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Metabolic profiling of valproic acid in patients using negative-ion chemical ionization gas chromatography–mass spectrometry

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SUMMARY

A negative-ion chemical ionization gas chromatographic–mass spectrometric method for the determination of valproic acid (VPA) and fourteen of its metabolites in a single chromatographic run is reported. The assay features the use of four internal standards and is applicable to the analysis of small serum and urine volumes. A combination of pentafluorobenzyl and trimethylsilyl derivatization resulted in the $[M-181]^-$ ion as the base peak for all the metabolites measured. When these ions were monitored sensitivities in the low picogram levels were achieved. The VPA metabolite profile was determined in pediatric patients on VPA monotherapy and on combined VPA therapy with either carbamazepine or clobazam. The recently characterized diene metabolite, (*E,E*)-2,3′-diene-VPA, was found to be a major serum metabolite of VPA. In the patient groups taking VPA in combination with carbamazepine, the induction of ω and $\omega-1$ pathways of VPA metabolism was apparent, while the levels of the β-oxidation products were significantly decreased.

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INTRODUCTION

Valproic acid (VPA) is a major antiepileptic drug widely used in the treatment of a variety of seizure types [1,2]. This short-chain fatty acid is metabolized in the body by a combination of mitochondrial, microsomal and cytosolic enzymes to produce at least sixteen known metabolites [3–6]. Studies in animals indicate that several of the metabolites contribute to both the therapeutic and toxic effects of the drug. Thus, the unsaturated metabolites, 2-ene-VPA [7,8] and 2,3′-diene-VPA [9] were found to have significant anticonvulsant activity in rodent models. The other unsaturated metabolites, namely 4-ene-VPA and 2,4-diene-VPA, are hepatotoxic in the rat [10] and are thought to be responsible for the rare but fatal hepatotoxicity associated with the drug [11,12].

Partly because of their large number, the wide concentration range at which they are found in biological fluids and similarities to endogenous compounds, the simultaneous quantitation of VPA metabolites is of considerable difficulty. Published gas chromatographic–mass spectrometric (GC–MS) methods for the analysis of VPA metabolites are based on either *tert.*-butyldimethylsilyl (t-BDMS) [13] or trimethylsilyl (TMS) [14–16] derivatives. Two methods which employ TMS derivatives [14,15] do not include all the important metabolites while in a third method [16] separation was achieved by using a much longer column and GC run time compared to the others. Although the previously reported assay from our laboratory [13] was satisfactory for most metabolites, there were chromatographic problems with some of the hydroxy metabolites.

In a previous communication [17], we described the use of negative-ion chemical ionization (NICI) GC–MS for the identification of VPA metabolites as their pentafluorobenzyl (PFB) derivatives. The present paper describes the application of the NICI methodology for the simultaneous quantitation of VPA and fourteen of its metabolites in a single chromatographic run time of 25 min. The assay makes use of the inherent sensitivity and soft ionization nature of electron-capture NICI for a successful quantitation of this large array of metabolites. By a combination of PFB (carboxyl group) and TMS (hydroxyl and 3-oxo moiety) derivatization and the use of a non-polar capillary column, adequate chromatographic separation and sensitivity were achieved. The assay was employed for metabolic profiling of VPA in pediatric patients on VPA monotherapy and on combined therapy with either carbamazepine (CBZ) or clobazam (CLBZ). Potential applications of this assay include metabolism studies of individual VPA metabolites in cellular fractions, tissue distribution studies of metabolites in animals and pulse dose studies of metabolite kinetics in patients.

EXPERIMENTAL

Materials

Diisopropylethylamine, 2-methylglutaric acid (2-MGA) and pentafluorobenzyl bromide (PFBB) were obtained from Aldrich (Milwaukee, WI, U.S.A.).

N-Methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) was purchased from Pierce (Rockford, IL, U.S.A.). VPA (Depakene®) was a gift from Abbott Labs. (Montreal, Canada).

The synthesis of the following VPA metabolites used as standards has been described elsewhere [4,18]: 2-propyl-4-pentenoic acid (4-ene-VPA), 2-propyl-3-pentenoic acid (stereochemistry not determined, 3-ene-VPA), 2-propyl-2-pentenoic acid (*E*- and *Z*-isomers, 2-ene-VPA), 2-propyl-3-hydroxypentanoic acid (3-OH-VPA), 2-propyl-4-hydroxypentanoic acid as the γ-lactone (4-OH-VPA), 2-(1′-propenyl)-2-pentenoic acid (*E,E*- and *E,Z*-isomers, 2,3′-diene-VPA), 2-propyl-2,4-pentadienoic acid (*E*- and *Z*-isomers, 2,4-diene-VPA), 2-propylglutaric acid (2-PGA) and 2-propylsuccinic acid (2-PSA). 2-Propyl-3-oxopentanoic acid (3-keto-VPA) was synthesized as the ethyl ester by the alkylation of ethyl pentanoate with propionyl chloride following the general procedure of the alkylation of ester enolates [19]. 2-Propyl-5-hydroxypentanoic acid (5-OH-VPA) was synthesized by the hydroboration of 4-ene-VPA [20]. 2-Propyl-4-oxopentanoic acid (4-keto-VPA) was synthesized by the palladium-catalyzed oxidation of 4-ene-VPA according to the method of Tsuji [21]. All the metabolites were obtained as free acids with the exception of 3-keto-VPA, 5-OH-VPA and 2,4-diene-VPA which were in the form of ethyl esters. Stock solutions were prepared from these esters by treating the required amounts with excess aqueous sodium hydroxide. Four internal standards were employed in the assay; di-([3,3,3-^{2}H]propyl)acetic acid ([$^2H_6$]VPA) [22], [3,5,5-^{2}H]3-heptene-4-carboxylic acid ([$^2H_3$]2-ene-VPA) [13], 2-MGA and [3′,3′,3′-^{2}H]2-propyl-3-oxopentanoic acid ([$^2H_3$]3-keto-VPA).

