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Note

Improved procedure for the high-performance liquid chromatographic determination of valproic acid in serum as its phenacyl ester

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Valproic acid (VPA) is clinically used as an anticonvulsant in the treatment of epilepsy, and the serum levels are frequently monitored for efficient control of seizures. In recent years, high-performance liquid chromatography has been increasingly employed for the analysis of drugs in serum. A sensitive assay of VPA by liquid chromatography is difficult because of the poor ultraviolet absorbance. In order to overcome this problem, derivatives by reaction with aryl bromomethyl ketones have been used as ideal derivatives having high ultraviolet absorbance properties for high-performance liquid chromatographic determination of VPA, such as phenacyl [1], 4-bromophenacyl [2] and 2-naphthacyl [3] esters.

Usually the derivatization was carried out under non-aqueous conditions after extraction of the acidified sample with organic solvent [1, 3]. The extraction technique was troublesome and the recovery of VPA from aqueous solution by solvent extraction was irreproducible, so a procedure for the derivative formation of VPA was proposed in aqueous conditions using 4-bromo-

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phenacyl bromide [2] and phenacyl bromide [4]. However, insufficient investigations on conditions for phenacylation of VPA were made.

We have chosen to use phenacyl bromide as derivatizing reagent since it was easier to obtain in this country, and triethylamine instead of crown-ether catalysis. The present paper describes evaluations of the factors affecting the yield in phenacylation under aqueous conditions, and a simple, effective procedure for phenacylation of VPA in serum has been established without recourse to a solvent extraction step.

EXPERIMENTAL

Reagents and standard

Sodium valproate (VPA) was obtained from Kyowa Hakko (Tokyo, Japan). Phenytoin, phenobarbital, hexobarbital, cyclohexane carboxylic acid (CCA), α-bromoacetophenone (phenacyl bromide) and triethylamine (TEA) were from Tokyo Kasei (Tokyo, Japan). Carbamazepine was purchased from Nippon Ciba-Geigy (Hyogo, Japan). All other chemicals were of analytical grade.

The esterification agent was a solution containing α-bromoacetophenone, 3 g/l in acetonitrile. The internal standard was CCA, 4 μg/ml in acetonitrile. The VPA standards were 1—100 μg (as valproic acid) per ml in drug-free serum. Aliquots of 10—1000 μg as valproic acid per ml in distilled water were diluted ten-fold with drug-free serum.

Procedure

Serum or VPA standard (50 μl) was added to a 1.5-ml tube containing 1.0 ml of internal standard. The tube was stoppered, mixed on a Vortex mixer (10 sec) and centrifuged. An 800-μl aliquot of the supernatant was transferred to a glass tube containing 200 μl of esterification agent and 100 μl of TEA. The mixture was heated in a dry heating block for 30 min at 80°C, keeping the tube open; on cooling 10-μl aliquots were injected into the liquid chromatograph.

High-performance liquid chromatography

Analyses were performed using a Japan Spectroscopic liquid chromatograph equipped with a Twincle pump and a Model Uvidec 100-IV UV (ultraviolet) variable-wavelength spectrophotometric detector, capable of monitoring at 245 nm. Chromatography was performed at 30°C on a 25 cm × 4.6 mm I.D. stainless-steel column packed with Sil $C_{18\text{-}5}$ (particle size 5 μm) (from Japan Spectroscopic, Tokyo, Japan) with acetonitrile—water (60:40, v/v) as mobile phase. The flow-rate was 1.0 ml/min.

Calculations

VPA concentrations were determined as free acid from graphs of peak height ratios against known concentrations constructed using VPA standards.

RESULTS AND DISCUSSION

The following factors affecting phenacylation of VPA in aqueous solution were investigated in detail. All examinations were performed through the entire

procedure described in *Procedure*, except that 1.0 ml of acetonitrile was added instead of internal standard and the reaction tube was heated keeping it tightly stoppered. The results were followed by measuring the average of the peak heights of phenacyl valproate obtained by duplicate injections.

Esterification agent and TEA concentrations

Using 50 μl of VPA standard (100 μg/ml), the necessary concentrations of esterification agent and TEA were examined. As shown in Fig. 1, the concentrations of α-bromoacetophenone and TEA sufficient to obtain a constant phenacylation were 0.2% (w/v) and 50 μl, respectively. As previously reported, phenacylation was carried out in aqueous solution with crown-ether catalysis [2, 4, 5]. However, it was found that the phenacyl ester of VPA was easy to prepare in aqueous conditions using TEA instead of crown ethers.

