Fluorinated Analogues as Mechanistic Probes in Valproic Acid Hepatotoxicity: Hepatic Microvesicular Steatosis and Glutathione Status†

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It is postulated that the hepatotoxicity of valproic acid (VPA) results from the mitochondrial β-oxidation of its cytochrome P450 metabolite, 2-propyl-4-pentenoic acid (4-ene VPA), to 2-propyl-(*E*)-2,4-pentadienoic acid ((*E*)-2,4-diene VPA) which, in the CoA thioester form, either depletes GSH or produces a putative inhibitor of β-oxidation enzymes. In order to test this hypothesis, 2-fluoro-2-propyl-4-pentenoic acid (α-fluoro-4-ene VPA) which was expected to be inert to β-oxidative metabolism was synthesized and its effect on rat liver studied in comparison with that of 4-ene VPA. Similarly, the known hepatotoxicant 4-pentenoic acid (4-PA) and 2,2-difluoro-4-pentenoic acid (F_2-4-PA) were compared. Male Sprague-Dawley rats (150–180 g, 4 rats per group) were dosed ip with 4-ene VPA (0.7 mmol/kg per day), 4-PA (1.0 mmol/kg per day), or equivalent amounts of their α-fluorinated analogues for 5 days. Both 4-ene VPA and 4-PA induced severe hepatic microvesicular steatosis (>85% affected hepatocytes), and 4-ene VPA produced mitochondrial alterations. By contrast, α-fluoro-4-ene VPA and F_2-4-PA were not observed to cause morphological changes in the liver. The major metabolite of 4-ene VPA in the rat urine and serum was the β-oxidation product (*E*)-2,4-diene VPA. The *N*-acetylcysteine (NAC) conjugate of (*E*)-2,4-diene VPA was also found in the urine. Neither (*E*)-2,4-diene VPA nor the NAC conjugate could be detected in the rats administered α-fluoro-4-ene VPA. In a second set of rats (3 rats per group), total liver GSH levels were determined to be depleted to 56% and 72% of control following doses of 4-ene VPA (1.4 mmol/kg) and equivalent α-fluoro-4-ene VPA, respectively. Mitochondrial GSH remained unchanged in the α-fluoro-4-ene VPA treated group but was reduced to 68% of control in the rats administered 4-ene VPA. These results strongly support the theory that hepatotoxicity of 4-ene VPA, and possibly VPA itself, is mediated largely through β-oxidation of 4-ene VPA to reactive intermediates that are capable of depleting mitochondrial GSH.

Introduction

The rare but sometimes fatal hepatotoxicity of the anticonvulsant agent valproic acid (VPA, 2-propylpentanoic acid)[1] has been investigated for more than a decade. The clinical syndrome includes lethargy, vomiting, and fever followed by hypoglycemia, hyperammonemic coma, and ultimately death (*1, 2*). Liver biopsies revealed microvesicular steatosis sometimes accompanied by necrosis (*1, 2*). The similarity of VPA induced hepatotoxicity to Reye's syndrome and Jamaican vomiting sickness led Gerber *et al.* (*3*) to postulate that 2-propyl-4-pentenoic acid (4-ene VPA) might be responsible for the liver injury because the structure was closely related to the known hepatotoxicants, hypoglycin and 4-pentenoic acid (4-PA). It was further confirmed experimentally that 4-ene VPA as well as its β-oxidation metabolite 2-propyl-2,4-pentadienoic acid (2,4-diene VPA) could produce severe hepatic microvesicular steatosis in rats (*4*). Studies into the microsomal metabolism of VPA demonstrated that desaturation to 4-ene VPA is a cytochrome P450 (P450)-mediated process (*5*). This finding is in accordance with clinical reports whereby patients who had taken VPA along with the P450 enzyme inducers, phenytoin, phenobarbital, or carbamazepine, were more vulnerable to VPA-induced liver injury than those under VPA monotherapy (*1, 2, 6, 7*). A proposed mechanism of the toxicity suggested that 4-ene VPA, in its CoA thioester form, undergoes β-oxidation to (*E*)-2,4-diene VPA-CoA to either deplete the crucial mitochondrial GSH pool (*8*) or yield a far more reactive electrophile, 2-propyl-3-oxo-4-pentenoic acid (3-keto-4-ene VPA), which alkylates and thereby irreversibly inhibits fatty acid β-oxidation enzyme(s) (*9*). In support of this mechanism, the GSH and *N*-acetylcysteine (NAC) conjugates of (*E*)-2,4-diene VPA (*8, 10*) and the GSH conjugate of 3-keto-4-ene VPA have been identified in 4-ene VPA-

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[1] Abbreviations: VPA, valproic acid, 2-propylpentanoic acid; P450, cytochrome P450; 4-ene VPA, 2-propyl-4-pentenoic acid; (*E*)-2,4-diene VPA, 2-propyl-(*E*)-2,4-pentadienoic acid; (*E*)-2-ene VPA, 2-propyl-(*E*)-2-pentenoic acid; 3-keto-4-ene VPA, 2-propyl-3-oxo-4-pentenoic acid; 3-ene VPA, 2-propyl-3-pentenoic acid; 2,3′-diene VPA, 2-(1′-propenyl)-2-pentenoic acid; 3-keto VPA, 2-propyl-3-oxopentanoic acid; α-fluoro-4-ene VPA, 2-fluoro-2-propyl-4-pentenoic acid; 4-PA, 4-pentenoic acid; F_2-4-PA, 2,2-difluoro-4-pentenoic acid; NAC, N-acetyl-L-cysteine; GR, glutathione reductase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTNB, 5,5′-dithiobis(nitrobenzoic acid); LDA, lithium diisopropylamide; MTBSTFA, *N*-methyl-*N*-(*tert*-butyldimethylsilyl)-trifluoroacetamide; HMPA, hexamethylphosphoramide; NFSi, *N*-fluorobenzenesulfonimide; H&E, hematoxylin–eosin.

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treated rats (*10*). The NAC conjugate of (*E*)-2,4-diene VPA was found in the urine of patients who were on VPA therapy (*8*). The activity of acetoacetyl-CoA thiolase was inhibited by 4-ene VPA in rat hepatocytes (*11*).

On the other hand, from clinical case studies of VPA-mediated hepatotoxicity in which VPA metabolite profiles in patients were determined, Siemes *et al.* suggested that the hepatotoxicity of VPA was the result of an impairment of β-oxidation (*12*). Higher than normal levels of 2-propyl-(*E*)-2-pentenoic acid ((*E*)-2-ene VPA), 2-propyl-3-pentenoic acid (3-ene VPA), and 2-(1′-propenyl)-2-pentenoic acid (2,3′-diene VPA) but much less 2-propyl-3-oxopentanoic acid (3-keto VPA) were detected in the serum of patients who had developed apparent drug-induced toxicity (*12*). These findings appear to describe a defect in the conversion of (*E*)-2-ene VPA-CoA to 3-keto VPA-CoA as a part of the hepatotoxicity (*12, 13*). The involvement of potential toxic metabolite(s) of VPA was ruled out based on a poor correlation of the hepatotoxicity with either the serum 4-ene VPA or (*E*)-2,4-diene VPA concentrations (*12*). A similar conclusion was reached following comparative studies of VPA and (*E*)-2-ene VPA in rats where the incidence of liver microvesicular steatosis was observed to be independent of plasma levels of 4-ene VPA and (*E*)-2,4-diene VPA. It was therefore suggested that these metabolites were not the decisive factor in the hepatotoxicity of VPA (*14*).

In order to clarify the roles of 4-ene VPA and its subsequent metabolite (*E*)-2,4-diene VPA in the VPA-related hepatotoxicity, a fluorinated 4-ene VPA analogue, 2-fluoro-2-propyl-4-pentenoic acid (α-fluoro-4-ene VPA), was synthesized in an effort to selectively avert β-oxidation. It is well-known that a fluorine substituent not only mimics hydrogen in size but the strong C−F bond is resistant to many biological reactions (*15, 16*). If the hepatotoxicity of VPA is induced through the formation of a reactive metabolite(s), preventing the metabolism of 4-ene VPA to (*E*)-2,4-diene VPA *via* the β-oxidation pathway should eliminate or markedly reduce the toxicity. This report presents the results from histopathological and biochemical studies of liver following acute and chronic administration of either α-fluoro-4-ene VPA or 4-ene VPA to rats. Similarly, the effect of 4-PA was compared to that of 2,2-difluoro-4-pentenoic acid (F_2-4-PA), since the mechanism proposed for the toxicity of 4-ene VPA is largely based on what is known for 4-PA (*3, 17*). Rat liver microvesicular steatosis was used as a marker of the toxicities, and the hepatic GSH status was investigated as a contributing factor.

