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Simultaneous determination of carbamazepine, phenytoin, phenobarbital, primidone and their principal metabolites by high-performance liquid chromatography with photodiode-array detection

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ABSTRACT

We have established a precise and accurate high-performance liquid chromatographic method for the simultaneous assay of carbamazepine, phenytoin, phenobarbital, primidone and their principal metabolites. This method has been used for the analysis of these drugs and the metabolites in serum, saliva and urine samples. Acetonitrile is used for the deproteinization of serum and saliva samples while solid-phase extraction is utilized for urine sample pretreatment. Samples of 2 μl are injected onto a 3-μm ODS-Hypersil column (250 mm × 2 mm I.D.) with a column temperature of 40°C. The drugs and metabolites are eluted with a mobile phase containing potassium phosphate buffer–acetonitrile–methanol (110:50:30, v/v/v) at a flow-rate of 0.2 ml/min. Signals are monitored by a photodiode-array detector at a sample wavelength of 200 nm with a bandwidth of 10 nm. These four commonly used antiepileptic drugs and their six metabolites are well separated from one another within 15 min. Within-day coefficients of variation (C.V.) are within 5% in most cases and between-day C.V. are from 2.32 to 4.75%. The recovery rates range from 95.12 to 104.42%. This method has the necessary sensitivity and linearity for routine therapeutic monitoring of both total and free drug levels and may be employed for pharmacokinetics studies of drug interactions and metabolism as well.

INTRODUCTION

Carbamazepine (CBZ), phenytoin (PHT), phenobarbital (PB) and primidone (PRM) are commonly used antiepileptic drugs (AEDs) [1]. The serum drug concentrations in epileptic patients administered with standardized doses of AEDs exhibit wide inter-patient variability [2,3]. In addition, despite the current trend to treat the patients with one drug, polytherapy is still common [4]. Polytherapy may be associated with pharmacokinetics interactions and unexpected toxicity [1,5,6]. These interactions, which can be associated with significant changes in blood level or the free fraction of a drug, make frequent drug measurements and dosage adjustments necessary [4].

Although drug monitoring may provide an important guide to clinical therapy, the relationship between parent drug concentration and therapeutic and/or toxic side-effects is complicated by the presence of metabolites which may also have anticonvulsant and toxic activities [7]. Furthermore, monitoring the metabolites will be an essential part of pharmacokinetics studies on AEDs [7,8].

Carbamazepine-10,11-epoxide (CBZ-E) is an active epoxide metabolite of CBZ [9]. CBZ-E is further metabolized to 10,11-dihydro-*trans*-dihy-

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droxycarbamazepine (CBZ-H). Abnormal ratios of CBZ-H to CBZ-E have been found to be useful clinical indicators of non-compliance in difficult cases [2]. PB and 2-phenyl-2-ethylmalonamide (PEMA) are two major metabolites of PRM [7,10], and both have been reported as having an antiepileptic effect. The major metabolite of PHT is 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (p-HPPH). A procedure which can resolve PHT and its hydroxylated metabolites p-HPPH and 5-(*m*-hydroxyphenyl)-5-phenylhydantoin (m-HPPH) is necessary for the accurate and precise evaluation of PHT metabolism [11]. PB is mainly metabolized by hydroxylation in the *para*-position to form *p*-hydroxyphenobarbital (p-HPB) [7]. The quantitation of p-HPB may be of interest in pharmacokinetics investigation [7,8].

Simultaneous multiple drug analysis is the most rapid and cost-effective approach of monitoring drug levels in epileptic patients with polytherapy [7]. Many high-performance liquid chromatographic (HPLC) methods have been reported for the determination of AEDs [12–23]. Either gradient elution and flow-step gradient or isocratic elution has been used for the assay of AEDs [8,15,16] with analysis time from 1.4 to 21.5 min [14,16]. The number of AEDs and related drugs assayed are from four to seventeen [7,12,17–20]. Unfortunately, less effort has been devoted to their metabolites [7,13]. We describe here an isocratic HPLC method for the simultaneous analysis of CBZ, PRM, PHT, PB and their six major metabolites in physiological fluid samples.

EXPERIMENTAL

Chemicals

Acetonitrile, methanol and distilled water were HPLC grade from Curtin Matheson Scientific (Houston, TX, USA). Monobasic and dibasic potassium phosphate were purchased from Aldrich (Milwaukee, WI, USA).

