PHARMACOKINETICS, TISSUE DISTRIBUTION, AND PHARMACODYNAMICS OF VALPROIC ACID AND ITS UNSATURATED METABOLITES IN RATS

by

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We accept this thesis as conforming
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ABSTRACT

Valproic acid (VPA), an antiepileptic drug, possesses a delay in maximum pharmacological response upon initial drug administration, and a prolonged duration of activity following discontinuation of the drug. Metabolites of VPA are thought to be involved as evidence from previous studies in mice demonstrated that (E)-2-ene VPA and (E,E)-2,3′-diene VPA, major products of VPA metabolism in serum, exerted some degree of anticonvulsant activity against pentylenetetrazole (PTZ)-induced seizures. Also associated with VPA therapy is a fatal idiosyncratic hepatotoxicity possibly involving two metabolites, 4-ene VPA and (E)-2,4-diene VPA. Preliminary tissue distribution studies had suggested that (E)-2-ene VPA may not be as hepatotoxic as VPA based solely on (E)-2-ene VPA concentrations in liver. The main objectives of this study were to investigate the kinetic and metabolic profiles, disposition, and anticonvulsant activity of VPA, (E)-2-ene VPA, and (E,E)-2,3′-diene VPA in rats. Results of these experiments were intended to provide insight into the possible contributions of these metabolites towards VPA activity or toxicity.

Synthesis of (E)-2-ene VPA and (E,E)-2,3′-diene VPA was accomplished by the regiospecific addition of propionaldehyde to an ester enolate, followed by nucleophilic elimination of the mesylate ester with 1,8-diazabicyclo[5.4.0]undec-7-ene or potassium hydride. The synthesis provided good yields and was stereoselective. The isomeric purity of the synthesized compounds was found to be 95 - 97% based on
nuclear magnetic resonance and gas chromatographic-mass spectrometric data.

The assay of VPA and its metabolites in rat plasma and tissue homogenate extracts was achieved by negative ion chemical ionization gas chromatography-mass spectrometry. This method proved to be selective, sensitive, reproducible, and amenable to automation.

In order to compare the disposition and pharmacokinetics of VPA and its analogues, VPA was administered intraperitoneally to rats and the kinetic profiles in plasma, liver, heart, lungs, and nine brain regions were determined. Selective binding of VPA to liver was observed with the liver/plasma ratio at 10 hours after dosing being 4.6. VPA did not persist in brain and the distribution in brain tissue appeared uniform. Metabolites of VPA also were not retained in brain. A most interesting observation was the absence of (E,E)-2,3'-diene VPA in brain while a minor plasma metabolite, (E,Z)-2,3'-diene VPA, was the only detectable diene. A stereoselective active transport mechanism could account for this unusual result. Present in plasma but not detected in liver was (E)-2,4-diene VPA, the hepatotoxic metabolite of VPA. It was proposed that the diene may be covalently bound to liver tissue.

Following single dose administration to rats, (E)-2-ene VPA appeared to persist in all tissues assayed following an initial decline phase. The prolonged terminal elimination phase may be attributed to the extensive plasma protein binding of (E)-2-ene VPA (>99%). No selective binding of (E)-2-ene VPA in brain was observed. Brain/plasma ratios at 10 hours after dosing did not exceed 0.03. Metabolites of (E)-2-ene VPA were mainly products of β-oxidation and reduction. Both hepatotoxic metabolites were observed in plasma with concentrations of 4-ene VPA in
liver higher than normally seen following VPA administration. Questions arise regarding the potential hepatotoxicity of (E)-2-ene VPA.

After single dose administration of (E,E)-2,3'-diene VPA to rats, clearance of the diene was rapid compared to that of VPA or (E)-2-ene VPA. Selective binding of the diene was observed in the superior and inferior colliculus and substantia nigra but the concentrations were too low to be considered clinically significant. Reduction of (E,E)-2,3'-diene VPA appeared to be the main route of metabolism. 4-Ene VPA and (E)-2,4-diene VPA were not detected in plasma or tissues suggesting (E,E)-2,3'-diene VPA may have a lower potential for liver toxicity.

The anticonvulsant activities of VPA, (E)-2-ene VPA, and (E,E)-2,3'-diene VPA were compared in rats by the PTZ-induced seizure test. Based on ED50 values, the anticonvulsant potencies of VPA and (E)-2-ene VPA were comparable and significantly greater than (E,E)-2,3'-diene VPA. The detection of (E,Z)-2,3'-diene VPA in brain following VPA administration led to the testing of this diene isomer. The potency of the (E,Z)-isomer was found to be equivalent to VPA and (E)-2-ene VPA. Sedation was a severe side effect of (E)-2-ene VPA and the (E,E)-2,3'-diene VPA was stereoselectively unique in causing skeletal muscle rigidity. Sedation was minimal and muscle rigidity was not a property of the (E,Z)-isomer over the dose range studied.

Based on the results of these studies, it can be concluded that neither (E)-2-ene VPA nor (E,E)-2,3'-diene VPA is responsible for the pharmacodynamic effects of VPA. From the metabolism of (E,E)-2,3'-diene VPA and the results of anticonvulsant testing, it was proposed that (E,Z)-2,3'-diene VPA may have potential as a relatively safe and useful anticonvulsant drug.
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Concentration-time plots of (E,E)-2,3'-diene VPA and its polar metabolites in liver following the administration of 150 mg/kg i.p. of (E,E)-2,3'-diene VPA (containing 3-5% (E,Z)-2,3'-diene VPA) to rats (n=8/time point, S.D. omitted for clarity).

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Proposed metabolic scheme of (E,E)-2,3'-diene VPA in rats
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<td></td>
</tr>
<tr>
<td>-------</td>
<td>------------</td>
<td></td>
</tr>
<tr>
<td>AUC</td>
<td>area under the curve</td>
<td></td>
</tr>
<tr>
<td>bp</td>
<td>boiling point</td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>corpus callosum</td>
<td></td>
</tr>
<tr>
<td>CER</td>
<td>cerebellum</td>
<td></td>
</tr>
<tr>
<td>CI</td>
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<td></td>
</tr>
<tr>
<td>Cl</td>
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<td></td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt;</td>
<td>peak drug concentration</td>
<td></td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
<td></td>
</tr>
<tr>
<td>CP</td>
<td>putamen</td>
<td></td>
</tr>
<tr>
<td>CSF</td>
<td>cerebral spinal fluid</td>
<td></td>
</tr>
<tr>
<td>d</td>
<td>doublet</td>
<td></td>
</tr>
<tr>
<td>DBU</td>
<td>1,8-diazabicyclo[5.4.0.]undec-7-ene</td>
<td></td>
</tr>
<tr>
<td>dd</td>
<td>doublet of doublets</td>
<td></td>
</tr>
<tr>
<td>dt</td>
<td>doublet of triplets</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>trans</td>
<td></td>
</tr>
<tr>
<td>EI</td>
<td>electron impact</td>
<td></td>
</tr>
<tr>
<td>FFA</td>
<td>free fatty acid</td>
<td></td>
</tr>
<tr>
<td>GABA</td>
<td>gamma aminobutyric acid</td>
<td></td>
</tr>
<tr>
<td>GABA-t</td>
<td>gamma aminobutyric acid-transaminase</td>
<td></td>
</tr>
<tr>
<td>GAD</td>
<td>glutaric acid decarboxylase</td>
<td></td>
</tr>
<tr>
<td>GC-MS</td>
<td>gas chromatography-mass spectrometry</td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>proton</td>
<td></td>
</tr>
<tr>
<td>HIP</td>
<td>hippocampus</td>
<td></td>
</tr>
<tr>
<td>i.d.</td>
<td>internal diameter</td>
<td></td>
</tr>
</tbody>
</table>
IC        inferior colliculus
i.p.     intraperitoneal
i.v.     intravenous
J        coupling constant
LES      low frequency electroshock stimulation
lit.     literature
m        meter/multiplet
M⁺       molecular ion
MED      medulla
MES      maximum electroshock seizures
MHz      megahertz
mmoles   millimoles
MSTFA    N-methyl-N-trimethylsilyl trifluoro acetamide
MW       molecular weight
m/z      mass/charge
NICI     negative ion chemical ionization
NMR      nuclear magnetic resonance
OLF      olfactory bulbs
PFB      pentafluorobenzyl
ppm      parts per million
PTZ      pentylentetrazole
q        quartet
s.c.     subcutaneous
SC       superior colliculus
SN       substantia nigra
t        triplet
t-BDMS   tertiary-butyldimethylsilane
<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>TIC</td>
<td>total ion chromatogram</td>
</tr>
<tr>
<td>t_{max}</td>
<td>time to reach peak drug concentration</td>
</tr>
<tr>
<td>TMS</td>
<td>trimethylsilane</td>
</tr>
<tr>
<td>VPA</td>
<td>valproic acid</td>
</tr>
<tr>
<td>[^{2}H]_{6}VPA</td>
<td>[^{2}H]_{6}valproic acid</td>
</tr>
<tr>
<td>WB</td>
<td>whole brain</td>
</tr>
<tr>
<td>Z</td>
<td>cis</td>
</tr>
<tr>
<td>2,3'-diene VPA</td>
<td>2-(1'-propenyl)-2-pentenoic acid</td>
</tr>
<tr>
<td>2,4-diene VPA</td>
<td>2-n-propyl-2,4-pentadienoic acid</td>
</tr>
<tr>
<td>2-ene VPA</td>
<td>2-n-propyl-2-pentenoic acid</td>
</tr>
<tr>
<td>[^{2}H]_{3}2-ene VPA</td>
<td>[^{2}H]_{3}2-n-propyl-2,4-pentadienoic acid</td>
</tr>
<tr>
<td>3-ene VPA</td>
<td>2-n-propyl-3-pentenoic acid</td>
</tr>
<tr>
<td>4-ene VPA</td>
<td>2-n-propyl-4-pentenoic acid</td>
</tr>
<tr>
<td>3-keto VPA</td>
<td>2-n-propyl-3-oxopentanoic acid</td>
</tr>
<tr>
<td>[^{2}H]_{3}3-keto VPA</td>
<td>[^{2}H]_{3}2-n-propyl-3-oxopentanoic acid</td>
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<tr>
<td>4-keto VPA</td>
<td>2-n-propyl-4-oxopentanoic acid</td>
</tr>
<tr>
<td>3-OH VPA</td>
<td>2-n-propyl-3-hydroxypentanoic acid</td>
</tr>
<tr>
<td>4-OH VPA</td>
<td>2-n-propyl-4-hydroxypentanoic acid</td>
</tr>
<tr>
<td>5-OH VPA</td>
<td>2-n-propyl-5-hydroxypentanoic acid</td>
</tr>
<tr>
<td>2-MGA</td>
<td>2-methylglutaric acid</td>
</tr>
<tr>
<td>2-PGA</td>
<td>2-propylglutaric acid</td>
</tr>
<tr>
<td>2-PMA</td>
<td>2-propylmalonic acid</td>
</tr>
<tr>
<td>2-PSA</td>
<td>2-propylsuccinic acid</td>
</tr>
</tbody>
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1. INTRODUCTION

1.1 VALPROIC ACID

1.1.1 Overview

Epilepsy is a common disorder occurring in at least 1 out of 200 people in the U.S. (Lechtenberg, 1990). Epilepsy cannot be defined as a single disease but rather as a group of disorders having similar pathophysiological properties (Lechtenberg, 1990). In fact, the term epileptic syndrome would be more appropriate rather than disease, since only a few diseases have been associated with epilepsy (Dreifuss et al., 1985). An epileptic syndrome is a group of signs and symptoms (clinical or ancillary) occurring together and may not have a common etiology or prognosis (Dreifuss et al., 1985; Levy et al., 1989). More specifically epilepsy is a symptom of excessive neuronal discharges resulting from either extra or intracranial disturbances.

The two common types of epilepsies are partial and generalized seizures, although other terms have been used in the literature (table 1)(Dreifuss et al., 1985; Levy et al., 1989). The initial protocol for the treatment of an epileptic episode is nearly always chemically based.

During the nineteenth century, bromide salts were introduced for the management of seizures but were later replaced in 1912 by phenobarbital (Lechtenberg, 1990). In 1938 phenytoin was marketed and quickly became the benchmark for future antiepileptics. The majority of the drugs used to manage epilepsy today are structurally similar to
Table 1: A summary of the two main types of epilepsies and their synonyms (Dreifuss et al., 1985).

<table>
<thead>
<tr>
<th>PARTIAL SEIZURES</th>
<th>GENERALIZED SEIZURES</th>
</tr>
</thead>
<tbody>
<tr>
<td>focal seizures</td>
<td>primary epilepsy</td>
</tr>
<tr>
<td>localizational-related epilepsy</td>
<td>idiopathic epilepsy</td>
</tr>
<tr>
<td>secondary epilepsy</td>
<td>cryptogenic epilepsy</td>
</tr>
<tr>
<td>symptomatic epilepsy</td>
<td></td>
</tr>
</tbody>
</table>
phenobarbital or phenytoin albeit with one exception, valproic acid (VPA)(figure 1).

VPA is an eight carbon branched chain fatty acid synthesized initially in 1881 for use as an organic solvent. In 1963 VPA was accidently discovered to have anticonvulsant activity in animals (Meunier et al., 1963). Soon after (1967) VPA was introduced into Europe as an antiepileptic agent and became available in the United States by 1978. In 1983 VPA was marketed as a syrup and gelatin capsule under the trade name Depakene® and since then has been used extensively for the successful management of primary generalized and some partial seizures (table 2)(Dulac and Arthuis, 1984; Chadwick, 1987; Bourgeois et al., 1987; Wilder, 1987; Dean and Penry, 1988).

Associated with VPA treatment is a delay in the onset of maximal anticonvulsant activity (Jeavons and Clark, 1974; Rowan et al., 1979) together with an extended duration of activity even after the disappearance of the parent compound from the circulation in both humans and animals (Lockard and Levy, 1976; Harding et al., 1978). It appears that the concentration of VPA in plasma does not correspond with anticonvulsant activity. Since VPA is extensively metabolized by the liver, perhaps one or more of these metabolites would be a better measure of anticonvulsant activity. The present study will investigate the potential contribution of a major VPA metabolite, (E,E)-2,3'-diene VPA, to these unusual pharmacodynamics of VPA.

1.1.2 Adverse Reactions

Adverse reactions of VPA can be classified into types A, B, and teratogenic effects (Dreifuss and Langer, 1988). The type A reactions
Figure 1: Chemical structures of some commonly used anticonvulsant agents.
Table 2: The drugs of choice for both generalized and localization-related seizures.

<table>
<thead>
<tr>
<th>Generalized Seizures</th>
<th>First Choice</th>
<th>Second Choice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Idiopathic (Primary)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>absence</td>
<td>VPA/ESM</td>
<td>CZP</td>
</tr>
<tr>
<td>atypical</td>
<td>VPA</td>
<td>CZP</td>
</tr>
<tr>
<td>myoclonic</td>
<td>VPA</td>
<td>PB</td>
</tr>
<tr>
<td>tonic</td>
<td>VPA</td>
<td>DPH</td>
</tr>
<tr>
<td>clonic</td>
<td>VPA</td>
<td>DPH</td>
</tr>
<tr>
<td>tonic-clonic</td>
<td>VPA</td>
<td>DPH</td>
</tr>
<tr>
<td>atonic</td>
<td>VPA</td>
<td>DPH</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Localization-related (Partial) Seizures</th>
<th>First Choice</th>
<th>Second Choice</th>
</tr>
</thead>
<tbody>
<tr>
<td>idiopathic (simple and complex)</td>
<td>CBZ/DPH</td>
<td>PRM/VPA/PB</td>
</tr>
<tr>
<td>secondary generalized</td>
<td>CBZ/DPH</td>
<td>VPA/PRM/PB</td>
</tr>
</tbody>
</table>

VPA=valproic acid; CZP=clonazepam; ESM=ethosuximide; PB=phenobarbital; DPH=phenytoin; CBZ=carbamazepine; PRM=primidone;
are dose-related and usually tend to occur at the upper end of the dose-response curve. Type A reactions will occur in most patients if the dose is high enough; however, the side effects usually subside when the dose is lowered. In general, a type A adverse reaction is not life threatening unless a fatal underlying disease is involved or the drug is taken in an overdose situation. In contrast, a type B adverse reaction is unpredictable, is not considered dose-related, and may be fatal. The causes of a type B reaction are usually unknown (idiosyncratic); however, the reaction sometimes involves a metabolic disorder (Zimmerman and Ishak, 1982; Dreifuss and Langer, 1988). Teratogenic effects can occur with a therapeutic and non-toxic dose to the mother but exposure of the fetus to VPA can result in a host of developmental complications.

1.1.2.1 Type A Adverse Effects of VPA

Gastrointestinal side effects are the most common complaints from patients on VPA. Up to 50% of patients on VPA therapy experience gastrointestinal disturbances (nausea, vomiting, anorexia) that can be managed by reducing the dose and/or by taking the drug with food (Wilder et al., 1983; Bruni and Wilder, 1979).

Central nervous system side effects such as sedation can occur in 50% of the patients on initial VPA treatment but after long-term use less than 1% of patients are affected by drowsiness (Jeavons et al., 1977; Gram et al., 1977; Lance and Anthony, 1977). Fine tremor has been associated with VPA therapy but this symptom can be alleviated by adjusting the dose or dosing regime such that the amplitude of the peak and trough plasma concentrations are reduced (Hyman et al, 1979).
Other adverse effects of VPA that are not as common and relatively less traumatic are weight gain (Dinessen et al., 1984), skin rash (Bruni and Albright, 1983), and hair changes (depigmentation, alopecia, perming effect) (Rauskanen et al., 1979; Gupta, 1988).

1.1.2.2 Type B Adverse Effects of VPA: Hepatotoxicity

The idiosyncratic hepatotoxicity associated with VPA therapy was initially thought to be a rare but fatal reaction with an overall incidence of 1:10000. As the use of VPA increased so did the rate of deaths, with an incidence of hepatotoxicity reported to be as low as 1:500 for high risk patients (Dreifuss et al., 1989). Today the number of fatalities has decreased dramatically since clinicians are becoming increasingly aware of the risk factors for hepatotoxicity associated with VPA. Fulminating hepatotoxicity associated with VPA use usually occurs within the first 4 to 6 months of therapy, with children under the age of 10 years representing 73% of all fatalities (Dreifuss et al., 1987). At highest risk were patients less than 2 years of age on multianticonvulsant treatment, i.e. VPA-polytherapy. For those patients over 2 years of age on VPA-monotherapy, the risk of developing fatal hepatotoxicity has decreased to 1:37000 (Dreifuss et al., 1989).

Some of the common pathological findings from patients exhibiting VPA-induced hepatotoxicity are increased body temperature, elevated liver enzymes, jaundice, hepatic microvesicular steatosis and necrosis with mitochondrial dissolution, increased intracranial pressure, and coma (Gerber et al., 1979; Itoh et al., 1982; Zimmerman and Ishak, 1982; Scheffner et al., 1988; Sugimoto et al., 1987). Although the mechanism of VPA hepatotoxicity is not known, there are similarities to Reye's
Syndrome, hypoglycin toxicity (Jamaican Vomiting Sickness) and 4-pentenoic acid toxicity (Kesterson et al., 1984). Both 4-pentenoic acid and methylenecyclopropylacetic acid (a metabolite of hypoglycin) are known steatogenic agents (Zimmerman and Ishak, 1982). Two metabolites of VPA, namely 4-ene VPA and 2,4-diene VPA are structurally similar to 4-pentenoic acid and methylenecyclopropylacetic acid (figure 2). Upon GC-MS analysis of the urine and plasma samples from patients succumbing to VPA-induced hepatotoxicity, abnormally high levels of 4-ene VPA and (E)-2,4-diene VPA and decreased levels of 3-keto VPA and 3-OH VPA, products of \( \beta \)-oxidation, were discovered (Scheffner et al., 1988). Evidence in humans, such as mitochondrial damage and a decrease in the \( \beta \)-oxidation products, 3-keto VPA and 3-OH VPA, indicated that \( \beta \)-oxidation was being inhibited. An inhibition of \( \beta \)-oxidation will result in a shunting of VPA metabolism to other pathways. This could explain the higher levels of 4-ene VPA and 2,4-diene VPA found in the plasma and urine of hepatotoxic patients, since the origin of these metabolites is via microsomal metabolism. Therefore, the disruption or interference of fatty acid \( \beta \)-oxidation by an unknown factor could be a prelude to VPA hepatotoxicity.

Isolated rat hepatocytes showed a dose-related toxicity to VPA (Kingsley et al., 1980). Rats administered a single dose of VPA showed altered hepatocytes and mitochondria (Jezequel et al., 1984). Rats administered either 4-ene VPA or 2,4-diene VPA were found to develop fatty livers, microvesicular steatosis and necrosis, and mitochondrial damage similar to that observed in patients displaying VPA hepatotoxicity (Kesterson et al., 1984).

The cause of VPA-induced hepatotoxicity is thought to involve possible reactive intermediates generated by common biotransformation
Chemical structures of two known hepatotoxic agents 4-pentenoic acid and methylenecyclopropylacetic acid and two metabolites of VPA suspected of being responsible for VPA hepatotoxicity.
processes. The metabolism of the hepatotoxic agent, 4-ene VPA, by cytochrome P-450 or β-oxidation to yield a potential electrophilic species is currently being investigated (Baillie, 1988). The metabolism of 4-ene VPA by the liver microsomal cytochrome P-450 enzymes generated a free radical intermediate that could alkylate heme, thereby destroying the mixed-function oxidase (Ortiz de Montellano and Correia, 1983). This route of 4-ene VPA metabolism is akin to a suicide-substrate. In addition, the free radical intermediate can give rise to an epoxide that could possibly alkylate key metabolizing enzymes (Baillie, 1988).

The bioactivation of 4-ene VPA via mitochondrial β-oxidation to a reactive metabolite was based on knowledge of 4-pentenoic acid metabolism (Schulz, 1983). Beta-oxidation of 4-pentenoic acid results in the formation of a highly reactive electrophile, 3-oxo-4-pentenoyl-CoA. This reactive species can alkylate 3-ketoacyl-CoA thiolase, the terminal enzyme in the β-oxidation pathway, and thereby inhibit β-oxidation. The 4-ene VPA could undergo a similar fate leading to a corresponding electrophilic species, 3-oxo-4-ene VPA (Porubek et al., 1989). However, from isolated perfused rat liver challenged with 4-ene VPA, 3-OH-4-ene VPA and 2,4-diene VPA were the only metabolites identified (Rettenmeier et al., 1985). The authors suggested that failure to detect the 3-oxo-4-ene VPA was in part due to the highly reactive nature of this compound.

Current work by Kassahun and Abbott (1989) suggests that the 3-oxo-4-ene VPA may not be the agent responsible for VPA hepatotoxicity. The metabolite found in the hepatotoxic patients, (E)-2,4-diene VPA may be the suspect compound. Although still speculative, the conjugation of 2,4-diene VPA with CoA to yield 2,4-diene valproyl CoA could proceed to a potentially reactive intermediate upon resonance rearrangement. This
reactive electrophile has the potential of interfering with liver metabolism and ultimately leading to severe hepatotoxicity.

1.1.2.3 Teratogenic Effects of VPA

The teratogenic effects of VPA encompass a host of malformations that include facial anomalies of the nose, lips, palate, eyes, and ears; digital anomalies such as polydactylysm and hypoplasia of the phalanges and nails; congenital heart defects such as ductus arteriosus and ventricular septal defects; and neural tube defects seen as lumbosacral meningocele and spina bifida (Nau et al., 1982; Kaneko et al., 1988; Carter and Stewart, 1989; Bertollini et al., 1985). Approximately 1% of fetuses exposed to VPA in utero develop spina bifida (Nau and Loscher, 1984). Unfortunately, the alternatives are either to use other anticonvulsants, which are also teratogenic, or to discontinue drug use and risk seizures.

Animal studies have shown that 4-ene VPA displayed enantioselective teratogenicity with the S-enantiomer being more teratogenic than VPA (Hauck and Nau, 1989). Conversely, the metabolite (E)-2-ene VPA was found to be less teratogenic than the parent drug when administered at comparable doses suggesting that perhaps (E)-2-ene VPA could be used in place of VPA when the risk of teratogenicity becomes relevant (Nau and Loscher, 1984).

1.1.3 Metabolism

The metabolism of VPA is highly complex for such a simple molecule (figures 3 and 4). In mammals, the fate of VPA is mainly hepatic metabolism, since only 1 - 3% of the dose is excreted unchanged
Figure 3: Metabolic pathways of valproic acid: (a) ω-oxidation, (b) dehydrogenation, (c) glucuronidation, and (d) (ω-1)-oxidation.
Figure 4: The β-oxidation pathway of valproic acid.
in the urine (Bruni and Wilder, 1979; Gugler and von Unruh, 1980; Bailer et al., 1985). Although several metabolic pathways exist, the two major routes are glucuronidation and β-oxidation with the relative contribution of the latter occurring to a greater extent in man when compared to rats (Granneman et al., 1984). Minor routes of VPA metabolism in both man and animals include ω-oxidation, (ω-1)-oxidation, dehydrogenation, reduction, glycine conjugation, epoxidation, and isomerization (Granneman et al., 1984; Abbott et al., 1986).

In humans, glucuronidation of VPA accounts for 10.8 - 68.3% of the dose (Bonora et al., 1979; Bruni and Wilder, 1979; Bailer et al., 1985; Dickinson et al., 1989). As the dose of VPA increases, the drug is shunted away from the β-oxidation pathway and more towards glucuronide conjugation thereby producing a net increase in glucuronidation (Granneman et al., 1984).

The second major route of VPA metabolism is β-oxidation (figure 4). Metabolites generated via β-oxidation are (E)-2-ene VPA, 3-OH VPA, and 3-keto VPA (Granneman et al., 1984) and, in total, account for approximately 12.5% of VPA in serum (Abbott et al., 1986; Dickinson et al., 1989; Kassahun et al., 1990). However, the use of (E)-2-ene VPA, 3-OH VPA, and 3-keto VPA to estimate the degree of VPA β-oxidation could be misleading. Several of the subsequent products from the β-oxidative metabolites of VPA occur endogenously as products of FFA metabolism. This could, in part, explain why 30% of the dose of VPA administered could not be accounted for on a mass balance basis using VPA metabolites (Granneman et al., 1984).

Products of ω-oxidation (figure 3) 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).

Dehydrogenation is thought to be responsible for the occurrence of several unsaturated metabolites such as 3-ene VPA, 4-ene VPA, (E)- and (Z)-2,4-diene VPA, and (E,E)- and (E,Z)-2,3'-diene VPA (Abbott et al., 1986; Kassahun et al., 1990). The hepatic distribution of 3-ene VPA and 4-ene VPA was similar to that of 2-ene VPA, 3-OH VPA, and 3-keto VPA and hence, it was assumed that these unsaturates were not microsomal but rather products of mitochondrial or peroxisomal metabolism (Granneman et al., 1984). However, Rettie et al. (1987) have shown that 4-ene VPA is a product of microsomal VPA metabolism and that the production of 4-ene VPA is susceptible to cytochrome P-450 enzyme induction or inhibition. The remaining unsaturated metabolites are considered to be secondary metabolites of VPA. The origin of these secondary metabolites is unclear, primarily as a result of multiple transformations and merging metabolic pathways.

The reduction of several unsaturated metabolites of VPA has been found to occur, albeit to a minor extent. In rats, following 2-ene VPA or 3-ene VPA administration, 2% of the dose was recovered as VPA (Granneman et al., 1984). Similar results were seen in rats following (E,E)-2,3'-diene VPA administration in which 3-ene VPA, (E)-2-ene VPA, and VPA were noted in the plasma (Abbott et al., 1987).

Two other minor routes of VPA metabolism are the epoxidation of the unsaturated metabolites to yield hydroxylactones, and glycine conjugation of which each accounts for <0.1% of the administered dose (Granneman et al., 1984; Rettenmeier et al., 1985; 1986).
1.1.4 Mechanism of Action

The mechanism of action of VPA is not well understood. Currently there are three proposed mechanisms of action: 1) VPA increases brain GABA levels (Godin et al., 1969); 2) VPA potentiates the postsynaptic response to GABA (MacDonald and Bergey, 1979); and 3) VPA exerts a direct membrane effect (Slater and Johnson, 1978). Although there is considerable evidence to support each hypothesis the mechanism still remains unclear. Each proposed mechanism of action of VPA will be briefly discussed below.

1.1.4.1 The GABA System

γ-Aminobutyric acid (GABA) functions as a neurotransmitter that inhibits the firing of an assortment of neurons by binding to the GABA\(_A\) receptor, thereby increasing the permeability of postsynaptic membranes to chloride ions. This causes the membrane to become hyperpolarized, increasing the threshold for the initiation of an action potential (Stryer, 1988). The GABA\(_A\) receptor is located predominantly in postsynaptic membranes and is associated with other binding sites that include the benzodiazepine, barbiturate, and picrotoxin (Cotariu et al., 1990).

GABA is widely distributed throughout the brain. On a cellular level, GABA uptake is greatest in the nerve terminals but there is some uptake by the neuronal cell bodies and glial cells. The substantia nigra contains the highest amount of GABA of all brain nuclei and 80% of this GABA is located in the nerve terminals (Iadarola and Gale, 1982).

