THE IDENTIFICATION, CHARACTERIZATION AND PROFILING OF NOVEL THIOL

AND AMINO ACID CONJUGATES OF VALPROIC ACID IN HUMANS AND ANIMALS

by

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ABSTRACT

Valproic acid (VPA) is an antiepileptic drug used for the management of generalized and absence seizures. It suffers from a rare but fatal hepatotoxicity side effect and its mechanism of action is not known. We demonstrated that the urinary thiol conjugates of (E)-2,4-diene VPA, a reactive intermediate arising from the further biotransformation of the metabolite 4-ene VPA, were significantly elevated in patients at high risk of developing the side effect. Therefore, these conjugates reflect a higher exposure towards the reactive intermediate of 4-ene VPA in high risk patients.

First, we characterized the structure of NAC II as 5-N-acetylcystein-S-yl-2-ene VPA by ^{1}H NMR. Then, using GC/MS and LC/MS/MS, we confirmed the identification of NAC I (previously detected in humans) and NAC II in patients on VPA therapy (n=40). The profiling of NAC I and II in urine samples of patients on VPA alone (group 1) or VPA in combination with non-enzyme inducing drugs (group 2), showed that patients ≤ 7.5 years old excreted significantly higher concentrations of the two conjugates compared to older patients (>7. 5 years) in both groups ($p<0.05$). Furthermore, patients on VPA polytherapy with enzyme inducing drugs (group 3 and > 7.5 years old), excreted significantly higher concentrations of NAC I and NAC II compared to patients in groups 1 and 2 and of similar age ($p<0.05$). There were no significant difference between the conjugates excreted by patients in group 3 and patients in groups 1 and 2 who were less than 7.5 years old. Therefore, age, dose, and polytherapy, the risk factors of VPA induced hepatotoxicity, were found to be associated with significantly higher concentrations of urinary NAC I and NAC II in patients on VPA therapy.

Furthermore, we reported the identification and characterization of novel valproyl glutamate (VPA GLU) and valproyl glutamine (VPA GLN) in the urine, serum and CSF samples of humans on VPA therapy, rats and rabbits treated with VPA. VPA GLU is the first glutamate conjugate of a xenobiotic discovered in humans, rats and rabbits. In addition, we were able to identify valproyl glycine (VPA GLY) in the urine and serum of humans on VPA therapy. These conjugates were not associated with the risk factors of VPA induced hepatotoxicity. The detection of VPA GLU in human CSF and at a concentration higher than the corresponding serum concentration suggests that the mechanism of action of VPA can be partially expressed through the conjugation of VPA with the excitatory neurotransmitter glutamic acid itself. Since, valproyl glycinamide, the amide of VPA GLY, was found to possess antiepileptic activity and is currently undergoing clinical trials (Hadad and Baieler, 1995), the detection of VPA GLU and VPA GLN in human CSF implies that both conjugates could have antiepileptic or CNS activity. The detection of VPA GLU in rabbit CSF supports our human findings.

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List of Abbreviations

AA	amino acid
AB	abnormal
ACN	acetonitrile
AMP	adenosine monophosphate
ANOVA	analysis of variance
APCI	atmospheric pressure chemical ionization
AST	amino aspartate transferase
ATP	adenosine triphosphate
CCNU	1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea
CID	collision induced dissociation
CBZ	carbamazepine
CI	chemical ionization
CL	clearance
CoA	coenzyme A thio ester
CLBZ	clobazam
Clonaz	clonazepam
CSF	cerebrospinal fluid
CYP	cytochrome P450
d	doublet (NMR)
DCI	desorption chemical ionization
DPH	diphenylhydantion (phenytoin)
DIPEA	diisopropylethylamine
E	*trans*

EI	electron impact
ER	endoplasmic reticulum
ES$^+$	positive ion electrospray
ES$^-$	negative ion electrospray
et al.	et alia
Ethosux	ethosuximide
EtOAc	ethyl acetate
f	female
FVPA GLN	2-fluoro-valproyl glutamine
GABA	γ-aminobutyric acid
GABA-T	GABA transaminase
GAD	glutamic acid decarboxylase
GC/MS	gas chromatography/mass spectrometry
GSH	glutathione
h	hour (s)
HPLC	high performance liquid chromatography
i.p.	intraperitoneal
I.S.	internal standard
J	coupling constant
kg	kilogram
lamo	lamotrigine
LC	liquid chromatography
LC/MS/MS	liquid chromatography tandem mass spectrometry
LC/UV	liquid chromatography/ultraviolet
m	male

M⁺	molecular ion
MeOH	methanol
mg	milligram
mh	mentally handicapped
mL	milliliter
MRM	multiple reaction monitoring
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MSTFA	N-methyl-N-trimethylsilyltrifluoroacetamide
MTBSTFA	N-methyl-N-t-butyldisilyltrifluoroacetamide
MW	molecular weight
m/z	mass to charge ratio
NAC	N-acetylcysteine
NAC I	5-N-acetylcystein-S-yl-3-ene VPA
NAC II	5-N-acetylcystein-S-yl-2-ene VPA
NAC III	4-hydroxy-5-N-acetylcysteinyl VPA-γ-lactone
NAC IV	3-keto-5-N-acetylcysteinyl VPA
NAC V	5-N-acetylcysteinyl-3-ene VPA glucuronide
NAC VI	dimethyl-N-acetylcysteinyl acrylate
NICI	negative ion chemical ionization
Nitraz	nitrazepam
NMDA	N-methyl-D-aspartate
NMR	nuclear magnetic resonance
4-PA	4-pentanoic acid
PB	phenobarbital

PCI	positive chemical ionization
PFBBr	pentafluorobenzyl bromide
PFB	pentafluorobenzyl
PPA	propionic acid
ppm	parts per million
psi	pounds per square inch
PPi	pyrophosphate
q	quartet
s	singlet
SIM	selected ion monitoring
SRM	selected reaction monitoring
t	triplet
t-BDMS	tertiarybutyldimethylsilyl
TFA	trifluoroacetic acid
TIC	total ion current
TMS	trimethylsilyl
t_R	retention time
TSP	thermospray
μl	microliter
V_D	volume of distribution
Viga	vigabatrin
VPA	valproic acid
VPA ASP	valproyl aspartate
VPA GLN	valproyl glutamine
VPA GLU	valproyl glutamate

VPA GLY	valproyl glycine
VPA GLYD	valproyl glycinamide
[^{7}H]VPA	[^{7}H]-valproic acid
(*E*)-2,4-diene VPA	*trans*-2-propyl-2,4-pentadienoic acid
2-ene VPA	(*E*)-2-propyl-2-pentenoic acid
4-ene VPA	2-propyl-4-pentenoic acid
[^{7}H]-3-keto VPA	[^{7}H]-2-propyl-3-oxopentanoic acid
3-keto VPA	2-propyl-3-oxopentanoic acid

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DEDICATION

To my family and friends for their constant support during my studies

1. INTRODUCTION

Valproic acid is a short chain fatty acid with a branched structure. It was first introduced in Europe in 1967, four years after its anticonvulsant properties were discovered serendipitously (Meunier *et al.,* 1963) and became available for therapy in North America in 1978. It is typically used for the management of generalized seizures (Wilder *et al.,* 1983; Gram *et al.,* 1985; Levy *et al.,* 1995) and is regarded as the treatment of choice for simple or complex seizures in pediatric patients (Jeavons, 1977). In contrast to other conventional antiepileptic drugs, it has a simple and unique chemical structure and can act either as a sole medication or adjunctively with other antiepileptic drugs in patients requiring multiple drug therapy (Jeavons *et al.,* 1977; Sato *et al.,* 1982; Dreifuss *et al.,* 1987).

Unlike most anticonvulsants, VPA is unique because of its broad spectrum of activity. Further, during the last decade, VPA has been beneficial as part of antipsychotic therapy (Chapman *et al.,* 1982; Penry and Dean, 1989; Lösher, 1993). However, VPA use is limited by a rare but fatal side effect. It is associated with hepatotoxicity, and children less than two years of age and on polytherapy are most at risk of the life-threatening complication. In addition, it is also associated with teratogenicity. Because the mechanism of action of VPA is not fully understood, the design of new analogues of VPA devoid of both side effects is complex. In both cases, it appears that the metabolism of VPA plays a crucial role.

The initial goal of this dissertation was to study the relationship between the thiol conjugates of VPA and the risk factors of VPA-induced hepatotoxicity through the

identification, characterization and profiling of the conjugates in pediatric patients belonging to various study groups. The progression of our first aim led to the study of amino acid (AA) conjugation of VPA, a pathway which has remained relatively unexplored until now. We provide below a review of several topics which are deemed pertinent to understanding the aims of this thesis.

1.1 Valproic Acid

1.1.1 Physical and chemical characteristics of VPA

VPA, 2-propylpentanoic acid, is a colorless liquid with a characteristic odor at room temperature. It is a short chain carboxylic acid as shown in figure 1. It has a low molecular weight of 144.21 and is slightly soluble in water with a pK_a of 4.8, but is readily soluble in organic solvents (Baillie and Sheffels, 1995).

COOH
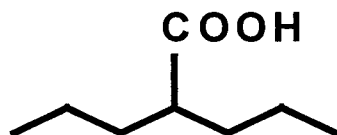
Figure 1. Structure of VPA

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1.1.2 Mechanism of action of VPA

The mechanism by which VPA exerts its pharmacological activity remains to be established. Its broad spectrum activity is believed to be the consequence of several modes of actions. In 1969, Godin *et al.* introduced the theory that the inhibitory neurotransmitter γ-aminobutyric acid (GABA) is elevated with VPA treatment. Since then, several studies have been conducted on that subject and reviewed (Lösher, 1993; Davis *et al.*, 1994). In brief, the elevation of GABA has been rationalized by either the reduction in the activity of GABA-transaminase (GABA-T) or of succinic semialdehyde dehydrogenase (SSA-DH), two enzymes involved in the degradation of GABA along the GABA shunt. Further, an increase in glutamic acid decarboxylase (GAD), the enzyme involved in the synthesis of GABA, can also lead to an increase in GABA levels. However, an elaborate structure activity study of VPA analogues conducted in our laboratory (Palaty, Ph.D. thesis, 1995) has cast doubt on the link between increased levels of GABA and the anticonvulsant effects of VPA and its analogues. Although the study verified that VPA increases GABA concentrations in rats, it failed to show any significant relationship between the level of GABA and the antiepileptic activity of the most potent analogue of VPA, cyclooctylideneacetic acid.

A second mechanism proposed to explain the effect of VPA on GABA relates to its ability to potentiate the post-synaptic response to GABA (MacDonald and Bergey, 1979), although this theory has subsequently been challenged (Lösher, 1993). Another proposed mechanism of action of VPA relates to its effect on the neuronal membrane. VPA was found to increase membrane conductance to potassium in Aplysia neurons (Slater and Johnson, 1978). Other reported effects of VPA include the inhibition of

sustained repetitive firing by blocking the voltage sensitive sodium channels (Franceschetti et al., 1986).

1.1.3 Clinical pharmacokinetics of VPA

The absorption, distribution and excretion characteristics of VPA in humans have been reviewed (Levy and Lai, 1982; Levy and Shen 1989, 1995). VPA is commercially available as the free acid or as the sodium salt. The bioavailabilty of an oral dose is close to unity and the drug binds extensively (mean 90%) to albumin at therapeutic concentrations. The apparent volume of distribution of VPA is reported to be 0.1-4 L/Kg (Von-Unruh, 1980).

The drug is rapidly absorbed and peak plasma levels are usually observed within 2 h. The therapeutic plasma levels vary considerably in patients. A therapeutic range of 50-100 μg/mL (Davis et al., 1994) is usually accepted for therapeutic drug monitoring purposes. For children between the ages of 9-18 years, a lower range of 30-80 μg/mL has been reported (Farrell et al., 1986). The elimination half-life of VPA in plasma ranges from 8-16 h in adult epileptics (Gugler and Von Unruh, 1980) and decreases considerably in children (Levy and Shen, 1995).

Similarly the clearance of VPA, which occurs predominantly by hepatic metabolism, is affected by age and polytherapy. Plasma clearance of VPA in adults ranges from 6-11 mL/h/kg (Levy and Shen, 1989) and increases by as much as 10 fold for children younger than 5 years (Dodson and Tasch, 1981). The range for adult patients on polytherapy was observed to be 14.4-15.5 mL/h/kg (Hoffman et al., 1981). For this

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reason, the dose of VPA is increased for younger children and those on polytherapy to maintain therapeutic concentrations of the drug (Levy and Shen, 1995).

Because of its short half life and the side effects associated with a single large dose, VPA is administered daily in 2-3 divided doses (Covanis and Jeavons, 1980; Löscher and Nau, 1984). For patients initiated on VPA therapy, steady state is reached within 3-4 days following an initial dose of 15 mg/kg/day. Although serum concentrations vary widely, therapeutic monitoring is recommended to ensure compliance and to monitor those who do not respond to the drug. The optimal time for blood sampling is recommended worthwhile to be immediately prior to the first morning dose (i.e. at "trough" level).

1.1.4 VPA-induced hepatotoxicity

At first, VPA was regarded as a safe drug devoid of any major debilitating side effects including sedation. In fact, VPA was reported to induce liveliness (Jeavons et al., 1977). However, over the past two decades, reports of hepatotoxicity associated with VPA have been a concern for clinicians. Two types of hepatotoxicity have been observed. The first one is a dose dependent reversible conditions whereas the second type is irreversible, non-dose related, and although rare, frequently fatal.

1.1.4.1 Risk factors associated with VPA-induced hepatotoxicity

The first fatal cases of VPA hepatotoxicity were reported in 1979 (Donat et al., 1979; Gerber et al., 1979; Suchy et al., 1979). Cumulative studies conducted over the years

indicate over 100 cases of VPA-associated fatal hepatotoxicity (Scheffner *et al.,* 1988; Dreifuss *et al.,* 1987, 1989; Bryant III and Dreifuss, 1996). The authors report that patients less that 2 years of age and on polytherapy are at highest risk of developing the disease. In the first study reported, the authors examined 37 fatal cases in the U.S. and found a risk factor of 1:500 for patients in that age group and on VPA polytherapy compared to patients more than 2 years of age and on VPA monotherapy (Dreifuss *et al.,* 1987).

Interestingly, in 1996, these same observations were reiterated in a third retrospective study of U.S. patients by Bryant III and Dreifuss, despite a decline in the use of VPA in young patients. The authors reported that the incidence of the side effect, in patients 0-2 years of age, did not decline dramatically and those patients continue to be most susceptible to the side effect (1:600) despite the increased awareness of the complication. Other risk factors associated with the disease are reported to be mental retardation, developmental delay and coincident metabolic disorders.

1.1.4.2 Incidence and histological manifestations of VPA-induced hepatotoxicity

Interest in understanding the cause of VPA-induced hepatotoxicity has prompted researchers to study the mechanism of this side effect. The difficulty arises as the fatal side effect is believed to be an idiosyncratic reaction (Zimmerman and Ishak, 1982; Dreifuss *et al.,* 1987, 1984; Bryant III and Dreifuss, 1996). The syndrome of hepatic injury observed with VPA is not characteristic of hypersensitivity reactions observed with other antiepileptics such as phenytoin (Zimmerman and Ishak, 1982).

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Histological studies have usually revealed that the hepatic injury observed in the fatal cases studied is characterized by hepatic microvesicular steatosis, often accompanied by centrizonal necrosis. These observations are consistent with the inhibition of fatty acid metabolism possibly due to toxic metabolites of VPA being produced. Other evidence, such as a delay in the onset and manifestation of the condition until at least a month of therapy, as well as the higher incidence of fatalities in the polytherapy groups (Rowan *et al.*, 1979; Jeavons, 1984) have collectively implicated the involvement of VPA metabolites.

Zimmerman and Ishak (1982) analyzed 23 cases associated with VPA-induced fatal hepatic liver failure. They found microvesicular steatosis as the most common histological finding in 17 of the 21 confirmed cases, indicative of impaired β-oxidation. Seven of those cases were accompanied by centrizonal necrosis and 2 had non zonal necrosis. A review of 67 cases by Jeavons (1984), including the cases mentioned above, indicated that steatosis was a common histological feature although he identified necrosis and cirrhosis as being present as well. Another analysis of 16 cases found microvesicular steatosis and necrosis at various degrees of severity in all cases (Scheffner *et al.*, 1988).

Zimmerman and Ishak (1982) suggested that the microvesicular steatosis is caused by a metabolite which inhibits key enzymes in the β-oxidation of fatty acid metabolism and the necrosis could be due to another reactive metabolite which binds covalently to cellular components in the centrolobular areas of the liver. The manifestation of VPA-induced hepatotoxicity resembles that of (a) Reye's syndrome, a metabolic disorder of fatty acid metabolism, (b) poisoning by hypoglycin in Jamaican vomiting sickness and

fatty acid metabolism and, (c) poisoning by 4-pentenoic acid (4-PA), a known hepatotoxin (Gerber, 1979). These observations of toxicity were consistent with the inhibition of fatty acid metabolism and fatty acid liver degeneration.

The similarity in chemical structure between 4-PA and 4-ene VPA was first pointed out by Gerber *et al.,* (1979). Both compounds have a five carbon chain with a terminal olefin separated from a carboxyl group by two carbons. It has been shown that 4-ene VPA induces steatosis in rats (Kesterson *et al.,* 1984; Jezequel *et al.,* 1984) and inhibits β-oxidation *in vitro* (Bjorge *et al.,* 1985) and *in vivo* (Granneman *et al.,* 1984b). It appears that 4-ene VPA has emerged as a putative metabolite responsible for the induction of VPA-hepatotoxicity (Baillie, 1988).

1.1.5 VPA metabolism

The metabolism of VPA has been reviewed by Baillie and Scheffels (1995). The study of VPA metabolism has revealed that VPA is excreted primarily via glucuronidation, ω-oxidation, (ω-1) oxidation and β-oxidation by a combination of microsomal, mitochondrial and cytosolic enzymes to produce a large number of metabolites as shown in scheme 1. It is the current thinking that the pathway which is initiated by the formation of 4-ene VPA induces VPA hepatotoxicity through the formation of reactive intermediates.

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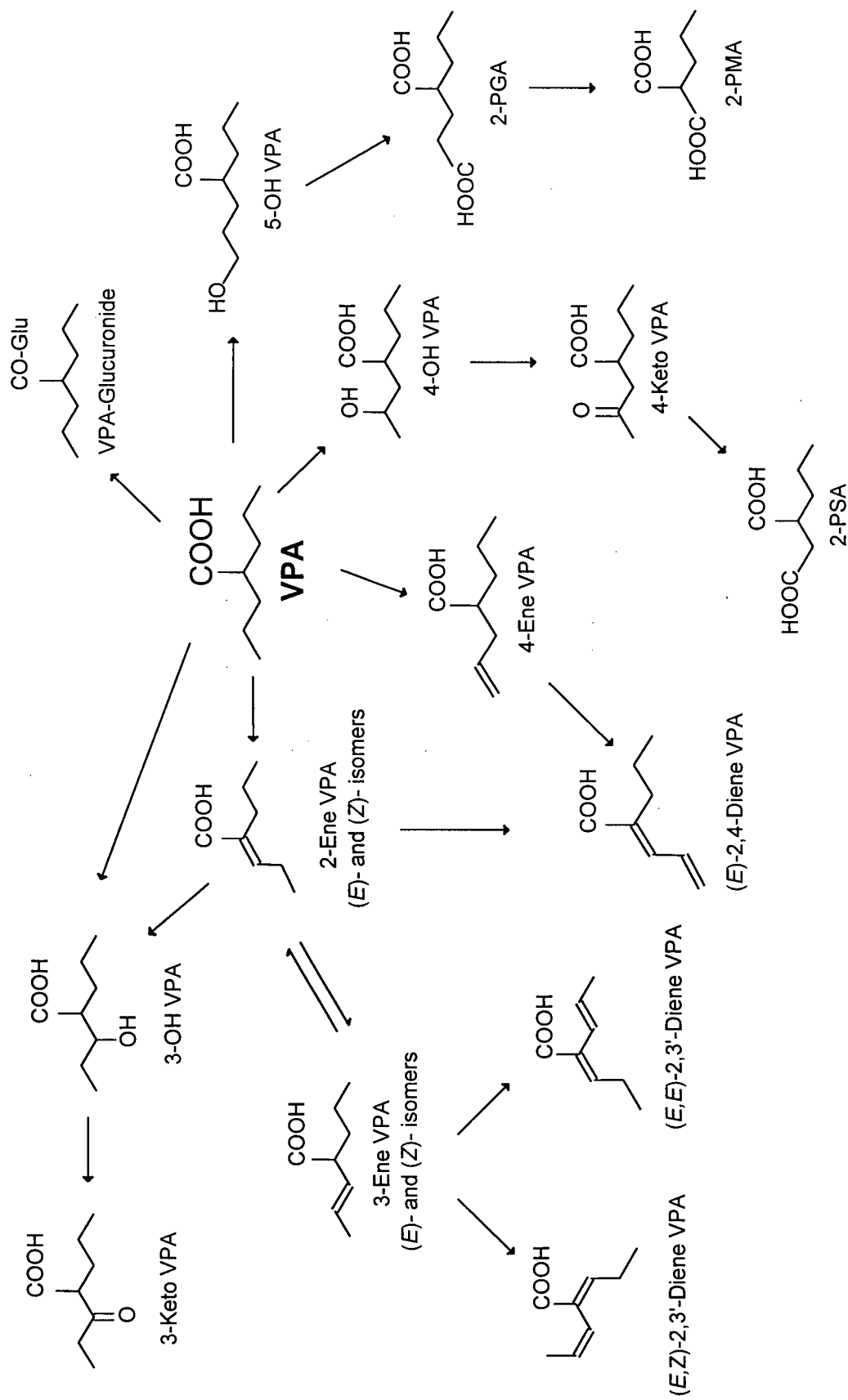
Scheme 1. Proposed metabolic pathways for the formation of phase I metabolites of VPA and VPA glucuronide in humans. (VPA=valproic acid, 2-PSA= 2-propylsuccinic acid, 2-PGA=2-propylglutaric acid)

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1.1.5.1. Origin of 4-ene VPA

VPA undergoes oxidation to produce a number of mono unsaturated metabolites. In contrast to 2-ene VPA and 3-ene VPA which are believed to originate in the mitochondria, it was demonstrated that 4-ene VPA is formed in the endoplasmic reticulum (ER) by the action of cytochrome (CYP) P450 enzyme systems (Rettie *et al.,* 1987, 1988, 1995). Although, the CYP2B isoforms could catalyze the desaturation process in the rat and rabbit, the specific isoform involved in humans is still not known.

Recently in our laboratory, we noted that the AUC of 4-ene VPA and its β-oxidation product, (*E*)-2,4-diene VPA in a patient on VPA was considerably decreased when the parent drug was administered with erythromycin, a known CYP3A4 inhibitor (Gopaul *et al.,* 1996a). These observations suggest indirectly that CYP3A4 could be involved in the induction of 4-ene VPA, although the involvement of other isoforms cannot be ruled out.

1.1.5.2. Origin of (*E*)-2,4-diene VPA

The (*E*)-2,4-diene metabolite is believed to originate from two sources: (1) mitochondria and (2) the ER. In mitochondria, 4-ene VPA undergoes β-oxidation to produce (*E*)-2,4-diene VPA. The process requires the activation of 4-ene VPA to its CoA derivative. Evidence for the formation of the diene formed in mitochondria was obtained when the dienoyl CoA metabolite was identified in rats treated with 4-ene VPA (Rettenmeier *et al.,* 1985).

(*E*)-2,4-Diene has also been found as a metabolite of (*E*)-2-ene VPA in the plasma and liver of rats treated with (*E*)-2-ene VPA *in vivo* (Lee, Ph.D thesis, 1991). *In vitro* studies have further supported the formation of the diene as a microsomal oxidation product of (*E*)-2-ene VPA (Kassahun and Baillie, 1992). The authors argue that the diene formed from (*E*)-2-ene is in the free acid form and is non-reactive. On the other hand, its formation as the CoA ester in mitochondria enhances its reactivity. Perhaps, this explains in part the observations of Kesterson *et al.,* (1984) who demonstrated that 4-ene VPA is toxic when administered to rats in contrast to an equivalent dose of (*E*)-2-ene VPA. Thus, the source of formation of the diene could be of importance in understanding its role in inducing hepatotoxicity.

1.1.5.3. Metabolic profiling of 4-ene and (*E*)-2,4-diene VPA as a means to predict hepatotoxicity

Because VPA-associated hepatotoxicity is apparently an idiosyncratic reaction with no clear biochemical changes, researchers have focussed on the serum concentrations of VPA metabolites in an effort to find one which could predict the onset of the side effect. One of the strongest arguments against the theory that 4-ene VPA and its β-oxidation product, (*E*)-2,4-diene VPA are potentially hepatotoxic, is the failure to observe a consistently high serum level of these metabolites in the patients experiencing hepatotoxicity.

The profiling of the 4-ene VPA and (*E*)-2,4-diene VPA metabolites is usually performed in combination with the other phase I metabolites of VPA to ensure chromatographic separation and avoid interference. The dilemma that one faces with the simultaneous

analysis of serum VPA and all of its metabolites is the drastic range of concentrations of the compounds, and their different chemical structures rendering derivatization and detection by GC/MS a challenging task. The difficulty is reflected by the conflicting results produced so far with regard to the metabolic profiling of phase I metabolites, in particular 4-ene VPA and (E)-2,4-diene VPA.

In 1983, Kochen et al., reported an abnormally high level of 4-ene VPA in the plasma and urine of a 7-year old boy who died of VPA-induced hepatic failure. In 1984, the same group reported the identification of (E)-2,4-diene VPA in increased amounts associated with the side effect (Kochen et al., 1984). However, these results were disputed shortly after when the metabolite could not be detected in a relatively small group of patients on VPA (Eadie et al., 1988; Tennison et al., 1988). In 1990, Levy et al. demonstrated that patients on VPA and in combination with P450 enzyme inducing drugs form significantly higher amounts of 4-ene VPA than those on monotherapy. Further, those on VPA and P450 enzyme inhibitors show a decrease in the formation of the monoene metabolite.

In 1992, Kondo et al. demonstrated a correlation between the risk factors for VPA-induced hepatotoxicity and serum 4-ene VPA in an extensive study of 106 epileptic patients. Although Fisher et al. (1992) were unable to correlate serum levels of 4-ene VPA with high doses of VPA, Anderson et al. (1992) claimed the contrary. In our own laboratory, serum levels of 4-ene VPA or (E)-2,4 diene VPA did not appear to be higher in patients on VPA polytherapy (Kassahun et al., 1989).

The most compelling argument against 4-ene VPA as a hepatotoxin in humans emanates from an elaborate study of 470 patients on VPA therapy who had a variety of clinical conditions (Siemes *et al.,* 1993). The authors were unable to correlate the unsaturated metabolite with any risk factors for hepatotoxicity and concluded that VPA itself may be the primary toxic agent. Since then, the focus on profiling studies of phase I metabolites has decreased and those that have been performed appear to corroborate the latter conclusion. One exception to this was the study which monitored the *t*-BDMS derivatives of VPA and its metabolites in 98 patients that was able to demonstrate that 4-ene VPA was significantly elevated in patients on polytherapy (Darius *et al.,* 1994). Whether the results are a reflection of the evolvement of analytical techniques and drug assays or whether the results were a function of a different group of patients is not known.

By and large, the metabolic profiling of the 4-ene VPA and (*E*)-2,4-diene VPA is difficult to assess and there is no clear indicator of hepatotoxicity. It has been proposed that if the reactive metabolites undergo further metabolism themselves, then it is perhaps reasonable to envisage that it is their end products which will correlate with incidence of hepatotoxicity and, therefore, better predict hepatotoxicity risk (Kassahun, Ph.D. thesis, 1991).

1.1.5.4 Bioactivation of 4-ene VPA and (*E*)-2,4-diene VPA

1.1.5.4.1. Glutathione conjugation

It is evident that glutathione (GSH) metabolism is vital to the elimination of the toxic metabolites of VPA. Most reactive species in the body are eliminated by GSH

metabolism to form GSH conjugates which undergo further metabolism and are subsequently eliminated as the *N*-acetylcysteine (NAC) or mercapturic acid conjugates (Ketterer *et al.*, 1993). Although research is now indicating that GSH conjugation can give rise to reactive metabolites (van Bladern PJ, 1988; Ketterer *et al.*, 1983), it is considered to be an important pathway in the detoxification of reactive metabolites by direct conjugation or assisted by GSH transferase (Ketterer *et al.*, 1983; Deleve *et al.*, 1991). GSH reacts readily with metabolites which are "soft" electrophiles, organic free radicals and those that produce oxidizing species such as O_2^-. In 1974, Gillette indicated that tissue necrosis could be due to the covalent binding of electrophiles to tissue components. By conjugation with GSH, reactive metabolites escape the covalent binding to proteins and nucleic acids which cause cytotoxicity effects such as mutations and cancer.

The GSH moiety itself is a tripeptide which consists of L-γ-glutamate, L-cysteine and glycine. The initial step in GSH conjugation consists of the nucleophillic attack of the sulfhydryl moiety on the electrophilic center of the reactive compound. The reaction is often assisted by GSH-S-transferase enzymes. GSH conjugates are not normally excreted unchanged. They undergo further metabolism to produce a variety of sulfur containing compounds among which are NAC conjugates (van Weilie, 1992; Boyland and Chasseaud, 1969). The metabolism of a GSH conjugate involves an attack by gamma glutamyltranspeptidase to remove the glutamyl moiety to yield a cysteinyl-glycine conjugate. The latter is further cleaved by a dipeptidase to give a cysteinyl conjugate which upon acetylation, mostly in the kidney, produces NAC conjugates which are then excreted in the urine. Whereas GSH conjugates are mostly found in the

bile, the NAC conjugates are mostly found in the urine (Deleve, 1991). GSH metabolism begins in the biliary tree but mostly occurs along the mercapturic pathway. Mercapturic acids are the metabolic end products of reactive xenobiotics (Norström *et al.*, 1986; Vermeulen *et al.*, 1989). They were first identified as sulfur containing metabolites after the administration of bromobenzene to dogs. Since their discovery, they have been used in many biotransformation, biological monitoring and toxicological studies (van Weilie *et al.*, 1992).

1.1.5.4.2. NAC conjugates

Urinary NAC conjugates, appropriately, reflect the chemical nature of the reactive electrophilic intermediate. In principle, the excretion of NAC conjugates can be viewed as indirect biochemical indices or as biomarkers in assessing exposure to reactive metabolites in a non-invasive manner. In occupational health studies, NAC conjugates are widely used to assess and monitor exposure of industrial workers to toxic compounds (Vermeulen *et al.*, 1989). In this study, this concept was used as a rational approach to demonstrate that NAC conjugates of reactive metabolites of VPA can be used to assess exposure to the hepatotoxic metabolites. Thus, patients at higher risk of developing VPA-associated hepatotoxicity can be identified. The tracing of reactive metabolites as their urinary NAC conjugates in patients has previously been used in our laboratory to assess the exposure of a cancer patient treated with CCNU to its reactive isocyanate metabolite (Borel and Abbott, 1993).

1.1.5.4.3. GSH conjugation of 4-ene VPA and (E)-2,4-diene VPA and their significance in VPA-induced hepatotoxicity

The reactivities of 4-ene VPA and (E)-2,4-diene VPA are difficult to determine directly in humans. However, there is evidence for their reactivity from mechanistic studies performed in animals. It is evident from the results of these studies that the biotransformation of the two phase I metabolites leads to their corresponding thiol conjugates which in turn validates their toxic characteristics in an indirect manner.

1.1.5.4.3.1. Animal studies

On the basis of information available for 4-PA, a known hepatotoxin, the bioactivation of 4-ene VPA is presumed to degrade to (E)-2,4-diene VPA, then to 3-keto-4-ene VPA which is capable of binding to 3-ketoacylCoA thiolase, a key enzyme in the β-oxidation pathway. Hence, researchers have focused on the search for the thiol conjugate of the unsaturated ketone metabolite in animals. Surprisingly, their initial efforts led to the discovery of the prominent 5-NAC-3-ene VPA (NAC I), the thiol conjugate of (E)-2,4-diene VPA in rats dosed with both the monoene and the diene itself. In fact, it was determined that NAC I constitutes 40% of a dose of (E)-2,4-diene VPA and is also a significant metabolite of 4-ene VPA (Kassahun et al., 1991). This represents the first concrete evidence for the reactivity of (E)-2,4-diene VPA and the formation of a reactive species in the metabolism of VPA.

Furthermore, parallel studies conducted between 4-ene VPA and 4-pentenoic acid in rats have found that both compounds formed the β-oxidation intermediate (E)-2,4-diene

VPA and (*E*)-2,4-pentadienoic acid (Kassahun *et al.,* 1993), respectively. However, the former diene intermediate binds to GSH more readily than the latter counterpart. The apparent increased reactivity of (*E*)-2,4-diene VPA is considered to be a fundamental difference between the metabolism of 4-ene VPA and 4-PA. On that basis, it is believed that the mechanism leading to hepatotoxicity may differ in both cases. The study provides strong evidence suggesting that (*E*)-2,4-diene VPA is a potential hepatotoxin itself.

Further evidence to support (*E*)-2,4-diene VPA as the reactive intermediate of 4-ene VPA was obtained in a study comparing the effects of the monoene metabolite with its fluorinated analogue, F-4-ene VPA (Tang *et al.,* 1995). Histological evaluation of the livers of rats dosed with F-4-ene VPA, which do not produce (*E*)-2,4-diene VPA, failed to show evidence of liver injury. In contrast, the livers of rats dosed with 4-ene VPA were characterized by microvesicular steatosis and mitochondrial alterations. Furthermore, the non-steatogenic effect of the fluorinated analogue of the monoene was not accompanied with a decrease in mitochondrial GSH as observed for 4-ene VPA. When assessed collectively, the authors also concurred that the bioactivation of 4-ene to reactive intermediates including (*E*)-2,4-diene VPA is an important step in the induction of hepatotoxicity of 4-ene VPA.

The major thiol conjugate of 4-ene VPA is believed to be formed from the epoxide metabolite of 4-ene VPA in the bile or urine of rats treated with a toxic amount of 4-ene VPA (Kassahun *et al.,* 1994). The study proposed that the site of formation of the metabolite epoxide is the ER. The epoxide is believed to bind covalently to hepatic proteins in rats. The accompanying decrease in cytosolic GSH observed is attributed

to the conjugation of the epoxide outside the mitochondria. These observations have been further corroborated by Tang *et al.* (1995).

The thiol conjugates of 3-keto-4-pentenoic acid, the ultimate metabolite believed to inhibit 3-ketoacyl-CoA thiolase, were identified in rats treated with 4-pentenoic acid VPA (Kassahun *et al.,* 1993). Similarly, the thiol conjugate of 3-keto-4-ene VPA was identified as a metabolite of 4-ene VPA in rats, albeit in minor concentrations (Kassahun *et al.,* 1994). The relatively low amount of thiol conjugates of 3-keto-4-ene VPA formed from 4-ene VPA suggests that the metabolite may not be detected as a metabolite of VPA in humans and its direct importance in VPA-induced hepatotoxicity will be difficult to determine. Conversely, the detection of this metabolite in significant amounts in urine of patients on VPA would be very strong evidence to implicate this metabolite in the inhibition of 3-ketoacylCoA thiolase with VPA therapy.

Furthermore, it is of importance to note that the GSH conjugate of (*E*)-2,4-diene VPA glucuronide has been reported in the bile of rats treated with (*E*)-2,4-diene VPA (Tang *et al.,* 1996b). The significance of this finding is that it suggests that the glucuronide ester of (*E*)-2,4-diene VPA formed outside mitochondria can be reactive and possibly be involved in the induction of liver toxicity. A proposed scheme for the formation of thiol conjugates derived from 4-ene VPA and (*E*)-2,4-diene VPA in rats is shown in Scheme 2.

4,5-Di-OH VPA Lactone

HO

P-450

COOH

4-Ene VPA

β-oxidation

COSCoA

(E)-2,4-Diene VPA-CoA

β-oxidation

COSCoA

O

3-Keto-4-Ene VPA-CoA

Mitochondrial GSH / GST

COSCoA

O

GS

3-keto-5-GS VPA-CoA

COOH

O

NAC

3-keto-5-NAC VPA

(NAC IV)

Mitochondrial GSH / GST

COSCoA

GS

5-GS-3-Ene VPA-CoA

COOH

NAC

5-NAC-3-Ene VPA + Isomer

(NAC I + NAC II)

4,5-Epoxy VPA

COOH

O

Cytosolic GSH / GST

O

C

O

GS

5-GS-4-OH VPA Lactone

O

C

O

NAC

5-NAC-4-OH VPA Lactone

(NAC III)

Scheme 2. Proposed biotransformation of 4-ene VPA leading to the formation of urinary NAC conjugates in rats (Kassahun et al., 1991, 1993, 1994; Tang et al., 1996b, 1997b). The formation of NAC V (5-NAC-3-Ene VPA Glucuronide, Tang et al., 1996b) formed from (E)-2,4-diene Glucuronide is not shown.

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1.5.4.3.2. Human studies

Information regarding the GSH conjugation of 4-ene VPA and (E)-2,4-diene VPA is obtained from the excretion of their mercapturic acids. Because some of the information regarding the formation of NAC conjugates is derived from mechanistic studies of 4-ene VPA in animals, it is crucial to investigate whether or not these metabolites are also products of VPA *in vivo* and in humans. It is equally important to investigate for new adducts which may not be formed in animal species.

To date, very little is known about the formation of thiol conjugates of VPA in humans. Only one study (Kassahun *et al.,* 1991) identified NAC I in pediatric epileptic patients treated with VPA. The investigation also revealed the presence of a second conjugate which appeared to be an isomer of NAC I but whose identity remains to be established. For the sake of this presentation, we refer to the metabolite as NAC II. Both NAC I and II have been detected in urinary samples of patients on VPA and in higher amounts in patients suffering from VPA-induced hepatotoxicity.

Consistent with observations from mechanistic studies, the synthesis of the GSH and hence the NAC conjugate of (E)-2,4-diene VPA occur by conjugation of GSH with the CoA ester of the intermediate (Kassahun *et al.,* 1991). It appears that the reaction is not feasible when the diene is not in the ester form, suggesting that the free acid itself is fairly innocuous *in vivo*. This line of reasoning has been used to explain the apparent lack of hepatotoxicity of (E)-2-ene VPA which produces the diene in the free acid form (Kassahun *et al.,*1993). Evidence has emerged that favors the conjugation of the CoA ester of (E)-2,4-diene VPA with mitochondrial GSH *in vivo*, prior to metabolism along

the mercapturic acid pathway (Tang *et al.*, 1996a). Hepatotoxicity could occur in situations when the liver is no longer capable of detoxifying the reactive intermediate which then binds to cellular components.

The formation of NAC conjugates of mitochondrial species provides indirect evidence for the consumption of mitochondrial GSH. This is supported by mechanistic studies in rats which demonstrated that VPA-induced hepatotoxicity was associated with selective mitochondrial GSH depletion (Tang *et al.*, 1995). The investigators proposed that a similar situation can also occur in humans. They supported their position by the fact that urinary NAC I was found at higher levels in hepatotoxic patients (Kassahun *et al.*, 1991) suggesting an imposition on mitochondrial GSH. Furthermore, they pointed out that patients suffering from VPA-associated hepatotoxicity recovered with supplements of NAC which is believed to increase intracellular GSH levels (Farrell and Abbott, 1991).

Since the NAC conjugates of 4-ene epoxide VPA (NAC III) and 3-keto-4-ene VPA (NAC IV) have been identified as metabolites of 4-ene VPA in rats, it is noteworthy to verify whether or not they are also metabolites of VPA in humans. Their presence in the urine of humans could provide evidence for the formation of the reactive epoxide *in vivo*. In addition, it is important to verify the formation of the NAC conjugate of (*E*)-2,4-diene glucuronide (NAC V) in patients. The detection of NAC IV and NAC V in those patients will validate the reactivity of (*E*)-2,4-diene outside the mitochondria. As mentioned earlier, Zimmerman and Ishak (1982) have alluded to the possibility of more than one reactive metabolite formed at other sites in the liver being implicated as well in VPA-induced hepatotoxicity.

1.1.6 Amino acid (AA) conjugation

1.1.6.1 General

AA conjugation is an important aspect in the elimination of xenobiotic carboxylic acids. Although the biotransformation of xenobiotics to AA conjugates has been known well before the mid century, the pharmacological and toxicological importance remains to be fully understood (Hutt and Caldwell, 1990). The reaction is believed to depend more on the chemical structure of the carboxylic acids and less on their physicochemical properties (Caldwell, 1978). AA conjugation is believed to be species dependent. In humans, it is more commonly observed with glycine, glutamine, and taurine although the conjugation with other amino acids such as serine, alanine and aspartic acid has been reported in other species and these have been reviewed (Hutt and Caldwell, 1990).

Although bile acids form conjugates with glycine and taurine by the action of microsomal enzymes, AA conjugation is believed to be a mitochondrial reaction occuring primarily in the liver or the kidney (Williams, 1989). The reaction is initiated by the activation of the acid to a CoA ester followed by the acyl transfer to an amino acid residue. The last step is catalyzed by N-acetyl transferases specific for each amino acid. Glycine N-acyltransferase (Tishler and Goldman, 1970) and glutamine N-phenylacetyltransferase (Webster et al., 1976) have been isolated from human liver mitochondria. The reactions are illustrated below (1.1-1.3). It is understood that the structural requirement for amino acid conjugation is defined by the final step.

$$RCOOH + ATP \rightarrow RCO \sim AMP + \text{pyrophosphate (PPi)} + H_2O \quad (1.1)$$

$$RCO \sim AMP + HSCoA \rightarrow RCO \sim SCoA + AMP \quad (1.2)$$

$$RCO \sim SCoA + NH_2CH_2CO_2H \rightarrow RCONHCH_2CO_2H + HSCoA \quad (1.3)$$
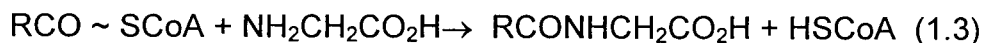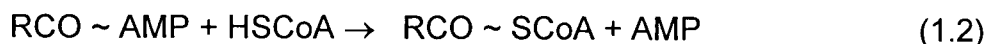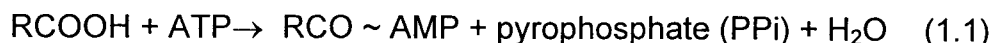
Conjugation with amino acids depends on the size and type of substituent adjacent to the carboxyl group of the xenobiotic. Branched aliphatic acids are believed to undergo glycine conjugation and/or glucuronidation (Williams, 1989). It is observed that substitution of the α-carbon favors glucuronidation rather than glycine conjugation. Benzoic acid and heterocyclic aromatic acids are principally conjugated with glycine in mammalian species (Hutt and Caldwell, 1990). A study conducted in rabbits clearly demonstrated that glycine conjugation with benzoic acid decreased with bulky substituents at the ortho position. Glutamine conjugation is known for phenylacetic acids and related arylacetic acids in humans and monkeys.

1.1.6.2 Glycine conjugation of VPA

The branched chain aliphatic structure renders VPA a suitable candidate for glycine conjugation. Earlier attempts to find this metabolite in humans have failed. Valproyl glycine (VPA GLY) was identified at concentrations less than 1% of a dose in rats treated with VPA (Granneman *et al.*, 1984a). In the same study, the unsaturated metabolites of VPA were also found to conjugate with glycine at greater quantities than VPA. Furthermore, when 4-ene VPA was administered to Rhesus monkeys, the glycine conjugate of (*E*)-2,4-diene VPA was found in relatively large amounts (Rettenmeier, 1986a). This indicates that glycine conjugation plays a more important role in the biotransformation of the unsaturated metabolites of VPA than the parent drug itself. So

far, no further attempts have been made to identify the glycine conjugate of VPA or (*E*)-2,4-diene VPA in humans.

1.1.7 Analytical techniques

1.1.7.1 GC/MS

Over the last decade, this laboratory has employed and refined several GC/MS NICI and EI assays for the identification and quantitation of VPA and its metabolites in various biological fluids (Acheampong *et al.,* 1983; Abbott *et al.,* 1986; Kassahun *et al.,* 1989, 1990, 1991, 1993; Yu *et al.,* 1993). The development of the NICI technique was initiated in our laboratory to investigate its suitability to identify and quantitate the pentafluorobenzyl (PFB) derivatives of VPA and metabolites (Kassahun *et al.,* 1989). This approach is routinely used for the identification and assay of fatty acids and prostaglandins (Min *et al.,* 1980; Strife *et al.,* 1984; Penttilä *et al,* 1985; Schweer *et al.,* 1985; Hughes H, 1988; van Rollins, 1995)

While the analysis of VPA and metabolites can be performed in both EI and NICI modes using different derivatization techniques, the NICI technique appears to have several advantages. First, the enhanced sensitivity allows for the facile detection of minor but significant metabolites such as 4-ene VPA in human samples. Second, the combination of PFB derivatization, the use of nonpolar columns and NICI detection allow for the complete separation of VPA from all its metabolites. Third, the PFB derivatization of VPA metabolites does not produce multiple products, complicating the analysis.

One drawback to the utilization of NICI methodology for the detection of VPA metabolites is the lengthy procedure which sometimes requires more than one derivatization step. In contrast, the EI methodology for the analysis of VPA metabolites only requires one derivatization step. Both ionization modes have been used quite efficiently for the structural elucidation of unknown metabolites of VPA. The analysis of NAC I has been achieved in our laboratory by GC/MS NICI (Kassahun *et al.*, 1991).

1.1.7.2 LC/MS/MS

The recent introduction of LC/MS/MS in our facility has shown very good promise as a tool for the analysis of phase II conjugates of VPA. The latter are difficult to analyze by GC/MS but analysis is facilitated by the combination of HPLC and MS/MS detection either under atmospheric chemical ionization (APCI) or electrospray (ES) conditions. The technique is highly selective and does not require the use of derivatization. LC/MS/MS enables detection through a number of experiments such as parent or product ion scanning, neutral loss scanning, multiple reaction monitoring (MRM) and selected reaction monitoring (SRM). The methodology has successfully been employed for the structural elucidation of thiol conjugates of VPA and isocyanates (Murphy *et al.*, 1992; Baillie and Davis, 1993; Borel *et al.*, 1993; Tang *et al.*, 1996c, 1997).

1.2 Rationale and objectives

1.2.1 Identification, characterization and profiling of thiol conjugates arising from VPA biotransformation

The histological observations of liver injury associated with VPA-induced hepatotoxicity, toxicological studies of 4-ene and (E)-2,4-diene VPA and the results of varied mechanistic studies of VPA-induced hepatotoxicity have implicated 4-ene, and more specifically (E)-2,4-diene VPA, as the putative metabolite involved in the life-threatening side effect both in humans and animals. However, numerous attempts to correlate the serum levels or urinary excretion of the two metabolites with the risk factors associated with the disease have been inconclusive or have failed.

The hypothesis of this thesis is that the observed liver injury is in part due to the ongoing metabolism of these unsaturated compounds. Therefore, it is believed that the metabolic profiling of NAC I and its isomer will be better indicators of VPA-induced hepatotoxicity. Thus, increased amounts of thiol conjugates of (E)-2,4-diene VPA in a polytherapy group of patients would mean a greater exposure of these patients to the reactive (E)-2,4-diene VPA. If this event occurs in mitochondria, these subjects should be more liable to hepatic injury and that may account for the increased incidence of VPA- associated liver failure in patients on polytherapy. Similarly, other thiol conjugates formed from the 4-ene pathway could contribute to the liver injury and should also be monitored.

1.2.2   Identification, characterization and profiling of AA conjugates of VPA biotransformation in humans

The investigation of AA conjugates of VPA in humans is based on the following facts:

(1) Urinary recovery of VPA and its metabolites accounts for about 85 % of a dose of VPA in humans (Baillie and Scheffner, 1995), suggesting that the parent drug may undergo further metabolism. The discovery of new AA conjugates of VPA would provide a more complete picture of its metabolism.

(2) The mechanism of VPA-induced hepatotoxicity is not established in humans but it is believed to be mediated by reactive metabolites. The identification and profiling of AA conjugates of VPA could provide valuable information in that regard.

(3) The mechanism of action of VPA is not known and VPA metabolites could be involved. Recently, researchers have reported that derivatives of VPA GLY appear to have potential as new antiepileptic or CNS drugs (Blotnik *et al.*, 1997). This provides a rationale to investigate the formation of AA conjugates in the CSF of humans for similar reasons.   So far, metabolites of VPA identified in human CSF have been found to be potential antiepileptic drugs (Abbott and Acheampong, 1988; Lösher, 1992). Identification of AA conjugates in human CSF could provide an explanation for the antiepileptic activity of VPA or lead to the development of new antiepileptic or CNS drugs.

1.3   Specific Objectives

1.3.1 NAC conjugates arising from VPA biotransformation

(a) Search for NAC I, NAC II, and NAC III in urinary samples of patients on VPA.

(b) Characterize the structure of NAC II in urinary samples of patients on VPA.

(c) Develop and validate GC/MS NICI and LC/MS/MS assays for the profiling of the identified NAC conjugates in urinary samples of patients on VPA.

(d) Perform a statistical comparison of the urinary levels of NAC conjugates in patients on monotherapy to those on polytherapy using the Mann-Whitney test.

1.3.2. Phase I metabolites arising from VPA biotransformation

(a) Modify the current GC/MS NICI assays for the metabolic profiling of all phase I metabolites in urine samples of patients on VPA.

(b) Determine the concentrations of free and total level 4-ene VPA and (E)-2,4-diene VPA in urine samples of patients on VPA.

(c) Compare the concentrations of free and total 4-ene and (E)-2,4-diene VPA in urine samples of patients on VPA monotherapy to those on polytherapy.

1.3.3 AA conjugates arising from VPA biotransformation in humans and animals

(a) Identify VPA AA conjugates in the urine, serum and CSF samples of patients on VPA using GC/MS and LC/MS/MS.

(b) Develop and validate assays to profile VPA AA in the urine, serum and CSF samples of patients on VPA

(c) Identify and profile VPA AA conjugates in biological fluids of rats dosed with VPA

(d) Identify and profile VPA AA conjugates in biological fluids of rabbits dosed with VPA

2. EXPERIMENTAL

2.1 Materials

2.1.1 Chemicals and materials obtained from outside sources

α-Bromo-2,3,4,5,6-pentafluorotoluene (PFBBr), diisopropylethylamine (DIPEA), and valproic acid (VPA) were purchased from Aldrich Chemical Co. (Milwaukee, WI).

Solid phase extraction cartridges were obtained from C.J.T. Baker Inc. (Phillipsburg, New Jersey). Bond Elut Certify (type LRC) solid phase extraction cartridges were purchased from Varian (Harbor city, CA).

Distilled in glass grade solvents (ethyl acetate, methanol, acetonitrile, hexane, dichloromethane, acetone) and chemicals (anhydrous sodium sulfate, sodium di-hydrogen orthophosphate, sodium hydroxide, hydrochloric acid, propionic acid) were purchased from BDH Chemicals (Toronto, Ontario) or Caledon Laboratories Ltd (Edmonton, Alta).

Polyethylene tubing PE-10 was purchased from Clay Adams (Parsippany, NJ).

Ammonium chloride and propionic acid were purchased from Fisher Scientific Co. (Fairlawn, New Jersey).

Acrodisc LC 13 PVDF (0.2 um) syringe filters were purchased from German Sciences Co, (Ann Arbor, MI).

N-Trimethylsilyl-*N*-methyltrifluoroacetamide (MSTFA), *N*-*tert*-butyldimethylsilyl-*N*-methyl-trifluoroacetamide (MTBSTFA) were purchased from Pierce Chemical Co (Rockford, IL). Trifluoroacetic acid was purchased from Sigma Chemical Co. (St. Louis, MO).

2.1.2 Chromatographic columns

Columns: DB101, (30 m x 0.32 mm (I.D.); film thickness 0.25 μm) and DB101, (30 m x 0.32 mm (I.D.); film thickness 0.25 μm) were purchased from J & W scientific (Folsom, CA); HP Ultra I, (12 m x 0.2 mm (I.D); film thickness 0.33 μm) was purchased from Hewlett Packard (Avondale, PA); C_8 column (100 mm x 2.1 mm, 5 μm) was purchased from Phenomenex (Torrance, CA); C_{18} column (25 cm x 4.6 mm x 5 μm) was purchased from Beckman (San Ramon, CA) and ODS column (250 x 4 mm, 5 μm) was purchased from Hewlett Packard (Avondale, PA).

2.1.3 Compounds synthesized in our laboratory

NAC I and the methyl ester of the NAC conjugate of methyl acrylate (di-methyl ester of NAC VI) were synthesized by Kassahun *et al.*, 1990 and Tang *et al.*, 1995; 5-cys-4-hydroxy VPA lactone (NAC III) was synthesized by Tang et al.,1996c.

α-Fluoro valproyl glutamine (FVPA GLN), valproyl glutamine (VPA GLN), valproyl glutamate (VPA GLU), valproyl glycine (VPA GLY), and valproyl aspartate (VPA ASP)

were all synthesized for the purpose of this study by Dr. Wei Tang following the procedure described for VPA GLN (Tang *et al.*, 1997a).

2-Propyl-2-pentenoic acid ((*E*)-2-ene VPA), 2-propyl-4-pentenoic acid (4-ene VPA) and (*E*)-2-propyl-2,4-pentadienoic acid ((*E*)-2,4-diene VPA), 2-propyl-3-oxopentanoic acid (3-keto VPA) were synthesized according to Acheampong *et al.*, 1983; Lee *et al.*, 1989; Palaty (Ph.D. thesis), 1995. [^{7}H$_2$] VPA and [^{7}H$_2$] 3-keto VPA were synthesized according to Zheng (M.Sc. thesis, 1993).

2.2 Instrumentation and Analytical Methods

2.2.1   Direct probe MS

Direct probe MS was performed on a Nermag R 1010-10C mass spectrometer in the Department of Chemistry, University of British Columbia.

Analysis was performed by desorption chemical ionization (DCI). The reagent gas used to induce chemical ionization was NH$_3$. The ion source was kept at 200° C. Samples were volatilized by ramping a current across a tungsten filament at 1 mamp/sec.

2.2.2 NMR spectroscopy

NMR spectra were obtained on Bruker WH-200 or Bruker WH-400, or Varian XL-300 (300 MHz) spectrometers in the Department of Chemistry, University of British Columbia.

2.2.3 LC/MS Thermospray (TSP)

TSP LC/MS analysis was performed on a Hewlett Packard (HP) 1090 series II liquid chomatograph (LC) coupled to a 5989 A HP mass spectrometer via a TSP interface operated under negative and positive TSP and by employing a direct injection technique. The interface settings were stem temperature 92°C, and the source temperature was kept at 225°C.

2.2.4. HPLC methods

Preliminary analysis of some compounds was performed on a HP 1050 liquid chomatograph equipped with a UV detector and the wavelength was set at 254 nm.

2.2.4.1 HPLC/UV method A

Column: HP ODS, 250 x 4 mm, 5 μm. The LC conditions: the mobile phase was composed of 0.01 M NaH_2PO_4 (A) and acetonitrile (B) delivered at 1.2 mL/min according to the following program: At time 0, the mobile phase was set at 5% B which increased to 25 % B at 10 min and held for 5 min. The UV detector was set at 254 nm.

This method was used for the analysis of the mixed products obtained from the synthesis described in scheme 3.

2.2.4.2 HPLC/UV method B

Column: Beckman C_{18} (25 cm x 4.6 mm x 5 μm). The LC conditions: The mobile phase was composed of methanol:water (43:57, 0.05 % TFA) delivered at a flow rate of 0.5 mL/min isocratically. The UV detector was set at 254 nm. This method was used to isolate, purify and separate the isomers of NAC I and NAC II from each other.

2.2.5. LC/MS/MS methods

Experiments were carried out on a Fisons VG Quattro triple quadrupole mass spectrometer (Fisons Instruments, Altrincham, England) interfaced to a HP 1090 II LC.

2.2.5.1. General approach

The HPLC conditions for both classes of metabolites (thiol and AA conjugates of VPA) were similar to each other. All experiments were conducted on a Phenomenex C_8 column (100 mm x 2.1 mm, 5 μm) operating at a flow rate of 0.1 mL/min. The two mobile phase systems used for LC/MS/MS experiments consisted of either methanol/water or acetonitrile/water. Ionization of molecules was facilitated by the addition of trifluoroacetic acid (TFA) or a combination of propionic acid (PPA) and TFA to improve sensitivity. The analyses of the conjugates of VPA were conducted using several HPLC mobile phase systems pumped at various time programs and flow rates.

The LC conditions and the MS/MS conditions for the various methods are described below.

2.2.5.2 LC/MS/MS method A

LC conditions: Mobile phase A consisted of methanol/water (43:57, 0.025 % of TFA and PPA each) and mobile phase B consisted of methanol. At time 0, mobile phase A was pumped isocratically at a flow rate of 0.1 mL/min for 30 min. Mobile phase B gradiently increased from 0 % to 100 % at a rate of 0.1 mL/min at time 30.1 min and held for 5 minutes, followed by a sharp gradient increase of mobile phase A to 100 % at time 35.1 min and at 0.1 mL/min. This method was employed for the identification, characterization and profiling of thiol conjugates of VPA in humans. It was also the first method used for the identification of amino acid conjugates of VPA in human urine.

2.2.5.3 LC/MS/MS method B

LC conditions: Mobile phase A consisted of acetonitrile/water (30:70, 0.05 % TFA) and mobile phase B consisted of acetonitrile. Mobile phase A was pumped at a flow of 0.1 mL/min for 25 min followed by a gradient increase of mobile phase B to 100% pumped at a rate of 0.1 mL/min at time 26 min, held for 5 min and reverted to 100 % A at 0.1 mL/min at time 30.1 min. This method was used to detect the amino acid conjugates of VPA in urinary matrices where a longer run time program was required to separate the peaks of interest from interfering chomatographic peaks.

2.2.5.4 LC/MS/MS method C

LC conditions: Mobile phase A consisted of acetonitrile/water (40:60, 0.05% TFA). A gradient program allowed 100 % of mobile phase A to be pumped for 14 min at a flow rate of 0.1 mL/min. Mobile phase B was then increased to 100 % at a flow rate of 0.2 mL/min at 15 min and held for 5 min at 0.2 mL/min. Subsequently, mobile phase A was increased to 100 % at time 20 min at 0.1 mL/min. This method was utilized for the identification and profiling of amino acid conjugates in human samples and animal samples.

2.2.5.5 ES$^+$ MS/MS conditions

The ionization energy of choice was positive electrospray although negative electrospray was also investigated. The MS/MS settings for the detection of various analytes were a modification of each other and were optimized for sensitivity. The HPLC eluent was introduced to the stainless steel capillary sprayer held at 3.5 kV. The mass spectrometer was operated for the purpose of precursor (parent) ion scanning, product (daughter) ion scanning or MRM. Multipliers 1 and 2 were set at 650 V for all thee experiments, cone voltage was set at 22 kV for the thiol conjugates of VPA and at 30 kV for the AA conjugates of VPA with skimmer offset by 5 V for both groups of metabolites. The low and high mass resolution were set at 12.5 for both precursor ion scanning and product ion scanning and at 5 for MRM. The precursor ion scanning determined the pseudomolecular ion or the precursor ion (MH$^+$) of the metabolite of interest, the product ion scanning determined the product ions of MH$^+$ using argon as the target gas for collision-induced dissociation (CID) and with collision energy typically

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set at 40 eV. During MRM, the pseudomolecular ion for each analyte was selected in the first quadrupole and subjected to CID with target gas argon set at a pressure of 3.0 x 10 $^{-4}$ mbar and collision energy was set at 50 eV to improve sensitivity. Specific fragment ions were then selected by the third quadrupole. For all the experiments, the source temperature was at 140°C.

Data analyses were processed using Mass Lynx® software (Fisons, Altrincham, England).

2.2.6. GC/MS methods

The GC/MS was a HP 5890 II GC coupled to a HP 5989 A mass spectrometer operating under both EI and NICI.

Various GC/MS methods were employed either for the identification, characterization or the profiling of the various conjugates both in humans and animals.

2.2.6.1 GC Method A

Column: J & W scientific DB101, (30 m x 0.32 mm (I.D.); film thickness 0.25 μm).

Oven temperature program: 50-150°C at 20°/min, held for 5 min; 150-300°C at 20°/min and held for 10 min.

Other conditions: The injection port was set at 240°C, the source temperature was at 200°C, the detector temperature was at 280°C and He was set at 10 psi. This method

was used initially for the identification and characterization of thiol conjugates of VPA and to develop the assay for the profiling of thiol conjugates of VPA. However, the method could not separate the epimers of NAC III.

2.2.6.2 GC Method B

Column: J & W scientific DB1701, (30 m x 0.32 mm (I.D.) with film thickness 0.25 μm).

Oven temperature program: 50-150°C at 20°/min, held for 5 min; 150-300°C at 10°/min and held for 5 min.

Other conditions: The injection port was 240°C, the source temperature was at 200°C, the detector temperature was at 280°C and He was set at 10 psi. This method was used for the identification, characterization and profiling of thiol conjugates of VPA in humans.

2.2.6.3. GC Method C

Column: HP Ultra I, (12 m x 0.2 mm (I.D) with film thickness 0.33 μm). Oven temperature program: 50°-200°C at 30°/min, held for 5 min and at 10°/min until 300°C.

Other conditions: The injection port temperature was at 300°C, the detector temperature was at 300 °C and He at 3 psi. This method was the first method employed for the characterization of NAC II.

2.2.6.4. GC Method D

Column: J & W Scientific DB1701 (30 m x 0.32 mm (I.D.) with film thickness 0.25 μm).

Oven temperature program: 50-150°C at 20°/ min, held for 5 min; 150-300°C at 20°/min and held for 20 min.

Other conditions: The injection port was 240°C, the source temperature was at 200°C, the detector temperature was at 280°C and He was set at 10 psi. This method was used for the identification, characterization of thiol conjugates in urine samples of humans under EI conditions.

2.2.6.5 GC Method E

Column: J & W Scientific DB1701, (30 x 0.32 mm (I.D.) with film thickness 0.25 μm).

Oven temperature: 50-150°C at 20°/min, held for 5 min; 150-300 at 5°/min and held for 20 min.

Other conditions: The ion source was at 200°C, the detector temperature was at 280 °C, and the injection port temperature was at 240°C and He was set at 10 psi. This method was used for the characterization of thiol conjugates by a slow oven temperature program under EI conditions.

2.2.6.6 GC Method F

Column: J & W Scientific DB1701, (30 x 0.32 mm (I.D.); film thickness 0.25 μm). Oven temperature: 100-200°C at 20°/min, held for 5 min; 150-300 at 15°/min and held for 5 min.

Other conditions: The ion source was at 200°C, the detector temperature was at 280°C; the injection port temperature was at 240°C and He was set at 10 psi. This method was used for the characterization of AA conjugates.

2.2.6.7 GC Method G

Column: J & W Scientific DB101, (30 x 0.32 mm (I.D.); film thickness 0.25 μm). Oven temperature: 50-140°C at 30°/min, held for 20 min; 140-240 at 30°/min and 240-300 at 10°/min and held for 10 min.   This method was used for the profiling of phase I metabolites.

Other conditions: The ion source was at 200°C, the detector temperature was at 280°C; the injection port temperature was at 240°C, and He was set at 10 psi.

2.2.6.8 MS conditions (scan and SIM modes) for GC methods

2.2.6.8.1 NICI analysis

The ion source temperature was kept at 200°C; and the interface was at 280°C. The reagent gas, methane, was maintained at 1 torr. The emission current was at 300 μA

and the ionization energy was 120 eV. For the purpose of identification and characterization of thiol and AA conjugates of VPA, we utilized both the scan mode and the selected ion monitoring (SIM) mode. On the other hand, the profiling of the derivatized thiol conjugates was conducted solely by SIM mode and based on the method employed previously in our laboratory for the analysis of NAC I (Kassahun *et al.*, 1990). The ions which correspond to the [M-181]$^-$ fragments of the PFB derivative of NAC I, NAC II (*m/z* 482), III (*m/z* 302) and that of the internal standard (I.S.), NAC VI, (*m/z* 414), respectively, were monitored for that purpose. For the analysis of 4-ene VPA, 2-ene VPA, (*E*)-2,4-diene VPA and 3-keto VPA we monitored *m/z* 144, 141, 139, 229, respectively, as described previously (Kassahun *et al.*, 1989). VPA and the I.S. [^{2}H$_7$] VPA were monitored by their respective isotopic ions [M-181+1]$^-$, *m/z* 144 and 151, to accommodate for their relatively higher concentration difference in comparison to the metabolites of VPA (Darius and Mayer, 1994). The ion monitored for the I.S. [^{2}H$_7$] 3-keto-VPA was 238 (Zheng, M.Sc. thesis, 1993).

2.2.6.8.2. EI analysis

The ion source temperature was at 200°C, the detector temperature was at 280°C, the emission current was at 300 μA and the ionization energy was at 70 eV. All analyses under EI were performed using full scan mode.

2.2.7 General approach for the identification and characterization of conjugates by GC/MS and LC/MS/MS

Prior to the identification process, the chomatographic and spectral characteristics of the standard reference sample were first determined. The results were then compared to the chomatographic and spectroscopic characteristics of the compounds in the biological fluid being studied or its extract.

2.2.7.1 GC/MS characterization of reference samples for the identification and profiling of experiments

The standard samples were derivatized for NICI or for EI analysis. The retention times of the derivatized compounds and their corresponding mass spectra were then compared to those observed in derivatized extracts of biological fluids of humans and animals under scanning mode. In some cases, identification could only be performed under SIM.

2.2.7.2 LC/MS/MS characterization of reference samples for the identification and profiling experiments

During the LC/MS/MS experiments, the HPLC methods needed to be modified to accommodate the chomatographic challenges associated with the analytes themselves or the matrices from which the analytes were extracted.

We directed our experiments using two approaches: (1) to confirm novel metabolites and new metabolites already elucidated by GC/MS such as the thiol conjugates and (2) to elucidate novel metabolites such as the AA conjugates which have not been studied by GC/MS.

Initial experiments were conducted to perform a full ES^+ scan to determine precursor ions. The latter were further fragmented under CID to obtain full product ion spectra. Elucidation of unknown metabolites was first conducted by comparing retention times and product ion spectra of synthetic reference samples to those observed from biological fluids of humans and animals or their corresponding extracts. When full product spectra could not be obtained, identification of metabolites was performed by comparing retention times and area ratio (i.e ratio of abundance of peaks at all transitions being monitored) of several characteristic product ions of the reference samples to those observed in biological fluids or their extracts.

2.2.8 Characterization of the synthetic mixture of NAC I and NAC II as obtained from the synthetic process described in scheme 3

2.2.8.1   Formation of the synthetic mixture

The ethyl methyl ester of NAC I was synthesized in our laboratory by Dr. A. Mutlib according to the procedure of Kassahun et al., 1991. The identity of the resulting product, (E)-5-NAC-3-ene VPA ethyl methyl ester, was confirmed by ^{1}H NMR. The di-ester was hydrolyzed under basic and reflux conditions resulting in a mixture of

products. The synthetic pathway followed by Dr. Mutlib is summarized in scheme 3. During the course of this project, we directed our efforts to identify the compounds in the resulting hydrolyzed mixture though a variety of spectroscopic and chomatographic means.

2.2.8.2 Analysis of the synthetic mixture of thiol conjugates

The resulting mixture obtained from scheme 3 was a yellow oily liquid which was subjected to a series of analyses to determine its characteristics.

2.2.8.2.1 Preliminary analysis of the synthetic mixture of thiol conjugates

The resulting mixture of products from scheme 3 was first analyzed by HPLC/UV as described in section 2.2.4.1, and direct probe MS analysis and LC/MS TSP as described in sections 2.2.1 and 2.2.3, respectively .
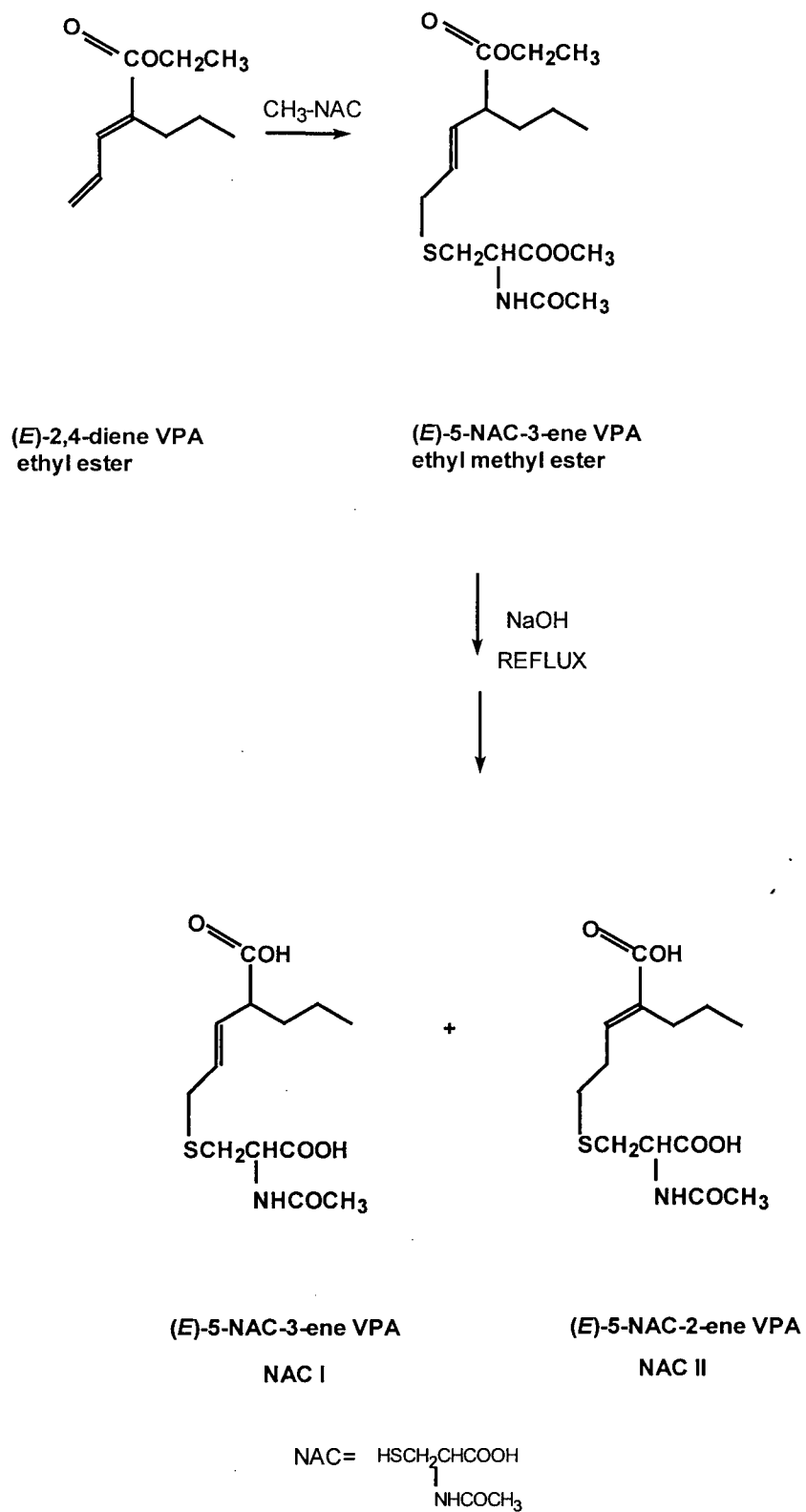
Scheme 3. Synthetic pathway leading to the formation of a mixture of NAC I and NAC II.

2.2.8.2.2 ^{1}H NMR analysis of the synthetic mixture of thiol conjugates

A solution of the compound (dissolved in CD$_3$OD) was submitted to the Department of Chemistry for ^{1}H NMR analysis.

2.2.8.2.3 Derivatization of mixture of thiol conjugates NAC I and II for GC/MS analysis

A solution of the mixture (0.25 μg/mL) was made in EtOAc. About 100 μL of the solution was derivatized with 10 μl of 40% PFBBr and 10 μL of DIPEA at 40°C and 1 μL of the solution was injected in the GC/MS system for NICI analysis according to GC/MS methods A, B and C. About 10 μL of the solution was also derivatized with 50 μL of MTBSTFA at 60°C and 1 μL of the solution was injected in the GC/MS system for EI analysis according to GC/MS methods D, and E. The derivatizations procedures described in this section were also employed for all GC/MS analysis.

2.2.8.3 Purification and isolation of NAC I and NAC II from the synthetic mixture

About half (~500 mg) of our synthetic product was dissolved in methanol and the isomers of NAC I and NAC II were isolated and purified according to the HPLC/UV method B described in section 2.2.4.2

2.2.9 Characterization of the isolated NAC II and the isomers of NAC I

The HPLC purified samples were each dissolved in D_2O and submitted for ^1H NMR analysis.

The purified compounds were each dissolved in water to make a stock solution of 1 mg/mL. A small aliquot of the stock solution was mixed in EtOAc to make a solution of 0.25 μg/mL and dried over anhydrous Na_2SO_4. About 100 μL of the solution was then derivatized with either PFBBr or MTBSTFA for GC/MS analysis under NICI or EI as described for the mixture.

2.2.10 Characterization of NAC III by GC/MS

A pure sample of NAC III was dissolved in water to prepare a 0.5 mg/mL solution. An aliquot of the stock solution was diluted with EtOAc to make a working solution of 0.25 μg/mL and dried under Na_2SO_4. About 100 μl of the solution was then derivatized with PFBBr in the presence of DIPEA and analyzed according to GC/MS method A, B, and D.

2.2.11 Characterization of NAC I, NAC II and NAC III by LC/MS/MS

The thee compounds were dissolved in water to form a solution of concentration equal to 0.25 μg/mL for each compound and 25 μL of the solution was injected onto the column. The compounds were analyzed according to LC/MS/MS method A. For each compound, a precursor ion spectrum and a product ion spectrum were obtained.

2.3 Human studies

2.3.1 Study groups

All patients involved in our study were being treated for epilepsy at the Seizure Clinic of British Columbia's Children's' Hospital, Vancouver, B.C., Canada. The diagnosis of each patient was either established or reviewed by the collaborating neurologist, Kevin Farrell (M.D., F.R.C.P.) who also assessed or reviewed all liver function tests and all other biochemical testing relevant to our studies. All human samples (serum, urine, CSF) were obtained from pediatric patients, by the research nurse and supervised by the research associate under the management of Dr. Farrell. Approval for all human studies was issued to Dr. K. Farrell by the U.B.C. Human Ethics Committee in 1995 (U.B.C. # C95-0412)

All patients involved in this study were on VPA and had reached steady state. Urine and serum samples were collected from patients before their morning dose of VPA. Samples were handed to us by the research associate and upon arrival at the laboratory were immediately assigned an identification number (UBC I.D #) in order of arrival. During our study, all samples were studied under their I.D. number. After analysis and treatment of data, the results were then studied in full knowledge of corresponding clinical information. In other words, full effort was made to remain blind to the identity of samples during analysis and initial data treatment.

