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VALPROIC ACID AND SEVERAL METABOLITES: QUANTITATIVE DETERMINATION IN SERUM, URINE, BREAST MILK AND TISSUES BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY USING SELECTED ION MONITORING

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SUMMARY

A method has been developed for the simultaneous quantitative determination of valproic acid (2-propylpentanoic acid) and its metabolites 2-propyl-2-pentenoic acid (*trans*), 2-propyl-3-pentenoic acid (*trans*), 2-propyl-4-pentenoic acid, 3-hydroxy-2-propylpentanoic acid, 4-hydroxy-2-propylpentanoic acid, 5-hydroxy-2-propylpentanoic acid, 3-oxo-2-propylpentanoic acid, and 2-propylglutaric acid. All compounds were extracted at pH 5.0 with ethyl acetate. The concentrated extracts were trimethylsilylated and the resulting mixtures analyzed by a gas chromatography—mass spectrometry—computer system operated in the selected ion monitoring mode. Linear calibration curves were obtained in the concentration ranges studied (0.1—20 μg/ml for metabolites, 0.1—150 μg/ml for valproic acid). The recoveries of the drugs were between 92 and 97%. The relative standard deviations were between 3.9 and 8.1% (analysis of multiple 10-μl samples of patient urine). The lower detection limits were found to be between 2.8 and 18 ng/ml using 200-μl serum samples. The derivatized extracts were stable for at least one week.

Applications of the method described include studies of placental transfer of valproic acid and metabolites in the human, the elimination of these substances by the neonate, their transfer via mother's milk, and their levels in mouse brain.

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INTRODUCTION

Valproic acid (VPA, 2-propylpentanoic acid*) is an anticonvulsant drug which is widely used for the control of a broad spectrum of epileptic seizures; for recent reviews see refs. 1 and 2. VPA is predominantly cleared by metabolic pathways and a number of metabolites have been identified [3—8]. Although numerous procedures have been developed for the determination of VPA [9—18], most of which rely on gas chromatography, quantitative methods exist for the assay of only a few VPA metabolites [4, 6, 7]. Also, these procedures require relatively large samples and are not sufficiently sensitive for the complete analysis of metabolites in human blood or urine samples.

Furthermore, some of the metabolites may be biologically active; and knowledge of their kinetics may therefore significantly contribute to the understanding of the pharmacological activity of VPA and possibly also of the side-effects observed.

We have therefore developed procedures for the simultaneous assay of VPA and the metabolites depicted in Fig. 1. The procedures were of sufficient sensitivity and selectivity for the analysis of small samples ($\leqslant$ 200 μl or mg) of serum, urine, saliva, breast milk, brain and fetal tissues. Analysis was performed by a gas chromatography—mass spectrometry—computer system (GC—MS—computer) operated in the selected ion monitoring mode (mass fragmentography). The high selectivity of the method enabled us to use very simple sample-handling procedures, thus minimizing the danger of labile substances decomposing.

MATERIALS AND METHODS

Chemicals and reagents

The metabolites of VPA (for their structures and abbreviations of their names see Fig. 1) have been prepared by chemical synthesis. The 3-hydroxy, 4-hydroxy, 5-hydroxy and 3-keto metabolites were synthesized as described previously [4, 6]. The 2-en (*trans*) metabolite was prepared by dehydrobromination of 2-bromo-2-propylpentanoic acid with N,N-diethylaniline. The 3-en (*trans*) metabolite, contaminated with 2-en (*trans*), was formed as a side-product of the above synthesis and purified by fractional distillation. The 4-en metabolite was synthesized by decarboxylation during boiling of a solution of 2-allyl-2-propylmalonic acid in a toluene—pyridine mixture. Details of these procedures will be published elsewhere. The internal standard used (2-ethyl-2-methylcaproic acid) was obtained from Fluka (Neu-Ulm, G.F.R.), acetonitrile and pyridine ("dried") were from Merck (Darmstadt, G.F.R.), ethyl acetate (Nanograde) was from Promochem (Wesel, G.F.R.), N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) from Pierce (Günter Karl OHG, Geisenheim, G.F.R.), and β-glucuronidase—arylsulfatase from Boehringer (Mannheim, G.F.R.).

*Although VPA is often administered as the sodium salt and, furthermore, is present at physiological pH mostly in its dissociated form, this compound is conventionally referred to as "acid".

