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Metabolic profiling of valproic acid by cDNA-expressed human cytochrome P450 enzymes using negative-ion chemical ionization gas chromatography–mass spectrometry

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Abstract

A sensitive negative ion chemical ionization (NCI) gas chromatographic–mass spectrometric (GC–MS) method was modified for the quantitation of valproic acid (VPA) metabolites generated from in vitro cDNA-expressed human microsomal cytochrome P450 incubations. The use of the inherent soft ionization nature of electron-capture NCI to achieve high sensitivity enabled us to conduct kinetic studies using small amounts of recombinant human P450 enzymes. The assay is based on the selective ion monitoring of the intense [M−181] fragments of pentafluorobenzyl (PFB) esters in the NCI mode, and has the following features: (1) a micro-extraction procedure to isolate VPA metabolites from small incubation volumes (100 μl); (2) a second step derivatization with *tert*.-butyldimethylsilylating reagents to enhance sensitivity for hydroxylated metabolites; (3) a short run-time (<30 min) while maintaining full separation of 15 VPA metabolites by using a narrow-bore non-polar DB-1 column plus a new temperature gradient; and (4) good reproducibility and accuracy (intra- and inter-assay RSDs <15%, bias <15%) by using seven deuterated derivatives of analytes as internal standards. The derivatives of mono- and diunsaturated metabolites, like the parent drug, produced abundant $[M-181]^-$ ions while the hydroxylated metabolites gave an ion at m/z of 273, corresponding to the $[M-181]^-$ ion of the *tert*.-butyldimethylsilyl ethers. In conclusion, the GC–NCI-MS analysis of valproate metabolites provided us with a high resolution and sensitivity necessary to conduct metabolic and kinetic studies of valproic acid in small volume samples typical of the in vitro cDNA-expressed micro-incubation enzymatic systems.

Keywords: Valproic acid; Cytochrome P450; Enzymes

1. Introduction

Valproic acid (VPA; 2-*n*-propylpentanoic acid) is a C-8 branched-chain carboxylic acid used widely for the treatment of a variety of seizure disorders [1]. Despite its simple molecular structure, valproic acid metabolism is extremely complex and a variety of overlapping phase I and II metabolic pathways are known to catalyze its biotransformation to over 50 known metabolites [2]. Moreover, the small molecular size and structural similarity of these metabolites along with the presence of several stereo- and geometric isomers have added a significant level of complexity to the identification and profiling of VPA

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metabolites. The advent of gas chromatographic–mass spectrometric (GC–MS) analytical methodologies has greatly facilitated the characterization and quantification of numerous VPA metabolites [3–12]. This has lead to the identification of the VPA metabolic pathways in various experimental animals and humans [6,12–14], although the enzymatic pathways involved in the formation of certain metabolites have yet to be fully understood. Such information is of great toxicological and clinical interest, as several VPA metabolites are known to exert anticonvulsant activity [1] or are considered to be responsible for valproate neurotoxic and hepatic side effects [15,16].

Recent advances in molecular biological techniques have provided a pure supply of cDNA-expressed human drug metabolizing enzymes, which have been used as valuable tools for high throughput screening of drug metabolizing enzymes as well as mechanistic and metabolic profiling studies. In this context, a preliminary in vitro metabolic study using a panel of 14 human cytochrome P450 enzymes and our earlier GC–negative ion chemical ionization (NCI) MS analytical technique [10] showed the possible involvement of a few human cytochrome P450 enzymes in the metabolic activation of VPA. However, the full enzyme kinetic metabolic studies could not be carried out in this in vitro model due to the low concentration of metabolites formed from the micro-incubation systems that contain low amounts of human cDNA-expressed enzymes. The purpose of the present study was to develop a highly sensitive and selective GC–MS analytical method, suitable for in vitro metabolic studies of valproic acid.

2. Experimental

2.1. Reagents

tert.-Butyldimethylsilyl chloride, diisopropylethylamine and pentafluorobenzyl bromide were obtained from Aldrich (Milwaukee, WI, USA). *N*-(*tert*.-Butyldimethylsilyl)-*N*-methyltrifluoroacetamide (MTBSTFA) and dimethylformamide were obtained from Pierce (Rockford, IL, USA). VPA and the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) were obtained from Sigma (St. Louis, MO, USA).

2.2. VPA metabolites

The VPA metabolites used as reference standards were synthesized in this laboratory. The syntheses of the following standards have been described elsewhere [5,17]: 2-propyl-3-hydroxypentanoic acid (3-OH-VPA), 2-propyl-4-hydroxypentanoic acid (4-OH-VPA), 2-propylglutaric acid (2-PGA), 2-propylsuccinic acid (2-PSA), 2-propyl-4-pentenoic acid (4-ene-VPA), 2-propyl-3-pentenoic acid (3-ene-VPA), 2-propyl-2-pentenoic acid (2-ene-VPA), 2-propyl-4-oxopentanoic acid (4-keto-VPA), 2-(1′-propenyl)-2-pentenoic acid (*E*,*Z*-, and *E*,*E*-isomers of 2,3′-diene-VPA). 2-Propyl-3-oxo-pentanoic acid (3-keto-VPA) was synthesized as the ethyl ester by the alkylation of ethyl pentanoate with propionyl chloride following the general procedure of alkylation of ester enolates [17]. 2-Propyl-5-hydroxypentanoic acid (5-OH-VPA) was synthesized by the hydroboration of 4-ene-VPA [18].

