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Liquid Chromatographic Determination of Six Antiepileptic Drugs and Two Metabolites in Microsamples of Human Plasma

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Summary: A simple, rapid, sensitive, and reproducible high-performance liquid chromatographic (HPLC) method for simultaneous determination of the antiepileptic drugs (ethosuximide, primidone, lamotrigine, phenobarbital, phenytoin, and carbamazepine) and two metabolites (carbamazepine-diol and carbamazepine-epoxide) in human plasma is described. The procedure involves extraction of the drugs from human plasma (100 μL) with ether using 9-hydroxymethyl-10-carbamyl acridan as an internal standard. The extract was evaporated and reconstituted with mobile phase and then injected onto the chromatograph. The drugs and the internal standard were eluted from a Supelcosil LC-18 stainless steel column at ambient temperature with a mobile phase consisting of a 0.01M phosphate buffer/methanol/acetonitrile (65/18/17, v/v/v) adjusted to a pH of 7.5 with phosphoric acid and a flow rate of 1 mL/min. The effluent was monitored at 220 nm. Quantitation was achieved by using peak area ratio of each drug to the internal standard. The intraassay and interassay coefficients of variation (CV) ranged from 2.43% to 6.25% and from 3.02% to 5.85%, respectively. The absolute (extraction) and relative (analytical) recoveries for the drugs ranged from 70.7% to 104.4% and from 88.3% to 106.1%, respectively. Stability tests showed that the drugs were stable in plasma for at least 4 weeks when stored at −20°C. The method was applied clinically for monitoring the AEDs in epileptic patients. **Key Words:** Antiepileptics—Metabolites—Human Plasma—High Performance Liquid Chromatography.

Lamotrigine, LTG [3,5-diamino-6 (2,3-dichlorophenyl)-1,2,4-triazine] is a new antiepileptic drug (AED) that is currently used as an add-on or monotherapy in patients with partial and secondary generalized seizures (1). When LTG is used as an add-on therapy, dosage depends on the type of concomitantly administered drugs. In the presence of hepatic enzyme inducers such as carbamazepine (CBZ), phenobarbital (PB), phenytoin (PT), and primidone (PR), higher doses of LTG may be required as a consequence of the drug's increased elimination. By contrast, a much lower dose of LTG may be needed with enzyme inhibitors such as valproic acid (VPA), which reduce LTG elimination (2). On the other hand, there are conflicting reports regarding the effect of LTG on either CBZ or its active metabolite, CBZ-10, 11-epoxide (CBZ-E). The initial report (3) that LTG elevated CBZ-E levels leading to neurotoxicity was not subsequently confirmed by other studies (4,5). For the further study of such potential interactions, to ensure compliance, and to reduce the likelihood of adverse effects, an analytic method that can reliably and simultaneously measure LTG and other AEDs with their active metabolites is highly desirable. Moreover, monitoring of AED concentrations in plasma is useful for the optimal drug therapy of epilepsy, because the therapeutic and toxic effects of most AEDs are better correlated with plasma concentrations than with administered doses (6).

The management of epilepsy has greatly benefited from the use of reliable and reproducible AED assays. Of

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these, high performance liquid chromatography (HPLC) stands out as highly efficient, rapid, less expensive, and versatile, and it has the potential for simultaneous measurements of most AEDs that are routinely administered together.

Very few HPLC methods exist for the determination of LTG simultaneously with other AEDs in plasma (7–9). Meyler et al (7) described a method for the determination of LTG with CBZ, PT, and PB. In that method, LTG coeluted with CBZ giving broad tailing peaks. Ramachandran et al (8) used dual wavelengths for the measurement of LTG with PB, PT, and CBZ but they were unable to separate LTG from the active metabolite of CBZ (CBZ-E). The method described by Lensmeyer and co-workers (9) for the determination of LTG in the presence of PT, CBZ, and CBZ-E is efficient. However, this assay can be rather tedious and expensive as it involves solid-phase extraction of the drugs from plasma as opposed to conventional extraction with organic solvents.

In this report we describe a new micro-method for the simultaneous determination of LTG with ethosuximide (ES), primidone (PR), PB, PT, CBZ, and two metabolites of CBZ [carbamazepine-diol (CBZ-D)] and CBZ-E in human plasma. The method is simple to perform, rapid, and of sufficient precision and accuracy for clinical applications.