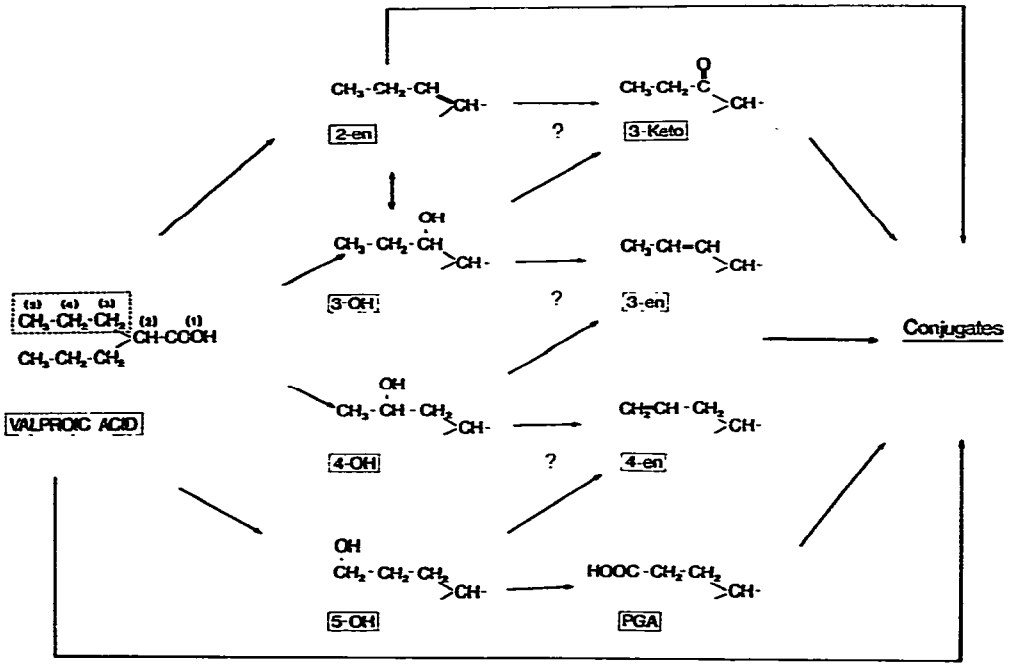

Fig. 1. Tentative metabolic scheme for valproic acid. Abbreviations: VPA = valproic acid (2-propylpentanoic acid); 2-en = 2-propyl-2-pentenoic acid (*trans*); 3-en = 2-propyl-3-pentenoic acid (*trans*); 4-en = 2-propyl-4-pentenoic acid; 3-OH = 3-hydroxy-2-propylpentanoic acid; 4-OH = 4-hydroxy-2-propylpentanoic acid; 5-OH = 5-hydroxy-2-propylpentanoic acid; 3-keto = 3-oxo-2-propylpentanoic acid; PGA = 2-propylglutaric acid. VPA, 2-en (*trans*) and 3-en (*trans*) are present in urine predominantly as conjugates and can be hydrolyzed at pH 5.0 with glucuronidase—arylsulfatase.

Hydrolysis of conjugated metabolites

A sample (2—200 μl) of urine, serum, breast milk or tissue homogenate was pipetted into a disposable 1.5-ml Eppendorf microtube and diluted to 200 μl if necessary; 50 μl of 1 *N* NaH_2PO_4 (adjusted to pH 5.0) and 30 μl of β-glucuronidase—arylsylfatase (5 U/ml) were added. This mixture was slowly agitated at 37°C for 1 h and then processed as described below.

Extraction procedure

A sample (2—200 μl, depending on availability) of serum, urine, breast milk, or tissue homogenate was pipetted into a disposable 1.5-ml Eppendorf microtube and diluted to 200 μl if necessary. Then 50 μl of 1 *N* NaH_2PO_4 buffer (adjusted to pH 5.0) and 1 ml of ethyl acetate containing the internal standard (1 μg) were added. The tube was shaken for 15 min and then centrifuged for 1 min in a 5012 Eppendorf centrifuge. An 800-μl portion of the supernatant organic phase was transferred to a 1.5-ml disposable glass reaction vial and, following the addition of 100 μl of acetonitrile, preconcentrated to 100—200 μl by a stream of nitrogen. The extraction was repeated using 1 ml of ethyl acetate. The combined extracts were evaporated at 20°C under a stream of nitrogen to a final volume of 10—20 μl. The sample was trimethylsilylated by

adding 30 μl of pyridine and 30 μl of MSTFA at room temperature. After at least 30 min, aliquots of 1—3 μl were injected into the GC—MS system.

