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The biotransformation of ketamine and its enantiomers

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THE BIOTRANSFORMATION OF KETAMINE AND ITS ENANTIOMERS

by

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DISSERTATION

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DISSERTATION ABSTRACT

The search for an analytical procedure for the quantitation of the optical isomers of ketamine started with the testing of various proline derivatizing reagents. (S)-N-Pentafluorobenzoylprolyl imidazolide was found to be inactive in the derivatization of both racemic ketamine and norketamine. (S)-N-Trifluoroacetylprolyl chloride successfully acylated norketamine, but it gave unequal diastereomeric yields with ketamine. A new reagent was developed, (S,S)-N-trifluoroacetylproline anhydride. The reaction characteristics of the anhydride were examined in an attempt to develop a quantitative GC assay of the enantiomers of the sterically hindered, chiral amine ketamine. With the aid of the individual enantiomers of ketamine and the corresponding synthetic N-trifluoroacetylprolyl amides, it was found that the derivatization reaction proceeds stereoselectively, in poor yield, and with some degree of racemization of the acylating reagent. The results indicate that care must be exercised when prolyl derivatizing reagents are chosen for assaying chiral amines.

The next step was to establish the in vitro metabolic fate of racemic ketamine as evaluated using microsomal preparations from rat liver. With the development of a rapid, non-selective metabolite extraction procedure and sample analysis by combined glass capillary gas chromatography-low (and high) resolution mass spectrometry, eight metabolites of the drug were identified, six of which have not been documented previously. The novel metabolites were products of alicyclic ring hydroxylation of ketamine and of N-desmethylketamine (norketamine). These metabolites are 5-hydroxyketamine, 6-hydroxyketamine, 6-hydroxy-

norketamine, two diastereomers of 5-hydroxynorketamine, and 4-hydroxynorketamine. Semiquantitative analysis of metabolites produced during microsomal incubation was achieved using glass capillary gas chromatography. The results from this study indicate that 5,6-dehydronorketamine, previously considered to be a major biotransformation product of ketamine in mammalian systems, is almost certainly a methodological artefact.

Finally, the metabolic fate of the enantiomers of ketamine and pseudoracemic mixtures were examined in rat and human liver microsomes. In rat experiments, S-ketamine produced S-norketamine, 5-hydroxyketamine and two diastereomers of 5-hydroxynorketamine. R-Ketamine formed R-norketamine, 6-hydroxyketamine, 6-hydroxynorketamine and 4-hydroxynorketamine. Human liver microsomes demonstrated similar results to the rat for R-ketamine, however from S-ketamine only S-norketamine and one isomer of 5-hydroxynorketamine were detected. The kinetic parameters of N-demethylation of the enantiomers were determined in the rat system. S-Ketamine possess a greater K_M and V_{max} than the R-isomer. Enantiomeric mixture experiments as well as pseudoracemic mixture studies demonstrated a chiral interaction in the N-demethylation of ketamine which favors the production of R-norketamine.

CHAPTER I. The Metabolism and Pharmacology of Ketamine and its Enantiomers: A Literature Review

When phencyclidine (PCP, Figure I.1) was first subjected to clinical studies in the late fifties, it was soon realized that the agent was not satisfactory for anesthetic use in man.[23] Its undesirable effects in humans include: convulsions, postoperative mania and delusions, inadequate anesthesia and persistent nystagmus.[60] Anesthesiologists were in need of a safe phencyclidine congener that would provide the desirable qualities of the agent. These qualities are: rapid onset of action, little cardiovascular and respiratory depression, and intravenous or intramuscular administration. Structural analogs were quickly developed to replace it. The most promising analog was ketamine, dl 2-(o-chlorophenyl)-2-(methylamino)cyclohexanone hydrochloride. Ketamine was synthesized in 1961 by the route shown in Figure I.2, and by 1965, clinical studies were already being reported.[17,21,22] Ketamine provided the desirable attributes of PCP as well as short duration of action, potent analgesia and less unpleasant emergence phenomena than associated with phencyclidine.[60]

Dissociative Anesthesia

The anesthetic state produced by PCP analogs is called dissociative anesthesia.[21] The state is characterized by open eyes with transient nystagmus, catalepsy, intact laryngeal and deep tendon reflexes, spontaneous breathing, amnesia, analgesia, coma, clonic movements, and muscular hypertonicity.[22] The intact reflexes prevent aspiration and reduce the need for endotracheal intubation. Muscle relaxants such as d-tubocurarine may be required for some procedures.[22] The most serious problem is that analgesia may not be adequate for abdominal and other equally painful operations. This creates the need for adjunct anesthetics such as nitrous oxide.[17]

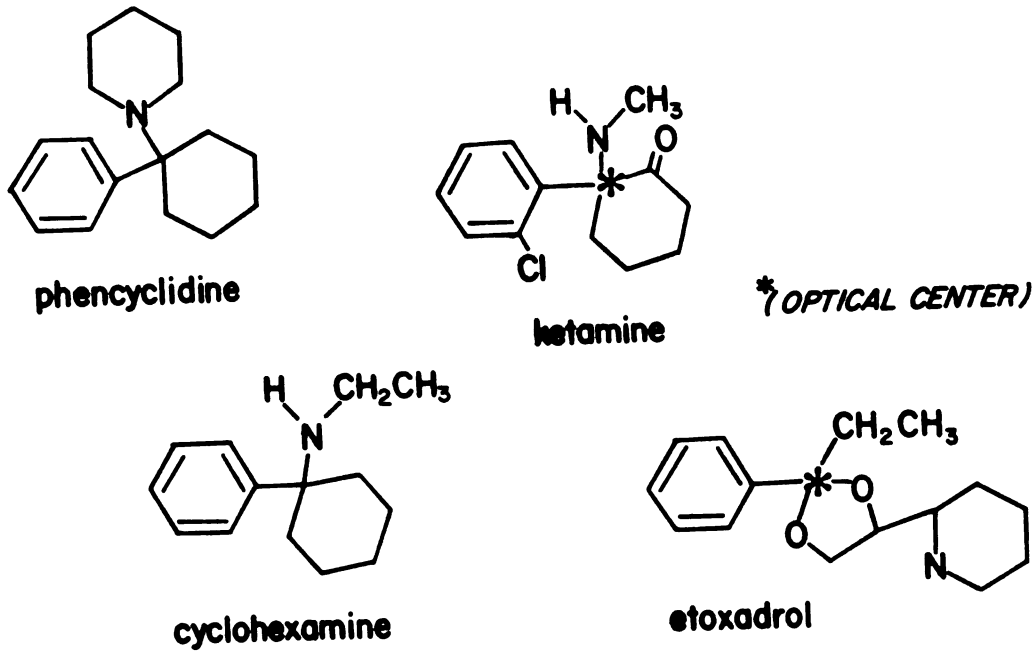


Figure I. 1 Dissociative Anesthetics

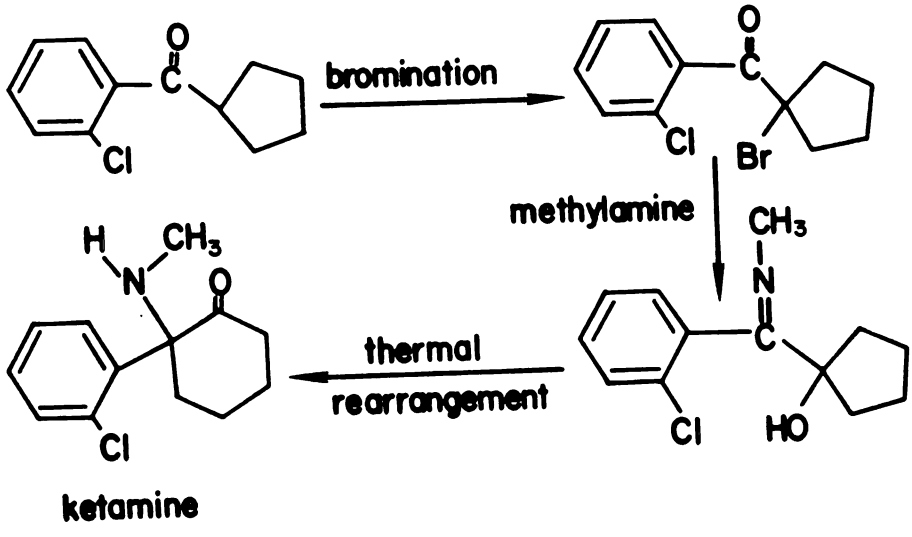


Figure I. 2

Ketamine was subjected to clinical trials in 18,000 patients before it was released for general use.[60] It gained in popularity quickly in the United States and Europe.[23] This enthusiastic use has now greatly diminished in this country due to unpleasant dreams or delusions upon emergence.

The search for substitute phencyclidine congeners continues in the hope of finding one with the desired effects of ketamine without the ensuant delusions. Besides phencyclidine and ketamine, other dissociative anesthetics that have been developed are cyclohexamine and etoxadrol (Figure I.1). These contain an aryl cycloalkylamine or a piperidinyl nucleus. Phencyclidine is a potent agent that is still used in veterinary procedures. Cyclohexamine has undergone a few clinical trials which demonstrated its unacceptability as an anesthestic in man. Inadequate anesthesia, emergence delirium and a high incidence of hallucinations were characteristic of its central nervous system actions.[60] Etoxadrol is now undergoing clinical trials.[72] In the rat, it causes a longer duration of hypnosis and posthypnotic hyperactivity (which may be analogous to emergence sequelae in man) than ketamine.[71,72] It also has a peculiar morphine-like characteristic of causing rigid tails in rats. The drug is used as the purified dextrorotatory isomer of etoxadrol hydrochloride. Ketamine is the most satisfactory agent tested so far because of the low incidence and short duration of hallucinations, rare convulsions and mania, profound analgesia, and adequate anesthesia.[60] It is administered as a racemic hydrochloride salt. It is possible that anesthesia with racemic ketamine may produce more side effects than the individual isomers will produce. Therefore,

pure enantiomeric preparations have been developed and tested in animals and man. A discussion of the findings will be presented later.

Pharmacology of Ketamine

Ketamine is a short duration, rapid onset, injectable general anesthetic.[22] It is a secondary amine with a pK_a of 7.5[15], melting point of HCl salt of 259°C,[17] and pressure reversibility. The drug is chiral and has been examined most frequently as the racemate. The general effects seen are hypnosis, catalepsy, coma, amnesia, hypertension, transient respiratory depression, sweating, lacrimation, salivation, intact reflexes, no increase in total peripheral resistance, and conversion of EEG alpha waves to theta waves.[17,21,22,77,81] Hallucinations are also common after operation, and may be seen in as many as 25% of all patients.[73] Children are reported to have less incidence of hallucinations after ketamine than adults.[24,73]

The obvious preponderance of central nervous system effects of ketamine has sparked a search for the areas of the brain that are involved. Anesthesia (coma) is undoubtedly due to a sensory input blockade at the association area of the cortex.[24,60] The spine and brainstem are probably not involved.[24] Proprioception is depressed but visual and auditory functions are less affected.[60] The disjunction of sensory input and central association is called "dissociation".[60]

Notably, ataxia is a centrally mediated effect of ketamine. This has been demonstrated since the agent causes no depression of muscle contractions in the isolated rat phrenic nerve, diaphram preparation.[51] Lack of a peripheral effect on the muscle or neuromuscular junction implies that ataxia is either mediated centrally in the spinal cord,

or afferently. Hypertension also appears to have a central component since it can be prevented by epidural nerve block prior to ketamine injection.[76] Another central phenomenon of ketamine is an opiate-like increase in locomotor activity in mice that is blocked by naloxone.[30] Blockade by naloxone may indicate that ketamine causes release of endogenous peptides (such as endorphins) in the brain or interacts directly with opiate receptors.

Central nervous system effects of ketamine are for the most part dependent upon nerve cell uptake of the drug.[68] The highly lipophilic drug should penetrate into the neuron quickly as the uncharged secondary amine. Within the slightly acid cytoplasm[15] it will exist largely in the charged form. It is the uncharged form which has been shown to be active in squid giant axons.[68] While in the nerve the drug decreases sodium and potassium conductance. Such a block in sodium conductance is similar in action to local anesthetics. The proposed mechanism of ketamine narcosis is an increase in intracellular sodium.[67,68]

In addition to general depression of nerve cell depolarization, ketamine may have interactions with CNS receptors. Early work on this problem involved ketamine assays with various convulsants.[14] Ketamine increases iproniazid induced central excitation as do the sympathomimetics. It has no activity against strychnine or pentylenetetrazol produced convulsions though. GABA receptor interaction and sedative hypnotic action therefore seem to be ruled out. Methiothepin, an antiserotonergic, was found to prolong ketamine hypnosis in cats.[28] An antidopamine agent (pimozide) decreased purposeless motion during coma.[28] Procyclidine, a central anticholinergic, also

exacerbated anesthesia.[28] These results may be due to additive effects of the drugs used or to mechanisms in no way related to the action of ketamine. The suggestion exists nonetheless that ketamine may interact with serotonin, dopamine and acetylcholine receptors in some direct or indirect manner. There have also been studies to investigate neurotransmitter turnover rates in the ketamine treated rat brain. The findings are that ketamine increases dopamine[72] turnover and decreases acetylcholine[59] and serotonin[72] turnover rates. This lends additional support to the idea that ketamine interacts with serotonergic, dopaminergic and cholinergic receptors.

Maayani et al[48,61,62,63,84] have postulated that PCP and its analogs are anticholinergic agents. This group studied the inhibition of AChE and BuChE as well as mydriatic activity in rodent eyes and spasmodic activity in guinea pig ileum smooth muscle assays produced by PCP and eleven aromatic substituted analogs. Inhibition of cholinesterases was potent and binding occurred at the active site, but there was no correlation between K_i and rotarod activity. Mydriasis proved to correlate with the CNS activity of the analogs much better than did smooth muscle contracture. Based upon the correlation between mydriatic and rotarod activity of this series, it was postulated that PCP acts as an anticholinergic agent. Elaborate quantum mechanical models were later established to show the compatibility of the interaction between PCP and the cholinergic receptor.

Ketamine has been found to interact with the opiate receptor in rat brain.[69] Ketamine has an IC_{50} in the range of antagonists, however, the reduction in naloxone displacement in the presence of so-

dium suggests that ketamine may be an agonist. It was also found that S-ketamine binds the receptor with a three times lower IC_{50} than R-ketamine. A structure activity study for phencyclidine analogs and the opiate receptor has been reported.[70] Although a clear correlation between biological activity and opiate receptor binding was not found, the rank order of receptor binding was similar to the rank order of rotarod and discriminative stimulus tests. It was of some interest that two PCP metabolites, the 4-cyclohexanol and the 4-hydroxypiperidine were not much less active than the parent compound.

A new class of analgesics has been found which is structurally related to PCP and ketamine.[40,82] The most potent of these are cyclohexanol derivatives which are named 4(m-hydroxyphenyl)-4-(dimethylamino)-1-alkylcyclohexan-1-ols. Analogs which have p-chlorophenyl substituents instead of m-hydroxyphenyl are also very potent. The alkyl functionality is trans and bears a phenyl ring. These compounds are more potent than morphine in displacing naloxone from the opiate receptor yet they are not opiate antagonists. More recently, Kalir's group has synthesized some analgesic PCP analogs that are substituted on the piperidine ring.[31] A compound was reported which is about twice as potent as morphine in the writhing test. This was 1-(1-phenylcyclohexyl)-4-phenyl-4-piperidinol, a hydroxylated PCP congener. The opiate receptor data for PCP analogs indicates that hydroxylated analogs retain CNS activity. This is strong evidence for active metabolites of both phencyclidine and ketamine.

A specific binding site in rat brain has been described for PCP.[78,80,92] This site is a saturable, noninteracting species and it does not bind molecules which are not PCP analogs (except N-allylnorcyclazocine). Receptor binding does not correlate well with

rotarod acitvity, it does correlate with discriminative stimulus tests, however. There are also PCP receptors in liver and kidney, but the binding activity at these sites does not correlate with biological activity. The specific brain receptors have been reported to be due to artifacts of the rapid filtration technique,[48] but this was later rebutted by Vincent et al.[79]

Ketamine Toxicity

Acute toxicity studies have all reported rather low lethality for ketamine. Mice seem to be less susceptible to the drug than rats with LD_{50} values of 616.6 mg/kg (PO), and 223.9 mg/kg (IP) for mice and 446.7 (PO), 223.9 (IP) and 58.9 (IV) for rats.[35,53] Differences between age groups have been found with respect to LD_{50}. Older mice (LD_{50} = 229 mg/kg [IP]) are more susceptible than neonates (LD_{50} = 275 mg/kg [IP]).[35] The opposite was found for rats where neonates (LD_{50} = 146 mg/kg [IP]) are more susceptible than young adults (LD_{50} = 248 mg/kg [IP]).[35] Subchronic studies have provided valuable data concerning the toxicity of ketamine. Rats dosed daily for six weeks with anesthetic doses showed no change in recovery time or organ system toxicity at necropsy.[35] Dogs subjected to a similar procedure demonstrated a decrease in recovery time and some liver necrosis.[35] The findings were hepatocellular vacuolization in the central vein region of the liver lobule with an increase in blood cholesterol, SGOT and SGPT indicative of possible hepatocyte death.[35,53] No organ system toxicity was discovered in monkeys given a similar regimen.[35,53] Leukocytosis and increased blood glucose and sedimentation rate were nonspecific findings.[35,53] The overall therapeutic safety of ketamine (therapeutic index) has been determined in rats as the ratio

of the dose causing serious side effects to anesthetic threshold dose (TI = 15) and also as the minimum lethal dose divided by the anesthetic dose (TI = 16). This assay was employed to compare ketamine with other injectable anesthetics. Ketamine was determined to be a safer agent than pentobarbital and thiamylal, and it was not distinguishable from phencyclidine.[53]

Differential enantiomeric toxicity has also been reported with ketamine. The (S) isomer has an LD_{50} of 35 mg/kg (IV) in the rat while the (R) form was 41.5 mg/kg (IV) and racemic ketamine was 35 mg/kg (IV).[50] Therapeutic indices calculated as LD_{50}/ED_{50} were: (R), 4.0; (R.S), 6.3; and (S), 10.0. The (S) isomer is therefore the more potent form and it has more of a margin of safety.

Biodisposition and Pharmacokinetics of Ketamine

The earliest study of ketamine metabolism was performed by Chang and Glazko.[11] Using *in vitro* systems, four metabolites were proposed (fig. I.3), although the only metabolites to be absolutely identified by chemical synthesis were MI (norketamine) and MII, the other metabolites were postulated based on evidence discussed later. In rat liver homogenates supplied with Mg^{+2}, NADPH and NADP, ketamine was converted to MI and MIII. There was no MII found as other studies have verified.[15,16,43,51] One study did find MII *in vitro*[87] for uncertain reasons. Monkey liver homogenates were capable of forming MI from ketamine and MII from MI. In figure I.3, reaction 1 is a cytochrome P450 oxidation with the mechanism shown in Figure I.4. The enzyme requires NADPH, NADPH-cytochrome P450 reductase, and O_2. Alkyl hydroxylation (3) is also a cytochrome P450 oxygen insertion reaction. Glucuronyl transferase catalyzes the formation of beta

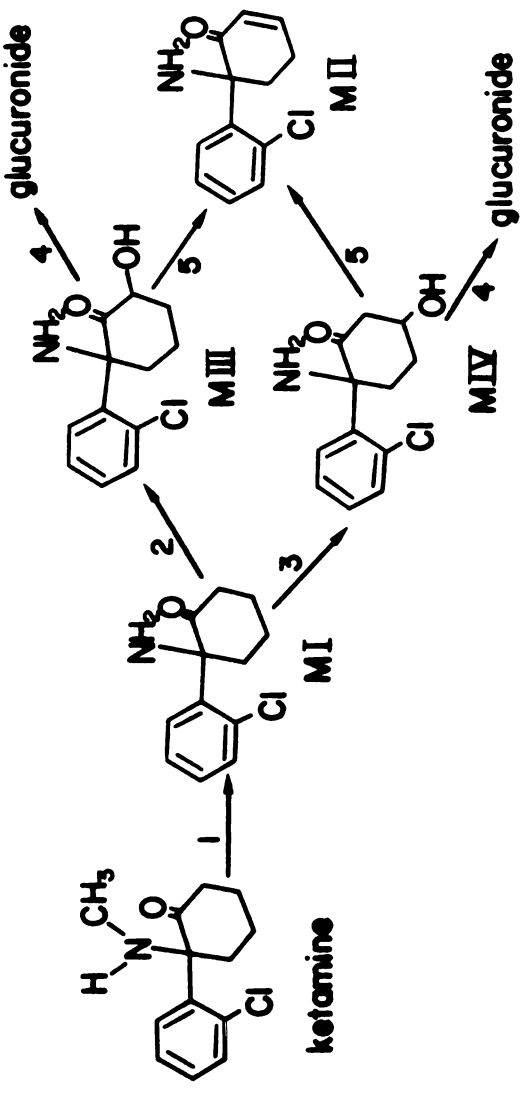


Figure I.3 Chang and Glazko metabolic pathway

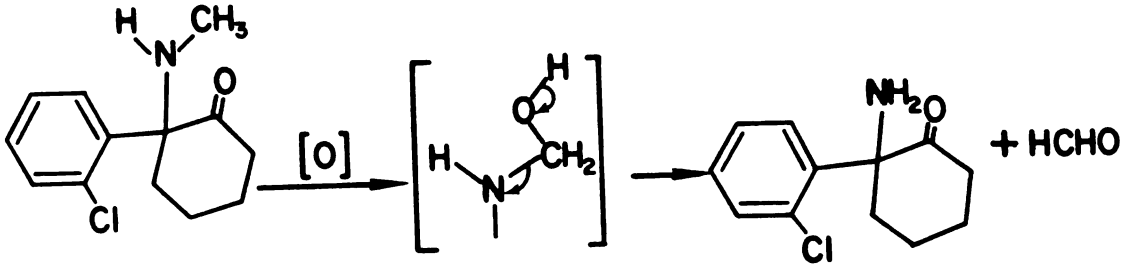


Figure I.4

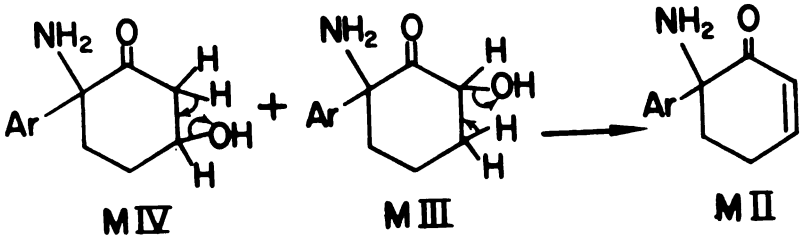


Figure I.5

glucuronides in the presence of UDPGA(4). Reaction 5 is either spontaneous or dehydrase catalyzed. The spontaneous mechanism is shown in Figure I.5. This type of uncatalyzed dehydration has been reported for a polycyclic compound.[6] MIV would be expected to convert to MII more quickly than MIII, due to the acidic nature of the alpha carbonyl protons. It should be mentioned that another possible route of metabolism of ketamine involves initial hydroxylation followed by N-demethylation and dehydration to produce MII as before.

Numerous in vivo metabolic studies of ketamine have analyzed plasma and urine and yielded four metabolites. In the rat, plasma samples one hour after dosing showed a preponderance of MIII with some MI and MII and no parent compound.[11] All metabolites were found in the liver and brain, and MIII was the major component of urine extracts. In monkey plasma, ketamine, MI, MII, MIII and MIV were detected. Monkey urine contained all of the metabolites seen in plasma, and acid hydrolysis of the urine resulted in an increase in detectable MIII. Bile also contained MIII and MIV (seen after acid treatment), leading the authors to propose an enterohepatic cycling of metabolites. Presumably, conjugated metabolites are secreted into the bile and then to the intestine. In the gut, the conjugates are cleaved and free metabolites are returned to the liver via the portal veins. Ketamine is also excreted into the feces. This route is unimportant in man and monkeys, for it accounts for less than 5% of the total dose administered. Rats may excrete as much as 30% of the drug metabolites in their feces. Tritiated ketamine given to man was quickly converted to MI and MII in the plasma. Acid labile conjugates appeared in the urine (as much as 80% of excreted forms)

which were hydrolyzed to MIII and MIV. The major free metabolite from man in urine and plasma appeared to be MII. Unknown polar metabolites in urine (unhydrolyzable) were also found to account for 20% of the injected tritium-labeled ketamine. In general, urinary excretion is the major means of ketamine metabolite elimination in all species studied, and unaltered ketamine is a minor excretion product in all animals.

