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CONCURRENT DETERMINATION OF VALPROIC ACID WITH OTHER ANTIEPILEPTIC DRUGS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

We describe a method for the simultaneous determination of valproic acid with four other antiepileptic drugs (phenobarbital, phenytoin, carbamazepine, and primidone) in plasma by high-performance liquid chromatography. These drugs are extracted from plasma by adding a small volume of acetonitrile following saturation with ammonium sulphate. An aliquot of the extract is then injected on a reversed-phase column with a methanol—water mobile phase. The total time required for the whole analytical process including the plasma pretreatment and chromatography is approximately 20 min. The assay method is simple, rapid, reproducible and specific, and considered, therefore, suitable for both emergency and routine uses in monitoring these antiepileptic drugs simultaneously.

INTRODUCTION

Therapeutic monitoring of antiepileptic drug(s) (AED) is an aid in the clinical management of patients with seizure disorders [1]. Monitoring AEDs is desirable mainly for adjusting dosage, avoiding side-effects and assessing patient compliance [2]. Among AEDs the use of valproic acid (VPA) to treat epilepsy is increasing, because it has a wider spectrum of activity [3—5] and less apparent central nervous system toxicity than other AEDs [5]. VPA can be used not only alone [6, 7] but also in combination with other AEDs [3]. Other AEDs are known to affect the pharmacokinetics of VPA [8—10].

The simultaneous assay of VPA with phenobarbital (PB) [11] or ethosuximide [12] by the use of high-performance liquid chromatography (HPLC) has been reported. To our knowledge, there has been no report of a method for the simultaneous HPLC determination of VPA and two or more

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AEDs. The physicochemical characteristics of VPA include poor ultraviolet absorption and easy volatility. These are in contrast to other AEDs and seem to preclude the simultaneous determination by usual HPLC methods. Therefore, in order to determine VPA and other AEDs simultaneously with an HPLC method, several modifications of the usual analytical procedures are required.

The purpose of this report is to describe a method for the simultaneous HPLC quantification of VPA, PB, phenytoin (PHT), carbamazepine (CBZ) and primidone (PRM).

MATERIALS AND METHODS

Reagents

Acetonitrile, methanol and tetrahydrofuran (all of chromatography grade), hydrochloric acid, phosphoric acid, sodium phosphate dibasic and ammonium sulphate (all of reagent grade) were purchased from Wako (Osaka, Japan).

A 1 *M* solution of hydrochloric acid, saturated with ammonium sulphate was prepared by adding sufficient granular ammonium sulphate to 1 *M* hydrochloric acid until no more could be dissolved, and by agitating for a few minutes. A phosphate buffer, 0.05 *M*, pH 5.9, was prepared by dissolving 17.9 g of $Na_2HPO_4 \cdot 12H_2O$ in 1 l of distilled water. The pH was adjusted to 5.9 with phosphoric acid.

Standards

VPA in the form of sodium valproate was donated by Kyowa Hakko Kogyo (Tokyo, Japan), PB, PHT, and PRM by Dainippon Pharmaceutical (Osaka, Japan), and CBZ by Ciba-Geigy (Osaka, Japan). 4-Methylprimidone was purchased from Aldrich (Milwaukee, WI, U.S.A.).

A stock solution containing 1000 μg/ml sodium valproate (which is converted to approximately 867.6 μg/ml as VPA), 200 μg/ml PB, and 100 μg/ml each of PHT, CBZ and PRM was prepared in methanol. This solution was further diluted with methanol to the required concentration for each drug. Another stock solution of 10 μg/ml of the internal standard, 4-methylprimidone, was prepared in acetonitrile. All solutions were stored at 4°C.

Apparatus

Our HPLC system was composed of an Altex Model 110A pump (Altex Scientific, Berkeley, CA, U.S.A.), a Rheodyne Model 7125 syringe-loading sample injector (Rheodyne, Cotati, CA, U.S.A.), and a UVILOG-5 III variable-wavelength ultraviolet detector (Oyo Bunko Kiki, Tokyo, Japan). Analyses were performed on a Hibar LiChrosorb RP-18 column (250 × 4 mm, I.D., 5 μm particle size). Detector output was recorded at 10 mV with a Hitachi Model 056 recorder (Hitachi, Tokyo, Japan). The chromatographic data were processed by a Model 5000E integrator (System Instruments, Tokyo, Japan). Other equipments included 1.5-ml stoppered conical polypropylene test tubes (Sarstedt), a bench-top vortex-type mixer, and centrifuge.

