SYNTHESIS AND APPLICATIONS OF DEUTERATED METHADONE
AND METABOLITES TO BIOTRANSFORMATION
AND DISPOSITION STUDIES

by

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ABSTRACT

Deuterium labeled methadone and deuterium labeled metabolites were synthesized to use in gas chromatography mass spectrometry (GCMS) studies of the metabolic pathways of methadone in rats. These compounds were also useful to develop sensitive and selective analytical methods to study the pharmacokinetics and disposition of methadone.

Synthesis of the deuterium labeled compounds was mainly achieved by using known procedures with special treatments required to provide label enrichment.

Using the labeled and unlabeled derivatives, mass fragmentation processes that are common to methadone and its metabolites were defined. Aryl ring migration was observed in a fragmentation process for 2-ethylidene-1,5-dimethyl-3,3-diphenyl pyrrolidine (EDDP). This aryl ring migration was not a favorable process for ring substituted EDDP analogs.

Various aspects regarding the optimization of the selected ion monitoring (SIM) analysis of methadone and its metabolites in biological fluids are described. The SIM analysis using deuterium labeled compounds as internal standards generally proved to be selective but not as sensitive as expected using electron impact ionization (EI) conditions of GCMS. One advantage of using SIM over GC analysis was described in terms of ratio analysis. Quantitation of methadone
in human plasma and saliva using SIM gave a lower limit of sensitivity of 20 ng/0.5 ml of sample by monitoring the base peak, m/e 72. The mean methadone ratios of saliva to plasma for two patients were 0.55 ± 0.15 (standard deviation) and 0.48 ± 0.10 (standard deviation).

Methadone metabolism studies emphasized the detection of minor metabolites using special extraction methods for rat bile and using labeled and unlabeled compounds. Comparison of the mass spectra from total ion current (TIC) profiles of metabolites from unlabeled compounds with those from labeled compounds run as separate experiments gave GCMS evidence for methadone nitrone (N-methylene-1-methyl-3,3-diphenyl-4-oxo-hexanamine-oxide). Possibilities for the metabolic formation of N-hydroxynormethadone and the pharmacological significance of the detection of methadone nitrone were described. A proposal for metabolic studies to examine the potential formation of other methadone metabolites resulting from metabolic oxidation of nitrogen was presented.

Structural evidence for the methadone nitrone molecule was obtained indirectly by chemical oxidation studies of methadone metabolites. m-Chloroperbenzoic acid treatment of EDDP perchlorate gave three products: methadone nitrone, 4,4-diphenyl-2,5-heptanedione (diketone), and 2-acetyl-5-methyl-3,3-diphenyl-1-pyrroline. These compounds were identified from their IR, NMR and mass spectral data. Mass fragmentation processes were defined for the methadone nitrone. Possible mechanisms for the formation of methadone nitrone and diketone from
chemical oxidation of EDDP are proposed.

Since diazepam is a drug widely abused by methadone maintenance patients, methadone-diazepam interaction studies were designed to analyze metabolites using deuterium labeled authentic compounds as internal standards. Metabolites in the conjugated fraction of rat bile were analyzed using deuterium labeled biosynthetic internal standards. Diazepam (5 mg/kg) was given to rats through a cannulated jugular vein and a subcutaneous dose of methadone (10 mg/kg) was given. Bile was collected through the cannulated bile duct over a period of 24 hours. The deuterium label was found to be stable even under severe conditions of incubation temperature and time. SIM analysis of bile sample extracts showed that concomitant administration of diazepam with methadone did not affect biliary excretion of EDDP nor the conjugated metabolites. This indicates that diazepam does not interact with methadone at the hepatic metabolism level and with transport of the metabolites by the biliary excretion route. Application of the use of a biosynthetic internal standard to drug metabolism and pharmacokinetic studies by means of ratio analysis was described with examples.

Signature of Thesis Supervisor

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ABBREVIATIONS

Ar. Phenyl
a.m.u. atomic mass unit
CI chemical ionization
DDP 1,5-dimethyl-3,3-diphenyl-2-pyrrolidone
DMSO dimethyl sulfoxide
dR deoxyribose
EDDP 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine
EI electron impact
EMDP 2-ethyl-5-methyl-3,3-diphenyl-1-pyrroline
EtOH ethanol
GC gas chromatography
GCMS gas chromatography mass spectrometry
GSH reduced glutathione
HPLC high pressure (performance) liquid chromatography
i.d. internal diameter
I.P. intraperitoneal
IR infrared spectroscopy (spectrophotometer)
I.S. internal standard
i.v. intravenous
MCPBA m-chloroperbenzoic acid
MeOH methanol
mp melting point
NMR  nuclear magnetic resonance (spectroscopy)
o.d.  external diameter
PFK  perfluorokerosene
s.c.  subcutaneous
SD  standard deviation
SIM  selected ion monitoring
t_{1/2}  biological half-life
TIC  total ion chromatogram
TLC  thin layer chromatography
TMCS  trimethylchlorosilane
TMS  tetramethylsilane
UV  ultraviolet

Note: Methadone nitrone is used for convenience to describe the nitrone found in metabolism studies of methadone and chemical oxidation of EDDP perchlorate. Specifically, the chemical is normethadone nitrone or N-methylene-1-methyl-3,3-diphenyl-4-oxo-hexanamine-oxide.
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DEDICATION

To my wife and daughters
INTRODUCTION

Methadone (1) was first synthesized by a German chemist during World War II and the drug was made clinically available in 1947 as a morphine substitute. The pharmacological properties of methadone are qualitatively identical to those of morphine.

The dose of methadone as an analgesic is in the same range as that of morphine. The drug has marked sedative effects in some patients upon repeated administration and therefore limited use was only allowed.

Methadone shows a considerable degree of analgesic action when given orally. While the withdrawal syndrome of methadone is similar to that of morphine, it develops slowly and is less intense and more prolonged. The drug has a long duration of action with an average $t_{1/2}$ of 25 hours compared with 2.5-3 hours for morphine. The possibility of
methadone treatment for rehabilitation of heroin addicts was first reported in 1965 by Dole and Nyswander. By 1977, in the United States alone, over 90,000 patients were enrolled in methadone maintenance program with costs between $4.00 and $5.50 a day per patient (1).

Metabolic pathways of methadone

Studies on metabolic pathways of methadone have been performed to determine contributions of specific pathways to the kinetics of methadone and to correlate the formation of specific metabolites to activity and toxicity of this drug. Beckett et al. (2), Pohland et al. (3), Sullivan et al. (4-6), Lynn et al. (7), and Ånggård et al. (8) showed that methadone undergoes demethylation to form EDDP (3) and in turn EMDP (4) which is then oxidized to ring hydroxylated metabolites. Methadone is reduced to methadol (7) and N-demethylmethadol. Fig. 1 is a summary of the metabolic pathways of methadone as found in rats and in humans.

Methadone and its major metabolite, EDDP have been analyzed to describe the pharmacokinetics of methadone. Conjugated metabolites (9, 10, 11) were also considered to be important with respect to interactions arising from concomitant drug administration with methadone (9).

No one has fully explained the activity and toxicity of methadone in relation to its metabolism. EDDP and EMDP were found to be inactive as analgesics (3). Methadol and demethylmethadol
Fig. 1. Metabolic Pathways of Methadone
account for only part of the analgesic activity (5). The postulate that the mechanism of action of methadone might be explained in relation to the binding of methadone and methadone metabolites to cellular components (10) has not been proved. Kreek et al. (11-12) evaluated the chronic toxicity of methadone and reported that methadone treatment caused minimal side effects without toxic effects. Medical complications (13) and hepatic damage (14) were studied in relation to narcotic addiction. This phenomenon is not limited to methadone but associated with narcotics in general. A specific metabolic route does not appear to be involved in the toxicity of methadone.

Synthesis of methadone and its analogs

Two methods are commonly available for the synthesis of methadone. The synthetic method outlined by Schultz et al. (15) used a condensation reaction of diphenylacetonitrile (12) with 2-dimethylaminoisopropyl chloride (13) in the presence of NaNH₂ to form two isomers, methadone nitrile (14) and isomethadone nitrile (15). Methadone nitrile was separated by fractional recrystallization from hexane.
Another method described by Easton et al. (16) involved the reaction of propylene oxide with diphenylacetonitrile to give 3,3-diphenyl-5-methyltetrahydro-2-furanone imine (16), which is further converted to 4-bromo-2,2-diphenylpentane nitrile (17). The bromo compound, 17 reacted with dimethylamine to give methadone nitrile (14).

\[
\begin{align*}
\text{(C}_6\text{H}_5\text{)}_2\text{C}^- \text{CH-CN} + \text{CH}_2 \text{CH-CN} & \rightarrow \text{NaNH}_2
\end{align*}
\]

\[
\begin{align*}
\text{(C}_6\text{H}_5\text{)}_2\text{C}^- \text{CH-CN} + \text{CH}_2 \text{CH-CN} & \rightarrow \text{NaNH}_2
\end{align*}
\]

\[
\begin{align*}
\text{(C}_6\text{H}_5\text{)}_2\text{C}^- \text{CH-CN} + \text{CH}_2 \text{CH-CN} & \rightarrow \text{NaNH}_2
\end{align*}
\]

The nitrile, 14 was converted to methadone and its analogs (18) by a Grignard reaction.
Two deuterium labeled methadones, methadone-$^2$H$_3$ (18, R=CH$_2$CD$_3$) and methadone-$^2$H$_5$ (1, Ar=$^2$H$_5$) have appeared in the literature. Both of them were synthesized by the method of Schultz et al. (15). Methadone-$^2$H$_3$ was prepared for use in measuring plasma levels and determining steady state kinetics of methadone (17). A possible use of methadone-$^2$H$_3$ as an in vivo marker for monitoring the methadone intake of a maintenance patient was also suggested (18). Methadone-$^2$H$_5$ was used as the internal standard in selected ion monitoring (SIM) analysis to determine plasma and urinary levels of methadone (19) and to study stereospecific metabolism of methadone (20). Kreek et al. dosed deuterium labeled R-(-)-methadone and S-(+)-methadone to methadone maintenance patients and monitored specifically labeled methadone, observing that the active R-(-)-enantiomer has a longer half life than the less active S-(+)-enantiomer.

The synthetic scheme adopted to obtain methadone-$^2$H$_3$ was unusual in that CD$_3$CD$_2$Br instead of CD$_3$CH$_3$Br was used, followed by a KOH catalyzed exchange to yield the desired compound (18, R=CH$_2$CD$_3$).
This could be due to an economical consideration in the synthesis. The synthesis of methadone-\(^2\)H\(_5\) was achieved with diphenylacetonitrile-\(^2\)H\(_5\) (12, Ar=\(^2\)H\(_5\)) which was obtained by Friedel-Crafts reaction of benzene-\(^2\)H\(_6\) and bromophenylacetonitrile in CS\(_2\) solvent.

Two methadone analogs, the octanone (18, R=CH\(_2\)CH\(_2\)CH\(_3\)) (17) and the nonanone (18, R=CH\(_2\)CH\(_2\)CH\(_2\)CH\(_3\)) (21) were prepared as the internal standards for the analysis of methadone. The synthesis of these compounds was simple, using either propyl bromide or butyl bromide for the Grignard reactions.

**Synthesis of methadone metabolites**

Synthetic methods for 4-dimethylamino-2, 2-diphenyl-pentanoic acid (5) and DDP (6) were published by Gardner et al. (22). Hydrolysis of methadone nitrile forms the acid, 5 which is demethylated by SOCl\(_2\) treatment to give the cyclized product, DDP.

\[
\begin{align*}
\text{H}_2\text{SO}_4, \text{H}_2\text{O} & \quad \text{COOH} \\
\text{SOCl}_2 & \quad \text{N-CH}_3
\end{align*}
\]

14 5 6

DDP is used as an intermediate to synthesize EDDP and EMDP. Treatment of DDP with C\(_2\)H\(_5\)Li gives EDDP. EDDP is demethylated by HI and heat to produce EMDP. Perchlorate
formation of 3 led to the conversion of the exocyclic structure, 3 to the endocyclic structure, 19 as shown by NMR studies (3, 23). EMDP (4) and its salt (20) both contain an endocyclic double bond (3, 23).

Methadol (7), a metabolite of methadone and an intermediate for the synthesis of 1-α-acetylmethadol (LAAM) was chemically prepared by platinum oxide hydrogenation, LiAlH₄ reduction, or sodium-propanol reduction (24) of methadone. The ratio of isomers varied depending upon the reagents with preferable formation of the α-isomer by the use of Adams catalyst or LiAlH₄. Synthesis of normethadol and dinormethadol was achieved by chemical N-demethylation procedures (25).

Methadone N-oxide was prepared by mild oxidation of methadone. Treatment of methadone with 2.5 mol of m-chloroperbenzoic acid gave EDDP (26). Permanganate oxidation of methadone was reported to produce a mixture of DDP and 21 (27). Methoxymethadone (22) was synthesized by using anisole instead
of benzene in the Friedel-Craft step of the reaction to synthesize methadone (28).

No report has appeared on the synthesis of deuterium labeled methadone metabolites. This is perhaps because of the fact that EDDP which has the most important implications for the pharmacokinetics of methadone, can be analyzed by gas chromatography.

Attempted synthesis of normethadone

Pohland et al. (25) treated DDP with EtLi in an attempt to obtain normethadone. The isolated product was EDDP because
of spontaneous cyclization of normethadone. Cyanogen bromide demethylation (29) of methadone did not give normethadone but yielded 2-ethylidene-5-methyl-3, 3-diphenyltetrahydrofuran (23) (30).

Analysis of methadone and metabolites in biological samples

Gas chromatography and SIM analysis are two major methods to analyze methadone and its metabolites in human samples. The sensitivity of gas chromatography for methadone in plasma and urine and EDDP in urine is of the order of 5-15 ng/ml (21, 31, 32). On the other hand, EMDP, a minor metabolite, could not be quantified in human urine because of a lack of sensitivity and selectivity of gas chromatography (33). A SIM assay by gas chromatography-mass spectrometry (GCMS) under electron impact conditions (EI) was described for the quantitation of methadone by monitoring m/e 294 (methadone), m/e 297 (methadone-$^2$H$_3$), and m/e 308 for the internal standard, 2-dimethylamino-4, 4-diphenyl-5-octanone (18, R=CH$_2$CH$_2$CH$_3$) (17). By this method, methadone in plasma was assayed with a sensitivity limit of 5 ng/ml. Methadone-$^2$H$_5$ has been utilized for the SIM assay of methadone in human plasma using chemical ionization (CI) (19). The sensitivity limit of this method was similar to that reported using the EI-SIM.
In animal experiments analytical difficulties are frequently encountered because of the small size of samples, in which the methods usually require ten times the sensitivity compared to those for human experiments. Use of a radioisotopic method is the method of choice in animal experiments. For example, the kinetics of methadone in rat plasma was studied by using $^{14}$C-labeled methadone (34) because of a lack of sensitivity generally shown in the use of gas chromatography (35). Sensitive analytical methods such as radioimmunoassay are still being developed to analyze methadone in plasma (36), by which a lower limit of sensitivity of 3 ng/ml with a sample size of 0.05 ml has been achieved.

Conjugated metabolites are important from both a qualitative and quantitative point of view. When drug interactions are studied the interaction may be reflected in quantitative changes of the conjugated metabolite(s) (9). Therefore, it is necessary to have a means to analyze conjugated metabolites accurately. Qualitative analysis of conjugated metabolites as intact molecules has been recently reviewed (37). Quantitative analysis is however achieved only by hydrolyzing the conjugate portions. The hydrolyzed metabolites are extracted quantitatively into organic solvents and chromatographed using GC or TLC. In the case of radioisotopic methods, TLC is the separation method of choice. In most cases, authentic compounds of conjugated metabolites are not available. When complicated bile samples are chromatographed by TLC, complete separation is a problem as emphasized by Roerig et al.
(38) and Whitehouse et al. (39) in their studies of methadone metabolism. Depending upon the solvent system, marked variation in the amount of conjugated and nonconjugated metabolites has been observed.

Pharmacokinetic aspects of methadone

Synthesis of methadone analogs continued from the 1940's to the early 1960's. The use of methadone for maintenance of addicts is based on accumulated pharmacological data to the middle of the 1960's. Studies of metabolite detection were initiated in the mid 1960's and continued for ten years to the mid 1970's. From 1970 to 1979, many references were published on the biopharmaceutic and clinical pharmacokinetic aspects of methadone.

Studies of methadone kinetics in humans were aimed at adjustment of dosage levels of maintenance patients. Holmstrand et al. (40) reported that the best record of rehabilitation was achieved with steady-state plasma concentrations above 200 ng/ml of methadone indicating that a pharmacokinetically optimized dosage regimen would be useful in increasing the effectiveness of methadone maintenance treatment. Horns et al. (41) reported that there was no relationship between plasma methadone level and patient's subjective symptom complaints.
The mechanism of tolerance to methadone was partly sought in metabolic tolerance by means of kinetic studies of methadone. Chronic administration of methadone to man shortens the half life of the drug. The acute primary $t_{1/2}$ of 14.3 hours in combination with the acute secondary $t_{1/2}$ of 54.8 hours was longer than the single exponential chronic $t_{1/2}$ of 22.2 hours (42). A recent paper by Liu et al. (43) reported that tolerance to methadone analgesia is due to both increased methadone metabolism and cellular adaptation to the drug in the brain.

The effect of disease states on methadone kinetics in maintenance patients was studied. No evidence was found for the accumulation of either methadone or its metabolites with renal disease, suggesting that methadone is an appropriate narcotic to use in patients with renal insufficiency (44). On the other hand, a decreased urinary excretion of methadone and its metabolites was observed in patients with liver disease (45).

**Methadone and drug interactions**

According to reported surveys (46-48), the drugs which are abused with methadone are ethanol, diazepam, other opiates, amphetamines, and barbiturates. The interactions of methadone with these drugs were studied either with human or with animal models.
An agonist-antagonist interaction occurred in maintenance patients between methadone and naloxone, with no changes in methadone disposition (49). Significant changes in methadone disposition occurred during combined treatment with rifampin (49), which was due to the enzyme induction property of rifampin (50). Enhanced metabolism of methadone by diphenylhydantoin was observed in studies of methadone maintenance patients (51). This is similar to the effect of diphenylhydantoin on dexamethasone (52). Biliary excretion of conjugated methadone metabolites was increased by phenobarbital pretreatment of rats (38). At doses of disulfiram suitable for the management of alcoholism there was no significant interaction between disulfiram and methadone (53). Desipramine showed inhibition of methadone metabolism in the rat liver (54).

Three papers appeared on methadone-ethanol interactions. The administration of ethanol to rats resulted in increased brain and liver concentrations of methadone and decreased biliary output of methadone (39, 55). No significant acute interaction was found between methadone and ethanol in studies of maintenance patients (56).

Diazepam was shown through \textit{in vitro} studies to be an effective inhibitor of the N-demethylation of methadone which may explain in part the enhanced effect of methadone observed in narcotic addicts when diazepam is taken in combination with methadone (57). Four \textit{in vivo} studies of methadone-diazepam interactions were published: one with rats (34), another with rhesus monkeys (58), and the others with mice.
Acute administration of diazepam to the rat prolonged the duration of methadone analgesia, increased the brain concentrations of total $^{14}$C and decreased the percent of total $^{14}$C in the liver or urine (34). Behavioral depression after diazepam was prolonged substantially in methadone maintained monkeys. Blood levels of diazepam and metabolites were not increased or prolonged in those animals (58). Enhanced hepatic levels of methadone for up to three hours by diazepam indicated an interference with methadone metabolism by this agent in mice (59). On the other hand, Shannon (60) reported that diazepam failed to enhance brain and plasma methadone levels in mice.

