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# CAPILLARY GAS CHROMATOGRAPHY—MASS SPECTROMETRY OF VALPROIC ACID METABOLITES IN SERUM AND URINE USING *tert.*-BUTYLDIMETHYLSILYL DERIVATIVES

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## SUMMARY

A quantitative method has been developed for valproic acid and twelve of its metabolites using capillary gas chromatography—mass spectrometry with selected-ion monitoring The method is applicable to serum or urine and all metabolites are measured in a single run Ions selected for quantitative purposes were the characteristic $(M - 57)^+$ ions of the *tert* -butyldimethylsilyl (tBDMS) derivatives The 4-hydroxyvalproic acid was measured as the $\gamma$-lactone Calibration curves were found to be linear and the sensitivities in the order of 0 1 $\mu$g/ml Patient data are presented A comparison of tBDMS and trimethylsilyl (TMS) derivatives showed that tBDMS gave superior sensitivity for the unsaturated metabolites and a shorter analysis time Mixed tBDMS—TMS derivatives were also investigated

## INTRODUCTION

Valproic acid (VPA, 2-propylpentanoic acid) is a widely used anticonvulsant drug which is eliminated mainly by metabolism [1, 2] Some VPA metabolites have anticonvulsant properties [3] and/or may be involved in the rare but severe cases of VPA hepatotoxicity [4, 5] It is therefore important to develop sensitive and selective methods for the quantitative analysis of VPA metabolites. To date these methods have consisted of gas chromatography (GC) and more frequently gas chromatographic—mass spectrometric (GC—MS) techniques. In 1981 Löscher [6] 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-

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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 [7] 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 eleven metabolites over an approximately 40-min run time [8] More selective GC—MS assays have included the analysis of TMS derivatives of mono- and diunsaturated VPA metabolites [9] More recently dienes VPA were measured in patient urine and plasma samples [10] The most complete GC—MS assay was reported by Nau et al [11] 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 [12]

The use of *tert.*-butyldimethylsilyl (tBDMS) derivatives for the GC—MS analysis of VPA metabolites has received little attention although earlier [13] and more recent [14, 15] studies have shown that tBDMS derivatives of fatty acids gave better sensitivity because of the intense $(M - 57)^+$ fragments formed in contrast to the less intense $(M - 15)^+$ fragments from TMS derivatives. Based on our previous experience with tBDMS derivatives for the analysis of VPA in saliva and serum [16, 17] and the use of these derivatives for the identification of VPA metabolites [18], the tBDMS derivatives were investigated as to their suitability for the quantitation of VPA metabolites Comparisons were made to TMS derivatives and mixed tBDMS—TMS derivatives were also briefly investigated

## EXPERIMENTAL

### *VPA metabolites*

Valproic acid (di-*n*-propylacetic acid) was obtained from K & K Fine Chemicals, ICN Pharmaceutical (Plainview, NY, U S A ) The metabolites used for the standard calibration curves were synthesized as reported elsewhere [18] These metabolites were namely 2-propyl-(*E*)-2-pentenoic acid [(*E*)-2-ene-VPA], 2-propyl-(*Z*)-2-pentenoic acid [(*Z*)-2-ene-VPA], 2-propyl-3-pentenoic acid (3-ene-VPA), 2-propyl-4-pentenoic acid (4-ene-VPA), 2-propyl-3-hydroxypentanoic acid (3-OH-VPA), the γ-lactone isomers of 2-propyl-4-hydroxypentanoic acid (4-OH-VPA lactones), 2-propyl-5-hydroxypentanoic acid (5-OH-VPA) as the δ-lactone, 2-propyl-3-oxopentanoic acid (3-keto-VPA), 2-propyl-4-oxopentanoic acid (4-keto-VPA), 2-propylsuccinic acid (2-PSA) and 2-propylglutaric acid (2-PGA) The synthesis of the diunsaturated metabolite 2-[(*E*)-1′-propenyl]-(*E*)-2-pentenoic acid [(*E*,*E*)-2,3′-diene-VPA] was recently reported [19]

The diunsaturated metabolite (*E*)-2,4-diene-VPA was a kind gift from Dr T. Baillie (University of Washington, School of Pharmacy, Seattle, WA, U S A )

### *Internal standards*

The 3-octanone (99%) was obtained from Aldrich (Milwaukee, WI, U S A ) The internal standard, [$^2H_3$]2-ene-VPA ([3,5,5-$^2$H]3-heptene-4-carboxylic acid), was prepared by dehydration and hydrolysis of the cyanohydrin obtained from [3,3,5,5-$^2$H]4-heptanone [$^2H_4$]-4-Heptanone was prepared

from 4-heptanone after several exchanges with solutions of $NaO^2H$ in $^2H_2O$. Percentage deuterium incorporated was 95%. A mixture of *cis*- and *trans*-[$^2H_3$]2-ene-VPA (1.4 ratio) was obtained, b.p 88—91°C, 0.4 mmHg, $^1$H-NMR ($C^2HCl_3$) relative to tetramethylsilane 0.8—1.1 (m,6), 1.2—1.7 (m,2), 2.0—2.6 (q,2H), 10—11.8 (br s, 1, COOH).

