Pharmacokinetics of Two Monounsaturated Metabolites of Valproic Acid in The Rat

by

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ABSTRACT

Valproic acid (VPA) is a broad spectrum antiepileptic agent used widely in the treatment of absence and tonic-clonic seizures. VPA is extensively metabolized and forms 17 metabolites in man. A monounsaturated metabolite, (E)-2-ene VPA, is at least as potent as the parent drug VPA in several animal models of epilepsy. Moreover, (E)-2-ene VPA appears to be free of two serious side effects of VPA, namely hepatotoxicity and teratogenicity. Another monounsaturated metabolite of VPA, 4-ene VPA, has been incriminated in the pathogenesis of fatal hepatic failure in children on VPA therapy.

This thesis describes the synthesis of (E)-2-ene VPA and 4-ene VPA and the development of a simple and sensitive capillary gas chromatographic-mass spectrometric (GCMS) assay method for the estimation of (E)-2-ene VPA and 4-ene VPA in the biological fluids of the rat. This thesis also describes the pharmacokinetics of (E)-2-ene VPA and 4-ene VPA at two dose levels of 20 and 100 mg/kg in normal and bile exteriorized rats.

A simple capillary GCMS assay method was developed that involves a single extraction of 80 μL of plasma, urine or bile with ethyl acetate followed by derivatization with MTBSTFA (N-tertiarybutyldimethylsilyl-N-methyl-trifluoroacetamide). For an 80 μL biological sample employed for extraction, the lowest detection limit for (E)-2-ene VPA was 60 ng/mL and for 4-ene VPA, 100 ng/mL. The calibration curves for (E)-2-ene VPA were linear over a fairly wide concentration range of 0.4-35 μg/mL in plasma and 2-200 μg/mL in urine of the rat. Standard curves for 4-ene VPA were prepared in concentration ranges of 0.5-45 μg/mL in plasma and 2-80 μg/mL

in urine. The assay method is reliable, reproducible, and is able to separate the diene metabolites of (E)-2-ene VPA.

For pharmacokinetic studies, a single intravenous (IV) bolus dose of either (E)-2-ene VPA or 4-ene VPA was administered to normal or bile-exteriorized rats. On increasing the dose from 20 to 100 mg/kg in normal rats, the apparent plasma clearance of (E)-2-ene VPA changed from 4.9 ± 1.7 (SD) to 3.0 ± 0.3 mL/min.kg, and of 4-ene VPA decreased from 8.7 ± 0.6 to 5.9 ± 0.5 mL/min.kg. A total (conjugates and unconjugates) of 32 ± 6% of the low dose and 50 ± 11% of the high dose of (E)-2-ene VPA was recovered in the urine of the rat. The second metabolite, 4-ene VPA, was eliminated in the urine to a relatively smaller extent (22 ± 3% of the low dose and 28 ± 6% of the high dose).

In bile-duct cannulated rats, the apparent plasma clearance of (E)-2-ene VPA was 7.7 ± 1.8 mL/min.kg at the low dose and 6.0 ± 1.1 mL/min.kg at the high dose. The corresponding values for 4-ene VPA were 11 ± 1.8 mL/min.kg and 7.4 ± 1.1 mL/min.kg, respectively. The apparent elimination half-life of (E)-2-ene VPA remained unchanged at 20-21 min at the two dose levels, compared to a 1.5 fold increase in the $t_{1/2}$ of 4-ene VPA from 13 ± 2 to 19 ± 3 min. The fraction of the low dose (29 ± 5%) eliminated in bile was significantly larger than at the high dose (21 ± 4%), when calculated as the sum of conjugated and unconjugated 4-ene VPA. The biliary elimination of (E)-2-ene VPA showed a non-significant change from 38 ± 10 to 31 ± 9% on increasing the dose.

Like the parent drug VPA, (E)-2-ene VPA and 4-ene VPA showed enterohepatic recirculation in the rat which produced secondary plasma peaks in normal animals. Moreover, both (E)-2-ene VPA and 4-ene VPA showed

a rapid but transient choleretic effect in the rat. The plasma protein binding of 4-ene VPA was apparently low (14-25%), in the concentration range of 20-350 μg/mL.

The results indicate that 4-ene VPA is cleared much faster from the plasma than (E)-2-ene VPA in the rat. The plasma levels of 4-ene VPA required to show a non-linear decline (>200 μg/mL) in the rat are two orders of magnitude higher than 4-ene VPA levels (<1 μg/mL) seen in patients on VPA therapy. It is, therefore, unlikely that 4-ene VPA is eliminated more slowly than VPA in man. On the other hand, the plasma elimination $t_{1/2}$ of (E)-2-ene VPA in bile-exteriorized rats is longer than that reported for VPA, indicating that (E)-2-ene VPA may have a longer lasting pharmacologic effect than VPA.

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SYMBOLS AND ABBREVIATIONS

AGA	allylglutaric acid
Amt	amount
AUC	area under the plasma concentration-time curve from 0-∞
B	biliary
BBB	blood brain barrier
conj	conjugated
Cl_B	apparent biliary clearance of unconjugated metabolite
Cl_{conj}	apparent clearance due to the formation of conjugates
Cl_{met}	apparent metabolic clearance
Cl_R	apparent renal clearance of unconjugated metabolite
Cl_T	apparent plasma clearance of unconjugated metabolite
cm	centimetre
Conc	concentration
C_o	plasma concentration of unconjugated metabolite at zero time
CSF	cerebrospinal fluid
CV	coefficient of variation
^oC	degree Celsius
DNBA	di-N-butylacetic acid
(E)	*trans* configuration
(E)-2-ene VPA	*trans* 2-n-propyl-2-pentenoic acid
EHC	enterohepatic circulation
EI	electron impact
4-ene VPA	2-n-propyl-4-pentenoic acid
eV	electron volt

GABA	γ-aminobutyric acid
GC	gas chromatograph
GCMS	gas chromatograph-mass spectrometer
GI	gastrointestinal
GIT	gastrointestinal tract
g	gram
HA	hexanoic acid
h	hour
I.D.	internal diameter
IP	intraperitoneal
IV	intravenous
k_1	apparent elimination rate constant in the log-linear phase between 0≈2 hr
k_{10}	apparent first-order elimination rate constant from compartment 1.
k_{12}	apparent first-order transfer rate constant from compartment 1 to compartment 2.
k_{21}	apparent first-order transfer rate constant from compartment 2 to compartment 1.
Lit.	literature
m/z	mass/charge
min	minute
mL	millilitre
mm	millimetre
MTBSTFA	N-tertiarybutyldimethylsilyl-N-methyl trifluoroacetamide
N	normal
na	not available

PE	polyethylene
PGA	propylglutaric acid
PTFE	polytetrafluoroethylene
SD	standard deviation
SGOT	serum glutamic oxaloacetic transaminase
SGPT	serum glutamic pyruvic transaminase
T	tau (time-lag)
t	time
t-BDMS	tertiary-butyldimethylsilyl
$t_{1/2}$	elimination half-life
THF	tetrahydrofuran
Torr	millimetre of mercury pressure
μ	micron
U	urinary
μA	microampere
unconj	unconjugated (unchanged)
UDP	uridine diphosphate
μg	microgram
μL	microlitre
VPA	2-n-propylpentanoic acid
V_1	volume of the central compartment
V_d	apparent volume of distribution
(Z)	*cis* configuration

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A. INTRODUCTION

A.1. VALPROIC ACID

A.1.1. Background

Valproic acid (VPA) is a small, branched-chain fatty acid (Fig 1). Its synthesis was first reported over a century ago (Burton 1882). Its anticonvulsant properties were only discovered in 1963 (Meunier *et al.*, 1963; Carraz *et al.*, 1964a; Carraz *et al.*, 1964b) in France. It was introduced as a drug (Depakene, Depakine, Epimyl or Ergenyl) in Europe in 1968 and in North America in 1978.

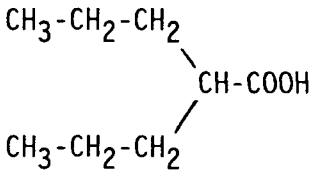


Fig.1 Valproic acid

VPA is a broad spectrum anticonvulsant used in the treatment of generalized absence, myoclonic and tonic-clonic seizures (Gram and Bentsen 1985; Dulac and Arthuis 1984; Feuerstein *et al.*, 1983; Covanis *et al.*, 1982). It is also effective in controlling fever convulsions (Lee *et al.*, 1986), partial seizures (Bruni and Albright 1983), status epilepticus (Vajda *et al.*, 1978) and photosensitive epilepsy (Harding *et al.*, 1978). It has been also employed to treat Lennox syndrome (Schobben *et al.*, 1980b) and epilepsies refractory to other anticonvulsants (Redenbaugh *et al.*, 1980). The drug is administered orally at a dose of 15-30 mg/kg daily. Its therapeutic blood levels are in the range of 50-100 μg/mL (Klotz and Schweizer 1980; Gram *et al.*, 1980).

A.1.2 Pharmacokinetics in Man

After oral administration, VPA is rapidly absorbed from the GI tract. Peak plasma levels are reached within 1-2 h of its administration (Gugler and von Unruh 1980). VPA is completely bioavailable without any evidence of first-pass elimination by the liver (Perucca *et al.*, 1978a; Klotz and Antonin 1977). Its plasma elimination half-life is between 10-16 h in healthy volunteers (Perucca *et al.*, 1978a; Gugler and von Unruh 1980), and 6-10 h in epileptic patients taking other anticonvulsants (Perucca *et al.*, 1978b; Gugler and von Unruh 1980). VPA follows linear pharmacokinetics exhibiting no change in its elimination $t_{1/2}$ after single or multiple dosing (Gugler *et al.*, 1977). Plasma concentrations of VPA increase linearly when its dose is increased within the therapeutic range and above, up to 60 mg/kg (Nutt and Kupferberg 1979). The plasma clearance of VPA is in the range of 5-10 mL/min (Gugler and von Unruh 1980). It has a small apparent volume of distribution of 0.1-0.4 L/kg (Gugler and von Unruh 1980). VPA is highly bound (>90%) to plasma proteins, especially to albumin (Loscher 1978; Patel and Levy 1979). The free-fraction of VPA is the same in serum and in heparin-treated or EDTA-treated plasma (Cramer *et al.*, 1983).

At therapeutic doses, CSF and brain levels of VPA, 3-33 and 7-27 μg/mL respectively, are directly proportional to free VPA concentration in the plasma (Rapeport *et al.*, 1983; Vajda *et al.*, 1981). Only low levels of VPA, between 1-3% of the maternal serum levels, are secreted into the mother's milk (Nau *et al.*, 1981a; Dickinson *et al.*, 1979b). The serum concentrations of VPA in newborn infants have been reported to be 1.4-1.7 times higher than the maternal serum levels (Nau *et al.*, 1981a; Dickinson *et al.*, 1979b). The elimination $t_{1/2}$ in neonates is 45-47 h, which is three

times longer than that in adults (Nau *et al.*, 1981a; Dickinson *et al.*, 1979b).

A.1.3 Pharmacokinetics in Animals

The disposition of VPA has been studied in many animal species including the pig (Bonora *et al.*, 1979), the monkey (Dickinson *et al.*, 1980), the dog (Loscher 1978; Loscher and Esenwein 1978), the rabbit (Ichimura *et al.*, 1985), the rat (Loscher 1978) and the mouse (Loshcer and Esenwein 1978). The plasma elimination $t_{1/2}$ of VPA in the pig, dog, rabbit and mouse has been reported to be 87, 61-84, 75 and 50 min respectively. The shorter $t_{1/2}$ of VPA in smaller animals has been attributed, in part, to lesser plasma protein binding of drug in such animals, thus providing a larger free-fraction for elimination. The free fraction (x100) of VPA in the dog, rat and mouse is 22, 37 and 88%, respectively (Loscher 1978). In an extensive study in the rat, Dickinson *et al.* (1979a) have reported that plasma elimination of VPA is linear at concentrations below 100 μg/mL, and non-linear at higher concentrations. VPA undergoes enterohepatic circulation (EHC) in the rat, which produces secondary rises in its blood levels after single dose administration. In bile-exteriorized rats, VPA is eliminated with a $t_{1/2}$ of 11.3 min after the low dose of 15 mg/kg, and 16.7 after the high dose of 100 mg/kg. Approximately 60% of the administered dose is eliminated in the bile of the rat. After oral administration, VPA is rapidly distributed in the body of the rat (Eymard *et al.*, 1971). The drug is mainly distributed in the liver, kidneys and testes of the rat (Dickinson *et al.*, 1979a). In the rabbit, the highest levels of VPA are found in kidneys, followed by liver, heart, GI tract and fat (Ichimura *et al.*, 1985).

Detailed disposition studies in the brain of the rat have shown that VPA is preferentially distributed in the cerebellum and hippocampus (Mesdjian *et al.*, 1982; Hariton *et al.*, 1984). In another study (Loscher and Nau 1983), the highest levels of VPA were found in substantia nigra of the rat after prolonged treatment. VPA is preferentially accumulated in hypothalamus and medulla of the dog after a constant infusion of VPA (Loscher and Nau 1983). The CSF levels of VPA are identical to its free levels in the plasma of dog (Frey and Loscher 1978). The authors have, however, shown that VPA is actively transported out of CSF, probably by the monocarboxylic acid transport system. The presence of an active transport system across BBB is also supported by experiments in cat (Hammond *et al.*, 1981), in which VPA is much more rapidly cleared from the brain ($t_{1/2}$ 41 min) than the plasma ($t_{1/2}$ 190 min)

VPA produces a choleretic effect in the rat, dog and monkey (Dickinson *et al.*, 1982), and cat (Marshall *et al.*, 1984). Bile flow rate increases by 2-3 times the basal flow, 30-60 min after a single dose of 40-60 mg/kg to the rat, cat and dog (Dickinson *et al.*, 1982, Watkins and Klaassen 1981; Marshall *et al.*, 1984). VPA-induced choleresis is primarily due to the osmotic activity (Watkins and Klaassen 1981; Dickinson *et al.*, 1982) of VPA conjugates excreted in bile.

A.1.4 Metabolism

VPA is extensively metabolized in man (Acheampong *et al.*, 1983; Kochen *et al.*, 1984; Schobben *et al.*, 1980a; Gugler and von Unruh 1980; Loscher 1981; Jakobs 1978;). The proposed metabolic pathway for VPA is shown in Fig 2. The major routes of its metabolism are glucuronidation and β-oxidation. Up to 20% of the administered dose is recovered as glucuronide in the urine (Granneman *et al.*, 1984b; Bialer *et al.*, 1985). β-oxidation of

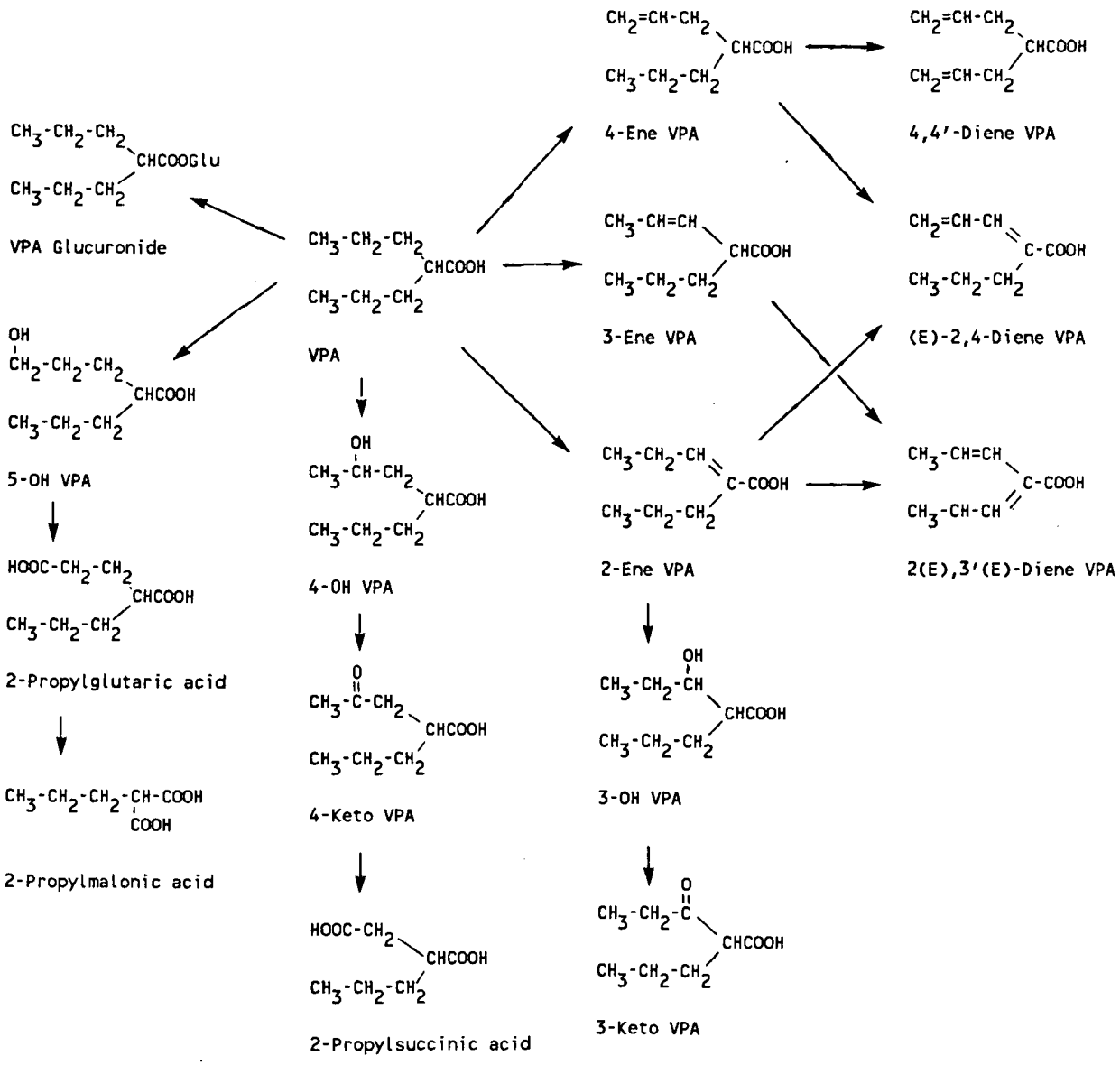


Fig. 2. Proposed metabolic pathway for VPA.

VPA produces cis and trans isomers of 2-ene VPA, and 3-keto VPA. Abbott *et al.* (1986) have reported that serum levels of trans 2-ene VPA and 3-keto VPA are 11.9 and 7.7%, respectively, of VPA serum levels at steady state in pediatric patients. The minor routes of VPA metabolism are ω-hydroxylation, ω-1 hydroxylation, γ-dehydrogenation and δ-dehydrogenation that produce 5-hydroxy VPA, 4-hydroxy VPA, 3-ene VPA and 4-ene VPA, respectively, as their first metabolites. A hydroxamate metabolite of VPA has been recently identified in the urine of patients on VPA therapy (Libert *et al.*, 1986). The serum levels of 12 metabolites of VPA in patients on VPA monotherapy (Abbott *et al.*, 1986) and their possible routes of formation are shown in Table 1.

The metabolic fate of VPA has been extensively studied in several animal species including rat, rabbit, mouse, dog and monkey (Matsumoto *et al.*, 1976; Jakobs and Loscher 1978; Ferrandes and Eymard 1977, Schobben *et al.*, 1980a). VPA metabolism in animals, especially in the rat, is similar to that in man. A total of 48 compounds have been identified as metabolic products of VPA and its intermediate metabolites in the rat (Granneman *et al.*, 1984c).

The major interest in the metabolites of VPA emerges from their possible contribution to the pharmacologic and toxicologic effects of this drug. The β-oxidation product, (E)-2-ene VPA, which is the major metabolite in the plasma of man, has been found to be as potent an anticonvulsant as VPA (Loscher *et al.*, 1984). This metabolite has been considered to contribute significantly to the total VPA activity. On the other hand, VPA-induced hepatic damage has been suggested to be caused by a minor metabolite, 4-ene VPA (Kesterson *et al.*, 1984).

TABLE 1. SERUM LEVELS OF VPA AND ITS METABOLITES IN PATIENTS ON VPA MONOTHERAPY[a]

Metabolite	Mean Conc (μg/mL)	% VPA Level
VPA[b]	46.4	100
(E)-2-ene VPA[c]	5.53	11.9
3-keto VPA[c]	3.59	7.7
(E)-2,3′-diene VPA[c,d]	2.95	6.4
3-ene VPA[d]	0.94	2.0
4-ene VPA[e]	0.67	1.4
4-keto VPA[f]	0.40	0.9
4-OH VPA[f]	0.38	0.8
(E)-2,4-diene VPA[c,e]	0.20	0.4
2-Propylglutaric acid[g]	0.20	0.4
(Z)-2-ene VPA[c]	0.19	0.4
5-OH VPA[g]	0.18	0.4
2-Propylsuccinic acid[f]	0.04	0.1

a, (Abbott et al., 1986); b, parent drug;
c, β-oxidation; d, γ-dehydrogenation;
e, δ-dehydrogenation; f, (ω-1) hydroxylation;
g, ω-hydroxylation.

A.1.5 Mechanism of Action

VPA increases whole brain GABA content in the mouse (Godin *et al.*, 1969; Nau and Loscher 1982) by inhibiting γ-aminobutyric acid transaminase (Fowler *et al.*, 1975), an enzyme that breaks down GABA. VPA is also a potent inhibitor of succinic semialdehyde dehydrogenase (Harvey *et al.*, 1975) and aldehyde reductase (Whittle and Turner 1978), enzymes present in the degradation pathway of the GABA shunt. It has been also reported that VPA administration raises GABA levels by increasing its synthesis from glutamate (Nau and Loscher 1982). When VPA is administered to mice, the brain GABA levels are significantly elevated, with a parallel increase in the enzymatic activity of glutamate decarboxylase, the enzyme responsible for GABA synthesis (Nau and Loscher 1982). The decline in GABA levels and glutamate decarboxylase activity is, however, much slower than the decline of VPA levels in the brain (Nau and Loscher 1982). The increase in GABA levels in different areas of the brain is not uniform (Hariton *et al.*, 1984). The highest GABA levels are found in the olfactory bulbs and hypothalamus of the rat (Hariton *et al.*, 1984).

VPA increases the threshold potential for excitation of nerve membrane by blocking sodium and potassium conductance (VanDongen *et al.* 1986). VPA has also been reported to depress the firing rate of spontaneously active cortical cells, whose time course is parallel to the onset of anticonvulsant activity in the rat (McLean and Macdonald, 1986; Kerwin and Olpe 1980). In another study, the pharmacologic activity was evident as early as 1 min after IP administration of VPA to rats (Schmutz *et al.*, 1979). Such a rapid onset of action was suggested to be due to the direct postsynaptic inhibitory effect of VPA that was independent of its ability to raise GABA levels (Schumtz *et al.*, 1979; Kerwin and Olpe 1980).

Another possible mechanism of action of VPA is ascribed to its ability to significantly decrease aspartate levels in the brain of mouse (Schechter *et al.*, 1978) and the rat Chapman *et al.*, 1982; Chapman *et al.*, 1983; Patsalos and Loscelles 1981). The time course of VPA-induced reduction in the cerebral aspartate levels coincides with the period of protection from audiogenic seizures in mice (Schechter *et al.*, 1978).

In actual practice, the antiepileptic activity of VPA may be due to its combined effect on neurotransmitters in the brain, and firing rate of subcortical cells.

A.1.6 Side Effects

VPA is considered a reasonably safe drug that shows mostly transient and mild side effects (Stefan *et al.*, 1984; Feuerstein *et al.*, 1983; Bruni and Albright 1983). The most common adverse effects are GI disturbances including nausea, vomiting, abdominal cramps, diarrhea and indigestion in some patients (Beran *et al.*, 1980; Simon and Penry 1975). VPA has been noticed to cause stomatitis (Russo 1981), pancreatitis (Wyllie *et al.*, 1984; Murphy *et al.*, 1981), change in appetite and weight gain (Feuerstein *et al.*, 1983; Bruni and Wilder 1979). VPA produces mild alopecia (Bruni and Albright 1983), and blood disorders such as thrombocytopenia (Winfield *et al.*, 1976) and neutropenia (Symon and Russell 1983). Several authors (Dulac and Arthuis 1984; Jeavons and Clark 1974) have reported increased alertness, improved behavior and improvement of intellectual functions following VPA administration. A very few patients on VPA monotherapy have experienced drowsiness (Stefan *et al.*, 1984) and reversible dementia (Zaret and Cohen 1986). VPA has been incriminated in causing resting and postural tremors (Coultar et al., 1980; Bruni and Wilder 1979;). VPA may produce hypothyroidism (Salvatoni *et al.*, 1983), in rare instances.

A serious toxic effect of VPA is teratogenicity (Lammer *et al.*, 1987; Di Carlo *et al.*, 1986). VPA is embryotoxic in the rabbit, rat (Whittle 1976) and mouse (Nau and Loscher 1984; Brown *et al.*, 1980). The most pronounced effects are neural tube defects (exencephaly), skeletal abnormalities, embryolethality and reduced fetal weight (Nau and Loscher 1984; Kao *et al.*, 1981). Expectant mothers on VPA therapy have a high risk (1-2%) of bearing offspring with minor abnormalities (Koch *et al.*, 1983) or major malformations including the neural tube defect spina bifida aperta (Lammer *et al.*, 1987; Robert 1983; Bjerkedal *et al.*, 1982; Jeavons 1984).

Hurd and coworkers (1981, 1982) have reported that VPA binds to zinc, and lowers zinc and selenium levels in the plasma of animals and man. The authors have suggested that VPA-induced deficiency of these rare metals may be responsible for the teratogenic effects of VPA. They (Hurd *et al.*, 1983) have further proposed that zinc supplements may reduce VPA-induced teratogenic effects. Trotz *et al.* (1987) have recently reported that folinic acid administration markedly reduces VPA-induced neural tube defects in the mouse.

A.1.7 Hepatotoxicity

The most serious side effect of VPA is fatal hepatotoxicity in young children (Zafrani and Berthelot 1982). Over 80 cases of fatal hepatic failure, on VPA therapy, have been reported (Bjorge and Baillie 1985). In these patients, typical symptoms of anorexia, vomiting, lethargy, jaundice, hepatic failure and terminal coma developed within 1-4 months of VPA therapy (Zimmerman and Ishak 1982). Autopsy of the liver showed microvesicular steatosis accompanied by cirrhosis or necrosis (Zimmerman and Ishak 1982). The hepatic injury and lesions caused by VPA (Keene *et al.*, 1982) are similar to those of Jamaican Vomiting Syndrome (JVS) and

Reye-like syndrome (RS) produced by hypoglycin A and 4-pentenoic acid (Glasgow and Chase 1975). Since 4-ene VPA, a VPA metabolite is structurally similar to the hepatotoxin, 4-pentenoic acid, VPA-induced hepatotoxicity has been ascribed to its metabolite, 4-ene VPA (Zimmerman and Ishak 1982). Delay in the onset of illness until after 1 month of drug administration in 80% of cases also suggests that a metabolic idiosyncracy rather than hypersensitivity reaction is the cause of hepatic failure. This hypothesis is supported by the lack of hallmarks of hypersensitivity such as rash, itch and urticaria in patients. Moreover, co-administration of other anticonvulsants such as phenytoin and phenobarbital, which are known enzyme inducers, enhances the hepatotoxicity of VPA in patients. In young children of 2 years or less in age, the incidence of fatal hepatic failure of 1:7000 in individuals on VPA monotherapy is increased to 1:500 on VPA polytherapy (Dreifuss *et al.*, 1987). Similarly, phenobarbital-treated rats show higher mortality and microvesicular steatosis with low doses of VPA compared to no deaths and no fatty liver in animals receiving VPA alone (Lewis *et al.*, 1982). These results suggest that enzyme inducers may enhance VPA metabolism to form a hepatotoxic metabolite (Granneman *et al.*, 1984a).

Several biochemical abnormalities have been reported in the literature, that may or may not be related to overt hepatotoxicity. VPA administration produces a temporary elevation of the hepatic enzyme SGOT in man (Willmore *et al.*, 1978) and rat (Cotariu *et al.*, 1987), but a decrease in the serum levels of SGPT in the rat (Kesterson *et al.*, 1984; Cotariu *et al.*, 1987). VPA, especially when co-administered with other anticonvulsants, produces hyperammonemia (Ratnaike *et al.*, 1986; Zaccara *et al.*, 1985). It inhibits gluconeogenesis in the rat (Turnbull *et al.*, 1983) and *in vitro* in isolated rat hepatocytes (Rogiers *et al.*, 1985; Turnbull *et al.*, 1983). VPA administration causes impaired fatty acid metabolism

including reduced fatty acid synthesis, and decreased β-oxidation (Becker and Harris 1983; Kesterson *et al.*, 1984). Rats receiving VPA have been reported to develop hypocarnitinemia and mitochondrial swelling of the liver cells (Sugimoto *et al.*, 1987). These abnormalities are corrected by administering supplements of L-carnitine (Sugimoto *et al.*, 1987). Chronic administration of VPA produces hyperglycinemia in the rat (Martin-Gallardo *et al.*, 1985; Cherruau *et al.*, 1981). It has been also reported to suppress plasma levels of corticotropin (ACTH) in children (Kritzler *et al.*, 1983).