The nature of ketamine metabolites has hitherto been investigated solely by Chang and Glazko.[11] Metabolites isolated by TLC and countercurrent extraction were compared to synthetic compounds by GC and TLC. In this manner, MI and MII were identified. Conjugates in the urine were identified as glucuronides of MIII and MIV due to their acid lability and cleavage by beta glucuronidase. The precise nature of MIII and MIV was not known since attempts to synthesize their possible forms were unsuccessful. Functional group analysis of undescribed nature of MIII demonstrated a hydroxy group alpha to the cyclohexanone carbonyl. Treatment with alkali converted MIV to MII (produced a double bond adjacent to the carbonyl), a characteristic of a beta carbonyl hydroxy group. Both MIII and MIV have been characterized only in TLC systems and are not apparent on the standard GC traces. So far, no evidence has been developed to dispute their proposed structures, however there are some problems that should be resolved before a definitive conclusion can be reached. MIII and MIV have to be adequately characterized chemically and pharmacokinetically. This may clarify the possible role of MIII and MIV as intermediates between MI and MII. The problem will have to be resolved using TLC, HPLC or mass spectrometry techniques due to the unstable nature of

MIV. In a hot environment (e.g. a GC injection port) this metabolite may spontaneously dehydrate and appear on the trace as MII. This mechanism may explain why some studies found MII after in vitro ketamine oxidation in rat liver microsomes. The hydroxylated metabolites will also have to be synthesized in order to provide authentic standards for the absolute identification of their structures. A generalized organ system scheme of ketamine biodisposition is proposed in Figure I.6. Metabolism is carried out for the most part in the liver. However, the gut and lungs may also be a source of significant amounts of metabolic enzymes. Dehydrase may be present in the gut wall which is capable of converting MIII and MIV to MII. This is especially pertinent in rats where MII is not reported to be formed by liver microsomes but is found, instead, in vivo in the plasma.[11]

Mass spectral studies have reported finding ketamine, norketamine, and dehydronorketamine in mouse, rat, dog, monkey and human blood and urine.[18,37,39] Metabolic products were extracted in the presence of strong base. In one study,[39] the samples were derivatized with heptafluorobutyric anhydride whereas the other studies assayed underivatized samples.[18,37] Since extraction with strong base and analyzing underivatized samples are two known methods of dehydrating hydroxylated metabolites, this is a possible explanation of why none of these studies found the proposed MIII or MIV. Fragments seen by GC-MS were identified as to chemical nature and a selected ion monitoring technique was developed for the quantification of metabolites.[39]

Three experimental approaches to the analysis of ketamine apearance and disappearance in an organism have been employed. The first

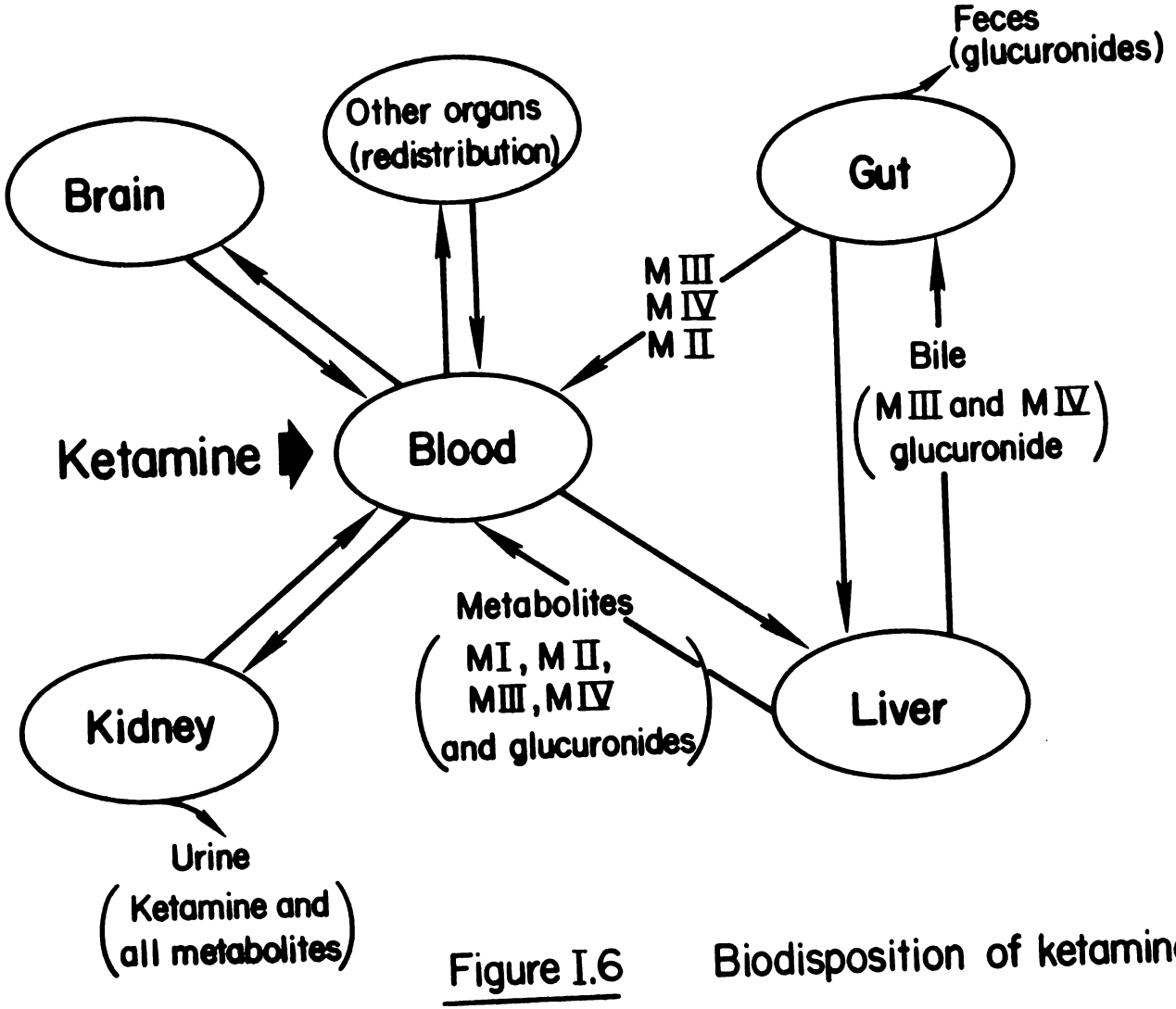


Figure I.6 Biodisposition of ketamine

involves a fluorometric analysis of ketamine and its first and second metabolites (MI and MII).[19] Radioactive (tritium) labeled ketamine has also been given to animals and man to analyze ketamine, MI and MII blood levels.[11,12] The most specific method is gas chromatographic analysis with identification of ketamine and each metabolite.[11,15] Fluorometric analysis of ketamine levels is of little use and has not been reported in recent references.[11,19,41] The first experiments with tritiated ketamine were done in dogs and monkeys.[11] In both species after I.V. or P.O. doses, an early high level of tritium activity (ketamine) rapidly diminishes, then a second peak develops. The second peak is composed of MI and MII.[11] An analogous two-peaked elimination curve has been reported in man dosed with radioactive ketamine.[12] Using gas chromatographic analysis of human plasma, the levels of free ketamine were shown to be biphasic.[41,88] Starting at initial high levels there is a quick drop (alpha phase) with a $t_{1/2}$ of 11 to 18 minutes. This soon changes to a slowly declining or beta phase ($t_{1/2}$ = 2.52 hours).[41,88] The alpha phase is assumed to be due to redistribution from the brain to less perfused organs. This is a possible mechanism of terminating ketamine anesthesia since the alpha phase and anesthesia have the same duration.[88] The beta phase is probably indicative of metabolism and excretion of ketamine from the body.

The kinetics of MI and MII have also been investigated in man after IV doses of ketamine.[11,41,88] MI appears at the end of the alpha phase of ketamine and rises to levels higher than ketamine.[11,41,88] It is then eliminated with a $t_{1/2}$ of 4.0 hours.[88] MII is evident at times after MI appears and levels of MII rise quickly to plateau

levels in the blood. These plateau levels may be higher or lower than MI plateau levels depending upon the clinical study.[11,41,88] Slow excretion follows with a half-life of 6.8 hours.[88] Volumes of distribution reported for ketamine are greatly in excess of plasma volume at all times.[34,66] This is perhaps due to quick tissue penetraton, since there is no significant plasma protein binding by the drug.[11,88]

Brain levels of ketamine have been examined in rats.[9,11,15,16,51] Due to the lipophilic nature of the drug, (benzene/water partition coefficient of 98 or 5 to 10 times greater than thiopental)[16], there is a rapid uptake and concentration in the brain.[15] Cohen et al[15] found a 6.5/1 brain to plasma difference five minutes after injection. Rapid brain uptake is partly due to an increase in cerebral blood flow caused by ketamine. Active transport is not a mechanism of uptake since 2,4-dinitrophenol and other metabolic inhibitors did not decrease brain levels in cerebral cortex slices. Within the brain, the highest concentrations of the anesthetic were in the cortex. MI was also studied in cerebral cortex slices. It has a benzene to water partition coefficient of 58, is concentrated in the brain 2.5/1 compared to plasma, and does not have a particular brain region in which it is concentrated.

Redistribution from the brain to other organs has been investigated as a mode of termination of ketamine anesthesia in the rat.[11,51] Near the end of the hypnosis phase, there occurs a quick rise in ketamine concentrations in muscle, fat, skin, gut, liver, kidneys and heart.[11,51] Tissue levels then rapidly fall except for liver and kidneys which remain high for hours.[11] MI has a similar pattern of

organ penetration.[51]

Metabolism of ketamine is a second major mechanism of termination of action. Indirect methods have been used to demonstrate the importance of metabolic degradation of ketamine, such as liver enzyme induction and inhibition studies. Induction studies have been performed treating rats with phenobarbital[16,49,51] or ketamine itself[42,43,49,51] in order to induce liver microsomal enzymes. Phenobarbital or ketamine pretreatment did not affect duration of hypnosis, although ataxia, analgesia, and hyperactivity were significantly reduced. One study did show a decrease in hypnosis in ketamine-pretreated animals. No pretreatment was able to effect plasma levels of ketamine during hypnosis,[11] but later levels were decreased by phenobarbital[51] and ketamine induction.[42,43,51] Metabolite levels (MI) were also decreased by enzyme induction.[51] Microsomal *in vitro* analysis after phenobarbital[16,51] and ketamine[43,51] pretreatment demonstrated an increase in MI formed from ketamine. The low *in vivo* levels of MI imply that there must be a stimulation of further oxidizing steps or an acceleration of alternate pathways of metabolism. SKF-525A inhibits microsomal enzymes and decreased ataxia,[16,51] analgesia,[51] and hyperactivity[16,51] while increasing plasma levels of ketamine and MI.[16,51] Ketamine induction of microsomal enzymes was found to be similar to the induction produced by phenobarbital; cytochrome P450 and its reductase are both induced.[49] In general, hypnosis appears to be terminated by tissue redistribution while ataxia, analgesia and hyperactivity are terminated by liver metabolism.

Urine acidification was found to decrease the excretion of ketamine and its metabolites, and alkalinizing the urine increased the

elimination of these compounds in rats.[45] This is the opposite of what is expected for an amine bearing drug, however it should be noted that animals with acidic urines have prolonged sleeping times.

Age and sex differences have been reported in the metabolic oxidation of ketamine in rats.[83] Sleeping time decreases and dehydronorketamine production increases from 1-16 weeks. Furthermore, at four or more weeks of age the male has a shorter sleeping time and produces more dehydronorketamine than the female.

In summary, pharmacokinetic models indicate that the metabolism of ketamine may be a deactivating process.[88] While analgesia and hypnosis are terminated by redistribution, posthypnotic hyperactivity appears to be stopped by metabolism and excretion. However very little is known about the activity of the proposed hydroxylated metabolites. These metabolites would be expected to appear in the plasma of patients about the same time as the delusions are observed. The recent structure-activity work done on the analgesic PCP analogs[31] demonstrates that hydroxylated PCP-like species can possess great activity in the central nervous system. It is possible that hydroxylation steps produce active metabolites and then glucuronidation is a deactivating process.

Pharmacology of the Enantiomers of Ketamine

Differential pharmacological activity of enantiomers is not an unknown phenomenon.[8,27,64,66] Ketamine has been found to possess such differential activity. Marietta et al[50] were first to investtigate the activity of the enantiomers. They found that in the rat the dextrotatory or S-isomer causes more ataxia and hypnosis (loss of righting reflex) than the levorotatory or R mirror image. Equi-

hypnotic doses of the two were also equiataxic. The measured ED_{50} values for hypnosis for (R), (R,S), and (S) were: 10.3, 5.6 and 3.5 mg/kg (I.V.). So, the (S) form is three times more potent than the (R) isomer. Locomotor activity was most sensitive to the (R) isomer, implying that the (S) enantiomer may be less psychotropic (this is because a correlation exists between hyperactivity in rodents and psychotropic potency in man[32,33]). Lethality was also assayed with the (R) form being less toxic than the (S) isomer. The LD_{50} values found were: (R), 41.5; (R,S), 35.0; (S), 35.0 mg/kg (I.V.). Therapeutic indices calculated from these data demonstrate the safety of the (S) isomer. The values are (R), 4.0; (R,S), 6.3; and (S), 10.0. Ryder et al[65] studied the effects of the isomers in mice. Analgesia produced by ketamine was measured by the phenylquinone writhing test. Resultant ED_{50} estimates produced were: (R), 11.0; (R,S), 6.5; and (S), 3.7 mg/kg (S.Q.). The (S) isomer is approximately three times more potent an analgesic than the (R) form. Hypnosis was also measured with the finding that the (S) is 1.5 times more active than the (R) isomer. Subcutaneous administration of ketamine was an inadequate route for determining LD_{50} due to volume restrictions for subcutaneous injections. Locomotor activity studies demonstrated that the (S) form was 1.8 times more active than the (R). An estimate of therapeutic efficacy was obtained by observing locomotor activity at equianalgetic doses. The (S) isomer produced much less hyperactivity than the (R) form. The results of these first reports on the differential activity of ketamine enantiomers demonstrate a clear superiority of the (S) isomer as a pharmacological agent.

Behavioral studies on the enantiomers of ketamine demonstrated a qualitative difference between the optical isomers.[56] In a fixed interval test, rats which were trained to barpress for food, exhibited a depressed response when injected with S-ketamine. R-Ketamine on the other hand, produced stimulant effects at doses above 15 mg/kg.

A recent clinical study of the enantiomorphs of ketamine demonstrated differences between the isomers themselves and the racemate.[86] Equianesthetic doses were given of each drug: 1 mg/kg, S-ketamine; 2 mg/kg, R,S-ketamine; and 3 mg/kg, R-ketamine. Upon termination of anesthesia at 35 minutes, the plasma levels of parent drug were determined. This established a potency ratio of 3.4/1 for R-ketamine: S-ketamine. Psychic emergence phenomena were reported as percentages of patients afflicted: 37%, R-ketamine; 15%, R,S-ketamine; and 5%, S-ketamine. Post-operative pain was not a problem with S-ketamine, whereas R-ketamine and the racemate exhibited this problem in 16% and 10% of patients. S-Ketamine was found to be the superior anesthetic by the anesthetists and it was more acceptable to the patients. The pharmacokinetics of ketamine and its metabolites did not differ between the optical isomers in the urine or plasma of the patients in this study. The authors therefore proposed that pharmacological differences between the isomers of ketamine are due to pharmacodynamic differences.

Stereoselective Metabolism of Ketamine

The subject of enantioselective metabolism of ketamine has been studied in the mouse[65] and rat.[50] In rat liver microsomes, the (S) isomer was found to have a slightly higher K_M and V_{max} than the (R) for N-demethylation.[46] Plasma samples from rats had similar MI levels after (R) and (S)-ketamine doses.[50] Brain MI levels were different

though, with higher values after (R) than (S).[50] Brain levels are probably more meaningful than plasma levels due to the concentrating of MI in the brain.[15] This is not due to brain cell metabolism of ketamine which does not occur in the rat.[15] Stereoselective brain uptake is probably not functioning here since (R) and (S) ketamine are found in the brain at identical levels.[50] Rather, the difference is attributed to differences in the hepatic biotransformation of the enantiomers. These results indicate that the (S) isomer might be metabolized quickly to MI then further oxidized to MIII and MIV. On the other hand, the (R) form seems to be metabolized to MI and probably little glucuronide is formed. The stereoselective steps therefore appear to be the ring hydroxylation with (S) ketamine forming the most alcohols and the N-demethylation step with (S) again being more active. In the mouse[60] there is a similar pattern except with the opposite isomers involved. Ketamine (R) isomer forms large amounts of MII in plasma while the (S) form gives high concentrations of MI. These results suggest that the (R) enantiomer is probably more active with cytochrome P450 for N-demethylation and subsequent ring hydroxylation which ultimately gives rise to more MII being detected. The (S) form is undoubtedly more slowly N-dealkylated and ring hydroxylated resulting in more MI in the plasma.

STATEMENT OF DISSERTATION OBJECTIVES

Ketamine (R,S-2-o-chlorophenyl-2-methylaminocyclohexanone) is a chiral anesthetic and both quantitative and qualitative pharmacological differences have been described for the individual optical isomers. Differences are found between the enantiomers for analgesic potency, hypnotic potency, lethality, and posthypnotic psychotomimetic effects in man. Such emergence phenomena are of interest since they occur at a time point when brain levels of the parent compounds have declined to low levels. This suggests the involvement of psychoactive metabolites in these phenomena. In addition, drugs known to decrease psychotomimetic effects (e.g. benzodiazepines) inhibit ketamine metabolism. Certain prospective metabolites of ketamine have close structural relationships to the dimethylcyclohexanols which have marked behavioral effects as well as analgesic potency.

Since the differences in the pharmacology of the isomers of ketamine could be in part due to differences in metabolism, it was decided to study the metabolic fate of the individual enantiomers separately and together in racemic mixtures. Some data was available that suggested differences in the metabolism of the enantiomers as has been discussed already in the dissertation. There are three basic techniques for studying the enantiomeric composition of metabolic products generated from racemic drugs that were tried: 1. derivatization of metabolites with a chiral reagent, thus forming diastereomers which are separable by various chromatographic techniques; 2. analysis of metabolites using a chiral phase capillary gas chromatograph column which will separate optically active eluates in a chiral environment; and 3. the use of pseudoracemic mixtures of a deuterium labeled enan-

tiomer and its unlabeled optical antipode where products from metabolic assays must be analyzed by gas chromatography-mass spectrometry in order to assess the ratio of deuterium labeled to unlabeled species. Chiral derivatizing reagents have been used with great success in the analysis of amphetamine, amino acids and certain other drugs. A promising reagent for the analysis of ketamine was pentafluorobenzoyl-S-proline imidazolide or any similar proline based reagent. A newly developed chiral phase capillary column that was used when chiral reagents failed was a siloxane copolymer of L-valine-t-butylamide (RSL 007). This column had been invented for use in the analysis of amino acids and results with primary amines looked promising. A last resort was a pseudoracemate study of ketamine. Similar studies have been conducted for many drugs including chlorpheniramine.

After developing an analytical technique, pathways of metabolism as well as specific metabolites were defined for the separate isomers of ketamine. Similar experiments were performed on racemic ketamine in order to assess the effects of the enantiomers upon each other.

In the course of this investigation, it became clear that prior methodology was not optimal for valid documentation of the metabolic fate of racemic ketamine. The goals of the dissertation were therefore modified to include a reevaluation of the metabolic profile of racemic ketamine and the positive identification of its metabolites.

CHAPTER II. Experimental Section to the Dissertation

EXPERIMENTAL

Materials

Samples of ketamine, norketamine and 5,6-dehydronorketamine, supplied as the hydrochloride salts, were furnished by the courtesy of Parke, Davis and Co., Ann Arbor, Michigan. The enantiomers of ketamine were prepared by recrystallization as the (+) or (-) tartaric acid salts:[7] the hydrochloride salts obtained gave $[\alpha]_D^{22}$ = +94.2° for S-ketamine and -87.4° for R-ketamine (c = 10 mg/ml, water).

Organic Synthesis

(S,S)-N-Trifluoroacetylproline Anhydride [(S,S)-IV]-- Trifluoroacetic anhydride (84 g, 0.40 mole) was added dropwise with stirring under nitrogen and external cooling to an ice cold suspension of (S)-proline (23 g, 0.20 mole). After 3 hr, the volatile components were removed under vacuum and the residual yellow mass was recrystallized 3 times from ether to yield 22.9 g (0.06 mole, 57%) of white crystals melting at 114-115° [lit.[74] mp 114-115°]: NMR ($CDCl_3$): 1.8-2.5 (m, 8H, CH_2), 3.79 (m, 4H, NCH_2) and 4.62 ppm (t, J=6.2 Hz, 2H, CH); IR (m.o. mull): 1822, 1760, 1690, 1460, 1380, 1360, 1350, 960, 940, 930, 910, 878, 840, 815, 795 and 768 cm^{-1}; EIMS (rel. int.): 404 (M+, 0.4), 376 (0.5), 211 (1.4), 194 (44), 166 (100), 98 (9.8), 96 (35) and 69 (82); $[\alpha]_D^{22}$= -98.6° (c 0.7 mg/ml, benzene).

Anal.- Calc. for $C_{14}H_{14}N_2O_5F_6$: C, 41.60; H, 3.49; N, 6.93. Found: C, 41.53; H, 3.51; N, 6.83.

The corresponding reaction with (R)-proline provided the enantiomeric anhydride [(R,R)-IV] in 36% yield: mp 114-115°; $[\alpha]_D^{22}$ = +106.5° (c 0.7 mg/ml, benzene).

(S,R)-N-Trifluoroacetylproline Anhydride [(S,R)-V]-- The reaction of (S)-proline (5.75 g, 0.05 mole) and trifluoroacetic anhydride (42.0 g, 0.20 mole) in 20 ml methylene chloride at room temperature for 48 hr yielded 2.5 g (0.006 mole, 25%) of the meso-anhydride (S,R)-V which was recrystallized from ether: mp 139-140° [lit.[85] mp 138-140°]; NMR and EIMS were indistinguishable from (S,S)-IV; $[\alpha]_D^{22}$= 0.0° (c 0.66 mg/ml, benzene); IR (m.o. mull): 1822, 1760, 1690, 1465, 1380, 1368, 1355, 940, 930, 914, 874, 812 and 760 cm.$^{-1}$

Anal.- Calc. for $C_{14}H_{14}N_2O_5F_6$: C, 41.60; H, 3.49; N, 6.93. Found: C, 41.77; H, 3.62; N, 6.96.

(S)-N-(N-Trifluoroacetylprolyl)-N-methylaniline [(S)-X]-- A mixture of (S, S)-IV (1.8 g, 4.5 mmole) and N-methylaniline (5 g, 46.7 mmole) was heated for 2 hr under nitrogen with stirring. The cooled reaction mixture was washed twice with 10 ml hexane and the residual oil was subjected to a short path distillation under reduced pressure. The distillate was crystallized from chloroform to yield 0.6 g (2.0 mmole, 22.4%) of a colorless solid: mp 136-138°; NMR ($CDCl_3$): 1.5-2.3 (m, 4H, CH_2), 3.28 (s, 3H, CH_3), 3.76 (m, 2H, NCH_2), 4.43 (t, J=6.3 Hz, 1H, CH), and 7.40 ppm (m, 5H, ArH); $[\alpha]_D^{22}$= -102.9° (c 0.7 mg/ml, benzene).

Anal.-Calc. for $C_{14}H_{15}N_2O_2F_3$: C, 56.00; H, 5.04; N, 9.33. Found: C, 55.65; H, 5.07; N, 9.28.

The same reaction carried out with (S,R)-V provided (S,R)-X in 17% overall yield: mp 102-104°; $[\alpha]_D^{22}$= 0.0° (c 0.7 mg/ml, benzene).

Anal.-Calc. for $C_{14}H_{15}N_2O_2F_3$: C, 56.00; H, 5.04; N, 9.33. Found: C, 56.05; H, 5.04; N, 9.31.

(S,S)-2-[N-(N-Trifluoroacetylprolyl)-N-methyl)]amino-2-o-chloro-

phenylcyclohexanone [(S)-N-Trifluoroacetylprolyl amide of (S)-ketamine, (S,S)-XIV]-- A solution of (S)-N-trifluoroacetylprolyl chloride (536 mg, 2.33 mmole in 23 ml methylene chloride) and (S)-ketamine[7] (500 mg, 2.11 mmole in 10 ml toluene) was heated at 75° with stirring under nitrogen for 1.5 hr. After cooling, the reaction mixture was stirred with 30 ml 0.2% NaOH, separated and washed with 10 ml 0.5% HCl. After drying with Na_2SO_4, hexane was added and the resulting solution was cooled to 5° to produce 300 mg (0.7 mmole, 33.2%) of a solid: $[\alpha]_D^{22}$ = +41.6° (c 0.77 mg/ml, benzene); mp 197-200°. The NMR, EIMS and GC retention time of the product were identical to the corresponding values for (S,R)-XV (see below) and therefore its structure is assigned as (R,S)-XV. Treatment of the mother liquor with an additional 5 ml hexane caused the precipitation of a second solid (45 mg, 0.2 mmole) which proved to be racemic N-trifluoroacetylproline: mp 53-55°; $[\alpha]_D^{22}$= 0.0° (c 0.7 mg/ml, benzene); CIMS, 212 (MH+).

Anal.-Calc. for $C_7H_8NO_3F_3$: C, 39.81; H, 3.79; N, 6.64. Found: C, 39.80; H, 3.88; N, 6.60.

The residue obtained from the above mother liquor filtrate was chromatographed on 10 g silica. Elution with benzene:ethanol (95:5) provided a solid which after crystallization from toluene/hexane yielded 7 mg (0.016 mmole, 0.8%) of the desired amide (S,S)-XIV: mp 184-186°; CIMS (m/e, rel. int.): 433 and 431 (MH+, 25, 100), 397 (10), 227 and 225 (50, 100), 211 and 209 (60, 100); GC retention time 16.8 min.

Anal.-Calc. for $C_{20}H_{22}N_2O_3F_3Cl$: C, 55.75; H, 5.15; N, 6.50. Found: C, 55.56; H, 5.30; N, 6.46.

(R)-2-[N-(N-Trifluoroacetyl-(S)-prolyl)-N-methyl]amino-2-o-chlorophenylcyclohexanone [(S)-N-Trifluoroacetylprolyl amide of (R)-ketamine,

(S,R)-XV]-- (S)-N-Trifluoroacetylprolyl chloride and (R)-ketamine were allowed to react under the same conditions as described above. After treatment with 0.2% NaOH, extraction with 0.5% HCl, and drying with Na_2SO_4, addition of hexane and cooling led to the crystallization of 490 mg (1.14 mmole, 54%) of (S,R)-XV: mp 200-202°; NMR ($CDCl_3$): 1.5-2.6 (m, 11H, CH_2), 3.24 (s, 3H, NCH_3), 3.3-3.5 (q, 1H, CH), 3.7-3.9 (m, 2H, CH_2), 5.09 (t, 1H, CH), and 7.1-7.5 ppm (m, 4H, ArH); $[\alpha]_D^{22}$= -41.2° (c 0.68 mg/ml, benzene); CIMS was identical to (S,S)-XIV; GC retention time 19.2 min.

Anal.-Calc. for $C_{20}H_{22}N_2O_3F_3Cl$: C, 55.75; H, 5.15; N, 6.50. Found: C, 55.70; H, 5.24; N, 6.32.