CBZ, CBZ-E, 5,5-diphenylhydantoin (PHT), p-HPPH, m-HPPH, PB, p-HPB, PRM, PEMA and nitrazepam (NT) were all purchased from Alltech-Applied Science (State College, PA, USA). We obtained CBZ-H from Ciba-Geigy (Basle, Switzerland). The working standards of drug and metabolite were prepared by diluting stock solutions of these compounds (1 mg/ml in methanol) with HPLC-grade water. The working standard solutions were stable for at least 45 days when kept at −80°C.

Apparatus and chromatographic conditions

The HPLC system used was a Hewlett-Packard HP 1090 M series, including a DR 5 solvent delivery system. Samples of 2 μl were injected by an autoinjector and autosampler onto a 3-μm ODS-Hypersil column (250 mm × 2 mm I.D.) from Keystone (Keystone Scientific, Bellefonte, PA, USA), which was preceded by a guard column (20 mm × 2 mm I.D.) with the same packing material. The column temperature was kept at 40°C. The mobile phase was a mixture of potassium phosphate buffer (0.01 *M*, pH 7.0)–acetonitrile–methanol (110:50:30, v/v/v) at a flow-rate of 0.2 ml/min. The mobile phase was filtered prior to use. Signals were monitored by an HP 1040A photodiode-array detector with a main sample wavelength of 200 nm and a bandwidth of 10 nm. Signals were also recorded at 215, 254 and 285 nm with the same bandwidth. The reference wavelength was 550 nm with a bandwidth of 100 nm. Data were collected and analyzed on an HP 79994A analytical workstation.

Sample preparation

Samples were collected from patients monitored for therapeutic drugs in the clinical laboratory of our hospital. To a 75 mm × 10 mm glass tube, 100 μl of serum and 200 μl of acetonitrile were added for deproteinization. This mixture was centrifuged at 1500 *g* for 5 min after vortex-mixing for 10 s. Finally, the supernatants were transferred to a vial, and 2 μl of the supernatants were injected into the column.

The serum ultrafiltrates for the analysis of free drug levels were prepared according to the ultrafiltration technique used for free valproic acid (VPA) assay [23].

Saliva samples were frozen at −80°C until

thawed and centrifuged prior to assay. Acetonitrile (50 μl) was added to 250 μl of the saliva. Supernatants of 2 μl were injected after centrifugation at 1500 *g* for 5 min.

Urine samples were treated by using a Sep-Pak column (Waters Assoc., Milford, MA, USA). The Sep-Pak column was attached to a 10-ml glass syringe and washed with 5 ml of acetonitrile followed by 20 ml of water. Then 2 ml of urine sample were added and the sample was pushed through the Sep-Pak. After washing the column again with 20 ml of water, 0.5 ml of acetonitrile was passed through the column to elute the AEDs. The eluent was collected in a sample vial, and 2 μl of eluents were injected.

RESULTS

Mobile phase optimization

The dependence of drugs and metabolites on different pH of the potassium phosphate buffer (6.70, 7.00 and 7.30) in the mobile phase was investigated. PHT and CBZ showed complete separation in all three different pH buffers. The capacity factor (k') was near 5.4 for CBZ and showed little variation in the pH range from 6.70 to 7.30. The k' of PHT was decreased as the pH of the buffer increased. The k' values of PHT were 5.04, 4.72 and 4.53 at pH 6.70, 7.00 and 7.30, respectively. The dependence of other drugs and metabolites on the pH of the phosphate buffer in the mobile phase is shown in Fig. 1. PEMA and p-HPB were not separated at pH 6.70, but these two components could be resolved when pH increased to 7.00 or 7.30.

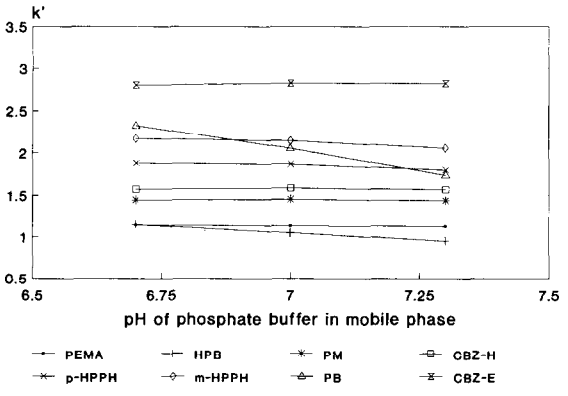

Fig. 1. Dependence of capacity factor (k') on the pH of potassium phosphate buffer in the mobile phase. Drug name abbreviations are: p-HPB = *p*-hydroxyphenobarbital; PEMA = phenylethylmalonamide; PM = primidone; CBZ-H = 10,11-dihydro-*trans*-dihydroxycarbamazepine; p-HPPH = 5-(*p*-hydroxyphenyl)-5-phenylhydantoin; PB = phenobarbital; m-HPPH = 5-(*m*-hydroxyphenyl)-5-phenylhydantoin; CBZ-E = carbamazepine-10,11-epoxide.