A.2. (E)-2-ENE VALPROIC ACID

A.2.1 Pharmacologic Activity

Ten metabolites of VPA have been tested for anticonvulsant activity in animals (Loscher and Nau 1985; Loscher 1981; Schafer *et al.*, 1980). Nine of the metabolites significantly raise threshold levels for maximal electroshock and/or pentylenetetrazole (PTZ)-induced seizures in mice (Loscher and Nau 1985; Loscher 1981). The two most active compounds, (E)-2-ene VPA and 4-ene VPA, are 80-90% as active as VPA on a molar basis after IP administration to mice (Loscher and Nau 1985). The ED_{50} for (E)-2-ene VPA is 66 mg/kg in rats exhibiting spontaneously occuring 'petit mal' seizures, 90 mg/kg in gerbils with generalized tonic-clonic seizures, and 225 mg/kg against PTZ-induced seizures in mice (Loscher *et al.*, 1984). The ratio of ED_{50} for (E)-2-ene VPA/VPA is 0.81, 1.23 and 0.69 for the above seizure models in the rat, gerbil and mouse, respectively. Loscher and Nau (1983) have reported that since the brain levels of (E)-2-ene VPA are slightly less than those of VPA after equimolar doses of the two, the relative pharmacologic activity of (E)-2-ene VPA is 1.3 times higher than that of VPA. After oral administration, (E)-2-ene VPA is approximately half

as potent as VPA in chemically induced seizure models in mice (Keane *et al.*, 1985). The low potency of (E)-2-ene VPA after oral administration may be due to its lesser absorption than VPA. Overall, (E)-2-ene VPA possesses a broad spectrum of anticonvulsant activity in a number of animal models.

The mechanism of action of (E)-2-ene VPA is not known, except that it elevates GABA levels in the brain to the same extent as VPA (Keane *et al.*, 1985).

A.2.2 Pharmacokinetics

The pharmacokinetics of (E)-2-ene VPA have not been well characterized. In some of the studies, the parent drug VPA was administered, and the plasma or tissue levels of (E)-2-ene VPA were monitored (Loscher and Nau 1982). On oral administration of VPA via drinking water for 12 days, the plasma levels of (E)-2-ene VPA (0.7 μg/mL) were 20% of VPA plasma levels (3-4 μg/mL) in mice (Loscher and Nau 1982). After an IP dose of VPA to mice, the plasma elimination $t_{1/2}$ of (E)-2-ene VPA was 130 min (Nau and Loscher 1982). On constant-rate administration of VPA for 7 days to mice, the elimination $t_{1/2}$ of (E)-2-ene VPA was found to be 70 min (Nau and Zierer 1982). In man, the apparent elimination $t_{1/2}$ of (E)-2-ene VPA has been reported to be 43 h (Pollack *et al.*, 1986). In human neonates, (E)-2-ene VPA is eliminated with a half-life of 47 h, which is identical to that for VPA (Nau *et al.*, 1981a).

Tissue distribution studies have shown that hepatic concentration of (E)-2-ene VPA in mice was much lower than that for VPA (Nau and Loscher 1985). The liver-to-plasma concentration ratio for (E)-2-ene VPA was 0.1-0.5 and for VPA, 1.5-3 in mice (Nau and Loscher 1985). The brain levels of (E)-2-ene VPA are 3% of its total plasma levels at steady-state in mice (Nau and Zierer 1982). CSF levels of (E)-2-ene VPA are much lower than its

free concentration in the plasma of dog, but its brain levels are higher than its CSF levels (Loscher and Nau 1983). These results suggest that the metabolite is probably bound to the brain tissues. This hypothesis is supported by studies in rats showing that, on prolonged administration of VPA, a marked increase of (E)-2-ene VPA levels occurs in some regions of the brain, especially hippocampus, substantia nigra, superior and inferior colliculus and medulla (Loscher and Nau 1983). Moreover, after the withdrawl of VPA, (E)-2-ene VPA is cleared much more slowly than VPA from the brain of mice (Loscher and Nau 1982; Nau and Loscher 1982). The apparent elimination $t_{1/2}$ of (E)-2-ene VPA in the brain of mice is 240 min, which is 5 times longer than the 50 min half-life for VPA (Nau and Loscher 1982).

In a few studies, (E)-2-ene VPA was administered to animals and its pharmacokinetic parameters were determined (Nau and Zierer 1982). When equal oral doses of (E)-2-ene VPA and VPA are given individually to mice, the maximum plasma concentration of (E)-2-ene VPA is approximately 70% of that of VPA (Nau and Loscher 1985). After a constant-rate administration of (E)-2-ene VPA to mice, its plasma elimination $t_{1/2}$ is reported to be 71 min, and plasma clearance, 339 mL/h.kg (Nau and Zierer 1982). In the dog, (E)-2-ene VPA is eliminated from the plasma with a $t_{1/2}$ of 1.8 h, and has an apparent volume of distribution of 0.25 litres/kg (Loscher and Nau 1983). A recent abstract (O'Connor *et al.*, 1986) has reported that in rats, after IV bolus doses of 25, 75 and 225 mg/kg of (E)-2-ene VPA, the serum clearance changes biphasically from 3.95 to 3.76 mL/min.kg. The apparent volumes of distribution are 471 and 718 mL/kg at the above doses, respectively (O'Connors *et al.*, 1986).

(E)-2-ene VPA is more highly bound than VPA to plasma proteins. In

the plasma of mouse, dog and man, (E)-2-ene VPA is bound up to 97, 97 and 99.5% (Nau and Loscher 1985; Loscher and Nau 1983) respectively.

Little information is available on the metabolism of (E)-2-ene VPA. Following oral administration of (E)-2-ene VPA to mice, three metabolites were detected in the plasma in the following decreasing order: 3-keto VPA, VPA, and 5-hydroxy VPA (Nau and Loscher 1985).

A.2.3 Side Effects

(E)-2-ene VPA shows no obvious side effects at doses required to control seizures in rats and gerbils (Loscher *et al.*, 1984). It is, however, more sedating than VPA in mice as determined by rotarod (Keane *et al.*, 1985) and chimney testing (Loscher *et al.*, 1984). The LD_{50} for (E)-2-ene VPA in mice is 760 mg/kg, which is similar to 810 mg/kg for VPA (Loscher *et al.*, 1984). (E)-2-ene VPA is free of embryotoxic effects in mice even at extremely high doses of 600 mg/kg (Loscher *et al.*, 1984). In whole embryo culture studies (Lewandowski *et al.*, 1986), (E)-2-ene VPA concentrations of 200 μg/g show no abnormal development of the embryo, whereas VPA levels of 40 μg/g and above clearly induce teratogenic effects. Moreover, at equimolar concentrations of (E)-2-ene VPA and VPA in the culture medium, (E)-2-ene VPA levels in the embryo are less than those of VPA (Lewandowski *et al.*, 1986).

Kesterson *et al.* (1984) have shown that (E)-2-ene VPA does not produce hepatic steatosis in the rat. On its administration to rats, the clinical features such as serum urea nitrogen, SGOT, SGPT, ammonia levels and ketone bodies are unaltered. These results strongly suggest that (E)-2-ene VPA may be free of hepatotoxicity seen with VPA. It may, therefore, be a suitable alternative antiepileptic agent free of serious side effects of VPA, namely embryotoxicity and hepatotoxicity.

A.3. 4-ENE VALPROIC ACID

A.3.1 Toxicity

Several investigators have reported the toxicity of 4-ene VPA. *In vitro* studies in rat hepatocytes have shown that 4-ene VPA significantly raises lactic dehydrogenase (LDH) index, a measure of hepatocyte toxicity (Kingsley *et al.*, 1983). The metabolite, 4-ene VPA inhibits gluconeogenesis in isolated rat hepatocytes (Rogiers *et al.*, 1985). It is also a strong inhibitor of β-oxidation of medium chain fatty acid in homogenates of rat liver (Bjorge and Baillie 1985). Kesterson *et al.* (1984) have reported that 4-ene VPA produces mitochondrial lesions in hepatocytes, and inhibits β-oxidation in the rat. It also causes severe microvesicular steatosis in the livers of animals. The authors (Kesterson *et al.*, 1984) concluded that two different mechanisms may be responsible for β-oxidation inhibition by VPA and 4-ene VPA in rats.

LD_{50} for 4-ene VPA in mice is 1000 mg/kg, on IV administration, compared to 630 mg/kg for VPA (Loscher and Nau 1985).

A.3.2 Metabolism

The metabolism of 4-ene VPA has been recently studied in animals (Rettenmeier *et al.*, 1986; Rettenmeier *et al.*, 1985). It is metabolized by the fatty acid β-oxidation complex, and by cytochrome P450-mediated hydroxylation and epoxidation pathways (Rettenmeier *et al.*, 1985). In isolated rat liver perfusion studies, eight metabolites of 4-ene VPA were identified namely 2,4-diene VPA, 3-hydroxy 4-ene VPA, 3′-oxo 4-ene VPA, 5′-hydroxy 4-ene VPA, 5-hydroxy, 4,5-di-hydroxy VPA lactone, AGA and PGA (Rettenmeier *et al.*, 1985). The metabolism of 4-ene VPA, in the liver

perfusion studies, was affected by the length of the perfusion time. When the duration of the experiment was short (20 min), approximately 58% of the 4-ene VPA dose was recovered unchanged and 15% was converted to metabolites in the perfusate. Only 2% of the dose was excreted in bile. In a longer perfusion time study (60 min), 29% of the injected 4-ene VPA was unchanged, and 13% was collected as the sum of all the metabolites in the perfusate. A higher proportion of dose, 12%, was eliminated in the bile.

Following 4-ene VPA administration to the monkey, a total of 20 metabolites were detected in the urine (Rettenmeier *et al.*, 1986). Three major metabolites were 4-ene VPA glucuronide (38.6%), (E)-2,4-diene VPA (8.9%) and 3'-oxo VPA (8.0%). Approximately 59% of the dose is recovered as unchanged 4-ene VPA and its metabolites, collectively, in the urine of the monkey. The proposed metabolic pathway for 4-ene VPA is shown in Fig 3.

A.3.3 Pharmacokinetics

Very little information is available on the disposition of 4-ene VPA in animals or man. Since a very small fraction of the dose of VPA is converted to 4-ene VPA, the plasma levels of the latter are either too low to determine its pharmacokinetics or are undetected. In children on VPA monotherapy, the serum levels of 4-ene VPA are between 0.16-1.22 μg/mL, which is only 1% of plasma VPA levels (Abbott *et al.*, 1986). Similarly in rats, only a small fraction (0.05%) of injected VPA is recovered as 4-ene VPA in the urine (Granneman *et al.*, 1984c). A recent article (Pollack *et al.*, 1986) has reported that the plasma elimination $t_{1/2}$ of 4-ene VPA in man, following VPA administration, is 50.7 h.

In one study, 4-ene VPA was administered to the monkey (Rettenmeier *et al.*, 1986). The plasma concentration-time curve in the monkey is bi-exponential with a terminal elimination half-life of 2.3-3.6 h, and a

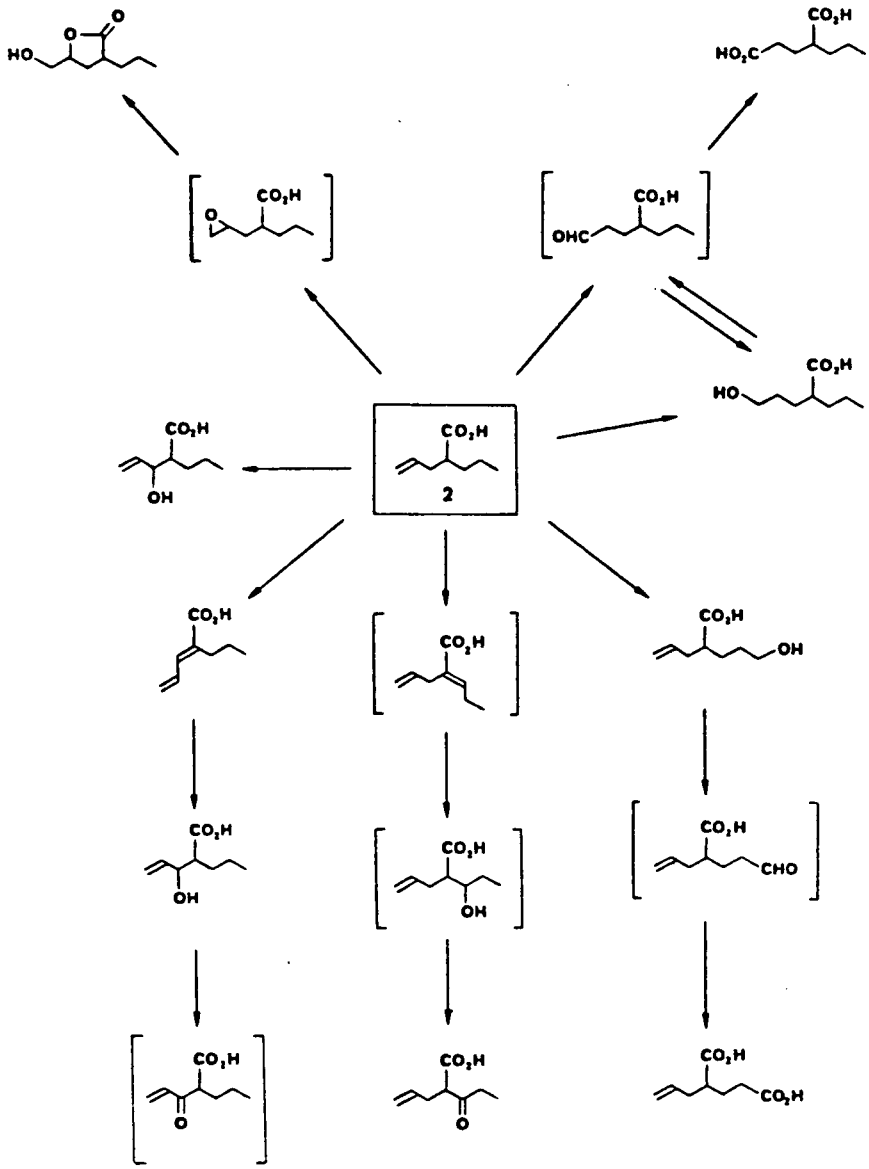


Fig. 3. Proposed metabolic pathway for 4-ene VPA[a]. (*a*, Rettenmeier *et al.*, 1985)

clearance of 2.8-2.0 mL/min.kg. A large proportion of the dose (39%) is eliminated as glucuronide and 5% is excreted unchanged in the urine of the monkey (Rettenmeier *et al.*, 1986). The plasma protein binding of 4-ene VPA in the monkey is 58-78%.

A.4. RATIONALE AND OBJECTIVES

I. The clinical effectiveness of valproic acid is not always related to its serum levels. Following its administration, a delayed onset of its antiepllileptic activity ranging from hours to weeks has been observed (Jeavons and Clark 1974; Rowan *et al.*, 1979). Similarly, after the drug is withdrawn, a carry-over effect both in animals and humans ranging from weeks to months has been reported (Pellegrini *et al.*, 1978; Harding *et al.*, 1978; Lockard and Levy 1976). A possible explanation for these temporal effects may be the formation of a pharmacologically active metabolite with longer half-life. (E)-2-ene VPA, a major mono-unsaturated metabolite of VPA, has been found to be as active as VPA in several animal models of epilepsy. A knowledge of its disposition would be needed to estimate its contribution to the overall action of VPA. Moreover, recent studies have shown that (E)-2-ene VPA appears to be free of any serious side effects associated with VPA, especially hepatotoxicity and embryotoxicity. It has been proposed (Nau *et al.*, 1984) that (E)-2-ene VPA may be used as an alternative anticonvulsant. Since it is not uncommon to develop more effective and safer drugs from drug metabolites, it appears that (E)-2-ene VPA may also join the ranks of now commonly used drugs such as oxazepam and acetaminophen, which were initially identified as metabolites of the 'parent' drugs diazepam and phenacetin, respectively. The pharmacokinetics of (E)-2-ene VPA have not been well characterized due to a lack of the

availability of pure compound in sufficiently large quantities. Dr. Abbott's laboratory has been a leader in the synthesis of several metabolites of VPA, including (E)-2-ene VPA. It was, therefore, most logical to use those facilities to synthesize (E)-2-ene VPA, develop a sensitive assay for (E)-2-ene VPA and study its disposition in the rat.

II. The metabolites of a drug are often responsible for drug-related toxicities. Hepatotoxicities of acetaminophen and iproniazid, caused by their respective metabolites, are classic examples of metabolite-induced side effects (Mitchell *et al.*, 1973; Nelson *et al.*, 1978). Similarly, Reye-like syndrome produced by VPA in children has been attributed to its metabolite, 4-ene VPA (Rettenmeier *et al.*, 1986; Kesterson *et al.*, 1984). The concentrations of this toxic metabolite, 4-ene VPA, in serum and urine of patients or animals receiving VPA, are normally either undetectable or very low. When a patient develops symptoms of severe hepatic damage, the serum levels of 4-ene VPA are increased several fold over the normal values (Kochen *et al.*, 1983). Moreover, there is a latent period of 1-4 months, after the initiation of VPA therapy, before clinical symptoms of hepatotoxicity appear. These observations suggest that the toxic metabolite levels may gradually build up during the course of therapy, or it may be rather slowly eliminated from the system. A pharmacokinetic study of 4-ene VPA, including its biliary elimination and/or enterohepatic circulation, may explain to some extent its role in VPA-induced hepatotoxicity.

The major objectives of this project were:

1. To synthesize (E)-2-ene VPA and 4-ene VPA in sufficiently large quantities to carry out disposition studies.
2. To develop capillary GCMS assay methods for the estimation of (E)-2-ene VPA and 4-ene VPA in the biological fluids of the rat.
3. To determine the effect of dose, at two dose levels, on the pharmacokinetics of (E)-2-ene VPA and 4-ene VPA in the rat.
4. To study the biliary elimination of (E)-2-ene VPA and 4-ene VPA in bile-exteriorized rats.
5. To develop a pharmacokinetic model that may be applied to drugs showing enterohepatic circulation.

B. EXPERIMENTAL

B.1. MATERIALS

B.1.1. Chemicals

Chemicals and solvents were reagent grade, and were obtained from the following sources.

a. Aldrich Chemical Company, Inc. (Milwaukee, WIS)

Triethylamine, potassium hydride (35% oil dispersion), diisopropylamine, n-butyllithium (1.6 M in hexane), hexanoic acid, MTBSTFA reagent.

b. BDH Chemicals (Toronto, Canada)

Bromine, sodium hydroxide pellets, concentrated sulfuric acid, anhydrous sodium sulfate (granular), hydrochloric acid.

c. Eastman Organic Chemicals (Rochester, N.Y.)

Propionaldehyde, methansulfonyl chloride.

d. Fisher Scientific Company (Fair Lawn, N.J.)

Quinoline, pyridine.

e. Mallinkrodt Chemicals (St. Louis, MI)

Para-toluenesulfonyl chloride.

f. Matheson Coleman and Bell Company, (Norwood, OH)

Phosphorous tribromide, 1-bromopropane.

g. Sigma Chemical Company, (St. Louis MO)

Valeric acid.

h. Caledon Laboratories (Georgetown, Canada)

Ethyl acetate (distilled-in-glass grade).

B.1.2. Instrumentation

Nuclear Magnetic Resonance Spectrometry

Proton NMR spectra were recorded on a Bruker WP-80 spectrometer at the Department of Chemistry, UBC, using deuterated chloroform as solvent and tetramethylsilane as the internal standard.

Gas Chromatography Mass Spectrometry

A Hewlett Packard 5987A GCMS (5880A GC) equipped with a 2623A HP terminal and a 59824A scanning interface was used for GCMS analysis. A fused silica capillary column (25 metre x 0.32 mm I.D.) coated with a bonded phase (0.25 μ film of OV-1701) was obtained from Quadrex Corporation, New Haven, Connecticut.

For packed column chromatography, a Hewlett Packard 5700 GC interfaced to a Varian Mat 111 mass spectrometer with an on-line Varian 620L data analysis computer system was used. A glass column (2 metre x 2 mm I.D.) packed with 3% Desxil 300 (carborane/silicone) on 100/120 Supelcoport (Supelco Inc., Bellefonte, Pennsylvania) was used.

B.2. METHODS

B.2.1. Synthesis

B.2.1.1. (E)-2-ene VPA

I. Valeric acid (1 mole), ethanol (3 moles), 1 mL conc. sulfuric acid and 400 mL benzene were refluxed overnight in a 1-litre round bottom flask fitted with a Dean-Stark apparatus. The contents were washed thoroughly with water, dried over anhydrous Na_2SO_4, and distilled to remove benzene at 66^oC. The residue was distilled to collect ethyl valerate (0.78 moles) at 140^o-143^oC (Lit. 145^o-146^oC, The Merck Index, 1976a).

A flame-dried 1-litre three-necked flask containing a magnetic stirring bar, and equipped with a dropping funnel with a septum inlet, a N_2 inlet, and an air condenser attached to a mercury bubbler was cooled in an ice-salt mixture. Diisopropylamine (0.25 moles, dried over calcium hydride, and distilled) and 200 mL tetrahydrofuran (THF) were placed in the flask under N_2 atmosphere. n-Butyllithium (0.25 moles) was added dropwise to the stirring mixture, followed by dropwise addition of ethyl valerate (0.25 moles) dissolved in 50 mL THF. The ice-salt mixture was replaced with a dry ice-acetone mixture. Propionaldehyde (0.25 moles) dissolved in 25 mL THF was added dropwise. The contents were stirred for 30 min, and the reaction quenched with 6N HCl (0.9 moles). The mixture was extracted with ether twice, the combined ether extracts washed with a weak solution of $NaHCO_3$ (5%) followed by three washings with water. The ethereal layer was dried over anhydrous Na_2SO_4, and the solvent removed using a flash-film evaporator. Distillation of the residue gave 3-hydroxy VPA ethyl ester, at 92^o-95^oC at 4.5 mm (Lit. 105^oC at 8 mm, Blaise and Bagard 1907)

3-hydroxy VPA ethyl ester (0.05 moles) dissolved in 40 mL methylene chloride (distilled in glass) was placed in a three-necked flask assembly

as described above, except that a drying tube was used in place of the mercury bubbler. A cold solution of triethylamine (0.075 moles) in 10 mL of methylene chloride was added gradually, followed by dropwise addition of methanesulfonyl chloride (0.052 moles) in 10 mL methylene chloride. The contents were stirred for 30 min, filtered and the precipitates were washed with ether. The washings were combined with the filtrate, and the solvent removed using a flash film evaporator. The residue was dissolved in 50 mL THF, and cooled to $0^{o}C$ in an ice bath. Clean potassium hydride (KH, 0.1 mole), carefully cleaned with petroleum ether to remove mineral oil, was added with the aid of THF. The flask was protected with a drying tube, and kept in ice for 2 h. The contents were stirred for 12 h at room temperature. Excess KH was decomposed by dropwise addition of cold glacial acetic acid (3 mL) in 5 mL THF, followed by gradual addition of water. The contents were extracted with ether, and analyzed on GCMS. Several peaks were obtained on the chromatogram indicating that the reaction did not proceed as expected.

II. To a 250 mL 3-necked flask equipped with a drying tube, a dropping funnel and a magnetic stirring bar was added 3-hydroxy VPA ethyl ester (0.1 mole) in 20 mL dry pyridine. The flask was cooled in an ice-salt mixture. Toluenesulfonyl chloride (0.15 mole) in 40 mL pyridine was added dropwise, and the contents stirred for 2 days at room temperature. After adding ice-water, the mixture was extracted with $CHCl_3$. The organic layer was washed first with dilute H_2SO_4, then with $NaHCO_3$ (5%) and finally with water. The extract was dried with anhydrous Na_2SO_4, the chloroform removed and the residue refluxed with 1N NaOH for 4 h. After stirring the mixture for 2 days at room temperature, it was neutralized with 2N HCl, extracted with ether and worked up as described in I. Distillation of the residue gave a

fraction (F1) at 110°-117°C/30 mm. This fraction was stirred overnight with 50 mL 4N NaOH, refluxed for 4 h, washed with ether, and acidified with 50% HCl. The acidified extract was re-extracted with ether and worked up as described in I. Distillation of the residue gave fraction (F2) at 125°C/4 mm. The product was identified to be 3-ene VPA (Lit. boiling point 116°C at 8 mm, Blaise and Bagard 1907).

III. Diisopropylamine (0.8 mole, dried and distilled) in 750 mL of dry THF was placed in a 1-litre three-necked flask as described above in I. n-Butyllithium (0.8 mole) was added dropwise, followed by dropwise addition of valeric acid (0.4 mole) in 50 mL THF. The mixture was stirred for 20 min, and the ice bath was removed. Propyl bromide (0.44 moles, dried and distilled) in 40 mL THF was added over a period of 5 min, and contents stirred for 3 h at room temperature. The reaction was quenched with 400 mL 6N HCl, extracted with ether twice and worked up as before. The residue was distilled at 123°-125°C at 14 mm (Lit 120°-121°C/14 mm, The Merck Index, 1976b) to obtain Valproic acid (0.22 mole).

Valproic acid (0.14 mole), bromine (0.15 mole) and PBr_3 (0.5 mole) were placed in a 250 mL flask equipped with a water reflux condenser attached to an all-glass gas absorption device, consisting of an inverted funnel dipped in water. The flask was heated in an oil bath at 70°C for 1 h, and then at 100°C for 3 h. Excess Br_2 and the reaction product HBr were completely removed by distillation under reduced pressure of a water pump. The residue was distilled under high vacuum at 73°C at 0.01 mm to obtain 2-bromo VPA (0.09 mole).

2-Bromo VPA (0.09 mole) and 100% ethanol (0.26 mole) were refluxed for 48 h with 20 mL benzene and 1 mL conc. H_2SO_4 in a round bottom flask fitted with a Dean-Stark apparatus. The contents were washed with $NaHCO_3$

(5%), water, and dried over anhydrous Na_2SO_4. Benzene and unreacted ethanol were removed by distillation, and 2-bromo VPA ethyl was collected by distillation under vacuum at 106^{o}-115^{o}C/11 mm.

2-Bromo VPA ethyl ester (0.06 mole) and quinoline (0.18 mole, dried, and distilled) were stirred rapidly in a 100 mL round bottom flask equipped with a Claissen still head fitted with an air condenser protected by a $CaCl_2$ guard tube. The flask was rapidly heated on a heating mantle to obtain fractions F1 at 183^{o}-186^{o}C and F2 at 192^{o}C at atmospheric pressure. F1 and F2 contained different proportions of cis and trans isomers of 2-ene VPA ethyl ester along with quinoline, a reactant. The fractions F1 and F2 were combined and the mixture was dissolved in ether. The organic layer was washed with 50 mL 1N HCl and then with water. The ethereal layer was dried over anhydrous Na_2SO_4, and ether removed using a flash film evaporator to obtain 2-ene VPA ethyl ester, as verified by GCMS.

The 2-ene VPA ethyl ester (0.045 mole) was refluxed for 6 h with 25 mL of 4N NaOH and 1 mL EtOH in a 100 mL flask. The contents were stirred overnight at room temperature, washed with ether twice, and acidified with 4N HCl. The mixture was extracted with ether and worked up as described earlier. Distillation of the residue at 112^{o}-114^{o}C at 2.4 mm (Lit 103^{o}C/1 mm for (E)-2-ene VPA, Neuman and Holmes 1971) gave 2-ene VPA (0.029 mole). GCMS analysis showed that it was a 3:1 mixture of trans:cis isomers of 2-ene VPA. The product was dissolved in a small volume of chloroform, and stored at -20^{o}C for several days to harvest crystals of (E)-2-ene VPA.

B.2.1.2. 4-Ene VPA

Diisopropylamine (0.4 mole) and 300 mL dry THF were stirred under a N_2 atmosphere in a 1-litre 3-necked flask equipped as described above in B.2.1.1.I. n-Butyllithium (0.4 mole) was added dropwise, followed by

dropwise addition of valeric acid (0.2 mole) in 75 mL of THF. The ice-bath was removed, and allyl iodide (0.2 mole) in 25 mL THF was added gradually over a period of 10 min. The reaction was allowed to proceed for 3 h at room temperature, and quenched with 200 mL of ice-cold 20% HCl. The mixture was extracted with ether twice, and the organic layer worked up as before. Distillation of the residue gave 4-ene VPA (0.12 mole) at 103^{o}-105^{o}C at 6.5 mm (Lit 95^{o}-100^{o}C at 5 mm, Campos and Amaral 1965).

B.2.2. Capillary GCMS Assay

B.2.2.1. Standards: Plasma, urine or bile standards for (E)-2-ene VPA and 4-ene VPA were separately prepared by adding 50 μL of appropriate stock solution in methanol to blank rat plasma, urine or bile, and the volume made up to 2 mL in volumetric tubes. Plasma, urine and bile standards of (E)-2-ene VPA were prepared in concentration ranges of 0.4-35, 2-200 and 1-150 μg/mL, respectively. The plasma standards for 4-ene VPA were prepared in the concentration range of 0.5-45 μg/mL, and urine and bile standards of 2-80 μg/mL. For the analysis of total (conjugated and unconjugated) (E)-2-ene VPA or 4-ene VPA in urine and bile, the same standards were used as described above.

The internal standard solutions for (E)-2-ene VPA samples in plasma, urine and bile were prepared by dissolving both DNBA and HA in 0.1N NaOH to give final concentrations of 20, 80 and 80 μg/mL of each, respectively. The internal standard solutions for 4-ene VPA in plasma, urine and bile contained 20, 40, and 40 μg/mL of DNBA and HA each in 0.1N NaOH, respectively. For the estimation of total (conjugates and unconjugates) (E)-2-ene VPA or 4-ene VPA in urine and bile, the internal standards were prepared by dissolving DNBA in 3N NaOH.