Patients and volunteers

Patients studied were regular visitors of the Seizure Clinic of British Columbia's Children's Hospital and included 26 receiving VPA only and 33 receiving VPA in combination with CBZ or CLBZ. The ages of the patients ranged from 1.0 to 19.8 years. The mean VPA daily doses were 32.9 mg/kg for the polytherapy group and 18.6 mg/kg for the monotherapy group. Metabolite concentrations were determined in serum (trough) and overnight urine samples. One healthy male volunteer weighing 70 kg participated in a study that sought to determine the washout kinetics, serum free and saliva concentrations of VPA metabolites. The volunteer was given an oral dose of 350 mg of VPA in the form of a solution for a total of six consecutive doses. Blood was collected in sterile, non-heparinized vacutainers at convenient intervals starting at 0.5 h and continuing to day 14 post-dose. The blood samples were allowed to clot and serum was obtained after centrifugation. Saliva samples were collected following stimulation with 5% citric acid solution and were taken simultaneous to blood samples up to 48 h post-dose.

Serum and urine standards

A reference standard was prepared by spiking VPA and the fourteen metabolites in either control serum or urine. Six standards including a blank sample were prepared for each run by taking aliquots of standard serum or urine and making up to a final volume of 250 μl with control serum or urine. The concentrations for the serum standards making up the calibration curves were as follows: 24.8, 49.6, 74.4, 99.2 and 124 μg/ml for VPA; 0.030, 0.300, 0.600, 0.900 and 1.20 μg/ml for 4-ene-VPA; 0.394, 0.788, 1.18, 1.58 and 1.97 μg/ml for 3-ene-VPA; 0.016, 0.162, 0.324, 0.486 and 0.648 μg/ml for (*Z*)-2-ene-VPA; 3.07, 6.16, 9.23, 12.3 and 15.4 μg/ml for (*E*)-2-ene-VPA; 3.10, 6.20, 9.30, 12.4 and 15.5 μg/ml for (*E,E*)-2,3′-diene-VPA; 0.016, 0.160, 0.320, 0.480 and 0.640 μg/ml for (*E,Z*)-2,3′-diene-VPA; 0.204, 0.408, 0.612, 0.816 and 1.02 μg/ml for (*E*)-2,4-diene-VPA; 0.380, 0.760, 1.14, 1.52 and 1.90 μg/ml for 4-OH-VPA; 0.196, 0.392, 0.588, 0.784 and 0.980 μg/ml for 3-OH-VPA; 0.320, 0.640, 0.960, 1.28 and 1.60 μg/ml for 5-OH-VPA; 0.208, 0.416, 0.624, 0.832 and 1.04μg/ml for 4-keto-VPA; 2.0, 4.0, 6.0, 8.0 and 10.0 μg/ml for 3-keto-VPA; 0.20, 0.40, 0.60, 0.80 and 1.0 μg/ml for 2-PGA; and 0.020, 0.198, 0.396, 0.594 and 0.792 μg/ml for 2-PSA.

Stock solutions of the internal standards were prepared in distilled water at a concentration of 50 μg/ml for each of [$^2H_3$]2-ene-VPA, [$^2H_3$]3-keto-VPA and [$^2H_6$]VPA and 10 μg/ml for 2-MGA. Equal volumes of each internal standard from the above solutions were mixed and a 100-μl aliquot was added to each sample.

The calibration curves were obtained by plotting the peak-area ratio of VPA or metabolite to that of the internal standard versus the concentration of the compound by a computer program written in our laboratory and which also provided a reading of sample concentration. [$^2H_6$]VPA and [$^2H_3$]2-ene-VPA served as the internal standards for VPA and the unsaturated metabolites, respectively. [$^2H_3$]3-Keto-VPA was used to quantitate 3-keto-VPA while 2-MGA served as the internal standard for the dicarboxylic acid metabolites. [$^2H_3$]2-Ene-VPA also served as the internal standard for the hydroxy metabolites and 4-keto-VPA.