The synthesis of the internal standard [$^2H_6$]VPA has previously been reported [17].

*Reagents*

Solvents were distilled-in-glass grade obtained from Caledone (Georgetown, Canada). Anhydrous sodium sulfate and sodium hydroxide were obtained from BDH Chemicals and hydrochloric acid from Fisher Scientific (Canada) *tert*-Butyldimethylsilyl chloride (tBDMCS), 97% purity, 4-dimethylaminopyridine (DMAP catalyst), 99% purity, pyridine, N-*tert*-butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA) were purchased from Aldrich N-Methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) was obtained from Pierce (Rockford, IL, U S.A.).

The tBDMCS reagent was prepared in pyridine to contain 5% DMAP 50 mg DMAP were dissolved in 1 ml dry pyridine and this mixture was then added to 1 g of tBDMCS.

*Instrumentation*

The assay was performed on a Hewlett-Packard 5987A gas chromatograph—mass spectrometer. Operating conditions were source temperature 200°C, transfer line temperature 240°C, injection port temperature 240°C and helium flow-rate 1 ml/min.

The mass spectrometer was operated with an electron ionization energy of 70 eV The capillary column used was an OV-1701 bonded phase, 25 m × 0.32 mm I.D , with a film thickness of 0 25 $\mu$m (Quadrex Scientific, New Haven, CT, U.S.A.) For tBDMS and TMS—tBDMS mixed derivatives the column oven initial temperature was 50°C (zero hold time) and programmed at 30°C/min to 100°C, then 8°C/min from 100°C to a final temperature of 260°C (with a 1-min hold time) Total run time was approx 22 min

For TMS derivatives, the initial oven temperature was 75°C (10-min hold time) and the rate of increase of temperature was 8°C/min from 75°C to 200°C then 30°C/min to a final temperature of 260°C (with a 5-min hold time) Total time required was approx 30 min

*Stock solutions of internal standards*

The stock solution concentration of internal standard [$^2H_3$]2-ene-VPA was 100 $\mu$g/ml either in 3 *M* sodium hydroxide for analysis of patient samples or in distilled water for analysis of reference standards Stock solution concentrations of the internal standards [$^2H_6$]VPA and 3-octanone were respectively 100 $\mu$g/ml and 1 mg/ml in distilled water

*Preparation of urine and serum standards*

Two sets of reference standards were prepared One set in 3 *M* sodium hydroxide was prepared by the addition of 3-OH-VPA, 4-OH-VPA $\gamma$-lactone,

5-OH-VPA δ-lactone and 3-keto-VPA ethyl ester to the alkaline solution and allowing these to dissolve with gentle shaking over five days

The other reference set was made in urine or serum and contained 4-ene-VPA, 3-ene-VPA, 2-ene-VPA, VPA, 4-keto-VPA, 2-PSA and 2-PGA.

For each calibration point 0.1 ml of standard in alkaline solution was added to 0 9 ml of its corresponding standard in serum or urine at the time of analysis The concentrations thus obtained for the calibration were 2.5, 10, 15, 30 and 45 μg/ml for 4-OH-VPA and 5-OH-VPA, 0 18, 0 45, 0 90, 4.5, 9 and 13 5 μg/ml for 4-ene-VPA, 3-ene-VPA, 4-keto-VPA and 2-PSA, 0 36, 0 90, 1.8, 9, 18 and 27 μg/ml for 2-ene-VPA, 18, 36, 54, 72, 108 and 144 μg/ml for VPA, 5, 10, 15, 50, 75 and 150 μg/ml for 3-keto-VPA, 0 36, 0 90, 1 80, 4.5, 15 and 18 μg/ml for 2-PGA and finally 2, 5, 10, 15, 20 and 25 μg/ml for 3-OH-VPA

The calibration curves were obtained by a plot of the area ratio of the metabolite or VPA peak to that of the internal standard versus the concentration of the metabolite [$^2H_6$]VPA was used as the internal standard for VPA while (*E*)-[$^2H_3$]2-ene-VPA was used as the internal standard for all the other metabolites. The 3-octanone was initially investigated as an internal standard for 4-OH-VPA γ-lactone but was eventually used to monitor the evaporation of the extract. A standard curve was run prior to each batch of patient samples

*Extraction and derivatization of standards and patient samples*

To 0 1 ml of standard in alkaline solution were added 0 9 ml of its corresponding standard in urine or serum and 100 μl of each of three internal standard solutions This mixture at pH 12 5–13 was incubated 1 h at 60°C to hydrolyze conjugated metabolites (mainly VPA-glucuronide). After cooling the mixture was brought to pH 2 with 110 μl of 4 *M* hydrochloric acid and left at room temperature for 15 min The mixture was then extracted with 3 ml of ethyl acetate by gentle rotation for 20 min. This procedure was repeated in the case of serum to ensure complete extraction. The organic phase was transferred and vortex-mixed with anhydrous sodium sulfate, then centrifuged (5 min, 1000 *g*, 25°C). The supernatant (approx. 2.4 ml) was transferred to a conical tube and concentrated to 200 μl under a nitrogen stream.