2.3. Internal standards

The following heptadeuterated compounds were prepared in this laboratory as described elsewhere [19]: 2-[$^2H_7$]propylpentanoic acid ([$^2H_7$]VPA), 2-[$^2H_7$]propyl-2-pentenoic acid (2-ene-[$^2H_7$]VPA), 2-[$^2H_7$]propyl-3-oxo-pentanoic acid (3-keto-[$^2H_7$]VPA), 2-[$^2H_7$]propyl-4-oxo-pentanoic acid (4-keto-[$^2H_7$]VPA), 2-[$^2H_7$]propyl-3-hydroxypentanoic acid (3-OH-[$^2H_7$]VPA), 2-[$^2H_7$]propyl-4-hydroxypentanoic acid (4-OH-[$^2H_7$]VPA), 2-[$^2H_7$]propyl-5-hydroxypentanoic acid (5-OH-[$^2H_7$]VPA).

2.4. Metabolic reaction conditions

Microsomal incubations were carried out for 15 min at 37°C, in a final volume of 0.1 ml in 1.5-ml Eppendorf tubes containing 10 μmol of potassium phosphate (pH 7.4), 0.02–1 μmol of VPA, 0.1 μmol of NADPH and 5 pmol of human cDNA-expressed cytochrome P450 enzymes. Incubation mixtures were equilibrated for 2 min at 37°C prior to the initiation of reaction with NADPH.

2.5. Derivatization

Samples of microsomal incubations were cooled on ice for 15 min and 100 μl of internal standards

was added to each Eppendorf tube. The mixture was acidified with 0.5 *M* HCl to pH 2–3 and extracted with ethyl acetate (300 μl) by gentle rotation of the phases for 30 min. The organic extract was transferred to another Eppendorf tube containing anhydrous sodium sulfate, vortex mixed, and centrifuged at 325×*g* for 5 min. The dried ethyl acetate extract (100–200 μl) was transferred to a conical Reacti-Vial for the PFB ester derivatization. Diisopropylethylamine (30 μl) and PFB bromide (30 μl of 30%, v/v, solution in ethyl acetate) were added to the mixture and heated at 40°C for 45 min. The mixture was cooled at room temperature and treated with 50 μl of the mixture of *tert.*-butyldimethylsilyl chloride (80 m*M*) and MTBSTFA (300 m*M*) dissolved in dimethylformamide and heated at 70°C for 3 h for the second step *tert.*-butyldimethylsilylation of hydroxyl groups.

2.6. Instrumentation

GC–MS analysis of VPA metabolites was carried out using a Hewlett-Packard 5890 II gas chromatograph (Hewlett-Packard, Avondale, PA, USA) interfaced to a Hewlett-Packard 5989A mass spectrometer. The gas chromatograph was equipped with a capillary splitless injector and a Hewlett-Packard 6890 autosampler. The operation of all instruments and mass spectrometric data acquisition were controlled with a Hewlett-Packard 59940 MS Chemsystem, a multitasking data handling and acquisition software based on the HP-UX operating system. This included a Hewlett-Packard 59970 ChemStation (V.06.01) software which was used to control the selection of the ion windows for single ion monitoring (SIM) (±0.2 Da) and to perform daily tuning of the MS system. Metabolite separation was carried out on a fused-silica narrow bore capillary gas chromatograph column (60 m×0.25 mm I.D., 0.25 μm film thickness) coated with the nonpolar bonded stationary phase DB-1 (J&W Scientific, Rancho Cordova, CA, USA). The carrier gas was helium with the column head pressure of 30 p.s.i. and a septum purge flow of 3 ml/min through the injector (1 p.s.i.=6894.76 Pa). Samples were injected in the splitless mode (injector temperature, 250°C) and cold-trapped on the column at 40°C. The column oven temperature was raised rapidly to 140°C and programmed linearly as follows: 2°C/min to 160°C, then 10°C/min to 270°C. The mass spectrometer was operated in the single ion monitoring NCI mode, with a fixed filament emission current and electron energy of 200 μA and 70 eV, respectively. The ion source temperature was set to 200°C and the gas chromatograph interface was held at 270°C. Methane was used as a reagent gas for NCI operation with the reagent gas pressure set to 1.5 Torr to gain maximum sensitivity (1 Torr=133.322 Pa).

