METABOLISM AND PHARMACOKINETIC STUDIES OF VALPROIC ACID USING STABLE ISOTOPE TECHNIQUES

by

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ABSTRACT

Valproic acid (VPA) is an anticonvulsant agent widely used in the treatment of several types of epileptic seizures. The drug is unique within its therapeutic class, in terms of its mechanism of action, its chemical structure, as well as its extensive biotransformation into at least 16 different metabolites. The interest in VPA metabolites has been stimulated by the potential of VPA to produce severe hepatotoxicity. Metabolites 4-ene VPA and 2,4-diene VPA are thought to be responsible for the rare but fatal hepatotoxicity associated with VPA. Thus, methodology to study the pharmacokinetics of VPA metabolites is important to an evaluation of the role that metabolites may play during VPA therapy.

Stable isotope techniques and gas chromatography mass spectrometry (GCMS) have been used in several areas of research on VPA. For example the application of a stable isotope labelled analog as a "pulse dose" in antiepileptic drug studies allows the elimination kinetics of the drug to be determined without discontinuing therapy and risking the exacerbation of seizures. In the present study, $[^2H_6]$VPA and $[^{13}C_4]$VPA were evaluated as to their applicability to pharmacokinetic studies of VPA. Pharmacokinetic parameters of VPA, $[^2H_6]$VPA were measured in a healthy human volunteer. Potential isotope effects of $[^2H_6]$VPA and $[^{13}C_4]$VPA were studied based on the urine recovery ratio or AUC ratio of VPA and its metabolites to their isotope labelled analogs. No apparent isotope effect was found in the metabolism of $[^{13}C_4]$VPA, which makes $[^{13}C_4]$VPA qualified for use in a "pulse dose" manner. Upon $[^2H_6]$VPA

administration, a large isotope effect was observed in the metabolic formation of $[^2H_5]$5-OH VPA and $[^2H_3]$2-PGA, and a small isotope effect was apparent for the formation of $[^2H_6]$(E)-2,4-diene VPA. Based on the latter observation it was proposed that the formation of 2,4-diene VPA might occur partly from 3-ene VPA. (E)- and (Z)-3-ene VPA were synthesized to test this proposal.

The use of stable isotope labelled analogs as internal standards can minimize the variance arising from extraction of VPA metabolites due to a slight pH change or incompleteness of derivatization due to time and temperature. In order to obtain optimal analytical results, eight deuterium labelled VPA metabolites were synthesized as internal standards, which included $[^2H_7]$VPA, $[^2H_7]$2-ene VPA, $[^2H_7]$3-keto VPA, $[^2H_7]$3-OH VPA, $[^2H_7]$4-ene VPA, $[^2H_7]$4-OH VPA, $[^2H_7]$4-keto VPA, and $[^2H_7]$5-OH VPA. These internal standards were applied to the analysis of VPA, $[^{13}C_4]$VPA and their metabolites in serum and urine samples collected from two nonpregnant sheep following single dose administration of VPA:$[^{13}C_4]$VPA (50:50). The elimination half-life of VPA in the sheep was estimated to be approximately 2.5-5 hours.

GCMS conditions for both electron ionization (EI) and negative chemical ionization (NCI) were optimized to obtain the best resolution and sensitivity for VPA metabolites. A single temperature program with a run time of 47 min was established for NCI analysis of PFB derivatives of VPA, $[^2H_6]$VPA and their metabolites. Two temperature programs were investigated for the EI analysis of t-BDMS derivatives. One run time of

35 min was used for VPA unsaturated metabolites, while a run time of 20 min was used for the more polar metabolites of VPA.

All the urine and serum samples were analyzed with both EI and NCI techniques. PFB derivatives of VPA metabolites analyzed by the NCI technique gave higher sensitivity and better resolution than t-BDMS derivatives of VPA metabolites analyzed by EI methods. All urine samples were hydrolyzed with glucuronidase and with NaOH solution. No difference was observed between the results obtained with the different hydrolysis methods, which indicated that there was little or no β-glucuronidase-resistant conjugate present in the urine samples of this human volunteer after urine samples were kept at -20 °C for about two months. The conjugated fractions were measured, more than 90% of VPA and its unsaturated metabolites were excreted into urine in the form of their glucuronic conjugates. Metabolites 3-OH, 4-OH and 5-OH VPA were excreted partly as glucuronides, while 3-keto, 4-keto VPA, 2-PSA and 2-PGA were excreted mostly as the free metabolites.

The present investigation reaffirmed the importance of GCMS assay techniques to studies of VPA pharmacokinetics and disposition. Stable isotope labelled internal standards improved the accuracy and precision of VPA metabolite analysis by GCMS. $[^{13}C_4]$VPA was ideal for "pulse dose" VPA studies of pharmacokinetics, in which techniques it was demonstrated that the pharmacokinetic parameters of VPA metabolites will be obtained for the first time in pediatric patients.

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LIST OF ABBREVIATIONS

AUC	Area Under the Curve
BDZ	Benzodiazepines
bp	Boiling Point
n-BuLi	n-Butyllithium
CL	Clearance
cm	Centimeter
CNS	Central Nervous System
CSF	Cerebral Spinal Fluid
d	Doublet
DBU	1,8-Diazabicyclo[5,4,0]undec-7-ene
E	Trans
EI	Electron Impact
EtOH	Ethanol
eV	Electron Volt
GABA	Gamma Aminobutyric Acid
GABA-T	Gamma Aminobutyric Acid Transaminase
GAD	Glutaric Acid Decarboxylase
GCMS	Gas Chromatography Mass Spectrometry
h	Hour
HMPA	Hexamethylphosphoramide
Hz	Hertz
i.d.	Internal Diameter
IDMS	Isotope Dilution Mass Spectrometry
I.S.	Internal Standard
i.v.	Intravenous

J	Coupling Constant in Hertz
K_E	Elimination Rate Constant
kg	Kilogram
L	Litre
LiICA	Lithium n-Isopropylcyclohexylamine
LDA	Lithium Diisopropylamide
m	Multiplet
M	Molarity
M^+	Molecular Ion
MeOH	Methanol
mg	Milligram
2-MGA	2-Methylglutaric Acid
MHz	Megahertz
MIDAS	Michigan Interactive Data analysis System
min	Minute
mL	Milliliter
mmoles	Millimoles
MSD	Mass Spectrometry Detector
MW	Molecular Weight
m/z	Mass/charge
NaOH	Sodium hydroxide
NCI	Negative Chemical Ionization
NMR	Nuclear Magnetic Resonance
PFB	Pentafluorobenzyl
2-PGA	2-Propylglutaric Acid
2-PSA	2-Propylsuccinic Acid
q	Quartet

SIM	Selective Ion Monitoring
SSA-DH	Succinic Semialdehyde Dehydrogenase
t	Triplet
$t_{1/2}$	Half Life
t-BDMS	*tertiary*-Butyldimethylsilyl
TMS	Trimethylsilyl
THF	Tetrahydrofuran
TIC	Total Ion Chromatogram
U	Unit
ug	Microgram
uL	Microlitre
V_d	Volume of Distribution
w	Wide
Z	Cis

DEDICATION

To Xudong and my parents

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1. Introduction

1.1 Overview of VPA

Valproic acid (VPA) is a relatively new antiepileptic compound whose pharmacological properties were discovered in 1963 (Meunier *et al.*, 1963). Since its first clinical use in France in 1964 (Carraz, 1964), valproate or VPA has rapidly established itself worldwide as a major antiepileptic drug against several types of seizures. In 1983, VPA was marketed as a syrup and gelatin capsule under the trade name Depakene^{R}. It was soon recognized as a highly effective first line drug against the primary generalized tonic-clonic, and myoclonic seizures. The drug is unique within its therapeutic class, in terms of both its mechanism of action and its chemical structure, and as a consequence, it has been the focus of much basic and applied research (Gram *et al.*, 1985; Chapman *et al.*, 1982).

In recent years, interest in VPA metabolites has been stimulated by the potential of VPA to produce severe hepatotoxicity (Bohan *et al.*, 1987; Dickinson *et al.*, 1985; Kuhara *et al.*, 1985; Zimmerman *et al.*, 1982). The risk of fatal hepatic dysfunction has been assessed at 1 in 37000 in patients receiving VPA as monotherapy, and as high as 1 in 500 in children younger than 2 years of age who are receiving VPA in combination with other anticonvulsants (Dreifuss *et al.*, 1987).

Although several studies of the pharmacokinetics of VPA and its metabolites in adults have been completed, no pharmacokinetic study of

VPA metabolites has been reported for children, the age group most susceptible to severe hepatotoxicity. Developing methodology that could be applied to a study of the pharmacokinetics of VPA metabolites in pediatric patients is one of the objectives of the present study.

1.2 Mechanism of Action

The mechanism of action of VPA is not clear. There are three proposed mechanisms of action: 1) VPA increases brain γ-aminobutyric acid (GABA) (Feriello *et al.*, 1983); 2) VPA potentiates the postsynaptic response to GABA (Macdonald and Bergey, 1979) and 3) VPA exerts a direct membrane effect (Slater and Johnson, 1978). A brief description for each hypothesis is presented as follows.

1.2.1 GABAergic hypothesis of VPA

VPA is able to antagonize seizures induced by GABA antagonists, bicuculline and picrotoxin (Frey and Loscher, 1976; Worms and Lloyd, 1981) and seizures induced by inhibitors of GABA synthesis, 3-mercaptopropionic acid, isoniazid and allyl-l-glycine (Dren *et al.*, 1979). Research indicated that GABA levels in the whole brain of rodents are elevated within 15-60 min of administration of VPA (Schechter *et al.*, 1978; Perry and Hansen, 1978) and these remain elevated for 3-8 hours (Nau and Loscher, 1982). Godin (1969) reported that VPA inhibits *in vitro* GABA - transaminase (GABA-T), the enzyme in the first step of GABA degradation. Harry *et al.*(1975) also presented their finding that VPA is a more potent inhibitor of *in vitro* succinic semialdehyde dehydrogenase (SSA-DH), the next enzyme in the GABA degradative pathway. Conversely, the activity of regional (Phillips and Fowler, 1982) and whole brain (Loscher, 1981) glutamic acid decarboxylase (GAD), the GABA synthesizing enzyme, is increased after VPA administration. The inhibited activities of GABA degradation enzymes and the increased GAD activity all result in increased GABA levels.

$$NH_2\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}COOH$$

Structure of GABA

In the neuron, GABA is contained in the synaptosomes of nerve terminals as well as in the neuronal metabolic pool of soma and glial cells. However, only the synaptosomal fraction is involved in neurotransmission. Therefore, unless a clear distinction is made between the two pools, it is impossible to establish the effectiveness of increased levels of whole brain GABA in increasing GABA-mediated inhibition (Balazs *et al.*, 1970).

Drugs which increase GABA function and elevate convulsant thresholds act as anticonvulsants. The fact that GABA levels in the brain increase after VPA administration has been the basis for the above hypothesis concerning the mechanism of action of this drug. However, the available evidence is still insufficient to make a final assessment of the validity of this hypothesis.

1.2.2 VPA potentiates the postsynaptic response to GABA

VPA has been shown to potentiate GABA-mediated postsynaptic inhibition *in vitro*, which is similar to the anticonvulsant benzodiazepines (BDZ) and barbiturates (MacDonald, 1986). The potentiation of GABA by VPA was also observed in the rat cortical neurons in the substantia nigra (*Kerwin et al.*, 1980). However, the concentration of VPA initially used to potentiate GABA response was higher than that seen *in vivo* (Harrison and Simmonds, 1982) and when the concentration was reduced to reflect serum levels, the results of potentiation could not be repeated. On the basis of *in vitro* binding studies, Ticku and Davis (1981) suggested that VPA action may be exerted at the picrotoxin binding site of the GABA receptor-chloride ionophore complex in the postsynaptic membrane. However, upon further investigation using a tritiated analogue, no evidence of binding to brain membranes was found (Morre *et al.*, 1984).

1.2.3 VPA action on Neuronal Membrane

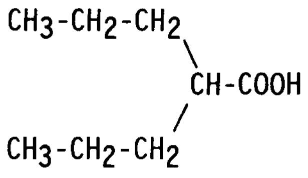
When VPA is at concentrations 15 to 50 times higher than clinical levels, an increase in membrane conductance to K^+ has been observed in the Aplysia neuron, a powerful hyperpolarizing mechanism (Slater and Johnson, 1978). Valproate at "therapeutic" cerebral spinal fluid (CSF) levels limits the depolarization-induced sustained repetitive firing to a few action potentials (McClean and MacDonald, 1986) through a blockage of voltage-sensitive Na^+ influx. Similarly, in studying hippocampal slices, Franceschetti *et al* (1986) found that VPA markedly depressed frequency potentiation and paired pulse facilitation. VPA also suppressed spontaneous epileptiform activity and prolonged the after discharge elicited by antidromic stimulation (Franceschetti *et al.*, 1986).

From the discussion above, VPA was shown to possess direct membrane effects at clinically obtainable levels. However, the relationship between these direct membrane effects in these test systems and VPA's anticonvulsant effect, although plausible, remain unknown.

Although there is considerable evidence to support each hypothesis, for the mechanism of action of VPA, none of the hypotheses can satisfactorily explain all of its anticonvulsant activity. It is therefore, probable that VPA acts through more than one mechanism in providing its broad anticonvulsant effects.

1.3 Metabolism

The structure of VPA (1) - a branched chain fatty acid, differs dramatically from the substituted heterocyclic ring structure which is common to other anticonvulsants.



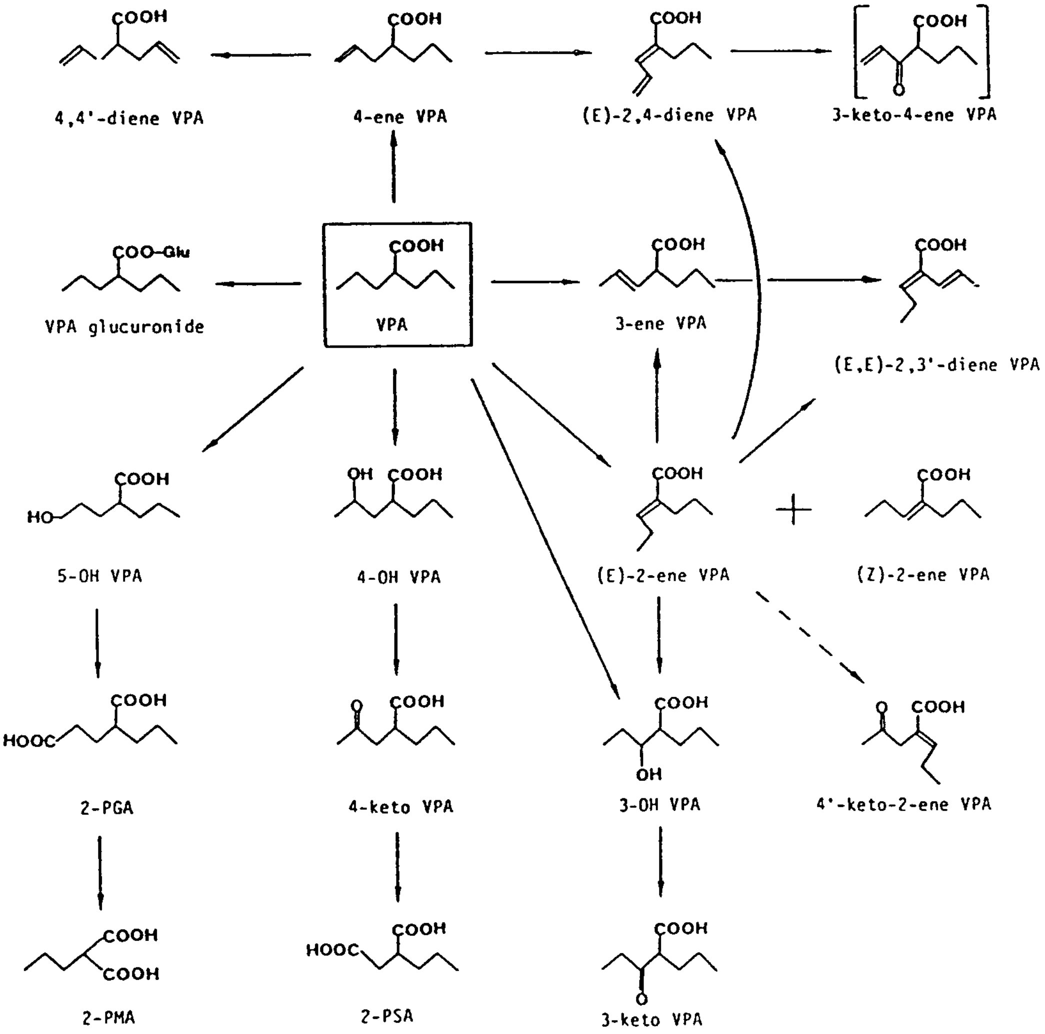
$$(CH_3\text{-}CH_2\text{-}CH_2)_2CH\text{-}COOH$$

(1)

Despite it structural simplicity, the metabolic fate of VPA is complex because of its branched chain structure. This short-chain fatty acid is metabolized in the body by a combination of mitochondrial, microsomal and cytosolic enzymes to produce at least sixteen known metabolites (Gugler *et al.*, 1980; Acheampong *et al.*, 1983, 1985; Kassahun et al., 1989; Rettie *et al.*, 1987). In mammals, the fate of VPA is mainly hepatic metabolism since only 1-3% of the dose is excreted unchanged in the urine (Gugler *et al.*, 1980; Bailer *et al.*, 1985). The major metabolic pathways of VPA include conjugation of VPA with glucuronic acid, β-oxidation, ω-oxidation and (ω-1)-oxidation. A series of unsaturated, hydroxyl and glucuronic acid conjugated metabolites are formed. Figure 1 summarizes the metabolic pathways of VPA in human (Kassahun *et al.*, 1989).

1.3.1 Conjugated metabolites of VPA

Direct conjugation of valproate with glucuronic acid is quantitatively the most important route of valproate biotransformation. Metabolism of valproate is dose-dependent, and, at least in humans and rats, glucuronidation accounts for progressively more of a dose as the dose or blood concentration increases (Dickinson *et al.*, 1979; Granneman *et al.*, 1984). In humans, glucuronidation of VPA varies between 20-70% of recovered dose (Abbott *et al.*, 1986; Chapman *et al.*, 1982). Dickinson *et al.* (1989) reported VPA glucuronide conjugation accounts for 59.3 ± 25.6 % of VPA dose, based on a study in 24 epileptic patients



4,4'-diene VPA
4-ene VPA
(E)-2,4-diene VPA
3-keto-4-ene VPA
VPA glucuronide
VPA
3-ene VPA
(E,E)-2,3'-diene VPA
5-OH VPA
4-OH VPA
(E)-2-ene VPA
(Z)-2-ene VPA
2-PGA
4-keto VPA
3-OH VPA
4'-keto-2-ene VPA
2-PMA
2-PSA
3-keto VPA

Fig. 1: Proposed metabolic pathways of VPA in humans. The broken line indicates a likely metabolic route not yet confirmed. The compounds in brackets have also not been confirmed (Kassahun *et al.*, 1989).

under steady state conditions. The corresponding conjugate, 1-O-acyl-β-linked ester glucuronide (Figure 2), is excreted into urine. This conjugate is also present at high concentrations in the bile of rats given VPA (Dickinson *et al.*, 1979), and is consistent with the finding that VPA undergoes enterohepatic recycling in rats (Dickinson *et al.*, 1985a).

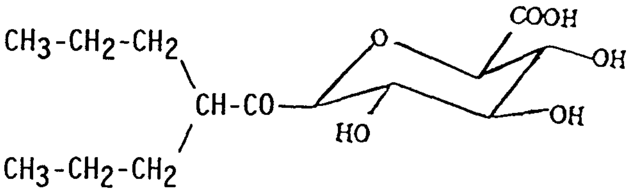


Fig. 2: The structure of 1-O-acyl-β-linked VPA glucuronide.

It should be noted that glucuronidation also represents an important pathway of biotransformation for primary metabolites of VPA that have been formed by initial oxidative processes (Rettenmeier *et al.*, 1985; 1986a; 1986b). In the case of hydroxylated VPA metabolites, both ether and ester glucuronides can result.

Other minor conjugation routes of VPA metabolism exist. Carnitine conjugates (Bohan *et al.*, 1984) were found in the urine of pediatric patients receiving prolonged administration of the drug. VPA glycine conjugates have been identified in rat urine, together with somewhat greater quantities of glycine conjugates of unsaturated VPA metabolites (Granneman *et al.*, 1984). The existence of a VPA conjugate with coenzyme A as a metabolic intermediate in liver tissue was proposed by the evidence from animal studies (Thurston *et al*., 1983; 1985), even though rigorous structural characterization of this conjugate has not been reported. A novel metabolite of VPA, 5-(N-acetylcystein-S-yl)3-ene VPA identified in rat and human urine has been reported by Kassahun *et al* (1991). In their studies, 5-(glutathion-S-yl)-3-ene VPA was detected in rat bile following the administration of either (E)-2,4-diene or 4-ene VPA, and it was assumed that GSH reacted *in vivo* with an activated form of the diene, namely with the CoA ester.

1.3.2 β-Oxidation pathway of VPA administration

The second major route of VPA metabolism is β-oxidation. Metabolites generated *via* this pathway are 2-ene VPA, 3-OH VPA and 3-keto VPA (Granneman *et al*., 1984). It is noteworthy that the structures of these three metabolites are formally analogous to the sequential intermediates of fatty acid β-oxidation (Prickett and Baillie, 1984). The available evidence indicates that VPA and endogenous lipids compete for the enzymes of β-oxidation (Bjorge and Baillie, 1985). In fact, VPA and its metabolites are thought to serve as competitive inhibitors of fatty acid β-oxidation, which causes the rare but fatal hepatotoxicity.

Recently, stable isotope labelling techniques have been employed to investigate the β-oxidation pathway of VPA metabolism in the rat. The findings indicated that have demonstrated that 3-OH VPA is not as was originally suspected an exclusive product of β-oxidation, but has a dual origin *in vivo* being derived largely by direct, cytochrome P450-

dependent hydroxylation of the parent drug (Rettenmeier *et al.*, 1987). This study also confirmed an earlier observation (Nau and Zierer, 1982) that 3-keto-VPA appeared to be formed mainly by oxidation of 2-ene VPA rather than derived from 3-OH VPA.

Recent studies on mitochondrial metabolism of VPA (Li *et al.*, 1991, Bjorge and Baillie, 1991) identified 3-keto VPA together with three unsaturated metabolites, *viz.* (E)-2-ene VPA, 3-ene VPA and (E,E)-2,3′ diene VPA after incubating VPA with freshly isolated rat liver mitochondria. The 3-ene VPA and (E,E)-2,3′-diene VPA were subsequently shown to be metabolites of 2-ene VPA. All three unsaturated metabolites were shown to serve as precursors of 3-keto VPA when incubated with mitochondrial preparations (Bjorge and Baillie, 1991). Metabolite 3-OH VPA was not detected as a metabolite of VPA in this *in vitro* system. However, trace amounts of 3-OH-VPA CoA were detected by HPLC. It was concluded that crotonase catalyzes the hydration of 2-ene-VPA CoA to 3-OH-VPA CoA, and that oxidation of the latter species to the corresponding 3-keto metabolite is mediated by a novel NAD^+-dependent 3-hydroxyacyl-CoA dehydrogenase (Li *et al.*, 1991).

1.3.3 ω-Oxidation and (ω-1) Oxidation

Products of ω-oxidation of VPA are 5-OH VPA, 2-propylglutaric acid (2-PGA) and 2-propylmalonic acid (2-PMA), while (ω-1) oxidation results in 4-OH VPA, 4-keto VPA and 2-propylsuccinic acid (2-PSA) (Granneman *et al.*, 1984).

As in the case of its endogenous counterparts, VPA undergoes hydroxylation at the 4 and 5 position by the action of cytochrome P-450 enzymes (Prickett and Baillie, 1984), mainly in liver tissue but also in other organs. A possible mechanism to account for the formation of 4-OH and 5-OH VPA was proposed. A carbon-centered VPA free radical was formed *via* hydrogen atom abstraction from position 4 or 5 by the perferryl oxygen of the heme prosthetic group, then followed by recombination of carbon radical-perferric hydroxide radical pairs to yield the isomeric alcohols (Rettie *et al.*, 1987). 4-Keto VPA is formed from 4-OH VPA (Granneman *et al.*, 1984), and was first identified as a human metabolite of VPA in a stable isotope "pulse dose" experiment (Acheampong *et al.*, 1983).

2-PGA and 2-PSA are believed to arise from further oxidation of 5-OH and 4-OH VPA, respectively (Granneman *et al.*, 1984).

1.4 Pharmacokinetics and Pharmacodynamics

VPA can be administered by intravenous (I.V.), oral and rectal routes. Among them, the oral route is by far the most widely used. Despite differences in the population (healthy volunteer versus epileptic patients) and formulation (oral solution, immediate release tablet, enteric-coated tablet), the absolute bioavailability of sodium valproate was consistently found to be close to unity (Levy and Shen, 1989). The various forms of oral VPA essentially differ only in the rate of absorption. The peak plasma levels are usually attained within

0.5-2 hours after administration of an oral solution or coated tablet of VPA.

VPA is highly bound (90%) to human plasma albumin at therapeutic concentrations (Levy and Lai, 1982). This property tends to keep most of the drug within the vascular compartment. A value of 0.1-0.4 L/Kg of V_d (volume of distribution) is an indication that the distribution of VPA is limited to the circulation and rapidly exchangeable extracellular water (Gugler and Von Unruh, 1980).

The clearance of VPA is independent of liver blood flow but is highly dependent on the free fraction. It has been recognized that the blood level-dose relationship for VPA is highly variable between patients. VPA is eliminated almost exclusively by hepatic metabolism (>96% of administered dose, Levy and Shen, 1989). The reported plasma (or metabolic) clearance in healthy volunteers is in the range of 6-11 mL/Kg/h (Levy and Shen, 1989). Children younger than five, 5 to 10 and 10 to 15 years of age have been reported to have mean VPA clearances of 48.3, 39.1 and 24.8 mL/Kg/h respectively (Dodson and Tasch, 1981). The clearance of VPA was found to increase (14.4-16.5 mL/Kg/h) in adults on polytherapy, and is thought to be the result of hepatic enzyme induction (Schappel *et al.*, 1980; Hoffman *et al.*, 1981).

The elimination half-life of VPA in plasma ranges from 8-16 hours in adult epileptics (Gugler and Von Unruh, 1980; Bowdle *et al.*, 1980) and 3-12 hr in children (Cloyd *et al.*, 1983).

Besides aging and coadministration of other drugs, pregnancy also affects the clearance of VPA. It was reported (Plasse *et al.*, 1979) that the blood level-to-dose ratio began to decline in the latter part of the second trimester in a pregnant woman and continued through the early part of the third trimester, finally reaching nadir within 3 weeks of delivery. Following parturition, VPA levels rose rapidly and regained pre-pregnancy values within 2-3 weeks. A similar experience in five pregnant patients was cited by Philbert and Dam (1982). Part of the reason for the apparent increase in clearance during late gestation is a decrease in maternal serum protein binding of VPA as a result of elevated nonesterified fatty acids and hypoalbuminemia (Nau and Krauer, 1986).

VPA exhibits several distinct pharmacodynamic characteristics as compared with traditional antiepileptic drugs. The anticonvulsant effect of VPA has been shown repeatedly to correlate poorly with the steady-state serum VPA concentration (Chadwick, 1984; Minns *et al.*, 1982). Also, a striking dissociation between serum VPA concentration and the time course of antiepileptic response has been demonstrated in patients (Rowan, 1979a; 1979b;) and in several experimental models of epilepsy (Lockard and Levy, 1976; Pellegrini *et al.*, 1978; Walter *et al.*, 1980).

Notably, maximal anticonvulsant effect is usually not observed during initial drug therapy until sometime after the attainment of steady-state serum VPA concentrations. In addition, following discontinuation of VPA administration, seizure control persists long after the intact drug has been cleared from the systemic circulation (Lockard and Levy, 1976; Harding *et al.*, 1978). Since VPA is extensively metabolized by the liver, one explanation for the above phenomena is that one or more of its metabolites may contribute significantly to the antiepileptic action of the drug and that these metabolites are eliminated more slowly than the parent drug.

In fact, the unsaturated metabolites 2-ene VPA, 3-ene VPA, 4-ene VPA (Loscher, 1981; Loscher *et al.*, 1985) and 2,3′-diene (Abbott *et al.*, 1988) were found to have significant anticonvulsant activity in rodent models.

1.5 Toxicity

Adverse reactions to VPA may be divided into physiological or dose-related side effects, metabolic effects, and unusual or rare drug reactions. Idiosyncratic reactions and effects other than those due to the pharmacology of the drug may be mediated by the formation of unusual or novel metabolites. Altered target organ responses may also result from genetic abnormalities. Finally, teratogenicity represents an adverse drug reaction which, in the case of valproate, appears to be dose-related (Jaeger-Roman *et al.*, 1986; Diliberti, *et al.*, 1984).

Among the idiosyncratic side effects of VPA, hepatotoxicity draws the most attention. A comprehensive, retrospective analysis of the cases reported in the United States from 1978 to 1984 has provided definitive information on the primary risk for fatal liver failure with valproate treatment (Dreifuss *et al.*, 1987). According to this study, patients at risk are children two years old or younger who receive valproic acid as part of anticonvulsant polypharmacy and who also have other medical problems besides severe epilepsy, e.g., mental retardation, developmental delay, and metabolic disorder. The incidence of hepatotoxicity found is as high as 1 in 500 in pediatric patients with multi- therapy. Outside of this group, the overall risk of fatal hepatic dysfunction with valproate (1 in 12,000 with polytherapy versus 1 in 45,000 with monotherapy) is still higher among patients receiving multiple anticonvulsants. In addition, no cases of hepatic failure were identified among those persons over the age of 10 who were administered valproate monotherapy (Dreifuss *et al.*, 1987).

The most common histopathological feature of VPA-induced liver injury is microvesicular steatosis, similar to that produced by the toxic metabolites of hypoglycin A and by 4-pentenoic acid (Rettie *et al.*, 1988) which is a potent inhibitor of mitochondrial fatty acid metabolism (Corredor et al., 1967). Evidence has been obtained from studies *in vivo* (Mortensen, 1980; Mortensen *et al.*, 1980; Kesterson *et al.*, 1984) and *in vitro* (Thurston *et al.*, 1983; Bjorge & Baillie, 1985; Thurston *et al.*, 1985) that VPA inhibits the β-oxidation of endogenous fatty acids.

Studies in animals have indicated that several metabolites contribute to the toxic effects of VPA. The 4-ene VPA and 2,4-diene VPA metabolites are hepatotoxic in rats (Kesterson *et al.*, 1984) and are thought to be responsible for the rare but fatal hepatotoxicity associated with VPA. These metabolites may cause damage to liver mitochondria, inhibit fatty acid β-oxidation activity and cause accumulation of hepatic lipids (Zimmerman *et al.*, 1982; Rettenmeier *et al.*, 1986a). Additional studies with 4-ene VPA served to reinforce the view that this terminal olefin, like 4-pentenoic acid, acts as a mechanism-based irreversible inhibitor of enzymes of the fatty acid β-oxidation complex, and then induces the microvesicular steatosis *in vivo* (Rettenmeier et al., 1985, 1986a). In fact, 4-ene VPA was shown to be the most toxic metabolite of VPA in the rat (Kingsley *et al.*, 1983). Since the formation of 4-ene VPA is mediated by cytochrome P450, the prevalence of 4-ene VPA may be increased by the presence of enzyme inducers (Rettie et al., 1987). This may in part be the explanation why patients on polytherapy are at considerably greater risk of developing fatal hepatic dysfunction than patients receiving valproate monotherapy.

Kassahun *et al.* (1990) reported the discovery of GSH 3-ene VPA in rat bile following the administration of either (E)-2,4-diene VPA or 4-ene VPA. Since 2,4-diene is considered to be a mitochondrial oxidation product of 4-ene VPA CoA (Rettenmeier *et al.*, 1985), it is conceivable then that, by virtue of its electrophilic nature, (E)-2,4-diene VPA CoA may bind to a nucleophilic site of a mitochondrial enzyme. This would account for the potent inhibition of mitochondrial β-oxidation of fatty acids observed for (E)-2,4-diene VPA in rats (Kesterson *et al.*, 1984). An alternate mechanism for the hepatotoxicity of the reactive (E)-2,4-diene VPA CoA ester, according to Kassahun's study could be the localized depletion of GSH in mitochondria.

Since β-oxidation of VPA would require that the drug be in the form of its CoA thioester (Bjorge and Baillie, 1991; Li *et al*., 1991), it appears likely that the effects of VPA on the hepatic lipid metabolism are mediated in part by the ability of the drug to sequester limited pools of CoASH, the obligatory cofactor for β-oxidation.