B.2.2.2. Extraction and Derivatization: To an 80 μL aliquot of the plasma, urine or bile was added 80 μL of the internal standard solution, 50 μL of 2N HCl and 200 μL of ethyl acetate in a 1 mL conical reaction vial with a PTFE-lined cap. The contents were mixed on a Fisher tumbler for 15 min, and centrifuged at 1000 g for 20 min. The top organic layer was dried over anhydrous Na_2SO_4, and 60 μL of dried organic extract was derivatized by heating at 60°C for 1 h with 20 μL of MTBSTFA reagent. A 1 μL sample was injected into the GCMS.

To determine total (E)-2-ene VPA or 4-ene VPA in urine and bile, an 80 μL sample was added to an 80 μL aliquot of internal standard solution in 3N NaOH, heated at 60°C for 1 h, acidified with 4N HCl and extracted with 200 μL ethyl acetate as detailed above.

B.2.2.3. Chromatography: A Hewlett-Packard GCMS, model 5987A, equipped with a fused silica capillary column, 25 m long and of 0.32 mm I.D., coated with a bonded stationary phase OV-1701 of 0.25 μ thickness (Quadrex Corporation, New Haven, Connecticut) was used. Other operating conditions were as follows: injection port temperature, 240°C; interface, 260°C; and ion source, 260°C. The injection mode was splitless. Helium was used as the carrier gas at a flow rate of 1 mL/min. GC oven temperature was programmed as follows: 50°-100°C at 30°C/min; 100°-160°C at 8°C/min, hold for 2 min; post run to 250°C at 30°C/min, and hold for 2 min. The mass spectrometer was operated in a positive-ion selected-ion-monitoring (SIM) mode and a source pressure of $3X10^{-6}$ Torr. Electron impact was the mode of ionization with an energy of 70 eV and emission current of 300 μA. The intense mass ions, at $(M-57)^+$, of tert-butyldimethylsilyl derivatives (t-BDMS) of (E)-2-ene VPA or 4-ene VPA, and the internal standard DNBA at 199 and 229, respectively, were monitored.

B.2.2.4. Optimum Derivatization Conditions: The effect of heating time on the derivatization of (E)-2-ene VPA or 4-ene VPA and the internal standards with MTBSTFA was studied as follows: Plasma aliquots of 80 μL, containing 200 μg/mL of either (E)-2-ene VPA or 4-ene VPA, were extracted as described above. To 60 μL of the dried organic extract was added 15 μL of MTBSTFA, and heated at 60°C for 0, 15, 30, 60 and 120 min. A 1 μL aliquot was injected into GCMS.

In another similar series of experiments, varying amounts of MTBSTFA, ranging from 5, 10, 15 to 20 μL, were added to 60 μL of the organic extract, and heated at 60°C for 1 h. A 1 μL aliquot was used for GCMS analysis.

B.2.2.5. Hydrolysis of Conjugates : Urine samples from a rat, following 4-ene VPA admininstration, were heated at 60° for 0.5, 1, 1.5 and 2 h with internal standard prepared in 3N NaOH. The samples were extracted, derivatized and analyzed as described before.

B.2.2.6. Calibration Curves: Peak area ratios of (E)-2-ene VPA or 4-ene VPA and the internal standard DNBA were plotted against concentration of (E)-2-ene VPA or 4-ene VPA in the standard sample. A linear least-squares regression analysis was performed to obtain a calibration curve. The concentration of the unknown sample was calculated from its peak area ratio and the regression equation of the calibration curve prepared on the same day.

B.2.2.7. Precision: Within-day precision of the assay method was estimated by the analysis of six individually prepared samples, at the same standard concentration, on the same day.

B.2.2.8. Extraction Efficiency: Plasma samples (80 μL) of different concentrations of either (E)-2-ene VPA or 4-ene VPA were acidified with HCl, and extracted with 200 μL ethyl acetate. An 80 μL aliquot of the top organic layer was mixed with an equal volume of internal standard solution of DNBA prepared in ethyl acetate. The mixture was dried over anhydrous Na_2SO_4, an aliquot derivatized and analyzed. The peak area ratio of analyte/internal standard in these extracted samples was corrected for by multiplying by a factor of 2.5. This factor compensates for the 2.5 fold difference (200/80 μL) in the volumes of ethyl acetate used for the preparation of extracted samples compared to the unextracted samples.

For the preparation of unextracted samples, solutions of (E)-2-ene VPA or 4-ene VPA were prepared in ethyl acetate at the same concentrations as the plasma samples. Aliquots (80 μL) of these solutions were mixed with an equal volume of the internal standard in ethyl acetate and analyzed as above. The corrected peak area ratios of analyte/internal standard in extracted samples were compared to those in unextracted samples to obtain extraction efficiency of ethyl acetate.

B.2.3. Rat Experiments

B.2.3.1. Animal Handling: Male Sprague-Dawley rats weighing 250-350 g were obtained from the UBC Animal Care Centre. The animals were allowed to acclimatize to the surroundings for 2-3 days. They were kept in a 12 h day-and-night cycle (6 a.m. to 6 p.m.) at 21.5^{o}C room temperature, and fed standard Purina rat chow and tap water *ad libitum*.

B.2.3.3. Jugular Vein Cannulation: The rat was anesthetized with ether, and the hair removed from about 3-cm square of the skin on the ventral side of the neck. The animal was placed on its dorsal side, and its

limbs were taped to a surgical board with adhesive tape. The animal was kept anesthetized with a nose cone containing an ether-soaked swab. A longitudinal incision, 2-3 cm long, was made above the mid-point of the right collar bone. About 1 cm of the external jugular vein was exposed. A glass rod or a flat smooth piece of metal, 3 cm x 3 mm x 0.5 mm, was inserted under the vein. The vein was ligated anteriorly with a 4-0 silk suture, and another suture was placed approximately 7 mm below the first. A small incision was made, 3 mm below the first suture, in the wall of the vein by piercing it with a 23 gauge needle. A 25 cm long PE-50 tubing, with a smooth, rounded beveled edge, was inserted and pushed gently with a rotating action about 2.5 cm towards the heart. The second suture was tied around the vein and the cannula inside. The knot was made tight enough to secure the cannula properly but not too tight to collapse the tubing. The loose ends of the first suture were tied to another silk suture that was glued 2.7 cm above the beveled end of the cannula. The cannula was checked for its patency by aspiration, filled with heparin solution, and its dorsal free end was plugged with a pin. The free end of the cannula was exited dorsally between the scapulae. The ventral incision was closed with 3-0 catgut and 4-0 silk sutures. A wider-bore (1 cm) hard plastic tubing, whose one end was tied with a silk suture to the back of the animal, was used as a protective covering for the emerging cannula. The animals were kept individually in steel metabolic cages.

The rats were allowed to recover for 1-2 days before dosing or bile-exteriorization. The cannula was kept patent by flushing and refilling it daily with heparin solution (20 units/mL).

B.2.3.3. Bile Duct Cannulation: The rat was anesthetized as described above, and shaved ventrally on the upper abdomen. The animal was

placed on its back and its limbs were taped on the surgical board. A midline abdominal incision, 3-cm long, was made posterior to the xiphoid cartilage. The duodenum and anterior segments of the small intestine were carefully pulled out to the left of the animal and placed on a pad, moistened with physiological saline, on the abdomen. The major lobes of the liver were pushed back towards the diaphragm or gently pulled out and placed on the chest wall and wrapped in a moist gauze. The bile duct above the pancreatic ducts was cautiously freed of the connective tissue with forceps. A 4-0 silk suture was tied tightly around the bile duct proximal to the pancreas. A second suture was kept loose at 4-5 mm in front of the first suture, towards the liver. A narrow glass rod was pushed under the bile duct between the two sutures. The free ends of the first suture were gently pulled towards the tail, and a 26 gauge needle was used to pierce into the bile duct, 2 mm in front of the suture knot. A 30 cm long PE-10 tubing with a beveled edge was pushed ~7 mm into the duct towards the liver. The second ligature was tightened around the duct and the cannula inside. The bile flow was checked for even and continuous flow through the catheter. The free ends of the first silk suture were tied to a second 4-0 silk suture glued to the cannula. The viscera were then gently returned to the abdominal cavity in their respective positions. The cannula was slightly bent to make a wide loop inside the abdominal cavity. A large-bore needle, from which the hub had been removed, was passed through the peritoneum on the dorsal side of the abdominal cavity and pushed subcutaneously to exit at the tip only, between the scapulae on the back of the rat. The free end of the cannula was passed through the needle to bring it out at the back of the neck. The needle was then removed. The bile flow was checked before the abdominal incision was closed with 3-0 catgut and 3-0 silk sutures. A hard plastic tubing was used to protect the external

portion of the cannula as described above.

The rats were kept in individual cages and allowed to recover for at least 2 h before dosing.

B.2.3.4. Pharmacokinetic Studies: Two separate solutions of 20 and 100 mg/mL of each metabolite, (E)-2-ene VPA or 4-ene VPA, were prepared in water as sodium salts and the pH adjusted to 7.4 with HCl. The concentration of each solution refers to the free acid. A single IV bolus dose of 20 or 100 mg/kg was administered *via* the jugular vein cannula. The cannula was then flushed with 250 μL of saline. Blood samples of about 0.2 mL were withdrawn typically at -10, 5, 15, 30, 60, 90, 120, 150, 180, 240, 300, 360, 420 and 480 min after the low dose; and at -10, 5, 15, 30, 60, 120, 180, 240, 300, 360, 435, 510, 585, 675 and 765 min following the high dose. In bile-exteriorized rats, blood samples were collected as described above for 6-8 h. After each blood sample, an equivalent volume of heparinized normal saline (20 units/mL) was administered into the cannula to prevent dehydration of the rat, and to prevent blood clotting in the cannula. The blood samples were immediately centrifuged in heparinized microhematocrit tubes (Caraway) and the plasma separated and stored at -20°C until analyzed.

Total urine output samples were collected at various time intervals for 24 h. The volumes of the urine samples were measured, and aliquots stored in a freezer at -20°C.

In bile-duct cannulated rats, 4 to 6 bile samples were collected usually at 0.5-1 h intervals during the first 6 h of the dose, and then at a longer interval of 6-24 h. The bile volume was measured, and samples stored as described for urine.

B.2.3.5. *In Vitro* Plasma Protein Binding: Aliquots of stock solutions of 4-ene VPA in methanol were diluted with pooled rat plasma to provide 4-ene VPA concentrations of 20, 50, 100, 150, 200, 250, 300 and 350 μg/mL. The final concentration of methanol in each spiked plasma sample was 2%. One mL of each plasma sample was added to the reservoir of an Amicon ultrafiltration unit equipped with a YMT (MPS-1) filter. The units were centrifuged in a fixed angle rotor at 1000 *g* for 10 min to yield approximately 140 μL of ultrafiltrate. The filtrate was stored at -20°C prior to analysis.

The concentration of bound 4-ene VPA was obtained as the difference between 4-ene VPA concentration in the plasma and 4-ene VPA concentration in the ultrafiltrate. Percent binding was calculated as the ratio of the bound over total 4-ene VPA concentration in the plasma multiplied by 100.

B.2.3.6. Metabolism of (E)-2-ene VPA: Urine and bile samples obtained from rats receiving 100 mg/kg of (E)-2-ene VPA were pooled separately. The samples (500 μL) were extracted without hydrolysis, and in another series after hydrolysis with 3N NaOH, as described above. The extracts were derivatized with MTBSTFA reagent. A 1 μL aliquot was injected into the GCMS, and the mass ions at m/z 98, 112, 114, 197, 199, 195, 173, 201, 213, 215, 217, 329, 331 and 343 corresponding to 4-hydroxy ene VPA lactone, Δ^6-3-heptanone, 3-heptanone, diene VPA, monounsaturated VPA, triene VPA, hexanoic acid, VPA, 3'-oxo-4-ene VPA, 3-keto VPA or 3-hydroxy ene VPA (mono derivative), 3-hydroxy VPA, 3-keto VPA (di derivative), 3-hydroxy VPA (di derivative) and allylglutaric acid (AGA), respectively, were monitored.

B.2.4. Pharmacokinetic Analysis: To describe the plasma profile of (E)-2-ene VPA or 4-ene VPA after the low dose administration to the rat, a time-lag pharmacokinetic model (Veng Pedersen and Miller 1980) was employed as shown in Fig. 4.

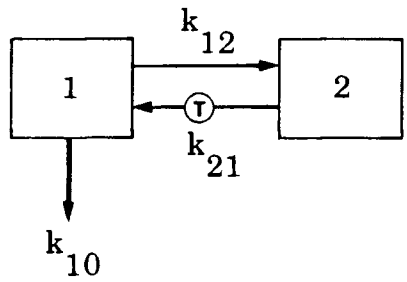


Fig. 4 Time-Lag Pharmacokinetic Model

The differential equations for this model are:

$$dC_1/dt = -(k_{10} + k_{12})\, C_1 + k_{21}C_2^*$$

$$dC_2/dt = k_{12}C_1 - k_{21}C_2$$

where: C_1 = concentration in compartment 1 at time t

C_2 = concentration in compartment 2 at time t

C_2^* = concentration in compartment 2 at time t - T

t = time after dosing

T (tau) = time-lag.

The equations were solved numerically by MULTI(RUNGE), a non-linear least squares regression program (Yamaoka and Nakagawa 1983). The overall elimination rate constant, K, was calculated from the slope of the log-linear regression line of the plasma concentration-time curve. The apparent half-life ($t_{1/2}$) was calculated as 0.693/K. The volume of the central compartment (V_1) was determined from dose (D) divided by the plasma

concentration at time zero (C_0), which was obtained by extrapolation of the log-linear regression line to zero time. The total body clearance Cl_T was calculated as dose divided by total area under the plasma concentration-time curve (AUC). Renal and biliary clearances, Cl_R and Cl_B, were obtained by multiplying the fractions of dose excreted unchanged (unconjugated) in the urine and bile, respectively, by Cl_T. The clearance due to the formation of conjugates Cl_{conj} was determined as the sum of the fractions of dose recovered as conjugates in urine and bile collectively, multiplied by Cl_T. The metabolic clearance Cl_{met} was obtained as the difference of Cl_T and Cl_R in normal rats.

Various pharmacokinetic parameters obtained after the low dose were compared to those after the high dose, in normal or bile-exteriorized rats, using unpaired two-tailed Students' t-test.

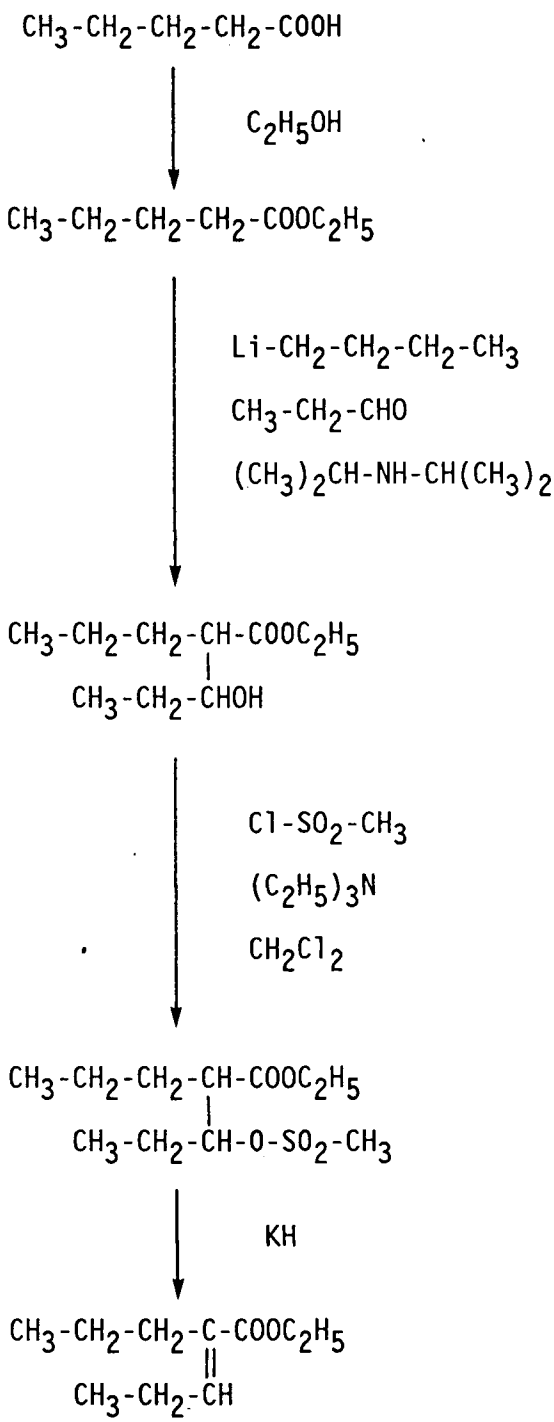
C. RESULTS

C.1. SYNTHESIS

C.1.1. (E)-2-ene VPA: The synthesis of (E)-2-ene VPA was attempted by dehydration of the 3-hydroxy VPA ethyl ester with methanesulfonyl chloride (Scheme 1) and with toluenesulfonyl chloride (Scheme 2). GCMS analysis of the reaction mixture, from the methanesulfonyl chloride reaction, showed at least 12 peaks (Appendix 1) with large amounts of 3-hydroxy VPA ethyl ester (peak 9), 3 condensation products (peaks 10, 11 and 12), several unidentified peaks, and extremely small quantities of 2-ene VPA ethyl ester (peak 3). When the same reaction was carried out in the presence of toluenesulfonyl chloride, a larger proportion of 3-ene VPA than 2-ene VPA along with 4-hydroxy VPA and other products was obtained. Washing the ethereal layer with alkaline solution and distillation under vacuum gave 3-ene VPA. NMR (Appendix 2) of the product showed: δ 0.8-1.1, triplet (3H, $C\underline{H}_3$-CH_2); 1.1-1.6, complex m (4H, -CH_2-CH_2-); 1.6-2.0, d (3H, CH_3-CH=); 2.7-3.3, m (1H, CH-C=O), 5.2-5.9, complex m (2H, CH=CH). A small amount of 2-ene VPA was present as indicated by 2.1-2.5, m (CH_2-C= from 2-ene VPA); 6.8-7.1, t (trans CH=C from 2-ene VPA).

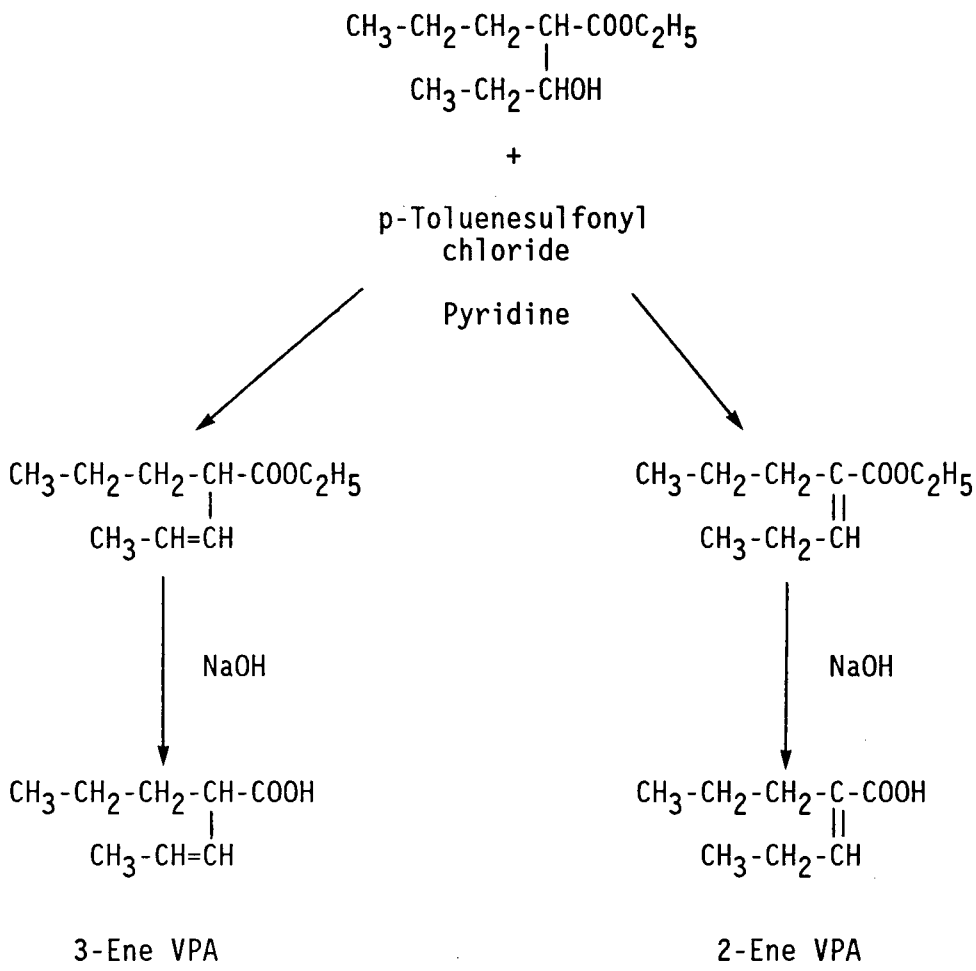
The desired product (E)-2-ene VPA was successfully synthesized by dehydrobromination, and then hydrolysis of 3-bromo VPA ethyl ester. After repeated fractional recrystallizations at -20^{o}C, the final product was found to be a mixture of 90% trans and 10% cis isomers of 2-ene VPA. Further attempts to increase the proportion of trans isomer of 2-ene VPA by recrystallization were unsuccessful. The purity of 2-ene VPA was confirmed by GCMS (Fig. 5). The smaller peak at 6.49 min was identified to be the cis isomer of 2-ene VPA and the larger peak at 7.11 min was the trans isomer of

Scheme 1

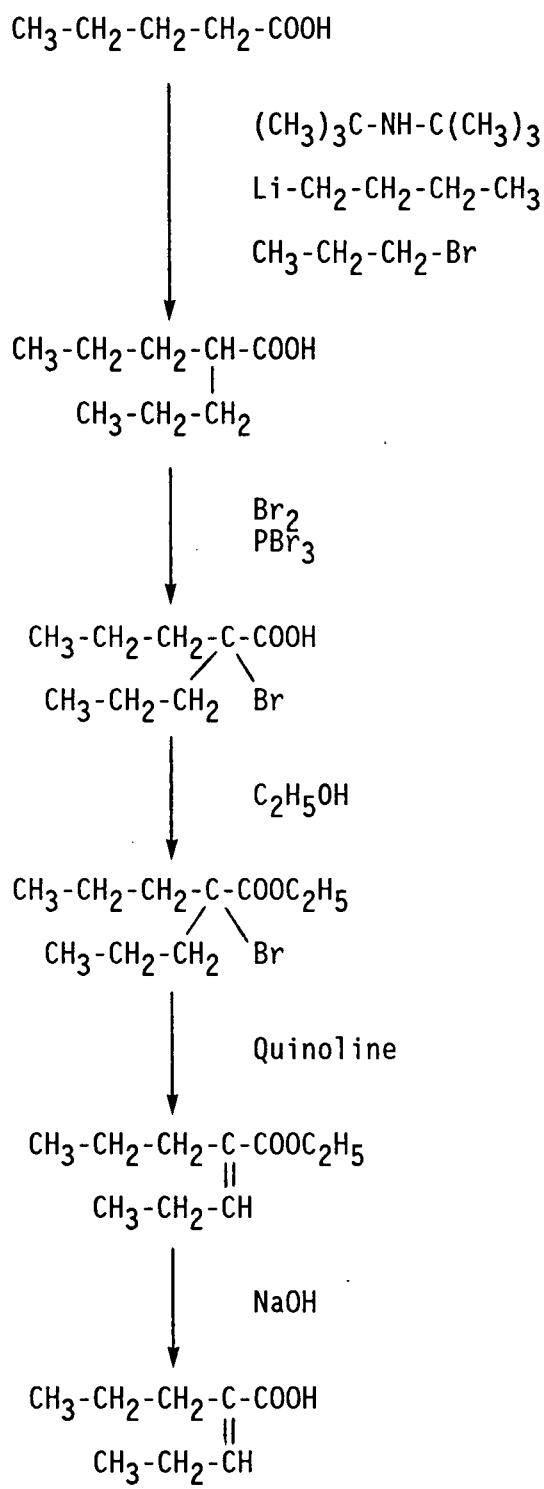


2-Ene VPA ethyl ester

Scheme 2

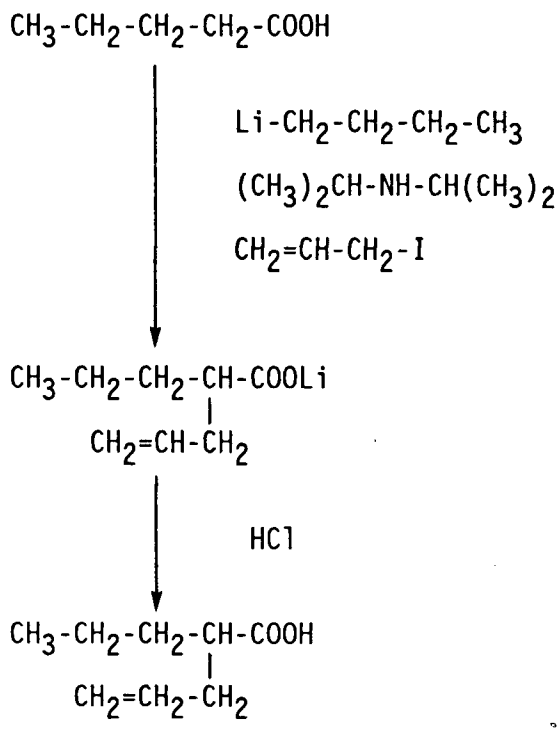

CH3-CH2-CH2-CH-COOC2H5
CH3-CH2-CHOH
+
p-Toluenesulfonyl chloride
Pyridine
CH3-CH2-CH2-CH-COOC2H5
CH3-CH=CH
NaOH
CH3-CH2-CH2-CH-COOH
CH3-CH=CH
3-Ene VPA
CH3-CH2-CH2-C-COOC2H5
CH3-CH2-CH
NaOH
CH3-CH2-CH2-C-COOH
CH3-CH2-CH
2-Ene VPA

Scheme 3



2-Ene VPA

Scheme 4



4-Ene VPA

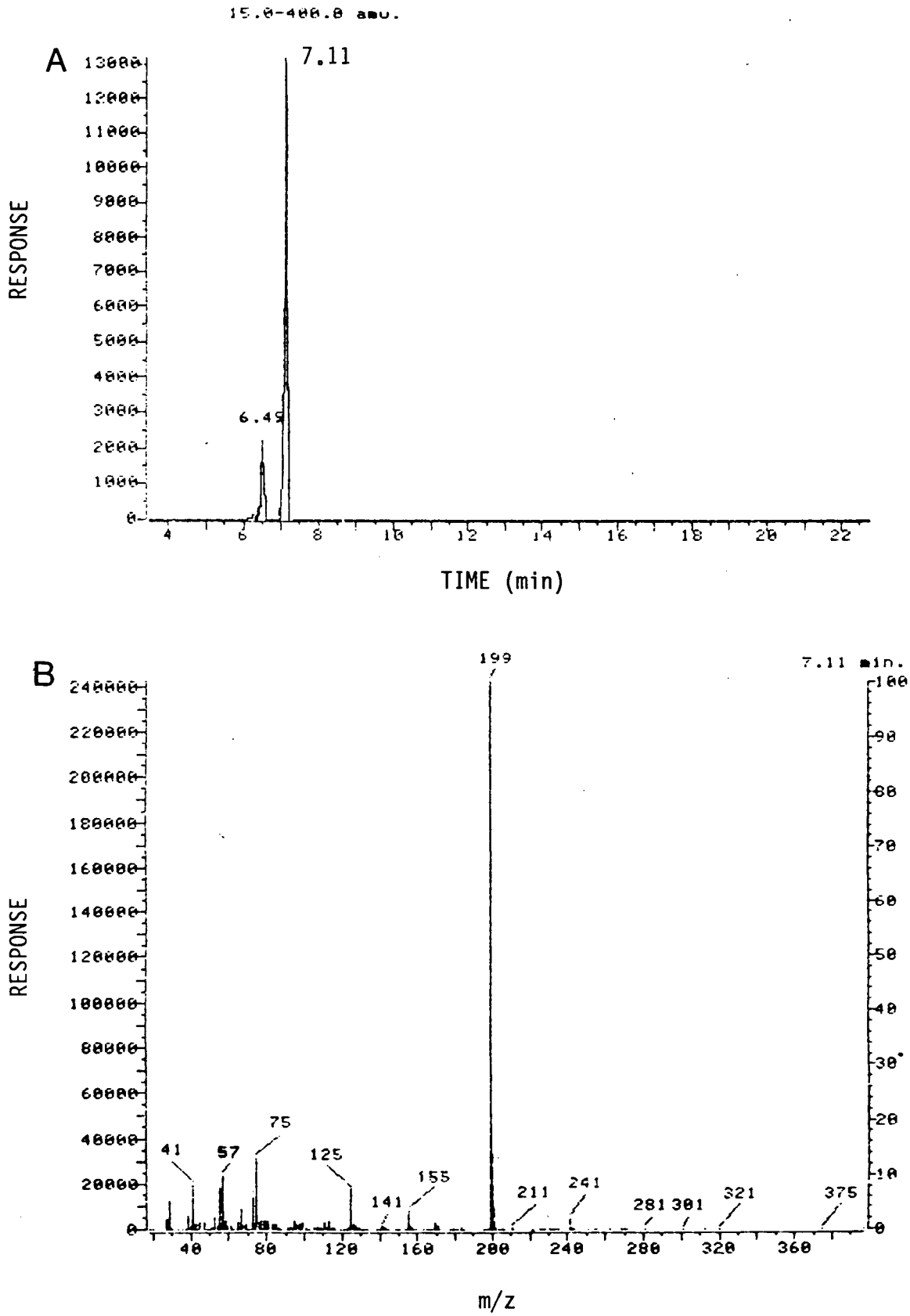


Fig. 5. Total ion chromatogram of t-BDMS derivative (A) of (Z)-2-ene VPA (6.49 min) and (E)-2-ene VPA (7.11 min), and EI-mass spectrum (B) of (E)-2-ene VPA.