3. Results and discussion

The inherent soft ionization nature of electron-capture negative ion chemical ionization and strong response of pentafluorobenzyl esters in the NCI mode make negative ion chemical ionization mass spectrometry the method of choice for analysis of trace levels of VPA-PFB derivatives [9,10]. The sensitivity of the analytical method is a major limiting step in determining enzyme kinetic parameters on trace levels of VPA metabolites, particularly when in vitro low volumes of recombinant enzyme incubations are used for in vitro studies. In this case the volume of each incubation was close to the injection volume and therefore concentrating the extracted samples before derivatization was not feasible. Moreover, the diverse array of metabolites generated from the extensive biotransformation of VPA results in close elution of some of the unsaturated structurally similar metabolites, e.g., VPA and 4-ene-VPA. The loss of chromatographic separation results in spillover of the VPA response into the lower mass channels (i.e., 4-ene-VPA), especially in the case of in vitro enzyme kinetic studies where the ratio of VPA to 4-ene-VPA is over five-orders of magnitude. Therefore due to the lack of specificity of mass spectral detection in single quadrupole mass spectrometry, a higher chromatographic resolution was required for adequate separation and specific detection of 4-ene-VPA when a high concentration of VPA is used. Lastly, some of the valproate metabolites have demonstrated low stability and pH-dependent degradation [3–20] and therefore the use of individual deuterated analytes as internal standards for unstable VPA metabolites was necessary for accurate estimation of these compounds.

The method described here has been modified to

achieve high specificity, sensitivity, and accuracy necessary to determine trace levels of desaturated and oxidized VPA metabolites generated from the in vitro micro-incubation of human cDNA-expressed enzymes.

Fig. 1 illustrates SIM chromatograms in the NCI mode of VPA and VPA metabolites following a two-step derivatization by PFB bromide followed by *tert*.-butyldimethylsilyl (TBDMS) reagents. Using a long, narrow-bore (60 m×0.25 mm I.D.) DB-1 column, a much higher chromatographic resolution was achieved for the separation of closely related VPA metabolites than those previously reported using medium-length or wide-bore columns [9,10,12,14]. All mono- and diunsaturated VPA metabolites and their corresponding *E* (*trans*) or *Z* (*cis*) geometric isomers were completely resolved, yet the analysis run time was kept below 30 min by using a new column temperature gradient program. This results in high specificity of the method and no cross-talk of analytes in selected ion monitoring mode of the single quadrupole GC–MS. Unlike semi-polar columns such as OV-1701 [12], a non-polar DB-1 column results in elution of the 4-ene-VPA peak before VPA, preventing any interference of VPA in the 4-ene-VPA channel even at the high VPA concentration used (Fig. 2).

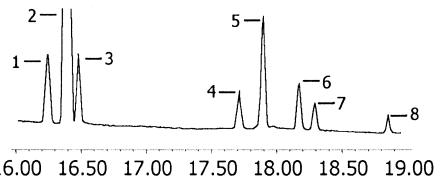

Fig. 2. Total ion chromatogram of VPA and VPA metabolites in NCI mode following two-step derivatization with PFB and TBDMS. GC–MS chromatographic conditions were as described in the Experimental section. The peaks, characterized based on their *m*/*z* ratio, are as follows: 1=4-ene-VPA; 2=VPA; 3=3-ene-VPA; 4=(*E*)-2-ene-[$^2H_7$]VPA; 5=(*E*)-2-ene-VPA; 6=(*E*,*Z*)-2,3′-diene-VPA; 7=(*E*)-2,4-diene-VPA; 8=(*E*,*E*)-2,3′-diene-VPA.

The heptadeuterated VPA analogues of the metabolites, whose chromatography is also illustrated in Fig. 1, were convenient and suitable internal standards for this analysis because their physicochemical and chromatographic characteristics were very simi-

