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HPLC Method for Simultaneous Determination of Five Antiepileptic Drugs in Rat Plasma

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A rapid, simple and sensitive method has been developed and validated for simultaneous determination of 5 antiepileptic drugs *i.e.*, lamotrigine (LTG), oxcarbazepine (OXC), carbamazepine (CBZ), its major metabolite carbamazepine 10,11-epoxide (CBZE) and phenytoin (PHT) in plasma of rat by high-performance liquid chromatography (HPLC) with spectrophotometric detection. After protein precipitation with acetonitrile, separation is achieved 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. The UV detector was set at 220 nm. The method was quantitatively evaluated in terms of linearity, accuracy, precision, limit of detection and limit of quantitation. which made it suitable for daily therapeutic drug monitoring.

Key Words: Antiepileptic drugs, Biomedical analysis, Rat plasma, Chromatographic separation.

INTRODUCTION

About 60 % of epileptic are greatly helped by the anti-seizure drugs. These drug-responsive patients are completely controlled by low drug dose and they lead normal lives, experiencing few side effects. If they are compliant, they may never experience another seizure. Although monotherapy is currently the preferred approach to epilepsy treatment, a large proportion of patients with refractory seizures still receive multiple antiepileptic drugs (AEDs), tentatively to achieve a better clinical control. This often leads to complex and unpredictable pharmacokinetic and pharmacodynamic interactions, with possible clinical consequences. Furthermore, antiepileptic therapy is chronic and may last for the whole patient's life[1].

Therapeutic drug monitoring (TDM) of antiepileptic drugs is necessary to optimize the patient's clinical outcome by managing their medication with the assistance of measured drug concentration[2]. Phenytoin (PHT) and carbamazepine (CBZ), are

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well-known classical antiepileptic drugs. Since 1990s a number of new antiepileptic drugs, such as lamotrigine (LTG) and oxcarbazepine (OXC) were registered worldwide and are currently used as an add-on or monotherapy in patients with epilepsy[3].

Lamotrigine is a second generation of antiepileptic drug from triazine class of chemical compounds. It has been approved for the treatment of partial and generalized epileptic seizures as an add-on agent or monotherapy and is generally well tolerated. Oxcarbazepine is a keto-analogue of carbamazepine and is rapidly converted to the racemic 10-monohydroxy derivative (MHD), which is the pharmacologically active metabolite[4]. In TDM of carbamazepine, determination of carbamazepine 10,11-epoxide (CBZE) and its parent drug carbamazepine was recommended, because carbamazepine 10,11-epoxide is found to have antiepileptic activity. It is also partially responsible for the side effects of carbamazepine therapy[5].

Different simple HPLC-UV procedures based on serum/plasma sample injection after deproteinization using different procedures or even without sample pretreatment have been reported for the determination of AEDs[2-9]. Patil and Bodhankar[2] employed a protein precipitation procedure using double centrifugation followed by filtration to prepare serum samples consisting of lamotrigine, phenobarbital (PHB), phenytoin and carbamazepine. They used a quaternary solvent mixture of mobile phase which eluted the analytes in 12 min. Carbamazepine 10,11-epoxide eluted between lamotrigine and phenobarbital with a small resolution factor. Vermeij and Edelbroek[3] developed an HPLC method equipped with Diode-Array Detector for simultaneous determination of carbamazepine, carbamazepine 10,11-epoxide, carbamazepine-10,11-(*trans*)-dihydrodiol (CBZD), phenobarbital, phenytoin, lamotrigine, zonisamide (ZNS) and oxcarbazepine active metabolite 10-monohydroxy derivative in serum after pretreatment with solid phase extraction (SPE). The separation column was 15 cm and all analytes eluted in about 18.3 min. Lamotrigine was determined individually by a high-performance thin-layer chromatographic (HPTLC) method in serum after being pretreated by a liquid-liquid extraction (LLE)[4]. Yoshida *et al.*[5] separated carbamazepine, carbamazepine 10,11-epoxide and zonisamide in 16 min and considered a signal-to-noise ratio of 5:1 (500 ng/mL for CBZ and 250 ng/mL for CBZE) as the lowest point of the calibration curves. Methanol used to prepare stock and standard solutions of the substances was evaporated under a nitrogen stream before mixing the standards with the blank serum. Contin *et al.*[6] separated lamotrigine, felbamate (FBM) and 10-monohydroxycarbazepine (MHD) in 4 min but higher LOD and LOQ values were obtained (*e.g.* LOD of 250 ng/mL and LOQ of 500 ng/mL for LTG). In a study conducted by Franceschi and Furlanut[7], an HPLC-UV method was applied for simultaneous quantification of oxcarbazepine, carbamazepine, their metabolites and lamotrigine in serum of epileptic patients. Each sample was pretreated by a SPE procedure which required a large volume of serum (500 µL) and lasted 30 min and a complex mobile phase consisting of five components (water-acetonitrile-methanol-acetic acid-triethanolamine) was used and the separation achieved within about 12 min. Greiner and