The various seizure types associated with the patients in the study are listed in Appendix a. The relevant clinical information of the patients including the type of VPA

therapy (monotherapy versus polytherapy) prescribed for them is given in Appendix b. Patients in Appendix b are sorted in ascending order according to their age.

2.3.1.1 Human control samples

Control blood and urine samples were obtained from healthy volunteers from the university of British Columbia. Control CSF samples were obtained form healthy volunteers and from patients suffering from multiple sclerosis.

2.3.2 Identification of NAC I , NAC II and NAC III conjugates in human urine

2.3.2.1 GC/MS experiment

One mL of urine sample (n=39) was treated according to scheme 4 and derivatized with PFBBr for NICI analysis (GC/MS method A, B, C) and EI analysis (GC/MS methods D, E).

For comparison, derivatized reference samples of NAC I, NAC II, and NAC III were also analyzed under identical GC/MS experimental conditions.

2.3.2.2 LC/MS/MS experiment

For each patient (n=34), a one mL of urine sample and a one mL of control urine sample were separately subjected to solid phase extraction according to scheme 5. The extracts were each dissolved in mobile phase and analyzed by LC/MS/MS method

A using MRM. A sample of the reference standard, dissolved in mobile phase, was analyzed at the same time under the same experimental conditions.

Scheme 4. Liquid/Liquid extraction procedure for the identification and profiling of thiol conjugates of VPA in urine samples for GC/MS NICI and EI

Urine sample (1mL)

↓

Adjust to pH 3-4 with H_3PO_4

↓

Add 1 gm of NH_4Cl

↓

Extract with 5 mL EtOAc (X2)

↓

Combine and concentrate organic layers to approximately 1 mL under N_2

↓

Dry over Na_2SO_4

↓

Derivatize with PFBBr (10 μL of 40% solution)/DIPEA (10 μL) for NICI analysis

OR

Derivatize with MTBSTFA (50 μL) for EI analysis
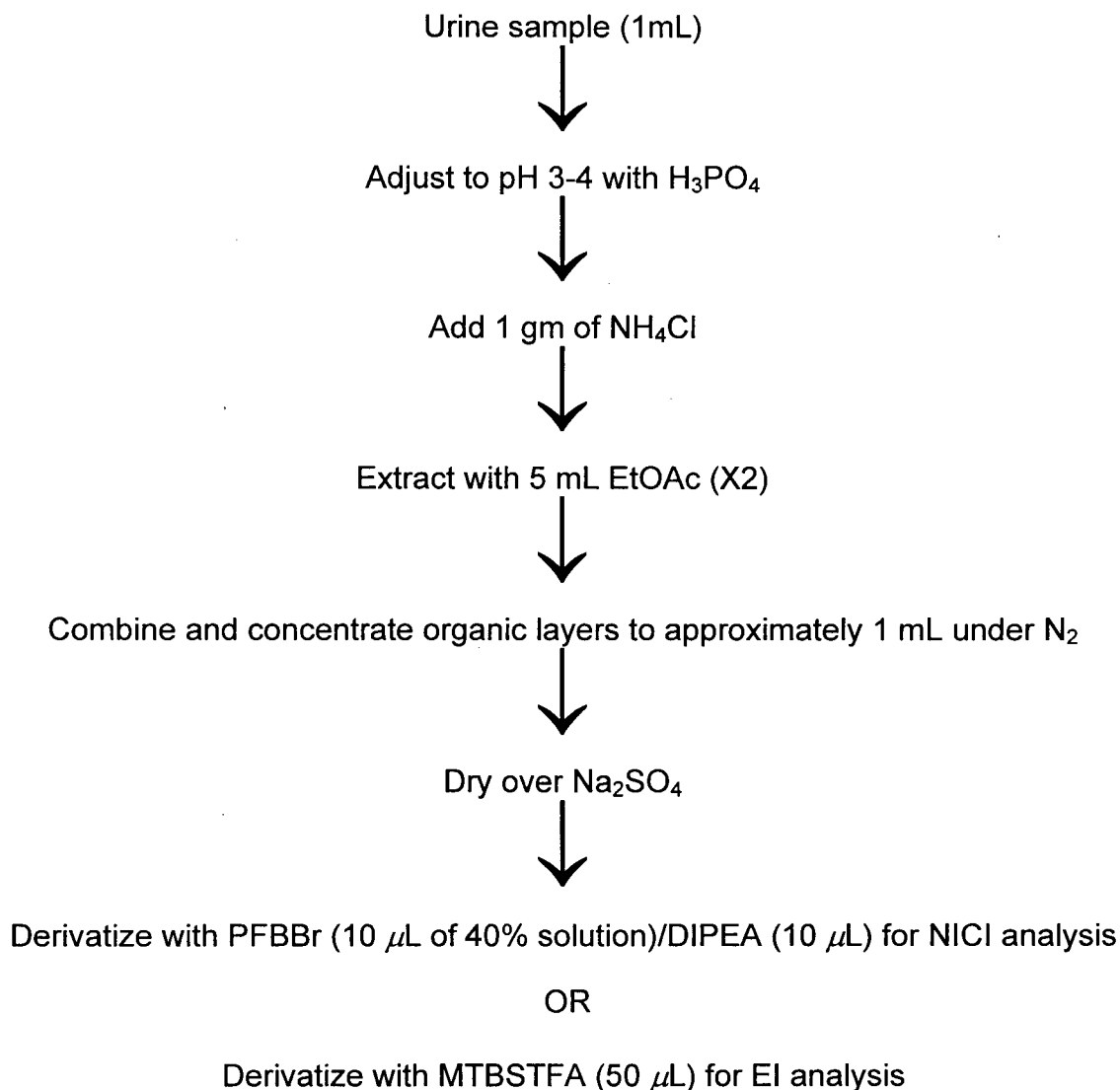
* Extraction was conducted at pH 3-4 to avoid hydrolysis of conjugates at lower pH.

Scheme 5. Solid phase extraction for the identification and profiling of thiol and amino acid conjugates of VPA in urine by LC/MS/MS

C_2 cartridges (Varian 500 mg)

↓

Conditioned with 3 mL MeOH, 3 mL H_2O

↓

Apply 1 mL urine (pH 3-4) to cartridge

↓

Wash with 3 mL H_2O:MeOH (95:5), 3 ml H_2O

↓

Elute analytes with 3 mL MeOH (x2)

↓

Combine and dry MeOH layers under N_2

↓

Reconstitute in 1 mL mobile phase (thiol conjugates) or 1 mL water (AA conjugates)
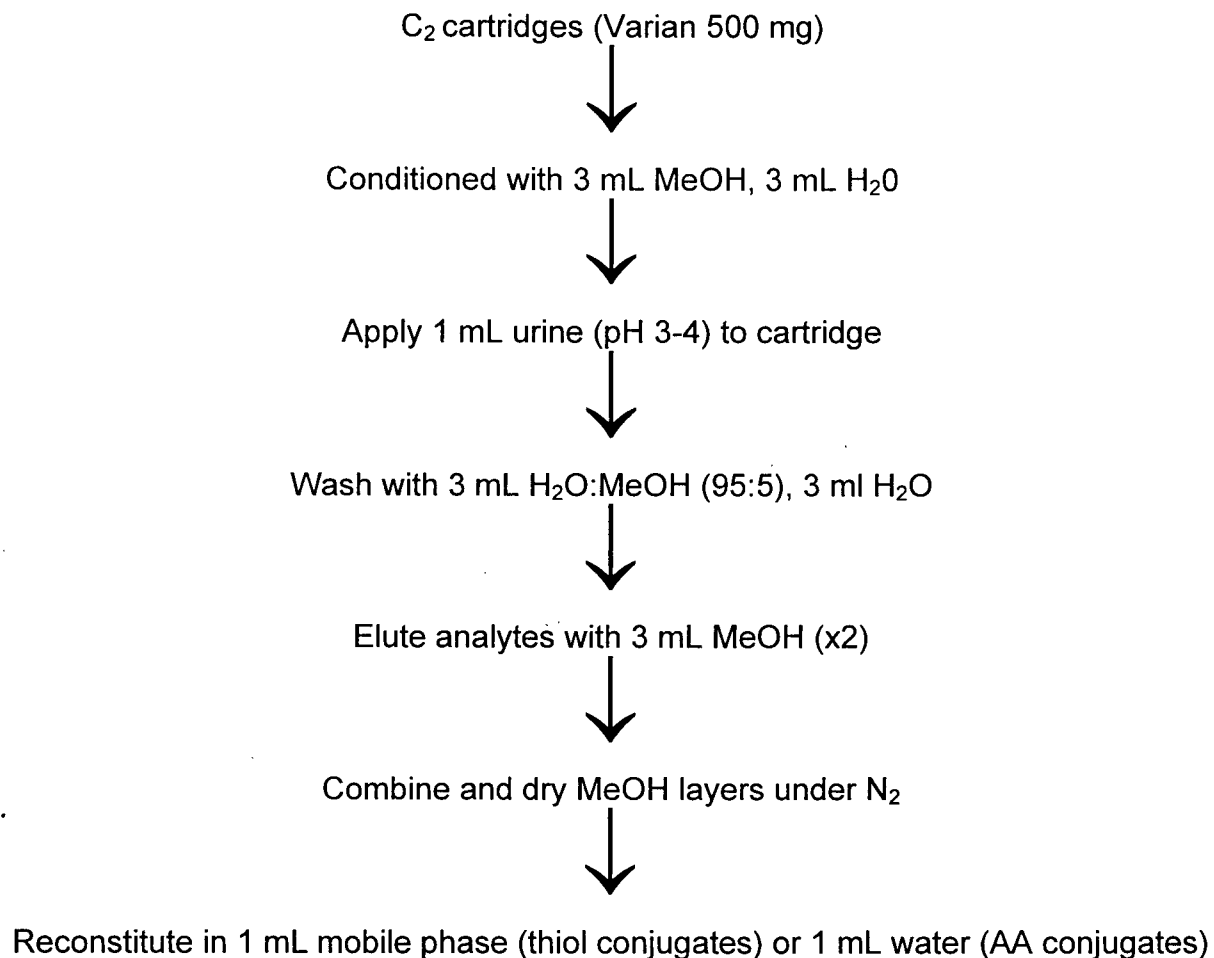
* Extraction was conducted at pH 3-4 to avoid the hydrolysis of conjugates at lower pH.

2.3.3. Identification of AA conjugates of VPA in the human

2.3.3.1 AA conjugates in the urine of patients on VPA therapy

A one mL of sample from each patient on VPA (n=29) was extracted according to scheme 5. The extract was dissolved in water and analyzed by LC/MS/MS methods A and B using MRM, product ion scanning. Reference samples of each amino acid conjugate dissolved in water were analyzed under the same experimental conditions.

2.3.3.2 AA conjugates of VPA in the serum of patients on VPA therapy

For each patient (n=6) on VPA, a two mL serum sample and a two mL of control serum sample were each adjusted to pH 3-4 with 3N HCl and each extracted with 5 mL of EtOAc (x2). The organic layers were dried and reconstituted in 200 μL of water and filtered though a membrane filter (0.2 μm, Acrodisc LC 13 PVDF, Gelman Sciences, Ann Arbor, MI, USA) and analyzed by LC/MS/MS method C. For comparison, reference samples were extracted from spiked blank serum samples, filtered and were analyzed under the same experimental conditions. Reference samples were also analyzed as unextracted and unfiltered compounds.

2.3.3.3 Identification of AA conjugates in the CSF of a human

2.3.3.3.1 Identification of AA conjugates of VPA in the CSF of a human by LC/MS/MS

A CSF sample of a patient on VPA, a blank CSF sample spiked with VPA GLU, VPA GLN, VPA GLY, VPA ASP and 10 control CSF samples (100 μL/sample) were filtered though a membrane filter as for the serum extract and analyzed under LC/MS/MS method C for VPA GLU, VPA GLN, VPA GLY, and VPA ASP.

2.3.3.3.2 Identification of AA conjugates of VPA in the CSF of a human by GC/MS

A CSF sample of a patient (50 μL) and a reference sample of VPA GLU (10 μL of 1 mg/mL) were subjected to solid phase extraction individually as described in scheme 5. The residue was dissolved in 500 μL of EtOAc and derivatized with PFBBr/DIPEA and analyzed according to scheme 4 and GC/MS method F. A non-extracted reference sample was derivatized with PFBBr/DIPEA and analyzed under the same analytical conditions as above.

2.3.4 Assay for the profiling of thiol conjugates in the urine of patients on VPA therapy by LC/MS/MS

2.3.4.1 Stock and working solutions of standards

FVPA GLN, synthesized in our laboratory, was chosen as the appropriate I.S. for this assay. The compound was dissolved in water to make a working solution of 0.08 mg/mL.

To prepare the standards, aliquots of the stock solution of NAC I, NAC II and NAC III were diluted with water to make working solutions of 0.1 mg/mL and 0.01 mg/mL.

2.3.4.2 Preparation of calibration curves

The calibration standards were prepared by adding appropriate amounts of working solutions to different test tubes to yield concentration standards of 0, 0.05, 0.1, 0.25, 0.50 and 1.0 μg/mL in 1 mL of control urine and were treated according to the extraction procedure described in scheme 5. Each extract was dissolved in 1 mL of mobile phase (MeOH:H$_2$O, 43:57, 0.05 % TFA and PPA) and analyzed by LC/MS/MS method A using the following MRM: m/z 304 >123, the characteristic transition for NAC I and NAC II, m/z 304 >130, the characteristic transition for NAC I, NAC II, and NAC III and m/z 291 > 130, the characteristic transition for FVPA GLN. The calibration curves were constructed by plotting area ratios of each analyte to the I.S for each concentration against the concentrations of the analyte.

2.3.4.3 Validation of LC/MS/MS assay for the thiol conjugates of VPA in urine samples of patients on VPA therapy

The validation results for NAC III are not described as it was not detected in any of the patients' samples treated.

2.3.4.3.1 Precision of the assay

The inter-assay variation was determined from the slopes of 10 calibration curves prepared and run on 10 consecutive analytical days and was expressed as the coefficient of variation (%). The inter-assay variation was further assessed by the triplicate runs of spiked samples at concentrations of 0.115, 0.22, 0.42, 0.5, and 0.80 μg/mL (expressed as % CV).

The intra-assay variation was assessed by three replicates of two spiked samples at concentrations of 0.115, and 0.80 μg/mL for both NAC I and NAC II and expressed as % CV.

2.3.4.3.2 Accuracy and recovery of the assay

The accuracy of the assay was assessed based on the concentrations of NAC I and NAC II observed for all spiked samples and expressed as a % of the expected values.

The recovery of the assay was determined by analyzing thee replicates of two samples at concentrations of 0.25, and 0.80 μg/mL which were extracted and compared to thee

replicates of two other samples at the same concentrations which were not extracted. Recovery was expressed as a % of the measured unextracted concentration.

2.3.4.4 Sample preparation for the quantitation of thiol conjugates by LC/MS/MS

A 1 mL aliquot of each overnight urine sample (n=34) and a set of calibration standards were extracted simultaneously as described in scheme 5 for the analysis of the conjugates by LC/MS/MS method A (section 2.2.5.2).

To determine the amount of the VPA dose recovered as thiol conjugates, 1 mL of either a 12 h or 24 h urine sample collected from four patients were also treated and analyzed for NAC I and II. The total amount of thiol conjugates was then calculated for the total volume of urine collected and the amount (%) of VPA recovered as thiol conjugates was calculated as shown in equation 1.

$$\% \text{ recovery of a VPA dose} = \frac{\text{Amount of thiol conjugates (moles) x 100}}{\text{Amount of VPA (moles)}} \qquad \text{equation 1}$$

2.3.5 Assay for the profiling of thiol conjugates in the urine samples of patients on VPA therapy by GC/MS

2.3.5.1 Stock and working solutions

To prepare the I.S, the dimethyl ester of NAC VI was synthesized by the method of Kassahun *et al.*, 1991. NAC VI was obtained by hydrolyzing the methyl NAC derivative of methyl acrylate in aqueous NaOH. NAC VI was confirmed by GC/MS.

Two working solutions of NAC I, NAC II, and III were prepared at concentrations of 0.1 mg/mL and 0.01 mg/mL and a working solution of NAC IV at 0.1 mg/mL was also prepared. These solutions were used to prepare calibration standards.

2.3.5.2 Preparation of calibration curves

The calibration curves were prepared by adding an appropriate amount of the working solutions in test tubes to yield standard concentrations of 0.05, 0.10, 0.25, 0.50, 0.75, 1.0, 2.5, 5.0 μg/mL of conjugates in 1 mL of control urine sample. Each tube was spiked with 20 μL of 0.1 mg/mL of NAC VI. The resulting mixture was then treated as described in scheme 4 for the liquid/liquid extraction and derivatization procedure for the thiol conjugates. The analysis was conducted by monitoring *m/z* 482 for NAC I and NAC II, *m/z* 302 for NAC III and, *m/z* 414 for NAC VI under SIM by GC/MS method B. The calibration curves were constructed by plotting the area ratios of each analyte to the I.S. for each concentration against the concentrations of the analyte.

2.3.5.3 Validation of GC/MS assay for the thiol conjugates of VPA in the urine samples of patients on VPA therapy

2.3.5.3.1 Precision of the assay

The inter-assay precision was determined from the variation observed for (1) the slopes of 8 calibration curves run on 8 consecutive analytical days and (2) the means of three or four replicates of four samples each spiked with NAC I and NAC II at concentrations equal to 0.115, 0.42, 0.80 and 2.50 μg/mL, respectively. The precision was expressed as the coefficient of variation (%).

The intra-assay precision was determined from the variation observed for (1) the slopes of one calibration curve run in four replicates, (2) triplicates of two samples at concentrations of 0.115 and 0.80 μg/mL, respectively, and was expressed as % CV.

2.3.5.3.2 Accuracy of the assay

The accuracy of the assay was assessed as the % of expected values observed for the spiked samples (n=4) described above.

2.3.5.3.3 Recovery of the assay

Thee replicates of 3 samples spiked with NAC I and NAC II to yield concentrations equivalent to 0.145, 1.50 and 3.5 μg/mL for NAC I and 0.435, 4.5 and 10 μg/mL for NAC

II were prepared. The samples were spiked with 20 μL of 0.1 mg/mL I.S. and subjected to the same extraction procedure as the calibration standards.

Three sets of control samples (1 mL each) were spiked with 20 μL of 0.1 mg/mL I.S. and extracted as for the calibration standards. The extracts were then spiked with the equivalent amount of NAC I and NAC II dissolved in EtOAc to yield concentrations equal to 0.15, 1.50 and 3.5 μg/mL for NAC I and 0.44, 4.50, and 10.00 μg/mL for NAC II analyzed in triplicates. They were unextracted samples.

All the extracts were made up to equal volume in 0.5 mL of EtOAc and derivatized with 10 μL of 40 % PFBBr in EtOAc and 10 μl of DIPEA. All the derivatized extracts were then analyzed using a calibration curve which was prepared simultaneously. Recovery of the assay was determined by the ratio of the concentration of the extracted samples to the unextracted concentrations calculated from the calibration curve and expressed as % recovery.

2.3.5.4   Sample preparation for the quantitation of thiol conjugates by GC/MS

The patients samples in our study were analyzed on several analytical days and in several batches. Each batch of sample was prepared with a set of calibration standards and quality control samples (spiked samples of known concentrations). A 1 mL sample from each patient specimen, calibration standards and quality control sample, were then extracted and derivatized as described for the preparation of calibration curves in section 2.3.5.2 and in scheme 4. All samples prepared on the same day were analyzed

in one batch by GC/MS method B monitoring for *m/z* 482, 302, and 414 as for the calibration standards.

To determine the recovery of VPA as the NAC conjugates in patients, 1 mL of the 12 h and 24 h urine samples from 4 patients collected immediately after their morning dose were also treated and analyzed. The amount of NAC conjugates recovered was calculated based on the volume of urine collected. The amount of VPA dose (%) recovered was calculated as described by equation 1 in section 2.3.4.4.

2.3.6 Determination of urinary creatinine levels

Urinary creatinine was determined in our laboratory using the routine Jaffe method (1886).

2.3.7 Statistical analysis of results for patients of all ages

(1) All urinary concentrations were normalized to their respective creatinine levels.

(2) Patients were divided into three categories:

    (a) Monotherapy: patients on VPA alone

    (b) Co-medication or non-enzyme-induced polytherapy: patients on VPA and non enzyme-inducing drugs such as lamotrigine, vigabatrin, nitrazepam, clobazam, clonazepam, ethosuximide, and flunarizine

    (c) Polytherapy: patients on VPA and enzyme-inducing drugs such as carbamazepine, phenytoin, and phenobarb, *as well as drugs that are not enzyme-inducers.*

(3) The means and ranges of NAC I and NAC II for each entire group were summarized.

(4) The mean ± SD of the entire group on monotherapy was then compared statistically to the mean ± SD of the co-medication group.

(5) Statistical analyses were performed using the Mann Whitney test for comparison of two means and one–way analysis of variance (Zar, 1984).   A value of $p < 0.05$ was considered significant thoughout.

2.3.8   Evaluating the effect of age on the excretion of NAC I and NAC II   in pediatric patients on VPA therapy

Each group was subdivided into two age categories:

    (a) Patients ≤ 7.5 years

    (b) Patients > 7.5 years.

The mean ± SD of each conjugate in each subgroup was determined and compared statistically within the same group and between the two groups.

2.3.9 Evaluating the effect of polytherapy on the excretion of NAC I and NAC II in different age groups

All patients on polytherapy were older than 7.5 years old. The range and mean ± SD of each conjugate for the polytherapy group was then compared to the range and mean ± SD of patients more than 7.5 years and less than or equal to 7.5 years in monotherapy and co-medication groups combined (non-induced polytherapy group).

2.3.10 Evaluating the effect of dose and age on the excretion of NAC I and NAC II

The dose for each patient was compared to the age of each patient and analyzed by simple linear regression.

2.3.11 Profiling of phase I metabolites of VPA in the urine samples of patients on VPA therapy

2.3.11.1 VPA and metabolites analyzed for this thesis

An assay has been modified and validated for the analysis of VPA and 14 of its metabolites in the serum of patients on VPA (Gopaul *et al.*, 1996b; Kassahun *et al.*, 1990). We provide below the details for the assay modified for the analysis of the same compounds in the urine of patients. For the purposes of this thesis, we focused on the analysis of VPA, 4-ene VPA and the β-oxidation metabolites (*E*)-2-ene VPA, 3-keto VPA and (*E*)-2,4-diene VPA.

2.3.11.2 Preparation of calibration curves

Calibration curves were prepared by spiking blank urine samples (100 μl) with VPA and metabolites to produce the following calibration ranges: 25-2500 μg/mL for VPA, 1.2-120 μg/mL for (*E*)-2-ene VPA, 0.025-2.5 μg/mL for 4-ene VPA, 0.3-30 μg/mL for (*E*)-2,4-diene VPA and 8-800 μg/mL for 3-keto VPA. The samples were spiked with the appropriate I.S. which included [^{2}H$_7$] VPA, and [^{2}H$_7$] 3-keto VPA.

2.3.11.3 Quantitating the total urinary levels of VPA and metabolites in patients on VPA therapy

Each calibration curve was composed of at least eight calibration standards which were prepared at the same time as 100 μL of each patient sample and control samples. Calibration standards, patients samples and control samples spiked with I.S were base-hydrolyzed with the appropriate amount of sodium hydroxide (pH 12-14) at 60° C for 1 h. The samples were adjusted to pH 2 with 3N HCl and extracted with 1 mL EtOAc (x2). The organic layers were combined and dried over anhydrous sodium sulphate. The organic phase was then derivatized with 20% PFBBr/DIPEA for 50 min at 40° C. The organic layer was dried gently under N_2 and reconstituted in 0.5 mL of water. The aqueous layer was extracted with 0.5 mL of hexane (x2) and the organic layers were again dried over anhydrous sodium sulfate and then derivatized with 100 μl of MSTFA. Then, 1μL of the resulting sample was analyzed by GC/MS method G.

2.3.12 Profiling of AA conjugates of VPA in the urine samples of patients on VPA therapy

2.3.12.1 Stock and working solutions

The internal standards chosen were VPA ASP (0.01 mg/mL) and FVPA GLN (0.01 mg/mL) dissolved in water. To prepare the standards, aliquots of a stock solution of 1 mg/mL of each VPA GLU, VPA GLN and VPA GLY were mixed and diluted to make working solutions at concentrations of 0.1 mg/mL and 0.01 mg/mL of all three compounds.

2.3.12.2 Preparation of calibration curves for urine analysis of AA conjugates

The calibration standards were prepared by spiking blank urine samples with the working solution of the thee AA conjugates to yield concentrations of 0, 0.05, 0.10, 0.25, 0.50, 1.0, 2.5 and 5.0 μg/mL in 1 mL of urine. The standards were then spiked with 25 μl of 0.08 mg/mL FVPA GLN and 10 μl of 0.01 mg/mL VPA ASP. The calibration standards were then subjected to the SPE extraction procedure described in scheme 5. The LC/MS/MS method was set to MRM for the following transitions: m/z 291>130 for FVPA GLN, 274>148 for VPA GLU, 273>147 for VPA GLN, 260>127 for VPA ASP and 201>57 for VPA GLY.

2.3.12.3 Validation of the assay for the profiling of AA conjugates of VPA in urine samples of patients

2.3.12.3.1 Precision of the assay

The inter-assay variation was assessed from the slopes of five calibration curves run consecutively and expressed as % CV.

The intra-assay was assessed by three replicate analyses of two samples at concentrations of 0.115 μg/mL and 1 μg/mL and expressed as % CV.

2.3.12.3.2 Accuracy of the assay

The accuracy of the assay was assessed as the % of expected values observed for the above spiked samples.

2.3.13 Analysis of AA conjugates in human samples by LC/MS/MS

2.3.13.1 Analysis of AA conjugates of VPA in the urine samples of patients on VPA therapy

A 1 mL aliquot from each of 29 overnight urine samples of patients in our study plus a set of calibration standards were extracted according to the procedure described in scheme 5 and analyzed by LC/MS/MS method B monitoring the same MRM transitions as described in section 2.3.12.2. To determine the recovery of VPA dose as VPA AA, 12 h or 24 h urine samples (n=4) were analyzed for each AA conjugate of VPA under the same conditions as all other samples. The % recovery of each identified conjugate was then calculated as described for the thiol conjugates by appropriately modified equation 1 in section 2.3.4.4.

The range and mean ± SD for each identified conjugate were determined and analyzed statistically (as for the thiol conjugates) to evaluate the effects of age and polytherapy.

2.3.13.2 Analysis of AA conjugates of VPA in the serum samples of patients on VPA

An SPE procedure could not be used to isolate the conjugates from serum samples. The liquid/liquid extraction procedure used to quantitate the amino acid conjugates in human serum was a modification of the one employed for the urinary thiol conjugates.

Instead of a one ml of urine sample, two ml of serum was treated according to scheme 4.

Solutions of VPA GLU, VPA GLN, VPA GLY were made at concentrations of 0.01 and 0.1 mg/mL. Blank serum samples were spiked with working solutions of the conjugates to make standard concentrations of 0, 0.01, 0.025, 0.05, 0.10, 0.25, 0.50 and 1.0 μg/mL in 2 mL of serum. The AA conjugates of VPA were then isolated from serum samples using the liquid-liquid extraction procedure similar to the one described in scheme 4. The standards and six serum samples were each spiked with 12.5 μl of 0.08 FVPA GLN and adjusted to pH 3-4 with 3N HCl. The samples were extracted twice with 5 mL EtOAc and the organic layers were dried under N_2 and reconstituted with 200 μL of water and filtered though a membrane filter (0.2 μm, Acrodisc LC 13 PVDF, Gelman Sciences, Ann Arbor, MI, USA). The filtrate was then analyzed by LC/MS/MS method C set for the MRM of m/z 291>130, 274>148, 273>147 and 201>57 for FVPA GLN, VPA GLU, VPA GLN, and VPA GLY, respectively.

2.3.13.3 Analysis of AA conjugates of VPA in one human CSF sample of a patient on VPA therapy

A solution of VPA GLU, VPA GLN and VPA GLY was made at concentrations equal to 0.001 mg/mL each. Control CSF samples were spiked with appropriate amounts of the working solution to yield standard concentrations of 0, 0.01, 0.025, 0.05 and 0.1 μg/mL in 100 μL of CSF. The calibration standards and 100 μl of the patient CSF were spiked with 5 μL of 0.001 μg/mL of FVPA GLN and were each filtered though a filter membrane and 60 μl of the filtrate was injected into the LC/MS/MS system to be analyzed using the

LC/MS/MS method C set for the MRM m/z 291>130, 274>148, 273>147 and 201>57 for FVPA GLN, VPA GLU, VPA GLN, and VPA GLY, respectively.

2.4 Animal studies

2.4.1 Identification and analysis of AA conjugates of VPA in animals

2.4.1.1 In vivo formation of AA conjugates of VPA in rats

Male Sprague-Dawley rats (Vancouver, B.C.) weighing 200-250 g were allowed free access to food (Purina Laboratory Chow, PMI Inc.) and water. They were housed in regular cages and exposed to a controlled 12 h cycle of light and darkness prior to the metabolic studies. VPA was administered as its sodium salt in water and the solution adjusted to pH 7.

2.4.1.1.1 Experiment 1

Five rats were administered a dose of 100 mg/kg of VPA dissolved in water (40 mg/mL, pH 7) i.p. for 3 consecutive days after first collecting control urine. The experiment was set up to collect 24 h urine from the rats shortly after the administration of the third dose. For control, a rat was dosed with water (pH 7) only and 24 h urine was also collected. The study rats were given a fourth i.p. dose of VPA 24 h later on day 4 and were sacrificed 1 h later by decapitation and about 10 mL of blood was collected.

All blood samples were centrifuged at 3000 g to separate the serum which was isolated and stored in non-heparinized blood collecting tubes at –20°C until analysis. All serum samples and urine samples were treated according to the procedures described in previous sections (2.3.3.1 and 2.3.3.2) for the identification of VPA AA in human urine and serum samples. Similarly, the quantitation of AA conjugates of VPA in serum and urine samples of rats were performed as described in sections 2.3.13.1 and 2.3.13.2, respectively.

2.4.1.1.2 Experiment 2

Four rats were anesthetized with urethane (1 g/kg) and their bile ducts cannulated with PE-10 tubing. Control bile was collected for 15 min. An aqueous solution (40 mg/mL, pH 7) of VPA (100 mg/kg) was administered to the animals by i.p. injection and bile was collected for 6 h. Surgical procedures for the collection of bile was conducted by Dr. W. Tang.

The bile samples were filtered though a membrane filter (0.2 μm, Acrodisc LC 13 PVDF, Gelman Sciences, Ann Arbor, MI, USA). The filtered samples were treated according to the procedure employed for the identification and quantitative analysis of VPA AA in the human CSF sample and described in sections 2.3.3.3.1 and 2.3.13.3.

2.4.1.1.3 Experiment 3

One rat was given an aqueous dose of VPA (700 mg/kg, 0.5 mL of 400 mg/mL solution, pH 7) by i.p. injection. One half hour later, the rat was sacrificed by decapitation and the brain was removed and 10 mL of blood was collected.

The brain was homogenized in water and centrifuged at 3000 g. The top aqueous layer and 1 mL of the corresponding serum sample were extracted separately at pH 3-4 with EtOAc twice. The organic layers were concentrated to 0.5 mL and derivatized with PFBBr/DIPEA at 50° C for GC/MS NICI analysis of VPG GLU using GC/MS method F under SIM of m/z 452.

2.4.1.2 Preliminary studies of *in vivo* formation of AA conjugates of VPA in rabbits

The purpose of investigating the formation of AA conjugates of VPA in rabbits was twofold. First, it was our intention to compare the AA conjugation of VPA in another species, and to make comparison to rat and human. Second, it was our goal (by studying the rabbit CSF) to provide further evidence to support our observation in one human CSF.

Male white New Zealand rabbits (n=4 or 5 per experiment) (Vancouver, B.C.) weighing 1.9-3.3 kg were given free access to food (Purina Laboratory Chow, PMI Inc) and water. They were housed in regular rabbit cages and were exposed to control 12 h cycle light

and darkness prior to metabolic studies. VPA was administered as its sodium salt in water and the solution adjusted to pH 7.

Surgical procedures for the collection of CSF and collection of blood were performed by Dr. A. Tabatabei in our laboratory according to standard procedure (Bivin, 1994). Prior to surgery, the dorsal cervical area and occipital area of the neck was shaved and scrubbed. One half h before CSF collection, each rabbit was anesthetized with xylene (5 mg/kg) and ketamine (35 mg/kg) i.p. as instructed by the Animal Care Center facility. Throughout experiments 1-4 described below, CSF (~0.5 mL from each animal) was collected from the anesthetized animals with the use of a 22-gauge 3.81 cm spinal needle from the cisterna magna and blood (3-5 mL from each animal) was collected from the ear with the use of a 25 gauge hypodermic needle. To determine the appropriate dose to conduct our study, we initiated our experiments with the same dose used for rats (i.e 100 mg/kg i.p.) and studied the conjugates at increasing doses. A dose of 300 mg/kg was the minimum dose at which the conjugates could be positively identified in the biological fluids of the rabbits.

2.4.1.2.1 Experiment 1

Four rabbbits (1.9-2.2 kg) were administered three daily doses of aqueous VPA (100 mg/kg) i.p. after control CSF was collected from each anesthetized animal. One h after the third dose, CSF was collected from each anesthetized rabbit.

2.4.1.2.2 Experiment 2

This experiment was conducted to determine the minimum dose at which VPA GLU and VPA GLN could be detected confirmatively in blood and CSF of rabbits.

Four rabbits (2.13-2.3 kg) were administered aqueous VPA i.p. as follows:

(1) Rabbit A received 300 mg/kg of VPA and CSF and blood were collected after 1.17h.

(2) Rabbit B received 500 mg/kg of VPA and CSF and blood were collected after 1 h.

(3) Rabbit C received 700 mg/kg of VPA and CSF and blood were collected after 1.6 h.

(4) Rabbit D received 500 mg/kg of VPA and CSF and blood were collected after 2.5 h.

(5) Rabbit E received 462 mg/kg of VPA and CSF and blood were collected after 4 h.

2.4.1.2.3 Experiment 3

To determine approximately the time for maximum detection of VPA GLU and VPA GLN, three rabbits were treated with 300 mg/kg of aqueous VPA (i.p., pH 7). CSF and blood were collected from each rabbit at either 2,3,or 4 h after dosing. An approximate time for maximum detection of VPA GLU and VPA GLN in CSF and blood was determined.

2.4.1.2.4 Experiment 4

To confirm the identity of VPA GLU and VPA GLN, five rabbits weighing 2.4-3.1 kg were dosed with 300 mg/kg of aqueous VPA (pH 7). CSF and blood were collected 2.5 h after dosing. CSF and blood were also collected from a rabbit dosed with saline (pH 7) and one rabbit which was not dosed at all.

2.4.1.2.5 Preparation of blood and CSF samples for the identification and quantitation of AA conjugates of VPA in rabbits by LC/MS/MS

Blood samples were centrifuged at 3000 g for 15 min and serum isolated and treated as for the identification and quantitation of AA conjugates of VPA in human samples (sections 2.3.3.2 and 2.3.13.2). Rabbit CSF samples were treated as the human CSF sample for the quantitative analysis as described in section 2.3.3.3.1.

3. RESULTS

3.1 Thiol conjugates of VPA

3.1.1 Synthesis of (*E*)-5-*N*-(acetylcystein-*S*-yl)-3-ene VPA (NAC I) and (*E*)-5-*N*-(acetylcystein-*S*-yl)-2-ene VPA (NAC II)

The ethyl methyl ester of 5-*N*-(acetylcystein-*S*-yl)-3-ene VPA (NAC I) was synthesized in our laboratory by Dr. A. Mutlib for the purpose of this project following the procedure previously designed for the di-methyl ester of NAC I (Kassahun *et al.*, 1991) and was the only product formed according to the ^{1}H NMR analysis. The goal was to produce NAC I in the free acid form by hydrolyzing the ester of NAC I according to scheme 3 of the experimental section. LC/MS molecular weight analysis was found to be consistent with that of NAC I. We initiated this thesis by performing ^{1}H NMR and GC/MS analysis of the product to confirm the identity and purity of NAC I. However, the results indicated that the hydrolysis step of scheme 3 produced more than one compound, NAC I and 5-*N*-(acetylcystein-*S*-yl)-2-ene (NAC II). Following the isolation and purification of each compound from the synthetic mixture, we confirmed for the first time the structure and identification of NAC II in human urine samples. NAC II was found at concentrations 2-3 times that of NAC I in the urine samples of patients on VPA.

3.1.2 Characterization of the synthetic mixture obtained from scheme 3

It appeared that the synthetic pathway for the formation of the ethyl methyl ester of NAC I produced one isomer while the base hydrolysis of the ester moieties afforded an isomeric mixture of NAC I and NAC II in the ratio of 1:3 as implied by ^{1}H NMR, GC/MS

and HPLC analyses of the product mixture. LC/MS/MS studies further gave indication that the diastereomers of NAC I were formed in equal amounts. The characteristics of NAC II in the synthetic mixture were similar to the unidentified GC/MS peak observed by Kassahun *et al.* (1991) in the urine samples of patients on VPA therapy. Therefore, we focused on the characterization of NAC II. Figure 2 shows the proposed structures of NAC I and NAC II as determined by the results of spectroscopic and chromatographic analyses (^{1}H NMR, GC/MS, HPLC/UV, and HPLC/MS/MS) performed on the synthetic products.

NAC I   NAC II

<div style="text-align:center">

$$\underset{5}{CH_2}\underset{6}{CH_2}\underset{7}{CH_3}$$
$$\underset{1}{CH_2}\underset{2}{CH}=\underset{3}{CH}\underset{4}{\overset{13}{C}H}COOH$$
$$\underset{S}{}-\underset{8}{CH_2}\underset{9}{C}\underset{10}{H}COOH$$
$$\underset{11}{NH}\underset{12}{C}OCH_3$$

</div>
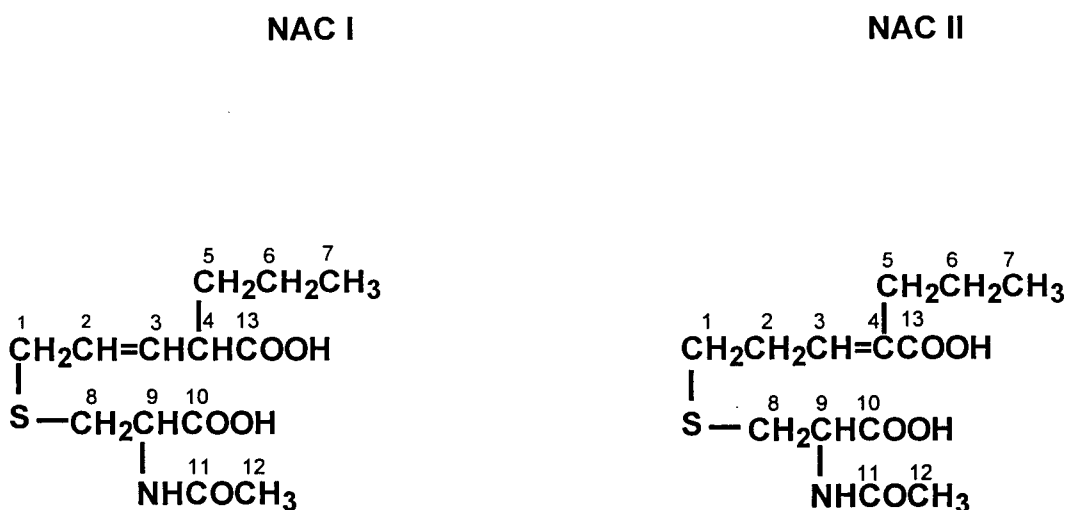
Figure 2. Structure of NAC I and proposed structure of NAC II based on ^{1}H NMR. Carbon assignments as given here were used in table 1 to describe proton chemical shift (δ) and multiplicity data.

74

Table 1. ^{1}H NMR data for NAC I and NAC II in the synthetic mixture.

δ (ppm)	Multiplicity	Carbon Assignment NAC I	Carbon Assignment NAC II
0.85	3H,t	7	7
1.1-1.5	2H,m	6	6
1.52-1.75	2H,m	5	
2.02	3H,s	12	12
2.25	2H,t		5
2.50	2H,m		2
2.60	2H,t		1
2.75	1H,m	8	8
2.90	1H,m	8	8
3.00	2H,d	1	
3.10-3.20	1H,m	4	
3.25	CD$_3$OD		
4.40-4.60	1H,m	9	9
5.10	H$_2$O		
5.50-5.65	2H,m	2,3	
6.70	1H,t		3

3.1.2.1. ¹H NMR analysis of synthetic mixture of thiol conjugates

The ¹H NMR of the synthetic products following base hydrolysis pointed to the presence of two compounds, one of which appeared to be NAC I based on previously reported results (Kassahun *et al.*, 1991) and the other compound was consistent with the structure of NAC II as proposed in figure 2. The chemical shifts observed are listed in table 1 and the ¹H NMR spectrum of the mixture is shown in Appendix c. To study the stability of the conjugates, the NMR tube containing our sample was stored at 0°C for three months and analyzed at regular intervals. There was no indication of either decomposition or interconversion of the isomers observed. The ¹H NMR spectrum obtained after three months is shown in Appendix d.

3.1.2.2 DCI analysis of mixture of thiol conjugates

Desorption chemical ionization of the synthetic mixture of thiol conjugates showed that the protonated molecular ion of the mixture was m/z 304 ([MH]$^+$) as depicted in figure 3. Ions at m/z 318 and m/z 332 could be the results of impurities arising from either the unhydrolyzed methyl ester $(M+15)^+$ or ethyl ester $(M+29)^+$ or ammonium adducts.

3.1.2.3 Direct chemical thermospray (TSP) analysis of the mixture of thiol conjugates

The positive TSP mass spectrum showed that the most abundant peak was at m/z 304 ([MH]$^+$) and the negative TSP mass spectrum was characterized by m/z 302 ([M-H]$^-$) as illustrated in figures 4a, and 4b, respectively
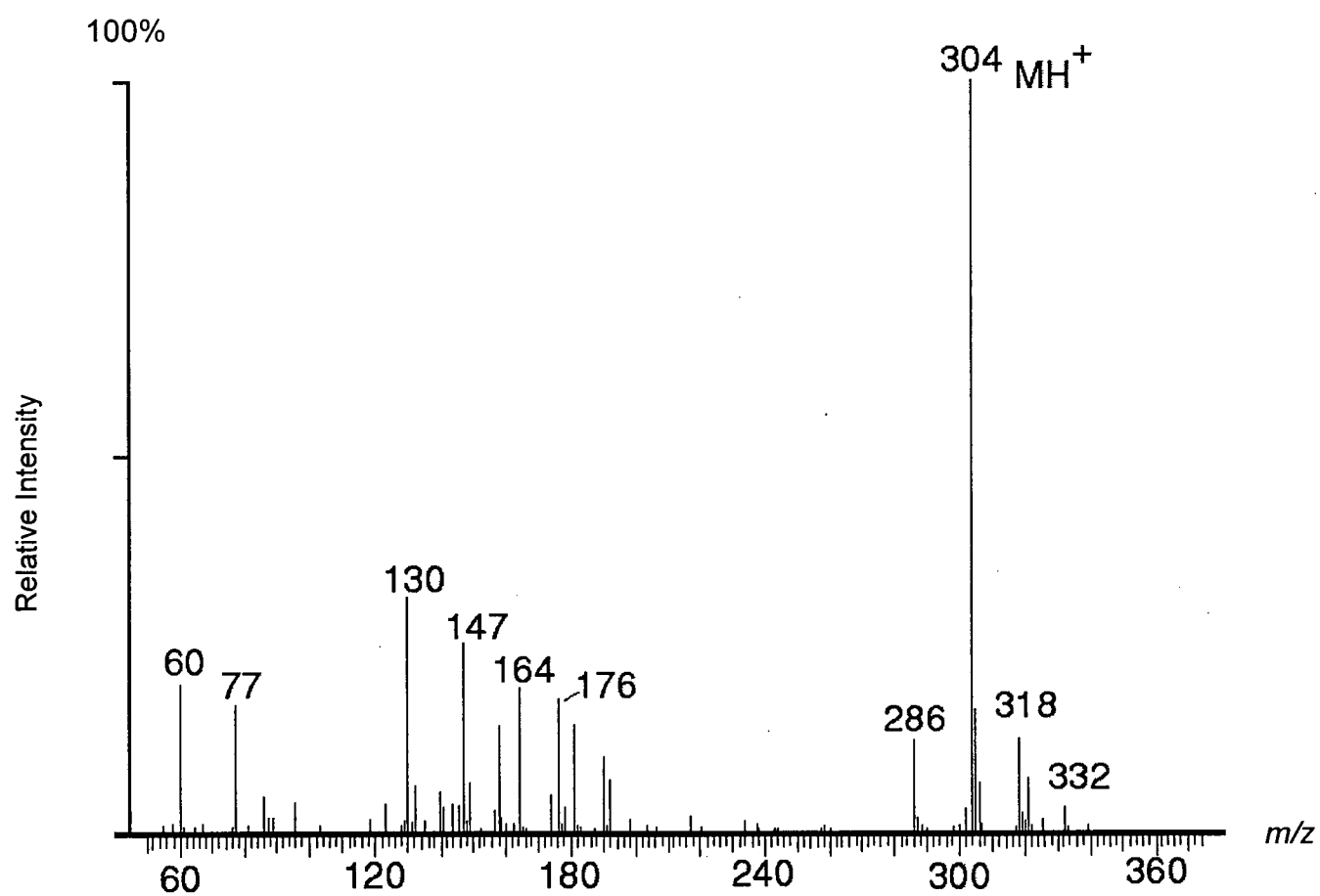
Figure 3. DCI spectrum of the mixture of thiol conjugates formed from the synthetic

pathway described in scheme 3 and using NH_3 to induce chemical ionization.
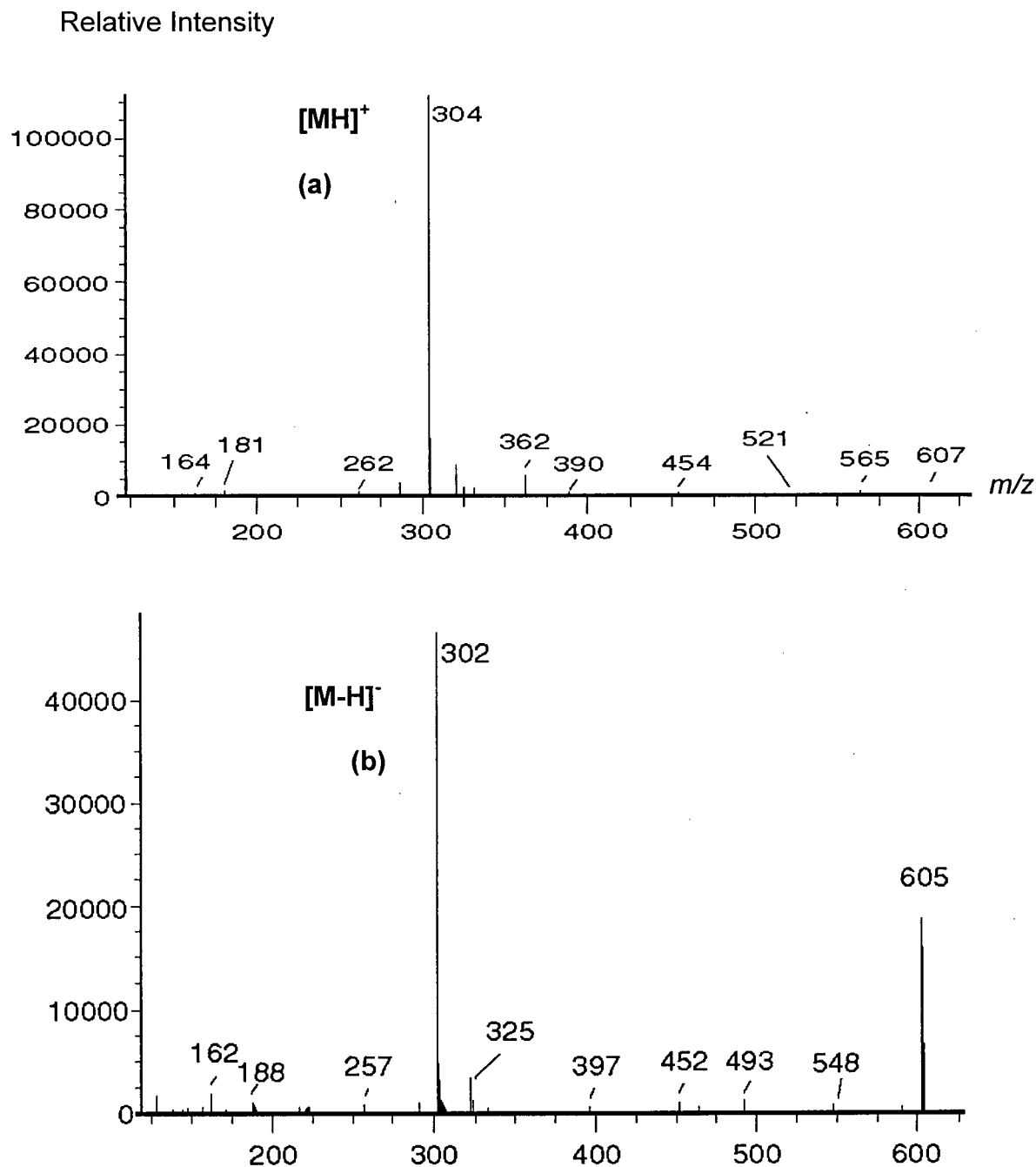
Figure 4. TSP spectra of product mixture of NAC I and NAC II under (a) positive and (b) negative ionization conditions employing direct injection technique. The interface settings were stem temperature 92°C and source temperature 225°C. Ion at *m/z* 605 is a dimer formed during the ionization.

3.1.2.4 GC/MS analysis of the PFB derivatives of the mixture thiol conjugates

Under the conditions of GC/MS method C, the TIC obtained for the PFB derivative of the synthetic mixture analyzed under NICI (figure 5a, below) showed two major peaks (t_R = 12.07 and 12.45 min) with a relatively minor one eluting at t_R = 11.89 min. All three peaks produced mass spectra characterized by the most abundant ion at m/z 482 ([M-181]⁻) as shown in figures 5 b, c, and d for peaks A, B, C, respectively.
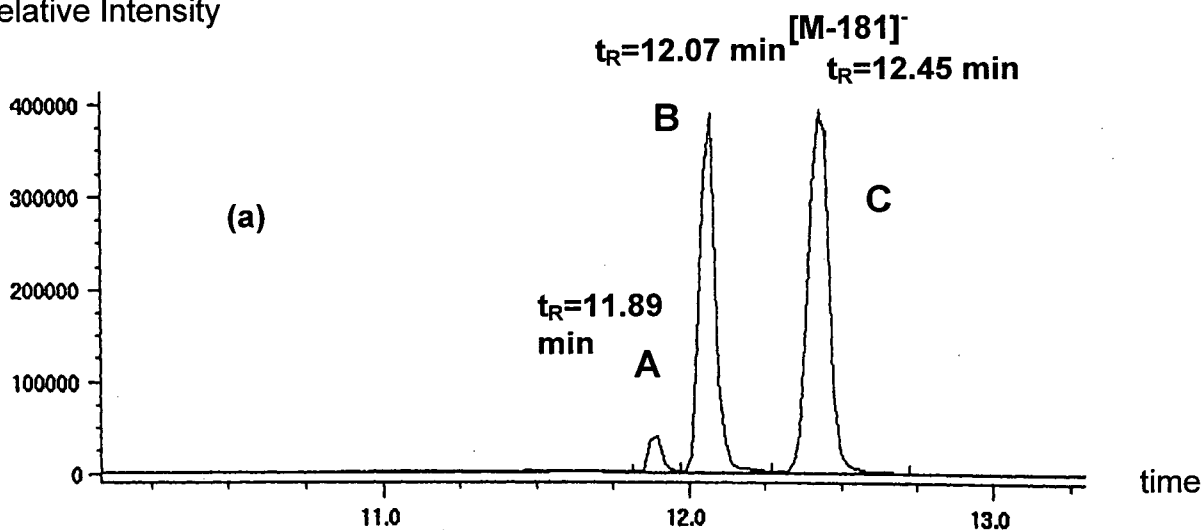
Relative Intensity

Relative Intensity

79

Relative Intensity

(c)

240000
200000
160000
120000
80000
40000
0

139 162 245 284 302 353 425 482 510 562

100 200 300 400 500 600   *m/z*

(d)

200000
160000
120000
80000
40000
0

139 162 245 256 302 353 425 482 510 562

100 200 300 400 500 600   *m/z*
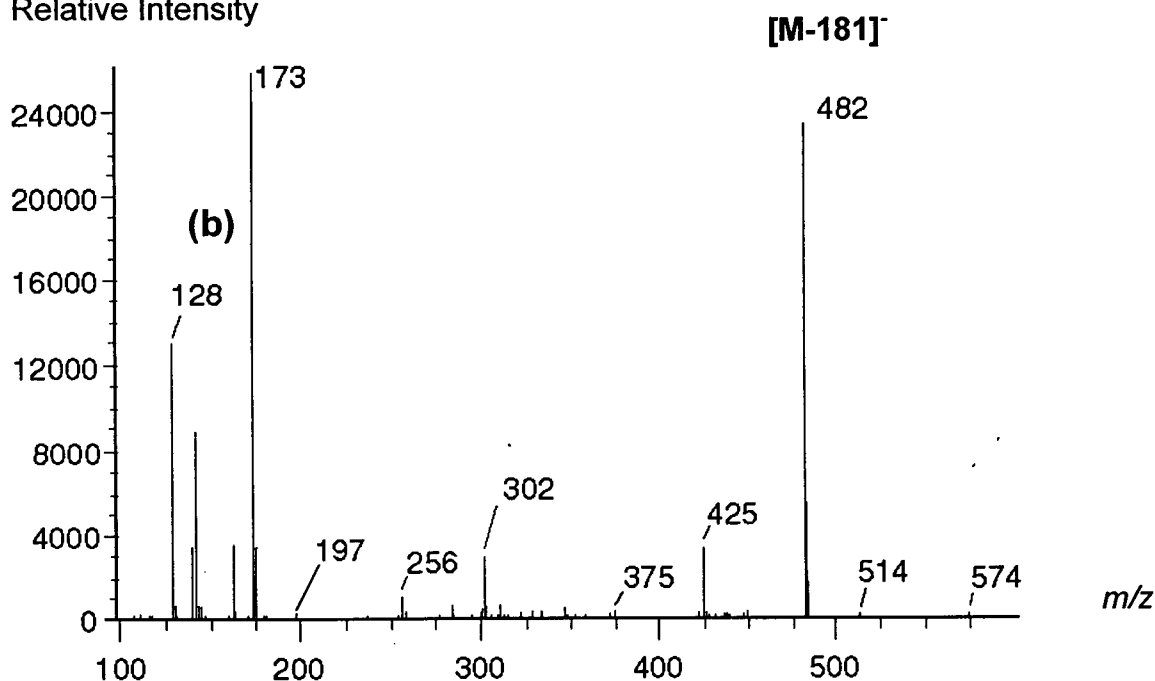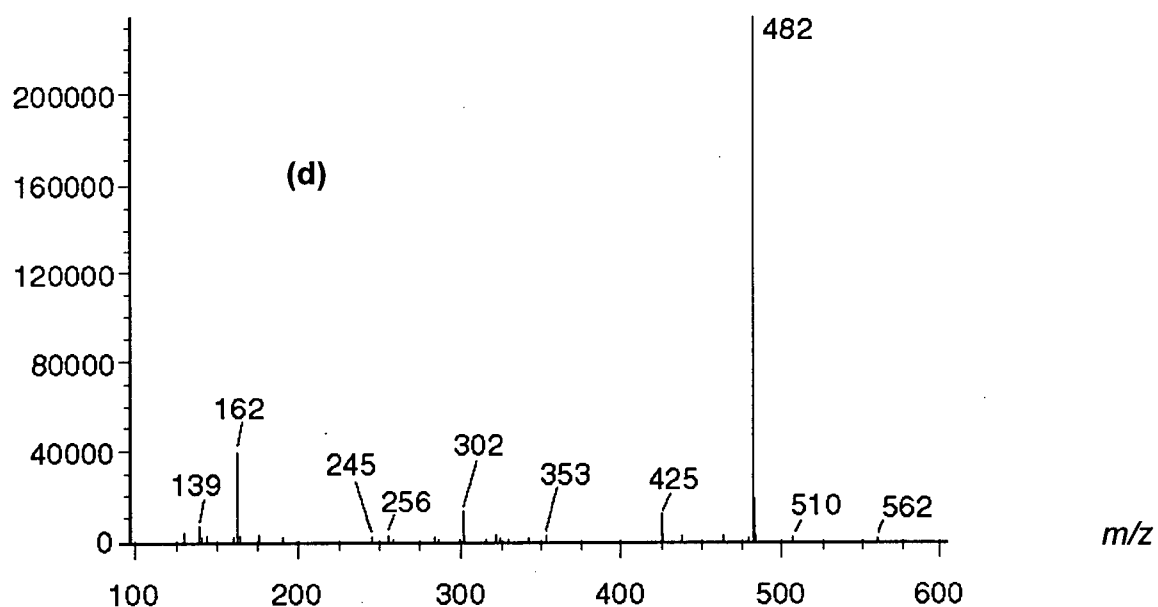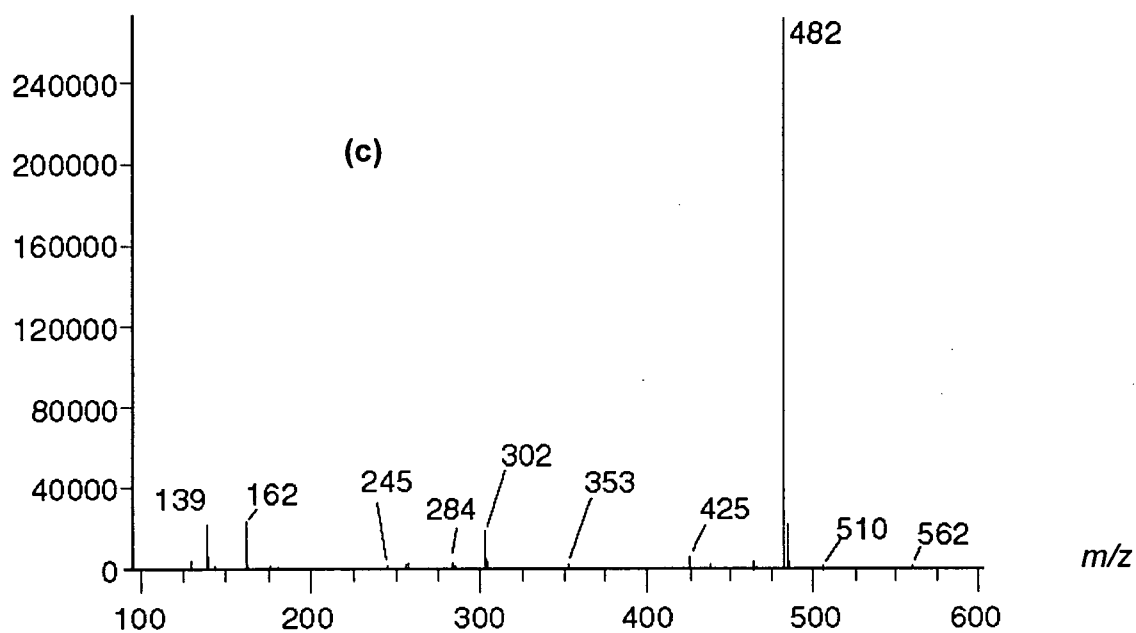
Figure 5(a) showing the TIC obtained for the scan of the PFB derivative of the thiol products under NICI mode using GC method C (See Experimental/Section 2.2.6.3) The corresponding mass spectra of peaks A, B, and C are shown in figures 5 b, c, and d, respectively.

3.1.2.5 GC/MS analysis of the t-BDMS-derivatized mixture of the thiol conjugates

Under the conditions of GC/MS method D, the TIC obtained for the mixture of thiol conjugates under EI mode (figure 6a) showed three peaks eluting at t_R = 19.44 min, 19.78 min and 20.34 min. The corresponding mass spectrum (figures 6b, c, d) of each peak included the characteristic [M-57]$^+$ ion fragment at m/z 474.
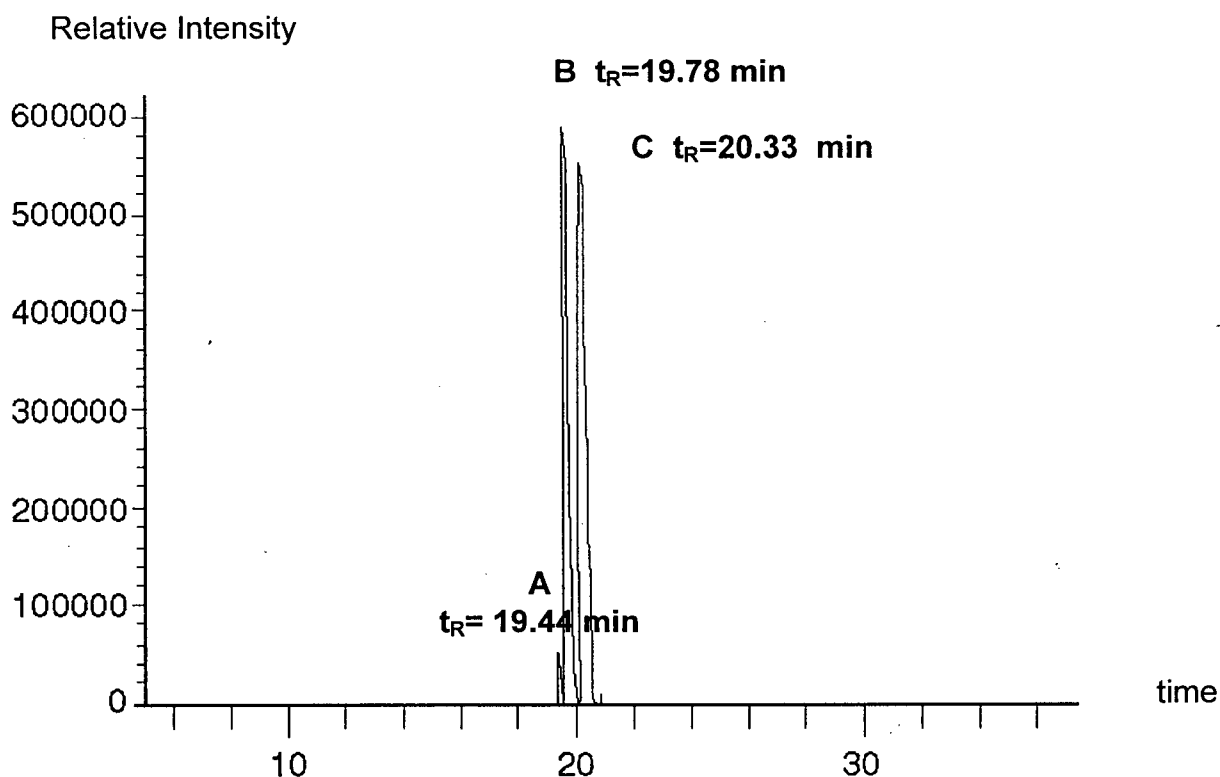
Figure 6a. TIC of the t-BDMS derivative of the synthetic mixture of NAC I and NAC II on a DB1701 column using GC method D. (See Experimental/Section 2.2.6.4)
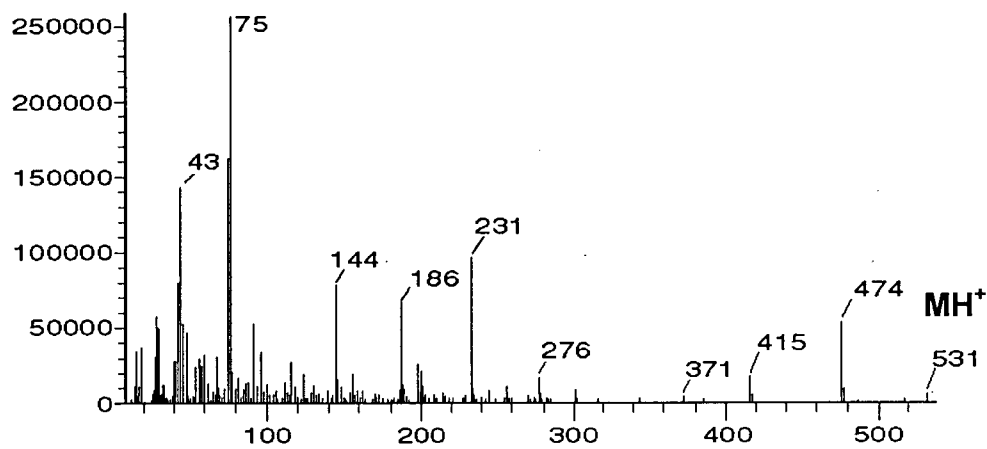
Figure 6b. The corresponding EI mass spectrum of peak A form figure 6a.
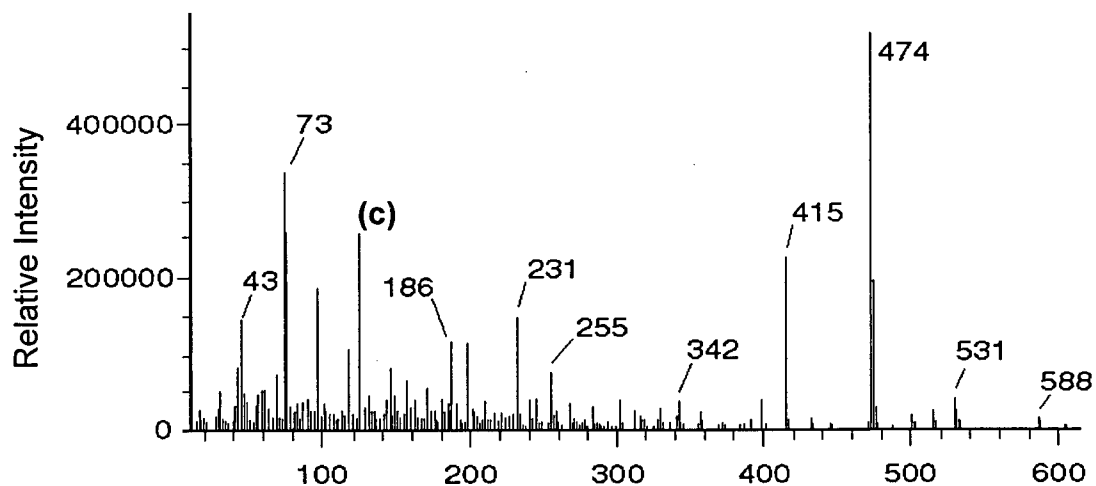
Figure 6c. The corresponding EI mass spectrum of peak B from figure 6a.
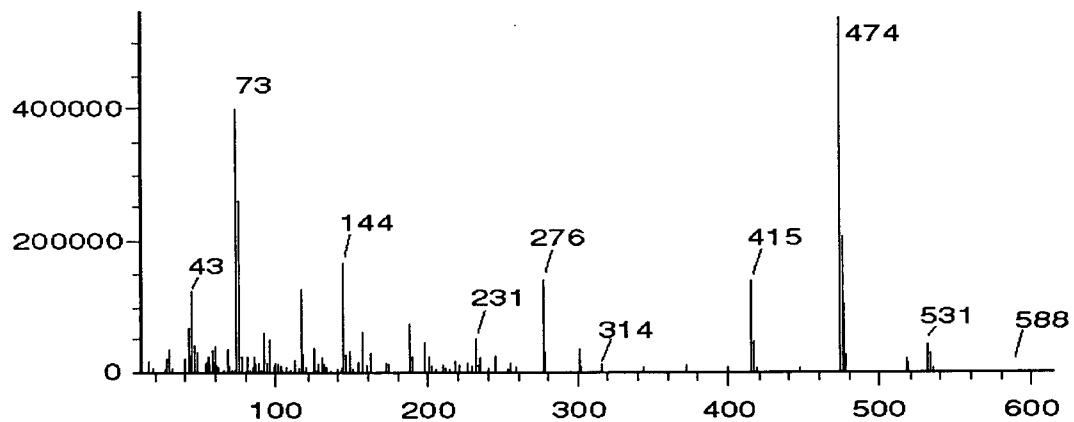
Figure 6d. The corresponding EI mass spectrum of peak C from figure 6a.

3.1.2.6 HPLC/UV analysis (LC/UV method A) of the mixture of thiol conjugates

The HPLC chromatogram of the product mixture as analyzed using LC/UV method A showed a prominent peak (80 % of total area) eluting at t_R = 10.39 min followed very closely by two minor peaks (17% of total area) at t_R = 10.55 and 10.71 min.

3.1.2.7 HPLC/UV analysis (LC/UV method B) of the mixture of thiol conjugates

The LC/UV method B was employed to separate and isolate all eluting compounds. A prominent peak eluted at t_R = 37.86 min and two separate and distinct peaks of equal area and height eluted at t_R = 40.50 min and 43.39 min (figure 7). All the peaks were fully resolved from each other and the latter two peaks had similar retention times as the diastereomers of pure NAC I synthesized in our laboratory *via* a different synthetic pathway (Tang *et al.*,1996a).

$t_R=37.86$ min

NAC II

NAC I b

$t_R=40.50$ min

$t_R=43.39$ min

a

34.0 min   45.0 min
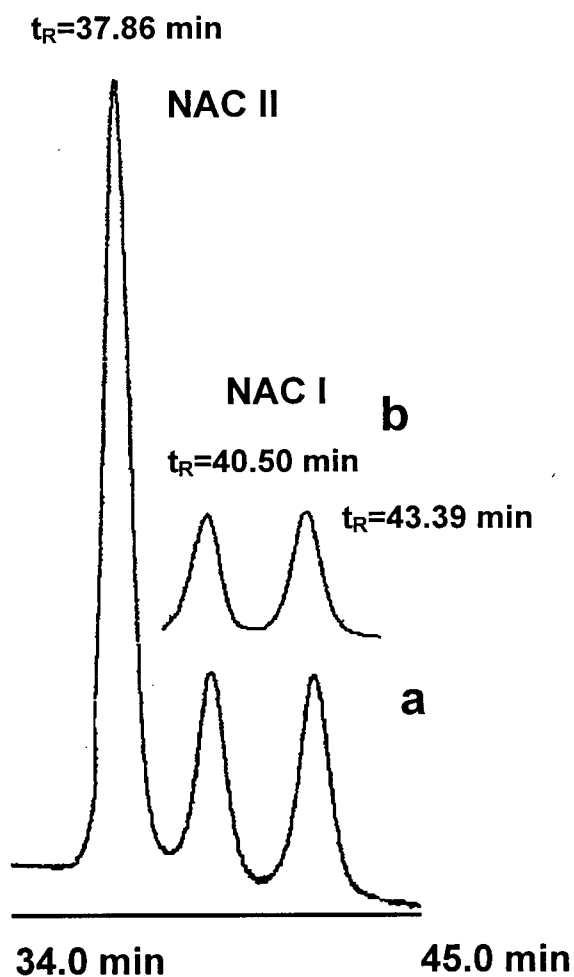
Figure 7. HPLC/UV chromatograms showing the elution of the thiol conjugates formed from the synthetic pathway described in scheme 3 in (a) and the elution of the isomers of pure NAC I in (b) under the conditions of HPLC/UV method B (See Experimental/section 2.2.4.2)

3.1.3 Separation, isolation and confirmation of HPLC-purified NAC I and NAC II

LC/UV method B allowed for the separation of all peaks at high concentration (0.1 g in 200 μL of water), thereby facilitating the isolation of each peak by preparative HPLC. Each fraction was collected individually and characterized using the various spectroscopic techniques described in the Experimental section. The HPLC chromatogram of isolated NAC II is shown in figure 8.

t_R=38.00 min

Isolated NAC II
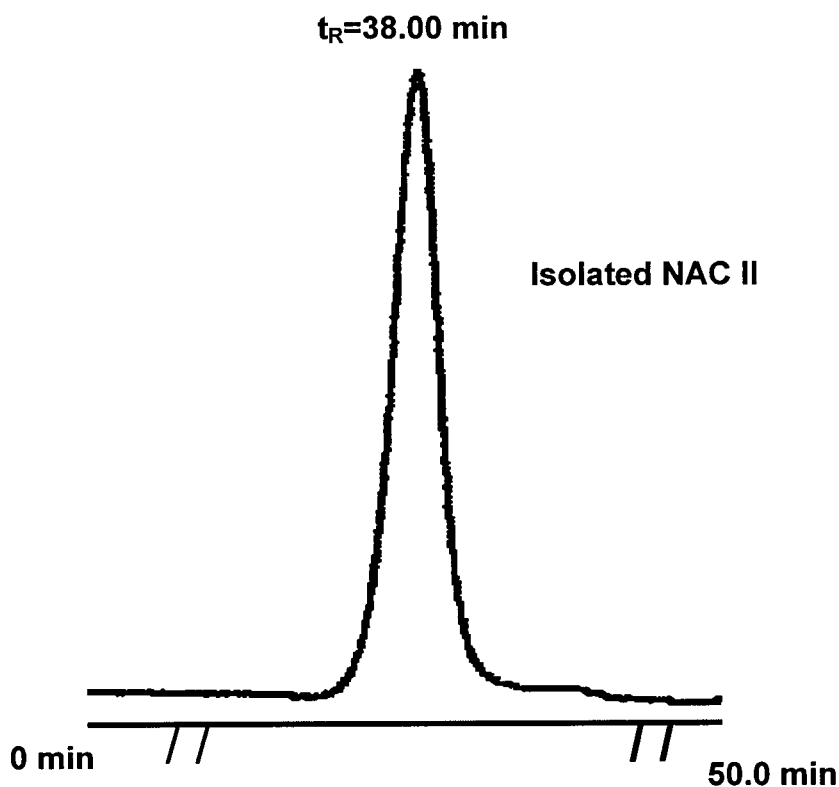
0 min
50.0 min

Figure 8. HPLC chromatogram (LC/UV method B) of NAC II following HPLC purification at t_R= 38.00 min under the conditions of LC/UV method B (See Experimental/Section 2.2.4.2).

3.1.3.1 ^{1}H NMR of HPLC-isolated (E)-5-NAC-2-ene VPA in D_2O

The ^{1}H NMR of NAC II is shown in figure 9.