Deuterium labeled compounds for studies of pharmacokinetics and drug metabolism

The steady state kinetics of a drug can be studied by a method in which stable isotope labeled and unlabeled drugs are administered and collected samples are analyzed concomitantly by using a third internal standard. Such an approach was demonstrated for methadone (61) and propoxyphene (62).

The same approach was applied to bioavailability studies of N-acetylprocainamide in humans (63). The method can provide ease in the analysis and compensate for differences in hepatic extraction resulting from different hepatic blood
flows at different times and in different individuals. The hepatic blood flow is variable even within a healthy population and varies between 0.5 and 3 l/min. (64).

When stable isotope labeled analogs are used for pharmacokinetic and metabolic studies of a drug, bioequivalence of the labeled analogs should be established. This was done by Hsia et al. (18) for methadone and methadone-$^2$H$_3$. Another report of [$_{13}$C$_{15}$N$_2$]-diphenylhydantoin published by Browne et al. (65) also showed careful consideration for kinetic equivalence. Bioequivalence is first studied using small animals, the results of which become a basis of the use of labeled compounds for human studies.

Recently published papers have ignored such a careful treatment of the toxicological and kinetic equivalence of stable isotope labeled compounds. For example, N-acetylpro-13C was used without such a test (63). Hachey et al. dosed pentadeuterated methadone to study stereoselective disposition of methadone in man (20). Benoxaprofen-$^2$H$_{17}$ was used only with acute toxicity data equivalent to unlabeled compound (66).

In order to see metabolic equivalence, mainly hepatic uptake, the liver perfusion method which was used to study hepatic uptake of propanolol (67) could be used.

Differences of pharmacological actions between deuterium labeled and protio drugs are mainly explained by differences in the biotransformation of the drugs. If the labeling is at the site where rate limiting metabolism occurs, an
isotope effect is observed. Marcucci et al. (68) studied
the metabolism and anticonvulsant activity of deuterated N-
demethyldiazepam and concluded that a significant shortening
of anticonvulsant activity was due to a reduced C₃-hydroxylation
of the compounds. Similar results were observed in the
metabolism and toxicity of phenacetin where hydrogen at the
position being oxidized was substituted with deuterium. It
was found that the labeling decreased hepatic necrosis due
to an oxidized metabolite and increased methemoglobinemia
resulting from the metabolites of the nondeethyalted pathway
(69).

In some cases it is difficult to conclude whether the
different pharmacological actions between deuterium labeled
and unlabeled drug are due to metabolic differences or differ-
ences in the physical properties which could influence transport
to the receptor site and receptor binding of the drug. A
significant reduction in toxicity and a decrease in sponta-
neous locomotor activity was observed for highly enriched
deuterated amphetamine (70). Further reports showing possible
reasons for this have not been published. An example of
the participation of the labeled site in receptor binding
was presented for N-CD₃ morphine. An explanation for the
decreased activity of N-CD₃ morphine compared to N-CH₃ morphine
was given by a clastic binding concept (71).
OBJECTIVES OF THE RESEARCH

The objectives of the research were to utilize stable isotope analogs of methadone and its metabolites together with GCMS techniques to investigate methadone disposition and metabolism. Methadone is a drug generally used on a long term basis. Therefore, a complete picture of metabolic pathways is essential to ascertain the potential long term toxicity, possibly that arising from minor metabolites. Extensive metabolic studies in rats using deuterium containing analogs were to concentrate on detecting and identifying new metabolites that might be implicated in potential toxicities.

Several pharmacokinetic studies of methadone have been completed in man to determine the nature of the varied dose requirements and whether blood levels of the drug correlate with good performance of the patient. Most of the dispositional studies are however incomplete because of deficient analytical methods. Therefore, the SIM methods developed with labeled compounds and GCMS were to be applied to pharmacokinetic studies. Saliva sampling and analysis were to be investigated as a noninvasive technique for pharmacokinetic studies and for monitoring methadone levels. A SIM method with deuterated analogs as internal standards was to be used in a drug interaction study in rats of diazepam and methadone.
to selectively identify any metabolic changes of methadone that might occur.

Synthesis of deuterated methadone and metabolites

The syntheses of methadone-\(^2\text{H}_{10}\), EDDP-\(^2\text{H}_{10}\), EMDP-\(^2\text{H}_{10}\), DDP-\(^2\text{H}_{10}\), 4-dimethylamino-2, 2-diphenylpentanoic acid-\(^2\text{H}_{10}\), EDDP-\(^2\text{H}_3\), and EMDP-\(^2\text{H}_3\) were designed. The primary choice for the synthesis of methadone and metabolites where both aromatic rings are deuterium labeled was based on the following reasons. First, the label was not likely to be lost since most metabolites of methadone can be expected to retain the phenyl rings. Secondly, under EI conditions major fragment ions of metabolites will frequently contain all or a portion of the label, and thirdly, the costs of the synthesis appeared reasonable.

The first point is important to detect metabolites and the second point for SIM analysis of methadone and metabolites under EI conditions. The compound labeled on one ring (\(1, \text{Ar}=\text{H}_5\)) which was used for stereoselective metabolism of methadone (19) has two diastereomers which can complicate metabolic studies.

The labeled compounds were also considered to be useful for two future research projects. According to the narcotic receptor model, which shows participation of the ring in the receptor binding (72), ring labeled methadone could be a good model to study such binding. The labeled
compounds could be used to investigate ring oxidation mechanisms. A study of isotope effects would reveal whether oxidation occurs via arene oxide formation such as in the case of diphenylhydantoin (73) or by direct oxygen insertion as shown in mono-o-demethylation of p-trideuteromethoxyanisole (74).

Mass fragmentation studies

Stable isotope labeled compounds were considered to be most useful in defining fragment ions in mass spectrometry. By comparing the mass spectra of the labeled and unlabeled derivatives, fragmentation processes can be described in more detail than previously available (6). Fragmentation studies can also be used to determine the structure of the new metabolites which may have very similar structures to known synthesized metabolites. In addition to the fragmentation information of mass spectrometry, spectroscopic studies such as IR and NMR can be described in more detail using deuterium labeled compounds.

SIM analysis of methadone and metabolites in biological samples

The GCMS, Varian MAT 111 was remodeled to do selected ion monitoring. The analytical methods for methadone and
metabolites were developed with the use of labeled methadone and metabolites as the internal standards and with a computer program which was improved to ensure high sensitivity and high precision. Human samples were used to investigate the applicability of the developed methodology to pharmacokinetic studies of methadone. Emphasis was also placed on the use of a high abundance ion of methadone to improve the sensitivity of methadone analysis in human or animal studies. The methodology was also applied to the investigation of the saliva-plasma relationship of methadone.

Studies of methadone-diazepam interaction

Methadone-diazepam interaction studies were designed which included a methodology for the use of biologically formed internal standards (biosynthetic internal standards). The use of biosynthetic internal standards for pharmacokinetic and drug metabolism experiments is possible only with deuterium labeled compounds and SIM methodology.

In view of the methadone-diazepam interaction study, the method was expected to accurately quantitate methadone and its metabolites, especially conjugated metabolites. A resolution problem in the TLC of the metabolites was encountered in previous studies (38, 39).
Detection of new methadone metabolites

Studies directed toward the detection of methadone metabolites have been performed over the past 15 years. Emphasis was therefore placed on the detection of minor metabolites making use of deuterated methadone and metabolites which have not previously been used for such a study. Special extraction procedures for rat bile samples were designed to ensure good recoveries of the metabolites and to have a relatively complete separation of endogenous materials from metabolites. GCMS with the use of labeled and unlabeled compounds provides separation of the metabolites from endogenous materials. Comparison of the mass spectra in peaks arising from unlabeled compounds with those from labeled compounds could provide important evidence of the formation of new metabolites.
EXPERIMENTAL

1. Materials

General chemicals and reagents
Benzene-$^2H_6$ (99.5% D, Merck Sharp & Dohme, Canada),
2-Dimethylaminoisopropyl chloride HCl (Aldrich Chemical),
$D_2O$ (99.7% D, SIGMA), $D_2SO_4$ (minimum isotopic purity 99 atom
% D, Merck Sharp & Dohme, Canada), $C^2H_3-CH_2Br$ (minimum isotopic
purity 99% D, Merck Sharp & Dohme, Canada), Glucurase$^R$ ($\beta$-glu-
curonidase, 5,000 sigma unit/ml, SIGMA), Glusulase$^R$ ($\beta$-glucuroni-
dase and aryl sulfatase, Endo products Inc., New York), $m$-Chlor-
operbenzoic acid (technical grade 85%, Aldrich Chemical),
Diazepam (active substance of Valium, Hoffmann-La Roche,
Montreal, Canada), 0.1 M sodium acetate buffer (pH 4.5), 0.1 M
disodium citrate buffer (pH 2.0), 0.25 M borate buffer (pH 9.0).
Diazomethane was prepared by the method of Levitt (75).

Solvents
Chloroform, hexane, methylene chloride, methanol;
Distilled in glass, Caledon Laboratories Ltd., Ontario.
Methanol, $H_2O$; HPLC grade, Fisher Scientific Co. Ether; ether
absolute, Caledon Laboratories.
Materials for animal surgery

Heparin (168 units/mg, SIGMA), Silastic\textsuperscript{R} (Dow Corning, 0.020 in. i.d., 0.037 in. o.d.), Polyethylene tubing-10 (Clay Adams, 0.011 in. i.d., 0.024 in. o.d.), Polyethylene tubing-50 (Clay Adams, 0.023 in. i.d., 0.038 in. o.d.).

2. Analytical methods

GCMS repetitive scanning

GCMS spectral data were obtained using a Varian MAT 111 gas chromatograph-mass spectrometer. Mass spectral data were recorded and processed using a Varian 620L computer. The electron ionization voltage was 70 eV with a source temperature of 285°C. A glass column (1.6 m x 2 mm i.d.) packed with 3% OV-17 on 80-100 mesh Chromosorb W (HP) was used with carrier gas (He) at 20 ml/min.

GCMS selected ion monitoring

SIM was performed using a Varian MAT 111 gas chromatograph-mass spectrometer with accelerating voltage supply of the mass spectrometer modified to permit scanning the accelerating voltage using a Varian 620L computer. Electron ionization voltage, source temperature, and column conditions were the same as those used for the repetitive scanning.
Gas chromatography

The GC analysis was carried out using a Hewlett-Packard 5830A model equipped with a hydrogen flame detector. The glass column, 1.8 m x 2 mm i.d. was packed with 3% OV-17 on 80-100 mesh Chromosorb W (HP). Injection temperature was 205°C, oven temperature 210°C, and detector temperature 300°C. The carrier gas (He) flow rate was 50 ml/min.

Chemical ionization GCMS

A Finnigan chemical ionization GCMS (Model 4000) was employed. The GC conditions were the same as those described for routine analysis except for the size of the glass column (1.6 m x 2 mm i.d.) and that methane was used as a carrier and reagent gas (flow rate 40 ml/min).

High resolution mass spectrometry

The KRATOS MS 50 high performance mass spectrometer (resolution 10,000) was used. Ionization voltage was 70 eV and source temperature, ~150°C.

High performance liquid chromatography

An ALTEX MODEL 153 high performance liquid chromatograph equipped with an ultraviolet detector set at 254 nm was employed. The column was a 4.6 mm i.d. x 25 cm packed with Ultrasphere-ODS (octadecylsilane) (dp, 5u). Pumping pressure and flow rate were 2000-3000 psi and 1 ml/min, respectively. The elution solvent was a mixture of methanol and H₂O (3:1).
NMR spectroscopy

The NMR spectroscopy was performed with the following instruments: Varian XL-100, Bruker WP-80, and Nicolet-Oxford H-270. TMS was used as the internal standard. Deuterated methanol and deuterated chloroform were used as solvents.

Infrared spectroscopy

A Beckman IR-10 infrared spectrometer and a Unicam SP 1000 infrared spectrometer (Pye Unicam) were used for IR spectroscopy. The spectra were recorded as the liquid films or as KBr discs.

3. Chemical studies

Synthesis of deuterium labeled methadone and metabolites

The degree of deuteration of the synthesized compounds was determined by NMR.

*Synthesis of methadone-$^2_{\text{H}}$$_{10}$*: A general procedure for the synthesis of deuterated diphenylacetonitrile (12) was taken from the literature (76). Benzene-$^2_{\text{H}}$$_6$ was used in place of benzene to obtain partially deuterated 12 (spectrum 1, p. 163). To obtain enriched diphenylacetonitrile-$^2_{\text{H}}$$_{10}$ (12, 2Ar-$^2_{\text{H}}$$_{10}$) (spectrum 2, p. 163), benzene-$^2_{\text{H}}$$_6$ (100 gm) and D$_2$O (4 drops) were added to a mixture of partially deuterated 12 (12.2 gm, 0.06 mol) and anhydrous AlCl$_3$ (12 g, 0.09 mol). The mixture was refluxed on a steam bath for 20 hours. AlCl$_3$ was destroyed by adding H$_2$O. The separated benzene layer was dried over anhydrous Na$_2$SO$_4$. 
Flash evaporation of the solvent gave a brownish compound (11.68 gm, 95.7%) which was used for subsequent reactions.

Methadone nitrile-$^2_{\text{H}}$ was prepared from enriched diphenylacetonitrile-$^2_{\text{H}}$ by the method of Attenburrow et al. (77) with minor modifications. A solution of enriched diphenylacetonitrile (9.0 gm, 0.047 mol) in dry benzene (75 ml) was treated with NaH (1.92 gm, 0.08 mol). The mixture was heated on a steam bath for 10 minutes and a drop of DMSO added as catalyst. 2-Dimethylamino-isopropyl chloride (7.59 gm, 0.048 mol) in benzene (90 ml) was prepared as described (76) and added to the mixture. The mixture was stirred for 45 hours at room temperature. Work up did not require a distillation but the extraction residue upon standing gave a mixture of methadone nitrile-$^2_{\text{H}}$ and isomethadone nitrile-$^2_{\text{H}}$ as a slightly yellow crystalline mass. The separation of methadone nitrile-$^2_{\text{H}}$ (4.7 gm, 36%) (spectrum 3, p. 164) from isomethadone nitrile-$^2_{\text{H}}$ was obtained by fractional recrystallization from hexane. The synthesis of methadone-$^2_{\text{H}}$ (1, 2Ar=$^2_{\text{H}}$) from methadone nitrile-$^2_{\text{H}}$ was also described by Attenburrow et al. (77). The product contained 97% labelling of the aromatic protons as determined by NMR (spectrum 5, p. 165).

Synthesis of 4-dimethylamino-2,2-diphenylpentanoic acid-$^2_{\text{H}}$ (5, 2Ar=$^2_{\text{H}}$ and DDP-$^2_{\text{H}}$ (6, 2Ar=$^2_{\text{H}}$) — the method was similar to that of Gardner et al. (22). Partially deuterated methadone nitrile-$^2_{\text{H}}$ obtained from the reaction of partially deuterated diphenylacetonitrile with 1-dimethylamino-2-chloropropane and NaH was hydrolyzed using D$_2$O and D$_2$SO$_4$ in a screw capped reaction bottle to give the bisulfate of 4-dimethylamino-2,2-diphenyl-
pentanoic acid\(^2\text{H}_{10}\). Hydrolysis of the bisulfate using 5% NaOH gave the free acid, which showed 97% labelling on the aromatic rings (spectrum 4, p. 164). The acid was dried at 100°C for 30 minutes prior to conversion to the pyrrolidone, DDP\(^2\text{H}_{10}\). The degree of deuteration of pyrrolidone was also 97% (spectrum 6, p. 166).

Synthesis of EDDP\(^2\text{H}_{10}\) (3, 2Ar=\(^2\text{H}_{10}\)) and EDDP\(^2\text{H}_3\) — A known procedure (3) was used. Starting with DDP\(^2\text{H}_{10}\), EDDP\(^2\text{H}_{10}\) was obtained with the aromatic rings 96% deuterated (spectrum 9, p. 167). DDP reacted with C\(^2\text{H}_3\text{-CH}_2\text{Br}\) to give EDDP\(^2\text{H}_3\) (99% D) (spectrum 8, p. 167).

Synthesis of EMDP\(^2\text{H}_{10}\) (2, 2Ar=\(^2\text{H}_{10}\)) and EMDP\(^2\text{H}_3\) — The procedure of Pohland et al. (3) was used. EMDP\(^2\text{H}_{10}\) and EMDP\(^2\text{H}_3\) were obtained from EDDP\(^2\text{H}_{10}\) and EDDP\(^2\text{H}_3\), respectively. The degree of ring deuteration of EMDP\(^2\text{H}_{10}\) was 96% (spectrum 7, p. 166). EMDP\(^2\text{H}_3\) showed more than 99% deuteration.

Synthesis of 2-dimethylamino-4,4-diphenyl-5-nonanone perchlorate

2-Dimethylamino-4,4-diphenyl-5-nonanone was prepared by the method of Lynn et al. (21). The product was not distilled but crystallized from ether solution as the perchlorate salt. Recrystallization from ether-EtOH gave crystals, mp 137-139°C.
Chemical oxidation studies

The compounds (EMDP HCl, EMDP base, EDDP perchlorate, EDDP base) were treated at 0-5°C with \textit{m}-chloroperbenzoic acid in chloroform. For EMDP HCl, EMDP base, and EDDP base 20% excess of \textit{m}-chloroperbenzoic acid was used. The reaction mixture was kept overnight at 0°C. The optimum molar ratio of \textit{m}-chloroperbenzoic acid and reaction time were studied with EDDP perchlorate. Three methods were used to work up the samples and all gave the same products:

1) The CHCl$_3$ solution was washed with 10% Na$_2$SO$_3$ until the presence of peroxide was not detected. The solution was further washed with saturated NaHCO$_3$. Final washing was made with H$_2$O. 2) The CHCl$_3$ solution was washed with saturated NaHCO$_3$. a) The CHCl$_3$ solution was directly analyzed by GCMS.

4. \textit{Selected ion monitoring (SIM) analysis of methadone and metabolites in human plasma, saliva, and urine samples}

Samples

Plasma, saliva, and urine samples were obtained from a pharmacokinetic study of four female methadone maintenance patients which was conducted by the Alcohol and Drug Commission, Vancouver. Maintenance dosage levels were for patient A, 30 mg;
B, 40 mg; C, 30 mg; and D, 90 mg/day. Plasma and saliva samples were taken at 0, 2, 4, 6, 8, 11, 12 and 24 hours after the usual dose. Urine samples were obtained at 1, 3, 5, 7, 9, 13, and 24 hours. After the oral dose of methadone was taken, the patients were instructed to rinse the mouth with 250 ml of water to remove traces of drug from the oral cavity. Patients were not allowed to eat or drink just prior to providing a saliva sample. The mouth was again rinsed with water before the sample was taken to reduce contamination from food substances. All samples were stored frozen until analyzed.

