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Sensitive Analytical Method for Serum Primidone and Its Active Metabolites for Single-Dose Pharmacokinetic Analysis in Human Subjects

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A sensitive method for determination of serum primidone (PRM) and its active metabolites, phenobarbital (PB) and phenylethylmalonamide (PEMA), was established to clarify the time-courses of serum PRM, PB and PEMA after a single oral administration of PRM to man. In this method, the serum was acidified and extracted with a mixture of chloroform–isopropanol (70 : 30). An aliquot of the organic phase was treated with 0.1 N sodium hydroxide to separate PRM and PEMA from PB and interfering substances. Then PRM and PEMA were simultaneously determined by reversed-phase high-performance liquid chromatography (HPLC) using a mixture of acetonitrile–water (12 : 88) as a mobile phase. PB was also determined by HPLC using a mixture of acetonitrile–phosphate buffer (19 : 81, pH 4.5) as a mobile phase after extraction with chloroform from the acidified aqueous alkaline phase. Sufficient accuracy and precision to determine submicrogram levels (50—1000 ng/ml) of serum PRM, PB and PEMA were obtained. By this method, serum PRM and its active metabolites were successfully measured for at least 5 d after a single 600 mg dose of PRM to a human subject.

Keywords——HPLC; primidone; active metabolite; human serum; phenobarbital; phenylethylmalonamide

Primidone (PRM), which is a drug for the treatment of tonic-clonic and complex partial seizures, is converted to two active metabolites, phenobarbital (PB) and phenylethylmalonamide (PEMA).[1] Because of the lack of sensitive and simple analytical procedures, there have been few investigations of PRM disposition kinetics (including the active metabolites) in humans. The conventional high-performance liquid chromatographic methods[2–4] or immunoassay[5] presently available are directed to therapeutic drug monitoring and are too insensitive to measure concentrations in the submicrogram range after a single oral dose. Although a gas chromatographic method[6] and a gas chromatographic-mass spectrometric method[7] recently developed have high sensitivity, these methods are technically limited.

For a human kinetic study, we have established a sensitive and convenient method for the assay of PRM, PB and PEMA in human serum by high-performance liquid chromatography (HPLC) with ultraviolet detection.

Experimental

Reagents and Standards——Sources of the drugs and the internal standards were as follows: PRM (Dainippon Pharmaceutical Co., Osaka, Japan), PB (Fujinaga Pharmaceutical Co., Tokyo, Japan), PEMA (Sigma Chemical Co., St. Louis, Mo. U.S.A.), barbital and phenacetin (Tokyo Kasei Kogyo Co., Tokyo, Japan). Acetonitrile (HPLC grade, Cica-Merck) was obtained from Kanto Chemical Co. (Tokyo, Japan). The water was double-distilled in an all-glass still after passage through an ion-exchange column. The other reagents were of analytical grade.

Stock solutions of the internal standards were prepared as follows: barbital was dissolved in methanol (1.0 μg/ml) and phenacetin was dissolved in distilled water (0.75 μg/ml).

Mobile phase A (pH = 4.5) was prepared by adding 2 ml of 0.1 M KH_2PO_4 and 300 μl of 0.1 M H_3PO_4 to 1000 ml of acetonitrile–water solution (19 : 81 (v/v)). Mobile phase B was a solution of 120 ml of acetonitrile in 880 ml of distilled water.

Apparatus and Conditions——We used a high-performance liquid chromatograph (model LC-3A, Shimadzu Corp., Kyoto, Japan) with a variable-wavelength ultraviolet absorbance detector (SPD-2A). A prepacked reversed-phase column (LiChrosorb RP-18, 7 μm, 4 × 250 mm, Cica-Merck, Kanto Chemical Co., Tokyo, Japan) was used.

For analysis of PRM and PEMA, the column temperature was maintained at 45 °C, the column was eluted with mobile phase B at a flow rate of 2.0 ml/min, and the column effluent was monitored at 210 nm. For PB analysis, the column temperature was maintained at 60 °C and the column was eluted with mobile phase A, while the other conditions were the same as those in the analysis of PRM and PEMA.

Extraction Procedure——A serum sample (200 μl) was pipetted into a 10-ml glass-stoppered centrifuge tube and 1 ml of 0.1 N hydrochloric acid and 6 ml of chloroform–isopropanol (70 : 30 (v/v)) were added. The tube was shaken for 10 min and then centrifuged to separate the layers. The aqueous phase (upper layer) was aspirated off and 5 ml of the organic phase (lower layer) was transferred to a 10-ml glass-stoppered centrifuge tube containing 1.2 ml of 0.1 N sodium hydroxide. The tube was shaken for 6 min and then centrifuged. Subsequently both the aqueous and the organic phases were processed as follows for analysis of PRM and its active metabolites.

