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Simultaneous determination of lamotrigine, phenobarbitone, carbamazepine and phenytoin in human serum by high-performance liquid chromatography

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Abstract

A simple reversed-phase high-performance liquid chromatography (HPLC) method was developed for the simultaneous estimation of the antiepileptic drugs (AEDs) lamotrigine (LTG), phenobarbitone (PB), carbamazepine (CBZ) and phenytoin (PHT) in human serum. The procedure involves extraction of the AEDs by mixing 200 μl of serum with 200 μl of acetonitrile containing 10 μg/ml of pentobarbitone as internal standard (IS). After centrifugation, 10 μl of the supernatant was injected onto a NOVA PAK C-18 column (250 mm × 4.6 mm, 5 μm Hypersil ODS) and eluted with a mobile phase consisting of phosphate buffer (10 mM)–methanol–acetonitrile–acetone in the ratio of 55:22:12:11 (v/v) adjusted to pH 7.0. A UV detector set at 210 nm was employed for detection. The AEDs were well resolved from the human serum constituents and the internal standard. The method can quantify LTG, PB, CBZ, and PHT at concentrations as low as 0.2 μg/ml. The method was quantitatively evaluated in terms of linearity, accuracy, precision, recovery, selectivity, sensitivity, and specificity. The method is simple, convenient, and suitable for the analysis of AEDs from human serum.

Keywords: Lamotrigine; Phenobarbitone; Carbamazepine; Phenytoin; Serum; High-performance liquid chromatography

1. Introduction

Therapeutic drug monitoring (TDM) of antiepileptic drugs is necessary to optimize the patient's clinical outcome by managing their medication regimen with the assistance of measured drug concentration [1]. Plasma concentration monitoring is widely used for the clinical management of epileptic patients receiving phenytoin (PHT), phenobarbitone (PB) and carbamazepine (CBZ) [2]. Lamotrigine (LTG), a new antiepileptic drug that is currently used as an add-on or monotherapy in patients with partial and secondary generalized seizures, also requires monitoring. There are large inter-individual variations in dose versus serum concentrations in patients on monotherapy, and pharmacokinetic variability plays a major role in the lamotrigine dosage requirements to achieve optimum serum concentrations depending on interacting AEDs comedication [3].

There are several HPLC methods reported for determination of lamotrigine in human serum or plasma [4–9] and there are various HPLC methods reported for the simultaneous determination of PHT, PB and CBZ [10–13]. For research work related to the study of drug interactions, pharmacokinetic studies and routine therapeutic drug monitoring, analytical methods that can reliably and simultaneously measure LTG and other AEDs are highly desirable. But there are very few HPLC methods for the determination of LTG simultaneously with other AEDs in plasma or serum [14–17].

Meyler et al. [14] described an HPLC method for the determination of LTG with PHT, PB and CBZ. In that method, LTG coeluted with CBZ showing broad tailing peaks. Ramachandran et al. [15] used dual wavelengths for the measurement of LTG with PHT, PB and CBZ, but they were unable to separate lamotrigine from interference with carbamazepine-

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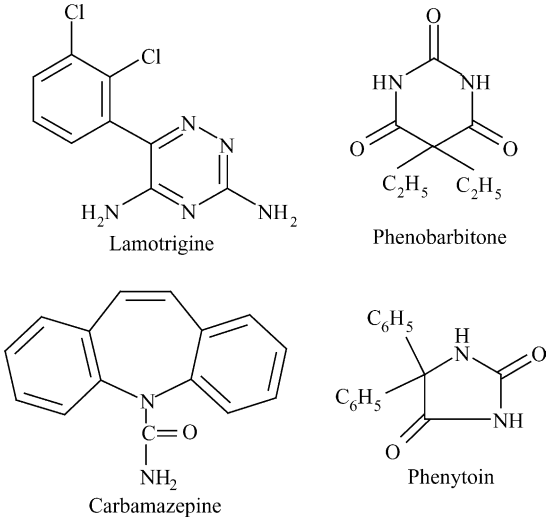

Fig. 1. Chemical structures of lamotrigine, phenobarbitone, carbamazepine, and phenytoin.