GABA is a simple amino acid synthesized from glutamate via the enzyme glutamic acid decarboxylase (GAD). Catabolism of GABA by the
enzyme GABA-α-oxoglutarate aminotransferase (GABA-T) yields succinic semialdehyde (SSA) which is further oxidized to succinate and then enters the Krebs cycle. The pathway for producing and eliminating GABA is known as the GABA shunt. Modulation of the enzymes within the GABA shunt can ultimately vary the amount of GABA and hence, influence neuronal activity. For example, an inhibitor of GABA-T, γ-vinyl GABA, has been shown to increase brain GABA levels up to five fold (Schecter et al., 1977). Thus an increase or potentiation of GABA activity in the brain by anticonvulsant agents seems to be a plausible mechanism of action.

1.1.4.2 Increase of Brain GABA

VPA has been shown to elevate GABA levels in the whole brain of rodents within 15 - 60 minutes of administration (Schechter et al., 1978; Perry and Hansen, 1978) and these remain elevated for 3 - 8 hours (Schechter et al., 1978; Nau and Loscher, 1982). The increase in GABA occurs primarily in the substantia nigra, an area of high GABA content, although the majority of this increase is seen in the nerve terminals (Iadarola and Gale, 1981). It should be noted that an increase in GABA levels is only suggestive of anticonvulsant action as researchers have yet to show that VPA directly results in an increased release of GABA.

The increase in brain GABA levels by VPA was thought to occur via the inhibition of GABA-T. GABA-T activity has been shown to decrease following VPA administration (Horton et al., 1977). However, inhibitors of GABA-T, i.e. γ-vinyl GABA and ethanolamine-O-sulfate, were not as potent as VPA in inhibiting MES or PTZ induced seizures in mice (Loscher, 1981) or rats (Iadarola and Gale, 1981). It has recently been
suggested that an increase in GABA is also associated with the effects of VPA on succinic semialdehyde dehydrogenase (SSADH), the enzyme that converts succinic semialdehyde (SSA) to succinate (Vayer et al., 1988). It was proposed that VPA inhibits SSADH thereby resulting in a pool of SSA that can either inhibit GABA-T, or upon transamination, be converted to GABA. Either route ultimately results in a net increase in GABA.

Conversely, elevation of GAD activity, the GABA synthesizing enzyme, has been observed in mice following continuous administration with VPA (Loscher and Nau, 1982; Nau and Loscher, 1982 and 1984). The increase in GAD activity was related to an increase in brain GABA level which corresponded to an elevation in the electroconvulsive threshold.

While these results suggest that the anticonvulsant activity of VPA is due to elevations of brain GABA, there are reports that have suggested that elevation of brain GABA by VPA may not be the mechanism of action. For example, correlation between brain GABA levels and the anticonvulsant activity of VPA using electroshock testing has been poor (Kerwin et al., 1980). At the lower doses of VPA used to block audiogenic or electrically induced seizures in mice, no changes in total GABA levels in the brain were observed (Kupferberg, 1980). Thus, other mechanisms of action for VPA should be considered until these findings can be rationalized.

1.1.4.3 Potentiation of Postsynaptic GABA

Based on studies employing isolated spinal cord neurons, VPA has been shown to potentiate GABA-mediated postsynaptic inhibition when applied microiontophoretically (MacDonald and Bergen, 1979). The potentiation of GABA by VPA was also observed in the rat cortical
neurons in the substantia nigra (Kerwin et al., 1980). Although VPA-mediated postsynaptic potentiation of GABA is a favored mechanistic explanation, it is not without shortcomings. 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 MacDonald and Bergen (1979) could not be repeated. Ticku and Davis (1981) had suggested that the interaction of VPA with the GABA receptor at the picrotoxin site might be the mechanism by which VPA interacts with GABA; however, upon further investigation using a tritiated analogue no evidence of binding to brain membranes was found (Morre et al., 1984).

1.1.4.4 Direct Membrane Effect

An increase in membrane conductance to potassium by VPA has been observed in the Aplysia neuron, an invertebrate model; however, the concentrations used were 15 - 50 times greater than the levels seen in patient serum (Slater and Johnston, 1978). Incubation of hippocampal slices in a low calcium and high magnesium bath blocked synaptic transmissions resulting in rhythmic discharges. These discharges can be reduced with VPA at concentrations similar to that seen in serum (Agopyan et al., 1986). Nevertheless, before this mechanism of VPA activity is to gain prominence, the involvement of potassium effluxes with epileptic activity must be determined.

1.1.5 Pharmacokinetics

After oral administration of VPA in man, the drug is rapidly and completely absorbed with peak plasma levels attained within 0.5 - 2
hours and the fraction of the dose absorbed close to 1 (Schobben et al., 1975; Loiseau et al., 1975; Meinardi et al., 1975; Perruca et al., 1978). Although the absorption of VPA can be delayed after a meal, the extent of absorption is not affected (Levy et al., 1980). The elimination half-life of VPA in plasma ranges from 8 - 16 hours in adult epileptics (Klotz and Antonin, 1977; Loscher, 1978; Bowdle et al., 1980; Gugler and von-Unruh, 1980) and 3 - 12 hours in children (Cloyd et al., 1983). However, patients on multiple antiepileptic drug therapy have significantly shorter half-lives of VPA, a result of hepatic enzyme induction (Gugler and von Unruh, 1980; Cloyd et al., 1983).

The clearance of VPA is independent of liver blood flow but is dependent on the free fraction. Thus, VPA is a restrictively cleared drug (Gugler and von Unruh, 1980; Levy, 1984). The clearance of VPA can vary with age and polytherapy. Children <5, 5 - 10, and 10 - 15 years of age have reported mean VPA clearances of 48.3, 39.1, and 24.8 mL/kg/h, respectively (Dodson and Tasch, 1981) whereas adult clearances of VPA range from 6 - 10 mL/kg/h (Klotz and Antonin, 1977; Wulff et al., 1977). The clearance of VPA was found to increase (14.4 - 16.5 mL/kg/h) in adults on polytherapy, again a result of hepatic enzyme induction (Schappel et al., 1980; Hoffman et al., 1981).

The volume of distribution of VPA is relatively small in both children and adults. A value of 0.1 - 0.4 L/kg is an indication that the distribution of VPA is limited to the circulation and rapidly exchangeable extracellular waters (Klotz and Antonin, 1977; Gugler and von Unruh, 1980). However, the volume of distribution of the unbound drug in plasma is approximately 1 L/kg, a reflection of the distribution of free VPA into the intracellular structures (Gugler et al., 1977).
Therapeutic plasma concentrations of VPA vary widely, although the recommended value for clinical purposes is between 50 - 100 ug/mL (Chadwick, 1985). The correlation between plasma level and dose has been poor (Loiseau et al., 1975; Levy, 1984). Graphically, the relationship between dose and steady-state total plasma concentration is curvilinear where an increase in the dose is followed by a disproportionate increase in drug plasma levels. The most likely explanation for this observation is an increase in free fraction in conjunction with the higher doses. At normal therapeutic levels VPA is 90 - 95% bound to plasma proteins (Bowdle et al., 1980; Gugler and von-Unruh, 1980). Plasma VPA concentrations of >100 ug/mL can result in a 50% increase in free drug in plasma as a result of the saturation of VPA binding to plasma proteins (Gugler and von-Unruh, 1980). The increase in free fraction can cause either an increase in drug clearance or an increase in the distribution of the drug into the intracellular compartments (May and Rambeck, 1985). The ultimate result is a net decrease of total VPA in plasma. The use of free plasma VPA concentrations rather than total concentrations to assess the appropriateness of dose appears to be a rational decision. However, a poor correlation was also found between free plasma VPA concentrations and the dose required to achieve seizure control (Farrell et al., 1986). Therefore, the ability to predict appropriate therapeutic levels of VPA based on dose could be difficult (Armijo et al., 1986).

1.1.6 Pharmacodynamics

The relationship between plasma VPA concentration and pharmacological effect has also been investigated. In a study of 61
children on VPA anticonvulsant mono- or polytherapy, 21 of the 24 seizure free-children had serum VPA concentrations between 20 - 60 ug/mL (Farrell et al., 1986). Attempts at correlating total and free plasma VPA concentrations (Gram et al., 1977; Schulz et al., 1979; Bowdle et al., 1980; Cramer et al., 1986) or brain (Loscher et al., 1988) and CSF (Loscher et al., 1988) levels with anticonvulsant activity in both man and animals have not been successful. This discrepancy between effect and concentration exists in-part because the antiepileptic effect of VPA develops independent of serum concentrations (Chadwick, 1985). Upon initial administration of VPA, a delay was noted in the onset of maximal anticonvulsant activity (Jeavons and Clark, 1974; Rowan et al., 1979), together with an extended duration of activity even after the disappearance of the parent compound from the circulation (Lockard and Levy, 1976; Harding et al., 1978). The duration of this post-drug effect has been reported to last for weeks in both patients and animals (Lockard and Levy, 1976; Harding et al., 1978). Since VPA is extensively metabolized by the liver, perhaps one or more metabolites may possess significant anticonvulsant activity and hence, serum concentrations of these metabolites may be a better indicator of activity.

1.1.7 Anticonvulsant Activity of VPA Metabolites

The slow onset of maximal anticonvulsant activity of VPA could in fact be due to the gradual accumulation of active metabolites, whereas the carry-over effect may be attributed to the metabolite(s) possessing a longer half-life. Several VPA metabolites have been evaluated for their potential to suppress either an electrically or chemically induced seizure in mice (Loscher, 1981; Loscher and Nau, 1985; Abbott and
Acheampong, 1988). Of the metabolites tested (E)-2-ene VPA, was shown to be 50–100% as potent as VPA. Furthermore, upon chronic administration of VPA to mice (Nau and Loscher, 1981; Loscher and Nau, 1982) and rats (Loscher and Nau, 1983), (E)-2-ene VPA was the only metabolite found in the brain. In addition, (E)-2-ene VPA remained in the brain and paralleled the prolonged anticonvulsant activity of VPA even after the disappearance of the parent drug from the plasma. In another study, the continuous i.v. infusion of VPA in the dog resulted in increasing levels of (E)-2-ene VPA in plasma and CSF over 7 hours (Loscher and Nau, 1983). In other words, the metabolite (E)-2-ene VPA appeared to be accumulating.

Upon the oral administration of VPA to healthy volunteers, at steady-state the unsaturated metabolites appeared to have longer plasma half-lives when compared to the parent drug (Pollock et al., 1986). These results were in keeping with the idea that a slowly accumulating metabolite with a longer half-life may be responsible for the unusual pharmacodynamic behavior of VPA.

On the other hand, the penetration of (E)-2-ene VPA into the CSF of paediatric patients on VPA therapy was found to be less than 1% of that in plasma (Loscher et al., 1988). Although actual brain levels of (E)-2-ene VPA were not determined, it could be concluded from the low levels of (E)-2-ene VPA found in the CSF that this metabolite does not contribute significantly to the anticonvulsant actions of VPA.

A diunsaturated metabolite, 2,3'-diene VPA, was shown to possess anticonvulsant activity towards PTZ-induced seizures in mice and was found to be equipotent to (E)-2-ene VPA (Abbott and Acheampong, 1988). Although there are four possible geometric isomers of this diene, only
two isomers have been identified in man, namely the (E,Z)- and (E,E)-isomers (Lee et al., 1989). The (E,E)-2,3'-diene VPA is considered to be a major metabolite of VPA in man with serum levels between 6.4 - 7.1% of that of VPA (Abbott et al., 1986; Kassahun et al., 1990).

1.2 ANTICONVULSANT EVALUATION

The current method of evaluating a new compound for its antiepileptic potential is by the use of animal models. An ideal model should possess all of the pathophysiological components of epilepsy and yet be able to assess a large number of compounds. Furthermore, the method should be able to evaluate the anticonvulsant activity for all types of epilepsies and must be cost and labour effective because of the large number of compounds to be screened. As expected, there is currently no such a test that fits all of the aforementioned criteria. 

In-vivo studies using mice and rats have become an attractive alternative for assessing anticonvulsant activity (Krall et al., 1978). Because there is not a single animal model to describe all the epilepsies seen in humans, a number of tests are performed for a complete assessment of the anticonvulsant activity of a new compound.

1.2.1 Electrically Induced Seizures

Electroshock induced seizures can be categorized into two types: threshold and maximal electroshock seizures. A threshold induced seizure elicits an electrical discharge in the brain that occurs regionally with the spread to adjacent areas kept to a minimum. Seizures of this type
are analogous to human absence epilepsy. Seizures elicited via maximal electroshock also stimulate neurons on a regional basis but unlike threshold induced seizures, the electrical activity spreads throughout the entire brain. A seizure produced by maximal electroshock is similar to a grand mal seizure. Therefore, the appropriate test should be applied when evaluating a compound that is targeted for a specific form of epilepsy.

Threshold seizures can be induced in mice by low frequency electroshock stimulation (LES) at 6 Hz for 3 sec via corneal electrodes (Swinyard, 1972). The application of a 23 V stimulus will stun the mouse whereas a 136 V stimulus will cause a seizure analogous to human absence epilepsy. A stimulation intensity >150 V will result in hindleg tonic-extension akin to generalized tonic-clonic seizures. Maximum electroshock seizures (MES) are elicited with voltages greater than that required for LES (Toman et al., 1946). Corneal application of a 60 Hz stimulus for 0.2 sec at 250 V will elicit tonic hindlimb extension (endpoint) in mice (Swinyard, 1972; Swinyard and Woodhead, 1982).

1.2.2 Chemically Induced Convulsions

A number of chemical convulsants are available for testing. These include picrotoxin, bicucullin, strychnine, and pentylenetetrazole (PTZ). Of these, PTZ is the most commonly used chemical convulsant.

Pentylenetetrazole can induce two types of seizures depending on the dose. Although the mechanism of action is not fully understood, PTZ is thought to be a synaptically acting convulsant which may directly activate excitatory synapses (Purpura and Gonzalez-Monteagudo, 1960).
Pentylenetetrazole apparently acts allosterically on the GABA-benzodiazepine receptor (Ramajaneyulu and Ticku, 1984).

Subcutaneous administration of 85 mg/kg of PTZ in mice or 75 mg/kg in rats produces clonic seizures which are considered to be a model for human absence seizures (Mares and Schickerova, 1980; Swinyard and Woodhead, 1982). This type of seizure is also termed a threshold convulsion. The clonic spasms are characterized initially by isolated myoclonic jerks within the first 5 minutes followed by clonus of the head and forelimb areas (Velisek et al., 1989; Yonekawa et al., 1980). A Straub tail phenomenon and loss of righting may also occur followed finally by a stupor phase or unusual posturing. Doses of >100 mg/kg s.c. in mice will elicit a generalized tonic-clonic seizure, similar to that induced by MES, and is usually fatal (Velisek et al., 1989).
1.3 OBJECTIVES

The pharmacokinetic and metabolic profiles of VPA have been extensively studied. However, similar work on the active metabolites of VPA is incomplete. The contribution of (E)-2-ene VPA and (E,E)-2,3'-diene VPA towards VPA anticonvulsant activity have so far been speculative. Detailed kinetic and distributive studies are lacking and need to be completed before commenting on the degree of involvement of these metabolites with VPA anticonvulsant activity.

The aim of this study was to provide the pharmacokinetic data and evaluate the anticonvulsant activity of VPA, (E)-2-ene VPA, and (E,E)-2,3'-diene VPA to adequately assess the role of these metabolites towards the anticonvulsant activity of VPA. The metabolism of VPA, (E)-2-ene VPA, and (E,E)-2,3'-diene VPA was also examined in order to determine if these unsaturates could possibly replace VPA as an anticonvulsant agent. The absence of toxic by-products from (E)-2-ene VPA or (E,E)-2,3'-diene VPA metabolism would be a major advantage over VPA.

1.3.1 Specific Aims

i. To stereoselectively synthesize sufficient amounts of both (E)-2-ene VPA and (E,E)-2,3'-diene VPA for pharmacokinetic and pharmacological evaluation.

ii. To develop a method for the analysis of VPA and its metabolites in rat tissue extracts by negative ion chemical ionization GC-MS.
iii. To determine and compare the tissue distribution of VPA, (E)-2-ene VPA, and (E,E)-2,3'-diene VPA in the rat after a single dose.

iv. To determine and compare the elimination kinetics of VPA, (E)-2-ene VPA, and (E,E)-2,3'-diene VPA in the rat.

v. To quantitate the metabolites formed in the various tissue fractions after the administration of VPA, (E)-2-ene VPA, and (E,E)-2,3'-diene VPA.

vi. To compare and quantitate the anticonvulsant activity of VPA, (E)-2-ene VPA, and (E,E)-2,3'-diene VPA in rats using the PTZ-induced seizure test.
2. EXPERIMENTAL

2.1 SUPPLIES

2.1.1 Chemicals and Reagents

Aldrich Chemical Co. (Milwaukee, WI)
\[ \alpha\text{-bromo-2,3,4,5,6-pentafluorotoulene, t-butyl-dimethylsilyl chloride, butyllithium (1.6 M in hexane), deuterochloroform, 1,8-diazabicyclo[5.4.0] undec-7-ene (DBU), Diazald}^R, \]
\[ \text{dichlorodimethylsilane, diisopropylamine, diisopropylethylamine, hexamethylphosphoramide, methanesulfonyl chloride, 2-methylglutaric acid, 1,5-pentamethylenetetrazole (pentylene-tetrazole, PTZ), (E)-2-pentenoic acid, triethylamine} \]

BDH Chemicals (Toronto, Ont.)
\[ \text{diethyl ether (anhydrous), hydrochloric acid, quinol, sodium chloride, sodium hydroxide, sodium sulfate, sulfuric acid, tetrahydrofuran (THF), toluene} \]

Caledon Laboratories Ltd. (Georgetown, Ont.)
\[ \text{dichloromethane, ethyl acetate} \]

Eastman Kodak Co. (Rochester, NY)
\[ \text{propionaldehyde} \]
ICN Pharmaceuticals Inc. (Plainview, NY)

di-n-propylacetic acid (valproic acid)

Manostat (New York, NY)

Chromerge®

Matheson Coleman and Bell Co. (Norwood, OH)

2-(2-ethoxyethoxy)ethanol

Pierce (Rockford, IL)

N-methyl-N-trimethylfluoroacetamide

2.1.2 VPA Metabolites and Internal Standards

The syntheses for the following VPA metabolites, used as standards for quantitation, has been described elsewhere (Acheampong et al., 1983): 3-ene VPA (stereochemistry not determined), 4-ene VPA, 3-OH VPA, 4-OH VPA, 5-OH VPA, 4-keto VPA, and 2-PGA. In addition, (E)-2,4-diene VPA was synthesized according to the procedure of Lee et al. (1989) while 3-keto VPA was synthesized by alkylation of ethyl valerate with propionyl chloride following the general procedure of Cregge et al. (1973).

The internal standards used for the assay of VPA and VPA metabolites were \([^2\text{H}]_6\text{VPA}\), synthesized previously by Acheampong et al. (1984); \([^2\text{H}]_3\text{ene VPA}\), synthesized by Abbott et al. (1986); \([^2\text{H}]_3\text{keto VPA}\) was a kind gift from Dr. T.A. Baillie (University of Washington, School of Pharmacy, Seattle, WA); and 2-methylglutaric acid (2-MGA).
2.1.3 Materials

Alltech Associates Inc. (Deerfield, IL)

1 mL amber crimp-top vials, 100 μL limited volume inserts

Amicon Corp. (Danvers, MA)

micropartition system MPS-1, ultrafiltration membrane type YMT

Becton Dickinson (Rutherford, NJ)

Yale hypodermic needle 23G 1, 1mL syringe (tuberculin)

J & W Scientific (Folsom, CA)

DB-1 capillary column (30m, 0.32mm i.d., 0.25 μm film thickness)

Restek Corp. (Bellefonte, PA)

phenyl-methyl deactivated guard column (5m, 0.32 mm i.d.)

Supelco Inc. (Bellefonte, PA)

3% Dexsil 300 on 100-120 mesh Supelcoport, GlasSealR connector

2.1.4 Animals

Male Sprague-Dawley rats weighing between 200 - 350 g were obtained from the Animal Care Facility at the University of British Columbia. The animals were housed in plastic cages with wood shavings used as bedding. The animals were allowed access to food (Purina Rat ChowR) and water ad libitum. Light was provided between the hours of
6:00 am to 9:00 pm daily while room temperature was kept at a constant 22°C.

2.2 INSTRUMENTATION

2.2.1 Nuclear Magnetic Resonance Spectrometry

High field proton NMR spectra were obtained on a Bruker WH-400 and a Varian XL-300 spectrometer in the Department of Chemistry, University of British Columbia NMR facility. Spectra were acquired in deuterated chloroform with tetramethylsilane used as an internal standard.

2.2.2 Packed Column Gas Chromatography - Mass Spectrometry

The identification of synthesized compounds was performed on a Hewlett Packard 5700A gas chromatograph interfaced to a Varian MAT-111 mass spectrometer equipped with a variable slit separator. Data was acquired and processed on a microcomputer. Data were recorded in scan mode with a mass range of 15 - 750 mass units collected every 5 seconds. The instrument was operated in electron impact mode with an emission current of 300 uA, ionization energy of 70 eV, and source pressure of 5 x 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 used 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.
2.2.3 Capillary Column Gas Chromatography - Mass Spectrometry

The quantitative analysis of biological samples for VPA and metabolites was performed on a Hewlett Packard 5987A GCMS with an open-split interface. Data recording and processing were managed with a HP-1000 on-line computer. Negative ion chemical ionization (NICI) was the method of ionization with ultra-high purity methane used as the reagent gas. The source pressure was 0.8 - 1.2 Torr, ionization energy 200 eV, and the emission current 250 μA. The instrument was programmed for selected-ion-monitoring (SIM) to enhance sensitivity. VPA and metabolites assayed and the ions monitored are listed in table 3.

Separation was achieved on a DB-1 fused-silica column connected to a phenyl-methyl deactivated 5 m guard column by way of a GlasSeal connector. The carrier gas used was helium with a head pressure of 10 psi and a resulting flow rate of 1 mL/min.

The oven temperature program was 110 - 140°C at 30°C/min, then 140 - 260°C at 8°C/min, and finally held at 280°C for 6 min for a total run time of 25 min.

A 1 μL aliquot of sample was injected using a Hewlett Packard 7673A automatic sampler.

2.2.4 Centrifuges

A Beckman centrifuge, model J2-21, equipped with a JA-20 45° rotor was used to separate the unbound drug from the plasma by ultrafiltration. A Damon/IEC centrifuge, model HN-SII fitted with a 45° rotor, was used to separate the organic and aqueous layers after
Table 3: Mass-to-charge ratios (m/z) for the internal standards (*), VPA, and VPA metabolites.

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[^2\text{H}_3]$2-ene VPA*</td>
<td>144</td>
</tr>
<tr>
<td>$[^2\text{H}_6]$VPA*</td>
<td>149</td>
</tr>
<tr>
<td>$[^2\text{H}_3]$3-keto VPA*</td>
<td>232</td>
</tr>
<tr>
<td>2-MGA*</td>
<td>325</td>
</tr>
<tr>
<td>(E,E)-2,3'-diene VPA</td>
<td>139</td>
</tr>
<tr>
<td>(E,Z)-2,3'-diene VPA</td>
<td>139</td>
</tr>
<tr>
<td>(E)-2,4-diene VPA</td>
<td>139</td>
</tr>
<tr>
<td>(E)-2-ene VPA</td>
<td>141</td>
</tr>
<tr>
<td>(Z)-2-ene VPA</td>
<td>141</td>
</tr>
<tr>
<td>3-ene VPA</td>
<td>141</td>
</tr>
<tr>
<td>4-ene VPA</td>
<td>141</td>
</tr>
<tr>
<td>VPA</td>
<td>143</td>
</tr>
<tr>
<td>p4-keto VPA</td>
<td>157</td>
</tr>
<tr>
<td>3-keto VPA</td>
<td>229</td>
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<tr>
<td>3-OH VPA</td>
<td>231</td>
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<tr>
<td>4-OH VPA</td>
<td>231</td>
</tr>
<tr>
<td>5-OH VPA</td>
<td>231</td>
</tr>
<tr>
<td>2-PGA</td>
<td>353</td>
</tr>
</tbody>
</table>
2.3 CHEMICAL SYNTHESSES

2.3.1 Synthesis of 2-n-propyl-(E)-2-pentenoic acid ((E)-2-ene VPA)

2.3.1.1 Synthesis of ethyl 2-n-propyl-3-hydroxypentanoate

In a 500 mL flask equipped with a dropping funnel, reflux condenser, and mechanical stirrer, n-butyllithium in hexane (0.025 mol, 156 mL of 1.6M in hexane) was added to a cooled solution of diisopropylamine (0.25 mol, 25.3g) in 200 mL of THF over ice. The mixture was stirred for 30 minutes and cooled to -78°C with a dry ice/acetone bath. Ethyl valerate (0.25 mol, 32.5g) in 10 mL THF was added dropwise over 10 minutes to the mixture and stirred a further 60 minutes. Upon quenching with 15% HCl to a pH of 1, the aqueous layer was extracted with 3 x 100 mL of ether and the ethereal portion washed consecutively with saturated NaHCO₃ and water. The ethereal solution was dried over anhydrous Na₂SO₄ and the ether removed by flash evaporation. Purification of the residue by fractional distillation afforded 22.8 g (48% yield) of ethyl 2-n-propyl-3-hydroxypentanoate, bp 94 - 96°C/2.5mm (lit. 70-72°C/0.2mm (Acheampong, 1982)).

Mass spectrum (MW=188) m/z(%): 101(100), 73(67), 55(50), 113(50), 130(27), 84(20), 159(20), 143(15).
2.3.1.2 Synthesis of 2-n-propyl-(E)-2-pentenoic acid

In a 250 mL flask equipped with a dropping funnel and mechanical stirrer, ethyl 2-n-propyl-3-hydroxypentanoate (48 mmol, 9g), triethylamine (72 mmol, 10 mL), and 150 mL of dichloromethane were cooled to 0°C with ice. Methanesulfonyl chloride (50 mmol, 4 mL) in 10 mL of dichloromethane was cooled to 0°C and added dropwise to the stirred mixture at room temperature. After 60 min the mixture was filtered by suction and the solvent removed by flash evaporation. The residue was reconstituted in 150 mL of dry THF, a solution of 1,8-diazabicyclo[5.4.0.]undec-7-ene (DBU, 48 mmol, 7 mL) in 10 mL of THF was added, and the contents gently refluxed for 2 h over an oil bath. The reaction was cooled to room temperature and quenched with 100 mL of distilled water. The aqueous fraction was extracted with 3 x 150 mL of ether and the combined organic portions washed consecutively with 100 mL of 1M HCl and 1M NaOH. The ethereal fraction was dried over anhydrous Na₂SO₄ and the solvent removed by flash evaporation.

To the residue, 15 mL of 3N NaOH and 30 mL of distilled water were added and stirred for 4 days at 60°C. The mixture was cooled and extracted with 50 mL of ether (discard). The aqueous layer was adjusted to a pH of 1 - 2 with 3N HCl and extracted with 3 x 100 mL of ether. The combined ethereal layers were dried over anhydrous Na₂SO₄, the ether was removed by flash evaporation, and the residue distilled. The clear distillate, bp 120°C/3mm (lit. 96-99°C/1.3mm (Acheampong et al., 1983)) yielded 2.45 g (36% yield) of 2-n-propyl-2-pentenoic acid. Upon methylation with diazomethane (Levitt, 1973), two isomers (95% E and 5% Z) were detected by GCMS and confirmed by NMR.
Mass spectrum of the (E)-isomer (as the methyl ester after derivatizing with diazomethane) m/z(%): 55(100), 95(83), 127(70), 156(M^+,42), 67(37), 113(10), 141(3). Mass spectrum of the (Z)-isomer (methyl ester, m/z(%)): 55(100), 95(100), 127(60), 67(45), 156(M^+,25), 113(8), 141(2).

400 MHz proton NMR (CDCl₃): 0.85(t,3H,CH₃-CH₂-CH₂), 1.0(t,3H,CH₃-CH₂-CH), 1.30-1.44(m,2H,CH₃-CH₂-CH₂), 2.1-2.25(m,2H,CH₃-CH₂-CH₂), 2.1-2.25(m,2H,CH₃-CH₂-CH, E), 2.4-2.49(m,2H,CH₃-CH₂-CH,Z), 5.95(t,1H,CH=,Z), 6.85(t,1H,CH=,E)(Comparable to lit. values of Acheampong et al., 1983).

2.3.2 Synthesis of 2-((Z)-1'-propenyl)-(E)-2-pentenoic acid ((E,Z)-2,3'-diene VPA)

2.3.2.1 Synthesis of ethyl 2-(1'-hydroxypropyl)-(Z)-3-pentenoate

In a 1 L flask equipped with a Dean-Stark apparatus, (E)-2-pentenoic acid (1.25 mol, 125 g), ethanol (3.75 mol, 219 mL), and concentrated H₂SO₄ (1.25 mL) in 500 mL of benzene were refluxed for 48 h. The mixture was cooled to room temperature, washed with a 200 mL solution of saturated NaHCO₃, and the organic portion was dried over MgSO₄. Fractionation afforded 100g (63% yield) of ethyl (E)-2-pentenoate, bp 159°C/760mm.

Mass spectrum m/z(%): 55(100), 29(70), 128(M^+,10), 83(10), 69(6), 100(5).

300 MHz proton NMR (CDCl₃): 1.09(t,3H,CH₃-CH₂-CH=), 1.3(t,3H,OCH₂-CH₃), 2.18-2.29(m,2H,CH₃-CH₂-CH=), 4.18(q,2H,OCH₂), 5.82(d,1H,CH=CH, J=14Hz), 7.02(m,2H,CH₃-CH₂-CH=).