Haen[8] injected serum samples directly to the HPLC system after centrifugation and separated lamotrigine, oxcarbazepine and 10-monohydroxy derivative in more than 16 min. Bhatti *et al.*[9] used an evaporation step to remove acetonitrile which was utilized to precipitate serum proteins and separated phenytoin, carbamazepine and carbamazepine 10,11-epoxide in 12 min using a 15 cm length column.

A sensitive, rapid and simple HPLC method with spectrophotometric detection for the simultaneous determination of lamotrigine, phenytoin, carbamazepine, carbamazepine 10,11-epoxide, and oxcarbazepine in plasma of rat was reported in this work and the method was validated according to the guidelines of the International Conference on Harmonisation (ICH)[10-12] for therapeutic drug monitoring in epileptic patients or pharmacokinetic studies conducted in humans or laboratory animals.

EXPERIMENTAL

Carbamazepine and oxcarbazepine were gifts from Sobhan pharmaceutical company (Rasht, Iran), carbamazepine 10,11-epoxide purchased from Sigma-Aldrich (St. Louis, USA), lamotrigine was gifted by Arasto Company (Tehran, Iran) and phenytoin was a gift from Alhavi pharmaceutical company (Tehran, Iran). Methanol and acetonitrile were obtained from Caledon (Georgetown, Canada). 2-Propanol and potassium dihydrogen phosphate and orthophosphoric acid purchased from Merck (Darmstadt, Germany). Doubly distilled water prepared using the Millipore-Q-plus water purification system (Millipore, Bedford, MA, USA) was used in this study.

Preparation of standard solutions: Stock solutions of each drug (1000 µg/mL) were prepared in methanol. Standard plasma solutions were prepared by spiking blank rat plasma with stock solutions of all drugs then standard plasma samples of the drugs were prepared by serial dilution of the stock plasma samples in the mobile phase (mixture of potassium dihydrogen phosphate buffer (pH 6.0), acetonitrile and 2-propanol (63:22:15, v/v/v)).

Blood sampling and plasma processing: Two hundred µL plasma aliquots were spiked with 200 µL of stock solution of drugs (1000 µg/mL), deproteinized by addition of 200 µL acetonitrile, vortex mixed for 1 min and then centrifuged at 10,000 rpm at room temperature for 7 min. Twenty µL of the clean upper layer was injected directly into the chromatographic system.

Chromatographic apparatus and conditions: HPLC analysis was carried out on a WellChrom Maxi-Star K-1000 pressure pump, a WellChrom K-2500 spectrophotometer, a 4 channel degasser K-5003 and a data processor EuroChrom 2000 software all from Knauer (Berlin, Germany). Separation was performed on a Nova-Pak® C_{18} analytical column (Milford, Ireland; 250 × 4.6 mm, 4 µm). The column temperature was set at 25 ºC with Grace Vydac space column heater (Worms, Germany). The mobile phase consisted of phosphate buffer, acetonitrile and 2-propanol (63:22:15, v/v/v) at pH adjusted to 6.0 with orthophosphoric acid. The mobile phase was prepared freshly and filtered using a Millipore vacuum filter system equipped

with 0.45 μm membrane filter and degassed for 15 min. The phosphate buffer was prepared by dissolving 1.78 g of potassium dihydrogen phosphate (KH_2PO_4) in 200 mL of doubly distilled water. Chromatography was performed at 25 ºC by pumping the mobile phase at a flow rate of 1 mL/min. The column effluent was monitored at 220 nm.