Sample preparation

Depending on the amount of available serum, 50–250 μl of serum sample were taken, internal standards added, and the pH was adjusted to 2 with 3 *M* hydrochloric acid. The sample was allowed to sit at room temperature for 10 min and extracted with 500 μl of ethyl acetate by gentle rotation of the phases for 20 min. To increase the recovery of the analytes the extraction step was repeated with another 500 μl of ethyl acetate. After each extraction step the sample was centrifuged at 450 *g* for 10 min in order to break the emulsion formed. The combined organic layer was then transferred to a 3.5-ml screw-cap vial containing anhydrous sodium sulfate, vortex-mixed and centrifuged

at 450 *g* for 20 min. The supernatant was then transferred to another vial and the volume reduced to about 200 μl under nitrogen.

Saliva samples (1 ml) were prepared in exactly the same way as serum samples. The unbound concentration in serum was determined after ultrafiltration. Ultrafiltration was carried out with YMT ultrafiltration membranes in an MPS-1 micropartition system (Amicon, Danvers, MA, U.S.A.). Centrifugation was carried out at 1000 *g* at ambient temperature.

To determine total urinary levels of VPA and metabolites, urine samples (50–250 μl) were treated with 3 *M* sodium hydroxide (pH 12–13) and incubated at 60°C for 60 min before their pH was adjusted to 2 with 3 *M* hydrochloric acid and extracted with ethyl acetate. For free urine concentration measurements, urine samples were treated as for serum except for a single extraction with ethyl acetate. The concentration of metabolites as conjugates was determined from the difference between total and free urine concentrations.

To form the PFB derivatives of the carboxyl groups the concentrated extract was transferred to a 1-ml conical reaction vial and 10 μl of diisopropylethylamine (neat) and 10 μl of 30% PFBB solution in ethyl acetate were added. The sample was heated in a heating block at 40°C for 45 min. The PFB esters were then treated with 50 μl of MSTFA and heated at 60°C for 30 min to silylate metabolites containing the hydroxyl and 3-oxo moiety.

Instrumentation

Capillary column GC–MS was performed on a Hewlett-Packard 5987A gas chromatograph–mass spectrometer fitted with a DB-1 column, 30 m × 0.32 mm I.D. with a film thickness of 0.25 μm (J. and W. Scientific, Folsom, CA, U.S.A.). The temperature program was: oven temperature, 110 to 140°C at 30°C/min, 140 to 260°C at 5°C/min; source temperature, 200°C; open split interface, 250°C; injection port temperature, 240°C. The carrier gas was helium at a head pressure of 68.9 kPa. MS operating parameters in the negative-ion mode were as follows: ionization energy, 200 eV; emission current, 250 μA; source pressure, 106–160 Pa; reagent gas, methane. Before each run negative-ion response was optimized first using the calibration gas and then a test sample of PFB-derivatized VPA. A 1-μl volume of the derivatized sample was injected into the GC–MS system in the splitless mode using an automatic sample injector (Hewlett-Packard Model 7673A). The total run time was 25 min.

RESULTS AND DISCUSSION

Negative-ion GC–MS properties of VPA metabolites

The PFB derivatives of the metabolites and the parent drug have excellent negative-ion response as reported earlier [17]. With the DB-1 column, however, the PFB derivatives of the hydroxy metabolites chromatograph poorly and sensitivity was compromised. Trimethylsilylation of the hydroxyl groups

after PFB derivatization of the carboxyl moiety greatly improved both peak shape and sensitivity of detection. The PFB derivative of 3-keto-VPA, unlike the other metabolites, fragments to yield the $[M-181-CO_2]^-$ (M−181 corresponds to the loss of the pentafluorobenzyl moiety) ion as the base peak [17]. This fragmentation was blocked with TMS derivatization of the 3-oxo moiety, resulting in the $[M-181]^-$ ion as almost the only ion in the mass spectrum. Hence, selected-ion monitoring (SIM) chromatograms were obtained by monitoring the $[M-181]^-$ anions which are the base peaks of all the metabolites studied, i.e. both PFB esters and O-TMS-PFB derivatives. The negative ions monitored are given in Table I. The negative-ion spectra obtained for VPA and its metabolites are typified by the example in Fig. 1, included here since the spectra of the O-TMS-PFB derivatives have not been reported. Thus, the combination of PFB and TMS derivatization produced suitable negative ions and chromatographic characteristics which allowed the development of a simultaneous assay of VPA and fourteen metabolites. Typical SIM chromatograms representing all the metabolites assayed are shown in Fig. 2.

The use of the DB-1 column and PFB derivatization enabled the complete separation of 4-ene-VPA from VPA as well as the separation of the monounsaturated metabolites as seen in Fig. 2A, *m/z* 141. PFB derivatization also re-

TABLE I

IONS MONITORED (*m/z*) IN THE NICI MODE FOR VPA AND METABOLITES DERIVATIZED WITH PFB AND TMS

Compound	$[M-181]^-$
(*E,Z*)-2,3′-Diene-VPA	139
(*E*)-2,4-Diene-VPA	139
(*E,E*)-2,3′-Diene-VPA	139
4-Ene-VPA	141
3-Ene-VPA	141
(*Z*)-2-Ene-VPA	141
(*E*)-2-ene-VPA	141
VPA	143
4-Keto-VPA	157
3-Keto-VPA[a]	229
4-OH-VPA[a,b]	231
3-OH-VPA[a]	231
5-OH-VPA[a]	231
2-PSA[c]	339
2-PGA[c]	353

[a]O-TMS-PFB derivatives.
[b]Two isomers.
[c]Di-PFB derivatives.