δ 0.85 (3t, 3H, CH_3 -), 1.30-1.50 (m, 2H, $C\underline{H}_2$ –CH_3), 2.02 (s, 3H, -NHCOC$\underline{H}_3$), 2.25 (t, 2H, -C$\underline{H}_2$- CH_2-CH_3), 2.55 (q, 2H, -CH_2-C$\underline{H}_2$-CH -), 2.75 (t, 2H, C$\underline{H}_2$, CH_2-CH-), 2.85-3.0 and 3.08-3.20 (m, 2H, -S-C$\underline{H}_2$ –CH), 4.4-4.6 (dd, 1H, S-CH_2-C$\underline{H}$ -), 6.75 (t, 1H, CH_2-C$\underline{H}$=C-)

3.1.3.2 ^{1}H NMR of HPLC-isolated NAC IA and B in D_2O

The ^{1}H NMR of NAC IA and NAC IB were identical in D_2O.

δ 0.90 (t, 3H, -CH_2-CH_2-C$\underline{H}_3$), 1.15-1.40 (m, 2H, CH_2-C$\underline{H}_2$-CH_3), 1.52 and 1.75 (2H, 2m, -C$\underline{H}_2$-CH_2-CH_3), 2.02 (s, 3H, -NH-CO-C$\underline{H}_3$), 2.78 and 3.00 (2H, 2m, S-C$\underline{H}_2$-CH-), 3.10 (1H,q, =CH-C$\underline{H}$-), 3.20 (2H, d, C$\underline{H}_2$-CH=), 4.45 (1H, dd, S-CH_2-C$\underline{H}$-), 5.50-5.65 (2H, m, CH_2-C$\underline{H}$=C$\underline{H}$). See appendix e,f.
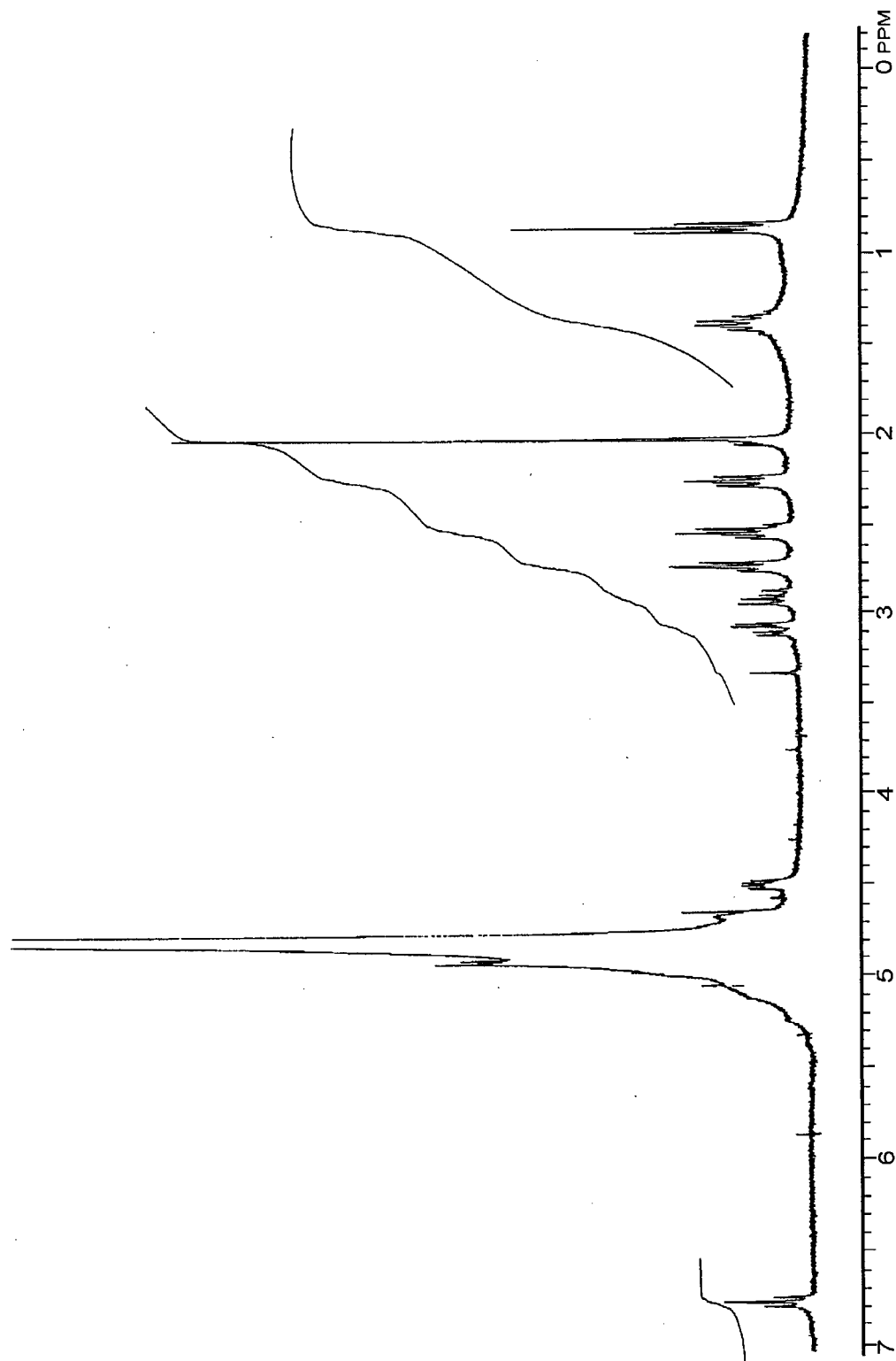
Figure 9. ^{1}H NMR of HPLC isolated NAC II in D_2O.

3.1.3.3 GC/MS EI analysis of the t-BDMS derivative of HPLC-isolated NAC II using GC/MS method E on a DB1701 column.

The *t*-BDMS derivative of NAC II eluted at t_R 37.27 min and had a corresponding mass spectrum identical to the proposed NAC II in the synthetic mixture as shown in figure 10. The characteristic fragments of the GC/MS spectrum were *m/z* 474 (100%) [M-57]$^+$, and 415 (30%) [M-116]$^+$.
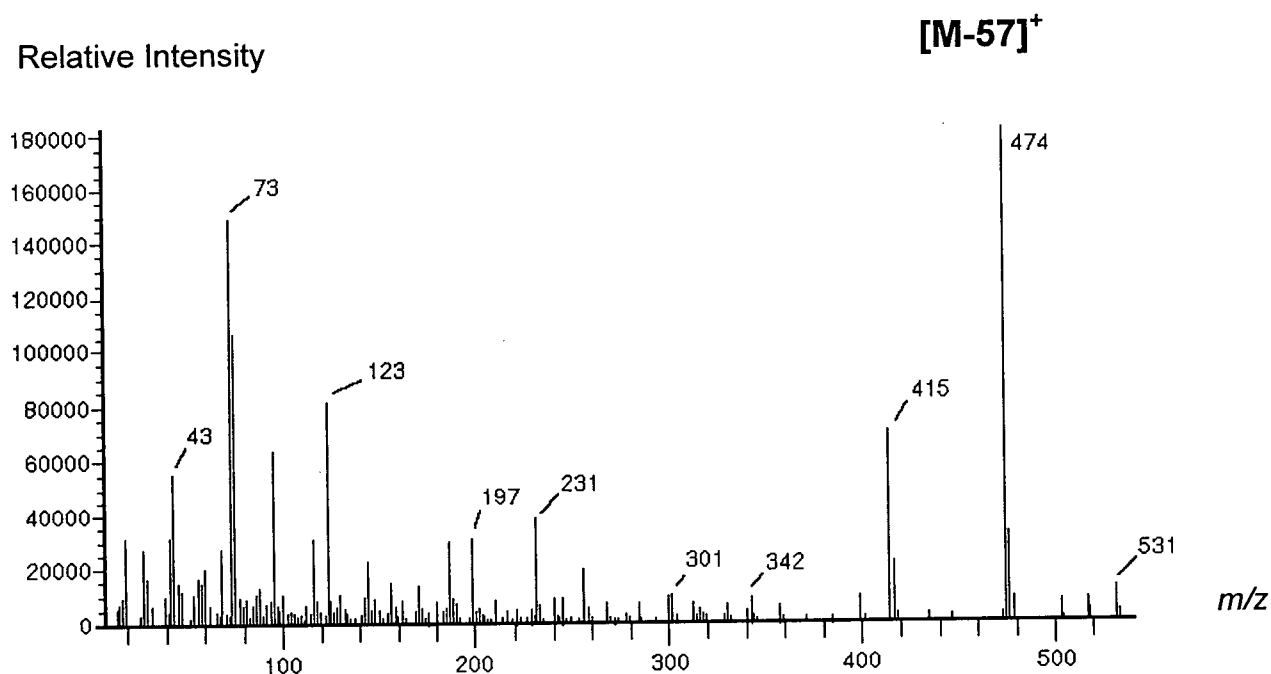
Figure 10. The GC/MS EI mass spectrum of the HPLC-purified and *t*-BDMS-derivatized NAC II using GC method E and a DB1701 column (See Experimental/Section 2.2.6.5).

3.1.3.4 GC/MS NICI analysis of the PFB derivative of HPLC-isolated NAC II

The PFB derivative of NAC II eluted at 19.79 min on a DB101 column under GC/MS method A. The mass spectrum was characterized by the [M-181]⁻ ion fragment at m/z 482.

The same derivative eluted at 30.90 min on a DB1701 column under conditions of GC/MS method B as shown in figure 11 a. Similarly, the mass spectrum was again characterized by the [M-181]⁻ ion at m/z 482 as shown in figure 11 b.

Relative Intensity
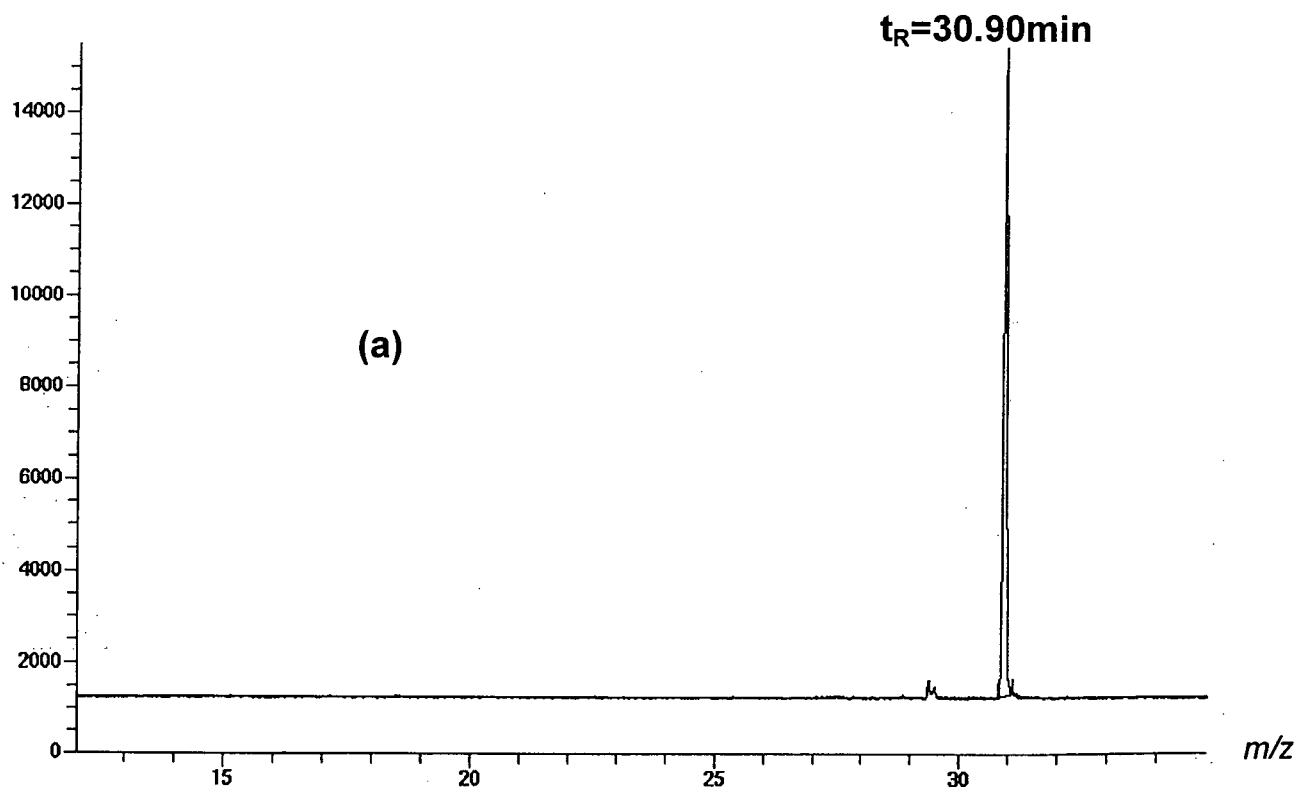
Figure 11 a. The TIC of the PFB derivative of NAC II on a DB1701 column by GC method B under NICI scanning (See Experimental/Section 2.2.6.2).
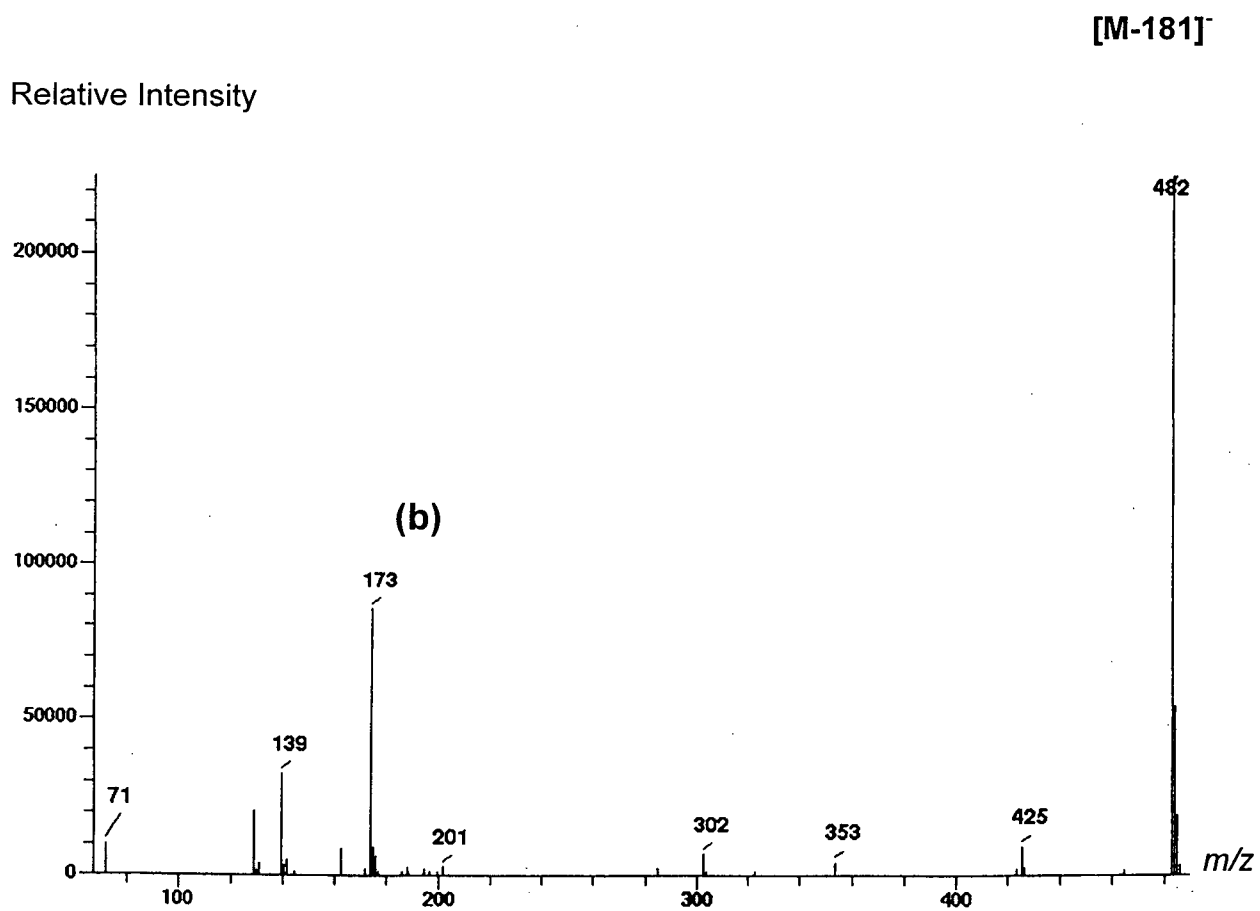
Figure 11 b. The NICI mass spectrum of the PFB derivative of the HPLC-purified NAC

II.

3.1.3.5 GC/MS EI analysis of the *t*-BDMS derivative of HPLC-isolated NAC I (A and B) and pure NAC II

Both the *t*-BDMS-derivatized samples of the HPLC fractions of NAC I and that of a previously purified sample of NAC I eluted at 36.27 min and produced mass spectra characterized by [M-57]$^+$ at *m/z* 474 and [M-116]$^+$ at *m/z* 415 on a DB1701 chromatographic column. Attempts to separate the diastereomers of NAC IA and B by a slow oven temperature program (GC/MS method E) were not successful using either DB1701 or DB101 columns. The TIC of the *t*-BDMS derivative of NAC IA is shown in figure 12 (a) and its corresponding mass spectrum in 12 (b). The same chromatographic and mass spectral observations were obtained for NAC IB.
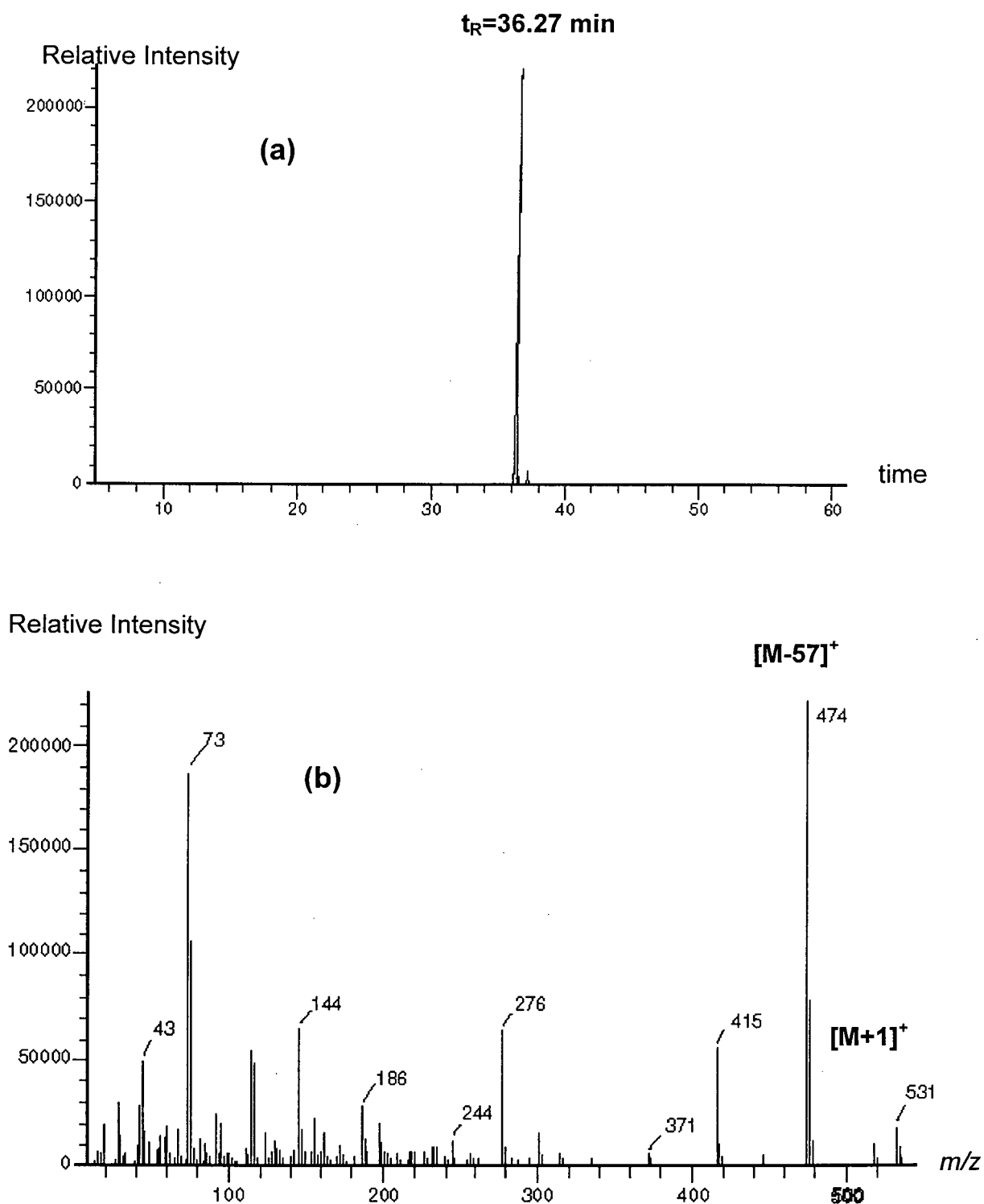
Figure 12. TIC of the *t*-BDMS derivative of NAC IA by GC method E on a DB1701 column in (a) and its corresponding EI mass spectrum in (b). NAC IB produced identical results (See Experimental/Section 2.2.6.5).

3.1.3.6 GC/MS NICI analysis of the PFB derivative of the HPLC-isolated NAC I A and B

The GC/MS NICI TIC extracted at *m/z* 482 observed for the PFB derivatives of both isomers of NAC I and a previously purified sample of NAC I prepared in our laboratory (Tang *et al.*, 1996a) and analyzed on a DB1701 chromatographic column, showed similar retention times and identical mass spectra characterized by [M-181]⁻ as illustrated in figures 13 a,b,c. The PFB derivative of HPLC-purified NAC IA eluted at 29.38 min under scan mode while HPLC-purified NAC IB eluted at 29.34 min under the conditions of GC/MS method B. On a DB101column, the elution times for both HPLC-purified isomers were identical at 19.39 min using GC/MS method A.

Relative Intensity t_R=29.38 min
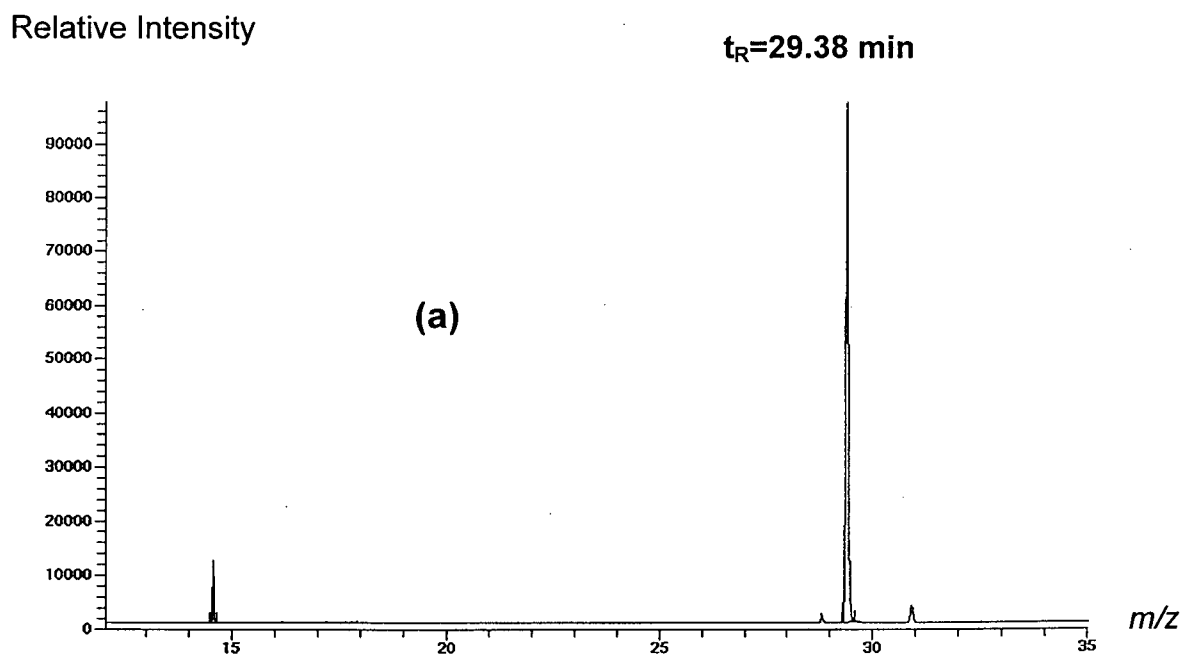
Figure 13 a. The TIC of the PFB derivative of isolated NAC IA on a DB1701 column (See GC method B in Experimental/Section 2.2.6.2).

t_R=29.34 min

(b)
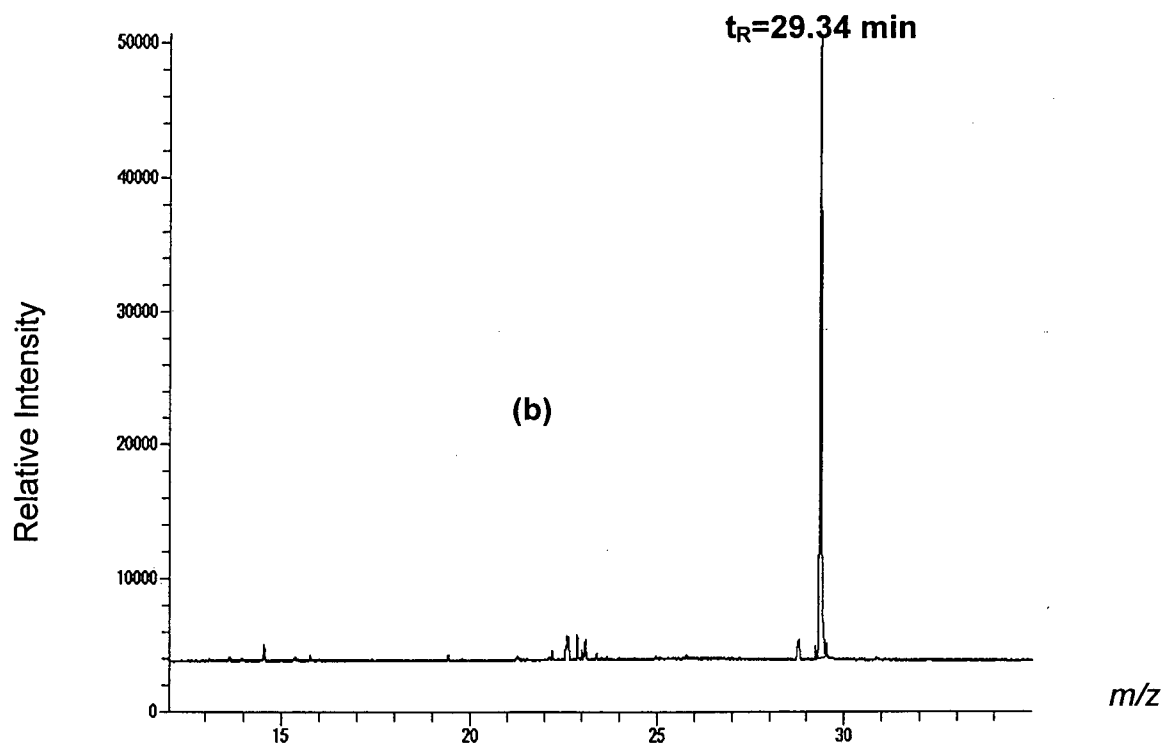
Figure 13b. The TIC of the PFB derivative of isolated NAC IB on a DB1701 column (See GC method B in Experimental/Section 2.2.6.2).

t_R=29.39 min

(c)
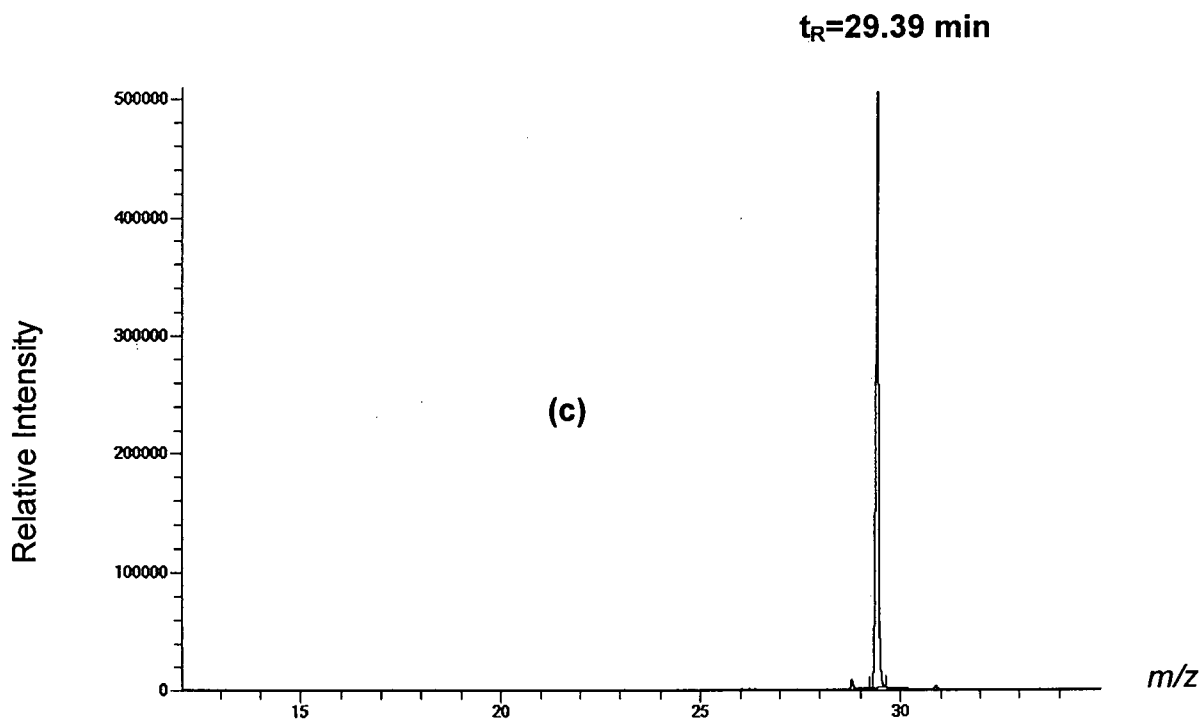
Figure 13c. The TIC of the PFB derivative of pure NAC I on a DB1701 column (See GC method B in Experimental/Section 2.2.6.2).

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3.1.3.7 LC/MS/MS characteristics of isolated NAC I and NAC II

Chromatographically on LC/MS/MS analysis (Experimental section 2.2.5.2), both isomers of NAC I eluted last at t_R = 25.64 min and 26.92 min and are labeled C and D in figure 14. NAC II eluted earlier as the peak at t_R = 23.37 min while NAC III (discussed in a later section) eluted first at t_R = 14.66 min.

Both NAC I and NAC II produced parent ion spectra (MS/MS) characterized by MH^+ 304 under positive electrospray. CID of m/z 304 for both NAC I and NAC II conjugates produced product ion spectra with similar fragmentation patterns as shown in figure 15 and appendices g and h. In all three MS/MS spectra, cleavage at the C-S bond appears to be common with the charge being retained either on the VPA or NAC moiety. A characteristic fragment ion at m/z 130 was the result of cleavage at the thioether bond of the NAC moiety or the loss of the protonated NAC followed by the neutral loss of hydrogen sulfide $[NAC + H^+ - H_2S]^+$ as proposed by Tang *et al.* (1996c). On the other hand, the loss of the NAC moiety and water $[MH^+ - NAC - H_2O]^+$ resulted in the dominant fragment m/z 123. The loss of the NAC and the COOH moieties from the protonated parent molecule led to the formation of the ion at m/z 95. The spectra of NAC IA and NAC IB did not produce any distinguishing MS/MS features which differentiated them from each other and from NAC II.
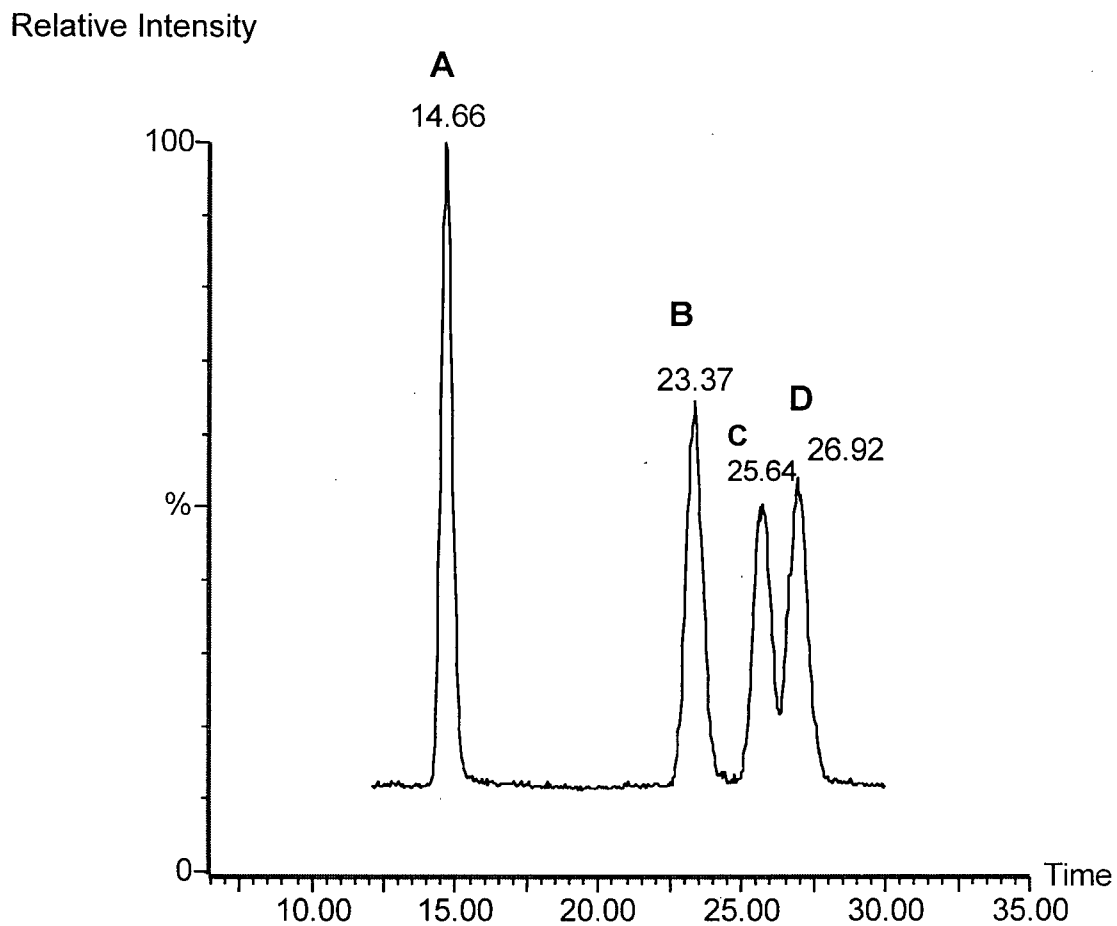
Figure 14. The elution of thiol conjugates arising from VPA biotransformation by LC/MS/MS and using mobile phase B under the conditions of LC/MS/MS method A and under product ion scanning (*m/z* 304) . NAC III(A), NAC II (B), NAC IA and NAC IB (C,D) . (See Experimental/Section 2.2.5.2)

NAC II

Relative Intensity

- H$_2$0, 123

O=COH

S CH$_2$CHCOOH
                |
            NHCOCH$_3$
---- 130

100 —

123

% —

95

130

146

MH$^+$

304

286

0 —

50   75   100   125   150   175   200   225   250   275   300   325   350   m/z
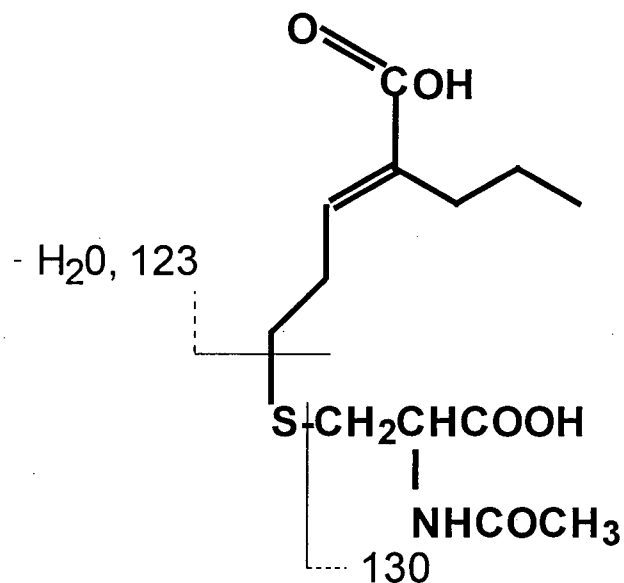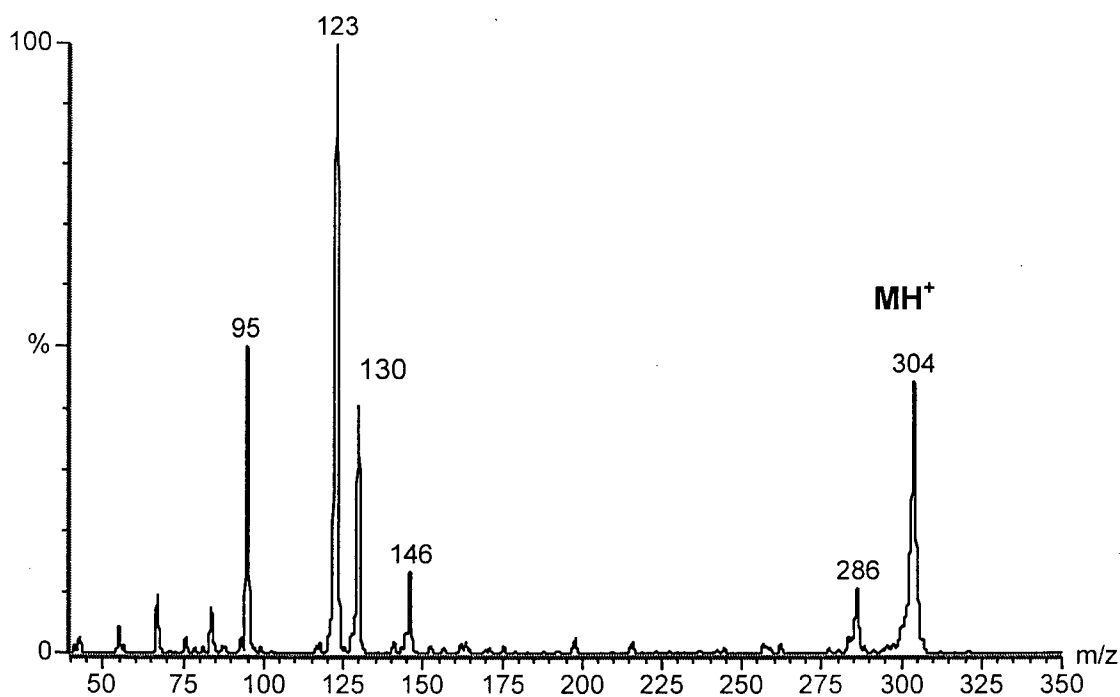
Figure 15. MS/MS product ion spectrum (MH$^+$ 304) of a standard of the HPLC-purified NAC II under ES$^+$.

3.1.4 Identification of NAC I and NAC II in the urine of patients on VPA therapy

3.1.4.1 Identification of NAC I and NAC II in patient samples by GC/MS NICI

The identification and characterization of NAC I in patients on VPA has been reported (Kassahun *et al.*, 1991) and we report here for the first time the identification and characterization of NAC II in humans.

A full NICI mass spectrum of the PFB derivative of NAC II could be obtained in some of the patients studied. Figure 16 shows a typical NICI spectrum of the derivative in the urine extract of one of the patients on VPA. The fragmentation pattern is similar to that of the authentic reference sample as depicted in figure 11 b. In both cases, the mass spectra were characterized by the most abundant $[M-181]^-$ fragment ion at *m/z* 482. However, the spectrum also indicates the presence of other relatively minor ions which probably arose as a result of other minor co-eluting peaks.

Further, under single ion monitoring at *m/z* 482, all urine extracts of patients on VPA (n=39) were positive for both NAC I and NAC II. Figures 17 a and b show typical selected ion current chromatograms in the NICI mode from the PFB-derivatized urine extract of a control sample spiked with NAC I and NAC II in (a) and of a urine sample from a patient on VPA therapy in (b). All samples were run by GC/MS using a DB1701 column under the conditions of GC/MS method B. Similar results were also obtained on a DB101 column as displayed in figures 18 a and b. On both columns, the retention times of the PFB-derivatized NAC I and NAC II in urine extracts of spiked control samples matched those found in the PFB-derivatized urine extracts of patients samples.

The identities of the peaks eluting at t_R =18.46 min and 19.00 min in figure 18 (b) are unknown and need to be investigated further. They did not appear to be present when the analysis was performed on a DB1701 column suggesting the peaks could be co-eluting with the conjugate.

Relative Intensity
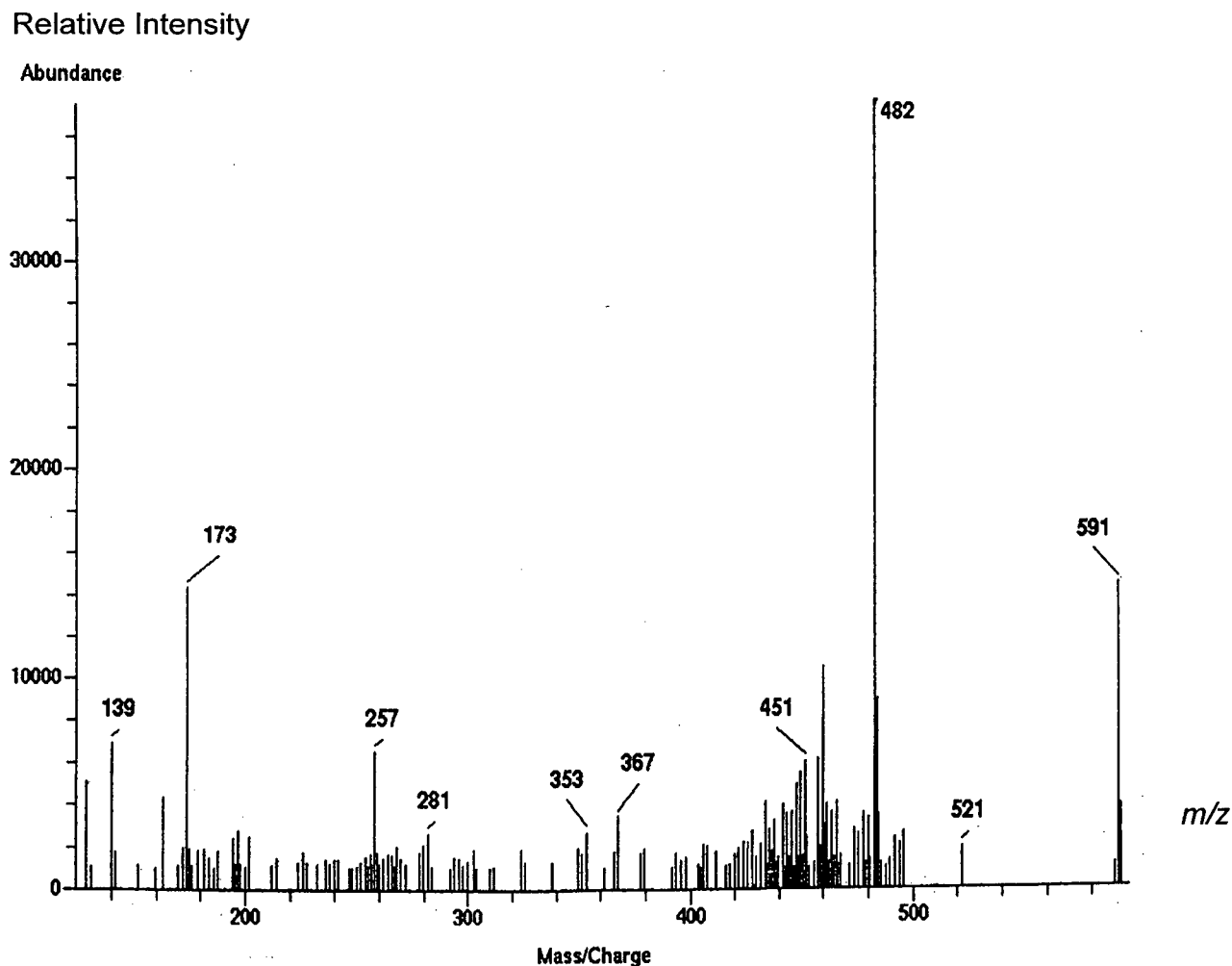
Figure 16. A typical GC/MS NICI mass spectrum of the PFB derivative of NAC II in a derivatized urine extract of a patient on VPA therapy.
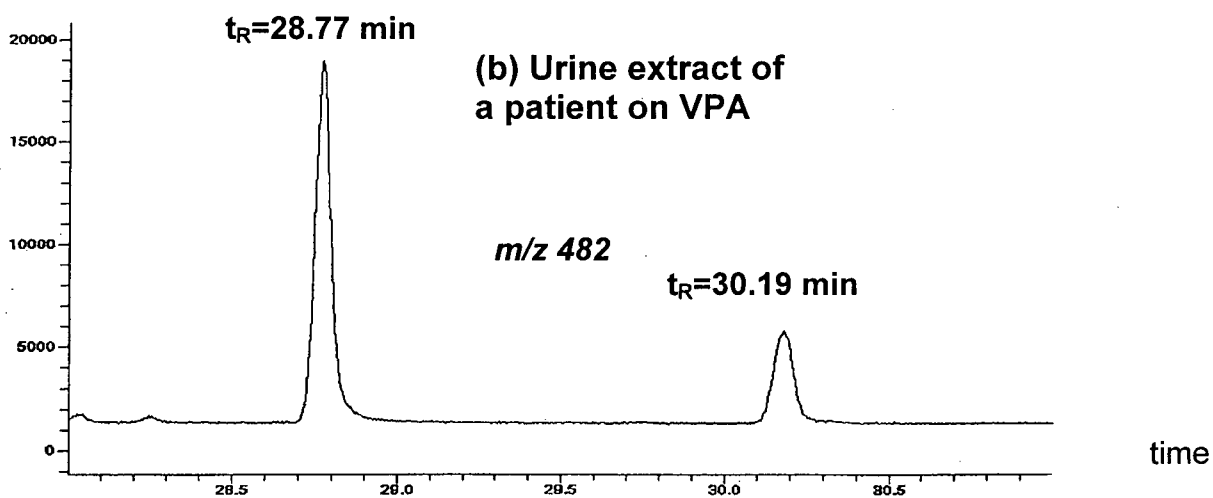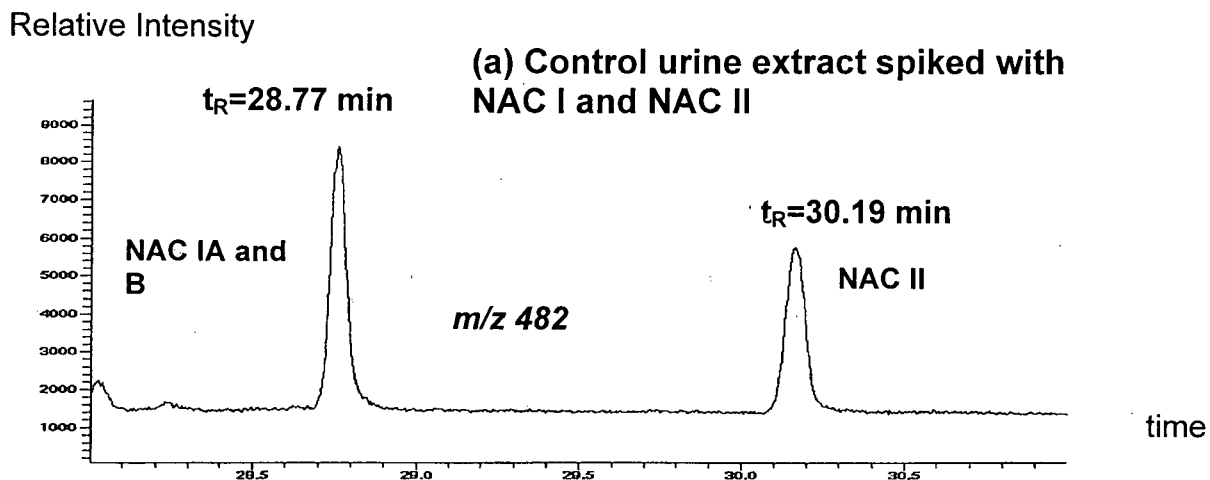
Figures 17. TIC of PFB derivatives of NAC I and NAC II in (a) a spiked control urine

extract and

(b) a urine extract of a patient on VPA and using GC method B and a DB1701 column in

NICI mode (See Experimental/Section 2.2.6.2)
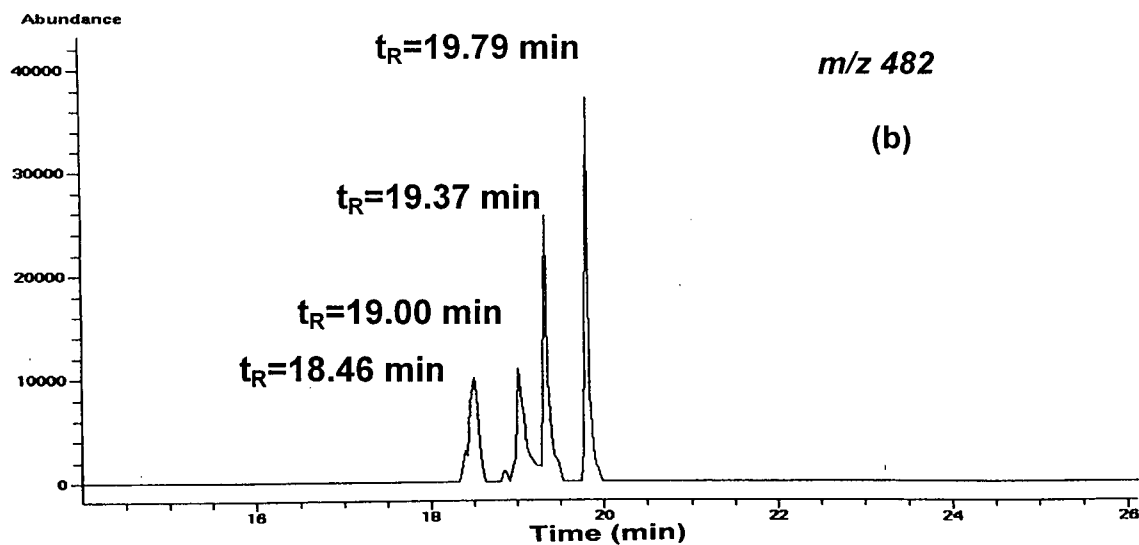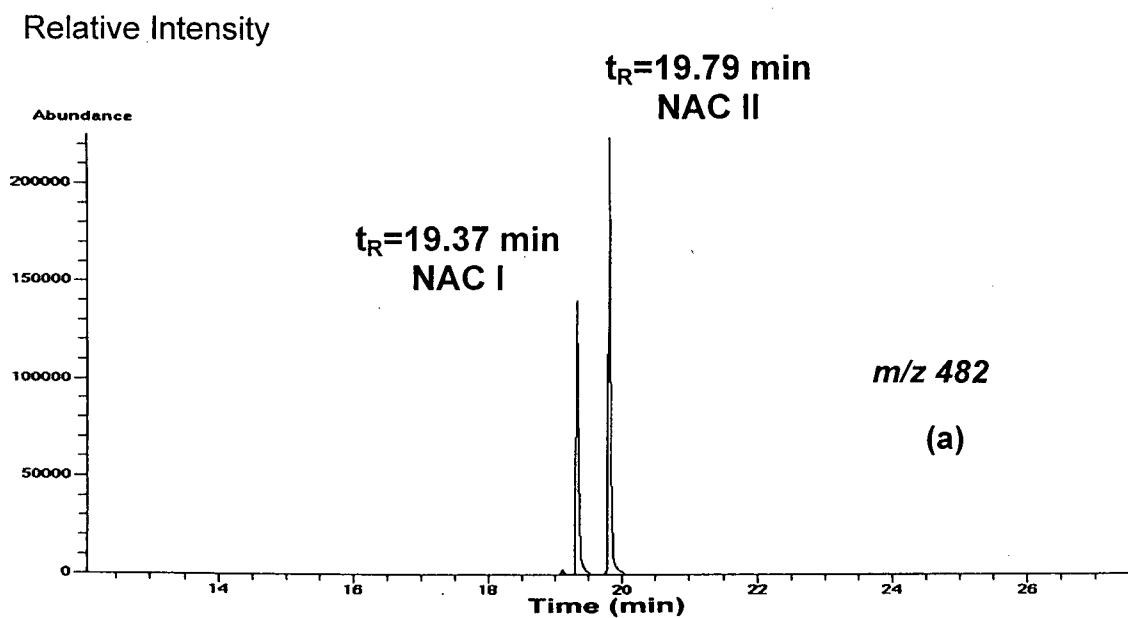
Figures 18. TIC of the PFB derivatives of NAC I and NAC II in (a) the extract of a spiked control urine sample and (b) a urine extract of a patient on VPA using GC method A and a DB101 column in NICI mode (See Experimental/Section 2.2.6.1).

3.1.4.2. Identification of NAC I and NAC II in patient samples by LC/MS/MS

LC/MS/MS analysis using MRM shows the characteristic product ions m/z 123 and 130 present in all of the extracted urine samples from patients (n=39) analyzed. At both MRM transitions, the peaks corresponding to NAC I and NAC II eluted at the same retention times and in the same ratio as observed for the authentic reference samples of both conjugates. The detection of both thiol conjugates in one urine extract of a patient sample is shown in figure 19.
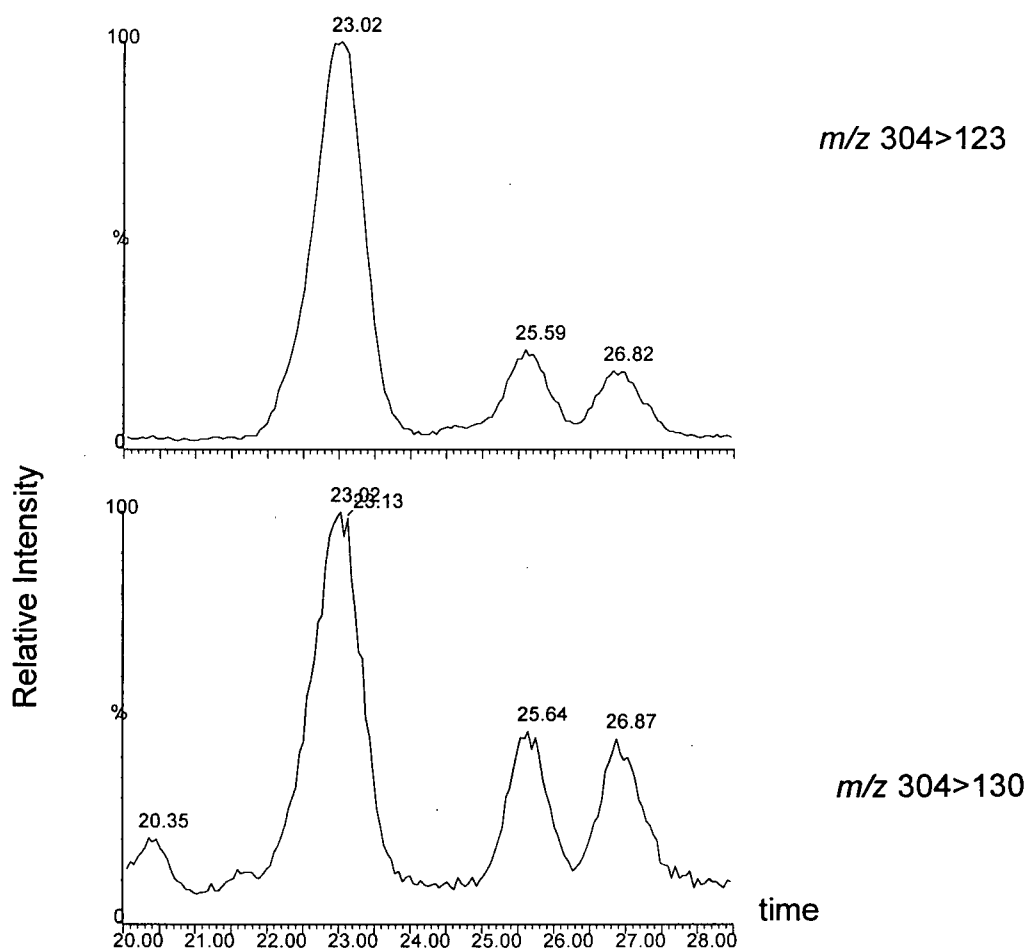
Figure 19. On-line LC/MS/MS detection of NAC II at 23.02 min and NAC I (A and B) at 25.59 and 26.82 min, respectively, in an extract of a urine sample of a patient on VPA therapy. (See Experimental/Section 2.2.5.2, ion transitions for MRM are m/z 304>123, 304> 130).

3.1.5 Attempts to detect 5-NAC-4-OH VPA-γ-lactone (NAC III) in human urine samples

NAC III was discovered as the predominant thiol conjugate of 4-ene VPA in rats dosed with 4-ene VPA (Kassahun *et al.*, 1994; Tang *et al.*, 1996c). To the best of our knowledge, the metabolite has not been identified in humans yet. In this study, the search for NAC III in humans was important in order to verify if a similar parallel exists between humans and rats.

3.1.5.1 GC/MS characteristics of NAC III

The PFB derivative of NAC III produced an NICI mass spectrum dominated by a single and most abundant [M-181]⁻ fragment at *m/z* 302. It appears that the lactone moiety of NAC III remained intact during our derivatization procedure despite the basic environment. The structure of the resulting derivatized product as shown in figure 20. The derivative eluted as one peak at t_R = 18.16 min on a DB101 column, a TIC and a mass spectrum are shown in figure 21. On the other hand, the epimers of the compound were partially resolved on a GC DB1701 column (figure 22) but not on the nonpolar DB101 column. NAC III eluted ahead of NAC I and NAC II when analyzed under identical experimental conditions. No attempt was made to study the fragmentation pattern of NAC III under EI mode conditions.
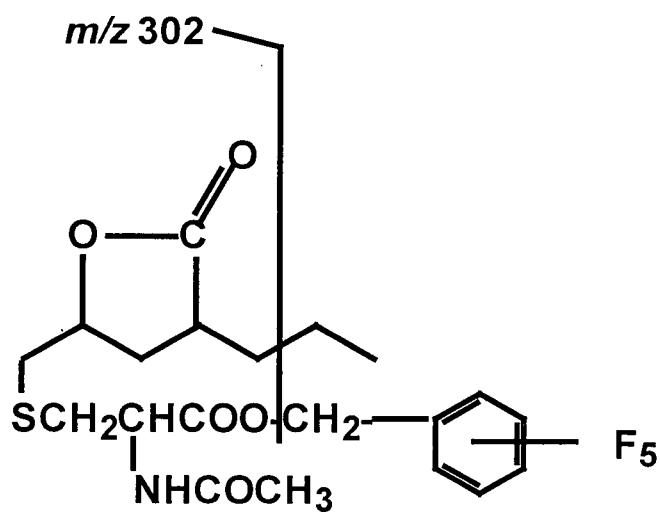
Figure 20. The structure of PFB derivative of NAC III showing the [M-181]⁻ fragment at m/z 302.

Relative Intensity

$t_R = 18.16$ min

(a)

time

Relative Intensity

128

302

(b)

71

173

245

322

380

437

554

m/z
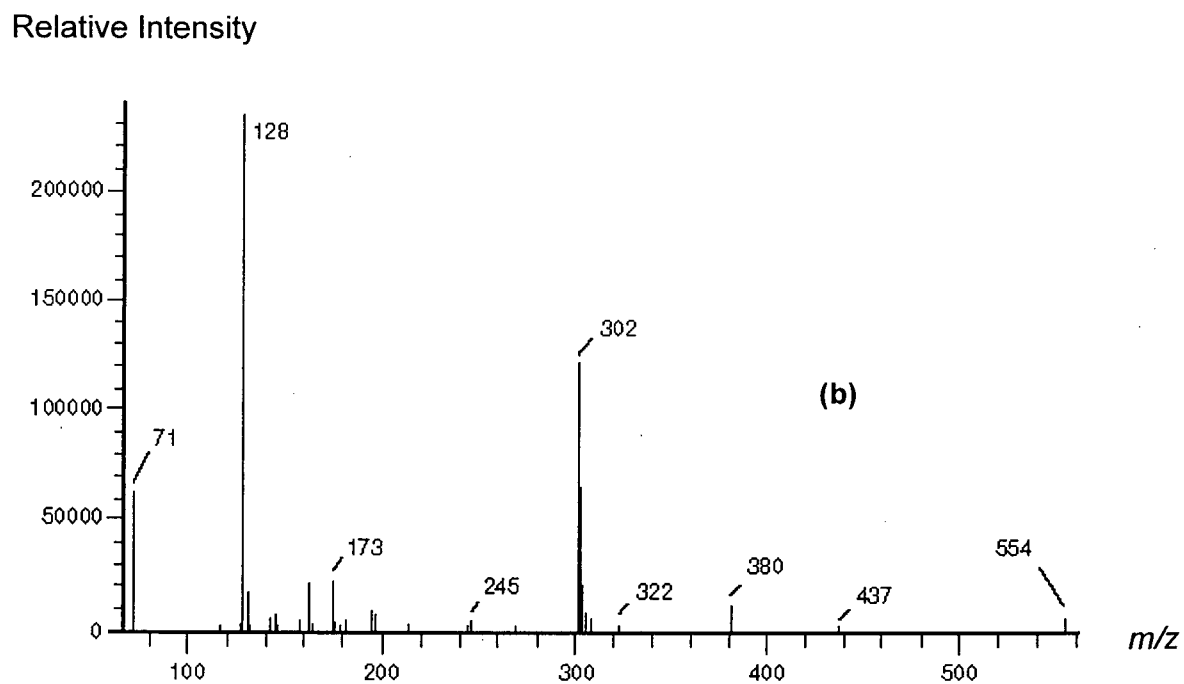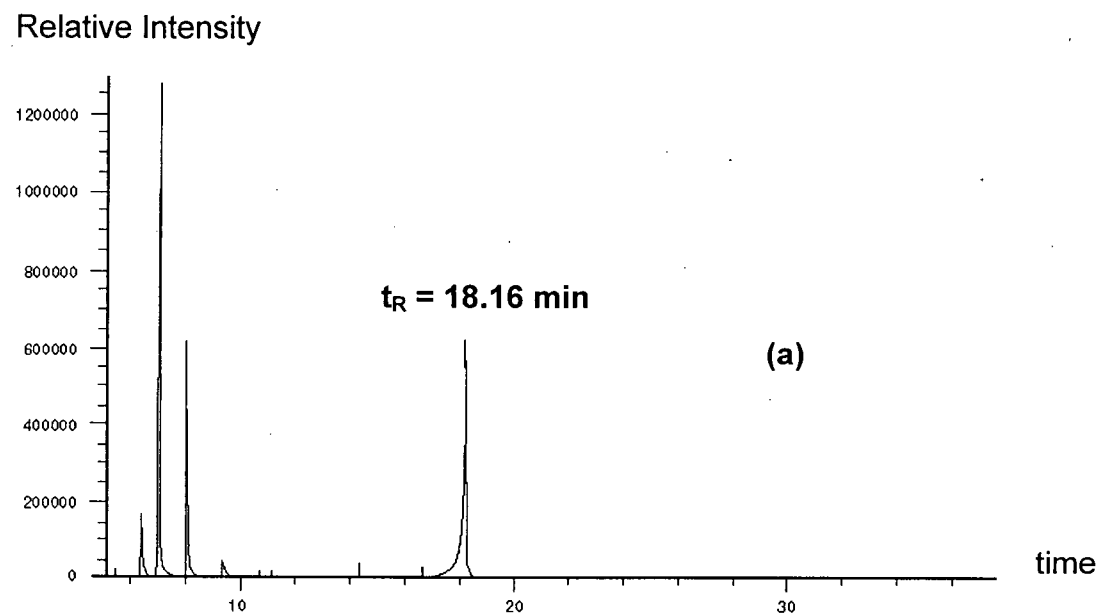
Figure 21 . The TIC of the PFB derivative of NAC III under NICI scan mode in (a) and

the corresponding mass spectrum in (b) using GC method A and a DB101 column in

NICI mode (See Experimental/Section 2.2.6.1).
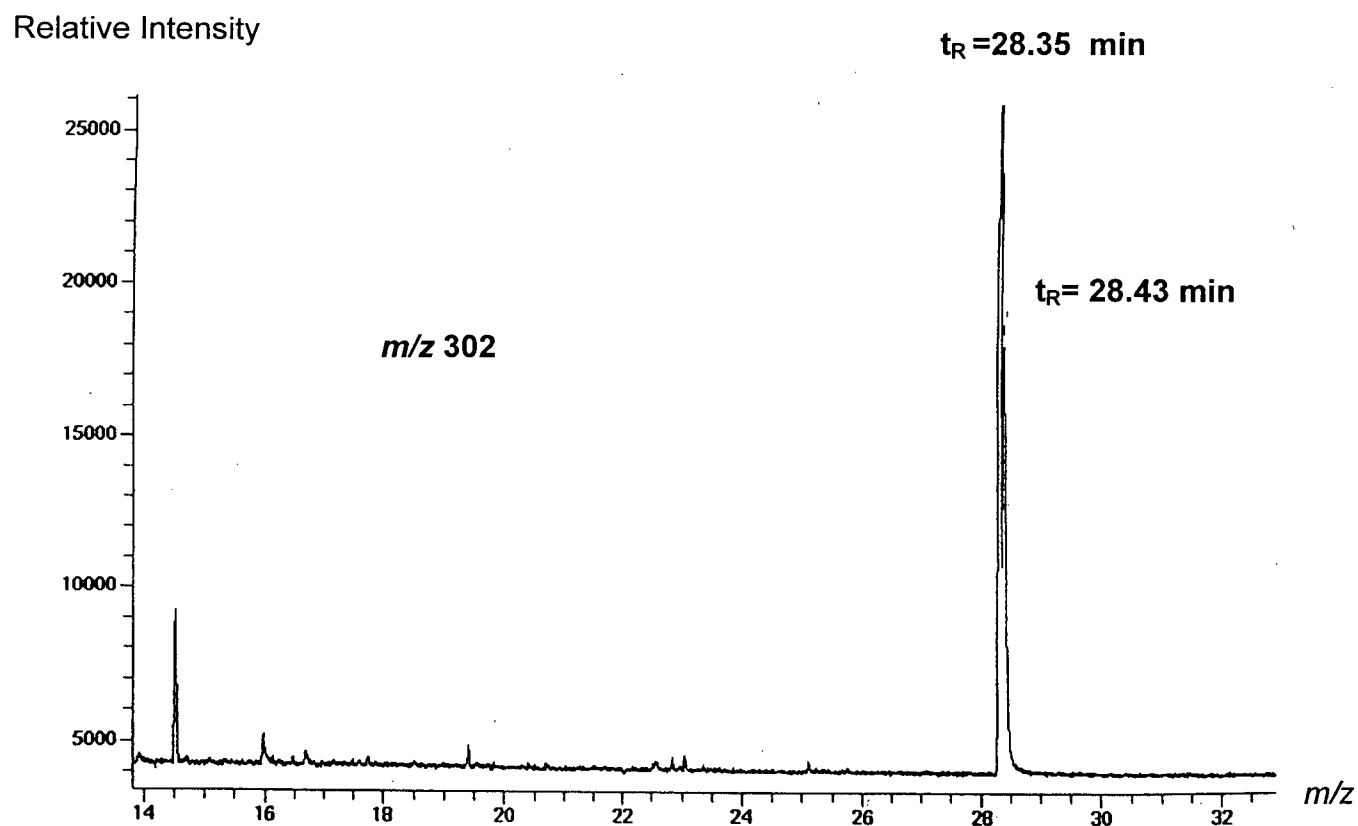
Figure 22. The TIC of the PFB derivative of NAC III under SIM of m/z 302 [M-181']

using GC method B and a DB1701 column in NICI mode. (See Experimental/Section

2.2.6.2)

3.1.5.2 LC/MS/MS analysis of the synthetic standard of NAC III

Under LC/MS/MS method A, NAC III eluted at an earlier retention time (t_R = 14.66 min) than the NAC conjugates of (E)-2,4-diene VPA (figure 14). As reported previously (Tang et al., 1996c), the CID product ion spectrum of MH^+ (m/z 304) of NAC III showed the distinct $[NAC\text{-}S]^+$ fragment at m/z 130. Another product ion at m/z 216 corresponds to the neutral loss of ketene and a terminal COOH group. Figure 23 shows the MS/MS spectrum of NAC III under ES^+.

Relative Intensity
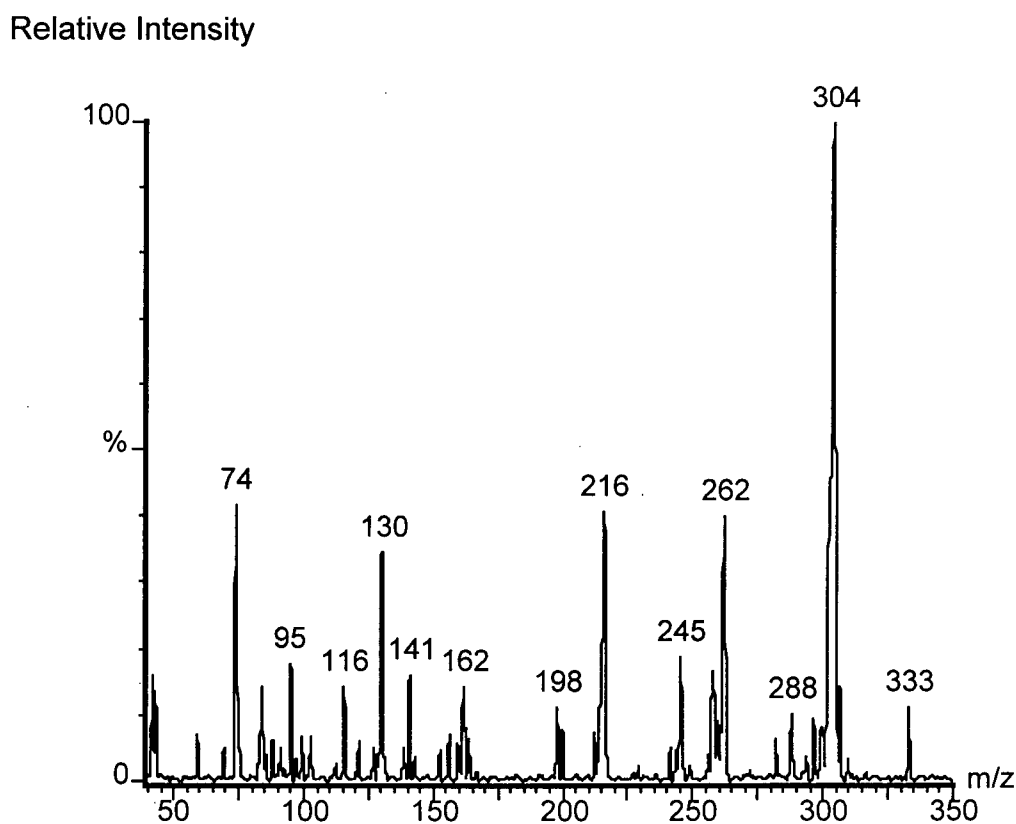
Figure 23. MS/MS product ion spectrum (MH^+ 304) of NAC III under ES^+ conditions.