MATERIALS AND METHODS

Reagents

Lamotrigine was kindly donated by Glaxo-Wellcome (London, UK). ES, PR, PB, PT, and CBZ were purchased from Sigma Chemical (St. Louis, MO, USA). CBZ-E, CBZ-D, and the internal standard (9-hydroxymethyl-10-carbamyl acridan) were gifts from Ciba-Geigy (Basle, Switzerland). Double distilled and deionized water was obtained with the aid of Milli-Q Water Purification System (Millipore, Bedford, MA, USA). Solvents used were of HPLC grade and all other chemicals and reagents were of analytic grade.

Apparatus

The analysis of the AEDs in human plasma was performed on a Waters Liquid Chromatograph (Waters, Milford, MA, USA). It consisted of a solvent delivery system (600E), an autosampler (M-717 plus), a dual wavelength absorbance detector (M-2487), and Millennium software (version 2.15) for chromatographic determination and evaluation. Chromatographic separations were performed using a Supelcosil LC-18 stainless steel column (150 mm × 4.6 mm i.d., 5 μm (Supelco, Bellefonte, PA, USA) coupled with a Waters Resolve C18-guard column. The column was maintained at ambient temperature.

Standard Solutions

Standard stock solution was prepared in methanol consisting of 2 mg/mL of each individual AED and the two metabolites. This solution was added to drug-free human plasma (10 mL) to prepare five nonzero concentrations (in the range of 20–200, 2–40, 0.5–15, 0.5–15, 2–60, 0.5–15, 2–40 and 2–30 mg/L for ES, PR, CBZ-D, LTG, PB, CBZ-E, PT and CBZ, respectively) and five quality control concentrations of the combined AEDs and their metabolites. Standard drug concentrations used for the preparation of the calibration curves were different from those employed in the quality control studies.

The internal standard, 9-hydroxymethyl-10-carbamyl acridan was selected from a list of potential standards because of its close structural similarity to some of the AEDs. It is extracted well with diethyl ether and has good UV absorption characteristics of the appropriate wavelength (220 nm). The drug was dissolved in methanol and a working solution of 50 mg/L was obtained by appropriate dilution of the stock (1 mg/mL).

Chromatographic Conditions

A mobile phase comprising K2HPO4 (0.01 M)-methanol-acetonitrile (65:18:17, v/v/v) at a pH adjusted to 7.5 with phosphoric acid was used. It was freshly prepared, then filtered by suction through a 0.45μm Millipore membrane filter (Millipore, Bedford, MA, USA) and degassed with helium. Chromatography was performed at ambient temperature by pumping the mobile phase isocratically at a flow rate of 1 mL/min. The column effluent was monitored at 220 nm, as all the AEDs presently assayed had optimum absorption at or around this band.

Extraction Procedure

100 μL of human plasma was thoroughly mixed with 20 μL of the IS working solution in a 2 mL microcentrifuge tube (Eppendorf, Hamburg, Germany). The content of the tube was extracted with 1.0 mL of diethyl ether. This involved vortex mixing for 30 sec, shaking on a rotary mixer for 5 min and subsequent centrifugation at 1000*g* for 10 min. After phase separation, the organic layer was transferred to another microcentrifuge tube and

allowed to evaporate to dryness at room temperature under a stream of purified nitrogen. The residue was redissolved in 100 μL of mobile phase, and 20 μL of the resulting solution was injected into the chromatograph.

Clinical Application

The clinical utility of the assay was assessed by collecting blood samples from epileptic patients receiving different AEDs. The method is now routinely applied in our clinical pharmacokinetic lab. (KKUH, Riyadh, SA) for monitoring AEDs as a replacement of TDX assays, which have been found to be fairly expensive compared to the HPLC system. More than 200 patient samples have already been analyzed using this method. The advantage of this method is its simultaneous determination of different AEDs with their active metabolites. Fig. 2 shows an example of such application in a patient receiving a combination of AEDs.

RESULTS AND DISCUSSION

The present procedure involves extraction of AEDs from the human plasma using diethyl ether rather than deproteinization with either acetonitrile or acids such as perchloric acid or trichloroacetic acid. The limitation of the latter procedure is that the plasma protein can not be entirely removed and consequently the column shelf life will be shortened. Instead, our procedure is preferable because of the low boiling point of diethyl ether and the injection of very clear solutions into the column, which may lead to prolongation of column shelf life compared to the deproteinization procedure.