In a 500 mL flask equipped with a dropping funnel, condenser, and mechanical stirrer, n-butyllithium (0.10 mol, 123 mL of 1.6M in hexane)
was slowly added to a cooled solution of diisopropylamine (0.19 mol, 26.6 mL) in 150 mL of THF over ice. The mixture was allowed to stir for 15 min and then cooled to -78°C with a dry-ice/acetone bath. A solution of hexamethylphosphoramide (0.19 mol, 33.1 mL) in 30 mL of THF was slowly introduced and after 15 min a solution of ethyl (E)-2-pentenoate (0.173 mol, 22.1 g) in 30 mL of THF was added dropwise to the reaction and stirred a further 30 min. Propionaldehyde (0.173 mol, 12.5 mL) in 25 mL of THF was added dropwise to the stirred mixture and allowed to react for 60 min. The mixture was quenched with 15% HCl to a pH of 1 and the aqueous layer extracted with 3 x 150 mL of ether. The combined organic fractions were consecutively washed with a saturated NaHCO₃ solution and water and dried over anhydrous Na₂SO₄. The solvent was removed by flash evaporation and the residue fractionated to afford 25.83 g (80% yield) of ethyl 2-(1'-hydroxylpropyl)-(Z)-3-pentenoate, bp 68 - 70°C/0.1 mm (lit. 85-90°C/0.25mm (Acheampong and Abbott, 1985)).

Mass spectrum (MW=186) m/z(%): 29(100), 55(87), 100(68), 128(70), 82(53), 113(4), 141(2), 157(2), 169(2).

400 MHz proton NMR (CDCl₃): 0.9(t,3H,CH₃-CH₂-CHOH), 1.25(t,3H,OCH₂-CH₃), 1.4-1.51(m,2H,CH₂-CHOH), 1.7(d,3H,CH₃-CH), 2.8 (broad s,1H,OH), 3.42(m,1H,CH-C=O), 3.8 (m,1H,CH-O), 4.15(q,2H,OCH₂CH₃), 5.3-5.61(m,1H,CH=CH, J=9Hz), 5.62-5.85(m,1H,CH=CH)(lit. Acheampong and Abbott, 1985).

2.3.2.2 Synthesis of 2-((Z)-1'-propenyl)-(E)-2-pentenoic acid

In a 500 mL flask equipped with a dropping funnel and stirrer ethyl 2-(1'-hydroxypropyl)-(Z)-3-pentenoate (118 mmol, 22g) and triethylamine (177 mmol, 26 mL) in 200 mL of dichloromethane were cooled
over ice. A cooled solution of methanesulfonyl chloride (124 mmol, 10 mL) in 10 mL of dichloromethane was then slowly added to the mixture. The ice was removed and the reaction was allowed to proceed at room temperature for 60 min. The mixture was filtered by suction, the solvent removed by flash evaporation and the residue reconstituted in 150 mL of THF. A solution of 1,8-diazabicyclo[5.4.0]undec-7-ene (118 mmol, 18 mL) in 20 mL of THF was added and the reaction was gently refluxed for 2 h. After cooling, the mixture was quenched with 100 mL of distilled water and the aqueous layer extracted with 3 x 100 mL of ether. The combined organic fractions were washed consecutively with 100 mL of 1M HCl and 1M NaOH and dried over anhydrous Na₂SO₄. The solvent was removed by flash evaporation, 40 mL of 3N NaOH and 20 mL of distilled water were added to the residue and the mixture was stirred for 48 h at 60°C. Upon cooling the aqueous mixture was extracted with 100 mL of ether (discard), the pH adjusted to 1 - 2 with 3N HCl and then extracted with 3 x 100 mL of ether. The combined ethereal layer was dried over anhydrous Na₂SO₄ and the solvent was removed by flash evaporation. The residue was distilled to afford 9.6 g (85% yield, bp 80°C/0.1 mm) of 2-((Z)-1′-propenyl)-(E)-2-pentenoic acid and 2-((E)-1′-propenyl)-(E)-2-pentenoic acid with an isomeric ratio of 20:1 as determined by GC-MS and NMR.

Mass spectrum (as the methyl ester after derivatizing with diazomethane), m/z(%): 95(100), 154(M⁺, 87), 79(82), 59(58), 122(58), 67(48), 107(20), 139(20).

400 MHz proton NMR (CDCl₃): 1.08(t,3H,CH₃-CH₂), 1.59(d,3H,CH₃-CH), 2.15(dq,2H,CH₂), 5.75-5.83(m,1H,CH=CH), 5.98(d,1H,CH=CH,J=11Hz), 6.95 (t,1H,CH=C=O,J=8Hz).
2.3.3 Synthesis of 2-((E)-1'-propenyl)-(E)-2-pentenoic acid ((E,E)-2,3'-diene VPA)

Details of the synthesis of 2-((E)-1'-propenyl)-(E)-2-pentenoic acid are recorded here and were recently published (Lee et al., 1989).

2.3.3.1 Synthesis of ethyl (Z)-2-pentenoic acid

(Z)-2-Pentenoic acid was synthesized by the Favorsky rearrangement of 1,3-dibromo-2-pentenone described briefly by Rappe and Adestrom (1965) and in detail by Rappe (1979). The ethyl (Z)-2-pentenoate was obtained by refluxing (Z)-2-pentenoic acid (23.3 g, 0.23 mol) with ethyl iodide (71.76 g, 0.46 mol), potassium carbonate (23.84 g, 0.17 mol), and 18-crown-6 (3 g, 0.05 M) in 230 mL of dry THF for 6 h. The mixture was filtered by suction and fractional distillation afforded 17.1 g of ethyl (Z)-2-pentenoate (yield 58%, bp 43 - 45°C/10 mm).

Mass spectrum m/z(%): 100(100), 83(95), 55(77), 29(65), 128(M⁺,30).

80 MHz proton NMR (CDCl₃): 1.1(t,3H,CH₃-), 1.3(t,3H,-CH₃), 2.65(q,2H,CH₂), 4.2(q,2H,OCH₂), 5.7(d, 1H,J=10Hz,CH=CH), 6.2-6.5(dt,1H,J_Cis=10Hz,J_gem=6Hz,HC=CH).

2.3.3.2 Synthesis of ethyl 2-(1'-hydroxypropyl)-(E)-3-pentenoate

In a 250 mL flask equipped with a dropping funnel, condenser, and mechanical stirrer, diisopropylamine (11.13 g, 0.11 mol) in anhydrous THF (90 mL) were cooled to 0°C over ice. n-Butyllithium (69 mL of 1.6 M in hexane, 0.11 mol) was then added dropwise over 15 min and allowed to react for 20 min. Upon cooling to -78°C, hexamethylphosphoramide (19.57 g, 0.11 mol) was added dropwise and the mixture was stirred for 15 min.
An aliquot of ethyl (Z)-2-pentenoate (12.8 g, 0.1 mol) in 10 mL of THF was added dropwise. After 30 min, propionaldehyde (5.8 g, 0.1 mol) in 10 mL of THF was slowly introduced and the mixture was stirred for 30 min. The reaction was quenched with 15% HCl until a pH of 1 was attained. The aqueous portion was extracted three times with ether and the combined organic fractions washed consecutively with saturated NaHCO₃ and water, then dried over anhydrous Na₂SO₄. Removal of the solvent by flash evaporation and fractional distillation of the residue afforded 11.2 g (yield 60%) of ethyl 2-(1'-hydroxypropyl)-(E)-3-pentenoate (bp 78°C/0.22 mm) (Lit. 95-100°C/1mm (Acheampong and Abbott (1985)).

Mass spectrum (MW=186) m/z(%): 29(100), 100(50), 55(49), 82(44), 128(40), 113(5), 141(2), 157(2).

300 MHz proton NMR (CDCl₃): 0.95(t,3H,CH₃-CH₂), 1.3(t,3H,CH₂-CH₃), 1.45(m,2H,CH₂), 1.7(d,3H,CH₃-CH=), 2.65(broad s,1H,OH), 3.0(m,1H,CH-C=O), 3.65-3.8(m,1H,CH-0), 4.15(q,2H,CH₂-CH₃), 5.47(m,1H,CH=CH, J=15Hz), 5.63 (m,1H,=CH-CH).

2.3.3.3 Synthesis of ethyl (E)-2-(1'-propenyl)-(E)-2-pentenoate

In a 150 mL flask equipped with a dropping funnel and mechanical stirrer, ethyl 2-(1'-hydroxypropyl)-(E)-3-pentenoate (9.3 g, 0.05 mol) was added to a solution of triethylamine (8.1 g, 0.08 mol) in 40 mL of dry dichloromethane and the mixture cooled to 0°C. Methanesulfonyl chloride (6.87 g, 0.06 mol) at 0°C was slowly added and the mixture was stirred for 60 min. The mixture was filtered by suction, the solvent removed by flash evaporation, and the crude mesyl ester, ethyl 2-(1'-hydroxypropyl)-(E)-3-pentenoate, analyzed by GCMS. Two peaks corresponding to the diastereomers gave identical mass spectra. GCMS
EI): m/z(%) 95(100), 67(55), 55(45), 123(30), 111(15), 139(15), 168(10), 153(5). The crude mixture of ethyl 2-(1'-hydroxypropyl)-(E)-3-pentenoate was reconstituted in 150 mL of anhydrous THF, cooled to 0°C, and potassium hydride (4.01 g, 0.1 mol) cautiously added. The mixture was then brought to 25°C and allowed to react for 12 h. Excess potassium hydride was carefully decomposed at -78°C with water and the aqueous layer extracted three times with ether. The combined organic fractions were consecutively washed with saturated NaHCO₃ and water, then dried over anhydrous Na₂SO₄. The solvent was removed by flash evaporation and fractional distillation of the residue afforded 3.9 g (yield 46%, bp 70°C/0.1 mm)(Lit. 65-70°C/0.1 mm (Acheampong and Abbott, 1985)) of an isomeric mixture that by GCMS was estimated to contain 81% of ethyl 2-((E)-1'-propenyl)-(E)-2-pentenoate and 19% of ethyl 2-((Z)-1'-propenyl)-(E)-2-pentenoate.

Mass spectrum of ethyl 2-((E)-1'-propenyl)-(E)-2-pentenoate, m/z(%): 95(100), 168(M⁺,90), 79(64), 67(49), 123(46), 140(37), 111(30), 153(7).

400 MHz proton NMR (CDCl₃): 1.0-1.1(t,3H,CH₃-CH₂), 1.32(t,3H,OCH₂-CH₃), 1.84(d,J=7Hz,3H,CH₃-CH=), 2.3(m,2H,CH₂-CH=), 4.2(q, 2H,OCH₂-CH₃), 5.75(trace,m,CH=CH,(E,Z)), 6.07(m,1H,CH=CH,(E,E)), 6.13 (d,J=17Hz,1H, CH=CH,(E,E)), 6.55(t,J=7Hz,1H,CH₂-CH=,(E,E)), 6.8(trace,CH₂-CH=,(E,Z)) (Lit. Acheampong and Abbott, 1985).

2.3.3.4 Synthesis of 2-((E)-1'-propenyl)-(E)-2-pentenoic acid

The free acids were obtained by stirring the mixture, ethyl 2-((E)-1'-propenyl)-(E)-2-pentenoate and ethyl 2-((Z)-1'-propenyl)-(E)-2-pentenoate, (2.7 g, 0.016 mol) in 25 mL of 2.2 M NaOH at 60°C for 48 h.
The mixture was extracted with 25 mL of hexane (discard) and the aqueous fraction adjusted to a pH of 1 with 10% HCl. The acidic solution was extracted three times with ether and the combined ethereal portions dried over anhydrous Na₂SO₄. Removal of the ether by flash evaporation and fractional distillation afforded 1.2 g (yield 52.1%, bp 84°C/0.05 mm) of 2-((E)-1'-propenyl)-(E)-2-pentenoic acid and 2-((Z)-1'-propenyl)-(E)-2-pentenoic acid with the (E,E)-isomer constituting approximately 94% of the mixture.

Mass spectrum of the t-BDMS ester derivative of 2-((E)-1'-propenyl)-(E)-2-pentenoic acid, m/z(%): 197(100), 75(35), 95(20), 123(10), 254(2), 139(2), 153(2), 167(2) (Lit. Abbott et al., 1986). Mass spectrum of the t-BDMS ester derivative of 2-((Z)-1'-propenyl)-(E)-2-pentenoic acid, m/z(%): 197(100), 75(20), 95(15), 123(10), 254(5), 139(2), 153(2), 167(2).

400 MHz proton NMR (CDCl₃): 1.09(t,J=8Hz,3H,CH₃-CH₂), 1.57(trace,d,CH₃-CH=,(E,Z)), 1.85(d,J=7Hz,3H,CH₃-CH=,(E,E)), 2.11(trace, m,CH₂,(E,Z)), 2.34(m,2H,CH₂,(E,E)), 5.83(trace,m,CH=CH,(E,Z)), 5.99 (trace, d,J=11Hz,CH=CH,(E,Z)), 6.02-6.11(m,1H,CH=CH,(E,E)), 6.15(d, J=16Hz,1H,CH=CH,(E,E)), 6.8(t,J=8Hz,1H,CH₂-CH=,(E,E)) (Lit. Acheampong and Abbott, 1985).
2.4 ANIMAL EXPERIMENTS

2.4.1 Pharmacokinetics and Tissue Distribution of VPA, (E)-2-ene VPA, and (E,E)-2,3′-diene VPA

Aqueous solutions of VPA, (E)-2-ene VPA, and (E,E)-2,3′-diene VPA for i.p. injection were prepared as their sodium salts. The concentrations used for VPA, (E)-2-ene VPA (corrected for the 5% (Z)-isomer content), and (E,E)-2,3′-diene VPA (corrected for the 3-5% (E,Z)-isomer content) were 75, 76, and 78 mg/mL, respectively. Each solution was prepared by the addition of an equivalent amount of 3N NaOH to a specified amount of each compound in distilled water to yield the sodium salt. The pH was then adjusted to 7.4 with 3N HCl if necessary and the mixture was made to a volume of 50 mL with distilled water.

Male Sprague-Dawley rats weighing between 250 - 350g were administered 150 mg/kg i.p of VPA and serially sacrificed at -15, 15, 30, 45, 60, 120, 240, 360, and 600 min with each time point consisting of eight rats. Before sacrifice, each rat was rendered unconscious by placing the animal in a carbon dioxide chamber for 30 seconds. The animal was then decapitated with a Harvard small animal decapitator. Blood was immediately collected in a heparinized test tube and later centrifuged to recover the plasma. The liver, kidneys, heart, and lung of each animal were quickly removed. With the aide of a dissecting microscope the brain was dissected by the use of a water aspirated micropipette and a small spatula. The brain regions collected were the hippocampus, superior colliculus, inferior colliculus, cerebellum, corpus callosum, olfactory bulbs, substantia nigra, medulla, and caudate putamen. The remaining rat brain was also kept for analysis. The liver,
kidneys, heart, lung and remaining brain were then weighed, frozen in liquid nitrogen, and stored at -80°C until assayed. Each brain section was pooled at each time point, weighed, and stored at -80°C. The plasma samples collected were also kept at -80°C.

The same experiment using the protocol above was used to obtain plasma and tissue samples for (E)-2-ene VPA and (E,E)-2,3′-diene VPA.

2.4.2 Anticonvulsant Evaluation

Aqueous solutions of VPA for i.p. injection were prepared at concentrations of 15, 37.5, 75, and 150 mg/mL, corresponding to doses administered of 30, 75, 150, and 300 mg/kg respectively. For (E,E)-2,3′-diene VPA solutions of 37.5, 75, 100, 150, and 200 mg/mL were prepared for dosages administered at 75, 150, 200, 300, and 400 mg/kg, respectively. Solutions of 37.5, 75, 100, and 150 mg/mL of both (E)-2-ene VPA and (E,Z)-2,3′-diene VPA were each prepared for anticonvulsant evaluation at dosages of 75, 150, 200, and 300 mg/kg, respectively. Solutions of each compound were prepared as their sodium salts by the addition of an equivalent amount of 3N NaOH. The pH was then adjusted to 7.4 if required with 3N HCl and the mixture made to volume with distilled water.

An aqueous solution of the convulsant pentylenetetrazole (PTZ, 3.5 g) was prepared daily by dissolving the compound in 100 mL of normal saline to yield a concentration of 35 mg/mL for s.c. injection.

Male Sprague-Dawley rats weighing between 200 - 250 g were administered solutions of either VPA, (E)-2-ene VPA, (E,E)-2,3′-diene VPA, or (E,Z)-2,3′-diene VPA i.p. at the various dosages prepared with each dose regimen requiring eight rats. Upon injection, each rat was
isolated by placing it into clear plastic cages for observation. At the approximate peak brain concentration for each compound, the convulsant PTZ was administered s.c. at a dose of 70 mg/kg. Each rat was observed for a further 30 minutes for signs of seizure activity.

The dose of PTZ administered was sufficient to elicit a minimum seizure also known as a clonic seizure. The seizure is first characterized by myoclonic jerks followed by a Straub tail phenomenon and then a clonic convulsion. A clonic convulsion in rats is depicted by whole body spasms followed by the arching of the spine and flexing of the jaw muscles. A paddling or swimming motion of the front paws is highly characteristic of a clonic convulsion with loss of righting sometimes occurring. After the convulsions, which last approximately 5 sec, the rat then enters a stupor or calm phase that can last for up to one hour.

A control group of eight rats was administered PTZ s.c. in order to characterize the seizure without the interference of the anticonvulsants and to determine the efficacy of the convulsant.

2.4.3 Protein Binding of (E,E) and (E,Z)-2,3'-diene VPA

The plasma protein binding of (E,E)- and (E,Z)-2,3'-diene VPA was determined from the rat plasma samples collected during the tissue distribution and kinetic studies. The total drug bound was determined by directly assaying the plasma samples for the dienes while the free drug was isolated by the method of ultrafiltration.

To obtain free drug concentration from total drug in plasma, a 500 ul aliquot of plasma was placed into upper chamber of an AmiconR micropartition apparatus fitted with a YMT membrane. The entire
micropartition apparatus was placed into a Beckman centrifuge equipped with a 45° rotor and spun at 1650 g for 20 min at 25°C. A 250 μL aliquot of the ultrafiltrate was then assayed to determine the free drug concentration.

2.5 ANALYTICAL

2.5.1 Homogenization of the Tissue Samples

All tissue samples collected (heart, lung, brain, liver, and kidneys) were homogenized in distilled water to uniformity and made to a specified volume or weight. The remaining brain, liver, and kidney samples were homogenized by three passes using a Potter-Elvehjem tissue grinder equipped with a Teflon pestle with each tissue made to volumes of 4, 35, and 6 mL, respectively. The heart and lung samples were homogenized for 20 sec over ice using a Polytron® equipped with a large cell disrupter and made to volumes of 4 and 5 mL, respectively. Since the sample size of each dissected brain section can be very small even after pooling, all measurements were determined by weight to minimize errors. The weight of distilled water added (0.2 - 1 g) depended on the weight of each brain section sample as the final concentration of brain tissue must be approximately 500 mg/g. Each of the pooled brain section samples were homogenized with water for 10 sec over ice using a Polytron® fitted with a small cell disrupter. Therefore, from the weight of brain section sample and the total weight of the homogenate, the concentration of brain material in solution can be accurately calculated.
Control tissues and plasma for the preparation of calibration curves were obtained from naive rats. The tissues were homogenized using the above procedures. The entire intact brain was homogenized and used as control brain tissue for the analysis of all brain components.

2.5.2 Internal Standards

A 100 mL internal standard solution was prepared by combining individual solutions of each compound into distilled water to yield final concentrations of 50 ug/mL for \([^{2}\text{H}]_{6}\text{VPA}\) and 25 ug/mL for \([^{2}\text{H}]_{3}\text{2-ene VPA}\), \([^{2}\text{H}]_{3}\text{3-keto VPA}\), and 2-MGA. The internal standard \([^{2}\text{H}]_{6}\text{VPA}\) was used to quantitate VPA; \([^{2}\text{H}]_{3}\text{2-ene VPA}\) was used to quantitate (E,E)- and (E,Z)-2,3′-diene VPA, (E)-2,4-diene VPA, (E)- and (Z)-2-ene VPA, 3-ene VPA, 4-ene VPA, and 4-keto VPA; \([^{2}\text{H}]_{3}\text{3-keto VPA}\) was used to quantitate 3-keto VPA; and 2-MGA was used to quantitate 3-OH VPA, 4-OH VPA, 5-OH VPA, and 2-PGA.

2.5.3 Calibration Curves

Calibration curves over a high concentration range were prepared for VPA, (E)- and (Z)-2-ene VPA, and (E,E)- and (E,Z)-2,3′-diene VPA. Calibration curves over a low concentration range were prepared for VPA, (E) and (Z)-2-ene VPA, and (E,E) and (E,Z)-2,3′-diene VPA in addition to (E)-2,4-diene VPA, 3-ene VPA, 4-ene VPA, 4-keto VPA, 3-keto VPA, 3-OH VPA, 4-OH VPA, 5-OH VPA, and 2-PGA.

For the preparation of the high concentration range calibration curves, approximately 50 mg of VPA, (E)-2-ene VPA (containing 5% (Z)-2-ene VPA), and (E,E)-2,3′-diene VPA (containing 3-5% (E,Z)-2,3′-diene VPA) and an equivalent amount of IN NaOH were combined and made to 10 mL
with distilled water to yield the main stock solution. A 200 uL aliquot of the main stock solution was added to 5 mL of control tissue homogenate to yield stock tissue solution #1. Dilution of stock tissue solution #1, as outlined in table 4, with control tissue homogenate affords the samples for the high concentration range calibration curve. The concentration ranges for each compound quantitated are listed in table 5. High concentration range calibration curves were prepared for each tissue assayed by spiking a 200 uL aliquot of main stock solution into the various tissues analyzed followed by the dilution scheme in table 4.

Stock tissue solution #2 was made by spiking 50 uL of stock tissue solution #1, varying amounts of each metabolite, and an equivalent amount of IN NaOH corresponding to the amount of metabolites added into approximately 5 mL of control tissue. Dilution of stock tissue solution #2, as detailed in table 4, yields the samples for the low concentration range calibration curves for VPA and its metabolites. The concentration ranges for each compound are listed in table 5. Similarly, the low concentration range calibration curves were prepared for each tissue assayed by using control tissue to dilute aliquots of stock tissue solution #1.

The calibration curves used for assaying the amount of free (E,E)- and (E,Z)-2,3′-diene VPA present in the plasma ultrafiltrate were prepared by using distilled water for dilution of the stock solutions. The final concentrations obtained were identical to those listed in table 5.
Table 4: Volumes of control tissue homogenate used for the dilution of stock tissue homogenate containing VPA and VPA metabolites to provide the samples required to produce a calibration curve.

<table>
<thead>
<tr>
<th>Sample</th>
<th>uL of Stock Solution</th>
<th>uL of Control Tissue</th>
<th>Total Volume uL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>250</td>
<td>250</td>
</tr>
<tr>
<td>2</td>
<td>50</td>
<td>200</td>
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<td>100</td>
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<td>5</td>
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<td>250</td>
</tr>
<tr>
<td>6</td>
<td>250</td>
<td>0</td>
<td>250</td>
</tr>
<tr>
<td>COMPOUND</td>
<td>LOW RANGE</td>
<td>HIGH RANGE</td>
<td></td>
</tr>
<tr>
<td>----------------------------</td>
<td>-------------------</td>
<td>------------------</td>
<td></td>
</tr>
<tr>
<td>VPA</td>
<td>0 - 2.00 ug/mL</td>
<td>0 - 200.00 ug/mL</td>
<td></td>
</tr>
<tr>
<td>(E,E)-2,3′-diene VPA</td>
<td>0 - 1.99 ug/mL</td>
<td>0 - 199.40 ug/mL</td>
<td></td>
</tr>
<tr>
<td>(E,Z)-2,3′-diene VPA</td>
<td>0 - 160 ng/mL</td>
<td>0 - 15.95 ug/mL</td>
<td></td>
</tr>
<tr>
<td>(E)-2-ene VPA</td>
<td>0 - 2.01 ug/mL</td>
<td>0 - 200.80 ug/mL</td>
<td></td>
</tr>
<tr>
<td>(Z)-2-ene VPA</td>
<td>0 - 261 ng/mL</td>
<td>0 - 26.10 ug/mL</td>
<td></td>
</tr>
<tr>
<td>3-ene VPA</td>
<td>0 - 3.94 ug/mL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-ene VPA</td>
<td>0 - 301 ng/mL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(E)-2,4-diene VPA</td>
<td>0 - 4.60 ug/mL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-keto VPA</td>
<td>0 - 4.10 ug/mL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-keto VPA</td>
<td>0 - 4.08 ug/mL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-OH VPA</td>
<td>0 - 2.83 ug/mL</td>
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<tr>
<td>4-OH VPA</td>
<td>0 - 11.46 ug/mL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-OH VPA</td>
<td>0 - 6.54 ug/mL</td>
<td></td>
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</tr>
<tr>
<td>2-PGA</td>
<td>0 - 3.98 ug/mL</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.5.4 Extraction and Derivatization

To a 250 uL aliquot of tissue homogenate or plasma or to 250 mg aliquot of each pooled brain section homogenate, 50 uL of internal standard solution, 100 uL of distilled water, and 55 uL of 1N HCl were added. The samples were vigorously mixed and extracted twice by gentle mechanical rotation with 1 mL of ethyl acetate for 20 minutes. After each extraction step the samples were centrifuged for 10 min at 1000 g. To the combined organic fractions, 400 uL of 1N NaOH was added and extracted by gentle mechanical rotation for 10 minutes. The organic layer was discarded and 85 uL of 4N HCl was added to the aqueous portion to yield a pH of 1 - 2. The acidic fraction was then extracted with 1 mL of ethyl acetate by gentle mechanical rotation for 10 min. Upon separation, the organic layer was dried over anhydrous Na2SO4 and the volume reduced to 50 uL with nitrogen. The extract was then transferred into a 1 mL Reacti-VialR.

To the residue, 10 uL of diisopropylethylamine, 10 uL of quinol (1 mg/mL in ethyl acetate), and 10 uL of α-bromo-2,3,4,5,6-pentafluorotoluene (PFB, 30% in ethyl acetate) were added and the mixture heated for 45 minutes at 60°C. The sample was cooled and 50 uL of N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) was added and heated for another 45 minutes at 60°C. Once cool, the derivatized extracts were transferred into 100 uL limited volume inserts which were placed into an auto-sample vial and sealed with an aluminum crimp cap. A 1 uL aliquot was injected via an autosampler.
2.5.5 Extraction Efficiency

The amount of VPA, (E)-2-ene VPA, and (E,E)-2,3'-diene VPA extracted from brain tissue homogenate was evaluated. Solutions in ethyl acetate were prepared by the combined addition of varying amounts of VPA, (E)-2-ene VPA, and (E,E)-2,3'-diene VPA into dry ethyl acetate such that the final concentration of each compound was 0.4, 0.8, 1.2, 1.6, and 2.0 µg/mL. Similar solutions at various concentrations of VPA, (E)-2-ene VPA, and (E,E)-2,3'-diene VPA in control brain tissue homogenate were prepared.

A 250 µL aliquot of brain tissue homogenate spiked with VPA, (E)-2-ene VPA, and (E,E)-2,3'-diene VPA at each concentration was extracted, according to the aforementioned procedure. To the extracted samples, internal standards in ethyl acetate were added and the mixtures derivatized and assayed based on the previously detailed procedure. A 250 µL aliquot from each sample concentration containing VPA, (E)-2-ene VPA, and (E,E)-2,3'-diene VPA in ethyl acetate was similarly derivatized and assayed. The peak area ratios obtained for the standard samples of VPA, (E)-2-ene VPA, and (E,E)-2,3'-diene VPA dissolved in ethyl acetate were used to generate a standard curve. The concentrations of VPA, (E)-2-ene VPA, and (E,E)-2,3'-diene VPA extracted from brain tissue homogenate were then determined from this standard curve. The ratio between the observed concentration in brain tissue homogenate to the theoretical concentration for each compound was used to determine the percent of compound extracted out of the brain tissue, i.e. the extraction efficiency.
2.6 CALCULATIONS AND STATISTICS

2.6.1 Calculation of Pharmacokinetic Parameters

The pharmacokinetic parameters such as systemic clearance (Cl), half-life ($t_{1/2}$), apparent elimination rate constant ($K_e$), and apparent volume of distribution ($V_d$) were calculated using the equations of Gibaldi and Perrier (1982). The area under the plasma and tissue concentration-time curves were determined by the trapezoidal rule (Gibaldi and Perrier, 1982). Because the apparent elimination rate constant was not available, the area under the curve after the last sample measured could not be calculated. Therefore, the area was expressed as area under the curve from 0 to 10 hours (AUC$_{0-10h}$). The plasma clearances for each drug were then reported as Cl$_{0-10h}$. Since the bioavailability (F) for each compound was not determined, clearance was expressed as Cl$_{0-10}$/F. The precision of the data was expressed as ± S.D.

2.6.2 Statistical Analysis

Differences in the normally distributed variables, i.e. pharmacokinetic parameters, were assessed by one-way analysis of variance. If a significant F ratio was encountered, an additional test, Newman Keul's multiple comparison test, was performed to determine which group was significantly different. All statistical tests used a P-value of 0.05 to assess significance.

