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# Determination of valproic acid and its metabolites using gas chromatography with mass-selective detection: application to serum and urine samples from sheep

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**Abstract**

An improved method for the quantitative determination of valproic acid (VPA) and sixteen of its metabolites has been developed using gas chromatography–mass spectrometry with selected-ion monitoring. The method is applicable to serum or urine and all metabolites are measured in a single chromatographic run of 29.5 min. Ions selected for quantitative purposes were the characteristic $[M-57]^+$ ions of the *tert.*-butyldimethylsilyl (tBDMS) derivatives. The method utilizes heptadeuterated VPA as well as six heptadeuterated metabolites as internal standards [i.e. 2-[$^2H_7$]propyl-2-pentenoic acid (2-ene[$^2H_7$]VPA), 2-[$^2H_7$]propyl-4-pentenoic acid (4-ene[$^2H_7$]VPA), 2-[$^2H_7$]propyl-3-oxopentanoic acid (3-keto[$^2H_7$]VPA), 2-[$^2H_7$]propyl-4-oxopentanoic acid (4-keto[$^2H_7$]VPA), 2-[$^2H_7$]propyl-3-hydroxypentanoic acid (3-OH[$^2H_7$]VPA), 2-[$^2H_7$]propyl-5-hydroxypentanoic acid (5-OH[$^2H_7$]VPA)]. The method demonstrates very good accuracy and precision over a large range of concentrations for VPA and all metabolites measured in both human and sheep biological fluids. The assay was applied to the analysis of VPA and metabolites in serum and urine samples collected from three non-pregnant ewes following intravenous bolus administration of a mixture of VPA and [$^{13}C_4$]VPA. Sheep were observed to produce measurable quantities of the majority of metabolites found in humans, with the notable exception of the di-unsaturated compounds (i.e. 2,3'-diene VPA and 2,4-diene VPA). The pharmacokinetics and metabolism of VPA and [$^{13}C_4$]VPA appear to be equivalent in the sheep model. The elimination half-life of VPA and [$^{13}C_4$]VPA in the ewe were estimated to be approximately $3.5 \pm 0.4$ and $3.2 \pm 0.4$ h, respectively.

## 1. Introduction

Valproic acid (VPA, 2-propylpentanoic acid) is an anticonvulsant agent widely used in the treatment of several types of epileptic seizures [1,2]. The elimination of VPA is characterized by its extensive biotransformation into at least 16 different metabolites in humans [3,4]. It has been proposed that some of these metabolites [e.g. 2-propyl-2-pentenoic acid (2-ene VPA) and 2-[1'-propenyl]-2-pentenoic acid (2,3'-diene VPA)], which possess anticonvulsant activity, may con-

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tribute to the effectiveness of VPA [5,6], while others [e.g. 2-propyl-4-pentenoic acid (4-ene VPA) and 2-propyl-2,4-pentadienoic acid (2,4-diene VPA)] may be responsible for the rare but serious hepatotoxicity associated with VPA therapy [7–10]. As a result, there has been considerable interest in the development of a simultaneous, sensitive assay for VPA and its metabolites. Attempts to develop such an assay have been hampered by the large number of metabolites, the large range of concentrations over which they are found in biological fluids, and the presence of endogenous substances similar in structure to VPA.

Recently, a method using capillary gas chromatography with ion-trap detection was reported which is capable of resolving VPA and all of its mono-unsaturated metabolites from serum in the form of methyl ester derivatives [11]. More commonly, several gas chromatographic–mass spectroscopic methods have been reported for VPA and its metabolites. These methods have employed both electron-impact ionization (EI) of the *tert.*-butyldimethylsilyl (tBDMS) [12–16] and trimethylsilyl (TMS) [8,17–20] derivatives as well as negative-ion chemical ionization (NICI) of the pentafluorobenzyl bromide (PFB) derivatives [21]. Although the EI/tBDMS assay previously reported by our laboratory [15] as well as more recently reported EI/TMS assays [19,20] may be used to successfully quantify most VPA metabolites, some separations have remained elusive [e.g. resolution of the E and Z isomers of 2-propyl-3-pentenoic acid (3-ene VPA)]. Although the NICI methods are adequate for the detection and quantitation of most of the known metabolites, analysis work for planned pharmacokinetic studies involving large numbers of samples would be best handled using a mass-selective detector with EI detection. As a result, we sought to improve our EI/tBDMS assay reported previously [15]. The present paper then, describes a convenient method for the separation and quantitation of VPA and 16 metabolites in a single chromatographic run with a run-time of 29.5 min. The assay utilizes the intense $[M-57]^+$ fragment of the tBDMS derivative which has been found to provide better sensitivity with fatty acids than the less intense $[M-15]^+$ fragment of TMS derivatives [12–14,16]. The assay also makes use of stable isotope labelled VPA and metabolites as internal standards because their structural similarity to the compounds of interest produces chromatographic characteristics akin to those of the compounds being examined [22,23].

In our research program we have examined the placental and non-placental clearances of a number of drugs [24,25] using the two-compartment model proposed by Szeto et al. [26]. Application of the model requires steady-state drug infusions to both the mother and fetus. To date, these infusions have been administered on separate occasions, typically 24–72 h apart. The use of unlabeled and stable-isotope labeled drug would allow these infusions to be conducted simultaneously thereby reducing time-dependent variations in drug disposition. Prior to using stable isotope labeled drug however, it is necessary to assess whether the pharmacokinetics and metabolism of the labeled and unlabeled drug are equivalent. To examine this possibility, the analytical technique described in the present report has been applied in the measurement of VPA and its metabolites in serum and urine obtained from three non-pregnant sheep following i.v. bolus administration of a 50% VPA and 50% [$^{13}C_4$]VPA mixture. The results of these studies are included in this report.

