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Note

Sensitive gas–liquid chromatographic method for determination of valproic acid in biological fluids

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Valproic acid (di-*n*-propyl acetate, VPA) has been shown to be effective against petit mal epilepsy and, in combination with phenobarbital, against grand mal epilepsy and other forms of epilepsy with absences and tonic–clonic seizures[1–4]. The exact mode of action is not yet clear, but hypothesis includes the inhibition of an enzymatic system that catalyses the transformation of γ-aminobutyric acid (GABA) in the central nervous system[5]. An elevation of GABA level, however, is suggested to provide protection against a number of forms of epileptic seizures[6]. Little information is available on the pharmacokinetics of VPA, including plasma protein binding behaviour, that can profoundly influence the distribution of the drug in the body[7,8]. All this is essential information, as attempts are made to define a therapeutic concentration range of VPA in plasma, as has been done with a number of drugs, particularly antiepileptic drugs[9].

Methods currently available for the determination of VPA in plasma or urine[10–13] are either time consuming or lack the sensitivity needed for pharmacokinetic studies where concentrations need to be followed for long periods after administration of the dose. Small concentrations may be encountered in particular in cerebrospinal fluid or in saliva if the drug is as highly protein bound as is suggested for VPA[14].

This paper describes a simple and sensitive gas–liquid chromatographic (GLC) method for the determination of VPA in plasma, saliva, spinal fluid and urine.

MATERIALS AND METHODS

Reagents

Sodium valproate was kindly supplied by Desitin-Werk Carl Klinke (Hamburg, G.F.R.). Caprylic acid (Fluka, Buchs, Switzerland) was used as an internal standard; the internal standard solution was prepared by dissolving 4 mg of caprylic acid in 100 ml of distilled water. All solvents used were of reagent grade (Merck, Darmstadt, G.F.R.).

Gas chromatography

A Hewlett-Packard Model 5736 A dual-column gas chromatograph was used, equipped with a flame-ionization detector. The silanized glass column (6 ft. × 2 mm I.D.) was packed with free fatty acid phase (FFAP, Applied Science Labs., State

College, Pa., U.S.A.) on 80–100-mesh Gas-Chrom Q and operated at 170°. The carrier gas (nitrogen) flow-rate was 30 ml/min and the injection port temperature was 250°. The detector was operated at 250° with a hydrogen flow-rate of 30 ml/min and an air flow-rate of 240 ml/min. Under these conditions, the retention times were 2.8 min for VPA and 4.3 min for caprylic acid. The results were recorded on a Hewlett-Packard Model 7123 A 10-in. recorder at a chart speed of 0.25 in./min. For data analysis, a Hewlett-Packard Model 3380 A integrator was also used.

Procedure

Blood samples (5–6 ml) were drawn by venopuncture from patients receiving VPA and centrifuged within 2 h at 4000 g for 10 min. VPA was stable for over 4 months when the plasma was kept at −20° until analysis. A 1-ml volume of plasma was transferred into a 12-ml glass tube and 1 ml of the internal standard solution containing 40 μg of caprylic acid were added. The mixture was acidified with 1 ml of 0.5 N hydrochloric acid and the compounds were extracted into 5 ml of organic solvent (*n*-hexane–chloroform, 1:1) containing 2% of methanol by shaking the tubes for 4 min. Following brief centrifugation, 3.5 ml of the organic layer were transferred into a second 12-ml centrifuge tube to which 2 ml of 0.5 N sodium hydroxide solution were subsequently added. After shaking and centrifuging, the organic phase was discarded and 1.5 ml of the aqueous layer were pipetted into a pointed tube, together with 1 ml of 3 N hydrochloric acid and 50 μl of chloroform. The tubes were vortexed for 30 sec and centrifuged for 2 min. A 1-μl volume of the organic (lower) phase was then injected on to the GLC column.

Determinations of VPA in urine, spinal fluid, saliva and dialysis buffer obtained from protein binding studies were carried out by using the same procedure as described for plasma. For analysis of the glucuronide metabolite of VPA, urine samples were diluted 1:5 with 0.2 M sodium acetate buffer of pH 5.0 and the solution was incubated for 24 h at 37° with 2000 Fishman units of a glucuronidase–arylsulphatase solution from *Helix pomatia* (Serva, Heidelberg, G.F.R.). A 1-ml volume of the buffer sample after hydrolysis was assayed as described above.

Calibration graphs for the determination of VPA in plasma or the other biological fluids were obtained by taking blank material to which known amounts of VPA were added. The calibration graphs were tested in the concentration ranges 0.5–150 μg/ml in plasma and 0.5–20 μg/ml in spinal fluid, saliva and buffer. The chromatograms were analyzed according to two different methods: (1) by plotting the peak-height ratio of VPA to the internal standard against the known concentrations; and (2) by using the integrator and plotting the ratio of the area under the curve of VPA to that of the internal standard against the known concentrations.

RESULTS AND DISCUSSION

A typical chromatogram of VPA is shown in Fig. 1. No interfering endogenous compounds are co-extracted with VPA as can be seen when blank plasma is carried through the procedure (C). The peaks of VPA and the internal standard are almost symmetrical and are well separated from each other.