Assay procedure

Each 100-μl plasma sample was added to a 1.5-ml Sarstedt centrifuge tube

containing 200 μl of 1 *M* hydrochloric acid saturated with ammonium sulphate and the tube was vortexed for 20 sec. Then 60 μl of acetonitrile containing the internal standard were added to the tube, which was stoppered, vortexed for 20 sec and then centrifuged at 2700 *g* for 5 min to separate the acetonitrile layer from the aqueous phase. A 5—10 μl aliquot of this acetonitrile layer was injected into the chromatograph.

The chromatographic conditions were set as follows: column temperature, 50° C; mobile phase, methanol—tetrahydrofuran—phosphate buffer, 0.05 *M*, pH 5.9 (44:1:55, v/v); flow-rate, 1.1 ml/min; wavelength, 210 nm; detector range, 0.005—0.16 absorbance unit full scale (a.u.f.s.).

Quantitation

Plasma standards were prepared by adding known amounts of AEDs to pooled plasma free of any drug to give concentrations required for each of their calibration curves. The calibration curve was constructed for each drug, using analyte/internal standard peak height ratios. The ratio for an unknown sample was converted to the concentration by use of this calibration curve.

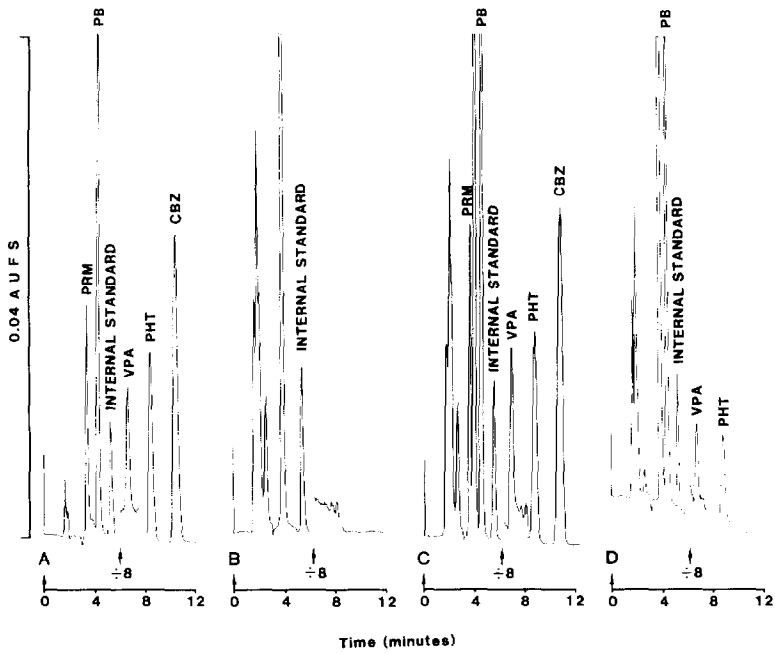

Fig. 1. Chromatograms obtained from: (A) a standard mixture of sodium valproate (1 μg), PB (0.2 μg), PHT (0.1 μg), CBZ (0.1 μg), PRM (0.1 μg) and internal standard (0.06 μg) without extraction; (B) blank plasma; (C) 100 μl of plasma spiked with sodium valproate (10 μg), PB (2 μg), PHT (1 μg), CBZ (1 μg) and PRM (1 μg); and (D) plasma of an epileptic patient, who was taking sodium valproate, PB and PHT concomitantly, with extraction according to the procedure. Plasma concentrations of AEDs of the patient are 45.5 μg/ml for VPA, 17.7 μg/ml for PB, and 4.9 μg/ml for PHT. Detector range was set at 0.04 a.u.f.s. except for VPA, for which it was set at 0.005 a.u.f.s. Multiplying the concentration of sodium valproate by the conversion factor of 0.8676 gives the concentration of VPA. PB = phenobarbital; PHT = phenytoin; CBZ = carbamazepine; PRM = primidone; VPA = valproic acid.