HPLC of biological fluids

Chromatograms of a pooled blank serum, a solution of authentic drugs and metabolites, and the blank serum sample spiked with standard drug and metabolite are shown in Fig. 2A, B and C, respectively.

Fig. 3 shows the chromatograms of serum samples from patients taking different drug regimens (A, CBZ; B, PHT plus CBZ; C, PRM plus CBZ).

The free drugs and metabolites in serum ultrafiltrates of patients taking PHT or PRM plus CBZ are illustrated in Fig. 4A or B.

The applications of the present method for the analysis of saliva and urine samples are shown in Fig. 5 (A, saliva CBZ and metabolites; B, urine PHT and p-HPPH).

Precision

To the pooled drug-free serum samples we added toxic, therapeutic and subtherapeutic concentrations of each drug and metabolite (n = 6, by series dilution). PEMA, p-HPB, p-HPPH and CBZ-E were prepared to yield final concentrations from 0.78 to 25 μg/ml. The concentrations of PRM, CBZ-H, m-HPPH, PHT and CBZ were from 1.56 to 50 μg/ml and the concentrations for PB were from 3.12 to 100 μg/ml. We assessed the precision by replicate analysis of these samples. Within-day coefficients of variation (C.V.) were found to be within 5% in most cases. The C.V. were larger (within 10%) at the lowest levels of CBZ, CBZ-E, CBZ-H, PRM and p-HPPH. The between-day C.V. were from 2.32 to 4.75% at the concentration of 7.00 μg/ml. Partial results of the within- and between-day C.V. are listed in Tables I, II and III.

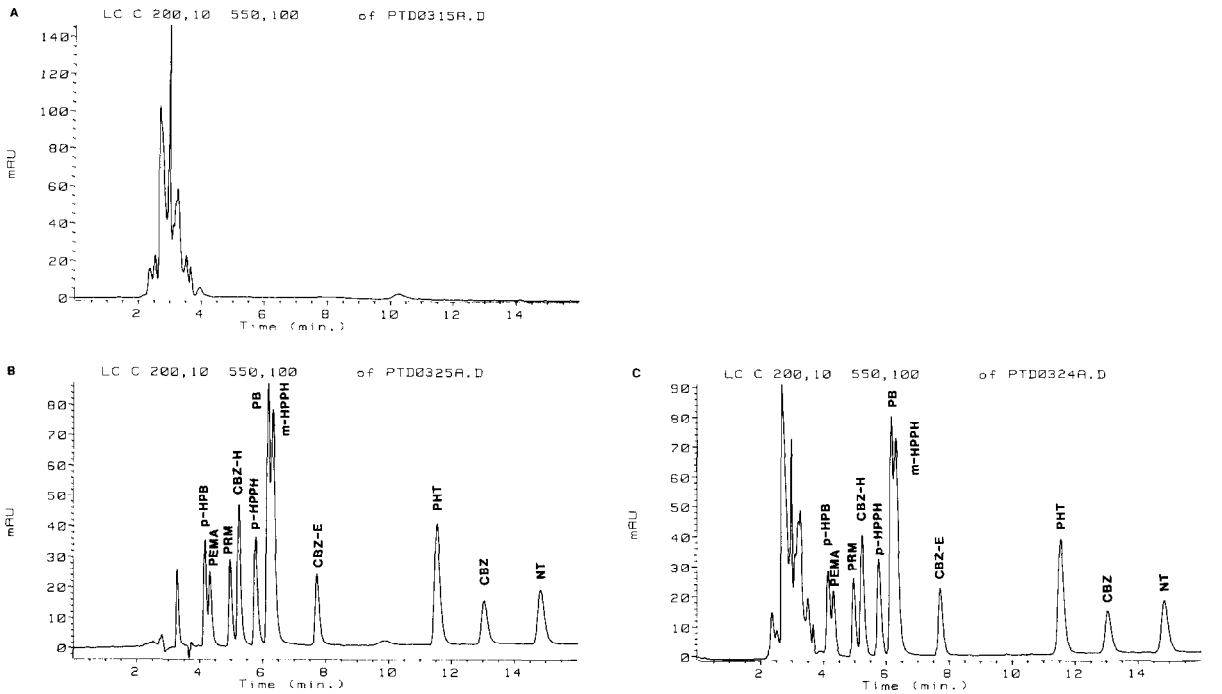

Fig. 2. Chromatograms of (A) a pooled blank serum, (B) a solution of authentic drugs and metabolites and (C) the drugs and metabolites added to the drug-free serum. Drug name abbreviations as in Fig. 1. Additional abbreviations are: PRM = primidone; PHT = phenytoin; CBZ = carbamazepine; NT = nitrazepam. The concentrations are 12.5 μg/ml for p-HPB, PEMA, p-HPPH and CBZ-E, 25 μg/ml for PRM, CBZ-H, m-HPPH, PHT, CBZ and NT and 50 μg/ml for PB.