10,11-epoxide (CBZ-E). The method described by Lensmeyer et al. [16] reported the simultaneous determination of LTG with PHT, CBZ and CBZ-E but not PB, which is also a widely used AED in concomitant therapy. This method involves the solid-phase extraction of drugs.

The method described by Matar et al. [17] is the only method which can be used for the simultaneous determination of LTG, PHT, PB, CBZ and CBZ-E. This method has limitation due to the long extraction procedure and also partial overlapping of peaks (LTG with PB and PHT with CBZ) was seen, which can reduce the accuracy of the measurements.

In the present work, we report a new isocratic reversed-phase HPLC-UV method for the simultaneous measurement of lamotrigine, phenobarbitone, carbamazepine and phenytoin (Fig. 1) in human serum.

2. Materials and method

2.1. Apparatus

The high-performance liquid chromatographic system was equipped with a JASCO solvent delivery pump (PU-1580), JASCO autosampler (AS-1555), JASCO UV/visible detector (UV-1575) and Borwin Chromatographic Software for data integration, all supplied by JASCO Corporation, Tokyo, Japan. Chromatographic separations were performed using a NOVA PAK C-18 stainless steel column (250 × 4.6 mm. 5 μm Hypersil ODS) supplied by Thermo Quest Hypersil Division, Mumbai, India. The main column was protected and preceded by a guard cartridge (7.5 mm × 4.6 mm, 5 μm Hypersil ODS) supplied by Flexit Jour Pvt. Ltd., Pune, India.

2.2. Drugs and chemicals

LTG was provided as a gift sample from RPG Life Sciences Ltd., Mumbai, India, and PHT and CBZ were provided as gift samples by Sun Pharmaceuticals (Mumbai, India). PB and pentobarbitone (IS) (Sigma Chemicals) and drug-free human serum was donated by Department of Pharmacology, B J Medical College and Sassoon Hospital, Pune, India. CBZ-E was purchased from Sigma–Aldrich Inc., St. Louis, USA. Solvents used were of HPLC grade and all other chemicals and reagents were of analytical grade.

2.3. Preparation of standard solutions

A stock solution containing 1 mg/ml each of LTG, PB, CBZ and PHT was prepared in methanol. The calibration standards (0.5, 1, 5, 10, 20 and 40 μg/ml) were prepared by further dilution of stock solution with drug-free human serum. Another stock solution containing 1 mg/ml pentobarbitone (IS) was prepared in methanol and was further diluted with acetonitrile to give a concentration of 10 μg/ml. All solutions were stored at −20 °C.

2.4. Chromatographic conditions

The mobile phase consisted of phosphate buffer (10 mM)–methanol–acetonitrile–acetone (55:22:12:11, v/v/v/v) at pH adjusted to 7.0 with 0.5 M NaOH. The mobile phase was always freshly prepared and was degassed and filtered by using a Millipore vacuum filter system equipped with 0.45 μm membrane filter. The 10 mM phosphate buffer was prepared by dissolving 1.36 g of potassium dihydrogen phosphate (KH_2PO_4) in 1 L of doubly distilled water. Chromatography was performed at ambient temperature by pumping the mobile phase at a flow rate of 1.2 ml/min. The column effluent was monitored at 210 nm.

2.5. Extraction procedure

Serum (200 μl) was transferred to polypropylene micro tubes (1.5 ml) and 200 μl of internal standard solution was added to it. The mixture was vortex-mixed for 30 s and centrifuged at 11,000 × *g* for 20 min. The supernatant was transferred to a clean, similarly labeled tube, and recentrifuged for 5 min. The supernatant was filtered through a filtration assembly equipped with 0.2 μm membrane filter, and 10 μl of filtrate was injected onto the column.