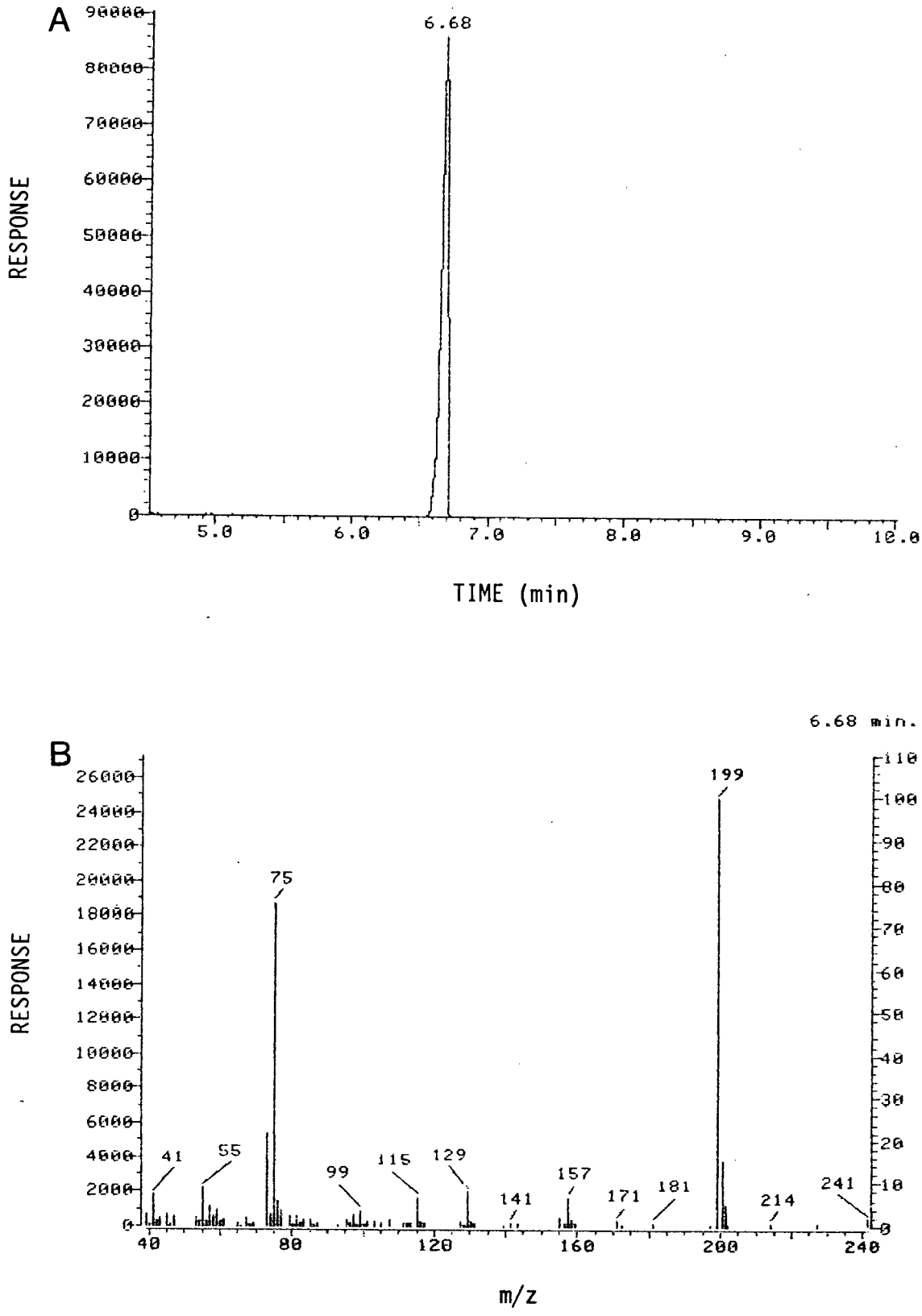


Fig. 6. Total ion chromatogram of t-BDMS derivative (A) and EI-mass spectrum (B) of 4-ene VPA.

2-ene VPA. From the relative sizes of these two peaks, the product was found to be 90% (E)-2-ene VPA. The mass spectrum of the tertiary butyldimethylsilyl derivative of (E)-2-ene VPA showed an intense and characteristic peak at $(M-57)^+$ mass ion (m/z) 199. NMR (Appendix 3) analysis showed: δ 0.8-1.1, complex m (6H, $2CH_3$), 1.2-1.7, m (2H, CH_3-$C\underline{H}_2$-CH_2-); 2.1-2.7, m (4H, $C\underline{H}_2$-CH= and -$C\underline{H}_2$-C=); 6.8-7.1, t (1H, -CH=C, trans, strong). Trace amounts of cis isomer were shown by 5.9-6.2, t (1H, -CH=C, cis, weak). In all the pharmacokinetic studies, the amounts refer to the trans isomer of free acid of 2-ene VPA.

C.1.2. 4-ene VPA: The synthesis of 4-ene VPA was carried out by the general procedure of Pfeffer *et al.* (1972). Valeric acid was treated with lithium diisopropylamide, and alkylated with allyl iodide to give 4-ene VPA. The purity of the compound was established by capillary GCMS analysis (Fig. 6A) that showed a single peak. The mass spectrum of the t-BDMS derivative of 4-ene VPA (Fig. 6B) showed an intense characteristic peak at mass ion (m/z) 199 obtained by the loss of 57 a.m.u. (atomic mass units) from the t-BDMS derivative. NMR (Appendix 4) analysis showed: sigma 0.8-1.1, t (3H, CH_3); 1.1-1.7, complex m, (4H, -CH_2-CH_2); 2.0-2.7, complex m (3H, -C$\underline{H}$-$C\underline{H}_2$-CH=); 4.9-5.3, m (2H, CH_2=), 5.5-6.1, complex m (1H, -CH=).

C.2. ASSAY

C.2.1. Chromatography:

A typical selected ion chromatogram obtained from an extract of spiked plasma sample is shown in Fig. 7. The retention times for (E)-2-ene VPA, 4-ene VPA, HA, DNBA, diene VPA I and diene VPA II were 7.50, 6.74, 5.72, 8.94, 7.90 and 8.44 min, respectively. The chromatographic peaks were sharp, symmetrical, well separated from each other and resolved at the base

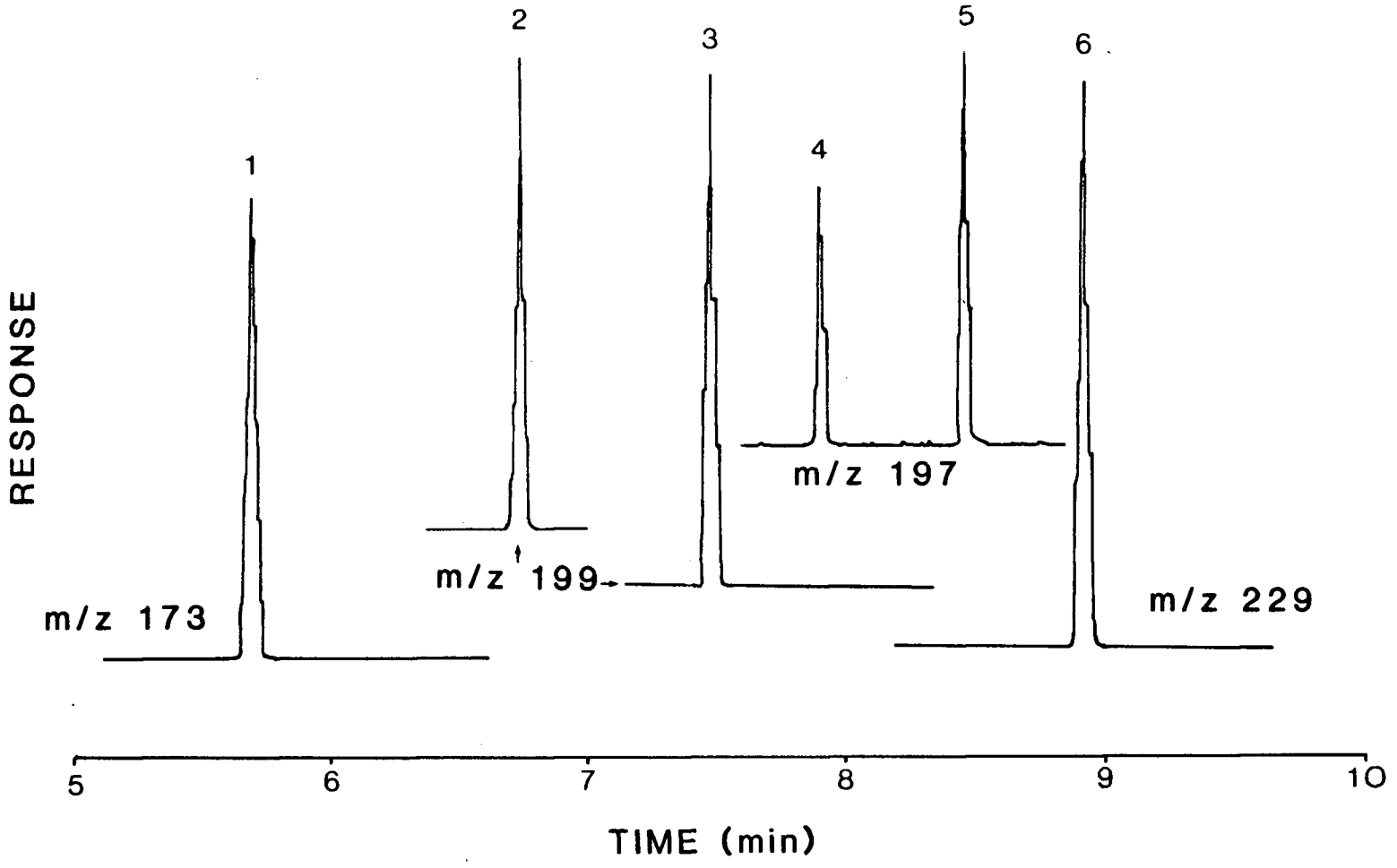


Fig. 7. Selected-ion-chromatogram of t-BDMS derivatives of an extract from a spiked plasma sample. Peaks 1, 2, 3, 4, 5, and 6 correspond to hexanoic acid, 4-ene VPA, (E)-2-ene VPA, (E)-2,4-diene VPA, (E)-2,3'-diene VPA and di-N-butylacetic acid, respectively.

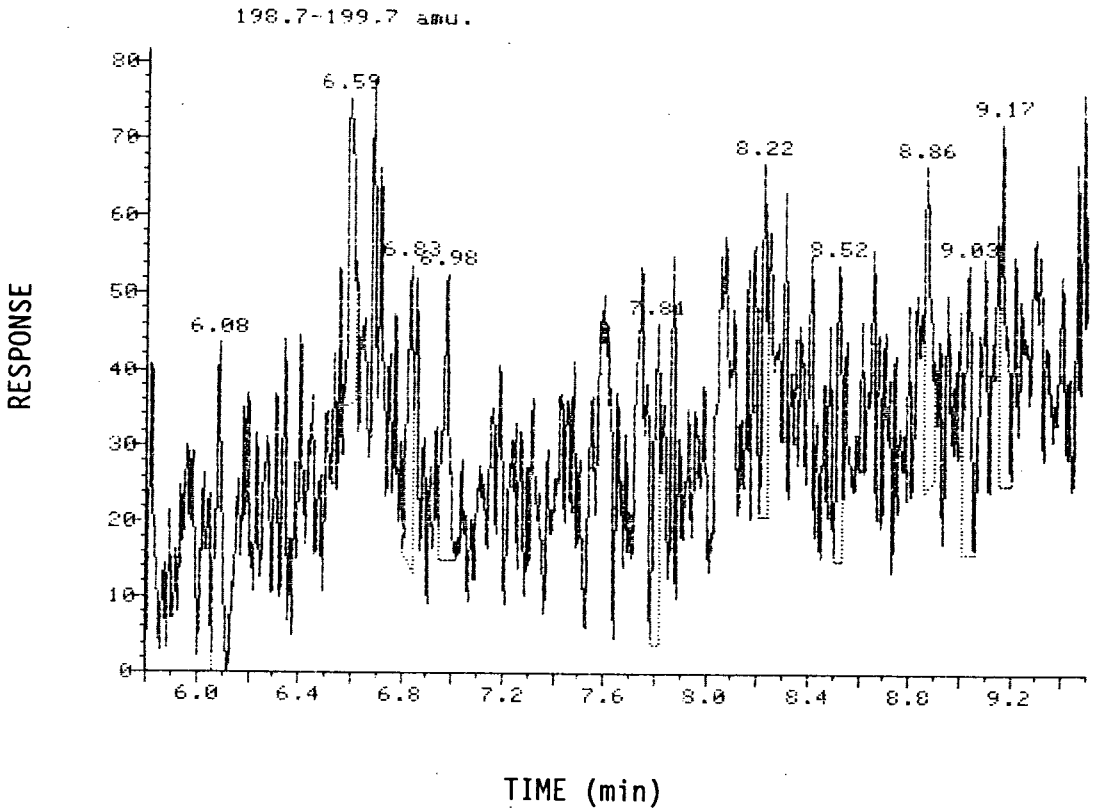


Fig. 8. Selected-ion-chromatogram of a blank plasma sample showing no interfering peaks at the lowest attenuation.

line. No interfering peaks were seen in the blank plasma (Fig. 8), urine and bile extracts, except for an extremely small endogenous component that contributed approximately 1% to the peak area of HA. The error in the estimation of peak area of HA was minimal. The chromatographic run was complete within 12 min.

Preliminary experiments with several other internal standards including 2-ethylhexanoic acid, heptanoic and 1-methyl-1-cyclohexanoic acids had shown an interference with cis-2-ene VPA, 4-ene VPA and trans-2-ene VPA, respectively. HA and DNBA were selected for further experiments.

C.2.2. Derivatization Kinetics

The effect of heating for 0, 15, 30, 60 and 120 min at 60°C showed that derivatization with MTBSTFA was extremely rapid. Even without heating, silylation was almost instantaneous (Table 2). To ensure complete derivatization, heating for 1 h was chosen for all subsequent studies.

The effect of varying amounts from 5, 10, 15 to 20 μL of MTBSTFA, added to 50 μL of ethyl acetate extract of plasma sample, showed no appreciable change in the peak areas of 4-ene VPA and the internal standards (Table 3). In the subsequent analysis of samples, 15 μL of MTBSTFA was added to the organic extracts.

C.2.3. Linearity and Reproducibility

Calibration curves of (E)-2-ene VPA were linear in the concentration range of 0.4-35 μg/mL in plasma, 2-200 μg/mL in urine and 1-150 μg/mL in bile (Tables 4-6). Similarly, the standard curves for 4-ene VPA were prepared at concentrations of 0.5-45 μg/mL in plasma, and 2-80 μg/mL in urine and bile (Tables 7-9). The correlation coefficient, r, was > 0.997 for all regression lines. Within-day coefficients of variation (CV) for

TABLE 2. EFFECT OF HEATING TIME ON DERIVATIZATION[a]

Time (min)	4-ene VPA	DNBA	HA	(E)-2-ene VPA	DNBA	HA
0	4770	3340	5650	7780	3440	5780
15	4760	3500	5540	8130	3610	5900
30	4200	2830	5070	7530	3320	5670
60	4710	3380	5540	9690	3770	6280
120	4880	3520	5790	9140	3480	5950

a, peak area x 10^3.
Each reading is a mean of 3 samples.

TABLE 3. EFFECT OF MTBSTFA ON THE PEAK AREAS[a]

MTBSTFA (μL)	4-ene VPA	DNBA	HA	(E)-2-ene VPA	DNBA	HA
5	4200	2810	4520	5790	2430	4820
10	4110	2780	5000	5610	2220	4660
15	4720	3550	5400	6340	2470	4670
20	3680	2460	4440	5490	2220	3880

a, peak area x 10^3

TABLE 4. CALIBRATION CURVE DATA FOR (E)-2-ENE VPA IN RAT PLASMA (N=5)

Added µg/mL	Found[1] µg/mL	CV[1] %	Found[2] µg/mL	CV[2] %
0.40	0.41±0.02	4.9	0.40±0.03	7.5
2.0	1.9±0.04	2.1	2.0±0.2	7.7
5.0	4.8±0.1	2.7	4.9±0.2	4.1
10	9.8±0.3	2.9	10±0.4	4.0
15	16±0.5	3.2	15±0.6	3.9
25	25±0.5	2.0	25±0.5	2.1
35	35±0.5	1.5	35±0.6	1.7

1=DNBA; 2=HA

TABLE 5. CALIBRATION CURVE DATA FOR (E)-2-ENE VPA IN RAT URINE (N=5)

Added µg/mL	Found[1] µg/mL	CV[1] %	Found[2] µg/mL	CV[2] %
2.0	2.0±0.1	5.0	2.1±0.2	7.8
5.0	5.2±0.1	1.9	5.0±0.4	7.3
30	29±0.7	2.3	31±1.2	4.0
60	59±1.8	3.1	61±1.9	3.1
100	97±6.0	6.1	102±4.3	4.2
150	154±3.4	2.2	150±2.5	1.7
200	199±2.9	1.4	199±3.9	2.0

1=DNBA; 2=HA

TABLE 6. CALIBRATION CURVE DATA FOR (E)-2-ENE VPA IN RAT BILE (N=3)

Added µg/mL	Found[1] µg/mL	CV[1] %	Found[2] µg/mL	CV[2] %
1.0	0.9±0.2	23	0.9±0.4	51
10	9.3±0.4	4	8.9±1.0	11
25	27±1.6	6	26±2.2	9
60	58±2.4	4	61±2.9	5
100	100±0.9	1	100±2.3	2
150	150±0.7	0.5	150±1.7	1

1=DNBA; 2=HA

TABLE 7. CALIBRATION CURVE DATA FOR 4-ENE VPA IN RAT PLASMA (N=6)

Added μg/mL	Found[1] μg/mL	CV[1] %	Found[2] μg/mL	CV[2] %
0.50	0.6±0.08	13	0.6±0.05	8.9
2.0	2.1±0.1	6.8	2.0±0.1	6.4
10	10±0.6	5.7	9.9±0.6	5.9
20	20±1.1	5.5	20±0.8	4.0
30	30±2.2	7.1	30±1.0	3.3
45	45±1.4	3.1	45±0.7	1.6

1=DNBA; 2=HA

TABLE 8. CALIBRATION CURVE DATA FOR 4-ENE VPA IN RAT URINE (N=6)

Added μg/mL	Found[1] μg/mL	CV[1] %	Found[2] μg/mL	CV[2] %
2.0	1.9±0.08	4.2	1.9±0.08	4.2
10	10±0.5	4.8	10±1.0	9.8
20	19±0.9	4.9	20±0.5	2.6
40	40±2.4	6.0	40±1.7	4.2
60	59±2.0	3.5	60±1.2	2.0
80	81±1.3	1.7	80±0.4	0.5

1=DNBA; 2=HA

TABLE 9. CALIBRATION CURVE DATA FOR 4-ENE VPA IN RAT BILE (N=3)

Added μg/mL	Found[1] μg/mL	CV[1] %	Found[2] μg/mL	CV[2] %
2.0	2.5±0.3	14	2.8±1.4	51
10	10±0.2	2	10±0.2	2
20	19±1.3	7	19±2.1	11
40	39±1.0	3	39±1.5	4
60	60±1.3	2	62±2.9	5
80	81±0.6	0.7	80±1.0	1

1=DNBA; 2=HA

TABLE 10. EFFECT OF HEATING TIME ON THE HYDROLYSIS OF CONJUGATES IN RAT URINE[a]

(Time, h)	0.5	1	1.5	2
4-ENE VPA	5880	6110	5920	6320
DNBA	2500	2390	2390	2690
Area Ratio	2.35	2.55	2.48	2.35

a, peak area x 100
Temperature = 60°C

(E)-2-ene VPA in plasma standards varied from 2-4% with DNBA as internal standard, and 2-6% when HA was used as internal standard. The corresponding values of within-day CV for 4-ene VPA plasma standards were 4-7% and 2-3%. The t-BDMS derivatives of the analyte and the internal standards showed no significant change in their peak area ratios when stored at -20°C for 2 weeks.

C.2.4. Hydrolysis of Conjugates

Table 10 shows that the maximum hydrolysis of the conjugates of 4-ene VPA, excreted in the urine of the rat, was achieved by heating with 3N NaOH at 60°C for 0.5 h. Further heating up to 2 h did not alter the peak area of 4-ene VPA or its peak area ratio with the internal standard.

C.2.5. Extraction Efficiency

Extraction efficiency studies were performed at three different plasma concentrations of 5, 20 and 40 μg/mL of 4-ene VPA. The average recoveries using ethyl acetate were 99, 102 and 102%, respectively. The recovery of (E)-2-ene VPA from plasma was also quantitative at concentrations of 5, 15 and 30 μg/mL and was 100, 99 and 100%, respectively.

C.3. PHARMACOKINETIC STUDIES

C.3.1. Pharmacokinetics in Normal Rats

Figures 9 and 10 represent the semilogarithmic plots of mean plasma concentrations of (E)-2-ene VPA and 4-ene VPA *versus* time following the low and high doses to normal rats. Following the low dose of 20 mg/kg, the plasma level of (E)-2-ene VPA and 4-ene VPA declined rapidly with a $t_{1/2}$

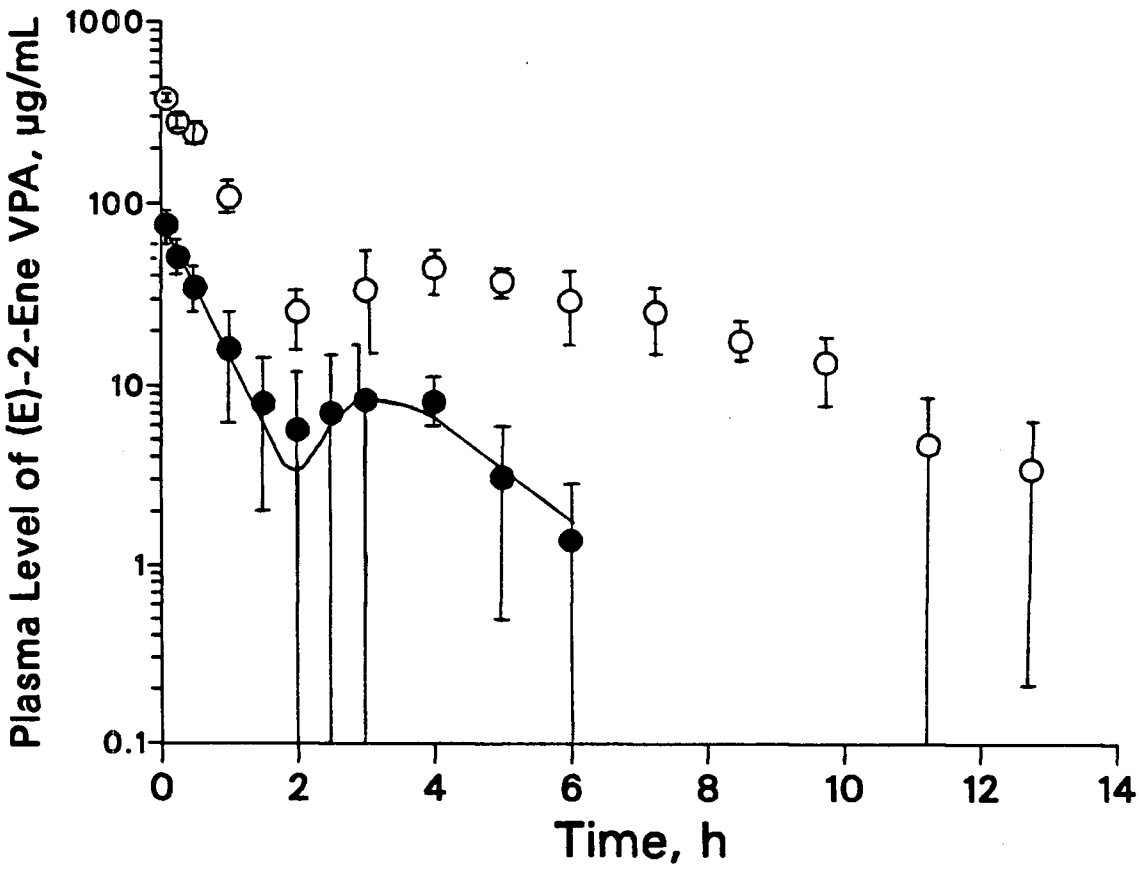


Fig. 9. Semilogarthimic plots of plasma concentrations of (E)-2-ene VPA *versus* time following IV dose of 20 (●) and 100 (○) mg/kg in normal rats. Each point represents mean ± 95% confidence limits (N=4). Solid line represents model-generated curve.

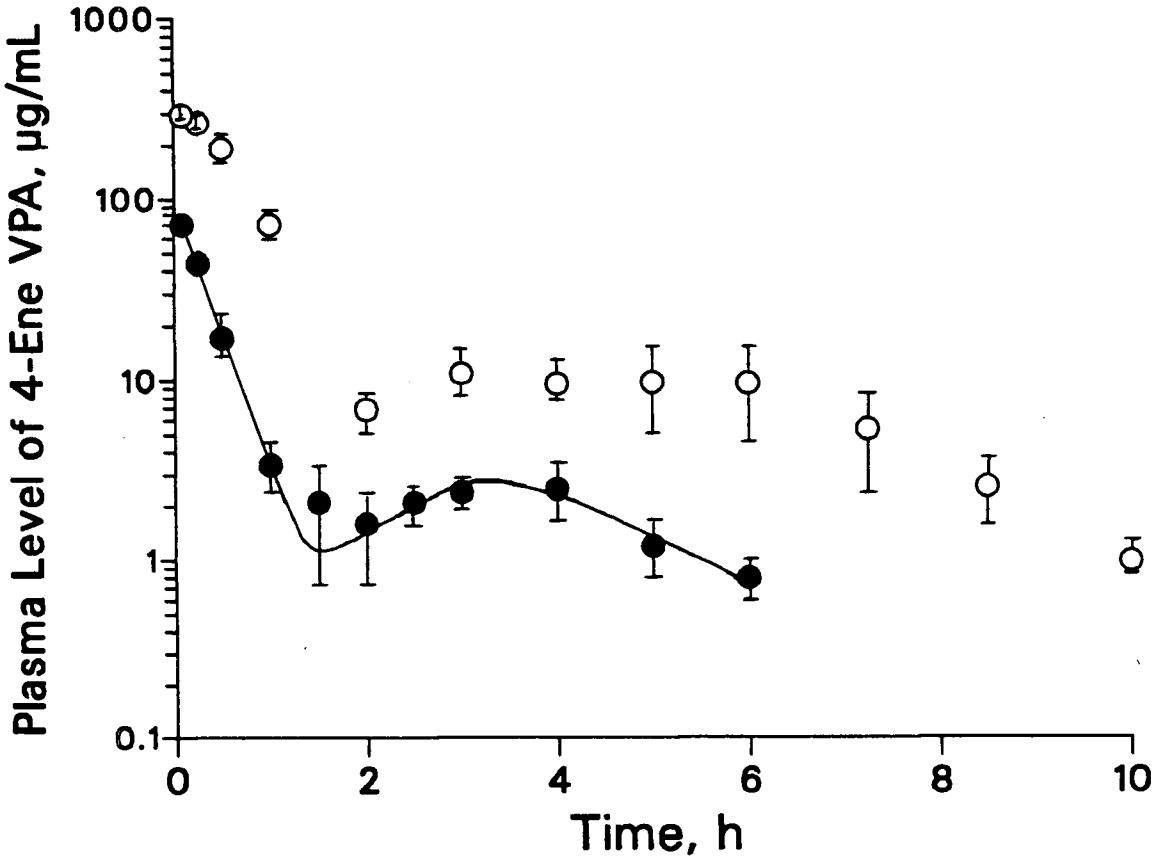


Fig. 10. Semilogarithmic plots of plasma concentrations of 4-ene VPA *versus* time following IV dose of 20 (●) and 100 (○) mg/kg in normal rats. Each point represents mean ± 95% confidence limits (N=6). Solid line represents model-generated curve.

of 23 ± 4 and 12 ± 2 min, respectively, during the first h. The plasma concentration of (E)-2-ene VPA reached trough levels of 5.7 ± 5.6 μg/mL and for 4-ene VPA, 1.6 ± 1.1 μg/mL, at approximately 2 h after the dose. Thereafter, the plasma levels of (E)-2-ene VPA and 4-ene VPA started to rise, showing a secondary peak at 4 h. This secondary peak was attributed to enterohepatic circulation of both the metabolites in the rat. After 4 h, (E)-2-ene VPA and 4-ene VPA were eliminated slowly from the plasma with apparent population $t_{1/2}$ of 55 and 73 min., respectively.

The plasma profile of (E)-2-ene VPA and 4-ene VPA, after the high dose of 100 mg/kg each, was similar to that of the low dose (Fig.9,10). There was, however, a short initial period of an apparently non-linear plasma decline of (E)-2-ene VPA and 4-ene VPA at concentrations above 200 μg/mL. In the log-linear phase, between 30 to 120 min, the plasma level of (E)-2-ene VPA and 4-ene VPA declined with apparent $t_{1/2}$ of 28 ± 6 and 18 ± 1.5 min to reach trough levels of 26 ± 11 and 6.9 ± 2.3 μg/mL, respectively. The plasma levels then started to rise indicating EHC of the administered metabolite. Following the secondary plasma peaks, (E)-2-ene VPA and 4-ene VPA were more slowly eliminated from plasma with apparent population $t_{1/2}$ of 99 and 73 min respectively. The plasma levels of (E)-2-ene VPA and 4-ene VPA in individual normal rats are tabulated in Appendices 5-8.

