NEGATIVE ION CHEMICAL IONIZATION

GCMS ANALYSIS OF VALPROIC ACID AND

ITS METABOLITES


by


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## ABSTRACT

Valproic acid (VPA) is a major anticonvulsant drug widely used in the treatment of absence seizures. VPA is extensively metabolized in humans. Several VPA metabolites possess anticonvulsant activity and other metabolites are implicated in rare but fatal cases of hepatotoxicity. A highly sensitive and more specific analytical method was required to analyze the large number of VPA metabolites, some of which are present at trace levels. The objective of this study was to develop such a method and to make a preliminary application of the method to the determination of trace VPA levels and to search for new VPA metabolites. The suitability of analyzing halogenated derivatives of VPA and its metabolites by negative ion chemical ionization (NICI) GCMS was evaluated for the desired sensitivity and specificity. An assay was thus developed for VPA in serum and saliva based on NICI-GCMS of the pentafluorobenzyl (PFB) derivative. The NICI spectrum of the PFB ester of VPA was dominated by a single fragment ion, the m/z 143 ([M-181]$^-$) ion. When the m/z 143 ion was monitored the lower limit of detection was 2 ng/mL of VPA in serum or saliva. Using [$^2$H$_6$]-VPA as the internal standard, the intra- and inter-assay variations were less than 10 % at serum VPA concentrations of 10 to 800 ng/mL. Linearity was observed over the concentration range of 10 ng/mL to 25 µg/mL.

The NICI assay was employed to quantitate VPA in serum (total and free) and saliva in five healthy volunteers who took part in a drug interaction study between VPA and carbamazepine (CBZ). A total of 63 paired saliva and serum samples were analyzed by NICI-GCMS; 33 before

the administration of CBZ and 30 after CBZ. The % decrease in the average VPA concentration after CBZ was 27.91 ± 3.48, 36.85 ± 13.64, and 48.13 ± 7.70, for serum total, serum free and saliva VPA, respectively. There was a significant reduction ($p < 0.025$) in the average VPA concentration in all three biological fluids. The average saliva to serum free VPA ratio was 18.92% ± 6.25 before CBZ and 16.37% ± 2.82 following CBZ. The average saliva to serum total VPA ratio was 2.43% ± 0.86 before CBZ and 1.67% ± 0.50 following CBZ, indicating that the saliva to serum total VPA ratio was concentration dependent. A strong correlation was found between saliva and both serum free ($r$ = 0.9035 ± 0.0784) and serum total VPA (0.9058 ± 0.0450) (after CBZ). The free fraction of VPA did not increase after CBZ administration suggesting that the decrease in VPA concentration after CBZ was not related to changes in the free fraction of VPA.

PFB derivative formation of VPA metabolites was facile and resulted in uniform derivatization of all metabolites studied. In the NICI mass spectra most of the ion current was carried by the $[M-181]^-$ fragment ion, the only exception being that of 3-keto VPA. The base peak in the NICI spectrum of PFB derivatized 3-keto VPA was $[M-181-CO_2]^-$. Isolated metabolites were identified with the help of twin ions (deuterated and undeuterated) in the mass spectra and by comparison of mass spectra and retention times with synthetic reference compounds. Urine or serum metabolites were analyzed in one chromatographic run and SIM chromatograms obtained. Serum and urine controls showed no interfering peaks and the analytical method appears suitable for a sensitive assay of VPA metabolites.

The NICI method employing PFB derivatives was sensitive enough to detect VPA metabolites in saliva. Seven metabolites were detected. The ratio of Z to E isomers of 2-ene VPA was much greater in saliva than in serum (3.82 vs. 0.458), suggesting differences in the transport or plasma protein binding properties of these two isomers.

A new VPA metabolite, assigned the structure 4'-keto-2-ene VPA was detected in urine. The mass spectrum and retention time of this new metabolite matched that of one compound which was present in a synthetic mixture containing 4'-keto-2-ene VPA. Another new metabolite which appears to be 2-(2'-propenyl)-glutaric acid was also detected in urine.

The synthesis of 4'-keto-2-ene VPA was attempted using two different synthetic methods. The first method which involved the dehydrogenation of the O-TMS dialkyl ketene acetal of ethyl 2-propyl-4-oxopentanoate apparently resulted in the formation of the positional isomer, 4-keto-2-ene VPA. The second synthetic route was based on the dehydration of 4-carboethoxy-2-ethylenethioketal-5-hydroxyheptane and produced 4'-keto-2-ene VPA. However, it was not possible to isolate sufficient product for NMR characterization.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| VPA | Valproic acid (2-propylpentanoic acid) |
| $[^2H_6]$-VPA | $[^2H_6]$-Valproic acid |
| 2,3'-diene VPA | 2-(1'-propenyl)-2-pentenoic acid |
| 2,4-diene VPA | 2-propyl-2,4-pentadienoic acid |
| 4,4'-diene VPA | 2-(2'-propenyl)-4-pentenoic acid |
| 2-ene VPA | 2-propyl-2-pentenoic acid |
| 3-ene VPA | 2-propyl-3-pentenoic acid |
| 4-ene VPA | 2-propyl-4-pentenoic acid |
| 3-keto VPA | 2-propyl-3-oxopentanoic acid |
| 4-keto VPA | 2-propyl-4-oxopentanoic acid |
| 3-OH VPA | 2-propyl-3-hydroxypentanoic acid |
| 4-OH VPA | 2-propyl-4-hydroxypentanoic acid |
| 5-OH VPA | 2-propyl-5-hydroxypentanoic acid |
| 4'-keto-2-ene VPA | 2-(2'-oxopropyl)-2-pentenoic acid |
| 4-keto-2-ene VPA | 2-propyl-4-oxo-2-pentenoic acid |
| 3'-keto-4-ene VPA | 2-(1'-oxopropyl)-4-pentenoic acid |
| 3-keto-4-ene VPA | 2-propyl-3-oxo-4-pentenoic acid |
| 4'-OH-2-ene VPA | 2-(2'-hydroxypropyl)-2-pentenoic acid |
| 4'-OH-4-ene VPA | 2-(2'-hydroxypropyl)-4-pentenoic acid |
| bis-TFMB | 3,5-bis(trifluoromethyl)benzyl |
| Bp | boiling point |
| CBZ | carbamazepine |
| CI | chemical ionization |
| CSF | cerebrospinal fluid |
| DDQ | 2,3-dichloro-5,6-dicyano-1,4-benzoquinone |

## LIST OF ABBREVIATIONS (CONT'D)

| | |
|---|---|
| DMAP | 4-dimethylaminopyridine |
| E | trans |
| ECD | electron capture detection |
| EI | electron impact |
| eV | electron volts |
| GC | gas chromatography |
| GCMS | gas chromatography mass spectrometry |
| I.D | internal diameter |
| IR | infrared |
| LDA | lithium diisopropylamide |
| Lit. | literature |
| m | multiplet |
| MHz | megahertz |
| MS | mass spectrometer |
| MSTFA | N-methyl-N-trimethylsilyltrifluoroacetamide |
| m/z | mass to charge ratio |
| NICI | negative ion chemical ionization |
| NMR | nuclear magnetic resonance |
| OA | octanoic acid |
| PFB | pentafluorobenzyl |
| PFBB | pentafluorobenzyl bromide |
| q | quadruplet |
| r | correlation coefficient |
| s | singlet |
| SIM | selected ion monitoring |
| $\delta$ | chemical shift |

LIST OF ABBREVIATIONS (CONT'D)

| | |
|---|---|
| t | triplet |
| t-BDMS | tertiarybutyldimethylsilyl |
| t-BDMSCl | tertiarybutyldimethylsilyl chloride |
| THF | tetrahydrofuran |
| TIC | total ion current |
| TMS | trimethylsilyl |
| Z | cis |

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# I. INTRODUCTION

Valproic acid (di-n-propylacetic acid, 2-propylpentanoic acid, VPA) is a major anticonvulsant drug now in use throughout the world. VPA has been known since 1881 but its anticonvulsant properties were demonstrated much later by Meunier et al. (1963). Structurally VPA is a simple branched fatty acid and hence it differs from the usual antiepileptic drugs in that it lacks nitrogen and a ring structure. Its structural formula is:

$$CH_3 - CH_2 - CH_2$$
$$CH - COOH$$
$$CH_3 - CH_2 - CH_2$$
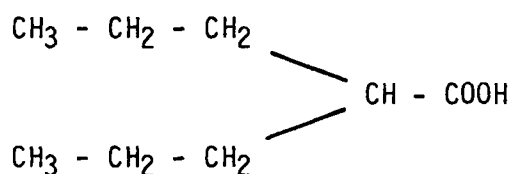
VPA is useful in a variety of seizures including primary generalized seizures of the petit mal and myoclonic types, tonic-clonic seizures and partial seizures. The precise mode of action of VPA remains uncertain, although it has been suggested that VPA exerts its actions through effects on gamma-aminobutyric acid. VPA is extensively metabolized in humans and experimental animals. The metabolism of VPA is very complex; so far 17 metabolites have been identified in humans. Several of these metabolites possess anticonvulsant properties and other metabolites are thought to be involved with rare but fatal hepatotoxicity associated with VPA therapy.

In view of the anticonvulsant activity and/or potential toxicity of the metabolites, there is a great deal of interest in studying VPA metabolism. Some of the VPA metabolites are found in minor quantities and trace metabolites might not have been identified because of the lack of

sensitivity of currently used analytical methods. Hence, there is a need for the development of convenient, highly sensitive and specific methods of analysis. The purpose of this work was to develop a highly sensitive and specific method of analysis and apply the method to measure trace VPA levels and to search for new VPA metabolites that may be present at trace levels.

A. Pharmacokinetics of VPA

The pharmacokinetics of VPA have been extensively studied both in humans and animals and have been reviewed by Gugler and von Unruh (1980), Schobben et al. (1980), Morselli and Franco-Morselli (1980), and Rimmer and Richens (1985). After oral administration, VPA is rapidly and almost completely absorbed, peak plasma levels being attained within one to four hours (Schobben et al., 1980). In terms of absolute bioavailability different formulations of the drug appear to be bioequivalent.

Therapeutic plasma levels are generally 50 to 100 μg/mL with some patients requiring plasma concentrations in excess of 100 μg/mL (Bruni and Wilder, 1979). There is a significant relationship between the reduction in the number of seizures and increasing serum VPA levels (Gram et al., 1979). It has also been found that the relationship between dose and plasma concentration is curvilinear, i.e. the plasma concentration to dose ratio decreases with increasing doses.

Studies in rodents have shown that VPA is distributed rapidly, reaching the brain in a few minutes (Vajda, 1983). The apparent volume of distribution is in the range of 0.1 to 0.4 L/kg (Gugler et al., 1977;

Perucca et al., 1978). This small volume of distribution indicates that VPA is distributed only to the circulation and rapidly exchangable extra-cellular water. VPA does not appear to be bound to intracellular proteins in brain nor is taken up selectively by the brain of humans (Goldberg and Todoroff, 1980). VPA is highly bound to plasma proteins (average 90%) (Gugler and Mueller, 1978; Loscher, 1978). Partly because of the high plasma protein binding, VPA concentration in the cerebrospinal fluid is 10% of that in plasma and the saliva concentration of VPA ranges from 0.4 to 6% of the plasma concentration. Protein binding of VPA is concentration dependent and the free fraction varies two-fold within the therapeutic range (Levy et al., 1986).

The plasma clearance of VPA ranges from 5 to 10 mL/min (Klotz and Antonin, 1977; Gugler et al., 1977). In children higher clearance values are found which may be explained by greater volumes of distribution (Schobben et al., 1980). The plasma elimination half-life is in the range of 9 to 18 hours in monotherapy and is 6 to 12 hours when VPA is administered with other antiepileptic drugs (Levy et al., 1986).

B.  Metabolism of VPA

Metabolism is the major means for the elimination of VPA and renal excretion of the unchanged drug accounts for less than 5% of the adminis-tered dose (Gugler et al., 1977). In spite of its simple structure the metabolism of VPA is very complex and complete elucidation of its metabo-lic pathways has proved elusive. The metabolism of VPA has been exten-sively studied in man and various animals and was initially reviewed by Gugler and von Unruh (1980). More recently, in this laboratory, the

metabolite 4-keto VPA was identified and 2-propylsuccinic acid and 2-propylmalonic acid were characterized as VPA metabolites using deuterated tracers and GCMS analysis (Acheampong et al., 1983). VPA undergoes glucuronidation, $\beta$-, $\omega$- and ($\omega$-1)-oxidation to produce a large number of metabolites (Loscher, 1981a; Granneman et al., 1984a). The human metabolic pathways of VPA are summarized in Figure 1. In both man and rat, $\beta$-oxidation and glucuronidation are the two primary pathways. In a single dose study in man 15 to 20% of the administered dose was excreted as VPA glucuronide (Bialer et al., 1985). $\beta$-Oxidation of VPA gives rise to 2-ene VPA, 3-OH VPA and 3-keto VPA with 2-ene VPA and 3-Keto VPA being the major metabolites in plasma (Nau and Loscher, 1984). 3-Keto VPA is also a major urinary metabolite and is considered to arise primarily as a result of mitochondrial oxidation.

The $\omega$- oxidation pathway leads to 5-OH VPA, 2-propylglutaric acid and 2-propylmalonic acid. The ($\omega$-1) - oxidation pathway results in the formation of 4-OH VPA, 4-keto VPA and 2-propylsuccinic acid. Products of $\omega$- and ($\omega$-1) -oxidation are found in minor quantities in the serum of epileptic patients (Abbott et al., 1986a). The $\omega$ and ($\omega$-1) pathways are cytochrome P-450 mediated (Prickett and Baillie, 1984). By administering 5-OH VPA to the rat, 2-propylglutaric acid was shown to be the terminal product of the $\omega$-oxidation pathway and similarly administration of 4-OH VPA resulted in the production of ($\omega$-1) metabolites (Granneman et al., 1984a). The unsaturated metabolites 3-ene VPA and 4-ene VPA were not observed after treatment with 4-OH VPA and 5-OH VPA and administration of these unsaturated metabolites produced negligible amounts of hydroxy metabolites. Thus 3-ene VPA and 4-ene VPA do not belong to the $\omega$ and

($\omega$-1) pathways and are thought to originate through dehydrogenation of VPA. These unsaturated metabolites are further metabolized to produce dienes VPA. One of these diene metabolites is a major serum metabolite and has been assigned the structure (E,E)-2,3'-diene VPA by Acheampong and Abbott (1985).

In addition to the above known metabolic pathways, multiple minor metabolic pathways are thought to operate in the metabolism of VPA. For example, Prickett and Baillie (1984) showed that incubation of VPA with rat liver microsomes led to the formation of 3-,4- and 5-OH VPA. The 3-OH VPA was thought to be a product of $\beta$-oxidation but the above study suggests that 3-OH VPA can also be formed by cytochrome P-450 dependent oxidation. More recently Rettie et al. (1987) have demonstrated that cytochrome P-450 catalyzes the formation of 4-ene VPA from VPA.

C. Anticonvulsant activity of VPA metabolites

VPA displays a late onset of antiepileptic effects (Rowan et al., 1979; Henriksen and Johannessen, 1980) and a carry-over effect after drug administration is discontinued (Lockard and Levy, 1976). These observations suggest that active metabolites may be formed which accumulate in the brain. In a study by Nau and Loscher (1982) of the pharmacokinetic and pharmacological properties of VPA and 2-ene VPA in the mouse, it was found that 2-ene VPA was cleared from the plasma and brain slower than the parent drug indicating that 2-ene VPA may contribute to the anticonvulsant effect of chronic VPA therapy.

The anticonvulsant activity of several VPA metabolites has been studied using different animal models of epilepsy (Loscher, 1981b; Loscher and Nau, 1983; Keane et al., 1985; Loscher and Nau, 1985). In one of
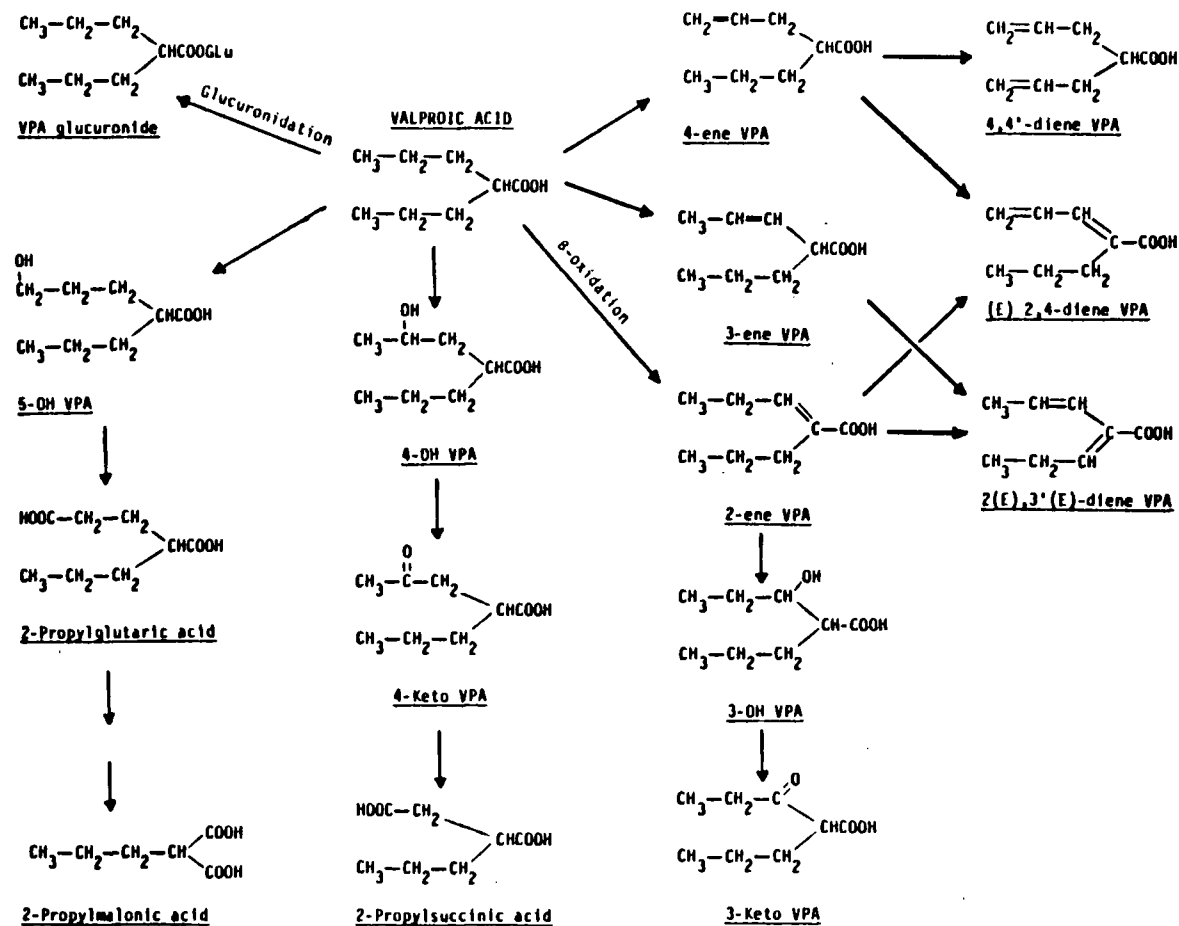
Figure 1.    Metabolic pathways of valproic acid in human (Abbott et al., 1986b).

these studies (Loscher, 1981b) the anticonvulsant activity of VPA metabolites was determined by measuring their effects on the thresholds for the maximal electroconvulsion and the pentylenetetrazole induced convulsions in mice. Of the tested metabolites 2-ene VPA and 4-ene VPA were the most potent displaying 50 to 90% of the potency of VPA. The other metabolites tested, i.e. 3-OH VPA, 3-keto VPA, 5-OH VPA, 2-propylglutaric acid, 3-ene VPA and 4-OH VPA also gave rise to significant threshold elevations. Another study (Loscher and Nau, 1983) has shown that although several VPA metabolites were present in plasma of dogs and rats after acute and long term treatment with VPA only 2-ene VPA was found in the brain of both animals. The 2-ene VPA accumulated in some brain regions during chronic treatment and was found to be 1.3 times more potent than the parent drug when calculation was based upon whole brain concentrations. Furthermore, studies with the trans isomer of 2-ene VPA indicate a comparable anticonvulsant profile with that of the parent drug without the potential for embryotoxicity associated with the latter (Loscher et al., 1984). In a recent study Acheampong and Abbott (unpublished data) have shown that 2,3'-diene VPA has an anticonvulsant activity comparable to that of 2-ene VPA whereas 4-keto VPA was inactive.

The anticonvulsant property of active VPA metabolites does not appear to be superior to that of VPA. However, 2-ene VPA or 2,3'-diene VPA could be an alternative to VPA if it can be shown that either is not associated with the hepatotoxicity caused by VPA therapy.

D. Toxicity of VPA and its metabolites

The common adverse effects with VPA therapy are nausea, vomiting, gastrointestinal disturbance, thrombocytopenia and behavioral disturbance

(Schmidt, 1984). Pancreatitis has also been related to VPA therapy (Wyllie et al., 1984). The most serious toxic effect of VPA is the hepatotoxicity which appears to be an idiosyncratic reaction in a small population of patients.

The VPA-induced hepatic toxicity can assume two different forms (Gram and Bentsen, 1983). The first form is associated with an increase in liver enzymes and appears to be dose related. The other form constitutes an irreversible liver damage. Its frequency has been estimated as 1 in 20,000 patients (Jaevons, 1984). The clinical symptoms of the liver toxicity include hepatocellular necrosis and microvesicular steatosis. The latter is similar to that observed in Reye's syndrome and Jamaican vomiting sickness (Gerber et al., 1979).

The hepatotoxicity of VPA is believed to be associated with the mono and/or doubly unsaturated metabolites of VPA. Kochen et al. (1984) noted an increased formation of diunsaturated metabolites in patients with side-effects as opposed to patients without side-effects. The 4,4'-diene VPA which has never been observed before was detected along with 4-ene VPA in one patient who died from hepatic failure (Kochen et al., 1983). Most fatal hepatotoxicity cases have been in multiple drug therapy (Dreifuss and Santilli, 1986). This may be due to an increased formation of one or more of the toxic metabolites. In rats, Granneman et al. (1984b) found that phenobarbital coadministration caused significant increases in the plasma levels of 4-ene VPA and 5-OH VPA.

The most likely metabolite to be involved with the liver toxicity is 4-ene VPA. The 4-ene VPA is structurally similar to the metabolite of

hypoglycin A that is responsible for Jamaican vomiting sickness, and 4-pentenoic acid which produces a fatty liver in the rat (Nau and Loscher, 1984). The mechanism by which 4-ene VPA may cause hepatotoxicity is not known. It has been postulated that VPA and unsaturated metabolites cause their hepatotoxicity by inhibition of the $\beta$-oxidation pathway (Kesterson et al., 1984). VPA causes a mild, transient inhibition of the $\beta$-oxidation pathway by sequestration of CoA, while 4-ene VPA is thought to cause a prolonged and potent inhibition due to the formation of 4-ene VPA-CoA. The 4-ene VPA-CoA might be a potent inhibitor of a specific enzyme(s) in the $\beta$-oxidation system. Furthermore, multiple biochemical reactions may result from the numerous VPA metabolites. High levels of unusual keto acids such as 3- and 4-keto VPA might also affect $\beta$-oxidation by interfering with the $\beta$-keto-acylthiolase enzyme (Kesterson et al., 1984). The metabolites, 4-ene VPA and 4-OH VPA have been found to be toxic in cultured rat hepatocytes (Kingsley et al., 1983).

In general terms VPA causes various metabolic disturbances because it inhibits several enzymes involved in intermediary cell metabolism. Thurston et al. (1985) have reported that a single therapeutic dose of VPA affects the metabolism of carbohydrates, fats and amino acids in infant mice. Also, a recent study (Turnbull et al., 1986) indicates that one gram of VPA given orally causes metabolic disturbances in normal humans.

In a study that sought to address the mechanism of the toxicity of 4-ene VPA Rettenmeier et al. have detected 3-OH-4-ene VPA as one of the metabolites of 4-ene VPA in the perfused rat liver (1985) and in the Rhesus monkey (1986a). These authors postulate that the detection of

3-OH-4-ene VPA is indirect evidence for the formation of 3-keto-4-ene VPA from 4-ene VPA. They suggest that 4-ene VPA is metabolically activated to 3-keto-4-ene VPA which is capable of alkylating mitochondrial proteins. The 3-keto-4-ene VPA like other $\alpha,\beta$-unsaturated carbonyl compounds is highly reactive and can undergo Michael additions to give covalent adducts of nucleophiles (Eder et al., 1982).

Teratogenicity is the second major toxic effect of VPA (Brown et al., 1985). VPA is teratogenic and embroyotoxic in rabbits, rats and mice (Petrere et al., 1986). VPA crosses the placenta and can affect the fetus (Dickinson et al., 1979). Published case reports of fetal malformation in epileptic mothers on VPA describe various malformations including spina bifida (Rimmer and Richens, 1985). The mechanism of VPA teratogenicity is unknown but Brown et al. (1985) have suggested that the biochemical mechanism of VPA teratogenicity differs from that of the hepatotoxicity.

E.  Interaction between VPA and carbamazepine

Carbamazepine (CBZ) drug interactions have been recently reviewed by Baciewicz (1986). Because the biotransformation of CBZ is inducible as well as susceptible to inhibition, and CBZ is protein bound to a large extent (Levy and Koch, 1982), interactions between VPA and CBZ are to be expected. To date there have been conflicting reports of possible interactions between the two. Bowdle et al. (1979) demonstrated a decrease in CBZ minimum steady-state concentration when VPA was administered concomitantly with CBZ. In another study (Levy et al., 1984a), where VPA was given for one week to seven epileptic patients receiving chronic CBZ, it was found that steady-state CBZ levels were reduced by 3-59% in six

patients and unchanged in one patient. On the other hand several studies (Pisani et al., 1981; Brodie et al.,1983; McKauge et al., 1981) did not find significant differences in plasma levels of CBZ when CBZ and VPA were administered concomitantly.

The effect of CBZ on VPA plasma levels has also been studied. Bowdle et al. (1979) observed that minimum steady-state concentrations of VPA declined and clearance increased when CBZ was given to normal volunteers on VPA steady-state. Similarly, Hoffman et al. (1981) found that the half-life of VPA was reduced from 15 to 6.9 hours and clearance increased from 8.0 to 13.7 mL/min when the two drugs were given together. In the present study the effect of CBZ on the protein binding (in vivo) of VPA will be investigated in five healthy volunteers as a part of a general study of the effect of CBZ on VPA metabolism.

F. Analytical methods for VPA and its metabolites

For the analysis of VPA, separation of the drug from biological fluids is required, usually by acidifying and extracting the serum or urine sample with organic solvents. The most efficient solvent for the extraction of VPA and its metabolites is ethyl acetate (Abbott et al., 1986a).

Many methods have been described in the literature for the analysis of VPA in biological fluids. These include high-performance liquid chromatography (Sutheimer et al., 1979; Alric et al., 1981; Kline et al., 1982; Moody and Allan, 1983; Nakamura et al., 1984; Kushida and Ishizaki, 1985), enzyme immunoassay (Higgins, 1983; Siegmund et al., 1981), gas chromatography, and gas chromatography - mass spectrometry.

GC determination of VPA has been by far the most common method. VPA has been assayed by GC underivatized (Loscher, 1977; Kwong et al., 1980; Freeman and Rawal, 1980; Berry and Clarke, 1978). The drug has also been quantitated as the methyl (Calendrillo and Reynoso, 1980), butyl (Hulshoff and Roseboom, 1979), trimethylsilyl (Loscher, 1981a), phenacyl (Gupta et al.,1979), hexafluoroisopropyl (Nishioka and Kawai, 1983) or t-butyldimethylsilyl ( Abbott et al., 1982) derivatives. The phenacyl ester of VPA has also been analyzed by GC with electron capture detection (Chan, 1980).

The GCMS methods include the quantitation of VPA as its methyl ester (von Unruh et al., 1980), identification of VPA metabolites using the methyl ester and t-butyldimethylsilyl derivatives (Acheampong et al., 1983), simultaneous analysis of VPA and eight of its metabolites using trimethylsilyl derivatives (Nau et al., 1981) and quantitation of 2-3- and 4-ene VPA as their trimethylsilyl ester (Rettenmeier et al., 1986b). A chemical ionization (CI) GCMS assay of the ethyl esters of VPA metabolites (Granneman et al., 1984a), a CI GCMS method for the determination of VPA (Balkon, 1979) and a direct insertion CI method (Schier et al., 1980) have also been reported.

The most complete GCMS assay is that of Abbott et al. (1986a) which enables the simultaneous determination of VPA and 12 metabolites in a single chromatographic run. This assay is based upon selected ion monitoring of the electron impact ionization of t-butyldimethylsilyl derivatives of the drug and its metabolites. The assay is relatively sensitive and specific. However, the large number of VPA metabolites,

some of which are present at very low concentrations (especially those implicated in hepatotoxicity), the possible interference of endogeneous fatty acids, make the search for an even more sensitive and specific method of analysis necessary. The above mentioned assay of Abbott et al. has a lower limit of detection of 0.1 µg/mL. The serum levels of some VPA metabolites (4-ene VPA, 3-ene VPA, 2,4-diene VPA, 5-OH VPA, 4-OH VPA) are frequently near the lower detection limits.

In this work a highly sensitive and more specific analysis for VPA and all its metabolites was to be developed. The method was to be based on the technique of electron capture negative ion chemical ionization GCMS. Because of this ionization technique, at least one order of magnitude increase in sensitivity over any current method was expected. The GCMS specificity would be further enhanced because of the soft ionization nature of negative ion chemical ionization.

The need for a more sensitive and specific analytical method cannot be overemphasized. Such a method will be valuable in the study of the metabolism of VPA metabolites in small animals in order to elucidate metabolic pathways and in the detection of intermediary metabolites which may be responsible for the hepatotoxicity of VPA therapy.

G.  Negative ion chemical ionization

In a number of laboratories there has been a recent interest in using negative ion chemical ionization (NICI) mass spectrometry coupled with GC for the analysis of certain fatty acids. This has been especially true for the determination of prostanoids as their pentafluorobenzyl derivatives. The technique of NICI mass spectrometry is a relatively new

technique and has been used to solve structural and analytical problems only during the last decade.

1. Ion forming reactions in NICI

Under conventional EI conditions, 70eV electrons, and source pressures in the range of $10^{-5}$ to $10^{-7}$ torr, formation of negative ions occurs by the ion pair mechanism and is dominated by low mass fragment ions (Hunt et al., 1976). Under CI conditions, i.e. at source pressures of about 1 torr, negative ions can be produced in two ways (Dougherty, 1981; Watson, 1985):

    a. Electron/molecule reactions

       i    Resonance electron capture

$$AB + e^- \longrightarrow AB^- \qquad (<0.1eV)$$
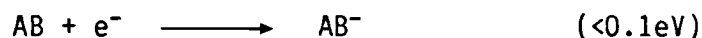
       ii   Dissociative electron capture

$$AB + e^- \longrightarrow A\cdot + B^- \qquad (0-15eV)$$
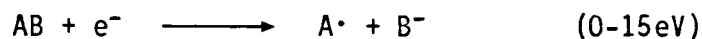
       iii  Ion-pair formation

$$AB + e^- \longrightarrow A^- + B^+ + e^- \qquad (>10eV)$$
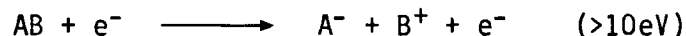
    b. Anion/molecule reactions

$$AB + C^- \longrightarrow ABC^- \quad or \quad (AB-H)^- + HC$$
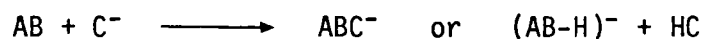
2. Negative ion reagent gas systems

    a. Bronsted-base reagent systems

These reagent substances play a similar role as the reagent gases in positive CI and result in ion/molecule reactions. The Bronsted-base

reagent systems include $H^-$, $NH_2^-$, $OH^-$, $O^-$, $O_2^-$, $CH_3O^-$, $F^-$ and $Cl^-$ (Harrison, 1983). They react either by proton abstraction or adduct formation.

b. Electron capture reagent systems

The capture of electrons by a molecule is a resonance process which requires electrons of near-thermal energy (Harrison, 1983). With a high source pressure the simplest type of process which leads to negative ion formation is where the reagent gas acts only as a moderating gas to produce a high population of thermal energy electrons which are captured by sample molecules with some electron affinity. The reagent gas can also act in the capacity of collisional stabilization of the newly formed negative ions (Hass, 1980).

Classes of compounds that have intrinsically high NICI sensitivity are generally oxidizing and alkylating agents (Dougherty, 1981). Positive electron affinities are observed for many halogenated compounds, quinones and nitro compounds (Howe et al., 1981). For molecules which lack electron capture capability, derivatization of the molecule with a suitable derivatizing agent that endows the molecule with positive electron affinity is possible. Derivatives such as pentafluorobenzaldehyde (to form a Schiff base with aromatic amines), pentafluorobenzoyl halide (for phenols and amines) and tetrafluorophthalic anhydride (for amines) have been used for NICI mass spectrometry. Pentafluorobenzyl bromide has been employed as the derivatizing reagent for NICI GCMS analysis of fatty acids and prostaglandins.

3. Sensitivity of NICI

It can be shown that the ion currents obtained in CI are generally as intense as those observed in EI (Harrison, 1983). Furthermore, the CI ion current may be concentrated in a few ions. The sensitivity of CI systems is dependent upon k, the rate constant of the CI reaction. Efficient CI reactions will show better or equal sensitivities to those of EI. Inefficient ionization reactions i.e. those with smaller rate constants will have lower sensitivities.

Compounds that are amenable to electron capture as opposed to ion/ molecule reactions in NICI can have high rate constants as a result of the high mobility of the electron. This can result in extraordinary sensitivity in that, when a molecule possesses both a positive electron affinity and large cross section for electron capture, the negative ion spectrum which depends upon electron capture can exhibit up to 100 times the sensitivity found with other ionization techniques.

4. Factors which determine the sensitivity of sample detection

in NICI.

The formation of negative ions by electron capture is strongly dependent upon the electron affinity of the analyte, the energy of the electrons effecting the ionization and the degree of collision of molecule ions with neutrals (Dougherty, 1981). The sensitivity with which a sample can be detected, therefore, depends upon the extent to which newly formed and excited anions can be stabilized by reagent gas molecules (Chapman, 1985). Collisional processes can also lead to electron ejection and hence there will be an optimal source pressure for maximum sensitivity. In addition, the relative importance of collisional stabilization to electron detachment will depend upon the internal energy of the reagent gas

molecules and hence sensitivity will be strongly influenced by source temperature (Hass, 1980). Furthermore, electron absorbing impurities such as halogenated solvents can deplete the thermal electrons in the ion source and this results in a drop in sensitivity. High sensitivities can not be maintained as the concentration of substrate molecules increases since the number of thermal electrons in the ion source is finite. Linear response ranges must be determined because non-linear response may start as low as 10 ng in some cases (Stout, 1984).

H.  Objectives

1.     The main objective of this study was to develop a highly sensitive and specific method for the detection and quantitation of trace level VPA and its metabolites. The suitability of halogenated derivatives and negative ion chemical ionization GCMS were to be evaluated for achieving the desired analytical method.

2.     The analytical method was to be used to measure VPA in serum (total and free) and in saliva in five healthy volunteers who participated in a drug interaction study between VPA and carbamazepine. The effect of carbamazepine on the free fraction of VPA was to be determined and the utility of measuring salivary concentration of VPA in a drug interaction study evaluated.

3.     A search for new VPA metabolites was to be done using the twin ion technique and employing both electron impact (t-butyldimethylsilyl derivatives) and negative ion chemical ionization (pentafluorobenzyl derivatives) mass spectrometry. Potential new VPA metabolites and known metabolites were to be synthesized (as required) for use as reference standards.

## II. EXPERIMENTAL

A. Chemicals and Materials.

1. General

Chemicals were reagent grade and obtained from the following sources.

    a. Aldrich Chemical Co. (Milwaukee, Wisconsin):

       3,5-Bis(trifluoromethyl)benzyl bromide, Boron trifluoride etherate, t-Butyldimethylsilyl chloride, n-Butyllithium (1.6 M in hexane), Calcium hydride, 18-Crown-6, 2,3-Dichloro-5,6-dicyano-1,4-benzoquinone, Diisopropylamine, Diisopropylethylamine, 4-Dimethylaminopyridine, 1,2-Ethanedithiol, Isopropylcyclohexylamine, Lithium aluminium hydride, Methanesulfonyl chloride, Pentanoic acid, Potassium hydride (35% dispersion in mineral oil), Propionyl chloride, Sodium hydride (50% dispersion in mineral oil), Tetrahydrofuran, Triethylamine.

    b. Alfa Products (Danvers, Massachusetts):

       Pentafluorobenzyl bromide

    c. BDH Chemicals (Toronto, Ontario):

       Acetone, Acetonitrile, Benzene, Citric acid (anhydrous), Ether (anhydrous), Hydrochloric acid, Potassium iodide, Sodium hydroxide, Sodium sulfate (anhydrous), Sulfuric acid.

    d. British Drug House (Poole, U.K.):

       Iodoethane, Pyridine.

e.  Caledon Laboratories Ltd. (Georgetown, Ontario):

Dichloromethane, Ethanol, Ethyl acetate.

f.  Eastman Kodak Co. (Rochester, New York):

Ethyl acetoacetate, 4-Oxopentanoic acid, Propionaldehyde.

g.  Fisher Scientific Co. (Fairlawn, New Jersey):

Bromine, t-Butanol, Cadmium carbonate.

h.  Mallinkrodt Chemicals (St. Louis, Missouri):

Potassium carbonate (anhydrous), Sodium bicarbonate.

i.  Matheson Coleman and Bell Co. (Norward, Ohio):

Chlorotrimethylsilane, Phosphorus tribromide, 2,4,6-Tri-methylpyridine.

j.  Nichols Chemical Company (Montreal, Canada):

Mercuric chloride.

k.  Pierce Chemical Company (Rockford, Illinois):

N-Methyl-N-trimethylsilyltrifluoroacetamide.

2.  VPA metabolites and internal standards

Di-n-propylacetic acid (VPA) was obtained from ICN Biochemicals Inc. K+K Labs. (Plainview, New York). The internal standard octanoic acid (OA) was purchased from Nutritional Biochemicals Corporation (Cleveland, Ohio). The synthesis of the other internal standard used, $[^2H_6]$-VPA has been reported (Acheampong et al., 1984). The VPA metabolites used as reference standards were obtained from this laboratory and their synthesis has been published (Acheampong et al., 1983). These metabolites included 4-ene VPA, 3-ene VPA, 2-ene VPA, 4-OH VPA, 5-OH VPA, 2-propylglutaric acid and 2-propylsuccinic acid. The synthesis of 2,4-diene VPA and 2,3'-diene VPA will be reported elsewhere.

## B. Instrumentation

### 1. Gas Chromatography Mass Spectrometry

#### a. Capillary Column GCMS

Capillary column GCMS analysis was performed on a Hewlett-Packard 5987A gas-chromatograph mass spectrometer with an RTE-6 data system. Electron impact spectra were obtained at electron energy of 70eV, and ion source pressure of $1.8 \times 10^{-6}$ Torr. EI GCMS analysis of the t-BDMS derivatives was done under the following conditions: OV-1701 bonded phase column, 25 m x 0.32 mm I.D. with a film thickness of 0.25 μ (Quadrex Scientific, New Haven, Connecticut); oven temperature, 50°C to 100°C at 30°/min, 100°C to 260°C at 8°/min; source temperature, 240°C; open split interface, 250°C; injection port temperature, 240°C; helium flow rate, 1 mL/min. Negative ion chemical ionization spectra were recorded at 120-170eV depending upon the value of the source pressure at which the instrument was tuned. Source pressure was about 1 torr. Operating conditions were: oven temperature, 50°C to 140°C at 30°/min; 140°C to 250°C at 5°/min; reagent gases, methane, ammonia, argon-methane; source temperature, 200°C; open split interface, 250°C; injection port temparture, 240°C; carrier gas, helium at a flow rate of 1 mL/min. One μL of sample was injected and the mode of injection was splitless.

#### b. Packed Column GCMS

Intermediates and end products of the synthetic reactions were monitored using a Hewlett-Packard 5700A gas chromatograph interfaced to a Varian Mat-111 mass spectrometer via a variable slit separator. Ionization energy was 70eV and source pressure $5 \times 10^{-6}$ torr.

Scanning range was 15-750 mass units with one scan taken every 5 seconds. Data was processed by an on-line Varian 620L computer system. Operating conditions: column (1.8 m x 2 mm I.D) packed with 3% Dexsil 300 on 100/200 mesh Supelcoport (Supelco, Inc., Bellefonte, Pennsylvania). Temperature program: initial 50°C, rate 8°C/min to 270°C, hold 5 min at 270°C.

2. GC - Electron Capture Detection

PFB derivatives of valproic and octanoic acids were analyzed by electron capture detection using a HP-5840A gas chromatograph, modified for capillary column use. Temperature program: 150°C (hold 4 min) then 20°C/min to 240°C, hold 3 min at 240°C. Argon/methane flow: 6 mL/min (column), 40 mL/min (detector). Column: the same as in 1a.

3. Other Instruments

The IR spectrum of ethyl 2-propyl-4-oxopentanoate was obtained as a neat liquid film on sodium chloride disks using a Unicam SP-1000 spectrophotometer. Proton NMR spectra were recorded on Bruker WP-80 and Nicolet Oxford-270 instruments at the NMR facility in the Department of Chemistry, U.B.C. NMR solvent was $CDCl_3$ and the internal standard tetramethylsilane.


C. Human Study


The blood, urine and saliva samples used for metabolite identification and quantitation of VPA were part of a drug interaction study between carbamazepine (CBZ) and VPA and were collected as part of the M.Sc. graduate research of Sukhbinder Panesar. Five healthy male volunteers participated in the study. Volunteers received an average of

16.4 mg/kg/day of VPA in syrup form. The drug was administered in two equal doses, one at 8 a.m. and the other 8 p.m. On day 9 of the study 100 mg CBZ twice daily was added to the dosing regimen which was increased to 200 mg for the evening dose on day 16. One volunteer, FA, also received six doses of 700 mg $[^2H_6]$-VPA on days 8, 9, 25, 26 (twice) and 27.

Blood was collected in sterile, non-heparinized vacutainers prior to the morning dose of days 7 and 23 after an overnight fast and at 0.5, 1, 1.5, 2, 2.5, 3, 5, 7, 9, 12, 24, 30, 36 and 48h after the dose. The samples were allowed to clot and serum obtained after centrifugation. Urine samples were collected in 2h blocks, other convenient blocks and also overnight. Total urine volume was recorded and a homogenous aliquot saved.

Saliva samples were collected following stimulation with 5% citric acid solution and were taken simultaneous to blood samples. Four mL of the citric acid solution was held in the mouth for 2 min and spat out. The saliva sample (3 to 5 mL) was then collected after 2 min. The pH of the saliva samples was not measured immediately after collection. However, during the collection of blank saliva following the same procedure the pH was measured and found to be constant at around 7.3 for a number of such samplings. All biological samples were stored at -20°C until analyzed.

## D. Analysis of Samples

### 1. Serum and Saliva Standards

Stock solutions of VPA (500 µg/mL), OA (250 µg/mL), and $[^2H_6]$-VPA

(24 µg/mL) in water were made by diluting concentrated solutions of the above substances in methanol with distilled water. For serum total calibration curves  drug free serum was added to an aliquot of VPA solution to give either 150, 30 or 10 µg/mL VPA in serum. For the NICI assay the appropriate volume was taken from either the 30 or 10 µg/mL VPA in serum to provide 24, 18, 12, 9, 6, 4, and 3 µg/mL VPA concentrations in a final volume of 100 µL of serum. For EI analysis the standards were prepared by pipetting the required volume from the 150 µg/mL VPA standard to give 90, 75, 60, 45, 30, and 15 µg/mL VPA concentrations in a final volume of 100 µL of serum.

Saliva standards were prepared by pipetting the appropriate volumes from either a 5 µg/mL or 0.5 µg/mL VPA standard in blank saliva. The calibration points prepared were 3, 1.5, 1, 0.5, 0.25, 0.1, and 0.05 µg/mL contained a final volume of 1 mL of saliva.

To determine serum free VPA concentrations, VPA solutions of 25 and 2.5 µg/mL in water were prepared and aliquots taken to provide 15, 10, 7.5, 5, 2.5, 1, and 0.5 µg/mL concentrations in water.  Final volume was 100 µL.

For purposes of investigating the precision of the NICI assay, standards of VPA over the concentration range of 800 to 10 ng/mL in serum were assayed three times on the day of the preparation of standards and repeated on days 4 and 7. Then the standard deviations of the slopes and  the coefficient of variation at each calibration point were calculated.

To determine the recovery of VPA in serum and saliva, serum and saliva samples spiked with the same amounts of VPA used for the standard curves were prepared.   These concentrations were then determined using a  standard curve of VPA prepared in water.

For the NICI assay the peaks at m/z 143 of the PFB ester of VPA and m/z 149 of the PFB ester of [$^2$H$_6$]-VPA (or m/z 143 of the PFB ester of OA) were monitored. The calibration curves were obtained by a plot of the area ratio of VPA to that of [$^2$H$_6$]-VPA or OA versus the known concentration of VPA in serum or saliva. The concentration of each serum or saliva sample was obtained using linear regression analysis. A new standard curve was prepared prior to the run of each batch of serum or saliva samples.

2. Sample preparation

To measure serum total VPA levels 100 μL of serum sample was taken and diluted five times with blank serum. Then 100 μL of the diluted sample was transferred into a 3.5 mL screw cap septum vial. Internal standard (40 μL from a 24 μg/mL solution of [$^2$H$_6$]-VPA) was added followed by 3N NaOH to make the pH 12-13. The samples were subsequently heated for 1 hour at 60°C. After cooling to room temperature the pH was brought down to 2 using 4N HCl and the samples allowed to sit at room temperature for 10 minutes. Then the samples were extracted with 500 μL of ethyl acetate by gentle rotation for 20 minutes. To increase the recovery of the drug the extraction step was repeated with another 500 μL of ethyl acetate. The organic layer was then transferred to a vial containing anhydrous Na$_2$SO$_4$, vortexed and centrifuged at 2000 rpm for 20 minutes. The supernatant was transferred to another vial and the volume reduced to about 200 μL under N$_2$. Finally the sample was derivatized to give either PFB or t-BDMS derivatives.