Solutions of all five analytes in drug-free plasma were assayed in triplicate. The concentration ranges employed were 10—500 μg/ml for sodium valproate (approximately 8.7—433.8 μg/ml as VPA), 2—100 μg/ml for PB and 1—50 μg/ml for each of PHT, CBZ and PRM. In all cases it was confirmed in the separate experiments that the calibration curves were linear for at least these concentration ranges. Linear regression analyses were performed for each analyte relative to the internal standard.

RESULTS

Chromatography

With our chromatographic conditions, VPA, PB, PHT, CBZ, PRM and the internal standard all exhibited symmetrical peaks. Fig. 1A shows a typical chromatogram for the drug standard. No interfering peak was observed when the blank plasma extract was analysed (Fig. 1B). Fig. 1C is a chromatogram from a spiked plasma sample containing known quantities of five AEDs [sodium valproate (100 μg/ml), PB (20 μg/ml), PHT, CBZ and PRM (10 μg/ml each)]. Fig. 1D is a chromatogram from plasma of an epileptic patient who was taking sodium valproate, PB and PHT concomitantly.

As can be seen in Fig. 1, it is necessary to switch the detector range from 0.04 to 0.005 a.u.f.s. to obtain an appropriate chromatographic peak for VPA. Except for VPA detected at 0.005 a.u.f.s., the peaks of the other four AEDs

TABLE I

PRECISION OF ASSAYS FOR ANTIEPILEPTIC DRUGS IN PLASMA

Drug	Concentration* (μg/ml)	C.V. (%)	
		Within-day (n = 5)	Day-to-day (n = 12)
Sodium valproate	500	4.2	5.6
	100	2.7	6.1
	25	3.0	7.2
Phenobarbital	100	1.6	2.1
	20	2.1	4.4
	5	1.0	2.7
Phenytoin	50	3.0	6.1
	10	4.5	6.0
	2.5	4.9	5.7
Carbamazepine	50	2.8	4.2
	10	3.5	5.0
	2.5	2.9	4.7
Primidone	50	2.9	5.3
	10	2.0	4.6
	2.5	4.2	4.4

*To convert concentrations of sodium valproate to those of valproic acid, multiply by 0.8676.

were adequate at 0.04 a.u.f.s. The capacity ratios (k') for PRM, PB, VPA, PHT, and CBZ were 1.00, 1.45, 2.75, 3.80, and 4.70, respectively, and 2.00 for the internal standard.

Analytical variables

Precision. We assessed the precision of the method by repeated analyses of plasma specimens containing known concentrations of the drugs being investigated. As given in Table I, the coefficient of variation (C.V.) for the within-day precision ranged from 1.0 to 4.9% (n = 5), and that of the day-to-day precision from 2.1 to 7.2% (n = 12).