The calibration graphs for VPA in plasma and urine are shown in Figs. 2 and 3. The concentration range covered is 1–150 μg/ml. To our knowledge, this is the
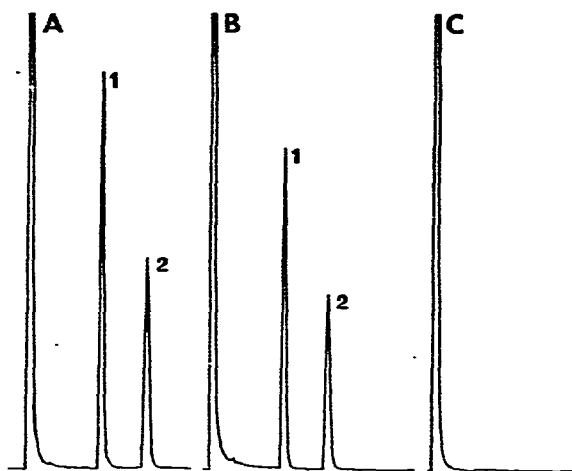
Fig. 1. Gas chromatograms for (A) extraction from blank plasma to which VPA (60 μg) and internal standard (40 μg) were added; (B) material extracted from plasma of an epileptic patient receiving VPA; (C) material extracted from blank plasma.
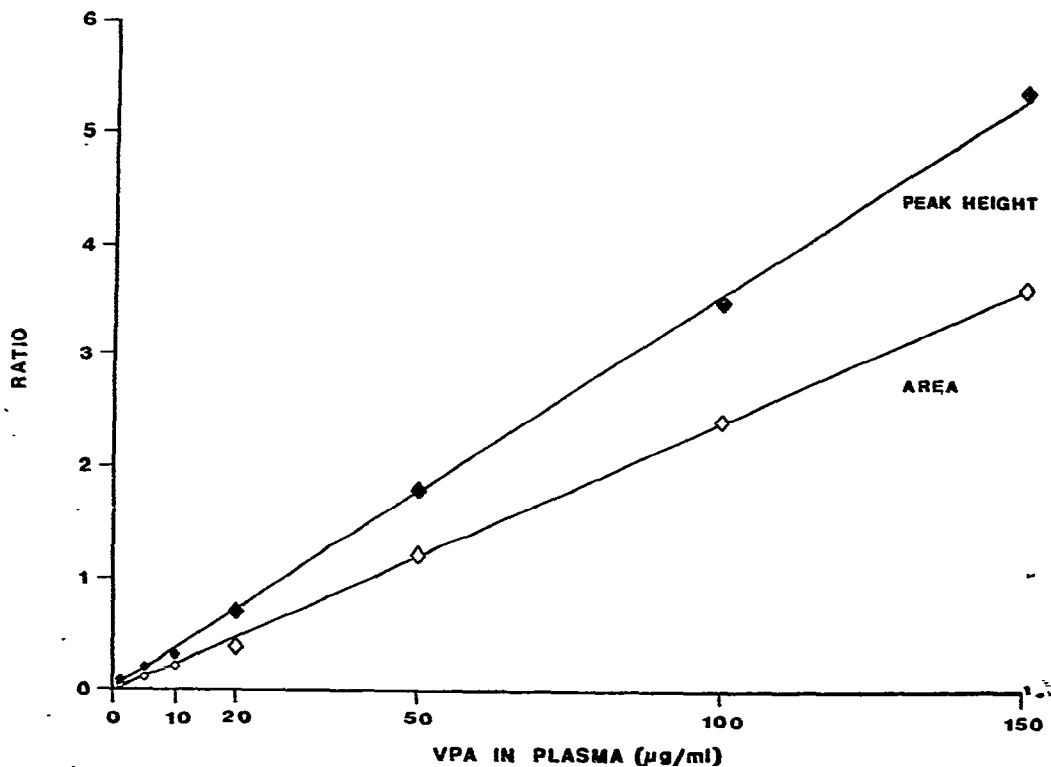