1.6 Chemical Derivatization and Analysis

Gas chromatography mass spectrometry (GCMS) is widely used for the analysis of VPA and its metabolites. The reported GCMS methods include electron impact (EI) of the *t*-butyldimethylsilyl (*t*-BDMS) and trimethylsilyl (TMS) derivatives (Abbott, *et al*., 1986; Nau *et al*., 1981; Rettenmeier *et al*., 1986b; 1989; Tatsuhara *et al*., 1987) and negative chemical ionization (NCI) of pentafluorobenzyl (PFB) derivatives (Abbott *et al*., 1987; Kassahun *et al*., 1989). A complete GCMS assay using EI techniques for VPA and its metabolites was reported by Abbott *et al*. (1986). With the EI method, *t*-BDMS derivatives of VPA and its metabolites have advantages over TMS derivatives except for that of 3-OH VPA. When derivatized with *t*-BDMS, VPA and its metabolites have increased sensitivity in EI mode because of the intense $(M-57)^+$ fragments formed in contrast to the less intense $(M-15)^+$ fragment from TMS derivatives. While very good sensitivity was obtained for the *t*-BDMS derivatized VPA unsaturated metabolites, problems arise in the analysis of the keto and hydroxy metabolites which can yield either mono- or di- derivatives depending upon the derivatization conditions used. The 3-OH VPA does not derivatize readily under the conditions suitable for derivatizing other metabolites with *t*-BDMS.

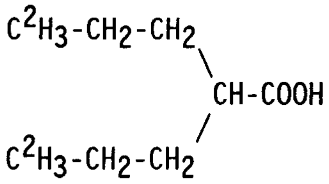
NCI GCMS is a very sensitive and specific assay method for the analysis of valproic acid metabolites. The PFB derivatives produce abundant $[M-181]^-$ ions except for 3-keto VPA which gave an $[M-181-CO_2]^-$ ion. However, when a combination of PFB and TMS (for hydroxy and 3-keto moiety) derivatization was used, 3-keto VPA formed an $[M-181]^-$ ion which was practically the only ion present in the mass spectrum. Hydroxy metabolites derivatized by this method gave significant improvement in both peak shape and sensitivity of detection (Kassahun *et al*., 1989, 1990). The PFB derivatives (NCI mode) proved to be 30-50 times more sensitive than the *t*-BDMS derivatives under the EI mode (Kassahun *et al*., 1989).

1.7 Stable Isotope Techniques

Stable isotope techniques have been used in different areas of VPA studies including steady-state kinetics (Acheampong *et al*., 1984), bioavailability studies (Strong *et al*., 1975), drug interaction studies (Von Unruh *et al*., 1980), drug metabolism studies (Acheampong *et al*., 1983), and mechanistic studies of drug metabolism (Rettie *et al*., 1988; Rettenmeier *et al*., 1987).

The identification of VPA metabolites is hindered by the chemical lability of some of the metabolites as well as the structural similarity of VPA to endogenous compounds. The use of stable-isotope labelled drugs has been widely applied to facilitate identification of drug metabolites (McMahon *et al*., 1973; Pohl *et al*., 1975). Usually an equimolar mixture of unlabeled and stable isotope-labelled drug is administered and biological fluids are examined by mass spectrometry for characteristic ion-doublets indicative of drug-derived metabolic products.

A stable isotope technique was used in this lab (Acheampong *et al.*, 1983) to search for new metabolites of VPA in human serum and urine. GCMS proved useful to clarify the identity of compounds described either as endogenous compounds or as VPA metabolites. In this study, a pulse dose of di-[(3,3,3-$^2H_3$)-propyl]-acetic acid ([$^2H_6$]VPA, (2)) was administered to a human volunteer at steady state serum concentrations of unlabeled VPA. The location of the deuterium on the terminal carbons provided ion doublets in the mass spectra of GC metabolite peaks with mass differences of either three or six amu.



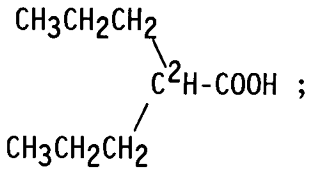
(2)

Stable isotope technique can also determine changes in the kinetic parameters of a drug in patients on multiple dose therapy (Sullivan *et al.*, 1975; Kapetanovic *et al.*, 1980). To determine kinetic parameters of an anticonvulsant in patients on multiple dosing or multi-therapy, a 'pulse' dose of stable isotope-labelled drug offers a convenient technique. Application of this method to antiepileptic drug studies allows the elimination kinetics of the drug to be determined without discontinuing therapy and risking the exacerbation of seizures. The labelled drug elimination phase can be followed for 3-4 half-lives during subsequent uninterrupted multiple dosing of unlabeled drug. This technique was applied first by Von Unruh *et al.* (1980) using di-[(2,3'-$^2H_2$) propyl]-acetic acid to study the elimination kinetics of VPA under steady state conditions in patients on combined antiepileptic drug therapy. Similar methodology was applied by Acheampong *et al.* in which a human volunteer was given a single dose of di-[(3,3,3-$^2H_3$)-propyl]-acetic acid (Acheampong *et al.*, 1984). In the latter case, an isotope effect was observed for metabolites in the ω-oxidation pathway.

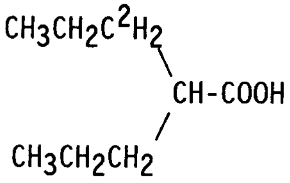
A stable isotope-labelled drug which shows a significant biological isotope effect is not appropriate for use in pharmacokinetic studies, particularly when metabolites are to be measured. Thus the position of the label in the drug should be optimal for minimizing isotope effects on metabolic reactions. In the present study, potential isotope effects in the *in vivo* metabolism of [$^{13}C_4$]VPA and [$^2H_6$]VPA will be investigated. If no significant isotope effect is found in [$^{13}C_4$]VPA as expected, [$^{13}C_4$]VPA will be used in pharmacokinetic studies of VPA and its metabolites in pediatric patients. Hence, pharmacokinetic parameters for VPA metabolites will be determined for the first time in children.

Stable isotope methodology, besides providing the identification of new metabolites and determining elimination kinetics under steady state conditions, also represents a powerful technique for studies on the origins of drug metabolites and for the elucidation of complex metabolic inter-relationships *in vivo*.

VPA or its metabolites are thought to serve as competitive inhibitors of fatty acid β-oxidation, which causes the rare but fatal hepatotoxicity. It is known that VPA is metabolized to three products (2-ene VPA, 3-OH VPA and 3-keto VPA) whose structures are formally analogous to the sequential intermediates of fatty acid β-oxidation (Prickett & Baillie, 1984). Rettenmeier and coworkers (1987) attempted to determine whether these three metabolites share a common metabolic origin *in vivo* and represent products of β-oxidation activity using stable isotope techniques. In that study, the metabolism of 2-[$^2H_1$]VPA (3) and 3,3-[$^2H_2$]VPA (4) was compared to unlabeled VPA. When 2-[$^2H_1$]VPA was administered to rats, the metabolite 3-OH VPA exhibited partial labeling (60%). This result demonstrated clearly that the 3-OH VPA formed in that experiment was not produced exclusively *via* β-oxidation. When [3,3-$^2H_2$]VPA was given to rats, the deuterium content of 3-keto-VPA was very close to that of 2-ene VPA, which suggested that 3-keto-VPA was formed solely *via* 2-ene VPA.



$(CH_3CH_2CH_2)_2C^2H\text{-}COOH$;



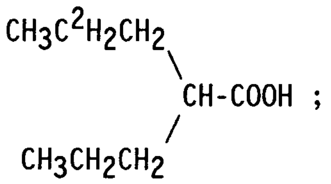
$(CH_3CH_2C^2H_2)(CH_3CH_2CH_2)CH\text{-}COOH$

2-[$^2H_1$]VPA (3)

3-[$^2H_2$]VPA (4)

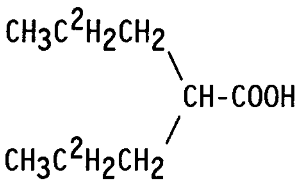
Since kinetic isotope effects can be associated with the formation of some metabolites, stable isotope techniques are useful in mechanistic studies of drug metabolism. Rettie and his coworkers (1988) used this technique in studying the formation of 4-ene VPA. The metabolite 4-ene VPA was shown to be the most toxic metabolite of VPA in rat hepatocytes in culture (Kingsley *et al.*, 1983) and to be considerably more potent as an inducer of steatosis in young rats than was the parent drug (Kesterson *et al.*, 1984).

Based on the supposition that a carbon-centered free radical, localized at either C-4 or C-5 is formed before eliminating hydrogen to yield 4-ene VPA (Rettie *et al.*, 1987), the mechanism of the desaturation reaction to form 4-ene was studied by inter- and intra- molecular deuterium isotope effect experiments using deuterium substitutions of VPA at either C-4 or C-5 (Compounds 5, 6, 7, 2, Rettie *et al.*, 1988).



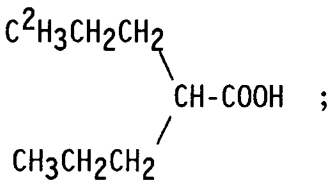
$$\begin{array}{l} CH_3C^2H_2CH_2 \\ \qquad\qquad \backslash \\ \qquad\qquad\quad CH\text{-}COOH\ ; \\ \qquad\qquad / \\ \ CH_3CH_2CH_2 \end{array}$$

4-[$^2H_2$]VPA (5)



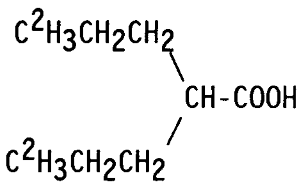
$$\begin{array}{l} CH_3C^2H_2CH_2 \\ \qquad\qquad \backslash \\ \qquad\qquad\quad CH\text{-}COOH \\ \qquad\qquad / \\ CH_3C^2H_2CH_2 \end{array}$$

4,4′-[$^2H_4$]VPA (6)



$$\begin{array}{l} C^2H_3CH_2CH_2 \\ \qquad\qquad \backslash \\ \qquad\qquad\quad CH\text{-}COOH\ ; \\ \qquad\qquad / \\ CH_3CH_2CH_2 \end{array}$$

5-[$^2H_3$]VPA (7)



$$\begin{array}{l} C^2H_3CH_2CH_2 \\ \qquad\qquad \backslash \\ \qquad\qquad\quad CH\text{-}COOH \\ \qquad\qquad / \\ C^2H_3CH_2CH_2 \end{array}$$

5,5′-[$^2H_6$]VPA (2)

A large primary kinetic isotope effect was obtained for the formation of 4-OH VPA from 4-[$^2H_2$]VPA and for the formation of 5-OH VPA from 5-[$^2H_3$]VPA. Conversely, some β-secondary kinetic isotope effects could be observed for the formation of 4-OH-VPA from 5-[$^2H_3$]VPA and for the formation of 5-OH VPA from 4-[$^2H_2$]VPA. After calculating the isotope effects of 4-ene VPA, 4-OH VPA and 5-OH VPA, the authors found that the formation of 4-ene VPA and 4-OH from hexadeuterated VPA 2 showed a small or no isotope effect, while high isotope effects were observed for 4-ene VPA and 4-OH VPA formed from compound 6. It was then concluded that removal of a hydrogen atom from the subterminal C-4 position of VPA to form a carbon-centered free radical at C-4 is rate limiting in the formation of both 4-ene and 4-OH, and the influence which the second hydrogen/deuterium exerts to form 4-ene on the overall kinetics of the reaction is small. Therefore, it is a carbon-centered free radical, localized at C-4 instead of C-5 that is formed before eliminating hydrogen to yield 4-ene VPA (Rettie *et al.*, 1988).

Another application of stable isotopes is also called isotope dilution mass spectrometry (IDMS). IDMS methods for organic analytes involve spiking a sample with a labelled version of the analyte as an internal standard, processing the sample, and then measuring the ratio of unlabeled to labelled analyte by using GCMS. IDMS has been the technique of choice for definitive methods at the National Institute of Standards and Technology, since it does not depend on sample recovery, shows high precision, and can be tested for bias and unknown interferences.

When stable-isotope labelled VPA and its metabolites were used as internal standards, calibration curves with high correlation coefficients and good reproducibilities were obtained (Abbott *et al.*, 1986; Kassahun *et al.*, 1990; Von Unruh *et al.*, 1980). The variance in extraction of VPA metabolites due to a slight pH change or incompleteness of derivatization due to time and temperature can be minimized by using stable isotope-labelled analogs as internal standards. In GCMS analysis, especially under CI conditions, the spectra depend on source pressure and temperature. More accurate measurements can be made with a stable isotope-labelled internal standard since the physicochemical properties of the internal standard closely approximate that of the analyte.

1.8 Specific objectives

1. To investigate potential isotope effects of the metabolism of $[^2H_6]$VPA and $[^{13}C_4]$VPA in a healthy human volunteer.

2. To determine the pharmacokinetics of $[^2H_6]$VPA, and its metabolites in a healthy volunteer, and compare the pharmacokinetic behavior of $[^2H_6]$VPA and its metabolites with those of unlabeled analogs.

3. The applicability of $[^{13}C_4]$VPA for pulse dose studies of the pharmacokinetics of VPA and metabolites requires the development of a

precise and sensitive assay technique. This project will focus on evaluating and improving GCMS methods for the assay of labelled and unlabeled VPA metabolites.

4. As part of the assay development, urine, serum and saliva samples are to be analyzed using different ionization methods (NCI and EI) to compare the sensitivities and reproducibilities of these two GCMS methods. The conjugated fraction of VPA and its metabolites in urine are to be measured by hydrolyzing conjugates with alkali and with glucuronidase, in order to examine if any glucuronidase resistant conjugates exist.

5. To synthesize stable isotope-labelled internal standards in order to facilitate the quantitative analysis of VPA, [$^{13}C_4$]VPA and their metabolites by GCMS. The labelled metabolites to be synthesized are:

[$^2H_7$]VPA; [$^2H_7$]2-ene VPA;
[$^2H_7$]3-keto VPA; [$^2H_7$]3-OH VPA;
[$^2H_7$]4-ene VPA; [$^2H_7$]5-OH VPA;
[$^2H_7$]4-OH VPA; [$^2H_7$]4-keto VPA

6. To perform preliminary study of the use of stable isotope labelled internal standards in a pharmacokinetics assay of [$^{13}C_4$] VPA and its metabolites in nonpregnant sheep.

2. Experimental

2.1 Chemicals and Instrumentation

2.1.1 Chemicals and Reagents

Chemicals were reagent grade and were obtained from the following sources:

Aldrich Chemical Co. (Milwaukee, WI):

Butyllithium (1.6 M in hexane), 1,8-diazabicyclo[5,4,0] undec-7-ene (DBU), Diazald^R, diisopropylamine, diisopropylethylamine, hexamethylphosphoramide, methanesulfonyl chloride, triethylamine, (E)-2-pentenoic acid, bromopropane, lithium aluminum hydride, propionyl chloride, 4-pentenoic acid, tetrahydrofuran, isopropylcyclohexylamine.

MSD Isotopes (Montreal, Canada):

1,2,3,1'-[$^{13}C_4$]-2-propylpentanoic acid, [$^2H_7$] propyl bromide.

BDH Chemicals (Toronto, Ontario):

Benzene, chloroform, ether, hydrochloric acid, magnesium sulfate, sodium hydroxide, sodium sulfate, sulfuric acid, hexane.

Caledon Laboratories Ltd. (Georgetown, Ontario):

Dichloromethane, ethanol, ethyl acetate.

ICN Pharmaceuticals Inc. (Plainview, NY):

Di-n-propylacetic acid (Valproic acid)

2.1.2 VPA metabolites and internal standards

The synthesis of the following VPA metabolites for use as analytical standards has been described elsewhere: 3-ene VPA (stereochemistry undetermined), 4-ene VPA, 3-OH VPA, 4-OH VPA, 5-OH VPA, 3-keto VPA, 4-keto VPA, 2-PSA and 2-PGA (Acheampong *et al.*, 1983); (E,E)-2,3′-diene VPA (Acheampong *et al.*, 1985); (E)-2,4-diene VPA (Lee *et al.*, 1989).

The internal standards used for the assay of VPA, $[^2H_6]$VPA and their metabolites were $[^2H_3]$2-ene VPA, synthesized by Abbott *et al.* (1986); $[^2H_3]$3-keto VPA, a kind gift from Dr. T.A. Baillie (University of Washington, School of Pharmacy, Seattle, WA); di-n-butylacetic acid; and 2-methylglutaric acid (2-MGA). In both the EI and NCI modes, di-n-butylacetic acid was used as the internal standard for VPA and $[^2H_6]$VPA; $[^2H_3]$3-keto VPA for 3-keto VPA and $[^2H_6]$3-keto VPA; 2-MGA for 2-PSA, 2-PGA and their deuterated analogs; $[^2H_3]$2-ene VPA for all other metabolites.

2.1.3 Nuclear Magnetic Resonance Spectrometry

High field ^1H NMR spectra were obtained on the Bruker WH-400 and Varian XL-300 spectrometers in the Department of Chemistry, University of British Columbia NMR facility. Spectra were acquired in CD_3Cl with tetramethylsilane as an internal standard. Carbon-13 NMR spectra were obtained on the Varian XL-300 spectrometer in the Department of Chemistry.

2.1.4 Centrifuges

Unbound drugs were separated from serum samples by the use of ultrafiltration. Protein becomes selectively partitioned into a fraction of the sample volume (retentate), while free ligand passes essentially unhindered through the membrane along with solvent into the ultrafiltrate. The physical separation does not change free ligand volume or concentration. Centrifree^{TM} micropartition system (Amicon, W.R. Grace & Co, Danvers, MA) and Beckman centrifuge, model J2-21, equipped with a 45° rotor were used to achieve this purpose. Serum samples were centrifuged at a speed of 3500 rpm for 30 minutes.

2.1.5 Packed Column Gas Chromatography - Mass Spectrometry

GCMS analysis of synthesized compounds was performed on a Hewlett Packard 5700A gas chromatograph interfaced to a Varian MAT-111 mass spectrometer using a variable slit separator. Electron impact (EI) data were recorded in scanning mode with a range of 15 - 750 mass units every 5 seconds, and the data were processed using a Packard Bell computer (IBM AT clone) and a program developed in our laboratory. Total ion current (TIC) plots were based on scanning range of m/z 50 - 500.

The instrument was operated with an emission current of 300 uA, ionization of 70 eV, and source pressure of 5×10^{-6} Torr. The column

(1.8 m x 2 mm i.d.) was packed with 3% Dexsil 300 on 100 - 120 mesh Supelcoport. The oven temperature program was 50 - 300 °C at 16 °C/min. Injection port temperature was 250 °C and the separator temperature set at 250 °C. The carrier gas, with a flow of 25 ml/min, was helium.

2.1.6 Capillary Column Gas Chromatography - Mass Spectrometry

The quantitative and qualitative analyses of VPA and its metabolites were performed on a Hewlett Packard 5987A GCMS having an open-split interface. Data recording and processing were managed with a HP-1000 on-line computer. EI techniques were used to analyze t-BDMS derivatives with an electron ionization energy of 70 eV. Operating conditions were: source temperature 200 °C, transfer line temperature 240 °C, injection port temperature 240 °C and helium flow-rate 1 ml/min. The capillary column was an OV-1701 bonded phase, 25 m x 0.32 mm i.d., with a film thickness of 0.25 um (Quadrex Scientific, New Haven, CT, U.S.A.). Two temperature programs were utilized for the GCMS, EI analysis. The temperature program used for VPA and its unsaturated metabolites was initiated at 50 °C, programmed at 30 °C/min to 110 °C, held for 18 min before being increased to 260 °C at rate of 8 °C/min. The temperature program used for the remaining polar metabolites of VPA was initiated at 50 °C, programmed to 100 °C at 30 °C/min, then increased to 250 °C at rate of 8 °C/min.

In the negative ion chemical ionization (NICI) mode, ultra-high purity methane was used as the reagent gas. The capillary column used was DB-1 (25m x 0.25mm, with a film thickness of 0.25 um, J&W

Scientific, Rancho Cordora, CA, U.S.A.). The source pressure was 0.8 - 1.2 Torr, ionization energy 200 eV, and the emission current 250 uA. The instrument was programmed for SIM as well. The initial oven temperature was 50 °C, programmed to 140 °C, held at 140 °C for 20 min before being increased to 260 °C at 5 °C/min.

2.1.7 Mass Selective Detector (MSD)

All samples obtained from the VPA study in sheep were extracted, derivatized with t-BDMS and analyzed with a HP 5890 GC interfaced to a HP 5971A MSD using EI mode. Data recording and processing were managed with a HP VECTRA^R 486 data system. Operation conditions were: injection temperature 250 °C, ion source temperature 180 °C, GCMS interface temperature 280 °C, and helium flow-rate 1 ml/min. The capillary column used was DB-1701 (30m x 0.25mm, with a film thickness of 0.25 um, J&W Scientific, Rancho Cordora, CA, U.S.A.). The initial oven temperature was 80 °C, programmed to 100 °C at rate of 10 °C/min, then to 130 °C at 2 °C/min, and finally to 260 °C at 30 °C/min and held for 8 minutes.

2.2 Chemistry:

2.2.1 Synthesis of [$^2H_7$]VPA

In a dry 250 ml 3-neck flask equipped with a mechanical stirrer, drying tube, dropping funnel, and reflux condenser, n-butyllithium in hexane (30 mL of 1.6 M, 48 mmol) was added dropwise to a solution of diisopropylamine (6.8 mL, 48 mmol distilled over CaH_2) in 40 mL of THF (distilled over $LiAlH_4$) at 0 °C under N_2 atmosphere and stirred for another 30 minutes. Distilled valeric acid (2.45 mL, 23 mmol) in 10 mL of THF was added dropwise so that the temperature of the reaction never exceeded 5 °C. The solution at first became milky, but when 5 mL of HMPA was added the solution became clear. The reaction was allowed to proceed for 30 min before $[^2H_7]$ bromopropane (18 mmol) was added to the mixture. The ice/water bath was then removed and the mixture stirred at room temperature for 3 h. The reaction was again cooled in ice and quenched with 15% HCl (cooled) until a pH of 1 was attained. The mixture was extracted with diethyl ether (50 mL x 3), the ethereal layer washed with 10% HCl and water, and then dried over anhydrous Na_2SO_4. The solvent was removed by flash evaporation and the residue was fractionally distilled (110-113 °C/10 mm Hg) to afford 1.2 g of pure $[^2H_7]$ VPA (yield 44%). A small portion of the sample was derivatized with diazomethane (Levitt, 1973) for GCMS analysis. GCMS mass spectrum of the methyl ester of $[^2H_7]$VPA, m/z(%): 89(100), 123(30), 117(28), 61(23), 134(5), 106(5).

^1H NMR of $[^2H_7]$VPA ($CDCl_3$): δ 0.93 (t, 3H, $C\underline{H}_3$), 1.37 (m, 2H, $CH_3C\underline{H}_2$), 1.46-1.62 (m, 2H, $C\underline{H}_2CH$), 2.39 (dd, 1H, $C\underline{H}COOH$), 8.0 (w, 1H, $COO\underline{H}$).

2.2.2 *Synthesis of $[^2H_7]$4-ene VPA*

In a dry 500 mL 3-necked flask equipped with a dropping funnel, condenser, mechanical stirrer, and drying tube, 90 mL of n-butyllithium (0.144 mol) in hexane was added dropwise to a solution of diisopropylamine (20.2 mL, 0.144 mol) in 120 mL of THF (distilled over $LiAlH_4$) in an ice/water bath under N_2 atmosphere. The mixture was stirred for 30 min. 4-Pentenoic acid (6.75 mL, 0.066 mol) was added dropwise over a period of 15 min, followed by 15 mL of HMPA (0.066 mol). The reaction was then allowed to proceed for 30 min. $[^2H_7]$ Bromopropane (6.2 g, 0.06 mol) was added and the ice/water bath removed. The mixture was stirred at room temperature for 2.5 hours before being quenched with 15% HCl solution. The aqueous layer was extracted 3 times with ether and the combined ether extracts dried over anhydrous Na_2SO_4. The solvent was removed by flash evaporation. A yield of 5.4 gram (77%) of $[^2H_7]$4-ene VPA was obtained by fractional distillation (110-120 °C/10 mm Hg). A small portion of the sample was derivatized with diazomethane (Levitt, 1973) for GCMS analysis.

GCMS mass spectrum of the methyl ester of 4-ene $[^2H_7]$VPA, m/z(%): 113(100), 59(67), 104(58), 115(37), 132(11), 163(M^+, 2).

^1H NMR of 4-ene $[^2H_7]$VPA ($CDCl_3$): δ 2.28 (m, 1H, $CH_2C\underline{H}$), 2.38-2.43 (m, 2H, $C\underline{H}_2CH$), 5.03 (dd, 1H, $C\underline{H}_2$=$CHCH_2$, J=9 Hz, cis), 5.08 (dd, 1H, $C\underline{H}_2$=$CHCH_2$, J=15 Hz, trans), 5.78 (m, 1H, CH_2=$C\underline{H}$), 8.5 (w, 1H, $COO\underline{H}$).

2.2.3 Esterification of $[^2H_7]$4-ene VPA

In a 100 mL round-bottomed flask equipped with Dean-Stark apparatus, drying tube, and a reflux condenser, $[^2H_7]$4-ene VPA (3.7 g, 0.027 mol), ethanol (6 mL), concentrated sulfuric acid (0.5 mL), and 12 mL of benzene were refluxed at 90 °C for 46 hours. The organic layer was consecutively washed with saturated $NaHCO_3$ solution and water until the wash was neutral, then dried over anhydrous Na_2SO_4. A yield of 3.75 g of ethyl [$^2H_7$]4-ene VPA was obtained by fractional distillation (80 °C/10 mm Hg, 85% yield).

GCMS mass spectrum of the ethyl ester of [$^2H_7$]4-ene VPA, m/z(%): 104(100), 127(88), 59(86), 99(73), 143(17), 189(M^+, 1).

2.2.4 Synthesis of [$^2H_7$]4-keto VPA

Benzoquinone (1.32 g) and $PdCl_2$ (0.033 g) were added to a mixture of 38.5 mL of dimethyformamide and 5.5 mL of water in a 50 mL round-bottomed flask equipped with a reflux condenser. To this stirred mixture, ethyl [$^2H_7$]4-ene VPA (1.87 g, 0.013 mol) was then added dropwise with a syringe over a period of 10 min and the reaction was allowed to proceed at room temperature for 22 h. The product was then poured into 20 mL of cold 10% HCl solution and extracted 3 times with ether (80 mL x 3). The combined organic layer was washed three times with 20 mL of 10% NaOH solution, once with saturated NaCl solution, and was then dried over anhydrous Na_2SO_4. The solvent was removed by flash evaporation and the residue was fractionally distilled to afford 1.18 g of pure ethyl [$^2H_7$]4-keto VPA (110-115 °C/10mm Hg, 60% yield).

To 0.54 mL of ethyl [$^2H_7$]4-keto VPA in a 25 ml round-bottomed flask, 1.55 g of NaOH in 6 mL of H_2O and 5 mL of CH_3OH was added and heated at 50 °C for 4 h. Using an oil bath at 120°C, methanol and ethanol were distilled off at atmospheric pressure. The remaining aqueous solution was washed with ether (3 x 20 mL) to extract any unhydrolyzed ethyl ester. The remaining aqueous solution was then acidified to pH 2 with diluted HCl and extracted with ether three times (3 x 40 mL). The combined ethereal layers were dried over anhydrous Na_2SO_4. The ether was evaporated under flash evaporation and the residue was further dried under vacuum (0.5 mm Hg) for 3 hours. $[^2H_7]$4-keto VPA (0.43 g) was obtained (93% yield). A small portion of the sample was derivatized with diazomethane (Levitt, 1973) for GCMS analysis.

GCMS mass spectrum of the methyl ester of $[^2H_7]$4-keto VPA, m/z(%): 43(100), 122(88), 87(40), 89(33) 75(28), 103(20), 148(17), 164(2), 179(M^+,1).

2.2.5 Synthesis of $[^2H_7]$4-OH VPA

To a well stirred mixture of 170 mg of $NaBH_4$ (4.47 mmol) in 8 mL of ethanol, 0.64 g (3.3 mmol) of ethyl $[^2H_7]$4-keto VPA was added dropwise over a period of 10 min. The reaction was allowed to proceed for 60 min at room temperature, and then quenched with saturated NH_4Cl solution. The aqueous solution was extracted 3 times (3 x 20 mL) with ether and the combined ethereal layers were dried over anhydrous Na_2SO_4. The solvent was removed under flash evaporation. The residue was fractionalized by distillation (115-118 °C / 8 mm Hg) and 170 mg (yield 26%) of ethyl $[^2H_7]$4-OH VPA was then obtained.

GCMS mass spectrum of ethyl [$^2H_7$]4-OH VPA, m/z(%): 101(100), 43(45), 134(8), 116(5), 151(2).

Ethyl [$^2H_7$]4-OH VPA (170 mg), 0.75 mL methanol and 0.3 g of NaOH in 3 mL of H_2O were heated at 50 °C for 2 hours. After being cooled, the solution was washed with ether to remove any unhydrolyzed ester.

2.2.6 Synthesis of [$^2H_7$] 5-OH VPA

To 0.75 g of ethyl [$^2H_7$]4-ene VPA (4.1 mmol) in dry THF in a 50 mL three neck round-bottomed flask cooled to 4 °C with ice. Under N_2, 1.5 mL of borane - THF was added dropwise. After the mixture was stirred at room temperature for 30 min, the flask was immersed into an ice/water bath and 100 μl of water was added to destroy excess hydride. To this was added after 5 min, 4 mL of 1.5 N NaOH followed with 0.5 mL of 30 % H_2O_2. The reaction temperature was maintained at 40-50 ^oC for 1 h. The reaction was then quenched by pouring into 20 mL of ice/water. The solution was extracted 3 times with 30 mL of ether. The combined ethereal layer was washed with water followed by saturated NaCl solution and then dried over anhydrous Na_2SO_4. The solvent was removed under flash evaporation. The residue was purified by flash column chromatography (Rettenmeier *et al.*, 1985) using a mobile phase of 5% methanol in ethyl acetate. A yield of 150 mg (19%) of ethyl [$^2H_7$]5-OH VPA was obtained.

GCMS mass spectrum of the ethyl ester of [$^2H_7$]5-OH VPA, m/z(%): 101(100), 57(28), 115(12), 150(7), 196(M^+, 1).

Ethyl [$^2H_7$]5-OH VPA (150 mg) was hydrolyzed by being heated with 1 mL of methanol and 0.35 g of NaOH in 4 mL of H_2O at 60 ^oC for 4 hours. After cooled, the solution was washed with ether to remove any unhydrolyzed ester.

2.2.7 Synthesis of [$^2H_7$]3-keto VPA

2.2.7a. Synthesis of ethyl 3-keto pentanoate

In a 1000 mL 3-necked flask equipped with a dropping funnel and mechanical stirrer, isopropylcyclohexylamine (40 mL) and THF (250 mL) under N_2 atmosphere were cooled to 0 ^oC with ice. N-butyllithium (150 mL, 0.24 mol, 1.6 M in Hexane) was added dropwise over a period of 10 min. The mixture was stirred for another 20 min before immersing the flask into a dry ice/acetone bath (-78 °C). Ethyl acetate (11 mL, 0.12 mol) dried over Na_2SO_4 was then added dropwise over a period of 10 min followed by propionyl chloride (9.3 mL). The reaction was allowed to proceed for an additional 15 min before being quenched by adding 4 N HCl slowly to a pH of 2. The product was extracted 3 times (100 mL x 3) with ether. The ethereal layer was washed with $NaHCO_3$ solution and then dried over anhydrous Na_2SO_4. The solvent was removed under vacuum using flash evaporation. The residue was fractionally distilled to afford 9.5 g of ethyl 3-keto pentanoate (70 °C/5 mm Hg, yield 55%).

GCMS mass spectrum of ethyl 3-keto pentanoate, m/z(%): 57(100), 29(95), 43(39), 98(14), 115(12.5), 144(M^+,12).

300 MHz ^{1}H-NMR of ethyl 3-keto pentanoate ($CDCl_3$): δ 1.1(t, 3H, $C\underline{H}_3$-CH_2), 1.3(t, 3H, $C\underline{H}_3$-CH_2O), 2.6(q, 2H, $CH_3C\underline{H}_2CO$), 3.45(s, 2H, $COCH_2CO_2$), 4.2(q, 2H, $C\underline{H}_2O$).

2.2.7b Alkylation of ethyl 3-keto-pentanoate with [$^2H_7$] bromopropane

Sodium metal (1.4 g, 0.06 mol) cut into small pieces was placed in a 50 mL 3-necked flask equipped with a dropping funnel filled with ethanol (20 mL) and protected from moisture with drying tubes. A wet towel was kept in readiness to control the vigor of the subsequent reaction. Absolute ethanol (10 mL) dried over Mg was added to the sodium producing a vigorous reaction. As the reaction subsides, more alcohol was introduced to maintain vigorous, but controllable refluxing. In this manner, most of the Na reacted rapidly. Finally the remainder of ethanol was added and the mixture refluxed by a heating mantle until the Na had reacted completely. Ethyl 3-keto pentanoate (8.6 g, 0.06 mol) was added dropwise over a period of 10 min, and the mixture was stirred and refluxed for another 20 min. [$^2H_7$] Bromopropane (7 g, 0.054 mol) was then introduced dropwise, and the reaction allowed to reflux for 7 hours. The solution was filtered to remove the sodium bromide after cooling. The filtered NaBr was washed with ethanol. The ethanol solutions were combined and the ethanol was evaporated by flash evaporation. Water was added to the residue and extracted with ether 4 times (4 x 50 mL). The combined organic layer was dried over anhydrous Na_2SO_4 over night. The ether was removed under flash evaporation and the residue fractionally distilled to yield 8.2 gram of ethyl [$^2H_7$]3-keto VPA (75-80 °C /3 mm Hg, 78% yield).

GCMS of ethyl [$^2H_7$]3-keto VPA, m/z(%): 57(100), 103(42), 137(18), 145(9), 164(3), 193(M^+,1).