The ED$_{50}$ values from the dose-response curves were determined using the method of Litchfield and Wilcoxin (1948). Differences between the ED$_{50}$ values for each compound administered were assessed using the method of Chi-squares.
3. RESULTS

3.1 CHEMICAL SYNTHESSES

3.1.1 2-n-Propyl-(E)-2-Pentenoic Acid ((E)-2-ene VPA)

Following the procedure outlined in scheme 1, 2-n-propyl-(E)-2-pentenoic acid was synthesized in good yield. The final product was found to contain two stereoisomers, both identified by GC-MS as methyl ester derivatives of 2-n-propyl-(Z)-2-pentenoic acid and 2-n-propyl-(E)-2-pentenoic acid (figure 5). The configuration of the two isomers was confirmed by NMR (figure 6). The mass spectrum of methyl 2-n-propyl-(E)-2-pentenoate revealed a prominent ion at m/z 156 corresponding to the molecular ion. In addition, a fragment ion at m/z 141 represented the loss of the methyl ester moiety, [M-15]+. From the NMR spectrum, the majority of the product possessed the (E)-configuration. A comparison of the integrated peaks at 6.8 ppm and at 5.9 ppm, corresponding to the proton at C3 for the (E)- and (Z)-isomers respectively, indicated that the product was 95% 2-n-propyl-(E)-2-pentenoic acid.

3.1.2 2-((Z)-1'-Propenyl)-(E)-2-Pentenoic Acid ((E,Z)-2,3' diene VPA)

The method used for the synthesis of 2-((Z)-1'-propenyl)-(E)-2-pentenoic acid (scheme 2) was similar to that of 2-n-propyl-(E)-2-pentenoic acid. The yield obtained from the synthesis of 2-((Z)-1'-propenyl)-(E)-2-pentenoic acid starting with the β-hydroxy-β',γ'-unsaturated ester was 85%. The product was identified by GC-MS as the methyl ester derivative (figure 7). Major ions at m/z 154 and 139
Scheme 1:  Synthesis of (E)-2-ene VPA:  

- **a** = EtOH, H₂SO₄, benzene, Δ;  
- **b** = [(CH₃)₂CH]₂NH, n-BuLi, CH₃CH₂CHO, THF, -78°C;  
- **c** = (C₂H₅)₃N, CH₃SO₂Cl, CH₂Cl₂, 0°C;  
- **d** = DBU, THF, Δ;  
- **e** = NaOH, Δ, HCl.
Figure 5: Total ion chromatogram and mass spectra of methyl 2-n-propyl-(E)-2-pentenoate and methyl 2-n-propyl-(Z)-2-pentenoate.
Figure 6: 400 MHz Proton NMR spectrum of 2-n-propyl-(E)-2-pentenoic acid (*=2-n-propyl-(Z)-2-pentenoic acid).
Scheme 2: Synthesis of (E,Z)-2,3'-diene VPA: a=EtOH, H₂SO₄, benzene, Δ; b=[(CH₃)₂CH]₂NH, n-BuLi, CH₃CH₂CHO, THF, -78°C; c=(C₂H₅)₃N, CH₃SO₂Cl, CH₂Cl₂, 0°C; d=DBU, THF, Δ; e=NaOH, Δ, HCl.
Figure 7: Total ion chromatogram and mass spectrum of methyl 2-((Z)-1'-propenyl)-(E)-2-pentenoate.
corresponded to the molecular ion and loss of the methyl ester derivative, respectively. The chemical structure was confirmed by NMR (figure 8) to be 95% isomerically pure for the 2-((Z)-1'-propenyl)-(E)-2-pentenoic acid.

3.1.3 2-((E)-1'-Propenyl)-(E)-2-Pentenoic Acid ((E,E)-2,3'-diene VPA)

The synthesis of 2-((E)-1'-propenyl)-(E)-2-pentenoic acid was based on the procedures of Lee et al. (1989) (scheme 3). The nucleophile, potassium hydride, was used to eliminate the mesyl group to yield the α,β-unsaturated ester. The synthesis of 2-((E)-1'-propenyl)-(E)-2-pentenoic acid had been completed before the availability of DBU as was used in the synthesis of 2-((Z)-1'-propenyl)-(E)-2-pentenoic acid.

To obtain sufficient material for the animal experiments, the synthesis of 2-((E)-1'-propenyl)-(E)-2-pentenoic acid was repeated several times to afford several grams of product. The final product was identified as the methyl ester derivative following GC-MS analysis (figure 9). From the mass spectrum, prominent ions at m/z 154, 139, and 95 corresponded to a molecular ion, loss of a methyl group, and [M-COOCH₃]+, respectively. The NMR spectrum for 2-((E)-1'-propenyl)-(E)-2-pentenoic acid (figure 10) compared favorably to that of published values (Acheampong and Abbott, 1985; Lee et al., 1989). The product was found to be 95 - 97% isomerically pure based on GC-MS and NMR data.
Figure 8: 400 MHz Proton NMR spectrum of 2-((Z)-1'-propenyl)-(E)-2-pentenoic acid (*=2-((E)-1'-propenyl)-(E)-2-pentenoic acid).
Scheme 3:  Synthesis of (E,E)-2,3'-diene VPA:  a=K₂CO₃, EtI, 18-crown-6, THF, Δ;  b=[(CH₃)₂CH]₂NH, n-BuLi, HMPA, CH₃CH₂CHO, THF, -78°C;  c=(C₂H₅)₃N, CH₃SO₂Cl, CH₂Cl₂, 0°C;  d=DBU, THF, Δ;  e=NaOH, Δ, HCl.
Figure 9: Total ion chromatogram and mass spectrum of methyl 2-((E)-1'-propenyl)-(E)-2-pentenoate.
Figure 10: 300 MHz Proton NMR spectrum of 2-((E)-1'-propenyl)-(E)-2-pentenoic acid (*=2-((Z)-1'-propenyl)-(E)-2-pentenoic acid).
3.2 ASSAY DEVELOPMENT

3.2.1 Extraction and Derivatization

The liver, heart, kidney, lung, and brain samples were uniformly homogenized in distilled water before extraction. The absolute extraction efficiencies for VPA, (E)-2-ene VPA, and (E,E)-2,3′-dienen VPA in brain tissue homogenates were within acceptable limits (table 6). The limits of detection achieved for VPA, (E)-2-ene VPA, and (E,E)-2,3′-dienen VPA in brain tissue homogenate (13 - 38 ng/mL) were 10 - 30 pg with a signal-to-noise ratio of less than 3. Similar limits of detection were observed for these compounds in the other tissues examined.

A two step derivatization procedure was employed to ensure good chromatographic separation of all compounds of interest from that of the background. A pentafluorobenzyl (PFB) ester on the carboxylic acid group was initially formed and was immediately followed by a second derivative to yield the trimethylsilyl (TMS) ester for those compounds possessing a hydroxyl or oxo moiety. The derivatization of the carboxylic acid group by the PFB reagent proceeded to completion since there was no trace of a TMS ester present for each compound assayed. That is, if the initial derivatization of the carboxylic acid group was not complete, the addition of the second derivatizing reagent (MSTFA) would have reacted with the remaining free acid to yield the TMS ester. Similarly, derivatization of the hydroxyl or 3-keto groups with TMS proceeded to completion since no ions were detected for these polar compounds at [M-181]−, i.e. loss of the PFB group from metabolites where the hydroxyl or 3-keto moiety was underivatized.
Table 6: The extraction efficiencies of VPA, (E)-2-ene VPA, (E,E)-2,3’-diene VPA from brain tissue homogenate at various concentrations (n=3).

<table>
<thead>
<tr>
<th>Concentration (ug/mL)</th>
<th>Extraction Efficiency (% S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VPA</td>
</tr>
<tr>
<td>0.4</td>
<td>85±6</td>
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<tr>
<td>0.8</td>
<td>73±3</td>
</tr>
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<td>1.2</td>
<td>80±4</td>
</tr>
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<td>82±1</td>
</tr>
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<td></td>
<td>82±23</td>
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<tr>
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<td>(E)-2-ene VPA</td>
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<tr>
<td>0.4</td>
<td>84±4</td>
</tr>
<tr>
<td>0.8</td>
<td>77±2</td>
</tr>
<tr>
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<td>80±3</td>
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<td>1.6</td>
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<td></td>
<td>(E,E)-2,3’-diene VPA</td>
</tr>
<tr>
<td>0.4</td>
<td>59±5</td>
</tr>
<tr>
<td>0.8</td>
<td>60±4</td>
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<tr>
<td>1.2</td>
<td>67±6</td>
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<tr>
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<td>2.0</td>
<td>61±8</td>
</tr>
<tr>
<td></td>
<td>57±21</td>
</tr>
</tbody>
</table>
3.2.2 Calibration Curves

The calibration curves obtained for VPA and the metabolites of interest from brain tissue homogenates showed linearity within the concentration ranges used (figures 11 - 20). Although not shown, similar results for calibration curves were obtained from the other tissue homogenates and plasma. The inter-assay variation was assessed based on the slopes of each calibration curve in each tissue assayed over an 18 month period and were found to be acceptable (table 7). The compounds demonstrating the best overall assay precision were those having a deuterated analogue of their respective structure as the internal standard, i.e. VPA, (E)-2-ene VPA, and 3-keto VPA. Compounds quantitated without a specific deuterated analogue as the internal standard tended to have a greater relative standard deviation. Since a calibration curve for each compound of interest was generated in-conjunction with each set of unknown samples, good inter-assay variability was not a critical factor.

3.2.3 Detection of VPA and Its Metabolites

The detection of VPA and its metabolites was by negative ion chemical ionization gas chromatography-mass spectrometry (NICI GC-MS) using selected-ion-monitoring (SIM). This method of ionization is a gentle technique and as a result fragmentation of derivatized compounds was minimal. The fragment ion monitored for each compound was the [M-181]⁻ base ion from loss of the PFB group from the derivatized carboxylic acid (table 8).

The mass chromatograms generated from the SIM of each compound of interest from brain tissue homogenate are illustrated in figure 21.
Figure 11: High and low range concentration calibration curves for VPA in brain tissue homogenate.
Figure 12: High and low range concentration calibration curves for (E,E)-2,3'-diene VPA in rat brain tissue homogenate.
Figure 13: High and low range concentration calibration curves for (E,Z)-2,3'-diene VPA in rat brain tissue homogenate.
Figure 14: Calibration curve for (E)-2,4-diene VPA in rat brain tissue homogenate.
Figure 15: High and low range concentration calibration curves for (E)-2-ene VPA in brain tissue homogenate.
Figure 16: High and low range concentration calibration curves for (Z)-2-ene VPA in rat brain tissue homogenate.
Figure 17: Calibration curves for 3-ene VPA and 4-ene VPA in rat brain tissue homogenate.
Figure 18: Calibration curves for 3-keto VPA and 4-keto VPA in rat brain tissue homogenate.
Figure 19: Calibration curves for 3-OH VPA and 4-OH VPA in rat brain tissue homogenate.
Figure 20: Calibration curves for 5-OH VPA and 2-PGA in rat brain tissue homogenate.
Table 7: Inter-assay variation based on the slopes of the calibration curves over an 18 month period for VPA and its metabolites. \( n \)

<table>
<thead>
<tr>
<th></th>
<th>BRAIN</th>
<th>PLASMA</th>
<th>LIVER</th>
<th>KIDNEY</th>
<th>HEART</th>
</tr>
</thead>
<tbody>
<tr>
<td>((E,E)-2,3'-diene) VPA</td>
<td>13.9%</td>
<td>15.0%</td>
<td>7.7%</td>
<td>1.7%</td>
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<td>((E,Z)-2,3'-diene) VPA</td>
<td>13.3</td>
<td>8.0</td>
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<td>6.0</td>
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<td>((E)-2,4-diene) VPA</td>
<td>17.9</td>
<td>12.7</td>
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<td>16.2</td>
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<td>4-ene VPA</td>
<td>17.2</td>
<td>4.8</td>
<td>19.1</td>
<td>7.2</td>
<td>7.7</td>
</tr>
<tr>
<td>VPA</td>
<td>5.5</td>
<td>10.8</td>
<td>0.9</td>
<td>2.7</td>
<td>7.9</td>
</tr>
<tr>
<td>4-keto VPA</td>
<td>18.7</td>
<td>16.9</td>
<td>23.0</td>
<td>1.5</td>
<td>5.0</td>
</tr>
<tr>
<td>3-keto VPA</td>
<td>6.2</td>
<td>2.4</td>
<td>13.1</td>
<td>2.2</td>
<td>8.9</td>
</tr>
<tr>
<td>3-OH VPA</td>
<td>13.3</td>
<td>20.8</td>
<td>21.6</td>
<td>9.6</td>
<td>14.0</td>
</tr>
<tr>
<td>4-OH VPA</td>
<td>1.3</td>
<td>5.6</td>
<td>3.6</td>
<td>11.6</td>
<td>1.7</td>
</tr>
<tr>
<td>5-OH VPA</td>
<td>10.1</td>
<td>13.0</td>
<td>15.5</td>
<td>15.9</td>
<td>3.0</td>
</tr>
<tr>
<td>2-PGA</td>
<td>15.1</td>
<td>10.2</td>
<td>11.4</td>
<td>3.6</td>
<td>9.3</td>
</tr>
</tbody>
</table>
Table 8: Ions monitored for the quantitative analysis and the retention times of internal standards(*), VPA and its metabolites.

<table>
<thead>
<tr>
<th>COMPOUNDS</th>
<th>ION MONITORED (m/z)</th>
<th>RETENTION TIME (MINUTES)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-ene VPA</td>
<td>141</td>
<td>7.48</td>
</tr>
<tr>
<td>[(^2)H(_6)] VPA*</td>
<td>149</td>
<td>7.50</td>
</tr>
<tr>
<td>VPA</td>
<td>143</td>
<td>7.55</td>
</tr>
<tr>
<td>3-ene VPA</td>
<td>141</td>
<td>7.66</td>
</tr>
<tr>
<td>(Z)-2-ene VPA</td>
<td>141</td>
<td>7.78</td>
</tr>
<tr>
<td>[(^2)H(_3)] 2-ene VPA*</td>
<td>144</td>
<td>8.62</td>
</tr>
<tr>
<td>(E)-2-ene VPA</td>
<td>141</td>
<td>8.65</td>
</tr>
<tr>
<td>(E,Z)-2,3′-diene VPA</td>
<td>139</td>
<td>8.47</td>
</tr>
<tr>
<td>(E)-2,4-diene VPA</td>
<td>139</td>
<td>8.78</td>
</tr>
<tr>
<td>(E,E)-2,3′-diene VPA</td>
<td>139</td>
<td>9.52</td>
</tr>
<tr>
<td>4-keto VPA</td>
<td>157</td>
<td>10.27</td>
</tr>
<tr>
<td>3-OH VPA</td>
<td>231</td>
<td>11.49</td>
</tr>
<tr>
<td>4-OH VPA</td>
<td>231</td>
<td>11.20/11.77</td>
</tr>
<tr>
<td>[(^2)H(_3)] 3-keto VPA*</td>
<td>232</td>
<td>12.77</td>
</tr>
<tr>
<td>3-keto VPA</td>
<td>229</td>
<td>12.80</td>
</tr>
<tr>
<td>5-OH VPA</td>
<td>231</td>
<td>13.31</td>
</tr>
<tr>
<td>2-MGA*</td>
<td>325</td>
<td>19.15</td>
</tr>
<tr>
<td>2-PGA</td>
<td>353</td>
<td>21.23</td>
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</table>
Figure 21: Mass chromatograms of VPA, VPA metabolites, and internal standards in rat brain homogenate following i.p. administration of 150 mg/kg of VPA (1), and mass chromatograms of internal standards added to control rat brain homogenate (2): a=4-ene VPA (7.48 min.); b=3-ene VPA (7.66); c=(Z)-2-ene VPA (7.78); d=(E)-2-ene VPA (8.65); e=D6-VPA (7.49); f=VPA (7.55); g=(E,Z)-2,3′-diene VPA (8.47); h=(E)-2,4-diene VPA (8.78); i=(E,E)-2,3′-diene VPA (9.52); j=D3-(E)-2-ene VPA (8.62); k=4-keto VPA (10.27); l/n=4-OH VPA (11.20/11.77); m=3-OH VPA (11.49); o=5-OH VPA (13.31); p=D3-3-keto VPA (12.77); q=3-keto VPA (12.80); r=2-MGA (19.15); s=2-PGA (21.23).
These selected ion mass chromatograms were well resolved and free of any interfering endogenous substances. Comparable results were obtained for the plasma and other tissue extracts.

3.3 PHARMACOKINETICS AND TISSUE DISTRIBUTION OF VPA

3.3.1 Profile of VPA in Plasma

Following i.p. administration of 150 mg/kg of VPA to rats, the drug was readily absorbed from the peritoneal cavity, reaching peak plasma (C\text{max}) concentrations within 30 minutes. The apparent maximum plasma concentration was $142.7 \pm 51.8 \text{ ug/mL}$. The elimination of VPA from plasma did not follow a typical log-linear decline but was interrupted by a temporary rise in plasma levels that occurred 4 - 6 hours following drug administration (figure 22).

The area under the curve (AUC) for VPA in plasma measured from 0 - 10 hours was calculated to be $455 \pm 68 \text{ ug\cdot h/mL}$. Plasma clearance of VPA was calculated to be $92 \pm 14 \text{ mL/h}$ and was expressed as Cl\text{0-10h}/F since the bioavailability (F) for VPA after i.p. administration in the rat was not known.

3.3.2 Profile of VPA in Peripheral Tissues

The kinetic profile of VPA was determined in liver, kidneys, heart, and lungs following i.p. administration. The distribution of VPA from the plasma to the tissues was not rapid with maximal tissue concentrations occurring within 30 - 45 minutes after injection. The C\text{max} values for VPA in the liver, kidneys, heart, and lungs were
Figure 22: VPA concentration-time curves in rat plasma, liver, kidneys, heart, and lungs following 150 mg/kg i.p. administration (n=8/time point, error bars=S.D.).
225.6 ± 63.1, 179.6 ± 32.0, 52.7 ± 13.3, and 93.2 ± 13.0 ug/g respectively. The elimination profiles of VPA in these tissues were similar to that seen in plasma including the temporary increase in VPA levels that occurred 4 - 6 hours following drug administration (figure 22). The concentration of VPA was highest in the liver and remained that way for the entire 10 hour period. The AUC₀₋₁₀h values for the liver, kidneys, heart, and lungs were calculated to be 854 ± 124, 698 ± 114, 138 ± 25, and 229 ± 39 ug h/g respectively.

A comparison of the tissue/plasma ratios at tₘₐₓ showed that VPA appeared to concentrate mainly in the liver and kidneys with ratios to plasma values of 1.8 and 1.3 respectively. At t₁₀h, the liver/plasma ratio increased to 4.6 while in kidneys the ratio increased slightly to 1.8. Thus, VPA appears to persist in the liver and kidney tissues independent of plasma levels. The tissue/plasma ratio of VPA in the heart and lungs at tₘₐₓ was calculated to be 0.3 and 0.9 respectively. These ratios decreased to 0.02 and 0.2, respectively at 10 hours.

3.3.3 Profile of VPA in Brain

The kinetic profile of VPA in brain tissue was determined in the whole brain (WB) and the following brain regions: hippocampus (HIP), superior colliculus (SC), inferior colliculus (IC), cerebellum (CER), olfactory bulbs (OLF), corpus callosum (CC), substantia nigra (SN), medulla (MED), and the caudate putamen (CP) (figure 23). The isolation of certain areas of the brain may indicate regional differences in the kinetic behavior of a compound that may otherwise be overlooked upon whole brain analysis.
Figure 23: VPA concentration-time curves in rat plasma (PLA), whole brain (WB), hippocampus (HIP), superior colliculus (SC), inferior colliculus (IC), cerebellum (CER), olfactory bulbs (OLF), corpus callosum (CC), substantia nigra (SN), medulla (MED), and putamen (CP) following 150 mg/kg i.p. administration (WB, n=8/time point; brain regions were pooled, n=8/time point).
The distribution of VPA into the brain was relatively fast with peak levels in all regions attained within 15 - 30 minutes. The concentration of VPA in the whole brain at $t_{\text{max}}$ was $50.6 \pm 13.0$ ug/g while in the various brain regions the concentrations were between 45.1 - 69.3 ug/g. The distribution of VPA throughout the brain appeared to be homogeneous. The elimination profile of VPA from the brain was similar to that in plasma with the recycling most evident for the brain fractions. The brief increase in VPA concentration observed in plasma also occurred in the brain but to a lesser degree. The AUC$_{0-10h}$ for VPA in whole brain was $105 \pm 23$ ug·h/mL whereas the AUC$_{0-10h}$ in the various regions of the brain were between 104.6 - 132.6 ug·h/mL.

Of all the tissues assayed, including peripheral tissues, the brain/plasma ratio for VPA was the lowest. The ratio of whole brain/plasma at $t_{\text{max}}$ was 0.3 while the tissue/plasma ratios for the individual brain sections were 0.3 - 0.4. Ten hours following the administration of VPA, these ratios were calculated to be in the range of 0.01 - 0.03. The brain/plasma ratios between whole brain sample and the individual brain sections appeared to be similar.

3.4 PHARMACOKINETICS AND TISSUE DISTRIBUTION OF (E)-2-ENE VPA

3.4.1 Profile of (E)-2-ene VPA in Plasma

Upon single dose i.p. administration of (E)-2-ene VPA, peak plasma concentrations of $143.0 \pm 50.0$ ug/mL were attained within 30 minutes. The elimination profile of (E)-2-ene VPA from plasma was interrupted by an increase in the plasma level 4 - 6 hours after drug administration
(figure 24). Thereafter, the plasma concentration of (E)-2-ene VPA maintained a plateau or equilibrium phase that appeared to continue well past the last sample at 10 hours. The AUC$_{0-10h}$ of (E)-2-ene VPA in plasma was determined to be $497 \pm 38$ ug·h/mL. Plasma clearance for (E)-2-ene VPA expressed as $Cl_{0-10h/F}$ was $82 \pm 6$ mL/h.

3.4.2 Profile of (E)-2-ene VPA in Peripheral Tissues

The absorption and elimination profiles of (E)-2-ene VPA in the liver, kidneys, heart, and lungs were similar to that in plasma (figure 24). The time taken to reach peak tissue levels was 30 - 45 minutes following administration. The $C_{max}$ values in the liver, kidneys, heart, and lungs were $244.5 \pm 64.9$, $99.5 \pm 29.5$, $66.3 \pm 29.8$, and $52.8 \pm 17.8$ ug/g respectively. Although the concentration of (E)-2-ene VPA in the liver was initially higher than that of plasma the AUC$_{0-10h}$ of $384 \pm 63$ ug·h/mL for the liver was less than that of plasma. Thus while the liver/plasma ratio at $t_{max}$ was 1.4, at $t_{10h}$ the ratio had decreased to 0.5, suggesting that (E)-2-ene VPA does not bind avidly to liver tissue.

The AUC$_{0-10h}$ values for (E)-2-ene VPA in the kidneys, heart and lungs were $273 \pm 41$, $79 \pm 16$, and $147 \pm 18$ ug·h/mL respectively. The tissue/plasma ratios determined at $t_{max}$ for kidneys, heart, and lungs were 0.7, 0.4, and 0.3 respectively. These ratios decreased to 0.5, 0.02, and 0.2 at $t_{10h}$.

3.4.3 Profile of (E)-2-ene VPA in Brain

The times required to reach peak brain levels of (E)-2-ene VPA were in the range of 15 - 30 minutes following administration. The mean $C_{max}$ in whole brain was $68.8 \pm 19.8$ ug/g while the $C_{max}$ values observed
Figure 24: (E)-2-ene VPA concentration-time curves in rat plasma, liver, kidneys, heart, and lungs following 150 mg/kg i.p. administration (n=8/time point, error bars=S.D.).
for the individual pooled brain sections were between 30.0 - 52.8 ug/g. The distribution of (E)-2-ene VPA throughout the brain appeared to be homogeneous as there were no areas found to contain unusually high levels of the drug. The elimination of (E)-2-ene VPA from the whole brain and various brain regions appeared to be rapid and linear for the initial 4 hours following drug administration. Thereafter a temporary increase in (E)-2-ene VPA levels occurred which was then followed by the slower elimination of the drug (figure 25). This transient rise in (E)-2-ene VPA levels in the brain corresponded to a similar effect observed in plasma.

The AUC₀⁻¹₀h value calculated for the whole brain following (E)-2-ene VPA administration was 48 ± 10 ug·h/g. The individual pooled brain regions had AUC₀⁻¹₀h values between 43 - 54 ug·h/g. The brain/plasma ratio for (E)-2-ene VPA in the whole brain at t_max was 0.3 which decreased to 0.04 after 10 hours. The brain/plasma ratios for the brain sections were in the range of 0.3 - 0.4 at t_max and decreased to 0.02 - 0.03 at 10 hours.

3.5 PHARMACOKINETICS AND TISSUE DISTRIBUTION OF (E,E)-2,3'-DIENE VPA

3.5.1 Profile of (E,E)-2,3'-diene VPA in Plasma

The absorption of (E,E)-2,3'-diene VPA into the plasma circulation was relatively rapid following i.p. administration of 150 mg/kg of the drug. The time taken to reach peak plasma levels was 15 minutes with a corresponding plasma concentration of 168.2 ± 13.9 ug/mL. The plasma levels for (E,E)-2,3'-diene VPA remained elevated for first hour
Figure 25: (E)-2-ene VPA concentration-time curves in rat plasma (PLA), whole brain (WB), hippocampus (HIP), superior colliculus (SC), inferior colliculus (IC), cerebellum (CER), olfactory bulbs (OLF), corpus callosum (CC), substantia nigra (SN), medulla (MED), and putamen (CP) following 150 mg/kg i.p. administration (WB,n=8/time point; brain regions were pooled,n=8/time point).
following administration but thereafter, the diene plasma levels began to decline (figure 26). The elimination of \((E,E)-2,3'-\text{diene VPA}\) was rapid although at the 4 - 6 hour period there appeared to be a brief interruption that resulted in a change in the rate of decline. The \(\text{AUC}_0-10\text{h}\) in plasma was \(406 \pm 46 \text{ ug}\cdot\text{h/mL}\) and the calculated clearance \((\text{Cl}_0-10\text{h}/F)\) was \(97 \pm 11 \text{ mL/h}\).

### 3.5.2 Profile of \((E,E)-2,3'-\text{diene VPA}\) in Peripheral Tissues

The distribution of \((E,E)-2,3'-\text{diene VPA}\) into the liver, kidneys, heart, and lungs was followed after i.p. administration of the drug (figure 26). The times required to reach peak tissue levels in the liver, kidneys, heart, and lungs were in the range of 45 - 60 minutes with corresponding tissue concentrations of 76.1 ± 19.0, 75.9 ± 6.7, 42.0 ± 12.6, and 80.8 ± 15.7 ug/g respectively. The elimination of \((E,E)-2,3'-\text{diene VPA}\) from these tissues was relatively rapid and similar to that in plasma. The \(\text{AUC}_0-10\text{h}\) for \((E,E)-2,3'-\text{diene VPA}\) in the lungs was highest at 135 ± 26 ug\cdot h/g and lowest in heart at 52 ± 8 ug\cdot h/g. The \(\text{AUC}_0-10\text{h}\) values for liver and kidneys were 133 ± 15 and 121 ± 30 ug\cdot h/g respectively.

The tissue/plasma ratios at maximum \((E,E)-2,3'-\text{diene VPA}\) concentrations in the liver, kidneys, heart, and lungs were 0.5, 0.5, 0.3, and 0.5, respectively. At \(t_{10\text{h}}\), the tissue/plasma ratios for kidneys and lungs had increased to values of 1.3 and 1.0, respectively while the tissue/plasma ratio at 6 hours for both liver and heart fell to 0.01. At 10 hours \((E,E)-2,3'-\text{diene VPA}\) in heart and liver was not readily detectable.
Figure 26: (E,E)-2,3'-diene VPA concentration-time curves in rat plasma, liver, kidneys, heart, and lungs following 150 mg/kg i.p. administration (n=8/time point, error bars=SD.).
3.5.3 Profile of (E,E)-2,3′-diene VPA in Brain

Following i.p. administration of (E,E)-2,3′-diene VPA, the drug quickly entered into the brain, reaching maximum concentrations in most regions within 15 minutes while in areas like the superior and inferior colliculus and the cerebellum the $t_{\text{max}}$ was 60 minutes (figure 27). In whole brain, the $t_{\text{max}}$ was 45 minutes and corresponded to a relatively high concentration of 56.2 ± 8.4 ug/g. The maximum concentrations of (E,E)-2,3′-diene VPA in the various pooled brain sections fell between 27.6 - 57.3 ug/g. The elimination profile of (E,E)-2,3′-diene VPA from all brain tissues was similar to that in plasma, including a delay in the decline of (E,E)-2,3′-diene VPA that occurred 4 - 6 hours following drug administration.

The AUC$_{0-10\text{h}}$ value for the whole brain was 56 ± 10 ug·h/g while the individual pooled brain sections gave areas in the range of 37 - 56 ug·h/g. The whole brain/plasma ratios at $t_{\text{max}}$ and $t_{10\text{h}}$ decreased from 0.3 to 0.1, respectively. However, the brain/plasma ratios for the individual brain regions at $t_{\text{max}}$ and $t_{10\text{h}}$ increased with these values ranging from 0.1 - 0.3 and 0.2 - 1.4, respectively.
Figure 27: (E,E)-2,3'-diene VPA concentration-time curves in rat plasma (PLA), whole brain (WB), hippocampus (HIP), superior colliculus (SC), inferior colliculus (IC), cerebellum (CER), olfactory bulbs (OLF), corpus callosum (CC), substantia nigra (SN), medulla (MED), and putamen (CP) following 150 mg/kg i.p. administration (WB,n=8/time point; brain regions were pooled,n=8/time point).
3.6 COMPARATIVE PHARMACOKINETICS AND TISSUE DISTRIBUTION OF VPA, (E)-2-ENE VPA, AND (E,E)-2,3’-DIENE VPA

3.6.1 Profiles in Plasma

The kinetic profiles of VPA, (E)-2-ene VPA, and (E,E)-2,3’-diene VPA in plasma were compared following i.p. administration of equivalent doses (figure 28). Similar absorption and elimination characteristics were noted for all three compounds during the initial 6 hours. Thereafter, the apparent increase in (E,E)-2,3’-diene VPA elimination resulted in the rapid decline of the diene from plasma. Common to each compound was an interruption in the decline from plasma seen 4 - 6 hours following administration. This interruption was illustrated by an increase in drug plasma concentrations that eventually lead to a plateau phase.

