STUDIES ON THE METABOLISM OF TOCAINIDE IN HUMANS

BY

DAVID W. K. KWOK

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Department of Pharmaceutical Chemistry

The University of British Columbia
1956 Main Mall
Vancouver, Canada
V6T 1Y3

Date April 30,1987.

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Abstract

Tocainide carbamoyl ester glucuronide (TOCG) (R-NHCO.O-GA) is a major metabolite of tocainide (Tonocard®). The structure of TOCG was first proposed by Elvin (35) based on the structure of 3-(2,6-xylyl)-5-methylhydantoin, a base hydrolyzed product of TOCG in urine. Due to the presence of two carbonyl groups on the hydantoin ring, TOCG was proposed to arrive from a novel metabolic pathway involving the addition of carbon dioxide to the terminal nitrogen of tocainide followed by glucuronic acid conjugation.

With the initial intention of carrying out a bioavailability study of tocainide using a deuterated pseudoracemic sample, the stereospecific synthesis of R(-)- and S(+)-trideuterated tocainide was attempted through two synthetic approaches.

This thesis describes a chemical reaction between tocainide and urea, a second pathway which leads to the formation of 3-(2,6-xylyl)-5-methylhydantoin through a tocainide ureide intermediate. With this observation, a tocainide N-ureide glucuronide structure (R-NHCO.NH-GA) was proposed for TOCG in support of the theory that an *in vivo* reaction between tocainide and urea may have resulted a tocainide N-ureide which can be further conjugated with glucuronic acid. Attempts were made to assign the correct structure of TOCG by identification of the theoretical tocainide carbamic acid (based on Elvin's proposed structure) or the tocainide N-ureide intermediate in urine.

This thesis also describes the preparative HPLC isolation and the structural characterization of this novel glucuronic acid conjugate. Evidence obtained as proof for the identity of TOCG as a conjugate was obtained from acid hydrolysis, basic hydrolysis, beta-glucuronidase hydrolysis, with or without the presence of sacchro-1,4-lactone, and a naphthoresorcinol color test. Structural evidence for the carbamoyl ester linkage of TOCG was obtained from proton-NMR and FAB analysis.

The 400-MHz proton NMR data of the isolated glucuronide provided partial evidence for the intact structure of TOCG. In FAB analysis, the [M+1] ion adduct at m/z 413, [M+Na] at m/z 435, and [M-H+2Na] at m/z 457 have provided positive evidence for the molecular ion of TOCG at m/z 412 in favor of the carbamoyl ester structure.

In addition to the hydrolysis of TOCG at pH > 12 to the hydantoin, this hydantoin was found to also undergo spontaneous first-order hydrolysis at pH > 12. To assay the levels of TOCG in urine as the hydantoin, a set of accurately timed calibration samples were employed in an assay protocol to take into account the spontaneous hydrolysis of the hydantoin.

Based on this analytical approach, the levels of TOCG were determined in three subjects both after an IV and oral dose of 200 mg tocainide HCl. The urinary excretion half-lives of TOCG of 13.86 hours and 13.33 hours, after an IV and oral dose respectively, were found to agree with literature values.

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2. Y-intercept of plot $\text{Log}\ \frac{dX_u}{dt}$ vs. $t_{mid} = \text{Log}\ k_e X_B^{\,o}$

where k_e = urinary excretion rate of tocainide.

$X_B^{\,o}$ = dose of tocainide. 149

3. $\text{Log}\ \frac{dM_u}{dt} = \text{Log}\ \frac{k_{mu} k_f X_B^{\,o}}{k_{mu} - K_E} - \frac{k_{mu}}{2.303}(t_{mid})$

the slope of the above equation as obtained by feathering $= \frac{-k_{mu}}{2.303}$

assuming $k_{mu} \gg K_E$ 151

4. $\text{Log}\ \frac{dM_u}{dt} = \text{Log}\ \frac{k_{mu}\ k_f\ X_B^{\,o}}{k_{mu} - K_E} - \frac{K_E\ (t_{mid})}{2.303}$

Y-intercept of plot $\log \frac{dM_u}{dt}$ vs. $t_{mid} = \text{Log}\ \frac{k_{mu} k_f X_B^{\,o}}{k_{mu} - K_E}$

where k_{mu} = urinary excretion rate of TOCG

$X_B^{\,o}$ = dose of tocainide in body

K_E = total elimination of tocainide 153

5. $X_u^{\infty} = \frac{X_B^{\,o} k_e}{K_E}$ 153

6. $\frac{M_u^{\infty}}{(F)\text{dose}} = \frac{k_f}{K_E}$ = ratio of dose excreted as metabolite in urine. 154

Symbols and Abbreviations

ACN	acetonitrile
BSTFA	Bis(trimethylsilyl)trifluoroacetamide
CBZCl	carbobenzyloxyl chloride
$CDCl_3$	deuterated chloroform
CD_3I	deuterated methyl iodide
CH_3I	methyl iodide
CI	chemical ionization
CNS	central nervous system
C.V. %	coefficient of variation
D_6-DMSO	deuterated dimethyl sulfoxide
DMSO	dimethyl sulfoxide
D_2O	deuterium oxide
D_2SO_4	deuterated sulphuric acid
EI	electron impact ionization
FAB	fast atom bombardment
GA	glucuronic acid
GC	gas chromatography
GCMS	gas chromatography interfaced with mass spectrometry
HBr	hydrogen bromide
HCl	hydrochloric acid
HFBA	heptafluorobutyic anhydride
HPLC	high performance liquid chromatography
HYD	3-(2,6-xylyl)-5-methylhydantoin
IR	infared spectroscopy
IV	intravenous

Symbols and Abbreviations (Contd)

J	coupling constant
K_E	elimination rate constant (tocainide)
k_e	urinary excretion rate constant (tocainide)
k_f	formation rate constant (TOCG)
k_{mu}	urinary excretion rate constant (TOCG)
M^+	molecular ion
m/z	mass to charge ratio
Mu^{∞}	total amount of TOCG excreted in urine
NaH_2PO_4	sodium phosphate monobasic
NMR	nuclear magnetic resonance
ODS	octadecylsilane
ppm	parts per million
PTFE teflon	polytetrafluoroethylene teflon
PVC	premature ventricular contractin
R_f	retention factor
RP	reverse-phase
TCF	trichloromethyl chloroformate
TIC	total-ion-chromatogram
TLC	thin layer chromatography
TMS	trimethylsilyl
TOC	tocainide
TOCG	tocainide carbamoyl ester glucuronide
t 1/2	half-life
UV	ultraviolet
Xu^{∞}	total amount of tocainide excreted in urine

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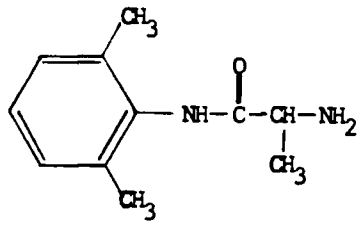
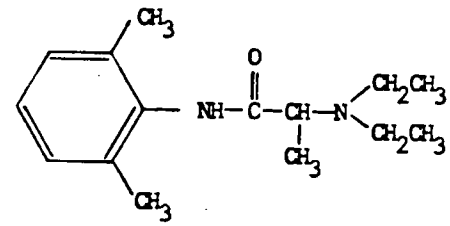
1 INTRODUCTION

A. Brief Overview of Tocainide

Tocainide, 2-amino-2',6'-propionoxylidide (I) is a new antiarrhythmic agent developed by Astra Pharmaceuticals. It is marketed in Canada as the hydrochloride salt (Tonocard ^{R}) in 400 mg and 600 mg tablets. The usual recommended dosing regimen is 400 mg to 600 mg every 8 hours up to 800 mg every 8 hours if necessary. At present, lidocaine (II) is the agent of choice for the treatment of acute myocardial infarction. However, lidocaine exhibits first-pass metabolism in the liver, thus rendering its oral bioavailability highly variable. Unlike lidocaine, tocainide is a primary amine analog which offers the advantage of both oral and intravenous routes of administration.



(I)



(II)

B. Electrophysiological Properties

According to Vaughan William's classification of antiarrhythmic drugs, class I compounds such as quinidine, procainamide, disopyramide, phenytoin and lidocaine, all possess a membrane-stabilizing action which interferes with sodium influx, thereby decreasing the rate of rise in phase zero of the cardiac action potential (1). In addition to this slowing of the depolarization rate, tocainide can also decrease impulse

excitability and automaticity in isolated dog Purkinje fibers (2). These electrophysiological properties all contribute to the antiarrhythmic activity of tocainide.

C. Pharmacodynamics

1. Clinical Efficacy

The pre-marketing clinical experience with tocainide in the United States can be summarized as follows. McDevitt and co-workers first reported a 60 % reduction in premature ventricular contractions (PVCs) in 5 out of 7 patients receiving more than 100 mg oral tocainide (3). Winkle and co-workers (4) subsequently demonstrated a 91 % ± 10 % reduction in PVCs in 11 out of 15 patients with oral tocainide therapy. Woosley and co-workers (5) later documented over a 75 % suppression of PVCs in 8 out of 12 patients with ventricular ectopic activity.

2. Efficacy in Refractory Arrhythmias

Tocainide was found to be effective in suppressing ventricular ectopic activity in 15 out of 19 patients refractory to conventional therapy with propranolol, quinidine or procainamide (6). Similar findings were reported by Ryan, W. (7), Haffajee,C.I. (8), and Moloney, J.D. (9).

3. Prophylactic Antiarrhythmic Therapy

Double-blind placebo studies were carried out to evaluate the usefulness of tocainide in the prophylactic therapy of ventricular arrhythmias after myocardial infarction (10-12). The results of these studies showed some preliminary beneficial effects of tocainide in abolishing ventricular tachycardia in patients with acute myocardial infarction, but further study in

this area would be required. At present, tocainide is proven to be an effective antiarrhythmic agent.

D. Pharmacokinetics and Pharmacodynamics

1. Bioavailability

In healthy human volunteers, oral tocainide hydrochloride was well absorbed with 90 to 100 % bioavailability (13). After a 400 mg dose of tocainide hydrochloride, peak plasma levels of 1.82 ug/mL were achieved in 1 to 2 hours in fasting subjects. Administration of the drug with food decreased peak levels and delayed the rate of absorption, but the overall bioavailability (13) was not affected.

2. Disposition Kinetics

Following zero-order infusion of 300 mg of tocainide over a 30 minute period, the peak plasma level was found to be 2.5 ug/mL. The biexponential plasma-drug concentration profile was characterized by a rapid distribution phase and a slower terminal elimination phase, with half-lives of 0.18 hour and 11.5 hours respectively (13). The apparent volume of distribution at steady state was found to vary between 1.46 L/Kg to 2.9 L/Kg in healthy subject (13,14). At steady state, 30 to 40 % of an administered dose was excreted unchanged in the urine with an average renal clearance of 70 mL/min (14,15).

3. Dose-response Pharmacodynamics

For tocainide, the plasma-drug concentration-antiarrhythmic response relationship was found to exhibit a large degree of intersubject variability (16). The reported effective therapeutic plasma concentrations of tocainide varied among

several studies (3,15,16), but the accepted effective range is considered to be from 4 to 10 ug/mL. An average steady-state plasma-tocainide concentration of 1.7 ug/mL was reached after an oral dosage regimen of 100 mg every 8 hours (15). Ventricular ectopic beats were suppressed in 70 % of the patients taking tocainide 400 mg orally every 8 hours with an average plasma concentration of 6 ug/mL. Doses greater than 800 mg every 8 hours were seldom tolerated due to adverse CNS effects (3).

4. Serum Protein Binding

Lalka (13) first reported that tocainide was 50 % bound to plasma proteins at clinically effective concentrations. Elvin (18) later reported a binding of only 4 to 12 % among 10 healthy human volunteers. In trauma patients with elevated alpha-1-acid glycoproteins, the percentage of drug bound was found to range from 10 to 20 %. Sedman (18) later examined the stereoselective binding of tocainide enantiomers. The extent of binding of the S(+)-tocainide enantiomer was slightly greater than that of the R(-)-enantiomer. Between the therapeutic concentration range of 4-12 ug/mL, there were no significant changes in the fraction bound for the two enantiomers, suggesting dose-independent binding. Nevertheless, the high free fraction of tocainide in plasma is not likely to create clinically significant binding displacement interactions.

5. The Effects of Metabolic Induction on Kinetics

Animal models were used to examine the effects of metabolic induction and inhibition on the disposition of tocainide (19,20). In the rat, with phenobarbital pretreatment,

a significant reduction in the area under the tocainide-plasma concentration versus time curve was observed after an intravenous dose. Pretreatment with SKF 525A, a known inhibitor of the enzyme responsible for glucuronide conjugation, was found to impair the elimination of tocainide as reflected by a reduction in clearance (19). However, studies in man failed to detect any alteration in the percent of unchanged drug recovered in the urine after pretreatment with phenobarbital, salicylamide, or clofibrate (21). Phenobarbital is a known enzyme inducer whereas salicylamide is a potent inhibitor of the glucuronidation of some drugs. Clofibrate is known to form metabolites that may be competitive inhibitors of the glucuronic acid conjugation pathway. However, none of these agents have affected the urinary excretion of the glucuronic acid conjugate of tocainide in man.

E. Stereoselectivity in Drug Metabolism

Tocainide is a chiral compound which can exist as two optically active enantiomers, R(-)- and S(+)-tocainide. It is well recognized that drug absorption, distribution, metabolism and excretion may all exhibit stereoselectivity or stereospecificity, particularly so when the process involves enzyme transformation.

Tocainide is marketed as a racemic mixture. In the literature, there are numerous examples of an stereoselective drug distribution and metabolism. An extensive review on stereochemical drug disposition was published by Jenner and Testa (23). For instance, the enantiomeric differences in distribution and metabolism of propranolol have been well

studied. In humans, racemic propranolol was shown to have a lower clearance than that of the vasoinactive R(+)-enantiomer (24). Stereoselectivity in aromatic hydroxylation, side chain hydroxylation, and glucuronidation were also reported for propranolol (25,26). Furthermore, stereoselectivity in the bioavailability of propranolol was also reported (25). The R(+)-enantiomer was found to have a lower oral bioavailability than the pharmacologically more active S(-)-enantiomer. Also, stereoselective binding of propranolol to alpha-1-acid glycoprotein was reported (27). The S(-)-propranolol was found to have a longer plasma half-life than the racemate in the rabbit (28). Recently, it was discovered that the vasoactive S(-)-enantiomer of propranolol has greater accumulation in the heart and brain of the rat (29). These studies have established the stereoselective differences in the disposition of the two enantiomers of propranolol in both humans and experimental animals.

In light of a possible stereoselective role in the disposition of tocainide, Gal and co-workers first studied the disposition of (R,S)-tocainide in rats and mice (30). S(+)-tocainide was reported in rat urine at higher levels than the (R)-antipode. However, in the mouse model, this observation was reversed. The enantiomers of tocainide have been subjected to pharmacological evaluation. The R(-)-enantiomer was found to be three times more potent than the S(+)-isomer in the chloroform-induced arrhythmic model in the mouse (31), but the difference in antiarrhythmic activities between the two enantiomers was smaller

in coronary-ligated dogs (31).

McErlane and Pillai (32) first reported the stereoselective disposition of tocainide in man. A similar study into the stereoselective disposition of tocainide in man revealed an elimination half-life for R(-)-tocainide and S(+)-tocainide to be 10.0 and 16.7 hours respectively (33). The half-life for tocainide carbamoyl glucuronide was found to be 10.3 hours suggesting that this glucuronic acid conjugate may have preferably formed from the R(-)-tocainide.

The stereoselective disposition of tocainide enantiomers into saliva was reported by Pillai *et al.*(34). The saliva levels of the individual enantiomers were measured after an intravenous dose of tocainide. The stereoselective disposition profiles of the enantiomers in saliva were compared with those in plasma and urine. Following an oral dose of 200 mg tocainide hydrochloride, the mean enantiomer concentration ratio of S(+)-tocainide/R(-)-tocainide in the plasma was 1.52 at 24 hours. Similar enantiomeric excretion profiles were observed in the urine. However, in saliva, the enantiomer concentration ratio was different in that the R(-)-enantiomer concentration was higher than that of the S(+)-enantiomer, and moreover, the levels of both enantiomers were higher than those found in the corresponding plasma samples. The authors suggested that a stereoselective pH-independent excretion process was responsible for the rapid distribution of the R(-)-enantiomer into the saliva.

F. Metabolism

In humans, 28 to 55 % of an oral dose of tocainide was excreted unchanged in the urine in 24 hours (35,36). It was suggested that another 15 to 30 % of the dose was metabolized via a novel pathway involving the addition of carbon dioxide to the primary amine nitrogen, followed by conjugation with glucuronic acid to form tocainide carbamoyl ester glucuronide (III) (35). Another 1 to 2 % of the dose was identified as a deaminated metabolite, lactoxylidide (IV), which could undergo further metabolism (36). A trace metabolite, tocainide oxime (V) has also been identified (37). The major metabolite, tocainide carbamoyl-O-β-D-glucuronide was reported to cyclize at pH > 12 to form 3-(2,6-xylyl)-5-methylhydantoin (VI) (35,37) as shown in Figure 1. However, in addition to the conjugation pathway, this hydantoin metabolite was also reported to form via a carbamic acid-urea intermediate as a metabonate in rats (38).

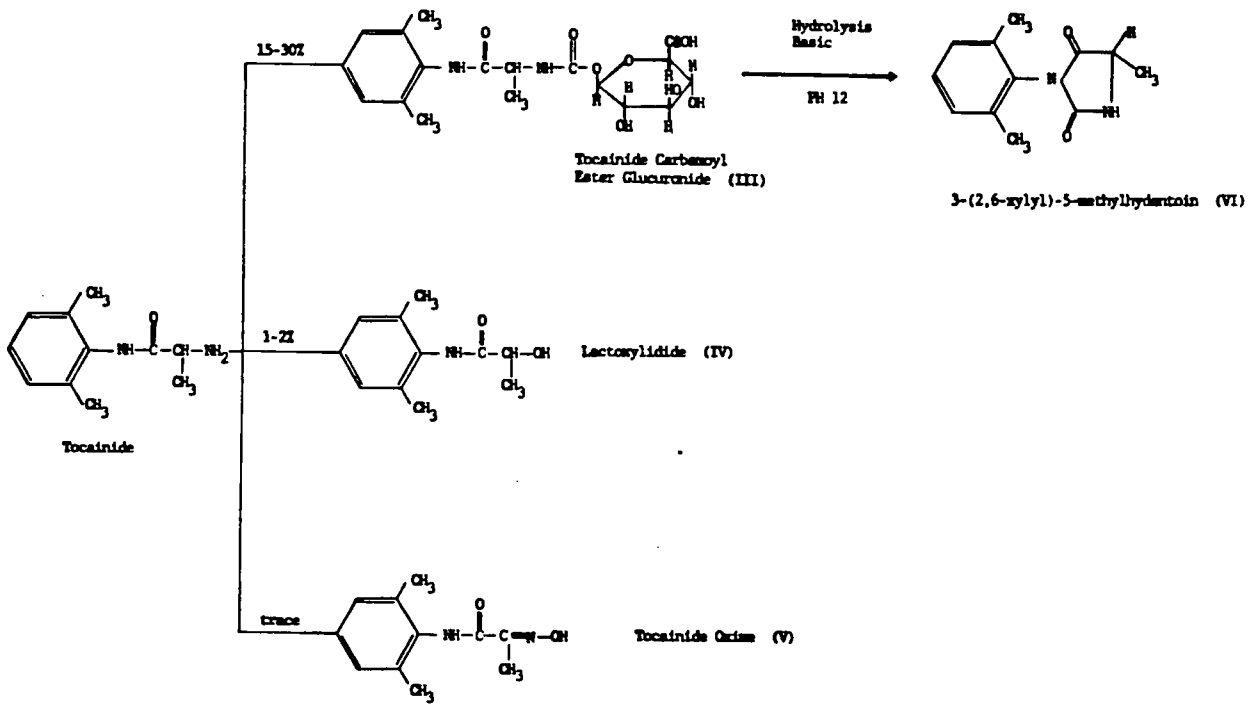
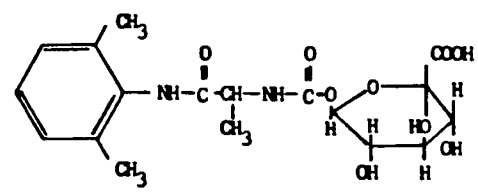


Figure 1. The metabolism of tocainide in humans.

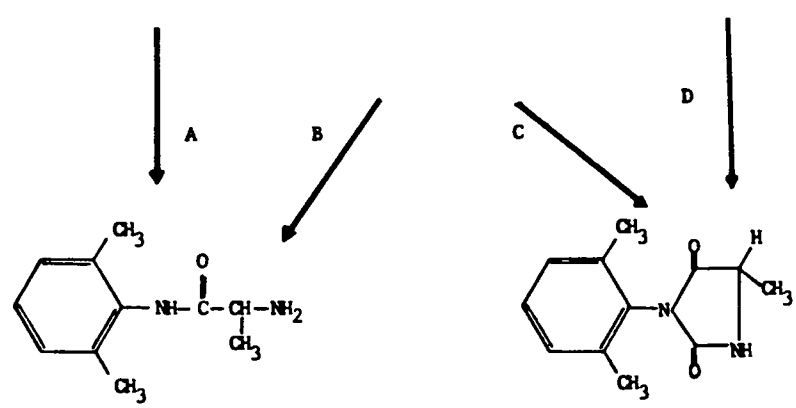
1. Observation of a Major Novel Metabolite of Tocainide

In 1979, Elvin and co-workers (35) identified a novel glucuronic acid conjugate of tocainide. The basis for elucidating the structure of this conjugated metabolite is described in Scheme 1.

A: Acid hydrolysis 1 M HCl/100°C.
B: Beta Glucuronidase hydrolysis.
C: Basic hydrolysis 1 M NaOH (pH 12).
D: Heat (100°C).



The Novel Tocainide Metabolite



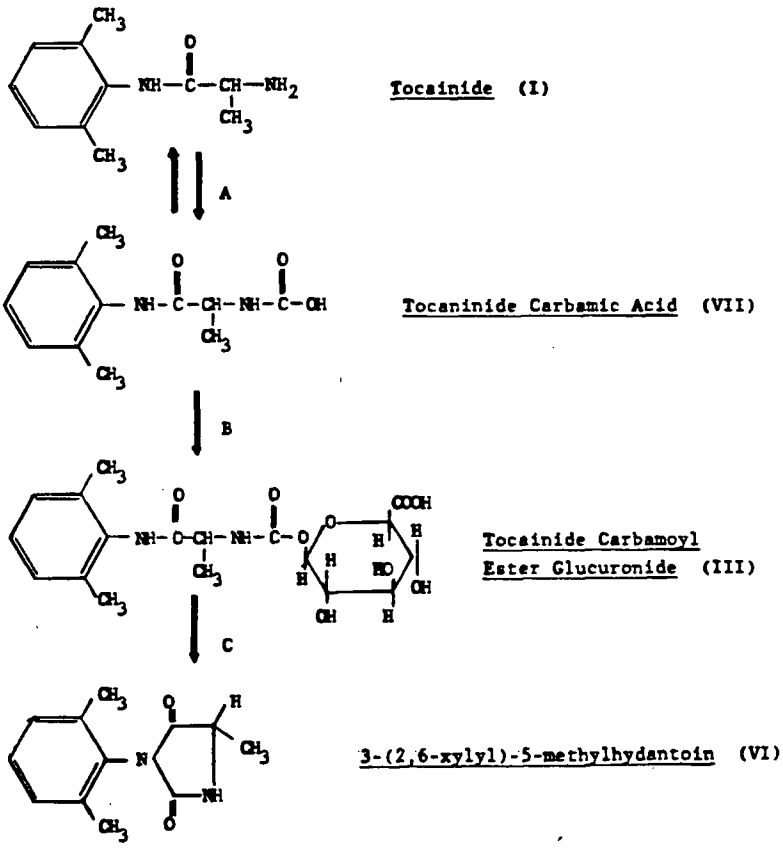
Tocainide Hydantoin

Scheme 1 : Various evidence for the presence of a novel tocainide carbamoyl ester glucuronide.

Acid hydrolysis (HCl) of urine samples produced free tocainide, probably released from a conjugated metabolite. Hydrolysis experiments with beta-glucuronidase produced similar amounts of free tocainide released likely from a glucuronic acid conjugate.

The initial interpretation of these data was to hypothesize a N-glucuronic acid conjugate of tocainide. However, the instability of this N-glucuronide in alkaline solution, forming a hydrolyzed product 3-(2,6-xylyl)-5-methylhydantoin, suggested that the structure of this novel metabolite contained a carbonyl on the primary amine possibly as a carbamoyl ester linkage with glucuronic acid.

Based on the proposed carbamoyl ester conjugated structure (III), tocainide must first be presented as a carbamic acid intermediate (VII) as shown in Scheme 2. This carbamic acid intermediate has not yet been isolated nor identified.



A: CO_2.

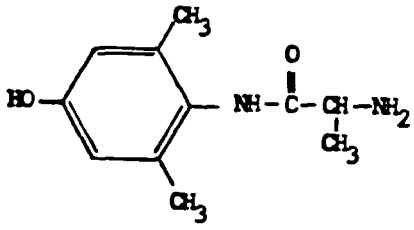
B: Uridine diphosphate/glucuronic acid.

C: NaOH.

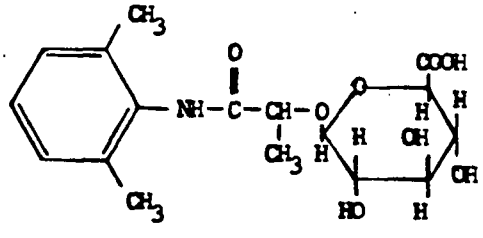
Scheme 2 : The proposed metabolic pathway leading to the formation of tocainide carbamoyl ester glucuronide.

Following this initial finding, an extensive investigation was undertaken with eight species of animals to establish an animal model for the toxicological evaluation of tocainide. However, the amount of tocainide carbamoyl glucuronide excreted in the urine, expressed as the percent of dose, was found to range from the lowest of < 1 % in the gerbil to the highest of 13 % in the rabbit (39). These figures are considered to be low compared to 30 to 40 % in humans. In humans, the elimination half-lives for tocainide carbamoyl ester glucuronide and lactoxylidide were reported to be 13.0 hours and 29.1 hours respectively (36). Although the lactoxylidide was known to accumulate during chronic therapy, both tocainide carbamoyl ester glucuronide and the lactoxylidide were shown not to possess antiarrhythmic activity. In addition, the two metabolites were shown not to possess cardiac nor CNS toxic effects (36).

From an animal study (37), four major metabolites of tocainide were identified, namely the 2,6-dimethylaniline (IV) from amide hydrolysis, an aldehyde adduct (VIII), an oxidative deaminated metabolite (IX), and the novel carbamoyl ester glucuronic acid conjugate of tocainide (III), as shown in Figure 2.



(X)



(XI)

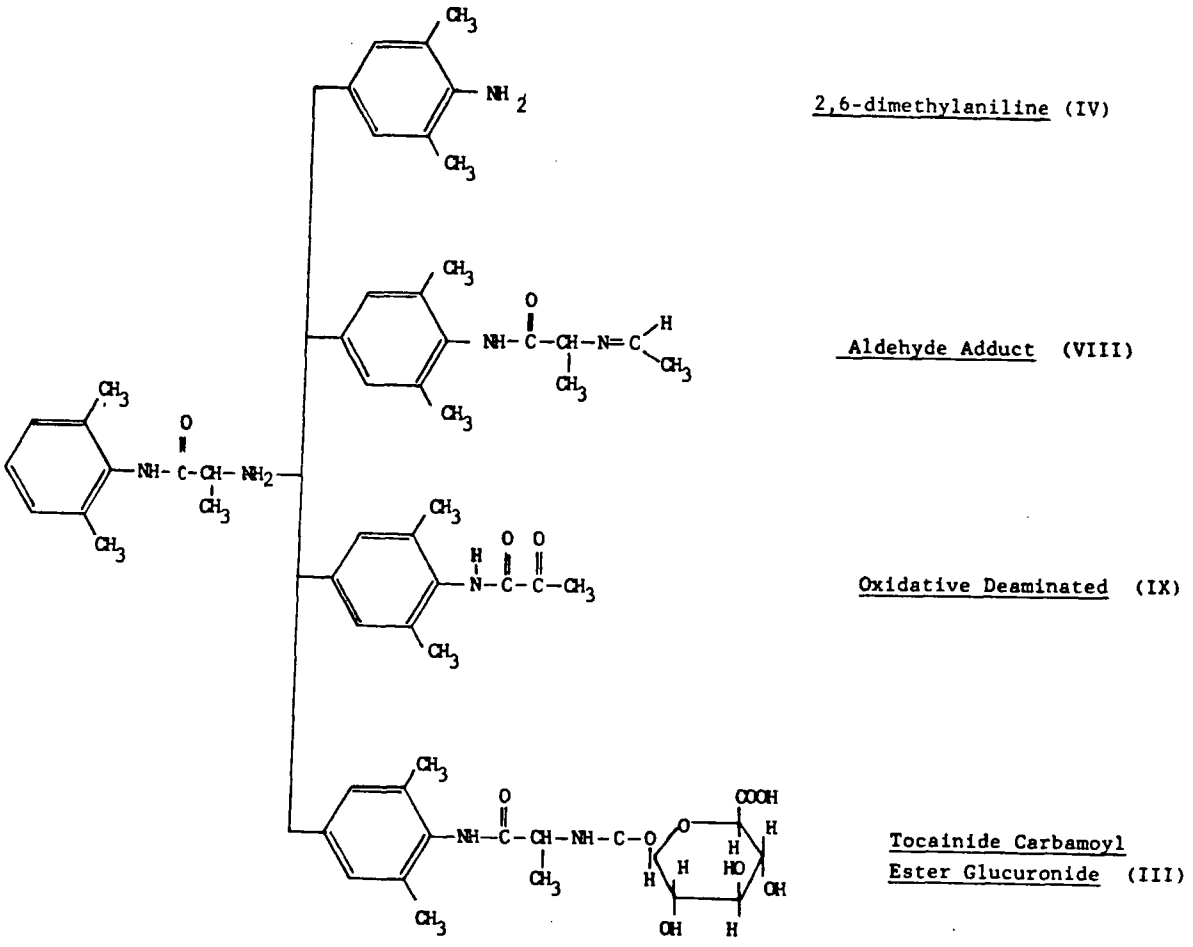


Figure 2 : The metabolism of tocainide in rats.

Three of these metabolites: the aniline, the ethanimine and the diketone, were not found in human urine. In addition to the identified metabolites in human urine, we suspected the presence of a 4'-hydroxypropionoxylidide (X) metabolite and possibly an

ether glucuronic acid conjugated metabolite (XI) being formed from the lactoxylidide.

2. Analysis of Glucuronic Acid Conjugates

It is generally recognized that glucuronidation is a stereoselective process. To date, only one study has used an indirect beta-glucuronidase enzyme hydrolytic method to assay the kinetic profile of the tocainide carbamoyl ester glucuronide (35). The disposition half-life of this glucuronide was found to be comparable to that of the R-(-)-enantiomer, suggesting that the glucuronide may have preferentially formed from the R-(-)-enantiomer of tocainide (35). However, analysis of the enantiomeric composition of the intact glucuronide of tocainide has not been reported.

The quantitative analysis of glucuronides usually involves acid or enzyme hydrolysis by beta-glucuronidase, followed by analysis of the liberated aglycone. This analytical approach is, however, subject to a number of drawbacks:

1. The hydrolytic reaction may not be specific for glucuronic acid conjugates; it usually requires verification by the use of a competitive enzyme inhibitor, saccharic acid 1,4-lactone.
2. The rate and extent of the hydrolytic reaction is dependent on the source of the enzyme, thus giving poor reproducibility of data between experiments.
3. The procedure is time consuming (up to 24 hours incubation) and does not distinguish between mono- and di-glucuronides.

4. The procedure may lack day to day reproducibillity due to a possible variation in enzyme activity with storage.

Due to the limitation of an enzyme hydrolytic assay, it was necessary to develop an assay for the direct resolution of the diasteromeric ester glucuronide of tocainide, and to examine the disposition profile of this glucuronide.

The metabolism and stereoselective disposition of tocainide may be effectively studied with the use of a deuterated pseudoracemic dose of tocainide (equal proportions of deuterated S(+)-enantiomer and unlabelled R(-)-enantiomer). Plasma, urine and saliva samples obtained from such a study in human subjects can then be used to study possible metabolic chiral inversion, *in vivo* racemization of the intact drug, metabolic substrate stereoselectivity and stereoselectivity in drug transport into saliva. Furthermore, the use of pseudoracemic dosing of tocainide will assist the identification of drug metabolites.

Hence, the initial aims of this project were three-fold:

1. Synthesis of enantiomerically pure R(-), or S(+)-3',4',5'-trideuterated tocainide for bioavailability measurements and metabolite isolation/identification using the deuterium-labelled compound.
2. Develop necessary analytical techniques to separate enantiomeric or diastereomeric metabolites of tocainide and to examine their kinetic profile.
3. Isolate the tocainide glucuronide and elucidate the intact structure of this novel metabolite.

3. Speculative Structures of the Novel Tocainide Glucuronide

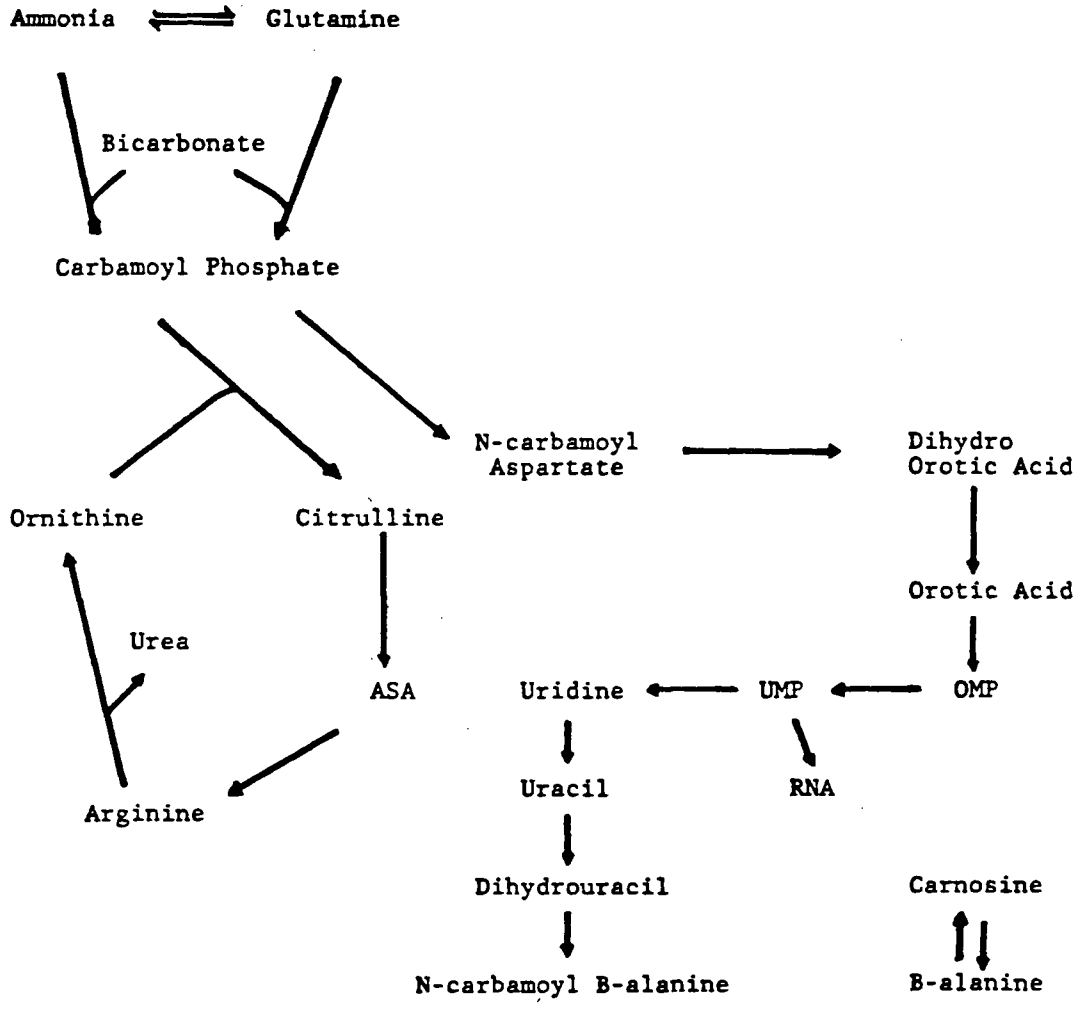
The structure of 3-(2,6-xylyl)-5-methylhydantoin (VI) indicated that the formation of the novel glucuronide involved the incorporation of a carbonyl group onto the primary amine nitrogen of tocainide followed by glucuronic acid conjugation. The true identity of this carbamoyl intermediate has not been established.

Theoretically, the terminal primary amine nitrogen can be carbamoylated through a number of biochemical pathways.

a. N-carbamoylation

High levels of N-carbamoyl-beta-alanine and N-carbamoylaspartate were found in the urine of patients with ornithine carbamoyl transferase deficiency (40). Scheme 3 outlines the role of carbamoylphosphate in the metabolism of urea.

Tocainide possesses an alanine moiety with a primary amine and can theoretically be N-carbamoylated by the enzyme carbamoylphosphate followed by glucuronic acid conjugation.



Scheme 3 : Pathways of ammonia metabolism. ASA, argininosuccinic acid; OMP, orotidine monophosphate; IMP, uridine monophosphate.

b. Carbonate Equilibrium

Carbon dioxide is present in the body as bicarbonates, carbonates and dissolved carbon dioxide gas. The formation of carbonates has been studied in human red cells and plasma as well as animal muscle and brain tissues (41). Carbon dioxide can react with the unionized amino group of amino acids to form carbonates as shown in Scheme 4:

$$ToC-NH_2 + CO_2 \rightleftharpoons ToC-NHCOO^- + H^+ \; ; \; k_c = \frac{[ToC-NHCOO^-]\,[H^+]}{[ToC-NH_2]\,[CO_2]}$$

$$ToC-NH_3^+ \rightleftharpoons ToC-NH_2 + H^+ \; ; \; k_a = \frac{[ToC-NH_2]\,[H^+]}{[ToC-NH_3^+]}$$

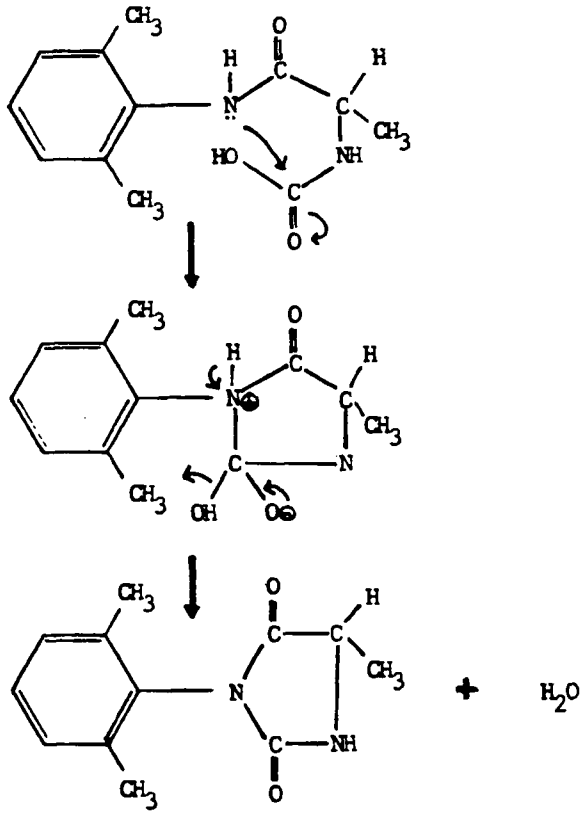
where k_c = carbamate equilibrium constant.

k_a = ionization constant of the amino group.

Scheme 4 : The theoretical equilibrium reactions of tocainide carbamate.

This carbon dioxide-carbonate equilibrium reaction has been studied in human plasma (41) with glycine and glycylglycine (pKa of 8.0 for both compounds). Tocainide, having a pKa of 7.8, may theoretically combine with carbon dioxide depending on the pH and carbon dioxide concentration of the metabolizing tissue.

In agreement with this theory, Venkataramanan and Axelson (38) demonstrated the formation of a small amount of the hydantoin (VI) when an aqueous solution of tocainide was incubated at 100 ^{o}C for 1 hour with solid carbon dioxide, possibly through the formation of carbamic acid intermediate followed by cyclization with the xylidide nitrogen as shown in Scheme 5:



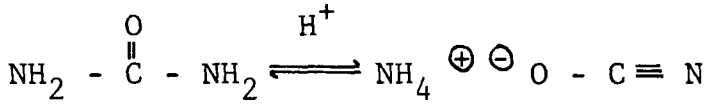
Scheme 5 : The mechanisms of carbamic acid cyclization to 3-(2,6-xylyl)-5-methylhydantoin.

C. Tocainide-urea Adduct Formation

Venkataramanan *et al.* (38) first observed the degradation of tocainide in the presence of urea in urine samples. However, no attempts were made to identify the degradation products. Our preliminary results from urea-tocainide hydrolysis experiments revealed the formation of the hydantoin (VI) in the presence of 8.0 M urea at 100 ^{o}C. The following sections will describe the two possible chemical pathways which can lead to the formation of this hydantoin through a reaction of tocainide with urea.

i. Urea-adduct Formation

An aqueous solution of urea can undergo isomeric transformation to ammonium cyanate as shown in Scheme 6:



Scheme 6 : The isomeric transformation of urea.