To obtain tBDMS derivatives 50 μl of tBDMCS reagent were added to the concentrated extract in a conical reaction vial and the mixture heated at 60°C for 4 h. TMS derivatives were obtained by addition of 50 μl of MSTFA reagent to the concentrated extract and the mixture heated at 60°C for 10– 20 min. Mixed derivatives were obtained by addition of 50 μl of MSTFA reagent to the tBDMS derivatives prepared from the reagent in pyridine and heating at 60°C for a further 10 min. The tBDMS derivatives obtained with MTBSTFA reagent were formed by adding 50 μl of the reagent to the concentrated extract and heating the mixture for 20 min at 60°C In all cases 1 μl of derivatized extract was injected into the gas chromatograph—mass spectrometer. Splitless mode was used.

For patient samples 1 ml of serum or urine was taken and 100 μl of [$^2H_3$]2-ene-VPA dissolved in 3 *M* sodium hydroxide and 100 μl of each of the other two internal standard solutions were added. Otherwise, extraction and derivatization procedures were carried out exactly as for the calibration standards In patient samples where the available volume of urine or serum was less than 1

ml, a known volume was pipetted and diluted with distilled water to a final volume of 1 ml. Corrections were made for the dilution factor in calculations

## RESULTS AND DISCUSSION

*Sample extraction*

The most efficient solvent for the recovery of VPA and its metabolites was ethyl acetate as reported by Nau et al [11] and the recovery was in the range 75–90%. Other solvents such as hexane, 10% ethyl acetate in hexane, chloroform, and dichloromethane were suitable for VPA and some of the unsaturated metabolites but recoveries were poor for the more polar metabolites.

For urine samples, 3 ml of ethyl acetate gave optimal results. In the case of serum a double extraction each for 20 min using gentle rotation prevented formation of intractable emulsions and gave good recoveries of VPA and its metabolites Alkaline hydrolysis (pH 12–13) of the conjugated metabolites was the preferred method since it was easy to perform and ensures hydrolysis of β-glucuronidase-resistant forms should they be present [20]. Deuterium was not lost from the internal standards under the alkaline conditions

An initial approach to the extraction and analysis of metabolites following alkaline hydrolysis was to adjust the solution pH to 2 and heat for 2 h Under these conditions 4-OH-VPA and 5-OH-VPA are converted to their γ- and δ-lactones, respectively, and 3-keto-VPA to 3-heptanone [21] This same approach was used by Löscher [3] in a GC method for the analysis of VPA metabolites In a similar way we attempted to measure 3-heptanone by GC—MS and monitoring *m/z* 114 [$^2H_4$]3-Heptanone was added at the acidification step to serve as internal standard. In many cases 3-heptanone was either lost by evaporation during the 2-h heating period in spite of sealed reaction tubes or was present at low concentrations because of incomplete decarboxylation of 3-keto-VPA. The tBDMS derivative of acetic acid resulting from the hydrolysis of ethyl acetate under the acidic conditions used interfered with the internal standard [$^2H_4$] 3-heptanone peak at *m/z* 118

The extraction and analysis of intact 3-keto-VPA was then investigated. It was found that bringing the sample to pH 2 for 15 min at room temperature gave optimal results. While some 3-keto-VPA is decarboxylated to form 3-heptanone a large part remains intact. Similar results were reported by Kochen and Scheffner [9]. The 3-keto-VPA upon derivatization can be assayed as the mono- or diderivative depending on the derivatizing reagent used These extraction conditions also converted 4-OH-VPA to its γ-lactone isomers which were extracted and analyzed by monitoring *m/z* 100

*Derivatization*

The choice of tBDMS derivatives of VPA and metabolites for SIM was based on the reported increased sensitivity of tBDMS derivatives compared to TMS [15] and our previous success of using tBDMS derivatives for measuring VPA and [$^2H_6$]VPA [16–18]. The mass spectra of the tBDMS derivatives of VPA metabolites contain typical and intense $(M-57)^+$ fragment ions corresponding to the loss of the *tert.*-butyl fragment Other characteristic ions from mass spectra obtained by GC—MS are given in Table I.