[^{3}H]Ketamine was prepared from the unlabeled compound by an acid-catalyzed exchange reaction (CF_3CO_2H, ^3H_2O, Pt) described by Blackburn and Ober.[19] Following removal of labile tritium introduced ortho to the carbonyl group by reflux (5 h) in aqueous base (H_2O:Et_3N; 5/2 v/v), the product was converted to the hydrochloride salt and purified by recrystallization from ethanol. This gave [^{3}H]ketamine hydrochloride with a specific activity of 545 Ci mole^{-1} and a radiochemical purity of more than 95% as judged by two-dimensional thin layer chromatography (TLC) and reversed phase high performance liquid chromatography (HPLC) analysis. A stock solution of this compound in water (0.46 mM) was prepared and stored at 3°. [^{2}H]Ketamine was synthesized by a similar procedure involving exchange in ^2H_2O; analysis of the product by GC-MS [pentaflouropropionyl (PFP) derivative] indicated the deuterium content to be 1.5% $^2H_0$, 3.4% $^2H_1$, 19.6% $^2H_2$, 67.2% $^2H_3$ and 8.3% $^2H_4$ (based on the [M-Cl-CO]+ cluster at m/z 320-324). Fragment ions at lower m/z values (m/z 102, 115, 125 and 152), which are known[39] to

retain the aromatic (but not the allicyclic) ring system of the ketamine molecule, displayed a similar labeling pattern, thus confirming the site of deuterium incorporation as the chlorobenzene moiety. Deuterium exchange and back exchange of facile label were also performed with the pure enantiomers of ketamine. Analysis of the final products by GC-MS of the trifluoroacetyl (TFA) derivatives indicated the deuterium content to be 1.1% $^{2}H_{0}$, 6.8% $^{2}H_{1}$, 32.8% $^{2}H_{2}$, 50.4% $^{2}H_{3}$, and and 8.8% $^{2}H_{4}$ (based on the [M-Cl-CO]+ cluster at m/z 270-274) for R-ketamine. For S-ketamine these values were 0.9% $^{2}H_{0}$, 2.3% $^{2}H_{1}$, 20.1% $^{2}H_{2}$, 64.4% $^{2}H_{3}$, and 12.3% $^{2}H_{4}$. Pseudoracemic mixtures were made by mixing S-ketamine and R-(^{2}H)ketamine in equal amounts. A similar mixture was prepared from R-ketamine and S-(^{2}H)ketamine. The ratio of unlabeled ketamine to deuterium labeled ketamine was determined in each case by GC-MS analysis of TFA derivatives of the mixtures. By summing the intensities of the ion at m/z 270 and 273 in all scans across the ketamine peak and dividing these numbers, the following ratios of 270/273 were found: 1.89 for R-ketamine, S-(^{2}H)ketamine and 1.64 for S-ketamine, R-(^{2}H)ketamine.

(1-Phenylcyclohexyl)-methylamine hydrochloride was synthesized for use as an internal standard in quantitative studies of the metabolism of the enantiomers of ketamine and mixtures of the isomers. The compound was made by reducing (lphenylcyclohexyl)-carbonitrile with lithium aluminum hydride by a published procedure (mp = 236-238° C, lit. mp = 238-240°).[34] Mass spectral analysis (EIMS) showed the following ions (rel. int.): 189 (M+, 2), 158 (80), 117 (65), 91 (100). A solution of the internal standard (2 mM in water) was made for use in liver microsomal metabolism assays.

Metabolic Experiments

Racemic Ketamine. Male Sprague-Dawley rats (average weight 380 g) were used for initial metabolic studies. The animals were starved for 12 h and then sacrificed by decapitation. The livers were removed, weighed, perfused with 3 volumes of cold 0.05 M Tris-HCl buffer/1.15% KCl solution, pH 7.4, and homogenized in 3 times the liver weight of cold buffer. Following two centrifugations at 9000xg for 20 min, the supernatant was centrifuged at 100,000xg for 70 min to sediment the microsomal pellet. The latter was washed with the original volume of cold buffer and resedimented by centrifugation at 100,000xg for 70 min. Microsomal incubations, which were performed in triplicate, were carried out at 37° for 30 min and contained the following reagents and co-factors: glucose-6-phosphate (9.6 mM), $MgCl_2$ (19.2 mM), NADP+ (0.77 mM) and glucose-6-phosphate dehydrogenase (2 I.U.). Stock solutions of these co-factors were prepared fresh prior to use and were made up in Tris buffer solution with the exception of $MgCl_2$, which was made up in distilled water. Racemic ketamine (or [^{2}H]ketamine) hydrochloride was employed as substrate at a concentration of 0.31 mM, together with a small amount of [^{3}H]ketamine hydrochloride (approx. 10^4 dpm/incubation). An aliquot (0.3 ml) of a suspension of microsomes in 0.05 M Tris-HCl buffer (pH 7.4; 8-10 mg protein ml^{-1}) was added to each incubation mixture, giving a final volume of 1.3 ml. Incubations were terminated by the addition of 1 M $NaHCO_3$ solution (1 ml) and the resulting mixture centrifuged briefly to remove precipitated material. The clear supernatants were applied to SEP-PAK cartridges (C18, Waters Associates, Milford, Connecticut), which were washed with water (10 ml) to remove inorganic salts and then with methanol

(10 ml) to elute metabolites. Aliquots of all incubation mixtures, and of washes and eluates from SEP-PAK cartridges, were taken for measurement of radioactivity. Following evaporation of the methanolic eluates *in vacuo* at 60°, metabolites were either subjected to purification by HPLC or converted directly to fluoroacyl derivatives for analysis by GC and GC-MS.

In the pseudoracemate experiments in chapter V, microsomes were prepared from male Sprague-Dawley rats as before. Metabolic incubations were performed at 37° for 30 min with the following ingredients: glucose-6-phosphate (7.4 mM), $MgCl_2$ (7.4 mM), NADP+ (0.6 mM), ketamine enantiomers or pseudoracemates (0.7 mM), and glucose-6-phosphate dehydrogenase (2 I.U.). The reaction was preincubated for 5 min then initiated by adding 0.3 ml of microsomal suspension (8-10 mg/ml protein in buffer, pH 7.4) to make a final reaction volume of 1.7 ml. Protein concentrations were determined according to Lowry.[48a] All reagents were made up in buffer except $MgCl_2$ which was in water. The incubations were terminated by the addition of 1 ml 0.1 M $NaHCO_3$ (pH 8.2). The mixtures were centrifuged to remove sediments and extracted on SEP-PAK C18 cartridges as described before.[2]

A normal human liver sample from biopsy was homogenized and microsomes were prepared as above. Microsomal metabolites were generated identically to the rat experiments. Duplicate incubations were done for pseudoracemates and pure enantiomers.

Phenobarbital (PB) induced rat liver microsomes were also prepared from a 300 g male Sprague-Dawley rat. The rat was injected IP with 40 mg/kg PB twice a day for three days, then with 50 mg/kg PB for two days. The liver was removed on the sixth day and microsomes were

prepared as before. One large incubation was performed in a 300 ml erlenmeyer flask by adding thirty times the usual volumes of each reagent as well as one million dpm of radioactive ketamine. The resultant mixture had all of the reagents present in the same concentrations as before. The reaction was stopped with 1 ml 1 N HCl which made the solution pH 2. Lipids were extracted with chloroform, the pH was adjusted to 8 with 4 N NaOH and metabolites were extracted with a SEP-PAK. Radioactivity was monitored at each step which demonstrated less than 15% overall loss of tritium in the extractions. The extract was then applied to an HPLC column to purify the metabolic products.

In kinetic studies of the N-demethylation of ketamine for the determination of K_M and V_{max} as well as in enantiomeric mixture experiments, the following concentrations of reagents were used: glucose-6-phosphate (5 mM), $MgCl_2$ (5 mM), NADP+ (1 mM), glucose-6-phosphate dehydrogenase (2 I.U.), the enantiomers of ketamine hydrochloride or tartrate or mixtures of the enantiomers (2 to 0.03 mM) and microsomes (approx. 3 mg protein). The incubations were made up to 3 ml with Tris buffer (pH 7.4 at 37°) and initiated with NADP. Time course and kinetic analyses were subjected to formaldehyde determinations according to Nash[58a] after reaction termination with zinc sulfate. Enantiomeric mixture incubations were terminated with 0.1 M $NaHCO_3$, 100 ul of internal standard was added, samples were SEP-PAK extracted and analyzed with the chiral capillary column.

Analytical Procedures

Radioactivity measurements were performed by liquid scintillation counting in a Packard Tri-Carb instrument using Insta-gel (Packard, Downers Grove, Illinois) as scintillation cocktail.

Thin Layer Chromatography. TLC was carried out on glass plates (20 cm X 20 cm), coated with 2 mm layers of silica gel 60 F_{254} (Merck, Darmstadt, Germany). For two-dimensional TLC, the plate was developed first in methanol/ammonium hydroxide (200:1.5, v/v) and following rotation through 90°, in tetrahydrofuran. Radioactive components on TLC were detected by use of a Varian Series 600 plate scanner.

High Pressure Liquid Chromatography. HPLC (chapter III) was performed using an Altex Model 320 system (Altex Scientific Inc., Berkeley, California), equipped with a reversed phase Ultrasil-ODS (10 u) column (24 cm x 4.6 mm i.d.) and a Schoeffel Model GM 770 variable wavelength UV detector operated at 254 nm. The mobile phase consisted of methanol/0.015 M phosphate buffer (1:1, v/v), containing Et_2NH (0.2% by vol.) and adjusted to a final pH of 5.0. The system was operated at a flow rate of 1 ml min^{-1} and the column effluent was collected in 1 ml fractions. Those fractions found to contain radioactivity were evaporated to dryness in vacuo (60°) and the residues dissolved in distilled H_2O for desalting on SEP-PAK cartridges as described above. The final phosphate-free samples were dried and converted to fluoroacyl derivatives for analysis by GLC and GC-MS.

Purification of metabolites from the PB induced rat was achieved with an Altex model 110A system (Altex Scientific Inc., Berkeley, CA), a reversed phase Ultrasphere ODS (5 u) column (25 cm by 10 mm) and a Hitachi model 100-10 variable wavelength detector set at 254 nm. The mobile phase was acetonitrile/0.015 M acetate buffer (1:1, v/v) containing 0.2% Et_2NH and the pH was adjusted to 5.6. The flow rate through the column was 4 ml/min and the effluent was collected in 2 ml fractions. Tritium containing fractions were lyophilized, dissolved in

2 ml water (pH 8) and extracted by SEP-PAK to remove residual buffer. Gas Chromatography GC analyses of the prolyl derivatized amines (chapter III) were recorded on a Hewlett-Packard 5700A machine equipped with a nickel electron capture detector. The column was a 3 m x 6 mm i.d. glass column packed with 3% SP 2250 coated on 100/120 mesh supelcoport. Argon-methane carrier gas flow rate was 30 ml/min and the column temperature was 250°. Peak area integration was done automatically by a Hewlett-Packard 3380A integrating recorder.

A Carlo-Erba Fractovap Model 2150 gas chromatograph was used in chapter III equipped with a Grob-type injector, flame ionization detector and a glass capillary column (34 m x 0.325 mm i.d.), wall-coated with OV-1 stationary phase. Helium (1 ml min^{-1}) was employed as carrier gas and analyses were carried out using a cold trapping technique in which samples (0.5-1.0 ul) were injected in the splitless mode with the column oven at ambient temperature. Upon elution of the solvent front, the oven temperature was raised rapidly to 120° and then programmed at 3° min^{-1} to 215°. The injector and detector block temperatures were maintained at 275° throughout. Ketamine and its metabolites were analyzed as their trifluoroacetyl (TFA) or PFP derivatives as described below. The retention times of derivatized metabolites were measured relative to those of a series of n-alkanes co-injected with each sample and are expressed as methylene unit (MU) values.

Semi-quantitative analyses were carried out using a similar procedure to that described above, with the exception that fixed amounts (50 ng each) of two n-alkanes (n-C_{16} and n-C_{22}) were added to the derivatized sample. The height of each peak of interest in the resulting chromatogram was related to that of the line (measured at the same

time point in the chromatogram) drawn between the tops of the two n-alkane peaks. This ratio was used to calculate the amount of the component present in the sample by reference to one of two standard curves; metabolites which had undergone N-demethylation were quantified using a standard curve constructed from norketamine, while metabolites which retained the N-methyl group of the parent drug were quantified using the standard curve for ketamine itself. TFA derivatives were used for quantitative analyses since batches of commercial pentafluoropropionic acid anhydride were frequently found to be contaminated with small amounts of trifluoroacetic acid anhydride, use of which thus gave rise to two types of fluoroacyl derivative for each component present.

A Varian 1440 gas chromatograph equipped with a Grob-type injector, flame ionization detector and a glass capillary column (36 m by 0.325 mm i.d.) which was wall coated with OV-1, was used for all retention time assessments in chapter IV. The temperature program used was 100-200° at 4° per min. A chiral phase capillary column was also used for the analysis of metabolites. The glass WCOT column was 25 m by 0.25 mm i.d. with RSL-007 stationary phase (Alltech Associates, Los Altos, CA). Near base line separation of the TFA derivatives of norketamine enantiomers was accomplished with a temperature program of 125-215° at 5° per minute.

Mass Spectrometry. CI mass spectra were taken in chapter II on an Associated Electronics Incorporated Model MS-902 double focus mass spectrometer equipped with a direct inlet system and modified for chemical ionization. The EI mass spectra were recorded on a Hitachi model M-52 instrument.

GC-MS. Low resolution ($m/\Delta m = 600$) mass spectra of derivatized metabolites were recorded in chapter III on an Hitachi M-52 GC-MS system modified for operation with capillary columns. The instrument was equipped with a 17m x 0.325 mm i.d. glass WCOT column coated with OV-1, which was connected directly to the mass spectrometer ion source via a glass-lined restrictor tube. Helium (1 ml min^{-1}) was employed as carrier gas and temperature programmed analyses were carried out from 120-220° at 5°/min. The injector, interface and ion source temperatures were maintained at 250°, 230° and 220° respectively, and mass spectra were recorded repetitively using a scan rate of 1.5 sec $decade^{-1}$, electron energy of 70 eV, accelerating voltage of 2 kV and trap current of 200 uA. Data acquisition and reduction were performed on-line using a Data General Nova II computer system.

Low resolution ($m/\Delta m = 600$) mass spectra of derivatized metabolites were recorded in chapter IV on a Kratos MS-25 GC-MS system modified for operation with capillary columns. The column used was a 33 m by 0.325 mm i.d. glass WCOT column (OV-1), which was connected to the mass spectrometer ion source by a glass restrictor tube. Helium (1 ml/min) was the carrier gas during temperature programming from 100-230° at 5° per min. The injector interface and ion source temperatures were 250°, 230° and 220° respectively. The other conditions were: scan rate 1.5 sec $decade^{-1}$, electron energy 70 eV, accelerating voltage 2 kV, and trap current 200 uA. Data were acquired and reduced on-line by a Data General Nova II computer system.

High resolution ($m/\Delta m = 10,000$) mass spectra of derivatized metabolites were also obtained under GC-MS conditions, using a modified AEI MS-902 instrument on-line to a Xerox Sigma 7 computer system.[55]

Analyses were performed using a 50 m x 0.5 mm i.d. glass WCOT capillary column, coated with SE-52 and temperature programmed from 100-220° at 2.5° min^{-1} with He (2 ml min^{-1}) as carrier gas. Mass spectra (650-70 amu) were recorded at a scan rate of 8 sec $decade^{-1}$, with an electron energy of 70 eV, accelerating voltage of 8 kV and trap current of 200 uA. The injector, interface and ion source temperatures were held at 250°, 230° and 220°, respectively.

Melting points were taken on a Thomas-Hoover apparatus and are uncorrected.

Infrared spectra were obtained using a Beckman Aculab 2 spectrophotometer.

NMR spectra were recorded on a Varian FT-80 instrument. Chemical shifts are reported in parts per million (ppm) relative to TMS ($CDCl_3$).

Specific rotation measurements were performed on a Perkin-Elmer 141 polarimeter.

Microanalyses were performed by the Microanalytical Laboratory, University of California at Berkeley.

Amine Derivatization

TFA and PFP Derivatives. Derivatives for capillary GC were prepared by reaction of the dried microsomal isolate with 100 ul of a mixture of trifluoroacetic acid anhydride or pentafluoropropionic acid anhydride and anhydrous ethyl acetate (1:1, v/v) at 60° for 30 min. Following evaporation of excess reagent under a gentle stream of nitrogen, the residue was taken up in anhydrous ethyl acetate for GC or GC-MS analysis.

Prolyl Derivatives. Derivatizations of racemic ketamine and the individual enantiomers of ketamine for GC analysis were performed under similar conditions except that the concentration of racemic ketamine was

twice that of the individual enantiomers. Reactions were carried out in 50 ul of toluene:methylene chloride:triethylamine (95:5:0.02) containing (R,S)-ketamine (1.68 mM) or the ketamine enantiomers (0.84 mM) and in each case a 30 molar excess of (S,S)IV or (S)-I. The reactions were performed in screw cap sealed, teflon lined reaction vials which were heated to 75-100° for 1-4 hr in a block heater. At the end of the reaction, the mixtures were washed with 0.2% NaOH (50 ul) followed by 0.5% HCl (50 ul) and then dried over a few mg Na_2SO_4. The solutions were carefully transfered with the aid of a Pasteur pipette to a second reaction vial and the solvent removed under a stream of dry N_2. The residues were dissolved in 100 ul toluene and 1 ul of the resulting solution was analyzed by GC. Retention times for (S,S)-XIV and (S,R)/(R,S)-XV were 16.8 and 19.2 min, respectively. Derivatization of (S)-α-methylbenzylamine with (S,S)-IV and (R,S)V proceeded in a similar fashion. GC retention times for (S,S)-VII and (S,R)-VIII were 15.5 and 13.3 min, respectively, using the same conditions as for the ketamine analyses described under GC.

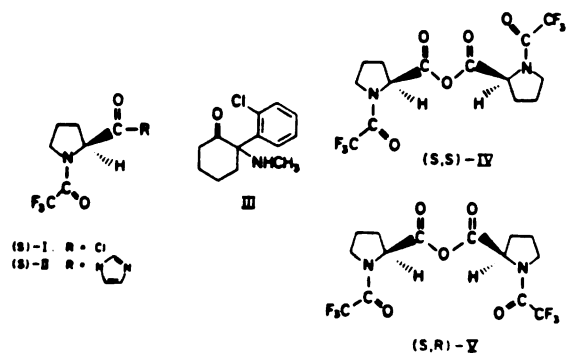
CHAPTER III. Derivatization of Chiral Amines with (S,S)-N-Trifluoroacetylproline Anhydride for Gas Chromatographic Estimation of Enantiomeric Composition

A. Introduction

B. Results and Discussion

Introduction

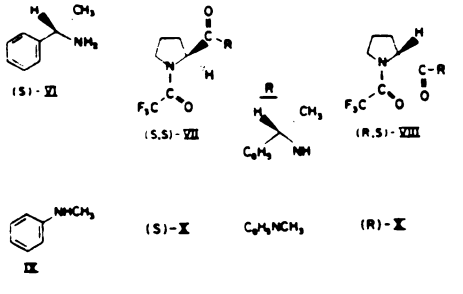
A primary objective of this dissertation work has been to examine the extent to which the enantiomers of ketamine may undergo enantioselective metabolic transformations in an effort to characterize the effects which such processes may have on the pharmacological and toxicological properties of the optical isomers. In the case of other chiral amines, quantitative estimations of enantiomeric composition have been achieved through GC analysis of the diastereoisomeric amides formed by derivatization with (S)-N-trifluoroacetylprolyl chloride [(S)-I].[25,47,54] Although commercially available as a solution in chloroform, this reagent is difficult to obtain in pure form and is susceptible to racemization.[75] The corresponding imidazolide, compound (S)-II, is a relatively stable, crystalline solid.[52] However, this derivative reacts sluggishly with sterically hindered amines such as ketamine (III).[3] In an attempt to obtain a derivatizing reagent which can be prepared in crystalline form and which might react more readily with ketamine, attention was turned to (S,S)-N-trifluoroacetylproline anhydride [(S,S)-IV]. An attractive feature of (S,S)-IV is that inversion about one of the two chiral centers present in this molecule leads to the meso-diastereoisomeric species (S,R)-V which, at least in theory, should be separable from (S,S)-IV. This chapter concerns the synthesis of (S,S)-IV and related compounds and assesses the suitability of the derivatizing reagent for the analysis of ketamine.



RESULTS AND DISCUSSION

The synthesis of (S,S)-IV from (S)-proline and trifluoroacetic anhydride was reported originally by Weygand in 1957.[85] Attempts to repeat this synthesis initially led to the isolation of a species (mp 114-115°) which proved to be an isomer of the Weygand compound; longer reaction times however provided the Weygand compound (mp 138-140°). The EI mass spectra and NMR spectra of these products were essentially identical which suggested that the two compounds were diastereoisomerically related. Since the high melting isomer did not rotate plane polarized light whereas the low melting isomer was strongly levorotatory, the low melting isomer was tentatively assigned the asymmetric structure (S,S)-IV and the Weygand compound the meso-structure (S,R)-V.

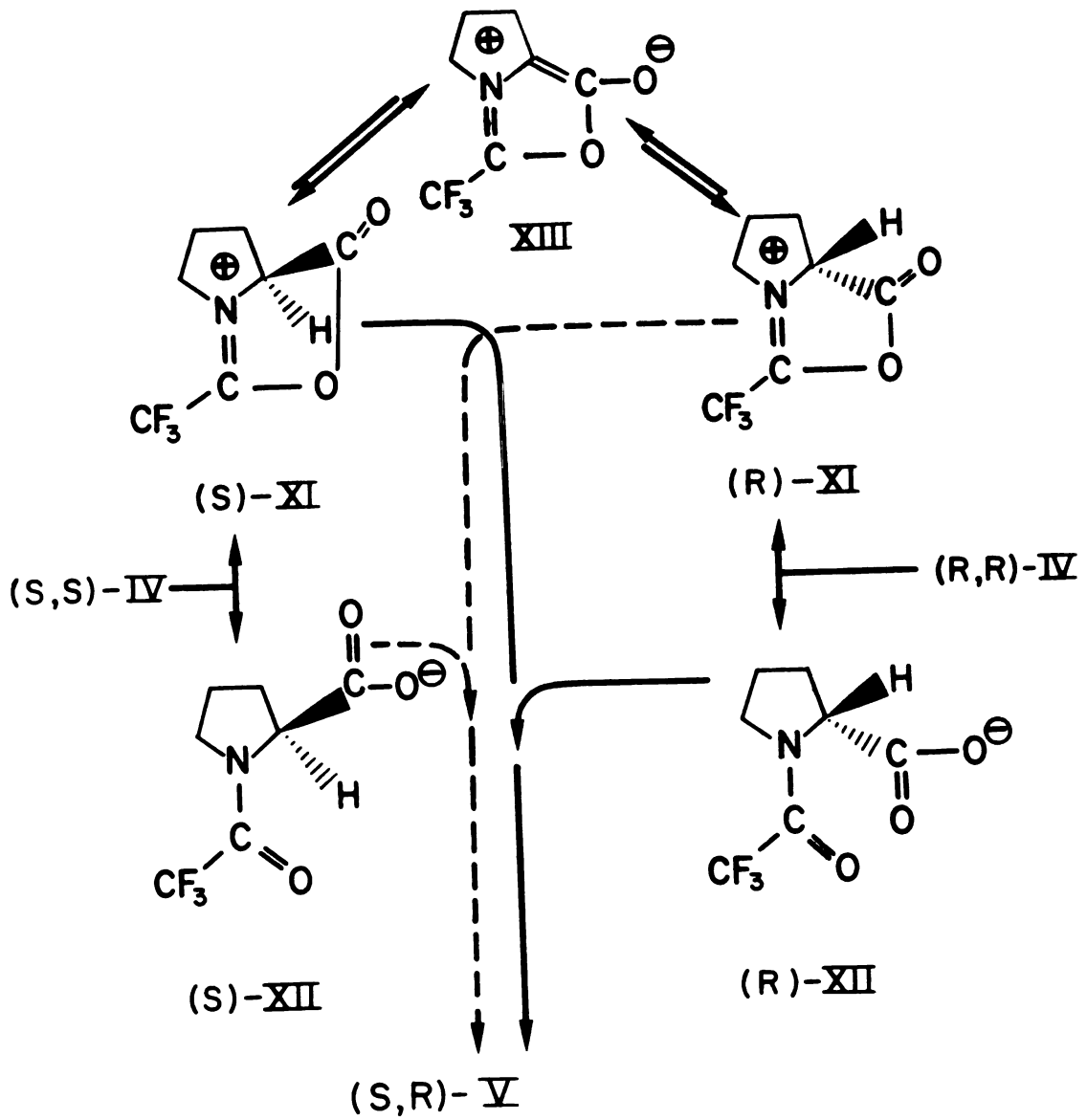
Consistent with these assignments, reaction of (S,S)-IV with (S)-methylbenzylamine [(S)-VI] yielded a product which displayed a single GC peak and which presumably corresponds to structure (S,S)-VII. Reaction of (S)-VI with (S,R)-V, on the other hand, yielded a product displaying two equally intense GC peaks corresponding to (S,S)-VII and (R,S)-VIII.[a] Additionally, reaction of (S,S)-IV with N-methylaniline (IX) yielded a levorotatory anilide [compound (S)-X] while the corresponding reaction with compound (S,R)-V yielded a racemic mixture of (S)-X and (R)-X.



[a]For all such designations the first symbol refers to the chirality of the N-trifluoroacetylprolyl moiety and the second symbol to the chirality of the amine.

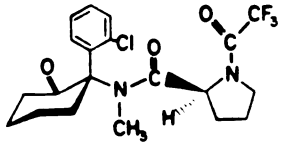
The experimental evidence described above is sufficient to assign unambiguously the structure of the low melting isomer. Similar experimental results have led Tomida and Kuwahara to the same conclusions.[74] The data supporting the structure assignment of the meso-isomer (S,R)-V however, are also consistent with a racemic mixture of (S,S)-IV and (R,R)-IV. As has been reported with related systems, the ability to distinguish between such species is often not a trivial task.[29] Therefore, attempts were made to prepare the racemate of IV by mixing equal amounts of (R,R)-IV [prepared from (R)-proline and trifluoroacetic anhydride] and (S,S)-IV. The product obtained after crystallization proved to be identical in every way to the high melting isomer. The IR spectra of the presumed racemate and the high melting isomer obtained from the Weygand procedure also were identical but were different from that of (S,S)IV. Based on these results and the facile interchange which is reported to occur with mixtures of acetic anhydride and trifluoroacetic anhydride,[91] it is likely that upon recrystallization the racemic mixture of IV undergoes disproportionation to yield the thermodynamically more stable meso-isomer (S,R)-V.

