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Ion trap detector — capillary gas chromatography of valproic acid and its mono-unsaturated metabolites in serum using methyl ester derivatives

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Abstract: A quantitative method was developed for valproic acid and five of its mono-unsaturated metabolites using capillary gas chromatography–mass spectrometry with selected ion monitoring. The method was applied to serum and all metabolites were measured in a single run. Methyl esters were synthesized as the derivatives suitable for gas chromatography. Calibration curves were found to be linear and the sensitivities in the order of 0.1 μg ml^{-1}. Patients' data are presented. By this method it is possible to separate the stereoisomers of 2-*n*-propyl-2-pentenoic acid and of 2-*n*-propyl-3-pentenoic acid.

Keywords: *Valproic acid; GC–MS; methyl ester derivatives; mono-unsaturated metabolites.*

Introduction

Idiosyncratic hepatotoxicity, although rare, is an ever present risk for patients under treatment with sodium valproate (sodium 2-*n*-propylpentanoate, VPA). Recent examination of up to date data has shown that youth, polytherapy and associated neurologic conditions [1] and poor nutrition and inanition all give rise to the risk of this catastrophic effect [2].

Some authors, working on animals, have proposed 2-*n*-propyl-4-pentenoic acid (4-ene-VPA) as the metabolite responsible for the toxicity because of its structural likeness to hypoglycin and 4-pentenoic acid, which give an hepatotoxicity similar to that produced by VPA [3].

It is therefore important to develop sensitive and selective methods for the quantitative analysis of VPA. To date, gas chromatographic (GC) and, more frequently, gas chromatographic–mass spectrometric (GC–MS) techniques have been used. In 1981, Loecher [4] reported a quantitative GC method using a differential extraction for the analysis of VPA, 2-propyl-3-oxopentanoic acid (3-keto-VPA), 2-propyl-4-hydroxypentanoic acid (4-OH-VPA), 2-propyl-2-pentenoic acid (2-ene-VPA), 2-propyl-3-hydroxypentanoic acid (3-OH-VPA) and 2-propyl-5-hydroxypentanoic acid (5-OH-VPA) in the plasma of epileptic patients. Prickett and Baillie [5] have reported the GC analysis of trimethylsilyl (TMS) derivatives of 3-, 4- and 5-OH-VPA isolated from rat liver microsomes. A capillary GC procedure of methyl ester derivatives separated VPA and at least 11 metabolites over a run time of approximately 40 min [6]. More selective GC–MS analysis has included the analysis of TMS derivatives of mono- and di-unsaturated VPA metabolites [7]. More recently, VPA dienes were measured in patient urine and plasma samples [8]. The most complete GC–MS assay was reported by Nau *et al.* [9] who achieved a simultaneous analysis of VPA and eight metabolites by selected-ion monitoring (SIM) of TMS derivatives. A chemical-ionization (CI) GC–MS assay of ethyl ester derivatives of VPA metabolites has also been reported [10]. Some authors have used *t*-butyldimethylsilyl (*t*BDMS) derivatives for GC–MS analysis of VPA metabolites because of better sensitivity owing to the intense $(M-57)^+$ fragments formed in contrast to the less intense $(M-15)^+$ fragments from TMS derivatives [11–17]. GC–MS methods using TMS or *t*BDMS derivatives, give poor chromatographic separation between *Z*(3-ene-VPA) and *E*(3-ene-VPA).

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In this work the authors describe a GC–MS method using methyl ester derivatives which give good separations of VPA and its mono-unsaturated metabolites and have interpretable mass spectra.

Experimental

VPA and its metabolites

Valproic acid (2-*n*-propylpentanoic acid) was obtained from Aldrich (Steinheim, Germany).