GC—MS—computer analysis

A Perkin-Elmer F-22 gas chromatograph was coupled via a Watson-Biemann separator to a Varian MAT CH-7A mass spectrometer. A glass column (2 m × 6 mm O.D. × 2.5 mm I.D.; Bodenseewerk Perkin-Elmer, Uberlingen, G.F.R.) packed with 3% OV-17 on 120—140 mesh Gas-Chrom Q was used (Applied Science Labs.). The trimethylsilylated samples were injected at a column temperature of 80°C. After an initial period of 1 min the column temperature was raised to 140°C at a rate of 12.5°C/min. During this period, the derivatives to be measured eluted from the GC column. The temperature was then raised quickly (30°C/min) to 250°C to elute the accompanying substances, and held there for 1 min. During this time, the selected ion records were plotted and the peak height ratios calculated. After the GC column had cooled down to the initial temperature, the next sample was injected. Four samples were analyzed in this way within 1 h.

The mass spectrometer was controlled by the SS-100 Varian data system and operated in the selected ion monitoring mode. The following ions were selected (see Fig. 1 for the structures of the metabolites): *m/e* 183 (3-keto), *m/e* 185 (5-OH, PGA), *m/e* 199 [4-en, 3-en (*trans*), 2-en (*trans*), 4-OH], *m/e* 201 (VPA), *m/e* 215 (internal standard), and *m/e* 275 (3-OH). The results were displayed on a Tektronix 4010 terminal and plotted on a Tektronix hardcopy unit.

Quantitation

Standard calibration graphs were obtained by the analysis of 200-μl portions of drug-free human serum or urine, to which known amounts of VPA and the metabolites indicated in Fig. 1 had been added. These samples were processed as described above. The stored samples were kept frozen at —30°C.

RESULTS AND DISCUSSION

We have evaluated a number of solvents and their mixtures for the extraction of the drugs at various pH values. It was found that ethyl acetate extraction at pH 5.0 led to good yields (Table I) without decomposition of any of the metabolites. At the same pH the conjugates could also be released by glucuronidase—arylsulfatase treatment. Therefore, hydrolysis of the conjugates and extraction of the aglycones could be performed at the same pH, further simplifying the experimental procedures. During the concentration of the extracts, loss of volatile compounds was avoided by the addition of acetonitrile as well as by the incomplete evaporation of the extracts (see Experimental).

Some of the metabolites could not be chromatographed without decomposition. Therefore, various derivatization procedures were evaluated and it was found that each of the drugs could be derivatized by trimethylsilylation into derivatives with excellent GC properties. The derivatization reaction was performed by simply adding the reagents. After a reaction time of 0.5 h (or overnight) the mixture was directly injected into the GC—MS system. The

TABLE I

RECOVERIES, RELATIVE STANDARD DEVIATIONS AND LOWER DETECTION LIMITS

Compound	Recovery of isolation procedure (%)	Lower detection limit* (ng/ml)	Relative standard deviation (%)**
VPA	97	3.6	4.5
2-en	94	2.9	
4-en	96	6.1	
3-OH	92	18.0	8.1
4-OH	96	5.5	4.8
5-OH	96	2.8	3.9
3-keto	96	3.0	5.6
PGA	95	4.6	7.6

*Signal-to-noise ratio = 2 using 200-μl serum samples.
**Ten 10-μl urine samples (daily dose of the epileptic patient: 1.5 g of VPA) were analyzed as described. Only the unconjugated metabolites were measured. Therefore, *trans*-2-en, *trans*-3-en, and 4-en, which are present in urine predominantly as conjugates, are not listed. For the concentrations of the drugs present see the legend to Fig. 4B.

derivatives were found to be stable for at least a week (see below). The hydroxylated metabolites did not form lactones as side-products during the isolation and derivatization procedure.

The mass spectra of the trimethylsilylated derivatives generated by electron impact are shown in Fig. 2. All intense ions were evaluated, but those indicated in Fig. 2 were selected in the final method by the criteria of favorable baselines and detection limits. The ion selected for the internal standard (*m/e* 215) was 14 mass units higher than that for VPA.