^1H NMR of ethyl [$^2H_7$]3-keto VPA ($CDCl_3$): δ 1.15(t, 3H, $C\underline{H}_3CH_2$), 1.26(t, 3H, $C\underline{H}_3CH_2O$), 2.55(m, 2H, $CH_3C\underline{H}_2$), 3.42(d, 1H, $COC\underline{H}CO_2$), 4.18(q, 2H, $CH_3C\underline{H}_2O$).

Ethyl [$^2H_7$]3-keto VPA (60 mg) was hydrolyzed by stirring with 1 mL of 3N NaOH and 0.4 mL of methanol at room temperature for two hours. Unhydrolyzed ester was extracted with hexane.

2.2.8 Synthesis of [$^2H_7$] 3-OH VPA

In a dry 100 mL flask, ethyl [$^2H_7$]3-keto VPA (5 g, 0.026 mol) was added dropwise to the well mixed $NaBH_4$ (1.425 g 0.0375 mol) in 60 mL of ethanol. The reaction was then allowed to proceed with stirring for 1 hour before being quenched with saturated NH_4Cl. The solution was extracted 5 times (5 x 50 mL) with ether, and the ethereal layer dried over anhydrous Na_2SO_4. The solvent was removed under flash evaporation and the residue further dried under vacuum (0.5 mm Hg). The reaction was found to be quantitative with 4.75 g (94% yield) of ethyl [$^2H_7$]3-OH VPA being obtained.

GCMS of the ethyl ester of [$^2H_7$] 3-OH VPA, m/z(%): 103(100), 75(52), 120(37),137(30), 166(25), 150(17), 196(M^+, 1).

Ethyl [$^2H_7$]3-OH VPA (45 mg) was hydrolyzed by refluxing with 1mL 3N NaOH and 0.6 mL methanol at 50 °C for two hours. Unreacted ester was extracted with 3 mL of hexane. The aqueous solution was then acidified with 4 N HCl solution to pH of 2 and extracted with ether (5mL x 3). The ethereal solution was then evaporated under flash evaporation to afford [$^2H_7$]3-OH VPA.

2.2.9 Synthesis of [$^2H_7$] 2-ene VPA

In a 50 mL flask equipped with a mechanical stirrer, ethyl [$^2H_7$]3-OH VPA (1.5g, 7.7 mmol), triethylamine (1.34 mL, 9.5 mmol) and dichloromethane (15 mL) were cooled to 0 °C in an ice bath. Methanesulfonyl chloride (0.83 mL, 9.9 mmol) in dichloromethane was cooled to 0 °C and added dropwise to the stirred mixture. The mixture was stirred for a further 60 min at room temperature. A few mL of ether were added to the mixture to precipitate the salt. The mixture was then filtered and the solvents removed by flash evaporation. The residue was reconstituted in 20 mL of dry THF, a solution of DBU (1,8-diazabicyclo [5.4.0] undec-7-ene, 1.43 mL, 9.5 mmol) was added and the contents gently refluxed for 3 hours. The reaction was quenched with water and the aqueous layer extracted 3 times (3 x 30 mL) with ether. The organic layer was washed first with 1M HCl, then with 1M NaOH solution and finally dried over anhydrous Na_2SO_4.

The product was purified by flash column chromatography (Rettenmeier *et al.*, 1985) using a mobile phase of 2-5% ethyl acetate in hexane. A yield of 260 mg of ethyl [$^2H_7$]2-ene VPA was obtained (19% yield). The product was confirmed with ^1H NMR to be mostly E isomer with a small portion of Z isomer.

GCMS of ethyl [$^2H_7$]2-ene VPA, m/z(%): 115(100), 57(95), 132(87), 177(M^+, 82), 97(42), 104(40), 148(37).

^1H NMR of ethyl [$^2H_7$]2-ene VPA ($CDCl_3$): 1.06(t, 3H, $C\underline{H}_3CH_2$), 1.3(t, 3H, $C\underline{H}_3CH_2O$), 2.2(m, 2H, $CH_3C\underline{H}_2$), 4.2(q, 2H, $CH_3C\underline{H}_2O$), 6.73(t, 1H, $C\underline{H}=C$).

Ethyl [$^2H_7$]2-ene VPA (50 mg) was hydrolyzed by heating with 1 mL of 3N NaOH and 0.3 mL of methanol at 80 °C for 20 hours.

2.2.10 Synthesis of (E)- 3-ene VPA

A flame-dried 250 mL three-necked flask equipped with a graduated separatory funnel and mechanical stirrer was flushed with N_2 and immersed in an ice-water bath. Diisopropylamine (5.6 mL, 0.04 mol) and 40 mL of THF were placed in the flask, 25 mL of n-butyllithium (1.6 M in hexane, 0.04 mol) was added dropwise from the funnel. The mixture was allowed to stir for 20 min. The flask was then immersed in a dry ice/acetone bath (-78 °C), HMPA (7.9 mL, 0.044 mol) was added and the mixture was stirred for 30 min. Ethyl (Z)-2-pentenoate (5.7 mL, 0.039 mol) was added to the mixture dropwise over a period of 15 min, followed by dropwise addition of bromopropane (4.4 mL, 0.048 mol). After stirring for 30 min, the reaction was quenched with 15% HCl solution to a pH of 2. The aqueous layer was extracted twice with 20 mL of ether. The combined organic layer was washed with saturated $NaHCO_3$ solution, with water, and then dried over anhydrous Na_2SO_4. The ester was then hydrolyzed by heating with 8 mL of 3N NaOH solution and 2 mL of methanol at 50 °C for 2 hours. Unhydrolyzed ethyl ester was extracted with hexane, and ethanol and methanol were distilled under atmospheric pressure. The aqueous solution was then acidified with 4N HCl solution to a pH of 1-2, and extracted three times (3 x 50 ml) with ether. Ether was removed under flash evaporation. The residue was fractionally distilled to afford 1 g of pure (E)-3-ene VPA (85 °C/1 mm Hg, yield 18%).

GCMS of the methyl ester of (E)-3-ene VPA, m/z(%): 55(100), 97(25), 113(18), 127(7), 156(M^+,2).

^1H NMR of (E)-3-ene VPA ($CDCl_3$): δ 0.9(t, 3H, $C\underline{H}_3CH_2$), 1.34(m, 2H, $CH_3C\underline{H}_2$), 1.53(m, 1H, $C\underline{H}_2CH$), 1.75(m, 1H, $C\underline{H}_2CH$), 1.70(d, 3H, $C\underline{H}_3C=$), 2.98(dd, 1H, $C\underline{H}COO$), 5.44(dd, 1H, J=15Hz, $=C\underline{H}CH$), 5.6(dt, 1H, J=15Hz, $CH_3C\underline{H}=$).

2.2.11 Synthesis of (Z)-3-ene VPA

(Z)-3-ene VPA was synthesized by the same method as described for the synthesis of (E)-3-ene VPA, but with the starting material being ethyl (E)-2-pentenoate.

GCMS of the methyl ester of (Z)-3-ene VPA, m/z(%): 55(100), 97(25), 113(18), 127(7), 156(M^+,3).

^1H NMR of (Z)-3-ene VPA (CD_3OD): δ 0.93(t, 3H, $C\underline{H}_3CH_2$), 1.34(m, 2H, $CH_3C\underline{H}_2$), 1.48(m, 1H, $C\underline{H}_2CH$), 1.72(m, 1H, $C\underline{H}_2CH$), 1.66(d, 3H, $C\underline{H}_3C=$), 3.35(dd, 1H, $C\underline{H}COO$), 5.36(dd, 1H, J=11Hz, $=C\underline{H}CH$), 5.6(dt, 1H, J=11Hz, $CH_3C\underline{H}=$).

2.3 Pharmacokinetic studies

2.3.1 Pharmacokinetic study with [$^2H_6$]VPA

A healthy male volunteer (my research supervisor) participated in a multiple-dose study of the pharmacokinetics of [$^2H_6$]VPA. A 700 mg dose consisting of VPA:[$^2H_6$]VPA (50:50) was given orally to the volunteer every 12 hours for a period of two and half days. Following the fifth and final dose, blood samples were withdrawn at 0, 0.5, 1, 1.5, 2, 2.5, 3, 5, 7, 9, 12, 24, 48, 96, 168, 240 and 336 h. Blood samples were allowed to clot and then centrifuged to provide serum samples, which were transferred to sterile vacutainers and stored at -20°C until analyzed. Saliva samples were taken at selected times convenient with the taking of blood samples. Saliva production was stimulated with a 5% citric acid solution rinse of the mouth. Following the first dose, urine samples were collected in 12 hour blocks for 2 days and then in 2 or 3 day blocks for another 8 days. Total urine volume and pH values were recorded and homogeneous aliquots were saved. Saliva and urine samples were also stored at -20°C. All samples were analyzed quantitatively by GCMS (HP 5987A) using EI analysis of the *t*-BDMS derivatives and NCI analysis of the PFB derivatives. Dibutylacetic acid, 2-methylglutaric acid and the stable isotope labelled metabolites, [$^2H_3$]2-ene VPA and [$^2H_3$]3-keto VPA were used as internal standards.

2.3.2 Pharmacokinetic study with [$^{13}C_4$]VPA

2.3.2a Human study:

A healthy human volunteer (my research supervisor, 70 Kg) was given a single oral dose consisting of 700 mg of VPA:[$^{13}C_4$]VPA (50:50). Blood samples were collected 0, 0.5, 1, 1.5, 2, 2.5, 3, 5, 7, 9, 12, 24, 48 and 72 h after the single dose and allowed to clot. The samples were centrifuged to obtain serum samples. Urine samples were collected at convenient blocks for three days after the dose. Total urine volume and pH values were recorded and homogeneous aliquots were saved. All samples were stored at -20°C until analyzed by GCMS using NCI techniques.

2.3.2b Animal study:

Two nonpregnant sheep (60 and 73.6 Kg respectively) were each administered i.v. a single dose of 1000 mg of VPA : [$^{13}C_4$]VPA (50:50). Blood samples were collected at 5 min before injection, 2, 6, 10, 15, 20, 30, 45, 60 min, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 10, 12, 24, 36, 48, 72 h after the single dose. All the samples were allowed to clot before centrifuging to collect serum samples. Urine samples were collected every half an hour, then in 1 hour, 2 hour, 12 hour, or 24 hour blocks.

Bile samples were taken at selected time 0-30 min, 60-90 min, 2.5-3.5 h, 5-5.5 h, 7-7.5 h, 11-11.5 h, 24-24.5 h, 36-36.5 h, 48-48.5 h, and 72-72.5 h after administration. The volume of urine and bile samples were recorded, and the pH values of urine samples were measured. All samples were stored at -20 ^{o}C until the time of analysis.

2.4 Metabolic studies of (E)- and (Z)-3-ene VPA

2.4.1 Study design

The metabolic studies of (Z)-3-ene VPA and (E)-3-ene VPA were performed in rats. The animals were kept in a restraint cage equipped with a funnel and a glass container to collect the urine.

2.4.2 Metabolism of (Z)- and (E)- 3-ene VPA

(Z)- and (E)-3-ene VPA (150 mg/kg dose) were separately administrated i.p. to two rats (adult male Wistar rats weighing 270 and 308 g). A blood sample from each rat was taken 2 h after the dose, allowed to clot, and centrifuged to obtain a serum sample. Urine samples were collected every 24 hours for two days. Samples were stored at -20 ^{o}C until derivatized and analyzed by GCMS using EI techniques.

2.5 Calibration Curves

To analyze VPA, [$^{2}H_{6}$]VPA and their metabolites in the steady-state study, calibration curves having different concentration ranges were prepared for urine, serum total, and serum free (saliva) samples respectively. Table 1 summarizes the concentrations of stock solutions of VPA and VPA metabolites used in the preparation of the different calibration curves.

Table 1: Stock solution concentrations (ug/mL) used for the preparation of calibration curves for VPA, [$^2H_6$]VPA and their metabolites.

COMPOUNDS	URINE	SERUM TOTAL	SERUM FREE (SALIVA)
VPA	509.2	124.4	12.4
4-ene VPA	1.5	1.5	0.15
(E+Z)-3-ene VPA	0.985	1.97	0.197
(Z)-2-ene VPA	4.188		
(E)-2-ene VPA	103.6	20.43	2.043
(E)-2,4-diene VPA	23	1.02	0.102
(E,E)-2,3′-diene VPA	49.2	5.03	0.503
3-keto VPA	300	10	1
4-keto VPA	40.8	1.04	0.104
3-OH VPA	28.3	0.98	0.098
4-OH VPA	52.8	1.908	0.1908
5-OH VPA	53.4	0.95	0.095
2-PSA	19.7	0.124	0.0124
2-PGA	99.6	1.004	0.1004

Calibration curves for VPA, [$^2H_6$]VPA and their metabolites were made from samples prepared by the following dilution of the stock solution described in Table 1.

Amount of stock solution(%)*	Control or water**(%)
100	0
80	20
60	40
40	60
20	80
0	100

* total amount: 1 mL for EI, 0.25 mL for NCI.

** water was used as a control for serum free.

A second set of calibration curves was prepared to analyze the samples from the pharmacokinetic study in sheep which were administered a single dose of VPA:[$^{13}C_4$]VPA (50:50). Table 2 summarizes the concentrations of stock solutions used for the dilution of VPA and VPA metabolites for the calibration standard samples.

Table 2: Stock solution concentrations (ug/mL) used for the preparation of calibration curves for VPA, [$^{13}C_4$] VPA and their metabolites.

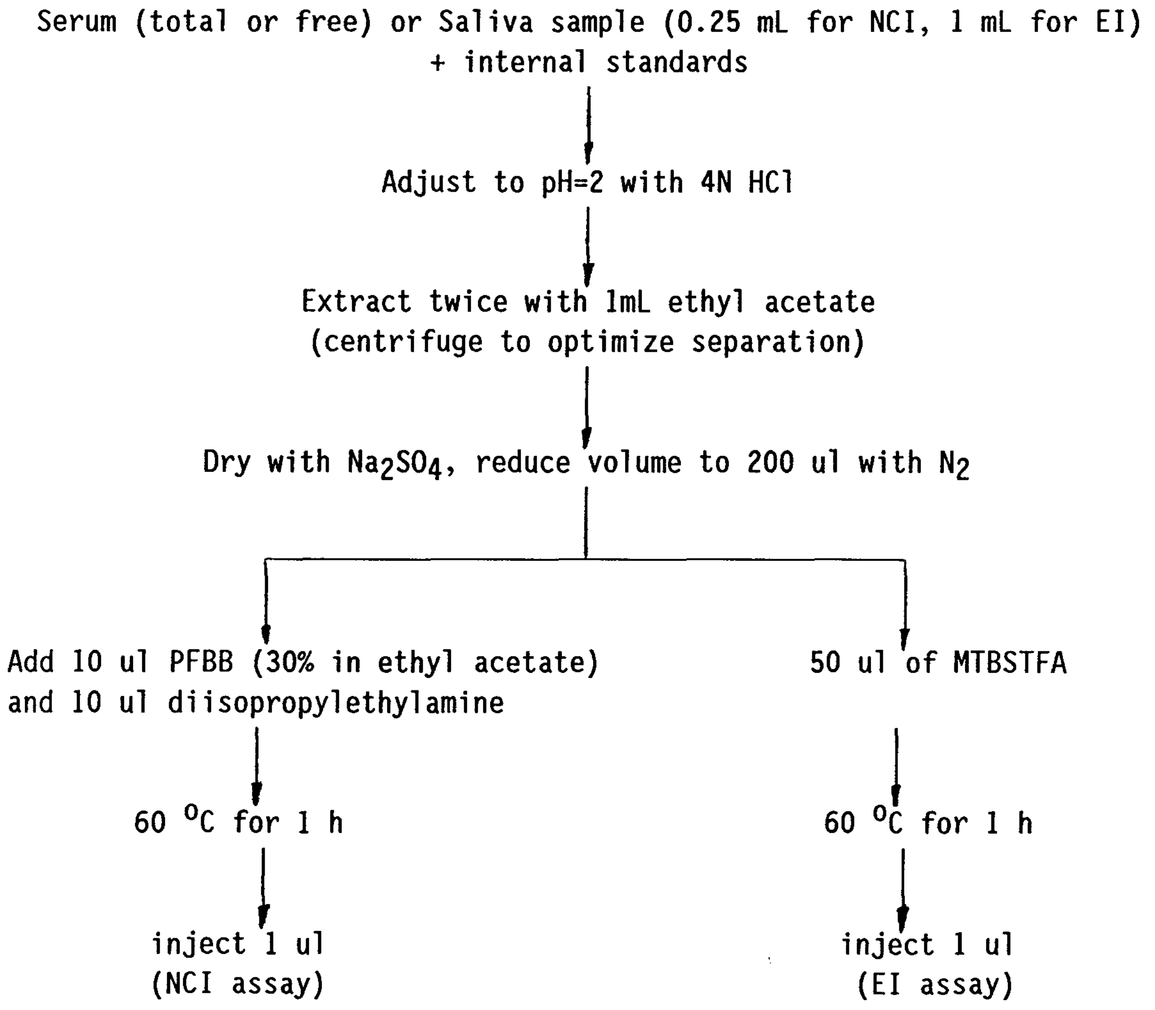
COMPOUNDS	Concentration (ug/mL)
VPA	20
4-ene VPA	2
(E+Z)-3-ene VPA	4
(Z)-2-ene VPA	20
(E)-2-ene VPA	20
(E)-2,4-diene VPA	4
(E,E)-2,3′-diene VPA	8
3-keto VPA	4
4-keto VPA	2
3-OH VPA	4
4-OH VPA	4
5-OH VPA	2
2-PSA	2
2-PGA	2

Calibration curves for VPA, [$^{13}C_4$]VPA and their metabolites were made from samples prepared by the following dilution of the stock solution described in Table 2.

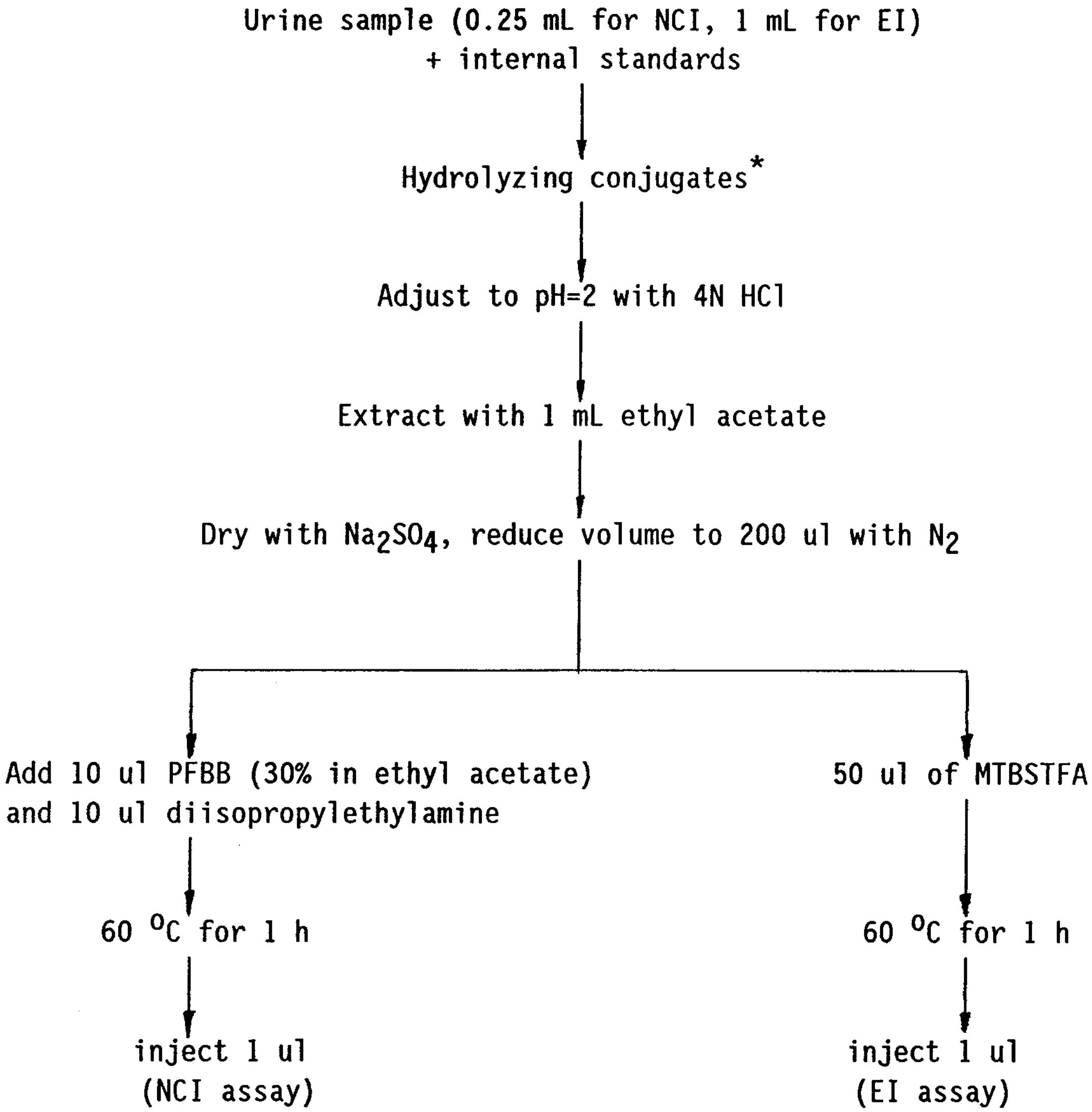
Amount of stock solution(%)*	Control or water**(%)
100	0
50	50
25	75
12.5	87.5
6.25	93.75
1.56	98.44

2.5 Extraction and Derivatization

Extraction and derivatization procedures for the biological samples are summarized in Schemes 1 and 2.


Serum (total or free) or Saliva sample (0.25 mL for NCI, 1 mL for EI)
+ internal standards
Adjust to pH=2 with 4N HCl
Extract twice with 1mL ethyl acetate
(centrifuge to optimize separation)
Dry with Na_2SO_4, reduce volume to 200 ul with N_2
Add 10 ul PFBB (30% in ethyl acetate)
and 10 ul diisopropylethylamine
50 ul of MTBSTFA
60 °C for 1 h
60 °C for 1 h
inject 1 ul
(NCI assay)
inject 1 ul
(EI assay)

Scheme 1: Sample handling procedure for serum (total and free) and saliva samples.


Urine sample (0.25 mL for NCI, 1 mL for EI)
+ internal standards
Hydrolyzing conjugates*
Adjust to pH=2 with 4N HCl
Extract with 1 mL ethyl acetate
Dry with Na2SO4, reduce volume to 200 ul with N2
Add 10 ul PFBB (30% in ethyl acetate)
and 10 ul diisopropylethylamine
60 °C for 1 h
inject 1 ul
(NCI assay)
50 ul of MTBSTFA
60 °C for 1 h
inject 1 ul
(EI assay)

Scheme 2: Sample handling procedure for urine samples.

* The glucuronide conjugates in urine were hydrolyzed using two techniques: (i). enzymatic hydrolysis by β-glucuronidase at $37^{o}C$ for 24 h (ii). alkaline (NaOH solution, pH=12) hydrolysis at $60^{o}C$ for 1 h.

All samples were run in selected ion monitoring (SIM) mode. Table 3 summarizes the ions of derivatized VPA and metabolites that were monitored for the EI or NCI ionization techniques.

Table 3: Mass to charge (m/z) for the internal standards (*), VPA, and VPA metabolites that were used for ion monitoring in the NCI (PFB derivatives) and EI (t-BDMS derivatives) mode.

COMPOUNDS	NCI $(M-181)^-$	EI $(M-57)^+$
Dibutyl acetic acid*	171	229
VPA	143	201
(E)-[$^2H_3$] 2-ene VPA*	144	202
4-ene VPA	141	199
(E+Z)-3-ene VPA	141	199
(Z)-2-ene VPA	141	199
(E)-2-ene VPA	141	199
(E)-2,4-diene VPA	139	197
(E,E)-2,3′-diene VPA	139	197
4-keto VPA	157	215
3-OH VPA	231	217
4-OH VPA	231	217
5-OH VPA	231 (monoderiv)	331 (dideriv)
[$^2H_3$]3-keto VPA*	232 (dideriv)	332 (dideriv)
	160 (monoderiv)	218 (monoderiv)
3-keto VPA	229 (dideriv)	329 (dideriv)
	157 (monoderiv)	215 (monoderiv)
2-MGA*	325	317
2-PSA	339	331
2-PGA	353	345

2.7 Calculation and Data Evaluation

2.7.1 Pharmacokinetic parameters

The pharmacokinetic parameters, area under the curve (AUC), half-life ($t_{1/2}$), elimination rate constant (K_E), distribution volume (V_d), and clearance (CL) of [$^2H_6$]VPA and some of its metabolites were calculated using the equations of Gibaldi and Perrier (1982) and compared with those of VPA and its metabolites.

2.7.2 Isotope effects

Isotope effects of [$^2H_6$]VPA were measured from a) serum data based on the AUC ratio of [$^2H_0$]VPA and its metabolites to [$^2H_6$]VPA and its deuterated metabolites, and b) urine data determined from the recovery ratio of [$^2H_0$]VPA and its metabolites to [$^2H_6$]VPA and its metabolites in urine samples collected for 12 hours after the final dose.

Isotope effects of [$^{13}C_4$]VPA was measured from serum data based on the concentration ratio of VPA and its metabolites to [$^{13}C_4$]VPA and its metabolites.

2.7.3 Conjugated fraction of VPA and its metabolites in urine samples

The glucuronide conjugates in urine were hydrolyzed using two techniques, enzymatic hydrolysis by β-glucuronidase at 37°C for 24 hours and alkaline (NaOH solution, pH=12) hydrolysis at 60°C for 1 hour. The efficacy of the two hydrolyzing methods and whether any β-glucuronidase-resistant conjugates exist or not were determined by comparing the results of the two hydrolysis methods. The conjugated fractions were determined from the concentration differences of free drug and metabolites in hydrolyzed and unhydrolyzed urine samples respectively.

2.7.4 Evaluation of Data

A MIDAS based computer program containing methodological statistics was used to evaluate the two analytical methods, NCI and EI, and to compare the efficiencies of the two hydrolysis methods, β-glucuronidase and treatment with alkali (NaOH solution, pH=12).

3. Results and Discussion

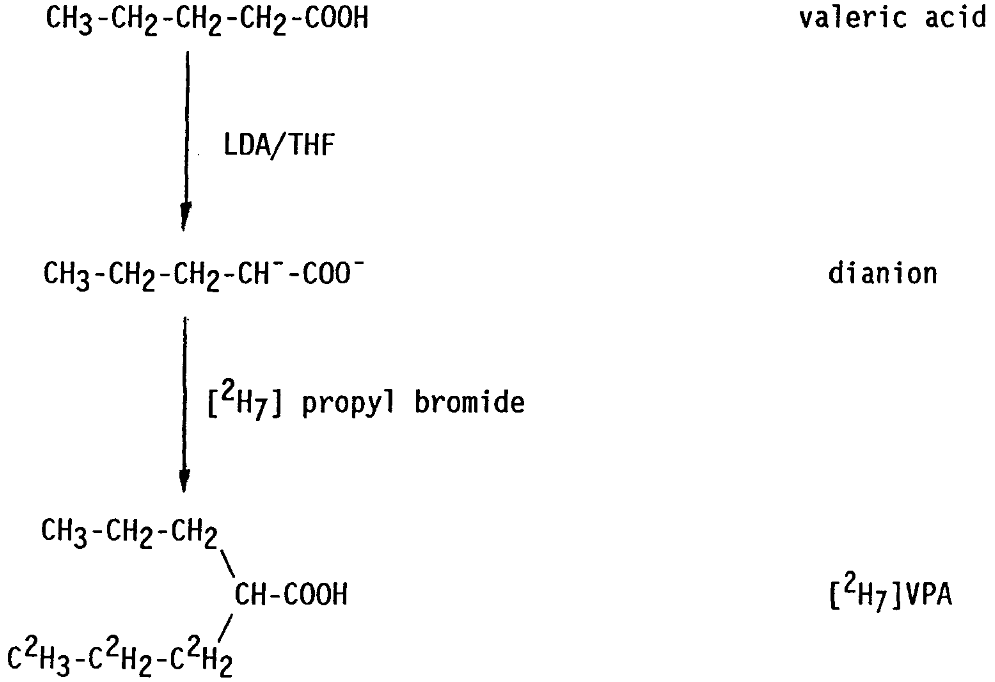
3.1 Synthesis of deuterium labelled internal standards

Stable isotope-labelled analogs provide optimal internal standards for GCMS assays. By using stable isotope-labelled analogs as internal standards, the lowest variance factors due to sample manipulation and instrumental errors was produced (Claeys *et al.*, 1977). Ideally the labelled internal standard should have a mass difference of at least 3 mass units from the analyte to minimize interference from natural isotopes and thus avoid correction for mass overlap. While $[^2H_6]$VPA is suitable for VPA analysis ($\Delta u = 6$), it is not a good internal standard for $[^{13}C_4]$VPA ($\Delta u = 2$). Based on the above principle, $[^2H_7]$VPA should provide an ideal internal standard for both $[^{13}C_4]$VPA and VPA analysis by GCMS. The previously reported assays (Abbott *et al.*, 1986; Acheampong *et al.*, 1983; Kassahun *et al.*, 1989) for VPA metabolites from this laboratory could also be improved by the availability of adequate stable isotope labelled internal standards of the metabolites. Thus, syntheses were carried out to produce several deuterium labelled VPA metabolites.

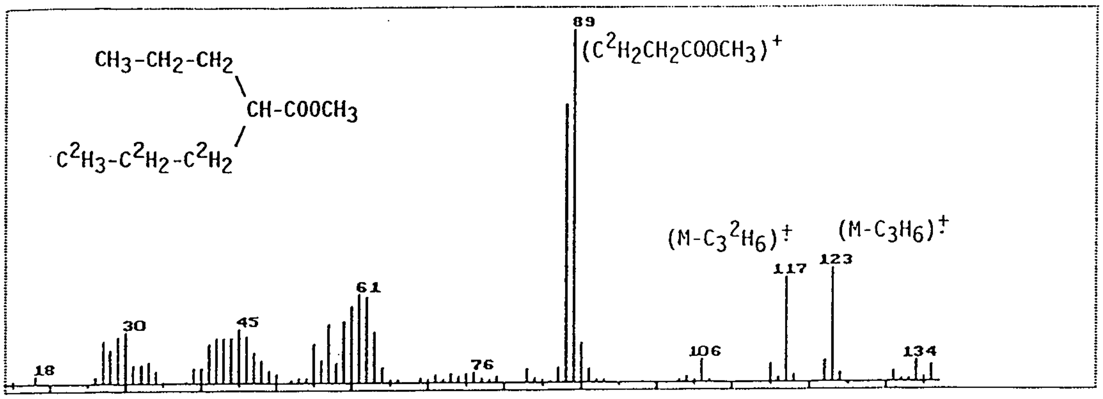
3.1.1 Synthesis of $[^2H_7]$VPA

$[^2H_7]$VPA was synthesized by a method for the alkylation of α-metalated aliphatic acids (Pfeffer *et al.*, 1972). The procedure is outlined in scheme 3. Distilled pentanoic acid was converted into the α-anion by means of two equivalents of lithium diisopropylamide in tetrahydrofuran (THF)-hexane solution. Hexamethylphosphoramide (HMPA) was added to the mixture to make the reaction more specific and efficient, due to its chemical and physical properties. First, the metalated straight-chain acids are insoluble in THF, but readily dissolve in the highly dipolar solvent, HMPA; second, because organolithium compounds are known to associate to higher molecular weight aggregates, the degree of polymerization (n=2-6) varying with solvent and structure (Mallan and Bebb, 1969), the aggregation state is disrupted by complexation with polar solvents in the formation of a solvent-separated ion pair of higher reactivity than the original aggregate. A strong dipolar solvent like HMPA should accordingly be more efficient in association with metalated carboxylates than THF by forming a complex of higher reactivity, presumably a solvent-separated ion pair (Pfeffer *et al.*, 1972). Pfeffer *et al.* (1972) also illustrated by comparative experiments that HMPA, in addition to solubilising dianions, also accelerates the alkylation rates, and quantitative conversion was observed in HMPA solution when 1.5 equivalents of alkyl bromide was added.

This method was first used to synthesize VPA in this lab by Acheampong (1985). The same method was applied in synthesizing $[^2H_7]$VPA, the only difference being the use of $[^2H_7]$propyl bromide. Since $[^2H_7]$propyl bromide is very expensive, we could not afford to redistill it before use, hence, the yield is less than the reference (91%, Lee, 1987). The final product was confirmed to be $[^2H_7]$VPA by comparing its GCMS mass spectrum and NMR spectrum with unlabeled VPA. The mass spectrum of the methyl ester derivative of $[^2H_7]$VPA which was prepared by derivatizing with diazomethane (Levitt, 1973) revealed fragment ions at m/z 136$(M-C_2H_5)^+$, 123$(M-C_3H_6)^+$, 117$(M-C_3{}^2H_6)^+$, and 89$(C^2H_2CH_2COOCH_3)^+$, corresponding to VPA fragments at m/z 129$(M-C_2H_5)^+$, 116$(M-C_3H_6)^+$, and 87$(CH_2CH_2COOCH_3)^+$ respectively (Figure 3). The ^1H NMR of the synthesized product also confirmed the $[^2H_7]$VPA structure (Figure 4).