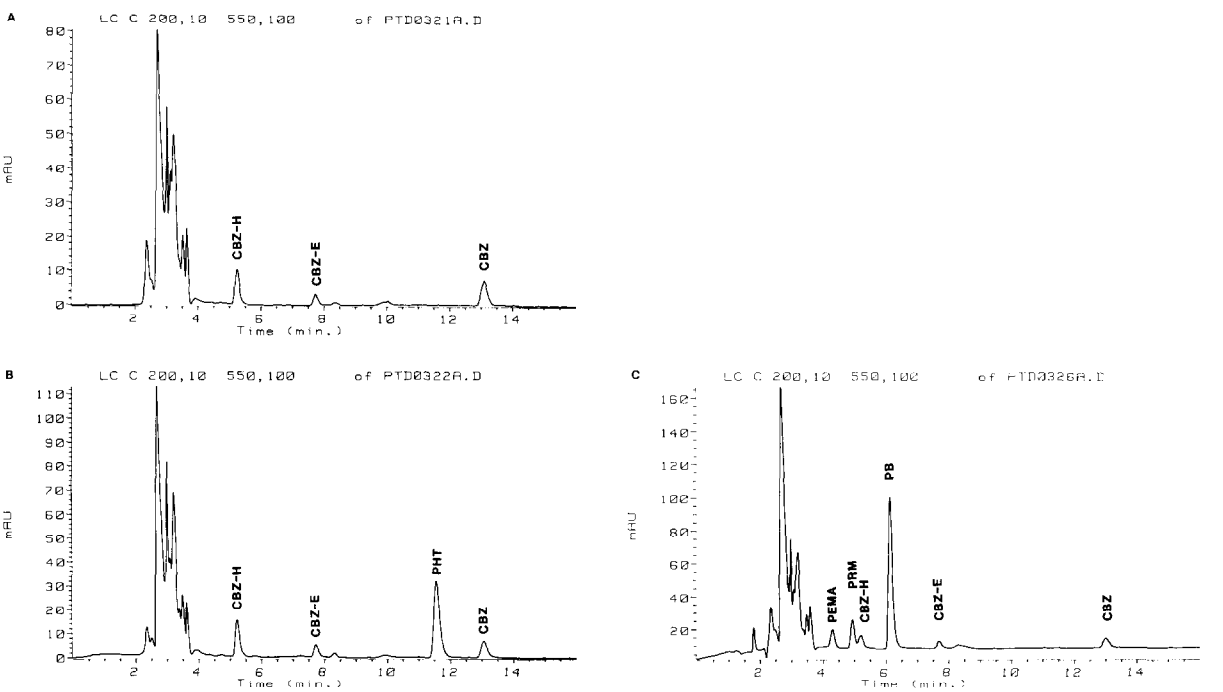

Fig. 3. Chromatograms of sera from patients receiving 600 mg of CBZ per day (A), 1700 mg of CBZ plus 400 mg of PHT per day (B) and 1600 mg of CBZ plus 800 mg of PRM per day (C). Drug name abbreviations as in Figs. 1 and 2.

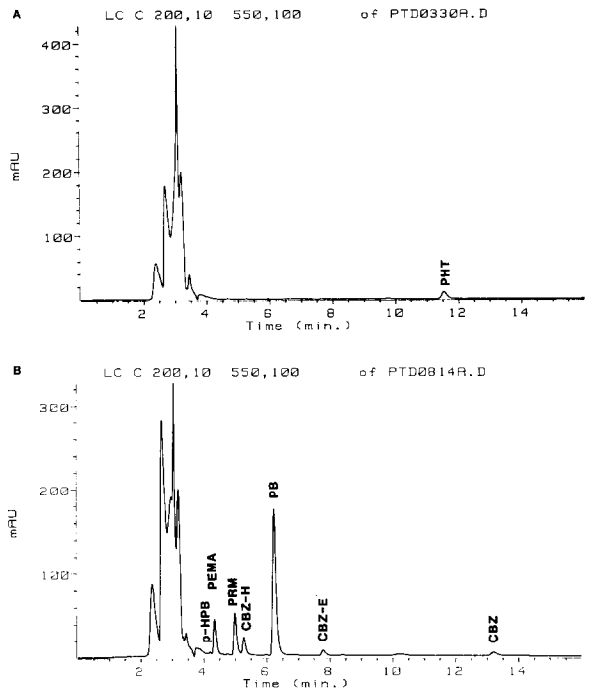

Fig. 4. Chromatograms of free PHT in a patient with a dose of 400 mg/day (A), and free CBZ plus PRM and their metabolites (B) in the serum ultrafiltrates from the same sample as in Fig. 3C. Drug name abbreviations as in Figs. 1 and 2.