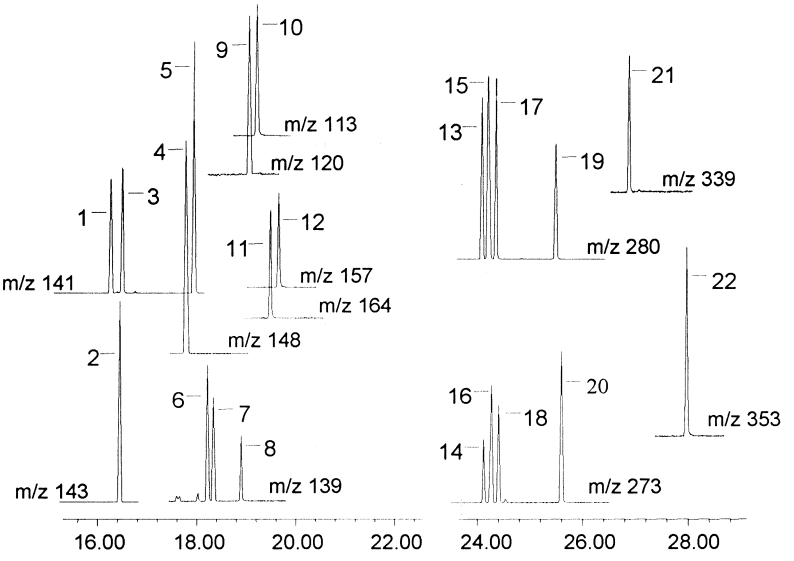

Fig. 1. SIM chromatograms of VPA and VPA metabolites in NCI mode following two-step derivatization with PFB and TBDMS. Chromatographic conditions were as described in the Experimental section. The peaks, characterized based on their *m*/*z* ratio, are as follows: 1=4-ene-VPA; 2=VPA; 3=3-ene-VPA; 4=(*E*)-2-ene-[$^2H_7$]VPA; 5=(*E*)-2-ene-VPA; 6=(*E*,*Z*)-2,3′-diene-VPA; 7=(*E*)-2,4-diene-VPA; 8=(*E*,*E*)-2,3′-diene-VPA; 9=3-keto-[$^2H_7$]VPA; 10=3-keto-VPA; 11=4-keto-[$^2H_7$]-VPA; 12=4-keto-VPA; 13=4-OH-[$^2H_7$]VPA (first isomer); 14=4-OH-VPA (first isomer); 15=3-OH-[$^2H_7$]VPA; 16=3-OH-VPA; 17=4-OH-[$^2H_7$]VPA (second isomer); 18=4-OH-VPA (second isomer); 19=5-OH-[$^2H_7$]VPA; 20=5-OH-VPA; 21=2-PSA; 22=2-PGA.

lar to those of the metabolites being quantitated. Moreover, the lack of stability of some of the VPA metabolites presents interesting problems with respect to their analysis. The 4-hydroxy-VPA metabolite (4-HO-VPA) readily forms a γ-lactone at low pH [3] and some laboratories find it difficult to obtain a proper derivative of 4-HO-VPA [12,21]. The 5-hydroxy-VPA (5-HO-VPA) also forms a lactone but only in strong acid. One must be careful to control conditions when extracting and derivatizing the 3-keto-VPA metabolite that readily undergoes heat and acid catalyzed decarboxylation to 3-heptanone. A stable isotope labeled internal standard of 3-keto-VPA is a valuable asset to reliable analysis of this metabolite [10,12].

The summary of diagnostic ions and retention times of all VPA metabolites and heptadeuterated analogues following PFB bromide/TBDMS derivatization is shown in Table 1. In the NCI mode, almost all of the ion current of the PFB esters of VPA metabolites is carried by a single fragment, the $[M-181]^-$ anion as the base peak [22,23]. The only exception was 3-keto-VPA (Fig. 3) as the presence of a keto group at position 3 facilitates a rearrangement (decarboxylation) reaction to yield m/z 113 as the base peak [9]. This finding is significant in light of the fact that the base peaks in the mass spectra of all PFB-derivatized acids reported thus far are the $[M-181]^-$ anions [22,23]. The mass spectrum of the PFB derivative of 3-keto-VPA also contains ions at $[M-181]^-$ and $[M-1]^-$. The latter appeared to be unique to 3-keto-VPA since it was not observed in the mass spectra of the other metabolites. A small fraction of the 3-keto-VPA formed a PFB/TBDMS di-derivative by reacting with the enolic form of 3-keto group, as previously reported [6]. This peak, which can be monitored at m/z 271, does not interfere with the accurate determination of 3-keto-VPA at m/z 113. The dicarboxylic acid VPA metabolites, 2-PSA and 2-PGA, gave diPFB derivatives with the usual base peak, i.e., $[M-181]^-$.

For double derivatized mono hydroxylated metabolites, the main fragment was still the $[M-181]^-$ anion, although the introduction of the *tert*.-butyldimethylsilyl ether group resulted in the formation of the new ion at m/z 273 (Fig. 4). Characteristic

Table 1
Diagnostic ions and retention times of PFB/TBDMS derivatives of VPA, VPA metabolites and deuteriated analogues from standard reference samples in rat liver microsomes

Compound	Ion monitored (m/z)	Retention time (min)
4-Ene-VPA	141	16.32
[$^2H_7$]VPA	150	16.36
VPA	143	16.48
3-Ene-VPA	141	16.55
(*E*)-2-Ene-[$^2H_7$]VPA	148	17.78
(*E*)-2-Ene-VPA	141	17.80
(*E*,*Z*)-2,3′-Diene-VPA	139	18.25
(*E*)-2,4-Diene-VPA	139	18.36
(*E*,*E*)-2,3′-Diene-VPA	139	18.92
3-Keto-[$^2H_7$]VPA	120	19.08
3-Keto-VPA	113	19.17
4-Keto-[$^2H_7$]VPA	164	19.55
4-Keto-VPA	157	19.64
4-OH-[$^2H_7$]VPA	280	24.18, 24.43
4-OH-VPA	273	24.23, 24.48
3-OH-[$^2H_7$]VPA	280	24.29
3-OH-VPA	273	24.36
5-OH-[$^2H_7$]VPA	280	25.45
5-OH-VPA	273	25.50
2-PSA	339	26.87
2-PGA	353	27.95