No significant differences were observed in plasma C\text{max} values for the three administered compounds (table 9) but there was a significant difference (p<0.05) in AUC\textsubscript{0-10h} values between (E)-2-ene VPA and (E,E)-2,3’-diene VPA (table 10). Thus, the rate and extent of absorption of VPA did not differ significantly from that of (E)-2-ene VPA or (E,E)-2,3’-diene VPA during the 10 hour period.

3.6.2 Profiles in Peripheral Tissues

The absorption and elimination profiles of VPA, (E)-2-ene VPA, and (E,E)-2,3’-diene VPA were compared in liver. From the concentration-time plots, the elimination of VPA and (E)-2-ene VPA from liver were comparable whereas (E,E)-2,3’-diene VPA appeared to clear at a faster
Figure 28: VPA, (E)-2-ene VPA, and (E,E)-2,3'-diene VPA concentration-time curves in plasma following 150 mg/kg i.p. administration of each compound to rats (n=8/time point, error bars=S.D.).
Table 9: The time to peak ($t_{\text{max}}$) and peak concentration ($C_{\text{max}}$) in tissues and plasma following VPA, (E)-2-ene VPA, and (E,E)-2,3'-diene VPA administration.

<table>
<thead>
<tr>
<th></th>
<th>$t_{\text{max}}$ (min)</th>
<th>$C_{\text{max}}$ (ug/g or mL, S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VPA</td>
<td>2-ene</td>
</tr>
<tr>
<td>PLASMA</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>LIVER</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>KIDNEY</td>
<td>45</td>
<td>30</td>
</tr>
<tr>
<td>HEART</td>
<td>45</td>
<td>30</td>
</tr>
<tr>
<td>LUNG</td>
<td>45</td>
<td>45</td>
</tr>
<tr>
<td>WHOLE BRAIN</td>
<td>30</td>
<td>30</td>
</tr>
</tbody>
</table>

Significant difference determined by one-way ANOVA and Newman-Keuls Test
(a) significance between VPA and 2,3'-diene ($p<0.05$)
(b) significance between 2-ene and 2,3'-diene ($p<0.05$)
(c) significance between VPA and 2-ene ($p<0.05$)
Table 10: AUC 0 to 10 h in tissues following i.p. administration of VPA, (E)-2-ene VPA, and (E,E)-2,3'-diene VPA.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>VPA (ug·h/g or mL, S.D.)</th>
<th>2-ene</th>
<th>2,3'-diene</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLASMA</td>
<td>455 (68)</td>
<td>497 (38) &lt;sup&gt;a&lt;/sup&gt;</td>
<td>401 (46)</td>
</tr>
<tr>
<td>LIVER</td>
<td>854 (124) &lt;sup&gt;b&lt;/sup&gt;</td>
<td>384 (63)</td>
<td>133 (15)</td>
</tr>
<tr>
<td>KIDNEY</td>
<td>698 (114) &lt;sup&gt;b&lt;/sup&gt;</td>
<td>273 (41)</td>
<td>121 (30)</td>
</tr>
<tr>
<td>HEART</td>
<td>138 (25) &lt;sup&gt;b&lt;/sup&gt;</td>
<td>79 (16)</td>
<td>52 (8)</td>
</tr>
<tr>
<td>LUNG</td>
<td>221 (39) &lt;sup&gt;cd&lt;/sup&gt;</td>
<td>147 (18)</td>
<td>135 (26)</td>
</tr>
<tr>
<td>WHOLE BRAIN</td>
<td>105 (23) &lt;sup&gt;cd&lt;/sup&gt;</td>
<td>48 (10)</td>
<td>56 (10)</td>
</tr>
</tbody>
</table>

Significant difference determined by one-way ANOVA and Newman-Keuls Test
(a) significance between 2-ene and 2,3'-diene (p<0.05)
(b) significance between VPA, 2-ene, and 2,3'-diene (p<0.05)
(c) significance between VPA and 2-ene (p<0.05)
(d) significance between VPA and 2,3'-diene (p<0.05)
rate (figure 29). Similar results were observed in the kidneys, heart, and lungs (figures 30 - 32).

Following the equivalent single dose administration of each compound, the peak concentration of (E,E)-2,3'-diene VPA attained in the liver was significantly less ($p<0.05$) than both VPA and (E)-2-ene VPA (table 9). The times required to reach peak liver concentrations for each compound were similar. A comparison of the $AUC_{0-10h}$ values in liver for each compound showed that the value for (E,E)-2,3'-diene VPA was significantly less ($p<0.05$) than that of VPA and (E)-2-ene VPA (table 10). Similar trends were found in the kidneys, heart, and lungs (tables 9 and 10).

The $AUC_{0-10h}$ values for VPA in the various peripheral tissues indicated that a large portion of the dose could be accounted for in the liver fraction whereas distribution of VPA into the heart and lungs were somewhat smaller. The distribution of (E)-2-ene VPA into the various peripheral tissues following single dose administration in decreasing order were the liver, kidneys, lungs, and heart. For (E,E)-2,3'-diene VPA, the distribution into the liver, kidneys, and lungs appeared to be equivalent whereas the heart received a smaller fraction of the dose.

3.6.3 Profiles in Brain

The concentration-time plots for VPA, (E)-2-ene VPA, and (E,E)-2,3'-diene VPA were compared in whole brain and no apparent differences in the elimination profiles were observed for each compound (figure 33). Similar results were observed from the concentration-time plots for each compound in the various pooled brain sections (appendices 1 - 9).
Figure 29: VPA, (E)-2-ene VPA, and (E,E)-2,3'-diene VPA concentration-time curves in liver following 150 mg/kg i.p. administration of each compound to rats (n=8/time point, error bars=S.D.).
Figure 30: VPA, (E)-2-ene VPA, and (E,E)-2,3'-diene VPA concentration-time curves in kidneys following 150 mg/kg i.p. administration of each compound to rats (n=8/time point, error bars=S.D.).
Figure 31: VPA, (E)-2-ene VPA, and (E,E)-2,3'-diene VPA concentration-time curves in heart following 150 mg/kg i.p. administration of each compound to rats (n=8/time point, error bars=S.D.).
Figure 32: VPA, (E)-2-ene VPA, and (E,E)-2,3'-diene VPA concentration-time curves in lungs following 150 mg/kg i.p. administration of each compound to rats (n=8/time point, error bars=S.D.).
Figure 33: VPA, (E)-2-ene VPA, and (E,E)-2,3'-diene VPA concentration-time curves in whole brain following 150 mg/kg i.p. administration of each compound to rats (n=8/time point, error bars=S.D.).
In the whole brain fraction and the various individual pooled brain sections the $t_{\text{max}}$ and $C_{\text{max}}$ values for VPA, (E)-2-ene VPA, and (E,E)-2,3'-diene VPA were compared (table 11). No significant differences were observed in the $C_{\text{max}}$ values of these compounds in the whole brain. Nor were there significant differences found in the $C_{\text{max}}$ values between VPA, (E)-2-ene VPA, and (E,E)-2,3'-diene VPA in the individual brain regions.

The AUC$_{0-10h}$ value for VPA in whole brain was significantly greater than either (E)-2-ene VPA or (E,E)-2,3'-diene VPA. The kinetic profiles and the AUC$_{0-10h}$ values from the individual pooled brain regions were similar to those in whole brain (table 12) and hence, the distribution of VPA throughout the brain appeared homogeneous. Similar results were observed for (E)-2-ene VPA and (E,E)-2,3'-diene VPA.

3.7 TISSUE DISTRIBUTION AND KINETIC PROFILES OF METABOLITES OF VPA

3.7.1 Profiles of VPA Metabolites in Plasma

The kinetic profile of VPA and its biotransformation products were monitored over a 10 hour period in plasma. For ease of presentation the metabolites were categorized into unsaturated and polar compounds.

Following VPA administration, seven unsaturated metabolites were detected in plasma (figure 34). The major unsaturated metabolites were (E)-2-ene VPA, 3-ene VPA, and (E,E)-2,3'-diene VPA (concentration exceeding 1.0 ug/mL). Minor metabolites were (Z)-2-ene VPA, (E,Z)-2,3'-diene VPA, (E)-2,4-diene VPA, and 4-ene VPA. The rate of decline for the unsaturated metabolites appeared to be similar to that of the parent
Table 11: The time to peak ($t_{\text{max}}$) and peak concentration ($C_{\text{max}}$) in brain tissues following either VPA, (E)-2-ene VPA, or (E,E)-2,3'-diene VPA administration.

<table>
<thead>
<tr>
<th></th>
<th>$t_{\text{max}}$ (min)</th>
<th></th>
<th>$C_{\text{max}}$ (ug/g,S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VPA</td>
<td>2-ene</td>
<td>2,3'-diene</td>
</tr>
<tr>
<td>WHOLE BRAIN</td>
<td>30</td>
<td>30</td>
<td>45</td>
</tr>
<tr>
<td>HIP</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>SC</td>
<td>15</td>
<td>30</td>
<td>60</td>
</tr>
<tr>
<td>IC</td>
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<td>30</td>
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</tr>
<tr>
<td>CER</td>
<td>15</td>
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</tr>
<tr>
<td>OLF</td>
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<td>30</td>
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</tr>
<tr>
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</tr>
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<td>SN</td>
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<tr>
<td>CP</td>
<td>30</td>
<td>30</td>
<td>15</td>
</tr>
</tbody>
</table>
Table 12: AUC from 0 to 10 hours in the individual pooled brain sections following i.p. administration of either VPA, (E)-2-ene VPA, or (E,E)-2,3'-diene VPA.

<table>
<thead>
<tr>
<th></th>
<th>AUC0-10h (ug·h/g)</th>
<th>VPA</th>
<th>2-ene</th>
<th>2,3'-diene</th>
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<tbody>
<tr>
<td></td>
<td>WHOLE BRAIN</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>VPA</td>
<td>105 (23)</td>
<td>48 (10)</td>
<td>56 (10)</td>
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<td>HIP</td>
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<td>106.4</td>
<td>44.2</td>
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<td>CER</td>
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<td>47.5</td>
<td>55.6</td>
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<td>OLF</td>
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<td>45.0</td>
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<td>118.0</td>
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<td>51.3</td>
<td>44.4</td>
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<td></td>
<td>106.8</td>
<td>47.1</td>
<td>52.7</td>
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Significant difference determined by one-way ANOVA and Newman-Keuls Test
(a) significance between VPA and 2-ene VPA (p<0.05)
(b) significance between VPA and 2,3'-diene VPA (p<0.05)
Figure 34: Concentration-time plots of VPA and its unsaturated metabolites in plasma following the administration of 150 mg/kg i.p. of VPA to rats (n=8/time point, S.D. omitted for clarity).
compound. The plateau phase associated with the plasma decline of VPA was also apparent for the metabolites.

The major polar metabolites observed in plasma were 5-OH VPA and 3-keto VPA with 2-PGA, 4-keto VPA, 3-OH VPA, and 4-OH VPA present in lesser amounts (figure 35). The elimination profiles of these polar metabolites appeared to correspond to the decline of the parent compound from plasma.

3.7.2 Profiles of VPA Metabolites in Peripheral Tissues

In the liver, five unsaturated metabolites were detected (figure 36) of which two, (E)-2-ene VPA and 3-ene VPA, were major metabolites while (E,E)- and (E,Z)-2,3'-diene VPA, and 4-ene VPA represented the minor metabolites. The major polar metabolites observed in liver following VPA administration were 2-PGA, 5-OH VPA, and the \( \beta \)-oxidation products 3-keto VPA and 3-OH VPA. The minor polar metabolites were 4-keto VPA and 4-OH VPA (figure 37).

The profile of the unsaturated metabolites of VPA observed in kidneys, heart, and lungs were similar to those found in liver (appendices 10 - 12). However, not present in liver but quantitated in kidneys and lungs was (E)-2,4-diene VPA. The concentration of (E)-2,4-diene VPA in these tissues was similar to that of (E,E)-2,3'-diene VPA. The polar metabolites observed in the kidneys, heart, and lungs following VPA administration were similar to those found in liver (appendices 13 - 15).
Figure 35: Concentration-time plots of VPA and its polar metabolites in plasma following the administration of 150 mg/kg i.p. of VPA to rats (n=8/time point, S.D. omitted for clarity).
Figure 36: Concentration-time plots of VPA and its unsaturated metabolites in liver following the administration of 150 mg/kg i.p. of VPA to rats (n=8/time point, S.D. omitted for clarity).
Figure 37: Concentration-time plots of VPA and its polar metabolites in liver following the administration of 150 mg/kg i.p. of VPA to rats (n=8/time point, S.D. omitted for clarity).
3.7.3 Profiles of VPA Metabolites in Brain

Following VPA i.p. administration, the quantitation of metabolites in the individual remaining whole brain fraction was not possible. Of the several metabolites detected the signals corresponding to these metabolites were below the limits of the assay and hence, only the parent compound could be quantitated (appendix 16).

Because of the small sections of rat brain being analyzed, pooling of individual regions from the 8 rats was necessary for the detection of metabolites. An area in the brain considered to be high in GABA activity is the substantia nigra. The unsaturated metabolites observed in the substantia nigra following VPA administration were 3-ene VPA and (E)-2-ene VPA, each at concentrations less than 0.1 ug/g, (E,Z)-2,3'-diene VPA at approximately 10 ng/g, and 4-ene VPA at 0.1 ng/g (figure 38). At no time was the major plasma metabolite, (E,E)-2,3'-diene VPA, detected in this region of the brain. The polar metabolites detected in the substantia nigra, in descending amounts, were 5-OH VPA, 4-OH VPA, 3-keto VPA, 2-PGA, 4-keto VPA, and 3-OH VPA (figure 39). The concentrations of the polar metabolites were similar but less than 1 ug/mL.

The other pooled brain sections assayed were the hippocampus, superior colliculus, inferior colliculus, cerebellum, olfactory bulb, corpus callosum, medulla, and caudate putamen. The nonpolar and polar metabolites observed in these areas were similar to those found in the substantia nigra (appendices 17 - 32).
Figure 38: Concentration-time plots of VPA and its unsaturated metabolites in substantia nigra following the administration of 150 mg/kg i.p. of VPA to rats (n=8 pooled/time point).
Figure 39: Concentration-time plots of VPA and its polar metabolites in substantia nigra following the administration of 150 mg/kg i.p. of VPA to rats (n=8 pooled/time point).
3.8 TISSUE DISTRIBUTION AND KINETIC PROFILES OF METABOLITES OF (E)-2-ENE VPA

3.8.1 Profiles of (E)-2-ene VPA Metabolites in Plasma

The kinetic profiles of (E)-2-ene VPA and its biotransformed products were determined in plasma following the i.p. administration of a dose of (E)-2-ene VPA containing 5% (Z)-2-ene VPA. The metabolites monitored by GC-MS were the same as those assayed after VPA administration. The (Z)-2-ene VPA quantitated in plasma and tissues following (E)-2-ene VPA administration was not considered a metabolite but instead as a by-product of (E)-2-ene VPA synthesis.

The major nonpolar metabolites found in plasma were 3-ene VPA, (E,E)-2,3'-diene VPA and VPA (figure 40). The minor unsaturated products (<1 ug/mL) detected were (E,Z)-2,3'-diene VPA, (E)-2,4-diene VPA, and 4-ene VPA. Of the several polar metabolites detected in plasma, 3-keto VPA was the major product while 3-OH VPA, 4-OH VPA, 5-OH VPA and 2-PGA were present in lesser amounts (figure 41). The elimination profile in plasma of the unsaturated and polar metabolites of (E)-2-ene VPA resembled the elimination profile of the parent compound.

3.8.2 Profiles of (E)-2-ene VPA Metabolites in Peripheral Tissues

The major unsaturated metabolite quantitated in the liver fraction after (E)-2-ene VPA administration was 3-ene VPA (figure 42). Somewhat surprisingly VPA, a product of (E)-2-ene VPA reduction, was the main metabolite in liver. In fact, the concentration of VPA attained levels greater than that of the parent compound at 6 hours following (E)-2-ene VPA administration. Thus, reduction of (E)-2-ene VPA appears to be a
Figure 40: Concentration-time plots of (E)-2-ene VPA, its unsaturated metabolites, and VPA in plasma following the administration of 150 mg/kg i.p. of (E)-2-ene VPA (containing 5% (Z)-2-ene VPA) to rats (n=8/time point, S.D. omitted for clarity).
Figure 41: Concentration-time plots of (E)-2-ene VPA and its polar metabolites in plasma following the administration of 150 mg/kg i.p. of (E)-2-ene VPA (containing 5% (Z)-2-ene VPA) to rats (n=8/time point, S.D. omitted for clarity).
Figure 42: Concentration-time plots of (E)-2-ene VPA, its unsaturated metabolites, and VPA in liver following the administration of 150 mg/kg i.p. of (E)-2-ene VPA (containing 5% (Z)-2-ene VPA) to rats (n=8/time point, S.D. omitted for clarity).
prominent metabolic pathway for this compound. Other minor unsaturated metabolites of (E)-2-ene VPA in liver were (E,E)- and (E,Z)-2,3'-diene VPA, and 4-ene VPA. The major polar metabolites observed following (E)-2-ene VPA administration were 3-keto VPA and 3-OH VPA, both of which are metabolic products of mitochondrial \( \beta \)-oxidation (figure 43). Minor polar metabolites were 4-OH VPA, 5-OH VPA, and 2-PGA.

The results observed in the kidneys, heart, and lungs were similar to that in the liver (appendices 33 - 38). In contrast to the results from liver was the absence of (E)-2,4-diene VPA, and the concentration of both 3-ene VPA and VPA in these peripheral tissues was approximately 10-fold less. For the polar metabolites, 4-OH VPA was not detected in the kidneys, heart, and lungs but was found in the liver.

### 3.8.3 Profiles of (E)-2-ene VPA Metabolites in Brain

The unsaturated metabolites detected in the remaining whole brain fraction following (E)-2-ene VPA administration, in order of decreasing brain concentration, were 3-ene VPA, (E,E)-2,3'-diene VPA, and (E,Z)-2,3'-diene VPA (appendix 39). VPA was present at levels equivalent to 3-ene VPA. The two polar products of (E)-2-ene VPA metabolism observed in the remaining whole brain were the \( \beta \)-oxidation products 3-keto VPA and 3-OH VPA also present in plasma (appendix 40). Polar products arising from microsomal metabolism such as 4-OH VPA and 5-OH VPA were below the levels of detection.

The metabolites of (E)-2-ene VPA were also quantitated in the hippocampus, superior colliculus, inferior colliculus, cerebellum, olfactory bulbs, corpus callosum, substantia nigra, medulla, and the
Figure 43: Concentration-time plots of (E)-2-ene VPA and its polar metabolites in liver following the administration of 150 mg/kg i.p. of (E)-2-ene VPA (containing 5% (Z)-2-ene VPA) to rats (n=8/time point, S.D. omitted for clarity).
caudate putamen. The nonpolar metabolites of (E)-2-ene VPA observed in the substantia nigra in order of decreasing brain concentration were 3-ene VPA, (E,E)-, and (E,Z)-2,3'-diene VPA (figure 44). VPA was present as the reduced product of (E)-2-ene VPA at levels similar to that of 3-ene VPA. The polar metabolites observed were the \( \beta \)-oxidation products, 3-keto VPA and 3-OH VPA, and 4- and 5-OH VPA (figure 45). The concentrations of the polar metabolites in the substantia nigra were similar to each other and were quantitated at submicrogram levels.

The unsaturated and polar metabolites observed in the other regions of the brain following (E)-2-ene VPA administration were comparable to the results found in the substantia nigra (appendices 41 - 56).

### 3.9 TISSUE DISTRIBUTION AND KINETIC PROFILES OF METABOLITES OF (E,E)-2,3'-DIENE VPA

#### 3.9.1 Profiles of (E,E)-2,3'-diene VPA Metabolites in Plasma

The metabolites of (E,E)-2,3'-diene were quantitated in plasma over a 10 hour period. The metabolites chosen for quantitation were those that appear as products of VPA metabolism. The (E,Z)-2,3'-diene VPA was not considered to be a metabolite but a by-product of (E,E)-2,3'-diene VPA synthesis, since the dose of (E,E)-2,3'-diene VPA contained 3 - 5% of the (E,Z)-isomer.

The metabolites observed in plasma following (E,E)-2,3'-diene VPA administration, in order of decreasing abundance, were 3-ene VPA, (E)-2-ene VPA, and a diene, and VPA (figure 46). At 10 hours following (E,E)-
Figure 44: Concentration-time plots of (E)-2-ene VPA, its unsaturated metabolites, and VPA in substantia nigra following the administration of 150 mg/kg i.p. of (E)-2-ene VPA (containing 5% (Z)-2-ene VPA) to rats (n=8 pooled/time point).
Figure 45: Concentration-time plots of (E)-2-ene VPA and its polar metabolites in substantia nigra following the administration of 150 mg/kg i.p. of (E)-2-ene VPA (containing 5% (Z)-2-ene VPA) to rats (n=8 pooled/time point).
Figure 46: Concentration-time plots of (E,E)-2,3'-diene VPA, its unsaturated metabolites, and VPA in plasma following the administration of 150 mg/kg i.p. of (E,E)-2,3'-diene VPA (containing 5% (E,E)-2,3'-diene VPA) to rats (n=8/time point, S.D. omitted for clarity).
2,3’-diene VPA administration, the concentration of (E)-2-ene VPA and 3-ene VPA in plasma exceeded that of the parent compound, suggesting that the half-lives of these metabolites were greater than that of the parent compound.

Only three polar metabolites were detected in plasma. The most abundant was 3-keto VPA, with smaller amounts of 3-OH VPA and 5-OH VPA also occurring (figure 47).

3.9.2 Profiles of (E,E)-2,3’-diene VPA Metabolites in Peripheral Tissues

Most notable in the liver fraction following (E,E)-2,3’-diene VPA administration, were the high levels of VPA, (E)-2-ene VPA, and 3-ene VPA respectively. All three of the reduced products of (E,E)-2,3’-diene VPA metabolism exceeded the level of the parent compound within 200 minutes of administration, suggesting a high turnover rate of this diene via reduction (figure 48). As in the case of plasma, polar metabolites found in liver were 3-keto VPA and 3-OH VPA with 5-OH VPA present in minor quantities (figure 49). The concentration of the polar metabolites surpassed the level of the parent compound within 240 minutes of administration.

The metabolites observed in the kidneys, heart, and lungs following (E,E)-2,3’-diene VPA administration were similar to those found in liver although in lesser quantities (appendices 57 - 59). The concentration of the metabolites did not at anytime exceed the level of the parent compound during the 10 hour period. The polar metabolites detected in the kidneys, heart, and lungs were comparable to the metabolites found in the liver (appendices 60 - 62). The decline of these polar products from the kidneys, heart, and lungs appeared to
Figure 47: Concentration-time plots of (E,E)-2,3'-diene VPA and its polar metabolites in plasma following the administration of 150 mg/kg i.p. of (E,E)-2,3'-diene VPA (containing 3-5% (E,Z)-2,3'-diene VPA) to rats (n=8/time point, S.D. omitted for clarity).
Figure 48: Concentration-time plots of (E,E)-2,3'-diene VPA, its unsaturated metabolites, and VPA in liver following the administration of 150 mg/kg i.p. of (E,E)-2,3'-diene VPA (containing 3-5% (E,Z)-2,3'-diene VPA) to rats (n=8/time point, S.D. omitted for clarity).
Figure 49: Concentration-time plots of (E,E)-2,3'-diene VPA and its polar metabolites in liver following the administration of 150 mg/kg i.p. of (E,E)-2,3'-diene VPA (containing 3-5% (E,Z)-2,3'-diene VPA) to rats (n=8/time point, S.D. omitted for clarity).
parallel the clearance of the parent compound. Although in some instances, as in the case of the heart tissue, clearance of (E,E)-2,3'-diene VPA was faster than the polar metabolites. The result was the concentrations of the polar metabolites exceeded the parent compound approximately 300 minutes following administration.

3.9.3 Profiles of (E,E)-2,3'-diene VPA Metabolites in Brain

VPA was the major metabolite observed in the remaining whole brain fraction following (E,E)-2,3'-diene VPA administration (figure 50). The concentration of VPA in the brain exceeded the level of the parent (E,E)-2,3'-diene VPA compound within 200 minutes after administration. The unsaturated metabolites detected in the remaining whole brain were 3-ene VPA, (E)-2-ene VPA, and another diene, although all were present in lesser amounts relative to VPA. The polar metabolites observed in the remaining whole brain were the β-oxidation products of VPA, namely 3-keto VPA and 3-OH VPA (appendix 63).

The metabolites of (E,E)-2,3'-diene VPA were also quantitated in the various pooled brain sections. As in the case of the remaining whole brain, the VPA concentration in the substantia nigra, an area of high GABA activity, exceeded that of (E,E)-2,3'-diene VPA after 350 minutes (figure 51). The unsaturated metabolites observed in decreasing concentrations were 3-ene VPA, (E)-2-ene VPA, and a diene. The elimination of the unsaturated metabolites appeared to be similar to that of (E,E)-2,3'-diene VPA. The polar metabolites of (E,E)-2,3'-diene VPA detected in the substantia nigra were the β-oxidation products of VPA and 5-OH VPA (figure 52). The elimination of (E,E)-2,3'-diene VPA from the substantia nigra appeared to be greater than the hydroxyl
Figure 50: Concentration-time plots of (E,E)-2,3'-diene VPA, its unsaturated metabolites, and VPA in whole brain following the administration of 150 mg/kg i.p. of (E,E)-2,3'-diene VPA (containing 3-5% (E,Z)-2,3'-diene VPA) to rats (n=8/time point, S.D. omitted for clarity).
Figure 51: Concentration-time plots of (E,E)-2,3'-diene VPA, its unsaturated metabolites, and VPA in substantia nigra following the administration of 150 mg/kg i.p. of (E,E)-2,3'-diene VPA (containing 3-5% (E,Z)-2,3'-diene VPA) to rats (n=8 pooled/time point).
Figure 52: Concentration-time plots of (E,E)-2,3'-diene VPA and its polar metabolites in substantia nigra following the administration of 150 mg/kg i.p. of (E,E)-2,3'-diene VPA (containing 3-5% (E,Z)-2,3'-diene VPA) to rats (n=8 pooled/time point).
metabolites as the concentration of these polar compounds exceeded that of the parent at approximately 400 minutes.

The metabolites observed in the other regions of the brain following single dose administration of (E,E)-2,3'-diene VPA were similar to those found in the substantia nigra (appendices 64 - 79). As in peripheral tissues, the major metabolite observed in the brain was VPA.

3.10 PLASMA PROTEIN BINDING OF (E,E)-2,3'-DIENE VPA AND (E,Z)-2,3'-DIENE VPA

When the elimination characteristics of VPA, (E)-2-ene VPA, and (E,E)-2,3'-diene VPA were compared in plasma, the clearance of the diene from plasma appeared to be fastest. Similar results were found for the peripheral tissues and brain. It was thought that variations in plasma protein binding between (E,E)-2,3'-diene VPA and VPA or (E)-2-ene VPA might explain these differences in tissue clearance. Knowing the degree of plasma protein binding of the isomers (E,Z)- and (E,E)-2,3'-diene VPA might also explain the presence of only the (E,Z)-isomer in the brain following VPA administration. While the plasma protein binding characteristics of VPA and (E)-2-ene VPA in rats is known, the binding of (E,E)-2,3'-diene VPA has not yet been established. Therefore, binding of (E,Z)- and (E,E)-2,3'-diene VPA was determined from the collected rat plasma samples.

Following the i.p. administration of (E,E)-2,3'-diene VPA containing 3 - 5% of the (E,Z)-isomer, plasma protein binding was
determined by ultracentrifugation from plasma samples collected over a 10 hour period. For the various total concentrations of (E,E)- and (E,Z)-2,3'-diene VPA measured in plasma over time, a corresponding free plasma concentration was determined. From this data, the degree of plasma protein binding of both (E,E)- and (E,Z)-2,3'-diene VPA was calculated (table 13). At an equivalent total plasma concentration of approximately 12 - 14 ug/mL, 96% of (E,E)-2,3'-diene VPA was bound to plasma protein compared to 35% for the (E,Z)-isomer. The binding isotherm for (E,E)-2,3'-diene VPA and (E,Z)-2,3'-diene VPA indicated that the binding of each compound was linear over the concentration ranges studied (figure 53).

3.11 ANTICONVULSANT EVALUATION OF VPA, (E)-2-ENE VPA, (E,E)-2,3'-DIENE VPA, AND (E,Z)-2,3'-DIENE VPA IN RATS

3.11.1 PTZ-Induced Seizure Test

Upon the s.c. administration of 75 mg/kg of PTZ to eight control rats, all animals exhibited clonic seizures within 30 minutes, the considered endpoint. The percentage of rats protected against PTZ-induced clonic seizures by either VPA, (E)-2-ene VPA, (E,E)-2,3'-diene VPA, or (E,Z)-2,3'-diene VPA at various doses are summarized in table 14. The (E,E)-2,3'-diene VPA at 200 mg/kg was found to be significantly less active (p<0.05) as an anticonvulsant agent when compared to VPA. In fact, the dose-response curves (figure 54) depicts (E,E)-2,3'-diene VPA as being less potent and less effective than VPA, (E)-2-ene VPA, or (E,Z)-2,3'-diene VPA. Quantitation of anticonvulsant activity
Table 13: Plasma protein binding of (E,E)-2,3'-diene VPA and (E,Z)-2,3’-diene VPA in rat plasma by ultracentrifugation; n=8 (S.D.).