Extraction procedures and standard curve preparation

Plasma and saliva. —— Saliva was centrifuged in order to remove solids. To plasma or saliva samples (0.5 ml) was added 0.2 ml of internal standard (I.S.), 2-dimethylamino-4, 4,4-diphenyl-5-nonanone perchlorate (a stock solution of I.S. was prepared to contain 10 mg/ml in methanol which was diluted with water to make a solution equivalent to 200 ng in 0.2 ml H₂O). The solution was diluted to 3 ml with H₂O and 0.1 ml of 1 N NaOH was added. After adding methylene chloride (15 ml), the solution was vortex mixed for 3 minutes. The aqueous layer was aspirated off and the methylene chloride layer was dried over anhydrous sodium sulfate. Dried methylene chloride (10 ml) was taken and evaporated under N₂. The residue was dissolved in 50-100 ul of CH₃OH. A 2-5 ul aliquot was injected onto the GCMS. Standard curves were prepared by spiking control samples of plasma and saliva (0.5 ml) with methadone in
the amounts of 0, 20, 40, 100, 200, and 500 ng. The peak area ratios of methadone/internal standard obtained by monitoring m/e 72 were plotted vs. the concentration of methadone.

**Urines.** --- After thawing the sample, 1 ml of urine was taken, to which was added 0.2 ml of solution containing the internal standards at concentrations of 20 ug methadone-\textsuperscript{2}H\textsubscript{10}, 10 ug EDDP-\textsuperscript{2}H\textsubscript{3}, 10 ug EMDP-\textsuperscript{2}H\textsubscript{10}, and 10 ug of DDP-\textsuperscript{2}H\textsubscript{10}/ml. The mixture was diluted to 5 ml with distilled water and the pH was adjusted to 7-8 with 0.1 N NaOH. The mixture was extracted by vortex mixing for 2 minutes with methylene chloride (15 ml). The methylene chloride extract (13 ml) was dried over anhydrous Na\textsubscript{2}SO\textsubscript{4} and taken to dryness using N\textsubscript{2}. The residue was taken up in MeOH (0.1-0.4 ml) and a 2-5 ul aliquot was injected onto the GCMS. For standard calibrations, varying amounts of methadone (0.5 ug to 50 ug), EDDP (0.5 ug to 50 ug), EMDP (0.05 ug to 1 ug), and DDP (0.05 ug to 1 ug) were added to control urines (1 ml). Blank samples containing only the deuterated internal standards in control urine (1 ml) were also prepared in order to subtract background interferences resulting from isotopic impurity and column bleeding. Calibration curves were prepared by plotting the peak area ratios of unlabeled/labeled compound at each of the ion pairs monitored vs. the known concentration ratio of unlabeled compound to its corresponding labeled internal standard. Monitoring ions were m/e 223/m/e 233 (methadone), m/e 277/m/e 280 (EDDP), m/e 208/m/e 218 (EMDP), and m/e 265/m/e 275 (DDP).
GC analysis

For the GC analysis of methadone and EDDP in urines 2-dimethylamino-4,4-diphenyl-5-nonanone perchlorate (10 ug in 0.2 ml H₂O) was used as the internal standard. The extraction procedures were essentially the same as those described for the SIM analysis of urine samples.

Stability of EDDP

Differential scanning calorimetry was used to determine mp of EDDP perchlorate; mp 175°C, when recrystallized from diethyl ether-EtOH (lit. (3) mp 167-168°C).

EDDP perchlorate stock solution (1.5 ml, 1 mg/ml in MeOH) was evaporated under N₂. After adjusting to pH 12 with 1N-NaOH, ether (25 ml) was added to extract the free base. An aliquot of the ether layer (20 ml) was transferred to a 25 ml volumetric flask and made up to 25 ml with the same solvent. Samples (0.2 ml) were taken for the analysis at 0, 1, 2, and 3 days after preparation of the sample which was kept on the bench at room temperature. An internal standard stock solution was prepared with MeOH to contain 10 ug of DDP⁻²H₁₀ and 20 ug of EDDP⁻²H₃ perchlorate per ml. Sample solution (0.2 ml) was mixed with internal standard solution (0.2 ml) and the mixture was analyzed for DDP and EDDP by monitoring m/e 265 and m/e 275 for DDP and m/e 277 and m/e 280 for EDDP. The standard curves were prepared using various concentrations of EDDP perchlorate and DDP dissolved in methanol.
The concentrations of EDDP were expressed as the free base.

5. Detection of methadone metabolites

Animal experiments

Male Wistar rats (250-350 gm) were anesthetized with ether during the surgical procedures. The common bile duct was isolated through a midline abdominal incision and cannulated with polyethylene tubing-10 for bile collection. The surgical area of the abdomen was sutured. The rat was placed in a restraining cage. After recovery from ether anesthesia, the rat was given the appropriate drug s.c. at a dose of 20 mg/kg (methadone, metabolites, deuterated methadone, deuterated metabolites). The bile was collected over a period of 24 hours.

Human experiments

The twenty four hour urine samples were obtained from methadone maintenance patients who were on dosages of 90 mg/day. Samples were supplied by the Alcohol and Drug Commission Laboratories, Vancouver.

Sample work up procedures

A bile sample (10 ml) was diluted with H₂O (10 ml) and the mixture centrifuged to remove solid substances. The bile sample was then extracted with methylene chloride (50
ml x 2). The methylene chloride layers were combined and kept for the analysis of nonconjugated metabolites. The aqueous layer was freeze dried.

Buffer (pH 4.5, 10 ml) was added to the freeze dried sample (final pH 5.0) and the mixture was incubated (37°C) in the presence of Glucurase<sup>R</sup> or Glusulase<sup>R</sup> (1 ml) for 24 hours. After incubation, the pH of the solution was adjusted to 8.0-8.5 by adding pH 9.0 borate buffer (10 ml). The solution was extracted with methylene chloride (75 ml x 3). The combined methylene chloride extracts were flash evaporated and the residue redissolved with methanol (1 ml). This solution was treated with diazomethane.

6. Methadone-diazepam interaction studies

Treatment of animals

Male Wistar rats (200-300 gm) obtained from Canadian Bio-Breeding Farm Laboratories (Montreal, Quebec) were used throughout. They were maintained on a standard diet of Purina Lab Chow (Ralston Purina Co. of Canada) and water ad libitum. The rat was jugular vein cannulated by the method of Upton (78). The external jugular vein was exposed using tissue forceps. A 3 cm piece of SILASTIC<sup>R</sup> tubing was connected to 3 cm of polyethylene tubing-50 by means of a piece of 22 gauge hypodermic needle. The vein was cut using a sharp blade or moria spring scissors. The heparinized saline was normal saline containing
heparin at a concentration of 20 units/ml.

After completion of jugular vein cannulation, bile duct cannulation was performed as described by Lambert (79). Iris forceps were used to hold the bile duct and to insert the cannula below the junction of the right and left hepatic ducts. A cut was made in the bile duct with a razor blade or moria spring scissors. Polyethylene tubing-10 was inserted into the common bile duct.

The total time spent for jugular vein and bile duct cannulation was 20-30 minutes. After bile duct cannulation, diazepam or vehicle only was given through the jugular vein at an infusion rate of 2 ml/hour. The time of infusion start was recorded. While the infusion was being performed, the surgical area of the bile duct cannulation was sutured. After infusion, the PE-50 tubing was cut short and the animal was resutured with the tubing inside the layer of the skin. Shortly before methadone dosing, one hour from the start of the infusion, the rat was placed in a restraining cage. Methadone HCl (10 mg/Kg in 2 ml saline) was given s.c.. Bile was collected from the cannulated bile duct in preweighed scintillation vials at different times (1, 2, 5, 11, and 23 hours). The bile which was collected before the methadone dose served as the blank.

The diazepam was dissolved in a solution of propylene glycol (40%), ethanol (10%), benzyl alcohol (1.5%), and sodium benzoate (5%) in water. Control animals received an equal volume of the vehicle.
Sample preparation procedures

The conjugated internal standards were prepared by dosing 3 rats with 20 mg/Kg EMDP-2H_{10} s.c. Bile was collected for 24 hours. The bile was extracted once with methylene chloride to remove EMDP-2H_{10}. The extracted bile was diluted to 100 ml with water and frozen in an Erlenmeyer flask at -20°C until used. This solution was used directly as the internal standard to measure conjugated metabolites.

The bile sample (0.2-0.4 ml) was mixed with 0.2 ml of the nonconjugated internal standard solution (20 ug EDDP-2H_{3}, 2 ug EMDP-2H_{10}, 5 ug methadone-2H_{3} in 0.2 ml H_{2}O) and 1 ml of the conjugated internal standard solution. The mixture (pH 9.0-9.5) was extracted with methylene chloride (15 ml). The aqueous layer was kept for the analysis of conjugated metabolites.

The methylene chloride fraction was evaporated under N_{2}. After adjusting the pH of the residue with pH 2.0 buffer (2.0 ml), the mixture was briefly extracted with CHCl_{3} (0.5 ml) to remove colored materials. The aqueous layer was made alkaline by adding 1 N NaOH (0.5 ml) and was extracted with methylene chloride (10 ml). The methylene chloride layer was dried over anhydrous Na_{2}SO_{4} and evaporated under N_{2}. The final samples for SIM analysis were prepared with methanol. Monitoring ions were m/e 277 (EDDP), m/e 280 (EDDP-2H_{3}), m/e 208 (EMDP), m/e 218 (EMDP-2H_{10}), m/e 294 (methadone), and m/e 297 (methadone-2H_{3}).
The aqueous layer was freeze dried. Buffer, pH 4.5 (1.5 ml) was added to the residue (final pH 5.0) and the mixture was incubated with Glucurase (0.2 ml) for 24 hours at 37°C. After incubation, the pH was adjusted with pH 9.0 borate buffer (2 ml) to pH 8.5-9.0. The mixture was extracted with methylene chloride (15 ml). The methylene chloride was dried over anhydrous Na$_2$SO$_4$ and taken to dryness. The residue was dissolved in 1 ml of methanol and treated with diazomethane. After methylation (sustained yellow color), methanol was evaporated and the mixture was vortex mixed with hexane (5 ml) after adjusting the pH with pH 2.0 buffer (1.5 ml) to remove endogenous materials. The pH of the aqueous part was adjusted to alkaline with 1 N NaOH (0.5 ml) and extracted with methylene chloride (10 ml). The methylene chloride was dried over Na$_2$SO$_4$ and taken to dryness under N$_2$. The final residue was dissolved in suitable volume of methanol for SIM analysis. Ions, m/e 247, 246, 238, and 237 were monitored for CH$_3$OEMDP and m/e 245, 244, 237, and 236 for DiCH$_3$OEMDP. The ratios, m/e 238/m/e 247 and m/e 237/m/e 245 were selected for the analysis of HOEMDP and DIHOEMDP, respectively.

Stability experiments

Nonconjugated metabolites —— A stock solution was prepared to contain 1 mg each of EDDP, EDDP-$^2$H$_{10}$, EDDP-$^2$H$_3$, EMDP, EMDP-$^2$H$_{10}$, methadone methadone-$^2$H$_{10}$, methadone-$^2$H$_3$, DDP, and DDP-$^2$H$_{10}$ in 1 ml of methanol. Stock solution (1 ml) was diluted to 50 ml with methanol in a volumetric flask and
2.5 ml of the solution (50 ug each) was taken to dryness. Using these dried samples solutions were made up to 5 ml with water at different pHs (1 N HCl, pH 2.0, pH 4.5, pH 9.0, and 0.1 N NaOH). After incubation at different times, the pH of each solution was adjusted to 9.0 and the solution extracted with 10 ml of methylene chloride. The methylene chloride fraction was dried over anhydrous Na₂SO₄ and evaporated under N₂. The residue was dissolved in methanol and injected into the GCMS. Ions, m/e 277 (EDDP), m/e 287 (EDDP-²H₁₀), m/e 280 (EDDP-²H₃), m/e 208 (EMDP), m/e 218 (EMDP-²H₁₀), m/e 223 (methadone), m/e 226 (methadone-²H₃), and m/e 233 (methadone-²H₁₀) were monitored.

Conjugated metabolites --- The conjugated internal standard (2 ml) was incubated with GlucuraseR (0.5 ml) at different times (12, 24, 48, and 96 hours) and the samples worked up following the same procedures for the conjugated fraction of the bile samples. Specific ions, m/e 247, 246, 245, 244 and m/e 247, 246, 238, 237 for CH₃O EMDP-²H₃; m/e 247, 246, 245, 244 and m/e 245, 244, 237, 236 for (CH₃O)₂ EMDP-²H₈ were monitored which would indicate stability of the deuterium labeling. See Table VIII and Table IX for details.

7. Pharmacokinetic and statistical analysis

The apparent elimination rate constants and half lives were calculated by using the NONLIN program (80). The use of
biosynthetic internal standards for pharmacokinetic studies was investigated by using the same program. Slope and intercept values of calibration equations were calculated by means of the computer program, Triangular Regression Package, Computing Center, the University of British Columbia. The statistical analysis was performed by Student's t test.
RESULTS AND DISCUSSION

1. Synthesis and mass spectrometry of deuterated methadone and metabolites

Deuterated diphenylacetonitrile

Treatment of phenylacetonitrile with benzene-$^2$H$_6$ by the method of Robb and Schultz (76) resulted in the isolation of diphenylacetonitrile labeled in both phenyl rings. As shown in the mass spectrum (Fig. 2a), deuteration of the phenyl rings was not complete. NMR analysis indicated 86% deuteration.

Labelling of both phenyl rings is probably the result of two processes. Acid catalyzed and or aluminum chloride catalyzed exchange (81) between benzene-$^2$H$_6$ and the ring protons of phenylacetonitrile or diphenylacetonitrile could account for the observed labelling. While this exchange process is indeed a contributing factor it does not totally determine the end result. For example, when unlabeled diphenylacetonitrile was treated with benzene-$^2$H$_6$ under the reaction conditions ($\text{AlCl}_3$, reflux, 2 hours), 55-60% of the expected exchange as measured by NMR had occurred. More than 20 hours reflux was required before the exchange approached the proportions achieved during the alkylation reaction. On the other hand, exchange between benzene and benzene-$^2$H$_6$ ($\text{AlCl}_3$, reflux)
Fig. 2. Mass spectra of deuterated diphenylacetonitriles
(a) prepared using an excess of benzene-$^2$H$_6$
(b) 50% deuterated prepared by the method of
Hachey et al. (19)
(c) after enrichment.
reached equilibrium within 20 minutes (as measured by mass spectrometry).

To explain the extent of labelling achieved by the alkylation reaction, it is suggested that the reversible nature of the Friedel Crafts reaction (82) is an additional factor to the exchange processes that occur. A suggested intermediate for the process illustrates that rapid exchange between benzene and labeled benzene plus the reversibility of the reaction leads to a product with the expected percent of label at equilibrium.

\[
\begin{align*}
\text{C}_6\text{H}_5\text{CN} & \rightleftharpoons \text{C}_6\text{H}_5\text{CN} \\
& \rightleftharpoons \text{C}_6\text{H}_5\text{CN} \\
\end{align*}
\]

\[
\begin{align*}
\text{AlCl}_3 & + \text{C}_6\text{D}_6 \\
\text{H}^+ & \text{CN} \\
\text{Br} & \\
\end{align*}
\]

The results are contrary to a report that diphenylacetonitrile synthesized using equimolar concentrations of phenylacetonitrile and benzene-$^2\text{H}_6$ in $\text{CS}_2$ as solvent gave a product with the five deuterium atoms contained in one ring (19). This experiment was repeated as described and the product obtained was found to be 50% deuterated by NMR. The mass spectrum (Fig. 2b) shows a cluster of the molecular ions for the isomers from $^2\text{H}_0$ to $^2\text{H}_{10}$ in approximately the expected distribution for 50% uniform labelling in both rings.
Diphenylacetonitrile-$^2\text{H}_{10}$ enriched with deuterium was prepared using aluminum chloride catalyst and benzene-$^2\text{H}_6$ as a deuterium source (81). A 1:20 molar ratio of diphenylacetonitrile-$^2\text{H}_{10}$ (86% ring deuteration) to benzene-$^2\text{H}_6$ gave the product with 98% ring deuteration as determined by NMR and mass spectrometry. Assuming equilibrium was achieved between the benzene-$^2\text{H}_6$ and partially labeled diphenylacetonitrile, 98.5% ring deuteration would be expected. The mass spectrum is shown in Fig. 2c. Deuterated diphenylacetonitrile prepared using exchange reactions was found to be preferable to using the alkylation reaction. The exchange reaction is simple with few if any side products produced. Methadone nitrile can not be labeled using these conditions.

Deuterated methadone

Methadone-$^2\text{H}_{10}$ prepared starting from enriched diphenylacetonitrile retained the same degree of labelling as in the starting material. The mass spectrum of methadone-$^2\text{H}_{10}$ (Fig. 3b) corresponds to that for methadone (83), the molecular ion appearing at m/e 319. Two ions that might be used in SIM appear at m/e 233 and m/e 304 corresponding to m/e 223 (25) and m/e 294 (27) for methadone (Fig. 3a). Sullivan et al. (17) reported that the application of methadone-$^2\text{H}_3$ to plasma determinations of methadone by monitoring m/e 294 for methadone and m/e 297 for methadone-$^2\text{H}_3$. While neither of these fragment ions is ideal for SIM studies, under our conditions of mass spectrometry, m/e 223 showed slightly higher abundance (1.6%).
Fig. 3. Mass spectra of (a) methadone and (b) methadone-$^{2}H_{10}$
than that of m/e 294 (1.0%) (Fig. 3a). Using methadone-\textsubscript{2H\textsubscript{10}} as internal standard, ions at m/e 223 and m/e 233 have formed the basis for a suitable ion monitoring assay of methadone in urine samples.

The ion at m/e 72 (24) was selected to quantify methadone in human saliva and plasma samples, and the ion at m/e 294 (27) for rat bile samples as described in the experimental.

By comparing the mass spectrum in Fig. 3a and 3b it becomes evident that besides the fragments at m/e 294 and 223 minor peaks at m/e 208, 193, 179, 178, and 165 appear as typical ions containing the two phenyl rings. The proposed
pathways to these fragment ions (31-35, 26) are described as part of the fragmentation processes of EMDP and DDP. However, the possibility that m/e 165 (26) might be derived from m/e 223 (25) can not be excluded. Diphenylacetonitrile which can not form m/e 208 also gave the ion m/e 165 (28).

\[ 
\begin{align*} 
\text{CH-CN} & \quad \text{m/e 165} \\
\text{H}^+ & \quad \text{m/e 173} \\
\text{H}_2\text{O} & \quad \text{m/e 203} \\
\text{M}^+ & \quad \text{m/e 193} \\
\end{align*} 
\]

Deuterated methadone metabolites

When partially deuterated methadone nitrile was hydrolyzed with $\text{H}_2\text{O}$ and $\text{H}_2\text{SO}_4$, a marked decrease in the degree of deuteration from 86% to 40% was observed in the product, 4-dimethylamino-2, 2-diphenylpentanoic acid. On the other hand, treatment of partially deuterated methadone nitrile with $\text{D}_2\text{O}$ and $\text{D}_2\text{SO}_4$ produced an increase in the degree of deuteration in the acid product to 97%. Starting with unlabeled methadone nitrile, hydrolysis with $\text{D}_2\text{O}$ and $\text{D}_2\text{SO}_4$
gave product containing only 60-70% deuteration as determined by NMR and it was therefore concluded that maximum deuteration of synthesized metabolites could not be achieved by this latter method. The labeled acid was decomposed in the GC inlet to the decarboxylated product, 1, l-diphenyl-3-dimethylaminobutane-\(H_{10}^{2}\) as determined from the mass fragmentation patterns with peaks at m/e 72 (base peak), m/e 263 (M\(^+\), 0.9%), m/e 177 (3.0%), and m/e 173 (1.3%) and DDP-\(H_{10}^{2}\) as determined from its retention time value and mass spectrum.