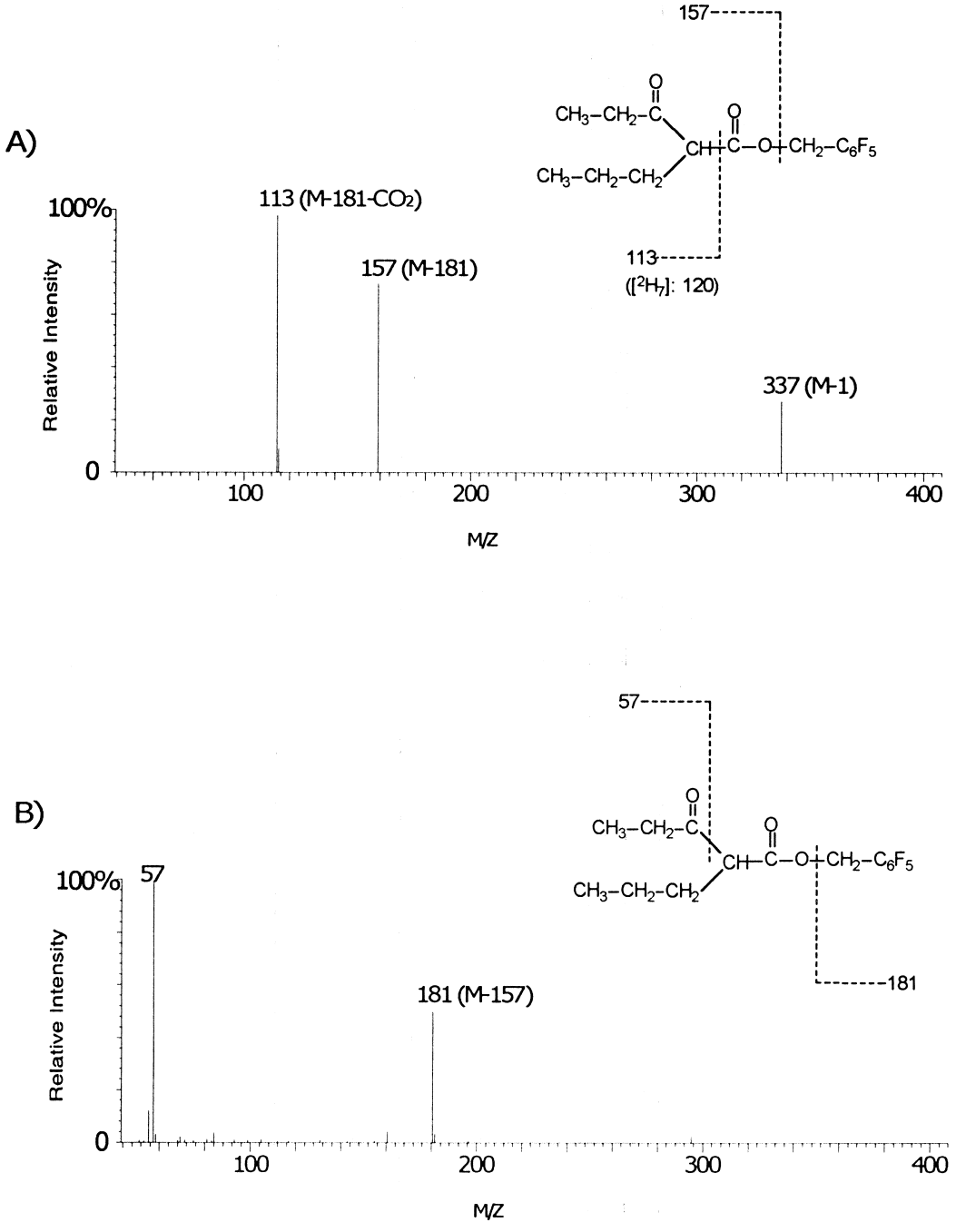

Fig. 3. Mass spectrum of the PFB derivative of 3-keto-VPA (peak 10, Fig. 1) in (A) negative ion chemical ionization, and (B) electron ionization scanning modes. A similar mass spectrum in NCI was obtained for 3-keto-[$^2H_7$]VPA (peak 9, Fig. 1) but with seven added mass units due to the deuterium substitution.