Materials and Methods

Chemicals. Trizma, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), sucrose, mannitol, 5-sulfosalicylic acid, 5,5′-dithiobis(nitrobenzoic acid) (DTNB), GSH, and glutathione reductase (GR) were obtained from Sigma Chemical Co. (St. Louis, MO). NADPH and GSSG were purchased from Boehringer (Mannheim, Germany). EDTA, acetonitrile, ammonium chloride, and petroleum ether were products of BDH Inc. (Vancouver, BC). Butyllithium (1.6 M in hexanes), hexamethylphosphoramide (HMPA), octanoic acid, chlorotrimethylsilane, allyl alcohol, chlorodifluoroacetic anhydride, calcium hydride, triethylamine, diisopropylamine, and 4-PA were obtained from Aldrich Chemical Co. (Milwaukee, WI). The 4-PA was distilled at 83−84 °C/12 mmHg prior to use. Diethyl ether and THF were obtained from Caledon Laboratories Ltd. (Georgetown, ON). The THF was distilled from calcium hydride before use. *N*-Fluorobenzenesulfonimide (NFSi) was a gift from Allied Signal Inc. (Buffalo, NY). Hydroxylamine hydrochloride was from Eastman Co. (Rochester, NY), and *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) was from Pierce Chemical Co. (Rockford, IL). Ethyl 2-propyl-4-pentenoate, 4-ene VPA, (*E*)-2,4-diene VPA, and the NAC conjugate of (*E*)-2,4-diene VPA were prepared as reported previously (*8*).

Instrumentation. NMR spectra were obtained on a Bruker WH-200 spectrometer in the Department of Chemistry at the University of British Columbia (UBC), and chemical shifts are expressed relevant to tetramethylsilane. Electron microscopic analyses were performed on an EM 10C/CR high resolution electron microscope of the Electron Microscopy Center, UBC. The quantitation of serum and urinary levels of 4-ene VPA, α-fluoro-4-ene VPA, and metabolite (*E*)-2,4-diene VPA was carried out on a HP 5971A mass selective detector (MSD) interfaced to a HP 5890II gas chromatograph. The NAC conjugate of (*E*)-2,4-diene VPA was identified using a HP 5989A mass spectrometer (MS) coupled with a HP 5890II gas chromatograph.

Chemical Synthesis. Synthesis of 2,2-Difluoro-4-pentenoic Acid (F_2-4-PA). F_2-4-PA was synthesized with modifications to a procedure described by Greuter *et al.* (*18*). To allyl alcohol (0.1 mol, 6.0 g) and triethylamine (0.1 mol, 10.1 g) in ether was added chlorodifluoroacetic anhydride (0.1 mol, 25 g) at 0 °C. The mixture was stirred at room temperature for an additional 2 h before partitioning between ether and water. Allyl chlorodifluoroacetate was obtained upon removal of ether and distilled at 100−103 °C; yield 90%.

To allyl chlorodifluoroacetate (75 mmol, 10 g) in acetonitrile (44 mL) was added dropwise chlorotrimethylsilane (88 mmol, 9.5 g) and stirred at room temperature for 1 h. Freshly activated zinc dust (4.5 g) was added, and the resultant mixture was heated to reflux at 100 °C for 20 h. Acetonitrile was rotor-evaporated under vacuum from the mixture to half of the original volume, and the residue was mixed with silica gel (40 g) in water (45 mL) and stirred at room temperature for 12 h. Silica gel was removed by filtration and the acetonitrile removed by rotor-evaporation under vaccum. The residue was partitioned between water and ether, and crude product was obtained upon removal of the ether layer by evaporation. The desired compound was obtained by fractional distillation (58−59 °C/5 mmHg, yield 80%). ^{1}H NMR ($CDCl_3$): δ 2.86 (2t, 2H, J_{HH} = 7 Hz, J_{FH} = 16 Hz, CH_2), 5.30 (d, 1H, J_{HH} = 10 Hz (*cis*); d, 1H, J_{HH} = 17 Hz (*trans*); CH=CH_2), 5.75 (tdd, 1H, J_{HH} = 7, 10, 18 Hz, CH=CH_2), 10.25 (s, 1H, COOH). ^{13}C NMR ($CDCl_3$): δ 38.89 (t, J_{CF} = 23.5 Hz, C-3), 114.98 (t, J_{CF} = 249.5 Hz, C-2), 122.31 (s, C-5), 126.38 (t, J_{CF} = 5.42 Hz, C-4), 168.71 (t, J_{CF} = 33.3 Hz, C-1). GC/MS mass spectrum (TMS derivative), *m*/*z* (%): 77 (100), 73 (30), 115 (17), 193 (17) (M^+ − 15).

Synthesis of 2-Fluoro-2-propyl-4-pentenoic Acid (α-Fluoro-4-ene VPA). To diisopropylamine (43 mmol, 6 mL) in THF (50 mL) was added butyllithium (43 mmol, 27 mL) at −78 °C. After stirring at −10 °C for 30 min, the mixture was cooled to −78 °C, HMPA (43 mmol, 7.5 mL) in THF (10 mL) was added, and the resultant mixture was stirred for 15 min. To this lithium diisopropylamide (LDA) THF/HMPA solution was added ethyl 2-propyl-4-pentenoate (30 mmol, 4 g) in THF (7 mL). The mixture was stirred for 60 min before NFSi (49 mmol, 16 g) in THF (50 mL) was added. The reaction was kept at −78 °C for 120 min, allowed to reach room temperature overnight, and quenched by adding saturated aqueous NH_4Cl. The mixture was acidified to pH 1 with 6 M HCl solution and then extracted with diethyl ether twice. The combined organic layer was washed consecutively with aqueous NH_2OH·HCl solution, saturated $NaHCO_3$ solution, and water, and then rotor-evaporated under vacuum to give ethyl 2-fluoro-2-propyl-4-pentenoate. The crude product was purified by flash chromatograpy (diethyl ether/petroleum ether = 10/90 v/v, yield 86%). GC/MS mass spectrum, *m*/*z* (%): 95 (100), 139 (65), 41 (65), 73 (57), 55 (44), 146 (44), 111 (39), 168 (26), 157 (11), 188 (M^+, 2). ^{1}H NMR ($CDCl_3$): δ 0.78 (t, 3H, J_{HH} = 7.0 Hz, CH_3), 1.07 (t, 3H, J_{HH} = 7.0 Hz, OCH_2CH_3), 1.15−1.45 (m, 2H, CH_2CH_3), 1.50−1.80 (m, 2H, $CFCH_2CH_2$), 2.60 (m, 2H, J_{HH} = 7.0 Hz, J_{FH} = 25 Hz, CH_2-CH=CH_2), 4.10 (q, 2H, J_{HH} = 7.0 Hz, OCH_2), 4.92 (dd, J_{HH} = 15, 11 Hz, CH=CH_2), 5.45−5.73 (m, 1H, CH=CH_2).

α-Fluoro-4-ene VPA was obtained upon alkaline hydrolysis of the ethyl ester and was purified by fractional distillation (56−

57 °C/0.05 mmHg, yield 80%). ^{1}H NMR ($CDCl_3$): δ 0.93 (t, 3H, J_{HH} = 7.0 Hz, CH_3), 1.20–1.65 (m, 2H, J_{HH} = 7 Hz, CH_2CH_3), 1.75–2.00 (m, 2H, $CFCH_2CH_2$), 2.61 (dd, 2H, J_{HH} = 7 Hz, J_{FH} = 20, 24 Hz, $CH_2CH{=}CH_2$), 5.17 (dd, 2H, J_{HH} = 11, 16 Hz, $CH{=}CH_2$), 5.80 (tdd, 1H, J_{HH} = 7, 10, 16 Hz, $CH{=}CH_2$). ^{13}C NMR ($CDCl_3$): δ 13.92 (s, C-5′), 16.51 (d, J_{CF} = 3.5 Hz, C-4′), 38.72 (d, J_{CF} = 21.8 Hz, C-3′), 41.43 (d, J_{CF} = 21.8 Hz, C-3), 96.93 (d, J_{CF} = 187.6 Hz, C-2), 119.99 (s, C-5), 130.31 (d, J_{CF} = 3.8 Hz, C-4), 177.23 (d, J_{CF} = 26.5 Hz, C-1). GC/MS mass spectrum (*tert*-butyldimethylsilyl derivative), *m*/*z* (%): 77 (100), 217 (48, $M^+ - 57$), 73 (47), 95 (33), 133 (10), 189 (3).

Animals. Male Sprague-Dawley rats (Vancouver, BC) weighing 130–150 g were randomly assigned into groups of 3–5 and allowed free access to food (Purina Laboratory Chow) and water. Rats were housed in regular cages during the first 4 days and then in metabolic cages during the 5th day in a controlled 12 h cycle of light and darkness.

Administration of Drugs. Doses used in the chronic study were selected from published data in which 100 mg/kg of either 4-PA or 4-ene VPA, when dosed to rats, was observed to cause severe hepatic microvesicular steatosis (*4*). Thus, 4-ene VPA (0.7 mmol/kg or 100 mg/kg per day) or equivalent α-fluoro-4-ene VPA (0.7 mmol/kg or 113 mg/kg per day), 4-PA (1.0 mmol/kg or 100 mg/kg per day) or equivalent F_2-4-PA (1.0 mmol/kg or 136 mg/kg per day) were administered in single daily doses to rats (4/group) for 5 consecutive days. Aqueous solutions of the sodium salts of the drugs (pH 7.2) were used for ip injection. Rats were fasted 24 h prior to their last dose, and urine was collected during this period. The animals were sacrificed 1 h after their 6th dose, and serum samples were prepared. Livers were rapidly removed, with a portion being fixed in 1% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 24 h. The remainder of the liver was used for the quantitation of total hepatic and mitochondrial GSH.

