels results and the dose rated blood levels of CBZ results were represented in Figure 3. CBZ was observed to be dose-dependent among individuals in the plasma samples of 30 patients. The presence of CBZ in plasma in a dose-dependent manner is also an indication that the drug is suitable for TDM.

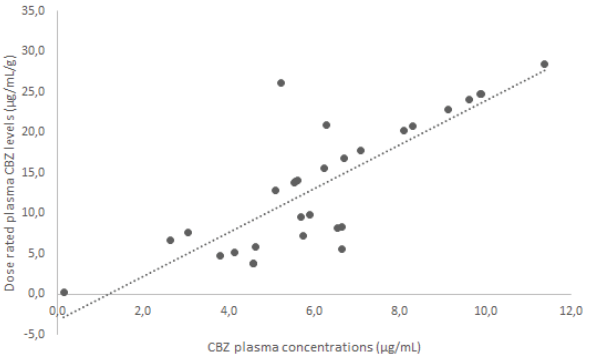

Figure 3. Comparision of the CBZ blood levels results and the dose rated blood levels of CBZ results.

Recommended blood plasma values for the treatment with CBZ is 6 and 12 µg/mL (Rani & Malik, 2012). The results of the study showed that 16 out of 30 patients treated with CBZ (53.3%) had blood concentrations below these values. This result showed that more than half of the patient population followed up did not reach an effective blood concentration, and continued their treatment. However, this is very important because the patients show that they continue to undergo treatment for CBZ at a lower level than recommended. This means that, despite drug intake, serious symptoms of the disease cannot be prevented.

Table 4. Number of samples included in the analysis, daily CBZ doses, serum concentrations and dose-based blood results.

Pateint number	Dose (mg)	Plasma result (µg/mL)	Plasma result / Dose	Pateint number	Dose (mg)	Plasma results (µg/mL)	Plasma result / Dose	Pateint number	Dose (mg)	Plasma results (µg/mL)	Plasma results / Dose
1	400	5.2	13.1	11	400	5.6	13.9	21	400	5.7	14.2
2	400	6.3	15.7	12	400	3.1	7.6	22	400	5.7	14.3
3	800	5.5	6.9	13	1200	9.9	8.2	23	400	6.5	16.3
4	1200	9.6	8.0	14	400	2.6	6.6	24	400	3.8	9.5
5	800	11.4	14.2	15	400	6.2	15.6	25	300	4.6	15.4
6	1200	5.6	4.7	16	800	9.9	12.4	26	800	6.6	8.3
7	800	8.3	10.4	17	800	9.1	11.4	27	200	4.1	20.7
8	600	7.1	11.8	18	400	6.7	16.7	28	400	4.6	11.5
9	400	8.1	20.3	19	600	5.9	9.8	29	400	6.7	16.6
10	400	5.1	12.8	20	600	0.1	0.2	30	400	4.6	11.4

Table 5. Descriptive statistics of real plasma results obtained from two different methods.

	1st metod results		2nd method results	
	Plasma result (µg/mL)	Plasma result (µg/mL)/Dose (g)	Plasma result (µg/mL)	Plasma result (µg/mL)/Dose (g)
Average	6.2	12.0	6.1	13.5
SD	2.4	4.5	2.2	8.0
RSD%	38.8	37.7	36.7	59.5

Abbrevisions: 1st method: Our developed analysis method, 2nd method: Analysis method routinely used by the hospital

Plasma concentrations of CBZ are significantly lower in polytherapy than in monotherapy (Koristkova, Bergman & Grundmann, 2006). The use of phenytoin and some barbiturates with CBZ in the treatment of epilepsy is frequently encountered in the clinic. This combined treatment (polytherapy) results in a significant auto-induction of CYP450 enzymes. This situation results in a decrease in plasma CBZ levels. Pregnancy is an important period that seriously affects the activity of CYP450 enzymes. During this term, plasma CBZ levels decrease, possibly due to increased activation of all the metabolic enzymes (Bertilsson, 1978). CYP3A4, which is responsible for the metabolism of more than 50% of therapeutic drugs, and also CBZ, show higher activity in women than in men. However, there are also studies reporting that there is no gender difference in the study rates of CYP enzymes. Many other CYP (CYP2C19, CYP2D6, CYP2E1) isozymes and conjugation (glucuronidation) activity, which is also so important, are reported to be higher in males than in females (Tanaka, 1999). According to this interpretation, drugs metabolized by these enzymes are observed in the plasma of women at a higher concentration than in men (Tanaka, 1999). Although smoking and alcohol consumption are more common in men, drug metabolism is severely affected by gender-specific factors (menopause, pregnancy and menstrual cycle) in women. It is thought that the factors given above may be the reason behind the significantly higher CBZ plasma levels of women compared to men.