The 4-OH metabolite was present in the synthesized sample, as well as in all patient samples encountered, as both diastereoisomeric forms, which were separated by the GC column (see Fig. 4). Since both forms yielded identical mass spectra, the total amount of the 4-OH present was calculated by adding both peaks (ion 199). The diastereoisomeric forms of the 3-OH were not separated by GC and eluted in a slightly broadened peak.

In the case of the 2-en metabolite, a pure standard of the *trans* isomer was available, and this isomer was predominantly present in patient serum and urine samples.

The oxo function of the 3-keto metabolite enolized during trimethylsilylation to yield *cis* and *trans* isomers which were not separated on the GC column used (see Fig. 4). Difficulties during quantitation were not encountered by this enolization.

The reproducibility of the method was evaluated by the analysis of urine samples of an epileptic patient treated with VPA. The relative standard deviations (between 3 and 8%) are listed in Table I. The lower detection limits, using 200-μl samples, were found to be in the low ng/ml range (Table I). Larger sample volumes further increased the sensitivity of the method.

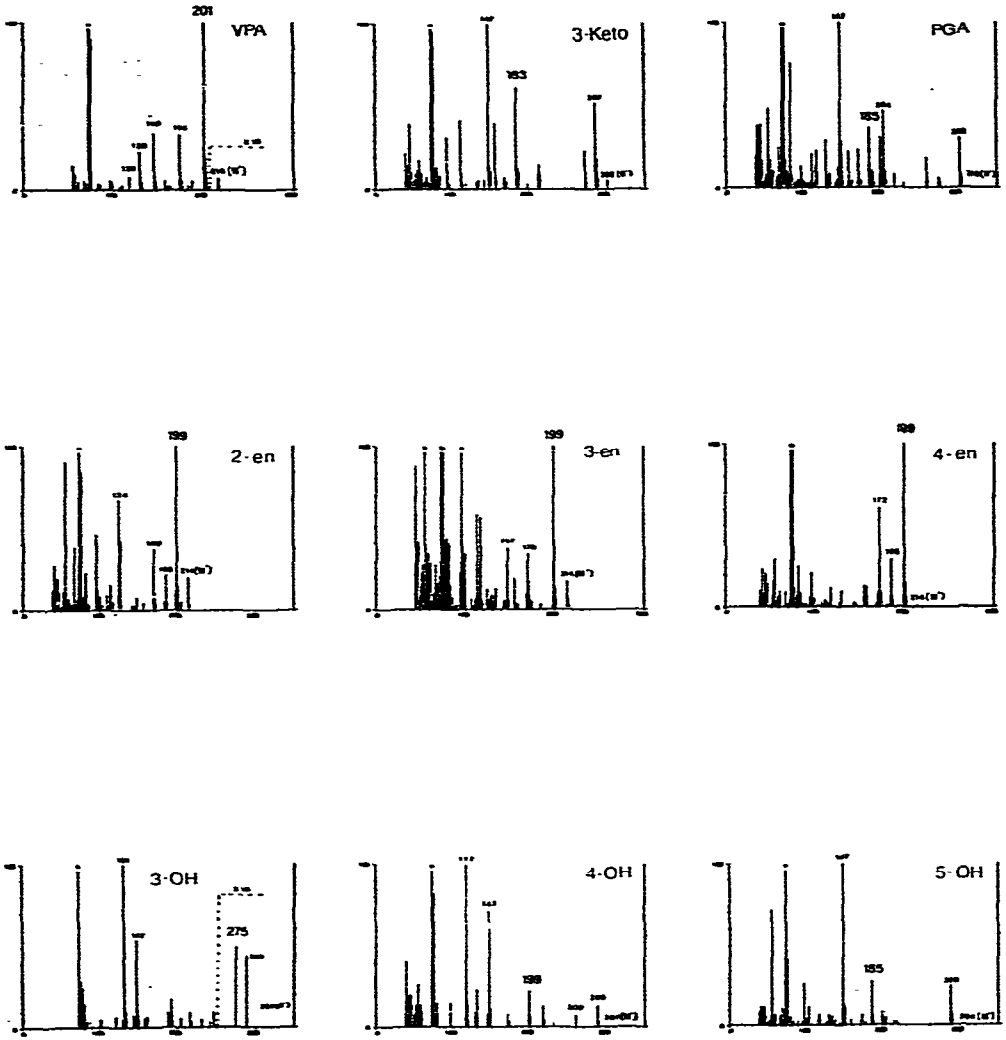

Fig. 2. Electron-impact mass spectra of the trimethylsilylated derivatives of VPA and metabolites. The abbreviated names of the compounds are spelled out in the legend of Fig. 1. The ions selected for mass fragmentography are indicated. All hydroxy and carboxy groups are trimethylsilylated. The 3-keto metabolite carries two trimethylsilyl groups, one on the carboxy and one on the hydroxy group (latter formed via enolization of the oxo function).