In the acute study, 4-ene VPA (1.4 mmol/kg or 200 mg/kg) and equivalent α-fluoro-4-ene VPA (1.4 mmol/kg or 225 mg/kg) were administered ip to rats (9/group), respectively. The rats were then sacrificed at 0.75, 2, and 4 h post-dose (3 rats per time point). Livers were quickly removed and kept ice-cold for the isolation of mitochondria.

Control rats in both the acute and chronic studies were injected ip with normal saline.

Histopathological Studies. Rat livers from the chronic treatment groups were subjected to histopathological examination. For light microscopic observations a section of the liver in paraformaldehyde/glutaraldehyde fixative was either stained with hematoxylin–eosin (H&E) or frozen and subsequently stained with Oil-Red-O. The following criteria were used for scoring: microvesicular steatosis of 0–10% of hepatocytes, 1+; 10–25% of hepatocytes, 2+; 25–50% of hepatocytes, 3+; and more than 50% of hepatocytes, 4+ (*4*).

Liver slices from 4-ene VPA, α-fluoro-4-ene VPA, and control groups were examined for ultrastructural changes. Liver tissue, which was fixed with paraformaldehyde/glutaraldehyde, was minced to 1 mm^3, fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4) for 1 h and in 2% uranyl acetate solution for 30 min, and then dehydrated with ethanol. The resultant samples were embedded in either epon/araldite or spurrs. Electron microscopic examinations were conducted on ultrathin sections stained with lead citrate.

Isolation of Mitochondria. The liver mitochondrial fraction was isolated by differential centrifugation at 4 °C (*19*). Briefly, the rat liver was washed in a Tris buffer (10 mM Trizma, 50 mM NaCl, pH 7.4) and homogenized in an isolation buffer (2 mM HEPES, 70 mM sucrose, 220 mM mannitol, 2 mM EDTA, pH 7.4). The liver homogenate was centrifuged twice at 1000*g* for 10 min to remove undisrupted tissue, cells, and nuclei. The resultant supernatant was centrifuged at 11000*g* for 15 min to pellet mitochondria. Crude mitochondria were then washed three times by repeatedly suspending the pellets in the isolation buffer and recentrifuging at 11000*g* for 15 min. The resultant mitochondrial pellet was resuspended in sucrose medium and kept at ice-cold temperature. The cytosolic contamination in the mitochondrial preparation was estimated from measurement of the activity of a cytosolic marker enzyme, lactate dehydrogenase (*20*), and expressed as the ratio of the total activity in the mitochondria to that in the homogenate. The respiratory control index and ADP/O ratio of the mitochondria were determined as a pooled sample of each treatment group. Polarographic measurements (Department of Biochemistry, UBC) were made with a Clark-type oxygen electrode using glutamate, in the presence of malate, as the substrate (*19*).

Biochemical Assays. Total hepatic and mitochondrial glutathione concentrations (a summation of GSH and GSSG, and expressed in GSH thereafter for simplicity) were determined by an enzymatic recycling assay immediately after the preparation of the homogenate and mitochondria (*21*). To liver homogenate (500 μL) or mitochondrial suspension (200 μL) was added with mixing an equal volume of 10% 5-sulfosalicylic acid solution. The mixture was vortexed and centrifuged in a microcentrifuge (13600*g*, 15 min) at 4 °C. A portion of the resultant supernatant was placed in a cuvette with NADPH and DTNB in phosphate/EDTA buffer (pH 7.5). The reaction was initiated by adding GR (45 units/mL, 10 μL). The amount of GSH was determined from the rate of change in absorbance at 412 nm. This assay was found reproducible using 5-sulfosalicylic acid treated supernatants which had been stored at −78 °C for up to 5 days.

Mitochondrial GR activity was determined by the method of Carlberg and Mannervik (*22*). The mitochondrial suspension was sonicated on ice and centrifuged in a microcentrifuge (13600*g*, 15 min) at 4 °C. Supernatant (50 μL) was placed in a cuvette containing water, EDTA, NADPH, and phosphate buffer (pH 7.0). The reaction was initiated *via* adding GSSG (20 mM, 100 μL), and the rate of change of NADPH concentration was recorded by following the decrease in absorbance at 340 nm. A nonspecific change of NADPH concentration was observed before adding GSSG. Therefore, the GR activities were reported as the difference between the two rates and expressed as μmol/(min·mg of protein).

Protein contents were quantified by a modified method of Lowry (*23*) using bovine serum albumin as a standard.

Detection of (*E*)-2,4-Diene VPA and the NAC Conjugate of (*E*)-2,4-Diene VPA. The presence of (*E*)-2,4-diene VPA and the corresponding NAC conjugate in the urine and serum of rats dosed with 4-ene VPA or α-fluoro-4-ene VPA was examined by GC/MS. Urine and serum samples (10–50 μL), to which octanoic acid (10 μg/mL, 100 μL) was added as internal standard, were hydrolyzed at pH 12.5, 65 °C for 60 min, then acidified to pH 2, or directly acidified and extracted with ethyl acetate (2 mL). The ethyl acetate extracts were dried over anhydrous Na_2SO_4, concentrated under nitrogen to 100 μL, and reacted with MTBSTFA (50 μL) at 60 °C for 1 h to obtain tBDMS derivatives.

The GC/MS parameters for the analysis of the parent drugs and the diene metabolite were as follows: GC: DB-1701 column (30 m × 0.25 mm i.d., 0.25 μm film thickness, J&W Scientific, Rancho Cordova, CA); helium carrier gas at a head pressure of 15 psi; temperature program: 80–100 °C (10 °C/min), 100–130 °C (2 °C/min), 130–260 °C (30 °C/min); and MSD: EI/Scan or SIM mode with an ion source temperature of 180 °C, an emission current of 300 mA, and an ionization energy of 70 eV (*24*).

The GC/MS parameters for the detection of the urinary NAC conjugate of (*E*)-2,4-diene VPA were as follows: GC: HP-1 column (12.5 m × 0.20 mm i.d., 0.33 μm film thickness); helium carrier gas at head pressure of 5 psi; temperature program: 50–200 °C (30 °C/min), 200–320 °C (5 °C/min); and MS: an emission current of 300 mA, an ion source temperature of 275 °C, and an ionization energy of 70 eV. Three ion fragments, *m*/*z* 474, *m*/*z* 415, and *m*/*z* 276, in EI/SIM mode were chosen as criteria for the identification of the tBDMS derivative of the NAC conjugate of (*E*)-2,4-diene VPA.

All data were expressed as mean ± standard deviation. Statistical comparison between control and drug-treated groups was made with an one-way Student's *t* test at a significance level of $p < 0.05$.

Table 1. Microvesicular Steatosis in the Livers of Rats Receiving Chronic Treatment[a]

		microvesicular steatosis			
treatment	no. of rats	0−1+	2+	3+	4+
control	3	3[b]	0	0	0
		(3)[c]	(0)	(0)	(0)
4-PA	3	0	0	0	3
		(0)	(0)	(0)	(3)
F_2-4-PA	4	4	0	0	0
		(0)	(4)	(0)	(0)
4-ene VPA	4	0	0	0	4
		(0)	(0)	(0)	(4)
α-F-4-ene VPA	4	2	2	0	0
		(4)	(0)	(0)	(0)

[a] The rats were administered ip with 4-PA (1.0 mmol/kg per day), F_2-4-PA (1.0 mmol/kg per day), 4-ene VPA (0.7 mmol/kg per day), and α-fluoro-4-ene VPA (0.7 mmol/kg per day), respectively, for 5 consecutive days, and sacrificed 1 h after their 6th dose. [b] Microvesicular steatosis was determined by the light microscopic examination of Oil-Red-O-stained liver samples. [c] The liver samples were stained with hematoxylin−eosin and examined by light microscopy.

Results

Chemical Synthesis. F_2-4-PA was formed through a Reformatsky−Claisen rearrangement of allyl chlorodifluoroacetate in the presence of chlorotrimethylsilane. The preliminary reaction of allyl chlorodifluoroacetate with chlorotrimethylsilane was essential to ensure the formation of the ketene acetal intermediate, as proposed by Greuter *et al.* for the mechanism of this reaction (*18*). Simply mixing chlorotrimethylsilane, allyl chlorodifluoroacetate, and zinc dust gave none of the desired product.

In order to prepare α-fluoro-4-ene VPA, an attempt was made to alkylate ethyl 2-fluoropentanoate with allyl bromide. This reaction was unsuccessful, and an alternative method of synthesis was sought by directly introducing a fluorine into the α-position of 4-ene VPA. NFSi which is a relatively new fluorinating reagent reported in the literature (*25*) proved useful in this reaction. Following generation of the lithium enolate of ethyl 2-propyl-4-pentenoate using LDA in the presence of HMPA, the reagent delivers one atom of fluorine per molecule into the α-position of the substrate. The presence of HMPA was necessary to prevent Michael addition of LDA to the vinylic bond (*26*). The use of HMPA also increased the solubility of the NFSi reagent at −78 °C.