TABLE I

CHARACTERISTIC IONS ($m/z$) IN THE MASS SPECTRA OF THE tBDMS DERIVATIVES OF VALPROIC ACID METABOLITES

For abbreviations and chemical names refer to text

| Compound | Base peak | $M^+$ (%) | $(M-15)^+$ $CH_3$ | $(M-57)^+$ $C_4H_9$ | $(M-99)^+$ $C_4H_9 + (CH_2)_3$ | $(M-101)^+$ $C_4H_9 + CO_2$ | $(M-131)^+$ tBDMSO | $(M-132)^+$ tBDMSOH | $(M-189)^+$ $C_4H_9$ + tBDMSOH | $(M-217)^+$ $C_4H_9$ + HCOOtBDMS | Other ions |
|---|---|---|---|---|---|---|---|---|---|---|---|
| (*E E*) 2 3 Diene | 197 | 254 (0) | 239 (3) | 197 (100) | – | 153 (3) | 123 (9) | – | – | – | 75 (29) |
| (*E*) 2,4 Diene* | 197 | 254 (0) | 239 (3) | 197 (100) | – | 153 (2) | 123 (7) | – | – | – | 75 (18) |
| 4 Ene* | 199 | 256 (0) | 241 (3) | 199 (100) | 157 (6) | – | – | – | | – | 75 (62) 129 (7) 171 (3) |
| 3 Ene | 199 | 256 (0) | 241 (4) | 199 (100) | 157 (2) | 155 (10) | – | – | - | – | 73 (76) 75 (72) 115 (40) |
| (*Z*) 2 Ene | 199 | 256 (0) | 241 (2) | 199 (100) | – | – | – | – | – | - | 73 (8) 75 (42) |
| (*F*) 2 Ene | 199 | 256 (0) | 241 (4) | 199 (100 | - | 155 (4) | 125 (9) | – | – | – | 73 (24) 75 (18) |
| VPA | 201 | 258 (0) | 243 (1) | 201 (100) | 159 (3) | – | – | - | – | – | 73 (25) 75 (82) 129 (13) |
| 3 Keto (monoderivative) | 75 | 272 (0) | – | 215 (32) | – | 171 (53) | – | – | – | – | 73 (21) 187 (5) |
| 3 Keto (diderivative) | 329 | 386 (0) | 371 (4) | 329 (100) | 287 (8) | – | 255 (28) | – | – | – | 73 (56) 75 (64) 147 (62) |
| 4 Keto | 75 | 272 (0) | - | 215 (93) | – | 171 (6) | – | – | - | – | 73 (30) 187 (45) |
| 3 OH | 75 | 274 (0) | 259 (1) | 217 (30) | – | – | – | – | – | – | 75 (100) 129 (15) 159 (25) |
| 5 OH* (diderivative) | 331 | 388 (0) | 373 (4) | 331 (100) | – | – | 257 (4) | – | 199 (13) | 171 (7) | 147 (91) 75 (64) 73 (88) |
| 2 PSA (diderivative) | 331 | 388 (0) | 373 (3) | 331 (100) | – | – | 257 (6) | – | – | 171 (17) | 73 (60) 75 (70) 147 (83) |
| 2 PGA* (diderivative) | 345 | 402 (0) | 387 (4) | 345 (100) | – | 301 (12) | 271 (3) | – | 213 (3) | 185 (18) | 73 (41) 75 (25) 129 (17) |

*Results similar to those reported by Rettenmeier et al [22]

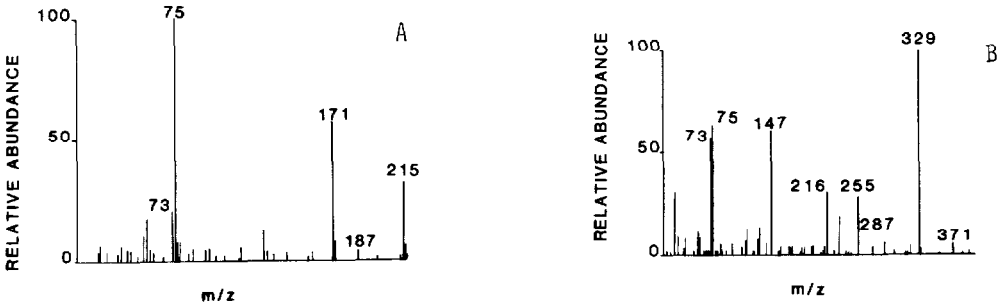



Fig 1 Mass spectra obtained by GC—MS of (A) the mono- and (B) the diderivatives of 3-keto-VPA The $m/z$ 215 and 329 are the $(M-57)^+$ ions, respectively

The majority of our analyses have been accomplished using tBDMS reagent prepared in pyridine catalyzed by 5% DMAP. This catalyst reduced reaction times but gave interfering background peaks when it exceeded 5% by weight of the reagent mixture. The reaction time of 4 h at 60°C was found to completely derivatize VPA and to give reproducible peaks for the metabolites. This reagent gave primarily the mono-tBDMS derivative of 3-keto-VPA ($m/z$ 215) Further storage at 0° C produces little or no formation of the diderivative (Fig 1) The 5-OH-VPA is readily derivatized and forms a diderivative ($m/z$ 331). On the other hand 4-OH-VPA remained largely as the γ-lactone and was analyzed as such ($m/z$ 100).

