Journal of Chromatography, 342 (1985) 89—96
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2597

IMPROVED DIRECT INJECTION METHOD AND EXTRACTIVE METHYLATION METHOD FOR DETERMINATION OF VALPROIC ACID IN SERUM BY GAS CHROMATOGRAPHY

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(First received October 26th, 1984; revised manuscript received February 20th, 1985)

SUMMARY

Two simple methods for the determination of valproic acid in serum by gas chromatography are described. One is a direct injection method. To the serum sample are added an acetonitrile solution including an internal standard and hydrochloric acid. The sample is deproteinized and after centrifugation valproic acid in the supernatant is measured in the free form by direct injection into the gas chromatograph. The other is an extractive methylation method. To the serum sample are added an internal standard solution and a counter-ion solution, and the mixture is extracted into methylene chloride containing methyl iodide. Valproic acid is extracted into methylene chloride and simultaneously the methylation reaction proceeds. After centrifugation, an aliquot of the lower layer without evaporation is injected into the gas chromatograph. Both methods have been applied successfully to monitoring routine serum levels of valproic acid.

INTRODUCTION

The determination of valproic acid (VPA) in serum by gas chromatography (GC) has been studied in epilepsy therapy for the efficient control of seizures.

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Most of the methods previously reported have required a pre-treatment step to separate the drug from serum or plasma, mainly extraction of the acidified serum sample with an organic solvent having been utilized [1—16], sometimes followed by derivatization of the drug by methods such as methylation [17, 18], butylation [19], trimethylsilylation [20], phenacylation [21—23] and hexafluoroisopropylation [24]. Most of these procedures carried out prior to GC separation are very complicated and time-consuming, and a rapid, sensitive and simple procedure is still required for the routine determination of VPA.

From this point of view, Jakobs et al. [25] reported a direct micro-method for the determination of VPA, in which the drug was assayed by direct injection of a serum sample acidified with hydrochloric acid into a gas chromatograph. We have examined thoroughly the deproteinization step and established an improved procedure using acetonitrile as a deproteinizing agent.

Among the derivatization methods used in the determination of VPA, extractive methylation as described by Gyllenhaal and Albinsson [26] is a very simple and convenient method, coupling extraction and derivatization. We investigated this procedure in detail with respect to the reaction conditions, especially the nature of the counter ion, and established an improved procedure involving direct injection of the resulting extract without evaporation.

In this paper, we describe the two methods mentioned above, a direct injection method and an extractive methylation method. Both methods are very simple, rapid and sensitive, and only a small volume of serum sample is required for analysis. These two methods were successfully applied to the measurement of routine serum levels of VPA in some epileptic patients who were receiving sodium valproate, and the data obtained from each method were compared.

EXPERIMENTAL

Reagents

Acetonitrile and hydrochloric acid were obtained from Wako (Osaka, Japan). Methylene chloride was obtained from Wako and methyl iodide from Tokyo Kasei (Tokyo, Japan). Tetramethylammonium chloride, tetrapropylammonium chloride, tetrabutylammonium chloride (TBA-Cl), cetyltrimethylammonium chloride and methyltriphenylphosphonium bromide were purchased from Tokyo Kasei, tetraethylammonium chloride and tetrapentylammonium chloride from Wako, tetrahexylammonium chloride from Kanto Chemical (Tokyo, Japan) and tetrabutylphosphonium bromide from Aldrich (Milwaukee, WI, U.S.A.). Octanoic acid was obtained from Tokyo Kasei; in the direct injection method it was dissolved in acetonitrile (50 μg/ml) and in the extractive methylation method in 50 mM phosphate buffer (pH 8) (50 μg/ml).

Valproic acid standard

Sodium valproate was obtained from Kyowa Hakko (Tokyo, Japan) and added to drug-free serum to give the required concentration.

Gas chromatography

A Shimadzu GC-4CPF gas chromatograph equipped with a hydrogen flame-ionization detector was used. In the direct injection method a glass column

(1.5 m × 3 mm I.D.) packed with 5% SP-1000 on 80–100-mesh Chromosorb W AW DMCS (Gasukuro Kogyo, Tokyo, Japan) was used with a column temperature of 160°C, a detector temperature of 180°C and an injection temperature of 200°C, and in the extractive methylation method a glass column (2.0 m × 3 mm I.D.) packed with 3% OV-1 on 80–100-mesh Chromosorb W AW DMCS (Gasukuro Kogyo) was used with a column temperature of 90°C, a detector temperature of 120°C and an injection temperature of 150°C. In both methods the carrier gas (nitrogen), hydrogen and air flow-rates were 40 ml/min, 30 ml/min and 1.5 l/min, respectively.