4-Ene-VAP (2-n-propyl-4-pentenoic acid). A portion of diethyl malonate (80 g) was added drop wise over 60–90 min to a solution of sodium metal (11.5 g) in ethanol at 50°C. After a period of 20 min, allyl bromide (60.5 g) was added and the reaction mixture was heated under gentle reflux for 2 h. Ethanol was removed under reduced pressure, water was added and the mixture was extracted twice with diethyl ether. The organic extracts were combined, washed with Na_2CO_3 solution and dried over Na_2SO_4. By evaporation of the solvent, the crude product as a yellow oil was obtained. Distillation of this material (b.p. 96–98°C at 12 mm Hg) afforded ethyl 2-carboxyethyl-4-pentenoate.

To a portion of this ester (48 g), *n*-propylbromide (29.6 g) was added as previously described for allyl bromide. Distillation of the yellow oil obtained (b.p. 110–112°C at 8 mmHg) yielded ethyl 2-carboxyethyl-2-*n*-propyl-4-pentenoate. A portion of this ester (32 g) was hydrolysed with aqueous alcoholic KOH solution under reflux conditions for 3 h. Ethanol was removed under reduced pressure and the residue, dissolved in water, was cooled in an ice bath and acidified to Congo red with diluted H_2SO_4. The product was extracted three times with diethyl ether and dried over Na_2SO_4. Evaporation of the solvent gave the crude product as a yellow oil. Distillation of this material (b.p. 91–93°C at 6 mmHg) afforded 2-*n*-propyl-4-pentenoic acid. IR and NMR spectroscopic data were in accordance with data reported by Rettenmeier *et al.* [18].

2-Ene-VPA (2-n-propyl-2-pentenoic acid). Thionyl chloride (5 ml) was added to valproic acid (3.5 g) and the mixture was stirred and gently heated for 2 h. The excess thionyl chloride was distilled under reduced pressure. Dry bromine (3 ml) was added drop wise (stirring and heating) to the obtained acid chloride. The mixture was stirred and heated for 6 h, then slowly poured into absolute ethyl alcohol, allowed to stand at room temperature overnight and then poured into water. The oily bottom layer was extracted with diethyl ether, then washed first with 2% $Na_2S_2O_5$ solution, and finally with several portions of water. The ether solution was dried over K_2CO_3 and the solvent was removed under reduced pressure. The reaction product, ethyl-2-Br-valproate, was refluxed with methanolic KOH for 2 h, allowed to stand at room temperature overnight then acidified with concentrated HCl; the 2-ene-VPA was then extracted with diethyl ether. The organic layer was separated, washed with water, dried over Na_2SO_4 and the ether evaporated under reduced pressure.

The NMR spectra of the product show two triplets at 5.9 and 6.9 $\delta(J = 7.5$ Hz) in the ratio 4:1 which, in accordance with Rettenmeier *et al.* [19], are attributable to —CH=C—, *Z* isomer and to —CH=C—, *E* isomer, respectively. A portion of the product, dissolved in ether, treated with diazomethane ethereal solution, and injected into the GC–MS instrument, showed the presence of unreacted VPA together with (*Z*)-2-ene-VPA and (*E*)-2-ene-VPA in the ratio 100:25:6.

Diazomethane reagent was obtained by means of the apparatus described by Fales *et al.* [20].

Internal standard

The internal standard, 2-ethyl esanoic acid of analytical grade, was obtained from Carlo Erba (Milan, Italy).

Reagents

Acetonitrile (CH_3CN), sodium, diethyl malonate, *n*-propyl bromide, allyl bromide, thionyl chloride, all of reagent grade, were obtained from Carlo Erba (Milan, Italy); 1-methyl-3-nitro-1-nitroso guanidine, of reagent grade, was obtained from Aldrich (Steinheim, Germany).

Instrumentation

DANI (Milan, Italy) 6500 Gas Chromatograph equipped with a silica capillary column SPB 20 (30 m × 0.25 mm i.d.) (Supelco, Springfield, NJ, USA): injector temperature, 250°C; carrier gas, 0.8 bar; split–splitless injection, 12 s; column temperature, 2 min at 50°C, then gradient 5°C min^{-1} to 150°C, then 2

min at 150°C, then gradient 10°C min^{-1} to 170°C, then 25 min at 170°C, then reset. Injection volume: 1 μl in all cases.