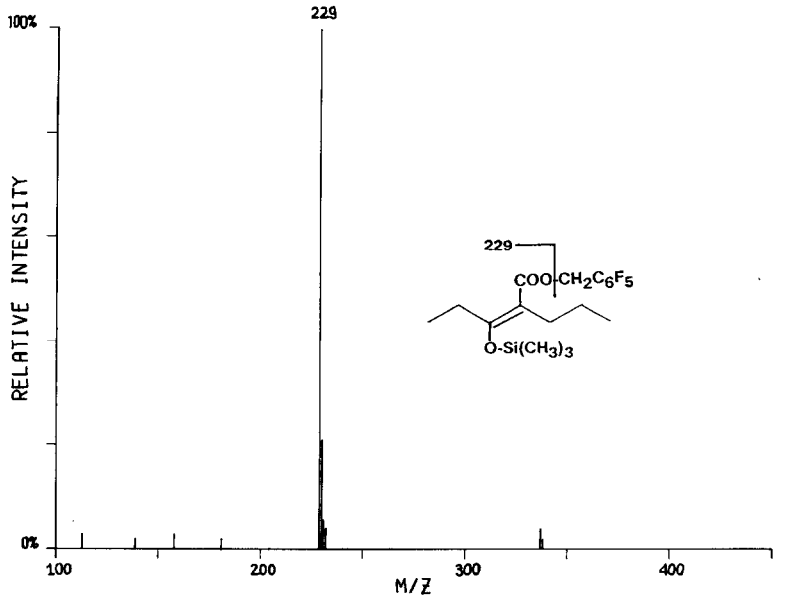

Fig. 1. NICI mass spectrum of the O-TMS-PFB derivative of 3-keto-VPA.

sulted in baseline separation of the geometric and/or positional isomers of the diunsaturated metabolites (Fig. 2A, *m/z* 139). The putative toxic metabolite, (*E*)-2,4-diene-VPA is clearly separated from (*E,Z*)-2,3′-diene-VPA. When the t-BDMS and TMS derivatives were used and analyzed under similar conditions, these two metabolites overlap and being isobaric are counted as one.

In the case of 4-OH-VPA only the open chain form was monitored since the γ-lactone would be transparent to NICI. Because the base diisopropylethylamine was used for the derivatization reaction, it was assumed that most of the 4-OH-VPA would be in the open chain form. Greater than 90% of the 3-keto-VPA formed a diderivative, i.e. O-TMS-PFB. Formation of a small amount of 3-keto-VPA monoderivative did not present a problem because a deuterated analogue was used as the internal standard. Even though the two geometric isomers of the O-TMS-PFB derivative of 3-keto-VPA were formed, only one isomer, which accounted for 93% of the ion current, was monitored.

Analytical parameters

Calibration curve linearity was observed over the concentration ranges tested. Non-linear response was evident in the case of VPA when serum volumes of greater than 250 μl were used. Correlation coefficients were typically greater than 0.99 for all standard curves. The method showed good precision with a day-to-day variation of less than 15% for all fifteen analytes. The day-to-day precision and accuracy of the assay for individual metabolites is shown in Ta-

ble II. The precision of the assay for analytes having deuterated analogues as internal standards (VPA, 2-ene-VPA, 3-keto-VPA) was especially high, although this could be attributable to the higher levels measured for these metabolites. For 4-OH-VPA it was necessary to maintain the extraction pH fairly constant in order to obtain reproducible results. A deuterated internal standard for 4-OH-VPA would eliminate this problem.

Trace metabolites such as 4-ene-VPA and (*E*,*Z*)-2,3′-diene-VPA were readily quantitated from 50 μl of serum. The lower limit of detection of the metabolites (signal-to-noise ratio >3) varied between 1 to 8 ng/ml based on a 250-μl sample of serum.

VPA metabolite profiles in pediatric patients

The assay was employed to determine the levels of VPA and fourteen of its metabolites in the serum and urine of 59 pediatric patients. The serum concentrations for 26 patients on VPA monotherapy are shown in Table III. The

