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Simultaneous determination of phenobarbital, phenytoin, carbamazepine and carbamazepine 10, 11-epoxide in plasma of epileptic patients

Azam Samadi[1], Maryam Khoubnasabjafari[2,*], Mohammad Barzegar[3], Shahram Sadeghvand[4], Shadi Shiva[5], Abolghasem Jouyban[1,6]

[1] *Pharmaceutical Analysis Research Center and Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran*

[2] *Tuberculosis and Lung Diseases Research Center, Tabriz University of Medical Science, Tabriz, Iran*

[3] *Pediatric Health Research Center, Tabriz University of Medical Sciences, Tabriz, Iran*

[4] *Student Research Committee, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran*

[5] *Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran*

[6] *Kimia Idea Pardaz Azarbayjan (KIPA) Science Based Company, Tabriz University of Medical Sciences, Tabriz, Iran*

* **Corresponding Author:** Maryam Khoubnasabjafari, Email: mkjafari2@yahoo.com.

ABSTRACT

Background: Quantitative analyses of antiepileptic drugs are required in clinic and to rational dosage adjustment, the clinician needs the blood levels of these drugs. A high-performance liquid chromatography with spectrophotometric detection has been developed and validated for simultaneous determination of some antiepileptic drugs in plasma of patients with epilepsy.

Methods: A simple procedure based on deproteinization by acetonitrile was used for pre-treatment of plasma samples. Liquid chromatographic analysis was carried out on a Nova-Pak® C_{18} analytical column, using a ternary mixture of potassium dihydrogen phosphate buffer (pH 6.0)-acetonitrile-2-propanol (63:22:15, v/v/v) as the mobile phase, at a flow rate of 1.0 mL min^{-1}.

Results: Calibration curves were linear over a range of 1–40 µg mL^{-1} for phenobarbital, 1–30 µg mL^{-1} for phenytoin, 0.3–15 µg mL^{-1} for carbamazepine and 0.5–6 µg mL^{-1} for carbamazepine epoxide.

Conclusion: The simple sample pre-treatment, combined with the fast chromatographic run was used for the determination of antiepileptic drugs for a large number of patient samples.

Keywords: Phenobarbital; Carbamazepine; Carbamazepine main active metabolite; Phenytoin; HPLC analysis; Therapeutic drug monitoring

Introduction

Antiepileptic drugs (AEDs) are a group of drugs used to treat neurological disorder such as epilepsy, neuropathic pain, and migraine. Epilepsy is a common and diverse set of chronic neurological disorders. It is characterized by seizures affecting about 1% of the population.[1,2] The pharmacotherapy with AEDs is the first choice for the treatment of epilepsy as it controls the occurrence of unpredictable epileptic seizures on approximately two thirds of the patients.[3] Approximately 30–40% of patients do not achieve seizure control with a single AED and rational polytherapy with the goal of finding combinations of AEDs that have favorable characteristics, has become of greater importance. [4] Before that one should ensure that the drug has reached its therapeutic range and there is no possibility to increase its daily dose. The first generation AEDs (phenobarbital (PB), phenytoin (PHT) and carbamazepine (CBZ)) have been widely used to treat epilepsy. Because of their narrow therapeutic range in adults (10–40 μg mL^{-1}, 5–20 μg mL^{-1} and 2–12 μg mL^{-1}, respectively for PB, PHT and CBZ) [5] and their complex pharmacokinetic properties, their blood levels should be monitored. Therapeutic ranges of PB, PHT and CBZ in children are 20-60 μg mL^{-1}, [3] 6-11 μg mL^{-1} [6] and 4-12 μg mL^{-1}, [7] respectively.