(E,E)-2,3'-diene VPA

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(E,Z)-2,3'-diene VPA

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<tr>
<td>3.9 (1.0)</td>
<td>3.0 (1.1)</td>
<td>28.7 (4.1)</td>
</tr>
<tr>
<td>1.2 (0.4)</td>
<td>0.8 (0.3)</td>
<td>43.2 (10.4)</td>
</tr>
<tr>
<td>1.2 (0.2)</td>
<td>0.3 (0.1)</td>
<td>75.8 (6.3)</td>
</tr>
</tbody>
</table>
Figure 53: Binding isotherm of (E,E)\(-2,3'\)-diene VPA and (E,Z)\(-2,3'\)-diene VPA in rat plasma as determined by ultracentrifugation (n=8).
Table 14: The percent of rats protected from PTZ-induced seizures after the i.p. administration of 70 mg/kg s.c. of PTZ 30 minutes post drug (n=8/dose).

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>30</th>
<th>75</th>
<th>150</th>
<th>200</th>
<th>300</th>
<th>400</th>
</tr>
</thead>
<tbody>
<tr>
<td>VPA</td>
<td>12.5%</td>
<td>25%</td>
<td>37.5%</td>
<td>87.5%</td>
<td>100%</td>
<td>-</td>
</tr>
<tr>
<td>(E,E)-2,3'-DIENE VPA</td>
<td>-</td>
<td>12.5%</td>
<td>12.5%</td>
<td>25%*</td>
<td>62.5%</td>
<td>75%</td>
</tr>
<tr>
<td>(E,Z)-2,3'-DIENE VPA</td>
<td>-</td>
<td>12.5%</td>
<td>12.5%</td>
<td>50%</td>
<td>100%</td>
<td>-</td>
</tr>
<tr>
<td>(E)-2-ENE VPA</td>
<td>-</td>
<td>0%</td>
<td>25%</td>
<td>62.5%</td>
<td>100%</td>
<td>-</td>
</tr>
</tbody>
</table>

(*) significant compared to VPA (p<0.05)
Figure 54: Dose-response curves for VPA, (E)-2-ene VPA, (E,Z)-2,3'-diene VPA, and (E,E)-2,3'-diene VPA following 150 mg/kg i.p. administration of each compound to rats (n=8/point).
from the dose-response curves demonstrated that, based on the ED$_{50}$ values, (E,E)-2,3'-diene VPA was significantly less potent (p<0.05) when compared to VPA (table 15). The ED$_{50}$ values for (E)-2-ene VPA and (E,Z)-2,3'-diene VPA did not differ significantly from that of VPA.

3.11.2 Observed Adverse Effects of VPA, (E)-2-ene VPA, (E,E)-2,3'-diene VPA, and (E,Z)-2,3'-diene VPA

The i.p. administration of 30 - 150 mg/kg of VPA did not produce any visible adverse effects, whereas doses of 200 and 300 mg/kg did elicit a slight sedative effect that was seen 10 minutes following administration. This was characterized by a quiet hypnotic-like state but without the loss of righting. Within an hour this VPA induced sedation had subsided and the animal appeared to regain movement.

Upon (E)-2-ene VPA administration at doses of 75 - 300 mg/kg, a marked sedative effect was noted which was characterized by a reduction in body movement and the loss of righting within 2 - 3 minutes. At 10 minutes post-injection, the animal appeared to be in a hypnotic-like motionless state which lasted for 2 - 3 hours.

Rats administered 75 - 150 mg/kg of (E,E)-2,3'-diene VPA displayed moderate sedation within 17 minutes, while doses of 200 - 400 mg/kg elicited a moderate to heavy sedative effect within 10 minutes of administration. In addition to the sedative/hypnotic effect, a stiffness or an increase in muscle tone of the hindlegs was noted following doses of 150 and 200 mg/kg. As the dose was increased to 300 and 400 mg/kg, whole body rigidity was observed. The tonicity of the muscles was severe enough to almost mask the seizure activity induced by PTZ. This apparent neurotoxic effect of (E,E)-2,3'-diene VPA was not permanent as the rats
Table 15: The mean effective doses against PTZ-induced seizures and the slopes of the log dose-response plots for each compound tested in rats

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>ED$_{50}$ (mmol/kg)</th>
<th>Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>VPA</td>
<td>1.1(1.0-1.3)</td>
<td>1.2(1.1-1.3)</td>
</tr>
<tr>
<td>(E,Z)-2,3'-dieno VPA</td>
<td>1.2(1.1-1.4)</td>
<td>1.2(0.8-1.8)</td>
</tr>
<tr>
<td>(E)-2-ene VPA</td>
<td>1.3(1.1-1.4)</td>
<td>1.2(1.0-1.3)</td>
</tr>
<tr>
<td>(E,E)-2,3'diene VPA</td>
<td>1.9(1.4-2.5)*</td>
<td>1.7(1.1-2.5)</td>
</tr>
</tbody>
</table>

() - 95% confidence limit
* - significant (p<0.05)
appeared to returned to their normal state of activity within 4 hours of injection.

The administration of (E,Z)-2,3'-diene VPA at doses of 150 - 300 mg/kg resulted in a mild sedative effect similar to that seen for VPA. No loss of righting occurred. Absent was the muscle rigidity and/or paralysis associated with the (E,E)-isomer.
4. DISCUSSION

4.1 CHEMICAL SYNTHESSES

The following compounds were successfully synthesized using methods that maximized both yield and structural selectivity. Previous methods used for the synthesis of VPA metabolites suffered from either low yields or poor isomeric selectivity. Because several grams of each substance under study were needed to perform both pharmacokinetic and anticonvulsant activity studies, modification of reported syntheses was undertaken to circumvent these limitations.

4.4.1 2-n-Propyl-(E)-2-Pentenoic Acid

The synthesis of 2-n-propyl-(E)-2-pentenoic acid by the method of Acheampong (1985) resulted in low yields (<10%) and the presence of a significant quantity of 2-n-propyl-(Z)-pentenoic acid. The synthesis required bromination of VPA in the \( \alpha \)-position followed by esterification of the carboxylic acid moiety. Upon isolation, the compound was debrominated and then rapidly distilled to yield the ethyl ester of 2-n-propyl-2-pentenoic acid. Liberation of the ester by alkaline hydrolysis followed by distillation afforded a mixture that consisted of 80% 2-n-propyl-(E)-2-pentenoic acid and 20% the (Z)-isomer. Because the synthesis of gram quantities of 2-n-propyl-(E)-2-pentenoic acid in an isomerically pure form using this method would prove too laborious and inefficient, an alternate method was investigated.
Syntheses of $\alpha,\beta$-unsaturated esters via the regioselective addition of electrophiles to ester enolates have in the past proved to be successful (Pfeffer and Silbert, 1971; Rathke and Sullivan, 1972; Pfeffer et al., 1973; Kajikawa et al., 1975; Kende and Toder, 1982). Based on this methodology, an attempt was made to synthesize 2-n-propyl-(E)-2-pentenoic acid by first reacting ethyl valerate with propionaldehyde in an aldol condensation reaction. The resulting $\beta$-hydroxysaturated ester was then mesylated with methanesulfonyl chloride. Nucleophilic elimination of the mesylate moiety with potassium hydride was thought to give rise to an $\alpha,\beta$-unsaturated ester; however, upon GC-MS analysis of the crude product, no trace of the unsaturated ester was present. The reaction was repeated several times with each attempt producing similar results.

In a recently published method for the synthesis of 2-n-propyl-(E)-2-pentenoic acid, toluenesulfonyl chloride was used to form a tosyl ester of ethyl $\beta$-hydroxypentanoate (Rettenmeier et al., 1989). Nucleophilic elimination of the tosyl group by 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) afforded the $\alpha,\beta$-unsaturated ester in quantitative yields. The final product consisted of 90% of the (E)-isomer. A reaction time of 72 hours for the formation of the tosyl ester in combination with difficulties in isolating the tosylated product from the solvent made this method less amenable for large scale synthesis required for our research.

Since the rate limiting step for the synthesis of 2-n-propyl-(E)-2-pentenoic acid by the method of Rettenmeier et al. (1989) was the formation of the tosylate, use of a mesylate would significantly reduce the reaction time. The formation of the mesyl ester from the $\beta$-
hydroxyunsaturated ester was completed within an hour in quantitative yields and with little or no side product contamination. Since the reaction was carried out in THF, isolation of the product by flash evaporation followed by distillation proved to be an efficient recovery method. Nucleophilic elimination of the mesylate moiety by refluxing with DBU followed by saponification of the ethyl ester afforded a product that was 95% isomerically pure.

From the NMR spectrum (figure 6), the product was determined to have the (E)-configuration based on the signal for the C3-proton. Depending on the configuration of the double bond at Δ2, the proton at C3 may be susceptible to the electronic effects of the π electron of the carbonyl moiety (figure 55). For 2-n-propyl-(E)-2-pentenoic acid, the proton at C3 is close to the carbonyl group thereby interacting with the π electrons, i.e. deshielded and hence, the signal for the proton occurs downfield at 6.8 ppm. For 2-n-propyl-(Z)-2-pentenoic acid, the C3-proton is away or shielded from the π electrons resulting in an upfield signal at 5.9 ppm. Since the integrated signal at 6.8 ppm was greater than the signal at 5.9 ppm by nearly 20-fold, it was concluded that the majority of synthesized product was 2-n-propyl-(E)-2-pentenoic acid.

Therefore, the synthesis of 2-n-propyl-(E)-2-pentenoic acid by the nucleophilic elimination of the mesyl ester by DBU proved to be both stereoselective and highly efficient for our purposes.

4.1.2 2-((Z)-1’-Propenyl)-(E)-2-Pentenoic Acid

The synthesis of 2-((Z)-1’-propenyl)-(E)-2-pentenoic acid was similar to that of 2-n-propyl-(E)-2-pentenoic acid. The electrophilic addition of propionaldehyde to an α,β-unsaturated ester enolate afforded
Figure 55: Chemical structures of 2-n-propyl-(E)-2-pentenoic acid and 2-n-propyl-(Z)-2-pentenoic acid illustrating the shielding and deshielding effects of the vinylic protons.
a β-hydroxy-β',γ'-unsaturated ester. The configuration of the double bond at Δ² will depend on the configuration of the initial reactant. In other words, to synthesize the (Z)-isomer the starting α,β-alkenyl ester must possess the (E)-configuration prior to geometric inversion (Kende and Toder, 1982). To synthesize ethyl 2-({1'-hydroxypropyl}-(Z)-3-pentenoate, the starting compound was (E)-2-pentenoic acid. Upon alkylation of the starting reactant with propionaldehyde, the double bond of the resulting hydroxy unsaturated ester was shifted to the 3-position by geometric inversion to the (Z)-configuration (scheme 2). Following mesylation of the hydroxyl group and nucleophilic elimination with DBU, saponification of the product gave good yields of 2-( (Z)-1'-propenyl)-(E)-2-pentenoic acid.

The configuration of the product was readily determined by NMR. Similar shielding and deshielding effects (figure 56) akin to 2-n-propyl-(E)-2-pentenoic acid were used to distinguish the positional isomers. The configuration at Δ² can also be rationalized by the behavior of the C₃-proton similar to that of 2-n-propyl-(E)-2-pentenoic acid. The proton at C₃ corresponds to a signal at 6.8 - 6.95 ppm when Δ² possesses the (E)-configuration (figure 56). The absence of a signal at 5.92 ppm (figure 55), corresponding to the shielded C₃-proton in the (Z)-configuration, indicates that the reaction was stereospecific for the (E)-isomer at Δ².

The proton at C₂' also produces a characteristic signal that differentiates between the (E)- and (Z)-configuration at Δ¹'. The signal at 5.8 ppm corresponds to the shielded C₂'-proton when Δ¹' has the (Z)-configuration (figure 56). However, the same proton when Δ¹' possesses the (E)-configuration results in a signal at 6.1 ppm. From the NMR
2-((E)-1'-propenyl)-(E)-2-pentenoic acid and 2-((Z)-1'-propenyl)-(E)-2-pentenoic acid

Figure 56: Chemical structures of 2-((E)-1'-propenyl)-(E)-2-pentenoic acid and 2-((Z)-1'-propenyl)-(E)-2-pentenoic acid illustrating the shielding and deshielding effects of the vinylic protons.
spectrum (figure 8), the intensity of the integrated signal at 5.8 ppm was 95% greater than the signal at 6.1 ppm. Therefore, the majority of the product was comprised of 2-((Z)-1'-propenyl)-(E)-2-pentenoic acid with a small fraction being 2-((E)-1'-propenyl)-(E)-2-pentenoic acid.

Although 2-((Z)-1'-propenyl)-(E)-2-pentenoic acid was previously identified as a contaminant of 2-((E)-1'-propenyl)-(E)-2-pentenoic acid synthesis (Acheampong and Abbott, 1985; Lee et al., 1989), it had not previously been stereoselectively synthesized as a major product. Therefore, the DBU elimination reaction of a mesylate intermediate proved to be an efficient synthesis for the desired product.

4.1.3 2-((E)-1'-Propenyl)-(E)-2-Pentenoic Acid

The synthesis of 2-((E)-1'-propenyl)-(E)-2-pentenoic acid was first described by Acheampong and Abbott (1985). The method was not stereoselective, since 3 to 4 isomeric dienoates were detected upon GC-MS analysis. In addition, the yield was low. Therefore, attempts were made to improve both the yield and the isomeric purity of this product by modifying the reported synthesis.

The method used for the synthesis of 2-((E)-1'-propenyl)-(E)-2-pentenoic acid was similar to that of 2-((Z)-1'-propenyl)-(E)-2-pentenoic acid. Since the configuration of the starting ester dictates the geometry of the final product, to synthesize 2-((E)-1'-propenyl)-(E)-2-pentenoic acid, the reacting ester must be ethyl (Z)-2-pentenoate (scheme 3). Synthesis of this ester required a catalyst, 18-crown-6. In the previous method for the synthesis of 2-((E)-1'-propenyl)-(E)-2-pentenoic acid by Acheampong and Abbott (1985), a catalyst was not employed which probably explains the poor yields achieved.
Mesylation of the hydroxy substituent followed by nucleophilic elimination with potassium hydride afforded mainly the ethyl 2-(((E)-1′-propenyl)-(E)-2-pentenoate. Potassium hydride was initially used for the elimination reaction until the utility of DBU was discovered. When KH was used, the purity of the ester product obtained depended on reaction conditions. With careful control of the reaction temperature and times, together with the use of re-distilled reagents, isomeric purity was achieved. Following saponification of the ester, fractional distillation gave a product that was 95% 2-(((E)-1′-propenyl)-(E)-2-pentenoic acid with a yield of 46%.

The stereochemistry and purity of the product was determined by NMR. The signal at 6.8 ppm corresponded to the deshielded proton at C3 when \( A^2 \) has the (E)-configuration (figure 56). In contrast to the NMR results of 2-(((Z)-1′-propenyl)-(E)-2-pentenoic acid, the vinylic proton signal at 6.1 ppm was approximately 20-fold greater than the signal at 5.8 ppm (figure 10). Therefore, the final product consisted mostly of 2-(((E)-1′-propenyl)-(E)-2-pentenoic acid.

The synthesis of 2-(((E)-1′-propenyl)-(E)-2-pentenoic acid was a significant improvement over the reported method of Acheampong and Abbott (1985). The isomeric purity of the final product was 95 - 97% as determined by both GC-MS and NMR. In addition, the yield was substantially increased thereby allowing the efficient synthesis of the diene for pharmacological study.
4.2 ASSAY DEVELOPMENT FOR VPA AND ITS METABOLITES IN RAT TISSUE

Successful GC-MS assays of VPA metabolites in various biological fluids have been reported for the ethyl (Granneman et al., 1984), TMS (Nau et al., 1981; Tatsuhara et al., 1987; Rettenmeier et al., 1989), or t-BDMS (Acheampong et al., 1984; Abbott et al., 1986) derivatives. Of the three derivatives, the greatest number of VPA metabolites separated within the shortest period of time during a single analysis was the t-BDMS esters. In addition, the relative sensitivity for VPA and unsaturated metabolites using the t-BDMS derivative was 20-fold greater than those observed for the TMS derivative (Abbott et al., 1986).

Two major drawbacks prevented use of the t-BDMS derivative for the analysis of VPA and its metabolites in rat tissue samples obtained from the current study. Analysis of t-BDMS derivatives by selected ion monitoring under EI conditions has a reported limit of detection of 0.1 ug/mL (signal-to-noise <3) (Abbott et al., 1986). In addition, the assay procedure for VPA and VPA metabolites using t-BDMS derivatives required a 1 mL tissue sample. Since individual pooled rat brain sections were to be assayed, the amount of brain tissue obtained would not allow for sample volumes of that size. If less than 1 mL of sample were to be assayed, the concentration of some VPA metabolites would fall below the limit of detection of the assay.

The second problem of the reported assay was the resolution of diene metabolites. As t-BDMS esters, resolution of (E,Z)-2,3'-diene VPA and (E)-2,4-diene VPA was not possible (figure 57). Previous studies that have quantitated VPA metabolites in the liver (Nau and Loscher, 1985) and brain tissue (Loscher and Nau, 1982) have measured only 7
Figure 57: Mass chromatogram at m/z 139 of t-BDMS derivatives of (A) peak 1=(E,Z)-2,3'-diene VPA and peak 2=(E,E)-2,3'-diene VPA in control human serum; (B) peak 3=(Z)-2,4-diene VPA and peak 4=(E)-2,4-diene VPA in control human serum; (C) serum sample of a patient on VPA therapy containing peaks 1 to 4, on an OV-1701 column.
metabolites in the brain and 4 in the liver. The diene detected in the brain was 2,3′-diene VPA with the stereochemistry not reported. The GC-MS assay employed TMS derivatives and while not known for certain, our experience indicated the method was likely not capable of separating the diene isomers. Thus, an alternative assay method having adequate sensitivity to detect trace amounts of VPA metabolites from small sample sizes and the ability to resolve the diene metabolites was required. A negative ion GC-MS assay was therefore investigated for our purposes.

A recent negative ion chemical ionization (NICI) method developed for the detection of VPA and VPA metabolites in human serum samples using a pentafluorobenzyl (PFB) derivative proved to be highly sensitive and selective for the diene metabolites (Kassahun et al., 1989). The limit of detection for VPA based on a 200 μL serum sample was 2 ng/mL (signal-to-noise >3) (Kassahun et al., 1990). Moreover, the sensitivity obtained with PFB derivatives of VPA and its metabolites under NICI conditions by selected ion monitoring was 30 - 50 times greater than that of their corresponding t-BDMS derivatives by EI GC-MS. In addition, separation of the diene metabolites, (E,Z)-2,3′-diene VPA and (E)-2,4-diene VPA, was also possible by NICI GC-MS (figure 58). Therefore, with this method appearing to meet the needs of our study, procedures for the analysis of VPA and VPA metabolites in rat tissue were devised.

In contrast to the simple extraction of acidified serum or urine (Abbott et al., 1986; Kassahun et al., 1989), the isolation of VPA and its metabolites from rat tissue homogenates required specific considerations. Because extracted tissue homogenates contained significantly more endogenous material than from either urine or serum samples, interference with the detection of the VPA metabolites
Figure 58: Mass chromatogram at m/z 139 of PFB derivatives of (A) peak 1=(E,Z)-2,3'-diene VPA and peak 2=(E,E)-2,3'-diene VPA in control human serum; (B) peak 3=(Z)-2,4-diene VPA and peak 4=(E)-2,4-diene VPA in control human serum; (C) serum sample of a patient on VPA therapy containing peaks 1 to 4, on a DB-1 column.
occurred. To remedy this problem a "clean-up" procedure was employed that consisted of a back extraction step for the removal of neutral compounds (figure 59). In addition, a 5 m pre-column was attached to the DB-1 chromatographic column to retain low volatile compounds that would otherwise accumulate on the analytical column. When the resolution for some VPA metabolites began to deteriorate, a 0.5 m portion of the pre-column at the inlet end was removed thereby restoring resolution (figure 60). Approximately 43 extracted and derivatized samples were analyzed before column performance significantly deteriorated and the pre-column modification was required.

The NICI GC-MS method for the analysis of VPA and VPA metabolites was not without its drawbacks. Because of the nature of the sample analyzed and the method used for producing negative ions, the integrity of the source appeared to be compromised over a relatively short period of time. Frequent source cleaning was required every 300 samples, thus making this method somewhat less amenable for routine analysis. However, since no other method achieved the sensitivity and separation capabilities needed for this study, regular maintenance of the source was an accepted inconvenience.

A second disadvantage, unrelated to instrumentation, was the unavailability of specific deuterium labelled VPA metabolites for use as internal standards. Deuterated analogues, similar to those used for VPA, (E)-2-ene VPA, or 3-keto VPA quantitation, were necessary in order to account for the physical or chemical changes characteristic of some individual metabolites during extraction. Failure to account for these particular changes may result in greater variations in the assay. Metabolites that may require the use of these deuterated analogues are
250 uL or 250 mg tissue

100 uL distilled water
50 uL internal standards
55 uL of 1 N HCl

Extracted with 2 x 500 uL of ethyl acetate

Discard aqueous fraction
Extract organic fraction with 400 uL of 1 N NaOH

Discard organic fraction
Add 85 uL of 4 N HCl to aqueous fraction
Extract with 1 mL of ethyl acetate

Dry organic fraction over sodium sulfate
Reduce volume to 100 uL under nitrogen

10 uL of diisopropylethylamine
10 uL of quinol (1 mg/mL in ethyl acetate)
10 uL of PFB (30% in ethyl acetate)
Heat 45 min at 60°C

50 uL of MSTFA
Heat 45 min at 60°C

Inject 1 uL

Figure 59: General extraction and derivatization scheme for the NICI GC-MS analysis of VPA and VPA metabolites in rat plasma and homogenized tissue.
Figure 60: Mass chromatogram at m/z 139 of (E,Z)-2,3'-diene VPA (a), (E)-2,4-diene VPA (b), and (E,E)-2,3'-diene VPA (c) in liver homogenate. Mass chromatogram one was obtained from a deteriorated DB-1 column fitted with a 5 m pre-column while mass chromatogram two was obtained following the removal of a 0.5 m portion of the pre-column.
the diene metabolites, the hydroxy metabolites, and dicarboxylic acid metabolites.

In spite of minor problems, the NICI GC-MS assay provided picogram or better sensitivity. A large number of VPA metabolites could be quantitated within a reasonably short period of time.

4.3 PHARMACOKINETICS AND TISSUE DISTRIBUTION OF VPA IN RATS

The pharmacokinetics of VPA in plasma has been thoroughly characterized in the rat following i.p. and i.v. doses of 15 - 600 mg/kg (Dickinson et al., 1979; Loscher et al., 1988). Distribution studies of VPA in peripheral tissues and brain have also been performed (Aly and Abdel-Latif, 1980; Nau and Loscher, 1982; Hariton et al., 1984). The present study of the pharmacokinetics and tissue distribution of VPA was undertaken to take advantage of our improved metabolite assay, and to provide valid comparison for similar studies of selected metabolites. The distribution and pharmacokinetics of pharmacologically active VPA metabolites were to be determined for several major organs, including various brain sections of rats and compared to VPA.

4.3.1 Profile of VPA in Plasma

Following a single dose of 150 mg/kg i.p. of VPA, peak plasma concentrations of 142.7 ± 51.8 ug/mL were attained within 30 minutes, thus absorption of the drug into the general circulation was not rapid. Similar results or findings have been reported (Hariton et al., 1984; Morton, 1984). The elimination phase of VPA in plasma displayed a
transient increase in the VPA concentration at 240 minutes following drug administration. Similar results for VPA were found in rats following either i.p. or i.v. administration (Dickinson et al., 1979; Lawyer et al., 1980; Ogiso et al., 1986). It appears that extensive enterohepatic recycling of VPA was responsible for the transient increase in plasma VPA concentration during the elimination phase. Bile exteriorized rats did not exhibit this secondary increase in VPA plasma concentration but gave the typical log-linear decline (Dickinson et al., 1979). It can be safely assumed that the observed secondary increase in VPA plasma concentration was a result of hepatobiliary recycling.

The non-linearity of VPA elimination from plasma does not allow for the determination of an apparent elimination rate constant by conventional pharmacokinetic techniques. The use of a complex multi-compartmental model is required but the present data were not sufficient for such rigorous modelling techniques.

Clearance of VPA from plasma was calculated from the AUC0-10h value and expressed as Cl0-10h/F since bioavailability (F) for VPA in rats was not determined. Bioavailability for VPA in man and animals after oral administration is close to 1 (Loiseau et al., 1975; Meinardi et al., 1975; Schobben et al., 1975). In addition, the literature value for AUC0-10h in rats following i.v. administration of 150 mg/kg of VPA was approximately 372 ug·h/mL (Dickinson et al., 1979). Since this value is comparable to the 455 ± 68 ug·h/mL obtained following i.p. administration in this study, F can be assumed to equal one. Clearance from 0 - 10 hours for VPA in plasma was then calculated to be 92 ± 14 mL/h.
Previous studies reporting pharmacokinetic parameters for VPA were determined from time zero to infinity. The current data were obtained between zero and ten hours as the observed enterohepatic recycling of VPA prevented the estimation of a half-life value. In order to compare our results with that of literature values, a half-life value for VPA from previous studies in rats, administered a similar dose, was used to calculate the residual decline of VPA after 10 hours following drug administration. The half-life value of VPA in the rat was approximately 15 minutes (Dickinson et al., 1979; Hariton et al., 1984; Ogiso et al., 1986). An apparent elimination rate constant was then calculated and used for the estimation of AUC and Cl to time infinity. Calculated AUC and Cl values from time zero to infinity following a single dose i.p. administration of 150 mg/kg of VPA were 460 ± 68 ug·h/mL and 5 ± 1 mL/min/kg, respectively. From the clearance value, a volume of distribution (Vd) of 119 ± 18 mL/kg was determined. These pharmacokinetic parameters in rat were comparable to those in the literature. Values for Cl and Vd were 4.17 mL/min/kg (Loscher, 1978) and 143.0 ± 7 mL/kg (Ogiso et al., 1986), respectively.

4.3.2 Profile of VPA in Peripheral Tissues

The analysis of VPA in peripheral tissues following drug administration has previously been limited to rat liver and kidneys and was based on the distribution of a radiolabelled analog of VPA (Aly and Abdel-Latif, 1980). Thus, it was felt that a comprehensive study of the tissue distribution and kinetics of VPA would provide useful information for the comparison of VPA with other metabolites to be studied.
Distribution of VPA into the liver, kidneys, heart, and lungs was found to reach peak drug concentrations within 30 - 45 minutes. The tissue profiles were very similar to that in plasma, including the secondary increase in VPA concentration due to enterohepatic recycling. Thus, the liver, kidneys, heart, and lungs along with plasma can be considered as a homogeneous unit generally termed the central compartment. The $t_{\text{max}}$ values for VPA in the peripheral tissues and in plasma were similar (table 9). After i.p. administration, the distribution of $[^{14}\text{C}]$VPA in rats gave maximum radioactivity in the liver within 30 minutes, which corresponded to peak plasma concentrations (Aly and Abdel-Latif, 1980). Similarly, following oral administration of VPA in the mouse, peak concentrations of VPA in plasma corresponded to peak concentrations in the liver (Nau and Loscher, 1985).

While the distribution and elimination profiles of VPA in the peripheral tissues were very similar, the concentrations were not. The concentration of VPA in liver was the highest, followed by kidneys, heart, and lungs. In fact, the concentration of VPA in liver and kidneys was greater than that in plasma. Thus, tissue/plasma ratios of VPA in liver and kidneys increased from 1.8 and 1.3 at 30 minutes to 4.6 and 1.8 at $t_{10h}$, respectively (figure 61). Liver/plasma ratios in mouse were reported to be in the range of 1.5 - 3.0 and it was suggested that an active transport mechanism may be functional (Nau and Loscher, 1985). Such an assumption is reasonable, since VPA at physiological pH is more than 99% ionized and hence, would not generally attain the high levels observed in liver by simple passive diffusion (Frey and Loscher, 1978). However, selective binding of VPA may also be contributing to the elevated ratios observed in liver tissue. Because the liver and the
Figure 61: Tissue/plasma ratios of VPA, (E)-2-ene VPA, and (E,E)-2,3'-diene VPA in whole brain (WB), liver (LIV), kidneys (KID), heart (HEA), and lungs (LUN) of rats at $t_{\text{max}}$ and $t_{10\text{h}}$ following 150 mg/kg i.p. administration of each compound ($n=8$, error bars=S.D.).
kidneys are the major organs responsible for the elimination of VPA (Bruni and Wilder, 1979; Gugler and von Unruh, 1980; Bailer et al., 1985), it would not be unusual to detect higher concentrations of drug in these tissues. The high concentration of VPA in liver has been implicated in drug-induced liver toxicity (Nau and Loscher, 1985).