Figure 1 shows typical chromatograms of drug-free human plasma and a plasma sample containing spiked standards of ES, PR, CBZ-D, LTG, PB, CBZ-E, PT, and

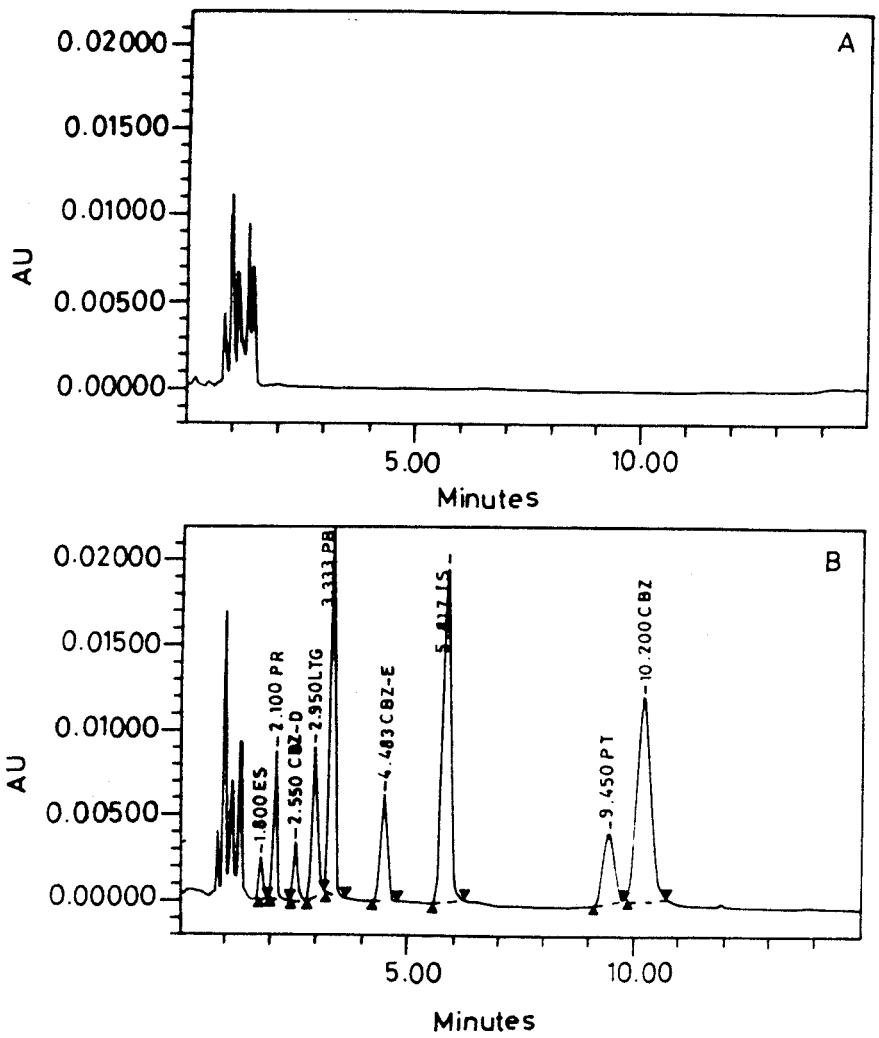

FIG. 1. Typical chromatograms. (A) drug-free human plasma. (B) human plasma extract spiked with 25 mg/L ethosuximide (ES), 2.5 mg/L primidone (PR), 1.0 mg/L carbamazepine-diol (CBZ-D), 1.0 mg/L lamotrigine (LTG), 5 mg/L phenobarbital (PB), 1.0 mg/L carbamazepine-epoxide (CBZ-E), 2.5 mg/L phenytoin (PT) and 2.5 mg/L carbamazepine (CBZ) standards. IS, is the internal standard (9-hydroxymethyl-10-carbamyl acridan).