Analysis of PB: One milliliter of the aqueous phase was immediately transferred to a 10-ml glass-stoppered centrifuge tube containing 200 μl of 1 N hydrochloric acid. Two hundred microliters of the internal standard solution of phenacetin (0.75 μg/ml) and 5 ml of chloroform were added, then the tube was shaken for 6 min and centrifuged. The aqueous phase was aspirated off and about 4 ml of the chloroform phase was evaporated to dryness *in vacuo* at ambient temperature. The residue was reconstituted with 200 μl of mobile phase A, and 80 μl of the solution was injected into the chromatograph.

Analysis of PRM and PEMA: Four milliliters of the organic phase was transferred to a 10-ml glass-stoppered tube and 100 μl of the internal standard solution of barbital (1.0 μg/ml) was added. Then the solvent was evaporated off *in vacuo* at ambient temperature. The residue was reconstituted with 200 μl of mobile phase B, and 80 μl of the solution was injected onto the chromatograph.

Experiments in Man——A single dose of 600 mg of PRM (Mysoline®, Dainippon Pharmaceutical Co., Osaka, Japan) was administered orally to a healthy male volunteer who had fasted for about 12 h prior to the experiment. The blood samples were obtained at selected intervals over a period of 10 d. The separated serum samples were stored at −20 °C until analysis.

Results and Discussion

PRM, PB and PEMA can be extracted with chloroform at acidic pH,[4] but the extraction ratios of PRM and PEMA were not adequate (less than 75%). To improve the extraction ratios of the drugs, we used a mixture of chloroform–isopropanol (70 : 30 (v/v)) as the first-step extracting solvent. This mixture gave good yields, and the absolute recoveries of PRM and PEMA were 94 and 90%, respectively.

The single-step extraction could not exclude serum components which interfered with the microanalysis of PRM and PEMA. Therefore we tried to wash the organic phase with 0.1 N sodium hydroxide. Previous investigators[8,9] have demonstrated that PRM and PB can be back-extracted into 0.5—1 N sodium hydroxide from the chloroform phase. In the case of the mixture of chloroform–isopropanol, we found that only PB was back-extracted into 0.1 N sodium hydroxide and more than 95% of PRM and PEMA remained in the organic phase. No apparent decomposition of PB in the alkaline solution was observed during at least an hour after the alkalinization.

Typical chromatograms of a human blank serum, a human blank serum spiked with drug standards and a human serum obtained from a volunteer who had received PRM orally are shown in Fig. 1. There were no interfering peaks of serum constituents, and adequate separation was obtained for determination of PRM and its metabolites.

Known amounts of PRM, PB and PEMA were added to drug-free human serum to yield standard samples, and each sample was then subjected to the assay. The peak height ratio of each drug to the internal standard was related to the serum concentration and the relationship