Method validation: Quantification experiments were based on peak areas. The method validation included selectivity, linearity, precision, accuracy, limit of detection (LOD) and limit of quantification (LOQ).

The selectivity of the method was confirmed by the analysis of a solution containing 5 μg/mL of each drug. The ability to separate all compounds in the sample was demonstrated by assessing the resolution factor between the peaks corresponding to various compounds. The identification was performed by comparing the retention time of major peaks in the chromatogram of each drug in standard solutions with those in the plasma samples. Linearity data was assessed by analyzing three standard solutions at each concentration. Standard solutions containing 0.25, 0.5, 1.0, 2.5, 5.0, 10.0 and 25.0 μg/mL of drugs were prepared and injected into HPLC column. The linearity was evaluated by linear regression analysis.

Accuracy was determined by spiking the rat plasma with tested drugs to obtain two different concentrations covering the medium and higher ranges of the calibration curves and comparing peak areas of each concentration of the drugs in plasma with peak areas obtained from calibration equations.

The precision of the HPLC method was determined by repeatability (intra-day) and intermediate precision (inter-day). We evaluated repeatability data of the method by 6 repeated analyses of plasma standard solutions at 2.5 μg/mL and intermediate precision by repeated analysis performed on 3 days using the same samples then calculating the relative standard deviation (RSD).

The LOD was defined as the concentration of drugs giving a signal to noise ratio of 3:1 and LOQ was estimated as the concentration of drugs giving a signal to noise ratio of 10:1 with the RSD of less than 20 %.

RESULTS AND DISCUSSION

Chromatography: Fig. 1 showed the chromatogram of drug-free (blank) rat plasma and Fig. 2 illustrated the chromatogram of spiked rat plasma with 5 μg/mL of lamotrigine (LGT), carbamazepine 10,11-epoxide (CBZE), oxcarbazepine (OXC), carbamazepine (CBZ) and phenytoin (PHT).

Chromatographic performance was good for all compounds with good peak shapes, acceptable retention times and reasonable resolution for routine activity (Table-1).

Linearity: The linearity of the developed method was checked by analyzing in calibration samples, in the range of 0.25 to 25 μg/mL. Linearity parameters were determined using the linear regression method and were given in Table-2, showing high linearity of the method.

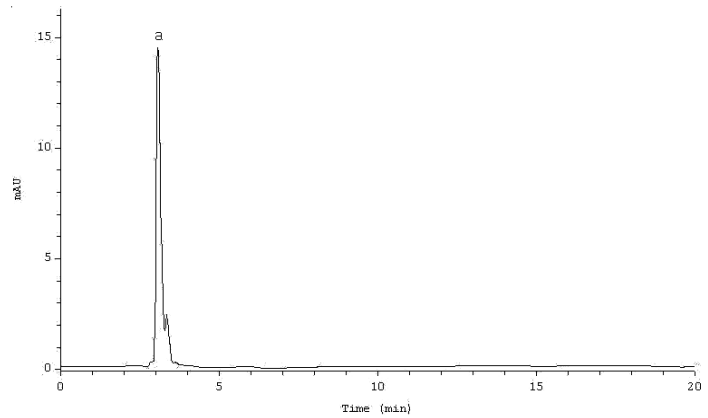

Fig. 1. Typical chromatogram obtained from a drug-free plasma of rat (a: plasma peak)

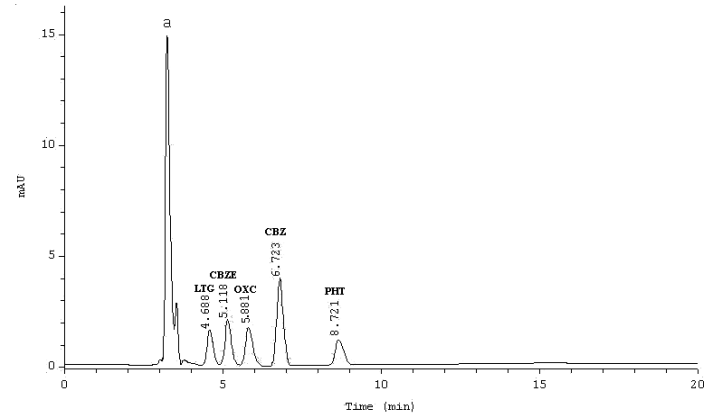

Fig. 2. Typical chromatogram obtained from a plasma of rat (a: plasma peak) spiked with 5 µg/mL of each drug