TABLE 1. *Retention times of some drugs and metabolites tested for potential interference*

Drug	Retention time (min)
Ethosuximide	1.80
Primidone	2.10
Carbamazepine-diol	2.55
Lamotrigine	2.95
Phenobarbital	3.33
Carbamazepine-epoxide	4.48
Phenytoin	9.45
Carbamazepine	10.20
Butabarbital	5.10
Secobarbital	13.45
Hexobarbital	7.95
P-Hydroxyphenobarbital	1.65
5-(P-Hydroxyphenyl)5-phenyl-hydantoin (p-HPPH)	3.62
Oxcarbazepine	5.62
10,11-Dihydro-10-hydroxycarbamazepine	3.54
Ranitidine	2.30
Cyclobarbital	4.96
Gabapentin	ND
Valproic acid	ND
Caffeine	ND
Glibenclamide	ND
Paracetamol	ND
Meclofenamate	ND
Theophylline	ND
Vigabatrin	ND
Diazepam	ND
Clonazepam	ND

ND, not detected within 30 minutes from injection.

CBZ. Figure 2 illustrates a representative chromatogram of a plasma sample of an epileptic patient receiving LTG, PB, PT, and CBZ using the described procedure. There was no interference from endogenous components of the plasma. The retention times of AEDs are shown in Table 1. The retention capacity factors (k') of ES, PR, CBZ-D, LTG, PB, CBZ-E, PT, CBZ, and the internal standard were 0.8, 1.1, 1.6, 2.0, 2.3, 3.5, 8.5, 9.2, and 4.8 min, respectively.

A number of solvents were examined for efficient extraction of the AEDs. Of these, only dichloromethane and diethyl ehter yielded sufficiently clear chromatograms. Diethyl ether was selected for our purpose because of its good extraction capacity and its boiling point, which affords a rapid evaporation at room temperature. This is particularly important because one of the AEDs to be determined (ES), is fairly volatile.

Quantitation

Quantitation of plasma AED levels was achieved by relating peak-area ratios to known concentrations in a calibration curve. To do this, linearity of the assay was first established by spiking drug-free human plasma with known amounts of each drug (n = 6). These were duly extracted and analyzed as described earlier. The standards were in the range of; 20–200, 2–40, 0.5–15, 0.5–15, 2–60, 0.5–15, 2–40 and 2–30 mg/L for ES, PR, CBZ-D, LTG, PB, CBZ-E, PT and CBZ, respectively. These standards were analyzed in replicates of ten over the ranges mentioned above. The slopes, intercepts and correlation coefficients (r) were determined using the least-squares linear regression analysis method. The results showed little variabilities in slopes (%CV in the range of 3.12–13.18) and had good correlation ($r > 0.99$) between peak area ratio and concentration over the specified ranges.

Sensitivity

The AEDs and metabolites under study were prepared in plasma at a concentration of 0.2 mg/L and analyzed.