3.1.5.3 Searching for NAC III and 5-OH-VPA-γ-lactone in the urine samples of patients on VPA therapy

NAC III was not detected in any of the patients samples (n=39) studied either by GC/MS under NICI (figure 24) or by LC/MS/MS using MRM transition 304 to 130 as indicated in figure 25. Our GC/MS method was capable of detecting NAC III concentrations below 10 ng/mL, whereas our LC/MS/MS method was sensitive for concentrations above 25 ng/mL. Furthermore, the TMS derivative of 5-OH-VPA lactone, the metabolic end product of 4-ene epoxide VPA in rats, was not detected in any of the urine samples of patients studied by GC/MS under EI mode.
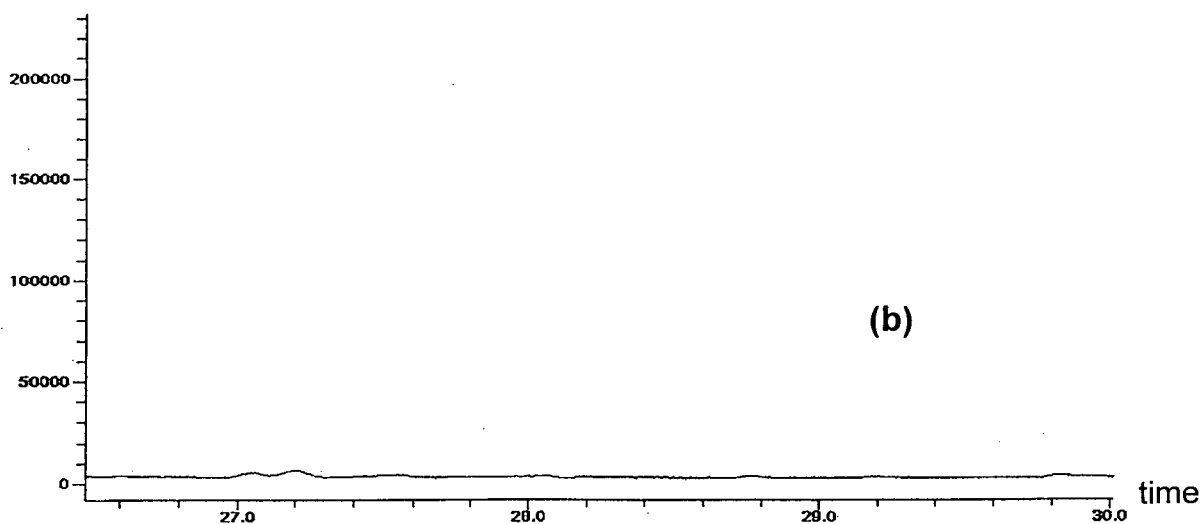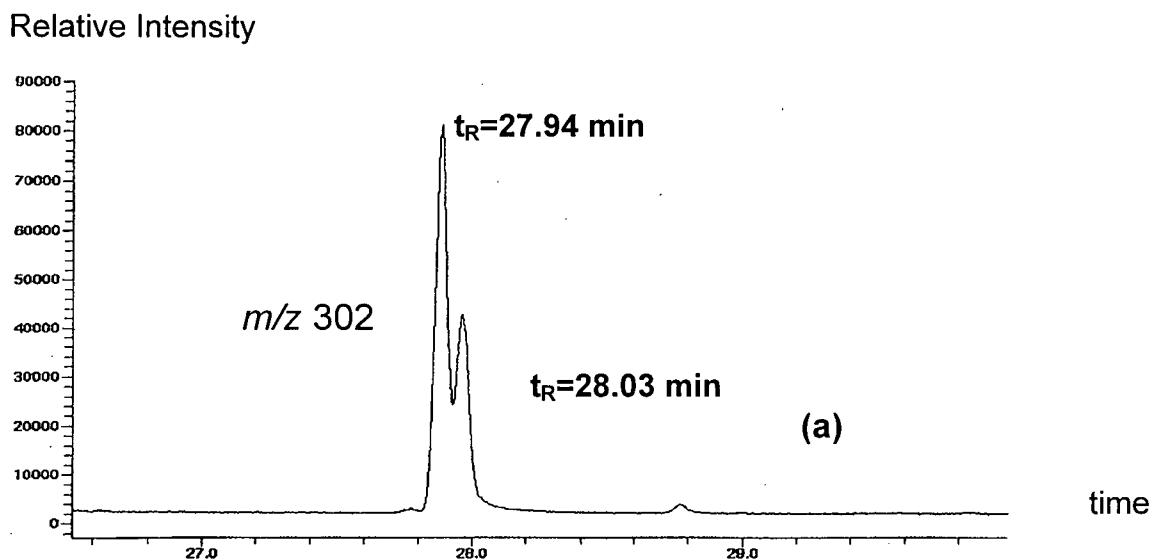
Figure 24. The expanded TIC (SIM, *m/z* 302) of a PFB-derivatized extract of a control

urine sample spiked with NAC III in (a) and the PFB-derivatized extract of a urine

sample of a patient on VPA in (b) using GC method B and a DB1701 (29 m) column in

NICI mode. (See Experimental/Section 2.2.6.2).
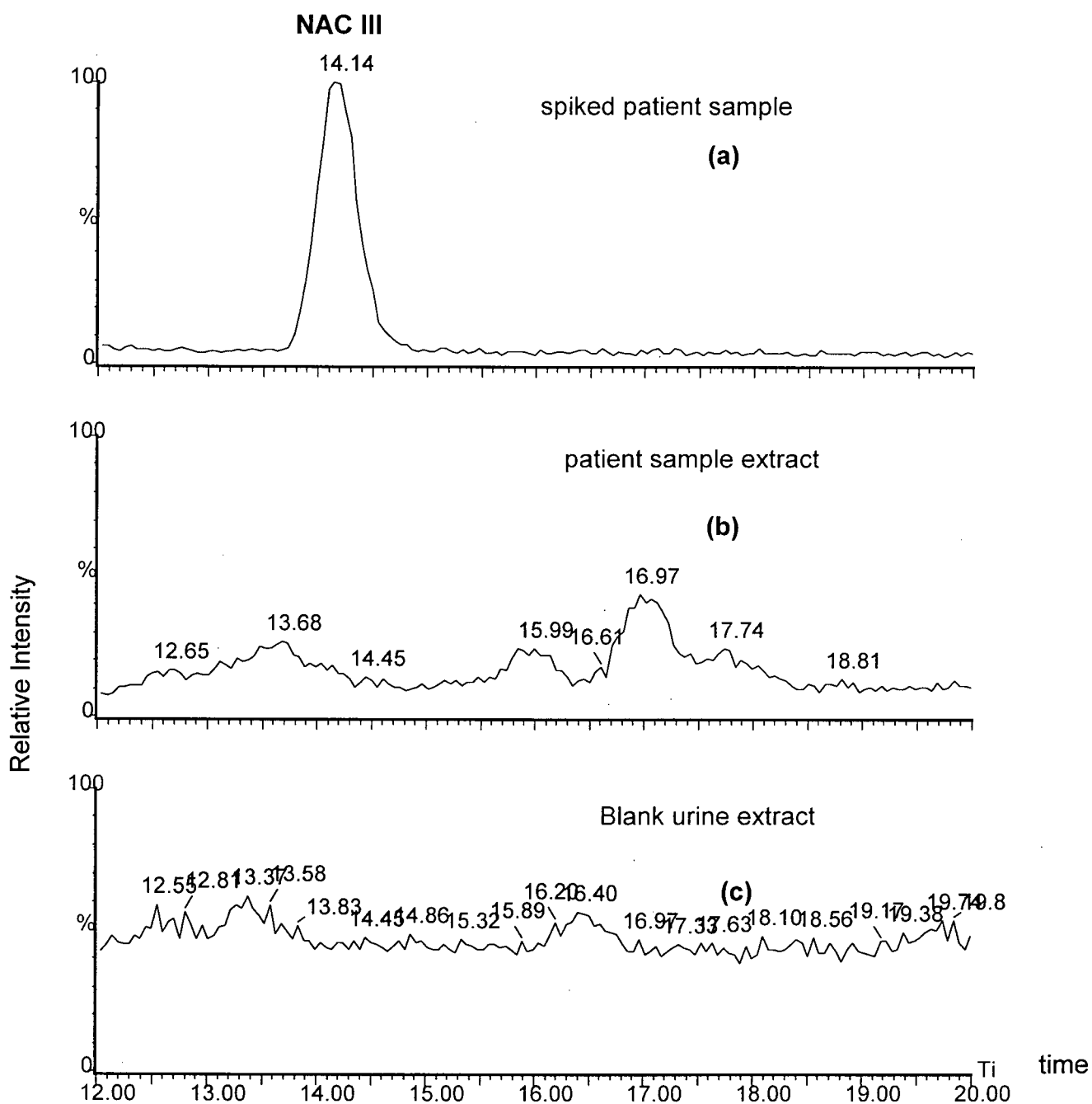
Figure 25. On-line LC/MS/MS monitoring of NAC III in (a) an extract of a control urine sample spiked with NAC III, (b) a urine extract of a patient on VPA and (c) an extract of a blank urine sample. The MRM transition *m/z* 304>130 was monitored. (See LC/MS/MS method A, Experimental/Section 2.2.5.2).

3.1.6 Development of an LC/MS/MS assay for the profiling of NAC I and NAC II in patients on VPA therapy.

The LC/MS/MS assay was a highly selective one developed and validated to be used primarily for the identification and confirmation of both thiol conjugates. However, the concentrations of the thiol conjugates observed in patient samples were below the linearity range of the assay (0.1-1 μg/mL). Therefore, the assay could only provide an estimate of the concentrations of the conjugates present in the samples studied here. The results were further confirmed quantitatively by GC/MS. Positive ion electrospray was the ionization of choice because of its higher sensitivity compared to negative ion electrospray.

The profiling of the conjugates was performed by monitoring the characteristic ES$^+$ MRM transition for m/z 304 to 130 for all three conjugates and a second MRM transition m/z 304 to 123 for NAC II and both isomers of NAC I. The assay allowed for the identification of NAC I and NAC II which was considered positive when the retention time of the eluting peaks at both transitions and their area ratio were similar to that observed for the reference standard as shown in figure 26. The identification of NAC III was based on the retention time of the conjugate at MRM transition m/z 304 to 130 only. Quantitation was based on the detection of the conjugates at MRM transition m/z 304 to 130 .

3.1.6.1. Extraction procedure for the LC/MS/MS assay of NAC I and II

The extraction procedure was designed to include the isolation of NAC I, NAC II and NAC III by solid phase extraction (SPE). The lowest detectable limit of our LC/MS/MS assay was approximately 500 pg (S/N >3), thereby requiring a high extraction efficiency to improve sensitivity. Utilization of C_2 cartridges and a procedure which involved sequential water washes allowed for the extraction of the three conjugates in a relatively clean methanol layer with a high extraction efficiency (100%).

| (A) blank urine extract | (B) patient urine extract | (C) spiked urine extract | MRM of 3 channels |
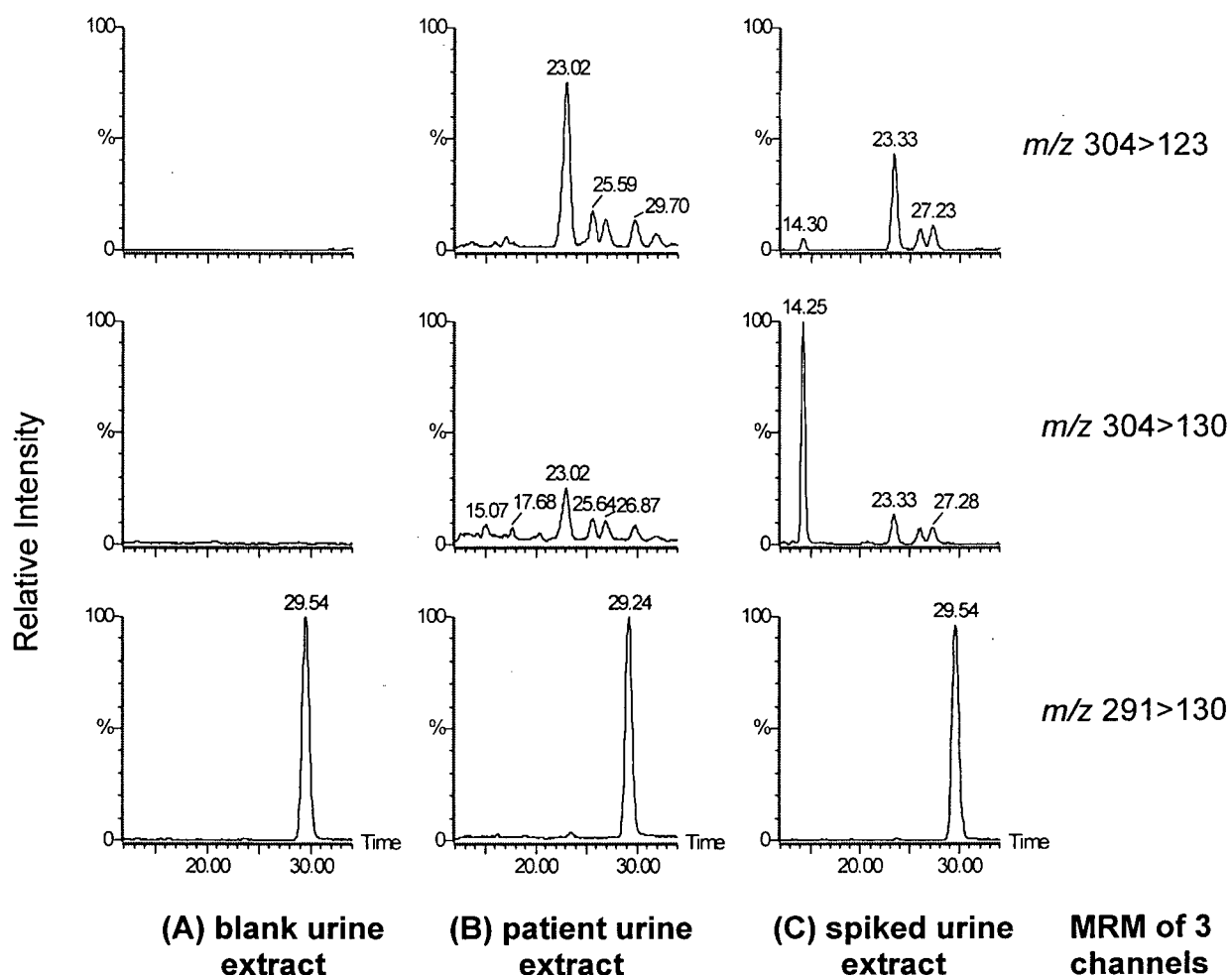
Figure 26. On line LC/MS/MS detection of NAC I and NAC II by MRM of m/z 304 to 123 and 304 to 130 during a quantitation analysis. NAC II eluted at t_R=23.33 min, NAC I at t_R= 27.23 and 27.28 min. FVPA GLN (I.S.) eluted at t_R = 29.54 min by MRM of m/z 291 to 130. (See LC/MS/MS method A in Experimental/Section 2.2.5.2).

Calibration curves for all LC/MS/MS assays for the NAC conjugates were assessed by simple linear regression, slope average regression and weighted ($1/y^2$) linear regression. Consistently, r^2 values were 0.999 or better for all three regressions. However, accuracy was considerably better and similar by slope average and weighted regression. Results obtained by the latter regression will be presented and discussed. Representative calibration curves for NAC I and NAC II are shown in figure 27. Since NAC III was not detected in any of the patient samples studied, we are only presenting validation data for the assay of NAC I and II.

3.1.6.2 Validation of the LC/MS/MS assay for the profiling of NAC I and NAC II in human urine

The precision of the LC/MS/MS assay was assessed by its inter-assay and intra-assay variation.

The inter-assay variation (% CV) based on the slopes of 10 calibration curves analyzed on 10 consecutive analytical days (table 2) was found to be 13.9 % and 15.4 % for NAC I and NAC II, respectively. The inter-assay variation based on quality control study (table 5) was 3-16 % for both conjugates.

The intra-assay variation (% CV) was based on three replicates of two samples run in one assay and four replicate runs of 1 calibration curve (tables 3, and 4) and was found to be less than 11 % for both conjugates.

The accuracy of the assay was based on the observed concentrations of the quality control samples which were found to be within 81-120 % of their expected values for both conjugates (table 5).

The recovery of both conjugates (expressed as % of the measured unextracted concentration) was found to be > 85 % for both conjugates (table 6) and at the two concentrations studied.

REPRESENTATIVE
CALIBRATION CURVE
NAC I *
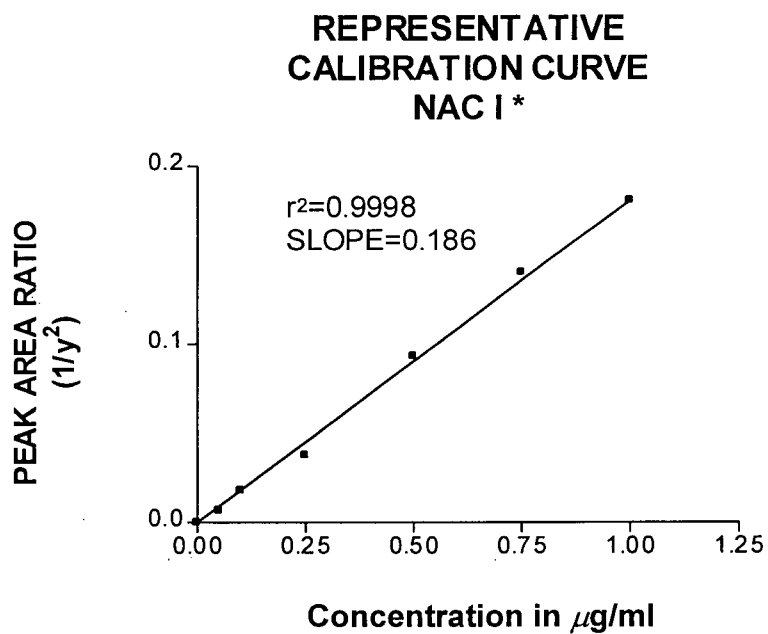
* both isomers of NAC I combined

REPRESENTATIVE
CALIBRATION CURVE
NAC II
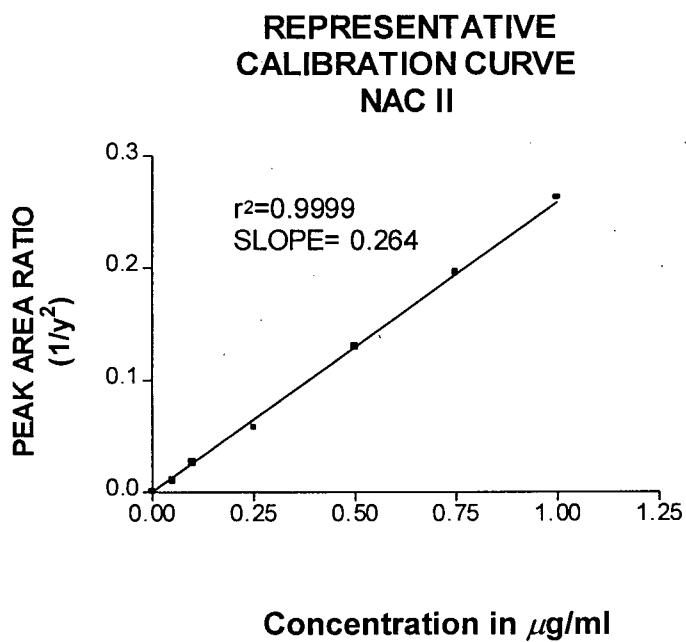
Figure 27. Representative calibration curves of NAC I (top) and NAC II (bottom).

Table 2. LC/MS/MS inter-assay variation based on calibration curves for NAC I and NAC II obtained on 10 consecutive analytical days.

	NAC I			NAC II		
	Slope	Intercept	r^2	Slope	Intercept	r^2
Day 1	0.186	-0.002	0.9996	0.264	-0.002	0.9999
Day 2	0.183	0	0.9999	0.248	0.001	0.9997
Day 3	0.163	0.0018	0.9991	0.249	0.001	0.9996
Day 4	0.162	0.0008	0.9988	0.190	0.002	0.9998
Day 5	0.160	-0.07	0.9998	0.236	0.001	0.9997
Day 8	0.164	0.004	0.9990	0.357	-0.009	1.0098
Day 9	0.132	0.002	0.9998	0.309	-0.004	0.9987
Day 10	0.116	0.003	0.9993	0.219	0.004	0.9997
Mean	0.158	0.0003	0.9994	0.259	-0.001	1.0001
SD	0.022	0.003	0.0004	0.049	0.004	0.0034
C.V%	13.9			15.4		

Table 3. Intra-assay variability of NAC I and NAC II by LC/MS/MS based on three replicates of two quality control samples (QC) of different concentrations obtained on one analytical day.

Spiked	NAC I (n=3)		NAC II (n=3)	
	0.115 μg/mL	0.800 μg/mL	0.115 μg/mL	0.800 μg/mL
QC 1	0.117	0.865	0.136	0.894
QC 2	0.122	0.906	0.126	0.884
QC 3	0.115	0.950	0.115	0.996
Mean	0.118	0.907	0.126	0.925
SD	0.003	0.055	0.009	0.070
C.V.%	2.5	6.25	7.3	7.8

Table 4. Intra-assay variability based on the slopes of one calibration curve run four consecutive times and on one analytical day.

	NAC I		NAC II	
	SLOPE	r^2	SLOPE	r^2
CAL 1	0.163	1.000	0.358	0.9987
CAL 2	0.126	0.995	0.347	1.0710
CAL 3	0.135	0.992	0.320	1.0760
CAL 4	0.125	0.999	0.311	1.1200
Mean	0.137	0.999	0.334	1.0640
SD	0.015	0.003	0.019	0.04
C.V.%	10.9	0.30	5.69	3.80

CAL= calibration curve

Table 5. Quality control study for NAC I and NAC IIby LC/MS/MS obtained over three analytical days for two or three samples of each concentration

NAC I	QC=0.115 μg/mL	QC=0.2207 μg/ml	QC=0.420 μg/ml	QC=0.500 μg/ml	QC=0.800 μg/ml	QC=1.00 μg/ml
Day 1	0.140	0.150	0.383	0.593	0.700	1.03
Day 2	0.143	0.212	0.466	0.554	0.865	0.98
Day 3	0.117	0.216	0.479	0.646	0.783	*
MEAN	0.133	0.192	0.443	0.598	0.783	1.00
SD	0.012	0.030	0.043	0.038	0.067	0.01
C.V. %	8.70	15.6	9.0	6.3	8.56	2.5

NAC II	QC=0.115	QC=0.220	QC=0.420	QC=0.500	QC=0.800	QC=1.00
DAY 1	0.119	0.165	0.426	0.593	0.650	1.00
DAY 2	0.115	0.242	0.413	0.588	0.800	0.92
DAY 3	0.136	0.216	0.501	0.661	0.894	*
MEAN	0.119	0.208	0.447	0.593	0.650	1.00
SD	0.009	0.032	0.039	0.033	0.100	0.04
C.V. %	7.3	14.8	8.7	5.4	12.8	4.0

*=no sample analyzed

Table 6. Percent recovery (%) of NAC I and NAC IIby LC/MS/MS

	NAC I		NAC II	
	0.25 μg/mL (n=3)	0.80 μg/mL (n=3)	0.25 μg/mL (n=3)	0.80 μg/mL (n=3)
Sample 1	113.6	106.7	113.6	100
Sample 2	125.0	100	86.2	97.6
Sample 3	100	100	106.8	98.8
Mean	112.9	102.2	102.2	98.8
SD	10.2	3.2	11.7	0.9
C.V.%	11.1	3.8	14	1.2

Calculated from the peak area ratio of NAC I and NAC II to I.S. in extracted *versus* non-extracted samples.

3.1.7 GC/MS assay for the profiling of NAC I, II and III

3.1.7.1 Development of a GC/MS assay for the quantitation of NAC I, II and III in urine samples of humans

We modified a pre-existing GC/MS assay (Kassahun *et al.*, 1991) for the conjugates of NAC I to include NAC II and NAC III. All three conjugates as well as the internal standard chosen (NAC VI) were easily extracted at pH 2. The assay was sufficiently sensitive enough to quantitate well below 50 pg for all three conjugates. A DB1701 GC column was used as it allowed for the separation of the epimers of NAC III. The epimeric properties of the conjugate were an added trait to recognize the presence or absence of NAC III in samples analyzed. Similar to our HPLC analysis, NAC III eluted ahead of NAC I and NAC II. However, in contrast, NAC I eluted ahead of NAC II. Furthermore, we were unable to separate the isomers of NAC I by GC/MS.

3.1.7.2 Validation results for the GC/MS assay of NAC I and NAC IIin urine samples of humans

The inter-assay variation (% CV) based on the slopes of calibration curves analyzed on eight analytical days was 8 % for NAC I and 25 % for NAC II (table 7). The variation based on quality control samples studied over three to four analytical days was less than 15 % for NAC I and less than 22 % for NAC II (table 8). The intra-assay variation (% CV) based on the replicate analysis of one calibration curve and two quality control samples analyzed on one analytical day was less than 10 % for both conjugates (tables 9 and 10).

Quality control samples of NAC I and NAC II were within 80-120 % of their expected values (table 10), with the exception of one quality control sample of NAC I and NAC II (0.8 μg/mL) which was only 79 and 70 % of the respective expected values.

The recovery of NAC I was 51 to 63 % of measured unextracted samples and that of NAC II was 43 to 69 % of measured unextracted samples (table 11).

The limit of quantitation was set at 0.05 μg/mL; however, precision studies were conducted at 0.115 μg/mL. Studies for the limit of detection were not formally done. The response of the instrument based on area counts showed that the assay could easily detect 10 pg of the compound (signal to noise > 3).

Table 7. **GC/MS calibration curves of NAC I and NAC II obtained on eight consecutive analytical days**

	NAC I			NAC II		
	Slope	r^2	Intercept	Slope	r^2	Intercept
Day 1	0.260	0.9998	0.0008	0.071	0.9999	0.0004
Day 2	0.321	0.9999	-0.0043	0.110	0.9998	-0.0019
Day 3	0.245	0.9999	-0.0036	0.152	0.9999	-0.0024
Day 4	0.274	0.9998	-0.0072	0.152	0.9999	-0.0038
Day 5	0.289	0.9996	-0.1312	0.189	0.9937	-0.0128
Day 6	0.296	0.9999	-0.0051	0.181	0.9999	-0.0025
Day 7	0.274	0.9998	-0.0062	0.133	0.9999	0.0014
Day 8	0.297	0.9995	0.0025	0.142	0.9993	-0.00001
Mean	0.282	0.9998	-0.0193	0.141	0.9990	-0.0027
SD	0.022	0.424	0.0424	0.035	0.002	0.0042
C.V%	7.9	0.01		25.0	0.2	

Table 8. Inter-assay variability based on quality control data obtained on three or four analytical days for four different concentrations.

	NAC I (μg/mL)					NAC II (μg/mL)			
	0.115	0.420	0.800	2.50		0.115	0.420	0.800	2.50
Run 1	0.115	0.324	0.720	2.40		0.090	0.490	1.059	3.59
Run 2	0.133	0.403	0.607	1.68		0.103	0.510	0.723	2.11
Run 3	0.113	0.437	0.752	2.16		0.092	0.543	0.957	2.67
Run 4	0.115		0.680					0.668	
MEAN	0.119	0.388	0.709	2.08		0.095	0.514	0.815	2.79
SD	0.008	0.047	0.059	0.30		0.006	0.022	0.162	0.61
C.V.%	6.7	12.1	8.3	14.4		6.3	3.9	18.8	21.9

Table 9. Intra-assay variation based on the slopes of one calibration curve run four times in one analytical day

	NAC I				NAC II		
	Slope	r^2	Intercept		slope	r^2	Intercept
Run 1	0.260	0.9996	-0.013		0.143	0.9937	-0.013
Rerun 1	0.281	0.9994	-0.018		0.185	0.9994	-0.115
Rerun 2	0.277	0.9995	-0.017		0.182	0.9993	-0.0113
Rerun 3	0.291	0.9996	-0.017		0.192	0.9995	-0.012
Rerun 4	0.280	0.9995	-0.016		0.181	0.9995	-0.010
Mean	0.278	0.9995	-0.0162		0.177	0.9983	-0.012
SD	0.0101	0.0001	0.0017		0.0172	0.002	0.001
C.V.%	3.6	0.01			9.6	0.2	

Table 10. Intra-assay variability and accuracy based on three replicates of two spiked samples of NAC I and II analyzed three or four times in one analytical day

NAC I					
0.115 μg/mL	0.115 μg/mL	0.115 μg/mL	0.800 μg/mL	0.800 μg/mL	0.800 μg/mL
Run 1	0.091	0.106	0.116	0.706	0.840	0.639
Run 2	0.119	0.10	0.123	0.714	0.869	0.643
Run 3	0.105		0.113	0.733	0.866	0.608
Run 4	0.104	0.088				
Mean	0.105	0.098	0.117	0.718	0.858	0.629
SD	0.010	0.008	0.004	0.011	0.013	0.015
C.V.%	9.45	7.64	3.4	1.58	1.52	2.4
% of expected value	91.30	92.5	101.74	89.75	107.3	78.6

NAC II					
0.115 μg/mL	0.115 μg/mL	0.115 μg/mL	0.800 μg/mL	0.800 μg/mL	0.800 μg/mL
Run 1	0.118	0.113	0.154		0.615	0.59
Run 2	0.116	0.132	0.128	0.670	0.906	0.542
Run 3	0.120		0.119	0.720	1.022	0.55
Run 4	0.092	0.120		0.655	1	
Mean	0.112	0.122	0.134	0.682	0.886	0.560
SD	0.011	0.008	0.015	0.028	0.162	0.021
C.V.%	10.12	6.47	10.40	4.08	18.00	3.74
% of expected value	97.4	106.1	116.5	85.3	110.8	70.0

Table 11. Percent recovery of NAC I and NAC II by the GC/MS method

	NAC I				NAC II		
	0.15 μg/mL	1.5 μg/mL	3.5 μg/mL		0.44 μg/mL	4.5 μg/mL	10.0 μg/mL
Run 1	64.0	48.6	59.2		71.9	30.9	71.6
Run 2	48.0	47.3	70.9		61.8	40.7	82.4
Run 3	48.0	57.1	58.2		53.3	57.3	52.3
Mean	53.4	51.0	62.8		62.3	42.9	68.8
SD	7.6	4.3	5.8		7.6	10.9	12.4
C.V. %	14.0	8.5	9.2		12.2	25.0	18.0

3.1.8 Profiling of patients on monotherapy, VPA co-medication and VPA polytherapy by GC/MS NICI and LC/MS/MS

The liver profiles of all the patients studied were assessed by the collaborating neurologist (K. Farrell, M.D., F.R.C.P.) who also assessed and/or reviewed the clinical diagnosis of each patient. Patients assessed with any abnormal liver function tests will be discussed individually. The GC/MS and LC/MS/MS results for the profiling of patients in our study are shown in table 12. The recovery of NAC I and NAC II in 4 patients was found to be less 1% of a VPA dose (table 13).

Table 12. Comparison of GC/MS and LC/MS/MS results (normalized to creatinine) for NAC I and NAC II in patients on VPA.

UBC #	NAC I (µg/kg creatinine) gc/ms	NAC1 (A and B) (µg/kg creatinine) lc/ms/ms	NAC II (µg/kg creatinine) gc/ms	NAC II (µg/kg creatinine) lc/ms/ms
1	0.93	0.74	0.90	1.65
2	0.22	nsq	0.40	nsq
5	0.24	0.20	1.36	0.72
6	1.84	1.07	5.63	2.46
7	0.24	0.33	0.47	0.52
8	0.58	0.59	0.89	0.60
9	0.07	0.08	0.14	0.11
10	0.07	nsq	0.09	nsq
11	0.14	0.17	0.30	0.33
12	0.24	0.29	0.29	0.37
13	0.44	0.46	0.76	0.70
14	0.44	0.45	0.88	0.79
15	0.21	0.28	0.34	0.30
16	0.08	0.16	0.10	0.32
17	0.06	0.11	0.07	0.18
18	0.18	0.21	0.25	0.35
19	0.24	0.30	0.28	0.56
20	0.07	0.11	0.07	0.18
21	0.14	0.23	0.16	0.41
22	0.36	nsq	0.48	nsq
23	0.20	0.23	0.39	0.38
24	0.22	0.28	0.36	0.46

nsq= not sufficient quantity for analysis

Table 12 (continue)

UBC #	NAC I (µg/kg creatinine) gc/ms	NAC1 (A and B) (µg/kg creatinine) lc/ms/ms	NAC II (µg/kg creatinine) gc/ms	NAC II (µg/kg creatinine) lc/ms/ms
25	0.55	0.61	0.61	0.99
26	0.06	0.15	0.08	0.23
27	0.49	0.45	0.60	0.68
qc	0.32	0.27	0.44	0.48
29	0.38	0.41	0.84	0.66
32	0.41	0.54	0.77	0.83
33	0.55	0.56	0.73	0.65
34	0.85	0.99	2.14	1.19
35	0.54	0.18	0.72	0.50
37	0.25	0.34	0.41	0.31
39	0.90	0.92	1.50	3.20
41	0.28	0.32	0.40	0.46
42	0.84	1.02	1.89	1.97
43	0.12	0.10	0.32	0.25
44	1.14	1.40	1.48	1.85

nsq= not sufficient quantity for analysis, qc=a previously analyzed patient sample which is used as a quality control (qc).

Table 13. % recovery of a VPA dose as NAC I and NAC II in four patients

#	NAC I GC/MS	NAC I LC/MS/MS	NAC II GC/MS	NAC II LC/MS/MS
003 a	0.154	0.185	0.246	0.328
003 b	0.211	0.239	0.263	0.337
036 c	0.017	0.014	0.022	0.029
043 a	0.017	0.026	0.030	0.033
043 b	0.055	0.100	0.015	0.017
044 a	0.089	0.137	0.200	0.166
044 b	0.125	0.093	0.138	0.134

a=first 12 h urine sample collected

b=second 12 h urine sample collected

c= 24 h urine sample collected
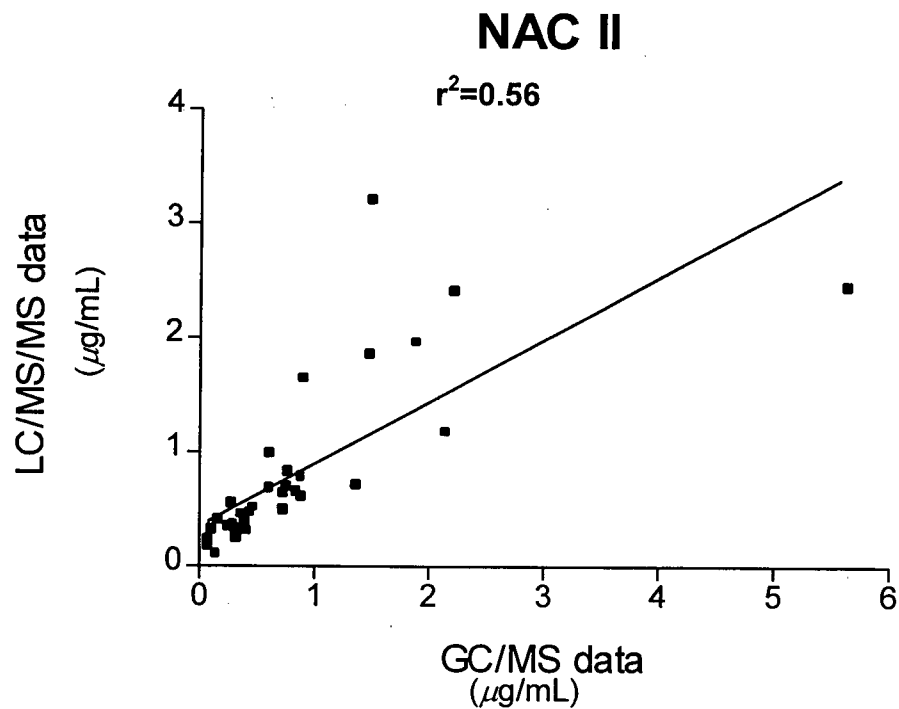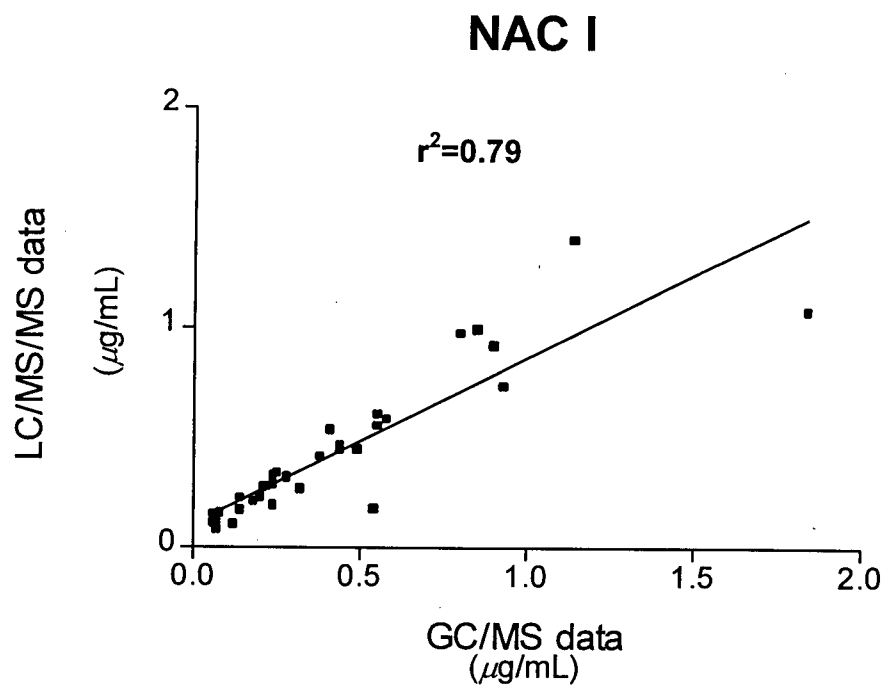
NAC I

NAC II

Figure 28. A graphical comparison of GC/MS *versus* LC/MS/MS data obtained from the profiling of NAC I and NAC II.

3.1.9 Comparison of GC/MS and LC/MS/MS results obtained from the profiling of NAC I and NAC II in patients on VPA therapy

The results obtained for the profiling of the two conjugates in our patient study are graphically compared in figure 28. Both the GC/MS and the LC/MS/MS assays confirmed the presence of NAC I and NAC II and failed to identify NAC III in all samples analyzed. For both NAC I and NAC II, there was little correlation between the GC/MS and LC/MS/MS results beyond 1 μg/mL, the upper limit for the LC/MS/MS assay. During the assay development, we observed a saturation of response for both conjugates at concentrations higher than 1 μg/mL by LC/MS/MS. In contrast, the GC/MS assay had a wider range of linearity (0.1-5 μg/mL) and quantitative analysis was therefore based on our GC/MS assay.

Results for all patients were categorized according to three groups: (1) monotherapy, (2) co-medication, and (3) polytherapy. Patients in our monotherapy group were using VPA as their sole medication, while patients belonging to the co-medication group were treated with VPA in combination with other none enzyme-inducing medications as explained in the Experimental section.

All of our study groups were composed of patients of varied ages. Treatment of the data clearly pointed out that the age associated factor needed to be considered in the interpretation of our results. Statistically, our results showed significant differences (p<0.05) for patients equal to or less that 7.5 years of age compared to those who were older than 7.5 years. Our polytherapy group was composed entirely of patients more than 7.5 years of age.

3.1.10 Summary of results obtained for the profiling of thiol conjugates by GC/MS in the three study groups

The data listed in table 12 were studied with respect to the type of VPA therapy of each patient. The ranges of NAC I and NAC II for each category are summarized in table 14 and 15.

Table 14. Summary of data for NAC I in the three study groups for patients of all ages

	Monotherapy (N=14)	Co-medication (N=12)	Polytherapy (N=8)
Range (μg/mg creatinine)	0.07-0.93	0.06-1.84	0.14-0.90
Mean	0.33	0.46	0.48
SD	0.26	0.51	0.10

Table 15. Summary of data for NAC II in the three study groups for patients of all ages

	Monotherapy (N=14)	Co-medication (N=12)	Polytherapy (N=8)
Range (μg/mg creatinine)	0.10-2.14	0.07-5.63	0.16-1.89
Mean	0.62	0.95	0.79
SD	0.53	1.47	0.56

3.1.11 Results of thiol conjugates observed for the monotherapy study group

The normalized concentrations of NAC I and NAC II obtained for the monotherapy groups are shown in table 16.

Table 16. Results of NAC I and NAC II for the monotherapy study group

U.B.C #	Dose mg/kg	Age Years	NAC I μg/mg creatinine	NAC II μg/mg creatinine	Total NAC μg/mg creatinine
1	35.7	3.75	0.93	0.9	1.83
34	31.45	4.80	0.85	2.14	2.99
5	29.26	5.36	0.24	1.36	1.60
32	35.71	6.87	0.41	0.77	1.18
33	24.50	6.92	0.55	0.73	1.28
15	16.67	8.50	0.21	0.34	0.55
18	9.71	10.75	0.18	0.25	0.43
22	22.94	11.33	0.36	0.48	0.84
2	20.90	12.36	0.22	0.40	0.62
43	7.74	14.67	0.12	0.32	0.55
41	25.32	14.92	0.28	0.40	0.68
16	13.60	16.30	0.08	0.10	0.18
9	3.88	16.60	0.07	0.14	0.21
11	6.20	17.33	0.14	0.30	0.44

Significant difference determined by Mann Whitney, one-way Anova and Newman-Keuls Tests

(a) The means observed for NAC I and NAC II in patients ≤ 7.5 years were significantly greater than those observed in patients > 7.5 years ($p<0.05$).

It appears that both dose and age play an important role in determining the observed concentration of NAC I and II. The results obtained for the monotherapy study group are summarized in table 16. The mean for NAC I observed for the entire study group was 0.33 ± 0.26 and NAC II was 0.62 ± 0.53 μg/mg creatinine (tables 14 and 15). The relationship between age and the urinary excretion of NAC I and NAC II for this study group is shown in figure 29. It is evident that an exponential trend existed between age and concentrations of both conjugates. The means for the conjugates excreted by patients less than or equal to 7.5 years of age were statistically higher ($p<0.05$) than those more than 7.5 year of age. The means for NAC I and NAC II for patients more than 7.5 years old were 0.18 ± 0.09 and 0.30 ± 0.12 μg/mg creatinine. For patients less than or equal to 7.5 years, the mean for NAC I and NAC II were 0.60 ± 0.26 and 1.18 ± 0.53 μg/mg creatinine, respectively. It should be noted however, that the younger patients were also on the higher doses of VPA. The effect of dose is apparent with patients 2, 22, 41 who were receiving a higher dose of VPA (20-25 mg/kg) than those of the same age group or older (<15 mg/kg). In these three cases, the concentrations of the conjugates were above that observed for other patients in their age group. NAC I was above 0.20 μg/mg of creatinine and NAC II was above 0.30 μg/mg creatinine, the mean concentrations observed for their age group. The liver function tests were assessed to be normal.

NAC I

$r^2=0.937$

NAC II

$r^2=0.638$
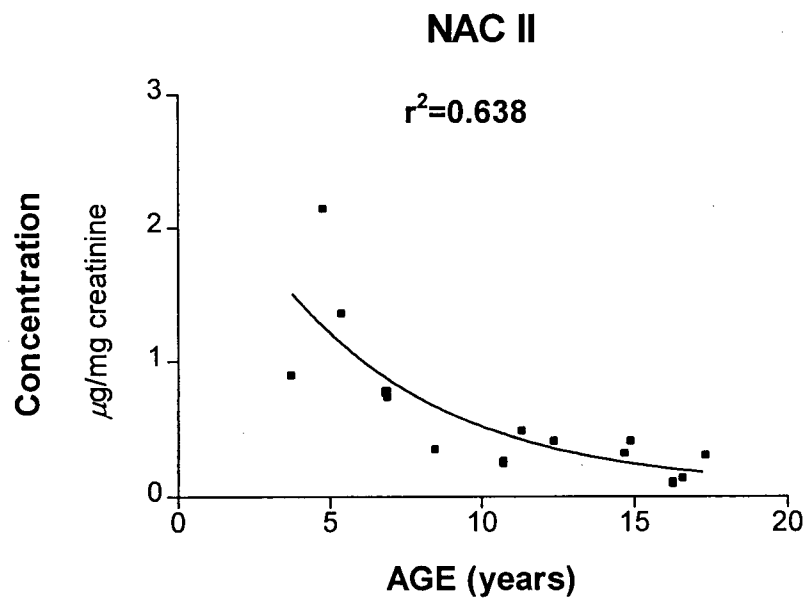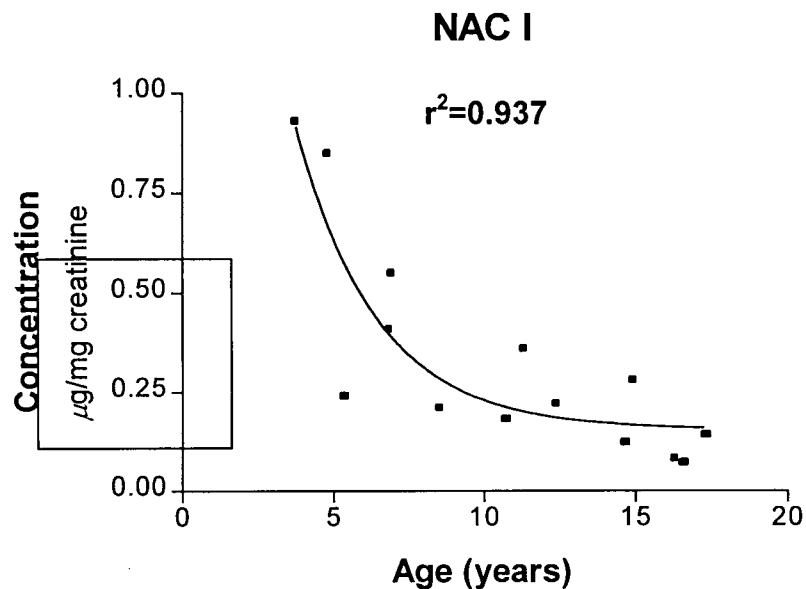
Figure 29. Exponential relationship between age and urinary concentrations of NAC I and NAC II in the monotherapy study group. Patients ≤ 7.5 years old excreted higher concentrations of NAC I and NAC II but they were receiving higher doses of VPA compared to patients > 7.5 years.

3.1.12 Results of the thiol conjugates observed in the co-medication study group

Details of the results obtained for NAC I and NAC II for the co-medication group are shown in table 17.

Table 17. Results of NAC I and NAC II for the co-medication group

U.B.C. #	Dose of VPA mg/kg	Age Years	NAC I μg/mg creatinine	NAC II μg/mg creatinine	Total NAC μg/mg creatinine
6	53.79	2.33	1.84	5.63	7.47
44	33.43	5.33	1.14	1.48	2.62
14	24.51	6.67	0.44	0.88	1.32
8	30.99	7.50	0.58	0.89	1.47
12	23.54	11.17	0.24	0.29	0.53
7	19.74	13.25	0.24	0.47	0.71
20	7.73	15.0	0.07	0.07	0.14
19	14.16	17.08	0.24	0.28	0.52
29	20.1	17.17	0.38	0.84	1.22
10	12.52	19.61	0.07	0.09	0.16
23	21.65	24.38	0.20	0.39	0.59
17	22.0	27.10	0.06	0.07	0.13

Significant difference determined by Mann Whitney test , one-way Anova and Newman-Keuls Tests

a) The means observed for NAC I and NAC II in patients ≤ 7.5 years were significantly higher than those in patients > 7.5 years ($p<0.05$).

The range of concentrations for NAC I and NAC II was 0.06–1.84 and 0.07-5.63 μg/mg of creatinine (tables 14 and 15). The corresponding means were 0.46 ±0.51 μg/mg creatinine for NAC I and 0.95 ± 1.47 μg/mg creatinine for NAC II. For patients ≤ 7.5 years of age, the mean for NAC I was 1 ± 0.55 μg/mg creatinine and for NAC II, the mean was 2.22 ± 1.98 μg/mg creatinine. For patients > 7.5 years of age, the mean of NAC I was 0.19 ± 0.11 μg/mg creatinine and that of NAC II was 0.31 ± 0.24 μg/mg creatinine. An exponential trend is also observed between age and the excretion of thiol conjugates in this study group as shown in figure 30. Among the patients > 7.5 years of age, three patients were observed to have concentrations of the thiol conjugates higher than expected for their age, as detailed below.

Patient # 29:

The concentration of the NAC conjugates for this 17 year old patient appeared to be abnormally high. There was no clinical abnormality reported to us to explain the observation made here. The urinary VPA metabolite profile (not included in this thesis) for this patient appeared to be normal. There was no indication that this patient was still in the absorption phase when the sample was collected. Under the conditions of our experiment, this patient appeared to be outside of the normal range for patients of similar age group when compared to both the monotherapy and co-medication study groups. The patient was on VPA and clobazam. A second patient (# 14), also on VPA and clobazam, showed a high level of the conjugates but the subject was less than 7.5 years of age.

NAC I

$r^2=0.937$

CONCENTRATION
µgmmg creatinine

AGE (years)

NAC II

$r^2=0.980$

CONCENTRATION
µgmmg creatinine

AGE (years)
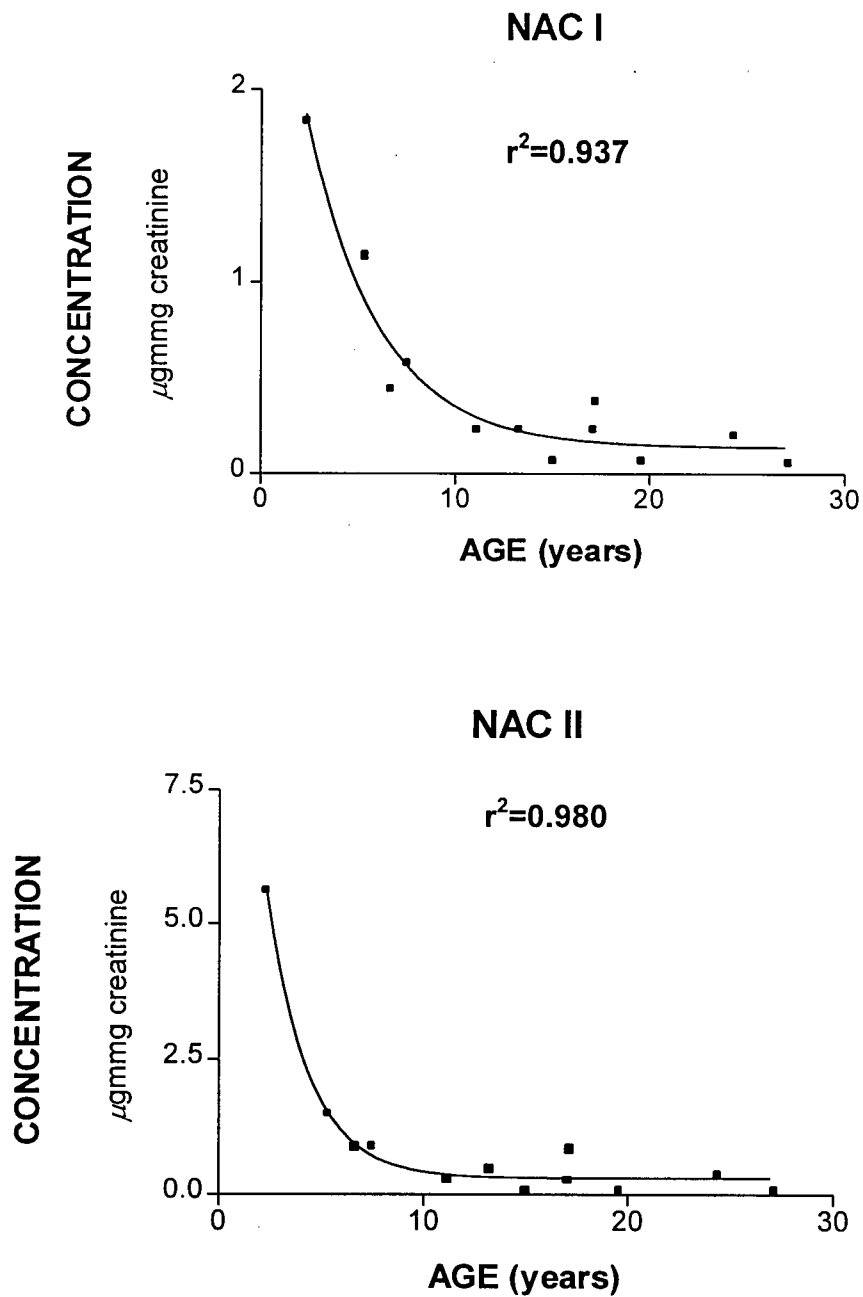
Figure 30. Exponential relationship between age and NAC I and NAC II in the co-medication study group. Patients ≤ 7.5 years old received higher doses of VPA and excreted higher concentrations of NAC I and II.

Patient # 19:

This 14 year old patient was on VPA and lamotrigine. In this case, the patient was known to be susceptible to mitochondrial disease, a condition which was common in his family. In May (1995), this patient showed a high urinary alanine concentration which is normally associated with this disease. Furthermore, this patient's seizure control was very poor while on both drugs. The phase I VPA urinary metabolite profile showed an unusually high concentration of 3-keto VPA (757 μg/mg creatinine). NAC I and NAC II in this patient were 0.24 and 0.28 μg/mg creatinine. Although, this result was not considered as an outlier, it was higher than the results for patients who are younger but within the same group (> 7.5 years).

Patient # 23:

At the time of the collection of the urine specimen, this 24 year old patient was treated with VPA, ethosuximide, lamotrigine and NAC. The biochemical profile of this patient provided to us showed an abnormally low glutathione peroxidase level and was considered clinically to be selenium-deficient. Furthermore, his biochemical profile also indicated a high lipid peroxide index, an indication of free radical burden. The profile did not show a compensation by GST nor GSSG reductase. The remainder of the profile was considered normal by the investigating neurologist. The patient's seizure activity was not being controlled by VPA and lamotrigine and following treatment with vitamin E, an antioxidant, the condition of the patient improved. It was the neurologist's opinion that this was the result of the new treatment. NAC was further added as part of his therapy. The patient's thiol conjugate levels were 0.20 μg/mg creatinine (NAC I) and

0.39 μg/mg creatinine (NAC II). Although, within the normal range in comparison to the monotherapy group, the results for this patient were higher than that for younger patients of the same group.

3.1.13 Effect of age on the excretion of NAC I and NAC II in the monotherapy and co-medication groups combined.

No statistical differences were observed between the means of the conjugates for the monotherapy and the co-medication groups when similar age groups were compared. It appeared that both groups are similar to each other and can be combined to increase sample size.   A graphical comparison of age *versus* concentration of NAC I and NAC IIin the combined group shows that age appeared to be exponentially related to the concentration of NAC I and NAC II (figure 31).

NAC I

$r^2=0.845$

NAC II

$r^2=0.873$
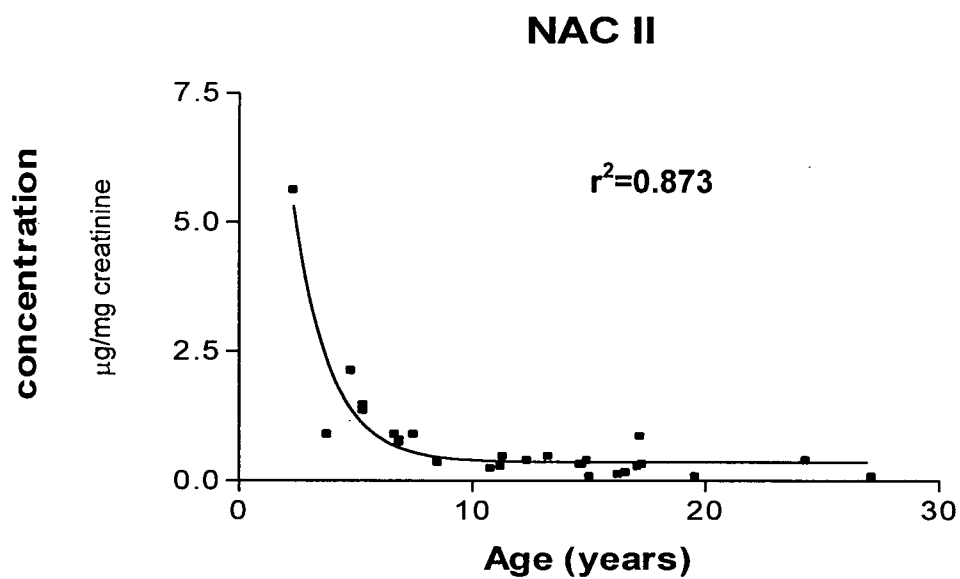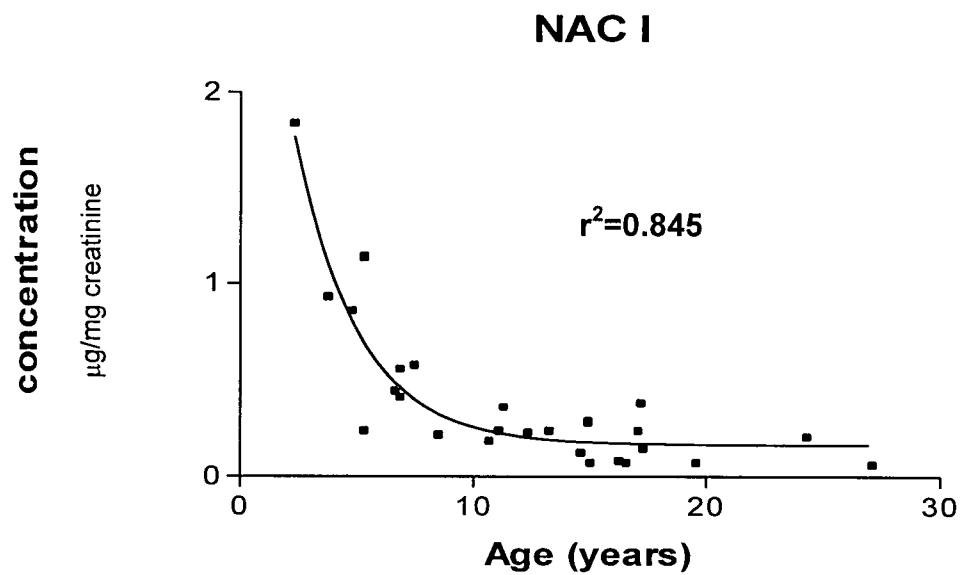
Figure 31. Exponential relationship between age and NAC I and NAC II in the monotherapy and co-medication groups combined.

3.1.14 Relationship between age and dose in the monotherapy and co-medication groups

Figure 32 shows that there was an inverse relationship between mg/kg dose and age and the youngest patients received the highest daily dose of VPA.
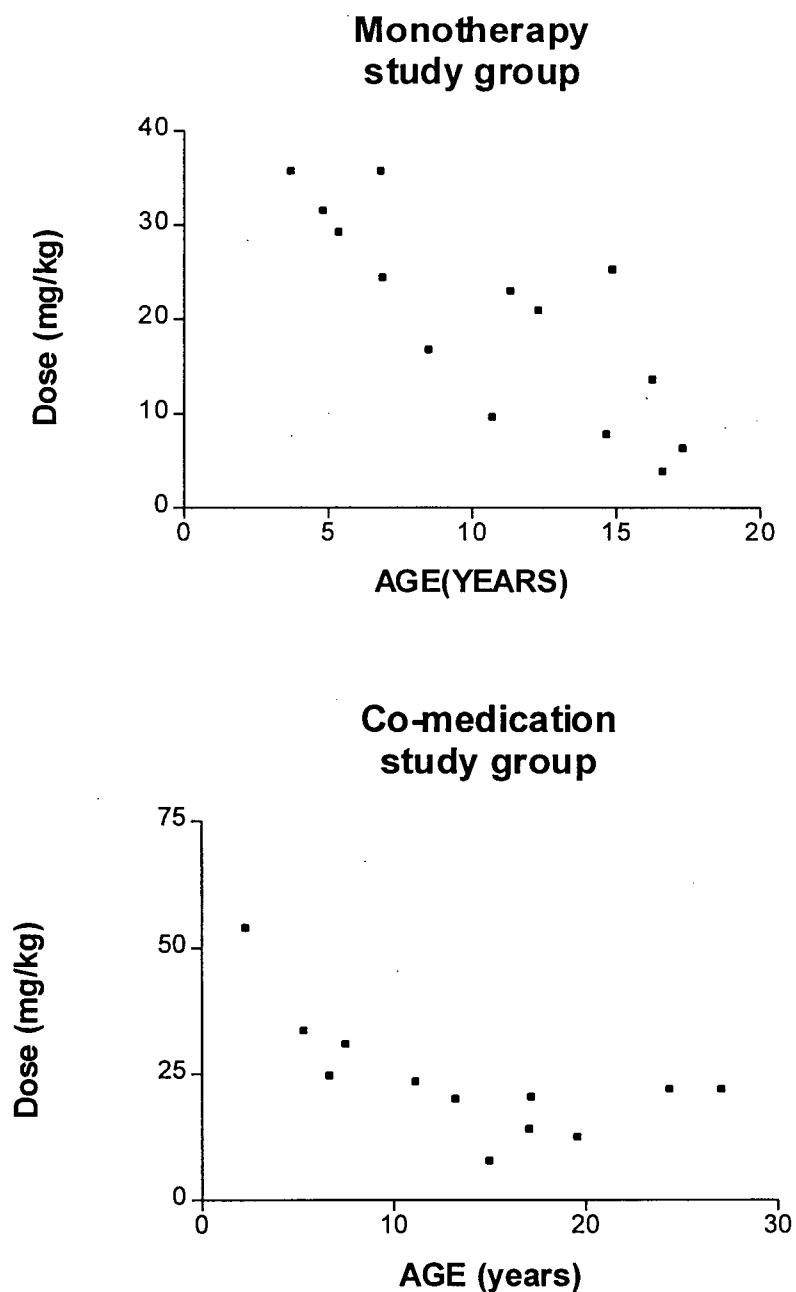
Figure 32. Inverse relationship between age and dose in the monotherapy (top) and co-medication groups (bottom).

3.1.15 Results of the thiol conjugates observed for the polytherapy study group

The concentrations of NAC I and NAC II measured in urine samples of patients on VPA polytherapy are shown in table 18.

Table 18. Results of NAC I and NAC II for polytherapy study group (> 7.5 years old)

UBC #	Dose of VPA (mg/kg)	Age years	NAC I μg/mg creatinine	NAC II μg/mg creatinine	Total NAC μg/mg creatinine
27	35.2	7.83	0.49	0.60	1.09
13	46.82	8.95	0.44	0.76	1.20
37	20.92	14.33	0.25	0.41	0.66
39	44.6	14.67	0.90	1.50	1.45
24	47.4	15.17	0.20	0.36	0.56
42	8.93	17.0	0.84	1.89	2.73
25	19.0	19.70	0.55	0.61	1.16
21	20.94	22.50	0.14	0.16	0.30

The ranges of concentrations observed for our polytherapy study group were 0.14-0.90 μg/mg of creatinine for NAC I and 0.16-1.89 μg/mg of creatinine for NAC II (tables 14 and 15).

The concentration of NAC I and NAC II obtained for both monotherapy and co-medication groups were studied statistically with respect to age. The results show that the means of the conjugates for patients equal to or less than 7.5 years of age were statistically greater ($p < 0.05$) than those observed for older patients (more than 7.5

years) in both study groups. The means of the conjugates for the polytherapy group were statistically greater than those observed for patients more than 7.5 years of age in the combined monotherapy and co-medication groups. A comparison of the ranges and means of the conjugates for the two groups are shown in tables 19 and 20.

The means of the conjugates for the patients on VPA polytherapy were not statistically different from the means of the conjugtaes for patients ≤ 7.5 years old in the combined monotherapy and co-medication groups.

Table 19. Comparison of data for NAC I for patients > 7.5 years in all the study groups

	Monotherapy + Co-medication (N=17)	Polytherapy (N=8)
Range (μg/mg creatinine)	0.06-0.38	0.14-0.49
Mean	0.19	0.48
SD	0.10	0.28
(Mann Whitney) $p=0.008$		

Significant difference determined by Mann Whitney

(a) The mean observed for NAC I was significantly higher in patients on VPA polytherapy compared to those on monotherapy and co-medication ($p<0.05$).

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Table 20. Comparison of data for NAC II for patients > 7.5 years in all the study groups

	Monotherapy + Co-medication (N=17)	Polytherapy (N=8)
Range (μg/mg creatinine)	0.07-0.40	0.16-1.89
Mean	0.31	0.79
SD	0.19	0.60
	p=0.011	

Significant difference determined by Mann Whitney test

(a) The mean observed for NAC II was significantly greater in patients on VPA polytherapy compared to those on monotherapy and co-medication ($p<0.05$).

3.2 Profiling of phase I metabolites of VPA in urine samples of patients on VPA

3.2.1 GC/MS assay for the phase I metabolic profiling of VPA

During the course of this research thesis we developed and validated GC/MS NICI assays for the profiling of VPA and 14 of its phase I metabolites in human serum and urine (Gopaul *et al.*, 1996a). For the purpose of this dissertation, we present only the data that serves to supplement the study of the thiol conjugates. Thus, we include here the results for the profiling of total urinary VPA, 4-ene VPA and the β-oxidation metabolites (*E*)-2,4-diene VPA, 2-ene VPA and 3-keto VPA.

3.2.2 Results of phase I metabolic profiling in urine samples of patients on VPA

The patients were categorized according to their type of VPA therapy. The results are tabulated below in order of increasing age (tables 21-23). There were several important observations with regard to the trends, the ranges and the means in each category. Although the youngest patients in the study showed high levels of VPA (211-5088 μg/mg of creatinine), those on polytherapy exhibited the highest concentrations of (*E*)-2,4-diene VPA and 4-ene VPA (Table 24). Interestingly, the highest concentrations of (*E*)-2-ene VPA and 3-keto VPA were observed in the youngest patients.

An evaluation of the trends (tables 21-23) showed that although in general, the observed ranges appeared to be lowest in the non-induced patients who were more than 7.5 years old (group C, table 24), the concentration of VPA and metabolites did not show a clear pattern with age within the latter group itself (table 22). In contrast, in the polytherapy patients and non-induced patients less than or equal to 7.5 years old

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(tables 21, 23), the concentrations of VPA and metabolites appeared to decrease with age. Based on trends observed, it appeared that polytherapy induced 4-ene VPA and (*E*)-2,4-diene VPA, but not 3-keto VPA and (*E*)-2-ene VPA. On the other hand, young age appeared to increase the formation of (*E*)-2-ene VPA and 3-keto VPA more than 4-ene VPA and (*E*)-2,4-diene VPA. However, a statistical analysis of the means (Mann Whitney) did not show a difference at $p < 0.05$. A comparison of 4-ene and (*E*)-2,4-diene VPA between the two age groups in the non-induced patients resulted in p values of 0.08 and 0.052, respectively (table 25). A comparison of the same metabolites between polytherapy patients and non-induced patients older than 7.5 years produced p values equal to 0.05 and 0.052. Perhaps with a larger sample size, a difference may be more statistically apparent.

Table 21.   Results of phase I metabolic profiling of VPA in monotherapy and co-medication patients ≤ 7.5 years old

UBC#	VPA (μg/mg creatinine)	4-ene VPA (μg/mg creatinine)	(*E*)-2,4-diene VPA (μg/mg creatinine)	(*E*)-2-Ene VPA (μg/mg creatinine)	3-keto VPA (μg/mg creatinine)
6	5088	1.67	4.7	94.40	1287.6
1	211	0.10	0.45	6.05	705.9
5	1466	0.39	2.39	36.08	1650.0
14	967.7	0.39	2.10	28.70	708.4
8	829.0	0.32	2.45	37.30	259.5
Range	211-5088	0.10-1.67	0.45-4.70	6.05-94.40	259.5-1650.0

Table 22. Results of phase I metabolic profiling of VPA in monotherapy and co-medication patients > 7.5 years old

UBC #	VPA (μg/mg creatinine)	4-ene VPA (μg/mg creatinine)	(E)-2,4-diene VPA (μg/mg creatinine)	(E)-2-Ene VPA (μg/mg creatinine)	3-Keto VPA (μg/mg creatinine)
15	0	0	0.42	0.64	105.8
18	580	0.20	1.06	15.56	369.0
12	554.4	0.47	2.46	22.4	528.4
22	488	0.39	3.94	34.99	517.7
2	555	0.37	1.57	24.98	609.4
7	330.6	0.05	0.28	0.77	534.1
20	192.4	0.09	0.65	5.78	452.7
16	85.6	0.04	0.78	8.92	279.8
9	24.5	0	0.06	1.05	189.3
19	1362.3	0.68	2.34	17.40	761.5
29	568.5	0.27	1.85	11.42	536.8
11	208	0.08	0.41	4.53	1448.7
10	86.34	0.10	0.04	2.27	91.4
23	331.5	0.21	1.22	10.54	47.6
17	686.6	0.36	1.72	19.67	570.7
Range	0-1362.3	0-0.68	0.04-3.94	0.64-34.99	47.6-1448.7

Table 23. Results of phase I metabolic profiling of VPA in polytherapy patients.

UBC #	VPA (μg/mg creatinine)	4-ene VPA (μg/mg creatinine)	(E)-2,4-diene VPA (μg/mg creatinine)	(E)-2-Ene VPA (μg/mg creatinine)	3-Keto VPA (μg/mg creatinine)
26	783.0	0.67	4.68	39	768.5
27	806.0	0.67	2.46	9.74	414.7
13	3717.0	4.78	5.65	25.62	684.1
24	3353.0	4.98	2.42	18.10	436.7
25	454.3	0.31	1.51	12.212	310.5
21	16.9	0.13	1.08	3.48	7.0
Range	16.9-3717	0.13-4.98	1.08-5.65	3.48-25.62	7.0-684.1

Note patient #26 is the only patient less than 7.5 years and the range established for this group does not include the results for patient # 26.

Table 24. Summary of ranges observed for VPA and phase I metabolites of VPA in 3 study groups.

	VPA (μg/mg creatinine)	4-ene VPA (μg/mg creatinine)	(E)-2,4-diene VPA (μg/mg creatinine)	(E)-2-ene VPA (μg/mg creatinine)	3-keto VPA (μg/mg creatinine)
A	211-5088	0.10-1.67	0.45-4.70	6.05-94.40	259.5-1650.0
B	0-1362.3	0-0.68	0.04-3.94	0.64-34.99	47.6-1448.7
C	16.9-3717	0.13-4.98	1.08-5.65	3.48-25.62	7.0-684.1

A=monotherapy and co-medication patients ≤ 7.5 years, B=monotherapy and co-medication patients > 7.5 years; C=polytherapy patients.

Table 25. Comparison of means observed for 4-ene VPA and (*E*)-2,4-diene VPA in the two study groups

	Monotherapy and co-medication (μg/mg creatinine)	Polytherapy (μg/mg creatinine)	Mann Whitney P
Patients $\leq$ 7.5 years			
4-ene VPA	0.57±0.56		
(*E*)-2,4-diene VPA	2.42±1.36		
Patients > 7.5 years			
4-ene VPA	0.21±0.20	2.18±2.22	0.08
(*E*)-2,4-diene VPA	1.25±1.05	2.62±1.60	0.052

3.3 Amino acid conjugation of VPA

The advent of LC/MS/MS was paramount for the identification and profiling of the newly discovered amino acid metabolites of VPA in both humans and animals. Only some of the results obtained by LC/MS/MS could be reproduced by GC/MS as well. This, perhaps, explained in part the reason why the amino acid conjugation pathway of VPA metabolism has remained relatively unexplored until now.

3.3.1 Characterization of AA conjugates of VPA by LC/MS/MS

3.3.1.1 HPLC characteristics of AA conjugates of VPA

Initial studies utilized a (LC/MS/MS method A) mobile phase of MeOH/water (43:57, 0.025 % TFA and PPA each) for the HPLC analysis of the conjugates and the same run time program as applied to the thiol conjugates. Because of the high sensitivity of the LC/MS/MS system for the detection of the AA conjugates (signal to noise ratio >> 3), the addition of PPA in the mobile phase was deemed non-essential. The time program utilized was a combination of an isocratic run followed by a fast gradient time program. AA conjugates of VPA eluted during the isocratic portion of the total run time at retention times varying from 22.97 to 30.70 min, a similar time range observed for the thiol conjugates. The individual retention time observed for each AA conjugate of VPA is shown in table 26. The analysis of these conjugates by LC/MS/MS method C, utilizing a mobile phase which was composed of ACN/water (40:60, 0.05 % TFA), reduced the observed retention time range to 7.86-11.99 min. The retention times of VPA GLN and VPA GLU were increased to 14.70 min and 23.20 min, respectively, by LC/MS/MS

method B (mobile phase ACN/water, 30:70, 0.05 % TFA), under our experimental conditions. LC/MS/MS method B was used to separate urinary interfering substances which eluted at the same retention time as VPA GLU at MRM transition (m/z 274 to 57) in human samples. All three LC/MS/MS methods were employed depending on the matrix under investigation. Retention times shifts were observed from matrix to matrix, but the shifts remained within 5% of the retention times observed for pure standards spiked in the matrix during any individual sequence run. Daily experiments were initiated by determining the retention times of the test samples of synthetic materials. Detection and separation of the individual AA conjugates of VPA from each other in a mixture were achieved by monitoring MRM transitions which were distinct and specific for each molecule using MS/MS under ES^+. The retention times of the conjugates increased in the following order with all the mobile phases: VPA GLN > FVPA GLN (I.S) > VPA ASP > VPA GLU > VPA GLY.

Table 26. Retention times (t_R) of AA conjugates of VPA with different mobile phases.

	LC/MS/MS A (t_R=min)	LC/MS/MS B (t_R=min)	LC/MS/MS C (t_R=min)
VPA GLN	22.97	14.70	7.86
VPA GLU	30.70	23.20	10.53
VPA GLY	30.30	24.0	11.99
VPA ASP		23.99	9.93
FVPA GLN			9.33

Mobile phase for LC/MS/MS A: methanol/water (43:57, 0.025% TFA and PPA each).
Mobile phase for LC/MS/MS B: acetonitrile/water (30:70, 0.05% TFA).
Mobile phase for LC/MS/MS C: acetonitrile/water (40:60, 0.05% TFA).

3.3.1.2. ES$^+$ MS/MS characteristics of the AA conjugates of VPA

Positive electrospray was chosen as the ionization mode for the analysis of the conjugates because of the high sensitivity achieved (signal to noise ratio > 3) and the characteristic fragmentation pattern observed which is summarized in table 27. CID of the precursor ions MH$^+$ produced product ion spectra (MS/MS spectra) typical of each conjugate.

Figure 33 depicts our proposed fragmentation pattern structurally while figures 34-37 show the product ion spectra obtained for each conjugate. The MS/MS spectral characteristics of the synthetic standards of the four AA conjugates of VPA studied showed a simple fragmentation pattern associated with both the VPA portion of the molecule, common to all four conjugates, and the AA portion of the molecule. The most abundant fragment at *m/z* 57 was shared by all four conjugates and is believed to be the protonated n-butyl group of VPA following cleavage on two sides of the tertiary carbon (C4) causing the loss of the formyl amino acid group and the n-propyl group as illustrated in figure 33. The product ion at *m/z* 99 resulted from the fragmentation at the carbonyl carbon (C8) causing the neutral loss of the *N*-formyl amino group leaving the protonated *N*-heptanyl chain of VPA. Cleavage of the amide bond gave rise to the protonated 2-propyl pentanal at *m/z* 127 for each conjugate. The corresponding protonated AA was also observed at *m/z* 147 for VPA GLN or *m/z* 148 for VPA GLU but not for VPA GLY (figures 34, 35). A small fragment at *m/z* 129 was observed for VPA GLN and assigned as the protonated fragment resulting from cleavage at the N-CH group and the subsequent neutral loss of the propyl pentanamide group. Similarly, *m/z*

130 was observed for VPA GLU. The same fragmentation pattern was not observed for

FVPA GLN (figure 38) as discussed by Tang *et al*, 1996a.

	R	R$_2$
GLN=	COOH	C$_2$H$_4$CONH$_2$
GLU=	COOH	C$_2$H$_4$COOH
GLY=	COOH	H
ASP=	COOH	CH$_2$COOH
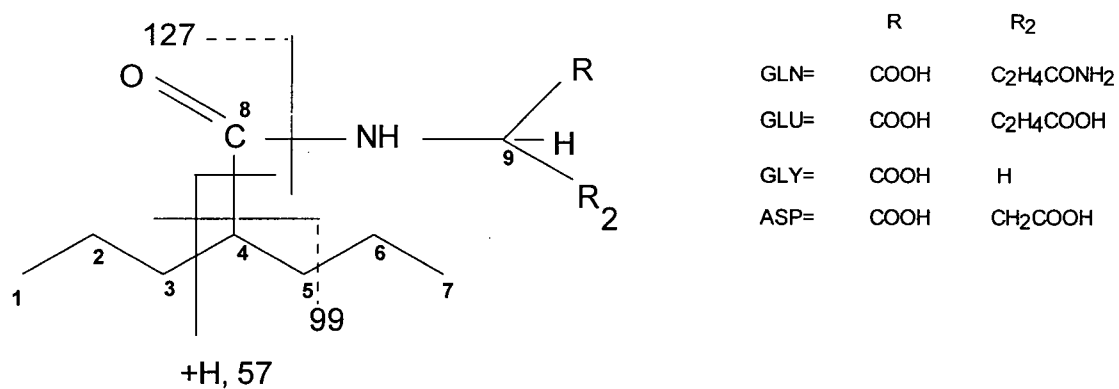
Figure 33.   MS/MS characteristics of AA conjugates of VPA under positive ion electrospray

Table 27. MS/MS characteristics of AA conjugates of VPA under positive ion electrospray. The protonated molecular ion (MH$^+$) and the relative abundance (%) of characteristic fragments observed for each conjugate are tabulated.