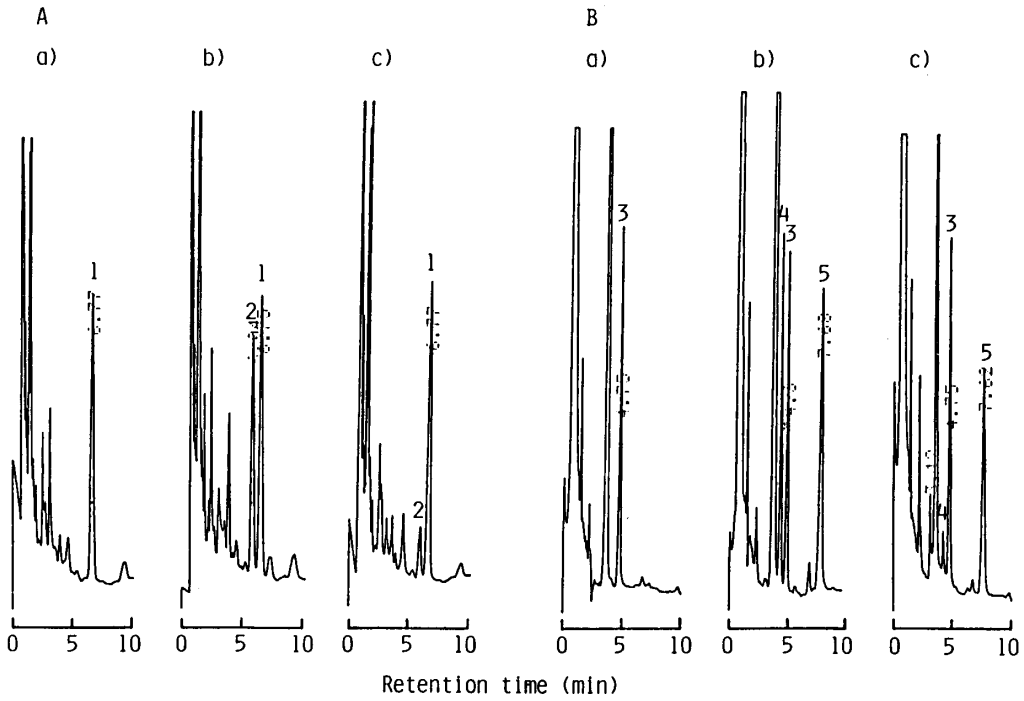

Fig. 1. Chromatograms of Serum Extracts

(A) PB analysis and (B) PRM and PEMA analysis; a) blank serum, b) serum spiked with PRM, PB and PEMA (1000 ng/ml) and c) serum obtained 72 h after a single oral administration of 600 mg of PRM to a healthy volunteer; (1) phenacetin (i.s.), (2) PB, (3) barbital (i.s.), (4) PEMA and (5) PRM.

TABLE I. Reproducibility of the Method for Analysis of PRM, PB and PEMA

Amount added	Amount found (ng/ml) (CV%)[a]		
(ng/ml)	PRM	PB	PEMA
50	49.9±3.79 (7.6)	49.5±2.97 (6.0)	50.0±1.30 (2.6)
100	101±5.05 (5.0)	101±4.46 (4.4)	102±4.85 (4.7)
200	201±5.69 (2.8)	198±8.51 (4.3)	200±4.97 (2.5)
500	503±3.98 (0.8)	494±11.8 (2.4)	505±10.3 (2.0)
1000	995±7.14 (0.7)	999±12.9 (1.3)	997±8.62 (0.9)

a) Mean±S.D. ($n=6$).

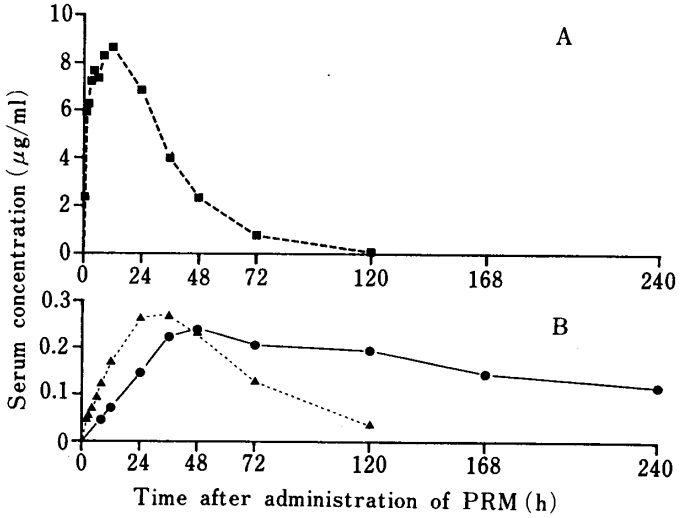

Fig. 2. Serum PRM (A) and Its Metabolites (B) after a Single Oral Administration of 600 mg of PRM to a Healthy Volunteer

PRM (■), PB (●) and PEMA (▲).

was found to be linear within the range of 50—1000 ng/ml (PRM: $Y=0.000857X+0.00869$, $r=0.99998$; PB: $Y=0.000890X+0.00634$, $r=0.99993$; PEMA: $Y=0.000938X+0.000640$, $r=0.99998$). Using these calibration curves, we evaluated the reproducibility of the method by analyses of samples of different human sera spiked with 50—1000 ng/ml of the drugs. As shown in Table I, the relative recoveries of the drugs were nearly 100% and the coefficients of variation were less than 7.6%. The minimum detection limits of the drugs estimated from the signal-to-noise ratio ($S/N=2$) were found to be about 10 ng/ml.

We have applied this method to the pharmacokinetic study of PRM in man. Figure 2 shows the serum concentration-time profiles of PRM, PB and PEMA following a single oral administration of 600 mg of PRM to a healthy volunteer. Peak serum concentrations of PRM, PB and PEMA were achieved about 12, 48 and 36 h after the administration. PEMA and PB were detectable in the serum up to 120 h and more than 240 h after the administration, respectively. Pisani *et al.*[10] reported that after the single 500 mg dose of PRM, only PEMA could be detected in the serum in normal subjects. Since our method is highly sensitive, we could follow the levels of both metabolites in the serum adequately for pharamacokinetic analysis. Details of the single-dose pharmacokinetic studies in healthy volunteers will be presented elsewhere.

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