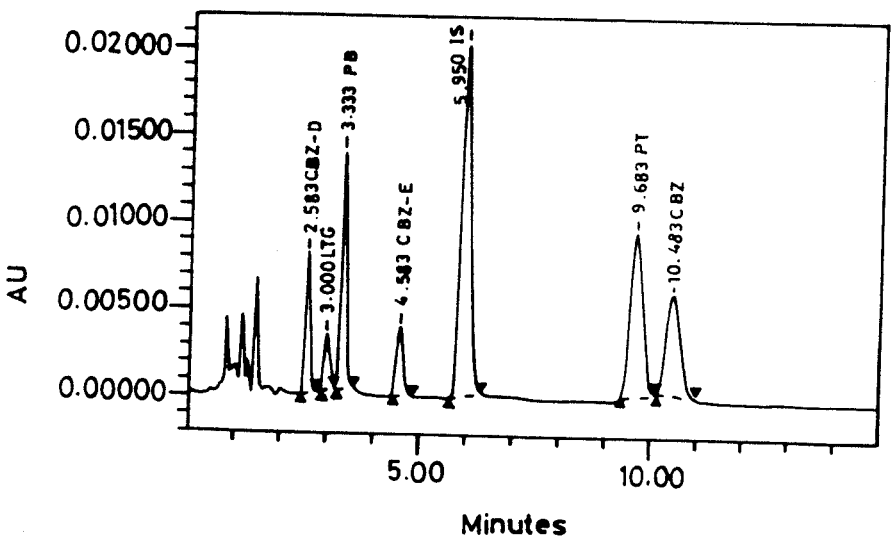

FIG. 2. Typical chromatogram of a plasma extract from an epileptic pediatric patient receiving a polypharmacy of AEDs: lamotrigine (LTG) 25 mg BID, phenobarbital (PB) 35 mg TDS, phenytoin (PT) 10 mg TDS, and carbamazepine (CBZ) 100 mg TDS). The measured concentration (trough) of each drug was 2.14 mg/L, CBZ-D; 0.34 mg/L, LTG; 3.0 mg/L, PB; 0.64 mg/L, CBZ-E; 5.62 mg/L, PT and 1.17 mg/L, CBZ.

TABLE 2. *Precision study of AEDs and metabolites* (n = 10)

Concentration added (mg/L)	Mean concentration determined ± SD (mg/L)	% CV	Mean concentration determined ± SD (mg/L)	% CV
	Intraassay		Interassay	
Ethosuximide				
25	22.80 ± 1.58	6.93	25.36 ± 2.10	8.28
50	48.87 ± 2.92	5.98	49.94 ± 3.56	7.13
75	73.40 ± 4.87	6.63	76.95 ± 4.47	5.81
100	90.02 ± 5.23	5.81	99.11 ± 4.93	4.97
150	139.93 ± 8.23	5.88	152.92 ± 4.71	3.08
Mean		6.25		5.85
Primidone				
2.5	2.44 ± 0.07	2.88	2.44 ± 0.14	5.70
5.0	5.02 ± 0.25	5.02	4.93 ± 0.16	3.16
10	9.33 ± 0.09	0.95	9.75 ± 0.29	2.96
20	19.83 ± 0.38	1.92	20.18 ± 0.64	3.15
30	29.68 ± 0.41	1.38	30.28 ± 0.91	3.02
Mean		2.43		3.60
Carbamazepine-diol				
1.0	109 ± 0.04	3.61	1.04 ± 0.04	3.87
2.5	2.58 ± 0.17	6.40	2.43 ± 0.14	5.68
5.0	5.13 ± 0.10	1.95	4.60 ± 0.14	3.04
7.5	7.55 ± 0.14	1.84	7.52 ± 0.13	1.69
10.0	10.25 ± 0.21	2.10	10.25 ± 0.15	1.46
Mean		3.18		3.15
Lamotrigine				
1.0	1.15 ± 0.05	4.58	1.08 ± 0.06	5.45
2.5	2.66 ± 0.17	6.20	2.43 ± 0.06	2.55
5.0	5.33 ± 0.20	3.85	4.38 ± 0.13	2.90
7.5	7.93 ± 0.16	2.04	7.67 ± 0.20	2.63
10.0	10.49 ± 0.28	2.65	10.19 ± 0.19	1.86
Mean		3.86		3.08
Phenobarbital				
5	5.14 ± 0.21	4.12	4.94 ± 0.30	5.97
10	10.67 ± 0.36	3.36	10.46 ± 0.33	3.15
15	15.17 ± 0.43	2.85	15.31 ± 0.36	2.35
25	25.06 ± 0.32	1.26	24.85 ± 0.96	3.88
50	50.03 ± 0.84	1.69	50.15 ± 0.90	1.79
Mean		2.66		3.43
Carbamazepine-epoxide				
1.0	1.13 ± 0.05	4.11	1.08 ± 0.06	5.49
2.5	2.35 ± 0.15	6.51	2.22 ± 0.05	2.45
5.0	4.65 ± 0.07	1.55	4.79 ± 0.13	2.78
7.5	7.32 ± 0.11	1.56	7.35 ± 0.18	2.49
10.0	10.24 ± 0.20	1.97	10.35 ± 0.20	1.90
Mean		3.14		3.02
Phenytoin				
2.5	2.43 ± 0.07	2.88	2.28 ± 0.11	4.82
5.0	4.89 ± 0.31	6.28	4.61 ± 0.14	2.97
10.0	9.88 ± 0.20	2.03	10.27 ± 0.42	4.09
20.0	19.96 ± 0.32	1.60	20.36 ± 0.50	2.44
30.0	29.19 ± 0.79	2.70	29.98 ± 0.34	1.15
Mean		3.10		3.09
Carbamazepine				
2.5	2.43 ± 0.10	3.97	2.31 ± 0.10	4.50
5.0	5.30 ± 0.35	6.70	4.94 ± 0.14	2.87
10.0	9.87 ± 0.25	2.53	9.97 ± 0.32	3.20
15.0	15.03 ± 0.22	1.44	15.09 ± 0.45	3.00
20.0	19.93 ± 0.46	2.33	20.16 ± 0.53	2.62
Mean		3.39		3.24