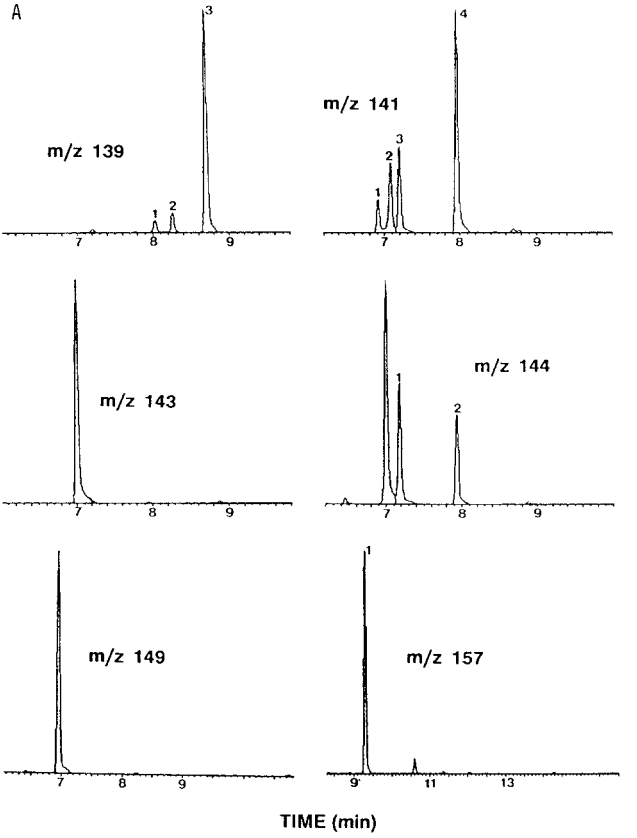

Fig. 2.

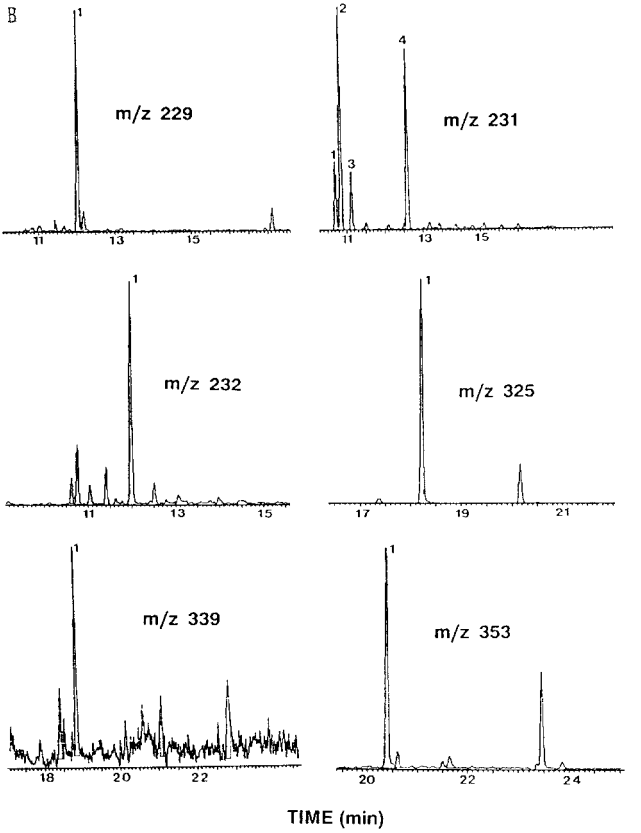

Fig. 2. (A) Typical SIM chromatograms obtained from the serum extract of a patient on VPA to which internal standards have been added. Peaks: *m/z* 139: 1 = (*E,Z*)-2,3′-diene-VPA; 2 = (*E*)-2,4-diene-VPA; 3 = (*E,E*)-2,3′-diene-VPA; *m/z* 141: 1 = 4-ene-VPA; 2 = 3-ene-VPA; 3 = (*Z*)-2-ene-VPA; 4 = (*E*)-2-ene-VPA; *m/z* 143: VPA; *m/z* 144: 1 = (*Z*)-[$^2H_3$]2-ene-VPA; 2 = (*E*)-[$^2H_3$]2-ene-VPA; *m/z* 149: [$^2H_6$]VPA; *m/z* 157: 1 = 4-keto-VPA. (B) Typical SIM chromatograms obtained from the serum extract of a patient on VPA to which internal standards have been added (same run as in A). Peaks: *m/z* 229: 1 = 3-keto-VPA; *m/z* 231: 1 and 3 = 4-OH-VPA; 2 = 3-OH-VPA; 4 = 5-OH-VPA; *m/z* 232: 1 = [$^2H_3$]3-keto-VPA; *m/z* 325: 1 = 2-MGA; *m/z* 339: 1 = 2-PSA; *m/z* 353: 1 = 2-PGA.

major metabolites in serum were (*E*)-2-ene-VPA, (*E,E*)-2,3′-diene-VPA and 3-keto-VPA. (*E*)-2-Ene-VPA and 3-keto-VPA are *β*-oxidation products [23], whereas the origin of (*E,E*)-2,3′-diene-VPA is unknown although its concentration pattern suggests it might be a derivative of (*E*)-2-ene-VPA probably via 3-ene-VPA [24]. The putative toxic metabolite, 4-ene-VPA, was present at low levels (mean = 0.20 *μ*g/ml). The mean concentration of the diene metabolite [(*E*)-2,4-diene-VPA] suspected to be a hepatotoxin was 0.65 *μ*g/ml. The serum concentration values obtained for most of the metabolites in this

TABLE II

DAY-TO-DAY PRECISION AND ACCURACY DATA FOR VPA AND METABOLITES IN SERUM ($n=5$)