DDP-\(H_{10}^{2}\) synthesized from the acid, 5 by thionyl chloride demethylation fully retained the deuterium label. The spectra of DDP is shown in Fig. 4. The fragmentation patterns for DDP were similar to those for EMDP being derived mainly from the m/e 208 fragment.

Mass spectra are shown in Fig. 5 for EDDP, EDDP-\(H_{10}^{2}\) (97% label), and EDDP-\(H_{3}^{2}\) (99% label). The relative abundance of m/e 208 (31) for EDDP was 0.9%. This implied that the fragmentation to produce m/e 208 which would involve the loss of CH\(_3\)-N=C=CHCH\(_3\)\(^{+}\) from the molecular ion is no longer a favored process.

The formation of m/e 105 (37) on the other hand is unique to EDDP and requires the migration of one phenyl ring to the unsaturated side chain. This was recognized from the appearance of the corresponding ions of m/e 110 and m/e 108 for EDDP-\(H_{10}^{2}\) and EDDP-\(H_{3}^{2}\), respectively. The mechanism of phenyl migration is similar to that reported for the compound, 38 (84).
Fig. 4. Mass spectra of (a) DDP and (b) DDP-$^{2}$H$_{10}$
Fig. 5. Mass spectra of (a) EDDP, (b) EDDP-\textsubscript{2}H\textsubscript{10}, and (c) EDDP-\textsubscript{2}D\textsubscript{3}.
Another process apparently unique to EDDP is the loss of \( \text{CH}_3-\text{CH}=\text{CH}_2 \) to form m/e 235 (40) which then can either form m/e 220 (41, 21%) or by further loss of a \( \text{CH}_3 \) form the fragment m/e 69 (42, 21%). In both cases EDDP-\(^2\text{H}_3\) proved valuable in determining the nature of these ions.
Mass spectra for EMDP-$^2\text{H}_3$ and EMDP are shown in Fig. 6. EMDP gave fragmentations resulting mainly from m/e 208. Since EMDP-$^2\text{H}_3$ loses the labeled methyl group when the m/e 208 fragment is formed, no difference in the mass spectra was observed between EMDP and EMDP-$^2\text{H}_3$.

Spectroscopic observations

NMR data for EDDP and EMDP were described earlier by Pohland et al. (3). NMR spectra were shown for EDDP perchlorate and EMDP HCl by Beckett et al. (2) without assignment of chemical shift values. The NMR data obtained for the methadone metabolites is summarized in Table I with a comparison of the C-4 protons which describe an AB system. The
Fig. 6. Mass spectra of (a) EMDP and (b) EMDP-\textsuperscript{2H\textsubscript{10}}
<table>
<thead>
<tr>
<th></th>
<th>EDDP (3) (trans)</th>
<th>EDDP (3) (cis)</th>
<th>EDDP perchlorate (19)</th>
<th>EMDP (4)</th>
<th>EMDP·HCl (20)</th>
<th>DDP (6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>δ Ha</td>
<td>2.24</td>
<td>2.24</td>
<td>2.60 (dd)</td>
<td>2.23 (dd)</td>
<td>2.51 (dd)</td>
<td>2.20 (dd)</td>
</tr>
<tr>
<td>J Ha-Hc</td>
<td>8.5</td>
<td>10.0</td>
<td>7.0</td>
<td>8.7</td>
<td>7.0</td>
<td>9.0</td>
</tr>
<tr>
<td>δ Hb</td>
<td>2.56</td>
<td>2.52</td>
<td>3.43 (dd)</td>
<td>2.67 (dd)</td>
<td>3.17 (dd)</td>
<td>2.95 (dd)</td>
</tr>
<tr>
<td>J Hb-Hc</td>
<td>5.3</td>
<td>7.0</td>
<td>8.0</td>
<td>6.5</td>
<td>8.0</td>
<td>6.0</td>
</tr>
<tr>
<td>J Ha-Hb</td>
<td>11.0</td>
<td>12.0</td>
<td>14.0</td>
<td>13.4</td>
<td>14.0</td>
<td>13.0</td>
</tr>
</tbody>
</table>

3, 4 as reported by Pohland et al. (3)
19, 20 Spectra obtained with the Varian XL-100
6 Spectra obtained with the Bruker WP-80
The solvent in all cases was CDCl₃.
chemical shift values for the C-4 protons in DDP are different from that in the reference by Singh et al (27), who reported that C-4 protons appeared at 2.05-2.40 as multiplets in CDCl$_3$ as solvent.

In the IR, labeled methadone and metabolites exhibit a C-D stretching vibration at 2270 cm$^{-1}$. The appearance of the C-D peak and disappearance of the aromatic C-H vibration could be used to indicate the extent of deuterium enrichment (85).

2. **SIM analysis of methadone and metabolites in biological samples**

Selected ion monitoring

The computer program for SIM using the MAT 111 GCMS was developed by Roland Burton in the Faculty of Pharmaceutical Sciences. The effective mass range for the instrument was designed to be ± 7% of the Hall probe mass setting. The limitation of the mass range results from the fact that ion optics and ion abundance are influenced by attenuation of the accelerating voltage when a magnetic sector instrument is used in SIM mode (86). In practice, it was found that the monitoring of ions was limited depending upon the total number and the masses of the ions of interest. When EMDP, EMDP-$_{2}$H$_{10}$, methadone, and methadone-$_{2}$H$_{10}$ were being monitored with a magnetic field setting of m/e 220, focussing of the ions was impossible although the ions being monitored, m/e 208, 218, 223, and 233
are all in the effective mass range. It was also found that methadone-$^2\text{H}_3$ is a preferable internal standard to methadone-$^2\text{H}_{10}$. Switching of the accelerating voltage over a large mass range results in large standard deviations in the analysis. Hence, EDDP-$^2\text{H}_3$ also proved to be a better internal standard than EDDP-$^2\text{H}_{10}$ for the analysis of EDDP.

The initial window for an ion being monitored was set with PFK. Final adjustment was made by injecting authentic samples to correct for mass defect. In practice, it was found that ion peak positions kept changing. The most important aspect in the computer program is to detect small changes in peak position and to correct for them. A method to adjust window positions to the peak position having the greatest ion current as measured by computer program appears ideal (87).

The method Roland Burton used for this program is as follows: Each ion is scanned from 0.5 amu below to 0.5 amu above its nominal mass. This scan is further subdivided into quarters. Intensity is calculated as the sum of the inner two quarters, minus the sum of the outer two quarters to correct for the baseline change or adjacent peak ions. The window position information is obtained by subtracting the second quarter from the third; if the number is positive, the window is too far to the left. The window is moved to correct accordingly.

Ion peak position was also found to change to the peak arising from column bleeding. Therefore, autofocussing was made to function over certain peak strengths. When we
found a sudden large change of the window position, readjustment of the function of autofocusimg was made. In most cases the autofocusimg was checked carefully at all times.

The sensitivity of the analysis is dependent on the ion abundance of ions being monitored and the contribution of background peaks. Several considerations were made to reduce background. Cleaning the ion source raised the sensitivity of the analysis, but in our experience, standard conditions for clean up of the ion source and changing filaments were difficult to obtain. Scan mode operation during a series of SIM analyses should be avoided in order to increase sensitivity.

Selection of column packing materials was also considered. It was found that m/e 209 from the bleed of an OV-17 column seriously interfered in the EMDP analysis where m/e 208 was monitored. Therefore, a well conditioned column was only used for the analysis of EMDP. The structure of OV-17 is shown with possible fragmentation ions. When m/e 72 was

\[
\begin{align*}
\text{CH}_3 & \quad \text{Si} & \quad \text{CH}_3 \\
\text{CH}_3 & \quad \text{Si} & \quad \text{CH}_3
\end{align*}
\]

\[
\text{m/e 73} \quad \text{m/e 209}
\]
monitored for methadone analysis, interference from silanizing agent which contains the m/e 73 ion was minimized by extensive conditioning of the column. Any solvent used in the extraction and which remained in the sample also contributed to the background. Methylene chloride was completely evaporated when m/e 72 was being monitored for methadone analysis.

One of the advantages of the use of labeled internal standards with the SIM system was reported to be a carrier effect of the labeled compound which could reduce column adsorption and decomposition of the unlabeled compound to be determined. Diphenoxylate (88) is such a case. Contrary to this finding, a lack of a carrier effect was observed in the case of octopamine-2H₃ (89). Generally impurities due to incomplete labelling limits the use of a large amount of internal standard to act as carrier. This was considered to be true in case of the [2H₁₀] labeled compounds. Moreover, the high concentrations required for the analysis of methadone using methadone-2H₁₀ as internal standard prevented any attempt to study a carrier effect.

Analysis of methadone in plasma and saliva

Methadone levels in plasma and saliva samples of methadone maintenance patients were frequently found to be too low to use the selective ion at m/e 223 for monitoring since with EI the relative abundance of this ion is only 1.6%. In order to enhance the sensitivity of the method the less selective but strong peak at m/e 72 was chosen for monitoring.
As shown in Fig. 7, SIM at m/e 72 showed high selectivity for methadone with 2-dimethylamino-4,4-diphenyl-5-nonanone perchlorate as the internal standard, the base peak of which is also m/e 72. The lower limit of reproducible quantitation of methadone in 0.5 ml of plasma or saliva taken for extraction was 20 ng.

The sensitivity obtained by this method is comparable to the methods which are routinely used to analyze methadone in human plasma. The disappointing sensitivity observed is contrary to expectations of using the base peak at m/e 72 for monitoring and is due to instrumental conditions, especially ion source conditions, variations of which were found to seriously limit sensitivity. Special care was therefore taken to reduce background. The temperatures of separator, inlet line, and ion source were elevated overnight to remove retained impurities before fresh samples were analyzed. In this way the method was found to be more than adequate to analyze patient saliva or plasma samples, the concentrations of which were found to vary over a wide range between 0.05-1.0 ug/ml during the 24 hour period of a study.

Monitoring drug concentrations in saliva might better reflect the time course of a drug at the receptor site. This was found to be the case for the drug procainamide for which a parallel relationship between time course of the drug in saliva and cardiac action of the drug was observed (90). This should especially be true for drugs which act on the central nervous system. For drugs such as methadone which are
Fig. 7. SIM chromatogram (m/e 72) of methadone from saliva

Fig. 8. Methadone concentration in plasma and saliva of a maintenance patient (90 mg/day dosage)
extensively bound to plasma protein, monitoring drug concentrations in saliva could better define the activity of a drug because the concentration of a drug in saliva reflects the unbound fraction of the drug which crosses the blood brain barrier. It appeared therefore useful to initiate monitoring of saliva levels of the drug in steady state maintenance patients especially in view of the fact that a lack in correlation between methadone concentrations in plasma and symptom complaints of patients have been reported (41).

The results for patients A and B were not obtained because problems with patients' compliance to the protocol were encountered. The mean ratio of saliva to plasma of patient C was 0.55 ± 0.15 (SD) with a range of 0.40-0.79. Patient D showed a mean ratio of saliva to plasma of 0.48 ± 0.10 (SD) with a range of 0.30-0.58. Salivary pH could possibly account for the intraindividual variation in the results but pH values were not available. Patient C had difficulty in providing saliva samples and chewing gum (Dentyne, Adams Brands Inc.) was used to stimulate saliva production. Interference from the chewing gum in the analysis was not observed. Adsorption of methadone to the gum was not proven but was considered to be minimal. If salivary concentrations of methadone in these two patients are a reflection of methadone in plasma then our results agree well with those reported by Horns et al. (41), in which 50% binding of methadone to plasma was reported. This contrasts with the results reported by Lynn et al. (91) where salivary concentrations were found to be
much higher than those obtained in whole blood. The result however was not adequate to draw pharmacokinetic conclusions because of the limited sample size and the number of samples.

**Analysis of methadone and metabolites in urine**

The analytical conditions were the same as those used for the analysis of methadone in plasma or saliva samples. Retention times by GC/MS (SIM) of methadone and metabolites are 3.24 (EMDP), 4.0 (EDDP), 4.78 (methadone), and 6.23 min. (DDP).

If we consider the fact that many different samples are dealt with when we analyze methadone and metabolites in urines, SIM might be a time consuming method compared with gas chromatographic methods. For example, a separate injection of the sample was required to analyze each metabolite in SIM analysis. On the other hand, methadone and metabolites were analyzed by one injection of the sample using one internal standard with GC.

SIM with deuterium labeled internal standards however provides ease in work up procedures. Sample dilutions and the size of the injection volume did not affect the observed ion ratios which were used to calculate the amount of methadone (Table II). Extractibility of labeled and unlabeled compounds was found to be the same in CH₂Cl₂ solvent. At pH 7.5 (n=4, 2.5 ug each in 1.0 ml), percent recovery of methadone, methadone-²H₁₀, EDDP and EDDP-²H₃ was 84.9 ± 2.6, 83.6 ± 0.2, 94.2 ± 3.8 and 93.9 ± 1.1, respectively. EDDP and EDDP-²H₃
were analyzed in the recovery studies using EDDP-$^2\text{H}_{10}$ as the internal standard; methadone and methadone-$^2\text{H}_{10}$ using methadone-$^2\text{H}_3$ as internal standard.

**TABLE II. Effect of Dilution and Injection Volumes Upon the Observed Ion Ratios**

<table>
<thead>
<tr>
<th>Methadone/ml MeOH</th>
<th>Injection Volume (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 µl</td>
</tr>
<tr>
<td>75 µg $^2\text{H}<em>0$, 50 µg $^2\text{H}</em>{10}$</td>
<td>1.91 ± 0.14</td>
</tr>
<tr>
<td>15 µg $^2\text{H}<em>0$, 10 µg $^2\text{H}</em>{10}$</td>
<td>1.99 ± 0.08</td>
</tr>
</tbody>
</table>

Injection was carried out by monitoring m/e 223 and m/e 233. The numbers denote m/e 223/m/e 233 (SD). Crude windows were set with PFK by monitoring m/e 231 which was later erased. Magnetic Hall setting was m/e 223.

The calibration equations prepared for methadone and EDDP are shown in Table III. The results indicate that calibration equations can be expressed by using only slope values because intercept values were found to be not significant. If we also know the ratio of unlabeled to labeled compound quantitation of the drug monitored is achieved by multiplying the standard ratio x reciprocal value of the amount of added internal standard x the observed ratio. Slope values of
TABLE III. Calibration Equations for Methadone and EDDP for Urine Analysis.

<table>
<thead>
<tr>
<th></th>
<th>Methadone</th>
<th>EDDP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slope</td>
<td>0.326</td>
<td>0.483</td>
</tr>
<tr>
<td>Slope Standard Error</td>
<td>0.0574</td>
<td>0.0033</td>
</tr>
<tr>
<td>Intercepts</td>
<td>0.0513</td>
<td>0.0054</td>
</tr>
<tr>
<td>Intercept Standard Error</td>
<td>0.0503</td>
<td>0.0117</td>
</tr>
<tr>
<td>Coefficient of Determination ($r^2$)</td>
<td>0.9957</td>
<td>0.9990</td>
</tr>
<tr>
<td>Standard Ratio (S.D.)$^b$</td>
<td>1.305 ± 0.1272</td>
<td>0.968 ± 0.0484</td>
</tr>
<tr>
<td>(n=6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slope Calculated from Standard Ratio$^c$</td>
<td>0.326</td>
<td>0.484</td>
</tr>
</tbody>
</table>

a. Slope and intercept values are calculated by means of computer program (Triangular Regression Package, Computing Centre, The University of British Columbia). Methadone (n=7), EDDP (n=10).

Ratio (Drug/Internal Standard) = Slope x Drug concentration + intercept

Internal Standards are Methadone-^{2}_H_{10} (4 μg) and EDDP-^{2}_H_{10} (2 μg).

b. Ratios of equal amount of Drug to Internal Standard (Standard Ratio).

c. Standard Ratio x 1/Internal Standard.
calibration curves and the slopes calculated from standard ratio were found to be the same. The concentration of EDDP was simply calculated by multiplying the ratio of m/e 277 to m/e 280 with the concentration of EDDP-\(^2\)H\(_3\) added as the internal standard. The calibration curve method was however used for the analysis for methadone because of instability of the measured ratio which occurred when ions of 10 mass units difference or more were monitored. The ratio was found to be changeable depending upon SIM conditions and in particular to the stability of the magnetic field. The coefficient of variation of the measured methadone standard ratio was twice (9.7%) that of EDDP (5.2%).

SIM analysis of methadone and EDDP in urine samples was compared with GC analysis. The two methods were found to be well correlated. Methadone (0.4 ug-6.8 ug/ml) was analyzed for patient B and C, EDDP (8 ug-32 ug/ml) for A and D. The correlation coefficient of determination (r\(^2\)) of the analysis of methadone (n=14) and EDDP (n=14) by SIM compared to GC were 0.963 and 0.962, respectively.

Previous experiments with the GC analysis of EMDP in the urine of maintenance patients indicated that the EMDP peak is frequently overlapped either with caffeine or with hydroxycotinine, a metabolite of nicotine (33). When an attempt was made to analyze maintenance patient urines for EMDP by monitoring m/e 208 for EMDP and m/e 218 for the internal standard, EMDP-\(^2\)H\(_{10}\), it was found that the amount of EMDP
appears to be less than 100 ng/ml. Because of the small amount of this metabolite, determination of EMDP was not necessary to study the demethylation mechanisms of methadone.

**Stability of EDDP**

In an attempt to obtain information on the minor metabolic pathways of methadone, the level of DDP in urine was determined by monitoring m/e 265 for DDP and m/e 275 for DDP-2H10 as internal standard. When the pH of the urine was adjusted to 10.4 for extraction, inconsistency in the reproducibility of the concentration of DDP in a urine sample was found. To investigate this, EDDP perchlorate solution was made alkaline and left to stand at room temperature. A TIC profile of the methylene chloride extract of the solution showed DDP as a significant product of EDDP decomposition. DDP was identified by TLC with an Rf 0.8 on Silica Gel F 254 (Brinkman) sheets with EtOH, HAC, H2O (6:3:1) as solvent. Further studies revealed that EDDP free base in ether solution is easily decomposed to DDP. Stoichiometric conversion of EDDP to DDP was observed when EDDP and DDP in ether solution were measured using labeled compounds as internal standards (Fig. 9). A similar result for the conversion of EDDP to DDP by apparent oxidation reactions was separately reported by Bowen et al. (92). A metabolic transformation of methadone to 2, 2-diphenyl-4-dimethylaminopentanoic acid (5) and further metabolism to DDP was suggested (6). 2, 2-Diphenyl-4-dimethylaminopentanoic acid was not detected in any of the urines of
Fig. 9. Decomposition of EDDP base to DDP (---EDDP, —DDP)
maintenance patients using mass chromatograms in which m/e 167 $\text{(C}_6\text{H}_5\text{)}_2\text{C}^+\text{H}$ for the acid and m/e 225 $\text{(C}_6\text{H}_5\text{)}_2\text{C}^+\text{COOCH}_3$ for the diazomethane-treated acid were monitored. The results strongly suggest that DDP is a metabolonate and that extreme pH adjustments should be avoided when biological samples are extracted to detect new metabolites.
3. **Detection of methadone metabolites**

**Extraction procedures for rat bile**

The polar solvent (methylene chloride) was used for extraction of rat bile samples. Clean-up procedures using column chromatography such as Amberlite XAD-2 and back extraction were not used. The sample was extracted and any precipitate was removed by centrifugation, followed by a direct injection of the sample extract into the GCMS. After initial evidence of the presence of metabolites was found, the sample was back extracted to see any difference between before and after the clean up procedure and to see any compounds which were transferred to the hexane layer used in the back extraction (Fig. 10).