Histopathological Studies. Because 4-PA and 4-ene VPA are known to induce microvesicular steatosis in rat liver (*4*), these compounds were selected for this study to serve as positive standards. At doses of 1.0 mmol/kg, two rats in the group administered 4-PA were dead within the first 2 days, and subsequently an additional animal was used for this treatment. In all other groups the animals survived the 5 day treatment.

Chronic administration of either 4-PA or 4-ene VPA to rats resulted in massive accumulation of lipid in their livers, with the whole liver appearance being quite similar to that reported previously (*4*). Upon sacrifice, the livers from 4-PA- and 4-ene VPA-treated animals were much paler than livers from controls. During the preparation of liver mitochondria from 4-PA- and 4-ene VPA-treated rats, larger amounts of adhering lipid as compared to controls were present in the supernatant when the liver homogenate was centrifuged. These two treatment groups were scored 4+ for microvesicular steatosis as determined by the light microscopic examination (Table 1). Liver sections stained with H&E and Oil-Red-O revealed that at least 85% of the hepatocytes were affected. The presence of lipid was diffuse throughout the panlobular area as illustrated in photomicrographs of the H&E-stained tissue (Figures 1A and 2A). Of the 3 rats that received 4-PA, two of the livers appeared to be affected less severely than that of the

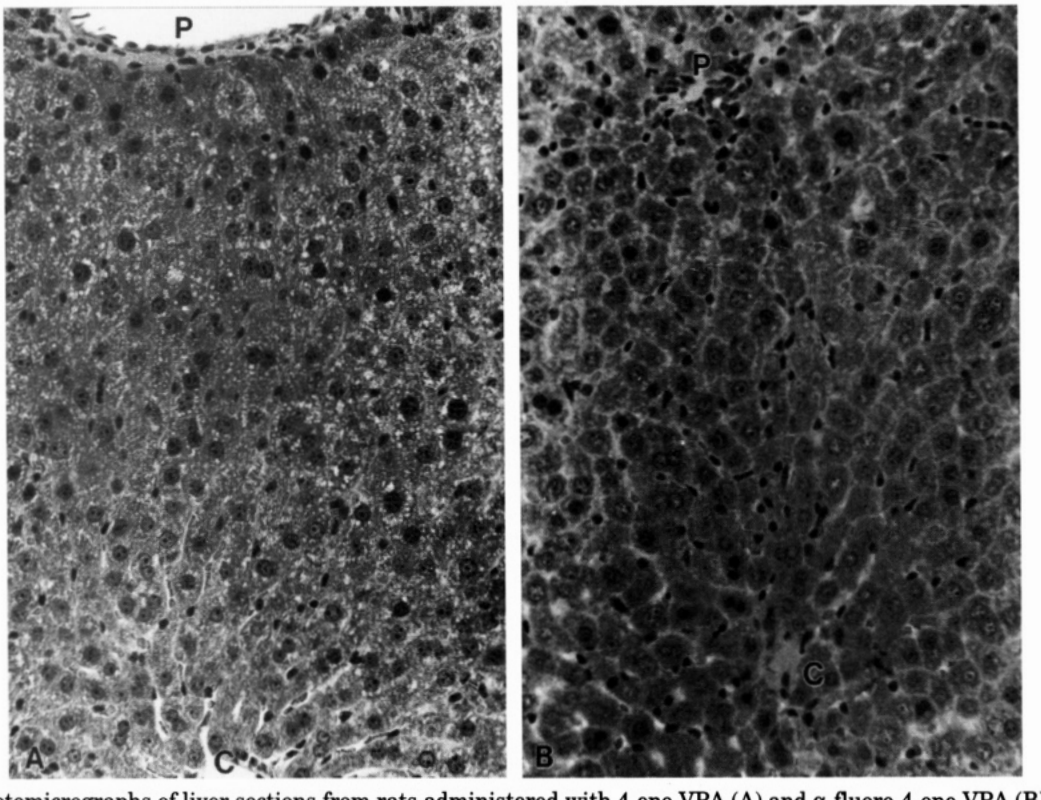


Figure 1. Photomicrographs of liver sections from rats administered with 4-ene VPA (A) and α-fluoro-4-ene VPA (B), respectively, at 0.70 mmol/kg daily for 5 days. Microvesicular steatosis was diffuse throughout the liver lobule in 4-ene VPA-treated animals but was absent in α-fluoro-4-ene VPA-treated rats (H&E; magnification 450×; reproduced at 90% of original size). P: portal vein; C: central vein.

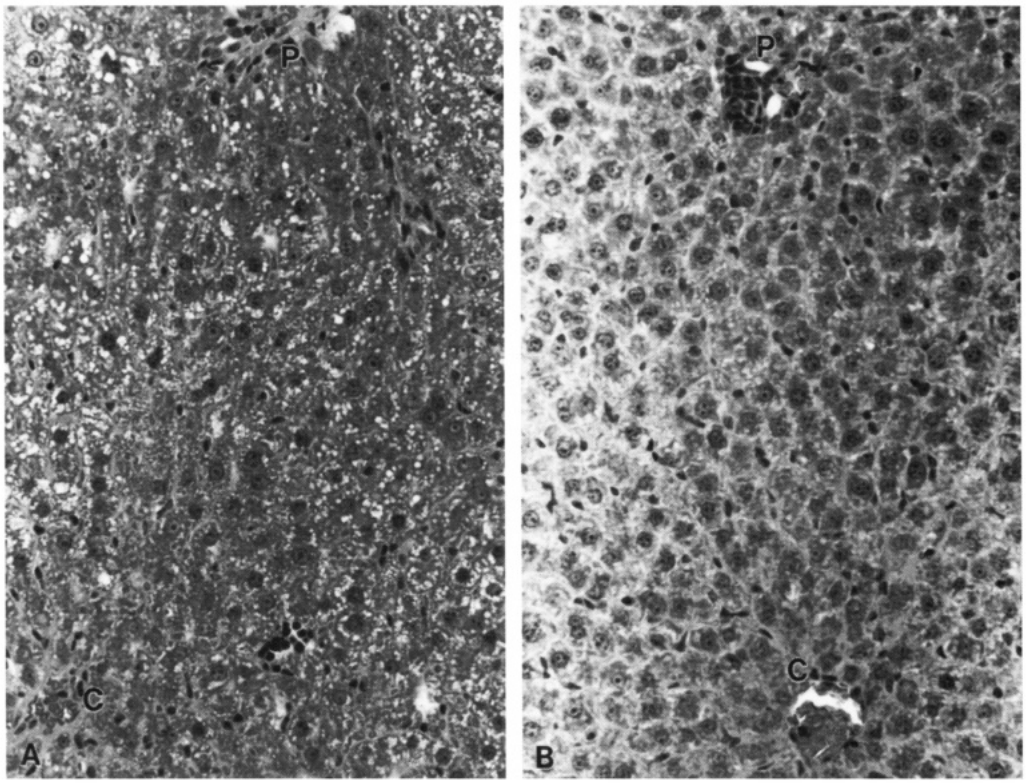


Figure 2. Photomicrographs of liver sections from rats treated with 4-PA (A) and F_2-4-PA (B), respectively, at 1.00 mmol/kg daily for 5 days. Microvesicular steatosis was diffuse throughout the liver lobule in 4-PA-treated animals but was absent in F_2-4-PA-treated rats (H&E; magnification 450×; reproduced at 90% of original size). P: portal vein; C: central vein.

4-ene VPA-treated rats; one liver from a 4-PA-treated rat was affected at the same level as 4-ene VPA-treated rats. In some livers, hypertrophy of the Kupffer cells could be observed.

In contrast, the livers from F_2-4-PA- and α-fluoro-4-ene VPA-treated rats were similar in appearance to controls, with much less adhering lipid present during the preparation of mitochondria. In prepared liver sections from treated rats, F_2-4-PA and α-fluoro-4-ene VPA-induced lipid deposits in less than 25% of the hepatocytes (≤2+ for steatosis, Table 1). The associated lipids were located only in the periportal areas of the affected livers. Half of the livers from rats in the α-fluoro-4-ene VPA groups and all livers from the F_2-4-PA group were judged to be unaffected through the examination of the Oil-Red-O-stained liver sections (0−1+ for steatosis, Table 1). Inspection of the H&E-stained liver sections revealed a slightly different result for these two treatment groups: all livers from the α-fluoro-4-ene VPA-treated group tested 0−1+ while those from the F_2-4-PA-treated group tested 2+ (Table 1). In any case the observed differences in the ability to induce microvesicular steatosis between 4-PA and F_2-4-PA and between 4-ene VPA and α-fluoro-4-ene VPA were apparent and significant.

Mitosis was occasionally observed in the livers from rats dosed with either fluorinated or nonfluorinated compounds and is considered to be normal among young rats. Very few lipid droplets were detected in the liver samples of the control group (0−1+ for steatosis, Table 1), and the distribution of the lipid appeared at random. In all groups, including those rats that developed severe microvesicular steatosis, the animals seemed to grow normally as estimated from their increased body weights (8−10 g per day). No hepatocyte necrosis could be clearly established in any of the treatment groups.

Electron microscopic examination confirmed the presence of microvesicular steatosis in the livers of rats administered 4-ene VPA. Lipid droplets were located throughout the cytoplasm. Myeloid bodies and mitochondrial matrix inclusions were also found in this group (Figure 3A). Livers of α-fluoro-4-ene VPA-treated rats, on the other hand, exhibited none of these manifestations. No ultrastructural differences in liver tissue between the control and α-fluoro-4-ene VPA groups could be distinguished (Figure 3B).