Fig. 3 shows the plots of the peak height ratios vs. amounts of drug and metabolites added [0.1—20 μg/ml (g) for the VPA metabolites and 0.1—150 μg/ml (g) for VPA]. The slopes of such plots (Fig. 3) indicate linear dependence of the peak height ratios vs. drug concentrations. The square of the correlation coefficient (r^2) exceeded 0.99.

The stability of the derivatives used was evaluated by the repeated analysis of the same set of standard serum samples. The data listed in Table II indicate that the derivatives were stable for at least a week if the samples were tightly capped and stored at 4°C.

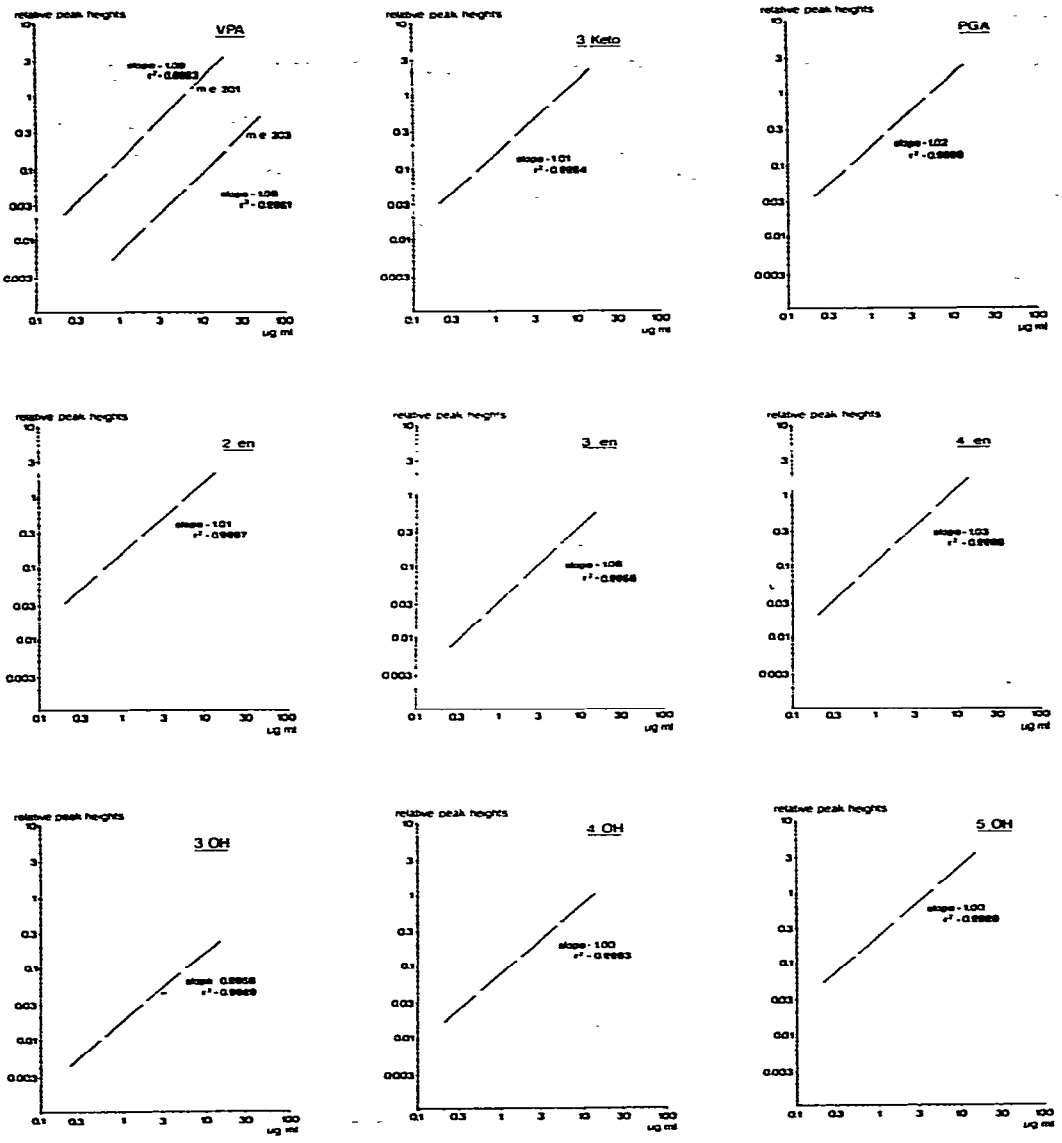

Fig. 3. Double logarithmic plots of the peak heights of the selected ions from trimethylsilylated VPA and metabolites (relative to the peak height of the trimethylsilylated internal standard used) vs. amounts of VPA and metabolites in spiked serum samples.

In spite of the simplicity of the sample-handling procedures, VPA and metabolites could be assayed in small samples of urine (Fig. 4B), serum (Fig. 4A), breast milk, and tissues due to the sensitivity and selectivity of the GC—MS method. Our method has now been in continuous operation for over a year. We have predominantly studied the metabolic profiles of VPA in human serum and urine, placental transfer, neonatal pharmacokinetic and transfer via mother's milk of VPA and metabolites as well as transfer of these substances into brain.

TABLE II