Mass spectrometer Finnigan Mat (San Jose, CA, USA) 700 ITD working at: acquisition mode full scan; scan range, 50–190 amu; scan time, 1.00 s; A.G.C. mode ON; multiplier, 1400 V; open split temperature, 120°C; transfer line temperature, 120°C; exit nozzle temperature, 150°C; manifold temperature, 220°C. Diazomethane generator (Pierce, Rockford, IL, USA).

Stock solutions

The stock solution concentrations are: VPA, 350 μg ml^{-1}; 2-ethyl esanoic acid (IS), 260 μg ml^{-1}; 4-ene-VPA, 240 μg ml^{-1}.

Preparation of reference samples

To four serum samples of healthy donor were added quantities of stock solution to obtain concentrations of IS (26 μg ml^{-1}) and concentrations from 0.2 to 2 μg ml^{-1} of 4-ene-VPA and from 3.5 to 35 μg ml^{-1} of VPA.

Calibration curves were obtained by plotting the ratio of areas of peaks of metabolite or of VPA to the area of IS peak versus the concentration of metabolite or VPA.

Extraction and derivatization of standards and patients' samples

Half a millilitre of serum was mixed with 40 μl of internal standard solutions, acidified with 0.5 ml of 0.4 M HCl and vortexed. The mixture was then extracted three times with 2 ml of ethyl ether by vortexing for 1 min, and the organic layer centrifuged for 5 min at 1000 rpm at room temperature. The supernatant (approximately 5.6 ml) was passed through a column filled with anhydrous Na_2SO_4 (1 × 2 cm) and the solvent removed under a stream of nitrogen in a conical reaction vial.

The methyl ester derivatives were obtained by addition of 0.4 ml of diazomethane reagent in anhydrous ethyl ether.

Results and Discussion

The extraction of VPA and its mono-unsaturated metabolites with ethyl ether gave recoveries of over 90% without formation of intractable emulsions.

Methyl ester derivatives used for GC–MS analysis proved useful for the identification and quantitation of investigated metabolites in plasma, even at low concentrations.

Derivatization by diazomethane is more selective than silylation and gave simpler chromatograms.

Mass spectra could be interpreted due to the presence of molecular peaks.

By using these methyl ester derivatives and comparing their retention times with those obtained by halogenation and dehydrohalogenation of VPA, we were able to correlate α–β unsaturated isomers with peaks at 12.42 and 14.08 min.

Comparing the ratio between the areas of these peaks and the results from NMR spectra (see Experimental), it was possible to assign the *Z* configuration to the component eluting at 12.42, and the *E* configuration to that eluting at 14.08 min.

By the examination of mass spectra one can observe that both *Z* and *E* isomers show high intensity fragments at *m/z* 95 and 127, and that in the *Z* isomer, the ratio between fragments *m/z* 95 and 127 is 1:1, whilst in the *E* isomer it is 0.6:1. On the other hand, chromatographic peaks at 12.22 and at 12.48 min show a *m/z* 127 fragment of low intensity and have instead 113 as the base fragment. These peaks should be assigned to the *Z* and *E* isomers of β–γ unsaturated VPA derivatives.

As the mass spectra of the *Z* and *E* isomers of the β–γ unsaturated VPA derivatives are identical, it was not possible to decide to which peak each geometrical isomer corresponded (Table 1).

The detection and quantitation of 4-ene-VPA was possible only by using SIM chromatography but not by total ion chromatography, although in our chromatographic conditions the peak of 4-ene-VPA was completely separated from the VPA peak (Fig. 1). These peaks are partially or completely overlapped in literature methods. For that reason we do not think that quantitation of this important toxic metabolite is possible simply by GC using a specific detector.