2.6. Application

The method was used for therapeutic drug monitoring in about 200 epileptic patients presently under the treatment of different antiepileptic drugs (LTG, PB, CBZ and PHT). The study was carried out after approval of protocol from Institutional Ethical Committee of B J Medical College and Sassoon Hospital, Pune, India. Venous blood sam-

ples were withdrawn from epileptic patients collected in centrifuge tubes and processed by centrifugation to obtain serum. The samples were analyzed using the above-described HPLC method.

3. Results and discussion

Fig. 2 shows representative chromatograms of drug-free (blank) human serum and a blank sample spiked with pentobarbitone (IS). Resolution for all the drugs was found to be adequate at pH 7 and the use of acetone in mobile phase played an important role in increasing the resolution of all the peaks, including shifting the carbamazepine peak before phenytoin (not seen in other methods). Fig. 3 illustrates a representative chromatograms of calibration standards viz. 0.5 μg/ml and 20 μg/ml of lamotrigine, phenobarbitone, carbamazepine and phenytoin. The same method can also be used to estimate carbamazepine-10,11-epoxide, an active metabolite of carbamazepine in patient serum. Fig. 4A depicts the chromatogram of a serum sample of epileptic patient receiving phenytoin, 300 mg/day, and carbamazepine, 600 mg/day. The estimated levels (trough) for phenytoin and carbamazepine were 5.49 and 7.54 μg/ml, respectively. Fig. 4B depicts the chromatogram of same patient's serum sample spiked with lamotrigine (11.74 μg/ml) and phenobarbitone (38.01 μg/ml). After each day, the column was washed with water and methanol. The column can be used for more than 300 injections.

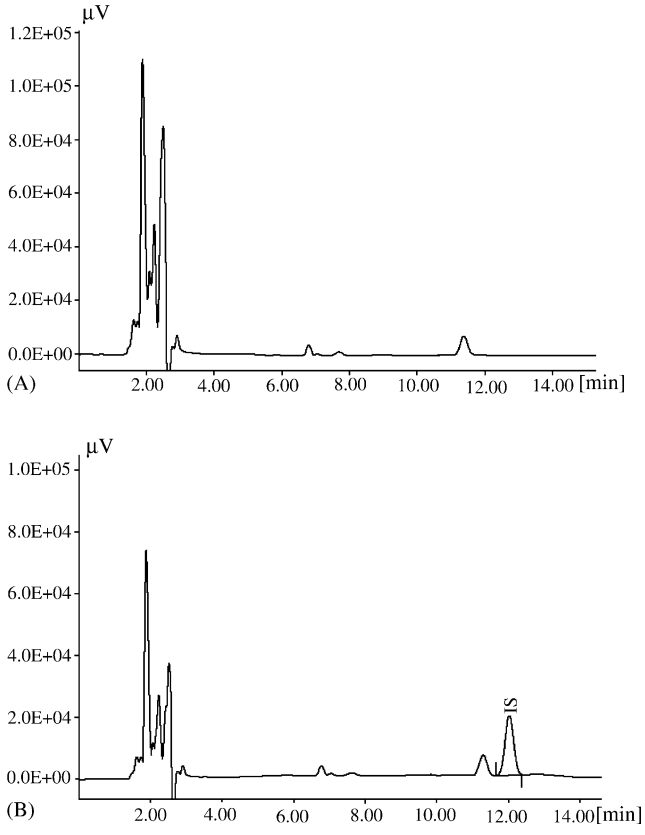

Fig. 2. Representative chromatograms of (A) blank human serum without IS, and (B) blank human serum with IS.