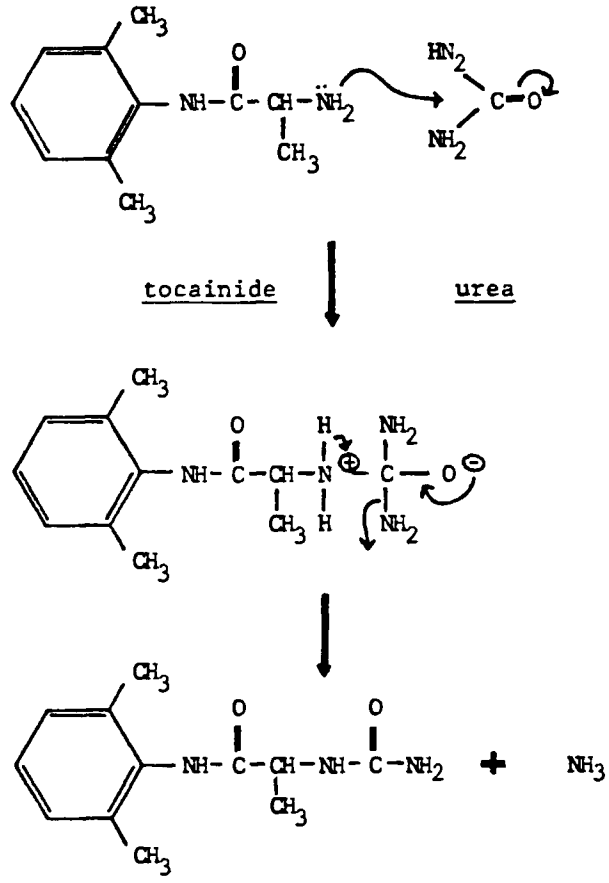
This spontaneously reversible reaction was studied by Dirnhuber & Schutz, 1948 (42). It was found that equilibrium is reached within 30 minutes at 100 ^{o}C, while at body temperature (38 ^{o}C), equilibrium is reached after several days. Also, in diluted urea solution, the reaction thermodynamically favours the dissociated cyanate species.

Based on this reaction, ammonium cyanate can break down at acidic urinary pH giving rise to carbon dioxide. The resultant carbon dioxide can combine with the amino group of tocainide to

form the carbamic acid intermediate as described.

ii. Urea Condensation with Tocainide

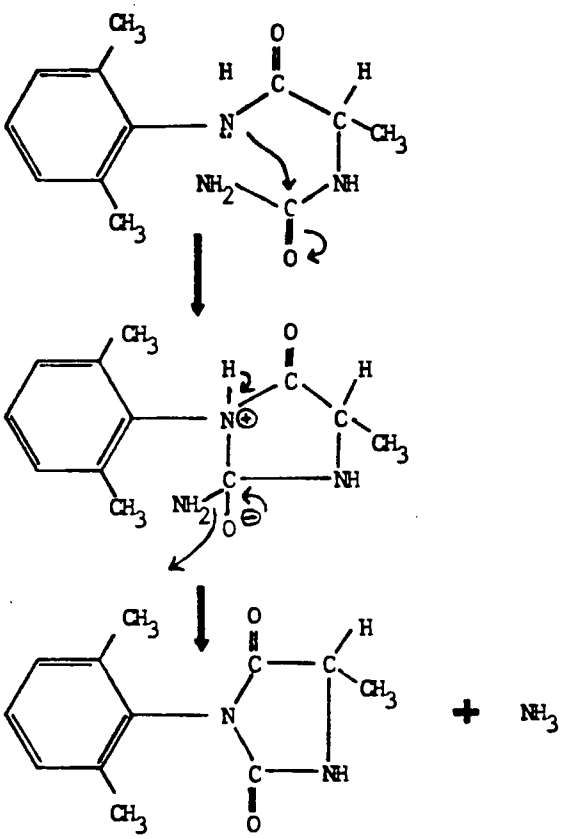
Alternately, tocainide can condense with urea to form a carbamate intermediate as proposed in Scheme 7:



tocainide ureide

Scheme 7 : The mechanisms of tocainide-ureide formation.

This tocainide ureide was found to undergo cyclization under basic conditions to form (VI). The mechanisms for the tocainide ureide cyclization are shown in Scheme 8.



Scheme 8 : The mechanisms of tocainide-ureide cyclization to 3-(2,6-xylyl)-5-methylhydantoin.

4. Drug Glucuronidation

The metabolic pathway and the types of xenobiotics giving rise to glucuronic acid conjugates have been extensively reviewed (43,44). According to the aforementioned metabolic

pathways leading to the formation of a phase I tocainide metabolite intermediate followed by GA conjugation, the intact structure of TOCG can either be a N-ureide glucuronide, (-NHCO.NH-GA), or a carbamoyl ester glucuronide, (-NHCO.O-GA).

a. Acyl-O-Glucuronides (Ester Glucuronides)

These conjugates are known to be unstable at alkaline conditions. The aglycone can be aryl, primary, secondary or tertiary aliphatic or heterocyclic compounds as in salicylic acid, indomethacin, iodopanoic acid, trimethylacetic acid and nicotinic acid respectively (43). The benzoate and salicylate ester glucuronides are known to hydrolyze completely when incubated with a pH 12 buffer at room temperature for 5 to 30 minutes (45).

b. N-glucuronides

The xenobiotics giving rise to N-glucuronic acid conjugates are summarized in Table 1:

Table 1 : Examples of various functional groups known to give rise to N-glucuronic acid conjugate in man.

Functional Groups	Examples
Aromatic amine	aniline
Azaheterocycle	sulphisoxazole
Carbamate	meprobamate
Sulphonamide	sulphadimethoxine
Hydroxylamine-N-	N-hydroxy-2-acetamidofluorene
Tertiary aliphatic	Cyproheptadile
Urea	Dulcin

The carbamate glucuronides (-NHCO.O-GA) are more stable than the amine conjugates (-NH-GA) at an acidic pH. Certain

N-glucuornides have been formed spontaneously *in vitro* through condensation of an aglycone with glucuronic acid at physiological pH and temperature. Meprobamate-N- (46), urethane-N- (47), and many arylamine-N- (48) and alkylamine-N-glucuronides (49) have been synthesized *in vitro* using a simple admixture procedure.

The theme of this thesis will centre on isolation of the tocainide glucuronide and on elucidation of the structure of a theoretically possible N- ureide glucuronide (-NHCO.NH-GA) or the proposed carbamoyl ester glucuronide (-NHCO.O-GA). Attempts will also be made to identify possible new conjugated or non-conjugated metabolites.

G. Toxicology

At the onset of this project, toxicological studies had not revealed any major toxic effects from tocainide nor from its two major metabolites in humans. A review report indicated that the common adverse effects of tocainide were gastrointestinal in nature. Other adverse effects included sporadic cases of lupus erythematosus (50) and interstitial pneumonitis (51) and the recently reported cases of blood dyscrasias (52). In early 1985, reports were released from Astra Pharmaceuticals summarizing the possible haematological toxicities which have resulted from the use of tocainide world wide. The first report was prepared in January 1984. At the time, 40 cases of haematological disorders (leucopenia and agranulocytosis) were reported from a 25,000 to 30,000 patient population (incidence of 1:750). In November 1984, with the approval of tocainide in the United States and in over 20 other countries, the number of cases of

tocainide related haematological toxicities reached a total of 125. In light of the increasing incidence of blood dyscrasias, it was important to study the metabolism of tocainide with reference to the toxicity of its metabolites.

As a result of the high incidence of agranulocytosis toxicities, Astra Pharmaceuticals withdrew tocainide from general use thus prohibiting the use of this drug in healthy human volunteers.

Due to the sudden change in drug regulation and the withdrawal of support from Astra Pharmaceuticals to continue basic research on tocainide, the initial aims of this project have since been revised as follows:

1. Synthesis of R(-) or S(+)-3',4',5'-trideuterated tocainide.
2. Attempt identification of new metabolites of tocainide from human urine samples collected during previous pharmacokinetic studies.
3. Isolation of the intact tocainide glucuronide and elucidation of the intact structure of this metabolite.
4. Large-scale isolation of the tocainide glucuronide for agranulocytosis toxicity studies.

H. Agranulocytosis

On the market today, numerous drugs are associated with granulocytopenia. Phenothiazines are widely used antipsychotic agents that can cause reversible granulocytopenia (53). Antithyroid medications, propylthiouracil and methimazole can

cause granulocytopenia in 0.3 % to 0.6 % of patients (54). The sulfonamides were reported to be agranulocytogenic through an immunological mechanism (54). The analgesic and anti-inflammatory drug aminopyrine was reported to cause granulocytopenia (54,55). Phenytoin was also reported to cause a 2 % incidence of granulocytopenia (53). The pencillins and cephalosporins were also occasionally connected with granulocytopenia in longterm, high-dose therapy (54).

In all cases of drug-induced granulocytopenia and neutropenia, the etiology of the disease can be explained by three general mechanisms (55):

1. The generation of reactive toxic metabolites of the structure R-N.
2. Interaction of the active metabolites with neutrophils and macromolecules.
3. Formation of antibodies to neutrophils.

The metabolism of aminopyrine can serve as an example to illustrate the generation of toxic metabolites. Aminopyrine is an enzyme inducer which can induce cytochrome P-450, cytochrome P-448 monooxygenases, and glucuronyl transferase. Aminopyrine was postulated to be metabolized through a two-step monooxygenase reaction followed by glucuronyl conjugation to yield a reactive R-N metabolite. This metabolic pathway, involving endogenous monooxygenase enzymes followed by a conjugation reaction with either glucuronyl transferase or sulfontransferase, is believed to be common to phenylbutazone, chlorpromazine, promazine, and chloramphenicol. Because of high chemical reactivity, the

glucuronic acid and sulphate conjugates of these drugs are labile molecules which can produce the reactive electrophilic structure R-N.

This species of reactive metabolite R-N generated from agranulocytotic drugs is capable of interacting with nucleophiles of protein molecules, thereby eliciting an immune response with the formation of antibodies. The R-N reactive molecules can also react with the nucleophiles of DNA and RNA disrupting DNA replication and protein synthesis. Bone marrow cells, being one of the most reactive cells undergoing replication in the body, are likely to suffer the most detrimental effects. With our knowledge of the reactive nature of the carbamoyl ester glucuronide of tocainide at basic pH, and the recent reports of agranulocytosis in the literature, we propose to examine whether the reported agranulocytosis toxicity of tocainide was elicited by the tocainide glucuronide.

2 EXPERIMENTAL

A. Chemicals and Materials

Chemicals are reagent grade (unless specified). The following suppliers are listed alphabetically:

1. Aldrich Chemical Co. (Milwaukee, Wisconsin, USA)

   D-alanine, benzyl chloroformate (carbobenzyloxyl chloride), 2,6-dimethylaniline, N-carbobenzyloxyl-D-alanine, Trichloromethyl chloroformate, 2,4,6-trimethylaniline, calcium hydride, sodium hydride (50% in mineral oil), sodium glucuronic acid, phosphoric acid, naphthoresorcinol (1,3-dihydroxynaphthalene)

2. Analytical International Inc. (Harbor city, CA, USA)

   Bond-elute^{R} C-18 extraction columns

3. Applied Science Laboratories. (State College, Pennsylvania, USA)

   Silar-10c liquid phase

4. Aristar^{R} BDH Chemical. (Poole, England)

   Sodium hydroxide (analytical grade)

5. Astra Pharmaceuticals Canada Ltd. (Mississauga, Ontario, Canada)

   Tocainide

6. Beckman Instruments Inc. (Berkeley, CA, USA)

   C-18 ODS 5um 4.6mm x 25cm HPLC column

7. BDH Chemicals. (Vancouver, B.C., Canada)

   HPLC grade solvents: methanol, water, acetonitrile, hexane, 2-propanol, methylene chloride.
   Reagent grade: chloroform, anhydrous sodium sulfate, ether, ethanol-100, toluene, dimethylsulfoxide, sodium phosphate monobasic, acetic acid

8. BrownLee Labs Inc. (Santa Clara, CA, USA)

   Ramnin 0.45 um on-line filter, ramnin C-18 guard column

9. Chromatographic Specialties. (Brockville, Ont., Canada)

   Chromosorb W-HP

10. Eastman Organic Chemicals. (Rochester, N.Y., USA)

    Dicyclohexylcarbodiimide

11. Fisher Scientific Co. (Fair Lawn, New Jersey, USA)

    Methyl iodide, 30-32% hydrogen bromide in acetic acid

12. J.T. Baker Ltd. (Philipsburg, New Jersey, USA)

    Silica gel (flash chromatography)

13. Mandel Scientific Company. (Nisku, Alberta, Canada)

    XAD-2 resin, SE-30 (bonded) 0.25um x 50m fused silica capillary column, Chirasil-Val^{R} 0.25um x 50m fused silica capillary column

14. Merk Sharp & Dohme. (isotopes) (Montreal, Quebec, Canada)

    Deuterated methyl iodide, D -DMSO

15. Parr Pressure Reaction Apparatus. (Moline, Illinois, USA)

    Variac^{R} pressure reaction vessel

16. Pierce Chemical Co. (Rockford, Illinois, USA)

    Pyridine, BSTFA

17. Sigma Chemical Co. (St. Louis, Missouri, USA)

    HFBA, beta-glucuronidase (glucurase), saccro-1,4-lactone, acetic anhydride, deuterium oxide (99.8 atom% D), deuterated chloroform (99.8% atom% D), deuterated sulphuric acid (99+ atom% D), L-alanine,

18. Supelco Ltd. (Bellafonte, Pennsylvania, USA)

    Dexsil-300 liquid phase

19. Union Carbide Canada Ltd. (Toronto, Ont., Canada)

    Helium, nitrogen, hydrogen, medical air

20. Whatman Inc. (Clifton, NJ., USA)

    Partisil-10 magnum-9 preparative column, 20 x 20cm KC-18F thin layer plates

B. Instrumentation

1. Fast Atom Bombardment

All FAB Spectra were recorded at the mass spectrometry facility in the Department of Chemistry, University of British Columbia.

Samples were analyzed either in glycerol or thioglycerol matrix with a Kratos/AeI Ms9 instrument. The accelerating voltage and the FAB were at 6 KV and 7 KV respectively. Collision gas was Xenon and the samples were analyzed on a copper probe tip.

2. Gas Chromatography Mass Spectrometry

GCMS analysis was carried out using either the Varian Magnetic-sector or the Hewlett-packard quadrupole instrument.

a. Varian Magnetic-sector GCMS

A Hewlett-packard 5700A Gas Chromatograph was interfaced to a Varian Mat-111 Mass Spectrometer. The electron-impact spectra were recorded on a Varian 620L computer and plotted on a chart-recorder. The filament current was 300 uA with an electron beam energy of 70 eV, ion source pressure at 8 X 10^{-6} torr, and scan from a cycle of 15 to 500 mass units every 5 seconds. Total-ion-chromatograms were obtained from a plot of m/z 50 to 500.

The gas chromatograph was operated in the splitless mode with GC inlet, injection port and separator temperature all at 250 ^{o}C. The following column conditions were employed:

1. A 3 % Silar 10C liquid phase was coated on chromosorb W-HP before packing into a 2 mm internal diameter X 2 m glass column. The oven temperature

was programmed from 150 to 270 ^{o}C at 8 ^{o}C/min. The column helium flow rate was 20 mL/min.

2. A 3 % Dexsil-300 liquid phase was coated on chromosorb W-HP before packing into a 2 mm internal diameter X 2 m glass column. The oven temperature was programmed from 150 to 300 ^{o}C at 8 ^{o}C/min. The column helium flow rate was 20 mL/min.

b. Hewlett-Packard Quadrupole GCMS

An HP 5880A gas chromatograph was interfaced with an 5987A mass spectrometer. Electron impact spectra were obtained with a electron beam energy at 70 eV and emission current at 300 uA. TIC were obtained from a plot of m/z 120 to m/z 800. Ion source temperature was at 240 ^{o}C. Chemical ionization was carried out using methane as the reagent gas with a source pressure of 1.45×10^{-4} Torr and emission current of 300 uA. Direct probe experiments were carried out either in the EI or CI mode.

The gas chromatograph was operated in the split mode with inlet temperature at 250 ^{o}C. The following chromatographic columns were employed:

1. A capillary SE-30 (bonded-phase), 0.25 mm X 50 m fused- silica (HP) was used with an oven temperature program from 40 to 300 ^{o}C at 30 ^{o}C/min. The column helium flow rate was 1 mL/min.
2. A Capillary Chirasil-Val^{R} , 0.25 mm X 50 m fused-silica column was used with an oven temperature program from 40 to 190 ^{o}C at 30 ^{o}C/min. Column helium flow rate was 1 mL/min.

3. Proton Nuclear Magnetic Resonance

The 80 MHz spectra were recorded on a Bruker WP-80 or a Varian XL-100 spectrometer. The 400 MHz experiments were carried out using a Bruker WP-400 spectrometer. All spectra were recorded at the NMR facility in the Department of Chemistry.

4. Gas Chromatography

Routine gas chromatographic analysis was carried out using either a Hewlett-Packard 5880A or 5830 GC with a flame ionization or electron-capture detector(^{63}Ni). The chromatographic columns used were as outlined for the quadrupole GCMS experiments. The flame ionization detector and the electron capture detector were operated at 275 ^{o}C and 300 ^{o}C respectively.

5. Liquid Chromatography

Liquid chromatographic analysis was carried out using a Hewlett-packard 1082B Liquid Chromatograph, with an Altex Variable wavelength detector and a 7000 Ultrorac^{R} automatic fraction collector. A C-18RP Partisil-10, magnum-9 preparative column was used with a Rainin 0.45 um on-line filter and a Rainin C-18 guard column. Flow rates were ranging from 2.3 to 3.5 mL/min with a column pressure between 13,790 KPa (2000 psi) to 17,237 KPa (2500 psi).

6. Infared Spectroscopy

IR spectra were obtained with sodium chloride disks as a nujol mull using an Unicam SP-1000 spectrometer.

7. Melting Point Determination

Melting Points were determined on a Thomas-Hoover^{R} capillary melting point apparatus and were uncorrected.

C. Synthesis of 3',4',5'-[$^2H]_3$-2-amino-2',6'-propioxylidide

The synthesis followed a modified method reported by Byrnes (31). The synthetic steps were similar to those utilized for peptide synthesis (62). The procedure involves the use of N-carbobenzyloxyl-D or L-alanine and 3,4,5-trideuterated-2,6-dimethylaniline in the presence of dicyclohexylcarbodiimide to form N-carbobenzyloxyl tocainide. The carbobenzyloxyl group was then removed by HBr/acetic acid yielding tocainide hydrogen bromide salt.

1. Synthesis of S(+)-3',4',5'-[$^2H]_3$-tocainide.

Initial attempts to synthesize the trideuterated tocainide were carried out using non-deuterated 2,6-dimethylaniline and commercially available N-carbobenzyloxyl-D-alanine.

N-Carbobenzyloxy-D-alanine (5.0 g, 0.0224 mole) and 2,6-dimethylaniline (2.72 g, 0.0224 mole) were dissolved in 20 mL of methylene chloride. Dicyclohexylurea was filtered off with suction and the filtrate was dried under vacuum to yield 3.08g of N-carbobenzyloxyl tocainide. A solution of 30 to 32 % hydrogen bromide in acetic acid was added in sufficient quantities to dissolve the tocainide derivative followed by vacuum removal of excess hydrogen bromide at room temperature. Then ether was added in small quantities for the crystallization of tocainide hydrogen bromide salt. The ethereal solution was left at 0 ^oC overnight to yield 2.83 g of tocainide hydrogen bromide salt (0.0147 mole, 65 % yield).

The hydrogen bromide salt was removed by extraction of

tocainide base in methylene chloride, dried under nitrogen, reconstituted with ethanol-100 and acidified with ethanol/hydrochloric acid solution. The salt was crystallized in ether at 0 ^{o}C overnight to yield 0.36 g tocainide hydrochloride salt.

Gas Chromatography (Chirasil-Val ^{R} column):

S(+)-tocainide (61 %),

R(-)-tocainide (39 %)

Melting Point:

266 ^{o}C uncorrected (ref: 264.5 - 265.5 ^{o}C) (31).

Infared (Nujol mull):

3300 cm^{-1}(NH stretch), 1540(C=O stretch), 1660(C=C stretch), 1150, 1090, 970, 780 (tri-substituted vicinal aromatic).

Mass Spectrum (GCMS):

m/z 192 (M^{+} 5 %), 44(100), 121(8), 105(5), 91(3).

2. Synthesis of 3,4,5-[^{2}H]$_3$-2,6-dimethylaniline

Deuteration of 2,6-dimethylaniline was carried out using a modified perdeuteration technique reported by Frischkorn (51). A sample of 2,6-dimethylaniline (2 mL, 0.016 mole) was added to 2 mL of D_2O (0.1 mole) followed by a sufficient amount of D_2SO_4 to form the aniline sulfate salt. The mixture was refluxed in a single condenser apparatus for 2 hours before the residual D_2O was removed by rotatory evaporation over a water bath at 100 ^{o}C. The dry aniline salt was reconstituted with 5 mL of D_2O/D_2SO_4 (pH 1) and heated in a Variac $^{(R)}$ reaction vessel for 18 hours at 250 ^{o}C with 4137 KPa (600 PSI)

cold nitrogen. The excess pressure in the reaction vessel was released periodically during the experiment to maintain the pressure below 13790 KPa (2000 PSI). After heating, the reaction mixture was basified to pH 10 with 1.0 M sodium hydroxide, extracted with chloroform, dried over anhydrous sodium sulfate, filtered and evaporated to dryness at room temperature, yielding 1.8 mL (90 %) of a dark brown liquid.

Mass Spectrum (TIC):

unresolved 3 major peaks:

SCAN 51: (1) m/z 126 (M^+ 100 %), 100(95), 80(35).

SCAN 61: (2) m/z 125 (M^+ 100 %), 109(55), 110(55), 80(20).

The reaction product in chloroform was then washed 3 times with 2 mL portions of water (for exchange of the amine deuterium atoms) before it was dried over anhydrous sodium sulfate, filtered, and evaporated to dryness over nitrogen.

The reaction product was then purified by distillation over an oil bath. The fraction collected at 218 ^{o}C was a light brown liquid (1.1 mL, chemical yield 50 %).

Mass Spectrum (TIC):

singular component:

SCAN 48: (1) m/z 124 (M^+ 100 %), 109(60), 80(20).

80 MHz-[^{1}H]-NMR ($CDCl_3$):

δ ppm, 2.2s(6H, $-CH_3$), 3.5s(2H, $-NH_2$),

6.65s(1H, para-aromatic), 6.95s(2H, meta-aromatic).

D. Attempted Synthesis of 1-[^{2}H]$_3$-2-amino-2',6'-propioxylidide

Due to damage of the Variac^R reaction vessel, the original synthetic route for synthesizing 3',4',5'-[^{2}H]$_3$-tocainide was abandoned in favor of an alternative method for the synthesis of 1-[^{2}H]$_3$-2-amino-2',6'-propioxylidide. This approach involves the synthesis of N-carbobenzyloxy-(D)-[^{2}H]$_3$-alanine from commerically available [^{2}H]$_3$-alanine and carbobenzyloxy chloride (CBZCL) followed by reaction with 2,6,-dimethylaniline to form 1-[^{2}H]$_3$-2-amino-2',6'-propioxylidide as described previously.

1. Synthesis of N-carbobenzyloxy-(+)-D$_3$-alanine

Initial attempts were carried out using unlabelled D-alanine. D-alanine (2.62 g, 0.0294 mole) was dissolved in 200 mL of saturated sodium bicarbonate solution with stirring. Carbobenzyloxy chloride (14.18 mL, 0.0294 mole) was added with stirring and under ice cooling. The reaction mixture was stirred under ice until all CBZCl was dissolved (6.0 hours). The reaction was terminated by the dropwise addition of concentrated hydrochloric acid until the solution is acidic to litmus paper. The white precipitate of N-CBZ-alanine was filtered and washed with 0.01 M hydrochloric acid before being vacuum-dried at room temperature, yielding 5.7 g of product (87.9 % chemical yield).

Melting Point:

71 - 72 ^{o}C (ref. 79.5 - 80 ^{o}C, Sigma Chemical Co.).

After crystallization in chloroform/ether at 0 ^{o}C for 24 hours, the reaction product yielded 3.2 g white powder (55.8 %).

Melting Point:

79 ^{o}C. (ref. 79.5 - 80 ^{o}C, Sigma Chemical Co.)

Infrared (Nujol mull):

1700 cm^{-1}(C=O stretch), 3000(O-H stretch), 3350(N-H stretch), 1540(N-H stretch), 1380(O-H stretch).

E. Synthesis of Potential Tocainide Metabolites

1. Synthesis of 2-Ethanimino-2',6'-propioxylidide

Tocainide base (1.0 mg, 5.2 x 10^{-6} mole) was added to 1 mL (2.3 x 10^{-5} mole) acetyl aldehyde at room temperature in a 5 mL reaction vial. The reaction proceeded at room temperature for 15 minutes before excess aldehyde was removed by drying under nitrogen. The reaction product was reconstituted with methanol for GCMS analysis.

Infrared (Nujol mull):

1680 cm^{-1}(C=O stretch), 3300(N-H stretch), 1670(C-N stretch), 700, 730 and 1030(tri-substituted vicinal aromatic).

Mass Spectrum (GCMS):

m/z 218 (M^{+} 10 %), 71(100), 56(35), 44(15), 190(10).

2. Synthesis of 3-(2,6-Xylyl)-5-methylhydantoin

Synthesis of this hydantoin involved the formation of a reactive isocynate derivative followed by internal cyclization to form the hydantoin ring. Synthesis of the isocyanate derivative of tocainide was carried out using a phosgene substitute, trichloromethyl chloroformate (TCF), as described by Ozaki (58) and Kurita (59).

To a stock solution of fresh TCF (0.1 mole/200 mL) in sodium dried toluene, tocainide base (1.0 g) and 0.1 g of active carbon was added in small quantities with stirring and gradual heating from 50 to 80 ^{o}C over a 30 minute period. Another 0.6 mL of TCF was added dropwise to the reaction mixture followed by heating from 80 to 100 ^{o}C over 2 hours. The white precipitate was filtered off and the filtrate was dried under vacuum at room temperature, yielding 1.5 g of white crystals. The reaction product was recrystallized in chloroform at 0 ^{o}C for 24 hours, yielding 0.5 g of the pure hydantoin. This recrystallized product was analyzed by IR, GCMS and NMR.

Melting Point:

138 - 140 ^{o}C (ref. 138 - 142.5 ^{o}C) (31).

Infrared (Nujol mull):

3300 cm^{-1}(N-H stretch), 2300(N-C=O stretch),

1795(C=O stretch), 1700(C=O stretch).

Mass Spectrum (GCMS):

m/z 218 (M^{+} 100 %), 147(90), 44(72), 119(30), 105(28),

132(25), 113(20), 203(10), 176(5).

400 MHz-[^{1}H]-NMR ($CDCl_3$):

δ ppm 1.54s(3H, 5-CH_3), 2.18s(3H, xylidine-CH_3), 2.22s(3H, xylidine-CH_3), 4.24dq(J=8Hz, 1H, 5-CH), 6.56s(1H, 1-NH), 7.1 to 7.3s(3H, aromatic-H).

3. Synthesis of 3-(2,4,6-Xylyl)-5-methylhydantoin

The synthetic procedures were similar to those described for the 2,6-xylyl analog. However, initial attempts were made to synthesize 2-amino-2',4',6'-propioxylidide (4'-methyl analog of tocainide).

a Synthesis of 2-Amino-2',4',6'-propioxylidide

The synthetic steps were similar to those described for the 2',6'-propioxylidide analog. N-carbobenzyloxyl-alanine (1.0 g, 0.0488 mole) was added to 6.05 g (0.0443 mole) of 2,4,6-trimethylaniline in 60 mL of dry methylene chloride. Dicyclohexylcarbodiimide (9.23 g, 0.0448 mole) was added slowly under stirring and the reaction mixture was left to stand at room temperature for 1 hour. The mixture was filtered and the filtrate was dried under vacuum. A solution of 30 to 32 % hydrogen bromide in acetic acid was added, and the hydrogen bromide salt of the 4'-methyltocainide analog was recovered and recrystallized by the addition of ether yielding 5.61 g of hydrogen bromide salt.

b. Reaction with Trichloromethyl Chloroformate

The 4'-methyltocainide hydrogen bromide salt (2.0 g, 0.0097 mole) was extracted in base with methylene chloride, dried with anhydrous sodium sulfate, and evaporated to dryness under a stream of nitrogen. The base was dissolved in 30 mL dry toluene,

followed by dropwise addition of TCF (1.16 mL, 0.0097 mole) with agitation and gradual heating from room temperature to 80 ^{o}C as described. The reaction mixture was then filtered, evaporated to dryness, and reconstituted in chloroform for recrystallization. The chloroform solution yielded 0.83 g of 3-(2,4,6-xylyl)-5-methylhydantoin.

Melting Point:

137.5 - 139 ^{o}C

Infrared (Nujol mull):

3300 cm^{-1}(N-H stretch), 2300(N-C=O stretch), 1795(C=O stretch), 1700(C=O) stretch).

Mass Spectrum (GCMS):

m/z 232 (M^{+} 100 %), 161(100), 44(40), 146(35), 119(25), 217(10).

80 MHz-[^{1}H]-NMR ($CDCl_3$):

δ ppm. 1.52s(3H, 5-CH_3), 2.12s(3H, xylidine-CH_3), 2.12s(3H, xylidine-CH_3), 2.32s(3H, 4'-xylidine-CH_3), 4.25dq(J=8 Hz, 1H, 5-CH_3), 6.15bs(1H, 1-NH), 6.9s(2H, aromatic-H).

F. Identification of Potential Tocainide Metabolites

1. Permethylation

The procedure involved generation of fresh sodium methyl sulfinylmethide carbanion as reaction catalyst/solvent followed by addition of CH_3I as outlined by Thompson and Desiderio (61).

a. Preparation of Dry Dimethylsulfoxide (DMSO)

Because of the hygroscopic nature of DMSO, it was distilled fresh with calcium hydride before each permethylation experiment. DMSO (20 mL) was added to 2.0 g of calcium hydride in a round bottom flask and distilled under vacuum at 70 ^{o}C until 15 mL of dry DMSO was collected.

b. Generation of Sodium Methylsulfinylmethide Carbanion (DMSO) Sodium

The salt was prepared with sodium hydride. Sodium hydride (250 mg, 50 % dispersed in mineral oil), an amount equivalent to 5 moles of sodium hydride, was washed rapidly three times with a total of 3 mL anhydrous ether. The washed sodium hydride was transferred quickly to a round bottom flask containing 5 mL of dry DMSO. The grey color suspension was then heated gently under nitrogen until the formation of hydrogen ceased. The resulting straw-color suspension was allowed to settle giving a clear solution. The filtered supernatant was stored under nitrogen at -20 ^{o}C.

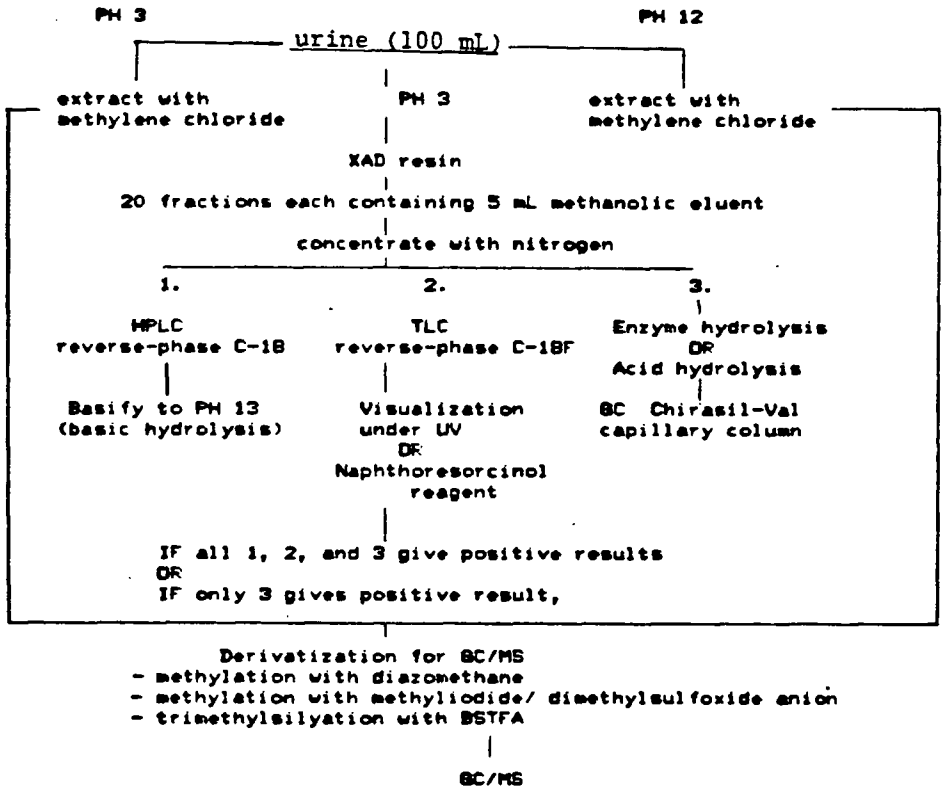
c. Permethylation

An aliquot of 200 uL of isolated metabolite sample in methanol (as discussed in the following experimental sections) was dried under nitrogen. The residue was then reconstituted with 50 uL of dry DMSO under nitrogen in a 5 mL screw-capped reaction vial. DMSO sodium carbanion (20 uL) was added, stirred in a vortex mixer, and let stand for 15 minutes at room temperature. Subsequently, 1.2 uL of methyliodide was added and the mixture was stirred as before. The reaction was allowed to

proceed at room temperature for 1 hour before it was terminated by the addition of 1 mL of water. The permethylated glucuronide was extracted with 1 mL of chloroform and back-washed three times with 1 mL of water. The chloroform extract was then concentrated under nitrogen before GCMS analysis.

2. Attempted Identification of Permethylated Urinary Tocainide Metabolites

Scheme 9 outlines the protocol used in screening both conjugated and non-conjugated metabolites.



Scheme 9 : Attempted identification of permethylated urinary tocainide metabolites.

a. Isolation of Tocainide-carbamoyl Ester Glucuronide

Total urine (1 to 10 hours), collected from a human volunteer after a 200 mg oral dose of tocainide, was pooled. A 2 cm x 20 cm XAD-2 resin column was used initially to trap urinary metabolites which were subsequently eluted with methanol. The crude methanolic sample thus collected from the XAD adsorption resin was applied as a narrow band onto a 20 x 20 cm KC18F TLC plate and the plate was eluted with a 60/40 methanol/water solvent system. After 15 cm of elution, the plate was divided into four fractions. The following Table 2 outlined the Rf values of the 13 bands separated from the crude extract. Various bands on the TLC were isolated together as a plate fraction, and thus four plate plate fractions were obtained as outlined below.

Table 2 : The Rf of urinary components separated on a KC-18F 20x20 cm TLC plate.

plate fraction			
1	2	3	4
0.84 0.81 0.74 0.70	0.64 0.61	0.57 0.53 0.50 0.43	0.38 0.34 0.30

The samples, from each plate fraction were again individually evaluated chromatographically on 20 x 20 cm KC18F plates using a 50/50 methanol/water solvent system. Band 1 of plate fraction 1, obtained from the 60/40 methanol/water solvent system, was resolved into bands 1a and 1b by the 50/50 methanol/

water solvent system. These two bands were isolated and labelled band 1a and 1b respectively. The Rf values of the band 1a and 1b are listed in Table 3.

Table 3 : The Rf three urinary components separated on a KC-18F TLC plate during a second purification.

	Band 1a	Band 1b	Band 2
Volunteer 2 Band 1	0.84	0.69	0.06

The rest of the 11 bands in plate fractions 1 to 4 were also isolated.

b. Acid and Enzyme Hydrolysis

A portion of each of the samples isolated from the TLC plates was hydrolyzed with hydrochloric acid or beta-glucuronidase enzyme. For acid hydrolysis, 0.5 mL of 1.0 M hydrochloric acid was added to the band fractions and than heated at 100^{o}C for 30 minutes. After treatment, the samples were basified, extracted with methylene chloride, and derivatized with 20 uL of HFBA in hexane at 55^{o}C for 30 minutes before analyzed with a gas chromatograph equipped with a 25 m x 0.31 mm id. Chirasil-Val^{R} capillary column. Samples isolated from the band fractions were also hydrolyzed with 0.5 mL of the beta-glucuronidase - glucurase^{R} in the presence of 1.0 mL

of acetate buffer at pH 5 in PTFE-lined screw-capped glass tubes. The tubes were incubated in a water bath at 37^{o}C for 12 hours. After hydrolysis, the samples were basified, extracted, derivatized with HFBA, and analyzed by GC as described. Band 1b yielded tocainide after both acid and enzymatic hydrolysis indicating that the fraction contained a glucuronic acid conjugate of tocainide.

c. Liquid Chromatographic Analysis of the Hydantoin Derived from the Glucuronic Acid Conjugate of Tocainide

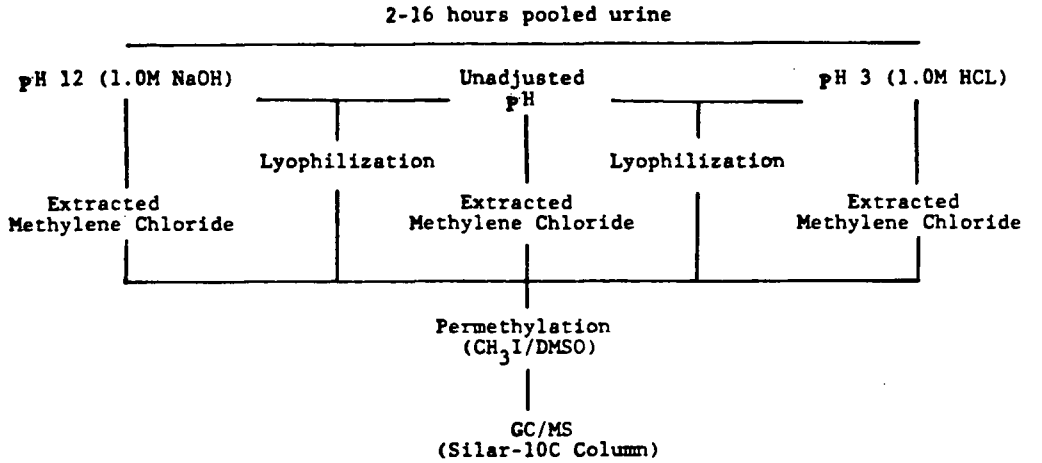
Band fractions isolated from reverse-phase TLC plates were subjected to pH 12 alkaline condition by the addition of 1.0 N sodium hydroxide solution. Upon basification to pH 12, tocainide carbamoyl glucuronide has been shown to cyclize to 3-(2,6-xylyl)-5-methylhydantoin which can be monitored by HPLC and compared with the synthesized material. After basification, the samples were extracted into methylene chloride and the solvent concentrated under nitrogen at 55 ^{o}C. Liquid chromatographic analysis was carried out on a 5 um C-18 ODS (25 cm x 4.6 mm) HPLC column with a 25 % acetonitrile in 0.05 M potassium phosphate solvent system.

d. Gas Chromatography/Mass Spectrometry Analysis of Permethylated Tocainide Carbamoyl Glucuronide

The suspected tocainide glucuronide was permethylated with methyliodide in dry dimethylsulfoxide (DMSO) in the presence of methylsulfinylmethide carbanion as described previously.

3. Attempted Identification of Urinary Tocainide Metabolites by Lyophilization and GCMS Analysis

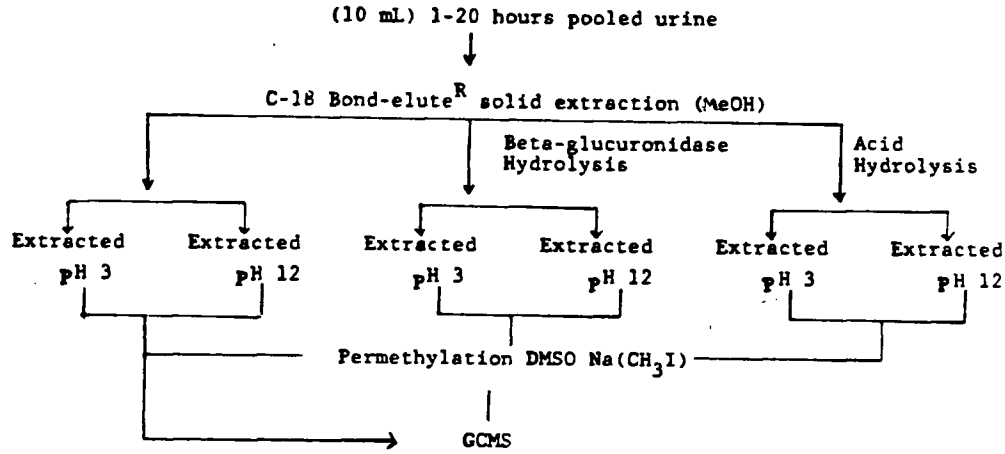
Urine samples were collected from a healthy volunteer between 2 to 16 hours after an oral dose of 200 mg tocainide HCl. The following Scheme 10 outlines the experimental protocol.



Scheme 10 : Attempted identification of urinary tocainide metabolites by lyophilization and GCMS analysis.

4. Attempted Isolation and Identification of Tocainide N-carbamic Acid or N-ureide Intermediate

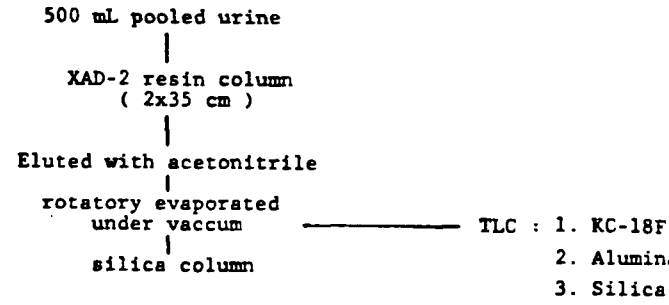
Urine samples from a healthy human subject were collected between 1 to 20 hours after an oral dose of 200 mg tocainide HCl. Scheme 11, as shown below, outlined the experimental design that was used in an attempt to isolate and identify the tocainide metabolite intermediates.



Scheme 11 : Attempted isolation and identification of tocainide N-carbamic acid or N-ureide intermediate.

5. Attempted Isolation and Identification of Urinary Metabolites by Flash Chromatography

A volume of 500 mL of urine, collected from 1 to 7 hours after an oral dose of 200 mg of tocainide in a human subject was used. As shown in Scheme 12, the pooled urine sample was first concentrated on a XAD-2 absorption resin column as described using acetonitrile as an eluant.



Scheme 12 : Attempted isolation and identification of urinary metabolites by flash chromatography.

A 6-inch silica gel column was dry-packed in a 1 cm id. glass cylinder with 0.5 cm silica sand forming a seal on the column ends. A 30/20/50 hexane/2-propanol/methanol combination solvent was flushed through the packing with 137 KPa (20 PSI) nitrogen at 1 mL/min until all gas bubbles were eluted. A volume of 5.0 mL of the XAD-2 urinary acetonitrile extract was delivered on top of the column and then eluted with the described solvent

system at 1 mL/min collecting 1 mL fractions. An aliquot of each fraction was spotted on a sil-UV_{254} 2 x 5 cm TLC plate and the plate was eluted with 30/20/50 Hexane/2-propanol/ methanol. Fractions 4 and 5, 6 to 11, and 12 to 16 were found to correspond to 3 distinct Rf values, and these various fractions were pooled into three samples. The solvent in each sample was evaporated to dryness and the residue was derivatizied with CH_3I or CD_3I in the presence of DMSO sodium, followed by GCMS analysis.

