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Note

New method for the gas chromatographic determination of valproic acid in serum

RYOTA NISHIOKA* and SATOSHI KAWAI

Gifu College of Pharmacy, Mitahora, Gifu 502 (Japan)

and

SHIGERU TOYODA

National Higashi Owari Hospital, Kikko, Moriyama-ku, Nagoya (Japan)

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Valproic acid is an anticonvulsant drug which is widely used in epilepsy. Determination of valproic acid in serum is required in epilepsy therapy for efficient control of seizures. Many gas chromatographic methods have been described for its determination. In some methods, valproic acid has been analysed after derivatization such as methylation [1—4], butylation [5], phenacylation [6, 7] and trimethylsilylation [8], and in others assayed in free form [9—16]. We have developed a novel method for measuring valproic acid in serum, in which valproic acid is derivatized into the hexafluoroisopropyl ester. This method gives a sharp peak of valproic acid on a gas chromatogram at low temperature, 80°C. It is possible to determine less than 0.5 μg of valproic acid using 0.1 ml of serum sample.

EXPERIMENTAL

Reagents and standards

Derivatizing reagents were hexafluoroisopropanol and trifluoroacetic anhydride which were obtained from Tokyo Kasei (Tokyo, Japan). The internal standard solution was octanoic acid (*n*-caprylic acid), 5 μg/ml in hexane. Standard solution of sodium valproate was prepared containing 5, 10, 20, 30, 50 and 100 μg/ml as valproic acid in water. For the extraction step hexane and 6 *N* hydrochloric acid were used.

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Apparatus and conditions

A Shimadzu GC-4BPF gas chromatograph equipped with a hydrogen flame ionization detector and a glass column 2.0 m × 3 mm I.D. packed with 3% OV-1 on 80—100 mesh were used, with a column temperature of 80°C, a detector temperature of 120°C and a carrier gas (nitrogen) flow-rate of 40 ml/min.

Standard procedure

To 0.1 ml of serum sample in a 1.5-ml centrifuge tube were added 0.3 ml of water, 1 drop of 6 *N* hydrochloric acid and 0.8 ml of hexane solution containing octanoic acid as an internal standard. The tube was shaken vigorously for 1 min and centrifuged at 7000 *g* for 4 min. The lower layer was removed. The hexane layer was dried over a small amount of anhydrous sodium sulphate, transferred to a tapered tube and evaporated just to dryness. To the residue were added 0.1 ml of hexafluoroisopropanol and 0.02 ml of trifluoroacetic anhydride, and the mixture was allowed to stand at room temperature for 30 min. One to 3 μl of the reaction mixture were injected into the gas chromatograph.

Drug-free serum spiked with standard solution of valproic acid (0.5—10 μg) was treated similarly to the standard procedure to establish calibration curves. The unknown concentration was determined by comparison of the valproic acid/octanoic acid peak height ratios with those of the calibration curve.

Procedure without derivatization (outline)

To 0.5 ml of serum were added 0.5 ml of water, 0.1 ml of 6 *N* hydrochloric acid and 0.8 ml of a mixture of chloroform—isobutanol (9:1, v/v) containing an internal standard (biphenyl). The mixture was vortexed for 1 min and then centrifuged at 1500 *g* for 10 min. The aqueous (upper) layer was removed and an aliquot (1—2 μl) of the organic phase was injected directly into the gas chromatograph. Gas chromatographic conditions: 10% SP-1000 (1-m long column), 160°C.

RESULTS AND DISCUSSION

A mixture of hexafluoroisopropanol and trifluoroacetic anhydride was used successfully for the derivatization of carboxylic acid where it was converted into hexafluoroisopropyl ester [17]. This technique was applied to the gas chromatographic determination of homovanillic acid [18, 19], vanillylmandelic acid [18, 19], bile acids [20, 21] and captopril [22]. In this reaction, trifluoroacetic anhydride acts as a catalyst, therefore the presence of a small amount of trifluoroacetic anhydride must be enough for derivatization. In most cases except one report [22], however, the proportion of trifluoroacetic anhydride to hexafluoroisopropanol in the reagent mixture is the same or twice as much. It should be noted that use of too much trifluoroacetic anhydride may result in a by-product. In the case of valproic acid, it had a tendency to give a side-peak on the chromatogram with increasing concentration of trifluoroacetic anhydride in the reagent mixture. This side-peak showed the same retention time as that in a case treated only with trifluoroacetic anhydride.

An appropriate concentration ratio of hexafluoroisopropanol/trifluoroacetic anhydride was 5:1.

Hexafluoroisopropyl ester has been considered to be superior with regard to simplicity of preparation, volatility and absence of artifacts. However, in most reports except for bile acids [20, 21], high reaction temperatures (50—75°C) have been used. It should be noted that a higher temperature does not give reproducible results, probably because of loss of reagents during derivatization. Esterification of valproic acid and octanoic acid with the reagent mixture proceeded readily at room temperature and was completed in 10 min. The derivatives were stable for at least one day at room temperature.

A mixture of 2,2,3,3,3-pentafluoro-1-propanol and pentafluoropropionic anhydride (1:4, v/v) was used in the gas chromatographic determination of indomethacin [23]. Recently, we found that a mixture of 2,2,2-trifluoroethanol and trifluoroacetic anhydride (5:1, v/v) also reacted readily with valproic acid to produce the corresponding ester, and no by-products were formed during derivatization. Compared with hexafluoroisopropanol, 2,2,2-trifluoroethanol is inexpensive, thus much more economical.