## 2. Experimental

### 2.1. VPA metabolites

The metabolites used for the standard calibration curves were synthesized as reported elsewhere [4,27]. These metabolites were (E)-2-propyl-2-pentenoic acid [(E)-2-ene VPA], (Z)-2-propyl-2-pentenoic acid [(Z)-2-ene VPA), (E) and (Z)-isomers of 2-propyl-3-pentenoic acid (3-ene VPA), 2-propyl-4-pentenoic acid (4-ene VPA), 2-propyl-3-hydroxypentanoic acid (3-OH-VPA), 2-propyl-4-hydroxypentanoic acid as γ-lactone isomers (4-OH-VPA), 2-[(E)-1′-propenyl]-(E)-2-pentenoic acid [(E,E)-2,3′-diene

VPA], 2-[(E)-1′-propenyl]-(Z)-2-pentenoic acid [(E,Z)-2,3′-diene VPA], (E)-2-propyl-2,4-pentadienoic acid [(E)-2,4-diene VPA], (Z)-2-propyl-2,4-pentadienoic acid [(Z)-2,4-diene VPA], 2-propylglutaric acid (2-PGA) and 2-propylsuccinic acid (2-PSA). The metabolites 2-propyl-3-oxopentanoic acid (3-keto-VPA) [21], 2-propyl-4-oxopentanoic acid (4-keto-VPA) [21] and 2-propyl-5-hydroxypentanoic acid (5-OH-VPA) [28] were also synthesized according to previously reported methods.

### 2.2. Internal standards

2-Methylglutaric acid (2-MGA) was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). The following heptadeuterated compounds were prepared as described elsewhere [29]: 2-[$^2H_7$]propylpentanoic acid ([$^2H_7$]VPA), 2-[$^2H_7$]propyl-2-pentenoic acid (2-ene[$^2H_7$]-VPA), 2-[$^2H_7$]propyl-4-pentenoic acid (4-ene[$^2H_7$]VPA), 2-[$^2H_7$]propyl-3-oxopentanoic acid (3-keto[$^2H_7$]VPA), 2-[$^2H_7$]propyl-4-oxopentanoic acid (4-keto[$^2H_7$]VPA), 2-[$^2H_7$]propyl-3-hydroxypentanoic acid (3-OH[$^2H_7$]VPA), 2-[$^2H_7$]propyl-5-hydroxypentanoic acid (5-OH[$^2H_7$]VPA).

### 2.3. Reagents

Solvents were distilled-in-glass grade obtained from Caledon (Georgetown, Ont., Canada). Reagent grade anhydrous sodium sulfate, sodium hydroxide and hydrochloric acid were obtained from BDH Chemicals (Toronto, Ont., Canada). The N-*tert.*-butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA) was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). 2-Propylpentanoic acid (di-*n*-propylacetic acid, valproic acid) was obtained from K and K Fine Chemicals (Plainview, NY, USA) while 2-propyl-1-$^{13}$C-pentanoic-1,2,3-$^{13}C_3$ acid ([$^{13}C_4$]VPA) was obtained from MSD Isotopes (Montreal, P.Q., Canada).

### 2.4. Instrumentation

The assay was performed on a Hewlett-Packard 5890 Series II gas chromatograph with a Hewlett-Packard 7673A autosampler coupled to a Hewlett-Packard 5971A mass-selective detector (Hewlett-Packard, Avondale, PA, USA). A DB-1701 fused-silica capillary column (30 m × 0.25 mm I.D., 0.25 μm film thickness; J and W Scientific, Rancho Cordova, CA, USA) was used with helium as carrier gas and a column head pressure of 104 kPa. Splitless injection was used with an injector temperature of 250°C. The initial column oven temperature was 80°C (held for 0.1 min) followed by an increase to 100°C at a rate of 10°C/min (0.1 min hold time), a 2°C/min increase to 130°C (0.1 min hold time) and a 30°C/min increase to 260°C (8 min hold time). The gas chromatograph interface temperature was held at 280°C. The detector was operated in the selected-ion monitoring (SIM) mode with an ion-source temperature of 180°C, an emission current of 300 μA and an ionization energy of 70 eV. The ions were monitored in groups according to their retention time with a dwell time of 100 ms per cycle within each group. The total run-time required was 29.5 min. The entire process, including data collection, was controlled by the Hewlett-Packard ChemStation Version A.02.01 on a Hewlett-Packard Vectra 486 with a Hewlett-Packard 7360 controller.

### 2.5. Stock solution of internal standards

Alkaline solutions of the internal standards were diluted with distilled water and combined to obtain a solution containing 4 μg/ml of 3-OH[$^2H_7$]VPA and 5-OH[$^2H_7$]VPA, 2 μg/ml of [$^2H_7$]VPA, 3-keto[$^2H_7$]VPA, 4-keto[$^2H_7$]VPA and 2-MGA, 1.7 μg/ml of (E)-2-ene[$^2H_7$]VPA, and 1 μg/ml of 4-ene[$^2H_7$]VPA.

### 2.6. Preparation of standard sample stock solutions

Alkaline stock solutions of VPA and its 16 metabolites were combined and diluted with distilled water to provide a concentrated standard stock solution. Subsequent serial dilutions of the stock with distilled water provided a series of seven standard stock solutions with the con-

Table 1
Concentration ranges, regression coefficients, diagnostic ions and retention times of TBDMS derivatives of VPA and metabolites from standard reference samples in human serum and urine

| Compound | Concentration range (μg/ml) | Regression coefficient ($r^2$) in serum/urine | Retention time (min) | Ion monitored ($m/z$) |
|---|---|---|---|---|
| VPA | 0.078–20.000 | 1.000/1.000 | 13.64 | 201 |
| (E)-2-ene-VPA | 0.074–19.076 | 1.000/0.999 | 16.58 | 199 |
| (Z)-2-ene-VPA | 0.004–0.924 | 1.000/0.999 | 14.64 | 199 |
| (E)-3-ene-VPA | 0.003–0.386 | 0.996/0.998 | 14.05 | 199 |
| (Z)-3-ene-VPA | 0.014–3.614 | 0.999/1.000 | 14.29 | 199 |
| 4-ene-VPA | 0.008–2.000 | 1.000/0.999 | 13.88 | 199 |
| (E)-2,4-diene-VPA | 0.012–3.044 | 0.999/1.000 | 17.78 | 197 |
| (Z)-2,4-diene-VPA | 0.004–0.956 | 0.999/1.000 | 17.05 | 197 |
| (E,E)-2,3′-diene-VPA | 0.027–7.032 | 0.999/0.999 | 18.71 | 197 |
| (E,Z)-2,3′-diene-VPA | 0.004–0.968 | 0.999/0.999 | 17.80 | 197 |
| 3-keto-VPA | 0.016–4.000 | 0.990/0.980 | 21.79, 21.99[a] | 329[b] |
| 4-keto-VPA | 0.008–2.000 | 0.999/0.999 | 20.03 | 215 |
| 3-OH-VPA | 0.016–4.000 | 0.995/0.995 | 19.66, 19.93[a] | 217 |
| 4-OH-VPA | 0.016–4.000 | 0.998/0.995 | 12.21, 12.41[a] | 100[c] |
| 5-OH-VPA | 0.008–2.000 | 0.999/0.995 | 22.14 | 331[b] |
| 2-PSA | 0.008–2.000 | 0.999/0.990 | 22.18 | 331[b] |
| 2-PGA | 0.008–2.000 | 1.000/0.999 | 22.50 | 345[b] |

[a] Isomers
[b] Diderivative
[c] γ-Lactone

centration ranges of VPA and VPA metabolites presented in Table 1.