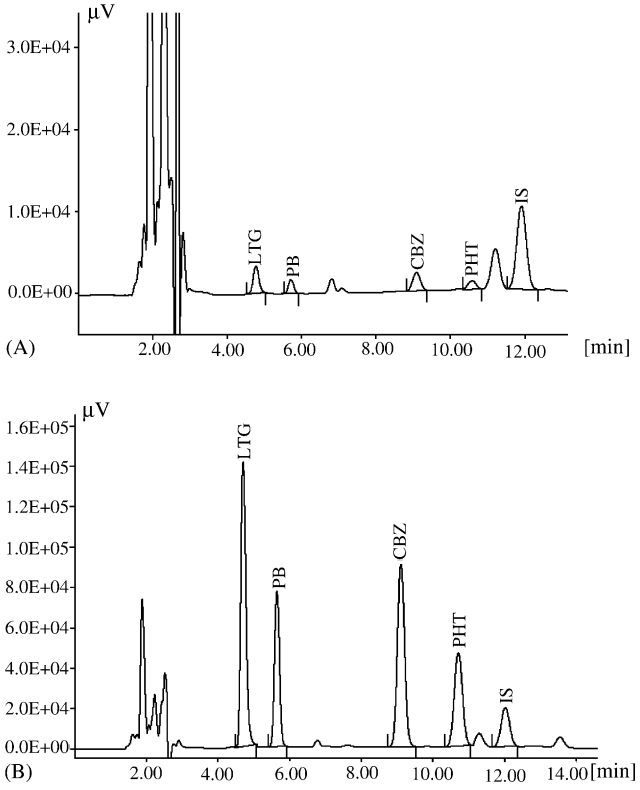

Fig. 3. Representative chromatograms of calibration standards (A) 0.5 μg/ml, and (B) 20 μg/ml.

3.1. Quantitation

Quantitation of serum AED concentrations for all the drugs was achieved by relating peak-area ratios of the drug to the IS with the known concentrations on the calibration curve. To do this, a working standard solution prepared by using blank serum was extracted as described earlier. The slope, intercept and correlation coefficient (r) was determined using the least-squares linear regression analysis method. Results from the linearity study (Table 1) shows little variation in slope (% CV in range of 3.65–7.01) and good correlation ($r > 0.998$) between peak-area ratio and concentration.

3.2. Sensitivity

Under the experimental conditions used, the detection limit (LOD) was approximately 0.1 μg/ml for all drugs, at a signal-to-noise ratio of 3:1. The limit of quantification (LOQ) is the lowest amount of analyte, which can be measured with defined precision and accuracy. This was found to be 0.2 μg/ml, with the coefficient of variation less than 8%.

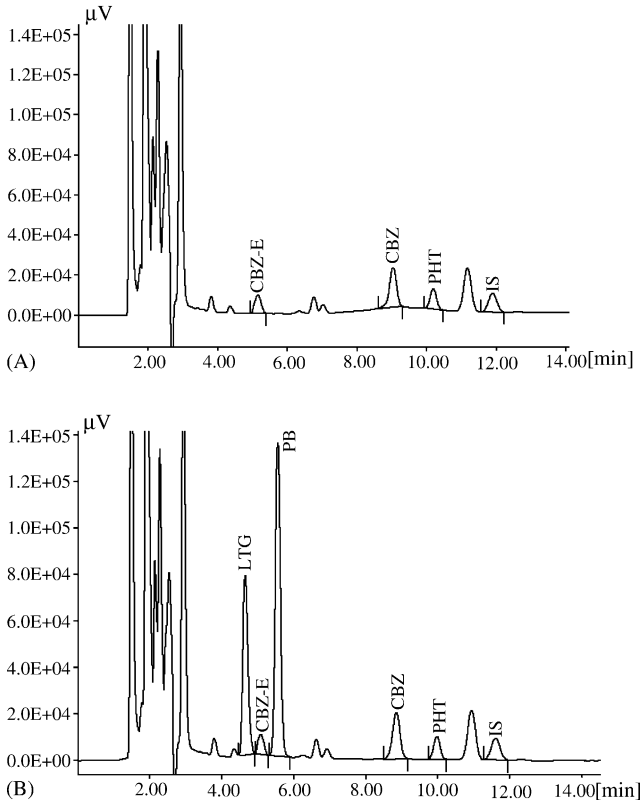

Fig. 4. Depicts the chromatograms (A) of serum of epileptic patient on phenytoin (300 mg/day) and carbamazepine (600 mg/day) therapy, and (B) of same patients serum spiked with lamotrigine (11.74 μg/ml) and phenobarbitone (38.01 μg/ml).