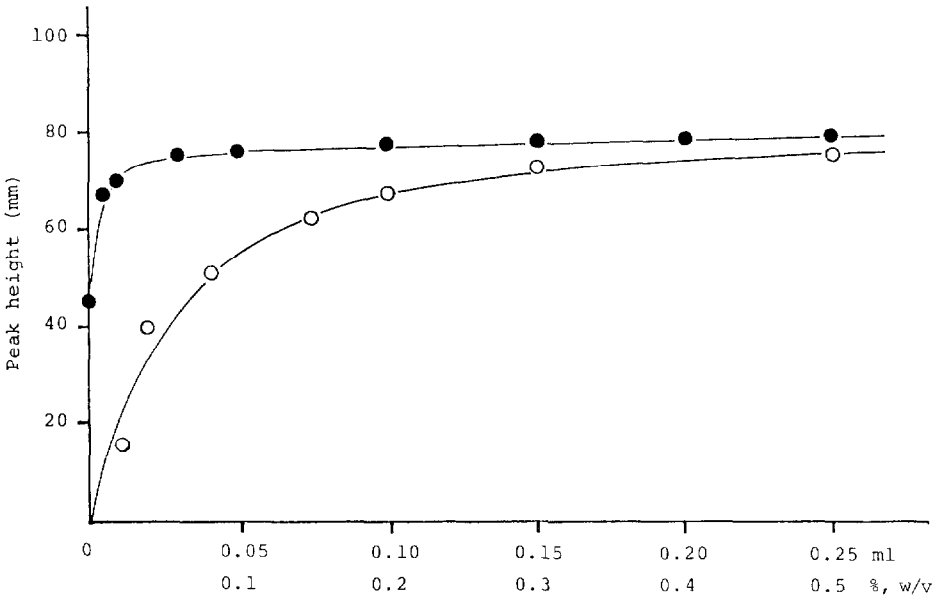

Fig. 1. Effect of α-bromoacetophenone and triethylamine concentrations on phenacylation. •, Quantity of added triethylamine (1 ml); ○, concentration of α-bromoacetophenone (%, w/v).

Reaction temperature and period

Using 50 μl of VPA standard (100 μg/ml), the effects of the reaction temperature and period were investigated through the entire procedure. The effects of these two factors were not so large. As regards the time course of derivatization, the reaction was sufficient in 30 min at 80°C to obtain a constant peak height of phenacyl valproate. The yield of phenacylation in these conditions was about 90% of that obtained in 72 h at 80°C.

Influence of water content in the reaction mixture on phenacylation

The water content of the reaction mixture seriously affected phenacylation. Using 50 μl of VPA standard (100 μg/ml), the influence of various water contents on phenacylation was investigated and the results are shown in Fig.

2. The phenacylation yield increased with decreasing water content and rose rapidly in the range of less than 10%. The same result was also obtained in the case of 4-bromophenacylation (Fig. 2). In the procedure, a relatively large volume of acetonitrile was added to the sample solution for the purpose of complete deproteinization and of obtaining a better phenacylation yield. The tubes were kept open during the phenacylation reaction. The sample solution diluted by the addition of this large amount of acetonitrile could be concentrated as phenacylation proceeded by heating the reaction mixture at 80°C for 30 min. The open system made the detection limit decrease markedly. Addition of CCA, which has properties similar to those of VPA, as an internal standard obviated the need for accurate estimation of reagent amounts, reaction temperature, reaction period and water content of the reaction mixtures which gave a slight influence on the phenacylation yield.

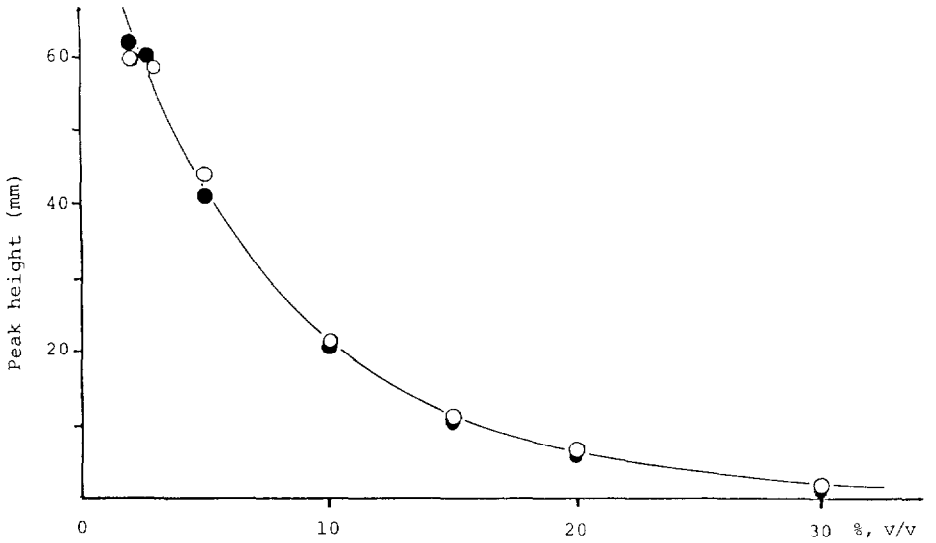

Fig. 2. Effect of water content of the reaction mixtures on phenacylation. ○, Phenacylation; ●, 4-bromophenacylation.