Differences of 4-Ene VPA and α-Fluoro-4-ene VPA in Producing (*E*)-2,4-Diene VPA and the NAC Conjugate of (*E*)-2,4-Diene VPA in Rat. The metabolite (*E*)-2,4-diene VPA was detected in the urine and serum of rats treated with 4-ene VPA (Figure 4, upper panel). Glucuronides of 4-ene VPA and (*E*)-2,4-diene VPA in urine were estimated from the differences in the free acid concentrations determined before and after alkaline hydrolysis. Thus, urinary recovery of 4-ene VPA was 5.9 ± 1.5 μmol as the free acid and 18.5 ± 1.3 μmol as the glucuronide. Urinary (*E*)-2,4-diene VPA accounted for ~2.6% of the 5th dose of 4-ene VPA, being 0.61 ± 0.068 μmol as the free acid and 2.1 ± 0.29 μmol as the glucuronide. Neither urine nor serum samples from rats administered α-fluoro-4-ene VPA were found to contain a detectable amount of (*E*)-2,4-diene VPA (Figure 4, lower panel). The differences in urinary α-fluoro-4-ene VPA before and after alkaline hydrolysis were minimal (44.4 ± 4.4 μmol *vs* 46.9 ± 3.1 μmol), suggesting that negligible amounts of the glucuronide were formed. The urinary recovery of α-fluoro-4-ene VPA during day 5 was ~37% of the dose administered on that day.

The 4-ene VPA metabolite (*E*)-2,4-diene VPA can be biotransformed to its GSH conjugate in the liver and subsequently through the mercapturate pathway be excreted in urine as the NAC conjugate (*8*). The NAC conjugate of (*E*)-2,4-diene VPA was detected in all urine

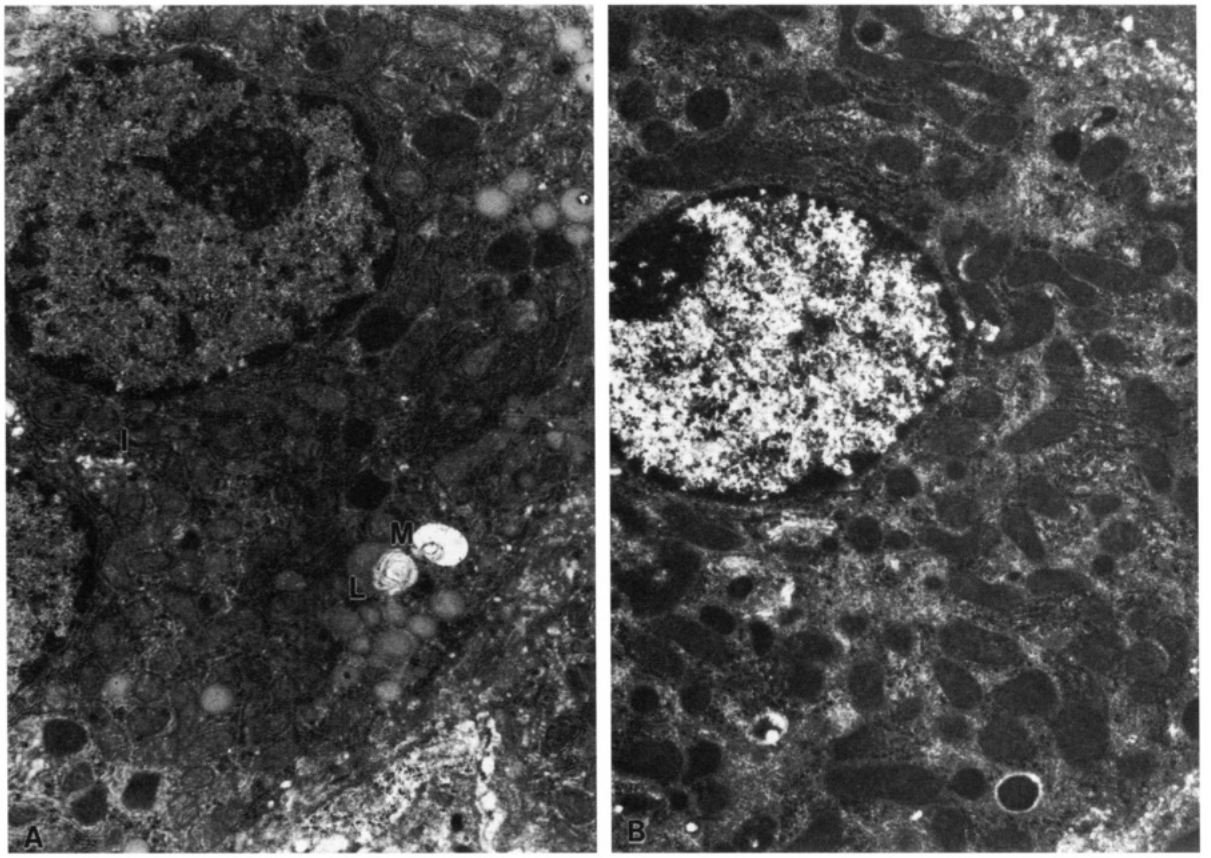


Figure 3. Electron micrographs of hepatocytes from rats administered 4-ene VPA (A) and α-fluoro-4-ene VPA (B), respectively, at 0.70 mmol/kg daily for 5 days. Numerous lipid vacuoles (L), myeloid bodies (M), and mitochondrial matrix inclusions (I) can be seen in 4-ene VPA-treated animals (magnification 1320×; reproduced at 90% of original size) but not in α-fluoro-4-ene VPA-treated rats (magnification 1120×; reproduced at 90% of original size).

samples from 4-ene VPA-treated rats (Figure 5, part a, part b, upper panel) but not in the urine of rats dosed with α-fluoro-4-ene VPA (Figure 5b, lower panel), further confirming that the metabolism of α-fluoro-4-ene VPA in rats does not lead to the formation of (*E*)-2,4-diene VPA.

GSH Status in the Liver of 4-Ene VPA- and α-Fluoro-4-ene VPA-Treated Animals. The hepatic mitochondria prepared from all treatment groups were found to be intact. There was no obvious inhibition of oxidative phosphorylation in any of the mitochondrial preparations based on measured respiratory control indexes of 5.1−12.0 and ADP/O ratios of 2.3−3.1 (*19*). The activity of the cytosolic marker enzyme, lactate dehydrogenase, in the mitochondrial preparations was 0.51% of that in the homogenates, indicating a negligible amount of cytosolic contamination in the mitochondrial preparations.

GSH levels in whole liver homogenates and in mitochondria were measured at 0.75, 2, and 4 h after the rats were administered 1.41 mmol/kg of either 4-ene VPA or α-fluoro-4-ene VPA. Control homogenate and mitochondrial GSH values were determined to be 33.6 nmol/mg of protein and 3.71 nmol/mg of protein, respectively, and were comparable to published values (*27*). Liver homogenate GSH levels at 4 h were depleted by 4-ene VPA and α-fluoro-4-ene VPA to 56% and 72% of control, respectively (Table 2). In the case of 4-ene VPA, the GSH level in the homogenate reached its lowest point at 2 h and was relatively constant without recovery during the subsequent 2 h period (Table 2). Hepatic mitochondrial GSH levels remained unchanged in rats dosed with α-fluoro-4-ene VPA, while 4-ene VPA treatment reduced mitochondrial GSH to 68% of control (Table 3). This decrease of mitochondrial GSH by 4-ene VPA was detected at 2 h post-dose and remained that way during the following 2 h (Table 3).

After 24 h of fasting, the 5th day control levels of hepatic GSH in the chronic treatment group decreased to 23.2 nmol/mg of protein (Table 2), most likely due to an enhanced rate of GSH efflux from the liver to other tissues (*28*). In contrast to the results of the acute treatment, neither 4-ene VPA nor α-fluoro-4-ene VPA showed any effect on the total hepatic GSH content following six daily doses of 0.70 mmol/kg of either drug (Table 2). After 5 days, mitochondrial GSH was elevated in 4-ene VPA-treated rats to twice that of control, but remained the same as the control in rats dosed with α-fluoro-4-ene VPA (Table 3). An inhibition of mitochondrial GR activity was detected in the 4-ene VPA-treated group of rats, the enzyme activity being reduced to 43% of control. No significant decrease in mitochondrial GR activity was observed in rats administered α-fluoro-4-ene VPA (Table 3).