3.3. Specificity

The method was evaluated for specificity by analyzing 10 different batches of drug-free human serum ($n = 3$) to check the interference of peaks of endogenous components of serum. All the different batches of serum tested were found to be free from interfering components at the retention times of the drugs and the IS.

Table 1
Standard curve summary

Drug		Intercept	Slope	Correlation (r)
Lamotrigine ($n = 6$)	Mean	0.0904	0.3836	0.9988
	S.D.	0.0108	0.0231	0.0017
	% CV	–	6.0208	0.1799
Phenobarbitone ($n = 6$)	Mean	0.0125	0.2303	0.9988
	S.D.	0.0024	0.0161	0.0012
	% CV	–	7.0169	0.1223
Carbamazepine ($n = 6$)	Mean	−0.0082	0.3821	0.9995
	S.D.	0.0029	0.0169	0.0002
	% CV	–	4.4384	0.0298
Phenytoin ($n = 6$)	Mean	0.0254	0.2345	0.9995
	S.D.	0.0037	0.0086	0.0024
	% CV	–	3.6543	0.24991

Table 2
Retention times of some tested drugs and metabolite

Drug	Retention time (min)
Lamotrigine	4.64
Phenobarbitone	5.55
Carbamazepine	8.85
Phenytoin	9.97
Carbamazepine-10,11-epoxide	5.08
Ethosuximide	3.56
Pentobarbitone	11.60
Diazepam	4.04
Quinidine	16.82
Alprazolam	14.45
Clonazepam	15.63
Thiopentone	15.35
Acetominophen	1.42
Chloramphenicol	6.16
Acetylsalicylate	1.15
Lidocaine	28.86
Theophylline	1.23
Caffeine	2.01
Valproic acid	ND
Oxcarbazepine	ND
Vigabatrin	ND
Gabapentine	ND

ND: not detected within 30 min from injection.

3.4. Selectivity

Commonly administered drugs, mainly taken by epileptic patients were tested for possible interference in the HPLC assay. Table 2 lists the retention times of the drugs and metabolite tested.

3.5. Precision and accuracy

The intraday precision was determined from replicate analysis of pooled human serum samples containing lamotrigine, phenobarbitone, carbamazepine and phenytoin at three different concentrations covering the low, medium and higher ranges of the calibration curve. Precision was expressed as the percent coefficient of variation (% CV), and accuracy was expressed as a percentage of the theoretical concentration (observed concentration × 100/theoretical concentration). The intraday precision ranged from 2.40 to 6.83% CV. Accuracy ranged from 97.76 to 104.83% (Table 3).

The interday precision was determined over a period of two weeks. The interday precision ranged from 2.78 to 7.89% CV. The accuracy ranged from 101.46 to 107.55% (Table 3).

3.6. Absolute recovery

The absolute recoveries for the AEDs and IS were determined by spiking known quantities of AEDs into drug-free human serum to obtain three different concentrations covering the low, medium and higher ranges of the calibration curve. The samples were then extracted and analyzed as described earlier. The absolute recovery was calculated by com-

Table 3
Intraday and interday precision of lamotrigine, phenobarbitone, carbamazepine and phenytoin in human serum