### 2.7. Preparation of standard serum and urine samples

Standard serum samples were prepared by combining 200 μl of standard solution, 100 μl of internal standard and 200 μl of control serum. The resulting solutions were mixed, adjusted to pH 2 with 1 *M* HCl and further adjusted to a total volume of 700 μl by the addition of distilled water. The solutions were then extracted twice with ethyl acetate (1.5 ml) by gentle rotation for 30 min. The combined organic phase was vortex-mixed with anhydrous $Na_2SO_4$, centrifuged (10 min, 1000 *g*, 25°C), transferred to a conical reaction vial and concentrated to approximately 50 μl under a nitrogen stream. MTBSTFA (50 μl) was added and the samples were derivatized at 60°C for 1 h. A 1-μl aliquot of sample was injected onto the GC–MSD system.

Standard urine samples prepared to quantify unconjugated VPA and metabolites were prepared as described above for the serum samples with the exception that 200 μl of control urine was used instead of control serum and 2 *M* HCl was used to adjust the samples to pH 2 before extraction.

In order to quantify total conjugated and unconjugated VPA and metabolites in urine, 50 μl of control urine, 100 μl of internal standard solution and 200 μl of standard solution were combined, adjusted to pH 12.6–13 with 4 *M* NaOH, and incubated for 1 h at 60°C. After cooling, the solutions were adjusted to pH 2 with 4 *M* HCl and distilled water was added to a final volume of 1 ml. These samples were then extracted and derivatized as described above for serum.

Calibration curves were obtained from a plot

of the area ratio of the VPA or metabolite peak to that of the internal standard versus the concentration of the compound of interest. For the experiments described in the present work, [$^2H_7$]VPA was used as the internal standard for VPA and 4-OH-VPA, 4-ene[$^2H_7$]VPA was used for 4-ene-VPA, (E)-2-ene[$^2H_7$]VPA was used to analyze all of the remaining monoenes as well as the dienes, 2-MGA was used for 2-PSA and 2-PGA, and 3-keto[$^2H_7$]VPA, 4-keto[$^2H_7$]VPA, 3-OH[$^2H_7$]VPA and 5-OH[$^2H_7$]VPA were used to quantitate the same corresponding unlabelled metabolites.

The accuracy of the assay in serum and urine was determined by analysis of six samples of spiked blank biofluid at both a high and low concentration of each compound of interest. The experimentally determined concentrations were then compared to the nominal (spiked) concentration of the sample. The intra-day variability of the analysis was determined by six-fold repeated analysis of spiked blank biofluid at both a high and low concentration of each compound of interest. The precision is reported as the coefficient of variation for each compound. The inter-day variability was determined by an eight-fold repeated analysis of a set of seven standard samples containing each of the compounds of interest, over a period of sixteen days.

### 2.8. Sheep experiments

Three non-pregnant sheep (mean weight of 71.9 ± 11.1 kg) of Suffolk or Dorset breeds were used in these experiments. The surgical procedure used in the catheterization of the sheep is described elsewhere [30]. Serial femoral arterial blood (serum) samples were collected for analysis at −5, 2, 6, 10, 15, 20, 30, 45, 60, 90 min, 2, 2.5, 3, 3.5, 4, 6, 8, 10, 12, 24, 48 and 72 h following intravenous bolus administration of a mean dose of 14.2 mg/kg body weight of VPA (50% VPA, 50% [$^{13}C_4$]VPA) through a femoral venous catheter. Cummulative urine samples were also obtained for the following time periods: −30–0, 0–30, 30–60 min, 1–2, 2–3, 3–4, 4–6, 6–8, 8–10, 10–12, 12–24, 24–36, 36–48 and 48–72 h. The samples were stored at −20°C prior to analysis.

### 2.9. Serum and urine samples from adult sheep

Samples (200 μl serum or urine for quantitation of unconjugated compounds, or 50 μl urine for quantitation of total VPA and metabolites) were combined with 100 μl of internal standard and 200 μl of distilled water, mixed, extracted and derivatized as described above for the standard sample preparation. The samples were analyzed for both unlabelled and $^{13}C_4$-labeled parent compound and respective metabolites.

## 3. Results and discussion

Fig. 1 shows a chromatogram of VPA and its metabolites extracted from sheep serum. The retention times and $m/z$ values of the $[M-57]^+$ diagnostic ions for VPA and its metabolites are listed in Table 1. The same information for the internal standards is provided in Table 2. As seen in Fig. 1, the chromatographic conditions provided resolution of VPA and its metabolites including all of the isomeric unsaturated compounds. For the first time, (E)- and (Z)-3-ene-VPA were completely resolved using tBDMS derivatives (Fig. 1, $m/z$ 199). Although complete resolution of (E)-2,4-diene-VPA and (E,Z)-2,3'-diene-VPA was not obtained, the separation was adequate for quantitative purposes (Fig. 1, $m/z$ 197). All compounds produced sharp, symmetrical peaks with the exception of 3-OH-VPA which tailed slightly. The heptadeuterated compounds, whose chromatography is also illustrated in Fig. 1, were convenient and suitable internal standards for this analysis because their physicochemical and chromatographic characteristics are very similar to those of the compounds being quantitated.

The calibration curves used for the quantitation of VPA and its metabolites in human and sheep serum and urine showed good linearity over the concentration ranges investigated as demonstrated by the correlation coefficients listed in Table 1 for analysis from human serum.