Saliva samples were prepared by taking 1 mL of sample followed by the addition of internal standard (30 μL of 12 μg/mL [$^2$H$_6$]-VPA solution). The pH of the samples was adjusted to about 2 and the

samples were extracted with 3 mL ethyl acetate and following centrifugation to break the emulsion, were treated as for serum samples.

For the determination of free serum VPA concentration, 1 mL of serum was centrifuged and 100 µL of the ultrafiltrate taken. The ultrafiltrate was then treated exactly as serum samples except that extraction was done once and that 40 µL of 12 µg/mL $[^2H_6]$-VPA solution was used as an internal standard.

3. Derivatization

To form the pentafluorobenzyl (PFB) derivative the concentrated serum or saliva extract was transferred into a 1 mL conical reaction vial and 10 µL of diisopropylethylamine (neat) was added followed by 10 µL of 30% pentafluorobenzyl bromide (PFBB) solution in ethyl acetate. The sample was then heated in a heating block for 45 minutes at 40°C. Samples for EI analysis were derivatized to give t-BDMS derivatives by adding 50 µL of t-BDMSCl in pyridine (containing 5% DMAP) and heating at 60°C for 4 hours. The TMS derivatives of urinary metabolites were prepared by adding 50 µL of MSTFA reagent and heating at 60°C for 30 minutes.

For GC-ECD analysis of the PFB ester of VPA using OA as the internal standard the following derivatization procedure was followed. Ten µL of each VPA (from 50 ng/mL to 10 µg/mL VPA) solution in ethyl acetate and 10 µL of OA (400 ng/mL OA solution in ethyl acetate) and 60 µL of 0.5% PFBB solution in benzene (containing 1.5 mg/mL 18-crown-6) were transferred to a reaction vial and a few crystals of potassium acetate added. The reaction vial was then allowed to sit at room temperature for 1 hour, following which 65 µL of the reaction mixture was pipetted into another reaction vial and the volume made to

200 µL with ethyl acetate. The solvent was then evaporated with $N_2$ to complete dryness and the residue reconstituted with 500 µL of ethyl acetate.

4. Serum free levels

Serum free levels of VPA were determined after ultrafiltration. Ultrafiltration was carried out with YMT ultrafiltration membranes in a MPS-1 micropartition system (Amicon Corp., Danvers, Massachusetts). The centrifuge was a Beckman Model J2-21. A 45° angle rotor was used which was equilibrated to 20°C before use. Centrifugation was carried out for 20 minutes at 3500 rpm.

5. Comparison of the sensitivity of EI(t-BDMS) with NICI (PFB, bis-TFMB)

To compare the sensitivity of the fluorinated derivatives (PFB, 3,5-bis(trifluoromethyl)benzyl (bis-TFMB)) relative to that of the t-BDMS derivative, a known amount of VPA was derivatized for EI and NICI analysis. The bis-TFMB derivative of VPA was prepared in the same way as the PFB derivative described above. Each derivative was prepared (octanoic acid used as internal standard) in such a way that the final concentration in the reaction vial was 5 µg/mL. The t-BDMS derivative was run in the EI mode and the other two derivatives under both EI and NICI conditions. The samples were run on five different days in two weeks time. The mean area ratios were taken and the ratio of NICI to EI calculated.

6. Identification of VPA metabolites

For this purpose urine samples and one serum sample from the volunteer on VPA steady state who had also been given six doses of [$^2$H$_6$]-VPA were used. Two mL urine samples were selected in such a way that the drug and metabolites were either mainly labelled, both labelled and unlabelled, or predominantly unlabelled. Samples analyzed included both before and after administration of CBZ. After the usual work up, each urine sample was derivatized to give the PFB, t-BDMS, TMS, and methyl ester derivatives. The retention times of all derivatives were recorded and mass spectra obtained in the NICI mode for PFB derivatives, and EI mode for the other derivatives. In addition to the above samples, derivatized extracts were also prepared without alkaline treatment of the urine samples prior to extraction. Metabolite peaks were identified by injecting synthetic standards.


E. Chemical Synthesis


1. Attempted synthesis of 2-(2'-oxopropyl)-2-pentenoic acid
   (4'-keto-2-ene VPA) via ethyl 2-propyl-4-oxopentanoate.
   a. Synthesis of ethyl 2-bromopentanoate

Pentanoic acid (20.4 g, 0.2 mol) was placed in a 500 mL flask with a reflux condenser whose top end was connected to a gas absorption device. Bromine (35 g, 0.22 mol) was added followed by 1 mL of

phosphorus tribromide. The mixture was stirred and heated with an oil bath at 70°C for 30 minutes and then at 100°C for 6 hours by which time all the bromine had reacted. The reaction mixture was subsequently distilled using a water pump in order to remove residual hydrogen bromide. The product was then distilled under reduced pressure. Bp 76-79°C/0.03 mm. [Lit. (Acheampong, Ph.D. thesis) bp 102°-105°C/2.5 mm]. Mass spectrum:(MW=181) m/z 55 (100%), 138(87%), 140(85%), 27(78%), 41(63%) 29(61%), 43(34%), 94(32%), 101(25%).

2-Bromopentanoic acid was converted to its ethyl ester by refluxing a mixture of 2-bromopentanoic acid (76g, 0.42 mol), ethanol (80 mL,1.36 mol), benzene (150 mL), and concentrated sulfuric acid (1.7 mL) for 12 hours using a Dean-Stark water separation unit. After washing the reaction mixture with saturated $NaHCO_3$ and water, pure ethyl 2-bromopentanoate was obtained by distillation. Bp 42-45°C/0.2 mm. [Lit. (Acheampong, Ph.D. thesis) bp 60-62°C/3.0 mm]. Mass spectrum:(MW=209) m/z 29(100%), 55(89%), 166(22%), 168(20%), 101(12%) 129(10%), 140(8%), 138(6%).

b. Synthesis of ethyl 2-propyl-4-oxopentanoate

To anhydrous THF (100 mL) (dried with lithium aluminium hydride) in a 250 mL flask, sodium hydride (5.76 g (50% dispersion), 0.12 mol) was added followed by dropwise addition of ethyl acetoacetate (13 g, 0.1 mol) over a period of 30 minutes. After stirring for an additional 15 minutes, ethyl 2-bromopentanoate (20.9 g, 0.1 mol) was added drop by drop and the solution refluxed for 6 hours. Distilled water (40 mL)

was then added and the resulting mixture filtered under suction. The organic layer was separated and the aqueous phase extracted three times with ether. The combined organic layer was dried over anhydrous $Na_2SO_4$ and the ether evaporated. The crude product was distilled to give ethyl 2-propyl-3-acetylsuccinate. Bp 118°C/0.2 mm. Mass spectrum: (MW=258) m/z 43(100%), 129(40%), 97(35%), 174(32%), 115(26%), 143(18%), 185(12%), 213(11%).

To obtain the 2-propyl-4-oxopentanoic acid a mixture of the acylsuccinate (9.9 g, 0.04 mol) and concentrated HCl (40 mL, 0.4 mol) was heated under reflux for 8 hours. The resulting mixture was extracted three times with ether and the extract dried over anhydrous $Na_2SO_4$. The solvent was then removed and the crude product distilled to give 2-propyl-4-oxopentanoic acid contaminated with a small amount of ethyl 2-propyl-4-oxopentanoate. Redistillation gave pure 2-propyl-4-oxopentanoic acid. Bp 115°C/0.07 mm. [Lit. (Acheampong et al., 1983) bp 133-136°C/2.5 mm]. Mass spectrum: (MW=158) m/z 43(100%), 58(19%), 101(16%), 83(15%), 73(14%), 140(8%), 158(1%).

The corresponding ethyl ester of the above acid was made by refluxing 18-crown-6 (0.3 g), ethyl iodide (1.56 g, 0.01 mol), 2-propyl-4-oxopentanoic acid (0.79 g, 0.005 mol) and $K_2CO_3$ (4 g) in THF for 6 hours. The mixture was filtered and THF removed. The residue was then fractionated by distillation and yielded pure ethyl 2-propyl-4-oxopentanoate which showed a single peak upon GCMS analysis. The esterification reaction was also carried out in a similar manner to

that of ethyl 2-bromopentanoate. Bp 97°C/1.7 mm.

Mass spectrum: (MW=186) m/z 43(100%), 129(32%), 101(30%), 141(16%), 29(14%), 73(12%).

IR Spectrum (neat film): 2900 cm$^{-1}$ (O-CH$_2$CH$_3$), 1735 cm$^{-1}$ (C=O).

NMR Spectrum: 0.9(t,3H,CH$_3$-CH$_2$-);1.1-1.6(m,4H,CH$_2$-CH$_2$);

1.2(t,3H,-CH$_3$); 2.1(s,3H,CH$_3$-CO), 2.3-3(m,3H,CH$_2$-CH);

4.2(q,2H,OCH$_2$).

c. Introduction of the double bond by dehydrogenation of the O-TMS dialkyl ketene acetal of ethyl 2-propyl-4-oxopentanoate with the keto function protected with TMS.

Lithium diisopropylamide (LDA) was prepared by dripping n-butyllithium (13.5 mL, 0.022 mol), to diisopropylamine (2.95 mL, 0.022 mol) in THF (25 mL) at 0°C over 20 min period. The mixture was then cooled in a dry ice acetone bath to -78°C and ethyl 2-propyl-4-oxopentanoate (1.86 g, 0.01 mol) added dropwise and allowed to react for 60 min, following which chlorotrimethylsilane (3.9 g, 0.036 mol) was added dropwise over a 10 min period. The temperature was allowed to attain 25°C and the mixture stirred for 60 min. The THF was distilled off and the residue reconstituted with 5 mL of dry benzene. Then dichlorodicyanobenzoquinone (DDQ) (2.27 g, 0.01 mol) was dissolved in benzene under N$_2$ and 2,4,6-trimethylpyridine (1 mL) in benzene was added dropwise to the DDQ solution. After 10 min this mixture was added to the silyl ether under N$_2$ and the mixture stirred for 2 hours. After diluting with ether the reaction mixture was washed with 1 M NaOH and the aqueous phase extracted three times with ether. The combined organic layer was washed successively with HCl, NaOH, and water and the extract dried over anhydrous Na$_2$SO$_4$. GCMS analysis of the crude

product indicated the presence of three components. One of these was the starting material. The other two were: 1) TMS enol ether of ethyl 2-propyl-4-oxopentanoate: Mass spectrum:(MW=258) m/z 73(100%), 75(65%), 185(55%), 130(44%), 115(42%) 97(20%), 45(18%), 213(10%), 143(15%), 215(8%), 243(5%), 258(3%).    2) TMS enol ether of the ethyl 2-propyl-4-oxo-2-pentenoate or ethyl 2-(2'-oxopropyl)-2-pentenoate. Mass spectrum: (MW=256) m/z 73(100%), 75(40%), 95(28%), 43(25%), 137(18%), 109(12%), 183(9%), 167(7%), 213(10%), 227(5%), 256(4%), 241(3%).

A small portion of the reaction mixture was taken and made alkaline with NaOH and stirred for three days at room temperature in order to effect hydrolysis of both the TMS enol ether and the ethyl ester of the product. The mixture was then acidified and extracted with ether. Following the evaporation of the ether a portion of the residue was derivatized to give the t-BDMS derivatives which were analyzed by capillary GCMS.   Two peaks were detected with mass m/z 213 (M-57)$^+$ and had similar mass spectra.

2.   Synthesis of 2-(2'-oxopropyl)-2-pentenoic acid starting with a protected 4-oxopentanoic acid

a. Protection of 4-oxopentanoic acid through a dithio ketal

Commercially available 4-oxopentanoic acid was purified by fractional distillation and was converted into its ethyl ester in a manner similar to that described for 2-bromopentanoic acid. Ethyl 4-oxopentanoate (11.5 g, 0.08 mol) was dissolved in dichloromethane (dried over calcium hydride) and 10 mL (0.12 mol) of 1,2-ethanedithiol was added followed by 2 mL of boron trifluoride etherate. The solution was stirred at room temperature overnight and 100 mL of 5% sodium

hydroxide added. The organic layer was separated and washed with water and dried over $MgSO_4$. Evaporation of the solvent and subsequent distillation of the residue afforded 15g (85%) of ethyl 4-ethylenethioketalpentanoate. Bp 112°C/0.5 mm.

Mass spectrum: (MW=220) m/z 119(100%), 59(20%), 175(17%), 29(15%), 61(14%), 87(13%), 115(13%), 220(12%), 87(10%), 205(2%).

NMR Spectrum: δ 1.3(t,3H,-CH$_3$); 1.8(s,3H,CH$_3$-); 2.25(t,2H,$\underline{CH}_2$-CH$_2$-CO);

2.6(t,2H,CH$_2$-$\underline{CH}_2$-CO); 3.3-3.4(m,4H,CH$_2$-CH$_2$-S); 4.7(q,2H,OCH$_2$).

b. Synthesis of 4-carboethoxy-2-ethylenethioketal-

5-hydroxyheptane

To diisopropylamine (8.2 mL, 0.06 mol) in 100 mL THF at 0°C was added dropwise n-butyllithium (38 mL, 0.06 mol). The mixture was stirred for 15 min and cooled to -78°C and ethyl 4-ethylenethioketal-pentanoate (11 g, 0.05 mol) in THF was added dropwise and the mixture stirred for 60 min. Then propionaldehyde (4 mL, 0.055 mol) was added and the reaction mixture allowed to stir for 2.5 hours. The mixture was then quenched with 15% HCl and extracted with ether. The etheral extract was washed with water and saturated $NaHCO_3$ and dried ($Na_2SO_4$). After evaporation of the ether the residue was distilled under reduced pressure and afforded 6 g of the product (thick yellowish liquid) which was shown to be homogeneous by GCMS.

Mass spectrum:(MW=278) m/z 119(100%), 29(35%), 59(30%), 185(27%), 43(25%), 111(22%), 61(21%), 139(20%), 120(15%), 121(14%), 220(10%), 159(8%), 186(7%), 205(4%), 233(2%), 278(1%).

c. Synthesis of 4-carboethoxy-2-ethylenethioketal-4-heptene

Dehydration of 4-carboethoxy-2-ethylenethioketal-5-hydroxyheptane was carried out using methanesulfonyl chloride and potassium hydride. Hydroxy compound (4 g, 0.015 mol), triethylamine (3 mL, 0.02 mol), and dichloromethane (40 mL) were cooled to 0°C. Methanesulfonyl chloride (1.6 mL, 0.02 mol) in dichloromethane was added dropwise and the mixture stirred for 60 min. The precipitate formed was filtered off and the solvent removed by flash evaporation. The mesylate was then taken up in dry THF and potassium hydride (1.2 g, 0.03 mol) added at 0°C and the reaction mixture stirred for 12 hours at room temperature following which unreacted potassium hydride was neutralized with t-butanol and water. The mixture was extracted with ether and dried ($Na_2SO_4$). GCMS analysis of the crude product showed five peaks. Two of these peaks in the TIC trace corresponded to the two geometric isomers of the desired product with one of these isomers being the major component. The other three peaks were not identified.

Mass spectrum: (MW=260) Isomer with the retention time of 19.18 min: m/z 127(100%), 29(58%), 199(45%), 132(22%), 45(21%), 119(21%), 59(20%), 99(20%), 41(19%), 61(18%), 74(15%), 200(14%), 155(10%), 159(9%), 187(6%), 260(5%), 215(3%).

Mass spectrum: Isomer with the retention time of 20.24 min: m/z 199(100%), 127(85%), 29(60%), 99(29%), 200(28%), 74(27%), 112(27%), 155(25%), 61(22%), 41(20%), 45(18%), 59(17%), 159(14%), 187(10%), 260(6%), 215(2%).

### d. Removal of the 1,3-dithiolane protecting group

Cleavage of the dithiolane group was accomplished with mercuric chloride in the presence of cadmium carbonate. A portion of the product mixture from above (c) (2.5 g) was dissolved in acetone (100 mL) and water (10 mL), mercuric chloride (2 g), and cadmium carbonate(2 g) were added and the mixture stirred at room temperature for 24 hours. At this stage additional mercuric chloride (0.8 g) and cadmium carbonate (0.8 g) were added and stirring continued for another 72 hours. Then the mixture was filtered and the acetone removed. The residue was dissolved in ether and the etheral solution washed successively with water, 10% potassium iodide, water and dried over anhydrous $Na_2SO_4$. After removal of the ether the residue was analyzed by GCMS. The TIC plot showed 9 peaks two of which (minor components) corresponded to the two isomers of the desired product.

Mass spectrum: (MW=184) Peak with the retention time of 9.06 min: m/z 111(100%), m/z 43(90%), 29(15%), 55(12%), 112(11%), 184(9%).

Mass spectrum: Peak with the retention time of 9.65 min: m/z 111(100%), 43(65%), 55(20%), 29(19%), 184(10%), 112(9%).

A portion of the product from above was then treated with alkali and stirred for 3 days following which it was acidified and extracted with ether. The ether was evaporated and two portions of the residue were taken and derivatized to give PFB and t-BDMS derivatives for NICI and EI analysis, respectively. The mass spectra and retention times were then compared to those from urine extract.

3. Synthesis of ethyl 2-propyl-3-oxopentanoate

To isopropylcyclohexylamine (16.9 g, 0.12 mol) in dry THF (80 mL), at 0°C, was added n-butyllithium (77 mL, 0.12 mol) dropwise and the mixture was stirred for 15 min. The mixture was cooled to -78°C and ethyl pentanoate (13 g, 0.1 mol) followed by propionyl chloride (9.3 g, 0.1 mol) in THF were added and stirring continued for 25 minutes. Then the reaction mixture was quenched with 15% HCl and extracted with ether. After the usual work-up and fractional distillation pure ethyl 2-propyl-3-oxopentanoate was obtained. Bp 86°C/0.1 mm.

Mass spectrum: (MW=186) m/z 57(100%), 29(72%), 101(33%), 55(20%), 73(19%), 130(10%), 144(9%), 43(8%), 157(4%).

4. Synthesis of ethyl 2-propyl-3-hydroxypentanoate

n-Butyllithium (77 mL, 0.12 mol) was added dropwise to diisopropylamine (12 mL, 0.12 mol) in dry THF at 0°C, over a 20 min period. The mixture was allowed to stir for a further 15 min and cooled to -78°C and ethyl pentanoate (15.6 g, 0.12 mol) in 10 mL THF was added dropwise and the mixture stirred for 60 min. Then propionaldehyde (7.4 g, 0.1 mol) was added and the stirring continued for 2 hours. The reaction mixture was neutralized with 15% HCl and extracted with ether. Following flash evaporation of the ether the residue was distilled to yield ethyl 2-propyl-3-hydroxypentanoate. BP 92°C/4 mm. [Lit. (Acheampong et al., 1983) bp 70-72°C/0.2 mm].

Mass Spectrum: (MW=188) m/z 101(100%), 73(85%), 55(65%), 29(45%), 57(38%), 130(32%), 113(30%), 84(20%), 41(15%), 159(12%), 143(10%).

## III. RESULTS AND DISCUSSION

### A. NICI GCMS assay development

A highly sensitive assay has been developed for VPA in serum and saliva based on the NICI-GCMS of the PFB ester. The assay was employed to quantitate VPA in serum and saliva in five volunteers at VPA steady state. The assay is also applicable to VPA metabolites, although no quantitation of the metabolites was made in the present study. The chromatographic characteristics of the PFB derivatives of all VPA metabolites have been determined and selected ion chromatograms obtained. The assay development is discussed below from the point of view of derivative formation, initial GC-ECD analysis, optimization of mass spectrometer parameters, and selection of a suitable internal standard.

### 1. Derivatization

Derivatization with PFBB was carried out by a modification of the method of Min et al. (1980) for the derivatization of a trimethyl prostaglandin $E_2$ analog. This is based upon the observation that the $C_1$ carboxyl group of prostaglandins undergoes facile reaction with benzylic halides. To form the PFB derivatives of VPA and its metabolites samples were heated at 40°C for 45 minutes in the presence of 10 µL of 30% PFBB solution in ethyl acetate and 10 µL of diisopropylethylamine and this procedure was used throughout the study. Longer reaction times or higher temperatures produced a yellow gummy

substance. Initially triethylamine was used, but was replaced with diisopropylethylamine, a bulky amine, which minimizes quaternary ammonium salt formation with PFBB (Wickramasinghe et al., 1973).

In most of the NICI work done with prostaglandins, solvent is removed after the extraction step and the residue is reconstituted with acetonitrile prior to derivatization. With VPA and its metabolites, such a procedure would not be desirable because of their low molecular weight compared to prostaglandins and hence complete solvent evaporation before derivatization was thought to result in loss of sample. Because of the presence of the extracting solvent (ethyl acetate), there was evidence of the esterification of a small amount of hydrolyzed ethyl acetate; however this was not a problem.

Removal of the excess PFBB prior to injection of derivatized sample into the GCMS did not prove possible without loss of peak intensities (see below). Because of the excess reagent solvent was diverted and scanning commenced 4 minutes after sample injection to reduce the amount of reagent entering the mass spectrometer ion source. The derivatizing procedure adopted appears to result in optimum derivatization since no additional peaks were observed upon attempts to methylate the components of the reaction mixture after the derivatization step with PFBB. A typical total ion chromatogram trace for the PFB esters of VPA and OA in the negative ion mode is shown in Figure 2.

2. GC-Electron Capture Detection (ECD)

Figure 3 shows typical GC-ECD chromatograms of the PFB derivatives of VPA and OA. Since GC with ECD is in principle similar to electron
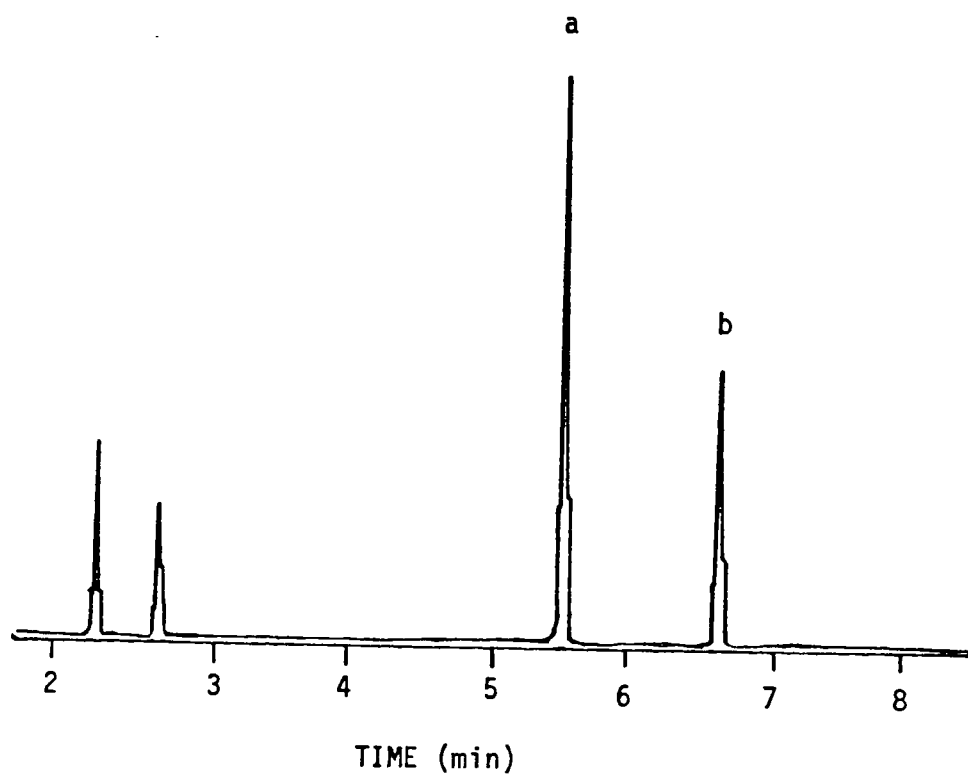
Figure 2.    Total ion current plot of the PFB esters of VPA(a) and OA(b) in the NICI mode.

capture NICI, it was of interest to initially evaluate the assay with GC. When the derivatization procedure outlined above was used and 1 or 2 μL of sample injected into the GC, there was a high background due to the excess derivatizing reagent. To remove most of the excess PFBB the following procedures were evaluated.

a. Treatment with alkali

After the derivatization step, solvent was removed under $N_2$ and the residue treated with 400 μL of 3N NaOH and/or 400 μL of 10N NaOH and extracted with ethyl acetate. This, however, did not remove excess reagent because the product presumably formed is pentafluorobenzyl alcohol which is soluble both in water and most organic solvents.

b. Evaporation of the reaction mixture at elevated temperature (40-60°C).

The reagent peak was significantly reduced but so were analyte and internal standard peaks. PFBB is not very volatile relative to VPA-PFB and conditions required to evaporate excess reagent invariably cause loss of the sample.

c. Selective solvent extraction

To selectively remove excess PFBB, different solvents (ethyl acetate, hexane, heptane, iso-octane etc.) were employed. The reaction mixture was made basic, extracted with one of the solvents and re-extracted with another solvent of differing polarity. It was found that both derivative and the PFBB reagent were soluble in all solvents.

d. Column chromatographic separation

Silica gel and sephadex LH-20 short columns were used in an attempt to separate the derivatives from PFBB; but, again separation was not possible.

When a different derivatization procedure that used 0.5% PFBB solution in the presence of 18-crown-6 and potassium acetate was employed, it was possible to obtain the chromatograms shown in Figure 3. An acceptable calibration curve for VPA in ethyl acetate using OA as internal standard was also obtained (Figure 4.) This procedure adopted from Rubio and Garland (1985) uses a lesser amount of PFBB and is supposedly advantageous in that phenols are not derivatized because of the use of the less basic potassium acetate.

### 3. Optimization of MS parameters for NICI

It is known that under CI conditions a number of factors influence the spectrum obtained and hence the sensitivity. During our initial work with NICI, we experienced lack of reproducible sensitivity. The area counts obtained for the same concentration of derivatized VPA on different runs and days varied at times by more than a factor of 100. There was also filament sag and the NICI conditions tended to destroy the electron multiplier. Efforts to manipulate instrument variables (electron energy, source pressure, etc) in order to get reproducible sensitivities did not prove successful. The lack of reproducibility at that time can be explained by the fact that it was not possible to maintain reagent gas flow at a reasonably constant value. The other problems such as filament sag were probably due to the fact that the pressure gauge reading did not reflect the actual pressure in the ion source.

With the instrument in apparently good working order, and with the tune values remaining reasonably constant, the effects of important
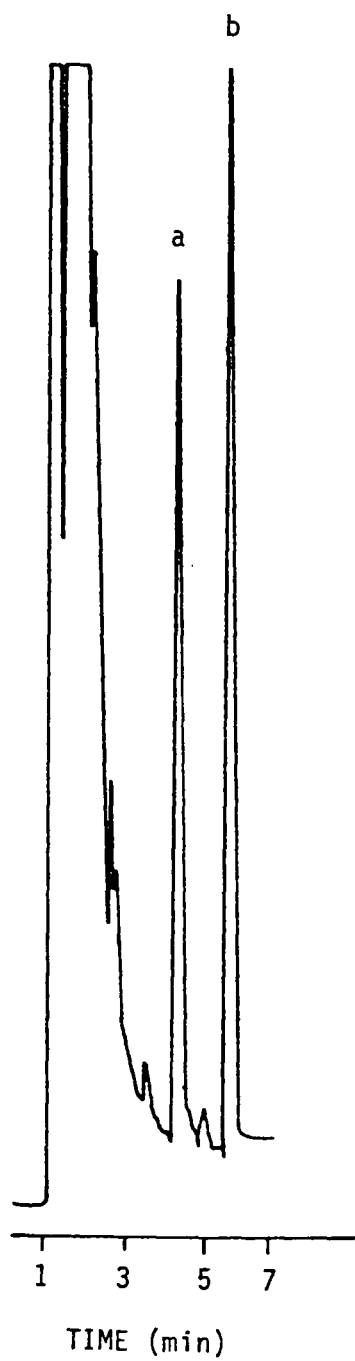
Figure 3.    Typical chromatograms of the PFB derivatives of VPA(a) and OA(b) obtained with GC-ECD.
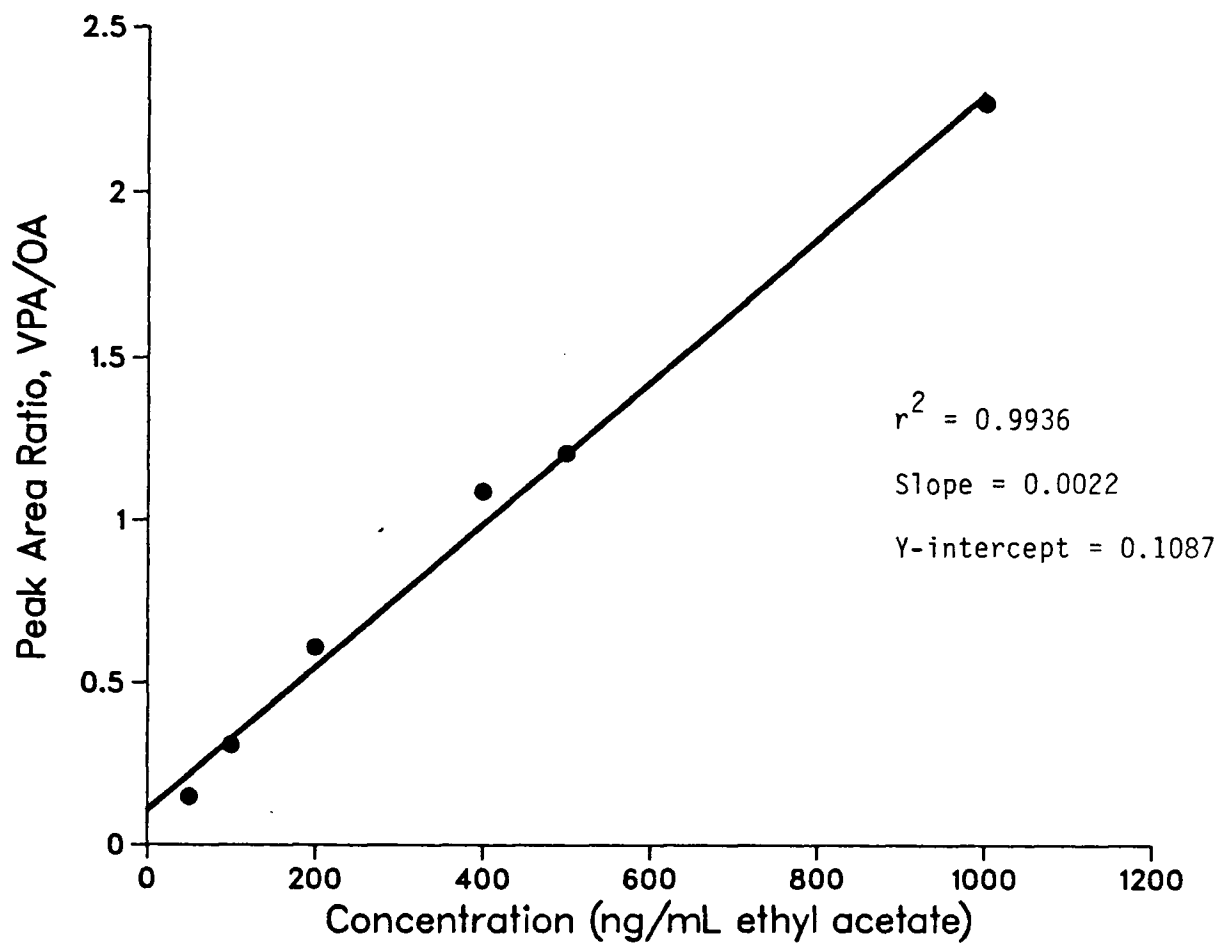
Figure 4.    Calibration curve for PFB derivatized VPA in ethyl acetate obtained using GC-ECD.

mass spectrometer variables on the sensitivity to VPA-PFB were determined. Source temperatures tried were 150, 190 and 240°C. Relative sensitivities of the VPA -PFB peak were 7.56, 2.94 and 1.0 respectively.

The sensitivity thus appeared to increase as the source temperature decreased, but for routine analysis a compromise must be made between an optimum low temperature and the consideration of the ion source becoming excessively contaminated at lower temperatures. Most of the analysis was, therefore, carried out at a source temperature of 200°C.

When varying the source pressure sensitivity was maximal at about the highest pressure tolerated by the instrument (1 torr). For pressures less than 1 torr, the sensitivity decreased dramatically until at low pressures i.e less than 0.3 torr there was no signal at all.

The highest response for the VPA-PFB derivative was obtained near the highest electron energy handled by the instrument (240eV). At electron energies less than 100eV the monitored ion, m/z 143⁻, was not detected.

All the above instrumental parameters are interrelated. For example, sensitivity depends upon the extent to which newly formed anions are stabilized by reagent gas molecules, but since collision can also lead to electron detachment, sensitivity will also depend upon the internal energy of the reagent gas so that sensitivity will be highly influenced by source temperature. The ionizing electron energy used also depends upon the value of the source pressure. Because it is

difficult to assess absolute enhancement of sensitivity, the effect of these interdependent parameters is measured approximately by varying one parameter while maintaining the others at some optimal value.

The effect of reagent gases on the sensitivity of the VPA-PFB was also investigated using methane, ammonia, and argon-methane (95:5). Ammonia gave an apparently greater sensitivity, which is similar to that reported by Miyazaki et al. (1984) for prostaglandins, but resulted in short filament life. There was no difference between methane and argon-methane with respect to sample response or background ions. No carbon containing ions of higher masses (ion/molecule adducts) were observed with methane and this gas was used throughout all subsequent experiments.

In spite of its high sensitivity the reported use of NICI is limited because in addition to other reasons (e.g. availablility of the instrument), poor reproducibility of NICI mass spectra is often mentioned as a restricting factor (Oehme et al., 1986). Furthermore, a drawback of most instruments with negative ion detection capability is that important parameters such as temperature and pressure can only be measured approximately. In our work with VPA we had difficulty getting reproducible sensitivity and some changes in the mass spectrometer were required to improve the reproducibility. The reagent gas flow controller of the mass spectrometer was replaced with a simple needle valve. That meant the source pressure gauge indicator gave closer values to the actual source pressure. A modification to the source heater was also made. These changes brought a dramatic improvement in reproducibility and, to a lesser extent sensitivity. In our experience,

we found ion source pressure to be a critical parameter for obtaining reproducible sensitivities.

4. Comparison of the relative sensitivity of derivatized VPA by EI and NICI

Once reproducible sensitivities were obtained, it was possible to determine the relative sensitivity of two fluorinated derivatives to that of the t-BDMS derivative. The NICI and EI spectra of the PFB and bis-TFMB derivatives of VPA are shown in Figures 5 and 6. The fluorinated derivatives were analyzed in both the EI and NICI modes while the t-BDMS derivative was analyzed by EI and the m/z 201 $(M-57)^+$ ion monitored. In the NICI spectra of both fluorinated derivatives, the base anion is m/z 143 corresponding to the loss of the pentafluorobenzyl and 3,5-bis(trifluoromethyl)benzyl moieties. In the EI spectra, the base peaks are m/z 181 (PFB) and m/z 227 (bis-TFMB) which are the complementary positive ions to those of the NICI. Since these ions represent moieties introduced by derivatization, the EI spectra of these derivatives lack specificity. All three derivatives were prepared in a manner that the final concentration in the reaction vial was an amount of derivative equivalent to 5 ng/µL of VPA. The result obtained after analyzing the samples is shown in Table 1. The area counts are the means of five determinations. In the EI mode, the sensitivity of all three derivatives was similar. In the NICI mode, the PFB derivative was found to be 30-50 times more sensitive than the t-BDMS derivative by EI. The PFB derivative (NICI mode) also proved to be about 5 times more sensitive than the similar fluorinated derivative, bis-TFMB.
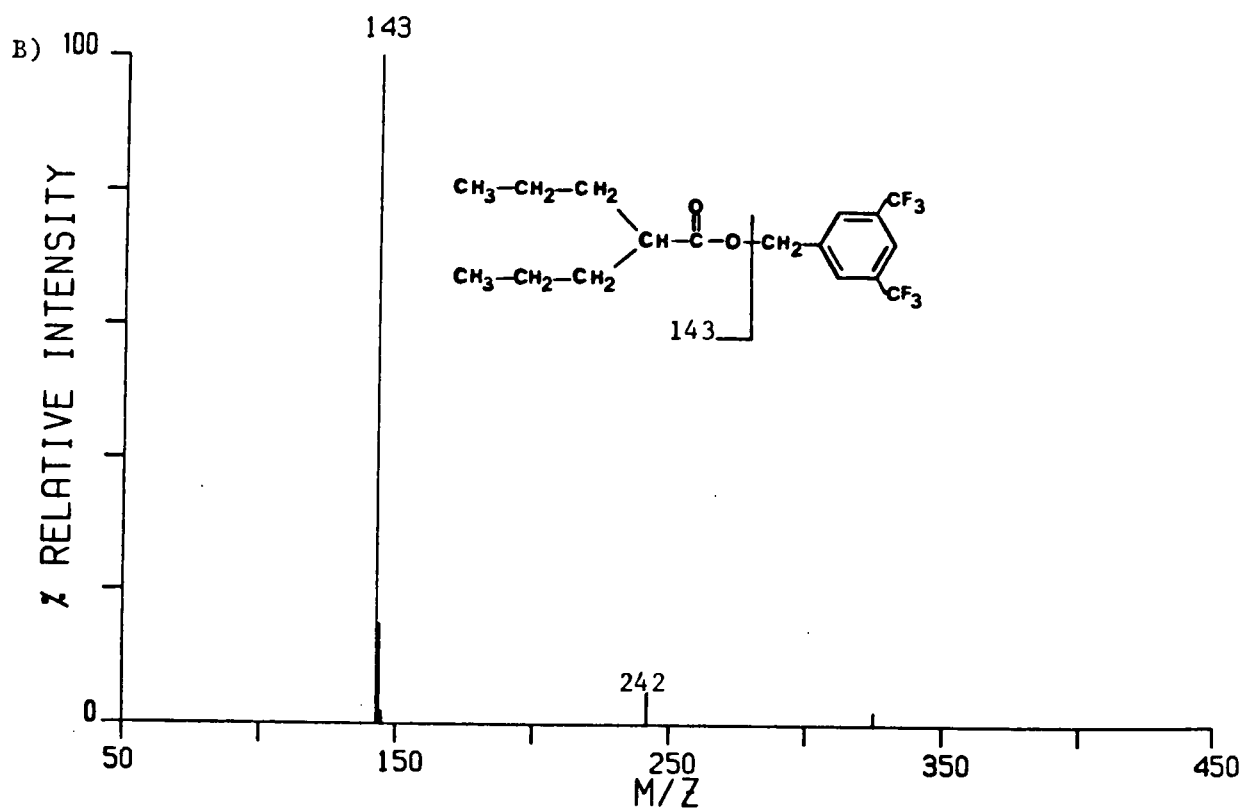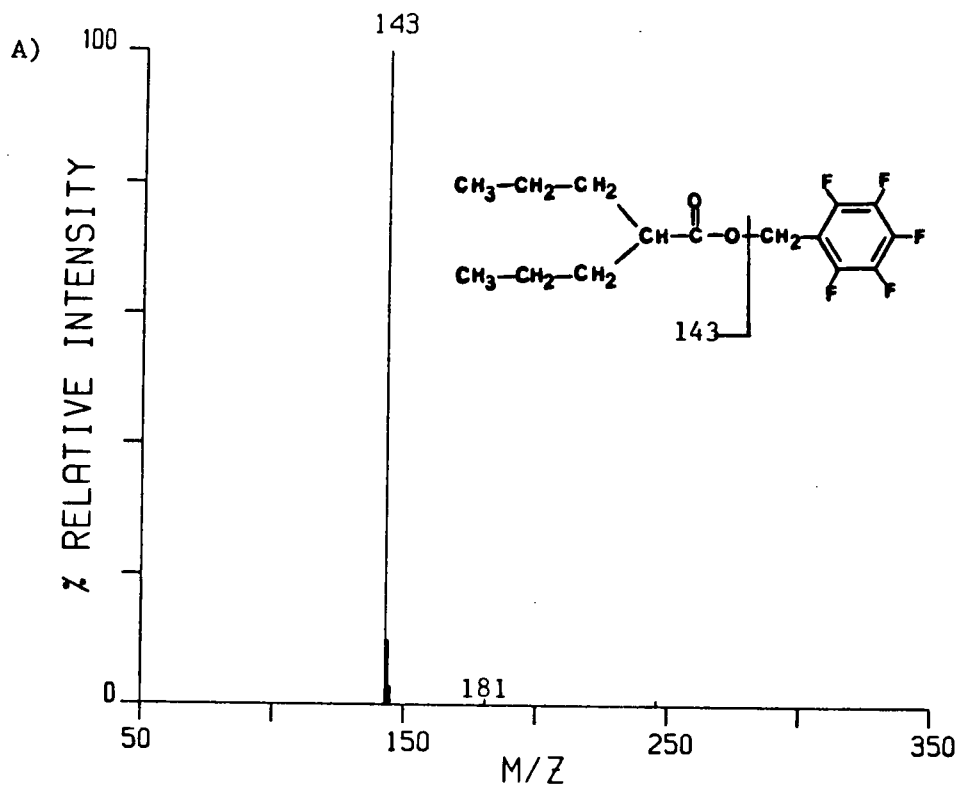
Figure 5.    NICI mass spectra of the   PFB(A) AND bis-TFMB(B) derivatives
of VPA.
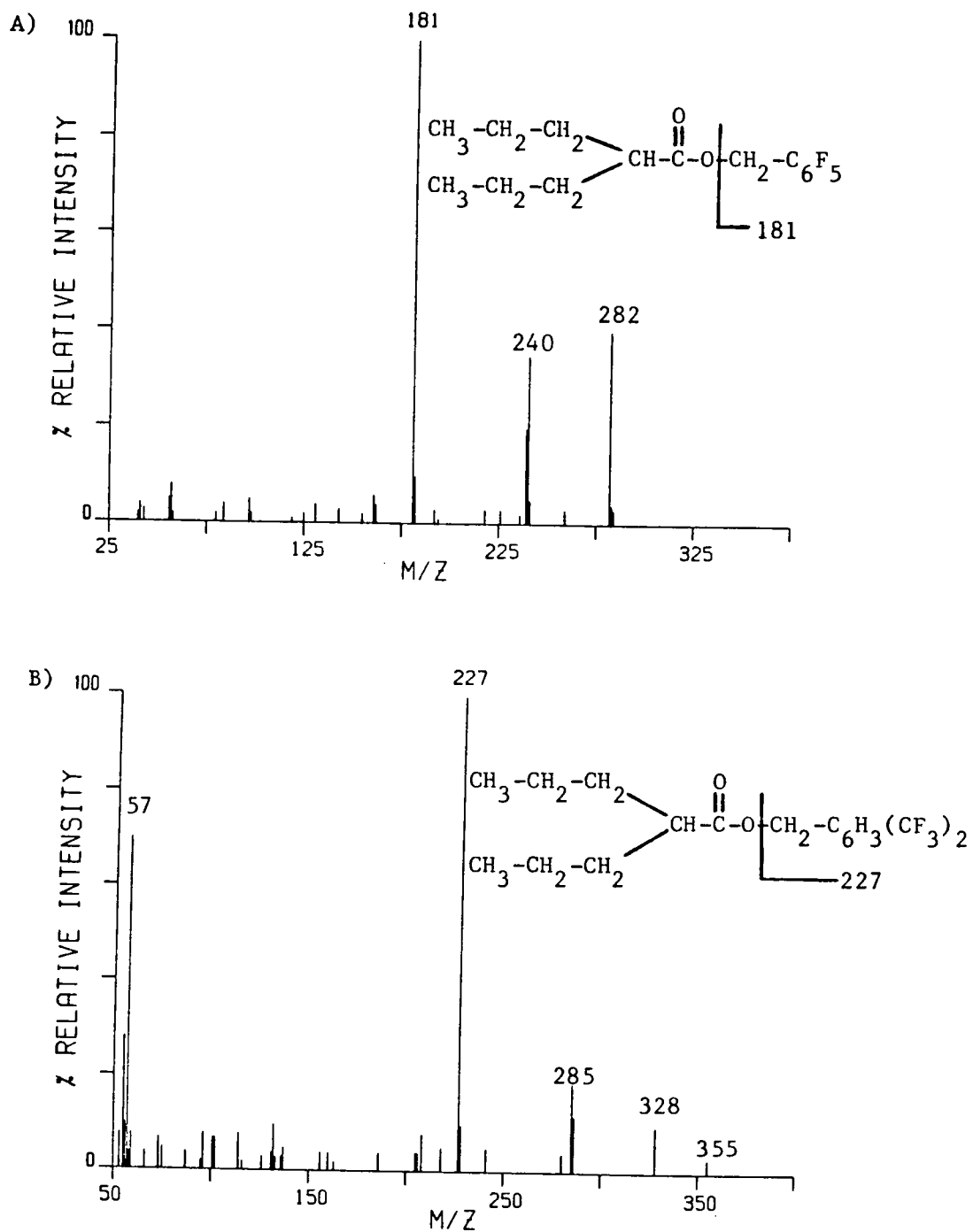
Figure 6. EI mass spectra of the PFB(A) and bis-TFMB(B) derivatives of VPA.

5. Quantitative analysis with the PFB derivative

    a. Internal standard

The internal standard used throughout the present study was $[^2H_6]$-VPA. The use of an internal standard that gives a common ion (OA) was also investigated. The result of the analysis of serum spiked with seven different concentrations (10 to 800 ng/mL) of VPA with the same amount of either $[^2H_6]$-VPA or OA as internal standard is shown in Table 2. The results are the means of three determinations. Figure 7 shows typical selected ion chromatograms of the PFB derivatives of VPA, $[^2H_6]$-VPA and OA extracted from serum.

As seen from Table 2, the calibration curve with $[^2H_6]$-VPA as the internal standard is superior to that with OA. The intercepts are much closer to the origin and the intra-assay variability is lower in the case of $[^2H_6]$-VPA. In GCMS assays using SIM, both stable isotope-labelled analogs and chemically related compounds have been used as internal standards. Claeys et al. (1977) evaluated the precision of assays using either internal standard and concluded that stable isotope-labelled internal standards produced the lowest variance factors due to sample manipulation and instrumental errors. On the other hand, Lee and Millard (1975) have argued that a substance is most accurately determined using an internal standard giving a common ion because of the advantage of monitoring a single ion and hence a gain in sensitivity and stability. Under CI conditions, though, because of the extreme dependence of the spectra on source pressure and temperature it can be said that more accurate measurements can be made with stable isotope-labelled internal standard since its physicochemical properties closely approximate that of the analyte. Our results seem to support this.