	VPA GLN	VPA GLU	VPA GLY	VPA ASP
MH+	273	274	202	260
m/z 57 [C$_4$H$_9$]$^+$	70-100	70-100	70-100	70-100
m/z 99 [C$_7$H$_{15}$]$^+$	35-45	35-45	30-45	35-45
m/z 127 [C$_8$H$_{15}$O]$^+$	20-30	20-30	20-30	20-30
m/z 129 [C$_5$H$_7$O$_3$N]$^+$	5	0	0	
m/z 147 C$_5$H$_9$O$_3$N$_2$	20	0	0	
m/z 148 C$_5$H$_{10}$O$_4$N	0	10	0	
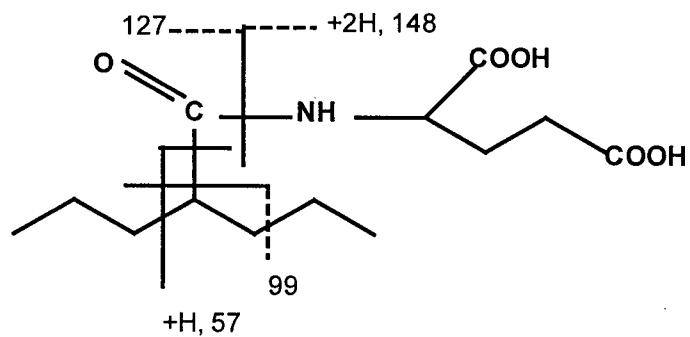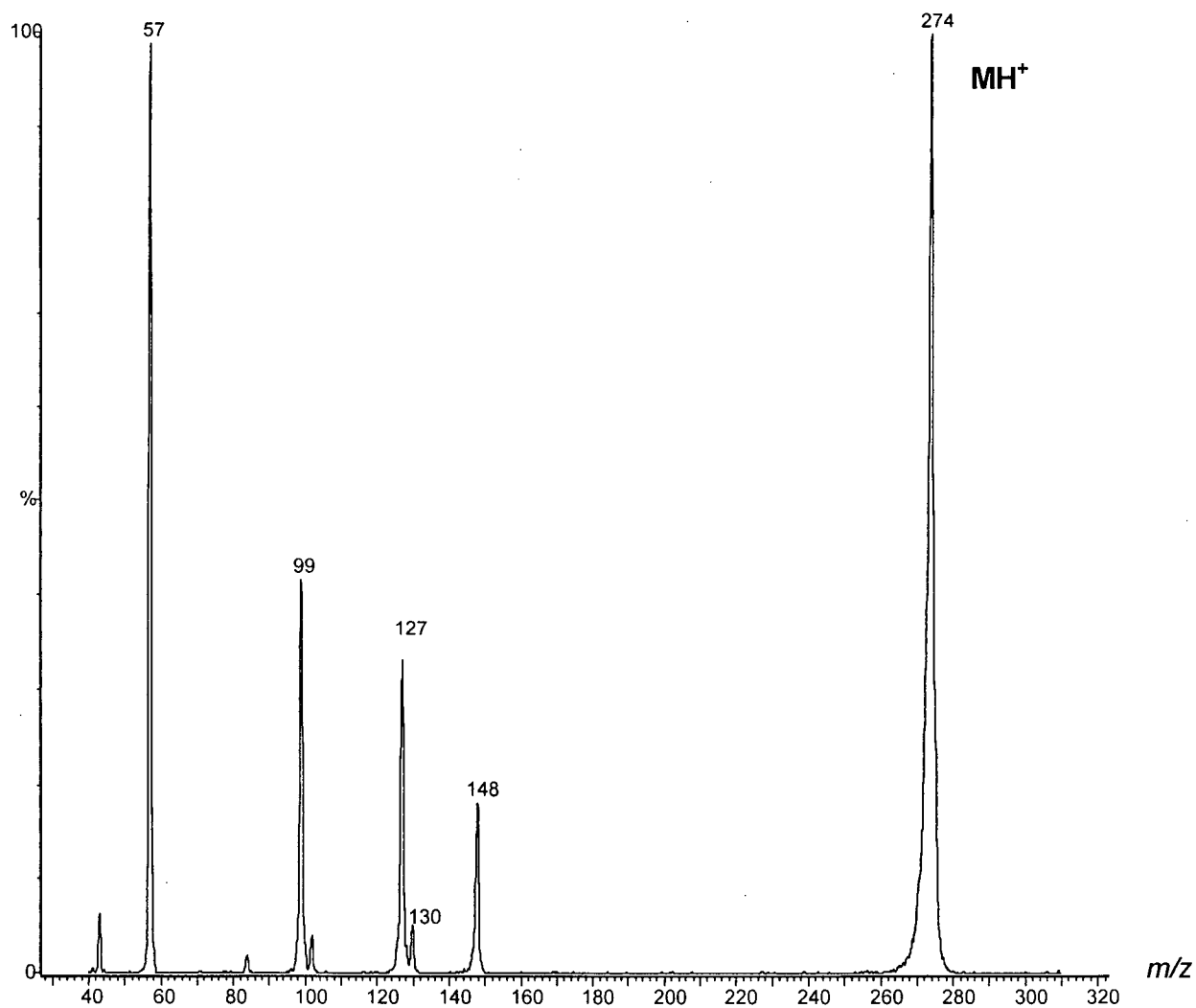
Figure 34. ES$^+$ MS/MS product ion mass spectrum of a reference standard VPA GLU (MH$^+$ 274).
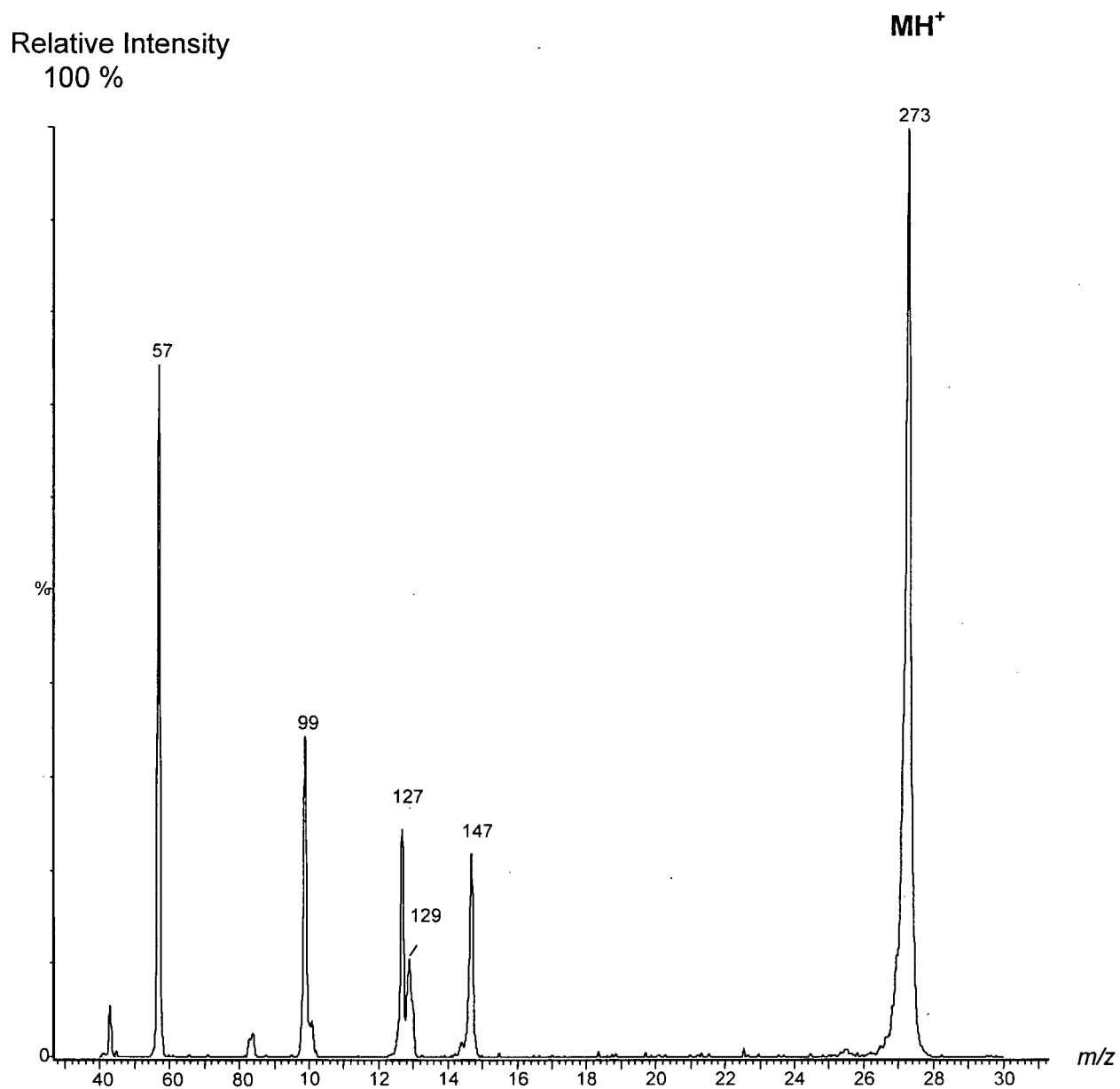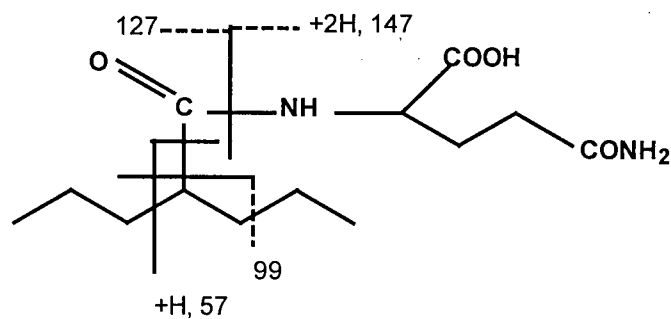
Figure 35. ES⁺ MS/MS product ion mass spectrum of a reference standard VPA GLN (MH⁺273).
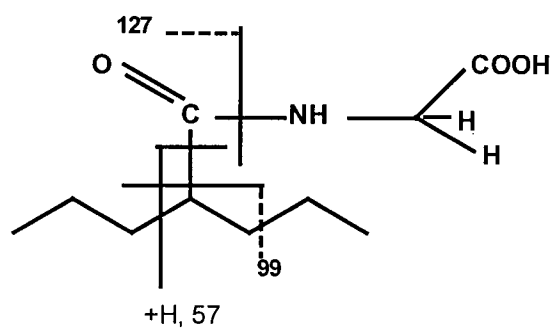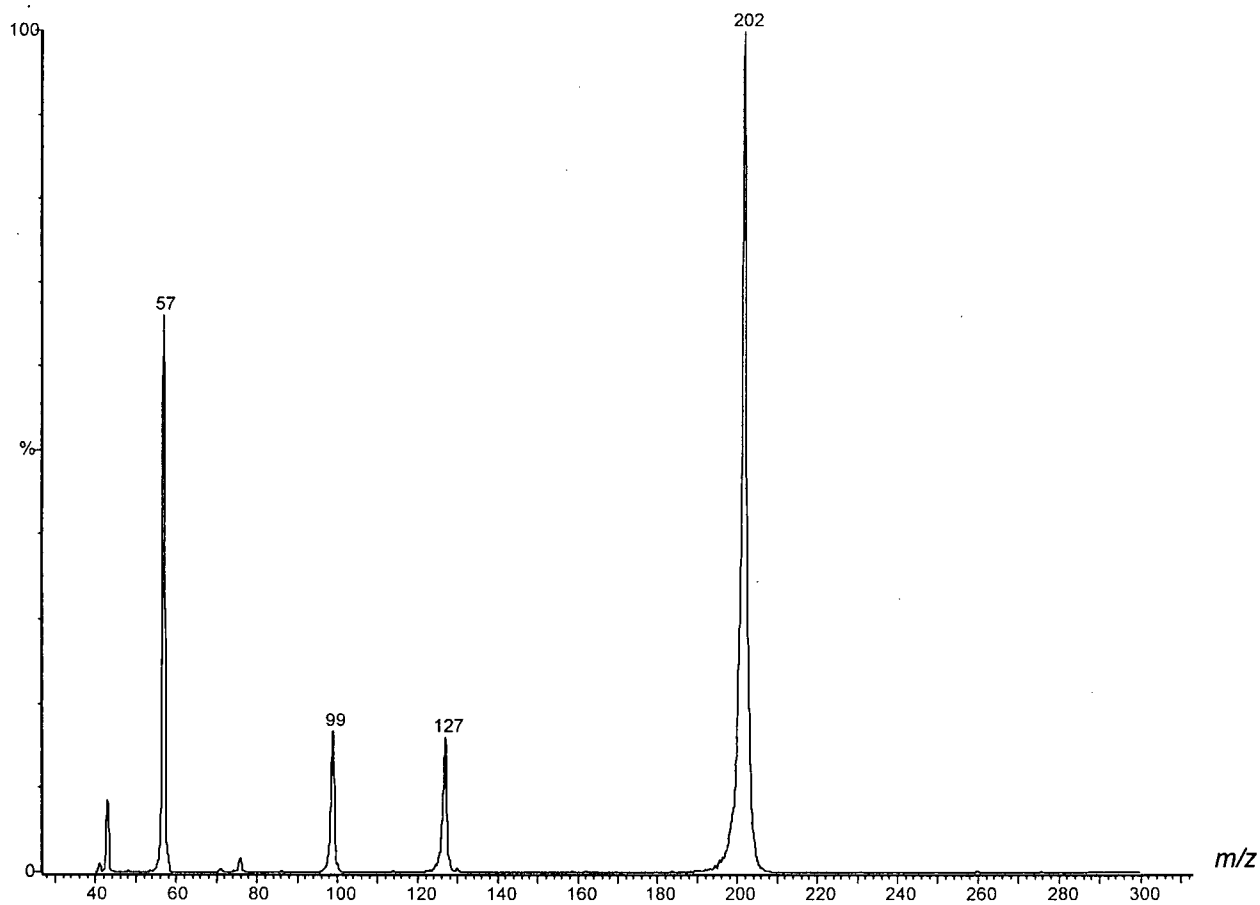
Figure 36. ES⁺ MS/MS product ion mass spectrum of a reference standard VPA GLY (MH⁺ 202).
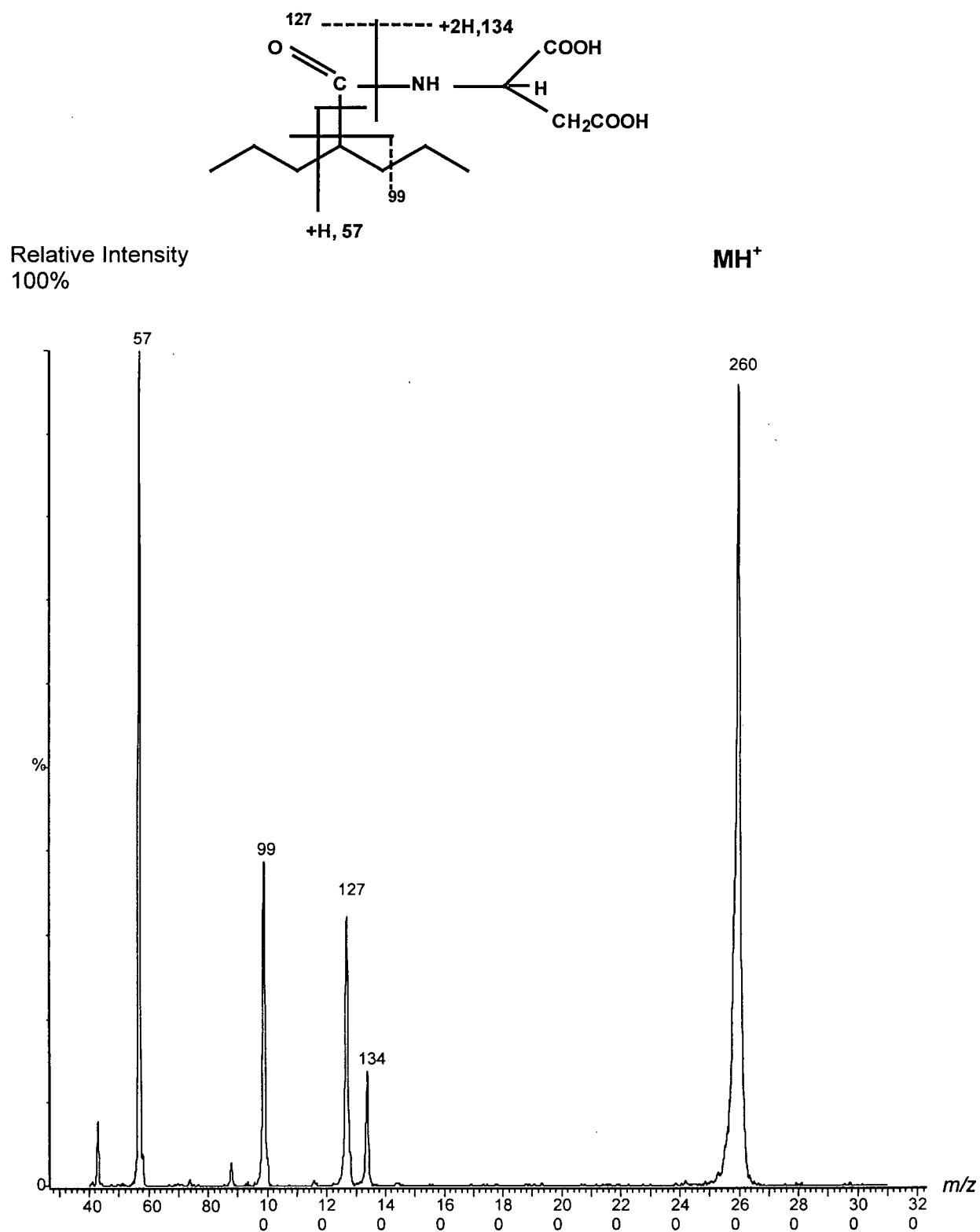
Figure 37. ES⁺ MS/MS product ion mass spectrum of a reference standard VPA ASP (MH⁺ 260).
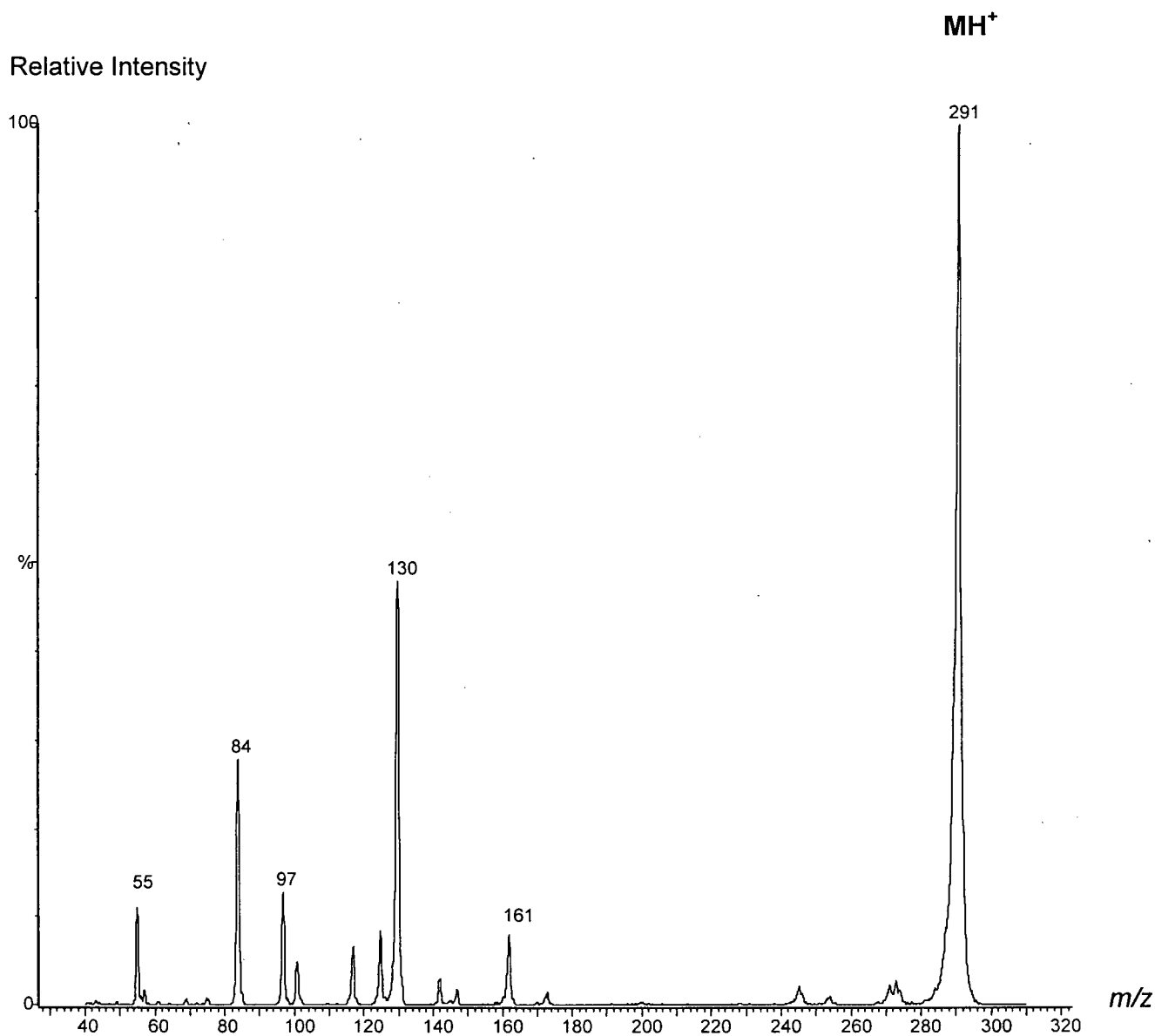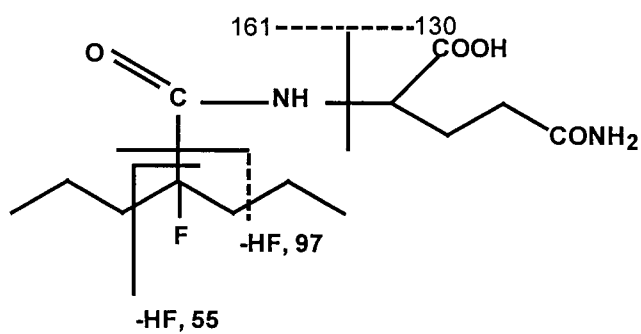
Figure 38. ES$^+$ MS/MS product ion mass spectrum of FVPA GLN (MH$^+$ 291).

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3.3.2 Characterization of AA conjugates of VPA by GC/MS NICI

GC/MS NICI analysis of AA conjugates of VPA in rats and humans was found to be a sensitive, convenient and a relatively facile technique to support and complement our LC/MS/MS findings.

Although the PFB derivatives of synthetic VPA GLN and VPA GLY were studied qualitatively, this approach was not developed quantitatively at this time. The PFB derivative of VPA GLU was studied to verify the presence of this conjugate in the CSF, brain and serum of both rat and human by a technique other than LC/MS/MS. The high sensitivity and soft ionization nature of NICI and the use of both DB101 or DB1701 columns allowed for the chromatographic separation of all the derivatized conjugates. Table 28 shows the individual retention times and $[M-181]^-$ fragments observed for each AA conjugate of VPA studied.

Table 28. GC/MS (method F) characteristics of AA conjugates of VPA

	VPA GLN	VPA GLU	VPA GLY	VPA ASP	FVPA GLN
[M-181]-	*m/z* 271	*m/z* 452	*m/z* 200	*m/z* 438	*m/z* 289
t_R (min)	15.10	16.42	8.98	15.71	14.78

The mass spectrum of each conjugate was characterized by a single most abundant $[M-181]^-$ ion which corresponded to the loss of a PFB group under soft negative ionization. A scan of VPA GLY, VPA GLN, and VPA GLU under NICI produced a TIC shown in

figure 39. The corresponding mass spectrum of the PFB derivative of each conjugate is shown in figures 40-42.
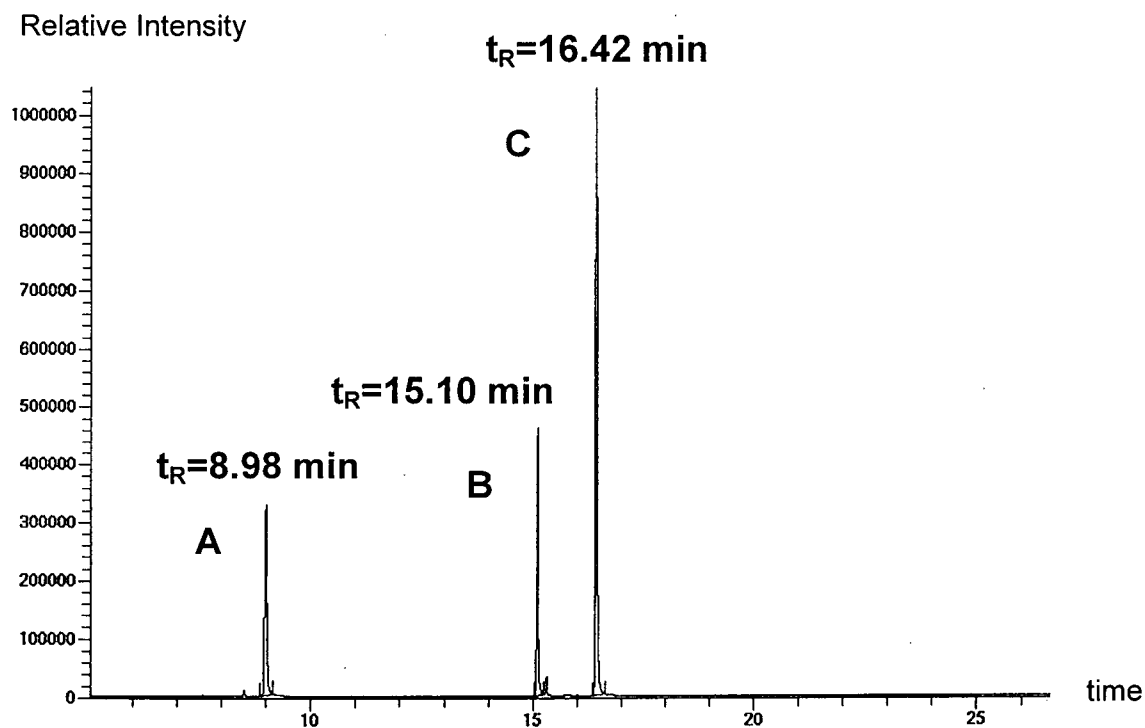
Figure 39. TIC of VPA GLY (A), VPA GLN (B), and VPA GLU (C) using GC/MS method F and a DB1701 column (See Experimental/Section 2.2.6.6).
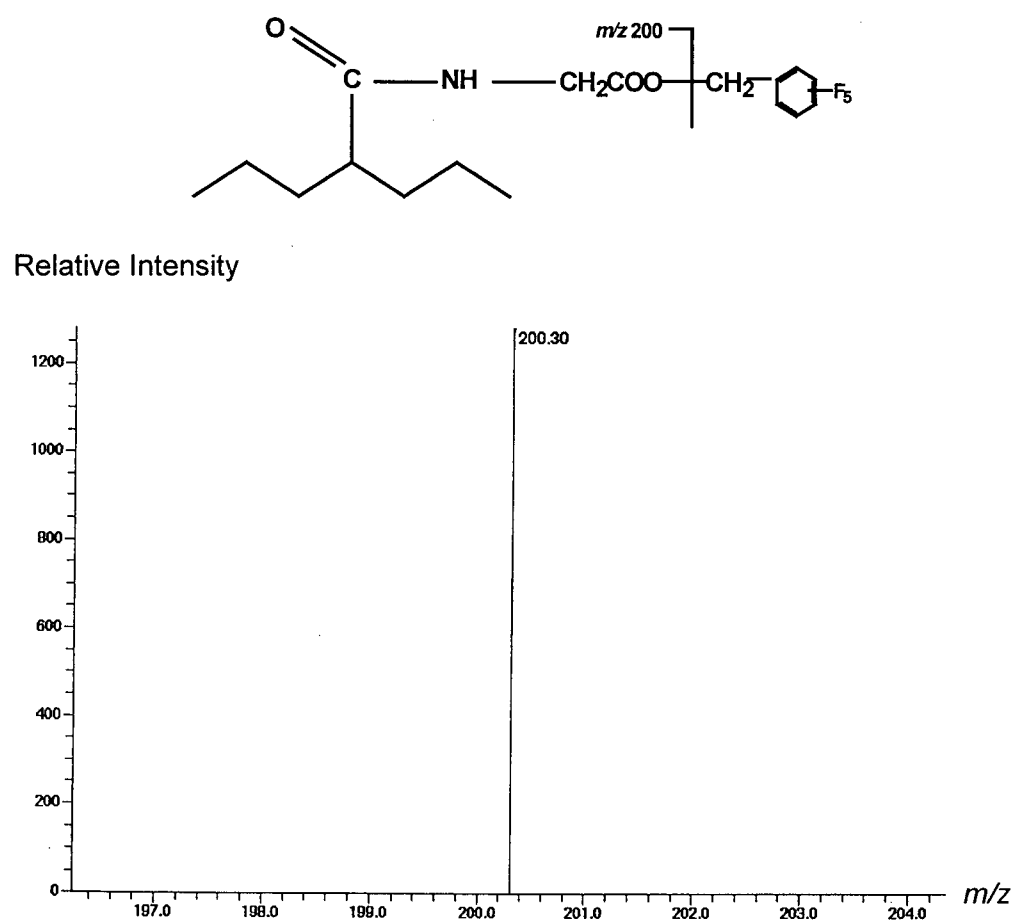
Figure 40. The GC/MS mass spectrum of the PFB derivative of VPA GLY (peak A in figure 39).
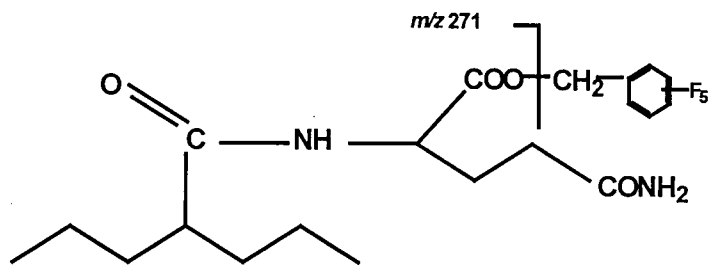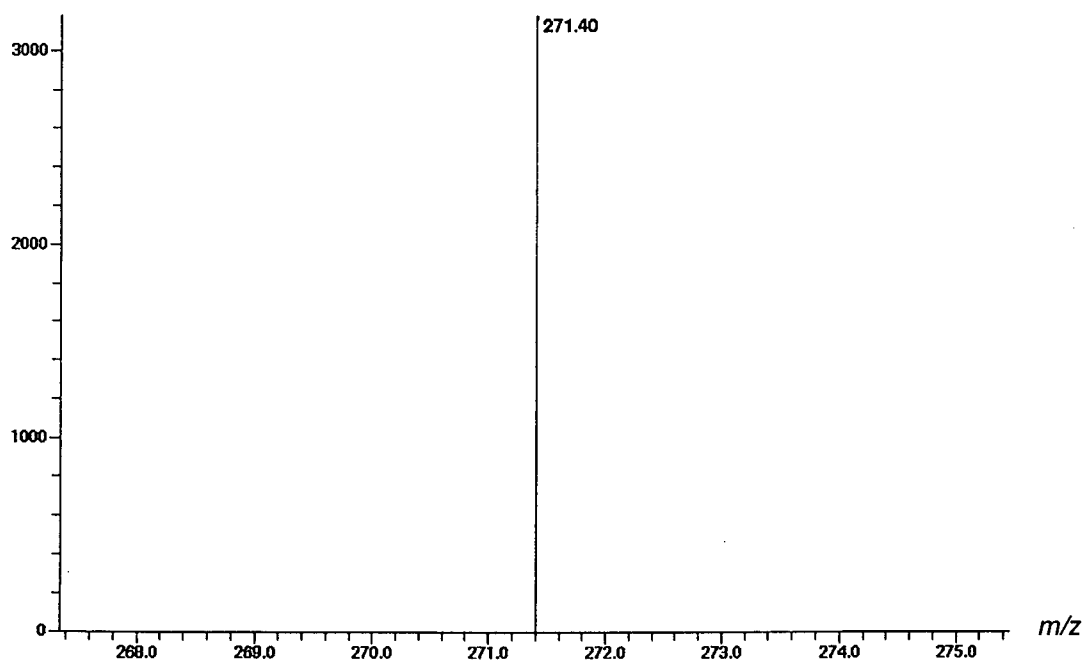
Figure 41. GC/MS mass spectrum of the PFB derivative of VPA GLN (peak B in figure 39).
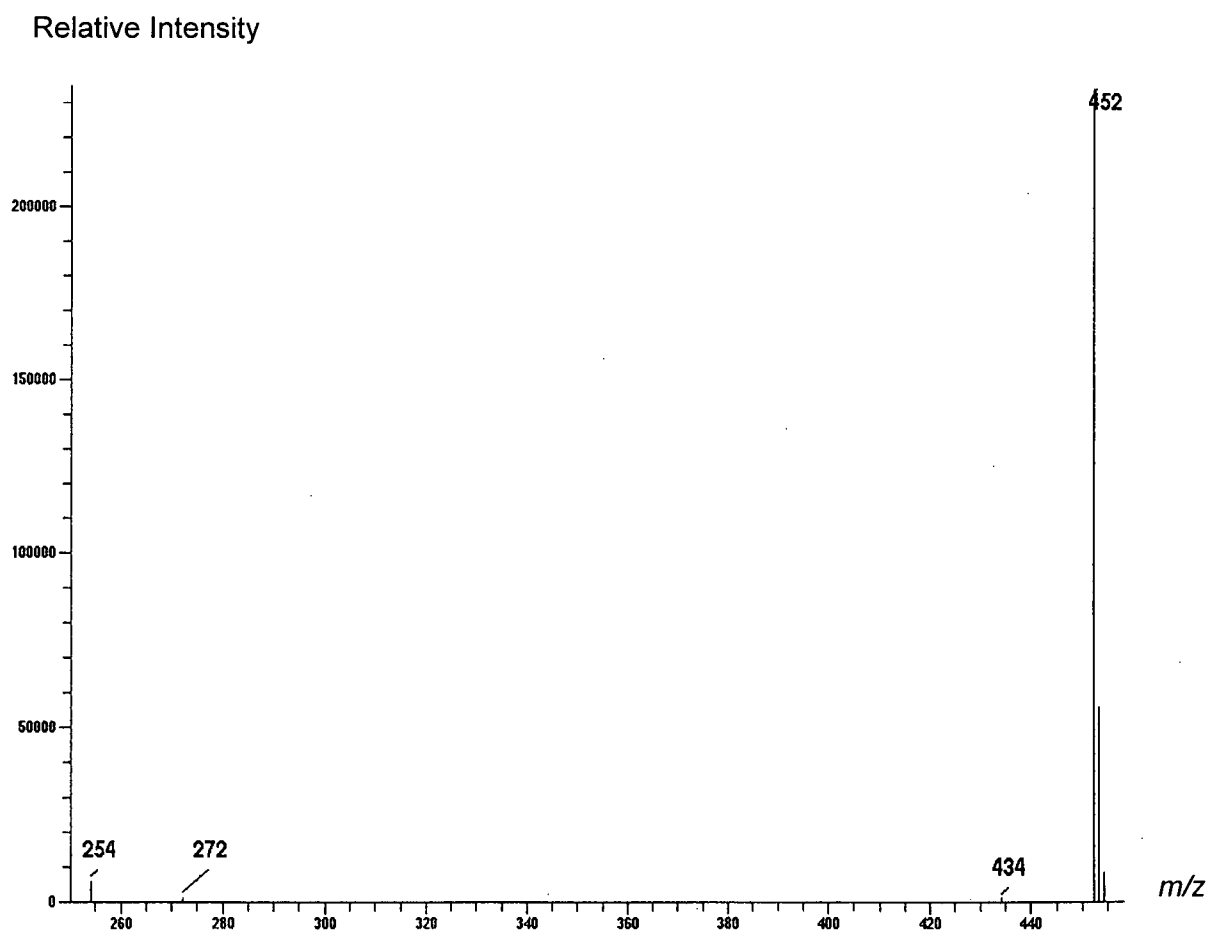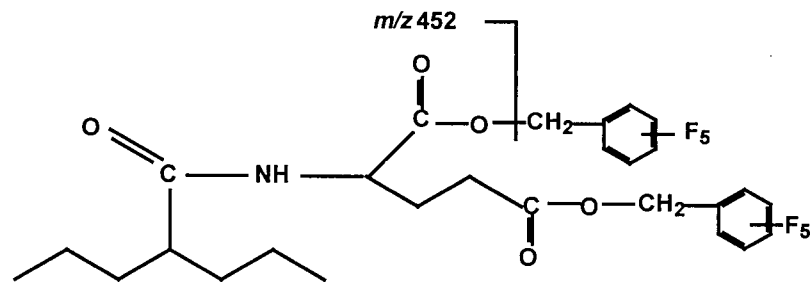
Figure 42. The GC/MS mass spectrum of the PFB derivative of VPA GLU (peak C in figure 39).

3.3.3 Investigation of AA conjugates of VPA in humans

3.3.3.1 Identification of AA conjugates of VPA in the urine of patients on VPA by LC/MS/MS

Both precursor ion scanning and product ion scanning for VPA GLU and VPA GLN were performed on some patient urine samples and the corresponding spectra obtained for both compounds are shown in figures 43 and 44.

Furthermore, urine samples of 29 patients on VPA belonging to the study group already described in the Experimental section were also examined. The identification of AA conjugates of VPA was performed by monitoring all the characteristic product ions of each amino acid conjugate and comparing their area ratio and retention times to that of synthetic reference samples as shown in figures 45-47. The identification of the three conjugates was verified and confirmed in urine using LC/MS/MS methods A, B, C. The same experiment failed to detect VPA ASP in any samples studied. The search for VPA taurine and VPA alanine was approached by MRM of the transition [MH]$^+$ to m/z 57. Neither of the latter conjugates were detectable in the urine extracts analyzed.
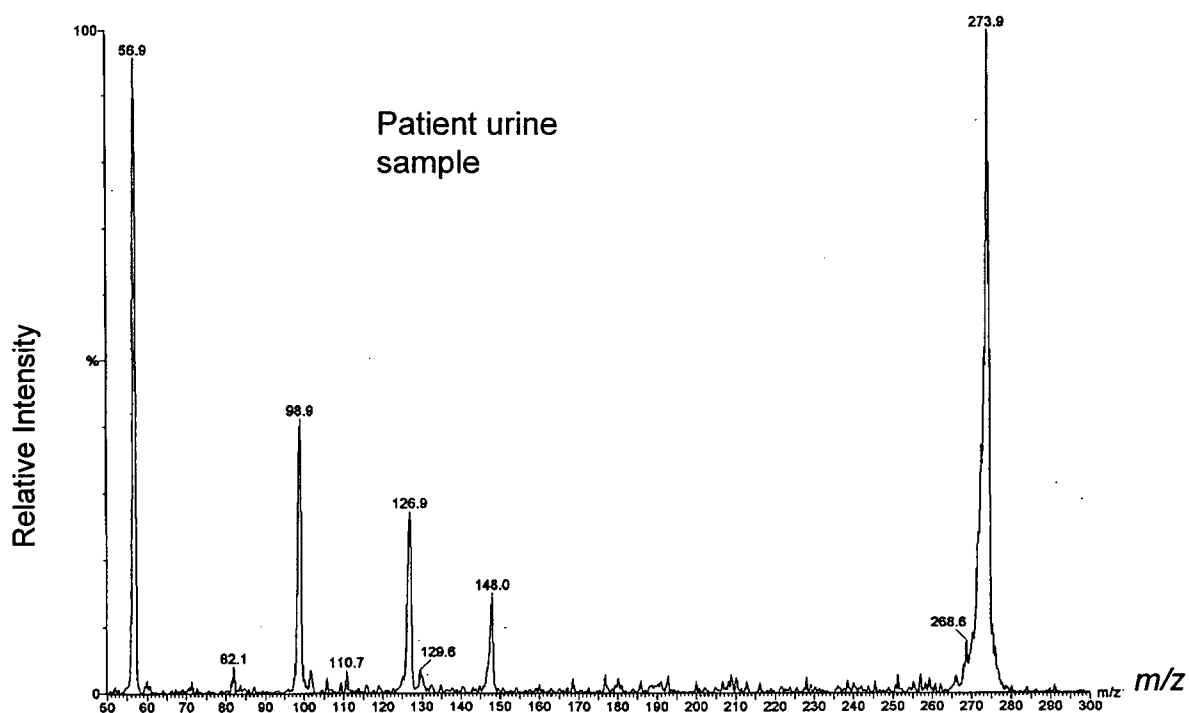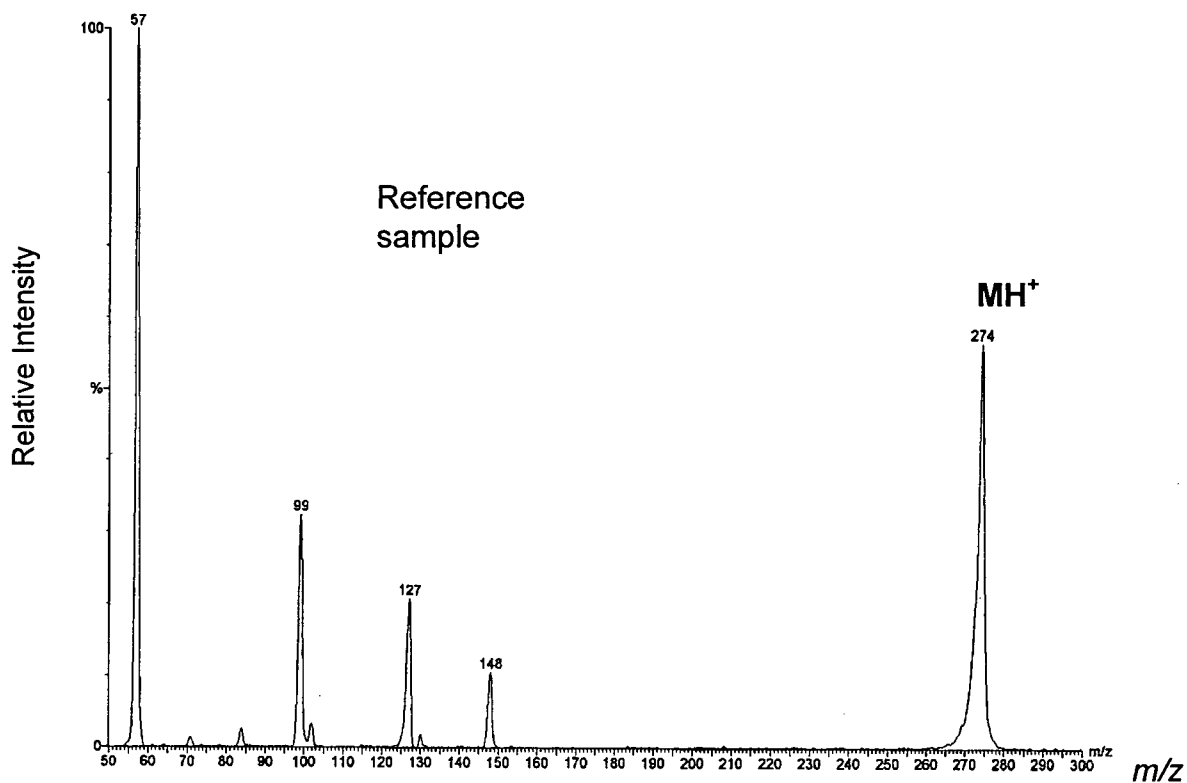
Figure 43. MS/MS product ion spectra of MH$^+$ 274 obtained for VPA GLU for a reference sample of VPA GLU (top) and in an extract of a urine sample of a patient on VPA therapy (bottom). Both spectra were obtained under the same analytical conditions.

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Figure 44. MS/MS product ion spectra of MH$^+$ 273 obtained for VPA GLN for a reference sample of VPA GLN (top) and in an extract of a urine sample of a patient on VPA therapy (bottom). Both spectra were obtained under the same analytical conditions.

m/z 274 > 57

m/z 274 > 99

m/z 274 > 127

m/z 274 >148

a   b   c   d

Figure 45. On-line LC/MS/MS monitoring of VPA GLU in: (a) an extracted urinary human control sample, (b) an extracted urinary sample of a patient on VPA, and (c) a synthetic standard of VPA GLU which eluted at t_R=23.53 min. The corresponding characteristic ion transitions monitored for MRM are shown in (d) (mobile phase: ACN (30%): H_2O (70%) and 0.05% TFA).

Figure 46. On-line LC/MS/MS monitoring of VPA GLN in: (a) an extracted urinary human control sample, (b) an extracted urinary sample of a patient on VPA, and (c) a synthetic standard of VPA GLN which eluted at t_R=14.77 min. The corresponding characteristic ion transitions monitored for MRM are shown in (d) (mobile phase: ACN (30%): H_2O (70%) and 0.05% TFA).

Figure 47. On-line LC/MS/MS monitoring of VPA GLY in: (a) an extracted urinary human control sample, (b) an extracted urinary sample of a patient on VPA, and (c) a synthetic standard of VPA GLY which eluted at t_R=12.65 min. The corresponding characteristic ion transitions monitored for MRM are shown in (d) (mobile phase : ACN (40%): H_2O (60%) and 0.05% TFA).

3.3.3.2 The profiling of VPA GLN, VPA GLU, and VPA GLY in human urine extracts

The assay of the conjugates employed the SPE method developed for the urinary thiol conjugates as described in scheme 3 of the Experimental section. The assay appeared to be sufficiently sensitive (signal to noise > 3) for the simultaneous analysis of all three amino acid conjugates present in humans. Furthermore, the range of the assay was linear over a wider concentration range than that observed for the thiol conjugates and hence, could be used for the quantitation of all the identified conjugates. VPA ASP was not detected in human samples and was subsequently chosen as one of the internal standards for the urinary quantitation. FVPA GLN was also suitable as an internal standard.

3.3.3.2.1 Assay validation for the profiling of AA conjugates of VPA in human urine

3.3.3.2.1.1 Accuracy and precision

The precision of the assay for the profiling of AA conjugates of VPA in urine was determined based on the inter-assay variation of the slopes of five calibration curves analyzed on five analytical days as shown in table 29. The coefficients of determination (r^2) of the calibration curves were 0.99 or better and the inter-assay variation was less than 7 %. Precision was also determined by the intra-assay variation of the observed concentrations of two in-house quality control samples (samples were spiked with known amount of the conjugates) analyzed in three to five replicates (Table 30). The

accuracy of the assay was determined by comparing the observed concentrations for the quality controls to their expected concentrations (Table 30).

The assay appeared to be linear over a range of 0.1-5.0 μg/mL for all three conjugates. The intra-assay study showed that at a concentration of 1 μg/mL, the coefficients of variation for the three conjugates were less than 10% whereas the coefficients of variation for 0.115 μg/mL were 11.4%, 28.8%, 18.5% for VPA GLU, VPA GLN and VPA GLY respectively. All quality control samples were within 83% to 122% of their expected values.

Table 29. Inter-assay variation of AA conjugates of VPA in human urine samples based on the slopes of calibration curves obtained on five consecutive analytical days.

	VPA GLU (0.1-5.0 μg/mL)		VPA GLN (0.1-5.0 μg/mL)		VPA GLY (0.1-5.0 μg/mL)	
	Slope	r^2	Slope	r^2	Slope	r^2
Day 1	0.312	0.998	0.279	0.997	0.510	0.998
Day 2	0.306	0.995	0.287	0.995	0.496	0.996
Day 3	0.294	0.999	0.253	0.988	0.536	0.999
Day 4	0.268	0.994	0.240	0.997	0.510	0.998
Day 5	0.303	0.999	0.281	0.998	0.586	0.999
Mean	0.296	0.997	0.268	0.995	0.528	0.997
SD	0.016	0.002	0.018	0.004	0.032	0.002
C.V.%	5.4	0.2	6.7	0.4	6.0	0.2

Table 30. Intra-assay variation of AA conjugates of VPA in human urine samples based on three to five replicate analyses of two concentrations analyzed on the same analytical day.

	QC=1.0 μg/mL			QC=0.115 μg/mL		
	VPA GLU	VPA GLN	VPA GLY	VPA GLU	VPA GLN	VPA GLY
Run 1	1.177	1.222	1.221	0.099	Reject	0.112
Run 2	1.700	1.245	1.147	0.082	0.092	Reject
Run 3	1.327	1.309	1.296	0.112	0.166	0.093
Run 4	1.127	1.000	1.174	0.091	0.095	0.085
Run 5	1.283	1.297	1.252			
Mean	1.217	1.215	1.218	0.096	0.118	0.097
SD	0.075	0.112	0.053	0.011	0.034	0.011
C.V.	6.10	9.18	4.35	11.4	28.8	11.3
% of expected value	122	122	122	83.5	102.6	84.4

Reject=sample spilt or test-tube broke

3.3.3.2.2 Recovery of a VPA dose as VPA AA in patients on VPA therapy

The total contribution of VPA AA conjugates to the recovery of a VPA dose was calculated by appropriately modifying equation (1) for thiol adducts in the Experimental section (2.3.4.4) for four patients and was found to be less than 1 % of a VPA dose. The results obtained are tabulated below (table 31).

Table 31. % of VPA dose recovered as VPA GLU, VPA GLN, and VPA GLY in four patients over 24 h.

U.B.C. #	VPA GLU	VPA GLN	VPA GLY	Total % Recovered
003 (a)	0.45	0.32	0.03	0.80
003 (b)	0.32	0.22	0.11	0.65
036 (c)	0.20	0.20	0	0.40
043 (a)	0.22	0.18	0.02	0.42
043 (b)	0.12	0.07	0.003	0.19
044 (a)	0.17	0.15	0	0.32
044 (b)	0.11	0.10	0	0.21

(a)=first 12 h urine collection; (b)=second h urine collection; (c)=24 h urine collection

3.3.3.2.3 Quantitation of AA conjugates of VPA in human urine

The quantitation of AA conjugates in urine samples from patients (n=29) was conducted as described in the experimental section (2.3.13.1). The ranges obtained for each conjugate are summarized in table 32.

Table 32. Summary of results for the profiling of VPA GLU, VPA GLN, VPA GLY in urine samples of patients on VPA.

	VPA GLU (μg/mg creatinine) n=29	VPA GLN (μg/mg creatinine) n=29	VPA GLY (μg/mg creatinine) n=29
Range	0.66-13.1	0.78-9.93	Trace-1.0
Mean	4.31	4.05	0.31
SD	2.76	2.50	0.37

3.3.3.3 Identification and profiling of AA conjugates of VPA in human serum

3.3.3.3.1 Identification of VPA GLU, VPA GLN, and VPA GLY in human serum

The serum from six of the patients studied above was also investigated for the presence of VPA GLU, VPA GLN, VPA GLY, and VPA ASP using LC/MS/MS under ES$^+$ and by GC/MS NICI. Results of preliminary GC/MS analysis under NICI of a PFB-derivatized

serum extract of a patient on VPA is shown in figure 48b. The SIM chromatogram at *m/z* 452, shows a peak at t_R =16.38 min similar to the retention time of the standard reference sample in figure 48a.

The identification of VPA GLU, VPA GLN, and VPA GLY in all six patient's serum extracts was further confirmed using LC/MS/MS and by comparing the retention times and area ratio of several characteristic product ions of each conjugate in all serum extracts to those of synthetic standards. Figures 49-51 show the presence of VPA GLU, VPA GLN and VPA GLY, respectively in one serum extract studied. VPA ASP was not detected in the six serum samples studied.

3.3.3.3.2 Profiling of VPA GLU, VPA GLN and VPA GLY in human serum

The liquid/liquid extraction procedure used to quantitate the amino acid conjugates in human serum was a modification of the procedure employed for the urinary thiol conjugates. Instead of one mL of urine sample, two mL of serum was treated according to scheme 4 of the

experimental section. The assay was suitable for the single LC/MS/MS analysis performed; the calibration curves were linear between 0.005-0.1 μg/mL with correlation coefficients of 0.99 or greater for each conjugate. The average levels of VPA GLU and VPA GLN were estimated at 0.008 ± 0.002 μg/mL and 0.005 ± 0.003 μg/mL, respectively for the six samples analyzed. The serum levels of VPA GLY were below our limit of quantitation (estimated at 0.005 μg/mL). The results obtained for each serum sample analyzed are shown in table 33.

Relative Intensity
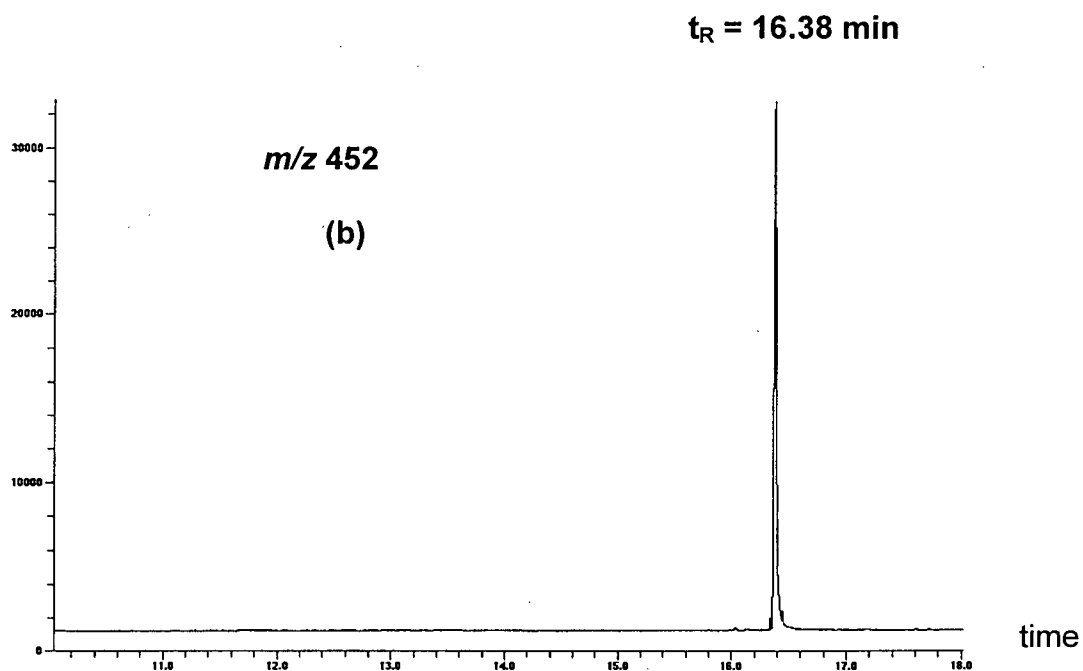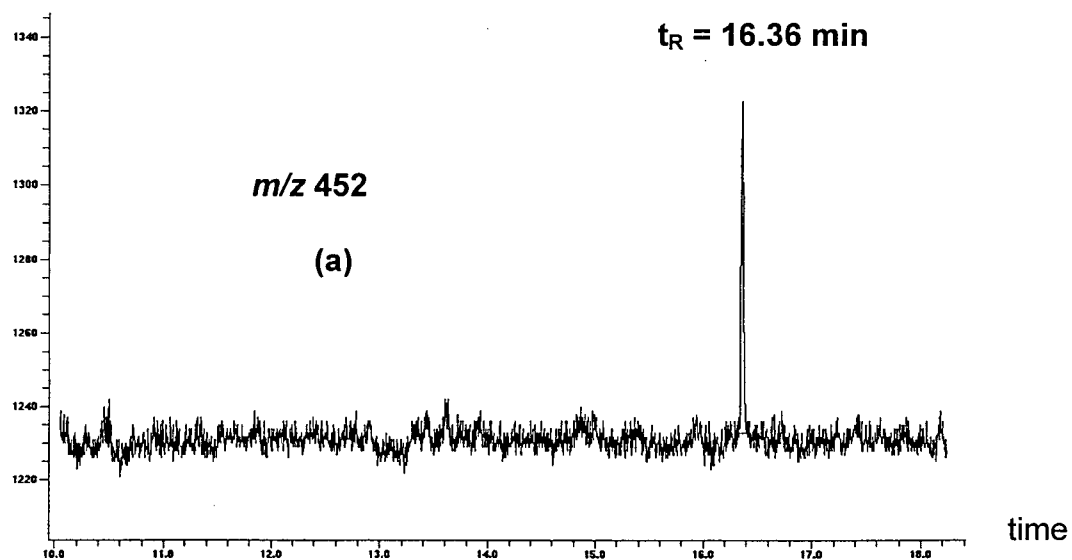
Figure 48. TIC of the PFB derivative of a standard reference sample of VPA GLU which eluted at 16.36 min in (a) and the TIC of a PFB-derivatized serum extract of a patient sample showing the elution of VPA GLU at 16.38 min in (b) under the conditions of GC method F. (See Experimental/Section 2.2.6.6).
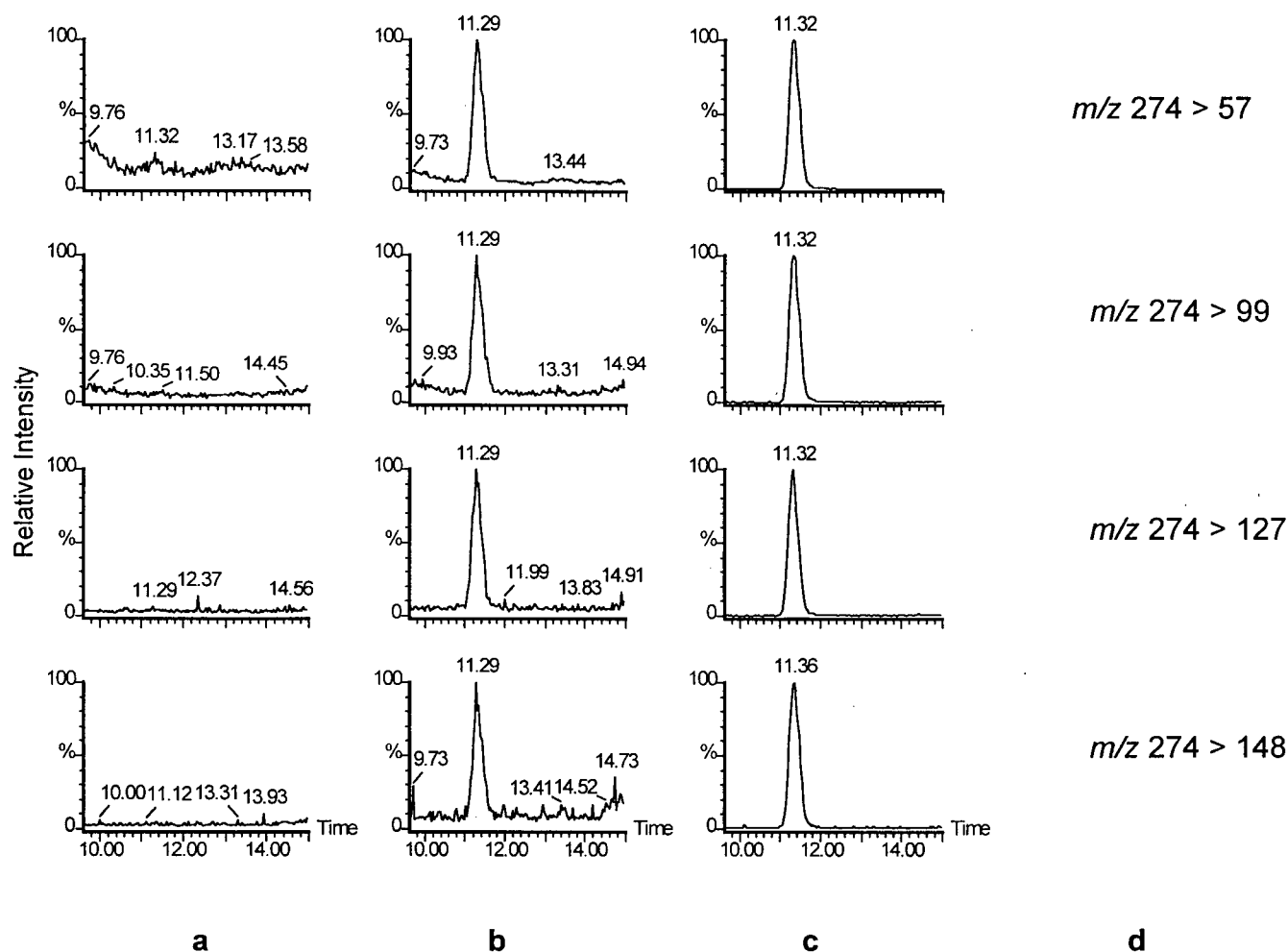
Figure 49. On-line LC/MS/MS monitoring of VPA GLU in: (a) an extracted human serum control sample, (b) an extracted serum sample of a patient on VPA, and (c) a synthetic standard of VPA GLU (0.025 μg/mL) which eluted at t_R=11.32 min. The corresponding characteristic ion transitions monitored for MRM are shown in (d) (mobile phase: ACN (40%): H_2O (60%) and 0.05% TFA).
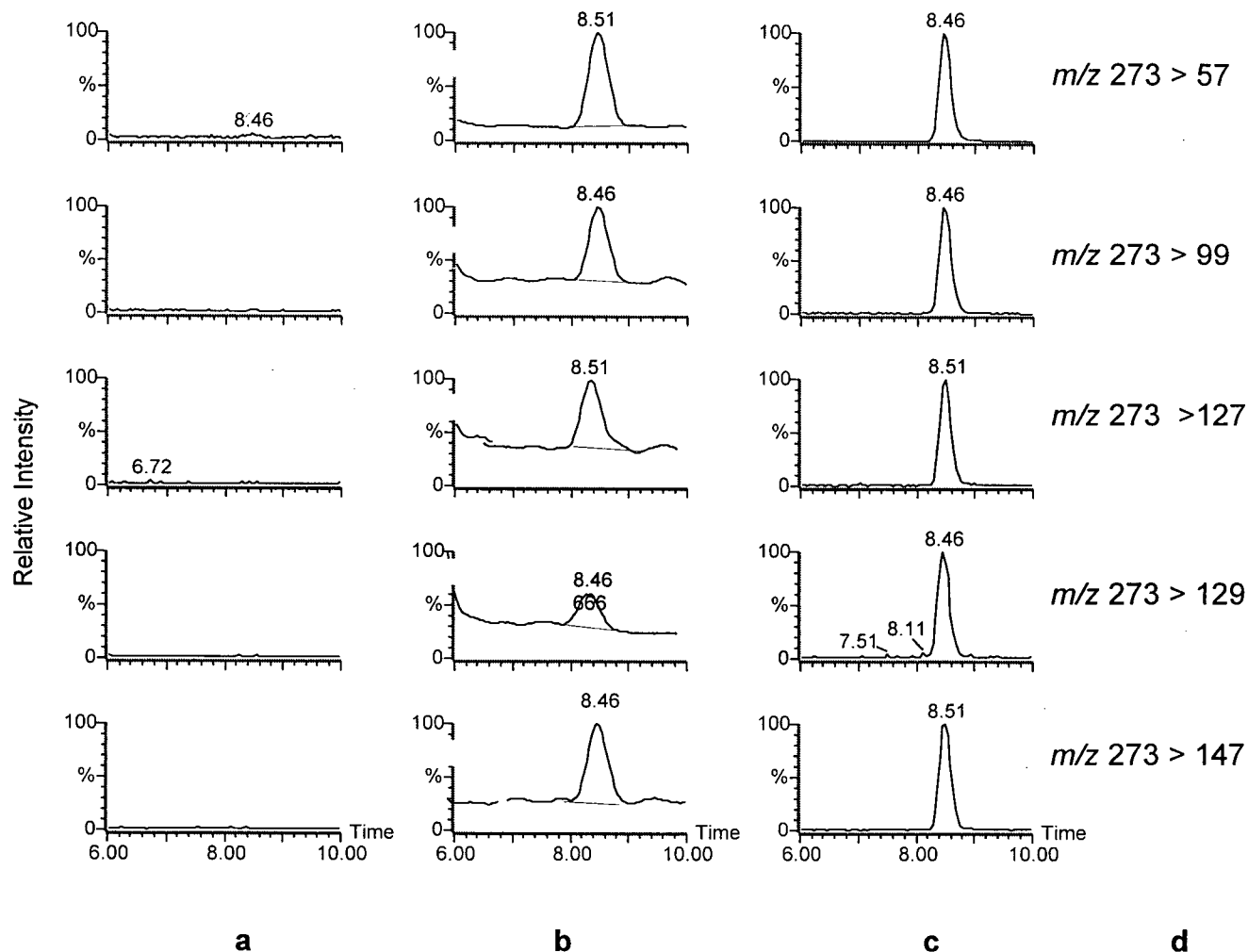
Figure 50. On-line LC/MS/MS monitoring of VPA GLN in: (a) an extracted human serum control sample, (b) an extracted serum sample of a patient on VPA, and (c) a synthetic standard of VPA GLN (0.025 μg/mL) which eluted at t_R=8.46 min. The corresponding characteristic ion transitions monitored for MRM are shown in (d) (mobile phase: ACN (40%): H_2O (60%) and 0.05% TFA).
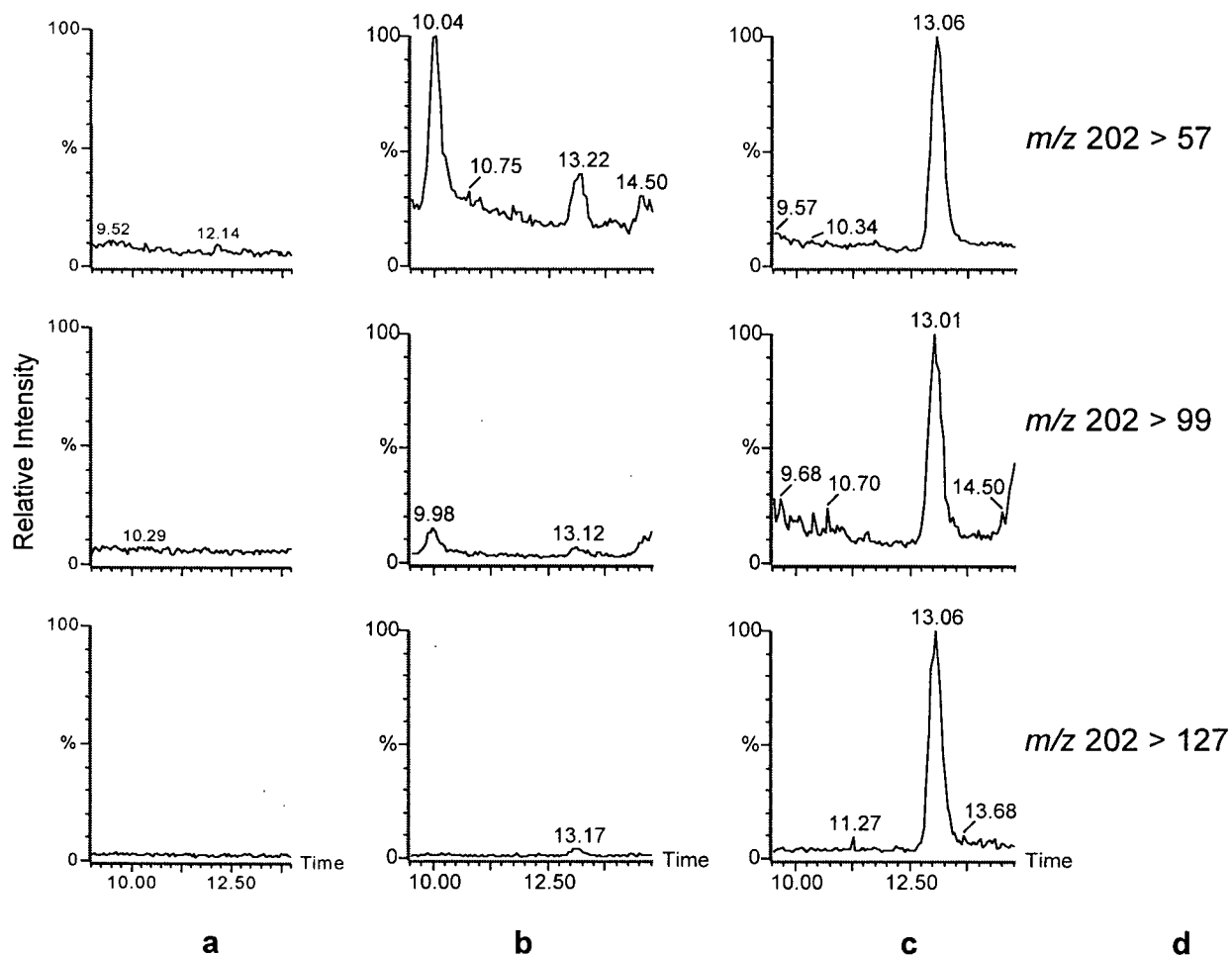
Figure 51. On-line LC/MS/MS monitoring of VPA GLY in: (a) an extracted human serum control sample, (b) an extracted serum sample of a patient on VPA, and (c) a synthetic standard of VPA GLY (0.025 μg/mL) which eluted at t_R=13.06 min. The corresponding characteristic ion transitions monitored for MRM are shown in (d), (mobile phase: ACN (40%): H_2O (60%) and 0.05% TFA).

Table 33. The serum concentrations of AA conjugates of VPA in six samples of patients on VPA.

Patient #	VPA GLU	VPA GLN	VPA GLY
	(μg/mL) n=1	(μg/mL) n=1	(μg/mL) n=1
# 10	0.008	0.007	0.002*
# 09	0.006	0.002*	0.001*
#14	0.011	0.005	0.002*
# 15	0.004*	0.002*	0.003*
#17	0.010	0.009	0.006
# 45	0.007	0.006	0.001*
Mean	0.008	0.005	0.003*
SD	0.002*	0.003*	0.002*

*=concentration below LOQ (0.005 ug/mL) and is an estimated value

3.3.3.4 Identification and quantitation of AA conjugates in human CSF

The detection of the AA conjugates in human urine and serum samples led us to question whether or not these conjugates possess antiepileptic activity and their significance in the mechanism of action of VPA. Therefore, during this study, we searched for VPA GLU, VPA GLN, VPA GLY and VPA ASP in a CSF sample of a patient (# 45) on VPA using GC/MS and LC/MS/MS.

Both VPA GLU and VPA GLN were positively identified in the human CSF sample when analyzed directly by LC/MS/MS method C under ES$^+$. Sensitivity was poor when analyzed by LC/MS/MS method A. Figures 52 and 53 show that all the characteristic product ions of both conjugates were present in the CSF sample of a VPA patient and in a control CSF sample spiked with the synthentic reference standards of the conjugates at a concentration of 0.025 μg/mL. The retention times of the eluting peaks at each transition and their area ratio were similar to those of the synthetic reference sample. All CSF samples were analyzed neat to avoid any possibility of contamination. VPA GLY (figure 54) and VPA ASP were not identified in the CSF sample.

The patient CSF sample was studied against ten control CSF samples from patients who were either healthy volunteers or suffering from multiple sclerosis. None of the product ions of VPA GLU, VPA GLN, VPA GLY and VPA ASP were detected in any of these control CSF samples.