Discussion

The hepatotoxicity of VPA is thought to be associated with a metabolic process that begins with the formation of the microsomal P450 metabolite 4-ene VPA, which is a structural analogue of the known hepatotoxicant 4-PA (*3*). The proposed mechanism for VPA toxicity suggests that the production of hepatotoxicity is highly dependent on the formation of reactive intermediates from 4-ene VPA in mitochondria, namely, (*E*)-2,4-diene VPA and 3-keto-4-ene VPA, the first of which is a potential GSH-depleting agent and the second is a putative inhibitor of β-oxidation enzymes (*8, 9*) (Scheme 1). This mechanism for VPA hepatotoxicity is based largely on prior knowl-

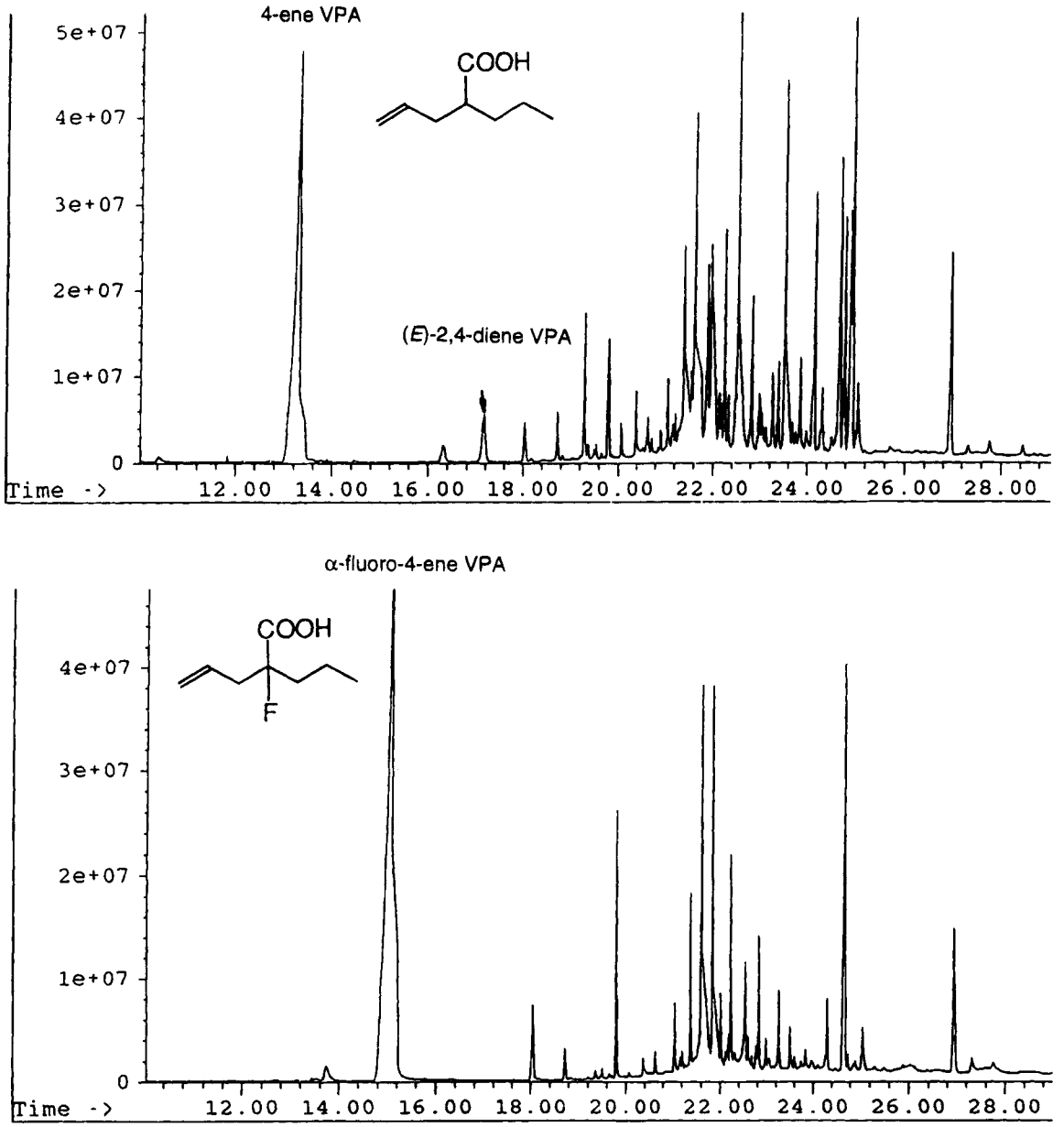


Figure 4. GC/MS total ion current chromatograms of tBDMS derivatives of extracted urine samples from 4-ene VPA (the upper panel) and α-fluoro-4-ene VPA (the lower panel) treated rats.

edge that 4-PA, when metabolized by β-oxidation *via* (*E*)-2,4-pentadienoic acid, produces 3-oxo-4-pentenoic acid that inhibits the enzyme(s) of that pathway in a suicidal manner (*17*). However, fundamental differences in the metabolism of 4-ene VPA from 4-PA in rats suggest that the mechanisms causing liver injury may not be identical (*29*). The (*E*)-2,4-diene VPA and its NAC conjugate are predominant metabolites of 4-ene VPA while evidence for 3-keto-4-ene VPA is difficult to obtain. The reverse is true for corresponding metabolites of 4-PA (*29*). But, apart from the details, it is reasonable to propose that bioactivation through β-oxidative metabolism is a common feature for both compounds and that hepatotoxicity of either 4-ene VPA or 4-PA can be prevented by averting their metabolism in mitochondria. In this study, it was confirmed that the major metabolites of 4-ene VPA excreted in rats were (*E*)-2,4-diene VPA, which is a key intermediate in the putative bioactivated toxification process (Scheme 1), and the NAC conjugate of (*E*)-2,4-diene VPA, which arises from the corresponding GSH conjugate (*8*). None of these species could be detected in the urine and serum of rats treated with the α-fluorinated compound. As a consequence of preventing the metabolism of α-fluoro-4-ene VPA by the β-oxidative pathway, this compound, as measured by liver steatosis, was determined to be nonhepatotoxic in rats. For example, in contrast to the more than 85% of hepatocytes affected in rats treated with 4-ene VPA, hepatic microvesicular steatosis was absent in the animals administered α-fluoro-4-ene VPA.

Parallel results were seen when 4-PA and F_2-4-PA were compared. Substitution of the α-hydrogens of 4-PA with two fluorine atoms was similarly expected to prevent metabolism of F_2-4-PA through the β-oxidation pathway. While the block of metabolism was not confirmed, F_2-4-PA, like α-fluoro-4-ene VPA, was ineffective in producing microvesicular steatosis in rat liver, even though 4-PA was a potent inducer of such lesions. Taken together, these results strongly suggest that reactive metabolites produced from β-oxidative processes in mitochondria are decisive factors in 4-PA- and 4-ene VPA-related liver toxicities.

In order to further clarify a basis for the apparent differences of fluorinated and nonfluorinated derivatives to produce liver toxicity in rats, the effects of 4-ene VPA and α-fluoro-4-ene VPA on hepatic GSH levels were