TABLE 1. Comparison of the relative sensitivities of three VPA derivatives*.

| Derivative | Mode of ionization | Ion monitored | Area count | Relative sensitivity NICI/EI |
|------------|--------------------|---------------|------------|------------------------------|
| PFB        | EI                 | 181           | 8,714      | 47                           |
|            | NICI               | 143           | 412,000    |                              |
| bis-TFMB   | EI                 | 227           | 8,228      | 13                           |
|            | NICI               | 143           | 103,500    |                              |
| t-BDMS     | EI                 | 201           | 10,184     |                              |

* Amount injected in all cases was an amount of derivative equivalent to 5 ng of VPA.

TABLE 2. Comparison of $[^2H_6]$-VPA and OA as internal standards*

|                          | $[^2H_6]$-VPA | OA        |
|--------------------------|---------------|-----------|
| Intra-assay Variation    | < 5%          | 8-15%     |
| $r^2$                    | 0.9986        | 0.9960    |
| y-intercept              | - 0.0027      | 0.1020    |
| x-intercept              | 2.2483        | - 12.0155 |

* Results obtained with seven different concentrations of VPA ranging from 10 to 800 ng/mL. The samples were assayed three times and values shown are means of the three determinations.
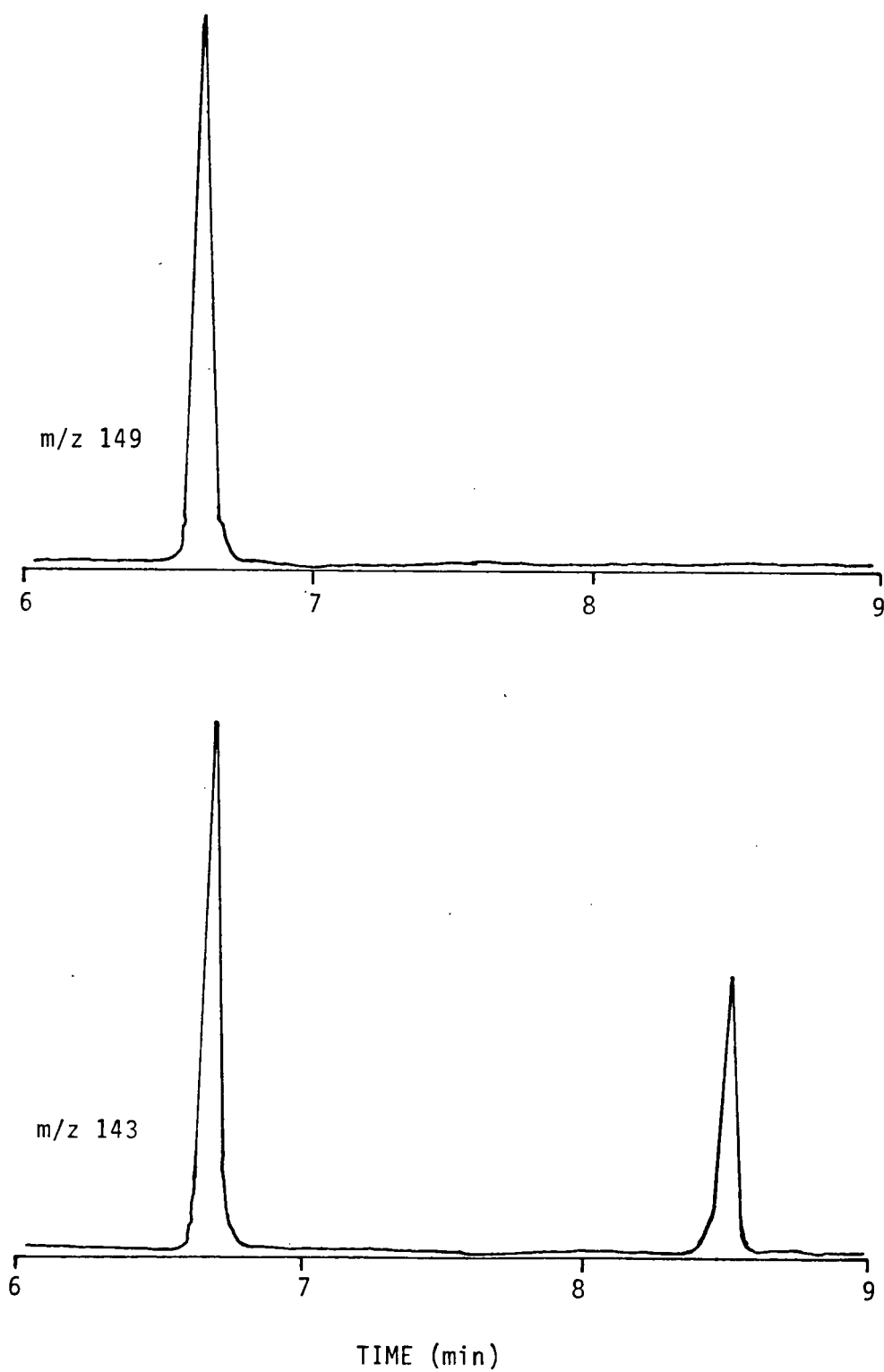
Figure 7.    SIM chromatograms of VPA, OA (m/z 143) and $[^2H_6]$-VPA (m/z 149)
from serum spiked with these substances.

b.  Analytical parameters

The extraction procedure employed was a modification of that of
Abbott et al. (1986a), modified to accommodate the small serum sample
volume (100 µL). For the extraction of saliva samples a similar
procedure was used, but samples were not treated with alkali prior to
acidification. The recovery of drug from serum and saliva was greater
than 95% compared to drug extracted from water.

The calibration curves for VPA using $[^2H_6]$-VPA as internal
standard were obtained by monitoring the intense peaks at m/z 143 and
149 which are the base ions in the NICI spectra of VPA-PFB and
$[^2H_6]$-VPA-PFB, respectively.  The coefficient of determinations,
$r^2$, were greater than 0.996 for both serum (free and total) and
saliva.  The calibration curves for serum  total, serum free, and
saliva VPA are shown in Figures 8, 9 and 10 respectively.  The
calibration curve for the EI(t-BDMS) determination of serum total VPA
is given in Figure 11.

The precision of the NICI assay using PFB derivatives was very
good with an intra-and inter- assay variation of less than 10%
(coefficient of variation) at 10-800 ng/mL of VPA in serum. The within
assay and between assay standard deviations of the slope of the
calibration curve were 0.000003 and 0.000021, respectively (C.V.=0.299%
and 1.92%). Linearity was observed over the concentration range of 10
ng/mL to 25 µg/mL. The lower limit of detection was 2 ng/mL of VPA
based on a 200 µL sample of serum. Figure 12 shows the SIM chromatogram
of VPA obtained with 10 pg of VPA extracted from serum. This limit of
detection is similar to most of the reported values for PFB derivatives
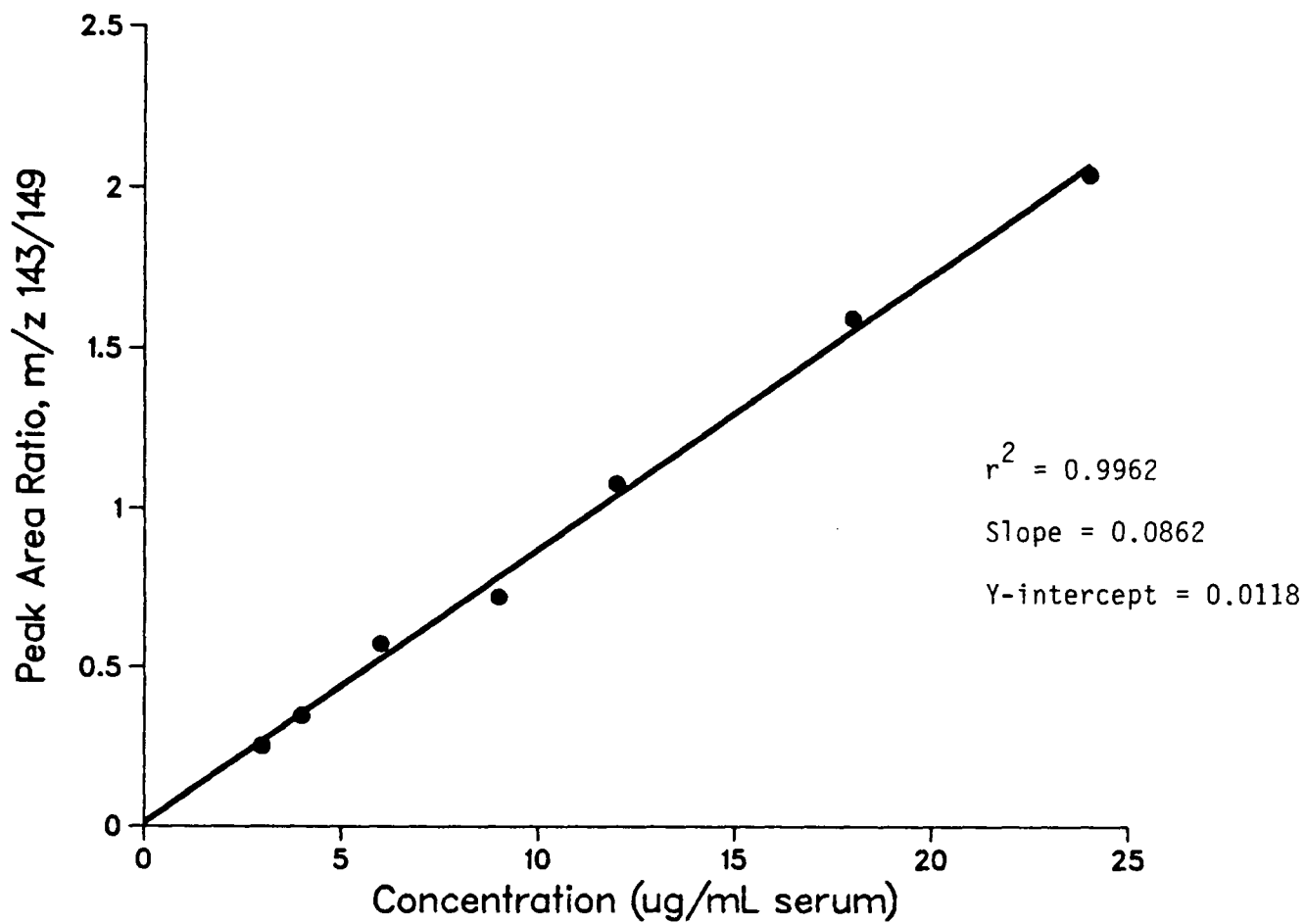
Figure 8.    Calibration curve for serum total VPA. Peak area ratio was
obtained by monitoring m/z 143 (VPA) and m/z 149 ($[^2H_6]$-VPA).
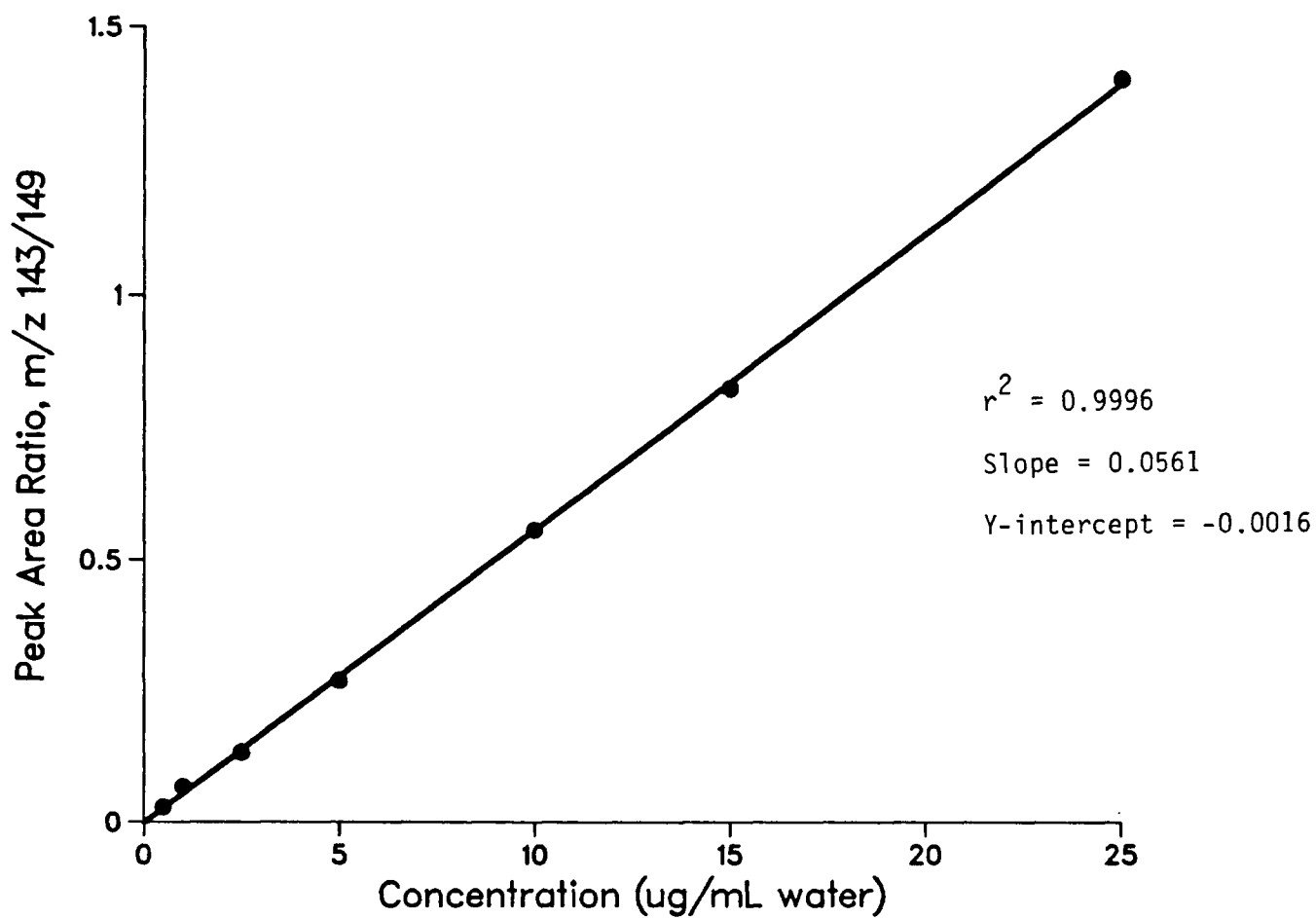
Figure 9.    Calibration curve for serum free VPA. Peak area ratio was obtained by monitoring m/z 143 (VPA) and m/z 149 ($[^2H_6]$-VPA).
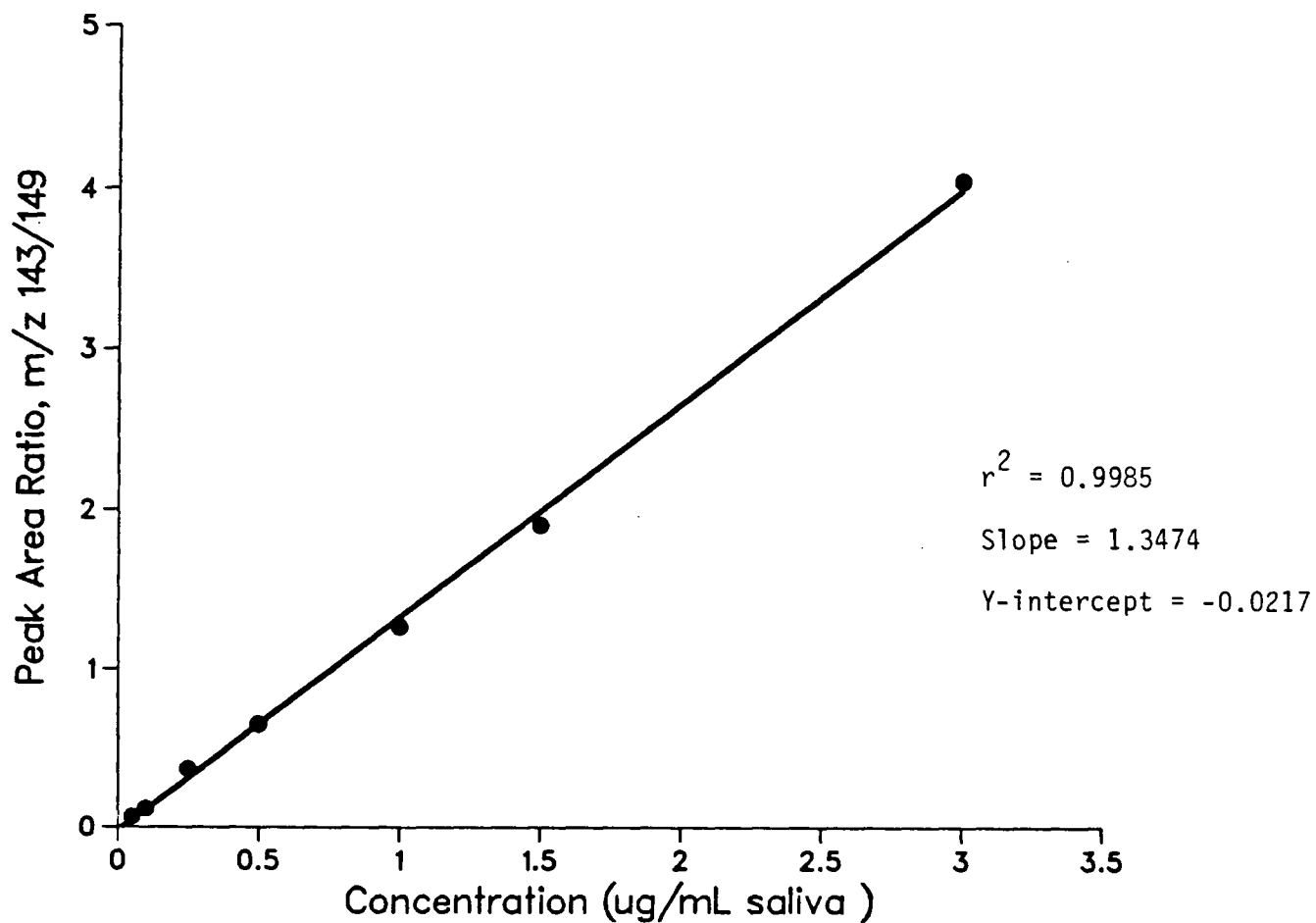
Figure 10.   Calibration curve for saliva VPA. Peak area ratio was obtained by monitoring m/z 143 (VPA) and m/z 149 ($[^2H_6]$-VPA).

$r^2$ = 0.9980

Slope = 0.0141

Y-intercept = -0.0344
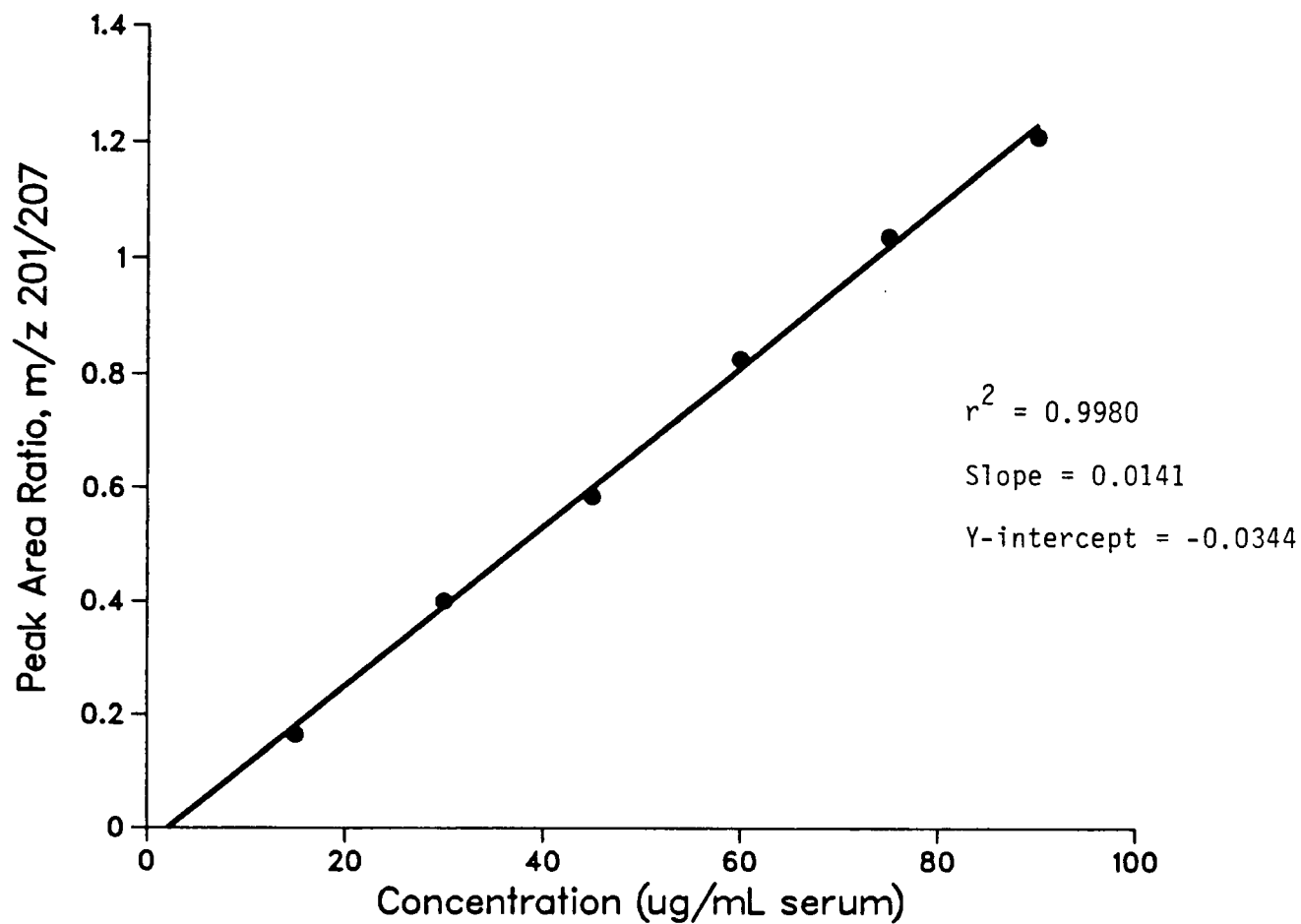
Figure 11.  Calibration curve for EI(t-BDMS) determination of serum total VPA. Peak area ratio was obtained by monitoring m/z 201 (VPA) and m/z 207 ($[^2H_6]$-VPA).

of prostanoids. In some cases, with prostaglandins limits of detection as low as 200 fg have been reported with ammonia as the reagent gas (Miyazaki et al., 1984). The lower limit of detection obtained with VPA-PFB is not as good as the above example probably because of the fact that VPA is a small molecule and hence with less surface area for electron capture.

To assess how well the developed NICI assay compared with an EI (t-BDMS) assay routinely used for quantitation of VPA in our laboratory, serum samples of two volunteers were measured by both EI and NICI GCMS methods. These values are given in Table 3, and Figure 13 shows the correlation of the serum values obtained by the two methods for one of the volunteers. As seen from Table 3 and Figure 13, agreement between EI and NICI methods for serum VPA is excellent. Mean values were 28.4 vs 27.1 (B.A) and 40.3 vs 40.2 (F.A) and correlations (r) were 0.98 and 0.99, respectively.

B. VPA levels in serum (free and total) and saliva before and after the administration of CBZ

A total of 63 paired saliva and serum samples were analyzed for VPA by NICI in SIM mode; 33 samples before CBZ and 30 after CBZ. The serum total, serum free and saliva levels of VPA in the five volunteers are presented in Tables 4 to 8. The relationships between serum total, serum free and saliva, as well as the correlations between each other are also given in these tables.

Tables 9 and 10 show the time-averaged ratios between serum free and serum total, saliva and serum total, and saliva and serum free
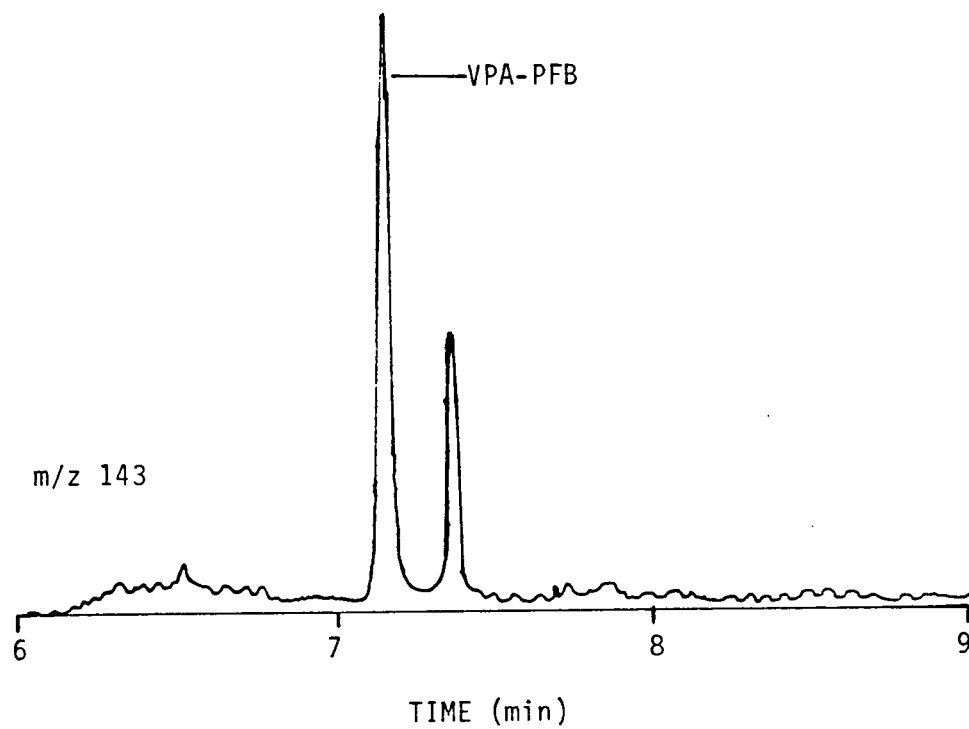
Figure 12.    SIM chromatogram of PFB derivatized VPA obtained with 10 pg
of VPA extracted from serum (the second peak is background
from serum).

TABLE 3. Serum VPA levels (µg/mL) in two subjects on VPA steady-state as measured by EI (t-BDMS) and NICI (PFB).

| | BA | | FA | |
| --- | --- | --- | --- | --- |
| EI | NICI | EI | NICI |
| 40.31 | 42.36 | 44.35 | 44.45 |
| 40.56 | 42.44 | 58.34 | 58.77 |
| 42.50 | 43.78 | 41.20 | 40.59 |
| 25.93 | 20.56 | 48.20 | 46.84 |
| 23.20 | 22.27 | 48.05 | 46.06 |
| 27.97 | 25.90 | 33.41 | 33.61 |
| 25.03 | 21.07 | 22.70 | 24.15 |
| 33.80 | 31.05 | 51.25 | 53.24 |
| 31.78 | 30.36 | 45.27 | 43.80 |
| 27.61 | 26.65 | 42.15 | 42.02 |
| 26.05 | 23.60 | 34.17 | 33.34 |
| 21.85 | 19.01 | 29.02 | 26.99 |
| 16.94 | 16.40 | 26.04 | 28.02 |
| 14.35 | 13.26 | | |

Mean: 28.42    27.053    40.319    40.145

Correlation(r):    0.9830    0.9913
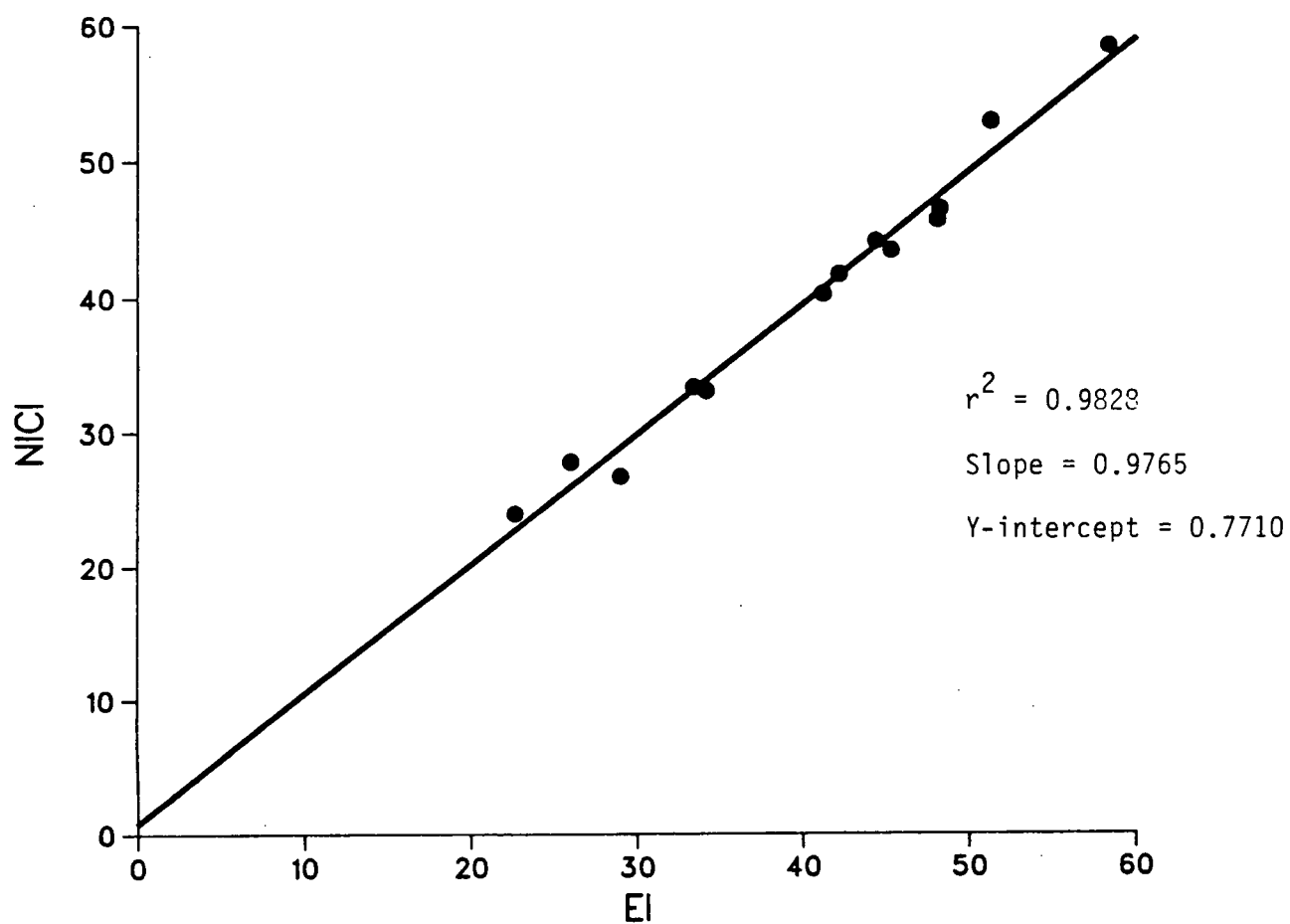
Figure 13.   Relationship between VPA concentrations (ug/mL) in serum
determined by EI(t-BDMS) and NICI (PFB).

while in Table 11 the % change in average VPA concentration in all three biological samples after CBZ administration is presented. Figure 14 is the saliva concentration-time profile for the five volunteers. In Figure 15 the concentration time curves for saliva, free, and total VPA for one volunteer are shown. Figures 16 and 17 are the concentration time curves for serum free and saliva VPA before and after CBZ in one volunteer. Curves showing the degree of correlation between saliva and both serum total and free VPA before and after CBZ for one of the volunteers are given in Figures 18 and 19. The relationship between serum total and saliva and between serum free and saliva for all the volunteers are shown in Figures 20 and 21.

1. Effect of CBZ on serum and saliva levels of VPA

The % decrease in average VPA concentration after CBZ was 27.91 ± 3.48, 36.85 ± 13.64, and 48.13 ± 7.70 (Table 11), for serum total, serum free, and saliva, respectively. There was a significant reduction (P<0.025) of VPA concentration in all three biological fluids. The % decrease is higher for serum free and saliva compared to serum total for all volunteers except one (R.M.). The greater % decrease of serum free VPA and saliva VPA after CBZ is because the free fraction declined with decreasing total serum VPA concentrations. The mean free fraction for the five subjects decreased from 0.1334 to 0.1072 after CBZ (Tables 9 and 10).

Because VPA is a highly protein-bound drug, VPA is likely to undergo drug interactions at the protein binding level. VPA is known to displace phenytoin from its binding site on serum albumin, and

TABLE 4a. Serum total, serum free and saliva concentrations (µg/mL) of VPA and their relationship to each other in volunteer W.T. before the administration of CBZ.

| Time (h) | Serum total | Serum free | Saliva | Saliva: total ratio | Saliva: free ratio | Free: total ratio |
|---|---|---|---|---|---|---|
| 1 | 52.95 | 6.75 | 0.891 | 0.017 | 0.132 | 0.127 |
| 2 | 54.57 | 5.29 | 1.020 | 0.019 | 0.193 | 0.097 |
| 3 | 47.50 | 4.60 | 1.114 | 0.023 | 0.242 | 0.097 |
| 5 | 41.30 | 4.36 | 0.756 | 0.018 | 0.173 | 0.106 |
| 7 | 38.50 | 3.15 | 0.472 | 0.012 | 0.150 | 0.082 |
| 9 | 32.47 | 2.45 | 0.323 | 0.010 | 0.132 | 0.076 |
| 12 | 25.80 | 1.88 | 0.345 | 0.013 | 0.184 | 0.073 |
| Mean | | | | 0.0160 | 0.1723 | 0.0940 |
| S.D. | | | | 0.0042 | 0.0361 | 0.0175 |
| C.V. | | | | 26.25% | 20.95% | 18.62% |

Correlation (r): Between serum total and saliva = 0.8945
: Between serum free and saliva = 0.8254
: Between serum total and free = 0.9400

TABLE 4b. Serum total, serum free and saliva concentrations (μg/mL) of VPA
and their relationship to each other in volunteer W.T. after the
administration of CBZ.

| Time (h) | Serum total | Serum free | Saliva | Saliva: total ratio | Saliva: free ratio | Free: total ratio |
|---|---|---|---|---|---|---|
| 1 | 41.26 | 3.83 | 0.697 | 0.017 | 0.181 | 0.093 |
| 2 | 35.97 | 3.19 | 0.683 | 0.019 | 0.214 | 0.089 |
| 3 | 31.71 | 3.08 | 0.499 | 0.016 | 0.162 | 0.097 |
| 5 | 29.75 | 2.15 | 0.427 | 0.014 | 0.198 | 0.072 |
| 7 | 23.02 | 1.62 | 0.273 | 0.012 | 0.168 | 0.070 |
| 9 | 20.71 | 1.20 | 0.219 | 0.011 | 0.182 | 0.058 |
| 12 | 16.44 | 0.81 | - | - | - | 0.049 |
| Mean | | | | 0.0148 | 0.1841 | 0.0754 |
| S.D. | | | | 0.0027 | 0.0175 | 0.0169 |
| C.V. | | | | 18.24% | 9.50% | 22.41% |

Correlation(r): Between serum total and saliva = 0.9793
: Between serum free and total = 0.9615
: Between serum total and free = 0.9831

TABLE 5a. Serum total, serum free and saliva concentrations (µg/mL) of VPA and their relationship to each other in volunteer M.S. before the administration of CBZ.

| Time (h) | Serum total | Serum free | Saliva | Saliva: total ratio | Saliva: free ratio | Free: total ratio |
|---|---|---|---|---|---|---|
| 1 | 92.63 | 8.33 | 1.533 | 0.017 | 0.184 | 0.090 |
| 2 | 63.60 | 9.48 | 1.020 | 0.016 | 0.108 | 0.149 |
| 3 | 65.31 | 9.25 | 1.274 | 0.020 | 0.138 | 0.142 |
| 5 | 50.74 | 6.48 | 1.093 | 0.022 | 0.169 | 0.128 |
| 7 | 51.06 | 8.74 | 1.029 | 0.020 | 0.118 | 0.171 |
| 9 | 51.57 | 7.94 | - | - | - | 0.154 |
| 24 | 21.48 | 1.75 | 0.343 | 0.016 | 0.196 | 0.081 |
| Mean | | | | 0.0185 | 0.1522 | 0.1307 |
| S.D. | | | | 0.0023 | 0.0330 | 0.0311 |
| C.V. | | | | 12.43% | 21.68% | 23.78% |

Correlation (r): Between serum total and saliva   = 0.9488
: Between serum free and saliva   = 0.8129
: Between serum total and free   = 0.7463

TABLE 5b. Serum total, serum free and saliva concentrations (µg/mL) of VPA and their relationship to each other in volunteer M.S. after the administration of CBZ.

| Time (h) | Serum total | Serum free | Saliva | Saliva: total ratio | Saliva: free ratio | Free: total ratio |
|---|---|---|---|---|---|---|
|  | 19.40 | 1.12 | 0.140 | 0.007 | 0.125 | 0.058 |
| 1 | 57.60 | 6.81 | - | - | - | 0.118 |
| 2 | 50.38 | 3.64 | 0.670 | 0.013 | 0.184 | 0.072 |
| 3 | 49.69 | 3.35 | 0.472 | 0.010 | 0.140 | 0.067 |
| 5 | 41.26 | 2.31 | - | - | - | 0.056 |
| 7 | 38.04 | 2.01 | 0.612 | 0.016 | 0.304 | 0.053 |
| 9 | 29.64 | - | 0.411 | 0.014 | - | - |
| 12 | 26.03 | 1.94 | 0.355 | 0.014 | 0.182 | 0.075 |
| Mean |  |  |  | 0.0123 | 0.1870 | 0.0713 |
| S.D. |  |  |  | 0.0030 | 0.0628 | 0.0205 |
| C.V. |  |  |  | 24.24% | 33.63% | 28.83% |

Correlation (r) : Between serum total and saliva = 0.8493
: Between serum free and saliva = 0.7500
: Between serum total and free = 0.8332

TABLE 6a. Serum total, serum free and saliva concentrations (μg/mL) of VPA and their relationship to each other in volunteer R.M. before the administration of CBZ.

| Time (h) | Serum total | Serum free | Saliva | Saliva: total ratio | Saliva: free ratio | Free: total ratio |
|---|---|---|---|---|---|---|
| 0 | 30.33 | 2.77 | 0.503 | 0.017 | 0.182 | 0.091 |
| 1 | 47.26 | 6.72 | 1.294 | 0.027 | 0.193 | 0.142 |
| 2 | 44.81 | 5.92 | 0.870 | 0.019 | 0.147 | 0.132 |
| 3 | 42.01 | 6.11 | 0.488 | 0.012 | 0.080 | 0.146 |
| 5 | 38.60 | 4.32 | 0.592 | 0.015 | 0.137 | 0.112 |
| 7 | 37.67 | 3.62 | 0.662 | 0.018 | 0.183 | 0.096 |
| 9 | 34.40 | - | - | - | - | - |
| 12 | 29.47 | 2.89 | - | - | - | 0.098 |
| Mean | | | | 0.0180 | 0.1536 | 0.1167 |
| S.D. | | | | 0.0046 | 0.0386 | 0.0213 |
| C.V. | | | | 25.66% | 25.15% | 18.30% |

Correlation (r) : Between serum total and saliva = 0.8412
: Between serum free and saliva = 0.6500
: Between serum total and free = 0.9556

TABLE 6b. Serum total, serum free and saliva concentrations (μg/mL)
of VPA and their relationship to each other in volunteer R.M.
after the administration of CBZ.

| Time (h) | Serum total | Serum free | Saliva | Saliva: total ratio | Saliva: free ratio | Free: total ratio |
|---|---|---|---|---|---|---|
| 0 | 22.79 | - | 0.175 | 0.008 | - | - |
| 1 | 42.43 | 6.69 | 0.590 | 0.014 | 0.089 | 0.157 |
| 2 | 39.35 | - | 0.388 | 0.010 | - | - |
| 3 | - | 6.82 | 0.757 | - | 0.111 | - |
| 5 | 30.56 | 3.10 | 0.430 | 0.014 | 0.139 | 0.101 |
| 7 | 27.61 | 2.56 | 0.268 | 0.010 | 0.105 | 0.092 |
| 9 | 24.10 | 1.66 | - | - | - | 0.069 |
| 12 | 18.11 | 1.20 | 0.185 | 0.010 | 0.154 | 0.066 |
| Mean | | | | 0.0110 | 0.1196 | 0.0970 |
| S.D. | | | | 0.0022 | 0.0235 | 0.0328 |
| C.V. | | | | 20.32% | 19.72% | 33.835% |

Correlation (r) : Between serum total and saliva = 0.9011
: Between serum free and saliva = 0.9549
: Between serum total and free = 0.9765

TABLE 7a. Serum total, serum free and saliva concentrations (µg/mL) of VPA and their relationship to each other in volunteer B.A. before the administration of CBZ.

| Time (h) | Serum total | Serum free | Saliva | Saliva: total ratio | Saliva: free ratio | Free: total ratio |
|---|---|---|---|---|---|---|
| 1 | 42.36 | 5.40 | - | 0.128 | - | - |
| 2 | 42.44 | 5.38 | 1.064 | 0.025 | 0.198 | 0.127 |
| 3 | 43.78 | 5.49 | 1.522 | 0.035 | 0.277 | 0.125 |
| 5 | 20.56 | - | 0.611 | 0.029 | - | - |
| 7 | 22.27 | 3.39 | 1.270 | 0.057 | 0.375 | 0.152 |
| 9 | 25.90 | 2.82 | 1.233 | 0.047 | 0.437 | 0.109 |
| 12 | 21.07 | 2.76 | 0.773 | 0.035 | 0.280 | 0.131 |
| Mean | | | | 0.0380 | 0.3134 | 0.1287 |
| S.D. | | | | 0.0108 | 0.0834 | 0.0126 |
| C.V. | | | | 28.63% | 26.61% | 9.79% |

Correlation (r) : Between serum total and saliva = 0.6017
: Between serum free and saliva = 0.4884
: Between serum total and free = 0.9733

TABLE 7b. Serum total, serum free and saliva concentrations (µg/mL) of VPA
and their relationship to each other in volunteer B.A. after the
administration of CBZ.

| Time (h) | Serum total | Serum free | Saliva | Saliva: total ratio | Saliva: free ratio | Free: total ratio |
|---|---|---|---|---|---|---|
| 1 | 31.05 | 4.60 | 0.940 | 0.030 | 0.204 | 0.148 |
| 2 | 30.36 | 3.80 | 0.585 | 0.019 | 0.154 | 0.125 |
| 3 | 26.65 | - | 0.549 | 0.021 | - | - |
| 5 | 23.60 | 3.37 | 0.529 | 0.022 | 0.157 | 0.142 |
| 7 | 19.01 | 1.90 | 0.391 | 0.021 | 0.206 | 0.099 |
| 9 | 16.40 | 1.62 | 0.360 | 0.022 | 0.222 | 0.099 |
| 12 | 13.26 | 1.61 | 0.292 | 0.022 | 0.181 | 0.121 |
| Mean | | | | 0.0224 | 0.1873 | 0.1223 |
| S.D. | | | | 0.0032 | 0.0254 | 0.0189 |
| C.V. | | | | 14.49% | 13.56% | 15.45% |

Correlation (r) : Between serum total and saliva  = 0.8727
: Between serum free and saliva   = 0.9354
: Between serum total and free    = 0.9614

TABLE 8a. Serum total, serum free and saliva concentrations (μg/mL) of VPA and their relationship to each other in volunteer F.A. before the administration of CBZ.

| Time (h) | Serum total | Serum free | Saliva | Saliva: total ratio | Saliva: free ratio | Free: total ratio |
|---|---|---|---|---|---|---|
| 0 | 44.45 | 8.21 | 0.889 | 0.020 | 0.108 | 0.185 |
| 1 | 64.66 | 13.71 | 2.963 | 0.046 | 0.216 | 0.212 |
| 2 | 56.58 | 13.11 | 2.432 | 0.043 | 0.186 | 0.231 |
| 3 | 58.77 | 11.04 | 3.230 | 0.055 | 0.293 | 0.188 |
| 5 | 40.59 | 9.18 | 1.218 | 0.030 | 0.133 | 0.226 |
| 7 | 46.84 | 7.99 | 1.209 | 0.026 | 0.151 | 0.171 |
| 9 | 46.06 | 7.88 | 0.483 | 0.011 | 0.061 | 0.171 |
| 12 | 33.61 | 6.45 | 0.559 | 0.017 | 0.087 | 0.192 |
| Mean | | | | 0.0310 | 0.1544 | 0.1979 |
| S.D. | | | | 0.0145 | 0.0706 | 0.0219 |
| C.V. | | | | 46.86% | 45.74% | 11.10% |

Correlation (r) : Between serum total and saliva = 0.8933
: Between serum free and saliva = 0.8818
: Between serum total and free = 0.9045

TABLE 8b. Serum total, serum free and saliva concentrations (µg/mL) of VPA and their relationship to each other in volunteer F.A. after the administration of CBZ.

| Time (h) | Serum total | Serum free | Saliva | Saliva: total ratio | Saliva: free ratio | Free: total ratio |
|---|---|---|---|---|---|---|
| 0 | 24.15 | 4.44 | 0.353 | 0.015 | 0.080 | 0.183 |
| 1 | 53.24 | 8.73 | - | - | - | 0.163 |
| 2 | 43.80 | 7.17 | 0.998 | 0.023 | 0.139 | 0.163 |
| 3 | 42.02 | 7.33 | 1.294 | 0.031 | 0.177 | 0.174 |
| 5 | 33.34 | 4.86 | 0.773 | 0.023 | 0.159 | 0.145 |
| 7 | 26.09 | 4.61 | 0.572 | 0.022 | 0.124 | 0.176 |
| 9 | 28.02 | 5.44 | - | - | - | 0.194 |
| Mean | | | | 0.0228 | 0.1405 | 0.1698 |
| S.D. | | | | 0.0051 | 0.0331 | 0.0149 |
| C.V. | | | | 22.26% | 23.60% | 8.76% |

Correlation (r) : Between serum total and saliva = 0.9265
: Between serum free and saliva = 0.9159
: Between serum total and free = 0.9654

TABLE 9. Time-averaged ratios (6-8 samples) and correlations between serum total, serum free and saliva VPA concentrations in five volunteers before the administration of CBZ.

| Volun-teer | Saliva: total ratio | Saliva: free ratio | Free: total ratio | Correlation (r) | | |
|---|---|---|---|---|---|---|
| | | | | Total: Saliva | Free: Saliva | Total: Free |
| W.T. | 0.0160 | 0.1723 | 0.0940 | 0.8945 | 0.8254 | 0.9400 |
| M.S. | 0.0185 | 0.1522 | 0.1307 | 0.9488 | 0.8129 | 0.7463 |
| R.M. | 0.0180 | 0.1536 | 0.1167 | 0.8412 | 0.6500 | 0.9556 |
| F.A. | 0.0310 | 0.1544 | 0.1970 | 0.8933 | 0.8818 | 0.9045 |
| B.A. | 0.0380 | 0.3134 | 0.1287 | 0.6017 | 0.4884 | 0.9733 |
| Mean | 0.0243 | 0.1892 | 0.1334 | 0.8945* | 0.7925* | 0.9039 |
| S.D. | 0.0086 | 0.0625 | 0.0343 | | | |
| C.V. | 35.63% | 33.06% | 25.76% | * excluding B.A.'s values | | |

TABLE 10. Time-averaged ratios (6-8 samples) and correlations between serum total, serum free and saliva VPA concentrations in five volunteers after the administration of CBZ.

| Volunteer | Saliva: total ratio | Saliva: free ratio | Free: total ratio | Correlation (r) | | |
|---|---|---|---|---|---|---|
| | | | | Total: Saliva | Free: Saliva | Total: Free |
| W.T. | 0.0148 | 0.1841 | 0.0754 | 0.9793 | 0.9615 | 0.9831 |
| M.S. | 0.0123 | 0.1870 | 0.0713 | 0.8493 | 0.7500 | 0.8332 |
| R.M. | 0.0110 | 0.1196 | 0.0970 | 0.9011 | 0.9549 | 0.9765 |
| F.A. | 0.0228 | 0.1405 | 0.1698 | 0.9265 | 0.9159 | 0.9654 |
| B.A. | 0.0224 | 0.1873 | 0.1223 | 0.8727 | 0.9354 | 0.9614 |
| Mean | 0.0167 | 0.1637 | 0.1072 | 0.9058 | 0.9035 | 0.9439 |
| S.D. | 0.0050 | 0.0282 | 0.0362 | | | |
| C.V. | 29.96% | 17.27% | 33.77% | | | |

salicylate in turn displaces VPA from its binding sites in plasma (Levy and Koch, 1982). VPA is also likely to compete with CBZ (75% bound) for protein binding sites. It has been shown in vitro that VPA reduces protein binding of CBZ, whereas addition of CBZ to VPA did not change the serum binding of VPA (Mattson et al., 1982; Patel and Levy, 1979). This was explained by the greater binding affinity of VPA compared to that of CBZ. Our in vivo results also confirm the above in vitro finding. In the present study the free fraction of VPA was actually slightly reduced (from 0.1334 to 0.1072) because of the lower serum total VPA concentrations after CBZ. Hence, in vivo, CBZ does not displace VPA from plasma protein binding sites.