Lethal concentrations of PB and PHT are 50 μg mL^{-1} for both drugs[5] and fatalities could be observed in CBZ serum concentrations of >20 μg mL^{-1} [5] or >39 μg mL^{-1}. [8] CBZ is metabolized to its metabolite, *i.e.* carbamazepine-10, 11-epoxide (CBZE), or simply the "epoxide" metabolite. The presence of this metabolite can have clinically significant implications in therapeutic drug monitoring (TDM) of CBZ. In addition, toxic symptoms may occur when plasma concentration of CBZE is >3.2 μg mL^{-1}. Thus CBZE quantification is importance in patients where CBZ has been ingested. [9,10] When each one of these three drugs fails to control seizures, a combination of the two most effective among them can be used. If the combination of CBZ and PHT fails, a combination

of PB with CBZ or PHT would then become a consideration.[11] Drug interactions of AEDs are very common and they can produce an inducing or inhibiting effect on the metabolism of other drugs, including antiepileptics; thus, these drugs can increase or decrease the effect of other drugs. Thus, it is necessary to evaluate the success of antiepileptic drugs during the treatment. TDM determines an individual reference concentration and improve treatment safety and efficacy. [12] Thus, several measurement methods were reported for optimizing the analytical signal, increasing sensitivity and selectivity of the determination of AEDs. [13] Efforts on developing analytical methods for quantification of AEDs in biological samples are ongoing. [14-20]

A number of factors should be taken into account in TDM of drugs in pediatrics. Differences exist in the pharmacokinetic behavior of drugs between children and adults, also between children in various development steps, and in many cases pediatrics drug dosage is scheduled based on personal experience of the pediatrician. [21] Recent findings on pharmacokinetic and drug interactions of AEDs in children and adolescents was reviewed by Lapadre et al. [22] Variations in pH value of gastrointestinal tract, slower gastric emptying, thinner stratum corneum, greater cutaneous perfusion, more hydration of epidermis, reduced skeletal-muscle blood flow, and insufficient muscular contraction may affect drug absorption in pediatrics. [21] Relatively larger total body water and lower fat tissue cause some variations in distribution of drugs in pediatrics. Some changes are observed in metabolism and elimination of drugs in pediatrics too. All these variations provide some changes in drug response and require the TDM of AEDs in children.

To continue our previous works on TDM of AEDs, [23,24] this work reports TDM of CBZ, PB, PHT and an active metabolite of CBZ, *i.e.* CBZE, in plasma samples of epileptic patients. A reported chromatographic method for quantification of AEDs (CBZ, CBZE, PHT, lamotrigine and oxcarbamazepine) in rat plasma was adopted for quantification of the drugs in children's plasma. The previously reported method was fully validated in human plasma samples and one more AED, i.e. PB, was added to the analytes for follow up of AEDs in patient's samples. When the response

to pharmacotherapy is not a proper response, a number of reasons could be considered. Refractory epileptic seizures are often observed in low drug dosage and/or drug resistance which could be compensated by dose increase and/or drug combination or drug replacement. This work focused on investigating the AED levels in plasma of refractory epileptic children who are candidate for dose increase and/or drug replacement.

Materials and methods

Chemicals and reagents

CBZ was a gift from Sobhan pharmaceutical company (Rasht, Iran), CBZE was purchased from Sigma Aldrich (St. Louis, USA), PB was a gift from Amin pharmaceutical company (Isfahan, Iran) and PHT was a gift from Alhavi pharmaceutical company (Tehran, Iran). Methanol, acetonitrile, 2-propanol and potassium dihydrogen phosphate and orthophosphoric acid were purchased from Merck (Darmstadt, Germany). Double distilled deionized water was prepared from Shahid Ghazi pharmaceutical company (Tabriz, Iran).

Blank human plasma samples were supplied by Iranian Blood Transfusion Research Center (Tabriz, Iran) and aliquot into polypropylene microtubes and frozen at -20 °C until analysis. All sample donor's parents were informed on details of the sample collection from children and signed a written consent form which has been approved by the Ethic Committee, Tabriz University of Medical Sciences under approval number of IR.TBZMED.REC.13953836. All patients' plasma samples were collected in K_2EDTA-treated tubes and stored at -80 °C.