$CH_3-CH_2-CH_2-CH_2-COOH$
valeric acid
LDA/THF
$CH_3-CH_2-CH_2-CH^--COO^-$
dianion
$[^2H_7]$ propyl bromide
$CH_3-CH_2-CH_2$
CH-COOH
$C^2H_3-C^2H_2-C^2H_2$
$[^2H_7]$VPA

Scheme 3: Synthesis of $[^2H_7]$VPA.



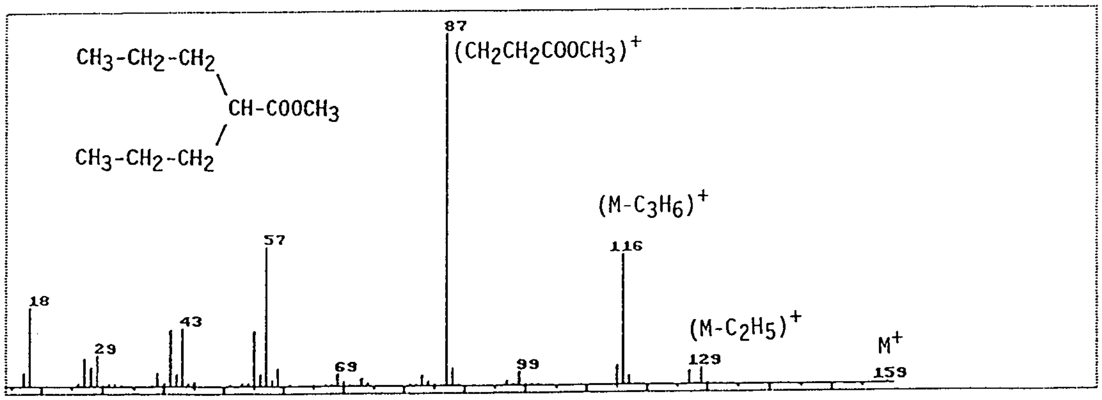


Fig. 3. GCMS mass spectra of the methyl esters of [$^2H_7$]VPA (top) and VPA (bottom).

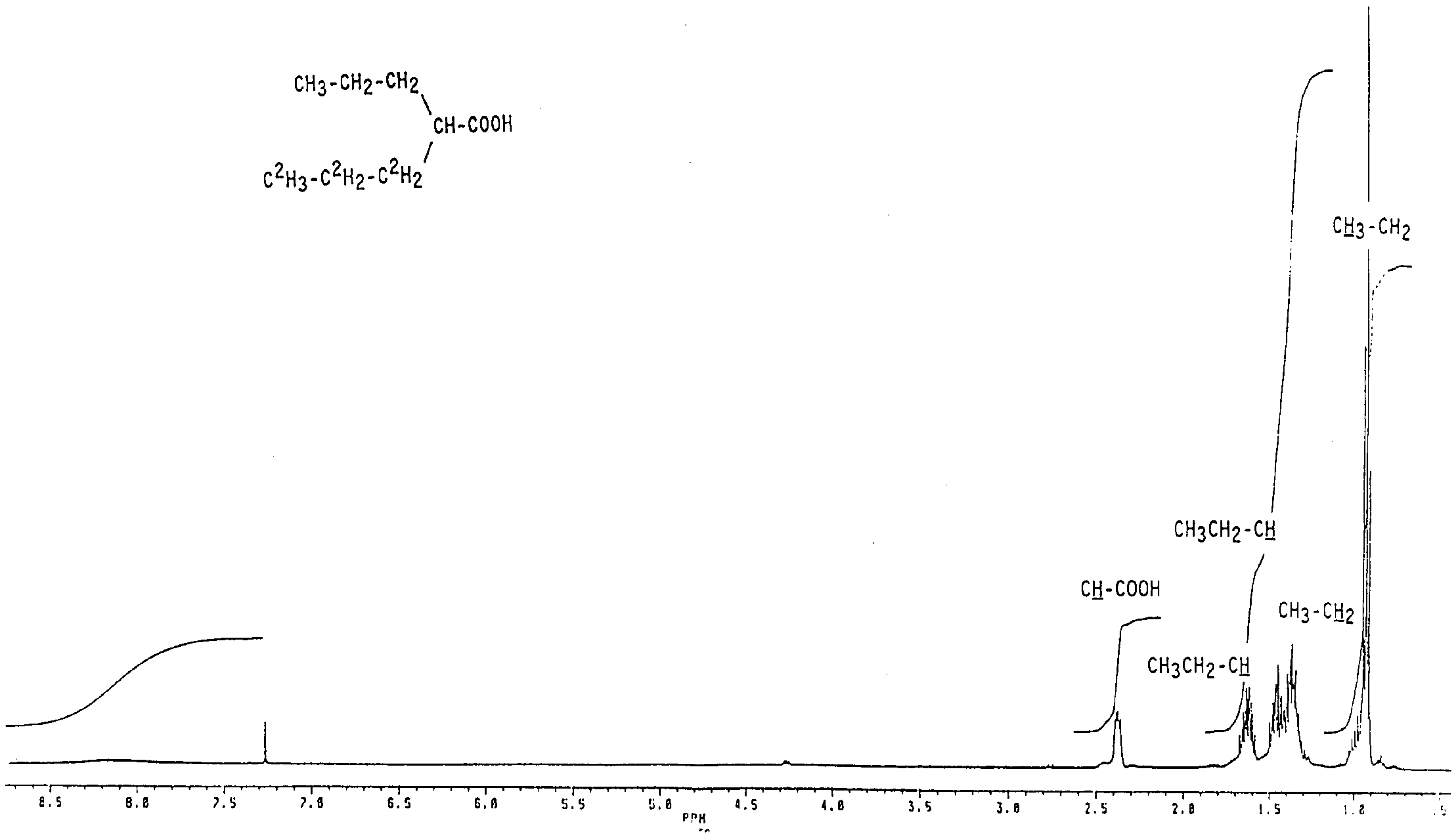
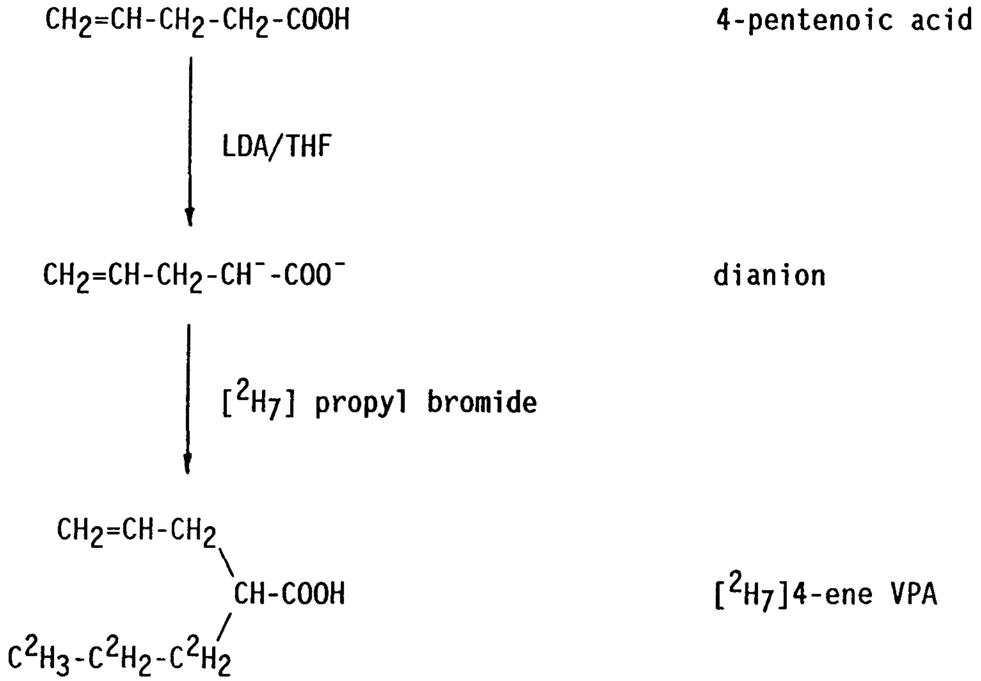


Fig. 4: ^1H NMR spectrum of $[^2H_7]$VPA.

3.1.2 Synthesis of [$^2H_7$]4-ene VPA

4-Ene VPA can usually be prepared *via* its ethyl ester, by reaction of ethyl valerate and allyl bromide according to the general method for the alkylation of esters at the α-carbon atom (Cregge, *et al.*, 1973). However, this method was not suitable for introducing the [$^2H_7$] deuterium labelled side chain. It turns out the α-metalation method was also applied by Pfeffer (1972) to olefinic acids. For example, 10-undecenoic and (Z)-9-octadecenoic acids, were converted to their α anions and then alkylated in good yields to the α-branched chain unsaturated acids. The isolated double bonds in these acids were found to be unaltered positionally or geometrically in the formation of their dianions by lithium diisopropylamide. An attempt to synthesize [$^2H_7$]4-ene VPA using this method was performed by starting first with the synthesis of unlabeled 4-ene VPA. Good yields were obtained for 4-ene VPA, and thus [$^2H_7$] 4-ene VPA was synthesized in this study starting from 4-pentenoic acid (Scheme 4) by Pfeffer's method.

[$^2H_7$]4-ene VPA was successfully purified by fractional distillation. The GCMS mass spectrum of the methyl ester of [$^2H_7$]4-ene VPA was compared with that of the methyl ester of 4-ene VPA (Figure 5). Fragments of [$^2H_7$]4-ene VPA occurred at m/z 163 (M^+), 129 $(M-C_2{}^2H_5)^+$, 113$(M-C_3{}^2H_7)^+$, and 104$(M-COOCH_3)^+$ and correspond to those of 4-ene VPA at m/z 156 (M^+), 127 $(M-C_2H_5)^+$, 113$(M-C_3H_7)^+$, and 97$(M-COOCH_3)^+$. ^{1}H-NMR (Figure 6) confirmed the presence of the terminal double bond: δ 5.78 (m, 1H, $CH_2=C\underline{H}$), 5.12 (d, 1H, J=17 Hz, proton at C-5 trans to proton at C-4), 5.08 (d, 1H, J=11 Hz, proton at C-5 cis to proton at C-4).


$CH_2=CH-CH_2-CH_2-COOH$
4-pentenoic acid
LDA/THF
$CH_2=CH-CH_2-CH^--COO^-$
dianion
$[^2H_7]$ propyl bromide
$CH_2=CH-CH_2$
CH-COOH
$C^2H_3-C^2H_2-C^2H_2$
$[^2H_7]$4-ene VPA

Scheme 4: Synthesis of $[^2H_7]$4-ene VPA.

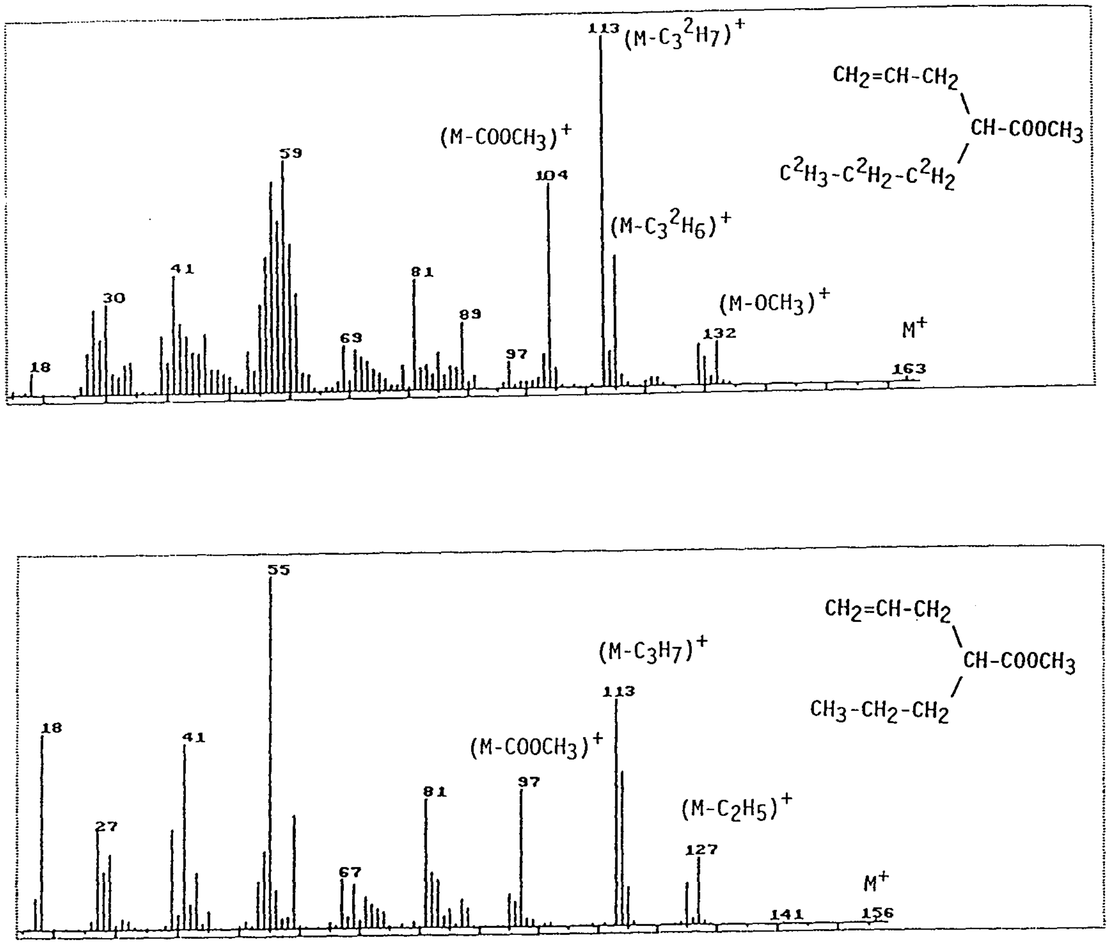


Fig. 5: GCMS mass spectra of the methyl esters of 4-ene [$^2H_7$]VPA (top) and 4-ene VPA (bottom).

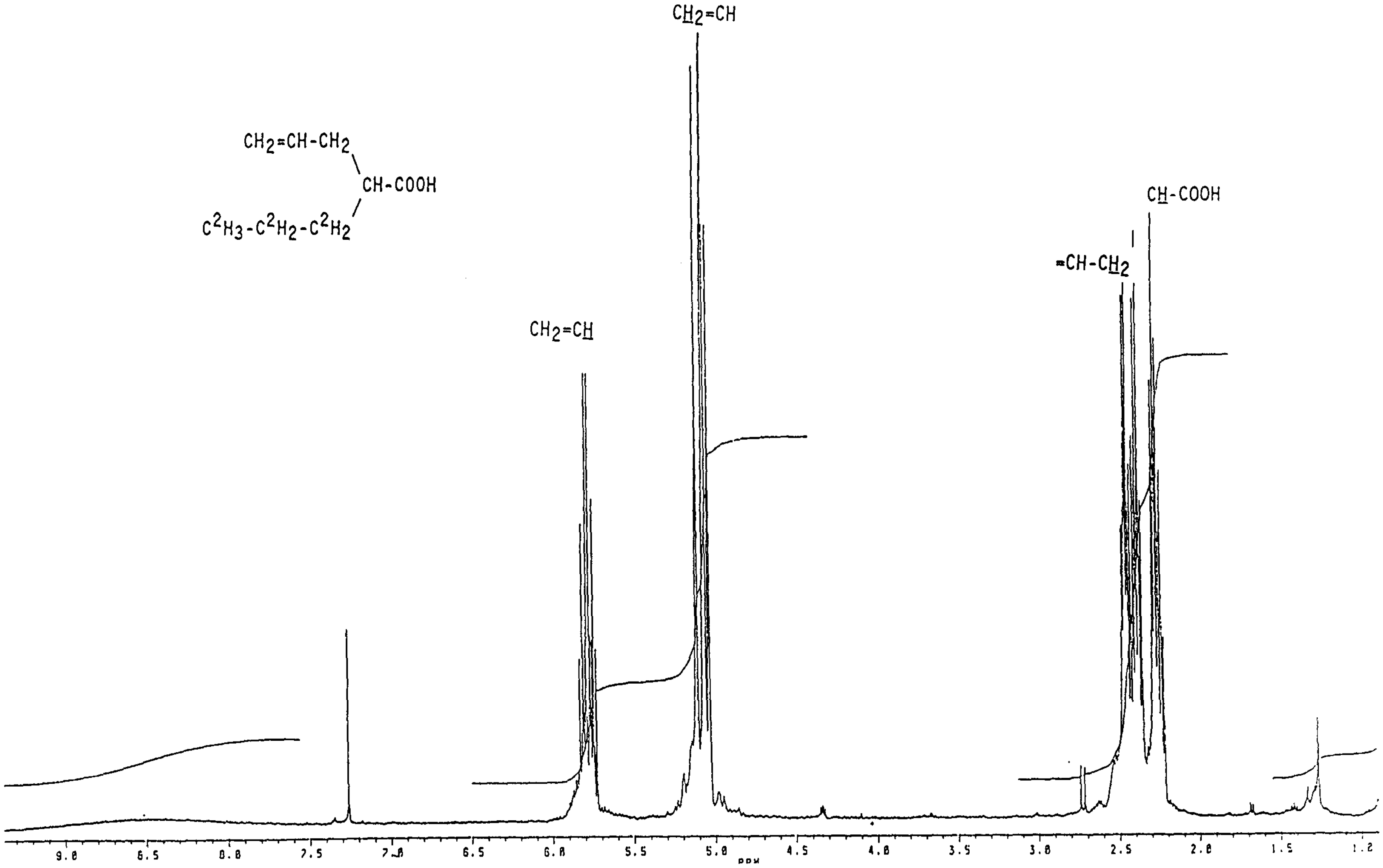
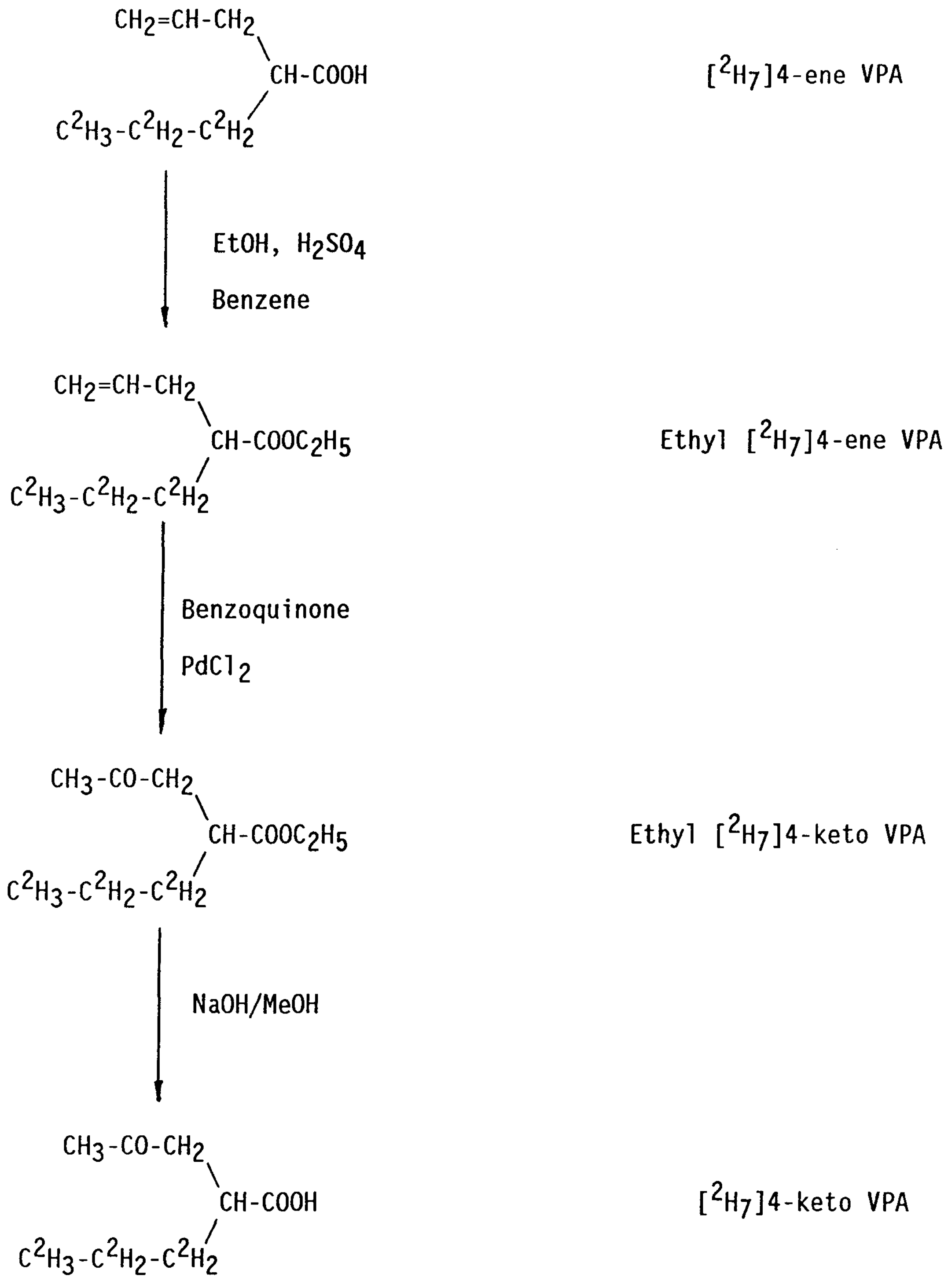


Fig. 6: ^1H NMR spectrum of $[^2H_7]$4-ene VPA.

3.1.3 Synthesis of [$^2H_7$]4-keto VPA

Ethyl [$^2H_7$]4-ene VPA was oxidized by benzoquinone to ethyl [$^2H_7$]4-keto VPA using palladium chloride as catalyst. This procedure is well-known as the Wacker process, and is one of the most important industrial processes employing transition metal catalysts (Smidt *et al.*, 1959). There are several methods which can selectively oxidize terminal double bonds to methyl ketones; of them, the palladium(II) chloride-catalyzed oxidation of terminal olefins seems to be one of the best (Tsuji, 1984). Since the oxidation proceeds under mild conditions, various functional groups, such as esters, carboxylic acids, aldehydes, are unchanged during the reaction (Tsuji, 1984). The conversion of ethyl [$^2H_7$]4-ene VPA to ethyl [$^2H_7$]4-keto VPA was quantitatively completed in 22 hours. Scheme 5 summarizes the synthesis procedure of [$^2H_7$]4-keto VPA.

The GCMS mass spectrum of ethyl [$^2H_7$]4-keto VPA (Figure 7) revealed a molecular ion (m/z 193) and other fragments, m/z 148 $(M\text{-}OCH_2CH_3)^+$, 136 $(M\text{-}CH_3COCH_2)^+$, and 108 $(C^2H_3C^2H_2C^2H_2CH_2COO)^+$, which correspond to ethyl 4-keto VPA fragments, m/z 186 (M^+), 141 $(M\text{-}OCH_2CH_3)^+$, 129 $(M\text{-}CH_3COCH_2)^+$, and 101 $(CH_3CH_2CH_2CH_2COO)^+$.


CH2=CH-CH2
CH-COOH
C2H3-C2H2-C2H2
[2H7]4-ene VPA
EtOH, H2SO4
Benzene
CH2=CH-CH2
CH-COOC2H5
C2H3-C2H2-C2H2
Ethyl [2H7]4-ene VPA
Benzoquinone
PdCl2
CH3-CO-CH2
CH-COOC2H5
C2H3-C2H2-C2H2
Ethyl [2H7]4-keto VPA
NaOH/MeOH
CH3-CO-CH2
CH-COOH
C2H3-C2H2-C2H2
[2H7]4-keto VPA

Scheme 5: Synthesis of $[^2H_7]$4-keto VPA.

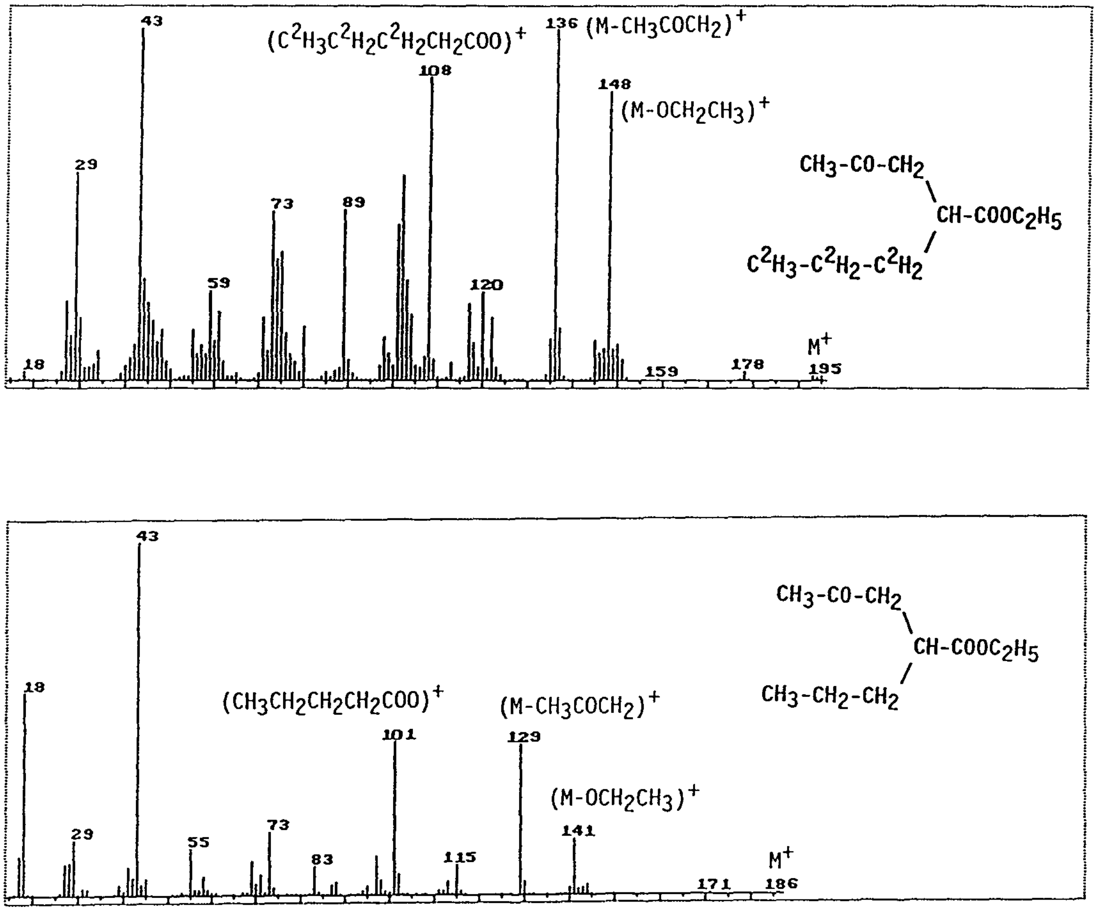
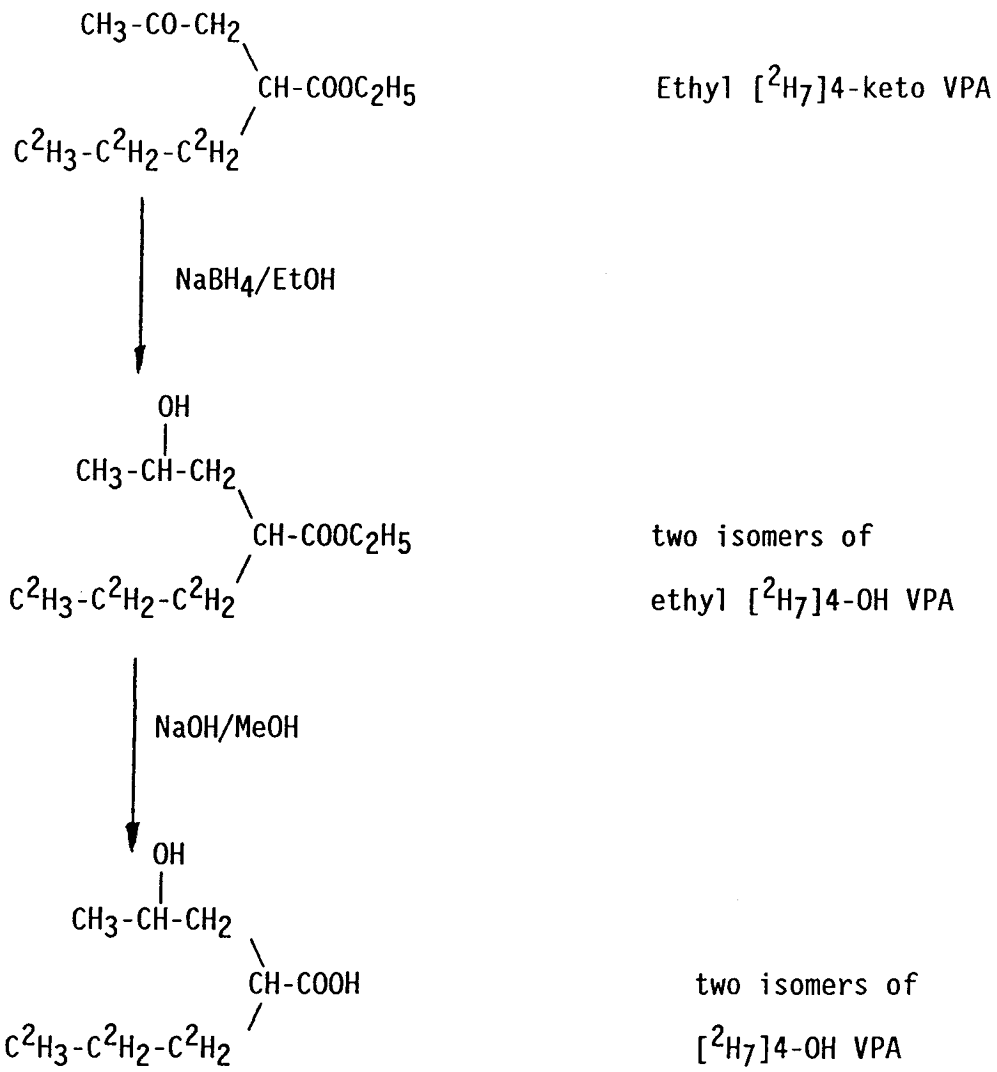


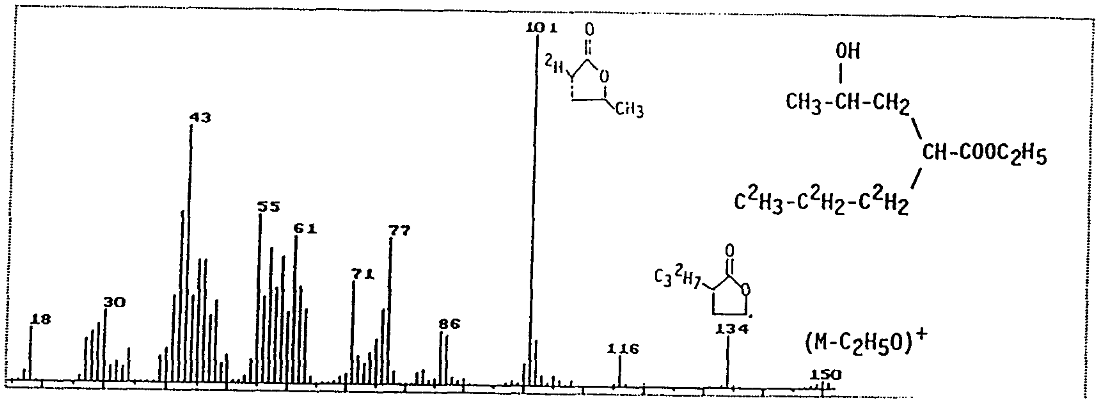
Fig. 7: GCMS mass spectra of ethyl esters of $[^2H_7]$4-keto VPA (top) and 4-keto VPA (bottom).

3.1.4 Synthesis of [$^2H_7$]4-OH VPA

The synthesis of [$^2H_7$]4-OH VPA was accomplished *via* its ethyl ester by reducing the ethyl ester of [$^2H_7$]4-keto VPA with $NaBH_4$. The reaction was quantitatively completed within 60 min at room temperature, when excess $NaBH_4$ was used. The steps in the synthesis are summarized in Scheme 6. The GCMS mass spectrum of ethyl [$^2H_7$]4-OH VPA was compared with that of unlabeled ethyl 4-OH VPA (Figure 8). The fragments of ethyl [$^2H_7$]4-OH VPA at m/z 150 ($M-C_2H_5O$), 134, 116 and 101 correspond to ethyl 4-OH VPA at m/z 143, 127, 113 and 110.


CH_3-CO-CH_2
CH-$COOC_2H_5$
C^2H_3-C^2H_2-C^2H_2
Ethyl [$^2H_7$]4-keto VPA
$NaBH_4$/EtOH
OH
CH_3-CH-CH_2
CH-$COOC_2H_5$
C^2H_3-C^2H_2-C^2H_2
two isomers of
ethyl [$^2H_7$]4-OH VPA
NaOH/MeOH
OH
CH_3-CH-CH_2
CH-COOH
C^2H_3-C^2H_2-C^2H_2
two isomers of
[$^2H_7$]4-OH VPA

Scheme 6: Synthesis of [$^2H_7$]4-OH VPA.