Compound	Spiked (μg/ml)	Found (μg/ml)	Coefficient of variation (%)
4-Ene-VPA	0.30	0.31	6.2
	1.20	1.24	1.4
3-Ene-VPA	0.39	0.38	10.2
	1.97	2.04	2.2
(*Z*)-2-Ene-VPA	0.16	0.15	6.0
	0.65	0.67	1.1
(*E*)-2-Ene-VPA	3.07	3.06	2.7
	15.39	15.52	0.8
(*E,E*)-2,3′-Diene-VPA	3.10	3.20	6.2
	15.50	16.26	1.8
(*E,Z*)-2,3′-Diene-VPA	0.18	0.21	11.0
	0.64	0.62	2.0
(*E*)-2,4-Diene-VPA	0.20	0.19	12.3
	1.02	1.08	3.7
VPA	24.88	27.70	2.0
	124.40	125.50	1.5
4-OH-VPA	0.38	0.37	11.8
	1.90	2.01	1.3
3-OH-VPA	0.19	0.22	6.1
	0.98	0.98	3.6
5-OH-VPA	0.80	0.95	13.8
	1.60	1.50	6.1
4-Keto-VPA	0.21	0.23	14.6
	1.04	1.04	3.9
3-Keto-VPA	2.00	2.50	2.8
	10.00	9.72	1.3
2-PSA	0.19	0.17	15.6
	0.60	0.58	8.5
2-PGA	0.20	0.20	15.3
	1.00	1.05	2.4

study compare favorably to those previously reported by Abbott et al. [13] and Tatsuhara et al. [15].

The concentrations of metabolites (normalized to dose) in the patient groups on combined therapy with other antiepileptic drugs is shown in Table IV. The serum levels of 4-keto-VPA and 5-OH-VPA were significantly ($P<0.05$) higher in the VPA plus CBZ group compared to the monotherapy group, suggesting the induction of ω (5-OH-VPA) and $\omega-1$ (4-keto-VPA) pathways, consistent with the fact that CBZ induces VPA metabolism [25]. Also, the serum

TABLE III

SERUM CONCENTRATIONS OF VPA METABOLITES IN 26 PEDIATRIC PATIENTS ON VPA MONOTHERAPY

Compound	Concentration (μg/ml)		Percentage of VPA
	Mean	Range	
4-Ene-VPA	0.20	0.04–0.40	0.2
3-Ene-VPA	1.80	0.72–3.30	2.0
(*Z*)-2-Ene-VPA	0.11	0.05–0.18	0.1
(*E*)-2-Ene-VPA	6.44	2.51–10.30	7.2
(*E*,*E*)-2,3′-Diene-VPA	6.31	2.47–13.50	7.1
(*E*,*Z*)-2,3′-Diene-VPA	0.28	0.06–0.38	0.3
(*E*)-2,4-Diene-VPA	0.65	0.15–1.16	0.7
VPA	89.2	39.8 –134.0	100
4-OH-VPA	0.71	0.14–1.64	0.8
3-OH-VPA	0.97	0.10–2.27	1.1
5-OH-VPA	0.45	0.13–0.96	0.5
4-Keto-VPA	0.60	0.16–1.27	0.7
3-Keto-VPA	3.63	0.78–8.24	4.1
2-PSA	0.02	Trace–0.15	0.02
2-PGA	0.16	0.02–0.30	0.2

TABLE IV

MEAN CONCENTRATIONS (μg/ml) NORMALIZED TO DOSE (mg/kg) OF VPA AND METABOLITES IN THE SERUM OF PATIENTS

Compound	VPA only ($n=26$)	VPA+CBZ ($n=21$)	VPA+CLBZ ($n=12$)
4-Ene-VPA	0.009	0.007	0.008
3-Ene-VPA	0.109[a]	0.042	0.086
(*Z*)-2-Ene-VPA	0.005[a]	0.002	0.005
(*E*)-2-Ene-VPA	0.311[a]	0.136	0.274
(*E*,*E*)-2,3′-Diene-VPA	0.304[a]	0.129	0.201
(*E*,*Z*)-2,3′-Diene-VPA	0.012[a]	0.004	0.007
(*E*)-2,4-Diene-VPA	0.035	0.029	0.029
VPA	4.88[a]	2.31	3.82
4-OH-VPA	0.053	0.051	0.046
3-OH-VPA	0.064	0.049	0.038
5-OH-VPA	0.022	0.031[a]	0.028
4-Keto-VPA	0.030	0.049[a]	0.026
3-Keto-VPA	0.311[a]	0.126	0.102
2-PSA	0.005	0.006	0.004
2-PGA	0.010	0.007	0.006

[a]Significantly different at $P<0.05$ (VPA only versus VPA+CBZ).