Chromatographic system

After protein precipitation with acetonitrile, HPLC analyses were performed using a LC apparatus (Knauer, Germany) on a Nova-Pak® C_{18} analytical column, using a ternary mixture of

potassium dihydrogen phosphate buffer (pH 6.0)-acetonitrile-2-propanol (63:22:15, v/v/v) as the mobile phase at a flow rate of 1.0 mL min^{-1}. The UV detector was set at 220 nm. [26]

Stock and working solutions

The standard stock solutions of PB, PHT, CBZ and CBZE were 1000 mg L^{-1} respectively and stored in brown bottles. Then different volumes of each stock solution were transferred, combined and diluted appropriately with methanol: water (50:50, v/v) for series of concentration. All the stock solutions were stored at -20 °C and the work solutions were stored at 4 °C. The calibration samples were prepared by spiking blank human plasma with respective working solutions. Calibration standards were made in the range of 1-40 µg mL^{-1} for PB, 1-30 µg mL^{-1} for PHT, 0.3-15 µg mL^{-1} for CBZ and 0.5-6 µg mL^{-1} for CBZE.

Two hundred microliters of plasma were pipetted to a polypropylene tube, 200 µL of acetonitrile as a precipitation solution was added and the tube was vortex-mixed for 30 s. The tube was then centrifuged for 5 min at 10,000 rpm and the supernatant was transferred to a vial and analyzed directly after filtration.

Results and discussion

The proposed method was validated for selectivity, linearity, precision, accuracy, limit of detection (LOD) and limit of quantification (LOQ). Fig. 1a and 1b represented HPLC chromatograms for the analysis of drug-free (blank) plasma and spiked human plasma with CBZE (6 µg mL^{-1}), PB (40 µg mL^{-1}), CBZ (15 µg mL^{-1}) and PHT (30 µg mL^{-1}). The developed HPLC method offers reasonable retention times and good resolution for four drugs as was reported in Table 1. Linearity was determined by analysis of spiked blank plasma samples in seven concentration levels in three replicates: 0.30, 0.6, 1.5, 3.0, 5.0, 10.0 and 15.0 µg mL^{-1} of CBZ, 0.5,

1.0, 1.5, 2.0, 3.0, 5.0 and 6.0 μg mL^{-1} of CBZE, 1.0, 2.0, 5.0, 10.0, 15.0, 20 and 40 μg mL^{-1} of PB and 1.0, 2.0, 5.0, 10.0, 15.0, 20 and 30 μg mL^{-1} of PHT. The linearity was estimated based on the regression curves (y= ax + b) and correlation coefficients (r) and the obtained results were shown in Table 2. For each drug, LOD and LOQ were calculated using equations, LOD = 3 × S/N and LOQ = 10 × S/N were presented in Table 3. The results of precision were reported in Table 4. The intra-day precision was determined from replicated analyses (five times) of plasma samples containing three quality control of each drug. The inter-day precision was determined using the same concentrations of four drugs over a period of 3 days. The acceptance criterion for precision was a RSD lower than 15%. The accuracy was defined for two definite concentrations of each compound and the obtained results were listed in Table 5. The relative error <20% is acceptable for biological samples according to FDA recommendations. [27]

The validated method was applied to quantification of real samples taken from children receiving AEDs and the obtained results were listed in Table 6. PB was found in 83 samples (83.8% of cases) with concentrations ranging from 1.63 to 62.23 μg·mL^{-1}. In 44 cases (44.4%), the patients were under monotherapy with PB. PHT was quantified in 17 samples (17.2% of cases) with the concentration range of 0.07 to 26.55 μg·mL^{-1}. In 2 cases (6.8%) PHT was used as a single drug to manage the epileptic patients and in 15 cases (51.7%), PB + PHT was administered. CBZ was found in the concentration range of 0.38 to 13.45 μg·mL^{-1} in 15 samples (15 % of cases) and the corresponding range for CBZE was 0.38 to 10.11 μg·mL^{-1} with the relative frequency of 25 % (25 cases). In 6 patients (21% of CBZ recievers), CBZE were more than the cut off concentration of 3.2 μg·mL^{-1} in which more side effects are expected. In 4 (14%) patients CBZ was used as a single drug to manage the epileptic seizures. In 11 cases, CBZ levels were < LOD and significant levels of CBZE were observed which could possibly be justified by the fast metabolism of CBZ

in these patients. CBZ induces the activity of cytochrome P450 enzymes and its dose adjustment should be done after achievement of autoinduction and before addition of the next drug to the therapeutic regimen. [22] In 22 cases (75.9% of CBZ receivers), PB + CBZ was co-administered.