The identification of VPA GLU in the CSF sample of patient # 45 was further investigated by GC/MS under NICI. A full mass spectrum of the PFB derivative of the
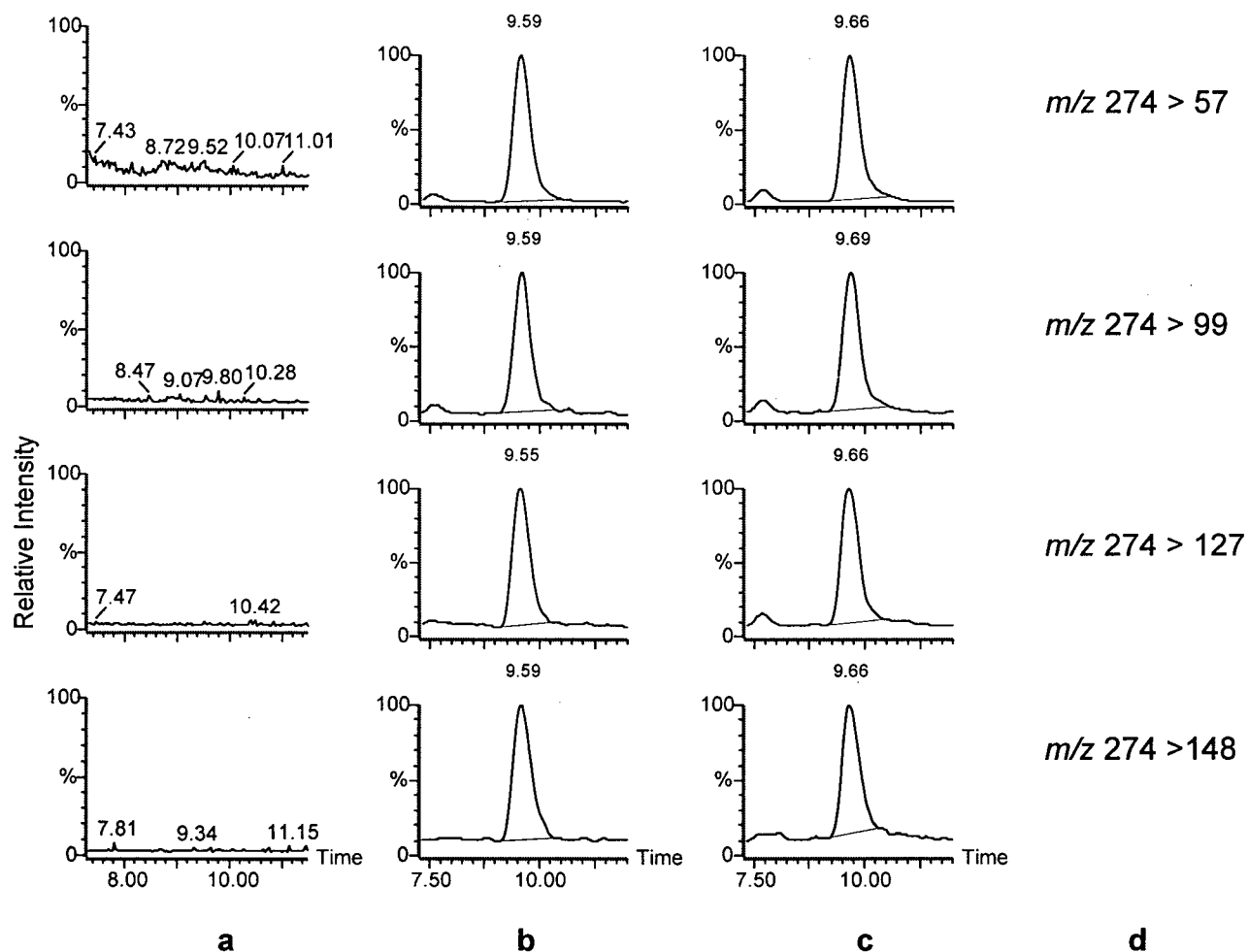
Figure 52. On-line LC/MS/MS monitoring of VPA GLU in (a) a control human CSF sample (b) a CSF sample of a patient treated with VPA and (c) a control human CSF sample spiked with VPA GLU which eluted at t_R=9.66 min. The corresponding characteristic ion transitions monitored for MRM are shown in (d) (mobile phase: ACN (40%): H_2O (60%) and 0.05% TFA).
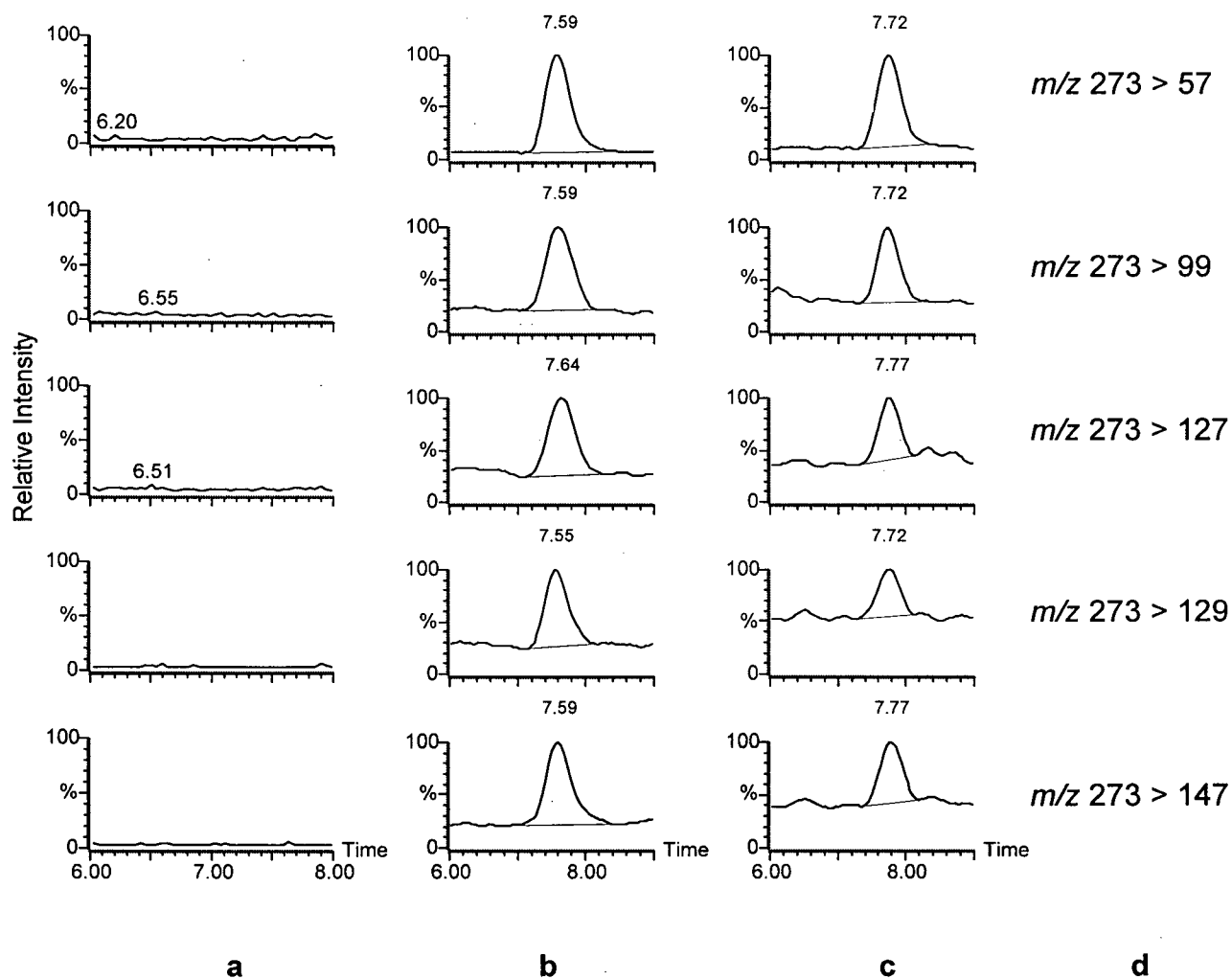
Figure 53. On-line LC/MS/MS monitoring of VPA GLN in (a) a control human CSF sample (b) a CSF sample of a patient treated with VPA and (c) a control human CSF sample spiked with VPA GLN which eluted at t_R=7.72 min. The corresponding characteristic ion transitions monitored for MRM are shown in (d) (mobile phase : ACN (40%): H_2O (60%) and 0.05% TFA).
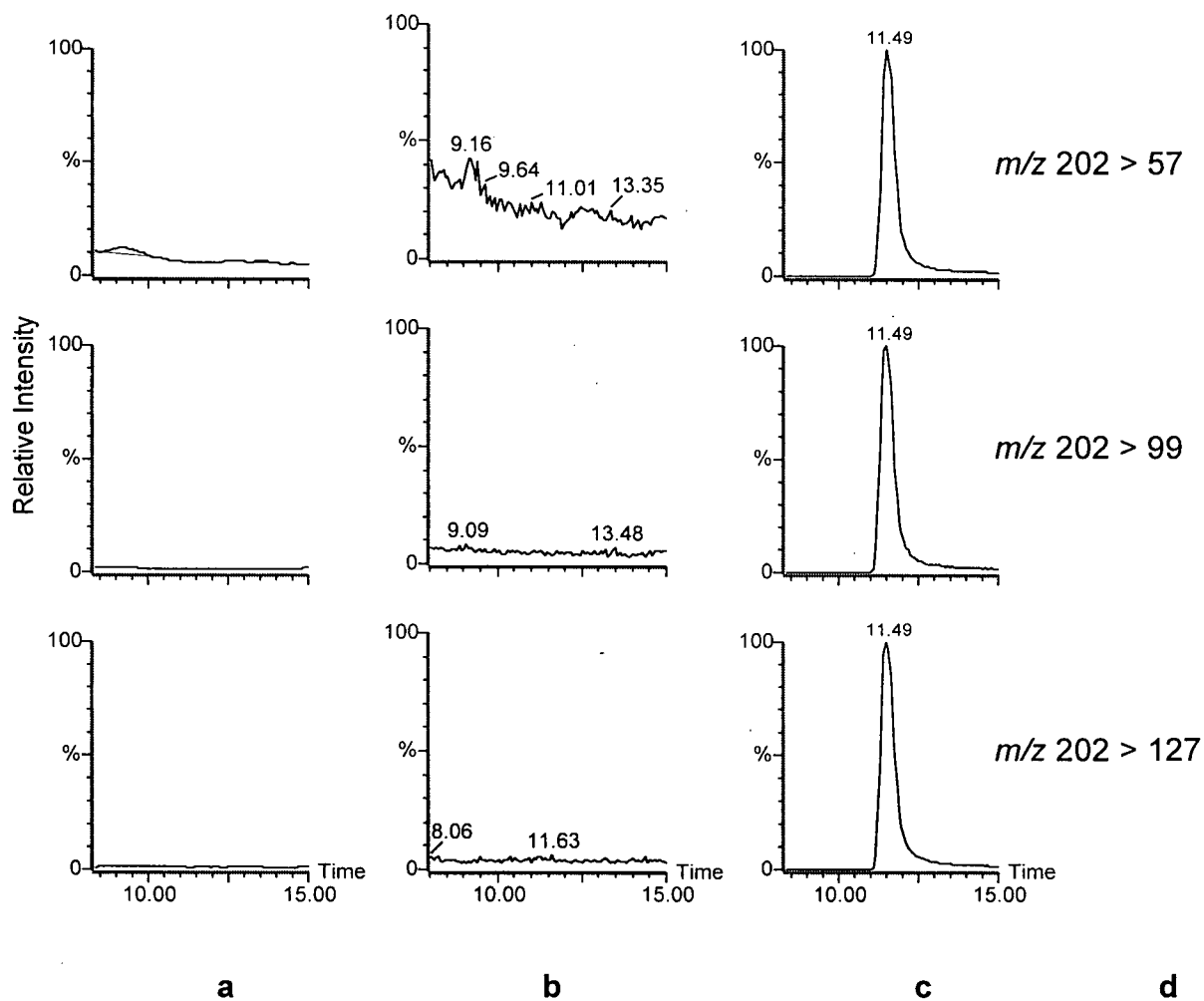
Figure 54. On-line LC/MS/MS monitoring of VPA GLY in (a) a control human CSF sample (b) a CSF sample of a patient treated with VPA and (c) a control human CSF sample spiked with VPA GLY which eluted at t_R=11.49 min. The corresponding characteristic ion transitions monitored for MRM are shown in (d) (mobile phase: ACN (40%): H_2O (60%) and 0.05% TFA).
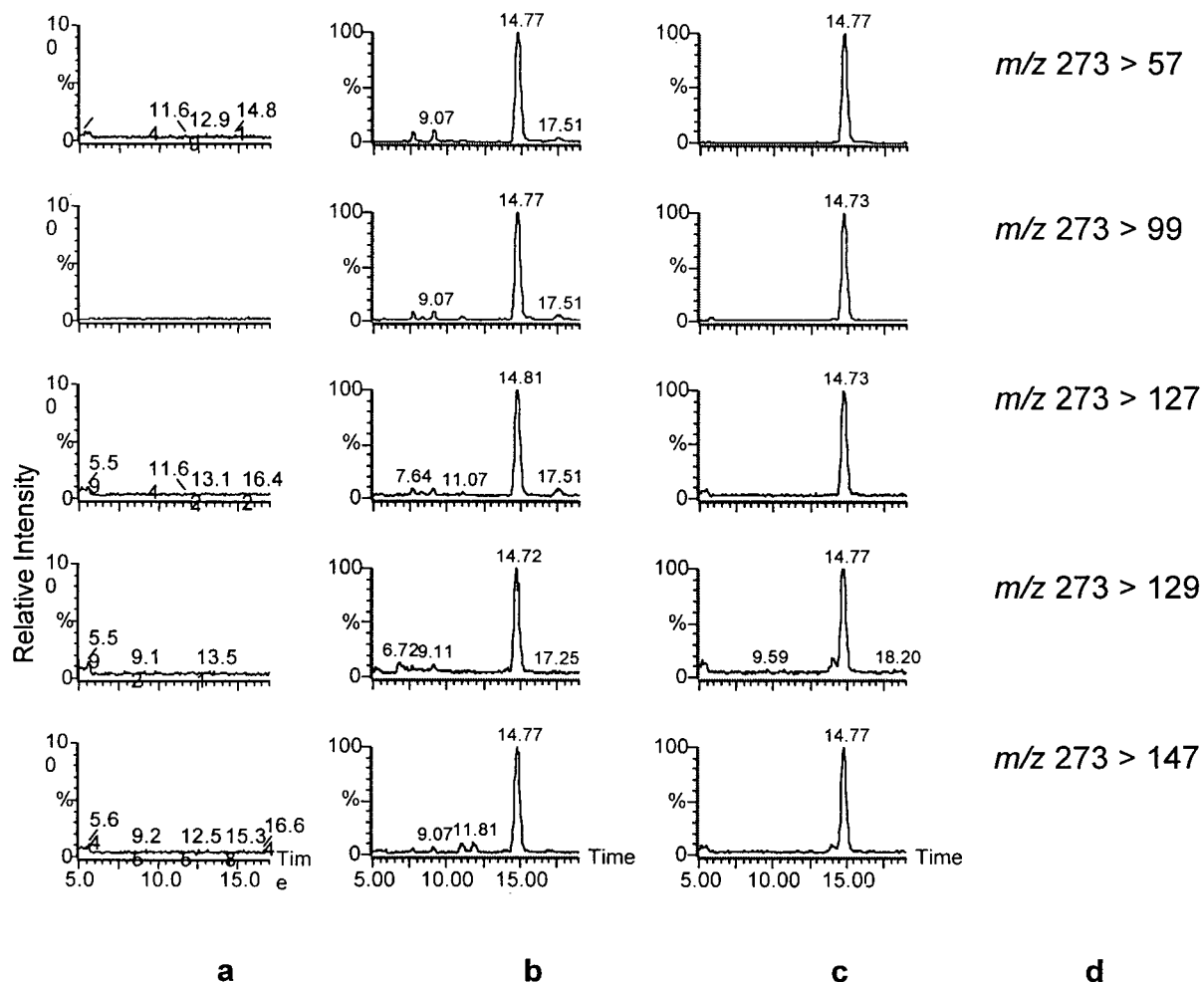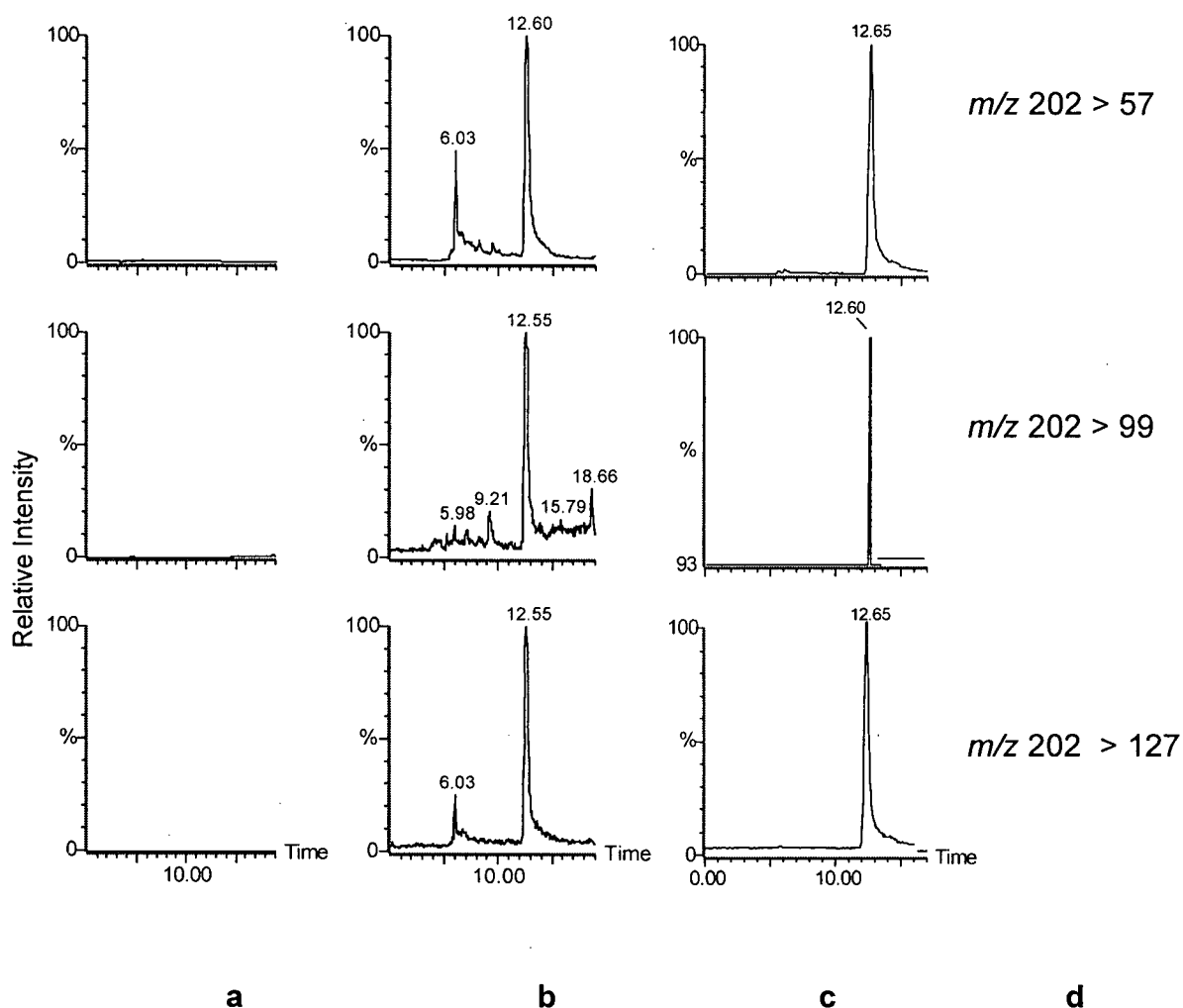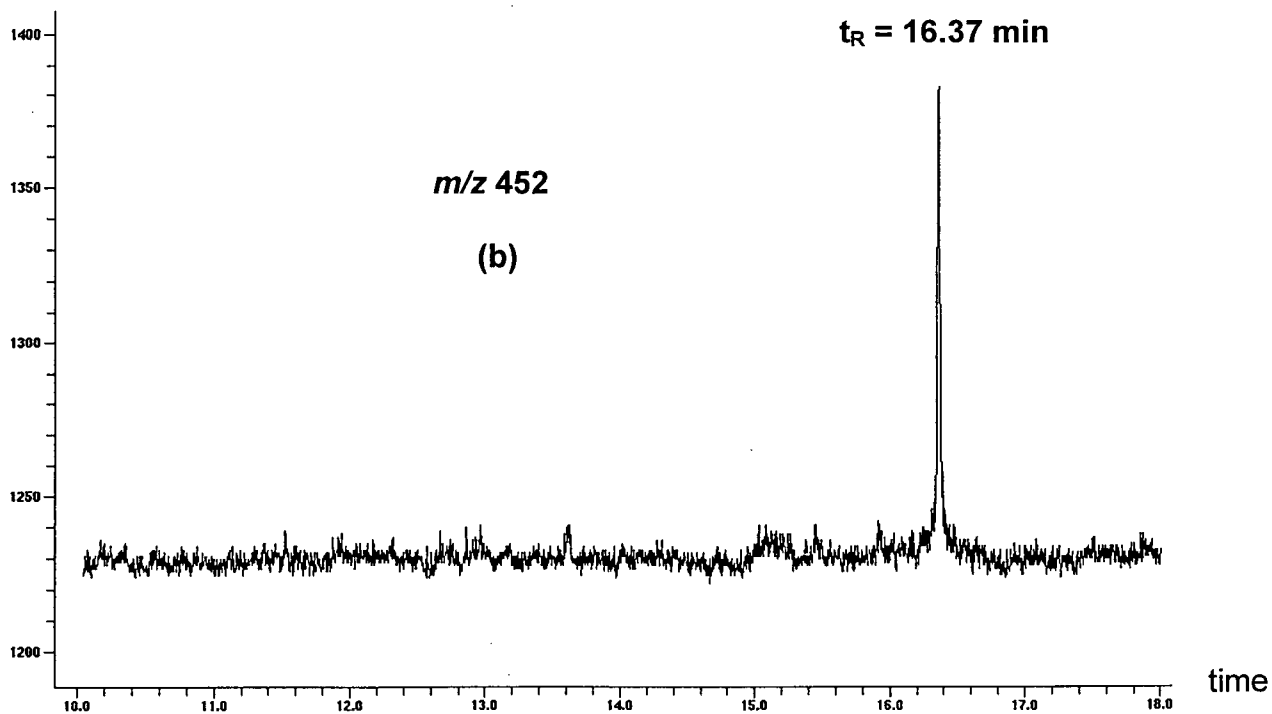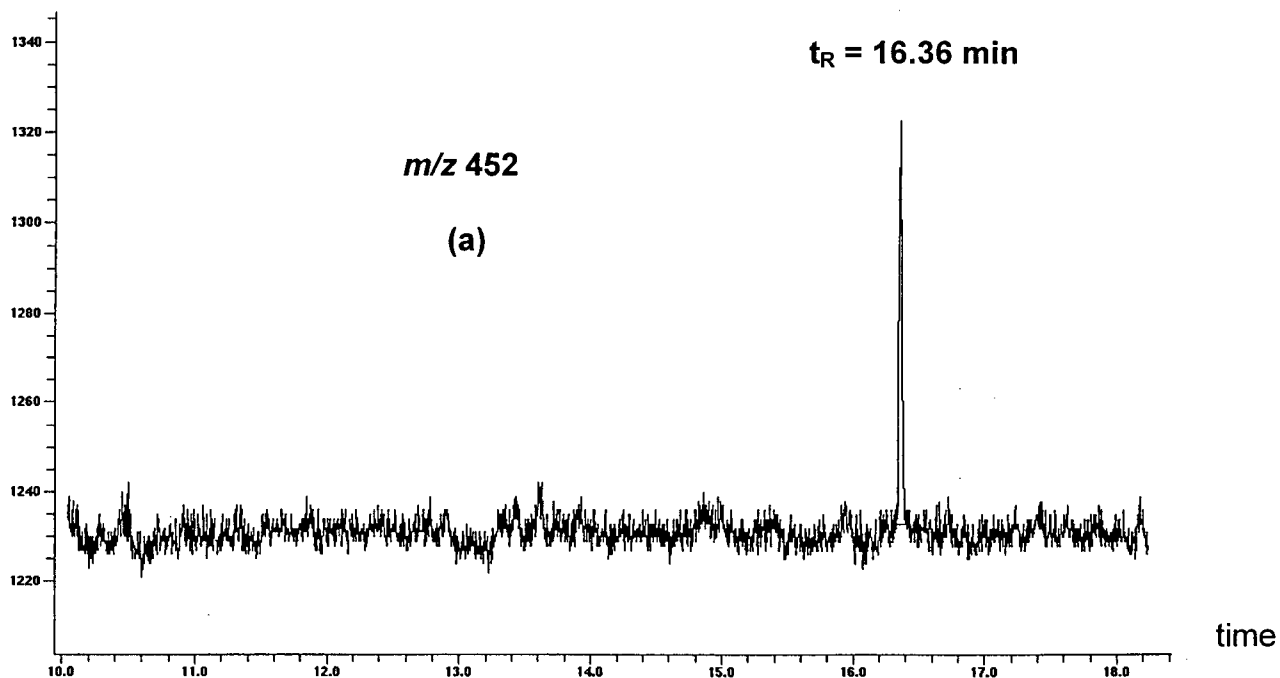
Figure 55. TIC of (a) PFB-derivatized standard of VPA GLU and (b) a PFB-derivatized extract of a CSF sample of a patient on VPA under the conditions of GC method F (See Experimental/Section 2.2.6.6).

reference standard in figure 42 shows that the most abundant ion is the characteristic [M-181]⁻ fragment at *m/z* 452.   Figures 55 a, b show typical selected ion current chromatograms in the NICI mode obtained for the PFB-derivatized CSF extract of the patient (50 μL of CSF) and a standard reference sample. The ion monitored was the [M-181]⁻ fragment carboxylate anion of the di-PFB derivative of the conjugate at *m/z* 452. As in the case of the PFB-derivatized patient serum extract, the chromatogram of the spiked CSF extract showed the elution of VPA GLU at t_R 16.36 min.

The quantitation of the CSF conjugates was relatively facile because no extraction procedure was required. The assay was designed to monitor one characteristic MRM transition for each conjugate simultaneously VPA ASP as the internal standard. To improve sensitivity, a higher volume (100 μL) of sample was introduced onto the column. The slight broadening of the peaks was not chromatographically significant. The range of the assay was 0.01-0.1 μg/mL. The calibration curves had correlation coefficients of 0.993, 0.979, 0.999 for VPA GLU, VPA GLN and VPA GLY, respectively. The concentration of VPA GLU was 0.06 μg/mL of CSF, while that of VPA GLN was 0.034 μg/mL of CSF.

3.3.4 Investigation of AA conjugates of VPA in animals

The interpretation of our human results should take into account that VPA is an antiepileptic drug with a mechanism of action as well as a mechanism of hepatotoxic side effect that are still being investigated. Therefore, it was important to verify that the AA conjugation of VPA also occurs in animals which have been, and continue to be, employed to study the antiepileptic and hepatotoxic effects of VPA.

3.3.4.1 Identification and profiling of AA conjugates of VPA in rats

3.3.4.1.1 Identification of AA conjugates of VPA in rat urine

Analytically, the approach taken to the identification of AA conjugates of VPA in rat urine was the same as that employed for the human studies. Similar to our human findings, VPA GLU, VPA GLN, and VPA GLY were all identified in the 24 h urine sample collected from rats (n=4) dosed with 100 mg/kg of VPA. Appendices i, j and k show the LC/MS/MS chromatograms of each of the conjugates in the urinary extracts of treated rats, control rats and reference standard samples. The total amount of the conjugates excreted over a 24 h period was 0.264 ± 0.088 % of a VPA dose. The contribution of each conjugate to the recovery of a VPA dose is detailed in table 34. Interestingly, unlike our human findings, VPA ASP was also detected in rat urine as shown in figure 56.
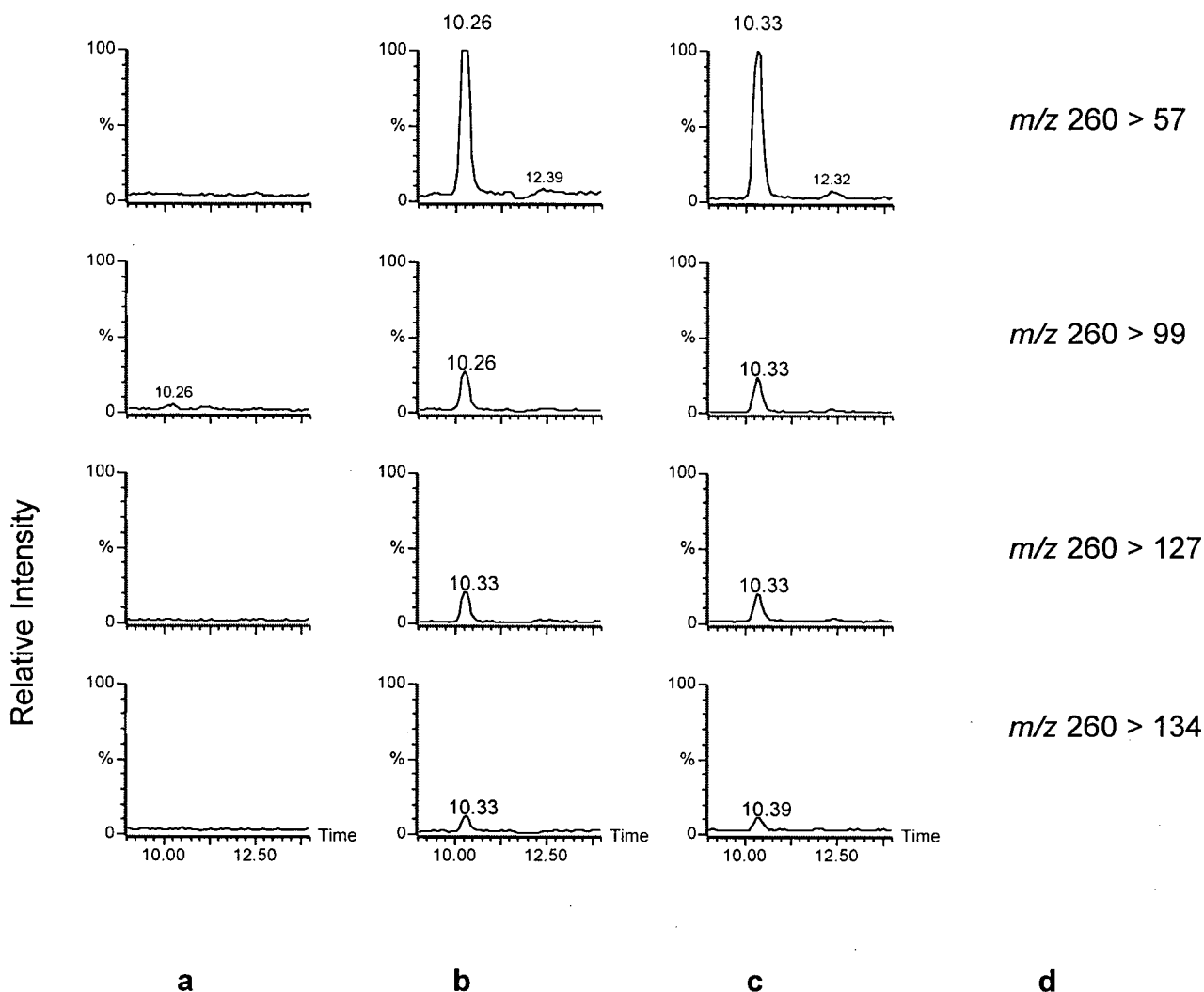
Figure 56. On-line LC/MS/MS monitoring of VPA ASP in: (a) an extracted rat urinary control sample, (b) an extracted urinary sample of a rat dosed with VPA, and (c) a synthetic standard of VPA ASP which eluted at t_R=10.39 min. The corresponding characteristic ion transitions monitored for MRM are shown in (d) (mobile phase: ACN (40%): H_2O (60%) and 0.05% TFA).

Table 34. **% of VPA dose recovered in urine as AA conjugates in rats.**

	VPA GLU	VPA GLN	VPA GLY	VPA ASP	Total
Rat # 1	0.157	0.089	0.130	0.005	0.381
Rat # 2	0.093	0.036	0.043	0.005	0.134
Rat # 3	0.158	0.049	0.066	0.004	0.277
Rat # 4	0.127	0.037	0.058	0.042	0.264
Mean	0.134	0.053	0.074	0.014	0.264
SD	0.027	0.022	0.033	0.016	0.088
C.V.%	20.2	41.2	44.6	114.3	33.3

3.3.4.1.2 Identification of AA conjugates of VPA in serum of VPA treated rats

The MRM chromatograms for the identification of VPA GLU, VPA GLN, and VPA GLY in treated rat serum of VPA treated rats are displayed in appendices l-n. VPA ASP was not detected in the serum of the rats studied (appendix o). Table 35 summarizes the concentrations of the conjugates found in the serum of the five rats sacrificed after four days of treatment with VPA. Blood was collected from the rats one half h after the fifth dose of VPA (100 mg/kg) was administered. While VPA GLN and VPA GLY were both detected in all samples at very low concentration (below 0.01 μg/mL), VPA GLU was found at a concentration (0.091 ± 0.044 μg/mL) corresponding to that observed for humans at steady state.

Table 35. The serum concentrations of VPA GLU, VPA GLN, VPA GLY and VPA ASP in VPA-treated rats.

	VPA GLU μg/mL	VPA GLN μg/mL	VPA GLY μg/mL	VPA ASP μg/mL
Rat # 1	0.167	<0.01	<0.01	nd
Rat # 2	0.096	<0.01	<0.01	nd
Rat # 3	0.066	<0.01	<0.01	nd
Rat # 4	0.091	<0.01	<0.01	nd
Rat # 5	0.035	<0.01	<0.01	nd
Mean	0.091±0.044	<0.01	<0.01	nd

nd=not detected; concentrations for VPA GLU and VPA GLN were below LOQ and and their concentrations were estimated.

3.3.4.1.3 Identification and quantitation of VPA AA in bile of VPA-treated rats

In 6 h bile samples collected from rats (n=4) following a dose of VPA (100 mg/kg), VPA GLU and VPA GLN appeared to be the predominent AA conjugate metabolites while VPA GLY was less significant.

Appendices p-r show the LC/MS/MS chromatograms depicting the identification of VPA GLU, VPA GLN and VPA GLY in bile. Similar to the observation made with respect to serum, the presence of VPA ASP was not positively detected in any of the biliary samples as depicted in appendix s.

As in the case of our CSF samples, biliary samples did not require an extraction step prior to analysis. Quantitation of the conjugates showed that over a 6 h period, less than 0.1 % of the VPA was recovered in bile as VPA GLU and VPA GLN. The conversion of VPA to VPA GLY and VPA ASP was minimal or not detected.

3.3.4.1.4 Identification of VPA GLU in rat brain by GC/MS

Preliminary analysis of VPA GLU in rat brain by GC/MS NICI was investigated with one rat. Figures 57 a-c show the m/z 452 total ion chromatogram for a PFB-derivatized extract of the brain homogenate, the corresponding PFB-derivatized serum extract and a PFB-derivatized standard of VPA GLU. The total ion current is carried by the peak determined to be the PFB derivative of VPA GLU at a similar retention time in all three cases. This experiment was a preliminary step in the design of a more controlled study involving CSF in an animal species, in order to follow up with our human findings.

Furthermore, our observation in this experiment revealed that VPA GLU is also found as a metabolite of VPA in the brain of an animal species which is routinely used to study the effects of the antiepileptic VPA.

However, attempts to analyze brain homogenates for AA conjugates of VPA by LC/MS/MS proved to be challenging. The high protein content of the brain interfered both chromatographically and mass spectrometrically after a single acidic extraction step. Therefore, this approach to the analysis of AA conjugates of VPA in rat brain was not pursued.
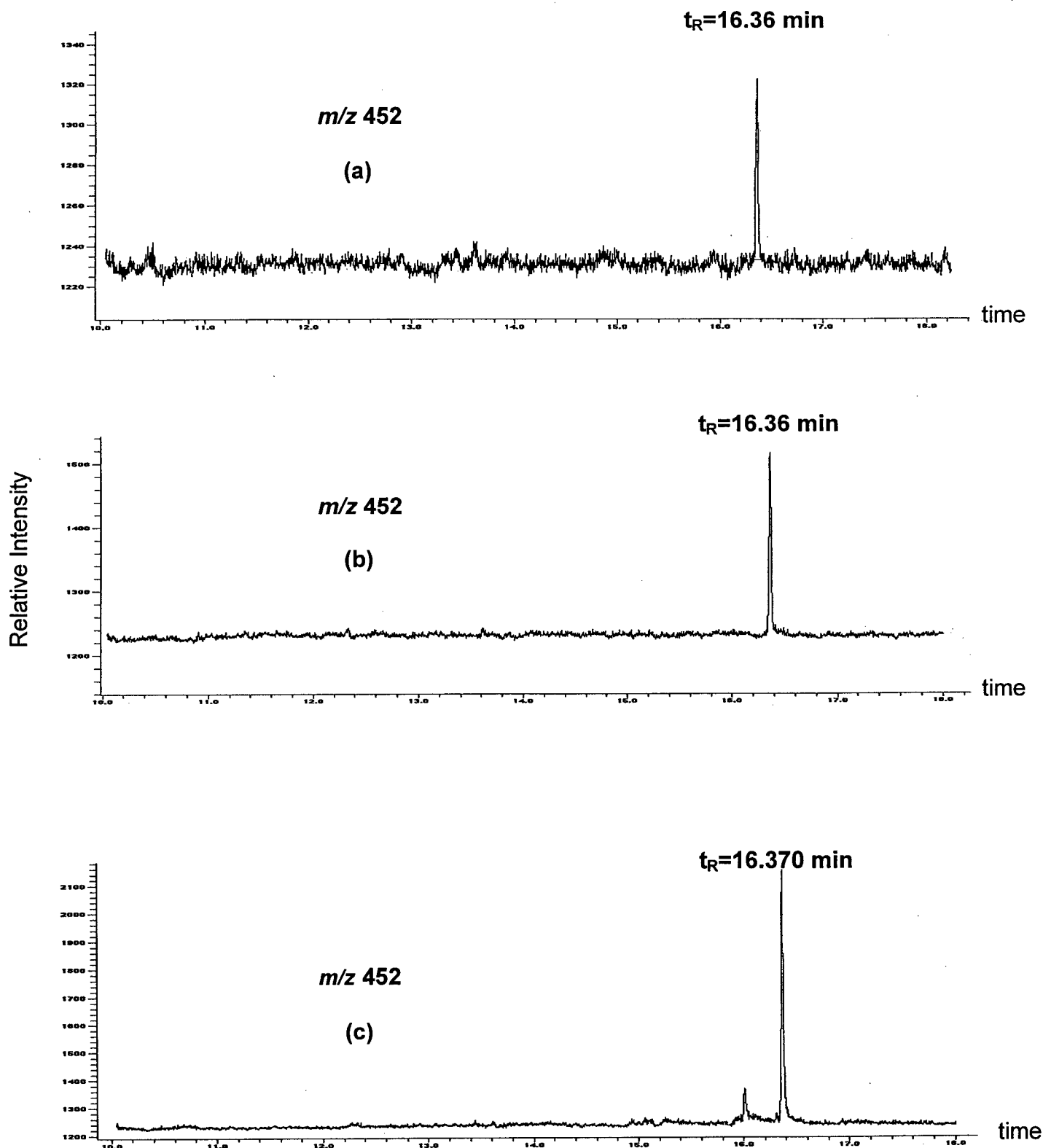
Figure 57. TIC of a PFB-derivatized standard of VPA GLU in (a), a PFB-derivatized brain homogenate of a rat dosed with VPA in (b), and a PFB-derivatized serum extract of a rat dosed with VPA in (c) under the conditions of GC method F (See Experimental/Section 2.2.6.6).

3.3.4.1.5 Hydrolysis of VPA GLU to VPA GLN in rats

Analysis of urine samples collected over 24 h from rats (n=2 for each compound) dosed with either VPA GLU (10 mg/kg) or VPA GLN (10 mg/kg) in a single experiment showed that there was a conversion of VPA GLN to VPA GLU. Based on urinary recovery area ratio, it appeared that less than 10% of VPA GLN had been converted to VPA GLU. From urinary analysis, conversion of VPA GLU to VPA GLN was not observed.

3.3.4.2 Identification and profiling of AA conjugates of VPA in rabbit

3.3.4.2.1 Preliminary observations

On LC/MS/MS analysis, all the characteristic MRM transitions of the VPA GLU conjugate were detected in rabbit CSF after VPA was administered to the animals at doses varying from 300-700 mg/kg. At 100 mg/kg, only one MRM transition i.e m/z 274 > 57 for the VPA GLU was discernable. At doses of 500-700 mg/kg, rabbits appeared to experience neurotoxic effects. In order to compensate for the lack of sensitivity of the LC/MS/MS technique and to minimize the side effects, the dose chosen for the rabbit pilot study was 300 mg/kg. A time profile of the CSF based on 3 time points (n=1 for each time point, figure 58) revealed that the maximum concentration of VPA GLU was formed after 2 h and declined after 3 h.

Time profile of CSF VPA GLU in rabbit
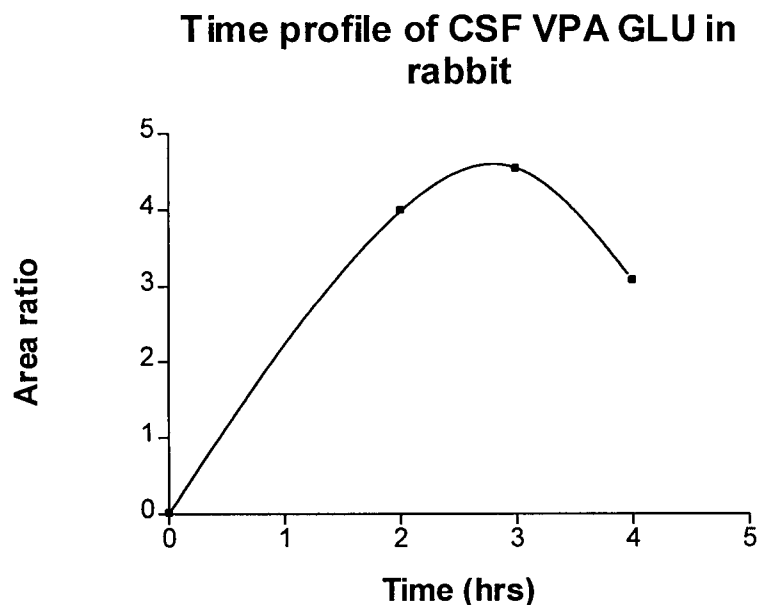
Figure 58. Time profile of CSF VPA GLU in rabbit after an i.p. dose of 300 mg/kg of VPA (n=1 for each time point).

3.3.4.2.2 Investigation of AA conjugates of VPA in the CSF of VPA-treated rabbits

An experiment was conducted such that CSF was collected at 2.5 h after an i.p. dose of 300 mg/kg of VPA was administered to the animals. The search for the VPA AA conjugates was based on the LC/MS/MS detection of all the characteristic MRM transitions monitored for VPA GLU, VPA GLN and VPA GLY. Only VPA GLU was identified in the CSF samples of rabbits. None of the control CSF collected from anesthesized rabbits showed the presence of VPA GLU.
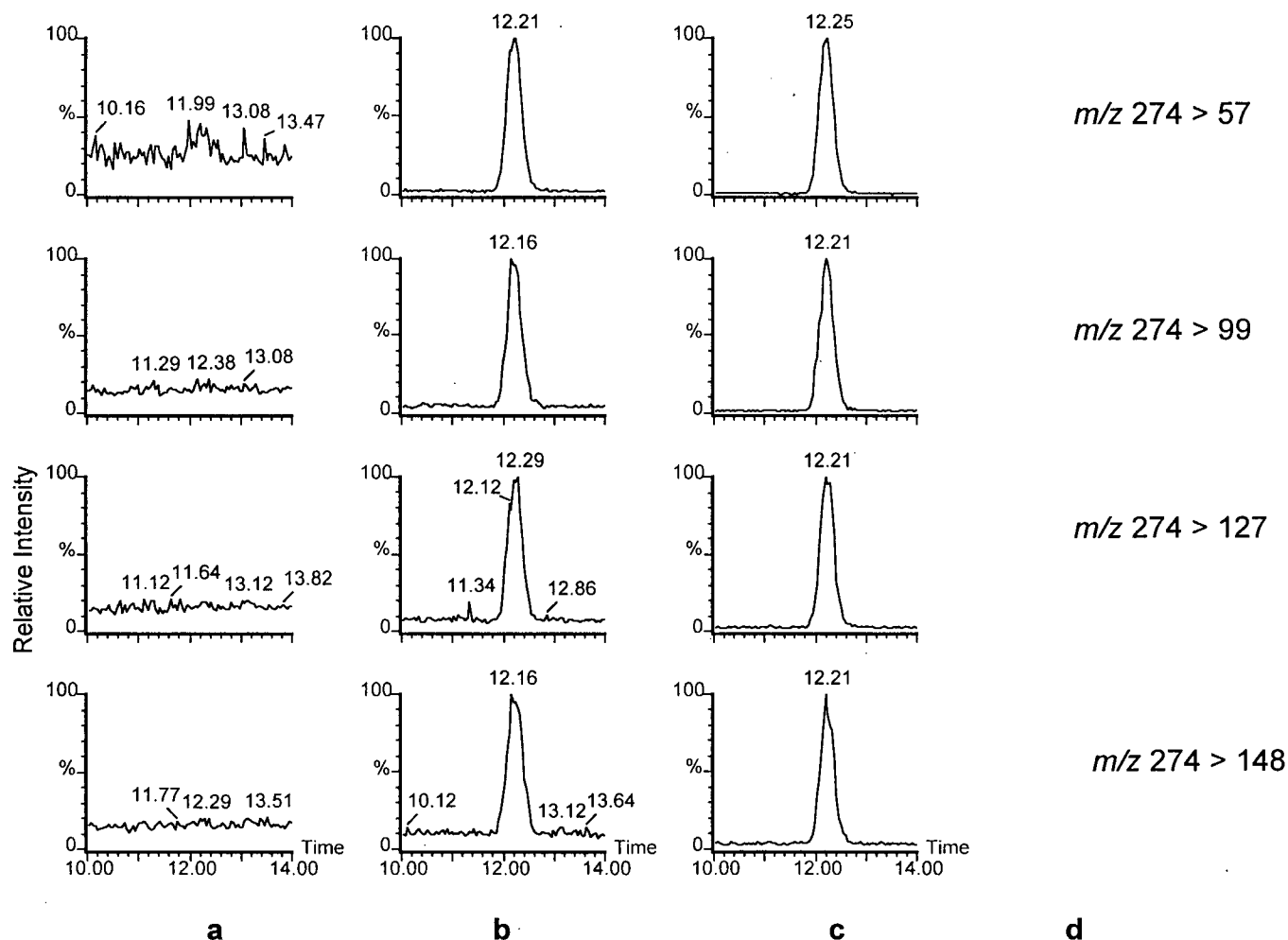
Figure 59. On-line LC/MS/MS monitoring of VPA GLU in: (a) a control rabbit CSF sample, (b) a CSF sample of a rabbit dosed with VPA, and (c) a synthetic standard of VPA GLU which eluted at t_R=12.25 min. The corresponding characteristic ion transitions monitored for MRM are shown in (d) (mobile phase: ACN (40%): H_2O (60%) and 0.05% TFA).

3.3.4.2.3 Identification VPA GLU, VPA GLN and VPA GLY in rabbit serum

Similar to our CSF samples, it appeared that the maximum concentration of serum VPA GLU was formed after 2 hours following an i.p. dose (300 mg/kg) of VPA. The abbreviated time profile for VPA GLU in serum for this dose is shown in figure 60.

Serum VPA GLU (300 mg/kg i.p. of VPA)
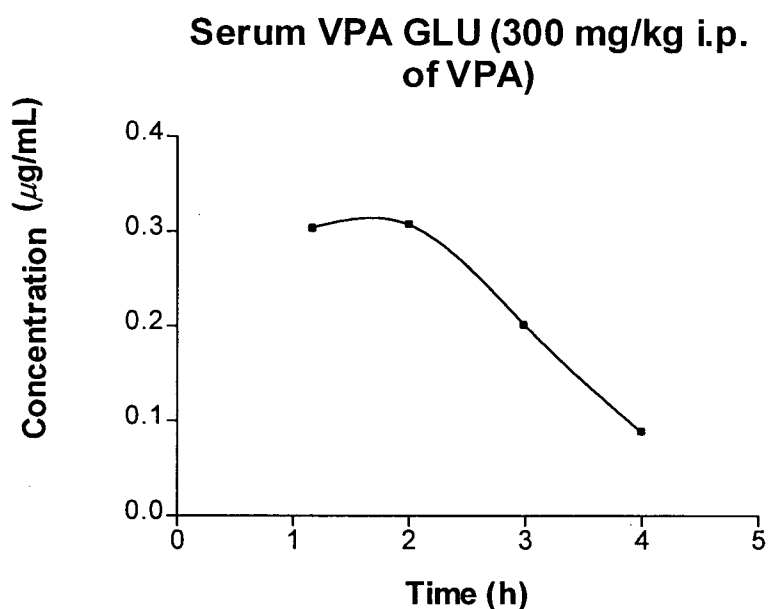
Figure 60. Time profile of VPA GLU in rabbit serum at following an i.p. dose of 300 mg/kg.

Individual serum samples collected from four rabbits 2.5 h following a single i.p. dose of VPA (300 mg/kg) were extracted according to a liquid-liquid extraction procedure employed for both human and rat serum (scheme 4, Experimental section) and analyzed by LC/MS/MS. Each conjugate was monitored individually in the serum

extracts. The characteristic MRM transitions to product ions for VPA GLU, VPA GLN and VPA GLY in the serum extracts are shown in figure 61.

a	b	c	d
Control serum extract of rabbit	Serum extract of rabbit dosed with VPA	Synthetic reference of 3 AA conjugates of VPA and I.S. (FVPA GLN)	Characteristic MRM transitions monitored for each conjugate
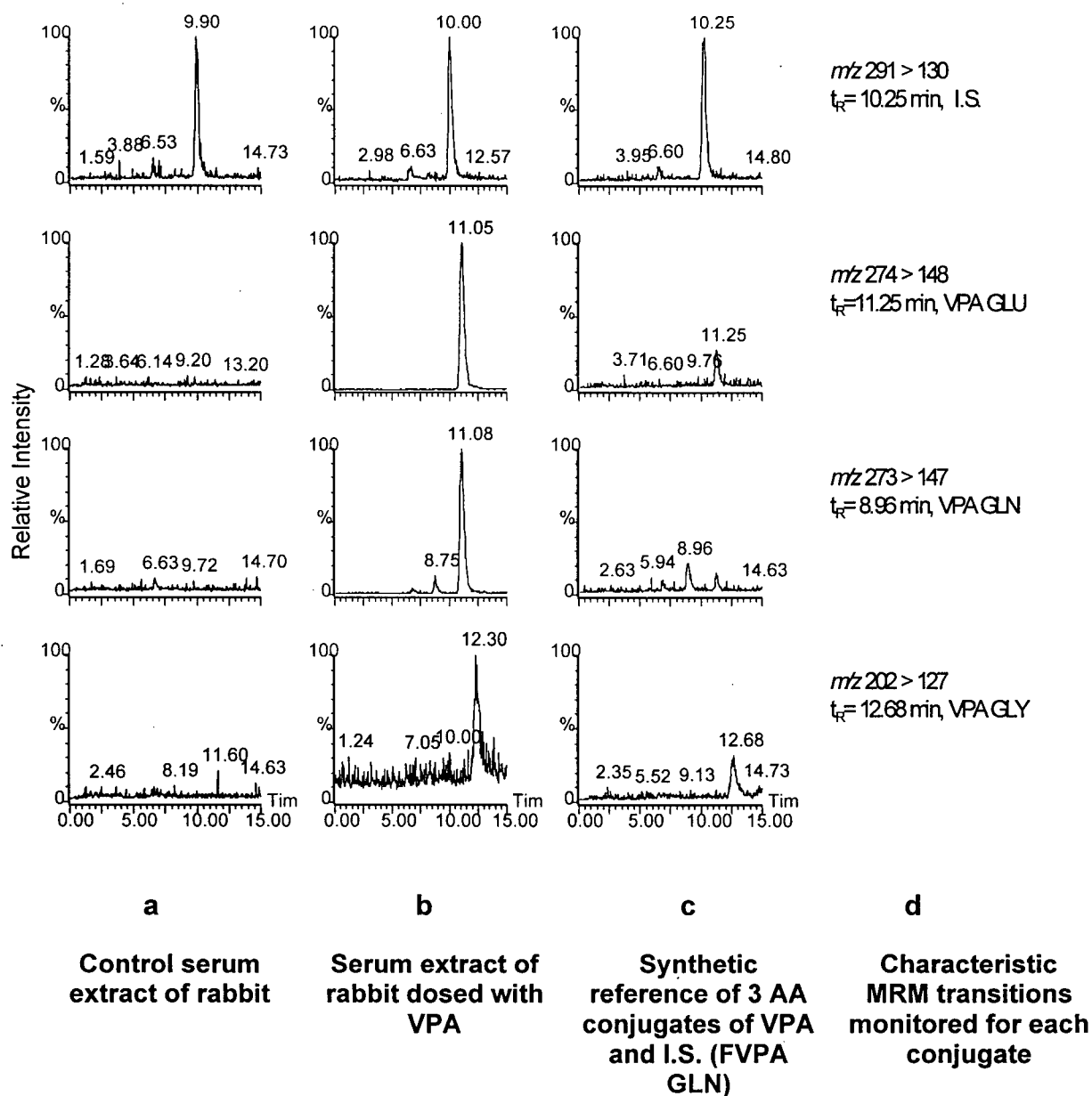
Figure 61. On-line LC/MS/MS monitoring of three AA conjugates of VPA and FVPA GLN (I.S.) in a serum extract of rabbit .

3.3.4.2.4 Profiling of VPA GLU, VPA GLN, VPA GLY in rabbit serum

As for rats and human, all rabbit serum samples were spiked with a FVPA GLN (I.S) and the conjugates were isolated by liquid-liquid extraction at pH 2. The profiling of all three AA conjugates was performed in one analytical run by an LC/MS/MS assay, monitoring one product ion for each AA conjugate. Three standard curves generated for VPA GLU, VPA GLN and VPA GLY over a concentration range of 0.01-0.5 μg/mL, demonstrated good correlation (r^2 = 0.9999) by weighted ($1/y^2$) linear regression.

Serum samples were collected following varying doses of VPA and at different time points. Initially, VPA was administered i.p. at a dose of 100 mg/kg for 3 days. Serum samples (n=1 for each animal) were collected on the third day one h after the third dose. The mean concentration of VPA GLU was quantitated and found to be 0.035 ± 0.019 μg/mL in the serum of four rabbits tested.

At 300 mg/kg of VPA, the serum concentration of VPA GLU (ranged from 0.088 μg/mL to 0.308 μg/mL from 1.17 h to 4 h following the dose.

The serum concentrations of VPA GLU following doses of 500 mg/kg and 700 mg/kg in the rabbit were also measured and the results are tabulated below (table 36).

Table 36. The serum concentration of VPA GLU (μg/mL) in rabbit following high doses of VPA.

Time	1.6 h	2.167 h	2.50 h	4 h
462 mg/kg				0.326
500 mg/kg		0.345	0.722	
700 mg/kg	0.409			

The concentrations of VPA GLU varied for both dose and at different time points. For doses of 462 and 500 mg/kg, the concentration of VPA GLU was highest at 2.5 hrs. Although this study was deficient statistically, the results obtained were of some use for the interpretation of the results obtained for our CSF study described in the previous section.

3.3.4.2.5 Profiling of VPA GLN and VPA GLY in rabbit serum

The calibration curves (r^2=0.99) for the quantitaion of VPA GLN and VPA GLY ranged from 0.01 to 0.5 μg/mL. The concentrations of VPA GLN and VPA GLY ranged from trace-0.05 μg/mL and trace-0.03 μg/mL, respectively. For all doses studied, the LC/MS/MS analysis of VPA GLN was observed at concentrations varying from trace amounts to 23% of serum VPA GLU. VPA GLY was detected at concentrations varying from trace levels to 14.3 % of VPA GLU.

4. DISCUSSION

4.1 Identification, characterization and profiling of thiol conjugates in humans

During the course of this study, we provided evidence to confirm the structure of a second thiol conjugate of (*E*)-2,4-diene VPA, a β-oxidation metabolite of VPA, in humans for the first time. NAC II was determined to be 5-(*N*-acetylcystein-*S*-yl)-2-ene VPA and was positively identified in all the urine samples of patients on VPA. The conjugate was more prominent than NAC I and was detected at 2-3 times the concentrations of NAC I in the urine samples studied by GC/MS NICI. The LC/MS/MS conditions employed to study the conjugates separated the isomers of NAC I. The latter were detected in the urine samples of all patients studied in approximately equal amounts. Since both NAC I and NAC II are the end products of the glutathione conjugates of (*E*)-2,4-diene VPA, the concentration of thiol conjugates in patients on VPA reflect the degree of exposure towards the toxic diene and its precursor, 4-ene VPA. Kassahun *et al.* (1991) proposed that patients on VPA polytherapy would excrete higher amounts of the conjugates compared to patients on VPA monotherapy. Studies so far have failed to associate the two metabolites of VPA, (*E*)-2,4-diene VPA and 4-ene VPA directly with the risk factors of hepatotoxicity. In this study we found that patients $\leq$ 7.5 years and on a high VPA dose as well as those on VPA polytherapy excreted significantly higher amounts of the conjugates than patients > 7.5 years old and on VPA monotherapy. This is an important finding because the two conjugates can be used as markers to predict hepatotoxicity in patients on VPA. NAC III, previously identified in

rats treated with 4-ene VPA (Kassahun *et al.*, 1994) was not detected in any patients' samples.

4.1.1 Characterization of NAC I and NAC II

The search for the thiol conjugates arising from VPA biotransformation in humans and animals was initiated in the laboratory (Kassahun *et al.,* 1991) and led to the discovery of two GC/MS peaks, one of which was positively identified and characterized as 5-(*N*-acetylcystein-*S*-yl)-3-ene VPA (NAC I) while the identity of the second one (NAC II) remained to be established. The characterization of NAC II was an important objective of this project.

It was also a goal of this study to identify and profile the thiol conjugates in patients on VPA in an attempt to assess their exposure to reactive metabolites. Consequently, it may be possible to determine the likelyhood of predicting the onset of VPA-induced hepatotoxicity by comparing the amounts of thiol conjugates excreted by patients at high risk of developing hepatotoxicity to those who are considered safe from this side effect as proposed by Kassahun *et al.* (1991).

Previously, NAC I was synthesized as the dimethyl ester derivative and for the purpose of this project, it was important to synthesize the conjugate in the free acid form. During the initial stages of this project, it was clear that the hydrolysis step used to produce NAC I led to a mixture of compounds. The pursuit to determine the identity and structure of the conjugates formed from the synthetic pathway depicted in scheme 3 of the Experimental section, was the recognition that the products formed synthetically

appeared to be formed *in vivo* as well. Therefore, a series of chromatographic and mass spectral analyses were performed in an attempt to obtain structural information to differentiate and separate the conjugates in the mixture of products. The most important structural information was eventually obtained by ^{1}H NMR as discussed in later sections. During this study, evidence is provided to confirm that the structure of the unidentified conjugate previously recognized by Kassahun *et al.* (1991) was *trans*-5-*N*-acetylcystein-*S*-yl-2-ene VPA (NAC II). Furthermore, it was evident that the formation of the conjugates synthetically could provide a clue to the mechanism by which the conjugates are formed *in vivo*.

4.1.1.1 Hydrolysis of ethyl methyl ester of NAC I

While the synthesis of NAC I and NAC II was performed by Dr. Mutlib for the purpose of this study, the isolation and characterization of the two conjugates became an important objective of this project. The hydrolysis of 5-NAC-3-ene VPA ethyl methyl ester under basic and refluxed conditions was an attempt to form NAC I in the free acid form. Instead, chromatographic and spectral analyses indicated that at least two major products were formed, one of which appeared to be NAC I. DCI mass spectral analysis of the mixture revealed a protonated molecular weight [M+H]$^+$ of 304 consistent with the molecular weight of NAC I. Thermospray mass spectral analyses also pointed towards one molecular weight of 303.

However, gas chromatographic analysis of the product clearly revealed that two major compounds and a minor one were formed and their mass spectral analyses under NICI and EI produced spectra which did not differentiate the compounds from each other

(figures 5 and 6). In EI mode, all three compounds produced spectra characterized by a fragment at m/z 474 consistent with the loss of a t-BDMS group as for NAC I (figures 6b-d). Similarly, in the NICI mode, the PFB derivatives of the mixture showed the separation of two major peaks and a minor one similar to the EI results (figures 5b-d). The NICI spectra of the three compounds were each dominated by the most abundant m/z 482 fragment, the result of the loss of a stable PFB group. The HPLC analysis using phosphate buffer showed a major compound with two minor peaks eluting at t_R 10.388, 10.550, 10.710 min, respectively. When evaluated collectively, the information obtained from the mass spectroscopic and chromatographic analyses suggested that the mixture might be a combination of geometric isomers of NAC I.

However, ^1H NMR analysis ruled out the possibility of geometric isomers but provided convincing evidence pointing to a mixture of two positional isomers. The proton chemical shift data are described in table 1. A comparison of the results with previously reported data from the laboratory (Kassahun et al., 1991) suggested that the chemical shifts consistent with the structure of NAC I were all present with new signals at 6.70 ppm (triplet), 2.25 (triplet), 2.50 (multiplet), and 2.60 ppm (triplet). The multiplicity and the chemical shifts of the last four signals were consistent with the structure of NAC II in which the double bond was at carbon 3,4 of the structure in figure 2 (Silverstein et al., 1981). The discovery made at this point provided compelling evidence to suggest for the first time that the unidentified GC/MS peak in patient urine samples (Kassahun et al., 1991) was 5-(N-acetylcystein-S-yl)-2-ene VPA (NAC II) and the findings were later acknowledged by Tang et al., 1996a.

Since the stability study by ^{1}H NMR ruled out the possibility of isomeric conversion over time, the hydrolysis of the di-ester of NAC I produced a mixture of NAC I and NAC II in the ratio of 1:3 as suggested by ^{1}H NMR and GC/MS analysis. This provided a reasonable explanation for a possible mechanism for the formation of the conjugates *in vivo*, as will be discussed in a later section (4.1.5). For all the reasons just stated, it was of prime importance to separate and confirm the characterization of NAC I and NAC II in the synthetic mixture in order to proceed with the identification and profiling of the conjugates in humans.

4.1.1.2 Isolated NAC I and NAC II

The analysis of the mixture by LC/UV method B facilitated the separation of three peaks (figure 7). A comparison of the mixture with a pure synthetic sample of NAC I showed that the last two eluting peaks were the diastereomers of NAC I. The HPLC conditions allowed for the isolation and purification of each compound in the reaction mixture. Figure 8 showed the HPLC chromatogram of the first eluting peak, NAC II.

The ^{1}H NMR spectrum of the compound (figure 9) confirmed the structure of NAC II, 5-(*N*-acetylcystein-*S*-yl)-2-ene VPA (figure 2). The presence of the double bond was confirmed by the downfield triplet signal at 6.75 ppm corresponding to the single proton at carbon 3. The vinylic proton was coupled to the adjacent -CH$_2$- group and was consistent with a trans double bond based on the chemical shift observed characteristic for this substituted alkene (Silverstein *et al.*, 1981). The absence of a signal for carbon 4 further confirmed the position of the double bond. Furthermore, protons at carbon 5 were coupled to the neighboring -CH$_2$- and were split into a triplet at 2.25 ppm. The

quartet at 2.55 ppm corresponded to the protons at carbon 2 and the triplet at 2.75 ppm was assigned to coupling of the protons on carbon 1 to the adjacent $-CH_2-$.

The presence of the double bond at C3, C4 in NAC II (figure 2) eliminated the structural presence of a chiral carbon in the VPA portion of the molecule and consequently no diastereomeric characteristic was observed for NAC II by HPLC. Both fractions assigned as the diastereomers of NAC I had identical ^{1}H NMR spectra as anticipated by Kassahun *et al.* (1991) for *trans* NAC I. The downfield signal at 5.50-5.65 ppm was a multiplet and consistent with the vinylic protons on carbons 2 and 3 of the structure of NAC I as depicted in figure 2. The quartet at 3.10 ppm was distinct for both isomers of NAC I and could be assigned to the single proton on C4. The doublet at 3.20 ppm was consistent with the protons of carbon 1. The upfield signal at 0.90 ppm corresponded to the terminal methyl proton on carbon 7. The signal at 1.15-1.4 ppm was a multiplet and corresponded to the protons of carbon 6 while the multiplet signal at 1.52-1.75 ppm corresponded to the protons of carbon 5.

The separation of the two diastereomers of NAC I was consistent with previous reports of the separation of diastereomeric mercapturates of bromoisovalerylurea in mobile phases containing ion-pairing reagent (te Koppele *et al.,* 1986,1988). Furthermore, chromatographic analysis of each individual compound was consistent with those observed for the synthetic mixture. The NICI spectra of the corresponding PFB derivatives of both fractions were characterized by the [M-181]$^-$ carboxylate anion. Similarly, in EI mode, the *t*-BDMS derivatives produced spectra characterized by the [M-57]$^+$ at *m/z* 474 as the most abundant fragment. The isomers of NAC I were not separable by GC/MS even under a slow oven temperature program on both DB1701

and DB101 columns. This can perhaps be achieved by using a chiral column and will be important to pursue in order to confirm that the isomerization of NAC I by HPLC does not occur in acidic solution (as an HPLC analysis with an acidic mobile phase). The elution of NAC I ahead of NAC II was consistent with the chromatographic characteristics of phase I metabolites whereby the PFB or *t*-BDMS derivatives of (*E*)-3-ene VPA elute ahead of the corresponding derivatives of (*E*)-2-ene VPA (Abbott *et al.*, 1986; Kassahun *et al.*, 1990).

4.1.2 Characterization of NAC III

This conjugate was first synthesized to serve as a reference sample to validate the formation of the compound as an end product of 4-ene VPA biotransformation in rats (Kassahun *et al.*, 1994). In this study, it was observed that the reaction of NAC III with PFBBr in basic medium resulted in the derivatization of the carboxylic group of the cysteine moiety leaving the lactone group intact. In contrast, the same reaction with 4-OH VPA, the phase I metabolite of VPA, causes the lactone moiety to open and the valproate carboxylic acid group is subsequently derivatized (Kassahun *et al.*, 1990). The observation regarding NAC III could be the result of steric hindrance or bulkiness of the molecule preventing the lactone to exist in the open form. Identification and profiling of the conjugates were carried out on the more polar DB1701 column which allowed for the separation of the epimeric isomers of NAC III which was not achievable on the comparatively nonpolar DB101 column.

4.1.3 GC/MS characteristic of 4,5 diOH VPA-γ-lactone

This compound was first synthesized to study the pathways by which 4-ene VPA is metabolized in rat liver (Rettenmeier *et al.*, 1985). The results were similar to and consistent with the results described in the latter study for the TMS derivative of the lactone.

The TIC of the TMS derivative of the 4,5-diOH VPA-γ-lactone showed that the ion current was carried by two major peaks at t_R 6.756 min and 6.611 min. The mass spectra were characterized by a prominent $[M-15]^+$ ion due to the loss of a methyl group and this fragment ion was used as the ion of choice for the SIM of the lactone in patient sample extracts. It was understood that the two major GC/MS peaks were the epimeric isomers of the compound.

4.1.4 LC/MS/MS characteristics of NAC I, NAC II, and NAC III

The polar characteristic of thiol conjugates allowed for their detection by LC/MS/MS without requiring derivatization. As expected, the more polar NAC III eluted ahead of both NAC I and NAC II under reverse phase chromatography. NAC II eluted ahead of the isomers of NAC I in contrast to the GC/MS results on two columns of different polarities. The presence of TFA in the mobile phase enhanced chromatographic peak shape and resolved the diastereomers of NAC I.

Tandem MS analysis of the synthetic standards reinforced the conclusion with regard to the structure of NAC II and the diastereomeric properties of NAC I. However, it

should be noted that this technique did not provide evidence to determine the position of the double bond in NAC I and NAC II. All three conjugates were characterized by the precursor ion *m/z* 304. Upon CID, the precursor ion produced identical ES$^+$ product ion spectra for NAC IA and B and similar to NAC II. The sensitivity of the conjugates based on signal to noise ratio (>3) decreased in the following order NAC III > NAC II > NAC I. However, the intensity of [MH]$^+$ of the product ion spectra under similar experimental conditions for the three conjugates were NAC III > NAC I > NAC II, as depicted by figures 14, 15, and 23 and appendices g and h and was dependent on the collision energy employed.

The fragmentation pattern for the positional isomers showed cleavage on either side of the sulfur bond characteristic and consistent with reported literature for thioethers and mercapturates (Deterding *et al.,* 1989; Odell *et al.,* 1991). Furthermore, the MS/MS fragmentation was consistent with the ES$^+$ characteristics reported for the GSH derivatives of NAC I, NAC II , and NAC III (Tang and Abbott, 1996c). The product ion at *m/z* 130 represented a typical fragment usually associated with NAC conjugates upon CID. Interestingly, under API or thermospray, the corresponding neutral loss fragment *m/z* 129 is usually observed (Murphy *et al.,* 1992; Baillie and Davis, 1993). The fragment at *m/z* 123 corresponded to VPA followed by the loss of a water molecule. Therefore, detection of NAC I and NAC II in patient samples was accomplished by the MRM of characteristic transitions MH$^+$ to *m/z* 130 and MH$^+$ to *m/z* 123, respectively.

While the position of the double bond in NAC I and NAC II did not alter the fragmentation pattern observed in their product ion spectra, the lactone moiety of NAC III produced spectra which lacked *m/z* 123 (figure 23). However, *m/z* 130 remained

distinct for NAC III indicating fragmentation at only one side of the thioether. Furthermore, *m/z* 95 which we presumed was the product ion resulting from the loss of the NAC and COOH groups was weak in contrast to NAC I and NAC II. The product ion at *m/z* 262 for NAC III has been proposed to result from the loss of the ketene group (Tang, Ph.D. thesis, 1996) while *m/z* 216 occurred following an additional loss of a COOH group. For the purpose of identification of NAC III in MRM, the transition MH^+ to *m/z* 130 served as the characteristic transition for the conjugate and the results were further verified by GC/MS.

4.1.5 Identification of thiol conjugates in patients on VPA therapy

We have reported the identification of the novel metabolite of 5-(*N*-acetylcystein-*S*-yl)-2-ene VPA (NAC II) in humans (Gopaul *et al.*, 1996c). Confirmation of the conjugate was achieved through a series of chromatographic and mass spectral analyses of urine samples of patients (n=39) on VPA therapy. The PFB derivatives of the urine extracts of patients were analyzed on two columns of different polarities. The GC/MS NICI peaks were characterized by the same retention times and mass spectral features of the PFB derivative of the synthetic standard of NAC II in SIM or scan mode under the same experimental conditions. The GC/MS results were confirmed by LC/MS/MS analyses of the intact conjugates by MRM of their characteristic product ions.

Similarly, NAC I was also identified in all of the patients studied. Although the identification of NAC I in human and rat has already been reported (Kassahun *et al.*, 1991), we were able to separate and isolate both isomers of NAC I by HPLC and determine their presence in equal amounts in patients on VPA by LC/MS/MS.

The GSH conjugation of (E)-2,4-diene VPA which eventually leads to the formation of NAC I and NAC II, is proposed to occur via a Michael-type addition reaction (Kassahun *et al.*, 1991; Friedman *et al.*, 1965), requiring that the conjugation proceed when the diene VPA is in the ester form. The nature of the ester is believed to be important for the reaction to occur (Delbressine *et al.*, 1981). *In vivo*, the diene can be formed as the Coenzyme A ester in mitochondria, the result of β-oxidation of 4-ene VPA or perhaps as the glucuronide ester, following the microsomal oxidation of (E)-2-ene in the cytosol (Kassahun *et al.*, 1994).

Although, the GSH conjugate of (E)-2,4-diene VPA glucuronide has been identified in the bile of rats treated with (E)-2,4-diene VPA (Tang *et al.*, 1996b), we were unable to detect the corresponding NAC derivative in urine samples of patients on VPA during this study. The latter finding further supports the theory that it is the CoA ester of (E)-2,4-diene VPA but not the glucuronide ester of the diene, which is the reactive ester involved in GSH conjugation in humans and that mitochondria is the site where the reaction occurs (Kassahun *et al.*, 1991).

Furthermore, comparative toxicological studies of 4-ene VPA and (E)-2-ene VPA do not support the latter metabolite as a potential hepatotoxin (Kesterson *et al.*, 1984) in rats ruling out (E)-2,4-diene VPA glucuronide formed from (E)-2-ene VPA as toxicologically significant. In contrast, evidence is mounting to implicate the metabolic pathway which leads to the formation (E)-2,4-diene CoA ester from 4-ene VPA in mitochondria to be responsible for VPA-induced hepatotoxicity (Kesterson *et al.*, 1984; Kassahun *et al.*, 1991, 1993, 1994; Tang *et al.*, 1996a, 1995). Hence, the (E)-2,4-diene VPA CoA is

believed to be the reactive metabolite which conjugates with GSH to form GSH conjugates and their respective NAC conjugates.

The discovery of NAC I (Kassahun *et al.,* 1991) and NAC II in humans lends further credence to the reactivity of (*E*)-2,4-diene VPA CoA in humans and their formation *in vivo* is a reflection of the exposure of humans to the toxic metabolite, (*E*)-2,4-diene VPA CoA. Based on the evidence obtained from the hydrolysis of the ethyl methyl ester of (*E*)-2,4-diene VPA, it is reasonable to infer that the formation of NAC II *in vivo* is the result of the rearrangement of the double bond of 5-GS-3-ene VPA-CoA following the conjugation of GSH with (*E*)-2,4-diene VPA , as we propose in scheme 6.

The conjugation of GSH with (*E*)-2,4-diene VPA gives rise to a 5-GS-3-ene VPA-CoA. The acidic proton alpha to the carbonyl group can be easily abstracted under basic conditions. The resulting product can exist in equilibrium with a second one where a more stable carbanion is in conjugation with an allylic double bond and that of a carbonyl group as shown in scheme 6. Upon acidification and hydrolysis, 5-GS-3-ene VPA and 5-GS-2-ene VPA are formed in amounts favoring the more stable 5-GS-2-ene VPA. These are subsequently subject to further metabolism along the mercapturic pathway to form NAC I and NAC II, respectively.