The pathway leading to the formation of the meso-compound is likely to involve the oxazolinium intermediate XI (Scheme III.1). Recombination of the (R)-N-trifluoroacetylprolyl anion [(R)-XII] with the (S)-oxazolinium intermediate [(S)-XI] leads to (S,R)-V. (S,R)-V may also be formed from (S)-XII and (R)-XI. It seems reasonable to speculate that the formation of compound (S,R)-V from (S)-proline and trifluoroacetic anhydride also proceeds through the oxazolinium intermediate which may undergo inversion to (R)-XI through reversible tautomerism to the symmetric mesoionic species XIII. A similar pro-

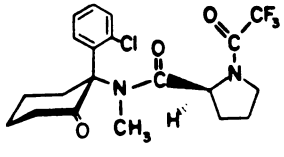


scheme III.1

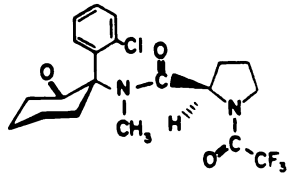
cess has been proposed for the racemization of (S)-N-p-nitrobenzoylproline in the presence of acetic anhydride and a trace of acid.[89] The utility of (S,S)-IV for the quantitative estimation of the enantiomeric composition of ketamine was examined next. GC analysis of the reaction product obtained between (S,S)-IV and racemic ketamine in the presence of triethylamine gave a pair of sharp peaks with baseline separation and mass spectra consistent with the expected amide structures (S,S)-XIV and (S,R)-XV.[b] With the aid of the pure ketamine enantiomers, the diastereoisomer with the shorter retention time was shown to be the (S)-N-trifluoroacetylprolyl amide of (S)-ketamine. Under a variety of reaction conditions however, product formation appeared to favor the diastereoisomer having the longer retention time, i.e. compound (S,R)-XV. For reasons discussed below, reaction of racemic ketamine with (S,S)-IV also may yield significant amounts of the enantiomeric species (R,S)-XV.



(S,S)- XIV



(S,R)- XV



(R,S)- XV

The two N-trifluoroacetylprolyl diastereoisomeric amides of (R)- and (S)-ketamine were obtained in analytically pure form from the reaction of (S)-N-trifluoroacetylprolyl chloride and individual ket-

[b]The absolute stereochemistry of ketamine free base has been established to be R(+) and S(-).

amine enantiomers. Although the reaction with (R)-ketamine proceeded smoothly, attempts to prepare the corresponding amide of (S)-ketamine were accompanied by extensive inversion of the derivatizing reagent. The main product obtained in this reaction was the diastereomeric (R)-N-trifluoroacetylprolyl amide of (S)-ketamine, i.e. compound (R,S)-XV. The N-trifluoroacetylproline recovered from the reaction mixture proved to be racemic which indicates that racemization of the prolyl reagent during the reaction with (S)-ketamine is extensive.

GC analysis of the two diastereoisomers showed that the detector responses of these compounds were essentially identical. Consequently, the different peak heights observed in the analysis of racemic ketamine must be due to the stereoselective formation of the (S)-N-trifluoroacetylprolyl amide of (R)-ketamine [and/or the (R)-N-trifluoroacetylprolyl amide of (S)-ketamine].

An additional frustration encountered in attempts to develop a ketamine assay with (S,S)-IV was the low yields realized in these reactions. Based on peak heights observed with the synthetic N-trifluoroacetylprolyl amides, the maximum combined yield of the two diastereoisomeric amides obtained upon reaction of racemic ketamine with (S,S)-IV was only 17% (Table III.1). The use of a large excess of the anhydride or introduction of additional anhydride during the course of the reaction did not influence the overall yield. Although somewhat better yields were obtained with the prolyl chloride reagent (S)-I, this reaction also was more stereoselective (Table III.1).

A pathway to account for these results would involve cleavage of the anhydride to the (S)-oxazolinium species [(S)-XI] and (S)-N-trifluoroacetylproline anion [(S)-XII]. Conversion of (S)-XI to the

TABLE III.1

GC ANALYSIS OF KETAMINE FOLLOWING DERIVATIZATION

WITH N-TRIFLUOROACETYLPROLYL REAGENTS[a]

Reactant	Reagent	Temp(Time)	Peak Areas[b] (S,S)-/(R,S)-XIV	Peak Areas[b] (S,R)-/(R,S)-XV	Yield[c]
(R,S)-III	(S,S)-IV	75° (2hr)	3	6	2%
(R,S)-III	(S,S)-IV	75° (2hr)	3	8	2%
(R,S)-III	(S,S)-IV	100° (1hr)	14	68	16%
(R,S)-III	(S,S)-IV	100° (2hr)	21	63	17%
(R,S)-III	(S,S)-IV	100° (4hr)	20	58	16%
(S)-III	(S,S)-IV	75° (2hr)	2	0	1%
(S)-III	(S,S)-IV	100° (2hr)	49	14	25%
(R)-III	(S,S)-IV	75° (2hr)	0	6	3%
(R)-III	(S,S)-IV	100° (2hr)	12	86	39%
(R,S)-III	(S)-I	75° (2hr)	33	206	48%
(R,S)-III	(S)-I	75° (4hr)	32	196	46%
(R,S)-III	(S)-I	100° (1hr)	49	211	52%
(R,S)-III	(S)-I	100° (2hr)	29	134	33%
(S)-III	(S)-I	75° (2hr)	27	19	18%
(R)-III	(S)-I	75° (2hr)	18	208	90%

[a]The assays were performed according to the procedure described in the experimental section. [b]Since (S,S)-IV and (S)-I are susceptible to inversion, the peak areas reported for reactions involving racemic ketamine represent the sum of the responses of the enantiomeric prolyl amides. [c]Yields were calculated on the basis of detector responses obtained with pure (S,S)-XIV and (R,S)-XV. Note that the individual enantiomers were run at 1/2 the concentration of racemic ketamine.

mesoionic compound XIII would deplete the acylating reagent and also generate a mole of acid. Although a 5/1 molar ratio of triethylamine to ketamine was used, the large excess of (S,S)-IV could result in adequate acid production to protonate (and hence inactivate) the ketamine. The yellow color which developed during the course of the reaction is consistent with the report that compound XIII is yellow.[26]

Analytical scale reactions with the individual isomers of ketamine again demonstrated the poor reactivity of (S)-ketamine with both (S,S)-IV and (S)-I (Table III.I). Extensive racemization occured during these reactions. Since the chiral center of ketamine is tetrasubstituted and therefore unlikely to racemize, the formation of the undesired diastereomer was attributed to inversion of the prolyl moiety. The reactions of (S,S)-IV and (S)-I with (R)-ketamine proceeded in reasonable yields and were accompanied by only a limited amount of inversion (Table III.1). If one assumes that the transition state energy for the formation of the prolyl amide with (S)-ketamine is greater than with (R)-ketamine, the competing reaction pathway (Scheme III.1) leading to isomerization of (S,S)-IV [and (S)-I] would proceed to a greater extent in the reaction with (S)-ketamine than with (R)-ketamine. Once inversion has occured, the energetically more favored reaction of (S)-ketamine with the (R)-prolyl reagent would occur preferentially.

The results obtained in this study clearly point to a number of difficulties with prolyl derivatizing reagents to which researchers should be alerted. Quantitative estimations based on GC analysis of reaction mixtures whenever possible should be based on detector responses measured with the pure synthetic diastereoisomeric amides. Secondly, the possibility of stereoselective reactions with chiral

amines and prolyl derivatizing reagents should be carefully evaluated. Finally, the stability of the derivatizing reagent with respect to racemization should be examined with the aid of the individual enantiomers of the amine. In the present study it has been found that the reaction of the anhydride reagent (S,S)-IV with ketamine proceeds stereoselectively, in poor yield, and is accompanied by inversion. Unfortunately, the corresponding reaction between the commercially available (S)-N-trifluoroacetylprolyl chloride suffers from similar limitations.

CHAPTER IV. STUDIES ON THE BIOTRANSFORMATION OF KETAMINE. I. IDENTIFICATION OF METABOLITES PRODUCED *IN VITRO* FROM RAT LIVER MICROSOMAL PREPARATIONS

A. Introduction

B. Results

C. Discussion

INTRODUCTION

This chapter describes a re-examination of the metabolic fate of racemic ketamine in mammalian systems in order to investigate any possible relationship between "active" metabolite formation and CNS side effects. This chapter concerns studies on the oxidative metabolism of ketamine by rat liver microsomal preparations, a model system which appeared to give rise to a profile of metabolites similar to that found in human plasma.[1] An analytical protocol involving a novel non-selective extraction procedure was developed. This was followed by sample analysis using combined gas chromatography-mass spectrometry (GC-MS). Six previously unidentified metabolites of ketamine were characterized and quantitative aspects of their formation were assessed using glass capillary gas chromatography (GC).

RESULTS

Identification of Metabolites

Metabolites isolated from microsomal incubations using the SEP-PAK extraction procedure detailed already were subjected to analysis, either directly by reverse-phase HPLC, or following conversion to TFA or PFP derivatives, by GC and GC-MS. Results from an HPLC analysis are shown in Fig. IV.1, which illustrates the profile of UV-absorbing (254 nm) components eluting from the column, together with the distribution of radioactivity among these components. The peak of radioactivity with the greatest retention volume (C) was found to coincide with the elution volume of ketamine itself, while the prominent radiolabeled component (B) eluting slightly ahead of ketamine possessed the chromatographic properties of norketamine. Positive identification of these two components as unmetabolized ketamine and norketamine, respectively, was subsequently obtained by mass spectral analysis of the material in appropriate HPLC fractions. The unresolved band of radioactivity (A) eluting ahead of norketamine represented a group of polar metabolites whose nature was investigated by GC-MS analysis.

Gas chromatographic analysis of the material present in extracts of incubation media led to the recognition of nine drug-related components which were not obtained from blank (no NADPH generation) incubations (Fig. IV.2). A representative gas chromatogram (following preparation of PFP derivatives) is illustrated in Fig. IV.3, which also shows the "bracketing" alkanes n-C_{16} and n-C_{22} used as external standards for semi-quantitative measurements. GC-MS analysis of this crude mixture of metabolites resulted in the identification of all nine components, as discussed below.

Figure IV.1. HPLC analysis of ketamine metabolites obtained from SEP-PAK extraction of a microsomal incubation product. The profile of both UV-absorbing (254 nm) and radioactive conponents is shown. Conditions of analysis are as reported in the text. Peak A corresponds to norketamine and Peak C corresponds to unmetabolized ketamine. The intensely UV- absorbing species eluting at 13-14 min represent endogenous microsomal components and NADP derived compounds.

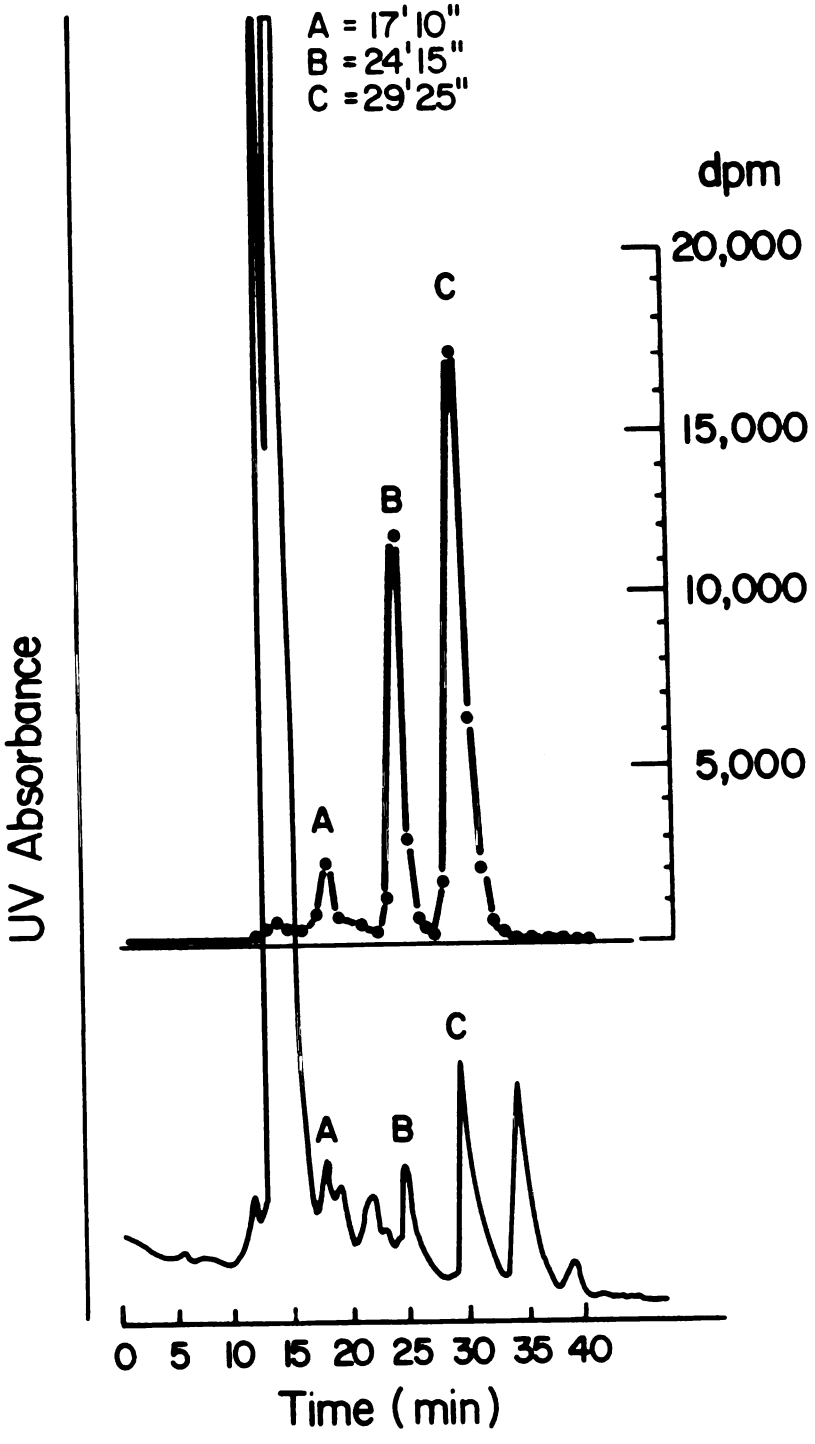

A = 17'10"
B = 24'15"
C = 29'25"
dpm
20,000
15,000
10,000
5,000
C
B
A
UV Absorbance
C
A
B
0 5 10 15 20 25 30 35 40
Time (min)

Figure IV.2. Gas chromatogram of ketamine microsomal isolates in an incubation with an inactive NADPH generating system. The two n-alkanes nC_{16} and nC_{22} and ketamine are the only peaks which are identifiable based upon retention time. Other peaks represent compounds endogenous to rat liver microsomes.

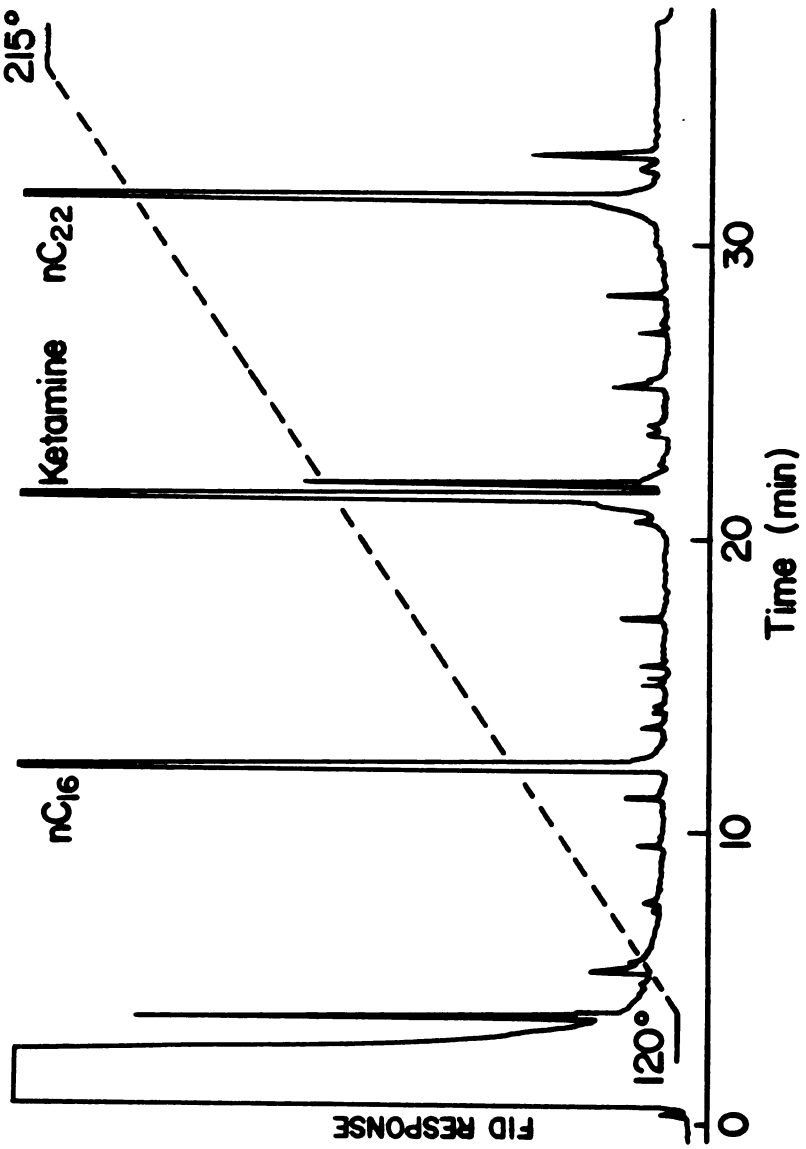
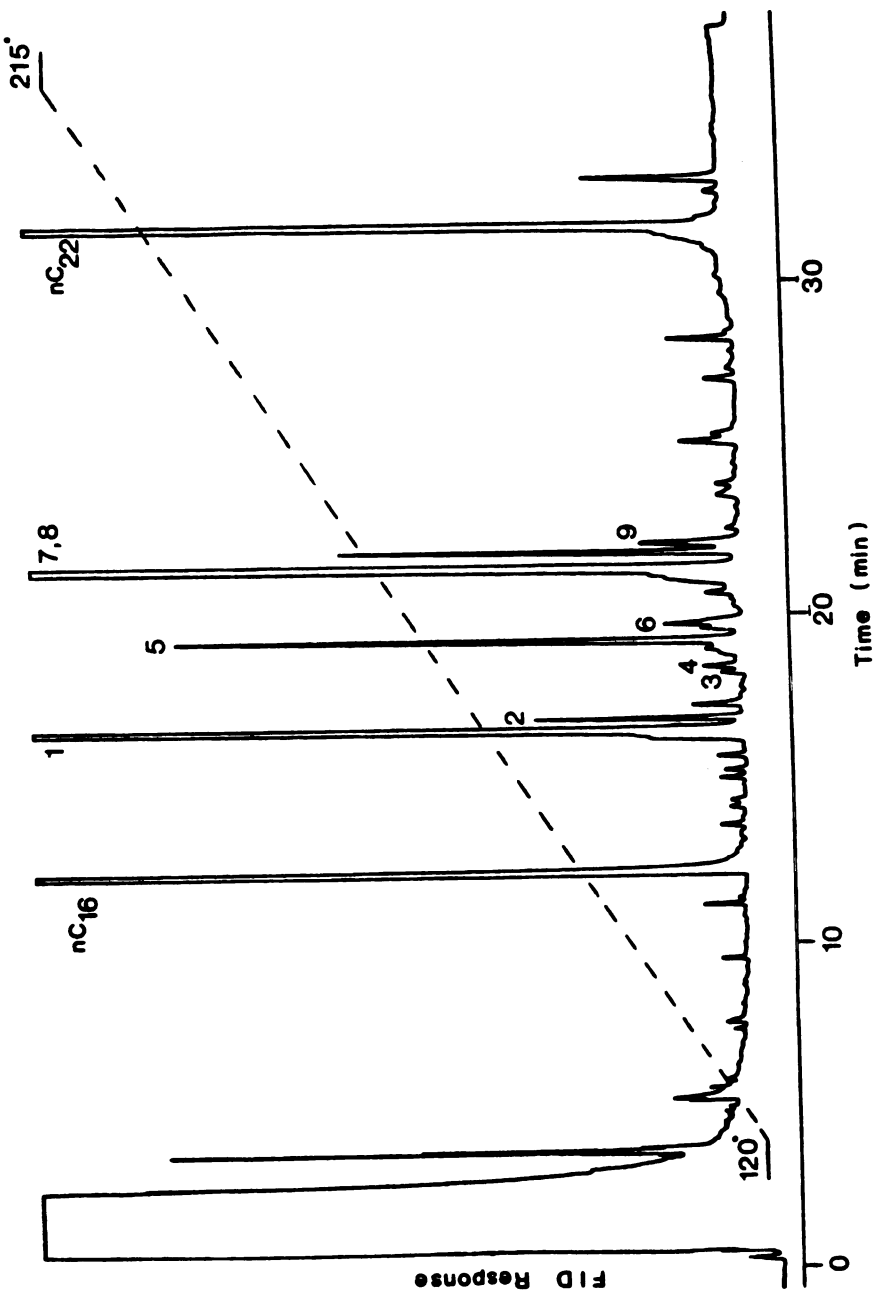

215°
nC22
Ketamine
nC16
120°
FID RESPONSE
0
10
20
30
Time (min)

Figure IV.3. Gas chromatographic separation of ketamine metabolites (as their PFP derivatives) isolated from a rat liver microsomal incubation. Peaks labeled $\underline{n}C_{16}$ and $\underline{n}C_{22}$ derive from $\underline{n}$-alkanes $C_{16}H_{34}$ and $C_{22}H_{46}$, respectively, which were co-injected with the samples. Numbered peaks denote the following metabolites: 1. Norketamine; 2. Hydroxynorketamine I (together with the TFA derivative of norketamine, resulting from the presence in the derivatizing reagent of small quantities of TFA anhydride); 3. Hydroxynorketamine II; 4. 5,6-Dehydronorketamine; 5. Hydroxynorketamine III; 6. Hydroxynorketamine IV; 7. Ketamine; 8. Hydroxyketamine I (not resolved from ketamine); 9. Hydroxyketamine II. Unnumbered peaks on the chromatogram are not drug related and were present in extracts of drug-free or inactive NADPH generation system incubations. GC conditions are given in the text.