Using the tBDMS reagent in pyridine, 3-OH-VPA does not derivatize readily and chromatographs poorly Longer reaction times were necessary to obtain reproducible peaks for the 3-OH-VPA monoderivative The di-tBDMS derivatives of 3-OH-VPA and 4-OH-VPA were rarely seen

More recently, a very reactive tBDMS reagent became available [23]. With this reagent, reaction times for the derivatization of VPA metabolites were shortened (20—30 min), and 3-OH-VPA was more readily derivatized However, 3-keto-VPA forms both the mono- and diderivatives (Fig 1) and on standing at 0°C continues to convert from mono- ($m/z$ 215) to the diderivative ($m/z$ 329) This suggests that short reaction times may be inadequate for reproducible derivative formation of 3-keto-VPA Ideally, reaction times should be sufficient to ensure complete formation of the 3-keto-VPA di-tBDMS derivative With the MTBSTFA reagent and our extraction conditions, 4-OH-VPA still remains largely as the γ-lactone. Similar results were found when MSTFA was used to form TMS derivatives

The stability of tBDMS derivatives prepared using the pyridine reagent was determined by the repeated analysis of the same set of standard urine samples over a one-week period The samples were first analyzed on the day of preparation, then three and seven days later while being stored at room temperature. It was found that the relative standard deviation of the slopes of the calibration curves did not exceed 6% When derivatized samples are tightly capped and stored at −20° C they were found to be stable for several weeks

*Selected-ion chromatograms*

The SIM chromatograms of VPA metabolites obtained for a patient urine and serum sample are shown in Figs 2 and 3, respectively All peaks of interest

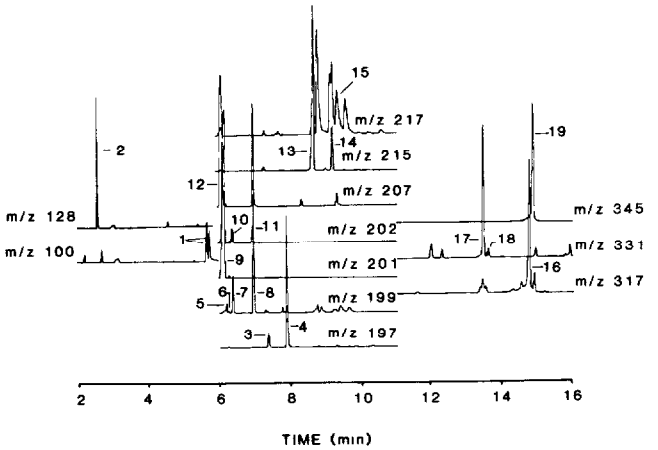



Fig 2 SIM chromatograms of tBDMS derivatives of VPA metabolites in a patient urine extract The tBDMS reagent in pyridine was used for the derivatization Chromatographic conditions capillary column, 25 m × 0 32 mm I D , OV-1701 bonded phase (phase or film thickness 0 25 $\mu$m), temperature program, 50 to 100°C at 30°C/min, then 100 to 260°C at 8°C/min, 1-min hold at 260°C Peaks 1 = 4-OH-VPA lactones, 2 = 3-octanone, internal standard, 3 = (*E*)-2,4-diene-VPA, 4 = (*E,E*)-2,3′-diene-VPA, 5 = 4-ene-VPA, 6 = 3-ene-VPA, 7 = (*Z*)-2-ene-VPA, 8 = (*E*)-2-ene-VPA, 9 = VPA, 10 = (*Z*)-[$^2H_3$]2-ene-VPA, internal standard, 11 = (*E*)-[$^2H_3$]2-ene-VPA, internal standard, 12 = [$^2H_6$]VPA, internal standard, 13 = 3-keto-VPA, 14 = 4-keto-VPA, 15 = 3-OH-VPA, 16 = adipic acid, 17 = 5-OH-VPA, 18 = 2-PSA, 19 = 2-PGA Adipic acid was measured to monitor the formation of dicarboxylic acids