In a study of the effects of carbamazepine on VPA kinetics in normal subjects, Bowdle et al. (1979) found that the minimum steady-state levels of VPA decreased significantly after CBZ. Our results also showed a significant decrease in VPA serum and saliva concentrations after two weeks on carbamazepine. An increase in the free fraction of a drug usually results in increased clearance rates, reduced steady-state total levels, and hence unchanged free concentrations (Koch-Weser and Sellers, 1976). However, the decrease in VPA total levels observed cannot be due to an increase in the free fraction since the free fraction did not increase after CBZ.

This work was part of a general study on the effect of CBZ on VPA metabolism and was done specifically to measure any effect of CBZ on the free fraction of VPA. The effect of CBZ on VPA metabolism was first studied by Sukhbinder Panesar (M.Sc., 1987). It was found that VPA volume of distribution did not change but that clearance and the elimination rate constant were increased significantly ($p < 0.05$)

in the five volunteers after CBZ administration. This suggests that CBZ induces the metabolism of VPA.

2. Serum free VPA levels

The serum free levels of VPA were measured employing the technique of ultrafiltration. We chose ultrafiltration over equilibrium dialysis because of its simplicity and a previous report that free levels of VPA obtained by ultrafiltration and equilibrium dialysis were found to be strongly correlated (Levy et al., 1984b). There is no adsorption of VPA to the filtration membranes or filtration device (Nau et al., 1984).

The free VPA concentrations in this study ranged from 13.71 to 0.81 µg/mL compared to total VPA concentrations of 92.63 to 13.26 µg/mL. The free concentrations were highly correlated with total concentration over the concentration range studied (Tables 4 to 8) (e.g., r=0.9439 ± 0.05, mean ± S.D., after CBZ, Table 10). The free fractions varied from 0.05 to 0.23 and were more or less concentration dependent. For all except one volunteer (B.A.) the free fractions decreased with a decrease in total VPA concentration, i.e. after CBZ. For B.A. there was no change in free fraction possibly because most of the total VPA concentration values were less than 30 µg/mL.

The overall mean free fraction was 0.1334 ± 0.0343 before CBZ and 0.1072 ± 0.0362 after CBZ (Tables 9 and 10). This concentration dependence of free fraction was evident even at VPA total concentrations of less than 50 µg/mL. The free fractions obtained in this study are in agreement with published data for epileptic patients (Otten et

TABLE 11. Decrease (%) in average VPA concentration after CBZ
administration.

| Volunteer | Serum Total | Serum Free | Saliva |
|-----------|-------------|------------|--------|
| W.T. | 32.15 | 44.24 | 38.85 |
| M.S. | 28.89 | 54.49 | 47.21 |
| R.M. | 21.94 | 13.91 | 40.85 |
| F.A. | 29.97 | 40.13 | 55.56 |
| B.A. | 26.58 | 31.49 | 58.20 |
| Mean | 27.91 | 36.85 | 48.13 |
| S.D. | 3.481 | 13.649 | 7.701 |

al., 1984; Garnett et al., 1983). The correlation between total VPA concentration and free fractions was much lower than that of the total to free concentration (0.336 vs. 0.787; calculation based on all values before and after CBZ for all volunteers). The plot of total VPA concentration versus free fraction (Figure 22) appears to curve upwards showing the concentration dependent binding of VPA.

Because of the saturable nature of VPA plasma protein binding, the free fraction is not constant over the range of VPA concentrations used therapeutically as demonstrated in this study and other studies (Levy et al., 1986). In addition, VPA binding is affected by free fatty acid concentration and salicylates, and can be modified by a number of disease conditions. For these reasons, total VPA levels do not reflect the free level which is assumed to be the pharmacologically active form. In spite of the strong correlation between total and free VPA, free concentrations cannot be accurately predicted from total VPA concentrations because of the high inter- and intra-subject variability. Hence, in situations where drug monitoring is required, the monitoring of the free levels rather than the total is likely to be more useful.

3. Saliva VPA levels

The saliva concentrations of VPA in the five volunteers ranged from 0.175 to 3.23 μg/mL compared to serum total VPA concentrations of 13.26 to 92.63 μg/mL. The time-averaged saliva to total and free VPA ratios are given in Tables 4 to 8. The mean ratios for the five
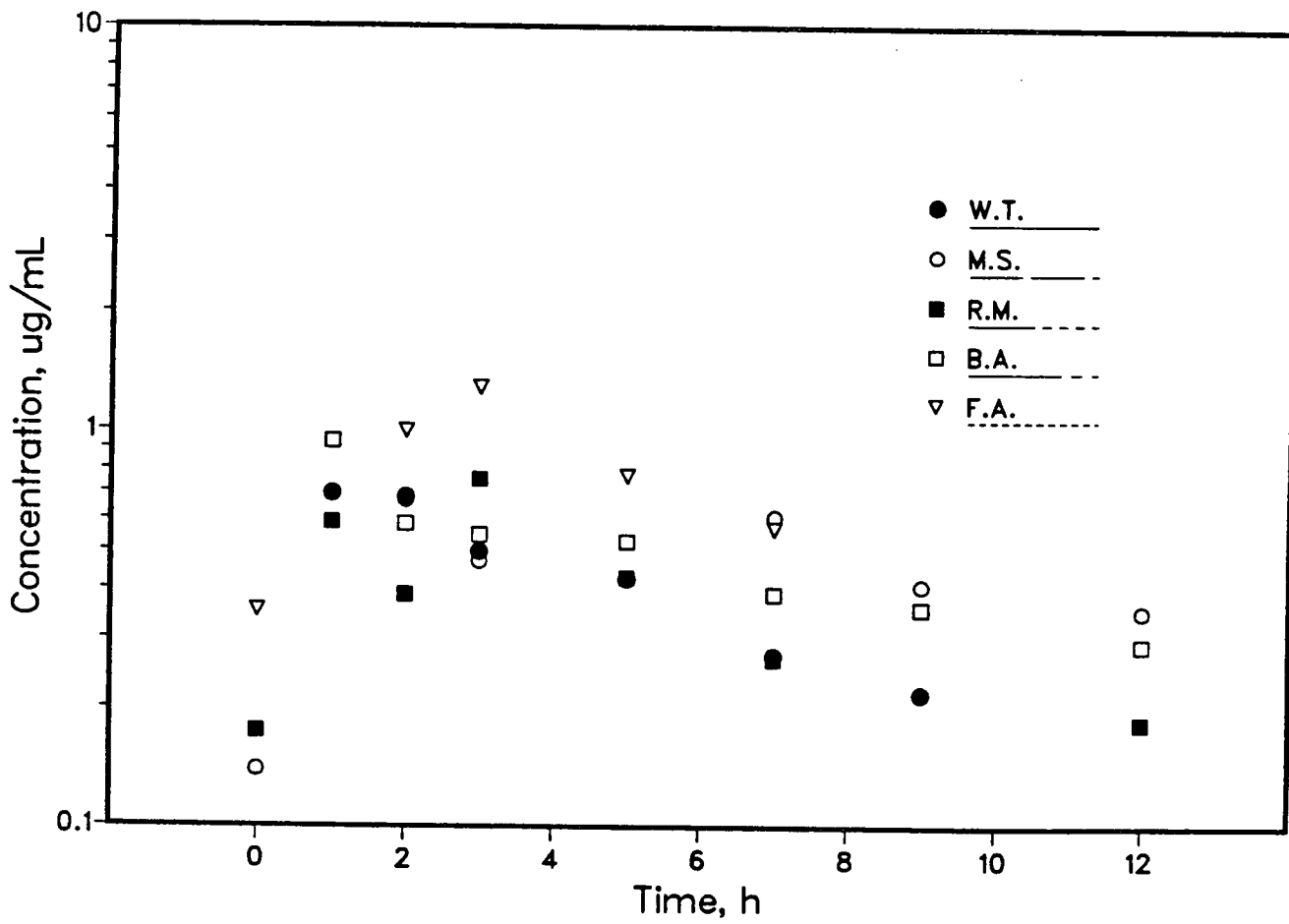
Figure 14.    Saliva concentration-time profiles for five volunteers at steady state VPA.
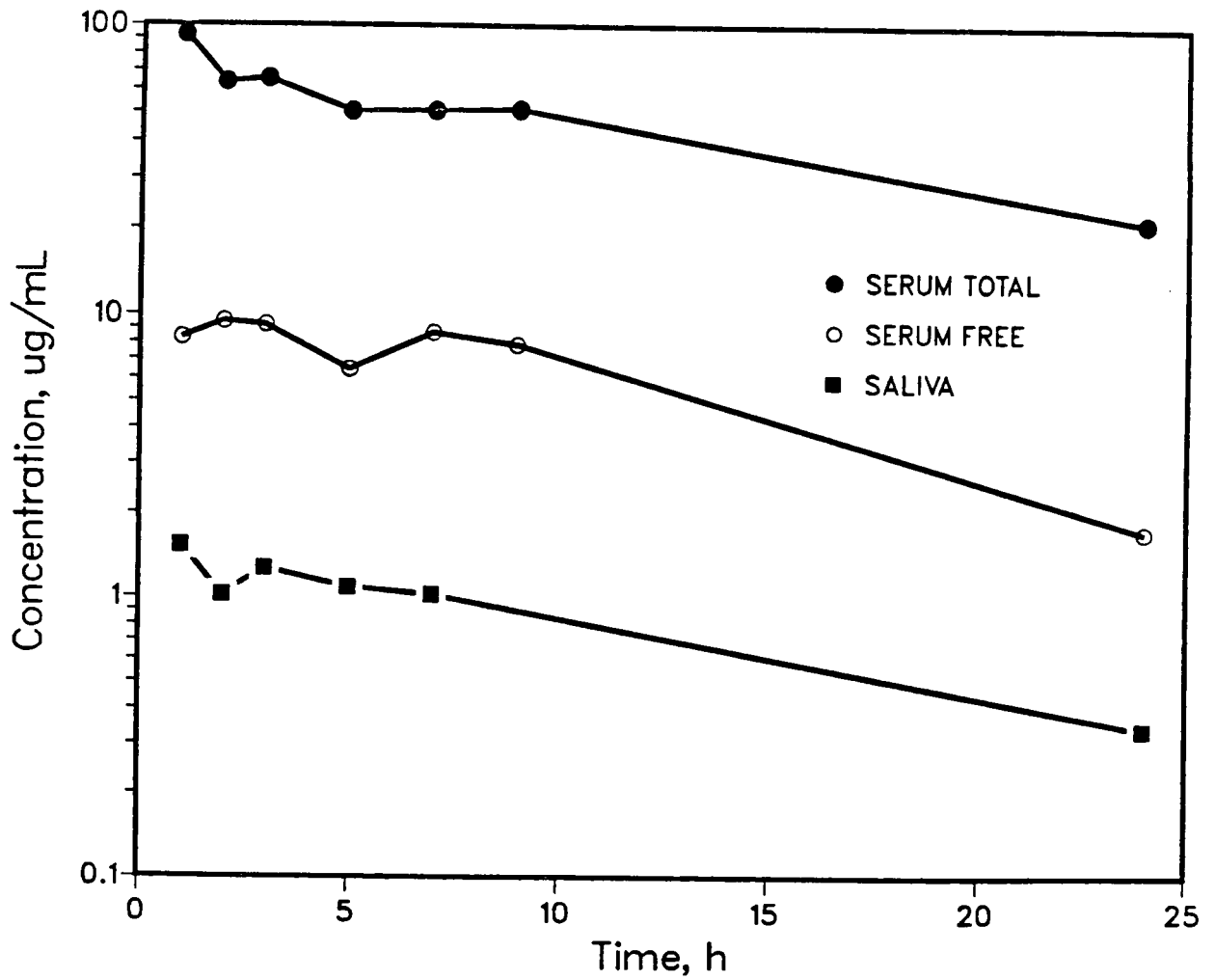
Figure 15.  Concentration-time curves for serum total, serum free and saliva VPA in one volunteer (M.S.).
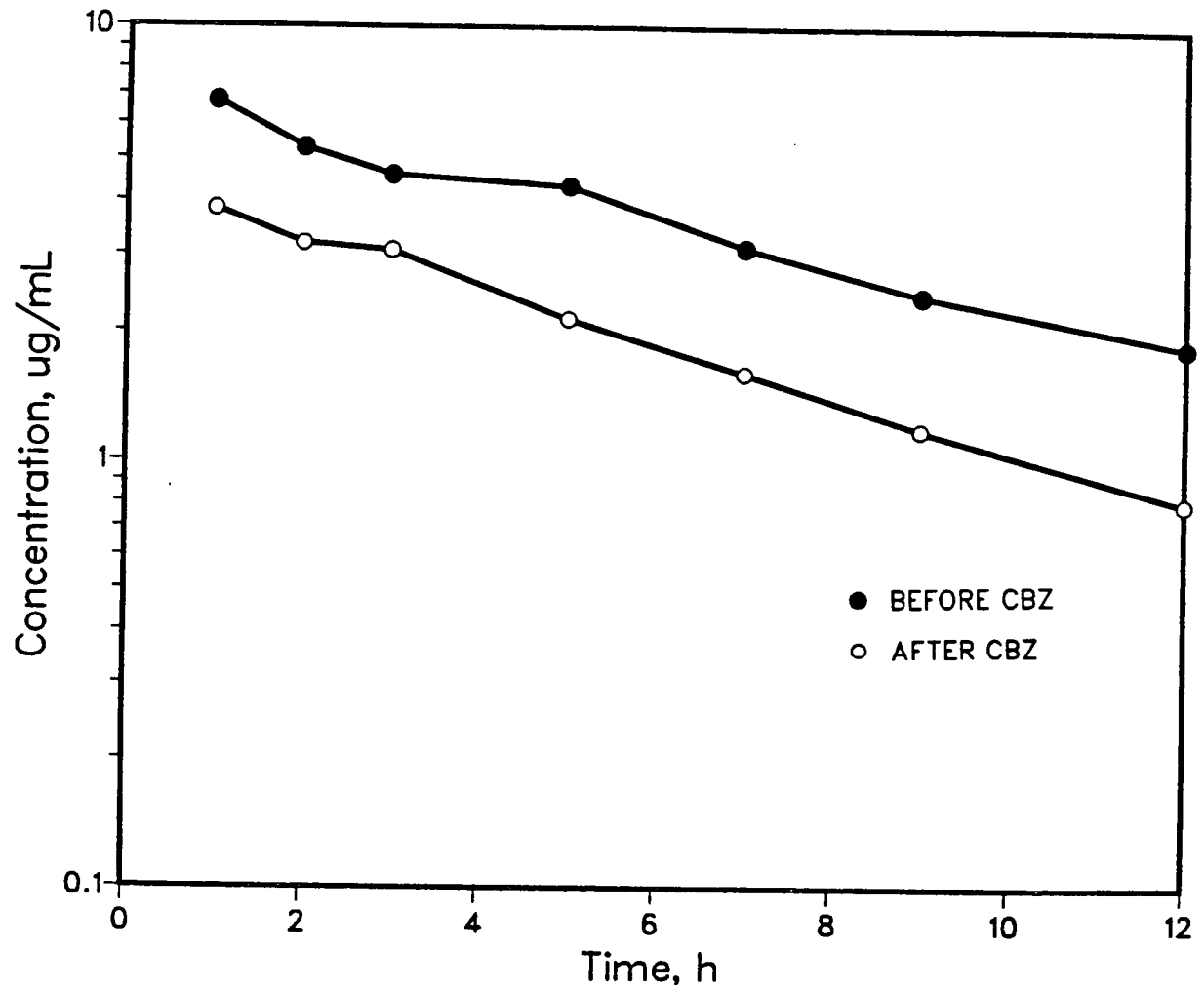
Figure 16.    Concentration-time curve for serum free VPA before and after
CBZ administration in one volunteer (W.T.).
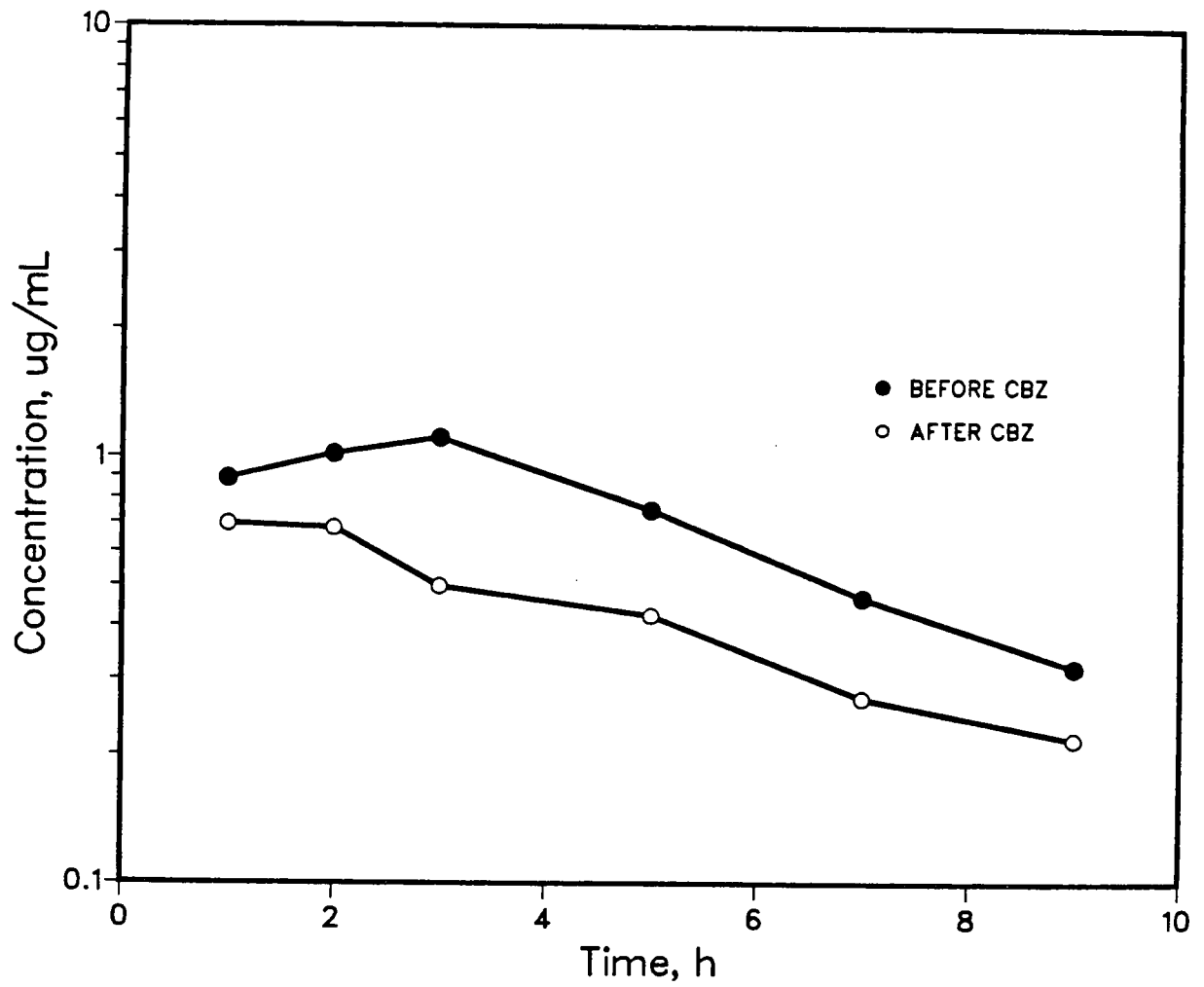
Figure 17. Concentration-time curve for saliva VPA before and after CBZ administration in one volunteer (W.T.).

The plot shows the following annotations:

N = 13

r = 0.9248, p < 0.00001

saliva = 0.0249 x total - 0.3182

Legend:
● Before CBZ
○ After CBZ

Axis labels:
- Y-axis: Saliva VPA, ug/mL (0, 0.2, 0.4, 0.6, 0.8, 1, 1.2)
- X-axis: Serum total VPA, ug/mL (0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55)

Figure 18.    Relationship between serum total and saliva VPA concentrations in one volunteer (W.T.).
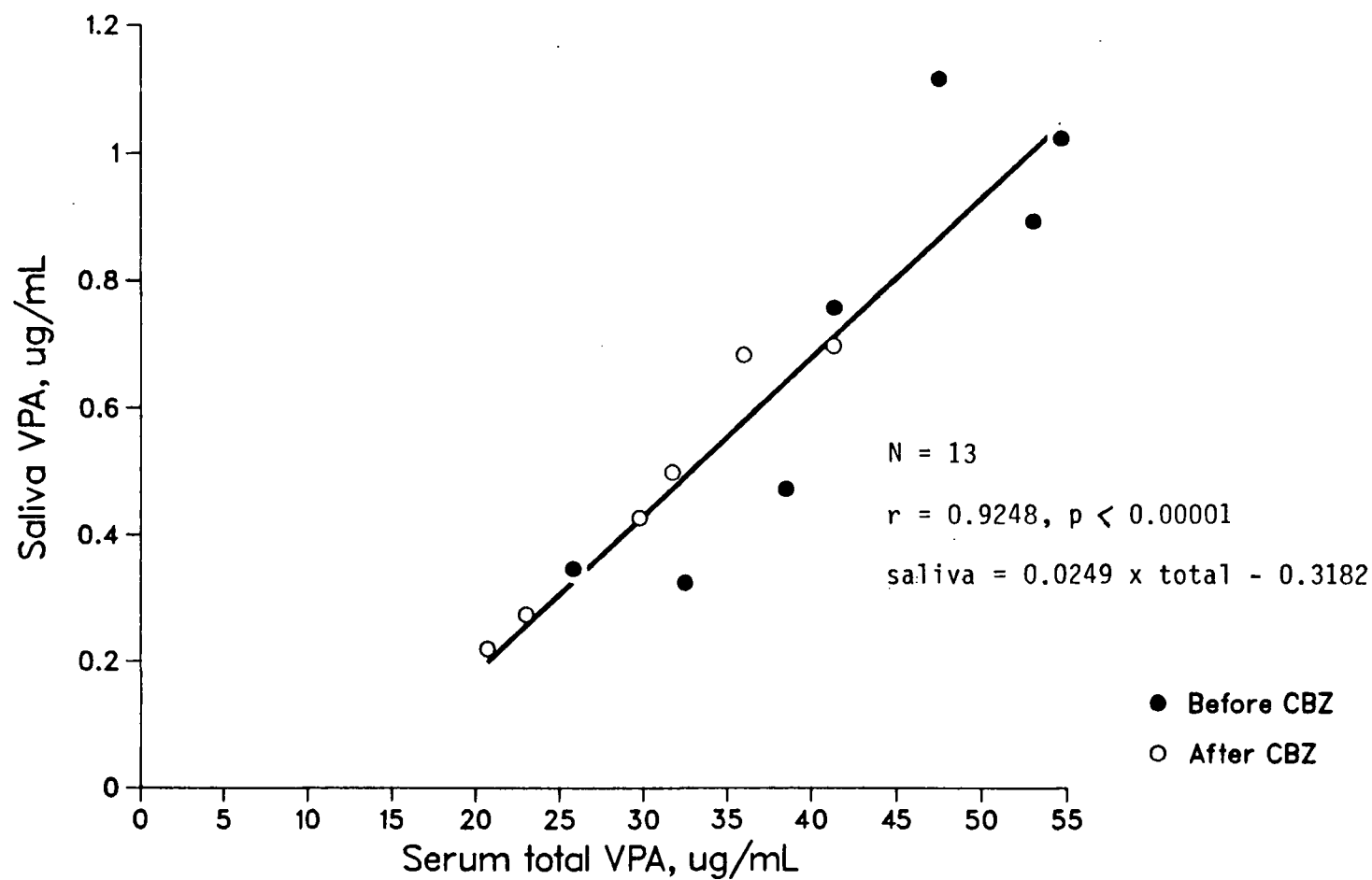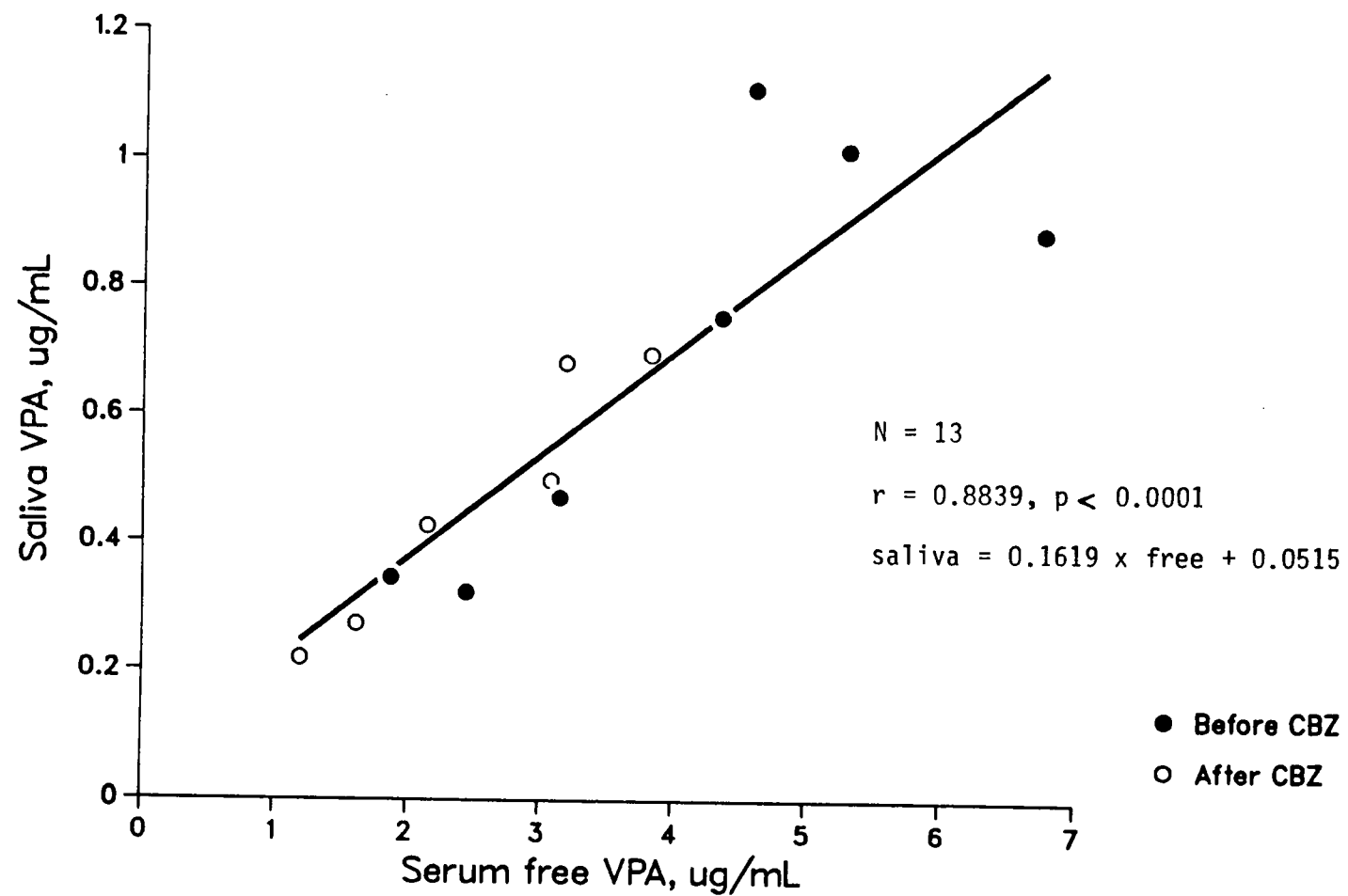
Figure 19.    Relationship between serum free and saliva VPA concentrations
in one volunteer (W.T.).

Figure 20.    The relationship between serum total and saliva VPA concen-
             trations in all five volunteers.
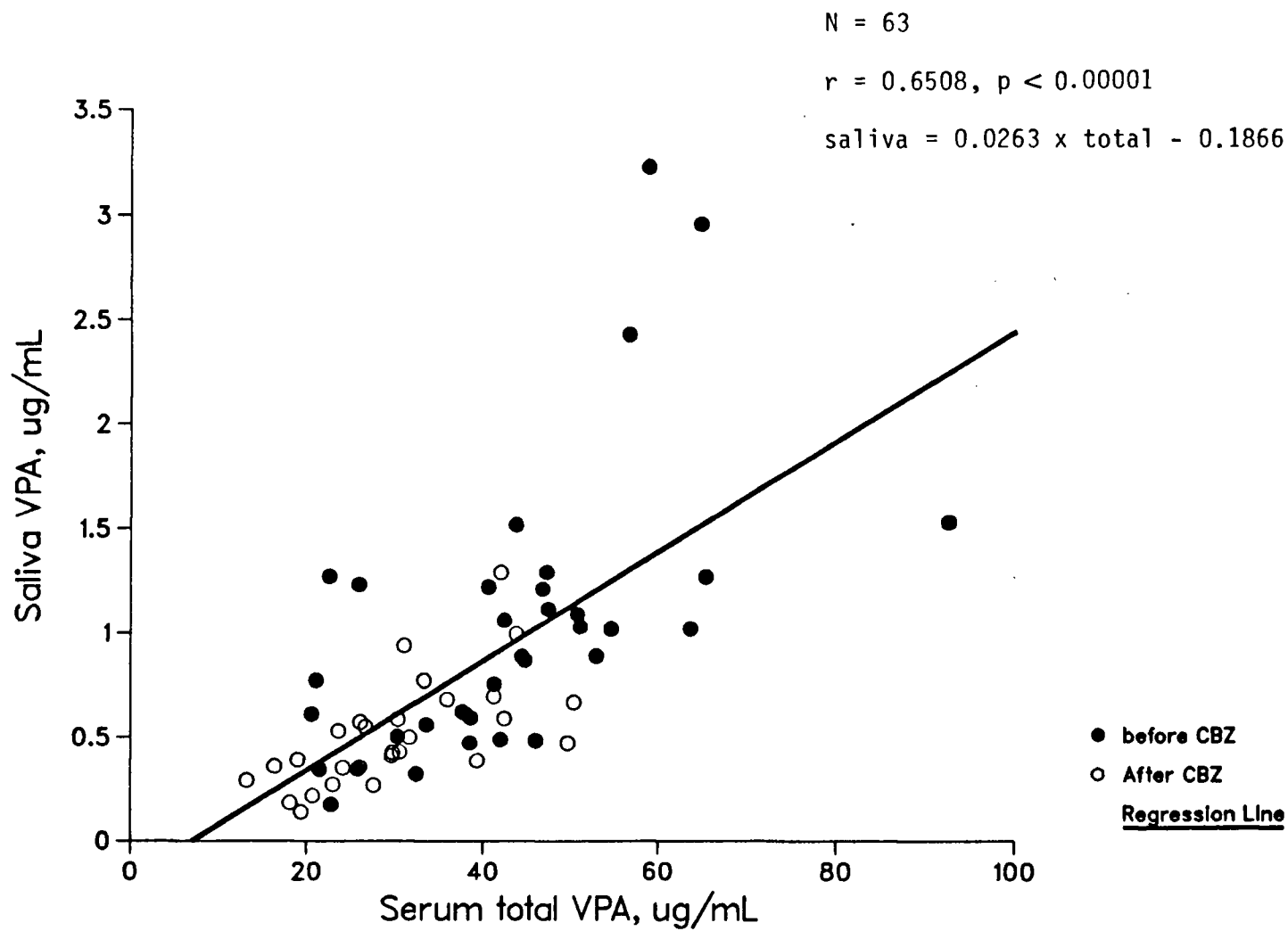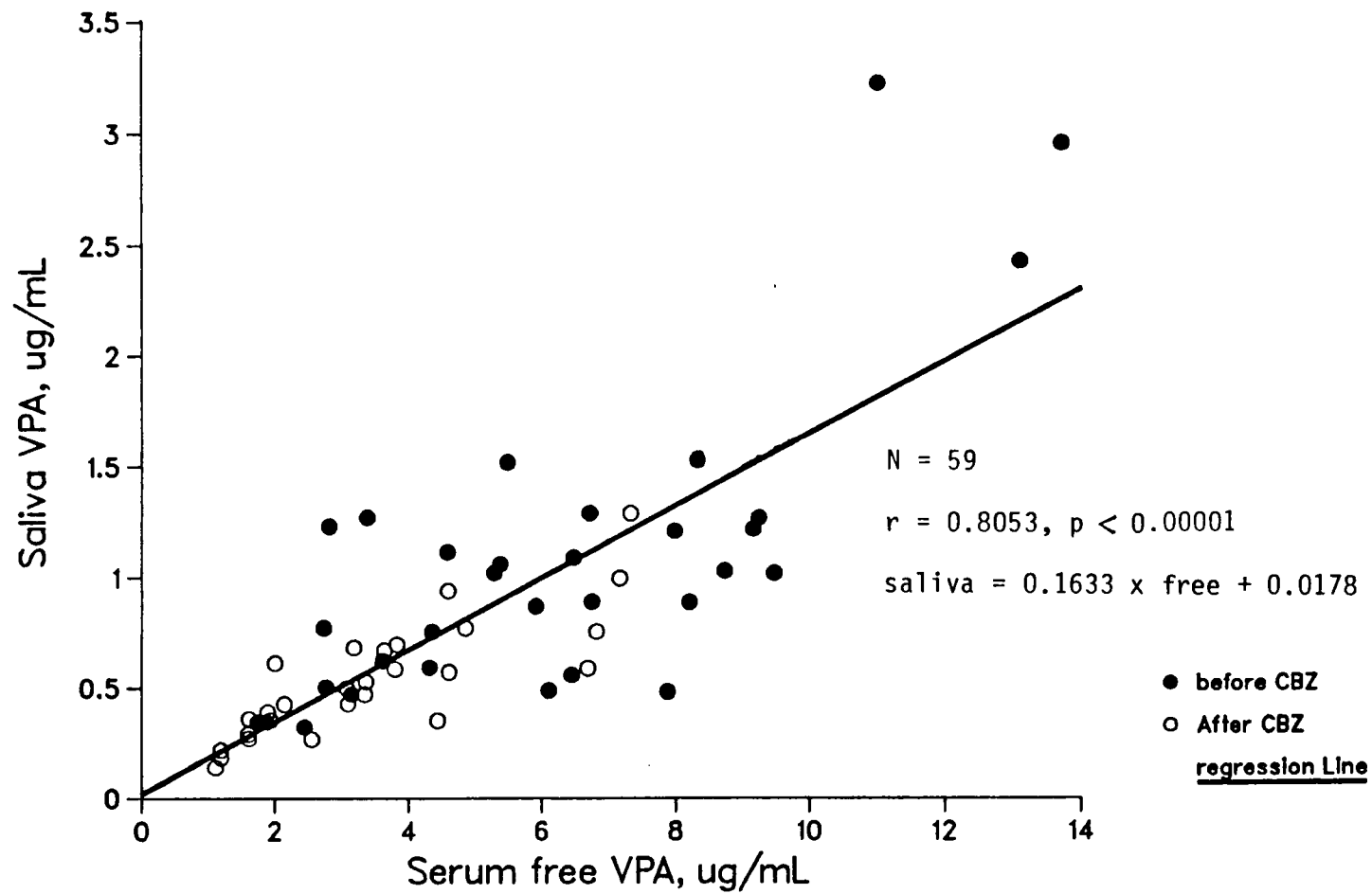
Figure 21.  The relationship between serum free and saliva VPA concentrations in all five volunteers.
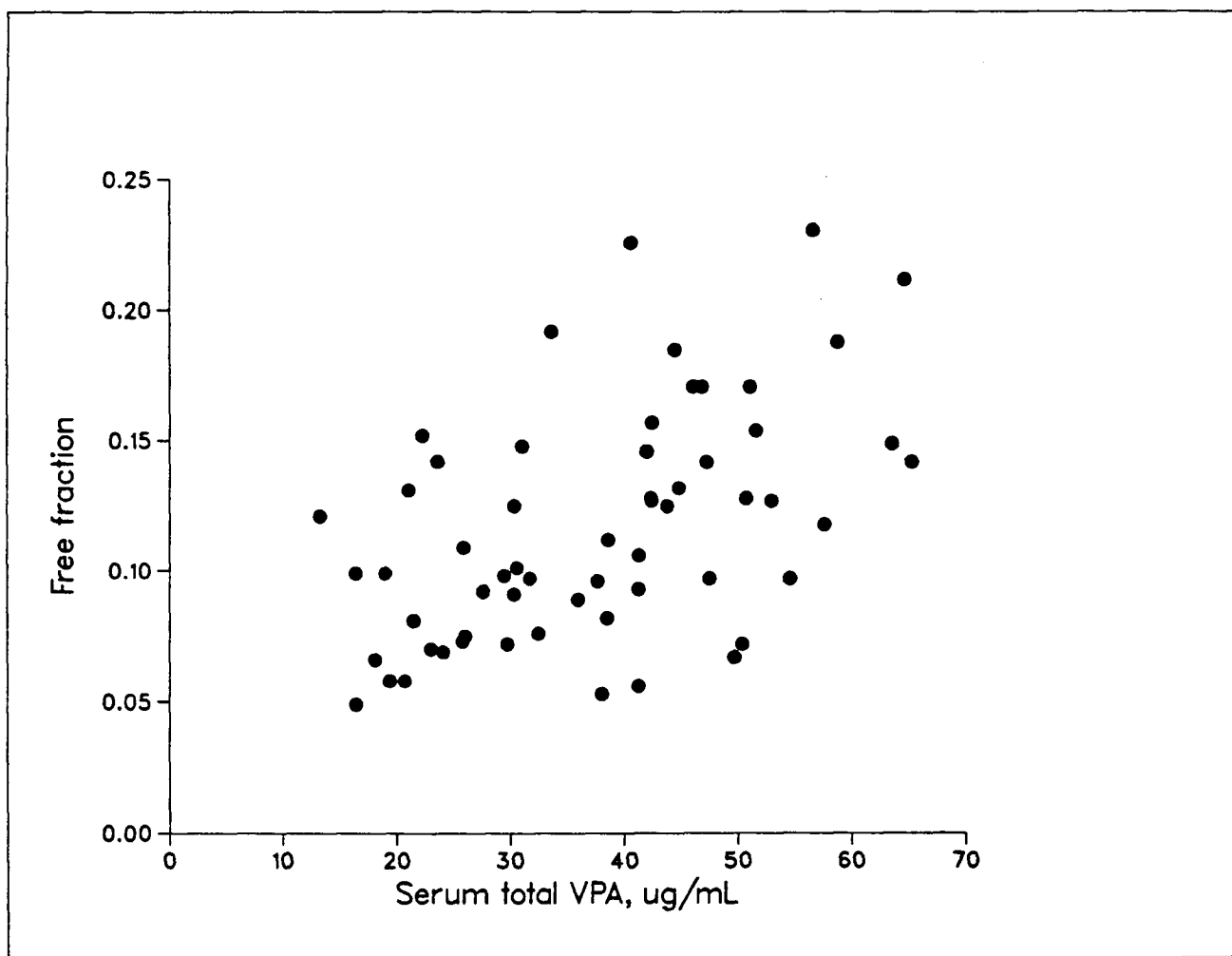
Figure 22.   A plot of free fraction versus serum total concentration of
VPA (all values before and after CBZ for all five volunteers).

volunteers were 0.0243 ± 0.0086, and 0.1892 ± 0.0625, for saliva to total and saliva to free, respectively (Table 9). These ratios were 0.0167 ± 0.0050 and 0.1637 ± 0.0282 after CBZ (Table 10). The time-averaged saliva to serum free ratios of four of the five volunteers were very similar (0.1723, 0.1522, 0.1536, 0.1544) as were the saliva to total ratios in three of the volunteers (0.0160, 0.0185, 0.0180) (Table 9). The saliva to free ratios in four of the volunteers, after CBZ (Table 10) were similar and did not change markedly from the values obtained before CBZ. However, this was not true of the saliva to total ratios.

The saliva to serum total VPA ratios were in general agreement with published data for healthy volunteers on multiple doses (Gugler et al., 1977) but were higher than those of Abbott et al. (1982) (single-dose study) and Acheampong et al. (1984) (multiple-dose study). The saliva to free ratios were similar to those reported by Abbott et al. (1982) and Acheampong et al. (1984) and were also comparable to values found for epileptic patients (Gugler et al., 1980).

The correlations between saliva VPA concentrations and both serum total and free concentrations were very good in the majority of cases (Tables 9 and 10). As seen in Figure 21 the overall correlation between serum free and saliva VPA concentration was also strong.

a. Analytical aspects of saliva VPA quantitation

Saliva VPA concentrations have been measured by a number of analytical techniques; GC (Blom and Guelen, 1977; Gugler et al., 1977; Fung and Ueda, 1982; Gugler et al., 1980); GCMS (Abbott et al., 1980; Acheampong et al., 1982); EMIT (Monaco et al., 1982). With the EMIT assay, no correlation was found between saliva and plasma VPA and in

more than 60% of the cases saliva levels were near zero. The correlations found by Blom and Guelen were poor. Gugler et al. (1977) also reported a high intra- and inter-subject variability of the saliva to plasma ratios.

The VPA saliva level work reported previously had shown that the saliva concentration of VPA is not equal to the unbound concentration in serum, saliva levels can often be erratic, a consistent ratio between saliva and serum total or free is not obtained, and there is a high intersubject variability. It has been suggested that because of its low pKa (4.9), VPA will be mostly ionized at plasma pH and hence little of it excreted into saliva and that the saliva concentration of VPA will be highly dependent on saliva pH. In addition, the low saliva concentration may prove difficult to correctly measure by most analytical techniques (Gugler et al., 1977).

Although saliva levels of VPA are not equal to the unbound concentration in plasma, it was of interest to find out whether a constant relationship between the two existed, so that the unbound concentration could be estimated from salivary levels. It was our intention to do the present study by incorporating two important factors. We used a highly sensitive and precise negative ion GCMS assay that can measure VPA in serum and saliva accurately down to about 2 ng/mL. The other important factor was the use of reproducible and standardized methods of collection of saliva samples.

b.  The effect of pH on the saliva concentration of VPA

Since the secretion of relatively strong acidic and basic drugs into saliva is dependent upon the pH of saliva and serum (Mucklow,

1982), it is generally assumed that saliva pH will affect the saliva concentration of VPA. However, Abbott et al. (1982) have observed highly inflated saliva levels of VPA which could not be explained by pH effects or free levels of the drug. Similarly, Acheampong et al. (1984) found discrepancies between the experimentally obtained values and those calculated using Matin's equation (Matin et al., 1974) for saliva levels of VPA. It is difficult to get an accurate measure of saliva pH by conventional means because of the possible difference in pH of mixed saliva compared to the pH of saliva in contact with the epithelial cells of the salivary glands (Koup et al., 1975). For diazepam, it has been found that although saliva to plasma ratios may show good correlation, salivary diazepam levels are higher than the free diazepam concentration in plasma (Gier et al., 1980). Since the pKa of diazepam (a weak base) is 3.3, the concentration of diazepam in saliva is not likely to be affected by salivary pH. In the case of salicylate, a relatively strong acid (pKa 3), a high intra-subject variation was found that was not related to saliva pH (Levy et al., 1980). It does not appear that the role of pH in the salivary excretion of individual drugs is fully understood. Against this background, it was felt that it was more important to standardize saliva collection by stimulation with citric acid and collecting sample over a given time interval rather than measuring whole mouth saliva pH which would either not be accurate (affected by citric acid) or otherwise would not reflect the pH in the salivary glands.

In a study (Abbott, unpublished data) of the relationship between stimulation of saliva flow with citric acid and ensuing parotid salivary pH, it was found that the saliva pH in six volunteers was reasonably constant following stimulation by 4 mL of 5% citric acid

solution retained in the mouth for 2 minutes. The parotid saliva pH plateaued between 2-3 minutes and was in the range of pH 7.3-7.6. This protocol was thus used for the collection of saliva samples throughout the present study, in the hope to minimize intra- and inter-subject variability.

c. The relationship between saliva and serum concentrations of VPA

In Tables 4 to 8 it can be seen that there are occasional saliva VPA values which are disproportionately high and thus contribute to decreased correlation (both free and total) and high variability in the saliva to total or free ratios. However, examination of Table 9 shows that in four of the five volunteers the mean saliva to free ratios are remarkably constant. The mean value for these four volunteers is 0.1581 with coefficient of variation of 5.2%. Interestingly, for four of the volunteers, similar ratios were observed after CBZ with a mean of 0.1747 and coefficient of variation of 11.3%, and for the three volunteers these values are similar to those before CBZ. This good agreement between the saliva to free ratios obtained two weeks apart, at least in three of the volunteers could be attributed to the standardized sampling protocol and precision of the assay.