For PB, PHT and CBZ in 78%, 85%, and 82% of the cases, the concentrations were less than therapeutic range in children. These observations are expected since all sample donors were children with poor epilepsy management and candidates for dose increase and/or drug replacement. The corresponding relative frequencies for within therapeutic range were 21%, 14% and 10%, respectively. In 1%, 5% and 4% of the cases, children received PB, PHT or CBZ in toxic levels. In one case (#4 in Table 6), the patient received both PB and PHT at higher levels.

The variability of carbamazepine pharmacokinetics is related to the intricate of pharmacokinetics and the thin therapeutic window of the carbamazepine, Thus level of serum CBZ are associated with neurologic side effects. This drug fast metabolized by the liver, with P450 enzyme system (CYP3A) and more than factors may affect serum levels of CBZ, and its metabolites. The age is an important. Parameter in children and elderly due to a decrease or increase activity of CYP3A4, on the other hand patient's received co-prescribed which affected CBZ concentrations. Thus quantification of CBZE is required [28]. In interpretation of TDM data, a number of parameters should be taken into account, including dose of drug, dosage schedule, type of biological sample, sampling time, pretreatments, co-administered drugs, analytical method used for drug quantification, start of drug therapy, age, sex, weight, disease severity, kidney and liver functions and genetics of the patients.

Conclusion

A validated reversed-phase HPLC–UV method for the separation, identification and simultaneous quantification of CBZ, CBZE, PB and PHT in human plasma was developed. The proposed

bioanalytical method was validated in terms of linearity, repeatability, accuracy and precision following the FDA guidelines and results demonstrated that the proposed approach is suitable for TDM of selected antiepileptic drugs in human plasma samples. Such methods could be used for dose adjustment of AEDS and provide more benefits to the patients.

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Table 1. Retention times and resolution factors for the investigated analytes

Analyte	Retention time (min)	Rs*
CBZE	8.4	7.6 (Plasma peak, CBZE)
PB	9.2	3.0 (CBZE, PB)
CBZ	14.1	24.3 (PB, CBZ)
PHT	15.2	3.4 (CBZ, PHT)

* Resolution factor= $(t_2-t_1) / 0.5 (w_1+w_2)$ in which t_2 and t_1 are the retention times of two analytes and their peak widths (w_1 and w_2).

Table 2. Linearity parameters of the drugs for the developed method based on peak height

Analyte	Linear range ($\mu g\ mL^{-1}$)	Linear equation	r
CBZE	0.5-6	y=1807.8x+187.32	0.9897
PB	1-40	y=446.04x+979.33	0.9821
CBZ	0.3-15	y=392.3+986.1	0.9852
PHT	1-30	y=442.48x-233.39	0.9895

Table 3. Limits of detection and limits of quantification of the investigated drugs

Compounds	LOD (μg mL^{-1})	LOQ (μg mL^{-1})
CBZE	0.49	1.63
PB	0.82	2.73
CBZ	0.02	0.07
PHT	0.28	0.93

Table 4. Precision of the method for each drug

Compounds	Concentration (μg mL^{-1})	Intraday (RSD%)	Interday (RSD%)
CBZE	0.5	6.7	13.1
	2.0	7.7	7.3
	6.0	9.6	5.0
PB	1.0	12.4	10.7
	15.0	10.4	14.0
	40	9.8	15.0
CBZ	0.3	6.2	9.0
	5.0	12.6	12.1
	15.0	7.6	13.6
PHT	1.0	2.5	8.4
	15.0	7.0	10.2
	30.0	8.3	12.5