There appears to be much less tissue binding of VPA to heart and lungs. Tissue/plasma ratios at $t_{max}$ and $t_{10h}$ were less than one (figure 61). The AUC$_{0-10h}$ values for heart and lungs were 50% that of plasma (table 10). It appears that the presence of VPA in heart and lungs may be of little clinical significance. Metabolism of VPA by these organs appear to be minimal based on their metabolite profiles being similar to that of plasma.

4.3.3 Profile of VPA in Brain

The binding or accumulation of VPA in brain could conceivably produce the "carry-over" effect of anticonvulsant activity that is observed following the discontinuation of VPA. Results of the current study indicate that VPA does not appear to bind or persist relative to plasma in whole brain tissue following single dose administration. The elimination of VPA was found to be rapid over the 10 hour period while the brain/plasma ratios at $t_{max}$ and $t_{10h}$ were 0.3 and 0.001, respectively (figure 61). Similar results were observed in rats following constant rate administration of VPA for up to 14 days via an osmotic pump. Brain/plasma ratios were in the range of 0.03 - 0.2 (Loscher and Nau, 1983). Following an i.p. dose of 200 mg/kg of VPA in mouse, the brain/plasma ratio was 0.2 at 30 minutes ($t_{max}$) and then fell below the limit of detection at $t_{8h}$ (Nau and Loscher, 1982).
The rapid clearance of VPA out of the brain, which was demonstrated to be twice the rate of its entry, is likely associated with an active transport mechanism such as the monocarboxylic acid carrier (Frey and Loscher, 1978; Cornford et al., 1985). Probenecid, a known inhibitor of the transport of monocarboxylic acid across the blood-brain barrier (Spector and Lorenzo, 1973; Pardridge and Oldendorf, 1977), can inhibit the outward movement of VPA from the CNS. Therefore, concentrations of VPA in the brain may not be an appropriate indicator for predicting anticonvulsant activity.

Because the analysis of VPA in whole brain does not provide information on the regional distribution of the drug, various brain sections were assayed independently following VPA administration. The areas of brain assayed were the hippocampus, superior colliculus, inferior colliculus, cerebellum, olfactory bulb, corpus callosum, substantia nigra, medulla, and the putamen. The brain regions chosen for analysis were based on similar studies carried out in the rat where these brain sections were selected for their potential GABA activity (Loscher and Nau, 1983; Hariton et al., 1984; Loscher et al., 1988). Of particular interest was the substantia nigra, as this is a known area of high GABA activity (Iadorola and Gale, 1982).

No regional differences in brain concentrations of VPA were found. The concentration of VPA in the brain sections were similar to that of whole brain (figure 62). The AUC0-10h values for each brain section analyzed did not differ from each other or from whole brain. Tissue/plasma ratios for the various areas of the brain were in the range of 0.3 - 0.4 at t_{max} and 0.01 - 0.03 at t_{10h} (figure 63). Similar ratios were obtained from whole brain analyses. In a previous study
Figure 62: Concentrations of VPA, (E)-2-ene VPA, and (E,E)-2,3'-diene VPA in hippocampus (HIP), superior colliculus (SC), inferior colliculus (IC), cerebellum (CER), olfactory bulbs (OLF), corpus callosum (CC), substantia nigra (SN), medulla (MED), putamen (CP), whole brain (WB), and plasma (PLA) of rats at $t_{\text{max}}$ and $t_{10h}$ following 150 mg/kg i.p. administration of each compound (WB&PLA$n=8$, HIP...CP pooled from 8 rats).
Figure 63: Tissue/plasma ratios of VPA, (E)-2-ene VPA, and (E,E)-2,3'-diene VPA in hippocampus (HIP), superior colliculus (SC), inferior colliculus (IC), cerebellum (CER), olfactory bulbs (OLF), corpus callosum (CC), substantia nigra (SN), medulla (MED), putamen (CP), whole brain (WB), and plasma (PLA) of rats at \( t_{\text{max}} \) and \( t_{10h} \) following 150 mg/kg i.p. administration of each compound (WB&PLA n=8, HIP...CP pooled from 8 rats).
following continuous administration of VPA to rats, comparable results were observed (Loscher and Nau, 1983). Concentrations of VPA in brain regions were found to be uniform and relatively low compared to that in plasma. Studies in humans have shown that patients on VPA therapy had brain/plasma ratios in the range of 0.068 - 0.28 (Vajda et al., 1981). Similarly, the CSF/plasma ratios for VPA in patients were in the range 0.054 - 0.11 (Loscher et al., 1988).

The appearance and decline of VPA from whole brain and brain sections following drug administration to the rat compared favorably with the results of previous studies. The data can now be used with confidence to evaluate results obtained for VPA metabolites.

4.4 PHARMACOKINETICS AND TISSUE DISTRIBUTION OF (E)-2-ENE VPA IN RATS

4.4.1 Profile of (E)-2-ene VPA in Plasma

In humans and animals, (E)-2-ene VPA is a major metabolite (Granneman et al., 1984; Abbott et al., 1986; Kassahun et al., 1990) that has been shown in rodents to possess anticonvulsant activity comparable to VPA (Loscher, 1981; Loscher and Nau, 1985; Abbott and Acheampong, 1988). Upon the discontinuation of VPA administration, (E)-2-ene VPA was still detected in rat brain while VPA was absent from plasma (Loscher and Nau, 1983). These results led researchers to propose that the anticonvulsant activity observed post-VPA administration may be due to the presence in brain of (E)-2-ene VPA. Thus, the pharmacokinetic profile of (E)-2-ene VPA was examined in detail.
The (E)-2-ene VPA reached peak plasma concentrations of 143.0 ± 50.0 ug/mL within 30 minutes following administration. Thereafter the plasma level of (E)-2-ene VPA began to decline but was characterized 240 minutes later by a secondary increase in (E)-2-ene VPA concentration similar to that observed for VPA. Hepatobiliary recycling of (E)-2-ene VPA would be the most likely explanation for this increase in (E)-2-ene VPA in plasma concentration. In a previous study similar effects were observed in rats following i.v. administration of (E)-2-ene VPA (Singh et al., 1990). This secondary increase in (E)-2-ene VPA plasma levels was absent in bile exteriorized rats.

Because an apparent elimination rate constant for (E)-2-ene VPA could not be obtained due to the non-linearity of the data from biliary effects, a literature half-life value of 28 minutes was taken to calculate estimates of AUC and Cl/F from time zero to infinity (Singh et al., 1990). Calculated values for AUC, Cl/F, and Vd were estimated to be 518 ± 40 ug·h/mL, 5 ± 0.4 mL/min/kg, and 213 ± 25 mL/kg respectively. These pharmacokinetic parameters for (E)-2-ene VPA in plasma were comparable to literature values of 3.0 - 6.1 mL/min/kg and 230 - 240 mL/kg for Cl and Vd, respectively following i.v. administration of 20 - 100 mg/kg of (E)-2-ene VPA (O’Connor et al., 1986; Singh et al., 1990).

What was particularly different about (E)-2-ene VPA was the kinetic profile of the terminal elimination phase. It appeared that (E)-2-ene VPA tended to persist longer in plasma compared to VPA. The slower decline of (E)-2-ene VPA from plasma may be due to the high degree of plasma protein binding by this compound. Plasma protein binding of (E)-2-ene VPA in rats is concentration-dependent and varies between 90 - >99% at plasma concentrations ranging from 10 - 120 ug/mL (Semmes and
An increase in free fraction from 0.3% to 6.7% was observed as plasma (E)-2-ene VPA concentration increased from 10 to 60 ug/mL. A similar trend was observed in mice. As the plasma concentration of (E)-2-ene VPA increased from 30 to 55 ug/mL, plasma protein binding was found to decrease from >98% to >93%, respectively (Nau and Loscher, 1985).

A similar rationale can be used to explain the results in the current study of (E)-2-ene VPA pharmacokinetic profiles in rat. Upon (E)-2-ene VPA administration, plasma concentrations reached a $C_{\text{max}}$ of approximately 140 ug/mL at which free drug concentrations can be expected to be > 7%. The initial phase of drug elimination appears to be rapid, but as drug plasma concentration decreases, the degree of protein binding increases until it reaches >99%. At that point with less than 1% of free (E)-2-ene VPA available in plasma, the terminal elimination phase would be prolonged.

Upon oral administration of 50 mg/kg of (E)-2-ene VPA to the mouse, a similar distribution phase as observed here in the rat, was reported but the prolonged terminal elimination phase was absent (Nau and Loscher, 1985). This observed difference between animal species was most likely a result of dissimilarities in plasma protein binding of (E)-2-ene VPA. Although no binding studies for (E)-2-ene VPA in mouse were available, binding of VPA in mouse was reported to be 11% (Loscher, 1978). By extrapolation, one can estimate that plasma protein binding of (E)-2-ene VPA in the mouse would be around 50% or less. Thus, a greater free fraction in mouse translates into more available drug in plasma for distribution or elimination as compared to that in the rat.
The persistence of (E)-2-ene VPA observed in rat plasma is in keeping with the idea of this unsaturated compound being the active metabolite responsible for the "carry-over" effect of VPA. In addition, the slow build-up of (E)-2-ene VPA in plasma following VPA administration could also explain the delay in anticonvulsant activity observed following an initial dose of VPA. One study has attempted to test this hypothesis. In a recent study involving amygdala-kindled rats administered 200 mg/kg of (E)-2-ene VPA i.p., no correlation between plasma concentration and anticonvulsant activity was found (Loscher et al., 1988). Therefore, it appears that the prolonged anticonvulsant effect of (E)-2-ene VPA was not a manifestation of drug persisting in plasma. Perhaps a correlation between drug level and pharmacological effect exists in other tissues such as the brain.

4.4.2 Profile of (E)-2-ene VPA in Peripheral Tissues

No indications were found to suggest that (E)-2-ene VPA persists or binds to peripheral tissues. The tissue/plasma ratios in liver, kidneys, heart, and lungs were found to decrease over the time of $t_{\text{max}}$ to $t_{10\text{h}}$ (figure 61). While the kinetic profiles of (E)-2-ene VPA in liver, kidneys, heart, lungs, and plasma were similar, the concentrations of drug in the tissues were consistently less than that of plasma (figure 24). There was a brief increase in (E)-2-ene VPA concentration in liver at 30 minutes that was most likely due to changes in plasma free drug concentrations. At higher drug concentrations the free fraction will increase thereby allowing more drug available for distribution. As the free fraction of (E)-2-ene VPA decreases in plasma the concentration in liver will also decrease. A comparison of the
liver/plasma ratios at \( t_{\text{max}} \) and \( t_{10h} \) showed a decrease from 1.4 to 0.5, respectively. Similar changes in the tissue/plasma ratios for kidneys, lungs, and heart were also noted.

The results of this study of (E)-2-ene VPA were comparable to previously reported work in which the distribution kinetics of (E)-2-ene VPA were determined in mouse (Nau and Loscher, 1985). The concentration of (E)-2-ene VPA in mouse liver was found to be less than that in plasma and the decline of (E)-2-ene VPA from liver paralleled the decline from plasma. Based on these results it was suggested that perhaps (E)-2-ene VPA could be used as an alternate anticonvulsant agent without the hepatotoxicity reactions associated with VPA (Nau and Loscher, 1985). Our results, from the metabolism of (E)-2-ene VPA that will be discussed later, show that this unsaturated metabolite may not be completely devoid of VPA-induced hepatotoxicity.

4.4.3 Profile of (E)-2-ene VPA in Brain

The uptake of (E)-2-ene VPA into the whole brain was found to reach a maximum concentration of 68.8 ± 19.8 ug/g within 30 minutes. The profile of decline of (E)-2-ene VPA from whole brain was similar to that in plasma and peripheral tissues. Concentrations of (E)-2-ene VPA in brain were lower than plasma with the AUC\(_{0-10h}\) values in whole brain being one-tenth the values obtained for plasma. Brain/plasma ratios for (E)-2-ene VPA in whole brain at \( t_{\text{max}} \) and \( t_{10h} \) were found to decrease from 0.3 to 0.04 respectively and hence, selective binding to brain tissue was not observed. These brain/plasma ratios for (E)-2-ene VPA in rat were comparable to values of 0.02 - 0.09 obtained in the dog 7 hours after i.v. administration of (E)-2-ene VPA (Loscher and Nau, 1983).
The kinetic profiles and distribution of (E)-2-ene VPA were also determined in various regions of the brain. The regions analyzed were the same as those monitored following VPA administration. The kinetic profiles of (E)-2-ene VPA in each brain section were similar as were the AUC\textsubscript{0-10h} values. Peak (E)-2-ene VPA levels in the brain regions were reached within 30 minutes with concentrations in the range of 30.0 - 52.8 ug/g (figure 62). Selective tissue binding was not observed as brain/plasma ratios for (E)-2-ene VPA in the various brain regions at t\textsubscript{max} and t\textsubscript{10h} were in the range of 0.3 - 0.4 and 0.02 - 0.03 respectively, similar to those found in whole brain (figure 63).

Distribution of (E)-2-ene VPA in the various regions of the brain appeared uniform, and none of the brain sections were seen to accumulate this drug. The persistence of (E)-2-ene VPA in brain was attributed to the prolonged and elevated mean plasma concentration of the drug observed at 10 hours. Even though (E)-2-ene VPA, at the lower concentrations, is eliminated more slowly than VPA from brain, the concentration of (E)-2-ene VPA observed at 10 hours was not consistent with effective anticonvulsant activity. Therefore, based on this single dose study in rat, it is highly unlikely that (E)-2-ene VPA is the metabolite responsible for the post-drug effect of VPA.

4.4.4 Evaluating The Differences Between (E)-2-ene VPA and VPA in Rats

Because the plasma protein binding of (E)-2-ene VPA is significantly greater than that of VPA, the kinetic profiles of the compounds in plasma can be expected to be dissimilar. This difference can be seen during the terminal elimination phase, 240 minutes after
drug administration, when the concentration of \((E)-2\text{-ene VPA}\) in plasma was greater than that of VPA (figure 28).

In peripheral tissues most notably in liver, the concentration of \((E)-2\text{-ene VPA}\) was lower than that of VPA following equivalent doses. The liver/plasma ratios for \((E)-2\text{-ene VPA}\) at \(t_{10h}\) were found to be significantly less than VPA - a possible indication that \((E)-2\text{-ene VPA}\) will be less hepatotoxic than VPA.

In brain, the mean concentrations of VPA and \((E)-2\text{-ene VPA}\) at \(t_{\text{max}}\) were similar (table 11). After 10 hours the concentrations of \((E)-2\text{-ene VPA}\) were twice those of VPA throughout the various regions of the brain (figure 62). The prolonged higher levels of \((E)-2\text{-ene VPA}\) in brain were merely a reflection of plasma concentrations and not selective tissue binding. The brain/plasma ratios at 10 hours between VPA and \((E)-2\text{-ene VPA}\) were comparable (figure 63).

4.5 PHARMACOKINETICS AND TISSUE DISTRIBUTION OF \((E,E)-2,3'\text{-DIENE VPA}\) IN RATS

4.5.1 Profile of \((E,E)-2,3'\text{-diene VPA}\) in Plasma

In humans \((E,E)-2,3'\text{-diene VPA}\) was found to be a major metabolite of VPA with serum concentrations between 6.4 - 7.1% that of VPA (Abbott et al., 1986; Kassahun et al., 1990). In rats \(2,3'\text{-diene VPA}\) was reported to be a major metabolite in urine and plasma following VPA administration although the stereochemistry of the diene was not determined (Granneman et al., 1984; Loscher et al., 1988). An isomeric mixture consisting of mainly \((E,E)\)- and \((E,Z)-2,3'\text{-diene VPA}\) in a 2:1
ratio was found to possess anticonvulsant activity in mouse (Abbott and Acheampong, 1988). It was therefore proposed that the unusual pharmacodynamic activity of VPA may be related to the metabolite (E,E)-2,3'-diene VPA. If the metabolism is sufficiently different the diene may also prove to be an alternative anticonvulsant drug to VPA.

Following an i.p. dose of 150 mg/kg of (E,E)-2,3'-diene VPA peak plasma concentrations of 168.2 ± 13.9 ug/mL were attained within 15 minutes. The plasma level of (E,E)-2,3'-diene VPA remained elevated for approximately 60 minutes which was then followed by a rapid decline (figure 26). Unlike VPA or (E)-2-ene VPA, a secondary increase in (E,E)-2,3'-diene VPA plasma concentration did not occur, although there was a flattening of the elimination curve at approximately 240 minutes post-dose. This was attributed to the fact that the enterohepatic recycling of (E,E)-2,3'-diene VPA in rats was not as extensive as VPA or (E)-2-ene VPA (Lee, 1987).

A half-life value for (E,E)-2,3'-diene VPA could not be determined because of the non-linear nature of the data. In order to estimate the pharmacokinetic parameters from time zero to infinity a half-life value of 28 minutes for the diene from a previous study in rats was used (Lee, 1987). The AUC, Cl/F, and Vd values from time zero to infinity were calculated to be 401 ug·h/mL, 6 mL/h/kg, and 378 mL/kg, respectively. These values were comparable to those obtained in the previous study (Lee, 1987). Values of 2.6 - 5.3 mL/h/kg and 172.5 - 279.4 mL/kg were reported for Cl and Vd, respectively.

Plasma data for (E,E)-2,3'-diene VPA should predict the tissue levels of this diene similar to that observed for VPA and (E)-2-ene VPA. Thus, the relatively rapid decline of (E,E)-2,3'-diene VPA compared to
VPA or (E)-2-ene VPA indicated that this diene was also eliminated promptly from tissues.

4.5.2 Profile of (E,E)-2,3′-diene VPA in Peripheral Tissues

Tissue distribution of the active VPA metabolite, (E,E)-2,3′-diene VPA, in man and animals has not previously been determined. Because (E,E)-2,3′-diene VPA has potential application as an anticonvulsant drug, the pharmacokinetic profiles of this agent in the major tissues of the rat were determined.

The kinetic profiles of (E,E)-2,3′-diene VPA in rat liver, kidneys, heart, lungs, and plasma were similar (figure 26). Distribution of (E,E)-2,3′-diene VPA to the tissues from plasma was not rapid as the $t_{\text{max}}$ values ranged from 15 - 60 minutes. The decline phase of (E,E)-2,3′-diene VPA in peripheral tissues appeared to parallel the rapid decline of the diene from plasma. The rate of decline of the terminal elimination phase for (E,E)-2,3′-diene VPA in peripheral tissues was faster than either that of VPA or (E)-2-ene VPA (figures 29 - 32). Because plasma protein binding of (E,E)-2,3′-diene VPA in rat was found to be <90% at plasma concentrations of approximately 60 ug/mL (table 13), the increased availability of free diene would account for its faster decline in plasma and ultimately in peripheral tissues.

A secondary increase in tissue diene concentrations was noted in lungs and to some extent in kidneys (figure 26), even though evidence for enterohepatic recycling in plasma was not very significant, unlike that of VPA or (E)-2-ene VPA. Thus, the brief increase in (E,E)-2,3′-diene VPA concentration in lungs during the terminal elimination phase was unusual and the mechanism is uncertain.
The relatively rapid decline of (E,E)-2,3'-diene VPA from liver, compared to VPA or (E)-2-ene VPA (figure 29), together with the small tissue/plasma ratio calculated at the 10 hour time point following drug administration suggests this diene may be potentially less hepatotoxic than VPA. Further evidence in support of this hypothesis will require an examination of the metabolites of (E,E)-2,3’-diene VPA in rats with the aim of identifying possible reactive products.

4.5.3 Profile of (E,E)-2,3’-diene VPA in Brain

There are presently no studies that describe the kinetics or distribution of (E,E)-2,3’-diene VPA in brain for purposes of establishing whether the diene favors or persists in discrete areas of the brain. Only one report in the literature examined the presence of 2,3’-diene VPA in the substantia nigra of rats following VPA administration (Loscher et al., 1988). Therefore, the kinetic profile of (E,E)-2,3’-diene VPA in brain was investigated with the aim of whether the diene could be associated with the unusual pharmacodynamic effects of VPA. No evidence was obtained to indicate any such involvement.

From the kinetic profile of (E,E)-2,3’-diene VPA in whole brain, elimination appeared to be rapid compared to that of VPA or (E)-2-ene VPA. Based on the AUC0-10h values the amount of (E,E)-2,3’-diene VPA present in whole brain was one-tenth that of plasma. Brain/plasma ratios were generally low (figure 61) and were found to decrease with time. In fact, the ratios obtained for the diene in brain were similar to that of heart (figure 61). No evidence was obtained to indicate that (E,E)-2,3’-diene VPA was bound to whole brain tissue.
Similar kinetic profiles were observed in the various sections of brain that were analyzed (figure 27). Distribution of (E,E)-2,3'-diene VPA throughout the brain appeared uniform as the C_{max} values obtained from the different regions were comparable to the value for whole brain (figure 62). What was different was an increase in the brain/plasma ratios at 10 hours in all regions examined except the putamen with the largest increases observed in the superior colliculus (0.6), inferior colliculus (1.4), and substantia nigra (0.8)(figure 63). Also noted in the brain sections was a transient increase in (E,E)-2,3'-diene VPA tissue concentration 60 minutes following drug administration. Although unexplained, the increase was not likely an analytical artifact since this did not occur in other tissues or plasma assayed.

A similar result was reported for repeated i.p. administration of VPA to rats for 3 days. The concentration of 2,3'-diene VPA, thought to be the (E,E)-isomer, in the substantia nigra was found to increase on the third day of VPA administration (Loscher et al., 1988). Increased electrical activity in the substantia nigra appears to be a common feature of generalized seizures (Gale, 1988). VPA has been shown to inhibit excessive neuronal activity (Waczck et al., 1986) and increase GABA concentration in the nerve terminals within the substantia nigra of rats (Iadarola and Gale, 1981; Loscher and Vetter, 1984).

The present study has shown that following (E,E)-2,3'-diene VPA administration to rats, this compound binds selectively to certain regions of the brain. Tissue-to-plasma ratios in these areas were found to increase over time. Thus, the persistence of (E,E)-2,3'-diene VPA in brain may in part explain the prolonged duration of activity observed following the discontinuation of VPA. However, one must consider that
brain levels of the diene decreased rapidly over the 10 hour period following the dose and concentrations in the brain sections at 10 hours were very low (<0.1 ug/g).

4.5.4 Evaluating Differences in the Tissue Distribution of (E,E)-2,3'-diene VPA to VPA and (E)-2-ene VPA

Following single dose (E,E)-2,3'-diene VPA administration, the decline of drug from plasma during the terminal elimination phase was faster than that of VPA. The central compartment for this diene appears to include tissues as well as plasma and hence, the elimination profiles of (E,E)-2,3'-diene VPA from these tissues parallels that in plasma. Based on tissue/plasma ratios, the binding of (E,E)-2,3'-diene VPA to liver tissues at the 10 hour time point was significantly less than that of VPA (figure 61). Since the persistence or binding of VPA to liver may be a prelude to an idiosyncratic hepatotoxic reaction, the transient nature of this diene in liver could avoid similar complications. Unfortunately, (E,E)-2,3'-diene VPA was seen to decline rapidly from whole brain and hence, the duration of pharmacological activity would be limited. There were some indications of site specific binding of (E,E)-2,3'-diene VPA to the superior colliculus, inferior colliculus, and substantia nigra which could be interpreted as potential areas of drug action. However, the absolute concentration of this diene in these areas appeared too low to exert any pharmacological effect.

Differences in the kinetic profiles between (E,E)-2,3'-diene VPA and (E)-2-ene VPA in plasma were similar to those found between (E,E)-2,3'-diene VPA and VPA. A striking difference is the terminal decline phase of (E,E)-2,3'-diene VPA from plasma being faster than either VPA
or (E)-2-ene VPA. The plasma protein binding properties of these compounds may largely be responsible for these differences.

4.6 TISSUE DISTRIBUTION AND KINETIC PROFILES OF VPA METABOLITES IN RATS

4.6.1 Profiles of VPA Metabolites in Plasma

The metabolism of VPA has been extensively studied (Ferrandes and Eymard, 1977; Acheampong et al., 1983; Turnbull et al., 1983; Granneman et al., 1984; Heinemeyer et al., 1985; Rettenmeier et al., 1987; Rogiers et al., 1988). Several pathways are involved in the metabolism of VPA (figures 3 and 4) although glucuronidation and β-oxidation are considered the main routes (Granneman et al., 1984). A major metabolite present in humans and animals that is not a direct product of either of the two major pathways is 2,3'-diene VPA (Abbott et al., 1986). An earlier study involving the identification of VPA metabolites in patients by GC-MS described the isolation of two diene metabolites postulated to have either the α,β'- or β,γ'-configuration (Acheampong et al., 1983). In a patient who had succumbed to VPA hepatotoxicity, five diunsaturated metabolites of VPA were observed in plasma and urine with two identified as 4,4'-diene VPA and (E)-2,4-diene VPA (Kochen et al., 1984). The remaining three compounds were labelled as dienes/2, /3, and /4. It was not until authentic samples of these unknown compounds were synthesized that the stereochemical and positional isomers were determined to be (E)- and (Z)-2,4-diene VPA and, (E,E)- and (E,Z)-2,3'-diene VPA (Acheampong and Abbott, 1985; Lee et al., 1989).
The metabolites identified following i.p. administration of 150 mg/kg of VPA to rats were similar to those reported by Loscher et al. (1988). In Loscher's investigations the only diene detected in plasma was reported to be a 2,3′-diene VPA with the stereochemistry not determined. In our study by using an improved method for the analysis of VPA metabolites and having synthetic standards available, three diene metabolites were identified. The metabolites detected in plasma were (E,E)-2,3′-diene VPA, (E,Z)-2,3′-diene VPA, and (E)-2,4-diene VPA (figure 21). The (E,Z)-2,3′-diene VPA was present in rat plasma at low concentrations (figure 34). This metabolite had not been previously identified in the rat and information regarding the anticonvulsant activity, toxicity, or pharmacokinetic characteristics of this diene were not known.

Unusually high amounts of 3-ene VPA in plasma comparable to that of (E)-2-ene VPA and (E,E)-2,3′-diene VPA were noted. Rats appear to produce a greater amount of 3-ene VPA from VPA compared to man. The 3-ene VPA is generally a minor metabolite of VPA in humans (Abbott et al., 1986; Kassahun et al., 1990). The origin of 3-ene VPA is presently unknown although it has been suggested that 3-ene VPA is a direct metabolite of (E)-2-ene VPA via an isomerase enzyme (Rettenmeier et al., 1987). The anticonvulsant activity of 3-ene VPA in the mouse was found to be 50% that of (E)-2-ene VPA and hence, the contribution of 3-ene VPA to VPA anticonvulsant activity would probably be insignificant (Loscher and Nau, 1985).
4.6.2 Profile of VPA Metabolites in Peripheral Tissues

The metabolites observed in liver, kidneys, heart, and lungs following VPA administration were very similar to those found in plasma. Of particular interest was the liver since VPA produces an idiosyncratic hepatotoxic reaction characterized by microvesicular steatosis and necrosis (Zimmerman and Ishak, 1982). The unsaturated metabolites (E)-2,4-diene VPA and 4-ene VPA were shown to induce a similar pathology when administered to rats (Kesterson et al., 1984). After VPA, very low concentrations of 4-ene VPA were seen in the liver (figure 36). In spite of (E)-2,4-diene VPA exceeding the concentration of 4-ene VPA in plasma, kidneys, and lungs (figure 34, appendices 10 and 12), no evidence of this diene could be found in the liver.

The inability to detect (E)-2,4-diene VPA from liver may be due to its covalent binding to endogenous liver components. Evidence obtained by Kassahun (1991) following the administration of (E)-2,4-diene VPA to rats suggests that the detoxification mechanism of the liver may be producing a highly reactive electrophilic intermediate potentially capable of irreversibly alkylating key liver enzymes. While not specifically looked for in the current study, the glutathione conjugate of (E)-2,4-diene VPA has been detected in rat bile and the N-acetylcysteine conjugate identified in the urine of patients on VPA. This identification of these conjugates is strong evidence in favor of the formation of a reactive electrophilic species. Thus, the absence of (E)-2,4-diene VPA in liver tissue may be an indication of irreversible tissue binding.
4.6.3 Profile of VPA Metabolites in Brain

Earlier studies had shown that following chronic oral administration of VPA to mice the only metabolite observed in whole brain was (E)-2-ene VPA. Two days following the discontinuation of VPA, (E)-2-ene VPA could still be detected in brain albeit at low levels (Loscher and Nau, 1982). A similar study in dogs in which VPA was administered as an i.v. bolus and in rats where VPA was dosed via an osmotic mini-pump, (E)-2-ene VPA was also found in the brain (Loscher and Nau, 1983). More specifically, in rat brain (E)-2-ene VPA was most prominent in hippocampus, superior and inferior colliculus, substantia nigra, and medulla.

It has been speculated that the substantia nigra may be the site of anticonvulsant action since this area is associated with high GABA activity (Iadarola and Gale, 1982). The accumulation of active VPA metabolites following VPA administration in the substantia nigra would support the above hypothesis. In a recent study in rats where multiple doses of 200 mg/kg of VPA was administered i.p., no accumulation of (E)-2-ene VPA in brain, including the substantia nigra, was observed (Loscher et al., 1988). This was in contrast to an earlier study in rats administered VPA by osmotic mini pumps. The differences in results were probably due to the low plasma levels of VPA achieved, 14 - 20 ug/mL, using the mini pump versus the i.p. administration (Loscher and Nau, 1983).

As detection methods for VPA metabolites improved and plasma concentrations of VPA were increased, a greater number of metabolites was observed in the brain. In rats administered multiple doses of VPA, six metabolites could be detected in the substantia nigra (Loscher et
al., 1988). These metabolites were, in order of decreasing concentration, 5-OH VPA, 3-keto VPA, 4-OH VPA, 3-ene VPA, 2,3'-diene VPA (stereochemistry not known), and (E)-2-ene VPA.