The accuracy and precision of the assay was assessed by analysis of multiple urine and serum samples which had been spiked with known amounts of VPA and metabolites. Tables 3 and 4 summarize the results of the assessment of the method in human urine and serum respectively. Similar results were observed in studies involving sheep serum and urine. As can be seen in Tables 3 and 4, the experimentally determined values for the spiked samples are in good agreement with the calculated values in most instances. Furthermore, the assay demonstrated excellent precision with coefficients of variation of $< 10\%$ for VPA and most metabolites. The analyses

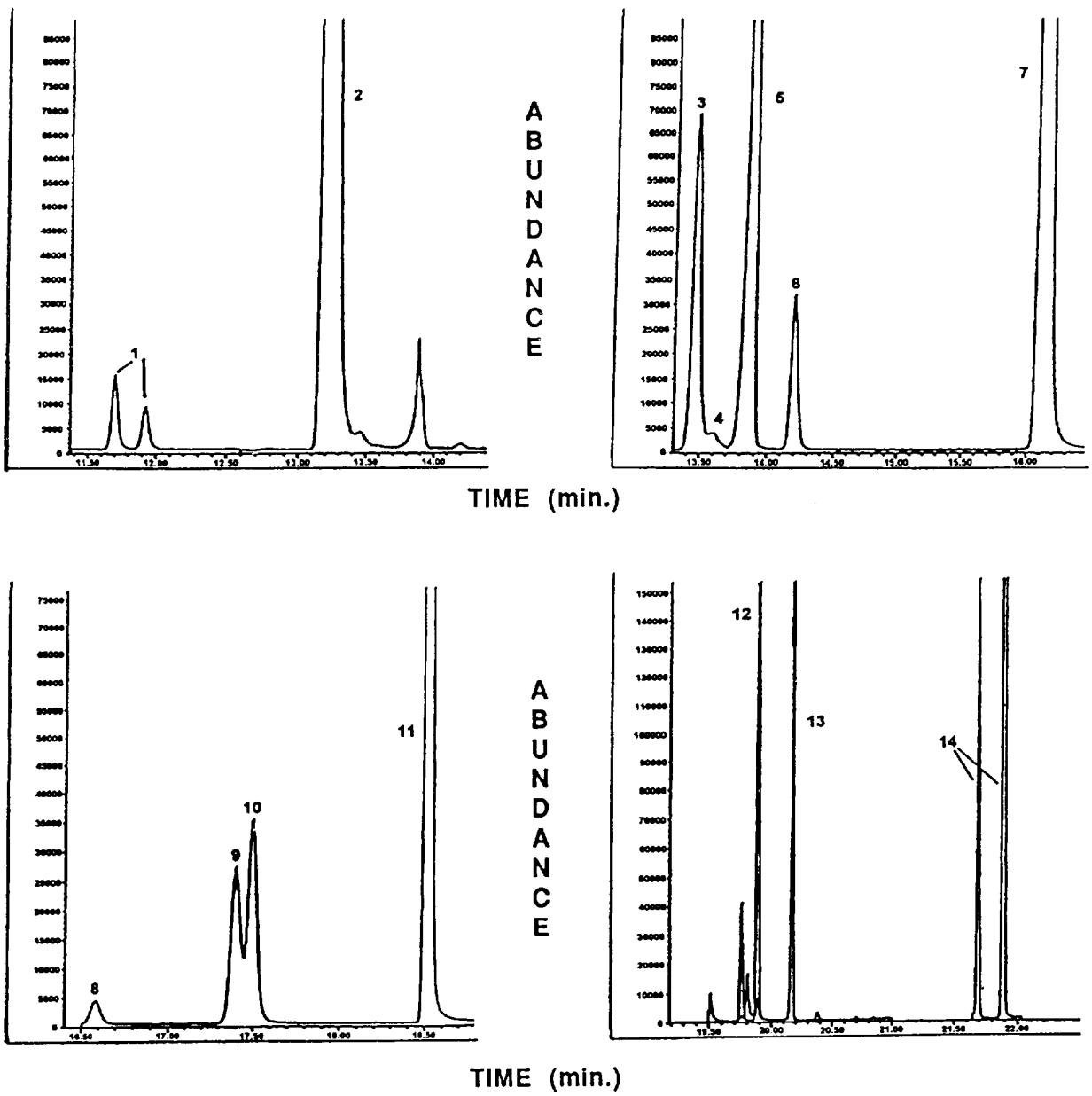


Fig. 1.