G. Reaction of Tocainide with Urea

Due to a preliminary observation that tocainide reacts with urea to form a hydantoin structure, experiments were carried out to study the reaction at different pH, temperature, and reaction time.

1. The Effect of Temperature and Reaction Duration

Tocainide base (50 mg) was added to 1 mL of 8.0 M urea solution. The solution was then heated in a block heater at 100^{o}C for 1 hour, 9 hours or 13 hours, or alternatively at 60^{o}C for 3 hours. The samples were subsequently basified by the addition of 200 uL of 1.0 M sodium hydroxide and then extracted with 5 mL methylene chloride. The solvent was dried over anhydrous sodium sulfate before being concentrated over a stream of nitrogen for GCMS analysis. In addition, a 500 mg sample of tocainide was added to a freshly collected drug-free urine sample and was either left at room temperature or heated at 100 ^{o}C for 1 hour or 9 hours. For reference, tocainide was added to distilled deionized water and heated at 100 ^{o}C for 13 hours.

2. The Effect of pH

Into three separate tubes, tocainide base (100) mg was dissolved in 1 mL of 8.0 M urea solution followed by pH adjustment to 2.3, 7.8 or 12.5 with 0.1 M hydrochloric acid or with 0.1 M sodium hydroxide. The samples were heated at 60 °C for 6 hours. The samples were then directly spotted on a 5 x 20 cm RPC18F plate and eluted with 80/20 methanol/water. Following elution on the TLC plate and visual identification under UV at 254 nm, the three reaction mixtures were then isolated separately by Bond-elut^{R} reverse phase solid extraction columns followed by permethylation with CH_3I in sodium DMSO and GCMS analysis as described previously.

TLC (KC-18F):

80/20 methanol/water: tocainide Rf = 0.45,
hydantoin Rf = 0.75,
N-ureide tocainide Rf = 0.60.

Mass Spectrum (GCMS):

permethylated N-ureide-tocainide,

m/z 191 (M^+ trace), 72(100), 129(45), 191(5).

3. Attempt Synthesis of Tocainide-N-ureide-glucuronide

Tocainide base (400 mg) and 300 mg sodium glucuronic acid were dissolved in 1.0 mL of 8 M urea. The solution was heated at 50 °C for either 1 or 2 hours followed by Bond-elut^{R} solid extraction isolation and permethylation with CH_3I/Na DMSO as described previously. In another experiment, tocainide was first heated at 50 °C for 1 hour in the presence of urea prior to the addition of sodium glucuronic acid. The reaction mixture was

then left at room temperature overnight before solid phase extraction and permethylation with CH_3I/Na DMSO as described.

4. Attempt Synthesis of 2,6-Dimethylaniline-N-glucuronide

2,6-Dimethylaniline (2.5 mL, 1.02 mole) was added to 3 mL of 2:1 acetone:water containing 0.5 g sodium glucuronic acid. The reaction mixture was adjusted to pH 5 with 1.0 M hydrochloric acid and mixed on a rotating rack overnight. The products were spotted on a 2 x 5 cm RPC-18F TLC plate and eluted using 40/60 methanol/water. Following TLC visualization under UV at 254 nm, the product was isolated by solid phase extraction and permethylated with CH_3I/Na DMSO, followed by GCMS analysis.

TLC (KC-18F):

40/60 methanol/water: 2,6-dimethylaniline Rf = 0.25,
N-glucuronide Rf = 0.45.

Mass Spectrum (GCMS):

permethylated N-glucuronide.

m/z 353 (m^+ 8 %), 103(100), 219(90), 134(72), 75(68), 85(40).

H. Isolation and Structural Elucidation of Tocainide Glucuronide

Urine samples were collected from a human subject who had received an intravenous dose of 200 mg of tocainide hydrochloride. An aliquot of 100 mL of the 14th hour urine collection was concentrated under vacuum to yield 5.9 mL of a yellow residue. This residue was adjusted to pH 3 with 0.1 M phosphoric acid and extracted with 10 mL of hexane prior

to evaluation by preparative HPLC.

In another experiment, 100 mL of the 7th hour urine from the same subject was concentrated by azeotropic evaporation at room temperature with 10% ACN under vacuum to yield 10 mL of a yellow residue. The residue was extracted 3 times with 10 mL methylene chloride at pH 7 followed by 3 repetitive extractions with 10 mL of hexane at pH 3. The extracted samples were then filtered through a 0.45 um syringe filter before preparative HPLC isolation.

1. Preparative HPLC Isolation of Tocainide Carbamoyl Ester Glucuronide

Initial selection of solvent systems using reverse-phase TLC indicated a high proportion of water in the mobile phase was required for the elution of tocainide glucuronide. Through a systematic approach, 10 % acetonitrile in 0.01 M sodium phosphate (monobasic) buffer (pH 3) solvent system delivered at 3 mL/min, was found to offer reasonable separation and retention of urinary components. The column eluant was monitored by UV at 220, 225, or 266 nm. Eighty 2.0 mL fractions were collected at the detector outlet with an automatic fraction collector. The absorbance of each fraction was measured by a UV spectrophotometer at the corresponding detection wavelength that was used to monitor the column eluant. Fractions containing major peaks were pooled and analyzed by base, acid and enzyme hydrolysis as well as by a naphthoresorcinol color test, as will be detailed in later sections.

Thirty-six injections of the azeotropically prepared

sample were made with a 200 uL sample injection loop using a 10 % acetonitrile in 0.01 M sodium phosphate (monobasic) buffer at pH 3 mobile phase. Fraction 4, which was identified to contain the tocainide glucuronide, was pooled and concentrated under vacuum. A second solvent system consisting of 3 % acetonitrile in 0.01 M sodium phosphate (monobasic) at pH 7 was used to re-purify the collected fraction 4 sample. The column eluant was again collected in fractions and tested for the presence of TOCG as described. Based on the volume of urine, containing 67 ug/mL of the hydantoin, that was used for this 2-step isolation, an estimate of 10 mg of pure tocainide glucuronide in sodium phosphate (monobasic) salt (63 mg) could be isolated.

The sodium phosphate salt contained in the eluate was removed by recycling the sample in 3 % acetonitrile in 0.3 % acetic acid (adjusted to pH 7 with 0.1 M sodium hydroxide). The TOCG-containing fraction was concentrated under vacuum, and the sodium acetate salt was removed by acidifying the sample to pH 3 with dilute hydrochloric acid followed by evaporation of the free acid under vacuum at room temperature. The resulted TOCG was isolated in NaCl.

UV Spectrum:

λ max 202.5 nm (λ max tocainide = 202.5 in 3 % acetonitrile in 0.01M sodium phosphate, pH 7).

Mass Spectrum (GCMS):

(BSTFA/pyridine derivatization leads to decomposition of TOCG)

m/z 218 (M^+ 100 %).

(acetic anhydride/diazomethane derivatization leads to decomposition of TOCG)

m/z 218 (M^+ 100 %).

400-MHz-[^{1}H]-NMR (D_6-DMSO):

δ ppm 1.38d(3H,1-CH_3), 2.11s(3H,xylidine-CH_3), 3.69d(glucuronic acid), 3.92d(glucuronic acid), 4.25t(1H,2-CH_3), 5.77s(glucuronic acid), 7.06s(3H,xylidine-H), 9.39s(1H,xylidine-NH).

Fast Atom Bombardment:

Glycerol: m/z 413 (M + 1), 435(M + Na), 457(M -H+2Na).

Thioglycerol: m/z 413 (M + 1), 435(M + Na), 457(M -H+2Na).

a. Acid Hydrolysis of TOCG with Hydrochloric Acid

Each pooled fraction, collected from the preparative HPLC, was concentrated to 5 mL under vacuum by azeotropic evaporation with acetonitrile. To each concentrated fraction, 1.0 mL of concentrated hydrochloric acid was added and the sample was then heated at 100 ^{o}C for 1 hour, following which, it was basified with 5.0 M sodium hydroxide, extracted with 2 mL of methylene chloride, concentrated under nitrogen and analyzed for the presence of tocainide by GC with a SE-30 capillary column.

b. Beta-glucuronidase Enzyme Hydrolysis

Each pooled fraction collected from the preparative HPLC was concentrated by azeotropic evaporation as described, followed by adjustment to pH 5 with 0.1 M sodium hydroxide or 0.1 M

hydrochloric acid, and hydrolyzed by the addition of 500 uL of bovine beta-glucuronidase enzyme in acetate buffer at pH 5. The hydrolysis was carried out at 37°C for 12 hours, followed by basification with sodium hydroxide and extraction with methylene chloride. The organic solvent was evaporated to dryness, reconstituted with methanol and analysed by GC for the presence of tocainide as described above. Each fraction collected from the liquid chromatograph was also treated in a similar fashion with the bovine beta-glucuronidase enzyme, but with the addition of 2 ug of saccro-1,4-lactone as an enzyme inhibitor.

c. Naphthoresorcinol Color Reaction

After concentration under vacuum, each HPLC fraction was spotted on a 2 x 5 cm RPC-18F TLC plate and sprayed with a 2 % naphthoresorcinol reagent in phosphoric acid/ethanol. The plates were then heated at 100°C for 10 minutes. A blue coloration of the resulting spots indicated the presence of a glucuronic acid moiety.

d. Sodium hydroxide Hydrolysis

Each concentrated HPLC fraction was basified to pH 12 with a 2.0 M sodium hydroxide solution. The sample was then extracted with methylene chloride. The organic layer was transferred to a clean tube, evaporated, and reconstituted with methanol prior to GC analysis for the presence of 3-(2,6-xylyl)-5-methylhydantoin.

I. Pharmacokinetics of Tocainide Carbamoyl Ester Glucuronide

The quantitation of tocainide glucuronide in urine was carried out using a modified indirect method as described by

Hoffmann (60). The procedure involved the hydrolysis of tocainide glucuronide in urine at pH > 12 forming 3-(2,6-xylyl)-5-methylhydantoin. Quantitation of the hydantoin derivative can provide an estimation for the tocainide glucuronide.

1. Hydrolysis Kinetics of Tocainide Glucuronide in Sodium Hydroxide

Into 7 tubes, 0.4 mL of urine collected at the 10th hour from a human subject who had received 200 mg of tocainide orally, and 0.1 mL of the internal standard etidocaine base (1.0 ug/mL) were added to 0.5 mL of 2.0 M, 1.0 M or 0.5 M sodium hydroxide with mixing. The reaction product was extracted at 0.5, 1, 2, 4, 6, 10 and 20 minutes by shaking with 5.0 mL of methylene chloride for 1 minute. The methylene chloride was then dried over anhydrous sodium sulfate, evaporated to dryness and reconstituted with 20 uL of methanol for GC analysis using a bonded phase SE-30 capillary column. The hydrolysis of tocainide carbamoyl ester glucuronide, as reflected by the formation of the hydantoin, was plotted as the peak height ratio of the hydantoin to that of etidocaine, over the timed intervals 1.5, 2, 3, 5, 7, 11 and 21 minutes.

2. Hydrolysis of 3-(2,6-Xylyl)-5-methylhydantoin

Into 7 tubes, 0.1 mL of an aqueous hydantoin solution (15 ug/mL) and 0.1 mL of etidocaine internal standard (10 ug/mL) solution were added to 0.2 mL of 2.0 M, 0.2 M or 0.1 M sodium hydroxide. The hydrolysis samples were extracted at accurately timed intervals from 0.5 to 40 minutes by shaking with 5.0 mL methylene chloride for 1 minute. The solvent was dried,

evaporated to dryness, and reconstituted with 2.0 uL methanol for GC analysis.

3. Hydrolysis of 3-(2,4,6-Xylyl)-5-methylhydantoin

The protocol was the same as described for the 2,6-xylyl analog.

4. 3-(2,6-Xylyl)-5-methylhydantoin Calibration Curve

All calibration samples were prepared in duplicate. Into 6 separate tubes, 100 uL of 1, 3, 6, 10, 15 and 20 ug/100 uL of aqueous tocainide base and hydantoin standard solution were added to 100 uL of 10 ug/100 uL etidocaine base internal standard solution. Into each sample, 100 uL of 4'-methylhydantoin internal standard (10 ug/100 mL) and 300 uL of blank urine were also added. An aliquot of 700 uL of 1.0 M sodium hydroxide was then added to each tube. The tubes were mixed on a vortex mixer for 10 minutes at room temperature, followed by extraction with 5 mL methylene chloride for 1 minute. The organic layer was transferred to a clean tube and evaporated to dryness under nitrogen. Each sample was reconstituted with 20 uL of methanol prior to GC analysis. Calibration samples were stored at 4 ^{o}C and a random sample was used to monitor daily instrumental variation. A new calibration curve was constructed if peak area ratios were found to deviate more than 10 % between daily analysis.

5. Inter-assay and Intra-assay Variability

Duplicate calibration samples of 6, 15 and 20 ug were each analyzed in duplicate. The average peak area ratio ± C.V. % was used to determine inter- and intra-assay sample variation.

A coefficient of variation of less than 10 % was considered acceptable.

6. Extraction Efficiencies of Tocainide and the Hydantoin into 5 mL of Methylene Chloride

Samples of 1 mg/mL of tocainide hydrochloride and etidocaine base were first prepared in methanol. The extraction efficiency of tocainide and the hydantoin in methylene chloride was evaluated in a concentration range of 6 to 20 ug/mL. Into 3 separate tubes, 100 uL of each of tocainide hydrochloride and the hydantoin, equivalent to 6, 10 and 20 mg of tocainide base and the hydantoin respectively, was added to 300 uL of blank urine. An aliquot of 500 uL of 1.0 M sodium hydroxide was then added to each tube immediately followed by extraction with 5 mL of methylene chloride for 1 minute. The organic layers were then transferred to clean tubes, evaporated to dryness under nitrogen and reconstituted with 100 uL of etidocaine as an external standard (1 ug/uL in methanol) prior to GC analysis. In another 3 separate tubes, 100 uL of each of tocainide base and the hydantoin in 6, 10 and 20 ug/100uL methanol was added to 100 uL of etidocaine (the same methanol internal standard solution). The samples were concentrated under nitrogen followed by GC analysis. The peak area ratios between tocainide and the hydantoin to that of etidocaine in the extracted samples were compared with the corresponding peak area ratios from the methanol samples. The percent difference in the peak area ratios between the two sets of samples provided the extraction efficiencies of tocainide and the hydantoin, assuming minimal

breakdown of the hydantoin during the short extraction period.

7. Analysis of Tocainide Glucuronide in Kinetic Studies

Urine samples were collected into polyethylene bags during previous pharmacokinetic studies in three fasted subjects who had received either a 200 mg oral or slow (20 minutes) intravenous dose of tocainide hydrochloride. Urine samples were collected hourly for the first 10 hours and as often as required thereafter for up to 96 hours. Urine volumes and pH were recorded immediately after collection. The samples were stored at -10 ^{o}C and were thawed into 10 mL screw-capped tubes, basified with sodium hydroxide, extracted with methylene chloride and analysed in the same manner as described for handling of the calibration samples. All work-up procedures and GC analyses were done on a daily basis.

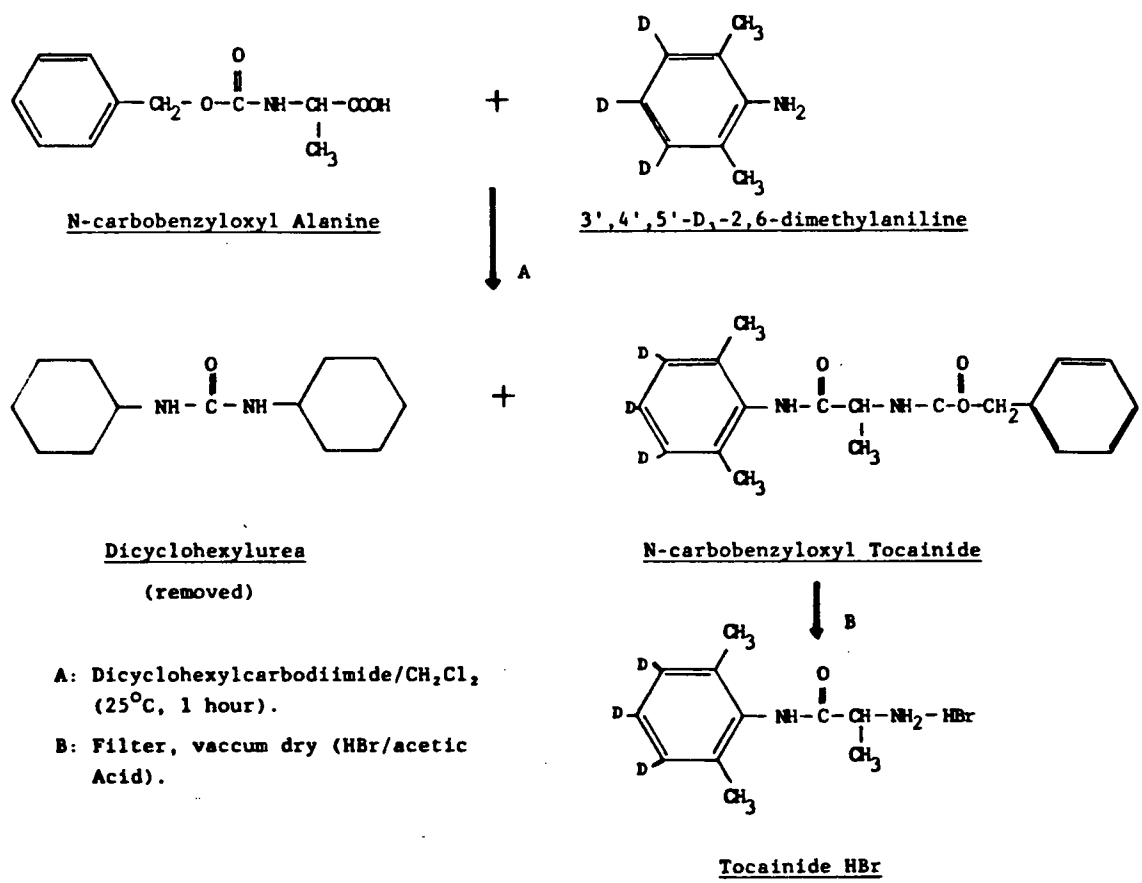
3 RESULTS AND DISCUSSION

A. Synthetic Pathways for 3',4',5'-Trideuterated Tocainide

The synthetic procedures for tocainide as described by Byrnes (31) are similar to those used for peptide synthesis. Since the 3',4',5'-positions on the xylidide ring of tocainide have not been reported to undergo metabolism, initial attempts were aimed at exchanging these protons with deuterium.

1. Synthesis of 3',4',5',-[$^2H]_3$-2-Amino-2',6'-propioxylidide

The synthetic approach as shown in Scheme 13 was modified from a method reported by Byrnes (31):



Scheme 13. The synthetic pathway for trideuterated tocainide.

a. 2,6-Dimethylaniline Deuteration

Initial discussion will describe the deuteration of the 3,4,5- positions of 2,6-dimethylaniline. The procedures followed a predeuteration method for labelling toluidines and xylidines as described by Frischkorn (57). The reaction mechanism is typically an electrophilic aromatic substitution (62). For 2,6-dimethylaniline, the amine is a strong activating group directing ortho- and para-substitution, while the 2,6-methyl groups are only weak activators. Preliminary attempts at refluxing the aniline with D_2O/D_2SO_4 yielded 70 % pure 4-[^{2}H]-2,6-dimethylaniline. The Frischkorn method, however, required heating at 250 ^oC in the presence of D_2O/D_2SO_4. To prevent vaporization of D_2O at 250 ^oC, a 4137 KPa (600 PSI) pressure was required. To achieve this condition, a Variac^R reaction vessel was pressurized at room temperature to 4137 KPa (600 PSI) with nitrogen prior to heating at 250 ^oC.

Mass Spectra:

Mass spectral analysis of the crude reaction product revealed a mixture of components with molecular ions m/z 126 and m/z 125 (see Figures 3 to 5). After exchange of the amino deuteriums with hydrogens, the loss of one mass unit from m/z 126 to m/z 125 suggested this ion contained the amino group. However, the ion fragment at and m/z 80 was unchanged, thus suggesting that the m/z 80 fragment did not contain the amino group (see Structures xii to xv). A mass spectrum of unlabelled 2,6-dimethylaniline was included in Figure 6 for comparsion.

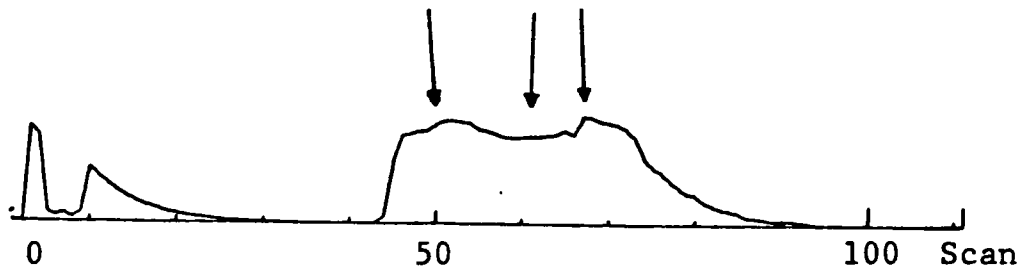


Figure 3 : Total-ion-chromatogram of crude deuterated 2,6-dimethylaniline product.

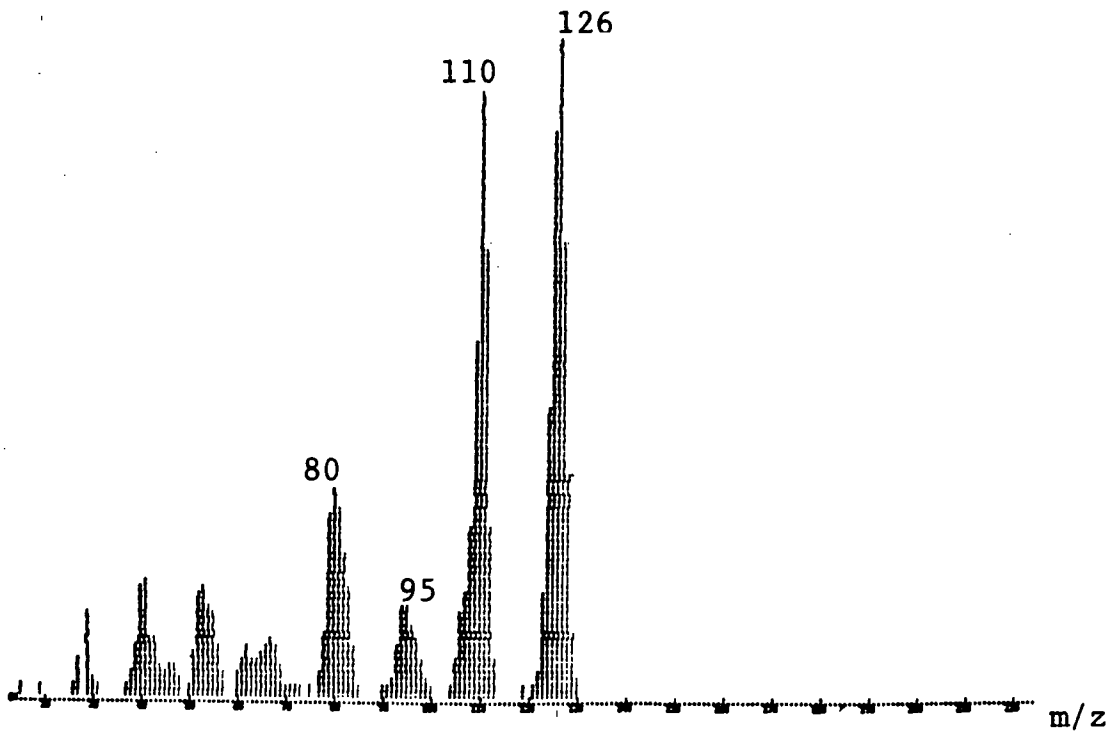


Figure 4 : The mass spectrum of crude deuterated 2,6-dimethylaniline at scan 51.

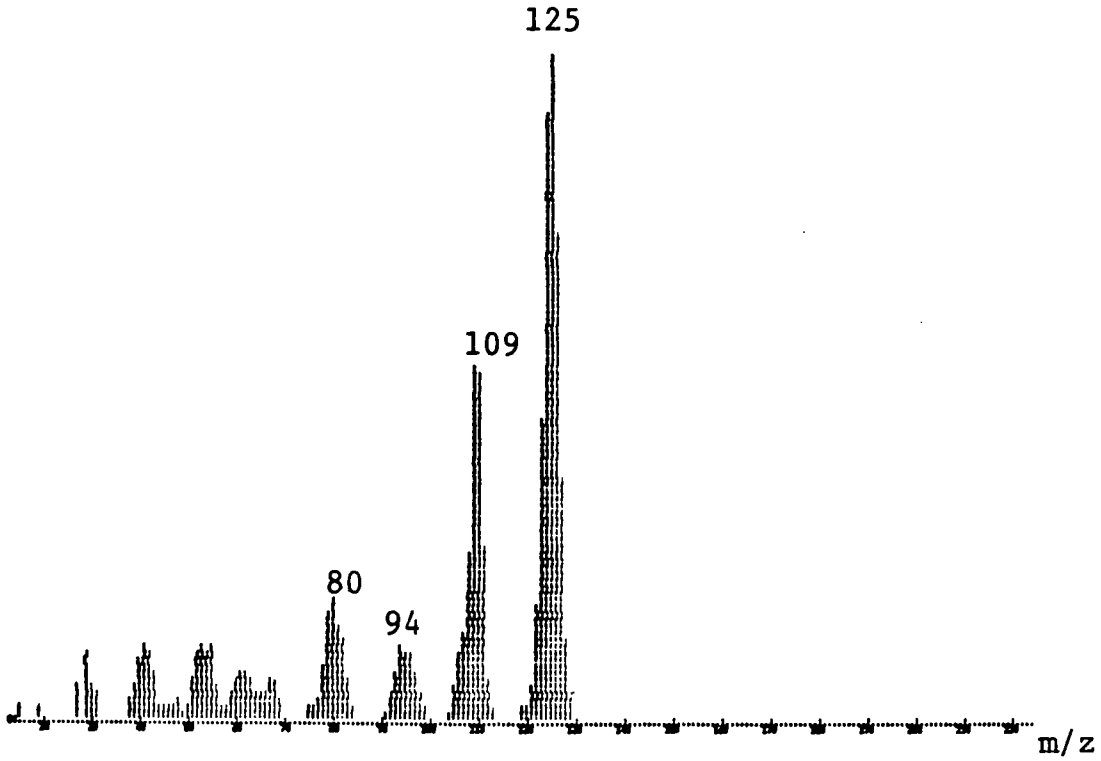


Figure 5 : The mass spectrum of crude deuterated 2,6-dimethylaniline at scan 61.

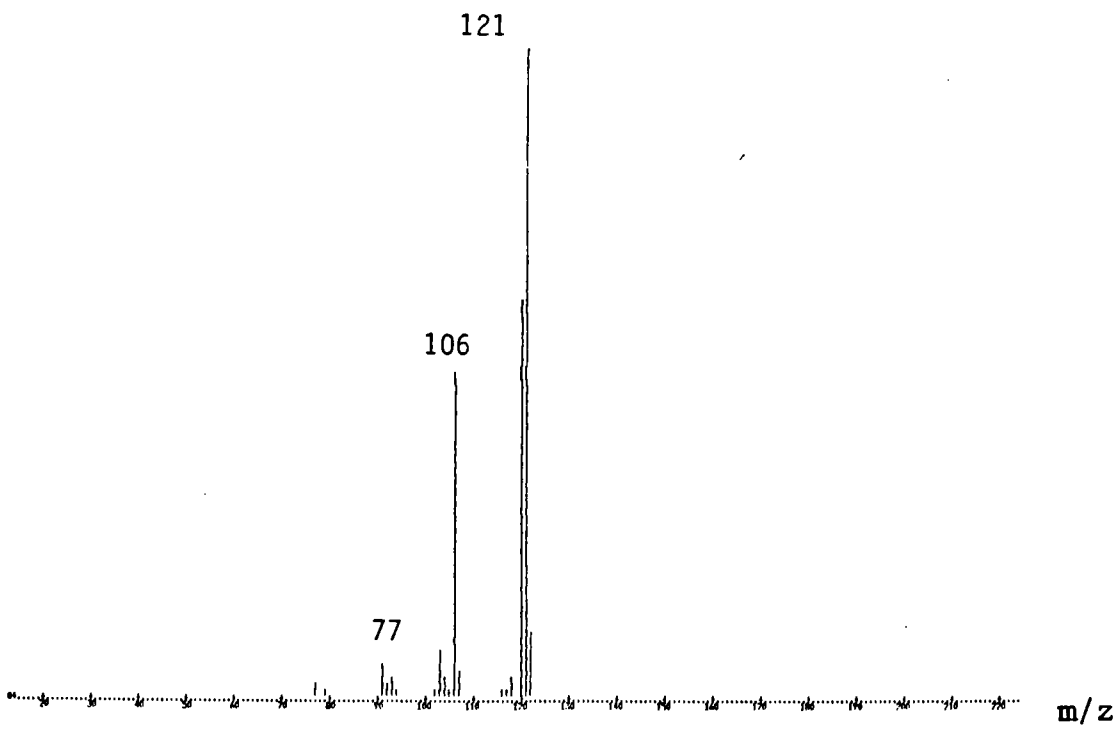
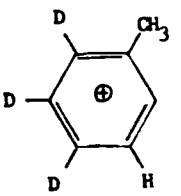
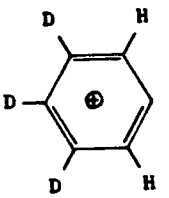


Figure 6 : The mass spectrum of unlabelled 2,6-dimethylaniline.



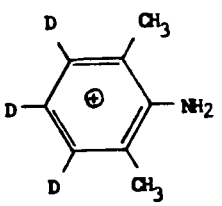
m/z 95

XII



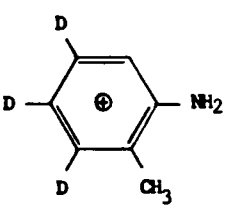
m/z 80

XIII



m/z 124

XIV



m/z 109

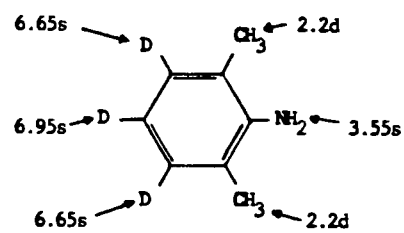
XV

The mass spectral data of the distilled product revealed a molecular ion of m/z 124 as well as the corresponding ion fragments which indicated deuteration at 3,4,5- ring positions (as shown in Figures 7 and 8, also see Structures xii to xv) After purification by distillation, the TIC revealed a single component with m/z 124 (M^+ d_3 100 %), 109 (M^+ $-CH_3$ d_3 60), 95 (M^+ $-NH-CH_3$ d_3 12), 80 (M^+ $2C_2H_6$ d_3 20)

80 MHz-[^{1}H]-NMR:

As shown in Figures 9 and 10, the proton NMR spectra of the deuterated product indicated only trace intensity of signals from the aromatic protons at around 7.00 ppm. The proton assignments are summarized on Table 4.

Table 4 : Summarized data from the 80-MHz-[^{1}H]-NMR spectrum of D_3-2,6-dimethylaniline.



Shift (ppm)	Decoupling	Integration (mm)	Proton(s)	Assignment
TMS (0.0)				
2.20	s	113	6H(19/H)	xylidine - CH_3
3.5	s	8	2H	xylidine - NH_2
6.65	bs	2	2H	aromatic - meta
6.95	bs	5	1H	aromatic - para

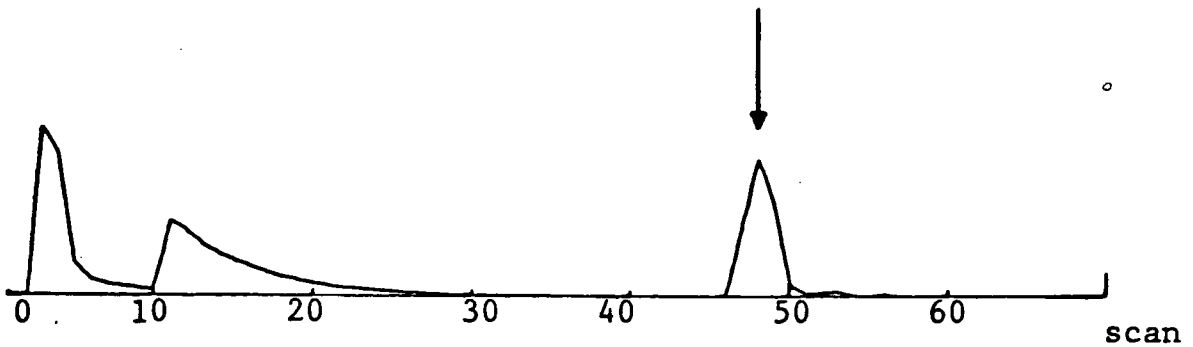


Figure 7 The total-ion-chromatogram of distilled D_3-2,6-dimethylaniline.

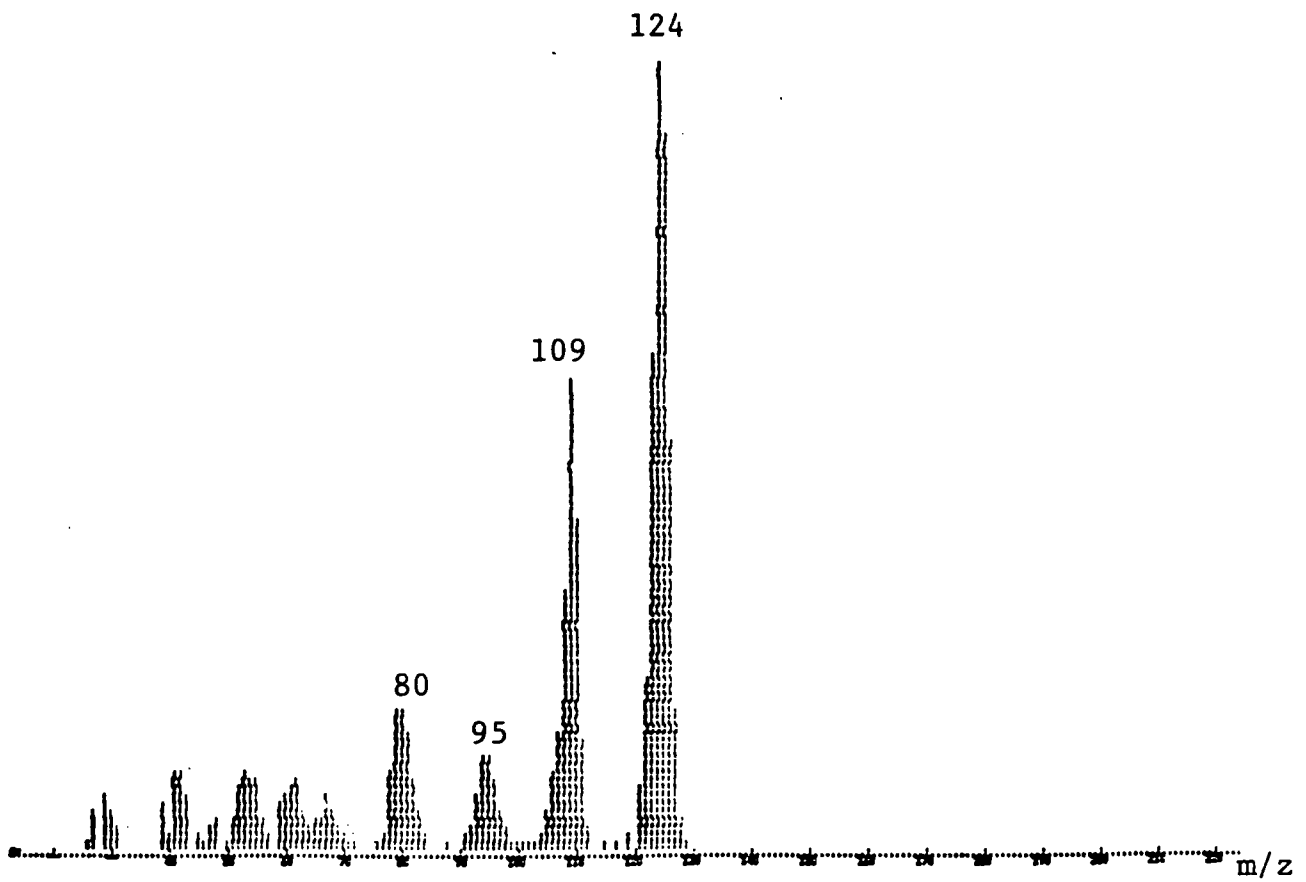


Figure 8 : The mass spectrum of distilled D_3-2,6-dimethylaniline.

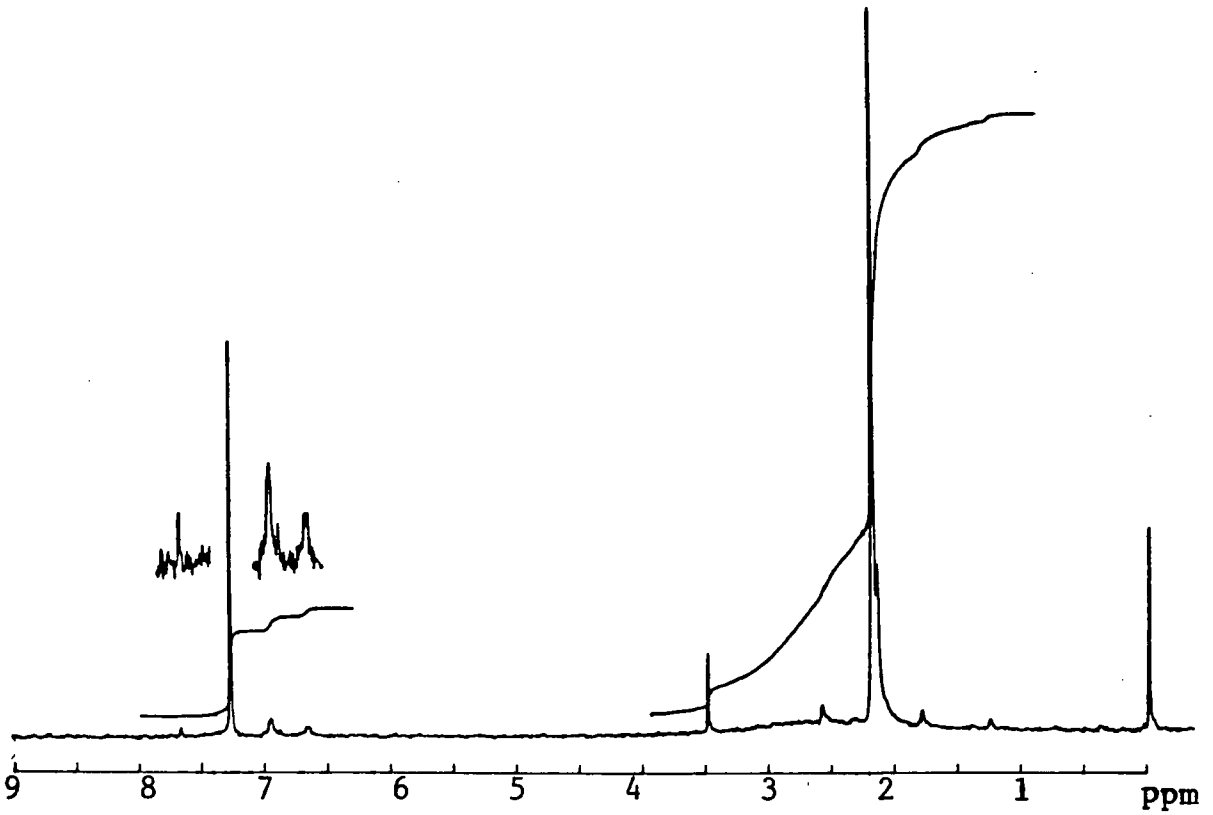


Figure 9 : 80 MHz-NMR spectrum of D_3-2,6-dimethylaniline ($CDCl_3$).

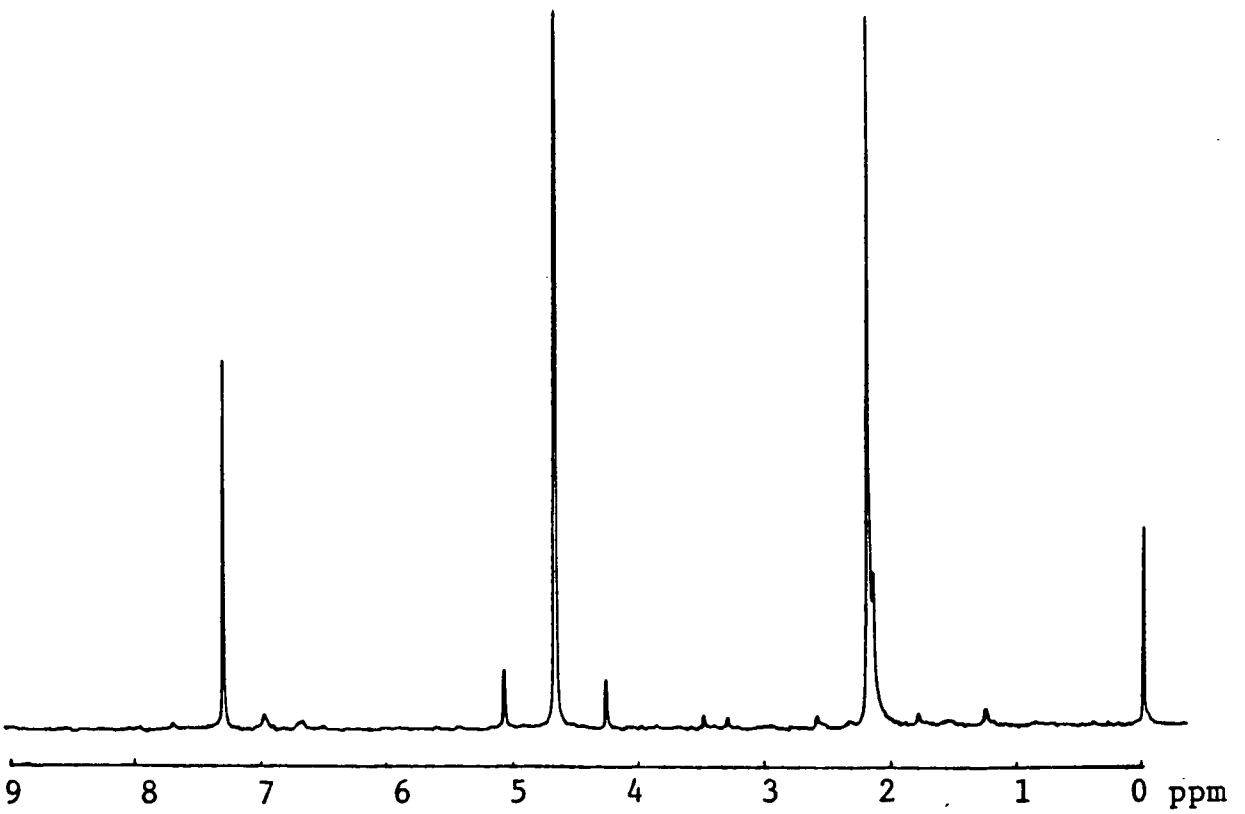


Figure 10 : 80 MHz-NMR spectrum of D_3-2,6-dimethylaniline after D_2O exchange.