We have evaluated a number of solvents — hexane, ethyl acetate, benzene, chloroform and their mixtures — for the extraction of the drug. It was found that hexane was the most satisfactory solvent because the best yield (about 70%) was obtained by a single extraction without salting-out.

TABLE I

COMPARISON OF THE PROPOSED PROCEDURE (METHOD A) WITH THE PROCEDURE IN THE UNDERIVATIZED FORM (METHOD B) FOR THE DETERMINATION OF VALPROIC ACID

Serum samples	No.	Valproic acid added (μg/ml)	Valproic acid found (μg/ml)	
			Method A	Method B
Drug-free sera	1	12	13.5	12.5
	2	24	28.8	26.4
	3	48	48.9	47.2
	4	54	54.5	52.8
	5	105	110.7	108.4
	6	135	135.2	136.4
	7	144	144.0	147.5
	8	150	151.5	163.6
	9	162	159.0	168.5
	10	180	171.6	181.5
Patients' sera	1		32.9	30.7
	2		33.3	33.3
	3		33.9	33.8
	4		39.0	33.9
	5		46.6	41.8
	6		46.9	45.5
	7		58.3	51.1
	8		62.6	60.0
	9		68.9	64.7
	10		69.2	68.2

The next step, evaporation of the extract to dryness, was effective with regard to concentrating the drug. In order to determine small amounts of valproate, the extract was evaporated to dryness under reduced pressure at room temperature by means of a rotatory evaporator, and the residue was derivatized. Valproic acid is volatile and we must be careful about loss by evaporation. However, use of octanoic acid, which has similar properties to valproic acid, as an internal standard can avoid the problem derived from loss by evaporation.

Linear calibration curves passing through the origin were obtained for plots of valproic acid/internal standard peak height ratio versus concentration in the range 0.5—10 μg per 0.1 ml of serum. The correlation coefficient was 0.9996 using the method of least squares. The minimal concentration detectable for valproic acid was 2 μg/ml of serum. The reproducibility of the method at the level 5 μg/ml of serum was evaluated by replicate analyses of an identical sample. The coefficient of variation was less than 3%.

Human drug-free serum samples spiked with valproic acid (range of concentrations: 12—180 μg/ml) were measured by the proposed procedure (Method A), and the values measured are compared with those obtained with a procedure without derivatization (Method B) in Table I. The comparison shows no significant difference in accuracy between results obtained by the two methods. The coefficient of correlation is 0.9986.

This method was applied to measure routine serum levels of valproic acid in some epileptic patients who were receiving sodium valproate. The results

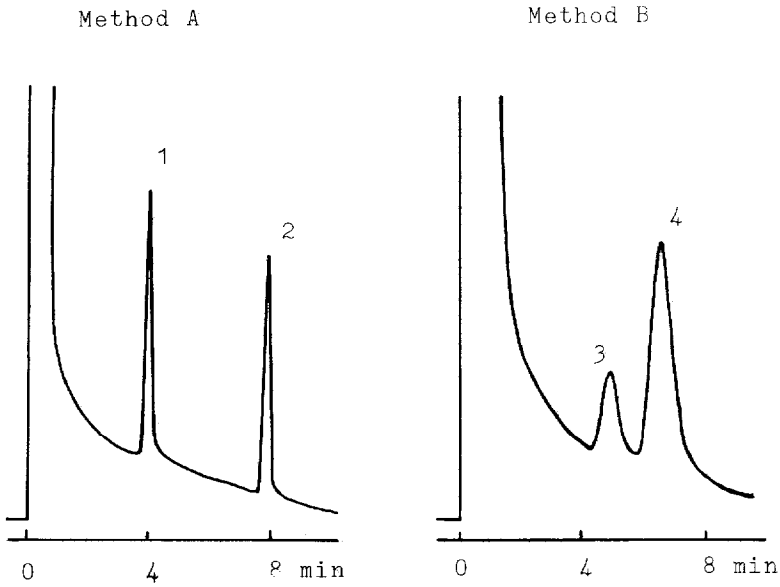

Fig. 1. Chromatograms from analyses for sodium valproate in a patient's serum by the proposed procedure (Method A) and the procedure in the underivatized form (Method B). Peaks: 1 = hexafluoroisopropyl valproate, 2 = hexafluoroisopropyl octanoate (internal standard), 3 = free valproic acid, 4 = biphenyl (internal standard). Peaks 1 and 3 correspond to about 33 μg of valproic acid in 1.0 ml of serum. Conditions: Method A, 3% OV-1, column 2 m long, 80°C, 0.1 ml of serum; Method B, 10% SP-1000, column 1 m long, 160°C, 0.5 ml of serum.

are also compared with those obtained with Method B in Table I and they almost agreed.

Fig. 1 shows chromatograms of a patient's serum by Method A and Method B. Compared with the procedure in the underivatized form, the proposed method was a little more time-consuming for routine use, but produced sharp, well-shaped peaks, improved the chromatographic properties of valproic acid and resulted in a reduction in the volume of serum samples required for analysis. The determination of valproic acid in serum at concentrations down to 0.2 μg per 0.1 ml was achieved. The method is therefore also applicable if the available sample volume is severely limited and can thus be used for determination of the drug in children, to whom it is frequently given.

A great improvement in sensitivity with electron-capture detection was expected for the derivatization. But, the sensitivity of the derivative toward electron-capture detection was found not to be as much as expected compared with that using the hydrogen flame ionization detector.

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