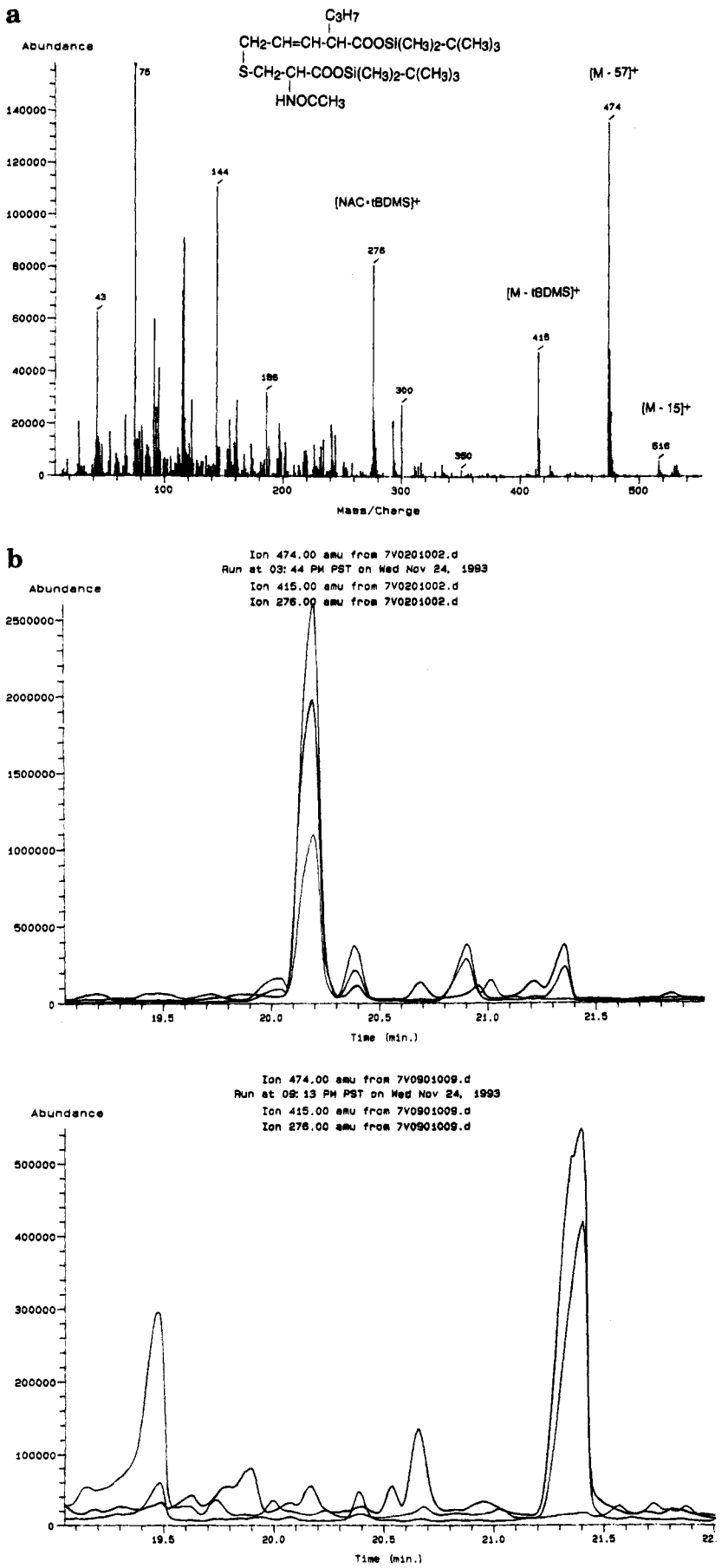


Figure 5. Part a: GC/MS mass spectrum of tBDMS derivative of the urinary NAC conjugate of (*E*)-2,4-diene VPA in 4-ene VPA-treated rats, which is identical to that of the synthesized reference compound. Part b: The NAC conjugate of (*E*)-2,4-diene VPA (retention time: 20.17 min) found in the urine of 4-ene VPA-treated rats (the upper panel) but not in the urine of α-fluoro-4-ene VPA-treated rats (the lower panel). Three fragment ions, *m*/*z* 474, *m*/*z* 415, and *m*/*z* 276, were used in the identification of the conjugate.

Table 2. Effects of 4-Ene VPA and α-Fluoro-4-ene VPA on Rat Hepatic Total Glutathione Levels

	hepatic glutathione, GSH + GSSG (nmol/mg of protein)				
	acute treatment[a]				chronic treatment[b]
test agent	0 (h)	0.75 (h)	2 (h)	4 (h)	5 (day)
control	33.64 ± 1.83 (100)				23.15 ± 4.55 (100)
4-ene VPA		24.46 ± 4.85[c] (72.7)	19.39 ± 1.10[c] (57.6)	18.92 ± 2.70[c] (56.2)	26.31 ± 2.99 (113.6)
α-F-4-ene VPA		27.04 ± 4.38 (80.3)	30.58 ± 3.08 (90.9)	24.37 ± 1.84[c] (72.4)	27.27 ± 4.65 (117.7)

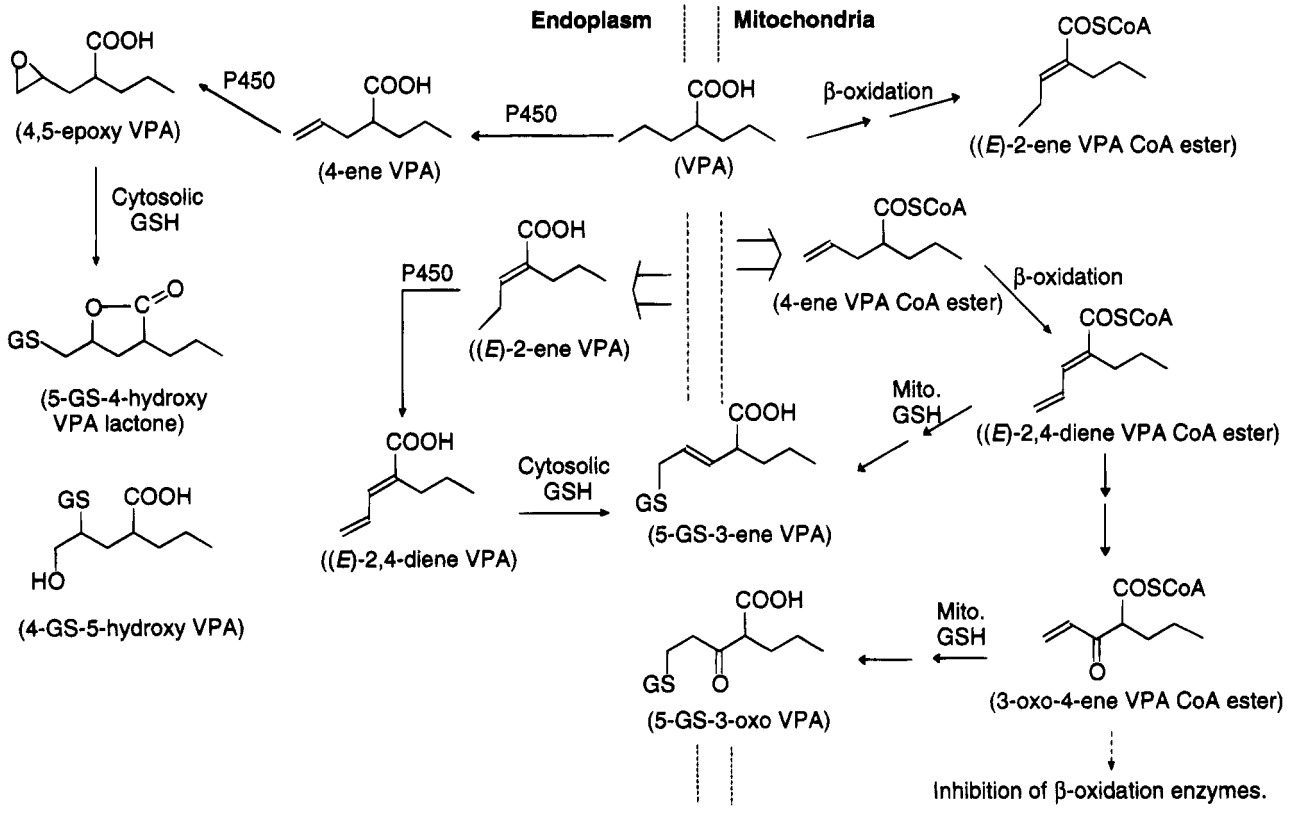
[a] Rats were administered 4-ene VPA and α-fluoro-4-ene VPA, respectively, at 1.41 mmol/kg, and liver glutathione levels were determined at 0, 0.75, 2.0, and 4.0 h following the dose (mean ± SD, with % of control expressed in the parentheses, $n = 3$ for each time point). [b] The rats were treated with 4-ene VPA or α-fluoro-4-ene VPA at 0.70 mmol/kg daily for 6 consecutive doses, and liver glutathione levels were determined 1 h following the 6th dose (mean ± SD, with % of control expressed in the parentheses, $n = 4$). [c] Significantly different from control as determined by one-way Student's t test ($p < 0.05$).

Table 3. Effects of 4-Ene VPA and α-Fluoro-4-ene VPA on Rat Liver Mitochondrial Glutathione Levels

	hepatic mitochondrial glutathione, GSH + GSSG (nmol/mg of protein)					
	acute treatment[a]				chronic treatment[b]	mitochondrial GR act.,[c]
test agent	0 (h)	0.75 (h)	2 (h)	4 (h)	5 (day)	% of control
control	3.73 ± 0.16 (100)				3.28 ± 0.81 (100)	100
4-ene VPA		3.75 ± 0.03 (100)	2.66 ± 0.21[d] (71.3)	2.54 ± 0.02[d] (68.1)	6.69 ± 0.77[d] (204)	43[d]
α-F-4-ene VPA		3.67 ± 0.36 (98.4)	3.89 ± 0.10 (104)	3.64 ± 0.26 (97.6)	3.06 ± 0.42 (93.4)	85

[a] Rats were administered 4-ene VPA and α-fluoro-4-ene VPA, respectively, at 1.41 mmol/kg, and liver mitochondrial glutathione levels were determined at 0, 0.75, 2.0, and 4.0 h following the dose (mean ± SD, with % of control expressed in parentheses, $n = 3$ for each time point). [b] The rats were treated with 4-ene VPA or α-fluoro-4-ene VPA at 0.70 mmol/kg daily for 6 consecutive doses, and liver mitochondrial glutathione levels were determined 1 h following the 6th dose. The results were pooled from two separate experiments (mean ± SD, with % of control expressed in parentheses, $n = 6$). [c] Mitochondrial glutathione reductase activity was measured in the liver of rats receiving the chronic treatment (pooled samples). [d] Significantly different from control as determined by one-way Student's t test ($p < 0.05$).

Scheme 1. Summary of the Metabolic Pathways of VPA Leading to Reactive Metabolites and Their GSH Conjugates



examined. There are two pools of cellular GSH, one located in cytosol and another in mitochondria (*30*), and it is important for mechanistic considerations of a toxic substance to distinguish which sources of GSH are being depleted. Because mitochondria are believed to lack catalase, GSH coupled with GSH peroxidase is considered to be the only antioxidant defense in this organelle (*30, 31*). Although recent data indicate that catalase is present in rat heart mitochondria (*32*), this enzyme cannot act as a defense against peroxides other than hydrogen peroxide. For example, it was reported that hepatic mitochondrial GSH was markedly reduced in rat hepatocytes by (*R,S*)-3-hydroxy-4-pentenoate (*33*). The causative species in this process is believed to be the metabolite 3-oxo-4-pentenoate (*33*), which is also a metabolite of 4-PA (*29*). As a consequence of the GSH depletion by 3-hydroxy-4-pentenoate, the toxicity of *tert*-butyl hydroperoxide to hepatocytes was significantly potentiated (*33*). In the present study, α-fluoro-4-ene VPA did in fact decrease total hepatic GSH, but it had

no effect on GSH within mitochondria. The acute administration of 4-ene VPA, on the other hand, significantly reduced mitochondrial as well as total hepatic GSH levels. A similar depletion effect of 4-ene VPA on liver GSH has recently been reported by Baillie and coworkers (*10*).