215°
120°
nC16
nC22
1
2
3
4
5
6
7,8
9
FID Response
0
10
20
30
Time (min)

The compounds giving rise to peaks 7, 1 and 3 (Fig. IV.3) were identified as the PFP derivatives of ketamine and its two known metabolites, norketamine and 5,6-dehydronorketamine, respectively. These structural assignments were made on the basis of both the mass spectra (low and high resolution) and the relative GC retention times of the derivatives which, in each case, were identical with those of the respective reference compounds. Low resolution mass spectra of the PFP derivatives are reproduced in Fig. IV.4. The availability of high resolution mass spectral data for these compounds (Table IV.1) was of particular value in predicting generalized pathways of fragmentation of ketamine metabolites under electron impact. The origin of some ions of diagnostic importance is outlined in Scheme IV.1. A common feature of the mass spectra of fluoroacyl derivatives of ketamine and related compounds was the appearance of M+• ions of low relative abundance, together with prominent fragment ions [M-Cl]+ and [M-Cl-CO]+. Cleavage of the cyclohexanone ring system, particularly involving the C_1-C_2 bond, was a major pathway of fragmentation in all cases, when charge retention was generally associated with the resulting benzylamide fragment. Ions formed by this process were of considerable diagnostic utility in that sites of metabolic attack were revealed on the basis of observed mass shifts of fragments formed by cleavage of the C_1-C_2 bond together with rupture of either the C_3-C_4, C_4-C_5 or C_5-C_6 bond. The base peak in the spectrum of ketamine PFP, m/z 160 (Fig. IV.4A), was found to have an elemental composition of $C_4H_3NF_5$ which can be accommodated by the structure proposed in Scheme IV.1; this ion appears to be diagnostic for PFP derivatives of compounds

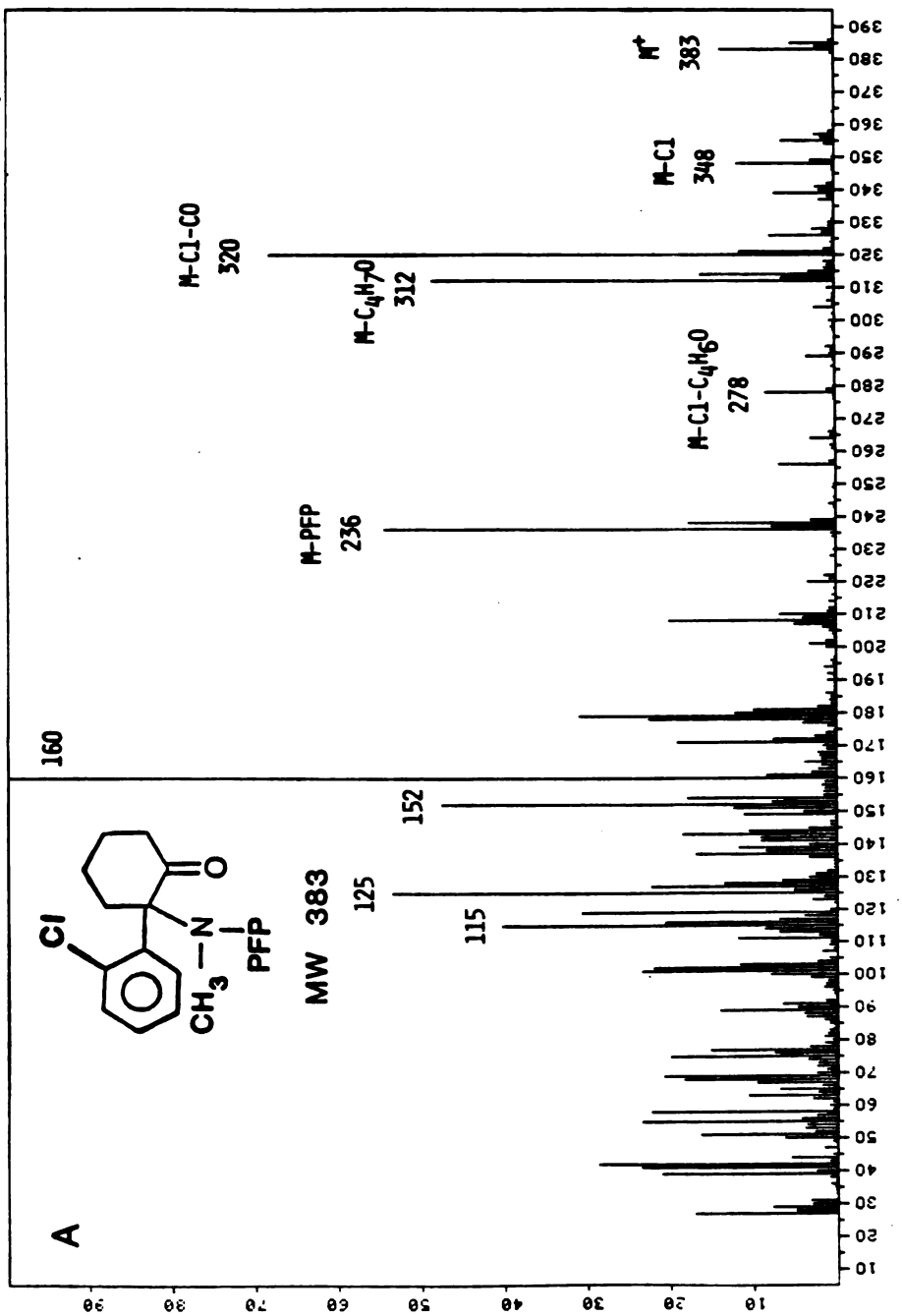
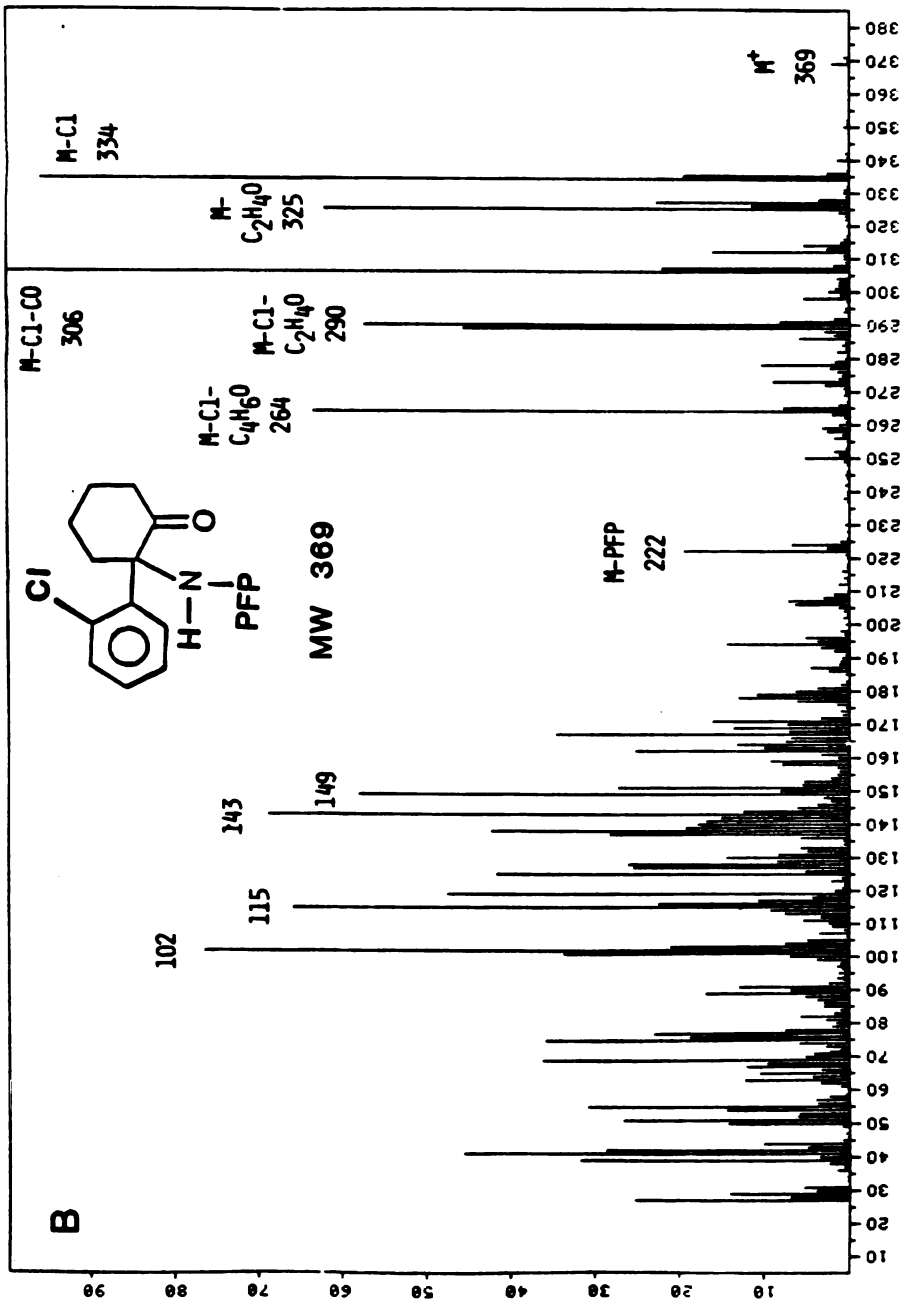


Figure IV.4. Low resolution mass spectra of the PFP derivatives of ketamine (A), norketamine (B) and 5,6-dehydronorketamine (C).


B
M+
369
M-Cl
334
M-
C2H4O
325
M-Cl-CO
306
M-Cl-
C2H4O
290
M-Cl-
C4H6O
264
M-PFP
222
MW 369
PFP
Cl
102
115
143
149

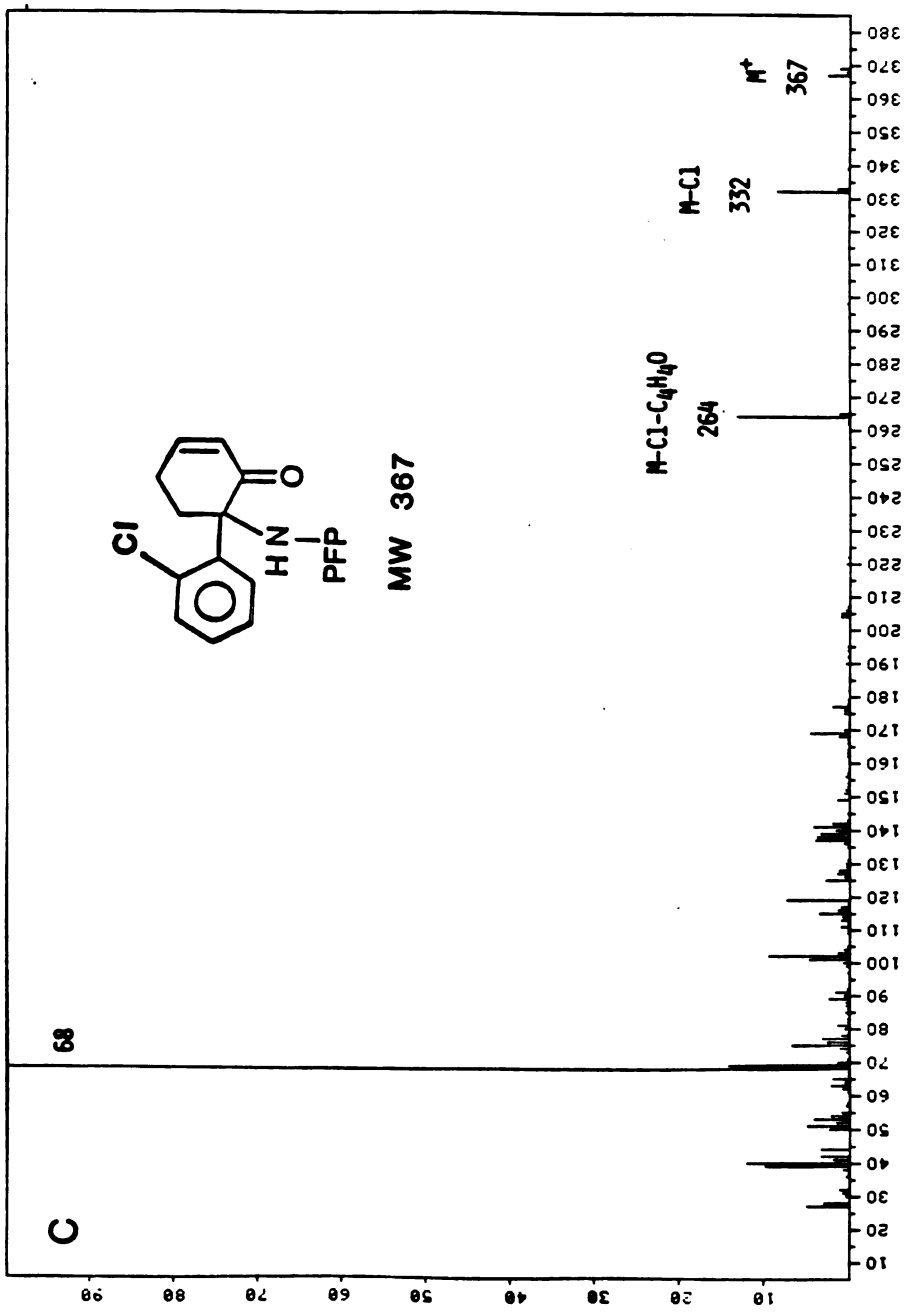

C
68
M+
367
M-Cl
332
M-Cl-C4H4O
264
MW 367
Cl
HN
PFP
O

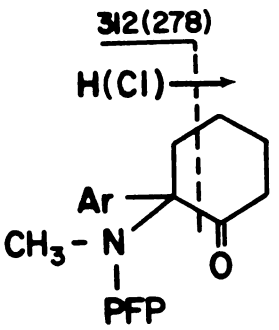
Table IV.1. Selected High Resolution Mass Spectral Data for PFP Derivatives of Ketamine, Norketamine and Dehydornorketamine.

Peak no.[a]/ MU value (OV-1)	Compound	Characteristic Ions			
		m/z Found	Elemental Composition	Error (ppm) from m/z Calculated	Neutral Lost
7	Ketamine	348.1036	$C_{16}H_{15}NO_2F_5$	+3.8	Cl
18.87		320.1082	$C_{15}H_{15}NOF_5$	+2.5	Cl + CO
		312.0203	$C_{12}H_8NOF_5{}^{35}Cl$	-3.9	C_4H_7O
		278.0588	$C_{12}H_9NOF_5$	-6.0	Cl + C_4H_6O
		160.0185	$C_4H_3NF_5$	-0.6	$C_{12}H_{12}O_2{}^{35}Cl$
1	Norketamine	334.0860	$C_{15}H_{13}NO_2F_5$	-2.0	Cl
17.33		325.0285	$C_{13}H_9NOF_5{}^{35}Cl$	-2.5	C_2H_4O
		312.0180	$C_{12}H_8NOF_5{}^{35}Cl$	-11.0	C_3H_5O
		306.0898	$C_{14}H_{13}NOF_5$	-6.2	Cl + CO
		290.0611	$C_{13}H_9NOF_5$	+2.4	Cl + C_2H_4O
		278.0615	$C_{12}H_9NOF_5$	+3.9	Cl + C_3H_4O
		264.0436	$C_{11}H_7NOF_5$	-4.6	Cl + C_4H_6O
3	5,6-Dehydro-	367.0340	$C_{15}H_{11}NO_2F_5{}^{35}Cl$	-16.0	-
	norketamine	332.0667	$C_{15}H_{11}NO_2F_5$	-12.9	Cl
		264.0444	$C_{11}H_7NOF_5$	-1.6	Cl + C_4H_4O

[a]Denotes number of peak on gas chromatogram, Fig. IV.3.

scheme IV.1

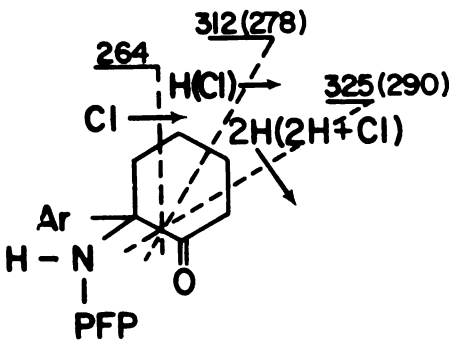
KETAMINE PFP



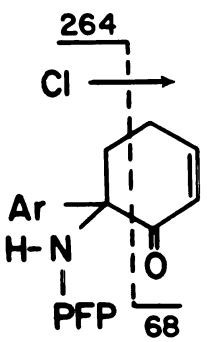
$CH_3-\overset{+}{N}\equiv C-C_2F_5$

m/z 160

NORKETAMINE PFP



5,6-DEHYDRONORKETAMINE PFP



which retain the N-methyl group of the parent drug. The mass spectrum of 5,6-dehydronorketamine PFP (Fig. IV. 4C) is dominated by an intense fragment ion at m/z 68, resulting from retro Diels-Alder cleavage of the cyclohexenone ring system; the presence of this ion was thus taken as strong evidence for ketamine derivatives which either possessed an $\alpha\beta$-unsaturated cyclohexenone moiety, or which could undergo elimination under electron impact to generate such a system.

The metabolites giving rise to peaks 2, 4, 5 and 6 (Fig. IV. 3) afforded the mass spectra illustrated in Figs. IV. 5a, 5b, 5c and 5d, respectively. The presence in each spectrum of prominent ions at m/z 496 and 468 suggested that these metabolites were isomeric hydroxynorketamines, the ions in question corresponding to [M-Cl]+ and [M-Cl-CO]+ fragments from the bis-PFP derivatives. This assumption was verified by high resolution mass measurements (Table IV.2) which supported the elemental composition assignments and, in two cases, by the presence of weak molecular ions at m/z 531. These metabolites will be referred to below as hydroxynorketamine isomers I-IV, as indicated in Table IV.2.

The mass spectrum of the bis-PFP derivative of hydroxynorketamine I (Fig. IV.5A) exhibited a fragment ion at m/z 264, the origin of which (Scheme IV.2) indicated that the site of hydroxylation could not be the aromatic moiety or the C-3 position of the cyclohexanone ring. The former conclusion was supported by the finding that when aromatic ring-labeled [$^2H_4$]ketamine was employed as metabolic substrate, no loss of deuterium occurred on formation of any of the hydroxylated metabolites. The ion at m/z 452 results from the combined loss from

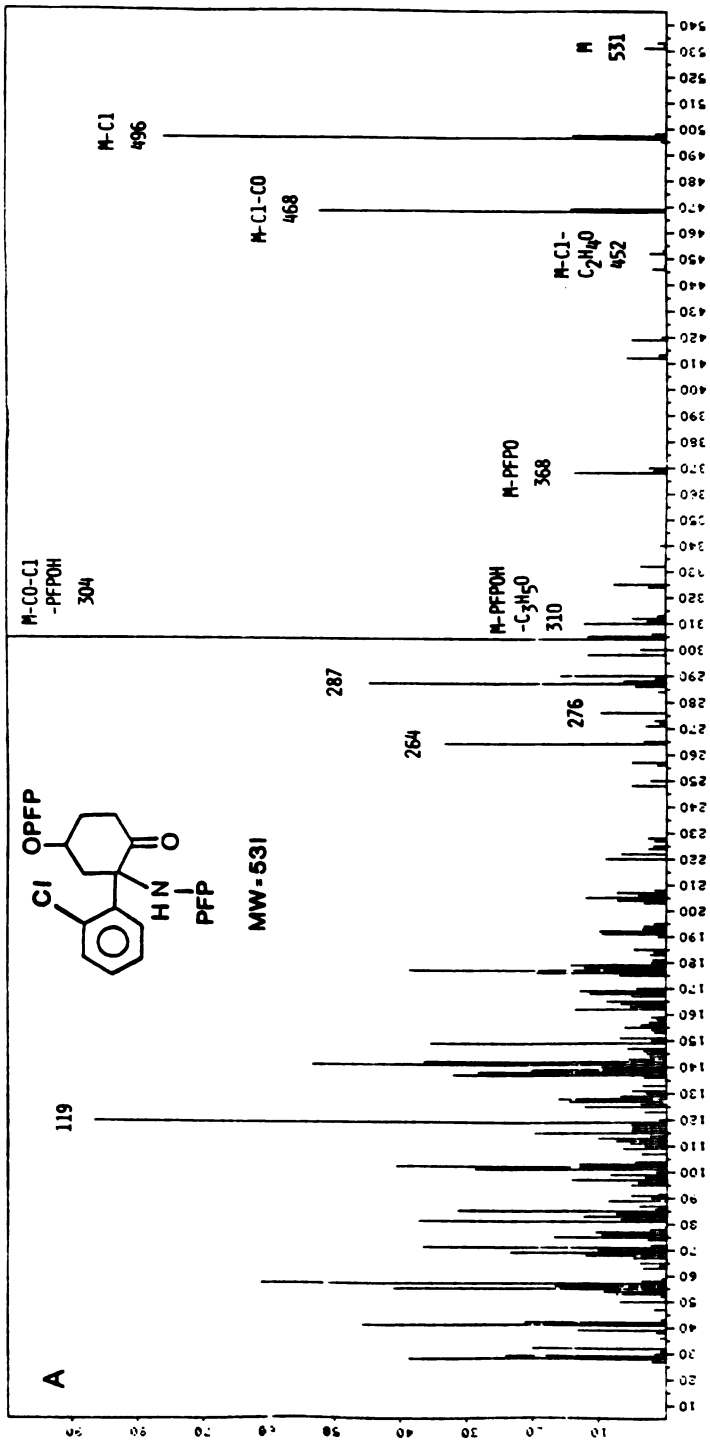
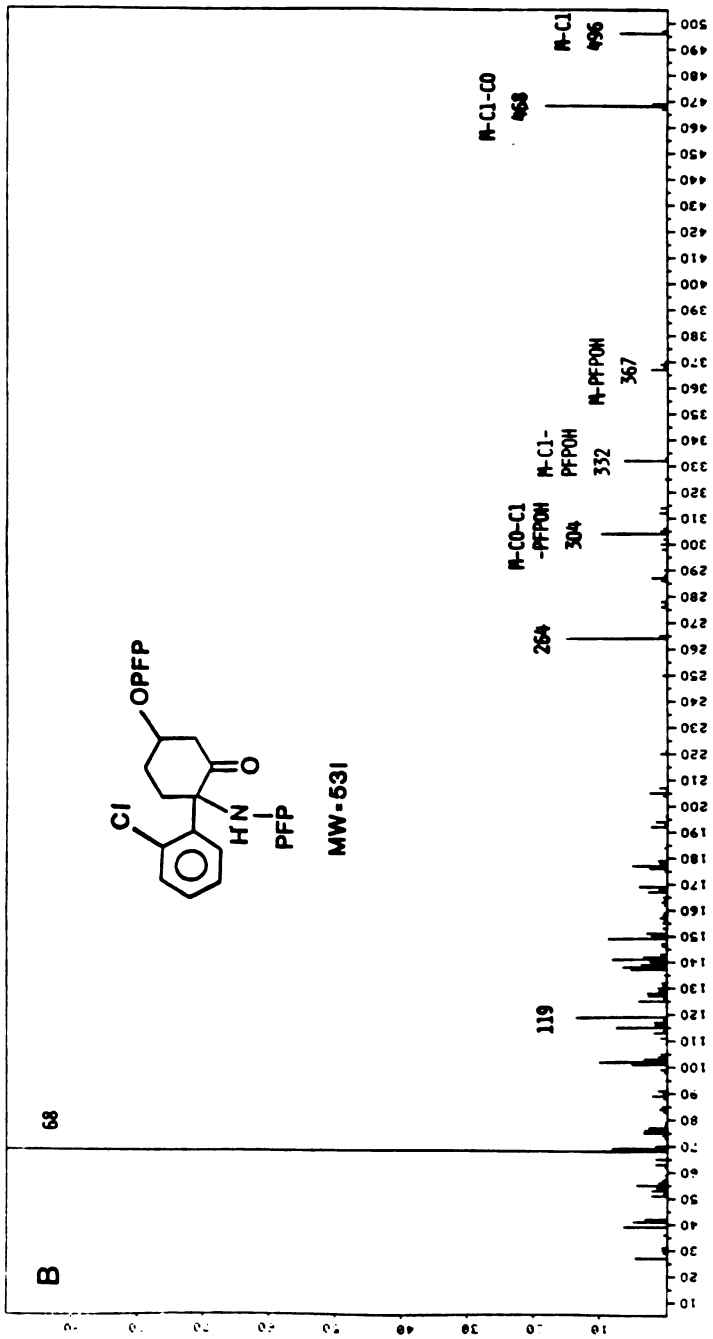
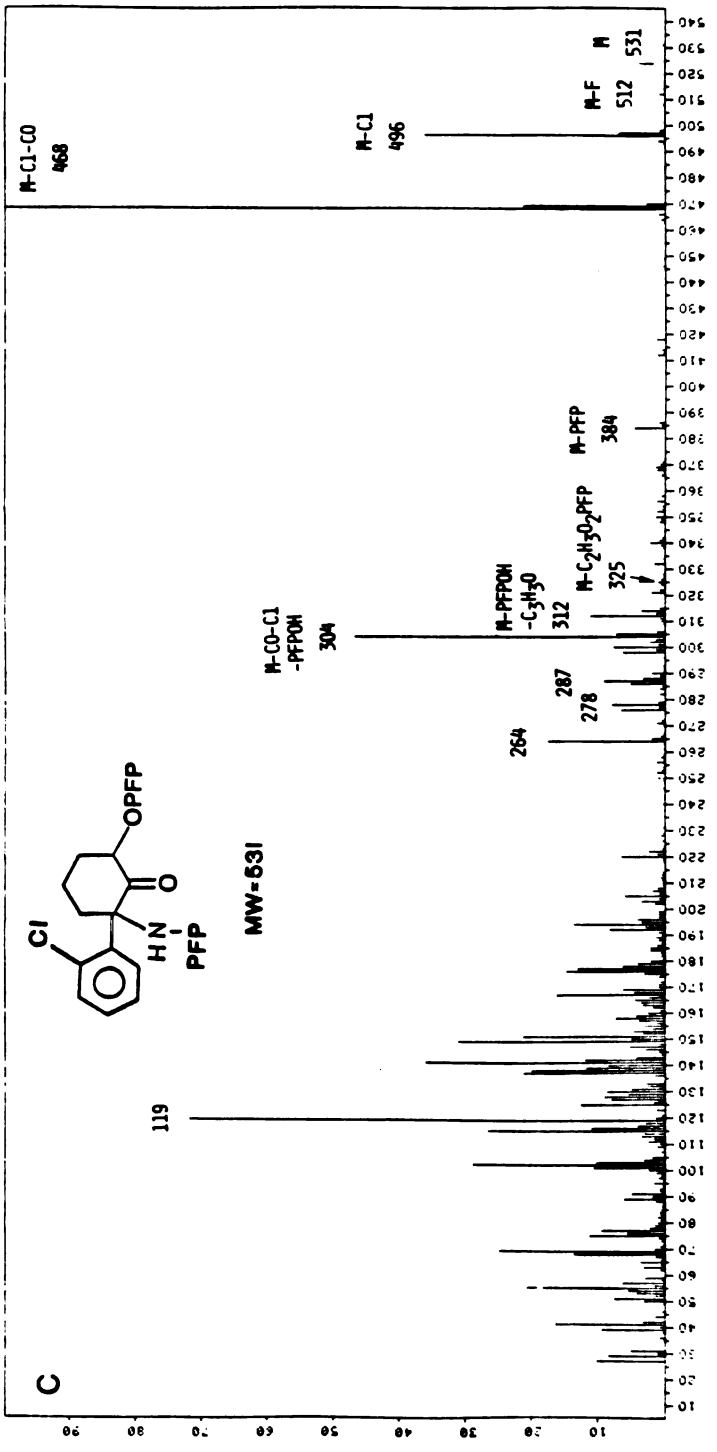


Figure IV.5. Low resolution mass spectra of the bis-PFP derivatives of hydroxymorketamine isomers I (A), II (B), III (C) and IV (D). No stereochemistry is implied in the structrual formulae illustrated.