Figure 1 shows that using the fragment *m/z* 113, one can render evident the 4-ene and 3-ene isomers, whilst using the fragment *m/z* 127, one can render evident the 2-ene isomers.

Calibration curves of VPA and 4-ene-VPA were obtained by adding known quantities of pure compounds to plasma, as described in the Experimental, and are linear within the concentration range selected, with correlation

Table 1
Chromatographic retention times and 10 of the major ions (m/z) in the mass spectra of the methyl derivatives of valproic acid metabolites. For abbreviations and chemical names refer to the text

Compound	Time (min)	Ions									
4-Ene-VPA	11.35	55 (100)	59 (15)	67 (16)	81 (40)	95 (10)	97 (25)	113 (72)	114 (55)	127 (24)	157 (16)
VPA	11.57	55 (32)	57 (31)	83 (6)	87 (100)	88 (11)	97 (5)	115 (12)	116 (42)	129 (8)	159 (28)
3-Ene-VPA	12.22	55 (100)	56 (20)	59 (18)	67 (13)	81 (15)	97 (25)	113 (53)	114 (22)	127 (25)	156 (9)
(*Z*)-2-Ene-VPA	12.42	55 (70)	57 (100)	67 (68)	71 (46)	85 (25)	95 (71)	125 (22)	127 (68)	156 (47)	157 (33)
3-Ene-VPA	12.48	55 (100)	56 (20)	59 (11)	67 (18)	81 (11)	97 (26)	113 (48)	114 (20)	127 (31)	156 (11)
(*E*)-2-Ene-VPA	14.08	55 (88)	59 (18)	67 (80)	81 (27)	95 (60)	113 (13)	125 (30)	127 (100)	156 (60)	157 (16)
IS	11.83	55 (40)	57 (46)	87 (100)	88 (16)	101 (31)	102 (93)	115 (15)	129 (16)	130 (14)	159 (27)

Table 2
Serum VPA and mono-unsaturated metabolite concentrations for 12 paediatric patients on VPA monotherapy

Compound	Concentration ($\mu g\ ml^{-1}$) Mean	Range	Percentage of VPA
4-Ene-VPA	0.34	±0.11	0.43
VPA	78.33	±27.71	100
3-Ene-VPA	1.13	±0.37	1.44
(*Z*)-2-Ene-VPA	0.59	±0.19	0.75
(*E*)-2-Ene-VPA	4.82	±3.06	6.15

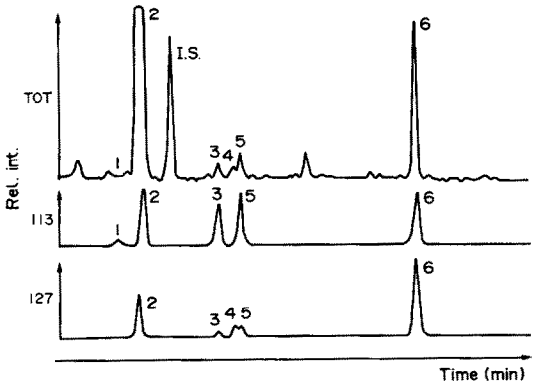

Figure 1
Total ion monitoring and SIM chromatograms of methyl ester derivatives of VPA and its metabolites from a sample of patient's serum. For peak numbers refer to Table 1.

coefficient (r^2) better than 0.98 and with the line passing through the origin in both cases.

The 3-ene and 2-ene isomers were quantified using the same response factor calculated for 4-ene; therefore, their quantitation is not absolute but indicative of possible variation in the clinical cases examined. The average values found by us are shown in Table 2 and are in very good accordance with values reported by Abbott *et al.* [17].

Our quantitations were made on 15 children under monotherapy by VPA, four times, every 4 months, and we did not find appreciable variations in the quantity of metabolites present in plasma.

We believe that our method is a valid alternative to the methods described in literature, as it shows that, in addition to the advantages outlined earlier, there is the capability of an easy identification of mono-unsaturated metabolites, even if one does not possess the corresponding pure standard.

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