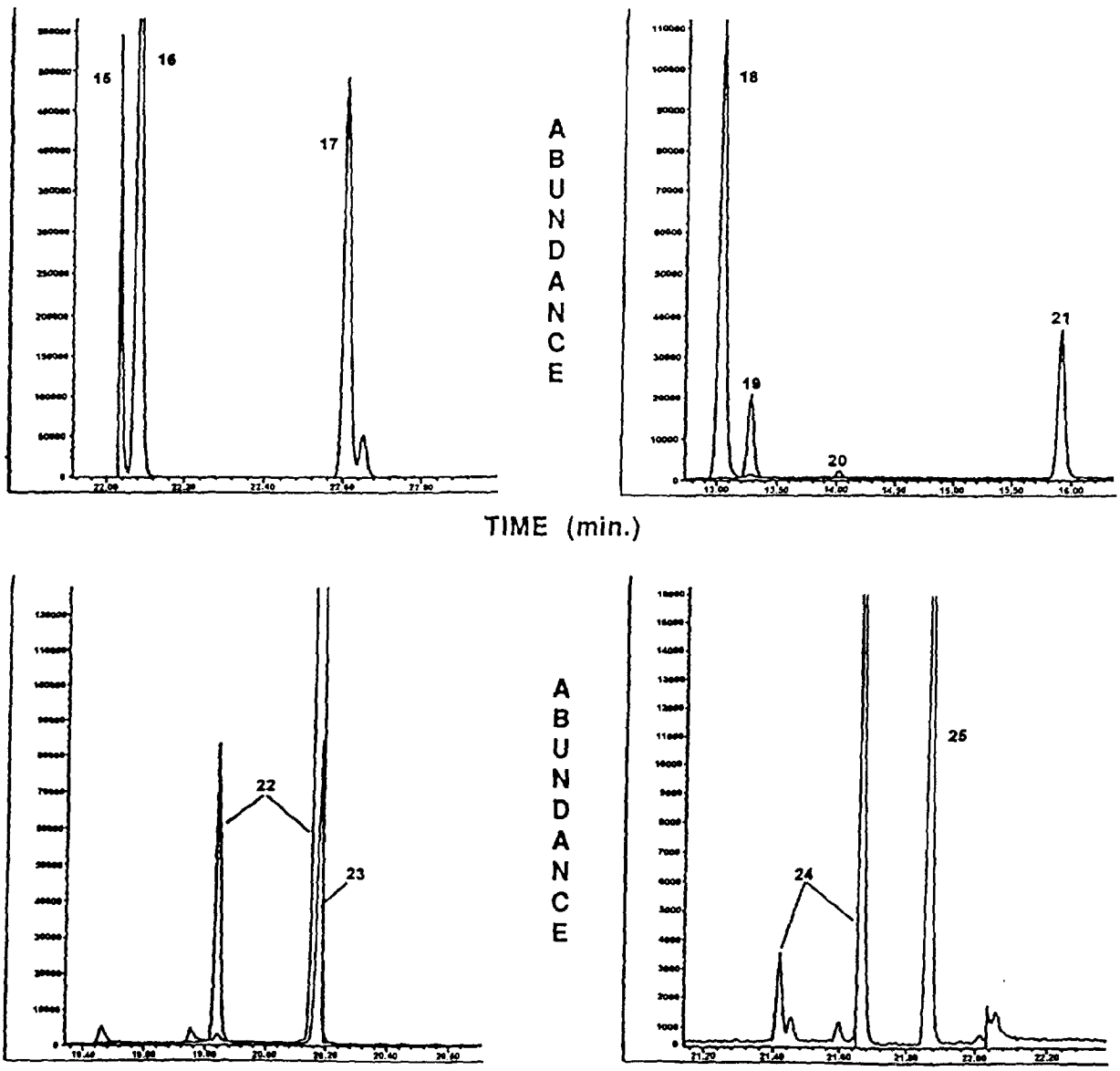


Fig. 1. SIM chromatograms of tBDMS derivatives of VPA and VPA metabolites in a standard human serum sample. The compounds detected and the ions monitored are as follows: 1 = 4-OH-VPA-γ-lactones (*m/z* 100); 2 = VPA (*m/z* 201); 3 = 4-ene-VPA (*m/z* 199); 4 = (E)-3-ene-VPA (*m/z* 199); 5 = (Z)-3-ene-VPA (*m/z* 199); 6 = (Z)-2-ene-VPA (*m/z* 199); 7 = (E)-2-ene-VPA (*m/z* 199); 8 = (Z)-2,4-diene-VPA (*m/z* 197); 9 = (E)-2,4-diene-VPA (*m/z* 197); 10 = (E,Z)-2,3'-diene (*m/z* 197); 11 = (E,E)-2,3'-diene (*m/z* 197); 12 = 3-OH-VPA (*m/z* 217); 13 = 4-keto-VPA (*m/z* 215); 14 = 3-keto-VPA (*m/z* 329); 15 = 5-OH-VPA (*m/z* 331); 16 = 2-PSA (*m/z* 331); 17 = 2-PGA (*m/z* 345); 18 = [$^2H_7$]VPA (*m/z* 208); 19 = 4-ene[$^2H_7$]VPA (*m/z* 206); 20 = (Z)-2-ene[$^2H_7$]VPA (*m/z* 206); 21 = (E)-2-ene[$^2H_7$]VPA (*m/z* 206); 22 = 3-OH[$^2H_7$]VPA (*m/z* 224); 23 = 4-keto[$^2H_7$]VPA (*m/z* 222); 24 = 3-keto[$^2H_7$]VPA (*m/z* 336); 25 = 5-OH[$^2H_7$]VPA (*m/z* 338).

Table 2
Diagnostic ions and retention times of the derivatized internal standard compounds

| Compound | Retention time (min) | m/z |
|---|---|---|
| [$^2H_7$]VPA | 13.44 | 208 |
| (Z)-2-ene[$^2H_7$]VPA | 14.43 | 206 |
| (E)-2-ene[$^2H_7$]VPA | 16.36 | 206 |
| 4-ene[$^2H_7$]VPA | 13.70 | 206 |
| 3-keto[$^2H_7$]VPA | 21.77, 21.97[a] | 336[b] |
| 4-keto[$^2H_7$]VPA | 19.99 | 222 |
| 3-OH[$^2H_7$]VPA | 19.61, 19.89[a] | 224 |
| 5-OH[$^2H_7$]VPA | 22.11 | 338[b] |
| 2-MGA | 22.10 | 317[b] |

[a] Isomers
[b] Diderivative

which resulted in larger degrees of error were typically associated with low levels of metabolite which approached the limits of detection of the assay. Using a signal-to-noise ratio of 3:1 as an indicator, the limits of quantitation (LOQ) for the metabolites ranged between 3 and 20 ng/ml in all of the biofluids studied. Table 5 describes the reliability of the assay by demonstrating stability in the slopes of calibration curves which were generated by repeated analysis of a set of standard samples prepared in human serum over a period of sixteen days. Coefficients of variation for these slopes did not exceed 10% except those of 4-OH-VPA, 4-keto-VPA and 5-OH-VPA which were $\leq 14\%$. The recoveries from this extraction procedure for the various compounds of interest were all satisfactory as has been noted previously [15].

### 3.1. VPA and metabolites in the ewe

The assay was employed to assess the levels of VPA and metabolites present over time in three non-pregnant ewes and to examine the effect of isotope labelling on VPA pharmacokinetics following i.v. bolus administration. A representative serum concentration–time profile for VPA and two of its metabolites in one ewe is illustrated in Fig. 2. The serum concentration–time profiles of VPA and [$^{13}C_4$]VPA followed a biexponential process with apparent terminal half-lives of approximately $3.5 \pm 0.4$ and $3.2 \pm 0.4$ h, respectively. The mean VPA/[$^{13}C_4$]VPA area under the serum concentration–time curve ratio ($AUC_{\infty,VPA}/AUC_{\infty,[^{13}C_4]VPA}$) was determined to be $1.03 \pm 0.02$ suggesting very similar pharmacokinetic behaviour. This was further supported by the similar amounts of labelled and unlabelled parent compounds recovered from urine (see Table 6). For comparison, the reported elimination half-life of VPA in adult humans ranges from 8 to 16 h [3,31,32]. This is consistent with the differences noted in the half-lives of other compounds, such as metoclopramide and diphenhydramine, which also show more rapid elimination in sheep compared to humans [24,33].