The observed distinction in mitochondrial GSH depletion between 4-ene VPA and α-fluoro-4-ene VPA could be due entirely to differences in metabolism. Conjugation of the mitochondrial metabolites of 4-ene VPA, namely, (*E*)-2,4-diene VPA and to a much lesser extent 3-keto-4-ene VPA, with GSH residing within mitochondria may account for the apparent depletion of this critical GSH pool. Similarly, the lack of effect of α-fluoro-4-ene VPA on mitochondrial GSH levels is in accord with the absence of (*E*)-2,4-diene VPA as a metabolite. Therefore, the nonsteatogenic properties of α-fluoro-4-ene VPA together with the absence of effects on mitochondrial GSH support the hypothesis that the selective depletion of mitochondrial GSH plays a critical role in the hepatotoxicity of 4-ene VPA, and, by extension, VPA. It has been reported, for example, that VPA was capable of decreasing GSH levels in rat liver (*34*) and in certain cell lines (*35*). The urinary NAC conjugate of (*E*)-2,4-diene VPA was found to be higher than average in patients who had developed liver toxicity while on VPA therapy (*8*), and in some cases patients with VPA-associated hepatotoxicity were successfully treated with supplements of NAC (*36*). The latter is believed to increase intracellular GSH levels (*36*).

The depletion of cytosolic GSH probably accounts for most of the decrease of total hepatic GSH observed for 4-ene VPA in this study (Table 2) and is largely the result of conjugation of cytosolic GSH to 2-(2′-carboxypentanyl)-oxirane (4,5-epoxy VPA), a microsomal P450-mediated oxidation product of 4-ene VPA metabolism (*10*) (Scheme 1). Likewise, metabolism of α-fluoro-4-ene VPA by microsomal P450 to 2-fluoro-4,5-epoxy VPA followed by conjugation with GSH would account for the apparent decrease of total hepatic GSH seen in rats treated with this fluorinated compound (Table 2). The epoxide metabolite of 4-ene VPA may also be important to the observed hepatotoxicity of 4-ene VPA although the results with the fluorinated derivative would suggest that the reaction of epoxide with GSH is of less significance to the production of steatosis. It is not fully understood at this time why, in the case of 4-ene VPA-treated rats, the decrease of mitochondrial GSH was not detected until at least 45 min post-dose whereas total GSH levels were already reduced to ~70% of control (Tables 2 and 3). One might assume that this is the time needed for 4-ene VPA to cross mitochondrial membranes, to be metabolized to (*E*)-2,4-diene VPA-CoA thioester, and to react with the residing GSH.

In contrast to the acute treatment, chronic administration of 4-ene VPA to rats for 5 days produced an elevation of the mitochondrial GSH levels (Table 3). This apparently paradoxical result can be interpreted in terms of the stimulation of either the cytosolic enzymes involved in the synthesis of GSH (*37*) or the transport system carrying GSH into mitochondia, or both, in response to the earlier depletion of cytosolic and mitochondrial GSH pools by electrophilic metabolites of 4-ene VPA. Such a phenomenon is not unusual for GSH-depleting agents. It was reported that after administration of either 1,2-dichloropropane (*38*) or 1,3-bis(2-chloroethyl)-1-nitrosourea (*39*) to rats an initial depletion led to a later rebound increase in hepatic GSH. An increase in the concentration of acetaminophen was seen to simultaneously elevate the synthesis rate of GSH in isolated rat hepatocytes (*40*).

The hepatotoxicity of 4-ene VPA may also be associated with an accumulation of GSSG in mitochondria, since an inhibition of mitochondrial GR activity was observed in 4-ene VPA-treated rats (Table 3). No export of GSSG from the mitochondria has been detected, the oxidized thiol apparently being reduced *in situ* by GR once it is formed (*41*). An accumulation of GSSG shifts the mitochondrial redox state and adds to the oxidative stress of the cell (*30*). This mechanism may in fact contribute to the cell toxicity observed for 4-ene VPA. The administration of α-fluoro-4-ene VPA did not produce any statistically significant inhibitory effect on the GR activity, which is again in accord with the observed nonhepatotoxic properties of the compound. Further studies exploring the mechanism of 4-ene VPA-induced inhibition of GR are warranted.

The strongest argument against the hypothesis of toxic metabolite(s) being responsible for VPA hepatotoxicity is that the toxicity is not correlated with the serum concentrations of either 4-ene VPA or (*E*)-2,4-diene VPA in humans (*12*) and in animals (*14*). The predication of such a relationship between drug metabolites and toxic effects is not necessarily to be expected. The biotransformation of VPA and its metabolites entails two phase I pathways, namely, mitochondrial β-oxidation and microsomal P450-catalyzed oxidation (Scheme 1) (*42*). The putative toxic metabolites may arise from either pathway. Microsomal dehydrogenation of VPA and (*E*)-2-ene VPA gives rise to 4-ene VPA (*5*) and (*E*)-2,4-diene VPA (*43*), respectively. Mitochondrial β-oxidation of 4-ene VPA also results in (*E*)-2,4-diene VPA (*42*), the difference being that the reactive intermediates such as (*E*)-2,4-diene VPA-CoA that are generated within mitochondria may immediately attack their targets to form covalent bonds. On the other hand, the (*E*)-2,4-diene VPA arising from the microsomal oxidation of (*E*)-2-ene VPA could have less impact on hepatotoxicity due to its localization in the cytosolic compartment (*10*) (Scheme 1). Experimentally, to differentiate in vivo whether (*E*)-2,4-diene VPA is formed in microsomes from (*E*)-2-ene VPA or in mitochondria from 4-ene VPA is very difficult to achieve when both of the precursors are present. Consequently, the toxic events are unlikely to be anticipated by the serum concentrations of either 4-ene VPA or (*E*)-2,4-diene VPA. It might even be argued that it is the attack of reactive species with their targets that is important to the expression of toxicity. Thus, reduced serum levels of 4-ene VPA or (*E*)-2,4-diene VPA could in fact be associated with more severe toxic consequences.

It is worthwhile here to mention one difference between our observations and that reported recently by Loscher *et al.* (*14*) regarding 4-ene VPA-induced microvesicular steatosis in rats. Doses of 4-ene VPA were the same in both experiments except that rats were treated for 8 days in the reported work, 3 days longer than the protocol for our experiment. Loscher *et al.* observed microvesicular steatosis in less than 50% of the treated animals, with only 20% having a 4+ score (*14*). This contrasts with 100% of the animals being affected with a score greater than 4+ in this study and in the earlier work by Kesterson *et al.* (*4*), which was also a 5 day study in vivo of 4-ene VPA hepatotoxicity. The reasons for these observed differences in experimental outcome are not readily apparent.

Because fluorine is a strong electron-withdrawing substituent, the introduction of fluorine into 4-ene VPA and 4-PA will change the physical–chemical properties

of these compounds. It is not impossible that the fluorinated compounds might have unique pharmacokinetic profiles that could lead to differences in the expression of toxicity. This possibility is currently under investigation.

Several VPA derivatives, including VPA and (*E*)-2-ene VPA, have been shown to inhibit fatty acid β-oxidation in rat liver mitochondria at levels comparable to that of 4-ene VPA (*44*). Yet, VPA and (*E*)-2-ene VPA are known to induce much less microvesicular steatosis than 4-ene VPA in experimental animals (*4*). Since reduced utilization of fatty acids in mitochondria is one of several possible mechanisms leading to a fatty liver, it may be concluded that a demonstrated in vitro inhibition of β-oxidation does not reflect the total picture of VPA hepatotoxicity. Although we were readily able to produce microvesicular steatosis in rat liver with 4-ene VPA treatment, necrosis, which is second to steatosis as a common lesion seen in VPA hepatotoxicity in humans (*1*), could not be clearly established. A fatty liver does not necessarily lead to the death of hepatocytes, and the liver may still function as normal (*45*). Further investigations into the mechanisms of VPA hepatotoxicity may reveal the intricacies of these observations.

In summary, the observed absence of hepatotoxicity in vivo in rats of α-fluoro-4-ene VPA and F_2-4-PA, when compared to their nonfluorinated counterparts, is strong evidence that bioactivation in mitochondria is an essential step leading to an expression of toxicity by either 4-ene VPA or 4-PA. The rationale for this claim is the fact that in 4-ene VPA-treated rats the potentially reactive (*E*)-2,4-diene VPA and the NAC conjugate of (*E*)-2,4-diene VPA were detected as metabolites, the latter being an indication for the in vivo reaction of (*E*)-2,4-diene VPA with GSH. Neither of these metabolites could be identified in rats treated with the nonhepatotoxic α-fluoro-4-ene VPA. Depletion of GSH and inhibition of GR in liver mitochondria were also selective for 4-ene VPA, which suggested that an impaired GSH redox system could contribute to the drug-associated hepatotoxicity.

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