A splitting of the signal at 2.12 ppm suggested that a small portion of the protons on the $-CH_3$ group might have been exchanged with deuterium. The integration at 2.2 ppm revealed 113 units/ 6 protons or 19 units/proton. In the same respect, the chemical shift at 3.5 ppm (2H, NH_2) was integrated for 4 units/proton which suggested that 78.9 % of the amino protons had exchanged. The meta- and para-aromatic protons at 6.65 ppm(2H, Ar-H) and 6.95 ppm(1H, Ar-H) were integrated for 1 unit/proton and 5 units/proton, suggesting a deuterium labelling purity of 94.8 % and 73.7 % respectively.

b. Reaction with Carbobenzyloxyl-alanine

With the successful synthesis of the deuterated-aniline, synthesis of trideuterated tocainide followed the method by Byrnes (31). Dicyclohexylcarbodiimide has been used widely for the synthesis of peptides and many dehydration reactions (63) involving an amino and a carboxylic acid group. The commonly cited by-product of these reactions is the insoluble dicyclohexylurea which can be easily filtered and removed. The reaction product after recrystallization yielded 0.36 g tocainide hydrochloric acid.

Infrared (Nujol Mull):

The infrared spectrum of the synthetic compound as shown in Figure 11 was superimposable to that obtained from reference tocainide. A prominent absorption at 3300 cm^{-1} indicated HN-stretching and C=O stretching at 1670 cm^{-1}.

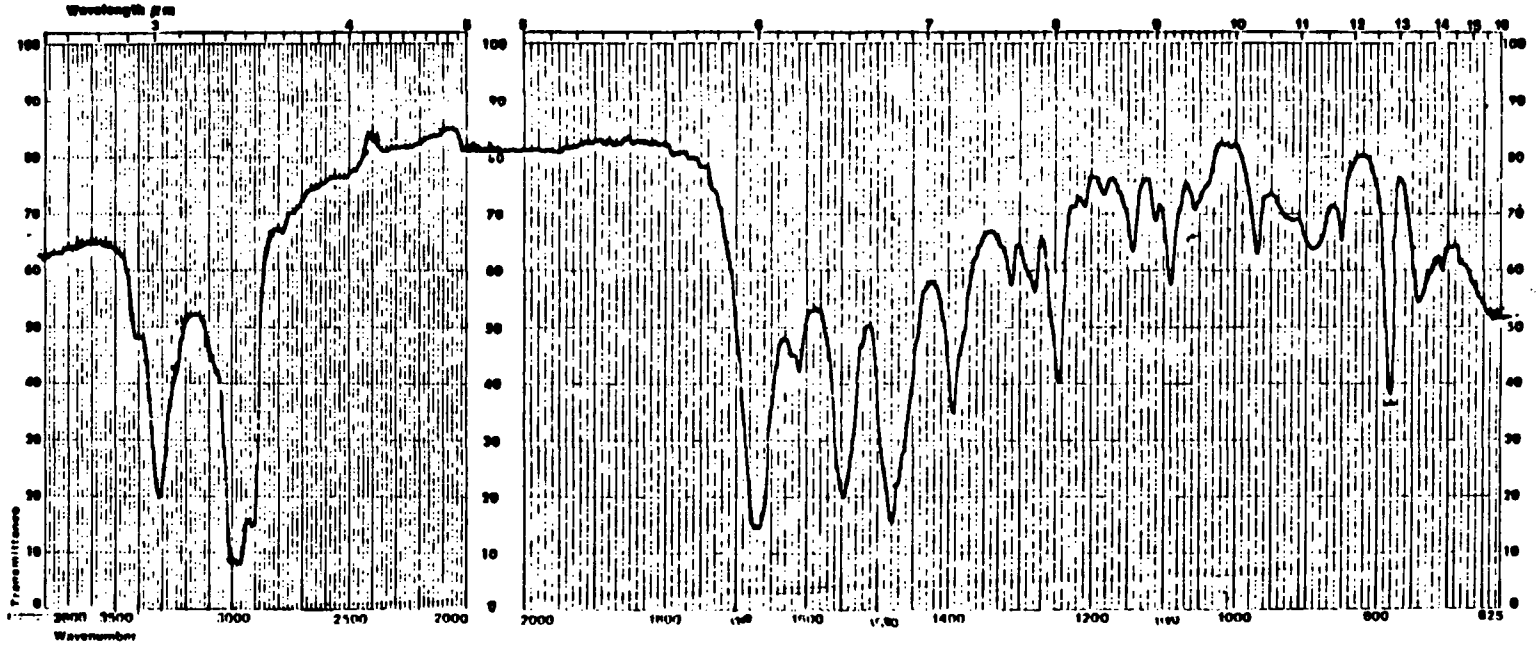


Figure 11 : The infared spectrum of synthetic tocainide (mull).

Mass Spectrum (GCMS):

Analysis was carried out using a 3 % silar-10C packed column. The TIC and Mass Spectrum are shown in Figures 12 and 13. The m/z 44 base peaks indicated a cleavage between the carbonyl and the chiral carbon. Fragmentation between the C-N bond of the xylidine nitrogen gave rise to m/z 106, while the fragment at m/z 121 was resulted from cleavage between the nitrogen and the C-3 carbonyl. Only 3 to 5 % of m/z 192 molecular ion was observed.

The Mass Spectrum with its fragmentation pattern agreed with data obtained from reference tocainide.

2. Attempted Synthesis of 1-[$^2H]_3$-2-Amino-2',6'-propioxylidide

This synthetic approach provided an alternative method of labelling three deuterium atoms on the chiral methyl group of tocainide using commerically available D_3-alanine. Unfortunately, during pilot attempts to synthesize the D_2-aniline, the Variac^R reaction vessel was damaged. Therefore, this alternative synthetic approach was designed and was found to satisfy the requirements of synthesizing trideuterated tocainide. Since carbobenzyloxyl-D_3-alanine is not commerically available, initial experiments were carried out to synthesize the carbobenzyloxy-alanine using unlabelled material as depicted in Scheme 14.

The reaction yielded 5.7 g (87.9 % chemical yield) of the crude CBZ-alanine. Recrystallization of the product in chloroform/ether offered a final yield of 3.2 g (55.8 %). In

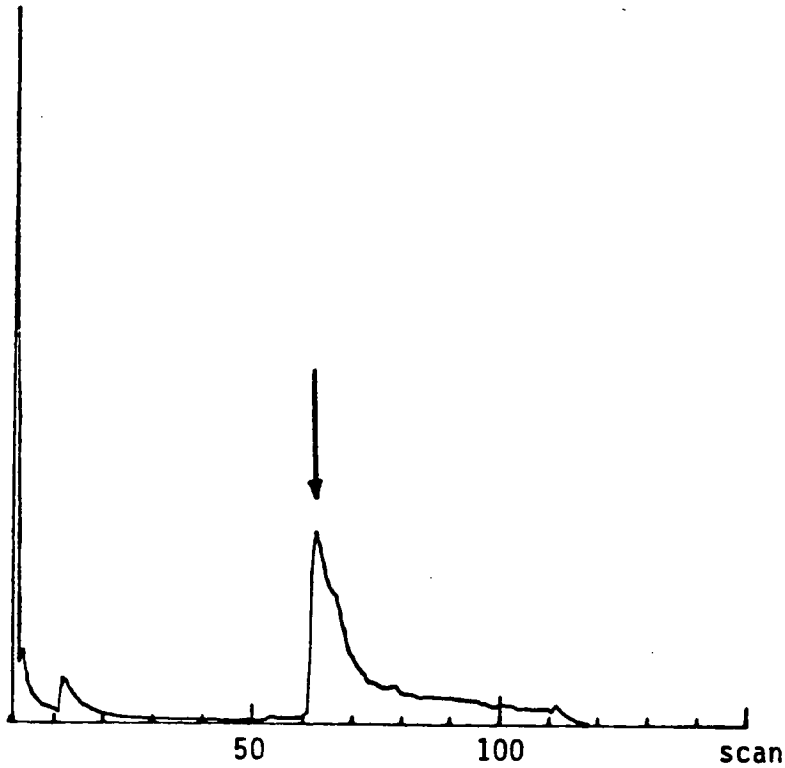


Figure 12 : The total-ion-chromatogram of synthetic tocainide.

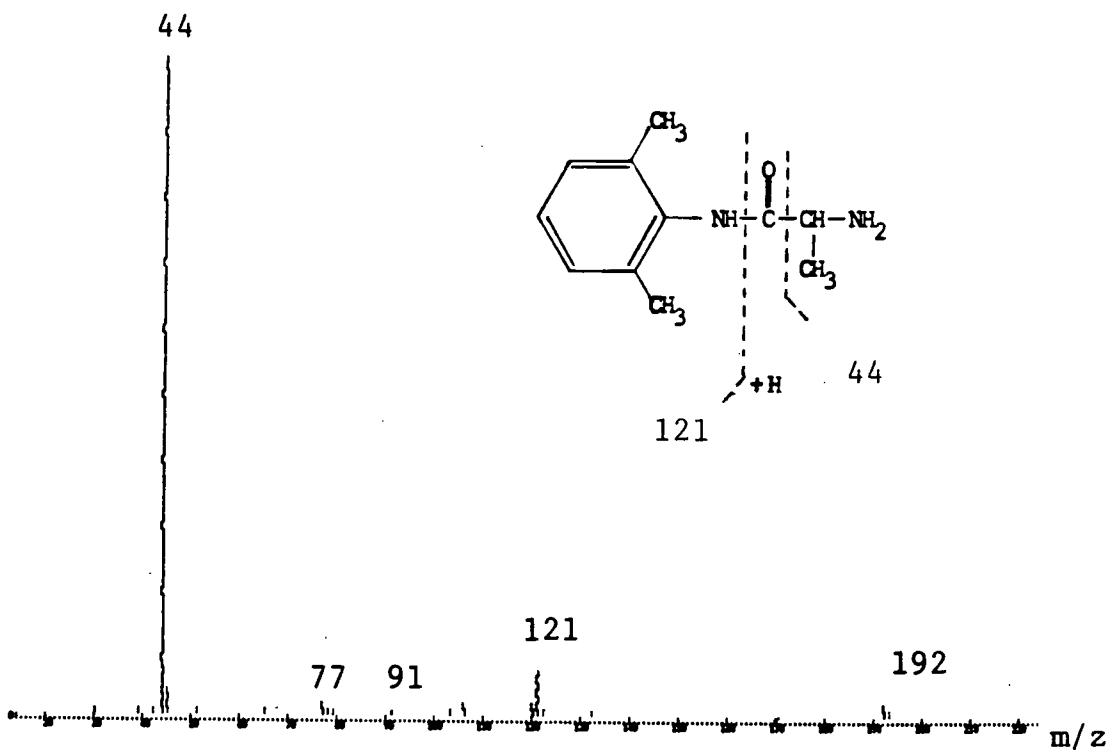
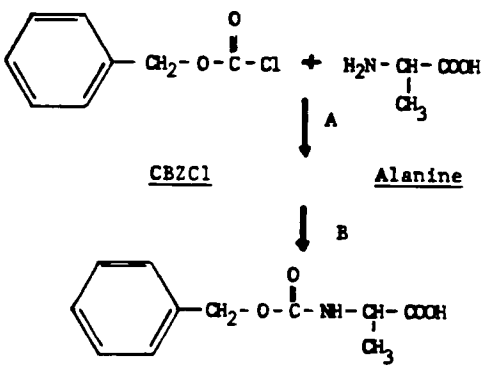


Figure 13 : The EI mass spectrum of synthetic tocainide.

accordance to this synthetic approach, the overall theoretical chemical yield for synthesis of this trideuterated tocainide from D3-alanine would offer approximately 6 % chemical yield.

Infrared (Nujol Mull) :

As shown in Figure 14, the absorption at 3350 cm^{-1} from the NH stretch of the amide was present. The broad -OH stretching vibration from the carboxylic acid give rise to the broad signal at 3000 cm^{-1}. overlaping the nujol -CH stretch. The two carbonyl stretching absorptions overlapped at 1700 cm^{-1}. Absorptions at 700 cm^{-1} and 750 cm^{-1} indicated the -CH rocking signals from the monosubstituted benzene ring.



A: Saturate $NaHCO_3$/ice cooling (6 hours).
B: 0.1 M HCl (PH 3).

Scheme 14 The synthesis of N-carbobenzyloxyalanine.

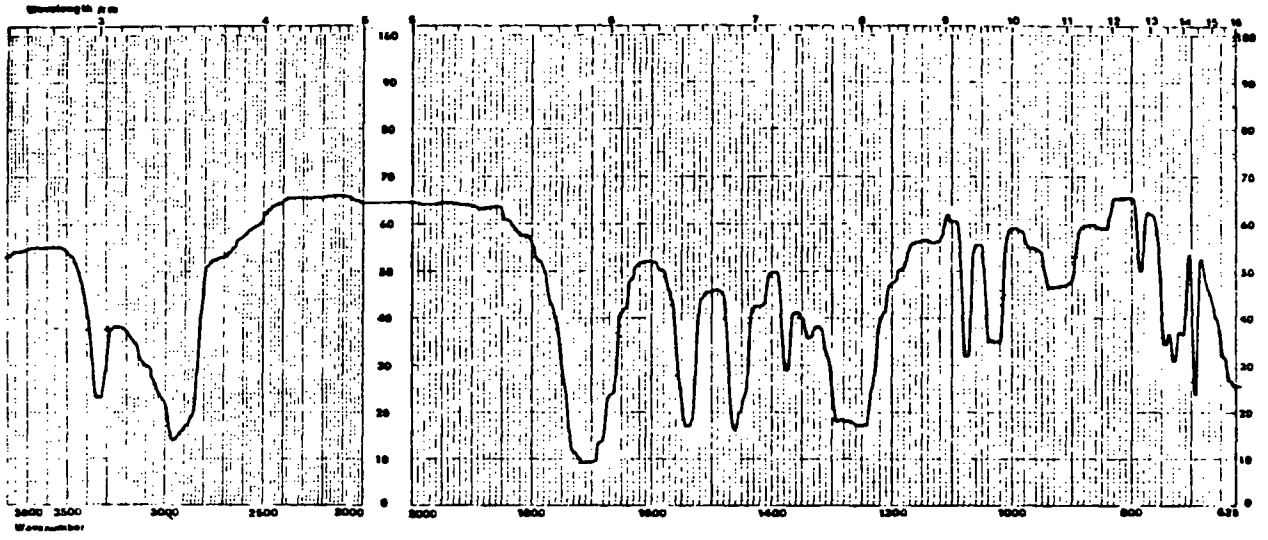


Figure 14 : The infared spectrum of N-carbobenzyloxy-alanine (mull).

B. Potential Tocainide Metabolites

1. 2-Ethanimino-2',6'-propioxylidide

This ethanimine was identified by Venkataramanan as a tocainide metabolite in rats (37). Since this metabolite has not been identified in humans, it was synthesized as a reference compound for subsequent metabolism studies. The TIC and mass spectrum are shown in Figures 15 and 16 respectively.

Infrared (Nujol Mull):

As shown in Figure 17, absorption at 3300 cm^{-1} indicated the presence of N-H stretching vibrations. The C=O and C-N stretching probably occurred at 1680 and 1670 cm^{-1} respectively. The aromatic absorptions occurred at 700 and 730 cm^{-1}.

Mass Spectrum (GCMS):

The mass spectrum of this ethanimine metabolite as shown in Figure 16 agreed with published data (37).

2. 3-(2,6-Xylyl)-5-methylhydantoin

The synthetic procedure reported by Elvin (35) was modified. Tocainide base was used in place of alanine and 2,6-dimethylaniline. A phosgene substitute, trichloromethyl chloroformate, was used in sodium dried toluene. The resulting product gave a single peak on the TIC from GCMS analysis.

Infrared (Nujol Mull):

As shown in Figure 18, absorption at 3310 cm^{-1} indicated the cyclic amide N-H stretching. The 2300 cm^{-1}(W) absorption

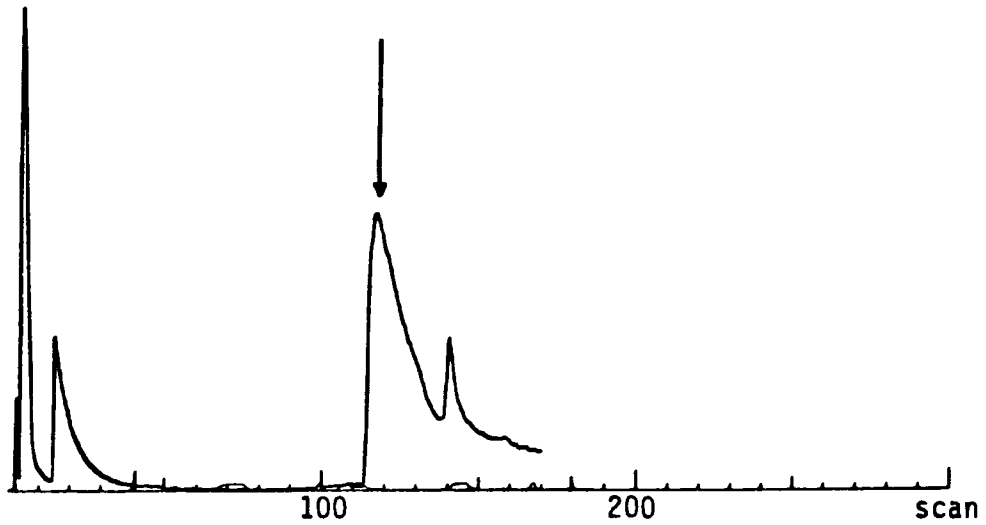


Figure 15. The total-ion-chromatogram of synthetic 2-ethanimine tocainide.

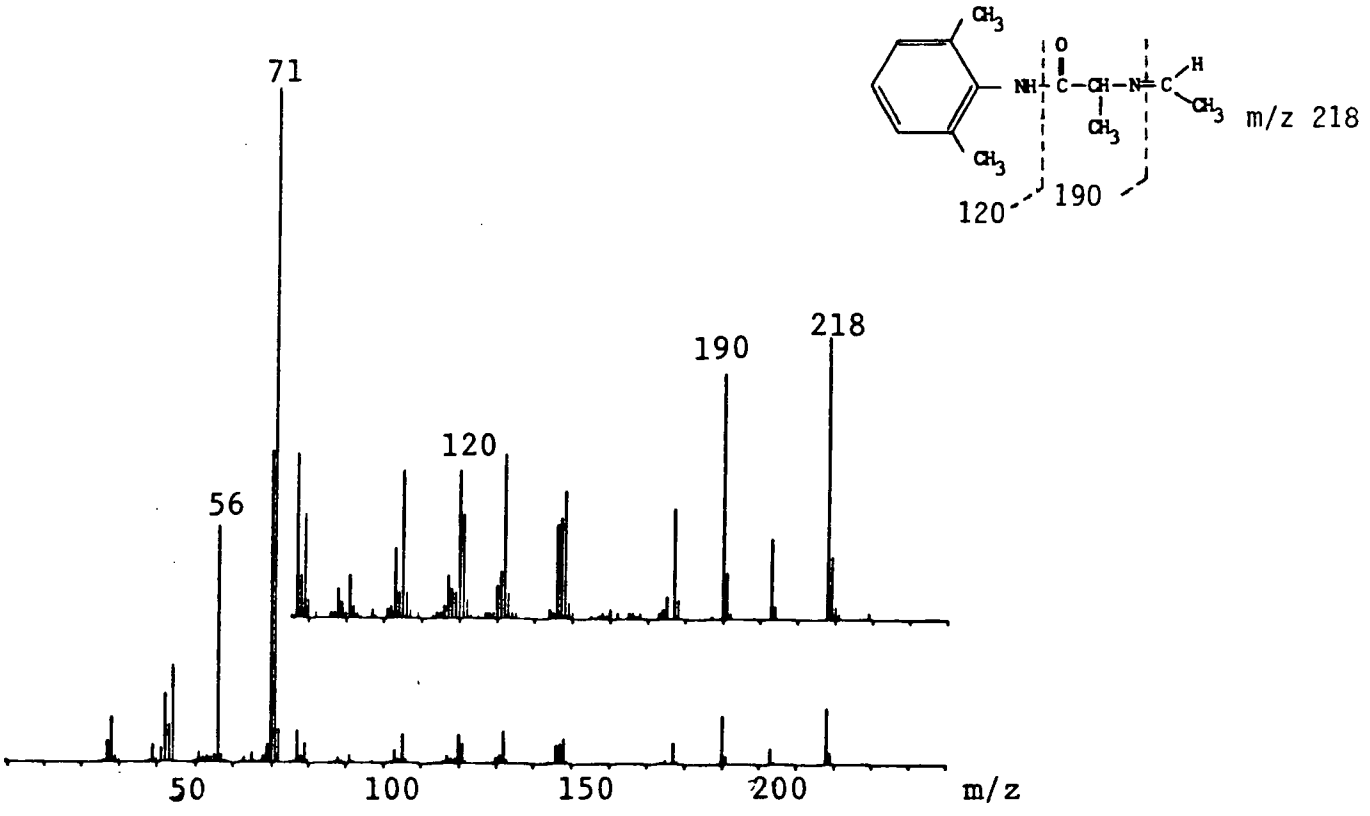


Figure 16. The EI mass spectrum of 2-ethanimine tocainide.

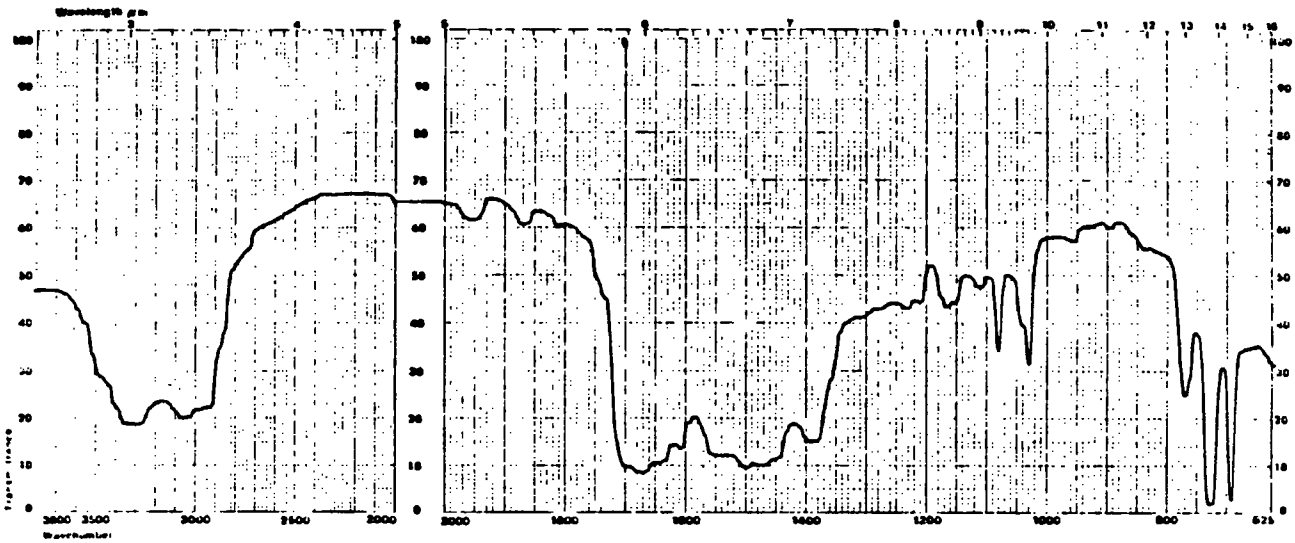


Figure 17 : The infared spectrum of tocainide ethanimine (mull).

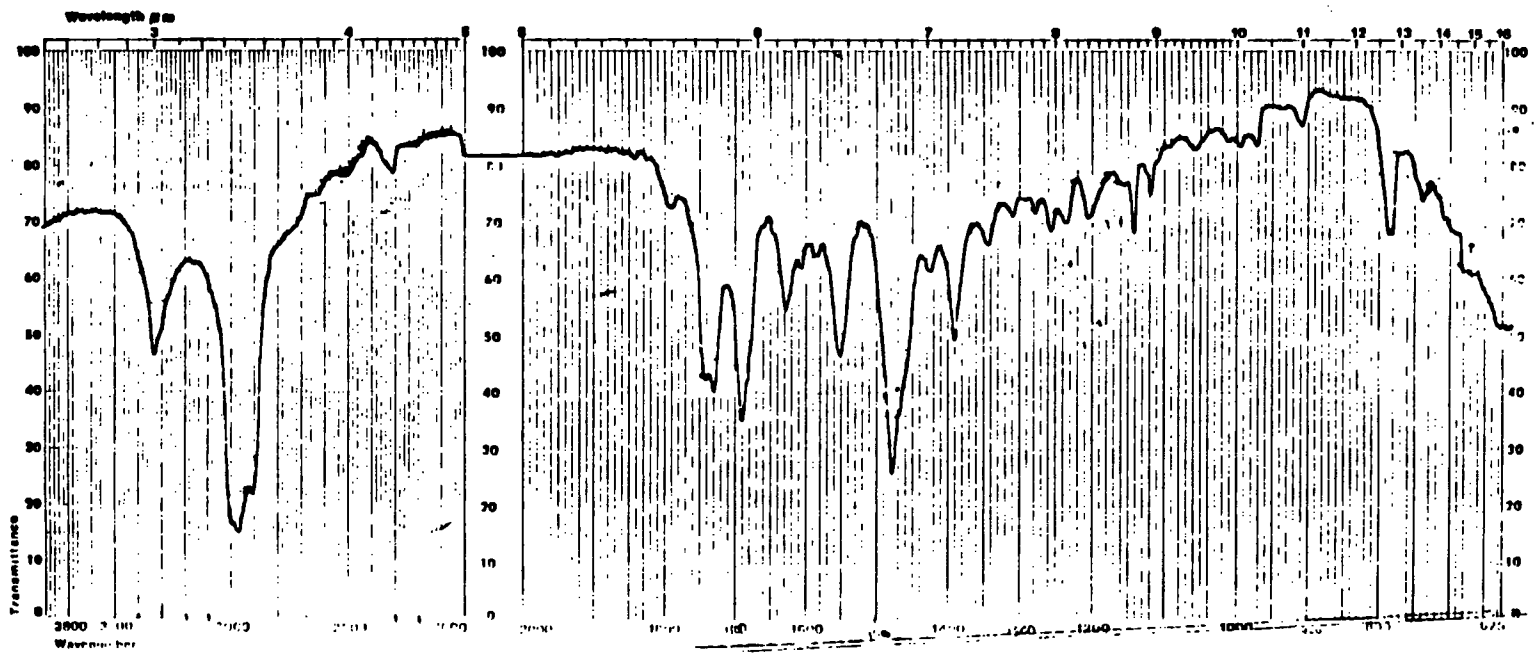


Figure 18 : The infared spectrum of 3-(2,6-xylyl)-5-methylhydantoin (mull).

was due to the isocyanate N-C=O stretching. Both 1795 cm^{-1} and 1700 cm^{-1} were from the hydantoin ring C=O stretching. These absorption values were in agreement with published data (35).

Mass Spectrum (GCMS):

As shown in Figures 19 and 20, the hydantoin M^+ at m/z 218 with the base peak at m/z 147, and the corresponding fragmentation were identical to Elvin's published spectrum (35).

400 MHz-[^{1}H]-NMR ($CDCl_3$):

Table 5 outlined the proton assignments. As shown in Figure 21, the 400 MHz spectrum revealed a doublet at 1.54 ppm due to splitting of the signal from the asymmetric C-5 methyl group protons. Further downfield, at shift 2.18 and 2.22 ppm, were the signals of the xylidide methyl protons. With molecular modelling, the conformation of the hydantoin ring in relation to the xylidine ring was such that the carbonyls were causing steric hindrance to one of the xylidine $-CH_3$ groups, thus rendering the two methyl groups non-equivalent. This interpretation proved to be helpful in determining the conformation of the tocainide glucuronide which will be discussed in later sections. The C-5 proton, at shift 4.25 ppm formed a quartet due to a small quadrupole coupling effect of the nitrogen at ring-1-position. The proton on the amide nitrogen was shifted downfield to 6.58 ppm into a broad singlet, again due to the quadrupole effect of the nitrogen atom. The aromatic protons were found between 7.1 and 7.3 ppm. The signal at 1.63 ppm was likely due to residual water present in the sample.

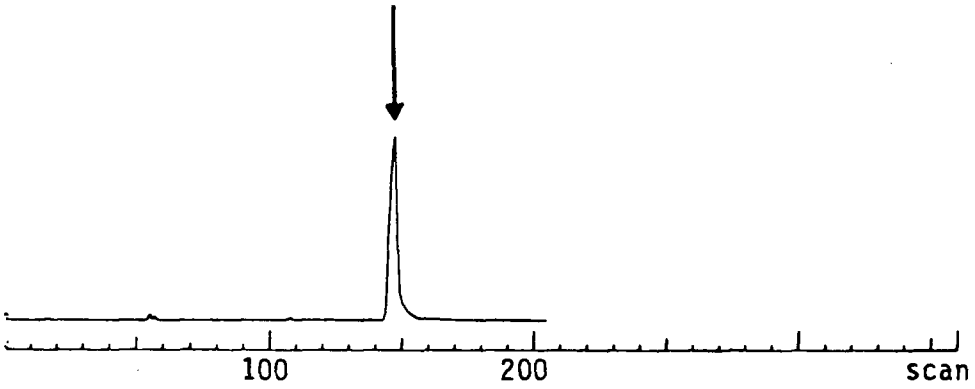


Figure 19 : Total-ion-chromatogram of the synthetic 3-(2,6-xylyl)-5-methylhydantoin.

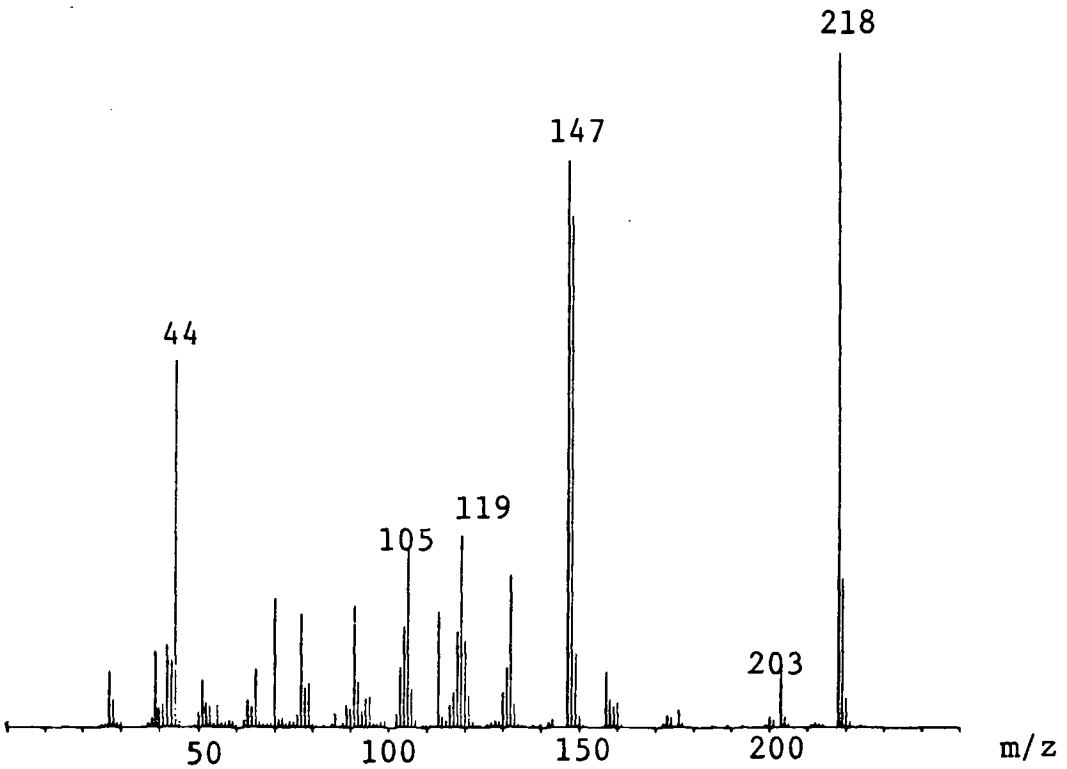


Figure 20 : The EI mass spectrum of the synthetic 3-(2,6-xylyl)-5-methylhydantoin.

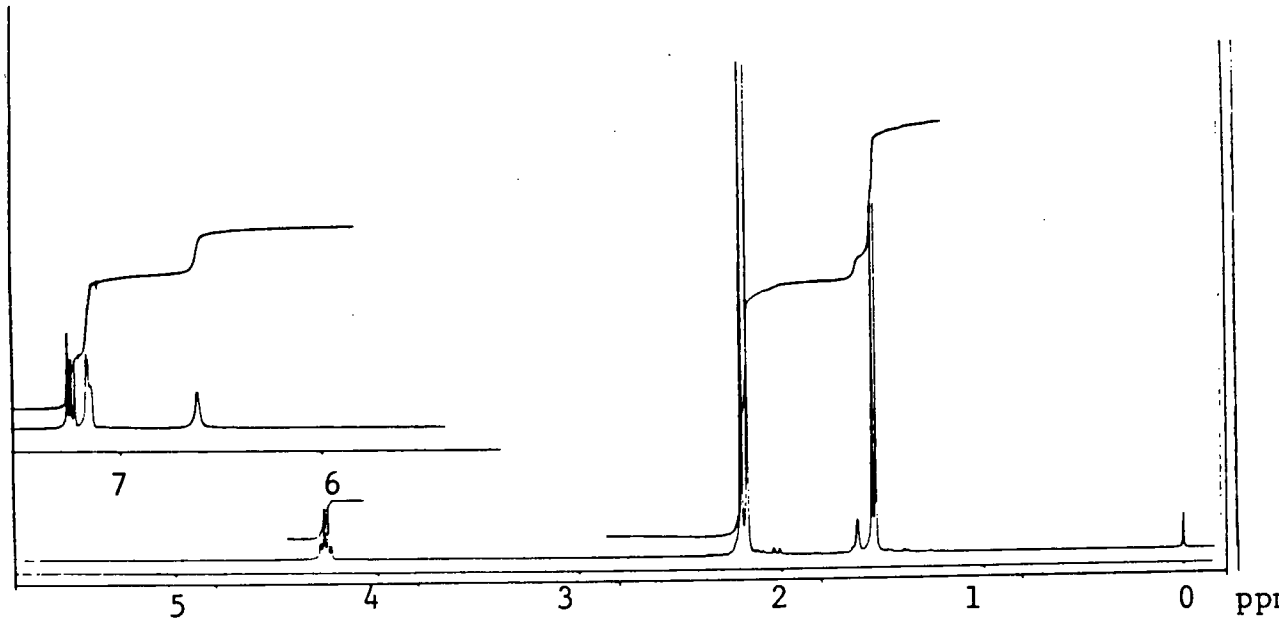
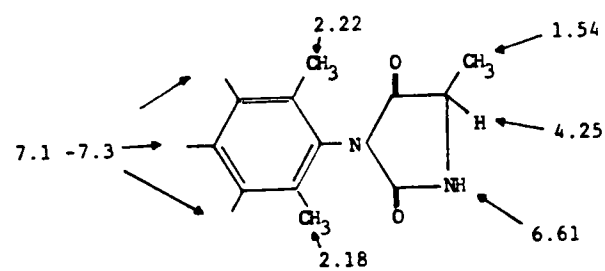


Figure 21 : 400 MHz-^{1}H-NMR spectrum of the synthetic 3-(2,6-xylyl)-5-methylhydantoin ($CDCl_3$).

Table 5 : Summarized data from the 400-MHz-[H]-NMR spectrum of 3-(2,6-xylyl)-5-methylhydantoin.



Shift (ppm)	Coupling	Integration (mm)	Proton(s)	Assignment
(TMS) 0.0				
1.54d	J = 6 Hz	60	3H(20mm/H)	5 - CH_3
2.18s*		63	3H	xylidine - CH_3
2.22s*		63	3H	xylidine - CH_3
4.25dq	J = 8 Hz	19	1H	5 - CH
6.61 bs		20	1H	1 - NH
7.1 - 7.3		61	3H	xylidine - H

Note:
* non-equivalent (H) due to ring conformational hinderance.

3. 3-(2,4,6-Xylyl)-5-methylhydantoin

The synthesis of 3-(2,4,6-xylyl)-5-methylhydantoin was carried out using a 2-step approach. 2-amino-2',4',6'-propioxylidide was first synthesized from 2,4,6-trimethylaniline and N-CBZ-alanine using the procedures as described for the synthesis of tocainide. This 2',4',6'-methyl analog of tocainide was then reacted with TCF to form the 3-(2,4,6-xylyl)-5-methylhydantoin analog using the procedure as described for the 3-(2,4-xylyl)-5-methylhydantoin.

Infrared (Nujol Mull):

As shown in Figure 22, the absorption at 3200 cm^{-1} indicated amide N-H stretching. The 1720 cm^{-1} and 1770 cm^{-1} absorptions were probably from ring C-O stretching. Furthermore, 700 cm^{-1} and 750 cm^{-1} were indicative of the aromatic absorption.

Mass Spectrum (GCMS)(silar-10C):

The recrystallized product appeared as a single peak in the TIC as shown in Figures 23. The mass spectrum in Figure 24 indicated that a molecular ion at m/z 232 was observed as the base peak. Fragments at m/z at 161, 144, 146, 119 and 217 were found to agree with the corresponding fragmentation as described for the 2,6-xylyl hydantoin analog.

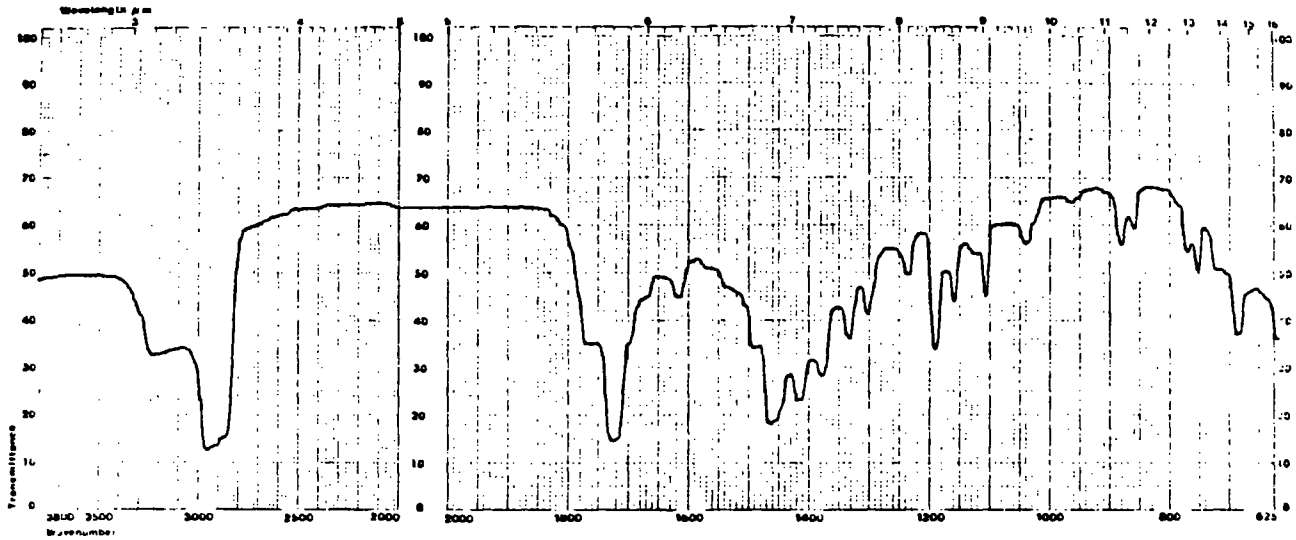


Figure 22 : The infared spectrum of 3-(2,4,6-xylyl)-5-methyl-hydantoin (mull).

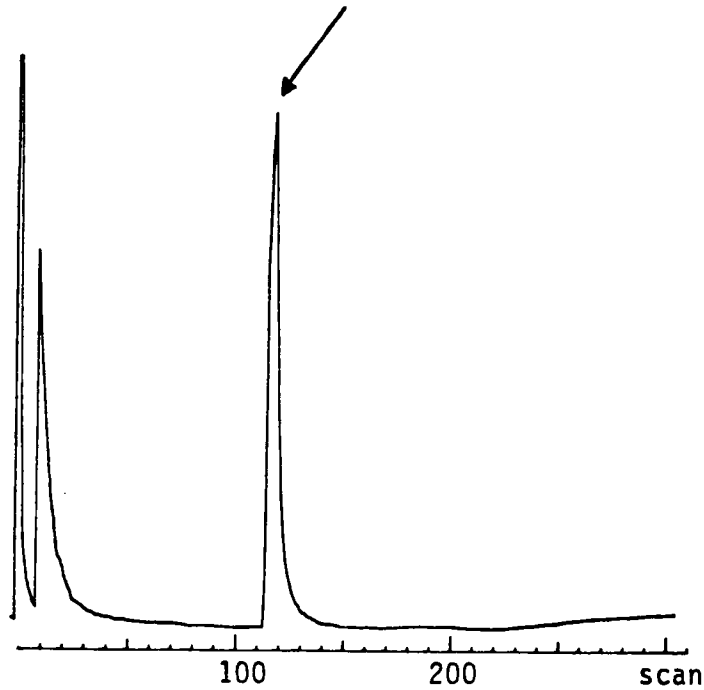


Figure 23 : Total-ion-chromatogram of the synthetic 3-(2,4,6-xylyl)-5-methylhydantoin.