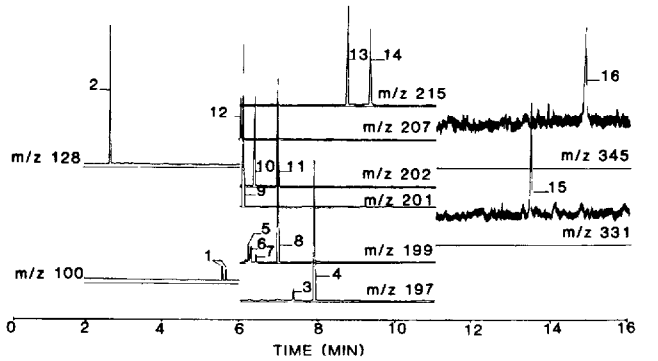



Fig 3 SIM chromatograms of tBDMS derivatives of VPA metabolites from a patient serum sample The tBDMS reagent in pyridine was used for the derivatization For chromatographic conditions, see Fig 2 Peaks 1 = 4-OH-VPA lactones, 2 = 3-octanone, internal standard, 3 = (*E*)-2,4-diene-VPA, 4 = (*E,E,*)-2,3′-diene-VPA, 5 = 4-ene-VPA, 6 = 3-ene-VPA, 7 = (*Z*)-2-ene-VPA, 8 = (*E*)-2-ene-VPA, 9 = VPA, 10 = (*Z*)-[$^2H_3$]2-ene-VPA, internal standard, 11 = (*E*)-[$^2H_3$]2-ene-VPA, internal standard, 12 = [$^2H_6$]VPA, internal standard, 13 = 3-keto-VPA, 14 = 4-keto-VPA, 15 = 5-OH-VPA 16 = 2-PGA

were suitably resolved, and serum and urine controls showed no interfering background peaks.

An OV-1701 capillary column was chosen for the analysis based on the resolution of 4-ene-VPA from VPA within a reasonable GC run time This was accomplished in approx 16 min.

The two major diene-VPA metabolites were readily seen at *m/z* 197 in serum

and urine. Other apparent dienes were present but the lack of authentic standards did not make their identification possible

While both the TBDMS mono- (*m/z* 215) and diderivative (*m/z* 329) of 3-keto-VPA are possible, the tBDMS diderivative of 3-keto-VPA is extensively seen only with MTBSTFA reagent The chromatogram for 3-OH-VPA obtained with tBDMS reagent in pyridine is not always reliable (Fig 2) Better results were obtained with MTBSTFA No peak for 4-OH-VPA monoderivative is evident since under the conditions of extraction 4-OH-VPA exists largely as the γ-lactone isomers (*m/z* 100, peaks 1)

In Fig 3, the ion chromatogram for 3-OH-VPA (*m/z* 217) was not included since the amount of 3-OH-VPA in serum was lower than the limits of detection In serum, 4-OH-VPA is again detected as the lactone and small amounts of 5-OH-VPA (*m/z* 331) and 2-PGA (*m/z* 345) are evident

*Quantitative analysis*

Calibration curves for VPA using [$^2H_6$]VPA as internal standard and for the metabolites using (*E*)-[$^2H_3$]2-ene-VPA as internal standard were linear over the ranges selected with coefficients of determination, $r^2$, exceeding 0 99 For example the calibration curve obtained for 4-ene-VPA spiked into urine and using (*E*)-[$^2H_3$]2-ene VPA as internal standard gave an $r^2$ of 0 9997 with the line passing through the origin The reproducibility of the method was checked over a period of five months. The relative standard deviation of the slopes obtained was found to be less than 8% for VPA, 3-keto-VPA, 4-keto-VPA, 4-ene-VPA, 3-ene-VPA, 2-ene-VPA and 5-OH-VPA The relative standard deviation of the slopes exceeded 10% for 2-PSA and 4-OH-VPA In the case of

TABLE II

SERUM VPA AND METABOLITE CONCENTRATIONS FOR 34 PEDIATRIC PATIENTS ON VPA MONOTHERAPY

All analyses were carried out using the tBDMS reagent

| Compound | Concentration (μg/ml) | | Percentage of VPA |
|---|---|---|---|
| | Mean | Range | |
| 4-OH-VPA | 0 38 | Tr*— 1 78 | 0 8 |
| (*E*)-2,4-Diene-VPA** | 0 20 | 0 02— 0 58 | 0 4 |
| (*E,E*)-2,3′-Diene-VPA** | 2 95 | 0 50— 7 29 | 6 4 |
| 4-Ene-VPA | 0 67 | 0 16— 1 22 | 1 4 |
| 3-Ene-VPA | 0 94 | 0 25— 1 86 | 2 0 |
| (*Z*)-2-Ene-VPA | 0 19 | 0 06— 0 40 | 0 4 |
| (*E*)-2-Ene-VPA | 5 53 | 0 95— 11 3 | 11 9 |
| VPA | 46 38 | 11 8 —105 | 100 |
| 3-Keto-VPA | 3 59 | 0 29— 15 60 | 7 7 |
| 4-Keto-VPA | 0 40 | 0 01— 1 29 | 0 9 |
| 5-OH-VPA | 0 18 | Tr— 1 25 | 0 4 |
| 2-PSA | 0 04 | Tr— 0 44 | 0 1 |
| 2-PGA | 0 20 | Tr— 1 23 | 0 4 |

*Tr = trace

**The (*E*)-2-ene-VPA standard curve was used for quantitation

2-PGA some improvement in reproducibility may be obtained by using a dicarboxylic acid as an internal standard. Because of the variability observed, standard curves were run prior to each batch of patient samples

Our method has been in use for over a year and patient results are shown in Table II The values obtained for 3-keto-VPA, 2-ene-VPA, 4-OH-VPA and 5-OH-VPA are in the same range as those obtained by Löscher [3] and Nau et al [11].