A radioisotope labeled compound and XAD-2 column chromatography for sample extraction was described in the detection of methadone metabolites from rat urine and brain (93). An XAD-2 column was also used to detect methadone metabolites from man and rat urine by Sullivan et al. (4). In this experiment, use of column chromatographic purification was not considered appropriate to detect minor metabolites because recovery of the metabolites could not be monitored.
Bile $\rightarrow$ H$_2$O $\rightarrow$ Centrifuge

\[ \text{pH 9.0} \]

Extraction with CH$_2$Cl$_2$ $\rightarrow$ Nonconjugate fraction

\[ \text{Back extraction, Derivatization.} \]

Freeze dry Residue

(Add MeOH, Centrifuge,
Evaporate)

\[ \text{Buffer, pH 5.0, } \beta\text{-glucuronidase, } 37^\circ\text{C} \]

\[ \text{pH 8.0-8.5} \]

Extract with CH$_2$Cl$_2$

Conjugate fraction $\rightarrow$ Derivatize with CH$_2$N$_2$

\[ \text{Back extraction} \]

Fig. 10. Extraction Procedure for Rat Bile.
The rat bile extraction method is similar to that used for detection of methadone metabolites from human urines by Sullivan et al. (6). Direct extraction, derivatization, and GCMS analysis were the methods used. When rat bile samples were analyzed, the bile salts were removed by acid precipitation (94). In our experiments, the amount of bile salt and protein was minimized by centrifugation and solvent (MeOH) precipitation. Two separate experiments with and without acid precipitation gave the same results.

Beckett et al. (2) carried out the detection of methadone metabolites from human urine by using normal solvent extraction and TLC separation. When authentic metabolite samples are not available, detection of minor metabolites among large amount of endogenous material by TLC is almost impossible. The use of color developing methods to detect specific metabolites has been attempted for methadone metabolites (95) and for N-oxidation metabolites (96). The approach however did not guarantee direct identification of the structure of metabolites. Therefore, TLC and color spray methods were not used in our experiments.

The GCMS computer techniques are a powerful tool to identify the molecular structure of compounds in mixtures. This technique is further aided by the use of stable isotope labeled compounds in the metabolic studies. The twin ion technique (ion doublet technique) has been used in the detection of metabolites for various drugs.
The $\text{[}^{2}\text{H}_{10}\text{]}$-labeled compounds synthesized for the methadone work display a difference in physical properties from the unlabeled compounds. The difference in the retention time observed by GC is presumably due to a decrease in polarity of the deuterated material. A similar difference of retention times for unlabeled and $\text{[}^{2}\text{H}_{16}\text{]}$-butylated hydroxytoluene was observed when a 3% OV-225 GC column was used (97). The deuterium labeled methadone and metabolites were found to have slightly shorter retention times than the unlabeled compounds. Therefore, twin ions were not readily observed in the GCMS scan data of extracts containing labeled and unlabeled methadone and/or metabolites. Comparison of the mass spectra of GC peaks resulting from unlabeled compounds with those from labeled compounds run as separate experiments gave important evidence for the formation of new metabolites.

**Nonconjugated fraction**

The nonconjugated fraction isolated from rat bile contained mainly EDDP and a small amount of DDP. Trace amounts of methadone and EMDP were also found. Kreek et al. (98) also reported the same result with trace amounts of methadone and EMDP found in human bile. The presence of DDP was accounted for by decomposition of EDDP.

The nonconjugated fractions of rat bile and human urine were dried in a vacuum desiccator. Silanization of the
samples (150 ul TMCS, 50 ul pyridine, 70°C for one hour) followed by GCMS did not indicate the presence of any dihydrodiol.

**Conjugated fraction**

Fig. 11 shows the TIC profile of the extract of the conjugated fraction isolated from rat bile. Table IV is a summary of the major fragments and ion abundance values for endogenous bile components and recovered metabolites. The presence of monohydroxy EMDP, dihydroxy EMDP, and hydroxy EDDP was evident from mass fragmentation studies of the GC
Fig. 11. TIC profile of diazomethane treated conjugate fraction from bile of methadone dosed rats. (GCMS: 150-280°C, 6°/min, hold at 280°C)
TABLE IV. Mass Spectral Data of TIC Obtained from Diazomethane Treated conjugate fraction from bile of methadone dosed rats

<table>
<thead>
<tr>
<th>Peak No.</th>
<th>Rt (Min)</th>
<th>M/e (Relative Intensity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11.2</td>
<td>87(50), 75(19), 74(100), 55(29), 43(36), 41(33)</td>
</tr>
<tr>
<td>2</td>
<td>12.8</td>
<td>73(87), 60(83), 57(80), 55(67), 43(100), 41(73)</td>
</tr>
<tr>
<td>3</td>
<td>16.0</td>
<td>109(25), 95(56), 80(85), 67(100), 55(87), 41(90)</td>
</tr>
<tr>
<td>4</td>
<td>19.7</td>
<td>93(49), 91(63), 79(100), 67(77), 55(43), 41(70)</td>
</tr>
<tr>
<td>5</td>
<td>24.5</td>
<td>93(44), 91(75), 79(100), 67(70), 55(39), 41(87)</td>
</tr>
<tr>
<td>6</td>
<td>27.6</td>
<td>270(16), 149(10), 134(100), 121(11), 119(14), 91(4)</td>
</tr>
<tr>
<td>7</td>
<td>34.7</td>
<td>326(5), 205(4), 159(5), 147(9), 122(100), 91(15)</td>
</tr>
<tr>
<td>8</td>
<td>37.5</td>
<td>107(43), 95(47), 81(57), 57(63), 55(80), 43(100)</td>
</tr>
</tbody>
</table>

`CH\textsubscript{3}OEMDP` 21.5
`CH\textsubscript{3}OEMDP\textsuperscript{-2}H\textsubscript{9}`
`CH\textsubscript{3}OEDDP\textsuperscript{-2}H\textsubscript{9}` 24.1

`CH\textsubscript{3}OEMDP\textsuperscript{-2}H\textsubscript{8}`

`Metabolite` 28.2
`Metabolite\textsuperscript{-2}H\textsubscript{10}`
peaks resulting from the metabolism of unlabeled and labeled methadone (44-49). Ions m/e 238 and m/e 247 were selected to monitor derivatized HOEMDP and HOEMDP-\(^{2}\text{H}_{9}\). Ions m/e 237 and m/e 245 were used to monitor derivatized DiHOEMDP and DiHOEMDP-\(^{2}\text{H}_{8}\). SIM analysis of conjugated metabolites using these ions was described in the study of methadone-diazepam interaction.

In addition to ring hydroxylated metabolites of methadone, the conjugated fraction from rat bile contained a peak having an abundant m/e 72 ion (Fig. 11, Table IV). Diazomethane treatment did not shift the GC peak position, implying that the compound does not contain a phenyl-hydroxyl group. The conjugated fraction was made acidic (pH 2.0) and extracted with hexane. The compound was transferred to the hexane layer, implying low water solubility and the absence of a basic nitrogen in the molecule. The mass spectrum of this GC peak is shown in Figure 12.

When the experiment for the detection of metabolites was repeated with methadone-\(^{2}\text{H}_{10}\), deuterated hydroxy EMDP, dihydroxy EMDP, and hydroxy EDDP were observed and the m/e 72 containing GC peak (Rt 28.2 min.) was found to retain deuterium on the benzene ring. The retention time of the compound is shown in Table IV with comparison to the other metabolites.

The peak with the abundant m/e 72 ion was not observed when EMDP and EDDP were dosed to rats. Urine samples from methadone maintenance patients did not indicate the presence of the m/e 72 compound. Nonconjugate fraction from rat bile was
Fig. 12. Mass spectrum of m/e 72 containing metabolite
subjected to the same work-up procedures as those for the conju-
gated metabolites. This procedure was to examine the possibil-
ity that methadone N-oxide or other metabolites which were not
extracted from the bile in the first extraction step might be
the source of the m/e 72 containing peak. The final extracts
did not give the m/e 72 containing peak.

Ring hydroxylation pathways of methadone metabolism

The presence of monohydroxy EM disdain and dihydroxy EM disdain
resulting from methadone metabolism was previously reported
by Sullivan et al. (4), Lynn et al. (7), and Ånggård et al.
(8). Roerig et al. (38) called the compounds "water soluble
metabolites" without differentiating between the mono- and
dihydroxy-compounds.

The peak height of dihydroxy EM disdain in two experiments
was found to be 1/3 that of monohydroxy EM disdain. When EDDP was
administered to a rat (20 mg/Kg), conjugated metabolites were
excreted in the bile in lesser amounts than when administering
methadone. The result is similar to the report of Roerig et
al. (99) and possible reasons could be sought in the different
metabolism or excretion mechanism of methadone compared to
EDDP.

An isotope effect in the metabolic hydroxylation of
labeled methadone was not studied mainly because methods to
calculate the small isotope effect were not available. The
mechanism of formation of the ring hydroxyl metabolites of methadone i.e. whether an epoxide intermediate (50) is involved (3→50→10 or 11), or direct insertion (3→10→11) is the oxidizing mechanism was therefore not studied. The fact that the dihydrodiol (51) was not detected does not necessarily imply that the epoxide intermediate (50) is not involved in the metabolism of methadone. If epoxide intermediate formation is a mechanism
there is a possibility for the formation of a reactive toxic intermediate as described for anticonvulsants (100).

An attempt was made to study stereochemical aspects of the ring hydroxylated metabolites since the configuration of hydroxy-metabolites has not been examined. It appeared useful to find a stereospecific fragmentation process (101) for methoxy EDDP. The major fragments to observe were the phenyl ring rearranged fragments analogous to m/e 105 (37) of EDDP. Mass spectra did not show any m/e 135 (\(\text{CH}_3\text{OC}_6\text{H}_4\text{CHCH}_3^+\)) or m/e 105, indicating that phenyl ring migration is not a favorable fragmentation process for methoxy EDDP.

Stereoselective hydroxylation and conjugation have been studied for drugs such as diphenylhydantoin (102), methylphenytoin (103), and hexobarbital (104). Stereoselectivity in the metabolism of EMDP to its ring hydroxylated metabolites is also very likely but this aspect of the work remains to be done.

EMDP was found to be pharmacologically inactive (3). The importance of hydroxy metabolites in the pharmacological action of methadone appears therefore to be minimal but may not be excluded. The metabolite \(p\)-hydroxyamphetamine for example takes part in the nerve terminal accumulation of the amine (105). Irreversible binding of estrogens and 2-hydroxy-estrogens to microsomal proteins requires oxidation to the catechol nucleus (106), indicating the importance of dihydroxy
molecules in the interaction with biomolecules. A semiquinone type interaction of adriamycin to rat liver microsomal proteins was also reported (107).

N-hydroxylated metabolic pathway of methadone

The metabolite which was identified by GCMS in the conjugated fraction of rat bile with m/e 72 for a base peak was temporarily assigned as N-methylene-1-methyl-3,3-diphenyl-4-oxo-hexanamine oxide (52). Since the postulated nitrone was found in the conjugated fraction, there was a strong possibility that the N-hydroxy metabolite (53) was detected as the methadone nitrone.

Methods to identify N-hydroxy metabolites have been extensively studied by Beckett et al. (108) and normally involve derivatization of the N-hydroxy group with TMCS and analysis of the silanized sample with GCMS. An attempt to
detect the N-hydroxy metabolite (53) using such methods was not successful meaning that this metabolite was absent or present in concentrations below the level of detection. Specific color reactions for N-hydroxy metabolites were described by Coutts et al. (96) but in this work were found to be difficult to use in the presence of many endogenous materials and without an authentic sample.

Earlier studies by Beckett et al. (109) described that secondary hydroxylamines are oxidized in aqueous solutions by atmospheric oxygen to nitrones, which suggests that methadone nitrone (52) is a decomposed or air oxidized product of N-hydroxynormethadone (53). The vulnerability of N-hydroxyamphetamine and N-hydroxyphehetmine to air oxidation was studied by Lindeke et al. (110).

Coutts et al. (111) described detection of nitrone metabolites (54) as cyclized forms (55) and by means of mass fragmentation studies and different TLC $R_f$ values.

$$\text{PhCH}_2\text{CH}_2\text{CH}_3 \rightleftharpoons \text{PhCH}_2\text{CH}_2\text{CH}_3$$

Application of this cyclization method to test for the presence of methadone nitrone (52) in rat bile was also considered to be difficult because of the small quantity of metabolite present in a sample. Therefore, the tentative assignment of
the new metabolite to a methadone nitrone structure is based on gas chromatographic retention time data and mass fragmentation patterns of the compound in GCMS studies of unlabeled and labeled methadone dosed rat bile.

Additional evidence for the methadone nitrone structure was obtained from chemical oxidation studies of EDDP perchlorate by m-chloroperbenzoic acid. A compound was obtained with the same fragmentation patterns and the same GC characteristics as the compound isolated from the conjugated fraction of bile from methadone dosed rats. Detailed determination of the nitrone structure is discussed in the section describing the chemical formation of methadone nitrone.

Possibilities for the metabolic formation of N-hydroxy metabolites of methadone

The formation of nitrone, 5, is shown as one part of possible metabolic routes for methadone (Fig. 13). Conjugation of the N-hydroxy metabolite of normethadone, 5, is a strong possibility based on other examples reported for N-hydroxy metabolites (96).

Normethadone (2) which is formed from methadone (1) may not all be spontaneously cyclized to EDDP. The cyclization is favored at pHs higher than the pKa of normethadone because the protonated amine does not function as a nucleophile. Although the pH at the site of enzymatic N-oxidation is not known, there is a strong possibility that under physiological conditions
Fig. 13. Possible metabolic pathways for methadone
normethadone is not all cyclized to EDDP.

A similar analogy can be found in the benzodiazepines. Konishi et al. (113) reported that 57 formed from 56 is not spontaneously cyclized to 58 but also forms 59 before cyclization occurs. At physiological pH of ~7.0 benzodiazepines (oxazepam and diazepam) favor cyclization over hydrolysis of the azomethine linkage (114). At pH values below its pKa, (68) is not subject to cyclization. On the other hand, in the higher pH region, facile cyclization occurs. Demoxepam (115) and nitrazepam (116) also form the same equilibrium with their hydrolyzed products. This means therefore that the pH at the metabolic site is important for the rate of formation of certain metabolites such as 59.
Another similar example was reported with N-hydroxyl-naphthylamine which forms adducts with DNA, one of which has the structure 69 or 70 (117). Under acidic conditions, 70 is the major species and the two forms were reported to be interconvertible by changing pH conditions.

In Fig. 13 a proposal was made for an equilibrium between normethadone and EDDP (3) at physiological conditions. The demethylated product, 2 can then be oxidized to 53 which is conjugated before excretion. Future work is required to
study a possible equilibrium between N-demethylmethadone and EDDP, perhaps by using UV or NMR and under different pH conditions. Similar work has been done by Hassal et al. (118) for benzodiazepine derivatives.

**Pharmacological significance of the detection of methadone nitrone**

The detection of an N-hydroxy metabolite of methadone and potential formation of methadone nitrone by a metabolic process from the N-hydroxy metabolite should be considered in terms of the possible binding of these metabolites and any metabolites which are further formed from the metabolite to cellular components.

Physical data on the compound formed from EDDP oxidation and thought to be a methadone nitrone has a high partition coefficient for this compound with high solubility in hexane, a long retention time on an OV-17 GC column relative to methadone or any of its other metabolites, and a long retention time with reversed phase HPLC.

N-demethylation occurs in the brain in the case of morphine (119) where N-demethylation of methadone and formation of N-hydroxynormethadone or methadone nitrone might also occur. N-hydroxynormethadone could be oxidized to nitrone and other metabolites in the body as are other hydroxylamines (120). The formed metabolites, e.g. methadone nitrone may then bind to proteins. This binding of the nitrone to proteins
is likely to be an electrophilic reaction as in the reaction of GSH with the nitrone, 71 (121).

\[
\begin{align*}
\text{71} & \quad \text{72} \\
\end{align*}
\]

The binding of N-hydroxy compounds and their metabolites to protein has been extensively studied in relation to the carcinogenicity of N-hydroxy-1-naphthylamine (73) (117) and N-hydroxy-N-methyl-4-aminoazobenzene (74) (112). Nitro and nitroxide metabolites are known to participate in cytochrome P-450 complex formation (121).

The pharmacological action resulting from N-hydroxy-normethadone or its metabolites, e.g. methadone nitrone binding to protein, one of which might be covalent bonding, is only speculative at the moment. The binding may cause carcinogenicity. The prolonged pharmacological activity of N-hydroxy metabolites compared to the parent drugs as reported by Gorrod
et al. (123) could mean that these metabolites are strongly bound to receptor structures. The tight binding of labeled material to rat brain tissue in studies of methadone by Misra et al. (10) might suggest involvement of N-hydroxynormethadone or methadone nitrore or their subsequent metabolic products in the tolerance or other pharmacological actions of methadone.

Possible metabolic pathways of methadone proposed in Fig. 13

Among the possible metabolites of methadone, N-hydroxy-normethadone (53), nitrones (52, 60), diketone (61), EMDP N-oxide (62), N-hydroxyamino compound (63), amino compound (64), nitroso-heptanone (65), and nitroheptanone (66) were not detected in the work. The limited amounts of the sample and detection limits prevented the possible detection of metabolites. This means that potentially active metabolites may be formed in the biological system without being detected. Evidence for 53 based on the postulated nitrore (52) suggests the possibility of the formation of its further metabolized products.

In order to examine the possible formation of such metabolites and their potential activity, model metabolism studies are suggested in this thesis. Model metabolism studies could be performed by using animal models or chemical reaction models for which chemically synthesized metabolites are essential. The metabolites e.g. N-hydroxynormethadone (53) which could be obtained by reduction of nitrore (52) are administered
to animals to study further metabolic pathways. The compounds, 52 and 62 could also serve as good starting materials.

Chemical reaction processes are involved in drug metabolism (124) and some of the metabolic processes such as nitrone hydrolysis and oxidation of N-hydroxy metabolites follow similar reaction patterns to those of chemical oxidation by \textit{m}-chloroperbenzoic acid. The metabolic formation of nitro and nitroso metabolites via nitrones and their hydrolyzed product found in N-hydroxyphentermine (120) and norbenzphentermine (121) could be reproduced by MCPBA oxidation of the N-hydroxy metabolites.