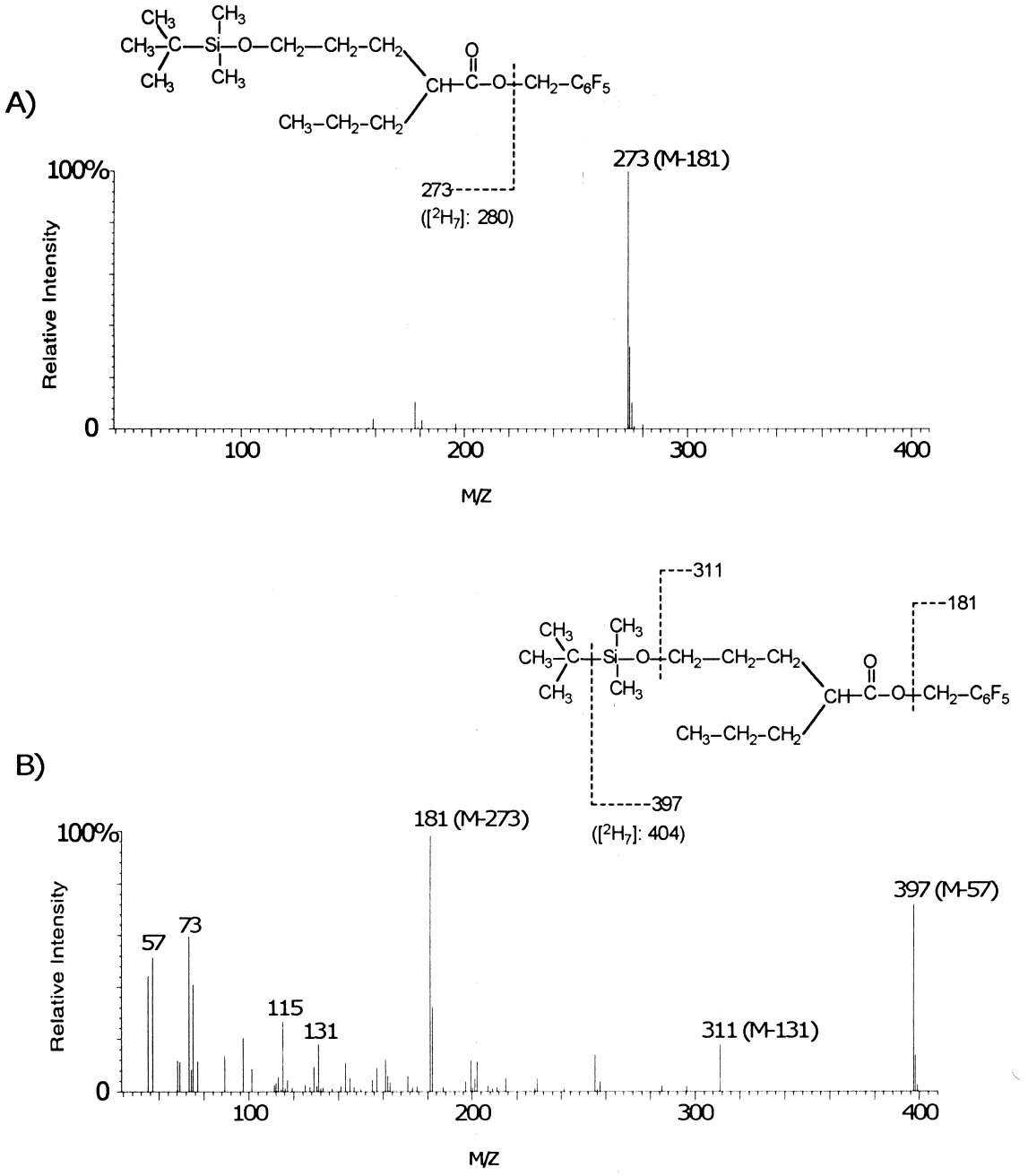

Fig. 4. Mass spectrum of the PFB/TBDMS derivative of 5-OH-VPA (peak 20, Fig. 1) in (A) negative ion chemical ionization, and (B) electron ionization scanning modes. Similar mass spectra were obtained for 4-OH-VPA (peaks 14 and 18, Fig. 1) and 3-OH-VPA (peak 16, Fig. 1). The deuterated monohydroxylated VPA metabolites also showed similar mass spectra but with seven added mass units due to the deuterium substitution.

fragments generated under scanning electron impact (EI) or NCI mode revealed the postulated structure of the double derivatized compound, being initially esterified at the carboxylic end by PFB followed by *tert*.-butyldimethylsilyl ether formation at the hydroxyl group (Fig. 4). A second derivatization step using silylating agents significantly improved both peak shape and sensitivity of detection for monohydroxylated VPA metabolites [6,10]. *N*-Methyl-*N*-trimethylsilyltrifluoroacetamide was shown to efficiently derivatize the intact hydroxyl groups following initial PFB derivatization of the valproate carboxyl group [10]. In our preliminary investigation, we found that the second-step derivatization of hydroxylated VPA metabolites by the *tert*.-butyldimethylsilyl group produces a better negative ion signal in NCI mode compared to derivatization with the trimethylsilyl group. This could be explained by the greater mass and thereby detection sensitivity of the derivatized metabolite by the *tert*.-butyldimethylsilyl group compared to the trimethylsilyl group as previously described under electron impact ionization mass spectroscopy of VPA metabolites [6]. Moreover, the samples following derivatization by *tert*.-butyldimethylsilyl chloride appear to be highly stable. This is not surprising as *tert*.-butyldimethylsilyl ethers are known to be 10^4 times more stable than trimethylsilyl ethers and the reaction by-products are also known to be neutral and volatile [24].