The detection limit was defined as a concentration of the drug giving a signal-to-noise ratio greater than 3:1. This method can detect all the AEDs and the two metabolites at levels as low as 0.2 mg/L.

Specificity

The specificity of the method was evaluated by analyzing six different batches of drug-free human plasma for potential interference from endogenous components of plasma. All the different batches of plasma tested (n = 6) were found to be free from interfering components at the retention times of the drugs and the internal standard.

Selectivity

The potential for chromatographic interference by other drugs in this assay was evaluated by inclusion of some of the more commonly encountered therapeutic drugs. Table 1 lists the retention times of those drugs and of certain metabolites tested. It is apparent that the PT-metabolite, 5-(p-hydroxyphenyl)5-phenyl-hydantoin (p-HPPH), eluted close to PB (retention time 3.62 min), but there was sufficient resolution between the two peaks. Most of the plasma samples collected from patients taking PT, however, failed to show detectable levels of the metabolite. Only a few samples showed very small levels of the metabolite.

Precision

The precision or reproducibility of the assay was assessed by determining the extent of within-run (intraassay) and between-run (interassay) variabilities. This was done by analyzing replicate samples of the AEDs together with the metabolites in human plasma at five different concentrations covering the range of subtherapeutic through therapeutic to toxic levels. The intraassay precision, expressed as the percent coefficient of variation (% CV), ranged from 2.43% to 6.25% with the same concentrations and the interassay precision evaluated over a period of 4 weeks was found to be between 3.02% and 5.85% (Table 2).

Recovery

Absolute (i.e., extraction) recoveries for the drugs and the internal standard were determined by supplementing drug-free human plasma with known amounts of the drugs and the internal standard to achieve the concentrations shown in Table 3. The samples were then extracted and analyzed as described. The recoveries from plasma were determined by comparing the resulting peak areas with those obtained from direct injection of the pure standards in mobile phase. The absolute recoveries for the AEDs and metabolites ranged from 70.7% to 104.4%

TABLE 3. *Mean recoveries (analytical and extraction) of AEDs and metabolites from human plasma (n = 10)*

Concentration (mg/L)	Analytical recovery (%, Mean ± SD)	Extraction recovery (%, Mean ± SD)
Ethosuximide		
25	101.29 ± 5.99	95.00 ± 6.88
50	101.89 ± 6.45	98.01 ± 4.31
75	102.51 ± 4.71	92.65 ± 6.07
100	99.47 ± 3.69	88.21 ± 10.26
150	101.07 ± 1.97	85.15 ± 6.29
Primidone		
2.5	99.70 ± 4.14	98.31 ± 3.10
5.0	99.85 ± 2.12	89.94 ± 7.04
10	98.61 ± 2.00	100.16 ± 1.66
20	101.64 ± 2.40	104.40 ± 2.11
30	100.17 ± 1.14	99.62 ± 1.69
Carbamazepine-diol		
1.0	103.00 ± 2.65	72.77 ± 3.62
2.5	97.89 ± 5.57	78.01 ± 5.83
5.0	92.49 ± 2.63	70.73 ± 3.07
7.5	100.6 ± 1.55	76.78 ± 1.49
10.0	102.14 ± 1.23	75.15 ± 1.73
Lamotrigine		
1.0	106.13 ± 5.28	95.91 ± 5.40
2.5	98.00 ± 2.07	103.98 ± 5.26
5.0	88.33 ± 2.39	90.88 ± 3.85
7.5	101.29 ± 2.02	104.11 ± 4.76
10.0	101.24 ± 1.45	102.13 ± 2.49
Phenobarbital		
5	96.48 ± 4.30	97.84 ± 4.37
10	103.55 ± 2.81	97.52 ± 7.48
15	101.49 ± 2.29	86.01 ± 2.46
25	100.84 ± 2.49	100.71 ± 1.18
50	99.92 ± 0.89	97.49 ± 1.71
Carbamazepine-epoxide		
1.0	104.88 ± 3.36	101.69 ± 3.84
2.5	90.15 ± 1.02	95.47 ± 7.49
5.0	96.98 ± 1.30	83.21 ± 1.78
7.5	98.93 ± 1.47	89.57 ± 1.15
10.0	102.74 ± 0.99	89.81 ± 2.06
Phenytoin		
2.5	92.35 ± 3.97	103.09 ± 3.62
5.0	93.10 ± 1.81	101.28 ± 5.04
10.0	102.71 ± 3.05	99.60 ± 1.81
20.0	100.86 ± 1.70	102.81 ± 1.79
30.0	100.20 ± 0.94	100.00 ± 2.69
Carbamazepine		
2.5	93.85 ± 2.59	95.69 ± 5.04
5.0	99.80 ± 2.12	94.57 ± 6.95
10.0	101.06 ± 1.56	98.55 ± 2.02
15.0	101.74 ± 2.07	100.08 ± 1.06
20.0	100.44 ± 1.68	97.40 ± 2.23
Internal Standard		80.85 ± 3.80