Concentration added (μg/ml)	Intraday[a]			Interday[b]		
	Measured concentration (mean ± S.D.)	CV (%)	Accuracy[c] (%)	Measured concentration (mean ± S.D.)	CV (%)	Accuracy
Lamotrigine						
1	1.048 ± 0.070	6.756	104.83	1.062 ± 0.069	6.552	106.222
5	4.918 ± 0.157	3.187	98.36	5.235 ± 0.245	4.682	104.711
10	10.252 ± 0.364	3.552	102.52	10.268 ± 0.419	4.082	102.688
Phenobarbitone						
1	1.025 ± 0.070	6.83	102.5	1.048 ± 0.082	7.89	104.888
10	9.906 ± 0.312	3.15	99.06	10.19 ± 0.299	2.94	101.90
20	20.25 ± 0.486	2.40	101.25	20.384 ± 0.568	2.78	101.92
Carbamazepine						
1	1.033 ± 0.054	5.28	103.33	1.075 ± 0.066	6.221	107.55
5	5.07 ± 0.194	3.844	101.40	5.144 ± 0.151	2.95	102.88
10	9.911 ± 0.302	3.055	99.11	10.407 ± 0.289	2.780	104.077
Phenytoin						
1	1.023 ± 0.030	2.942	102.33	1.05 ± 0.073	6.998	105
5	4.888 ± 0.189	3.873	97.76	5.073 ± 0.17	3.36	101.46
10	10.103 ± 0.268	2.657	101.03	10.32 ± 0.366	3.54	103.24

[a] Mean values represent six different serum samples for each concentration.
[b] Interday was determined from nine different runs over two-week period. The concentration of each run was determined from a single calibration curve run on the first day of the study.
[c] Accuracy = 100 (observed concentration/theoretical concentration).

paring the resultant peak areas with those obtained from pure standards in methanol of the drugs and the internal standard at the same concentrations. The absolute recoveries of lamotrigine, phenobarbitone, carbamazepine and phenytoin ranged from 94.135 ± 3.35 to 102.05 ± 2.62%, while the absolute recovery for IS was 96.58 ± 3.62% (Table 4). The relative recovery of the AEDs was calculated by comparing the concentrations of the drug-spiked serum with the actual added concentration. The relative recovery of the AEDs ranged from 97.76 ± 3.78 to 104.83 ± 7.08% (Table 4).

Table 4
Absolute and relative recoveries of lamotrigine, phenobarbitone, carbamazepine and phenytoin

Concentration (μg/ml)	Absolute recovery[a] (% mean ± S.D.)	Relative recovery[a] (% mean ± S.D.)
Lamotrigine		
1	95.90 ± 5.33	104.83 ± 7.08
5	94.13 ± 3.35	98.36 ± 3.13
10	96.07 ± 1.99	102.51 ± 3.64
Phenobarbitone		
1	95.24 ± 4.18	102.5 ± 7.00
10	99.23 ± 2.60	99.06 ± 3.12
20	102.05 ± 2.62	101.25 ± 2.43
Carbamazepine		
1	95.99 ± 2.91	103.33 ± 5.46
5	97.65 ± 3.09	101.40 ± 3.89
10	98.20 ± 1.20	99.11 ± 3.03
Phenytoin		
1	95.65 ± 3.84	102.33 ± 3.01
5	96.39 ± 3.08	97.76 ± 3.78
10	100.9 ± 1.92	101.03 ± 2.68
Internal standard		
10	96.58 ± 3.62	–

[a] Mean values represent six different serum samples for each concentration.

3.7. Stability

The antiepileptic drugs are stable in serum/plasma when stored at −20 °C for at least four weeks [17]. Stability of the extracted drug samples from serum samples with AED concentrations covering the low, medium and higher ranges of calibration curve was tested. The extracted drug samples ($n = 4$) were stored at room temperature (25 °C) for 24 h prior to analysis. The results showed that the percent coefficient of variation was in range of 1.07–2.91, and thus, the extracted samples were stable at 25 °C for at least 24 h.

4. Conclusion

A simple, sensitive, specific, accurate, and precise HPLC method for simultaneous determination of lamotrigine, phenobarbitone, carbamazepine and phenytoin in human serum using a simple single-step extraction procedure is reported. The method is suitable for therapeutic drug monitoring studies and can also be used for pharmacokinetic studies conducted in humans.

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