B
68
119
264
M-CO-Cl -PFPOH
304
M-Cl-PFPOH
332
M-PFPOH
367
M-Cl-CO
468
M-Cl
496
OPFP
Cl
HN
PFP
O
MW-531


C
MW=531
OPFP
Cl
HN
PFP
O
M-Cl-CO
468
M-Cl
496
M-F
512
M
531
M-CO-Cl
-PFPOH
304
M-PFPOH
-C3H3O
312
M-C2H3O2PFP
325
M-PFP
384
287
278
264
119

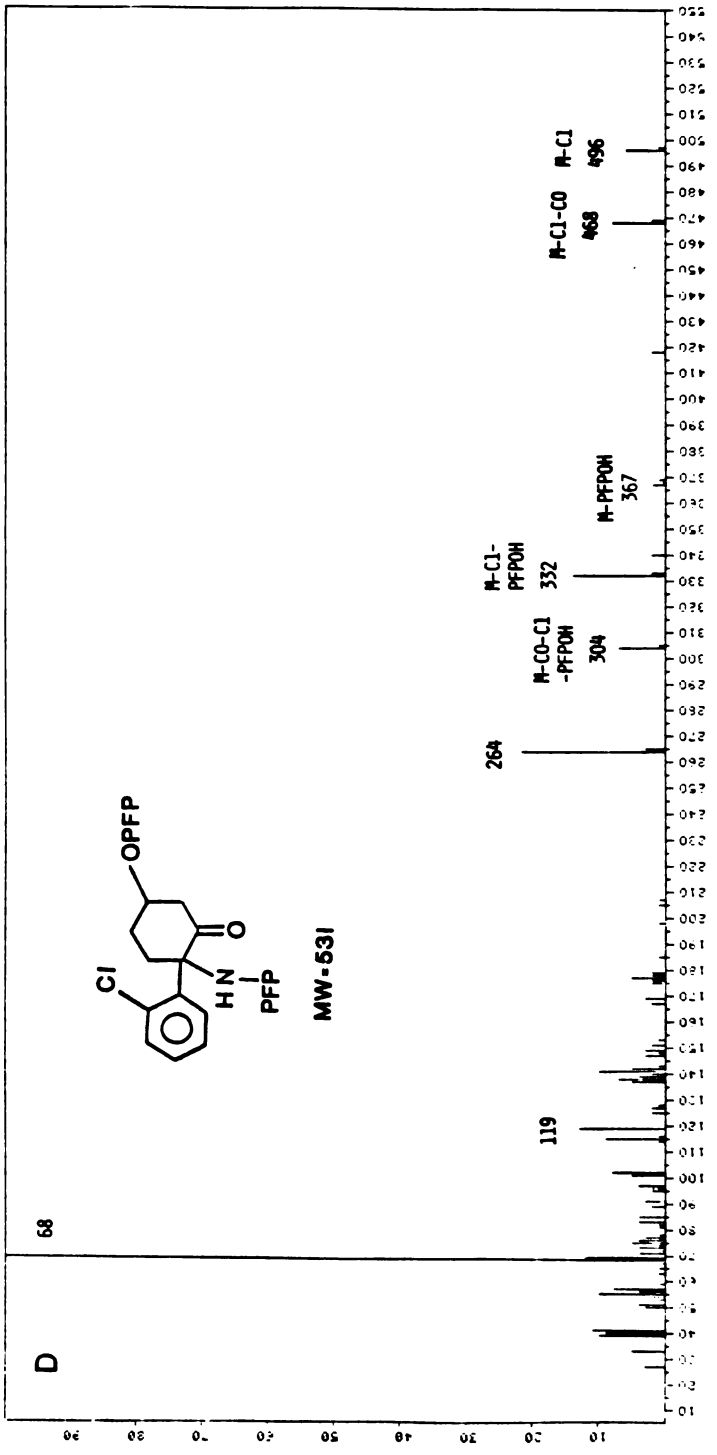

D
68
119
264
M-CO-Cl
-PFPOH
304
M-Cl-
PFPOH
332
M-PFPOH
367
M-Cl-CO
468
M-Cl
496
OPFP
Cl
O
HN
PFP
MW-531

Table IV.2. Selected High Resolution Mass Spectral Data for PFP Derivatives of Hydroxynorketamine Isomers

Peak no.[a]/ MU value (OV-1)	Compound	Characteristic Ions: m/z Found	Elemental Composition	Error (ppm) from m/z Calculated	Neutral Lost
2	Hydroxynor-ketamine I	496.0599	$C_{18}H_{12}NO_4F_{10}$	-1.7	Cl
17.44		468.0637	$C_{17}H_{12}NO_3F_{10}$	-4.5	Cl + CO
		452.0315	$C_{16}H_8NO_3F_{10}$	-6.6	Cl + C_2H_4O
		310.0012	$C_{12}H_6NOF_5{}^{35}Cl$	-14.9	PFPOH + C_3H_5O
		276.0417	$C_{12}H_7NOF_5$	-11.2	Cl + PFPOH + C_3H_4O
		264.0427	$C_{11}H_7NOF_5$	-7.7	Cl + PFPOH + C_4H_4O
4	Hydroxynor-ketamine II	496.0506	$C_{18}H_{12}NO_4F_{10}$	-20.3	Cl
17.91		468.0689	$C_{17}H_{12}NO_3F_{10}$	+6.8	Cl + CO
		264.0442	$C_{11}H_7NOF_5$	-2.1	Cl + PFPOH + C_4H_4O
5	Hydroxynor-ketamine III	496.0655	$C_{18}H_{12}NO_4F_{10}$	+9.8	Cl
18.17		468.0620	$C_{17}H_{12}NO_3F_{10}$	-8.0	Cl + CO
		325.0326	$C_{13}H_9NOF_5{}^{35}Cl$	+10.1	$C_2H_3O_2PFP$
		312.0200	$C_{12}H_8NOF_5{}^{35}Cl$	-4.6	PFPOH + C_3H_3O
		290.0645	$C_{13}H_9NOF_5$	+14.0	Cl + $C_2H_3O_2PFP$
		278.0622	$C_{12}H_9NOF_5$	+6.1	Cl + PFPOH + C_3H_2O
		264.0426	$C_{11}H_7NOF_5$	-8.2	Cl + PFPOH + C_4H_4O

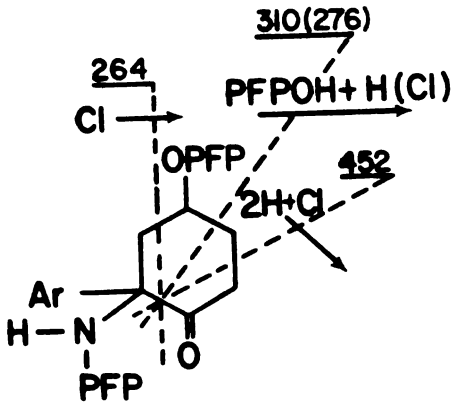
continued

Table IV.2. (continued)

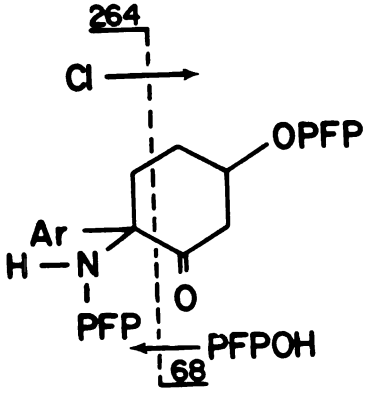
Peak no.[a]/ MU value (OV-1)	Compound	Characteristic Ions			
		m/z Found	Elemental Composition	Error (ppm) from m/z Calculated	Neutral Lost
6	Hydroxynor-ketamine IV	496.0532	$C_{18}H_{12}NO_4F_{10}$	-15.2	Cl
18.34		468.0700	$C_{17}H_{12}NO_3F_{10}$	+9.1	Cl + CO
		264.0429	$C_{11}H_7NOF_5$	-7.1	Cl + PFPOH + C_4H_4O

[a]Denotes number of peak on gas chromatogram, Fig. IV.3.

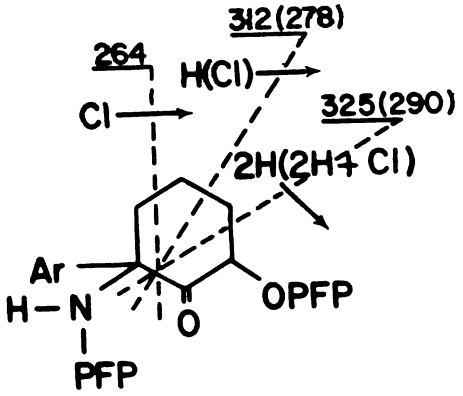
HYDROXYNORKETAMINE I bis-PFP



HYDROXYNORKETAMINE II , IV bis-PFP



HYDROXYNORKETAMINE III bis-PFP



scheme IV.2

the parent ion of the elements of Cl and acetaldehyde; an analogous process is evident in the spectrum of norketanine PFP itself and indicates absence of substitution at the C-6 position. Cleavage of the cyclohexanone ring by rupture of the C_1-C_2 and C_4-C_5 bonds, however, gives rise to ions at m/z 310 and 276 in the spectrum of this metabolite, whereas the corresponding ions in the spectrum of norketamine PFP are found at m/z 312 and 278. High resolution mass measurements (Table IV.2) showed that these pairs of ions (m/z 276/278 and 310/312) differed from one another in elemental composition by two hydrogen atoms; this finding is rationalized as shown in Scheme IV.2, and involves introduction of a double bond at the 3,4 position by elimination of the elements of PFPOH, prior to ring cleavage. Since the C-3 position does not carry the PFP substituent, the mass spectral data point to C-4 as the site of hydroxylation in this isomer.

The mass spectra of the bis-PFP derivatives of hydroxynorketamine isomers II and IV (Figs. IV.5B, D) were closely similar, each displaying an intense ion at m/z 68 and complementary fragment at m/z 264 (Scheme IV.2). As discussed above, the m/z 68 ion is indicative of a 5-1-keto precursor fragment and thus locates the hydroxyl substituent in these metabolites at either C-5 or C-6. Electron impact induced elimination from the molecular ion of the elements of PFPOH yields a daughter (m/z 367), formally equivalent to the $M^{+\cdot}$ ion of 5,6-dehydronorketamine PFP which, in turn, undergoes retro Diels-Alder cleavage to give m/z 68. Although it is not possible to distinguish, by mass spectrometry, between 5- and 6-hydroxylation in the case of these two metabolites, it would appear that the observed elimination of PFPOH would occur much more readily for the derivatized 5-hydroxy ketone in

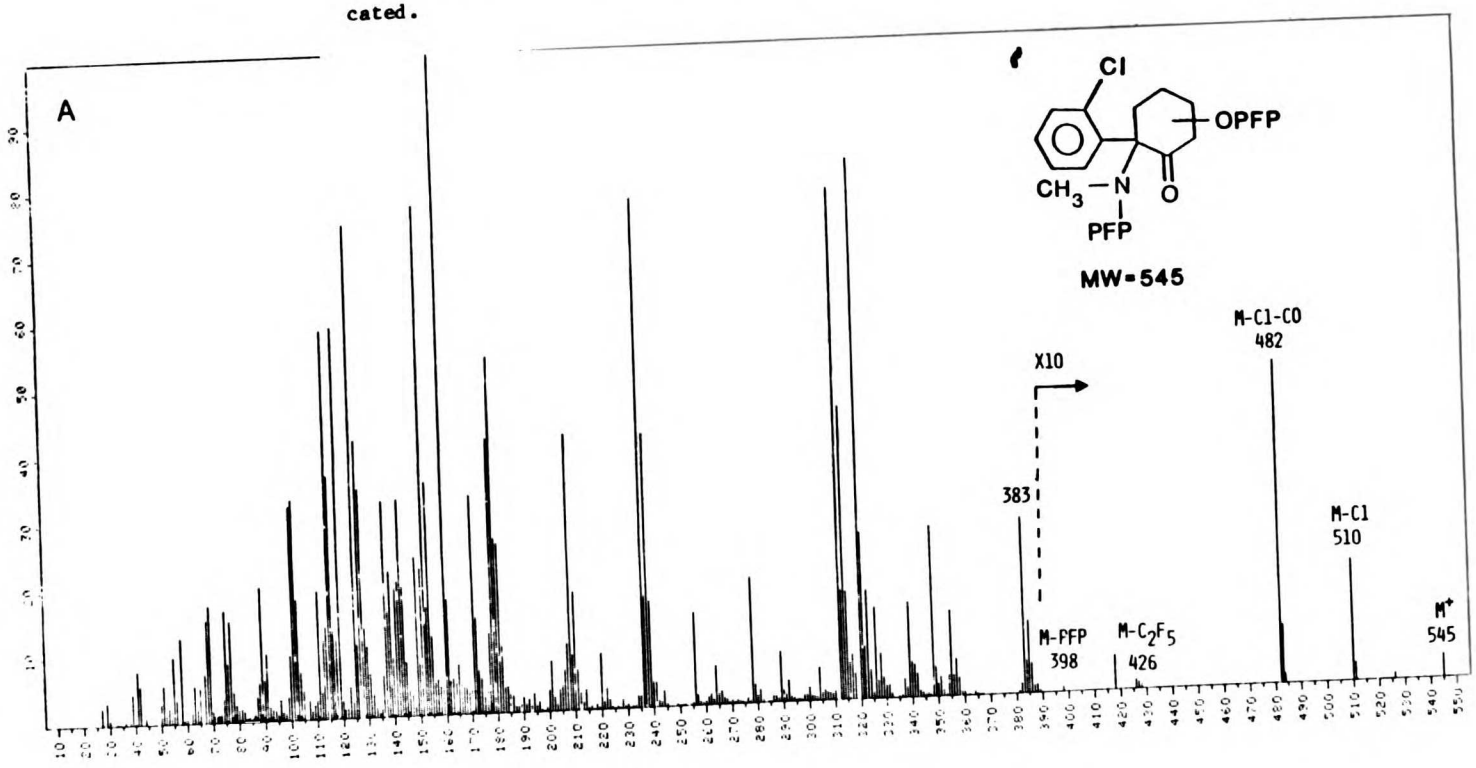
view of the relatively high acidity of the C-6 protons which are -to the ketone group. Hydroxynorketamines II and IV are thus tentatively assigned a 5-hydroxy structure, and differ from one another in stereochemistry at either of the two chiral centers, C-2 or C-5.

The mass spectrum of the bis-PFP derivative of hydroxynorketamine III (Fig. IV.5C) exhibits a prominent ion at m/z 264 which indicates that the C-3 position does not carry the hydroxyl substituent (Scheme 2). Furthermore, cleavage of the C_1-C_2 and C_4-C_5 bonds to give fragments at m/z 312 and 278 rules out C-4 as the site of substitution. Two pieces of indirect evidence favor a 6-hydroxynorketamine structure for this isomer, viz. (i) the absence of an ion at m/z 452, whose presence would have demonstrated an intact methylene group at C-6 (see discussion above for hydroxynorketamine I), and (ii) the absence of an intense ion at m/z 68, which would have pointed to hydroxylation at C-5 (see discussion above from hydroxynorketamines II and IV). Further indication of C-6 as the site of hydroxylation was provided by the presence of weak ions at m/z 290 and 325, which result from elimination of carbons 5,6 and their substituents, with and without, respectively, the accompanying loss of chlorine (Scheme IV.2). This isomer is thus tentatively assigned the structure 6-hydroxynorketamine.

The metabolites responsible for GLC peaks 8 and 9 (Fig. IV.3) were found to be hydroxylated analogs of the parent drug itself and were designated hydroxyketamine I and hydroxyketamine II, respectively. The bis-PFP derivative of the former metabolite had a GLC retention time identical with that of ketamine PFP on the non-polar stationary phase (OV-1) employed in this study and the mass spectrum recorded at the maximum of the peak with an MU value of 18.87 was thus a composite

spectrum of the two species (Fig. IV.6A). In addition to a molecular ion (m/z 545) of low relative intensity, characteristic [M-Cl]+ and [M-Cl-CO]+ fragments from the hydroxylated product were evident, whose accurate mass values were found to be consistent with the required elemental compositions (Table IV.3). In order to obtain high resolution mass measurements on ions derived from this metabolite, free of interference from the co-eluting ketamine derivative, a sample of the crude incubation extract was subjected to purification by HPLC and a "polar" fraction consisting of all material eluting prior to the retention volume of norketamine (Fig. IV.2) was collected. Following desalting and derivatization with PFP anhydride, this fraction was analyzed by GC-high resolution MS. Despite the very low levels of hydroxyketamine I present in the original extract, coincident peaks were obtained (at MU = 18.87) in the reconstructed ion current profiles for m/z 160, 312 and 482; the accurate masses and elemental composition assignments for these ions are given in Table IV.3 and the origin of the m/z 160 and 312 fragments is as indicated in Scheme IV.3. It may be concluded that the site of hydroxylation in this metabolite is at C-4, C-5 or C-6 of the cyclohexanone ring, although insufficient data are available for a more precise structural assignment. Comparison of the set of ions present in the upper mass region (m/z 390) of the spectrum of this derivative with those in the corresponding segments of the mass spectra of the hydroxynorketamine derivatives reveals a striking similarity between the fragmentation patterns of hydroxyketamine I (Fig. IV.6A) and hydroxynorketamine III (Fig. IV.5C). It is tempting to speculate, therefore, that both of these metabolites may be hydroxylated at the C-6 position, although further studies will be

Figure IV.6. Low resolution mass spectra of the bis-PFP derivatives of hydroxyketamine isomers I (A) and II (B). Spectrum A also contains ions from the PFP derivative of ketamine (MW = 383, Figure 4A) which co-elutes with the bis-PFP of hydroxyketamine I on the OV-1 stationary phase. No stereochemistry is implied in the structural formulae indicated.



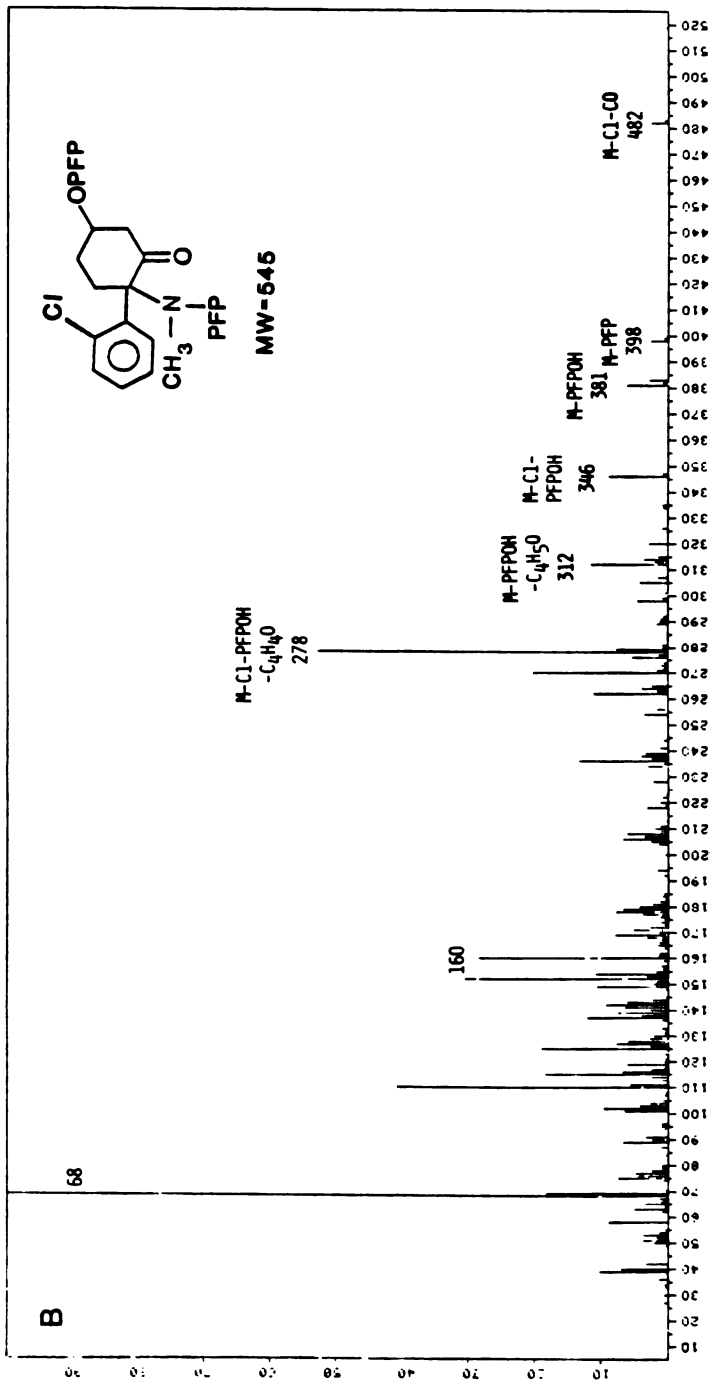

B
MW=645
OPFP
Cl
CH3—N—PFP
M-Cl-CO
482
M-PFP
398
M-PFPOH
381
M-Cl-
PFPOH
346
M-PFPOH
-C4H5O
312
M-Cl-PFPOH
-C4H4O
278
160
68

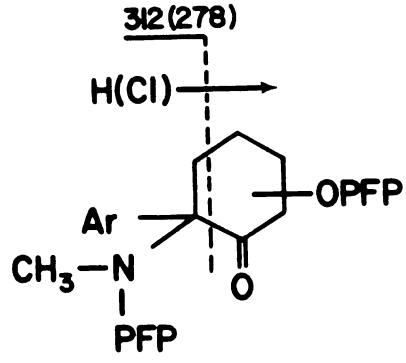
Table IV.3. Selected High Resolution Mass Spectral Data for PFP Derivatives of Hydroxyketamine Isomers

Peak no.[a]/ MU value (OV-1)	Compound	Characteristic Ions: m/z Found	Elemental Composition	Error (ppm) from m/z Calculated	Neutral Lost
8	Hydroxyketamine I	482.0885	$C_{18}H_{14}NO_3F_{10}$	+14.8	Cl + CO
18.87		312.0262	$C_{12}H_8NOF_5{}^{35}Cl$	+15.1	PFPOH + C_4H_5O
		160.0197	$C_4H_3NF_5$	+6.8	$C_{15}H_{11}O_4F_5{}^{35}Cl$
9	Hydroxyketamine II	482.0865	$C_{18}H_{14}NO_3F_{10}$	+10.6	Cl + CO
19.07		312.0184	$C_{12}H_8NOF_5{}^{35}Cl$	-9.9	PFPOH + C_4H_5O
		278.8597	$C_{12}H_9NOF_5$	-2.8	Cl + PFPOH + C_4H_4O
		160.0177	$C_4H_3NF_5$	-5.8	$C_{15}H_{11}O_4F_5{}^{35}Cl$

[a]Denotes number of peak on gas chromatogram, Fig. IV.3.

scheme IV.3

HYDROXYKETAMINES I, II bis-PFP



$CH_3\overset{+}{N}\equiv C-C_2F_5$

m/z 160

necessary to resolve this issue.

In contrast to the case of hydroxyketamine I, the PFP derivative of isomer II was well resolved on GLC from other ketamine metabolites and afforded the mass spectrum shown in Fig. III.6B. No M+• or [M-Cl]+ species were observed, although the [M-Cl-CO]+ ion was present and possessed an accurate mass consistent with the proposed elemental composition (Table IV.3). The presence of ions at m/z 312 and 278 again pointed to substitution at position C-4, C-5 or C-6 (Scheme IV.3), although C-5 was strongly favoured in this instance by the intense m/z 68 peak which is taken to be indicative of substitution - to the cyclohexanone carbonyl group (see discussion above for hydroxy-norketamine isomers II and IV). This metabolite is thus tentatively identified as 5-hydroxyketamine.

Semi-Quantitative Analysis of Metabolites

The efficiency of SEP-PAK cartridges for the isolation of drug-related material from aqueous media was evaluated by monitoring the radioactivity recovered from solutions (10 ml) of [^{3}H]ketamine in water, pH 8. Mean recoveries ($\pm$ S.D.) from five replicate experiments were: initial aqueous effluent, 8.2 $\pm$ 0.4%; water wash, 1.5%; methanol eluant, 88.3 $\pm$ 3.9%. As expected, retention of ketamine on passage through the SEP-PAK cartridge was found to be somewhat pH dependent and decreased on acidification of the aqueous solution. On the other hand, increasing the pH above 8 had no effect on the recovery of ketamine from aqueous media.

Initial studies on the quantification of ketamine metabolites formed in rat liver microsomes centered on the use of 2-(o-bromophenyl)-2-(methylamino)-cyclohexanone (CL394), the o-Br analog of ketamine, as

internal standard. Although fluoroacyl derivatives of this compound were found to be well separated on GLC analysis from the corresponding derivatives of ketamine and its metabolites, fluoroacylation of CL394 proved troublesome in that incomplete derivatization was observed frequently under the reaction conditions employed in this study. It should be noted, however, that the same o-Br analog has been employed by other workers as an internal standard in assay procedures for ketamine in which heptafluorobutyryl derivatives were employed.[10,39,41] In view of the above difficulties, a semiquantitative approach was adopted in which two n-alkanes, n-$C_{16}$and n-C_{22}, were employed as external standards in the gas chromatographic analysis. Calculation of the quantity of each metabolite produced in incubations was carried out by reference to the appropriate standard curve (Fig. IV.7), as outlined in the Experimental section. Results obtained from five replicate incubations of ketamine with pooled rat liver microsomal preparations from five animals are given in Table IV.4. Within experimental error, the total mass of drug-related material present in microsomal extracts, as determined by GLC, was equal to that calculated from radioactivity measurements. Thus, all ketamine metabolites eluted from SEP-PAK cartridges could be accounted for in terms of the eight compounds identified in this study.

DISCUSSION

The present investigation has led to the identification of eight *in vitro* metabolites of ketamine, produced by rat liver microsomal preparations, six of which have not been characterized previously in any of the mammalian species studied, including the rat. These novel

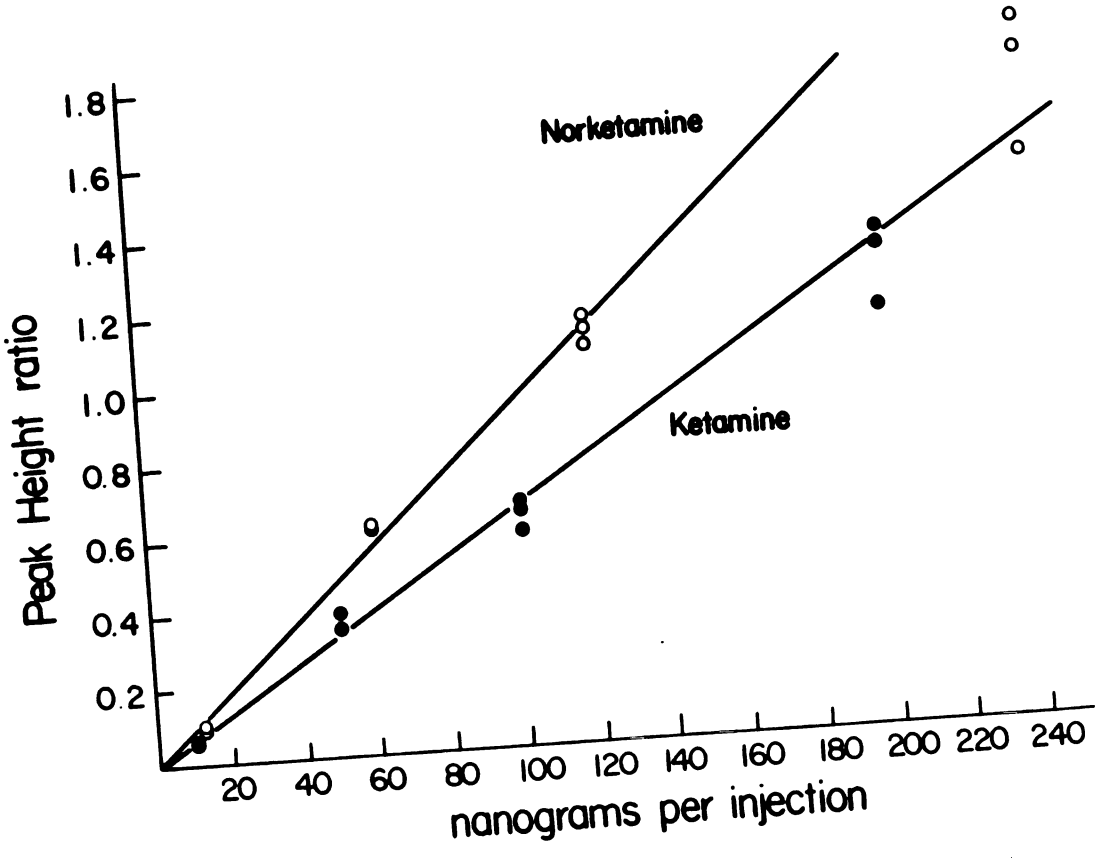


Figure IV.7. Standard curves for the semi-quantitative GLC analysis of ketamine, norketamine and related metabolites.