Table 5. Accuracy of the method for each drug

Compounds	Concentration (µg mL^{-1})	Intraday accuracy (RE%)	Interday accuracy (RE%)
CBZE	0.5	-2.0	8.0
	2.0	9.5	13.0
	6.0	4.9	9.6
PB	1.0	10.0	11.0
	15.0	-12.0	-4.0
	40	12.4	14.2
CBZ	0.3	6.0	14.0
	5.0	-9.0	11.0
	15	7.6	13.6
PHT	1.0	10	12.0
	15.0	7.6	9.6
	30	5.9	16.1

Table 6. The results of analysis of plasma samples from patients treated with PB, PHT and CBZ

Sample	Age (years)	Weight	PB(μg mL^{-1})	PHT(μg mL^{-1})	CBZ(μg mL^{-1})	CBZE(μg mL^{-1})
1	10	32	17.39	7.55	-	-
2	3.5	11	2.37	-	<LOD	1.34
3	12	30	26.57	-	<LOD	1.19
4	NR	NR	62.23	26.55	-	-
5	NR	NR	-	0.47	-	-
6	4.5	12	20.24	-	<LOD	3.53
7	8	15	21.27	-	<LOD	<LOD
8	2.5	11	10.23	-	-	-
9	4.5	12	20.10	-	6.64	1.40
10	20 months	10	-	-	<LOD	1.82
11	8	22	-	-	5.75	6.87
12	14	40	-	-	2.53	0.58
13	14	91	16.78	0.07	-	-
14	7	17	42.23	1.33	-	-
15	8	26	6.56	5.91	-	-
16	3	13	5.29	2.15	-	-
17	4.5	17	-	-	3.13	1.15
18	3.5	16	20.05	-	<LOD	0.98
19	4	17	-	-	2.77	5.08
20	2	10	5.96	4.69	-	-
21	3	13	20.25	-	-	-
22	13	36	19.64	-	<LOD	<LOD
23	6	16	7.04	-	3.93	0.8
24	10	26	17.91	<LOD	-	-
25	10	26	16.10	-	1.31	0.46
26	11	14	20.97	-	-	-
27	2	8	6.20	-	<LOD	1.30
28	9	39	6.82	2.40	-	-
29	5	22	8.93	3.92	<LOD	1.72
30	9	32	9.52	-	2.46	2.23
31	14	19	-	-	5.06	1.12
32	4	15	-	-	<LOD	0.57
33	2	10	7.74	-	0.256	0.44
34	9	30	8.56	-	<LOD	0.38
35	10	36	4.97	-	1.76	1.21
36	4	21	9.54	-	<LOD	2.45
37	10	16	20.31	1.16	-	-
38	NR	NR	13.00	-	2.48	3.94
39	NR	NR	15.05	-	-	-
40	21 months	8	10.23	-	-	-
41	8.5	26	-	1.15	-	-
42	NR	NR	12.74	-	6.23	5.71
43	13	50	20.28	5.95	-	-
44	5	13	21.82	0.48	-	-
45	NR	NR	16.95	-	13.45	10.11
46	11 months	5	4.14	-	-	-
47	NR	NR	21.11	-	-	-
48	22 months	10	17.77	<LOD	<LOD	0.43
49	14	55	18.54	0.81	-	-
50	8.5	23	24.04	5.06	-	-
51	22 months	10	19.83	<LOD	0.38	<LOD
52	10	36	14.39	4.23	-	-