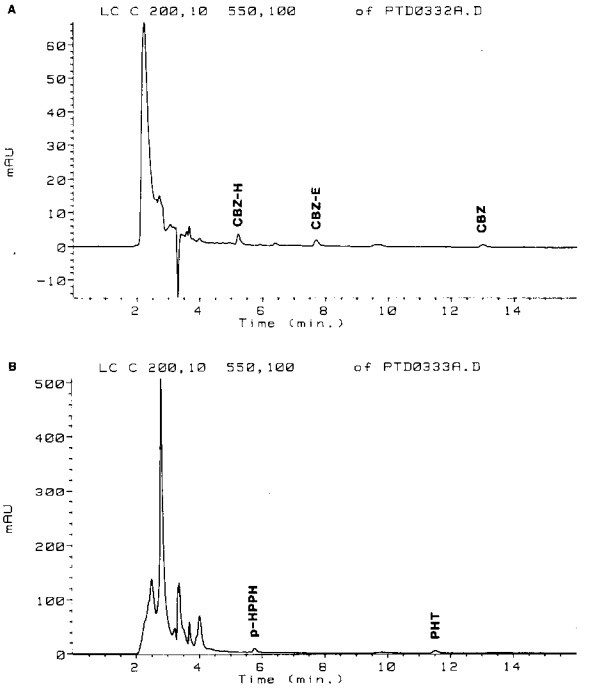

Fig. 5. Chromatograms of a saliva sample from the same patient as in Fig. 3A (A) and of urine sample from the same patient as in Fig. 4A showing PHT and p-HPPH (B). Drug name abbreviations as in Figs. 1 and 2.

Recovery

To three drug-free serum samples were added 12.50 μg/ml of each drug and metabolite. Then the samples were analyzed after deproteinization by acetonitrile. Relative recovery was calculated by the values obtained from drug-supplemented serum and the added concentration. The recovery rate ranged from 95.12 to 104.42% (Table IV).

Linearity

Standard curves for all of these drugs and metabolites were linear in each case, with the coefficients of correlation greater than 0.9998. The concentration ranges were from 0.39 to 25 μg/ml for PEMA, p-HPB, p-HPPH and CBZ-E. The concentrations of PRM, CBZ-H, m-HPPH, PHT and CBZ were from 0.78 to 50 μg/ml and PB ranged from 1.56 to 100 μg/ml (Table V).

Ratios of peak areas

The photodiode-array detector we used can monitor a sample at more than one wavelength. The main sample wavelength of 200 nm was chosen because this wavelength gave the greatest sensitivity for most of the compounds. Signals were also recorded at 215, 254 and 285 nm with the same bandwidth and reference. The ratios of peak areas obtained at 215, 254 and 285 nm to 200 nm are listed in Table VI. The results showed that each component had characteristic peak-area ratios.

Correlation with enzyme immunoassay

Linear regression analysis of the results of CBZ, PRM, PHT and PB obtained from our HPLC method and enzyme immunoassay technique (EIA, by using SYVA reagents on a Monarch 2000 chemistry system) is shown in Table

TABLE I

WITHIN- AND BETWEEN-DAY PRECISIONS OF PRIMIDONE, PHENOBARBITAL AND THEIR METABOLITES

Concentration (μg/ml)	Within-day (n = 5)		Between-day (n = 7)	
	Mean ± S.D. (μg/ml)	C.V. (%)	Mean ± S.D. (μg/ml)	C.V. (%)
Primidone				
3.12	2.98 ± 0.10	3.37		
7.00			7.09 ± 0.21	2.96
25.00	25.34 ± 0.87	3.46		
50.00	49.73 ± 1.15	2.32		
Phenylethylmalonamide				
3.12	2.98 ± 0.06	2.17		
7.00			7.02 ± 0.16	2.32
12.50	12.40 ± 0.44	3.51		
25.00	25.11 ± 0.33	1.32		
Phenobarbital				
3.12	3.28 ± 0.10	2.93		
7.00			6.91 ± 0.23	3.36
50.00	51.44 ± 1.70	3.31		
100.00	98.96 ± 0.48	0.49		
p-Hydroxyphenobarbital				
3.12	3.20 ± 0.07	2.13		
7.00			6.71 ± 0.31	4.62
12.50	12.69 ± 0.14	1.13		
25.00	24.84 ± 0.28	1.13		