Recovery. We measured the absolute analytical recovery from plasma of the

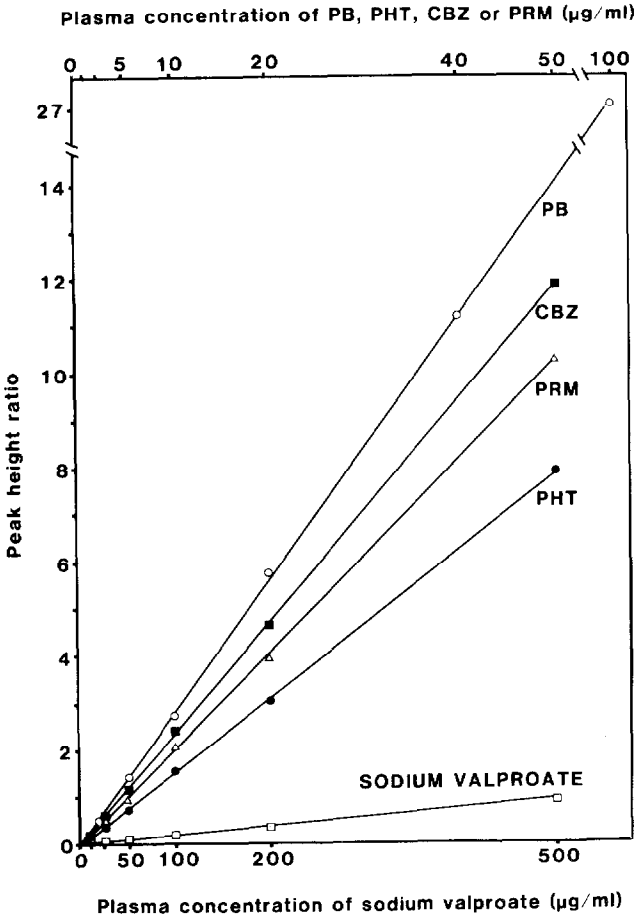

Fig. 2. Calibration curves for sodium valproate, PB, PHT, CBZ and PRM in plasma. A three-point standard curve was prepared by plotting on the ordinate the ratio of each compound's peak height to that of the internal standard for each concentration. Linear regression analysis of calibration curve data indicated no significant deviation from linearity (r = 0.9986–0.9997). In addition, intercept values did not significantly differ from zero. (□) Sodium valproate; (○) phenobarbital (PB); (●) phenytoin (PHT); (■) carbamazepine (CBZ); (△) primidone (PRM). Multiplying the concentration of sodium valproate by the conversion factor of 0.8676 gives the concentration of valproic acid.

five drugs in the following way. The drugs were added to drug-free plasma to achieve the midpoint concentrations as given in Table I. This plasma was then analysed by our method. Carefully measured aliquots of the acetonitrile layer were then injected and their peak heights measured. Absolute recovery was calculated by comparing these peak heights with the peak heights obtained by the direct injection of the pure drug standards. Absolute recoveries of AEDs were 89.6%, 104.0%, 79.2%, 83.9%, and 91.8% for VPA, PB, PHT, CBZ, and PRM, respectively.

Linearity and sensitivity. Plasma standards were prepared containing various known amounts of each drug. A constant amount of the internal standard was added to each sample. Concentration and peak height ratio correlated linearly with each other for all AEDs examined. All the calibration curves passed through the origin (Fig. 2).

With the 100-μl volume of sample used in the present assay method, the sensitivity was such that the drugs can be detected at plasma concentrations as low as 3 μg/ml for sodium valproate (approximately 2.6 μg/ml for VPA), and at least as low as 0.05 μg/ml for the other four AEDs.

Interference. To determine the potential clinical usefulness of our assay method, we examined the possible chromatographic interference from several other compounds including pharmacologically active metabolites derived from their parent drugs (CBZ and PRM) and clinically commonly used drugs, which may be administered concurrently with VPA. Table II summarizes the data for capacity ratios of the tested compounds. None of the compounds showed any potential interference with the present assay method.

TABLE II

CAPACITY RATIOS FOR SELECTED COMPOUNDS

Compound	Capacity ratio k'	Compound	Capacity ratio k'
Acetaminophen	0.21	4-Methylprimidone	2.00
Salicylic acid	0.25	Valproic acid	2.75
Ethylphenylmalonamide	0.38	Methylphenobarbital	3.10
Theophylline	0.39	Phenytoin	3.80
Caffeine	0.45	Glutethimide	4.37
Ethosuximide	0.53	Pentobarbital	4.42
Primidone	1.00	Carbamazepine	4.70
Chloramphenicol	1.35	Lidocaine	7.80
Phenobarbital	1.45	Diazepam	15.25
Carbamazepine-10,11-epoxide	1.57		

DISCUSSION

Various HPLC techniques for simultaneously determining the plasma concentrations of AEDs have been reported [11—22]. However, to our knowledge, there have been only two reports where VPA and PB [11] or VPA and ethosuximide [12] were measured simultaneously by HPLC.

There seem to be several reasons that simultaneous assays for VPA and other AEDs have rarely been developed. First, VPA has a poor absorbance in the