MCPBA oxidation of methadon may lead to the formation of methadon nitrone (75) and further oxidation of methadon nitrone gave the nitroheptanol (77) (125).

\[
\begin{align*}
\text{CHOH-CH}_2\text{CH}_3 & \quad \xrightarrow{\text{CH}_2\text{CH}-\text{N-CH}_2\text{CH}_3} \quad \text{CHOH-CH}_2\text{CH}_3 & \quad \xrightarrow{\text{CH}_2\text{CH}-\text{N}=\text{O}} \quad \text{CHOH-CH}_2\text{CH}_3
\end{align*}
\]

75 \quad 76 \quad 77

A similar example was proposed in the MCPBA oxidation of isoxazolidine, 78 to form oxazine, 79 which was further oxidized to nitroso, 82 and nitro compound, 83 via the hydrolyzed product, 81 (126).
Chemical hydrolysis of nitrones is common in nitrone chemistry (127, 128). Therefore, hydroxyamino compound, 63 could be formed from methadone nitrone, 52 and EMDP N-oxide, 62. There is also a possibility that 63 could be formed from amino compound, 64 which is a hydrolyzed product of protonated EMDP (20).
4. Chemical oxidation studies

Chemical oxidation of methadone metabolites

The formation of DDP from air oxidation of EDDP base is consistent with the fact that EDDP base has an exocyclic double bond (3). This does not exclude the possibility that DDP might be derived from thermal cleavage of the air oxidized EDDP on the GC column. Detection of DDP by TLC is however more indicative of the oxidative cleavage of the double bond.

In order to see the importance of the double bond character of EDDP and EMDP at different pH conditions, the free base and salt forms of EDDP and EMDP were oxidized with m-chloroperbenzoic acid.

Oxidation of EDDP base follows a mechanism involving oxidative double bond breakage (129) and results in the formation of DDP. Oxidation of EDDP perchlorate resulted in the formation of three major products including the methadone nitrone product (52) which is discussed separately in a later section.

Oxidation of EMDP base gave 2-ethyl-5-methyl-3,3-diphenylpyrrolidyl-1,2-oxaziran (84) identified by spectroscopic methods.
Four peaks were shown in the oxidation mixture of EMDP HCl: GCMS, 150-280°C, 8°/min.; Rt 4 min., diphenylethylene; 9.4 min., EMDP; 11.6 min., m/e 208 base peak; 12.6 min., m/e 208 base peak. Attempts to isolate the two unknown compounds using column chromatography and TLC in order to elucidate their structures failed because of the unstable nature of these compounds.

Chemical oxidation of EDDP perchlorate

The TIC profile of the oxidation products from EDDP perchlorate is shown in Fig. 14. The same oxidation experiment was repeated with EDDP-$^2$H$_{10}$ perchlorate and EDDP-$^2$H$_{3}$ perchlorate to collect additional information on mass fragmentation. Three major components (A, B, and C) from the EDDP perchlorate oxidation products were collected using preparative GC. High resolution mass spectra were obtained on these isolated fractions. Chemical ionization GCMS of the oxidation mixture also gave additional data for molecular ions.
Fig. 14. TIC profile (a; GCMS, 200-280°C 8°/min.) and HPLC (b) of EDDP perchlorate oxidized products
Two major components from oxidation mixture were isolated in pure form using HPLC (Fig. 14 B and C). Compound A was not isolated in pure form and was thus identified by GC retention time and mass fragmentation patterns. A possible structure for A was 2-acetyl-5-methyl-3,3-diphenyl-1-pyrroline (85). NMR and IR of compounds, B and C gave positive identification as 4,4-diphenyl-2,5-heptan...
TABLE V. High Resolution Mass Fragmentation Data for Methadone Nitrone (52)

<table>
<thead>
<tr>
<th>Methadone nitrone</th>
<th>$^{2}\text{H}_3$</th>
<th>$^{2}\text{H}_{10}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>m/e 309</td>
<td>C$<em>{20}$H$</em>{23}$NO$_2$</td>
<td>m/e 312</td>
</tr>
<tr>
<td>m/e 253</td>
<td>C$<em>{17}$H$</em>{19}$NO</td>
<td>m/e 253</td>
</tr>
<tr>
<td>m/e 208</td>
<td>C$<em>{16}$H$</em>{16}$</td>
<td>m/e 207</td>
</tr>
<tr>
<td>m/e 207</td>
<td>C$<em>{16}$H$</em>{15}$</td>
<td>m/e 207</td>
</tr>
<tr>
<td>m/e 193</td>
<td>C$<em>{15}$H$</em>{15}$</td>
<td></td>
</tr>
<tr>
<td>m/e 181</td>
<td>C$<em>{14}$H$</em>{13}$</td>
<td></td>
</tr>
<tr>
<td>m/e 180</td>
<td>C$<em>{14}$H$</em>{12}$</td>
<td></td>
</tr>
<tr>
<td>m/e 179</td>
<td>C$<em>{14}$H$</em>{11}$</td>
<td></td>
</tr>
<tr>
<td>m/e 178</td>
<td>C$<em>{14}$H$</em>{10}$</td>
<td></td>
</tr>
<tr>
<td>m/e 165</td>
<td>C$_{13}$H$_9$</td>
<td></td>
</tr>
<tr>
<td>m/e 130</td>
<td>C$<em>{10}$H$</em>{10}$</td>
<td></td>
</tr>
<tr>
<td>m/e 129</td>
<td>C$_{10}$H$_9$</td>
<td>m/e 129</td>
</tr>
<tr>
<td>m/e 115</td>
<td>C$_9$H$_7$</td>
<td></td>
</tr>
<tr>
<td>m/e 91</td>
<td>C$_7$H$_7$</td>
<td></td>
</tr>
<tr>
<td>m/e 73</td>
<td>C$_3$H$_7$NO</td>
<td></td>
</tr>
<tr>
<td>m/e 72</td>
<td>C$_3$H$_6$NO</td>
<td></td>
</tr>
<tr>
<td>m/e 57</td>
<td>C$_3$H$_5$O</td>
<td>m/e 60</td>
</tr>
<tr>
<td>m/e 44</td>
<td>m/e 44</td>
<td>m/e 44</td>
</tr>
</tbody>
</table>

Comparable ion fragments as seen by low resolution GCMS are given for the $^{2}\text{H}_3$ and $^{2}\text{H}_{10}$ analogs.
Formation of the m/e 292 fragment (87 or 89) appears to be the preferable route under chemical ionization conditions whereby protonation easily occurs. The six membered ion, 86 appears
more stable than the seven membered ion, $88$ and thus the formation of $86$ appears to be a favorable route. As an example the reaction intermediate, $94$ of 3-carboxypyridine-1-oxide and propionic anhydride gave $96$, which is a case of electrophilic attack at the site of methylene carbon (130). Similarly,

*methadone nitrone* (52) forms cyclized fragment, $86$.

The fragment, m/e 253 (92) is important in the fact that the deuterium labeled $\text{[}^{2}\text{H}_{3}\text{]}$-compound was a key to the interpretation of this fragment (Table V).

The second fragmentation pathway for the methadone nitrone yields ions, m/e 72 (99), m/e 44 (97), m/e 207 (101), and m/e 57 (98) in the order of relative intensity. Fragmentation processes for the formation of m/e 72 and m/e 57 commonly occur in methadone analogs. The pathway leading to the formation of m/e 207 (101) is a common process for nitroxide derivatives. Further fragmentation leads to ions, m/e 130 (102) and m/e 129 (103).
The formation of fragment ion m/e 44 is similar to the observed fragmentation of nitrones, 104 to 105 (131) and 106 to 107 (132).
The third fragmentation pathway provides additional information on the fragmentation of diphenyl type compounds. The high resolution mass spectrometry data (Table V) provides a basis for the explanation of the fragmentation processes described for DDP and EMDP (31-35). Such ions are m/e 193 \(^{(108)}\), m/e 180 \(^{(32)}\), m/e 181 \(^{(111)}\), m/e 115 \(^{(109)}\), and m/e 179 \(^{(33)}\). The fragments m/e 91 \(^{(112)}\) and m/e 89 \(^{(113)}\) could be derived from m/e 180 \(^{(32)}\); m/e 181 \(^{(111)}\) was from m/e 208 \(^{(31)}\).
New proposal for fragmentation pathways for methamphetamine nitrone

Mass fragmentation processes for the nitrone metabolite (54) of N-methylamphetamine were shown in two papers published by Coutts et al. (133, 134). It was proposed that under EI conditions the nitrone, 54 forms the cyclized intermediate of m/e 163 (115), which further fragments to m/e 148 (117) and m/e 132 (118). To explain the formation of m/e 104 (116), a retro Diels-Alder type fragmentation process was proposed. Oxygen expulsion was involved in the formation of 118 from 117. These ions were defined with deuterium labeled amphetamine derivatives.
The fragmentation processes proposed for methadone nitrone are different from those described for N-methylamphetamine nitrone by Coutts et al. (134). It was found that the fragment ions reported for this amphetamine nitrone could also be explained following our proposal for the methadone nitrone fragmentations.

Thus, formation of the fragments, m/e 148 (120) and m/e 132 (121) follows from the cyclized intermediate 119. Fragment 121 has m/e 132 corresponding to the fragment of 118 proposed by Coutts et al. (134).

\[
\text{N-methylamphetamine nitrone fragments to the ion m/e 118 (122) which is a similar process to the formation of m/e 207 (101) in methadone nitrone (52). The ion m/e 118 (122) was further fragmented to m/e 91 (124), m/e 117 (125),}
\]
and m/e 104 (126). A hydrogen scrambling process is involved in the formation of 124, 125, 126 which is suggested by the reported mass spectra (134) which were prepared by using unlabeled and labeled compounds. The fragment, m/e 104 (126) was previously explained by a retro Diels-Alder process. Our proposal describes formation of m/e 104 (126) from m/e 118 (123).
The NMR spectrum of methadone nitrone is shown in Fig. 15. The peaks were assigned as follows: Two doublets of doublets at 2.25 δ and 2.73 δ for C-5 protons are typical of nitrone type compounds. Methylene protons appear at 3.1 δ and 3.55 δ as two doublets. The other chemical shift values are shown below together with the structural assignment. Peaks from impurities derived from HPLC solvents and CHCl₃ are shown at 1.0 - 1.6 δ.

Table VI compares the C-5 and C-6 protons and methylene protons of methadone nitrone with those of N-(1-(3',4'-dimethoxyphenyl) prop-2-yl) nitrone (127) published by Morgan et al. (135). The methylene protons of 52 (δ 3.1 and 3.55) were shown to be more shielded than those of 127 (δ 6.04 and 6.24).

The coupling constants, JHa-Hc (4.0 Hz) and JHb-Hc (4.0 Hz) suggest a preferred conformation of 52 in which the
Fig. 15. NMR spectrum of methadone nitrope
TABLE VI. NMR of C-5 and geminal protons of methadone nitrone (52) and 3.4-dimethoxyamphetamine nitrone (127)

<table>
<thead>
<tr>
<th></th>
<th>52</th>
<th>127</th>
</tr>
</thead>
<tbody>
<tr>
<td>δ CH₂ (5)</td>
<td>2.25 (dd)</td>
<td>2.73 (dd)</td>
</tr>
<tr>
<td>δ Ha</td>
<td>2.25 (dd)</td>
<td>2.73 (dd)</td>
</tr>
<tr>
<td>J Ha-Hc</td>
<td>4.0</td>
<td>5.6</td>
</tr>
<tr>
<td>δ Hb</td>
<td>2.73 (dd)</td>
<td>3.18 (dd)</td>
</tr>
<tr>
<td>J Hb-Hc</td>
<td>4.0</td>
<td>10.0</td>
</tr>
<tr>
<td>J Ha-Hb</td>
<td>14.0</td>
<td>14.0</td>
</tr>
<tr>
<td>δ Hc</td>
<td>1.75 (m)</td>
<td>4.05 (m)</td>
</tr>
</tbody>
</table>

+ \( \text{N} \equiv \text{C} \) Ha

<table>
<thead>
<tr>
<th></th>
<th>52</th>
<th>127</th>
</tr>
</thead>
<tbody>
<tr>
<td>δ Ha</td>
<td>3.1 (d)</td>
<td>6.04 (d)</td>
</tr>
<tr>
<td>δ Hb</td>
<td>3.55 (d)</td>
<td>6.24 (d)</td>
</tr>
<tr>
<td>J Ha-Hb</td>
<td>11.0</td>
<td>8.0</td>
</tr>
</tbody>
</table>

Oxygen of the ketone group is oriented towards the methylene group. This interaction may contribute to an increase of electron density on the methylene group which could lead to the electron shift causing loss of polarity of the nitrone nitrogen. This overall effect may explain the stability of this methadone nitrone and the high shielding observed for the C-6 proton (δ 1.75; δ 4.05 for 127) and that of the methylene protons.
An alternative explanation of the high shielding observed for the C-6 proton and the methylene protons is that instead of structure 52 the compound isolated has the oxaziridine structure 128. The methylene protons of oxaziridines are known to be more shielded than those of nitrones (135). While the structure 128 cannot be excluded on the basis of spectroscopic evidence (MS, NMR, IR) there is no precedence for the formation of such a compound by in vivo metabolism. Upon this basis we will tend to prefer the nitrone structure 52.

**IR spectroscopy of methadone nitrone and related compounds**

The IR spectrum and a summary of the data for the methadone nitrone are shown in Fig. 16. The C-H vibrations described are assigned on the basis of IR data collected from
methadone analogs and their deuterated counterparts and should be quite useful for describing the IR of other diphenyl containing compounds.

**Formation of diketone (61) from oxidation of EDDP perchlorate**

The compound which has m/e 43 as a base peak (Fig. 14(a), B and Fig. 14(b), B) was assigned to the diketone, 61. NMR and IR spectra shown in Fig. 17 and mass fragmentation data were consistent with the diketone structure.

![Chemical structure of diketone 61](image)

Isolation of diketone, 61 would support the proposed mechanism (Fig. 18) of m-chloroperbenzoic acid oxidation of EDDP perchlorate.
\[ \nu (=\text{CH}) \ 3020, \ 3040, \ 1600, \ 1490 \ \text{cm}^{-1} \ \ \ \delta (=\text{CH}) \ 1140, \ 1100, \ 1030, \ 930, \ 760, \ 700 \\
\nu (\text{CH}) \ 2960, \ 2920 \ \delta (\text{CH}, \text{CH}_2, \text{CH}_3) \ 1450, \ 1440, \ 1370, \ 1340, \ 820, \ 800 \\
\nu (\text{C}=\text{O}, \ \text{N}=\text{C}) \ 1710 \ \ \ \nu (\text{N} + \text{O}) \ 1550, \ 1250 \\

\text{Fig. 16. IR (liquid film) of methadone nitrone} \]
Fig. 17. (a) NMR and (b) IR (KBr pellet) of 4,4-Diphenyl-2.5-heptanedione (61).
A possible mechanism for the formation of methadone nitrone (52), or oxaziridine (128), and diketone (61) from the chemical oxidation of EDDP perchlorate is presented in Fig. 18. More than 2 moles of MCPBA was required for the formation of 52, or 128, and 61 which is illustrated by the proposed intermediate, 129, formed by N-oxide formation and an addition of a second mole of MCPBA to the double bond. Nitrone, 52, or oxaziridine (128) were not formed selectively.

The diketone, 61 can be seen to be formed through the intermediate 130. This chemical hydrolysis has a similar mechanism to the metabolic formation of the ketone 132 from the nitrone 131 (136).

\[
\begin{align*}
R - CH_2 - C = N^+ - CH_2R' & \rightarrow R - CH_2 - C = O \\
\text{CH}_3 & \quad \text{CH}_3
\end{align*}
\]
Fig. 18. Proposed mechanisms for the formation of methadone nitrone (52) or oxaziridine (128) and 4,4-diphenyl-2,5-heptanedione (61) by MCPBA oxidation of EDDP perchlorate.
Proposed structure for compound A (Fig. 13)

High resolution mass spectra of compound A gave fragments, m/e 208 (24.29%), m/e 207 (12.87%), m/e 193 (22.53%), m/e 179 (13.09%), m/e 178 (14.34%), m/e 165 (11.27%), m/e 130 (19.53%), and m/e 115 (19.82%). These ions were common to the diphenyl butane containing compound. The compounds also gave ions, m/e 277 (1.32%) (C\textsubscript{19}H\textsubscript{19}NO), m/e 235 (11.63%) (C\textsubscript{16}H\textsubscript{13}NO, \textsuperscript{2}H\textsubscript{3} m/e 238) and m/e 43 (base peak) (C\textsubscript{2}H\textsubscript{3}O, \textsuperscript{2}H\textsubscript{3} m/e 46). On the basis of these ions the compound was assigned the structure, 85.

Compound B was also detected by GCMS when old urine samples were analyzed and proved to result from air oxidation of EDDP. When EDDP base was air oxidized in CHCl\textsubscript{3} and analyzed by GCMS (150 - 280°C at 10°/min.), the major product DDP was eluted at 245°C and another product eluting at 225°C close to the EDDP peak was identical to the acetyl compound, 85.

A possible mechanism to the formation of 85 either by chemical oxidation of EDDP perchlorate or by air oxidation of EDDP base is shown in 3, 19 through 85 (p. 115).
MCPBA oxidation of EMDP base gave an oxidized product, which was eluted at 250°C (120-270°C at 10°/min.) by GCMS.
Mass fragmentation data were compared with deuterium labeled compounds obtained by oxidation of EMDP-$^2$$^1$H$_{10}$:

A molecular ion appeared at m/e 279 (28.3%) ($^2$H$_{10}$ m/e 289). M$^+$-CH$_2$CH$_3$ (m/e 250 (36.0%), $^2$H$_{10}$ m/e 260) and M$^+$-phenyl (m/e 202 (16.7%), $^2$H$_{10}$ m/e 207) were major fragments. The ions commonly derived from m/e 208 were also present with high relative intensity.

The structure was not absolutely proved but is most likely EMDP oxaziran (84) rather than EMDP N-oxide (62). High abundant m/e 279, m/e 250, and m/e 202 indicated the stable nature of the oxygen in the molecule of 84. On the other hand, N-oxide may easily lose oxygen to give high abundant m/e M-16. An attempt to obtain cyclized product, 135, was not successful.

![Chemical structures](image)

The chemical forms of 62 and 84 are reported to be interconvertible either by acidic conditions or by UV irradiation (137). The oxidized product was hydrolyzed in acid (10% HCl, 70°C, 2 hours) and a mixture of products which has peaks
on GCMS containing m/e 208 fragment as base peak was obtained.
Further attempts to determine the structures were not performed.

NMR data for the proposed oxidized product of EMDP is shown below along with the structure. The chemical shift values for the C-8, C-7, and C-6 protons were similar to those of EMDP (C-8 1.07 δ, C-7 2.09 δ, and C-6 1.37 δ) (3).

The IR spectrum of this compound had strong peaks at 1250 and 1550 cm⁻¹ corresponding to N - O stretching vibrations.
5. **Interaction of methadone and diazepam**

Stability of the labeling of the compounds which were used as internal standards to analyze nonconjugate and conjugate metabolites was examined to ensure the stability of the labeling during incubation and work up procedures. Paired experiments were performed with four rats for methadone treatment and another four rats for methadone and diazepam treatment.

**Stability studies**

The stability of the deuterium labeled methadone and the labeled EDDP and EMDP was explored over a wide range of pH's and under conditions of prolonged incubation (Table VII). As can be seen from the ion ratios monitored which remain constant, the deuterium was stable in all cases. The result implies that the deuterium labeled compounds which were used as internal standards in this interaction study are stable during the equilibration and work up procedures for the analysis.