*Comparison of tBDMS and TMS derivatives*

VPA metabolites were also derivatized using MSTFA, and the ion chromatograms using the same column as for the tBDMS derivatives are shown in Fig. 4 In order to separate 4-ene-VPA and VPA a longer chromatographic time and different conditions than used for the tBDMS derivatives were required. Using our extraction and derivatization conditions, 4-OH-VPA lactones are present (peaks 1, m/z 100) as well as isomers of the 4-OH-VPA monoderivative (peaks 16, *m/z* 217) The isomers of 3-OH-VPA readily appeared as mono-TMS derivatives in contrast to tBDMS derivatives and gave sharp peaks (peaks 15,

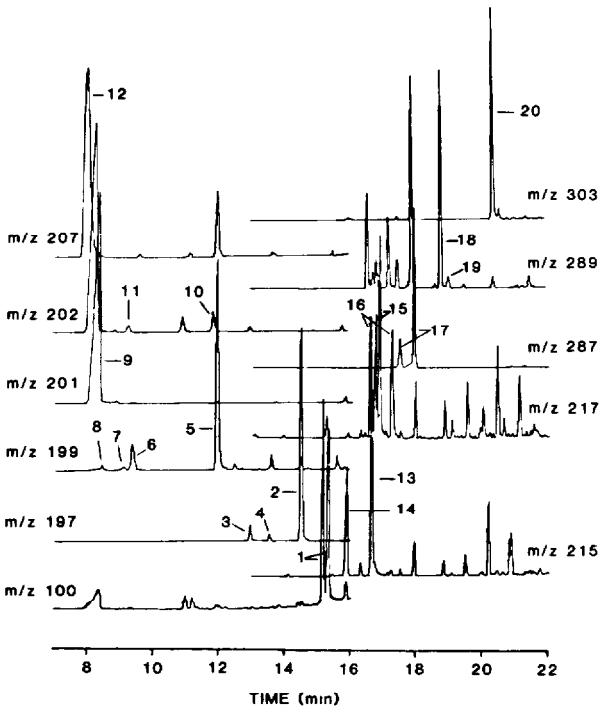



Fig 4 SIM chromatograms of TMS derivatives of VPA metabolites from a patient urine sample MSTFA reagent was used for derivatization Chromatographic conditions capillary column, 25 m × 0 32 mm I D , OV-1701 bonded phase (film thickness 25 $\mu$m), temperature program, 75° C (10 min), 75 to 200° C at 8° C/min, then 200 to 260° C at 30° C/min, 5-min hold at 260° C Peaks 1 = 4-OH-VPA lactones, 2 = (*E,E*)-2,3′-diene-VPA, 3 and 4 = dienes-VPA, 5 = (*E*)-2-ene-VPA, 6 = (*Z*)-2-ene-VPA, 7 = 3-ene-VPA, 8 = 4-ene-VPA, 9 = VPA, 10 = (*E*)-[$^2H_3$]2-ene-VPA, internal standard, 11 = (*Z*)-[$^2H_3$]2-ene-VPA, internal standard, 12 = [$^2H_6$]VPA, internal standard, 13 = 4-keto-VPA, 14 = 3-keto-VPA, TMS monoderivative, 15 = 3-OH-VPA (two isomers), 16 = 4-OH-VPA (two isomers), 17 = 3-keto-VPA, TMS diderivative (two isomers), 18 = 5-OH-VPA, 19 = 2-PSA, 20 = 2-PGA

*m/z* 217). 3-Keto-VPA was present as both the monoderivative (peak 14) and the diderivative (peaks 17) and upon standing the monoderivative 3-keto-VPA was found to decrease with a corresponding increase of the diderivative isomers

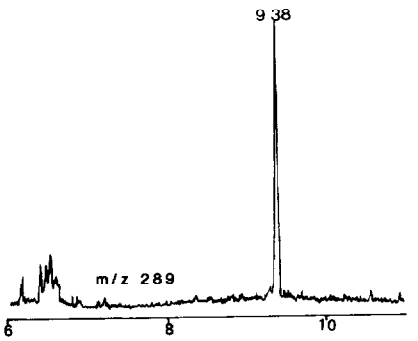
TABLE III

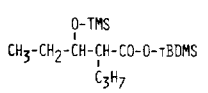
COMPARISON OF THE RELATIVE SENSITIVITY OF tBDMS AND TMS DERIVATIVES OF VPA AND METABOLITES WHEN MEASURED BY SIM

Peaks were the $(M - 57)^+$ and $(M - 15)^+$ ions, respectively For each metabolite $n = 12$

| Metabolite | Relative sensitivity tBDMS/TMS* |
|---|---|
| Dienes-VPA, (*Z*)-2-ene-VPA, 3-ene-VPA, 4-ene-VPA | 10 —20 |
| VPA, (*E*)-2-ene-VPA, 4-keto-VPA, 2-PSA, 2-PGA | 5 —10 |
| 5-OH-VPA, 3-keto-VPA | 0 3— 2 |