TABLE V

URINARY CONCENTRATIONS OF THE CONJUGATED AND UNCONJUGATED FRACTIONS OF VPA METABOLITES IN NINE PEDIATRIC PATIENTS ON VPA MONOTHERAPY

Compound	Concentration (μg/mg of creatinine)			
	Conjugated fraction		Unconjugated fraction	
	Mean	Range	Mean	Range
4-Ene-VPA	0.25	0.02-1.53	0.03	Trace-0.17
3-Ene-VPA	0.21	0.03-0.93	0.05	Trace-0.28
(*Z*)-2-Ene-VPA	0.94	0.10-2.77	0.03	Trace-0.22
(*E*)-2-Ene-VPA	21.5	1.0 -54.3	0.92	Trace-5.73
(*E,E*)-2,3′-Diene-VPA	17.2	3.85-56.9	1.12	Trace-6.12
(*E,Z*)-2,3′-Diene-VPA	1.23	0.24-3.56	0.16	Trace-0.26
(*E*)-2,4-Diene-VPA	3.68	0.18-11.5	0.52	Trace-3.17
VPA	262	15.6 -1191	15.3	0.43-96
4-OH-VPA	17.2	0.45-79.4	29.7	3.16-94.9
3-OH-VPA	10.6	0.83-26.7	10.1	5.01-18.4
5-OH-VPA	5.21	Trace-16.30	12.6	2.56-34.8
4-Keto-VPA	0.51	Trace-3.60	9.64	1.09-28.1
3-Keto-VPA	7.93	Trace-28.0	160	33.9 -362
2-PSA	0.23	Trace-1.48	1.57	0.23-5.66
2-PGA	6.45	1.49-25.1	32.0	4.26-66.2

concentrations of VPA and its β-oxidation products were lower in the VPA plus CBZ group. The decrease in the serum concentration of 2-ene-VPA when VPA was coadministered with CBZ was also documented in a study in adult volunteers [26]. CLBZ had no significant effect on the VPA metabolite profile.

In urine the major metabolites were 3-keto-VPA, 4-OH-VPA and 2-PGA (Table V). The unsaturated metabolites were excreted mainly as conjugates, while the keto metabolites were predominantly excreted in the free form. The hydroxy metabolites and 2-PGA were found in urine in both the conjugated and free forms.

Wash-out profile of VPA metabolites in a volunteer

The wash-out serum concentration–time course for VPA and its metabolites is shown in Fig. 3. This was obtained after four days of VPA administration totaling six doses. Because of the excellent sensitivity of the assay the levels of three of the metabolites could be followed up to fourteen days and that of most of the other metabolites up to seven days after the last VPA dose. The elimination half-lives of the active metabolites (*E*)-2-ene-VPA and (*E,E*)-2,3′-diene-VPA were not different from the parent drug in this volunteer. Even though no inference can be made about the half-lives of the metabolites be-

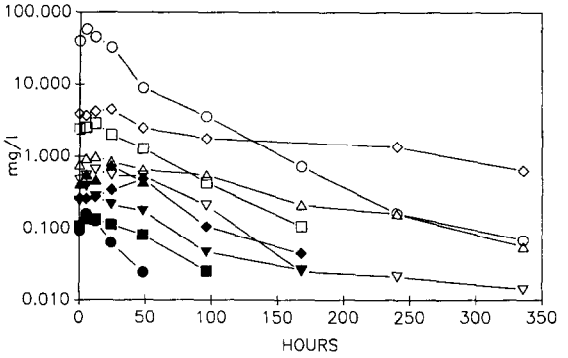

Fig. 3. Wash-out profile of VPA and its metabolites in the serum of a volunteer following the last dose of a regimen consisting of 350 mg of VPA twice daily for three days. (○) VPA; (●) 4-ene-VPA; (△) 3-ene-VPA; (▲) (*Z*)-2-ene-VPA; (□) (*E*)-2-ene-VPA; (■) (*E*)-2,4-diene-VPA; (▽) (*E,E*)-2,3′-diene-VPA; (▼) 4-keto-VPA; (◇) 3-keto-VPA; (◆) 3-OH-VPA.

TABLE VI

TIME-AVERAGED ($n=7$) CONCENTRATIONS OF VPA AND ITS METABOLITES IN SERUM AND SALIVA

Compound	Concentration (μg/ml)			Free/total ratio	Saliva/free ratio
	Total	Free	Saliva		
4-Ene-VPA	0.140	0.025	0.005	0.1786	0.2000
3-Ene-VPA	0.793	0.022	0.007	0.0277	0.3182
(*Z*)-2-Ene-VPA	0.486	0.074	0.024	0.1523	0.3243
(*E*)-2-Ene-VPA	2.57	0.009	0.003	0.0035	0.3333
(*E,E*)-2,3′-Diene-VPA	0.586	0.041	0.007	0.0700	0.1707
(*E*)-2,4-Diene-VPA	0.123	0.008	0.003	0.0650	0.3750
VPA	56.1	4.03	1.14	0.0720	0.2829
4-OH-VPA	0.310	0.134	0.128	0.4323	0.9552
3-OH-VPA	0.243	0.053	0.042	0.2181	0.7925
5-OH-VPA	0.106	0.050	0.020	0.4717	0.4000
4-Keto-VPA	0.293	0.106	0.020	0.3618	0.1887
3-Keto-VPA	3.66	0.750	0.380	0.2049	0.5067
2-PGA	0.160	0.058	–	0.3625	–

cause this study was done in but one volunteer, this example demonstrates the suitability of the assay for use in an expanded study to determine the wash-out kinetics of the metabolites.