Based on a signal-to-noise ratio of 3:1, the limits of detection for VPA and its monohydroxylated metabolites were 0.2 and 2 ng/ml, respectively. These indicate a 4–10-fold enhanced sensitivity of detection compared to the previously reported NCI assays [9,10], which are known to be 30–50-times more sensitive than GC–EI-MS assays [6,12]. The enhanced detectability under NCI mode observed here may be explained by the increased bulk of the double-derivatized metabolites as well as excellent chromatographic peak shapes for all metabolites that were separated with high efficiency by a long narrow-bore column. The reagent gas pressure, which is known as the main determinant of sensitivity in NCI mode, was also found to be optimal at 1.5 Torr when the column head pressure was adjusted to 30 p.s.i. for the long narrow-bore column.

The calibration curves used for the quantitation of VPA and its metabolites in control cDNA-expressed microsomes showed good linearity over the concentration ranges investigated as demonstrated by the coefficients of determination listed in Table 2. The limit of quantitation (LOQ), i.e., the lowest con-

Table 2
Calibration curves for the quantitation of VPA and its metabolites[a]

Compound	Concentration range (ng/ml)	Slope	Coefficient of determination (r^2)
4-Ene-VPA	2–200	0.055	0.996
VPA	0.2–4000	0.361	0.990
(*E*)-3-Ene-VPA	1–200	0.071	0.998
(*E*)-2-Ene-VPA	2–200	0.068	0.996
(*E*,*Z*)-2,3′-Diene-VPA	2–200	0.047	0.994
(*E*)-2,4-Diene-VPA	2–200	0.025	0.994
(*E*,*E*)-2,3′-Diene-VPA	2–200	0.034	0.992
3-Keto-VPA	5–200	0.032	0.991
4-Keto-VPA	0.3–200	0.061	0.990
4-OH-VPA	2–200	0.018	0.994
3-OH-VPA	2–200	0.022	0.992
5-OH-VPA	2–200	0.099	0.999
2-PSA	1–200	0.008	0.991
2-PGA	0.3–200	0.031	0.990

[a] Peak area ratio of each metabolite to its appropriate deuteriated internal standard was used to establish the calibration curve for each metabolite. [$^2H_7$]VPA was used as the internal standard for VPA, (*E*)-2-ene-[$^2H_7$]VPA was used for 4-ene-VPA, (*E*)-3-ene-VPA, (*E*)-2-ene-VPA, (*E*,*Z*)-2,3′-diene-VPA, (*E*)-2,4-diene-VPA, and (*E*,*E*)-2,3′-diene-VPA. 3-Keto-[$^2H_7$]VPA was used for 3-keto-VPA. 4-Keto-[$^2H_7$]VPA was used for 4-keto-VPA. 4-OH-[$^2H_7$]VPA was used for 4-OH-VPA, 3-OH-[$^2H_7$]VPA for 3-OH-VPA, and 5-OH-[$^2H_7$]VPA for 5-OH-VPA and all of the remaining dicarboxylic acid metabolites.

Table 3
Intra-assay precision and accuracy of the method[a]

Compound	LOQ		Middle		High	
	RSD (%)	Bias (%)	RSD (%)	Bias (%)	RSD (%)	Bias (%)
4-Ene-VPA	5.7	10.1	3.0	4.3	−3.3	2.7
VPA	8.3	−9.1	4.7	−6.6	6.4	3.6
(*E*)-3-Ene-VPA	4.5	3.4	1.5	4.2	7.3	1.1
(*E*)-2-Ene-VPA	3.8	−5.9	3.5	−2.3	5.8	2.8
(*E*,*Z*)-2,3′-Diene-VPA	4.5	1.9	1.9	3.4	2.4	2.3
(*E*)-2,4-Diene-VPA	3.8	−3.0	3.9	−2.5	6.4	2.5
(*E*,*E*)-2,3′-Diene-VPA	5.7	6.3	2.1	−1.4	3.6	4.3
3-Keto-VPA	7.3	−5.4	5.6	−0.7	9.4	−7.8
4-Keto-VPA	5.2	−5.5	2.9	−8.9	5.3	−3.1
4-OH-VPA	6.2	−2.9	7.8	−2.5	2.5	−3.2
3-OH-VPA	7.4	−7.6	1.1	−0.3	6.2	2.3
5-OH-VPA	4.9	8.3	5.6	4.6	1.5	6.6
2-PSA	3.3	−3.3	0.8	−3.1	5.4	−3.1
2-PGA	2.9	4.3	1.2	−1.9	3.6	−1.9

[a] Intra-assay precision or the percentage of RSD was estimated from consecutive analysis of six aliquots of blank microsomal sample spiked with metabolites at the LOQ, middle, and high concentration range of their respective standard curve. Inaccuracy of the method or the bias was calculated by obtaining the difference of the nominal concentration from the back-calculated concentration and dividing it by the value of the nominal concentration.

centration on the calibration curve measured precisely (RSD<15%) and accurately (% error <15%), ranged between 0.2 and 5 ng/ml for VPA and its metabolites.