For one of the volunteers (B.A) the saliva to free VPA ratio decreased from 0.3134 to 0.1873 after CBZ. This appears to contradict the finding of Acheampong et al. (1984) who reported that low saliva to free ratios were observed at high serum concentrations of VPA. In fact, saliva to free ratios should not change depending upon VPA serum concentration since any changes in free VPA should bring a proportional

change in saliva levels. This is consistent with our results since there is essentially no difference between the saliva to serum free ratio in all subjects before and after CBZ except for B.A. whose ratio decreases after CBZ.

The saliva to serum total ratio did not show the good agreement exhibited by the saliva to free serum ratio before and after CBZ. After CBZ there was a significant decrease in the saliva to total ratio at least in four of the volunteers indicating that the saliva to total ratio was concentration dependent, higher ratios being found at higher serum total concentrations. Since serum total VPA concentrations decreased by 27.91% ± 3.48 (Mean ± S.D.) after CBZ (Table 11) the % free decreases (36.85) and therefore, results in a corresponding % decrease in saliva concentrations (48.13). The correlation between saliva and serum total or free was better for the samples after CBZ (Table 9 and 10). Similarly, the intraindividual variability was less after CBZ. There was no difference between the variability of saliva to total and saliva to free ratios.

In summary, despite intraindividual variability the saliva VPA to serum free ratio in three of the five volunteers was reasonably constant over two different sampling periods, two weeks apart. For the saliva VPA to total ratios such a relationship was not found because of the decreased serum concentration and accompanying decrease in free fraction following CBZ administration.

From this work it appears the time-averaged saliva to free ratio once determined could be used for assessing serum free VPA by measuring VPA in saliva. The good correlations found between saliva and both serum total and free VPA concentrations suggest that measuring saliva

VPA by this method would be suitable for pharmacokinetic and drug interaction studies. Salivary measurement of VPA could be useful for therapeutic drug monitoring but it is unlikely to be of value using routine assay methods that do not have the sensitivity and accuracy of NICI. For example, Fung and Ueda (1982) were not able to detect any VPA in some of their saliva samples using GC.

d. The concept of salivary clearance in the salivary excretion of drugs

Zuidema and van Ginneken (1983a) and Kido (1982) have introduced the concept of salivary clearance in an attempt to explain some of the irregularities observed with salivary levels of drugs. When a drug is poorly lipophilic and hence with a low extraction ratio, permeation of the drug across the epithelial membrane is rate-determining. The saliva clearance is then independent of blood flow whereas saliva flow remains dependent on the blood flow. The salivary concentration of the drug will decrease with increasing blood and saliva flow since the drug is not able to equilibrate between saliva and blood at high flow because of its poor lipid solubility. Such substances as urea and primidone, show flow dependent salivary excretion (Bartels et al., 1979). Since drugs with good lipophilicity have a high extraction ratio, salivary clearance will be proportional to blood flow and as a consequence saliva flow and clearance will change proportionally. In a study (Kido, 1982) with some anticonvulsant drugs, where the kinetics of the drugs in saliva were studied after stimulation with pilocarpine, salivary levels of phenobarbital showed maximum values between 10 and 30 minutes. This observation could not be explained with Matin's equation.

With the addition of the clearance parameter, however, the phenobarbital levels were better explained.

Saliva to plasma ratios greater than one can be explained by either active transport or ionization in saliva (relatively strong bases). An active transport mechanism appears to be true for penicillin (Zuidema and van Ginneken, 1983b).

In the present study saliva was obtained following stimulation with citric acid. Whereas this helps to eliminate variations in resting saliva pH between sampling periods, a new variable can be introduced - saliva flow rate. This may partly explain the erratic saliva VPA levels observed. VPA is poorly lipophilic compared to other antiepileptic drugs such as CBZ, phenytoin and phenobarbital (Goldberg and Todoroff, 1980). However, since the concentration of VPA was found to be higher in the citric acid stimulated saliva compared to non-stimulated saliva (Abbott, unpublished observation), other factors such as facilitated transport of VPA may be responsible for the inconsistent saliva levels of VPA.

It has been stressed that little VPA is secreted into saliva because of its low pKa. On the other hand the cerebrospinal fluid (CSF) to serum VPA concentration ratio has been found to be 0.10 (Blom and Guelen, 1977), 0.11, (Monaco et al., 1982) and 0.08 (Kido, 1982). These concentrations in CSF are more or less equal to the unbound concentration in serum and it is generally accepted that the CSF concentration reflects the free drug in plasma. The normal pH of the CSF is 7.32 (West, 1985). In the study by Abbott (unpublished data) it was found that following stimulation of saliva flow with citric acid in six volunteers the parotid saliva pH varied very little after 2 minutes of the initial stimulation by citric acid (7.46 ± 0.14, n=20). Thus there

is little difference between stimulated saliva and CSF pH whereas there is almost a 5-10 fold difference in the concentration of VPA in the two tissues.

Cornford et al. (1985) have suggested that a fraction of the VPA entering the capillaries in the protein bound form has the capacity to equilibrate with brain because of enhanced drug dissociation from albumin in the brain circulation and that VPA is actively transported out of the brain. It has also been demonstrated in dogs that VPA is transported out of the CSF by the same anion efflux mechanism that transports γ-aminobutyric acid and probenecid out of the CSF (Loscher and Frey, 1982). In spite of this it was found in humans that the concentration of VPA in CSF was 7.6 to 25.0% of the total plasma concentrations in the range of 35.5 to 150.4 µg/mL (Vajda et al., 1981). This CSF concentration apparently reflects the free fraction, in plasma, although free levels were not determined in this study. From the above considerations, it is conceivable that there is an active transport of VPA out of saliva which would explain the low concentration in saliva relative to free serum and CSF concentrations.

C. Identification of VPA metabolites using NICI-GCMS of their PFB derivatives

The total ion current chromatogram plot of the PFB derivatized urine extract from one volunteer at VPA steady state administered selected doses of [$^2H_6$]-VPA is shown in Figure 23. This TIC plot contains peaks for VPA and 14 VPA metabolites: 3-ene VPA, (Z)-2-ene VPA, (E)-2-ene VPA, (E,Z)-2,3'-diene VPA, 2,4- diene VPA, (E,E)-2,3'-diene VPA, a new VPA metabolite (peak 10), 3-keto VPA, 3-OH VPA diastereomers, 4-keto VPA, 4-OH VPA, 5-OH VPA, 2-PSA and 2-PGA. All
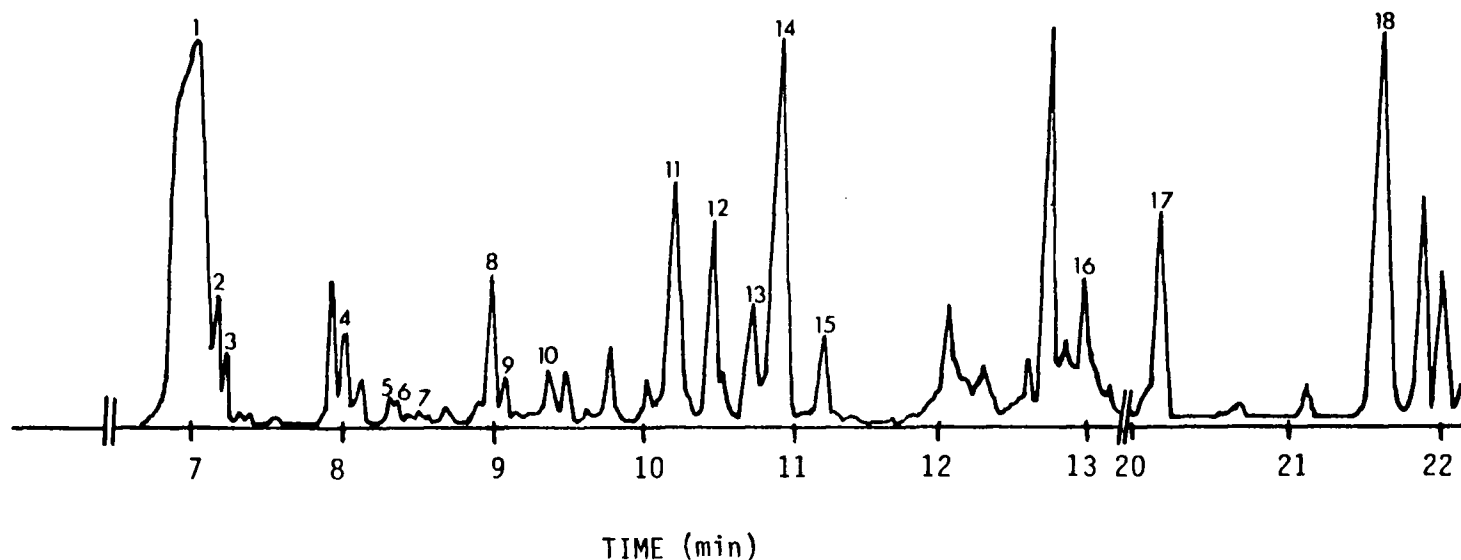
Figure 23.   Total ion current plot, in the NICI mode, of the PFB derivatized urine extract from a volunteer on VPA steady state, also given selected doses of $[^2H_6]$-VPA. Peak numbers correspond to: 1= VPA, 2= 3-ene VPA, 3= (Z)-2-ene VPA, 4= (E)-2-ene VPA, 5= (E,Z)-$[^2H_6]$-2,3'-diene VPA, 6= (E,Z)-2,3'-diene VPA, 7= 2,4-diene VPA, 8= (E,E)-$[^2H_6]$-2,3'-diene VPA, 9= (E,E)-2,3'-diene VPA, 10= 4'-keto-2-ene VPA, 11= 3-keto VPA, 12= 3-OH VPA, 13= 3-OH VPA, 14= 4-keto VPA, 15= 4-OH VPA, 16= 5-OH VPA, 17= 2-PSA, 18= 2-PGA.

peaks of interest were suitably resolved except those of 4-ene VPA and VPA. The 4-ene VPA peak is swamped by the huge VPA peak. The existence of the 4-ene VPA peak under the VPA peak was verified by obtaining the mass chromatograms at m/z 141. One of these peaks had identical retention time to that of injected synthetic 4-ene VPA (see later in Figure 37).

The identification of the isolated metabolites was facilitated by the doublet fragment ions (deuterated and undeuterated) in their negative ion spectra and with the help of synthetic reference compounds. The negative ion spectra of these urinary metabolites along with their synthetic standards are illustrated in Figures 24 through 36. Since the urine sample analyzed contained mainly deuterated VPA and metabolites, the intensities of labelled and unlabelled ions were not equal in the spectra obtained for the drug and metabolites.

1. Negative ion spectra of PFB derivatized VPA metabolites

In the negative ion mass spectra of VPA and its metabolites almost all of the ion current is carried by a single fragment anion, [M-pentafluorobenzyl (181)]$^-$ anion. This highly abundant, resonance stabilized carboxylate anion is formed under dissociative electron capture by cleavage of the PFB-oxygen bond. Since excess energy from the ionization process is dissipated by bond cleavage, this electron capture NICI technique is a highly efficient soft ionization process.

In Figures 24 to 37 the negative ion spectra of VPA and its metabolites are presented. All the metabolites and the parent drug have the [M-181]$^-$ ion as their base peak, the only exception being 3-keto
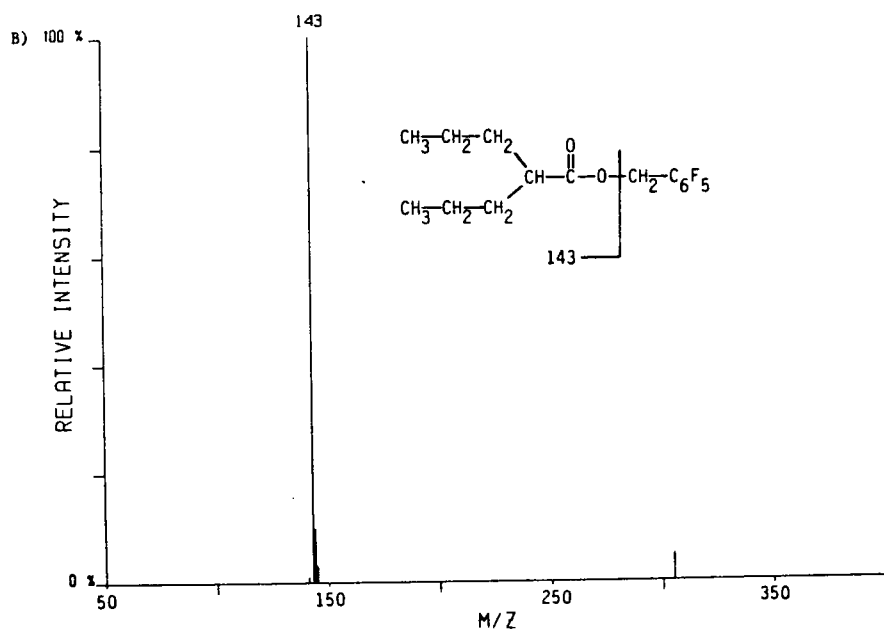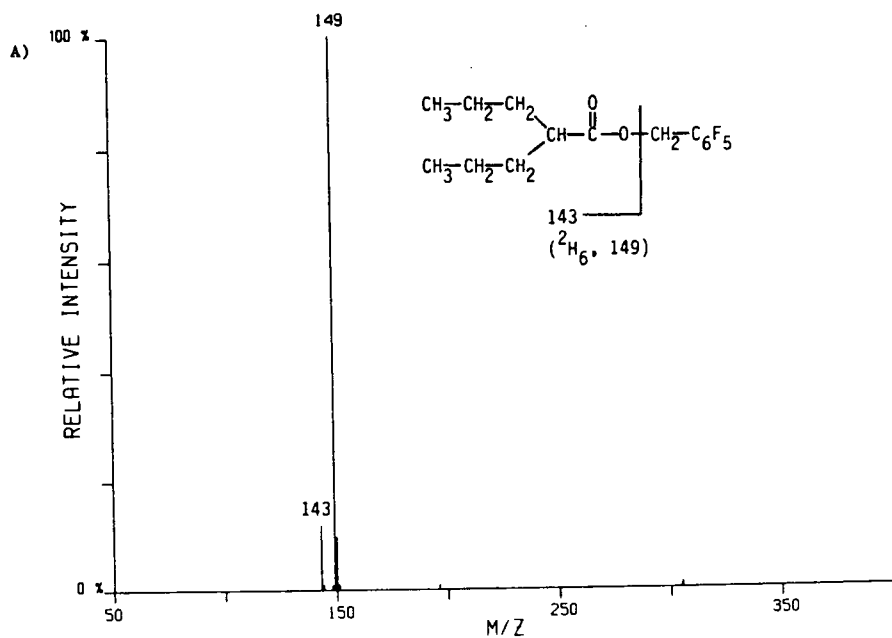
Figure 24.   NICI mass spectrum of A) VPA-PFB ($^2H_0$ + $^2H_6$) (peak 1, Fig. 23) and B) synthetic VPA-PFB.
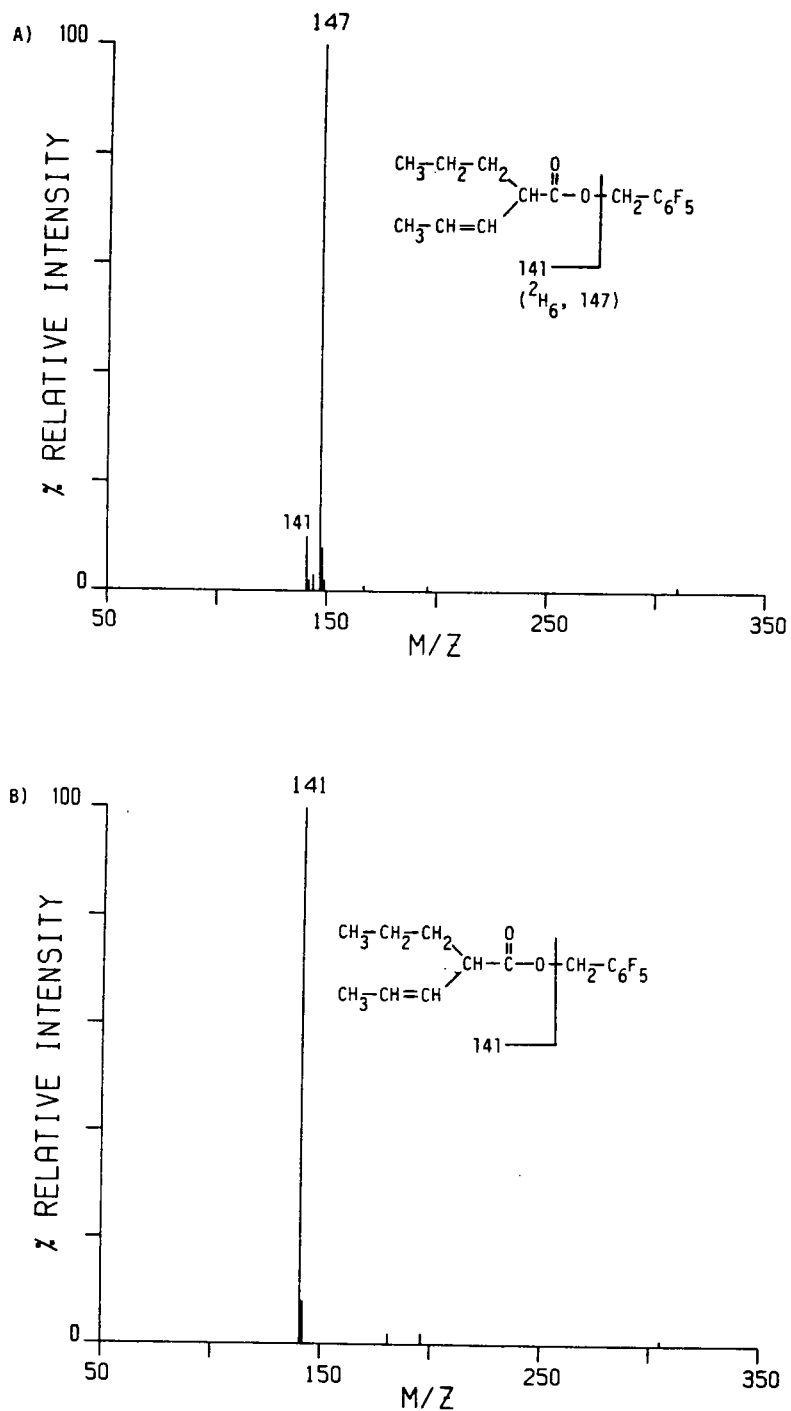
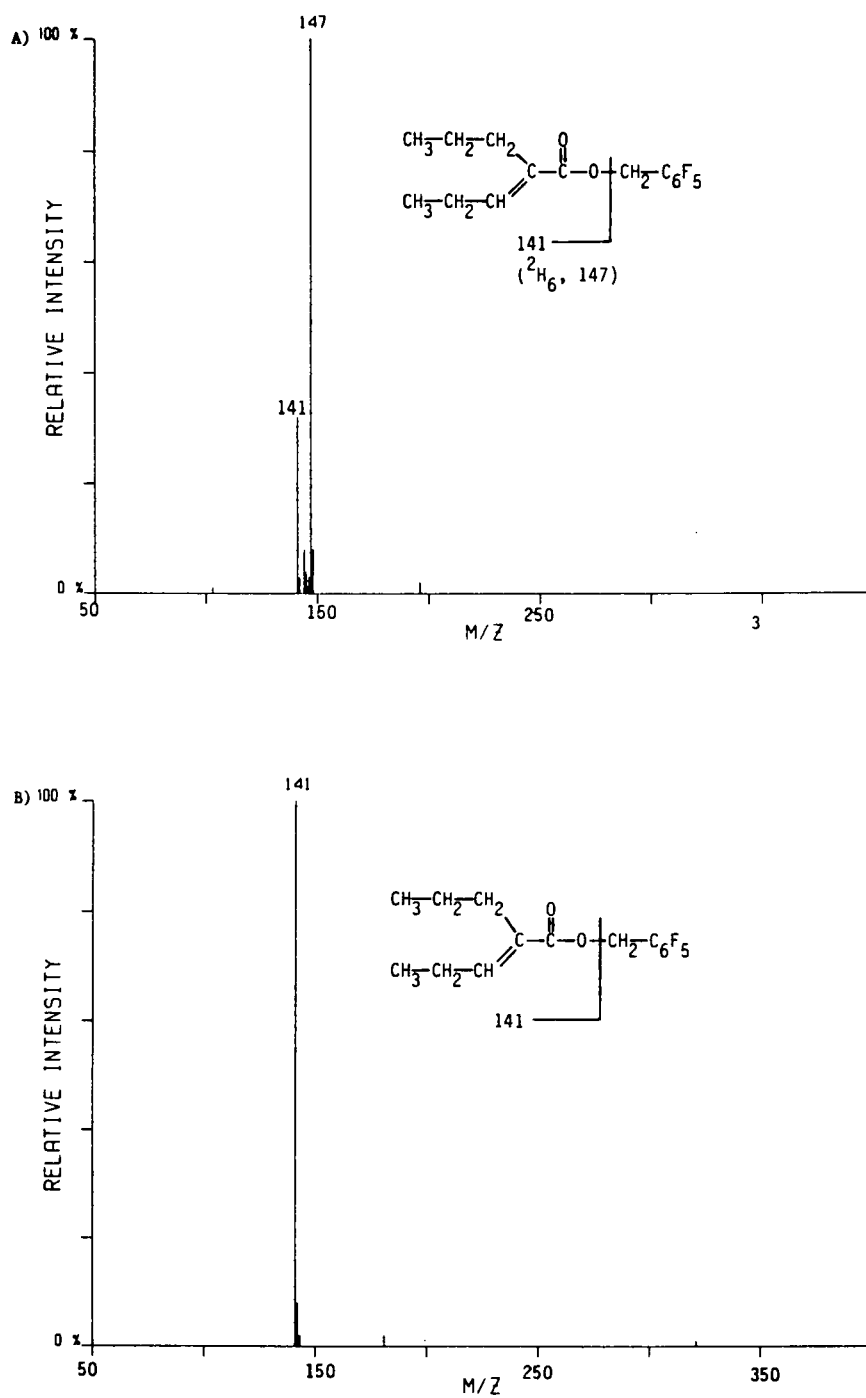Figure 25. NICI mass spectrum of A) 3-ene VPA-PFB ($^2H_0$ + $^2H_6$) (peak 2, Fig. 23) and B) synthetic 3-ene VPA-PFB.

Figure 26.   NICI mass spectrum of A) (E)-2-ene VPA-PFB ($^2H_0$ + $^2H_6$) (peak 4, Fig. 23) and B) synthetic (E)-2-ene VPA-PFB.

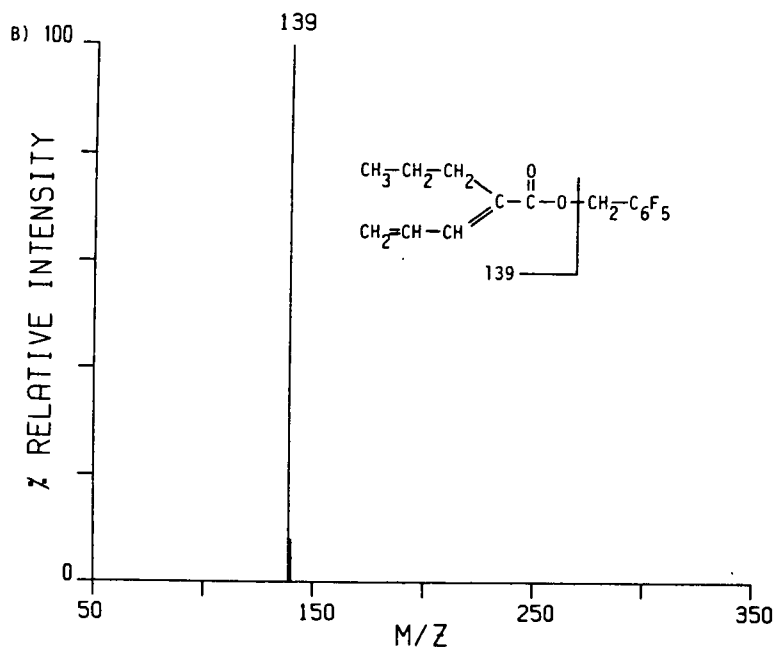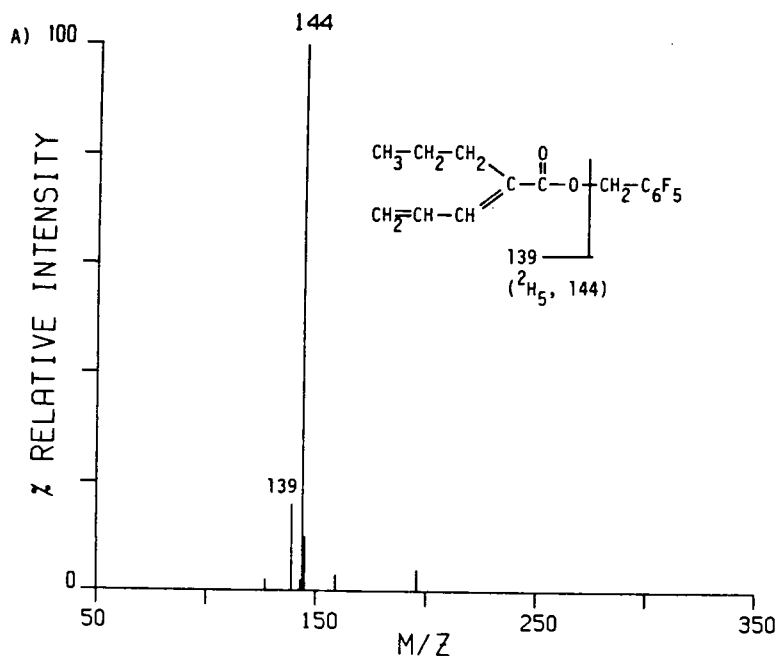Figure 27.   NICI mass spectrum of A) 2,4-diene VPA-PFB ($^2H_0$ + $^2H_5$) (peak 7, Fig. 23) and B) synthetic 2,4-diene VPA-PFB.
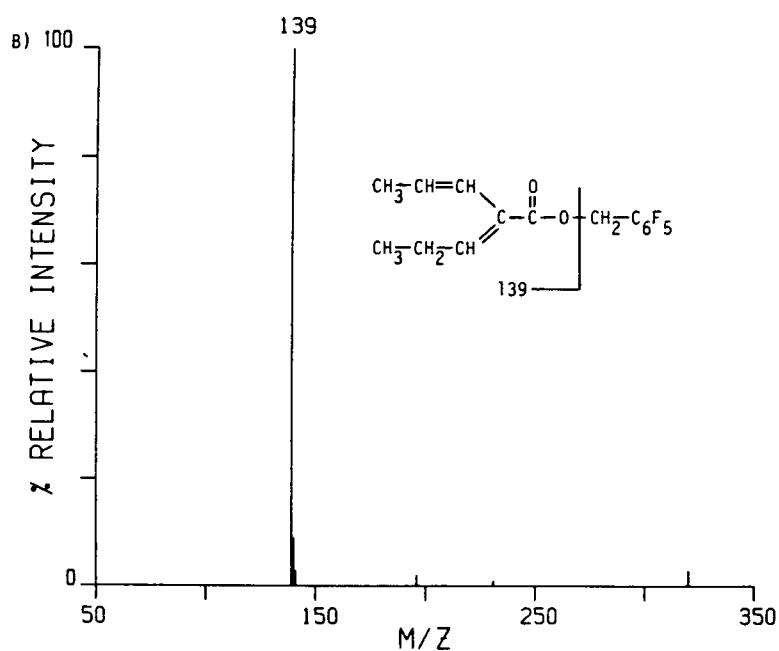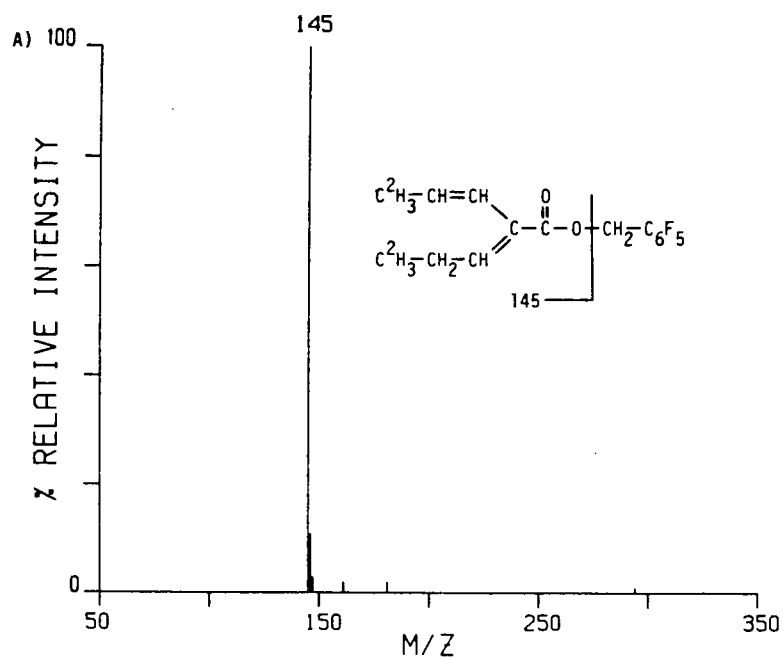
Figure 28. NICI mass spectrum of A) (E,E)-2,3'-diene VPA-PFB ($^2H_6$) (peak 8, Fig.23) and B) synthetic (E,E)-2,3'-diene VPA-PFB.[6]
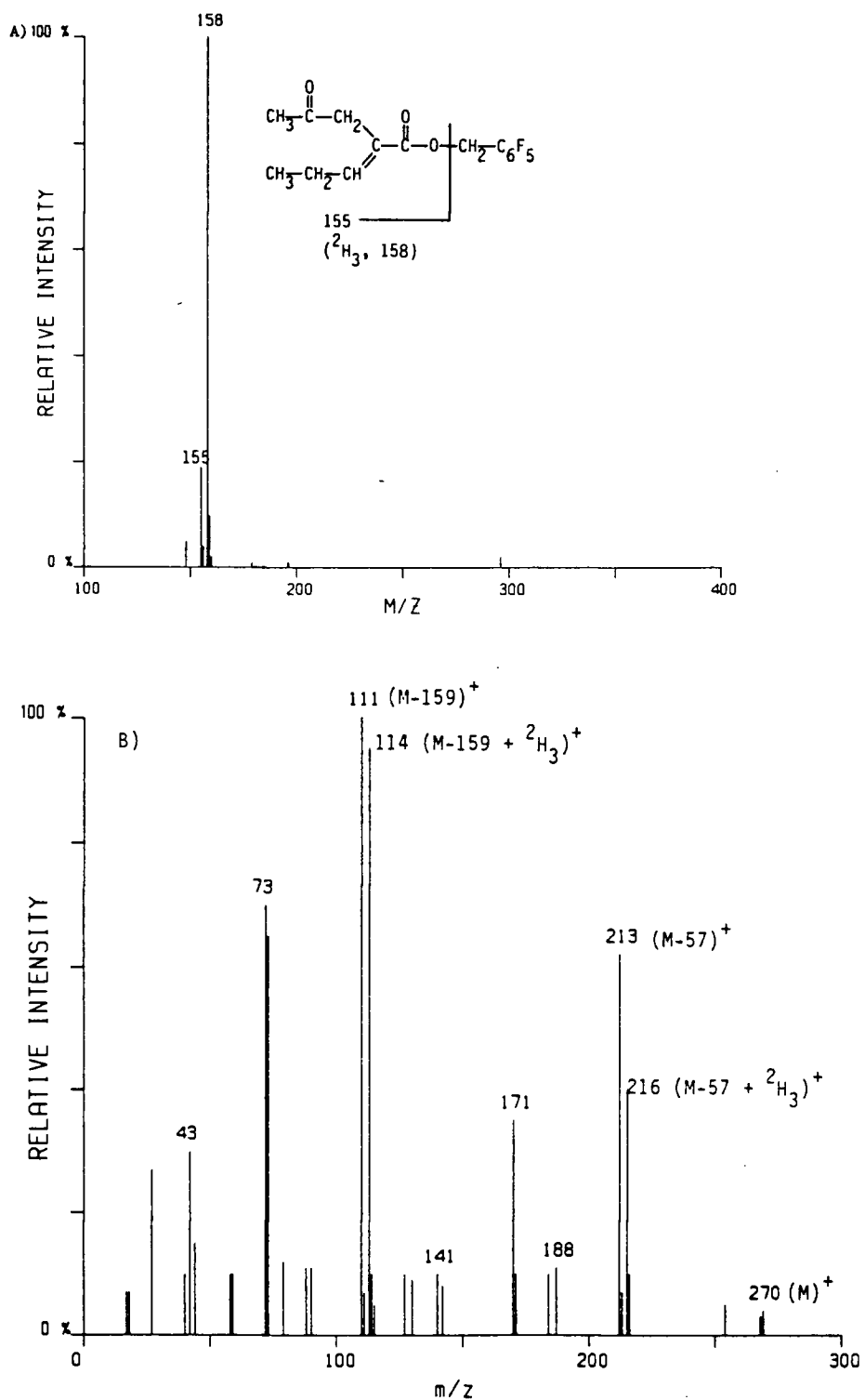
Figure 29. A) NICI mass spectrum of 4'-keto-2-ene VPA-PFB ($^2H_0$ + $^2H_3$) (peak 10, Fig.23), B) EI(t-BDMS) mass spectrum of 4'-keto-2-ene VPA.
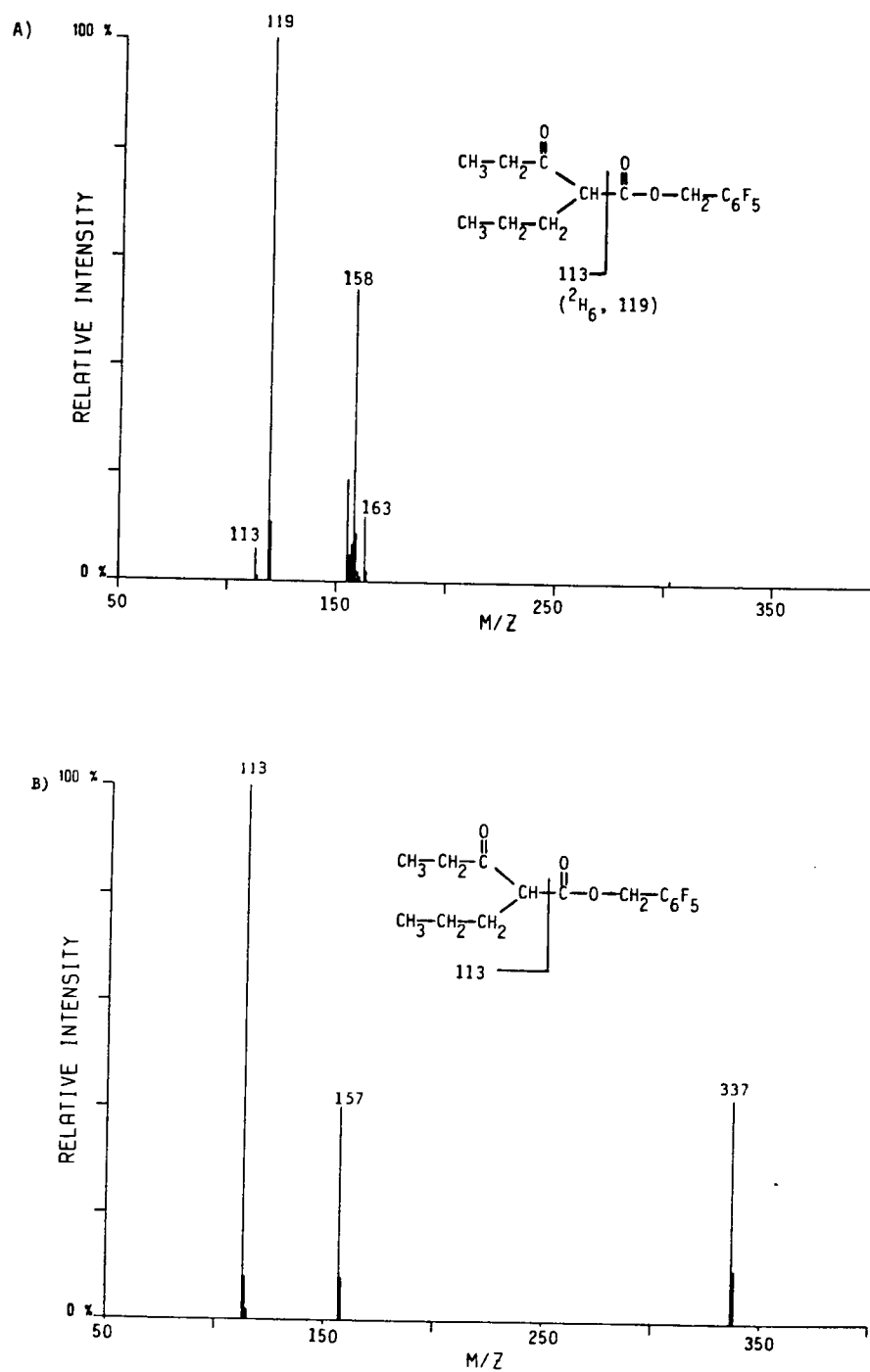
Figure 30. NICI mass spectrum of A) 3-keto VPA-PFB ($^2H_0$ + $^2H_6$) (peak 11, Fig. 23) and B) synthetic 3-keto VPA-PFB.
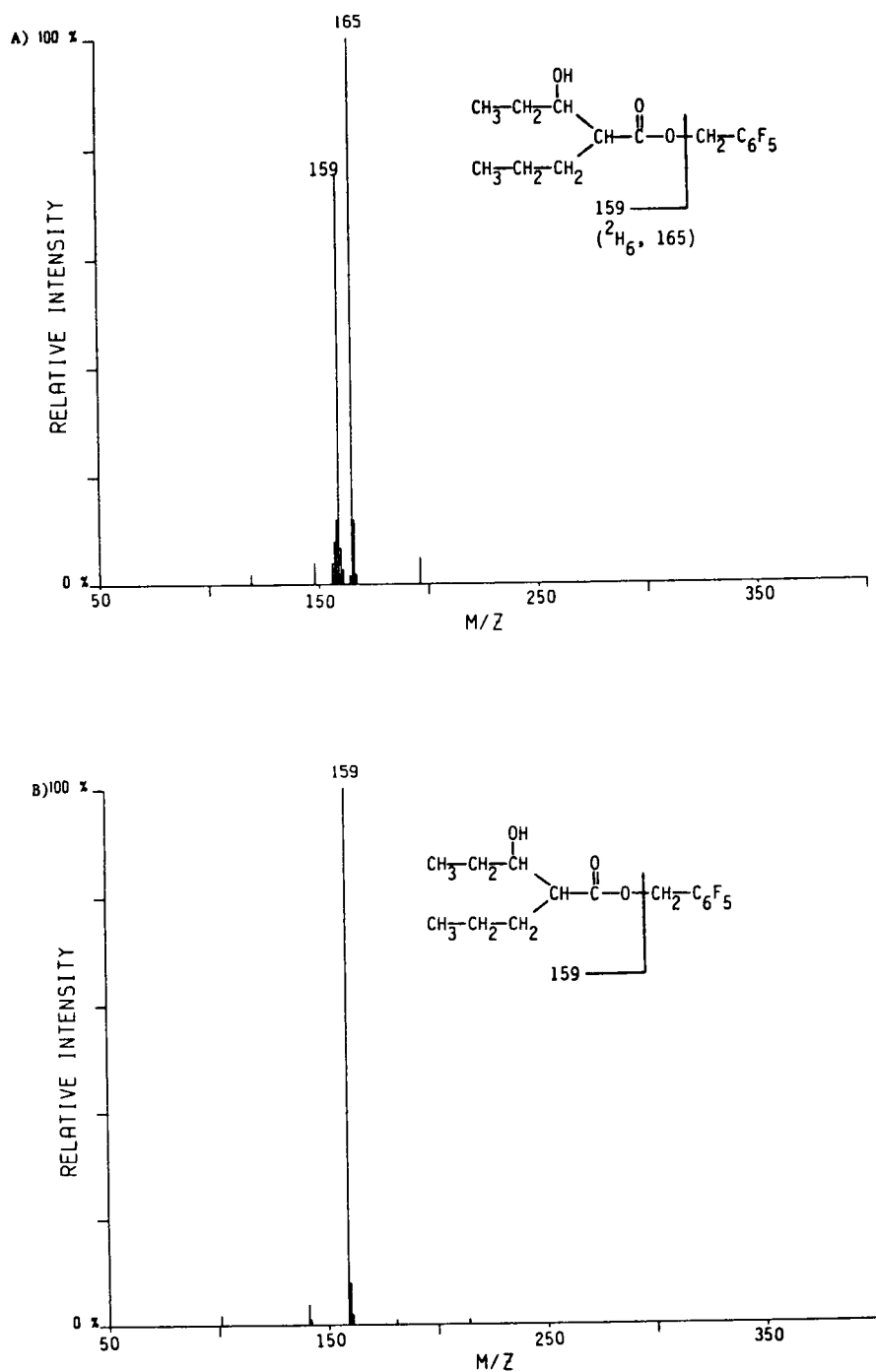
Figure 31    NICI mass spectrum of A) 3-OH VPA-PFB ($^2H_0 + {}^2H_6$) (peaks 12 and 13, Fig. 23) and B) synthetic 3-OH VPA-PFB.
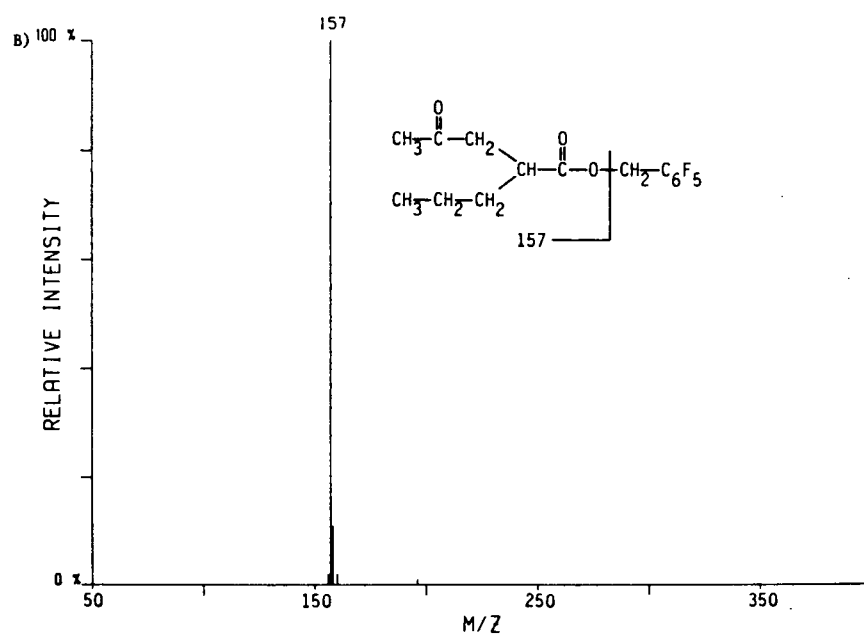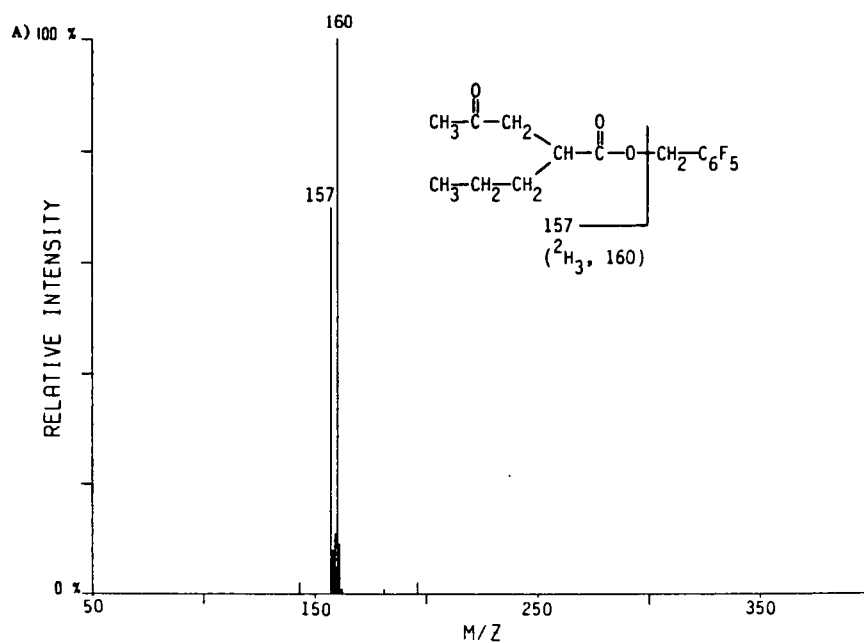
Figure 32. NICI mass spectrum of A) 4-keto VPA-PFB ($^{2}H_{0}$ + $^{2}H_{3}$) (peak 14, Fig. 23) and B) synthetic 4-keto VPA-PFB.
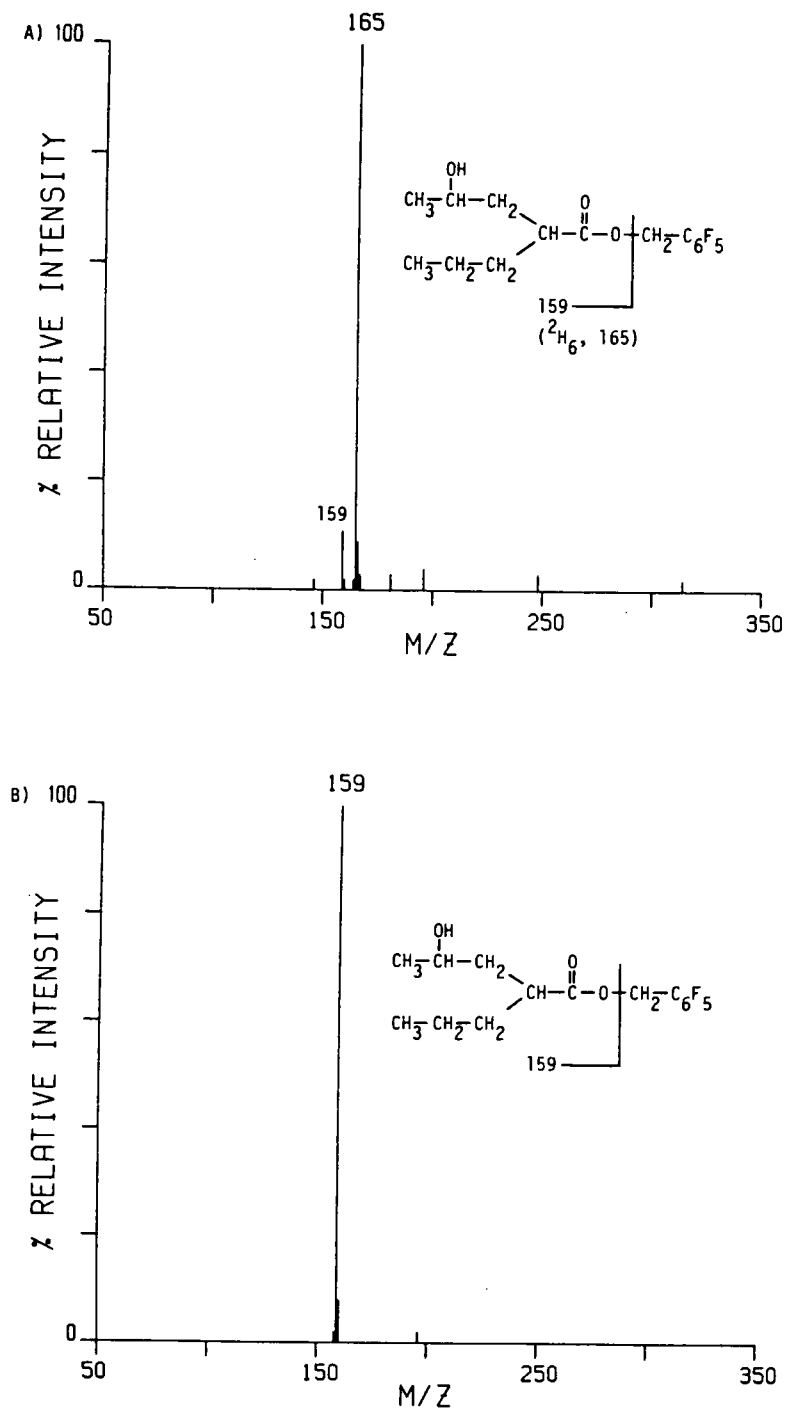
Figure 33. NICI mass spectrum of A) 4-OH VPA-PFB ($^2H_0$ + $^2H_6$) (peak 15, Fig. 23) and B) synthetic 4-OH VPA-PFB.