Also, drug-drug interactions and diet can affect the plasma CBZ levels. For example, vigabatrin decreases the plasma concentration of CBZ by increasing its clearance, not catabolism (Sánchez-Alcaraz, Quintana & López, 2002. Topiramate interferes with the plasma level of CBZ (Grunze et al., 2001). In some patients., zonisamide may increase CBZ serum levels. Topiramate clearance was 70% higher in patients treated with CBZ, and was found to increase with age (Mimaki, 1998). However, not all interactions are pharmacokinetic. CBZ plus stiripentol (a newer anticonvulsant) interacts pharmacodynamically, and its benefits may outweigh the usual disadvantages of polytherapy. As expected, fluvoxamine, an inhibitor of CYP4503A4, significantly increases plasma CBZ levels (Cottencin et al., 1995). However, fluoxetine, metabolised primarily by CYP2D6, does not interact with CBZ (Sproule, Naranjo & Brenmer, 1997) Similarly, pomegranate juice, which inhibits cytochrome P4503A4, significantly increases the AUC of CBZ in rats (Misaka et al., 2011).

It was thought that the effects of CBZ on CYP3A4 and CYP2B6 were important factors for the emergence of the results, that we got from the study. Also, individual metabolism rate differences observed in CYP3A4 and CYP2B6 enzymes are considered as important factors affecting the CBZ metabolism rate. These factors are capable of changing blood CBZ levels. Our study is related to the fact that female CBZ levels are significantly higher than male results, and that sex-specific factors (menopause, pregnancy and menstrual cycle) are significantly effective in CBZ metabolism.

The CBZ levels in the blood samples of 30 patients included in our study were analyzed by an immunoassay based approach. In this method, called cloned enzyme donor immunoassay (CEDIA), 1 mL of serum or plasma sample can be used in the analysis. Reported analytical measuring range was 0.5-20 µg/mL. This control analysis is a routine periodic application, and is performed at the initial stage of drug treatment or at the dose increase or decrease stages of the drug. With this analysis method, the plasma CBZ concentration can be determined in 1 hour in total, with other blood biochemical analyzes performed simultaneously. This follow-up may also be needed during combined drug therapy or because of complaints related to epilepsy.

Plasma-CBZ analysis results performed by the Faculty of Medicine were compared statistically with the results obtained by our suggested method. No significant difference was found between the results of blood drug levels obtained from both methods ($p>0.05$). Blood CBZ results from the two methods were rearranged according to the drug doses administered to the patients, and no significant difference was observed in the results from the two methods ($p>0.05$).

CONCLUSION

This HPLC method, which was developed and validated for quantitatif analysis of CBZ, was simple, rapid and reliable. The precision and accuracy test results of the method, which are RSD% ≤4.2 and RE% value were between (-5.6) and 3.6 respectively, showed good repeatability. Sample extraction was simple, rapid and provided reliable recovery values between 82.4% and 105.7%. Due to the simplicity of the sample preparation, the short analysis time (<6 min) and the high sensitivity, this technique was ideal for the quantification of CBZ in human plasma.

We recommend this validated method to be used in routine therapeutic drug analysis of CBZ, and it could be adapted for monitoring overdose/poisoning with this drug in suicide cas-

es. Also, this method can be directly applied in routine TDM studies of CBZ. It can be utilized in bioequivalence, pharmacovigilance and pharmacokinetics studies.

Although the recommended blood values in the CBZ treatment are 6-12 µg/mL, low drug levels observed in the blood values of the volunteers showed that it is necessary to perform TDM by a reliable method for the treatment of CBZ. Because of the significant difference ($p<0.05$) observed in drug plasma levels in patient plasma CBZ levels, the determination of polymorphism rates of CYP3A4 and CYP2B6 subtypes responsible for CBZ metabolism was planned as future work of this study.

Ethics Committee Approval: The ethical permission protocol was approved by The Local Ethics Committee with 2018-01/20 decision number on 10th January 2018. It was conducted in accordance with the Declaration of Helsinki and its subsequent revisions. Information consent forms were obtained from the volunteers before being included in the study.

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