Table IV.4. Semi-Quantitative Analysis of Metabolites of Ketamine in Rat Liver Microsomes.

GLC Peak No.[a]	Compound	Amount Formed (% Total Drug-Related Material)					
		Expt. 1	Expt. 2	Expt. 3	Expt. 4	Expt. 5	Mean ± S.D.
7	Ketamine	65.8	75.3	58.7	66.0	70.7	67.3 ± 6.2
1	Norketamine	27.3	20.9	30.8	28.4	23.6	26.2 ± 3.9
2	Hydroxynorketamine I	0.2	0.2	0.3	0.3	0.2	0.2 ± 0.05
3	5,6-dehydronorketamine	1.0[b]	0.3[b]	0.6[b]	0.4[b]	1.1[b]	0.7 ± 0.4
4	Hydroxynorketamine II						
5	Hydroxynorketamine III	3.9	2.6	6.7	3.8	3.2	4.0 ± 1.6
6	Hydroxynorketamine IV	0.6	0.2	0.6	0.3	0.2	0.4 ± 0.2
8	Hydroxyketamine I	N.D.[c]	N.D.[c]	N.D.[c]	N.D.[c]	N.D.[c]	—
9	Hydroxyketamine II	1.1	0.6	2.3	0.9	0.9	1.2 ± 0.7

[a]Number of peak on gas chromatogram illustrated in Fig. IV.3.

[b]Values calculated from composite peak height of unresolved doublet.

[c]N.D. means "not determined" due to coincidence in GLC retention times between fluoroacyl derivatives of ketamine and hydroxyketamine I. Based on GC-MS evidence, it was estimated that the latter compound accounted for less than 0.5% of total drug-related material in incubation products.

metabolites all proved to be products of alicyclic ring hydroxylation, either of the parent drug itself or of norketamine, the product of metabolic N-demethylation of ketamine. Although it has been speculated that polar compounds of this type might be produced *in vitro* and *in vivo* during metabolism of ketamine and may be partly responsible for that fraction of the dose which cannot be accounted for,[11,20] this report provides the first experimental evidence for the formation of such products *in vitro*. Hydroxylation was found to be restricted to the cyclohexanone ring system, where carbons 4,5 and 6 were sites of metabolic attack; no evidence was obtained in this investigation for the formation of phenolic metabolites of the drug. Although hydroxylation of the alicyclic ring led to the introduction of a second chiral center in each of these metabolites, no attempt was made in the present study to define the stereochemistry at sites of oxidation. Failure of previous studies on the biotransformation of ketamine to characterize hydroxylated metabolites has likely been due to a combination of factors, *viz*. (i) earlier methods for the isolation of metabolites from biological samples involved extraction with non-polar solvents, e.g., benzene,[10] which may have resulted in poor recovery of polar metabolites; (ii) published solvent extraction procedures employed in studies of ketamine metabolism entailed the addition to biological samples of strong alkali prior to recovery of basic constituents into the organic phase;[10] such an approach may have led to partial dehydration of base-labile metabolites, such as the three 5-hydroxy ketones (hydroxyketamine II and hydroxynorketamines II and IV) identified in the present study; (iii) early reports on the identification of ket amine metabolites by GLC[9] and GC-MS[37] methods were based on analytical

protocols which did not include a derivatization step; without such derivative formation, hydroxylated metabolites of norketamine undergo extensive thermal degradation on GLC and GC-MS analysis to yield the dehydration product, 5,6-dehydronorketamine.

The analytical approach adopted in the present investigation was designed to avoid these shortcomings, and entailed the development of a nonselective extraction procedure and subsequent fractionation step using HPLC; in both cases, reversed phase chromatographic conditions were employed in which metabolites were maintained in an extremely "mild" environment with respect to both temperature and pH. Thermal dehydration of hydroxylated metabolites on GLC or GC-MS analysis was effectively prevented by the use of PFP or TFA derivatives; by this approach, 5,6-dehydronorketamine, previously considered to be a major metabolite of ketamine in several species including the rat,[9] was shown to represent a very minor component in microsomal incubation products. It seems likely, therefore, that this unsaturated species may have been an artefact of the analytical procedures employed in its characterization, since it is clearly present in much smaller amounts than originally assumed.

The use of glass capillary GLC columns was found to be essential for adequate resolution of the ketamine metabolites identified in this study, whose mass spectra were therefore obtained under GC-MS conditions employing WCOT capillary columns. In addition, the availability of specialized glass capillary GC/high resolution MS instrumentation[55] permitted accurate mass data and elemental conposition assigments to be obtained for each metabolite; these proved to be of great value in establishing respective fragmentation pathways which led, in turn, to

the sites of metabolic attack.

Semi-quantitative analyses of metabolite formation in rat liver microsomal preparations confirm previous reports that N-demethylation of ketamine is the major pathway of biotransformation,[11] the norketamine produced accounting for some 26% of drug-related material. Isomers of hydroxynorketamine together accounted for a further 5% of the dose, while two isomers of hydroxyketamine were formed as minor metabolites (1.5%).

On the basis of the qualitative and quantitative data derived from this study, a revised scheme for the metabolic fate of ketamine in rat liver may be proposed (Fig. IV.8). The major pathways of metabolism involves N-demethylation of the parent conpound to give norketamine which, in turn, may undergo further oxidation to yield 4-, 5- and 6-hydroxynorketamine. A minor route of metabolism *in vitro* involves direct oxidation of ketamine at positions C-5 and C-6 to afford the isomeric hydroxyketamine derivatives I and II. Formation of 5,6-dehydronorketamine probably reflects non-enzymatic dehydration of one or both isomers of 5-hydroxynorketamine during sample work-up and analysis. Finally, it should be stressed that while the mass spectral data strongly support the structural assignments indicated in Fig. III.8, confirmation of these assignments must await synthesis of the authentic reference compounds.

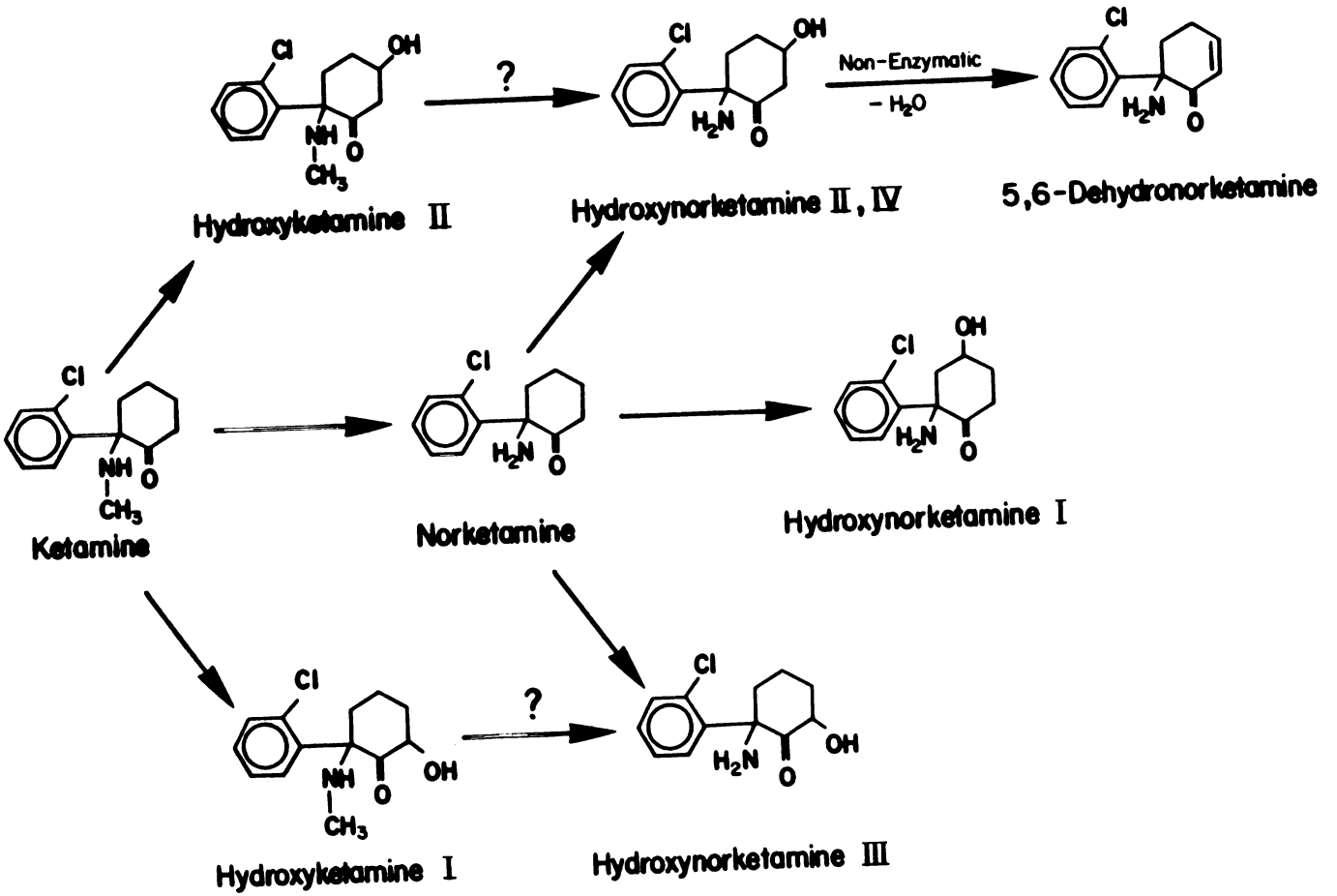


Figure IV.8. Proposed scheme for the metabolism of ketamine in rat liver microsomal preparations. Pathways indicated by a question mark are considered less likely to occur but cannot be eliminated on the basis of current information.

CHAPTER V. STUDIES ON THE BIOTRANSFORMATION OF KETAMINE II. STEREOCHEMICAL ASPECTS AND PSEUDORACEMATE STUDIES IN LIVER MICROSOMES

A. Introduction

B. Results

C. Discussion

Introduction

The objective of this part of the dissertation was to examine the metabolic fate of the pure enantiomers and pseudoracemic mixtures of ketamine (R,S-2-o-chlorophenyl-2-methylaminocyclohexanone). A pseudoracemic mixture contains two enantiomers in equal amounts, however one is labeled with a stable isotope (i.e. deuterium). The heavy isotope serves as a stereochemical marker and the ratio of labeled to unlabeled molecules provides a direct measure of the optical purity of the compound being studied. Of interest was the possibility of stereoselectivity and enantiomeric interactions in the metabolic oxidation of the drug. Stereoselective differences in the metabolism of pure optical isomers have been described for many cytochrome P450 mediated reactions.[31a,47] Enantiomeric interactions in which the metabolism of the enantiomers as a mixture is not predictable from the metabolism of the pure enantiomers due to an interaction between the enantiomers have also been reported for these types of reactions.[47]

The possibility of differential metabolism of the individual enantiomers of ketamine has been suggested by *in vivo* studies. In both the rat[50] and mouse,[65] plasma or brain levels of N-desmethylketamine (norketamine) were different from one another after the administration of R or S-ketamine in identical doses. The kinetic parameters of N-demethylation have also been investigated *in vitro* with the finding that S-ketamine posesses a slightly greater K_M and V_{max} than R-ketamine.[50]

It was decided to evaluate the *in vitro* formation of metabolites from the pure enantiomers and from racemic ketamine based upon the recently redefined metabolic profile of ketamine as described in the

preceding chapter.[2] It is possible that one or more of the six newly described hydroxylated metabolites is a psychoactive species that may be responsible for the delusional state seen in some patients after ketamine anesthesia. A clinical study of the optical isomers has revealed a considerable difference between the isomers for the production of these delusions.[86] This finding suggests that the enantiomers may be excellent probes for the characterization of possible active metabolites.

Capillary gas chromatography and gas chromatography-mass spectrometry were employed to identify the metabolites of the enantiomers of ketamine. Pseudoracemate experiments were performed with the deuterium labeled enantiomers in order to assess the metabolism of the enantiomers as a mixture. In these experiments equal concentrations of deuterium labeled (S)-ketamine and unlabeled (R)-ketamine (or deuterium labeled (R)-ketamine and unlabeled (S)-ketamine) are incubated with hepatic microsomes. The resultant metabolites are separated by established chromatographic methods and their nature and enantiomeric source can then be identified by mass spectrometry by virtue of mass differences due to the deuterium enantiomeric marker. Such pseudoracemic mixture procedures have been used to establish the existence of enantiomeric interactions for other drugs such as propoxyphene, warfarin and amphetamine.[3a]

RESULTS

Metabolism of the Individual Enantiomers of Ketamine by Rat Liver Microsomes

Analysis by GC-MS of microsomal incubations demonstrated that R-ketamine is metabolized to norketamine, 6-hydroxynorketamine, 4-hydroxy-norketamine and 6-hydroxyketamine. No other metabolites were detected (fig. V.1). Characteristic ions in the mass spectra of these products are presented in table V.1. S-Ketamine produces only norketamine, 5-hydroxyketamine and the two epimers of 5-hydroxynorketamine (fig. V.1 and table V.1). The metabolites were identified both by GC retention index (as compared to the n-alkanes C-16 and C-22, fig. V.2) and by GC-EIMS fragmentation patterns as detailed in the experimental section of this dissertation.

Metabolism of Racemic Ketamine by Rat Liver Microsomes

HPLC purified fractions of metabolites of racemic ketamine (figure V.3) produced by microsomes of the PB induced rat were prepared and analyzed by GC. The chiral column demonstrated adequate separation of the enantiomers of norketamine. The ketamine enantiomers however were not separated and no standards were available to determine the chromatographic behavior of the hydroxylated metabolites of either enantiomer (figure V.4). The stereochemical identity of the norketamine peaks as assigned in figure V.4 was determined by analysis of the norketamine produced by the pure isomers of ketamine. Both unpurified and HPLC purified metabolic norketamine were 59.7% R and 40.3% S norketamine as determined by the ratios of peak A'/peak A. The observation that metabolically produced norketamine was enriched in the R-

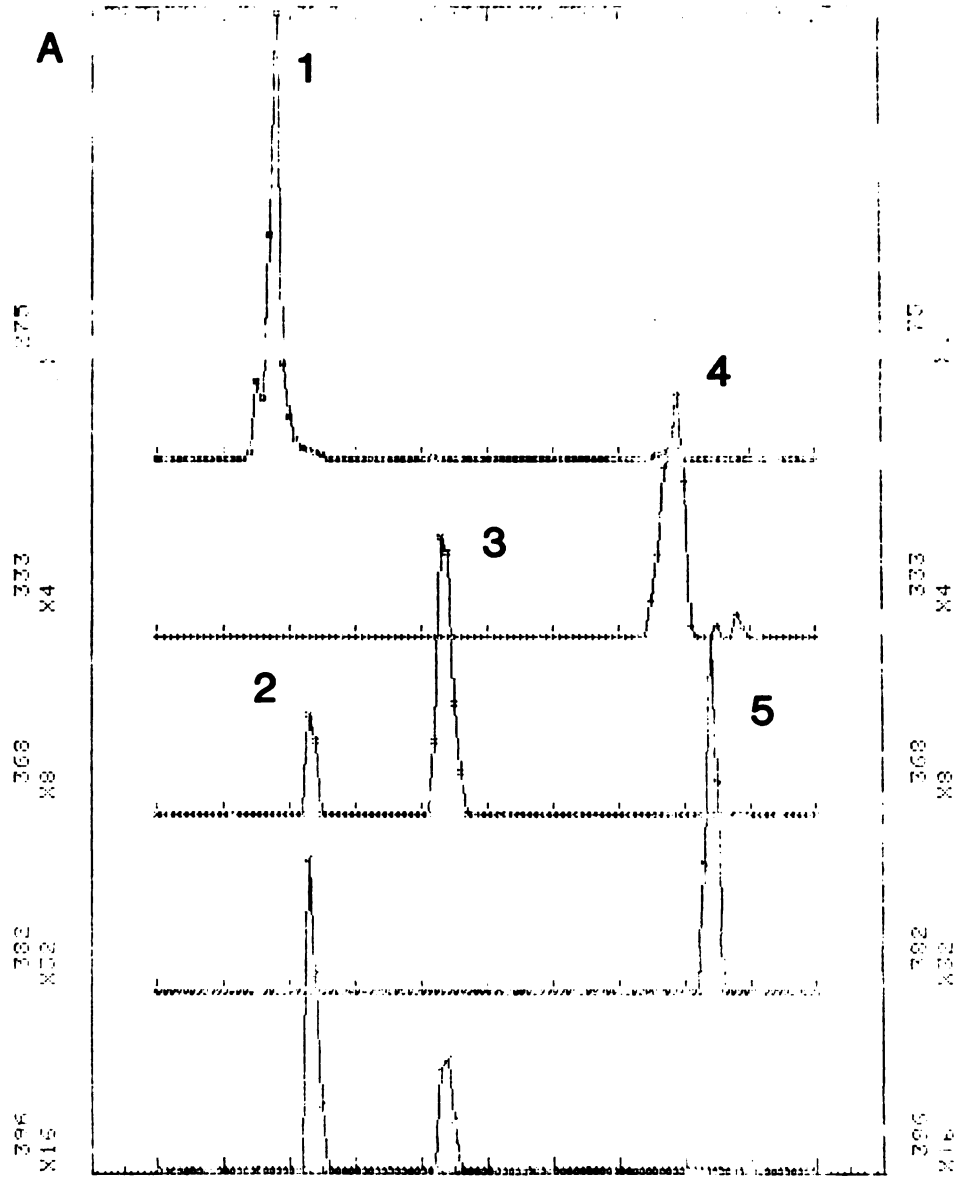


Figure V.1. Computer generated GC-MS mass chromatograms of R-ketamine (A) and S-ketamine (B) metabolic products from rat liver microsomes showing ion currents of interest. The numbered peaks are as follows: 1, norketamine; 2, 4-hydroxynorketamine; 3, 6-hydroxynorketamine; 4, ketamine; 5, 6-hydroxyketamine; 6, 5-hydroxynorketamine; 7, 5-hydroxynorketamine; 8, 5-hydroxyketamine.

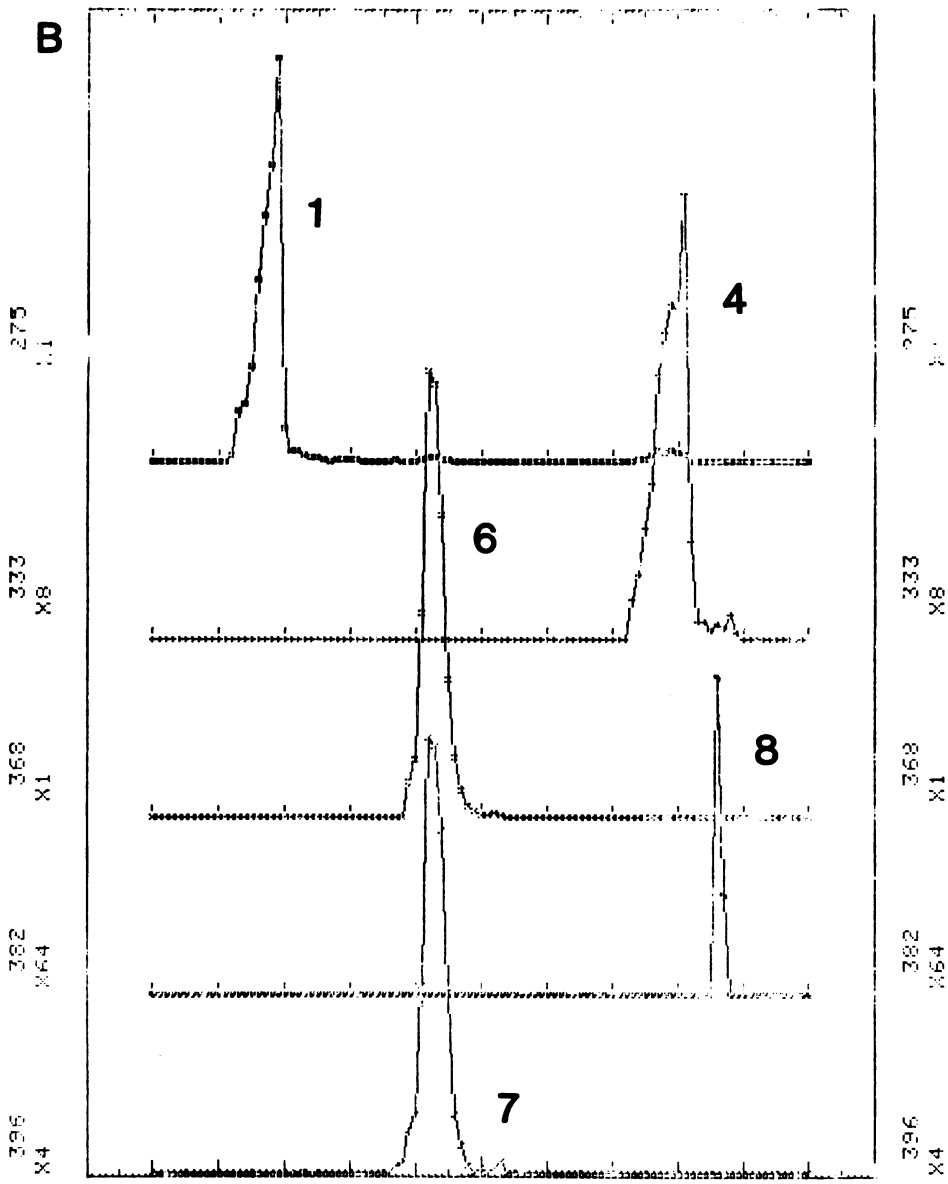

B
1
4
6
8
7
275
X1
333
X8
368
X1
382
X64
396
X4

Table V.1 Mass spectral data for TFA derivatives of ketamine metabolic products

GC peak #[a] and MU value	Compound	ketamine enantiomeric source	characteristic ions m/z	relative intensity (%)
6	ketamine	R,S	110	100
19.52			262	40
			270	70
			298	20
			333	7
1	norketamine	R,S	214	60
17.90			240	50
			256	100
			262	20
			275	70
			284	90
			319	3
2	4-hydroxynor-ketamine	R	69	100
18.06			214	50
			228	20
			262	25
			368	55
			396	75
3	5-hydroxynor-ketamine-1	S	214	30
18.55			368	100
			396	25

continued

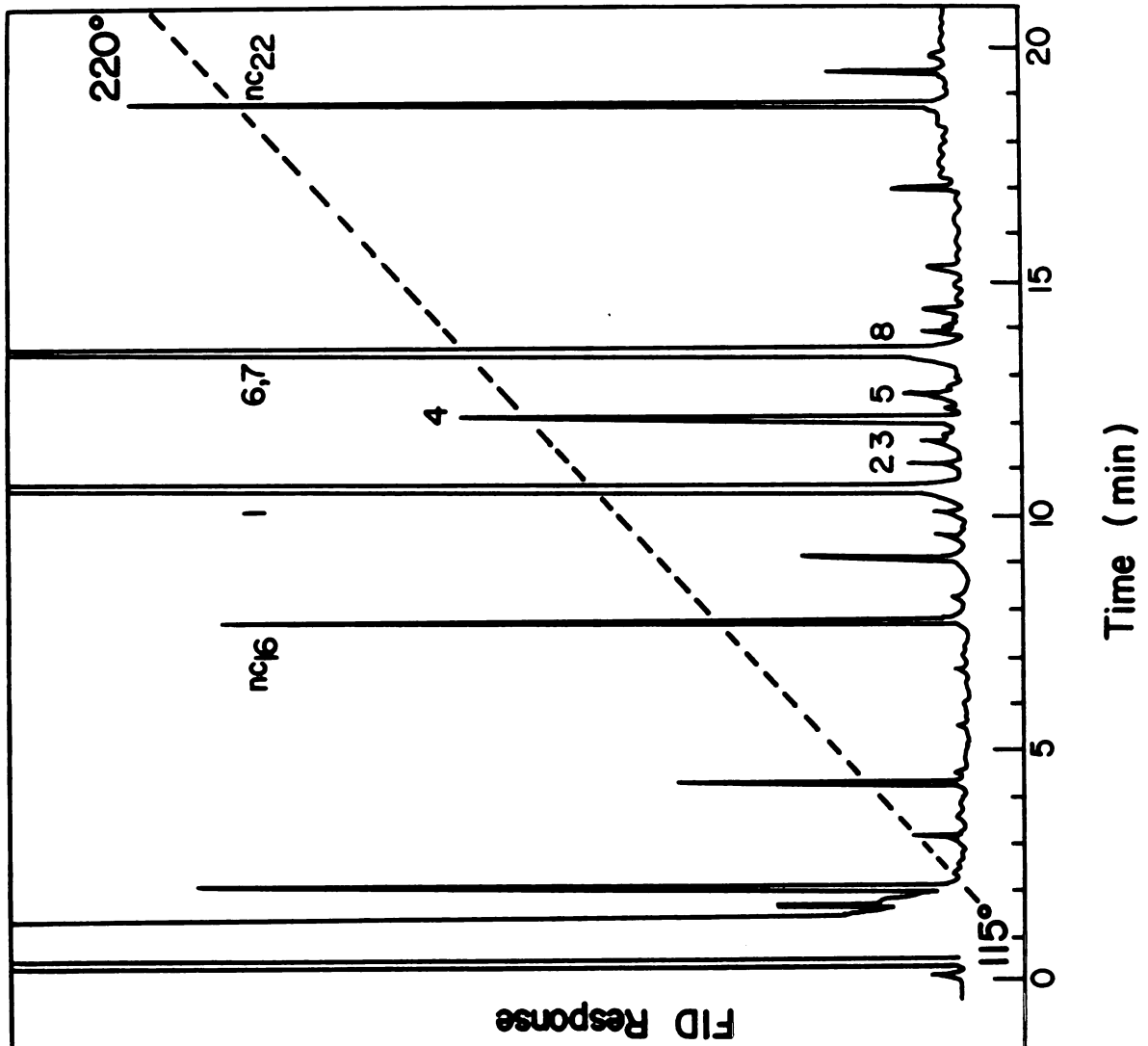
Table V.1 continued

GC peak # and MU value	Compound	ketamine enantiomeric source	characteristic ions m/z	relative intensity (%)
4	6-hydroxynor-ketamine	R	214	50
18.65			226	15
			240	7
			260	20
			368	100
			396	25
5	5-hydroxynor-ketamine-2	S	68	100
18.80			214	70
			368	90
			396	20
7	6-hydroxyketamine	R	110	*
19.57			262	20
			382	85
			410	30
8	5-hydroxyketamine	S	68	100
19.59			110	*
			228	40
			262	5
			382	10

[a]Peak number from figure V.3

*Spectra were generated by subtracting ketamine ions from the scans, so no m/z 110 ions were detected in the resulting difference spectra due to the large amounts of ketamine present at this retention time.