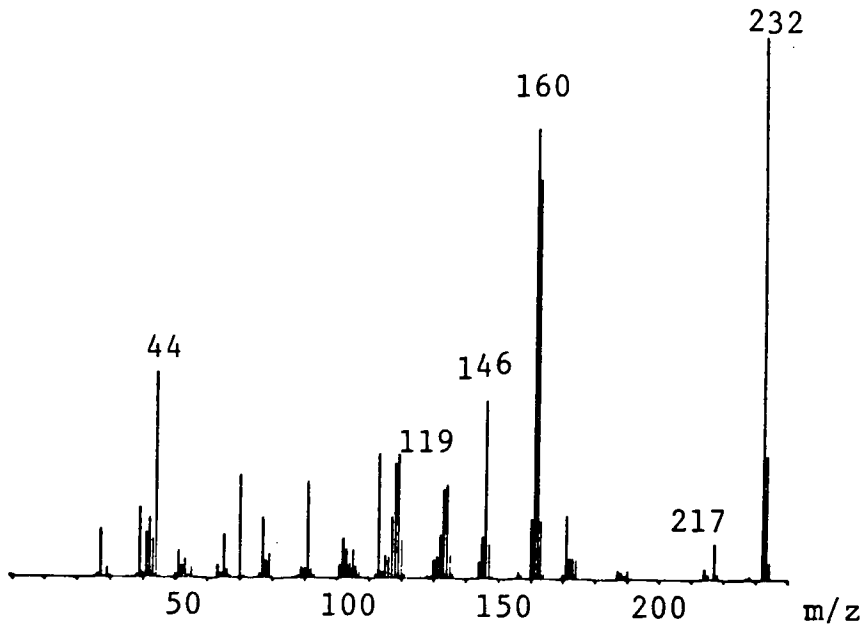
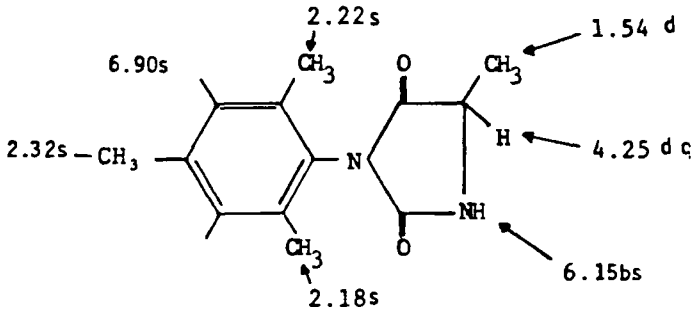


Figure 24 : The EI mass spectrum of the synthetic 3-(2,4,6-xylyl)-5-methylhydantoin.

80 MHz-[^{1}H]-NMR:

As shown in Figure 25, the 80 MHz spectrum revealed similar proton shift characteristics as described for the 2,6-xylyl-5-methylhydantoin analog. Table 6 outlines the proton assignments.

Table 6. Summarized data from the 80-MHz proton NMR spectrum of 3-(2,4,6-xylyl)-5-methylhydantoin.



Shift (ppm)	Coupling	Integration (mm)	Proton(s)	Assignment
(TMS) 0.00				
1.54 d	J=6 Hz	60	3H	5-CH_3
2.18s		63	3H	xylidine - CH_3
2.20s		63	3H	xylidine - CH_3
2.32s		63	3H	xylidine-CH_3
4.25 d q	J=8 Hz	19	1H	5-CH
6.15bs		20	1H	1-NH
6.90s		40	2H	xylidine - H

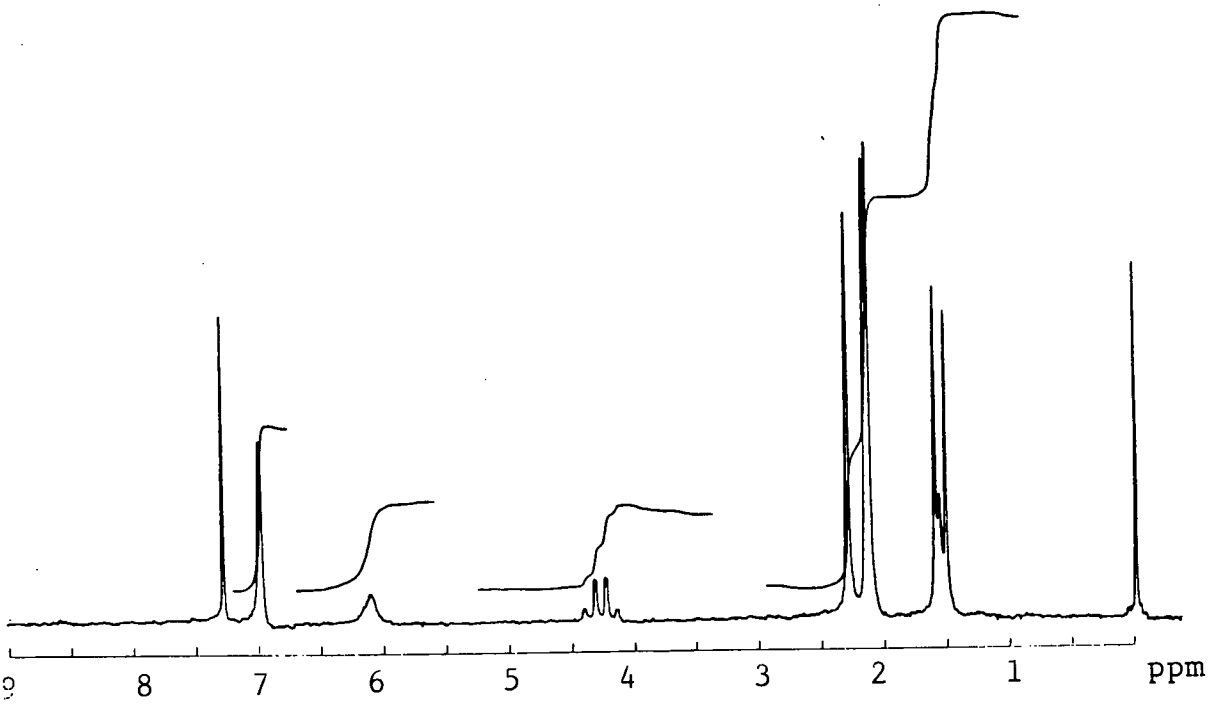


Figure 25 : The 80-MHz-[H]-NMR spectrum of the synthetic 3-(2,4,6-xylyl)-5-methylhydantoin ($CDCl_3$).

C. Tocainide Metabolites

1. Derivatization Techniques for Glucuronides

A permethylation method has been described by Thompson and Desiderio (61) for the analysis of intact ether glucuronides. This permethylation procedure, using dimethylsulfinylmethide sodium ($DMSO^-$) and methyl iodide (CH_3I), is a useful technique for the analysis of saccharides, oligopeptides and uronic acid residues in polysaccharides (64,65).

Various derivatization techniques have been published for GC or GCMS analysis of intact glucuronides. Fenselau (65) and Johnson reviewed these techniques for use with the appropriate functional groups on the aglycone, as well as on the glucuronic acid moiety. The types of derivatization reagents used for GC analysis of glucuronides are:

1. Acetic anhydride/methanesulfonic acid/ diazomethane.
2. Trifluoroacetic anhydride/pyridine/ diazomethane.
3. Methyl Iodide/$DMSO^-$ (permethylation).
4. Bis(trimethylsilyl)-trifluoroacetamide (BSTFA).
5. BSTFA/diazomethane.
6. Tri-Sil-Z^R.

Initial derivatization experiments of raw urine samples with Tri-Sil-Z, BSTFA and BSTFA with diazomethane did not yield useful information for various known metabolites of tocainide. However, the use of reference compounds, P-nitrophenol glucuronide and D-glucose, in these reactions to monitor the derivatization conditions proved the usefulness of these derivatization

techniques to obtain molecular weight information for compounds of high molecular weight. The mass spectra of these reference compounds are shown in Figures 26 and 27.

With these data from the derivatized sugar and P-nitrophenol glucuronide, we speculated that the carbamoyl- or ureido-glucuronide of tocainide were probably hydrolyzed either during reaction or in the GC inlet.

2. Identification of Tocainide Glucuronide by TLC Isolation and CH_3I/Permethylation

Band 1b sample, obtained after a 2-step TLC isolation, revealed a substantial quantity of free tocainide after acid hydrolysis. Enzyme hydrolysis of the same two bands revealed a slightly reduced quantity of free tocainide compared to that from acid hydrolysis. The presence of free tocainide in the sample after enzyme hydrolysis indicated that the sample contained a glucuronic acid conjugate of tocainide.

After the isolated band 1b was adjusted to pH 13 with sodium hydroxide, the sample was analyzed by reverse-phase HPLC using a mobile phase of 25 % acetonitrite in 0.05 M potassium acetate. This sample revealed the presence of 3-(2,6-xylyl)-5-methylhydantoin having an identical retention time to that of the synthetic standard.

The presence of tocainide after beta-glucuronidase hydrolysis, and the presence of the hydantoin derivative after base hydrolysis suggest that the sample contained a glucuronic acid conjugate of tocainide. In an attempt to examine the intact structure of this glucuronide, band 1b was permethylated with

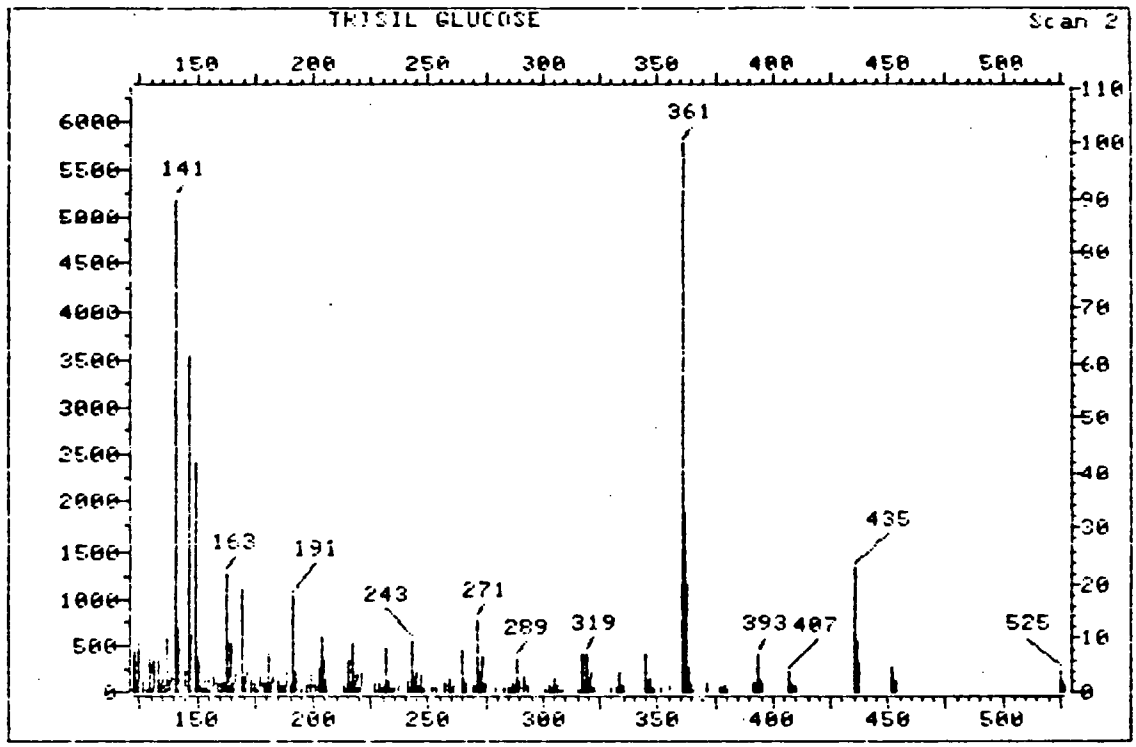


Figure 26 : The CI mass spectrum of TMS-derivative of glucose.

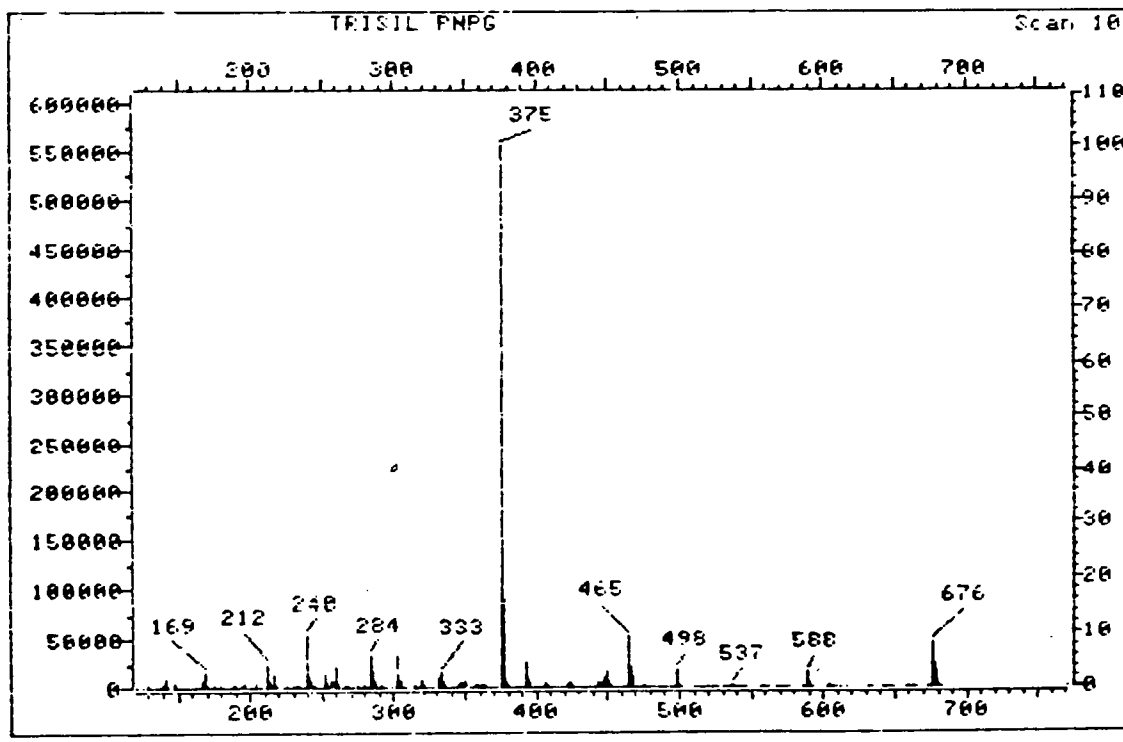


Figure 27 : The CI mass spectrum of TMS-derivative of p-nitrophenol glucuronide.

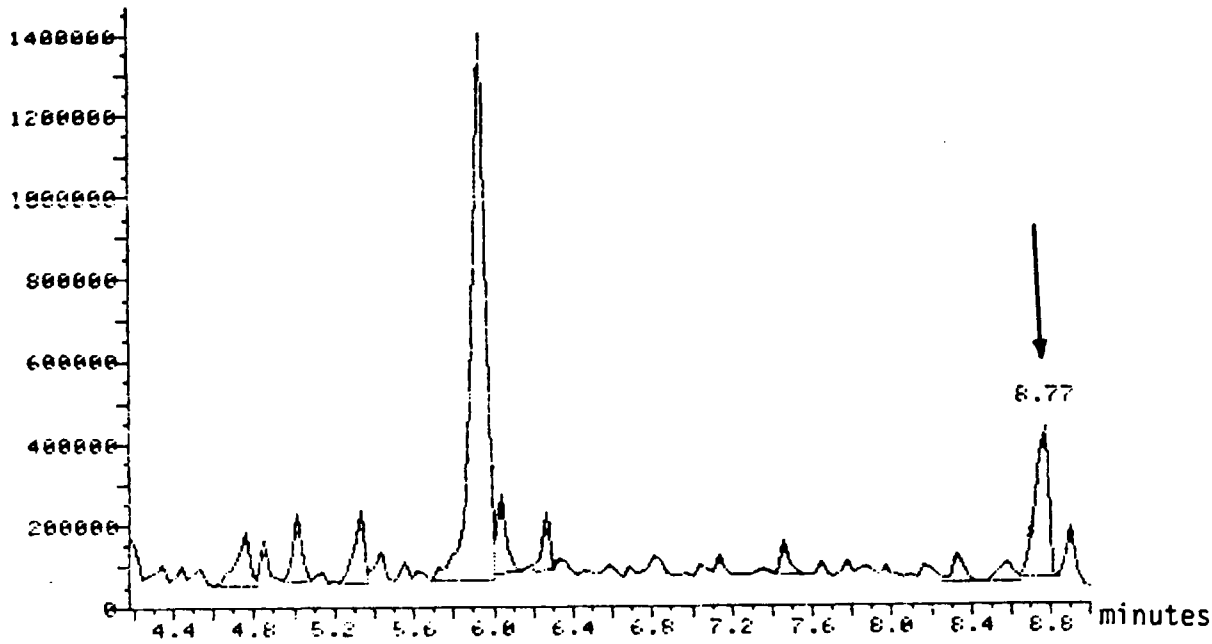


Figure 28 : Total-ion-chromatogram of permethylated XAD/crude urine extract isolated band 1b sample.

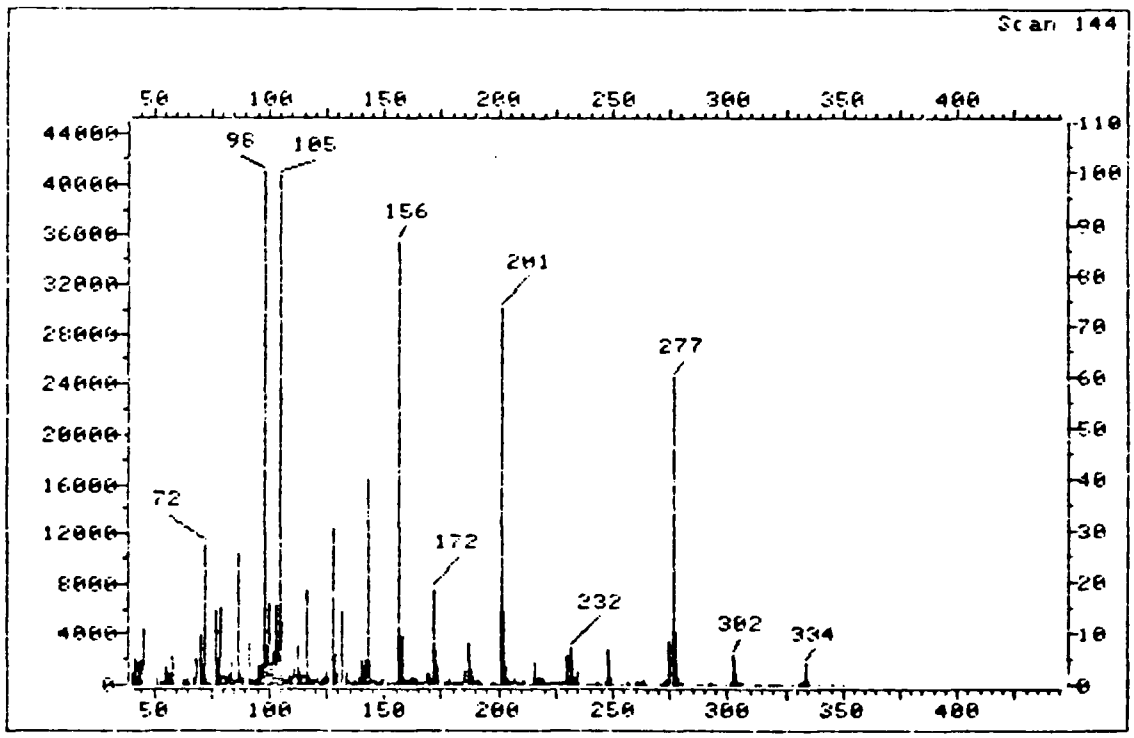


Figure 29 : Mass spectrum of permethylated tocainide carbamoyl ester glucuronide (electron impact ionization).

methyliodide in dry DMSO as described. The TIC and the mass spectrum of the permethylated glucuronide are shown in Figures 28 and 29.

The possible mass ion fragments resulted from the permethylated intact tocainide carbamoyl ester glucuronide are outlined in Figure 30.

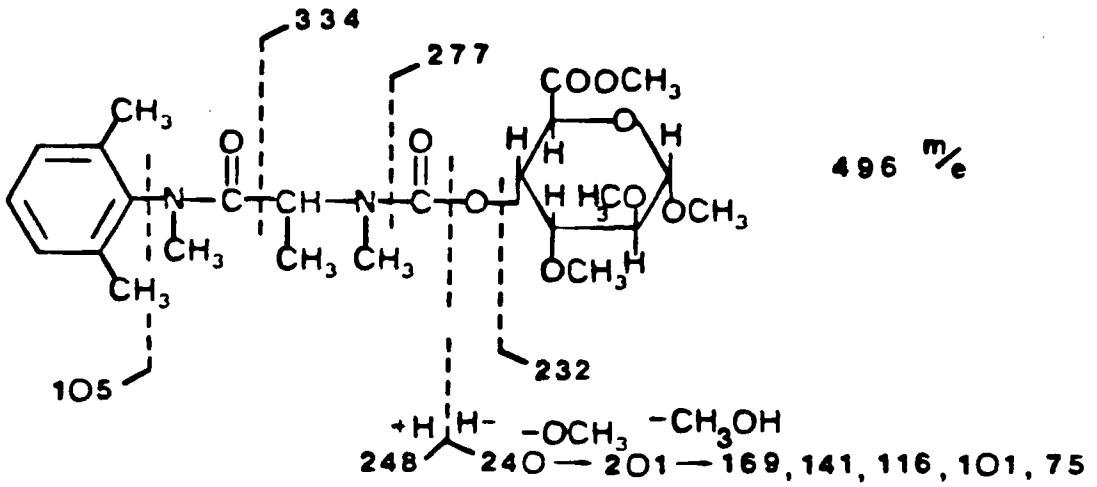


Figure 30 : Fragmentation pattern of the proposed tocainide carbamoyl ester glucuronide.

Although the molecular ion of the tocainide carbamoyl glucuronide was not observed, the m/z 277 ion was present. This ion was most likely formed via the cleavage of the amide linkage at the site of conjugation. The m/z 334 ion may have been resulted from the cleavage of the carbonyl bonding within the tocainide moiety. The m/z 232 ion was probably resulted via cleavage of the glucosidic bond accompanied by proton transfer from the permethylated glucuronide moiety to the aglycone.

The spectrum containing the mass ions m/z 232, 201, 169, 141, 161, 101 and 75 suggested that the permethylated sample was a glucuronide. These ions, resulted from the cleavage of the glucuronic acid moiety, are diagnostic for the identification of permethylated glucuronic acid.

During mass spectral analysis, p-nitrophenol glucuronide was used as a reference compound to confirm our results with published data. For the permethylated p-nitrophenol glucuronide, the mass spectral analysis was carried out using the CI mode with methane as the reagent gas. The mass spectrum and total-ion chromatogram are shown on Figures 31 and 32.

The striking features which were common to the spectra of these compounds were the pair of ions m/z 201 and 101. One of these two ions was present in every spectrum. These ions have proved to be the most diagnostic ions in identifying permethylated glucuronides (65-68).

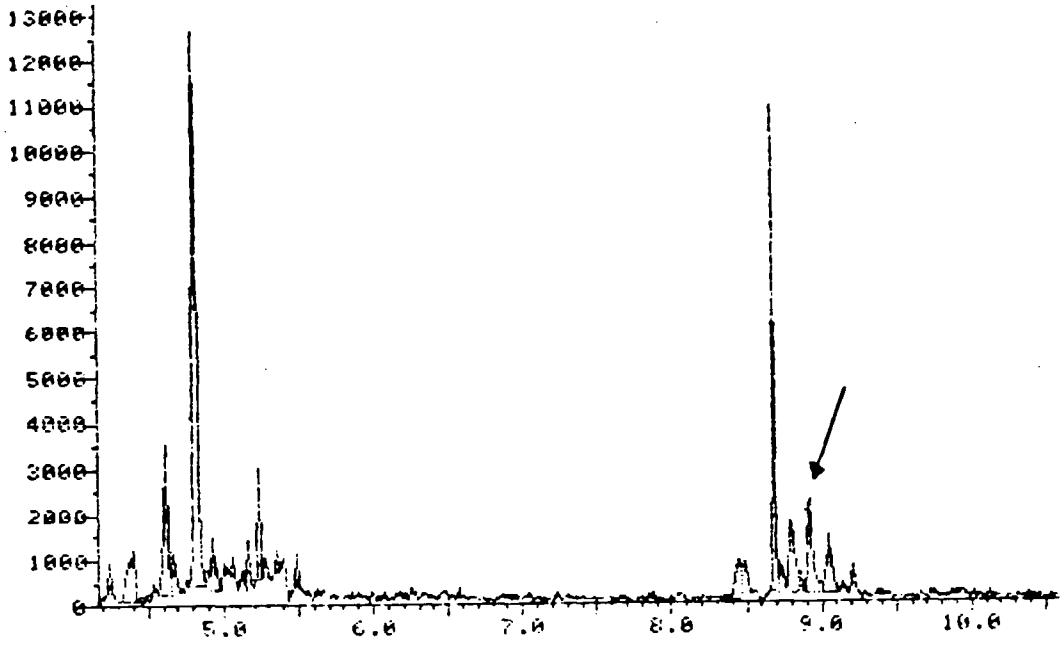


Figure 31 : Total-ion-chromatogram of permethylated para-nitrophenol glucuronide.

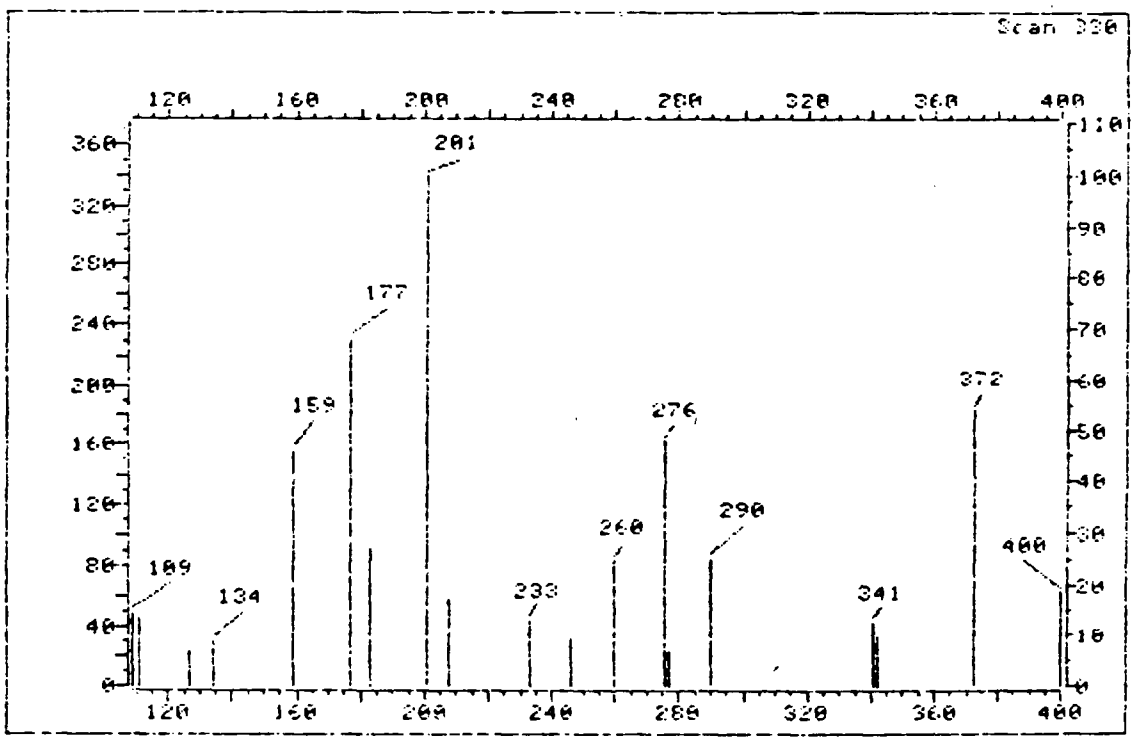


Figure 32 : The CI mass spectrum of the permethylated para-nitrophenol glucuronide.

The molecular ion m/z 372 of the permethylated p-nitrophenol glucuronide was present. The m/z 400 ion was formed from the C_2H_5 adduct. The m/z 340 ion was probably resulted from the cleavage of a methoxy group, and m/z 308 was probably resulted from the cleavage of a CH-OH group. Again the fragment at m/z 201 was present along with ions at m/z 169 and 232. All these three ions must have resulted from the fragmentation of the permethylated glucuronic acid moiety.

As pointed out by Thompson (66), the M^+ ions of permethylated glucuronides are not always present or detected by GCMS techniques. The peaks of m/z 277, 334 and 248 were mostly likely resulted from the cleavages of the intact carbamoyl glucuronide. These ion fragments were of important diagnostic value for the identification of permethylated tocainide carbamoyl ester glucuronide. The m/z 105 ion was a prominent ion from deamination of the xylidide portion of tocainide. This latter ion fragment, together with the former ones discussed, suggested the glucuronide was a tocainide carbamoyl ester glucuronide.

3. Attempted Identification of Tocainide Metabolites by Lyophilization Followed by GCMS (Permethylation)

The purpose of this experimental protocol was to identify conjugated metabolites which were sufficiently polar to prevent solvent extraction. From the TIC of the permethylated blank urine sample, hippuric acid was identified as its methyl ester. From the underivatized lyophilized sample, a molecular ion at m/z 218 was identified to be the 3-(2,6-xylyl)-5-methylhydantoin. No further useful data were obtained during

this experiment.

4. Attempted Identification of Metabolic Intermediates of Tocainide

According to earlier discussions, formation of the tocainide glucuronide could take place through a carbamoyl or ureido intermediate. This section describes the attempted identification of these intermediates from human urine.

As outlined previously in Scheme 11, beta-glucuronidase enzyme was used to produce the ionized carbamic acid or urea-adduct intermediate. Following hydrolysis, acidic extraction with methylene chloride, and permethylation, the observation of the carbamic acid intermediate by GCMS should be theoretically possible. Conversely, after basic extraction with methylene chloride and permethylation, the observation of the urea-adduct intermediate should be possible. Later sections will discuss the synthesis of this urea-adduct and the GCMS data of this permethylated hypothetical intermediate.

In studying the formation of unstable intermediates using enzyme hydrolysis, the site of the enzymatic cleavage must be considered. Eisenberg, F. Jr. (69) studied the cleavage of benzoyl glucuronic acid and methyl glucosiduronic acid in oxygen-labelled acetate buffer. From the distribution of O^{18} in the hydrolyzed products, it was inferred that in both substrates the glucuronosyl-O bond was cleaved. Variations in pH from 4 to 6 had no effect on the isotope distribution in the enzymatic reaction. Non-enzymatic hydrolysis of both substrates in strong acids also resulted in glucuronosyl-O-cleavage (69).

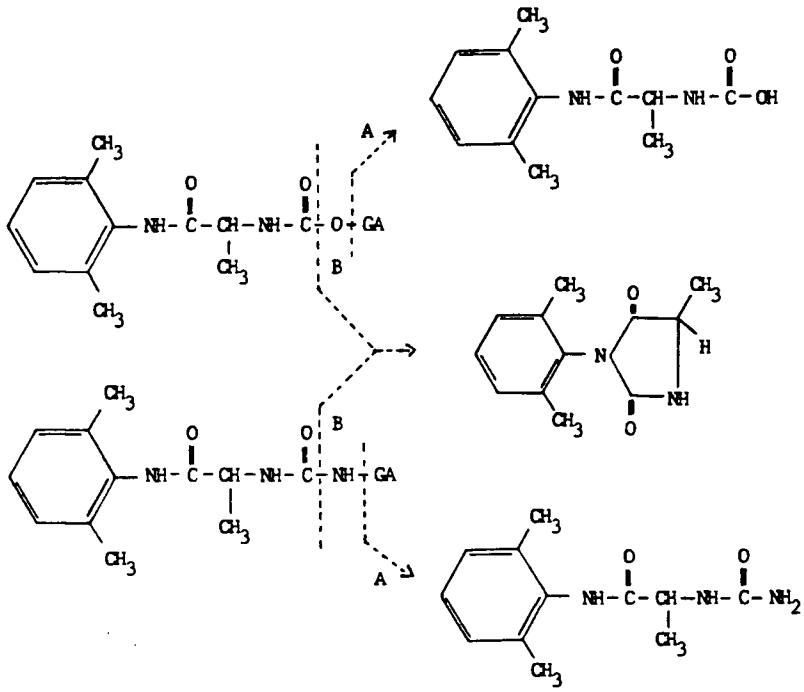
The implications of these findings, as they reflect on the possible identification of the tocainide metabolic intermediates, are outlined in Scheme 15.

With the possible enzymatic and non-enzymatic cleavages in mind, if the splitting occurs at the C-O or the C-N bond, then either the carbamoyl ester or the ureido glucuronide would give rise to the same hydrolyzed product.

During this experiment, the mass spectra of the permethylated tocainide, para-nitrophenol glucuronide, 2,6-dimethylaniline, lactoxylidide and the hydantoin were obtained as reference spectra for positive identification. In Figures 33 and 34, the molecular ion of permethylated tocainide at m/z 244 suggested a permethylation of 3 $-CH_3$ groups, replacing 2 hydrogens from the primary amine nitrogen and 1 hydrogen from the amide nitrogen. The base peak at m/z 72 corresponded to the cleavage between C-2 and C-3 bond of permethylated tocainide.

In Figure 35, the mass spectrum of the permethylated 2,6-dimethylaniline revealed a similar fragmentation pattern as was obtained from earlier deuteration experiments. The molecular ion at m/z 149 indicated an addition of 2 $-CH_3$ groups on the aniline nitrogen.

The permethylated lactoxylidide molecular ion, as shown in Figures 36 and 37, was observed with a molecular ion at m/z 221. The presence of m/z 134, as found in the spectrum of permethylated tocainide, suggested a permethylated xylidine moiety. In addition, m/z 206 suggested that a $[M-15]^+$ ion was lost due to the cleavage of a $-CH_2$ on the permethylated lactic



A: Beta-glucuronidase enzyme hydrolysis.
B: Basic hydrolysis (PH 12).

Scheme 15. The theoretical tocainide intermediates derived from the hydrolysis of the tocainide glucuronide.

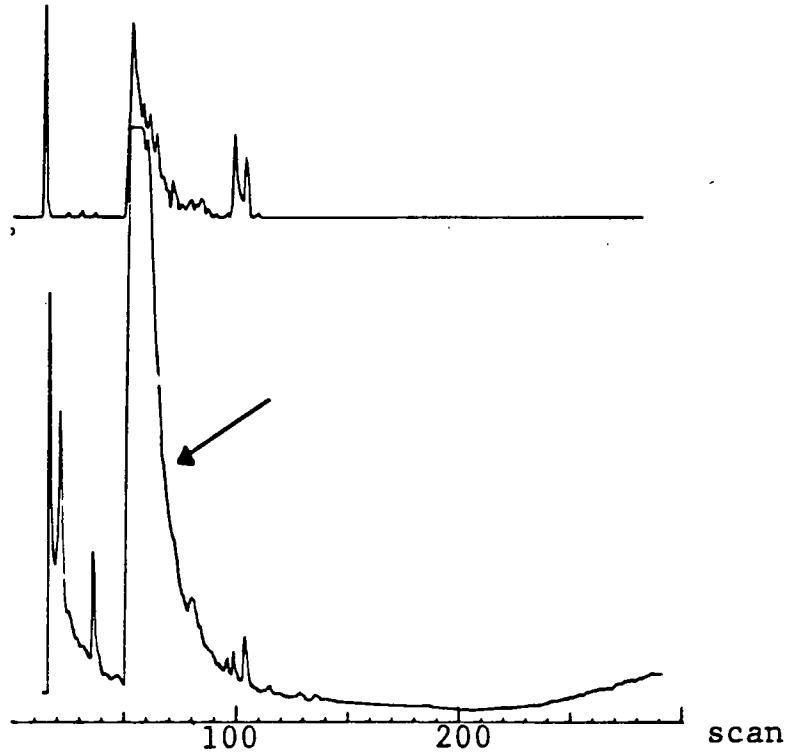


Figure 33 : The total-ion-chromatogram of permethylated tocainide.

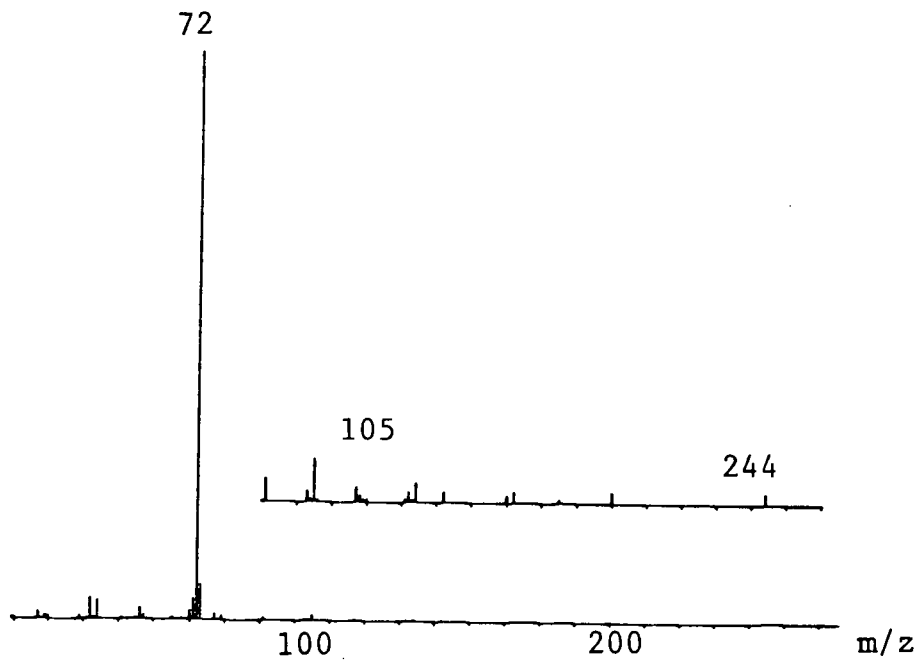


Figure 34 : The EI mass spectrum of the permethylated tocainide.

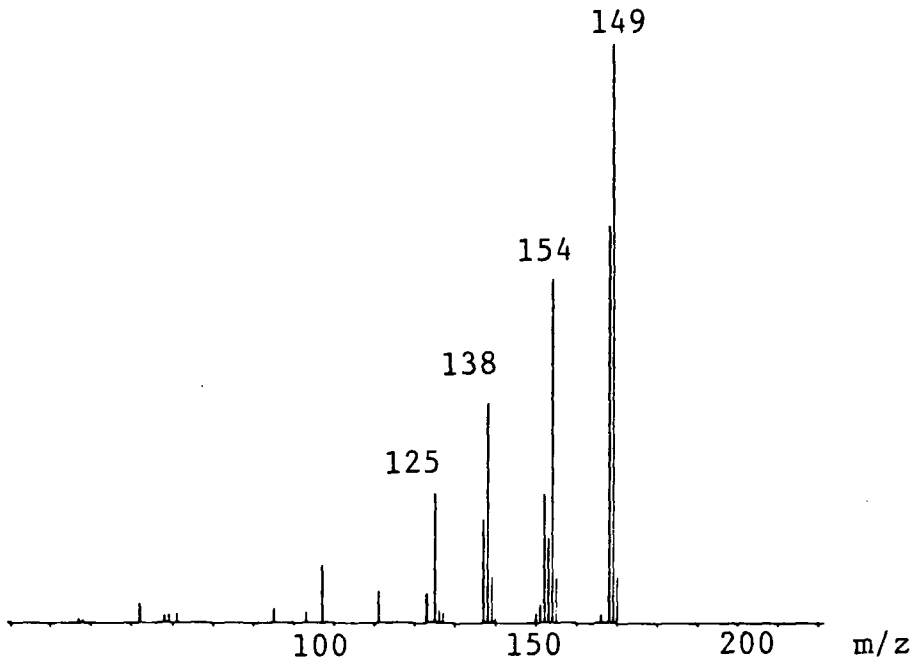


Figure 35 : The EI mass spectrum of the permethylated 2,6-dimethylaniline.

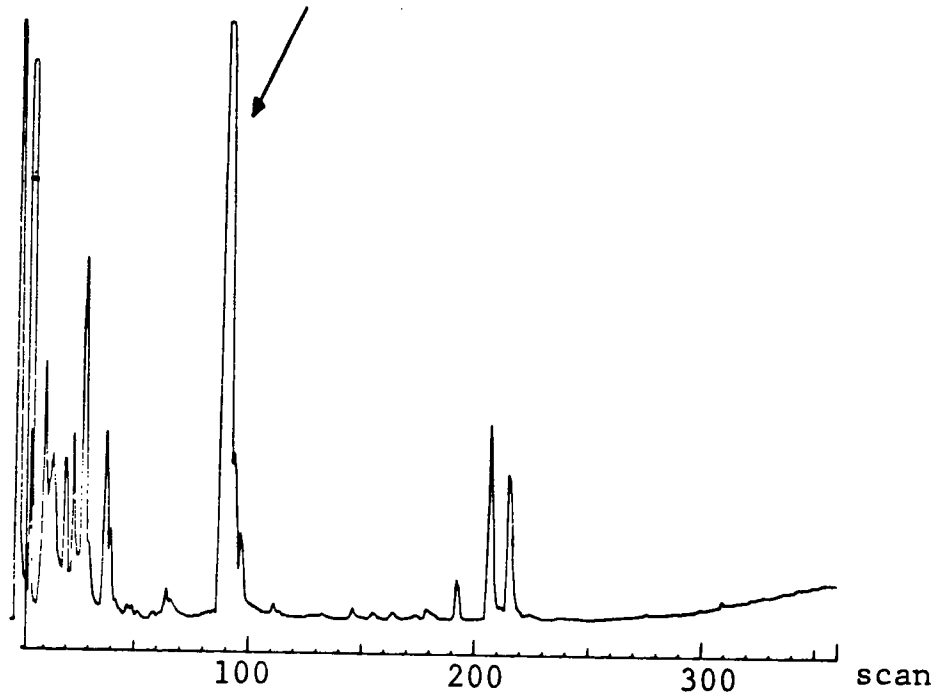


Figure 36 : The total-ion-chromatogram of the permethylated lactoxylidide.