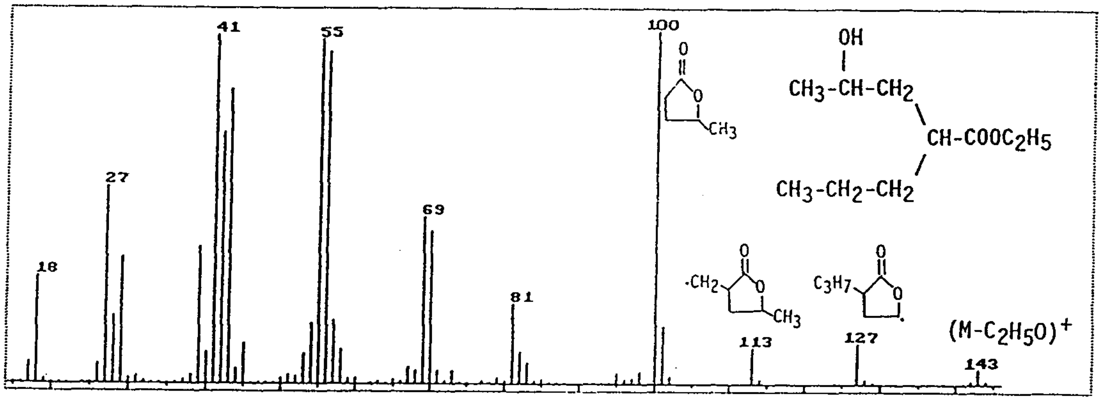
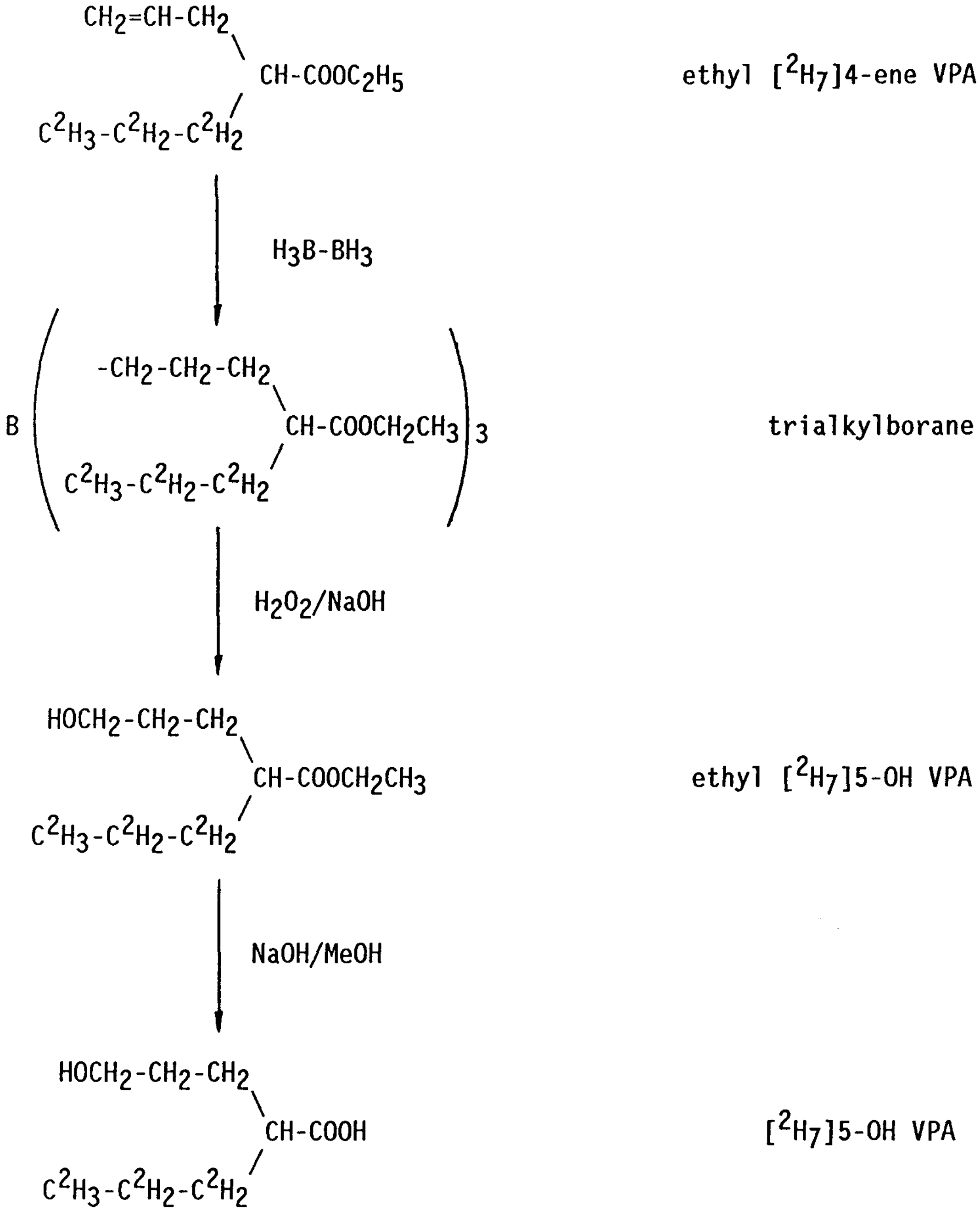


Fig. 8: GCMS mass spectra of ethyl esters of [$^2H_7$]4-OH VPA (top) and 4-OH VPA (bottom).

3.1.5 Synthesis of [$^2H_7$] 5-OH VPA

The ethyl ester of [$^2H_7$]5-OH VPA was prepared from ethyl [$^2H_7$]4-ene VPA by the same method that Rettenmeier *et al.*(1985) used to synthesize 5-OH VPA. Scheme 7 summarizes the synthesis procedure. Ethyl [$^2H_7$]4-ene VPA was hydroborated into the corresponding trialkylborane, which was then oxidized with alkaline hydrogen peroxide to provide ethyl [$^2H_7$]5-OH VPA. The GCMS mass spectrum (Figure 9) of ethyl [$^2H_7$]5-OH VPA indicates the molecular ion at m/z 195 and other fragments at m/z 164 $(M-CH_3OH)^+$, 150 $(M-C_2H_5OH)^+$, 115, and 101, corresponding to fragments of ethyl 5-OH VPA at m/z: 157 $(M-CH_3OH)^+$, 143 $(M-C_2H_5OH)^+$, 113, and 100.


ethyl [2H7]4-ene VPA
H3B-BH3
trialkylborane
H2O2/NaOH
ethyl [2H7]5-OH VPA
NaOH/MeOH
[2H7]5-OH VPA

Scheme 7: Synthesis of $[^2H_7]$5-OH VPA.

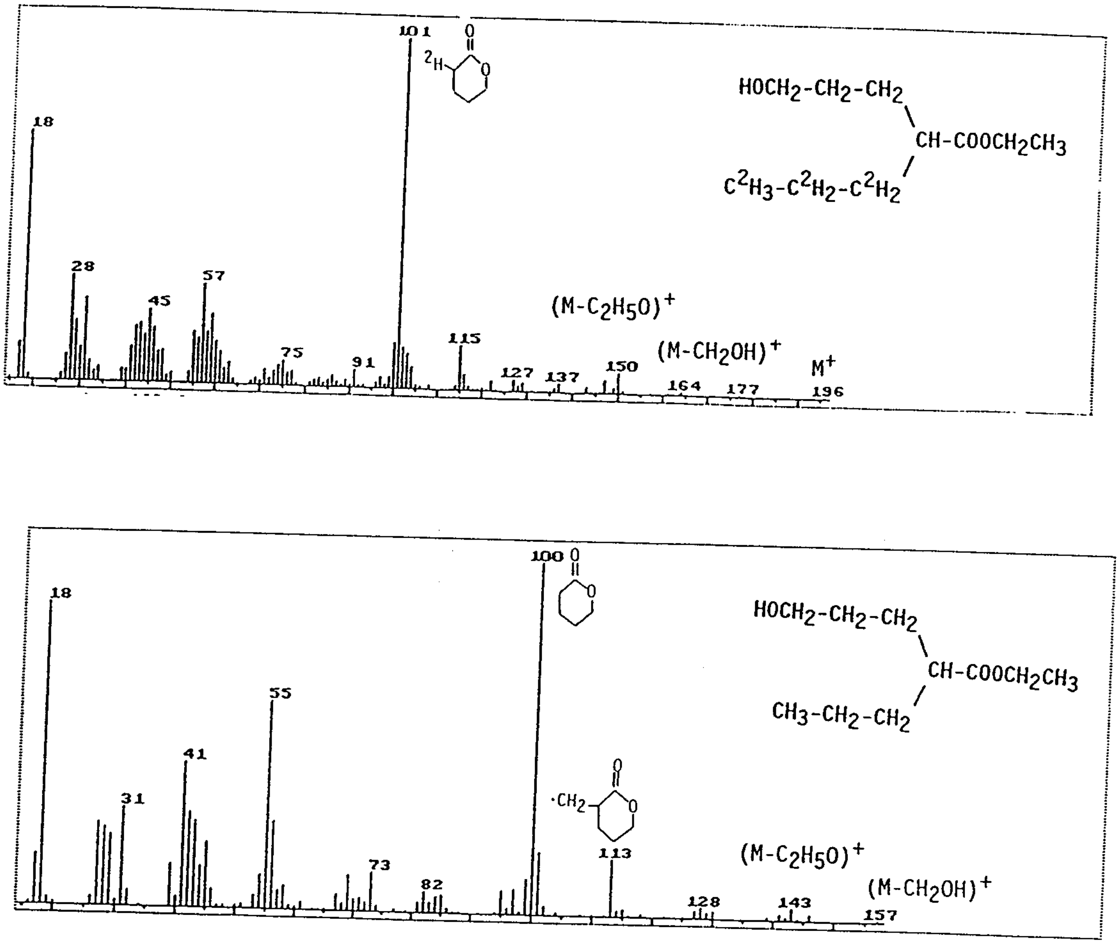


Fig. 9: GCMS mass spectra of the ethyl esters of [$^2H_7$]5-OH VPA (top) and 5-OH VPA (bottom).

3.1.6 Synthesis of [$^{2}H_{7}$]3-keto VPA

Ethyl 3-keto VPA is usually synthesized by alkylating the enolate ester of valeric acid with propionyl chloride, a very well established method for the synthesis 3-keto VPA (Acheampong, 1982). However, [$^{2}H_{7}$]pentanoic acid would have to be prepared first if this method were applied in the synthesis of [$^{2}H_{7}$]3-keto VPA. This adds to increased costs and a lower yield based on the availability of [$^{2}H_{7}$]bromopropane. It is optimal to introduce the [$^{2}H_{7}$]propyl side chain in the final step of the synthesis for considerations of expense and efficacy. Accordingly, we designed a synthesis of ethyl 3-keto pentanoate, which could then be alkylated with [$^{2}H_{7}$]bromopropane to produce the desired product.

3.1.6a Synthesis of ethyl 3-keto pentanoate:

Ethyl 3-keto pentanoate was synthesized according to the method published by Micheal *et al.*(1971) for the preparation of β-keto esters. Scheme 8 summarizes the procedure. Ethyl acetate was converted into the corresponding lithium enolate ester by reaction with lithium N-isopropylcyclohexylamide (LiICA). The enolate ester was then condensed with propionyl chloride to form the desired β-keto ester without significant self-condensation. However, attack of the enolate ester at the ketone carbonyl of the product could lead to decreased yields. This possibility was minimized by using an extra equivalent of the generating base, LiICA, thereby converting the product into the relatively inert β-keto enolate ester. The product gave only one peak in the TIC upon GCMS analysis and proved to be ethyl 3-keto pentanoate from the GCMS mass spectrum and from ^1H NMR, after purification by fractional distillation. GCMS mass spectrum (Figure 10) of the product shows a molecular ion of ethyl 3-keto-pentanoate at m/z 144, and other fragments m/z 115$(M-C_2H_5)^+$, 98$(M-C_2H_5OH)^+$, 87$(M-CH_3CH_2CO)^+$, 69$(M-CH_3CH_2COOH)^+$, 57$(CH_3CH_2CO)^+$. ^1H NMR (Figure 11) also confirmed the structure of the product to be ethyl 3-keto-pentanoate.

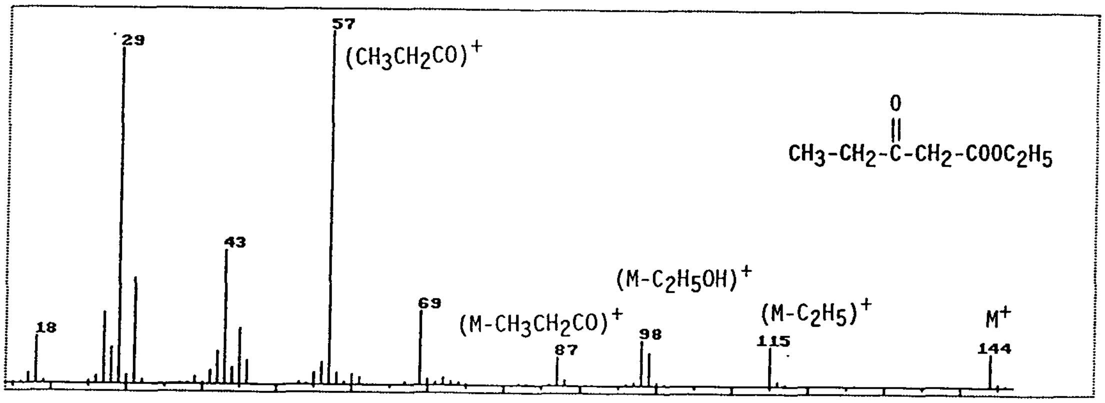


Fig. 10: GCMS mass spectra of ethyl 3-keto-pentanoate.

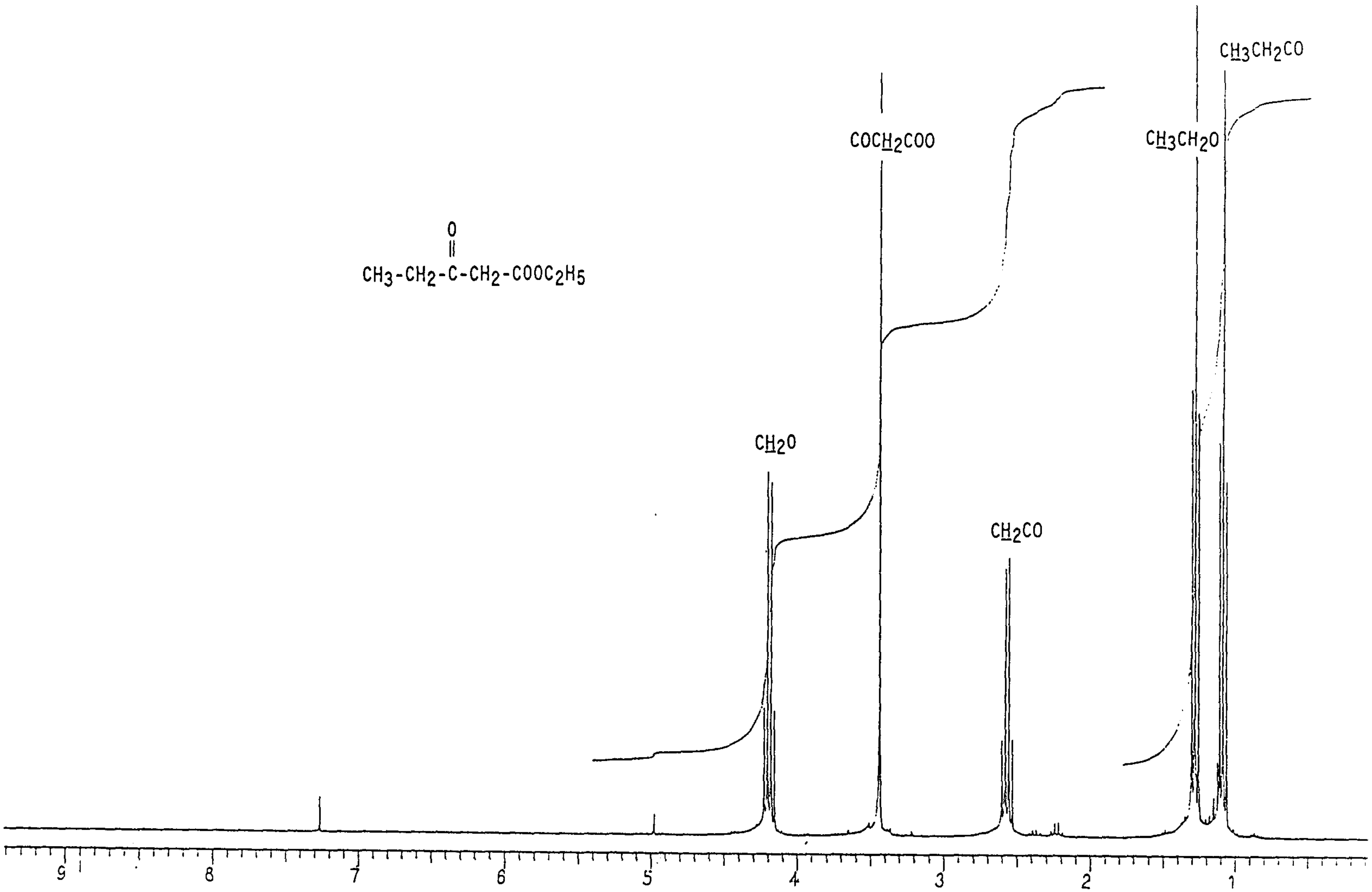
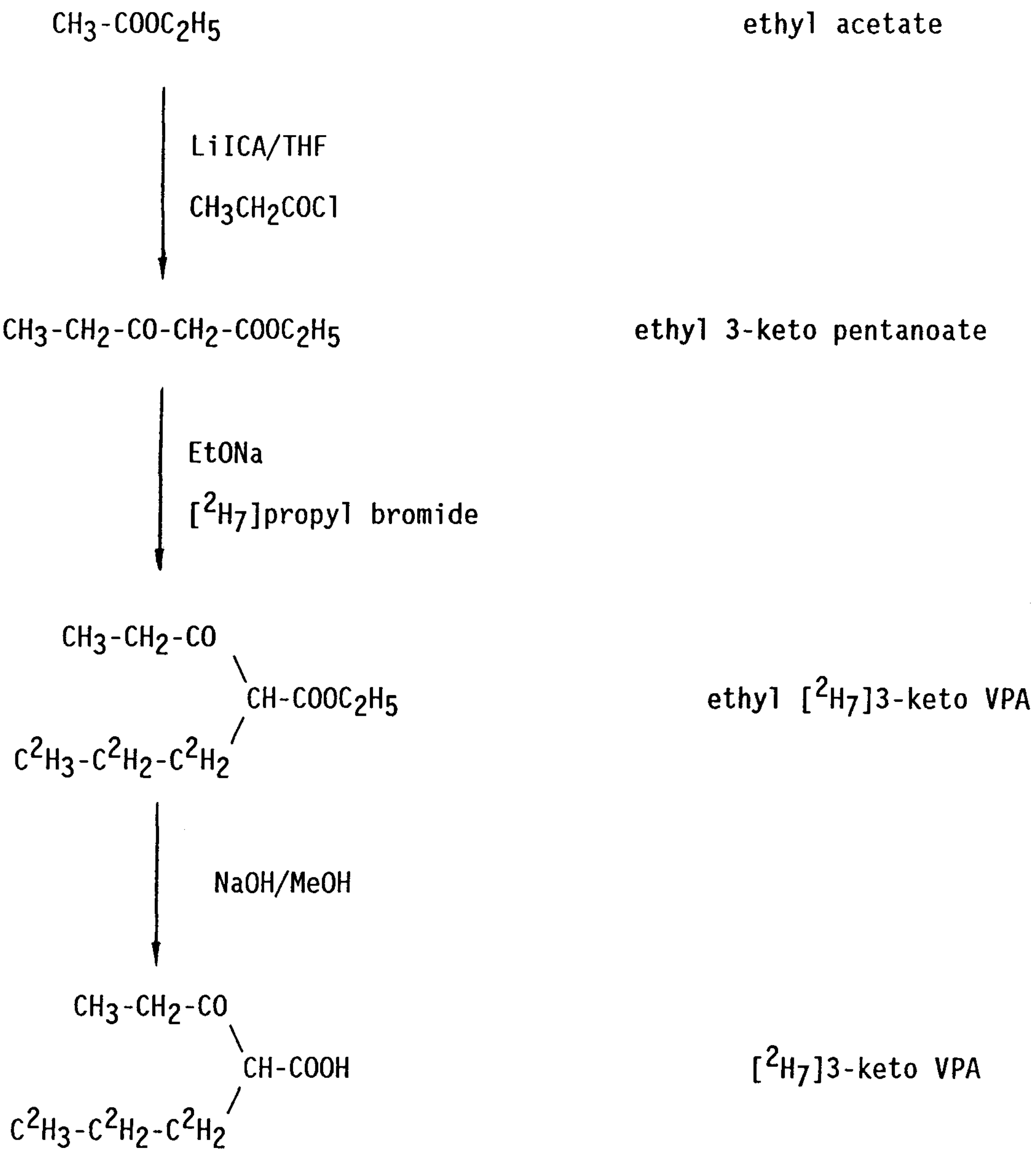


Fig. 11: ^{1}H NMR spectrum of ethyl 3-keto-pentanoate.

3.1.6b Alkylation of ethyl 3-keto pentanoate with [$^2H_7$]bromopropane

Ethyl 3-keto-pentanoate was converted into the corresponding sodium β-keto enolate ester with sodium ethoxide which was prepared by adding absolute alcohol to freshly cut Na metal. The enolate was then alkylated with [$^2H_7$]bromopropane to afford a high yield of ethyl [$^2H_7$]3-keto VPA (Scheme 8). [$^2H_7$]3-keto VPA was purified by fractional distillation. Figure 12 shows the mass spectra of ethyl [$^2H_7$]3-keto VPA and ethyl 3-keto VPA. The molecular ion (m/z 193) and other fragments of [$^2H_7$]ethyl 3-keto VPA at m/z 164 $(M-C_2H_5)^+$, 145 $(M-C_3{}^2H_6)^+$, 137 $(M-CH_2CH_2CO)^+$, 103 $(C^2H_2CH_2COOC_2H_5)^+$, correspond to those of ethyl 3-keto VPA at m/z 186 (M^+), 157 $(M-C_2H_5)^+$, 144 $(M-C_3H_6)^+$, 130 $(M-CH_2CH_2CO)^+$, 101 $(CH_2CH_2COOC_2H_5)^+$. ^1H NMR (Figure 13) of ethyl [$^2H_7$]3-keto VPA confirmed the structure and purity of product.


$CH_3-COOC_2H_5$
ethyl acetate
LiICA/THF
CH_3CH_2COCl
$CH_3-CH_2-CO-CH_2-COOC_2H_5$
ethyl 3-keto pentanoate
EtONa
[$^2H_7$]propyl bromide
CH_3-CH_2-CO
$CH-COOC_2H_5$
$C^2H_3-C^2H_2-C^2H_2$
ethyl [$^2H_7$]3-keto VPA
NaOH/MeOH
CH_3-CH_2-CO
$CH-COOH$
$C^2H_3-C^2H_2-C^2H_2$
[$^2H_7$]3-keto VPA

Scheme 8: Synthesis of [$^2H_7$]3-keto VPA.

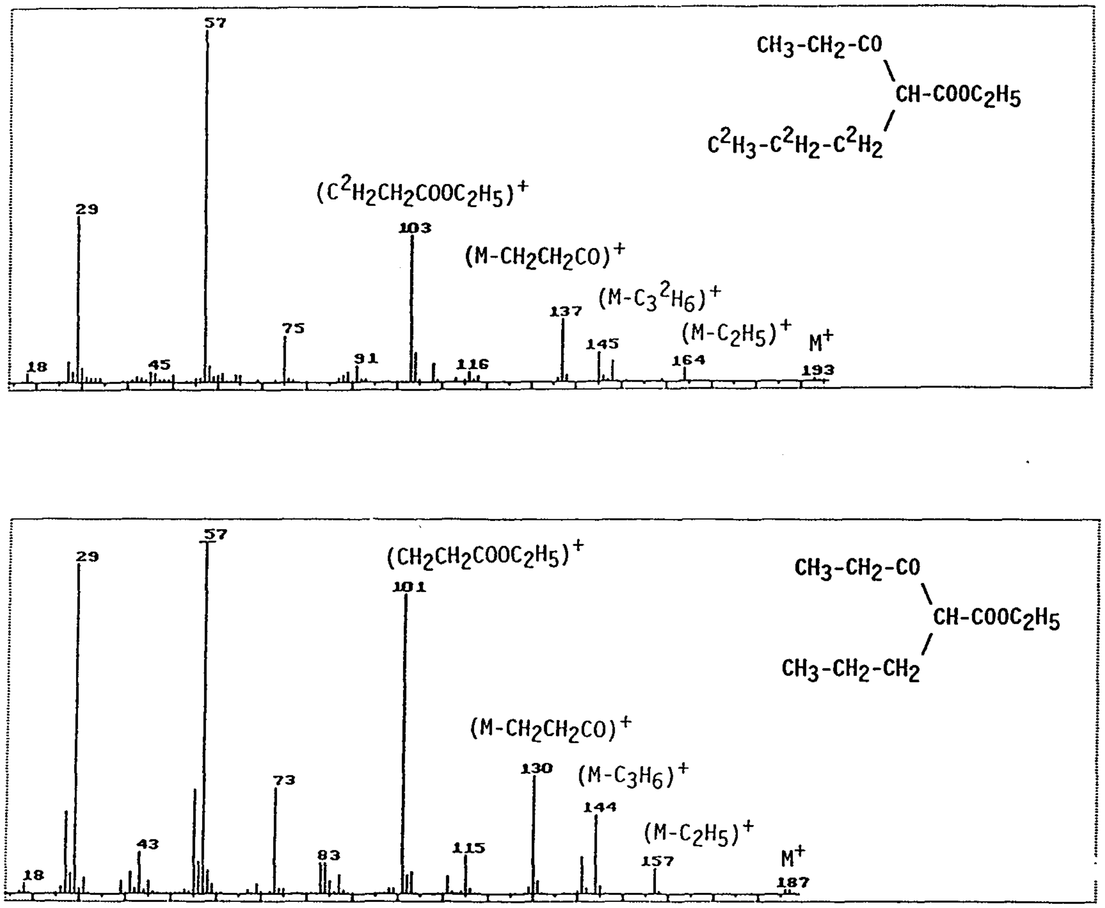


Fig. 12: GCMS mass spectra of the ethyl esters of $[^2H_7]$3-keto VPA (top) and 3-keto VPA (bottom).

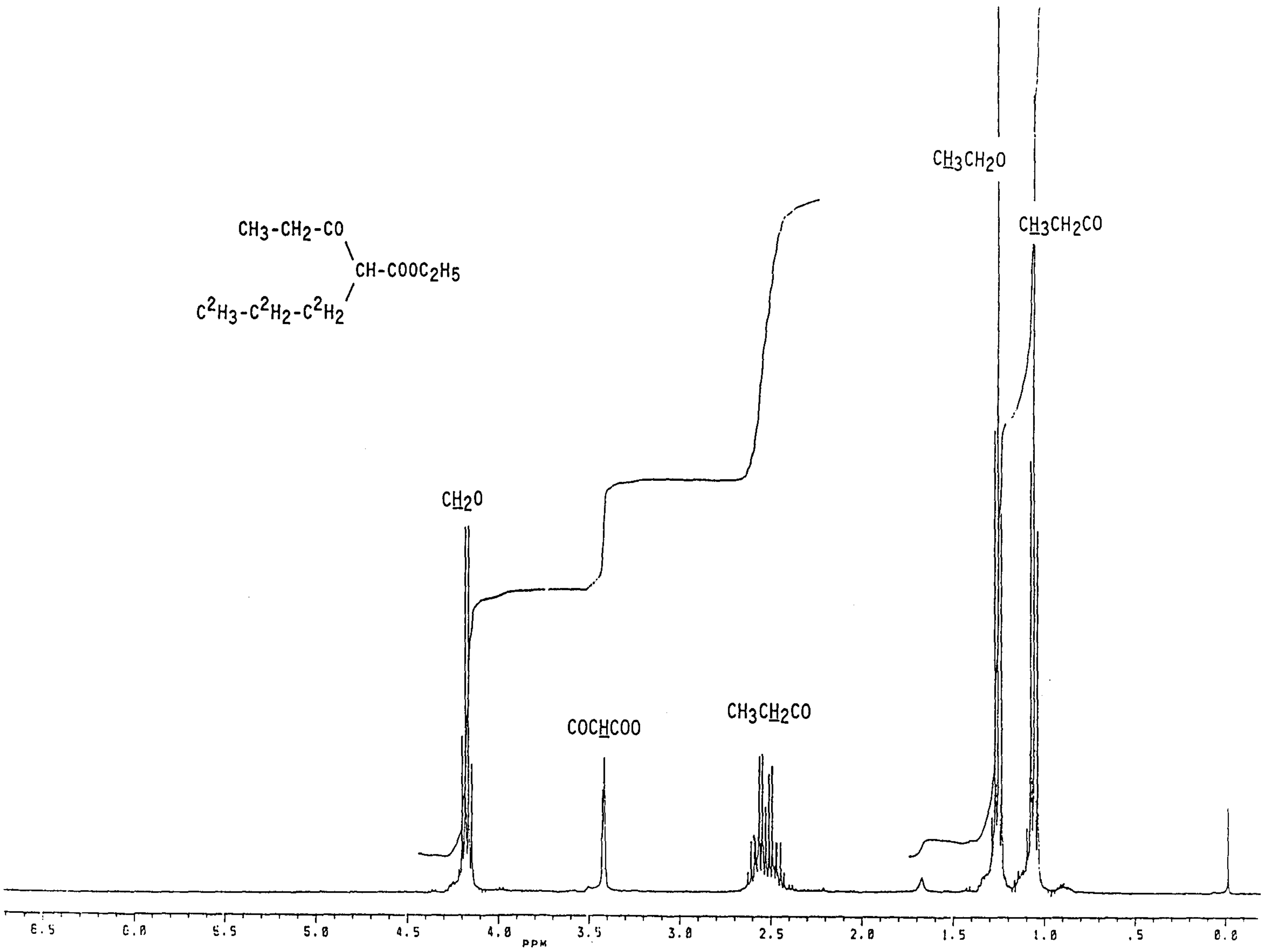
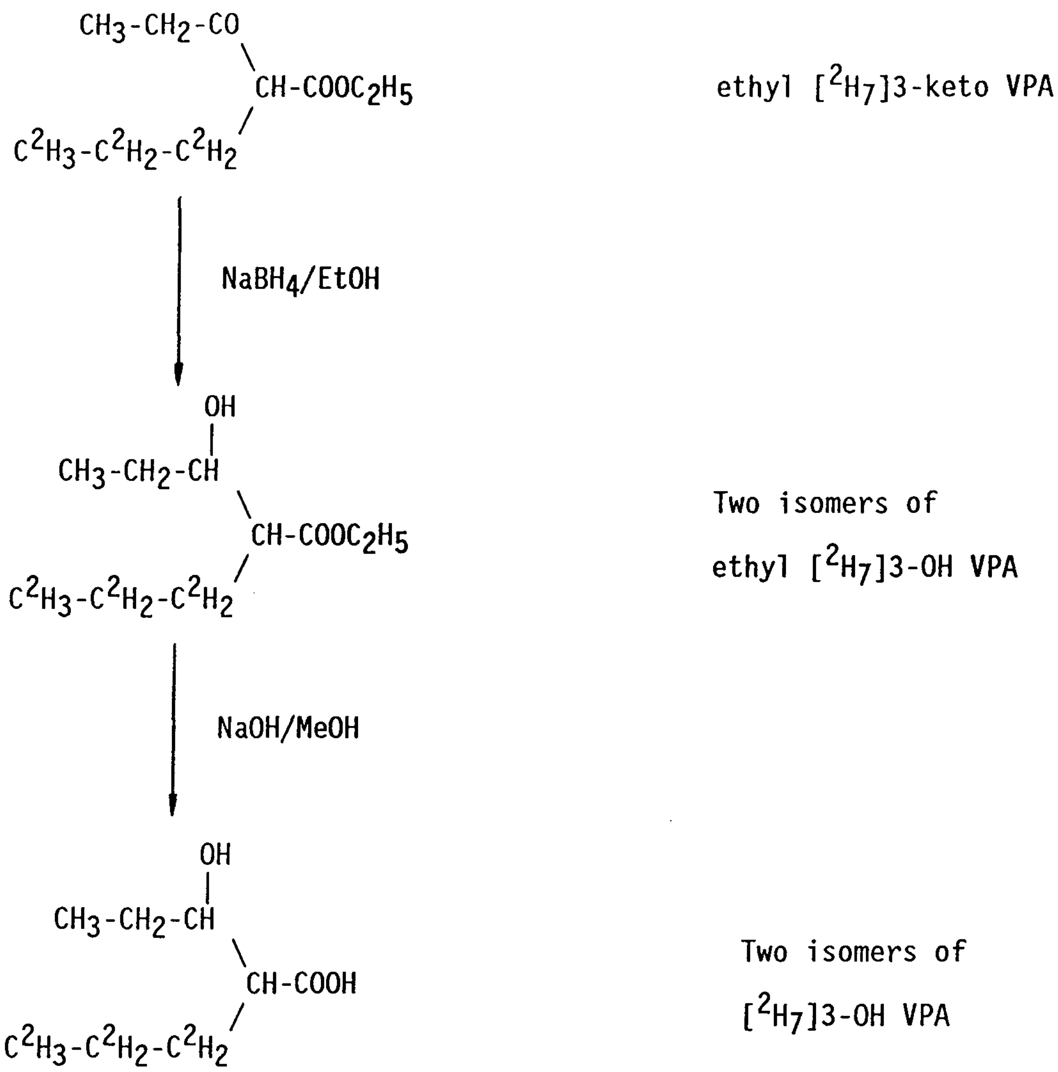


Fig. 13: ^{1}H NMR spectrum of ethyl ester of [$^2H_7$]3-keto VPA.