The accuracy and the precision of the assay was assessed by analysis of the multiple control microsomal samples which had been spiked with known amounts of VPA and metabolites corresponding to

Table 4
Inter-assay precision and accuracy of the method[a]

Compound	LOQ		Middle		High	
	RSD (%)	Bias (%)	RSD (%)	Bias (%)	RSD (%)	Bias (%)
4-Ene-VPA	3.4	5.2	4.4	3.5	−5.6	3.6
VPA	4.6	−6.4	4.5	−4.3	3.2	−7.4
(*E*)-3-Ene-VPA	7.7	2.5	3.2	3.8	4.0	4.6
(*E*)-2-Ene-VPA	8.4	−6.6	1.3	−7.2	7.8	6.4
(*E*,*Z*)-2,3′-Diene-VPA	5.3	4.9	2.2	1.2	2.4	−4.7
(*E*)-2,4-Diene-VPA	3.2	−2.4	7.0	−6.7	2.5	3.5
(*E*,*E*)-2,3′-Diene-VPA	5.1	5.1	2.1	−2.1	1.5	8.4
3-Keto-VPA	3.7	−8.4	1.2	8.7	5.7	−2.1
4-Keto-VPA	1.3	−4.7	3.3	−4.4	3.6	−5.3
4-OH-VPA	6.8	−3.4	1.2	−5.5	7.2	−6.4
3-OH-VPA	9.6	−6.4	2.6	−1.4	5.9	2.5
5-OH-VPA	5.3	6.4	1.1	9.2	2.6	4.6
2-PSA	2.7	−4.5	0.9	−2.6	4.5	−0.9
2-PGA	3.4	3.2	3.5	−1.9	4.3	−2.5

[a] Inter-assay precision or the percentage of RSD was estimated from replicate analysis on six separate days of the blank microsomal sample spiked with metabolites at the LOQ, middle, and high concentration range of their respective standard curve. Inaccuracy of the method or the bias was calculated by obtaining the difference of the nominal concentration from the back-calculated concentration and dividing it by the value of the nominal concentration.

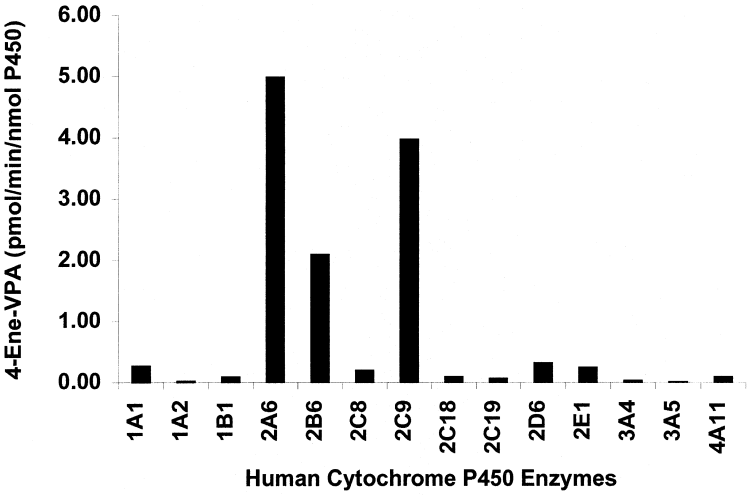

Fig. 5. A panel of 14 human cDNA-expressed P450 enzymes was screened for the formation of 4-ene-VPA as described in the experimental procedures.

the LOQ, a middle and a high concentration over the range of their standard curves. The assay demonstrated excellent precision as relative standard deviations (RSDs) in both intra- (Table 3) and inter-assay (Table 4) analyses were $<15\%$ for all three concentration ranges. The percentages of bias between the nominal and measured concentrations in both intra- and inter-assay analyses were below 15% at the three concentration range, indicating high accuracy and reproducibility of the method.

Following the validation study, a panel of fourteen human cDNA-expressed P450 enzymes was screened for the formation of 4-ene-VPA, a well-known hepatotoxic metabolite of VPA. Fig. 5 summarizes the results of 4-ene-VPA formation in the whole panel of human P450 enzymes, with cytochrome P450 2B6, 2A6, and 2C9 appearing to play a major role in this novel desaturation reaction. The kinetic studies on the formation of 4-ene-VPA and screening the human P450 panel for the formation of other potentially cytotoxic metabolites is still under investigation.

4. Conclusions

In conclusion, the present paper describes a robust, reliable, and accurate method for the separation and quantification of VPA and its 14 metabolites in a single chromatographic run time of 30 min. The method was found suitable for high-throughput in vitro enzyme kinetic and metabolite profiling of valproic acid using human cytochrome P450 enzymes.

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