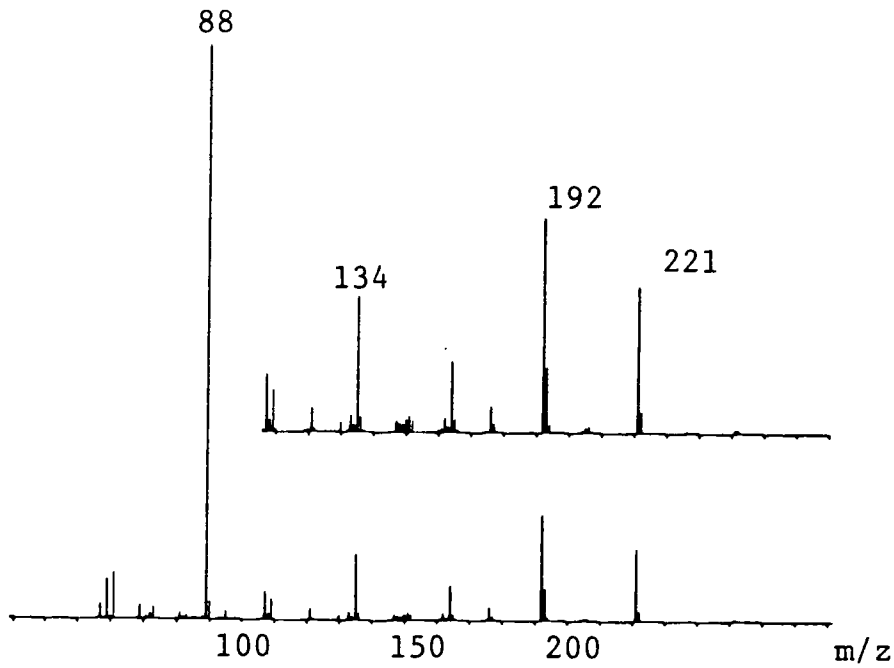


Figure 37 : The EI mass spectrum of the permethylated lactoxylidide.

acid moiety.

The TIC and the mass spectrum of the permethylated 3-(2,6-xylyl)-5-methylhydantoin, as shown in Figures 38 and 39, could not be interpreted. The TIC revealed two symmetrical peaks of equal intensity and a mass spectral fragmentation pattern which suggested the presence of a pair of diastereoisomers. The base peak of m/z 135 may have resulted from the permethylated xylidine, and the m/z 162 ion may have been due to the N-carboxyl fragment (xylidine- NCH_3.CO-). However, an ion at m/z 282 did not correspond to any hypothetical structure.

For ease of interpretation of the following data in this section, discussion of the experimental results will follow the corresponding numbering as shown in Table 7.

Table 7. The samples obtained from the experimental protocol used for identifying tocainide glucuronide intermediates.

Sample	Experimental (pooled urine sample)
1	Extraced by RPC-18 elute-bond^R Column
2	Extracted at PH 12
3	Extracted at PH 3
4	Enzyme hydrolyzed, extracted at PH 3
5	Enzyme hydrolyzed, extracted at PH 12
6	Acid hydrolyzed
7	Basic extracted PH 9, underivatized
8	Acidic extracted PH 3
9	Blank urine

Note: All samples were permethylated with $CH_3I(CD_3I)$/ DMSO Na except Sample 7 and 8.

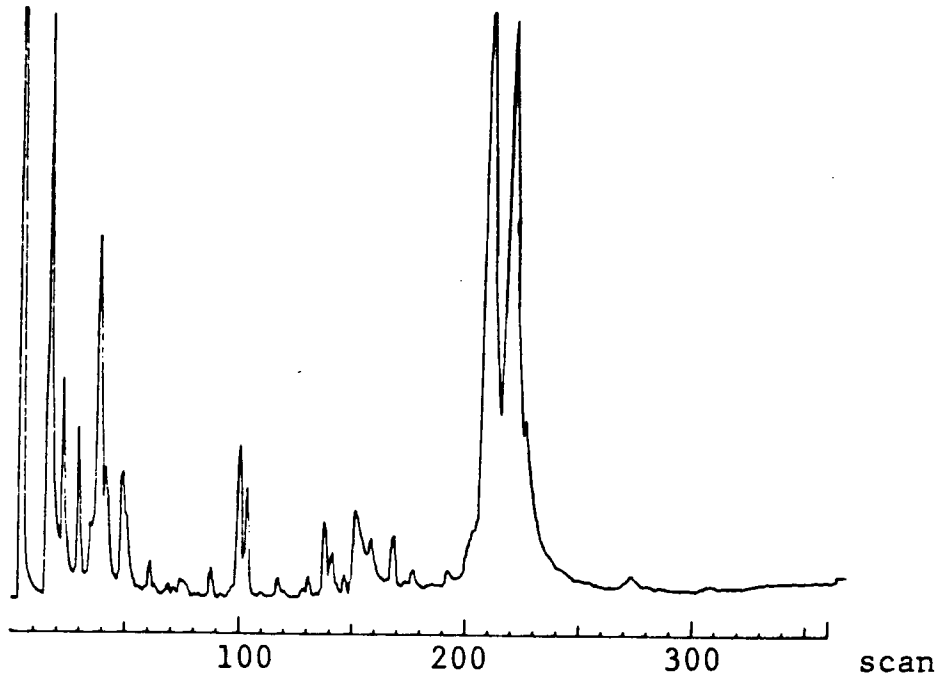


Figure 38 : The total-ion chromatogram of the permethylated 3-(2,6-xylyl)-5-methylhydantoin.

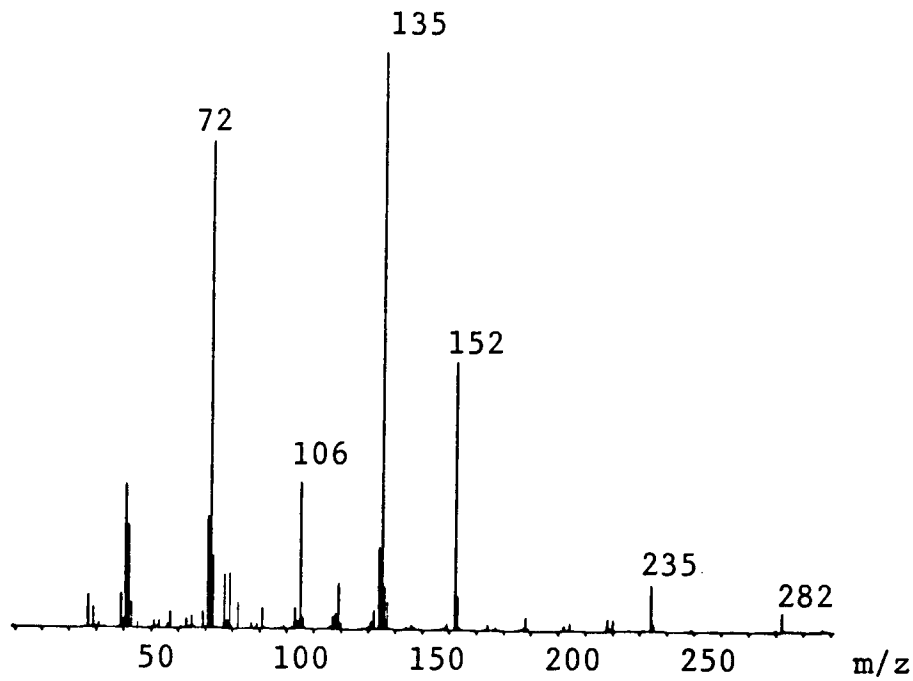


Figure 39 : The EI mass spectrum of the permethylated 3-(2,6-xylyl)-5-methylhydantoin.

Sample 1 (extracted by RP-18 Bond-elute^{R} column) and sample 9 (blank urine) revealed a major component with a base peak at m/z 105. This compound was identified to be permethylated hippuric acid when compared with the Sadlter^{R} (70) reference spectrum. Caffeine was also identified from its base peak at m/z 194. From sample 1, neither tocainide nor its metabolites could be identified, possibily due to lack of sensitivity.

The TIC from Sample 2 revealed two components, both with molecular ion at m/z 135 and a base peak at m/z 88. These two components have also been observed with identical retention times to those from the permethylated hydantoin sample. A component at Scan 136 was found to contain the m/z 162 ion. This was suspected to be the xylidide-NCH_3.CO- fragment, as observed from the permethylated hydantoin spectrum. However, the identity of this compound could not be conculsively determined.

The acidic-extracted, and permethylated Sample 3, was found to contain caffeine as the only identifiable component.

Both Samples 4 and 5 again were found to contain hippuric acid methyl ester, with a molecular ion at m/z 206, and caffeine, with a molecular ion at m/z 194. Attempts to identify the tocainide-urea, or the tocainide-carbamic acid intermediate, were not successful.

In the acid hydrolyzed Sample 6, hippuric acid methyl ester was present. Again, neither of the tocainide conjugated intermediates were observed.

In the underivatized Sample 7, tocainide and the hydantoin were identified with molecular ions m/z 192 and m/z 218, respectively. Caffeine with a molecular ion at m/z 194 was also observed. The underivatized, acid-extracted Sample 8, revealed caffeine as the only identifiable compound.

5. Identification of Tocainide Glucuronide by Flash Chromatography and CH_3I/CD_3I Permethylation

Initial attempts to separate the colored urinary components from drug metabolites by the use of different chromatographic methods led to the use of flash chromatography. This method has been shown to provide quick separations of non-polar compounds and is widely used in the purification of synthetic products.

Initial chromatographic evaluation of the Acetonitrile - XAD urine extract with three TLC plates: KC-18F, alumina and silica gel, revealed that silica gel separated the polar urinary components, hence, flash chromatography was carried out using a dry-packed silica gel column. The crude ACN-XAD extract was eluted from this column as described in the Experimental Section.

The crude ACN-XAD extract, prior to evaluation by flash chromatography, revealed a prominent m/z 232 with a fragmentation pattern suggesting that the permethylated hydantoin was present. No other significant ions were identified in this the sample.

After fraction 4 and 5 were collected from the silica gel column, pooled and permethylated as decribed, the resulting mass ion m/z 105 was found to be present in two peaks in the TIC. A molecular ion at m/z 206 from one peak indicated the presence of hippuric acid. Another total ion peak with a molecular ion at

m/z 221 indicated the presence of the lactoxylidide. The use of CD_3I during another permethylation experiment confirmed the structures of these compounds.

The TIC and mass spectrum of the permethylated, pooled fractions 6 to 12 are shown in Figures 40 and 41. At Scan 66 and 99, caffeine and hippuric acid were identified respectively. At Scan 142, the base peak at m/z 147, with a molecular ion m/z 218, was identified to be the hydantoin. Lastly, at Scan 182, as shown in Figure 41, a base peak of m/z 98, with highest mass detected at m/z 334, was found to be identical to the proposed fragmentation pattern of the permethylated tocainide ester glucuronide as described in earlier discussions. Permethylation experiments with CD_3I confirmed the presence of 6 replacable hydrogens on the tocainide glucuronide according to the carbamate structure. As shown in Figure 42, the structure of the tocainide glucuronide offered 4 replacable hydrogens from the hydroxyl groups of glucuronic acid moiety, plus two amide hydrogens from tocainide. The CD_3I permethylated sample is shown in Figure 43. The fragment at m/z 334 suggested cleavage between the C_2 and C_3 carbon of tocainide containing 5 replacable hydrogens. CD_3I permethylation revealed a corresponding 15 mass unit increase to m/z 349, suggesting the labelling of 5 methyl groups. Again, the ion fragment at m/z 277 from the (N-CO) cleavage containing 4 replaceable hydrogens, showed a corresponding 12 mass unit increase to m/z 288. The ion fragment at m/z 105 from the xylidine portion of tocainide contained no replaceable hydrogens, did not show any change in mass after permethylation

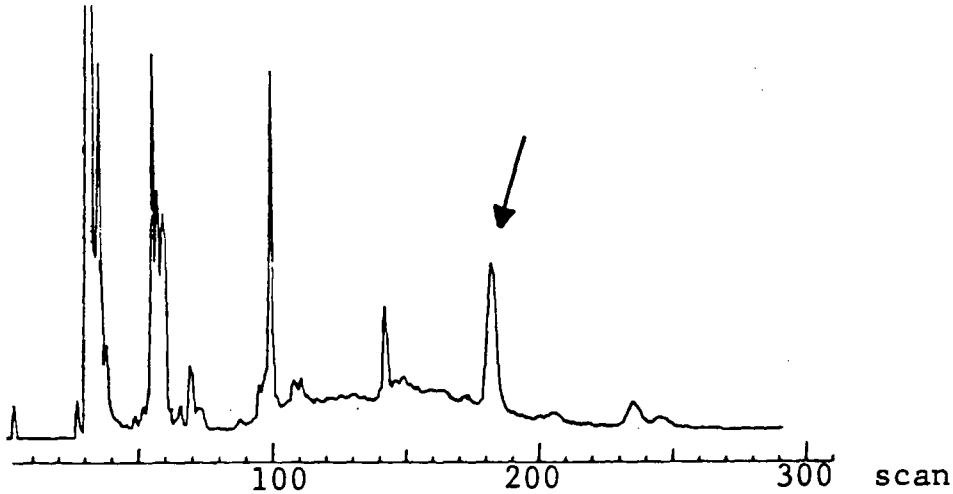


Figure 40 : Total-ion-chromatogram of the permethylated urine.

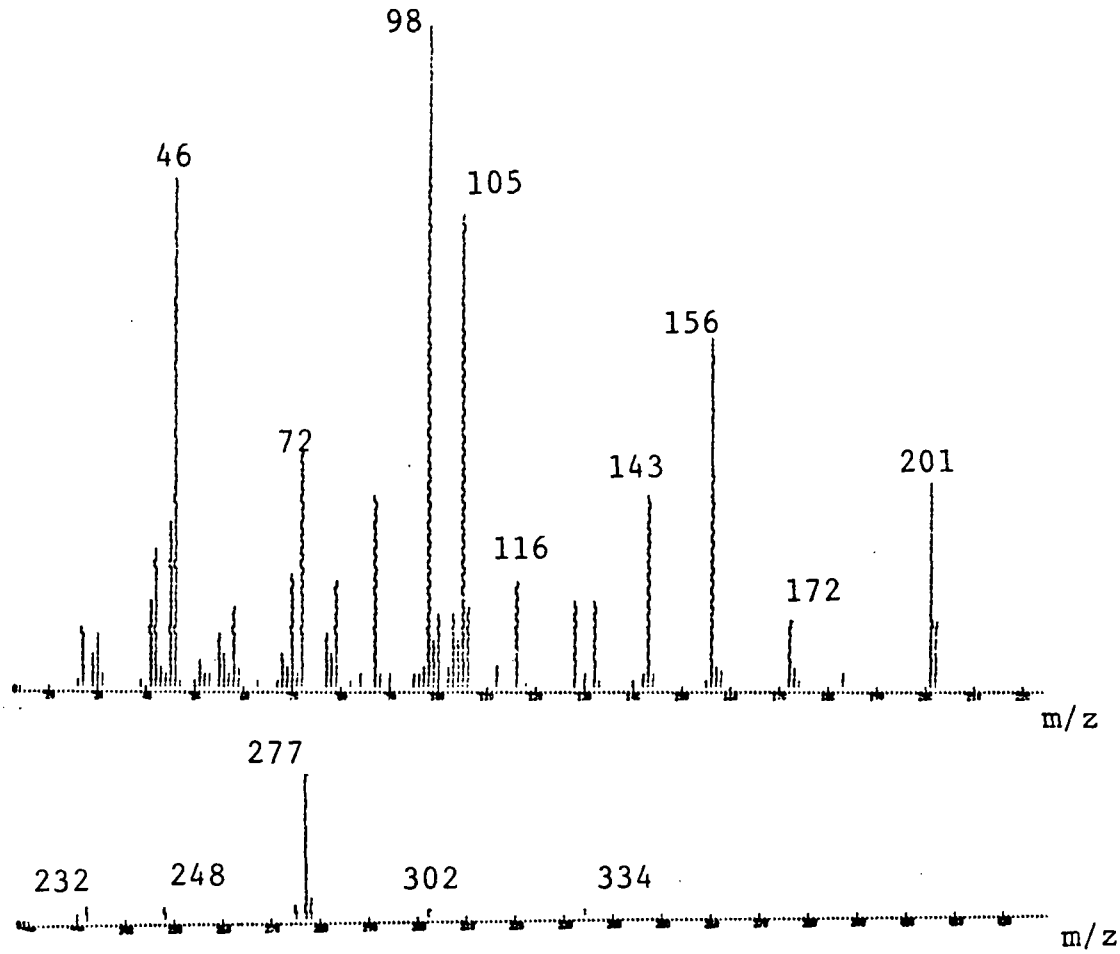


Figure 41 : The EI spectrum of permethylated tocainide carbamoyl ester tocainide (CH_3 I).

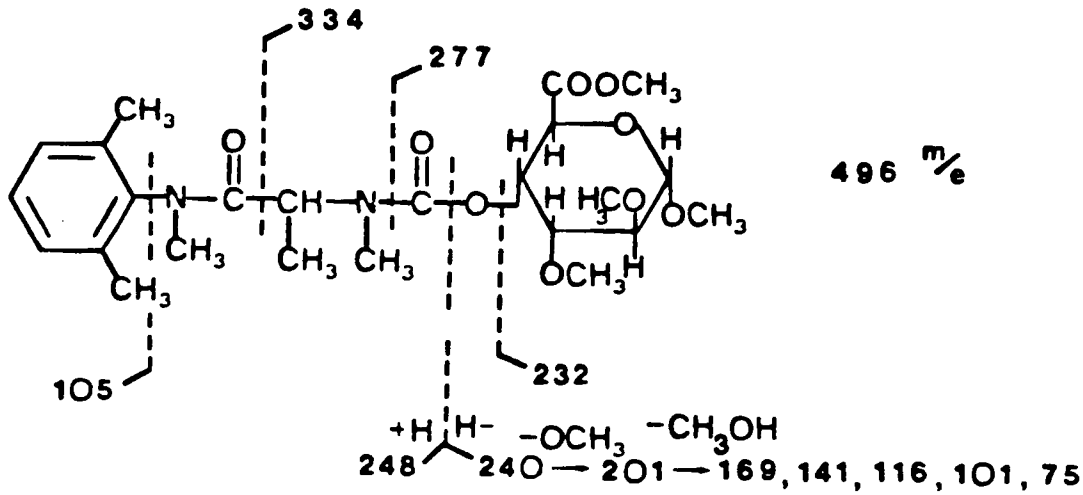


Figure 42. The EI fragmentation pattern of permethylated tocainide carbamoyl ester glucuronide.

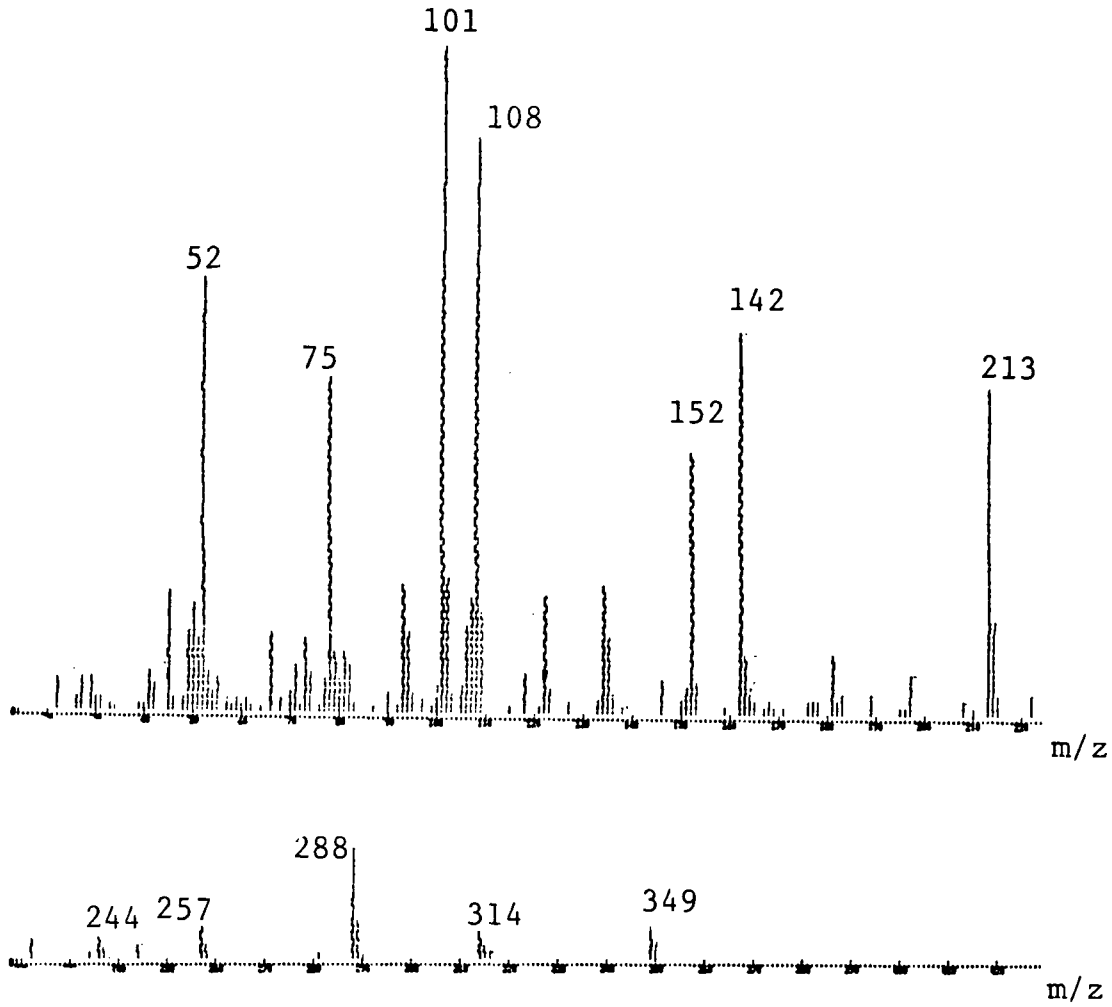


Figure 43. The EI mass spectrum of permethylated tocainide carbamoyl ester glucuronide (CD_3I).

with CD_3I. Lastly, the permethylated glucuronic acid moiety, with ion fragment m/z 232 containing 4 replaceable hydrogens, showed an increase of 12 mass units to m/z 248, corresponding to the 4 methyl groups labelled.

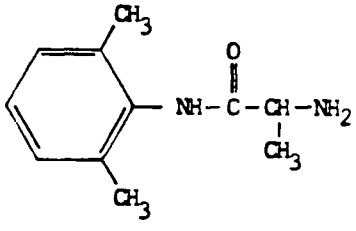
The absence of a molecular ion from these experiments, as described before, was probably due to the instability of this glucuronide under GC conditions. It should be noted that the samples were also analyzed in the CI mode without observing the molecular ion of the permethylated TOCG.

Other fractions collected from the silica-gel column did not reveal any additional useful information.

6. Tocainide-urea Reaction

The reaction between tocainide and urea was examined at different urea concentrations and at different temperatures. The experimental results are summarized in Table 8.

Table 8 : The Reaction of Tocainide with Urea at Different Temperatures

		Temp.	Time	GCMS Data	
				Tocainide (m/Z 192)	Hydantoin (m/Z 218
Tocainide (base) 	Urea (8.0 M)	100°C	1 hr.	+	+
			9 hrs.	+	+
			13 hrs.	-	+
		60°C	3 hrs.	+	+
	Blank Urine	25°C	10 hrs.	+	-
		100°C	1 hr.	+	+
		100°C	9 hrs.	+	+
	Distilled Water	100°C	13 hrs.	+	-
Mexilitine (base)	Urea	100°C	13 hrs.	Mexilitine Methanimine m/Z	
	Distilled Water	100°C	13 hrs.	Mexilitine	

In fresh, drug-free urine at room temperature (25 ^{o}C), tocainide did not react with urea to any measurable extent. Thus, the hydantoin was not likely to arise as an artifact formed during sample storage. In the presence of 8 M urea at 100 ^{o}C for 13 hours, almost quantitative conversion of tocainide to the hydantoin was observed. In addition, when tocainide was heated at 100 ^{o}C for 1 hour in fresh drug-free urine, the hydantoin could also be detected.

As shown in Table 9, formation of the hydantoin favored neutral to alkaline conditions. At acidic pH, the ionized terminal amine of tocainide probably repelled the equally charged amino group of urea preventing condensation.

Table 9 : The Reaction of Tocainide With Urea at Different pH

	R_f* (80/20: Methanol/H_2O)		
pH	Tocainide	Hydantoin	Suspected Tocainide-N-Ureide
2.5	0.45	0.75	-
7.8	0.45	0.75	0.6
12.5	0.45	0.75	0.6

Note:
* KC-18 TLC.

From these experiments, the formation of the hydantoin was repeatedly demonstrated. This hydantoin was thought to be evolved through a tocainide ureide intermediate. Subsequently, permethylation of the reaction mixture, which contained this intermediate, revealed the presence of the tocainide-N-ureide as shown in Figure 44. The molecular ion of the permethylated tocainide-N-ureide at m/z 291 was observed with trace intensity. As shown in Figure 45, a base peak at m/z 72 indicated the permethylated (-CO.NCH_3CH_3-) fragment, whereas m/z 129 corresponded to the permethylated C_2-C_3 cleavage product of the tocainide-N-ureide. The identification of this tocainide-N-ureide suggested that such an intermediate can occur at near neutral pH.

7. Synthesis of N-glucuronides

The attempted chemical synthesis of tocainide-N-ureide glucuronide following the procedures of Arita (46), Tsakamoto (47), Ishidate (48) and Takitani (49) was not successful, as evidenced by permethylation followed by GCMS analysis. These methods have previously been shown to be suitable for N-glucuronide condensation (section 4b).

Experiments with 2,6-dimethylaniline using the published procedures of Arita (46) yielded the permethylated 2,6-dimethylaniline-N-glucuronide molecular ion at m/z 353 as shown in Figures 46 and 47. However, this permethylated aniline glucuronide was not observed in any permethylated urine samples from volunteers who had ingested tocainide.

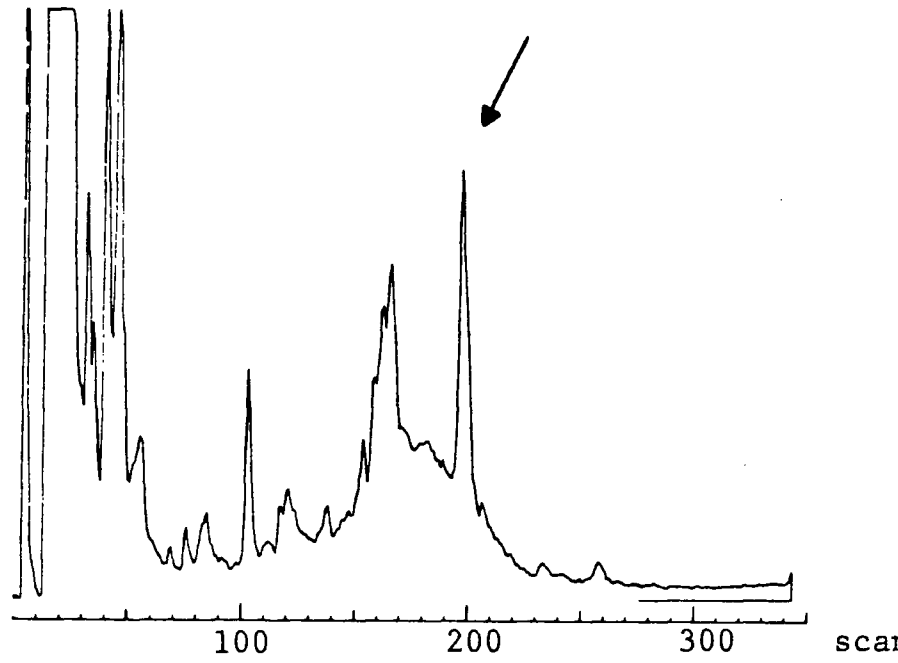


Figure 44 : Total-ion-chromatogram of the permethylated tocainide-N-ureide.

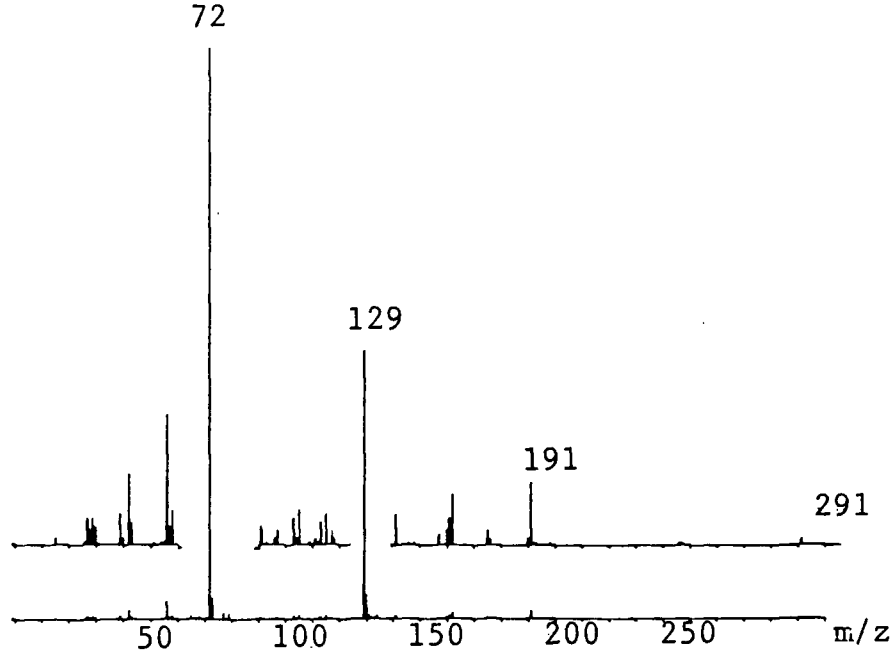


Figure 45 : The EI mass spectrum of the permethylated tocainide-N-ureide.

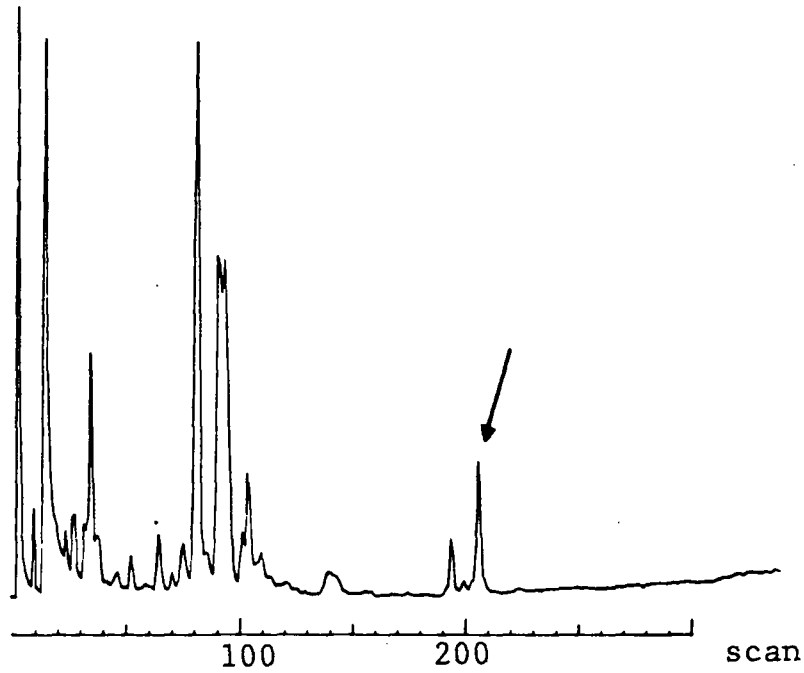


Figure 46 : The total-ion-chromatogram of the permethylated 2,6-dimethylaniline-N-glucuronide.

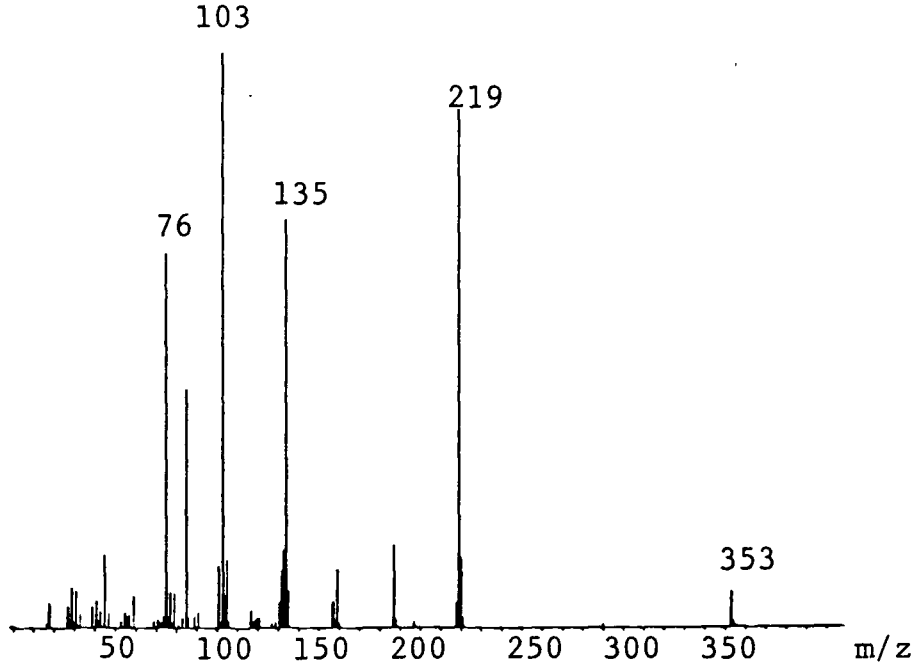


Figure 47 : The EI mass spectrum of the permethylated 2,6-dimethylaniline-N-glucuronide.

D. HPLC Isolation and Structural Elucidation of Tocainide Glucuronide

Thus far, from previouly discussed mass spectral data, the structure of TOCG was established based on the mass fragments from the permethylated TOCG. The intact molecular structure of TOCG was not observed. Therefore, preparative HPLC was employed to isolate larger quantities of this conjugate for NMR and FAB spectral measurements.

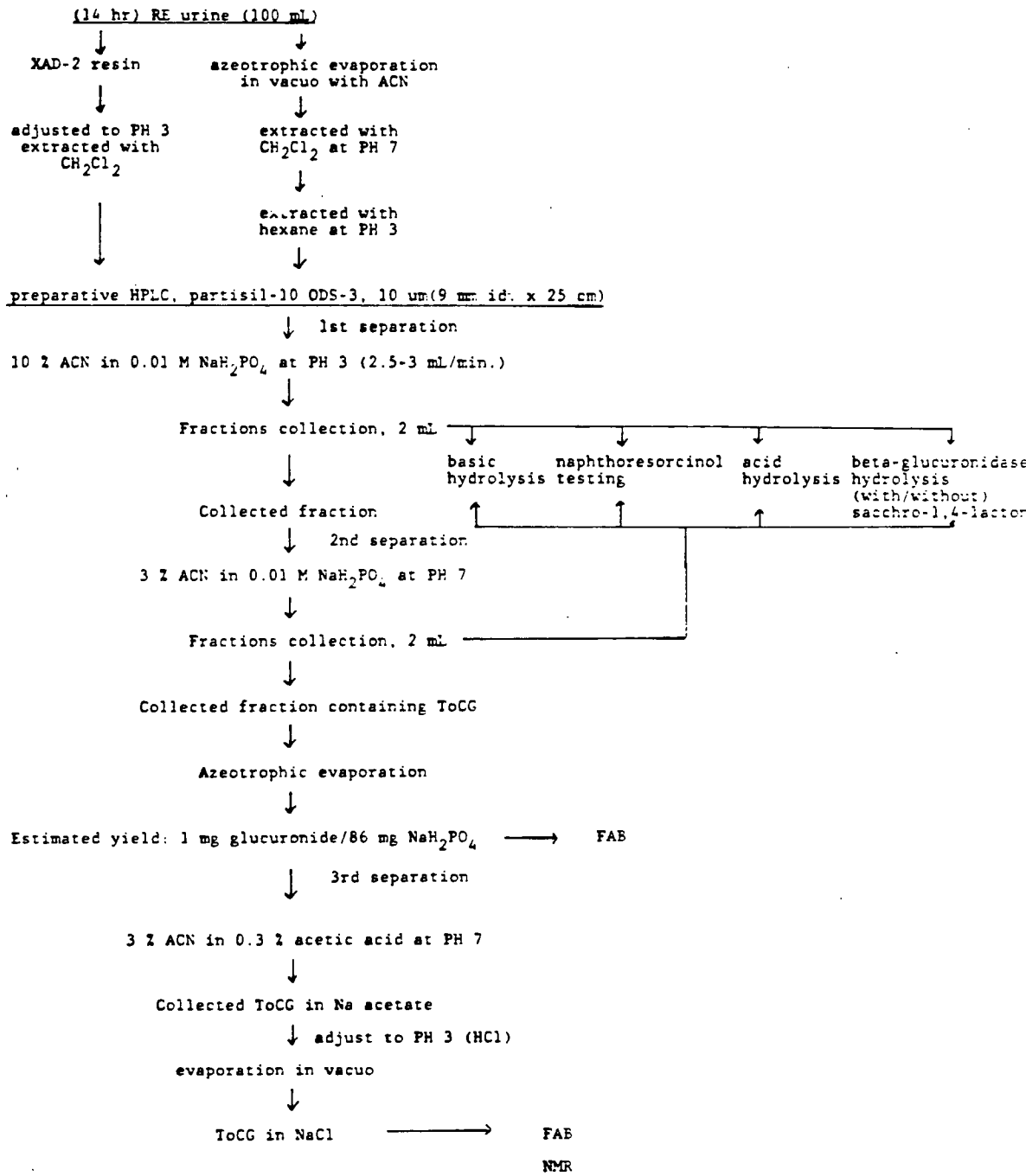
Scheme 16 summarizes the experimental steps involved in the isolation procedure.

1. Preparative HPLC Isolation

To concentrate urine samples for preparative HPLC, 10 % acetonitrile was added for azeotropic evaporation of the urine sample under vacuum. The solvent admixture offered reasonable time (2 hours) to concentrate 100 mL urine to 10 mL, compared to lyophilization which required 12 hours for the same volume.

Chromatographic evaluation of the concentrated urine sample was carried out using a mobile phase of 10 % acetonitrile in 0.01 M sodium phosphate buffer, adjusted to pH 3 with phosphoric acid. Detection was at UV 226 nm and column eluant was collected in 2 mL fractions. Figure 48 and 49 show the chromatogram at 226 nm and the UV absorbance of each individual fraction at 226 nm, respectively. Fraction 4 was found to yield the hydantoin after alkaline hydrolysis. Testing of fraction 4 by naphthoresorcinol reaction on a TLC plate revealed a blue color, indicating the presence of a glucuronic moiety.

Subsequent to this finding, fraction 4 was collected over



Scheme 16 : The experimental steps involved in isolating the tocainide glucuronide.

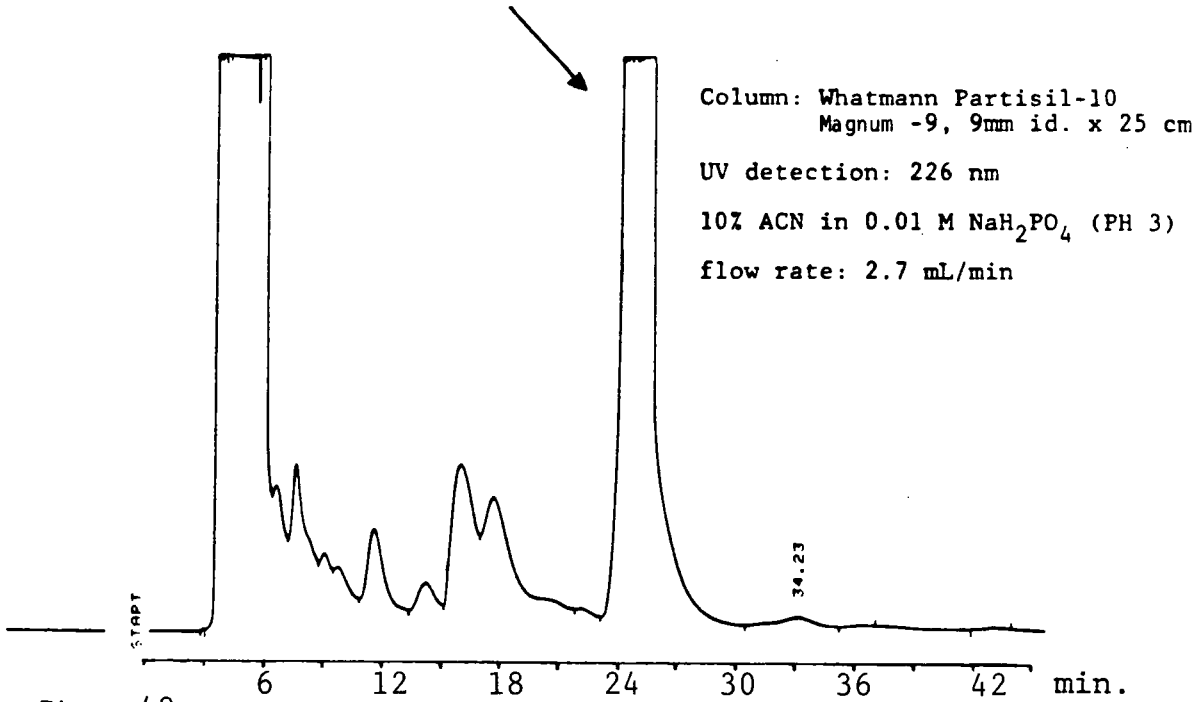
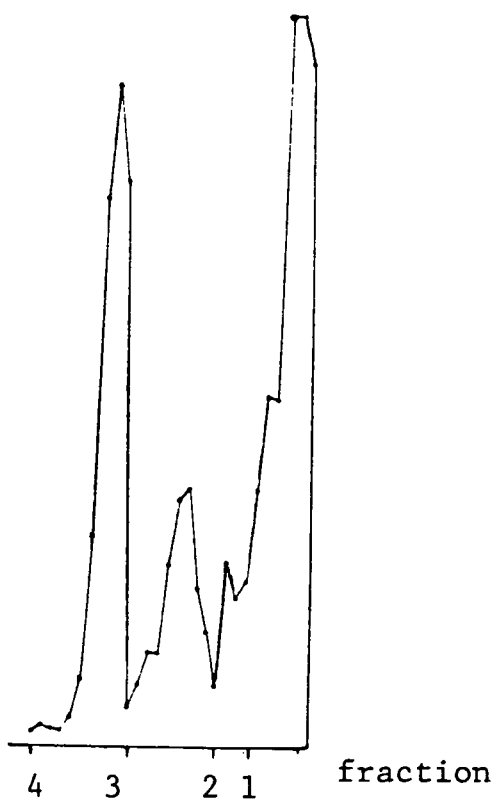


Figure 48 : Preparative HPLC chromatogram of crude urine.



2 mL fractions

UV absorbances: 225 nm

Figure 49 : UV absorbance of 2 mL fractions collected from preparative HPLC.