The mechanism proposed by Kassahun *et al.* (1991) for the conjugation reaction of GSH with (*E*)-2,4-diene CoA also involved the formation of a tertiary carboxyl anion as in scheme 6 and presumably could also lead to the formation of 5-GS-2-ene VPA CoA. Alternatively, the formation of NAC II could be explained by invoking the participation of isomerase, the enzyme responsible for converting a 3-ene double bond

$$\text{GS-CH}_2\text{-CH=CH-CH-}\overset{\overset{\displaystyle O}{\|}}{\text{C}}\text{-S-CoA}$$
$$|$$
$$R$$

$$\downarrow \text{OH}^-$$

$$\text{GS-CH}_2\text{-CH=CH-}\overset{-}{\text{C}}\text{-}\overset{\overset{\displaystyle O}{\|}}{\text{C}}\text{-S-CoA} \quad\longleftrightarrow\quad \text{GS-CH}_2\text{-}\overset{-}{\text{C}}\text{H-CH=C-}\overset{\overset{\displaystyle O}{\|}}{\text{C}}\text{--S-CoA}$$

$$\downarrow \text{H}^+$$

$$\text{GS-CH}_2\text{-CH=CH-CH-}\overset{\overset{\displaystyle O}{\|}}{\text{C}}\text{-OH} \qquad + \qquad \text{GS-CH}_2\text{-CH}_2\text{-CH=CH-}\overset{\overset{\displaystyle O}{\|}}{\text{C}}\text{-OH}$$

$$\downarrow \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad \downarrow$$

$$\text{NAC-CH}_2\text{-CH=CH-CH-}\overset{\overset{\displaystyle O}{\|}}{\text{C}}\text{-OH} \qquad\qquad \text{NAC-CH}_2\text{-CH}_2\text{-CH=CH-}\overset{\overset{\displaystyle O}{\|}}{\text{C}}\text{-OH}$$

$$R = C_3H_7$$
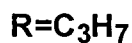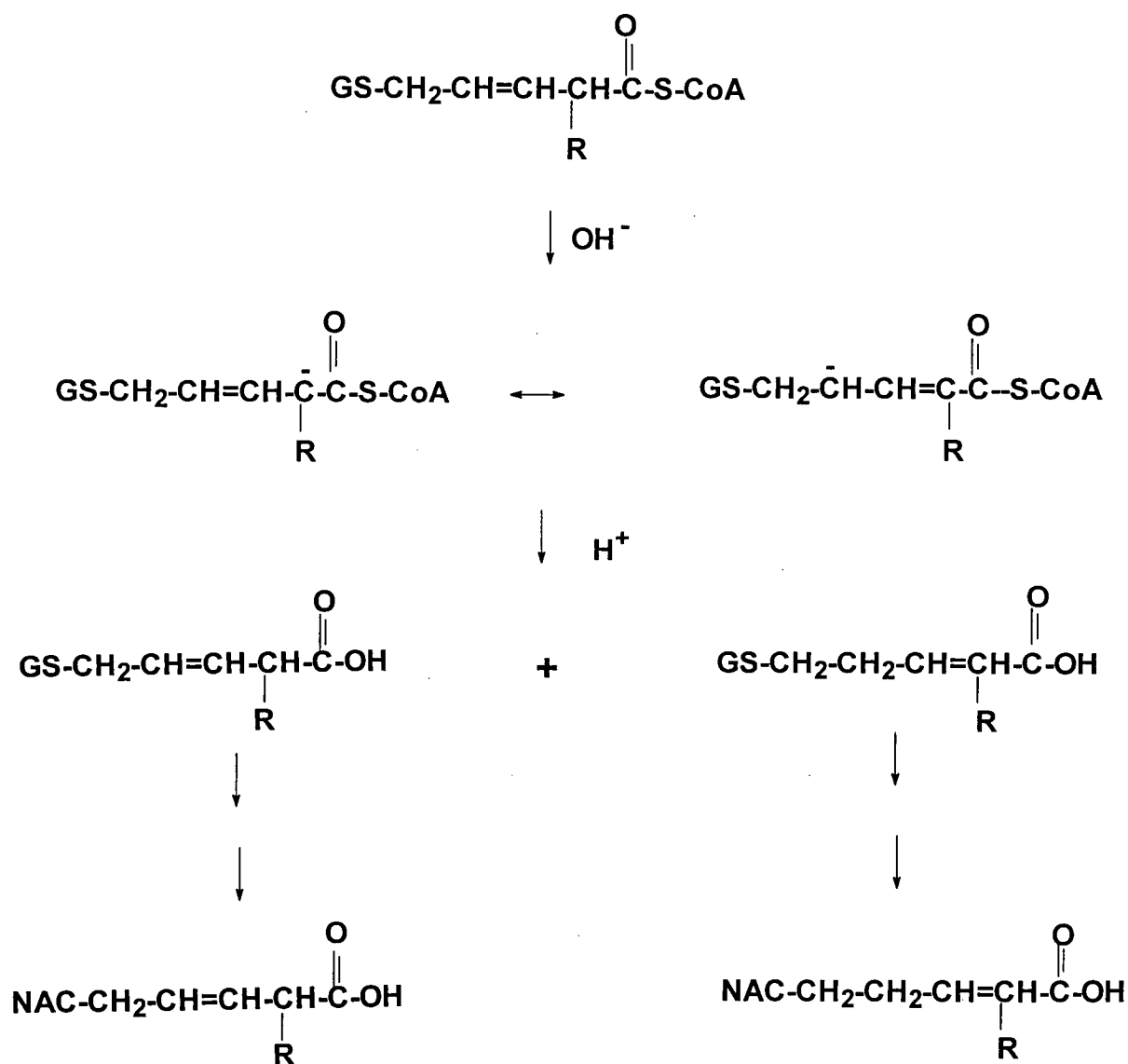
Scheme 6. Proposed metabolic pathway leading to the formation of NAC I and NAC II *in vivo*.

to a trans 2-ene double bond (Osmundsen and Hovik, 1988; Kassahun *et al.,* 1993), converting NAC I to NAC II. The action of 2,3-enoyl CoA isomerase has been invoked to explain the origin of 2-ene VPA and 3-ene VPA (Granneman *et al.,* 1984a; Rettenmeier *et al.,* 1987), both of which are believed to be formed in mitochondria (Bjorge and Baillie, 1991). This would support the theory that mitochondria is the site of formation for the GSH conjugates of (*E*)-2,4-diene VPA which are further metabolized to NAC I and NAC II. Hence, hepatic mitochondria are more susceptible to injury as a result of exposure to reactive metabolites of VPA.

4.1.6 Search for NAC III in urine samples of patients on VPA therapy

NAC III was not detected in any of the patients studied (n=39) either by GC/MS or LC/MS/MS. Chromatographic and mass spectral characteristics of a reference sample of NAC III were absent in all the urine extracts of the samples studied. The non-reactive side product of 4,5-epoxy VPA, *i.e.* 4,5-diOH VPA-γ-lactone, was also searched for in two of the patients studied and was not found. It appeared that the metabolic pathway of 4-ene VPA which leads to the formation of the epoxide as proposed previously for rats (Rettenmeier *at al.,* 1985) may not be a significant pathway in patients, at least those not exhibiting overt toxicity.

That we were unable to detect the thiol conjugate of 4-ene VPA or its side product suggests that 4-ene VPA is immediately excreted as the glucuronide or metabolized to (*E*)-2,4-diene CoA in mitochondria. This reasoning supports the argument that 4-ene VPA formed in patients on VPA therapy appears to be less reactive than (*E*)-2,4-diene VPA although the mono-unsaturated metabolite has proven to be more steatogenic in

rats than the diene (Kesterson *et al.*, 1984). This observation was rationalized by the proposition that (*E*)-2,4-diene VPA is more harmful when formed as a CoA ester *in situ* and at the site of toxicity in mitochondria (Kasshun *et al.*, 1991). Furthermore, most of the urinary 4-ene VPA (> 90%) was recovered as its hydrolyzed product suggesting that most of 4-ene VPA was formed as its glucuronide ester.

However, the results cannot eliminate the possibility that the lack of detection of NAC III was because 4,5-epoxy VPA spontaneously binds to macromolecules as demonstrated in rats (Kassahun *et al.*, 1994) and escaped detection by the methods. Investigators demonstrated previously that in rats dosed with VPA, NAC III accounted for 11.5 % of a 4-ene VPA dose (100 mg/kg). Thereafter, a study conducted in the laboratory, investigating the biotransformation of 4-ene VPA in rats and guinea pigs, showed that the formation of NAC III from 4-ene VPA was higher in newborn guinea pigs than in young guinea pigs when treated with 4-ene VPA (100 mg/kg, Tang *et al.*, 1996). The activation of the pathway leading to the formation of NAC III in VPA-induced hepatotoxicity is unlikely to occur, however, it cannot be ruled out and therefore NAC III is a metabolite that remains to be studied in hepatotoxic patients.

4.1.7 Profiling of NAC I and NAC II by LC/MS/MS in urine samples of patients on VPA therapy

The assay employed for the quantitation of NAC I and NAC II was a combination of an efficient SPE extraction procedure and MS/MS detection under ES^+ which allowed for the simultaneous screening of the conjugates with a high degree of specificity and quantitation over the range of 0.1-1 μg/mL. This HPLC assay had the added capability

of separating both isomers of NAC I from each other and from the only isomer of NAC II based on two MRM transitions without requiring any derivatization steps. Furthermore, the SPE extraction procedure had a high recovery (98-112%, table 6) and was most appropriate for the less sensitive detection of the LC/MS/MS system (LOD~500 pg) compared to the GC/MS system (LOD~20 pg).

The SPE extraction procedure was fast and convenient, requiring very few steps. A combination of C_2 SPE cartridges, a water/methanol (95:5) wash and employment of a C_8 HPLC column proved highly efficient in removing urinary endogenous substances and stabilizing retention time shifts considerably while producing a high recovery of the analytes. The addition of the modifier TFA to the mobile phase at 0.05 % separated the isomers of NAC I but at the expense of sensitivity. On the other hand, the addition of TFA in combination with PPA as modifiers at 0.05 % increased sensitivity without compromising separation of the peaks. Because the analytes were being detected in positive ES, the sensitivity reduction with TFA could be attributed to the ion-pairing effect of the modifier (CF_3COO^-) (Zheng, Ph.D thesis, 1997).

Quantitation was based on weighted linear regression ($1/y^2$) analyses of the calibration curves. The latter were linear over a concentration range 0.1-1 μg/mL and afforded coefficients of determination (r^2) of 0.999 or better for both conjugates. The inter-assay variation based on the slopes of the calibration curves were 14 % for NAC IA and B and 15.4 % for NAC II (table 2). The intra-variation of the assay based on slopes of calibration curves and samples spiked at a low and high concentration yielded a CV of less than 11% (tables 3 and 4). Quality control samples run in triplicate throughout the

study indicated a variation ranging from 2.5-15.6 % for six different concentrations and for both conjugates (table 5).

Overall, validation studies showed that the assay could only quantitate reliably within a range that was too narrow for the study. Therefore, the LC/MS/MS assay served as the primary tool for the identification and screening of the conjugates and provided an estimate of the amount of conjugates which was then confirmed by the GC/MS assay under SIM. A comparison of the results by LC/MS/MS and GC/MS (figure 28) for the concentrations of NAC I and NAC II clearly showed that beyond 1 μg/mL, there was little correlation between the two assays. Therefore, the results obtained by LC/MS/MS provided support for the GC/MS assay. The LC/MS/MS assay confirmed that both isomers of NAC I were always present in equal amounts in patients on VPA and that NAC II was confirmed at concentrations consistently higher than NAC I as observed by GC/MS. In most cases, the intensity of the peak for NAC II was observed to be approximately 2-3 times higher than the peaks for both isomers of NAC I. The assay remained a useful quantitative tool for biological fluids containing the conjugates within the 0.1-1 μg/mL concentration range and has been employed for other studies performed in this laboratory involving the biotransformation of 4-ene VPA in animals (Tang *et al.,* manuscript in preparation)

4.1.8 Profiling of NAC I and NAC II by GC/MS

4.1.8.1 GC/MS assay for the profiling of NAC I, NAC II and NAC III in urine of patients on VPA therapy

The GC/MS assay employed here was a modification of the assay developed earlier in the laboratory (Kassahun *et al.*, 1991) to include the quantitation of NAC II and III. This assay utilized a liquid-liquid extraction procedure at pH 3-4 followed by extraction with EtOAc. The extract was derivatized with PFBBr in the presence of DIPEA at 50°C and showed no sign of thermal decomposition. Although in the original assay the pH was adjusted 2 with HCl (3N), it was observed that the extraction of the conjugates and the internal standard, NAC VI, were more reproducible when phosphoric acid was used. Similar to previous findings (Kassahun *et al.*, 1991), the derivatization of the conjugates with PFBBr to form either the mono-PFB derivative of NAC III or the di-PFB derivatives of NAC I and NAC II was facile. Derivatization was found to be most efficient at 50°C.

The analysis was performed in the SIM mode, monitoring for *m/z* 482 for the NAC I and NAC II, *m/z* 302 for NAC III and *m/z* 414 for NAC VI (I.S.) and proved to be highly reliable and robust over a range of 0.1-5 μg/mL. The PFB derivatives of the conjugates and I.S. were well separated from each other producing sharp chromatographic peaks facilitated by a slow oven temperature program to ensure separation from any endogenous interferences. Furthermore, the temperature program was chosen to enable the analysis of phase I metabolites in the future. Sensitivity based on signal to noise > 3 was abundant and highly convenient for the purposes.

The assay showed good precision and accuracy. The inter-assay variability based on slopes was better for NAC I (8%) than NAC II (25 %) (table 7). An evaluation of the slopes suggested that while the response of NAC I remained fairly stable on a day to day basis, that of NAC II increased, an event which is often observed for more polar compounds. Possibly NAC II may be more bound to active sites on the column, a phenomenon which improved over time.

Intra-assay variability studies based on slopes or spiked samples (tables 9 and 10) did not appear to show the same trend and showed variability of less than 10 % for each sample of NAC I and less than 20 % for each sample of NAC II studied. Samples spiked with NAC I were found to be within 90-110 % of their expected values. Samples spiked with NAC II were within 85-120 % of their expected values with the exception of one sample. Although recovery in the assay was within 53-63% for NAC I and 43-69% for NAC II (table 11), the sensitivity, the precision and the accuracy of the assay were reliable during the study.

4.1.8.2 Quantitation of NAC I and NAC II in urine samples of patients on VPA by GC/MS

4.1.8.2.1 Recovery of thiol conjugates in urine of patients on VPA therapy

The thiol conjugates recovered in the urine of 4 patients formed an average about 0.23 % of a VPA dose (table 13). While the amount of the conjugates recovered constituted a relatively small percent of a VPA dose, one needs to consider that a daily dose of VPA ranged between 500-2000 mg for the patients we studied. Hence, the results suggest

that a non-hepatotoxic patient belonging to a non high risk category, may be exposed to at least 1.15-4.6 mg of 4-ene VPA and (E)-2,4-diene VPA CoA. It is conceivable that a patient, less than 2 years of age and on polytherapy, may be exposed to relatively higher amounts of 4-ene VPA plus (E)-2,4-diene VPA CoA at every dose as a result of increased metabolism due to age and enzyme induction. In such cases, over a period of 90 days or less, given the duration within which VPA-induced hepatotoxicity is known to occur, it is quite possible that the patient may be exposed to a cumulative amount of both metabolites which are toxic to both rats and humans. Evidence to support this theory is that various toxicological and mechanistic studies which have shown that when adults rats are dosed with 10 mg of 4-ene VPA (200 g, 100 mg/kg, i.p), the animals will either succumb or develop VPA-induced hepatotoxicity within five days or less (Kesterson et al., 1984; Kassahun et al., 1991, 1993, 1994; Tang et al., 1995).

4.1.8.2.2 Profiling of thiol conjugates by GC/MS

The GC/MS assay employed enabled confirmation that NAC II was the more prominent conjugate in the urine samples of patients on VPA, at a concentration 2-3 times higher than NAC I. These data were consistent with the LC/MS/MS observations. These human results are also consistent with those observed for the hydrolyzed products of the di-ester of NAC I during synthesis. This further supports the proposed mechanism for the origin of these conjugates, namely, that they result from the hydrolyzed products of the CoA ester of the GSH conjugate of (E)-2,4-diene VPA, the precursor of NAC I and NAC II as described in scheme 6.

The results obtained during this study were consistent with the results previously obtained in this laboratory (Kassahun *et al.*, 1991). The purpose of the profiling study was to compare the excretion of the conjugates in patients on VPA monotherapy and on combined therapy with enzyme-inducing and non-inducing drugs. It was the hypothesis to show that the excreted NAC conjugates of the reactive metabolite (*E*)-2,4-diene VPA CoA will reflect the degree of exposure to these metabolite for patients in the various study groups. As a result, those patients sustaining the highest exposure should be most liable to hepatic injury. This may account for the increased incidence of VPA-associated liver failure in patients on polytherapy (Dreifuss *et al.*, 1987, 1989; Bryant and Dreifuss,1996).

Quantitative urinary analysis of drugs is rare and complicated as it involved many limitations such as ensuring complete timed 24 h urine collections and compliance (Dickinson *et al.*, 1989). For this reason, for the purpose of patient profiling, we opted for a urine sample collected from patients just before their morning VPA dose and normalized their urinary levels to creatinine, a practice routinely employed in the laboratory (Kassahun *et al.*, 1989). The assumption made was that urine flow was normal because none of the patients was suffering from renal disease. Most of the patients were mentally handicapped and their prescribed drug dosage was administered by a care giver, thus ensuring compliance.

Age and polytherapy are risk factors associated with irreversible VPA-induced hepatotoxicity (Jeavons, 1984), whereas the use of high doses is not clearly implicated with the side effect (Dreifuss, 1987; Eadie *et al.*, 1988; Anderson *et al.*, 1992). The

analysis of the results showed that all three factors (age, high dose, and polytherapy) were associated with higher levels of excretion of thiol conjugates of VPA metabolites.

4.1.9 Age associated effect on the excretion of thiol conjugates

A statistical comparison of the means of NAC I and NAC II concentrations in urine that were observed for all three groups of all ages studied showed no difference among the groups. However, an assessment of each group individually revealed that the urinary levels of NAC I and NAC II decreased with age for both the monotherapy group and the co-medication group (figures 29 and 30), a clear indication that the younger patients were excreting higher amounts of the thiol conjugates. The youngest patient of the study who belonged to the co-medication group was observed to excrete the highest concentration of thiol conjugates. The age associated effect was further evident statistically when patients were categorized in age groups and the means of the conjugates were compared within the same study groups and cross-compared between the study groups.

The means of the conjugates for patients ≤ 7.5 years of age in both the monotherapy and co-medication groups were statistically higher than the means of those > 7.5 years of age and within the same group ($p < 0.05$). The monotherapy and co-medication subgroups were not statistically different when the same age groups were cross-compared. However, the means for the different age subgroups compared between the two groups were statistically different. The results indicate that the monotherapy and co-medication group were similar and that non enzyme-inducing drugs given in combination with VPA in the co-medication group did not appear to increase the

formation of thiol conjugates significantly. Hence, for the purpose of this study, the polytherapy group could be compared to the combined monotherapy and co-medication groups and be referred to as the non-induced polytherapy group. These results support the findings of retrospective studies performed in the U.S. (Dreifuss *et al.,* 1989, 1987; Bryant III and Dreifuss.,1996). The authors reported that the rate of mortality associated with the hepatotoxic side effect was highest in children less than 2 years of age (1:618) and that those belonging to the 3-10 year range had the second highest death rate (1:8,307).

Of interest is that the comparative increase in thiol conjugates of (E)-2,4-diene observed in young patients was consistent with an apparent increase in the urinary excretion of base- hydrolyzed (E)-2,4-diene VPA and 4-ene VPA in the same age group for the first 26 patients analyzed. The results (table 24) showed that the range of total 4-ene VPA and (E)-2,4-diene VPA in the urine for non-polytherapy patients $\leq$ 7.5 years (n=5) was 0.10-1.67 μg/mg creatinine and 0.45-4.7 μg/mg creatinine, respectively. On the other hand, patients older than 7.5 years excreted the two metabolites at lesser concentration ranges of trace-0.68 μg/mg creatinine for 4-ene VPA and 0.04-3.94 μg/mg creatinine for (E)-2,4-diene VPA. The trend for both metabolites clearly indicated that the two metabolites decreased with increasing age similar to the observations made for the thiol conjugates. However, statistical comparison of the means of both metabolites (table 25) for patients more than 7.5 years failed to demonstrate a significant difference at $p <$ 0.05 (Mann Whitney, 4-ene VPA p=0.08 for 4-ene VPA, p=0.052 for (E)-2,4-diene VPA). These results need to be confirmed with a larger sample size. It should be noted that the results for the thiol conjugates were initially evaluated on the same 26 patients and showed a clear statistical difference (Gopaul *et al.,* 1996b).

While these results for 4-ene VPA and (*E*)-2,4-diene VPA here were in accordance with some studies (Kondo *et al.,* 1992; Darius and Meyer, 1994), they were in contradiction with earlier reports which were clearly unable to correlate 4-ene VPA or (*E*)-2,4-diene VPA levels with age (Tennison *et al.,* 1988; Kassahun *et al.,* 1991; Seimes et al 1993). An evaluation of 2-ene VPA and 3-Keto VPA, in the same group of patients we studied, indicated that the two β-oxidation metabolites were higher among the younger patients ($\leq$ 7.5 years) than older patients, suggesting a more active β-oxidation activity in younger patients.

A comparison of the thiol conjugates with the corresponding (*E*)-2,4-diene VPA and 4-ene VPA clearly indicated that the hydrolyzed metabolites were excreted at higher amounts than the thiol conjugates. This suggests that the two phase I metabolites, while being produced at increasing amounts in younger children, were not all conjugated with GSH and were eliminated by other conjugation pathways as well. Furthermore, the higher concentration of hydrolyzed (*E*)-2,4-diene VPA compared to the thiol conjugates further supports the contention that the diene can be the outcome of both mitochondrial and microsomal metabolism. It also confirms that the (*E*)-2,4-diene VPA formed in the microsome does not undergo glutathione conjugation. As discussed before, reports have shown that the diene can be formed as a direct β-oxidation product of 4-ene VPA in mitochondria (Rettenmeier *et al.,* 1986a,b) or as a microsomal oxidation of 2-ene VPA (Kassahun *et al.,* 1992). This emphasized the rationale for determining a correlation between the thiol conjugates which are more indicative of exposure to the reactive metabolite and associated risk factors of VPA.

4.1.10 Effect of dose on the excretion of the thiol conjugates

Dose has not been defined clearly as a risk factor for the idiosyncratic nature of VPA-induced hepatotoxicity but it is clear from the result of this study that a higher dose of VPA can lead to an increase in the formation of reactive metabolites. Therefore, the results need to be interpreted in relation to the higher dose of VPA being administered to younger patients involved in this study. We observed that the therapeutic dose of VPA (mg/kg) increased linearly with decreasing age and the thiol conjugates increased exponentially with decreasing age. This suggests that the higher formation of thiol conjugates in younger children perhaps resulted from a combination of age and higher dose, but not the sole effect of the latter.

The linear regression correlation coefficient (r) for the thiol conjugates *versus* dose in patients of all ages was 0.92 for the monotherapy group, 0.78 for the co-medication group and 0.81 when the two non-polytherapy groups were combined. The linear correlation coefficients between dose and 4-ene and (*E*) 2,4-diene VPA for 26 of the patients studied were 0.59 and 0.72, respectively for the non-polytherapy patients. It is apparent that in the non-polytherapy patients, the unsaturated metabolites demonstrated less linear correlation with dose than the thiol conjugates. The increase in the excretion of 4-ene VPA and (*E*)-2,4-diene VPA with dose is corroborated by previous reports (Levy *et al.*, 1990; Anderson *et al.*, 1992).

The effect of dose on the excretion of thiol conjugates was apparent in three of the monotherapy patients. Patient 2, 22, and 41, who were 11.33, 12.36 and 14.92 years old, respectively, and receiving a higher dose of VPA (>15 mg/kg) for their age group,

excreted a higher level of thiol conjugates compared to patients who were younger and receiving a lower dose. The effect observed for patient 41 could also be attributed to the consumption of a high protein and low fat diet diet. In the co-medication group, patient 20 and 10 were both receiving a low dose of VPA (<13 mg/kg) and excreted low amounts of the thiol conjugates.

4.1.11 Effect of polytherapy on the excretion of thiol conjugates

The effect of VPA dose was not linearly correlated with the excretion of thiol conjugates (r=0.013) in the polytherapy patients, although the correlation coefficient for the unsaturated metabolites *versus* dose was 0.92 and 0.72 for 4-ene VPA and (*E*)-2,4-diene VPA, respectively

The effect of dose did not appear to be the main factor in the observation for the polytherapy patients although it cannot be excluded. We were able to demonstrate that levels of the urinary thiol conjugates were associated with polytherapy and age. The means of the conjugates for the polytherapy patients who were > 7.5 years were statistically higher than the means for patients in the non-polytherapy group and within the same age group (Mann Whitney, $p < 0.05$). Interestingly, no statistical difference was observed when the means for patients on polytherapy were compared with the means for patients ≤ 7.5 years in the non-polytherapy study group. It appears that the addition of PB, CBZ or DPH with VPA therapy increased the excretion of the thiol conjugates significantly in patients more than 7.5 years. Therefore, it can be argued that the patients on polytherapy in that age group were exposed to significantly more of the reactive metabolite (*E*)-2,4-diene VPA CoA compared to non-polytherapy patients of

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similar age. In accord with the findings, the range of 4-ene VPA (0.13-4.98 μg/mg creatinine) and (E)-2,4-diene VPA (1.08-5.65 μg/mg creatinine) observed for the first 6 polytherapy patients in the study appeared to be higher than the range observed for the 15 non-polytherapy patients within the same age group (> 7.5 years). On the other hand, the range of 2-ene VPA and 3-keto VPA did not appear to be higher in the polytherapy patients more than 7.5 years old when compared to non-polytherapy patients.

These results are in agreement with the studies of Levy et al., 1990 and Kondo et al., (1990,1992). In all three studies, it appears that P-450 inducers common to the present study increased the level of 4-ene VPA, the precursor of (E)-2,4-diene VPA. However, the induced human isozyme responsible for the metabolism of VPA to 4-ene VPA remains to be established. Recently, a report from this laboratory involved the investigation of the role of erythromycin, a CYP3A4 inhibitor on the metabolism of VPA in one patient on VPA (Gopaul et al., 1996a). We observed that 4-ene VPA and (E)-2,4-diene VPA decreased by 21 % and 26 %, respectively, in the presence of the inhibitor in that patient. In parallel, the total % recovery of the thiol conjugates calculated for the patient over a 12 h period in that study was increased by about 15% upon withdrawal of erythromycin. If confirmed, the latter observation was evidence indicating the possible involvement of CYP3A4 in the metabolism of VPA to 4-ene VPA and its β-oxidation product (E)-2,4-diene VPA. This is in agreement with reports which have demonstrated that PB, CBZ and DPH, which are all inducers of CYP3A4 (Albani et al., 1991; Sachedo et al., 1994; Reidelberg et al., 1995), induce the metabolism of VPA and felbamate, a substrate for CYP3A4, inhibits the metabolism of VPA (Glue et al., 1997). It would be of considerable interest to understand the involvement of the specific

isozymes involved in each metabolic pathway of VPA. It was interesting to note that patient # 29 also showed the highest level of thiol conjugates among non-polytherapy patients > 7.5 years. The corresponding urinary level of 4-ene VPA and (E)-2,4-diene VPA were also elevated. The only plausible explanation of the observation is an interaction of clobazam with VPA. It appeared that the interaction of the two antiepileptic drugs led to an increase in thiol formation in patient # 29. It has been reported that clobazam interacts with VPA to decrease its protein binding ability and increase VPA serum level (Thies *et al.*, 1997). Perhaps this allows VPA to become more available for metabolism. The authors recommend that patients on both VPA and clobazam should be carefully monitored.

4.1.12 Effect of other clinical conditions on the excretion of thiol conjugates

The results need to be interpreted within the clinical features and underlying disease associated with VPA-induced hepatotoxicity. Mental retardation and abnormal neurologic conditions in children have been reported to be associated with fatal liver hepatotoxicity (Dreifuss *et al.*, 1987; Scheffner *et al.*, 1988). All of the patients in the study suffered from epilepsy of varied types. The majority of the patients (n=32) were mentally handicapped and only 8 had normal cognitive ability. Furthermore, two of the younger patients had abnormal plasma AST levels and all other liver tests were assessed as normal. Patient # 5 was 5.36 years old had high AST levels and excreted a high level of thiol conjugates. Similarly, patient # 6, the youngest patient in the study (2.33 years) who also had a high AST level excreted the highest amount of thiol conjugates observed among the non-polytherapy groups. Whether or not the abnormal

liver function and the high thiol excretion were directly related to each other cannot be confirmed.

4.1.13 Effect of metabolic disturbances on the excretion of thiol conjugates

The possible effect of mitochondrial disease as observed by an increased urinary alanine excretion appears to be associated with an elevated concentration of thiol conjugates as observed in patient 19. An evaluation of the urinary phase I metabolic profile of the patient showed the highest level of (E)-2,4-diene VPA (2.34 μg/mg creatinine) and 4-ene VPA (0.68 μg/mg creatinine) excreted for that particular age group (table 22). The comparison of the two unsaturated metabolites was made against 15 other patients, all older than 7.5 years. The increased metabolism of VPA to 4-ene VPA appeared to increase β-oxidation of the metabolite to (E)-2,4-diene VPA, as reflected by a higher level of thiol conjugate of the metabolite and free (E)-2,4-diene VPA.

In the case of patient 23, the elevated level of thiol conjugates was consistent with urinary 4-ene VPA and (E)-2,4-diene VPA which were higher than observed in younger patients within the same group. The biochemical profile of the patient suggested an activated mitochondrial β-oxidation of fatty acid which led to an over production of lipid peroxide and depletion of glutathione peroxidase. Cellular membrane alteration caused by high lipid peroxidation has been demonstrated to be an effect of VPA (Perlman et al., 1984). The antioxidant, vitamin E, has been shown to decrease toxicity of VPA in rat hepatocytes, emphasizing the role of lipid peroxidation in VPA-induced hepatotoxicity (Buchi et al., 1984; Scheffner et al., 1988).

It has been suggested that VPA competes with endogenous fatty acids for the enzymes of β-oxidation (Koch *et al.,* 1989b; Siemes *et al.,* 1993). In the case of patient 41 who was on a low fat, high protein diet, it could be argued that mitochondrial β-oxidation enzymes were available for the metabolism of 4-ene VPA leading to a higher formation of thiol conjugates of (*E*)-2,4-diene VPA in comparison with patients within the same age group. Patient 37 who was on VPA in combination with CBZ was on a similar diet. Although, both patients were of similar age (14.3 and 14.9 years) and similar doses (21, 25 mg/kg), the patient on polytherapy excreted similar amounts of thiol conjugates as the non-polytherapy patient. Whether or not decomposition of the thiol conjugates to (*E*)-2-4-diene VPA occurred in the polytherapy case is not known.

The data strongly suggest that polytherapy, and age are both significant contributing factors associated with an increased formation of NAC I and NAC II in patients on VPA. The findings provide compelling evidence that younger patients and those on polytherapy are more exposed to reactive metabolites of VPA, and hence are at higher risk of developing VPA-induced hepatotoxicity. The effect of underlying disease appears to increase the production of the conjugates. To the best of our knowledge, this was the first evidence showing a clear correlation between reactive VPA metabolites and the associated risk factors of VPA-induced hepatotoxicity in humans. Previous studies in search of similar evidence (Siemes *et al.,* 1993) were negative or inconclusive when the phase I metabolites of VPA, 4-ene VPA and (*E*)-2,4-diene VPA were studied. The findings support the theory of Kassahun *et al.,* 1991, that the thiol conjugates of (*E*)-2,4-diene VPA which represent the end product of the proposed hepatotoxic pathway would be a better predictor of the side effect.

4.1.14 Significance of the excretion of thiol conjugates in patients on VPA.

The identification and characterization of the novel metabolite NAC II in humans further reinforced the reactive nature of (E)-2,4-diene VPA as the CoA ester *in vivo*. From a mechanistic point of view, the results suggest that NAC I and NAC II are positional isomers of each other and are formed as a result of the metabolism of 5-GS-2-ene VPA and 5-GS-3-ene VPA. The two GS conjugates are isomers of each other as proposed for 2-ene VPA and 3-ene VPA and formed at the same site following conjugation of GSH with (E)-2,4-diene VPA CoA in mitochondria. The results for the profiling of thiol conjugates in patients is consistent with recently reported epidemiological findings (Dreifuss *et al.*, 1987; Bryant III and Dreifuss 1996) that age and polytherapy continue to be risk factors associated with VPA-induced hepatotoxicity. Furthermore, this study also points out that apart from patients who are less than or equal to 7.5 years and on polytherapy, those suffering from metabolic disturbances are also vulnerable to hepatotoxicity and should be monitored. Results reported previously (Kassahun *et al.*,1991) have shown that NAC I was about 5 times higher in hepatotoxic patients compared to patients who are not suffering from the disease. The results clearly show that NAC I and NAC II can be used as biochemical markers to predict onset of VPA-induced hepatotoxicity.

Although the exact mechanism of VPA-induced hepatotoxicity is not known, all the studies performed and discussed in this report have led to the theory that the potentially fatal side effect could be attributed to several factors. The β-oxidation of 4-ene VPA involves the consumption of CoA at the expense of the oxidation of fatty acids. The metabolism of increased amounts of 4-ene VPA competes with the enzymes of the β-

oxidation pathway to inhibit the metabolism of fatty acids which accumulate in the liver. The formation of toxic metabolites in mitochondria may deplete the limited GSH mitochondrial pool and lead to oxidative stress.

In this study, although results of the phase I analysis of metabolites need to be confirmed with larger sample size, the total amount of 4-ene VPA and (E)-2,4-diene VPA excreted in the urine appears to be elevated in high risk patients consistent with the observations of the thiol conjugates. Furthermore, both these phase I metabolites appear to be present in higher amounts than the thiol conjugates of (E)-2,4-diene. It is reasonable to suggest that not all of the diene formed from 4-ene VPA remains in the reactive CoA form but a significant amount may to be hydrolyzed and excreted *via* a different conjugation pathway, presumably the glucuronidation route. If such is the case, it suggests that glucuronidation is also increased when the metabolism of VPA to 4-ene VPA is induced, an important factor to consider in assessing the induction of VPA-induced hepatotoxicity in the various high risk groups. Moreover, the increased formation of (E)-2,4-diene VPA can be due in part to 2-ene VPA metabolism. The lack of detection of NAC III in all patients studied indicates that the metabolism of 4-ene VPA to form 4,5-epoxy VPA is not important in non-hepatotoxic patients.

The results show that the thiol conjugates of (E)-2,4-diene VPA might be useful as a non-invasive marker for hepatotoxicity. The failure to correlate the increase of 4-ene VPA and (E)-2,4-diene VPA in high risk patients in some previous studies appears to be related, in part, to the complexity of the analytical techniques as well. In the present study, we have employed a technique which is highly selective, sensitive and very efficient at demonstrating a clear correlation between exposure to reactive metabolites

and high risk factors of VPA-induced hepatotoxicity. A GC/MS NICI assay employed to study the urinary excretion of phase I metabolites, 4-ene VPA and (*E*)-2,4-diene VPA independently supports the findings regarding the conjugates. The assay for the phase I metabolites is more laborious and involves detection of metabolites at very low level, whereas the assay for the thiol conjugates provides an easier means to determine exposure to the reactive metabolites of VPA. The assay is simple and robust. Collection of urine samples for the assay is practical and non-invasive, an important aspect to consider in dealing with pediatric patients.

4.2 *Amino acid conjugation of VPA in humans and animals*

4.2.1 Summary of findings

We report here the first glutamate conjugate of a xenobiotic in human, rabbits and rats. Valproyl glutamate (VPA GLU) was identified in the urine, serum and CSF of patients treated with VPA. The conjugate was also identified in the urine, serum and brain homogenate of rats dosed with VPA. We further identified the conjugate in the CSF and serum of rabbits dosed with VPA.

Moreover, we also report the first identification of valproyl glutamine (VPA GLN) in the biological fluids mentioned above. Valproyl glycine (VPA GLY) which has already been reported as a metabolite of VPA and its metabolite, (*E*)-2,4-diene VPA, in rats and monkeys (Granneman *et al.,* 1984a; Rettenmeier, 1986a,b) was identified in human serum and urine for the first time. VPA GLY could not be identified in the CSF of a human or rabbits and was not investigated in rat brain homogenate. Furthermore,

valproyl aspartate (VPA ASP) was sought after in the biological fluids of humans, rats and rabbits and could only be identified in the serum, bile and urine of rats. Similarly, valproyl taurine (VPA TAU) and valproyl alanine (VPA ALA) did not appear to be present in humans.

Amino acid conjugates of xenobiotics are known in humans, the most commonly known being those of glycine, glutamine, taurine and ornithine (Hutt and Caldwell, 1990). Reports in the literature indicate that glycine and to a lesser extent glutamine are the most likely amino acids to conjugate with a xenobiotic (Hutt and Caldwell, 1990). In contrast, the study showed that VPA GLU was the more predominant conjugate compared to VPA GLN in humans, rats and rabbits. Furthermore, VPA GLY was a relatively minor conjugate in all three species studied. The discovery of these rare conjugates of VPA could be largely attributed to the utilization of newer and more powerful detection techniques such as LC/MS/MS. The discussion of the results is based on the implication of these conjugates with regard to both VPA-induced hepatotoxicity and the mechanism of action of VPA.

4.2.2 LC/MS/MS characteristics of AA conjugates of VPA

The identification of the amino acid conjugates in most of the biological fluids in this study was determined using LC/MS/MS as the primary spectral tool which offered a unique means of characterizing various amino acid conjugates of VPA. The combination of chromatographic properties under reverse phase HPLC and the mass spectral characteristics of the compounds under ES^+ facilitated the structural elucidation of the compounds. For the purpose of confirming the identification of the conjugates in

their respective biological fluids, the chromatographic and mass spectral characteristics were compared to those of the synthetic standards of the conjugates which were synthesized in this laboratory and characterized by ^{1}H NMR and LC/MS/MS.

Both mobile phases employed in this study were suitable for the chromatography of the conjugates and are similar to the ones employed for the analysis of VPA GLY and VPA glycinamide (Blotnik *et al.,* 1997). The more polar compound, VPA GLN, had the shortest retention time by both methods while VPA GLY eluted last. All AA conjugates had sharp symmetrical peak shapes with the exception of VPA GLY, which was characterized by a tailing peak suggesting the carboxylic moiety of the conjugate remained partially ionized during chromatography.

Although, all the conjugates studied here had a terminal carboxylic group, they were all very sensitive under positive electrospray (signal to noise >3). The MS/MS fragmentation of all four AA conjugates studied showed characteristic fragmentation patterns associated with both the VPA portion of the molecule and their respective amino acid group, as shown in figure 33. A common and abundant product ion observed was *m/z* 57 for all the product ion spectra. Hence, this facilitated the preliminary search for other AA conjugates of VPA such as VPA TAU and VPA ALA by use of MRM of MH$^+$ to *m/z* 57 in biological fluids without requiring a synthetic reference sample. All four conjugates of VPA shared common MS/MS ES$^+$ features such as an intense MH$^+$ ion characteristic of the molecular weight of each conjugate. The product ion *m/z* 99 which reflected fragmentation at the tertiary carbon and *m/z* 127, corresponding to the product following cleavage of the peptide bond, were both characteristic of the VPA moiety of the conjugates. The product ion spectra of each

conjugate were distinguishable from each other by the ions resulting from their branched amino acid moiety. In the case of VPA GLY, where the amino acid group was non-substituted, only product ions from VPA were detected.

In contrast, when a fluorine group was introduced at the tertiary carbon of VPA as in FVPA GLN, the resulting product ion spectrum was distinctly different. The two bond cleavage at the tertiary carbon (C4, figure 33) of VPA produced only a weak ion at *m/z* 57. The loss of the *N*-formyl glutamine followed by the loss of hydrogen fluoride produced a weak ion at *m/z* 97. The spectrum of FVPA GLN showed another fragment characteristic of the VPA portion at *m/z* 84 which was assigned to the protonated product ion resulting from the loss of the amino acid moiety and a propyl group and following a further loss of hydrogen fluoride. Only one product ion characteristic of the amino acid moiety was observed. The ion at *m/z* 130 corresponded to the loss of the fluorinated 2-propylpentanamide group. In the non-fluorinated analogue, VPA GLN, the corresponding ion was non-protonated and weak (*m/z* 129). Similar to the non-fluorinated analogues, the spectrum of FVPA GLN showed a fragment which corresponded to the formation of 2-fluoro-2-propylpentanamide at *m/z* 161.

4.2.3 GC/MS characteristics of amino acid conjugates of VPA

The GC/MS characteristics of the conjugates were investigated for the purpose of identification when liquid chromatography was not suitable for analysis as in the case of rat brain homogenate. In addition, GC/MS provided evidence to support and verify the LC/MS/MS results.

Similar to the thiol conjugates, all AA conjugates had a terminal carboxylic acid group. Upon derivatization with PFBBr and analysis under NICI, each conjugate produced mass spectra dominated by a single characteristic [M-181]⁻ fragment as shown in figures 40-42. Under the oven temperature program employed, VPA GLY eluted first at t_R 8.980 min, whereas FVPA GLN, VPA GLN, VPA ASP, and VPA GLU all eluted fairly close to each other between 14.78 min to 16.42 min (table 28). The order of elution on a DB1701 column appeared to be dependent on both molecular weight and polarities of the PFB derivatives.

4.2.4 Development of analytical assays for the AA conjugates of VPA

4.2.4.1 Profiling of VPA AA in biological fluids

The detection of AA conjugates of VPA in biological fluids of humans and animals necessitated the development of assays for the profiling of the compounds in urine, serum, bile and CSF of patients on VPA and rats and rabbits treated with VPA.

Reports for the profiling of AA conjugates of xenobiotics are rare in humans and, to the best of our knowledge, only the quantitation of VPA GLY have been performed so far in rats by GC/MS in the EI mode (Granneman *et al.,* 1984a) and by HPLC/UV (Blotnik *et al.,* 1997). In this study, an LC/MS/MS assay was developed for the profiling of VPA GLU, VPA GLN, and VPA GLY for the first time. FVPA GLN was a suitable internal standard on the basis of its similar chemical properties to the analytes being assayed. Furthermore, it possessed chromatographic and mass spectral characteristics which were highly compatible with the analysis of the conjugates. For similar reasons, VPA

ASP which was not identified in patients studied served as a second internal standard. In analysis involving rats and rabbits, only FVPA GLN was utilized as the internal standard.

4.2.4.2 Urine assay of AA conjugates of VPA

The conjugates were isolated from rat or human urine by using the SPE procedure employed for the thiol conjugates and depicted in scheme 5 of the Experimental section. For identification purposes, the extracts were dissolved in mobile phase and analyzed by MRM of several characteristic product ions, full product ion or precursor ion scanning. For quantitation, one relatively heavy and abundant product ion was monitored under MRM for each conjugate and internal standard.

The focus of the studies involving AA conjugates of VPA was to screen and quantitate the identified conjugates simultaneously in 29 human urine samples. Hence, the developed assay needed to be validated. Calibration curves generated for concentrations ranging from 0.1 μg/mL to 5 μg/mL showed good coefficients of determination (r^2=0.99 or better) for VPA GLU, VPA GLN, and VPA GLY. Overall, the inter-assay variation was minimal (less than 10%). The intra-assay variation was also minimal (10 %) for higher concentrations (*1* μg/mL) for all three conjugates. At 0.115 μg/mL, VPA GLN showed the largest intra-assay variation at 29 %, whereas VPA GLU and VPA GLY had CVs less than 15% (table 30). The larger variation was observed at the lower limit of quantitation (0.1 μg/mL) and was acceptable for the present studies, especially considering that patients' samples contained VPA GLU and VPA GLN at

concentrations which were well above the limit of quantitation (0.1 μg/mL). The concentrations observed for spiked samples (Q.C) were within 85-122% of their expected values suggesting that the accuracy was adequate for the intended purposes. The conjugates were isolated and quantitated from urine samples of VPA-treated rats using the assay just described.

4.2.4.3 Serum quantitation of AA conjugates of VPA

The identified conjugates were detected at very low levels and therefore 2 mL of serum sample was required for quantitation. The large volume of serum required for the assay was not appropriate for an SPE extraction and consequently a liquid-liquid extraction was necessary. The conjugates were isolated from the serum with organic solvent at acidic pH and this proved adequate for human, rat and rabbit serum quantitation.

The extraction procedure was very similar to that employed for the extraction of VPA metabolites for GC/MS analysis (Kassahun *et al.,* 1990; Yu *et al.,* 1995). Calibration curves afforded good coefficients of determination (r^2 >0.99) for all the conjugates.

4.2.4.4 CSF and bile assay of AA conjugates of VPA

The assays of the conjugates in CSF and bile were relatively facile requiring no extraction procedure. The fluid was spiked with internal standard and filtered to remove proteins. CSF samples from patients on VPA are rare and the benefit of the approach to analyze CSF samples without an extraction procedure was to minimize sample loss

and minimize contamination during sample workup. For both biological fluids, calibration curves afforded good correlation of determination (r^2 >0.99) and quantitation was feasible.

4.2.5 Identification of VPA AA in humans

To the best of our knowledge, VPA GLU identified in the present study is the first glutamate conjugate of a xenobiotic reported for humans, rats or rabbits. In this study, two novel metabolites, VPA GLU and VPA GLN, were identified in the urinary extracts of patients on VPA. Both conjugates were positively identified in the urine extracts of all 29 epileptic patients on VPA by ES$^+$ MS/MS MRM of all their characteristic transitions. The concentrations of VPA GLU and GLN were high enough that their full precursor spectra and daughter ion spectra could be obtained for some patients. Furthermore, the metabolite VPA GLY identified previously in rats (Granneman et al., 1984a) was detected in all the samples studied. The profiling of the conjugates revealed that VPA GLU was a more abundant AA conjugate (0.66-13.1 μg/mg creatinine) than VPA GLN (0.78-9.93 μg/mg creatinine). However, in comparison to the other two metabolites, the range of concentrations of VPA GLY (trace-1.0 μg/mg creatinine) placed it as a less abundant metabolite. Although the role of the amino acid conjugation pathway for VPA has not been largely explored, the conjugation of VPA with the amino acids glutamic acid, glutamine, and glycine in humans could bear toxicological significance. This is important to consider because VPA metabolites have been implicated in the induction of hepatotoxicity by the drug in humans and animals.

Urinary excretion of the conjugates suggests that the metabolites could be formed in the liver. The amount of the amino acid conjugates recovered in the urine samples of non-hepatotoxic patients was about 1% of a VPA dose. Although, the recovery of the conjugates of VPA itself did not account for a large percent of an administered VPA dose, perhaps the AA conjugates of phase I metabolites of VPA metabolites could account for a higher recovery of VPA. In support of this view, it was found that although conjugation with glycine was a minor route of metabolism for VPA in rats, it had more significance for the unsaturated metabolites (Granneman et al., 1984a). Furthermore, the glycine conjugate of (E)-2,4-diene VPA was identified in "relatively large amounts " in Rhesus monkeys dosed with VPA and 4-ene VPA (Rettenmeier et al., 1986a,b).

Conjugation of xenobiotics with amino acids is believed to involve the acyl transfer of CoA thioester to a particular amino acid (Hutt and Caldwell, 1990). By this reasoning, it can be expected that 4-ene CoA and (E)-2,4-diene CoA formed in mitochondria can conjugate with GLU, GLN, and GLY as well. Hence, it would appear that in the case of VPA, although the amino acid conjugation of the parent drug and possibly its metabolites is a relatively minor pathway, it could represent a detoxification pathway rather than an imposition on the CoA pool. Amino acid conjugation would restore free CoA levels and render VPA and metabolites more water soluble, and hence more readily excreted.

Furthermore, the inability of VPA and 4-ene VPA to conjugate with glutamine to a large extent as was observed in rats has been argued to be a factor that may assist in the induction of the hepatotoxic side effect of the drug (Tang et al., 1997a,b). The authors argued that the CoA ester of the P450 metabolite 4-ene VPA preferentially undergoes

β-oxidation to produce reactive metabolites instead of conjugating with glutamine as observed for the non-hepatotoxic and fluorinated analogue of VPA. It was reported that in contrast to VPA, 33.3 % of a dose of the fluorinated analogue of VPA appears to be metabolized to its corresponding glutamine conjugate in rats *in vivo*. Furthermore, it was proposed that the CoA ester of F-4-ene VPA is less able to undergo β-oxidation and, therefore, preferentially conjugates with glutamine to form non-reactive metabolites. Therefore, based on the present results, it is reasonable to speculate that FVPA, a potential non-hepatotoxic antiepileptic drug, and its metabolite F-4-ene VPA would metabolize to FVPA GLU and F-4-ene VPA GLU, respectively, to a larger extent than their corresponding GLN conjugates in rats and humans. It appears that metabolism by conjugation with amino acids protects the compounds from producing reactive metabolites.

The protective role of glycine to rat hepatocytes was linked to the removal of harmful acyl-CoA esters which accumulate upon exposure to VPA (Vance *et al.,* 1994). Furthermore, hypoglycin toxicity was decreased by the administration of glycine (Al-Bassam and Sherratt, 1981), presumably the result of removal of putative acyl-CoA responsible for the toxicity. Furthermore, glycine was found to have a beneficial effect in isovaleryl acidemia for similar reasons (Naglak *et al.,* 1988). By the same reasoning, it can be expected that glutamine, which appear to be more readily conjugated to VPA, may have a more protective role than glycine. To that end, it would be of benefit to determine if glutamic acid and glutamine would decrease or abolish the toxicity of VPA and its metabolites 4-ene VPA and (*E*)-2,4-diene VPA in rats.

Similar to the observation in urine samples, VPA GLU, VPA GLN, and VPA GLY were positively identified in the serum of patients on VPA. In the six samples studied, VPA GLU identified by both LC/MS/MS and GC/MS was at the highest concentration (although at relatively low concentration, < 0.01 μg/mL), while VPA GLN was present at a lesser concentration and VPA GLY was detected only at trace levels. In comparison, the serum concentration range usually observed for (E)-2,4-diene VPA is 0.06-0.38 μg/mL while its corresponding urine concentration range (0.18-11 μg/mg creatinine) is similar to that of VPA GLU (0.66-13.1 μg/mL). That the serum concentrations of the amino acid metabolites are considerably lower than their urinary levels suggests that the metabolites are either readily excreted upon formation in the liver or that they may be formed at other sites as well, such as the kidney (Hutt and Caldwell, 1990).

Perhaps the detection of VPA GLU and VPA GLN in the CSF of a patient (# 45) on VPA was the most interesting discovery in that it bring insight to the mechanism of action of VPA. The CSF concentration of VPA GLU and VPA GLN were measured at 0.06 and 0.034 μg/mL of CSF, whereas the level of VPA itself was found at a concentration below 15 μg/mL. Because CSF concentrations of VPA and unsaturated VPA metabolites are believed to correspond to those in the brain (Vajda et al., 1981; Lösher et al., 1988), it is reasonable to assume that the levels of the conjugates reflect their respective brain concentrations. Furthermore, the present results are consistent with the levels of VPA metabolites which are found at concentrations less than 15% of VPA in human brain (Adkinson e t al., 1995).

However, the corresponding serum concentrations of VPA GLU and VPA GLN were 0.007 and 0.006 μg/mL (table 33), respectively for patient # 45 and were consistent with the results for serum samples of six other patients who were also at steady state. Unlike the unsaturated VPA metabolites which have been identified in brain and CSF of patients, the concentrations of both conjugates were 6-10 fold higher in the CSF than the serum, suggesting that the metabolites could be formed in the brain itself as a result of conjugation of VPA with glutamic acid and glutamine. Furthermore, the concentration of VPA GLU was higher than GLN suggesting that the conjugation with glutamic acid is more prevalent perhaps because of its higher abundance in the brain. Then, the presence of VPA GLU and VPA GLN in the CSF provides a rationale to suggest that the mechanism of action of VPA could in part be the result of conjugation of VPA with the excitatory neurotransmitter glutamic acid itself or its precusor glutamine .

The interpretation of the results was based on several relevant findings regarding the detection of VPA metabolites in the CSF and brain. Since the CSF is the extracellular fluid in the brain, the detection of the metabolites therein can be indicative of the presence and amount of VPA and its metabolites in the brain tissue itself. It has been demonstrated that VPA and its metabolites in the CSF correlate well with their respective brain concentrations in humans (Vajda, 1982; Adkinson *et al.,* 1995). Further support for the proposed theory is based on the CSF metabolites of VPA, which have been found to possess antiepileptic activity and to similarly conjugate with glutamic acid or glutamine *via* their terminal carboxyl group. This suggests that the metabolites such as 2-ene VPA, which bear structural similarity to VPA, may exert their antiepileptic effect by the same mechanism.

Furthermore, glutamic acid is believed to play a key role in the generation of epileptic seizures (Bradford and Dodd, 1976) and glutamate concentrations, but not GABA have been observed to be elevated in human epileptogenic foci (Perry and Hansen, 1986) and by 28 % in the CSF of newly diagnosed epileptic patients untreated with anticonvulsants (Kälviäinen *et al.,* 1993). The latter study further demonstrated that the antiepileptic drug, vigabatrin, a GABA-T inhibitor, increases CSF GABA levels and decreases CSF glutamate levels. In addition, VPA has been found to reduce glutamate levels in the brain of infant mice (Thurston *et al.,* 1981).

The neurotransmitter glutamic acid is believed to act at glutamate receptors and studies have found that VPA suppresses NMDA-evoked transient depolarizations in rat neocortex (Zeise *et al.,* 1991) and blocks seizures induced by NMDA in rodents (Czuczwar *et al.,* 1985). Moreover, a study on the effects of VPA on amino acid concentrations in seizure prone mice led to the suggestion that the seizure-inhibiting effect of VPA could be related to enhanced GABA release or to inhibition of excitatory glutamic acid release (Vriend *et al.,* 1996).

In addition, a prolonged antiepileptic effect is observed in patients well after the discontinuation of VPA and this phenomenon could be related to an unidentified metabolite (Adkinson *et al.,* 1995) and it could be the result of desensitization of glutamate receptors. Alternatively, the decrease in excitatory glutamic acid concentrations by VPA could reach a level which remains stabilized well after the drug has been removed. Although the low concentration of the conjugates relative to VPA in the CSF could argue against the proposed theory, the concentration of the conjugates (~0.4 μM) in relation to those of glutamic acid and glutamine which are present in the

μM range in the human brain supports it. Failure to detect VPA GLY in the CSF is consistent with the theory that VPA conjugates in the brain readily with the excitatory neurotransmiter glutamic acid but not with the inhibitory neurotransmitter glycine.

Most of the identified metabolites of VPA in the CSF of epileptic patients have been observed to possess antiepileptic activity (Löscher, 1981; Löscher and Nau 1984; Nau and Löscher, 1984; Abbott and Achaempong, 1988; Lee R, 1991), although they have been detected at concentrations which are less than 1% that of VPA in CSF . (E)-2-ene VPA, a primary phase I metabolite was found to be twice as potent as VPA itself (Siemes and Shen, 1991; Lösher et al., 1991) and it is currently undergoing clinical trials as an anticonvulsant. The monoene metabolite was found at 0.8% of the concentration of VPA in CSF of patients on VPA (Lösher et al., 1988).

Therefore, it is conceivable that the two amino acid conjugates of VPA detected in patient CSF could also have potential as antiepileptic drugs. Their contribution to the antiepleptic activity of VPA itself will depend on their potency in comparison to VPA. Evidence to support VPA GLU and VPA GLN as potential antiepileptics is the report that valproyl glycinamide (N-(2-n-propylpentanoyl)glycinamide, VPA GLYD) has expressed anticonvulsant activity in rodents (Hadad and Bialer, 1995) and it is currently undergoing clinical trials. The compound emerged from structure-pharmacokinetic-pharmacodynamic activity relationship studies of a series of N-valproyl derivatives of GABA and glycine. VPA GLY was found to have no anticonvulsant activity, while the glycinamide derivative was a better anticonvulsant than VPA itself and did not appear to be a pro-drug of VPA as did valpromide. The development of VPA GLYD was based on the study demonstrating that amide derivatives of VPA analogues could also exhibit

pharmacological effects. The latter depend on the extent of the hydrolysis of the amide to its homologous carboxylic acids causing their rapid excretion (Haj-Yehia and Bialer, 1990). Finally, if VPA GLN is hydrolyzed to VPA GLU and the latter possess pharmacological activity, it can be investigated as a prodrug of VPA GLU.

The potential for antiepileptic activity of both conjugates will depend largely on their ability to be distributed in the brain and their disposition. It has been argued that the lack of antiepileptic activity of VPA GLY in rodents is related to its extensive first pass metabolism upon oral administration and its high degree of ionization at physiological pH when administered intravenously thereby preventing its distribution to the brain (Blotnik *et al.,* 1997). On the other hand, the glycinamide derivative appears to be readily distributed in the brain and the liver in rodents, a property which the authors relate to the favorable anticonvulsant activity of the compound. By the same analogy, it can be expected that VPA GLN will also behave in a similar fashion to VPA GLYD. In contrast to VPA GLY, VPA GLU appeared to be more lipophilic and may penetrate the blood brain barrier more readily.

The detection of VPA GLU at higher concentrations than VPA GLN in the CSF could also be the result of an active transport system which carries VPA GLN out of the brain at a faster rate than VPA GLU. Evidence to support this argument stems from the fact that VPA, itself a mono carboxylic acid which is completely ionized at physiological pH, is believed to penetrate into the CSF and brain rapidly and is cleared out at a faster rate by active transport (Nau and Lösher, 1984). There is evidence to support the participation of active transport in the clearance of VPA from the CNS of rats and rabbits (MacMillan *et al.,* 1987; Adkinson *et al.,* 1995; Cornford *et al.,* 1985).

We proposed earlier that the mechanism of action of VPA is partly the result of the conjugation of VPA with glutamic acid. If so, from a chemical point of view, it is possible that the amide of a VPA analogue such as VPA GLYD and VPA GLN will have more antiepileptic activity than VPA by virtue of its ability to form peptide bonds with the carboxyl group rather than the amino group of glutamic acid. The antiepileptic activity of VPA GLN and VPA GLU could be further enhanced because each molecule of the conjugate can bind to more than one molecule of glutamic acid. VPA GLN can form two peptide bonds with both its carboxyl and amino moieties and the carboxylic groups of glutamic acid. VPA GLU can conjugate with the amino group of glutamic acid again at both of its carboxylic groups to form two peptide bonds. In comparison, VPA and its unsaturated metabolites can only form one peptide bond with the one amide group of glutamic acid. Arguments against the proposal will depend on the chemical structural requirement for antiepileptic activity.

To provide further support to the interpretation of the human results, we investigated the formation of the AA metabolites in other species which have served as models for the study of VPA as an antiepileptic drug .

4.2.6 Identification of AA conjugates in rats

LC/MS/MS studies of 24 h urine samples collected from four rats dosed with VPA (100 mg/kg) confirmed the presence of VPA GLU, VPA GLN, VPA GLY and VPA ASP. VPA GLU was the predominant AA conjugate being detected at concentrations varying from 2 to 4 times the urinary concentrations of VPA GLN (table 34). It appears that the

conjugation of VPA with GLN in rats is not as important as in humans. This is in support of other studies performed in this laboratory (Tang, Ph.D. thesis, 1995; Tang *et al.*, 1997a). Interestingly, VPA GLY was found at concentrations approximately similar to VPA GLN. Contrary to the human observations, VPA ASP was detected in the urine of treated rats but at very minimal concentrations. The dose of VPA administered to the rats was higher than the average dose of VPA given to a patient and hence could explain the detection of VPA ASP. Alternatively, the detection of VPA ASP could be indicative of a species difference. With respect to the identification of AA conjugates, the results obtained for rats complement the human findings indicating that VPA GLU is the predominant AA metabolite found in the urine.

The corresponding serum concentrations of VPA GLU in rats varied from 0.066-0.17 μg/mL, while VPA GLN and VPA GLY were observed at trace concentrations and VPA ASP was not detected. VPA GLU appears to be a more dominant serum metabolite in rats than in humans. This is probably related to the sample collection time. Serum samples were collected from patients at steady state and prior to their morning dose of VPA, whereas the blood samples were collected from rats one half h after a fifth dose of VPA. However, since a time-profile study for the formation of the metabolite in rats was not performed, the significance of the observation cannot be completely interpreted. Although the maximum concentration of serum VPA is usually reached within one half h after dosing (i.p.) in rats (Tang, Ph.D. thesis, 1995) , it is likely all three conjugates were still being formed at the time of collection. The concentration at which VPA GLU could be detected suggests that it is readily formed in the liver. On the other hand, serum VPA GLN was detected at considerably lower levels than in humans, suggesting again that the glutamine conjugation with VPA is relatively minor in rats. On the other hand,

the trace amount of VPA GLY appears to be consistent with the finding in humans that glycine conjugation with VPA is a relatively minor route of metabolism in rats. The lack of detection of serum VPA ASP is consistent with trace amounts of VPA ASP recovered in the urine of treated rats indicating that ASP conjugation with VPA is also relatively minor. The results are consistent with the findings of Thurston et al., 1985 who were unable to explain the observed decrease in liver glutamate and glutamine levels following the administration of a single dose of VPA to infant mice.

The bile samples collected for 6 h after dosing revealed again that the conjugates were formed in the liver and VPA GLU was the predominant AA conjugate. The total amount of conjugates formed (~0.02 % of a VPA dose) was considerably less than that recovered in the urine over a 24 h period. Whether or not, this is an indication that the metabolites are not formed solely in the liver but also in the kidney needs to be investigated further.

The hydrolysis of VPA GLN to VPA GLU in vivo as determined by the urinary excretion of VPA GLN after 10 mg/kg i.p. dose of VPA GLN was less than 10% in two rats studied. This provided some evidence to support the view that VPA GLU is not primarily the result of the hydrolysis of VPA GLN but mostly the conjugation product of VPA with glutamic acid. Furthermore, if the tendency of VPA GLN to be hydrolyzed to the corresponding carboxylic acid is confirmed to be relatively small, this would further support the suggestion to investigate the glutamate conjugate itself as an antiepileptic or CNS drug. Identification of VPA GLU by GC/MS in the brain of one rat dosed with a high dose of VPA is consistent with the claim that the metabolite is possibly formed in the brain of rats and supports the proposed theory for the mechanism of action of VPA.

Alternatively, the metabolite also could be transported to the brain. The finding would need to be further verified with more animals.

Overall, the detection of VPA GLU in rats appear to be similar to the observations in humans. However, the lower concentration of VPA GLN detected indicates that in rats conjugation of VPA with glutamine is less significant than in humans. The detection of VPA GLY is similar to a previous report (Granneman *et al.*, 1984a). This preliminary rat study was performed primarily to obtain data on the identification of AA conjugates in rats. A complete pharmacokinetic study will need to be performed in order to understand the disposition and distribution of the conjugates as a means of investigating the potential of any of the conjugates as antiepileptic or CNS drugs.

4.2.7 Identification of AA conjugates of VPA in rabbits

The rabbit experiments were conducted primarily to support and supplement the human and rats studies. Rabbit was chosen as a third species to verify the formation of AA conjugates of VPA by LC/MS/MS. The presence of VPA GLU was confirmed in the CSF samples of four rabbits treated with a dose of 300 mg/kg of VPA. Thus, VPA GLU was detected in the of CSF of human and rabbit. Consequently, if the mechanism of action of VPA is the result of its conjugation with the excitatory neurotransmitter glutamic acid, the formation of VPA GLU in the CSF of rabbits is in agreement with this theory. Unlike human, VPA GLN was not detected in rabbit CSF.

This experiment was designed to detect VPA GLU at the time of its maximum formation. The maximum concentration of CSF VPA GLU appeared to be formed at about 3 h after

a dose of 300 mg/kg of VPA. Interestingly, the maximum serum concentration of VPA GLU appeared to be between 1-2 h after dosing. The time difference could be related to the accumulation of VPA in the brain. Furthermore, the fact that we detected VPA GLU solely in the CSF is further indication that the metabolite is a conjugation product of VPA and glutamic acid but not the hydrolyzed product of VPA GLN. That VPA GLN and VPA GLY were both detected in the serum but not in the CSF could be related to species difference.

4.2.8 Significance of the detection of AA conjugates in humans and animals

VPA GLU was detected as the major amino acid conjugate of VPA in the urine, serum and CSF of patients treated with VPA. This is the first glutamate conjugate of a xenobiotic identified in humans. To the best of our knowledge, the glutamate conjugates of benzoic acid in the Indian fruit bat (Idle *et al.,* 1975) and *p*-nitrobenzoic acid in spiders (Smith, 1962) are the only two reports of glutamate conjugates in the literature.

The presence of VPA GLU in CSF of one patient on VPA and CSF samples of rabbits (n>4) allows the proposal that VPA exerts it antiepileptic activity partly by conjugating with the excitatory neurotransmitter glutamic acid. The detection of VPA GLU in the only brain homogenate from a single rat dosed with VPA also supports this theory, although the results would need to be repeated for confirmation. If proved true, this novel approach to study the mechanism of action VPA can be adopted to design new antiepileptic drugs. Since it was first introduced (Godin *et al.,* 1969), the theory for the mechanism of action of VPA has been leaning towards the enhancement of the inhibitory (GABA mediated) processes. Whereas the proposal here does not refute the

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GABA theory, it suggests that VPA could also reduce excitatory transmission by preventing excitatory glutamic acid binding to glutamate receptors. Furthermore, if such is the case, this will be the first direct evidence of VPA binding to a cellular component (glutamic acid) believed to be involved in triggering epilepsy. To date, there is no evidence that has emerged to indicate that VPA binds to any receptors to exert a direct antiepileptic effect.

In addition, the conjugation of VPA with metabolic glutamate, the precursor of GABA, would lead to a decrease in GABA levels. The fact that the opposite effect is observed to occur (Lösher and Nau, 1982; Nau and Lösher, 1982, 1984 ; Lösher and Siemes, 1984, 1985), reinforces the theory that VPA GLU in CSF results from the conjugation of VPA with the excitatory neurotransmitter glutamic acid but not metabolic or gaba-ergic glutamic acid. This is possible as metabolic and gaba-ergic glutamate are believed to be compartmentalized and kept separate from the excitatory neurotransmitter (Fonnum et al, 1986; Otterson et al., 1992).

Furthermore, if glutamine and glutamic acid are rapidly converted to GABA (Ottersen et al., 1992), the conjugation of VPA with the metabolic amino acid may be unlikely. Alternatively, presynaptic GABA elevation may not be a direct effect of VPA at all but perhaps the consequence of a feedback mechanism resulting in increased synthesis or decreased degradation of the inhibitory neurotransmitter (Taberner et al., 1980; Lösher, 1989) .

Evidence has emerged to indicate that there is suppression of NMDA-evoked transient depolarizations in rat neocortex by VPA (Zeise et al., 1991). Anticonvulsant drugs

which may exert some of their antiepileptic activity by decreasing brain levels of excitatory amino acids are vigabatrin (Halonen *et al.,* 1990) and lamotrigine (Leach *et al.,* 1986). Felbamate, another broad spectrum anticonvulsant is believed to exert its pharmacological activity at the NMDA receptor (McCabe *et al.,* 1993) and at the GABA receptor as well (Rho *et al.,* 1990). Undoubtedly, efforts to understand the mechanism of action of antiepileptic drugs should enhance knowledge of the underlying causes of epilepsy disorders themselves and provide the opportunity for designing better treatment in the future (Upton, 1994).

Both VPA GLU and VPA GLN, similar to other VPA metabolites, may themselves possess antiepileptic activity. If both conjugates can be delivered to the brain via transport or diffusion, they too could conjugate with glutamic acid to form AA conjugates. Their pharmacokinetic and pharmacodynamic profile would need to be studied to assess their potential as anticonvulsant or other CNS drugs. The low serum concentrations tend to indicate the potential of rapid excretion of the drug which could be a factor that should be considered.

Interestingly all three species studied produced VPA GLU in the CSF or in the brain. Hence, both rabbits and rats could be utilized to study the significance of the conjugate. However, the absence of VPA GLN in the CSF of rabbit suggests that there may be some species difference and its relevance needs to be studied.

The excretion of these conjugates in the urine could have implications in studying the induction of hepatotoxicity if formed in the liver. The results did not reveal any clear correlation between risk factors of hepatotoxicity and urinary profiling of the conjugates

as observed for the thiol conjugates. A larger sample size of patients is required to confirm the observations.

5. SUMMARY and CONCLUSION

5.1 Investigation of thiol conjugates of VPA in humans

It was attempted to determine whether or not the thiol conjugates of VPA formed from the 4-ene VPA pathway could be used to assess exposure towards reactive metabolites and predict the onset of VPA hepatotoxicity in high risk patients. The NAC conjugates of 4-ene VPA and (E)-2,4-diene VPA were studied qualitatively and quantitatively in the urine of patients belonging to various therapeutic groups. NAC I was previously identified as a thiol conjugate discovered in patients on VPA.

The first goal was to determine the structure of a second thiol conjugate (NAC II) also observed in patients on VPA. NAC II was characterized as 5-(N-acetylcystein-S-yl)-2-ene VPA in patients on VPA using several spectroscopic and chromatographic techniques. Furthermore, the LC/MS/MS conditions employed enabled the separation of NAC I into its diastereomers which were found in equal amounts in all patients studied (n=39).

NAC III, the NAC conjugate of 4,5-epoxy VPA discovered as a metabolite of 4-ene VPA in rats was not detected in any of the patients studied. The metabolite 4,5-diOH VPA-γ-lactone which is the end product of 4,5-epoxy VPA metabolism in rats, was also searched for but not found. These findings led to the following possible interpretations: (1) the metabolism of 4-ene VPA to 4,5 epoxy VPA is not significant in humans and (2) 4,5-epoxy VPA is formed in a minimal amount which binds to cellular components and escapes detection in non-hepatotoxic patients.

An LC/MS/MS assay was developed and validated in order to identify and screen for NAC I, NAC II and NAC III in MRM. The assay utilized an SPE extraction and had low inter-assay and intra- assay variability. However, the narrow linearity range (0.10-1.0 μg/ml) was not adequate for the wider concentration ranges observed for the patients. Hence, the assay could only provide an estimate of the concentrations of the identified conjugates. The assay monitored for specific product ions of the molecular ion m/z 304 common to NAC I, NAC II and NAC III.

Furthermore, a second GC/MS NICI (SIM) method was developed and fully validated for the quantitation of the identified conjugates. The assay utilized liquid/liquid extraction and was linear over a range of 0.1-5 μg/ml. It was robust, reproducible and had low inter-assay and intra-assay variation, and good precision and accuracy. NAC II was observed to be 2-3 times the concentrations of NAC I in the urine samples studied. Interestingly, the two conjugates were also formed in the same ratio when the ethyl methyl ester of NAC I was base-hydrolyzed *in situ*. Consequently, the similarity of the two events prompted the rationalization that NAC I and NAC II might be formed from one pathway and presumably in the mitochondria following the hydrolysis of 5-GS-3-ene VPA-CoA and 5-GS-2-ene VPA-CoA . Therefore, this suggests that the mitochondrion is the site most exposed to the reactive metabolite (*E*)-2,4-diene VPA CoA.

The patients studied were categorized according to whether they were receiving VPA as monotherapy (group 1, VPA alone), co-medication (group 2, VPA in combination with non-enzyme-inducing drugs) and polytherapy (group 3, VPA in combination with enzyme-inducing drugs). Furthermore, the patients were also studied according to their age. It was found that young age ($\leq$ 7.5 years), high dose and polytherapy were

associated with an increase in the level of thiol conjugates of (E)-2,4-diene VPA excreted in the urine.

In group 1, the age of the patients ranged from 3.75 years to 17.33 years. In group 2, the ages ranged from 2.33 years to 27.10 years and in group 3, the patients ages were 7.83 years to 22.50 years. The respective ranges of NAC I and NAC II were 0.07-0.93 μg/mg creatinine and 0.10-2.14 μg/mg creatinine for group 1, 0.07-1.84 μg/mg creatinine and 0.07-5.63 μg/mg creatinine for group 2 and 0.14-0.90 μg/mg creatinine and 0.16-1.89 μg/mg creatinine for group 3.

The means of the thiol conjugates were statistically higher for patients $\leq$ 7.5 years compared to older patients in groups 1 and 2. The means of the conjugates were also significantly higher in patients $\leq$ 7.5 years than older patients when groups 1 and 2 were combined. The youngest patient in our study (2.33 years) belonged to group 2 and excreted the highest concentration of NAC I and NAC II. It should be noted however, that a higher dose of VPA was also associated with higher concentrations of thiol conjugates excreted. The younger patients in groups 1 and 2 received a higher dose of VPA than older patients and their results were interpreted as the effect of age and dose.

On the other hand, dose did not appear to have an effect on the excretion of thiol conjugates in group 3. The means of the conjugates were statistically higher in group 3 compared to patients in groups 1 and 2 and belonging to the same age group (i.e. > 7.5 years). The means of the conjugates for patients $\leq$ 7.5 years in groups 1 and 2

combined were not statistically different from those in group 3 where the patients were more than 7.5 years old.

In summary, it appeared that young patients ($\leq$ 7.5 years), those on polytherapy and those receiving a high dose of VPA are more exposed to the reactive (*E*)-2,4-diene VPA and could be at higher risk of developing hepatic injury. Other conditions such as metabolic diseases also appear to cause an increase of the conjugates. To the best of our knowledge, this is the first study demonstrating a relationship between the risk factors of VPA and the putative metabolite (*E*)-2,4-diene VPA. In a previous study, NAC I was observed to be at least 3-4 times the concentrations observed in non hepatotoxic patients (Kassahun *et al*, 1991). Hence, NAC I and NAC II can be used as markers to predict the onset of VPA hepatotoxicity.

5.2 The profiling of Phase I metabolites of VPA in humans

A second objective was to compare the urinary concentrations of 4-ene VPA, (*E*)-2,4-diene VPA, 3-keto VPA and (*E*)-2-ene VPA in the patients studied above. To that end, we modified an existing GC/MS NICI assay for the profiling of VPA and 14 of its metabolites to ensure chromatographic separation. The assay was reproducible and robust and the analyses of the first 26 patients were performed independently of the thiol conjugates. The results for 4-ene VPA and (*E*)-2,4-diene VPA support the findings for the thiol conjugates. An analysis of the trend observed when the patients were categorized as group 1, 2 and 3 above showed that 4-ene VPA and (*E*)-2,4-diene VPA also increased with polytherapy. The results need to be confirmed with a larger sample size.

5.3 Investigation of amino acid conjugation of VPA in humans and animals

A third objective was to investigate the formation of amino acid conjugates of VPA in humans, rats and rabbits. The utilization of LC/MS/MS supplemented with GC/MS allowed for the identification and characterization of the novel metabolites valproyl glutamate (VPA GLU) and valproyl glutamine (VPA GLN) in the urine, serum and CSF in humans on VPA. Furthermore, VPA GLU is the first glutamate conjugate of a xenobiotic discovered in humans. In addition, we were able to identify valproyl glycine (VPA GLY) in the urine and serum of humans on VPA.

The identification and characterization of the conjugates were performed using LC/MS/MS procedures such as precursor ion scanning, product ion scanning and MRM. An LC/MS/MS assay was developed and validated for the profiling of the conjugates in urine samples in MRM mode. The assay was linear over a range 0.1-5 μg/ml and produced good precision and accuracy. The amount of the three conjugates appeared to account for less than 1% of a VPA dose. The profiling of the conjugates in 29 patients indicated that VPA GLU was a more prominent metabolite (0.66-13.1 μg/mg creatinine) than VPA GLN (0.78-9.93 µg/mg creatinine) and VPA GLY was a minor metabolite (trace-1.0 μg/mg creatinine). Statistical analysis of the results did not show a correlation between urinary AA conjugates and the risk factors of VPA hepatotoxicity.

The mean serum concentrations of VPA GLU, VPA GLN and VPA GLY (n=6) were all less than 0.01 μg/ml. The identification of VPA GLU and VPA GLN in the CSF of one patient on VPA and at steady state was confirmed by GC/MS and LC/MS/MS. The

concentrations of CSF VPA GLU and VPA GLN were found to be 0.06 μg/ml and 0.034 μg/ml, respectively.

In rats treated with five repeated doses of VPA (100 mg/kg, i.p.), VPA GLU was a more prominent AA conjugate identified in the urine than VPA GLN and VPA GLY. VPA ASP was detected at trace concentrations. All four conjugates accounted for less than 1% of a VPA dose. In the corresponding serum samples, VPA GLU was the most prominent AA metabolite and was present at a concentration of 0.091±0.044 μg/ml after the fifth VPA dose. VPA GLN and VPA GLY were identified at concentrations below 0.01 μg/ml. VPA ASP was not detected in the serum. All identification procedures utilized LC/MS/MS as in humans. VPA GLU was also identified in the brain homogenate of a rat dosed with 700 mg/kg by GC/MS NICI.

In rabbits (n>4) treated with VPA, VPA GLU was found to be a more prominent serum AA conjugate than VPA GLN. Serum VPA GLY was found at only trace levels. The concentrations of VPA GLU in the serum of rabbits dosed with 300 mg/kg (i.p.) and collected over 1.17-4 h ranged from 0.088 μg/ml to 0.304 μg/ml. Interestingly, only VPA GLU was identified positively in the CSF of rabbits (n=4) dosed with 300 mg/kg (i.p.).