TABLE 4. *Stability of AEDs and metabolites at −20°C, room temperature (25°C), and effect of freeze–thaw*

Concentration (mg/L)	Mean ± SD, % CV (n = 10) −20°C	Mean ± SD, % CV (n = 5) 25°C	Mean ± SD, % CV (n = 5 cycles) Freeze–Thaw
Ethosuximide			
25	25.32 ± 1.50, 5.92	25.33 ± 0.81, 3.19	22.87 ± 1.81, 7.91
50	50.94 ± 3.23, 6.34	50.42 ± 0.78, 1.55	46.40 ± 4.71, 10.20
75	77.05 ± 2.72, 1.80	75.00 ± 1.36, 1.81	76.80 ± 3.90, 5.08
100	99.47 ± 3.69, 3.71	99.96 ± 3.54, 3.54	91.73 ± 5.22, 5.69
150	151.03 ± 2.72, 1.80	150.03 ± 1.23, 0.82	148.93 ± 6.58, 4.42
Primidone			
2.5	2.49 ± 0.10, 4.18	2.30 ± 0.03, 1.33	2.50 ± 0.22, 8.80
5.0	4.94 ± 0.17, 3.54	5.00 ± 0.06, 1.17	5.06 ± 0.12, 2.29
10	9.86 ± 0.20, 2.03	9.57 ± 0.05, 0.52	10.13 ± 0.37, 3.63
20	20.25 ± 0.40, 1.99	20.33 ± 0.21, 1.02	20.64 ± 0.97, 4.68
30	30.08 ± 0.34, 1.14	30.28 ± 0.37, 1.22	31.42 ± 1.05, 3.34
Carbamazepine-diol			
1.0	1.03 ± 0.03, 2.57	1.01 ± 0.02, 2.33	1.03 ± 0.06, 6.24
2.5	2.45 ± 0.14, 5.67	2.39 ± 0.03, 1.23	2.35 ± 0.14, 6.04
5.0	4.62 ± 0.13, 2.84	4.60 ± 0.03, 0.58	4.78 ± 0.24, 5.08
7.5	7.55 ± 0.12, 1.55	7.58 ± 0.07, 0.89	7.70 ± 0.18, 2.34
10.0	10.21 ± 0.21, 1.20	10.22 ± 0.07, 0.64	10.51 ± 0.34, 3.19
Lamotrigine			
1.0	1.06 ± 0.05, 5.08	1.05 ± 0.01, 1.34	1.12 ± 0.08, 6.85
2.5	2.45 ± 0.05, 2.11	2.39 ± 0.03, 1.38	2.50 ± 0.12, 4.80
5.0	4.42 ± 0.12, 2.69	4.70 ± 0.09, 1.93	4.47 ± 0.23, 5.21
7.5	7.60 ± 0.15, 1.99	7.64 ± 0.14, 1.77	7.67 ± 0.10, 1.28
10.0	10.12 ± 0.15, 1.43	10.02 ± 0.13, 1.32	10.57 ± 0.59, 5.59
Phenobarbital			
5	4.82 ± 0.20, 4.17	4.71 ± 0.04, 0.92	5.00 ± 0.55, 10.90
10	10.36 ± 0.28, 2.71	10.46 ± 0.09, 0.87	10.64 ± 0.25, 2.36
15	15.22 ± 0.34, 2.25	15.00 ± 0.09, 0.60	15.85 ± 0.46, 2.91
25	25.21 ± 0.62, 2.48	24.96 ± 0.23, 0.91	25.25 ± 1.42, 5.62
50	49.96 ± 0.44, 0.89	50.25 ± 0.31, 0.62	51.77 ± 1.40, 2.70
Carbamazepine-epoxide			
1.0	1.06 ± 0.04, 3.36	1.06 ± 0.01, 1.26	1.15 ± 0.08, 7.13
2.5	2.24 ± 0.03, 1.52	2.23 ± 0.01, 0.60	2.26 ± 0.05, 2.09
5.0	4.85 ± 0.06, 1.34	4.71 ± 0.02, 0.35	4.96 ± 0.17, 3.55
7.5	7.42 ± 0.11, 1.48	7.40 ± 0.06, 0.77	7.51 ± 0.26, 3.46
10.0	10.27 ± 0.10, 0.97	10.34 ± 0.07, 0.66	10.66 ± 0.23, 2.15
Phenytoin			
2.5	2.31 ± 0.10, 4.29	2.41 ± 0.07, 2.78	2.39 ± 0.20, 8.49
5.0	4.66 ± 0.09, 1.95	4.59 ± 0.03, 0.55	4.75 ± 0.13, 2.74
10.0	10.27 ± 0.30, 2.97	10.02 ± 0.07, 0.67	10.64 ± 0.32, 2.98
20.0	20.17 ± 0.34, 1.69	20.57 ± 0.17, 0.84	20.67 ± 1.59, 7.69
30.0	30.09 ± 0.28, 0.94	30.17 ± 0.59, 1.95	30.89 ± 1.57, 5.08
Carbamazepine			
2.5	2.35 ± 0.06, 2.76	2.38 ± 0.03, 1.27	2.34 ± 0.18, 7.69
5.0	4.99 ± 0.11, 2.12	4.84 ± 0.04, 0.85	5.08 ± 0.10, 1.97
10.0	10.11 ± 0.16, 1.54	9.60 ± 0.09, 0.91	10.44 ± 0.29, 2.74
15.0	15.25 ± 0.31, 2.03	14.93 ± 0.14, 0.97	15.52 ± 0.86, 5.57
20.0	20.09 ± 0.34, 1.68	19.87 ± 0.16, 0.80	21.10 ± 0.68, 3.23