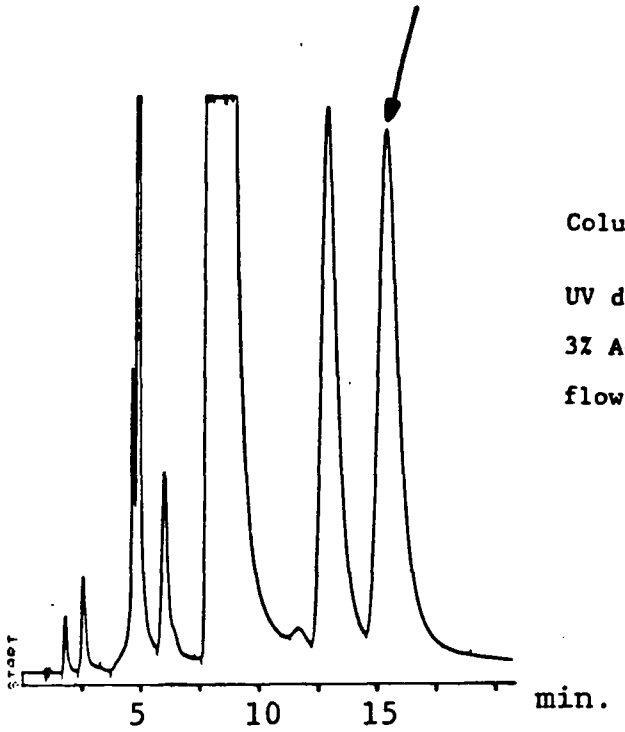
36 repetitive analyses.

To further resolve the urinary components within this collected fraction, a mobile phase composition of 3 % acetonitrile in 0.01 M sodium phosphate buffer, adjusted to pH 7 with sodium hydroxide, was used. The resulting chromatogram using UV detection at 220 nm is shown on Figure 50. Fraction 4 was found to give positive results with basic, acidic and enzymatic hydrolysis, as well as showing positive naphthoresorcinol color test. Furthermore, the UV spectrum of fraction 4 was found to be identical to that obtained from tocainide with a λ max at 205 nm.

Subsequently, fraction 4 was collected over 42 injections, concentrated by azeotropic evaporation, and rechromatographed as shown in Figure 51, showing 91 % purity. After evaporation to dryness under vacuum, the sample yielded 62 mg of tocainide glucuronide/sodium phosphate salt.

Initial GC assay of the urine sample estimated a concentration of 67 ug/mL (30 mole/mL) of the hydantoin. According to the 100 mL urine used for isolation, 12.3 mg (30 umoles) of tocainide glucuronide could theoretically be isolated.

To further remove the phosphate salt, the salt sample was reconstituted in water and chromatographed using 10 % acetontrile in 0.03 % acetic acid, adjusted to pH 4.3 using 1.0 M sodium hydroxide. UV detection was at 225 nm as shown in Figure 52. The resulting conjugate was isolated in sodium acetate salt. The acetate was removed as its free acid by



Column: Whatmann Partisil-10
Magnum -9, 9mm id. x 25 cm

UV detection: 220 nm

3% ACN in 0.01 M NaH_2PO_4 (PH 7)

flow rate: 2.7 mL/min

Figure 50 : Preparative HPLC chromatogram of recycled TOCG containing fraction.

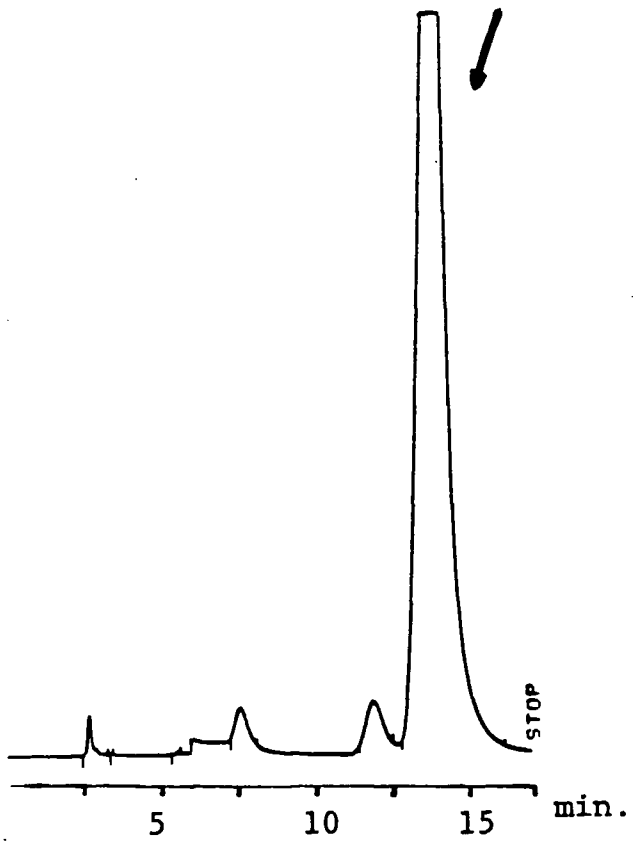


Figure 51 : Preparative HPLC chromatogram of recycled TOCG in sodium phosphate salt.

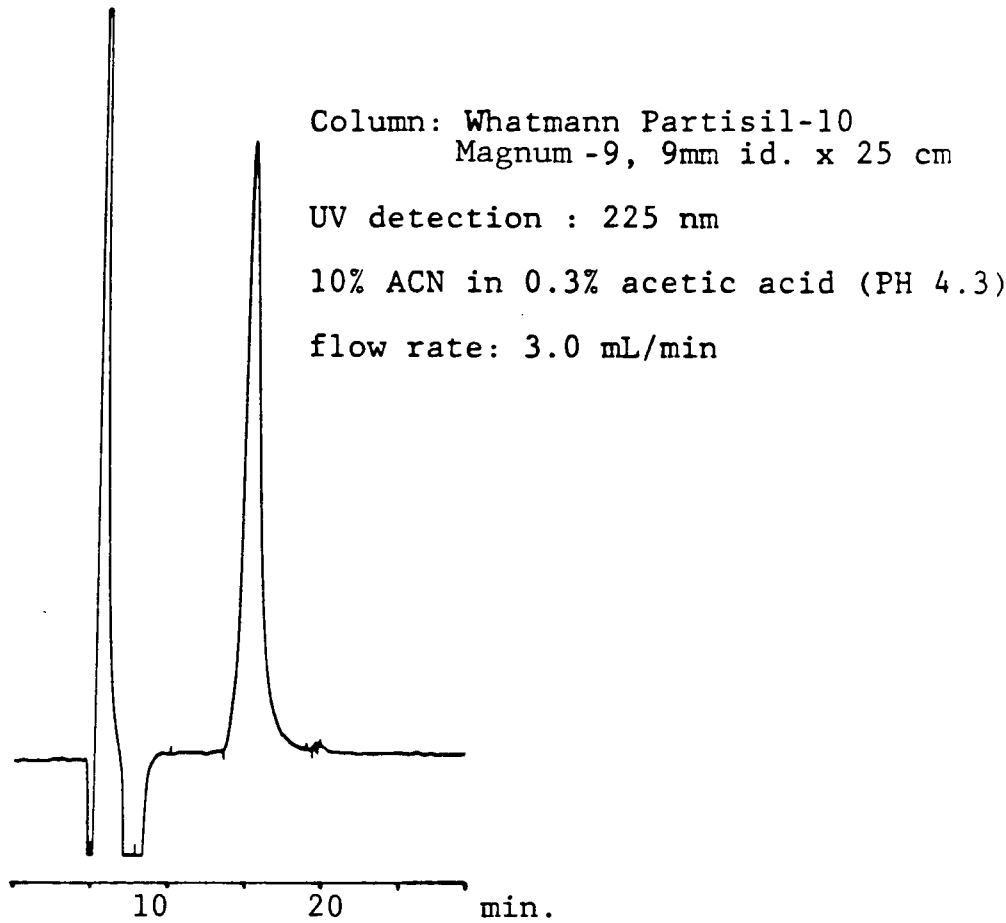


Figure 52 : Preparative HPLC chromatogram of recycled TOCG in sodium chloride salt.

adjusting the pH to 2.8 with 1.0 M hydrochloric acid and evaporating under vacuum. This sample was stored desiccated at -10 ^{o}C.

2. EI Mass Spectral Analysis of the Tocainide Glucuronide

Attempts to elucidate the structure of the isolated glucuronide by GCMS derivatization methods were not successful.

3. 400 MHz Proton Nuclear Magnetic Resonance Experiment

The interpretation of the tocainide glucuronide NMR spectrum was simplified with the aid of the reference spectra of tocainide and glucuronic acid obtained in the same solvent. Figure 53 and 54 show the 400 MHz proton spectra of tocainide before and after deuterium exchange. Figure 55 shows the spectrum of glucuronic acid. Table 10 summarizes the proton assignments of the tocainide spectrum.

Figure 56 and 57 show the 400 MHz proton NMR spectrum of the isolated tocainide glucuronide in D_6-DMSO. The proton-NMR assignments are summarized in Table 11. The doublet signal observed at 1.38 ppm was integrated for 3 protons and its chemical shift was found to correspond to the chiral methyl group of tocainide (1.54 ppm).

The singlet at 2.11 ppm was integrated for 6 methyl groups, accounting for the two xylidine CH_3 groups. Chemical shift at 3.69 ppm, 3.92 ppm, and 5.77 ppm were found to correspond to the signals obtained from the reference glucuronic acid spectrum. Furthermore, the signals at 7.06 ppm and 9.39 ppm were assigned as the aromatic protons of the xylidine ring and the xylidine-NH group, respectively.

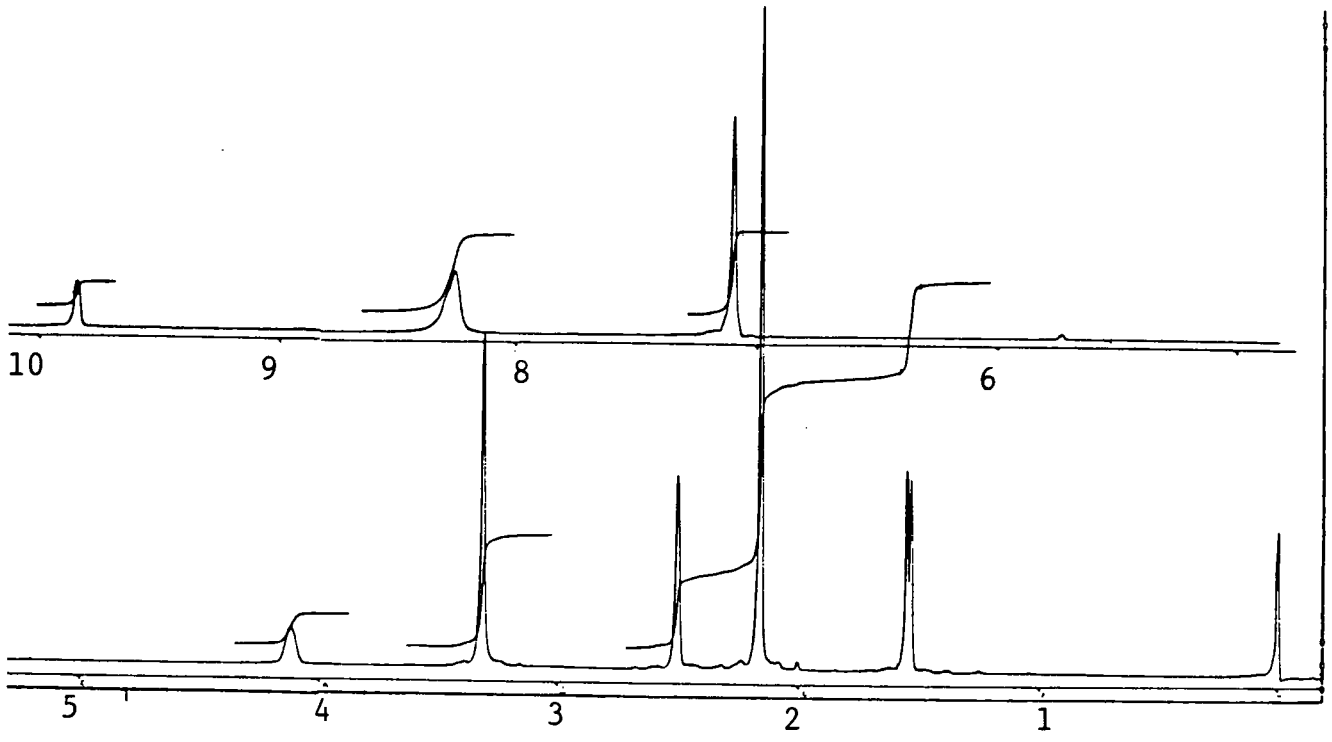


Figure 53 : 400-MHz ^{1}H-NMR spectrum of tocainide (D_6-DMSO).

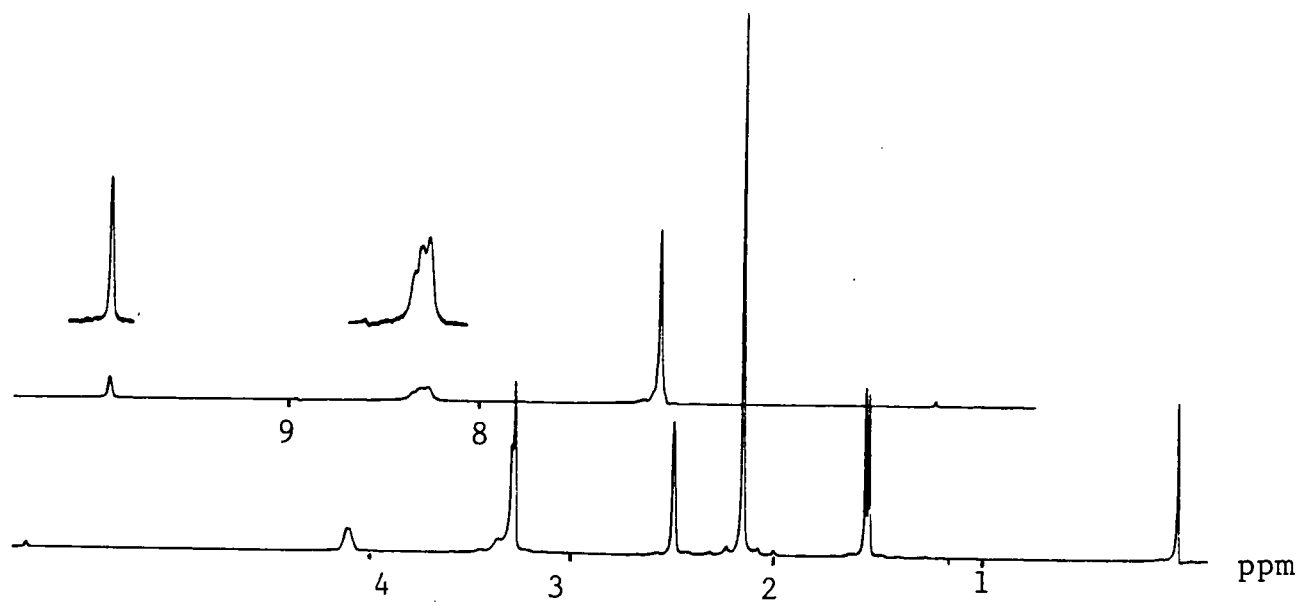


Figure 54 : 400-MHz-^{1}H-NMR spectrum of tocainide after D_2O exchange (D_6-DMSO).

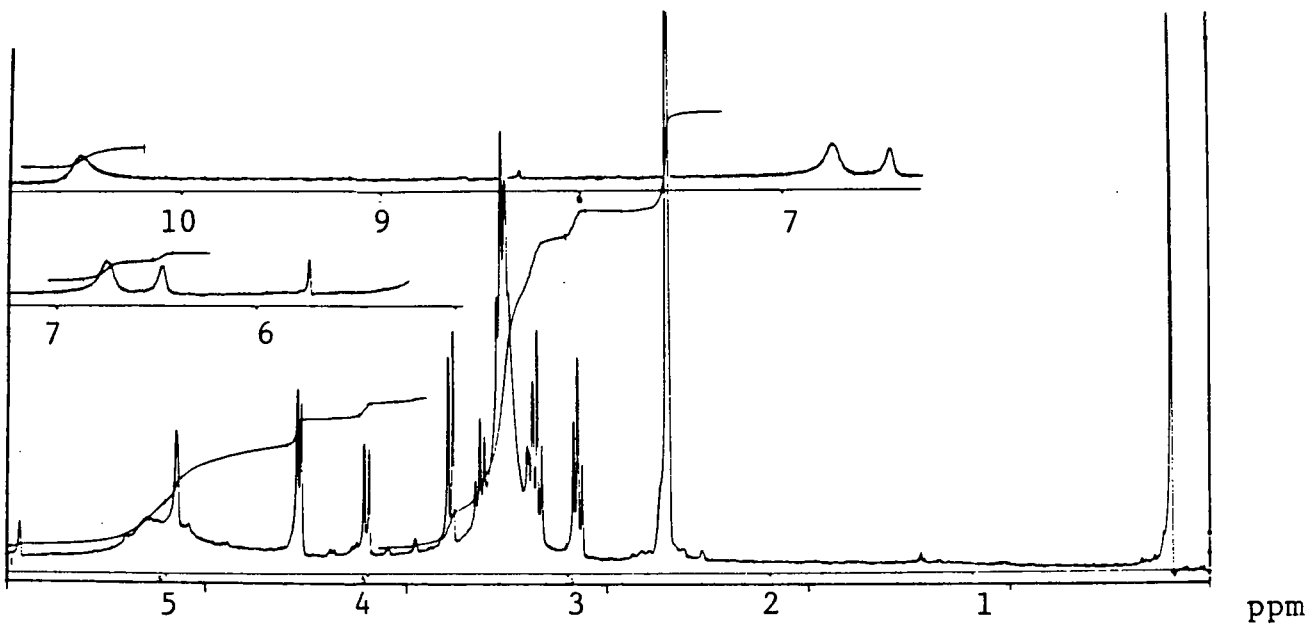
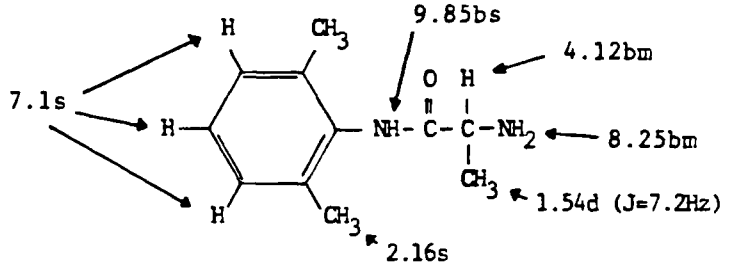


Figure 55 The 400-MHz proton NMR spectrum of glucuronic acid.

Table 10 Summarized data from the 400-MHz proton NMR spectrum of tocainide.



Shift (ppm)	Coupling	Integration (mm)	Proton(s)	Assignment
TMS (0.0)				TMS
1.54d	J = 7.2 Hz	36	3H(12mm/H)	1 - CH_3
2.16s		72	6H	xylidine - CH_3
2.51s				DMSO
3.30s				H_2O
4.12bm		12	1H	2 - CH (due to quadrapole broadening by -N)
7.10s		35	3H	xylidine - H
8.25 bm		32	2H	exchange with D_2O (2-amino-NH_2)
9.85bs		10	1H	exchange with D_2O (xylidine -NH)

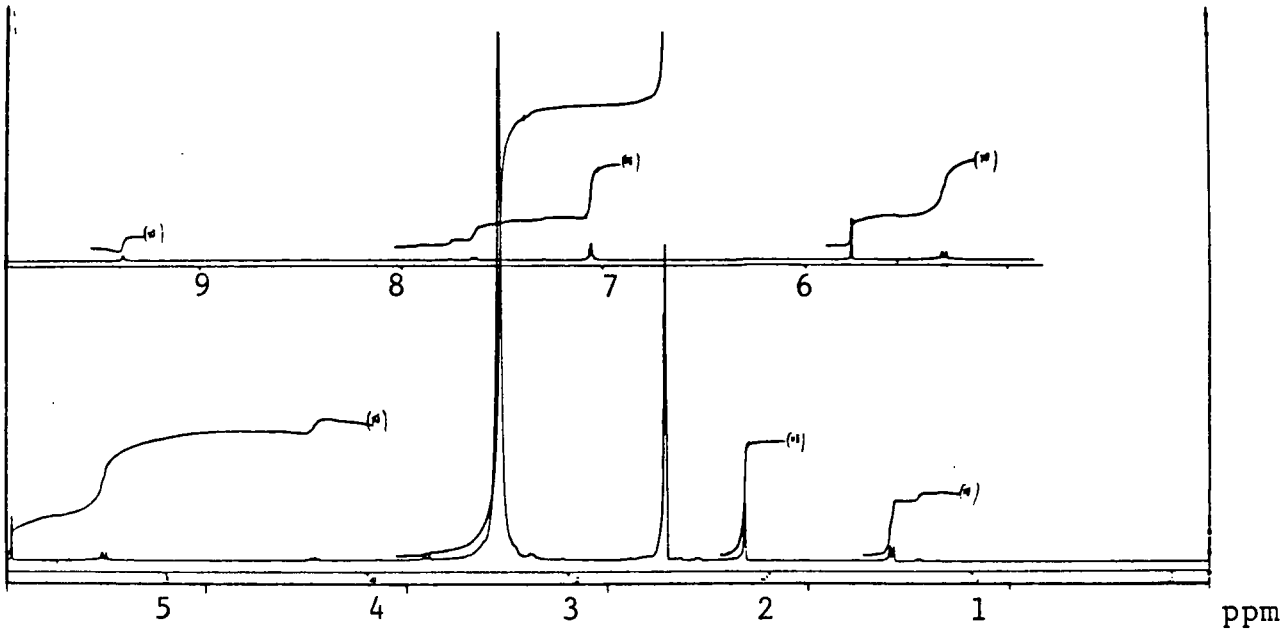


Figure 56 : 400 MHz-^{1}H-NMR spectrum of tocainide carbamoyl ester glucuronide (D_6-DMSO).

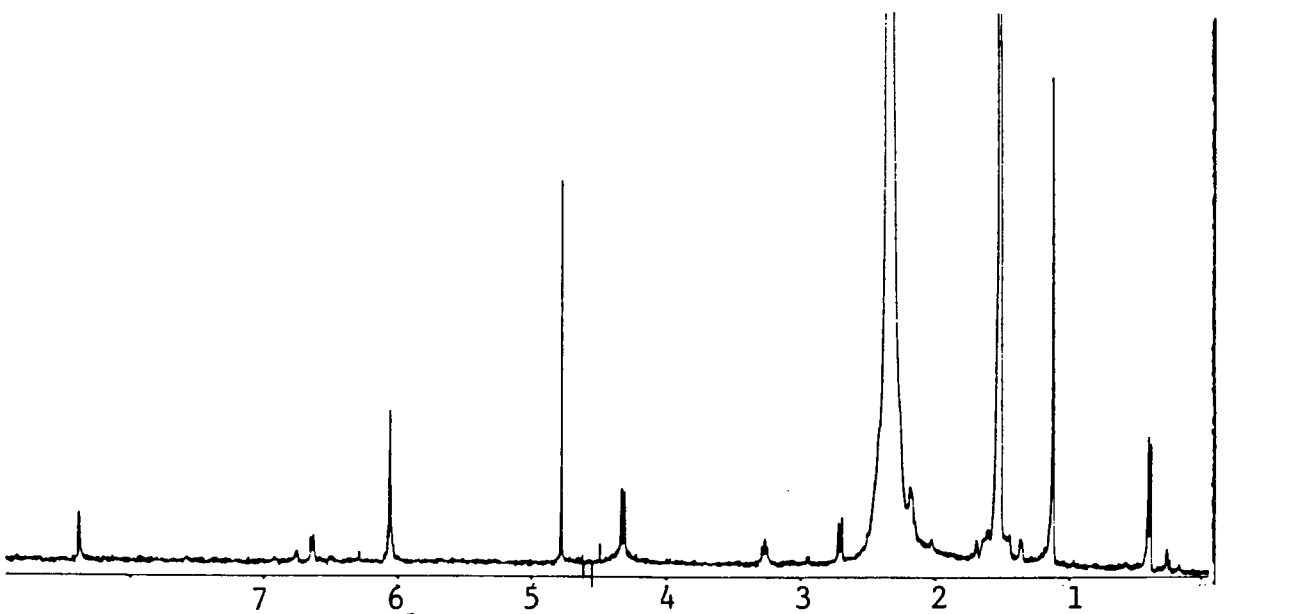
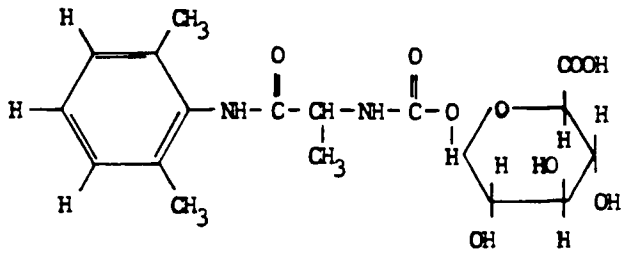


Figure 57 : 400 MHz-^{1}H-NMR spectrum of tocainide carbamoyl ester glucuronide (8 X intensity / D_6-DMSO).

Table 11 Summarized data from the 400-MHz proton NMR spectrum of the isolated tocainide carbamoyl ester glucuronide.



Shift (ppm)	Coupling	Integration (mm)	Proton(s)	Assignment
1.38d	J = 6.6 Hz	26	3H(8mm/H)	1 - CH_3 (Tocainide)
2.11S		56	6H(8mm/H)	xylidine - CH_3[1]
2.51		DMSO		
3.34		H_2O		
3.68, 3.71, 3.69d	J = 9 Hz	16		Glucuronic acid 3.55, 3.58[2]
3.92d	J = 9 Hz	trace		Glucuronic acid 3.99, 4.33[2]
4.25t		7	1H	2 - CH (Tocainide)
5.30d	J = 8 Hz	16		
5.77s		10		Glucuronic acid 5.72[2]
7.06bs		27	3H	xylidine - H
7.63d	J = 7.2 Hz	9		
7.77bs		3		
9.39bs		8	1H	xylidine - NH

Note: 1. Singlet, indicating straight chain conformation.
2. Clin Chem 26/9: 1323-1335 (1980) (Wu, et. al.).

These proton NMR signals from the isolated glucuronide have partially suggested the presence of a glucuronic acid conjugate of tocainide. However, the anomeric proton signal was not determined. Moreover, as shown in Figure 55, the reference glucuronic acid spectrum contained many broad singlets from 6 to 11 ppm which were absent in the reference glucuronic spectrum.

One plausible explanation for the loss of the glucuronic acid signals was the insufficient concentration of TOCG in the sample submitted for analysis.

4. Fast Atom Bombardment Analysis

The isolated tocainide glucuronide in sodium chloride submitted for FAB analysis in both glycerol and thioglycerol matrices. Figures 58 and 59 show the FAB spectra of the conjugate in glycerol and thioglycerol, respectively. In both spectra, a prominent intensity of the 457 m/z ion adduct was observed, suggesting an addition of 2 sodium ions onto the $[m-H]^+$ molecular ion of 411 m/Z. The equally intense ion at 435 m/z from the two spectra suggested the presence of the $[M+Na]^+$ adduct. In the spectra, the $[M+1]^+$ adduct at 413 m/Z was also observed.

As shown in Figure 60, the fragmentation pattern of the conjugate would likely to give rise to the successive mass ions at m/Z 235, 236, 237, and 239. These ion fragments were of diagnostic value in determining the carbamoyl ester versus the ureide structure.

As shown in Figure 61, when the mass analyzer was adjusted to observe the low mass ions, the fragment ion at m/z 221 was

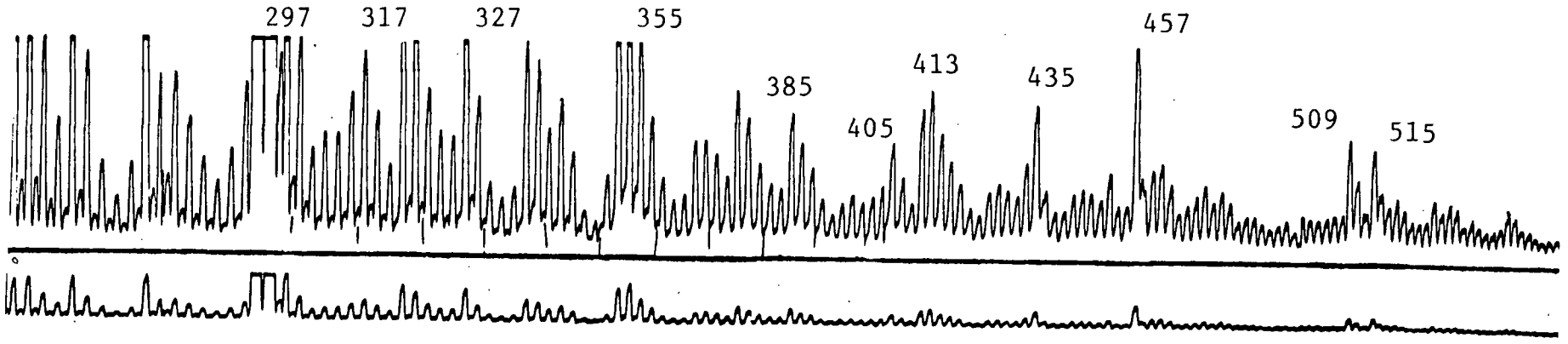


Figure 58 : The fast atom bombardment spectrum of tocainide carbamoyl ester glucuronide in glycerol matrix with sodium chloride.

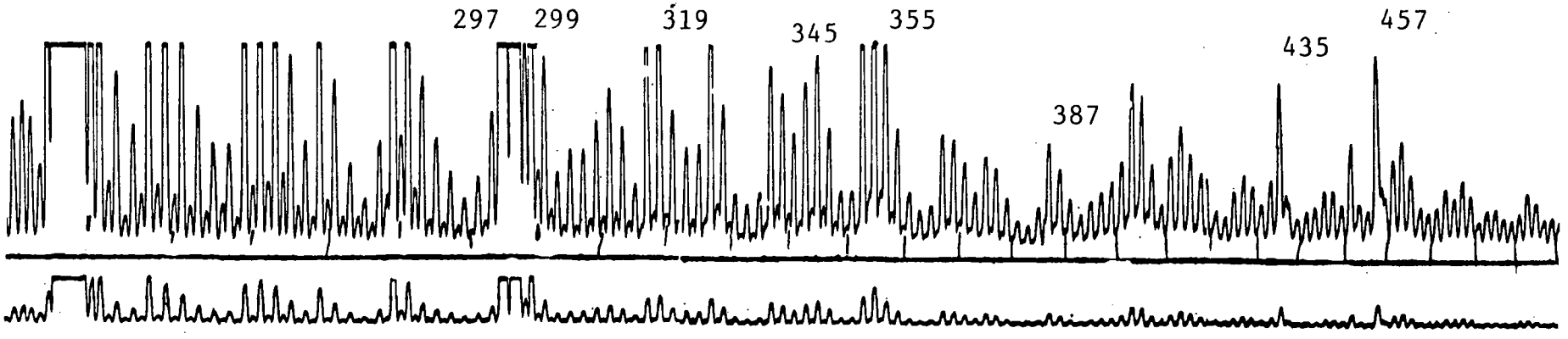


Figure 59 : The fast atom bombardment spectrum of tocainide carbamoyl ester glucuronide in thioglycerol matrix with sodium chloride.

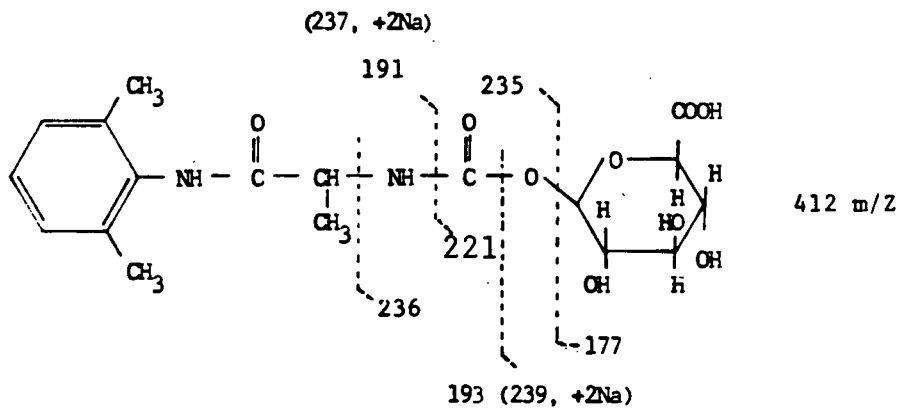


Figure 60 The fragmentation pattern of tocainide carbamoyl ester glucuronide in fast atom bombardment mass spectrometry.

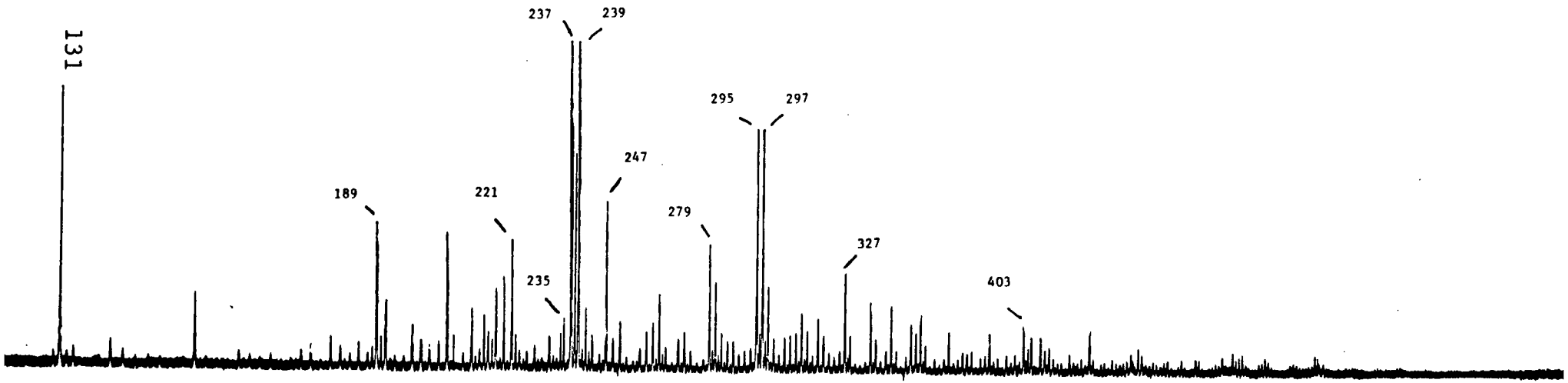


Figure 61 : The fast atom bombardment spectrum of tocainide carbamoyl ester glucuronide in thioglycerol matrix with low mass ion monitoring.

found to correspond to the ester glucuronic moiety (-CO.O-GA). Ions at m/z 237 and m/z 239 were found to correspond to the 2Na ion adduct of tocainide (toc-H+2Na) and of glucuronic acid (O-GA+2Na) respectively. Correspondingly, m/z 295 and m/z 297 were probably arrived from the (2Na+NacL) ion adducts.

These FAB fragmentation ions from the isolated TOCG have provided definitive evidence for a carbamoyl ester structure for TOCG. In addition, the observation of the intact molecular ion at m/z 412 with its characteristic ion adducts at m/z 435, and m/z 457 agreed with the carbamoyl ester structure of molecular weight 412.395 g/mole.

E. Pharmacokinetics of Tocainide Glucuronide

To follow the kinetic profile of tocainide glucuronide in urine, an indirect basic hydrolysis assay was used as described by Hoffmann (60). The method required initial hydrolysis of the tocainide glucuronide to its hydantoin at pH 12, followed by quantitation of the hydantoin.

1. Tocainide Glucuronide Basic Hydrolysis Kinetics

The hydrolysis-time profile of TOCG in a urine sample obtained from a volunteer who had been given 200mg tocainide orally is shown in Figure 62. From the semi-log plot, as shown in Figure 63, it can be inferred that the hydrolysis of tocainide glucuronide followed the reaction Scheme 17 below. Both k_1 and k_2 are first-order rate constants.

$$\text{ToCG} \xrightarrow{k_1} \text{hydantoin} \xrightarrow{k_2} \text{degradation product}$$

Scheme 17 Schematic representation of the kinetics of ToCG hydrolysis in the presence of NaOH.

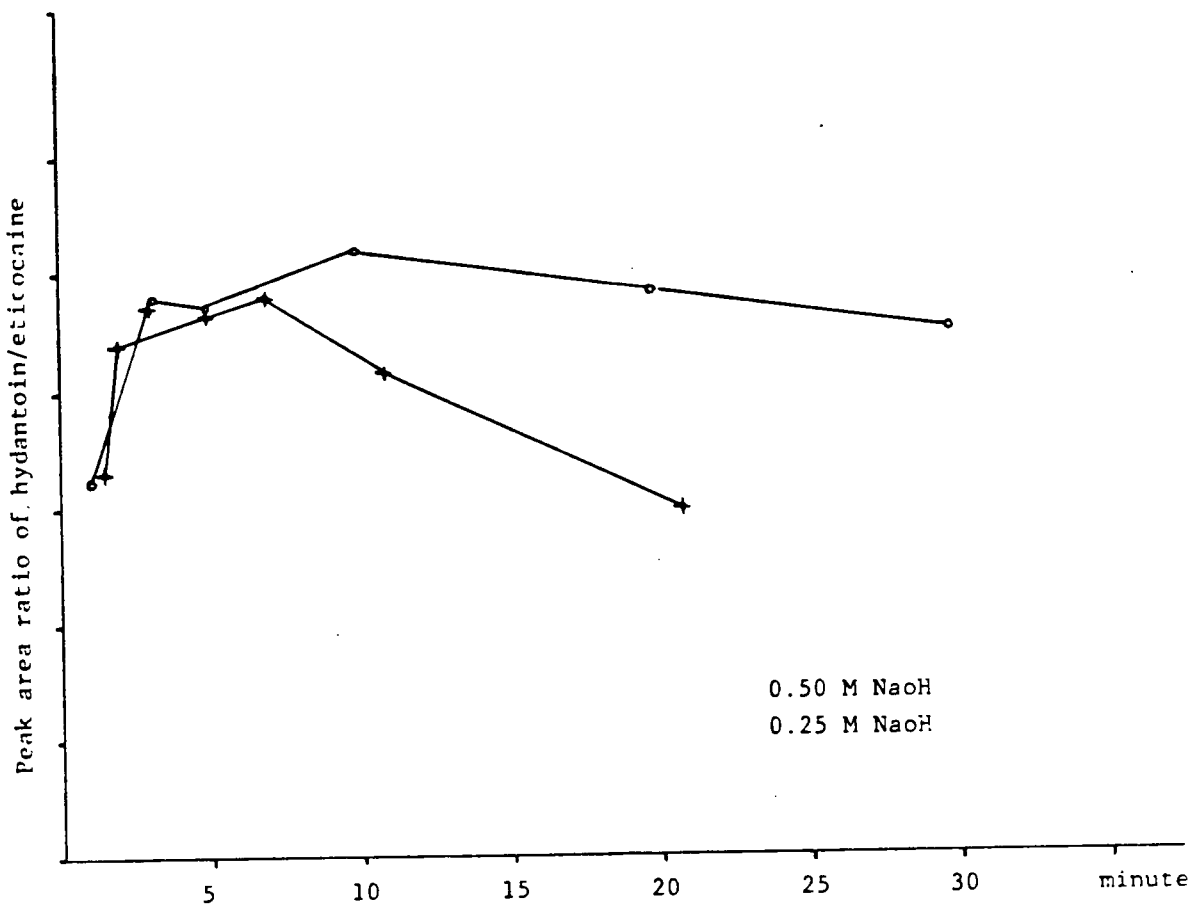


Figure 62 The hydrolysis profile of tocainide glucuronide and the hydantoin in the presence of sodium hydroxide.

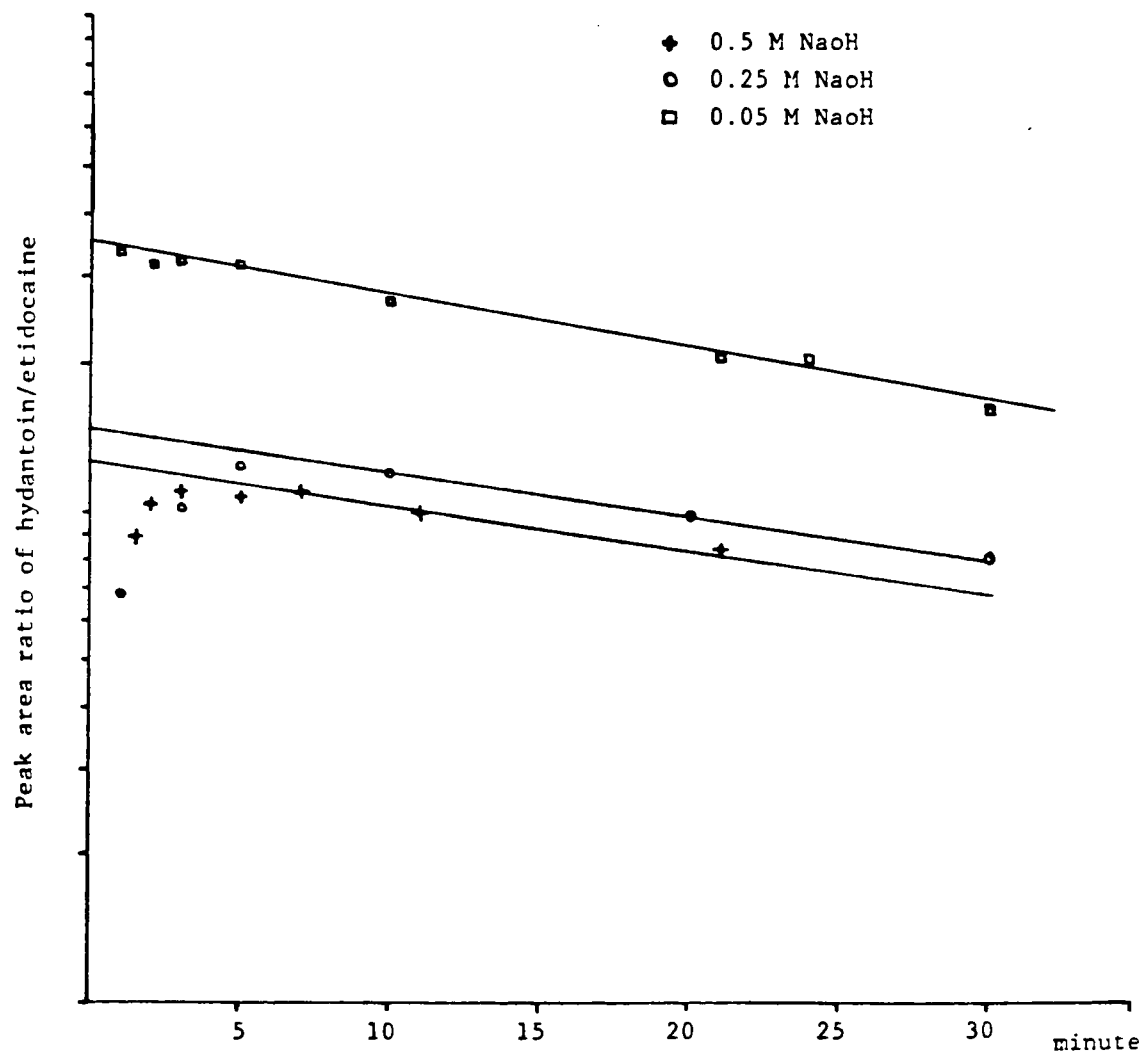


Figure 63 Semi-log plot of the tocainide glucuronide and the hydantoin hydrolysis kinetics in various concentrations of sodium hydroxide.