Tables 11 and 12 summarize the pharmacokinetic parameters calculated from individual animals describing the above data. For (E)-2-ene VPA, the apparent volume of the central compartment was unaltered at the two dose levels (Table 31). There was no significant change in the metabolic and total plasma clearances of (E)-2-ene VPA, and in the fraction of dose excreted as unconjugated, conjugated and total (E)-2-ene VPA in the urine of the rat when the dose was increased by 5 fold (Tables 31, 13, 14). There

TABLE 11. PHARMACOKINETIC PARAMETERS OF (E)-2-ENE VPA IN NORMAL RATS (DOSE=20 mg/kg)

Parameter	1a	2a	3a	4a	Mean
Weight[a]	270	285	290	300	286
Dose[b]	5.4	5.7	5.8	6.0	5.7
C_o[c]	90	84	98	66	85
k_1[d]	300	390	250	300	310
$t_{1/2}$[e]	23	18	28	23	23
V_1[f]	220	240	200	300	240
AUC[g]	4700	3300	7400	3100	4600
		% DOSE EXCRETED IN URINE			
Unconj	2.4	7.0	9.8	4.2	5.9
Conj	22	22	23	35	26
Total	25	29	33	39	32
		CLEARANCE (mL/min.kg)			
Cl_R	0.10	0.43	0.27	0.27	0.27
Cl_{met}	4.2	5.7	2.4	6.2	4.6
Cl_T	4.3	6.1	2.7	6.4	4.9

a, g; *b*, mg/kg; *c*, μg/mL; *d*, $x10^{-5}$ min^{-1}; *e*, min; *f*, mL/kg; *g*, μg.min/mL

TABLE 12. PHARMACOKINETIC PARAMETERS OF (E)-2-ENE VPA IN NORMAL RATS (DOSE=100 mg/kg)

Parameter	1b	2b	3b	4b	Mean
Weight[a]	260	260	310	320	288
Dose[b]	26	26	31	32	29
C_o[c]	410	460	480	390	430
k_1[d]	320	260	190	260	260
$t_{1/2}$[e]	22	27	36	27	28
V_1[f]	250	220	210	260	230
AUC[g]	29000	33000	35000	35000	33000
		% DOSE EXCRETED IN URINE			
Unconj	11	12	10	4.7	9.4
Conj	31	52	32	46	40
Total	42	64	42	51	50
		CLEARANCE (mL/min.kg)			
Cl_R	0.38	0.35	0.30	0.13	0.29
Cl_{met}	3.1	2.7	2.6	2.7	2.8
Cl_T	3.4	3.0	2.9	2.8	3.0

a, g; *b*, mg/kg; *c*, μg/mL; *d*, $x10^{-5}$ min^{-1}; *e*, min; *f*, mL/kg; *g*, μg.min/mL

TABLE 13. URINARY EXCRETION OF UNCONJUGATED (E)-2-ENE VPA IN NORMAL RATS
(DOSE=20 mg/kg)

Rat	Time (h)	Volume (mL)	Conc (μg/mL)	Amount (mg)	Time (h)	Cumulative Amt (mg)	Cumulative % of Dose
1a	0-2.5	2.68	32 (400)	0.09 (1.1)	0-2.5	0.09 (1.1)	1.7 (20)
	2.5-8	2.44	6.9 (89)	0.02 (0.22)	0-8	0.11 (1.3)	2.0 (24)
	8-24	6.60	2.9 (3.3)	0.02 (0.02)	0-24	0.13 (1.3)	2.4 (25)
2a	0-3	3.63	99 (360)	0.36 (1.3)	0-3	0.36 (1.3)	6.3 (23)
	3-9	1.60	27 (190)	0.04 (0.30)	0-9	0.40 (1.6)	7.0 (28)
	9-24	9.50	- (5.0)	- (0.05)	0-24	0.40 (1.6)	7.0 (29)
3a	0-3.25	3.61	140 (480)	0.52 (1.7)	0-3.25	0.52 (1.7)	8.9 (30)
	3.25-8	1.40	36 (140)	0.05 (0.19)	0-8	0.57 (1.9)	9.8 (33)
	8-24	15.0	- (-)	- (-)	0-24	0.57 (1.9)	9.8 (33)
4a	0-8	10.1	22 (230)	0.22 (2.3)	0-8	0.22 (2.3)	3.7 (39)
	8-24	15.2	2.2 (2.9)	0.03 (0.04)	0-24	0.25 (2.4)	4.2 (39)

Numbers inside brackets indicate total (sum of conjugated and unconjugated) (E)-2-ene VPA.

TABLE 14. URINARY EXCRETION OF UNCONJUGATED (E)-2-ENE VPA IN NORMAL RATS
(DOSE=100 mg/kg)

Rat	Time (h)	Volume (mL)	Conc (μg/mL)	Amount (mg)	Time (h)	Cumulative Amt (mg)	Cumulative % of Dose
1b	0-1.5	3.0	860 (1900)	2.6 (5.8)	0-1.5	2.6 (5.8)	10 (22)
	1.5-8.5	2.28	64 (1600)	0.14 (3.6)	0-8.5	2.7 (9.4)	11 (36)
	8.5-11.5	2.00	29 (560)	0.06 (1.1)	0-11.5	2.8 (11)	11 (40)
	11.5-24	4.65	13 (79)	0.06 (0.37)	0-24	2.9 (11)	11 (42)
2b	0-6.5	3.99	480 (3700)	1.9 (15)	0-6.5	1.9 (15)	7.3 (57)
	6.5-12.5	2.92	360 (580)	1.1 (1.7)	0-12.5	3.0 (17)	12 (64)
	12.5-24	4.50	14 (15)	0.06 (0.06)	0-24	3.0 (17)	12 (64)
3b	0-1	2.15	130 (1600)	0.27 (3.4)	0-1	0.27 (3.4)	0.87 (11)
	1-8.5	2.50	960 (3500)	2.4 (8.7)	0-8.5	2.7 (12)	8.6 (39)
	8.5-24	4.86	120 (190)	0.56 (0.91)	0-24	3.2 (13)	10 (42)
4b	0-0.5	1.05	150 (480)	0.16 (0.50)	0-0.5	0.16 (0.50)	0.50 (1.6)
	0.5-2	1.50	240 (5300)	0.36 (7.9)	0-2	0.52 (8.4)	1.6 (24)
	2-11.5	3.61	240 (2100)	0.87 (7.4)	0-11.5	1.4 (16)	4.3 (49)
	11.5-24	3.56	34 (110)	0.12 (0.41)	0-24	1.5 (16)	4.7 (51)

Numbers inside brackets indicate total (sum of conjugated and unconjugated) (E)-2-ene VPA.

TABLE 15. PHARMACOKINETIC PARAMETERS OF 4-ENE VPA IN NORMAL RATS (DOSE=20 mg/kg)

Parameter	1c	2c	3c	4c	5c	6c	Mean ± SD
Weight[a]	290	280	280	270	320	335	296 ± 26
Dose[b]	5.8	5.6	5.6	5.4	6.4	6.7	5.9 ± 0.5
C_o[c]	94	110	83	75	100	120	98 ± 17
k_1[d]	610	490	590	490	620	680	580 ± 80
$t_{1/2}$[e]	11	14	12	14	11	10	12 ± 1.7
V_1[f]	210	180	240	270	190	170	210 ± 40
AUC[g]	2200	2500	2200	2100	2400	2500	2300 ± 160
			% DOSE EXCRETED IN URINE				
Unconj	9.3	3.3	3.6	5.5	6.1	4.0	5.3 ± 2.3
Conj	16	18	16	18	19	14	17 ± 2.1
Total	25	22	19	24	25	18	22 ± 3.1
			CLEARANCE (mL/min.kg)				
Cl_R	0.85	0.26	0.32	0.51	0.51	0.35	0.47 ± 0.21
Cl_{met}	8.3	7.8	8.8	8.8	7.9	7.7	8.2 ± 0.51
Cl_T	9.2	8.1	9.1	9.4	8.4	8.0	8.7 ± 0.60

a, g; *b*, mg/kg; *c*, μg/mL; *d*, $x10^{-5}$ min^{-1}; *e*, min; *f*, mL/kg; *g*, μg.min/mL

TABLE 16. PHARMACOKINETIC PARAMETERS OF 4-ENE VPA IN NORMAL RATS (DOSE=100 mg/kg)

Parameter	1d	2d	3d	4d	5d	6d	Mean ± SD
Weight[a]	280	256	262	260	305	295	276 ± 20
Dose[b]	28	26	26	26	31	30	28 ± 2.1
C_o[c]	280	320	330	350	310	290	310 ± 24
k_1[d]	360	400	410	330	370	410	380 ± 34
$t_{1/2}$[e]	19	18	17	21	19	17	18 ± 1.5
V_1[f]	350	320	300	290	320	350	320 ± 25
AUC[g]	17000	15000	15000	19000	19000	18000	17000 ± 1600
			% DOSE EXCRETED IN URINE				
Unconj	4.3	4.9	6.6	2.5	2.4	3.3	3.8 ± 1.7
Conj	21	31	21	19	32	22	24 ± 5.7
Total	25	36	28	21	34	25	28 ± 5.7
			CLEARANCE (mL/min.kg)				
Cl_R	0.26	0.32	0.43	0.13	0.13	0.14	0.23 ± 0.12
Cl_{met}	5.8	6.2	6.1	5.2	5.2	5.6	5.7 ± 0.43
Cl_T	6.0	6.5	6.5	5.3	5.3	5.7	5.9 ± 0.54

a, g; *b*, mg/kg; *c*, μg/mL; *d*, $x10^{-5}$ min^{-1}; *e*, min; *f*, mL/kg; *g*, μg.min/mL

TABLE 17. URINARY EXCRETION OF UNCONJUGATED 4-ENE VPA IN NORMAL RATS
(DOSE=20 mg/kg)

Rat	Time (h)	Volume (mL)	Conc (μg/mL)	Amount (mg)	Time (h)	Cumulative Amt (mg)	Cumulative % of Dose
1c	0-3	3.70	97 (230)	0.36 (0.86)	0-3	0.36 (0.86)	6.2 (15)
	3-7	3.40	22 (110)	0.08 (0.39)	0-7	0.43 (1.3)	7.5 (22)
	7-24	23.3	4.5 (8.6)	0.10 (0.20)	0-24	0.54 (1.5)	9.3 (25)
2c	0-2.5	2.23	78 (450)	0.18 (0.99)	0-2.5	0.18 (0.99)	3.1 (18)
	2.5-5	1.24	6.9 (142)	0.008 (0.18)	0-5	0.18 (1.2)	3.3 (21)
	5-8	1.35	- (24)	- (0.033)	0-8	0.18 (1.2)	3.3 (22)
	8-24	8.00	- (-)	- (-)	0-24	0.18 (1.2)	3.3 (22)
3c	0-0.5	1.47	78 (280)	0.12 (0.41)	0-0.5	0.12 (0.41)	2.0 (7.2)
	0.5-4	2.59	22 (220)	0.06 (0.58)	0-4	0.17 (0.98)	3.1 (18)
	4-8	1.90	4.7 (37)	0.009 (0.069)	0-8	0.18 (1.1)	3.2 (19)
	8-24	8.50	2.1 (2.2)	0.018 (0.019)	0-24	0.20 (1.1)	3.6 (19)
4c	0-2	3.00	61 (250)	0.18 (0.75)	0-2	0.18 (0.75)	3.4 (14)
	2-8	7.40	15 (74)	0.11 (0.54)	0-8	0.30 (1.3)	5.5 (24)
	8-24	8.55	- (-)	- (-)	0-24	0.30 (1.3)	5.5 (24)
5c	0-8	3.50	110 (440)	0.39 (1.6)	0-8	0.39 (1.6)	6.1 (24)
	8-24	11.20	- (3.1)	- (0.035)	0-24	0.39 (1.6)	6.1 (25)
6c	0-8	2.95	90 (370)	0.27 (1.1)	0-8	0.27 (1.1)	4.0 (16)
	8-24	15.50	- (6.3)	- (0.098)	0-24	0.27 (1.2)	4.0 (18)

Numbers inside brackets indicate total (sum of conjugated and unconjugated) 4-ene VPA.

TABLE 18. URINARY EXCRETION OF UNCONJUGATED 4-ENE VPA IN NORMAL RATS
(DOSE=100 mg/kg)

Rat	Time (h)	Volume (mL)	Conc (μg/mL)	Amount (mg)	Time (h)	Cumulative Amt (mg)	Cumulative % of Dose
1d	0-3	3.07	330 (1400)	1.0 (4.4)	0-3	1.0 (4.4)	3.6 (16)
	3-7.5	2.63	62 (0.85)	0.16 (2.2)	0-7.5	1.2 (6.6)	4.2 (24)
	7.5-12	2.37	11 (0.13)	0.025 (0.31)	0-12	1.2 (6.9)	4.3 (25)
	12-24	3.28	3.2 (0.018)	0.010 (0.06)	0-24	1.2 (7.0)	4.3 (25)
2d	0-1	1.85	260 (2600)	0.48 (4.9)	0-1	0.48 (4.9)	1.9 (19)
	1-6	1.29	530 (2700)	0.68 (3.4)	0-6	1.2 (8.3)	4.5 (33)
	6-10	2.14	25 (350)	0.054 (0.75)	0-10	1.2 (9.1)	4.8 (36)
	10-24	3.40	14 (21)	0.047 (0.073)	0-24	1.3 (9.2)	4.9 (36)
3d	0-1	0.35	230 (290)	0.08 (0.10)	0-1	0.079 (0.10)	0.3 (0.4)
	1-2	2.35	610 (2200)	1.4 (5.2)	0-2	1.5 (5.3)	5.8 (21)
	2-8.5	2.27	85 (700)	0.19 (1.6)	0-8.5	1.7 (6.9)	6.6 (27)
	8.5-11	1.59	5.5 (52)	0.009 (0.083)	0-11	1.7 (7.0)	6.6 (27)
	11-13	1.62	11 (68)	0.018 (0.11)	0-13	1.7 (7.1)	6.6 (27)
	13-24	4.52	- (9.3)	- (0.042)	0-24	1.7 (7.2)	6.6 (28)
4d	0-1	0.51	110 (450)	0.057 (0.23)	0-1	0.057 (0.23)	0.2 (0.9)
	1-2	1.50	250 (1400)	0.38 (2.1)	0-2	0.43 (2.4)	1.7 (9.1)
	2-10	1.29	130 (2200)	0.16 (2.9)	0-10	0.60 (5.2)	2.3 (20)
	10-12	1.30	9.1 (160)	0.012 (0.21)	0-12	0.61 (5.4)	2.3 (21)
	12-24	3.54	8.9 (37)	0.032 (0.13)	0-24	0.64 (5.6)	2.5 (21)
5d	0-6	5.30	110 (1500)	0.57 (8.1)	0-6	0.57 (8.1)	1.9 (27)
	6-24	18.50	8.5 (130)	0.16 (2.4)	0-24	0.73 (11)	2.4 (34)
6d	0-6	3.10	290 (1800)	0.91 (5.6)	0-6	0.91 (5.6)	3.1 (19)
	6-24	7.88	9.9 (230)	0.078 (1.8)	0-24	0.99 (7.4)	3.3 (25)

Numbers inside brackets indicate total (sum of conjugated and unconjugated) 4-ene VPA.

was, however, a trend towards smaller metabolic and total clearance values at the high dose than at the low dose.

For 4-ene VPA, the apparent volume of the central compartment increased 1.5 times with a five fold increase in the dose (Tables 15,16). The fraction of dose excreted as conjugates and total 4-ene VPA in urine increased significantly with the dose (Table 32). However, the apparent total plasma, renal and non-renal (metabolic) clearances decreased significantly with an increase in the dose of 4-ene VPA (Table 32). More than 95% of 4-ene VPA excreted in urine was recovered within 12 h of the dose (Tables 17,18).

C.3.2. Pharmacokinetic Model

The plasma data of (E)-2-ene VPA and 4-ene VPA obtained from normal rats receiving the low dose (Fig 9,10) were fitted to the proposed time-lag pharmacokinetic model using an optimal tau (T) value of 1.75 for (E)-2-ene VPA and 4-ene VPA. The calculated values of first-order transfer rate constants, k_{10}, k_{12} and k_{21} for (E)-2-ene VPA were 1.0, 0.69 and 1.3 h^{-1} and for 4-ene VPA, 2.4, 0.89 and 0.50 h^{-1}, respectively. The model-generated curve (solid line) showed an acceptable fit with most of the actual data points. At the trough levels, however, the predicted values were lower than the observed plasma levels. The residual sum of squares was found to be the least, 63 and 12 μg/mL, for (E)-2-ene VPA and 4-ene VPA, respectively, at a time-lag value of 1.75 h. Since the proposed model assumes first-order transfer processes between the compartments, it was not applied to the plasma profile after the high dose since that showed a short non-linear phase at concentrations > 200 μg/mL.

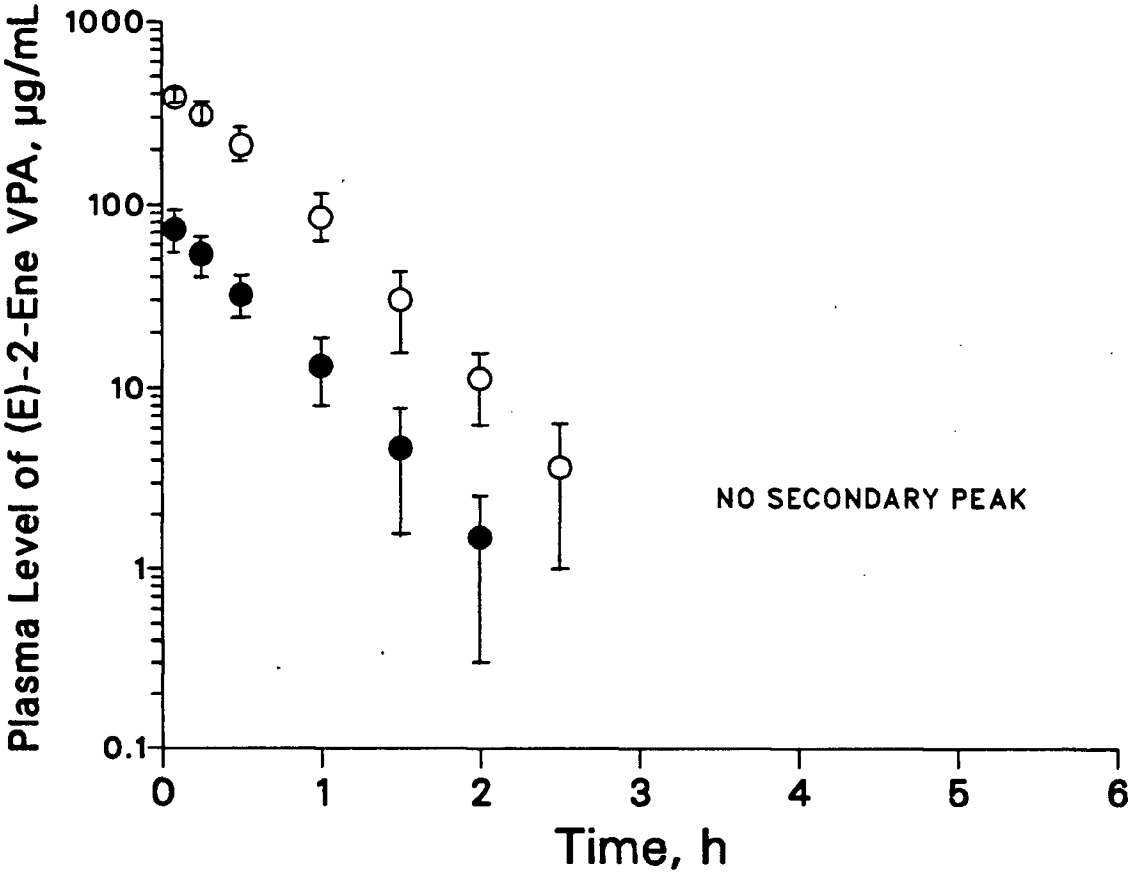


Fig. 11. Semilogarthmic plots of plasma concentrations of (E)-2-ene VPA *versus* time following IV dose of 20 (●) and 100 (○) mg/kg in bile-exteriorized rats. Each point represents mean ± 95% confidence limits (N=4).

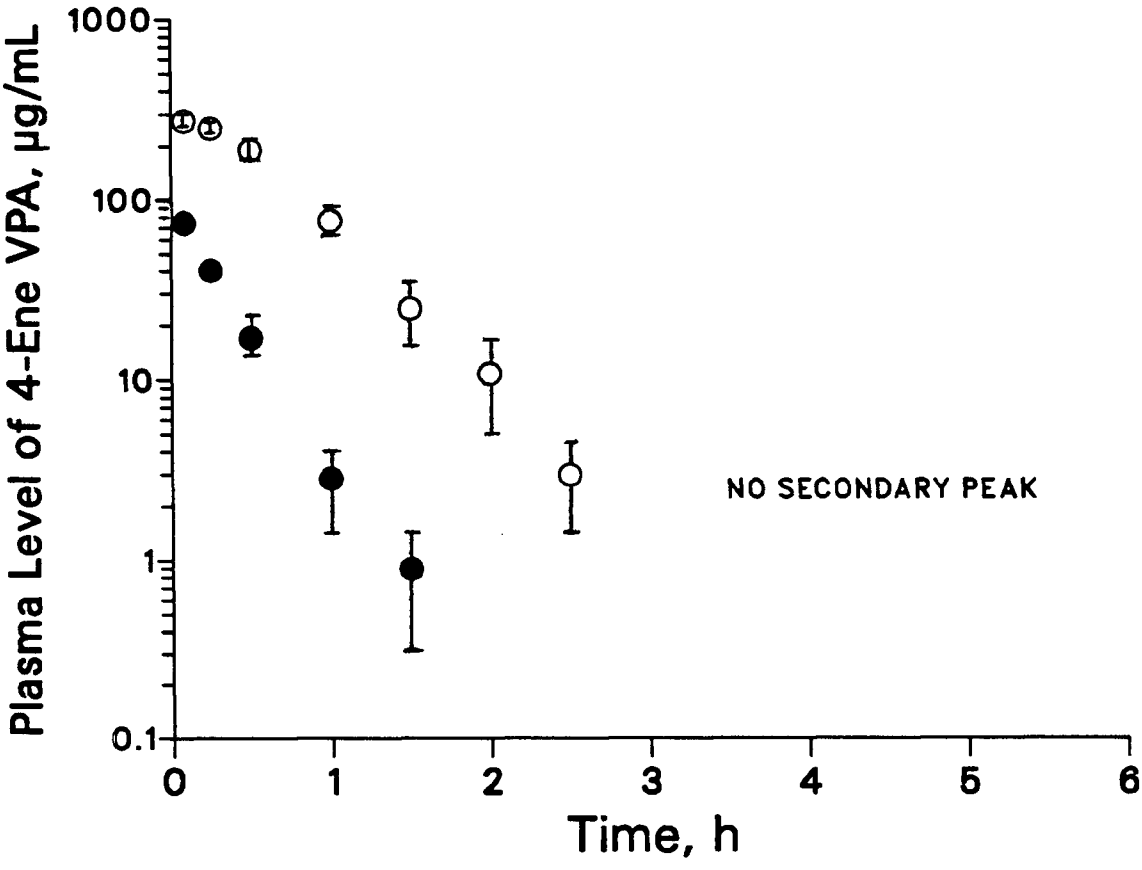


Fig. 12. Semilogarthmic plots of plasma concentrations of 4-ene VPA *versus* time following IV dose of 20 (●) and 100 (○) mg/kg in bile exteriorized rats. Each point represents mean ± 95% confidence limits (N=6).

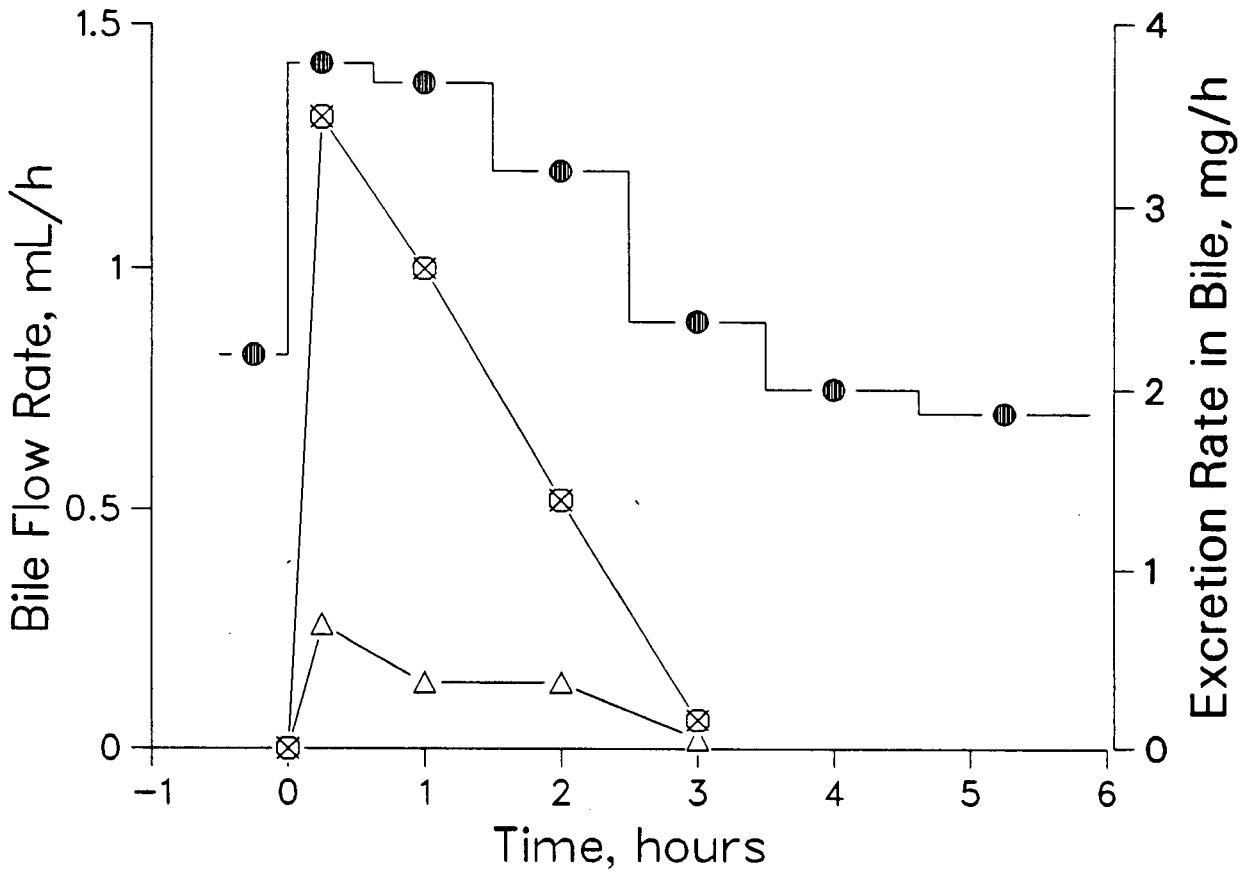


Fig. 13. A typical choleretic effect, and excretion rate plot of 4-ene VPA in the bile of a rat after high dose of 100 mg/kg. Bile flow rate (●), and conjugated 4-ene VPA (⊗) and unconjugated 4-ene VPA (△) in bile.

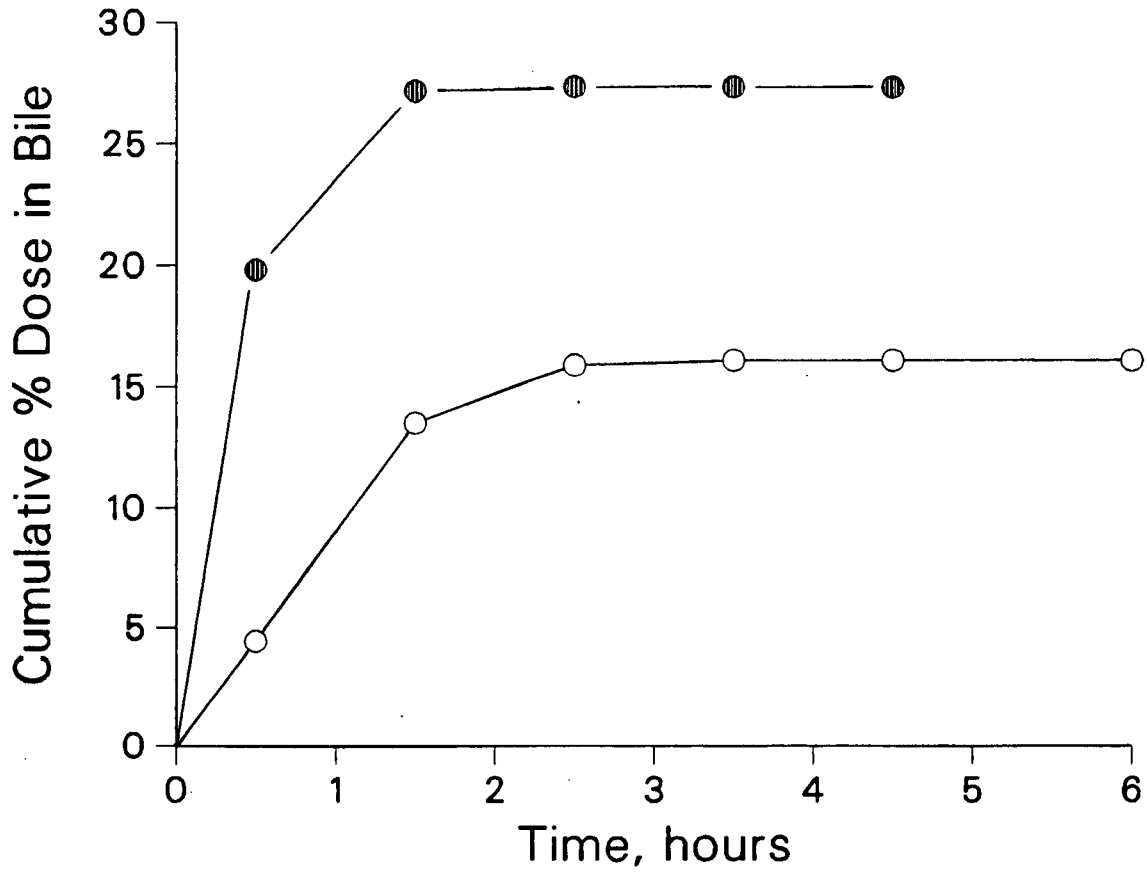


Fig. 14. A typical cumulative biliary excretion plot of 4-ene VPA *versus* time after 20 (●) and 100 (○) mg/kg.