REPEATED ANALYSIS OF THE SAME SET OF STANDARD SERUM SAMPLES

Known amounts of VPA and metabolites were added to serum (0.1—30 μg/ml) for the preparation of calibration curves. GC—MS analysis was performed following the extraction and derivatization procedures. The samples were then stored at 4°C and re-analyzed three and seven days later. The slopes of the experiments on the three different days were used to document stability of the derivatives as well as reproducibility of the assay. The correlation coefficient exceeded 0.99 for all compounds.

Compound	Slope of calibration data				
	Day of analysis			Mean	Relative standard deviation (S.D. x 100/mean)
	0	3	7		
VPA	0.089	0.092	0.093	0.091	2.2
2-en	0.163	0.168	0.171	0.167	2.4
4-en	0.156	0.163	0.170	0.163	4.3
3-OH	0.023	0.022	0.021	0.022	4.5
4-OH	0.113	0.112	0.110	0.112	1.3
5-OH	0.170	0.165	0.162	0.167	2.6
PGA	0.105	0.100	0.095	0.100	5.0

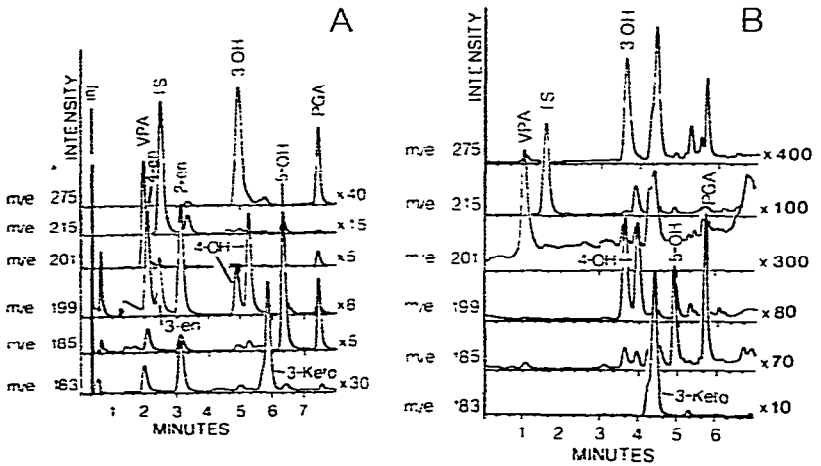

Fig. 4. Selected ion records of (A) a 200-μl serum sample to which VPA and metabolites had been added (3 μg/ml) and (B) of a 10-μl urine sample of a pregnant epileptic woman treated with VPA monotherapy (1.5 g of VPA per day). The concentrations found are: VPA, 4.0 μg/ml; 3-OH, 32.0 μg/ml; 4-OH, 32.1 μg/ml; 5-OH, 10.4 μg/ml; 3-keto, 184 μg/ml; PGA, 25.8 μg/ml. The conjugates (for example, of VPA and 2-en) had not been released by enzymatic treatment of the sample, which explains the absence of the 2-en and 3-en and the low levels of VPA.