In the current study comparable results were obtained. Because the sensitivity and stereoselectivity of our assay was significantly improved, several more metabolites were observed in brain. These were 4-keto VPA, 3-OH VPA, 2-PGA, and 4-ene VPA. No evidence of VPA metabolites persisting in the substantia nigra over the 10 hour period was observed. The decline of the metabolites from the substantia nigra was similar to that of the parent compound (figures 38 and 39). Quantitation of VPA metabolites in other regions of the brain, such as the hippocampus, superior colliculus, inferior colliculus, cerebellum, olfactory bulbs, corpus callosum, medulla, and putamen, gave results identical to that of the substantia nigra (appendices 17 - 32).

The kinetic profiles of VPA metabolites in brain were similar to those in plasma, although (E)-2,4-diene VPA was absent in brain. Most surprising was the detection in brain of (E,Z)-2,3'-diene VPA, a minor diene metabolite in plasma. Also surprising was the complete absence of (E,E)-2,3'-diene VPA, the major metabolite of VPA in plasma.

In an attempt to explain this unusual finding the following possibilities were considered. The (E,Z)- and (E,E)-isomers of the diene appear to illustrate distinct stereochemical properties. Perhaps (E,Z)-2,3'-diene VPA binds favorably to brain tissue while the (E,E)-isomer has a low affinity or fails even to cross the blood brain barrier. However, upon subsequent administration of the (E,E)-2,3'-diene VPA to rats, this diene was readily detected in brain and could still be detected 10 hours following the dose.
Another possibility for the presence of (E,Z)-2,3'-diene VPA and not the (E,E)-isomer in the substantia nigra could be due to differences in plasma protein binding. The (E,Z)-2,3'-diene VPA was 28.7 - 75.8% bound to plasma proteins compared to 74.2 - 97.1% for the (E,E)-isomer (table 13). The amount of free (E,Z)-2,3'-diene VPA available in plasma for distribution into brain would be greater than that of (E,E)-2,3'-diene VPA. However, if protein binding was the major factor responsible for the absence of (E,E)-2,3'-diene VPA in brain, this phenomenon should also occur in the other tissues assayed. Furthermore, since (E)-2-ene VPA is highly plasma protein bound (Semmes and Shen, 1990) this metabolite should also be absent from brain tissues. This was not the case. Thus, the absence of (E,E)-2,3'-diene VPA in brain cannot be easily rationalized based on plasma protein binding.

A third possibility was that (E,E)-2,3'-diene VPA was isomerizing to the (E,Z)-isomer in brain. The likelihood of this having occurred was small. Following the administration of (E,E)- and (E,Z)-2,3'-diene VPA in a 19:1 ratio respectively to rats, similar ratios of the dienes were observed throughout the brain.

A likely explanation for the absence of (E,E)-2,3'-diene VPA from brain following VPA administration, is the presence of a selective transport mechanism responsible for rapidly removing the diene out of the brain, similar to that proposed for VPA (Cornford et al., 1985). This would account for the rapid removal of (E,E)-2,3'-diene VPA from brain following administration of the diene itself. Upon (E,E)-2,3'-diene VPA administration, the initially high diene plasma concentrations would most likely saturate this transport mechanism thereby allowing for some of the compound to initially remain in brain.
The differences in decline for (E,E)- and (E,Z)-2,3'-diene VPA from whole brain following the simultaneous administration of both compounds in a 19:1 ratio, respectively is supportive of this hypothesis (figure 50). The decline of (E,E)-2,3'-diene VPA from whole brain was clearly faster than that of the (E,Z)-isomer with the decline curves having crossed after 200 minutes following administration. On the other hand, the decline profiles (parallel curves) of both dienes appeared to be similar in the substantia nigra (figure 51) and in all other brain regions (appendices 64 - 71). This could be expected because decline of the dienes from individual brain regions may not represent drug clearing from the brain itself, whereas whole brain analysis better reflects the elimination characteristics of the isomers from the brain. Studies examining the transport of both dienes across the blood-brain barrier bears further investigation.

4.7 TISSUE DISTRIBUTION AND KINETIC PROFILES OF METABOLITES OF (E)-2-ENE VPA IN RATS

4.7.1 Profiles of (E)-2-ene VPA Metabolites in Plasma

The VPA metabolite, (E)-2-ene VPA is currently undergoing clinical trials in Europe with the aim of marketing this compound as an alternate anticonvulsant drug to VPA. While hepatotoxicity (Dreifuss et al., 1989) and embryotoxicity (Bertollini et al., 1985; Kaneko et al., 1988; Carter and Stewart, 1989) are negative keynote features of VPA, (E)-2-ene VPA was found to have a significantly decreased teratogenic effect in mice (Loscher et al., 1984; Loscher and Nau, 1985). Furthermore, following
(E)-2-ene VPA administration to mice, the concentration of this compound in liver was significantly lower than that of VPA when given at comparable doses (Nau and Loscher, 1985). However, since 4-ene VPA and (E)-2,4-diene VPA are the metabolites implicated in VPA hepatotoxicity (Kassahun et al., 1989; Porubek et al., 1989) the degree of biotransformation of (E)-2-ene VPA to these toxic species may be a more appropriate indicator of potential toxicity.

For (E)-2-ene VPA to be considered less hepatotoxic than VPA, virtually little or no 4-ene VPA or (E)-2,4-diene VPA should arise from (E)-2-ene VPA metabolism. But in fact following (E)-2-ene VPA administration to rats both 4-ene VPA and (E)-2,4-diene VPA were detected in plasma (figure 40) at concentrations similar to that following VPA administration (figure 34). Thus if (E)-2,4-diene VPA is the key factor in VPA hepatotoxicity, one would predict similar problems for the (E)-2-ene VPA.

A summary of the metabolism for (E)-2-ene VPA determined from the metabolites detected in plasma following drug administration is illustrated in figure 64. The main pathways for the metabolism of (E)-2-ene VPA, based on the presence of each metabolite, appear to be β-oxidation, reduction, dehydrogenation, and isomerization. These pathways account for most of the metabolites detected in plasma following (E)-2-ene VPA administration. Since the β-oxidation pathway is a known major pathway for VPA metabolism (Abbott et al., 1986), and (E)-2-ene VPA is the initial metabolite of this pathway, it was not surprising to see that (E)-2-ene VPA was being metabolized by this route.

The reduction of (E)-2-ene VPA and other unsaturated fatty acids mediated by reductases in rats has previously been reported (Granneman
Figure 64: Proposed metabolic scheme for (E)-2-ene VPA in rats (dotted line represents a possible pathway).
et al., 1986). The formation of 3-ene VPA and (E,E)-2,3'-diene VPA following (E)-2-ene VPA is not clear. Two possible routes of metabolism have been proposed. The dehydrogenation of (E)-2-ene VPA to (E,E)-2,3'-diene VPA followed by reduction to 3-ene VPA (Abbott et al., 1986) or the isomerization of (E)-2-ene VPA to 3-ene VPA followed by β-oxidation to (E,E)-2,3'-diene VPA (Rettenmeier et al., 1987). Both routes appear to be possible as 3-ene VPA and (E,E)-2,3'-diene VPA were present in similar amounts in plasma, following (E)-2-ene VPA administration. In addition, the administration of (E,E)-2,3'-diene also resulted in both 3-ene VPA and (E)-2-ene VPA.

Polar metabolites such as 4-OH VPA, 5-OH VPA, and 2-PGA, were likely secondary metabolites of (E)-2-ene VPA derived directly from VPA. In other words the VPA produced via the reduction of (E)-2-ene VPA underwent a second phase of metabolism to yield products normally observed following VPA biotransformation. Other products from VPA metabolism not detected following (E)-2-ene VPA administration were most likely below the limits of the assay.

4.7.2 Profiles of (E)-2-ene VPA Metabolites in Peripheral Tissues

VPA was the major metabolite observed in liver following (E)-2-ene VPA administration (figure 42). The concentration of VPA in liver was above 10 μg/mL over the entire 10 hour period, 10-fold greater than that in plasma (figure 40). The 4-ene VPA concentration in liver following (E)-2-ene VPA administration was approximately 1000 times greater than that observed in liver following VPA administration (figure 36). This suggests that 4-ene VPA is formed by a different route rather than via microsomal oxidation of VPA. There is little reason to think 4-ene VPA
should be higher in liver exposed to lesser amounts of VPA unless (E)-2-ene VPA was saturating β-oxidation, thereby shunting VPA into the 4-ene VPA pathway. Elevated 4-ene VPA liver concentrations following (E)-2-ene VPA administration may have toxicological implications.

No (E)-2,4-diene VPA was detected in liver following (E)-2-ene VPA administration similar to that observed upon VPA administration. As previously discussed, this does not infer that (E)-2,4-diene VPA was not present in liver as (E)-2,4-diene VPA is a known metabolite of 4-ene VPA (Rettenmeier et al., 1985) and appears here to be a direct microsomal metabolite of (E)-2-ene VPA. The diene metabolite may in fact have been irreversibly conjugated to liver components or reacted with glutathione and thus not amenable to detection.

The detection of unusually high concentrations of 4-ene VPA in liver and the relatively high plasma levels of both 4-ene VPA and (E)-2,4-diene VPA following (E)-2-ene VPA administration suggests that (E)-2-ene VPA may not be entirely free of hepatotoxicity. On the contrary, (E)-2-ene VPA may pose a potentially greater hazard than that of VPA.

4.7.3 Profiles of (E)-2-ene VPA Metabolites in Brain

The metabolites detected in whole brain and the various brain regions were similar to those observed in plasma. The kinetic profiles of these metabolites appear to be similar to the parent compound indicating that the concentration of the metabolites was dependent on (E)-2-ene VPA concentration. There was no evidence indicating the persistence of metabolite(s) in brain following (E)-2-ene VPA administration as the concentration of each metabolite at 10 hours was less than that of the parent compound.
VPA was also detected as a metabolite of (E)-2-ene VPA in the brain at levels similar to that of 3-ene VPA. Perhaps the anticonvulsant activity associated with (E)-2-ene VPA (Abbott and Acheampong, 1988) was due to VPA. However, the concentration of VPA in brain tissue was less than 1 ug/mL. This appeared to be too low to be clinically significant since amygdala-kindled rats with VPA brain concentrations of 1 ug/mL did not display any anticonvulsant activity (Loscher et al., 1988).

4.8. TISSUE DISTRIBUTION AND KINETIC PROFILES OF METABOLITES OF (E,E)-2,3'-DIENE VPA IN RATS

4.8.1 Profiles of (E,E)-2,3'-diene VPA Metabolites in Plasma

The reduction of (E,E)-2,3'-diene VPA appears to be the main route of metabolism as the major metabolites observed in plasma were (E)-2-ene VPA and 3-ene VPA (figure 65). Other metabolites identified in similar amounts were VPA and 3-keto VPA, products of secondary metabolism of (E)-2-ene VPA. The mean plasma concentration of VPA observed following (E,E)-2,3'-diene VPA administration was less than that observed upon (E)-2-ene VPA administration. VPA was further metabolized to yield the polar compounds, 4-OH VPA and 5-OH VPA.

Other biotransformation products of VPA, such as 4-ene VPA and (E)-2,4-diene VPA, were not detected possibly because the concentrations of the logical precursors, VPA and (E)-2-ene VPA in plasma were initially low. This would suggest a lower potential for liver toxicity with this diene over either VPA or (E)-2-ene VPA.
Figure 65: Proposed metabolic scheme for (E,E)-2,3'-diene VPA in rats (dotted line represents possible metabolites or pathways).
A metabolite observed in plasma following (E,E)-2,3′-diene VPA administration that was not previously detected was a diene of unknown configuration. GC-MS analysis indicated that this compound chromatographed prior to the two other known dienes. The retention time for this unknown diene was approximately 8.01 minutes. Although the unknown diene could be any number of possible dienoic isomers, based on the reported GC characteristics of diene isomers of VPA, this compound is likely to be (Z,Z)-3,3′-diene VPA (Acheampong and Abbott, 1985). Until a synthesized standard for (Z,Z)-3,3′-diene VPA is available, positive identification by GC-MS and NMR was not possible.

Identification of possible metabolites predicted for (E,E)-2,3′-diene VPA metabolism, as illustrated in figure 65 was attempted, but no GC-MS peaks were detected that corresponded to the appropriate base ions. Since synthetic standards for most of these potential metabolites were not available, positive identification would not have been possible. Only the 3-OH-3′-ene VPA metabolite was synthesized but, upon analysis of plasma samples, no peak corresponding to this metabolite was observed.

4.8.2 Profiles of (E,E)-2,3′-diene VPA Metabolites in Peripheral Tissues

The elimination of the unsaturated and polar metabolites from liver following (E,E)-2,3′-diene VPA administration was slower than that of the parent compound. The concentration of diene fell to a level less than that of its metabolites within 180 minutes. Although the main metabolites detected in plasma (figures 46 and 47) were also found in liver (figures 48 and 49), the mean VPA concentration in liver was 100 times that in plasma. However, this was not unusual as similar findings
for VPA in liver were observed following VPA or (E)-2-ene VPA administration (figure 42). What was interesting was the absence of 4-ene VPA and presumably (E)-2,4-diene VPA in liver. (E)-2,4-diene VPA or 4-ene VPA are not direct by-products of (E,E)-2,3'-diene VPA biotransformation like that of VPA (figure 3) or (E)-2-ene VPA (figure 64).

The metabolites observed in kidneys, heart, and lungs following (E,E)-2,3'-diene VPA administration were similar to the liver although the amount of VPA present in these tissues was less than that of liver.

In summary, the metabolites of (E,E)-2,3'-diene VPA observed in peripheral tissues did not differ from those observed in plasma. The majority of the metabolites detected appeared to be similar to those observed following VPA administration. Of particular interest was the absence of 4-ene VPA and (E)-2,4-diene VPA in liver following (E,E)-2,3'-diene VPA administration, suggesting that this diene may be free of the hepatotoxic effects associated with VPA.

4.8.3 Profiles of (E,E)-2,3'-diene VPA Metabolites in Brain

The metabolites detected in whole brain and in the various brain regions following (E,E)-2,3'-diene VPA administration were similar to those observed in plasma (figures 46 and 47). Slightly higher concentrations of VPA were observed in the substantia nigra (figure 51) and other brain regions (appendices 64 - 71) compared to that of plasma. The higher concentrations of VPA found in brain relative to plasma suggests that reduction of (E,E)-2,3'-diene VPA was occurring in brain, but similar results were not observed following (E)-2-ene VPA
administration. Another possibility was that VPA was binding to brain tissue although this was not observed following VPA administration.

VPA, 5-OH VPA, and 3-keto VPA concentrations in the substantia nigra were found to increase over the 10 hour period (figures 51 and 52). Similar results were observed in the other brain regions analyzed. In spite of the persistence of 5-OH VPA and 3-keto VPA in brain, these compounds are not considered to be potent anticonvulsants (Loscher, 1981).

The question arises whether VPA and/or (E)-2-ene VPA, major metabolites in brain, could have contributed to the anticonvulsant activity of (E,E)-2,3'-diene VPA observed in mice (Abbott and Acheampong, 1985). The concentrations of these metabolites in the various brain regions were all below 1 ug/mL, and any significant clinical effects arising from these products would be highly unlikely. No anticonvulsant activity was observed in rats when VPA or (E)-2-ene VPA brain concentrations were below 1 ug/mL (Loscher et al., 1988).

4.9 ANTICONVULSANT EVALUATION OF VPA, (E)-2-ENE VPA, (E,E)-2,3'-DIENE VPA, AND (E,Z)-2,3'-DIENE VPA IN RATS

The anticonvulsant activities of VPA and (E)-2-ene VPA have been evaluated in mouse. The potency for (E)-2-ene VPA was found to be 50 - 100% that of VPA against pentylenetetrazole (PTZ)-induced seizures (Loscher, 1981; Loscher et al., 1984; Loscher and Nau, 1985; Abbott and Acheampong, 1988).
In the current study the anticonvulsant activities of VPA, (E)-2-ene VPA, and (E,E)-2,3′-diene VPA in rats were evaluated using the PTZ-induced seizure test. However, unlike previous studies in which PTZ was injected 15 minutes following the administration of the anticonvulsant compound, in our study the convulsant was administered at the time when brain concentrations of the anticonvulsant were at their highest.

The anticonvulsant potencies of VPA and (E)-2-ene VPA in rats were found to be comparable as no significant differences in their ED50 values of 1.1 and 1.3 mmol/kg, respectively were observed (table 15). Similar results were noted from the dose-response curves for VPA and (E)-2-ene VPA (figure 54). These findings were in agreement with Loscher’s observation of (E)-2-ene VPA activity in mice.

The dose-response curve for (E,E)-2,3′-diene VPA shows that potency and intrinsic activity were less than that of VPA and (E)-2-ene VPA (figure 54). An ED50 value of 1.9 mmol/kg was obtained for (E,E)-2,3′-diene VPA which was significantly less than that of VPA or (E)-2-ene VPA (table 15). The results for (E,E)-2,3′-diene VPA were not in keeping with previous findings of Abbott and Acheampong (1988) where the potency of the diene in mice was reported to be comparable to that of (E)-2-ene VPA and 50% that of VPA. The contrasting results might be due to several reasons. Firstly, different species were used to evaluate the anticonvulsant activity of these compounds. Secondly, the administration of the convulsant 15 minutes after the administration of 2,3′-diene VPA in mice may not have corresponded to peak brain levels of the diene. Finally, the anticonvulsant testing of the diene by Abbott and Acheampong (1988) used a solution containing a mixture of (E,E)- and (E,Z)-2,3′-diene VPA in a 2.5:1 ratio. In the current study a solution
containing 97% (E,E)-2,3'-diene VPA was employed. Perhaps the activity observed from the mixture of (E,E)- and (E,Z)-2,3'-diene VPA in the mouse was mainly attributed to the (E,Z)-isomer. Evidence obtained from this study supports this claim. Following the administration of VPA to rats, the only diene detected in brain was (E,Z)-2,3'-diene VPA (figure 38). Therefore, based on these findings the (E,Z)-2,3'-diene VPA was synthesized and evaluated for its anticonvulsant activity. The purity of the sample tested was found to be 95% the (E,Z)-isomer.

Using identical testing procedures, the anticonvulsant activity of (E,Z)-2,3'-diene VPA was found to be significantly more potent against PTZ-induced seizures than (E,E)-2,3'-diene VPA (figure 54). An ED$_{50}$ value of 1.2 mmol/kg for the (E,Z)-2,3'-diene VPA was comparable to both VPA and (E)-2-ene VPA, and significantly less than that of (E,E)-2,3'-diene VPA. It appears then that the reported anticonvulsant activity in mice for a mixture containing the two diene isomers may have largely been due to (E,Z)-2,3'-diene VPA.

Following the administration of (E)-2-ene VPA to rats, a marked sedative effect was noted. Similar CNS effects were also observed in mice (Loscher et al., 1984; Abbott and Acheampong, 1988). Since the (E)-2-ene VPA is currently being investigated as a possible novel anticonvulsant agent for its apparent lack of hepatotoxicity and embryotoxicity in mice, a second major foreseeable drawback with this compound is the undesirable CNS effects. As stated previously, the presence of significant quantities of VPA and 4-ene VPA in liver following (E)-2-ene VPA administration brings to question the apparent non-hepatotoxic nature of this compound.
The use of (E,E)-2,3'-diene VPA as a substitute anticonvulsant agent to VPA has also been suggested (Abbott and Acheampong, 1988). However, the current results indicate that (E,E)-2,3'-diene VPA is not as potent as VPA and the diene may produce some serious side effects. Following (E,E)-2,3'-diene VPA administration at doses of 150 - 400 mg/kg to rats, rigidity of the skeletal muscles occurred within 10 minutes. This toxicity was characterized by the hyperextension of the hindlimbs followed by rigidity of the back muscles. The toxic effects were transient as the animal appeared to recover completely within 4 hours. The basis for this toxicity is not understood although it does appear to resemble strychnine poisoning (Franz, 1980). There appears to be no relationship between the two toxicities. Strychnine acts at the spinal cord level as a competitive antagonist to the inhibitory neurotransmitter glycine (Curtis et al., 1967), while the pharmacological effect of VPA is thought to be associated with the central inhibitory neurotransmitter GABA (Godin et al., 1969; MacDonald and Bergey, 1979).

The (E,Z)-2,3'-diene VPA could prove to be a better alternative to VPA than either (E,E)-2,3'-diene VPA or (E)-2-ene VPA. The (E,Z)-2,3'-diene VPA offers several advantages. No muscle toxicity was observed following the administration of (E,Z)-2,3'-diene VPA at doses of 75 - 300 mg/kg. Nor was there a marked sedative effect with the (E,Z)-2,3'-diene VPA. Anticonvulsant activity of the diene was comparable to that of VPA and (E)-2-ene VPA against PTZ-induced seizures. Finally, the (E,Z)-2,3'-diene VPA may not be hepatotoxic as no 4-ene VPA was detected in plasma or liver following diene administration. If the metabolites
observed for (E,Z)-2,3'-diene VPA are similar to those of the (E,E)-
isomer, the potential for hepatotoxicity should be minimized.

Future studies should determine the pharmacokinetics, tissue
distribution, and metabolism of (E,Z)-2,3'-diene VPA in rats and compare
the data to the current study. Favorable results may eventually lead to
the clinical use of (E,Z)-2,3'-diene VPA as an effective anticonvulsant.
5. SUMMARY AND CONCLUSIONS

The synthesis of three unsaturated metabolites of VPA, (E)-2-ene VPA, (E,E)-2,3′-diene VPA, and (E,Z)-2,3′-diene VPA, was successfully completed. The procedure required addition of an electrophile to an ester enolate followed by nucleophilic elimination of a mesyl ester. The alkylating step was found to be regiospecific and the elimination of the mesylate was highly stereoselective. Nucleophilic elimination of the mesylate was initially accomplished using potassium hydride although careful control of reaction time and temperature was required to prevent side reactions from occurring. For this reason, potassium hydride was replaced with DBU, a reagent much easier to handle and which gave comparable if not better yields. Thus, the general synthetic procedure was much improved over previous methods. Increased yields and greater stereoselectivity were achieved. Isomeric purities of 95 - 97%, as determined by NMR and GC-MS, were attained for the synthesized compounds. Several grams of each compound were synthesized.

A negative ion chemical ionization (NICI) GC-MS analytical procedure was developed for the separation and quantitation of VPA and 13 of its metabolites extracted from rat plasma and homogenized tissue samples. The analysis of extracts of tissue homogenates presented a particular problem because column integrity was rapidly compromised. A back-extraction step together with the use of a pre-column extended the viability of the column. The NICI assay was much improved, giving resolution of the diene metabolites and sensitivity that exceeded
previous assays. VPA metabolites could be quantitated reproducibly in the picogram range.

After a single i.p. dose, the kinetic profile of VPA was characterized in rat plasma, liver, kidneys, heart, lungs, and in nine regions of the brain. Enterohepatic recycling of VPA was observed causing a transient increase in VPA plasma concentrations 240 minutes following the dose. Comparable kinetic profiles of VPA were observed between plasma and the peripheral tissues. Mean concentrations of VPA in liver throughout the 10 hour time period exceeded that in plasma with a tissue/plasma ratio of 4.6, determined at 10 hours following the dose. This may have significance to the potential of VPA to be hepatotoxic. Selective binding of VPA to kidney tissue was also observed although this not did not occur to the same extent as found in liver. No evidence was found for selective binding of VPA in the brain regions examined nor did the drug persist in whole brain.

Similar tissue profiles were determined for (E)-2-ene VPA following single dose i.p. administration to rats. Enterohepatic recycling of (E)-2-ene VPA in plasma, similar to that of VPA, was observed producing a brief increase in drug concentration 240 minutes following administration. The (E)-2-ene VPA appeared to persist in plasma for a longer period of time compared to that of VPA. This was attributed to differences in plasma protein binding as (E)-2-ene VPA is highly protein bound compared to VPA. The persistence of (E)-2-ene VPA in peripheral tissues and brain was also observed. This was a reflection of (E)-2-ene VPA concentrations in plasma since tissue/plasma ratios did not at anytime exceed unity. The affinity of (E)-2-ene VPA for the liver was less evident than for VPA but hepatotoxic implications must also
take into account the metabolites that are formed. Distribution of (E)-2-ene VPA in the brain sections analyzed was found to be uniform.

The kinetic profiles of (E,E)-2,3'-diene VPA in plasma, peripheral tissues, and various brain regions of rats were determined for the first time. There was less evidence for enterohepatic cycling of the diene and clearance from plasma, peripheral tissues, and brain was more rapid than VPA and (E)-2-ene VPA. At very low concentrations (<0.02 ug/g), the diene was selectively bound to brain regions with the superior colliculus, inferior colliculus, and substantia nigra showing ratios to plasma of 0.6, 1.4, and 0.8 respectively. There appears to be little significance of this property of the diene to the prolonged pharmacological activity observed with VPA.

Metabolite profiles were determined and compared in plasma, peripheral tissues, and nine regions of the brain for VPA, (E)-2-ene VPA, and (E,E)-2,3'-diene VPA. Two major objectives of this study were to determine if pharmacologically active metabolites in rats selectively bind and localize in brain, and the degree of formation of potentially hepatotoxic metabolites.

Based on the kinetic profiles of VPA metabolites in brain, it was concluded that metabolites were not being retained in brain. The only diene detected in brain following VPA administration was (E,Z)-2,3'-diene VPA, a minor plasma metabolite. The absence of (E,E)-2,3'-diene VPA from brain suggests an active transport mechanism is involved whereby the diene is stereoselectively being cleared from brain at a rate greater than its (E,Z)-isomer. The suspected hepatotoxic metabolite of VPA, (E)-2,4-diene VPA, was not detected in liver. The absence of (E)-2,4-diene VPA was attributed to the covalent binding of this diene
to liver tissue. The metabolite 4-ene VPA, a precursor to (E)-2,4-diene VPA, was present in liver.

The metabolites observed following single dose administration of (E)-2-ene VPA in plasma, peripheral tissues, and brain were similar to those found after a single dose of VPA. The major pathways of (E)-2-ene VPA metabolism in rats were β-oxidation, reduction, and isomerization. The main metabolites detected were VPA, 3-keto VPA, and 3-ene VPA. Although (E)-2,4-diene VPA was present in plasma, it was not detected in liver. The concentration of 4-ene VPA was higher in liver after (E)-2-ene VPA dosing than after VPA. Based on the known hepatotoxicity of 4-ene VPA and (E)-2,4-diene VPA, the (E)-2-ene VPA may not be free of hepatotoxicity.

Upon (E,E)-2,3'-diene VPA administration, the main route of metabolism was via reduction to VPA, (E)-2-ene VPA, and 3-ene VPA. Since VPA was a major metabolite of (E,E)-2,3'-diene VPA in brain, the possibility of VPA contributing to the activity of this diene was considered. Concentrations of VPA throughout the various brain sections were less than 1 µg/mL over the entire 10 hour period, and activity arising from such low levels would be highly unlikely. Two metabolites not detected in plasma, peripheral tissues, or brain were 4-ene VPA and (E)-2,4-diene VPA. This suggests that the diene may have a lower potential for liver toxicity compared to that of VPA or (E)-2-ene VPA.

The anticonvulsant activity of VPA, (E)-2-ene VPA, and (E,E)-2,3'-diene were compared in rats using the standardized PTZ-induced seizure test. The ED50 values obtained from the dose-response curves were used to compare the relative potencies. VPA and (E)-2-ene VPA had comparable ED50 values and were significantly more potent than (E,E)-2,3'-diene
VPA. The presence of (E,Z)-2,3'-diene VPA in brain following VPA administration prompted the testing of the (E,Z)-isomer. The potency of (E,Z)-2,3'-diene VPA was found to be equivalent to that of VPA and (E)-2-ene VPA.

Sedation was a common observation for all compounds tested with (E)-2-ene VPA eliciting the most severe response. An unusual pharmacological effect was observed for (E,E)-2,3'-diene VPA which appeared to resemble strychnine toxicity. Although the effect was transient, this response in man would severely limit the usefulness of this compound. The (E,Z)-2,3'-diene VPA was free of this muscular effect once again pointing out that there is considerable stereoselectivity in the pharmacological and pharmacokinetic properties of these 2 diene isomers.

Although premature, the (E,Z)-2,3'-diene VPA has potential for use as an anticonvulsant agent. Future investigation should include single dose pharmacokinetic, distribution, and metabolic studies in rats, similar to that of VPA, (E)-2-ene VPA, and (E,E)-2,3'-diene VPA, in order to establish whether this diene is retained in brain or if potential hepatotoxic metabolites are formed. If the results are favorable, perhaps multiple dose studies might be considered to establish whether this diene accumulates in tissues, particularly the brain.
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7. APPENDICES
Appendix 1: VPA, (E)-2-ene VPA, and (E,E)-2,3'-diene VPA concentration-time curves in hippocampus following 150 mg/kg i.p. administration of each compound to rats (each data point=8 pooled samples)
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Appendix 58: Concentration-time plots of (E,E)-2,3'-diene VPA, its unsaturated metabolites, and VPA in heart following the administration of 150 mg/kg i.p. of (E,E)-2,3'-diene VPA to rats (n=8/time point, S.D. omitted for clarity).
Appendix 59: Concentration-time plots of (E,E)-2,3'-diene VPA, its unsaturated metabolites, and VPA in lungs following the administration of 150 mg/kg i.p. of (E,E)-2,3'-diene VPA to rats (n=8/time point, S.D. omitted for clarity).
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