Figure V.2. Capillary GC trace of the n-alkanes C16 and C22 and trifluoroacetyl derivatized rat liver microsomal metabolic products from racemic ketamine.


220°
nc22
6,7
4
8
5
23
1
nc16
115°
0
5
10
15
20
Time (min)
FID Response

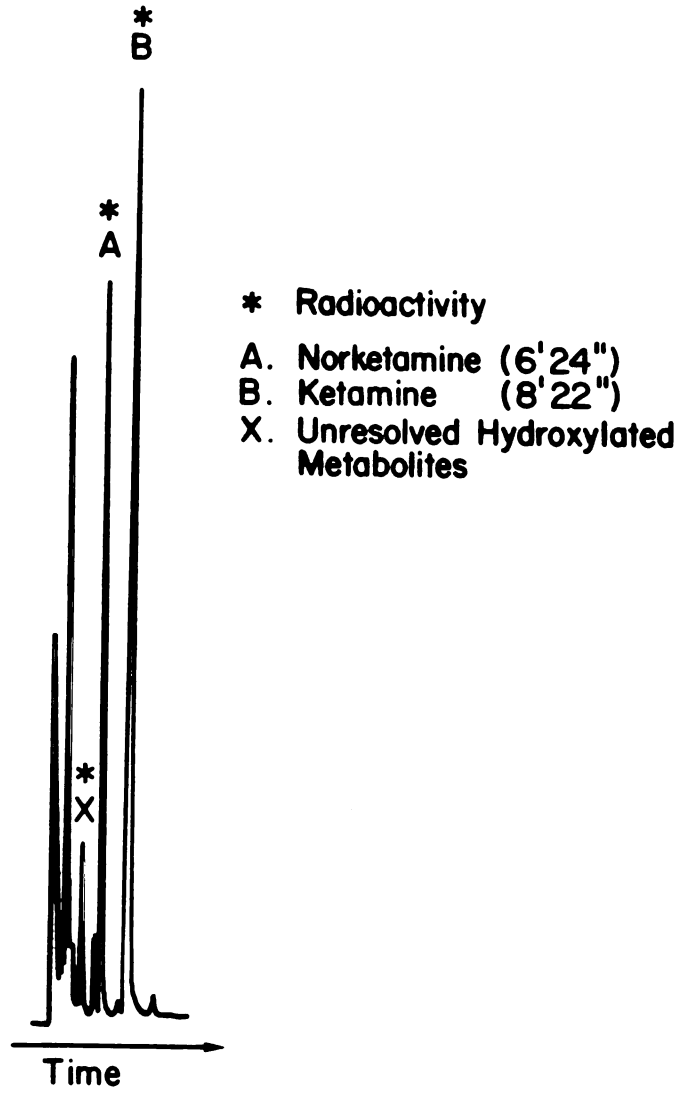
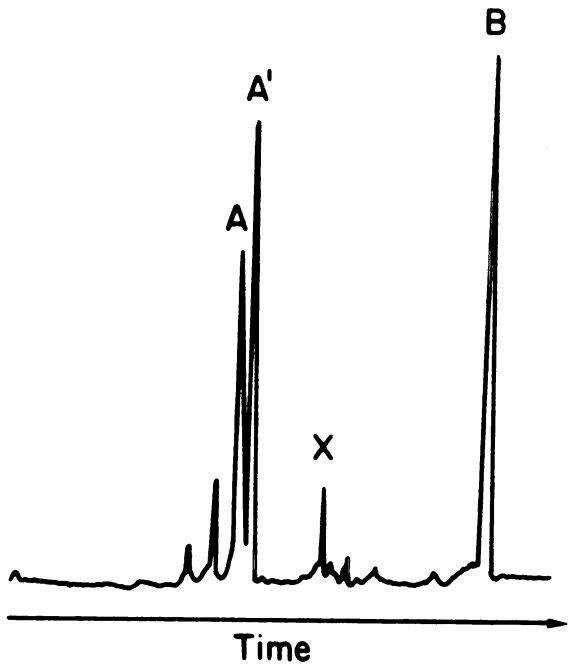


Figure V.3. HPLC trace of products from a rat liver microsomal incubation with racemic ketamine.

Figure V.4. Chiral capillary GC column assay of trifluoroacetyl derivatized rat liver microsomal metabolic products from racemic ketamine.

A. S-Norketamine
A'. R-Norketamine (12.5 min)
B. Ketamine (unresolved) (15.1 min)
X. Hydroxylated Metabolites



optical isomer suggested the possibility of enantiomeric differences at the level of N-demethylation. Since the chiral phase column would not be satisfactory for the analysis of the ketamine enantiomers themselves as a racemic mixture, pseudoracemate experiments were performed.

The optical purity of the metabolites from pseudoracemate incubations with rat microsomal preparations was investigated by GC-MS. The norketamine formed was found to be a mixture of deuterated and non-deuterated molecules. The hydroxylated metabolites however, all proved to originate from individual enantiomeric sources. The configurations at the 2-position of 6 and 4-hydroxynorketamines and 6-hydroxyketamine were R. The opposite configuration (S) at C-2 was found for 5-hydroxyketamine and the two 5-hydroxynorketamine derivatives based upon the presence or absence of deuterium in the metabolite. This was done by searching in the total ion current for ions at m/z 268 or 271 (M-Cl-CO+) as well as ions at m/z 396 or 399 (M-Cl+) in the case of the hydroxynorketamines. For the hydroxyketamines, m/z 382 or 385 (M-Cl-CO+) were sought. In each pair of ions, the lighter ion is unlabeled and the heavier ion contains deuterium atoms as a stereochemical marker. The optical purity of norketamine was evaluated precisely by measuring the area under the peaks in the reconstructed ion current profiles for $^{2}H_{0}$- or $^{2}H_{3}$-norketamine (evaluated as the [M-Cl]+ cluster at m/z 284 and 287). The ratio of $^{2}H_{0}/^{2}H_{3}$ was calculated and the percentages of each enantiomer were estimated by constructing a straight line semi-log curve of percent composition versus theoretical peak height ratios. Norketamine was found to be approximately a 56.5/43.5% mixture with enrichment of the R enantiomer in both pseudoracemates (table V.2). The equal percentages for the optical isomers of norketamine in both pseudoracemates demonstrates a lack of deuterium

Table V.2 Norketamine enantiomeric enrichment from rat liver microsomal incubations of pseudoracemic ketamine

Substrate	$^2H_0/^2H_3$ Ratio of Norketamine[a]				Stereochemical Composition of Norketamine Mean % ± S.D.	
	Rat 1	Rat 2	Rat 3	Rat 4	R	S
S-(^{2}H),R-Ket	2.42	2.34	2.43	2.70	55.8 ± 0.8	44.2 ± 0.8
S,R-(^{2}H)-Ket	1.27	1.31	1.27	1.24	56.0 ± 0.3	44.0 ± 0.3

[a]Standard S-(^{2}H),R-ketamine had a $^2H_0/^2H_3$ ratio of 1.89 and the ratio for standard S,R(^{2}H)-ketamine was 1.64 in this assay.

Table V.3 Norketamine enantiomeric enrichment from human liver microsomal incubations of pseudoracemic ketamine

Substrate	$^2H_0/^2H_3$ Ratio of Norketamine[a]	Stereochemical Composition of Norketamine	
		R	S
S-(^{2}H),R-Ket	1.64	45.5	54.5
S,R-(^{2}H)-Ket	2.37	42.0	58.0

[a]Standard S-(^{2}H),R-ketamine gave a $^2H_0/^2H_3$ ratio of 1.93 in this assay and the ratio for standard S,R-(^{2}H)-ketamine was 1.73.

isotope effect upon the metabolism of ketamine pseudoracemates. Ketamine was also analyzed for possible enantiomeric enrichment by calculating the $^{2}H_{0}/^{2}H_{3}$ ratio from the [M-Cl-CO]+ cluster at m/z 270 and 273. The ketamine isolated from microsomes was racemic within experimental error. This could suggest that there is no stereoselectivity in the metabolism of ketamine itself. A more likely explanation is that the original substrate concentration was not changed radically during the conditions of incubation so that any differences between the remaining ketamine enantiomers would not be readily detected.

Metabolism of the Enantiomers of Ketamine by Human Liver Microsomes

Human liver microsomes metabolize the pure optical isomers in a manner qualitatively similar to that observed with rat liver microsomes. The results from GC-MS assays showed that R-ketamine is oxidized to 4- and 6-hydroxynorketamine (which is interfered with by an endogenous impurity) and 6-hydroxyketamine. S-Ketamine produces 5-hydroxynorketamine, but only one isomer (the one with the shorter retention time) was detected. Sensitivity limitations may account for the inability to detect the other diastereomer. These results are presented in figure V.5. Rates of formaldehyde production were also determined in human liver microsomes. This data is discussed later in the chapter.

Metabolism of Pseudoracemic Ketamine by Human Liver Microsomes

Contrary to the rat, human liver microsomes produce norketamine enriched in the S-isomer (table V.3). The ratio of S/R found was 56.2 %/43.8 % of the total norketamine peak. Ketamine proved to remain racemic throughout the metabolic process probably for the reasons given above.

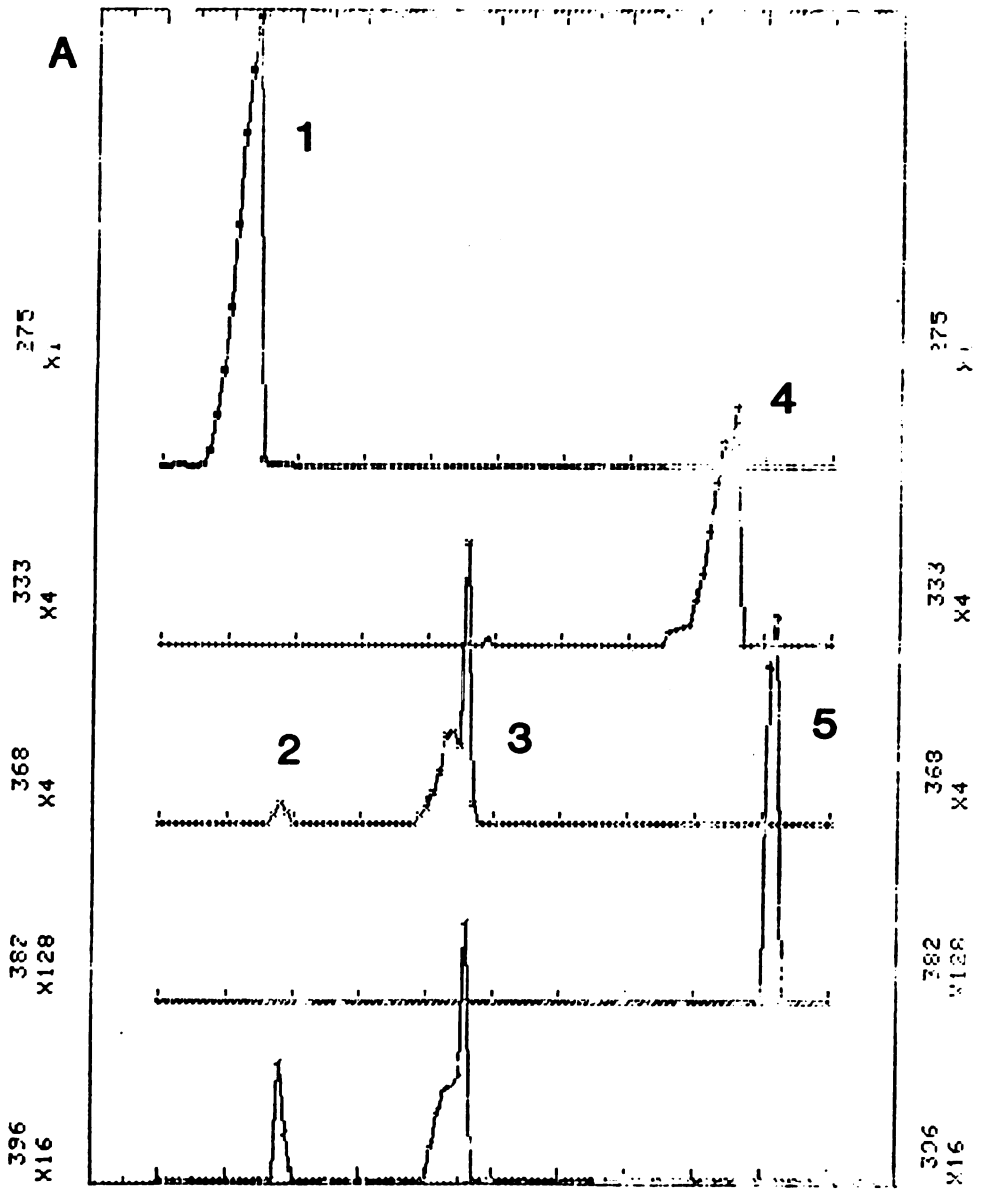
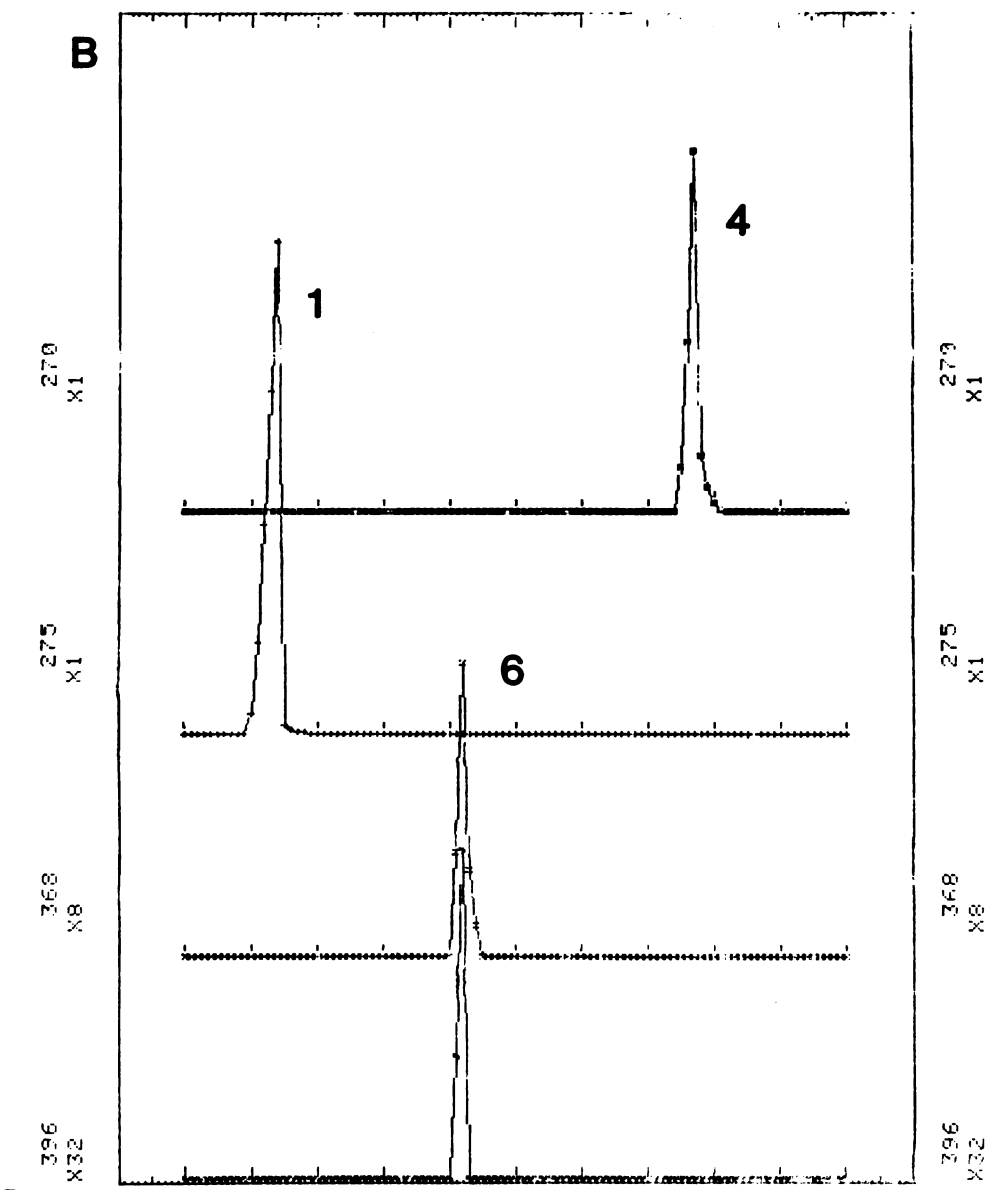


Figure V.5. Computer generated GC-MS mass chromatograms of R-ketamine (A) and S-ketamine (B) metabolic products from human liver microsomes showing ion currents of interest. The numbered peaks correspond to the numbers in fig. V.1.


B
1
4
6
270
X1
275
X1
368
X8
396
X32
270
X1
275
X1
368
X8
396
X32

The Mechanism of Norketamine Enrichment in Enantiomeric Mixtures

The enrichment of norketamine could have been due to enantiomeric interactions at the levels of N-demethylation of ketamine or of the oxidation of norketamine, or simply differential rates of production or degradation of the enantiomers of norketamine. In order to explore these possibilities, the kinetic parameters of formaldehyde production from the individual enantiomers of ketamine were established in rat liver microsomes (table V.4). An enantiomeric mixture experiment was also performed. The mixtures consisted of one isomer at a substrate level close to its apparent K_M, and the other isomer at three times that concentration. The two mixtures were compared to the pure enantiomers at concentrations close to their apparent K_M values by analysis using the chiral capillary column. The incubations were performed for 10 minutes which was found to be in the linear portion of the norketamine production curve and at which time very little of the hydroxynorketamine products were detected. The kinetic parameters of the enantiomers were found to be similar to those reported previously with the S-isomer having a greater apparent K_M and V_{max} than R-ketamine.[50] The results of the mixture experiment (table V.5) demonstrate a significant depression of N-demethylation in both mixtures. The S-isomer inhibits the N-demethylation of the R by 65.5% and R depresses the oxidation of S by 57.3%. These data suggest that both optical isomers compete with the N-demethylation of the other.

DISCUSSION

The results presented here demonstrate that in both rat and human liver microsomes, ketamine is metabolized with substrate stereoselec-

Table V.4 Kinetic parameters for the N-demethylation of the enantiomers of ketamine

Substrate	K_M (mM)	V_{max} (m/min/mg protein)
R-ketamine	0.165	3.17
S-ketamine	0.268	4.11

Table V.5 Norketamine levels following incubations of the enantiomers of ketamine and mixtures of the enantiomers

Conditions[a]	Norketamine (mean g ± S.D.) R	S	R/S	S/R
1	129.6 ± 18.5[b]			
2	44.8 ± 8.4[b]	94.4 ± 13.5		2.13 ± 0.24
3		84.9 ± 5.8[c]		
4	129.0 ± 51.0	37.0 ± 15.7[c]	3.5 ± 0.13	

[a]The conditions used were as follows: all assays were incubated for 10 min at 37° C and pH 7.4

1 0.21 mM R-ketamine

2 0.21 mM R and 0.63 mM S-ketamine

3 0.26 mM S-ketamine

4 0.26 mM S and 0.78 mM R-ketamine

[b] significantly different with 95% confidence

[c] significantly different with 95% confidence

tivity in the production of norketamine and substrate-product stereospecificity in the hydroxylation of norketamine and ketamine. In chapter IV, no conclusions could be reached concerning the absolute configurations at either chiral center of the hydroxylated metabolites. The present study allows the assignment of the configuration at C-2 in these metabolites. The stereochemistry of the second chiral center in the hydroxylated diastereomers remains to be established. S-Ketamine forms 5-hydroxyketamine and two diastereomers of 5-hydroxynorketamine. R-Ketamine produces the other metabolites, 4- and 6-hydroxynorketamine and 6-hydroxyketamine. These qualitative differences between the two enantiomers in terms of sites of hydroxylation in the cyclohexanone ring are observed following *in vitro* studies with microsomes from both rat and human liver. It would appear therefore that in this regard the rat may represent an adequate model for metabolism of the ketamine isomers in humans. The extent to which such stereoselective hydroxylation occurs *in vivo* remains to be determined.

Although R and S-ketamine are stereoselectively hydroxylated in a similar manner by both rat and human liver microsomes, the rates of N-demethylation of the enantiomers by such microsomes appears to be quantitatively different. Thus one observes an enrichment of R-norketamine after incubation of racemic ketamine with rat liver microsomes. Conversely, incubation of human microsomes with racemic mixtures leads to enrichment of S-norketamine over its optical antipode.

The observation of differences in rates of N-demethylation of the individual enantiomers of ketamine lead to considerations of possible mechanisms involved. This included interactions between the substrates (or metabolic products) at the level of cytochrome P450. First the kinetic parameters for N-demethylation of individual enan-

tiomers were established. Then enantiomeric mixtures of the ketamine enantiomers were made as described above. Under such conditions, both enantiomers caused significant inhibition of the N-demethylation of the other. This represents a chiral (or enantiomeric) interaction that could be due to direct competition between substrates for the enzyme (or enzymes) active site or to indirect inhibitory effects of products including norketamine or a hydroxylated metabolite.

The kinetic data for formaldehyde production from the pure enantiomers of ketamine present some difficulties in interpretation. The high affinity form (R) demonstrates a lower apparent V_{max} than the low affinity S-isomer in Michaelis-Menten kinetics. Also, in all of the enantiomeric mixtures and pseudoracemates studied in the rat, the R-isomer of norketamine is enriched. This might not be expected from the lower V_{max} of N-demethylation of R-ketamine, though it is in accord with a lower apparent K_M i.e. a higher affinity for the enzyme. It therefore appears that the classic Michaelis-Menton kinetics are not readily applicable to interpretations of these experiments. Nonetheless, an examination of formaldehyde production rates (table V.6) demonstrates a more rapid rate of N-demethylation of the R-enantiomer over the S-isomer at any given substrate concentration. This is a possible explanation of why the R-isomer is enriched in mixture experiments in rat microsomal systems. In human liver microsomes, the S-enantiomer of ketamine shows the higher rate of formaldehyde formation which may explain the enrichment of S-norketamine in mixture studies. One possible reason for the inapplicability of the conventional kinetic parameters would be if there were more than one form of cytochrome-P450 involved in the N-demethylation of ketamine in rat

Table V.6. Formaldehyde generation rates from the enantiomers of ketamine in rat and human liver microsomal incubations

Rat	Rate (um/min/mg protein)					
[R-ketamine]	0.06 mM		0.10		0.50	
[S-ketamine]		0.06		0.10		0.50
	0.80±0.10		1.51±0.34		2.64±0.66	
		0.70±0.31		1.44±0.33		2.41±0.40

Human						
[R-ketamine]	0.16 mM		0.24		0.32	
[S-ketamine]		0.16		0.24		0.32
	0.70±0.02		1.05±0.07		1.21±0.02	
		0.88±0.02		1.18±0.06		1.58±0.02

liver microsomes. If this were true, the typical kinetic analysis which assumes enzyme homogeneity would be irrelevant. This has been found to be the case with the metabolism of the enantiomers of warfarin wherein stereoselective and regioselective hydroxylation were attributable to different species of cytochrome P450.[24a] One piece of experimental evidence that suggests the existence of multiple enzyme forms is that of the PB induction study. In this study with racemic ketamine, the enrichment of R-norketamine was increased over that observed with uninduced animals. Thus, it is possible that Pb induces species of cytochrome P450 that metabolize R-ketamine more efficiently than it induces other forms of the enzyme.

The metabolism of ketamine involves hydroxylation as well as N-demethylation. This may complicate matters when establishing kinetic parameters by formaldehyde production. However, a previous study reported very similar kinetic parameters to those reported here for N-demethylation of ketamine after examining norketamine production by a gas chromatographic method.[50]

In summary it is clear that the <u>in vitro</u> metabolism of the individual enantiomers of ketamine is qualitatively different, and that the metabolism of racemic ketamine is significantly depressed from that predicted from the enantiomers in both rodent and human hepatic tissues. Though such differences are yet to be confirmed in <u>in vivo</u> studies, they form a rational basis for observed differences in pharmacological properties between the two enantiomers of ketamine. In particular these differences may explain the differences between R and S-ketamine in the incidence of delayed psychotomimetic emergence phenomena.[86] Though qualitative similarities exist between rodent and human metabolism of the enantiomers of ketamine, there are differ-

ences in N-demethylation rates. In the rat R-norketamine is formed to a greater extent than S, and the reverse is true in human liver microsomes. Therefore, the rat may not be a suitable model for the human in all cases and future in vivo work may have to be performed with patients. The enantiomers act to inhibit the respective metabolism of each other either directly by competition with enzymes as cosubstrates or via formation of active metabolites that can inhibit N-demethylation by microsomal enzymes. In this regard, the hydroxylated metabolites of ketamine should be synthesized and tested in hepatic in vitro systems.

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