ultraviolet range. Derivatization for detection enhancement [23—26] or other technical alterations (i.e. a colorimetric procedure based on variation in colour of a solution of bromocresol purple [11] or controlled evaporation using the Technicon Evaporation-to-Dryness Module and a low wavelength of 210 nm for adequate detection sensitivity [12]) seem to be necessary. In contrast, AEDs other than VPA show good ultraviolet absorbance at low wavelengths, and no derivatization is required. Secondly, since VPA is a volatile compound [12, 26, 27], extreme care must be taken to avoid losses due to volatilization when concentrating sample extracts. In general, when compounds have a low ultraviolet absorbance, one would attempt to derivatize them for detection enhancement and/or to evaporate the extract for their enrichment. Obviously, the volatility of VPA precludes any evaporation procedure. Thirdly, the chemical structure of VPA differs from that of other AEDs, VPA being a branched-chain carboxylic acid and lacking nitrogen and a ring moiety [3]. The optimum conditions for separating VPA from other AEDs on a chromatogram, where VPA and other AEDs are simultaneously detected within a given time period and the method used for clinical application, seem somewhat difficult to be set because of their different chemical structures and associated physicochemical properties.

In order to overcome the several difficulties associated with the simultaneous determination of VPA and the four other AEDs as discussed above, we attempted several modifications. First, we tried to derivatize VPA according to the various methods reported [23—26]. However, those methods were found not to be specific for VPA; namely, the structural portion (>C=O) of VPA and the other AEDs reacted on derivatization with phenacyl bromide [23, 26], 1-chlormethylisatin [24] and 4-bromophenacyl bromide [25]. We cannot find any other method by which VPA is specifically derivatized. Even if such method were found, obtaining adequate capacity ratios of VPA and other AEDs on one chromatogram would be very difficult. Secondly, we tried to extract VPA and other AEDs simultaneously by using organic solvents such as diethyl ether and ethyl acetate and then extracting back into an alkaline aqueous phase without evaporation to avoid loss of VPA due to its easy volatility. However, this procedure resulted in a low recovery of the five analytes (approximately 41—75%). Thirdly, we pretreated samples simply by adding a water-soluble organic solvent to the plasma, and injected the supernatant into the chromatograph. However, this pretreatment method was considered to be inadequate since the volume required becomes larger and the concentration of VPA is decreased.

Therefore, we tried to separate the mixture of water and water-soluble organic solvent into two layers. AEDs except for CBZ are acidic drugs and they should, therefore, be extracted into a water-soluble organic layer when plasma is acidified (with 1 *M* hydrochloric acid). CBZ was also considered likely to dissolve in a water-soluble organic layer. In order to achieve the separation of water and water-soluble organic solvent mixture into the two phases, we subjected the plasma samples to salting-out procedure. The salts tested were sodium chloride, potassium chloride, sodium dihydrogen phosphate, zinc sulphate, magnesium sulphate and ammonium sulphate. Water-soluble organic solvents tested were methanol, ethanol and acetonitrile. The combination of

ammonium sulphate and acetonitrile yielded the best extraction efficiency for all the analytes. In order to increase the enrichment of VPA with this separation step, we set the detector wavelength rather low (210 nm, Fig. 1). Furthermore, we adjusted the pH of the mobile phase to 5.9 since AEDs except for CBZ are acidic drugs with different pK_a values. Finally, we added tetrahydrofuran to the mobile phase to adjust the AEDs' capacity ratios. These procedures were found to be necessary for obtaining satisfactory chromatograms of all the analytes examined.

The extraction technique described for analytes in plasma is simple, rapid, reproducible and specific enough for application to therapeutic monitoring of AEDs. The time required for the pretreatment procedure is 8 min and that for the chromatographic run is 12 min, resulting in a total of 20 min. The coefficients of variation (C.V., %) for both within-day and day-to-day analyses (Table I) were within the performance limits required for medical management [28]. Furthermore, pharmacologically active metabolites that are derived from CBZ and PRM, and several drugs that may be co-administered with VPA and/or AEDs, did not interfere with our assay. The absolute recovery of all the analytes from plasma was > 79%. An excellent linear calibration curve (r = 0.9986—0.9997), passing through the origin, was obtained for each analyte (Fig. 2).

The common use of VPA with other AEDs [3] and the consequent need not only to monitor plasma concentrations of VPA with [3] or without other AED(s) [6, 7] but also to detect any interactions between VPA and other AED(s) [3, 8—10] makes multiple drug analyses in one HPLC system an attractive option. Finally, the HPLC method reported here is the first for simultaneously determining VPA and two or more AEDs. Our method appears to be specific for VPA, PB, PHT, CBZ and PRM, and is simple, rapid, and of sufficient precision for clinical application.

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