Figure 34.   NICI mass spectrum of A) 5-OH VPA-PFB ($^2H_0$ + $^2H_5$) (peak 16, Fig. 23) and B) synthetic 5-OH VPA-PFB.

Figure 35. NICI mass spectrum of A) 2-PSA-diPFB ($^2H_0$ + $^2H_3$) (peak 17, Fig. 23) and B) synthetic 2-PSA-diPFB.

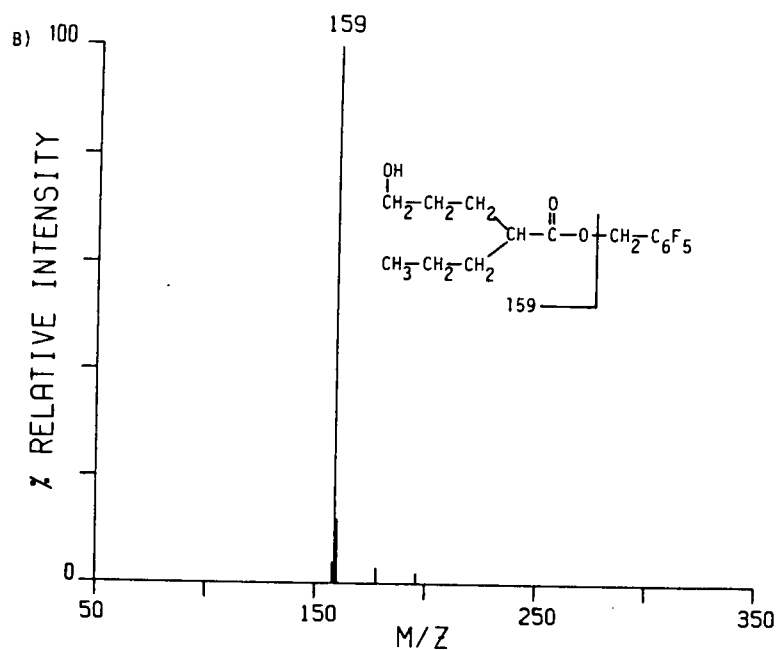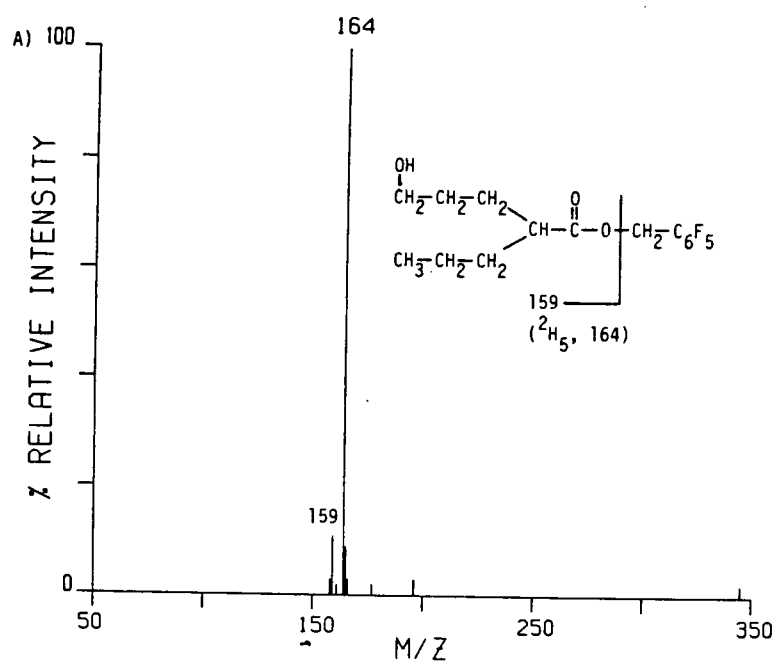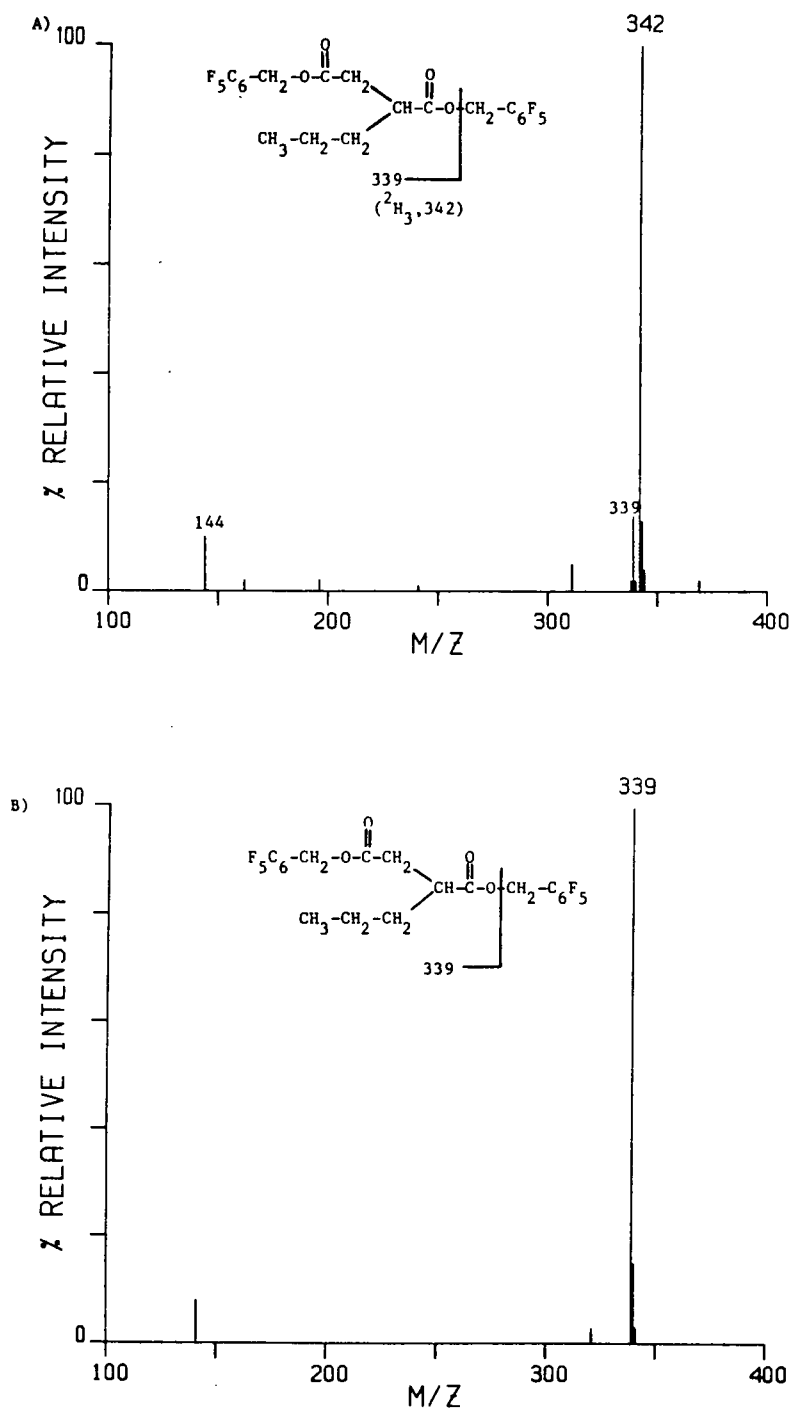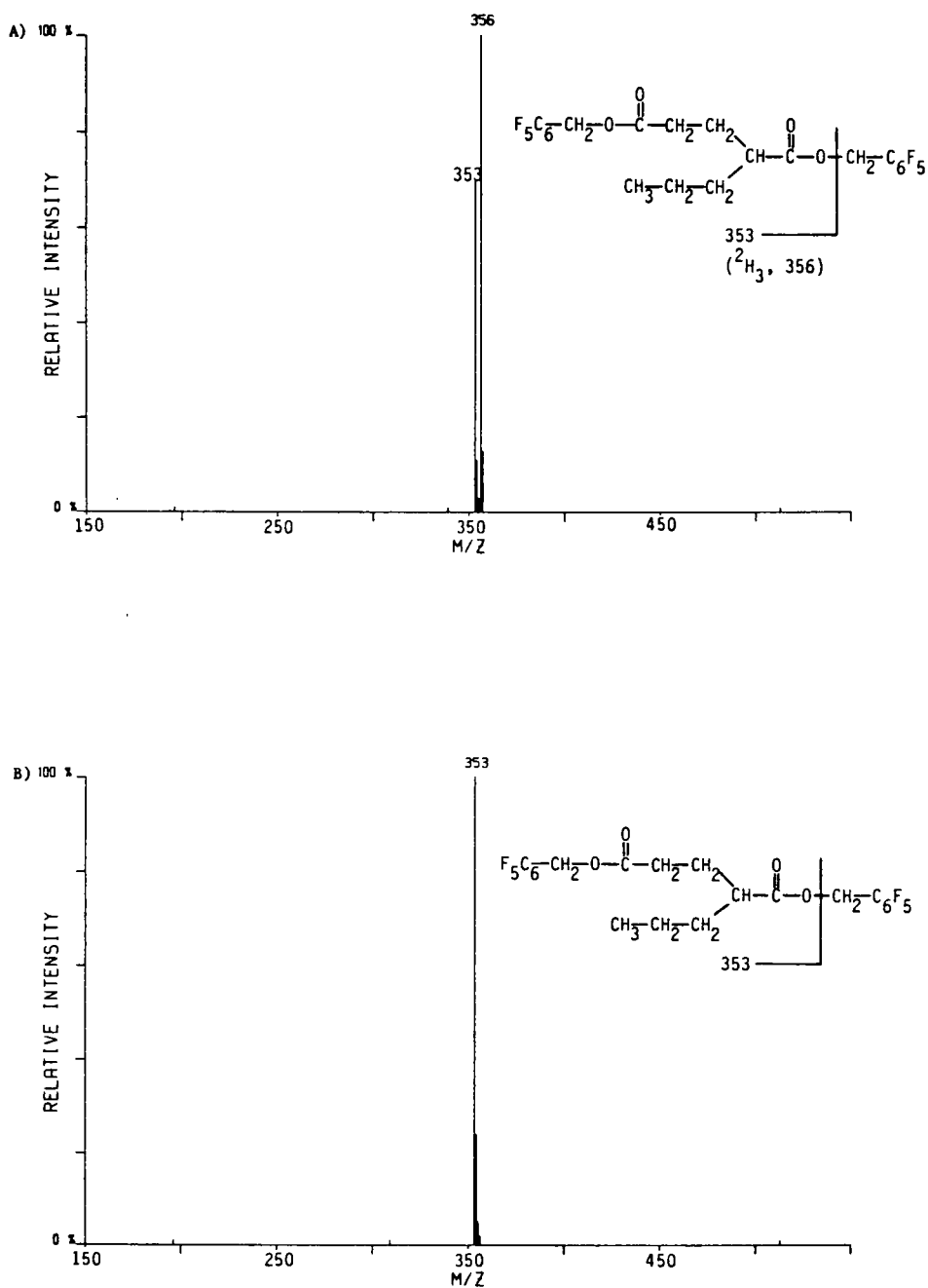Figure 36.   NICI mass spectrum of A) 2-PGA-diPFB ($^2H_0$ + $^2H_3$) (peak 18, Fig. 23) and B) synthetic 2-PGA-diPFB.

A)



B)
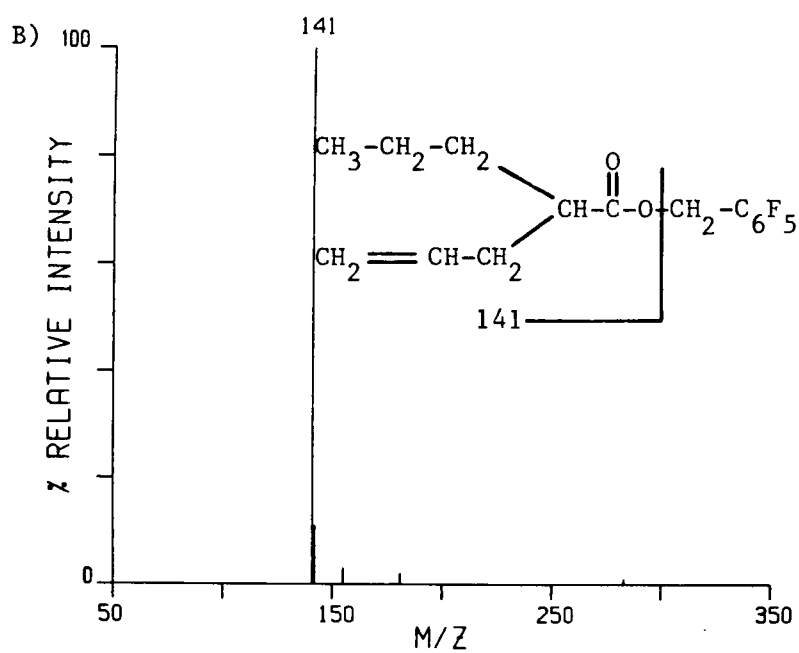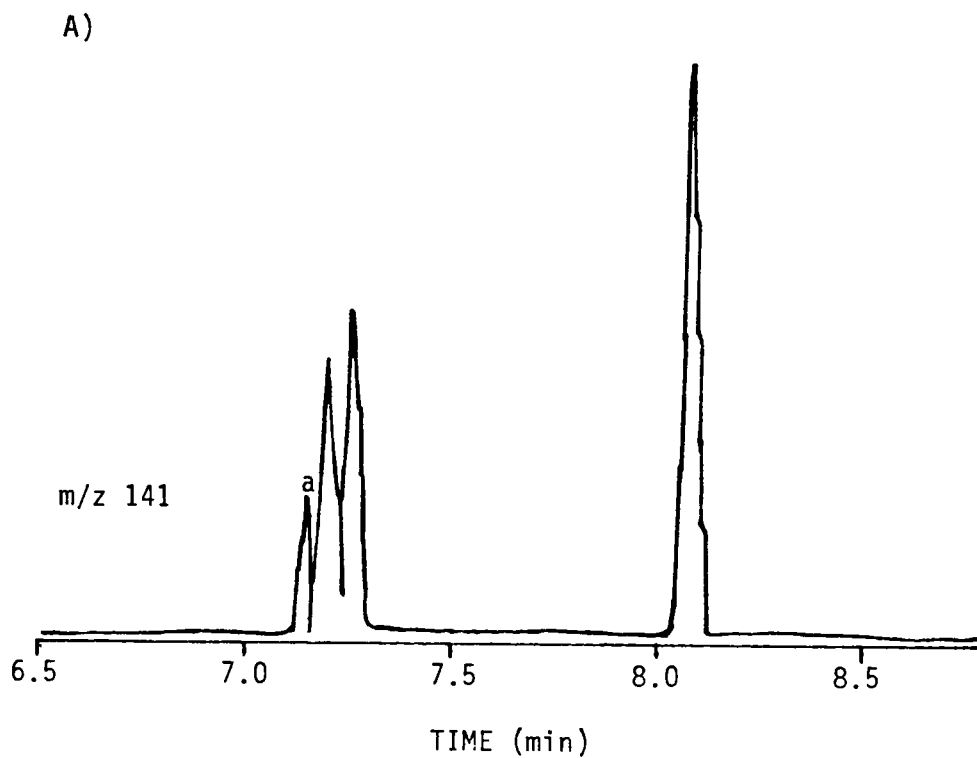


Figure 37. A) mass chromatograms at m/z 141 from Fig. 23 (peak a = 4-ene VPA), B) NICI mass spectrum of synthetic 4-ene VPA-PFB.

VPA. The negative ion spectrum of this particular metabolite is more complex than that of similar compounds derivatized with PFB. The base peak is m/z 113 (m/z 119, $^2H_6$), and there are two other major fragments; m/z 157 (M-181) and 337 (M-1) (Figure 30). The fact that the major peak is m/z 113 rather than m/z 157 can be explained by the presence of a 3-keto group that facilitates a rearrangement (decarboxylation) of the fragment ion to give the m/z 113 anion. The mechanism of the formation of this anion is given in Scheme 1.

Under the conditions of derivatization employed, there was no evidence of the derivatization of keto and hydroxyl groups. Hence, keto and hydroxyl metabolites of VPA yielded only mono-derivatives. This is in agreement with the report of Strife and Murphy (1984) who noted the non- reactivity of the hydroxy function of hydroxyeicosanoids towards PFBB. For 6-oxo-prostaglandins it was possible to obtain di-derivatives via the 6-keto function (using pentafluorobenzyl-hydroxylamine) and the carboxyl group i.e. PFB oxime and PFB ester (Waddell and Blair, 1983). Introduction of two electron capturing groups into a molecule should increase detection sensitivity. In this case, however, the sensitivity was less than the mono-PFB derivative because of a fragmentation pathway that gave rise to less abundant high mass ions of the di-derivative. The dicarboxylic acid VPA metabolites, 2-PSA and 2-PGA, gave di-PFB derivatives with the usual base peak i.e. [M-181]⁻ (Figures 35 and 36). In the TIC trace (Figure 23), 2-PGA appears to be the most prominent metabolite based on its peak height and the level of 2-PSA also looks high. Since 2-PGA is not the major urinary metabolite of VPA the enhanced sensitivity observed is because of the presence of two PFB moieties. Despite the increased sensitivity
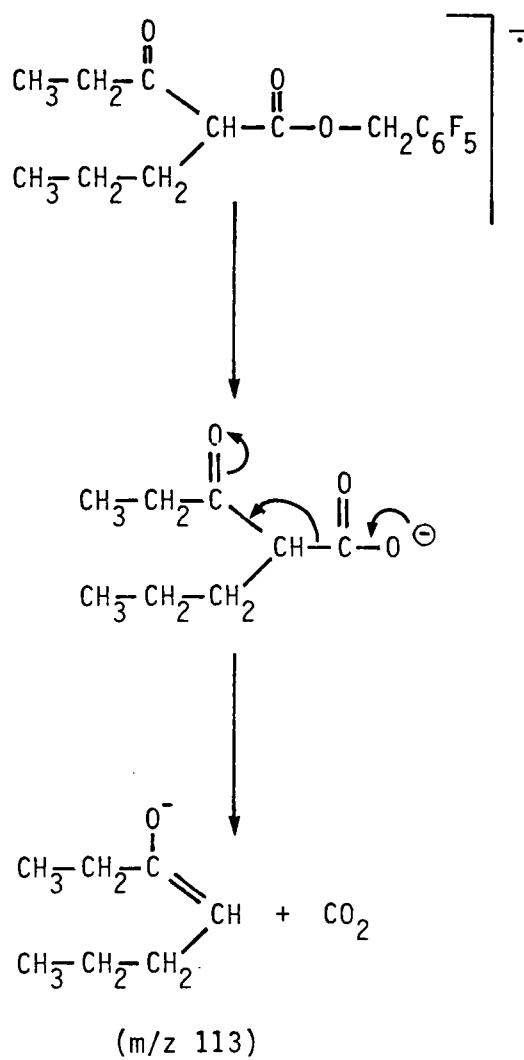
Scheme 1. Origin of the m/z 113 anion in the NICI mass spectrum of PFB
derivatized 3-keto VPA.

observed for the two dicarboxylic acids, 2-PGA and 2-PSA, a third dicarboxylic acid metabolite, 2-PMA, was not detected. 2-Propylmalonic acid (2-PMA) has been characterized as a VPA metabolite by Acheampong et al. (1983).

2. PFB as an electron capture NICI GCMS derivative for VPA metabolites

The PFB derivatives of VPA and its metabolites have good chromatographic properties giving sharp peaks in a reasonable GC run time. As with other carboxylic compounds derivatized with PFB (mainly prostanoids) the NICI spectra of the PFB derivatized VPA metabolites is typified by the $[M-181]^-$ anion. This ion results from cleavage of the PFB-oxygen bond. The exact mechanism of this process is not known (Waddell and Blair, 1983).

GCMS SIM is a very specific technique because analysis is carried out on the basis of two parameters; gas chromatographic retention time and the mass of the monitored ion(s). In addition, since fragmentation with PFB derivatives is almost always directed away from the intact analyte molecule (base peak is the intact molecule less 1), there is enhanced specificity. Because of the inherent sensitivity of electron capture NICI and since there is little fragmentation the system is very sensitive and hence ideal for SIM.

The PFB derivative is also superior to perfluoroalkyl derivatives because with the latter, the majority of the ion current is carried by small fragments from the derivatizing moiety. For example, Stan and Reich (1980) have reported a detection limit of 1fg by NICI for the

heptafluorobutyrate derivatives of hydroxy fatty acid methyl esters. But the ions monitored were those arising from the derivatizing molecule and are less specific. With bis-TFMB, however, an identical fragmentation pattern to that of the PFB was obtained (Figure 5B).

In contrast to PFB and bis-TFMB, pentafluorobenzoyl acyl derivatives of compounds containing free hydroxy groups and/or secondary amines produce molecular anions with virtually no fragmentation (Ramesah and Pickett, 1986). This is thought to be due to the increased stability of the PFB acyl derivative compared to PFB ester derivatives.

Less that 2% (Figure 5A) of the PFB ion (m/z 181), which is the base peak in the EI spectrum of PFB derivatized VPA, is observed in the NICI spectrum. The PFB anion is possibly not as stable as the resonance stabilized carboxylate anion (Strife and Murphy, 1984). The stability of the benzyl cation in EI, which rearranges to the more stable tropylium ion is well known and this explains the striking abundance of the m/z 181 in the EI spectrum of VPA-PFB. However, the number of $\pi$ electrons in a cycloheptatrienyl anion (tropylium anion) would not fit the $(4n+2)$ $\pi$ electron rule and it would be antiaromatic (Morrison and Boyd, 1973) and hence only a small amount of the m/z 181 anion was observed in the NICI spectra of VPA and its metabolites.

### 3. NICI (PFB) versus EI (t-BDMS) spectra of VPA metabolites

VPA metabolites have been assayed in our laboratory (Abbott et al., 1986a) as their t-BDMS derivatives by monitoring the characteristic [M-57]+ fragment ion which constitutes the base peak for VPA and many of the unsaturated metabolites. Nevertheless,

the [M-57]$^+$ ion is not the most intense ion for the polar metabolites, 3-OH VPA, 3-keto VPA and 4-keto VPA, most of the ion current being carried by fragment ions including m/z 73 and m/z 75 from the derivatizing moiety. This is illustrated for 3-OH VPA in Figure 38. In addition to the increased sensitivity, NICI analysis of PFB derivatized metabolites appears to be superior to the EI (t-BDMS) analysis in many ways. The [M-181]$^-$ ion is the base anion for all metabolites except for 3-keto VPA and this enables more specific and sensitive detection for all VPA metabolites. The keto and hydroxy metabolites can give either mono or di-derivatives of t-BDMS depending upon the derivatization conditions. With the t-BDMS reagent in pyridine, 3-OH VPA does not derivatize readily and chromatographs poorly. With PFB, 4-OH VPA derivatizes and gives NICI spectrum whereas with the t-BDMS method a derivative of 4-OH VPA is not seen and 4-OH VPA is analyzed as the underivatized $\gamma$-lactone. Finally, PFB derivative formation is facile and the time is short compared to the 4 hours required for t-BDMS derivative formation.

## 4. Selected ion chromatograms

Selected ion chromatograms obtained by monitoring the appropriate ions for all VPA metabolites extracted from a urine sample are illustrated in Figure 39. The deuterated analogs are also presented since the sample contained [$^2$H$_6$]-VPA and its metabolites. The ions monitored are summarized in Table 12. Similar SIM chromatograms for serum VPA and [$^2$H$_6$]-VPA metabolites are presented in Figure 40.
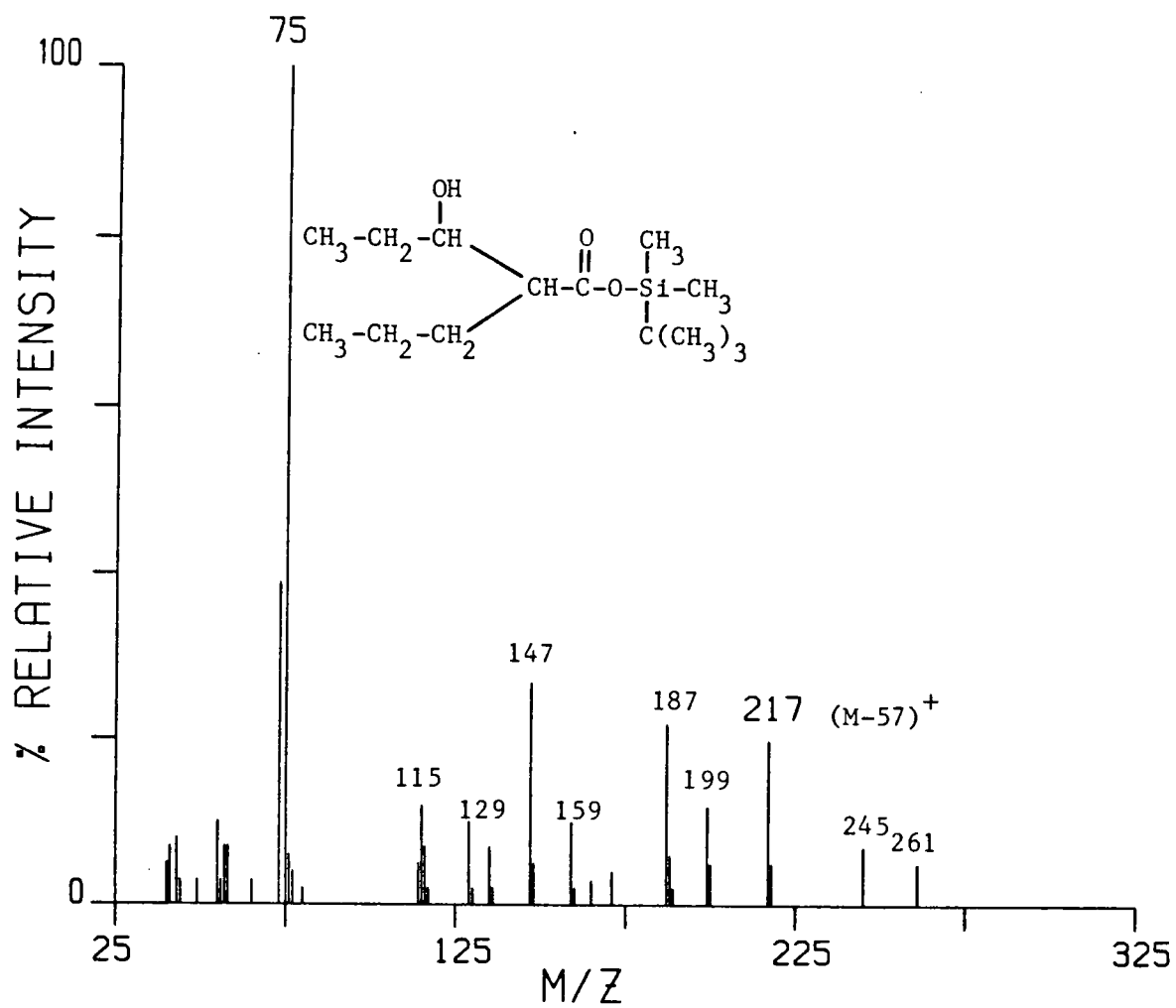
Figure 38.   EI (t-BDMS) mass spectrum of 3-OH VPA.

TABLE 12. Ions (m/z) monitored in NICI mode for VPA and [$^2$H$_6$]-VPA metabolites derivatized with PFB.

| COMPOUND | UNDEUTERATED | DEUTERATED |
| --- | --- | --- |
| 3-keto VPA | 113 | 119 |
| 2,4-diene VPA | 139 | 144 |
| (E,E)-2,3'-diene VPA | 139 | 145 |
| (Z)-2-ene VPA | 141 | 147 |
| (E)-2-ene VPA | 141 | 147 |
| 4-ene VPA | 141 | 146 |
| 3-ene VPA | 141 | 147 |
| VPA | 143 | 149 |
| 4'-keto-2-ene VPA | 155 | 158* |
| 4-keto VPA | 157 | 160* |
| 3-OH VPA | 159 | 165 |
| 4-OH VPA | 159 | 165 |
| 5-OH VPA | 159 | 164 |
| 2-PSA | 339 | 342** |
| 2-PGA | 353 | 356** |

* When alkali is used to hydrolyze conjugates, otherwise 161 and 163

** di-derivatives

Figure 39. SIM chromatograms of the PFB derivatives of VPA and [$^2$H$_6$]-VPA metabolites in a urine extract. Peaks: 1= 2,4-diene VPA, 2= (E,E)-2,3'-diene VPA, 3= 4-ene VPA, 4= 3-ene VPA, 5= (Z)-2-ene VPA, 6= (E)-2-ene VPA, 7= VPA, 8= [$^2$H$_5$]-2,4-diene VPA, 9= (E,E)-[$^2$H$_6$]-2,3'-diene VPA, 10= [$^2$H$_6$]-3-ene VPA, 11=(Z)-[$^2$H$_6$]-2-ene VPA, 12= (E)-[$^2$H$_6$]-2-ene VPA, 13= [$^2$H$_6$]-VPA, 14= 4'-keto-2-ene VPA, 15 and 18= unidentified peaks interfering with 3-keto VPA peaks, 16= 4-keto VPA, 17= [$^2$H$_3$]-4'-keto-2-ene VPA, 19= 3-OH VPA, 20= 3-OH VPA, 21= 4-OH VPA, 22= 5-OH VPA, 23= [$^2$H$_3$]-4-keto VPA, 24= [$^2$H$_5$]-5-OH VPA, 25= [$^2$H$_6$]-3-OH VPA, 26= [$^2$H$_6$]-3-OH VPA, 27= [$^2$H$_6$]-4-OH VPA, 28= 2-PSA, 29= [$^2$H$_3$]-2-PSA, 30= 2-PGA, 31= [$^2$H$_3$]-2-PGA

Figure 40. SIM chromatograms of the PFB derivatives of serum VPA and
$[^2H_6]$-VPA metabolites. Peaks: 1= 3-keto VPA, 2= $[^2H_6]$-3-keto
VPA, 3= 2,4-diene VPA, 4= (E,E)- 2,3'-diene VPA, 5= 4-ene VPA,
6= 3-ene VPA, 7= (Z)-2-ene VPA, 8= (E)-2-ene VPA, 9= VPA,
10= $[^2H_5]$-2,4-diene VPA, 11= (E,E)-$[^2H_6]$-2,3'-diene VPA,
12= $[^2H_6]$-3-ene VPA, 13= (Z)-$[^2H_6]$-2-ene VPA, 14= (E)-$[^2H_6]$-
2-ene VPA, 15= $[^2H_6]$-VPA, 16= 4'-keto-2-ene VPA, 17= 4-keto
VPA, 18= $[^2H_3]$-4'-keto-2-ene VPA, 19= 3-OH VPA, 20= 3-OH VPA
21= 4-OH VPA, 22= 5-OH VPA, 23= $[^2H_3]$-4-keto VPA, 24= $[^2H_5]$-
5-OH VPA, 25= $[^2H_6]$-3-OH VPA, 26= $[^2H_6]$- 3-OH VPA, 27= $[^2H_6]$-
4-OH VPA, 28= 2-PSA, 29= $[^2H_3]$-2-PSA, 30= 2-PGA, 31= $[^2H_3]$-
2-PGA.

Urine and serum controls showed no interfering peaks, however, the origin of the m/z 158 ion in the NICI mass spectrum of PFB derivatized 3-keto VPA (Figure 30A) is not clear since this ion is absent in the spectrum of synthetic 3-keto VPA (Figure 30B).

In summary GCMS-NICI using PFB derivatives appears to be superior, in terms of derivative formation and sensitivity to other currently available methods for the analysis of VPA metabolites. All metabolites gave mono-derivatives (dicarboxylic acid metabolites formed di-derivatives) and were analyzed simultaneously in one chromatographic run. The derivative formation and chromatographic characteristics have been defined and SIM chromatograms obtained. The analysis has not yet been used to quantitate metabolites, but it is only a matter of selecting appropriate internal standards and applying the method.

## 5. VPA Metabolites in Saliva

Because of the high sensitivity of the NICI method with PFB derivatives it was possible to detect and identify VPA metabolites in saliva. The metabolites detected were 4-ene VPA, 3-ene VPA, (Z)-2-ene VPA, (E)-2-ene VPA, (E,E)-2,3'-diene VPA, 3-keto VPA and 4-keto VPA. The metabolites were first detected by SIM, but it was also possible to detect them under linear scan conditions. The latter was performed on a saliva sample that also contained metabolites of $[^2H_6]$-VPA. The metabolites were positively identified with the help of their twin ions and by injecting standards for retention time comparison. The SIM chromatograms of fourteen salivary metabolites of VPA and $[^2H_6]$-VPA are shown together in Figure 41. Interestingly, none of the more polar
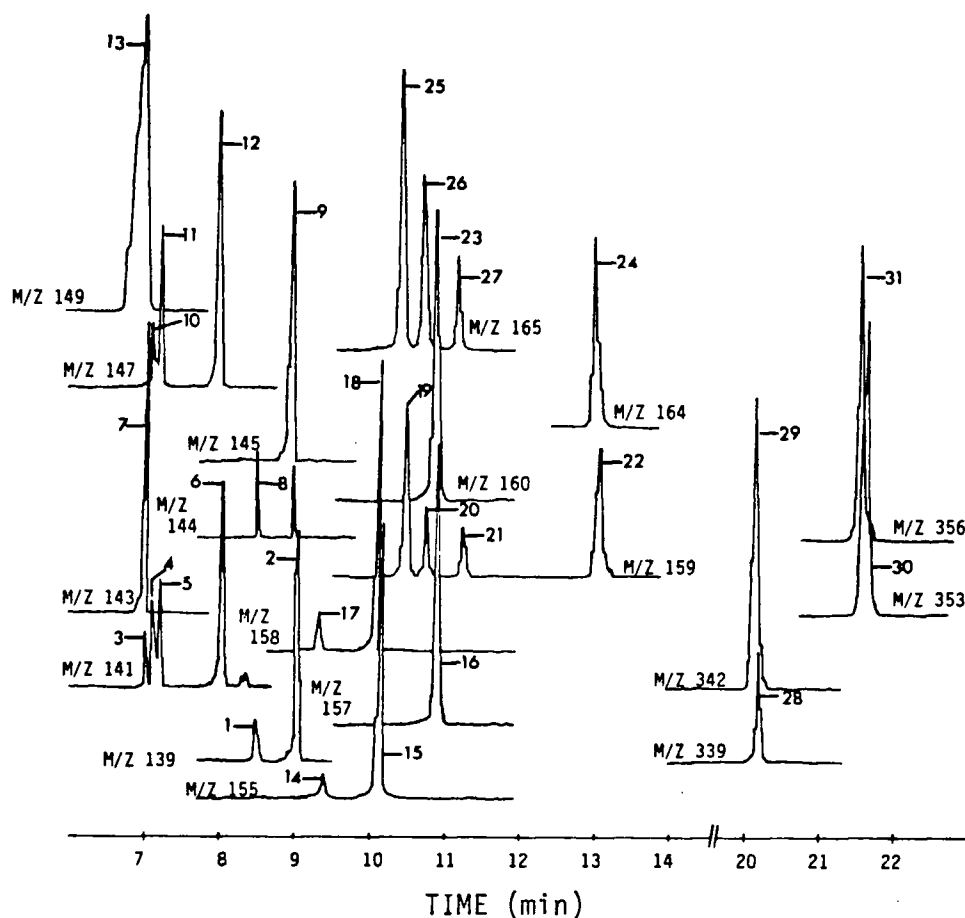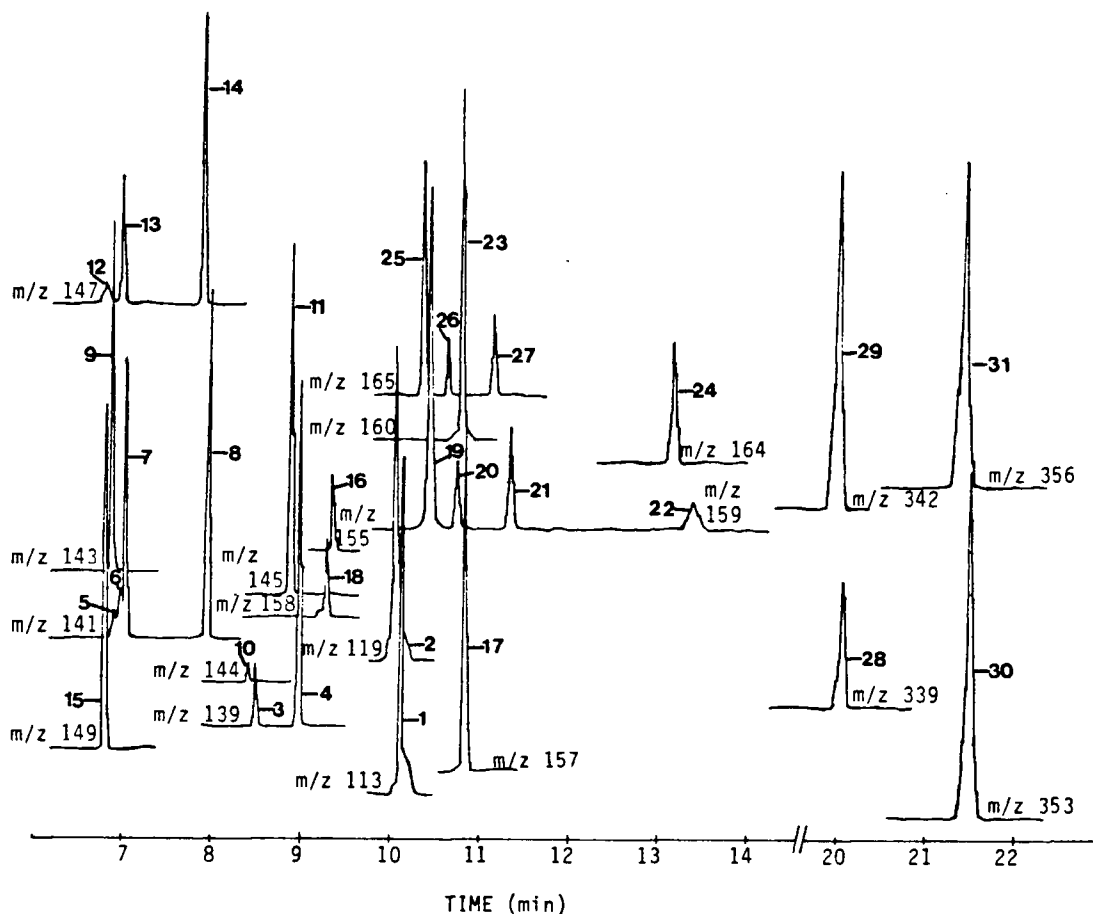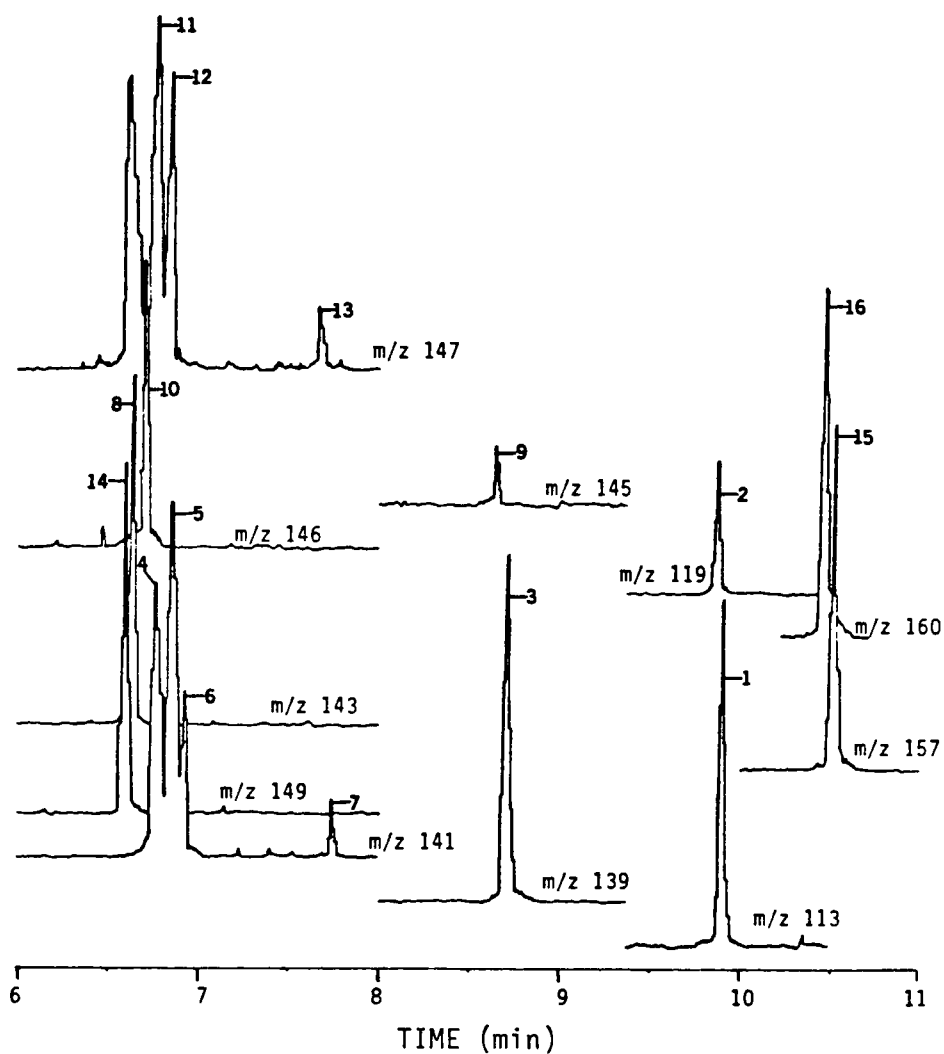
Figure 41. SIM chromatograms of the PFB derivatives of VPA and $[^2H_6]$-VPA metabolites in a saliva extract. Peaks: 1= 3-keto VPA, 2= $[^2H_6]$-3-keto VPA, 3= (E,E)-2,3'-diene VPA, 4= 4-ene VPA, 5= 3-ene VPA, 6= (Z)-2-ene VPA, 7= (E)-2-ene VPA, 8= VPA, 9= (E,E)-$[^2H_6]$-2,3'-diene VPA, 10= $[^2H_5]$-4-ene VPA, 11= $[^2H_6]$-3-ene VPA, 12= (Z)-$[^2H_6]$-2-ene VPA, 13= (E)-$[^2H_6]$-2-ene VPA, 14= $[^2H_6]$-VPA, 15= 4-keto VPA, 16= $[^2H_3]$-4-keto VPA.

hydroxy metabolites were detected in saliva. The levels of all metabolites appear to be higher than that of (E)-2-ene VPA which is the major VPA metabolite in serum. This is not surprising since this metabolite is bound to plasma proteins in excess of 98% (Nau et al.,1984). What is intriguing, however, is the fact that there is a higher level of (Z)-2-ene in saliva than (E)-2-ene. Under the experimental conditions employed for the extraction of VPA metabolites including the isomers of 2-ene VPA from serum, it does not appear that there is a conversion of (E)-2-ene VPA to (Z)-2-ene VPA, and it is unlikely that there will be an interconversion during the extraction of these compounds from saliva. In a recent study of VPA metabolite levels in the serum of pediatric patients by Abbott et al.(1986a) it was found that the concentration of (E)-2-ene was about 30 times that of (Z)-2-ene. In the present study the ratio of Z to E isomers of 2-ene VPA was much greater in saliva than in serum (3.82 vs. 0.458), suggesting differences in the transport or plasma protein binding properties of these two isomers.