3.1.7 Synthesis of [$^2H_7$]3-OH VPA

The ethyl ester of [$^2H_7$]3-OH VPA was synthesized by reducing the ethyl ester of [$^2H_7$]3-keto VPA with $NaBH_4$, the same method that was used in the synthesis of [$^2H_7$]4-OH VPA. Scheme 9 presents the synthetic procedure.

The product was confirmed to be ethyl [$^2H_7$]3-OH VPA by comparison of its GCMS mass spectrum with that of ethyl 3-OH VPA (Figure 14). The fragments of ethyl [$^2H_7$]3-OH VPA at m/z: 166 $(M\text{-}C_2H_5)^+$, 150 $(M\text{-}OC_2H_5)^+$, 137 $(M\text{-}CH_3CH_2CHO)^+$, 120 $(CH_3CH_2COCHC_3{}^2H_7)^+$, 103 $(C^2H_2CH_2CO_2C_2H_5)^+$, correspond to the fragments of ethyl 3-OH VPA at m/z: 159 $(M\text{-}C_2H_5)^+$, 143 $(M\text{-}OC_2H_5)^+$, 130 $(M\text{-}CH_3CH_2CHO)^+$, 113 $(CH_3CH_2COCHC_3H_7)^+$, and 101 $(CH_2CH_2CO_2C_2H_5)^+$.


CH3-CH2-CO
CH-COOC2H5
C2H3-C2H2-C2H2
ethyl [2H7]3-keto VPA
NaBH4/EtOH
OH
CH3-CH2-CH
CH-COOC2H5
C2H3-C2H2-C2H2
Two isomers of
ethyl [2H7]3-OH VPA
NaOH/MeOH
OH
CH3-CH2-CH
CH-COOH
C2H3-C2H2-C2H2
Two isomers of
[2H7]3-OH VPA

Scheme 9: Synthesis of [$^2H_7$]3-OH VPA

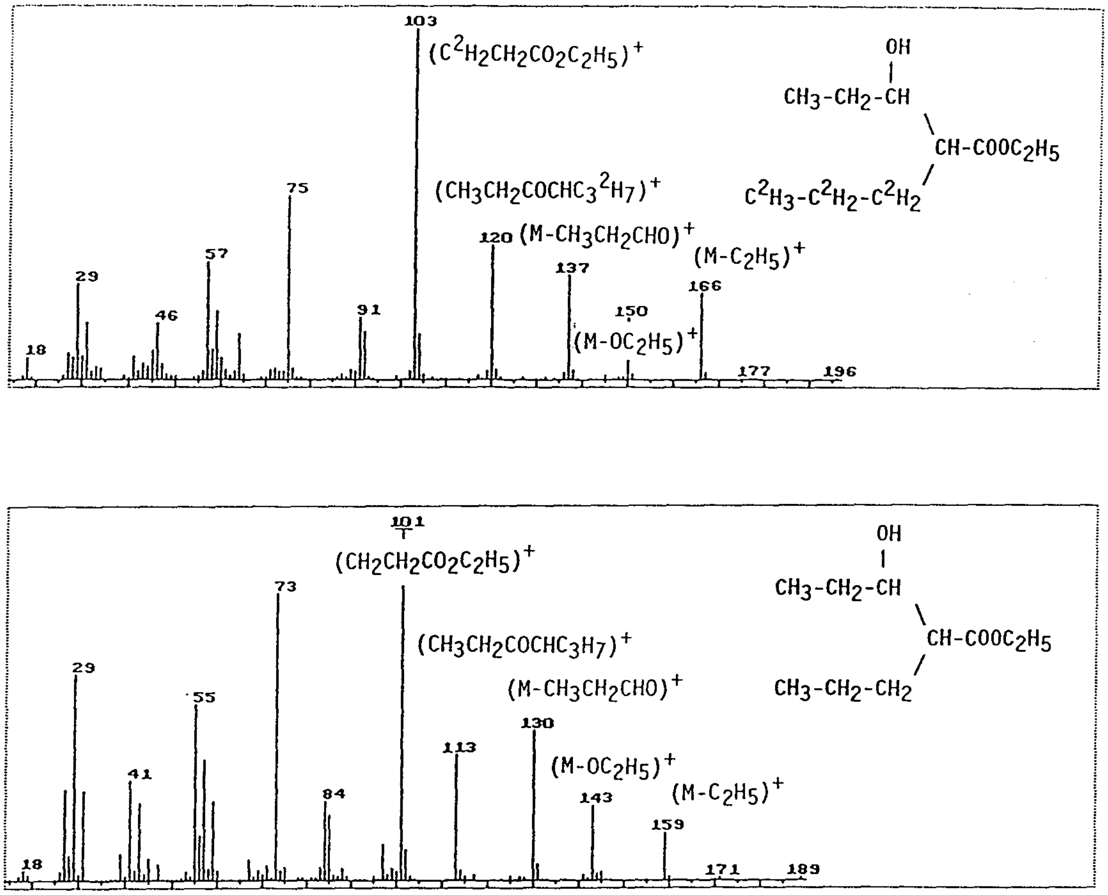
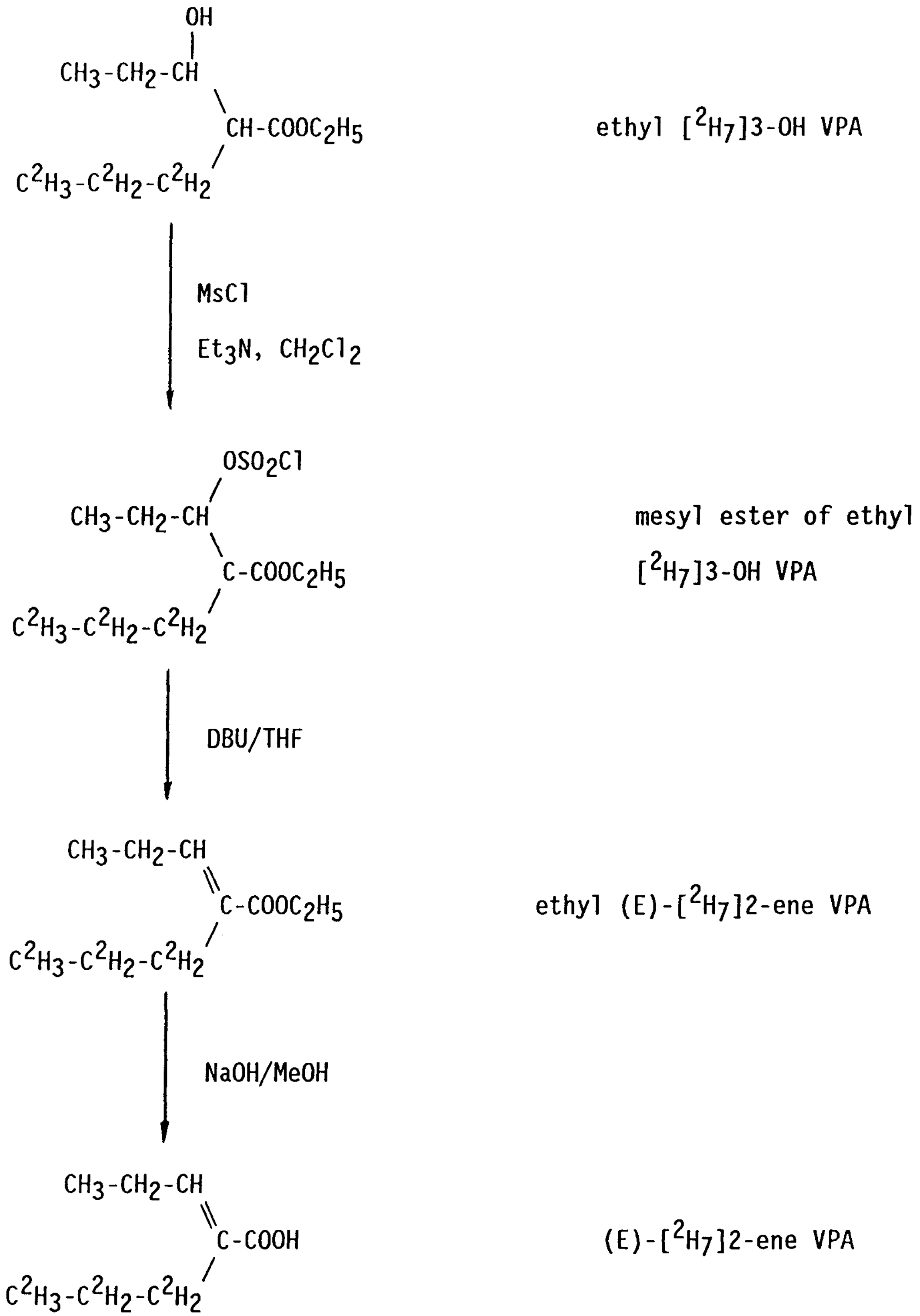


Fig. 14: GCMS mass spectra of the ethyl esters of $[^2H_7]$3-OH VPA (top) and 3-OH VPA (bottom).

3.1.8 Synthesis of (E)-[$^2H_7$]2-ene VPA

Ethyl (E)-[$^2H_7$]2-ene VPA was synthesized from ethyl [$^2H_7$]3-OH VPA according to the procedure in scheme 10, and based on the method of Lee *et al.*, (1989) to synthesize (E)-2-ene VPA. The formation of the mesyl ester of [$^2H_7$]3-OH VPA was completed within one hour after methanesulfonyl chloride was added. Nucleophilic elimination of the mesyl group by 1,8-diazabicyclo [5.4.0]undec-7-ene (DBU) afforded ethyl [$^2H_7$]2-ene VPA. The separation and purification of product was performed by column chromatography. The main impurity was unreacted ethyl [$^2H_7$]3-OH VPA, which combined with the stationary phase tightly because of the hydroxyl group. The impurity eluted only when using a highly polar solvent, therefore, it was easy to separate the relatively non-polar ethyl [$^2H_7$]2-ene VPA from it. Moreover, being an α,β-unsaturated ester, ethyl [$^2H_7$]2-ene VPA was visible under UV light, which facilitated monitoring the separation. The solvent was removed under flash evaporation from the fraction shown to contain the 2-ene VPA, and the residue was dried under vacuum. The ^1H NMR (Figure 16) confirmed the product to be ethyl [$^2H_7$](E) 2-ene VPA, with a small portion of (Z) isomer, which agrees with Lee's result (1989). Figure 15 shows the mass spectra of labelled and unlabeled ethyl (E)-2-ene VPA. The fragments of ethyl [$^2H_7$]2-ene VPA at m/z 177 (M^+), 148 $(M-C_2H_5)^+$, 132 $(M-OC_2H_5)^+$, 115 $(CH_2=C(C^2H_2)COOC_2H_5)^+$, and 97 $(CH_3CH=CHC(CO)=C^2H_2)^+$, correspond to those of ethyl 2-ene VPA at m/z 170 (M^+), 141 $(M-C_2H_5)^+$, 125$(M-OC_2H_5)^+$, 113$(CH_2=C(CH_2)COOC_2H_5)^+$, and 95$(CH_3CH=CHC(CO)=CH_2)^+$.


ethyl [2H7]3-OH VPA
MsCl
Et3N, CH2Cl2
mesyl ester of ethyl [2H7]3-OH VPA
DBU/THF
ethyl (E)-[2H7]2-ene VPA
NaOH/MeOH
(E)-[2H7]2-ene VPA

Scheme 10: Synthesis of (E)-[$^2H_7$]2-ene VPA.

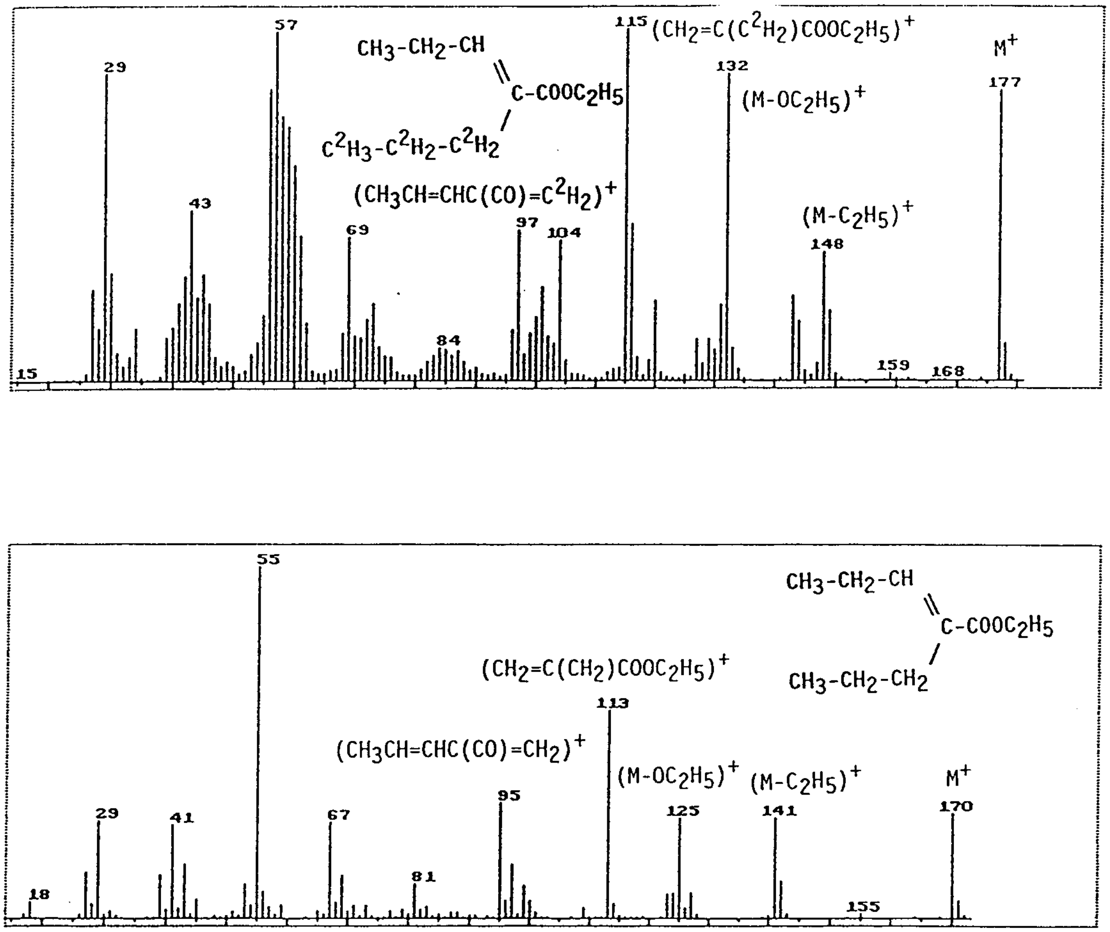


Fig. 15: GCMS mass spectra of the ethyl esters of (E)-[$^2H_7$]2-ene VPA (top) and (E)-2-ene VPA (bottom).

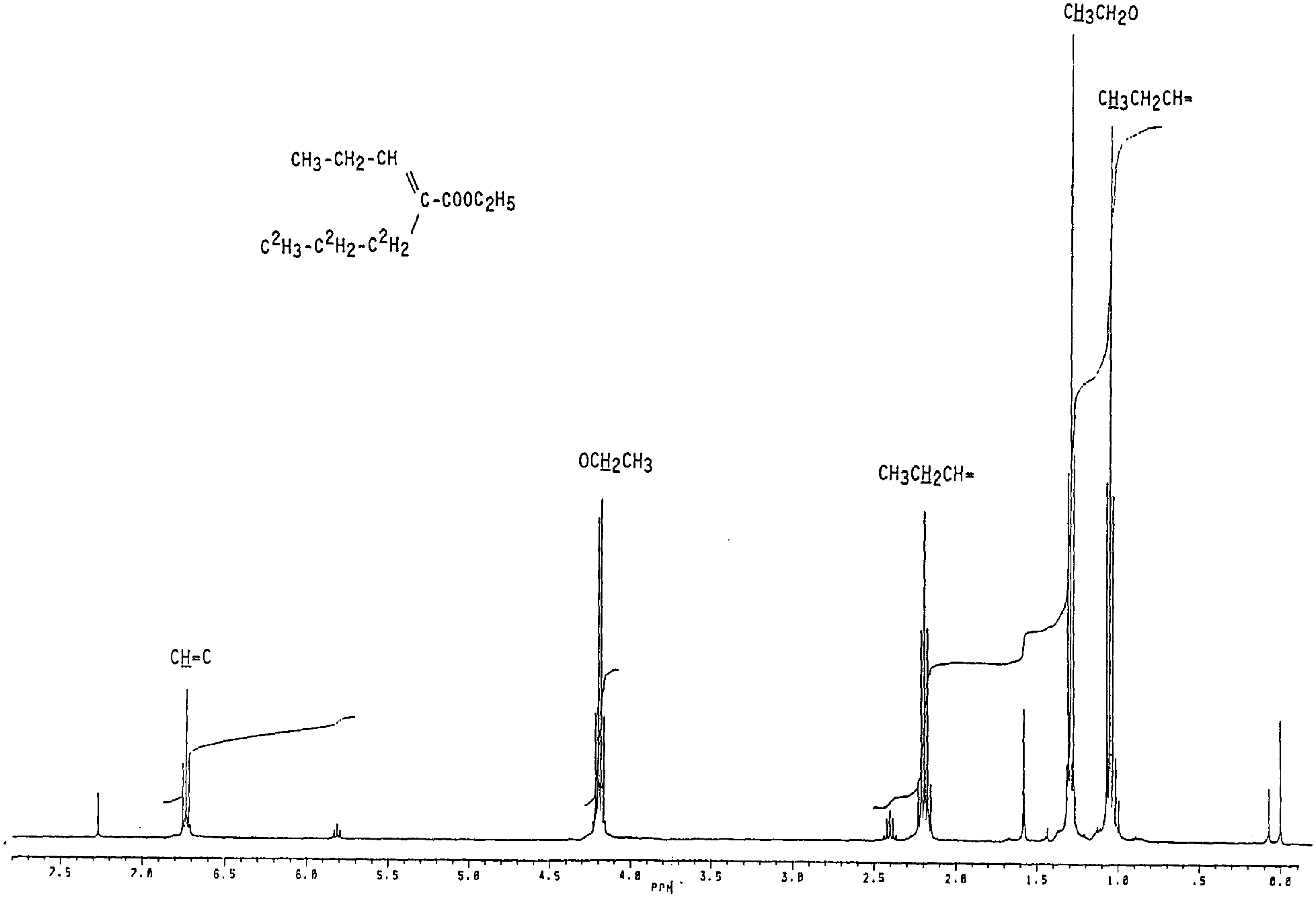


Fig. 16: ^{1}H NMR spectrum of the ethyl ester of [$^2H_7$]2-ene VPA.

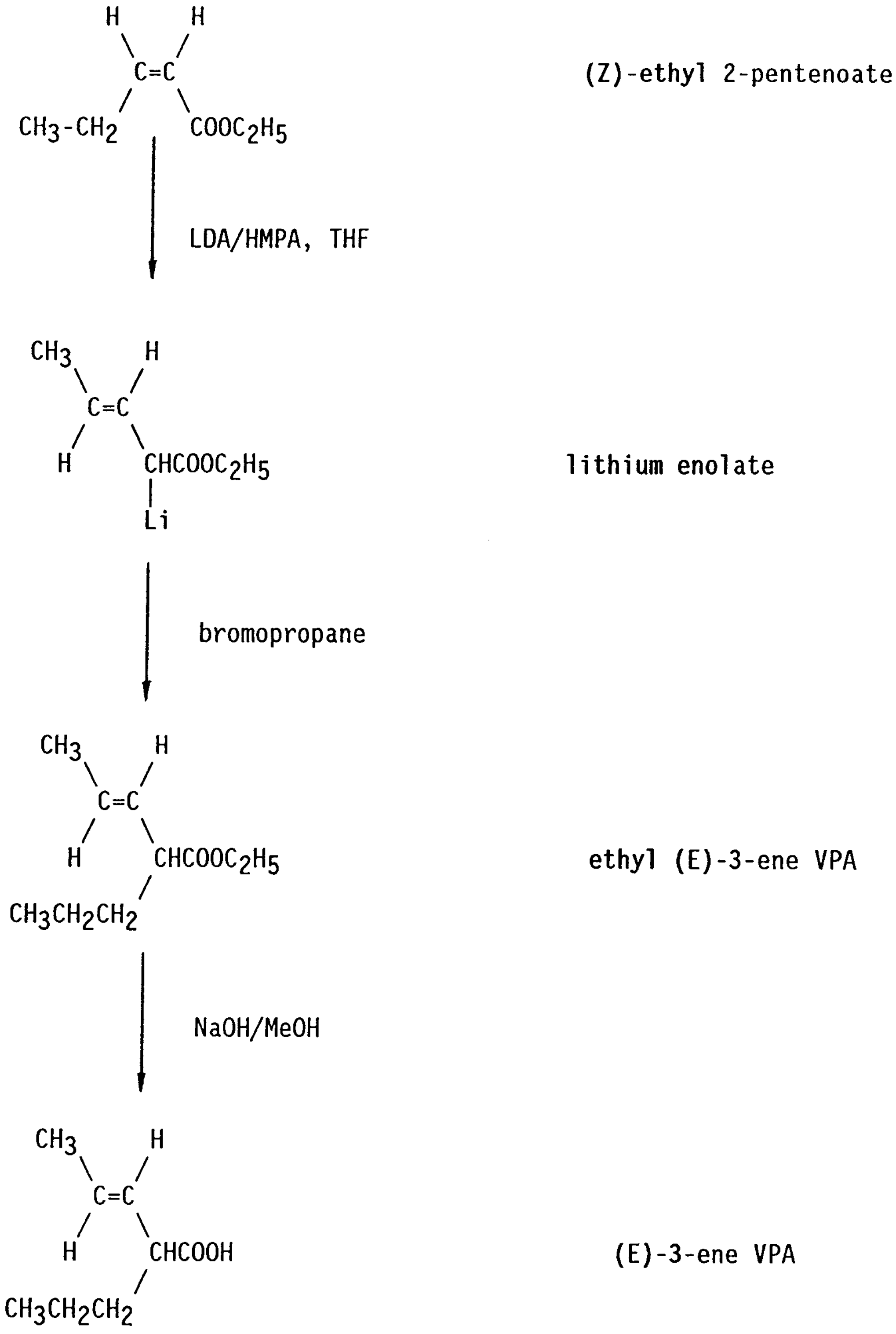
3.1.9 Stereoselective syntheses of (E)- and (Z)-3-ene VPA

(E)-3-ene VPA was synthesized as outlined in scheme 11, by a method based on general procedures described by Herrmann *et al.* (1973). According to the research of Kende and Toder (1982), alkylation of ethyl (Z)-2-alkenoate yields stereospecifically ethyl (E)-3-alkenoate. The α,β-unsaturated ester ethyl (Z)-2-pentenoate was converted into the lithium enolate by the non-nucleophilic form of lithium diisopropylamide (LDA) which is a 1:1 complex of LDA and HMPA. Besides being employed as base, LDA might also act as a nucleophile and conjugatively add to the unsaturated ester at a rate competitive with proton abstraction. After applying HMPA to modify LDA, no Micheal addition to (Z)-2-pentenoate was observed. The lithium enolate was then alkylated by bromopropane to afford ethyl (E)-3-ene VPA. Ethyl (E)-3-ene VPA was hydrolyzed with base and the free acid purified by fractional distillation. The product was confirmed by GCMS and ^{1}H-NMR to be pure (E)-3-ene VPA. GCMS mass spectrum (Figure 17) of 3-ene VPA after methylation affords ions at m/z 156, 127, 113, 97, 55, corresponding to the molecular ion (M^+) and fragments $(M\text{-}C_2H_5)^+$, $(M\text{-}C_3H_7)^+$, $(M\text{-}COOCH_3)^+$, and $(CH_3CH{=}CHCH_2)^+$ respectively.

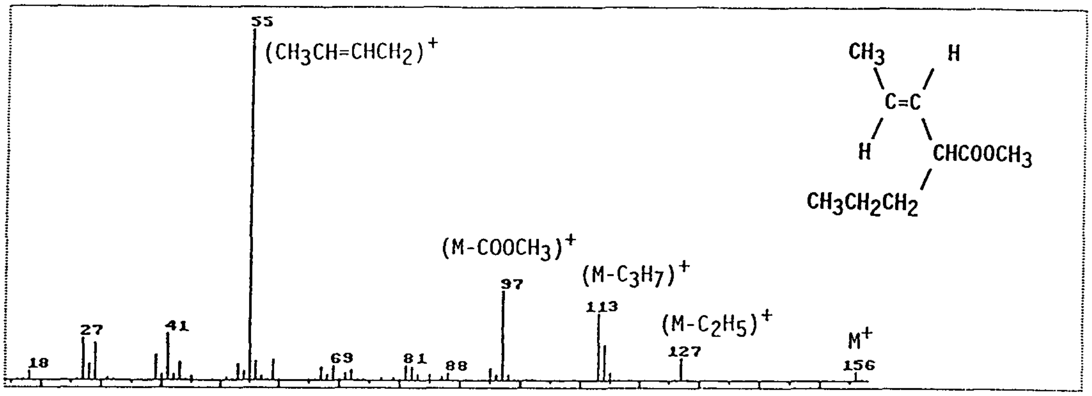
The NMR spectrum (Figure 18) confirmed the stereoselectivity of this reaction. The proton at C-4 has a chemical shift value at 5.6 ppm, and is split into a 16 peak multiplet with coupling constants of 15 Hz (with the proton at C-3), 6.5 Hz (with CH_3), and 1 Hz (with the proton at C-2); the proton at C-3 has a chemical shift value of 5.43 ppm, and is split into a 16 peak multiplet with coupling constants of 15 Hz (with the proton at C-4), 8.6 Hz (with the proton at C-2) and 2 Hz (with CH_3).

(Z)-3-ene VPA was synthesized by the same method as (E)-3-ene VPA except the starting material was ethyl (E)-2-pentenoate. The GCMS mass spectrum (Figure 17) of the methyl ester of (Z)-3-ene VPA was identical with that of the methyl ester of (E)-3-ene VPA. However, ^1H NMR can distinguish between these two isomers. Figure 19 shows the NMR spectrum for (Z)-3-ene VPA. The signal for the vinylic proton at C-4 appears at 5.6 ppm, and occurs as a 16 peak multiplet with coupling constants of 11 Hz (with the proton at C-3), 6.5 Hz (with CH_3), and 1 Hz (with the proton at C-2); the vinylic proton at C-3 shows a signal at 5.36 ppm, and is split into a 16 peak multiplet with coupling constants of 11 Hz (with the proton at C-4), 9.5 Hz (with the proton at C-2), and 2 Hz (with CH_3 adjacent to the C-4).

The purity of both (E)- and (Z)-3-ene VPA were checked by GCMS and by NMR, before they were used in the metabolism studies.


(Z)-ethyl 2-pentenoate
LDA/HMPA, THF
lithium enolate
bromopropane
ethyl (E)-3-ene VPA
NaOH/MeOH
(E)-3-ene VPA

Scheme 11: Synthesis of (E)-3-ene VPA.



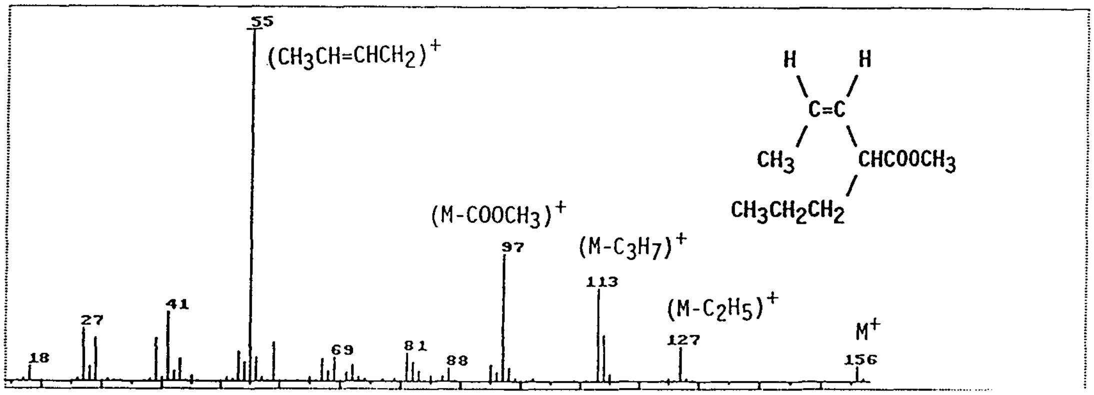


Fig. 17: GCMS mass spectra of the methyl esters of (E)- (top) and (Z)-3-ene VPA (bottom).

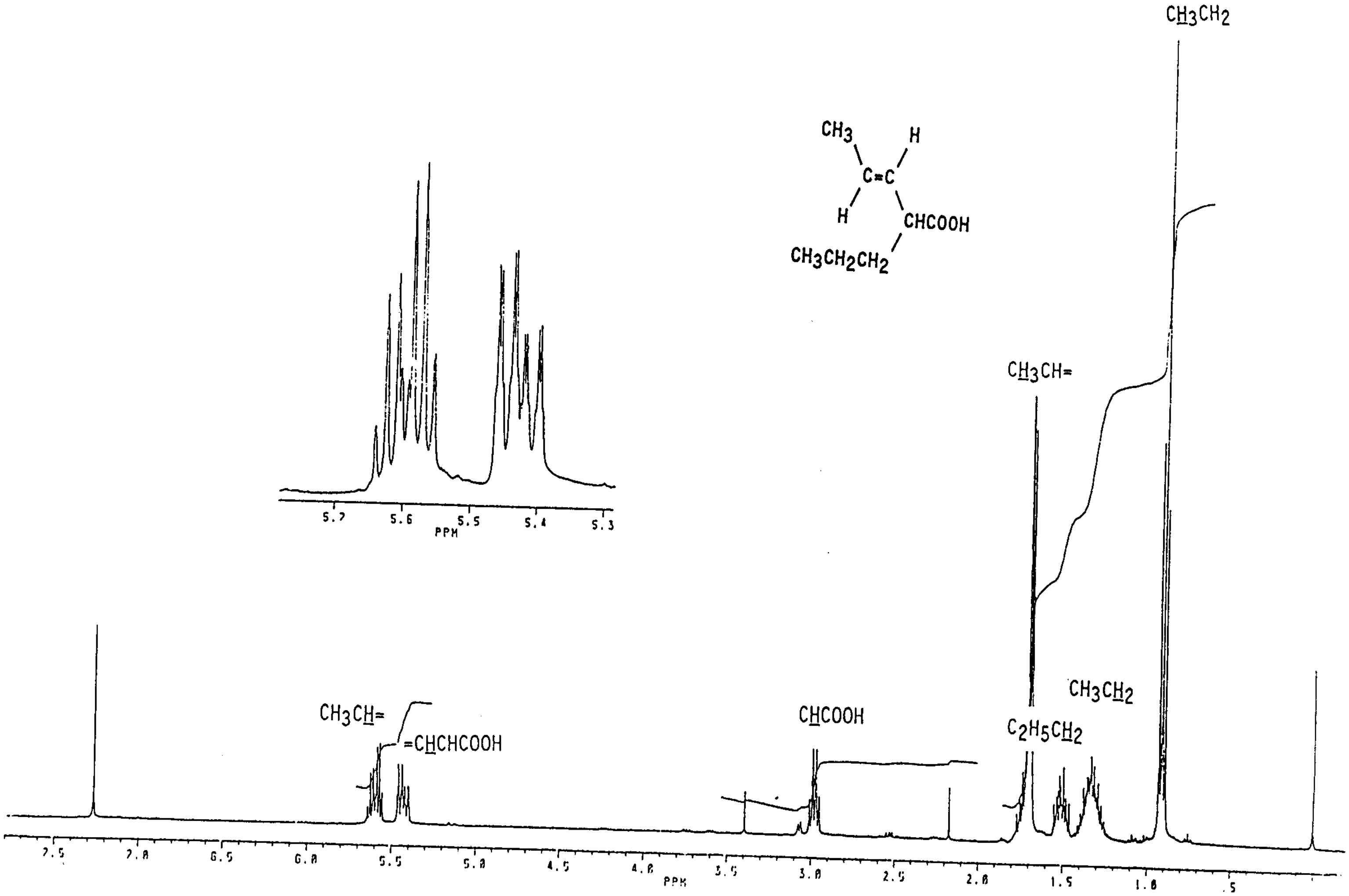


Fig. 18: ^1H NMR spectrum of (E)-3-ene VPA.