C.3.3. Pharmacokinetics in Bile-Exteriorized Rats

The semilogarithmic plots of average plasma levels of (E)-2-ene VPA and 4-ene VPA *versus* time in bile exteriorized rats are shown in Fig. 11 and 12, respectively. After the 20 mg/kg dose, (E)-2-ene VPA and 4-ene VPA plasma profile followed an open one-compartment model with first-order elimination $t_{1/2}$ of 20 $\pm$ 3.4 and 13 $\pm$ 1.8 min, respectively. Following the high dose, a brief period of non-linear plasma decline was observed at concentrations above 200 μg/mL for both the metabolites. Thereafter, (E)-2-ene VPA and 4-ene VPA were eliminated with an apparent $t_{1/2}$ of 21 $\pm$ 1.7 and 19 $\pm$ 3.1 min, respectively. No secondary plasma peaks were seen in these bile-exteriorized rats. The plasma levels of (E)-2-ene VPA and 4-ene VPA in individual bile-exteriorized rats are shown in Appendices 9-12.

Various pharmacokinetic parameters of (E)-2-ene VPA at the low and the high dose in bile-duct cannulated rats are shown in Tables 19 and 20. The fraction of (E)-2-ene VPA dose, measured as a sum of unconjugated and conjugated (E)-2-ene VPA, in the urine showed a slight but non-significant increase with the dose. The percentage of (E)-2-ene VPA dose eliminated in bile showed a non-significant decrease with an increase in the dose (Table 31). A total of 65% of the low dose and 66% of the high dose were eliminated in the urine and bile, collectively (Tables 21-24). Plasma clearance of (E)-2-ene VPA decreased by 22%, when the dose was increased by 5 fold.

In the case of 4-ene VPA, there was a significant increase in the apparent volume of the central compartment, and in the fraction of dose excreted as conjugated and total 4-ene VPA in urine with an increase in the administered dose (Tables 32, 25, 26). In contrast, the fraction of dose recovered as conjugated and total 4-ene VPA in bile, the total plasma clearance and the metabolic clearance decreased by approximately 40% with a

TABLE 19. PHARMACOKINETIC PARAMETERS OF (E)-2-ENE VPA IN BILE-EXTERIORIZED (DOSE=20 mg/kg)

Parameter	1e	2e	3e	4e	Mean
Weight[a]	320	285	280	310	299
Dose[b]	6.4	5.7	5.6	6.2	6.0
C_o[c]	100	79	100	81	91
k_1[d]	390	400	280	340	350
$t_{1/2}$[e]	18	17	25	20	20
V_1[f]	200	250	200	240	220
AUC[g]	2600	2100	3800	2500	2700
	% DOSE EXCRETED IN URINE				
Unconj	1.7	2.8	4.1	5.5	3.5
Conj	16	25	24	30	24
Total	17	28	29	36	27
	% DOSE EXCRETED IN BILE				
Unconj	12	8.4	6.3	5.9	8.1
Conj	39	29	29	22	29
Total	51	37	34	28	38
	CLEARANCE (mL/min.kg)				
Cl_R	0.13	0.33	0.22	0.44	0.28
Cl_B	0.93	0.81	0.33	0.47	0.64
Cl_{conj}	4.2	5.2	2.8	4.2	4.1
Cl_T	7.8	9.7	5.3	8.0	7.7

a, g; *b*, mg/kg; *c*, μg/mL; *d*, $x10^{-5}$ min^{-1}; *e*, min; *f*, mL/kg; *g*, μg.min/mL

TABLE 20. PHARMACOKINETIC PARAMETERS OF (E)-2-ENE VPA IN BILE-EXTERIORIZED RATS (DOSE=100 mg/kg)

Parameter	1f	2f	3f	4f	Mean
Weight[a]	270	290	320	270	288
Dose[b]	27	29	32	27	29
C_o[c]	440	440	380	440	430
k_1[d]	360	300	320	340	330
$t_{1/2}$[e]	19	23	22	20	21
V_1[f]	230	230	260	230	240
AUC[g]	13000	20000	18000	16000	17000
	% DOSE EXCRETED IN URINE				
Unconj	4.7	3.5	1.3	3.5	3.3
Conj	44	26	38	20	32
Total	49	29	39	24	35
	% DOSE EXCRETED IN BILE				
Unconj	6.0	8.4	2.5	7.4	6.1
Conj	25	34	17	25	25
Total	31	42	19	32	31
	CLEARANCE (mL/min.kg)				
Cl_R	0.35	0.18	0.07	0.21	0.20
Cl_B	0.45	0.43	0.13	0.45	0.37
Cl_{conj}	5.2	3.0	3.0	2.8	3.5
Cl_T	7.5	5.0	5.4	6.1	6.0

a, g; *b*, mg/kg; *c*, μg/mL; *d*, $x10^{-5}$ min^{-1}; *e*, min; *f*, mL/kg; *g*, μg.min/mL

TABLE 21. URINARY EXCRETION OF UNCONJUGATED (E)-2-ENE VPA IN BILE-EXTERIORIZED RATS (DOSE=20 mg/kg)

Rat	Time (h)	Volume (mL)	Conc (μg/mL)	Amount (mg)	Time (h)	Cumulative Amt (mg)	Cumulative % of Dose
1e	0-6	1.52	71 (730)	0.11 (1.1)	0-6	0.11 (1.1)	1.7 (17)
	6-24	20.0	- (-)	- (-)	0-24	0.11 (1.1)	1.7 (17)
2e	0-6	3.4	47 (450)	0.16 (1.5)	0-6	0.16 (1.5)	2.8 (27)
	6-24	21.5	- (3.4)	- (0.07)	0-24	0.16 (1.6)	2.8 (28)
3e	0-5.5	2.17	97 (720)	0.21 (1.6)	0-5.5	0.21 (1.6)	3.8 (28)
	5.5-24	8.20	2.9 (3.0)	0.02 (0.02)	0-24	0.23 (1.6)	4.1 (29)
4e	0-5.5	4.90	69 (450)	0.34 (2.2)	0-5.5	0.34 (2.2)	5.5 (36)
	5.5-24	4.80	- (2.2)	- (0.01)	0-24	0.34 (2.2)	5.5 (36)

Numbers inside brackets indicate total (sum of conjugated and unconjugated) (E)-2-ene VPA.

TABLE 22. URINARY EXCRETION OF UNCONJUGATED (E)-2-ENE VPA IN BILE-EXTERIORIZED RATS (DOSE=100 mg/kg)

Rat	Time (h)	Volume (mL)	Conc (μg/mL)	Amount (mg)	Time (h)	Cumulative Amt (mg)	Cumulative % of Dose
1f	0-6.5 6.5-24*	3.20	400 (4100)	1.3 (13)	0-6.5	1.3 (13)	4.7 (49)
2f	0-6	1.93	58 (1700)	0.11 (3.3)	0-6	0.11 (3.3)	0.4 (12)
	6-24	11.80	77 (430)	0.91 (5.1)	0-24	1.0 (8.4)	3.5 (29)
3f	0-6.5	11.33	35 (1100)	0.39 (12)	0-6.5	0.39 (12)	1.2 (38)
	6.5-24	5.60	7.9 (45)	0.04 (0.25)	0-24	0.43 (13)	1.3 (39)
4f	0-6	2.25	190 (2000)	0.42 (4.5)	0-6	0.42 (4.5)	1.6 (17)
	6-24	7.80	66 (230)	0.51 (1.8)	0-24	0.94 (6.3)	3.5 (24)

Numbers inside brackets indicate total (sum of conjugated and unconjugated) (E)-2-ene VPA.
*, accidentally lost.

TABLE 23. BILIARY ELIMINATION OF UNCONJUGATED (E)-2-ENE VPA IN RATS
(DOSE=20 mg/kg)

Rat	Time (h)	Volume (mL)	Conc (μg/mL)	Amount (mg)	Time (h)	Cumulative Amt (mg)	Cumulative % of Dose
1e	0-0.5	1.24	440 (1900)	0.54 (2.4)	0-0.5	0.54 (2.4)	8.4 (38)
	0.5-1.5	1.56	140 (520)	0.22 (0.81)	0-1.5	0.75 (3.2)	12 (50)
	1.5-2.5	1.12	12 (32)	0.013 (0.035)	0-2.5	0.77 (3.2)	12 (51)
	2.5-3.5	1.07	- (1.3)	- (0.001)	0-3.5	0.77 (3.2)	12 (51)
	3.5-4.5	1.10	- (-)	- (-)	0-4.5	0.77 (3.2)	12 (51)
	4.5-5.5	1.05	- (-)	- (-)	0-5.5	0.77 (3.2)	12 (51)
2e	0-0.5	1.15	330 (1400)	0.38 (1.7)	0-0.5	0.38 (1.7)	6.6 (29)
	0.5-1.5	1.53	65 (300)	0.10 (0.45)	0-1.5	0.48 (2.1)	8.3 (37)
	1.5-2.5	1.10	2.6 (8.0)	0.003 (0.009)	0-2.5	0.48 (2.1)	8.4 (37)
	2.5-3.5	0.91	- (-)	- (-)	0-3.5	0.48 (2.1)	8.4 (37)
	3.5-4.5	0.86	- (-)	- (-)	0-4.5	0.48 (2.1)	8.4 (37)
	4.5-5.5	0.74	- (-)	- (-)	0-5.5	0.48 (2.1)	8.4 (37)
3e	0-0.5	0.84	240 (1400)	0.20 (1.2)	0-0.5	0.20 (1.2)	3.5 (21)
	0.5-1.5	1.01	140 (750)	0.14 (0.76)	0-1.5	0.34 (1.9)	6.1 (34)
	1.5-2.5	0.87	12 (60)	0.010 (0.015)	0-2.5	0.35 (1.9)	6.1 (35)
	2.5-3.5	0.54	1.2 (2.8)	0.001 (0.001)	0-3.5	0.35 (1.9)	6.3 (35)
	3.5-4.5	0.78	- (-)	- (-)	0-4.5	0.35 (1.9)	6.3 (35)
	4.5-5.5	0.64	- (-)	- (-)	0-5.5	0.35 (1.9)	6.3 (35)
4e	0-0.5	1.05	220 (1200)	0.23 (1.3)	0-0.5	0.23 (1.3)	3.8 (21)
	0.5-1.5	1.21	100 (350)	0.13 (0.43)	0-1.5	0.36 (1.7)	5.8 (28)
	1.5-2.5	1.00	5.1 (23)	0.005 (0.023)	0-2.5	0.36 (1.7)	5.9 (28)
	2.5-3.5	0.94	- (1.1)	- (0.001)	0-3.5	0.36 (1.7)	5.9 (28)
	3.5-4.5	0.86	- (-)	- (-)	0-4.5	0.36 (1.7)	5.9 (28)
	4.5-5.5	0.81	- (-)	- (-)	0-5.5	0.36 (1.7)	5.9 (28)

Numbers inside brackets indicate total (sum of conjugated and unconjugated) (E)-2-ene VPA.

TABLE 24. BILIARY ELIMINATION OF UNCONJUGATED (E)-2-ENE VPA IN RATS
(DOSE=100 mg/kg)

Rat	Time (h)	Volume (mL)	Conc (μg/mL)	Amount (mg)	Time (h)	Cumulative Amt (mg)	Cumulative % of Dose
1f	0-0.5	0.84	870 (4400)	0.73 (3.7)	0-0.5	0.73 (3.7)	2.7 (14)
	0.5-1.5	1.42	590 (3100)	0.84 (4.4)	0-1.5	1.6 (8.1)	5.8 (30)
	1.5-2.5	0.78	75 (290)	0.058 (0.23)	0-2.5	1.6 (8.3)	6.0 (31)
	2.5-5.0	1.54	1.6 (5.7)	0.002 (0.01)	0-5	1.6 (8.3)	6.0 (31)
	5.0-6.5	1.23	- (-)	- (-)	0-6.5	1.6 (8.3)	6.0 (31)
2f	0-0.5	1.01	580 (2500)	0.58 (2.5)	0-0.5	0.58 (2.5)	2.0 (8.7)
	0.5-1.0	1.18	670 (3900)	0.80 (4.6)	0-1.0	1.4 (7.1)	4.8 (24)
	1.0-1.5	1.04	570 (3200)	0.59 (3.4)	0-1.5	2.0 (11)	6.8 (36)
	1.5-2.5	1.15	360 (1400)	0.41 (1.6)	0-2.5	2.4 (12)	8.2 (41)
	2.5-3.5	0.82	54 (180)	0.044 (0.15)	0-3.5	2.4 (12)	8.4 (42)
	3.5-4.5	0.60	10 (36)	0.006 (0.022)	0-4.5	2.4 (12)	8.4 (42)
	4.5-6.0	0.67	2.4 (8.7)	0.002 (0.006)	0-6.0	2.4 (12)	8.4 (42)
3f	0-0.5	0.87	320 (2300)	0.28 (2.0)	0-0.5	0.28 (2.0)	0.9 (6.2)
	0.5-1.5	1.12	330 (2900)	0.37 (3.3)	0-1.5	0.64 (3.3)	2.0 (10)
	1.5-2.5	0.87	150 (1100)	0.13 (0.93)	0-2.5	0.77 (6.2)	2.4 (19)
	2.5-3.5	0.77	14 (46)	0.011 (0.035)	0-3.5	0.79 (6.2)	2.4 (19)
	3.5-5.0	0.98	- (2.6)	- (0.002)	0-5.0	0.79 (6.2)	2.4 (19)
	5.0-6.5	0.98	- (1.2)	- (0.001)	0-6.5	0.79 (6.2)	2.4 (19)
4f	0-0.5	0.93	730 (2500)	0.68 (2.3)	0-0.5	0.68 (2.3)	2.5 (8.5)
	0.5-1.5	1.32	620 (3300)	0.81 (4.3)	0-1.5	1.5 (6.6)	5.5 (24)
	1.5-2.5	1.07	400 (1700)	0.43 (1.8)	0-2.5	1.9 (8.4)	7.1 (31)
	2.5-3.5	0.84	87 (260)	0.073 (0.22)	0-3.5	2.0 (8.6)	7.4 (32)
	3.5-4.5	0.83	5.4 (17)	0.004 (0.14)	0-4.5	2.0 (8.8)	7.4 (32)
	4.5-6.0	1.09	- (-)	- (-)	0-6.0	2.0 (8.8)	7.4 (32)

Numbers inside brackets indicate total (sum of conjugated and unconjugated) (E)-2-ene VPA.

TABLE 25. PHARMACOKINETIC PARAMETERS OF 4-ENE VPA IN BILE-EXTERIORIZED RATS (DOSE=20 mg/kg)

Parameter	1g	2g	3g	4g	5g	6g	Mean ± SD
Weight[a]	270	285	290	310	265	305	288 ± 18
Dose[b]	5.4	5.7	5.8	6.2	5.3	6.1	5.8 ± 0.4
C_0[c]	85	88	100	96	74	100	91 ± 11
k_1[d]	560	550	620	420	560	590	550 ± 70
$t_{1/2}$[e]	12	13	11	16	12	12	13 ± 1.8
V_1[f]	220	210	180	200	270	190	210 ± 30
AUC[g]	1700	1700	1800	2400	1400	1800	1800 ± 310
			% DOSE EXCRETED IN URINE				
Unconj	0.62	0.36	1.4	1.3	2.6	2.9	1.5 ± 1.0
Conj	19	12	22	19	12	14	16 ± 4.0
Total	19	12	23	20	15	17	18 ± 3.9
			% DOSE EXCRETED IN BILE				
Unconj	5.3	5.4	4.3	10	7.8	4.0	6.2 ± 2.4
Conj	33	27	22	14	21	23	23 ± 6.5
Total	38	32	26	24	29	27	29 ± 5.2
			CLEARANCE (mL/min.kg)				
Cl_R	0.08	0.04	0.15	0.11	0.36	0.33	0.18 ± 0.14
Cl_B	0.64	0.63	0.48	0.87	1.1	0.44	0.69 ± 0.24
Cl_{conj}	6.3	4.5	4.8	2.8	4.6	4.2	4.5 ± 1.1
Cl_T	12	12	11	8.5	14	11	11 ± 1.8

a, g; *b*, mg/kg; *c*, μg/mL; *d*, $x10^{-5}$ min^{-1}; *e*, min; *f*, mL/kg; *g*, μg.min/mL

TABLE 26. PHARMACOKINETIC PARAMETERS OF 4-ENE VPA IN BILE-EXTERIORIZED RATS (DOSE=100 mg/kg)

Parameter	1h	2h	3h	4h	5h	6h	Mean ± SD
Weight[a]	290	325	302	295	280	300	299 ± 15
Dose[b]	29	33	30	30	28	30	30 ± 1.5
C_o[c]	290	320	310	250	270	310	290 ± 28
k_1[d]	430	300	310	380	400	440	380 ± 58
$t_{1/2}$[e]	16	23	22	18	17	16	19 ± 3.1
V_1[f]	340	320	320	400	370	320	350 ± 35
AUC[g]	15000	16000	16000	12000	12000	12000	14000 ± 2100
			% DOSE EXCRETED IN URINE				
Unconj	2.6	3.5	2.5	2.8	0.96	1.4	2.3 ± 0.94
Conj	23	31	22	24	23	15	23 ± 5.1
Total	26	35	24	27	24	17	25 ± 5.8
			% DOSE EXCRETED IN BILE				
Unconj	4.7	8.4	3.8	3.9	6.1	4.5	5.5 ± 1.6
Conj	15	18	16	12	16	15	15 ± 2.0
Total	20	27	20	16	22	19	21 ± 3.5
			CLEARANCE (mL/min.kg)				
Cl_R	0.18	0.23	0.15	0.22	0.08	0.19	0.16 ± 0.06
Cl_B	0.32	0.53	0.30	0.32	0.53	0.38	0.40 ± 0.11
Cl_{conj}	2.6	3.2	2.3	2.9	3.4	2.6	2.8 ± 0.42
Cl_T	6.8	6.4	6.1	8.0	8.7	8.4	7.4 ± 1.1

a, g; *b*, mg/kg; *c*, μg/mL; *d*, $x10^{-5}$ min^{-1}; *e*, min; *f*, mL/kg; *g*, μg.min/mL

TABLE 27. URINARY EXCRETION OF UNCONJUGATED 4-ENE VPA IN BILE-EXTERIORIZED RATS
(DOSE=20 mg/kg)

Rat	Time (h)	Volume (mL)	Conc (μg/mL)	Amount (mg)	Time (h)	Cumulative Amt (mg)	Cumulative % of Dose
1g	0-6	3.80	8.8 (270)	0.03 (1.0)	0-6	0.03 (1.0)	0.6 (19)
	6-24	18.5	- (-)	- (-)	0-24	0.03 (1.0)	0.6 (19)
2g	0-6	3.00	6.9 (230)	0.02 (0.68)	0-6	0.02 (0.68)	0.4 (12)
	6-24	4.70	- (-)	- (3.5)	0-24	0.02 (0.70)	0.4 (12)
3g	0-6	4.30	19 (300)	0.08 (1.3)	0-6	0.08 (1.3)	1.4 (23)
	6-24	5.70	- (3.8)	- (0.02)	0-24	0.08 (1.3)	1.4 (23)
4g	0-6	2.80	28 (440)	0.08 (1.2)	0-6	0.08 (1.2)	1.3 (20)
	6-24	3.84	- (4.1)	- (0.02)	0-24	0.08 (1.3)	1.3 (20)
5g	0-6	3.15	33 (220)	0.10 (0.69)	0-6	0.10 (0.69)	2.0 (13)
	6-24	4.38	7.8 (21)	0.03 (0.09)	0-24	0.14 (0.78)	2.6 (15)
6g	0-6	3.75	35 (260)	0.13 (0.98)	0-6	0.13 (0.98)	2.2 (16)
	6-24	5.14	9.2 (15)	0.05 (0.08)	0-24	0.18 (1.1)	2.9 (17)

Numbers inside brackets indicate total (sum of conjugated and unconjugated) 4-ene VPA.

TABLE 28. URINARY EXCRETION OF UNCONJUGATED 4-ENE VPA IN BILE-EXTERIORIZED RATS
(DOSE=100 mg/kg)

Rat	Time (h)	Volume (mL)	Conc (μg/mL)	Amount (mg)	Time (h)	Cumulative Amt (mg)	Cumulative % of Dose
1h	0-5	4.66	150 (1500)	0.70 (7.0)	0-5	0.70 (7.0)	2.4 (24)
	5-24	9.70	4.8 (46)	0.047 (0.45)	0-24	0.75 (7.4)	2.6 (26)
2h	0-6	3.80	280 (2800)	1.1 (11)	0-6	1.1 (11)	3.3 (34)
	6-24	9.50	6.2 (46)	0.059 (0.44)	0-24	1.1 (11)	3.5 (35)
3h	0-6	6.70	96 (1000)	0.64 (6.9)	0-6	0.64 (6.9)	2.1 (23)
	6-24	18.0	5.7 (20)	0.10 (0.36)	0-24	0.74 (7.3)	2.5 (24)
4h	0-6	2.80	220 (2900)	0.62 (4.9)	0-6	0.62 (4.9)	2.1 (17)
	6-24	4.70	39 (600)	0.18 (2.8)	0-24	0.81 (7.7)	2.8 (27)
5h	0-7	4.40	56 (1400)	0.25 (6.1)	0-7	0.25 (6.1)	0.9 (22)
	7-24	9.70	2.4 (55)	0.023 (0.53)	0-24	0.27 (6.7)	1.0 (24)
6h	0-7	3.96	100 (1200)	0.40 (4.8)	0-7	0.40 (4.8)	1.3 (16)
	7-24	8.45	2.9 (31)	0.025 (0.26)	0-24	0.42 (5.0)	1.4 (17)

Numbers inside brackets indicate total (sum of conjugated and unconjugated) 4-ene VPA.

TABLE 29. BILIARY ELIMINATION OF UNCONJUGATED 4-ENE VPA IN RATS
(DOSE=20 mg/kg)

Rat	Time (h)	Volume (mL)	Conc (µg/mL)	Amount (mg)	Time (h)	Cumulative Amt (mg)	Cumulative % of Dose
1g	0-0.5	1.10	200 (1500)	0.22 (1.7)	0-0.5	0.22 (1.7)	4.1 (31)
	0.5-1.5	1.43	45 (280)	0.064 (0.41)	0-1.5	0.28 (2.1)	5.2 (38)
	1.5-2.5	1.19	2.3 (4.9)	0.003 (0.01)	0-2.5	0.29 (2.1)	5.3 (38)
	2.5-3.5	1.15	- (-)	- (-)	0-3.5	0.29 (2.1)	5.3 (38)
	3.5-4.5	1.00	- (-)	- (-)	0-4.5	0.29 (2.1)	5.3 (38)
2g	0-0.5	1.10	220 (1300)	0.24 (1.5)	0-0.5	0.24 (1.5)	4.2 (26)
	0.5-1.5	1.40	43 (240)	0.06 (0.34)	0-1.5	0.03 (1.8)	5.3 (32)
	1.5-2.5	1.28	2.8 (4.7)	0.004 (0.01)	0-2.5	0.31 (1.8)	5.4 (32)
	2.5-3.5	1.31	- (-)	- (-)	0-3.5	0.31 (1.8)	5.4 (32)
	3.5-4.5	1.23	- (-)	- (-)	0-4.5	0.31 (1.8)	5.4 (32)
3g	0-0.5	1.08	170 (1100)	0.18 (1.2)	0-0.5	0.18 (1.2)	3.1 (20)
	0.5-1.5	1.60	41 (200)	0.065 (0.31)	0-1.5	0.25 (1.5)	4.2 (26)
	1.5-2.5	1.38	2.2 (4.5)	0.003 (0.01)	0-2.5	0.25 (1.5)	4.3 (26)
	2.5-3.5	1.17	- (-)	- (-)	0-3.5	0.25 (1.5)	4.3 (26)
	3.5-4.5	1.08	- (-)	- (-)	0-4.5	0.25 (1.5)	4.3 (26)
4g	0-0.5	0.79	410 (990)	0.32 (0.78)	0-0.5	0.32 (0.78)	5.2 (13)
	0.5-1.0	0.62	360 (930)	0.22 (0.58)	0-1.0	0.55 (1.4)	8.8 (22)
	1.0-2.25	1.12	76 (97)	0.085 (0.11)	0-2.25	0.63 (1.5)	10 (24)
	2.25-3.25	0.65	2.5 (3.0)	0.002 (0.002)	0-3.25	0.64 (1.5)	10 (24)
	3.25-4.75	0.99	- (-)	- (-)	0-4.75	0.64 (1.5)	10 (24)
	4.75-6.0	0.85	- (-)	- (-)	0-6.0	0.64 (1.5)	10 (24)
5g	0-0.5	1.06	290 (1100)	0.31 (1.2)	0-0.5	0.31 (1.2)	5.7 (23)
	0.5-1.5	1.47	73 (220)	0.11 (0.33)	0-1.5	0.41 (1.5)	7.8 (29)
	1.5-2.5	1.22	2.4 (3.5)	0.003 (0.004)	0-2.5	0.42 (1.5)	7.8 (29)
	2.5-3.5	1.00	- (-)	- (-)	0-3.5	0.42 (1.5)	7.8 (29)
	3.5-4.5	0.91	- (-)	- (-)	0-4.5	0.42 (1.5)	7.8 (29)
6g	0-0.5	0.95	190 (1300)	0.18 (1.2)	0-0.5	0.18 (1.2)	2.9 (20)
	0.5-1.5	1.25	51 (360)	0.064 (0.45)	0-1.5	0.24 (1.7)	4.0 (27)
	1.5-2.5	1.11	2.1 (11)	0.002 (0.012)	0-2.5	0.25 (1.7)	4.0 (27)
	2.5-3.5	1.02	- (-)	- (-)	0-3.5	0.25 (1.7)	4.0 (27)
	3.5-4.5	0.78	- (-)	- (-)	0-4.5	0.25 (1.7)	4.0 (27)

Numbers inside brackets indicate total (sum of conjugated and unconjugated) 4-ene VPA.