Albumin, the most abundant protein in plasma is the most important drug binding protein (Sjoholm, 1984). Albumin has a broad binding specificity with compounds of widely different structures including fatty acids, bilirubin and many drugs binding with high affinity. For fatty acids, binding appears to be dependent upon the structure of the fatty acid (Spector and Fletcher, 1978). The strength of binding increases as the chain length of the fatty acid increases. Also for a given chain length the presence of a single cis double bond increases the strength of the binding (oleate > stearate). This report is not

consistent with our observation for the isomers of 2-ene VPA, but Spector and Fletcher (1978) did not compare the binding of cis oleate to that of its trans isomer. Drug metabolites are generally more polar and less protein bound than their parent drugs (Drayer, 1984). This is not, however, true with trans-2-ene VPA which is more protein bound and has a similar elimination half-life to that of the parent drug (Loscher et al., 1984). Because of its high protein binding trans-2-ene VPA is likely to have a displacing effect on VPA binding and may result in drug metabolite interaction.

The binding of acidic drugs to albumin or to other human plasma proteins is to some extent stereoselective with respect to enantiomers (Drayer, 1984; van Ginneken et al., 1983). To our knowledge, there is no report in the literature with respect to stereoselective protein binding of geometric isomers. There are some examples of stereoselective metabolism of trans and cis isomers (Vermeulen and Breimer, 1983). In rat adipocytes, cis-unsaturated fatty acids stimulated lipogenesis whereas saturated or trans-unsaturated fatty acids were ineffective (Shechter and Henis, 1984). From a structural point of view, cis isomers of fatty acids have a bend at the double bond in contrast to trans isomers and saturated fatty acids which extend in a linear conformation (Morrison and Boyd, 1973). For this reason, cis isomers fit with each other or other molecules very poorly. This may explain why cis-2-ene VPA can be less protein bound than trans-2-ene VPA.

The higher saliva level of the cis isomer of 2-ene VPA might as well be due to a stereoselective transport to saliva. There may be a facilitated transport of the cis isomer into saliva or conversely, the

trans isomer may be stereoselectively transported out of saliva.

In order to delineate the mechanism of the apparent higher saliva level of the cis isomer of 2-ene VPA compared to the trans isomer, more experiments are required, including in vitro and in vivo protein binding studies of the pure isomers. Should the serum protein binding properties prove to be markedly different, it is likely that the cis isomer will also have different pharmacokinetic and pharmacodynamic properties from that of the trans isomer.

### 6. Detection of new VPA metabolites

Four derivatives, namely, TMS, methyl ester, t-BDMS and PFB (NICI) were employed for the detection of new VPA metabolites. The latter two derivatives proved to be more useful because of their superior sensitivity and typical diagnostic fragment ions. New metabolites including one which appears to be 2-(2'-propenyl)-glutaric acid and an unsaturated keto metabolite were apparent. In Figure 42 are shown the EI and NICI spectra of a VPA related compound in urine (absent in control urine) that appears to be 2-(2'-propenyl)-glutaric acid. The structural assignment for this apparent new metabolite is made difficult by the fact that the intensity of the deuterated ions in both the EI and NICI spectra are much less than those of other metabolites. The 2-(2'-propenyl)-glutaric acid has been characterized as a metabolite of 4-ene VPA in the Rhesus monkey (Rettenmeier et al., 1986a).

The mass spectra of the unsaturated keto metabolite contains characteristic twin fragment ions that help reveal its identity with
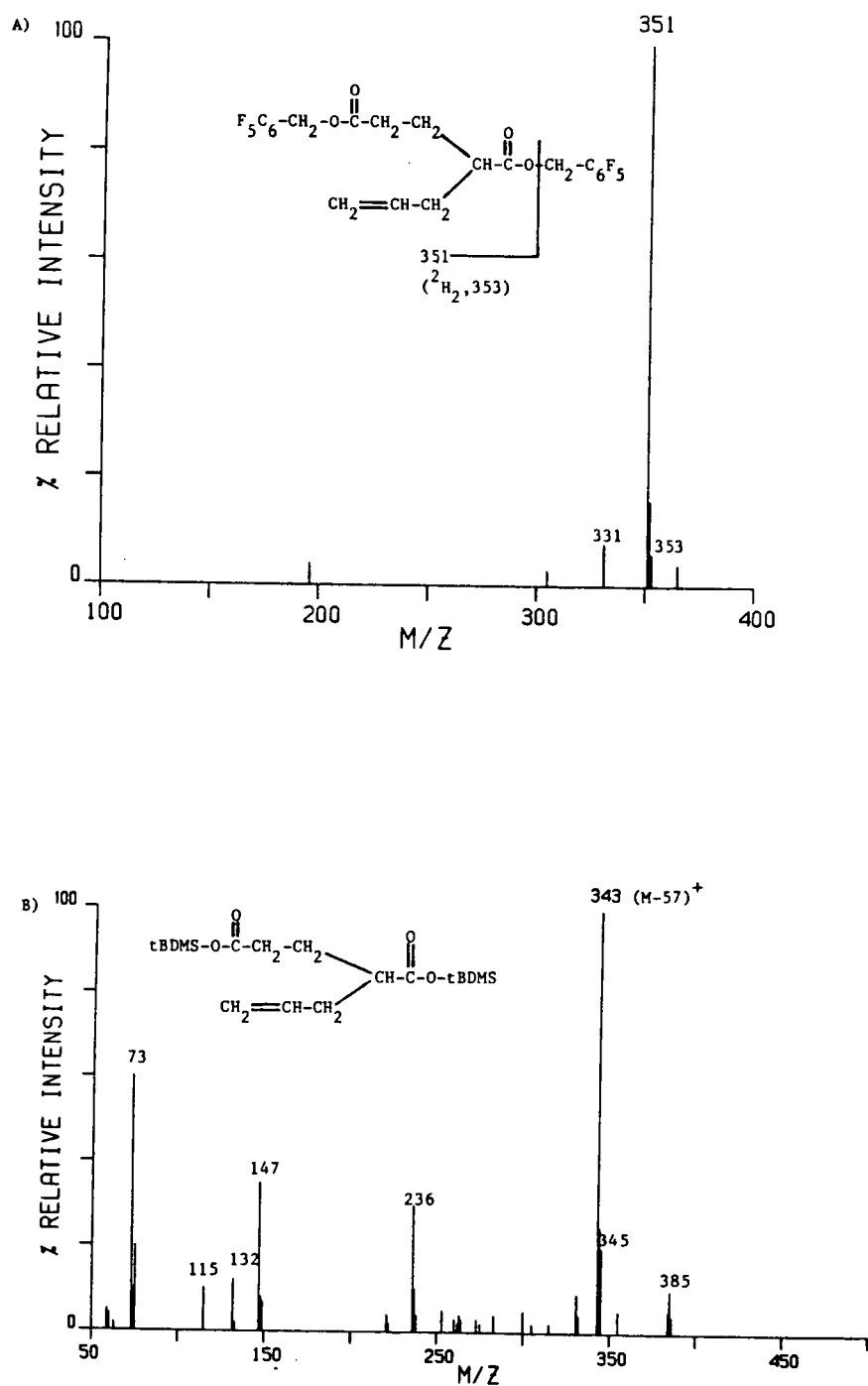
Figure 42.    NICI (A) and EI (B) mass spectra of VPA related material in urine that appears to be 2-(2'-propenyl)-glutaric acid.

some certainty. Diagnostic fragment ions and ion doublets in the mass spectra of the derivatives of the new metabolite were compared to those of known metabolites to aid in identification. The EI and NICI mass spectra of these derivatives are presented in Figure 29. In the EI mass spectrum m/z 213 ($^2H_3$, 216) corresponds to [M-57]$^+$, m/z 111 ($^2H_3$,114) is [M-159]$^+$ and m/z 270 is the molecular ion. The fragmentation pathway proposed for the t-BDMS derivative of the new metabolite is shown in Scheme 2. In the NICI mass spectrum, m/z 155 ($^2H_3$, 158) is the [M-181]$^-$ anion. From these spectra it was apparent that the new metabolite was an unsaturated keto compound (the [M-57]$^+$ ion of the t-BDMS derivatives of 3-keto and 4-keto VPA is 215 and the [M-181]$^-$ ion of the PFB derivative of 4-keto VPA is 157). Since the twin ions were separated by only three daltons instead of six, three of the deuterium atoms must have been lost from the metabolite. With compounds like methadone-$D_5$ (Hsia et al., 1976) having a keto group α to $C^2H_2$, the deuterium atoms can readily exchange for hydrogen in alkaline solution. In the work-up procedure, the urine sample from which the spectra in Figure 29 were obtained was treated with alkali in order to hydrolyze conjugates.

Figures 43 and 44 are the EI and NICI mass spectra respectively, of the derivatives of the new metabolite and 4-keto VPA obtained without alkaline treatment of the urine sample. In both the EI and NICI spectra of the new metabolite and 4-keto VPA the twin ions are now separated by six mass units ((111, 117), (213, 219), (155, 161), (215, 221), (157, 163)). This indicates that the keto group is at position 4 and hence the new metabolite must be a 4-keto
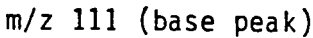
Scheme 2. Proposed fragmentation pathway for the t-BDMS derivative of a new VPA metabolite assigned the structure 4'-keto-2-ene VPA.
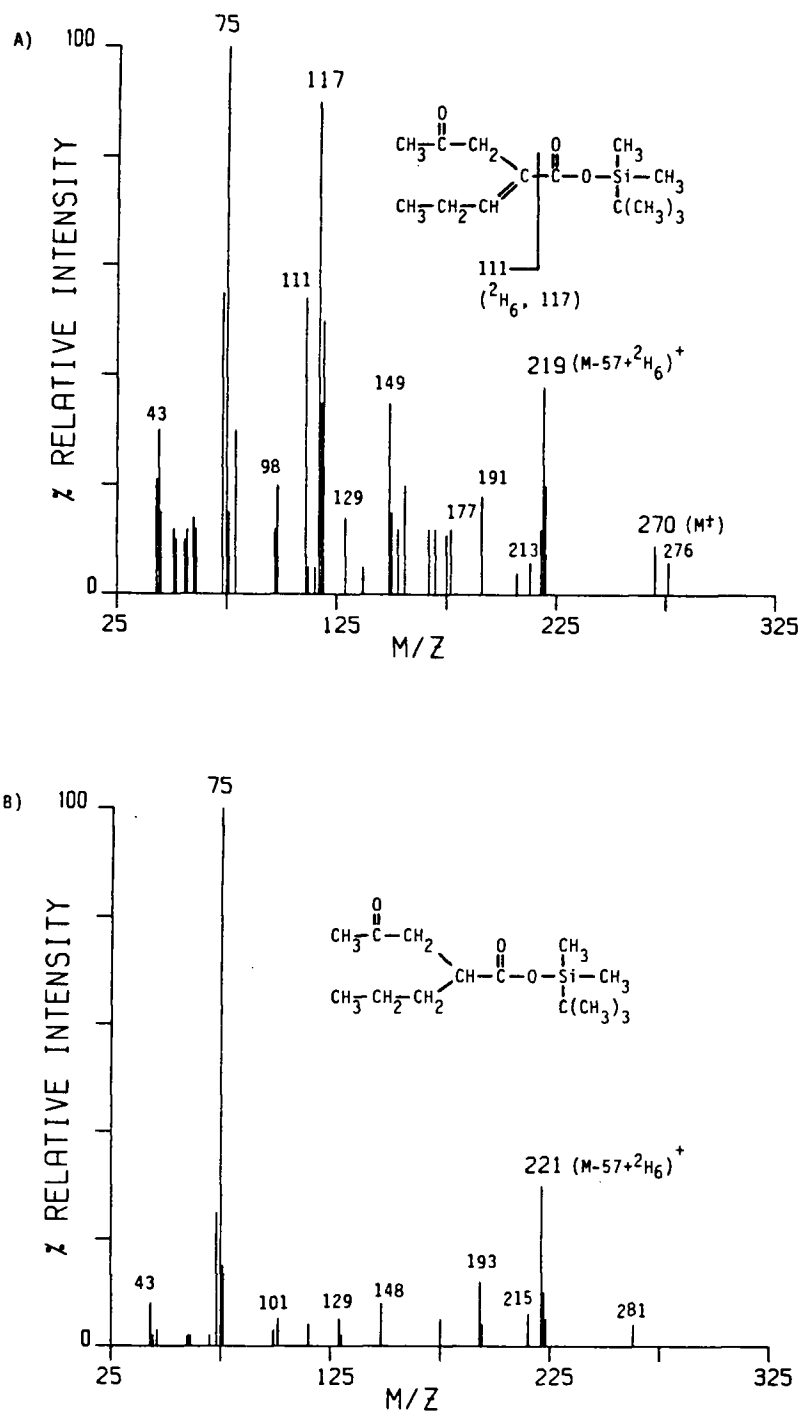
Figure 43. EI mass spectra of A) 4'-keto-2-ene VPA and B) 4-keto VPA extracted from urine without alkaline treatment.
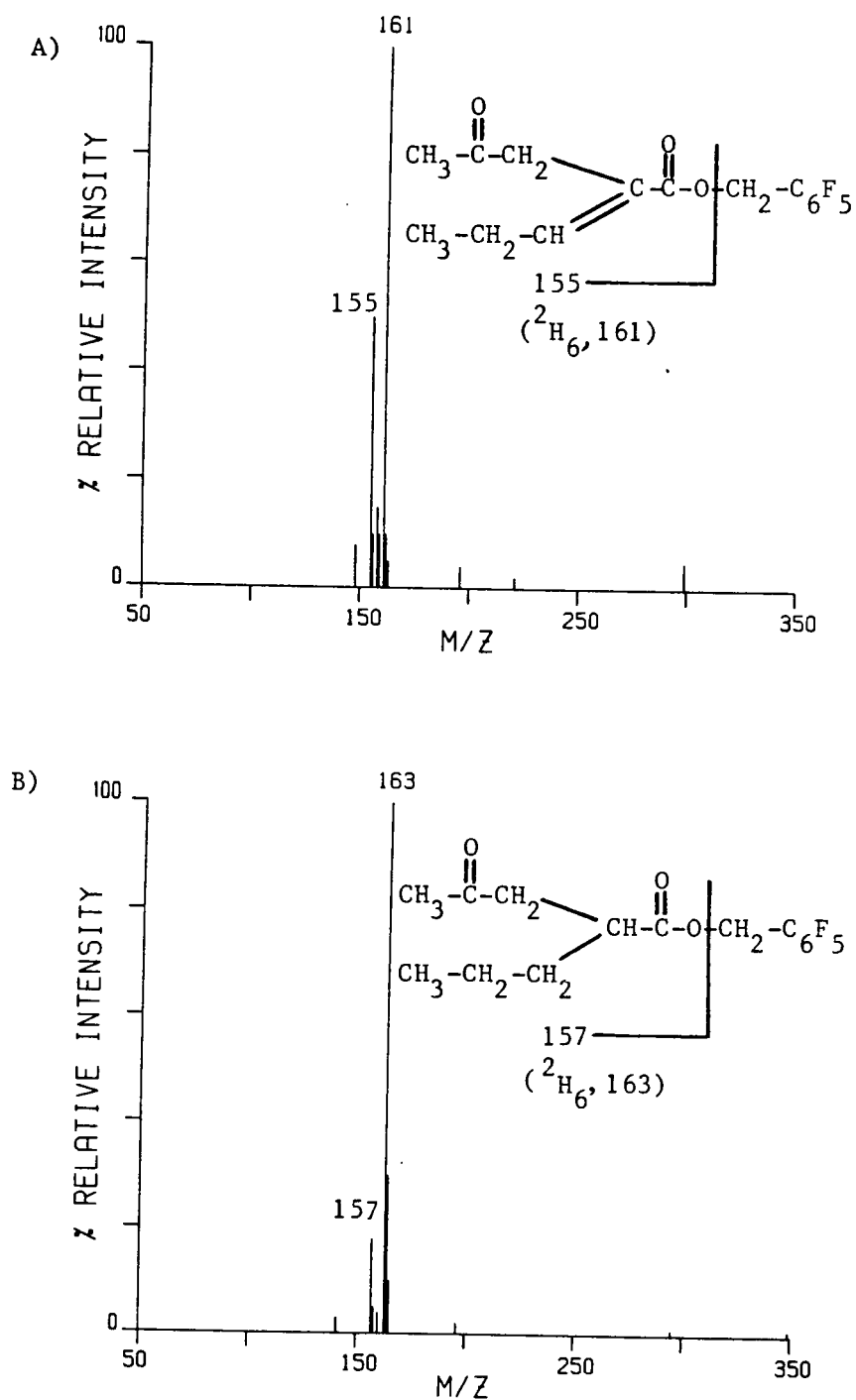
Figure 44.   NICI mass spectra of A) 4'-keto-2-ene VPA and B) 4-keto VPA
extracted from urine without alkaline treatment.

compound. The double bond cannot be at position 4' because there is no evidence for the loss of a deuterium atom. Therefore, the new metabolite may be one of the following: 4'-keto-2-ene VPA, 4-keto -2-ene VPA or 4'-keto-3-ene VPA. The latter possibility is unlikely since the new metabolite would most likely be a derivative of the major unsaturated metabolites in serum (i.e. 2-ene VPA or 2,3'-diene VPA).

From the peak height in Figure 23 (peak 10) the new metabolite is a prominent peak and has been detected in all urine samples analyzed. Its level in urine appears to be higher than 2,4-diene VPA. It was detected both before and after induction by CBZ. The new metabolite is related to 3'-keto-4-ene VPA which was detected as a VPA metabolite in the Rhesus monkey (Rettenmeier et al., 1986b), in that it is an unsaturated keto metabolite of VPA.

The new metabolite was detected both by EI and NICI GCMS. The complimentary nature of the two ionization methods in terms of the identification of VPA metabolites cannot be overemphasized. NICI with PFB in most cases gave the diagnostic and abundant $[M-181]^-$ ion which corresponds to the intact molecular anion less one. Once the NICI structural information was obtained, the t-BDMS derivative under EI gave more fragment ions and the characteristic ion, $[M-57]^+$, which aided in the postulation of a structure for the unknown compound. Thus, the two GCMS systems were highly complimentary to each other in identifying known VPA metabolites and the new VPA metabolite.

The new VPA metabolite appears to be 4'-keto-2-ene VPA although this structural assignment should be considered tentative (see section D). For the sake of convenience the new metabolite has been referred

to as 4'-keto-2-ene VPA in the text. The 4'-keto-2-ene VPA could be detected in urine under linear scan conditions but in serum by SIM only. One possible origin of 4'-keto-2-ene VPA is ($\omega$-1)-hydroxylation of the saturated side chain of 2-ene VPA followed by dehydrogenation of its precursor 4'-OH-2-ene VPA, by the enzyme alcohol dehydrogenase in a manner analogous to that of the conversion of ($\omega$-1)-hydroxy fatty acids to ($\omega$-1)-keto fatty acids (Bjorkhem, 1972). The possible precursor i.e. 4'-OH-2-ene VPA, however was not detected in serum or urine. This possible metabolic origin of 4'-keto-2-ene VPA is similar to the report by Rettenmeier et al. (1986a) where 4'-OH-4-ene VPA was apparently found to be a minor metabolite of 4-ene VPA in the Rhesus monkey.

The 4'-keto-2-ene VPA could also arise from the hydration of the 3'-double bond of 2,3'-diene VPA followed by oxidation. Addition of water across a double bond was evident in one study of the metabolism of unsaturated VPA metabolites (Granneman et al., 1984a). In rats given 2,3'-diene VPA, however, no evidence was seen for the formation of 4'-keto-2-ene VPA (Ron Lee, M.Sc. thesis). Regardless, 4'-keto-2-ene VPA must be derived from either a mono or di-unsaturated VPA metabolite and this confirms that unsaturated VPA metabolites are likely to give rise to potentially toxic oxidation products. The detection of 4'-keto-2-ene VPA also demonstrates the complex nature of VPA metabolism which involves a variety of enzymes and multiple minor pathways such as dehydrogenation, hydration, reduction and hydroxylation.

D. Synthesis

From the spectral data discussed above and metabolic considerations, it was felt that the new VPA metabolite was 4'-keto-2-ene VPA and the synthesis of this compound was attempted employing literature methods.

1. Attempted synthesis of 2-(2'-oxopropyl)-2-pentenoic acid (4'-keto-2-ene VPA) via ethyl 2-propyl-4-oxopentanoate.

2-Propyl-4-oxopentanoic acid, I, was synthesized from ethyl 2-bromopentanoate and ethylacetoacetate. 2-Bromopentanoic acid was prepared by bromination of pentanoic acid according to standard procedures. Ethyl 2-bromopentanoate and ethyl acetoacetate were then condensed in the presence of NaH (Scheme 3). This synthetic route was adopted from that of Acheampong et al. (1983) which is based on the method of Lawessen et al. (1962) for the preparation of γ-keto acids. The final product was obtained by decarboxylation and hydrolysis of the acylsuccinate intermediate. Distillation of the crude product yielded 2-propyl-4-oxopentanoic acid contaminated with small amounts of its ethyl ester. Redistillation yielded pure 2-propyl-4-oxopentanoic acid. The acid was converted to its ethyl ester initially by using ethyl iodide in the presence of potassium carbonate and 18-crown-6 (Fedorynoki et al., 1978). However, because the keto function was not affected by acid, ethyl alcohol in the presence of sulfuric acid was used for the esterification of most of the synthesized acid since this procedure resulted in a higher yield. The identity of ethyl 2-propyl-4-oxopentanoate was established by GCMS, IR and NMR (see appendix).

$$CH_3-CH_2-CH_2-CH_2-\overset{\overset{\displaystyle O}{\|}}{C}-OH$$

$$\downarrow Br_2$$
$$PBr_3$$

$$CH_3-CH_2-CH_2-\underset{\underset{\displaystyle Br}{|}}{CH}-\overset{\overset{\displaystyle O}{\|}}{C}-OH$$

$$\downarrow EtOH$$
$$H^+$$

$$CH_3-CH_2-CH_2-\underset{\underset{\displaystyle Br}{|}}{CH}-\overset{\overset{\displaystyle O}{\|}}{C}-OC_2H_5$$

$$CH_3-\overset{\overset{\displaystyle O}{\|}}{C}-CH_2-\overset{\overset{\displaystyle O}{\|}}{C}-OC_2H_5 \quad + \quad CH_3-CH_2-CH_2-\underset{\underset{\displaystyle Br}{|}}{CH}-\overset{\overset{\displaystyle O}{\|}}{C}-OC_2H_5$$

$$\downarrow \text{NaH} \mid \text{THF} \quad \triangle$$

$$CH_3-\overset{\overset{\displaystyle O}{\|}}{C}-\underset{\underset{\displaystyle CH_3-CH_2-CH_2}{|}}{\overset{\overset{\displaystyle COOC_2H_5}{|}}{CH}}\diagdown\underset{}{CH-\overset{\overset{\displaystyle O}{\|}}{C}-OC_2H_5}$$

$$\downarrow \text{Conc. HCl} \quad \triangle$$

$$CH_3-\overset{\overset{\displaystyle O}{\|}}{C}-CH_2\diagdown\underset{\underset{\displaystyle CH_3-CH_2-CH_2}{|}}{CH-\overset{\overset{\displaystyle O}{\|}}{C}-OH} \qquad (I)$$
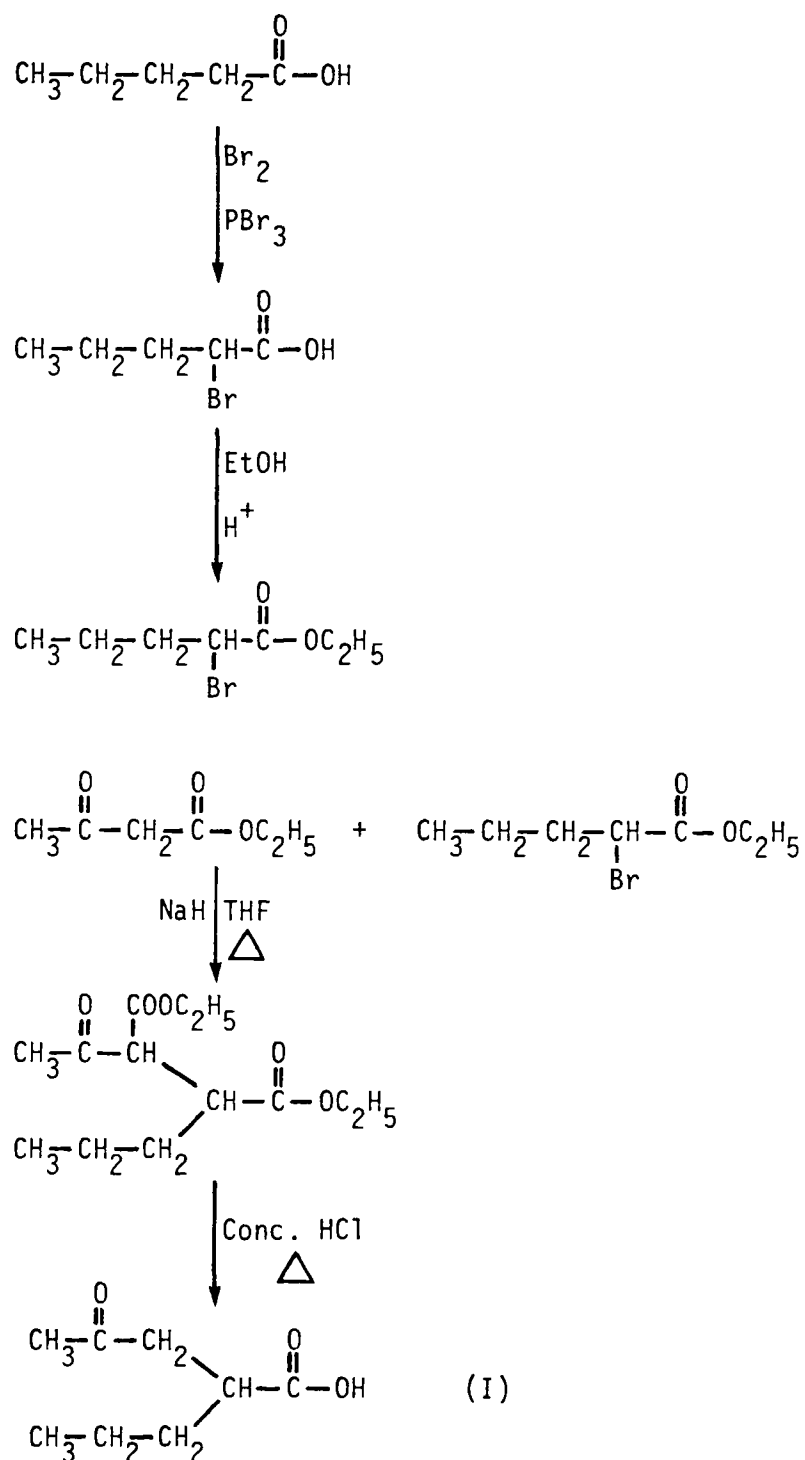
Scheme 3. Synthetic route for 2-propyl-4-oxopentanoic acid.

Introduction of the double bond at the 2 or 2' position was attempted by the oxidation of the trimethylsilyl enol ether using the hydride abstracting reagent DDQ according to Jung and Pan (1977). The synthetic strategy was to use two equivalents of LDA and chlorotrimethylsilane since both the keto and carboxyl carbonyls will form the TMS enols (Scheme 4). The presence of TMS containing compounds, a mono- and di-TMS derivatives, was observed by GCMS before the reaction with DDQ. The mono -TMS enol was preferentially formed as indicated by a 3:1 ratio of the mono-TMS enol to the di-TMS enol. Following the reaction with DDQ, GCMS analysis of the crude product indicated the presence of three components in about equal quantities. These were the starting material, the TMS enol ether of ethyl 2-propyl-4-oxopentanoate, and the TMS enol ether of ethyl 2-propyl-4-oxo-2-pentenoic acid (or the TMS enol ether of ethyl 2-(2'-oxopropyl)-2-pentenoic acid).

The yields of oxidation products of enol ethers are generally moderate and oxidation is almost all of the time incomplete resulting in considerable starting material remaining at the end of the reaction (Jung and Pan, 1977). When the enolate product of 4-keto VPA is trapped as a silyl enol ether structures A and B are possible.

$$O - TMS \atop | \atop CH_2=C-CH_2R \qquad\qquad O - TMS \atop | \atop CH_3-C=CHR$$

A                                                                 B
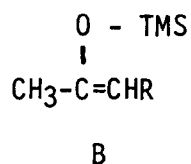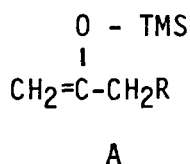
The composition of the regioisomeric enolate mixture of unsymmetrical ketones is governed by kinetic or thermodynamic factors (Carey and Sundberg, 1983). By appropriate selection of experimental conditions
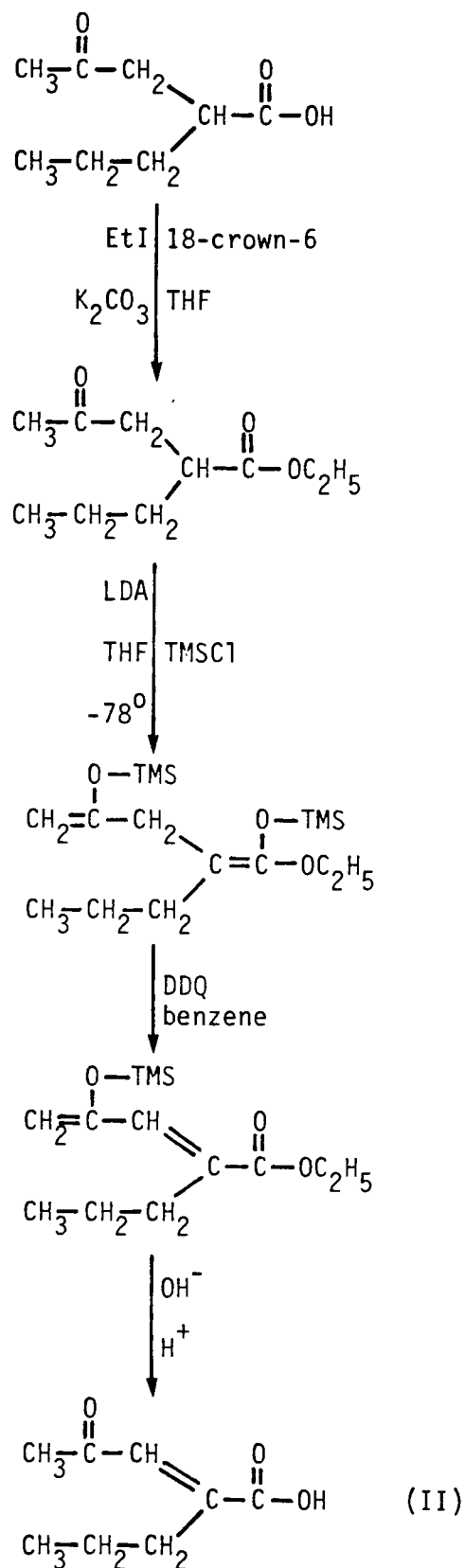
Scheme 4. Attempted synthesis of 2-(2'-oxopropyl)-2-pentenoic acid.

under which an enolate is formed from a ketone it is possible to establish either kinetic or thermodynamic control. If the base is strong and sterically bulky and if aprotic solvents are used, the major product formed will be the product of kinetic control. In the present reaction LDA and THF were used and hence, the dominant enol ether will be A which is the product of kinetic control. Under these conditions therefore, the final product will have the double bond at either positions 2 or 2'. The formation of the double bond on the same chain as the keto function will be favored because of conjugation.

A portion of the product mixture was treated with alkali to effect hydrolysis of both the TMS enol ether and the ester. Following acidification and extraction the extract was derivatized to give the t-BDMS ester. The mass chromatograms at m/z 213 $[M-57]^+$ are shown in Figure 45 (two peaks) and the corresponding mass spectra in Figure 46. The mass spectra as well as the retention times for the two products were different from that of the new metabolite extracted from urine. Since the mass spectra were different from that of 4'-keto-2-ene VPA (III), these compounds are possibly the two geometric isomers of 2-propyl-4-oxo-2-pentenoic acid (II).

2. Synthesis of 4'-keto-2-ene VPA starting with a protected 4-oxopentanoic acid

The 4-oxopentanoic acid was converted to its ethyl ester and the keto function protected by means of 1,3-dithiolane (Scheme 5) according to Hatch et al. (1978). The ethyl 4-ethylenethioketalpentanoate was
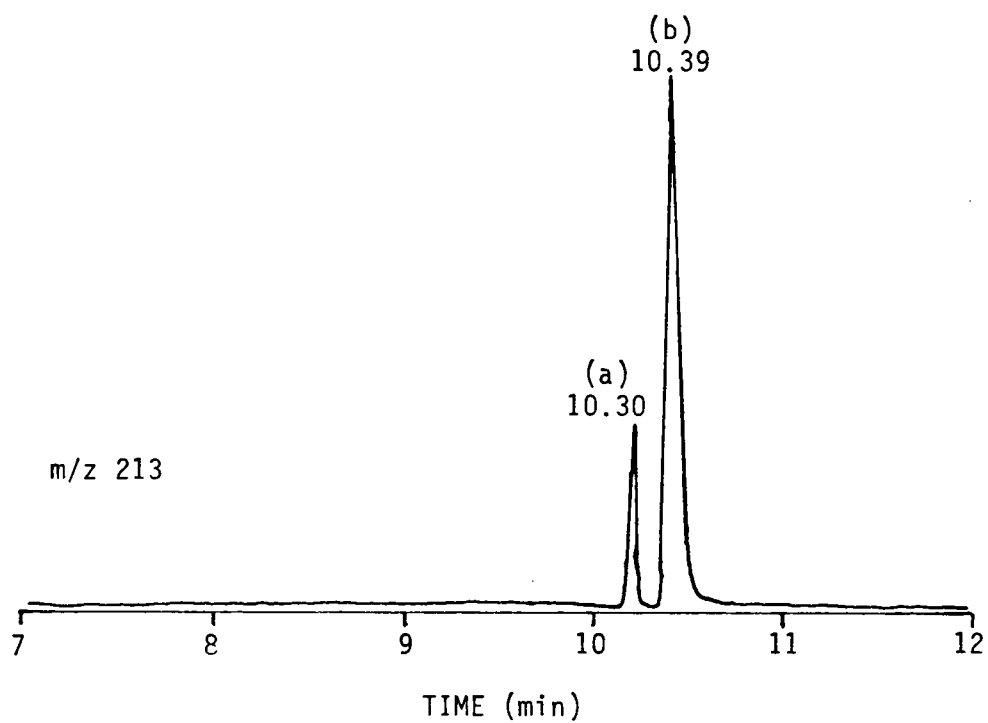
Figure 45.    Mass chromatograms (m/z 213) of the t-BDMS derivatives of
              synthesized 2-propyl-4-oxo-2-pentenoic acid (4-keto-2-ene
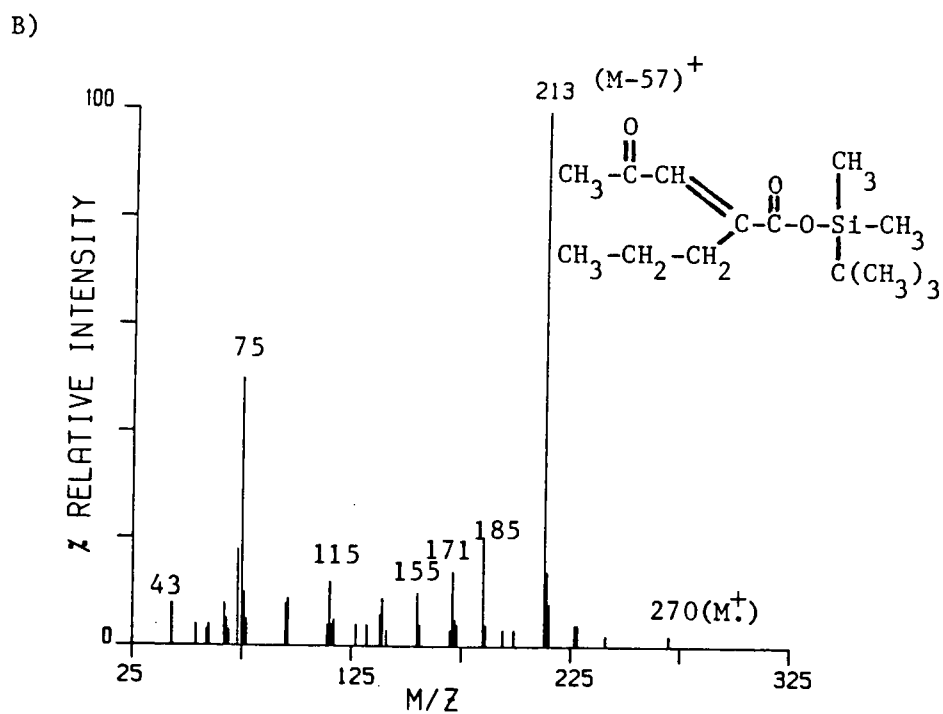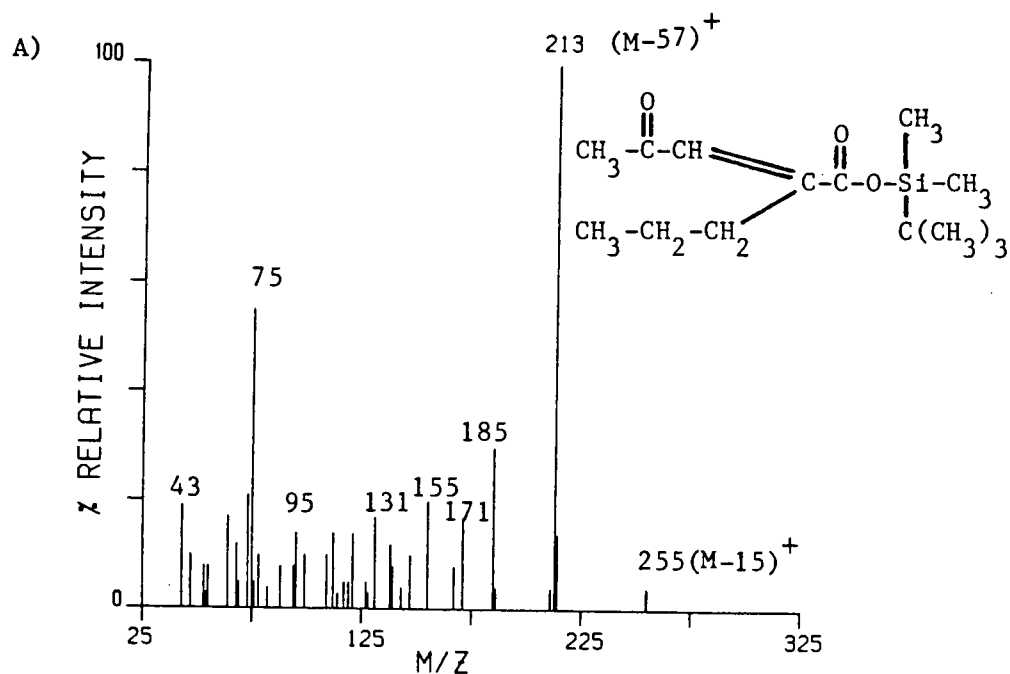              VPA).

Figure 46    Mass spectra of A) peak a and B) peak b in Figure 45.

obtained in good yield (85%) and its identity confirmed by GCMS and NMR (for NMR spectrum see appendix). The synthetic route employed was an aldol condensation of ethyl 4-ethylenethioketalpentanoate with propionaldehyde. The enolate formed with LDA was condensed with propionaldehyde to form the β-hydroxy ester which was then dehydrated with methane sulfonyl chloride followed by potassium hydride. This synthetic route was adopted from the method for preparing β-hydroxy, β', γ'-unsaturated esters (Kende and Toder, 1982; Acheampong and Abbott, 1985). The 4-carboethoxy-2-ethylenethioketal-5-hydroxyheptane was obtained in a moderate yield and was shown to be homogeneous by GCMS. The hydroxy compound was then mesylated and treated with potassium hydride. After the dehydration step, GCMS analysis of the crude product showed five peaks. Two of these peaks (one of them was the major component) in the TIC plot corresponded to the two geometric isomers of the desired product with the protecting group (for EI spectra see appendix). The three compounds could not be identified from mass spectral data.

Since it was not possible to isolate the product, the product mixture as such was treated with mercuric chloride in the presence of cadmium carbonate in order to cleave the 1,3-dithiolane protecting group (Pappas and Nace, 1959). The TIC plot of the resulting product mixture showed nine peaks, two of which (minor components) were the two isomers of the ethyl ester of 4'-keto-2-ene VPA. Their mass spectra are shown in Figure 47. Even though Hg(II) salts have been used for the cleavage of the 1,3-dithiolane derivatives of a number of compounds, in the present case the deprotection step was inefficient and resulted in the appearance of additional compounds. The protecting group itself
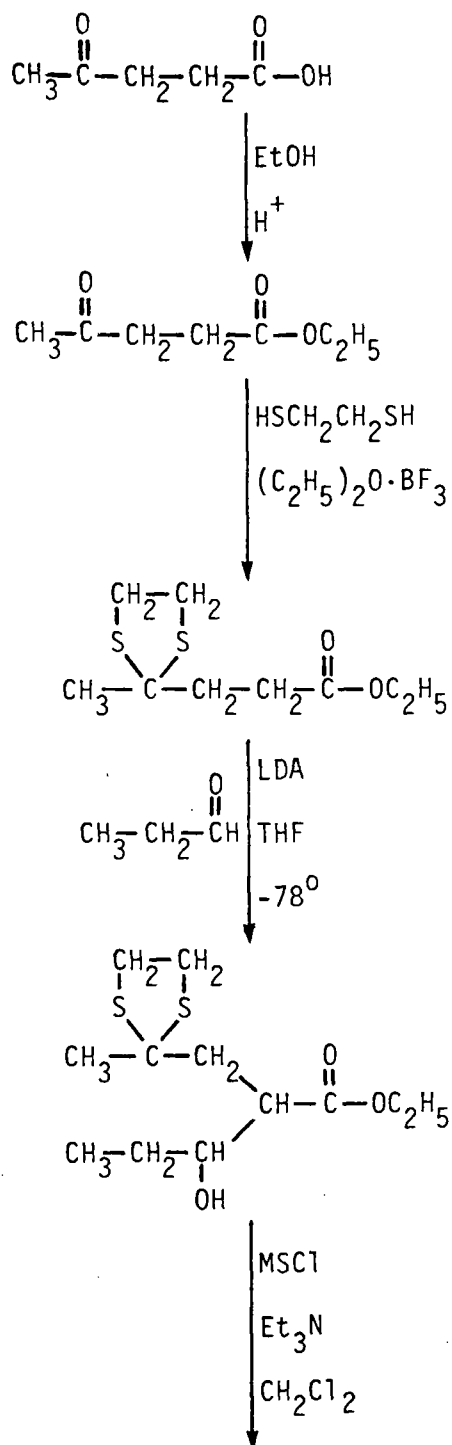
Scheme 5. Synthetic route for 2-(2'-oxopropyl)-2-pentenoic acid.
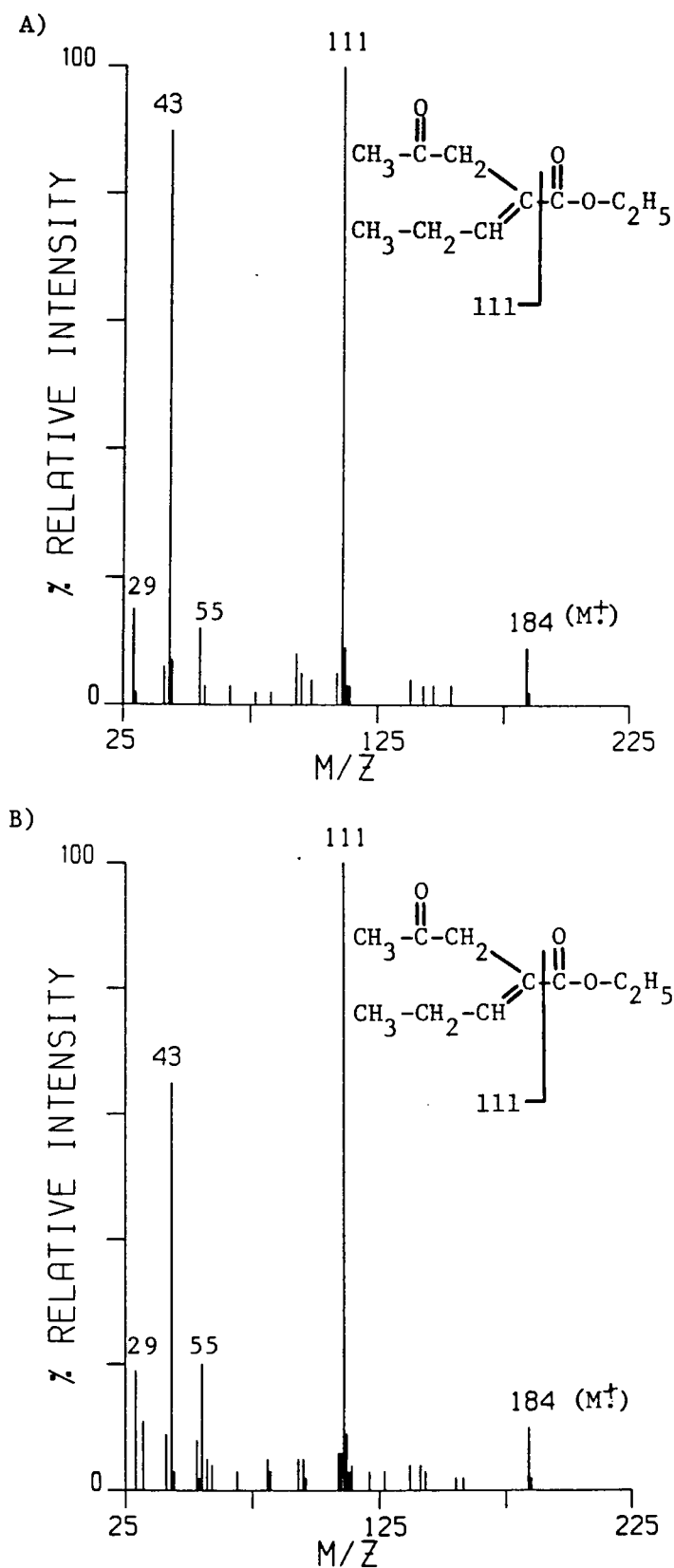
Scheme 5. (Continued)



(III)
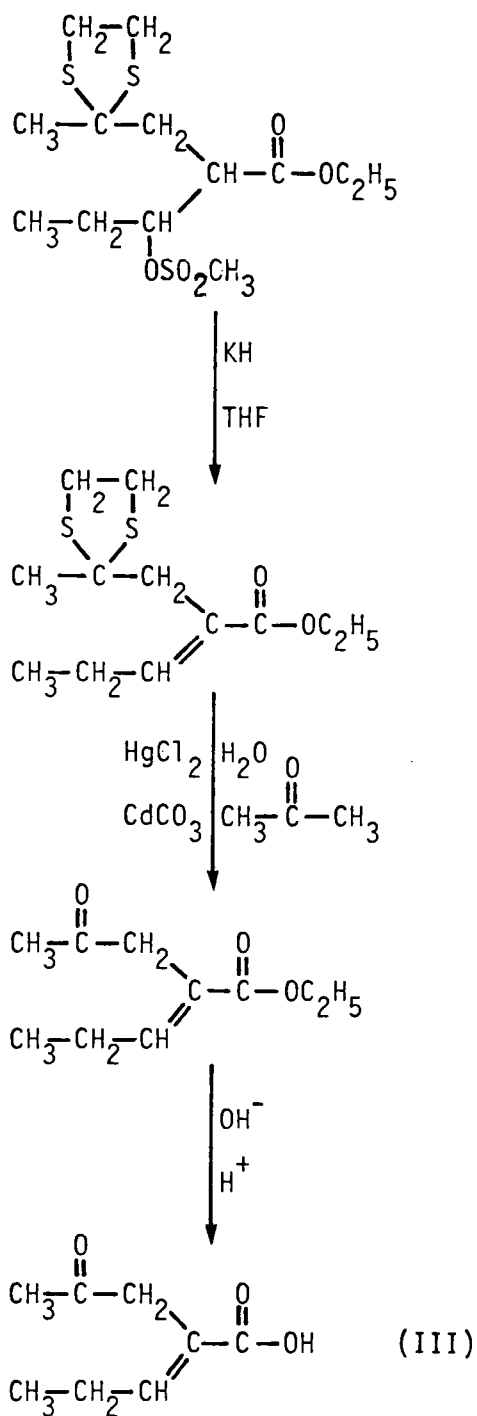
Figure 47.    EI mass spectra of the isomers of the ethyl ester of 4'-keto-
2-ene VPA
A) Isomer with the shorter retention time.
B) Isomer with the longer retention time.

might have caused a complication in the synthesis since three unknown compounds were observed after the dehydration step with potassium hydride. As a result, the crude product mixture was analyzed by GCMS. After hydrolysis of the ester, a portion of the product mixture was derivatized to give the t-BDMS and PFB derivatives. The mass chromatograms at m/z 213 $[M-57]^+$ are shown in Figure 48 along with that from the urine extract. Figure 49 shows the mass spectra of peaks a and b from Figure 48. The mass spectra of peaks a and b are identical and their retention times are almost the same (8.12 minutes versus 8.15 minutes). Therefore, the chromatographic and mass spectral data of the new metabolite match one synthetic product, which is considered to be 4'-keto-2-ene VPA on the basis of intermediates that led to its formation and the synthetic route used.