The metabolites identified in serum and urine in both the unlabelled and $^{13}C_4$-labelled form were the (E) and (Z)-isomers of both 2-ene-VPA and 3-ene-VPA, 4-ene-VPA, 3-keto-VPA, 4-keto-VPA, 3-OH-VPA, 4-OH-VPA, 5-OH-VPA and 2-PGA. Fig. 3 shows ion chromatograms for five metabolites of VPA detected in urine collected from a non-pregnant ewe following i.v. bolus administration of VPA (50% VPA, 50% [$^{13}C_4$]VPA). The ion peaks for the $^{13}C_4$-analogues of these metabolites were virtually superimposable on the illustrated peaks in most instances. Tables 7 and 8 illustrate that the amounts of labelled and unlabelled metabolites detected and recovered from serum and urine respectively are equivalent suggesting that the metabolism of the two compounds follows similar pathways. Therefore, [$^{13}C_4$]VPA appears to be equivalent to VPA in the sheep model based on comparisons of the $AUC_\infty$ values, terminal elimination half-lives and recovery of metabolites. [$^{13}C_4$]VPA is thus suitable for use in simultaneous administration experiments with unlabeled VPA to study the pharmacokinetics and placental transfer of this drug in the pregnant sheep model.

Analysis of urine indicated that approximately 47% of the dose was excreted as VPA plus conjugates while 2.6 and 1.8% were excreted as 3-keto-VPA and 3-OH-VPA, respectively.

The major metabolites observed in human

Table 3
Accuracy and precision of assay with respect to analysis of VPA and metabolites extracted from human urine[a]

| Compound | Nominal concentration (μg/ml) | Mean measured concentration (μg/ml) | Coefficient of variation (%) | Relative difference[b] (%) |
|---|---|---|---|---|
| VPA | 20.000 | 19.146 | 0.44 | 4.3 |
| | 0.156 | 0.165 | 9.1 | 5.7 |
| (E)-2-ene-VPA | 6.736 | 6.714 | 0.76 | 0.33 |
| | 0.053 | 0.057 | 8.8 | 7.7 |
| (Z)-2-ene-VPA | 1.264 | 1.346 | 1.7 | 6.5 |
| | 0.010 | 0.013 | 7.7 | 28 |
| (E)-3-ene-VPA | 0.386 | 0.393 | 1.9 | 1.8 |
| | 0.003 | 0.003 | 3.3 | 7.2 |
| (Z)-3-ene-VPA | 3.614 | 3.382 | 2.0 | 6.4 |
| | 0.028 | 0.028 | 7.1 | 1.1 |
| 4-ene-VPA | 4.000 | 4.117 | 1.1 | 2.9 |
| | 0.031 | 0.034 | 5.9 | 10 |
| (E)-2,4-diene VPA | 3.044 | 3.279 | 2.7 | 7.7 |
| | 0.025 | 0.028 | 7.1 | 17 |
| (Z)-2,4-diene VPA | 0.956 | 1.045 | 3.0 | 9.3 |
| | 0.007 | 0.012 | 17 | 66 |
| (E,E)-2,3'-diene-VPA | 7.032 | 6.798 | 2.4 | 3.3 |
| | 0.055 | 0.058 | 6.9 | 5.0 |
| (E,Z)-2,3'-diene-VPA | 0.968 | 1.047 | 1.8 | 8.2 |
| | 0.008 | 0.009 | 11 | 14 |
| 3-keto-VPA | 4.000 | 3.872 | 3.6 | 3.2 |
| | 0.031 | 0.033 | 12 | 6.7 |
| 4-keto-VPA | 2.000 | 2.010 | 1.0 | 0.01 |
| | 0.016 | 0.020 | 5.0 | 27 |
| 3-OH-VPA | 4.000 | 3.887 | 1.9 | 2.8 |
| | 0.031 | 0.035 | 17 | 12 |
| 4-OH-VPA | 4.000 | 3.914 | 9 | 2.1 |
| | 0.031 | 0.032 | 19 | 2.2 |
| 5-OH-VPA | 4.000 | 3.844 | 1.2 | 3.9 |
| | 0.031 | 0.031 | 9.7 | 0.70 |
| 2-PSA | 2.000 | 2.018 | 2.4 | 0.9 |
| | 0.016 | 0.018 | 11 | 17 |
| 2-PGA | 2.000 | 2.079 | 3.2 | 4.0 |
| | 0.016 | 0.017 | 5.9 | 5.7 |

[a] Based on analysis of six samples of spiked blank human urine.
[b] Relative difference is defined as the difference between the measured mean and nominal concentration of a sample divided by the nominal concentration.

Table 4
Accuracy and precision of assay with respect to analysis of VPA and metabolites extracted from human serum[a]