TABLE 30. BILIARY ELIMINATION OF UNCONJUGATED 4-ENE VPA IN RATS
(DOSE=100 mg/kg)

Rat	Time (h)	Volume (mL)	Conc (µg/mL)	Amount (mg)	Time (h)	Cumulative Amt (mg)	Cumulative % of Dose
1h	0-0.5	0.70	600 (2000)	0.42 (1.4)	0-0.5	0.42 (1.4)	1.5 (4.1)
	0.5-1.5	1.24	490 (2600)	0.61 (3.2)	0-1.5	1.0 (4.6)	3.6 (16)
	1.5-2.5	1.07	290 (1000)	0.32 (1.1)	0-2.5	1.4 (5.7)	4.6 (20)
	2.5-3.5	0.88	25 (50)	0.022 (0.044)	0-3.5	1.4 (5.8)	4.7 (20)
	3.5-4.5	0.80	3.8 (6.6)	0.003 (0.005)	0-4.5	1.4 (5.8)	4.7 (20)
	4.5-5.5	0.85	- (-)	- (-)	0-5.5	1.4 (5.8)	4.7 (20)
2h	0-0.5	1.15	610 (2800)	0.70 (3.2)	0-0.5	0.70 (3.2)	2.2 (10)
	0.5-1.0	1.30	630 (2800)	0.82 (3.6)	0-1.0	1.5 (6.8)	4.8 (21)
	1.0-1.5	1.10	450 (680)	0.50 (0.75)	0-1.5	2.0 (7.6)	6.3 (24)
	1.5-2.0	1.24	260 (480)	0.32 (0.59)	0-2.0	2.3 (8.2)	7.3 (26)
	2.0-3.5	1.34	250 (270)	0.33 (0.36)	0-3.5	2.7 (8.5)	8.3 (27)
	3.5-5.0	1.57	4.8 (3.2)	0.008 (0.005)	0-5.0	2.7 (8.5)	8.4 (27)
	5.0-6.0	0.87	- (-)	- (-)	0-6.0	2.7 (8.5)	8.4 (27)
3h	0-0.5	0.71	480 (2500)	0.34 (1.7)	0-0.5	0.34 (1.7)	1.1 (5.8)
	0.5-1.5	1.38	260 (2000)	0.36 (2.8)	0-1.5	0.70 (4.5)	2.3 (15)
	1.5-2.5	1.23	300 (1100)	0.37 (1.4)	0-2.5	1.1 (5.9)	3.6 (20)
	2.5-3.5	0.89	70 (170)	0.063 (0.15)	0-3.5	1.1 (6.0)	3.8 (20)
	3.5-4.5	0.75	7.6 (15)	0.006 (0.011)	0-4.5	1.1 (6.1)	3.8 (20)
	4.5-6.0	1.08	- (3.0)	- (0.003)	0-6.0	1.1 (6.1)	3.8 (20)
4h	0-0.5	0.76	500 (1700)	0.38 (1.3)	0-0.5	0.38 (1.3)	1.3 (4.4)
	0.5-1.5	1.23	420 (2100)	0.52 (2.6)	0-1.5	0.90 (3.9)	3.1 (14)
	1.5-2.5	0.75	290 (930)	0.22 (0.70)	0-2.5	1.1 (4.6)	3.8 (16)
	2.5-3.5	0.55	42 (120)	0.023 (0.065)	0-3.5	1.1 (4.7)	3.9 (16)
	3.5-4.5	0.49	6.3 (12)	0.003 (0.006)	0-4.5	1.1 (4.7)	3.9 (16)
	4.5-6.0	1.03	- (2.0)	- (0.002)	0-6.0	1.1 (4.7)	3.9 (16)
5h	0-0.5	1.05	500 (2100)	0.53 (2.2)	0-0.5	0.53 (2.2)	1.9 (8.0)
	0.5-1.5	2.04	520 (1800)	1.1 (3.6)	0-1.5	1.6 (5.8)	5.7 (21)
	1.5-2.5	1.46	75 (240)	0.11 (0.35)	0-2.5	1.7 (6.2)	6.1 (22)
	2.5-3.5	1.29	5.7 (16)	0.007 (0.020)	0-3.5	1.7 (6.2)	6.1 (22)
	3.5-4.5	1.05	- (3.4)	- (0.003)	0-4.5	1.7 (6.2)	6.1 (22)
	4.5-5.5	0.97	- (-)	- (-)	0-5.5	1.7 (6.2)	6.1 (22)
6h	0-0.5	0.95	480 (2000)	0.45 (1.9)	0-0.5	0.45 (1.9)	1.5 (6.4)
	0.5-1.5	2.00	350 (1600)	0.70 (3.2)	0-1.5	1.2 (5.2)	3.8 (17)
	1.5-2.5	1.38	110 (310)	0.16 (0.43)	0-2.5	1.3 (5.6)	4.4 (19)
	2.5-3.5	1.12	15 (160)	0.016 (0.18)	0-3.5	1.3 (5.8)	4.4 (19)
	3.5-4.5	1.04	8.3 (25)	0.009 (0.026)	0-4.5	1.3 (5.8)	4.5 (19)
	4.5-5.5	0.91	- (-)	- (-)	0-5.5	1.3 (5.8)	4.5 (19)

Numbers inside brackets indicate total (sum of conjugated and unconjugated) 4-ene VPA.

5-fold increase in the dose. For both the low and the high dose, a total of 47-46% was excreted in the urine and bile collectively (Tables 27-30). The fraction of dose excreted as unconjugated 4-ene VPA in urine and bile was not altered significantly (Table 32).

C.3.4. Choleretic Effect

A typical choleretic effect of the high dose of 4-ene VPA in the rat is shown in Fig. 13. The normal bile flow rate of 0.82 mL/h increased rapidly to 1.4 mL/h within the first 0.5 h of 4-ene VPA injection. The bile flow declined slowly for the next hour and rapidly thereafter to normal values within 4 h. The excretion rate of 4-ene VPA in bile samples plotted against the mid-points of the time intervals for sample collection is also shown in the Fig. 13. The maximal excretion rate of conjugated 4-ene VPA (3.48 mg/h) in bile was observed within the first half-hour of the dose. The excretion rate of conjugated 4-ene VPA in bile then declined almost parallel to the bile flow rate. Approximately 95% of the biliary elimination of 4-ene VPA was complete within 3 h of the dose (Fig. 14). Only low levels of unconjugated 4-ene VPA were found in the bile. A similar choleretic effect was observed after the administration of low dose of 4-ene VPA (Table 29). The duration of maximal bile flow rate after the low dose was, however, shorter than after the high dose.

A similar choleretic effect of (E)-2-ene VPA on the bile flow rate, and its elimination in the bile of the rat was observed (Tables 23, 24) as described above for 4-ene VPA.

C.3.5. *In Vitro* Protein Binding: The plasma protein binding of 4-ene VPA was apparently low (14-25%), at various concentrations ranging from 20 to 350 μg/mL (Table 33).

TABLE 31. COMPARISON OF PHARMACOKINETIC PARAMETERS OF (E)-2-ENE VPA IN THE RAT

Parameter	NORMAL 20 mg/kg[a]	NORMAL 100 mg/kg[a]	p-Value[b]	Parameter	BILE EXTERIORIZED 20 mg/kg[a]	BILE EXTERIORIZED 100 mg/kg[a]	p-Value[b]
k_1[c]	310±60	260±50	NS	k_1[c]	350±55	330±27	NS
$t_{1/2}$[d]	23±4.2	28±5.8	NS	$t_{1/2}$[d]	20±3.4	21±1.7	NS
V_1[e]	240±40	230±20	NS	V_1[e]	220±29	240±17	NS
AUC/D[f]	810±350	1160±74	NS	AUC/D[f]	460±150	590±77	NS
Cl_R[g]	0.27±0.13	0.29±0.11	NS	Cl_R[g]	0.28+0.13	0.20+0.11	NS
Cl_{met}[g]	4.6±1.7	2.8±0.21	NS	Cl_B[g]	0.64±0.28	0.37±0.16	NS
Cl_T[g]	4.9±1.7	3.0±0.27	NS	Cl_{conj}[g]	4.1±1.0	3.5±1.1	NS
	% Dose Excreted in Urine			Cl_T[g]	7.7±1.8	6.0±1.1	NS
Unconj	5.9±3.3	9.4±3.2	NS		% Dose Excreted in Urine		
Conj	26±6.4	40±11	NS	Unconj	3.5±1.6	3.3±1.4	NS
Total	32±6.3	50±11	NS	Conj	24±6.1	32±11	NS
				Total	27±7.6	35±11	NS
					% Dose Excreted in Bile		
				Unconj	8.1+2.8	6.1+2.6	NS
				Conj	29±6.8	25±6.8	NS
				Total	38±9.5	31±9.3	NS

a, Dose; *b*, p≥0.05=Nonsignificant (NS);

c, $x10^{-5}$ min^{-1}; *d*, min; *e*, mL/kg;

f, μg.min/mL.mg; *g* , mL/min.kg

Each value is a mean of 4 observations ± SD

TABLE 32. COMPARISON OF PHARMACOKINETIC PARAMETERS OF 4-ENE VPA IN THE RAT

Parameter	NORMAL 20 mg/kg[a]	NORMAL 100 mg/kg[a]	p-Value[b]	Parameter	BILE EXTERIORIZED 20 mg/kg[a]	BILE EXTERIORIZED 100 mg/kg[a]	p-Value[b]
k_1[c]	580±80	380±34	S	k_1[c]	550±68	380±60	S
$t_{1/2}$[d]	12±1.7	18±1.5	S	$t_{1/2}$[d]	13±1.8	19±3.1	S
V_1[e]	210±37	320±25	S	V_1[e]	210±31	350±35	S
AUC/D[f]	390±27	620±53	S	AUC/D[f]	310±37	460±60	S
Cl_R[g]	0.47±0.21	0.23±0.12	S	Cl_R[g]	0.18±0.14	0.16±0.06	NS
Cl_{met}[g]	8.2±0.51	5.7±0.43	S	Cl_B[g]	0.69±0.24	0.40±0.11	S
Cl_T[g]	8.7±0.60	5.9±0.54	S	Cl_{conj}[g]	4.5±1.1	2.8±0.42	S
	% Dose Excreted in Urine			Cl_T[g]	11±1.8	7.4±1.1	S
Unconj	5.3±2.3	3.8±1.7	NS		% Dose Excreted in Urine		
Conj	17±2.1	24±5.7	S	Unconj	1.5+1.0	2.3+0.94	NS
Total	22±3.1	28±5.7	S	Conj	16±4.0	23±5.1	S
				Total	18±3.9	25±5.8	S
					% Dose Excreted in Bile		
				Unconj	6.2±2.4	5.5±1.6	NS
				Conj	23±6.5	15±2.0	S
				Total	29±5.2	21±3.5	S

a, Dose; *b*, p≥0.05=Nonsignificant (NS),

b, p < 0.05 Significant (S);

c, $x10^{-5}$ min^{-1}; *d*, min; *e*, mL/kg;

f, μg.min/mL.mg; *g* , mL/min.kg

Each value is a mean of 6 observations ± SD

TABLE 33. PLASMA PROTEIN BINDING OF 4-ENE VPA

4-Ene Conc (μg/mL)	Ultrafiltrate Conc (μg/mL)	Bound Conc (μg/mL)	% Bound
20	15 ± 1.7	5.1 ± 1.7	26 ± 8.3
50	38 ± 2.2	12 ± 2.2	24 ± 4.4
100	82 ± 4.9	18 ± 4.9	18 ± 4.9
150	120 ± 1.3	30 ± 1.3	20 ± 0.8
200	170 ± 5.9	31 ± 5.9	16 ± 3.0
250	210 ± 1.5	36 ± 1.5	14 ± 0.6
300	240 ± 8.3	58 ± 8.3	19 ± 2.8
350	300 ± 2.6	53 ± 2.6	16 ± 1.2

Each value is a mean ± SD of 3 observations.

C.3.6. Metabolism of (E)-2-ene in the rat

The presence of several potential metabolites of (E)-2-ene VPA was investigated in the urine and bile of the rat. Only two diene VPA metabolites (I and II) were found in conjugated form in the urine and bile of the rat. Diene VPA I may be either 2(E),4-diene VPA or 2(E),3'(Z)-diene VPA or a mixture of the two, since both isomers have identical retention times. Diene VPA II was tentatively assigned the structure of (E)-2,3'-diene VPA, based on the same retention time as of an authentic synthetic sample. No conclusive evidence was found for the reverse conversion of (E)-2-ene VPA to VPA. Nau and Loscher (1985) have reported that the major metabolites of (E)-2-ene VPA in the plasma of mice are 3-keto VPA, VPA and 5-hydroxy VPA, but no diene VPA metabolites were mentioned. These results may be explained by the difference in the metabolic pathways of (E)-2-ene VPA in different species.

D. DISCUSSION

D.1. CHEMISTRY

The 3-hydroxy VPA, VPA and 4-ene VPA were prepared by the treatment of an alkylcarboxylic acid with strong base, lithium diisopropylamide (LDA), to generate dianions which on alkylation with appropriate alkyl derivative such as alkyl halide or aldehyde gave the alpha-subsituted aliphatic acid in good yield (Pfeffer *et al.*, 1972).

Dehydration of 3-hydroxy VPA gave a mixture of 3-ene VPA and 2-ene VPA as reported by Blaise and Bagard (1907). 3-Ene VPA was separated from 2-ene VPA by double reverse extractions, and vacuum distillation.

2-Ene VPA was obtained by dehydrobromination of 2-bromo-2-propylvaleric acid ethyl ester with quinoline, followed by saponification and acidification (Thallandier *et al.*, 1977).

D.2. ASSAY

The metabolites (E)-2-ene VPA and 4-ene VPA have been quantitated, along with other VPA metabolites, by GC (Loscher, 1981) and GCMS (Nau *et al.*, 1981b; Kochen *et al.*, 1983; Granneman, *et al.*, 1984c) assay methods. The major drawbacks of these methods are poor sensitivity (Loscher, 1981), long and tedious extraction procedures for sample preparation (Nau *et al.*, 1981b; Kochen *et al.*, 1983) and long retention time (Kochen *et al.*, 1983). A capillary GCMS procedure has been used to measure 4-ene VPA as its trimethylsilyl derivative (Rettenmeier *et al.*, 1985). (E)-2-ene VPA has also been determined by GCMS using chemical ionization (Granneman *et al.*, 1984c; Schobben *et al.*, 1980a). Abbott *et al.* (1986) have successfully employed silylation, with t-BDMS derivatizing reagents, for the estimation of VPA and its metabolites in human saliva, serum and urine. The t-BDMS

derivatives are very stable (de Jong *et al.*, 1980), and provide higher sensitivity than TMS derivatives for the estimation of VPA metabolites. In the initial stages of the development of this assay method, t-BDMS chloride was employed to study optimum conditions for derivatization of VPA, since the drug was available in large quantities to carry out experimentation. The reagent was dissolved in dry pyridine at a concentration of 50% w/v. The reagent solution derivatized the analyte very slowly, and took 6 h of heating at 60^{o}C for complete esterification. To increase the speed of this reaction, various concentrations of a strongly basic catalyst, DMAP (dimethylaminopyridine) were added to the reagent solution. At a DMAP concentration of 20%, the reaction rate was enhanced several fold and was complete within 30 min. Chromatographic analysis showed peaks of the reagent which did not interfere with the peak of VPA, and had different retention time than that of either of the metabolites studied in this project. Not long after that, MTBSTFA was introduced to prepare t-BDMS derivatives of alcohols and carboxylic acids (Mawhinney and Madson 1982). With MTBSTFA, derivatization was instantaneous on its addition to a carboxylic acid. Moreover, no extraneous peaks were seen since the reagent peak emerges very early along with the solvent peak under the present GC conditions. This reagent also offers the convenience of being sold in a ready to use form. Addition of a large excess of MTBSTFA (20 μL or greater) gradually decreased the peak areas on repeated injections into GCMS.

The t-BDMS derivatives provide a large $(M-57)^{+}$ mass ion peak on electron impact MS. Thus, selected ion monitoring of m/z 199, corresponding to (E)-2-ene VPA and 4-ene VPA, and 229 for DNBA were carried out to estimate the amount of the analyte. Monitoring a higher mass offers the advantage of reducing the chances of interference by endogenous and extraneous substances.

Conjugates of (E)-2-ene VPA and 4-ene VPA, in urine and bile, were hydrolyzed at pH > 12 by heating with 3N NaOH to ensure complete hydrolysis. Dickinson *et al.* (1984) have reported that glucuronide ester conjugates of VPA undergo rearrangement to resistant forms, which do not hydrolyze on incubation with the enzyme, β-glucuronidase. In alkaline medium, however, various kinds of conjugate esters of VPA, including glucuronide and sulfate esters, are hydrolyzed (Dickinson *et al.*, 1984; Dickinson *et al.*, 1979a).

The salient features of this assay method are that it is an extremely simple procedure that involves a single extraction of the biological sample, unlike the long procedure of Nau *et al.* (1981). The extraction efficiency is virtually 100%. There is no concentration step involved. Using an 80 μL plasma sample, concentrations as low as 60 and 100 ng/mL of (E)-2-ene VPA and 4-ene VPA, respectively, were detected with a signal-to-noise ratio of 4. The assay method provide higher sensitivity than the GC method of Loscher (1981). Calibration curves are linear over a wide range of concentrations in the plasma, urine and bile of the rat. The assay method is able to separate the diene VPA metabolites of (E)-2-ene VPA and 4-ene VPA without any interference. The assay method is sensitive, specific and requires only 12 min for elution of the metabolites and the internal standard after an injection into the GCMS (Singh *et al.*, 1987).

D.3. PHARMACOKINETICS

The pharmacokinetics of (E)-2-ene VPA and 4-ene VPA in the rat were similar to those reported for VPA (Dickinson *et al.*, 1979a). Both (E)-2-ene VPA and 4-ene VPA were, initially, rapidly cleared from the plasma within the first 1-2 h after the dose, followed by recirculation into plasma due to enterohepatic circulation. Thus, the plasma levels were maintained for

several hours after a single injection. Like the parent drug VPA, (E)-2-ene VPA and 4-ene VPA were mainly eliminated as conjugates in urine and bile.

D.3.1. Pharmacokinetics in Normal Rats

D.3.1.1. Plasma Profile: Both (E)-2-ene VPA and 4-ene VPA showed a non-linear plasma decline at concentrations above 200 μg/mL, whereas VPA exhibited similar non-linear decline at plasma levels > 100 μg/mL (Dickinson *et al.*, 1979a). The much higher concentration of (E)-2-ene VPA and 4-ene VPA in the collected plasma samples compared to the highest plasma standard in the calibration curve may contribute to the non-linear plasma profile of these metabolites. However, this non-linear behavior was observed in every animal and was, therefore, assumed to be due to the saturation of one or more of the metabolic pathways and/or an excretory process(s) at the above concentrations. The plasma $t_{1/2}$ of (E)-2-ene VPA was 23 min during the first hour following the 20 mg/kg dose and it increased by 1.2 times to 28 min following the 100 mg/kg dose. The plasma profile of 4-ene VPA also showed a similar increase of 1.5 fold in the $t_{1/2}$, from 12 to 18 min, during the first h following the two doses. These results are similar to those reported for VPA, which showed a 3.6-fold longer $t_{1/2}$, from 12 to 41 min, when the dose was increased from 15 to 150 mg/kg (Dickinson *et al.*, 1979a). There was a time lag of at least 1 h, before plasma levels of (E)-2-ene VPA, 4-ene VPA and the parent drug VPA (Ogiso *et al.*, 1986; Dickinson *et al.*, 1979a) started to rise due to EHC in the rat. After the appearance of a secondary peak at 3-4 h, the metabolites were slowly eliminated from the plasma. This terminal, slow elimination of (E)-2-ene VPA and 4-ene VPA from plasma cannot be called a β-phase, as has been pointed out by Dickinson *et al.* (1979a), since reabsorption of the xenobiotic would still be occuring from the GIT of the rat. Experiments in

bile-exteriorized rats in the present study have confirmed that if (E)-2-ene VPA or 4-ene VPA is prevented from entering the GIT of the rat via the bile, secondary plasma peaks and the subsequent slow elimination phase are abolished.

Several drugs including acetaminophen (Watari *et al.*, 1983), VPA (Dickinson *et al.*, 1979a) and xenobiotics such as 4-ene VPA (Rettenmeier *et al.*, 1986) are extensively conjugated with glucuronic acid before elimination. Glucuronide conjugates are secreted into bile which flows into the intestine *via* the duodenum. Inside the lumen of the GIT of the rat, as well as other animals, glucuronic acid esters are hydrolyzed by β-glucuronidase, an enzyme produced mainly by resident microorganisms (Clark *et al.*, 1969; Hill and Drasar 1975). The unconjugated moiety (drug or xenobiotic) is then reabsorbed through the intestinal wall into the systemic circulation (Klaassen and Watkins 1984). The β-glucuronidase enzyme activity is negligible in the duodenum and ileum, and is maximal in the cecum of the rat (Marsh *et al.*, 1952). The lack of glucuronidase activity in the proximal part of the intestine explains, in part, the time lag associated with the appearance of a secondary peak of (E)-2-ene VPA and 4-ene VPA in the plasma of the rat. Moreover, the processes of deconjugation and absorption of unconjugated molecules through the GI wall may also add to the delay in the circulation. Ogiso and coworkers (1986) have also shown that deconjugation of VPA is the rate limiting step during its enterohepatic circulation in the rat.

D.3.1.2. Pharmacokinetic Modelling: In the early stages of the pharmacokinetic modelling for drugs undergoing EHC, simple two compartment models were proposed to describe their plasma profiles (Harrison and Gibaldi 1976; Chen and Gross 1979), especially in the rat

which does not have a gall bladder and therefore secretes bile more or less continously into the GIT. However, such models are not suitable for the plasma data of (E)-2-ene VPA, 4-ene VPA or VPA in the rat due to the delay in the transfer of the molecules from bile to blood as described above. To solve this problem, Dahlstrom and Paalzow (1978) successfully applied a multi-segment-loop intestine model to describe the EHC of morphine in the rat. The drug, in bile, enters into 3-5 serially connected "intestinal compartments" before entering the blood compartment. Such a model essentially adds to the time required for the recirculating drug to reach the systemic circulation. A second approach was taken by other investigators including Veng Pedersen and Miller (1980), Steimer *et al.* (1982), Colburn (1984), and more recently by Shepard *et al.* (1985). According to this modelling technique, a continuous time-lag function is introduced in the solution to the differential equations. These models provide the freedom to alter the time-lag (tau), and also to study the effect of such a change on the overall disposition of a drug undergoing EHC. In addition, more than one time-lag function can be chosen to describe repetitive recycling (Colburn, 1984). The plasma data, after the low dose administration of (E)-2-ene VPA and 4-ene VPA to the rat, were fitted to a relatively simple version of the model proposed by Veng Pedersen and Miller (1980) and Colburn (1984). The differential equations were solved by MULTI(RUNGE), a non-linear least squares regression program (Yamaoka and Nakagawa 1983). The time-lag value was chosen to provide a best fit of the model-predicted curve to actual plasma data points, as measured by the least sum of residual squares. The calculated values of microconstants k_{10}, k_{12} and k_{21} were used to determine the effective clearance (Cl_{eff}) and net clearance (Cl_{net}) as reported by Colburn (1984). Cl_{eff} may be calculated as $V_1.(k_{10} + k_{12})$ to describe the intrinsic ability of liver to remove (E)-2-

ene VPA or 4-ene VPA from the blood. Cl_{net} is obtained by $V_1.k_{10}$ to estimate the permanent elimination of the metabolite from the body of the rat. The calculated values of Cl_{eff} for (E)-2-ene VPA was 6.9 and for 4-ene VPA, 11 mL/min.kg. The Cl_{eff} values, calculated from the plasma data of normal rats, are close to Cl_T in bile-exteriorized rats, which suggests that the model-generated values for microconstants are fairly accurate. This pharmacokinetic model, however, does not explain all the plasma data points, especially at the trough levels approximately 2 h after the dose. Therefore, its applicability is partially limited.

D.3.1.3. Pharmacokinetic Parameters: The pharmacokinetic parameters of (E)-2-ene VPA in the rat determined in this study are different from those reported by O'Connor *et al.* (1986) in several respects. The total plasma clearance of (E)-2-ene VPA decreased from 4.9 to 3.0 mL/min.kg on increasing the dose from 20 to 100 mg/kg. In contrast, O'Connor *et al.* (1986) have reported that the serum clearance of (E)-2-ene VPA increased from 4.0 to 6.1 mL/min.kg when the dose was raised from 25 to 75 mg/kg in the rat. The value of Cl_T at the low doses are close to each other (4.9 *versus* 4.0 mL/min.kg). However, the effect of increasing the dose is opposite in these two studies. This discrepency is not easy to explain due to the lack of information in their abstract on their experimental design, the schedule for blood sampling and the duration of blood collection. It may be speculated that the difference in the strains of the rat used could produce different results. Secondly, a short duration of blood collection, up to 2 h or less, could miss the recirculation of (E)-2-ene VPA in the blood, and erroneously give a smaller value of AUC and a larger Cl_T. On the other hand, if the blood sampling is infrequent, a

recycled drug may appear to exhibit a two-compartment model profile (Colburn 1984). As an example, in a pharmacokinetic study of VPA in the rat in which blood samples were collected infrequently, the plasma data were fitted to a two compartment model by Loscher (1978). The pharmacokinetic experiments in which blood samples were withdrawn frequently (Dickinson *et al.*, 1979; Ogiso *et al.*, 1986) have shown that VPA in fact undergoes substantial EHC in the rat. Therefore, the elimination half-life (4.6 h) and $V_d(\beta)$, 657 mL/kg for VPA as calculated by Loscher (1978) are different from the pharmacokinetic parameters such as V_1 (143 mL/kg) of Ogiso *et al.* (1986). Similarly O'Connor *et al.* (1986) may have used a different experimental protocol or different modelling techniques from ours to arrive at their results.

A comparison of the total apparent plasma clearance of (E)-2-ene VPA in different animals species shows that the Cl_T in the rat (4.9 mL/min.kg), following the low dose, is similar to that in the mouse (5.7 mL/min.kg) (Nau and Zierer 1982).

4-ene VPA is cleared much faster from the plasma of the rat than in the monkey (Rettenmeier *et al.*, 1986). Cl_T in the rat at the low dose of 4-ene VPA (8.7 mL/min.kg) was on an average 3.7 times faster than that in the monkey, probably due to the higher metabolic rate in smaller animals. The urinary elimination of 4-ene VPA in the unconjugated form was 5% in the rat, which is identical to that reported for the monkey (Rettenmeier *et al.*, 1986). The rat, however, excreted less than half as much 4-ene VPA in conjugated form in the urine as does the monkey.

D.3.2. Pharmacokinetics in Bile-Exteriorized Rats

D.3.2.1. Plasma Profile: In bile-exteriorized rats, (E)-2-

ene VPA and 4-ene VPA were eliminated with respective plasma $t_{1/2}$ of 20 and 13 min at the low dose, 21 and and 19 min at the high dose. The elimination half-life remained unaltered for (E)-2-ene VPA and increased 1.5 times for 4-ene VPA with a five fold increase in their respective doses. A similar increase of 1.5 times, from 11 to 17 min, was reported for VPA plasma half-life in bile-exteriorized rats given doses of 15 and 150 mg/kg (Dickinson *et al.*, 1979a). No secondary plasma peaks were observed in these rats.

D.3.2.2. Biliary Elimination: The biliary elimination of (E)-2-ene VPA decreased from 38 to 31% of the administered dose, and 29 to 21% for 4-ene VPA, with a 5 times increase in the dose. The urinary excretion, however, increased from 27 to 35% of (E)-2-ene VPA dose, and 18 to 25% of 4-ene VPA dose with a five fold increase in their respective doses. These results are similar to those of VPA which showed an enhanced urinary elimination from 4 to 15%, when the dose was raised 10 times (Dickinson *et al.*, 1979a). These observations suggest that as the biliary excretion of VPA or one of its monounsaturated metabolites approaches saturation at the high dose, urinary elimination is enhanced as a complementary excretory pathway.

The marked differences in the extent of biliary excretion of these chemically similar compounds of almost identical molecular weights, may be due to the subtle structural changes introduced by the presence and position of a double bond in the molecule. A molecular weight threshold of approximately 200 Daltons for quaternary ammonium compounds and 300-325 Daltons for aromatic anions is essential for the elimination of a molecule in the bile of the rat (Welling 1986; Hirom *et al.*, 1972b). However, a large molecule or size is not the sole factor in determining the extent of elimination in the bile. Molecules of similar size, but of slightly

different structures, have been reported to be eliminated to significantly different extents in the bile of the rat (Hirom *et al.*, 1972a).

D.3.2.3. Conjugation: The conjugation of (E)-2-ene VPA and 4-ene VPA seems to be the major route of metabolism and subsequent elimination in the rat. For the administered low dose (20 mg/kg), 29% of (E)-2-ene VPA and 23% of 4-ene VPA were eliminated as conjugates in the bile. For the high dose (100 mg/kg), 25% of (E)-2-ene VPA and 15% of 4-ene VPA were eliminated as conjugates in the bile. In contrast, a much larger percentage (58-61%) of VPA doses (15 and 150 mg/kg) was recovered as conjugates in the bile of the rat (Dickinson *et al.*, 1979a). Approximately 53% of the low dose and 57% of the high dose of (E)-2-ene VPA was recovered in conjugated form collectively in the bile and urine. The corresponding values for 4-ene VPA were 39% and 38% , and for VPA, 62% and 76% in the rat (Dickinson *et al.*, 1979a). Thus, conjugation, which is the most prominent route of elimination in the rat decreases in the order: VPA > (E)-2-ene VPA > 4-ene VPA. The difference in the extent of conjugation may be attributed to one of the following factors: The organic anions, including fatty acids (Renaud *et al.*, 1978), are transported into hepatocytes by carrier-mediated active transport mechanisms. A reduced transport uptake of the monounsaturated metabolites compared to VPA may be responsible for this difference in the extent of conjugation amongst these compounds. This hypothesis is also supported by the experiments carried out by other investigators. Rettenmeier *et al.* (1985) have reported that in isolated rat liver perfusion studies, approximately 4 times larger amounts of conjugated 4-ene VPA were recovered in the bile when the length of the perfusion time was increased from 20 to 60 min. These results suggest that 4-ene VPA is transferred to hepatocytes in a time-dependent fashion, probably mediated

by a transport system. Similarly, Nau and Loscher (1985) have reported that (E)-2-ene VPA appears to enter the liver by an active transport mechanism in the mouse.

The second factor that may be partly responsible for varying extents of conjugation is the nature of the enzyme, especially substrate affinity. The enzyme UDP-glucuronyltransferase (UDPGT) exists in two separate substrate-specific and inducer-selective forms (Watkins and Klaassen 1982; Dutton and Burchell 1977). A higher affinity of UDPGT enzyme for VPA than (E)-2-ene VPA, which in turn is greater than the affinity for 4-ene VPA, may contribute to this variance in the degree of conjugation of these compounds.

An interesting observation is that, with an increase in the dose of VPA (Dickinson *et al.*, 1979a) and (E)-2-ene VPA, the fraction of the dose excreted as conjugates in the urine and bile, collectively, also increased in bile-exteriorized rats. This increase in the conjugation of VPA (62% *versus* 79% of the dose) is significant (Dickinson *et al.*, 1979a) and only slight (53% *versus* 57% of the dose) for (E)-2-ene VPA. Practically no change was observed in the fraction of 4-ene VPA dose (39% *versus* 38%) eliminated as conjugates between the low and high dose. These results indicate that metabolic pathways other than conjugation and/or excretory routes for the elimination of VPA and (E)-2-ene VPA may be approaching saturation at the high dose. Thus, a larger fraction of the high dose may be available for conjugation.