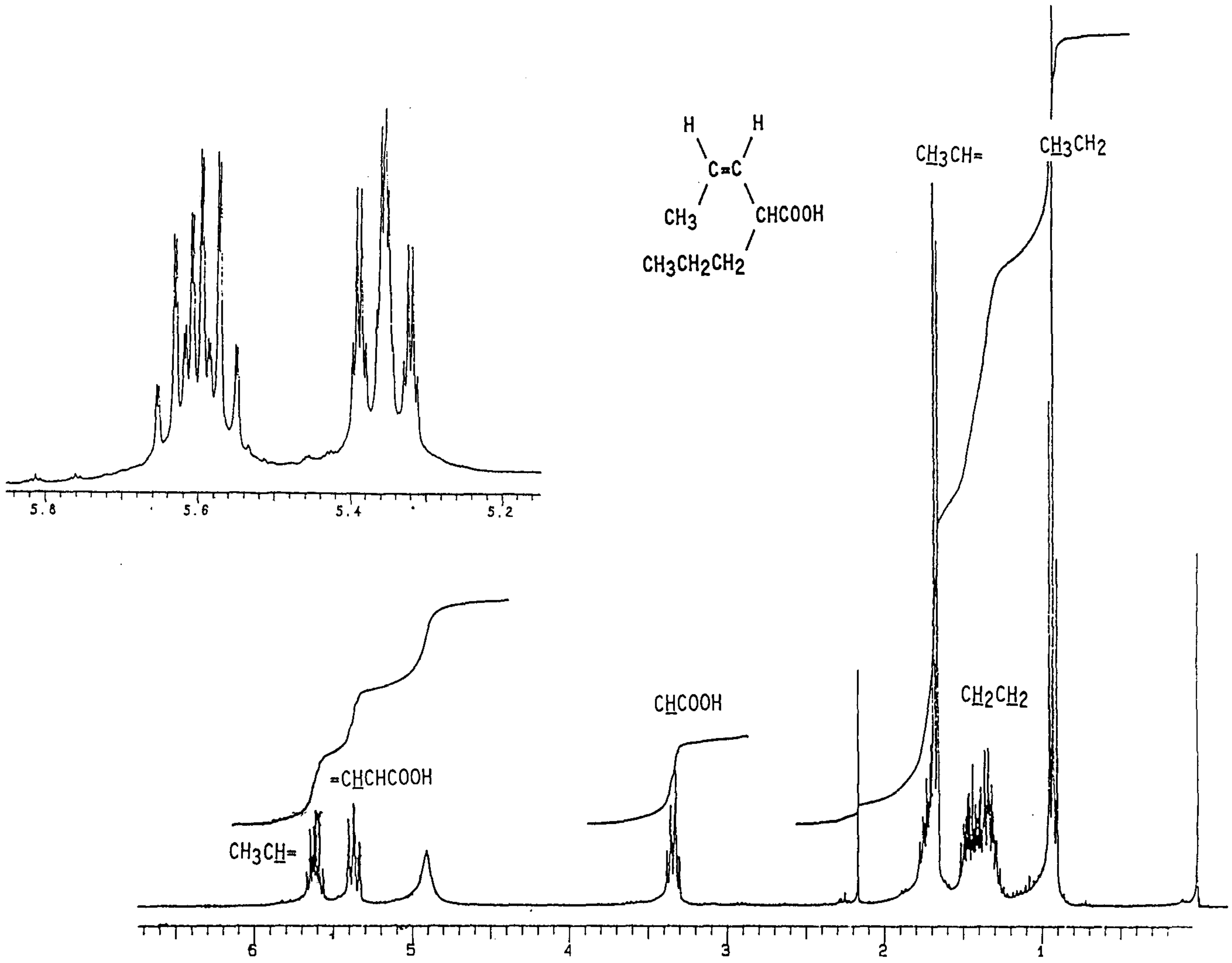


Fig. 19: ^{1}H NMR spectrum of (Z)-3-ene VPA.

3.2 Optimizing GCMS conditions for the analysis of VPA metabolites in EI (t-BDMS derivatives) and NCI (PFB derivatives) modes

When a mixture of VPA and stable isotope labelled VPA is administered to a subject, the number of metabolite peaks to be quantitated by GCMS will double. Adequate resolution of metabolite peaks becomes even more critical. Under a constant carrier gas flow rate (1 ml/min), oven temperature is certainly the most important factor for good resolution when using either EI or NCI techniques.

By performing several experiments with different oven temperature programs, we obtained relatively satisfying results for both the EI and NCI methods. Table 4 lists the retention times of the PFB derivatives of VPA, $[^2H_6]$VPA and their metabolites analyzed by NCI technique, with a GC oven temperature initiated at 50 °C, programmed to 140 °C at 30°C/min, held for 20 min, and then increased to 260 °C at 8 °C/min.

The PFB derivatives of VPA, $[^2H_6]$VPA and their metabolites have good chromatographic properties and give sharp and symmetric peaks even during a relatively long GC run time. All of the derivatized metabolites gave an abundant ion at $[M-181]^-$ corresponding to the loss of the PFB moiety. These ions were monitored in NCI SIM mode for the quantitation of metabolites.

Table 4: List of the negative ions monitored and retention times for the PFB derivatives of VPA, [$^2H_6$]VPA, their metabolites and internal standards (I.S.) for the NCI analysis mode.

COMPOUNDS	NEGATIVE ION MONITORED (m/z)	t_R (min)
Dibutyl acetic acid (I.S.)	171	27.0
[$^2H_3$](Z)-2-ene VPA (I.S.)	144	15.35
[$^2H_3$](E)-2-ene VPA (I.S.)	144	18.50
[$^2H_3$]3-keto VPA (I.S.)	232	32.31
2-MGA (I.S.)	325**	43.23
VPA	143	14.67
[$^2H_6$]VPA	149	14.46
4-ene VPA	141	14.34
[$^2H_6$]4-ene VPA	146	14.14
3-ene VPA	141	14.92
[$^2H_6$]3-ene VPA	147	14.71
(Z)-2-ene VPA	141	15.44
[$^2H_6$](Z)-2-ene VPA	147	15.21
(E)-2-ene VPA	141	18.62
[$^2H_6$](E)-2-ene VPA	147	18.33
2,4-diene VPA	139	19.93
[$^2H_5$]2,4-diene VPA	144	19.21
(E,Z)-2,3′-diene VPA	139	18.93
[$^2H_6$](E,Z)-2,3′-diene VPA	145	18.60
(E,E)-2,3′-diene VPA	139	22.43
[$^2H_6$](E,E)-2,3′-diene VPA	145	22.03
4-keto VPA	157	25.03
[$^2H_6$]4-keto VPA	160	24.87
3-OH VPA	231	29.69
[$^2H_6$]3-OH VPA	237	29.53
4-OH VPA	231	29.30, 30.36*
[$^2H_6$]4-OH VPA	237	29.14, 30.21*
5-OH VPA	231	33.28
[$^2H_5$]5-OH VPA	236	33.19
3-keto VPA	229	32.36
[$^2H_6$]3-keto VPA	235	32.26
2-PSA	339**	41.76
[$^2H_3$]2-PSA	342**	41.71
2-PGA	353**	43.43
[$^2H_3$]2-PGA	356**	43.38

* isomers; ** di-derivatives

Good resolution of all peaks was obtained by using the GC condition described above. The 4-ene VPA peak which is frequently overlapped by high concentrations of VPA was well separated from VPA with retention time difference of about 0.34 min. (E,Z)-2,3′-diene VPA, which is usually missed by EI analysis of the t-BDMS derivative, was detected and well separated from (E)-2,4-diene VPA with a retention time difference of up to 1 minute.

Two different temperature programs were investigated for t-BDMS derivatives of VPA and metabolites when analyzed by GCMS in EI mode. Table 5 summarizes retention times of the t-BDMS derivatives of the unsaturated metabolites of VPA and $[^2H_6]$VPA with the GC temperature initiated at 50 °C, programmed at 30 °C/min to 110 °C, held for 18 min and then increased to 260 °C at a rate of 10 °C/min. All unsaturated metabolites were eluted by 26 min and gave good resolution and peak shapes by this temperature program. However, the run time was too long for the keto and hydroxyl metabolites and these did not produce sharp and symmetric peak shapes because of their high polarity. Thus, another temperature program was set to analyze polar metabolites. Table 6 shows retention times of t-BDMS derivatives of VPA, $[^2H_6]$VPA and their keto and hydroxyl metabolites with the GC temperature initiated at 50 °C, programmed to 100 °C at 30 °C/min, then increased to 250 °C at a rate of 8 °C/min. Total run time was less than 20 min.

Table 5: Positive ions monitored and the retention times of the t-BDMS derivatives of VPA, [$^2H_6$]VPA, their unsaturated metabolites, and the internal standards (I.S.) in the EI analysis mode.

COMPOUNDS	ION MONITORED (m/z)	t_R (min)
[$^2H_3$](Z)-2-ene VPA (I.S.)	202	16.79
[$^2H_3$](E)-2-ene VPA (I.S.)	202	20.41
VPA	201	15.71
[$^2H_6$]VPA	207	15.66
4-ene VPA	199	15.68
[$^2H_6$]4-ene VPA	204	15.53
3-ene VPA	199	15.88
[$^2H_6$]3-ene VPA	205	15.62
(Z)-2-ene VPA	199	16.86
[$^2H_5$](Z)-2-ene VPA	205	16.61
(E)-2-ene VPA	199	20.59
[$^2H_6$](E)-2-ene VPA	205	20.19
2,4-diene VPA	197	22.88
[$^2H_5$]2,4-diene VPA	202	22.71
(E,E)-2,3′-diene VPA	197	24.91
[$^2H_6$](E,E)-2,3′-diene VPA	203	24.71

Table 6: Positive ions monitored and the retention times of the t-BDMS derivatives of VPA, [$^2H_6$]VPA, their keto and hydroxyl metabolites and internal standards in the EI analysis mode.

COMPOUNDS	ION MONITORED (m/z)	t_R (min)
Dibutyl acetic acid (I.S.)	229	11.02
[$^2H_3$](E)-2-ene VPA (I.S.)	202	9.45
[$^2H_3$]3-keto VPA (I.S.)	218	11.33
VPA	201	8.55
[$^2H_6$]VPA	207	8.48
4-keto VPA	215	11.96
[$^2H_6$]4-keto VPA	221	11.89
3-OH VPA	217	11.50, 11.83*
[$^2H_6$]3-OH VPA	223	11.43, 11.77*
4-OH VPA	217	11.37, 11.94*
[$^2H_6$]4-OH VPA	223	11.30, 11.90*
5-OH VPA	331**	16.25
[$^2H_5$]5-OH VPA	336**	16.21
3-keto VPA	332**	15.85
[$^2H_6$]3-keto VPA	335**	15.83
2-MGA (I.S.)	317**	16.16
2-PSA	331**	16.39
[$^2H_3$]2-PSA	334**	16.36
2-PGA	345**	17.70
[$^2H_3$]2-PGA	348**	17.67

* isomers, ** di-derivatives

3.3 Pharmacokinetics of [$^2H_6$]VPA and its metabolites in a healthy volunteer

A 700 mg dose consisting of VPA:[$^2H_6$]VPA (50:50) was given orally to a healthy human volunteer every 12 hours for a period of two and half days to perform a multiple-dose study of the pharmacokinetics of VPA and [$^2H_6$]VPA. Following the final dose, blood samples were withdrawn at certain times and serum samples were then obtained by centrifugation. Saliva samples were taken at selected times convenient with the taking of blood samples after stimulation with a 5% citric acid solution rinse of the mouth. Following the first dose, urine samples were collected in 12 hour blocks for 2 days and then in 2 or 3 day blocks for another 8 days.

All samples were analyzed quantitatively by GCMS (HP 5987A) using EI analysis of the *t*-BDMS derivatives and NCI analysis of the PFB derivatives with the GC condition we discussed in section 3.2.. Urine or serum samples (0.25 mL) were extracted and derivatized with PFB and then TMS to be made ready for NCI analysis, while 1 mL of urine or serum was required to be derivatized with t-BDMS for EI analysis. Dibutylacetic acid was used as an internal standard to analyze VPA and [$^2H_6$]VPA, 2-methylglutaric acid for 2-PSA and 2-PGA, [$^2H_3$]3-keto VPA for 3-keto VPA and [$^2H_6$]3-keto VPA, and [$^2H_3$]2-ene VPA for the rest of VPA metabolites and their deuterium labeled analogs. Calibration curves with different standard concentrations were made for urine, serum total, serum free and saliva respectively, and run for both PFB and t-BDMS derivatives. Table 7 and 8 summarize the coefficients of determination for calibration curves of VPA metabolites.

Good linearity of calibration curves was obtained for most of the unsaturated VPA metabolites and 3-keto VPA, since [$^2H_3$]2-ene VPA and [$^2H_3$]3-keto VPA were used as internal standards. However, the linearity could be improved for 4-keto and hydroxyl metabolites, if adequate stable isotope labelled internal standards were used for those metabolites.

Table 7: Linearity of calibration curves for quantitative assays of VPA, VPA metabolites and their [$^2H_7$] labelled analogues which were isolated from urine samples of a human volunteer administered with 700 mg of VPA:[$^2H_7$]VPA (50:50) every 12 hours for two and half days.

	r^2	
Metabolites	NCI	EI
VPA	0.986	0.984
4-ene	0.999	0.997
3-ene	0.997	0.997
(Z)-2-ene	0.999	0.999
(E)-2-ene	1.000	0.996
(E)-2,4-diene	0.998	0.992
(E,Z)-2,3′-diene	0.971	N.D.
(E,E)-2,3′-diene	0.993	0.980
4-keto	0.987	0.987
3-keto	0.998	0.998
3-OH	0.996	0.987
4-OH	0.984	0.983
5-OH	0.990	0.978
2-PSA	0.990	0.999
2-PGA	0.997	0.998

Table 8: Linearity (r^2) of calibration curves for quantitative assays of VPA, VPA metabolites and their [$^2H_7$] labelled analogues in serum total, serum free and saliva samples of a human volunteer administered with 700 mg of VPA:[$^2H_7$]VPA (50:50) every 12 hours for two and half days.

	Serum Total		Serum Free & Saliva	
Metabolites	NCI	EI	NCI	EI
VPA	0.999	0.997	0.998	0.999
4-ene	0.995	0.997	0.992	0.993
3-ene	0.996	N.D.	0.992	0.991
(Z)-2-ene	0.999	1.000	1.000	0.998
(E)-2-ene	1.000	1.000	1.000	0.999
(E)-2,4-diene	0.991	0.999	0.984	0.993
E,Z 2,3′-diene	0.994	N.D.	0.992	N.D.
E,E 2,3′-diene	0.994	0.998	0.992	0.994
4-keto	0.997	0.953	0.961	0.994
3-keto	0.998	0.999	0.998	0.999
3-OH	0.995	0.960	0.992	N.D.
4-OH	0.962	0.906	N.D.	N.D.
5-OH	0.971	N.D.	0.995	N.D.
2-PSA	N.D.	0.998	N.D.	N.D.
2-PGA	0.994	0.996	0.876	0.985

Pharmacokinetic parameters of VPA and its metabolites were obtained based on the serum and saliva data. The elimination rate constants (K_E) were obtained from the slopes of the log serum or saliva concentration *vs* time plot using statistical linear regression program. Total body clearance (CL) and volume of distribution (V_d) were obtained from the following equation where area under the curve (AUC) over 12 hours after the final dose was calculated using a computer program.

$$CL = K_E \times V_d = \frac{\text{Dose}}{\text{AUC}}$$

Figure 20 shows elimination curves of VPA and $[^2H_6]$VPA in serum total, serum free and saliva which were measured with NCI techniques.

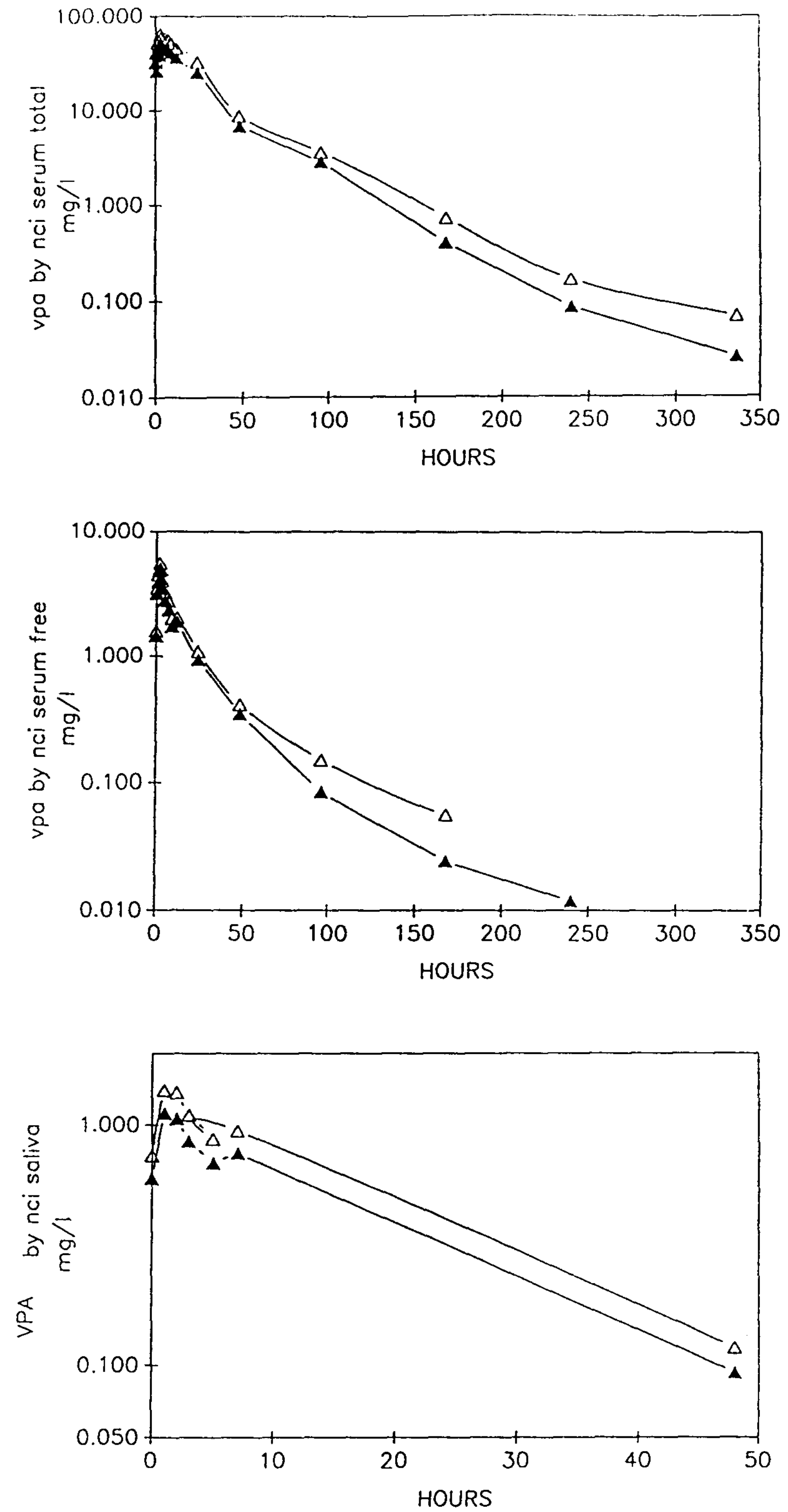


Fig. 20: Elimination Curves of VPA (Δ) and [$^2H_6$]VPA (▲) in serum total (top), serum free (middle) and saliva (bottom) which were measured with NCI techniques.

From the elimination curves shown in figure 20, [$^2H_6$]VPA shows very similar pharmacokinetic behavior to VPA. Table 9 presents the pharmacokinetic parameters of both VPA and [$^2H_6$]VPA measured in this volunteer under steady state conditions.

These [$^2H_6$]VPA (Table 9) parameters are in agreement with the results that Acheampong *et al.* obtained for a pulse dose of [$^2H_6$]VPA (1984). The pharmacokinetic equivalency of the labelled and unlabeled VPA might indicate that the two main metabolic pathways, glucuronidation and β-oxidation, cannot be affected by primary deuterium isotope effects in the metabolism of [$^2H_6$]VPA.

To help demonstrate the equivalency of VPA and [$^2H_6$]VPA with respect to metabolism *via* the β-oxidation metabolic pathway, the time course (12 hours) of both labelled and unlabeled β-oxidative metabolites of VPA are illustrated in Figure 21.

Table 10 lists the terminal elimination half-life and elimination constant values of the β-oxidation and other metabolites of VPA and [$^2H_6$]VPA. Deuterium labelled 2-ene, 3-keto and 3-OH VPA have very similar elimination behaviors as their unlabeled analogues.

Table 9: Pharmacokinetic Parameters of VPA(I) and [$^2H_6$]VPA(II) measured by NCI technique in serum and saliva samples of one subject after 5 oral doses of 700 mg of VPA:[$^2H_6$]VPA (50:50).

	$t_{1/2}$ (h)	K_E (h^{-1})	CL (L/h/Kg)	AUC (mg.h/L)	V_D (L/Kg)
Serum Total					
I	19.8	0.035	0.0079	636	0.226
II	20.4	0.034	0.0078	642	0.229
Serum Free					
I	15.1	0.046	0.134	37.22	2.92
II	14.7	0.047	0.154	32.38	3.29
Saliva					
I	13.6	0.051			
II	13.6	0.052			

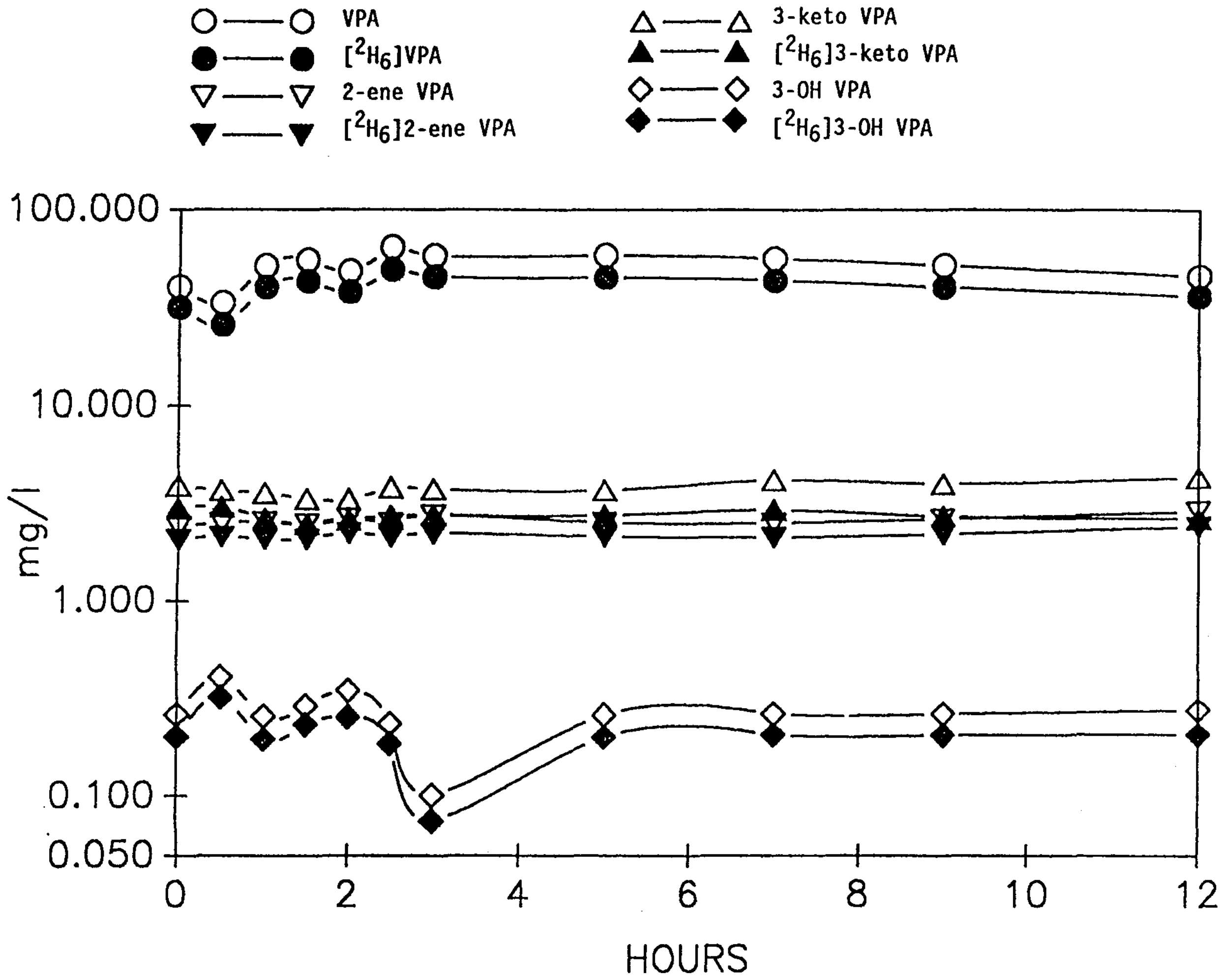


Fig. 21: Time courses (12 hours after last dose) of labelled and unlabeled β-oxidation metabolites of VPA in the subject administered 5 doses of 700 mg of VPA:[$^2H_7$]VPA (50:50).

Table 10: Pharmacokinetic Parameters of some deuterium labelled and unlabeled metabolites of VPA measured in a healthy volunteer under steady state conditions, all data were based on NCI results.

Metabolites of VPA	Apparent $t_{1/2}$ (h)	Apparent K_E (h^{-1})
4-ene	15.0	0.046
$[^2H_6]$4-ene	14.7	0.047
3-ene	80.4	0.0086
$[^2H_6]$3-ene	75.2	0.0092
(E)-2-ene	31.2	0.0222
(E)-$[^2H_6]$2-ene	29.7	0.0237
2,4-diene	50.1	0.0139
$[^2H_5]$2,4-diene	41.0	0.0169
(E,E)-2,3′-diene	27	0.0259
$[^2H_6]$(E,E)-2,3′-diene	26	0.0269
3-keto	28.5	0.024
$[^2H_6]$3-keto	32.1	0.0216
4-keto	32.7	0.0212
$[^2H_6]$4-keto	34.8	0.0199
3-OH	35.7	0.0194
$[^2H_6]$3-OH	32.8	0.0211

The half-life values of 3-keto and 2-ene VPA determined in this study are very close to the reported values of Pollack *et al.*'s studies (1986). Basically, elimination of the metabolites of VPA were slow compared with the parent drug. If one or more of these metabolites exerted a significant anticonvulsant action, their presence might explain the general clinical observation of a slow onset of maximal anticonvulsant effect and a prolonged duration of action of VPA. As discussed in the introduction, the unsaturated metabolites 2-ene VPA, 3-ene VPA, 4-ene VPA (Loscher, 1981; Loscher *et al.*, 1985) and 2,3′-diene (Abbott *et al.*, 1988) were found to have significant anticonvulsant activity in rodent models.

3.4 Isotope Effects of $[^2H_6]$VPA Metabolism.

When used as a 'pulse' dose to investigate the pharmacokinetic parameters of a drug, the stable isotope labelled analogue should not show isotope effects with respect to metabolic reactions and should have the same pharmacokinetic behavior as the unlabeled analogue. There are two main reasons for these requirements. First, the pharmacokinetics of the labelled analogue must represent those of the unlabeled drug if the pulse dose method is to prove successful. Secondly and most important, the labelled drug should not be expected to switch metabolic pathways as a result of introducing a stable isotope, otherwise, severe toxicity may be caused. Because isotope effects are possible, these should be evaluated based on the labelling position of stable isotopes and mechanism of the drug metabolism, and confirmed with experiments before use of the labelled drug in patients.

Since [$^2H_6$]VPA has been labelled with deuterium on the terminal carbons, the two main metabolic pathways, β-oxidation and glucuronidation should not be affected by any deuterium isotope effect. However, for ω-oxidation which occurs at terminal protons, isotope effects were expected for this metabolic pathway.

Potential isotope effects of [$^2H_6$]VPA were investigated in the healthy volunteer from a) serum data based on the AUC ratio of [$^2H_0$]VPA and its metabolites to [$^2H_6$]VPA and its deuterated metabolites, and b) urine data based on the recovery ratio of [$^2H_0$]VPA and its metabolites to their stable isotope labelled counterparts during the 12 hours following the last dose. All urine samples were measured by both EI and NCI. Tables 11 and 12 show data of the serum AUC ratios and urine recovery ratios of VPA and its metabolites to their deuterium analogues. Several conclusions can be drawn from the results.

Table 11: Area under curve (AUC) ratios of VPA and VPA metabolites to their deuterium labelled analogs over 12 hours after the final dose in serum samples of a healthy volunteer administered 5 doses of 700 mg VPA:[$^2H_6$]VPA (50:50). All values were based on NCI results.

	AUC (mg·h/l)		AUC Ratio
Metabolites	$^2H_0$	$^2H_6$	$^2H_0/^2H_6$
VPA	636.26	493.8	1.28
4-ene	1.675	1.294	1.29
3-ene	10.08	6.768	1.49*
(Z)-2-ene	6.021	4.827	1.25
(E)-2-ene	31.45	26.26	1.20
2,4-diene	1.538	1.03	1.49*
(E,E)-2,3′-diene	7.365	6.663	1.10
4-keto	3.424	1.78	1.93*
3-keto	46.84	33.11	1.41
3-OH	3.063	2.363	1.29
4-OH	3.402	2.663	1.28
5-OH	1.075	0.028	38.4*
2-PGA	1.268	N.D.	N.D.

*: Potential isotope effects based on AUC ratio.

Table 12: Steady state urinary recovery molar ratio of VPA and its metabolites to their deuterium labelled analogs, [$^2H_6$]VPA and metabolites in a healthy human volunteer administered 5 doses of 700 mg of VPA:[$^2H_6$]VPA (50:50), based on 12 hour urine collected following the final dose*.

Metabolites	Ratio $^2H_0/^2H_6$			
	NCI + Base[1]	NCI + Enzyme[2]	EI + Base[3]	EI + Enzyme[4]
VPA	1.12	1.21	1.10	1.09
4-ene	1.08	1.25	N.D.	N.D.
3-ene	1.29	1.20	1.34	1.22
(Z)-2-ene	0.97	1.28	1.27	1.23
(E)-2-ene	0.97	1.13	1.30	1.27
(E)-2,4-diene	1.36	1.42	1.59	1.63
(E,Z)-2,3′-diene	0.87	1.03	N.D.	N.D.
(E,E)-2,3′-diene	1.00	1.04	1.26	1.23
4-keto	0.90	1.20	0.90	1.13
3-keto	1.14	1.45	3.23	3.12
3-OH	1.10	1.15	1.27	1.67
4-OH	1.00	1.22	2.33	2.98
5-OH	6.50	6.38	6.04	5.89
2-PSA	1.11	1.00	1.20	1.20
2-PGA	15.5	16.3	14.6	15.2
N-Acetyl Cysteine Conjugate of (E)-2,4-diene (NCI)				1.54

* Steady state dose consisted of 2.43 mmol VPA and 2.33 mmol [$^2H_6$]VPA.

1. Urine samples were hydrolyzed by alkali and analyzed by NCI.
2. Urine samples were hydrolyzed by glucuronidase and analyzed by NCI.
3. Urine samples were hydrolyzed by alkali and analyzed by EI.
4. Urine samples were hydrolyzed by glucuronidase and analyzed by EI.

From serum data, $[^2H_5]$5-OH VPA shows prominent isotope effects, while deuterated 3-ene, 2,4-diene and 4-keto also show slight isotope effects based on this AUC ratios.

According to the urine data, a large isotope effect was observed in the metabolic formation of $[^2H_5]$5-OH VPA and $[^2H_3]$2-PGA, which agrees with the finding of Acheampong *et al.* (1984). This result was expected because 5-OH VPA and 2-PGA are the products of metabolic ω-oxidation by mixed function oxidative enzymes. As mentioned in the introduction, 5-OH VPA is formed via abstraction of a hydrogen from position 5 to form a carbon-centered free radical(Rettie *et al.*, 1987). An isotope effect is predicted if the extraction of hydrogen is the rate limiting step.

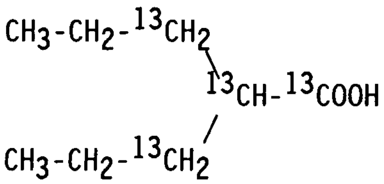
Since 5-OH VPA and 2-PGA only account for a small portion of the total metabolites, the decrease in the formation of $[^2H_5]$5-OH VPA and $[^2H_3]$2-PGA did not markedly affect the elimination kinetics of $[^2H_6]$VPA.

No major isotope effects were observed in the other metabolic pathways, based on urine data, including the formation of 4-ene VPA, where one deuterium is lost to form the product. This result supports a recently reported mechanism for the cytochrome P-450 desaturation metabolism (Rettie *et al.*, 1988) of VPA in which a carbon centered radical at C-4 serves as an intermediate, and this step is rate limiting in the formation of 4-ene VPA.

Surprisingly, a small isotope effect was apparent for the formation of $[^2H_6]$(E)-2,4-diene VPA, from both serum and urine data. It is believed that 2,4-diene VPA is formed *via* 2-ene and 4-ene VPA (Porubek *et al.*, 1988). The formation of $[^2H_6]$(E)-2,4-diene VPA should not show any isotope effect since the formation of $[^2H_6]$4-ene VPA does not. It was also found by my colleague Dr. Kassahun that the ratio of the N-acetylcysteine conjugate of (E)-2,4-diene VPA to its deuterium counterpart was as high as 1.54 (Table 10), when the same urine samples were analyzed. Based on this information and our findings of a small isotope effect for 2,4-diene VPA formation from $[^2H_6]$VPA, we therefore proposed that the formation of 2,4-diene VPA might occur partly from 3-ene VPA. Removal of a deuterium atom from the C-5 position in $[^2H_6]$3-ene VPA may be the first step in the formation of 2,4-diene VPA. Consequently, an isotope effect is expected for this pathway, and thus might explain the observed result. An experiment was designed to test this proposal, and the results of that experiment will be discussed later.