TABLE II

WITHIN- AND BETWEEN-DAY PRECISIONS OF PHENYTOIN AND ITS METABOLITES

Concentration (μg/ml)	Within-day (n = 5)		Between-day (n = 7)	
	Mean ± S.D. (μg/ml)	C.V. (%)	Mean ± S.D. (μg/ml)	C.V. (%)
Phenytoin				
3.12	2.94 ± 0.03	1.11		
7.00			6.88 ± 0.25	3.68
25.00	25.43 ± 0.78	3.07		
50.00	49.65 ± 0.28	0.57		
5-(p-Hydroxyphenyl)-5-phenylhydantoin				
3.12	2.96 ± 0.05	1.87		
7.00			7.10 ± 0.23	3.23
12.50	12.69 ± 0.40	3.18		
25.00	24.86 ± 0.28	1.11		
5-(m-Hydroxyphenyl)-5-phenylhydantoin				
3.12	3.26 ± 0.07	2.21		
7.00			6.74 ± 0.22	3.21
25.00	25.16 ± 0.49	1.94		
50.00	49.83 ± 0.52	1.04		

TABLE III

WITHIN- AND BETWEEN-DAY PRECISIONS OF CARBAMAZEPINE AND ITS METABOLITES

Concentration (μg/ml)	Within-day (n = 5)		Between-day (n = 7)	
	Mean ± S.D. (μg/ml)	C.V. (%)	Mean ± S.D. (μg/ml)	C.V. (%)
Carbamazepine				
3.12	3.31 ± 0.16	4.84		
7.00			6.90 ± 0.33	4.75
25.00	25.42 ± 0.97	3.83		
50.00	49.74 ± 0.21	0.42		
10,11-Dihydro-trans-dihydroxycarbamazepine				
3.12	2.97 ± 0.07	2.41		
7.00			6.71 ± 0.24	3.54
25.00	25.41 ± 0.84	3.29		
50.00	49.68 ± 0.51	1.02		
Carbamazepine-10,11-epoxide				
3.12	3.01 ± 0.02	0.66		
7.00			6.85 ± 0.17	2.54
12.50	12.71 ± 0.25	1.94		
25.00	24.86 ± 0.07	0.28		

VII. Correlations between these two different assays were satisfactory. The HPLC procedure yielded slightly lower results for PHT and PB than the EIA method.

Interference

A number of other drugs were analyzed for the evaluation of potential interference. Table VIII lists the retention times of drugs studied in this method. No interference was observed.

TABLE IV

RECOVERY RATE OF DRUGS AND METABOLITES ADDED TO DRUG-FREE SERUM

Drug[a]	Expected level	Actual level (mean ± S.D., n = 3)	Recovery[b] (mean ± S.D., n = 3) (%)
p-HPB	12.50	12.32 ± 0.41	98.36 ± 3.23
PEMA	12.50	12.60 ± 0.42	100.72 ± 3.40
PRM	12.50	11.91 ± 0.40	95.72 ± 3.07
CBZ-H	12.50	11.82 ± 0.28	95.12 ± 2.13
p-HPPH	12.50	12.32 ± 0.41	98.65 ± 3.21
PB	12.50	13.06 ± 0.27	104.42 ± 2.27
m-HPPH	12.50	11.98 ± 0.09	95.91 ± 0.71
CBZ-E	12.50	12.30 ± 0.25	98.45 ± 1.96
PHT	12.50	11.87 ± 0.36	95.02 ± 2.75
CBZ	12.50	12.14 ± 0.32	97.21 ± 2.46

[a] p-HPB = *p*-hydroxyphenobarbital; PEMA = phenylethylmalonamide; PRM = primidone; CBZ-H = 10,11-dihydro-*trans*-dihydroxycarbamazepine; p-HPPH = 5-(*p*-hydroxyphenyl)-5-phenylhydantoin; PB = phenobarbital; m-HPPH = 5-(*m*-hydroxyphenyl)-5-phenylhydantoin; CBZ-E = carbamazepine-10,11-epoxide; PHT = phenytoin; CBZ = carbamazepine.

[b] Recovery rate = (actual level/expected level) × 100.