In the EI mass spectra of 4'-keto-2-ene VPA the m/z 111 ion appears to be a characteristic ion. The m/z 111 is the base peak in both the t-BDMS (native and synthetic) (Figure 49) and ethyl (Figure 47) esters of 4'-keto-2-ene VPA. There are qualitative as well as quantitative differences in the mass spectra of 4'-keto-2-ene VPA and 2-propyl-4-oxo-2-pentenoic acid (4-keto-2-ene VPA). In the mass spectrum of the former (Figures 47 and 49) m/z 111 is the base peak whereas in the latter (Figure 46) this ion is absent. In addition, the m/z 213 ion is the base peak in the mass spectrum of 2-propyl-4-oxo-2-pentenoic acid, but has an intensity of less than 50% in that of 4'-keto-2-ene VPA.

In the m/z 155 mass chromatogram obtained by NICI of synthesized 4'-keto-2-ene VPA there are four peaks whose mass spectra are the same

Figure 48.  A) Mass chromatograms at m/z 213 of t-BDMS derivatized syn-
thetic 4'-keto-2-ene VPA.
B) Mass chromatograms at m/z 213 of t-BDMS derivatized native
4'-keto-2-ene VPA.

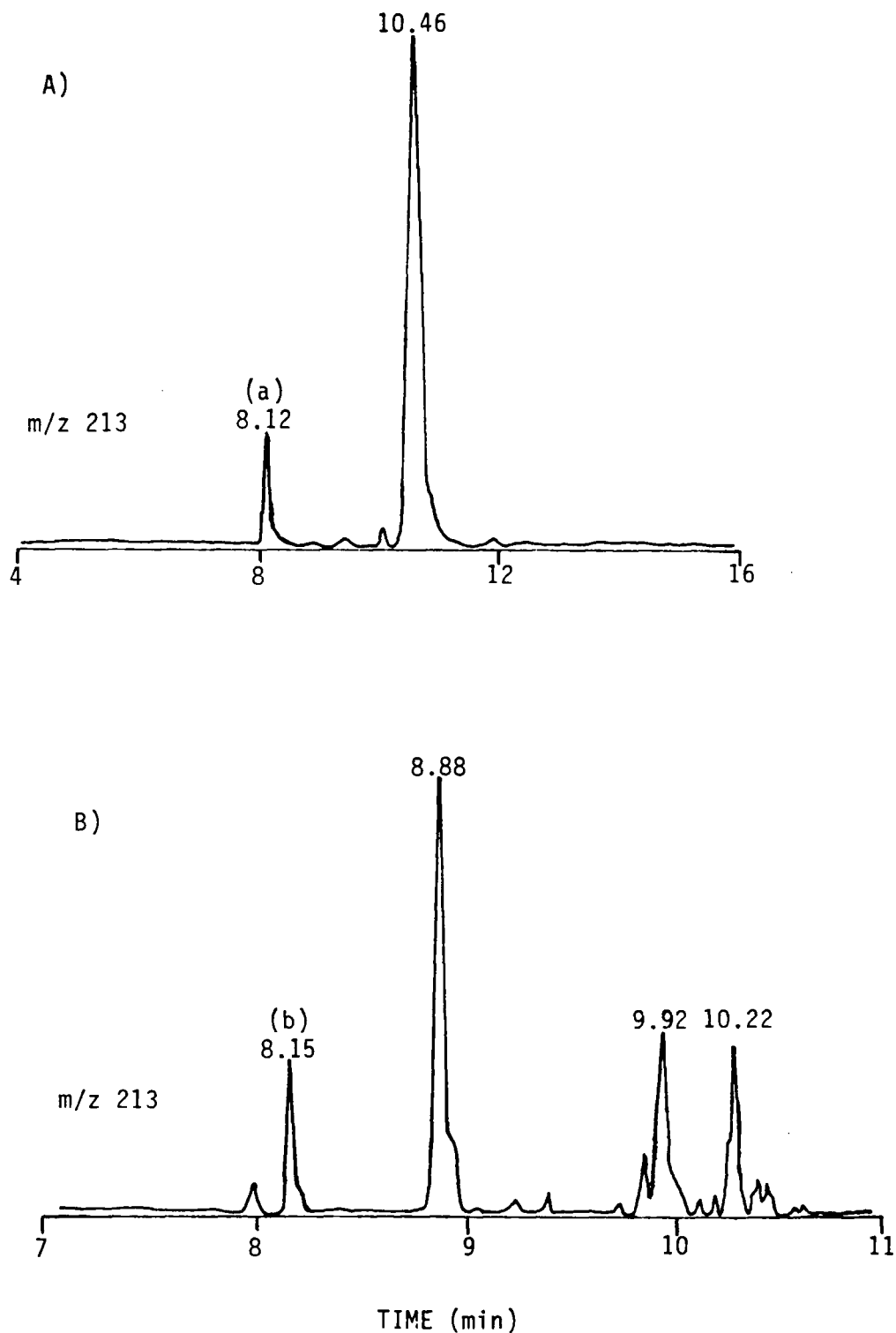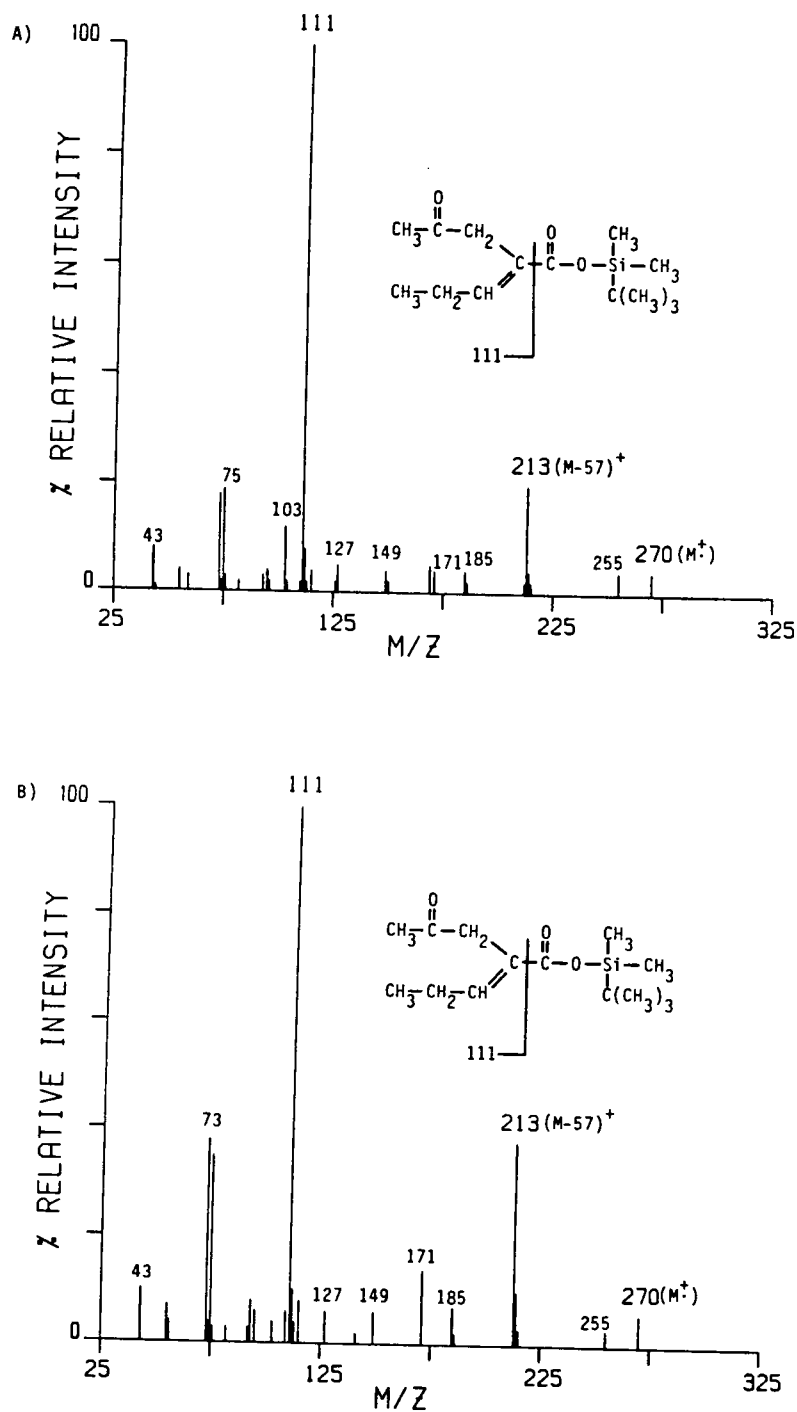Figure 49.    Mass spectra of A) peak a and B) peak b in Figure 48.

A)



B)



TIME (min)
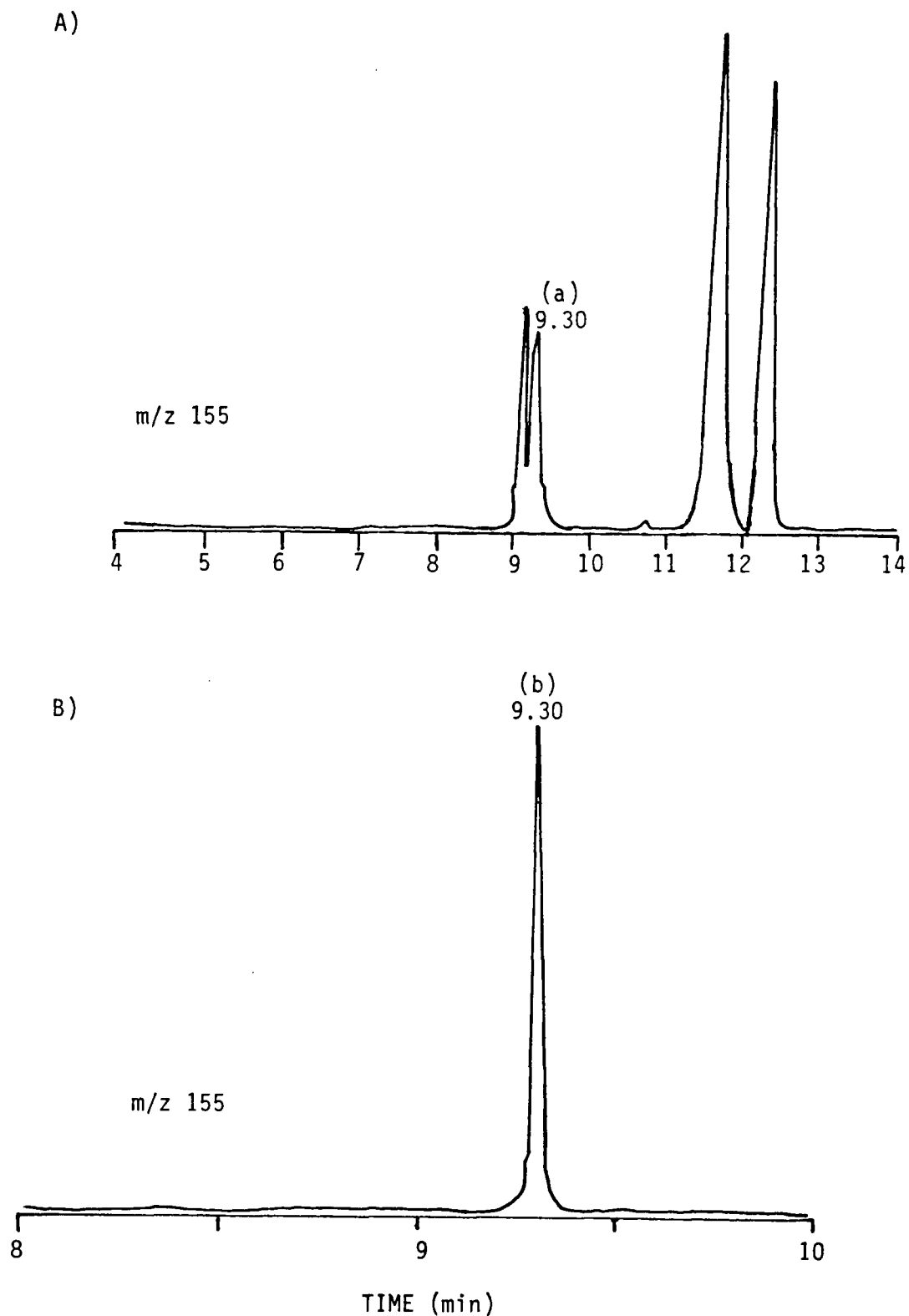
Figure 50.   A) Mass chromatograms at m/z 155 of PFB derivatized synthetic 4'-keto-2-ene VPA.
B) Mass chromatogram at m/z 155 of PFB derivatized 4'-keto-2-ene VPA from a urine extract.

with m/z 155 as the base peak. The retention time of one of these peaks matches that from the urine extract (Figure 50). The two other peaks could be the isomers of 4'-keto-3-ene VPA since it is possible that small amounts of this positional isomer may be formed as a result of the removal of a γ proton from the mesylate derivative during the dehydration step. It is difficult, however, to say for certain which of the peaks are those of 4'-keto-2-ene VPA or 4'-keto-3-ene VPA since it is the smaller peak that has identical retention time to that of the metabolite extracted from urine. It is also possible that the two larger peaks in Figure 50A could be due to side products in the synthetic product mixture. Because the only ion of significance in the mass spectra of the peaks in Figure 50 is 155, an inference about these compounds can not be made on the basis of their NICI spectra.

The synthetic strategy for 4'-keto-2-ene VPA worked well except at the deprotection step. The keto group was protected with 1,3-dithiolane group because of the availability of the reagents at that time. From this experience, it appears that a 1,3-dioxolane would be a better protecting group because of its ease of removal.

In summary, the new VPA metabolite is most likely 4'-keto-2-ene VPA. This structural assignment must be considered tentative however, until a successful synthesis provides sufficient product to obtain NMR data.

## SUMMARY AND CONCLUSIONS

1.  The PFB derivatives of VPA and its metabolites produced intense
    $[M-181]^-$ ions ($[M-181-CO_2]^-$ for 3-keto VPA), which are
    suitable for SIM analysis as well as identification of
    metabolites.

2.  Three VPA derivatives (PFB, bis-TFMB and t-BDMS) were compared
    with respect to detection sensitivity. In the EI mode, all three
    derivatives had similar sensitivities. In NICI, the PFB
    derivative was found to be 30-50 times more sensitive than the
    t-BDMS derivative by EI. The PFB derivative (NICI mode) also
    proved to be about five times as sensitive as the similar
    fluorinated derivative, bis-TFMB.

3.  Comparison of a stable isotope-labelled internal standard
    ($[^2H_6]$-VPA) and an internal standard that gives a common ion
    (OA) showed that the $[^2H_6]$-VPA was a superior internal
    standard for the NICI assay of VPA.

4.  A highly sensitive and precise NICI assay was developed that can
    quantitate VPA in serum and saliva accurately down to 2 ng/mL.
    Serum VPA concentrations obtained by NICI were in excellent
    agreement with those obtained by an EI (t-BDMS) assay used for
    routine VPA quantitation in this laboratory.

5.  Paired saliva and serum samples were assayed for VPA using the NICI assay developed, in five healthy volunteers both before and after CBZ administration.  In spite of the considerable intra-subject variability, the time-averaged saliva to serum free ratios were remarkably similar in three volunteers both before and after CBZ administration.  The time-averaged saliva to serum total ratios decreased after CBZ indicating that the saliva to serum total ratio was concentration dependent.

6.  An active transport of VPA out of saliva is invoked to explain the lower concentration of VPA in saliva ($18.92\%  \pm 6.25$ of serum free) compared to serum free and CSF concentrations.

7.  The good correlations found between saliva and both serum total and free VPA concentrations suggest that measuring VPA in saliva by the NICI method would be suitable for drug interaction and pharmacokinetic studies.

8.  The conditions for derivative formation and the chromatographic behavior of the PFB derivatives of VPA metabolites have been determined and SIM chromatograms obtained.  This analytical method appears to be superior in terms of ease of derivative formation and sensitivity to currently available GCMS methods for the analysis of VPA metabolites.

9.  Seven VPA metabolites were identified in saliva.  There appears to be a stereoselective plasma protein binding or transport of the geometric isomers of 2-ene VPA as indicated by their saliva levels relative to those in serum.

10. A new VPA metabolite which from mass spectral and chromatographic
    data appears to be 4'-keto-2-ene VPA was detected in human urine.
    The detection of this metabolite, apparently arising from the
    oxidation of 2,3'-diene VPA or 2-ene VPA, confirms that
    unsaturated VPA metabolites are likely to give rise to potentially
    toxic oxidation products. Another new metabolite that appears to
    be 2-(2'-propenyl)-glutaric acid was also detected in urine.

11. The synthesis of 4'-keto-2-ene VPA was attempted using two
    different synthetic routes. The synthetic route in which
    4-oxopentonoate protected through dithio ketal was used as the
    starting material yielded the protected 4'-keto-2-ene VPA. The
    deprotection step, however, was inefficient and consequently, it
    was not possible to isolate sufficient product for obtaining NMR
    data.

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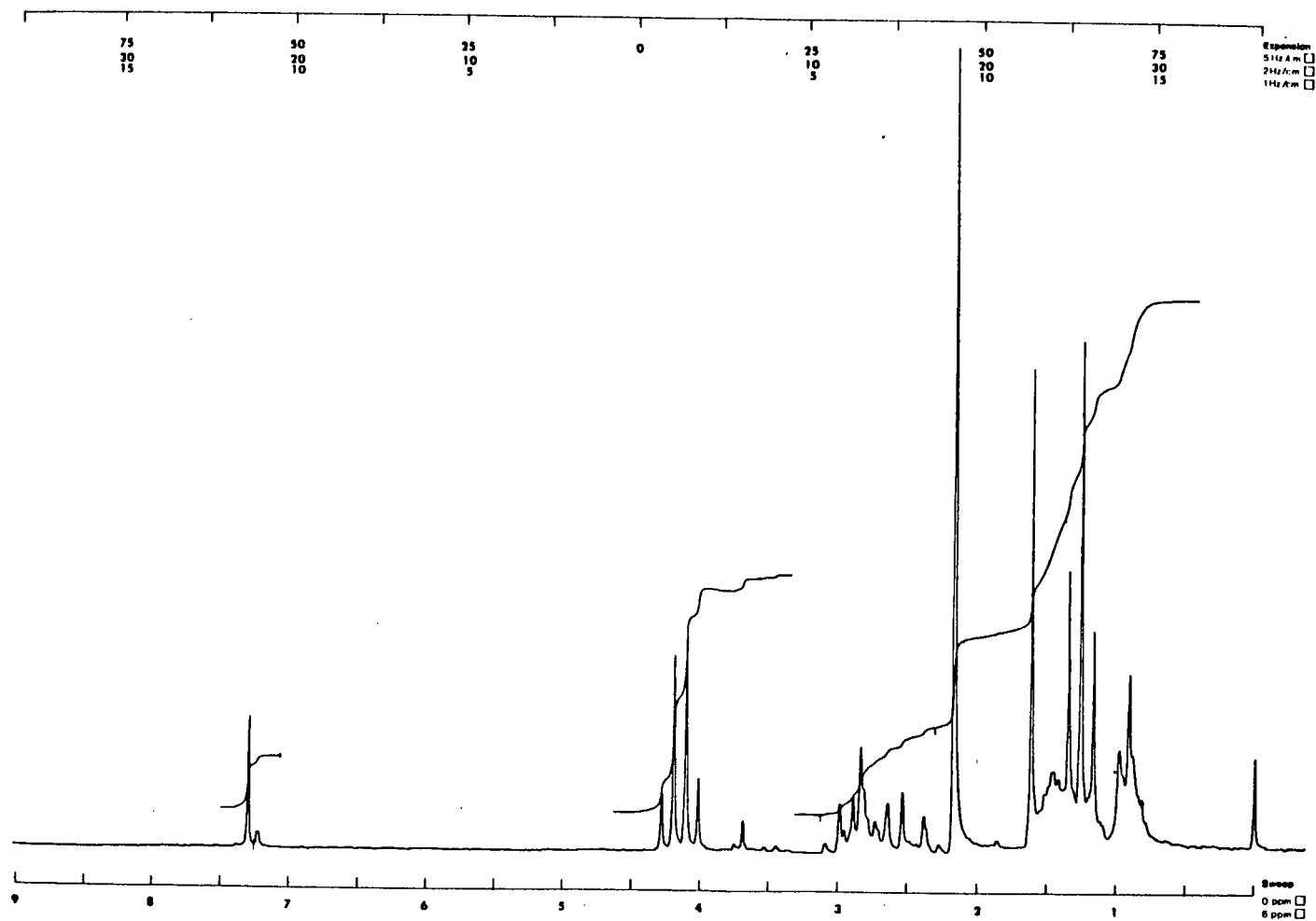
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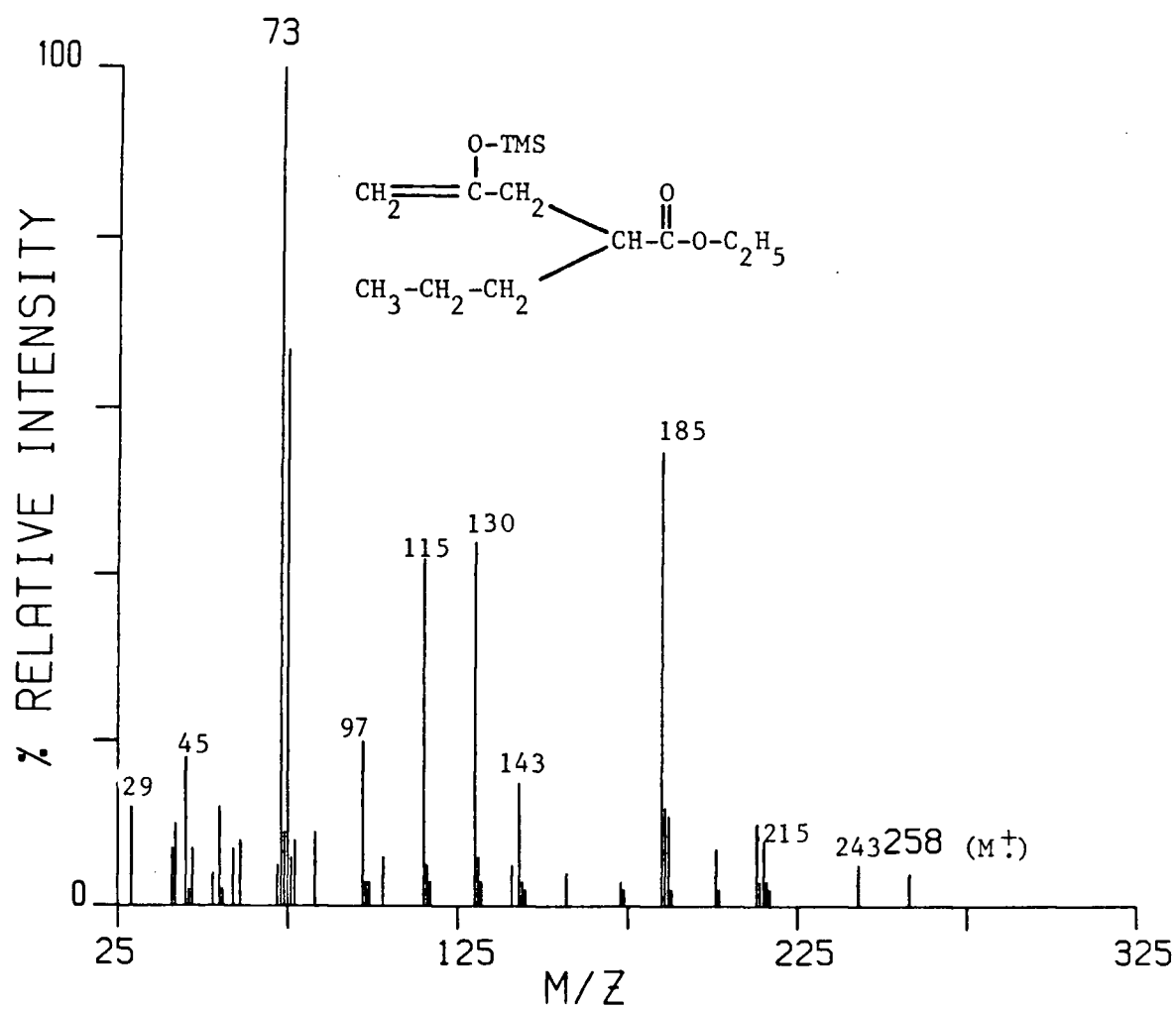
## APPENDIX
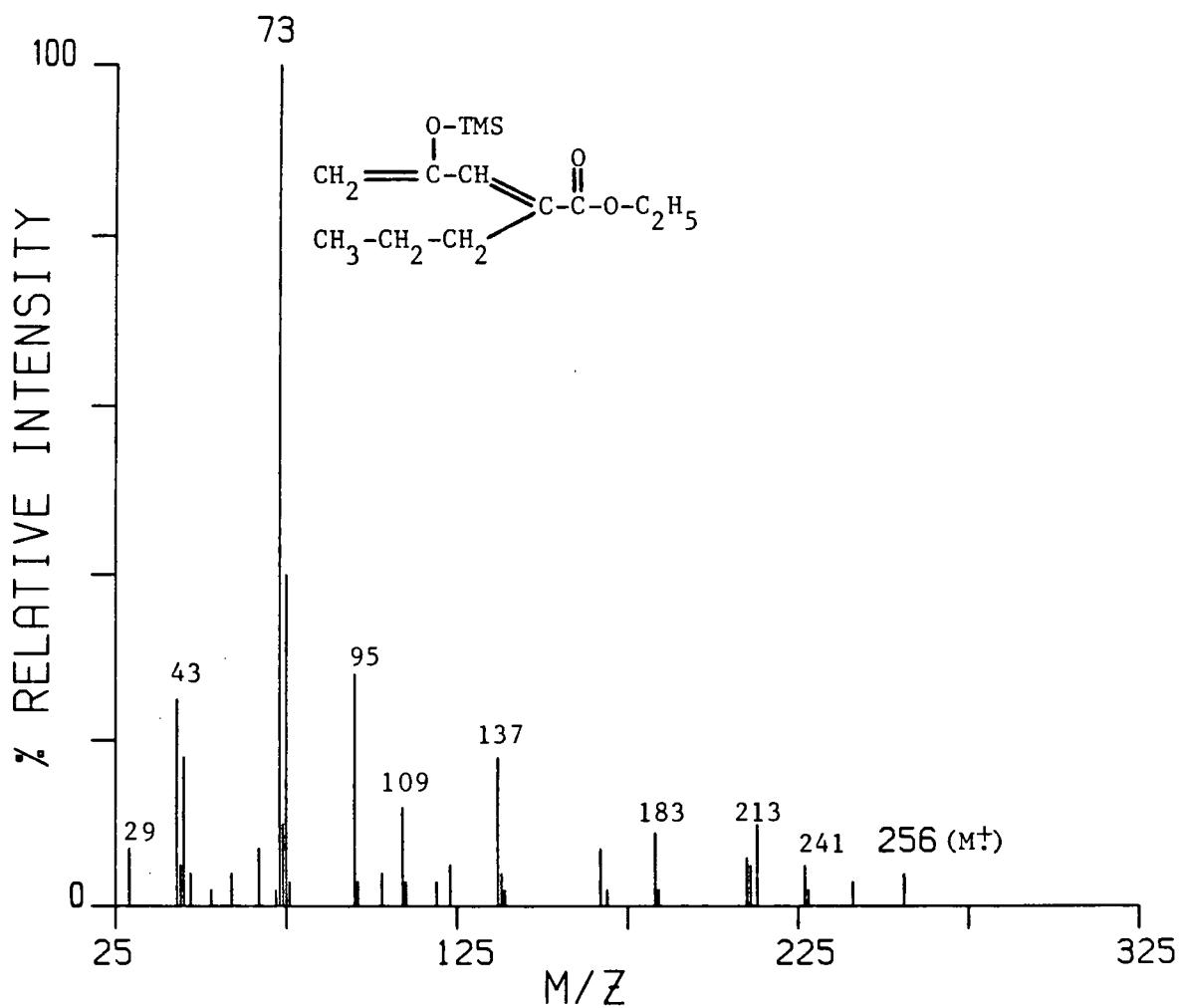
NMR, IR and mass spectra of some of the synthesized compounds.
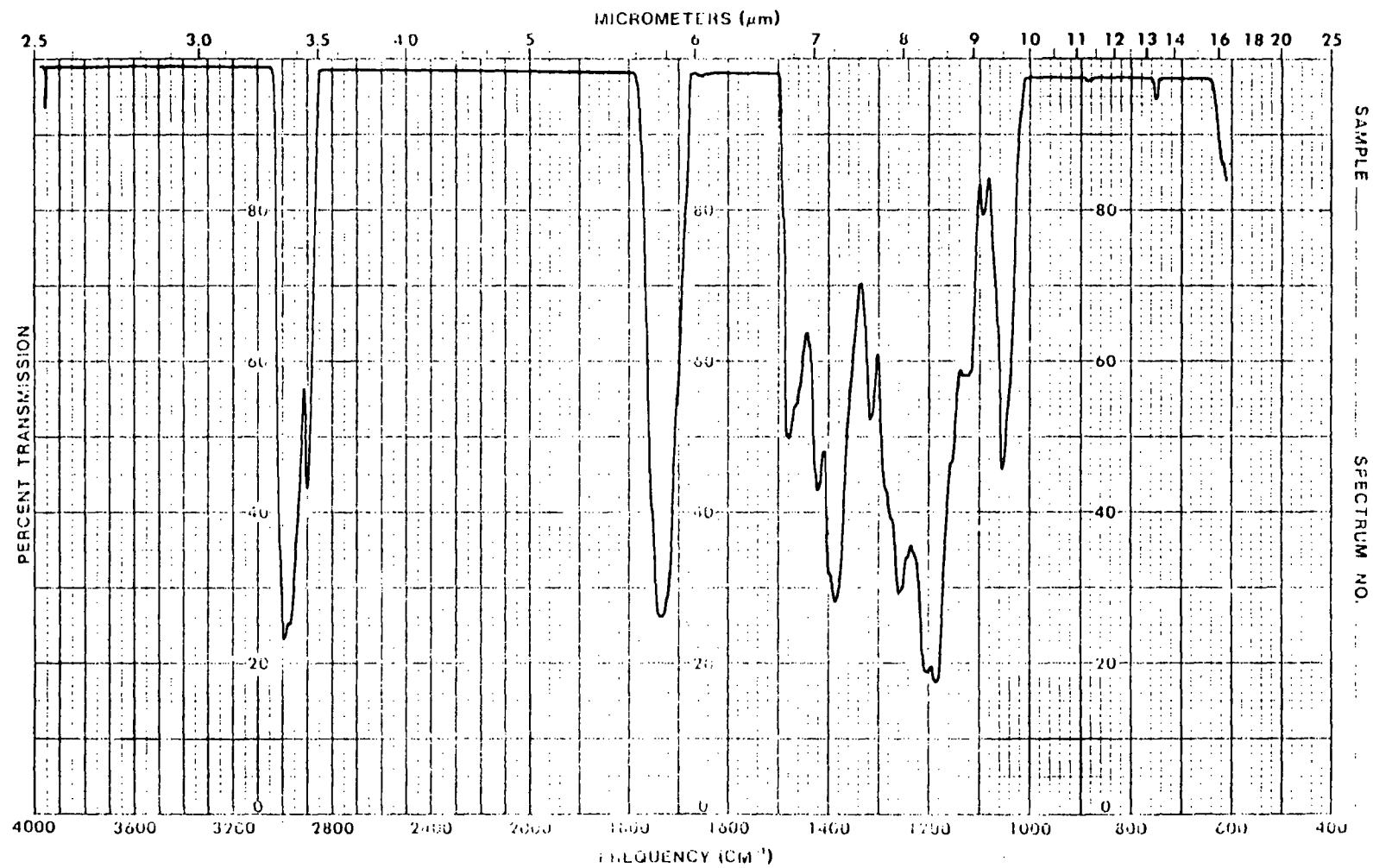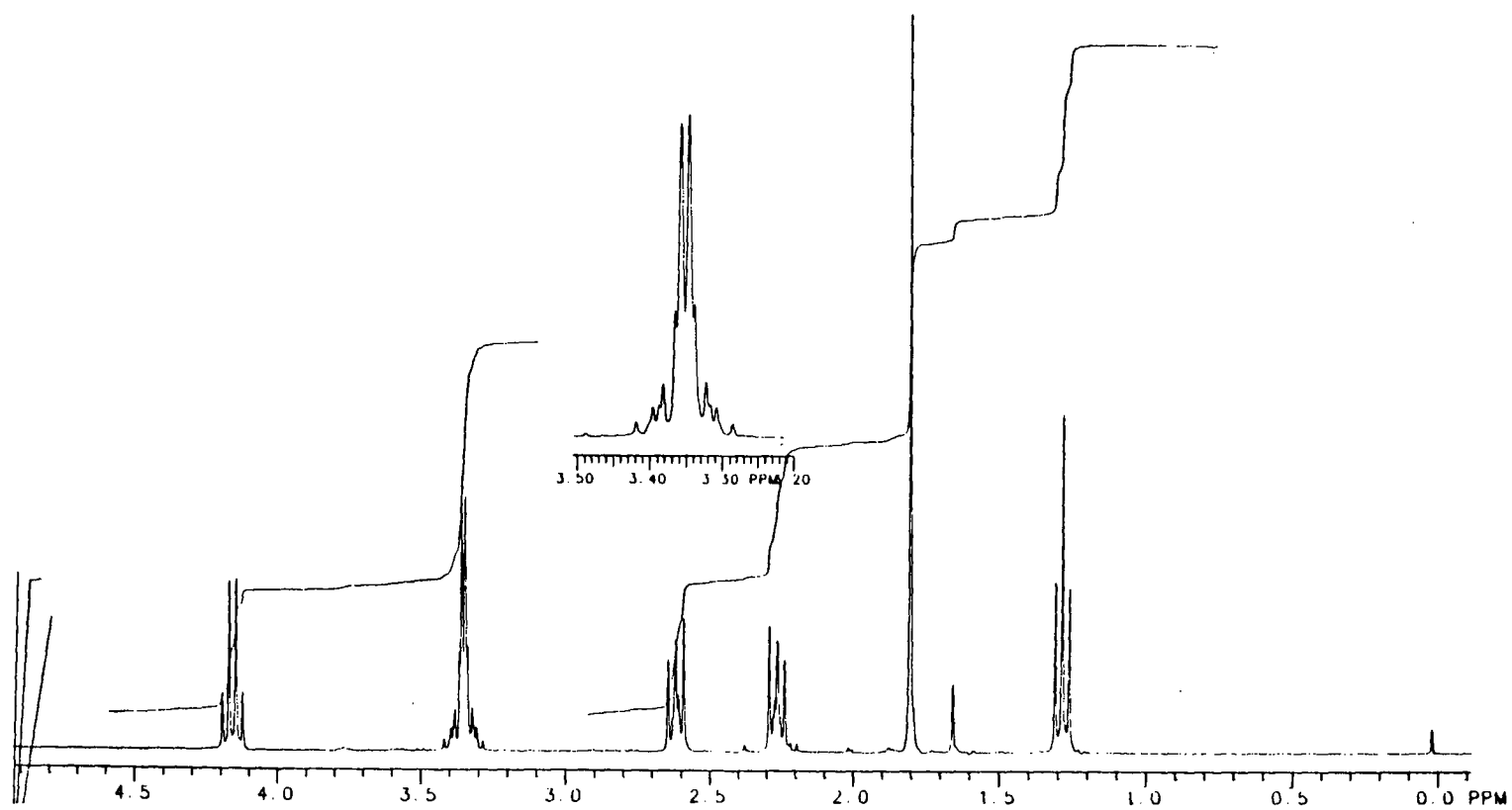
IR spectrum of ethyl 2-propyl-4-oxopentanoate.

$^1$H-NMR (80 MHz) spectrum of ethyl 2-propyl-4-oxopentanoate.

EI spectrum of the TMS enol ether of ethyl 2-propyl-4-oxopentanoate.

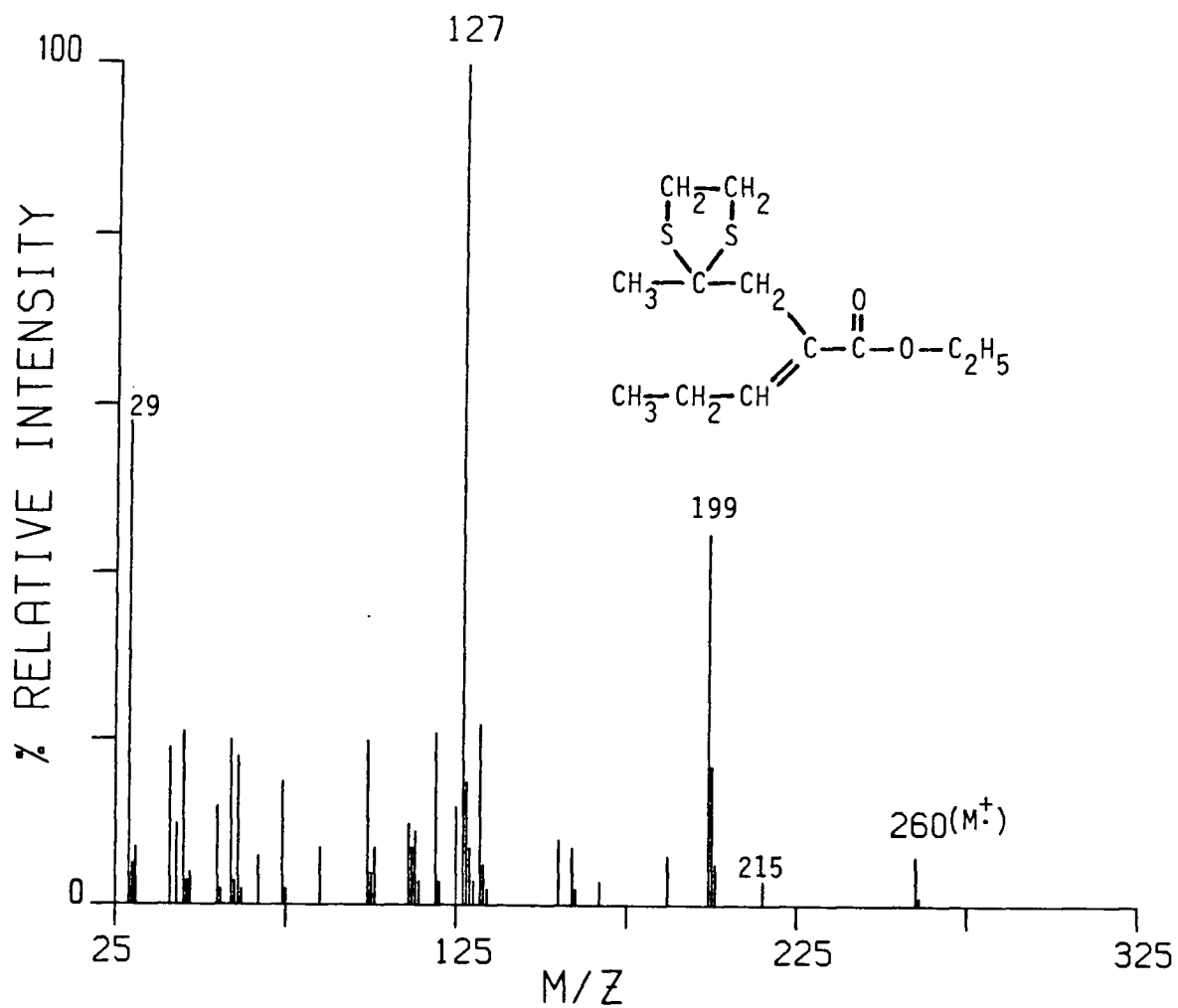EI spectrum of the TMS enol ether of ethyl 2-propyl-4-oxo-2-pentenoate.
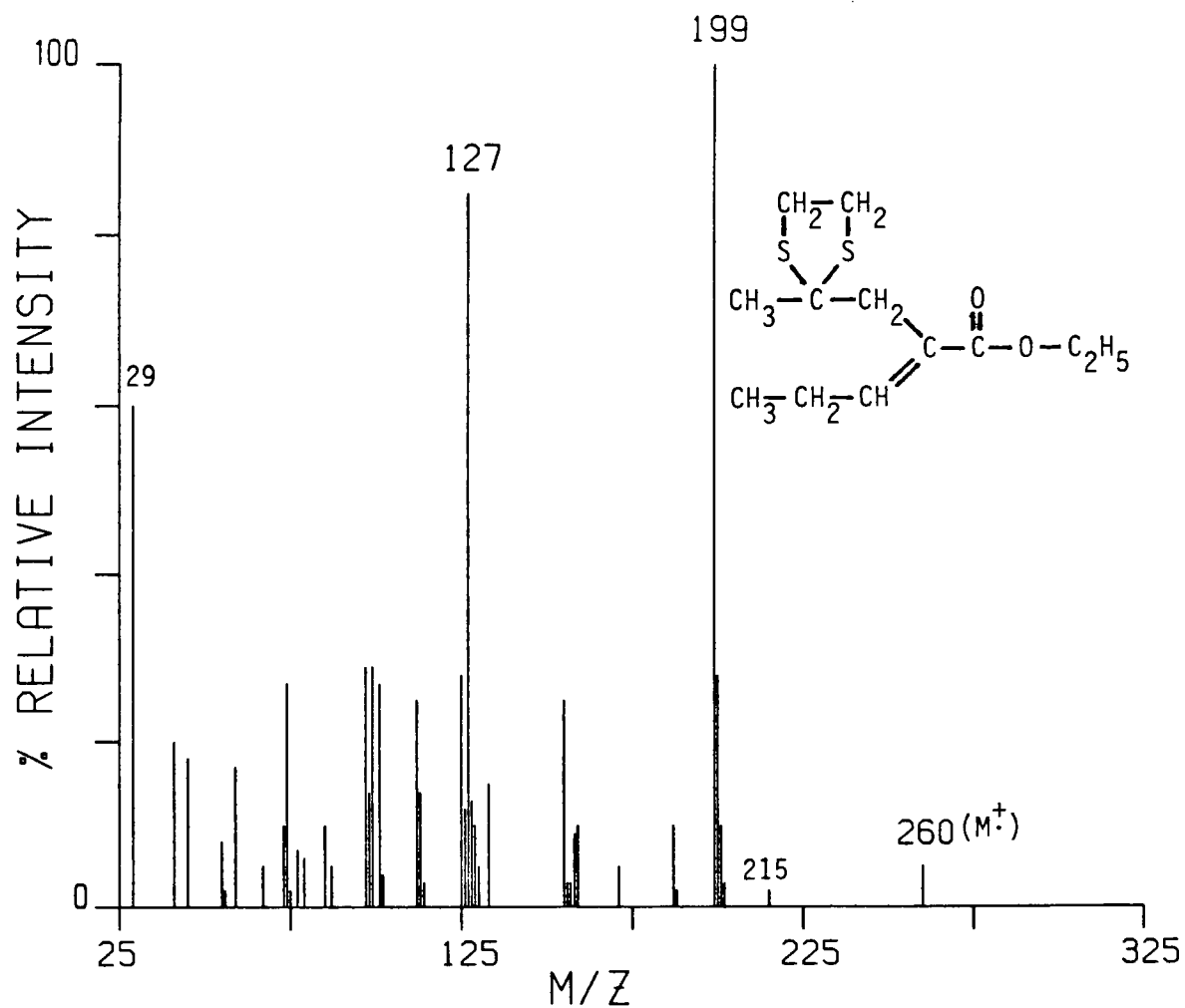
$^1$H-NMR (300 MHz) spectrum of ethyl 4-ethylenethioketalpentanoate.

EI mass spectrum of 4-carboethoxy-2-ethylenethioketal-4-heptene (isomer with the shorter retention time).

EI mass spectrum of 4-carboethoxy-2-ethylenethioketal-4-heptene (isomer with the longer retention time).