3.5 Isotope effects with respect to $[^{13}C_4]$VPA metabolism:

A healthy human volunteer participated in this study. He was given a single oral dose consisting of 700 mg of VPA:$[^{13}C_4]$VPA (50:50). The structure of $[^{13}C_4]$VPA (8) is illustrated below.



(8)

Thirteen blood samples were collected at 0, 0.5, 1, 1.5, 2, 2.5, 3, 5, 7, 9, 12, 24, 48 and 72 h after the single dose and allowed to clot. The blood samples were centrifuged to obtain serum samples. A few urine samples were collected at convenient time blocks. All urine and serum samples were analyzed using NCI techniques, and the potential isotope effects of $[^{13}C_4]$VPA were studied based on the concentration ratio of VPA and its metabolites to $[^{13}C_4]$VPA and its metabolites in urine or serum samples. The results are presented in Table 13. No apparent isotope effects were observed, thus qualifying $[^{13}C_4]$VPA to be used in pharmacokinetic studies of VPA metabolites in pediatric patients. The ratios of $[^{13}C_0/^{13}C_4]$VPA for 4-OH VPA and 5-OH VPA wre different from unity, but these differences were accounted for by a high back ground for the unlabeled VPA metabolites.

Table 13: Metabolic equivalence of [$^{13}C_4$]VPA and VPA based on mean TIC peak area ratio of VPA metabolites to their [$^{13}C_4$]-labelled analogs (13 serum samples and a urine sample collected 3-9 hr after the dose from a healthy human volunteer administered a single dose of 700 mg of VPA:[$^{13}C_4$]VPA (50:50) were analyzed by NCI techniques).

Metabolites	Ratio ($^{13}C_0/^{13}C_4$) Serum	Urine
4-ene VPA	0.954	0.974
3-ene VPA	0.982	1.154
(Z)-2-ene VPA	0.780	0.884
(E)-2-ene VPA	1.018	1.110
VPA	1.006	0.987
2,4-diene VPA	1.020	1.112
(E,E)-2,3′-diene VPA	1.041	1.104
4-keto VPA	1.041	1.045
3-OH VPA	1.038	1.122
4-OH VPA	1.413*	1.335*
3-keto VPA	0.891	0.956
2-PGA	0.934	0.894
5-OH VPA	6.331*	1.675*

* Area ratio of 4-OH and 5-OH VPA are over 1 due to high background at the same retention time as 4-OH and 5-OH VPA when monitoring ion m/z 231.

3.6 Urinary recoveries of VPA and its metabolites

The urinary recoveries of VPA, [$^2H_6$]VPA and their metabolites in the study of multiple doses (one dose every 12 hr for 2 and half days) were measured in urine samples collected for 12 hours after the final dose.

This study found that VPA glucuronide and 3-keto VPA are the predominant urinary metabolites, which is consistent with previous studies (Abbott *et al.*, 1986; Pollack *et al.*, 1986; Dickinson *et al.*, 1989) on VPA metabolites in humans. About 50% of the VPA dose is recovered as conjugated metabolites, while 25% is recovered *via* the β-oxidation pathway. The remaining oxidative metabolites in urine, the unsaturated and hydroxyl metabolites, together account for only a small fraction of the recovered VPA-derived products in urine.

Tables 14 to 17 present the metabolite concentrations measured by EI or NCI after hydrolysis with either glucuronidase or sodium hydroxide solution. The conjugated fraction will be discussed in section 3.7.

Table 14: Steady state urinary recoveries of VPA, [$^2H_6$]VPA and metabolites (free plus conjugated) in the urine collected 12 hr following the final dose*, hydrolyzed with NaOH solution, and analyzed by NCI GCMS.

Metabolites	Recoveries(% of dose)**	
	$^2H_0$	$^2H_6$
VPA	59.9	53.3
4-ene	0.056	0.052
3-ene	0.036	0.028
(Z)-2-ene	0.037	0.038
(E)-2-ene	0.89	0.92
(E)-2,4-diene	0.99	0.73
(E,Z)-2,3′-diene	1.66	1.90
(E,E)-2,3′-diene	1.26	1.26
4-keto	1.64	1.83
3-keto	26.6	23.4
3-OH	2.03	1.84
4-OH	2.83	2.84
5-OH	1.24***	0.19
2-PSA	0.30	0.27
2-PGA	3.10***	0.20

* Steady state dose consisted of 2.43 mmol VPA and 2.33 mmol [$^2H_6$]VPA.

** Recoveries are calculated on a molar basis.

*** Qualify as an isotope effect.

Table 15: Steady state urinary recoveries of VPA, [$^2H_6$]VPA and metabolites (free plus conjugated) in urine sample collected 12 hours following the final dose*, hydrolyzed with glucuronidase and analyzed by NCI GCMS.

Metabolites	Recoveries(% of dose)**	
	$^2H_0$	$^2H_6$
VPA	48.3	39.8
4-ene	0.045	0.036
3-ene	0.024	0.020
(Z)-2-ene	0.041	0.032
(E)-2-ene	0.87	0.77
(E)-2,4-diene	0.94	0.66
(E,Z)-2,3′-diene	2.09	2.02
(E,E)-2,3′-diene	1.11	1.07
4-keto	2.01	1.67
3-keto	16.4	11.3
3-OH	2.09	1.81
4-OH	4.40	3.60
5-OH	1.34***	0.21
2-PSA	0.29	0.29
2-PGA	3.26***	0.20

* Steady state dose consisted of 2.43 mmol VPA and 2.33 mmol [$^2H_6$]VPA.

** Recoveries are calculated on a molar basis.

*** Qualify as an isotope effect.

Table 16: Steady state urinary recoveries of VPA, [$^2H_6$]VPA and Metabolites (free plus conjugated) in the urine collected 12 hours following the final dose*, hydrolyzed with NaOH solution, and analyzed by EI GCMS.

Metabolites	Recoveries(% of dose)**	
	$^2H_0$	$^2H_6$
VPA	45.6	41.6
4-ene	0.065	N.D.
3-ene	0.055	0.041
(Z)-2-ene	0.047	0.037
(E)-2-ene	1.13	0.86
(E)-2,4-diene	0.54	0.34
(E,E)-2,3′-diene	1.45	1.15
4-keto	2.25	2.50
3-keto	29.8	9.22
3-OH	2.70	2.12
4-OH	3.39	1.45
5-OH	5.80***	0.96
2-PSA	0.24	0.20
2-PGA	2.92***	0.20

* Steady state dose consisted of 2.43 mmol VPA and 2.33 mmol [$^2H_6$]VPA.

** Recoveries are calculated on a molar basis.

*** Qualify as an isotope effect.

Table 17: Steady state urinary recoveries of VPA, [$^2H_6$]VPA and metabolites (free plus conjugated) in the urine collected 12 hours following the final dose*, hydrolyzed with glucuronidase, and analyzed by EI GCMS.

Metabolites	Recoveries(% of dose)**	
	$^2H_0$	$^2H_6$
VPA	42.1	39.2
4-ene	0.055	N.A.
3-ene	0.028	0.023
(Z)-2-ene	0.042	0.034
(E)-2-ene	1.00	0.79
(E)-2,4-diene	0.49	0.30
(E,E)-2,3′-diene	1.15	0.93
4-keto	1.31	1.16
3-keto	18.7	5.98
3-OH	2.11	1.26
4-OH	3.70	1.24
5-OH	1.59***	0.27
2-PSA	0.30	0.25
2-PGA	3.80***	0.25

* Steady state dose consisted of 2.43 mmol VPA and 2.33 mmol [$^2H_6$]VPA.

** Recoveries are calculated on a molar basis.

*** Qualify as isotope effect.

3.7 Conjugated Fraction of VPA and Its Metabolites in Urine Samples

VPA and most of its metabolites undergo phase II conjugation prior to excretion. The most important conjugate in urine is the glucuronic acid conjugate, which is susceptible to hydrolysis with β-glucuronidase, and with alkali or strong acid (Dickinson *et al.*, 1985a).

Studies by Dickinson *et al.* (1979; 1982) of the disposition of VPA in the rat, monkey and dog revealed that, in bile and urine samples, a proportion of the total conjugated VPA, as determined by alkaline hydrolysis, was resistant to cleavage by β-glucuronidase, suggesting that nonglucuronide conjugates were present. Further studies by Dickinson *et al.* (1984) indicated that acid- and base- catalyzed intramolecular acyl migration of the valproate moiety in valproate glucuronides away from the C-1 position could occur. In the subsequent process of ring-opening mutarotation and lactonization, six structural isomers and lactones could be formed which were not substrates for β-glucuronidase, but could be hydrolyzed in strong alkaline media. The disposition of these rearranged glucuronides *in vivo* differs from that of the primary isomer (Dickinson *et al.*, 1985a; 1985b; 1986), and it is of some interest that abnormally high concentrations of VPA conjugates, consisting largely of the rearranged glucuronide isomers, were detected in the plasma of a patient diagnosed with VPA-associated hepatobiliary and renal dysfunction (Dickinson *et al.*, 1985b).

In the present study, we used both enzyme β-glucuronidase and alkali NaOH solution to hydrolyze VPA and its metabolite conjugates.

Whether any glucuronidase-resistant conjugates exist or not were determined based on results of these two hydrolysis methods. The conjugated fractions were obtained from the concentration differences of recovered drug and metabolites in hydrolyzed (total) and unhydrolyzed (free) urines. All urine samples were analyzed with both the EI and NCI methods. Table 18 shows the conjugated fractions of VPA and its metabolites obtained with the different hydrolysis and assay methods.

About 95% of VPA in urine was in the form of the glucuronide conjugate and all unsaturated metabolites were excreted mainly as their glucuronide conjugates. However, other metabolites of VPA particularly polar metabolites show relatively low or no conjugation. These results are in agreement with the findings of Kassahun *et al*. (1989) for the excretion of VPA and metabolites in the urine of pediatric patients.

Table 18: Conjugated fraction (%) of VPA and its metabolites in urine samples collected for 12 hours after final dose measured by different hydrolysis and assay methods.

Metabolites	Conjugated Fraction (%)			
	Enzyme + NCI*	Base + NCI*	Enzyme + EI*	Base + EI*
VPA	96	97	93	93
4-ene	96	97	89	91
3-ene	79	86	61	80
(Z)-2-ene	99	99	92	92
(E)-2-ene	99	99	89	90
(E)-2,4-diene	98	98	84	86
(E,Z)-2,3′-diene	96	95	N.D.	N.D.
(E,E)-2,3′-diene	96	97	81	85
4-keto	34	19	0	35
3-keto	0	0	0	0
3-OH	47	45	44	56
4-OH	22	0	65	62
5-OH	22	15	0	49
2-PSA	0	0	20	0
2-PGA	7	0	24	0

* Base: Hydrolyzed with NaOH solution,
Enzyme: Hydrolyzed with glucuronidase,
NCI: Analyzed by NCI GCMS,
EI: Analyzed by EI GCMS.

No apparent differences were observed between the β-glucuronidase and alkaline catalyzed hydrolysis (Table 18), as measured by a paired t-test. Table 19 presents the t-test results. No significant difference was indicated between glucuronidase and alkaline hydrolysis (p-values: 0.4069, 0.1141), with samples analyzed with either NCI or EI. We can therefore, assume that there was little glucuronidase-resistant conjugate present in the urine samples of this subject after being stored at -20 °C for about 2 months. A greater number of subjects would be required to give this finding statistical significance.

Table 19: P-values of paired t-test over different hydrolysis and analysis methods.

	*Enzyme + NCI	*Base + EI
*Enzyme + EI	0.7776	0.1141
*Base + NCI	0.4069	0.8115

* Base: Hydrolyzed with NaOH solution,
Enzyme: Hydrolyzed with glucuronidase,
NCI: Analyzed by NCI GCMS,
EI: Analyzed by EI GCMS.

3.8 Comparison of analysis and hydrolysis methods

As discussion above, all urine samples from the multiple dose study were hydrolyzed with both NaOH solution and glucuronidase, and analyzed by both EI and NCI techniques. The data from different method combinations were compared to see if the same results were obtained. Table 19 presents the t-test results of comparison of four groups of urine data measured by different method (hydrolysis and analysis) combination. No significant difference (p-value >> 0.05) among those four groups of data was indicated by this statistic results.

Further comparison of these four groups of data were performed on a MIDAS based computer program containing methodological statistics. Table 20 lists the correlation coefficients r^2 between those four groups of data.

Alkaline and glucuronidase hydrolysis gave very good consistency, when samples were analyzed by the NCI technique. The results from the NCI and EI methods also gave good consistency except for 5-OH VPA, when urine samples were hydrolyzed with enzyme. The poor agreement for 5-OH VPA could be due to EI analysis, since very good agreement was obtained between the two hydrolysis methods when 5-OH VPA was analyzed by NCI. Comparing these four groups of data of total urine VPA and metabolites, we can say that the combined method of base hydrolysis and EI analysis is not as good as the other three.

Table 20: Coefficients of determination (r^2, n=11) between concentrations of VPA urine metabolites measured by different hydrolysis (base or enzyme) and analysis (NCI or EI) methods. Urine samples were collected from a human volunteer participated in the multiple doses study (700 mg of VPA : [$^2H_6$]VPA (50:50) every 12 hr for 2.5 days).

Metabolites	Correlation Coefficients				
	NCI*	EI*	Base*	Enzyme*	Unhydrolyzed*
4-ene	0.98968	0.99668	0.98718	0.97977	0.67333**
3-ene	0.97400	0.97742	0.96690	0.95566	0.79158**
2-ene (Z)-	0.96690	0.99339	0.97621	0.98726	0.35921**
2-ene (E)-	0.99391	0.99846	0.98287	0.99561	0.84806**
VPA	0.97912	0.98785	0.98547	0.96284	0.81675**
2,4-diene	0.99594	0.99777	0.98856	0.99397	0.54398**
2,3′-diene	0.97460	0.99190	0.94098	0.94878	0.29505**
4-keto	0.95869	0.89630	0.95808	0.94769	0.97706
3-OH	0.98686	0.89371	0.95017	0.96591	0.91851
4-OH	0.95681	0.59668**	0.91728	0.95648	0.87108
3-keto	0.96737	0.99358	0.99383	0.96563	0.99271
5-OH	0.99854	0.52438**	0.61801**	0.62679**	0.95798
2-PSA	0.95541	0.99230	0.95195	0.95161	0.98570
2-PGA	0.99005	0.99584	0.99193	0.99827	0.98440

* NCI: Correlation between alkaline and glucuronidase hydrolysis results, when measured by NCI GCMS.

EI: Correlation between alkaline and glucuronidase hydrolysis, when measured by EI GCMS.

Base: Correlation between NCI and EI analysis methods when hydrolyzed with NaOH solution.

Enzyme: Correlation between NCI and EI analysis methods when hydrolyzed with glucuronidase.

Unhydrolyzed: Correlation between NCI and EI analysis results of non-conjugated components in urine samples.

** Poor correlation

For unhydrolyzed (free) urine samples, two analysis methods gave different correlation values for different metabolites. VPA and its unsaturated metabolites which exist mainly as conjugates in urine show poor agreement between the two analysis methods. Other metabolites which are not conjugated or have low levels of conjugates in urine show very good consistency when analyzed by the two different methods. Since strong acidic solution can dissociate the conjugates, it could be possible that some conjugates were hydrolyzed when urine samples were adjusted to pH=2 during extraction. The partial hydrolysis results in the variation of free fraction of VPA and its unsaturated metabolites that are excreted into urine mostly as their conjugated forms.

3.9 A Pharmacokinetic study of VPA in sheep using [$^{13}C_4$]VPA

The pharmacokinetics of VPA in sheep were studied by giving a single dose (I.V.) of 1000 mg of VPA:[$^{13}C_4$]VPA (50:50) to each of two sheep. Blood samples were collected and allowed to clot before centrifuging to collect serum samples. All serum samples were then analyzed by EI GCMS quantitatively, using the [$^{2}H_7$]VPA and its metabolites that were synthesized as internal standards. When stable isotope-labelled analogs are applied as internal standards, SIM mode is the ideal method for quantitative analysis. Figure 21 illustrates the SIM chromatograms of VPA [$^{13}C_4$]VPA and [$^{2}H_7$]VPA. These three analogs show very sharp peaks and can be readily differentiated based on their ion masses. As mentioned in the introduction, there should be no natural isotope interference since the mass difference is equal or higher than 3 mass units. The separation and quantitation of VPA, VPA metabolites and their C-13 labelled analogs were completed in a single chromatographic run of 29.5 minutes in length, C-13 labelled metabolites had very similar retention time as their unlabeled analogs, with about 0.01 min difference. Table 21 presents the retention times and m/z values of the $(M-57)^+$ diagnostic ions for VPA and its metabolites.

Table 21: The retention time and m/z values of the $(M-57)^+$ diagnostic ions of VPA and its metabolites isolated from serum samples of sheep dosed with single dose of 1 g of VPA:[$^{13}C_4$]VPA (50:50).

Metabolites[*]	Retention time (min)	Ion monitored (m/z)
VPA	13.64	201
4-ene VPA	13.88	199
(E)-3-ene VPA	14.05	199
(Z)-3-ene VPA	14.29	199
(Z)-2-ene VPA	16.58	199
(E)-2-ene VPA	14.64	199
(Z)-2,4-ene VPA	17.05	197
(E)-2,4-diene VPA	17.78	197
(E,Z)-2,3′-diene VPA	17.80	197
(E,E)-2,3′-diene VPA	18.71	197
3-keto VPA	21.79	329[b]
4-keto VPA	20.03	215
3-OH VPA	19.66, 19.93[a]	217
4-OH VPA	12.21, 12.41[a]	100[c]
5-OH VPA	22.14	331[b]
2-PSA	22.18	331[b]
2-PGA	22.50	345[b]

[a] Two isomers

[b] Diderivatives

[c] γ-Lactone

[*] [$^{13}C_4$]VPA and its metabolites were monitored by SIM with diagnostic ions which were four mass units higher than those of their unlabeled analogs.

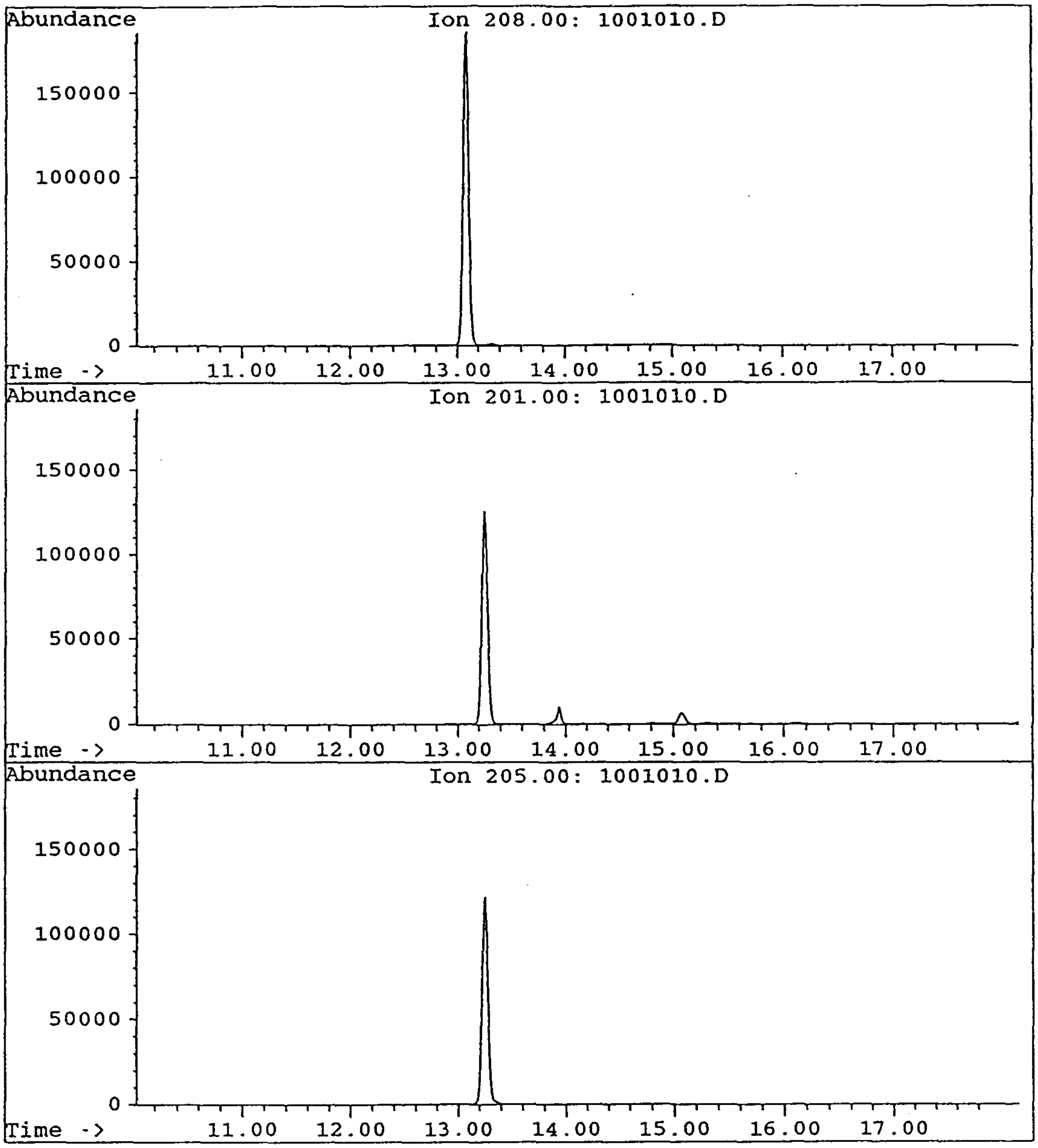


Fig. 22: SIM chromatograms of [$^{2}H_{7}$]VPA (top, internal standard), VPA (middle), and [$^{13}C_{4}$]VPA (bottom).

Excellent standard curves were obtained for most of the metabolites. The coefficients of determination obtained for calibration curves of metabolites isolated from standard urine samples are presented in Table 22.

As Table 22 shows, the calibration curves have very good linearity. The coefficients of determination r^2 for all metabolites, were greater than 0.994, except for 3-OH and 3-keto VPA. Calibration curves for 3-OH and 3-keto VPA could be expected to improve had $[^2H_7]$3-OH VPA and $[^2H_7]$3-keto VPA been used as internal standards. These two internal standards were not synthesized until after this analysis had been completed. Our results thus support the contention that optimal calibration curves are obtained by using stable isotope-labelled analogues as internal standards when samples are analyzed with GCMS. Unfortunately, $[^2H_7]$4-ene VPA could not be used in this assay because of interference from $[^{13}C_4]$VPA with a mass difference of only 1 mass units. Retention times were very close and the sample contained a high concentration of $[^{13}C_4]$VPA. Thus to apply $[^2H_7]$4-ene VPA as an internal standard, some modification in the GC conditions would be necessary to adequately separate VPA and the 4-ene VPA peaks. The NCI method should be suitable for application of $[^2H_7]$4-ene VPA as an internal standard because the 4-ene precedes VPA in retention time.

Table 22: Linearity of calibration curves for the quantitative assays of VPA, VPA metabolites and their C-13 labelled analogues isolated from urine samples of sheep dosed I.V. with a single dose of 1000 mg of VPA:[$^{13}C_4$]VPA (50:50).

Metabolites*	r^2	Internal Standards
VPA	1.000	[$^2H_7$]VPA
4-ene VPA	0.999	[$^2H_7$]VPA
3-ene VPA	0.999	[$^2H_7$]VPA
(Z)-2-ene VPA	0.999	[$^2H_7$]VPA
(E)-2-ene VPA	0.999	[$^2H_7$]VPA
(E)-2,4-diene VPA	0.999	[$^2H_7$]VPA
(E,Z)-2,3′-diene VPA	0.999	[$^2H_7$]VPA
(E,E)-2,3′-diene VPA	0.996	[$^2H_7$]VPA
3-keto VPA	0.975	[$^2H_7$]4-keto VPA
4-keto VPA	0.999	[$^2H_7$]4-keto VPA
3-OH VPA	0.988	[$^2H_7$]VPA
4-OH VPA	0.996	[$^2H_7$]VPA
4-OH VPA isomer	0.990	[$^2H_7$]VPA
5-OH VPA	0.994	[$^2H_7$]5-OH VPA
2-PGA	0.997	[$^2H_7$]VPA

* [$^{13}C_4$]VPA and its metabolites were analyzed using the calibration curves for the unlabeled analogs.

Pharmacokinetic parameters for VPA, $[^{13}C_4]$VPA and the major serum metabolites, 2-ene VPA and $[^{13}C_4]$2-ene VPA were determined for the two sheep that were studied. The results are presented in table 23.

Table 23: Pharmacokinetic Parameters for VPA(I), $[^{13}C_4]$VPA(II), (E)-2-ene VPA (III) and $[^{13}C_4]$(E)-2-ene VPA (IV) measured in two sheep dosed I.V. with a single dose of 1000 mg of VPA:$[^{13}C_4]$VPA (50:50). Serum samples were analyzed by EI GCMS.

	$t_{1/2}$ (h)	K_E (h^{-1})	CL (L/h/Kg)	AUC (mg.h/L)	V_D (L/Kg)
Sheep #1					
I	5.09	0.136	0.122	55.9	0.897
II	4.98	0.139	0.127	53.42	0.913
III	5.63	0.123			
IV	5.63	0.123			
Sheep #2					
I	2.42	0.286	0.159	52.5	0.556
II	2.41	0.287	0.160	52.0	0.557
III	4.33	0.160			
IV	3.89	0.178			

No apparent isotope effect of $[^{13}C_4]$VPA metabolism was observed in these two sheep based on AUC ratio of VPA to $[^{13}C_4]$VPA, which is in agreement with the result we obtained from the study in a healthy human volunteer.

Since few studies of VPA pharmacokinetics have been carried out in sheep, very little information is available on the metabolic fate of valproic acid in this species. Nevertheless, this initial study of $[^{13}C_4]$VPA indicates that this compound should prove ideal for the study of placental transfer and the determination of drug pharmacokinetics in both mother and fetus.

3.10 Metabolic studies of (Z)-and (E)-3-ene VPA

As discussed in the section 3.4., it was suggested that, 2,4-ene VPA may be partially formed from 3-ene VPA. To further investigate this hypothesis, we synthesized (Z)-3-ene and (E)-3-ene VPA for metabolism studies in rats.

The syntheses of (E)- and (Z)-3-ene VPA were discussed in section 3.1.9. Both (E)-and (Z)-3-ene VPA were analyzed by GCMS and by NMR and determined to be of very high purity before they were used in metabolism studies.

(Z)- and (E)-3-ene VPA (150 mg/kg dose) were separately administrated i.p. to two rats (adult male Wistar rats weighing 270 and 308 g). A blood sample (about 200 ul) from each rat was taken 2 h after the dose, allowed to clot, and centrifuged to obtain a serum sample. Urine samples were collected every 24 hours for two days. Samples were stored at -20 °C until derivatized and analyzed by GCMS using EI techniques.

In the SIM mode used, m/z 197 and 199 were monitored to see the diene VPA metabolites and the parent drugs (E)- and (Z)-3-ene VPA. From the assay results, two diene VPA metabolites were present in urine samples of both sheep, one metabolite was confirmed to be (E,E)-2,3′-diene VPA. The other diene VPA could be either (E,Z)-2,3′-diene VPA or 2,4-diene VPA since they had very close retention time under EI analysis conditions. It was likely that (E,E)-2,3′-diene VPA was the main diene metabolite of (E)-3-ene VPA, while (E,Z)-2,3′-diene or 2,4-diene VPA accounts for most of the diene metabolites of (Z)-3-ene VPA. Table 24 shows the peak area of all the ions monitored in urine samples. Since (E)-2,4-diene VPA and (E,Z)-2,3′-diene VPA were not differentiated well under that condition, it is not apparent that (E)-2,4-diene VPA is one of metabolites of 3-ene VPA. Further analysis by using NCI mode has to be done before making a final conclusion.

The t-BDMS derivative of (Z)-3-ene VPA had a slightly longer retention time than that of (E)-3-ene VPA, when run on an OV-1701 column. It is usually the (E)-isomer of unsaturated fatty acids that has the longer retention time with this stationary phase. The reason for this reversal of elution order by the (E)- and (Z)- isomers of 3-ene VPA is not readily apparent.

Table 24: Retention time and peak area of monitored ions m/z 199 and 197 which represent parent drug 3-ene VPA and diene metabolites respectively isolated from urine of rats dosed with either (Z)- or (E)-3-ene VPA (150 mg/kg).

	(Z)-3-ene VPA		(E)-3-ene VPA	
Metabolites (t_R)	0-24h	24-48h	0-24h	24-48h
(Z)-3-ene VPA (8.53 min)	178442944	21974892	N.D.	N.D.
(E)-3-ene VPA (8.38 min)	N.D.	N.D.	243174752	9520370
*(E,Z)-2,3′-diene VPA (10.82 min)	66036848	3494949	4874712	118144
(E,E)-2,3′-diene VPA (12.83 min)	21874212	1503089	17302344	548160

* or (E)-2,4-diene VPA

4. Summary and Conclusions

4.1 GCMS conditions for both EI and NCI were optimized to obtain optimal resolution and sensitivity for VPA metabolites and their stable isotope-labelled analogs. A single temperature program with a run time of 47 min was established for NCI analysis of PFB derivatives of VPA, VPA metabolites and their [$^2H_6$] labelled analogs. Two temperature programs were investigated for t-BDMS derivatives of VPA and VPA metabolites and their [$^2H_6$] labelled analogs, one with a run time of 35 min was used for VPA unsaturated metabolites, the other with a run time of 20 min was for more polar metabolites of VPA.

4.2 In the 12h period immediately following the dose, $t_{1/2}$ and AUC values for VPA and [$^2H_6$]VPA in the human volunteer were not different. AUC values and urinary recoveries of unlabeled and labelled metabolites over the same time period were equivalent except for 5-OH VPA and 2-propylglutaric acid (2-PGA). Significant isotope effects were observed for 5-OH VPA and 2-PGA. No apparent isotope effect was observed for 4-ene VPA, which supported the mechanism that a carbon centered radical at C-4 of VPA serves as an intermediate, and this step is rate limiting in the formation of 4-ene VPA.

A small isotope effect was observed for 2,4-diene VPA. It was then proposed that the formation of 2,4-diene VPA might occur partly from 3-ene VPA. An experiment was designed to test this proposal.

4.3 (E)- and (Z)- 3-ene VPA were synthesized. Both were used in metabolic studies in rats. The metabolites diene VPA and parent drug 3-ene VPA were monitored in urine samples of rats dosed with either (E)- or (Z)-3-ene VPA by SIM EI mode. Both (E)- and (Z)- isomers of 3-ene VPA were shown to have two diene VPA metabolites. One is confirmed to be (E,E)-2,3′-diene VPA, the other could be either 2,4-diene VPA or (E,Z)-2,3′-diene VPA since they had the same retention time. The (E)-3-ene VPA had a higher level of metabolite (E,E)-2,3′-diene VPA, while (Z)-3-ene VPA was metabolized into more (E,Z)-2,3′-diene VPA (or 2,4-diene VPA).

4.4 More than 90% of VPA and its unsaturated metabolites (2-ene, 3-ene, 4-ene, 2,4-diene and 2,3′-diene-VPA) were in the form of their glucuronic acid conjugates when excreted into urine. Metabolites 3-OH, 4-OH and 5-OH VPA were excreted partly as glucuronides, while, 3-keto, 4-keto VPA, 2-PSA and 2-PGA were excreted mostly as free metabolites.

4.5 No difference was observed between the concentrations of total VPA and its metabolites when urine samples were hydrolyzed with glucuronidase and alkali NaOH solution. Therefore, it seems that there was no glucuronidase-resistant conjugate present in the urine samples of this healthy human volunteer after urine samples were kept at -20 °C for about two months.

NCI and EI analyzing techniques show very good agreements for most metabolites in urine samples. PFB derivatives of VPA metabolites analyzed by NCI technique give higher sensitivity and better resolution than t-BDMS derivatives of VPA metabolites analyzed by EI methods.

4.6 No apparent isotope effect was observed in the metabolism of $[^{13}C_4]$VPA based on concentration ratios of VPA and its metabolites to their $[^{13}C_4]$ labelled analogs in serum and urine samples of a healthy human volunteer. Thus it would appear that $[^{13}C_4]$VPA is suitable for the pharmacokinetic studies of VPA metabolites in pediatric patients when given as a "pulse" dose.

4.7 Eight deuterium labelled compounds that included $[^{2}H_7]$VPA, $[^{2}H_7]$4-ene, $[^{2}H_7]$4-keto, $[^{2}H_7]$4-OH, $[^{2}H_7]$5-OH, $[^{2}H_7]$3-keto, $[^{2}H_7]$3-OH, and $[^{2}H_7]$2-ene VPA were synthesized and used as internal standards in a GCMS assay of VPA, $[^{13}C_4]$VPA and their metabolites for the pharmacokinetic studies in sheep.

4.8 Pharmacokinetics of VPA was studied in sheep dosed with VPA:$[^{13}C_4]$VPA (50:50). No isotope effect was observed for the $[^{13}C_4]$VPA. The elimination half-life of VPA in these two sheep was estimated to be approximately 5.0 and 2.4 hr respectively.

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