During the initial 7 minutes of hydrolysis, it was evident that the amount of the hydantoin was increasing, thereby indicating a positive k_1. However, as the amount of tocainide glucuronide was depleted, and as k_1 approached zero, the hydrolysis of the hydantoin became apparent. To demonstrate that k_2 represented the degradation rate constant of the hydantoin, a synthetic sample, hydrolyzed as described, revealed an identical k_2 value compared to that obtained from urine samples. Hydrolysis experiments of the synthetic hydantoin in 1.0 M, 0.1 M and 0.05 M NaOH revealed a parallel pseudo-first-order degradation kinetics, with rate constants ranging from 0.0122 to 0.0241 min^{-1} as shown in Figure 64.

2. Hydrolysis of 3-(2,4,6-Xylyl)-5-methylhydantoin

According to Hoffmann's published assay, if the internal standard 3-(2,4,6-xylyl)-5-methylhydantoin (4'-methylhydantoin) followed the same pseudo-first-order hydrolysis kinetics as the TOCG derived hydantoin, then the change in relative amounts of the metabolite hydantoin versus the 4'-methylhydantoin, as reflected by the peak height ratio, would reveal little change with time. However, Hoffmann was not aware of the hydrolytic nature of the hydantoin in base.

To demonstrate this relationship, the 3-(2,4,6-xylyl)-5-methylhydantoin was synthesized and subjected to the same hydrolysis experiment as described, but using etidocaine as an internal standard. The results are also shown in Figure 65. The 4'-methylhydantoin was found to exhibit pseudo-first-order degradation kinetics with a rate constant of 0.0122 min^{-1}.

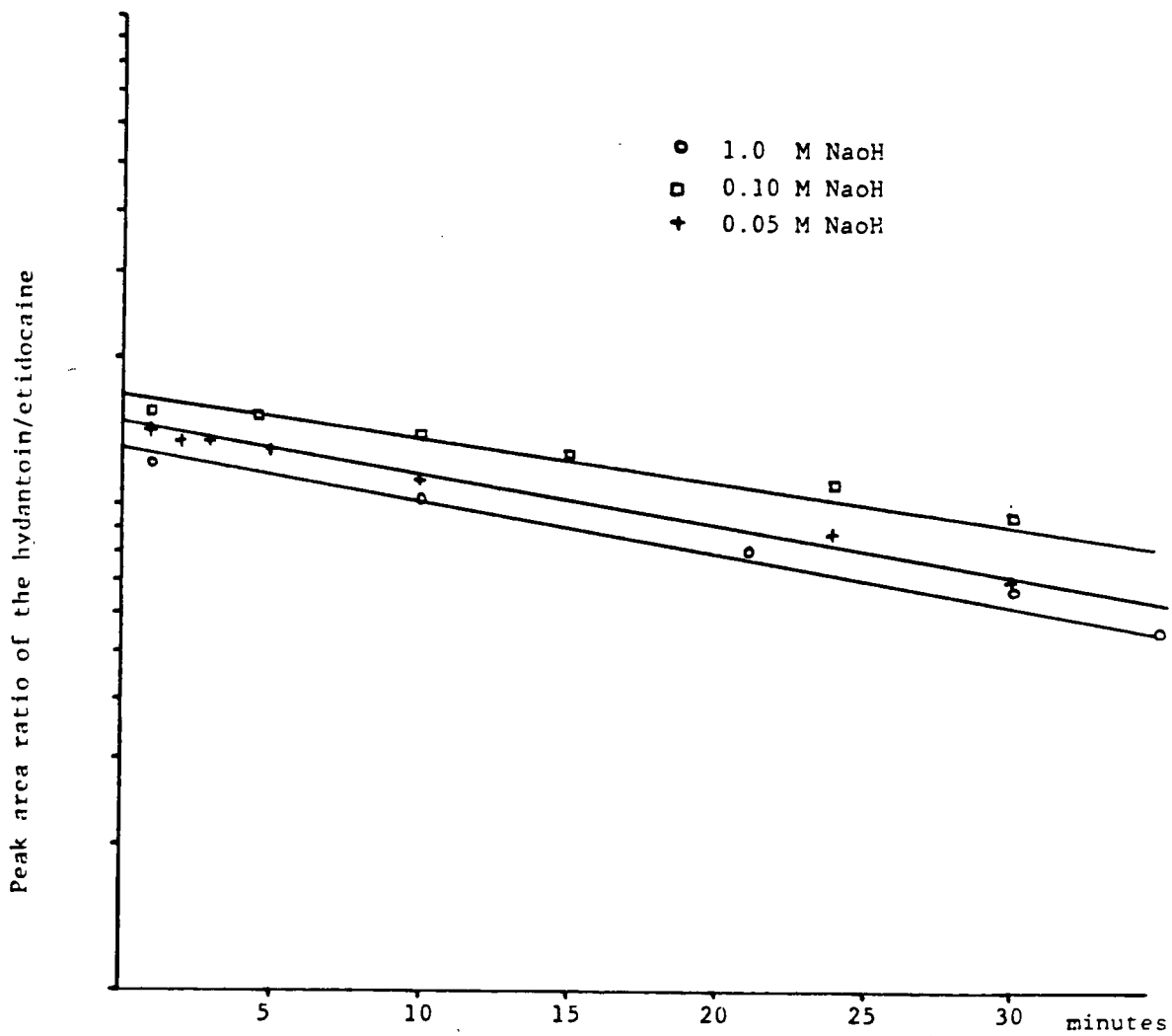


Figure 64 Semi-log plot of the hydrolysis of 3-(2,6-xylyl)-5-methylhydantoin in various concentrations of sodium hydroxide.

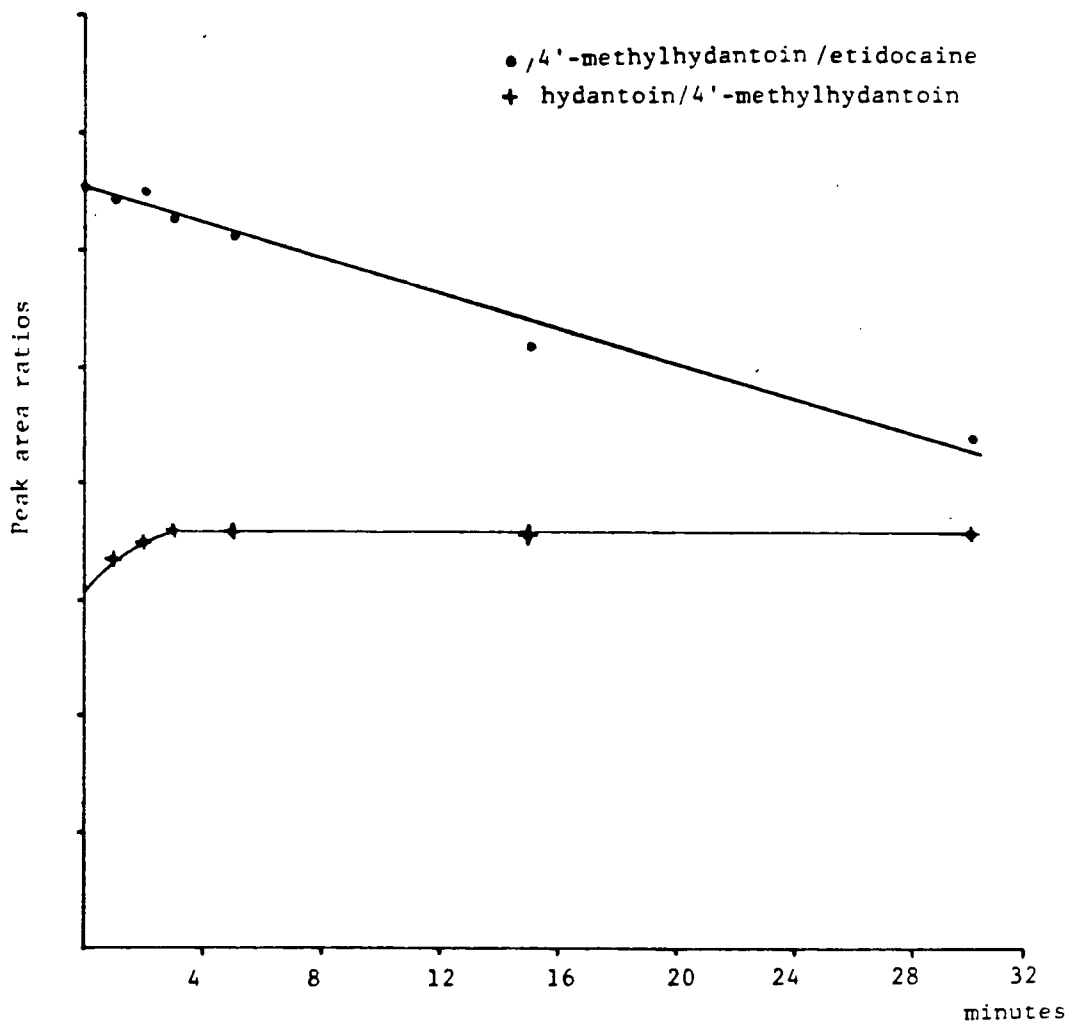


Figure 65 The hydrolysis of tocainide glucuronide and the 4'-methylhydantoin in the presence of 0.5M sodium hydroxide.

similar to those obtained from the metabolite-derived hydantoin. The peak height ratio of the hydantoin/4'-methylhydantoin obtained from the urine of a volunteer who had been given tocainide, revealed an initial hydantoin formation phase, at which k_1 is very much greater than k_2. As k_1 approached zero, the peak height ratio of the hydantoin to the 4'-methylhydantoin internal standard approached a constant as shown in Figure 65.

In essence, the peak height ratio of the hydantoin/4'-methylhydantoin over time should theoretically experience a gradual decline depending on the rate of hydrolysis of the two compounds. As shown in Figure 66A, the parallel first-order decline of the hydantoin and 4'-methylhydantoin would reflect a very gradual fall in peak area ratio of the hydantoin depending on the steepness of the first-order decline as depicted in Figure 66B.

3. Calibration Curve Data

Etidocaine was found to be stable at pH 12 and was used as an internal standard. To maintain reproducibility and to assay the levels of the hydantoin in the presence of its spontaneous hydrolysis, an accurately timed assay protocal was required. Since urine samples contained varying amounts of tocainide glucuronide, a predetermined hydrolysis time had to be choosen for both the calibration curve and the kinetic urine samples. As shown in Figure 66D, at any time > t, the quantity of hydantoin in a urine sample can be approximated by an identically timed calibration sample, as depicted in Figure 66C. Since at time

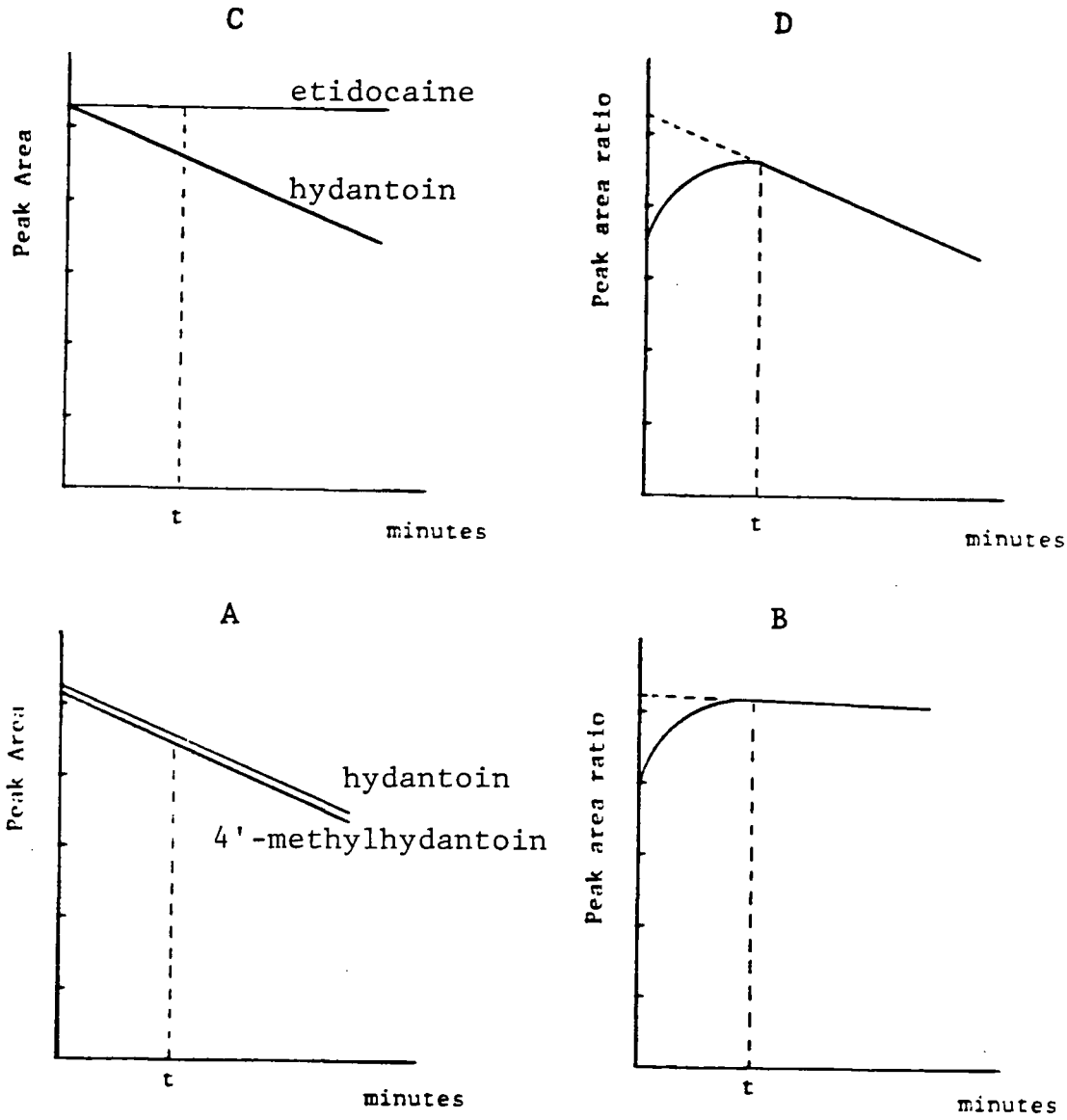


Figure 66 Theoretical profiles of the 3-(2,6-xylyl)-5-methylhydantoin hydrolysis using both etidocaine and 3-(2,4,6-xylyl)-5-methylhydantoin as internal standards. A: Peak area-time profile of the hydantoin and the 4'-methylhydantoin. B: The peak area ratio of the hydantoin to the 4'-methyl-hydantoin. C: Peak area-time profiles of etidocaine and the hydantoin. D: The peak area ratio of the hydantoin to etidocaine.

zero, in a urine sample, unlike a calibration sample, only a small portion of the theoretical amount of hydantoin was subjected to hydrolysis, this accurately timed protocol provided a close approximation for the levels of hydantoin in urine. If the hydrolysis of the hydantoin was not accounted for by the use of an accurately timed calibration samples, then the levels of TOCG in the urine, as reflected by the levels of the hydantoin, coulld not be correctly determined.

Calibration data are tabulated in Table 12. The assay included the use of 4'-methylhydantoin as a second internal standard to quantitate the amount of hydantoin in the urine. Linear regression of the 3 calibration curves revealed a coefficient of determination of 0.9974, 0.9997 and 0.9943 for tocainide/etidocaine, hydantoin/etidocaine, and hydantoin/4'-methylhydantoin respectively.

Inter- and intra-assay variability were determined using the calibration samples. The data are tabulated in Tables 13 and 14. For tocainide, intra-assay variations were expressed as % C.V. between duplicate determinations of 6 samples with concentrations ranging from 6 to 20 ug/mL. The % C.V. was found to range from 0.05 % to 1.76 %. Inter-assay variations expressed as % C.V. from 3 samples in the same concentration range were found to range from 0.04 % and 1.75 %. For the hydantoin, intra-assay variability was higher from 0.13 to 0.05 %, whereas inter-assay variability was ranged from 1.94 % to 8.59 %. This higher variability in the hydantoin assay of the hydantoin was probably due to the time dependent variation in the quantity of the

Table 12 Calibration curve data for tocainide and the hydantoin using etidocaine and the 4'-methylhydantoin internal standards.

Concentration[1] (ug/mL)	Tocainide[2] (avg.±S.D.)	Hydantoin[2] (avg.±S.D.)	Hydantoin[3] (avg.±S.D.)
1	0.064±0.005	0.047±0.010	0.067±0.011
3	0.226±0.009	0.166±0.022	0.251±0.008
6	0.477±0.014	0.318±0.015	0.496±0.006
10	0.877±0.014	0.580±0.011	0.840±0.010
15	1.385±0.002	0.963±0.008	1.299±0.005
20	1.927±0.049	1.358±0.065	1.746±0.001
	r^2=0.9974	r^2=0.9997	r^2=0.9943

1. n=2
2. peak area ratio using etidocaine (internal standard)
3. peak area ratio using 4'-methylhydantoin (internal standard)

Table 13 Intra-assay variability of tocainide and the hydantoin at 6, 15, 20 ug/mL concentrations.

Sample (ug)	Tocainide[1] (avg.±S.D.)	C.V.%[2]	Hydantoin[1] (avg.±S.D.)	C.V.%[2]
6	0.472±0.007	1.49	0.319±0.016	5.05
6	0.481±0.008	1.76	0.339±0.015	4.42
15	1.385±0.001	0.05	0.964±0.007	0.72
15	1.385±0.002	0.16	0.957±0.001	0.13
20	1.946±0.022	1.12	1.397±0.009	0.68
20	1.898±0.003	0.16	1.359±0.066	4.80

1. peak area ratio using 10ug etidocaine (internal standard)
2. n=2

Table 14 Intra-assay variability of tocainide and the hydantoin at 6, 15, 20 ug/mL concentrations.

Sample (ug)	Tocainide[1] (avg.±S.D.)	C.V.%[2]	Hydantoin[1] (avg.±S.D.)	C.V.%[2]
6	0.476±0.006	1.28	0.329±0.014	4.38
15	1.385±0.001	0.04	0.960±0.083	8.59
20	1.922±0.034	1.75	1.378±0.027	1.94

hydantoin metabolite during hydrolysis.

The extraction efficiencies of 6, 10 and 20 ug/ml of tocainide and the hydantoin in 5 mL methylene chloride, as outlined in the experimental section, were determined to range from 77.8% to 81.2 % for tocainide and 56.6% to 64.6 % for the hydantoin. Tabulation of the data is shown in Table 15.

Concentration (ug/mL)[2]	% recovery[1]	
	tocainide base	hydantoin
20	77.8	63.6
10	80.1	64.6
6	81.2	56.6

1 in 5 mL of methylene chloride.
2 duplicate determinations.

Table 15 Extraction efficiences of tocainide and the hydantoin in methylene chloride.

4. The Elimination Kinetics of Tocainide Glucuronide

The kinetics of tocainide and the hydantoin in urine, after an IV and oral dose of 200 ug tocainide, were examined in three healthy human volunteers. The urinary excretion rates of tocainide and the hydantoin after an IV and oral dose from one of the three subjects are shown in Table 16 and 17. The urinary excretion rate data from the two other subjects are shown in Appendixes 1 and 2.

The pharmacokinetic data from the three subjects are summarized in Table 18. The semi-log urinary excretion rates of tocainide and the hydantoin were plotted against mid-time of collection using the one-compartment Wagner-Nelson ^{R} kinetics program on an Apple ^{R} II plus computer.

The elimination rate constants (K_E) of tocainide from the subjects, calculated from the terminal slope of the excretion rate plots using Equation 1, were found to range from 0.037 to 0.059 hr^{-1} after an intravenous dose. These values were comparable to the range of 0.036 to 0.057 hr^{-1} after an oral dose suggesting that the net rates of overall elimination of tocainide between oral and intravenous routes of administration are similar (assuming 100 % bioavailability and with equal doses).

$$\text{Log}\ \frac{dX_u}{dt} = \text{Log}\ k_e X_B^{\,o} - \frac{K_E}{2.303}(t_{mid})$$

$$\text{Terminal slope of the plot Log}\ \frac{dX_u}{dt}\ \text{vs.}\ t_{mid} = \frac{-K_E}{2.303}$$

...Equation 1

Table 16 Urinary excretion rates of tocainide and the hydantoin after a 200 mg IV dose of tocainide HCL in a healthy human volunteer.

hours	urine vol (mL)	Toc[2] ug/mL	dtoc/dt	ug/mL HYD[2]	dHYD/dt	HYD[3]
0						
1	203	6.56	1331.68	8.21	1666.63	8.18
2	521	3.91	2037.11	5.45	2839.45	5.87
3	135	8.19	1105.65	14.29	1929.15	16.01
5	96	12.31	590.88	38.32	1839.26	36.99
7	93	23.63	1098.80	67.91	3157.82	61.78
10	146	28.23	1373.86	44.71	2175.89	41.92
14	175	44.55	1949.06	59.82	2617.13	49.84
48	40	6.08	243.20	5.46	218.40	4.34
53	198	2.41	95.44	5.69	225.32	4.49
59	180	7.61	228.30	5.68	170.40	4.35
62	101	6.96	234.32	4.86	163.62	3.53
71	302	3.97	133.22	2.30	77.18	1.83
72	56	2.72	152.32	1.60	89.60	1.31
76	202	1.38	69.69	1.55	78.28	1.24
82	345	1.67	96.03	1.41	81.08	1.15
87	378	1.86	140.62	-	-	-
95	270	1.46	49.28	-	-	-
96	148	-		-	-	-

Note: 1. Averaged value from duplicate determinations.
2. Using etidocaine as internal standard.
3. Using 4'-methylhydantoin as internal standard.

Table 17 Urinary excretion rates of tocainide and the hydantoin after a 200 mg oral dose of tocainide HCL in a healthy human volunteer.

hours	urine vol (mL)	(ug/mL)[1] tocainide[2]	dToc dt	(ug/mL)[1] HYD[2]	dHYD dt	(ug/mL)[1] HYD[3]
0						
1	44	52.35	2303.40	50.63	2227.72	46.97
2	264	12.87	3397.68	15.34	4049.76	14.72
3	132	17.38	2360.16	17.84	2354.88	22.17
5	68	50.01	1700.34	77.55	2636.70	67.64
7	106	28.60	1515.80	55.35	2933.55	51.34
10	130	36.73	1591.63	46.10	1997.67	47.68
14.5	140	37.90	1179.11	47.49	1477.47	43.03
23.5	336	37.79	1410.83	38.19	1425.76	43.03
24.5	50	15.48	774.0	17.43	871.50	13.27
28	190	5.14	279.03	14.11	765.97	13.29
32	212	11.87	629.11	16.34	866.02	12.72
38	222	13.37	494.69	14.54	537.98	10.86
47	246	16.85	460.57	10.04	274.43	9.11
48	34	5.35	181.90	5.39	183.26	4.76
52	158	2.08	82.15	4.32	170.64	-
58	220	7.52	275.73	5.13	188.10	4.12
62	130	7.26	235.95	3.91	127.08	3.23
72	294	4.14	121.72	2.54	74.68	2.12
76	183	-	-	-	-	-
83	233	3.51	116.83	2.53	84.21	-

Note: 1. Average value from duplicate samples.
2. Using etidocaine as internal standard.
3. Using 4'-methylhydantoin as internal standard.

Table 18 The summarized pharmacokinetic data for tocainide and the hydantoin in three healthy human volunteers after both an IV and oral dose.

	SR		CK		RE		AVERAGE (n=3)	
	IV	ORAL	IV	ORAL	IV	ORAL	IV	ORAL
KE (hr^{-1})	0.057	0.059	0.036	0.044	0.037	0.037	0.044	0.047
t 1/2	12.25	11.84	19.27	15.78	18.73	18.75	15.75	14.85
Kmu (hr^{-1})	0.055	0.054	0.043	0.047	0.052	0.055	0.050	0.052
t 1/2	12.63	12.72	15.93	14.63	13.36	12.57	13.86	13.33
X_u^{∞} (mg)	195.43	52.51	49,57	24.23	24.87	53.8	-[4]	-[4]
M_u^{∞} (mg)	91.24	40.58	33.15	10.92	37.87	61.81	-[4]	-[4]
Mu^{∞} (mg)	93.35	39.45	39.11	9.91	34.50	54.39	-[4]	-[4]
k_e (hr^{-1})	0.028	-[5]	0.014	-[5]	0.010	-[5]	0.0172	-[5]
k_f (hr^{-1})	-[6]	-[5]	0.0089	-[5]	0.0061	-[5]	0.0075[7]	-[5]
% dose excreted intact	47.97	-[5]	31.37	-[5]	25.68	-[5]		
% dose excreted as ToCG	-[6]	-[5]	24.72	-[5]	16.49	-[5]		

Note: 1. May represent underestimation of true X_u^{∞} due to incomplete urine collection.
2. Using etidocaine as I.S. (may represent underestimation due to incomplete urine collection).
3. Using 4-methylhydantoin as I.S. (may represent underestimation due to incomplete urine collection).
4. Cannot be determined due to incomplete urine collection.
5. Cannot be determined due to lack of absolute bioavailability data.
6. Cannot be determined due to $k_{mu} < KE$.
7. n=2.

The elimination half-lives of tocainide among the three subjects averaged 15.75 hours (oral) and 14.85 hours (intravenous), both of which are in agreement with literature reported half-lives (33).

The urinary excretion rate constants of tocainide (ke) from the three subjects after an IV dose, as determined by an extrapolation plot as shown in Figure 67A using Equation 2, were averaged to be 0.0172 $hour^{-1}$. Due to incomplete urine collection from all three subjects, the extrapolation method based on equation 2 is the only reasonable estimate for the urinary excretion rate constant (ke). In addition, due to the lack of oral bioavailability data, the extrapolation method cannot estimate the excretion rate constants after the oral doses.

$$\text{Y-intercept of plot Log } \frac{dX_u}{dt} \text{ vs. } t_{mid} = \text{Log } k_e X_B^{\,o}$$

where k_e = urinary excretion rate of tocainide.

$X_B^{\,o}$ = dose of tocainide.

.....Equation 2

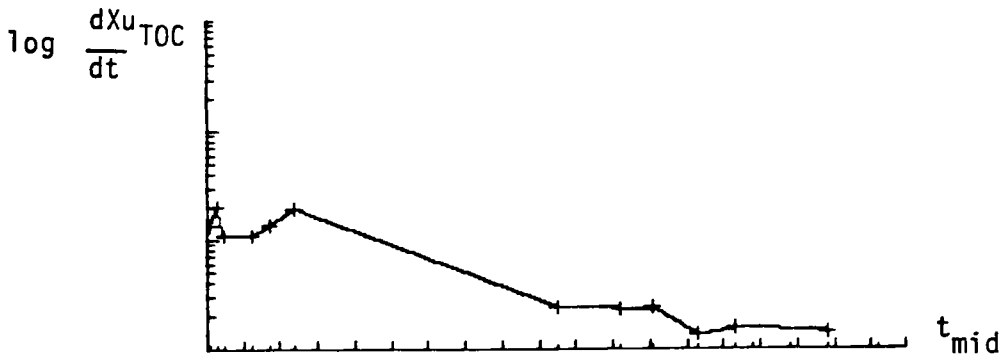


Figure a: Urinary excretion rate profile of tocainide.

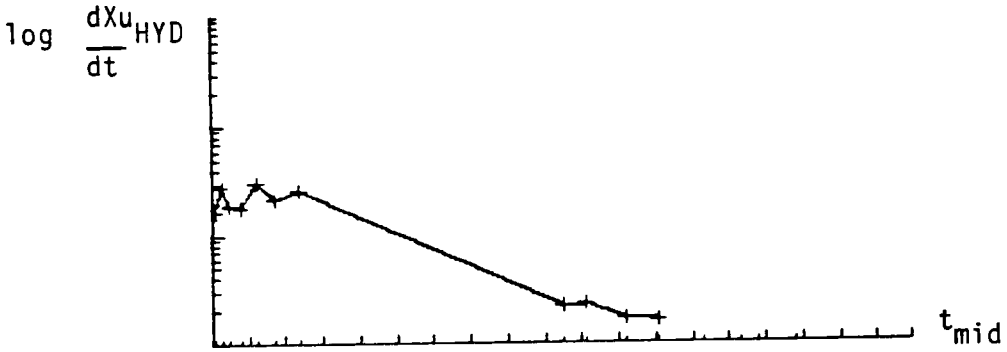


Figure b: Urinary excretion rate profile of hydantoin.

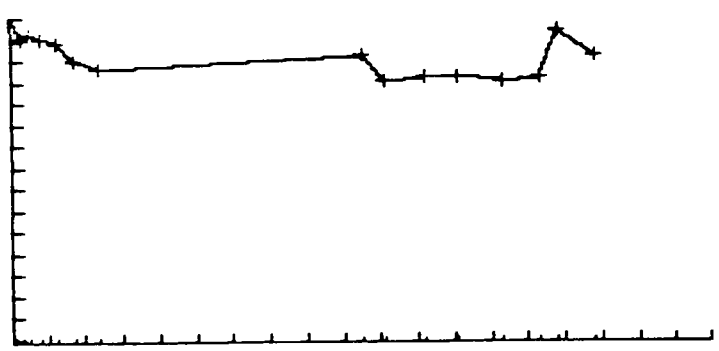
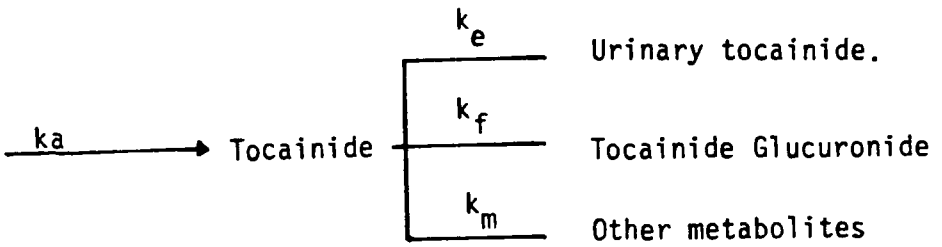


Figure c: Urinary PH.

Figure 67 The semi-log plot of the urinary excretion profile of tocainide (a) and the hydantoin (b) after receiving a 200mg IV dose of tocainide.



Scheme 18 Schematic representation of the elimination of tocainide in humans.

According to Scheme 18 shown above, the elimination of tocainide can be broken down into various pharmacokinetic parameters described by the corresponding first-order rate constants. The urinary excretion rate constants (Kmu) for the glucuronide, as calculated by the method of feathering, as shown in Figure 67B and 68B using Equation 3, were averaged to be 0.050 $hour^{-1}$ (oral) and 0.052 $hour^{-1}$ (intravenous). The averaged urinary excretion half-lives for the glucuronide from the three subjects were calculated to be 13.86 and 13.33 hours after receiving an oral and an intravenous dose respectively, and are in agreement with published literature values (33).

$$\text{Log}\ \frac{dM_u}{dt} = \text{Log}\ \frac{k_{mu} k_f X_B^{\,o}}{k_{mu} - K_E} - \frac{k_{mu}}{2.303}(t_{mid})$$

the slope of the above equation as obtained by feathering $= \frac{-k_{mu}}{2.303}$

assuming $k_{mu} \gg K_E$

.....Equation 3

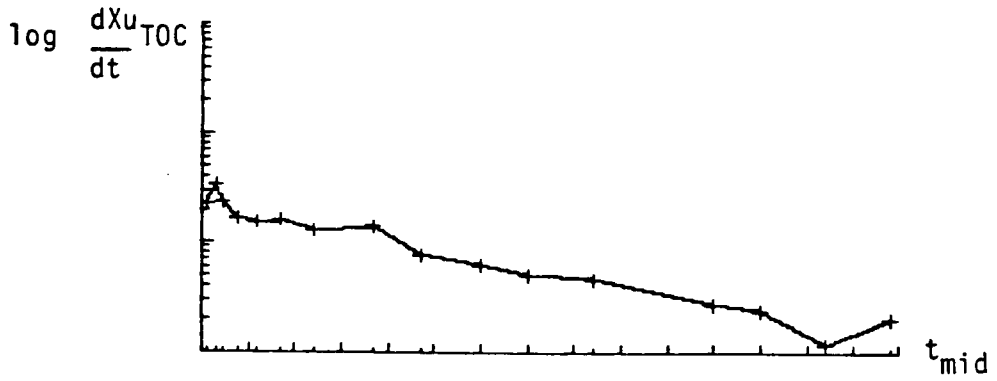


Figure a: Urinary excretion rate profile of tocainide.

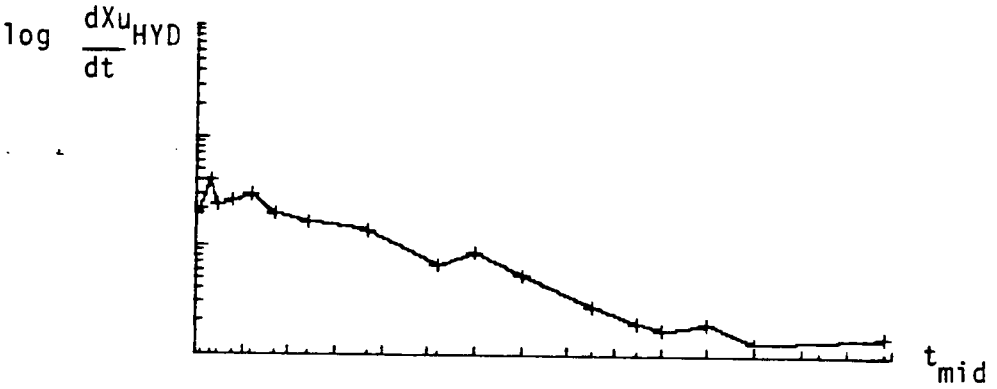


Figure b: Urinary excretion rate profile of hydantoin.

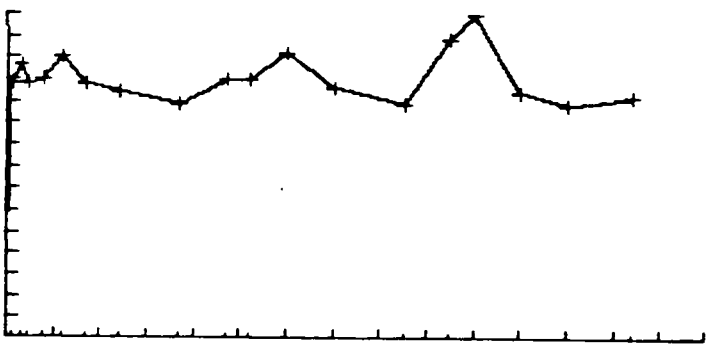


Figure c: Urinary PH

Figure 68 The semi-log plot of the urinary excretion profile of tocainide (a) and the hydantoin (b) after receiving a 200mg oral dose of tocainide.

The formation rate constants (kf) of the tocainide glucuronide from the three subjects were again estimated by extrapolation using Equation 4.

$$\text{Log } \frac{dM_u}{dt} = \text{Log } \frac{k_{mu}\ k_f\ X_B^{\,o}}{k_{mu} - K_E} - \frac{K_E\ (t_{mid})}{2.303}$$

$$\text{Y-intercept of plot log } \frac{dM_u}{dt} \text{ vs. } t_{mid} = \text{Log } \frac{k_{mu} k_f X_B^{\,o}}{k_{mu} - K_E}$$

where k_{mu} = urinary excretion rate of TOCG

$X_B^{\,o}$ = dose of tocainide in body

K_E = total elimination of tocainide

.... Equation 4

The formation rate constants of the glucuronide from the three subjects were averaged to 0.0075 $hour^{-1}$ after an IV dose. Due to the lack of absolute bioavailability data from the three subjects, kf cannot be estimated by the extrapolation method after receiving an oral dose.

The total amount of tocainide excreted in the urine (Xu^{∞}) after an IV dose, as calculated by Equation 5 , was found to range from 95.93 mg (47.97 % dose) to 51.35 mg (25.68 % dose) among the three subjects.

$$X_u^{\infty} = \frac{X_B^{\,o}\ k_e}{K_E}$$

... Equation 5

The percent of dose excreted in the urine as tocainide glucuronide was calculated by the ratio kf/K_E as shown in Equation 6.

$$\frac{M_u^{\infty}}{(F)dose} = \frac{k_f}{K_E} = \text{ratio of dose excreted as metabolite in urine.}$$

.... Equation 6

The total amount of the glucuronide expressed as % dose was calculated to 24.72 % and 16.49 % in two subjects.

In summary, the pharmacokinetic data calculated from the three healthy human volunteers are in agreement with published literature values.

SUMMARY AND CONCLUSIONS

The oral bioavailability of tocainide can be conveniently determined by the use of a single pseudo-racemic dose. With the previous objective for such a study, the stereospecific synthesis of R(-)- and S(+)-trideuterated tocainide was designed through two approaches.

Tocainide carbamoyl ester glucuronide was known to cyclize to 3-(2,6-xylyl)-5-methylhydantoin at pH > 12. This thesis described a novel reaction between tocainide and urea, a second pathway which led to the formation of 3-(2,6-xylyl)-5-methylhydantoin through a tocainide N-ureide intermediate. This finding led to the speculation of an alternative tocainide-N-ureide glucuronide structure for TOCG. However, structural evidence for the carbamoyl ester conjugation structure was obtained from permethylation experiments (CH_3I/CD_3I) and GCMS analysis. Furthermore, a preparative HPLC assay was developed to isolate TOCG for NMR and FAB spectral measurements. The 400-MHz proton NMR data of the isolated glucuronide provided only partial evidence for the intact structure of TOCG. However, FAB analysis of the sample in both glycerol and thioglycerol revealed [M+1], [M+Na] and [M-H+2Na] ion adducts at m/z 413, 435 and 457 respectively, indicating the intact molecular ion of TOCG at m/z 412.

The levels of TOCG in urine can be conveniently assayed as the hydantoin after hydrolysis of TOCG at pH > 12. However, the hydantoin was found to also undergo first-order hydrolysis at pH > 12 conditions. To account for the spontaneous hydrolysis of

the hydantoin, a set of accurately timed calibration sample used in order to approximate the true levels of TOCG in urine. Based on this analytical scheme, the levels of TOCG in urine were examined in three subjects after IV and oral dosing of 200 mg tocainide hydrochloride. The urinary excretion half-lives of TOCG were found to be 13.86 hours and 13.33 hours after an IV and oral dose respectively. These data were in agreement with literature values.

These studies have conclusively established the structural identity of TOCG as a carbamoyl ester glucuronide. In addition, a novel chemical reaction between tocainide and urea was observed to give rise to a ureide intermediate. This urea condensation reaction has not been reported in drug metabolism. Nevertheless, such a reaction between urea and other primary amine xenobiotics remains possible.

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APPENDIX 1

	IV			ORAL	
	dToC	dHYD		dToC	dHYD
hours	dt	dt	hours	dt	dt
1	4071.04	1517.33	1	845.05	648.46
2	2275.38	1423.92	2	-	-
3	3630.96	2700.69	3	-	-
5	2084.13	1696.41	5	-	-
7	2383.54	1589.66	7	-	-
9	1693.74	1059.68	9	2110.81	1402.30
11	1068.69	1198.91	10	-	-
12.5	498.04	-	16	-	-
13.5	506.27	639.11	20	1424.20	388.04
19	157.62	1047.77	24	-	-
21	1208.67	471.26	30	491.92	341.46
24	506.81	337.59	32	275.62	160.25
27	1681.42	806.70	35	278.83	159.42
28	1427.28	-	37	463.11	208.71
30	505.12	283.52	43	369.29	218.13
32.5	688.53	279.36	45	442.37	170.97
37	1085.19	515.04	48	278.73	160.61
43	529.08	249.48	54	224.08	-
46	425.80	186.77	57	211.72	117.34
53	-	-	61	-	-
58	-	-	64	356.24	-
63	-	-	66	186.32	-
68	-	-	69	135.56	-
72	-	-	72	54.51	55.06
78	89.79	69.62	81	29.57	-
82	-	-	83	-	-
86	-	-			
96	-	-			

Appendix 1 : The urinary excretion rates of tocainide and the hydantoin after a 200mg oral and IV dose of tocainide HCl in a healthy volunteer CK.

APPENDIX 2

hours	IV dToC dt	IV dHYD dt	hours	ORAL dToC dt	ORAL dHYD dt
1	1082.43	1328.04	1		
2	-	-	2	4107.88	4623.46
3	1638.20	1273.64	3	5377.42	5056.92
5	4363.81	2447.44	5	3455.85	3090.57
7	3194.67	2183.09	7	336.73	254.41
10	39469.54	19853.81	10	5664.46	3614.07
15.5	2177.26	951.77	15.5	-	-
23	1998.95	1033.72	23	1428.36	902.41
24	2716.40	1299.98	26	291.55	361.90
28	223.92	191.00	30	507.58	416.91
30	157.24	62.29	31.5	806.87	644.47
32	1528.67	637.25	36	-	-
35	7182.43	312.76	38	-	-
39	377.92	271.74	44	-	-
48	-	-	48	-	-
50	-	-	50	-	-
54	-	-	52	-	-
58	267.29	171.83	56	225.23	171.05
62	150.54	98.66	61	174.59	144.19
71	-	-	64	130.83	106.54
72	-	-	72	137.25	114.54
79	-	-	76	-	-
84	-	-	82	-	-
85	-	-	87	-	-
92	-	-	94	-	-
96	-	-	96	-	-

Appendix 2 : The urinary excretion rates of tocainide and the hydantoin after a 200mg oral and IV dose of tocainide HCl in a healthy volunteer RE.