TABLE V

CORRELATION BETWEEN DRUG CONCENTRATIONS AND PEAK AREAS

Drug[a]	Concentration range (n = 7) (μg/ml)	Intercept	Slope	r^2	$S_{y \cdot x}$
p-HPB	0.39 – 25.00	−0.04	17.56	0.9999	1.45
PEMA	0.39 – 25.00	0.08	14.75	0.9999	1.31
PRM	0.78 – 50.00	−0.05	7.92	0.9999	1.26
CBZ-H	0.78 – 50.00	0.06	14.69	0.9999	2.73
p-HPPH	0.39 – 25.00	0.03	23.82	0.9999	1.58
PB	1.56 – 100.0	−4.87	11.04	0.9999	3.19
m-HPPH	0.78 – 50.00	−2.14	31.64	0.9998	8.97
CBZ-E	0.39 – 25.00	0.02	17.90	0.9999	1.29
PHT	0.78 – 50.00	0.10	20.15	0.9999	4.68
CBZ	0.78 – 50.00	0.02	8.12	0.9999	1.21

[a] Drug name abbrevations as in Table IV.

TABLE VI

PEAK-AREA RATIOS AMONG UV SIGNALS

Drug[a]	UV 200 nm	UV 215/200	UV 254/200	UV 285/200
p-HPB	1.0	0.3299	0.0770	0.0346
PEMA	1.0	0.3499	0.0122	0.0002
PRM	1.0	0.4040	0.0087	0.0001
CBZ-H	1.0	0.6302	0.0446	0.0042
p-HPPH	1.0	0.3189	0.0572	0.0180
PB	1.0	0.4337	0.0581	0.0018
m-HPPH	1.0	0.3889	0.0224	0.0380
CBZ-E	1.0	0.8296	0.0434	0.0021
PHT	1.0	0.3791	0.0238	0.0006
CBZ	1.0	1.1495	0.2714	0.4724

[a] Drug name abbreviations as in Table IV.

TABLE VII

CORRELATION BETWEEN DRUG LEVELS MEASURED BY EIA AND THE PRESENT HPLC METHOD

Drug[a]	n	Intercept	Slope	r	$S_{y \cdot x}$
CBZ	26	0.66	1.01	0.990	0.98
PHT	24	0.92	0.94	0.981	1.21
PB	22	1.27	0.96	0.985	1.67
PRM	15	0.43	1.03	0.976	1.04

[a] CBZ = carbamazepine; PHT = phenytoin; PB = phenobarbital; PRM = primidone.

TABLE VIII

RETENTION TIMES OF SOME DRUGS BY THIS METHOD

Drug[a]	Retention time (min)
p-HPB	4.221
PEMA	4.375
PRM	5.016
CBZ-H	5.320
p-HPPH	5.838
PB	6.215
m-HPPH	6.397
CBZ-E	7.783
PHT	11.563
Hexobarbital	12.098
CBZ	13.073
Nitrazepam	15.485
Clonazepam	16.983
Oxazepam	>17.000
Nordiazepam	>17.000
Cyheptamide	>17.000
Diazepam	>17.000
Prezapam	>17.000
Temazepam	>17.000
Lorazepam	>17.000
Chlordiazepoxide	>17.000

[a] Drug name abbrevations as in Table IV.

DISCUSSION

Separation

Many HPLC methods have been reported for the simultaneous determination of commonly used AEDs [12–22]. An ideal method for the monitoring of AEDs should be able to simultaneously analyze these drugs as well as their principal metabolites. Chromatographic peaks of parent drugs and their metabolites may overlap, and their resolution is essential to the accuracy of the determination [15].

Isocratic elution has been chosen for the present experiment since the gradient elution usually needs a long analysis time and requires gradient equipment. These restrictions are less likely to appeal to most clinical laboratories for the routine drug monitoring. We have found that the methanol in the mobile phase is important for the separation of PHT and CBZ. It has been reported that the changes in pH of the phosphate buffer of the mobile phase have the greatest influence on PB, PHT and p-HPB [8,15]. A mobile phase with a buffer at pH 7.0 gave optimal separation in our experiment. An analytical column with 3 μm particle size packing material was used in this study, which resulted in higher efficiency and greater sensitivity than conventional packing, thus allowing the better separation for these compounds.

Quantitation

Although excellent results can be obtained by single-wavelength detection [7], the photodiode-array detector used in this method provides additional advantages of multi-signal detection, together with the confirmation of peak identity and assessment of peak homogeneity. The ratios of peak areas at 215, 254 and 285 nm to 200 nm indicate that each component has characteristic peak-area ratios. Since the absorbance ratio for a pure component is constant, a co-eluting impurity peak will lead to the deviation from these peak-area ratios.