53	18	36	39.18	-	-	-
54	NR	NR	20.86	-	-	-
55	NR	NR	8.83	-	-	-
56	8	31	8.91	-	-	-
57	13	35	2.43	-	-	-
58	11 months	10	21.54	-	-	-
59	2.5	9	<LOD	<LOD	-	-
60	11	35	14.70	-	-	-
61	3.5	18	-	<LOD	-	-
62	8	23	13.54	-	-	-
63	8 months	6	6.67	<LOD	-	-
64	10	18	14.23	-	-	-
65	11	40	-	<LOD	-	-
66	2	14	9.67	-	-	-
67	NR	NR	13.63	-	-	-
68	12	35	16.70	-	<LOD	<LOD
69	10	26	11.05	-	-	-
70	18 months	9.5	5.47	-	-	-
71	5	18	7.61	-	-	-
72	2.5	14.5	1.63	-	-	-
73	9	38	5.58	-	-	-
74	7	32	11.46	-	-	-
75	8	34	13.84	-	-	-
76	15	41	15.07	-	-	-
77	14	43	10.51	<LOD	-	-
78	10 months	7	3.88	-	-	-
79	3.5	18	9.72	-	-	-
80	13 months	15	16.66	-	-	-
81	8	24	5.38	-	-	-
82	2	11.5	15.87	<LOD	-	-
83	2.5	11	19.15	-	-	-
84	14 months	9.5	10.55	-	-	-
85	5	25	6.65	-	-	-
86	9	22	9.27	-	-	-
87	10	48	5.78	-	-	-
88	4	15	11.44	-	-	-
89	9 months	9	5.37	<LOD	-	-
90	6	12	29.99	<LOD	-	-
91	11 months	12	16.38	-	-	-
92	6.5	16	5.55	-	-	-
93	13 months	15	15.65	-	-	-
94	NR	NR	8.56	-	-	-
95	8	23	-	<LOD	-	-
96	11	64	<LOD	-	-	-
97	NR	NR	<LOD	-	-	-
98	8 months	7.5	<LOD	-	-	-
99	17 months	25	10.86	-	-	-

NR: Not recorded

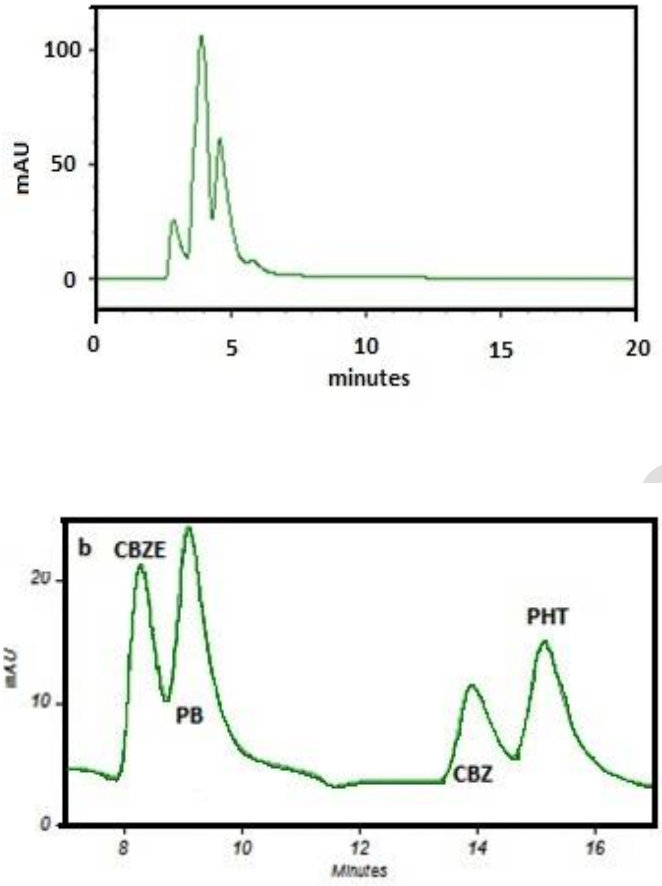

Fig 1. (a) Typical chromatogram obtained from a drug-free human plasma (b) Typical chromatogram obtained from a human plasma spiked with CBZE (6 µg mL^{-1}), PB (40 µg mL^{-1}), CBZ (15 µg mL^{-1}) and PHT (30 µg mL^{-1}).

Graphical Abstract