whereas the absolute recovery for the internal standard was 80.9% (Table 3). The relative (analytical) recoveries of the drugs were calculated by comparing the concentrations obtained from the drug-spiked plasma with the actual (i.e., added) concentrations. As shown in Table 3, the mean relative recoveries ranged from 88.3% to 106.1%.

Stability

Stability of AEDs in human plasma was determined through five freeze–thaw cycles (–20°C to room temperature). Frozen plasma samples were allowed to stand at room temperature for 2 hr to allow complete thawing before being processed for analysis. The results showed that all the drugs and the metabolites were stable after five cycles of freeze–thaw (Table 4).

Moreover, the effect of storage at –20°C on the stability of AEDs in human plasma was studied over a 4-week period. In addition, stability of the extracted drugs which were reconstituted and stored at room temperature (25°C) over 24 hr was also studied. The latter was carried out because most of the times large numbers of samples were kept in the autosampler to be chromatographed overnight.

The results showed that the AEDs and the metabolites were stable in plasma when stored at –20°C for at least 4 weeks and that the reconstituted samples were stable at 25°C (room temperature) for at least 24 hrs (Table 4).

CONCLUSION

A simple, sensitive, specific, robust, precise, and rapid HPLC method for simultaneous determination of ethosuximide, primidone, lamotrigine, phenobarbital, phenytoin, carbamazepine, and two metabolites (carbamazepine-diol and carbamazepine-epoxide) in human plasma is described. The method is suitable for therapeutic drug monitoring of AEDs and for pharmacokinetic studies, particularly in pediatric patients because of the small sample size required (100 μL).

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