Procedure for direct injection method

To 0.1 ml of serum sample in a 1.5-ml stoppered centrifuge tube were added 0.1 ml of acetonitrile containing octanoic acid as an internal standard (50 μg/ml) and 0.03 ml of 6 *M* hydrochloric acid. The tube was shaken vigorously for 1 min and centrifuged at 7000 *g* for 5 min. An aliquot of the supernatant was injected directly into the gas chromatograph.

Procedure for extractive methylation method

To 0.1 ml of serum sample in a 1.5-ml stoppered centrifuge tube were added 0.1 ml of 0.18 *M* tetrabutylammonium chloride solution in 50 m*M* phosphate buffer (pH 8.0), 0.1 ml of 50 m*M* phosphate buffer (pH 8.0) containing 50 μg/ml octanoic acid as an internal standard and 0.5 ml of 1.0 *M* methyl iodide solution in methylene chloride. The tube was shaken vigorously for 30 min and centrifuged at 7000 *g* for 5 min. An aliquot of the lower layer without evaporation was injected into the gas chromatograph.

RESULTS AND DISCUSSION

Procedures for the extraction and derivatization of VPA prior to chromatographic analysis are troublesome and have variable yields. In the method reported by Jakobs et al. [25], the drug was neither extracted nor derivatized; serum samples were merely acidified with hydrochloric acid without even deproteinization. This is a very simple and rapid procedure, but when it was attempted to apply it for routine clinical purposes, it was found to be difficult to draw the resulting serum sample into a micro-syringe because of its high viscosity. Therefore, acetonitrile was added to the serum sample as a deproteinizing agent followed by centrifugation and a small volume of 6 *M* hydrochloric acid was added in order to suppress the dissociation of VPA. The use of acetonitrile lowered the viscosity of the sample solution, facilitated the suction of the sample and increased the sensitivity considerably. The effect of the addition of acetonitrile was examined as follows: (A) 0.1 ml of serum containing VPA (100 μg/ml) was treated as described under *Procedure for direct injection method*, omitting the use of an internal standard; (B) 0.1 ml of distilled water was added instead of acetonitrile. Mean peak heights of VPA (n = 3) were 11.9 cm for A and 6.6 cm for B. It seems that the addition of acetonitrile to the serum sample is not only useful as a deproteinizing agent, but also promotes the isolation of VPA in the free form from protein.

Next, the influence of the amount of acetonitrile added to the serum sample

was examined as described under *Procedure for direct injection method* with different amounts of acetonitrile added and without an internal standard. Too small amount of acetonitrile was ineffective for deproteinization but the use of a large amount decreased the sensitivity because of dilution of the sample. As a result, a suitable ratio of the amount of acetonitrile to that of the serum sample, which gave the highest peak of VPA, was 1:1. We also investigated the influence of the amount of 6 *M* hydrochloric acid added on the peak height of VPA; there was no difference with amounts ranging from 0.01 to 0.05 ml, although the peak height decreased slightly because of dilution of the solution as the amount of 6 *M* hydrochloric acid added increased.

Complete deproteinization was not always necessary in this method, because a small amount of protein remaining in the supernatant did not interfere in the GC analysis. The injection of a sample solution that contains a high-molecular-weight substance into a gas chromatograph causes deterioration of the column, but in this instance the column lasted for at least 100 analyses and, as Jakobs et al. [25] suggested, only the first 10 cm of the column had to be renewed. Linear calibration graphs passing through the origin were obtained in the range 4—100 μg/ml VPA in serum, the correlation coefficient being 0.998. The reproducibility of the method at a level of 50 μg/ml in serum was evaluated by five replicate analyses of an identical sample, and the coefficient of variation was 4.7%. The recovery of VPA (30 μg/ml) added to a patient's serum was 98.0% ($n = 3$).