D.3.2.4. Choleretic Effect: The choleretic effect of (E)-2-ene VPA and 4-ene VPA may be partly due to increased osmotic pressure in the bile canaliculi, created by large quantities of conjugated moieties. This hypothesis is supported by the observation that the bile flow rate was

directly proportional to the excretion rate of 4-ene VPA conjugates in the bile (Fig 13). Moreover, the duration of maximal flow rate was dose-dependent, being shorter (0.5 h) after the low dose and longer (>2 h) after the high dose of either of the metabolites. These results are similar to those observed for VPA which has been shown to induce choleresis due primarily to osmotic activity in the bile (Dickinson *et al.*, 1982; Watkins and Klaassen 1981). The bile flow rate increased by approximately 19 μL/μmole of 4-ene VPA excreted in bile. Since this value was greater than the predicted increase in the bile flow (7 μL/μmole) due to osmotic pressure alone (Watkins and Klaassen 1981), either fluid absorption is inhibited in the ductular tract or an electrolyte transport mechanism is stimulated by unknown mechanism. In addition, these monounsaturated metabolites of VPA may have a direct effect on a hormone such as secretin, which regulates canalicular secretion of bile. Moreover, (E)-2-ene VPA and 4-ene VPA are partially metabolized into dienes, which are largely excreted as conjugates in the bile. The presence in the bile of dienes and other possible metabolites of 4-ene VPA (Rettenmeier *et al.*, 1985) and (E)-2-ene VPA may also contribute to the total choleretic effect seen after the administration of monounsaturated metabolites.

After an initial choleresis produced by (E)-2-ene VPA or 4-ene VPA, the bile flow rate decreased steadily over the period of study. The flow rate invariably decreased to values slightly smaller than those observed before the administration of the metabolite. A similar observation was reported for VPA-induced choleresis in the rat (Dickinson *et al.*, 1982). Depletion of the bile acid pool may be responsible for reduction in the bile formation, and the consequent reduced bile flow rate in the rat.

D.3.2.5. Pharmacokinetic Parameters: After the low dose, the

plasma elimination of both the metabolites follows an open one-compartment model: thus, the apparent volume of distribution of the central compartment (V_1) is the same as the volume of distribution (V_d). The values of V_d for (E)-2-ene VPA and 4-ene VPA were, respectively, ~220 and 210 mL/kg, which are almost half the V_d of 430 mL/kg for VPA in the rat receiving a dose of 15 mg/kg (Dickinson *et al.*, 1979a). The smaller volume of distribution of (E)-2-ene VPA may be due to higher plasma protein binding than VPA in the rat (Loscher and Nau 1983). A simple calculation of blood volume of ~15 mL and a total body water volume of ~200 mL (Altman and Dittmer) in a 300 g rat suggests that both the metabolites may not be restricted to the body fluids, but they appear to penetrate and perhaps bind to some degree to the tissues of the rat.

The AUC of (E)-2-ene VPA or 4-ene VPA, due to the EHC phase alone, may be estimated by subtracting the average $AUC_{bile\text{-}exteriorized}$ from the AUC_{normal}. The contribution of EHC to the total AUC of (E)-2-ene VPA was 40% at the low dose and 49% at the high dose. The corresponding values for 4-ene VPA were 23% and 24%. These results suggest that the extent of recirculation for (E)-2-ene VPA was twice as much as that of 4-ene VPA, an observation which agrees with the greater biliary elimination of (E)-2-ene VPA than 4-ene VPA, at the low dose, in the rat.

D.3.3. *In Vitro* Protein Binding: 4-ene VPA is bound *in vitro* to a very small extent (14-25%) to the plasma proteins of the rat. Rettenmeier *et al.* (1986) have reported that 4-ene VPA binding is 58-78% in the monkey. These results suggest that the binding of 4-ene VPA decreases with the size of the animal. A similar observation was made with VPA, which is highly bound (90%) to the plasma proteins of the man and monkey (Levy *et al.*, 1977), but is only 63% bound in the rat (Loscher 1978).

The plasma protein binding of VPA has been studied by equilibrium dialysis, ultrafiltration and ultracentrifugation (Barre *et al.*, 1985). The results obtained with ultrafiltration were similar to those obtained with equilibrium dialysis (Barre *et al.*, 1985). Thus, it is assumed that the use of ultrafiltration would yield reasonably accurate results with 4-ene VPA.

The plasma levels of a drug are often correlated to its pharmacologic response in man and animals. Occasionally, the correlation is poor due to several factors such as the presence of an active metabolite, tolerance to drug, delayed response because of the time required for equilibration of drug in plasma to that at the site of action, and time delays due to indirect pharmacologic activity. The poor correlation in the plasma level of VPA and its anticonvulsant activity has been speculated to be due to the formation of an active metabolite, (E)-2-ene VPA. The present results obtained in the rat show that (E)-2-ene VPA is cleared more slowly than VPA (Loscher 1978) from the body of the animal (3.0 *versus* 4.2 mL/min.kg). These results suggest that (E)-2-ene VPA may be responsible for the carry-over effect seen after withdrawl of VPA. Moreover, the plasma, urine and biliary profiles of (E)-2-ene VPA in the rat indicate that the metabolite exhibits dose-dependent pharmacokinetics, probably due to saturation of its metabolism at the high dose. Only 10% or less of the administered dose is recovered unchanged in the urine. Thus, 90% of the metabolite is expected to be metabolized. It is possible that (E)-2-ene VPA may be further metabolized to an active (or toxic) metabolite. The presence of (E)-2,3 diene VPA, an active diene metabolite of (E)-2-ene VPA, in the plasma of the rat further substantiates the contribution of (E)-2-ene VPA to the pharmacologic activity of the parent drug VPA.

In addition, (E)-2-ene VPA undergoes extensive enterohepatic circulation in the rat, which delays its elimination from the body of the animal. Recirculated (E)-2-ene VPA is redistributed to the site of action, the brain, which prolongs its pharmacologic activity in the animal. This was apparent during experimentation where rats were sedated within 5-10 min of the dose and remained sedated for 10 h after the high dose. The duration and intensity of sedation was less at the low dose.

Paradoxical as it may be, the plasma levels of (E)-2-ene VPA may not be easily correlated with pharmacologic activity due to the same factors that were applied to VPA above. While this work was in progress, Loscher and Nau (1983) have shown that on repeated administration, (E)-2-ene VPA is gradually accumulated at the site of action, the brain of the rat. Moreover, (E)-2-ene VPA is eliminated much more slowly than VPA from the brain of animals. Therefore, continued pharmacologic activity of (E)-2-ene VPA should be expected even after its blood levels have declined below analytical limits.

It is suggested that to evaluate the pharmacologic activity of VPA, it is imperative to monitor the parent drug, and its active metabolites, especially (E)-2-ene VPA and (E)-2,3-diene VPA. Unfortunately, the site of action, the brain in this case, is not accessible for the measurement of a drug/metabolite; instead blood and urine samples are the only biological fluids that can be collected without causing bodily harm. Thus, when the plasma levels of (E)-2-ene VPA have declined below the limits of the assay method, urinary recovery of the metabolites may indicate whether or not the metabolite is still present in the body.

The delayed hepatotoxic reaction of VPA has been speculated to be caused by another metabolite, 4-ene VPA. The delay in the onset of toxicity symptoms may be due to slow elimination of the toxicant. The present work, however, suggests that 4-ene VPA is cleared 1.4 times as fast as VPA (Loscher 1978) from the plasma of the rat (5.9 *versus* 4.2 mL/min.kg). The plasma elimination of 4-ene VPA is dose-dependent in the rat. The plasma levels required to show dose dependency (>200 μg/mL) are two orders of magnitude higher than 4-ene VPA levels (<1ug/mL) seen in patients on VPA therapy. It is, therefore, unlikely that 4-ene VPA is eliminated more

slowly than VPA in man. Like its parent drug, 4-ene VPA produces a choleretic effect in the rat, and undergoes enterohepatic circulation. The role of these effects in the hepatic damage suspected of 4-ene VPA is uncertain.

Of the administered dose, only 5% is excreted unchanged in the urine and the rest of it is probably metabolized. At the most 47-46% of the low and the high dose was recovered as a total of conjugated and unconjugated 4-ene VPA in the urine and bile, collectively. Thus, more than 50% of the dose was either metabolized to other compounds and/or excreted in the feces or stored in the liver (Rettenmeier *et al.*, 1985). Our animal studies do not rule out the possibility of irreversible binding of 4-ene VPA or one of its metabolic toxic species to the liver.

SUMMARY AND CONCLUSIONS

1. A simple, sensitive and selective capillary GCMS assay method was developed that could detect concentrations as low as 60 ng/mL of (E)-2-ene VPA and 100 ng/mL of 4-ene VPA in the biological fluids of the rat.
2. (E)-2-ene and 4-ene VPA apparently follow linear pharmacokinetics at the low dose of 20 mg/kg. Non-linear plasma decline was observed at plasma levels greater than ~200 μg/mL, after the high dose of 100 mg/kg.
3. The plasma protein binding of 4-ene VPA was apparently low (14-25%) in the concentration range of 20-350 μg/mL, indicating that binding may not be the cause of dose-dependent elimination of 4-ene VPA in the rat.
4. For the first time, enterohepatic circulation of (E)-2-ene VPA and 4-ene VPA has been documented in the rat. Secondary plasma peaks observed in normal rats following an IV bolus dose of either metabolite were abolished in bile-exteriorized rats, confirming the existence of EHC.
5. Following the low dose of (E)-2-ene and 4-ene VPA, the plasma profile including the appearance of secondary plasma peaks can be described by a time-lag pharmacokinetic model.
6. Conjugation of (E)-2-ene and 4-ene VPA, followed by excretion in the urine and bile was the major mode of elimination for both the metabolites in the rat.
7. Less than 10% of the administered dose of (E)-2-ene or 4-ene VPA was excreted unchanged in the urine.

8. A marked but transient choleretic effect was observed within the first hour of the administration of (E)-2-ene or 4-ene VPA in the rat. The choleretic effect was thought to be partly due to an osmotic pressure effect similar to that seen for the parent drug, VPA.
9. (E)-2-ene VPA is further metabolized to diene metabolites, which were detected but not quantitated in the plasma, urine and bile of the rat.

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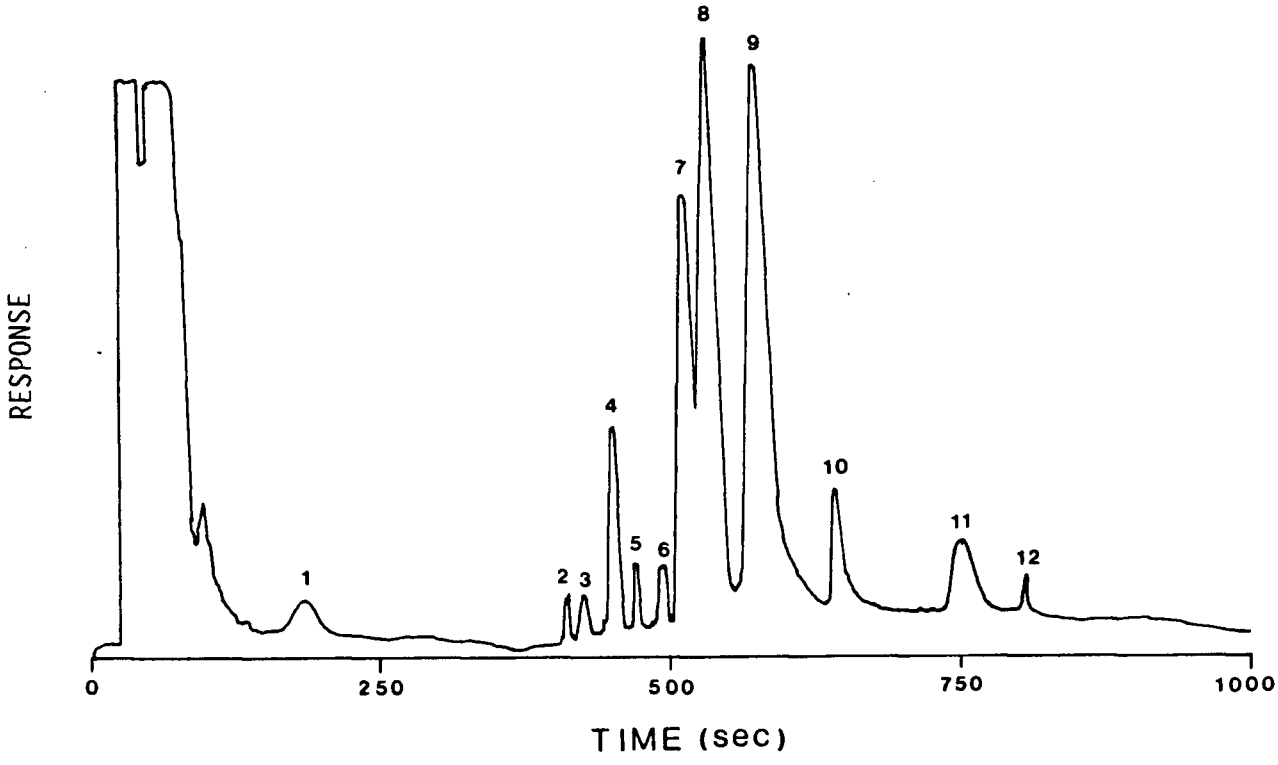
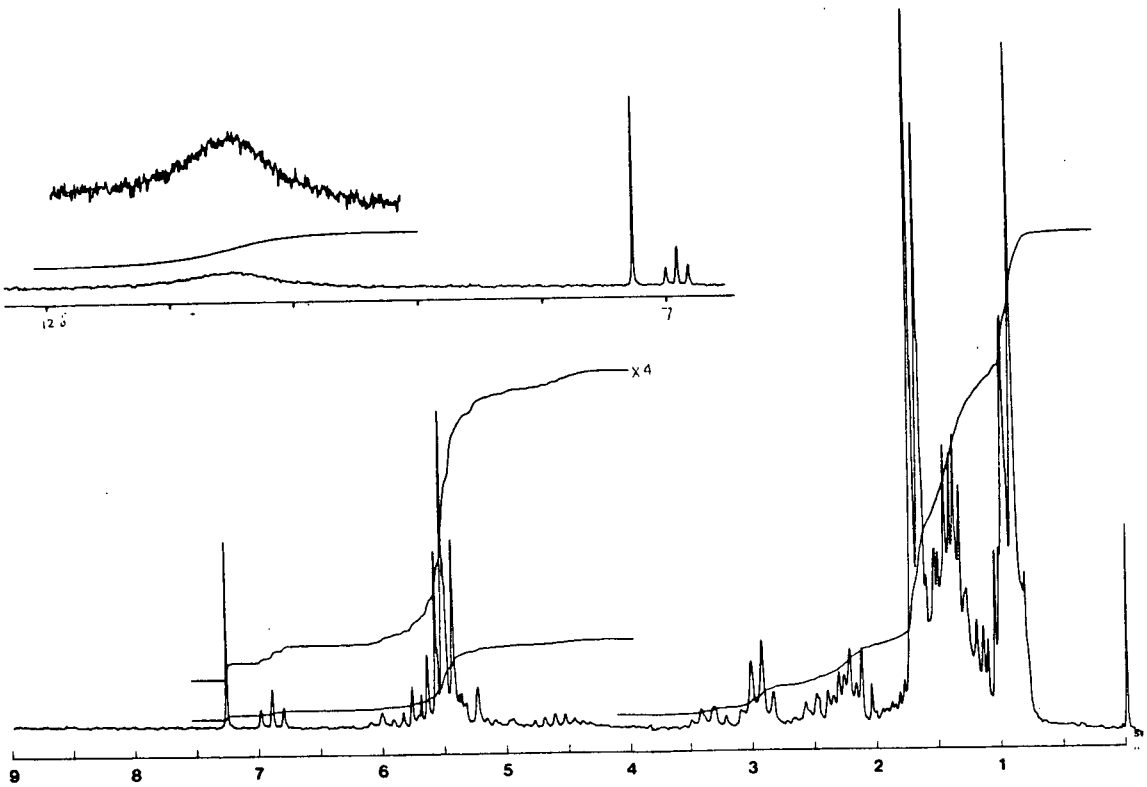
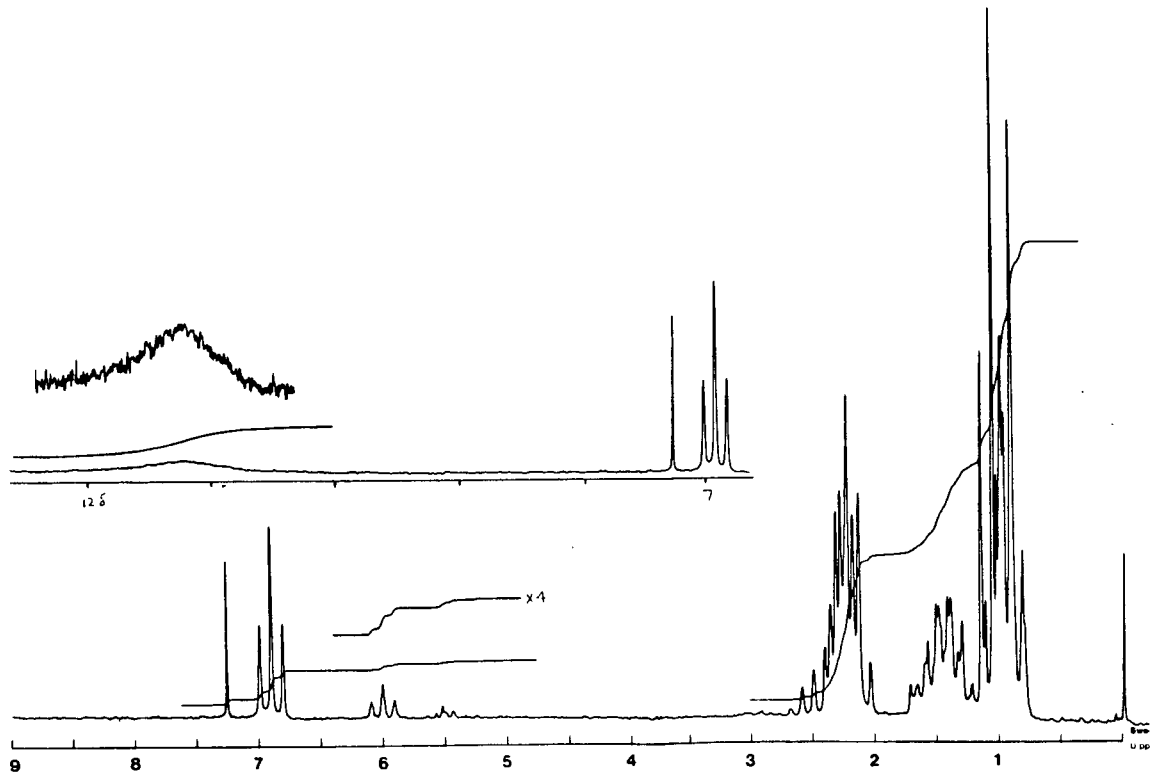
APPENDIX



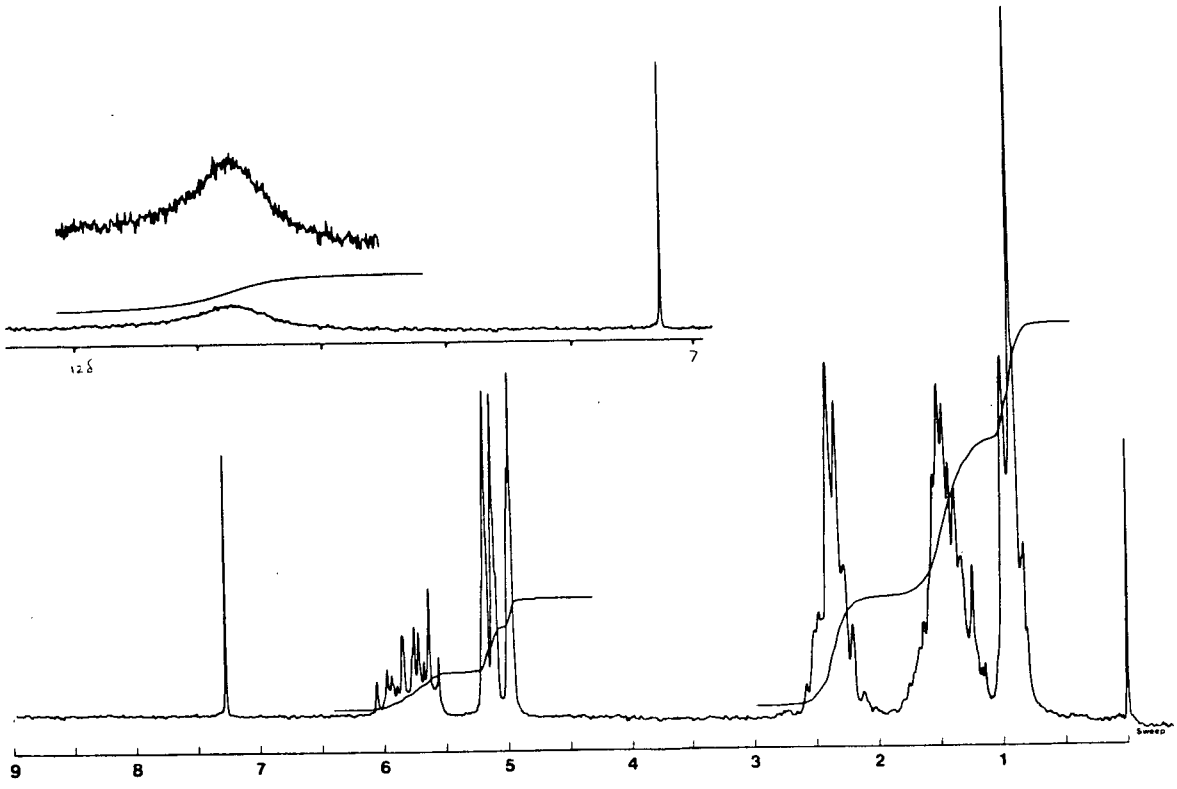
Appendix 1. Total ion chromatogram of ether layer of methanesulfonyl chloride (Scheme 1) reaction. Glass column (2 metre x 2 mm I.D.) was packed with 3% Dexsil 300 on 100/120 Supelcoport. The temperature was programmed to hold for 2 min at 50°C and was then raised to 270°C at a rate of 16°C/min. Peak 3 = 2-ene VPA ethyl ester, peak 9 = 3-OH VPA ethyl ester, peaks 10-12 were condensation products.



Appendix 2. NMR (80 MHz) spectrum of 3-ene VPA in $CDCl_3$.



Appendix 3. NMR (80 MHz) spectrum of 2-ene VPA in $CDCl_3$.



Appendix 4. NMR (80 MHz) spectrum of 4-ene VPA in $CDCl_3$.

APPENDIX 5. PLASMA LEVELS OF (E)-2-ENE VPA IN NORMAL RATS, μg/mL (DOSE = 20 mg/kg)

Time (min)	1a	2a	3a	4a	Mean
0*	90	84	98	66	85
5	79	70	90	na	77
6.5	na	na	na	56	
15	53	46	64	42	51
30	39	26	48	26	35
60	14	7.9	30	11	16
90	7.5	2.9	17	4.9	8.0
120	5.0	2.3	14	1.7	5.7
150	4.9	2.5	20	1.1	7.1
180	3.8	2.7	22	5.0	8.4
240	11	8.0	8.2	5.3	8.2
300	1.6	3.1	6.9	1.0	3.1
360	0.4	1.0	2.9	-	1.4
420	-	0.6	1.2	-	
480	-	-	0.5	-	

*, extrapolated to time zero; na, plasma sample not available.

APPENDIX 6. PLASMA LEVELS OF (E)-2-ENE VPA IN NORMAL RATS, μg/mL (DOSE = 100 mg/kg)

Time (min)	1b	2b	3b	4b	Mean
0*	410	460	480	390	430
5	370	390	390	360	380
15	na	280	260	320	280
30	na	260	230	240	240
39	180	na	na	na	
60	82	120	120	120	110
120	13	25	39	25	26
180	14	na	45	43	34
240	33	47	46	56	45
300	39	43	38	33	38
360	28	na	42	21	30
435	15	20	30	40	26
510	20	14	23	15	18
585	17	6.1	16	16	14
675	12	0.7	2.8	4.0	4.8
765	6.8	-	1.5	2.1	3.5

*, extrapolated to time zero; na, plasma sample not available.

APPENDIX 7. PLASMA LEVELS OF 4-ENE VPA IN NORMAL RATS, μg/mL (DOSE = 20 mg/kg)

Time (min)	1c	2c	3c	4c	5c	6c	Mean ± SD
0*	94	110	83	75	104	120	98 ± 17
5	64	82	59	66	76	86	72 ± 11
15	43	54	39	37	43	46	44 ± 6.0
30	17	29	15	14	15	14	17 ± 5.7
60	2.8	5.7	2.7	4.5	2.6	2.1	3.4 ± 1.4
90	1.4	na	1.7	4.9	1.4	0.9	2.1 ± 1.6
120	1.3	0.6	1.2	3.7	1.3	1.4	1.6 ± 1.1
150	2.4	1.4	na	2.8	1.7	2.0	2.1 ± 0.6
156	na	na	1.5	na	na	na	
180	2.9	-	2.8	1.9	2.0	2.4	2.4 ± 0.5
240	2.7	-	4.2	0.8	2.5	2.2	2.5 ± 1.2
300	1.4	-	na	0.6	1.5	1.3	1.2 ± 0.4
360	-	-	0.7	-	-	0.9	

*, extrapolated to time zero; na, plasma sample not available.

APPENDIX 8. PLASMA LEVELS OF 4-ENE VPA IN NORMAL RATS, μg/mL
(DOSE = 100 mg/kg)

Time (min)	1d	2d	3d	4d	5d	6d	Mean ± SD
0*	280	320	330	350	310	290	310 ± 24
5	280	na	300	320	290	280	300 ± 16
8	na	290	na	na	na	na	
15	270	na	na	280	250	270	270 ± 12
16	na	270	na	na	na	na	
22	na	na	220	na	na	na	
30	180	190	-	230	200	210	190 ± 24
35	na	na	160	na	na	na	
57	na	na	75	na	na	na	
60	71	47	-	68	96	75	72 ± 16
120	7.1	5.1	5.2	11	7.8	5.4	6.9 ± 2.3
180	14	15	6.4	14	5.6	13	11 ± 4.3
240	11	8.5	11	11	7.1	9.6	9.6 ± 1.5
300	5.4	4.4	4.5	9.2	18	8.8	9.8 ± 6.3
360	3.9	5.4	4.3	9.3	15	5.8	9.7 ± 6.6
435	4.1	2.8	2.5	5.5	4.2	5.1	5.4 ± 4.1
510	2.1	-	-	1.5	3.2	3.6	2.6 ± 0.9
600	0.9	-	-	0.9	1.0	1.1	1.0 ± 0.1

*, extrapolated to time zero; na, plasma sample not available.

APPENDIX 9. PLASMA LEVELS OF (E)-2-ENE VPA IN BILE-EXTERIORIZED RATS, μg/mL (DOSE = 20 mg/kg)

Time (min)	1c	2c	3c	4c	Mean
0*	101	79	102	81	91
5	na	62	85	62	73
8	76	na	na	na	na
15	na	42	65	48	54
17	54	na	na	na	na
30	29	25	44	31	32
60	10	8.2	21	12	13
90	3.1	2.2	9.2	4.1	4.7
120	0.8	0.6	3.3	1.2	1.5
150	-	-	1.5	0.5	
180	-	-	-	-	-

*, extrapolated to time zero; na, plasma sample not available.

APPENDIX 10. PLASMA LEVELS OF (E)-2-ENE VPA IN BILE-EXTERIORIZED RATS, μg/mL (DOSE = 100 mg/kg)

Time (min)	1d	2d	3d	4d	Mean
0*	440	440	380	440	430
5	370	410	na	390	390
8	na	na	360	na	
15	260	340	340	310	310
30	170	260	na	220	210
39	na	na	210	na	
60	61	108	99	76	86
90	13	48	34	27	31
120	7.5	18	11	8.5	11
150	na	na	5.4	2.4	
180	na	2.9	na	1.5	
187	na	na	1.7	na	
240	-	-	na	-	
262	na	na	-	na	

*, extrapolated to time zero.; na, plasma sample not available.

APPENDIX 11. PLASMA LEVELS OF 4-ENE VPA IN BILE-EXTERIORIZED RATS, μg/mL (DOSE = 20 mg/kg)

Time (min)	1g	2g	3g	4g	5g	6g	Mean ± SD
0*	85	88	103	96	74	103	91 ± 11
5	77	73	70	80	67	80	74 ± 5.5
15	38	36	42	49	33	45	40 ± 5.9
30	13	16	18	28	12	15	17 ± 5.7
60	2.1	3.1	2.4	6.4	2.0	1.1	2.8 ± 1.8
90	0.7	0.6	-	2.3	0.6	0.5	1.0 ± 0.8
120	0.4	-	-	-	-	-	
150	-	-	-	-	-	-	

*, extrapolated to time zero; na, plasma sample not available.

APPENDIX 12. PLASMA LEVELS OF 4-ENE VPA IN BILE-EXTERIORIZED RATS, μg/mL (DOSE = 100 mg/kg)

Time (min)	1h	2h	3h	4h	5h	6h	Mean ± SD
0*	290	320	310	250	270	310	290 ± 28
5	290	290	300	240	260	290	280 ± 25
15	280	240	280	220	230	250	250 ± 24
30	180	215	230	200	160	160	190 ± 29
60	90	97	95	65	59	58	77 ± 19
90	38	37	34	19	13	8.0	25 ± 13
120	9.5	20	19	10	4.9	2.3	11 ± 7.2
150	3.9	6.1	4.3	1.8	0.8	1.1	3.0 + 2.1
180	0.7	na	na	-	-	-	
188		4.2	2.0				

*, extrapolated to time zero; na, plasma sample not available.