It was found that 3-keto and 2-en (*trans*) were the major VPA metabolites in the serum of epileptic patients undergoing VPA therapy, while the 3-keto and the VPA conjugate were the major urinary metabolites. All other VPA metabolites measured were also present in serum and urine, albeit in lower

concentrations [19, 20]. Placental transfer studies indicate that VPA and some metabolites are present in cord blood in higher concentrations than in maternal blood [20]. These findings are rather unusual because most drugs are found in cord blood in concentrations that are equal to or lower than maternal blood levels. The reason for fetal accumulation of VPA and metabolites is not known. The half-lives of VPA and the two main metabolites (40—50 h) in the neonates [20] were much longer than those of adult epileptic patients (8—16 h).

Breast feeding can be performed with apparent safety because only approximately 3% of the maternal VPA levels and 7% of the maternal 3-keto levels were found in breast milk.

The methods described are also applicable without modification to the analysis of brain tissue homogenates for a study of brain levels of VPA and metabolites [21]. Also, our method can be directly applied to the analysis of VPA pharmacokinetics in patients during steady-state therapy; following administration of 1,2-^{13}C-labelled VPA the kinetics of this drug and metabolites can be studied in detail in blood and urine [19].

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REFERENCES

1 R.M. Pinder, R.N. Brogden, T.M. Speight and G.S. Avery, Drugs, 13 (1977) 81.
2 R. Gugler and G.E. von Unruh, Clin. Pharmacokin., 5 (1980) 67.
3 W. Kochen, H. Imbeck and C. Jakobs, Arzneim.-Forsch., 27 (1977) 1090.
4 H. Schäfer and R. Lührs, Arzneim.-Forsch., 28 (1978) 657.
5 C. Jakobs and W. Löscher, Epilepsia, 19 (1978) 591.
6 H. Schäfer, R. Lührs and H. Reith, in S.I. Johannessen (Editor), Antiepileptic Therapy: Advances in Drug Monitoring, Raven Press, New York, 1980, pp. 103—110.
7 F. Schobben, T.B. Vree, E. van der Kleijn, R. Claessens and W.O. Renier, in S.I. Johannessen (Editor), Antiepileptic Therapy: Advances in Drug Monitoring, Raven Press, New York, 1980, pp. 91—102.
8 T. Kuhara, Y. Hirohata, S. Yamada and I. Matsumoto, Eur. J. Drug Metab. Pharmacokin., 3 (1978) 171.
9 W. Löscher, Epilepsia, 18 (1977) 225.
10 A.J. Fellenberg and A.C. Pollard, Clin. Chim. Acta, 81 (1977) 203.
11 C. Jakobs, M. Bojasch and F. Hanefeld, J. Chromatogr., 146 (1978) 494.
12 J.-C. Libeer, S. Scharpé, P. Schepens and R. Verkerk, J. Chromatogr., 160 (1978) 285.
13 R.N. Gupta, F. Eng and M.L. Gupta, Clin. Chem., 25 (1979) 1303.
14 S. Willox and S.E. Foote, J. Chromatogr., 151 (1978) 67.
15 O. Gyllenhaal and A. Albinsson, J. Chromatogr., 161 (1978) 343.
16 A. Hulshoff and H. Roseboom, Clin. Chim. Acta, 93 (1979) 9.
17 G.A. Peyton, S.C. Harris and J.E. Wallace, J. Anal. Toxicol., 3 (1979) 108.
18 D.J. Freeman and N. Rawal, Clin. Chem., 26 (1980) 674.
19 H. Nau, H. Schäfer, D. Rating and H. Helge, in L. Bossi, M. Dam, H. Helge, D. Janz, A. Richens and D. Schmidt (Editors), Epilepsy, Pregnancy and the Child, Raven Press, New York, 1981, pp. 367—372.

20 H. Nau, W. Wittfoht, D. Rating, H. Schäfer and H. Helge, in L. Bossi, M. Dam, H. Helge, D. Janz, A. Richens and D. Schmidt (Editors), Epilepsy, Pregnancy and the Child, Raven Press, New York, 1981, pp. 131—139.
21 H. Nau, W. Löscher and H. Schäfer, unpublished results.