Derivatization of VPA has mainly been achieved after extraction from an acidified serum sample with an organic solvent, except in one report [26], in which VPA was assayed using extractive methylation. Extractive methylation is a very convenient technique for the derivatization of acidic compounds, in which the extraction is performed in an alkaline solution and the derivatization reaction proceeds simultaneously. This technique has been applied to the GC determination of several carboxylic acids [27—29]. In the extractive alkylation method, the extract containing the derivative was generally evaporated before injection into the gas chromatograph in order to stop the reaction and prevent further alkylation from occurring in the injector [29]. However, with VPA, the evaporation might cause a loss of sample because of the high volatility of methyl valproate formed and also owing to the complicated procedure.

In order to evaluate the influence of evaporation of the extract in the extractive methylation of VPA, the two procedures, with and without evaporation, were examined and the coefficients of variation from five replicate analyses of an identical sample solution (50 μg/ml) were compared. Procedure A (without evaporation) was carried out as described under *Procedure for extractive methylation method*. In procedure B (with evaporation), the extract was transferred into a tapered tube and evaporated under reduced pressure just to dryness. The residue was dissolved in 0.1 ml of methylene chloride and injected into the gas chromatograph. The coefficients of variation of the measured values ($n = 5$) were 4.4% for A and 7.8% for B. From these results, it seems to be unnecessary to consider the further alkylation in the injector; on the contrary, variation of the measured values resulting from the evaporation of the extract seems to be a serious problem. The procedure without evaporation of the extract is much simpler, only one tube being required through overall

procedure. For these reasons, the present method was adopted, although the evaporation of the extract resulted in about a five-fold increase in the sensitivity.

The detection limit of VPA by the proposed procedure was 3 μg/ml in serum. Linear calibration graphs passing through the origin were obtained in the range 3—100 μg/ml VPA in serum and the correlation coefficient was 0.997. The recovery of added VPA (30 μg/ml) was 98.5% (n = 3) using patient's serum.

Nine kinds of counter ion for the extractive methylation of VPA were evaluated. Chlorides of quaternary ammonium cations were used. As shown in Table I, the best result was obtained with tetrabutylammonium chloride.

TABLE I

EFFECT OF COUNTER IONS ON EXTRACTIVE METHYLATION

A 10-μg amount of VPA per 0.1 ml was treated as described under *Procedure for extractive methylation method*, using each counter ion and omitting the internal standard solution. All counter ions: 0.18 M in 50 mM phosphate buffer (pH 8.0).

Counter ion	Peak height of methyl valproate*
Tetramethylammonium chloride	0
Tetraethylammonium chloride	0
Tetrapropylammonium chloride	0.12
Tetrabutylammonium chloride	1.00
Tetrapentylammonium chloride	0.92
Tetrahexylammonium chloride	0.86
Cetyltrimethylammonium chloride	0.56
Tetrabutylphosphonium bromide	0.78
Methyltriphenylphosphonium bromide	0.87

*Mean values (n = 3) relative to that obtained using tetrabutylammonium chloride.

In the extractive alkylation process, extraction and alkylation should be discussed separately. There is no doubt that the larger the alkyl chain of the counter ion, the more the extraction coefficient increases, because the lipophilic character of the counter ion is greater. However, with regard to the alkylation, when the length of alkyl chain of counter ion is longer than C_4 (butyl), it seems probable that steric hindrance by the bulky alkyl group interferes with the alkylation reaction. This seems to be the reason why the reaction yield showed little tendency to decrease as the alkyl chain of the counter ion increased in length beyond C_4. We decided to investigate the use of quaternary phosphonium ion, which is a new type of counter ion. However, the reaction yields were not as high as expected (Table I).

We also studied the influence of methyl iodide and counter ion concentrations and the extraction time on the yield in the extractive methylation of VPA. The concentrations of methyl iodide and tetrabutylammonium chloride sufficient to obtain a constant reaction yield were 0.8 M in methylene chloride and 0.1 M in phosphate buffer (pH 8.0), respectively. Therefore, 1.0 M methyl iodide and 0.18 M tetrabutylammonium chloride were used. With regard to

extraction time, the methylation yield became constant after 20 min. It was decided to shake the reation solution for 30 min to obtain reproducible results. All these results were in good agreement with those reported by Gyllenhaal and Albinsson [26].