Fig. 2. Calibration graphs for VPA following extraction from plasma. Graphs are shown for analysis with peak-height and peak-area ratios.
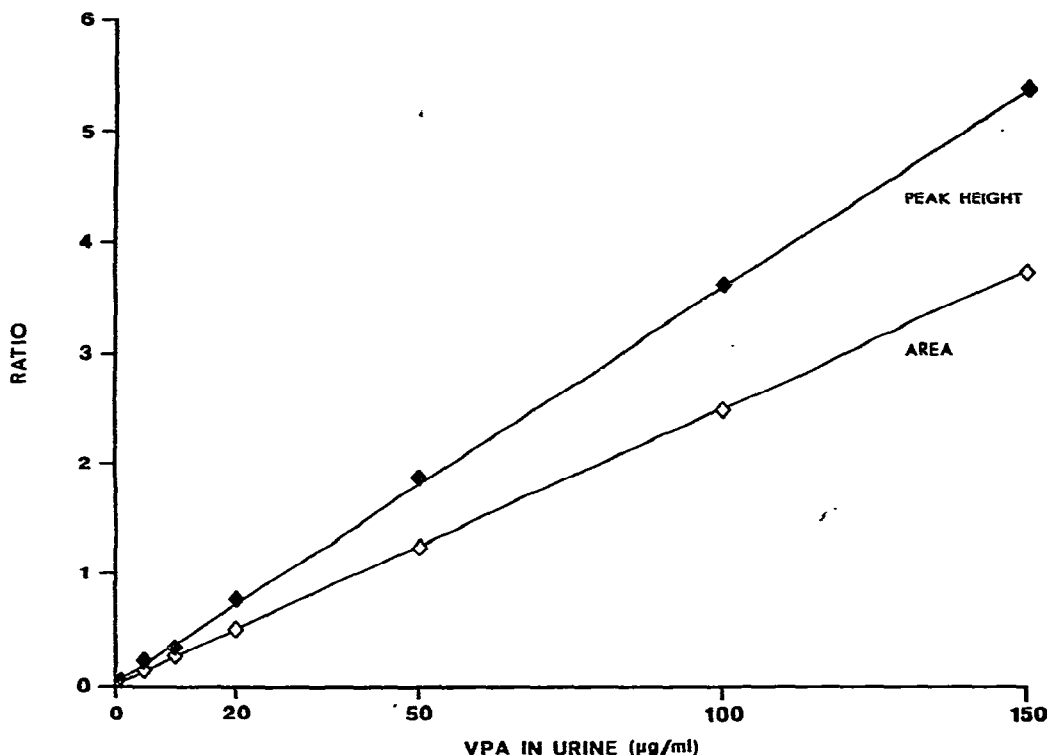
Fig. 3. Calibration graphs for VPA following extraction from urine. Graphs are shown for analysis with peak-height and peak-area ratios.

only method that is sensitive to the 1-μg level or even below. All other reported studies[10-14] are based on methods with sensitivity limits of about 5 μg/ml in plasma. Although under steady-state conditions of treatment with VPA therapeutic concentrations in plasma commonly range between 50 and 120 μg/ml[15-18], we found it necessary to measure concentrations as low as 0.5 μg/ml in pharmacokinetic studies[19] when the time course of the plasma level was followed over 72 h. This is particularly important for VPA, as in one pharmacokinetic study the investigators had calculated half-lives on the basis of plasma concentrations measured over 34 h only[16], although the terminal slope of the plasma level time curve does not begin before 24 h. Therefore, the half-lives reported were shorter than the predominant half-lives of VPA when based on observations over longer time periods.

    A sensitive method for the determination of VPA is needed for several reasons: (1) preliminary results indicate that the plasma protein binding of VPA is 90–95%, so that concentrations of 2–4 μg/ml in buffer need to be detected if protein binding is measured by equilibrium dialysis; with highly protein-bound drugs, the degree of binding may be of value for the interpretation of plasma levels in the individual patient[20]; (2) in saliva, VPA concentrations appear to be even lower than the free concentrations in plasma[19]; (3) in spinal fluid, VPA may be present in low concentrations, if concentrations correspond to the free level in plasma as has been shown with phenytoin[21].

All calibration graphs were linear over the concentration range shown (Figs. 2 and 3). If the integrator is used to calculate the results, however, the same amount of internal standard can be used for all concentrations of VPA, whereas with the peak-height comparison a smaller amount of internal standard (2 μg) is appropriate for quantifying adequately concentrations below 2 μg/ml.

The coefficient of variation was different when the integrator and the peak-height ratio were used. In plasma and urine the coefficient of variation was determined by assaying 10 samples each of concentrations 1, 5, 10, 20, 50 and 100 μg/ml. The results were 3.1 % in plasma and 2.0% in urine by use of the peak-height ratio but only 2.7% in plasma and 1.6% in urine by use of the integrator system. The reproducibility of the method is good and can even be improved if data are obtained by computerized integration of peak areas.

When identical samples were assayed on 10 different days, the reproducibility was 3.8% in plasma and 3.0% in urine. Although three extraction steps are necessary in order to achieve pure samples for analysis, the recovery is still good, being 91.7% in plasma, 92.2% in urine, 94.9% in spinal fluid and buffer and 94.5% in saliva ($n = 3$).

The accuracy of the method was tested by preparing plasma samples with amounts of VPA added to the plasma that were unknown to the operator. The results show that the concentrations measured by this method agree well with the actual concentrations (Table I). Plasma taken from patients receiving phenobarbital, carbamazepine, phenytoin, primidone, ethosuximide, clonazepam and digoxin was tested and no interfering peaks were found when the drugs were administered in therapeutic doses. Twenty-five plasma samples can easily be assayed in one working day with the proposed method.

TABLE I

ANALYSIS OF PLASMA FOR VPA CONTENT WHEN KNOWN AMOUNTS OF THE DRUG WERE ADDED TO SAMPLES

Valproic acid (μg/ml)	
Added	Found
1	0.9
5	5.1
5	5.3
10	9.7
20	20.7
40	40.9
50	48.2
80	79.4

ACKNOWLEDGEMENT

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