The time-averaged serum total, serum free and saliva concentrations of VPA and metabolites are shown in Table VI. The serum free fraction was lowest for the two unsaturated metabolites, 3-ene-VPA and (*E*)-2-ene-VPA. The other unsaturated metabolites had similar free fractions to the parent drug. The hy-

droxy and to a certain extent the keto metabolites had relatively higher free fractions, with 5-OH-VPA showing a free fraction of nearly 0.5.

The saliva concentrations of the majority of the metabolites and the parent drug were in the range of 17–40% of the serum free concentration. The hydroxy metabolites, 3-OH-VPA and 4-OH-VPA, exhibited the highest saliva to serum free ratios (79 and 95%, respectively). For 3-keto-VPA and 4-OH-VPA the saliva-to-serum total ratio was very similar to the cerebrospinal fluid (CSF) to serum total ratio reported by Loscher et al. [27] in epileptic children, suggesting that the saliva concentrations of these metabolites reflect their CSF concentrations.

In summary the NICI assay described here is applicable for routine metabolic screening of patients on VPA therapy since it is highly sensitive, includes most of the known metabolites, and separation of the metabolites including the various diunsaturated metabolites is achieved in a reasonable run time.

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REFERENCES

1 P. Loiseau, Epilepsia, 25 (Suppl.1) (1984) S65.
2 O. Dulac, D. Steru, E. Rey, A. Perret and M. Arthuis, Brain Dev., 8 (1986) 47.
3 R. Gugler and G.E. von Unruh, Clin. Pharmacokin., 5 (1980) 67.
4 A. Acheampong, F. Abbott and R. Burton, Biomed. Mass Spectrom., 10 (1983) 586.
5 A. Acheampong and F.S. Abbott, J. Lipid Res., 26 (1985) 1002.
6 A.E. Rettie, A.W. Rettenmeier, W.N. Howald and T.A. Baillie, Science, 235 (1987) 890.
7 W. Loscher, Arch. Int. Pharmacodyn., 249 (1981) 158.
8 W. Loscher and H. Nau, Neuropharmacology, 24 (1985) 427.
9 F.S. Abbott and A.A. Acheampong, Neuropharmacology, 27 (1988) 287.
10 J.W. Kesterson, G.R. Granneman and J.M. Machinist, Hepatology, 4 (1984) 1143.
11 H.J. Zimmerman and K.G. Ishak, Hepatology, 2 (1982) 591.
12 A.W. Rettenmeier, W.P. Gordon, K.S. Prickett, R.H. Levy and T.A. Baillie, Drug Metab. Dispos., 14 (1986) 454.
13 F.S. Abbott, J. Kassam, A. Acheampong, S. Ferguson, S. Panesar, R. Burton, K. Farrell and J. Orr, J. Chromatogr., 375 (1986) 285.
14 H. Nau, W. Wittfoht, H. Schafer, C. Jakobs, D. Rating and H. Helge, J. Chromatogr., 226 (1981) 69.
15 T. Tatsuhara, H. Muro, Y. Matsuda and Y. Imai, J. Chromatogr., 399 (1987) 183.
16 A.W. Rettenmeier, W.N. Howald, R.H. Levy, D.J. Witek, W.P. Gordon, D.J. Porubek and T.A. Baillie, Biomed. Environ. Mass Spectrom., 18 (1989) 192.

17 K. Kassahun, R. Burton and F.S. Abbott, Biomed. Environ. Mass Spectrom., 18 (1989) 918.
18 R.D. Lee, K. Kassahun and F.S. Abbott, J. Pharm. Sci., 78 (1989) 667.
19 R.J. Cregge, J.L. Herrmann, C.S. Lee, J.E. Richman and R.H. Schlessinger, Tetrahedron Lett., 26 (1973) 2425.
20 A.W. Rettenmeier, K.S. Prickett, W.P. Gordon, S.M. Bjorge, S.L. Chang, R.H. Levy and T.A. Baillie, Drug Metab. Dispos., 13 (1985) 81.
21 J. Tsuji, Synthesis, 5 (1984) 369.
22 A.A. Acheampong, F.S. Abbott, J.M. Orr, S.M. Ferguson and R.W. Burton, J. Pharm. Sci., 73 (1984) 489.
23 W. Loscher, Epilepsia, 22 (1981) 169.
24 A.W. Rettenmeier, W.P. Gordon, H. Barnes and T.A. Baillie, Xenobiotica, 17 (1987) 1147.
25 S.K. Panesar, J.M. Orr, K. Farrell, R.W. Burton, K. Kassahun and F.S. Abbott, Br. J. Clin. Pharmacol., 27 (1989) 323.
26 S.K. Panesar, J.M. Orr, K. Farrell, R.W. Burton and F.S. Abbott, Br. J. Clin. Pharmacol., submitted for publication.
27 W. Loscher, H. Nau and H. Siemes, Epilepsia, 29 (1988) 311.