The two improved methods for the measurement of VPA in serum were applied to monitoring routinely serum levels of VPA in 31 epileptic patients who were receiving sodium valproate. Fig. 1 shows chromatograms obtained from a patient's serum by each method; the chromatograms obtained from blank serum did not show any interfering peaks in either method. Fig. 2 shows the relation between the measured values obtained by the two methods. The correlation coefficient was 0.970 and the regression curve obtained by the least-squares method was $y = 1.03x + 0.17$. With a few exceptions, there was a good correlation between the two methods. The reason why several results deviated from the regression curve is not clear. However, when a frozen serum sample containing VPA is thawed for determination, thorough mixing is very important. If not, the concentration of VPA in the lower layer was about five times greater than that in the upper layer. Generally, it is probable that there is such a variation between the data obtained by the two different methods.

For the determination of VPA, the direct injection method is superior to the

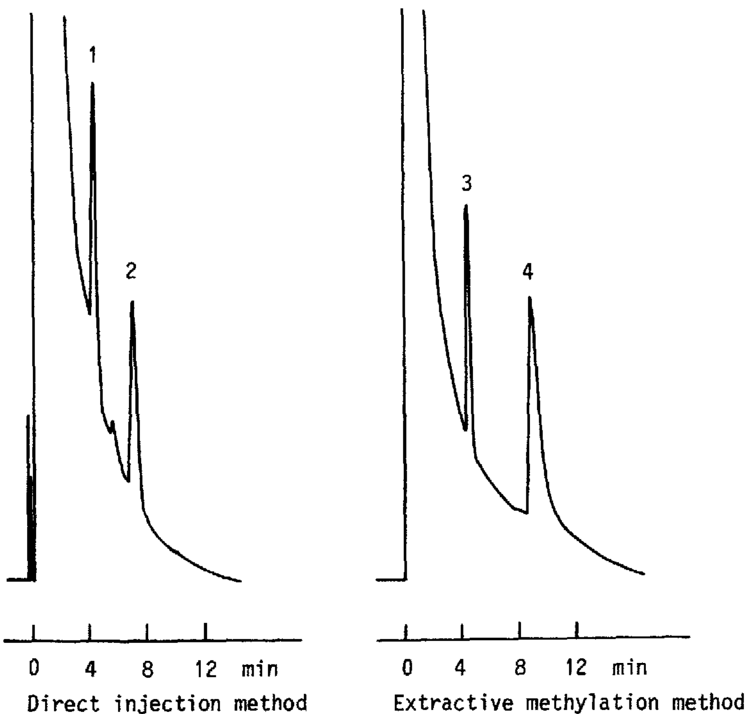

Fig. 1. Chromatograms obtained from analyses of VPA in a patient's serum using the direct injection method and the extractive methylation method. Peaks: 1 = free valproic acid; 2 = free octanoic acid (internal standard); 3 = methyl valproate; 4 = methyl octanoate (internal standard). Peaks 1 and 3 correspond to about 58 μg of VPA in 1.0 ml of serum. Conditions: direct injection method, 5% SP-1000, column 1.5 m long, 160°C, flame-ionization detector; extractive methylation method, 3% OV-1, column 2 m long, 90°C, flame-ionization detector.