TABLE-1
RETENTION TIMES OF INVESTIGATED COMPOUNDS

Compounds	Retention time (min)	Rs*
CBZ	6.765 ± 0.03	1.68 (OXC, CBZ)
CBZE	5.205 ± 0.06	1.23 (LTG, CBZE)
OXC	5.914 ± 0.02	1.66 (CBZE, OXC)
PHT	8.820 ± 0.07	3.63 (CBZ, PHT)
LTG	4.659 ± 0.02	1.95 (Plasma peak, LTG)

*Resolution factor = $(t_2 - t_1) / 0.5 (w_1 + w_2)$.

Limits of detection and quantification: For each drug, the LOD and LOQ were calculated based on signal to noise ratio and were presented in Table-3.

Intra- and Inter-day precision: The results of precision were reported in Table-4. The intra-day precision was determined from replicated analyses of plasma samples containing CBZ, CBZE, OXC, LTG and PHT at 2.5 µg/mL. The inter-day precision was determined over a period of 3 d.

TABLE-2
LINEARITY PARAMETERS OF THE DRUGS IN
CONCENTRATION RANGE: 0.25-25 µg/mL FOR FIVE DRUGS

Compounds	Linearity	r[a]
CBZ	$y^b = 0.3106\ x^c + 0.0206$	0.999
CBZE	y = 0.0808 x - 0.0012	0.999
OXC	y = 0.0323 x + 0.0062	0.998
PHT	y = 0.0535 x - 0.0066	0.999
LTG	y = 0.1123 x + 0.0651	0.997

[a]Regression coefficient; [b]Peak area; [c]Drug concentration (µg/mL).

TABLE-3
LIMITS OF DETECTION AND LIMITS OF QUANTIFICATION
OF THE INVESTIGATED DRUGS

Compounds	LOD (ng/mL)	LOQ (ng/mL)
CBZ	100	330
CBZE	115	350
OXC	70	706
PHT	125	322
LTG	80	250

TABLE-4
PRECISION OF THE METHOD FOR EACH DRUG AT 2.5 µg/mL

Compounds	Intra-day RSD %[a]	Inter-day RSD %[b]
CBZ	2.59	2.81
CBZE	2.56	3.94
OXC	0.26	3.93
PHT	1.89	2.96
LTG	2.96	4.02

[a]Intra-day RSD %: relative standard deviation, as calculated on six independent assays carried out on the same day (repeatability).
[b]Inter-day RSD %: relative standard deviation, as calculated on three groups that each group containing six independent assays carried out on the same day (precision).

Accuracy: Accuracy for each compound was tested through analyses of two different concentrations of plasma calibration samples, 2.5 and 25 µg/mL and the obtained results were listed in Table-5.

TABLE-5
ACCURACY OF THE METHOD FOR EACH DRUG

Compounds	Accuracy (%)*	
	2.5 µg/mL	25.0 µg/mL
CBZ	95.57	104.83
CBZE	99.20	108.50
OXC	107.35	100.45
PHT	94.17	91.37
LTG	93.59	97.90

*(Found concentration / Added concentration) × 100 %.

Conclusion

An HPLC method with UV detection for the separation, identification and simultaneous quantification of CBZ, CBZE, OXC, LTG and PHT in rat plasma was developed. Although other methods exist for the measurement of AEDs, in this method the extraction process is simple requiring only 200 µL of plasma and run times are relatively shorter than previous methods. The experimental results indicated that this method was selective (resolution factor: 1.23 to 3.63), linear ($r \geq 0.998$), accurate (91.37 to 108.5) and precise (intra-day RSD: 0.26 to 2.96, inter-day RSD: 2.81 to 4.02). The obtained results of LOD (70 to 125 ng/mL) and LOQ (250 to 706 ng/mL) demonstrated the reliability and applicability of the procedure for the analysis of AEDs for TDM studies and pharmacokinetic studies conducted in humans and animals.

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