This HPLC method can achieve the relative recovery rate of 95.12–104.42% and within-day and between-day C.V. of 5% or less (the exception is the C.V. at the lowest levels of CBZ, CBZ-E, CBZ-H, PRM and p-HPPH, which are within 10%). These ranges satisfy the desirable limits defined by Szabo and Browne [13]. The concentrations we have tested cover the subtherapeutic, therapeutic and toxic serum levels.

NT was used as an internal standard in our experiment at the stage of search for optimal separation (Fig. 2B and C). Since the present method has a high precision and accuracy and with near 100% recovery of the drugs and metabolites added to serum, the quantitative results calculated by the internal standard or external standard method are very close (data not shown). This suggests that the internal standard is not necessary, and we discontinued the use of the internal standard method in the subsequent quantitation studies. Bock and Ben-Ezra [17] also reported an HPLC method for the analysis of AEDs without

the use of an internal standard. Of course, the proper function of the injecting system must be assured if no internal standard is used [17].

Sample preparation

The advantages of protein precipitation are the speed of operation, very low cost, micro-scale application and near 100% relative recoveries [7,12]. Acetone is significantly more UV-absorptive at the low UV wavelength than acetonitrile, and this can lead to an unstable baseline [13]. Thus we prefer to use acetonitrile rather than acetone for the protein precipitation. Ou and Rognerud [15] used a ratio of 1:1 of acetonitrile to serum while a ratio of 3.75:1 was used by Asberg and Haffner [18] to obtain a more complete protein precipitation. However, injection of this mixture resulted in the shifting of the retention time due to the eluting property of this acetonitrile-rich solution [18]. We used a 2:1 ratio of acetonitrile to serum and obtained a satisfactory protein precipitation of the serum without the adverse influence on retention time.

The disadvantage of protein precipitation is problems with potential interfering substances since all water-soluble impurities of the samples are injected together with drugs into the column [7,12]. Another shortcoming of the protein precipitation is that the sample is diluted by deproteinization instead of being concentrated as is the case with solvent extraction [7]. Nevertheless, we have not observed significant impurity interference from the pooled blank serum and during the practical applications. The sensitivity of our method is also sufficient for use with the diluted serum and saliva samples.

Many techniques have been used to study the binding of drugs to proteins and to measure the free drug fraction [7]. The ultrafiltration technique is one of the most likely candidates for adoption in a clinical laboratory [24]. Although the measured concentrations that have been reported are not critically dependent on the exact conditions of ultrafiltration (rotor angle, speed of centrifugation and amount of sample filtered) [17], we and other authors have found a lower concentration of free VPA and PHT in the ultrafiltrates collected at 5 min than in the fractions collected after longer periods of the centrifugation [23,25].

Saliva samples are frozen at −80°C until thawed and centrifuged prior to assay. This procedure has been reported to be necessary to overcome difficulties resulting from the high viscosity of freshly obtained saliva [20]. A volume of 50 μl acetonitrile is added to 250 μl of saliva sample since saliva contains less protein, and the drug and metabolite levels in saliva are lower than that in the serum. The quantitation results of the drug and metabolite added to the saliva sample are similar to that of the serum samples (data not shown).

Solid-phase extraction has been used in this study for the treatment of urine samples. We have not fully investigated the quantitation results of AEDs and their metabolites in the urine samples at this stage.

Applications

Not all of the published methods are amenable to the analysis of unbound drug because the free fraction may represent only a small percentage of the total concentration, and the sensitivity becomes the limiting factor [7]. Our method can determine both total and free drugs and their metabolites. Actually, the concentrations of most free drugs and metabolites are relatively higher in the undiluted ultrafiltrates than the total levels in the diluted supernatants of the serum.

Our HPLC method can simultaneously analyze PHT and its metabolites p-HPPH and m-HPPH, PB and its metabolite p-HPB, PRM and its metabolites PEMA and PB, and CBZ and its metabolites CBZ-E and CBZ-H. Our methodology has been in clinical use for six months, and we have processed hundreds of samples from patients on a wide variety of drug regimens. We have not found any substance, compound or metabolite that interferes with this analysis. It is obvious that the number of possible comedications is quite limitless, and it is therefore very important to maintain a list of tested substances and possible interferences [7].

In conclusion, we have established a precise

and accurate method for the simultaneous measurement of CBZ, PHT, PB, PRM and their principal metabolites in serum, saliva and urine samples. This procedure has the necessary sensitivity and linearity for routine therapeutic monitoring of both total and free drug levels and may be employed for pharmacokinetics studies of drug interactions and metabolism.

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