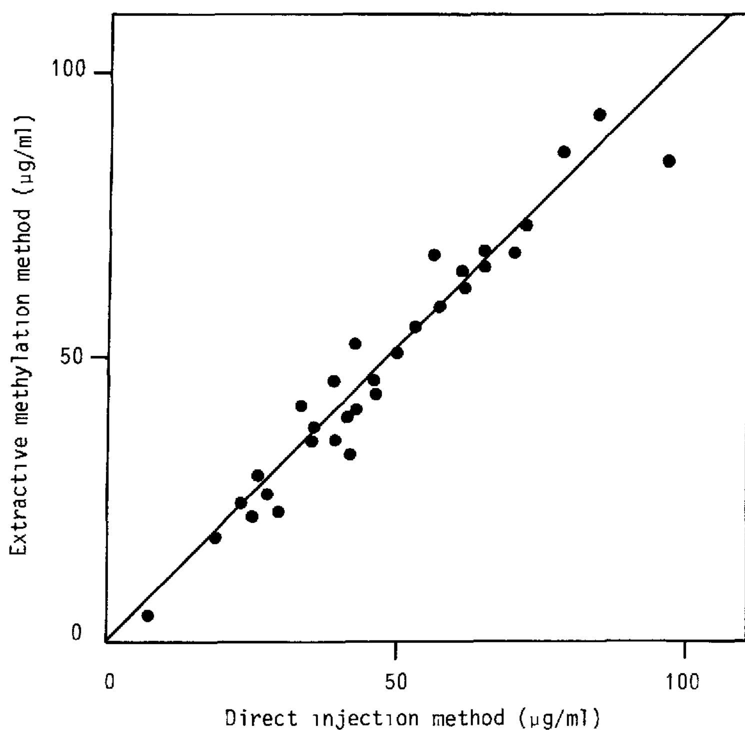

Fig. 2. Relationship between the direct injection method and the extractive methylation method: VPA in sera from 31 patients was measured by each method. Regression curve: $y = 1.03x + 0.17$; correlation coefficient: $r = 0.970$.

extractive methylation method from the viewpoint of simplicity, and therefore it is of more practical use for routine analyses. It is also possible to measure VPA in less than 50 µl of serum using a 0.2-ml micro-centrifuge tube. On the other hand, the extractive alkylation method is applicable to the alkyl derivatization of many kinds of acidic compounds and is valuable in a wide variety of applications. For this reason, the conditions for extractive methylation were examined in detail using VPA.

REFERENCES

1 L.J. Dusci and L.P. Hackett, J. Chromatogr., 132 (1977) 145.
2 A.J. Fellebberg and A.C. Pollard, Clin. Chim. Acta, 81 (1977) 203.
3 D.J. Berry and L.A. Clarke, J. Chromatogr., 156 (1978) 301.
4 J.-C. Libeer, S. Scharpé, P. Schepens and R. Verkerk, J. Chromatogr., 160 (1978) 285.
5 R.H. Levy, L. Martis and A.A. Lai, Anal. Lett., B11 (1978) 257.
6 N. Grgurinovich and J O. Miners, J. Chromatogr., 182 (1980) 237.
7 A. Sioufi, D. Colussi and F. Marfil, J. Chromatogr., 182 (1980) 241.
8 D.J. Freeman and N. Rawal, Clin. Chem., 26 (1980) 674.
9 W.R. Külpmann, Fresenius Z. Anal. Chem., 301 (1980) 108.
10 M.I.A. Peña, J. Chromatogr., 225 (1981) 459.
11 R.O. Fullinfaw and J.J Marty, Clin. Chem., 27 (1981) 1776.
12 C. Manfredi and L. Zinterhofer, Clin. Chem., 28 (1982) 246.

13 A. Wu, M.L. Pearson, S.K. Mertens, D.D. Bretl and G.S. Wolffe, Clin. Chem., 28 (1982) 544.
14 R. Riva, F. Albani and A. Baruzzi, J. Pharm. Sci., 71 (1982) 110.
15 M.H. Wood, D.C. Sampson and W.J. Hensley, Clin. Chim. Acta, 77 (1977) 343.
16 A. Sengupta and M.A. Peat, J. Chromatogr., 137 (1977) 206.
17 S.L. Braun, A. Tausch, W. Vogt, K. Jacob and M. Knedel, Clin. Chem., 27 (1981) 169.
18 S. Willox and S.E. Foote, J. Chromatogr., 151 (1978) 67.
19 A. Hulshoff and H. Roseboom, Clin. Chim. Acta, 93 (1979) 9.
20 H. Nau, W. Wittfoht, H Schäfer, C. Jakobs, D. Rating and H. Helge, J. Chromatogr., 226 (1981) 69.
21 R.N. Gupta, F. Eng and M.L. Gupta, Clin. Chem., 25 (1979) 1303.
22 S.C. Chan, Clin. Chem., 26 (1980) 1528.
23 A.B. Rege, J.J.L. Lertora, L.E. White and W.J. George, J. Chromatogr., 309 (1984) 397.
24 R. Nishioka, S. Kawai and S. Toyoda, J. Chromatogr., 277 (1983) 356.
25 C. Jakobs, M. Bojasch and F. Hanefeld, J. Chromatogr., 146 (1978) 494.
26 O. Gyllenhaal and A. Albinsson, J. Chromatogr., 161 (1978) 343.
27 Y. L'Emeillat, J.F. Ménez, F. Berthou and L. Bardou, J. Chromatogr., 206 (1981) 89.
28 J. Shen, Anal. Chem., 53 (1981) 475.
29 O. Gyllenhaal, H. Brötell and P. Hartvig, J. Chromatogr., 129 (1976) 295.