*Determined from the peak area ratios of the respective $(M - 57)^+$ or $(M - 15)^+$ *m/z* to the *m/z* 160 of diethyl 1-methylbutylmalonate



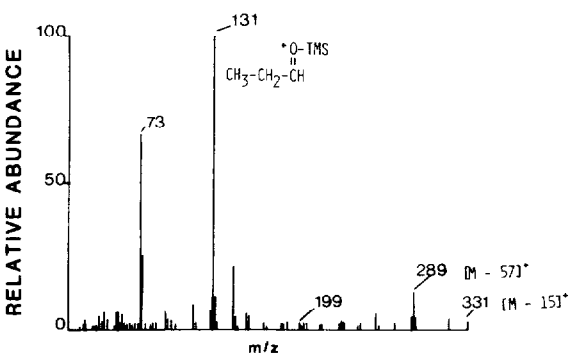




Fig 5 Mass spectrum and corresponding mass chromatogram of the mixed tBDMS—TMS derivative of 3-OH-VPA in urine extract For chromatographic conditions see Fig 2

In order to compare the relative sensitivity of detection of the tBDMS and TMS derivatives, a calibration standard, a patient urine sample and a patient serum sample were each extracted and divided with one part derivatized by MTBSTFA reagent and the other half by MSTFA Diethyl 1-methylbutylmalonate served as a non-derivatized internal standard. The samples were run on three different days over a period of one week All samples were stored at −18°C and found to be stable The results are given in Table III Except for 3-keto-VPA, the tBDMS derivatives in all cases gave greater sensitivity. This was particularly true and has special significance for the unsaturated metabolites 3- and 4-ene-VPA which are frequently present in concentrations nearing the lower detection limits. Barring differences in the chromatography and the degree of derivatization, the greater sensitivity observed with the ion monitoring of the tBDMS derivatives is very similar to the comparisons seen by Woollard [15] for TMS and tBDMS derivatives of $C_{13}$ and $C_{20}$ straight-chain fatty acids

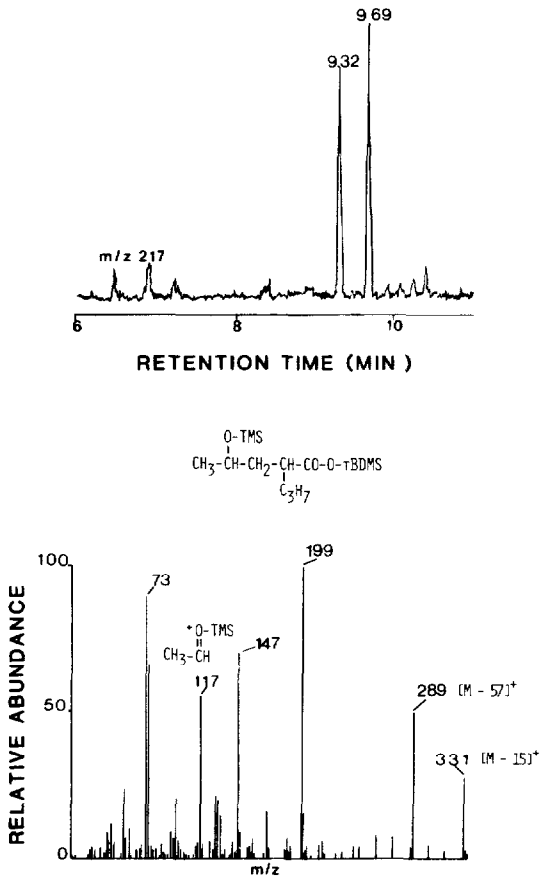



Fig 6 Mass spectrum and corresponding mass chromatogram of the mixed tBDMS—TMS derivative of 4-OH-VPA isomers in urine extract For chromatographic conditions see Fig 2

*Mixed derivatives*

Mixed tBDMS—TMS derivatives as described by Woollard [15] for hydroxy fatty acids were obtained for 3-OH-VPA and 4-OH-VPA. The chromatographic conditions were identical to that used for the tBDMS derivatives Ion chromatograms and the mass spectra are shown in Figs 5 and 6. The isomers of 3-OH-VPA appear as a single peak The most characteristic ion is *m/z* 131 and confirms the position of the TMS substituent

The 4-OH-VPA isomers appeared as two peaks and gave several characteristic ions The base peak was *m/z* 199 (M — TMSOH — $C_4H_9$). If a fairly unreactive tBDMS reagent is to be used, then formation of mixed derivatives has potential for the analysis of VPA metabolites These initial results suggest that a combination of reagents might provide optimal derivatives for the GC—MS--SIM analysis of VPA metabolites, i e sensitive and selective ions for the unsaturated (tBDMS) and mixed derivatives for the hydroxy metabolites that are poorly derivatized using tBDMS reagent alone

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