| Compound | Nominal concentration (μg/ml) | Mean measured concentration (μg/ml) | Coefficient of variation (%) | Relative difference[b] (%) |
|---|---|---|---|---|
| VPA | 20.000 | 19.136 | 2.1 | 4.3 |
| | 0.156 | 0.153 | 5.2 | 1.9 |
| (E)-2-ene-VPA | 6.736 | 6.576 | 1.1 | 2.4 |
| | 0.053 | 0.055 | 9.1 | 3.8 |
| (Z)-2-ene-VPA | 1.264 | 1.312 | 1.8 | 3.8 |
| | 0.010 | 0.009 | 33 | 10 |
| (E)-3-ene-VPA | 0.386 | 0.376 | 3.7 | 2.6 |
| | 0.003 | 0.003 | 3.5 | 0 |
| (Z)-3-ene-VPA | 3.614 | 2.800 | 10 | 22 |
| | 0.028 | 0.030 | 10 | 7.1 |
| 4-ene-VPA | 4.000 | 4.187 | 3.1 | 4.7 |
| | 0.031 | 0.032 | 3.1 | 3.2 |
| (E)-2,4-diene-VPA | 3.044 | 3.279 | 2.7 | 7.7 |
| | 0.025 | 0.020 | 25 | 0.4 |
| (Z)-2,4-diene-VPA | 0.956 | 1.045 | 3.0 | 9.3 |
| | 0.007 | 0.010 | 7.9 | 43 |
| (E,E)-2,3'-diene-VPA | 7.032 | 6.798 | 2.4 | 3.3 |
| | 0.055 | 0.055 | 3.6 | 0 |
| (E,Z)-2,3'-diene-VPA | 0.968 | 1.047 | 1.8 | 8.2 |
| | 0.008 | 0.008 | 0 | 0 |
| 3-keto-VPA | 4.000 | 4.030 | 0.67 | 0.75 |
| | 0.031 | 0.034 | 5.9 | 9.7 |
| 4-keto-VPA | 2.000 | 2.017 | 0.55 | 0.85 |
| | 0.016 | 0.015 | 17 | 6.2 |
| 3-OH-VPA | 4.000 | 4.354 | 5.4 | 8.8 |
| | 0.031 | 0.028 | 7.1 | 9.7 |
| 4-OH-VPA | 4.000 | 3.892 | 5.7 | 2.7 |
| | 0.031 | 0.049 | 27 | 58 |
| 5-OH-VPA | 4.000 | 4.067 | 0.74 | 0.02 |
| | 0.031 | 0.032 | 9.4 | 3.2 |
| 2-PSA | 2.000 | 1.990 | 4.8 | 0.5 |
| | 0.016 | 0.015 | 16 | 6.2 |
| 2-PGA | 2.000 | 1.894 | 8.2 | 5.3 |
| | 0.016 | 0.022 | 14 | 38 |

[a] Based on analysis of seven samples of spiked blank human serum.
[b] Relative difference is defined as the difference between the measured mean and nominal concentration of a sample divided by the nominal concentration.

Table 5
Assessment of inter-assay variability[a]

| Compound | Mean slope | Coefficient of variation (%) |
|---|---|---|
| VPA | 5.104 | 2.03 |
| (E)-2-ene-VPA | 0.829 | 7.81 |
| (Z)-2-ene-VPA | 2.76 | 8.25 |
| (E)-3-ene-VPA | 0.897 | 7.92 |
| (Z)-3-ene-VPA | 0.891 | 8.32 |
| 4-ene-VPA | 1.023 | 7.67 |
| (E)-2,4-diene-VPA | 6.244 | 6.72 |
| (Z)-2,4-diene-VPA | 8.634 | 7.74 |
| (E,E)-2,3'-diene-VPA | 1.511 | 7.04 |
| (E,Z)-2,3'-diene-VPA | 1.723 | 7.90 |
| 3-keto-VPA | 0.194 | 5.45 |
| 4-keto-VPA | 0.264 | 11.1 |
| 3-OH-VPA | 1.812 | 2.00 |
| 4-OH-VPA | 34.249 | 10.2 |
| 5-OH-VPA | 0.353 | 14.0 |
| 2-PSA | 1.469 | 4.23 |
| 2-PGA | 1.652 | 11.1 |

[a] Determined by an eight-fold replicate analysis of a set of seven standard samples prepared in human serum.

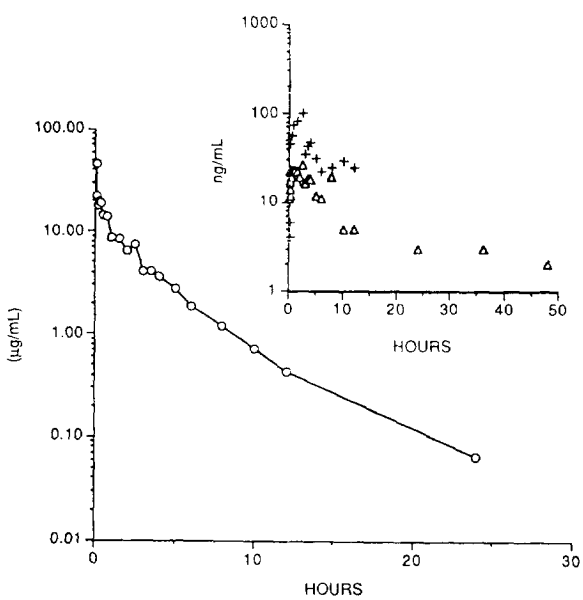


Fig. 2. Representative concentration–time profiles for VPA (○), 4-ene-VPA (△) and 3-keto-VPA (+) in serum from a non-pregnant ewe following i.v. bolus administration of 13.6 mg/kg body weight of VPA (50% VPA, 50% [$^{13}C_4$]VPA).

Table 6
Mean percentage of dose excreted as parent drug in urine following i.v. bolus administration of a [$^{13}C_4$]VPA/VPA 50:50 mixture

| Compound | Mean percentage excreted | | |
|---|---|---|---|
| | Unconjugated | Conjugated | Total |
| VPA | 9.2 ± 0.7 | 37.8 ± 1.1 | 47.1 ± 0.3 |
| [$^{13}C_4$]VPA | 8.5 ± 0.9 | 39.2 ± 1.2 | 47.7 ± 0.3 |

Table 7
Time-averaged peak-area ratios from serum for some of the more prominent metabolites detected following i.v. bolus administration of a 50% [$^{13}C_4$]VPA/50% VPA mixture (1 g dose)

| Metabolite | Peak-area ratio |
|---|---|
| 4-ene-VPA/ 4-ene[$^{13}C_4$]VPA | 0.97 ± 0.22 |
| (E)-3-ene-VPA/ (E)-3-ene[$^{13}C_4$]VPA | 1.02 ± 0.18 |
| (E)-2-ene-VPA/ (E)-2-ene[$^{13}C_4$]VPA | 1.14 ± 0.18 |
| 3-keto-VPA/ 3-keto[$^{13}C_4$]VPA | 1.09 ± 0.13 |
| 5-OH-VPA/ 5-OH[$^{13}C_4$]VPA | 1.18 ± 0.10 |
| 2-PGA/ 2-[$^{13}C_4$]PGA | 1.29 ± 0.17 |