The advantages of using deuterium labeled compounds as internal standards was further demonstrated by EDDP when
TABLE VII. Stability of Deuterated Methadone, EDDP and EMDP

<table>
<thead>
<tr>
<th></th>
<th>Methadone</th>
<th>EDDP</th>
<th>EMDP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>233/223</td>
<td>226/223</td>
<td>287/277</td>
</tr>
<tr>
<td>1N HCl</td>
<td>0.633</td>
<td>0.954</td>
<td>0.532</td>
</tr>
<tr>
<td>pH 2.0</td>
<td>0.643</td>
<td>0.968</td>
<td>0.532</td>
</tr>
<tr>
<td>pH 4.5</td>
<td>0.657</td>
<td>0.946</td>
<td>0.525</td>
</tr>
<tr>
<td>pH 9.0</td>
<td>0.615</td>
<td>0.945</td>
<td>0.513</td>
</tr>
<tr>
<td>0.1N NaOH</td>
<td>0.656</td>
<td>0.963</td>
<td>0.528</td>
</tr>
<tr>
<td>Control</td>
<td>0.673</td>
<td>0.956</td>
<td>0.532</td>
</tr>
</tbody>
</table>

The values denote ion ratios of ions monitored: m/e 223 (methadone), m/e 226 (methadone-\textsuperscript{2}H\textsubscript{3}), m/e 233 (methadone-\textsuperscript{2}H\textsubscript{10}), m/e 277 (EDDP), m/e 280 (EDDP-\textsuperscript{2}H\textsubscript{3}), m/e 287 (EDDP-\textsuperscript{2}H\textsubscript{10}), m/e 208 (EMDP), and m/e 218 (EMDP-\textsuperscript{2}H\textsubscript{10}). The values represent the mean of three determinations. The aqueous samples were incubated for 48 hours at the respective pH's. The control was prepared in MeOH.
under alkaline conditions (Table VII). Because deuterium labeled EDDP could compensate for the chemical instability of EDDP, the ratio of unlabeled to labeled EDDP was the same notwithstanding significant decomposition of EDDP under alkaline conditions. Total integration values for EDDP in 0.1 N NaOH were less than 1/3 that of control samples prepared in MeOH. This example of the advantage of using a deuterium labeled internal standard could be extended to the analysis of conjugate metabolites. For example, the instability of phenol and catechol type molecules could be compensated for by using deuterium labeled standards.

Stability of the deuterium label was also shown by the conjugate metabolites following 96 hours of incubation time (Tables VIII, IX). Even in the presence of an activating group such as the hydroxyl group on the ring, the deuterium was stable under all conditions of incubation pH and temperature. The effect of activating groups on the stability of labeling was described for a hydroxyl group (138) and an amino group (139).

This result is consistent with a report that the deuterium label on the ring of p-hydroxyephedrine obtained by metabolic processes was found to be stable during the glucuronide formation and the subsequent enzyme hydrolysis of the conjugate (140).

The stability studies of conjugate metabolites (Tables XIII and IX) also gave some insight into the contribution of
### TABLE VIII. Stability of Deuterium in HOEMDP-\(^2\)H\(_9\)

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>246/247</th>
<th>238/247</th>
<th>237/247</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>0.435</td>
<td>-0.0062</td>
<td>0.0005</td>
</tr>
<tr>
<td>24</td>
<td>0.439</td>
<td>0.0060</td>
<td>0.0062</td>
</tr>
<tr>
<td>48</td>
<td>0.432</td>
<td>0.0054</td>
<td>0.0041</td>
</tr>
<tr>
<td>96</td>
<td>0.441</td>
<td>-0.0070</td>
<td>-0.0001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>246/247</th>
<th>245/247</th>
<th>244/247</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>0.446</td>
<td>0.359</td>
<td>0.118</td>
</tr>
<tr>
<td>24</td>
<td>0.437</td>
<td>0.403</td>
<td>0.113</td>
</tr>
<tr>
<td>48</td>
<td>0.461</td>
<td>0.407</td>
<td>0.113</td>
</tr>
<tr>
<td>96</td>
<td>0.437</td>
<td>0.407</td>
<td>0.125</td>
</tr>
</tbody>
</table>

Samples were monitored twice with a different set of ions being monitored each time. The first set of ions was m/e 247, m/e 246, m/e 238, and m/e 237 and the second, m/e 247, m/e 246, m/e 245, and m/e 244. The stability of the label, the presence of impurities and the stability of the ion focussing were followed using the ratio of ions, m/e 246 to m/e 247. The ion, m/e 238 was chosen for CH\(_3\)OEMDP and m/e 247 for CH\(_3\)OEMDP-\(^2\)H\(_9\). The values were the average from two incubations at each incubation time (12, 24, 48, and 96 hours). Negative values result from background subtraction.
Samples were monitored twice with a different set of ions being monitored each time. The first set of ions was $m/e$ 245, $m/e$ 244, $m/e$ 237, and $m/e$ 236 and the second, $m/e$ 247, $m/e$ 246, $m/e$ 245, and $m/e$ 244. The stability of the label, the presence of impurities, and the stability of the ion focusing were followed using the ratio of ions, $m/e$ 244 to $m/e$ 245. The ion $m/e$ 237 was chosen for $\text{DiCH}_3\text{OEMDP}$ and $m/e$ 245 for $\text{DiCH}_3\text{OEMDP-}^2\text{H}_8$. The values were the average from two incubations at each incubation time (12, 24, 48, and 96 hours).

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>244/245</th>
<th>237/245</th>
<th>236/245</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>0.363</td>
<td>0.0088</td>
<td>0.0091</td>
</tr>
<tr>
<td>24</td>
<td>0.359</td>
<td>0.0069</td>
<td>0.0052</td>
</tr>
<tr>
<td>48</td>
<td>0.364</td>
<td>0.0059</td>
<td>0.0060</td>
</tr>
<tr>
<td>96</td>
<td>0.369</td>
<td>0.0066</td>
<td>0.0077</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>247/245</th>
<th>246/245</th>
<th>244/245</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>0.0296</td>
<td>0.215</td>
<td>0.359</td>
</tr>
<tr>
<td>24</td>
<td>0.0341</td>
<td>0.211</td>
<td>0.356</td>
</tr>
<tr>
<td>48</td>
<td>0.0266</td>
<td>0.214</td>
<td>0.359</td>
</tr>
<tr>
<td>96</td>
<td>0.0250</td>
<td>0.207</td>
<td>0.359</td>
</tr>
</tbody>
</table>
background to the analytical procedure. The stability studies of conjugate metabolites which were performed with deuterium labeled compounds showed that the ion ratios, m/e 238/247, m/e 237/247, m/e 237/245, and m/e 236/245 were less than 1 percent. This meant that there was an absence of impurities in endogenous bile and no contribution from labeled metabolites which might interfere in the analysis of unlabeled metabolites.

The mass spectrum for CH$_3$OEMDP-$^2$H$_9$ did not show any ions at m/e 238, 237 and the mass spectrum of DiCH$_3$OEMDP-$^2$H$_8$ did not show ions at m/e 237 and m/e 236.

A method to examine the potential overestimation of methadone conjugate metabolites arising from analytical interferences such as from endogenous materials and from conjugate metabolites of diazepam was also studied. If there is no interference, then monitoring of consecutive ions should give constant ratios. The ions monitored were m/e 246/247 for CH$_3$OEMDP-$^2$H$_9$ and m/e 244/245 for DiCH$_3$OEMDP-$^2$H$_8$. A sudden change of the ratios indicated in most cases disruption of the ion focussing arising from either power failure or an unstable magnetic field. The fact that blank samples which were collected for the first one hour after diazepam dose did not interfere with the ion ratios indicated an absence of interference from diazepam metabolites. It has been shown that during the first one hour, 45% of the radioactivity injected in the form of [5-$^{14}$C]-diazepam into the rat appeared in the bile (141).
Stability studies were also aimed at measuring the accuracy and precision of the developed SIM system. Independent of the set of ions monitored (m/e 247, 246, 245, 244 and m/e 247, 246, 238, 237), the ratio of m/e 246 to m/e 247 was the same (0.43) for CH₃OEMDP⁻²Hg. Consecutive ion monitoring i.e. m/e 247, 246, 245, 244 and monitoring four separate ions i.e. m/e 245, 244, 237, 236 also gave the same m/e 244/245 value of 0.36 for DiCH₃OEMDP⁻²Hg, implying precision of the methodology.

Accuracy of the ion monitoring method was difficult to evaluate. Ion ratios calculated from the relative intensity of mass spectral scan data were compared with those from integration values of specific ion being monitored in SIM mode. The ion ratios of m/e 245 to m/e 247 and m/e 246 to m/e 247 obtained by scan mode for CH₃OEMDP⁻²Hg were 0.46 and 0.36, respectively. On the other hand, the ratios obtained by SIM were 0.40 and 0.44. In the case of DiCH₃OEMDP⁻²Hg, the ratios from scan mode were 0.366 and 0.220 for m/e 244 to m/e 245 and m/e 246 to m/e 245, respectively. SIM ratios were 0.360 and 0.215. The difference in the ratios between SIM and repetitive scanning is due to the different data acquisition methods.
Bile collection

Table X describes the bile volumes collected from each rat in the methadone-diazepam interaction study. The total amount of collected bile averaged 50 ml/kg for 23 hours, which was a little below the bile flow (60-100 ml/kg/24 hour) described by Johnson et al. (141). Taking into account that fluid replacement by infusion was not carried out, the bile flow appeared adequate to obtain reliable data for the drug interaction studies.

Diazepam did not influence bile flow during the 23 hour time period of the study (p>0.20). A similar result was reported by El-Hawari et al. (143) in their studies of the effects of diazepam on biliary excretion of diphenylhydantoin.

Analysis of nonconjugated metabolites from rat bile

As shown in individual SIM chromatograms of EMDP, methadone, and EDDP (Fig. 19) prepared from spiked samples, analysis of methadone and EDDP had high specificity because high masses (m/e 294 and m/e 297 for methadone, m/e 277 and m/e 280 for EDDP) were monitored. Monitoring ions, m/e 208 and m/e 218 was found to lack specificity for EMDP but the
TABLE X. Effect of Diazepam Treatment on the Bile Flow of Rats

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>1-M</th>
<th>1-DM</th>
<th>2-M</th>
<th>2-DM</th>
<th>3-M</th>
<th>3-DM</th>
<th>4-M</th>
<th>4-DM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-2</td>
<td>3.85</td>
<td>4.04</td>
<td>4.28</td>
<td>4.07</td>
<td>3.52</td>
<td>4.41</td>
<td>3.72</td>
<td>5.22</td>
</tr>
<tr>
<td>2-11</td>
<td>26.00</td>
<td>28.08</td>
<td>13.81</td>
<td>20.89</td>
<td>23.18</td>
<td>21.92</td>
<td>19.37</td>
<td>26.79</td>
</tr>
<tr>
<td>11-23</td>
<td>29.90</td>
<td>27.48</td>
<td>23.72</td>
<td>17.69</td>
<td>25.17</td>
<td>25.66</td>
<td>17.97</td>
<td>18.85</td>
</tr>
<tr>
<td>Total</td>
<td>57.75</td>
<td>59.60</td>
<td>41.81</td>
<td>42.65</td>
<td>51.87</td>
<td>51.99</td>
<td>41.06</td>
<td>50.86</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>M</th>
<th>DM</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-2</td>
<td>3.842±0.321</td>
<td>4.435±0.549</td>
<td>&gt;0.10</td>
</tr>
<tr>
<td>2-11</td>
<td>20.361±5.627</td>
<td>24.420±3.546</td>
<td>&gt;0.20</td>
</tr>
<tr>
<td>11-23</td>
<td>24.190±4.915</td>
<td>22.420±4.872</td>
<td>&gt;0.20</td>
</tr>
<tr>
<td>Total</td>
<td>48.393±8.900</td>
<td>51.275±6.930</td>
<td>&gt;0.20</td>
</tr>
</tbody>
</table>

Paired experiments were performed using four rats for methadone treatment (M) and another four rats for methadone and diazepam treatment (DM).

The values denote the amount of bile (g/kg rat) collected over the time periods 0-2, 2-11, and 11-23 hours following the methadone dose. The values in the lower part of the Table represent mean ± SD obtained for each of the two groups. The value p was calculated by using Student's t test.
Fig. 19. SIM chromatograms for EMDP (a), EDDP (b), and methadone (c).
chromatographic separation of peaks was sufficiently selective to allow analysis of EMDP.

For the analysis of nonconjugated metabolites, authentic samples and deuterium labeled internal standards were available. Calibration curves were prepared for EDDP, methadone, and EMDP by spiking EDDP (5-80 ug), methadone (0.05-0.8 ug), and EMDP (0.1-1.6 ug) in 0.4 ml of control bile and by working up the samples as described in the experimental using EMDP-\(^2\)\(^{10}\) (2 ug), methadone-\(^2\)\(^3\) (5 ug) and EDDP-\(^2\)\(^3\) (20 ug) as the internal standards. Under the above calibration conditions, 200 ng of methadone in 0.4 ml of bile, 400 ng of EMDP in 0.4 ml of bile, and 5 ug of EDDP in 0.4 ml of bile were reproducibly analyzable.

Methadone excreted in rat bile was not detectable under the conditions of the analysis. Assuming that the lowest concentration of methadone that can be positively measured is 200 ng in 0.4 ml of bile, then less than 0.2% of the administered methadone dose is excreted into the bile, a trace amount. This result was consistent with a report that a very small amount of unchanged methadone and EMDP was present in human bile (98) and rat bile (144). This meant that the analysis of methadone can not be used for studies of the methadone-diazepam interaction.

EMDP levels in rat bile samples were also analyzed and concentrations below 400 ng/0.4 ml were observed. This result is the same as that reported by Kreek et al. (98)
and Baselt et al. (145). Therefore, analysis of EMDP would not be a good indicator of a metabolic interaction between methadone and diazepam.

The low level of methadone and EMDP in bile can be explained in that liver has a high intrinsic activity for the metabolism of methadone and EMDP. The extent of biliary excretion of a drug is proportional to the polarity of the drug which is reflected by its partition coefficient. The percentages reported excreted in rat bile 4 hours after the i.v. injection were 36% for EDDP (partition coefficient 0.04) and 0.2% for EMDP (partition coefficient 13.4)(94).

EDDP levels found in bile samples in the interaction study are shown in Table XI where 20-30% of administered methadone was found to be excreted into the bile as EDDP.

Table XI clearly indicates that biliary excreted EDDP does not show any difference between methadone only and methadone-diazepam treated rats (p >0.10).

Analysis of conjugated metabolites from rat bile

SIM chromatograms of mono and dihydroxy EMDP isolated from rat bile are shown in Fig. 20. The ions (45, 48) chosen to monitor were the base peaks for the compounds and had high selectivity.

Diazomethane treatment and selective extraction of the diazomethane treated samples provided several advantages
TABLE XI. Excretion of EDDP from Methadone and Methadone-Diazepam Treated Rats

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>1-M</th>
<th>1-DM</th>
<th>2-M</th>
<th>2-DM</th>
<th>3-M</th>
<th>3-DM</th>
<th>4-M</th>
<th>4-DM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-2</td>
<td>0.365</td>
<td>0.386</td>
<td>0.282</td>
<td>0.486</td>
<td>0.352</td>
<td>0.395</td>
<td>0.379</td>
<td>0.496</td>
</tr>
<tr>
<td>2-11</td>
<td>1.967</td>
<td>1.932</td>
<td>1.784</td>
<td>1.758</td>
<td>1.723</td>
<td>1.785</td>
<td>2.002</td>
<td>1.727</td>
</tr>
<tr>
<td>11-23</td>
<td>0.435</td>
<td>0.146</td>
<td>0.227</td>
<td>0.942</td>
<td>0.234</td>
<td>0.330</td>
<td>0.099</td>
<td>0.204</td>
</tr>
<tr>
<td>Total</td>
<td>2.767</td>
<td>2.464</td>
<td>2.293</td>
<td>3.186</td>
<td>2.309</td>
<td>2.510</td>
<td>2.480</td>
<td>2.427</td>
</tr>
<tr>
<td>Dose %</td>
<td>25.31</td>
<td>22.54</td>
<td>20.98</td>
<td>29.15</td>
<td>21.12</td>
<td>22.96</td>
<td>22.69</td>
<td>22.20</td>
</tr>
</tbody>
</table>

$\text{Time (h)} \quad M \quad \text{DM} \quad P$

| 0-2      | 0.344 ± 0.0431 | 0.440 ± 0.0582 | <0.05 |
| 2-11     | 1.869 ± 0.1364 | 1.800 ± 0.0908 | >0.20 |
| 11-23    | 0.248 ± 0.1388 | 0.405 ± 0.3658 | >0.20 |
| Total    | 2.462 ± 0.220 | 2.646 ± 0.361 | >0.10 |

Paired experiments were performed using four rats for methadone treatment (M) and another four rats for methadone and diazepam treatment (DM). The values denote the amount of EDDP (shown as EDDP perchlorate mg/kg rat) excreted over the time periods, 0-2, 2-11, 11-23 hours after the methadone dose. The values in the lower part of the Table represent mean ± SD obtained for each of the two groups. The value $p$ was calculated by using Student's t test. Dose % refers to the percentage of the dose excreted as EDDP.
Fig. 20.: SIM chromatograms for (a) monohydroxy EMDP and (b) dihydroxy EMDP analysis
in the analysis. Underivatized dihydroxy EMDP was not
detectable in the TIC profile of the conjugate fraction.
Diazomethane treatment and hexane extraction of the sample
totally removed any interferences (Fig. 21). Derivatization
by diazomethane methylation also had advantages in terms of
preventing oxidation of the phenolic and catechol type meta-
bolites.

Calibration curves were prepared by using one of
the bile samples in different volumes (Table XII). The ratios
obtained from natural sample analysis were within the calibra-
tion range. The ratio of m/e 246 to m/e 247 and that of
m/e 244 to m/e 245 were 0.43-0.46 and 0.36-0.37, indicating
absence of interference in the analysis i.e. the values were
the same as those shown in Tables VIII and IX.