The detection of VPA GLU in human CSF at a concentration higher than that observed in serum and at a concentration comparable to the level of glutamic acid in the brain suggests that the conjugate is the product of VPA conjugation with glutamic acid in the brain. If so, this suggests that the mechanism of action of VPA is at least partially expressed through the excitatory neurotransmitter itself. This represents a novel

approach to the study of the mechanism of action of an antiepileptic drug and could lead to the development of new antiepileptic drugs. Furthermore, the detection of VPA GLU and VPA GLN in the CSF of human also implies that both conjugates could have potential as antiepileptic or CNS drugs.

6. Future Research Plans

An LC/MS/MS assay would be most valuable for the analysis of thiol conjugates of VPA as it is fast and does not require any derivatization. The LC/MS/MS assay employed for the analysis of NAC I and NAC II was not linear over a range wide enough for quantitation of the conjugates. This appeared to be due an analytical problem which could perhaps be overcome by using less sample or reconstituting the extracts in a larger volume of mobile phase and optimizing the operating conditions of the instrument. The assay should perhaps be conducted using negative ion electrospray.

Although NAC III was not detected in any patient samples studied, it is important to confirm that the conjugate is not detected in samples of hepatotoxic patients as well. Similarly, the amount of NAC I and NAC II observed in our study needs to be compared to that excreted by patients with VPA-induced hepatotoxicity. The study of thiol conjugates of VPA has shown that age, dose and polytherapy are associated with an elevated amount of NAC I and NAC II being excreted by patients on VPA therapy. It would be of considerable clinical value to set up a study in which patients of the same age who are receiving similar doses of VPA and are on the same type of antiepileptic drug regimen be studied. To confirm that non-inducing anticonvulsants do not elevate the amount NAC I and NAC II excreted by patients, it would be important to conduct a study where patients on monotherapy are compared to patients (n>>5) on VPA and each of the non- enzyme-inducing anticonvulsant and patients on VPA and each of the enzyme inducing- anticonvulsants.

In this study, the AA conjugates of VPA were identified for the first time. While the conjugates of the parent drug did not account for a large percentage of the recovery of VPA, the AA conjugates of the phase I metabolites of VPA could be formed in larger amounts. Based on the present observations and other evidence in the literature, there is a high probability of detecting the AA conjugates of phase I metabolites of VPA in the urine, serum and possibly CSF of humans. The significance of these conjugates should then be investigated with respect to the recovery of the parent drug and their potential as antiepileptics. Both VPA GLU and VPA GLN are solids, and unlike VPA can likely be easily pressed into tablets without converting them to their sodium salt.

The antiepileptic activity of VPA GLU and VPA GLN should be assessed to determine if the two conjugates should be considered as potential antiepileptic drugs. Furthermore, the conjugates could also possess other beneficial CNS activity and the pharmacokinetic studies of the conjugates need to be undertaken to determine their distribution in the brain and other tissues.

The study of the AA conjugates of VPA in humans led to the identification of VPA GLU and VPA GLN in the CSF of one human subject. VPA GLU was later identified in the CSF (n>4) of rabbits treated with VPA. To determine whether or not VPA GLU is the conjugation product of VPA and the excitatory neurotransmitter glutamic acid, the first step is to conduct *in vitro* animals studies to investigate whether VPA GLU is formed in the brain. The conjugate has been observed in at least one rat brain and should be investigated in other VPA-treated rats (n=5) for confirmation. Then, for practical reasons, *in vitro* studies investigating the formation of VPA in rat brain can be an initial step in achieving an understanding of the mechanism of action of VPA.

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8. Appendices

Appendix a: Classificaton of seizure types

Table 1.0 Seizure types	Code #
Atypical absence	1
Myoclonic	2
tonic clonic	3
clonic	4
complex partial	5
secondary tonic clonic	6
simple partial,	7
secondary generalized	8
typical absence	9
atonic	10
absence	11
partial	12
atypical	13
tonic	14
Atypical drop	15
Spasms	16

Appendix b. Relevant clinical information of patients involved in our study.

Monotherapy

#	Dose mg/kg	Age Yrs	Sex	Epilepsy & Syndrome	Cognitive Ability	Seizure type (from table 1)	liver function tests
1	35.7	3.75	M		mh	1,2,3,4	
34	31.45	4.8	F	Temporal lobe epilepsy	mh	5,6	
5	29.26	5.36	M	Benign myoclonic epilepsy	mh	2,	Hi AST
32	35.71	6.87	F	Control temp lobe epilepsy	normal	7,8	
33	24.5	6.92	M	Severe myoclonic epilepsy	mh	1,2,3	
15	16.67	8.5	M	Lennox-gastaut syndrome	normal	1,2,3	
18	9.71	10.75	F	Idiopathic	normal	9,	
22	22.94	11.33	M	Symptomatic generalized epilepsy	mh	1,2,10	
2	20.9	12.36	F	Epilepsy with myoclonic absences	mh	1,	
43	7.74	14.667	M	Juvenile myoclonic epilepsy	normal	2,3	
41	25.32	14.92	F	Symptomatic generalized epilepsy	mh	1,	
16	13.6	16.3	F	Juvenile myoclonic epilepsy	normal	1,2,11	
9	3.88	16.6	M	Juvenile myoclonic epilepsy	mh	1,2	
11	6.2	17.33	M	Chidhood absence epilepsy	mh	3,9	

Co-medication

#	Dose mg/kg	Age Yrs	Sex	Epilepsy & Syndrome	Cognitive Ability	Seizure type (from table 1)	liver function tests
6	53.79	2.33	M	Symptomatic generalized epilepsy	mh	2,3	Lamo — AST level AB /other normal
44	33.43	5.33	F	Unclassified	mh	2,3,12	Clonaz
14	24.51	6.67	M	Temporal lobe epilepsy	mh	12,	Clobaz
8	30.99	7.5	M	Symptomatic generalized epilepsy	mh	13,	Lamo
12	23.54	11.17	M	Symptomatic generalized epilepsy	mh	1,2,10	Ethosux
7	19.74	13.25	F	Electrographic status epilepticus In sleep (ESES),no seizure in 4 yrs	mh	1,12	Ethosux
20	7.73	15	F	Lennox-gastaut syndrome	mh	3,12,14	Lamo Viga Nitra
19	14.16	17.08	M	Symptomatic generalized epilepsy	mh	1,3	Ethosux
29	20.1	17.17	F	Temporal lobe epilepsy	normal	12,	Clobaz
10	12.52	19.61	F	Symptomatic generalized epilepsy	mh	2,3	Clonaz
23	21.65	24.38	M	Progressive myoclonic epilepsy	mh	2,3	ethosux, Lamo NAC
17	22	27.1	M	Lennox-gastaut syndrome	mh	1,14	lamo, Flun

Polytherapy

| #	Dose mg/kg	Age Yrs	Sex	Epilepsy & Syndrome	Cognitive Ability	Seizure type (from table 1)	liver function tests
26	52.1	4.78	F	West syndrome	mh	12,16	CBZ
27	35.2	7.83	F	Frontal lobe epilepsy	mh	12,	CBZ, Viga
13	46.82	8.95	F	Frontal lobe epilepsy	normal	12,	CBZ
37	20.92	14.33	F	Symptomatic generalized epilepsy	mh	14,15	CBZ
39	44.6	14.67	M	Symptomatic generalized epilepsy	mh	2,12,14	PB, Viga
24	47.4	15.17	M	Lennox-gastaut syndrome	mh	1,14,10	DPH
42	8.93	17	F		mh	3,	CBZ
25	19	19.7	M	West syndrome epilepsy	mh	2,12,16	CBZ Clobaz
21	20.94	22.5	F	Frontal lobe epilepsy	normal	12	CBZ,Lamo

mh=mentally handicap M=male, F=female, Lamo=lamotrogine, Viga=vigabatrin, Nitraz=nitrazepam, Ethosux=ethosuximide, Clobaz=clobazam, Clonaz=clonazepam, CBZ=CBZamazepine, DPH=phenytoin,PB=phenobarb, Hi=high, AB=abnormal
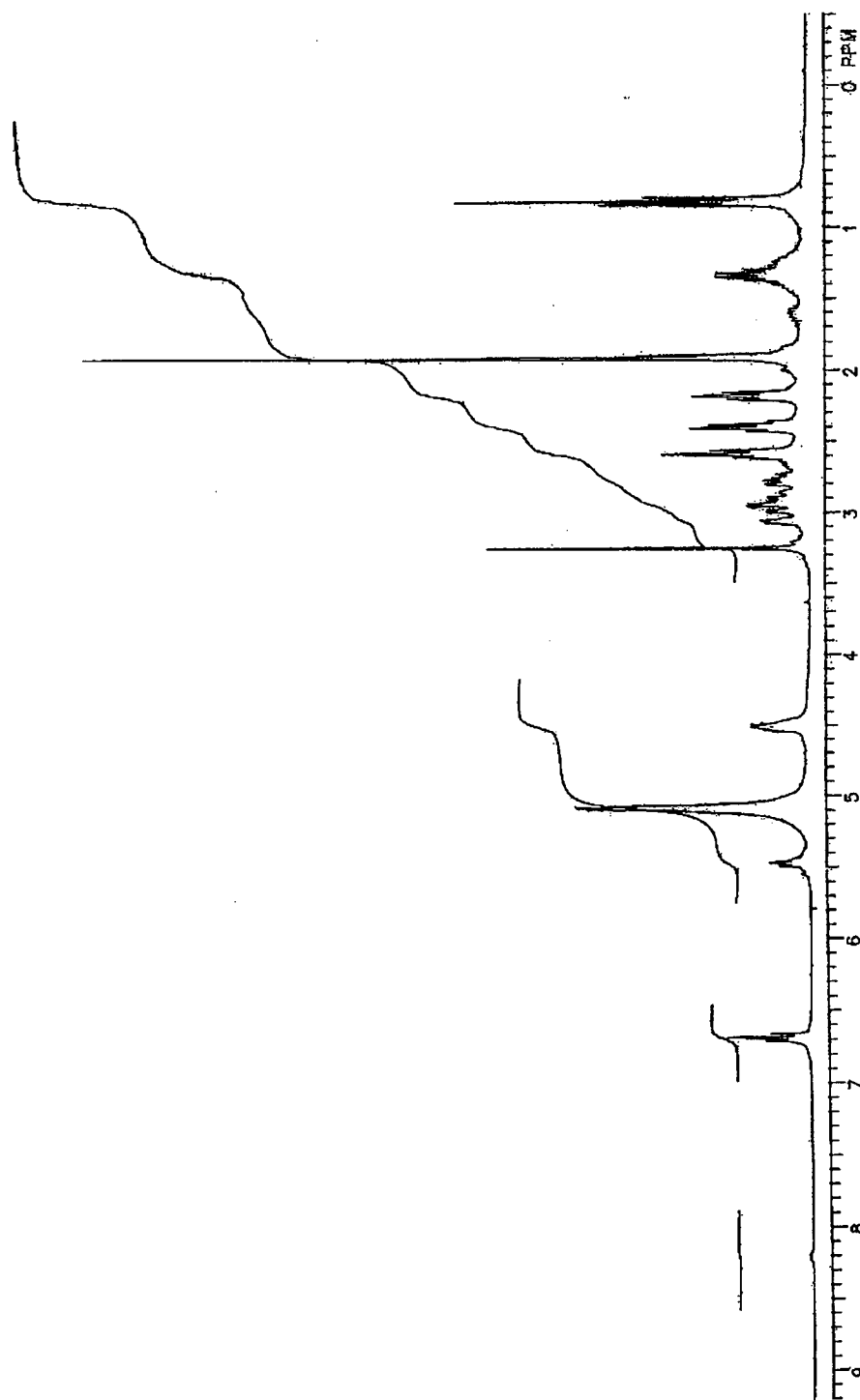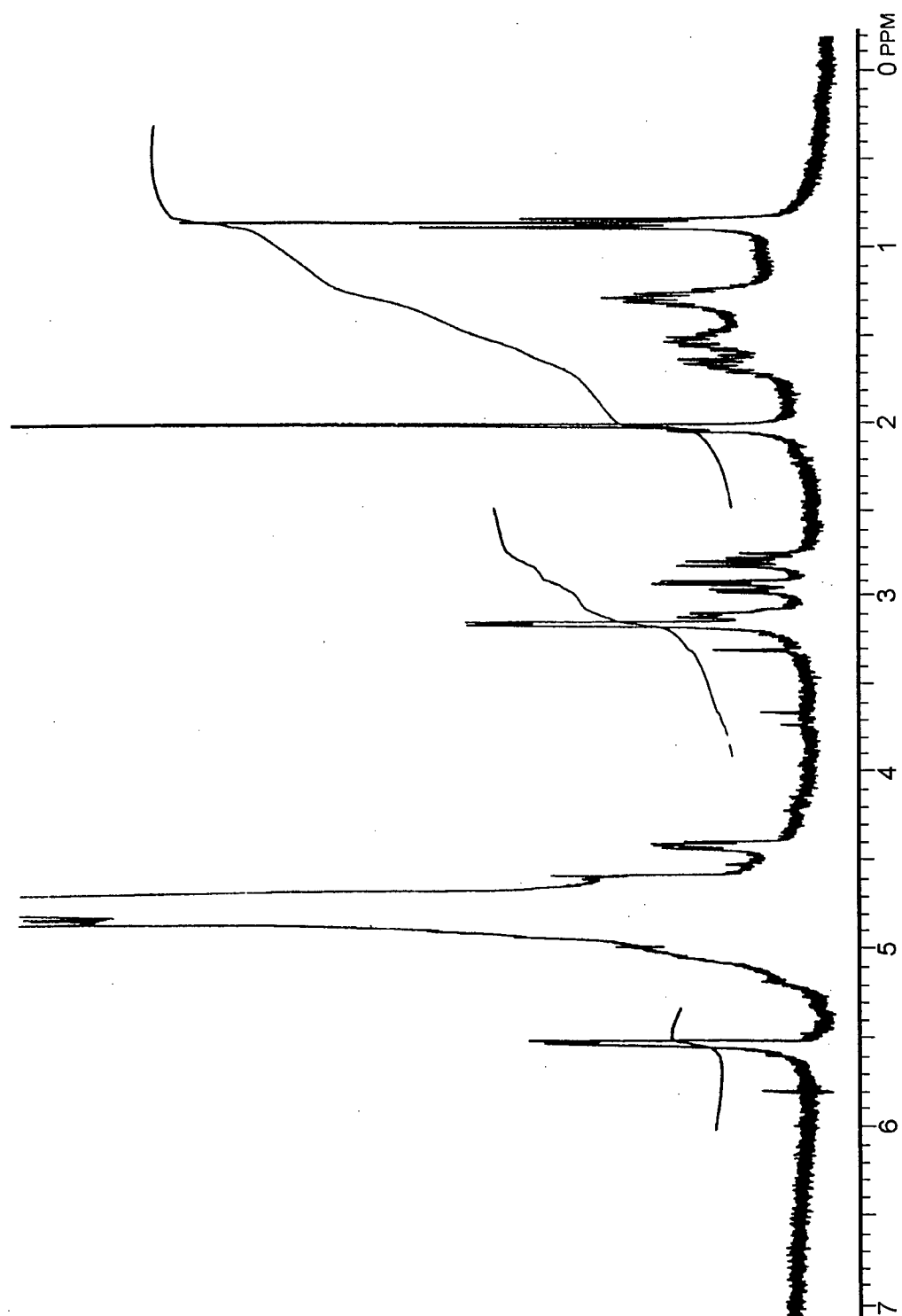
Appendix c. ^{1}H NMR of mixture of thiol conjugates formed from the synthetic pathway described in scheme 3.

288
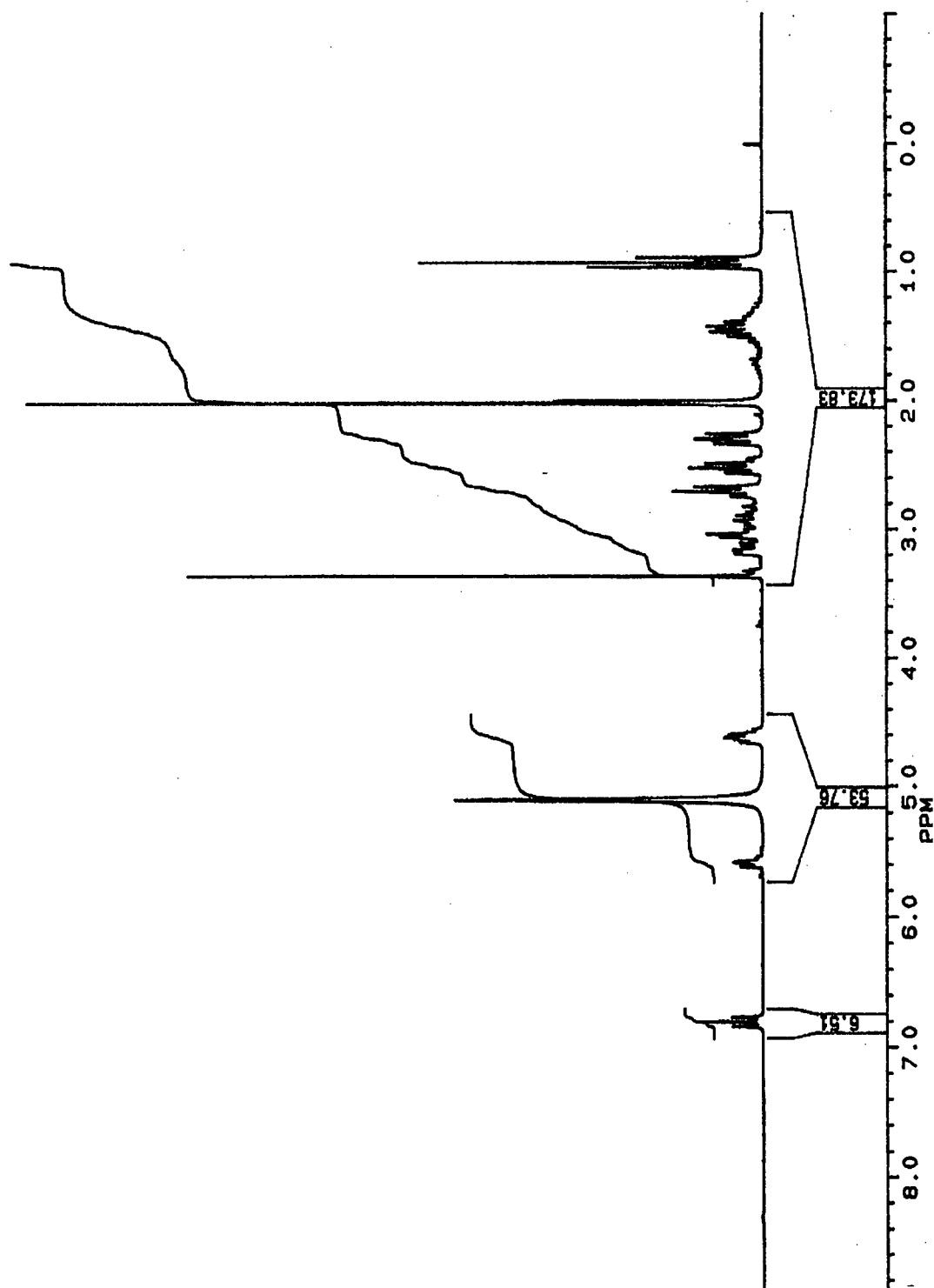
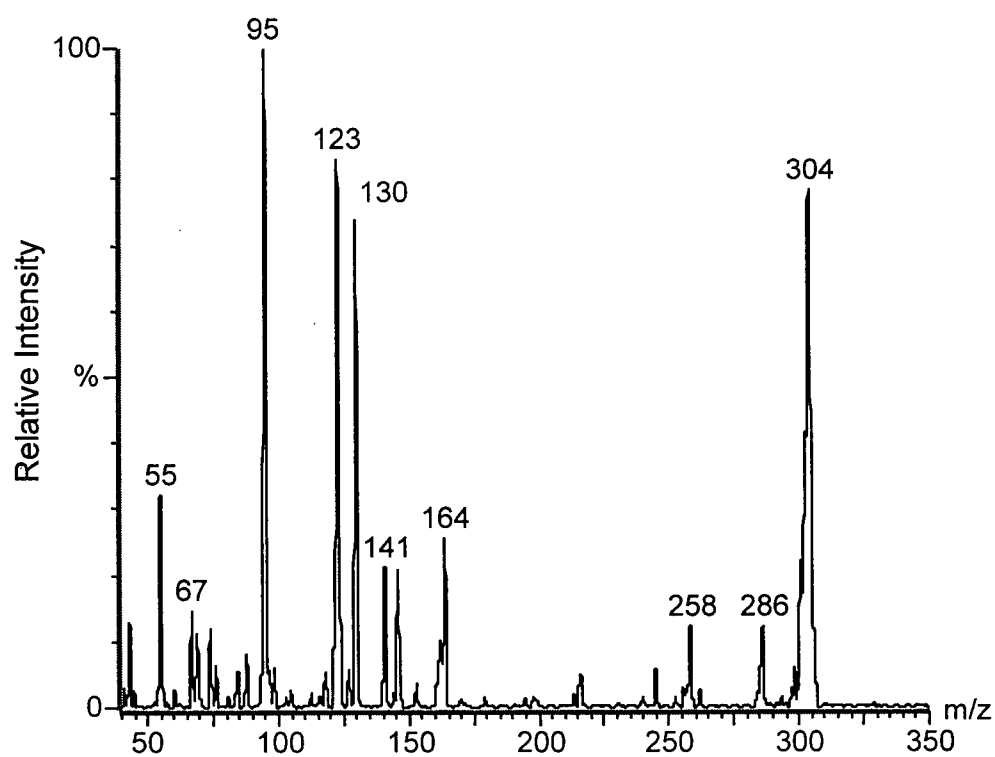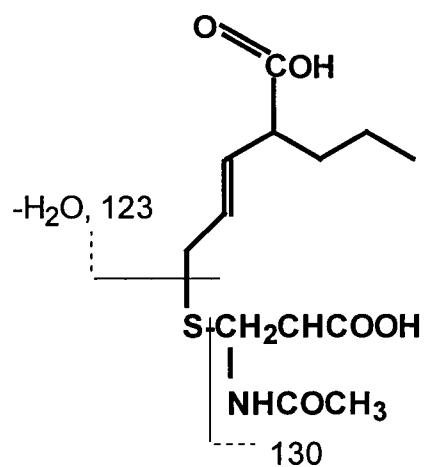Appendix d. ^1H NMR of mixed products obtained three months after initial analysis.
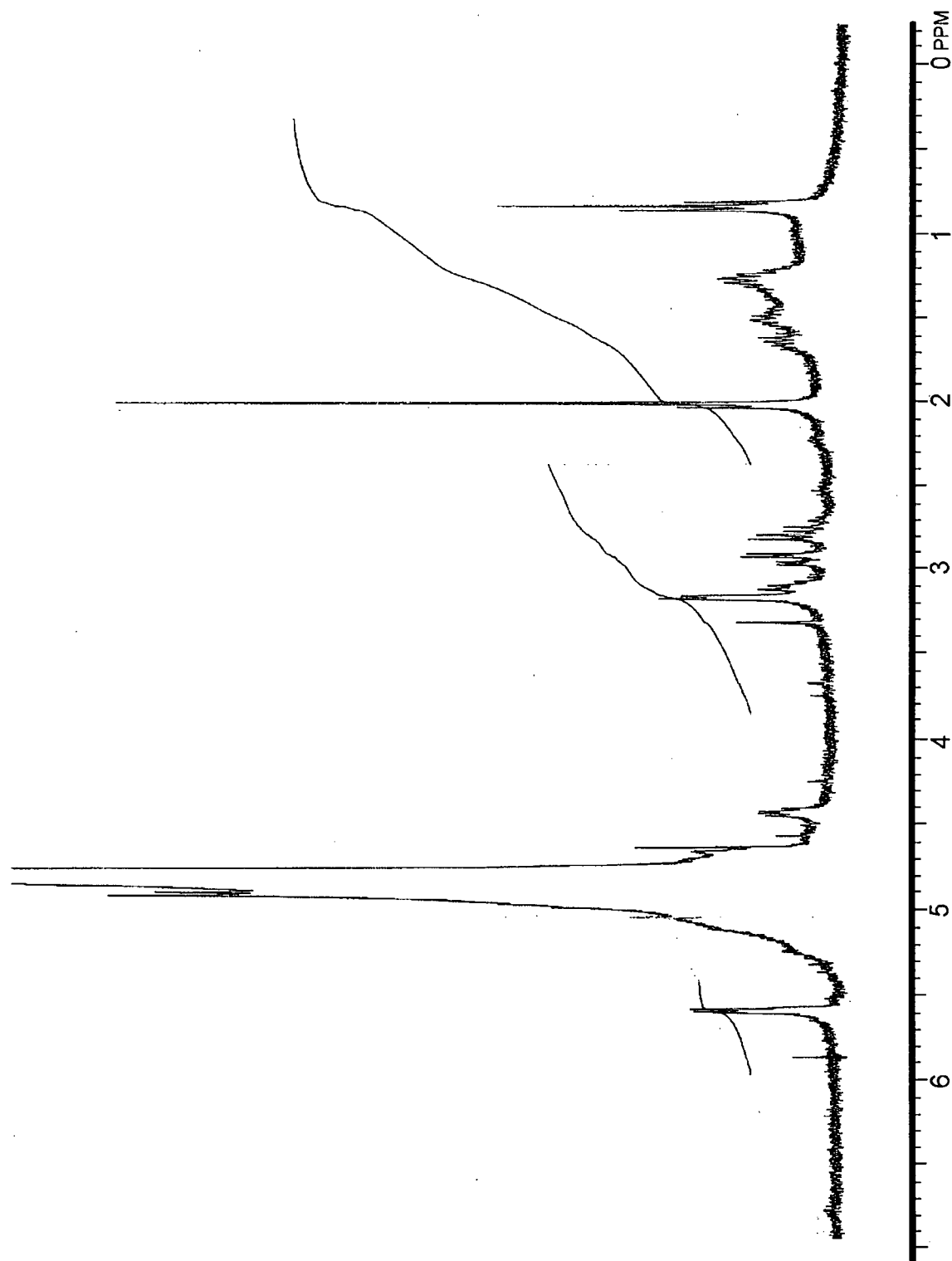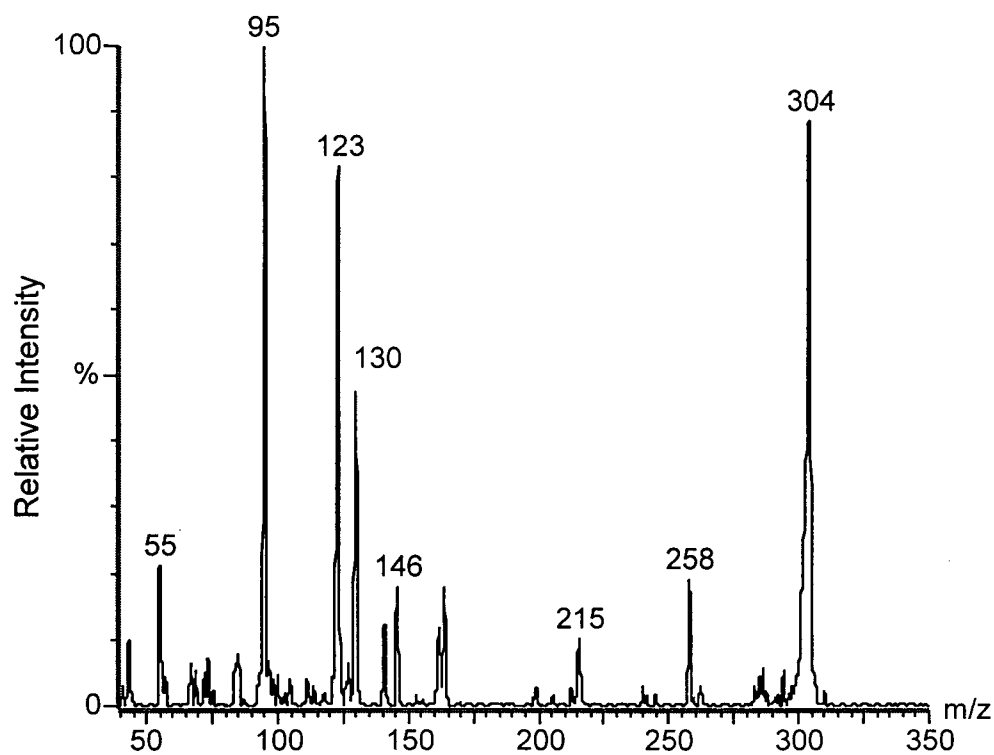
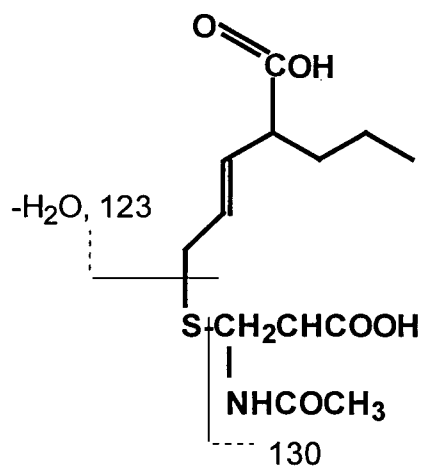Appendix e. ^{1}H NMR of HPLC isolated isomer A of NAC I

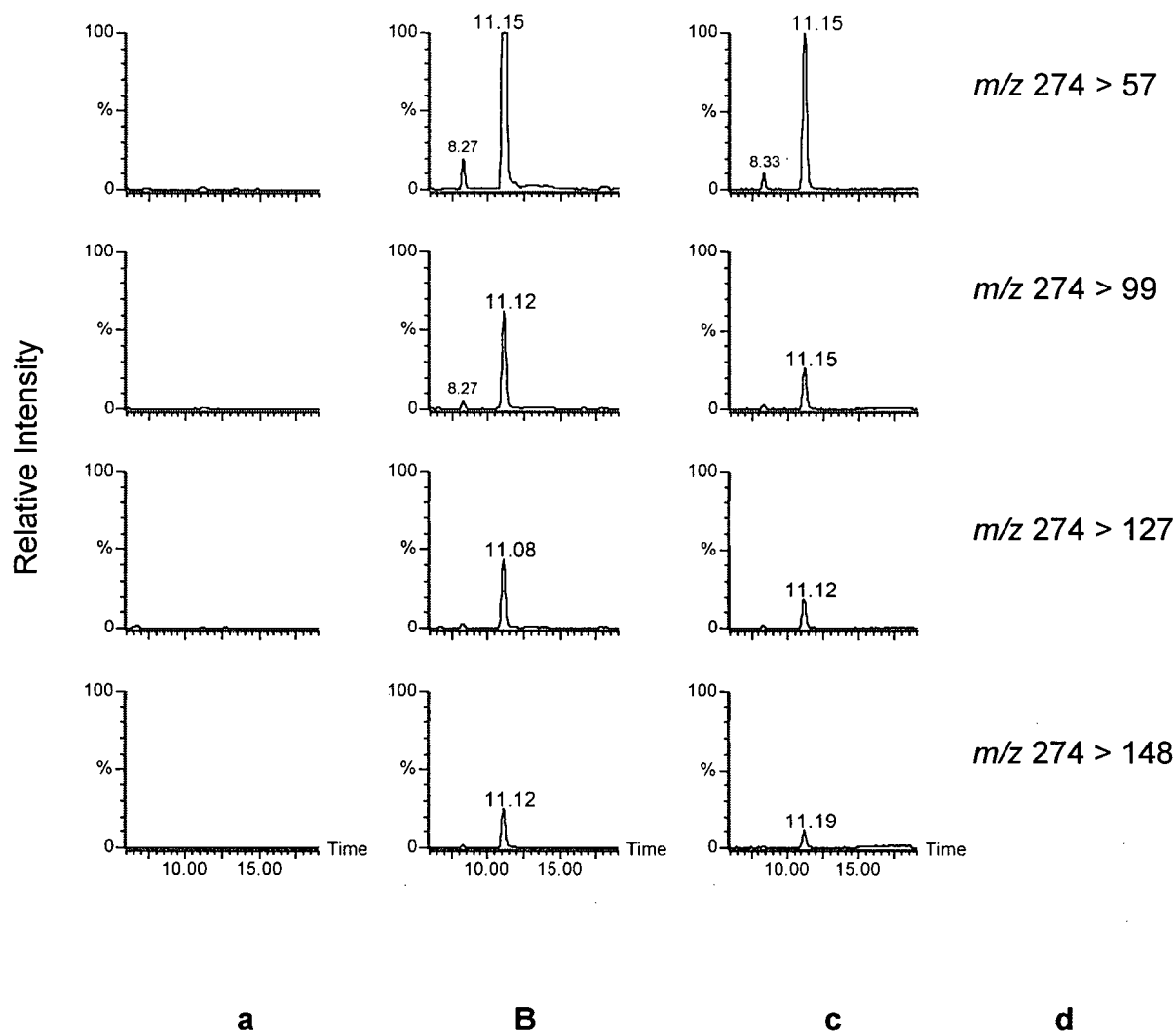Appendix f. ^{1}H NMR of HPLC isolated isomer B of NAC I

NAC I A

-H$_2$O, 123

COH

O

S-CH$_2$CHCOOH

NHCOCH$_3$

130

Appendix g. MS/MS product ion spectrum of HPLC purified NAC IA under positive elecrospray (*m/z* 304).

292

NAC I B

Appendix h. MS/MS product ion spectrum of HPLC purified NAC I B under positive electrospray *(m/z 304)*.
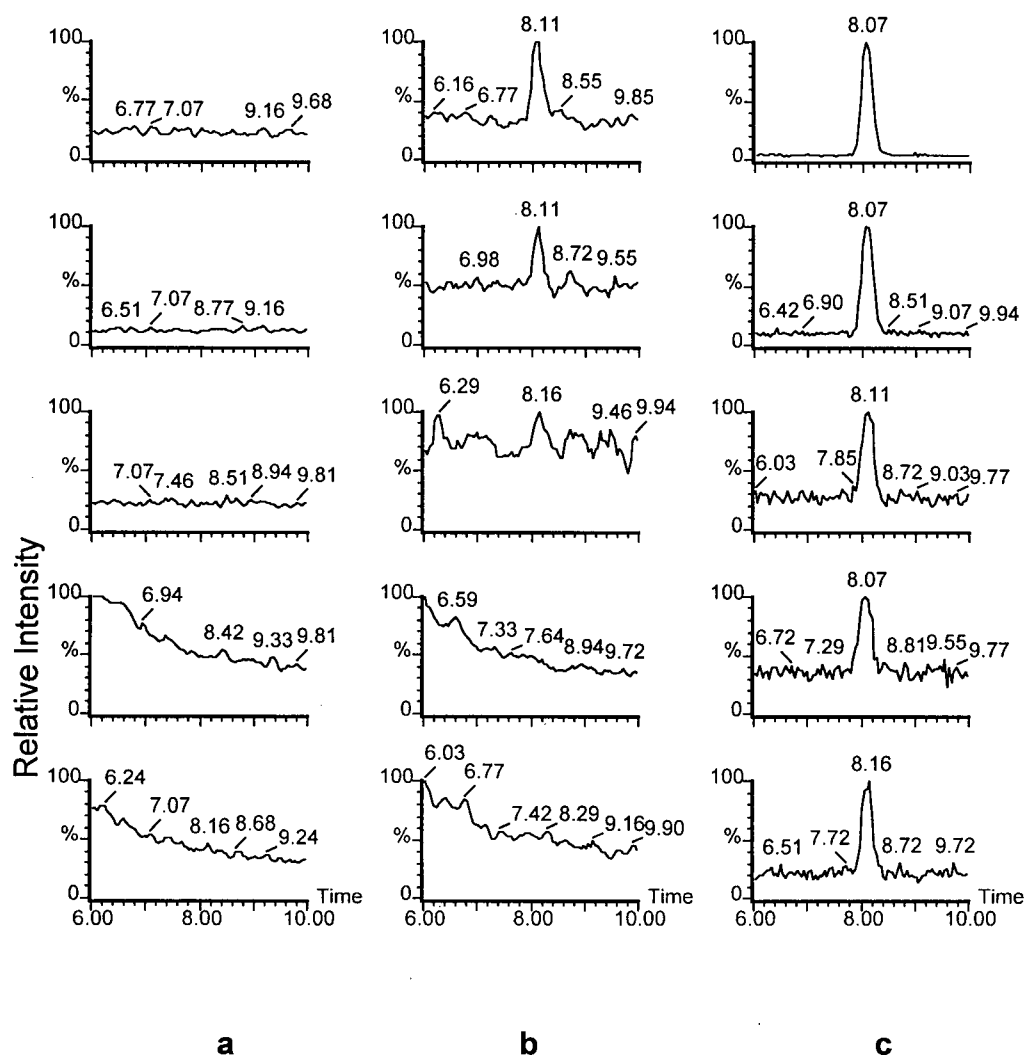
Appendix i. On-line LC/MS/MS monitoring of VPA GLU in: (a) an extracted rat urinary control sample, (b) an extracted urinary sample of a rat dosed with VPA, and (c) a synthetic standard of VPA GLU which eluted at t_R=11.15 min. The corresponding characteristic ion transitions monitored for MRM are shown in (d) (mobile phase : ACN (40%): H_2O (60%) and 0.05% TFA).

m/z 273 > 57

m/z 273 > 99

m/z 273 > 127

m/z 273 > 129

m/z 273 > 147
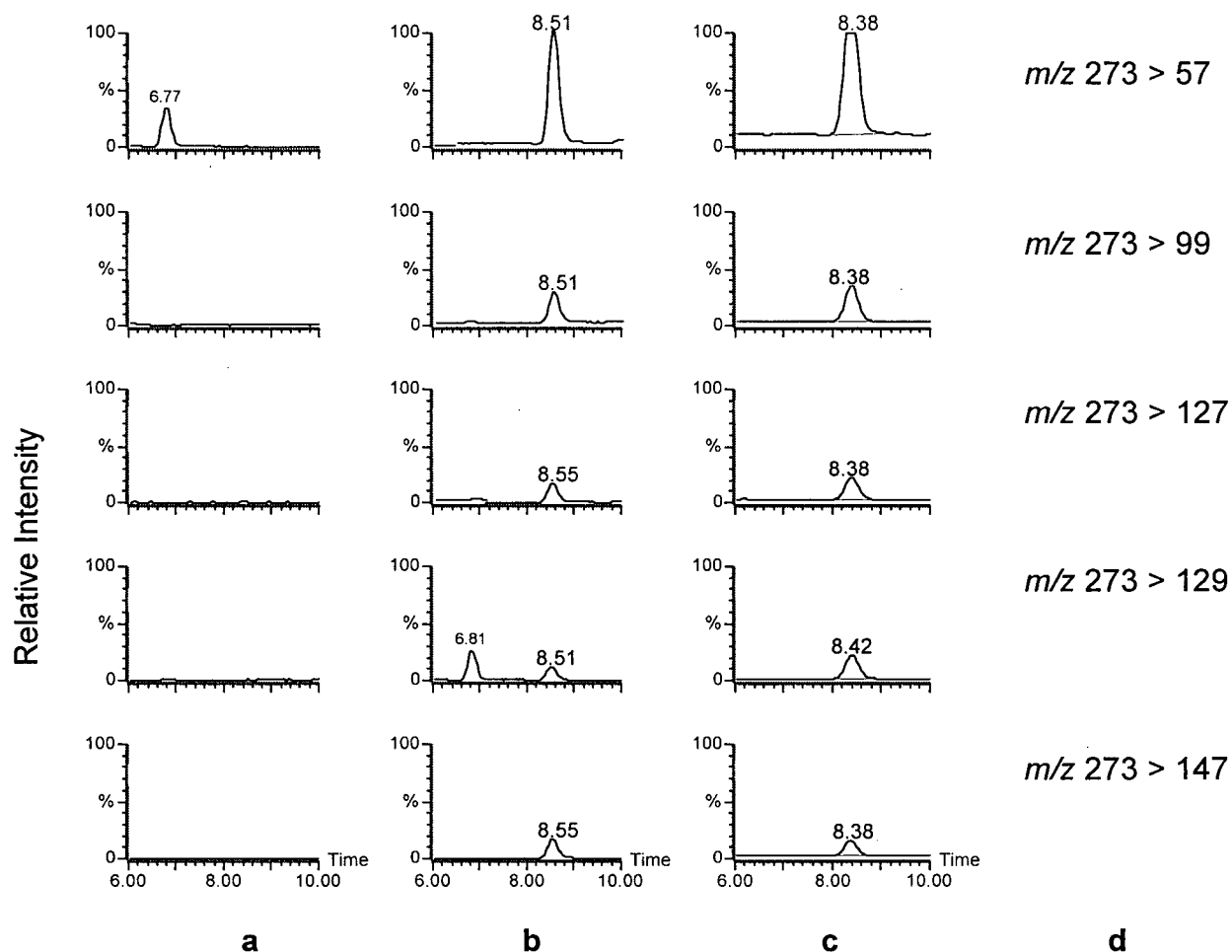
Appendix j. On-line LC/MS/MS monitoring of VPA GLN in: (a) an extracted rat urinary control sample, (b) an extracted urinary sample of a rat dosed with VPA, and (c) a synthetic standard of VPA GLN which eluted at t_R= 8.38 min. The corresponding characteristic ion transitions monitored for MRM are shown in (d) (mobile phase : ACN (40%): H_2O (60%) and 0.05% TFA).
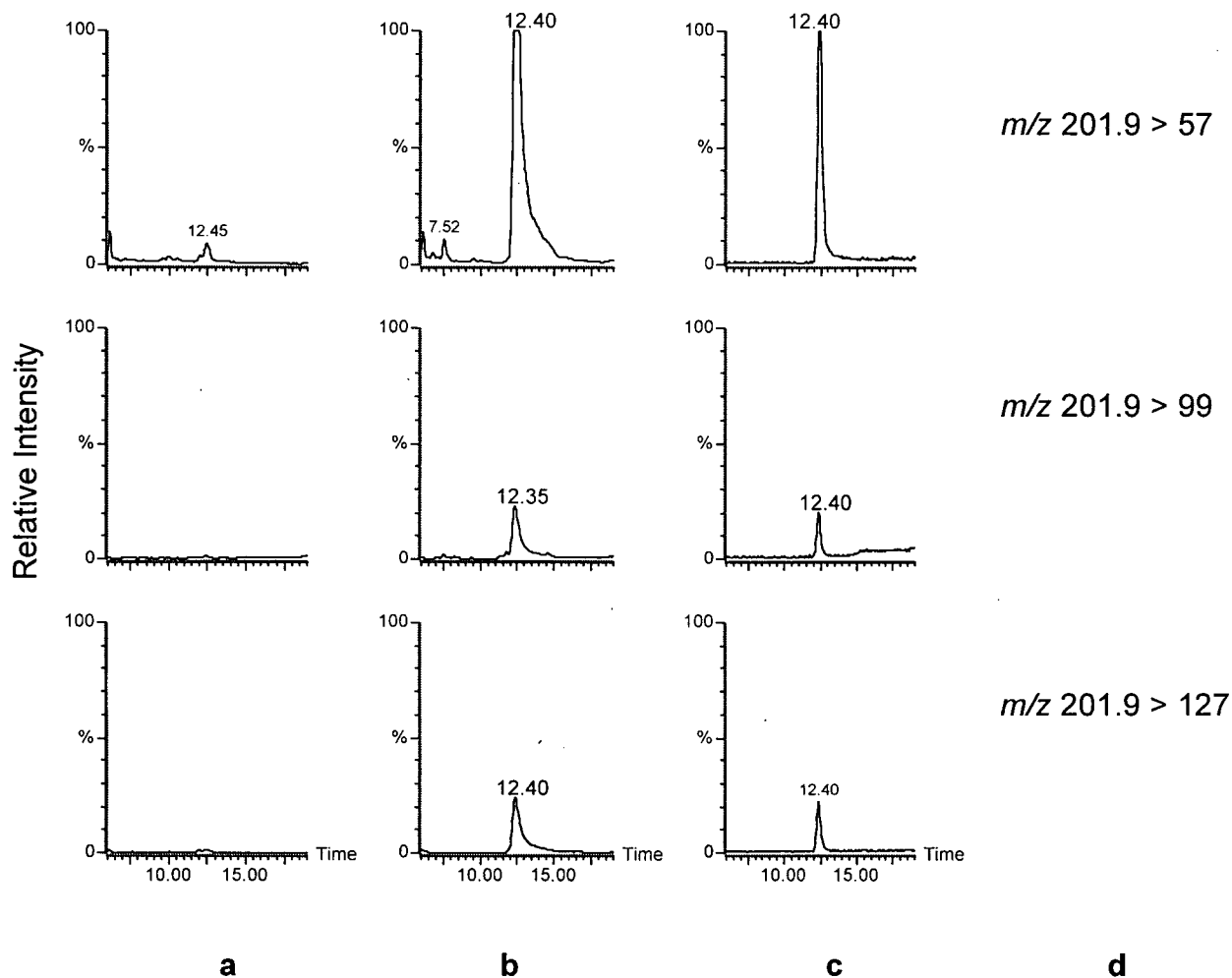
Appendix k. On-line LC/MS/MS monitoring of VPA GLY in: (a) an extracted rat urinary control sample, (b) an extracted urinary sample of a rat dosed with VPA, and (c) a synthetic standard of VPA GLY which eluted at t_R=12.40 min. The corresponding characteristic ion transitions monitored for MRM are shown in (d) (mobile phase : ACN (40%): H_2O (60%) and 0.05% TFA).
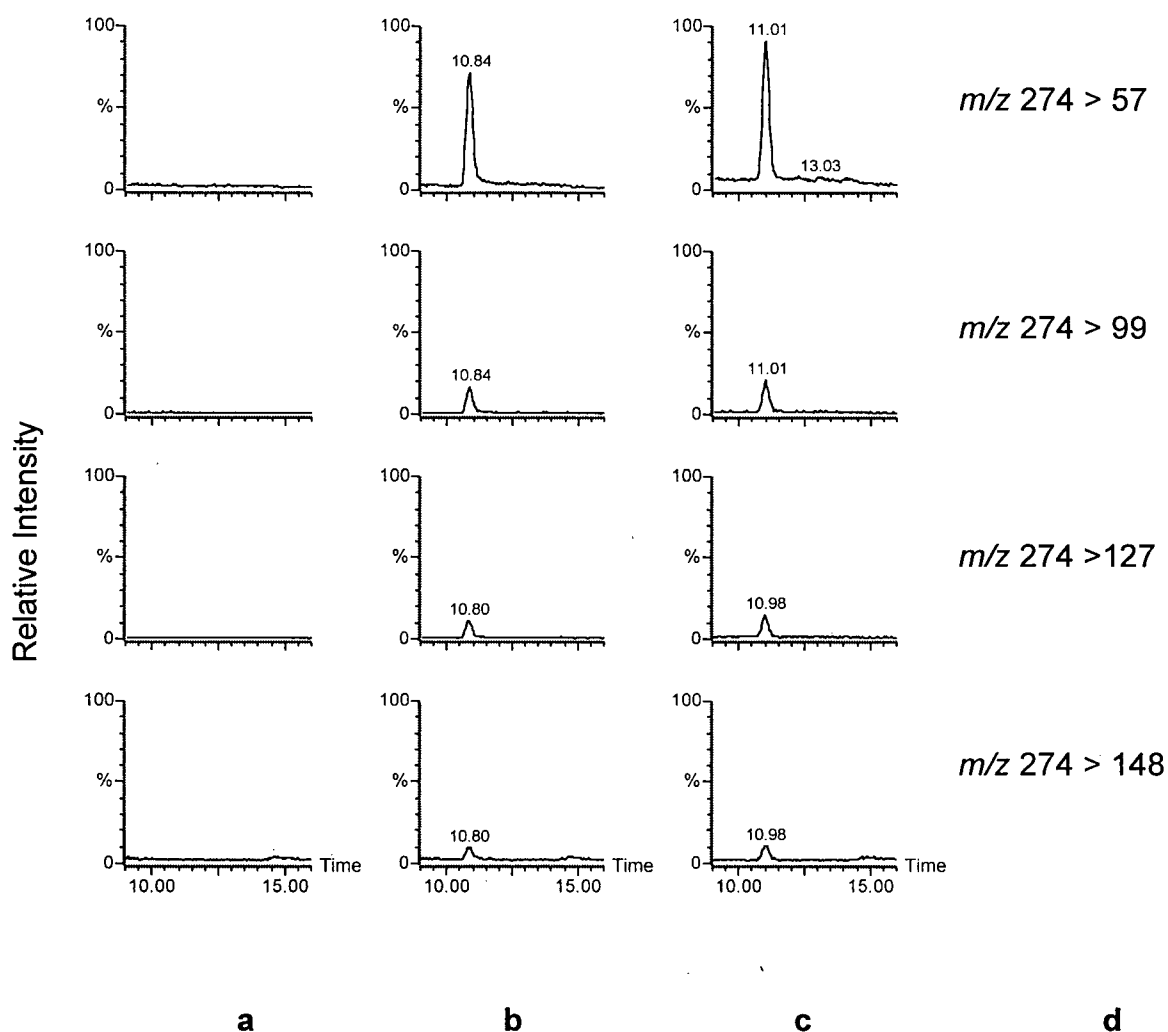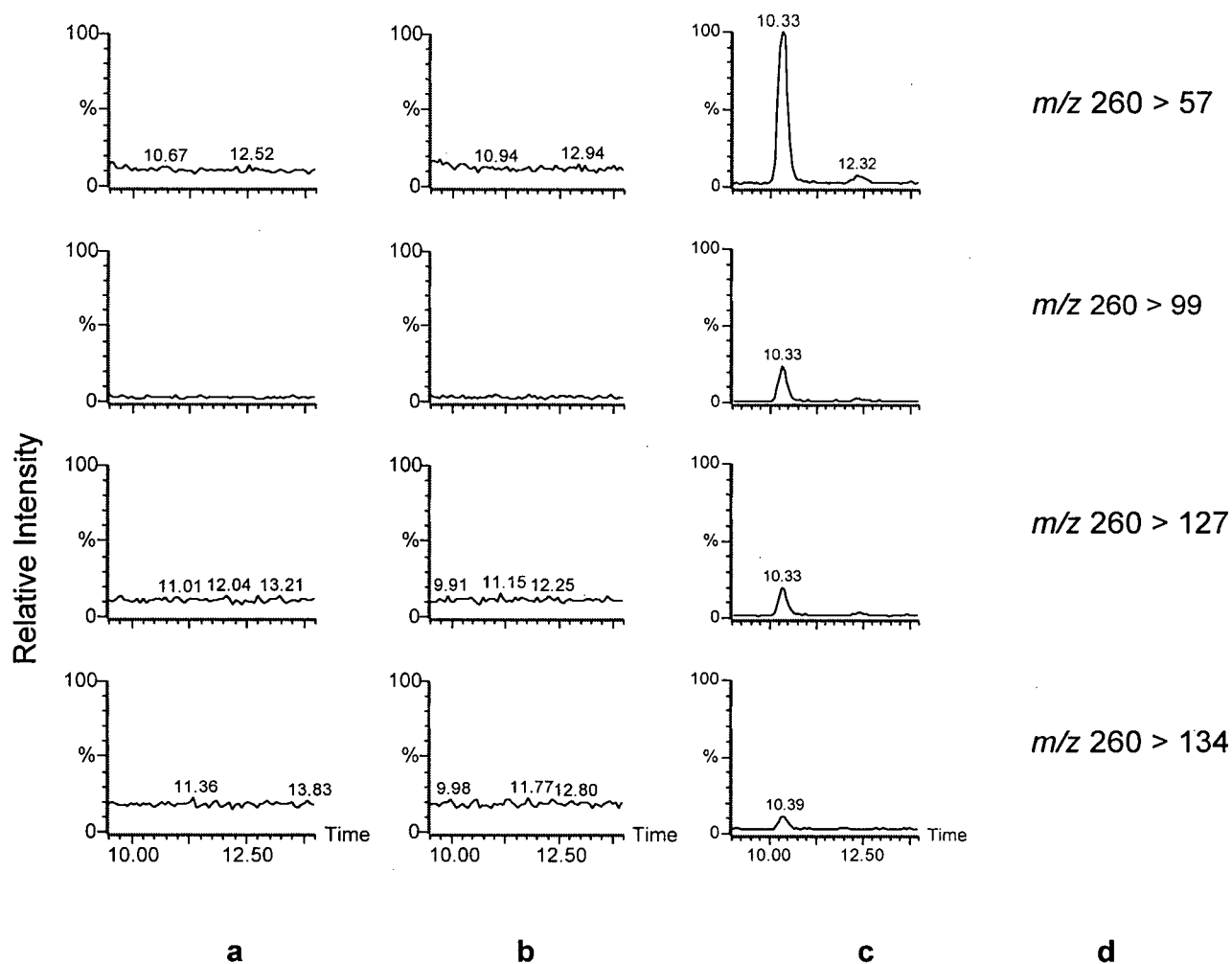
Appendix I. On-line LC/MS/MS monitoring of VPA GLU in: (a) an extracted rat serum control sample, (b) an extracted serum sample of a rat dosed with VPA, and (c) a synthetic standard of VPA GLU which eluted at t_R=11.01 min. The corresponding characteristic ion transitions monitored for MRM are shown in (d) (mobile phase: ACN (40%): H_2O (60%) and 0.05% TFA).
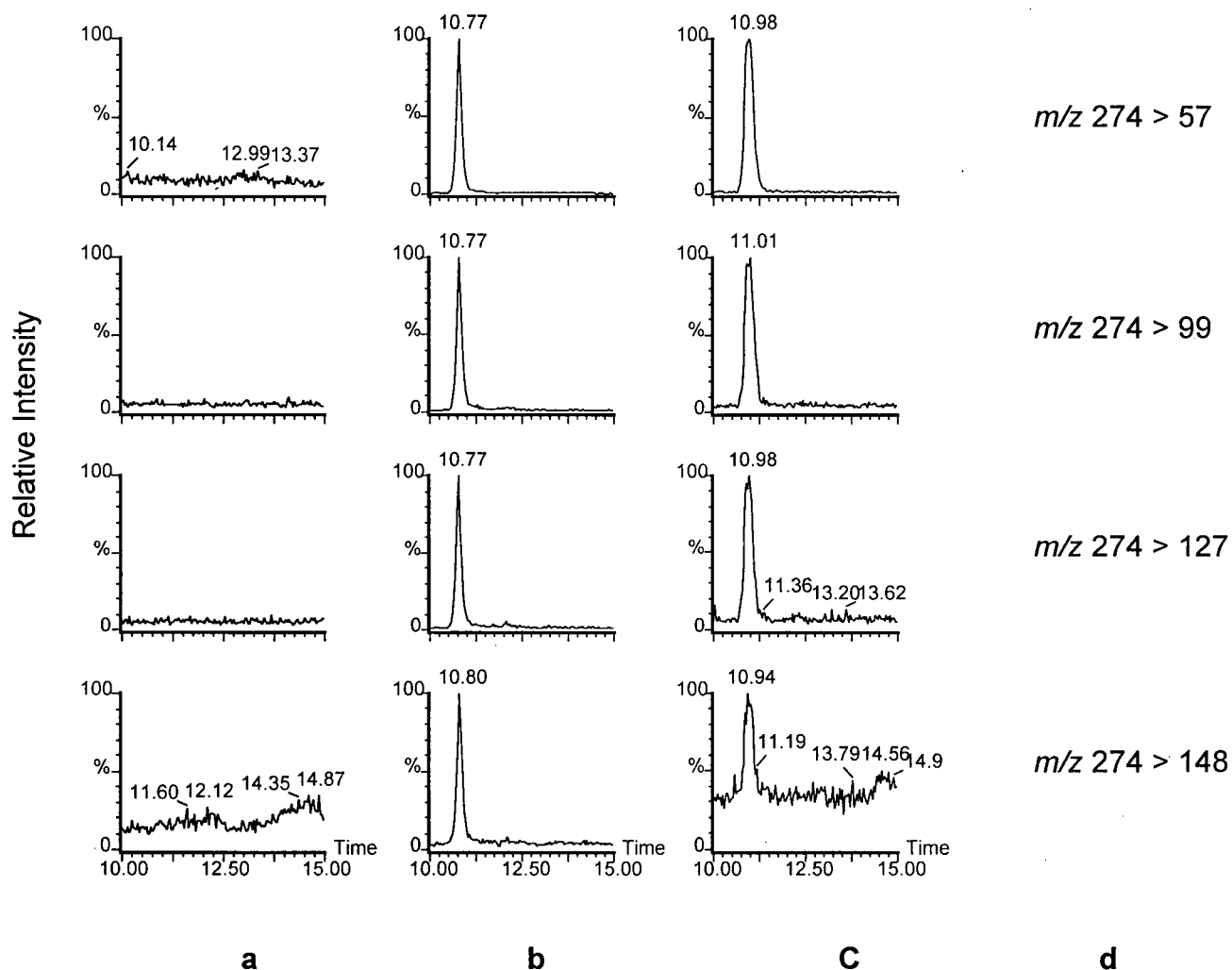
297

Appendix m. On-line LC/MS/MS monitoring of VPA GLN in: (a) an extracted rat serum control sample, (b) an extracted serum sample of a rat dosed with VPA, and (c) a synthetic standard of VPA GLN which eluted at t_R=8.07 min. The corresponding characteristic ion transitions monitored for MRM are shown in (d) (mobile phase : ACN (40%): H_2O (60%) and 0.05% TFA).

m/z 202 >57

m/z 202 > 99

m/z 202 > 127

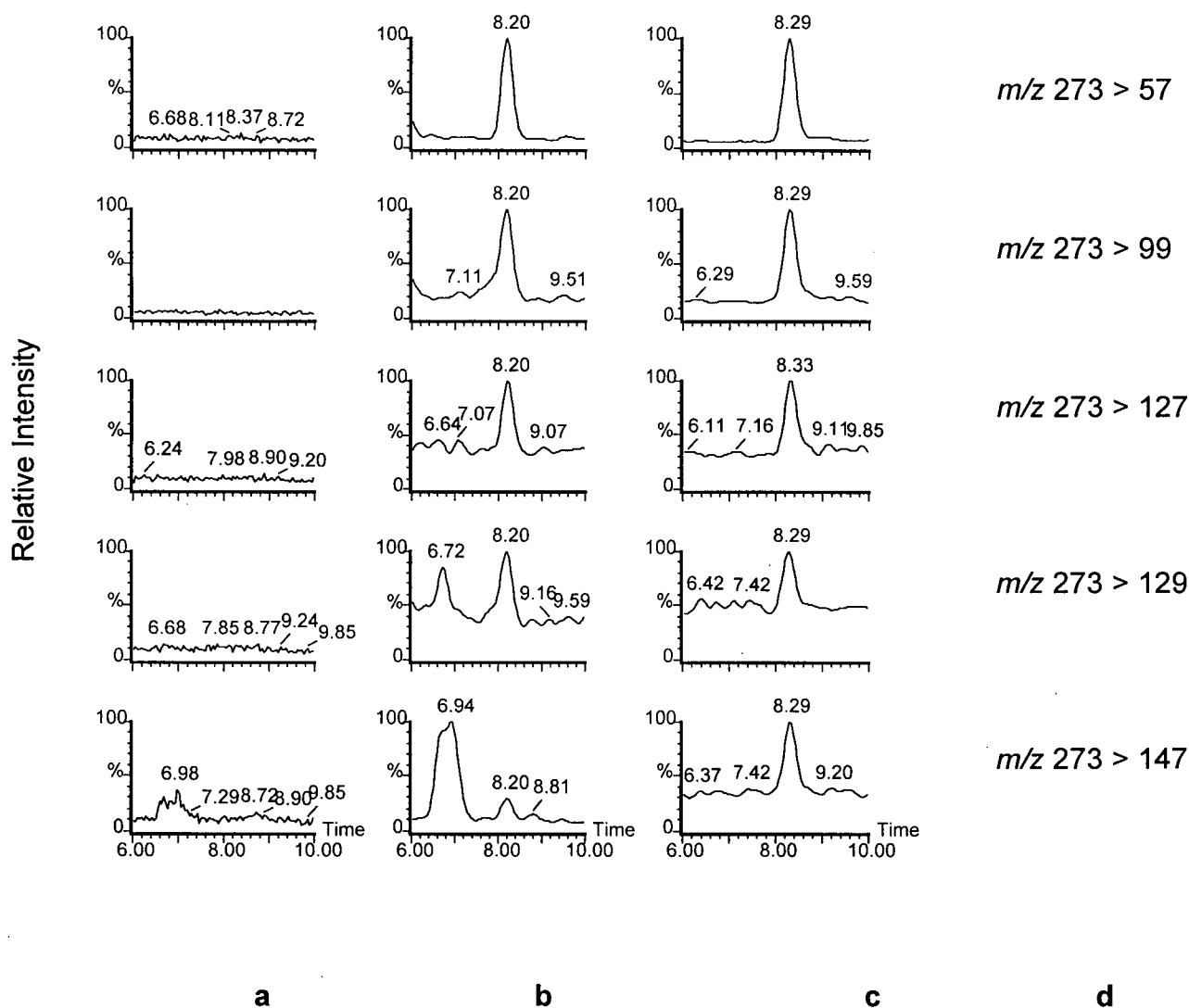a   b   c   d
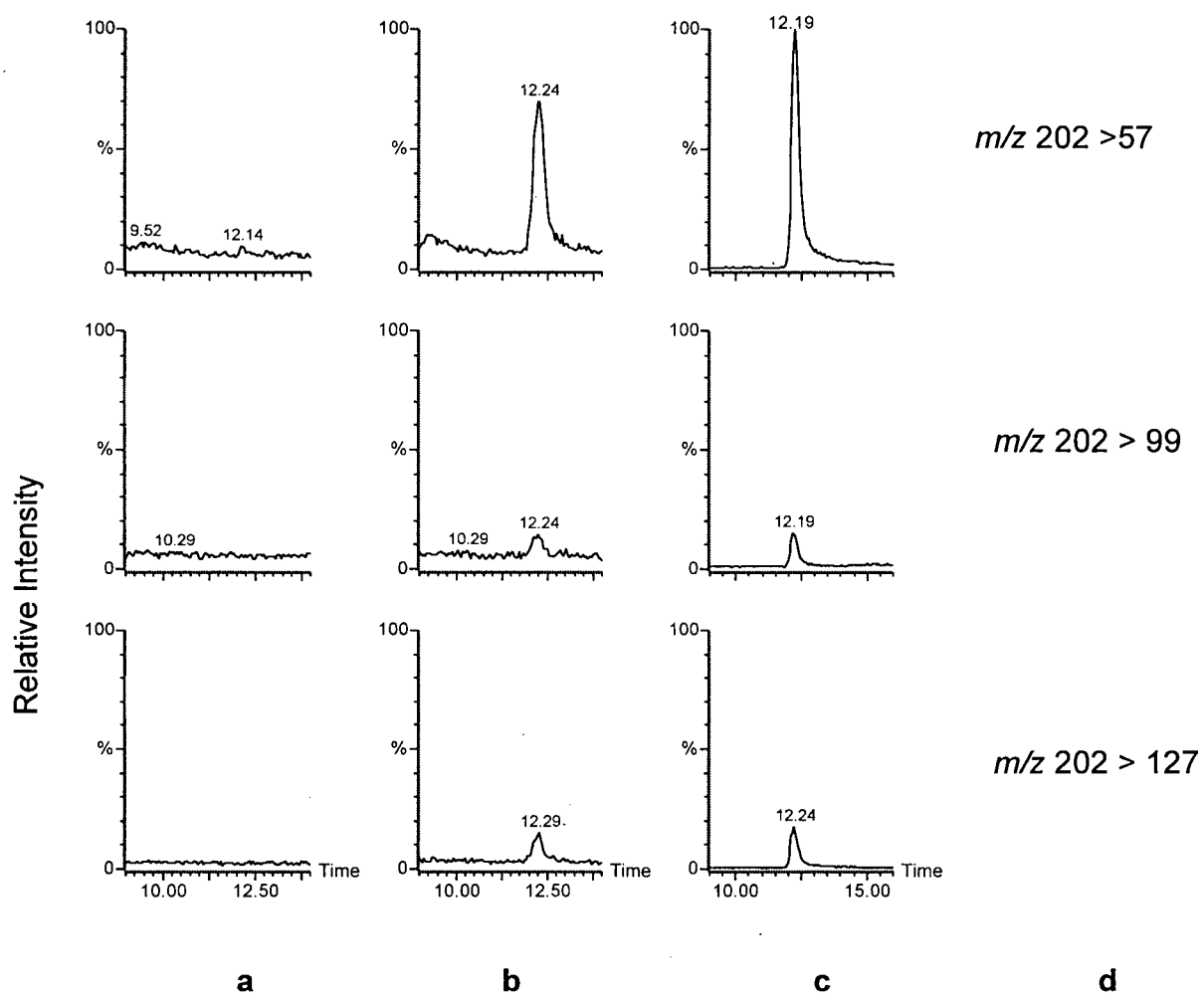
Appendix n. On-line LC/MS/MS monitoring of VPA GLY in: (a) an extracted rat serum control sample, (b) an extracted serum sample of a rat dosed with VPA, and (c) a synthetic standard of VPA GLY which eluted at t_R=12.19 min. The corresponding characteristic ion transitions monitored for MRM are shown in (d) (mobile phase : ACN (40%): H_2O (60%) and 0.05% TFA).
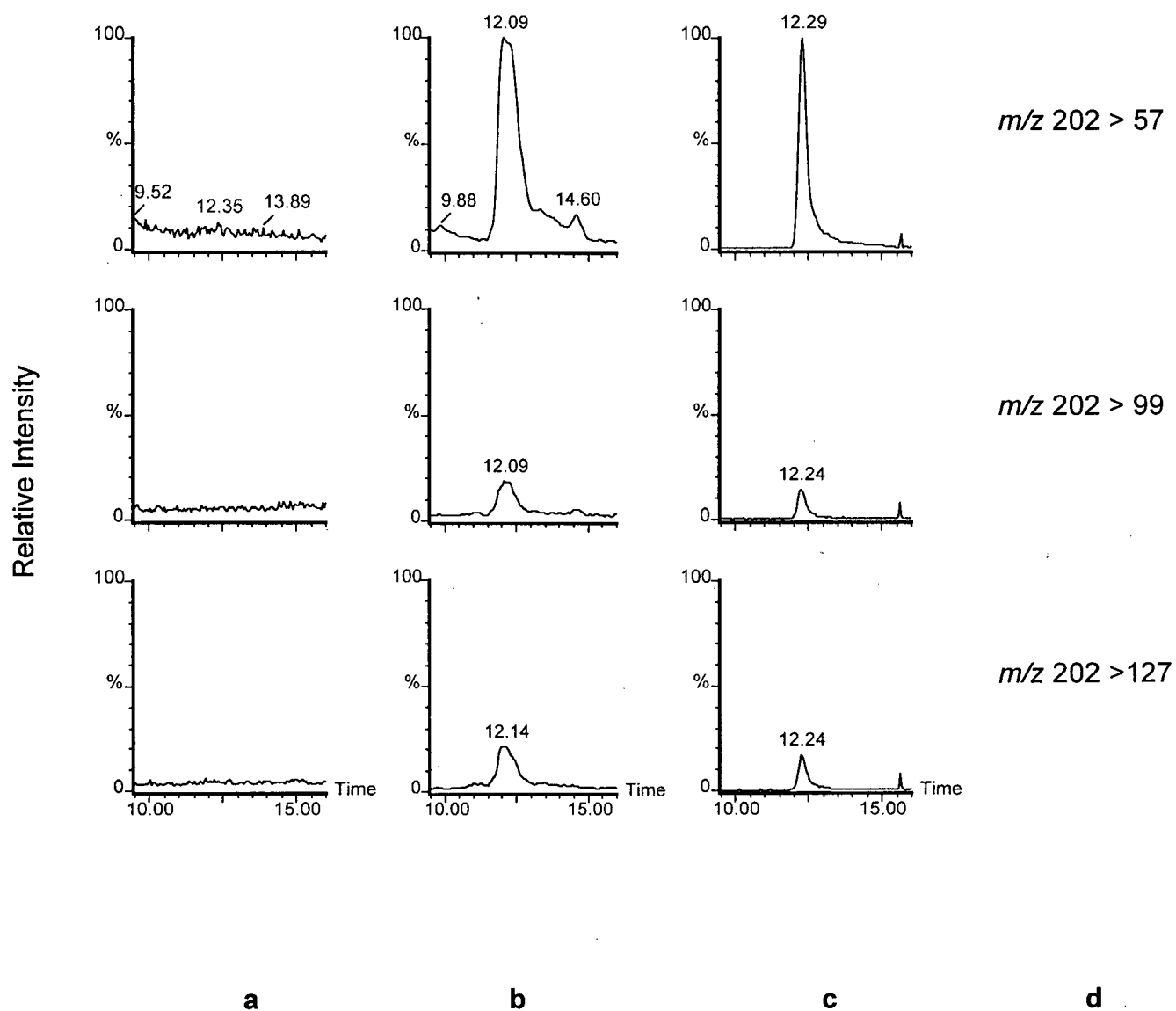
Appendix o. On-line LC/MS/MS monitoring of VPA ASP in: (a) an extracted rat serum control sample, (b) an extracted serum sample of a rat dosed with VPA, and (c) a synthetic standard of VPA ASP which eluted at t_R=10.33 min. The corresponding characteristic ion transitions monitored for MRM are shown in (d) (mobile phase : ACN (40%): H_2O (60%) and 0.05% TFA).
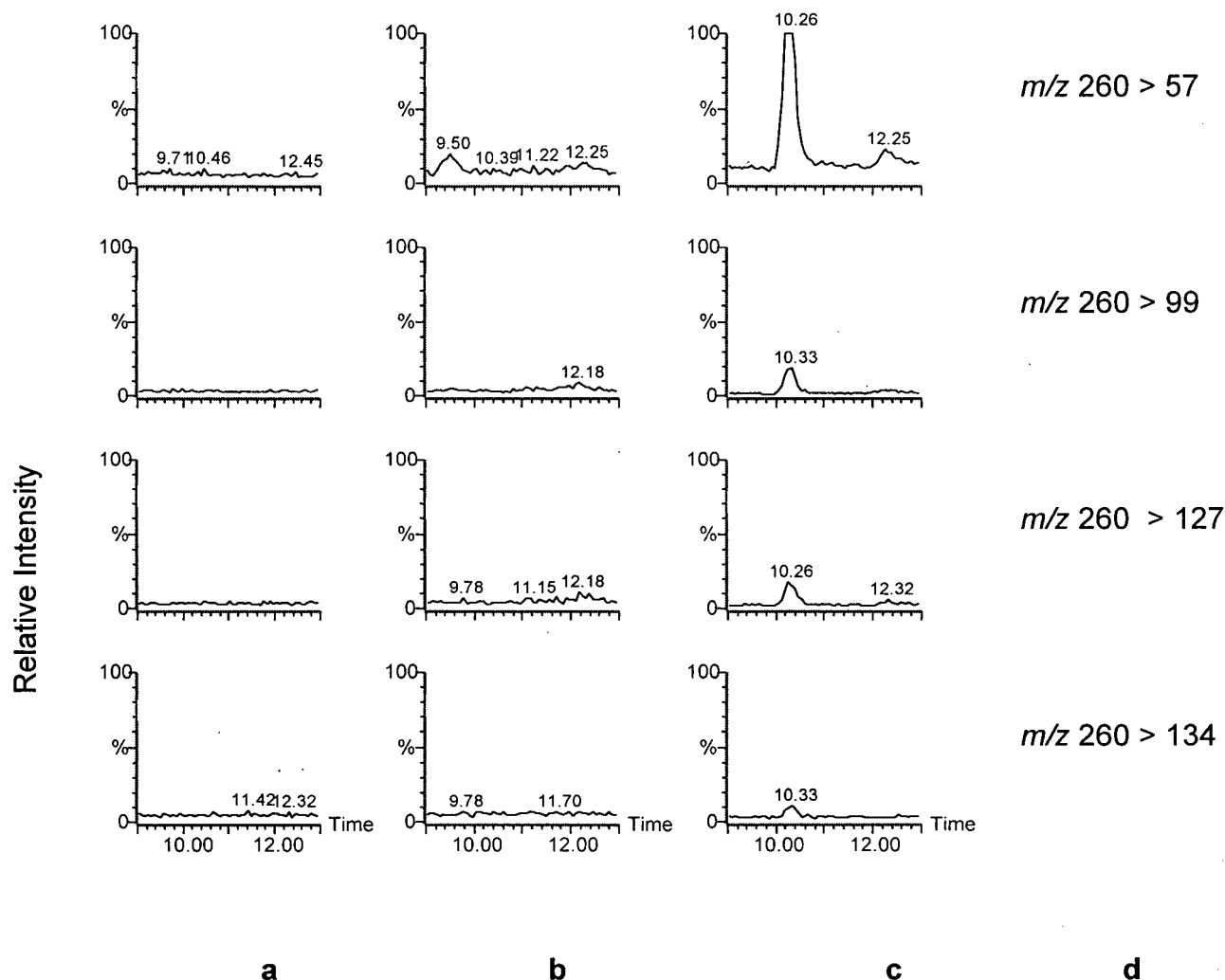
Appendix p. On-line LC/MS/MS monitoring of VPA GLU in: (a) a control rat bile sample, (b) a biliary sample of a rat dosed with VPA, and (c) a synthetic standard of VPA GLU which eluted at t_R=10.98 min. The corresponding characteristic ion transition monitored for MRM are shown in (d) (mobile phase : ACN (40%): H_2O (60%) and 0.05% TFA).

Appendix q. On-line LC/MS/MS monitoring of VPA GLN in: (a) a control rat bile sample, (b) a biliary sample of a rat dosed with VPA, and (c) a synthetic standard of VPA GLU which eluted at t_R=8.29 min. The corresponding characteristic ion transitions monitored for MRM are shown in (d) (mobile phase: ACN (40%): H_2O (60%) and 0.05% TFA).

m/z 202 > 57

m/z 202 > 99

m/z 202 >127

Relative Intensity

a   b   c   d

Appendix r. On-line LC/MS/MS monitoring of VPA GLY in: (a) a control rat bile sample, (b) a biliary sample of a rat dosed with VPA, and (c) a synthetic standard of VPA GLY which eluted at t_R=12.29 min. The corresponding characteristic ion transitions monitored for MRM are shown in (d) (mobile phase: ACN (40%):H_2O (60%) and 0.05% TFA).

Appendix s. On-line LC/MS/MS monitoring of VPA ASP in: (a) a control rat bile sample, (b) a biliary sample of a rat dosed with VPA, and (c) a synthetic standard of VPA ASP which eluted at t_R=10.26 min. The corresponding characteristic ion transitions monitored for MRM are shown in (d) (mobile phase : ACN (40%): H_2O (60%) and 0.05% TFA).