The calibration curve was linear from 1 to 100 μg/ml VPA in serum. The precision of the method was examined by following the procedure at three different concentrations: 20, 40 and 80 μg/ml VPA in serum. The coefficients of variation for three concentrations of VPA (n = 6 each) were 3.4% for 80 μg/ml, 2.7% for 40 μg/ml and 5.3% for 20 μg/ml. The procedure was found to be satisfactorily reproducible. The minimum concentration for quantitation of VPA was 0.5 μg/ml for a 50-μl specimen.

Fig. 3 shows a chromatogram of serum from a patient who was receiving VPA. The peak corresponded to about 50 μg/ml in serum. We selected an ODS column as the stationary phase for the analysis of phenacyl esters because it is commonly used in the assay of drugs. Peaks of coexisting substances in serum and of reagents were eluted early, while there was excellent separation of phenacyl valproate and internal standard from each other and from reagent peaks.

When serum from a patient receiving various antiepileptics (hexobarbital,

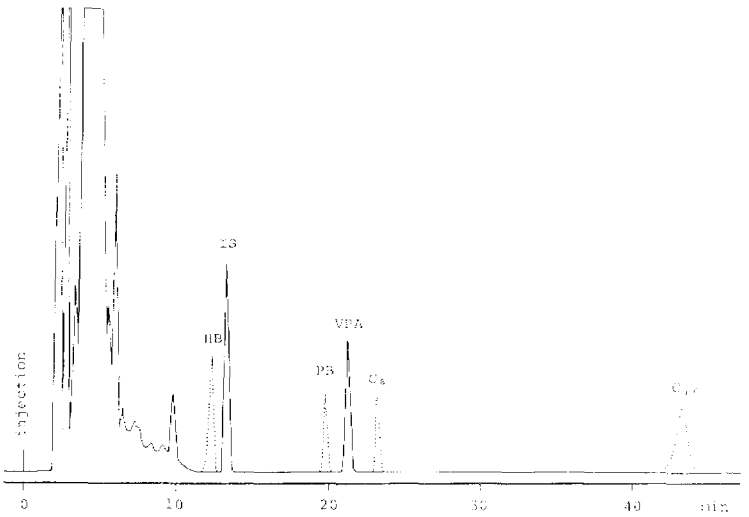

Fig. 3. Chromatogram of serum from a patient receiving valproate. HB = Hexobarbital; IS = internal standard (cyclohexane carboxylic acid); PB = phenobarbital; VPA = valproic acid; C_8 = octanoic acid; C_{10} = decanoic acid. (As their phenacyl derivatives.) Conditions: column, Finepak Sil C_{18-5}, 25 cm × 4.6 mm I.D.; eluent, acetonitrile—water (60:40, v/v); temperature, 30°C; flow-rate, 1.0 ml/min; detector, 245 nm.

phenobarbital, phenytoin, carbamazepine) is measured by the present method, serious trouble may arise. Retention values of some phenacylated drugs are shown by the dotted line in Fig. 3. Hexobarbital and phenobarbital gave no interference and produced sharp, symmetrical peaks. Alric et al. [3] reported an incomplete separation of 2-naphthacyl derivatives of phenobarbital and VPA using the mixture acetonitrile—water (83:17, v/v) as mobile phase, while a separation of phenacyl esters of these two drugs was complete using the present solvent mixture (acetonitrile—water, 60:40, v/v). Phenytoin and carbamazepine did not show any peak. These two drugs did not react with bromomethyl naphthyl ketones [3]. Probably the two drugs do not react with α-bromoacetophenone either. They themselves eluted early, close to reagent peaks, using the present mobile phase and no interference was encountered in specimens from patients receiving these drugs.

Some factors affecting phenacylation of VPA have been examined in detail. The phenacyl esters were easily prepared and proved to be satisfactory for analysis. The most important advantages of the present procedure are direct and effective derivatization in sample solution by adding a large volume of acetonitrile, concentration of the reaction mixtures with the open system during heating for phenacylation and excellent sensitivity permitting measurement of 0.5 μg/ml VPA using only 50 μl of serum.

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