As shown in Tables XIII and XIV, an increased excre-
tion of conjugate metabolites was found during the first
2 hours of bile excretion in the methadone-diazepam treated
rats. For HOEMDP 5.7% and 16.8% was excreted during the
first 2 hours in control and diazepam treated rats, respective-
ly. In the case of DiHOEMDP, the percentages excreted were
5.3% and 10.7% for control and diazepam treated rats, respec-
tively. However, when the excretion of conjugate metabolites
was followed over the entire 23 hour period, there was no
difference observed between the methadone only treated
and methadone-diazepam treated rats (HOEMDP, p >0.20,
DiHOEMDP p >0.20).
Fig. 21. TIC profile (a) and Mass chromatogram (b) of the conjugate fraction obtained from methadone dosed rat bile before back extraction and TIC profile (c) after back extraction.
TABLE XII. Calibration Curve Data for Conjugate Metabolites

<table>
<thead>
<tr>
<th>HOEMDP</th>
<th>m/e 246/m/e 247</th>
<th>m/e 238/m/e 247</th>
</tr>
</thead>
<tbody>
<tr>
<td>ml of sample</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.6</td>
<td>0.443</td>
<td>1.440</td>
</tr>
<tr>
<td>0.8</td>
<td>0.431</td>
<td>0.712</td>
</tr>
<tr>
<td>0.4</td>
<td>0.461</td>
<td>0.359</td>
</tr>
<tr>
<td>0.2</td>
<td>0.448</td>
<td>0.162</td>
</tr>
<tr>
<td>0.1</td>
<td>0.466</td>
<td>0.092</td>
</tr>
</tbody>
</table>

Slope, 0.903; intercept, -0.00683; $r^2$, 0.9998

<table>
<thead>
<tr>
<th>DiHOEMDP</th>
<th>m/e 244/m/e 245</th>
<th>m/e 237/m/e 245</th>
</tr>
</thead>
<tbody>
<tr>
<td>ml of sample</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.6</td>
<td>0.373</td>
<td>1.204</td>
</tr>
<tr>
<td>0.8</td>
<td>0.365</td>
<td>0.619</td>
</tr>
<tr>
<td>0.4</td>
<td>0.366</td>
<td>0.341</td>
</tr>
<tr>
<td>0.2</td>
<td>0.367</td>
<td>0.153</td>
</tr>
<tr>
<td>0.1</td>
<td>0.377</td>
<td>0.074</td>
</tr>
</tbody>
</table>

Slope, 0.748; intercept, 0.014; $r^2$, 0.9992

1 ml of internal standard (as prepared in Experimental, p. 36) was added.
TABLE XIII. Excretion of Monohydroxy EMDP from Methadone and Methadone-diazepam Treated Rats

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>1-M</th>
<th>1-DM</th>
<th>2-M</th>
<th>2-DM</th>
<th>3-M</th>
<th>3-DM</th>
<th>4-M</th>
<th>4-DM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-2</td>
<td>0.447</td>
<td>3.654</td>
<td>0.961</td>
<td>3.275</td>
<td>2.893</td>
<td>5.092</td>
<td>0.874</td>
<td>4.316</td>
</tr>
<tr>
<td>11-23</td>
<td>6.270</td>
<td>2.966</td>
<td>9.827</td>
<td>5.469</td>
<td>2.848</td>
<td>4.554</td>
<td>5.995</td>
<td>5.454</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>1-M</th>
<th>1-DM</th>
<th>2-M</th>
<th>2-DM</th>
<th>3-M</th>
<th>3-DM</th>
<th>4-M</th>
<th>4-DM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-2</td>
<td>1.293 ± 1.089</td>
<td>4.084 ± 0.797</td>
<td>&lt;0.01</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-11</td>
<td>15.044 ± 4.499</td>
<td>15.534 ± 2.988</td>
<td>&gt;0.20</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11-23</td>
<td>6.235 ± 2.853</td>
<td>4.611 ± 1.177</td>
<td>&gt;0.20</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>22.583 ± 2.951</td>
<td>24.229 ± 2.584</td>
<td>&gt;0.20</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Paired experiments were performed using four rats for methadone treatment (M) and another four rats for methadone and diazepam treatment (DM). The values denote the ratios (ratio/kg rat) of m/e 238 to m/e 247 with average of two determinations. The samples were collected over the time periods 0-2, 2-11, and 11-23 hour after the methadone dose and were monitored by GCMS at m/e 247, m/e 246, m/e 238 and m/e 237. The values in the lower part of the Table represent mean ± SD obtained for each of the two groups. The value p was calculated by using Student's t test.
TABLE XIV. Excretion of Dihydroxy EMDP from Methadone and Methadone-diazepam treated rats

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>1-M</th>
<th>1-DM</th>
<th>2-M</th>
<th>2-DM</th>
<th>3-M</th>
<th>3-DM</th>
<th>4-M</th>
<th>4-DM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-2</td>
<td>1.794</td>
<td>3.302</td>
<td>0.509</td>
<td>2.009</td>
<td>1.840</td>
<td>2.218</td>
<td>0.726</td>
<td>2.028</td>
</tr>
<tr>
<td>11-23</td>
<td>7.079</td>
<td>2.376</td>
<td>11.171</td>
<td>7.526</td>
<td>2.122</td>
<td>5.150</td>
<td>7.243</td>
<td>3.711</td>
</tr>
<tr>
<td>Total</td>
<td>25.383</td>
<td>20.646</td>
<td>22.217</td>
<td>23.695</td>
<td>22.23</td>
<td>21.74</td>
<td>21.48</td>
<td>22.41</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>M</th>
<th>DM</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-2</td>
<td>1.217 ± 0.698</td>
<td>2.389 ± 0.615</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>2-11</td>
<td>14.707 ± 3.404</td>
<td>14.875 ± 2.356</td>
<td>&gt;0.20</td>
</tr>
<tr>
<td>11-23</td>
<td>6.903 ± 3.706</td>
<td>4.690 ± 2.203</td>
<td>&gt;0.20</td>
</tr>
<tr>
<td>Total</td>
<td>22.825 ± 1.783</td>
<td>22.122 ± 1.275</td>
<td>&gt;0.20</td>
</tr>
</tbody>
</table>

Paired experiments were performed using four rats for methadone treatment (M) and another four rats for methadone and diazepam treatment (DM). The values denote the ratios (ratio/kg rat) of m/e 237 to m/e 245 with an average of two determinations. The samples were collected over the time periods, 0-2, 2-11, and 11-23 hour after the methadone dose, and monitored by GCMS at m/e 245, m/e 244, m/e 237, and m/e 236. The values in the lower part of the Table represent mean ± SD obtained for each of the two groups. The value p was calculated by using Student's t test.
Discussion of the methadone-diazepam interaction

Drug interactions are generally shown by analyzing drug and metabolite levels in various biological samples, i.e. plasma, urine, bile and tissues such as brain and liver. The analysis of methadone and metabolites excreted through the biliary route can be used to assess drug interactions at the hepatic level. The drug interaction could depend on the metabolizing activity of the liver, transport of the drug to the biliary route, hepatic blood flow, and drug binding to protein (145). Excretion of metabolites by the urinary route was considered not to be significant to analyze because less than 10% of methadone and metabolites is excreted through the urinary route (144). Methadone levels in rat plasma could not be quantitated even by monitoring the high abundance m/e 72 ion because of a limited analytical sensitivity of our SIM procedure.

The research was designed to examine the applicability of using ratio analysis to the drug metabolism study. Analysis of conjugate metabolites by the method of ratio measurement was expected to provide accurate data on the change in metabolite levels resulting from a concomitant administration of a large dose of diazepam (5 mg/kg) with methadone (10 mg/kg s.c.).

Analysis of EDDP showed the absence of a methadone-diazepam interaction (Table XI). This result was consistent
with a report by Roerig et al. (38) that methadone levels in plasma and methadone and EDDP concentrations in rat urine did not show any methadone-diazepam interaction.

Shah et al. (59) showed in their experiment using mice (20 mg/kg i.p. diazepam and 5 mg/kg i.p. methadone) that plasma and brain levels of methadone were increased by concomitant administration of diazepam. Their result was contradictory to the report of Shannon et al. (60), whose experiment with 10 mg/kg i.p. diazepam and 0.6 mg/kg i.p. methadone failed to show any interaction in mice when plasma and brain levels of methadone were measured.

Since methadone in plasma was not analyzed in this study an attempt was made to calculate an apparent elimination rate constant using EDDP data from rat bile (5 data points) by means of NONLIN (80). A similar calculation of a rate constant using bile samples was reported for tripamide metabolites (146) but in this work reliability of the calculation was found to be heavily dependent on small differences in bile flow with time so that bile data could not be used for pharmacokinetic calculations.

The result obtained by analyzing conjugate metabolites by GCMS was quite different from that reported by Roerig et al. (38), in that water soluble metabolites from methadone in urine and liver were significantly decreased by diazepam administration. Their result indicated that the drug interaction between methadone and diazepam was reflected in a decreased concentration of conjugate metabolites and that
this decrease of conjugate metabolites was indirect evidence
for an increase of methadone levels in brain.

The reason for the transient increase of metabolites
in the initial 2 hour period was not sought but could be
due to competition by diazepam with methadone plasma protein
binding. An effect of diazepam on increasing free drug levels
was indicated in studies of diazepam-diphenylhydantoin (143).
But the effect of an increase of free drug might be minimal
because in the case of the drug which has high hepatic extrac-
tion, drug binding to the plasma is not a critical factor in
the hepatic metabolism (147).

In conclusion, the concomitant administration of
diazepam with methadone in rats did not affect biliary excre-
tion of EDDP and conjugate metabolites. Diazepam did not
interact with methadone at the hepatic metabolism level nor
on the transport of metabolites by the biliary excretion
route. This result is not consistent with that reported by
Roerig et al. (38) in which a decrease of water soluble metabo-
lite levels in urine and liver was considered to indicate
a metabolic methadone-diazepam interaction.
Potential applications of ratio analysis to drug metabolism and pharmacokinetic studies

A biosynthetic internal standard was used in this work to measure relative changes of the conjugated metabolites of methadone due to a concomitant administration of diazepam. This use of a simple measured ratio of a metabolite to its biosynthetic internal standard without knowing the absolute quantity of a substance can be a valuable technique for pharmacokinetic and drug metabolism studies.

This method is especially useful to analyze drug and metabolites for which authentic standards are not available. A stable isotope labeled precursor of the drug produces an ideal internal standard to measure the relative concentration of their metabolites. When the radioisotope - TLC method fails to separate isolated metabolites, SIM using a biosynthetic internal standard is a very convenient method to separate and quantitate the drug and its metabolites.

In addition to this application, many metabolites in the same mixture can be analyzed using an internal standard mixture obtained by using a labeled precursor. This approach is applicable to the biphenyl metabolism studies published by Benford et al. (148) and Halppap-Wood et al. (149).

Pharmacokinetic studies: When we measure the concentration of any drug by GCMS, drug concentration = $a \times$ peak height ratio (unlabeled/labeled) + $b$. In the case of SIM analysis
when using labeled analogs as internal standards, \( b = 0 \) can be achieved by subtracting the background contribution. This was proved in the SIM analysis of methadone and metabolites (Table III). Therefore, drug concentration = \( a \times \) peak height ratio. The optimal dynamic range of the ratio is usually 0.1-10. This range is further adjustable by changing the amount of labeled internal standards. Even if we do not know the value of \( a \), we can use the ratio of unlabeled to labeled for kinetic studies of a drug.

In order to see the applicability of the methodology to a two-compartment model (150), the equation of the model is rewritten as:

\[
R = \frac{C}{a} = \frac{X_0}{aV_C} \left[ \frac{(a - k_{21})}{(a - \beta)} e^{-at} + \frac{(k_{21} - \beta)}{(a - \beta)} e^{-\beta t} \right]
\]

for methadone plasma data reported by Swanson et al. (151). All kinetic constants, \( a \), \( \beta \), \( k_{21} \), \( \frac{X_0}{aV_C} \) are calculable independently of \( a \) with ratios \( R \) at times \( t \) by the NONLIN program (80).

**Kinetic approach to the studies of hydroxylation mechanism:** As an example of the application of ratio analysis to pharmacokinetic studies, this method can be used to study the mechanism of formation of phenol and catechol metabolites. A general scheme for the formation of hydroxy metabolites is shown in the section of ring hydroxylation pathways of methadone metabolism (p. 79). If the metabolites can be analyzed in plasma, the following proposal can be made.
Deuterium labeled drug A produces deuterium labeled monohydroxy (M1) and dihydroxy metabolites (M2). The mixture serves as an internal standard. After collecting a series of samples from drug A which is a mixture of monohydroxy and dihydroxy metabolites at different times, the samples are mixed with equal amount of labeled internal standard (biosynthetic internal standard). After working up the samples, the ratios $R_1$ for M1 (10) and $R_2$ for M2 (11) of the metabolites to the labeled internal standard at different times (6) are obtained.

The best fit for the equation which shows one of the pathways is derived by means of NONLIN program. The equations for the formation of monohydroxy and dihydroxy metabolites by three possible mechanisms, direct insertion, epoxide formation [1] and another epoxide formation mechanism [2] are shown in Table XV.
TABLE XV. Pharmacokinetic equations for the formation of monohydroxy and dihydroxy metabolites

Direct insertion mechanism

\[ A(3) \xrightarrow{k_1} M_1(10) \xrightarrow{k_2} M_2(11) \]

\[
R_1 = \frac{M_1}{a} = \frac{A_0 k_1}{(k_2 - k_1)} \left[ e^{-k_1 t} - e^{-k_2 t} \right]
\]

\[
R_2 = \frac{M_2}{a} = \frac{A_0 k_1 k_2}{k_2} \left[ \frac{1}{k_1 k_2} + \frac{1}{k_1(k_1-k_2)} e^{-k_1 t} - \frac{1}{k_2(k_1-k_2)} e^{-k_2 t} \right]
\]

Epoxide formation mechanism (1)

\[ A(3) \xrightarrow{k_1} M_1(10) \xrightarrow{k_2} M_2(11) \]

\[
R_1 = \frac{M_1}{a} = \frac{A_0 k_1}{(k_1 + k_2)} \left[ 1 - e^{-(k_1+k_2)t} \right]
\]

\[
R_2 = \frac{M_2}{a} = \frac{A_0 k_2}{(k_1 + k_2)} \left[ 1 - e^{-(k_1+k_2)t} \right]
\]

Epoxide formation mechanism (2)

\[ A(3) \xrightarrow{k_1} M_1(10) \xrightarrow{k_2} M_2(11) \xrightarrow{k_3} \]

\[
R_1 = \frac{M_1}{a} = \frac{A_0 k_1}{(k_1 + k_2 + k_3)} \left[ e^{-k_3 t} - e^{-(k_1+k_2)t} \right]
\]

\[
R_2 = \frac{M_2}{a} = A_0 \left[ 1 + \frac{k_1}{k_3 k_1 - k_2} e^{-k_3 t} + \frac{(k_2 - k_3)}{k_3 k_1 - k_2} e^{-(k_1+k_2)t} \right]
\]
SUMMARY AND CONCLUSIONS

1. Friedel Crafts reaction of bromophenylacetonitrile with benzene-$^2$H$_6$ gave diphenylacetonitrile labeled in both phenyl rings. The labeling process was found to be a combination of aluminum chloride catalyzed exchange between benzene-$^2$H$_6$ and the ring protons of either phenylacetonitrile or diphenylacetonitrile and of the reversible nature of the Friedel Crafts reaction.

2. Methadone-$^2$H$_{10}$ was synthesized from enriched diphenylacetonitrile-$^2$H$_{10}$. The metabolites, EDDP-$^2$H$_{10}$, EMDP-$^2$H$_{10}$, and DDP-$^2$H$_{10}$ were synthesized from 4-dimethylamino-2,2-diphenylpentanoic acid-$^2$H$_{10}$ prepared using D$_2$SO$_4$ to maintain label enrichment. Methadone-$^2$H$_3$, EDDP-$^2$H$_3$, and EMDP-$^2$H$_3$ were synthesized using C$_2$H$_3$CH$_2$Br as starting material. All compounds except for methadone-$^2$H$_3$ are new compounds.

3. Methadone and its metabolites fragmented using EI mass spectrometry to give ions common to diphenylbutane-containing compounds. These were useful to identify new metabolites of methadone. Mass spectra of the deuterium labeled analogs provided confirmation of the ion structures. Aryl ring migration was observed in the fragmentation processes for EDDP.
4. Methadone levels in plasma and saliva were analyzed by monitoring m/e 72 with 2-dimethylamino-4,4-diphenyl-5-nonanone perchlorate as the internal standard. The lower limit of reproducible quantitation of methadone in 0.5 ml of plasma or saliva taken for extraction was 20 ng. The mean ratios of saliva to plasma for two patients were 0.55 ± 0.15 (SD) and 0.48 ± 0.10 (SD).

5. Analysis of methadone and EDDP in patients' urine showed that the calibration equations prepared for the analysis were consistent with those calculated by using the ratios of equal amount of drug to internal standard (standard ratio), indicating that the concentration of the compounds can be analyzed without constructing calibration curves.

6. The deuterium labeled analogs proved to be very useful for the GCMS detection and identification of methadone metabolites. The conjugated fraction from rat bile was found to contain a new m/e 72 containing compound. On the basis of GCMS data the new metabolite was assigned the structure, N-methylene-1-methyl-3,3-diphenyl-4-oxo-hexamethamine oxide (methadone nitrone). The nitrone appeared to result from decomposition of N-hydroxynormethadone during work up procedures. The detection of the nitrone implies that methadone is not all spontaneously cyclized to EDDP at the site of enzymatic N-oxidation.

7. Indirect evidence for the structure of the new metabolite was obtained from chemical oxidation of EDDP perchlorate
with m-chloroperbenzoic acid in which 4,4-diphenyl-2,5-heptanedione and the methadone nitrone were obtained as products. Mass spectral data, NMR, and IR were used to identify these compounds. A mechanism for the formation of nitrone and the diketone from oxidation of EDDP perchlorate was proposed.

8. Three processes for the EI fragmentation of methadone nitrone were proposed. The proposed fragmentation processes were also found to apply to amphetamine nitrone.

9. Air or chemical oxidation of EDDP base gave DDP as the main product and the evidence suggests that DDP is not a true metabolite of methadone. MCPBA oxidation of EMDP base gave 2-ethyl-5-methyl-3,3-diphenylpyrrolidyl-1,2-oxazirane.

10. The feasibility of using biosynthetic internal standards and SIM was derived for drug metabolism and kinetic studies.

Drug concentration = slope x ratio of drug to internal standard + intercept, where the intercept is zero and the slope is calculable using the standard ratio which is a drug dependent value. Therefore, ratio = drug concentration/a (drug dependent constant value). The change of drug concentration is reflected on the ratio. Pharmacokinetic constants can be obtained by using these ratio values.

11. A methadone-diazepam interaction study in rats was designed in which methadone, EDDP, and EMDP were analyzed by SIM using deuterium labeled analogs as internal standards and conjugated metabolites using deuterium labeled biosynthetic internal standards. The deuterium labeling of the internal standards was found to be stable over a wide range of pH and
under conditions of prolonged incubation. A significant in­
crease in the excretion of conjugated metabolites was found ... 
during the first 2 hours of bile excretion in the methadone-
diazepam treated rats. When the excretion of conjugated 
metabolites was followed over the 23 hours period, there 
was no difference observed between the methadone only treated 
and methadone-diazepam treated rats. The reason for the 
transient increase of the conjugated metabolites in the ini­
tial 2 hour period could be due to the competition by diazepam 
with methadone plasma protein binding. Diazepam did not 
interact with methadone at the hepatic metabolism level nor 
on the transport of metabolites by the biliary excretion 
route.
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APPENDIX

NMR SPECTRA OF DEUTERATED COMPOUNDS
1

Partially deuterated diphenylacetonitrile-$^2$H$_{10}$
Solvent, CDCl$_3$; 86\% labelling.

2

Enriched diphenylacetonitrile-$^2$H$_{10}$
Solvent, CDCl$_3$; 98\% labelling
3. Methadone nitrile-$^2\text{H}_{10}$
   Solvent, CD$_3$OD; 97% labelling.

4. 4-Dimethylamino-2,2-diphenylpentanoic acid-$^2\text{H}_{10}$
   Solvent, CDCl$_3$; 97% labelling.
5. Methadone-$^2\text{H}_{10}$

Solvent, CD$_3$OD; 97% labelling.
6. DDP-\textsuperscript{2}H\textsubscript{10}
   Solvent, CDCl\textsubscript{3}; 97% labelling

7. EMDP-\textsuperscript{2}H\textsubscript{10}
   Solvent, CD\textsubscript{3}OD; 96% labelling.
8. EDDP$^{-2}_3$H$_3$
Solvent, CDCl$_3$; >99% labelling

9. EDDP$^{-2}_3$H$_{10}$
Solvent, CD$_3$OD; 96% labelling.