Table 8
Ratio of amount of unlabeled VPA metabolite to amount of [$^{13}C_4$]VPA metabolite recovered from urine for some of the more prominent metabolites following i.v. bolus administration of a 50% [$^{13}C_4$]VPA/50% VPA mixture (1 g dose)

| Metabolite | Metabolite ratio |
|---|---|
| (E)-2-ene-VPA/ (E)-2-ene[$^{13}C_4$]VPA | 0.90 ± 0.10 |
| 3-keto-VPA/ 3-keto[$^{13}C_4$]VPA | 1.08 ± 0.02 |
| 4-keto-VPA/ 4-keto[$^{13}C_4$]VPA | 1.16 ± 0.31 |
| 5-OH-VPA/ 5-OH[$^{13}C_4$]VPA | 1.13 ± 0.14 |
| 2-PGA/ 2-[$^{13}C_4$]PGA | 1.13 ± 0.04 |

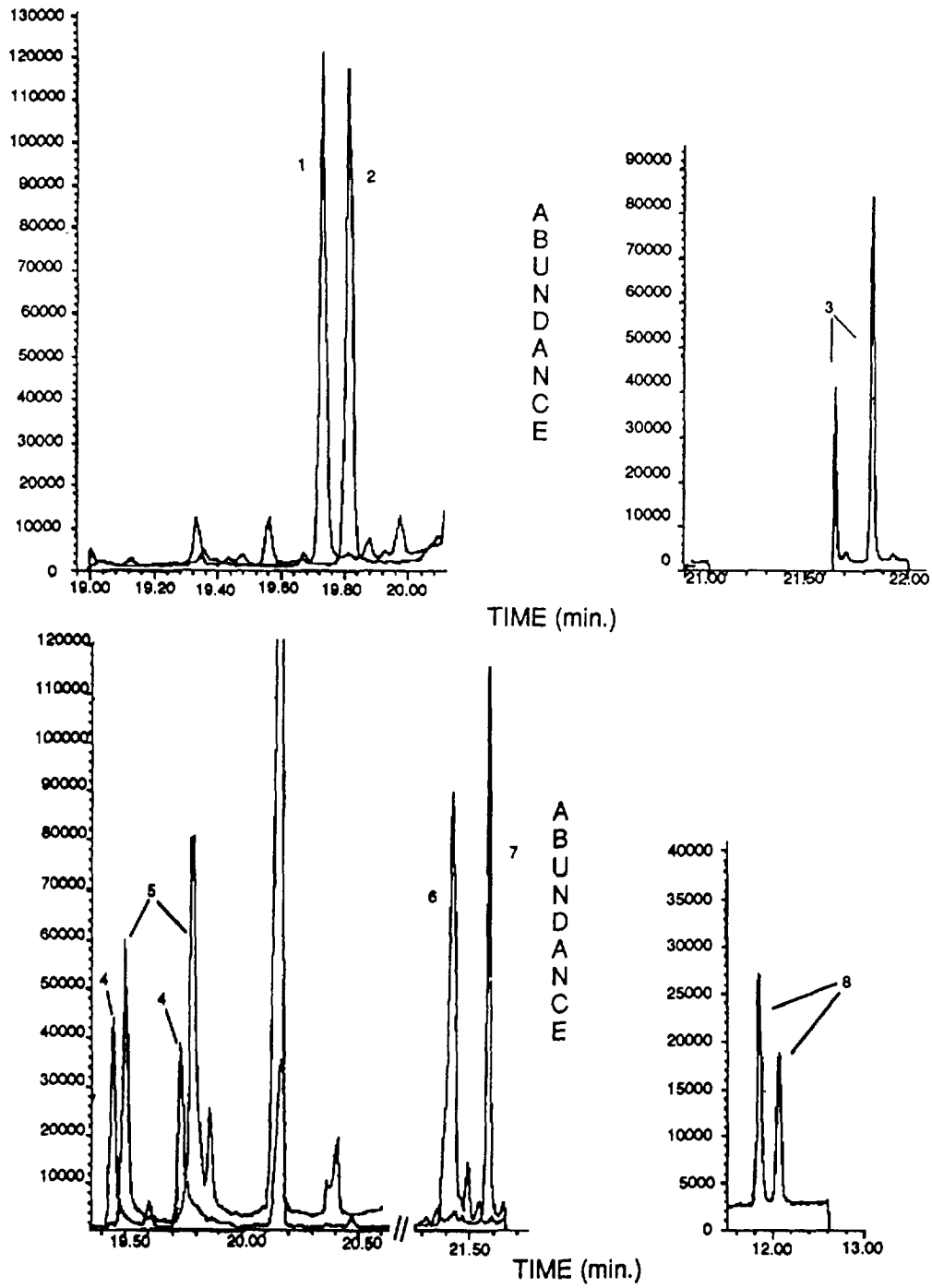


Fig. 3. SIM chromatograms of tBDMS derivatives of some representative VPA metabolites collected in urine from a non-pregnant ewe following i.v. bolus administration of 13.6 mg/kg body weight of VPA (50% VPA, 50% [$^{13}C_4$]VPA). SIM chromatograms of tBDMS derivatives of some internal standards are also shown. The compounds presented and the ions monitored are as follows: 1 = 4-keto[$^2H_7$]VPA (*m/z* 222); 2 = 4-keto-VPA (*m/z* 215); 3 = 3-keto-VPA (*m/z* 329); 4 = 3-OH[$^2H_7$]VPA (*m/z* 224); 5 = 3-OH-VPA (*m/z* 217); 6 = 5-OH[$^2H_7$]VPA (*m/z* 338); 7 = 5-OH-VPA (*m/z* 331); 8 = 4-OH-VPA-γ-lactones (*m/z* 100).

serum are the β-oxidation products (E)-2-ene-VPA and 3-keto-VPA as well as (E,E)-2,3′-diene-VPA and 3-ene-VPA [21,34]. The ewe appears to produce all of these compounds with the exception of the (E,E)-2,3′-diene. In fact, none of the diunsaturated metabolites were found to be present in the ewe in measurable quantities. Furthermore, while the potential hepatotoxin 4-ene-VPA is only a minor metabolite in humans, it was found to be one of the more prominent serum metabolites in sheep. In a fashion similar to humans, the oxidation products 3-OH-VPA, 4-OH-VPA, 5-OH-VPA, 3-keto-VPA, 4-keto-VPA and 2-PGA were all found in significant amounts in the urine of the ewe.

## 4. Conclusions

An improved quantitative assay employing GC–MS has been developed for the measurement of VPA and sixteen of its metabolites in serum and urine (human and sheep). The method is reliable and reproducible and is being used for further studies of VPA and its metabolites in pregnant sheep (ewe and fetus) and in humans.

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